# *European Commission*

**Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) N° 1107/2009**
**and**
**Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008**

# Eugenol
# 2-methoxy-4-(prop-2-en-1-yl)phenol

## Volume 3 – B.6 (AS)

Rapporteur Member State : Spain
Co-Rapporteur Member State : Greece

# Version History

| When | What |
|---|---|
| July 2022 | Initial DRAR-RMS Spain |
| November 2022 | DRAR after CoRMS and Applicant comments |
| February 2023 | RAR updated after EFSA CoCh received on 24/01/2023 |

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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# B.6. TOXICOLOGY AND METABOLISM DATA

## B.6.1. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION IN MAMMALS

*A total of eight studies have been submitted to address this point for the renewal process, six of which were presented in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC. These studies were deemed acceptable following evaluation and peer review at EU level. For the renewal process, a comparative in vitro metabolism study has been submitted to meet the data requirements of the Commission Regulation (EU) 283/2013, and a pharmacokinetic study in the rat has also been provided.*

*All available information has been evaluated (or re-assessed) according to the current guidelines.*

### B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

**B.6.1.1-01 Human: Absorption, metabolism and excretion of eugenol**

| | |
|---|---|
| **Data point** | CA 5.1.1 (EU data requirement) |
| **Report author** | Fischer, I.U., von Unruh, G.E. and Dengler, H.J. |
| **Report year** | 1990 |
| **Report title** | The metabolism of eugenol in man |
| **Report No** | Not applicable, published study (*Xenobiotica*, Vol 20 (2), 209-222) |
| **Document No** | KCA 5.1.1/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol); Purity: not specified; Lot/Batch No.: not specified |
| **Test system** | Four male and four female healthy volunteers (23-29 years old) |
| **Test method** | Healthy volunteers received 150 mg of eugenol each (3 gelatine capsules of 50 mg eugenol each) together with a standard breakfast consisting of tea *ad lib*. and two biscuits. The 24-hour urine was collected in 0-3, 3-6, 6-12 and 12-24h periods. Venous blood samples were obtained by an indwelling cannula at 0, 15, 20, 25, 30, 40, 50, 60, 80, 100 and 120 min. The 6h bile samples were obtained from patients with indwelling T-tubes. Urinary metabolites were extracted according to the method published by Bakke and Scheline (1969). Aliquots (10 mL) of urine were extracted as follows:<br>One portion was acidified with 4 M HCl to pH 1 and extracted three times with ether (20 mL, 2 x 10 mL). The combined ether extracts were shaken three times with 5 % aqueous $NaHCO_3$ (20 mL, 2 x 10 mL) to remove acidic compounds, dried over $MgSO_4$ and evaporated to dryness (referred to as the '*phenolic fraction*'). The phenolic acids were recovered from the combined $NaHCO_3$ extracts after acidification and extraction with ether (referred to as the '*acidic fraction*').<br>Another aliquot of the urine was extracted directly three times with ether at pH 5. Combined extracts were dried over $MgSO_4$ and evaporated to dryness.<br>A third portion of the urine (20 ml) was hydrolysed enzymatically at pH 4 (0.1 M sodium acetate buffer). Hydrolysis was performed at 37°C for 5 h with 1 mL β-glucuronidase/arylsulphatase from *Helix pomatia*. The incubation was divided in two equal parts and extracted as described above: one aliquot after acidification with HCl, the other one directly at pH4.<br>3,4-Dimethoxy-styrene was added as internal standard (IS) to the aliquots of urine. The IS was separated from eugenol and its metabolites in the GC and HPLC systems used. |

| | |
|---|---|
| | The recovery was the same as for eugenol during the work-up for phenols. The extraction for eugenol and the IS was similar. |

**Results**

Eugenol was rapidly absorbed and metabolised after oral administration and was almost completely excreted in the urine within 24 h. Un-metabolised eugenol excreted in urine amounted to less than 0.1 % of the dose.

All metabolites detected in human urine together with mean recoveries are displayed in Table B.6.1.1-01. The urine contained conjugates of eugenol and nine metabolites. A trace of unchanged eugenol (less than 0.1% of the dose) but no metabolites were extracted from untreated urine. Recovery in urine of 95% of the administered dose was obtained, most of which (>99 %) consisted of phenolic conjugates. Over 53 % recovery (mean from eight volunteers, range 38-62%) of applied dose corresponded to eugenol conjugated metabolites (glucuronide and sulphate conjugates). A quantitatively relevant metabolite was identified as a thiol derivative (compound X) that amounted a total of 11% of the applied dose. Other metabolic routes observed were the epoxide-diol pathway accounting for 13% of the applied dose (metabolites XI and XII), synthesis of a substituted propionic acid *via* allylic oxidation (metabolite IX, 5% of applied dose) and migration of the double bond (*cis/trans* isoeugenol, metabolites II and III, 7% of applied dose). The methoxy group of eugenol was resistant to cleavage in human.

**Table B.6.1.1-01. Urinary excretion of eugenol and its metabolites after oral dose to human volunteers**

| Compound | % Dose (Mean) | % Dose (Min) | % Dose (Max.) |
|---|---|---|---|
| ***Metabolites excreted in 0-3h Urine*** | | | |
| Unconjugated eugenol (I) | 0.05 | 0.03 | 0.09 |
| Conjugated eugenol | 42.2 | 30.2 | 52.8 |
| Conjugated metabolites: | | | |
| *cis*-Isoeugenol (II) | 0.8 | 0.5 | 1.5 |
| *trans*-Isoeugenol (III) | 4.7 | 2.8 | 7.9 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propylene-l,2-oxide (IV) | 1.5 | 0.5 | 3.3 |
| HCl-artefact (VI) | 4.0 | 1.4 | 7.4 |
| 3-Hydroxy-3-(4-hydroxy-3-methoxy)-allylbenzene (V) | 0.6 | 0.3 | 1.0 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propionic acid (IX) | 3.6 | 1.3 | 5.4 |
| 3-(6?-Mercapto-4-hydroxy-3-methoxyphenyl)-propane (X) | 8.9 | 6.2 | 10.2 |
| 2-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-propionic acid (XI) | 2.6 | 2.0 | 3.3 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propane-l,2-diol (XII) | 2.4 | 0.5 | 6.1 |
| *Percentage of dose excreted within the first 3 hours* | **71.3** | **69.9** | **74.0** |
| ***Metabolites excreted in 3-6h Urine*** | | | |
| Unconjugated eugenol (I) | 0.01 | 0.003 | 0.02 |
| Conjugated eugenol | 9.3 | 2.8 | 13.4 |
| Conjugated metabolites: | | | |
| *cis*-Isoeugenol (II) | 0.04 | 0.01 | 0.06 |
| *trans*-Isoeugenol (III) | 1.1 | 0.8 | 1.4 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propylene-l,2-oxide (IV) | 0.1 | 0.04 | 0.13 |
| HCl-artefact (VI) | 0.7 | 0.3 | 1.4 |
| 3-Hydroxy-3-(4-hydroxy-3-methoxy)-allylbenzene (V) | 0.1 | 0.02 | 0.17 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propionic acid (IX) | 1.0 | 0.3 | 1.7 |
| 3-(6?-Mercapto-4-hydroxy-3-methoxyphenyl)-propane (X) | 2.4 | 1.5 | 3.0 |
| 2-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-propionic acid (XI) | 0.7 | 0.5 | 0.9 |
| 3-(4-Hydroxy-3-methoxyphenyl)-propane-l,2-diol (XII) | 0.6 | 0.1 | 1.4 |
| *Percentage of dose excreted within the first 6 hours* | **87.4** | **82.0** | **89.6** |
| ***Metabolites excreted in 6-24h Urine*** | | | |
| Unconjugated eugenol (I) | 0.01 | 0.01 | 0.01 |
| Conjugated eugenol | 4.0 | 0.8 | 6.7 |
| Estimated amounts of metabolites | 3.4 | 0.4 | 5.2 |
| ***Percentage of dose recovered in 24 hours*** | **94.8** | **83.2** | **101.0** |

Figure B.6.1.1-01 displays the chemical structures of identified metabolites of eugenol in urine. The structures of these metabolites elucidated using GC-MS, and by comparison with synthetic reference compounds, were identified as: Eugenol, 4-hydroxy-3-methoxyphenyl-propane, *cis*- and *trans*-isoeugenol, 3-(4-hydroxy-3-methoxyphenyl)-propylene-1,2-oxide, 3-(4-hydroxy-3-methoxy-phenyl)-propane-1,2-diol and 3-(4-hydroxy-3-methoxyphenyl)-propionic acid. The structures of the following metabolites were tentatively deduced from mass spectra only, as reference compounds were not available: 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-allylbenzene, 3-(6-mercapto-4-hydroxy-3-methoxy- phenyl)-propane, and 2-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-propionic acid.

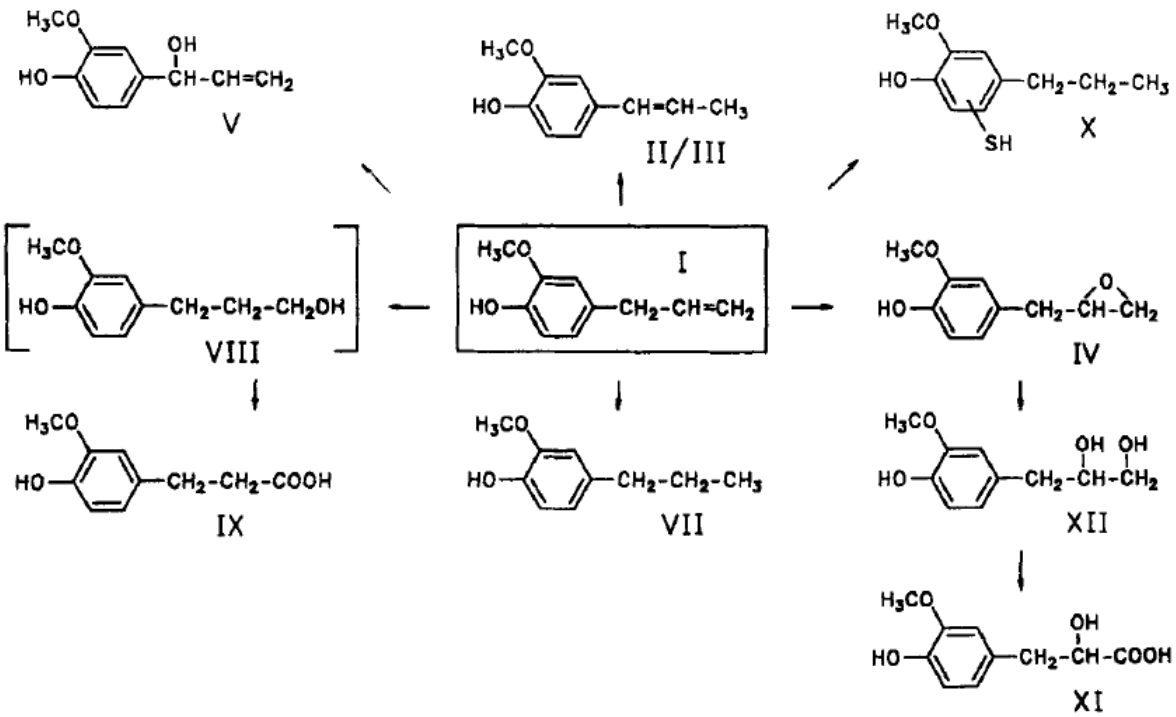


**Figure B.6.1.1-01. Proposed structures of metabolites and metabolic pathways of eugenol in man**

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.<br>EFSA (EFSA Journal 2012;10(11):2914) agreed endpoint: eugenol is rapidly and extensively absorbed, >90% is excreted in urine in 24h, and accumulation is unlikely. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This study is a scientific publication from a peer-reviewed journal. The study is pre-guideline and a compliance check with the current OECD TG 417 (2010) has identified the following deviations: no characterisation of the test item, the selection of the dose was not justified and only one dose was tested in the study, urine was only collected within a 24h period or no individual results reported (instead mean results). The latter deviation could be relevant to evaluate the recovery of conjugated eugenol, which ranged from 38 to 62%, and to evaluate sex differences if there were. Furthermore, the analytical method followed in this study (Bakke and Scheline, 1969) may be off dated and it is currently not in use in standard urinary analyses. Based on these deficiencies in the study, the RMS deems the study as supporting information to evaluate the metabolism and excretion of eugenol in humans. |

**B.6.1.1-02 Rat: Excretion of eugenol**

| | |
|---|---|
| **Data point** | CA 5.1.1 (EU data requirement) |

| | |
|---|---|
| **Report author** | Sutton, J.D., Sangster, S.A. and Caldwell, J. |
| **Report year** | 1985 |
| **Report title** | Dose-dependent variation in the disposition of eugenol in the rat |
| **Report No** | Not applicable, published study (*Biochem. Pharmacol*, Vol 34 (3), 465-466) |
| **Document No** | KCA 5.1.1/02 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 417 (2010)** | -Test material not characterised (batch and purity not reported).<br>-The selection of the dose was not justified<br>-Number of animals used in the study per dose not stated<br>-Results from urine collected 0-24h only.<br>-Very limited detail given. No individual results data were provided, for example % conjugates per dose. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information (see deviations) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | [ring-$^{14}$C] Eugenol, Purity: not specified; Lot/Batch No.: not specified |
| **Solvent** | Trioctanoin |
| **Test system** | Female Wistar albino rats |
| **Test method** | Radiolabelled eugenol dissolved in trioctanoin was administered to rats by stomach tube at dose levels of 0.5. 5, 50 and 1000 mg/kg bw (10-15 µCi/animal in a total volume of 0.8 mL/animal). Urine and faeces were collected daily for three days. Excreted $^{14}$C from urine and faeces was analysed by liquid scintillation counting. Urinary metabolites were separated by HPLC before and after treatment of the urine with β-glucuronidase or sulfatase, and characterised by GC-MS as such and as methylated or trimethylsilyl (TMS) derivates. |

**Results**

Urinary excretion of ranged between 75-80% of the total administered $^{14}$C and 10% was excreted in faeces. The rate and route of excretion was no dependent on dose level.

Conjugated eugenol (~ 50% of dose) was the major excretion product at all dose levels although the nature of the conjugate was dependent on dose level. The sulphate conjugate predominated at 0.5-50 mg/kg bw dose and the glucuronide conjugate predominated at the top dose (1000 mg/kg bw).

The metabolites identified in urine were formed by reduction of the side chain and O-demethylation, which correspond to 4-hydroxy-3-methoxypropylbenzene and 3,4-dihydroxypropyl benzene, respectively. These metabolites were excreted as sulphate or glucuronide conjugates and the relative proportion of these conjugates was equivalent to eugenol conjugates, i.e. sulphate at low doses and glucuronide at top doses. The variation in the excretion of eugenol conjugates and metabolites is displayed in Table B.6.1.1-02. No metabolites were identified in the top dose group. The author states that approximately 10% of the administered dose at each dose level remained unidentified.

**Table B.6.1.1-02. Urinary excretion of eugenol and its main metabolites following oral administration**

| **Substance** | **% Radioactivity in 0-24 hour urine of rats given doses (mg/kg bw)** | | | |
|---|---|---|---|---|
| | **0.5** | **5** | **50** | **1000** |
| **Eugenol (sulphate and glucuronide conjugates)** | 50 | 60 | 55 | 60 |
| **3,4-dihydroxypropyl benzene** | 15 | 5 | 15 | ND |
| **4-hydroxy-3-methoxypropyl benzene** | 3 | 1 | 1 | ND |

ND: not detected (< 0.5 % urinary $^{14}$C)

It was also shown that 4-hydroxy-3-methoxypropyl benzene and 3,4-dihydroxypropyl benzene could be formed by incubation of rat faecal contents with $^{14}$C-eugenol under anaerobic, but not aerobic conditions.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and was accepted as supporting information. The study is considered acceptable for the purpose of renewal.<br>EFSA (EFSA Journal 2012;10(11):2914) agreed endpoint: eugenol is rapidly and extensively absorbed, >90% is excreted in urine in 24h, and accumulation is unlikely. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This study is a scientific publication from a peer-reviewed journal. The study is pre-guideline and compliance with the current OECD TG 417 (2010) has been assessed. A few deviations from guidance have been identified: no characterisation of the test item, the selection of the dose was not justified, single sex tested, number of animals not stated, results displayed only for the urine collected 0-24 h, no individual results data provided (% glucuronide vs sulphate conjugates, for example) and overall very limited detail given in the study. Based on these method deficiencies of the study, the RMS deems the study as supporting information to evaluate the metabolism and excretion of eugenol in rats. |

**B.6.1.1-03 Rat: Disposition of eugenol *in vivo* and *in vitro*.**

| | |
|---|---|
| **Data point** | CA 5.1.1 (EU data requirement) |
| **Report author** | Delaforge, M., Janiaud, P., Levi, P. and Morizot, J.P. |
| **Report year** | 1980 |
| **Report title** | Biotransformation of allylbenzene analogues *in vivo* and *in vitro* through the epoxide-diol pathway |
| **Report No** | Not applicable, published study (*Xenobiotica*, Vol 10 (10), 737-744) |
| **Document No** | KCA 5.1.1/04 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 417 (2010)** | - No characterisation of test item<br>-Poorly described method (number of animals, dose selection, single sex study)<br>-Poorly description of results (no individual results provided, limited data reported)<br>-Qualification but not quantification of the formed metabolites |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Batch/Lot No.: not stated |
| **Vehicle** | Corn oil |
| **Test system *in vivo*** | Male Wistar rats |
| **Test system *in vitro*** | Rat liver microsomes and adult epithelial liver cells |
| **Test method** | *In vivo* study:<br>A single dose of 200 mg/kg bw of eugenol in corn oil (0.5 mL/200 g) was administered intraperitoneally to rats. Urine was collected every 2 hours during a 24-hour period. Animals were killed 24h after dose administration and the liver was removed.<br>*In vitro* study:<br>Liver microsomes were prepared from rats pre-treated i.p. with phenobarbital (80 mg/kg bw; injected once per day for 3 days). Livers were excised and homogenates prepared.<br>Adult rat liver cell cultures were prepared according to the method described by Janiaud *et al.*, 1976. About 6 x $10^6$ cells were incubated for 24h at 37ºC with 1mg of eugenol in 5 mL of HAM F10 medium supplemented with 10 % human serum and 10 % foetal calf serum. |

| | |
|---|---|
| | Analysis of all samples is performed using gas-liquid chromatography and mass spectrometry. |

**Results**

Table B.6.1.1-03 displays the formation of epoxide derivatives in various biological samples. Following administration of a single dose of eugenol to rats the epoxides were detected in urine, both catechol and allyl epoxide and also the dihydrodiols.

**Table B.6.1.1-03. Detection of metabolites in biological samples following exposure to eugenol**

| Compound | Metabolites | | | |
|---|---|---|---|---|
| | Urine | Liver homogenates from rats pre-treated with eugenol | Rat liver microsomes incubation with eugenol | Rat liver cells incubated with eugenol |
| **Eugenol epoxide** | Yes | Yes | Yes | Yes |
| **Allylcatechol epoxide** | Yes | - | Yes | - |
| **Dihydrodihydroxy eugenol** | Yes | Yes | Yes | Yes |
| **Dihydrodihydroxy allylcatechol** | Yes | - | Yes | - |

*In vitro* incubation of rat liver cells with eugenol resulted in the formation of the allyl epoxide. Hydration of the resulting eugenol-epoxide to form the 2',3'-dihydrodiol derivative was observed following incubation of eugenol with hepatic microsomes. The detection of dihydrodiols result from hydration of epoxides can be considered as indirect evidence of the epoxide-diol pathway.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is a scientific publication from a peer-reviewed journal. The study is pre-guideline and upon comparison with the current OECD TG 417 (2010) a few deviations have been identified: no characterisation of the test item, poor description of the method (number of rats, dose selection, one sex only used in the study), poor reporting of results, and poor description of results (no individual data reported). Furthermore, the study only covers the identification but not quantification of these metabolites. Based on the method deficiencies, the RMS deems the study as supporting information to evaluate the metabolism and excretion of eugenol in rats.

**B.6.1.1-04 *In vitro*: Metabolism in rat and mouse liver microsomes.**

| | |
|---|---|
| **Data point** | CA 5.1.1 (EU data requirement) |
| **Report author** | Swanson, A.B., Miller, E.C. and Miller, J.A. |
| **Report year** | 1981 |
| **Report title** | The side-chain epoxidation and hydroxylation of the hepatocarcinogens safrole and estragole and some related compounds by rat and mouse liver microsomes |
| **Report No** | Not applicable, published study (*Biochim. Biophys. Acta*, 673, 504-516) |
| **Document No** | KCA 5.1.1/03 |
| **Guidelines followed in study** | Not stated/No specific testing regulations/guidelines |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Batch/Lot No.: not stated |
| **Solvent** | Ethanol |
| **Test system** | Young adult Fischer rat liver microsomes<br>CD-1 Mice liver microsomes |
| **Test method** | *Microsomes preparation*:<br>Animals were fasted for 12-15h before they were killed. Livers were extracted, finely minced and 20% homogenates (w/v) were prepared in 0.25 M sucrose buffered with 5 mM Tris-HCl (pH 8.0 at 5ºC). The supernatant fraction obtained by centrifugation of the homogenate at 13000 x *g* for 10min was further centrifuged at 40000 x *g* for 40min. The microsome-containing pellet was resuspended in 0.25 M sucrose buffered with tris-Hcl and the suspension was centrifuged at 43000 x *g* for 60 min. Microsomes in the pellet were isolated and resuspended in Tris-buffered sucrose.<br>*Incubation conditions*:<br>The reactions were carried out in a 5 mL volume that contained 2.0 mM eugenol (added in 0.1 mL of ethanol)/5.0 mM $MgCl_2$/1.0 mM EDTA/0.5 mM NADP/5.0 mM glucose-6-phosphate/10 u of glucose-6-phosphate dehydrogenase/100 mM bis-(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane buffer (pH 7.4) and a freshly prepared microsome suspension containing 7-12 mg proteins. Trichloro-propylene oxide was added at a final concentration of 4.0 mM.<br>Each run included control incubations that consisted of the same reaction mixture without eugenol. The reaction time was 20 min. Upon stopping the reaction, the metabolites were extracted with diethyl ether and analysed by HPLC (reverse phase). Qualification and quantification of the metabolites was also performed using reference standards. |

**Results**

Under the conditions of this study, the 2',3'-epoxidation of eugenol was just detectable (< 1nmol/mg protein/h). Table B.6.1.1-04 displays the amount per species recovered of the 2'3-oxide metabolite.

**Table B.6.1.1-04. Formation of epoxy-eugenol derivative following incubation of eugenol with rat and mouse liver microsomes fortified with an NADPH-generating system**

| Substrate | Metabolite (product) | Yield (nmol product formed per mg protein per h) | | | |
|---|---|---|---|---|---|
| | | Mouse | | Rat | |
| | | Male | Female | Male | Female |
| Eugenol | Eugenol-2',3'-oxide | 0.7 ± 0.3 (7) | ≤ 1.3* (4) | ≤ 1.9* (4) | 0.4 ± 0.1 (4) |
| Values are expressed as mean ± standard deviation. Numbers in parenthesis are the numbers of pools of mouse liver (5-18 livers per pool) or the numbers of individual rat livers analysed.<br>*Limit of detection for the experiment | | | | | |

The 1'-hydroxylation of eugenol was not investigated due to unavailable reference compounds.

Eugenol-2',3'-oxide was also very susceptible to hydrolysis by microsomal epoxide hydrase. For example, on addition of this oxide to female mouse liver microsomes only about 7% was recovered when no trichloropropylene oxide was included in the incubation mixture, while about 70 % was recovered on addition of 4mM trichloropropylene oxide (inhibitor of epoxide hydrase).

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with the notifier assessment and conclusion and considers the study relevant to evaluate the metabolism *in vitro* of eugenol with liver microsomes.

This study is a scientific publication from a peer-reviewed journal. There is no guideline available for this type of *in vitro* study. Under the conditions of the study, eugenol is a poor substrate for epoxidation when incubated with rat and mouse liver microsomes.

**B.6.1.1-05 *In vitro*: Rat liver and lung microsomes.**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Thompson, D., Constantin-Teodosiu, D., Egestad, B., Mickos, H. and Moldeus, P. |
| **Report year** | 1990 |
| **Report title** | Formation of glutathione conjugates during oxidation of eugenol by microsomal fractions of rat liver and lung |
| **Report No** | Not applicable, published study (*Biochem. Pharmacol.* Vol 39 (10), 1587-1595) |
| **Document No** | KCA 5.1.1/05 |
| **Guidelines followed in study** | Not stated/No specific testing regulations/guidelines |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol); Lot/Batch No. or Purity not stated<br>[$^3$H]Eugenol; Lot/Batch No. or Purity not stated |
| **Test system** | Male rat (Sprague-Dawley) liver and lung microsomes |
| **Test method** | This study investigates the metabolism of eugenol by rat liver and lung microsomes to form reactive intermediates that can be trapped with glutathione.<br>Synthesis of eugenol-glutathione conjugates:<br>The eugenol-glutathione conjugates were synthesised *via* the reaction of eugenol quinone methide with glutathione. Eugenol (1.5 mmol; 0.25 g) was added to 40 mL carbon tetrachloride. Silver (I) oxide (6.5 mmol 1.5 g) was added and the mixture was stirred vigorously at 65°C for 10 min. The reaction was filtered and allowed to cool. Glutathione (1.5 mmol; 0.46 g in 40 mL of 0.1M phosphate, pH 8) was added to the filtrate (quinone methide) and the mixture was stirred at 37°C for 3 h. Water (50 mL) was added and the mixture decanted into a separation funnel. The aqueous layer was washed twice with 50 mL carbon tetrachloride. The residue was dissolved in 1-2 mL water and the eugenol-glutathione conjugates were collected and purified by HPLC, first with the semi-preparative column, and second with repeated chromatography on an analytical column. The samples were then analysed by mass spectrometry and NMR.<br>Preparation of microsomes:<br>Microsomes were prepared from male SD rats. Animals were pre-treated i.p. with corn oil (control, 3 mL of corn oil injected once), phenobarbital (80 mg/kg; 0.4 mL if 40 mg/mL solution in saline, injected once per day for 3 days) or 3-methylcholanthrene (50 mg/kg; 2 mL of 5 mg/mL solution in corn oil, injected once). Homogenates (25%) of liver and lung tissues were made in 0.25 M sucrose and centrifuged at 10.000 g for 20 min.<br>Incubation conditions:<br>The standard incubation conditions consisted of 1 mg/mL microsomal protein, 1 mM eugenol, 5 mM glutathione and a NADPH-regenerating system consisting of 5 mM isocitrate. 10 µL (0.2 units) isocitrate dehydrogenase, and 1 mM NADP in a total |

| | |
|---|---|
| | volume of 1 mL of 0.1 M Tris buffer, pH 7.5. The reaction mixture was pre-incubated at 37°C for 5 min prior to the addition of eugenol to start the reaction. Reactions were stopped by the addition of 50 µL of 100 % trichloroacetic acid and centrifuged for 5 min at 12000 rpm to pellet the protein. Samples (200 µL) were then analysed for conjugate formation by HPLC.<br>The same procedure was followed to identify the covalent binding of reactive intermediates from [$^3$H]eugenol (0.5 µCi, equivalent to 1mM eugenol) to microsomal (liver and lung) proteins. |

**Results**

Rat hepatic and pulmonary microsomes catalysed the formation of at least three distinct glutathione conjugates with eugenol identified as peaks a, b and c (Figure B.6.1.1-05/1). These three conjugates were identical to the products obtained from the chemical reaction of synthetic eugenol quinone-methide and glutathione, *i.e*. eugenol-glutathione conjugates. The replacement of NADPH-regenerating system with cumene hydroperoxide generated an extra metabolite (peak e) although the structure of this compound remains unidentified.

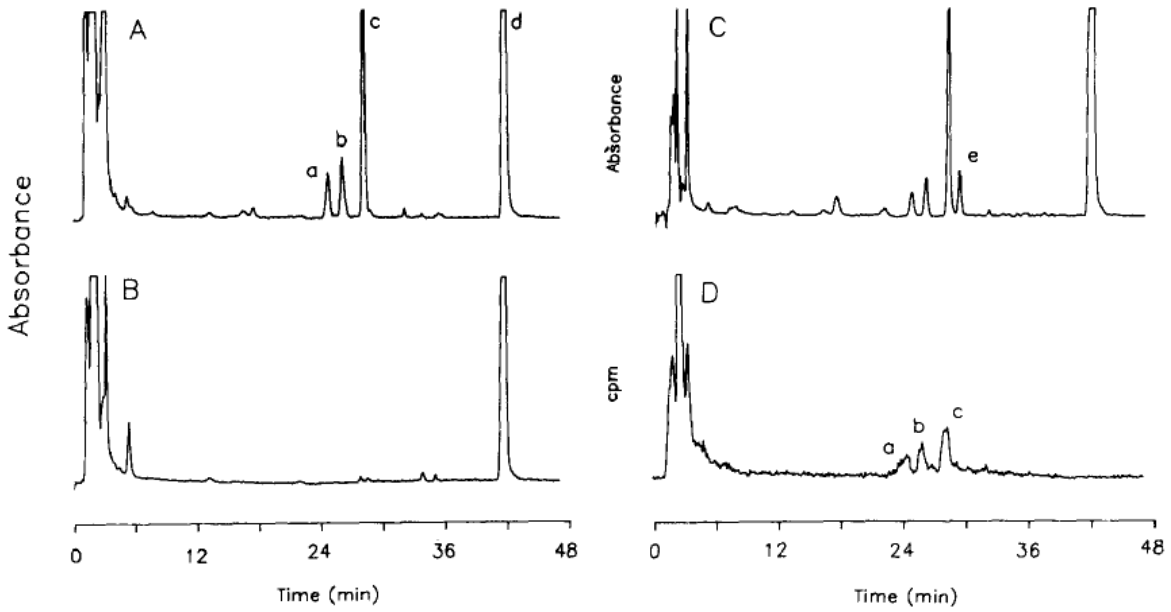


**Figure B.6.1.1-05/1. HPLC analysis of products formed during microsomal incubation with eugenol. Panel A: complete system; Panel B: complete system except glutathione; Panel C: cumene hydroperoxide instead of NADPH-regenerating system; Panel D: complete system with [$^3$H]glutathione.**

The chemical structures of peaks a and c were elucidated using NMR spectroscopy (Figure B.6.1.1-05/2). These compounds are the result of nucleophilic reaction at the benzylic carbon (peak a) and the allylic terminal carbon (peak c).

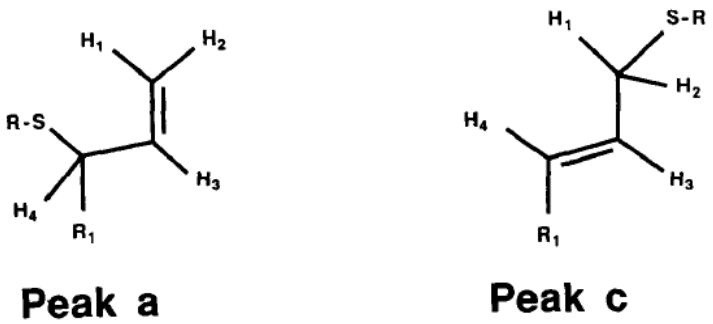


**Figure B.6.1.1-05/2. Proposed structures of two eugenol-glutathione conjugates (peaks a and c), where R1 corresponds to the phenyl moiety of eugenol**

The microsomal reaction was dependent on NADPH and oxygen and it was inhibited by cytochrome P450 inhibitors such as metyrapone, 2-diethylaminoethyl-2,2'diphenylvalerate (SKF 525-A), ∝-naphthoflavone and piperonyl butoxide.

The enzyme responsible for eugenol oxidation was inducible with 3-methylcholanthrene but not with phenobarbital pre-treatment. The rate of formation of conjugates was not affected by the presence of glutathione-depleted cytosol, which contained active glutathione transferase, even at low glutathione concentrations, suggesting that conjugation occurs non-enzymatically with an electrophilic metabolite of eugenol. Covalent binding to microsomal protein was observed using [$^3$H]eugenol in the absence of glutathione (Table B.6.1.1-05), which suggest the reactive intermediate reacts with sulphidryl groups of proteins.

**Table B.6.1.1-05. Covalent binding of [$^3$H]eugenol to rat liver and lung microsomal protein**

| Tissue | Eugenol bound (pmol/min/mg) | |
|---|---|---|
| | - GSH | + GSH |
| Liver | 196 ± 10 | 1 ± 0 |
| Lung | 78 ± 10 | 9 ± 0.3 |

Cumene hydroperoxide catalysed the formation of these same glutathione conjugates *via* the formation of a quinone methide-like intermediate, which was detected by spectroscopic means.

**Conclusion**

The results suggest that eugenol is oxidised by cytochrome P450 to a reactive quinone methide intermediate which can then covalently modify protein or conjugate with glutathione.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This study is a scientific publication from a peer-reviewed journal. It investigates the formation *in vitro* of glutathione conjugates of eugenol in rat liver and lung microsomes. The outcome of the study concludes the reaction mechanism involves the formation of a highly reactive quinone methide intermediate that is susceptible to react with proteins/glutathione. The RMS deems the study as acceptable to evaluate the glutathione conjugation *in vitro* of eugenol. |

**B.6.1.1-06 *In vitro*: Metabolism and covalent binding in rat hepatocytes.**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Thompson, D.C., Constantin-Teodosiu, D. and Moldeus, P. |

| | |
|---|---|
| **Report year** | 1991 |
| **Report title** | Metabolism and cytotoxicity of eugenol in isolated rat hepatocytes |
| **Report No** | Not applicable, published study (*Chem. Biol. Interactions*, Vol 77, 137-147) |
| **Document No** | KCA 5.1.1/06 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (reference item); Lot/Batch No: Not stated, Purity: not stated<br>[$^3$H]-Eugenol (labelled on methoxy group); Radiopurity/specific activity: 3.4 mCi/mmol; Lot/Batch No.: not stated |
| **Test systems** | Male Sprague-Dawley rat hepatocytes |
| **Test method** | Preparation and incubation of hepatocytes:<br>Male Sprague Dawley rats were used for these experiments. Hepatocytes were isolated by collagenase perfusion of the liver. Cells prepared for use in these experiments were 85-90% viable. Cells were incubated at a concentration of 1 x $10^6$ cells/mL in rotating round bottom flasks at 37°C in Krebs-Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes.<br>Eugenol was dissolved in DMSO before addition to the incubations in a volume not exceeding 1% (v/v). In some experiments, cells were pre-treated with 0.5 mM diethylmaleate for 30 min to deplete intracellular glutathione. The cells were washed and re-suspended in buffer before use in subsequent experiments.<br>Glutathione measurement:<br>Glutathione was measured by HPLC using the thiol-specific reagent monobromobimane. Aliquots (0.1 mL) were taken from hepatocyte incubations at various time points and mixed immediately with an equal volume of 8 mM monobromobimane. The reaction was carried out in the dark for 5 min after which 10 µL of 100 % trichlororacetic acid was added. These samples were assayed directly for glutathione by HPLC.<br>Covalent binding:<br>Covalent binding of eugenol metabolites was measured in hepatocyte incubations containing [$^3$H]-eugenol. The protein pellets were subsequently washed 2 x with 5 % trichloroacetic acid and 8 x with methanol to remove all non-covalently bound radioactivity. The final pellet was dissolved in 1 mL of 1 M NaOH with heating at 60°C for 1 h. An aliquot (0.25 mL) was neutralised with HCl and counted in a liquid scintillation counter. Another aliquot (50 µL) was used to measure protein.<br>HPLC Analysis of eugenol metabolites:<br>Aliquots (1.0 mL) of hepatocyte incubations were removed at various time points and the reactions terminated by the addition of 50 µL of 100 % trichloroacetic acid. |

**Results**

*In vitro* metabolism:

Incubation of hepatocytes with eugenol resulted in the identification of three major metabolites: sulphate, glucuronic acid and glutathione conjugates. The major metabolite formed at a concentration of 1mM eugenol was the glucuronic acid conjugate whereas the sulphate and glutathione conjugates were formed in much lower amounts (Figure B.6.1.1-06). Approximately 200 nmol of eugenol-glucuronide was formed after 5h incubations, compared with 25nmol of both glutathione and sulphate conjugates.

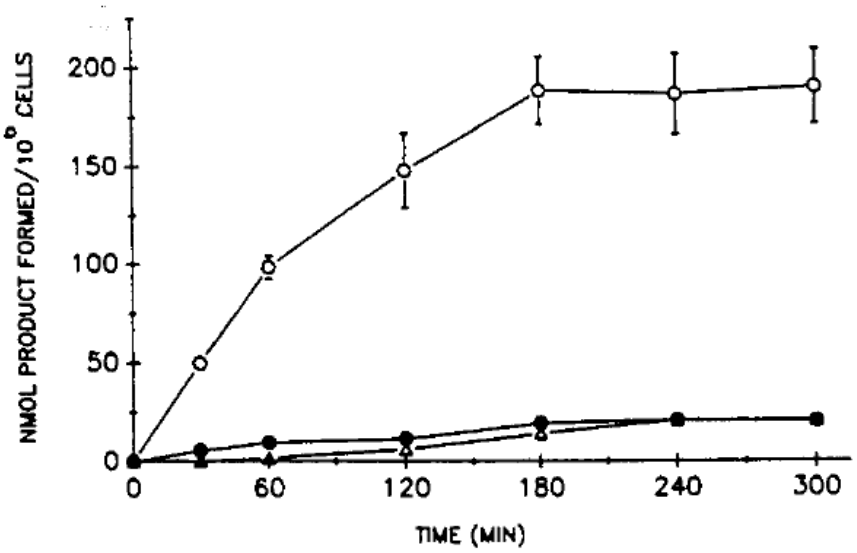


**Figure B.6.1.1-06. Metabolism of eugenol (1mM) in isolated rat hepatocytes up to 5h [○ Eugenol-glucuronide; ●Eugenol-Sulphate; ▲ Eugenol-glutathione].**

Cytotoxicity:

Concentrations of 1 mM eugenol caused a loss of over 90% of intracellular glutathione and resulted in approximately 85 % cell death over a 5-h incubation period compared with 35% cell death in control incubations. The loss of the majority of glutathione occurred prior to the onset of cell death (2 h) and was less than 30% of control values after 2h incubation.

The occurrence of cell death with 1mM eugenol was prevented in the presence of 1mM of *N*-acteylcysteine. Furthermore, pre-treatment with diethylmaleate resulted in an increase in cytotoxicity effects of eugenol (1mM), which was observed approximately 2h earlier than in control cells.

Covalent binding:

Covalent binding to cellular protein was observed using [$^3$H]-eugenol. Loss of intracellular glutathione and cell death were also observed in these incubations. The effects of eugenol were concentration-dependent.

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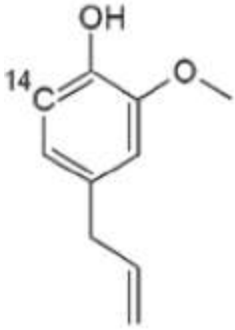
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** The RMS deems the study as acceptable to evaluate the metabolism *in vitro* of eugenol in rat hepatocytes. Based on the results of the study, three major metabolites were identified in rat hepatocytes at 1mM eugenol concentration for 5h. Based on the results of the study, the eugenol-glucuronide conjugate was the major metabolite whereas the sulphate and glutathione conjugates were formed in much less concentration. |

**B.6.1.1-07 *In vitro*: Comparative metabolism using liver and lung microsomes from human, mouse and rat.**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Minet, E.F., Gentile, D., Clive, M. and Massey, E.D. |
| **Report year** | 2012 |
| **Report title** | A comparative *in vitro* kinetic study of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol activation and detoxification in human, mouse and rat liver and lung fractions. |
| **Report No** | Not applicable, published study (*Xenobiotica*, Vol 42 (5), 429-441) |

| | |
|---|---|
| **Document No** | KCA 5.1.1/07 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study (*in vitro* comparative metabolism study) |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Labelled Eugenol (test item)<br>[$^{14}$C]Eugenol; Lot/Batch No.: not stated, Radiopurity/specific activity: not stated<br>  <br><br>Eugenol (reference item); Lot/Batch No: Not stated, Purity: not stated |
| **Test systems** | Microsomes and S9 fractions from human, rat and mouse purchased from commercial suppliers.<br>**Human**:<br>Liver microsomes: 1 ♂, 2 ♀, batch SHH/JVT<br>Liver S9 : 2 ♂, 1 ♀, batch FRA259048<br>Lung microsomes : : 5 ♂, 5 ♀, batch APT<br>Lung S9 : 2 ♂, batch FRA50406<br>**Mouse**:<br>Liver microsomes : 150 ♂, batch ZLR<br>Liver S9 : 100 + ♂, batch VLH<br>Lung microsomes : 6 ♂, batch CMM<br>Lung S9 : 6 ♂, batch CMS<br>**Rat** :<br>Liver microsomes: 1 ♂, batch MIC254015<br>Liver S9 :15 ♂, batch FRA254002<br>Lung microsomes : 10 ♂, MIC501005<br>Lung S9 : 12 ♂, batch FRA501007 |
| **Test method** | Incubations with [$^{14}$C]-eugenol (20 µM, 1.8 µCi) were performed in triplicate in microfuge tubes at 37ºC with hepatic microsomes (1 mg/mL) or S9 fractions (1 mg/mL), reaction buffer (50 mM potassium phosphate, pH 7.4) and relevant cofactors: NADPH (0.6 U isocitrate-dehydrogenase), 250 µM $NADP^+$, 2.5 mM DL isocitric acid and 5 mM $MgCl_2$, UDPGA (2 mM), GSH (500 µM). The incubation time was set to 180 min.<br>Covalent binding to protein:<br>Covalent binding was investigated as an indirect method to measure the formation of the quinone methide intermediate. Control experiments with glutathione (GSH) were performed to assess the protein binding. Samples were stored at -20ºC overnight to aid protein precipitation and centrifuged at 10000 g for 10min. Extraction of the protein pellets was done with one acetonitrile:methanol (2:1 v/v) and two with 70 % methanol washes followed by sonication and centrifugation. The supernatant was combined and analysed by LSC.<br>Glucuronidase treatment: |

| | |
|---|---|
| | Supernatants from UDPGA incubations were submitted for glucuronidation treatment. Deconjugation reactions were performed overnight at 37ºC with β-glucuronidase (*Helix pomatia*, 10.4 µL of 10 mg/mL).<br>Metabolite identification & quantification:<br>Metabolites were identified using HPLC analyses of post-incubate samples with a radio UV-HPLC. The identity of the 1'-hydroxymetabolite was confirmed by chromatography with synthetic 1'-hydroxyeugenol. The identities of other metabolites in the profile were not investigated for the purpose of this study, which focused on the 1'-hydroxy and quinone methide metabolites.<br>Quantification of peak metabolites was done by integration as a % of the total soluble radioactivity. The total soluble radioactivity (supernatant) is a % of the total dose recovered, which is a percentage of the total dose recovered.<br>Kinetics:<br>Km and Vmax were established using the incubation conditions described above. Incubation time was 10 min for human and mouse and 30 min for rat. The following substrate concentrations were tested: 5, 7.5, 10, 12.5, 15, 20, 25, 30, 40, 50, 75, 100, 125, 150, 200 and 250 µM. Km and Vmax were calculated by non-linear regression with Prism V.4. |

**Results**

The chromatographic profile of [$^{14}$C]-eugenol in human hepatic microsomes and S9 fractions is displayed in Figure B.6.1.1-07/1. The formation of 1'-hydroxy and phenoxy-glucuronide conjugates were identified, whereas the identities of other soluble metabolites were not investigated. These metabolites were identified in all three species following incubation with hepatic microsomes.

Covalent binding of [$^{14}$C]-eugenol to liver microsomal proteins after incubations with NADPH was significantly reduced in the presence of glutathione (Figure B.6.1.1-07/2).

The kinetics and quantification of 1'-hydroxy, covalent binding and glucuronidated (phase II) metabolites across the three species in both liver microsomes and S9 fractions are displayed in Table B.6.1.1-07. The percentage of 1'-hydroxy metabolite formation was highest in the mouse, 22.0 % versus 16.4 % in human or 7.1 % in rat liver microsomes. Eugenol *in vitro* clearance through the formation of this metabolite appeared similar in human and mouse and it was three times lower in rats. Covalent binding, that is the formation of the quinone methide metabolite intermediate, was relatively equal amongst the three species, being the mouse the highest percentage of formation. With regards to phase II metabolites, [$^{14}$C]-eugenol was extensively glucuronidated in human and mouse and to a lesser extent in rat both in the presence and absence of NADPH.

The formation of the 1'-hydroxy and covalent binding of [$^{14}$C]-eugenol is limited in the lung compared to liver, whereby oxidation is approximately 20 % to 30 % to what is observed in the liver. Little [$^{14}$C]-eugenol phenoxy glucuronidation activity was detected in lung S9 fractions supplemented with UDPGA only or with UDPGA and NADPH in human and mouse but not in rat, in which significant glucuronidation was observed in rat lung S9 fractions.

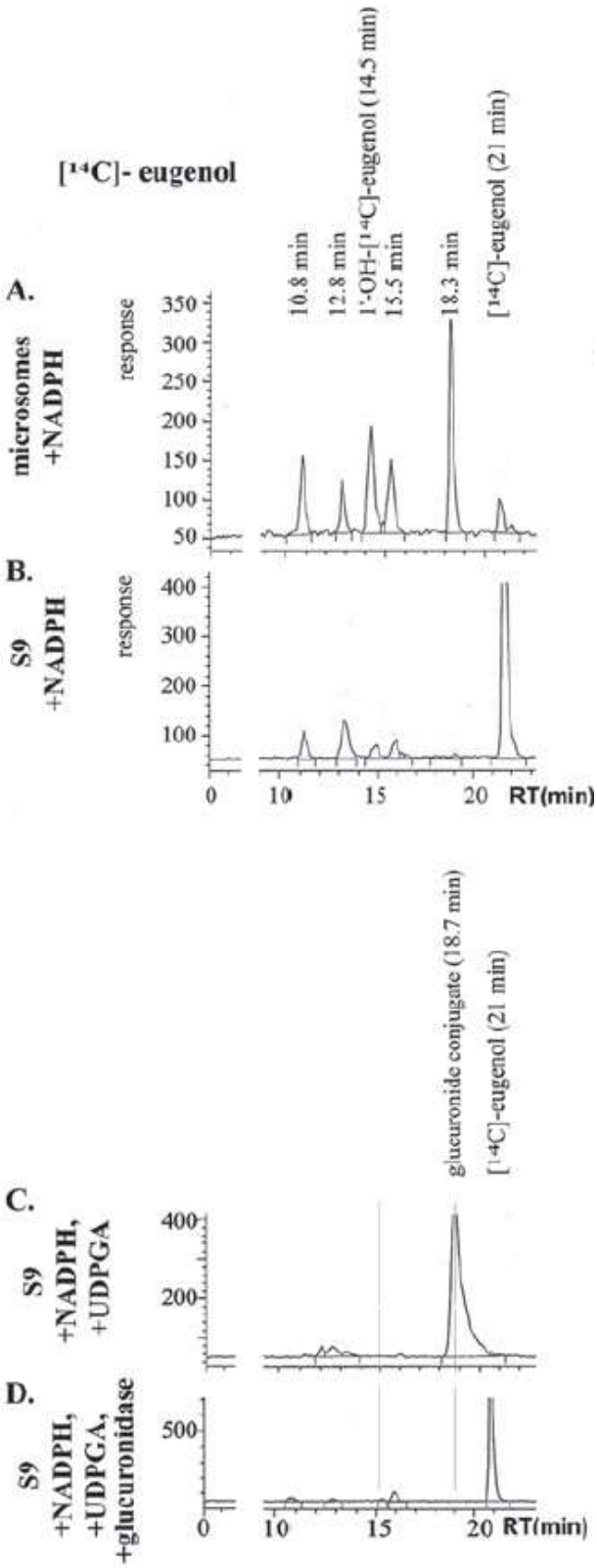



**Figure B.6.1.1-7/1. Chromatograms of metabolites of [$^{14}$C]-eugenol in microsomes with NADPH (A), in S9 fractions supplemented with NADPH (B), in S9 supplemented with NADPH and UDPGA (C) and in S9 fractions supplemented with NADPH and UDPGA.**

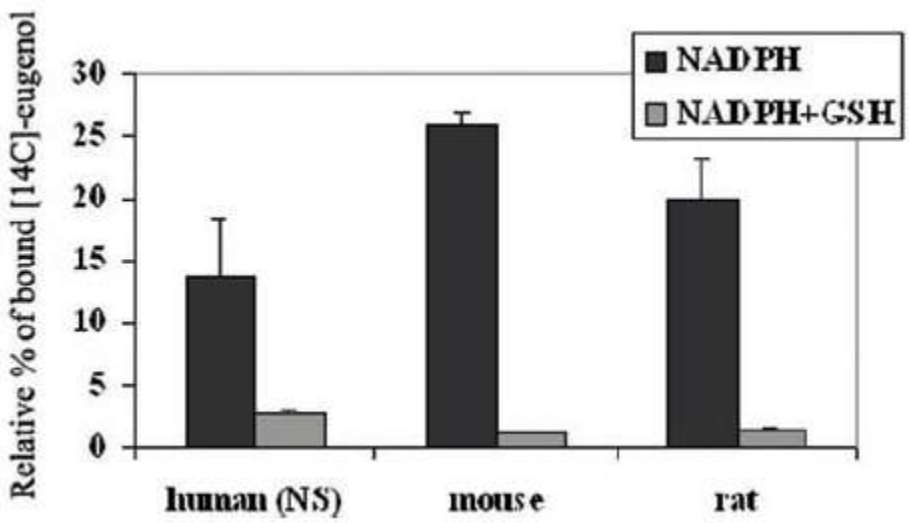



**Figure B.6.1.1-07/2. Covalent binding of [$^{14}$C]-eugenol to microsomal proteins after incubation in the presence of NADPH ± GSH.**

**Table B.6.1.1-07. Relative percentage and kinetic values ($K_m$, $V_{max}$, CLint) for 1'-hydroxylation (A), covalent binding (CB) (B) and phenolic glucuronidation (C,D) of [14C]-eugenol in human, mouse and rat hepatic microsomes + NADPH, and S9 fractions ± NADPH + UDPGA.**

| **A** | | **Microsomes + NADPH** | | |
|---|---|---|---|---|
| Species | 1'-hydroxylation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 16.4 % ± 0.4 | 109.9 | 0.58 | 11.4 |
| Mouse | 22.0 % ± 0.8 | 33.2 | 0.45 | 13.6 |
| Rat | 7.1 % ± 0.5 | 18.5 | 0.07 | 3.8 |
| **B** | | **Microsomes + NADPH** | | |
| Species | CB | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 13.8 % ± 4.5 | 165.7 | 1.7 | 10.3 |
| Mouse | 25.9 % ± 1.0 | 233.0 | 2 | 8.6 |
| Rat | 20.0 % ± 3.3 | 168.7 | 0.7 | 4.1 |
| **C** | | **S9 + UDPGA** | | |
| Species | Glucuronidation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 84.3 % ± 1.3 | 37.2 | 3.3 | 88.7 |
| Mouse | 93.7 % ± 2.6 | 96.3 | 9.9 | 106.1 |
| Rat | 55.5 % ± 4.5 | 115.0 | 3.7 | 32.2 |
| **D** | | **S9 + NADPH + UDPGA** | | |
| Species | Glucuronidation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 78.1 % ± 1.1 | 45.3 | 5.7 | 125.8 |
| Mouse | 91.9 % ± 0.4 | 127.6 | 15.7 | 123.0 |
| Rat | 47.2 % ± 1.9 | 128.9 | 5 | 38.8 |

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable with restrictions).

In liver, methyleugenol generates a significant amount of the 1-hydroxy proximate carcinogen while eugenol glucuronidation prevents the formation of both 1-hydroxyeugenol and the quinone methide.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS considers the study as relevant to address a top-level comparison of metabolism of eugenol in various species. Two metabolic routes (phase I - covalent binding and 1'-hydroxylation and phase II -glucuronidation) are investigated in both liver and lung. Based on the results of the study, eugenol is comparably metabolised in mouse and human whereas slight differences were observed in rats such as the extent of glucuronidation (47-55 % in rats against 78-84 % in humans) and formation of 1'-hydroxy-eugenol (7.1 % in rats against 16.4 % in humans). Covalent binding was detected in each of the species, implying the formation of the quinone methide reactive intermediate, which was depleted by the addition of glutathione. Overall, the RMS considers that, under the test conditions of the study, the metabolism *in vitro* of eugenol is comparable in mouse and humans whereas rat metabolism appears to differ in glucuronidation rate.

**B.6.1.1-08 Pharmacokinetic study in rats**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Guénette, S.A., Ross, A., Marier, J.F., Beaudry, F. and Vachon, P. |
| **Report year** | 2007 |
| **Report title** | Pharmacokinetics of eugenol and its effects on thermal hypersensitivity in rats |
| **Report No** | Not applicable, published study (*Eur. J. Pharmacol.*, 562, 60-67) |
| **Document No** | KCA 5.1.1/08 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 417 (2010) |
| **Deviations from current test guideline OECD TG 417 (2010)** | -Non-radiolabelled material used<br>-Limited detail on experimental method and results |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No: Not stated, Purity: not stated |
| **Test systems** | Male Sprague-Dawley rats |
| **Route** | Oral gavage |
| **Number of animals** | 6 animals per experiment |
| **Test method** | Pharmacokinetic study:<br>A group of 6 rats each was used to assess the plasma and red blood cells pharmacokinetics, respectively, following the administration of a single oral dose of eugenol (40 mg/kg bw). Blood samples were collected from the jugular vein at predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post-dose. Plasma was collected following blood centrifugation for 10 min at 3200 x *g*. Hematocrit was fixed to a nominal value of 0.45 based on results found in the literature for this species.<br>Analysis of the content of eugenol in plasma and blood was done using LC/MS/MS according to methods using dansyl chloride as a derivatising agent.<br>Pharmacokinetic parameters:<br>The following pharmacokinetic parameters were calculated in intact animals: area under the curve from time zero to the last detectable concentration ($AUC_{0\text{-}t}$), maximum observed concentration ($C_{max}$), time of maximum concentration ($T_{max}$), terminal elimination half-life ($T_{1/2}$) and apparent oral clearance (CL/F, calculated as Dose/$AUC_{0\text{-}inf}$). All pharmacokinetic parameters were calculated using Kinetica® version 4.1.1 and statistical analysis were performed using SPSS Inc. |

**Results**

The mean concentration profiles of eugenol in plasma and red blood cells are displayed in Figure B.6.1.1-08. The mean plasma concentration profile of eugenol displayed double peaks at 0.25 and 4 hours after dosing and then declined very slowly over the 24 h kinetic study. The sudden increase in plasma concentration may be associated with enterohepatic recirculation of eugenol.

Eugenol profile in blood included an initial rapid decline followed by a steady decrease afterwards.

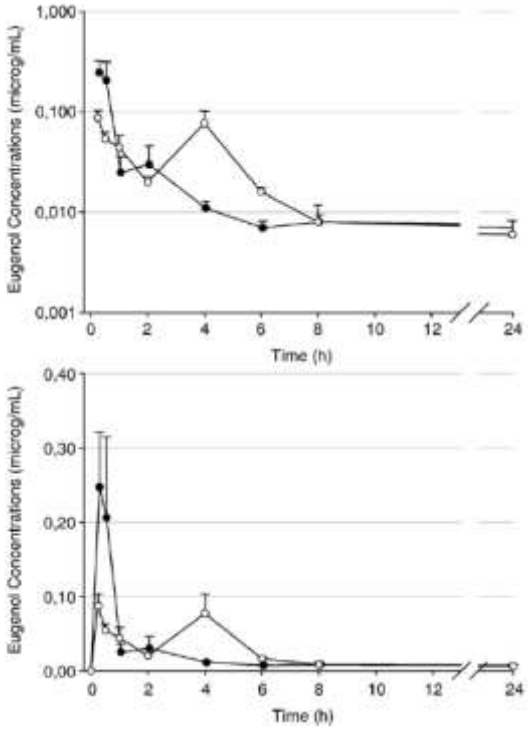



**Figure B.6.1.1-08: Mean (± S.E.M) concentrations of eugenol in plasma (○, n = 6) and blood (●, n = 6) following oral administration of eugenol on semi-log scale (top graph) and linear scale (bottom graph)**

Mean pharmacokinetic parameters of eugenol in plasma and blood are displayed in Table B.6.1.1-08.

**Table B.6.1.1-08. Mean pharmacokinetic parameters of eugenol in plasma and blood following oral administration of eugenol (40 mg/kg bw) in male rats**

| PK parameters | Plasma | Blood |
|---|---|---|
| $AUC_{0\text{-}t}$ (µg h/mL) | 0.384 (42.4 %) | 0.342 (31.0 %) |
| $AUC_{0\text{-}inf}$ (µg h/mL) | 0.577 (51.0 %) | 0.518 (38.8 %) |
| $C_{max}$ (µg/mL) | 0.123 (25.7 %) | 0.270 (80.5 %) |
| $T_{max}$ (h)* | 2.13 (0.25-4.00) | 0.25 (0.25-0.50) |
| $T_{1/2}$ (h) | 14.0 (58.3 %) | 18.3 (37.3 %) |
| CL/F (L/h/kg) | 86.7 (50.0 %) | 86.8 (34.9 %) |

Mean (%CV) values, n = 6 blood and n = 6 plasma
*Median (Minimum-Maximum)

**Assessment and conclusion by applicant:**

This new study is considered acceptable as supporting information for the renewal of eugenol; the results are broadly consistent with available data showing extensive excretion within 24 h (reliable with restrictions; only one dose level tested, only measured blood, not conducted to GLP).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS deems the study as supporting information to assess the blood kinetics of eugenol in the rat. Based on the results of the study, plasma levels of eugenol peaks at 0.5 h and 4h, which suggests entero-hepatic recirculation.

### B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

No studies of eugenol by other routes have been submitted.

There is no concern for toxicity following dermal exposure to eugenol as acute dermal toxicity is expected low compared to acute toxicity following oral exposure. Studies by dermal route are not required.

As for the inhalation route, eugenol has a vapour pressure of 12.2 Pa measured at 25ºC and it was considered volatile in the original DAR (2011). No studies are required using this route of exposure as based on the proposed uses (application is post-harvest indoor drenching or dipping application) it is not regarded as a relevant route of exposure.

In summary, studies by the dermal or inhalation routes are not required.

## B.6.2. ACUTE TOXICITY

*Acute toxicity studies presented in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC are re-assessed according to current test guidelines and requirements.*

*No new data have been submitted for the renewal process with the exception of one skin sensitisation in vitro study. Furthermore, the RMS has summarised and reviewed a mouse IgE study (B.6.2.6.3) to cover the respiratory endpoint. Eugenol does not fulfil the criteria laid in Regulation (EC) 283/2013 for phototoxicity testing and therefore, no phototoxicity study is available.*

### B.6.2.1. Oral

#### B.6.2.1.1 Acute oral toxicity study in rats, mice and guinea pigs

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. and Fitzhugh, O.G. |
| **Report year** | 1964 |
| **Report title** | Food Flavourings and Compounds of Related Structure I. Acute Oral Toxicity |
| **Report No** | Not applicable, published study (*Fd Cosmet. Toxicol*., **2**, 327-343) |
| **Document No** | KCA 5.2.1/01 |
| **Guidelines followed in study** | This study is pre-guideline. |
| **Deviations from current test guideline OECD TG 420 (2001)** | Test material not characterised (batch and purity not reported). Very limited information provided in the study (method, dosing and results poorly described; no necropsy). Mice were not fasted prior to treatment. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP) |
| **Acceptability/Reliability** | Supportive only (methodological deficits) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol); Lot/Batch No.: not specified, Purity: not specified |
| **Vehicle** | - |
| **Test system** | Male and female young adult Osborne-Mendel rats<br>Male and female guinea pigs<br>Mice (strain not indicated) |
| **Test method** | Eugenol (undiluted) was administered orally by intubation to groups of 10 young adult rats evenly divided by sex, guinea pigs (male and female) and mice. Rats and guinea pigs were fasted 18 h prior to treatment. Mice were treated on full stomachs. Animals were observed usually for 2 weeks during which time the development of toxic signs was followed and time of death recorded. Such observation was continued until animals appeared normal and showed weight gain. In a few cases, where non-acute toxic signs were seen, the animals were observed for only one week.<br>The $LD_{50}$ was calculated according to Litchfield & Wilcoxon. |

**Results**

The $LD_{50}$ slope function and their confidence limits, together with toxic signs and time of death are recorded for eugenol in Table B.6.2.1.1.

**Table B.6.2.1.1. Results of acute oral studies with eugenol**

| Compound | Species | $LD_{50}$ with 95% confidence limits (mg/kg) | Slope function with 95% confidence limits | Toxic signs and time of death |
|---|---|---|---|---|
| **Eugenol** | Rat | 2680<br>(2420-2970) | 1.2<br>(1.1-1.4) | Coma soon after treatment.<br>Time of death approx. 1h |
| | Mouse | 3000<br>(2400-3750) | 1.8<br>(1.3-2.3) | Severe depression immediately after treatment.<br>Time of death: few min – 2 days |
| | Guinea pig | 2130<br>(1860-2430) | 1.3<br>(1.2-1.5) | Depression.<br>Time of death: 4 h – 2 days. |

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The oral $LD_{50}$ identified in this study was 2680 mg/kg bw in the rat; the EU-agreed acute oral $LD_{50}$ in rats is 1930 mg/kg bw (EFSA Journal 2012;10(11):2914). |
| **Assessment and conclusion by RMS:**<br>This study was carried out prior to OECD Test Guidelines on acute oral toxicity, and several relevant differences with internationally accepted guidelines have been detected (see deviations point). Therefore, RMS deems this study as supportive, but not acceptable for classification purposes. |

**B.6.2.1.2. Acute oral toxicity study in rats**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Sober, H.A., Hollander, F. and Sober, E.K. |
| **Report year** | 1950 |
| **Report title** | Toxicity of Eugenol: Determination of $LD_{50}$ on Rats |
| **Report No** | Not applicable, published study (*Proc. Soc. Exp. Biol. Med.*, **73** (1), 148-151) |
| **Document No** | KCA 5.2.1/02 |
| **Guidelines followed in study** | This study is pre-guideline. |
| **Deviations from current test guideline OECD TG 420 (2001)** | Test material not characterised (batch and purity not reported). |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP) |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol); Lot/Batch No.: not specified, Purity: not specified |
| **Vehicle** | - |
| **Test system** | Male and female Albino rats |
| **Test method** | A preliminary dose-range study was carried out with several groups of 2 rats each. Based on the outcome of these observations, single doses of eugenol (undiluted) were administered orally *via* intra-gastric instillation to groups of 12 animals each at doses of 1.5, 1.6, 1.75, 1.9, 2.0, 2.1 and 2.2 mL/kg, equivalent* to 1597.5, 1704, 1863.75, 2023.5, 2130, 2236.5 and 2343 mg/kg bw. The animals were fasted 24 h prior to treatment and for about 4 h after dosage. A total of 91 animals were included in the final dosage-mortality analysis. All animals were observed closely throughout the day of administration of eugenol and were checked frequently for at least 10 days thereafter. Animals were autopsied as soon after death as possible and tissues from representative animals were taken for microscopic examination. These rats, in which *post-mortem* changes were considerable, because of death during the night, were discarded.<br>The $LD_{50}$ was calculated according to Litchfield & Wilcoxon. |

*eugenol has a relative density of 1.065 g/mL which has been used to calculate the dose equivalents

**Results**

**Mortality:** All but two of the deaths occurred during the first two days after treatment. Time of death varied from 1 to 48 hours, 41 % of all the deaths occurred within the first 6 hours, 33% in the following 18 hours and 22% during the second day after administration of eugenol. Mortality is summarised in Table B.6.2.1.2.

**Table B.6.2.1.2. Mortality in rats after oral administration of eugenol**

| Group No. | Dose (mL/kg bw) | No. Rats per group | No. of deaths following administration (by days) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | | 2 | 3 | 4-10 | Total | % |
| T-13 | 1.5 | 12 | 2 | (2) | 2 | - | - | 4 | 33 |
| 20 | 1.6 | 12 | 6 | (3) | 1 | - | - | 7 | 58 |
| 14 | 1.75 | 11 | 0 | (0) | 2 | - | 1[a] | 3 | 27 |
| 16 | 1.75 | 11 | 1 | (0) | 1 | 1 | - | 3 | 27 |
| 17 | 1.9 | 12 | 3 | (1) | 1 | - | - | 4 | 33 |
| 15 | 2.0 | 11 | 7 | (5) | 1 | - | - | 8 | 73 |
| 22 | 2.1 | 11 | 8 | (6) | 1 | - | - | 9 | 82 |
| 21 | 2.2 | 11 | 9 | (3) | 2 | - | - | 11 | 100 |
| | **Total** | **91** | **36** | **(20)** | **11** | **1** | **1** | **49** | |
| | | | 74% | | 22% | 2% | 2% | 54%[b] | |
| **$LD_{50}$: 1.8 ml/kg (1930 mg/kg)** | | | | | | | | | |

*Values in parenthesis give number of deaths in first 6 h after treatment.*
[a] *This death occurred on day 6 after dosage*
[b] *This represents the proportion of all deaths among all the animals treated, whereas the other percentage on this line gives the proportions of all the deaths which occurred in the groups indicated.*

**Clinical signs:** The first observable effect after the administration of eugenol was a weakness of the hind legs, which was seen in 100% of the animals. As a rule, this appeared in about 5 min, and by 15 min. there was complete paralysis of the lower extremities. Simultaneously, the lower jaw was relaxed and remained in an open position for a variable period of time in 70 (86%) of the animals. Prostration was observed in 64 (70%) animals, of which 54 of these went on to complete coma. Only 5 animals survived the coma. During coma, breathing was irregular, changing from slow, deep, gasping excursions of the chest to rapid, weak, abdominal respiration. In those animals in which the acute symptoms subsided, the animals remained lethargic, showed signs of urinary incontinence and frequently haematuria, and gave evidence of impaired function of the hind legs for several days. By the fifth day, the survivors had essentially recovered.

**Necropsy:** In 38 (95 %) of the 40 animals which were autopsied, eugenol odour was detected in the stomach, the duodenum, or both. The blood vessels in the peritoneum and mesentery were engorged, as were those in the diaphragm, ribs, and genital organs. The peritoneal wall was glistening and diffusely inflamed, varying from pink to bright red, and fluid was present in the peritoneal cavity. There was diffuse congestion of the kidneys, and the medulla as often pink and poorly delineated. The liver also was usually congested and darker than normal. The glandular stomach contained mucus, clotted blood, and occasionally some areas of mucosal erosion: the fore-stomach appeared normal. Blood clots were often found in the lumen of the small intestine. The lungs were usually mottled and redder than normal. In many animals opened promptly after death, the auricles were still beating: in all instances, the ventricles were engorged and the vessels were injected. The general impression was that of a diffuse inflammation with congestion in the viscera.

The microscopic studies yielded evidence of pathologic change, especially following the use of the highest dosages (2.1 and 2.2 ml/kg). Gross and microscopic observations of the tissues suggested that profound changes in fluid distribution had occurred in response to acute irritation of the gastrointestinal tract. The net impression of the effect of eugenol, from microscopic evidence, was essentially that of circulatory collapse with resultant congestion.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

The EU-agreed acute oral $LD_{50}$ in rats is 1930 mg/kg bw (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with the applicant. The calculated acute oral $LD_{50}$ is 1930 mg/kg bw for male and female rats.

This study was carried out prior to OECD Test Guidelines on acute oral toxicity, and several relevant differences with internationally accepted guidelines have been detected (see deviations point). Furthermore, the clinical observations indicative of neurotoxicity are reported as number of animals/percentage of animals observed with effects, and those numbers do not add up.

In the absence of additional valid studies, it is considered acceptable to assess the acute oral toxicity of eugenol. Based on the results of the study and according to the criteria of Regulation (EC) No 1272/2008, classification of eugenol as **Acute (oral) Toxicity, Category 4 (Acute Tox. 4; H302)** is required.

**B.6.2.1.3. Acute oral toxicity study in rats**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | National Toxicology Program (NTP) |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats and $B6C3F_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.2.1/03 |
| **Guidelines followed in study** | This study is pre-guideline. |
| **Deviations from current test guideline OECD TG 420 (2001)** | -No necropsy performed<br>-Limited level of reporting (*i.e.* clinical signs) |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP) |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol), Purity: > 99 %, Lot/Batch No.: 36483 |
| **Vehicle** | 1 % carboxymethylcellulose in saline |
| **Test system** | Male and female F344/N rats |
| **Test method** | Eugenol was administered by gavage to groups of 5 rats/sex at single doses of 150, 250, 500, 1000 and 2000 mg/kg bw as a suspension in 1% carboxymethylcellulose in saline. Surviving animals were killed on day 16. Body weights were measured at the start and end of the study but no necropsy was performed. |

**Results**

*Mortality*

One female rat from the highest test dose group died on day 2 and one female in the 250 mg/kg dose group died by accidental death as a result of gavage error (Table B.6.2.1.3/1).

**Table B.6.2.1.3/1. Mortality in rats after oral administration of eugenol**

| Dose level (mg/kg bw) | Mortality / animals treated | | | |
|---|---|---|---|---|
| | Males | Females | Total | Time of death |
| 150 | 0/5 | 0/5 | 0/10 | NA |
| 250 | 0/5 | 1/5* | 0/10 | NA |
| 500 | 0/5 | 0/5 | 0/10 | NA |
| 1000 | 0/5 | 0/5 | 0/10 | NA |
| 2000 | 0/5 | 1/5 | 1/10 | Day 2 |

| Dose level (mg/kg bw) | Mortality / animals treated | | | |
|---|---|---|---|---|
| | Males | Females | Total | Time of death |
| *Accidental death by gavage error<br>Untreated controls were not included in this test | | | | |

The lethal acute dose appears to be > 1000 mg/kg bw with an acute oral $LD_{50}$ value > 2000 mg/kg bw.

*Body weight*

Changes in body weights of rats were not affected by treatment in any groups (Table B.6.2.1.3/2).

**Table B.6.2.1.3/2. Mean body weight changes of rats treated with a single dose of eugenol**

| Dose level (mg/kg bw) | Body weight changes (g)[a] | |
|---|---|---|
| | Males | Females |
| 150 | 55 ± 0.8 | 33 ± 1.3 |
| 250 | 63 ± 2.2 | 34 ± 2.1 |
| 500 | 61 ± 3.5 | 30 ± 2.0 |
| 1000 | 54 ± 4.6 | 41 ± 1.9 |
| 2000 | 56 ± 3.7 | 29 ± 1.4 |
| [a] Mean ± standard error of the mean | | |

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

No $LD_{50}$ was identified in this particular study; the EU-agreed acute oral $LD_{50}$ in rats is 1930 mg/kg bw.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with the applicant. Based on the results of the study the estimated acute oral $LD_{50}$ is > 2000 mg/kg bw for male and female rats.

No guideline was stated in the study although the study was comparable to the OECD TG 401 (1987). A few deviations from current internationally-accepted guidelines have been detected (see deviations point) and for this reason, the RMS deems the study as supporting information but not acceptable for classification purposes.

**B.6.2.1.4. Acute oral toxicity study in mice**

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | National Toxicology Program (NTP) |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats and $B6C3F_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.2.1/03 |
| **Guidelines followed in study** | This study is pre-guideline. |
| **Deviations from current test guideline OECD TG 420 (2001)** | -No necropsy performed<br>-Limited level of reporting (*i.e.* clinical signs) |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP) |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol (4-allyl-2-methoxyphenol), Purity: > 99 %, Lot/Batch No.: 36483 |
| **Vehicle** | 1 % carboxymethylcellulose in saline |
| **Test system** | Male and female B6C3F1 mice |
| **Test method** | Eugenol was administered by gavage to groups of 5 animals/sex at doses of 180, 375, 750, 1500 and 3000 mg/kg bw as a suspension in 1% carboxymethylcellulose in saline. Surviving animals were killed on day 16. Body weights were measured at the start and end of the study but no necropsy was performed. |

**Results**

*Mortality*

Mortality was observed at 750 mg/kg group dose (one male) and at the highest dose group, 3000 mg/kg in which 2 males and 5 females died. (Table B.6.2.1.4/1). The time of death occurred at days 1 and 2 at the highest dose group.

**Table B.6.2.1.4/1. Mortality in mice after oral administration of eugenol**

| Dose level (mg/kg bw) | Mortality / animals treated | | | |
|---|---|---|---|---|
| | Males | Females | Total | Time of death |
| 180 | 0/5 | 0/5 | 0/10 | NA |
| 375 | 0/5 | 0/5 | 0/10 | NA |
| 750 | 1/5 | 0/5 | 1/10 | Day 6 |
| 1500 | 0/5 | 0/5 | 0/10 | NA |
| 3000 | 2/5 | 5/5 | 7/10 | Days 1 and 2 |

The acute oral $LD_{50}$ value in mice is estimated between 1500 and 3000 mg/kg bw.

*Body weight*

Body weights of mice were not affected by treatment in any groups (Table B.6.2.1.4/2).

**Table B.6.2.1.4/2. Mean body weight changes of rats treated with a single dose of eugenol**

| Dose level (mg/kg bw) | Body weight changes (g)[a] | |
|---|---|---|
| | Males | Females |
| 180 | 5 ± 0.4 | 4 ± 0.5 |
| 375 | 5 ± 0.5 | 4 ± 0.4 |
| 750 | 5 ± 0.9 | 4 ± 0.7 |
| 1500 | 4 ± 0.4 | 3 ± 0.4 |
| 3000 | 4 ± 1.2 | - |
| [a] Mean ± standard error of the mean | | |

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

No $LD_{50}$ was identified in this particular study; the EU-agreed acute oral $LD_{50}$ in rats is 1930 mg/kg bw.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with the applicant. Based on the results of the study the estimated acute oral $LD_{50}$ is between 1500 and 3000 mg/kg bw for female mice.

No guideline was stated in the study although the study was comparable to the OECD TG 401 (1987). A few deviations from current internationally-accepted guidelines have been detected (see deviations point) and for this reason, the RMS deems the study as supporting information but not acceptable for classification purposes.

### B.6.2.1.5. Acute oral toxicity study in dogs

| | |
|---|---|
| **Data point** | CA 5.2.1 (EU data requirement) |
| **Report author** | Lauber, F.U. and Hollander, F. |
| **Report year** | 1950 |
| **Report title** | Toxicity of the Mucigogue, Eugenol, Administered by Stomach Tube to Dogs |
| **Report No** | Not applicable, published study (*Gastroenterology*, 15 (5), 481-486) |
| **Document No** | KCA 5.2.1/04 |
| **Guidelines followed in study** | This study is pre-guideline. |
| **Deviations from current test guideline OECD TG 420/423 (2001)** | Test material not characterised (batch and purity not reported). No specific guideline followed in the study. No control groups were used. Five animals used in 20 experiments (four dose groups). |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP) |
| **Acceptability/Reliability** | Supportive only (methodological deficits) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified |
| **Vehicle** | Aqueous emulsion stabilised with 5% gum acacia |
| **Test system** | Female Mongrel dogs |
| **Test method** | Five healthy female dogs were employed for the 20 experiments of this study. The food was withheld for 18-24 hours and water was removed from the cage about 15 hours before the experiment. The dogs were given single doses of 2% and 5% concentration of eugenol emulsion stabilised with 5% gum acacia at a volume of 50 or 100 ml, equivalent to 100, 200, 250 and 500 mg/kg bw. The emulsion was allowed to flow by gravity into the stomach *via* a Levin tube. Following instillation of the test item, observations were made on pulse, respiration, temperature, retching, vomiting, character of stools, faltering gait and general alertness, at least every half hour for 5-7 hours. |

**Results**

**Mortality:** Two animals died at the top dose (5% eugenol). One animal died at 7 hours after treatment whereas the second dog died within 24 hours after treatment. When dosage was reduced by half (50 ml, 5 % eugenol), there were no deaths.

**Table B.6.2.1.5. Mortality and toxic effects following a single intra-gastric administration of eugenol**

| Eugenol dosage | No of Experiments | No. of dogs | Incidence of reactions (no. experiments) | | |
|---|---|---|---|---|---|
| | | | Vomiting | Ataxia | Death |
| 100 cc – 5 % (5.0 g) | 6 | 4 | 4 | 3 | 2 |
| 50 cc – 5 % (2.5 g) | 7 | 4 | 2 | None | None |
| 100 cc – 2 % (2 g) | 6 | 3 | None | None | None |
| 50 cc – 2 % (1 g) | 1 | 1 | None | None | None |
| Total | 20 | 5 | 6 | 3 | 2 |

**Clinical signs:**

Pulse and respiratory rate - Control (pre-treatment) pulse values were generally in the range of 84 – 140. In two experiments, however, the control values were 60-64, and in a third, they were 144 – 156. There was frequently a difference of as much as 20 beats per minute in the three measurements taken half-hourly interval on the same day. The pulse rates taken during the 5-hour post-eugenol period were in essentially the same range (84-160) as the control values, although there were two particularly high values of 216 and 224. The mean for the 59 pre-treatment values was 103.6 ± 3.1 (S.D. m), whereas for 65 values following treatment, it was 117.7 ± 3.1 – a difference which is significant at the 1% level of probability.

The observation on respiratory rates failed to show any definite effect of the eugenol except in one dog during the pre-mortal period. The 53 control values were 12 to 50 (mean 26.5 ± 1.1), whereas, the 55 experimental values varied from 10 to 36 (mean 23.8 ± 0.77). The difference between these two means is not significant at the 1 % level of probability.

Temperature – Eugenol administration resulted in a slight depression of body temperature. Examination of the data for individual experiments revealed no apparent correlation between temperature changes with volume or concentration of eugenol, nor with other variable.

Systemic reactions –Severe reactions were noted in 5 of the 6 experiments performed with the highest dose (5% eugenol). In three of these experiments, retching and vomiting occurred between 1.5 and 4.5 hours after administration of the emulsion. Only one animal experienced no reaction at this dosage. Furthermore, motor dysfunction (ataxia) occurred in 3 of the 6 experiments (3 dogs) at the highest dose (5% eugenol). These neurological symptoms started from 2h following administration of eugenol. Effects included “drunken” gait, impaired movement of hind legs and paralysis. None of these effects were observed at lower dosage levels.

In only one of the 14 experiments at the low dosages there was an episode of considerable vomiting, whereas in the others there was a minor episode of vomiting. At none of the dose levels tested were there any signs of diarrhoea or constipation.

Cumulative effects – Since the above experiments demonstrated that eugenol in a single 2 g dose had no immediate effect on dogs, 2 experiments were carried out in which 10 successive 2 g doses were administered at intervals of 48 to 72 hours. In general, the procedure was the same as before. The dogs acted normally after each administration, with non gastro-intestinal, motor or other systemic reactions noted at any time. Forty-eight hours after the last administration – on the twenty-third day – the animal was sacrificed. The autopsy and histological findings were essentially negative.

**Necropsy:** Effects in the liver included congestion, hyperaemia and enlargement of cells. Congestion of kidneys, hyperaemia of the glomeruli and parenchyma was also observed. Stomach and duodenum had a mild haemorrhagic appearance. Histological examination of the liver, stomach, duodenum, kidney and adrenal gland revealed no additional abnormalities other than a mild venous congestion of the liver.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

No $LD_{50}$ was identified in this particular study; the EU-agreed acute oral $LD_{50}$ in dogs is ~ 500 mg/kg bw (EFSA Journal 2012; 10(11): 2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study was carried out prior to OECD Test Guidelines on acute oral toxicity, and several relevant differences with internationally accepted guidelines have been detected (see deviations point). Therefore, RMS deems this study as supportive, but not acceptable for classification purposes.

Based on the results of the study, the $LD_{50}$ cannot be established.

Comments: The study uses 5 female dogs for 20 experiments. It is not clear which animal was tested at what doses.

### B.6.2.2. Dermal

Applicant:

No data are available. OECD 402 (2017) states that regulatory classifications based on acute oral studies were equivalent to or more severe than those derived from dermal data in more than 98% of cases; the agreed acute oral $LD_{50}$ in the rat is 1930 mg/kg bw, therefore no specific concern is expected for acute toxicity *via* the dermal route. In addition, eugenol is classified as a skin irritant (Skin Irrit., Cat 2, H315) and testing by the dermal route should be avoided on animal welfare grounds. Therefore, acute toxicity studies *via* the dermal route are not considered necessary for eugenol. This was confirmed by EFSA (EFSA Journal 2012;10(11):2914). In addition, a GLP and guideline-compliant study is available for the representative formulation; the acute dermal $LD_{50}$ was >2000 mg/kg bw in the rat.

Classification is not proposed for acute toxicity by the dermal route.

*RMS Comments:*
*The RMS does not agree with the applicant. The acute oral toxicity of eugenol is 1930 mg/kg bw, hence it does not exceed the 2000 mg/kg bw cut-off value defined in the criteria of Regulation (EC) 283/2013 that excludes the requirement for this type of study. However, the oral $LD_{50}$ is close enough to the cutoff value of 2000 mg/kg bw to expect a low acute dermal toxicity. Furthermore, eugenol has shown to be severely irritant to the rabbit skin (refer to section B.6.2.4). Taking a weight of evidence approach, the RMS deems the acute dermal toxicity testing not necessary and no classification for this hazard class is proposed in this evaluation in compliance with Regulation (EC) 1107/2009. In the absence of data, a harmonised classification according to Regulation (EC) 1272/2008 cannot be proposed.*

### B.6.2.3. Inhalation

| | |
|---|---|
| **Data point** | CA 5.2.3 (EU data requirement) |
| **Report author** | Clark, G.C. |
| **Report year** | 1988 |
| **Report title** | Acute inhalation toxicity of eugenol in rats |
| **Report No** | Not applicable, published study (*Arch. Toxicol.*, 62, 381-386) |
| **Document No** | KCA 5.2.3/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 403 (2009)** | Test material not characterised (batch number not reported). |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: 99% |
| **Test system** | Male and female Sprague-Dawley rats |
| **Test method** | Three groups of 10 rats each (5 males and 5 females) were exposed in a whole-body exposure system for 4 hours to a submicron aerosol eugenol at a concentration of 2.58 (group 1), 1.37 (group 2) and 0.77 mg/L (group 3). A control group (of equal size) was exposed to an inhalation atmosphere of air under the same conditions. |

| Treatment group | Parameter | Value determined |
|---|---|---|
| Group 1 | Nominal concentration | 4.48 mg/L |
| | Mass Median Aerodynamic Diameter (MMAD) | 0.82 µm |
| | Geometric standard deviation | σg 2.26 |
| | Mean temperature (ºC) | 23.7ºC |

| | | |
|---|---|---|
| | Mean Humidity (%) | 58 % |
| Group 2 | Nominal concentration<br>Mass Median Aerodynamic Diameter (MMAD)<br>Geometric standard deviation<br>Mean temperature (ºC)<br>Mean Humidity (%) | 2.39 mg/L<br>0.88 µm<br>σg 2.05<br>23.3ºC<br>50.8 % |
| Group 3 | Nominal concentration<br>Mass Median Aerodynamic Diameter (MMAD)<br>Geometric standard deviation<br>Mean temperature (ºC)<br>Mean Humidity (%) | 1.53 mg/L<br>0.9 µm<br>σg 1.87<br>22.8ºC<br>55.2 % |

The animals were examined for clinical signs of toxicity and mortality during and after exposure and daily thereafter until day 14. Individual body weights were recorded from the day of arrival until the end of the observation period.
Necropsy was performed on all animals and they were subjected to macroscopic examinations. Particular attention was given to the respiratory tract. Histopathology of the lungs was performed.

**Results**

**Mortality:** No mortality occurred.

**Clinical signs:** During exposure-treatment, animals in the high dose group showed increased salivation and restlessness. Some gasping was noted which disappeared within 2 h, as did all clinical signs except restlessness at the lower eugenol levels. Immediately following exposure, all high dose rats were lethargic and many high and intermediate dose rats had wet snouts and red-brown staining of their fur. Animals in the low dose group and control animals were normal in appearance and behaviour. During the 1st day of the 14-day observation period, all clinical signs disappeared in all groups and all rats appeared normal. Control rats were normal throughout the study.

**Body weight:** Both male and female rats exposed to eugenol lost weight (ca. 7%) overnight following exposures; however, this was not concentration related. Subsequently, the weight gain of the eugenol-exposed groups was similar to or greater than that of the control group such that, the body weights of all groups were similar by the end of the 14-day observation period.

**Food consumption:** There was a marked reduction (ca. 80%) in all eugenol-exposed groups overnight following exposure, with little difference in response between the groups. During the following 2-3 days, food intake rapidly returned towards comparability with the control group intake.

**Water consumption:** There was a marked reduction (ca. 40- 70%) in water consumption in all eugenol-exposed groups overnight following exposure with a slightly less marked effect in the low dose (0.77 mg/L). Subsequently, with the exception of the mid dose (1.37 mg/L) in which consumption was generally elevated, the water intake by all groups was comparable.

**Necropsy:** Macroscopic abnormalities consisting of dark red slightly raised areas, were detected in the lungs of only two animals in the highest and lowest eugenol concentrations and in three control animals. Such abnormalities are not uncommon in the lungs of laboratory-maintained rats and their presence with equal incidence in control animals suggests that they are unlikely to be related to inhalation of eugenol.

Lung weights: The individual and group mean lung weight to body weight ratio data are tabulated below (Table B.6.2.3). Group mean values within each sex were similar.

Histopathological examination of the lungs indicated there were no significant histopathological findings in the lungs of individual animals. No treatment-related changes were detected.

**Table B.6.2.3. Mean lung and relative lung weights**

| Group | | Lung weight (g) | Body weight (g) | Relative lung weight |
|---|---|---|---|---|
| Males | | | | |
| Control | Mean | 1.34 | 314 | 0.43 |

| Group | | Lung weight (g) | Body weight (g) | Relative lung weight |
|---|---|---|---|---|
| | SD | 0.15 | 18.2 | 0.054 |
| Low dose (0.77 mg/L) | Mean | 1.18 | 286 | 0.41 |
| | SD | 0.07 | 3.9 | 0.026 |
| Mid dose (1.37 mg/L) | Mean | 1.29 | 303 | 0.42 |
| | SD | 0.05 | 2.6 | 0.015 |
| High dose (2.58 mg/L) | Mean | 1.25 | 306 | 0.41 |
| | SD | 0.05 | 21.1 | 0.027 |
| Females | | | | |
| Control | Mean | 1.11 | 230 | 0.48 |
| | SD | 0.04 | 12.1 | 0.015 |
| Low dose (0.77 mg/L) | Mean | 1.10 | 228 | 0.48 |
| | SD | 0.06 | 6.0 | 0.032 |
| Mid dose (1.37 mg/L) | Mean | 1.13 | 232 | 0.48 |
| | SD | 0.07 | 3.4 | 0.026 |
| High dose (2.58 mg/L) | Mean | 1.07 | 221 | 0.49 |
| | SD | 0.05 | 16.2 | 0.031 |

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed 4-hour acute inhalation $LC_{50}$ is >2.58 mg/L in rats (EFSA Journal 2012;10(11):2914)

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with the applicant. This study is considered relevant to evaluate the acute inhalation toxicity of eugenol in rats. Under the test conditions, the acute inhalation $LD_{50}$ was > 2.58 mg/L (maximum attained concentration) for male and female rats. Therefore, according to the current EU Criteria, no classification is required for eugenol.

Comments: There is a significant difference between the nominal and measured concentrations, although the ratios between these concentrations across the treatment groups are reported to be within the same magnitude: 0.58, 0.57 and 0.50, respectively.

## B.6.2.4. Skin irritation

### B.6.2.4.1. Skin irritation in rabbits

| | |
|---|---|
| **Data point:** | **CA 5.2.4 (EU data requirement)** |
| **Report author** | Motoyoshi, K., Toyoshima, Y., Sato, M. and Yoshimura, M. |
| **Report year** | 1979 |
| **Report title:** | Comparative studies on the irritancy of oils and synthetic perfumes to the skin of rabbit, guinea pig, rat, miniature swine and man |
| **Report No.** | Not applicable, published study (*Cosmetics & Toiletries*, **94** (8), 41-48) |
| **Document No.** | KCA 5.2.4/01 |
| **Guidelines followed in study** | Not stated (pre-guideline) |
| **Deviations from current test guideline OECD TG 404 (2015)** | -Test chemical not fully characterised<br>-Dose level was 0.1 g of test chemical (instead of 0.5 mL/0.5 g required by guidance)<br>-Exposure to the chemical exceeded the 4h recommended by guidance (24 to 72 h exposure)<br>-Exposure to test item was not done in a patch under occlusion<br>-Multiple application of the test item onto the skin<br>-Observations were not performed following guidance (reversibility of effects were not assessed)<br>-Non-standard scoring method<br>-No individual results provided, only the summary of the overall result |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/ reliability** | Supportive only (methodological deficits) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Purity: > 95 %, Lot/Batch No.: not stated |
| **Vehicle** | - |
| **Positive control** | n-Hexadecane |
| **Test system** | Albino angora rabbits |
| **Test method** | Eugenol technical (0.1 g) was applied undiluted using a glass tuberculin syringe directly to the central area of the skin of 6 rabbits. The test compound applied was spread over the whole area immediately. The exposure to the chemical was 24 hour and the skin was scored for irritation after this time. After clipping the hair of the test areas after scoring, a second dose of eugenol was applied 30min later. A second set of reading and application was made 48 h later. After the 72 h reading, all the hair on the dorsal surface of each animal was clipped and Evans blue dye dissolved in physiological saline was injected intravenously into each animal one hour before sacrifice. Erythema, oedema and rate of dye penetration (as a result of increased capillary permeability) were scored, with the scores being combined to give an overall severity classification (Table below).<br><br>Evaluation of skin reactions: |

| On the living skin | On the removed skin | Total score |
|---|---|---|
| Reddening rate (erythema):<br>-1<br>±1<br>+1<br>++1<br>+++1<br>Others: scaling, crust formation, loss of elasticity and fissures | -Dilating rate<br>-Swelling rate (edema)<br>-Bluing rate (increased capillary permeability)<br>-Bleeding rate | Total score (72h reading): Dilating rate + swelling rate+bluing rate + redding rate |

| | |
|---|---|
| | Compounds producing the total score (primary irritation index) of 4 or less were only mildly irritating whereas those with indexes from 4 to 8 were moderate irritants and those with scores above 8 were considered severe irritants. |

**Results**

Eugenol produced a relative irritancy **score of 3**, which is classified as severely irritating to the rabbit skin.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: skin irritant (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** Under the conditions of the study, eugenol was scored by the study authors as severely irritant to the rabbit skin.

The RMS disagrees with the previous assessment (DAR 2011) and the study is deemed as supporting information but not acceptable for classification purposes.

The study is a publication of a peer reviewed journal. Deviations from the current OECD TG 404 (2015) include, amongst others, non-standard scoring of the lesions, no assessment of reversibility of effects, exposure longer than 4 hours and multiple application of the test item in the study. Based on these methodological deficiencies, the study is not considered acceptable for classification purposes according to the criteria of Regulation EC 1272/2008. However, in the absence of further evidence, in this study eugenol is reported to cause severe irritation to rabbit skin with the undiluted test substance (maximum score given in the test: grade 3 of 3), which is the most sensitive species to assess skin corrosion. This finding is in line with the irritation observed in human skin (grade 2 of 3, B.6.2.4.4) when applied at a 32% concentration. For these reasons, and in the absence of any other data, the RMS considers that eugenol may be considered as **Skin irritant – Category 2 (Skin Irrit. 2; H315)**.

### B.6.2.4.2. Skin irritation in guinea pigs

| **Data point:** | **CA 5.2.4 (EU data requirement)** |
|---|---|
| **Report author** | Motoyoshi, K., Toyoshima, Y., Sato, M. and Yoshimura, M. |
| **Report year** | 1979 |
| **Report title:** | Comparative studies on the irritancy of oils and synthetic perfumes to the skin of rabbit, guinea pig, rat, miniature swine and man |
| **Report No.** | Not applicable, published study (*Cosmetics & Toiletries*, **94** (8), 41-48) |
| **Document No.** | KCA 5.2.4/01 |
| **Guidelines followed in study** | Not stated (pre-guideline) |
| **Deviations from current test guideline OECD TG 404 (2015)** | -Test item not fully characterised<br>-Dose level was 0.1 g of test item (instead of 0.5 mL/0.5 g required by guidance)<br>-24h exposure to the chemical instead of 4h<br>-Exposure to test item was not done in a patch under occlusion<br>-Multiple application of the test item onto the skin<br>-No observation at 30 min was performed.<br>-Reversibility of effects was not assessed (no observation period of 14 days)<br>-Non-standard scoring method and non-standard species<br>-No individual results provided, only summary of overall result |

| | |
|---|---|
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/ reliability** | Supportive only (methodological deficits) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Purity: > 95 %, Lot/Batch No.: not stated |
| **Vehicle** | - |
| **Positive control** | n-Hexadecane |
| **Test system** | Male Hartley guinea pigs |
| **Test method** | Eugenol technical (0.1 g) was applied undiluted using a glass tuberculin syringe directly to 1 of the two areas of the dorsal, mid-lumbar region of each animal (6 animals/test) that were clipped 24 h before. The other side was left untreated. The test compound applied was spread over the whole area immediately. The exposure to the chemical was 24 hour and the skin was scored for irritation after this time. After clipping the hair of the test areas after scoring, a second dose of eugenol was applied for 30min and readings were performed 48 h and 72 hours later. After the 72 h reading, all the hair on the dorsal surface of each animal was clipped and Evans blue dye dissolved in physiological saline was injected intravenously into each animal one hour before sacrifice. Erythema, oedema and rate of dye penetration (as a result of increased capillary permeability) were scored, with the scores being combined to give an overall severity classification (Table below).<br><br>Evaluation of skin reactions:<br>**On the living skin**: Reddening rate (erythema): -1, ±1, +1, ++1, +++1; Others: scaling, crust formation, loss of elasticity and fissures<br>**On the removed skin**: -Dilating rate; -Swelling rate (edema); -Bluing rate (increased capillary permeability); -Bleeding rate<br>**Total score**: Total score (72h reading): Dilating rate + swelling rate+bluing rate + redding rate<br><br>Compounds producing the total score (primary irritation index) of 4 or less were only mildly irritating whereas those with indexes from 4 to 8 were moderate irritants and those with scores above 8 were considered severe irritants. |

**Results**

Eugenol produced a relative irritancy **score of 2** in guinea pigs, which corresponds to an overall assessment of moderately irritant.

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: skin irritant (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** Under the conditions of the study, eugenol was scored by the study authors as moderately irritant to guinea pig skin.

The RMS disagrees with the previous assessment (DAR 2011) and the study is deemed as supporting information but not acceptable for classification purposes.

The study is a publication of a peer reviewed journal. Deviations from the current OECD TG 404 (2015) include, amongst others, non-standard scoring of the lesions, no assessment of reversibility of effects, exposure longer than 4 hours and multiple application of the test item in the study. Based on these methodological deficiencies, the study is not considered acceptable for classification purposes according to the criteria of Regulation EC 1272/2008. However, in the absence of further evidence, eugenol is reported to cause severe irritation to rabbit skin with the undiluted test substance (maximum score given in the test: grade 3 of 3, B.6.2.4.1), which is the most sensitive species to assess skin corrosion. This finding is in line with the irritation observed in human skin (grade 2 of 3, B.6.2.4.4) when applied at a 32% concentration For these reasons, and in the absence of any other data, the RMS considers that eugenol may be considered **as Skin irritant – Category 2 (Skin Irrit. 2; H315)**.

### B.6.2.4.3. Skin irritation in miniature swine

| **Data point:** | **CA 5.2.4 (EU data requirement)** |
|---|---|
| **Report author** | Motoyoshi, K., Toyoshima, Y., Sato, M. and Yoshimura, M. |
| **Report year** | 1979 |
| **Report title:** | Comparative studies on the irritancy of oils and synthetic perfumes to the skin of rabbit, guinea pig, rat, miniature swine and man |
| **Report No.** | Not applicable, published study (*Cosmetics & Toiletries*, **94** (8), 41-48) |
| **Document No.** | KCA 5.2.4/01 |
| **Guidelines followed in study** | Not stated (pre-guideline) |
| **Deviations from current test guideline OECD TG 404 (2015)** | -Test item not fully characterised<br>-Dose level was 0.05 g of test item (instead of 0.5 mL/0.5 g required by guidance)<br>-48h exposure to the chemical instead of 4h<br>-No observation at 30 min was performed.<br>-Reversibility of effects was not assessed (no observation period of 14 days)<br>-Non-standard scoring method and non-standard species<br>-No individual results provided, only summary of overall result |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/ reliability** | Supportive only (methodological deficits) |

**Materials and Methods**

| **Test chemical** | Eugenol; Purity: > 95 %, Lot/Batch No.: not stated |
|---|---|
| **Vehicle** | - |
| **Test system** | Miniature Pitman-Moore swine (one month old) |
| **Test method** | The day before the experiment, the hair on the whole dorsal region of six miniature swine was removed with a clipper and the area was cleansed with warm water to remove the dirt.<br>The method consisted of introducing 0.05g of the test item undiluted under a 15mm diameter patch. The patches were secured in place by adhesive tape. The entire trunk of the animal was wrapped with rubberised cloth for the 48h period exposure after which time the patches were removed and the resulting reactions were evaluated on the basis of the scores in the following table:<br><br>Evaluation of skin reactions: |

| **On the living skin** | **On the removed skin** | **Total score** |
|---|---|---|

| | | | |
|---|---|---|---|
| | Reddening rate (erythema):<br>-1<br>±1<br>+1<br>++1<br>+++1<br>Others: scaling, crust formation, loss of elasticity and fissures | -Dilating rate<br>-Swelling rate (edema)<br>-Bluing rate (increased capillary permeability)<br>-Bleeding rate | Total score (72h reading):<br>Dilating rate + swelling rate+bluing rate + redding rate |
| | Compounds producing the total score (primary irritation index) of 4 or less were only mildly irritating whereas those with indexes from 4 to 8 were moderate irritants and those with scores above 8 were considered severe irritants. | | |

**Results**

Eugenol produced a relative irritancy **score of 1**, which corresponds to an overall conclusion of mildly irritation to the miniature swine skin.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: skin irritant (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** Under the conditions of the study, eugenol was scored by the study authors as midly irritant to the miniature swine skin.

The RMS disagrees with the previous assessment (DAR 2011) and the study is deemed as supporting information but not acceptable for classification purposes.

The study is a publication of a peer reviewed journal. Deviations from the current OECD TG 404 (2015) include, amongst others, non-standard scoring of the lesions, no assessment of reversibility of effects, exposure longer than 4 hours and multiple application of the test item in the study. Based on these methodological deficiencies, the study is not considered acceptable for classification purposes according to the criteria of Regulation EC 1272/2008. However, in the absence of further evidence, eugenol is reported to cause severe irritation to rabbit skin with the undiluted test substance (maximum score given in the test: grade 3 of 3, B.6.2.4.1), which is the most sensitive species to assess skin corrosion. This finding is in line with the irritation observed in human skin (grade 2 of 3, B.6.2.4.4) when applied at a 32% concentration. For these reasons, and in the absence of any other data, the RMS considers that eugenol may be considered as **Skin irritant – Category 2 (Skin Irrit. 2; H315)**.

#### B.6.2.4.4. Skin irritation in humans

| | |
|---|---|
| **Data point:** | **CA 5.2.4 (EU data requirement)** |
| **Report author** | Motoyoshi, K., Toyoshima, Y., Sato, M. and Yoshimura, M. |
| **Report year** | 1979 |
| **Report title:** | Comparative studies on the irritancy of oils and synthetic perfumes to the skin of rabbit, guinea pig, rat, miniature swine and man |
| **Report No.** | Not applicable, published study (*Cosmetics & Toiletries*, **94** (8), 41-48) |
| **Document No.** | KCA 5.2.4/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/ reliability** | Supportive only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Purity: > 95 %, Lot/Batch No.: not stated |
| **Vehicle** | Acetone |
| **Positive control** | n-Hexadecane |
| **Test system** | Human volunteers |
| **Test method** | Eugenol technical was applied to a panel of 50 volunteers and individuals with known allergic reactions were excluded. Using a glass tuberculin syringe, 0.05 mL of test solution (32% eugenol in acetone) was transferred to lint placed in contact with the skin on the back of each subject during 48 h. The patches were then removed and the sites were swabbed with dry gauze to remove any residual test material. Reading of skin reactions was done 30 min after patch removal and the test sites were evaluated on the basis of scores in the following criteria:<br>-: Negative reading<br>±: Questionable erythema not covering the entire patch area<br>+: Definite erythema of the entire patch area<br>++: Erythema and edema<br>+++: Erythema, edema and vesicles (or papules)<br>++++: bullous reaction<br><br>If necessary, 72, 96 and 120 h readings were performed.<br><br>Compounds producing the percentages of positive reactions (including questionable) of 10% or less were considered almost non-irritating; those with percentages from 10%; those with percentages from 10% to 40 % were mildly irritating; those with percentages from 40% to 70 % were moderately irritating; and those with percentages above 70% were severely irritating. |

**Results**

Eugenol produced a relative irritancy **score of 2**, which corresponds to the overall assessment of moderately irritating to the human skin.

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: skin irritant (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** Under the conditions of the study, eugenol was scored by the study authors as moderately irritating to human skin.

The RMS disagrees with the previous assessment (DAR 2011) and the study is deemed as supporting information but not acceptable for classification purposes.

The study is a publication of a peer reviewed journal. No guideline has been stated in the study for this type of study but based on the results, eugenol at 32 % is a moderate irritant to human skin. The study may not be considered acceptable for classification purposes according to criteria of Regulation EC 1272/2008. However, in the absence of further evidence, eugenol is reported to cause severe irritation to rabbit skin with the undiluted test substance (maximum score given in the test: grade 3 of 3, B.6.2.4.1), which is the most sensitive species to assess skin corrosion. This finding is in line with the irritation observed in this study (grade 2 of 3) when applied at a 32% concentration. For these reasons, and in the absence of any other data, the RMS considers that eugenol may be considered as **Skin irritant – Category 2 (Skin Irrit. 2; H315)**.

## B.6.2.5. Eye irritation

Justification by applicant:
No data is available for eugenol and none is considered necessary on the basis that *in vivo* data are available demonstrating that the substance is skin irritant.

Eugenol is considered as an eye irritant based on the skin irritation potential, and a precautionary classification as Eye Irrit., Cat.2, H319 is proposed according to CLP (EFSA Journal 2012;10(11):2914).

*RMS comments:*
*The RMS agrees with the applicant that based on skin irritation properties of eugenol, it is expected that at minimum, eugenol may cause irritation to the eye. However, eugenol is a severe irritant to the rabbit skin (reported in the rabbit skin irritation study, B.6.2.4.1) and therefore, the potential to cause severe eye damage cannot be disregarded. For this reason, in the absence of suitable data to determine the extent of the damage to the eye, a data requirement is established for this hazard class. The RMS recommends that for a more certain assessment, a tiered testing strategy as specified in Section 5.2.5 of Regulation (EU) No. 283/2013 should be followed.*

## B.6.2.6. Skin sensitization

### B.6.2.6.1. Local lymph node assay

#### B.6.2.6.1.1. Study I

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Takeyoshi, M., Noda, S., Yamazaki, S., Kakishima, H., Yamasaki, K. and Kimber, I. |
| **Report year** | 2004 |
| **Report title** | Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the Local Lymph Node Assay |
| **Report No** | Not applicable, published study (*J. Appl. Toxicol.*, **24**, 77-81) |
| **Document No** | KCA 5.2.6/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 442B (2018)** | -No positive control used<br>-Injection of BrdU was done on day 4 instead of day 5 as required per guidance<br>-Limited level of reporting (clinical signs, body weights) |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: > 95%, Batch/Lot No.: EG0704 |
| **Vehicle** | 4:1 acetone:Olive oil (AOO) |
| **Positive control** | Not used |
| **Negative control** | 4:1 acetone:Olive oil (AOO) |
| **Test system** | Female CBA/JN strain mice |
| **Test Method** | Groups of four mice each were exposed topically on the dorsum of both ears to 25 µL of eugenol in 4:1 acetone:olive oil (AOO) at concentrations of 1%, 6%, 15% or 30% solutions, daily for three consecutive days. The concentration range was decided according to the sensitization potencies from the GPMT study (B.6.2.6.2.1). A single intraperitoneal injection of BrdU was given on Day 4. On Day 5, the draining auricular lymph nodes were collected and lymph node proliferation was measured by bromodeoxyuridine incorporation.<br><br>Statistical analysis was performed by calculation of means and standard errors for the labelling index obtained for each treatment group. The $EC_3$ value was derived by linear interpolation as described by Basketter *et al.* (2000) by interpolating between two points on the SI axis, one immediately above and one immediately below the SI value of 3. |

**Results and Conclusion**

A dose-dependent induction of lymph node cell proliferative activity was observed indicative of skin sensitisation at 15% concentration or greater (Table B.6.2.6.1.1).

A positive response was obtained for the 30% concentration with a SI = 3.3. The $EC_3$ value was 25.1%. A positive result was concluded.

**Table B.6.2.6.1.1. Results of non-radioisotopic LLNA with eugenol**

| Concentration tested (%) | BrdU labelling index | | |
|---|---|---|---|
| | Mean | SI[a] | $EC_3$ |
| Control (AOO) | 0.107 | - | - |
| 1 | 0.187 | 1.7 | 25.1 |
| 6 | 0.161 | 1.5 | |
| 15 | 0.251* | 2.3 | |
| 30 | 0.355** | 3.3 | |

*[a]The SI was calculated by dividing the mean value obtained in each treatment group by that of the control group.*
** P < 0.05; ** P< 0.01*

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914)

**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:** RMS agrees with the applicant and considers this study acceptable to evaluate the skin sensitisation potential of eugenol. Based on the results of the study, eugenol is positive in the local lymph node assay.

The study is a publication from a peer reviewed journal. Deviations from the current OECD TG 442B (2017) guideline include limited level of reporting, injection of BrdU was done on day 4 instead of day 5 of the test and no positive control used. The RMS deems the study as supporting information and cannot be used for classification purposes.

#### B.6.2.6.1.2. Study II

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Lalko, J., Isola, D. and Api A.M. |
| **Report year** | 2004 |
| **Report title** | Ethanol and diethyl phthalate: vehicle effects in the Local Lymph Node Assay |
| **Report No** | Not applicable, published study (*Int. J. Toxicol.*, **23**, 171-177) |
| **Document No** | KCA 5.2.6/02 |
| **Guidelines followed in study** | Method described by Kimber *et al.* (1992); Comparable to OECD TG 429 (2002) |
| **Deviations from current test guideline OECD TG 429 (2010)** | -Male instead of female mice used<br>-No positive control used<br>-Limited level of reporting (clinical signs, body weights) |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: 99.9 %, Batch No.: not stated |
| **Vehicle** | Diethyl phthalate (DEP), 1:3 ethanol:DEP, 3:1 EtOH:DEP and Ethanol |
| **Positive control** | Not stated |
| **Negative control** | Vehicle |
| **Test system** | Male CBA/Ca strain mice |
| **Test Method** | Mice from groups of 4 animals received a 25 µL volume of eugenol at 0%, 1%, 3%, 10%, 30% and 50% in one of the 4 vehicles to be tested. A 25 µL volume of each vehicle served as a negative control. The topical application was done daily for 3 consecutive days. On Day 6, 250 µL of phosphate-buffered saline containing 20 µCi of [$^3$H] methyl thymidine was injected intravenously. Five hours later, auricular lymph nodes were collected and lymph node proliferation was measured by [$^3$H] methyl thymidine incorporation rate.<br><br>For each concentration, the stimulation index (SI) value was calculated by dividing the group mean cpm at a given dose level by the mean cpm of the vehicle control group. A material was considered a sensitiser if at least one concentration of the test material was observed to have an SI value of 3 or more. Linear interpolation was used to derive the SI and the EC3 values calculated by the equation by Basketter *et al.* (1996). |

**Results and Conclusion**

The LLNA dose-response data are displayed in Table B.6.2.6.1.2. Positive responses were obtained with eugenol in every vehicle at concentrations over 30 % except with the vehicle 3:1 EtOH:DEP that was over 10%.

**Table B.6.2.6.1.2. Results of the LLNA with eugenol using various vehicles**

| Concentration tested (%) | Stimulation Index per vehicle | | | |
|---|---|---|---|---|
| | DEP | 1:3 EtOH:DEP | 3:1 EtOH:DEP | EtOH |
| **Control** | / | / | / | / |
| **1.0** | 2.0 | 0.8 | 1.1 | 1.6 |
| **3.0** | 1.1 | 1.7 | 2.2 | 1.4 |
| **10.0** | 1.8 | 2.8 | 4.7 | 2.7 |
| **30.0** | 6.5 | 9.2 | 8.6 | 10.3 |
| **50.0** | 6.4 | 10.6 | 18.7 | 15.1 |

The derived LLNA $EC_3$ values (w/v) for eugenol in the various vehicles are the following: 15.1 (DEP), 10.5 (1:3 EtOH:DEP), 5.3 (3:1 EtOH:DEP) and 10.7 (EtOH).

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914) |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** RMS agrees with the applicant and considers this study acceptable to evaluate the skin sensitisation potential of eugenol. Based on the results of the study, eugenol is positive in the local lymph node assay.<br>The study is a publication from a peer reviewed journal. Deviations from the current OECD TG 429 (2010) guideline include the use of males instead of female CBA/Ca mice, limited level of reporting or no positive control in the study. These deviations however, are not expected to alter the outcome of the study. Based on the results, eugenol is a mild/moderate sensitizer and therefore, based on criteria of Regulation (EC) No 1272/2008, classification of eugenol as **Skin Sensitisation, Category 1 (Skin Sens. 1; H317)** is required. |

#### B.6.2.6.1.3. Study III

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Hilton, J., Dearman, R.J., Fieding, I., Basketter, D.A. and Kimber, I. |
| **Report year** | 1996 |
| **Report title** | Evaluation of the sensitising potential of Eugenol and Isoeugenol in mice and guinea pigs. |
| **Report No** | Not applicable, published study (*J. Appl. Toxicol.*, **16** (5), 459-464) |
| **Document No** | KCA 5.2.6/03 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 429 (2010)** | Mice strain used was BALB/c as opposed to CBA/Ca strain. Test item not characterised. No positive control used. No Stimulation Index or EC3 values calculated. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/Reliability** | Supporting information (see method deficiencies) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Lot/Batch No.: not stated |
| **Vehicle** | 4:1 Acetone:Olive oil (AOO) |
| **Positive control** | Not used |
| **Test system** | Female BALB/c mice |
| **Test Method** | Groups of four mice each were exposed topically on the dorsum of both ears to 25 µL of eugenol in 4:1 acetone:olive oil (AOO) to give 5%, 10% or 25% solutions or to an equal volume of AOO alone, daily for three consecutive days. Five days following the initiation of exposure all mice were injected intravenously *via* the tail vein with 20µCi of [$^3$H]methyl thymidine in 250µL of phosphate-buffered saline (PBS). Five hours later mice were killed and the draining auricular lymph nodes were excised and pooled for each experimental group. |

| | |
|---|---|
| | A single-cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation through 200-mesh stainless steel gauze. The LNC were washed twice with an excess of PBS and precipitated in 5% trichloroacetic acid (TCA). Twelve hours later, pellets were resuspended with 1mL of TCA and transferred to 10mL of scintillation fluid (Optiphase MP, LKB, UK). Incorporation of $^{3}$HTdR was measured by β-scintillation counting and expressed as mean disintegrations per minute (dpm) per node for each experimental group. |

**Results and Conclusion**

A dose-dependent induction of lymph node cell proliferative activity was observed indicative of skin sensitising potential (Figure B.6.2.6.1.3).

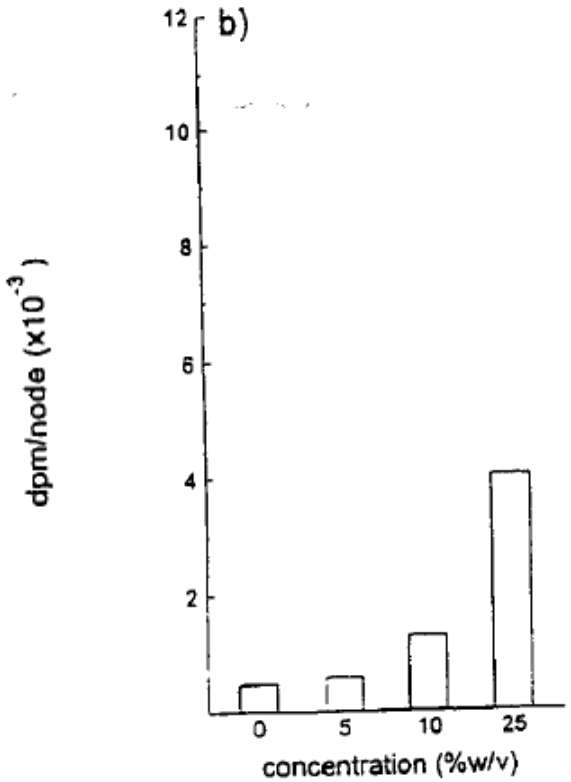



**Figure B.6.2.6.1.3. Activity of eugenol in the local lymph node assay (dpm per node).**

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914). |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** RMS agrees with the applicant and considers this study acceptable to evaluate the skin sensitisation potential of eugenol. Eugenol is deemed positive in the LLNA.<br>Due to the deviations from the current OECD TG 429 identified in the study (see deviations from current guidance above), the study is considered as supporting information. Based on the available data, eugenol is a sensitiser and therefore, based on criteria of Regulation (EC) No 1272/2008, classification of eugenol as **Skin Sensitisation, Category 1 (Skin Sens. 1; H317)** is required. |

### B.6.2.6.2. Guinea Pig Maximisation Test

#### B.6.2.6.2.1. Study I

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Takeyoshi, M., Noda, S., Yamazaki, S., Kakishima, H., Yamasaki, K. and Kimber, I. |
| **Report year** | 2004 |
| **Report title** | Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the Local Lymph Node Assay |
| **Report No** | Not applicable, published study (*J. Appl. Toxicol.*, **24**, 77-81) |
| **Document No** | KCA 5.2.6/01 |
| **Guidelines followed in study** | Method by Magnusson & Kligman (1969), comparable to OECD TG 406 (1992) |
| **Deviations from current test guideline OECD TG 406 (2021)** | -No detailed information of the preliminary dose-range finding test<br>-Adjuvant not stated<br>-No control group tested<br>- Individual results not reported |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/Reliability** | Supporting information (see method deficiencies) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: > 95%, Batch/Lot No.: EG0704 |
| **Vehicle** | Olive oil |
| **Positive control** | Not used |
| **Test system** | Female Hartley guinea pigs |
| **Test Method** | Guinea pigs (10 animals) received a series of intradermal injections of eugenol in the shoulder region to induce sensitisation. After 6-8 days sensitisation was boosted by a 48h occluded patch of the test item placed over the injection sites. Two weeks later, the animals were challenged on a shaved flank by a 24h occluded patch containing the same compound. All induction and challenge concentrations were set at 5 % (maximum non-irritant concentration) in olive oil.<br><br>The chemical was classified by the sensitisation rate according to the criteria given by Magnusson & Kligman: weak sentiser 0-8%, mild sensitiser 9-28%, moderate sensitiser 29-64%, strong sensitiser 65-80%, extreme sensitiser 81-100%. |

**Results and Conclusion**

Under the assay conditions, eugenol elicited a sensitisation response rate of 20 %. According to the criteria stablished by Magnusson & Kligman, eugenol is a mild skin sensitiser.

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with the applicant and considers this study acceptable to evaluate the skin sensitisation potential of eugenol.

This study is a publication from a peer-reviewed journal. Deviations from the current OECD TG 406 (2021) include very poor level of detail, no information of the adjuvant used, no control used and no individual results reported. Based on these method deficiencies the RMS deems the study as supporting information only.

**B.6.2.6.2.2. Study II**

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Hilton, J., Dearman, R.J., Fieding, I., Basketter, D.A. and Kimber, I. |
| **Report year** | 1996 |
| **Report title** | Evaluation of the sensitising potential of Eugenol and Isoeugenol in mice and guinea pigs. |
| **Report No** | Not applicable, published study (Journal of Applied Toxicology, Vol 16 (5), 459-464) |
| **Document No** | KCA 5.2.6/03 |
| **Guidelines followed in study** | Comparable to OECD TG 406 (1992) |
| **Deviations from current test guideline OECD TG 406 (2021)** | -Test item not characterised<br>-Adjuvant not stated<br>-No individual results are reported<br>-No positive control used in the test |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Lot/Batch No.: not stated |
| **Vehicle** | 0.01 % dodecyloxybenzene sulphate (DOBS) in 0.9 % sodium chloride |
| **Positive control** | Not used |
| **Test system** | Female albino Dunkin-Hartley guinea pigs |
| **Test Method** | *Preliminary irritation tests* were conducted to determine suitable assay concentrations.<br>*Principal test*<br>A group of 10 guinea pigs were treated with a series of 6 intradermal injections of 0.1 % eugenol in 0.01 % dodecyloxybenzene sulphate in 0.9 % sodium chloride, in the shoulder region to induce sensitisation. After 6-8 days, sensitisation was boosted by a 48-h occluded patch of test substance placed over the injection sites (undiluted). Two weeks later, these animals and a vehicle-treated control group (5 animals), were challenged with a 24-h occluded patch at the maximum non-irritant concentration of the test material (25% in acetone/PEG). Challenge sites were scored for erythema (scale 0-3) 24h after removal of the patches. The response rate was calculated by assessing the percentage of test guinea pigs exhibiting a response after making allowance for any irritant responses observed in control group animals. |

**Results and Conclusion**

Under the assay conditions, eugenol elicited a positive response of 30 % and a mean erythema score from positive animals of 0.8. No dermal responses were observed in guinea pigs that had received vehicle alone and were subsequently challenged with eugenol.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914). |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** RMS agrees with the applicant and considers this study acceptable to evaluate the skin sensitisation potential of eugenol. Despite the deviations from the current OECD TG 406 identified in the study (see deviations from current guidance above), they do not affect the outcome of the study. Based on the results, eugenol is a mild/moderate sensitiser and therefore, based on criteria of Regulation (EC) No 1272/2008, classification of eugenol as **Skin Sensitisation, Category 1 (Skin Sens. 1; H317)** is required. |

**B.6.2.6.3. Mouse IgE assay**

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Hilton, J., Dearman, R.J., Fieding, I., Basketter, D.A. and Kimber, I. |
| **Report year** | 1996 |
| **Report title** | Evaluation of the sensitising potential of Eugenol and Isoeugenol in mice and guinea pigs. |
| **Report No** | Not applicable, published study (*J. Appl. Toxicol.*, **16** (5), 459-464) |
| **Document No** | KCA 5.2.6/03 |
| **Guidelines followed in study** | No guideline |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Lot/Batch No.: not stated |
| **Test system** | Female BALB/c strain mice |
| **Vehicle** | 4:1 Acetone:olive oil |
| **Positive control** | Trimellitic anhydride (TMA) |
| **Negative control** | 2,4-Dinitrochlorobenzene (DNCB) |
| **Test Method** | Group of six mice received 50 µl of Eugenol, DNCB or TMA on both shaved flanks at concentrations 2.5%, 5% and 10%. A control group of 10 mice received 50 µl of vehicle alone. Seven days later, 25 µl of chemical at half the concentration dosed previously, or vehicle alone, was applied to the dorsum of both ears.<br>Fourteen days following the initiation of exposure, mice were exsanguinated by cardiac puncture and serum prepared and stored at –20°C until analysis.<br>Serum IgE was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Plastic microtiter plates were coated with 2.5 µg/ml$^{-1}$ rat monoclonal anti-mouse IgE antibody in 0.1 M carbonate buffer (pH 9.6) by overnight incubation at 4°C. The plates were then blocked by treatment for 30 min at 37° with 5 % normal goat serum (NGS) in PBS. Individual test serum samples and mouse monoclonal IgE anti-dinitrophenol (DNP) antibody (clone SPE-7; sigma), diluted to various extents in 0.5 % NGS in PBS, were added to triplicate wells and the plates incubated for 3 h at 4°C. Subsequently, plates were incubated for 3 h at 4°C with a 1:2000 dilution of goat anti-mouse IgE. Enzyme substrate (o-phenylenediamine and urea hydrogen peroxide) was added and the reaction terminated after 10 min by addition of 0.5 M citric acid. Between each incubation, the plates were washed three times with PBS containing 0.05 % Tween 20. Substrate conversion was measured as optical density at 450 nm using an automated reader. The concentration of IgE in test serum samples was derived from a standard curve for monoclonal mouse IgE. |

**Results**

The outcome of the test is shown in Table B.6.2.6.3. Treatment of mice with concentrations of eugenol of 5 % or 10 % failed to cause any significant change in IgE concentration. In contrast, however, treatment with 2.5 % eugenol was associated with a statistically significant elevation of IgE levels. TMA elicited a significant increase in the serum concentration of IgE consistent with possession of respiratory sensitisation potential whereas treatment of mice with DNCB did not influence significantly serum IgE levels.

**Table B.6.2.6.3. Activity of eugenol in the mouse IgE test**

| Serum IgE concentration (µg mL$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|
| **Values** | **AOO** | **Eugenol** | | | **DNCB** | **TMA** |
| | | 2.5% | 5% | 10% | 1% | 25% |
| Mean values | 0.401 | 0.856* | 0.295 | 0.492 | 0.567 | 2.951* |
| SD | ± 0.042 | ± 0.218 | ± 0.022 | ± 0.062 | ± 0.078 | ± 0.309 |
| **Significantly different from control (AOO) values; P < 0.01* | | | | | | |

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914).

**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:** RMS agrees with notifier assessment and conclusion. Based on the outcome of the study, eugenol applied topically produced a statistically significant increase in IgE at 2.5 % whereas higher concentrations did not have any effect in serum IgE levels, hence this effect is considered not of biological relevance.

This study is considered as supporting information to evaluate the respiratory sensitisation of eugenol because the route of application does not involve inhalation but dermally application.

**B.6.2.6.4. *In vitro* skin sensitisation study**

| | |
|---|---|
| **Data point** | CA 5.2.6 (EU data requirement) |
| **Report author** | Maeda, Y., Takeyoshi, M., Chuma, T. and Iwata, H. |
| **Report year** | 2020 |
| **Report title** | α-Sens: The improved ARE-Nrf2-based sensitization screening assay using normalized transcriptional activity |
| **Report No** | Not applicable, published study (*Toxicology*, **439**:152476) |
| **Document No** | KCA 5.2.6/04 |
| **Guidelines followed in study** | Comparable to OECD TG 442D (2018) |
| **Deviations from current test guideline OECD TG 442D (2018)** | -Non-standard assay (new methodology proposed), i.e. not currently approved within the scope of the guidance.<br>-Limited level of reporting. |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supportive only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol, Purity: not stated; Lot/Batch No.: not stated |
| **Test system** | PHK 16-0b cell line (immortalised human keratinocyte cell line) |

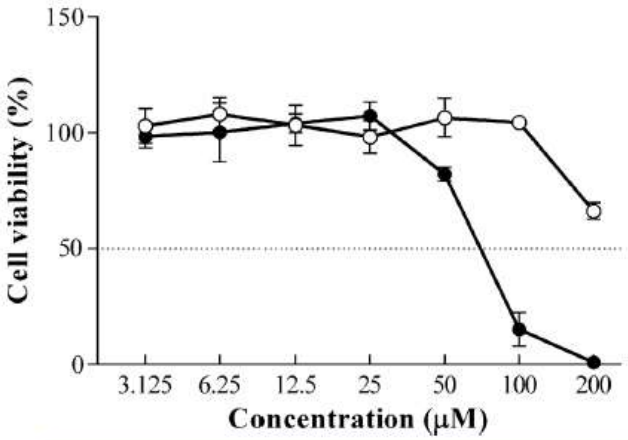
| | |
|---|---|
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Positive control** | Cinnamaldehyde |
| **Reporter gene** | Firefly luciferase (Luc) and Renilla luciferase (rLuc) |
| **Test Method** | This study describes and develops a simple and highly accurate skin sensitisation methodology (named as α-Sens), based on a dual luciferase system, that measures simultaneously antioxidant response and cytotoxicity.<br>This method evaluates simultaneously the antioxidant response element (ARE) Nrf2-mediated transcriptional activity using the *Firefly* luciferase reporter gene, and cytotoxicity through a second vector containing *Renilla* luciferase reporter gene. Further application of luciferase-based cell viability determination was used to calculate and normalize the ARE-Nrf2- mediated transcriptional activity for evaluating the skin sensitization potential of chemicals (named normalized ARE Activity -nAA).<br><br>*Establishment of cell line for the α-Sens assay*<br>The PHK-16Ob human keratinocyte cell line was used as cell host for two lentiviral constructs.<br>• The first lentiviral vector used to measure the antioxidant response was:<br>-pLVSIN-ARE-AUG-Luc. This vector contains α2-globulin promoter-driven the antioxidant response element (ARE) *AKR1C2* coupled to *firefly* luciferase reported gene.<br>• The second lentiviral vector used to measure cell viability was:<br>-pLVSIN-TK-rLuc: This vector contains thymidine kinase (TK) promoter-driven *Renilla* luciferase (rLUC) reporter gene.<br><br>PHK 16-0b cells were infected with the lentiviral vector carrying ARE-AUG-Luc or TK-rLuc, and the infected cells were selected in the presence of 50 µg/mL of hygromycin and 2.5 µg/mL of puromycin. The surviving cells were used for the subsequent assay.<br><br>*Cytotoxicity assays*<br>The cytotoxicity tests based on the mitochondrial reductase activity employed in the KeratinoSens and LuSens assays were compared with the transcriptional activity of *rLuc* for cinnamic aldehyde, the positive control substance for OECD TG442D.<br>This study conducted the water soluble tetrazolium salts-8 (WST-8) assay as the mitochondrial reductase activity-based cytotoxicity test, which uses the same principle as the traditional methylthiazolyl tetrazolium (MTT) assay adopted in the KeratinoSens and LuSens protocol.<br><br>Thus, the cell viability was calculated as follows:<br>**Cell viability (%) = absorbance of a test well at 450nm/mean absorbance for the vehicle control at 450nm× 100.**<br><br>Thereafter, the result of the cytotoxicity tests based on the mitochondrial reductase activity (WST-8 assay) was compared with the result of the rLuc based assay, and afterwards the IC50 value was calculated.<br><br>*α-Sens Assay*<br>All the chemicals were serially diluted at a common ratio of two from 200mM with DMSO, then diluted 50-fold using the cell culture medium. The vehicle control wells were also prepared with DMSO.<br><br>The Relative Light Unit (RLU) value normalized by the number of active cells (nRLU) was calculated by dividing the RLU value of ARE mediated luciferase activity of each well by the RLU value of the TK driven *Renilla* luciferase activity in a concurrent well. Then, the normalized ARE Activity (nAA) was calculated using the following formula:<br><br>**nAA=nRLU of each well/mean nRLU of the vehicle control group.** |

| | |
|---|---|
| | Fold induction was calculated for the *Firefly* luciferase activity using the following formula:<br><br>**Fold induction=RLU of luciferase in the test well/mean RLU of luciferase in the vehicle control wells.**<br><br>Cell viability was also calculated based on the *Renilla* luciferase activity using the following formula:<br><br>**Cell viability (%) = (RLU of *rLuc* in the test well/mean RLU of *rLuc* in the vehicle control wells) × 100.**<br><br>All chemicals were tested in 12 concentrations for three runs, and the maximum nAA values for each chemical were recorded in each run, then the mean maximum nAA value for each chemical and the standard deviation (SD) were calculated.<br><br>*Optimal cutoff value to distinguish sensitizers from non-sensitizers*<br>The optimal cutoff value was determined as the value providing the highest correct outcome (%) exceeding the highest mean nAA + 2SD of the non-sensitizers.<br><br>*Predictive performance of α-Sens, KeratinoSens™, and LuSens*<br>The sensitivity (percentage of positive outcomes in α-Sens against positives in LLNA or based on human data), specificity (percentage of negative outcomes in α-Sens against negatives in LLNA or based on human data) and accuracy (overall percentage of correct predictions) of the α-Sens assay were calculated according to Cooper statistics (Cooper *et al.*, Br. J. Cancer, 1979, 39, 87). These performance parameters were also calculated for KeratinoSens and LuSens. |

**Results**

**Comparison of the results of the cytotoxicity tests based on mitochondrial reductase activity and *Renilla* luciferase activity**

The IC50 value for cinnamic aldehyde was calculated as 61.9 µM, and the cell viability at 200 µM was 0.9 % in the *rLuc* activity-based cytotoxicity test. However, the IC50 value in the WST-8 assay was estimated as being over 200 µM and the cell viability at 200 µM was >50 % (Figure B.6.2.6.4/1).



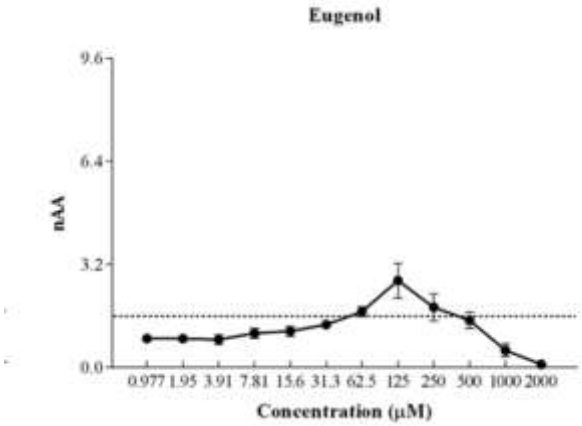

*Open circle cell viability based on mitochondrial reductase activity, closed circle cell viability based on Renilla luciferase activity*

**Figure B.6.2.6.4/1. Comparison of the outcome of cytotoxicity tests of cinnamic aldehyde using the reporter gene assay and the mitochondrial reductase activity.**

**α-Sens assay**

The optimal candidate cutoff value to distinguish sensitizers from non-sensitizers was determined as 1.6, which exceeded the highest nAA (1.32) of non-sensitizers + 2SD (=1.57), as compared to the correct outcomes (%) in human data and LLNA of 100.0 and 96.4, respectively.

The mean maximum nAA±SD for eugenol was 2.7±0.52 at 125 µM with a cell viability of 63% (Figure B.6.2.6.4/2).




*Broken line Cutoff (nAA=1.6).*

**Figure B.6.2.6.4/2. Dose-related changes in nAA of eugenol.**

**Predictive performance of α-Sens, KeratinoSens and LuSens**

The α-Sens displayed positive results for eugenol, chemical for which KeratinoSens yielded a false-negative outcome (table B.6.2.6.4/1).

The accuracy, sensitivity and specificity of α-Sens against LLNA were calculated as 96.4 %, 95.0 %, and 100.0 %, respectively, and these against human data were all 100%. The corresponding values for KeratinoSens against LLNA were calculated as 89.3 %, 85.0 %, and 100.0 %, respectively, with the values against human data being 92.9 %, 89.5 %, and 100.0 %, respectively. In regard to LuSens, the values against LLNA were calculated 89.3%, 90.0 %, and 87.5 %, respectively, and these against human data were 92.9 %, 94.7 %, and 88.9 %, respectively (table B.6.2.6.4/2).

**Table B.6.2.6.4/1. Summary of the outcomes of skin sensitisation assays for eugenol.**

| | LLNA | KeratinoSens | LuSens | Human data | α-Sens |
|---|---|---|---|---|---|
| **Eugenol** | + | - | + | + | + |

**Table B.6.2.6.4/2. Comparison of predictive performance of α-Sens, KeratinoSens, and LuSens.**

| vs. LLNA | | α-Sens | | KeratinoSens | | LuSens | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative | Positive | Negative |
| LLNA | Positive | 19 | 1 | 17 | 3 | 18 | 2 |
| | Negative | 0 | 8 | 0 | 8 | 1 | 7 |
| Sensitivity (%) | | 95.0 | | 85.0 | | 90.0 | |
| Specificity (%) | | 100.0 | | 100.0 | | 87.5 | |
| Accuracy (%) | | 96.4 | | 89.3 | | 89.3 | |
| vs. Human data | | α-Sens | | KeratinoSens | | LuSens | |

| | | Positive | Negative | Positive | Negative | Positive | Negative |
|---|---|---|---|---|---|---|---|
| **Human** | **Positive** | 19 | 0 | 17 | 2 | 18 | 1 |
| | **Negative** | 0 | 9 | 0 | 9 | 1 | 8 |
| **Sensitivity (%)** | | 100.0 | | 89.5 | | 94.7 | |
| **Specificity (%)** | | 100.0 | | 100.0 | | 88.9 | |
| **Accuracy (%)** | | 100.0 | | 92.9 | | 92.9 | |

*Sensitivity (%) = TP/(TP+FP)×100.*
*Specificity (%) =TN/(TN+FN)×100.*
*Accuracy (%) =(TP+TN) / (TP+FP+TN+FN)×100.*
*TP True positive, FP False positive, TN True negative, FN False negative.*

**Assessment and conclusion by applicant:**

This new study is considered acceptable as supporting information for the renewal of eugenol (reliable with restrictions: the paper is a review of previously conducted studies, original data for all the assays are not available).

The EU-agreed endpoint is: sensitising (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** RMS agrees with notifier assessment and conclusion.

The study is a scientific publication from a peer reviewed journal. This study describes a new skin sensitization method that measures simultaneously antioxidant response and cytotoxicity.

The RMS deems that the fact that antioxidant response and cytotoxicity can be measured at same time, does not save so much time compared with previous methods (KerationSens and LuSens), due to it´s useful, before conduct ARE assay, to know the cytotoxic concentration of a chemical for a specific cell line, and these assays could be performed follow each other.

On the other hand, this method uses lentiviral vectors, and this approach facilitates cell culture due to it is not necessary to maintain antibiotics in cell medium after clone selection, due to transgene sequence is integrated into the host genome upon viral transduction, instead of plasmids used in KeratinoSens and LuSens that are independent host cell genome elements that require a continuous antibiotic amount in the medium, and in most cases, the cell host can lost the plasmid expression.

Additionally, the authors must explain the huge difference regarding cytotoxicity values comparing mitochondrial reductase activity approach (MTT or WST-8) of KerationSens/LuSens methods, with Thymidine kinase (TK) promoter- *Renilla* luciferase (rLUC) reporter gene. It seems that TK-rLuc approach is much more sensitive than MTT/WST-8, so it should be clarified if the copy number of lentiviral sequence, or any other technical feature could affect sensitivity.

Moreover, the summary of results of the α-Sens assay for eugenol reported a mean maximum nAA±SD value of 2.7±0.52 at 63% of cell viability; however, according OECD TG 442D guideline, the test chemical concentrations for measuring antioxidant response should have > 70% viability.

Importantly, this study displayed better values of accuracy, sensitivity, and specificity than KeratinoSens and LuSens to LLNA or human data.

Therefore, RMS considers that α-Sens methodology described in this study could be taken into account as an alternative approach to currently approved guidelines. However some technical clarifications are needed.

### B.6.2.7. Phototoxicity

Phototoxicity testing is required by Regulation (EU) 283/2013 where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L × $mol^{-1}$ × $cm^{-1}$, no toxicity testing is required.

| |
|---|
| **Assessment and conclusion by applicant:**<br>Eugenol absorbs at >290 nm only at pH 11.6 (ε 3780) [EFSA Journal 2012;10(11):2914)]. Therefore, phototoxicity testing is not triggered for this active substance and no study has been performed. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier. According to Regulation (EU) No 283/2013, no phototoxicity testing is required based on the results of UV/Visible spectroscopy performed with the active substance eugenol (purity 98.8%) that showed no absorption > 290 nm in acidic and neutral conditions (White G.A., 2007). Minor absorption at 297 nm under alkaline conditions. |

## B.6.3. SHORT-TERM TOXICITY

*Six studies have been presented to address short-term toxicity of the active substance eugenol. All these studies were included in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC, and have been reassessed for the renewal process. Therefore, no new studies with eugenol have been provided.*

*The studies presented were two 14-day studies in mice and rat, two 90-day studies in mice and rat, one study of 21-day conducted in dogs, and one 19-week study in rats. All these studies were non-GLP compliant and presented methodological deficits according to the OECD guidelines (haematology, clinical chemistry, necropsy, organ weights, or histopathological examination). Therefore, these studies have been deemed as supportive information.*

### B.6.3.1. Less than 28-day study

#### B.6.3.1.1. Oral 14-day study in rats

| | |
|---|---|
| **Data point** | CA 5.3 (No EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 407 (2008). |
| **Deviations from current test guideline OECD TG 407 (2008)** | -No control group was used.<br>-14 days treatment was assayed instead of 28 days.<br>- Clinical signs, food consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology not performed.<br>- Statistical analysis was not performed in bodyweight. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483, Purity: >99% |
| **Test system** | Male and Female Fisher F344/N rats |
| **Test method** | Five groups of 5 male and 5 female Fisher F344/N rats of 6-7 weeks of age were tested *via* feed diet with eugenol at 0, 6000, 12500, 25000, 50000 and 100000 ppm (equivalent to equivalent to 0, 720, 1500, 3000, 6000 and 12000 mg/kg bw/day) for 14-day. All surviving animals were killed at day 15. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Survival:** All female rats that received the high dose died (on day 7,8,8,9 and 10) , whereas only 1 male died (on day 9) at high dose group.

**Table B.6.3.1.1/01. Survival in 14-day rats study**

| | % Survival | |
|---|---|---|
| **Dose (ppm)** | **Males** | **Females** |
| **6000** | 5/5 (100%) | 5/5 (100%) |
| **12500** | 5/5 (100%) | 5/5 (100%) |
| **25000** | 5/5 (100%) | 5/5 (100%) |
| **50000** | 5/5 (100%) | 5/5 (100%) |
| **100000** | 4/5 (80%) | 0/5 (0%) |

**Bodyweight:** A reduction in terminal bodyweights was recorded in high dose male group. Due to all females died in the high dose level, no bodyweight data was recorded in that group. On the other hand, no decreases were observed in the other dose groups for both sexes. Bodyweight gain decreases were noted from 12500 ppm male group and from 25000 ppm female group, compared with low dose group. Statistical analysis was not performed.

**Table B.6.3.1.1/02. Summary of bodyweight and bodyweight gain in 14-day rats study**

| | Males (grams) (Mean ± SE) | | | Females (grams) (Mean ± SE) | | |
|---|---|---|---|---|---|---|
| **Dose (ppm)** | **Initial** | **Final (#)** | **bwg** | **Initial** | **Final (#)** | **bwg** |
| **6000** | 82 ± 2.3 | 128 ± 2.6 **(↑56%)** | 46 ± 2.3 | 89 ± 3.0 | 121 ± 3.4 **(↑36%)** | 32 ± 3.1 |
| **12500** | 91 ± 6.6 | 133 ± 5 **(↑46%)** | 42 ± 2.6 **(↓9%)** | 85 ± 3.3 | 118 ± 1.5 **(↑39%)** | 33 ± 2.7 |
| **25000** | 92 ± 4.5 | 128 ± 5.9 **(↑40%)** | 36 ± 3.1 **(↓22%)** | 79 ± 4.2 | 101 ± 3.4 **(↑28%)** | 22 ± 1.2 **(↓31%)** |
| **50000** | 90 ± 6.5 | 103 ± 8.2 **(↑14%)** | 13 ± 3.3 **(↓72%)** | 74 ± 1.8 | 82 ± 3 **(↑11%)** | 8 ± 2.2 **(↓75%)** |
| **100000** | 98 ± 5.8 | 72 ± 3.8 **(↓27%)** | -26 ± 5.1 **(↓157%)** | 77 ± 3.6 | | |

*bwg= bodyweight gain*
*# = (% variation initial weight)*

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and was accepted as supporting information (range-finder). No NOAEL was identified in this particular study; the EU-agreed 90-day oral NOAEL is 600 mg/kg bw/d in the rat.

**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:** This study is a range-finding study to select appropriate doses for chronic toxicity studies. It is non-GLP compliant and presents several deviations, as no control group was included, absence of clinical signs, food consumption, haematology, clinical chemistry, organ weights and histopathology data. Therefore, this study is considered acceptable as supportive.

**B.6.3.1.2. Oral 14-day study in mice**

| | |
|---|---|
| **Data point** | CA 5.3 (No EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |

| | |
|---|---|
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 407 (2008). |
| **Deviations from current test guideline OECD TG 407 (2008)** | -No control group was used.<br>-14 days treatment was assayed instead of 28 days.<br>- Clinical signs, food consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology not performed.<br>- Statistical analysis was not performed in bodyweight. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483, Purity: >99% |
| **Test system** | Male and Female B6C3F$_1$ |
| **Test method** | Five groups of 5 male and 5 female B6C3F$_1$ mice of 6-7 weeks of age were tested *via* feed diet with eugenol at 0, 6000, 12500, 25000, 50000 and 100000 ppm (equivalent to 0, 1200, 2500, 5000, 10000 and 20000 mg/kg bw/day) for 14-day. All surviving animals were killed at day 15. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Survival:** All male and female mice that received the high dose died (on days 11,11,12,12, 13 for males and 7,7,7,7,8 for females, respectively). At 50000 ppm 3/5 males died (on day 10, 10 and 15), however, no deaths occurred at 50000 ppm female group.

**Table B.6.3.1.2/01. Survival in 14-day mice study**

| | % Survival | |
|---|---|---|
| **Dose (ppm)** | **Male** | **Females** |
| **6000** | 5/5 (100%) | 5/5 (100%) |
| **12500** | 5/5 (100%) | 5/5 (100%) |
| **25000** | 5/5 (100%) | 5/5 (100%) |
| **50000** | 2/5 (40%) | 5/5 (100%) |
| **100000** | 0/5 (0%) | 0/5 (0%) |

**Bodyweight:** At 50000 ppm dose groups, male mice lost the 35% of the initial bodyweights, whereas a decrease of 30% was recorded in females. At 25000 ppm dose groups, male mice lost 15% and females a 12% compared with the initial bodyweights. At 12500 ppm dose groups, male mice lost 15%, whereas females gain a 6% compared with the initial bodyweights. On the other hand, bodyweight gain decreases were noted from 12500 ppm male group and from 25000 ppm female group, compared with low dose group. Statistical analysis was not performed.

**Table B.6.3.1.2/02. Summary of bodyweight and bodyweight gain in 14-day mice study**

| | Males (grams) (Mean ± SE) | | | Females (grams) (Mean ± SE) | | |
|---|---|---|---|---|---|---|
| **Dose (ppm)** | **Initial** | **Final (#)** | **bwg** | **Initial** | **Final (#)** | **bwg** |
| **6000** | 19 ± 0.7 | 22 ± 1 **(↑16%)** | 3 ± 1 | 17 ± 0.6 | 18 ± 0.5 **(↑6%)** | 1 ± 0.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **12500** | 21 ± 0.6 | 20 ± 1 **(↓5%)** | -1 ± 0.9 **(↓133%)** | 17 ± 0.6 | 18 ± 0.4 **(↑6%)** | 1 ± 0.2 |
| **25000** | 20 ± 0.5 | 17 ± 1 **(↓15%)** | -3 ± 0.9 **(↓200%)** | 17 ± 0.4 | 15 ± 0.7 **(↓12%)** | -2 ± 1 **(↓300%)** |
| **50000** | 20 ± 0.4 | 13 ± 0.9 **(↓35%)** | -7 ± 0.9 **(↓333%)** | 17 ± 0.5 | 12 ± 0.4 **(↓30%)** | -5 ± 0.7 **(↓600%)** |
| **100000** | 21 ± 0.5 | | | 18 ± 0.4 | | |

*bwg= bodyweight gain*
*# =( % variation initial weight)*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information (range-finder). No NOAEL was identified in this particular study; the EU-agreed 90-day oral NOAEL is 900 mg/kg bw/d in the mouse.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is a range-finding study to select appropriate doses for chronic toxicity studies. It is non-GLP compliant and presents several deviations, as no control group was included, absence of clinical signs, food consumption, haematology, clinical chemistry, organ weights and histopathology data. Therefore, this study is considered acceptable as supportive.

### B.6.3.1.3. Oral 21-day toxicity study in dog

| | |
|---|---|
| **Data point** | CA 5.3 (No EU data requirement) |
| **Report author** | Lauber, F.U., Hollander, F. |
| **Report year** | 1950 |
| **Report title** | Toxicity of the Mucigogue, Eugenol, Administered by Stomach Tube to Dogs |
| **Report No** | Not applicable, published study (Gastroenterology, 15 (5), 481-186) |
| **Document No** | KCA 5.2.1/04 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 407 (2008). |
| **Deviations from current test guideline OECD TG 407 (2008)** | -Test material not characterised (batch and purity not reported).<br>-Dogs were used instead of rodents.<br>-No control groups were used.<br>-Only one dose level was tested.<br>-Test substance was administered in dose-intervals of 48-72 h.<br>-No bodyweight, food consumption, clinical chemistry and haematology were performed.<br>-Data of necropsy, organ weights and histopathology were not presented in the study. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No (prior to the enforcement of GLP). |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified |
| **Test system** | Female Mongrel dogs |
| **Test method** | 2g of eugenol/dog were administered at intervals of 48-72 hours for 10 times to 1-3 healthy female dogs *via* Levin tube. Forty-eight hours after the last administration, at day 23, the dogs were sacrificed and subjected to autopsy. Histopathology analysis was performed. |

| | |
|---|---|
| **Statistical analysis of results** | Not stated. |

**Results**

**Survival:** No mortality or morbidity signs were observed during the study.

**Clinical signs**: No clinical signs were observed during the study.

**Necropsy (data not shown in the study)**: No relevant findings were reported.

**Histopathology (data not shown in the study):** No relevant findings were reported.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and was accepted as supporting information. The EU-agreed oral NOAEL is 100 mg/kg bw/d in the dog (EFSA Journal 2012;10(11):2914. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This study is non-GLP compliant and presents important deviations. According the available information, necropsy and histopathological analysis were performed in the animals. However, no data has been presented in the study. Therefore, this study is considered acceptable as supportive.<br>No cumulative effects after eugenol administration were observed in dogs. A NOAEL of 100 mg/kg bw/day could be established based on no adverse effects detected. |

## B.6.3.2. Oral 28-day study

### B.6.3.2.1. Oral 28-day study in rat

| | |
|---|---|
| **Data point** | CA 5.3 (EU data requirement) |
| **Report author** | 1. Sipes, I.G., Mattia, A.<br>2. Hirose, M., Masuda, A., Maida, K., Kagawa, M., Tsuda, H. and Ito, N. |
| **Report year** | 1.2006<br>2.1986 |
| **Report title** | 1. Eugenol and related hydroxyallylbenzene derivates.<br>This report includes a summary from the following published study:<br>2. Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. |
| **Report No** | 1. WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200.<br>2. None, published study (Jpn. J. Cancer Res.; 78, 317-321). |
| **Document No** | KCA 5/05 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 407 (2008). |
| **Deviations from current test guideline OECD TG 407 (2008)** | -Test substance not characterised (purity, batch number and certificate of chemical analysis were not reported).<br>- Only 5 male rats were used.<br>- Only one dose level was assayed.<br>- Food consumption, haematology and clinical chemistry were not performed.<br>- Only livers and stomachs were subjected to gross examinations and histopathological analysis.<br>-Bodyweight and histopathology data not presented. |
| **Previous evaluation** | No |

| | |
|---|---|
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified |
| **Test system** | Male Fisher F344/N rats |
| **Test method** | Five male Fisher F344/N rats of 6 weeks of age were tested *via* powdered basal diet with 2% eugenol (equivalent to 2000 mg/kg bw/day) for 28-day. Five control animals received diet without any addition. The rats had free access to food and water and were weighed weekly. The rats were killed for autopsy at the end of experimental week 4. The liver was weighed, and buffered formalin solution was injected into the stomach. Then, the anterior and posterior walls of the forestomach were each cut into 6 to 7 strips for histological examination. |
| **Statistical analysis of results** | Not stated. |

**Results**

**Survival:** No mortality or morbidity signs were observed during the study.

**Clinical signs**: No clinical signs were observed during the study.

**Bodyweight (data not shown in the study):** A reduction of 10-15% was observed in rats treated with 2% eugenol.

**Liver weight (data not shown in the study):** No differences were described between treated group and controls**.**

**Histopathology (data not shown in the study):** No gross or histological changes were found in the forestomach.

| |
|---|
| **Assessment and conclusion by applicant:**<br>Not provided |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** In this review it is included a summary with the main short term and long term published studies with eugenol. This published study is non-GLP compliant and presents important deviations. Therefore, this study is considered acceptable as supportive.<br>No adverse effects in forestomach and liver were observed in rats after 28-day diet treatment with 2% eugenol (equivalent to 2000 mg/kg bw/day). A LOAEL = 2000 mg/kg bw/day could be established based on bodyweight reduction, so a NOAEL cannot be derived. |

## B.6.3.3. Oral 34-day study

### B.6.3.3.1. Oral 34-day study in rat

| | |
|---|---|
| **Data point** | CA 5.3 (No EU data requirement) |
| **Report author** | 1. Sipes, I.G., Mattia, A.<br>2.Hagan, EC., Jenner, P.M., Jones, W.I., Fitzhugh, O.G., Long, E.L., Brouwer, J.G. and Webb, W.K. |
| **Report year** | 1.2006<br>2.1965 |
| **Report title** | 1. Eugenol and related hydroxyallylbenzene derivates.<br>This report includes a summary from the following published study:<br>2.Toxic properties of compounds related to safrole. |

| | |
|---|---|
| **Report No** | 1. WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200.<br>2. None, published study (Toxicology and applied pharmacology, 18-24). |
| **Document No** | KCA 5/05 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 407 (2008). |
| **Deviations from current test guideline OECD TG 407 (2008)** | -Test substance not characterised (purity, batch number and certificate of chemical analysis are not reported).<br>-Only male rats were assayed.<br>-The dose pattern used between the dose-range applied is not specified.<br>-The dosage levels in which the adverse effects were described are not stated.<br>-No clinical biochemistry analysis was performed.<br>-No data of bodyweights, food consumption, haematology, necropsy, organ weights and histopathological analysis were presented. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified |
| **Test system** | Osborne-Mendel rats |
| **Test method** | Twenty male weanling Osborne-Mendel rats were given 1400 mg/kg bw per day of eugenol by stomach tube, and the dose was gradually increased to 4000 mg/kg bw per day during the 34-day study. Gross necropsy, macroscopic and histopathological examinations were not detailed. |
| **Statistical analysis of results** | Not stated. |

**Results**

**Survival (data not shown in the study):** A few deaths were reported at 2000 mg/kg bw/day, and the number increased with increasing doses. Eight animals survived at termination study (40%).

**Clinical signs**: No clinical signs were observed during the study.

**Bodyweight (data not shown in the study):** No differences were observed between treated groups and controls.

**Food consumption (data not shown in the study):** No differences were observed between treated groups and controls.

**Haematology (data not shown in the study):** No differences were observed between treated groups and controls.

**Necropsy (data not shown in the study):** Eugenol treatment caused slight adrenal enlargement, with marked yellow discolouration, and slight enlargement of the liver, which was found microscopically to be due to slight liver-cell enlargement. Macroscopic examination of the forestomach showed that the mucosa contained coalescent areas covered with a thick, flaky, white material punctuated with minute ulcers.

**Histopathology (data not shown in the study):**

*Forestomach:*

Moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration.

*Liver:* Slight liver-cell enlargement.

*Bones:*

Mild osteoporosis.

| |
|---|
| **Assessment and conclusion by applicant:**<br>Not provided |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** In this review it is included a summary with the main short term and long term published studies with eugenol. This published study is non-GLP compliant and presents important deviations. Therefore, this study is considered acceptable as supportive.<br>Thirty four-day gavage administration of eugenol caused adverse effects in forestomach (moderately-severe hyperplasia and hyperkeratosis), slight observable effects in liver (enlargement due to slight liver-cell enlargement) and in adrenal glands (enlargement and discolouration). The doses in which effects were observed had not been specified in the study. The MTD is probably exceed in this study, so reliability of findings should be taken cautiously. |

## B.6.3.4. Oral 90-day study

### B.6.3.4.1. Oral 13-week study in rats

| | |
|---|---|
| **Data point** | CA 5.3 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 408 (2018). |
| **Deviations from current test guideline OECD TG 408 (2018)** | - Food consumption, haematology, clinical chemistry and ophtalmological examination were not performed.<br>- Statistical analysis was not performed.<br>- Organ weights were not recorded.<br>- Data of necropsies and histopathology were not presented.<br>- Circulating thyroid hormones (T4, T3, TSH) levels were not measured. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (refer to the assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483, Purity: >99% |
| **Test system** | Male and Female Fisher F344/N rats |
| **Test method** | Six groups of 10 male and 10 female Fisher F344/N rats of 6-7 weeks of age were tested *via* feed diet with eugenol at 0, 800, 1500, 3000, 6000, 12500 ppm (equivalent to 0, 72, 135, 270, 540 and 1125 mg/kg bw/day) for 13 weeks (91 days). Observations for clinical signs or mortality were made twice daily and animals were weighed weekly. At the end of the 91-day study, survivors were killed and necropsies were performed on all animals. Tissues from the control and the highest dose groups were taken for histopathologic analysis. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Survival:** No differences in survival were noted between treated groups and controls.

**Table B.6.3.4.1/01. Survival in 13-week rat study**

| | % Survival | |
|---|---|---|
| **Dose (ppm)** | **Males** | **Females** |
| **0** | 10/10 (100%) | 10/10 (100%) |
| **800** | 9/9 (100%) | 10/10 (100%) |
| **1500** | 10/10 (100%) | 10/10 (100%) |
| **3000** | 10/10 (100%) | 10/10 (100%) |
| **6000** | 10/10 (100%) | 9/9 (100%) |
| **12500** | 10/10 (100%) | 10/10 (100%) |

**Bodyweight:** A decrease in bodyweight and bodyweight gain (10% and 12%, respectively) were recorded in the top dose male group compared with controls. On the other hand, no differences were noted in the female dose groups.

**Table B.6.3.4.1/02. Summary of bodyweight and bodyweight gain in 13-week rat study**

| | Males (grams) (Mean ± SE) | | | Females (grams) (Mean ± SE) | | |
|---|---|---|---|---|---|---|
| **Dose (ppm)** | **Initial** | **Final (% of controls)** | **bwg** | **Initial** | **Final (% of controls)** | **bwg** |
| **0** | 69 ± 3.8 | 334 ± 5.4 | 265 ± 3.4 | 71 ± 1.8 | 190 ± 1.9 | 119 ± 1.8 |
| **800** | 66 ± 2.6 | 330 ± 4.9 **(98%)** | 264 ± 3.6 | 68 ± 2.9 | 188 ± 2.4 **(99%)** | 120 ± 2.5 |
| **1500** | 68 ± 2.6 | 324 ± 5.2 **(97%)** | 256 ± 5.4 | 71 ± 2.1 | 188 ± 3.4 **(99%)** | 117 ± 2.3 |
| **3000** | 68 ± 3.4 | 324 ± 6.1 **(97%)** | 256 ± 5.4 | 65 ± 1 | 184 ± 2.4 **(97%)** | 119 ± 2.5 |
| **6000** | 63 ± 2.4 | 309 ± 3.8 **(92%)** | 246 ± 3.4 | 69 ± 1.8 | 184 ± 2.0 **(97%)** | 115 ± 2.7 |
| **12500** | 68 ± 3.1 | 300 ± 3.9 **(90%)** | 232 ± 4.4 **(↓12%)** | 66 ± 1.8 | 178 ± 2.2 **(94%)** | 112 ± 2.5 |

*Statistical analysis not performed*

**Necropsy (data not shown in the study):** No differences in gross necropsy examination were detected between high dose group and controls.

**Histopathology (data not shown in the study):** No differences in tissue histopathology were observed between high dose group and controls.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information. The EU-agreed oral NOAEL is 600 mg/kg bw/d (13-week) in the rat, based on reduced body weight (≥10%) in males (EFSA Journal 2012;10(11):2914)..

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is non-GLP compliant and presents several deviations such as absence of food consumption, haematology and clinical chemistry data. Therefore, this study is considered acceptable as supportive. This study was conducted to determine the most appropriate doses for the 2-year study.

Necropsy and histopathology examinations did not reveal differences between treated groups and controls.

A **NOAEL** could be established in 6000 ppm (540 mg/kg bw/day) based on the decreased bodyweight and bodyweight gain in males at study termination.

**B.6.3.4.2. Oral 13-week study in mice**

| | |
|---|---|
| **Data point** | CA 5.3 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 408 (2018). |
| **Deviations from current test guideline OECD TG 408 (2018)** | - Food consumption, haematology, clinical chemistry and ophtalmological examination were not performed.<br>- Statistical analysis was not performed.<br>-Organ weights were not recorded.<br>- Data of necropsies and histopathology were not presented.<br>- Circulating thyroid hormones (T4, T3, TSH) levels were not measured. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483, Purity: >99% |
| **Test system** | Male and Female B6C3F$_1$ mice |
| **Test method** | Six groups of 10 male and 10 female B6C3F$_1$ mice of 6-7 weeks of age were tested *via* feed diet with eugenol at 0, 400, 800, 1500, 3000 and 6000 ppm (equivalent to 0, 80, 160, 300, 600 and 1200 mg/kg bw/day) for 13 weeks (91 days). Observations for clinical signs or mortality were made twice daily and animals were weighed weekly. At the end of the 91-day study, survivors were killed and necropsies were performed on all animals. Tissues from the controls and the highest dose group were taken for histopathologic analysis. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Survival:** No differences in survival were noted between treated groups and controls.

**Table B.6.3.4.2/01. Survival in 13-week mice study**

| | % Survival | |
|---|---|---|
| **Dose (ppm)** | **Males** | **Females** |
| **0** | 10/10 (100%) | 10/10 (100%) |
| **400** | 10/10 (100%) | 10/10 (100%) |
| **800** | 10/10 (100%) | 10/10 (100%) |
| **1500** | 10/10 (100%) | 10/10 (100%) |
| **3000** | 10/10 (100%) | 10/10 (100%) |
| **6000** | 10/10 (100%) | 10/10 (100%) |

**Bodyweight:** A decrease in bodyweight gain was detected in the top male dose group (10%) and in 400, 1500 and 3000 ppm female groups (14%), compared with controls.

**Table B.6.3.4.2/02. Summary of bodyweight and bodyweight gain in 13-week mice study**

| | Males (grams) (Mean ± SE) | | | Females (grams) (Mean ± SE) | | |
|---|---|---|---|---|---|---|
| **Dose (ppm)** | **Initial** | **Final (% of controls)** | **bwg** | **Initial** | **Final (% of controls)** | **bwg** |
| **0** | 21 ± 0.5 | 31 ± 0.5 | 10 ± 0.5 | 17 ± 0.3 | 24 ± 0.4 | 7 ± 0.3 |
| **400** | 22 ± 0.6 | 32 ± 0.7 **(103%)** | 10 ± 0.9 | 18 ± 0.4 | 24 ± 0.7 **(100%)** | 6 ± 0.4 **(↓14%)** |
| **800** | 22 ± 0.6 | 33 ± 0.5 **(106%)** | 11 ± 0.6 **(↑10%)** | 17 ± 0.4 | 24 ± 0.5 **(100%)** | 7 ± 0.4 |
| **1500** | 22 ± 0.7 | 32 ± 0.5 **(103%)** | 10 ± 0.4 | 17 ± 0.4 | 23 ± 0.5 **(96%)** | 6 ± 0.2 **(↓14%)** |
| **3000** | 21 ± 0.4 | 31 ± 0.5 **(100%)** | 10 ± 0.6 | 17 ± 0.4 | 23 ± 0.5 **(96%)** | 6 ± 0.3 **(↓14%)** |
| **6000** | 22 ± 0.5 | 31 ± 0.5 **(100%)** | 9 ± 0.5 **(↓10%)** | 17 ± 0.3 | 24 ± 0.3 **(100%)** | 7 ± 0.3 |

*Statistical analysis not performed*

**Necropsy (data not shown in the study):** No differences in gross necropsy examination were detected between high dose group and controls.

**Histopathology (data not shown in the study):** No differences in tissue histopathology were observed between high dose group and controls.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information. The EU-agreed oral NOAEL is 900 mg/kg bw/d (13-week) in the mouse (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is non-GLP compliant and presents several deviations such as absence of food consumption, haematology and clinical chemistry data. Therefore, this study is considered acceptable as supportive. This study was conducted to determine the most appropriate doses for the 2-year study.

Necropsy and histopathology examinations did not reveal differences between treated groups and controls.

A **NOAEL** could be established in 3000 ppm (600 mg/kg bw/day) based on the decreased bodyweight gain in males at study termination.

## B.6.3.5. More than 90- day study

### B.6.3.5.1. Oral 19-week study in rats

| | |
|---|---|
| **Data point** | CA 5.3 (No EU data requirement) |
| **Report author** | Hagan, E.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones W.I. |
| **Report year** | 1967 |
| **Report title** | Food Flavourings and Compounds of Related Structure. 11. Subacute and Chronic Toxicity*”*. |
| **Report No** | Not applicable, published study (Fd. Cosmet. Toxicology, 1967; 5*:*141-157). |
| **Document No** | KCA 5.3.2/03 |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 408 (2018). |
| **Deviations from current test guideline OECD TG 408 (2018)** | - Test substance not characterised (purity, batch number and certificate of chemical analysis are not reported).<br>- Only two dose levels were assayed.<br>- The doses used between the dose-range applied are not specified. |

| | |
|---|---|
| | - No clinical biochemistry analysis was performed.<br>- No data of bodyweights, food consumption, haematology, necropsy, organ weights and haematological analysis were presented. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified<br>In the case of eugenol (which was included in the diet) neither purity nor batch were indicated. According to the publication: *Commercially available materials, rather than pure chemicals, were used since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives*. |
| **Test system** | Osborne-Mendel rats |
| **Test method** | Three groups of 10 male and 10 female weanling Osborne-Mendel rats were tested *via* feed diet with eugenol at 0, 1000 and 10000 ppm (equivalent to 0, 90 and 900 mg/kg bw/day) for 19 weeks (133 days). Weekly measurements of body weight, general condition and food intake were performed. All surviving animals were killed, and haematological examinations (white and red cell counts, haemoglobin concentration and erythrocyte volume fraction) were performed. At necropsy, liver, kidneys, spleen, heart and testes were weighed. These organs, the abdominal and thoracic viscera, one hind leg, for bone, bone marrow and muscle, were preserved in 10% buffered formalin-saline solution for histopathological examination. |
| **Statistical analysis of results** | Not stated. |

**Results**

**Survival:** No mortality or morbidity signs were observed during the study.

**Clinical signs**: No clinical signs were observed during the study.

**Bodyweight (data not shown in the study):** No differences were observed between treated groups and controls.

**Food consumption (data not shown in the study):** No differences were observed between treated groups and controls.

**Haematology (data not shown in the study):** No differences were observed between treated groups and controls.

**Necropsy (data not shown in the study):** No differences were observed between treated groups and controls.

**Histopathology (data not shown in the study):** No differences were observed between treated groups and controls.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and was accepted as supporting information. The study NOAEL is the highest dose tested (~500 mg/kg bw/d; dietary exposure). The EU-agreed oral NOAEL is 600 mg/kg bw/d (13-week) in the rat. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This published study is non-GLP compliant and presents several deviations. Therefore, this study is considered acceptable as supportive. |

No adverse effects were detected in rats after repeated oral exposure of eugenol throughout 19 weeks.

For this renewal assessment, the conversion of concentrations in feed into daily doses has been performed following the Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data, 2012 (EFSA Journal 2012;10(3):2579).

Following this guideline, a default factor of 0.09 for rats should be used for subchronic studies starting usually at week 5 to 7 (in this case, weanling rats were used). Therefore, administered doses in this study have been calculated as 90 and 900 mg/kg bw/day in diet.

According to the guideline, the converting factor of 0.05 that was used in the previous DAR (calculated by the Notifier using default conversion factors), and by the applicant in this renewal assessment should be applied for chronic studies (or subacute or subchronic studies starting in a later age).

It should be noted that the results obtained in this study were obtained with the commercially available eugenol used as food additive, instead of the technical active substance.

A NOAEL of **10000 ppm (900 mg/kg bw/day**) could be established based on no adverse effects observed at the high dose level tested.

### B.6.3.6. Other routes

No repeat- dose studies *via* dermal route have been submitted for the renewal process of eugenol. According to the criteria of Regulation (EC) 283/2013, no dermal studies need to be considered if the active substance is a severe irritant. The available data reported for eugenol indicate that it is a severe/moderate/mild irritant in rabbit, guinea pig, miniature swine, and human (B.6.2.4). Furthermore, eugenol is a skin sensitiser. For all these reasons, the RMS is of the opinion that no dermal repeat-dose toxicity studies are deemed necessary.

No inhalation repeat-dose studies have been submitted with eugenol. Eugenol has a vapour pressure of 12.2 Pa measured at 25ºC, hence it is a volatile compound. The RMS does not consider inhalation studies necessary based on the following:

- Eugenol did not produce any mortality at the maximum attainable dose in the acute toxicity inhalation study ($LC_{50}$> 2.58 mg/L, for male and female rats). Furthermore, acute toxicological inhalation study performed with the plant protection product (Mevalone, 3.2% eugenol) displayed a $LC_{50}$ > is 2.280 mg/L (high dose tested).
- Mevalone is applied through outdoor foliar spraying against *Botrytis* on table and wine grapes. Application is *via* an encapsulated formulation, so although eugenol is volatile, operators will not be exposed to high concentrations of eugenol because it will be encapsulated and hence not available for volatilization. Moreover, human exposure also occurs *via* inhalation from the presence of eugenol in cosmetics and in essential oils used for aromatherapy. Overall, inhalation is not a relevant route for the proposed uses.

## B.6.4. GENOTOXICITY

*Most data to address this point were presented in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC and were deemed acceptable following evaluation and peer review at EU level. A total of 29 studies have been submitted for the renewal process to evaluate the genotoxicity of eugenol, of which 16 correspond to studies in vitro and 13 correspond to studies in vivo. Three new genotoxicity studies have been submitted for the renewal process to address the genotoxicity of eugenol (one in vitro chromosome aberration, one in vitro comet assays and one in vivo micronucleus assay). All studies have been re-evaluated following current guidelines for the renewal process.*

*In Commission Regulation (EU) No. 283/2013 in vitro photomutagenicity studies may be indicated by the structure of a molecule. If the ultraviolet/visible (UV/VIS) molar extinction/absorption coefficient of the active substance and its major metabolites is less than 1000 L x mol $^{-1}$ x cm $^{-1}$ photomutagenicity testing is not required. In the case of eugenol (alkaline), the molar extinction/absorption coefficient is 3780 L x mol$^{-1}$ x cm$^{-1}$ at 297 nm, although maximum absorption occurs at 206 nm (30461 L x mol$^{-1}$ x cm$^{-1}$) (White, G.A., 2007). The neutral or acid forms of eugenol do not absorb at UV/vis spectrum. Due to the minimal absorption of the alkaline form in the UV/vis spectrum no photomutagenicity testing is considered to be necessary.*

### B.6.4.1. *In vitro* studies

#### B.6.4.1.1. Bacterial gene mutation

##### B.6.4.1.1.1 Study I

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Ishidate, M., Sofuni, T., Yoshikawa K., Hayashi, M., Nohmi, T., Sawada, M. and Matsuoka, A. |
| **Report year** | 1984 |
| **Report title** | Primary mutagenicity screening of food additives currently used in Japan. |
| **Report No** | Not applicable, published study (*Food Chem. Toxic*. 22 (8), 623-636) |
| **Document No** | KCA 5.4.1/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 471 (2020)** | - Batch of test chemical not reported<br>-No individual test results and statistics have been provided, just overall result.<br>- Despite the use of 5 strains, the recommended strain TA102 or WP2*uvr*A have not been tested.<br>-Positive controls not reported<br>-No historical control data are reported<br>- No repeat assay was performed. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 100.0 %; Batch No.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Not specified |
| **Test system** | *S. typhimurium* TA92, TA1535, TA1537, TA94, TA98 and TA100 |
| **Metabolic activation** | S9 from Fischer rat liver induced with Kaneclor KC-400 |
| **Test method** | A pre-incubation test was conducted according to the test method reported by Ames *et al.* (1975). Eugenol was tested at 6 different concentrations up to 2000 µg/plate in *S. typhimurium* strains TA92, TA1535, TA1537, TA94, TA98 and TA100. Metabolic activation was derived from Fischer rat liver induced with Kaneclor KC-400. Duplicate plates were used for each concentration.<br><br>The maximum dose represents the highest non-cytotoxic dose used in the experiment in case of a negative result. |

| | |
|---|---|
| | No statistical analysis was stated. |
| **Criteria for assessment of results** | A negative result indicates that no significant increases in the number of revertant colonies were detected in any *S. typhimurium* strains at the maximum dose.<br>The result was considered positive if the number of colonies found was twice the number in the control (exposed to the appropriated solvent or untreated). If no reasonable dose response was detected, additional experiments using different doses or induced mutation frequency assays were performed (Yoshikawa, Nakadate, Watabe *et al.* 1980). |

**Results**
A negative result was reported for eugenol up to 2000 µg/plate with and without metabolic activation. This indicates that no significant increases in the number of revertant colonies were detected in any of the *S. typhimurium* strains tested compared to the control.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion.<br>Under the conditions of the study, eugenol is negative in *S. typhimurium strains* TA92, TA1535, TA1537, TA94, TA98 and TA100, in the presence and absence of metabolic activation.<br>The study is a scientific publication from a peer-reviewed journal. The study follows the Ames method reported by Ames *et al.* (1975), which is comparable to OECD TG 471 (1983). Deviations from current OECD TG 471 (2020) include no characterisation (batch no.) and stability determination of the test item. Despite five *S. typhimurium* strains tested no TA102 or WP2*uvr*A were tested. The results are provided as a summary, hence no individual test results are reported, nor positive controls and historical control data.<br>Based on the lack of information available from the study report, the RMS deems the study as supporting information only. |

**B.6.4.1.1.2 Study II**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Haworth, S., Lawlor, T., Mortelmans, K., Speck, W. and Zeiger, E. |
| **Report year** | 1983 |
| **Report title** | Salmonella mutagenicity test results for 250 chemicals |
| **Report No** | Not applicable, published study (*Environ. Mutagen. Suppl.* 1, 3-142) |
| **Document No** | KCA 5.4.1/02 |
| **Guidelines followed in study** | Modification of pre-incubation procedure test (Yahagi *et al.* 1975*)*. Previous to OECD TG 471 (1983) |
| **Deviations from current test guideline**<br>**OECD TG 471 (2020)** | -Four *S. typhimurium* strains tested (instead of five strains)<br>-Statistical analysis not reported<br>-No historical control data<br>-An additional indicator of S9-mix efficacy should be used in combination with 2-aminoanthracene |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: C8A |
| **Solvent** | Not stated |

| | |
|---|---|
| **Negative control** | Solvent |
| **Positive control (-S9)** | 4-Nitro-o-phenylenediamine (NOPD) for TA98<br>Sodium azide for TA100 and TA1535<br>9-Aminoacridine (9AA) for TA1537 |
| **Positive control (+S9)** | 2-Aminoanthracene (2AA) for *S. typhimurium*: TA98, TA100, TA1535 and TA1537 |
| **Test system** | *S. typhimurium* TA98, TA100, TA1535 and TA1537 |
| **Metabolic activation** | Liver S9 was prepared from male Sprague Dawley rats (RLI) and Syrian hamsters (SLI) induced with Aroclor 1254 (500 mg/kg bw) |
| **Test method** | The pre-incubation procedure was the chosen method with some modifications (Yahagi *et al.* 1975).<br>A preliminary dose range-finding/toxicity study was carried out using *S. typhimurium* TA100 up to a concentration of 10000 µg/plate or the limit of solubility, both in the presence and absence of S9 metabolic activation (+/- S9). As indication of toxicity different parameters were chosen: reduced numbers of revertant colonies per plate and/or thinning or absence of the bacterial lawn. If toxicity was observed, the high dose chosen tested for the experiment exhibited some degree of toxicity.<br>Liver S9 was prepared from Sprague-Dawley rats and Syrian hamsters with Aroclor 1254. The S9 mix was prepared immediately prior to each assay.<br>At least five concentrations of the test material were tested on each strain, TA98, TA100, TA1535 and TA1537 both in the presence and absence of metabolic activation.<br>Cultures were prepared as Ames *et al.*, 1975 described. After the pre-incubation period and the overlay solidification, the plates were inverted and incubated for about 48 hours at 37 °C. Three plates were used and the experiment was repeated no less than 1 week after completion of the initial test.<br>Statistical analysis is based on the models presented by Margolin *et al.* (1981) |
| **Criteria for assessment of results** | The test substance is considered to be positive if in at least one strain a reproducible dose-related increase in the mean number of revertants per plate above negative control is demonstrated, whether it be two-fold over background or not. Inconclusive or questionable are applied to results that showed a definite trend but with which the investigator did not feel comfortable in making a positive or negative decision. It also includes tests in which an elevated revertant colony yield occurred at only a single dose level. |

**Results**

Under the conditions of this study, eugenol is not mutagenic in the *S. typhimurium* strains TA98, TA100, TA1535 and TA1537. Positive controls gave a satisfactory response in the presence and absence of metabolic activation (rat and hamster).

Average revertant counts for TA98 and TA100 are displayed in the Table B.6.4.1.1.2/1; data for TA1535 and TA1537 are shown in Table B.6.4.1.1.2/2.

**Table B.6.4.1.1.2/1. Average revertants per plate in tester strains TA98 and TA100**

| Concentration (µg/plate) | Revertants/plate (mean ± SD) | | | | | |
|---|---|---|---|---|---|---|
| | TA98 | | | TA100 | | |
| | -S9 | +S9 (rat) | +S9 (hamster) | -S9 | +S9 (rat) | +S9 (hamster) |
| Vehicle (control) | 27 ± 3.1 | 35 ± 2.3 | 37 ± 4.7 | 99 ± 5.2 | 113 ± 2.0 | 115 ± 8.7 |
| 3.3 | 21 ± 2.3 | 37 ± 2.3 | 34 ± 3.3 | 85 ± 3.2 | 105 ± 3.7 | 124 ± 11.3 |
| 10 | 20 ± 2.6 | 33 ± 6.2 | 44 ± 1.7 | 80 ± 5.8 | 104 ± 4.0 | 111 ± 11.0 |
| 33.3 | 17 ± 1.2 | 46 ± 2.2 | 36 ± 2.1 | 85 ± 5.3 | 108 ± 2.6 | 111 ± 10.5 |
| 100.0 | 19 ± 4.2 | 36 ± 1.9 | 37 ± 2.0 | 73 ± 3.6 | 107 ± 2.6 | 103 ± 8.5 |
| 333.3 | 13 ± 2.3 | 36 ± 4.0 | 41 ± 1.5 | 77 ± 2.2* | 109 ± 3.0 | 107 ± 10.3 |

| Concentration (µg/plate) | Revertants/plate (mean ± SD) | | | | | |
|---|---|---|---|---|---|---|
| | TA98 | | | TA100 | | |
| | -S9 | +S9 (rat) | +S9 (hamster) | -S9 | +S9 (rat) | +S9 (hamster) |
| Positive control (S9 from rat and hamster)<br>TA98/-S9=5.0<br>TA98/+S9=1.0<br>TA100/-S9=1.0<br>TA100/+S9=1.0 | 636 ± 3.4[a] | 288 ± 3.0[b] | 1258 ± 130.5[b] | 625 ± 28.8[c] | 460 ± 15.3[b] | 963 ± 104.3[b] |

[a] *: 4-Nitro-o-phenylenediamine (NOPD);* [b]*: 2-Aminoanthracene(2-AA);* [c]*: Sodium azide (SA)*; *Cytotoxicity

**Table B.6.4.1.1.2/2. Average revertants per plate in tester strains TA1535 and TA1537**

| Concentration (µg/plate) | Revertants/plate (mean ± SD) | | | | | |
|---|---|---|---|---|---|---|
| | TA1535 | | | TA1537 | | |
| | -S9 | +S9 (rat) | +S9 (hamster) | -S9 | +S9 (rat) | +S9 (hamster) |
| Vehicle (control) | 20 ± 1.0 | 13 ± 3.0 | 13 ± 0.6 | 8 ± 1.0 | 14 ± 1.9 | 12 ± 2.7 |
| 3.3 | 18 ± 3.8 | 9 ± 1.0 | 17 ± 3.5 | 10 ± 0.9 | 9 ± 0.9 | 11 ± 2.0 |
| 10 | 16 ± 1.8 | 10 ± 1.0 | 10 ± 1.2 | 7 ± 1.5 | 13 ± 1.5 | 11 ± 0.7 |
| 33.3 | 21 ± 1.5 | 7 ± 0.3 | 12 ± 2.3 | 8 ± 1.8 | 9 ± 3.2 | 14 ± 3.3 |
| 100 | 22 ± 4.3 | 11 ± 1.0 | 13 ± 2.6 | 6 ± 0.9 | 11 ± 1.8 | 11 ± 2.2 |
| 333.3 | 21 ± 1.5 | 9 ± 1.9 | 13 ± 2.9 | 4 ± 1.2* | 9 ± 1.7 | 14 ± 1.7 |
| Positive control (S9 from rat and hamster)<br>TA1535/-S9=1.0<br>TA1535/+S9=2.5<br>TA1537/-S9=50.0<br>TA1537/+S9=2.5 | 444 ± 23.0[a] | 305 ± 3.2[b] | 268 ± 34.8[b] | 710 ± 64.7[c] | 158 ± 10.6[b] | 244 ± 5.8[b] |

[a]*: Sodium azide;(SA);* [b] *: 2-Aminoanthracene (2AA);* [c] *: 9-Aminoacridine (9-AAD)* ; *Cytotoxicity

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**<u>Assessment and conclusion by RMS</u>:**

The RMS agrees with the notifier's assessment and conclusion.

Under the conditions of the study, eugenol is negative in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, in the presence and absence of metabolic activation. Cytotoxicity was observed at 333.3 µg/plate in TA 100 and TA1537 strains without metabolic activation.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 471 (2020) guideline include the use of four instead of five strains (no cross linking mutagenicity tested in the study), cytotoxicity is not achieved at some top doses under the conditions of this study, no statistical analysis or the use of an additional indicator of S9 mix efficacy in combination with 2-amminoanthracene. However, these deviations are not considered to have an impact on the outcome of the study. The RMS deems the study as supporting information to evaluate the bacterial gene mutation of eugenol.

**B.6.4.1.1.3 Study III**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Green, N.R. and Savage, J.R. |
| **Report year** | 1978 |
| **Report title** | Screening of safrole, eugenol, and their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. |
| **Report No** | Not applicable, published study (*Mutat. Res.* 57, 115–121) |
| **Document No** | KCA 5.4.1/03 |
| **Guidelines followed in study** | Not stated. Previous to OECD TG 471 (1983) |
| **Deviations from current test guideline**<br>**OECD TG 471 (2020)** | - No characterisation and stability determination of the test item<br>- Only four *S. typhimurium* tested strains although none of them are recommended by the guideline<br>- The enzyme-inducing agent for S9 is not reported<br>- Very limited information on the study reports, no individual data provided<br>- No historical control data |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No, pre-GLP requirement |
| **Acceptability/ reliability** | Not acceptable (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | Not stated |
| **Negative control** | Not stated |
| **Positive control** | Direct bacterial assay: N-methyl-N-nitrosoguanidine<br>Microsomal and host-mediated assays: Dimethylnitrosamine |
| **Test system** | Direct bacterial asay and microsomal assay: *S. typhimurium* strains TA1530, TA1531, TA1532 and TA1964.<br>Host-mediated assay: male mice (C3H/HeJ) and *S. typhimurium* strains TA1534, TA1950, TA1951 and TA1952. |
| **Metabolic activation** | Microsomal assay: liver fraction prepared from male mice (C3H/HeJ) (enzyme-inducing agent not reported) |
| **Test method** | Three assays were performed with eugenol:<br>**Direct bacterial assay**:<br>Method described in Ames, B.N. and Yanofsky, C. (1971), where media and incubation mixture are described. Concentration range was 1000-5000 µg/plate. The plates were incubated at 37ºC for two days, after which time the number of revertant colonies appearing was counted.<br><br>**Microsomal Assay**:<br>Method described by Malling H.V. *et al.* (1971) and it consists of mouse-liver post-mitochondrial fraction for activation of the test item and the Salmonella strains for mutagen detection.<br>Homogenised livers were obtained from three male mice (C3H/HeJ). The test compound was incubated with the liver fraction, NADPH + $H^+$ solution, $MgCl_2$ $6H_2O$ solution and a bacterial suspension of $2x10^9$ bacteria/mL of saline (*S. typhimurium* TA1530, TA1532, TA1532 and TA1964). The concentration of test item is 0.02 and 0.2 M. After incubation, the suspension was diluted and incorporated into an overlay media and poured on agar plates. After a two-day incubation period, the number of revertant/$10^6$ survivors was determined.<br><br>**Host-mediated Assay**:<br>Method described by Legator, M.S. and Malling, H.V. (1971).<br>Male mice (C3H/HeJ) were injected with 2 mL of overnight growth of one of the *S. typhimurium* strains. The test compound (0.1 mL at a concentration 200 mg/kg) was given to the mice by intramuscular injection. Three hours later, they were sacrificed. Each mouse received a 1 mL injection of isotonic saline and the peritoneum fluid was |

| | |
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| | plated following the method described by Legator and Malling (1971). The mutant frequency was studied following a two-day incubation at 37ºC. |
| **Criteria for assessment of results** | The test substance is considered to be positive if in at least one strain a reproducible dose-related increase in the mean number of revertants per plate above negative control is demonstrated, whether it be two-fold over background or not. Inconclusive or questionable are applied to results that showed a definite trend but with which the investigator did not feel comfortable in making a positive or negative decision. It also includes tests in which an elevated revertant colony yield occurred at only a single dose level. For the host-mediated assay, a compound is mutagenic if the mutant frequency increased by a factor of 10 over the control value. |

**Results**

Average revertant counts for the three assays are displayed in Table B.6.4.1.1.3. Eugenol is not mutagenic with or without S9. Positive controls gave a satisfactory response.

**Table B.6.4.1.1.3. Average revertant counts in the three tester strains**

| **Direct Bacterial Assay (-S9)** | | | | |
|---|---|---|---|---|
| Concentration | Revertants/plate in *S. typhimurium* strains | | | |
| | **TA1530** | **TA1531** | **TA1532** | **TA1964** |
| Vehicle (control) | 20,23,24 | 3,3,4 | 12,12,14 | 6,7,9 |
| Eugenol (1000-5000 µg/plate) | 21,22,26 | 1,3,3 | 12,8,12 | 7,9,5 |
| Positive control N-methyl-N-nitrosoguanidine | 300,300,300 | 6,4,2 | 31,42,44 | 26,24,30 |
| Revertants per plate refers to the number of mutants to histidine independence after 2 days incubation at 37ºC | | | | |
| **Microsomal Assay (+S9)** | | | | |
| Concentration | Reversions/$10^6$ survivors *S. typhimurium* strains | | | |
| | **TA1530** | **TA1531** | **TA1532** | **TA1964** |
| Control | 0.39 (100) | 0.19 (100) | 0.56 (100) | 0.31 (100) |
| Eugenol 32841 (µg/mL) | 0.53 (78.3) | 0.23 (80.1) | 0.61 (79.2) | 0.45 (83.4) |
| Eugenol 3284 (µg/mL) | 0.48 (85.3) | 0.25 (92.2) | 0.63 (83.2) | 0.42 (89.2) |
| Positive control Dimethylnitrosamine | 15.50 (87.5) | 0.88 (90.2) | 8.23 (80.3) | 4.40 (89.4) |
| Reversions/$10^6$ survivors correspond to mean values; Number in parenthesis refers to percent survival | | | | |
| **Host-mediated Assay** | | | | |
| Concentration | Mutant frequency (x $10^{-8}$) *S. typhimurium* strains | | | |
| | **TA1950** | **TA1951** | **TA1952** | **TA1534** |
| Control | 2.0 | 0.6 | 1.9 | 4.3 |
| Eugenol 200000 (µg/kg) | 1.7 | 0.8 | 4.6 | 5.6 |
| Positive control Dimethylnitrosamine | 34.4 | 0.9 | 24.2 | 28.2 |

Mutant frequency obtained from an average of 3 mice per treatment

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**<u>Assessment and conclusion by RMS</u>:**

The RMS agrees with the notifier's assessment and conclusion.

Under the conditions of the study, eugenol is negative in *S. typhimurium* strains TA1530, TA1531, TA1532 and TA1964 with and without metabolic activation. Eugenol is also negative in the host-mediated assay using *S. typhimurium* strains TA1550, TA1951, TA1952 and TA1534.

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| The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 471 (2020) guideline include the use of four instead of five strains (none the recommended strains by the guideline), no individual results provided, solvent not indicated or historical control data. Based on the deviations from the guideline and since none of the strains are the standard recommended, the RMS deems the study as not acceptable to evaluate the bacterial mutation of eugenol. |

**B.6.4.1.1.4 Study IV**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Florin, I., Rutberg, L., Curvall, M. and Enzell, C.R. |
| **Report year** | 1980 |
| **Report title** | Screening of tobacco smoke constituents for mutagenicity using the Ames test |
| **Report No** | Not applicable, published study (*Toxicology*, 15(3), 219-232) |
| **Document No** | KCA 5.4.1/04 |
| **Guidelines followed in study** | Not stated but comparable to OECD TG 471 (1983) |
| **Deviations from current test guideline OECD TG 471 (2020)** | - Batch of test chemical not reported<br>- No justification provided for the use of ethanol as solvent<br>- Only four *S. typhimurium* tested strains instead of five<br>- One concentration used instead of a range of concentrations<br>- No individual data provided, just the result<br>- No historical control data |
| **Previous evaluation** | Yes, evaluated and classified as supplemental in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No, pre-GLP requirement |
| **Acceptability/ reliability** | Not acceptable (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity ≥ 97%; Batch No.: not stated |
| **Solvent** | Ethanol |
| **Negative control** | Not stated |
| **Positive control** | (-S9): N-methyl-N-nitrosoguanidine<br>(+S9): 2-aminoanthracene |
| **Test system** | *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 |
| **Metabolic activation** | Rat liver S9 derived from male Sprague-Dawley rats induced with Aroclor 1254 or 3-methylcholanthrene |
| **Test method** | The test substance was tested in spot tests using the Salmonella strains TA98, TA100, TA1535 and TA1537 with and without metabolic activation. A single concentration was tested (3µmol/plate, equivalent to 450µg/plate). |
| **Criteria for assessment of results** | Not stated |

**Results**

No increase in revertants in any strain was reported for eugenol. A negative result can be concluded from this limited assay although no information regarding the interpretation of the results is stated in the publication.

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|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and was accepted as supporting information.<br>The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion. It is acknowledged that a spot test of this nature with a single test concentration is of much lower sensitivity than an Ames test conducted according to the current OECD TG 471. |

Eugenol is reported negative when tested in Salmonella strains TA98, TA100, TA1535 and TA1537.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 471 (2020) guideline include the use of four instead of five strains, no individual results provided, one concentration tested, justification for the use of ethanol as solvent not provided or historical control data. Due to the deviations from the guideline and since only one concentration was tested, the RMS deems the study as not acceptable to evaluate the bacterial gene mutation potential of eugenol.

**B.6.4.1.1.5 Study V**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Azizan, A. and Blevins, R.D. |
| **Report year** | 1995 |
| **Report title** | Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay |
| **Report No** | Not applicable, published study (*Arch. Environ.Contam.Toxicol.*, 28, 248-258) |
| **Document No** | KCA 5.4.1/05 |
| **Guidelines followed in study** | Not stated but comparable to OECD TG 471 (1983) |
| **Deviations from current test guideline OECD TG 471 (2020)** | - No characterisation and stability determination of the test item<br>- Only three *S. typhimurium* tested strains instead of five<br>- Incubation period extended to 120 min<br>- Concentrations not stated<br>- No individual data provided, just the result<br>- No information on S9<br>- No positive controls and historical control data were provided |
| **Previous evaluation** | Yes, evaluated and classified as supplemental in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Not acceptable (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | DMSO |
| **Positive control** | Not stated |
| **Test system** | *S. typhimurium* strains TA97, TA98 and TA100 |
| **Metabolic activation** | S9 (no further information) |
| **Test method** | Eugenol was tested for mutagenicity using *Salmonella typhimurium* strains TA97, TA98 and TA100, with or without S9, in triplicate plates (tested concentrations not reported but indicated to be non toxic). Eugenol was diluted in DMSO to give a final concentration of 1mg/mL. The incubation time was extended to 120 min. Values for the mean revertants per plate and the mutagenic ratio (mean revertants per plate/mean spontaneous revertants per plate) were determined. Negative control was tested using the solvent (DMSO). |
| **Criteria for assessment of results** | A mutagenic ratio greater than two inferred a positive mutagenic response. |

**Results**

Eugenol gave a mutagenic ratio lower than 2 in all assays at non-toxic concentrations. No dose-related response was observed.

No mutagenic response was observed for eugenol under the conditions of the study.

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| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and was accepted as supporting information.<br>The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion.<br>Under the conditions of the study, eugenol did not show mutagenic response when tested in *S.typhimurium* strains TA97, TA98 and TA100.<br>The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 471 (2020) guideline include the use of three instead of five strains, no individual results provided or information about the concentrations tested, no information on S9, no positive control and historical control data were provided. Due to the deviations from the guideline, the RMS deems the study as not acceptable to assess the bacterial gene mutation potential of eugenol. |

**B.6.4.1.2. Mammalian cell gene mutation**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Myhr, B.C. and Caspary, W.J. |
| **Report year** | 1991 |
| **Report title** | Chemical mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells: results for 31 coded compounds in the National Toxicology Program |
| **Report No** | Not applicable, published study (*Environ.Mol. Mutagen.*, 18, 51-83) |
| **Document No** | KCA 5.4.1/13 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 490 (2016)** | - No characterisation and stability determination of the test item<br>- The assay was carried out in the absence of metabolic activation only<br>- Acceptability criteria not met (mutant frequency of untreated/solvent control is outside the valid range)<br>- No historical control data were provided |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Ethanol |
| **Positive control (-S9)** | Methyl methanesulphonate (MMS) |
| **Positive control (+S9)** | Not stated |
| **Test system** | L5178Y $TK^{+/-}$ |
| **Metabolic activation** | None |
| **Test method** | *Preliminary cytotoxicity assay*<br>Preliminary solubility and cytotoxicity studies were conducted. For the solubility test, the solvents used in order of preference were water, ethanol, DMSO, DMF (dimethylformamide). The solubility of eugenol was examined without cells. Eugenol toxicity to 24-hr cell suspension growth was determined for 4-hr treatments with a range of dose up to a maximum of 5000 µg/mL.<br>*Mutation assay*<br>Eugenol (in ethanol) was tested in the L5178Y $TK^{+/-}$ mouse lymphoma mutagenesis assay in the absence of metabolic activation. |

| | |
|---|---|
| | Two experiments were conducted. In the first experiment, an appropriate range of doses was chosen such that the relative total growth (RTG) values for clonable cultures would fall in an expected range of approximately 10–100 %. Six application rates were selected (20, 30, 40, 60, 80, 120 nL/mL). In a second experiment, doses were adjusted to allow better coverage of the toxicity range of greatest interest. Three application rates were selected (30, 40, 60 nL/mL). Ethanol was used as the negative control and methyl methanesulphonate as the positive control (without metabolic activation). Concentrations were tested in triplicate.<br>The treated cells were incubated on a roller drum for a 4-hour treatment period, at 37°C. After the treatment, the cells were washed and re-suspended in medium and returned to the roller for a 2-day expression and growth period. Viable cell densities were determined by haemacytometer using trypan blue exclusion. On day 2, cultures showing an increase in density were diluted and cloned in soft agar medium. The cultures were incubated for 11-12 days at 37°C with 5% $CO_2$/humidified air. Colonies were counted by an electronic counter. |

**Results**

Eugenol was mutagenic in the two trials without metabolic activation (Table B.6.4.1.2/1).

In the first experiment, high toxicity (mean of 8% RTG) was obtained at 120 nL/mL, which induced a 4.2-fold increase in MF compared to the negative control ethanol. In the second experiment, treatment with 60 nL/mL was greater toxic than in the first experiment (mean of 4% RTG) but induced a 2.2-fold increase in MF. The different results obtained in the two experiments could not be explained by the authors of the study. The mutation frequencies for cells treated with eugenol were statistically greater than negative control ones. Also, both large and small mutant colonies were detected but there was a slight increase in the proportion of the small colonies (Table B.6.4.1.2/2). The positive control showed large mutation frequencies, confirming the validity of the study. The effect of S9 mix was not determined.

**Table B.6.4.1.2/1. Mutation frequency after treatment with eugenol under non-activation conditions**

| Concentration (nL/mL) | Cloning efficiency (CE) | Relative total growth (RTG) | Total number of mutant colonies[1] | Mutant frequency /$10^6$ cells (MF) | Average mutant frequency |
|---|---|---|---|---|---|
| **First experiment – without metabolic activation system** | | | | | |
| 0 (Ethanol) | 110 | 97 | 82 | 25 | 27 |
| | 86 | 67 | 61 | 24 | |
| | 101 (A) | 145 | 79 | 26 | |
| | 87 | 90 | 86 | 33 | |
| Control + GEF | 27 + 90 = **117** | | | | |
| 20 | 96 | 84 | 104 | 36 | **41*** |
| | 81 | 73 | 107 | 44 | |
| | 93 | 72 | 118 | 43 | |
| 30 | 99 | 88 | 112 | 38 | **55*** |
| | 88 | 57 | 187 | 71 | |
| | 78 | 74 | 131 | 56 | |
| 40 | 98 | 61 | 136 | 46 | **49*** |
| | 107 | 62 | 137 | 43 | |
| | 84 | 48 | 144 | 57 | |
| 60 | 63[2] | 35 | 142 | 75 | **63*** |
| | 99 | 64 | 123 | 41 | |
| | 77 | 38 | 171 | 74 | |
| 80 | 90 | 16 | 222 | 82 | **84*** |
| | 65 | 28 | 180 | 92 | |
| | 88 | 27 | 210 | 79 | |
| 120 | 61 | 11 | 180 | 98 | **114*** |
| | 50 (A) | 5 | 215 | 142 | |
| | 51 | 8 | 159 | 103 | |
| MMS (5 nL/mL) | 49 | 40 | 558 | 384 | **438*** |

| Concentration (nL/mL) | Cloning efficiency (CE) | Relative total growth (RTG) | Total number of mutant colonies[1] | Mutant frequency /$10^6$ cells (MF) | Average mutant frequency |
|---|---|---|---|---|---|
| | 43 | 24 | 616[2] | 479 | |
| | 48 | 44 | 649 | 452 | |
| **Second experiment – without metabolic activation system** | | | | | |
| 0 (Ethanol) | 99 | 89 | 54 | 18 | 26 |
| | 93 (A) | 104 | 95 | 34 | |
| | 108 | 107 | 85[2] | 26 | |
| | (C) | - | 97 | - | |
| Control + GEF | 26 + 90 = **116** | | | | |
| 30 | 115 | 37 | 125 | 36 | **43*** |
| | 102 | 46 | 115 | 38 | |
| | 90 | 32 | 148 | 55 | |
| 40 | 95 (A) | 30 | 180 | 63 | **54*** |
| | 100 | 19 | 163 | 55 | |
| | 71 | 5 | 94 | 44 | |
| 60 | 76 | 4 | 119 | 52 | **57*** |
| | 71 | 4 | 131 | 62 | |
| | Lethal | | | | |
| MMS (5 µL/mL) | 73 | 25 | 696 | 316 | **290*** |
| | 74 | 37 | 577 | 262 | |
| | 89 | 51 | 777 | 290 | |

* results greater than solvent control ($P \leq 0.05$)
GEF: Global Evaluation Factor; OECD TG 490 (2016) The value for the agar version of MLA is 90 x $10^{-6}$
[1]Total number of mutant colonies in the three dishes
[2] Loss of one culture due to contamination
(A) Culture used to determine the proportion of large and small mutant colonies (Refer to Table B.6.4.1.2/2)

**Table B.6.4.1.2/2. Incidence of small and large colonies after treatment with eugenol under non-activation conditions**

| Concentration of eugenol | Mutant colony count and cloning efficiency | | | Mutant frequency | | Change in mutant frequency | |
|---|---|---|---|---|---|---|---|
| | Large | Small | Cloning efficiency | Large | Small | Large | Small |
| First experiment – without metabolic activation system | | | | | | | |
| 0 (Ethanol) | 47 | 36 | 100 | 16 | 12 | 4.3 | 6.2 |
| 120 | 101 | 112 | 50 | 67 | 74 | | |
| Second experiment – without metabolic activation system | | | | | | | |
| 0 (Ethanol) | 51 | 44 | 93 | 18 | 16 | 1.3 | 2.3 |
| 40 | 66 | 104 | 95 | 23 | 37 | | |

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion.

Under the conditions of the study, eugenol induced a dose-response increase in mutation frequency in mouse lymphoma cells without metabolic activation. According to OECD TG 490 (2016) a test chemical is considered clearly positive if the increase in MF above the concurrent background exceeds the GEF. The GEF calculated for the first and second experiment area 117 and 116, respectively. The MF does not exceed these values in any of the assays. Furthermore, in the first experiment high cytotoxicity occurs at high doses and

according to paragraph 67 of the current guideline, the result would not be considered positive if the increase in MF occurred only at or below 10 % RTG. Additionally, the mutant frequency of the untreated/solvent control does not fulfil the criteria and it falls outside the range given (35-140 x $10^{-6}$). Based on the available information and taking into consideration current guidelines, eugenol is deemed negative in the L5178Y TK $^{+/-}$ mouse lymphoma mutagenesis assay in the absence of metabolic activation.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 490 (2016) guideline include no characterisation of the test item, study conducted only in the absence of metabolic activation, acceptability criteria not met for untreated/solvent control (outside the valid range) and no historical control data were provided. For these reasons, the RMS deems the study as supporting information only to evaluate the mammalian gene mutation of eugenol.

### B.6.4.1.3. Mammalian chromosome aberration

#### B.6.4.1.3.1 Study I

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Ishidate, M., Sofuni, T., Yoshikawa K., Hayashi, M., Nohmi, T., Sawada, M. and Matsuoka, A. |
| **Report year** | 1984 |
| **Report title** | Primary mutagenicity screening of food additives currently used in Japan. |
| **Report No** | Not applicable, published study (*Food Chem. Toxic*. 22 (8), 623-636) |
| **Document No** | KCA 5.4.1/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 473 (2016)** | - Characterisation of the test substance not provided<br>- Test concentrations not reported<br>- Short-term treatments (±S9) were not performed.<br>- Only 100 metaphases per concentration were evaluated<br>- Gaps were not excluded from evaluation<br>- Number of replicates not reported<br>- No tabulated results provided<br>- No negative/positive control data shown, nor historical control data |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 100.0 %; Batch No.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Not specified |
| **Test system** | Chinese hamster lung fibroblast (CHL) cell line |
| **Metabolic activation** | Not used |
| **Test method** | Protocol described by Ishidate & Odashima (1977).<br>The cells were exposed to each sample at three different doses for 48 h. No metabolic activation was used. The maximum dose for each sample was selected by a preliminary test in which the $IC_{50}$ for growth inhibition was determined.<br>The maximum eugenol concentration tested was 0.125 mg/mL. Colcemid (final concentration 0.2 µg/mL) was added to the culture 2 h before harvesting. The cells were spread onto clean glass slides and were stained with Giemsa solution (1.5 % at pH 6.8) after air-drying. A hundred well-spread metaphases were observed under the microscope. The incidence of polyploid cells as well as of cells with structural chromosomal aberrations (chromatid or chromosome gaps, breaks, exchanges, ring formations, fragmentations and others) were recorded on each culture plate.<br>Untreated cells and solvent-treated cells served as negative controls, in which the incidence of aberrations was usually less than 3.0 %.d. |

| | |
|---|---|
| **Criteria for assessment of results** | Results were considered negative if the incidence was less than 4.9 % equivocal if it was between 5.0 and 9.9 % and positive if it was more than 10.0 %. |

**Results**

The results obtained from the cytogenicity assay on CHL showed that eugenol induced 0 % polyploid cells and 17.0 % of the cells had structural aberrations after 48 h exposure to the test substance. The maximum dose tested was 0.125 mg/mL, which represents the highest non-cytotoxic dose used in the experiment.
Based on the results of the study and according to the assessment criteria, eugenol is clastogenic in Chinese hamster lung fibroblasts in the absence of metabolic activation.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion.<br>Under the conditions of the study, eugenol is clastogenic in Chinese hamster lung cells without metabolic activation.<br>The study is a scientific publication from a peer-reviewed journal and detailed experimental results are not provided. No guideline was reported in the study and it was compared against the current OECD TG 473 (2016). The RMS deems the study as supporting information based on the lack of experimental results and deviations from the current guideline, no short-term treatments (±S9) performed, no information on test concentration range, less than 300 metaphases scored (only 100 in the study), gaps were not excluded and no historical control data were available. |

**B.6.4.1.3.2 Study II**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Stich, H.F., Rosin, M.P, Wu, C.H. and Powrie, W.D. |
| **Report year** | 1981 |
| **Report title** | The action of transition metals on the genotoxicity of simple phenols, phenolic acids and cinnamic acids |
| **Report No** | Not applicable, published study (*Cancer Lett.* 14, 251-260) |
| **Document No** | KCA 5.4.1/07 |
| **Guidelines followed in study** | Not stated. Comparable to OECD TG 473 (1983) |
| **Deviations from current test guideline**<br>**OECD TG 473 (2016)** | - Characterisation of the test substance not provided<br>- Only two concentrations tested<br>- Only 200 metaphases per concentration were scored<br>- Evaluation of aberrations types in chromatids only (not chromosomes). Gaps were not excluded.<br>- Number of replicates not reported<br>- Criteria for the assessment of the test results is not reported<br>-No positive control data shown, nor historical control data |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No, pre-GLP requirement |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |

| | |
|---|---|
| **Solvent** | 2.5% Eagle´s Minimal Essential Medium (MEM) |
| **Negative control** | Solvent |
| **Positive control** | Not specified |
| **Test system** | Chinese hamster ovary (CHO) cell line |
| **Metabolic activation** | S9 mixture from Aroclor 1254 induced male Fischer rat livers |
| **Test method** | The chromosome aberration test was performed by a short treatment method in which Chinese hamster ovary cells were continuously treated over a 3h-exposure period with two different doses of eugenol (0.05 and 0.4 mg/mL) with or without a metabolic activation system. Following the exposure time, the medium was removed and 0.1 mL of colchicine (0.01 % in 2.5 % MEM) was added at 16 h post-exposure and left for 4 h. Then, the cells were fixed in ethanol/acetic acid (3:1), then air-dried, and slides were stained with orcein in 50% acetic acid/water. For determination of chromosome aberrations, 200 cells were scored. The frequency of two types of chromosome aberrations was examined: chromatid breaks and chromatid exchanges.<br>In addition, the modulating of the ubiquitous transition metals $Cu^{2+}$ and $Mn^{2+}$ on the clastogenicity was examined. Metal solutions of 0.1M of cupric sulphate or manganese chloride and 0.5M of glycine in distilled water were prepared. The metal stock solutions were then prepared by mixing glycine with the metal stock 10:1 molar ratio, followed by dilution with 2.5% MEM. A 0.5 mL aliquot to these mixtures was added to Chinese hamster ovary cells treated with 0.2 mg/mL eugenol. The same protocol described above was followed. |

**Results**

Eugenol induced increases in chromosome aberrations without S9 at the highest concentration tested, and with S9 at the lowest concentration tested, compared to the control (Table B.6.4.1.3.2/1).

**Table B.6.4.1.3.2/1. Clastogenic activity of eugenol in CHO cells**

| Compound | Concentration (mg/mL) | % metaphase with chromosome aberration | | Chromatid breaks per cell | | Chromatid exchanges per cell | |
|---|---|---|---|---|---|---|---|
| | | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Eugenol | 0.4 | 21.5 | MI | 0.08 | 0.00 | 0.20 | 0.00 |
| | 0.05 | 0.5 | 13.9 | 0.01 | 0.17 | 0.00 | 0.24 |

Concurrent control frequencies for cells receiving culture medium only were 0.7 % metaphase plates with chromosome aberrations with an average of 0.01 breaks/cell and 0.00 exchanges/cell.
MI: Mitotic inhibition

Both transition metals did not induce enhancement of the clastogenic activity of eugenol (Table B.6.4.1.3/2).

**Table B.6.4.1.3.2/2. Effect of transition metals on the clastogenic activity of eugenol in CHO cells**

| Compound | Concentration (mg/mL) | % metaphase with chromosome aberration | | |
|---|---|---|---|---|
| | | No metals | $Cu^{2+}$ ($10^{-4}$M) | $Mn^{2+}$ ($10^{-4}$M) |
| Eugenol | 0.2 | 5.3 (0.06) | 3.9 (0.04) | 5.2 (0.06) |
| Control (Solvent) | - | 0.5 (0.00) | 0.7 (0.01) | 0.8 (0.00) |

Numbers in parentheses are average number of chromatid exchanges per cell

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion.

| |
|---|
| Under the conditions of the study, eugenol is clastogenic in Chinese hamster ovary cells with and without metabolic activation.<br><br>The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 473 (2016). The RMS deems the study as supporting information based on the lack of experimental results and deviations from the current guideline, which include no characterisation of the test item, only two concentrations tested, less than 300 metaphases scored (only 200 in the study) and no positive control or historical control data were available. |

**B.6.4.1.3.3 Study III**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B. and Zeiger, E. |
| **Report year** | 1987 |
| **Report title** | Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals |
| **Report No** | Not applicable, published study (*Environ. Mol. Mutagen*. 10 (Suppl 10), 1-175) |
| **Document No** | KCA 5.4.1/08 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 473 (2016)** | - No characterisation of the test substance<br>- Solvent not stated specifically<br>- Positive control not stated specifically<br>- Only 100 metaphases were scored (instead of the recommended 300)<br>- Method and results poorly described, for example type of aberrations not described<br>- No historical data were reported<br>- The cells were exposed to the chemical only 2 hours (3-6 hours are recommended) |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | Not stated; substances were dissolved in water, DMSO, ethanol or acetone in this order |
| **Negative control** | Solvent (not stated) |
| **Positive control** | Triethylenemelamine. mitomycin C or cyclophosphamide |
| **Test system** | Cloned Chinese hamster ovary (CHO-W-B1) cell line |
| **Metabolic activation** | S9 mixture from Aroclor 1254 induced male Sprague-Dawley rat livers |
| **Test method** | An *in vitro* mammalian chromosome aberrations test developed by Galloway *et al.* 1985 test was conducted in 108 coded chemicals.<br>The top dose selection was based on a preliminary growth inhibition test.<br>Eugenol was dissolved in water, DMSO, ethanol or acetone in that order of preference although not specifically stated in the method.<br>Cloned CHO cells (CHO-W-B1) were grown in McCoy's 5a medium with 10 % foetal calf serum. Tests were carried out with and without metabolic activation (S9 mix). For the cell cultures without metabolic activation eugenol was left in culture until the colcemid was added. For the one with metabolic activation eugenol was added with S9 mix for only 2h at the begening of the test.<br>Doses were chosen based on a preliminary test of cell survival 24 h after treatment. The doses were spaced more closely than half-log series and extended into the toxic range.<br>Non-toxic doses were not always tested because the chosen doses were in the toxic range and only 100 cells per dose were sampled. |

| | |
|---|---|
| | Cells were exposed to the test chemical for 2 h in the presence and absence of S9 throughout the incubation period.<br>The control data (Margolin *et al.* 1986) were valuable in forming the decision scheme for data evaluation and were useful in deciding whether to repeat an assay. Harvest time was 10.5 and delayed harvest time was 19.5-26 h. |
| **Criteria for assessment of results** | A positive response has the adjusted P value ≤ 0.05 from the binomial sampling assumption adjusted by Dunnett's method. |

**Results**

Eugenol did not induce any chromosome aberration without S9, even after a 24-h incubation period and up to cytotoxic concentrations (300 µg/mL). With S9, cells fixed at about 10.5 hours showed a small increase in aberrations at the highest dose, and when the assay was repeated with a harvest time of 20 hours, there was a clear increase in all types of aberrations. The results are shown in Table B.6.4.1.3.3.

**Table B.6.4.1.3.3. Results of the chromosome aberration test of eugenol**

| | -S9 | +S9 | Summary Result |
|---|---|---|---|
| **Range (µg/mL)** | 198-300 | 201-324 | Positive |
| **Result** | -[d] | +W/+W[*d] | |
| **LEC** | - | 300/324 | |

*Least effective concentration tested (LEC) is the lowest dose to give a statistically significant increase (P≤0.05) in aberrations.*
*"d" delayed harvest; "+w" 0.015 > P ≥. 0.003; "+w*" P < 0.0005 but due to primarily to a statistically significant increase at a single dose (usually the highest).*

The aberration test without S9 was negative up to toxic doses 300 µg/mL, even at a 24 h fixation time. For the first test with S9, 10.5 h was the fixation time. The cells showed a small increase in aberrations at the highest dose. When the harvest time was increased to 20 h, there was a clear increase in all type of aberrations.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS agrees with the applicant assessment and conclusion.

Eugenol was negative at 198-300 µg/mL without metabolic activation and weakly positive with metabolic activation at 201-324 µg/mL under the conditions of the reported chromosomal aberration test (2 hours of exposure).

The study is a scientific publication from a peer-reviewed journal. The RMS considers that the study lacks of important information, as the characterisation of the test substance, method and results poorly described, only 100 metaphases were scored, no positive control or historical data were reported, and cells were exposed to the chemical for two hours only with metabolic activation. Due to these deviations from guideline, the RMS considers the study as supporting information to evaluate the potential clastogenicity of eugenol.

**B.6.4.1.3.4 Study IV**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Maralhas, A., Monteiro, A., Martins, C., Kranendonk, M., Laires, A., Rueff, J. and Rodrigues, A.S. |
| **Report year** | 2006 |
| **Report title** | Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol |
| **Report No** | Not applicable, published study (*Mutagenesis*, **21** (3), 199-204) |
| **Document No** | KCA 5.4.1/09 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 473 (2016)** | - No characterisation of the test substance<br>- Only 100 metaphases were scored (instead of the recommended 300)<br>- Average test results but not individual were reported<br>- The mitotic index should not be use to evaluate cytotoxicity<br>- No historical control data were provided |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 99 %; Batch No.: not stated |
| **Solvent** | Dimethylsuphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Mitomycin C (MMC) (-S9), cyclophosphamide (CP) (+S9) and ICRF-193 |
| **Test system** | V79 Chinese hamster cells |
| **Metabolic activation** | S9 mixture from Aroclor 1254 induced rat livers |
| **Test method** | An *in vitro* chromosome aberration test was performed in V79 Chinese hamster cells. Twenty-four hour cultures (~ $5x10^5$ cells/flask) were treated for 3h with different doses of eugenol (100-3000 µM) in DMSO both in the presence and absence of metabolic activation (500 µL of 10% S9 mix). Chromosome preparations were made after an additional incubation period of 13 h in fresh medium. Colchicine (6.5 mM) was added to the culture 3 h before cell harvesting. The cells were then trypsinized and suspended in hypotonic KCl solution (0.075M). After cell fixation with ice-cold methanol/acetic acid (3:1), slides were stained with Giemsa solution. A hundred well-spread metaphases were observed under a microscope and scored for gaps, exchanges, ring formation, fragmentation, polyploidy and other abnormal chromosomal structures. Two independent experiments were done. The statistical analysis was performed using the two-tailed Student's *t*-test.<br><br>The effect of eugenol in endoreduplication was also investigated. Cells for this test were prepared as for the chromosome aberration test, with 5 different concentrations of eugenol diluted in DMSO (500, 1000, 1500, 2000, 2500 µM). ICRF-193 was used as a positive control for endoruplication, at 4 different concentrations (0.1, 0.2, 0.3, 0.5 µM). |

**Results**

Eugenol induced a dose-dependent increase of aberrant cells excluding gaps in V79 cells up to 3000 µM in the presence of metabolic activation (Table B.6.4.1.3.4/1). There was a less marked increase in the absence of metabolic activation. Cytotoxicity (reduced mitotic index) was recorded at the highest concentration (3000 µM). Chromatid breaks and chromatid exchanges both showed clear increases. The positive controls gave the appropriate results validating the study.

**Table B.6.4.1.3.4/1. Chromosome aberrations in V79 cells (average value of two experiments).**

| Treatment | Dose | No. cells with structural aberrations (per 100 cells) | % ACEG | | MI (%) |
|---|---|---|---|---|---|

| | µM | Ctg | Csg | Ctb | Csb | Exch | Dic+ Oth | >10 | | CAEG / cell | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **3-hour exposure, without S9** | | | | | | | | | | | |
| Solvent (DMSO) | 0.5% v/v | 3.5 | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 ± 0 | 0 | 2.1 ± 0.9 |
| Eugenol | 100 | 6.5 | 0.5 | 0 | 0.5 | 0 | 0 | 0.5 | 1.0 ± 1.4 | 0.01 | 1.1 ± 0.1 |
| | 1000 | 4.0 | 0 | 0.5 | 0 | 0 | 0 | 0.5 | 1.0 ± 1.4 | 0.01 | 1.1 ± 0.1 |
| | 2500 | 7.0 | 0.5 | 1.0 | 1.0 | 0 | 1.0 | 0.5 | **3.5 ± 0.7*** | 0.04 | 0.6 ± 0.2 |
| | 3000 | 13.0 | 0 | 6.0 | 0 | 2.0 | 0 | 4.0 | 12.0 | 0.12 | - |
| MMC (Positive control) | 0.36 | 11.5 | 1.0 | 9.5 | 0.5 | 2.5 | 0.5 | 0 | 10.4 ± 4.8 | 0.13 | 3.8 ± 1.1 |
| **3-hour exposure, with S9** | | | | | | | | | | | |
| Solvent (DMSO) | 0.5% v/v | 4.0 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0.5 ± 0.7 | 0.01 | 5.7 ± 4.7 |
| Eugenol | 100 | 2.5 | 0 | 3.0 | 0 | 0.5 | 0.5 | 0 | 3.5 ± 5.0 | **0.04** | 7.9 ± 9.6 |
| | 1000 | 4.0 | 0 | 3.0 | 0 | 0.5 | 0.5 | 0 | **6.5 ± 0.7**** | **0.07** | 4.0 ± 4.0 |
| | 2500 | 3.0 | 0 | 7.5 | 0 | 0.5 | 0.5 | 0 | **15.0 ± 1.4***** | **0.15** | 2.3 ± 2.6 |
| | 3000 | 4.0 | 1.0 | 6.0 | 1.0 | 9.0 | 9.0 | 0 | 18.0 ± 12.7 | 0.18 | 0.4 ± 0.5 |
| CP (Positive control) | 21.5 | 3.0 | 0 | 3.5 | 0.5 | 0 | 0 | 0 | 4.5 ± 2.1 | 0.05 | 7.8 ± 0.6 |

***Ctg**: Chromatid gap; **Csg**: Chromosome gap; **Ctb**: Chromatid break; **Csb**: Chromosome break; **Exch**: Triradial, quadriradial and other chromatid exchanges; **Dic + other**: Dicentric chromosomes and other aberrations (eg. rings), **>10**: Multiaberrant cells – included in the % aberrant cells score but the individual aberrations are not included in the scores for aberrations per 100 cells **%ACEG**: % Aberrant cells excluding gaps (average ± standard deviation), **CAEG/cell**: Chromosomal aberrations excluding gaps/cell; **MI**: Mitotic index; **MMC**: Mitomycin C; **CP**: Cyclophosphamide*
** P<0.05, ** P<0.01, ***P<0.005*

Eugenol induced a dose-dependent increase in the percentage of endoreduplicated cells in the presence and absence of metabolic activation (Table B.6.4.1.3.4/2) at doses from 1500 µM. High levels of cytotoxicity were observed at the top dose (2500 µM).

**Table B.6.4.1.3.4/2. Induction of endoreplications in V79 cells following treatment with eugenol**

| Treatment | Dose (µM) | Endoreplications per 2000 metaphases | % endoreduplication | MI (%) |
|---|---|---|---|---|
| **3-hour exposure, without S9** | | | | |
| Solvent (DMSO) | 0.5% v/v | 0 ± 0 | 0 ± 0 | 7.10 ± 0.57 |
| Eugenol | 500 | 3.5 ± 3.5 | 0.18 ± 0.18 | 3.95 ± 0.21 |
| | 1000 | 6.0 ± 4.2 | 0.30 ± 0.21 | 3.40 ± 0.99 |
| | 1500 | 22.0 ± 1.4* | 1.10 ± 0.07 | 3.20 ± 0.99 |
| | 2000 | 45.0 ± 1.4* | 2.25 ± 0.07 | 2.65 ± 0.35 |
| | 2500 | [a] | [a] | 0.05 ± 0.07 |
| Solvent (DMSO) | 0.5% v/v | 0 ± 0 | 0 ± 0 | 6.80 ± 1.22 |
| ICRF-193 | 0.1 | 26.5 ± 0 | 1.33 ± 0.67 | 6.00 ± 2.18 |
| | 0.2 | 45.5 ± 13.4* | 2.28 ± 0.95 | 4.47 ± 1.78 |
| | 0.3 | 59.5 ± 19.1 | 2.98 ± 1.38 | 3.57 ± 1.24 |
| | 0.5 | 69.5 ± 21.9 | 3.48 ± 1.10 | 3.07 ± 1.16 |
| **3-hour exposure, with S9** | | | | |
| Solvent (DMSO) | 0.5% v/v | 0 ± 0 | 0 ± 0 | 6.56 ± 0.55 |
| Eugenol | 500 | 13.0 ± 7.0 | 0.65 ± 0.35 | 3.73 ± 1.01 |
| | 1000 | 30.5 ± 7.8 | 1.53 ± 0.39 | 3.40 ± 0.00 |
| | 1500 | 59.3 ± 10.6* | 2.97 ± 0.53 | 2.90 ± 0.28 |
| | 2000 | 101.3 ± 39.4* | 5.07 ± 1.97 | 2.10 ± 0.44 |
| | 2500 | [a] | [a] | 0.70 ± 0.42 |
| Solvent (DMSO) | 0.5% v/v | 0 ± 0 | 0 ± 0 | 8.60 ± 1.41 |
| ICRF-193 | 0.1 | 20.5 ± 0.7* | 1.03 ± 0.04 | 5.85 ± 0.78 |
| | 0.2 | 44.0 ± 0* | 2.20 ± 0 | 4.70 ± 0.42 |
| | 0.3 | 58.0 ± 4.2* | 2.90 ± 0.21 | 4.05 ± 0.35 |

| | | | | |
|---|---|---|---|---|
| | 0.5 | 62.0 ± 7.1** | 3.10 ± 0.35 | 3.65 ± 0.50 |

[a)] High levels of cytotoxicity were observed at this concentration
*$P < 0.05$ (Student's *t*-test) when compared with the control
**$P = 0.051$ (Student's *t*-test) when compared with the control

In conclusion, in both assays, eugenol showed genotoxic activity. It induced a dose-dependent increase in chromosome aberrations in V79 cells, especially in the presence of metabolic activation. The marked increases were recorded at relatively high concentrations (2500 µM concentrations and above) accompanied by cytotoxicity. It was shown that eugenol induces dose-related increases in endoreduplicated cells (to a greater extent in the presence of metabolic activation)..

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS disagrees with the applicant assessment and conclusion. Eugenol induced chromosomal aberrations in the presence of metabolic activation. In the absence of metabolic activation, a positive response was observed at 2500 µM although this response was associated with high cytotoxicity. Eugenol also induced endoreduplication in the presence and absence of metabolic activation.

The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 473 (2016). The RMS deems the study as supporting information based on the deviations from the current guideline, which include no characterisation of the test item, less than 300 metaphases scored (only 100 in the study), no historical control data provided and the test results presented are shown as average of two assays.

#### B.6.4.1.3.5 Study V

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Hikiba, H., Watanabe, E., Barrett, J.C. and Tsutsui, T. |
| **Report year** | 2005 |
| **Report title** | Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syriam hamster embryo cells |
| **Report No** | Not applicable, published study (*J. Pharmacol. Sci*, **97**, 146-152) |
| **Document No** | KCA 5.4.1/10 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 473 (2016)** | - No characterisation of the test substance<br>- Only 100 metaphases were scored (instead of the recommended 300)<br>- No indication as to how the S9 fraction was derived<br>- Cytotoxicity was not measured in the main experiment<br>- No positive control or historical control data were provided |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: > 95 %; Batch No.: not stated |
| **Solvent** | Dimethylsuphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Not stated |
| **Test system** | Syrian hamster embryo (SHE) cells (doubling time 16 h) |
| **Metabolic activation** | Rat liver post-mitochondrial supernatant (S9 mix) |

| | |
|---|---|
| **Test method** | An *in vitro* chromosome aberration test was performed in Syrian hamster embryo (SHE) cells. A preliminary cytotoxicity assay was carried out to determine the colony-forming efficiencies of SHE cells treated with eugenol. SHE cells were treated with eugenol at varying concentrations (not stated) for 24 hours. After harvesting with 0.1% trypsin, the cells were incubated in triplicate for 7 days for colony formation. The relative colony-forming efficiency was expressed as the number of colonies in the treated dishes divided by the number in the control dishes.<br>In the main experiment, 24-hour cultures of SHE cells (~ $5x10^5$ cells/flask) were treated with eugenol for 24h at concentrations of 65, 195 and 650 µM without S9 and at 6.5, 20 and 65 µM with S9 for 3h. Colcemid (0.2 µg/mL) was added to the culture 3h before cell harvesting. Then, the cells were harvested with 0.1% trypsin for chromosome preparation. For determination of chromosome aberrations, 100 metaphases were scored per experimental group. Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid. If there was displacement, they were scored as breaks. |

**Results**

Eugenol induced statistically-significant increases in the levels of chromosome aberrations in SHE cells, both in the presence and absence of metabolic activation (Table B.6.4.1.3.5). The clastogenic activity was enhanced in the presence of post-mitochondrial supernatant (PMS) compared with an absence of exogenous metabolic activation.

**Table B.6.4.1.3.5. Induction of chromosomal aberrations in SHE cells following treatment with eugenol**

| Eugenol concentration (µM) | Relative colony forming efficiency (%) | Number of metaphases scored | Type of aberration (%) | | | | | | | | Aberrant metaphases (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CG | ICG | CB | ICB | E | O | D | F | |
| **24-hour exposure, without metabolic activation (post-mitochondrial supernatant)** | | | | | | | | | | | |
| 0 | 100 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 65 | 82 | 100 | 2 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | **4.0** |
| 195 | 77 | 100 | 4 | 5 | 2 | 1 | 2 | 0 | 0 | 0 | **12.0**** |
| 650 | 58 | 100 | 10 | 12 | 0 | 0 | 1 | 0 | 0 | 0 | **18.0**** |
| **3-hour exposure, with metabolic activation (post-mitochondrial supernatant)** | | | | | | | | | | | |
| 0 | nr | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6.5 | nr | 100 | 1 | 0 | 4 | 0 | 3 | 0 | 0 | 0 | **5.0** |
| 20 | nr | 100 | 4 | 1 | 12 | 3 | 10 | 0 | 0 | 0 | **20.0**** |
| 65 | nr | 100 | 5 | 0 | 17 | 4 | 19 | 0 | 0 | 0 | **27.0**** |

**nr**: Note recorded; **CG**: Chromatid gaps; **ICG**: Isochromatid gaps; **CB**: Chromatid breaks; **ICB**: Isochromatid breaks; **E**: Exchanges; **O**: Ring chromosomes; **D**: Dicentric chromosomes; **F**: Fragmentations
**Significantly different from control ($P<0.01$, $\chi^2$ test).

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**<u>Assessment and conclusion by RMS</u>:**

RMS agrees with the applicant assessment and conclusion.

Eugenol induced chromosomal aberrations in the presence and absence of metabolic activation. A dose-related increase in the frequency of aberrant metaphases at concentrations with acceptable relative colony forming efficieny is observed, which could be used as an estimation of the cytotoxic potential of the test item.

The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 473 (2016). The RMS deems the study as supporting

| |
|---|
| information based on the deviations from the current guideline, which include: no characterisation of the test item, less than 300 metaphases scored (only 100 in the study), no indication as to how S9 was derived, no positive control in this study and no historical control data provided. |

**B.6.4.1.3.6 Study VI**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Someya, H., Higo, Y., Ohno, M., Tsutsui, T.W. and Tsutsui, T. |
| **Report year** | 2008 |
| **Report title** | Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells. |
| **Report No** | Not applicable, published study (*Mutat. Res.*, **650**, 39-47) |
| **Document No** | KCA 5.4.1/11 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 473 (2016)** | - No characterisation of the test substance<br>- No metabolic activation test performed<br>- Only 100 metaphases were scored (instead of the recommended 300)<br>- No historical controls provided<br>- No appropriate positive control for no metabolic activation conditions was used. |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: > 95 %; Batch No.: not stated |
| **Solvent** | Dimethylsuphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Ciclophosphamide |
| **Test system** | Human dental pulp cells (D824) |
| **Metabolic activation** | No metabolic activation |
| **Test method** | An *in vitro* chromosome aberration test was performed in human dental pulp cells (D824) extracted from a healthy female volunteer.<br>A preliminary cytotoxicity assay was carried out to select the concentrations for the main test. Cytotoxicity was determined as the number of cells treated with eugenol relative to the number of cells in the control cultures*100. Triplicate cultures of D824 cells were treated with eugenol for 3h at various concentrations (not stated). After two washings with 2 mL of fresh medium, cells were incubated for a further 27 h, and the number of cells was counted after harvesting with 0.25% trypsin (GIBCO/Invitrogen). In the second experiment, cells were treated with test agents for 30 h and then harvested for counting.<br>Two main experiments were performed: 3h and 30h exposure to eugenol, respectively. The concentrations were selected on the basis of that induced cytotoxicity that were greater than those of 50% of the control cultures were used in principle in all experiments for assessing chromosome aberrations. In the first experiment, triplicate cultures of D824 cells were treated with eugenol for 3 h at 100, 300, 1000 µM, then washed and incubated for a further 27 h (1.4 cell cycles). Three hours before the end of incubation, colcemid (GIBCO/Invitrogen) was administered at 0.02 g/mL, and metaphase chromosomes were prepared. In a second experiment, cells were treated with the test agent for 30 h. Three hours before the end of treatment, colcemid was administered and metaphase chromosomes were prepared. In the activation assays, after treatment, cells were washed twice with 5 mL fresh medium and then incubated for 28 h. Three hours before the end of incubation, colcemid was added. As a negative control, cells were treated with the medium and the solvent. No metabolic activation assays were performed in this study.<br>The aberrations scored were chromatid gaps, chromosome gaps, chromatid breaks, chromosome breaks, chromatid exchanges, dicentric chromosomes, ring chromosomes |

| | |
|---|---|
| | and fragmentations. Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid; if there was displacement, these were scored as breaks.<br>Statistical analysis was performed by $\chi^2$ test to assess the significance of the difference in the incidences of chromosome aberrations between control and treated cultures. |

**Results**

No increases in the levels of chromosome aberrations were observed in cells treated for 3 h with eugenol, which also failed to induce polyploidy and endoreduplication in D824 cells. A statistically-significant increase in the levels of chromosome aberrations was observed for cells treated for 30 h; the increase was concentration dependent. No statistically significant increases in the percentage of cells with polyploidy or endoreduplication were elicited by eugenol (see table B.6.4.1.3.6). A concentration-dependent growth inhibition was observed. The positive control gave a positive response in the assay.

**Table B.6.4.1.3.6. Induction of chromosomal aberrations in D824 cells following treatment with eugenol.**

| Concentration (µM) | Relative cell number | Number of metaphases scored | Type of structural aberration[a] (%) | | | | | | | | Aberrant metaphases (%) | Polyploidy and endoreduplication (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ctg | csg | ctb | csb | cte | D | O | F | | |
| **3 h treatment without metabolic activation** | | | | | | | | | | | | |
| 0 | 100 | 500 | 0.8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.8 | 2.0 |
| 100 | 99 | 100 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.0 | 0 |
| 300 | 97 | 100 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.0 | 4.0 |
| 1000 | 96 | 100 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.0 | 4.0 |
| **30 h treatment without metabolic activation** | | | | | | | | | | | | |
| 0 | 100 | 500 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.0 | 2.0 |
| 100 | 89 | 100 | 4 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 5.0* | 3.0 |
| 300 | 67 | 100 | 13 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 15.0** | 1.0 |
| 1000 | 58 | 100 | 35 | 0 | 18 | 0 | 0 | 0 | 0 | 0 | 43.0** | 0 |
| **Positive control, with or without metabolic activation (2h)** | | | | | | | | | | | | |
| 50 (-PMS) | 87 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.0 |
| 50 (+PMS) | 47 | 100 | 20 | 0 | 10 | 0 | 3 | 0 | 0 | 0 | 35.0** | 0 |

[a] **ctg**: chromatid gaps; **csg**: chromosome gaps; **ctb**: chromatid breaks; **csb**: chromosome breaks; **cte**: chromatid exchanges; ***D***: dicentric chromosomes; ***O***: ring chromosomes; ***F***: fragmentations.
* Statistically significant difference from control ($p < 0.05$; $\chi 2$-test).
** Statistically significant difference from control ($p < 0.01$; $\chi 2$-test).

**<u>Assessment and conclusion by applicant</u>:**

This new study is considered to be acceptable as supporting information for the renewal of eugenol (reliable with restrictions; study is similar to current OECD guideline but is not GLP and there are some deficiencies in the methodology).

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**<u>Assessment and conclusion by RMS</u>:**

RMS agrees with the applicant assessment and conclusion.

Eugenol failed to induce chromosome aberrations in human D824 at 3h. However, it induced chromosomal aberrations following a 30-hour exposure in the absence of metabolic activation.

The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 473 (2016). The RMS deems the study as supporting information based on the deviations from the current guideline, which include: no characterisation of the test item, less than 300 metaphases scored (only 100 in the study), non-activation experiments only, and no historical control data provided.

### B.6.4.1.4. DNA Damage

#### B.6.4.1.4.1 Study I

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Jansson, T., Curvall, M., Hedin, A. and Enzell, C.R |
| **Report year** | 1986 |
| **Report title** | *In vitro* studies of biological effects of cigarette smoke condensate. II. Induction of sister-chromatid exchanges in human lymphocytes by weakly acidic, semivolatile constituents |
| **Report No** | Not applicable, published study (*Mutat. Res.*, **169**, 129-139) |
| **Document No** | KCA 5.4.1/12 |
| **Guidelines followed in study** | No guideline |
| **Deviations from current test guideline OECD TG 479 (deleted in 2014)** | - No characterisation of the test substance<br>- No metabolic activation test performed<br>- Very limited level of reporting |
| **Previous evaluation** | Yes, evaluated and classified as supplemental in DAR 2011 |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Not acceptable (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 99 %; Batch No.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Styrene-7,8-oxide |
| **Test system** | Human lymphocyte cells |
| **Metabolic activation** | None |
| **Test method** | Lymphocytes extracted from human non-smoker volunteers were incubated for 24 h at 37°C. Eugenol was added to the cultures at various concentrations from 0-0.5 mM range. In the tests of pure compounds, the potent SCE inducer styrene-7,8-oxide was used as a positive control.<br>The final concentrations of the solvent, DMSO, did not exceed 0.7%. After 88-90 h, the cells were treated consecutively with colchicine (50 ng/ml, 2 h) and hypotonic KC1 (0.075 M, 5-10 min) and they were then fixed in methanol/acetic acid (3:1) for 1 h. After the culture time used, the portion of cells that have divided more than 2 times is about 20-30%. Chromosome preparations were made by applying the cell suspension to wet cooled slides. The staining procedure was mainly according to Wolff and Perry (1974). The slides were stained with Hoechst dye (0.5 µg/ml) for 12 min and then rinsed in Mcllvaines buffer (pH 7.0). They were then exposed to UV-light (Philips TUV 30W G30T8, 10 cm) for 10 min, incubated in 0.3 M NaCI/0.3 M sodium citrate for 2 h at 60°C and stained with Giemsa dye (4%, pH 6.8) for 20 min. Well-spread metaphases were scored on coded slides and 25 metaphases from one culture were analysed for each concentration tested. |

**Results**

Eugenol failed to induce the incidence of sister chromatid exchanges in human lymphocytes. The calculated regression coefficient and correlation coefficient for eugenol were respectively -2.4 SCE/cell/mM and -0.49.

The positive control Styrene-7,8-oxide induced statistically significant increases in the levels of sister chromatid exchanges over the dose range tested.

**<u>Assessment and conclusion by applicant</u>:**

| |
|---|
| The study has been previously reviewed at EU level and was accepted as supporting information.<br>The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>RMS agrees with the applicant assessment and conclusion.<br>Eugenol failed to induce sister chromatid exchanges in the absence of metabolic activation at the concentrations tested in the study.<br>The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 479 (deleted in 2014). The RMS deems the study as not acceptable based on method deficiencies (non-activation test only) and based on the poor level of reporting. |

**B.6.4.1.4.2 Study II**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B. and Zeiger, E. |
| **Report year** | 1987 |
| **Report title** | Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals |
| **Report No** | Not applicable, published study (*Environ. Mol. Mutagen*. 10 (Suppl 10), 1-175) |
| **Document No** | KCA 5.4.1/08 |
| **Guidelines followed in study** | No guideline stated in the study. |
| **Deviations from current test guideline**<br>**OECD TG 479 (deleted in 2014)** | - No characterisation of the test substance<br>- Solvent not stated specifically<br>- Positive control not stated specifically<br>- Method and results poorly described<br>- No cytotoxicity measurements made<br>- No historical data were reported |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | Not stated; substances were dissolved in water, DMSO, ethanol or acetone in this order |
| **Negative control** | Solvent (not stated) |
| **Positive control** | Triethylenemelamine. mitomycin C or cyclophosphamide |
| **Test system** | Cloned Chinese hamster ovary (CHO-W-B1) cell line |
| **Metabolic activation** | S9 mixture from Aroclor 1254 induced male Sprague-Dawley rat livers |
| **Test method** | The sister chromatid exchange test was performed by a long treatment method in which Chinese hamster ovary cells were continuously treated for 25 hours with different doses of eugenol (concentration range not specified but up to solubility limit or 5 mg/mL) without metabolic activation system or for 2 hours with metabolic activation system. 5-Bromodeoxyuridine (10 µM) was used to label chromatid arms and was added 2 hours after addition of eugenol. Colcemid (0.1 µg/mL) was added to the culture 2-3 h before cell harvesting. Then, the cells were harvested and fixed and slides were stained with Giemsa and Hoechst 33258. For determination of sister chromatid exchanges, 50 cells per dose were scored from the 3 highest dose at which sufficient M2 cells were available. |

**Results**

Weak induction of sister chromatid exchange was reported in Chinese hamsters ovary cells incubated with 11-123 µg/mL of eugenol in absence of metabolic activation whereas positive results were observed after incubation with 273-326 µg/mL in the presence of metabolic activation (Table B.6.4.1.4.2). This increase in sister chromatid exchange occurred at doses that caused severe cell cycle delay.

**Table B.6.4.1.4.2. Sister chromatid exchanges following exposure to eugenol (LEC µg/mL)**

| | -S9 | +S9 | Overall result |
|---|---|---|---|
| LEC* | 107/75 | < 273 | + |
| RESULT | $+w^d/+$ | $+^d$ | |
| RANGE (µg/mL) | (11-123) | (273-326) | |

**Least effective concentration tested (**LEC**) is the lowest dose to give a 20% increase in SCE "d" delayed harvest. Harvest time was after 28.5 to 37.3 h in BrdUrd; "+w" 0.005 > P; 1 dose increased 20% over control; +: 2 or more doses increased 20% over control.*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS agrees with the applicant assessment and conclusion.

Eugenol induced sister chromatid exchanges both in the presence and absence of metabolic activation at the concentrations tested in the study.

The study is a scientific publication from a peer-reviewed journal. No guideline was reported in the study and it was compared against the current OECD TG 479 (deleted in 2014). The RMS deems the study as supporting information based on deviations from the guideline that include non-characterisation of the test item and poor level of reporting (no individual results provided).

**B.6.4.1.4.3 Study III**

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Bodell, W.J., Ye, Q., Pathak, D.N. and Pongracz, K. |
| **Report year** | 1998 |
| **Report title** | Oxidation of eugenol to form DNA adducts and 8-hydroxy-2'-deoxyguanosine: Role of quinone methide derivative in DNA adduct formation. |
| **Report No** | Not applicable, published study (*Carcinogenesis* 19 (3), 437-443) |
| **Document No** | KCA 5.4.1/14 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and classified as supplemental in DAR 2011 |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | DMSO |
| **Test system** | HL-60 cells |

| | |
|---|---|
| **Test method** | The formation of DNA adducts in HL-60 cells following treatment with eugenol was investigated. Also, the generation of 8-OH-dG in calf thymus DNA treated with eugenol and its oxidation products was studied.<br><br>*DNA Adduct formation:*<br>Eugenol diluted in DMSO was administered to cultured HL-60 cells at 4 different dose rates (100 µM, 250 µM, 500 µM, 1000µM) without $H_2O_2$ or at 100 µM with $H_2O_2$ (100 µM) during 24 hours. Following treatment, the cells were collected by centrifugation and resuspendend in cold Hanks balanced salt solution. DNA was isolated by a modified Marmur procedure.<br>The measurement of 8-OH-dG was carried out with HPLC-EC.<br><br>*Formation of 8-OH-dG:*<br>Incubation of 500 µg of purified calf thymus DNA with 100 µM eugenol was used in combination with either 15 U horseradish peroxidase (HRP) or 0.5 U myeloperoxidase (MPO) and 100 µM $H_2O_2$ in 10 mM $KH_2PO_4$. The final volumes of the incubation were 2 mL. Some of the incubations were additionally treated with either 500 µM acid ascorbic (AA) or reduced glutathione (GSH). The mixtures were incubated at 37ºC for 2h. The reactions were stopped with 1mL ice-cold chloroform and subsequent extraction was done with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 4M sodium acetate and cold ethanol and dissolved in 1mM sodium chloride and 1.5 mM sodium acetate.<br>For the copper sulphate activation assay, 100 µM eugenol was incubated with 500 µg DNA and 10 µM $CuSO_4$ in phosphate buffer. The final volumes were adjusted to 2mL.<br><br>Following the treatments, the cells were collected by centrifugation and DNA was isolated before $^{32}$P-labelling for analysis and relative adduct levels (RAL) calculation. |

**Results**

In HL-60 cells treated with eugenol, the level of DNA adducts formed was significantly correlated with the treatment concentration. Also, compared with treatment with 100 µM eugenol alone, the combination of eugenol and $H_2O_2$ potentiated the level of DNA adducts formed in HL-60 cells by 14-fold. (Table B.6.4.1.4.3/1). The negative control DMSO showed no DNA adduct formation, as expected. Treatment with 100 µM $H_2O_2$ alone did not produce any DNA adducts.

**Table B.6.4.1.4.3/1. Formation of DNA adducts in HL-60 cells treated with eugenol**

| Compound | Concentration (µM) | RAL x $10^{-7}$ (*n* = 3) |
|---|---|---|
| DMSO | - | - |
| Eugenol | 100 | 0.5 ± 0.3 |
| | 250 | 1.3 ± 0.3 |
| | 500 | 3.0 ± 1.2 |
| | 1000 | 6.2 ± 2.6 |
| $H_2O_2$ | 100 | Undetectable |
| Eugenol + $H_2O_2$ | 100 + 100 | 7.3 ± 3.0 |

*Adduct values are mean ± standard deviations*

*In vitro* activation of eugenol with HRP or MPO and $H_2O_2$ produced 3 DNA adducts whereas no DNA adducts were observed in reactions without HRP and $H_2O_2$. Addition of AA or GSH to the activation system inhibited the formation of DNA adducts.

The DNA adducts formed in HL-60 cells treated with eugenol were the same as those formed by *in vitro* peroxidase activation. Peroxidase activation of eugenol resulted in increased levels of 8-OH-dG (Table B.6.4.1.4.3/2). In addition to adduct formation, peroxidase activation of eugenol produced a 2- to 3-fold increase in the level of oxidative base damage.

**Table B.6.4.1.4.3/2. Formation of 8-OH-dG upon activation of eugenol with peroxidase enzymes**

| Activation system | 8-OH-dG (pmol/µg DNA) (*n* = 6) |
|---|---|
| DNA | 0.02 ± 0.02 |
| DNA + eugenol | 0.12 ± 0.05 |
| DNA + eugenol + $H_2O_2$ | 0.38 ± 0.06 |
| DNA + eugenol + $H_2O_2$ + MPO | 0.82 ± 0.10 |
| DNA + eugenol + $H_2O_2$ + HRP | 1.05 ± 0.24 |
| DNA + eugenol + $H_2O_2$ + HRP + mannitol | 0.09 ± 0.04 |

*Each of the incubations contained 100 µM of eugenol and 500 µg of DNA. To some of the incubations, 15 U HRP, 0.5 U MPO, 100 µM $H_2O_2$ and 100 mM mannitol were added as indicated. 8-OH-dG values are mean ± standard deviation.*

*In vitro* activation of eugenol with $CuSO_4$ also produced 2 DNA adducts and several minor adducts. Incubations of eugenol plus $CuSO_4$ significantly increased the formation of 8-OH-dG (Table B.6.4.1.4.3/3).

**Table B.6.4.1.4.3/3. Formation of 8-OH-dG upon activation of eugenol with $CuSO_4$**

| Activation system | 8-OH-dG (pmol/µg DNA) (*n* = 3) |
|---|---|
| DNA + eugenol | 0.12 ± 0.05 |
| DNA + eugenol + $CuSO_4$ | 11.4 ± 2.2 |
| DNA + eugenol + $CuSO_4$ + 100 µM BCS | 3.87 ± 0.48 |
| DNA + eugenol + $CuSO_4$ + 150 U catalase | 1.04 ± 0.70 |
| DNA + eugenol + $CuSO_4$ + 100 mM mannitol | 1.02 ± 0.70 |

*All of the incubations contained 500 µg of DNA plus 100 µM of eugenol. To some of the reactions 10 µM and $CuSO_4$ either BCS, catalase or mannitol were added as indicated. 8-OH-dG values are mean ± standard deviation.*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and was accepted as supporting information.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS agrees with the applicant assessment and conclusion.

The RMS deems the study as supporting information as the study is not a validated method to evaluate DNA reactivity.

### B.6.4.1.4.4 Study IV

| | |
|---|---|
| **Data point** | CA 5.4.1 (EU data requirement) |
| **Report author** | Martins, C., Doran, C., Laires, A., Rueff, J. and Rodrigues, A.S. |
| **Report year** | 2011 |
| **Report title** | Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells |
| **Report No** | Not applicable, published study (*Food Chem. Toxicol.* 49, 385-392) |
| **Document No** | KCA 5.4.1/15 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Solvent** | DMSO |
| **Test system** | Chinese Hamster ovary AA8 (repair proficient) |
| **Metabolic activation** | None |
| **Negative control** | Solvent |
| **Positive control** | $H_2O_2$ |
| **Test method** | *MTT cell viability assay*<br>Eugenol diluted in DMSO was applied to CHO cells for 3 and 24 h periods at doses ranging from 50 to 2000 µM. DMSO was added to cells without eugenol as control cultures. After each treatment cells were washed with culture medium and MTT was added to each well at a concentration of 0.5 mg/ml. Cells were grown for a further period of 3 h and then carefully washed with PBS. At the end of the incubation period, the media were discarded and DMSO (200 µL) was added to each well to dissolve the formazan crystals. Absorbance values presented by control cultures, correspond to 100% cell viability. At least two independent experiments were performed and eight replicates were used in each independent experiment.<br><br>*In vitro alkaline comet assay*<br>Eugenol diluted in DMSO was applied to CHO cells at doses ranging from 50 to 500 µM for 1h in F-10 medium supplemented with 10% foetal bovine serum and 1% antibiotic solution, at 37°C and 5% $CO_2$. Cells with DMSO were used as negative control, and 200 l M $H_2O_2$ treatment was used as a positive control. At least 50 cells were analysed per slide (two slides per independent experience giving a total of 100 cells for each concentration per experiment) and at least three independent experiments were performed.<br><br>*γ-H2AX assay*<br>Detection of double strand breaks (DSB) in DNA was carried out by immunofluorescence. Eugenol diluted in DMSO was applied to CHO cells at 250 and 750 µM for 1 h, washed and fixed before incubation for 2 h with the γ-H2AX primary antibody (mouse anti-c-H2AX (ser139), Stressgen, bioreagents Corp., Canada). γ-H2AX foci/nucleus were counted in order to distinguish between cells with numerous foci and cells with large foci. At least two independent experiments with two replicates per experiment for each dose were performed. At least 20 nuclei were analysed per experiment per dose. |

**Results**

*MTT cell viability assay*
Eugenol displayed a dose dependent decreases in viability. For a 3-hour exposure there were no significant reductions in cell viability, either in AA8 nor EM9 cells. For a 24 h exposure, the cell viability was reduced below 50% when cells were treated with concentrations higher than 500 µM or 1000 µM, for AA8 and EM9 cells, respectively. Therefore, for the comet assay, a maximum concentration of 500 µM and an incubation period of 1 h were chosen in order to minimize the influence of cell death in these assays.

*Alkaline comet assay*
CHO AA8 and EM9 cells were exposed to a range of concentrations from 50 to 500 µM for 1 h and the percentage DNA in tail was calculated. In AA8 cells, DNA damage was induced by eugenol, but with no statistical significance (one-way ANOVA, P = 0.0528). However, there was a significant linear trend for DNA damage (P = 0.0132) (Figure B.6.4.1.4.4/1).

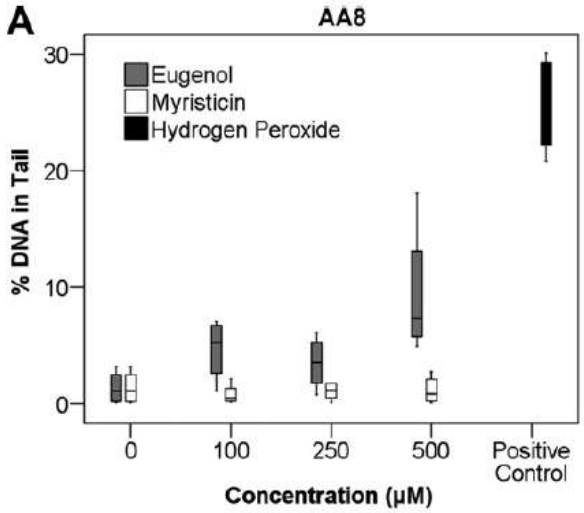



**Figure B.6.4.1.4.4/1: DNA damage induced by Eugenol in AA8 cells using the alkaline comet assay**

*γ-H2AX assay*

Eugenol induced a significant response in DSBs after 1-hour exposure period in AA8 cells (one-way ANOVA, P = 0.0263) and results displayed a significant linear trend (P = 0.0097) (Figure B.6.4.1.4.4/2). To confirm the analysis authors performed a Student t-test comparing the control with individual doses, and eugenol showed a significant response at 750 µM in these cells (P = 0.0255).

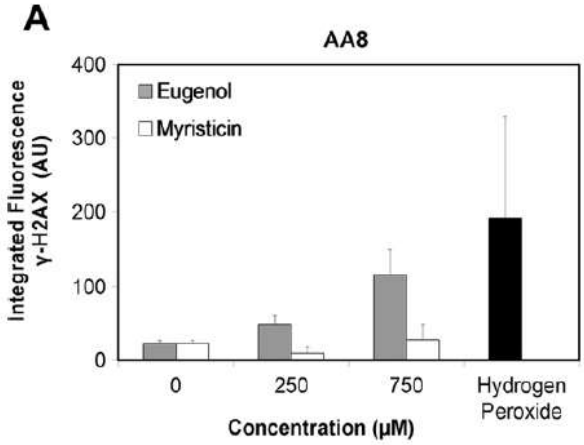



**Figure B.6.4.1.4.4/2: Histone H2AX phosphorylation in AA8 cells treated with eugenol**

In all assays, eugenol showed genotoxic activity. In AA8 cells, after 1 h incubation, eugenol induces DNA strand breaks as measured by the comet assay and measured by the γ-H2AX assay. Authors indicated the positive results with the γ-H2AX assay for eugenol are in accordance with their previous results with the CA assay (Maralhas *et al.*, 2006; B.6.4.1.3.4), showing that eugenol produces DSBs in the absence of an exogenous biotransformation system.

**<u>Assessment and conclusion by applicant</u>:**

This new study is considered to be acceptable as supporting information for the renewal of eugenol on the basis that it follows a non-standard study design (reliable with restrictions; the study is not GLP or guideline-compliant, however methodology is scientifically acceptable).

| |
|---|
| The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion.<br>The RMS deems the study as supporting information only as the study is not a validated method to evaluate DNA reactivity. |

## B.6.4.2. *In vivo* studies in somatic cells

### B.6.4.2.1. Mammalian micronucleus test

#### B.6.4.2.1.1. Study I

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Hayashi, M., Kishi, M., Sofuni, T. and Ishidate, M. |
| **Report year** | 1988 |
| **Report title** | Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals |
| **Report No** | Not applicable, published study (*Fd. Chem. Toxic*., 26, 487-500) |
| **Document No** | KCA 5.4.2/01 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 474 (2016)** | -Batch number is not reported<br>-No data about animal housing<br>-Intraperitoneal injection is not a recommended route of administration<br>-Only 1000 PCE were scored per animal (4000 are recommended in the current guideline)<br>-Limited level of reporting of results |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: > 98 %; Batch No.: not stated |
| **Vehicle** | Olive oil |
| **Negative control** | Vehicle (olive oil) |
| **Positive control** | Mitomycin C |
| **Test system** | Eight week old male ddY mice |
| **Test method** | A preliminary maximum tolerated dose test was carried out prior to the main study (details not given in the publication). Two male mice per group were used in the pilot study and six in full-scale tests.<br>Eugenol was diluted in olive oil and was administered *via* intraperitoneal injection to mice (6 male mice/group) once at dose levels of 100, 200, 400 and 800 mg/kg bw or four times at 24h at 400 mg/kg bw. Negative controls received vehicle (olive oil) only. The animals were sacrificed by cervical dislocation 18h (single injection) or 24h after the 4$^{th}$ injection. Samples of the femoral marrow were flushed out with foetal bovine serum.<br>1000 Polychromatic erythrocytes per animal were scored and the micronucleated polychromatic erythrocytes (MNPCE) were recorded. The proportion of polychromatic erythrocytes (PCEs) among the total erythrocytes was also evaluated by observing 1000 erythrocytes on the same slide.<br>Before evaluating the test data statistically, the frequencies of MNPCEs in concurrent negative and positive control groups were compared with the historical data (not reported). |

| | |
|---|---|
| **Criteria for assessment of results** | For the test with single treatment: A positive result was recorded when one or more treatment group(s) showed a statistically significant difference (P< 0.01) from the spontaneous level of MNPCEs and the trend test indicated a positive dose response (p< 0.05).<br>For the multiple treatment test only the first step is applied. |

**Results**

Eugenol gave negative results both in the single and multiple-dose treatments. No induction of micronuclei or alteration of proportion of PCEs compared to the negative control was observed (Table B.6.4.2.1.1).

Eugenol did not cause cytogenetic damage in mice in the *in vivo* micronucleus test. Whilst eugenol did not cause toxicity in the bone marrow cells, indications of systemic toxicity (including mortality) were observed at the highest dose (800 mg/kg bw). This observation may be considered as evidence that there is sufficient exposure to the bone marrow (according to OECD TG 474 (2016).

**Table B.6.4.2.1.1. Results of the micronucleus test using mouse bone marrow cells**

| Compound | Number of dose | Time between doses (hour) | Sampling time (hour) | Dose level (mg/kg) | MNPCE (%) | PCE (%) | Mortality |
|---|---|---|---|---|---|---|---|
| Mitomycin C (positive control) | 1 | - | - | 2.0 | 6.93 ± 2.20* | 41.7 ± 6.9 | 0/6 |
| Olive oil (negative control, vehicle) | 1 | - | 18 | 0 | 0.23 ± 0.14 | 48.3 ± 6.0 | 0/6 |
| Eugenol | 1 | - | 18 | 100 | 0.17 ± 0.12 | 52.3 ± 3.4 | 0/6 |
| | | | | 200 | 0.25 ± 0.16 | 53.4 ± 5.7 | 0/6 |
| | | | | 400 | 0.27 ± 0.12 | 53.4 ± 2.2 | 0/6 |
| | | | | 800 | 0.25 ± 0.21 | 40.8 ± 1.7 | **4/6** |
| | 4 | 24 | 24 | 400 | 0.18 ± 0.12 | 54.5 ± 7.2 | 0/6 |

MNPCE = Number of Micronucleated Polychromatic Erythrocytes; PCE = Polychromatic Erythrocytes; MMC = Mitomycin C; NS = Not significant

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Based on the available data, eugenol did not induce micronuclei in mice. Mortality was observed at the top dose (800 mg/kg), which may be an indication of systemic exposure to this compound.

The study is a scientific publication from a peer-reviewed journal. Some deviations from the current OECD TG 474 (2016) have been identified, such as characterisation of the test item, only 1000 PCE are studied, single sex animals used and overall very limited detail of reporting. Furthermore, the intraperitoneal route is not the recommended route of administration of the test item. For these reasons, the RMS deems the study as supporting information to evaluate the clastogenicity *in vivo* of eugenol.

#### B.6.4.2.1.2. Study II

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Woolverton, C., Fotos, P., Mokas, M. and Mermigas, M |
| **Report year** | 1986 |
| **Report title** | Evaluation of eugenol for mutagenicity by the mouse micronucleus test |
| **Report No** | Not applicable, published study (*J. Oral Pathol*. 15, 450-453) |
| **Document No** | KCA 5.4.2/02 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline**<br>**OECD TG 474 (2016)** | - No characterisation of the test substance<br>- No data about animal housing<br>- 3 different concentrations should be tested<br>- Oral doses exceed the 2000 mg/kg bw recommended by guidance<br>- Only 1000 PCE were scored per animal (4000 are recommended for the current guideline)<br>- No historical control data provided |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011). |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Sterile physiologic saline |
| **Negative control** | Vehicle (sterile physiological saline) |
| **Positive control** | Quinacrine dihydrochloride |
| **Test system** | Adult male Swiss-Webster mice |
| **Route** | Intraperitoneal and oral |
| **Test method** | A preliminary toxicity test was conducted to determine the maximum tolerated dose of eugenol. The dose selection was based on the determination of $LD_{50}$ described by Reed and Meunch (1938). Eugenol diluted in sterile pyrogen-free saline was administered intraperitoneally at a volume of 250 µL (i.p) or orally at a volume of 100 µL (cannula into the esofagus) at 8 dose levels ranging from 0-14794 mg/kg. Hourly observations for mortality were made over 3 days.<br>The main assay was carried out in accordance with the US EPA's Gene-Tox Program (Heddle *et al.*, 1983). Eugenol diluted in sterile pyrogen-free saline was administered intraperitoneally to a group of 12 male mice at 80% (739.7 mg/kg bw) and 25% (147.9 mg/kg bw) of the $LD_{50}$ (1109.6 mg/kg bw) in 250 µL determined in the preliminary test. Undiluted eugenol was administered by gavage to a separate group of 12 animals. A second dosing was administered after 24h to examine long-term anaphase mutations in erythrocyte stem cells in both dosing routes.<br>The positive control was quinacrine dihydrochloride, and the negative was the pyrogen-free saline used as the diluent and vehicle.<br>Mice were sacrificed at 30h post initial treatment. The bone marrow from the epiphyses was extracted and 1000 PCEs were scored for micronuclei. |
| **Statistical analysis** | ANOVA statistical analysis was performed to detect significant differences or similarities between positive and negative controls versus eugenol test doses |

**Results**

*Preliminary test*
The intraperitoneal $LD_{50}$ was found to be 1109.6 mg/kg bw (7.5% eugenol in saline); mortality was observed in all treated groups (Table B.6.4.2.1.2/1). There were no mortalities following oral exposure.

**Table B.6.4.2.1.2/1. $LD_{50}$ determination for eugenol (i.p. route)**

| Eugenol (mg/kg) | Eugenol in saline* | Surviving mice (%) |
|---|---|---|
| 0 | 0% | 12 (100 %) |
| 961.6 | 6.6% | 10 (83 %) |
| 1035.6 | 7.0% | 8 (67 %) |
| 1109.6 | 7.5% | 6 (50 %) |
| 1183.5 | 8.0% | 5 (42 %) |
| 1849.3 | 12.5% | 2 (25 %) |
| 3698.6 | 25% | 1 (8 %) |
| 7397.2 | 50% | 0 (0 %) |
| 14794.0 | 100% | 0 (0 %) |

**Dilution in physiologic saline*

*Micronucleus test*
No mortality occurred in this study.
Eugenol induced a statistically-significant increase in micronucleus formation over the negative control both in intraperitoneal and oral administrations, with a more important frequency by ip dosing (Table B.6.4.2.1.2/2). Negative controls showed low frequency of spontaneous micronucleus formation in mice and a low increase with saline. The results with the positive control indicate the validity of the study.

**Table B.6.4.2.1.2/2. Induction of micronuclei in polychromatic erythrocytes by intraperitoneal eugenol**

| Compound | Dose (mg/kg bw) | Frequency (‰) mean ± standard deviation | No. cells/sample | Analysis of variance (p) |
|---|---|---|---|---|
| **Intraperitoneal administration** | | | | |
| - | - | 1.40 ± 0.89 | 8000/8 | - |
| Saline | - | 2.00 ± 0.76 | 8000/8 | - |
| Quinacrine | 100.0 | 58.5 ± 2.88 | 8000/8 | 0.001 |
| Eugenol | 739.7 | 20.5 ± 5.79 | 12000/12 | 0.001 |
| Eugenol | 147.9 | 18.5 ± 4.96 | 12000/12 | 0.001 |
| **Oral administration** | | | | |
| - | - | 1.40 ± 0.89 | 8000/8 | - |
| Saline | - | 1.75 ± 1.71 | 8000/8 | - |
| Quinacrine | 100.0 | 9.25 ± 2.06 | 8000/8 | 0.001 |
| Eugenol | 14794.4 | 5.70 ± 2.54 | 12000/12 | 0.003 |

‰ Frecuency per 1000 cells scored

The author explained that the gastric inactivation could be the cause of the decrease in mutagenicity by oral route. Very small volumes of 100% eugenol could reach systemic distribution.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways.

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Eugenol caused cytogenetic damage in mice in the *in vivo* micronucleus test both by the oral and intraperitoneal routes.

Note from Co-RMS: since the frequency of micronuclei is statistically-significantly increased, then the test item reached bone marrow and no other proof is needed. The increased micronuclei frequency in orally-administered animals (recommended route of exposure) was caused by 14794.4 mg/kg bw of eugenol, a far higher dose than the recommended (2000 mg/kg bw) by the OECD TG 474 (2016).

| |
|---|
| The study is a scientific publication from a peer-reviewed journal. The study was assessed for compliance with the current OECD TG 474 (2016) and a few deviations have been found such as scoring of 1000 PCEs (instead of the recommended 4000), no historical control data were provided and no characterisation of the test item. Due to these method deficiencies, the RMS considers the study as supportive information only.<br>In conclusion, the RMS agrees with Co-RMS that oral doses exceed the recommended dose by Guidance and therefore, the positive outcome may be considered as supporting information only. |

**B.6.4.2.1.3. Study III**

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Rompelberg, C.J.M., Stenhuis, W.H., de Vogel, N., Van Osenbruggen, W.A., Schouten, A. and Verhagen, H. |
| **Report year** | 1995 |
| **Report title** | Anti-mutagenicity of eugenol in the rodent bone marrow micronucleus test |
| **Report No** | Not applicable, published study (*Mutat. Res.* 346, 69-75) |
| **Document No** | KCA 5.4.2/03 |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline OECD TG 474 (2016)** | -Incomplete characterisation of the test item<br>-3 different concentrations should be tested<br>-Only 1000 PCE were scored per animal (4000 are recommended for the current guideline)<br>-Limited level of reporting of results (no individual results reported) |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 99 %; Batch No.: not stated |
| **Vehicle** | Sterile physiologic saline |
| **Negative control** | Diet free-eugenol |
| **Positive control** | Not applicable |
| **Test system** | Adult male Swiss-Webster mice |
| **Route** | Oral (eugenol) and intraperitoneal (mutagens) |
| **Test method** | The antimutagenic effect of eugenol on the mutagenicity of cyclophosphamide (CP), mitomycin C (MMC), ethyl methylsulphonate (EMS) and benzo[a]pyrene (B[a]P) was investigated in an *in vivo* micronucleus test.<br>Two groups of 30 Swiss mice were fed with two different diets: one group were fed with pelleted purified diet (control) and the other group were fed pelleted purified diet containing 0.6 % eugenol (nominal concentration) for 15 days. After 14 days, the groups were divided and 5 animals per group were treated i.p. with one of the following known mutagens: CP (25 mg/kg bw), MMC (1.5 mg/kg bw), EMS (300 mg/kg bw) and B[a]P (250 mg/kg bw). CP, MMC and EMS were dissolved in saline; B[a]P was suspended in olive oil. Control animals were given the solvents only (physiological saline or olive oil). At 24 hours, the animals were killed by cervical dislocation. From the dissected femur, bone marrow was collected. For each animal, the number of micronucleated polychromatic erythrocytes (MPE) per 1000 polychromatic erythrocytes (PE) and the number of PE per 1000 erythrocytes were determined. |
| **Statistical analysis** | Data were analysed by two-way analysis of variance (ANOVA). If the result was significant, ($P < 0.05$) asymptotic pairwise t-tests were performed (2-tailed). |

**Results**

There was no statistically significant difference in food intake of mice on the eugenol diet (0.4% actual concentration) (mean ± sd; 5.02 ± 0.56 g/mouse/day) versus control diet (mean ± sd; 5.27 ± 0.59 g/mouse/day). For eugenol group the average dose was approximately 680 mg/kg bw/day. No differences in bodyweight were noted.

No mortality occurred in the main micronucleus assay. There was no statistically significant difference in food intake of mice on the eugenol diet versus mice on the control diet. Also, there was no significance in body weight between the two groups of diet.

Eugenol has no effect on the frequency of MPEs.

The incidence of micronucleated polychromatic erythrocytes (MPE) is significantly different in mutagens from solvent controls. Eugenol apart from cyclophosphamide (CP) itself had no effect on the frequency.

Pre-treatment with eugenol for 15 days resulted in a significant decrease in the frequency of MPEs induced by CP to 15.6 ± 3.7 MPE/1000 PE (p<0.01), but not the other mutagens (Table B.6.4.2.1.3/1). Remarkably, in the eugenol diet group an increase in the percentage of polychromatic erythrocytes is detected in all of them, especially in CP and EMS (p>0.01). In this group, there is an increase in the percentage of PE % with saline, 50.1±2.6 and with olive oil 51.1±4.9. The results are shown in Table B.6.4.2.1.3/2.

**Table B.6.4.2.1.3/1. Summary of micronuclei induction of polychromatic erythrocytes in mice**

| Compound | MPE/1000PE | |
|---|---|---|
| | Control diet (no eugenol) | Diet containing eugenol |
| Mitomycin C (MMC) | 66.0 ± 14.5 | * |
| Cyclophosphamide (CP) | 28.4 ± 8.7 | 15.6 ± 3.7 ($p < 0.01$) |
| Ethyl methanesulfonate (EMS) | 27.6 ± 6.7 | * |
| Benzo[a]pyrene (B[a]P) | 11.2 ± 4.1 | * |
| Saline | 1.6 ± 1.5 | * |
| Olive oil | 1.6 ± 1.8 | * |

*Values correspond to mean ± standard deviation*
**No statistical difference compared to control diet group (numbers not reported in the publication)*

**Table B.6.4.2.1.3/2. Summary of percentage of polychromatic erythrocytes in mice**

| Compound | % Polychromatic erythrocytes | |
|---|---|---|
| | Control diet (no eugenol) | Diet containing eugenol |
| Mitomycin C (MMC) | 42.5 ± 6.3 % | 49.5 ± 13.1 % |
| Cyclophosphamide (CP) | 41.7 ± 9.2 % * | 58.7 ± 5.8 % |
| Ethyl methanesulfonate (EMS) | 29.9 ± 5.7 % * | 59.3 ± 13.1 % |
| Benzo[a]pyrene (B[a]P) | 40.0 ± 12.5 % | 50.5 ± 6.2 % |
| Saline | 46.7 ± 8.9 % | 50.1 ± 2.6 % |
| *Olive oil* | 42.5 ± 3.7 % | 51.1 ± 4.9 % |

*Values correspond to mean ± standard deviation*
**Statistically-significant (P < 0.01)*

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**<u>Assessment and conclusion by RMS</u>:**

RMS agrees with the applicant's assessment. Eugenol had a modulatory effect on cyclophosphamide induction of micronuclei.

The study is a scientific publication from a peer-reviewed journal. The aim of the study was to evaluate the anti-mutagenic effects of eugenol in mice treated with known mutagens. Based on the deviations from current OECD TG 474 (2016), which include only 1000 PCE studied, incomplete characterisation of the test item, limited detail of reporting of results or no historical control data provided, the RMS deems the study as supporting information.

#### B.6.4.2.1.4. Study IV

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Shelby, M.D., Erexson, G.L., Hook, G.J. and Tice R.R. |
| **Report year** | 1993 |
| **Report title** | Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals |
| **Report No** | Not applicable, published study (*Environ. Mol. Mutagen.*, 21, 160-179) |
| **Document No** | KCA 5.4.2/04 |
| **Guidelines followed in study** | Not stated (Comparable to OECD TG 474 (1983)) |
| **Deviations from current test guideline OECD TG 474 (2016)** | -Incomplete characterisation of the test item<br>-Only 2000 PCE were scored per animal (4000 are recommended for the current guideline)<br>- The proportion of polychromatic erythrocytes (PCEs) was determined among 200 erythrocytes (instead of a minimum of 500 recommended by guidance)<br>-No clinical signs reported<br>-No results reported for positive controls (only for HCD)<br>-Route of administration of test substance is not recommended by the guideline<br>-Limited level of reporting of results (no individual data provided) |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: 210426 |
| **Vehicle** | Corn oil |
| **Negative control** | Vehicle (corn oil) |
| **Positive control** | 7,12-Dimethylbenzanthracene (DMBA) and Mitomycin C (MMC) |
| **Test system** | Male B6C3F1 mice |
| **Test method** | This study evaluates the potential cytogenetic damage of eugenol in an *in vivo* micronucleus test using B6C3F1 male mice.<br><br>*Preliminary test*<br>A preliminary study was carried out to select the maximum dose for the main study. No detailed information is give although the general criteria included mortality, administrations characteristics or cytotoxicity (depression in the percentage of bone marrow PCE > 15%). The initial doses tested were generally 200, 1000 and 2000 mg/kg bw/d however specific information for eugenol is not reported. The test substances were administered to groups of 5 mice on three consecutive days, surviving mice were sacrificed 48 h after the third treatment for preparation of bone marrow and peripheral blood smears (two slides/tissue/mouse). The percentage of PCE among 200 erythrocytes was determined. Based on the results obtained doses were selected for the main test (results were not reported).<br><br>*Main study*<br>Eugenol diluted in corn oil was administered for three consecutive days *via* intraperitoneal injection to groups of 5-7 animals/dose group at a volume of 0.4 mL and dose levels of 150, 300 and 600 mg/kg. Corn oil was used as a negative control and a weakly active dose of DMBA in corn oil was used as the positive control. The animals in every group were sacrificed by $CO_2$ exposure 24 hours after the last of the 3 injection. Femoral marrow cells were collected, fixed with methanol and stained with acridine orange. For each mouse, 2000 polychromatic erythrocytes were scored for micronuclei and the proportion of polychromatic erythrocytes (PCEs) was determined among 200 erythrocytes. |

| | |
|---|---|
| **Criteria for assessment of results** | Data were analysed by two-way analysis of variance (ANOVA). If the result was significant, (P < 0.05) asymptotic pairwise t-tests were performed (2-tailed). |

**Results**
Eugenol did not induce micronuclei in polychromatic erythrocytes up to the highest dose tested (600 mg/kg) (Table B.6.4.2.1.4). No induction of micronuclei or alteration of proportion of PCEs compared to the negative control was observed.

**Table B.6.4.2.1.4. Summary of micronuclei induction of polychromatic erythrocytes in mice after a three-day exposure to eugenol (intraperitoneal)**

| Compound | Dose level (mg/kg) | Micronuclei / 1000 PCE | PCE (%) | Mortality |
|---|---|---|---|---|
| Corn oil | 0 | 3.50 ± 0.82 | 68.0 | 0/5 |
| Eugenol | 150 | 3.70 ± 0.96 | 58.8 | 0/5 |
| | 300 | 3.20 ± 0.74 | 60.4 | 0/5 |
| | 600 | 3.29 ± 0.46 | 59.0 | 0/7 |
| DMBA (positive control) | 12.5 | 7.93 ± 1.69* | Micronuclei/1000 PCE Range 4.40 – 10.60 over 17 groups | |
| MMC (positive control) | 0.2 | 6.85 ± 2.26* | Micronuclei/1000 PCE Range 3.80 – 11.20 over 8 groups | |

** Historical control data*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Eugenol did not cause cytogenetic damage in mice in a multidose *in vivo* micronucleus (ip route).

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 474 (2016) include no characterisation of the test item (purity not sated), 2000 instead of 4000 PCE analysed and poor level of reporting (no individual results provided, only summary). Therefore, the RMS deems the study as supporting information only.

### B.6.4.2.1.5. Study V

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Ellahueñe, M.F., Perez-Alzola, L.P., Orellana-Valdebenito, M., Muñoz, C. and Lafuente-Indo, N. |
| **Report year** | 1994 |
| **Report title** | Genotoxic evaluation of eugenol using the bone marrow micronucleus assay |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 320, 175-180) |
| **Document No** | KCA 5.4.2/05 |
| **Guidelines followed in study** | Not stated (Comparable to OECD TG 474 (1983)) |
| **Deviations from current test guideline OECD TG 474 (2016)** | - Incomplete characterisation of the test item<br>- Only 1000 PCE were scored per animal (4000 are recommended for the current guideline)<br>-The proportion of immature among total erythrocytes accounted only for 300 instead of a minimum of 500 recommended by guidance<br>-No mortality should be observed at the highest dose tested<br>- No historical data provided<br>- Route of administration is not the recommended by guidance (*i.e.* oral) |

| | |
|---|---|
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Corn oil |
| **Negative control** | Vehicle (corn oil) |
| **Positive control** | Methyl methanesulphone (MMS) |
| **Test system** | Adult male CF1 mice |
| **Test method** | This study evaluates the potential cytogenetic damage of eugenol in an *in vivo* micronucleus test using CF1 male mice.<br><br>*Preliminary test – $LD_{50}$ determination*<br>A preliminary study was carried out to select the maximum dose for the main study. Eugenol diluted in corn oil was administered intraperitoneally (i.p.) at 8 dose levels (400, 500, 600, 700, 800, 900, 1000, 1500 mg/kg bw) to groups of 10 mice. Mortality was observed for 7 days post-treatment.<br><br>*Main study*<br>Eugenol diluted in corn oil was administered intraperitoneally to groups of 8 male mice at three dose levels (100, 400, 600 mg/kg bw) based on the preliminary test ($LD_{50}$ = 700 mg/kg bw). The dose was adjusted with a maximum volume of 25 mL/kg. The negative control (corn oil) and the positive control (methyl methane-sulfonate (MMS)) were also administered intraperitoneally to groups of 8 mice. All mice were sacrificed 30 hours post-treatment.<br>In a post-treatment sampling time, eugenol was administered intraperitoneally once to groups of 8 mice at 400 mg/kg bw. The animals in every group were sacrificed by cervical dislocation 24- or 48-hours post-treatment. The negative control was sampled a single time at 30 hours. Femoral marrow cells were collected, fixed, stained with Giemsa and examined. The frequency of micronuclei was determined as the number of micronucleated polychromatic erythrocytes (MnPCE) in 1000 PCEs per mouse. Cytotoxicity was determined as the ratio of polychromatic/normochromatic erythrocytes PCE/NCE in 300 erythrocytes (ratio ≤ 0.1 indicates induced cytotoxicity). |

**Results**

*Preliminary study*

The $LD_{50}$ as determined at 7 days following treatment was 700 mg/kg (Table B.6.4.2.1.5/1).

**Table B.6.4.2.1.5/1. Determination of LD50 at 7 days following intraperitoneal dose of eugenol**

| Eugenol (mg/kg) | % Survival |
|---|---|
| 400 | 100 % |
| 500 | 100 % |
| 600 | 70 % |
| 700 | 50 % |
| 800 | 30 % |
| 900 | 10 % |
| 1000 | 20 % |
| 1500 | 30 % |

*Main study*

Eugenol induces a statistically significant increase in micronucleus formation at 400 and 600 mg/kg bw (57% and 87% $LD_{50}$ respectively) compared to the negative control (Table B.6.4.2.1.5/2). The positive control MMS induced

statistically significant increase in micronucleus formation over the negative control, validating the study. For both treatment and control groups, the ratio PCE/NCE was in the normal range showing no cytotoxicity.

**Table B.6.4.2.1.5/2. Incidence of micronucleated polychromatic erythrocytes at 30h sampling**

| Compound | Dose (mg/kg bw) | MnPCE Frequency (%) mean ± standard deviation | PCE/NCE |
|---|---|---|---|
| Corn oil | - | 0.25 ± 0.07 | 1.15 |
| Eugenol | 100 | 0.36 ± 0.07 | 0.87 |
| | 400 | **0.75 ± 0.18*** | 0.39 |
| | 600 | **0.97 ± 0.46*** | 0.81 |
| MMS | 100 | **10.75 ± 1.99*** | 0.27 |

In a separate study, intraperitoneal administration of 400 mg/kg bw eugenol to groups of 8 mice and further evaluation of MPCE, eugenol induced statistically-significant increases in micronucleus formation at all post-treatment sampling time (24, 30, 48 h) (Table B.6.4.2.1.5/3). No differences have been observed between the various sampling times.

**Table B.6.4.2.1.5/3. Incidence of micronucleated polychromatic erythrocytes at various sampling times following a 400 mg/kg bw dose of eugenol**

| Sampling time | MnPCE Frequency (%) [mean ± standard deviation] | PCE/NCE |
|---|---|---|
| 24 h | 0.55 ± 0.18* | 1.44 |
| 30 h | 0.75 ± 0.18* | 0.39 |
| 48 h | 0.61 ± 0.19* | 0.84 |

**Statistically significant at p < 0.01 compared with corn oil at 30 h post-treatment*

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**<u>Assessment and conclusion by RMS</u>:**

RMS agrees with the applicant's assessment. Eugenol induced micronuclei in polychromatic erythrocytes following i.p administration at doses over 400 mg/kg bw. Sampling time did not seem to affect the outcome of the study.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 474 (2016) include no characterisation of the test item (purity not sated), 1000 instead of 4000 PCE analysed and no historical control data. Therefore, the RMS deems the study as supporting information only.

### B.6.4.2.1.6. Study VI

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Rompelberg, C.J.M., Evertz, S.J.C.J., Bruijntjes-Rozier, G.C.D.M., van den Heuvel, P.D. and Verhagen, H. |
| **Report year** | 1996a |
| **Report title** | Effect of eugenol on the genotoxicity of established mutagens in the liver |
| **Report No** | Not applicable, published study (*Fd. Chem. Toxic.*, 34 (1), 33-42) |
| **Document No** | KCA 5.4.2/06 |
| **Guidelines followed in study** | Not stated (Comparable to OECD TG 474 (1983)) |
| **Deviations from current test guideline OECD TG 474 (2016)** | - Incomplete characterisation of the test item<br>- Only 1000 PE were scored per animal (4000 are recommended for the current guideline) |

| | |
|---|---|
| | - Limited level of reporting (results, clinical observations)<br>- No historical data provided |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 99%; Batch No.: not stated |
| **Vehicle** | Corn oil |
| **Negative control** | Vehicle (corn oil) |
| **Positive control** | Mitomycin C (MMC) |
| **Test system** | Adult male Wistar rats |
| **Test method** | A dose-range finding study was carried out prior to the main experiment.<br>Eugenol dissolved in corn oil was administered by gavage for 10 days at daily to groups of 8 rats at dose levels of 500 and 1000 mg/kg bw (at a volume of 10 mL/kg bw). The control group (4 animals) was given 10 mL/kg bw of corn oil. The positive control mitomycin C was administered once intraperitoneally at 3 mg/kg bw 24 h before sacrifice. The animals in all test groups were sacrificed 24 hours after dosing. Femoral marrow cells were collected, fixed with methanol and stained with May-Grünwald-Giemsa stain. For each animal, 1000 polychromatic erythrocytes (PE) were scored for micronuclei (MPE) and the proportion of polychromatic erythrocytes (PEs) was determined among 1000 erythrocytes.<br>Statistical analysis was carried out by one-way analysis of variance, followed by Dunnett's multiple comparison test and an orthogonal polynomial test. The MPE data were transformed before analysis by use of a square-root transformation to 'normalize' the counts. If results were significant ($P \leq 0.05$), asymptotic pairwise *t*-tests were performed. |

**Results**

Eugenol had no effect on the incidence of MPE in the bone marrow cells compared to the negative control group (Table B.6.4.2.1.6).

**Table B.6.4.2.1.6. Incidence of MPE in bone marrow cells of rats treated orally for 10 days with eugenol**

| Compounds | Dose (mg/kg bw) | MPE/1000 PE | % PE | Mortality |
|---|---|---|---|---|
| Corn oil | - | 1.25 ± 0.89 | 54.0 ± 8.3 | 0/8 |
| Eugenol | 500 | 1.50 ± 0.93 | 54.5 ± 12.6 | 0/8 |
| | 1000 | 1.14 ± 1.07 | 60.2 ± 9.2 | 1/8 |
| Mitomycin C | 3 | 39.50 ± 3.7* | 47.3 ± 8.2 | 0/4 |

*% PE: percentage of polychromatic erythrocytes of total erythrocytes.*
** P < 0.001*

The percentage of PE showed a slight increase in rats at the top dose group (1000 mg/kg bw) although no statistical significance against control. The positive control substances induced micronuclei formation.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Eugenol did not induce an increase in the frequency of micronucleated polychromatic erythrocytes following oral daily doses of eugenol for 10 days.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 474 (2016) include incomplete characterisation of the test item, 1000 instead of 4000 PCE analysed, very limited level of reporting of results and clinical observations and no historical control data. Furthermore, one rat died at the top dose group. The evidence of bone marrow exposure is not reported in this study. Data from other studies in rat, namely *in vivo* micronucleus test (B.6.4.1.8, study VIII) and ADME data (pharmacokinetic study in rats, B.6.1.1-08), are available to support the evidence for bone marrow exposure. The RMS deems the study as supporting information only based on the deviations from guideline.

**B.6.4.2.1.7. Study VII**

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Maura, A., Pino, A. and Ricci, R. |
| **Report year** | 1989 |
| **Report title** | Negative evidence *in vivo* of DNA-damaging, mutagenic and chromosomal effects of eugenol |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 227, 125-129) |
| **Document No** | KCA 5.4.2/07 |
| **Guidelines followed in study** | Not stated (Comparable to OECD TG 474 (1983)) |
| **Deviations from current test guideline OECD TG 474 (2016)** | - Incomplete characterisation of the test item<br>- Only 4 rats/group dose (recommended a minimum of 5 animals/group)<br>- Sampling was only done 6h after the last dose (minimum of 24h after dose). Also, split dosing was done at 24h instead of the recommended 2-3 h<br>- Only 1000 PE were scored per animal (4000 are recommended for the current guideline)<br>- Limited reporting of results, clinical observations<br>- No information on the proportion of immature erythrocytes given<br>- No historical data provided |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Physiological saline |
| **Negative control** | Vehicle (water) |
| **Positive control** | Triethylenemelamine |
| **Test system** | Female Sprague-Dawley rats |
| **Test method** | Dose selection was based on the acute oral toxicity study by Jenner *et al.* (1964) and the administered doses increase serially by a factor of 2, with a maximum equal to ½ $LD_{50}$.<br><br>Eugenol diluted in physiological saline was administered orally to groups of 4 female rats at three dose levels (335, 670, 1340 mg/kg bw). Half the dose was given 30 h and the remainder 6 h prior to sacrifice. Triethylenemelamine, administered intraperitoneally with the same dosage schedule served as positive control. A minimum of 1000 polychromatic erythrocytes (PCEs) was scored for micronuclei per animals.<br>Data was analysed statistically by the $\chi^2$ test. |

**Results**

Eugenol did not induce a statistically-significant increase in micronucleus formation at the 3 tested doses (Table B.6.4.2.1.7). The positive control triethylenemelamine induced a statistically significant increase in micronucleus formation over the negative control.

**Table B.6.4.2.1.7. Incidence of MPE in bone marrow cells of rats treated orally with eugenol**

| Compound | Dose (mg/kg bw) | Number of rats | MnPCE Frequency (%) [mean ± standard deviation] |
|---|---|---|---|
| Negative control | - | 4 | 0.12 ± 0.13 |
| Eugenol | 335 | 4 | 0.22 ± 0.15 |
| | 670 | 4 | 0.32 ± 0.29 |
| | 1340 | 4 | 0.05 ± 0.06 |
| Triethylenemelamine | 1 | 4 | 5.28 ± 1.24* |

**Statistically-significant (p<0.001)*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Based on the results of this study, eugenol did not induce an increase in the frequency of micronucleated polychromatic erythrocytes.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 474 (2016) include incomplete characterisation of the test item, 1000 instead of 4000 PCE analysed, very limited level of reporting of results and clinical observations and no historical control data. Furthermore, sampling was carried out 6h the last dose, which also corresponded to half the dose of the study, and the split dosing time exceeded the 2-3h recommended by guidance (24h in the study). Evidence of the exposure to bone marrow has not been reported in this study. Data from other studies in rat, namely *in vivo* micronucleus test (B.6.4.1.8, study VIII) and ADME data (pharmacokinetic study in rats, B.6.1.1-08), are available to support the evidence for bone marrow exposure. Therefore, the RMS deems the study as supporting information only.

#### B.6.4.2.1.8. Study VIII

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Chen, Y., Hou, J., Liu, Y., Zeng, Z., Zhu, X., Chen, X., Wu, R. and Chen, J. |
| **Report year** | 2021 |
| **Report title** | Development of a novel flow cytometry-based approach for reticulocytes micronucleus test in rat peripheral blood |
| **Report No** | Not applicable, published study (*J.Appl. Toxicol.*, 41, 595-606) |
| **Document No** | KCA 5.4.2/12 |
| **Guidelines followed in study** | OECD TG 474 (2016) |
| **Deviations from current test guideline OECD TG 474 (2016)** | - Incomplete characterisation of the test item<br>- Limited reporting of results, clinical observations |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Corn oil |
| **Negative control** | Sodium saccharin in 1% sodium carboxymethyl cellulose and vehicle (corn oil) |
| **Positive controls** | Ethylmethanesulphonate (EMS), cyclophosphamide (CP), colchicine (COL) and ethyl nitrosourea (ENU) |
| **Test system** | Male Sprague-Dawley rats |
| **Test method** | Eugenol diluted in corn oil was orally administered *via* gavage to male rats (5 animals per group dose) for two consecutive days at dose levels of 0, 400, 800 and 1200 mg/kg bw/day. Peripheral blood samples from the tail were taken at 0 (prior to treatment) and |

| | |
|---|---|
| | 48 h after dosing for MN analysis for eugenol and negative controls. Additional samplings were made for the positive control substances, for which also a different set of doses were used (preliminary dose range finding assay- no details given):<br>- EMS: 0, 50, 100 and 200 mg/kg bw/day<br>- CP: 0, 5, 10 and 15 mg/kg bw/day<br>- COL: 0, 4, 6 and 8 mg/:kg bw/day<br>- ENU: 0, 20, 40 and 80 mg/kg bw/day<br>- Sodium saccharin: 0, 2500, 5000 and 7500 mg/kg bw/day<br><br>Samples were placed in EP tubes containing sodium heparin solution and maintained at 4°C before analysis. For flow cytometry (FCM) analysis, anticoagulant blood and staining systems (CD71-FITC and DRAQ5) were used. FCM analysis was carried out with a flow cytometer which has 488- and 633-nm lasers. FSC and SSC channels of the flow cytometer were adjusted to isolate the erythrocyte population and the APC channel was used to eliminate interference of nuclear cells and define the range of micronucleus. The APC channel (capturing the DRAQ5 fluorescence signal, which represents DNA content) and the FITC channel (labelled with CD71) were set to identify different cell populations. A blank control was required for each test and more than 20000 RETs were detected per sample.<br>Erythrocyte micronucleus frequency (‰MNRBC), RET micronucleus frequency (‰MNRET), and RET frequency (%RET) were calculated as follows:<br>‰MNRBC= (MNRBC / (RBC +MNRBC)) × 1000<br>‰MNRET= (MNRET / (RET + MNRET)) × 1000<br>%RET = ((RET + MNRET) / (RBC + RET +MNRBC + MNRET)) × 100<br>In the formula, MNRBC, RBC, MNRET, and RET are the number of cells in the respective gate labelled.<br>Manual counting was carried out using Giemsa dye solution as described in the OECD guideline 474. 4000 Polychromatic erythrocytes (PCEs) per rat were scored for micronuclei and the number of micronucleated polychromatic erythrocytes (MNPCEs) was recorded. The proportion of PCEs was also calculated among 2000 cells.<br>The data were analysed by SPSS 17.0. The ‰MNRBC and ‰MNRET were tested by the Kruskal–Wallis rank-sum test. If the difference was statistically significant, Wilcoxon rank-sum test was used for comparison between the dose group and the control group (α = 0.05, unilateral). ANOVA was used to test the %RET, and Dunnett-t was used to compare the dose group and control group if the difference was statistically significant (α = 0.05, bilateral). When the correlation between the two methods was analysed, the Spearman rank correlation coefficient was performed on ‰MNRBC and ‰MNRET, and the Pearson product distance correlation coefficient was performed on %RET.<br>The historical control data of the lab were used for comparison with the concurrent control data as part of the assay acceptance criteria. |

**Results**

Eugenol and the negative control sodium saccharin gave negative results 48 h after treatment. No increase of ‰MNRBC and ‰MNRET was observed. In addition, the percentage of reticulocytes decreases at the highest eugenol concentration. Positive controls induced significant dose-related increases in MNRET and decreases in %RET.

**Table B.6.4.2.1.8. Micronucleus test results of sodium saccharin and eugenol with FCM method**

| Compound | Dose (mg/kg bw/day) | ‰MNRBC | | ‰ MNRET[a] | | % RET | |
|---|---|---|---|---|---|---|---|
| | | 0 h | 48 h | 0 h | 48 h | 0 h | 48 h |
| Eugenol | 0 | 0.08 ± 0.06 | 0.04 ± 0.02 | 0.35 ± 0.16 | 0.33 ± 0.22 | 2.96 ± 0.50 | 1.95 ± 0.44 |
| | 400 | 0.05 ± 0.02 | 0.05 ± 0.02 | 0.39 ± 0.18 | 0.35 ± 0.32 | 2.53 ± 0.45 | 1.68 ± 0.26 |
| | 800 | 0.04 ± 0.02 | 0.02 ± 0.03 | 0.53 ± 0.13 | 0.31 ± 0.13 | 2.76 ± 0.73 | 1.54 ± 0.45 |
| | 1200 | 0.07 ± 0.04 | 0.04 ± 0.02 | 0.33 ± 0.16 | 0.23 ± 0.18 | 2.44 ± 0.60 | 0.79 ± 0.27* |
| | 0 | 0.05 ± 0.03 | 0.03 ± 0.02 | 0.22 ± 0.07 | 0.34 ± 0.18 | 3.40 ± 0.43 | 2.66 ± 0.20 |

| Compound | Dose (mg/kg bw/day) | ‰MNRBC | | ‰ MNRET[a] | | % RET | |
|---|---|---|---|---|---|---|---|
| | | 0 h | 48 h | 0 h | 48 h | 0 h | 48 h |
| Sodium saccharin | 2500 | 0.04 ± 0.01 | 0.02 ± 0.02 | 0.32 ± 0.20 | 0.38 ± 0.38 | 2.99 ± 0.53 | 2.60 ± 0.51 |
| | 5000 | 0.05 ± 0.02 | 0.03 ± 0.03 | 0.42 ± 0.17 | 0.35 ± 0.17 | 2.66 ± 0.90 | 1.62 ± 0.27* |
| | 7500 | 0.03 ± 0.02 | 0.03 ± 0.01 | 0.40 ± 0.18 | 0.36 ± 0.18 | 2.90 ± 1.09 | 1.02 ± 0.20* |
| CP | 15 | 0.02 ± 0.01 | 0.05 ± 0.03 | 0.28 ± 0.18 | 2.72 ± 0.36* | 2.90 ± 0.35 | 0.45 ± 0.19* |

[a]FCM method analyses 20000 RETs
* $p < 0.05$

**Assessment and conclusion**

Eugenol is a nongenotoxic chemical and was confirmed to be negative with the double-staining FCM method, which implies the specificity of the FCM method. Eugenol did not cause cytogenetic damage in rats in the *in vivo* micronucleus test.

**Assessment and conclusion by applicant:**

This new study is considered acceptable as supporting information for the renewal of eugenol; the results are consistent with available data showing no signs of genotoxicity (reliable with restrictions; no details on the materials, brief results on eugenol based on the FCM method but no results from the manual count, not conducted to GLP, some apparent editorial errors in dose reporting in results tables).

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Based on the results of this study, eugenol did not induce an increase in the frequency of micronucleated polychromatic erythrocytes.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 474 (2016) include incomplete characterisation of the test item, very limited level of reporting (both dose selection and results) and no clinical observations reported. There is a 59% reduction on the percentage of RET induced by eugenol (statistically-significant) at the top dose, which could serve as a justification of the doses tested plus evidence that tissue toxicity occurred. Evidence of bone marrow exposure is not reported in this study. Data from other studies in rat, namely *in vivo* micronucleus test (B.6.4.1.8, study VIII) and ADME data (pharmacokinetic study in rats, B.6.1.1-08), are available to support the evidence for bone marrow exposure. Based on these deviations, the RMS deems the study as acceptable to evaluate the clastogenicity of eugenol *in vivo*.

### B.6.4.2.2. Gene mutation *in vivo*

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Rompelberg, C.J.M., Steenwinkel, M-J.S.T., van Asten, J.G., van Delft, J.H.M., Baan, R.A. and Verhagen, H. |
| **Report year** | 1996b |
| **Report title** | Effect of eugenol on the mutagenicity of benzo[*a*]Pyrene and the formation of benzo[a]Pyrene-DNA adducts in the λ-lacZ-transgenic mouse |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 369, 89-96) |
| **Document No** | KCA 5.4.2/08 |
| **Guidelines followed in study** | Not stated. |
| **Deviations from current test guideline OECD TG 488 (2020)** | -Non-standard assay<br>-No reason given for dose selection<br>-Very poor level of reporting<br>-No estimation/evidence of liver exposure to eugenol |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: 99%; Batch No.: not stated<br>Benzo[a]pyrene (B[a]P), Purity: not stated; Batch No.: not stated |
| **Vehicle** | Olive oil (ip injection of B[a]P) |
| **Negative control** | Diet (pelleted purified diet)<br>Olive oil |
| **Positive control** | Not used |
| **Test system** | λ-lacZ-transgenic mouse strain 40.6 (Muta Mouse) |
| **Test method** | The effect of eugenol on the mutagenicity and DNA adduct formation of Benzo[a]pyrene was investigated in the mouse transgenic assay.<br><br>Two groups of 8 mice were fed a diet containing 0 (control) or 0.6% w/w eugenol for 58 days. Diet and water were available *ad libitum*. On day 10, 4 animals per group were given an i.p. dose of 100 mg/kg bw Benzo[a]Pyrene (B[a]P) in olive oil at a volume of 10 mL/kg. The other animals were given olive oil only.<br>After 58 days exposure, the mice were killed by $CO_2$. Livers were collected and DNA was isolated following the method described by Roggeband *et al.* (1993). The extracted DNA was incubated with a culture of *Escherichia coli* strain C lacZ$^-$ recA$^-$ galE$^-$ harbouring a galK$^-$ and galT$^-$ expressing plasmid. The resulting mutants were counted. $^{32}$P-postlabelling was carried out on remaining DNA and the DNA was analysed for adducts by fast protein liquid chromatography (FPLC) analysis.<br>Statistical analysis of the data was determined by a two-way analysis of variance (ANOVA). Before analysis, the data were transformed by log transformation to stabilise the variance. When the result was significant ($p \leq 0.05$), asymptotic pairwise t-tests were performed. |

**Results**

*Diet preparation and analysis*
The diet containing eugenol was 0.6% w/w nominal concentration with an actual 0.4% w/w concentration. At room temperature, the eugenol content of the diet had decreased by 13% and 15% after 3 and 7 days respectively.

*Mortality and clinical observations*
Not reported.

*Food intake and body weight*
There was no statistically significant difference in food intake of mice on the eugenol diet, calculated as 4.21 ± 0.4 g/mouse per day, versus mice in the control group (4.26 g ± 0.51 g/mouse per day). The calculated dose of eugenol in the diet, taking into account the weight of the mouse (25 g) is estimated to be 673.60 mg/kg bw/day. No statistical differences in body weight were observed, however there was a trend for lower body weight gain in treated mice compared to controls.

*Mutation assay*
The established mutagen B[*a*]P significantly increased the lacZ mutant frequency in liver of mice fed the control diet (Table B.6.4.2.2/1). No effect of eugenol was demonstrated on the lacZ mutant frequency induced by B[*a*]P. Eugenol itself had no effect on the lacZ mutant frequency.

**Table B.6.4.2.2/1. *lac*Z mutant frequencies in liver of λ-lacZ-transgenic mice fed a diet containing 0.4% eugenol/control diet and treated with a single dose of B[*a*]P**

| B[*a*]P (mg/kg bw) | Eugenol (% diet) | No. Phages analysed | No. mutants | MF (x10$^6$) | Group mean [MF ± SD] n = 4 |
|---|---|---|---|---|---|
| 0 | 0.0 | 521550 | 24 | 46.0 | 71.3 ± 25.3 |
| | | 226062 | 24 | 16.2 | |
| | | 330030 | 23 | 69.7 | |

| B[*a*]P (mg/kg bw) | Eugenol (% diet) | No. Phages analysed | No. mutants | MF (x10$^6$) | Group mean [MF ± SD] n = 4 |
|---|---|---|---|---|---|
| | | 458280 | 29 | 63.3 | |
| 0 | 0.4 | 266760 | 19 | 71.2 | 63.9 ± 22.1 |
| | | 347130 | 12 | 34.6 | |
| | | 320625 | 20 | 62.4 | |
| | | 389025 | 34 | 87.4 | |
| 100 | 0.0 | 301815 | 36 | 119.3 | 185.2 ± 66.7* |
| | | 195453 | 29 | 148.4 | |
| | | 184509 | 50 | 271.0 | |
| | | 415530 | 84 | 202.2 | |
| 100 | 0.4 | 309510 | 47 | 151.9 | 206.5 ± 48.6** |
| | | 205884 | 54 | 262.3 | |
| | | 460161 | 105 | 228.1 | |
| | | 239400 | 44 | 183.8 | |

**Statistically-significant difference versus control (same diet, no B[a]P) at p ≤ 0.05*
***Statistically-significant difference versus control (same diet, no B[a]P) at p ≤ 0.01*
*MF : Mutant frequency*

*DNA-adducts formation*
In liver DNA from mice fed eugenol diet without B[*a*]P, one specific adduct spot was detected. No spot was detected in the control diet. With B[*a*]P the same adduct quantity was observed. Four B[a]P-induced DNA adducts were observed in eugenol and control diet (table B.6.4.2.2/2).

**Table B.6.4.2.2/2. DNA adduct levels (x10$^8$ nucleotides) in liver DNA of λ-lacZ-transgenic mice fed with a diet containing 0.4% eugenol/control diet and treated with a single dose of B[*a*]P**

| B[*a*]P (mg/kg bw) | Eugenol (% diet) | Adduct 1 | Adduct 2 | Adduct 3 | Adduct 4 | Total adduct level |
|---|---|---|---|---|---|---|
| 0 | 0 | 0.5 ± 0.3 (38%) | 0.4 ± 0.2 (31%) | 0.1 ± 0.2 (9%) | 0.3 ± 0.2 (22%) | 1.3 ± 0.8 (100 %) |
| 0 | 0.4 | 0.4 ± 0.2 (41%) | 0.3 ± 0.2 (32%) | 0.1 ± 0.1 (12%) | 0.2 ± 0.1 (15%) | 1.0 ± 0.5 (100%) |
| 100 | 0 | 6.6 ± 2.9 (36%) | 1.9 ± 1.0 (10%) | 8.7 ± 4.6 (48%) | 1.1 ± 0.8 (6%) | 18.2 ± 8.2 (100%) |
| 100 | 0.4 | 7.4 ± 2.6 (43%) | 1.7 ± 0.7 (10%) | 6.9 ± 1.3 (41%) | 1.7 ± 0.7 (6%) | 17.1 ± 4.4 (100%) |

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Based on the results of this study, eugenol did not show any anti-mutagenic effect on the mutagenicity of B[*a*]P in λ-lacZ-transgenic mice (Muta Mice). Furthermore, eugenol-fed group did not show an increase of mutant frequency compared to control animals.

The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 488 (2020) include incomplete characterisation of the test item, very limited level of reporting both on dose selection or clinical observations and no estimation or evidence has been provided on eugenol exposure to liver. Therefore, the RMS deems the study as supporting information only to evaluate gene mutation *in vivo* of eugenol.

**B.6.4.2.3. DNA strand breaks – Comet assay *in vivo***

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Maura, A., Pino, A. and Ricci, R. |
| **Report year** | 1989 |
| **Report title** | Negative evidence *in vivo* of DNA-damaging, mutagenic and chromosomal effects of eugenol |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 227, 125-129) |
| **Document No** | KCA 5.4.2/07 |
| **Guidelines followed in study** | Alkaline elution assay *in vivo* according to Brambilla *et al.* (1978) |
| **Deviations from current test guideline OECD TG 489 (2016)** | -No characterisation of the test item<br>-Poor description of the method<br>-Very limited level of reporting<br>-No evidence of exposure to liver and kidney provided |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Physiological saline |
| **Negative control** | Vehicle (water) |
| **Positive control** | N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)<br>N-Nitrosodimethylamine (DMNA) |
| **Test system** | Female Sprague-Dawley rats (Organs: liver and kidney) |
| **Test method** | Dose selection was based on the acute oral toxicity study by Jenner *et al.* (1964).<br>The alkaline elution assay was carried out with eugenol according to Brambilla *et al.* (1978).<br><br>Eugenol diluted in physiological saline was administered by gavage to groups of 6 or 8 female rats at 1340 mg/kg bw (half the $LD_{50}$ value). At 2, 24 or 48 hours after dosing, rats were killed, liver and kidneys were removed and the level of DNA fragmentation was determined by alkaline elution. DMNA was used as a positive control. The percentage of eluted DNA in treated groups was compared to the controls.<br>Statistical analysis of the data was calculated using a 2-tailed 2-sample Wilcoxon test. |

**Results**

Eugenol did not induce DNA fragmentation in liver and kidneys of rats at 1340 mg/kg bw at any of time interval after treatment. The positive control DMNA clearly showed DNA damage at 1.2 mg/kg bw.

**Table B.6.4.2.1.7. DNA fragmentation in liver and kidney after oral administration of eugenol**

| Compounds | Dose (mg/kg bw) | Tissue | Time interval after treatment (h) | Number of rats | % DNA eluted[a] |
|---|---|---|---|---|---|
| Eugenol | 1340 | Liver | 2 | 8 | 30.7 ± 3.83 |
| | | | 24 | 6 | 31.2 ± 4.08 |
| | | | 48 | 8 | 30.0 ± 2.76 |
| | | Kidney | 2 | 8 | 29.0 ± 4.40 |
| | | | 24 | 6 | 34.2 ± 4.39 |
| | | | 48 | 8 | 31.9 ± 7.23 |
| DMNA | 1.2 | Liver | 6 | 4 | 60.4 ± 5.96* |
| Negative control (physiological saline) | - | Liver | - | 10 | 24.8 ± 6.84 |
| | - | Kidney | - | 10 | 31.7 ± 4.05 |

[a]*Mean ± SD*
** statistically significant, p = 0.002*

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways |
| **Assessment and conclusion by RMS:**<br>RMS agrees with the applicant's assessment. Based on the results of this study, eugenol did not induce DNA fragmentation in liver and kidney in the alkaline elution assay.<br>The study is a scientific publication from a peer-reviewed journal. Deviations from current OECD TG 489 (2016) include no characterisation of the test item, very limited level of reporting and poor description of the method. Furthermore, paragraph 34 of the guidance indicates that supporting evidence (direct or indirect) of toxicity or exposure to the target tissue(s) should be considered when a negative result is obtained. Based on the available data from the study, the RMS deems the study as supporting information only. |

**B.6.4.2.4. DNA binding**

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Phillips, D.H. |
| **Report year** | 1990 |
| **Report title** | Further evidence that eugenol does not bind to DNA *in vivo* |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 245, 23-26) |
| **Document No** | KCA 5.4.2/10 |
| **Guidelines followed in study** | No guideline available. Protocol by Miller *et al.*, 1983 |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated |
| **Vehicle** | Not stated |
| **Negative control** | Not stated |
| **Positive control** | Safrole |
| **Test system** | Male B6C3F1 mice |
| **Test method** | Eugenol was administered intraperitoneally to newborn B6C3F1 mice over the first 22 days of life, on day 1 (0.25 µmoles), on day 8 (0.5 µmoles), on day 15 (1.0 µmoles) and on day 22 (3.0 µmoles), making a total of 4.25 µmoles. Animals were sacrificed on day 23.<br>Eugenol was also administered intraperitoneally at a single dose (2 or 10 mg in 0.1 mL trioctanoin). Animals were sacrificed 24 h after dosing. Safrole was used as a positive control and administered as for eugenol. DNA from liver cells were isolated before $^{32}$P-labelling and analysis of DNA adducts by thin layer chromatography (TLC). |

**Results**

The positive control safrole showed DNA adduct formation in both assays with 3 intense spots of radioactivity on the chromatograms of newborn mice and young mice (6 weeks old). For eugenol-treated mice, DNA did not reveal

any radioactive spots in comparable regions of the chromatograms, for both assays compared to the negative control group. The limit of detection was estimated to be 1 adduct in $10^9$ nucleotides.

The covalent binding of safrole and eugenol to mice liver DNA is displayed in Table B.6.4.2.4.

**Table B.6.4.2.4. Covalent binding of safrole and eugenol to mouse liver DNA**

| Compound | Dose (µmoles) | Mouse | fmoles adducts/µg DNA |
|---|---|---|---|
| Safrole | 4.75 | Newborn B6C3F1 | 14.1 |
| Eugenol | | | ND |
| Safrole | 61.7 | Adult Parkes | 2.69 |
| Eugenol | 60.9 | | ND |

*ND: Not detected*

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways |
| **Assessment and conclusion by RMS:**<br>RMS agrees with the applicant's assessment. Under the experimental conditions of the study, safrole but not eugenol produced detectable DNA adducts in both newborn and young adult mice.<br>The study is a scientific publication from a peer-reviewed journal. No guidance is available for this type of study, and therefore, the RMS deems the study as supporting information to evaluate the DNA binding of eugenol *in vivo*. |

**B.6.4.2.5. Other studies**

**B.6.4.2.5.1. Study I**

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Rompelberg, C.J.M, Vogels, J.T.W.E, de Vogel, N., Bruijntjes-Rozier, G.C.D.M., Stenhuis, W.H., Bogaards, J.J.P. and Verhagen, H. |
| **Report year** | 1996c |
| **Report title** | Effect of short-term dietary administration of eugenol in humans |
| **Report No** | Not applicable, published study (*Hum. Exp. Toxicol.*, 15, 129-135) |
| **Document No** | KCA 5.4.2/09 |
| **Guidelines followed in study** | No guideline available |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: food-grade; Batch No.: not stated |
| **Vehicle** | Dried starch |
| **Negative control** | Placebo |
| **Test system** | Human healthy volunteers (21-26 years old, 68-88 kg) |
| **Test method** | Human healthy volunteers were randomly assigned to either treatment group (n = 5) or control group (n = 5). The treatment group (group 1) received capsules containing 50 mg eugenol in dried starch to be taken orally three times per day (at 8am, 4pm and 11pm) during 7 days. Control group (group 2) received the placebo. On days 7-14 none |

| | |
|---|---|
| | of the groups ingested capsules (wash-out period). On days 14-21 the groups were crossed so that Group 1 received placebo and Group 2 received capsules containing 50 mg eugenol three times per day. Blood samples were taken on days 8 and 22 and analysed for clinical biochemical parameters (numbers of leucocytes (WBC) and platelets (PLT), haemoglobin content (HBG), haematocrit (HCT), levels of L-aspartate aminotransferase (ASAT), L-alanine aminotransferase (ALAT), γ-glutamyl transpeptidase (γ-GT), lactate dehydrogenase (LDH), alkaline phosphatase (AP)) and phase-II biotransformation enzymes activities (glutathione S-transferase GST levels).<br><br>A paracetamol test described by Miners *et al.* (1983) was performed. On days 8 and 22, after fasting overnight volunteers ingested 500 mg paracetamol with a glass of water. Blood was sampled after 5 hours and levels of paracetamol in plasma was measured to calculate clearance of paracetamol.<br><br>*Cytogenetic analysis*<br>For the assessment of micronucleated binucleates and chromosome aberrations, cultured blood samples collected from each of the volunteers on day 8 and 22 were treated with known positive control substances increasing micronucleus frequency (vinblastine and mitomycin C). Control cultures were exposed to the solvent control (saline) only. After incubation, sides were fixed and stained with Giemsa. A total of 500 binucleated lymphocytes (from two slides) was scored for the incidence of micronucleated binucleates and a total of 50 metaphases (from two slides) was scored for the incidence of chromosome aberrations.<br><br>*Urine analysis*<br>On day 7 and 21 the volunteers collected urine after the first morning urine up to an including the urine of the next morning. Samples were filtered and analysed by proton NMR spectroscopy.<br><br>Statistical analysis was performed by analysis of variance. The standard deviation is replaced by the least significant difference at the 5% level (LSD). |

**Results**

No significant differences in the clinical biochemical parameters between eugenol-treated or placebo groups (Table B.6.4.2.5.1/1).

**Table B.6.4.2.5.1/1. Clinical biochemical parameters of volunteers treated with eugenol for 7 days**

| Treatment | HGB (mM) | HCT (L/L) | WBC (*$10^9$/L) | PLT (*$10^9$/L) | ASAT (U/L plasma) | ALAT (U/L plasma) | γ-GT (U/L plasma) | LDH (U/L plasma) | AP (U/L plasma) |
|---|---|---|---|---|---|---|---|---|---|
| Placebo | 9.8 | 0.4 | 5.5 | 232 | 21.7 | 19.3 | 17.4 | 331 | 79.0 |
| Eugenol | 9.6 (0.2) | 0.4 (0.0) | 5.7 (0.6) | 236 (17) | 23.0 (4.8) | 19.0 (3.9) | 17.7 (3.5) | 333 (32) | 78.2 (4.1) |

*HGB: Haemoglobin content; HCT: Haematocrit; WBC: No. leucocytes; PLT: Platelets; ASAT: L-Aspartate aminotransferase; ALAT: L-Alanine aminotransferase; γ-GT: γ-Glutamyl transpeptidase; LDH: Lactate dehydrogenase; AP: Alkaline phosphatase.*
*Blood samples were collected 8h after the last dose. Least Significance difference (5%) is given in parenthesis.*

Eugenol had no effect on the clearance of paracetamol and on GST activity in erythrocytes (Table B.6.4.2.5.1/2). By contrast, administration of eugenol caused a significant decrease of 26% in levels of α-class GST in plasma.

**Table B.6.4.2.5.1/2. Phase II biotransformation activities in blood samples of volunteers treated with eugenol**

| Treatment | GST (U/L blood) | GST-α (µg/L plasma) | $CL_{pc}$ (mL/min) |
|---|---|---|---|
| Placebo | 619 | 5.7 | 473 |
| Eugenol | 611<br>(37) | 4.2*<br>(1.3) | 496<br>(30) |

*GST: Glutathione S-transferase activity; α-class GST; $CL_{pc}$: Clearance paracetamol*
**Statistically-different from placebo group*
*Least Significance difference (5%) is given in parenthesis*

Vinblastine and mitomycin C significantly induced the incidence of micronucleated binucleates *in vitro* and mitomycin C significantly induced the percentage of lymphocytes with chromosome aberrations *in vitro*, as compared to the control group, as expected. Administration of eugenol had no effect on the incidence of micronucleated binucleates and on the percentage of lymphocytes with chromosome aberrations (Figure B.6.4.2.5.1).

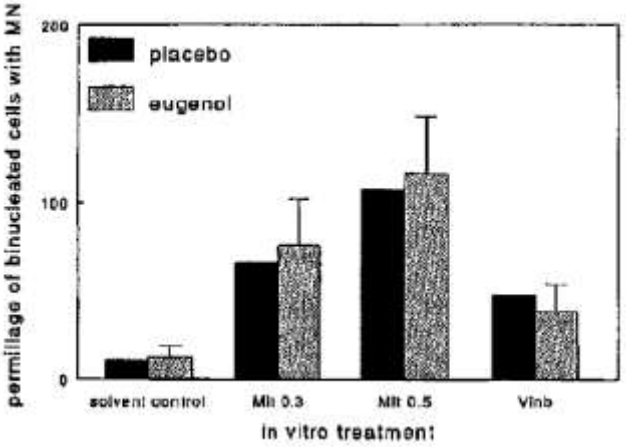



**Figure B.6.4.2.5.1. Permillage of binucleated lymphocytes with micronuclei (500 cells scored) in blood samples of volunteers who ingested 150 mg eugenol during 7 days**

Eugenol was detected in the proton NMR spectra of the 24-h urine samples of all volunteers confirming systemic exposure. No eugenol was detected in the urine of the volunteers in the placebo group.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways

**Assessment and conclusion by RMS:**

RMS agrees with the applicant's assessment. Based on the results of this study, eugenol did not show significant differences to control when assessed for biochemical or haematological parameters. Erythrocytes taken from volunteers following exposure to eugenol for 7 days did not show an increase in background level of micronuclei or chromosomal aberration when treated with vinblastine and mitomycin C.

The study is a scientific publication from a peer-reviewed journal. It is not a standard assay and the RMS deems it as supporting information only.

**B.6.4.2.5.2. Study II**

| | |
|---|---|
| **Data point** | CA 5.4.2 (EU data requirement) |
| **Report author** | Green, N.R. and Savage, J.R. |
| **Report year** | 1978 |
| **Report title** | Screening of safrole, eugenol, their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity |
| **Report No** | Not applicable, published study (*Mutat. Res.*, 57, 115-121) |
| **Document No** | KCA 5.4.2/11 |
| **Guidelines followed in study** | No guideline available |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in the DAR (2011) |
| **GLP/Officially recognised testing facilities** | No, pre-GLP requirement |
| **Acceptability/ reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemicals** | Eugenol, Purity: not stated; Batch No.: not stated<br>Eugenol metabolite II [3-piperidyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone], Purity: not stated; Batch No.: not stated<br>Eugenol metabolite III [3-pyrrolidinyl-1-(3'-methoxy-4'-hydroxyphenyl)1-propanone], Purity: not stated; Batch No.: not stated |
| **Vehicle** | Not stated |
| **Negative control** | Not stated |
| **Positive control** | Dimethylnitrosamine (DMN) |
| **Test system** | Male mice C3H/HeJ |
| **Test method** | Male mice (3 per treatment) were treated intraperitoneally with 2 mL of solution containing *S. typhimurium* strains TA1530, TA1531, TA1532 and TA1964. Also, each mouse received a 0.1 mL intramuscular injection of eugenol or its metabolites, corresponding to a dose rate of 200 mg/kg bw. Three hours after intramuscular injection, the mice were killed. Each mouse received then 1 mL (i.p.) of isotonic saline and as much fluid as possible was removed from the peritoneum. Then, the extracted samples were incubated at 37°C for 2 days before colonies counting. The result was considered positive, and therefore, the test substance was considered mutagenic, if the number of colonies found was 10-fold the number in the control (appropriate solvent or untreated). |

**Results**

Eugenol and its metabolites were mutagenic in the host-mediated assay (Table B.6.4.2.5.2).

**Table B.6.4.2.5.2. Effect of compounds in the host-mediated assay**

| Compound | Mutant Frequency ($x10^{-8}$) in Salmonella strains | | | | |
|---|---|---|---|---|---|
| | Concentration (mg/kg) | TA1950 | TA1951 | TA1952 | TA1964 |
| None (control) | - | 2.0 | 0.5 | 1.9 | 4.3 |
| Eugenol | 200 | 1.7 | 0.8 | 4.6 | 5.6 |
| Eugenol metabolite II | 200 | 1.9 | 0.5 | 1.3 | 5.4 |
| Eugenol metabolite III | 200 | 1.9 | 0.7 | 6.1 | 5.8 |
| Positive control (DMN) | 100 | 34.4* | 0.9 | 24.2* | 28.2* |

*Mutant frequency obtained from an average of 3 mice per treatment except for the control which are an average of 8 mice per treatment*
**Compound were mutagenic if the mutant frequency increased by a factor of 10 over the control value*

**Assessment and conclusion by applicant:**

| |
|---|
| The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br><br>The EU-agreed endpoint is: unlikely to be genotoxic at exposures that do not result in cytotoxicity and saturation of conjugation pathways |
| **Assessment and conclusion by RMS:**<br><br>RMS agrees with the applicant's assessment. Under the conditions of this study, eugenol or its metabolites (II or III) did not induce an increase in mutant frequency of any of the salmonella strains tested.<br><br>The study is a scientific publication from a peer-reviewed journal. It is not a standard assay and the RMS deems it as supporting information only. |

### B.6.4.3. *In vivo* studies in germ cells

*In vivo* studies in germ cells are required by Regulation (EU) 283/2013 on a case by case basis, where any result of an *in vivo* somatic cell study is positive. Eugenol did not induce any genotoxicity in *in vivo* studies in somatic cells (see CA 5.4.2), and in addition this substance is used in food, cosmetics etc. without adverse effects. Therefore further *in vivo* studies in germ cells are not considered necessary based on the available data.

## B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

*The long-term toxicity and carcinogenicity studies contained in this section were all included in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC, and had been reassessed to support the renewal of the active substance. Two studies were conducted in mice (18-months and 2-year, respectively) and one in rats (2-year).*

*These three studies in mouse and rat present several deviations, such as the testing only one or two dose levels, or the lack of important measurements (haematology, clinical chemistry, urinalysis and organ weights). Consequently, these studies were considered acceptable as supportive information.*

### B.6.5.1. Carcinogenicity study in rats (2-year)

| | |
|---|---|
| **Data point** | CA 5.5 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. Prior to OECD Test Guidelines for combined chronic toxicity/carcinogenicity studies. The study was checked for compliance with OECD TG 453 (2018). |
| **Deviations from current test guideline OECD TG 453 (2018)** | -No satellite groups to monitor the reversibility of toxicological changes were incorporated.<br>-Only 40 animals per sex were used in control group (OECD TG 453 requires at least 50 animals/sex/group).<br>- Only two dose levels were assayed, when at least three dose levels should be used.<br>- No haematology, urinalysis and clinical chemistry were performed.<br>- Organ weight data was not recorded.<br>-Individual bodyweight data were not presented.<br>- Statistical analysis was not performed in bodyweight, food consumption and non-neoplastic lesions. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483 and 26068, Purity: >99% |
| **Test system** | Male and Female F344/N rats |
| **Test method** | Groups of 50 male and 50 female F344/N rats of 6-7 weeks of age were tested *via* feed diet with two different dose levels of eugenol for 105 weeks. Dose levels selected for the two year studies were based on 13-week (91-day) studies. Males were tested at 3000 and 6000 ppm. The equivalence values in mg/kg bw/day adjusted by RMS according to EFSA Guidance (EFSA Journal 2012;10(3):2579) were 128 and 260 mg/kg bw/day for males (3000 and 6000 ppm, respectively). The default values in mg/kg bw/day were 150 (3000 ppm) and 300 mg/kg bw/day (6000 ppm). On the other hand, females were tested at 6000 and 12500 ppm. The equivalence values in mg/kg bw/day adjusted by RMS according to EFSA Guidance (EFSA Journal 2012;10(3):2579) were 302 and 648 mg/kg bw/day for females (6000 and 12500 ppm, respectively). The default values in mg/kg bw/day were 300 (6000 ppm) and 625 mg/kg bw/day (12500 ppm). . A control group of 40 rats of each sex were used. All animals were observed twice daily for morbidity or mortality. Clinical signs were recorded monthly. Individual animals were weighed weekly for the first 13 weeks, then monthly to week 93, and every 2 weeks thereafter. Necropsies were performed on all animals not excessively autolyzed or cannibalized. Examinations for grossly visible lesions were performed on major tissues or organs. Tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with |

| | |
|---|---|
| | hematoxylin and eosin. The following tissues were examined microscopically: tissue masses, abnormal lymph nodes, skin, mandibular lymph nodes, mammary gland, salivary gland, thigh muscle, sciatic nerve, bone marrow, costochondral junction (rib), thymus, larynx, trachea, lungs and bronchi, heart, thyroid, parathyroid, esophagus, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph nodes, liver, pancreas, spleen, kidneys, adrenals, bladder, seminal vesicles/prostate/testes or ovaries/uterus, nasal cavity, brain, pituitary, and spinal cord. The incidence of neoplastic or non-neoplastic lesions has been given as the ratio of the number of animals bearing such lesions at a specific anatomic site to the number of animals in which that site was examined. |
| **Statistical analysis of results** | Dose-related effect on survival was analysed using Cox method (1972) for testing two groups for equality; and Tarone's (1975) extensions of Cox's methods for testing for a dose-related trend. Statistical analysis of tumor incidence data used two different methods for adjusting intercurrent mortality. Each used the classical methods for combining contingency tables developed by Mantel and Haenszel (1959). The first method of analysis is the life table method of Cox and Tarone and assumed that all tumors of a given type observed in animals dying before the end of the study were "fatal". According to this approach, the proportions of tumor-bearing animals in the dosed and control groups were compared at each point in time at which an animal died with a tumor of interest. The second method of analysis assumed that all tumors of a given type observed in animals dying before the end of the study were "incidental". According to this approach, the proportions of animals found to have tumors in dosed and control groups were compared in each of five time intervals: 0-52 weeks, 53-78 weeks, 79-92 weeks, week 93 to the week before the terminal kill, and the terminal kill period. The individual time interval comparisons were then combined by the previously described methods to obtain a single overall result.<br>Another set of statistical analyses was carried out and reported in the tables analysing primary tumors; the Fisher exact test for pairwise comparisons and the Cochran-Armitage linear trend test for dose-response trends. These tests were based on the overall proportion of tumor-bearing animals. All reported P-values are one-sided. |
| **Historical control data** | In order to completeness, all the available information is presented, however, HCD from Southern Research Institute was the only accepted by RMS, due to it was the laboratory in which the study was conducted, and the time frame is within the 5-recommended years. Hence, the following historical control data (HCD) of neoplastic lesions are presented (Table B.6.5.1/1):<br><br>**Table B.6.5.1/1. Historical control data from NTP Carcinogenesis Program and Southern Research Institute (1983)** |

| | HCD from NTP feeding carcinogenicity studies (1990-1997) | | | | | |
|---|---|---|---|---|---|---|
| | Male | | | Female | | |
| | nº animals /total | Rate (%)# | Range (%) | nº animals /total | Rate (%)# | Range (%) |
| **Alveolar/bronchiolar adenoma / carcinoma** | 45/1352 | 3.3 | 0-14 | 26/1351 | 1.9 | 0-10 |
| **Alveolar/bronchiolar adenoma** | 35/1352 | 2.6 | 0-14 | 22/1351 | 1.6 | 0-10 |
| **Alveolar/bronchiolar carcinoma** | 10/1352 | 0.7 | 0-4 | 4/1351 | 0.3 | 0-2 |
| **Thyroid C-cell adenoma/carcinoma** | 199/1347 | 14.8 | 0-35 | 184/1347 | 13.6 | 0-22 |
| **Thyroid C-cell adenoma** | 175/1347 | 13 | 2-35 | 158/1347 | 11.7 | 4-22 |
| **Thyroid C-cell carcinoma** | 24/1347 | 1.8 | 0-6 | 26/1347 | 1.9 | 0-4 |
| | – | – | – | 199/1351 | 14.7 | 0-30 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Uterine endometrial stromal polyp / sarcoma** | | | | | | |
| **Uterine endometrial stromal polyp** | – | – | – | 192/1351 | 14.2 | 2-30 |
| **Uterine endometrial stromal sarcoma** | – | – | – | 7/1351 | 0.5 | 0-4 |
| **Mammary gland fibroadenoma** | 58/1354 | 4.3 | 0-12 | 556/1351 | 41.2 | 8-60 |
| | **HCD from NTP feeding carcinogenicity studies (1983)** | | | | | |
| **Alveolar/bronchiolar adenoma / carcinoma** | 55/2305 | 2.4 | n.a. | 27/2354 | 1.2 | n.a. |
| **Alveolar/bronchiolar adenoma** | 35/2305 | 1.5 | n.a. | 18/2354 | 0.8 | n.a. |
| **Alveolar/bronchiolar carcinoma** | 20/2305 | 0.9 | n.a. | 9/2354 | 0.4 | n.a. |
| **Thyroid C-cell adenoma/carcinoma** | 198/2230 | 8.9 | n.a. | 192/2265 | 8.5 | n.a. |
| **Thyroid C-cell adenoma** | 114/2230 | 5.1 | n.a. | 111/2265 | 4.9 | n.a. |
| **Thyroid C-cell carcinoma** | 84/2230 | 3.8 | n.a. | 81/2265 | 3.6 | n.a. |
| **Uterine endometrial stromal polyp / sarcoma** | – | – | – | 449/2318 | 19.4 | n.a. |
| **Uterine endometrial stromal polyp** | – | – | – | 424/2318 | 18.3 | n.a. |
| **Uterine endometrial stromal sarcoma** | – | – | – | 25/2318 | 1.1 | n.a. |
| **Mammary gland fibroadenoma** | 51/2320 | 2.2 | n.a. | 572/2370 | 24.1 | n.a. |
| | **HCD from Southern Research Institute (1983)** | | | | | |
| | **Male** | | | **Female** | | |
| | **nº animals /total** | **Rate (%)#** | **Range (%)** | **nº animals /total** | **Rate (%)#** | **Range (%)** |
| **Alveolar/bronchiolar adenoma / carcinoma** | 15/438 | 3 | n.a. | n.a. | n.a. | n.a. |
| **Uterine endometrial stromal polyp / sarcoma** | – | – | – | 66/438 | 15 | n.a. |
| **Mammary gland fibroadenoma** | n.a. | n.a. | n.a. | 120/439 | 27 | n.a. |

*# percentage of animals with tumors*

**Results**

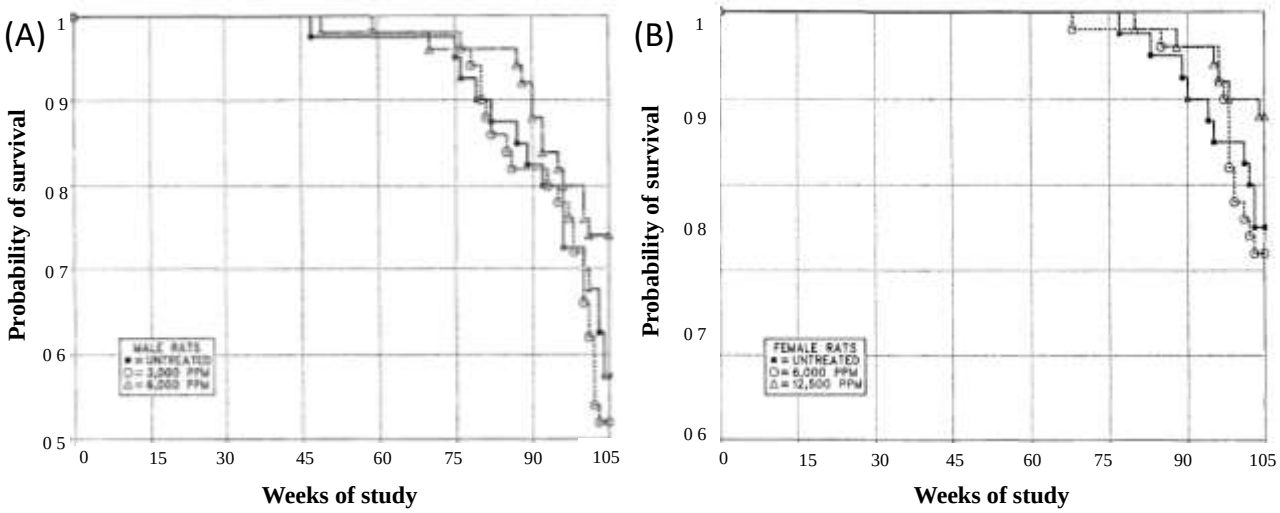
**Survival:** No statistically significant differences were detected between treated groups and controls in both sexes throughout study (Table B.6.5.1/2 and Figure B.6.5.1).

**Table B.6.5.1/2. Survival at termination of study (105 weeks)**

| | survival | |
|---|---|---|
| | ♂ | ♀ |
| **Control** | 58% (23/40) | 75% (30/40) |
| **Low dose (3000 ppm ♂/ 6000 ppm ♀)** | 52% (26/50) | 72% (36/50) |
| **High dose (6000 ppm ♂/ 12500 ppm ♀)** | 74% (37/50) | 88% (44/50) |

*No statistically significant differences*

**Figure B.6.5.1. Kaplan-Meier survival curves of males (A) and females (B) groups.**




**Bodyweight:** No differences in mean bodyweights were detected in male treated groups. In the female high dose group a reduction >10% (10-15%) was observed in the mean bodyweight from week 51 to the end of the study (week 102), compared with controls (see tables B.6.5.1/3 and B.6.5.1/4).

**Table B.6.5.1/3. Bodyweight of male rats in the 2-year feed study with eugenol**

| | Males | | | | |
|---|---|---|---|---|---|
| | Controls | 3000 ppm | | 6000 ppm | |
| weeks | average weight (g) | average weight (g) | % of controls | average weight (g) | % of controls |
| 0 | 94 | 93 | 98.9 | 90 | 95.7 |
| 4 | 195 | 199 | 102.1 | 193 | 99 |
| 8 | 259 | 260 | 100.4 | 253 | 97.7 |
| 13 | 307 | 308 | 100.3 | 306 | 99.7 |
| 17 | 333 | 335 | 100.6 | 331 | 99.4 |
| 21 | 365 | 365 | 100 | 363 | 99.5 |
| 25 | 377 | 386 | 102.4 | 375 | 99.5 |
| 28 | 391 | 397 | 101.5 | 385 | 98.5 |
| 34 | 404 | 405 | 100.2 | 381 | 94.3 |
| 38 | 411 | 410 | 99.8 | 399 | 97.1 |
| 42 | 408 | 419 | 102.7 | 406 | 99.5 |
| 46 | 435 | 430 | 98.9 | 417 | 95.9 |
| 51 | 428 | 427 | 99.8 | 415 | 97 |
| 55 | 432 | 434 | 100.5 | 418 | 96.8 |
| 59 | 444 | 439 | 98.9 | 423 | 95.3 |
| 64 | 439 | 440 | 100.2 | 413 | 94.1 |
| 68 | 447 | 439 | 98.2 | 421 | 94.2 |
| 72 | 442 | 439 | 99.3 | 424 | 95.9 |
| 77 | 453 | 437 | 96.5 | 430 | 94.9 |
| 81 | 442 | 427 | 96.6 | 423 | 95.7 |
| 86 | 443 | 430 | 97.1 | 417 | 94.4 |
| 90 | 438 | 433 | 98.9 | 417 | 95.2 |
| 94 | 437 | 422 | 96.6 | 410 | 93.8 |
| 98 | 432 | 418 | 96.8 | 407 | 94.2 |
| 102 | 422 | 413 | 97.9 | 402 | 95.3 |
| 104 | 418 | 413 | 98.8 | 404 | 96.7 |

*Statistical analysis not performed*

**Table B.6.5.1/4. Bodyweight of female rats in the 2-year feed study with eugenol**

| | Females | | | | |
|---|---|---|---|---|---|
| | Controls | 6000 ppm | | 12500 ppm | |
| weeks | average weight (g) | average weight (g) | % of controls | average weight (g) | % of controls |
| 0 | 83 | 85 | 102.4 | 85 | 102.4 |
| 4 | 140 | 136 | 97.1 | 131 | 93.6 |
| 8 | 168 | 167 | 99.4 | 158 | 94 |
| 13 | 188 | 186 | 98.9 | 175 | 93.1 |
| 17 | 188 | 189 | 100.5 | 183 | 97.3 |
| 21 | 203 | 199 | 98 | 196 | 96.6 |
| 25 | 205 | 203 | 99 | 196 | 95.6 |
| 28 | 214 | 212 | 99.1 | 203 | 94.9 |
| 34 | 214 | 208 | 97.2 | 199 | 93 |
| 38 | 218 | 217 | 99.5 | 207 | 95 |
| 42 | 220 | 216 | 98.2 | 208 | 94.5 |
| 46 | 227 | 221 | 97.4 | 211 | 93 |
| 51 | 232 | 220 | 94.8 | 208 | **89.7 (↓10.3%)** |
| 55 | 239 | 223 | 93.3 | 213 | **89.1 (↓10.9%)** |

| | | | | | |
|---|---|---|---|---|---|
| 59 | 240 | 226 | 94.2 | 216 | 90 |
| 64 | 244 | 230 | 94.3 | 221 | 90.6 |
| 68 | 254 | 241 | 94.9 | 223 | **87.8 (↓12.2%)** |
| 72 | 258 | 244 | 94.6 | 225 | **87.2 (↓12.8%)** |
| 77 | 269 | 255 | 94.8 | 235 | **87.4 (↓12.6%)** |
| 81 | 274 | 253 | 92.3 | 234 | **85.4 (↓14.6%)** |
| 86 | 280 | 266 | 95 | 242 | **86.4 (↓13.6%)** |
| 90 | 281 | 272 | 96.8 | 247 | **87.9 (↓12.1%)** |
| 94 | 287 | 273 | 95.1 | 245 | **85.4 (↓14.6%)** |
| 98 | 289 | 274 | 94.8 | 251 | **86.9 (↓13.1%)** |
| 102 | 291 | 281 | 96.6 | 253 | **86.9 (↓13.1%)** |
| 104 | 290 | 293 | 101 | 271 | 93.4 |

*Statistical analysis not performed*

**Food consumption**: No differences in food consumption were observed in treated groups for both sexes compared with controls. The average daily feed consumption was 99% and 96% for low and high dose male groups, and 98% and 95% for low and high dose female groups, respectively, compared with controls (see tables B.6.5.1/5 and B.6.5.1/6).

**Table B.6.5.1/5. Feed consumption of male rats in the 2-year feed study with eugenol**

| | Males | | | | |
|---|---|---|---|---|---|
| | Controls | 3000 ppm | | 6000 ppm | |
| weeks | g feed/day (a) | g feed/day (a) | % of controls | g feed/day (a) | % of controls |
| 8 | 19 | 19 | 100 | 18 | 100 |
| 13 | 16 | 18 | 110 | 17 | 110 |
| 17 | 20 | 18 | 90 | 19 | 100 |
| 21 | 16 | 17 | 110 | 17 | 110 |
| 25 | 17 | 18 | 110 | 16 | 90 |
| 28 | 21.9 | 20.6 | 90 | 23.1 | 110 |
| 34 | 14.6 | 13.7 | 90 | 15.4 | 110 |
| 38 | 21.9 | 19.4 | 90 | 21.9 | 100 |
| 42 | 13.4 | 12.6 | 90 | 12.6 | 90 |
| 46 | 15 | 16 | 110 | 15 | 100 |
| 51 | 18 | 18 | 100 | 17 | 90 |
| 55 | 23 | 17 | 70 | 17 | 70 |
| 59 | 17 | 17 | 100 | 17 | 100 |
| 64 | 17 | 17 | 100 | 16 | 90 |
| 68 | 17 | 17 | 100 | 16 | 90 |
| 72 | 17 | 17 | 100 | 16 | 90 |
| 77 | 17 | 16 | 100 | 16 | 90 |
| 81 | 17 | 16 | 100 | 16 | 90 |
| 86 | 17 | 16 | 100 | 16 | 90 |
| 90 | 16 | 16 | 100 | 15 | 90 |
| 94 | 16 | 16 | 100 | 15 | 90 |
| 98 | 15 | 18 | 120 | 16 | 110 |
| 102 | 17.7 | 17.8 | 100 | 17.8 | 100 |
| 104 | 14.9 | 14.9 | 100 | 14.9 | 100 |
| **mean±SD** | **17.3±2.4** | **16.9±1.7** | **99±0.1** | **16.7±2.2** | **96±0.1** |

*a) grams of feed consumed per animal per day*
*Statistical analysis not performed*

**Table B.6.5.1/6. Feed consumption of female rats in the 2-year feed study with eugenol**

| | Females | | | | |
|---|---|---|---|---|---|
| | Controls | 6000 ppm | | 12500 ppm | |
| weeks | g feed/day (a) | g feed/day (a) | % of controls | g feed/day (a) | % of controls |
| 8 | 15 | 13 | 90 | 13 | 90 |
| 13 | 11 | 13 | 120 | 11 | 110 |
| 17 | 14 | 11 | 80 | 10 | 70 |
| 21 | 12 | 9 | 80 | 11 | 100 |
| 25 | 12 | 10 | 80 | 10 | 90 |
| 28 | 14.2 | 16.8 | 120 | 11.6 | 90 |
| 34 | 9.4 | 11.1 | 120 | 7.7 | 90 |
| 38 | 13.4 | 12.1 | 90 | 12.1 | 100 |
| 42 | 8.6 | 7.9 | 90 | 8.6 | 110 |
| 46 | 11 | 9 | 80 | 8 | 80 |
| 51 | 12 | 11 | 100 | 10 | 90 |
| 55 | 12 | 10 | 90 | 10 | 80 |
| 59 | 13 | 11 | 90 | 11 | 80 |
| 64 | 12 | 12 | 110 | 11 | 90 |
| 68 | 12 | 12 | 110 | 11 | 90 |
| 72 | 12 | 12 | 110 | 11 | 90 |
| 77 | 12 | 11 | 100 | 12 | 100 |
| 81 | 12 | 11 | 100 | 12 | 100 |
| 86 | 12 | 11 | 100 | 12 | 100 |
| 90 | 13 | 12 | 90 | 12 | 90 |
| 94 | 13 | 12 | 100 | 12 | 90 |
| 98 | 12 | 13 | 110 | 13 | 110 |
| 102 | 12.4 | 13.3 | 100 | 15.5 | 120 |
| 104 | 12.1 | 11.1 | 90 | 13 | 110 |
| **mean±SD** | **12.3±1.4** | **11.5±1.8** | **98±0.1** | **11.2±1.7** | **95±0.1** |

*a) grams of feed consumed per animal per day*
*Statistical analysis not performed*

**Histopathology:**

**Neoplastic changes (table B.6.5.1/7)**

Lung: Statistically significant increased in the alveolar/bronchiolar adenomas and carcinomas (combined) incidences was observed in the 3000 ppm dose male group compared with controls (10% vs 0% in controls). In the high dose male group, the incidence was non-statistically significant compared to controls, and displayed lower values than noted in the 3000 ppm dose group (4%), so a dose-related trend was not observed. On the other hand, the incidences of alveolar/bronchiolar adenomas and carcinomas were not significantly increased in female rats at any dose tested.

The incidence of alveolar/bronchiolar adenomas and carcinomas (combined) noted in the low dose male group (10%) was higher than mean incidence of HCD reported by Southern Research Institute (3%), and NTP Carcinogenesis Program (2.4% for 1983 period and 3.3% for 1990-1997 period), but within the range of the NTP Carcinogenesis Program 1990-1997 (0-14%) [Haseman *et al.*, *Toxicol Pathol.*, 1984: 12(2): 126-35; and 1998, 26(3):428-41].

Thyroid gland: Statistically significant increase incidence of thyroid C-Cell adenoma was observed in the 6000 ppm female dose group compared with controls (22% vs 8% in controls). In the 12500 ppm dose female group, the incidence displayed lower values than noted in the 6000 ppm dose group and controls (4%), so a dose-related trend was not observed. No differences were detected in thyroid C-Cell carcinomas in any female dose groups. On the other hand, the incidences of thyroid C-Cell adenoma or carcinomas were not increase in male rats at any dose level.

The incidence of thyroid C-Cell adenomas noted in the low dose female group (22%) was higher than mean incidence and in the range limit of the HCD reported by NTP Carcinogenesis Program (mean incidence= 4.9%; range: n.a. for 1983 period; and mean incidence=11.7%; range= 4-22% for 1990-1997 period).

In contrast, a statistically significant decrease in the incidence of thyroid C-Cell adenoma (10%, 10% and 0% for controls, low and high dose groups) and adenoma/carcinoma combined (18%, 16% and 4% for controls, low and high dose groups) was detected in the thyroids of high dose male group compared with controls.

Uterus: Statistically significant increase trend in the incidence of uterine endometrial stromal polyp or sarcoma was observed in the high dose female group (15%, 12% and 34% for controls, low and high dose groups, respectively). In the 12500 ppm dose female group, the incidence was non-statistically significant compared to controls (p=0.051), but as noted, the p value was just in the border of the statistical significance. However, this value was higher than mean incidence of HCD reported by Southern Research Institute (15%) and NTP Carcinogenesis Program (19.4% for 1983 period and 14.7% for 1990-1997 period), and out of the range recorded by NTP Carcinogenesis Program 1990-1997 (0-30%). If we analyse the incidences individually, the incidence of uterine endometrial stromal polyps was 15, 12 and 32% for control, low and high dose female groups, respectively. The occurrence in the top dose female group was also higher than mean and out of the range of HCD reported by NTP Carcinogenesis Program (mean incidence= 18.3%; range: n.a. for 1983 period; and mean incidence=14.2%; range 2-30% for 1990-1997 period). On the other hand, the incidence of uterine endometrial stromal sarcoma was 0, 0 and 2% for control, low and high dose female groups, respectively. The result observed in the high dose female group was higher than mean, but within the HCD reported by NTP Carcinogenesis Program (mean= 1.1%; range = n.a. for 1983 period; and mean=0.5%; range 0-4% for 1990-1997 period).

Mammary gland: Statistically significant decreases in the incidence of mammary gland fibroadenomas were recorded in the low and high dose female group compared with controls (35%, 14% and 12% for control, low and high dose group, respectively).

Other organs/tissues: No differences were detected between treated-groups and controls in other tissues or organs.

**Table B.6.5.1/7. Summary of neoplastic incidences in the rat 2-year feed study with eugenol**

| | Neoplastic incidences | | | | | |
|---|---|---|---|---|---|---|
| | Control (%) | | low dose (%) (3000 ppm ♂/ 6000 ppm ♀) | | high dose (%) (6000 ppm ♂/ 12500 ppm ♀) | |
| | males | females | males | females | males | females |
| **Alveolar/bronchiolar adenoma / carcinoma** | 0/40 (0%) | 1/39 (3%) | **5/49 (10%)* (↑10%)** | 1/50 (2%) | 2/50 (4%) | 0/50 (0%) |
| **Alveolar/bronchiolar adenoma** | 0/40 (0%) | 0/39 (0%) | 2/49 (4%) | 1/50 (2%) | 2/50 (4%) | 0/50 (0%) |
| **Alveolar/bronchiolar carcinoma** | 0/40 (0%) | 1/39 (3%) | 3/49 (6%) | 0/50 (0%) | 0/50 (0%) | 0/50 (0%) |
| **Thyroid C-cell adenoma/carcinoma** | **7/40 (18%)#** | 7/40 (18%) | 8/50 (16%) | 12/49 (24%) | **2/50 (4%)* (↓14%)** | 6/50 (12%) |
| **Thyroid C-cell adenoma** | **4/40 (10%)#** | 3/40 (8%) | 5/50 (10%) | **11/49 (22%)* (↑14%)** | **0/50 (0%)* (↓10%)** | 2/50 (4%) |
| **Thyroid C-cell carcinoma** | 3/40 (8%) | 4/40 (10%) | 3/50 (6%) | 1/49 (2%) | 2/50 (4%) | 4/50 (8%) |
| **Uterine endometrial stromal polyp /sarcoma** | – | **6/40 (15%)#** | – | 6/50 (12%) | – | 17/50 (34%) |
| **Uterine endometrial stromal polyp** | – | 6/40 (15%) | – | 6/50 (12%) | – | 16/50 (32%) |
| **Uterine endometrial stromal sarcoma** | – | 0/40 (0%) | – | 0/40 (0%) | – | 1/50 (2%) |
| **Mammary gland fibroadenoma** | 0/40 (0%) | 14/40 (35%) | 2/50 (4%) | **7/50 (14%)* (↓21%)** | 2/50 (4%) | **6/50 (12%)* (↓23%)** |

** p<0.05*

*# p<0.05 (Cochran-Armitage trend test)*

**Non-neoplastic changes (table B.6.5.1/8)**

Uterus: Increased incidence of cystic hyperplasia was detected in the uterus of high dose female group (3%, 4% and 22% for control, low and high dose groups, respectively).

Spleen: Increased incidence of spleen haemosiderosis was detected in high dose female group (2%, 2% and 16% for control, low and high dose groups, respectively).

Kidney: A slight increase of kidney chronic inflammation incidences was found in male dose groups (73%, 92% and 86% for control, low and high dose group, respectively).

Thyroid: A slight increase of C-cell hyperplasia was detected in top dose male group (3%, 2% and 8% for control, low and high dose group, respectively). This finding presented low incidence (<10%), and was not related with an increase of C-cell adenomas or carcinomas.

Prostate: Prostate inflammation suppurative and chronic incidences were increased in low dose male group. However, the relevance of these findings was discarded due to the absence of a dose-response pattern.

No statistical analysis was performed in non-neoplastic changes in the study, so these results are not supported by statistical significance.

- No other relevant non-neoplastic lesions were detected in the female and male rats at any dose level.

**Table B.6.5.1/8. Summary of non-neoplastic incidences in the rats 2-year feed study with eugenol**

| | non-neoplastic incidences | | | | | |
|---|---|---|---|---|---|---|
| | Control (%) | | low dose (%) (3000 ppm ♂/ 6000 ppm ♀) | | high dose (%) (6000 ppm ♂/ 12500 ppm ♀) | |
| | males | females | males | females | males | females |
| **Uterus cystic hyperplasia** | – | 1/40 (3%) | – | 2/50 (4%) | – | 11/50 (22%) |
| **Spleen haemosiderosis** | 0/40 (0%) | 1/40 (2%) | 0/50 (0%) | 1/50 (2%) | 0/50 (0%) | 8/50 (16%) |
| **Chronic inflammation kidney** | 29/40 (73%) | 4/40 (10%) | 46/50 (92%) | 3/50 (6%) | 43/50 (86%) | 2/50 (4%) |
| **Thyroid hyperplasia C-cell** | 1/40 (3%) | 6/40 (15%) | 1/50 (2%) | 7/49 (14%) | 4/50 (8%) | 5/50 (10%) |
| **Prostate inflammation suppurative** | 3/40 (8%) | – | 9/50 (18%) | – | 5/47 (11%) | – |
| **Prostate inflammation chronic suppurative** | 0/40 (0%) | – | 5/50 (10%) | – | 2/47 (4%) | – |

*Statistical analysis not performed*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: no carcinogenic potential in humans. The NOAEL in the rat was 300 mg/kg bw/d. Target / critical effect (rat): reduced body weights, spleen haemosiderosis, uterine cystic hyperplasia (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is non-GLP compliant and presents several deviations, as using only two dose levels and the absence of haematology, urinalysis, clinical chemistry and organ weights data. Therefore, this study is considered acceptable as supportive.

There was no evidence for carcinogenicity after administration of eugenol at 3000 and 6000 ppm in males, or at 6000 and 12500 ppm in females to F344/N rats. The increased incidences of alveolar/bronchiolar adenomas and carcinomas (combined), and thyroid C-Cell adenomas observed in the low dose male and female groups, respectively, did not show a dose-related pattern due to high dose male and females groups exhibited lower values than respective low dose groups.

On the other hand, the incidence of uterine endometrial stromal polyp or sarcoma in high dose female group was in the border of statistical significance (p=0.051), and was higher than mean of the HCD recorded by Southern Research Institute. In addition, and increase incidence of cystic hyperplasia was also noted in the uterus of high dose female group. The cystic hyperplasia effect noted in high dose female groups is not supported by statistical significance, and the HCD incidence was not provided.

**NOAEL for carcinogenicity** was considered to be **6000 ppm (**equivalent to 260/302 mg/kg bw/day for males/females), based on no evidence of carcinogenicity. **NOAEL for toxicity** was considered to be **6000 ppm (**equivalent to 260/302 mg/kg bw/day for males/females), based on increase incidences of cystic hyperplasia in uterus, spleen haemosiderosis, and reduced bodyweights in females.

### B.6.5.2. Carcinogenicity study in mice (2-year)

| | |
|---|---|
| **Data point** | CA 5.5 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 1983 |
| **Report title** | Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) |
| **Report No** | NTP-80-068. NIH Publication No. 84-1779 (CAS No. 97-53-0). NTP TR 223 |
| **Document No** | KCA 5.3.1/01 |
| **Guidelines followed in study** | None. Prior to OECD Test Guidelines for combined chronic toxicity/carcinogenicity studies. The study was checked for compliance with OECD TG 453 (2018). |
| **Deviations from current test guideline OECD TG 453 (2018)** | -No satellite groups to monitor the reversibility of toxicological changes were incorporated.<br>- Only two dose levels were assayed, when at least three dose levels should be used.<br>- No haematology, urinalysis and clinical biochemistry were performed.<br>- Organ weight data was not recorded.<br>-Individual bodyweight data were not presented.<br>- Statistical analysis was not performed in bodyweight, food consumption and non-neoplastic lesions.<br>-Animal cages were not arranged in such a way that possible effects due to cage placement were minimized. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 36483 and 26068, Purity: >99% |
| **Test system** | Male and Female B6C3F$_1$ mice |
| **Test method** | Three groups of 50 male and 50 female B6C3F$_1$ mice of 6-7 weeks of age were tested *via* feed diet with eugenol at 0, 3000 ppm and 6000 ppm for 106 weeks. Dose levels selected for the two year studies were based on 13-week (91-day) studies. The equivalence values in mg/kg bw/day adjusted by RMS according to EFSA Guidance (EFSA Journal 2012;10,3:2579) were 632/784 for males/females (3000 ppm) and 1250/1546 for males/females (6000 ppm). The default values in mg/kg bw/day were 450 (3000 ppm) and 900 (6000 ppm) mg/kg bw/day. All animals were observed twice daily for morbidity or mortality. Clinical signs were recorded monthly. Individual animals were weighed weekly for the first 13 weeks, then monthly to week 93, and every 2 weeks thereafter. Necropsies were performed on all animals not excessively autolyzed or cannibalized. Examinations for grossly visible lesions were performed on major tissues or organs. Tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The following tissues were examined microscopically: tissue masses, abnormal lymph nodes, skin, mandibular lymph nodes, mammary gland, salivary gland, thigh muscle, sciatic nerve, bone marrow, costochondral junction (rib), thymus, larynx, trachea, lungs and bronchi, heart, thyroid, parathyroid, esophagus, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph nodes, liver, gallbladder, pancreas, spleen, kidneys, adrenals, bladder, seminal vesicles/prostate/testes or ovaries/uterus, nasal cavity, brain, pituitary, and spinal cord. The incidence of neoplastic or non-neoplastic lesions has been given as the ratio of the number of animals bearing such lesions at a specific anatomic site to the number of animals in which that site was examined. |
| **Statistical analysis of results** | Dose-related effect on survival was analysed using Cox method (1972) for testing two groups for equality, and Tarone's (1975) extensions of Cox's methods for testing for a dose-related trend. Statistical analysis of tumor incidence data used two different methods for adjusting intercurrent mortality. Each used the classical methods for combining contingency tables developed by Mantel and Haenszel (1959). The first method of |

analysis is the life table method of Cox and Tarone and assumed that all tumors of a given type observed in animals dying before the end of the study were "fatal". According to this approach, the proportions of tumor-bearing animals in the dosed and control groups were compared at each point in time at which an animal died with a tumor of interest. The second method of analysis assumed that all tumors of a given type observed in animals dying before the end of the study were "incidental". According to this approach, the proportions of animals found to have tumors in dosed and control groups were compared in each of five time intervals: 0-52 weeks, 53-78 weeks, 79-92 weeks, week 93 to the week before the terminal kill, and the terminal kill period. The individual time interval comparisons were then combined by the previously described methods to obtain a single overall result.

Another set of statistical analyses was carried out and reported in the tables analysing primary tumors; the Fisher exact test for pairwise comparisons and the Cochran-Armitage linear trend test for dose-response trends. These tests were based on the overall proportion of tumor-bearing animals. All reported P-values are one-sided.

**Historical control data**

In order to completeness, all the available information is presented, however, HCD from Southern Research Institute was the only accepted by RMS, due to it was the laboratory in which the study was conducted, and the time frame is within the 5-recommended years. Hence, the following historical control data (HCD) of neoplastic lesions are presented (Table B.6.5.2/1):

**Table B.6.5.2/1. Historical control data of liver and thyroid gland tumors from NTP carcinogenesis program and Southern Research Institute**

| | | HCD from NTP feeding carcinogenicity studies (1990-1997) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Male | | | Female | | |
| | | nº animals /total | Rate (%)# | Range (%) | nº animals /total | Rate (%)# | Range (%) |
| Liver | Adenoma/ carcinoma | 638/1350 | 47.3 | 4-60 | 347/1350 | 25.7 | 0-50 |
| | Adenoma | 397/1350 | 29.4 | 4-60 | 234/1350 | 17.3 | 2-50 |
| | Carcinoma | 241/1350 | 17.9 | 6-29 | 113/1350 | 8.4 | 0-20 |
| Thyroid gland | Follicular cell adenoma/ carcinoma | 26/1343 | 1.9 | 0-4 | 26/1340 | 1.9 | 0-8 |
| | Follicular cell adenoma | 20/1343 | 1.5 | 0-4 | 24/1340 | 1.8 | 0-8 |
| | Follicular cell carcinoma | 6/1343 | 0.4 | 0-2 | 2/1340 | 0.1 | 0-2 |
| | | HCD from NTP feeding carcinogenicity studies (1983) | | | | | |
| | | Male | | | Female | | |
| | | nº animals /total | Rate (%)# | Range (%) | nº animals /total | Rate (%)# | Range (%) |
| Liver | Adenoma/ carcinoma | 743/2386 | 31 | 0-58 | 208/2519 | 8 | 0-18 |
| | Adenoma | 242/2386 | 10 | 0-22 | 102/2519 | 4 | 0-18 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Carcinoma | 501/2386 | 21 | 6-36 | 106/2519 | 4 | 0-15 |
| Thyroid gland | Follicular cell adenoma/ carcinoma | 27/2178 | 1.2 | n.a. | 46/2203 | 2.1 | n.a. |
| | Follicular cell adenoma | 22/2178 | 1 | n.a. | 40/2203 | 1.8 | n.a. |
| | Follicular cell carcinoma | 5/2178 | 0.2 | n.a. | 6/2203 | 0.3 | n.a. |
| | | HCD from Southern Research Institute (1983) | | | | | |
| | | Male | | | Female | | |
| | | nº animals /total | Rate (%)# | Range (%) | nº animals /total | Rate (%)# | Range (%) |
| Liver | Adenoma/ carcinoma | 145/446 | 32 | n.a. | 30/445 | 7 | n.a. |
| | Adenoma | 42/446 | 9 | n.a. | 14/445 | 3 | n.a. |
| | Carcinoma | 103/446 | 23 | n.a. | 16/445 | 4 | n.a. |

*# percentage of animals with tumors*

**Results**

**Survival:** No statistically significant differences were detected between treated groups and controls throughout study for both sexes. Survival in the high dose male group was slightly lower than low dose and control group after week 38, whereas the survival in the low dose female group was lower than high dose and control group after week 80 (see Kaplan-Meier survival curve, Figure B.6.5.2). A reduction in survival was detected in low and high dose male groups (12%), and in low dose female group (6%), compared with controls at termination of the study (table B.6.5.2/2).

**Table B.6.5.2/2. Survival at termination of study (106 weeks)**

| | Survival (%) | |
|---|---|---|
| | ♂ | ♀ |
| **Control** | 82% (41/50) | 86% (43/50) |
| **3000 ppm** | 70% (35/50) | 80% (40/50) |
| **6000 ppm** | 70% (35/50) | 90% (45/50) |

*No statistically significant differences*

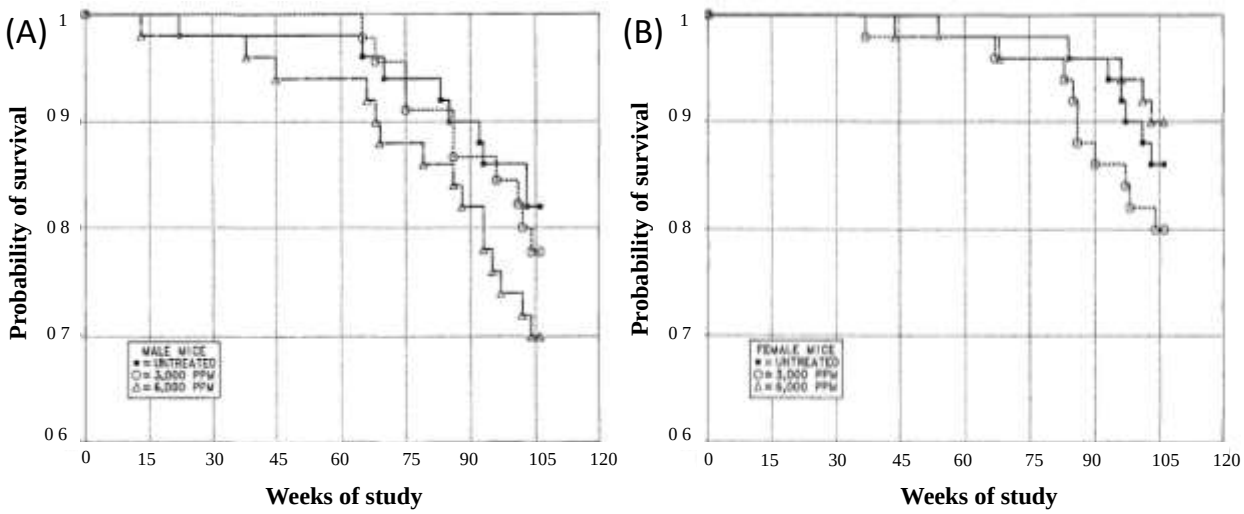



**Figure B.6.5.2. Kaplan-Meier survival curves of males (A) and females (B) groups.**

**Bodyweight:** No differences in mean bodyweights were detected in male treated groups. In the female high dose group a reduction >10% was observed in the mean bodyweight at weeks 101 (14%) and 104 (11%), respectively compared with controls (see tables B.6.5.2/3 and B.6.5.2/4).

**Table B.6.5.2/3. Bodyweight of male mice in the 2-year feed study with eugenol**

| | Males | | | | |
|---|---|---|---|---|---|
| | Controls | 3000 ppm | | 6000 ppm | |
| weeks | average weight | average weight | % of controls | average weight | % of controls |
| 0 | 18 | 19 | 105.6 | 19 | 105.6 |
| 7 | 28 | 29 | 103.6 | 28 | 100 |
| 11 | 32 | 31 | 96.9 | 31 | 96.9 |
| 15 | 32 | 32 | 100 | 32 | 100 |
| 20 | 34 | 34 | 100 | 33 | 97.1 |
| 24 | 35 | 35 | 100 | 34 | 97.1 |
| 28 | 36 | 36 | 100 | 35 | 97.2 |
| 32 | 36 | 37 | 102.8 | 36 | 100 |
| 36 | 35 | 36 | 102.9 | 36 | 102.9 |
| 41 | 37 | 36 | 97.3 | 37 | 100 |
| 46 | 37 | 36 | 97.3 | 36 | 97.3 |
| 49 | 36 | 37 | 102.8 | 35 | 97.2 |
| 53 | 38 | 37 | 97..4 | 37 | 97.4 |
| 58 | 39 | 38 | 97.4 | 37 | 94.9 |
| 62 | 40 | 38 | 95 | 38 | 95 |
| 66 | 41 | 39 | 95.1 | 39 | 95.1 |
| 71 | 40 | 38 | 96 | 37 | 92.5 |
| 75 | 41 | 39 | 95.1 | 38 | 92.7 |
| 79 | 40 | 39 | 97.5 | 38 | 95 |
| 84 | 40 | 39 | 97.5 | 39 | 97.5 |
| 88 | 39 | 39 | 100 | 38 | 97.4 |
| 93 | 40 | 38 | 95 | 38 | 95 |
| 97 | 39 | 38 | 97.4 | 38 | 97.4 |

| | | | | | |
|---|---|---|---|---|---|
| 101 | 39 | 38 | 97.4 | 38 | 97.4 |
| 104 | 39 | 38 | 97.4 | 38 | 97.4 |

*Statistical analysis not performed*

**Table B.6.5.2/4. Bodyweight of female mice in the 2-year feed study with eugenol**

| | Females | | | | |
|---|---|---|---|---|---|
| | Controls | 3000 ppm | | 6000 ppm | |
| weeks | average weight | average weight | % of controls | average weight | % of controls |
| 0 | 16 | 16 | 100 | 16 | 100 |
| 7 | 22 | 22 | 100 | 22 | 100 |
| 11 | 25 | 24 | 96 | 24 | 96 |
| 15 | 25 | 24 | 96 | 25 | 100 |
| 20 | 26 | 25 | 96.2 | 25 | 96.2 |
| 24 | 27 | 27 | 100 | 26 | 96.3 |
| 28 | 29 | 28 | 96.6 | 27 | 93.1 |
| 32 | 29 | 28 | 96.6 | 28 | 96.6 |
| 36 | 28 | 27 | 96.4 | 28 | 100 |
| 41 | 28 | 29 | 103.6 | 28 | 100 |
| 46 | 29 | 29 | 100 | 28 | 96.6 |
| 49 | 30 | 29 | 96.7 | 28 | 93.3 |
| 53 | 31 | 30 | 96.8 | 29 | 93.5 |
| 58 | 31 | 31 | 100 | 29 | 93.5 |
| 62 | 32 | 31 | 96.9 | 30 | 93.8 |
| 66 | 31 | 31 | 100 | 30 | 96.8 |
| 71 | 34 | 34 | 100 | 31 | 91.2 |
| 75 | 34 | 34 | 100 | 31 | 91.2 |
| 79 | 33 | 32 | 97 | 31 | 93.9 |
| 84 | 34 | 33 | 97.1 | 31 | 91.2 |
| 88 | 33 | 32 | 97 | 31 | 93.9 |
| 93 | 35 | 35 | 100 | 32 | 91.4 |
| 97 | 35 | 34 | 97.1 | 32 | 91.4 |
| 101 | 36 | 35 | 97.2 | 31 | **86.1 (↓13.9%)** |
| 104 | 35 | 33 | 94.3 | 31 | **88.6 (↓11.4%)** |

*Statistical analysis not performed*

**Food consumption:** No differences in food consumption were observed in treated groups for both sexes compared with controls. The average daily feed consumption was 97% and 93% for low and high dose male groups, and 106% and 90% for low and high dose female groups, respectively, compared with controls (see tables B.6.5.2/5 and B.6.5.2/6).

**Table B.6.5.2/5. Feed consumption of male mice in the 2-year feed study with eugenol**

| | Males | | | | |
|---|---|---|---|---|---|
| | Controls | 3000 ppm | | 6000 ppm | |
| weeks | g feed/day (a) | g feed/day (a) | % of controls | g feed/day (a) | % of controls |
| 7 | 10 | 10 | 100 | 10 | 100 |
| 11 | 8 | 8 | 100 | 7 | 90 |
| 15 | 8 | 7 | 90 | 7 | 90 |
| 20 | 7 | 8 | 110 | 8 | 110 |
| 24 | 8 | 8 | 100 | 9 | 110 |

| | | | | | |
|---|---|---|---|---|---|
| 28 | 7.7 | 7.7 | 100 | 8.7 | 110 |
| 32 | 9.3 | 8.3 | 90 | 8.3 | 90 |
| 36 | 9 | 8 | 90 | 8 | 90 |
| 41 | 9 | 8 | 90 | 8 | 90 |
| 46 | 9.7 | 7.7 | 80 | 8.7 | 90 |
| 49 | 8.4 | 8.4 | 100 | 8.4 | 100 |
| 53 | 7.7 | 7.7 | 100 | 6.8 | 90 |
| 58 | 9 | 8 | 90 | 9 | 100 |
| 62 | 9 | 9 | 100 | 9 | 100 |
| 66 | 9 | 9 | 100 | 9 | 100 |
| 71 | 9 | 9 | 100 | 8 | 90 |
| 75 | 9 | 9 | 100 | 9 | 100 |
| 79 | 6 | 6 | 100 | 5 | 80 |
| 84 | 6 | 6 | 100 | 5 | 80 |
| 88 | 6 | 6 | 100 | 5 | 80 |
| 93 | 6 | 6 | 100 | 5 | 80 |
| 97 | 6 | 6 | 100 | 5 | 80 |
| 101 | 6 | 6 | 100 | 5 | 80 |
| 104 | 6 | 6 | 100 | 6 | 100 |
| **mean±SD** | **7.9±1.4** | **7.6±1.2** | **97±0.1** | **7.4±1.7** | **93±0.1** |

*a) grams of feed consumed per animal per day*
*Statistical analysis not performed*

**Table B.6.5.2/6. Feed consumption of female mice in the 2-year feed study with eugenol**

| | **Females** | | | | |
|---|---|---|---|---|---|
| | **Controls** | **3000 ppm** | | **6000 ppm** | |
| **weeks** | **gr feed/day (a)** | **gr feed/day (a)** | **% of controls** | **gr feed/day (a)** | **% of controls** |
| 7 | 10 | 10 | 100 | 10 | 100 |
| 11 | 10 | 8 | 90 | 9 | 90 |
| 15 | 9 | 8 | 110 | 7 | 80 |
| 20 | 10 | 8 | 100 | 8 | 80 |
| 24 | 9 | 9 | 100 | 9 | 100 |
| 28 | 7.7 | 7.7 | 100 | 7.7 | 100 |
| 32 | 9.4 | 8.3 | 100 | 8.3 | 90 |
| 36 | 8 | 8 | 100 | 8 | 100 |
| 41 | 8 | 8 | 100 | 8 | 100 |
| 46 | 8.7 | 9.7 | 110 | 8.7 | 100 |
| 49 | 9.4 | 7.3 | 90 | 8.4 | 90 |
| 53 | 9.6 | 8.7 | 110 | 7.7 | 80 |
| 58 | 9 | 8 | 100 | 8 | 90 |
| 62 | 9 | 8 | 100 | 8 | 90 |
| 66 | 8 | 9 | 110 | 8 | 100 |
| 71 | 8 | 8 | 110 | 7 | 90 |
| 75 | 8 | 9 | 110 | 8 | 100 |
| 79 | 7 | 6 | 100 | 6 | 90 |
| 84 | 6 | 6 | 120 | 5 | 80 |
| 88 | 6 | 6 | 120 | 5 | 80 |
| 93 | 6 | 6 | 120 | 5 | 80 |
| 97 | 5 | 5 | 130 | 4 | 80 |
| 101 | 5 | 5 | 130 | 4 | 80 |
| 104 | 6 | 5 | 100 | 5 | 80 |
| **mean±SD** | **8±1.6** | **7.6±1.5** | **106±0.1** | **7.2±1.7** | **90±0.1** |

*a) grams of feed consumed per animal per day*
*Statistical analysis not performed*

**Histopathology:**
**Neoplastic changes (see table B.6.5.2/7)**

Liver: Statistically significant increase in the hepatocellular adenomas and carcinomas incidences was recorded in the 3000 ppm male dose group (26% and 40% for adenomas and carcinomas, respectively) compared with controls (8% and 20% for adenomas and carcinomas, respectively). In the high dose male group, the incidences were non-statistically significant compared to controls, and displayed lower values than noted in the 3000 ppm dose group (20% and 18% for adenomas and carcinomas, respectively), so a dose-related trend was not observed.

The incidence of hepatocellular adenomas noted in the low dose male group (26%) was higher than mean, and slightly outside the range of the historical control datasets documented until 1983 by the NTP Carcinogenesis Program (mean=10%; range=0-22%) and the Southern Research Institute (mean= 9%; range= n.a.). Conversely, and taken into account a further HCD published by the NTP Carcinogenesis Program encompassing period 1990-1997 (Haseman *et al.*, *Toxicol Pathol.*, 1998, 26(3):428-41), the occurrence observed in the 3000 ppm male group was lower than mean incidence and within the range of this HCD (mean= 29.4%; range= 4-60%).

On the other hand, the incidence of hepatocellular carcinomas observed in the low dose male group (40%) was higher than mean incidence and out of the range of the three reported historical control datasets (see material and methods section).

In addition, the incidence of both combined incidences in the low dose male group displayed statistically significant results compared with controls (56% vs 28% in controls). The result was higher than mean reported in the three HCD, but was within the range of the datasets reported by the NTP Carcinogenesis Program in 1983 (range=0-58%) and through 1990-1997 (range=4-60%). Besides, the incidence noted in the high male dose group (37%), was lower than observed in the low dose group, so a dose-related trend was not considered.

Moreover, the incidences of hepatocellular adenomas and carcinomas (combined) were increased in female mice at low and high dose level (14% and 18% for low and high dose group), displaying a statistically significant trend compared with controls (4%), however, only incidences at high dose level showed statistical significance. These incidences were higher than overall historical mean incidence of the Southern Research Institute (mean= 7%) and NTP Carcinogenesis Program (mean=8%), reported until 1983. However, these incidences were lower than mean of the HCD recorded by NTP Carcinogenesis Program through 1990-1997 (mean: 25.7%) and within the overall historical range of NTP Carcinogenesis Program during both periods 1983 (range: 0-18%) and 1990-1997 (range: 0-50%), respectively.

Lesions diagnosed as hepatocellular adenomas consisted of solid nodules of well-differentiated hepatocytes and compressed adjacent hepatic parenchyma. Hepatocytes in these lesions were often larger, with cytoplasm that was more vacuolated and was often basophilic. On the other hand, hepatocellular carcinomas had a more disorderly arrangement of hepatocytes, usually with evidence of invasive growth into adjoining hepatic tissue. A key criterion for diagnosing hepatocellular carcinoma was the arrangement of hepatocytes into trabeculae. One male mouse in the low dose group had a liver tumor that had some areas characteristic of hepatocellular carcinoma, as well as areas consisting of a disorderly proliferation of structures resembling bile ducts. That tumor was classified as a mixed hepatocellular/cholangiocarcinoma.

On the other hand, the authors of the study explained that the location of housing during the 2-year feeding period might affect the incidence of hepatocellular adenomas and carcinomas. It is common practice in the conduct of long-term studies to cage mice together and to locate cages of mice receiving the same treatment contiguously, rather than randomly. This practice was observed during this study. Nevertheless, it was postulated that local room effects might have been associated with the occurrence of hepatic tumours, particularly in male mice at the lower dose. In a cage-by-cage analysis, the incidence of hepatic lesions in male mice in cages 1-5 was found to be higher (80%) than that of hepatic tumours in the rest of the mice in the study (32%). Furthermore, on the basis of National Toxicology Program studies in mice reviewed by Haseman (Haseman, J.K. *et al.*, Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.*,1984, 12, 126-135), the incidence of hepatic carcinoma in control groups was ≤36%, whereas the incidence of hepatic carcinoma in cages 1-5 housing male mice at the lower dose was 64%. Young concluded that, as the cages were located systematically and as the increase in proliferative hepatic lesions, primarily carcinoma, was concentrated in the first 5 of 10 contiguous cages housing mice at the lower dose, the effect might have been due to room location and not eugenol (Young, S.S. *et al.*, Are there local room effects on hepatic tumors in male mice? An examination of the NTP eugenol study. *Fundam. Appl. Toxicol.*, 1987, 8, 1-4).

Thyroid gland: A slightly increased incidence of follicular cell adenomas of the thyroid gland was observed in the high dose male mice. This incidence did not show statistically significant differences compared with controls, although a statistically significant increased trend was noted (0%, 0% and 6% for control, low and high dose

groups, respectively). The incidence of follicular cell adenomas in the high dose male group was higher than the mean incidence recorded by NTP Carcinogenesis program (1% and 1.5% for 1983 and 1990-1997 period, respectively), and slightly greater than the range (0-4%) of HCD recorded by the NTP Carcinogenesis Program during period 1990-1997.

**Table B.6.5.2/7. Summary of neoplastic incidences in the mice 2-year feed study with eugenol**

| | neoplastic incidences (%) | | | | | |
|---|---|---|---|---|---|---|
| | Control | | 3000 ppm | | 6000 ppm | |
| | males | females | males | females | males | females |
| **Hepatocellular adenoma** | 4/50 (8%) | 0/50 (0%) | **13/50 (26%)* (↑18%)** | 4/49 (8%) | 10/49 (20%) | 3/49 (6%) |
| **Hepatocellular carcinoma** | 10/50 (20%) | 2/50 (4%) | **20/50 (40%)* (↑20%)** | 3/49 (6%) | 9/49 (18%) | 6/49 (12%) |
| **Hepatocellular adenoma or carcinoma** | 14/50 (28%) | **2/50 (4%)#** | **28/50 (56%)* (↑28%)** | 7/49 (14%) | 18/49 (37%) | **9/49 (18%)* (↑14%)** |
| **Thyroid follicular cell adenoma** | **0/48 (0%)#** | 2/48 (4%) | 0/49 (0%) | 0/47 (0%) | 3/49 (6%) | 1/49 (2%) |

** p<0.05*

*# p<0.05 (Cochran-Armitage trend test)*

Other organs/tissues: No differences were detected between treated-groups and controls in other tissues or organs.

**Non-neoplastic changes (see table B.6.5.2/8)**

Kidney: Increased incidence of focal inflammation of the kidney was described in high dose males (16% vs 0% in controls). On the other hand, a high decrease in nephrosis incidences were noted in the top dose male groups compared with low and control groups (61%, 52% and 8% for control, low and high male dose groups, respectively).

Lung: Increased incidence of focal granulomatous inflammation (51% vs 36% in controls), and hyperplasia adenomatous (54% vs 44% in controls) of the lung was noted in high dose females.

Ovary: A slight increase of ovary follicular cyst incidence was seen in top dose female group compared with low and control groups (22%, 22% and 33% for control, low and high dose female group, respectively).

Thyroid: Non-neoplastic alterations were found in the thyroid of dose groups. On the other hand, a decrease in cystic degeneration was described in male dose groups (23%, 6% and 0% for controls, low and high dose groups).

Skin: A slight increase of chronic inflammation of the skin was noted in the low male dose groups compared with controls (22% vs 14%). However, any top dose male showed this alteration, so a dose-related trend was not observed. On the other hand a decrease was noted in female groups (14%, 0%, 0% for control, low and high dose groups).

No statistical analysis was performed in non-neoplastic changes in the study, so these results are not supported by statistical significance.

-No relevant alterations were detected in other organs or tissues.

**Table B.6.5.2/8. Summary of non-neoplastic incidences in the mice 2-year feed study with eugenol**

| non-neoplastic incidences (%) |
|---|

| | Control | | 3000 ppm | | 6000 ppm | |
|---|---|---|---|---|---|---|
| | males | females | males | females | males | females |
| **Thyroid, cystic degeneration** | 11/48 (23%) | – | 3/49 (6%) | – | 0/49 (0%) | – |
| **Thyroid, follicular cell hyperplasia** | 1/48 (2%) | – | 1/49 (2%) | – | 0/49 (0%) | – |
| **Thyroid, ultimobranchial cyst** | – | 0/48 (0%) | – | 0/47 (0%) | – | 1/49 (2%) |
| **Ovary, follicular cyst** | – | 11/50 (22%) | – | 10/45 (22%) | – | 15/46 (33%) |
| **Skin, chronic inflammation** | 7/49 (14%) | 7/50 (14%) | 11/49 (22%) | 0/49 (0%) | 0/50 (0%) | 0/49 (0%) |
| **Kidney, focal inflammation** | 0/49 (0%) | 0/50 (0%) | 0/50 (0%) | 0/49 (0%) | 8/49 (16%) | 0/49 (0%) |
| **Kidney, nephrosis** | 30/49 (61%) | 18/50 (36%) | 26/50(52%) | 16/49 (33%) | 4/49(8%) | 13/49 (27%) |
| **Lung focal granulomatous inflammation** | 12/49 (24%) | 18/50 (36%) | 14/49 (29%) | 19/49 (39%) | 0/50 (0%) | 25/49 (51%) |
| **Lung adenomatous hyperplasia** | 17/50 (35%) | 22/50 (44%) | 21/50 (43%) | 22//49 (45%) | 18/50 (36%) | 26//49 (54%) |

*Statistical analysis not performed*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: no carcinogenic potential in humans. The NOAEL in the mouse was 450 mg/kg bw/d. Target / critical effect: reduced body weights and food consumption, inflammation in the lungs and kidneys (EFSA Journal 2012; 10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is non-GLP compliant and presents several deviations, as using only two dose levels and the absence of haematology, urinalysis, clinical chemistry and organ weights data. Therefore, this study is considered acceptable as supportive information.

There was no evidence for carcinogenicity after administration of eugenol at 3000 and 6000 ppm to $B6C3F_1$ mice. The incidence of hepatocellular adenomas and carcinomas observed in the low dose male group (26% and 40% for adenomas and carcinomas, respectively) did not show a dose-response pattern due to the incidence noted in the top dose group was lower than observed in the low dose group.

The increase incidences of hepatocellular adenomas and carcinomas (combined) in the high dose female group was considered an equivocal evidence of carcinogenicity due to both incidences did not display statistical significant results when were analysed individually.

As pointed in the study evaluation, the house location of animal´s cages might affect the incidence of hepatocellular adenomas and carcinomas.

Regarding follicular cell adenoma in thyroid gland, a positive trend was observed in high dose male group, but the value was not statistically significant compared with controls and the incidence was low.

On the other hand, increase incidences of ovary follicular cyst were noted in female top dose group. This finding was not support by statistical analysis and historical control data were not provided.

Thus, eugenol was not carcinogenic in mice based on the high spontaneous control incidence of neoplastic lesions described in the $B6C3F_1$ mice strain, the absence of a clear dose response, and the lacking of statistical significance.

**NOAEL for carcinogenicity** was considered to be **6000 ppm,** equivalent to 1250 mg/kg bw/day, based on no evidences of carcinogenicity at high dose tested. **NOAEL for toxicity** was considered to be **3000 ppm,** equivalent to 632 mg/kg bw/day, based on increase incidences of focal inflammation of the kidney in males, and focal granulomatous inflammation and adenomatous hyperplasia of lung, and ovary follicular cysts in females.

### B.6.5.3. Carcinogenicity study in mice (18-month)

| | |
|---|---|
| **Data point** | CA 5.5 (EU data requirement) |
| **Report author** | Miller, E.C., Swanson, A.B., Phillips, D.H., Fletcher, T.L., Liem, A.and Miller, J.A. |
| **Report year** | 1983 |
| **Report title** | Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivates related to safrole and estragole. |
| **Report No** | Not applicable, published study (*Cancer Research*, 43:1124-1134) |
| **Document No** | KCA 5.5/03 |
| **Guidelines followed in study** | None. Prior to OECD Test Guidelines for combined chronic toxicity/carcinogenicity studies. The study was checked for compliance with OECD TG 453 (2018). |
| **Deviations from current test guideline OECD TG 453 (2018)** | -Test substance not characterised (batch number and certificate of chemical analysis are not reported).<br>-The duration of the experiment was not 24 month for carcinogenicity assessment.<br>-No satellite groups to monitor the reversibility of toxicological changes were incorporated.<br>-Only 30 female mice/dose were used (OECD TG 453 requires at least 50 animals/sex/dose).<br>- Only one dose level was assayed, when at least three dose levels should be used.<br>- Bodyweight and food consumption were not measured. Bodyweight gain was only measured at 1, 4 and 8 month.<br>- No haematology, urinalysis and clinical chemistry were performed.<br>- Organ weight data was not recorded.<br>- Only liver, lung, skin, pleural and peritoneal cavity were subjected to necropsy and histopathological examinations.<br>-Statistical analysis not performed in survival and bodyweight gain measurements.<br>-Individual data were not presented. |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No, published study. |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol ( 1 -allyl-4-hydroxy-3-methoxybenzene); purity: >98% |
| **Test system** | Female CD-1 mice |
| **Test method** | Four groups of 30 female CD-1 mice of 8 week of age were tested with grain diet without any supplement (negative control), 0.5% eugenol (equivalent to 750 mg/kg bw/day), 0.5% eugenol + 0.05% phenobarbital (PB), and 0.05% PB (positive control). Eugenol was administered for 12 month in the diet followed by 6-month recovery period. The phenobarbital was administered in the drinking-water for 18 month. Bodyweight gain was measured at 1, 4 and 8 month, respectively. Necropsies were performed on all animals found dead or moribund and, at the termination, on all of the surviving animals. Liver, lung, skin, pleural and peritoneal cavity were further subjected to gross and histopathological examinations. |
| **Statistical analysis of results** | Comparisons of tumor incidences were made by Fisher's exact test. Differences in tumor multiplicity were examined by the Mann-Whitney (Wilcoxon) test. |

**Results**

**Survival:** Survival at 18 month was comparable between eugenol-fed animals without phenobarbital and control animals. At study termination, a reduction in survival (20%) was detected between eugenol-fed animals with phenobarbital (PB) and control animals (table B.6.5.3/1).

**Table B.6.5.3/1. Survival of mice in the 18-month study with eugenol**

| | Survival (no mice alive) | | |
|---|---|---|---|
| | **start** | **10 month (%)** | **18 month (%)** |
| **control** | 30 | 30 (100%) | 30 (100%) |
| **Phenobarbital (PB)** | 30 | 30 (100%) | 29 (97%) |
| **Eugenol** | 30 | 29 (97%) | 29 (97%) |
| **Eugenol + PB** | 30 | 25 (83%) | 24 (80%) |

*Statistical analysis not performed*

**Bodyweight gain:** Bodyweight gains of treated mice measured at 1 month was comparable to controls. However, at 4 month eugenol treated group showed a decrease of 13%, whereas eugenol + PB dose group displayed a reduction of 20%. At 8 month measurement, eugenol dose group showed a decrease of 15%, whereas eugenol + PB dose group exhibited a reduction of 23%. (see table B.6.5.3/2).

**Table B.6.5.3/2. Bodyweight gain of mice**

| | Bodyweight gain (g/mouse) | | |
|---|---|---|---|
| | **1 month (% vs control)** | **4 month (% vs control)** | **8 month (% vs control)** |
| **control** | 4.9 | 9.9 | 13 |
| **Phenobarbital (PB)** | 5.5 (112%) | 8.5 (85%) | 11 (85%) |
| **Eugenol** | 5.1 (104%) | 8.6 (87%) | 11 (85%) |
| **Eugenol + PB** | 6 (122%) | 7.9 (80%) | 10 (77%) |

*Statistical analysis not performed*

**Neoplastic incidences**: One pulmonary adenoma (3%), two thymic lymphomas (7%) and one mammary adenoacanthoma (3%) were found in animals given eugenol without phenobarbital, while control mice without phenobarbital treatment had one pulmonary adenoma (3%). Phenobarbital-treated controls also had one case each of pulmonary adenoma and liver haemangioendotheliosarcoma. All these findings did not display statistically significant results (table B.6.5.3/3).

**Table B.6.5.3/3. Neoplastic incidences of mice**

| | Neoplastic incidences | | | |
|---|---|---|---|---|
| | **control** | **Phenobarbital (PB)** | **Eugenol** | **Eugenol + PB** |
| **Pulmonary adenoma** | 1/30 (3%) | 1/30 (3%) | 1/30 (3%) | 0 |
| **Haemangioendotheliosarcoma** | 0 | 1/30 (3%) | 0 | 0 |
| **Thymic lymphoma** | 0 | 0 | 2/30 (7%) | 0 |
| **Mammary adenoacanthoma** | 0 | 0 | 1/30 (3%) | 0 |

*Statistically significant differences not found*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

No effects were observed in this study; in all cases the NOAEL was the highest dose tested. The EU-agreed endpoint is: no carcinogenic potential in humans.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is non-GLP compliant and presents several deviations, as using only one dose level and the lack of haematology, urinalysis, clinical chemistry, organ weights data and a complete histopathology examination, so that an overall evaluation of both chronic toxicity and carcinogenicity performance of eugenol, cannot be carried out. Therefore, this study is considered acceptable as supportive.

Administration of eugenol at 0.5% (equivalent to 750 mg/kg bw/day) to CD-1 mice showed a slight increase incidence of thymic lymphomas and mammary gland adenocanthomas compared with controls. However, these incidences were low and did not display statistical significance. These findings reported in the eugenol treated group were not reproduced in the further two-year study in mice, therefore, they were considered not relevant for overall long-term toxicity assessment. **A NOAEL** cannot be established based on the increased incidences of thymic lymphomas and mammary gland adenocanthomas at single dose tested (750 mg/kg bw/day).

## B.6.6. Reproductive toxicity

*For the assessment of the reproductive toxicity potential of eugenol, no multi-generation studies had been provided. Regarding developmental toxicity, four studies conducted with the botanic substance clove oil in rats, rabbits, mice and hamsters, have been submitted. These four studies were previously included in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC. Furthermore, two additional developmental toxicity studies conducted with eugenol (one in rat and one in rabbit), that were previously evaluated and accepted in the Conclusion on the peer review of eugenol (EFSA Journal 2012;10(11):2914), have been presented to address this point as part of the renewal assessment of eugenol.*

### B.6.6.1. Generational studies

No studies submitted.

**Assessment and conclusion by applicant:**

Reproductive toxicity studies are not available for eugenol. Eugenol had no effects on reproductive organs in chronic studies in rats and mice. Eugenol forms part of a normal diet due to its presence in fruits, vegetables, herbs and spices; it is also approved for use as a food additive and has GRAS (generally recognised as safe) status in the USA. Eugenol is also used in medicines, cosmetics and consumer products. Due to the widespread human exposure without significant effects or concern, further *in vivo* testing is not considered necessary and has not been performed.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** No multi-generation studies were presented. RMS agrees with EFSA conclusion that, based on the available data, it was not considered necessary (*EFSA Journal 2012;10(11):2914, and "Peer review report on eugenol, October 2012"*). Additionally, a multi-generational study performed with isoeugenol was available in the public US EPA Health & Environmental Research Online (HERO)- National Technical Reports Library database. Study ID: 5923934. https://hero.epa.gov/hero/index.cfm/reference/details/reference_id/5923934, and consequently, this study was included by RMS in order to completeness as supporting information for the renewal process of the active substance eugenol.

The study was performed with isoeugenol and it was included by the applicant in the renewal dossier of the active substance clove oil, in order to be used in a read-across approach to eugenol (which is the main component of clove oil). Spain, as RMS of clove oil DRAR, did not find enough justification in this read-across and this point of view was included in the recently submitted DRAR of clove oil. However, in anticipation of a hypothetical acceptance of such read-across during the clove oil renewal process, the RMS has added the study and the conclusion drawn to the eugenol DRAR.

#### B.6.6.1.1. Two-generation reproductive toxicity study in rats with isoeugenol.

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2002 |
| **Report title** | Isoeugenol: Reproductive assessment by continuous breeding when administered to Sprague Dawley rats by gavage. |
| **Report No** | TherImmune Research Corporation Study No. 7244-203. NTP-RACB-97-004. |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Non stated. The study was checked for compliance with OECD Guideline 416 (2001). |
| **Deviations from current test guideline OECD TG 416 (2001)** | -The length of P animals premating period was only one week, instead of 10 weeks (one complete spermatogenic cycle for males and several oestrus cycles for females).<br>-Food consumption measurements were not performed weekly.<br>-Food consumption measurements were not performed in the premating periods of F1 generation.<br>-Pups bodyweights were not measured during lactation period. |

| | |
|---|---|
| | -No of implantations, corpora lutea and post-implantation loss were not analysed.<br>-Brain, thyroid, pituitary and adrenal glands weights were not measured.<br>-Statistical analysis not performed in histopathological findings. |
| **Previous evaluation** | Provided by RMS for the purpose of renewal. |
| **GLP** | Yes |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Isoeugenol; Lot/Batch No.: 46928, CAS No.: 97-54-1, Purity: 99.1% |
| **Test system** | Male and Female Sprague-Dawley (CD® –Crl : (SD) BR) rats. |
| **Test method** | Groups of 20 adult male and female Sprague-Dawley rats were exposed daily to 0, 70, 230, or 700 mg/kg bw/day of isoeugenol *via* oral gavage. The corresponding formulations were prepared on corn oil. The $F_0$ cohabitation period began on study day 8. During the mating period, three litters ($F_{1a}$, $F_{1b}$, and $F_{1c}$) were produced. $F_{1c}$ litters were further used as parental $F_2$ generation.<br><br>In parental $F_0$ animals, bodyweights were collected weekly and food consumption was measured during weeks 1, 6, 12 (males only), and 14 (males only). During the lactation period for the $F_{1c}$ litters, bodyweights of $F_0$ parental females were measured on PND 1, 4, 7, 14, and 21, and food consumption was measured on PND 1-4, 4-7, 7-11, 11-14, 14-18, and 18-21. Physical examinations were performed weekly. Post dose observations were performed at 15, 30, 60, and 120 minutes in parental animals, and at 15 and 30 minutes in pups.<br><br>The three litters produced ($F_{1a}$, $F_{1b,}$ and $F_{1C}$) were counted, and examined for external malformations at PND1. Only $F_{1c}$ litters were weighed at PND 21. After PND1, $F_{1a}$ and $F_{1b}$ litters were euthanized by sodium phenobarbital overdose and discarded without necropsy.<br><br>The $F_{1c}$ litters were reared by the dam until weaning on PND 21. On PND 1, 4, 7, 14, and 21, the pups were counted, weighed, and examined for external malformations (PND 1 only). AGD was measured in the three $F_1$ litters on PND 1. Observations of testicular descent (starting on PND 16), vaginal opening (starting on PND 25), and preputial separation (starting on PND 35) were conducted. Upon completion of the lactation phase (at least 4 days after the last weaning), vaginal cytology was conducted for 14 days on the P dams.<br><br>On PND 16, two males and two females of the $F_{1c}$ litters were randomly selected for rearing subsequent generation. On PND 21, selected $F_{1c}$ animals for the cohabitation were housed separately from the dam and were exposed to the first dose of isoeugenol. The remaining pups were euthanized and discarded without necropsy.<br><br>$F_{1c}$ weanlings selected for the $F_1$ cohabitation were reared in same sex groups until PND 81±10 days of age, when 20 animals of each sex in each dose group were randomly assigned to breeding pairs (avoiding sibling mating), and cohabited for 9 weeks (or 14 days after the delivery of the $F_{2b}$, pups). Once cohabited, weekly physical examinations and bodyweights were obtained and feed consumption was measured during weeks 1, 3, 6, 12 (males only), and 14 (males only), after weaning.<br><br>Three litters ($F_{2a}$, $F_{2b}$, and $F_{2c}$) were obtained. On PND 1, the $F_{2a}$, $F_{2b}$, and $F_{2c}$, pups were counted, weighed, and examined for external malformations. Four (2/sex, when available) randomly selected pups had AGD measured. Then, the pups were euthanized and discarded without necropsy.<br><br>Vaginal cytology was conducted on the $F_{1c}$ dams for 14 days beginning at least 4 days after delivery of the last $F_{2c}$ litters. Following delivery of the $F_{2c}$ pups, $F_{1c}$ males and females were necropsied. Terminal bodyweights and organ weights were obtained, sperm analysis was conducted in males and tissues were saved.<br><br>Due to a decrease in the number of males in the F, pups, a one-week outbreeding cohabitation was performed on the control and high-dose $F_0$ animals (up to 20 |

| | |
|---|---|
| | animals/sex/group) in order to determine the affected sex. The outbreeding cohabitation was initiated on the day after completion of the vaginal cytology sample collection. $F_0$ males and females from each group were paired with naive animals **(**1:1 ratio). All F0 animals were maintained on the same dosing regimen throughout cohabitation. The animals were separated upon confirmation of vaginal sperm or after **7** days of cohabitation. Bodyweights and physical examinations of the $F_0$, animals were collected weekly, at cohabitation, and at littering (bodyweights only). Naive animals had bodyweights collected at cohabitation, at littering, and on weeks 4 and **5** of the outbreeding period (naive females only). Naive animals were examined weekly for clinical signs until necropsy. On PND 1, pups were counted, weighed, and examined for external malformations. Four (2/sex, when available) randomly selected pups had AGD measured. The pups were euthanized by sodium pentobarbital overdose and discarded without necropsy. After the last female littered, the naive animals were weighed and euthanized by carbon dioxide asphyxiation. The naive males were discarded without necropsy. A complete uterine examination was performed on the naive females and included the number of implantation sites. No tissues were preserved from the naive females.<br><br>The following organ weights were obtained: kidneys, liver, testis (right), epididymis (right), ventral prostate, dorsolateral prostate, cauda epididymis (right), seminal vesicles/coagulating glands, uterus and spleen.<br><br>The following tissues were subjected to histopathological examinations: Adrenals, cervix, liver, kidneys, spleen, left testis, left epididymis, ovaries, dorsolateral prostate, seminal vesicles/coagulating, stomach, vagina and uterus.<br><br>The carcasses will be discarded without necropsy after data collection on PND 21. |
| **Statistical analysis of results** | Most hypotheses (including but not limited to pup weights, body weights, food consumption, organ weights, sperm parameters, and cycle length) are tested using the nonparametric multiple comparisons procedure of Dunn (1964) or Shirley (1977), as modified by William (1986). Shirley's test was designed to detect treatment-related differences when the response to treatment consistently increased (or decreased) with increasing dose. Dunn's test was more appropriate whether the departure from monotonicity was severe.<br><br>Jonckheere's test (1954) was used to ascertain whether there was sufficient evidence of a dose-related response to apply Shirley's test. If the p-value from Jonckheere's test was less than 0.01, Shirley's test was used; otherwise, Dunn's test was applied.<br><br>For data expressed as a proportion, such as number fertile/number cohabitated, the Cochran-Armitage test (Armitage, 1971) was used to 'test for a dose-related trend, and pairwise comparisons were performed using a chi-square test (Conover, 1971).<br><br>The ratio of the number of pups born alive to the total number of pups carried to full term was computed for all fertile pairs. The sex ratio, expressed as the proportion of males, was computed for all fertile pairs with at least one live pup. Shirley's or Dunn's test was used to compare dosed groups to controls based on Jonckheere's test, as described above.<br><br>Since the number of pups in a litter may influence the average pup weight, a parametric analysis of covariance (Neter and Wasserman, 1974) was used to test overall equality in average pup weight, after adjustment for average litter size. The covariant used was average litter size, including live and dead pups. Least squares estimates of dose group means adjusted for litter size were computed and tested for overall equality using an F-test. Pairwise comparisons were performed using Dunnett's test (1955).<br><br>To examine potential differences in treatment effects on males and females, number of male pups, number of female pups, and total number of pups in litters in treated groups were compared to controls.<br><br>Feed consumption data were analysed as g/animal/day and g1kg body weight/day. Both absolute organ weights and organ weights relative to body weight were analysed. |

| | |
|---|---|
| | The relative time spent in estrous cycle stages was analysed using a multivariate analysis of variance (Wilks criterion) to test for the simultaneous equality of measurements across dose levels (Morrison, 1976). Before applying the test, an arcsine transformation was performed to bring the data into closer conformance with normality assumptions. |
| **Historical control data (HCD)** | HCD are not provided in the study report. |

**Results**

**P animals**

**Clinical signs and mortality:** No relevant clinical signs were noted in the treated groups compared with controls. Only urine stains were increased in both sexes compared with controls (*males*: 20%, 10% and 25% for low, mid and high dose groups vs 5% in controls; *females*: 20%, 35% and 55% low, mid and high dose groups vs 15% in controls).

Mortality was observed in four P males and ten P females:

*Males*:

-One 70 mg/kg bw/day male was killed moribund on starting day (SD) 138 because of severe clinical signs including dyspnoea, rales, few faeces, rough hair coat, and languid behaviour.

-Three males (one 70 mg/kg bw/day, one 230 mg/kg/day, and one 700 mg/kg bw/day) were found dead on SD179, SD 10, and SD 154, respectively. Each animal appeared normal prior to death.

*Females*:

-Two 230 mg/kg bw/day females were found dead, one on SD 114 and one on SD 133. One female appeared normal prior to death. The other female apparently died as a result of gavage error.

- Eight 700 mg/kg/day females were found dead. Two deaths appear to be the result of gavage errors. Three deaths occurred due to parturition difficulties. Three females appeared normal prior to their deaths.

**Bodyweight:** Bodyweights were statistically significant decreased in the mid (3-8%) and high (10-18%) male dose groups throughout weeks 2-26.

In female dose groups, a statistically significant decrease was observed in the low dose group (9-20%) throughout weeks 21-26. In the mid dose female group, a significant decrease (6-21%) was recorded during weeks 11, 12, and 20-26, whereas in the high dose group, a significant decrease (4-12%) was observed throughout weeks 2-7, 9-14, and 17-26, respectively (Table B.6.6.1/1).

**Table B.6.6.1/1. Bodyweights of P animals**

| | Males dose groups (Mean ± SE) | | | | Females dose groups (Mean ± SE) | | | |
|---|---|---|---|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| 1 | 387.3 ± 2.54 | 385.7 ± 3.06 | 385.4 ± 3.56 | 380.5 ± 2.91 | 254 ± 3.44 | 255.3 ± 2.28 | 250.6 ± 2.71 | 250.5 ± 2.57 |
| 2 | 425.7 ± 3.73 | 424.1 ± 4.16 | ***** 411.6 ± 6.09 (↓3%)** | ***** 381.3 ± 5.02 (↓10%)** | 267.9 ± 4.15 | 269.3 ± 3.26 | 261.8 ± 2.45 | **** 256.3 ± 2.66 (↓4%)** |
| 3 | 446.3 ± 4.12 | 440.9 ± 5.38 | ***** 434 ± 6.11 (↓3%)** | ***** 401.6 ± 4.36 (↓10%)** | 296.6 ± 4.16 | 291.3 ± 3.36 | 289.8 ± 2.64 | **** 281 ± 2.93 (↓5%)** |
| 4 | 480.4 ± 5.04 | 473 ± 6.48 | ***** 461.6 ± 7.33 (↓4%)** | ***** 429.3 ± 5.23 (↓11%)** | 333 ± 4.31 | 323.8 ± 4.25 | 323.2 ± 3.57 | ***** 310.9 ± 3.66 (↓7%)** |
| 5 | 503.2 ± 5.77 | 500.6 ± 7.52 | ***** 485.3 ± 8 (↓4%)** | ***** 443.3 ± 5.44 (↓12%)** | 409.5 ± 7.03 | 394.6 ± 8.12 | 394.8 ± 7.57 | ***** 364.8 ± 7.72 (↓11%)** |
| 6 | 521.5 ± 7.12 | 513.2 ± 8.01 | ***** 497 ± 8.62 (↓5%)** | ***** 459.3 ± 6.16 (↓12%)** | 340.7 ± 6.04 | 331.6 ± 5.78 | 336.1 ± 5.49 | *** 319.5 ± 5.07 (↓6%)** |
| 7 | 547.7 ± 7.67 | 537.5 ± 9.02 | ***** 520.6 ± 8.91 (↓5%)** | ***** 486.8 ± 6.62 (↓11%)** | 368.9 ± 5.82 | 359.3 ± 6.31 | 353 ± 4.9 | **** 350.8 ± 8.99 (↓5%)** |
| 8 | 553 ± 8.26 | 549.9 ± 9.05 | 541.2 ± 10.18 | ***** 499.5 ± 7.61 (↓10%)** | 395.9 ± 7.09 | 390.7 ± 10.44 | 394.8 ± 7.97 | 382.3 ± 8.92 |
| 9 | 576.8 ± 8.46 | 565.4 ± 9.36 | ***** 550.5 ± 10.46 (↓5%)** | ***** 505.2 ± 7.02 (↓12%)** | 392.5 ± 8.43 | 372.4 ± 9.32 | 371.8 ± 7.36 | *** 363.8 ± 8.54 (↓7%)** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 10 | 602.1 ± 9.89 | 585 ± 9.87 | ***** 561.7 ± 9.37 (↓7%)** | ***** 521.5 ± 7.32 (↓13%)** | 399.5 ± 9.7 | 381 ± 7.15 | 386.8 ± 11.26 | ***** 362 ± 9.41 (↓9%)** |
| 11 | 615.1 ± 10.24 | 597.4 ± 10.45 | ***** 576.3 ± 10.19 (↓6%)** | ***** 539.4 ± 7.39 (↓12%)** | 424.1 ± 7.32 | 410.7 ± 8.25 | ***** 397.6 ± 7.36 (↓6%)** | ***** 382.1 ± 7.57 (↓10%)** |
| 12 | 633.1 ± 10.33 | 617 ± 10.38 | ***** 590.4 ± 10.78 (↓7%)** | ***** 542.7 ± 8.08 (↓16%)** | 461.8 ± 16.45 | 439.5 ± 14.58 | **** 411 ± 9.68 (↓11%)** | **** 407.7 ± 11.27 (↓12%)** |
| 13 | 643.6 ± 10.74 | 625 ± 10.63 | ***** 603.4 ± 11.13 (↓6%)** | ***** 550.9 ± 8.81 (↓16%)** | 424.1 ± 9.07 | 400.7 ± 7.89 | 406.3 ± 10.05 | **** 381.7 ± 9.25 (↓10%)** |
| 14 | 657.4 ± 10.68 | 633.9 ± 10.34 | ***** 616.1 ± 1.35 (↓6%)** | ***** 564.4 ± 8.9 (↓16%)** | 404.9 ± 6.89 | 404.9 ± 6.33 | 388.1 ± 6.54 | **** 379.2 ± 8.02 (↓6%)** |
| 15 | 673.7 ± 11.36 | 650.2 ± 11.35 | ***** 627.2 ± 12.53 (↓7%)** | ***** 569.6 ± 8.96 (↓15%)** | 389.1 ± 8.44 | 383.5 ± 8.28 | 375 ± 7.27 | 376.8 ± 5.53 |
| 16 | 681.2 ± 11.66 | 652.8 ± 11.46 | ***** 633.7 ± 12.57 (↓7%)** | ***** 571.9 ± 10.57 (↓16%)** | 371.5 ± 8.45 | 359.5 ± 6.67 | 355.4 ± 6.07 | 348.5 ± 7.59 |
| 17 | 696.3 ± 12.31 | 663.7 ± 12.26 | ***** 645.4 ± 12.34 (↓7%)** | ***** 576.8 ± 10.79 (↓17%)** | 387.1 ± 8.42 | 375.1 ± 5.89 | 365.2 ± 6.22 | **** 357.9 ± 7.47 (↓8%)** |
| 18 | 700.8 ± 12.39 | 665.4 ± 12.71 | ***** 644.8 ± 13.17 (↓8%)** | ***** 583.3 ± 10.86 (↓17%)** | 392.7 ± 8.66 | 375.8 ± 5.04 | 373.9 ± 6.48 | **** 361.6 ± 8.48 (↓8%)** |
| 19 | 699.4 ± 14.22 | 674.3 ± 12.99 | ***** 656.1 ± 13.36 (↓6%)** | ***** 588.9 ± 8.99 (↓16)%** | 397.9 ± 9.06 | 381.2 ± 5.82 | 377.5 ± 6.84 | **** 361.3 ± 8.65 (↓9%)** |
| 20 | 707.3 ± 13.39 | 683.6 ± 13.18 | ***** 657.0 ± 14.03 (↓7%)** | ***** 594.3 ± 10.27 (↓16)%** | 400.3 ± 9.18 | 381.8 ± 6 | **** 375.3 ± 7.59 (↓6%)** | **** 360.7 ± 9.01 (↓10%)** |
| 21 | 713.2 ± 13.74 | 695.8 ± 15.29 | ***** 667.3 ± 13.78 (↓6%)** | ***** 588.8 ± 11.21 (↓17%)** | 418.4 ± 8.78 | ***** 381.8 ± 6.33 (↓9%)** | ***** 379.1 ± 6.93 (↓9%)** | ***** 375 ± 9.45 (↓10%)** |
| 22 | 726.3 ± 14.2 | 709.5 ± 15.06 | ***** 683.1 ± 13.97 (↓6%)** | ***** 599 ± 10.95 (↓18%)** | 442.5 ± 10.01 | ***** 388.8 ± 6.45 (↓12%)** | ***** 384.4 ± 6.83 (↓13%)** | ***** 394.1 ± 8.84 (↓11%)** |
| 23 | 739.2 ± 14.68 | 718.6 ± 15.02 | ***** 692.5 ± 14.71 (6%)** | ***** 605.4 ± 12.51 (↓19%)** | 491.3 ± 14.64 | **** 392.6 ± 6 84 (↓20%)** | **** 386.4 ± 6.87 (↓21%)** | **** 437.4 ± 14 (↓11%)** |
| 24 | 743.6 ± 15.78 | 724.7 ± 15.12 | ***** 698.3 ± 14.99 (↓6%)** | ***** 617.1 ± 12.9 (↓17%)** | 439 ± 11.71 | ***** 394.8 ± 7 2 (↓10%)** | ***** 389.8 ± 7.49 (↓11%)** | ***** 386.6 ± 11.06 (↓12%)** |
| 25 | 764.5 ± 16.26 | 733.8 ± 15.79 | ***** 709.2 ± 16.02 (↓7%)** | ***** 627.9 ± 13.57 (↓18%)** | 444.8 ± 12.7 | **** 396.4 ± 7.42 (↓11%)** | **** 394.8 ± 7.71 (↓11%)** | **** 391.2 ± 12.86 (↓12%)** |
| 26 | 778 ± 16.73 | 744.6 ± 16.75 | ***** 721.3 ± 16.4 (↓7%)** | ***** 637.7 ± 13.86 (↓18%)** | 449.6 ± 12.51 | **** 406.4 ± 8.63 (↓10%)** | **** 398.7 ± 8.12 (↓11%)** | **** 401 ± 13.3 (↓11%)** |

**p<0.05; **p<0.01; ***p<0.001*

**<u>Food consumption</u>:** In high dose male group, absolute and relative food consumption was statistically significantly lower at week 1 (27% and 25%, respectively) and 14 (12%), however, an increase in relative food consumption was observed at week 12 (9%). On the other hand, both absolute and relative food consumption was decreased at week 1 in mid and high female dose groups (8% and 20%, respectively) (see Table B.6.6.1/2).

**Table B.6.6.1/2. Food consumption of P animals**

| **P generation : Food consumption (g/animal/day) (Mean ± SE)** | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **Males** | | | | |
| 1 | 25.86 ± 0.53 | 26.02 ± 0.52 | 24.02 ± 0.94 | *****18.96 ± 0.75 (↓27%)** |
| 12 | 22.39 ± 0.52 | 20.66 ± 0.53 | 20.63 ± 0.44 | 20.89 ± 0.49 |
| 14 | 22.13 ± 0.50 | 21.81 ± 1.86 | 21.30 ± 0.64 | ***19.42 ± 1.03 (↓12%)** |
| **Females** | | | | |
| 1 | 17.32 ± 0.49 | 17.58 ± 1.22 | ***** 15.89 ± 0.40 (↓8%)** | ***** 13.88 ± 0.32 (↓20%)** |
| **Males and females** | | | | |
| 6 | 21.09 ± 0.37 | 20.53 ± 0.35 | 20.44 ± 0.38 | 20.20 ± 0.41 |

| **P generation : Food consumption (g/Kg bw/day) (Mean ± SE)** | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |

| | | | | |
|---|---|---|---|---|
| **Males** | | | | |
| 1 | 66.70 ± 1.04 | 67.44 ± 1.19 | 62.26 ± 2.06 | ***** 49.69 ± 1.71 (↓25%)** |
| 12 | 35.36 ± 0.54 | 33.54 ± 0.81 | 35.00 ± 0.63 | **** 38.51 ± 0.72 (↑9%)** |
| 14 | 33.67 ± 0.58 | 34.13 ± 2.49 | 34.59 ± 0.90 | 34.43 ± 1.72 |
| **Females** | | | | |
| 1 | 68.26 ± 1.82 | 68.60 ± 4.30 | ***** 63.31 ± 1.82 (↓8%)** | ***** 55.52 ± 1.40 (↓20%)** |
| **Males and females** | | | | |
| 6 | 48.97 ± 0.78 | 48.65 ± 0.75 | 49.14 ± 1.02 | 51.67 ± 0.74 |

**p<0.05; **p<0.01; ***p<0.001*

**Necropsy**

The following non-treatment related findings were observed during necropsy examinations:

*Males*

-Dorsal ulcer in the body surface in the high dose group (10% vs 0% in controls).

-Right thorax ulcer in the body surface in the low dose group (10% vs 0% in controls).

*Females:*

-Discoloured stomach in the high dose female group (10% vs 0% in controls).

**Organ weights**

The following organ weights were recorded for both sexes in each dose groups (Table B.6.6.1/3).

*Males:*
- Decrease terminal bodyweight in high dose male group (19%).
- Decrease (26%) of the absolute spleen weight in the high male dose group.
- Increase in mean relative organ weights in the high dose group:
  .Right epididymis (14%)
  .Kidney (14%)
  .Right testis (19%)

*Females*:
-Decreased terminal bodyweight in all the dose groups (11%, 10%, and 13% for the low, mid and high dose groups, respectively).
- Decrease in absolute ovary weight in mid dose group (29%).
- Increase in relative liver weight (10%, 14%, and 20% for the low, mid and high dose groups, respectively).
- Increase in relative kidney weight in the low and high dose groups (10% and 14%, respectively).

**Table B.6.6.1/3. Male and female organ weights of P animals**

| | | P: Male organ weights (Mean ± SE) | | | | P: Female organ weights (Mean ± SE) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Dose groups | | | | Dose groups | | | |
| | | 0 mg/kg bw/day | 70 mg/kg bw/day | 230 mg/kg bw/day | 700 mg/kg bw/day | 0 mg/kg bw/day | 70 mg/kg bw/day | 230 mg/kg bw/day | 700 mg/kg bw/day |
| **BODY (g)** | | 784.5 ± 16.97 | 769.7 ± 25.87 | 730.6 ± 29.56 | *****635.5 ± 21.74 (↓19%)** | 447.4 ± 12.54 | ****398.5 ± 12.58 (↓11%)** | ****401.3 ± 7.86 ) (↓10%)** | ****389.4 ± 13.1 (↓13%)** |
| **LIVER (g)** | Absolute | 28.04 ± 0.73 | 27.70 ± 1.09 | 27.34 ± 1.58 | 24.77 ± 0.92 | 15.34 ± 0.5 | 15.12 ± 0.64 | 15.78 ± 0.57 | 16.03 ± 0.6 |
| | Relative | 35.8 ± 0.69 | 36.12 ± 1.25 | 37.43 ± 1.44 | 39.03 ± 0.95 | 34.38 ± 0.76 | *****37.85 ± 0.66 (↑10%)** | ***** 39.25 ± 0.83 (↑14%)** | *****41.20 ± 0.87 (↑20%)** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **KIDNEYS (mg)** | Absolute | 4901 ± 116.78 | 4678.7 ± 112.82 | 4764.9 ± 133.23 | 4510 ± 147.98 | 2774.4 ± 58.21 | 2720.1 ± 49.19 | 2642.2 ± 80.34 | 2759.7 ± 100.96 |
| | Relative | 6.26 ± 0.12 | 6.11 ± 0.15 | 6.56 ± 0.17 | ****7.12 ± 0.18 (↑14%)** | 6.26 ± 0.16 | ****6.87 ± 0.18 (↑10%)** | 6.58 ± 0.13 | ****7.13 ± 0 26 (↑14%)** |
| **SPLEEN (mg)** | Absolute | 1033.8 ± 53.79 | 970.7 ± 55.46 | 990 ± 49.5 | **** 760.90 ± 52.76 (↓26%)** | 710.95 ± 18.54 | 629.7 ± 34.04 | 649 ± 35.06 | 663.3 ± 30.61 |
| | Relative | l. 33 ± 0.07 | 1.26 ± 0.06 | l. 36 ± 0.04 | 1.2 ± 0.08 | 1.6 ± 0.04 | 1.59 ± 0.07 | 1.62 ± 0.08 | l.71 ± 0.07 |
| **DORSOLATERAL PROSTATE (g)** | Absolute | 0.64 ± 0.03 | 0.59 ± 0.03 | 0.63 ± 0.04 | 0.6 ± 0.07 | | | | |
| | Relative | 0.82 ± 0.04 | 0.78 ± 0.05 | 0.87 ± 0.05 | 0.96 ± 0.11 | | | | |
| **VENTRAL PROSTATE (g)** | Absolute | 0.74 ± 0.03 | 0.65 ± 0.05 | 0.66 ± 0.07 | 0.65 ± 0.04 | | | | |
| | Relative | O. 96 ± 0.05 | 0.85 ± 0.06 | 0.91 ± 0.1 | 1.02 ± 0.06 | | | | |
| **RIGHT CAUDA EPIDIDYMIS (mg)** | Absolute | 372.6 ± 9.25 | 361.8 ± 13.8 | 383.18 ± 11.58 | 338.5 ± 14.29 | | | | |
| | Relative | 0.48 ± 0.02 | 0.48 ± 0.03 | 0.53 ± 0.02 | 0.54 ± 0.03 | | | | |
| **RIGHT EPIDIDYMIS (mg)** | Absolute | 867.75 ± 19.43 | 894.2 ± 34.12 | 850.6 ± 31.76 | 803 ± 23.36 | | | | |
| | Relative | 1.11 ± 0.03 | 1.17 ± 0.06 | 1.18 ± 0.05 | ****1.27 ± 0.05 (↑14%)** | | | | |
| **SEMINAL VESICLES (mg)** | Absolute | 1876.5 ± 91.85 | 1767.4 ± 157.08 | 1914.2 ± 100.96 | 1888.2 ± 139.07 | | | | |
| | Relative | 2.42 ± 0.14 | 2.32 ± 0.24 | 2.67 ± 0.19 | 2.97 ± 0.21 | | | | |
| **RIGHT TESTIS (mg)** | Absolute | 2030.5 ± 49.16 | 1988 5 ± 48.78 | 2056.5 ± 41.86 | 1962.2 ± 43.53 | | | | |
| | Relative | 2.61 ± 0.08 | 2.61 ± 0.12 | 2.86 ± 0.14 | ****3.11 ± 0.11 (↑19%)** | | | | |
| **OVARIES (mg)** | Absolute | | | | | 0.14 ± 0.01 | 0.12 ± 0.01 | ***0.10 ± 0.01 (↓29%)** | 0.15 ± 0.01 |
| | Relative | | | | | 0.32 ± 0.02 | 0.3 ± 0.03 | 0.25 ± 0.03 | 0.38 ± 0.02 |
| **UTERUS (mg)** | Absolute | | | | | 1265.5 ± 79.47 | 1269.7 ± 59.9 | 1229.7 ± 61.91 | 1195.6 ± 62.49 |
| | Relative | | | | | 2.86 ± 0.18 | .23 ± 0.21 | 3.08 ± 0.18 | 3.10 ± 0 18 |

**p<0.05; **p<0.01; ***p<0.001*

## Histopathology

Histopathological analysis of the P animals revealed dose-related changes in the non-glandular stomachs. These observations were described in all the dose level of both sexes, and their incidence and/or severity was related to dose (Table B.6.6.1/4). These alterations consisted of hyperkeratosis and hyperplasia of the squamous epithelium (acanthosis) and were characterized by an increased thickness of the non-keratinized (hyperplasia) and keratinized (hypkeratosis) layers of the epithelium. All other tissues microscopic changes were commonly detected in laboratory rats and were unrelated to treatment.

**Table B.6.6.1/4. Histopathological stomach findings in P animals**

| | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| **Group (mg/kg bw/day)** | **0** | **70** | **230** | **700** | **0** | **70** | **230** | **700** |
| **number examined** | **10** | **10** | **10** | **10** | **10** | **10** | **10** | **10** |
| **Hyperkeratosis** | 0 | 4 (40%) | 9 (90%) | 8 (80%) | 0 | 6 (60%) | 7 (70%) | 10 (100%) |
| *Minimal* | 0 | 3 (30%) | 1 (10%) | 3 (30%) | 0 | 3 (30%) | 1 (10%) | 4 (40%) |
| *Mild* | 0 | 1 (10%) | 6 (60%) | 2 (20%) | 0 | 3 (30%) | 4 (40%) | 0 |
| *M0derate* | 0 | 0 | 2 (20%) | 3 (30%) | 0 | 0 | 2 (20%) | 6 (60%) |
| **Hyperplasia** | 0 | 3 (30%) | 9 (90%) | 7 (70%) | 0 | 5 (50%) | 7 (70%) | 9 (90%) |
| *Minimal* | 0 | 2 (20%) | 8 (80%) | 2 (20%) | 0 | 5 (50%) | 5 (50%) | 3 (30%) |
| *Mild* | 0 | 1 (10%) | 1 (10%) | 3 (30%) | 0 | 0 | 2 (20%) | 5 (50%) |
| *Moderate* | 0 | 0 | 0 | 2 (20%) | 0 | 0 | 0 | 1 (10%) |

**Sperm analysis:** Computer-assisted sperm motion analysis using Hamilton Thorne Integrated Visual Optic System revealed no changes in mean path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, motile percentage, and progressive percentage of the treated groups as compared to the control. Also, no changes were seen in epididymal sperm density, percent abnormal sperm, number of spermatids per cauda epididymis, and the total number of spermatids per testis (see Table B.6.6.1/5-6).

**Table B.6.6.1/5. Sperm analysis in P males**

| | Dose group (Mean ± SE) | | | |
|---|---|---|---|---|
| | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **COMPUTER ASSISTED SPERM ANALYSIS (VAS DEFERENS)** | | | | |
| EPIDIDYMAL SPERM DENSITY (a) | 399.13 ± 14.15 | 401.73 ± 23.73 | 393.10 ± 20 .62 | 408.49 ± 24.47 |
| EPIDIDYMAL SPERM MORPHOLOGY( % ABNORMAL) (b) | 0.43 ± 0 .10 | | | 0.65 ± 0.11 |
| SPERMATID/mg TESTIS (c) | 132.53 ± 4 .58 | | | 152.68 ± 10.09 |
| TOTAL SPERMATID/TESTIS (d) | 254.47 ± 6 .53 | | | 267.13 ± 12.35 |
| TOTAL SPERMATID/CAUDA (d) | 139.85 ± 6.38 | 136.13 ±7.83 | 130.75 ± 7 .44 | 134.53 ± 6.25 |

*(a) Sperm density expressed as 1000 sperm per mg right caudal tissue.*
*(b) Dose group means and standard errors are computed only from samples with at least 200 sperm.*
*(c) Spermatid heads per mg testis (x 1000) = (total number of spermatid heads per right testis) I (right testis weight in mg)*
*(d) Total number of spermatid heads (x 1000000) = (average number of spermatid heads x 2.5 x 100) / 0.0001*

**Table B.6.6.1/6. Summary of Hamilton-Throne sperm analysis data in P males**

| | Dose group (Mean ± SE) | |
|---|---|---|
| | 0 mg/kg bw/day | 700 mg/kg bw/day |
| COMPUTER ASSISTED SPERM ANALYSIS (VAS DEFERENS) | | |
| BT/CS FREQ (hz/sec) | 17.38 ± 0.43 | 17.41 ± 0.67 |
| LATERAL AMPLITUDE (um) | 16.62 ± 0.31 | 16.57 ± 0.62 |
| LINEARITY | 34.45 ± 0.55 | 34.4 ± 0 9 |
| MOTILE PERCENTAGE | 94.1 ± 0.52 | 94.6 ± l.01 |
| PROGRESSIVE VELOCITY | 91.49 ± 2.45 | 90.35 ± 3 59 |
| PATH VELOCITY | 159.23 ± 3.4 | 156.24 ± 4.84 |
| STRAIGHTNESS | 55.95 ± 0.6 | 56.3 ± 1.16 |
| TRACK SPEED | 275.8 ± 6.04 | 271 ± 9.6 |

**Oestrus cycle:** Vaginal cytology was performed on $F_0$ dams for 14 days after weaning of the $F_1$c litters. No changes were revealed in the number of females with regular cycles, cycle length, number of cycles, and in number of cycling females across the dose groups as compared to the control females. There was no evidence that any treated females differed from the control females in the relative amount of time spent in estrous stages (see Table B.6.6.1/7).

**Table B.6.6.1/7. Summary of stages and length of estrous cycle in P females**

| | Estrous stage % (a) | | | | | Cycle length (days) | Number cycles | No cycling females (b) | No of females W/regular cycle (c) |
|---|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | P | E | M | D | N | Mean±S E. | Mean±S.E. | | |
| 0 | 20 | 29.6 | 21.8 | 28.6 | 0 | 4.12 ± 0.09 | 2.60 ± 0.13 | 20 /20 (95%) | 17 /20 (85%) |
| 70 | 18.2 | 34.6 | 21.4 | 25.7 | 0 | 4.60 ± 0.32 | 2.70 ± 0 13 | 20 /20 (100%) | 17 /20 (85%) |
| 230 | 18.5 | 29.8 | 23 | 28.7 | 0 | 4 .19 ± 0 .13 | 2.58 ± 0 14 | 19 19 (100%) | 16/19 (84%) |
| 700 | 19.7 | 30.6 | 21.8 | 28.0 | 0 | 4 .41 ± 0.15 | 2.57 ± 0 14 | 14 /14 (100%) | 12/14 (86%) |

*a P=Proestrus, E=Estrus, M=Metestrus, D=Diestrus, N=Not Clear, Poor Quality, or Insufficient Number of Cells*

*b Number of cycling females / number of females sampled (percent cycling).*

*c Number of females with a regular cycle / number of females sampled (percent regular).*

**Reproductive performance (P→$F_1$):**

A summary of the main reproductive parameters are listed below (see also Tables B.6.6.1/8-11) :

**700 mg/kg bw/day**

-Decreases of dam bodyweights during lactation (7-13%) of the $F_1$c litter (PND 1, 4, 7, 14).

-Increase in relative food consumption was recorded in high dose lactating dams during the $F_{1c}$ litter throughout PND 1-4 (23%).

-Decrease in the number of live male pups in all $F_1$ litters (21%).

-Decrease in the number of combined live male and female pups in all $F_1$ litters (15%).

-Decrease in the number of live male pups in $F_{1a}$ litter (22%).

-Decrease in the number of combined live male and female pups in $F_{1a}$ litter (16%).

-Decreases in dam bodyweights (8-13%) at delivery of $F_1$ litters ($F_{1a}$, $F_{1b}$ and $F_{1c}$).

-Decrease in absolute female anogenital distance in the $F_{1c}$ litter (8%).

-Increase in the female anogenital distance (AGD) to bodyweight ratio in the $F_{1a}$ litter (10%).

**230 mg/kg bw/day**

-Deceases of AGD/pup bodyweight ratio in females of $F_{1c}$ litter (14%).

**70 mg/kg bw/day**

-Deceases absolute female anogenital distance in $F_{1c}$ litter (7%).

All other reproductive parameters during the P cohabitation phase were comparable to the controls.

**Table B.6.6.1/8. Summary of pregnancy index (P→$F_1$)**

| SUMMARY OF PREGNANCY INDEX OF BREEDING PAIRS | | | | |
|---|---|---|---|---|
| **Litter** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| $F_{1a}$ | 20/20 (100%) | 20/20 (100%) | 20/20 (100%) | 19/20 (95%) |
| $F_{1b}$ | 18/20 (90%) | 19/20 (95%) | 19/19 (100%) | 18/19 (95%) |
| $F_{1c}$ | 17/20 (85%) | 17/20 (85%) | 16/19 (84%) | 16/19 (84%) |

**Table B.6.6.1/9. Dam bodyweight during $F_{1c}$ lactation**

| Dam bodyweight during F1c generation (Mean ± SE) | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| 1 | 418 3 ± 9.48 | 404 3 ± 9.72 | 393.3 ± 4.99 | ***** 364.7 ± 10.64 (↓13%)** |
| 4 | 407.8 ± 8.28 | 400 5 ± 8.37 | 390.6 ± 4.4 | ***** 368.2 ± 9.12 (↓10%)** |
| 7 | 409 1 ± 7.96 | 405.6 ± 8.43 | 396 ± 5.04 | **** 378.8 ± 8.88 (↓7%)** |
| 14 | 396 9 ± 7.94 | 397 2 ± 8.58 | 390.5 ± 3.97 | **** 370.2 ± 7.39 (↓7%)** |
| 21 | 382 1 ± 6.29 | 388 5 ± 6.02 | 368.3 ± 5.05 | 368 ± 5.48 |

**p<0.05; **p<0.01; ***p<0.001*

**Table B.6.6.1/10. Dam food consumption during $F_{1c}$ lactation**

| Dam food consumption (g/kg bw/day) during $F_{1c}$ lactation (Mean ± SE) | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| 1-4 | 58.13 ± 3.44 | 62.64 ± 3.39 | 66.54 ± 5.76 | *** 71.83 ± 4.62 (↑23%)** |
| 4-7 | 92.35 ± 7.37 | 106.29 ± 4 | 101.12 ± 4.79 | 99.32 ± 6.2 |
| 11-14 | 133.44 ± 12 37 | 139 .42 ± 11.99 | 120.11 ± 12.75 | 117.68 ± 14.66 |
| 18-21 | 224.81 ± 10.06 | 227.5 ± 7.87 | 221.62 ± 15.43 | 232.01 ± 10 |

**p<0.05*

**Table B.6.6.1/11. Reproductive performance during F1 generation (P→$F_1$)**

| | | Dose groups mg/kg bw/day | | | |
|---|---|---|---|---|---|
| | | 0 | 70 | 230 | 700 |
| **Average litters per pair** | **All $F_1$ litters** | 2.8 ± 0.14 | 2.8 ± 0.12 | 2.8 ± 0.12 | 2.7 ± 0.18 |
| **Live pups per litter** | | | | | |
| **All $F_1$ litters** | Male | 7.2 ± 0.28 | 7.1 ± 0.30 | 6.6 ± 0.41 | *** 5.7 ± 0.43 (↓21%)** |
| | Female | 7.2 ± 0.40 | 6.9 ± 0.33 | 7.0 ± 0.37 | 6.7 ± 0.46 |
| | Combined | 14.5 ± 0.52 | 14.0 ± 0.43 | 13.6 ± 0.62 | ***12 3 ± 0.72 (↓15%)** |
| **$F_{1a}$** | Male | 7.8 ± 0.61 | 8.0 ±0.41 | 7.4 ±0.43 | ***6.1 ±0.64 (↓22%)** |
| | Female | 7.6 ± 0.53 | 7.4 ± 0.52 | 7.4 ± 0.42 | 6.7 ± 0.70 |
| | Combined | 15.3 ± 0.61 | 15.3 ± 0.50 | 14.7 ± 0.65 | ***12 8 ± 1.01 (↓16%)** |
| **$F_{1b}$** | Male | 7.0 ± 0.60 | 7.1 ± 0.55 | 7.1 ± 0.78 | 6.1 ± 0.42 |
| | Female | 7.1 ± 0.53 | 6.9 ± 0.55 | 6.7 ± 0.62 | 7.9 ± 0.67 |
| | Combined | 14.1 ± 0.81 | 14.1 ± 0.59 | 13 .8 ± 0.97 | 13.9 ± 0.73 |
| **$F_{1c}$** | Male | 6.8 ± 0.54 | 6.4 ± 0.61 | 4.9 ± 0.73 | 5.3 ± 0.74 |
| | Female | 6.9 ± 0.88 | 5.9 ± 0.48 | 6.7 ± 0.78 | 5.7 ± 0.74 |
| | Combined | 13.7 ± 0.97 | 12.3 ± 0.86 | 11.6 ± 1.17 | 11 ± 1.11 |
| **Proportion of pups born alive** | **All $F_1$ litters** | 0.99 ± 0.01 | 0.99 ± 0.01 | 0.99 ± 0.01 | 0 96 ± 0.02 |
| | **$F_{1a}$** | 0.99 ± 0.01 | 1.00 ± 0.00 | 0.98 ± 0.01 | 0 93 ± 0.04 |
| | **$F_{1b}$** | 0.99 ± 0 | 0.99 ± 0 | 0.99 ± 0 | 0 99 ± 0.01 |
| | **$F_{1c}$** | 0.99 ± 0.01 | 0.98 ± 0.02 | 0.99 ± 0.01 | 0 93 ± 0.06 |
| **Sex of pups born alive (Males/Total)** | **All F1 litters** | 0.51 ± 0.02 | 0.51 ± 0.02 | 0.48 ± 0.02 | 0.46 ± 0.02 |
| | **$F_{1a}$** | 0.50 ± 0.03 | 0.52 ± 0.03 | 0.51 ± 0.03 | 0.49 ± 0.04 |
| | **$F_{1b}$** | 0.49 ± 0.03 | 0.51 ± 0.03 | 0.49 ± 0.03 | 0.44 ± 0.03 |
| | **$F_{1c}$** | 0.53 ± 0.05 | 0.51 ± 0.03 | 0.41 ± 0.04 | 0.48 ± 0.05 |
| **Live pup weight (grams)** | | | | | |
| **All $F_1$ litters** | Male | 6.53 ± 0.09 | 6.75 ± 0.08 | 6.62 ± 0.09 | 6 54 ± 0.11 |
| | Female | 6.18 ± 0.07 | 6.34 ± 0.08 | 6.33 ± 0.10 | 6 18 ± 0.11 |
| | Combined | 6.36 ± 0.08 | 6.55 ± 0.08 | 6.46 ± 0.09 | 6 35 ± 0.11 |
| **$F_{1a}$** | Male | 6.43 ± 0.10 | 6.55 ± 0.11 | 6.39 ± 0.10 | 6 22 ± 0.12 |
| | Female | 6.07 ± 0.10 | 6.17 ± 0.11 | 6.03 ± 0.09 | 5 96 ± 0.12 |
| | Combined | 6.25 ± 0.10 | 6.36 ± 0.11 | 6.21±0.09 | 6.05 ± 0.12 |
| **$F_{1b}$** | Male | 6.58 ± 0.13 | 6.84 ± 0.09 | 6.77 ± 0.12 | 6.68 ± 0.1 |
| | Female | 6.28 ± 0.14 | 6.41 ± 0.09 | 6.41 ± 0.12 | 6 35 ± 0.08 |
| | Combined | 6.43 ± 0.13 | 6.64 ± 0.08 | 6.58 ± 0.11 | 6.5 ± 0.08 |
| **$F_{1c}$** | Male | 6.88 ± 0.15 | 7.09 ± 0.14 | 7.29 ± 0.24 | 7.05 ± 0.24 |

| | | | | | |
|---|---|---|---|---|---|
| | Female | 6.47 ± 0.14 | 6.6 ± 0.13 | 6.89 ± 0.22 | 6 54 ± 0.19 |
| | Combined | 6.69 ± 0.15 | 6.85 ± 0.13 | 7.06 ± 0.22 | 6.77 ± 0.21 |
| **Anogenital Dist. (PND 1)** | | | | | |
| **All $F_1$ litters** | Male | 2.79 ± 0.05 | 2.78 ± 0.05 | 2.76 ± 0.07 | 2.8 5± 0.06 |
| | Female | 1.2 6± 0.02 | 1.23 ± 0.02 | 1.23 ± 0.02 | 1 2 6 ± 0.02 |
| **$F_{1a}$** | Male | 2.73 ± 0.04 | 2.88 ± 0.06 | 2.7 ± 0.07 | 2.83 ±0.08 |
| | Female | 1.22 ± 0.02 | 1.24 ± 0.02 | 1.23 ± 0.02 | 1 28 ± 0.03 |
| **$F_{1b}$** | Male | 2.71 ± 0.05 | 2.60 ± 0.05 | 2.59 ± 0.06 | 2.69 ± 0.04 |
| | Female | 1.22 ± 0.02 | 1.22 ± 0.02 | 1.21 ± 0.03 | 1 26 ± 0.03 |
| **$F_{1c}$** | Male | 2.93 ± 0.07 | 2.86 ± 0.04 | 2.99 ± 0.09 | 3.04 ± 0.07 |
| | Female | 1.34 ± 0.03 | *** 1.25 ± 0.04 (↓7%)** | 1.25 ± 0.03 | *** 1.24 ±0.02 (↓8%)** |
| **Ratio AGD/pup wt. (PND 1)** | | | | | |
| **All $F_1$ litters** | Male | 0.42 ± 0.01 | 0.41 ± 0.01 | 0.41 ± 0.01 | 0.43 ± 0.01 |
| | Female | 0.21 ± 0.01 | 0.19 ± 0.01 | 0.19 ± 0.01 | 0 2 ± 0.01 |
| **$F_{1a}$** | Male | 0.43 ± 0.01 | 0.44 ± 0.01 | 0.43 ± 0.01 | 0.46 ± 0.02 |
| | Female | 0.2 ± 0 | 0.2 ± 0 | 0.21 ± 0 | *** *0.22 ± 0.01 (↑10%)** |
| **$F_{1b}$** | Male | 0.41 ± 0.01 | 0.38 ± 0.01 | 0.39 ± 0.01 | 0.40 ± 0.01 |
| | Female | 0.20 ± 0.01 | 0.19 ± 0.00 | 0.19 ± 0.01 | 0 20 ± 0.01 |
| **$F_{1c}$** | Male | 0.43 ± 0.01 | 0.41±0.01 | 0.41 ± 0.01 | 0.43 ± 0.01 |
| | Female | 0.21 ± 0.01 | 0.19 ± 0.01 | ***0.18 ± 0.01 (↓14%)** | 0 19 ± 0.01 |
| **Average dam weight (grams)** | **All $F_1$ litters** | 384.5 ± 8.55 | 372.6 ± 7.96 | 366.7 ± 5.84 | *****344 2 ± 7.28 (↓11%)** |
| | **$F_{1a}$** | 347.1 ± 7.38 | 337 .3 ± 6.18 | 330.9 ± 4.25 | *****317.7 ± 4.98 (↓8%)** |
| | **$F_{1b}$** | 388.2 ± 8.79 | 376.3 ± 7.98 | 375.9 ± 8.29 | ***** 350.4 ± 6.22 (↓13%)** |
| | **$F_{1c}$** | 418.3± 9.48 | 404.3 ± 9.72 | 393.3 ± 4.99 | *****364.7 ± 10.64 (↓13%)** |

**p<0.05; **p<0.01; ***p<0.001*

## $F_1$ parental

**Clinical signs:** No relevant clinical signs were noted in the treated groups compared with controls. Urine stains were increased in both sexes compared with controls (*males*: 10%, 25% and 55% for low, mid and high dose groups vs 5% in controls; *females*: 15%, 25% and 45% low, mid and high dose groups vs 0% in controls). Abrasion was increased in low and mid dose male groups (40% and 20% respectively, vs 10% in controls), and alopecia was increased in low dose male group (45% vs 20% in controls).

Mortality was observed in six $F_{1c}$ adult males and five adult females:

*Males*:

- One 70 mg/kg/day male was killed moribund on PND 171 because of severe clinical signs including rough hair coat, thinness, hunched posture, diarrhoea, few faeces, red discharge from the mouth, and anorexia.

- One 230 mg/kg/day male was found dead on PND 50. Each animal appeared normal prior to its death.

- One 230 mg/kg/day male was killed moribund on PND 193 because of severe clinical signs including rales, dyspnoea, red discharge from the mouth, and hypoactivity. This death was a result of a gavage error.

- Two 700 mg/kg/day males were found dead on PND 66. Each animal appeared normal prior to its death.

- One 700 mg/kg/day male was found dead on PND 171. The animal appeared normal prior to its death.

*Females*:

- One 230 mg/kg/day female was found dead on PND 107. The death was a result of a gavage error.

- One 230 mg/kg/day female was found dead on PND 124. The animal appeared normal prior to its death.

- One 700 mg/kg/day female died ten minutes after dosing on PND 74. The time of death suggests a possible aspiration of compound as the cause of death.

-Two 700 mg/kg/day females were found dead on PND 109 and PND 153. The first animal died immediately after dosing of possible aspiration of compound. The second animal appeared normal prior to its death

***$F_1$ premating phase***

**Bodyweights:** Increase in bodyweight was recorded in low dose male group during the whole adolescent growing phase (PND 30±10 - PND 79±10), although data only were statistically significant throughout PND 30-44 ± 10 (10-12%). However, a statistically significant decrease was noted in high dose male group throughout PND 51 - 79 ± 10 (7-10%), and in mid dose male group on PND 79 ± 10 (5%) (see Table B.6.6.1/12)

On the other hand, no differences were observed in mean female bodyweights during the adolescent growing phase compared with controls (see Table B.6.6.1/12).

**Table B.6.6.1/12. Bodyweights of $F_1$ parental animals during premating phase**

| | Males dose groups (Mean ±SE) | | | | Females dose groups (Mean ±SE) | | | |
|---|---|---|---|---|---|---|---|---|
| **Post natal day** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **30** | 141.3 ± 5.63 | ***157 9 ± 4.53 (↑12%)** | 137 ± 6.88 | 142.9 ± 3.62 | 125.8 ± 4.48 | 137.8 ± 3.93 | 133.1 ± 5.43 | 126.7 ± 2.65 |
| **37** | 198.4 ± 6.58 | ***217 8 ± 5.3 (↑10%)** | 191 ± 7.17 | 197.7 ± 4.37 | 160.6 ± 3.76 | ***174.6 ± 3.87 (↑9%)** | 171.3 ± 4.85 | 163.4 ± 2.5 |
| **44** | 253.4 ± 6.24 | ***283 ± 6.22 (↑12%)** | 253.2 ± 8.09 | 245.4 ± 5.92 | 192.5 ± 3.32 | 203.3 ± 3.53 | 201.9 ± 4.52 | 190.9 ± 2.55 |
| **51** | 318.8 ± 6.26 | 343 ± 7.1 | 307.4 ± 7.91 | ***** 295 ± 5.52 (↓7%)** | 218 ± 2.9 | 228 ± 3.53 | 223.3 ± 4.33 | 217.8 ± 2.54 |
| **58** | 363.9 ± 5.35 | 378 ± 7.27 | 351.7 ± 6.67 | ***** 337.8 ± 5.54 (↓7%)** | 236.9 ± 2.6 | 244.9 ± 3.57 | 241.4 ± 4.03 | 235.9 ± 2.49 |
| **65** | 411.7 ± 5.15 | 423.8 ± 7.28 | 393.5 ± 5.98 | ***** 374.7 ± 6.19 (↓9%)** | 252 .3 ± 3 .03 | 259.3 ± 3.81 | 255.7 ± 3.9 | 250 ± 2.58 |
| **72** | 445.2 ± 5.67 | 455.2 ± 7.65 | 425.8 ± 5.87 | ***** 403.3 ± 6.59 (↓9%)** | 266.6 ± 2.89 | 270.8 ± 3.88 | 269.1 ± 3.84 | 262.9 ± 2.65 |
| **79** | 476.2 ± 5.88 | 484.6 ± 8.02 | *****450.5 ± 6.54 (↓5%)** | ***** 428.4 ± 7.08 (↓10%)** | 280.1 ± 3.09 | 283.9 ± 4.15 | 278.7 ± 3.93 | 270.3 ± 2.78 |

**p<0.05; **p<0.01; ***p<0.001*

**Reproductive organ parameters:** Sexual developmental parameters as testicular descent, preputial separation, and vaginal opening were comparable between dose groups and controls (see Table B.6.6.1/13).

**Table B.6.6.1/13. Reproductive organ parameters of $F_1$ parental animals during premating phase**

| Dose groups (Mean ±SE) | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **Day of Vaginal Opening** | 33.32 ± 0.46 | 33.22 ± 0.33 | 32.97 ± 0.30 | 33.18 ± 0.38 |
| **Day of Prepuce Separation** | 44.74 ± 0.39 | 44.14 ± 0.43 | 43.68 ± 0.52 | 43.75 ± 0.4 |
| **Day of Testicular Descent** | 25.53 ± 0.35 | 25.17 ± 0.27 | 25.16 ± 0.37 | 25.80 ± 0.29 |

### *$F_1$ mating phase*

**Bodyweight:** Statistically significant decrease in bodyweight were detected in high dose male group (12-22%) throughout weeks 1-15, and in mid dose male group throughout weeks 2-15 (6-12%). On the other hand, a statistically significant decrease in bodyweight was detected in high dose female group (9-14%) during throughout weeks 2-15 (except week 11). See Table B.6.6.1/14.

**Table B.6.6.1/14. Bodyweights of $F_1$ parental animals during mating phase**

| | Males dose groups (Mean ±SE) | | | | Females dose groups (Mean ±SE) | | | |
|---|---|---|---|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| 1 | 482.4 ± 8.94 | 479.4 ± 10.78 | 458.4 ± 7.11 | ***** 425.3 ± 9.77 (↓12%)** | 284.5 ± 5.45 | 278.5 ± 4.41 | 283.1 ± 5.29 | 271.3 ± 3.56 |
| 2 | 513.5 ± 9.11 | 508.3 ± 11.07 | ***** 485.2 ± 7.38 (↓6%)** | ***** 440.4 ± 10.73 (↓14%)** | 311.4 ± 5.41 | 305.3 ± 4.74 | 311.6 ± 5.69 | 293.9 ± 3.85 |
| 3 | 547.8 ± 10.15 | 536.7 ± 11.07 | ***** 511.8 ± 8.45 (↓7%)** | ***** 461.0 ± 11.16 (↓16%)** | 353.9 ± 6.15 | 340.2 ± 5.62 | 345.2 ± 5.55 | ***** 323.4 ± 4.29 (↓9%)** |
| 4 | 571.1 ± 11.45 | 555.7 ± 10.95 | ***** 525.4 ± 8.86 (↓8%)** | ***** 473.6 ± 10.67 (↓17%)** | 427 ± 8.29 | 402.4 ± 9.12 | 412.7 ± 8.66 | ***** 378.5 ± 7.22 (↓11%)** |
| 5 | 584.4 ± 11.56 | 564.4 ± 10.73 | ***** 534.0 ± 9.99 (↓9%)** | ***** 482.0 ± 11.05 (↓18%)** | 363.2 ± 7.28 | 348.1 ± 6.93 | 346.l ± 6.98 | ***** 322.3 ± 4.26 (↓11%)** |
| 6 | 608.7 ± 12.38 | 587.6 ± 11.54 | ***** 550.5 ± 10.06 (↓10%)** | ***** 492.8 ± 10.90 (↓19%)** | 381.2 ± 5.93 | 370.4 ± 7.33 | 377.6 ± 8.32 | ***** 336.8 ± 3.91 (↓12%)** |
| 7 | 626.6 ± 13.45 | 605.0 ± 11.76 | ***** 564.3 ± 9.87 (↓10%)** | ***** 510.6 ± 10.86 (↓19%)** | 417.6 ± 7.51 | 408.1 ± 9.53 | 421.7 ± 9.33 | **** 378.9 ± 5.84 (↓9%)** |
| 8 | 639.3 ± 15.32 | 614.1 ± 12.62 | ***** 570.6 ± 10.42 (↓11%)** | ***** 515.2 ± 11.48 (↓19%)** | 407 ± 10.29 | 405.7 ± 13.87 | 402.4 ± 15.27 | *** 368.3 ± 12.49 (↓10%)** |
| 9 | 657.6 ± 14.80 | 629.4 ± 12.45 | ***** 587.0 ± 10.47 (↓11%)** | ***** 530.4 ± 11.89 (↓19%)** | 412.l ± 11.17 | 393.7 ± 9.7 | 398.8 ± 9.48 | **** 365.2 ± 8.41 (↓11%)** |
| 10 | 670.0 ± 14.96 | 642.2 ± 13.24 | ***** 597.5 ± 11.53 (↓11%)** | ***** 533.2 ± 13.24 (↓20%)** | 419 ± 9.32 | 413.8 ± 8.93 | 425.9 ± 9.5 | **** 383.9 ± 10.59 (↓8%)** |
| 11 | 681.7 ± 6.73 | 657.4 ± 13.28 | ***** 610.2 ± 10.90 (↓11%)** | ***** 542.6 ± 14.53 (↓20%)** | 431.9 ± 11.96 | 420.7 ± 12.48 | 451.7 ± 15.16 | 409.2 ± 10.46 |
| 12 | 705.5 ± 16.78 | 671.1 ± 14.97 | ***** 626.8 ± 11.74 (↓11%)** | ***** 557.8 ± 14.87 (↓21%)** | 414.2 ± 10.57 | 396.3 ± 10.23 | 411.9 ± 10.73 | *** 364.3 ± 7.07 (↓12%)** |
| 13 | 720.0 ± 16.62 | 676.8 ± 19.02 | ***** 637.9 ± 12.59 (↓11%)** | ***** 570.8 ± 14.55 (↓21%)** | 407.2 ± 8.57 | 400 ± 8.49 | 414.9 ± 13.11 | **** 356.5 ± 6.45 (↓12%)** |
| 14 | 730.7 ± 16.78 | 696.2 ± 16.07 | ***** 644.1 ± 13.31 (↓12%)** | ***** 581.7 ± 14.94 (↓20%)** | 404.2 ± 9.04 | 394.4 ± 8.34 | 408.5 ± 10.03 | **** 352.4 ± 7.54 (↓13%)** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 15 | 752.7 ± 17.22 | 716.9 ± 16.60 | ***** 659.7 ± 14.21 (↓12%)** | ***** 586.7 ± 15.17 (↓22%)** | 410.3 ± 8.61 | 398.5 ± 9.17 | 408.6 ± 9.83 | ***** 354.4 ± 6.4 (↓14%)** |

**p<0.05; **p<0.01; ***p<0.001*

**Food consumption:** Statistically significant decrease in absolute food consumption was recorded in low (12%), mid (14%) and high (15%) dose male groups at week 12, whereas a decrease of 15% and 23% was noted at week 14 in mid and high dose male groups, respectively.

On the other hand, a statistically significant increase in relative food consumption was observed in low dose female group (16%).

Take into account both sexes, a statistically significant decrease of 8% was described in absolute food consumption in mid and high dose groups during weeks 3 and 6, whereas an increase of 9% was described in relative food consumption in high dose groups at week 6 (See Table B.6.6.1/15).

**Table B.6.6.1/15. Food consumption of $F_1$ parental animals during mating phase**

| **F1 generation : Food consumption (g/animal/day) (Mean ±SE)** | | | | |
|---|---|---|---|---|
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **Males** | | | | |
| 12 | 26.64 ± 0.64 | ***** 23.39 ± 1.34 (↓12%)** | ***** 23.04 ± 0.61 (↓14%)** | ***** 22.76 ± 0.64 (↓15%)** |
| 14 | 28.24 ± 0.73 | 26.53 ± 0.74 | ***** 24.02 ± 0.66 (↓15%)** | ***** 21.86 ± 0.68 (↓23%)** |
| **Females** | | | | |
| 12 | 18.50 ± 0.45 | 20.74 ± 0.82 | 19.55 ± 0.78 | 19.03 ± 1 23 |
| 14 | 18.50 ± 0.59 | 18.24 ± Í.04 | 16.83 ± 0.48 | 16.76 ± 0.81 |
| **Males and females** | | | | |
| 3 | 23.80 ± 0.30 | 22.47 ± 0.80 | ***** 21.79 ± 0.62 (↓8%)** | ***** 21.78 ± 1.40 (↓8%)** |
| 6 | 23.15 ± 0.52 | 22.81 ± 0.58 | 21.89 ± 0.53 | ***** 21.17 ± 0.33 (↓8%)** |
| **$F_1$ generation : Food consumption (g/kg bw/day) (Mean ±SE)** | | | | |
| **Week** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **Males** | | | | |
| 12 | 38.02 ± 1.08 | 34.68 ± 1.90 | 36.66 ± 0.70 | 40.49 ± 0.86 |
| 14 | 38.76 ± 0.82 | 38.19 ± 0.85 | 37.30 ± 0.71 | 37.72 ± 1.02 |
| **Females** | | | | |
| 12 | 45.83 ± 1.74 | ***53.38 ± 1.62 (↑16%)** | 48.26 ± 2.14 | 52.50 ± 3.73 |
| 14 | 46.64 ± 1.81 | 47.01 ± 2.55 | 41.93 ± 1.32 | 47.92 ± 2 37 |
| **Males and females** | | | | |
| 3 | 52.85 ± 0.61 | 51.51 ± 2.03 | 50.98 ± 1.48 | 55.36 ± 2 98 |
| 6 | 47.13 ± 1.17 | 47.80 ± 1.36 | 47.49 ± 1.16 | ****51 37 ± 0.81 (↑9%)** |

**p<0.05; **p<0.01; ***p<0.001*

**Organ weights**

The following organ weights were recorded for both sexes in each dose groups (see Table B.6.6.1/16).

*Males*

-Decreases terminal bodyweights in the mid and high dose groups (9% and 22% respectively).

-Increases were seen in the following relative organ weight in the high dose male group compared with controls:
.Liver (19%),
.Cauda epididymis (24%).
.Right epididymis (20%).
.Kidneys (20%).
.Spleen (17%).
.Right testis (21%).

*Females*

-Decreases terminal bodyweights in the high dose group (12%).
-Increases were seen in the following relative organ weight of the high dose male group compared with controls:
.Liver (24%),
.Ovaries (18%).
.Kidneys (15%),

-Increases relative liver weight in mid dose group (6%).

**Table B.6.6.1/16. Male and female organ weights of F1 parental animals**

| | | P: Male organ weights (Mean ±SE) | | | | P: Female organ weights (Mean ±SE) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Dose groups | | | | Dose groups | | | |
| | | 0 mg/kg bw/day | 70 mg/kg bw/day | 230 mg/kg bw/day | 700 mg/kg bw/day | 0 mg/kg bw/day | 70 mg/kg bw/day | 230 mg/kg bw/day | 700 mg/kg bw/day |
| **BODY (g)** | | 761.5 ± 17.35 | 745.6 ± 22.29 | ***** 690.7 ± 25.23 (↓9%)** | *****591.6 ± 22.75 (↓22%)** | 411.2 ± 8.91 | 415.8 ± 10.61 | 421.3 ± 14.6 | *** 360.7 ± 8.53 (↓12%)** |
| **LIVER (g)** | Absolute | 27.26 ± 0.8 | 27.22 ± 0.96 | 25.74 ± 0.94 | 25.32 ± 1.34 | 14.02 ± 0.34 | 14.81 ± 0.72 | 15.28 ± 0.5 | 15 29 ± 0.43 |
| | Relative | 35.81 ± 0.70 | 36.58 ± 1.04 | 37.37 ± 0.95 | *****42.73 ± 1.27 (↑19%)** | 34.12 ± 0.54 | 35.52 ± 1.14 | *****36.33 ± 0.67 (↑6%)** | *****42.4 ± 0.84 (↑24%)** |
| **KIDNEYS (mg)** | Absolute | 4591.95 ± 100.98 | 4549.4 ± 151.74 | 4339.1 ± 140.98 | 4292.5 ± 175.7 | 2472 ± 49.24 | 2512.8 ± 58.86 | 2632.4 ± 97.93 | 2524.1 ± 114.51 |
| | Relative | 6.06 ± 0.14 | 6.12 ± 0.16 | 6.32 ± 0.2 | *****7.27 ± 0.16 (↑20%)** | 6.04 ± 0.13 | 6.07 ± 0.18 | 6.26 ± 0.15 | ***** 6.97 ± 0.16 (↑15%)** |
| **SPLEEN (mg)** | Absolute | 932.05 ± 31.54 | 897 ± 28.72 | 908 ± 59.51 | 858.8 ± 67.03 | 656.7 ± 20.33 | 605.5 ± 28.92 | 696 ± 32.68 | 604.4 ± 31.1 |
| | Relative | l.23 ± 0.04 | 1.21 ± 0.05 | 1.31 ± 0.05 | ***1.44 ± 0.07 (↑17%)** | 1.62 ± 0.07 | 1.46 ± 0.06 | 1.66 ± 0.08 | 1.68 ± 0.08 |
| **DORSOLATERAL PROSTATE (g)** | Absolute | 792.07 ± 44.98 | 771.72 ± 56.27 | 793.29 ± 65.17 | 661.54 ± 67.25 | | | | |
| | Relative | 1.04 ± 0.06 | 1.05 ± 0.1 | 1.15 ± 0.09 | 1.14 ± 0.13 | | | | |
| **VENTRAL PROSTATE (g)** | Absolute | 701.74 ± 30.22 | 750.46 ± 45.77 | 669.47 ± 41.01 | 617.8 ± 27.2 | | | | |
| | Relative | 0.93 ± 0.04 | 1.02 ± 0.07 | 0.97 ± 0.05 | 1.06 ± 0.06 | | | | |
| **RIGHT CAUDA EPIDIDYMIS (mg)** | Absolute | 349.39 ± 7.56 | 339.65 ± 7.96 | 333.6 ± 10.85 | 332.11 ± 8.06 | | | | |
| | Relative | 0.46 ± 0.01 | 0.46 ± 0.02 | 0.49 ± 0.02 | ***** 0.57 ± 0.02 (↑24%)** | | | | |
| **RIGHT EPIDIDYMIS (mg)** | Absolute | 738.55 ± 33 .49 | 726. 90 ± 40.11 | 701.6 ± 44.23 | 683.6 ± 44 .13 | | | | |
| | Relative | 0.98 ± 0.05 | 0.98 ± 0.06 | 1.02 ± 0.07 | **** 1.17 ± 0.08 (↑20%)** | | | | |
| **SEMINAL VESICLES (mg)** | Absolute | 2080.15 ± 65.43 | 2124 9 ± 149.77 | 2058.1 ± 135.23 | 1823.3 ± 76.97 | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Relative | 2.74 ± 0.08 | 2.87 ± 0.21 | 3.01 ± 0.22 | 3.09 ± 0.1 | | | | |
| **RIGHT TESTIS (mg)** | Absolute | 2017.3 ± 46.89 | 2053.4 ± 45.56 | 1943.1 ± 55.79 | 1897.7 ± 45.71 | | | | |
| | Relative | 2.67 ± 0.08 | 2.78 ± 0.1 | 2.84 ± 0.11 | ***** 3.23 ± 0.09 (↑21%)** | | | | |
| **OVARIES (mg)** | Absolute | | | | | 0.15 ± 0.01 | 0.15 ± 0.01 | 0.17 ± 0.02 | 0.16 ± 0.01 |
| | Relative | | | | | 0.38 ± 0.02 | 0.38 ± 0.03 | 0.41 ± 0.04 | *** 0.45 ± 0.02 (↑18%)** |
| **UTERUS (mg)** | Absolute | | | | | 1168.95 ± 69.39 | 1021.1 ± 64.07 | 1236.2 ± 84.7 | 1164.3 ± 74.47 |
| | Relative | | | | | 2.86 ± 0.18 | 2.45 ± 0.14 | 2.97 ± 0.23 | 3 23 ± 0.21 |

**p<0.05; **p<0.01; ***p<0.001*

**Necropsy**

Incidental gross findings were only observed in males dose groups at necropsy:

-Dilated pelvis of the right kidney in the low dose group (10% vs 0% in controls).

-Dark lumen fluid in the urinary bladder in the low dose group (10% vs 0% in controls).

-Thickened pericardium of the heart adherent to lungs in the high dose group (10% vs 0% in controls).

-Bloated stomach, duodenum, jejunum, ileum, and cecum in the high dose male group (10% vs 0% in controls).

There were no gross findings in the females.

**Histopathology**

Treatment-related changes were found in the non-glandular stomach and were comparable to changes observed in the P animals. These changes were found in all treated groups and their incidence and/or severity were dose related. All other tissue microscopic changes were commonly detected in laboratory rats and were unrelated to treatment (see Table B.6.6.1/17).

**Table B.6.6.1/17. Histopathological stomach findings in $F_1$ parental animals**

| | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| **Group (mg/kg bw/day)** | **0** | **70** | **230** | **700** | **0** | **70** | **230** | **700** |
| **number examined** | **10** | **10** | **10** | **10** | **10** | **10** | **10** | **10** |
| **Hyperkeratosis** | 0 | 9 (90%) | 10 (100%) | 10 (100%) | 0 | 6 (60%) | 9 (90%) | 10 (100%) |
| *Minimal* | 0 | 4 (40%) | 2 (20%) | 0 | 0 | 2 (20%) | 2 (20%) | 0 |
| *Mild* | 0 | 4 (40%) | 3 (30%) | 1 (10%) | 0 | 4 (40%) | 4 (40%) | 2 (20%) |
| *Moderate* | 0 | 1 (10%) | 5 (50%) | 9 (90%) | 0 | 0 | 3 (30%) | 8 (80%) |
| **Hyperplasia** | 0 | 9 (90%) | 9 (90%) | 9 (90%) | 0 | 5 (50%) | 9 (90%) | 10 (100%) |
| *Minimal* | 0 | 5 (50%) | 4 (40%) | 2 (20%) | 0 | 5 (50%) | 4 (40%) | 1 (10%) |
| *Mild* | 0 | 4 (40%) | 5 (50%) | 5 (50%) | 0 | 0 | 4 (40%) | 1 (10%) |
| *Moderate* | 0 | 0 | 0 | 2 (20%) | 0 | 0 | 1 (10%) | 8 (80%) |

**Sperm analysis:** Computer-assisted sperm motion analysis using Hamilton Thome Integrated Visual Optic System revealed no changes in mean path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, motile percentage, and progressive percentage of the treated groups as compared

to controls. Also, no changes were seen in epididymal sperm density, percent abnormal sperm, number of spermatids per cauda epididymis, or the total number of spermatids per testis (see tables B.6.6.1/18-19).

**Table B.6.6.1/18. Sperm analysis in $F_1$ parental males**

| | Dose group (Mean ± SE) | | | |
|---|---|---|---|---|
| | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| **COMPUTER ASSISTED SPERM ANALYSIS (VAS DEFERENS)** | | | | |
| EPIDIDYMAL SPERM DENSITY (a) | 399.13 ± 14 15 | 401.73 ± 23.73 | 393 10 ± 20.62 | 408.49 ± 24.47 |
| EPIDIDYMAL SPERM MORPHOLOGY( % ABNORMAL) (b) | 0.43 ± 0.10 | | | 0.65 ± 0 .11 |
| SPERMATID/mg TESTIS (c) | 132.53 ± 4.58 | | | 152.68 ± 10.09 |
| TOTAL SPERMATID/TESTIS (d) | 254.47 ± 6.53 | | | 267.13 ± 12.35 |
| TOTAL SPERMATID/CAUDA (d) | 139.85 ± 6.38 | 136.13 ± 7.83 | 130.75± 7.44 | 134.53 ± 6.25 |

*(a)Sperm density expressed as 1000 sperm per mg right caudal tissue.*
*(b) Dose group means and standard errors are computed only from samples with at least 200 sperm.*
*(c)Spermatid heads per mg testis (x 1000) = (total number of spermatid heads per right testis) I (right testis weight in mg)*
*(d) Total number of spermatid heads (x 1000000) = (average number of spermatid heads x 2.5 x 100) / 0.0001*

**Table B.6.6.1/19. Summary of Hamilton-Throne sperm analysis data in P males**

| | Dose group (Mean ± SE) | |
|---|---|---|
| | **0 mg/kg bw/day** | **700 mg/kg bw/day** |
| **COMPUTER ASSISTED SPERM ANALYSIS (VAS DEFERENS)** | | |
| **BT/CS FREQ (hz/sec)** | 16.69 ± 0.44 | 16.78 ± 0.42 |
| **LATERAL AMPLITUDE (um)** | 17.16 ± 0.45 | 17.96 ± 0.47 |
| **LINEARITY** | 34.63 ± 0. 71 | 35.4 ± 0.76 |
| **MOTILE PERCENTAGE** | 88.11 ± 1.09 | 86.8 ± l.93 |
| **PROGRESSIVE VELOCITY** | 94.75 ± 3.01 | 97.41 ± 3.13 |
| **PATH VELOCITY** | 162.23 ± 3.33 | 162.57 ± 4.69 |
| **STRAIGHTNESS** | 56.79 ± 0.77 | 59.4 ± 1.05 |
| **TRACK SPEED** | 278.98 ± 5.39 | 283.97 ± 5.97 |

**Oestrus cycle:** Vaginal cytology was performed on $F_1$ dams for 14 days after weaning of the $F_{1c}$ litters. There was no evidence of any differences between the dosed and control groups in cycle length, in number of cycles, or in relative length of time spent in estrous stages. There was an increase in the number of females with regular cycles in the high dose group (29%) (see Table B.6.6.1/20).

**Table B.6.6.1/20. Summary of stages and length of estrous cycle in $F_1$ parental females**

| | Estrous stage % (a) | | | | | Cycle length (days) | Number cycles | No cycling females (b) | No of females W/regular cycle (c) |
|---|---|---|---|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **P** | **E** | **M** | **D** | **N** | **Mean±S.E.** | **Mean±S.E.** | | |
| 0 | 16.1 | 30.0 | 19.3 | 34.6 | 0 | 4.87 ± 0.33 | 2.26 ± 0.13 | 19 /20 (95%) | 13 /20 (65%) |
| 70 | 12.1 | 28.9 | 20.0 | 38.9 | 0 | 4.33 ± 0.10 | 2.47 ± 0.12 | 19 /20 (95%) | 16 /20 (80%) |
| 230 | 12.7 | 27.0 | 21.4 | 38.9 | 0 | 4.42 ± 0.17 | 2.47 ± 0.12 | 17 /18 (94%) | 14 /18 (78%) |
| 700 | 14.3 | 29.0 | 22.2 | 34.5 | 0 | 4.54 ± 0.15 | 2.22 ± 0.13 | 18 /18 (100%) | ***17 /18 (94%)** |

*a P=Proestrus, E=Estrus, M=Metestrus, D=Diestrus, N=Not Clear, Poor Quality, or Insufficient Number of Cells*

*b Number of cycling females / number of females sampled (percent cycling).*
*c Number of females with a regular cycle / number of females sampled (percent regular).*

**$F_2$ offspring**

**Reproductive performance**

A summary of the main reproductive parameters are listed below (see also Tables B.6.6.1/21-22) :

**700 mg/kg bw/day**

-Increase in pregnancy index for $F_{2b}$ litter (100% vs 80% in controls).

-Decreases of the whole average live male pup weight in $F_2$ litters (6%).

-Decreases of the whole average live female pup weight in $F_2$ litters (4%).

-Decreases of the whole average combined male and female pup weight of $F_2$ litters (5%).

-Decreases of male live pup weight in $F_{2a}$ litter (5%).

-Decreases of female live pup weight in $F_{2a}$ litter (5%)

-Decreases of male and female live pup weight in $F_{2a}$ litter (5%).

-Decreases of male live pup weight in $F_{2b}$ litter (7%).

-Decreases of female live pup weight in $F_{2b}$ litter (6%)

-Decreases of male and female live pup weight in $F_{2b}$ litter (6%).

-Decreases in dam bodyweights (13-16%) at delivery in $F_2$ litters ($F_{2a}$, $F_{2b}$ and $F_{2c}$).

-Increase in the female AGD/bodyweight ratio during the $F_{2a}$ litter (12%).

**230 mg/kg bw/day**

-Increase in pregnancy index for $F_{2c}$ litter (83% vs 45% in controls).

**70 mg/kg bw/day**

-Increase in pregnancy index for $F_{2b}$ litter (100% vs 80% in controls)**.**

During the $F_1$ cohabitation, there was an unexplained decrease in pregnancy index in control animals. Twenty pairs were pregnant for the $F_{2a}$ litters, whereas in $F_{2b}$ and $F_{2c}$ litters, the pregnancy index decreased to 16/20 (80%), and to 9/20 (45%), respectively. Based on historical results in this laboratory, the pregnancy index would be expected to be between 15/20 and 20/20. This decrease is unexplained since there were no adverse effects noted in oestrous cyclicity or sperm endpoints.

**Table B.6.6.1/21. Summary of pregnancy index during $F_2$ litters**

| | Dose groups (a) | | | |
|---|---|---|---|---|
| **Litter** | **0 mg/kg bw/day** | **70 mg/kg bw/day** | **230 mg/kg bw/day** | **700 mg/kg bw/day** |
| $F_{2a}$ | 20/20 (100%) | 19/20 (95%) | 18/20 (90%) | 20/20 (100%) |
| $F_{2b}$ | 16/20 (80%) | ***20/20 (100%)** | 17/19 (89%) | ***19/19 (100%)** |
| $F_{2c}$ | 9/20 (45%) | 14/20 (70%) | ***15/18 (83%)** | 14/19 (74%) |

**p<0.05*

**Table B.6.6.1/22. Reproductive performance during $F_2$ generation ($F_1$-$F_2$)**

| | | Dose groups mg/kg bw/day [a] | | | |
|---|---|---|---|---|---|
| | | **0** | **70** | **230** | **700** |
| **Average litters per pair** | **All $F_1$ litters** | 2.3 ± 0.18 | 2.7 ± 0.13 | 2.5 ± 0.22 | 2.7 ± 0.13 |
| **Live pups per litter [c,d]** | | | | | |
| **All $F_1$ litters** | Male | 6.7 ± 0.30 | 6.3 ± 0.29 | 7.1 ± 0.41 | 7.2 ± 0.33 |
| | Female | 7.2 ± 0.43 | 6.9 ± 0.52 | 7.0 ± 0.29 | 6.9 ± 0.34 |
| | Combined | 13 .9 ± 0.46 | 13.2 ± 0.69 | 14.1 ± 0.29 | 14.1 ± 0.36 |
| **$F_{2a}$** | Male | 7.8 ± 0.53 | 6.4 ± 0.50 | 7.1 ± 0.54 | 7.6 ± 0.62 |
| | Female | 7.5 ± 0.55 | 7.1 ± 0.59 | 8.2 ± 0.68 | 7.2 ± 0.56 |
| | Combined | 15.3 ± 0.40 | 14.2 ± 0.66 | 15.3 ± 0.42 | 14.8 ± 0.29 |
| **$F_{2b}$** | Male | 6.2 ± 0.68 | 6.7± 0.53 | 7.8 ± 0.68 | 7.1 ± 0.70 |
| | Female | 7.1 ± 0.59 | 6.9 ± 0.68 | 6 .6 ± 0 .45 | 7.1 ± 0.49 |
| | Combined | 13.3 ± 1.04 | 13.6 ± 0.97 | 14.5 ± 0.71 | 14.2 ± 0.62 |
| **$F_{2c}$** | Male | 5.2 ± 0.92 | 5.9 ± 0.69 | 6.6 ± 0.68 | 7.1 ± 0.73 |
| | Female | 5.3 ± 0.96 | 6.9 ± 0.77 | 5.7 ± 0.74 | 5.6 ± 0.66 |
| | Combined | 10.6 ± 1 50 | 12.8 ± 1.18 | 12.3 ± 0.88 | 12.7 ± 1.01 |
| **Proportion of pups born alive** | **All $F_1$ litters** | 0.98 ± 0.01 | 0.98 ± 0.01 | 0.97 ± 0.01 | 0.97 ± 0.01 |
| | **$F_{2a}$** | 0.99 ± 0.01 | 0.99 ± 0.00 | 0.96 ± 0.02 | 0.99 ± 0.01 |
| | **$F_{2b}$** | 0.99 ± 0.01 | 0.96 ± 0.03 | 0.98 ± 0.01 | 0.96 ± 0.02 |
| | **$F_{2c}$** | 0.96 ± 0.03 | 0.98 ± 0.01 | 0.98 ± 0.01 | 0.94 ± 0.04 |
| **Sex of pups born alive (Males/Total)** | **All $F_1$ litters** | 0.49 ± 0.02 | 0.49 ± 0.02 | 0.50 ± 0.02 | 0.51 ± 0.02 |
| | **$F_{2a}$** | 0.51 ± 0.03 | 0.48 ± 0.02 | 0.47 ± 0.04 | 0.51 ± 0.04 |
| | **$F_{2b}$** | 0.45 ± 0.03 | 0.51 ± 0.03 | 0.53 ± 0.03 | 0.49 ± 0.04 |
| | **$F_{2c}$** | 0.50 ± 0.07 | 0.48 ± 0.05 | 0.55 ± 0.04 | 0.57 ± 0.04 |
| **Live pup weight (grams)** | | | | | |
| **All $F_1$ litters** | Male | 6.7 ± 0.06 | 6.7 ± 0.11 | 6.71 ± 0.08 | ***6.33 ± 0.08 (↓6%)** |
| | Female | 6.36 ± 0.05 | 6.34 ± 0.10 | 6.31 ± 0.07 | ***6.09 ± 0.08 (↓4%)** |
| | Combined | 6.52 ± 0.05 | 6.51 ± 0.10 | 6.51 ± 0.07 | ***6.21 ± 0.08 (↓5%)** |

| | | | | | |
|---|---|---|---|---|---|
| **$F_{2a}$** | Male | 6.66 ± 0.08 | 6.72 ± 0.12 | 6.48 ± 0.10 | ****6.33 ± 0.07 (↓5%)** |
| | Female | 6.28 ± 0.08 | 6.33 ± 0.09 | 6.05 ± 0.09 | ****5.99 ± 0.07 (↓5%)** |
| | Combined | 6.48 ± 0.07 | 6.51 ± 0.10 | 6.26 ± 0.09 | ****6.17 ± 0.07 (↓5%)** |
| **$F_{2b}$** | Male | 6.94 ± 0 13 | 6.69 ± 0.15 | 6.74 ± 0.09 | ***6.44 ± 0.13 (↓7%)** |
| | Female | 6.52 ± 0 13 | 6.33 ± 0.14 | 6.36 ± 0.09 | ***6.13 ± 0.12 (↓6%)** |
| | Combined | 6.70 ± 0 12 | 6.52 ± 0.15 | 6.56 ± 0.09 | ***6 .27 ± 0.12 (↓6%)** |
| **$F_{2c}$** | Male | 6.80 ± 0 10 | 6.78 ± 0.16 | 7.03 ± 0 .12 | 6.33 ± 0.15 |
| | Female | 6.36 ± 0 11 | 6.40 ± 0.18 | 6.74 ± 0 .17 | 6.15 ± 0.15 |
| | Combined | 6.62 ± 0 10 | 6.64 ± 0.15 | 6.89 ± 0.14 | 6.28 ± 0.15 |
| | | | | | |
| **Anogenital Dist. (PND l)** | | | | | |
| **All F1 litters** | Male | | | | |
| | Female | | | | |
| **$F_{2a}$** | Male | 2.68 ± 0.05 | 2.71 ± 0.06 | 2.59 ± 0.05 | 2.66 ± 0.04 |
| | Female | 1.08 ± 0.02 | 1.12 ± 0.03 | 1.09 ± 0.02 | 1.12 ± 0.02 |
| **$F_{2b}$** | Male | 2.52 ± 0.06 | 2.60 ± 0.08 | 2.59 ± 0.05 | 2.44 ± 0.05 |
| | Female | 1.07 ± 0.03 | 1.09 ± 0.03 | 1.10 ± 0.03 | 1.07 ± 0.03 |
| **$F_{2c}$** | Male | 2.67 ± 0.05 | 2.66 ± 0.06 | 2.63 ± 0.06 | 2.58 ± 0.04 |
| | Female | 1.07 ± 0.02 | 1.09 ± 0.03 | 1.13 ± 0.03 | 1.15 ± 0.04 |
| | | | | | |
| **Ratio AGD/pup wt. (PND l)** | | | | | |
| **All $F_1$ litters** | Male | 0.39 ± 0.01 | 0.39 ± 0.00 | 0.38 ± 0.01 | 0.40 ± 0.01 |
| | Female | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.18 ± 0.01 |
| **$F_{2a}$** | Male | 0.41 ± 0.01 | 0.40 ± 0.01 | 0.40 ± 0.01 | 0.41 ± 0.01 |
| | Female | 0.17 ± 0.00 | 0.18 ± 0.01 | 0.18 ± 0.00 | ****0.19 ± 0.00 (↑12%)** |
| **$F_{2b}$** | Male | 0.37 ± 0.01 | 0.39 ± 0.01 | 0.38 ± 0.01 | 0.39 ± 0.01 |
| | Female | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.01 |
| **$F_{2c}$** | Male | 0.39 ± 0.01 | 0.39 ± 0.01 | 0.38 ± 0.01 | 0.42 ± 0.01 |
| | Female | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.01 | 0.19 ± 0.01 |
| | | | | | |
| **Average dam weight (grams)** | **All $F_1$ litters** | 407.5 ± 9.3 | 389.5 ± 8.9 | 389 ± 10 | *****344.2 ± 6.2 (↓16%)** |
| | **$F_{2a}$** | 366.5 ± 6.76 | 349.7± 6.46 | 344.8 ± 7.07 | *****317.7 ± 4.72 (↓13%)** |
| | **$F_{2b}$** | 417.5 ± 9.05 | 394.1 ± 8.00 | 398.5 ± 10.51 | *****348.7 ± 6.27 (↓16%)** |

| | | | | | |
|---|---|---|---|---|---|
| | **$F_{2c}$** | 438.4 ± 12.31 | 424.8 ± 12.44 | 423.7± 12.53 | ***366.3 ± 7.74 (↓16%)** |

**p<0.05; **p<0.01; ***p<0.001*

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** No generational studies with eugenol have been provided. Instead, a generational study with isoeugenol has been included by RMS in this dossier as supportive information for the purpose of renewal.

Isoeugenol and eugenol are isomeric compounds in which the structural difference relies on the position of the double bond in the propenyl substituent. The physicochemical properties, such as water solubility, of these compounds differ slightly. The Log P values, however, are within the same range. The metabolic pathway of both compounds may differ based on the susceptibility of the terminal double bond of eugenol to form oxidation products as observed in the metabolism studies described in *section 6*. Moreover, this eugenol metabolism studies showed that isoeugenol is not a relevant metabolite (quantified at ~ 7% accounting for cis and trans isomerisation metabolites). The percentage of allylic oxidation metabolites in humans detected in urine 3h after oral administration of eugenol accounts for 9 %, which indicates this metabolic pathway is relevant in eugenol. Despite the lack of metabolism studies performed with isoeugenol, based on poor water solubility reported for this compound and the absence of a reactive terminal double bond, its ADME properties are expected to differ from eugenol.

Isoeugenol triggers the occurrence of hyperkeratosis and hyperplasia in the non-glandular stomach of dams at all the dose levels tested, although no relevant adverse effects were described in offspring in absence of maternal toxicity. Thus, parental toxicity has been observed in the low dose level, so a NOAEL cannot be derived.

Therefore, taking into account above considerations, RMS deems that the multi-generational study conducted with isoeugenol could not be used as supporting substance for eugenol, in agreement with EFSA conclusions (EFSA Journal, 2009: ON-965, 1-53).

**Parental NOAEL**:Parental NOAEL could not be stablished based on effects observed at low dose level (hyperkeratosis and hyperplasia in the non-glandular stomachs in both sexes at low dose level in P and $F_1$ parental animals).

**Offspring NOAEL:** 230 mg/kg bw/day based on decreased male and females pup weights in $F_2$ generation.

**Reproductive toxicity NOAEL**: 230 mg/kg bw/day based on decreased number of male pups per litter in $F_1$ generation.

## B.6.6.2. Developmental toxicity studies

### B.6.6.2.1. Developmental toxicity study in rat with clove oil

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | Morgareidge K. |
| **Report year** | 1973a |
| **Report title** | Teratologic evaluation of FDA 71-30 (Clove oil) in rats |
| **Report No** | Food and Drug Research Laboratories Study No. 1574c |
| **Document No** | KCA 5.6.2/01 |
| **Guidelines followed in study** | Non-stated/pre-guideline. |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Eugenol technical was not tested.<br>-Food consumption was not measured.<br>-The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>-The degree of resorption should be described (early, late).<br>-Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. |

| | |
|---|---|
| | -Statistical analysis was not performed.<br>-Dam necropsies were not reported.<br>-Individual foetal data was not reported.<br>- Historical control data were not provided. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP** | No (prior to the enforcement of GLP) |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Clove oil: FDA-71-30. Lot/Batch No.: Non-stated, Purity: Non-stated |
| **Test system** | Wistar rats (females) |
| **Test method** | Clove oil was tested at dose levels of 0, 2.8, 13, 60 or 280 mg/kg bw/day, in which 23-25 mated females were assigned to each test group. Clove oil (diluted in corn oil) was administered by oral gavage from gestation day 6 to gestation day 15. Negative controls were treated with the vehicle at a level equivalent to the group receiving the highest test dose. An additional group of 23 mated females received aspirin (250 mg/kg bw/day) as a positive control. Animals were examined daily and bodyweights were recorded on days 0, 6, 11, 15 and 20 of gestation. A caesarean section was performed on all rats on day 20 of gestation and the numbers of implantation sites, resorption sites, live and dead foetuses were recorded. The urogenital tract of each dam was also examined for abnormalities. All live foetuses were weighed and all foetuses were examined externally for the presence of abnormalities. One third of the foetuses underwent detailed visceral examination and the remaining two thirds were examined for skeletal defects. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Maternal toxicity**

**Survival:** All pregnant dams survived at study termination.

**Clinical signs**: No adverse clinical signs were recorded throughout the study.

**Bodyweights:** No relevant differences in bodyweights were observed between clove oil-treated groups and negative controls in dams. The bodyweight gain was increased in some doses of GD11 and GD15 but there was not dose-relationship and the increment was not observed at GD20 (See tables B.6.6.2.1/1-2).

**Table B.6.6.2.1/1. Summary of average bodyweights (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **11** | **15** | **20** |
| **0** | 220 | 240 | 258 | 274 | 341 |
| **2.8** | 217 (99%) | 241 (100%) | 259 (100%) | 279 (102%) | 344 (100%) |
| **13** | 227 (103%) | 248 (103%) | 265 (103%) | 288 (105%) | 356 (104%) |
| **60** | 225 (102%) | 248 (103%) | 267 (103%) | 288 (105%) | 357 (105%) |
| **280** | 214 (97%) | 236 (98%) | 252 (98%) | 272 (99%) | 333 (98%) |
| **250 (aspirin)*** | 216 (98%) | 234 (97%) | 248 (96%) | 267 (97%) | 313 (92%) |

**positive control*
*(% referred to negative control)*
*Statistical analysis not performed*

**Table B.6.6.2.1/2. Summary of average bodyweight gain (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **11** | **15** | **20** |
| **0** | 0 | 20 | 38 | 54 | 121 |
| **2.8** | 0 | 24 (↑20%) | 42 (↑10%) | 62 (↑14%) | 127 (↑5%) |
| **13.0** | 0 | 21 (↑5%) | 38 | 61 (↑13%) | 129 (↑7%) |
| **60.0** | 0 | 23 (↑15%) | 42 (↑10%) | 63 (↑17%) | 132 (↑9%) |
| **280.0** | 0 | 22 (↑10%) | 38 | 58 (↑7%) | 119 (↓2%) |
| **250 (aspirin)*** | 0 | 18 (↓10%) | 32 (↓16%) | 51 (↓6%) | 97 (↓20%) |

**positive control*
*(% increase/decrease bwg compared with negative control)*
*Statistical analysis not performed*

**Reproductive performance**

A summary of the main reproductive adverse parameters that present an incidence >10% (considered as potentially relevant by RMS) compared with negative controls are listed below (see table B.6.6.2.1/3):

280 mg/kg bw/day

-Decrease in the number of implantations per litter (11%).
-Decrease in live foetuses per litter (12%).
-Decrease in the male/female pups sex ratio (33%).

60 mg/kg bw/day

-Increase in the number of resorptions per litter (79%).
-Decrease in the male/female pups sex ratio (14%).

13 mg/kg bw/day

-Decrease in the male/female pups sex ratio (23%).

2.8 mg/kg bw/day

-Decrease in the male/female pups sex ratio (26%).

Positive control group

-Increase in the number of resorptions per litter (2953%).
-Decrease in live foetuses per litter in the top dose level (17%).
-Decrease in average foetus weight (36%).

As shown in table B.6.6.2.1/3, the negative control group presented a lower pregnancy index (84%) compared with the treated groups. No effect of treatment on foetal deaths was found, and the decrease in live foetus observed in the high dose group could be accounted for by the slightly lower number of implantations at this dose level. Moreover, all treated groups showed a reduction in the male/female pups sex ratio (14-33%) compared with negative controls, however, a dose-related trend was not observed. On the other hand, an increase of resorptions was noted in the 60 mg/kg bw/day dose group (79%), but a dose-relationship was not observed in the treated groups, and no further effect on live foetuses mean was noted.

Therefore, as noted in table B.6.6.2.1/3, no dose-response relationship was observed in the clove oil-treated groups regarding these reproductive parameters, and importantly, no statistical analysis was performed in the study, so these results are not supported by statistical analysis.

**Table B.6.6.2.1/3. Summary of reproductive performance**

| | Dose (mg/kg bw/day) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **2.8** | **13** | **60** | **280** | **250*** |
| **Mated** | 25 | 25 | 25 | 25 | 25 | 23 |
| **Pregnancies** | | | | | | |
| *Total* | 21 | 23 | 25 | 23 | 24 | 21 |
| *pregnancy index* | **84%** | **92%** | **100%** | **92%** | **96%** | **91%** |
| *Died or aborted* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dams to term* | 21 | 23 | 25 | 23 | 24 | 21 |
| **Litters examined** | 21 | 23 | 25 | 23 | 24 | 21 |
| **Live litters** | 21 (100%) | 23 (100%) | 25 (100%) | 23 (100%) | 24 (100%) | 19 (90%) |
| **Implant sites** | | | | | | |
| *Total* | 253 | 257 | 294 | 275 | **257** | 254 |
| *average/litters examined* | 12 | **11.2 (↓7%)** | **11.8 (↓2%)** | **12** | **10.7 (↓11%)** | **12.1 (↑1%)** |
| **Resorptions** | | | | | | |
| *Total* | 2 | 2 | 2 | 4 | 2 | 61 |
| *resorptions/litters examined* | 0.095 | **0.087 (↓8%)** | **0.08 (↓16%)** | **0.17 (↑79%)** | **0.083 (↓13%)** | **2.9 (↑2953%)** |
| **Dead foetuses** | | | | | | |
| *Total* | 0 | 0 | 2 | 1 | 0 | 2 |
| *dams with ≥1* | 0 | 0 | 1 | 1 | 0 | 2 |
| *dams with all dead* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dead foetuses/litters examined* | 0 | **0** | **0.08** | **0.043** | **0** | **0.095** |
| **Live foetuses** | | | | | | |
| *Total* | 251 | 255 | 290 | 270 | 255 | 191 |
| *average/litters examined* | 12 | **11.1 (↓7%)** | **11.6 (↓3%)** | **11.7 (↓2%)** | **10.6 (↓12%)** | **9 (↓25%)** |
| *sex ratio (♂/♀)* | 1.06 | **0.78 (↓26%)** | **0.82 (↓23%)** | **0.91 (↓14%)** | **0.71 (↓33%)** | **1.07 (↑1%)** |
| **Average foetus weight** | 3.91 | 3.98 **(↑2%)** | 4.17 **(↑7%)** | 4 **(↑2%)** | 3.95 **(↑1%)** | 2.52 (↓36%) |

**positive control*
*Statistical analysis not performed*

## Necropsy

Increased incidences regarding ossification of ribs (mostly wavy), incomplete ossification of vertebrae and incomplete closure of skull were noted in treated groups, the latter being the only parameter displaying a dose-response pattern (see table B.6.6.2.1/4). However, without a proper statistical analysis, the whole findings are difficult to assess. On the other hand, no increase of visceral malformations was found in the treated groups compared with negative controls. The positive control aspirin-treated group showed high incidences of skeletal findings and head malformations.

**Table B.6.6.2.1/4. Summary of skeletal findings(*)**

| Skeletal finding | Dose group (mg/kg bw/day) | | | | | |
|---|---|---|---|---|---|---|
| | **Negative control** | **Clove oil** | | | | **Positive control (aspirin)** |
| | **0** | **2.8** | **13** | **60** | **280** | **250** |
| **Litters examined** | **21** | **23** | **25** | **23** | **24** | **19** |
| Live foetuses examined | 175/21 | 177/23 | 201/25 | 187/23 | 181/24 | 133/19 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Sternebrae** | | | | | | |
| Incomplete ossification | 49/15 | 63/19 | 53/16 | 51/17 | 57/20 | 108/19 |
| Bipartite | 0 | 2/2 | 1/1 | 3/3 | 0 | 21/9 |
| Extra | 0 | 1/1 | 0 | 0 | 0 | 0 |
| Missing | 4/2 | 9/6 | 4/3 | 5/5 | 4/4 | 108/19 |
| **Total incidences per litters examined** | **2.5** | **3 2 (↑28%)** | **2 3 (↓8%)** | **2.5** | **2.5** | **12.5 (↑400%)** |
| **Ribs** | | | | | | |
| Incomplete ossification | 0 | 0 | 0 | 0 | 1/1 | 2/2 |
| Fused/split | 0 | 0 | 1/1 | 0 | 0 | 17/7 |
| Wavy | 9/6 | 18/10 | 28/14 | 18/9 | 20/12 | 62/16 |
| More than 13 | 2/2 | 0/0 | 2/2 | 5/3 | 3/3 | 104/19 |
| **Total incidences per litters examined** | **0.5** | **0 8 (↑60%)** | **1.2 (↑140%)** | **1 (↑100%)** | **1 (↑100%)** | **9.7 (↑1840%)** |
| **Vertebrae** | | | | | | |
| Incomplete ossification | 8/7 | 15/8 | 6/5 | 15/5 | 14/9 | 127/19 |
| **Incidences per litters examined** | **0.4** | **0.6 (↑50%)** | **0 2 (↓50%)** | **0.6 (↑50%)** | **0.6 (↑50%)** | **6.7 (↑1575%)** |
| **Skull** | | | | | | |
| Incomplete closure | 23/12 | 26/12 | 38/12 | 45/11 | 54/17 | 84/18 |
| **Incidences per litters examined** | **1.1** | **1.1** | **1 5 (↑36%)** | **2 (↑82%)** | **2.2 (↑100%)** | **4.4 (↑300%)** |
| **Extremities** | | | | | | |
| Incomplete ossification | 0 | 1/1 | 2/2 | 0 | 1/1 | 7/5 |
| **Incidences per litters examined** | **0** | **0.04** | **0.08** | **0** | **0.04** | **0.37** |
| **Miscellaneous** | | | | | | |
| Hyoid missing | 27/11 | 40/15 | 17/9 | 42/12 | 25/12 | 78/18 |
| Hyoid reduced | 25/13 | 8/6 | 29/13 | 20/11 | 34/17 | 7/6 |
| **Total incidences per litters examined** | **2.5** | **2 (↓20%)** | **1 8 (↓28%)** | **2.7 (↑8%)** | **2.5** | **4 5 (↑80%)** |

** Results expressed as number of foetuses affected / number of litters affected*
*Statistical analysis not performed*

**<u>Assessment and conclusion by applicant</u>:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

Administration of clove oil to pregnant rats did not induce any maternal toxicity, any effect on litter parameters and any effect on foetal development, even at the highest dose level tested. The NOAEL for maternal and developmental effects was therefore the highest dose tested: 280 mg/kg bw/day clove oil. Assuming clove oil contains 90% eugenol this dose is equivalent to 252 mg/kg bw/day eugenol. There was no evidence that eugenol had a teratogenic effect in this study.

The NOAEL in this study was the highest dose tested; ~252 mg/kg bw/day (based on clove oil contains 90% eugenol). The EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rat and rabbit) and the developmental NOAEL was 250 mg/kg bw/d (rat and rabbit). It was concluded there is no evidence of teratogenicity; target / critical effect: decreased foetal weight and delayed ossification (rat), post-implantation loss (rabbit), maternal clinical signs in both species, with reduced food consumption in rabbits (EFSA Journal 2012;10(11):2914).

**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:** This study presents important deviations such as it was not conducted with the active substance eugenol, the duration of treatment, food consumption was not measured, historical control data were not presented, and importantly, no statistical analysis was performed, therefore, it was deemed as supportive information.

Equivocal evidence of reduction in live foetus, likely caused by the decreased number of implantations was found in the top dose level. In addition, a reduction in the male/female sex ratio was noted in all dose levels. Both findings did not show a dose-response relationship. Regarding reproductive development, increased incidence of incomplete closure of skull with a dose-response pattern was found in the treated groups from 13 mg/kg bw/day dose group. However, the absence of statistical analysis and the incidence of the historical control data, make the results difficult to assess.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

**B.6.6.2.2. Developmental toxicity study in rabbit with clove oil**

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | Morgareidge K. |
| **Report year** | 1973b |
| **Report title** | Teratologic evaluation of FDA 71-30 (Clove oil) in rabbits |
| **Report No** | Food and Drug Research Laboratories Study No. 1576c |
| **Document No** | KCA 5.6.2/02 |
| **Guidelines followed in study** | Non-stated/pre-guideline. |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Eugenol technical was not tested.<br>-Food consumption was not measured.<br>-The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>-The degree of resorption should be described (early, late).<br>-Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.<br>-Statistical analysis was not performed.<br>-Dam necropsies were not reported.<br>-Individual foetal data was not reported.<br>- Historical control data were not provided. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP** | No (prior to the enforcement of GLP) |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Clove oil: FDA-71-30. Lot/Batch No.: Non-stated, Purity: Non-stated |
| **Test system** | Dutch-belted rabbits (female) |
| **Test method** | Groups of 11-15 virgin, adult, Dutch-belted female rabbits given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) *via* the marginal ear vein (day 0). Three hours later, each doe was artificially inseminated with 0.3 ml of diluted semen from a proven donor buck using approximately 20 x $10^6$ motile sperm. Clove oil (diluted in corn oil) was administered by oral gavage to pregnant females at 0, 1.72, 7.99, 37.1 or 172 mg/kg bw/day from gestation day 6 to gestation day 18. An additional group of 12 rabbits received 6-aminonicotinamide (6-AN) at 2.5 mg/kg bw/day as positive control group. Animals were examined daily and bodyweights were recorded on days 0, 6, 12, 18 and 29 of gestation. A caesarean section was performed on all rabbits on day 29 of gestation and the numbers of corpora lutea, implantation sites, resorption sites and live and dead foetuses were recorded. The urogenital tract of each animal was examined in detail for normality. In addition, all foetuses underwent a detailed gross examination for the |

| | |
|---|---|
| | presence of external congenital abnormalities. The live foetuses of each litter were then placed in an incubator for 24 hours for the evaluation of neonatal survival. All surviving pups were sacrificed, and all pups examined for visceral abnormalities (by dissection). All foetuses were then cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Maternal toxicity**

**Survival:** One pregnant dam from low clove oil dose group and another from positive control-group aborted on day 17 and 16, respectively, without evidence of adverse clinical signs.

**Clinical signs**: No adverse clinical signs were recorded throughout the study.

**Bodyweights:** No differences in bodyweights were observed between clove oil-treated groups and negative controls (See table B.6.6.2.2/1). Regarding bodyweight gain, the 7.99 mg/kg bw/day dose group showed lower bodyweight gain values throughout gestation days 0-29 (8-90%) compared with controls, but a dose-relationship was not observed (table B.6.6.2.2/2).

**Table B.6.6.2.2/1. Summary of average bodyweights (kg)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **12** | **18** | **29** |
| **0** | 2.17 | 2.3 | 2.27 | 2.31 | 2.41 |
| **1.72** | 2.36 (109%) | 2.5 (107%) | 2.51 (111%) | 2.58 (112%) | 2.61 (108%) |
| **7.99** | 2.09 (96%) | 2.12 (92%) | 2.1 (92%) | 2.12 (92%) | 2.31 (96%) |
| **37.1** | 2.09 (96%) | 2.41 (105%) | 2.4 (106%) | 2.46 (106%) | 2.62 (109%) |
| **172** | 2.16 (99%) | 2.29 (99%) | 2.3 (101%) | 2.34 (101%) | 2.48 (103%) |
| **2.5 (6-AN)*** | 2.28 (105%) | 2.42 (105%) | 2.43 (107%) | 2.52 (109%) | 2.63 (109%) |

**positive control*
*(% referred to negative control)*
*Statistical analysis not performed*

**Table B.6.6.2.2/2. Summary of average bodyweight gain (kg)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **12** | **18** | **29** |
| **0** | 0 | 0.13 | 0.1 | 0.14 | 0.24 |
| **1.72** | 0 | 0.14 (↑8%) | 0.15 (↑50%) | 0.22 (↑57%) | 0.25 (↑4%) |
| **7.99** | 0 | 0.03 (↓77%) | 0.01 (↓90%) | 0.03 (↓79%) | 0.22 (↓8%) |
| **37.1** | 0 | 0.32 (↑146%) | 0.31 (↑210%) | 0.37 (↑164%) | 0.53 (↑121%) |
| **172** | 0 | 0.13 | 0.14 (↑40%) | 0.18 (↑29%) | 0.32 (↑33%) |
| **2.5 (6-AN)** | 0 | 0.14 (↑8%) | 0.15 (↑50%) | 0.24 (↑71%) | 0.35 (↑43%) |

**positive control*
*(% increase/decrease bwg compared with negative control)*
*Statistical analysis not performed*

**Reproductive performance**

A summary of the main reproductive adverse parameters that present an incidence >10% (considered as potentially relevant by RMS) compared with negative controls are listed below (see table B.6.6.2.2/3):

172 mg/kg bw/day

-Increase in the male/female pups sex ratio (26%).

37.1 mg/kg bw/day

-Decrease in the male/female pups sex ratio (25%).

7.99 mg/kg bw/day

-Increase in the number of resorptions per litter (55%).
-Decrease in the male/female pups sex ratio (21%).

1.72 mg/kg bw/day

-Decrease in the male/female pups sex ratio (45%).

Positive control group

-Decrease in the male/female pups sex ratio (34%).
-Decrease in the average pup weight (18%).

As shown in table B.6.6.2.2/3, all treated groups showed a reduction in the male/female pups sex ratio (21-44%), except the high dose group, so a dose-response was not shown. On the other hand, a non-dose-relationship increase of resorptions was noted in the 7.99 mg/kg/bw day dose group (55%), however, did not effect on the live foetuses mean.

**Table B.6.6.2.2/3. Summary of reproductive performance**

| | **Dose (mg/kg bw/day)** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **1.72** | **7.99** | **37.1** | **172** | **2.5* (6-AN)** |
| **Mated** | 11 | 13 | 12 | 13 | 15 | 12 |
| **Pregnancies** | | | | | | |
| *Total* | 11 | 13 | 10 | 12 | 12 | 12 |
| *pregnancy index* | **100%** | **100%** | **83%** | **92%** | **80%** | **100%** |
| *Aborted* | 0 | 1 | 0 | 0 | 0 | 1 |
| *Dams to term* | 11 | 12 | 10 | 12 | 12 | 11 |
| **Litters examined** | 11 | 12 | 10 | 12 | 12 | 11 |
| **Live litters** | 10 (90%) | 12 (92%) | 9 (90%) | 12 (100%) | 12 (100%) | 11 (92%) |
| **Corpora lutea** | | | | | | |
| *Total* | 140 | 156 | 146 | 174 | 209 | 179 |
| *average/litters examined* | 12.7 | **13 (↑2%)** | **14.6 (↑15%)** | **14.5 (↑14%)** | **17.4 (↑37%)** | **16.2 (↑27%)** |
| **Implant sites** | | | | | | |
| *Total* | 50 | 70 | 57 | 77 | **64** | 81 |
| *average/litters examined* | 4.5 | **5.8 (↑29%)** | **5.7 (↑27%)** | **6.4 (↑42%)** | **5.3 (↑18%)** | **7.3 (↑62%)** |
| **Resorptions** | | | | | | |
| *Total* | 5 | 4 | 7 | 2 | 4 | 5 |
| *resorptions/litters examined* | 0.45 | **0.33 (↓27%)** | **0.7 (↑55%)** | **0.16 (↓65%)** | **0.33 (↓27%)** | **0.45** |
| **Dead foetuses** | | | | | | |
| *Total* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dead foetuses/litters examined* | 0 | **0** | **0** | **0** | **0** | **0** |
| **Live foetuses** | | | | | | |
| *Total* | 45 | 65 | 50 | 75 | 60 | 76 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *average/litters examined* | 4 | **5.4 (↑35%)** | **5 (↑25%)** | **6.25 (↑56%)** | **5 (↑25%)** | **6.9 (↑72%)** |
| *sex ratio (♂/♀)* | 1.37 | **0.76 (↓45%)** | **1.08 (↓21%)** | **1.03 (↓25%)** | **1.73 (↑26%)** | **0.9 (↓34%)** |
| **Average foetus weight (g)** | 40 | 39.9 (↓0.2%) | 40.6 (↑1%) | 40.2 (↑0.5%) | 40.4 (↑1%) | 32.8 (↓18%) |

**positive control*
*Statistical analysis not performed*

**Necropsy**

Skeletal findings revealed an increase of ossification incidences on sternebrae (mostly incomplete ossification, bipartite, fused and extra) in all treated groups compared with negative controls, however no clear dose-response relationship was observed.

On the other hand, the incidence of another external, visceral and skeletal abnormalities was broadly similar between clove oil treated groups and negative controls (data not shown).

The positive control 6-AN group showed high incidences of skeletal findings (scramble and scoliosis of vertebrae) and visceral malformations (mostly anopia, medial rotation of hind limbs, and short tail; data not shown).

**Table B.6.6.2.2/4. Summary of skeletal findings**

| Skeletal finding | Dose group (mg/kg bw/d)* | | | | | |
|---|---|---|---|---|---|---|
| | Negative control | Clove oil | | | | Positive control 6-AN |
| | **0** | **1.72** | **7.99** | **37.1** | **172** | **2.5** |
| **Litters examined** | **10** | **12** | **9** | **12** | **12** | **11** |
| Live foetuses examined | 45/10 | 65/12 | 50/9 | 75/12 | 60/12 | 76/11 |
| **Sternebrae** | | | | | | |
| Incomplete ossification | 0 | 5/4 | 3/3 | 3/3 | 2/2 | 4/3 |
| Bipartite | 0 | 0 | 2/2 | 2/2 | 1/1 | 0 |
| Fused | 0 | 4/2 | 0 | 2/2 | 0 | 4/3 |
| Extra | 0 | 3/2 | 0 | 1/1 | 5/4 | 4/2 |
| Missing | 1/1 | 1/1 | 0 | 0 | 0 | 0 |
| **Total incidences per litters examined** | **0.1** | **1.08 (↑980%)** | **0.55 (↑450%)** | **0.66 (↑560%)** | **0.66 (↑560%)** | **1.09 (↑990%)** |
| **Ribs** | | | | | | |
| Fused/split | 0 | 1/1 | 0 | 0 | 0 | 11/4 |
| **Total incidences per litters examined** | **0** | **0.83** | **0** | **0** | **0** | **1** |
| **Vertebrae** | | | | | | |
| scrambled | 0 | 1/1 | 0 | 0 | 0 | 13/4 |
| scoliosis | 0 | 1/1 | 0 | 0 | 0 | 17/7 |
| **Incidences per litters examined** | **0** | **0.17** | **0** | **0** | **0** | **2.73** |
| **Skull** | | | | | | |
| Incomplete closure | 0 | 1/1 | 1/1 | 0 | 0 | 0 |
| **Incidences per litters examined** | **0** | **0.083** | **0.11** | **0** | **0** | **0** |

** Results expressed as number of foetuses affected / number of litters affected*
*Statistical analysis not performed*

**Assessment and conclusion by applicant:**

| |
|---|
| The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br><br>Administration of clove oil to pregnant rabbits did not induce any maternal toxicity, any effect on litter parameters and any effect on foetal development, even at the highest dose level tested. The NOAEL for maternal and developmental effects was therefore the highest dose tested: 172 mg/kg bw/day clove oil. Assuming clove oil contains 90% eugenol this dose is equivalent to 155 mg/kg bw/day eugenol. There was no evidence that eugenol had a teratogenic effect in this study.<br><br>The NOAEL in this study was the highest dose tested; ~155 mg/kg bw/d. The EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rat and rabbit) and the developmental NOAEL was 250 mg/kg bw/d (rat and rabbit). It was concluded there is no evidence of teratogenicity; target / critical effect: decreased foetal weight and delayed ossification (rat), post-implantation loss (rabbit), maternal clinical signs in both species, with reduced food consumption in rabbits (EFSA Journal 2012;10(11):2914). |
| **Assessment and conclusion by RMS:**<br><br>**Outcome and conclusion of the study:** This study presents important deviations such as it was not conducted with the active substance eugenol, the duration of treatment, food consumption was not measured, historical control data were not presented, and importantly, no statistical analysis was performed, therefore, it was deemed as supportive information.<br><br>No effects on reproductive parameters were observed after clove oil oral administration to female rabbits. On the other hand, no teratogenic effects were noted on skeleton and viscera. Only increased ossification incidences on sternebrae were recorded in all treated group, in which no clear dose-response relationship was observed. These observations were likely due to the low mean incidence of negative controls, but without HCD incidences and statistical analysis, a proper assessment it is difficult to conduct.<br><br>NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established. |

**B.6.6.2.3. Developmental toxicity study in mice**

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | Morgareidge K. |
| **Report year** | 1973c |
| **Report title** | Teratologic evaluation of FDA 71-30 (Clove oil) in mice |
| **Report No** | Food and Drug Research Laboratories Study No. 1573c |
| **Document No** | KCA 5.6.2/03 |
| **Guidelines followed in study** | Non-stated/pre-guideline. |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Eugenol technical was not tested.<br>-Food consumption was not measured.<br>-The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>-The degree of resorption should be described (early, late).<br>-Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.<br>-Statistical analysis was not performed.<br>-Dam necropsies were not reported.<br>-Individual foetal data was not reported.<br>- Historical control data were not provided. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP** | No (prior to the enforcement of GLP) |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Clove oil: FDA-71-30. Lot/Batch No.: Non-stated, Purity: Non-stated |

| | |
|---|---|
| **Test system** | Albino CD-1 (females) mice |
| **Test method** | Clove oil was tested at dose levels of 0, 2.2, 10, 46 or 215 mg/kg bw/day, in which 23-25 mated females were assigned to each test group. Clove oil (diluted in corn oil) was administered by oral gavage from gestation day 6 to gestation day 15. Negative controls were treated with the vehicle at a level equivalent to the group receiving the highest test dose. An additional group of 24 mated females received aspirin (150 mg/kg bw/day) as a positive control. Animals were examined daily and bodyweights were recorded on days 0, 6, 11, 15 and 17 of gestation. A caesarean section was performed on all mice on day 17 of gestation and the numbers of implantation sites, resorption sites, live and dead foetuses were recorded. The urogenital tract of each dam was also examined for abnormalities. All live foetuses were weighed and all foetuses were examined externally for the presence of abnormalities. One third of the foetuses underwent detailed visceral examination and the remaining two thirds were examined for skeletal defects. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Maternal toxicity**

**Survival:** All pregnant dams survived at study termination.

**Clinical signs**: No adverse clinical signs were recorded throughout the study.

**Bodyweights:** No differences in bodyweights were observed between clove oil-treated groups and negative controls (See table B.6.6.2.3/1). Regarding bodyweight gain, an extensive decrease > 10% was recorded in all clove-oil treated groups throughout gestation days 0-11 (13-45%) compared with controls. During the last gestation week, bodyweight gain decrease was less marked (6-14%) in the clove oil treated groups than first 11 days of gestation, being >10% only in the 46 mg/kg bw/day dose group. Overall, no dose-response pattern was noted throughout measurements points (table B.6.6.2.3/2).

**Table B.6.6.2.3/1. Summary of average bodyweights (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **11** | **15** | **17** |
| **0** | 31.2 | 35.4 | 38 | 46.2 | 51.9 |
| **2.2** | 31.1 (99.6%) | 33.4 (94.3%) | 35 (92.1%) | 48.1 (104.1%) | 50.6 (97.5%) |
| **10.0** | 31.4 (100.6%) | 33.7 (95.2%) | 37 (97.4%) | 45.3 (98%) | 50.9 (98%) |
| **46.0** | 30.5 (97.8%) | 33.6 (95.2%) | 36 (94.7%) | 43.4 (94%) | 48.6 (93.6%) |
| **215.0** | 30.8 (98.7%) | 34.1 (96.3%) | 36.7 (96.6%) | 45.8 (99.1%) | 49.4 (95.2%) |
| **150 (aspirin)*** | 30.1 (96.5%) | 34.4 (97.1%) | 36.3 (95 5) | 44.4 (96.1%) | 49.7 (95.7%) |

**positive control*
*(% referred to negative control)*
*Statistical analysis not performed*

**Table B.6.6.2.3/2. Summary of average bodyweight gain (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **11** | **15** | **17** |
| **0** | 0 | 4.2 | 6.8 | 15 | 20.7 |
| **2.2** | 0 | 2.3 (↓45%) | 3.9 (↓43%) | 17 (↑13%) | 19.5 (↓6%) |
| **10.0** | 0 | 2.3 (↓45 %) | 5.6 (↓18%) | 13.9 (↓7%) | 19.5 (↓6%) |
| **46.0** | 0 | 3.1 (↓26%) | 5.5 (↓19%) | 12.9 (↓14%) | 18.1 (↓13) |
| **215.0** | 0 | 3.3 (↓21%) | 5.9 (↓13%) | 15 | 18.6 (↓10%) |

| | | | | | |
|---|---|---|---|---|---|
| **150 (aspirin)*** | 0 | 4.3 (↑2%) | 6.2 (↓9%) | 14.3 (↓5%) | 19.6 (↓5%) |

**positive control*
*(% increase/decrease bwg compared with negative control)*
*Statistical analysis not performed*

**Reproductive performance**

A summary of the main reproductive adverse parameters that present an incidence >10% (considered as potentially relevant by RMS) compared with negative controls are listed below (see table B.6.6.2.3/3):

215 mg/kg bw/day

-Increase the number of dead foetuses per litter (211%; 3 dead foetuses vs 1 dead foetus in controls).

46 mg/kg bw/day

-Increase the number of dead foetuses per litter (84%; 2 dead foetuses vs 1 dead foetus in controls).

10 mg/kg bw/day

-Increase the number of dead foetuses per litter (93%; 2 dead foetuses vs 1 dead foetus in controls).
-Decrease in the male/female pups sex ratio (21%).

Positive control

-Increase the number of resorptions (38%).

As shown in table B.6.6.2.3/3, the negative control and low clove oil dose group presented a lower pregnancy index (88%) compared with the rest of treated groups. No effect of treatment on resorptions and average foetus weight were recorded. A slight increase on total deaths and foetal deaths per litter were observed, however, no effect in live foetuses per litter was noted.

**Table B.6.6.2.3/3. Summary of reproductive performance**

| | **Dose (mg/kg bw/day)** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **2 2** | **10** | **46** | **215** | **150*** |
| **Mated** | 25 | 25 | 23 | 25 | 24 | 24 |
| **Pregnancies** | | | | | | |
| *Total* | 22 | 22 | 23 | 24 | 22 | 23 |
| *pregnancy index* | **88%** | **88%** | **100%** | **96%** | **92%** | **96%** |
| *Died or aborted* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dams to term* | 22 | 22 | 23 | 24 | 22 | 23 |
| **Litters examined** | 22 | 22 | 23 | 24 | 22 | 23 |
| **Live litters** | 22 (100%) | 22 (100%) | 23 (100%) | 24 (100%) | 22 (100%) | 23 (100%) |
| **Implant sites** | | | | | | |
| *Total* | 264 | 241 | 277 | 268 | **246** | 274 |
| *average/litter* | 12 | **11 (↓8%)** | **12** | **11.2 (↓7%)** | **11.2 (↓7%)** | **12** |
| **Resorptions** | | | | | | |
| *Total* | 14 | 10 | 3 | 11 | 3 | 20 |
| *resorptions/litter* | 0.63 | **0.45 (↓28%)** | **0.13 (↓79)** | **0.46 (↓27%)** | **0.14 (↓78%)** | **0.87 (↑38%)** |
| **Dead foetuses** | | | | | | |
| *Total* | 1 | 1 | 2 | 2 | 3 | 1 |
| *dams with ≥1* | 1 | 1 | 2 | 2 | 3 | 1 |
| *dams with all dead* | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Dead foetuses/litter* | 0.045 | **0.045** | **0.087 (↑93%)** | **0.083 (↑84%)** | **0.14 (↑211%)** | **0.043 (↓4%)** |
| **Live foetuses** | | | | | | |
| *Total* | 249 | 230 | 272 | 255 | 240 | 253 |
| *average/litter* | 11.3 | **10.5 (↓7%)** | **11.8 (↑4%)** | **10.6 (↓6%)** | **10.9 (↓4%)** | **11 (↓3%)** |
| *sex ratio (♂/♀)* | 0.98 | **1.08 (↑10%)** | **0.77 (↓21%)** | **1.08 (↑10%)** | **0.89 (↓9%)** | **0.85 (↓13%)** |
| **Average foetus weight (g)** | 0.9 | 0.94 **(↑4%)** | 0.87 **(↓3%)** | 0.9 | 0.86 **(↓4%)** | 0.85 (↓6%) |

**positive control*
*Statistical analysis not performed*

**Necropsy:** No effect of treatment was found regarding skeletal alterations (table B.6.6.2.3/4). On the other hand, no visceral findings were described in the clove oil-treated groups (data not shown).

**Table B.6.6.2.3/4. Summary of skeletal findings**

| **Skeletal finding** | **Dose group (mg/kg bw/d)*** | | | | | |
|---|---|---|---|---|---|---|
| | **Negative control** | **Clove oil** | | | | **Positive control (aspirin)** |
| | **0** | **2.2** | **10** | **46** | **215** | **150*** |
| **Litters examined** | **22** | **22** | **23** | **24** | **22** | **23** |
| Live foetuses examined | 176/22 | 161/22 | 186/23 | 180/24 | 168/22 | 177/23 |
| **Sternebrae** | | | | | | |
| Incomplete ossification | 91/18 | 37/14 | 74/19 | 61/20 | 67/17 | 73/20 |
| Bipartite | 9/8 | 2/2 | 3/3 | 4/3 | 6/5 | 4/4 |
| Missing | 14/8 | 11/7 | 26/11 | 17/12 | 27/9 | 27/12 |
| **Total incidences per litters examined** | **5.2** | **2.3 (↓56%)** | **4.5 (↓13%)** | **3.4 (↓35%)** | **4.5 (↓13%)** | **4.5 (↓13%)** |
| **Ribs** | | | | | | |
| Fused/split | 0 | 0 | 0 | 0 | 1/1 | 0 |
| More than 13 | 45/16 | 53/18 | 46/16 | 49/17 | 38/16 | 34/12 |
| **Total incidences per litters examined** | **2** | **2.4 (↑20%)** | **2** | **2** | **1.8 (↓10%)** | **1.5 (↓25%)** |
| **Vertebrae** | | | | | | |
| Incomplete ossification | 5/4 | 4/2 | 6/4 | 3/2 | 2/2 | 6/2 |
| **Incidences per litters examined** | **0.22** | **0.18 (↓18%)** | **0.26 (↑18%)** | **0.12 (↓43%)** | **0.09 (↓59%)** | **0.26 (↑18%)** |
| **Skull** | | | | | | |
| Incomplete closure | 4/3 | 0 | 1/1 | 0 | 0 | 3/2 |
| **Incidences per litters examined** | **0.18** | **0** | **0.043 (↓76%)** | **0** | **0** | **0.13 (↓28%)** |
| **Extremities** | | | | | | |
| Incomplete ossification | 6/4 | 3/2 | 5/5 | 5/3 | 2/2 | 5/1 |
| **Incidences per litters examined** | **0.27** | **0.14 (↓48%)** | **0.22 (↓19%)** | **0.21 (↓22%)** | **0.09 (↓67%)** | **0.22 (↓19%)** |
| **Miscellaneous** | | | | | | |
| Hyoid missing | 37/15 | 23/12 | 38/13 | 35/17 | 44/15 | 59/18 |
| Hyoid reduced | 36/14 | 29/16 | 19/9 | 20/12 | 27/16 | 26/13 |
| **Total incidences per litters examined** | **3.3** | **2.4 (↓27%)** | **2.5 (↓24%)** | **2.3 (↓30%)** | **3.2 (↓3%)** | **3.7 (↑12%)** |

** Results expressed as number of foetuses affected / number of litters affected*

*Statistical analysis not performed*

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>Administration of clove oil to pregnant mice did not induce any maternal toxicity, any effect on litter parameters and any effect on foetal development, even at the highest dose level tested. The NOAEL for maternal and developmental effects was therefore the highest dose tested: 215 mg/kg bw/day clove oil. Assuming clove oil contains 90% eugenol this dose is equivalent to 194 mg/kg bw/day eugenol. There was no evidence that eugenol had a teratogenic effect in this study.<br>The NOAEL in this study was the highest dose tested; ~194 mg/kg bw/d. The EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rat and rabbit) and the developmental NOAEL was 250 mg/kg bw/d (rat and rabbit). It was concluded there is no evidence of teratogenicity; target / critical effect: decreased foetal weight and delayed ossification (rat), post-implantation loss (rabbit), maternal clinical signs in both species, with reduced food consumption in rabbits (EFSA Journal 2012;10(11):2914). |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This study presents important deviations such as it was not conducted with the active substance eugenol, the duration of treatment, food consumption was not measured, historical control data were not presented, and importantly, no statistical analysis was performed, therefore, it was deemed as supportive information.<br>No effects on reproductive parameters were observed after clove oil oral administration to female mice. On the other hand, no teratogenic effects were noted on skeleton and viscera.<br>NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established. |

**B.6.6.2.4. Developmental toxicity study in hamsters with clove oil**

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | Morgareidge K. |
| **Report year** | 1973d |
| **Report title** | Teratologic evaluation of FDA 71-30 (Clove oil) in hamsters |
| **Report No** | Food and Drug Research Laboratories Study No. 1575c |
| **Document No** | KCA 5.6.2/04 |
| **Guidelines followed in study** | Non-stated/pre-guideline. |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Eugenol technical was not tested.<br>-Food consumption was not measured.<br>-The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>-The degree of resorption should be described (early, late).<br>-Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.<br>-Statistical analysis was not performed.<br>-Dam necropsies were not reported.<br>-Individual foetal data was not reported.<br>- Historical control data were not provided. |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP** | No (prior to the enforcement of GLP) |
| **Acceptability/ reliability** | Supportive only (See Assessment and conclusion by RMS) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Clove oil: FDA-71-30. Lot/Batch No.: Non-stated, Purity: Non-stated |
| **Test system** | Golden hamsters (females) |
| **Test method** | Clove oil was tested at dose levels of 0, 1.8, 8.2, 38.2 or 177 mg/kg bw/day, in which 25-28 mated females were assigned to each test group. Clove oil (diluted in corn oil) was administered by oral gavage from gestation day 6 to gestation day 15. Negative controls were treated with the vehicle at a level equivalent to the group receiving the highest test dose. An additional group of 25 mated females received aspirin (250 mg/kg bw/day) as a positive control. Animals were examined daily and bodyweights were recorded on days 0, 6, 8, 10 and 14 of gestation. A caesarean section was performed on all hamsters on day 14 of gestation and the numbers of implantation sites, resorption sites, live and dead foetuses were recorded. The urogenital tract of each dam was also examined for abnormalities. All live foetuses were weighed and all foetuses were examined externally for the presence of abnormalities. One third of the foetuses underwent detailed visceral examination and the remaining two thirds were examined for skeletal defects. |
| **Statistical analysis of results** | Not performed. |

**Results**

**Maternal toxicity**

**Survival:** All pregnant dams survived at study termination.

**Clinical signs**: No adverse clinical signs were recorded throughout the study.

**Bodyweights:** No relevant differences in bodyweights were observed between clove oil-treated groups and negative controls (See table B.6.6.2.4/1). Regarding bodyweight change, loss of bodyweight was observed in the 1.8 and 8.2 mg/kg bw/day dose groups at gestation day 6. Moreover, in these two groups, bodyweight gain values were lower (>10%) than controls throughout gestation period, although the difference with controls was gradually reduced over time. On the other hand, in 38.2 and 177 mg/kg bw/day dose groups, the bodyweight gains was broadly similar between clove oil treated groups and negative controls, being slightly higher in the gestation day 6 (table B.6.6.2.4/2). As described, a dose-relationship was not shown.

**Table B.6.6.2.4/1. Summary of average bodyweights (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **8** | **10** | **14** |
| **0** | 122 1 | 122 2 | 126.6 | 136.4 | 157.7 |
| **1.8** | 120 (98.3%) | 118.6 (97%) | 120.1 (95%) | 131.5 (96.4%) | 154.5 (98%) |
| **8.2** | 122.5 (100.3%) | 122.1 (99.9%) | 124.5 (98.3%) | 134.7 (98.7%) | 154 (97.6%) |
| **38.2** | 122.6 (100.4%) | 123.6 (101.1%) | 125.7 (99.3%) | 136.7 (100.2%) | 157.3 (99.7%) |
| **177.0** | 119.5 (97.8%) | 121.3 (99.2%) | 124.5 (98.3%) | 135 (99%) | 154.8 (98.2%) |
| **250 (aspirin)*** | 127.8 (104.7%) | 128.4 (105%) | 132.6 (104.7%) | 144.2 (105.7%) | 168.6 (107%) |

**positive control*
*(% referred to negative control)*
*Statistical analysis not performed*

**Table B.6.6.2.4/2. Summary of average bodyweight gain (grams)**

| | Gestation day | | | | |
|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **0** | **6** | **8** | **10** | **14** |
| **0** | 0 | 0.1 | 4.5 | 14.3 | 35.6 |

| | | | | | |
|---|---|---|---|---|---|
| **1.8** | 0 | -1.4 (↓1500%) | 0.1 (↓98%) | 11.5 (↓20%) | 34.5 (↓3%) |
| **8.2** | 0 | -0.4 (↓500%) | 2 (↓56%) | 12.2 (↓15%) | 31.5 (↓12%) |
| **38.2** | 0 | 1 (↑900%) | 3.1 (↓31%) | 14.1 (↓1%) | 34.7 (↓3%) |
| **177.0** | 0 | 1.8 (↑1700%) | 5 (↑11%) | 15.5 (↑8%) | 35.3 (↓1%) |
| **250 (aspirin)*** | 0 | 0.6 (↑500%) | 4.8 (↑7%) | 16.4 (↑15%) | 40.8 (↑15%) |

**positive control*
*(% increase/decrease bwg compared with negative control)*
*Statistical analysis not performed*

**Reproductive performance**

A summary of the main reproductive adverse parameters that present an incidence >10% (considered as potentially relevant by RMS) compared with negative controls are listed below (see table B.6.6.2.4/3):

177 mg/kg bw/day

-Increase the sex ratio male/female (10%).

38.2 mg/kg bw/day

-Increase the number of resorptions per litter (250%).
-Increase the number of death foetuses per litter (212%; 3 death foetuses vs 1 death foetus in controls).
-Increase the sex ratio male/female (21%).

8.2 mg/kg bw/day

-Increase the number of resorptions per litter (115%)
-Increase the number of death foetuses per litter (588%; 7 death foetuses vs 1 death foetus in controls).
-Increase the sex ratio male/female (49%).

1.8 mg/kg bw/day

-Increase the number of resorptions per litter (35%).
-Increase the sex ratio male/female (30%).

Positive control

-Increase the number of resorptions per litter (45%).

As shown in table B.6.6.2.4/3, the negative control and clove oil treated groups displayed a pregnancy index between 71-88%. In the positive control group, a dam had a premature natural birth at gestation day 13 with alive foetuses. However, the authors of the study did not account this litter in the overall assessment.

No differences in live litters, mean of live foetuses per litter and pups bodyweights were described, compared with negative controls. An increased number of resorptions per litter were observed in the 1.8, 8.2 and 38.2 mg/kg bw/day (35, 115 and 250%, respectively) dose groups, but no at high dose group, so a dose-reponse was not noted. Moreover, the total death foetuses and the average per litter were increased in the 8.2 and 38.2 mg/kg bw/day dose group. These observations were primarily due to one dam of the 8.2 mg/kg bw/day dose group had 5 late resorptions and the whole 5 foetuses of the litter were still-born, and another dam of 38.2 mg/kg bw/day dose group that had 8 resorptions and consequently, no offspring was bred. However, neither the increase in the number of resorptions nor the increased number of death foetuses, affect the number and rate of live foetuses.

On the other hand, the sex ratio male/female was increased in all the treated groups, including positive control group treated with aspirin, compared with negative controls. These could be explained by the low sex ratio displayed by the group treated with vehicle (0.6). However, a dose-response was not observed in treated groups.

**Table B.6.6.2.4/3. Summary of reproductive performance**

| | Dose (mg/kg bw/day) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **1.8** | **8.2** | **38.2** | **177** | **250*** |
| **Mated** | 27 | 25 | 26 | 28 | 25 | 25 |
| **Pregnancies** | | | | | | |
| *Total* | 21 | 22 | 21 | 20 | 21 | 25 |
| *pregnancy index* | **77%** | **88%** | **80%** | **71%** | **84%** | **100%** |
| *Premature births* | 0 | 0 | 0 | 0 | 0 | 1 |
| *Dams to term* | 21 | 22 | 21 | 20 | 21 | 24 |
| **Litters examined** | 21 | 22 | 21 | 20 | 21 | 24 |
| **Live litters** | 21 (100%) | 22 (100%) | 20 (95%) | 19 (95%) | 21 (100%) | 25 (100%) |
| **Implant sites** | | | | | | |
| *Total* | 270 | 292 | 276 | 278 | 270 | 359 |
| *average/litters examined* | 12.8 | **13.3 (↑4%)** | **13.1 (↑2%)** | **13.9 (↑9%)** | **12.8** | **15 (↑17%)** |
| **Resorptions** | | | | | | |
| *Total* | 4 | 6 | 9 | 14 | 3 | 7 |
| *resorptions/litters examined* | 0.2 | **0.27 (↑35%)** | **0.43 (↑115%)** | **0.7 (↑250%)** | **0.14 (↓30%)** | **0.29 (↑45%)** |
| **Dead foetuses** | | | | | | |
| *Total* | 1 | 0 | 7 | 3 | 1 | 1 |
| *dams with ≥1* | 1 | 0 | 3 | 3 | 1 | 1 |
| *dams with all dead* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dead foetuses/litters examined* | 0.048 | **0** | **0.33 (↑588%)** | **0.15 (↑212%)** | **0.048** | **0.042 (↓12%)** |
| **Live foetuses** | | | | | | |
| *Total* | 265 | 286 | 260 | 261 | 266 | 351 |
| *average/litters examined* | 12.6 | **13 (↑3%)** | **12.4 (↓2%)** | **13 (↑3%)** | **12.7 (↑1%)** | **14.6 (↑16%)** |
| *sex ratio (♂/♀)* | 0.61 | **0.79 (↑30%)** | **0.91 (↑49%)** | **0.74 (↑21%)** | **0.67 (↑10%)** | **0.76 (↑25%)** |
| **Average foetus weight (g)** | 1.79 | 1.77 **(↓1%)** | 1.78 **(↓1%)** | 1.76 **(↓2%)** | 1.80 **(↑1%)** | 1.72 (↓4%) |

**positive control*
*Statistical analysis not performed*

**Necropsy:** A summary of the main adverse skeletal incidences >10% are listed below (see table B.6.6.2.4/4):

177 mg/kg bw/day

-Increase ossification incidences in the sternebrae (31%).
-Increase incomplete ossification in the extremities (80%).
-Increase hyoid missing or reduced incidences (48%).

38.2 mg/kg bw/day

-Increase ossification incidences in the sternebrae (40%).

8.2 mg/kg bw/day

-Increase ossification incidences in the sternebrae (27%).

1.8 mg/kg bw/day

-Increase ossification incidences in the sternebrae (20%).
-Increase more than 13 ribs incidences (22%).
-Increase incomplete ossification in the extremities (24%).

Positive control

-Increase ossification incidences in the sternebrae (89%).
-Increase incomplete ossification in the extremities (72%).

Increased no clearly dose-related ossification incidences in the sternebrae were described in the clove oil-treated groups. Moreover, increased incomplete ossification incidences of the extremities were described in the low and top dose groups (24 and 80%, respectively), whereas increased incidences of hyoid reduced or missing were noted only in the top dose group (48%).

On the other hand, no visceral findings were described in the clove-oil treated groups (data not shown).

**Table B.6.6.2.4/4. Summary of skeletal findings**

| Skeletal finding | Dose group (mg/kg bw/d)* | | | | | |
|---|---|---|---|---|---|---|
| | Negative control | Clove oil | | | | Positive control (aspirin) |
| | 0 | 1.8 | 8.2 | 38.2 | 177 | 250* |
| **Litters examined** | **21** | **22** | **20** | **19** | **21** | **24** |
| Live foetuses examined | 1184/21 | 195/22 | 178/20 | 179/19 | 186/21 | 244/24 |
| **Sternebrae** | | | | | | |
| Incomplete ossification | 70/17 | 91/20 | 95/20 | 86/18 | 101/18 | 160/23 |
| Bipartite | 19/13 | 24/17 | 27/14 | 29/13 | 20/12 | 31/8 |
| Fused | 0 | 0 | 0 | 0 | 0 | 1/1 |
| Extra | 2/2 | 3/2 | 0 | 9/6 | 0 | 1/1 |
| Missing | 25/9 | 27/10 | 19/8 | 22/10 | 30/8 | 56/17 |
| **Total incidences per litters examined** | **5.5** | **6.6 (↑20%)** | **7 (↑27%)** | **7.7 (↑40%)** | **7.2 (↑31%)** | **10.4 (↑89%)** |
| **Ribs** | | | | | | |
| Fused/split | 0 | 0 | 0 | 0 | 1/1 | 0 |
| More than 13 | 68/18 | 86/20 | 55/14 | 45/16 | 52/18 | 55/19 |
| **Total incidences per litters examined** | **3.2** | **3.9 (↑22%)** | **2.8 (↓13%)** | **2.4 (↓25%)** | **2.5 (↓22%)** | **2.3 (↓28%)** |
| **Vertebrae** | | | | | | |
| Incomplete ossification | 7/4 | 3/2 | 1/1 | 3/2 | 7/2 | 0 |
| Fused | 1/1 | 0 | 0 | 0 | 0 | 0 |
| Scoliosis | 0 | 0 | 0 | 0 | 0 | 2/2 |
| **Incidences per litters examined** | **0.38** | **0.14 (↓63%)** | **0.05 (↓87%)** | **0.16 (↓58%)** | **0.33 (↓13%)** | **0.083 (↓78%)** |
| **Skull** | | | | | | |
| Incomplete closure | 0 | 0 | 0 | 1/1 | 0 | 1/1 |
| **Incidences per litters examined** | **0** | **0** | **0** | **0.053** | **0** | **0.041** |
| **Extremities** | | | | | | |
| Incomplete ossification | 6/3 | 8/4 | 4/2 | 4/2 | 11/4 | 12/7 |
| **Incidences per litters examined** | **0.29** | **0.36 (↑24%)** | **0.2 (↓31%)** | **0.21 (↓28%)** | **0.52 (↑80%)** | **0.5 (↑72%)** |
| **Miscellaneous** | | | | | | |
| Hyoid missing | 2/2 | 3/1 | 0 | 0 | 4/1 | 2/2 |
| Hyoid reduced | 8/5 | 6/5 | 6/4 | 4/4 | 11/5 | 7/6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Total incidences per litters examined** | **0.48** | **0.4 (↓17%)** | **0.3 (↓38%)** | **0.21 (↓56%)** | **0.71 (↑48%)** | **0.37 (↓23%)** |

** Results expressed as number of foetuses affected / number of litters affected*
*Statistical analysis not performed*

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

Administration of clove oil to pregnant hamsters did not induce any maternal toxicity, any effect on litter parameters and any effect on foetal development, even at the highest dose level tested. The NOAEL for maternal and developmental effects was therefore the highest dose tested: 177 mg/kg bw/day clove oil. Assuming clove oil contains 90% eugenol this dose is equivalent to 159 mg/kg bw/day eugenol. There was no evidence that eugenol had a teratogenic effect in this study.

The NOAEL in this study was the highest dose tested; ~159 mg/kg bw/d. The EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rat and rabbit) and the developmental NOAEL was 250 mg/kg bw/d (rat and rabbit). It was concluded there is no evidence of teratogenicity; target / critical effect: decreased foetal weight and delayed ossification (rat), post-implantation loss (rabbit), maternal clinical signs in both species, with reduced food consumption in rabbits (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study presents important deviations such as it was not conducted with the active substance eugenol, the duration of treatment, food consumption was not measured, historical control data were not presented, and importantly, no statistical analysis was performed, therefore, it was deemed as supportive information.

Increased number of resorptions and foetal death were described in the 1.8, 8.2 and 38.2 mg/kg bw/day dose groups, without compromising the number and rate of live foetuses. A dose-related pattern was not seen due to these incidences were not reproduced in the top dose level. Regarding skeletal incidences, equivocal ossification incidences in the sternebrae were recorded in the clove-oil treated groups. In addition, increased incidences of hyoid reduced or missing were noted in the top dose group. However, the absence of statistical analysis and the incidence of the historical control data, make the results difficult to assess.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

### B.6.6.2.5. Developmental toxicity study in rats with eugenol

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | [redacted] |
| **Report year** | 2004 |
| **Report title** | Eugenol: Oral gavage pre-natal developmental toxicity study in the rat. |
| **Report No** | [redacted] Project number: 1620/034 |
| **Document No** | KCA 5.6.2/05 (Study included by the RMS, extracted from Clove oil renewal dossier and used for the first time in the Clove oil DAR-2008) |
| **Guidelines followed in study** | OECD Guideline 414 (2001). |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Purity of test substance not stated.<br>-The following developmental endpoints were not measured: resorptions, anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>- Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.<br>- Historical control data sources are not detailed in the study report. |
| **Previous evaluation** | No, not included in DAR 2011. Taked into account in the peer review of eugenol (EFSA Journal 2012; 10,11: 2914) |

| | |
|---|---|
| **GLP** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 29424, purity not specified. |
| **Test system** | Sprague-Dawley CD rats |
| **Test method** | Four groups of 25 mated females Sprague-Dawley CD rats were dosed with 0, 100, 250, 600 mg/kg bw/day throughout day 5-19 of gestation.<br><br>The test material was suspended daily in 1% carboxymethyl cellulose (vehicle), and subsequently administered *via* oral gavage using a rubber catheter and plastic syringe at a constant dose volume of 10 ml/kg bodyweight.<br><br>*Morbidity/Mortality:* All females were checked twice daily during the normal working week and once daily at weekends for signs of morbidity.<br><br>*Clinical Observations:* All females were observed once daily, in the morning throughout gestation and, additionally, one hour after dosing, throughout the dosing period.<br><br>*Bodyweight:* All females were weighed on gestation day 3, 6- 9, 12, 15, 18 and 20.<br><br>*Food Consumption:* Food consumption for individual animals was recorded during days 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20.<br><br>*Necropsy*: All surviving females were killed on day 20 of gestation by carbon dioxide asphyxiation, followed by cervical dislocation. Each animal was examined externally and internally for macroscopic abnormalities. The ovaries and uteri of pregnant females were removed, examined and the following data recorded:<br><br>Gravid uterus weight, pregnancy status, number of corpora lutea, number position and type of intrauterine implantation, foetal sex, external foetal appearance, foetal weight and placental weight.<br><br>The foetuses were killed by subcutaneous injection of sodium pentobarbitone. Except where conditions dictate otherwise, alternate foetuses were identified using an indelible marker and placed in Bouin's fixative. Foetuses were transferred to 90% industrial methylated spirits (IMS) in distilled water and examined for visceral anomalies under a low power binocular microscope The remaining foetuses were identified using colour coded wires and placed in 70% IMS in distilled water. The foetuses were eviscerated, processed and the skeletons stained with alizarin red. The foetuses were examined for skeletal development and anomalies.<br><br>*Evaluation of Data*<br><br>Data were processed to give litter mean values, group mean values and standard deviations. |
| **Statistical analysis of results** | The following parameters were analysed statistically, where appropriate using the test methods outlined below:<br><br>Female bodyweight change and food consumption: Bartlett's test for homogeneity of variance and one way analysis of variance; followed by Dunnetts multiple comparison test; or if unequal variances were observed, an alternative multiple comparison test was performed. All caesarean necropsy parameters, foetal parameters: Kruskal-Wallis non-parametric analysis of variance; and a subsequent pairwise analysis of control values against treated values using the Mann-Whitney 'U' test, where significance was seen. Foetal evaluation parameters, including skeletal or visceral findings: Kruskal-Wallis nonparametric analysis of variance and Mann-Whitney 'U' test. |
| **Historical control data (HCD)** | HCD sources are not detailed in the study report., so its validity cannot be determined.<br><br>**Visceral findings of HCD in rat teratology studies.**<br><br>[illegible] |

| | **% *( range from most recent studies)** |
|---|---|
| **Foetus small** | 0-2.8 |

| | |
|---|---|
| **Foetus pale** | 0-0.3 |
| **Foetus oedema** | 0-0.5 |
| **Agnathia/astomia** | 0-0.4 |
| **Placenta fused** | 0-0.7 |
| **Excess amniotic fluid** | 0-0.9 |
| **Marked subcutaneous oedema** | 0-0.9 |

*• = Mean per litter*
*Note = a foetus may appear in more than one category*

**Skeletal findings of HCD in rat teratology studies.**

| | | **%*(range from most recent studies)** |
|---|---|---|
| Number of ribs | 13/13 | 89.1-95.2 |
| | 13/14 | 3-8.3 |
| | 14/14 | 1.2-4.2 |
| Number of fully ossified stemebrae | <4 | 0.7-19.3 |
| | 4 | 19.4-66.1 |
| | >4 | 14.7-80 |
| Number of post lumbar vertebral centra | < 7 | 0-19.8 |
| | ≥ 7 | 80.2-100 |
| Number of post lumbar vertebral arches | <5 | 9 3-30.1 |
| | ≥5 | 69.9-90.7 |
| Number of metacarpals | <6 | 0-8.7 |
| | 6 | 44-89.8 |
| | >6 | 7.7-55.3 |
| Number of metatarsals | <6 | 0-0 |
| | 6 | 1 1-30.7 |
| | >6 | 69.3-100 |
| Fontanelle size | Small | 4 3-17.4 |
| | Medium | 82.6-95.2 |
| | High | 0-2.8 |
| **HEAD/NECK** | | |
| Incomplete ossification of one cranial bone | | 8 2-29.0 |
| Incomplete ossification of more than one cranial bone | | 6 2-13.4 |
| Incomplete ossification of one facial bone | | 0-3.4 |
| Incomplete ossification of more than one facial bone | | 0.6-6.2 |
| Irregular ossification of one cranial bone | | 0 5-10.4 |
| Irregular ossification of more than one cranial bone | | 0.6-6.3 |
| Irregular ossification of one facial bone | | 0.0-2.7 |
| Irregular ossification of more than one facial bone | | 0-2.1 |
| No ossification of hyoid | | 7.0-19.3 |
| incomplete ossification of hyoid | | 0.0-7.4 |
| Irregular ossification of hyoid | | 0.0-1.7 |
| Enlarged frontal/nasal suture | | 0.0-0.9 |
| No ossification of lower jaw | | 0.0-0.8 |
| **RIBS** | | |
| Unilateral/bilateral rudimentary 13th rib(s) | | 0.0-0.5 |
| Unilateral/bilateral rudimentary 14th rib(s) | | 4.0-10.9 |
| Unilateral/bilateral short 14th rib(s) | | 0.0-0.6 |

| | |
|---|---|
| Unilateral/bilateral wavy ribs | 0.0-1.7 |
| Bilateral thickened ribs | 0.0-0.7 |
| Cervical ribs | 0.0-1.3 |
| Unilateral/bilateral short rib(s) (excludes 14th rib) | 0.0-1.2 |
| Bifurcated 12th rib | 0.0-0.8 |
| **VERTEBRAE** | |
| One thoracic vertebral centrum bipartite | 0.0-3.9 |
| More than one thoracic vertebral centrum bipartite | 0.0-4.1 |
| One thoracic vertebral centrum semi-bipartite | 5.4-27.3 |
| More than one thoracic vertebral centrum semi-bipartite | 1.2-8.5 |
| One lumbar vertebral centrum semi-bipartite | 0.0-1.4 |
| More than one lumbar vertebral centrum semi-bipartite | 0.0-0.6 |
| One thoracic vertebral centrum not ossified | 0.0-0.6 |
| One thoracic ·vertebral centrum hemi-centric | 0.0-0.8 |
| One thoracic vertebral centrum asymmetrically ossified | 0.0-0.6 |
| One thoracic vertebral centrum irregular ossification | 0.0-0.8 |
| One lumbar vertebral centrum asymmetrically ossified | 0.0-0.6 |
| Incomplete ossification of one thoracic vertebral centrum | 0.0-0.9 |
| **PELVIC GIRDLE** | |
| Pelvis-shift- 27-pre-sacral vertebrae | 0-1.3 |
| Incomplete ossification of pubis(es) | 0.8-3.8 |
| Irregular ossification of pubis(es) | 0-2.8 |
| No ossification of pubis(es) | 0-3.6 |
| Incomplete ossification of ischium(a) | 0-1.3 |
| No ossification of ischium(a) | 0-0.8 |
| Small pubis | 0-0.7 |
| **STERNEBRAE 1-4** | |
| One small stemebrae | 0-1.9 |
| Incomplete ossification of one stemebrae | 0-5.4 |
| Irregular ossification of one stemebrae | 0-2.7 |
| Irregular ossification of more than one stemebrae | 0-3.6 |
| No ossification of one stemebrae | 0-4.9 |
| No ossification of more than one stemebrae | 0-3.2 |
| Incomplete ossification of more than one stemebrae | 0-3.3 |
| One stemebrae semi-bipartite | 0-1.3 |
| More than one stemebrae bipartite | 0-0.5 |
| More than one stemebrae misaligned | 0-0.8 |

*• = Mean per litter*
*Note = a foetus may appear in more than one category*

## Results

### Mortality

Only one female was found dead throughout the whole study. This was located in the high dose group and was found dead one hour after dosing on gestation day 5. There were no clinical signs of morbidity on the day of observation of death. Post mortem macroscopic findings included fluid within the thorax and haemorrhagic lungs. These findings are consistent with dosing trauma but are not evidence of systemic toxicity.

**Clinical signs**

-At 600 mg/kg bw/day there was evidence of treatment-related clinical signs of toxicity 1h post dose, such as pilo-erection (72%), ataxia (12%), prostration (8%), increased lachrymation (8%), lethargy (4%), noisy respiration (4%) and increased salivation (4%). With the exception of one occasion, these findings were resolved within 24 hours. These clinical findings were seen in the majority of females at the highest dose level.

-At 250 mg/kg there were also incidents of clinical signs 1h post dose, particularly pilo-erection (16%), hunched posture (4%) and increased salivation pre dose (8%) (Table B.6.6.2.5/1).

The clinical signs observed at mid and high dose groups appeared after the fifth day of treatment.

No clinical signs were observed in low dose and control groups.

**Table B.6.6.2.5/1. Summary of clinical signs**

| Clinical observations | Number of Animals with Clinical observations | | | |
|---|---|---|---|---|
| | Group 1 (0 mg/kg bw/day) | Group 2 (100 mg/kg bw/day) | Group 3 (250 mg/kg bw/day) | Group 4 (600 mg/kg bw/day) |
| | N=25 | N=25 | N=25 | N=25 |
| Pilo erection pre dose | 0 | 0 | 0 | 1 |
| Pilo erection 1h post dose | 0 | 0 | 4 (16%) | 18 (72%) |
| Hunched posture | 0 | 0 | 1 (4%) | 0 |
| Increased salivation pre dose | 0 | 0 | 2 (8%) | 0 |
| Ataxia | 0 | 0 | 0 | 3 (12%) |
| Lethargy | 0 | 0 | 0 | 1 (4%) |
| Prostrate | 0 | 0 | 0 | 2 (8%) |
| Noisy respiration | 0 | 0 | 0 | 1 (4%) |
| Increased lachrymation | 0 | 0 | 0 | 2 (8%) |
| Found dead | 0 | 0 | 0 | 1 (4%) |
| Increased salivation 1h post dose | 0 | 0 | 0 | 1 (4%) |
| **No. animals with clinical signs** | **0** | **0** | **7 (28%)** | **19 (76%)** |

*(% referred to total animals of each group)*
*Statistical analysis not performed*

**Bodyweights**

No statistically significant differences in bodyweight were observed in treated groups compared with controls (Table B.6.6.2.5/2). A slight decrease was noted in high dose group compared with controls throughout post coitum day 6-20 (3-5%).

**Table B.6.6.2.5/2. Summary of bodyweight (grams) (mean ± standard deviation)**

| Dose Level (mg/kg/day) | | Day post coitum | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 7 | 8 | 9 | 12 | 15 | 18 | 20 |
| 0 | Mean± SD | 239.13 ± 19.82 | 254.71 ± 19.06 | 258.54 ± 18.36 | 263.21 ± 20.04 | 267.46 ± 19.15 | 284.79 ± 19.78 | 302.42 ± 19.92 | 338.88 ± 23.07 | 366.33 ± 24.54 |
| 100 | Mean± SD | 244.68 ± 20.45 | 256.8 ± 21.24 | 261.4 ± 21.09 | 266.84 ± 21.77 | 270.84 ± 21.79 | 288.68 ± 23.72 | 308 ± 25.21 | 344.76 ± 28.64 | 371.68 ± 31.59 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **250** | **Mean± SD** | 246.72 ± 20.08 | 257.44 ± 20.76 | 261.84 ± 21.49 | 266.64 ± 21.00 | 270.24 ± 21.62 | 287.92 ± 23.39 | 307.64 ± 25.11 | 342.6 8 ± 29.87 | 367.8 ± 32.96 |
| **600** | **Mean± SD** | 245 ± 17.09 | 247.26 ± 18.44 | 248.09 ± 19.00 | 250.04 ± 20.05 | 256.43 ± 21.13 | 272.6 5± 20.24 | 294.57 ± 20.81 | 328.87 ± 23.72 | 354.17 ± 25.41 |

*No statistically significant diferences*

**Food consumption**

-Statistically significant reduction in food consumption was detected through gestation days 3-6 (12%), 6-9 (23%) and 9-12 (11%) in the high dose group compared with controls. On the other hand, no differences were detected in food consumption in the mid and low dose groups (Table B.6.6.2.5/3).

**Table B.6.6.2.5/3. Summary of food consumption (mean ± standard deviation)**

| Dose level (mg/kg/day) | | Day *post coitum* | | | | | |
|---|---|---|---|---|---|---|---|
| | | **3 – 6** | **6 – 9** | **9 – 12** | **12 – 15** | **15 – 18** | **18 - 20** |
| **0** | **Mean± SD** | 23.54± 2.16 | 23.88± 2.04 | 25.86± 2.04 | 27.21± 2 | 29.94± 2.61 | 28.54± 2.33 |
| **100** | **Mean± SD** | 23.2± 2.43 | 24.37± 2 29 | 25.91± 2.41 | 27.88± 2.64 | 30.65± 2.5 | 28.1± 2.73 |
| **250** | **Mean± SD** | 22.37± 2.53 | 22.75± 3.44 | 25.53± 2.86 | 27.87± 2.96 | 30.08± 3.45 | 27.4± 3.84 |
| **600** | **Mean± SD** | **20.64 ± 2.77* (↓12%)** | **18.43 ± 4.85* (↓23%)** | **23.03 ± 2.91* (↓11%)** | 26.23 ± 3.19 | 29.28 ± 3.03 | 28.13 ± 8.39 |

**p<0.001*

**Adult necropsy**

No differences were detected between treated groups and controls.

**Foetal data**

In the high dose group, a statistically significant decrease in the mean foetal weight was noted compared with controls (6%). On the other hand, there were no observable effects in other foetal parameters in the treated groups (Table B.6.6.2.5/4).

Regarding skeletal alterations, a statistically significant reduction in the percentage of foetuses with more than 6 ossified metatarsals was recorded in the high dose group (9%). No other skeletal alteration was recorded in the high dose group.

At 250 and 100 mg/kg bw/day dose groups, there were no statistically significant effects on skeletal or visceral development, and there were no treatment-related effects upon the type or incidence of skeletal or visceral findings observed. (see Tables B.6.6.2.5/5-6).

**Table B.6.6.2.5/4. Summary of foetal data (mean ± standard deviation)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **0** | **100** | **250** | **600** |
| **Mated** | 25 | 25 | 25 | 25 |
| **Pregnant** | | | | |
| *Total* | 24 | 25 | 25 | 23 |
| *Pregnancy index* | 96% | 100% | 100% | 92% |
| **Corpora lutea** | 14.21±1.8 | 14.68±1.7 | 14±2.1 | 14.83±1.5 |

| | | | | |
|---|---|---|---|---|
| **Total nº of live implants** | 12.38±1.7 | 12.76±1.7 | 12.08±2.7 | 12.74±2.5 |
| **Total nº of implant** | 12.58±1.6 | 12.84±1.8 | 12.36±2.5 | 13.26±1.7 |
| **% male foetuses** | 55.97±14.4 | 47.61±11.9 | 48.88±15.5 | 57.64±12.5 |
| **Embryonic/foetal deaths** | | | | |
| *Early* | 0.17±0.5 | 0.04±0.2 | 0.24±0.5 | 0.39±1 |
| *Late* | 0.04±0.2 | 0.04±0.2 | 0.04±0.2 | 0.13±0.5 |
| *Total* | 0.21±0.5 | 0.08±0.3 | 0.28±0.6 | 0.52±1.4 |
| **Implantation loss %** | | | | |
| *Pre* | 11.13±7.6 | 12.44±8 | 11.98±12.7 | 10.53±7.8 |
| *Post* | 1.7±4 | 0.57±2 | 2.53±5.6 | 4.49±11.9 |
| **Mean foetal weight (g)** | 3.73±0.2 | 3.77±0.3 | 3.81±0.3 | **3.50±0.3 (↓6%)*** |
| **Total litter weight (g)** | 46.12±6.7 | 48.08±7.6 | 45.61±9.1 | 44.58±9.4 |
| **Mean placental weight (g)** | 0.53±0.1 | 0.52±0.1 | 0.54±0.1 | 0.52±0.1 |
| **Total placental weight (g)** | 6.52±1 | 6.58±1.1 | 6.47±1.3 | 6.52±1.3 |
| **Gravid uterus weight (g)** | 72.65±10.3 | 73.72±10 | 71.54±13.6 | 70.21±13 |

** p<0.05*

**Table B.6.6.2.5/5. Summary of foetal visceral findings**

| External findings | Dose level (mg/kg bw/day) (no. of foetuses examined) | | | |
|---|---|---|---|---|
| | 0 (143) | 100 (152) | 250 (149) | 600 (140) |
| | % ¥ | % ¥ | % ¥ | % ¥ |
| Total number affected | 4.6 | 4.1 | 3.3 | 6.3 |
| Unilateral/bilateral kinked ureter | 3.5 | 1.2 | 2.5 | 5.4 |
| Unilateral/bilateral dilated ureter | 4.0 | 1.2 | 2.5 | 6.3 |
| Unilateral/bilateral increased renal pelvic cavitation | 3.5 | 1.2 | 2.5 | 4.3 |
| Unilateral/bilateral small renal papilla | 1.8 | - | 0.5 | 2.7 |
| Unilateral/bilateral ovoid lens | 0.5 | 0.5 | - | - |
| Undescended lobe(s) of thymus | 0.6 | 0.7 | - | - |
| Ventricle dilated | - | 1.7 | - | - |
| Extended lobular pattern on more than 1 lobe of lungs | - | - | 0.8 | - |

*¥= group mean per litter; - = not applicable*
*No statistically significant differences*

**Table B.6.6.2.5/6. Summary of foetal skeletal findings**

| Skeletal finding | | Dose level (mg/kg bw/day) (no. foetuses examined) | | | |
|---|---|---|---|---|---|
| | | 0 (143) | 100 (152) | 250 (149) | 600 (140) |
| | | %¥ | %¥ | %¥ | %¥ |
| Number of ribs | 13/13 | 100 | 100 | 100 | 99.3 |
| | 13/14 | - | - | - | 0.7 |
| Number of fully ossified sternebrae | <4 | 0.8 | 1.8 | 0.5 | 2.0 |

| | | | | | |
|---|---|---|---|---|---|
| | 4 | 24.1 | 26.5 | 25.6 | 40.5 |
| | >4 | 75.0 | 71.7 | 73.9 | 56.8 |
| Number of post lumbar vertebral centra | <7 | 3.9 | 5.2 | 3.8 | 13.9 |
| | ≥7 | 96.1 | 94.8 | 92.2 | 86.1 |
| Number of post lumbar vertebral arches | <5 | 11.5 | 15.1 | 21.4 | 23.9 |
| | ≥5 | 88.5 | 84.9 | 74.6 | 76.1 |
| Number of metacarpals | <6 | - | 2.4 | - | 4.2 |
| | 6 | 74.3 | 58.8 | 63.5 | 80.6 |
| | >6 | 24.7 | 38.8 | 36.5 | 15.2 |
| Forelimb phalanges | ≤2 | 100 | 98.4 | 99.4 | 100 |
| | >2 | - | 1.6 | 0.6 | - |
| Number of metatarsals | <6 | - | - | - | 0.7 |
| | 6 | - | 1.3 | - | 8.0 |
| | >6 | 100 | 98.7 | 100 | **91.3* (↓9%)** |
| Fontanels | Small | 7.0 | 3.9 | 1.8 | 2.7 |
| | Medium | 92.2 | 94.2 | 94.4 | 94.5 |
| | Large | 0.8 | 1.9 | 3.8 | 2.8 |
| | | **Dose level (mg/kg bw/day) (no. foetuses examined)** | | | |
| | | **0 (143)** | **100 (152)** | **250 (149)** | **600 (140)** |
| | | **%¥** | **%¥** | **%¥** | **%¥** |
| **HEAD/NECK** | | | | | |
| Incomplete ossification of one cranial bone | | 2.3 | 1.7 | 4.3 | 1.6 |
| Incomplete ossification of more than one cranial bone | | 0 | 2.6 | 3.7 | 3.4 |
| Irregular ossification of one cranial bone | | 2.1 | 0.6 | 1.3 | 1.2 |
| Incomplete ossification of hyoid | | 0 | 0 | 0.6 | 0 |
| Hyoid not ossified | | 0.7 | 1.8 | 1.1 | 0 |
| **RIBS** | | | | | |
| Unilateral/bilateral shortened 13th ribs(s) | | 0.7 | 1.8 | 1.5 | 2.0 |
| Unilateral/bilateral irregular ossification of 13th rib(s) | | 0.8 | 0 | 0 | 0 |
| Unilateral/bilateral rudimentary 14th rib(s) | | 0 | 0 | 0 | 0.7 |
| **VERTEBRAE** | | | | | |
| One thoracic vertebral centrum semi-bipartite | | 6.6 | 7.6 | 14.7 | 8.6 |
| More than one thoracic vertebral centrum semi-bipartite | | 2.9 | 4.3 | 6.0 | 3.1 |
| One thoracic vertebral centrum bipartite | | 2.2 | 3.4 | 3.4 | 0 |
| More than one thoracic vertebral centrum bipartite | | 0 | 0 | 0.7 | 0.7 |
| Asymmetric ossification of one thoracic vertebral centrum | | 0.5 | 0 | 0 | 0 |
| lncomplete ossification of more than one thoracic vertebral centrum | | 0.6 | 1.5 | 0.5 | 1.3 |
| Unilateral ossification of one thoracic vertebral centrum | | 0 | 0 | 0 | 0.6 |
| Precocious ossification of lumbar region | | 0 | 0.8 | 0 | 0 |
| **STERNEBRAE** | | | | | |
| lncomplete ossification of stemebrae | | 2.7 | 1.8 | 1.2 | 1.2 |
| Incomplete ossification of more than one stemebrae | | 0 | 0.7 | 1.3 | 2.7 |
| Two or more stemebrae fused | | 1.3 | 2 | 0.5 | 0 |
| No ossification of one stemebrae | | 1.4 | 0.7 | 0.5 | 0.7 |
| No ossification of more than one stemebrae | | 0 | 1.2 | 0 | 1.2 |
| Asymmetric ossification of more than one stemebrae | | 0 | 0.7 | 0 | 0 |
| **OTHER** | | | | | |
| lncomplete ossification of ischia | | 0 | 0 | 0 | 0.7 |
| lncomplete ossification of ilia | | 0 | 0 | 0 | 0.7 |

| | | | | |
|---|---|---|---|---|
| Pelvic shift 27 (PSU) | 0.6 | 0 | 0 | 0 |

*¥= group mean per litter*
*- = not applicable*
** p<0.05*

**Assessment and conclusion by applicant:**

Eugenol was not teratogenic to rats at dose levels up to 600 mg/kg bw/d. The NOAEL for foetotoxicity / development is 250 mg/kg bw/d based on slight effects on foetal weight and metatarsal ossification at 600 mg/kg bw/d. The NOAEL for maternal toxicity is 100 mg/kg bw/d based on clinical signs shortly after dosing at 250 mg/kg bw/d and above.

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rat) and the developmental NOAEL was 250 mg/kg bw/d (rat). It was concluded there is no evidence of teratogenicity; target / critical effect: decreased foetal weight and delayed ossification, and maternal clinical signs in rats (EFSA Journal 2012;10(11):2914). The EU-agreed ADI and AOEL was derived from the developmental NOAEL obtained in this study, a safety factor of 100 was applied to the NOAEL to give an overall ADI / AOEL of 1.0 mg/kg bw/d (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with applicant conclusion. This study has been conducted according to required guideline, and does not present important deviations. Therefore, this study is considered acceptable.

Decreases in mean foetal bodyweight and in the number of ossified metatarsals were noted in offspring at high dose level (6% and 9%, respectively).

On the other hand, several post-dose clinical signs were described in dams of mid and high dose groups. Additionally, decreased in dam food consumption was noted throught gestation day 3-12 at high dose level, which slightly affected dams bodyweight.

**NOAEL** $_{\textbf{developmental toxicity}}$: 250 mg/kg bw/day based on decreased mean foetal bodyweight and in the number of ossified metatarsals.

**NOAEL** $_{\textbf{maternal toxicity}}$: 100 mg/kg bw/day based on clinical signs noted in dams.

**B.6.6.2.6. Developmental toxicity study in rabbits with eugenol**

| | |
|---|---|
| **Data point** | CA 5.6 (EU data requirement) |
| **Report author** | [redacted] |
| **Report year** | 2004 |
| **Report title** | Eugenol: Oral gavage pre-natal developmental toxicity study in the rabbit. |
| **Report No** | [redacted] Project number: 1620/035 |
| **Document No** | KCA 5.6.2/06 (Study included by the RMS, extracted from Clove oil renewal dossier and used for the first time in the Clove oil DAR-2008) |
| **Guidelines followed in study** | OECD Guideline 414 (2001). |
| **Deviations from current test guideline OECD TG 414 (2018)** | -Purity of test substance not stated.<br>-The following developmental endpoints were not measured: resorptions, anogenital distance, and indication of incomplete testicular descent/cryptorchidism.<br>-Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.<br>- Historical control data sources are not detailed in the study report. |
| **Previous evaluation** | No, not included in DAR 2011. Taked into account in the peer review of eugenol (EFSA Journal 2012; 10,11: 2914) |
| **GLP** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: 29424, purity not specified. |
| **Test system** | Female New Zealand White rabbits. |
| **Test method** | Four groups of 24 mated females New Zealand White rabbits were dosed with 0, 100, 250, and 500/350 mg/kg bw/day throughout day 5-28 of gestation.<br><br>The test material was suspended daily in 1% carboxymethyl cellulose (vehicle), and subsequently administered *via* oral gavage using a rubber catheter and plastic syringe at a constant dose volume of 4 ml/kg bodyweight. As a result of adverse toxicity at the highest dose level, 5 females were found dead or killed *in extremis*, consequently, the high dose tested was reduced to 350 mg/kg bw/day.<br><br>*Morbidity/Mortality:* All females were checked twice daily during the normal working week and once daily at weekends for signs of morbidity.<br><br>*Clinical Observations:* All females were observed once daily, in the morning throughout gestation and, additionally, one hour after dosing, throughout the dosing period.<br><br>*Bodyweight:* All females were weighed on gestation day 3, days 6-9, 12, 15, 18, 21, 24, 27 and 29.<br><br>*Food Consumption:* Food consumption for individual animals was recorded during days 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 21-24, 24-27 and 27-29.<br><br>*Incidental Deaths/Kills:* Moribund or aborting females were killed and necropsied. Dead animals were also necropsied. Intact foetuses were examined externally and discarded if there were no visible abnormalities. All surviving females were killed on day 29 of gestation by intravenous injection of sodium pentobarbitone *via* the marginal ear vein. The foetuses were killed by intrathoracic injection of sodium pentobarbitone.<br><br>*Necropsy*: Each animal was examined externally and internally for macroscopic abnormalities. The ovaries and uteri of pregnant females were removed, examined and the following data recorded:<br><br>Gravid uterus weight, pregnancy status, number of corpora lutea, number position and type of intrauterine implantation, foetal sex, external and internal foetal appearance, foetal weight and placental weight. |

| | |
|---|---|
| | All foetuses were dissected and examined for visceral abnormalities macroscopically. The heads of alternate foetuses were removed and identified using an indelible marker and placed in Bouin's fixative. After a minimum of fourteen days, the heads were transferred to 90% industrial methylated spirits (IMS) in distilled water and examined for visceral anomalies under a low power binocular microscope. All foetuses were identified using colour coded wires and placed in 70% IMS in distilled water. The foetuses were eviscerated, processed and the skeletons stained with alizarin red. The foetuses were also examined for skeletal development and anomalies.<br><br>*Evaluation of Data*<br><br>Data were processed to give litter mean values, group mean values and standard deviations. |
| **Statistical analysis of results** | The following parameters were analysed statistically, where appropriate using the test methods outlined below:<br><br>Female group mean bodyweight, bodyweight and food consumption: Analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons will be performed between control and treated groups. For homogenous datasets, Dunnett's multiple comparison test will be used. For heterogeneous datasets an adjusted 't' test will be used.<br><br>All foetal parameters, skeletal development, group incidence of specific visceral and skeletal anomalies: Kruskal-Wallis non-parametric analysis of variance followed by pairwise analysis of control values against treated values using the Mann-Whitney 'U' test where appropriate. |
| **Historical control data (HCD)** | HCD sources are not detailed in the study report., so its validity cannot be determined.<br><br>**Visceral findings of HCD in rabbit teratology studies.**<br><br>**% *( range from most recent studies)**<br>**Enlarged third ventricle** 1.4 -2.8<br>**Ovoid lens(es) of eye(s)** 0.0-7.8<br>**Short brachiocephalic trunk** 0.0-0.6<br>**Gastroschisis** 0.0-0.9<br>**Umbilical hernia** 0.0-0.6<br>**Malrotated hind limb, short tail** 0.0-3.I<br><br>*• = Mean per litter*<br>*Note = a foetus may appear in more than one category*<br><br>**Skeletal findings of HCD in rabbit teratology studies.**<br><br>**%*(range from most recent studies)**<br>Number of ribs 12/12 26.9 -31.7<br>Number of ribs 12/13 11.2-23.6<br>Number of ribs 13/13 49.5-57.1<br>Number of fully ossified stemebrae ≤4 7.9- 15.8<br>Number of fully ossified stemebrae >4 84.2-92.1<br>Number of post lumbar vertebral centra < 19 1.3-6.6<br>Number of post lumbar vertebral centra ≥ 19 93.4-98.7<br>Number of post lumbar vertebral arches ≤10 36.6-40.4<br>Number of post lumbar vertebral arches >10 59.6-63.4<br>Number of metacarpals 8 100.0<br>Number of phalanges < 16 7.5-28.l<br>Number of phalanges 16 71.9-92.5<br>Number of polleces <4 4.4-6.2 |

| | | |
|---|---|---|
| | 4 | 93.8-95.6 |
| Number of metatarsals | 8 | 100.0 |
| Number of phalanges | <16 | 0.0-1.5 |
| | 16 | 98.5- 100.0 |
| Number of tarsals | <4 | 0.0-1.1 |
| | 4 | 98.9-100.0 |
| Fontanelle size | Small | 22.0-28.3 |
| | Medium | 71.7 -78.0 |
| **HEAD/NECK** | | |
| One cranial bone incomplete ossification | | 0.0-2.9 |
| More than one cranial bone irregular ossification | | 0.0-1.3 |
| Hyoid irregular ossification | | 3.1-7.l |
| Hyoid incomplete ossification | | 0.0-1.8 |
| Saggital suture enlarged | | 0.0-2.8 |
| Atlas irregular ossification | | 0.0-0.6 |
| **VERTEBRAE** | | |
| One thoracic vertebral centrum misshapen and misaligned | | 0.0-0.6 |
| More than one thoracic vertebral centra semi-bipartite | | 0.0-3.1 |
| No ossification of post thoracic vertebrae | | 0.0-3.1 |
| One thoracic vertebral centrum bi-partite | | 0.0-3.1 |
| Extra vertebral arch resulting in scoliosis | | 0.0-1.0 |
| **STERNEBRAE** | | |
| One stemebrae bipartite | | 0.0-1.3 |
| One stemebrae asymmetric | | 0.0-3.6 |
| One stemebrae semi-bipartite | | 0.0-1.1 |
| One stemebrae misaligned | | 0.0-1.0 |
| More than one sternebra semi-bipartite | | 0.0-0.5 |
| Two stemebrae fused | | 0.0-1.9 |
| More than two stemebrae fused | | 0.0-1.4 |
| Two or more misaligned stemebrae | | 1.0-1.6 |
| **RIBS** | | |
| Unilateral/bilateral short 13th rib | | 17.6-19.8 |
| Unilateral/bilateral 13th rudimentary ribs | | 2.3-9.0 |
| Unilateral/bilateral no ossification of rib proximal to vertebrae | | 3.3-5.6 |
| One pair of ribs articulate to same vertebra -ribs forked | | 0.0-3.l |
| Irregular articulation of 12th rib pair | | 0.0-3.l |
| Rib irregularly ossified | | 0.0-0.9 |
| **PELVIC GIRDLE** | | |
| Pelvic shift (25 presacral vertebrae) | | 0.0-0.9 |
| Pelvic shift (27 presacral vertebrae) | | 0.0-0.6 |
| Pelvic shift | | 0.0-1.8 |
| **OTHERS** | | |
| One digit of forepaw small | | 0.0-1.8 |

*• = Mean per litter*
*Note = a foetus may appear in more than one category*

**Results**

**Clinical signs**

-At 500 mg/kg bw/day dose level, relevant signs of toxicity were observed in females up to 1h post dose, such as ataxia (17%) and prostration (12.5%). Low incidence (4%) pallor of the extremities, eyes and ears, together with respiratory difficulties were also noted. As a result of this toxicity, four females died and other female was killed *in extremis*. Consequently, the high dose level was reduced to 350 mg/kg bw/day (see Table B.6.6.2.6/1).

The gestation day where there was a change in dose level varies between gestation day 10 and 13 for animals (numbers 73 to 84) within the first group used. On the other hand, animals (numbers 85-96) from group 2 were dosed at 350 mg/kg bw/day (see Table B.6.6.2.6/2). One dam dosed at 350 mg/kg bw/day aborted at gestation day 25 and was killed *in extremis*.

-At 250 mg/kg bw/day dose level, ataxia 10 min post dose (8%), abortions (4%), laboured respiration (4%) and noisy respiration (4%) were observed during treatment.

The clinical signs observed at mid and high dose groups appeared after the sixth day of treatment.

-At control group, one female aborted on day 25 of gestation. There were no clinical signs of morbidity and no significant macroscopic post mortem findings. It was concluded that the abortion of offspring was a spontaneous event.

No relevant clinical signs were observed at low dose level.

**Table B.6.6.2.6/1. Summary of clinical signs**

| **Clinical observations** | **Number of Animals with clinical observations** | | | |
|---|---|---|---|---|
| | **Group 1 (0 mg/kg/day)** | **Group 2 (100 mg/kg/day)** | **Group 3 (250 mg/kg/day)** | **Group 4 (500/350) mg/kg/day)** |
| | n=24 | n=24 | n=24 | n=24 |
| Animal aborted | 1 (4%) | 0 | 1 (4%) | 1 (4%) |
| Ataxia up to 1h post dose | 0 | 0 | 2 (8%) | 4 (17%) |
| Laboured respiration up to 1h post dose | 0 | 0 | 1 (4%) | 1 (4%) |
| Noisy respiration | 0 | 0 | 1 (4%) | 0 |
| Decreased respiration up to 1h post dose | 0 | 0 | 0 | 1 (4%) |
| Prostrate up to 1h post dose | 0 | 0 | 0 | 3 (12.5%) |
| Pallor of the extremities up to 1h post dose | 0 | 0 | 0 | 1 (4%) |
| Eyes pale | 0 | 0 | 0 | 1 (4%) |
| Ears pale | 0 | 0 | 0 | 1 (4%) |
| **No. animals with clinical signs** | **1 (4%)** | **0** | **4 (17%)** | **6 (25%)** |

*(% referred to total animals of each group)*
*Statistical analysis not performed*

**Table B.6.6.2.6/2. High dose levels adjustments**

| Animal number | Dose Level | | |
|---|---|---|---|
| | 500mg/kg bw/day | Off dose | 350 mg/kg bw/day |
| | Gestation days | Gestation day | Gestation days |
| 73 | 5-11 | 12 | 13-28 |
| 74* | 5-11 | - | - |
| 75* | 5-11 | - | - |
| 76* | 5 | - | - |
| 77 | 5-10 | 11 | 12-28 |
| 78* | 5-9 | - | - |
| 79 | 5-9 | 10 | 11-28 |
| 80 | 5-9 | 10 | 11-28 |
| 81* | 5-6 | - | - |
| 82 | 5-8 | 9 | 10-28 |
| 83 | 5-8 | 9 | 10-28 |
| 84 | 5-8 | 9 | 10-28 |
| 85 to 96 | - | - | 5-28 |

** = All animals found dead/killed in extremis*

**Mortality:**

500/350 mg/kg bw/day dose group

-Two out five dead females were found dead after one and two doses respectively, without showing any clinical sign of morbidity or reaction to treatment.

-Three out five dead females received between five and seven doses of eugenol and showed clinical signs of reaction to treatment, particularly ataxia and/or prostration post dosing, during the day(s) prior to death.

No consistent post mortem macroscopic findings were observed for the decedent females but gastric changes were observed in 2/5 females. The findings included thickening with/without congestion and sloughing of the gastric mucosa.

-One further female was killed *in extremis* following abortion of her offspring on day 25 of gestation. There were no significant post mortem macroscopic changes (table B.6.6.2.6/3).

250 mg/kg bw/day dose group

-One female was found dead on day 12 of gestation. This female showed ataxia 10 min post dosing on day 10 of gestation. Macroscopic post mortem examination showed colourless fluid around the mouth. With no significant findings, an association with test material toxicity could not be discounted.

-One female was killed *in extremis* on day 8 of gestation due to laboured and noisy respiration. Macroscopic post mortem examination showed a portion of the dosing catheter in the oesophagus. The cause of death was therefore considered to be a dosing accident.

-One female was killed *in extremis* following the abortion of offspring on day 23 of gestation. There were no significant macroscopic post mortem findings.

100 mg/kg bw/day dose group

At 100 mg/kg bw/day dose group, there were no mortalities.

0 mg/kg bw/day dose group

-One female was killed *in extremis* following the abortion of offspring on day 25 of gestation. There were no clinical signs of morbidity and no significant macroscopic post mortem findings, so it was concluded that the abortion of offspring was a spontaneous event.

**Table B.6.6.2.6/3. Survival in dose groups**

| | Dose group (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **0** | **100** | **250** | **500/350** |
| **no animals at initiation** | **24** | **24** | **24** | **24** |
| **Total Deaths/ killed *in extremis*** | **1** | **0** | **3** | **6** |
| **Deaths with clinical signs associated** | 0 | 0 | 1 | 3 |
| **Deaths without clinical signs associated** | 0 | 0 | 0 | 2 |
| **killed *in extremis* associated to abortions** | 1 | 0 | 1 | 1 |
| **killed *in extremis* associated to dosing errors** | 0 | 0 | 1 | 0 |
| **Survival (gestation day 29)** | 23 | 24 | 21 | 18 |
| **% survival (gestation day 29)** | 96% | 100% | 88% | 75% |

*Statistical analysis not performed*

**Bodyweights**

There were no statistically significant differences in dams mean bodyweights between eugenol dose groups and controls throughout gestation (Table B.6.6.2.6/4). ). A slight decrease was noted in high dose group compared with controls throughout post coitum day 6-29 (3-6%).

**Table B.6.6.2.6/4. Summary of bodyweights (grams) (mean ± standard deviation)**

| Dose Level (mg/kg/day) | | Day post coitum | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 7. | 8 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 29 |
| **0** | **Mean ± SD** | 3459.7 ± 288.39 | 3552 ± 270.91 | 3582 ± 278.28 | 3595.2 ± 276.61 | 3603.7 ± 294.16 | 3670.6 ± 296.16 | 3736.1 ± 318.91 | 3786.5 ± 337.70 | 3832.7 ± 334.16 | 3833.2 ± 328.93 | 3863.9 ± 311.05 | 3900.1 ± 319.81 |
| **100** | **Mean ±** | 3444 ± | 3537.2 ± | 3536.1 ± | 3521.7 ± | 3535.4 ± | 3638.6 ± | 3720.4 ± | 3755.7 ± | 3810.7 ± | 3820.7 ± | 3845. I ± | 3890.7 ± |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SD** | 287.47 | 306.97 | 310.94 | 316.29 | 318.37 | 317.23 | 294.82 | 309.54 | 333.41 | 316.35 | 281.28 | 300.36 |
| **250** | **Mean ± SD** | 3427.6 ± 294.58 | 3457.1 ± 319.90 | 3466 ± 337.88 | 3455 ± 334.01 | 3488.6 ± 320.30 | 3523.3 ± 339.58 | 3581.8 ± 358.20 | 3638 ± 392.97 | 3704.2 ± 407.82 | 3780.8 ± 307.90 | 3766.7 ± 269.56 | 3784.8 ± 281.30 |
| **500 (350)** | **Mean ± SD** | 3414.3 ± 248.01 | 3428.3 ± 285.41 | 3403.2 ± 293.36 | 3386.3 ± 303.96 | 3399.7 ± 319.49 | 3561.9 ± 211.55 | 3641.6 ± 234.16 | 3655.7 ± 231.95 | 3695.2 ± 250.75 | 3712.8 ± 270.15 | 3772.2 ± 224.17 | 3794 ± 251.96 |

*No statistically significant diferences*

## Food consumption

At 500 (350) mg/kg bw/day dose group, there was a decreased trend in mean food consumption throughout gestation day 3-18 (16-40%). The difference was statistically significant through gestation days 6-9 (40%) and 9-12 (26%) compared to controls (Table B.6.6.2.6/5).

At 250 mg/kg bw/day dose group, there was also a slight reduction in group mean female food consumption throughout gestation days 3-18 (16-29%). However, the difference was only statistically significant through gestation days 9-12 (26%).

**Table B.6.6.2.6/5. Summary of food consumption (mean ± standard deviation)**

| **Dose Level (mg/kg/day)** | | **Day *post coitum*** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **3-6** | **6-9** | **9-12** | **12-15** | **15-18** | **18-21** | **21-24** | **24-27** | **27-29** |
| **0** | Mean ± SD | 155.7±57.9 | 176±52.9 | 166.1±48.6 | 158±52.8 | 165.9±53.8 | 154.4±49.8 | 105.8±42 9 | 79.3±26.3 | 88.7±31.6 |
| **100** | Mean ± SD | 151.8±57.1 | 148.9±65.8 | 160.3±42.8 | 148.7±40.9 | 160.2±38.8 | 166.2±34.4 | 115.1±40 9 | 86.1±43 | 96.1±40.9 |
| **250** | Mean ± SD | 124.9±75.8 | 125.8±63.9 | **123.4±61.1* (↓26%)** | 132.3±62.9 | 133.5±75.7 | 161.7±64.6 | 126.1±41.6 | 84±34.3 | 87.1±37.3 |
| **500/350** | Mean ± SD | 130.5±59.5 | **105.4±65.5** (↓40%)** | **123.3±56* (↓26%)** | 129.6±42 | 129±52.4 | 140.5±54.1 | 108.7±43.6 | 95.1±46.2 | 94.4±49.6 |

**p<0.05*
***p<0.001*

## Adult necropsy

At 500 mg/kg bw/day dose group, two decedent females showed gastric changes that may have been associated with test material administration. The reduction of dose level to 350 mg/kg bw/day subsequently resulted in no significant macroscopic findings.

## Foetal data

At 500 (350) mg/kg bw/day dose group, there was an increase in post implantation loss (275%) value compared to controls. This finding was non-statistically significant and presented a wide standard deviation. This data was limited primarily to two females with significant numbers of late embryonic deaths, and would suggest evidences of treatment-related toxicity (see table B.6.6.2.6/6).

On the other hand, there were a slight reduction, without statistically significant differences, on placental weight (7% and 9%, for mid and high dose groups, respectively) and in gravid uterus weight (4% and 7% for mid and high dose groups, respectively), compared with controls.

Regarding skeletal and visceral alterations, at high dose group, there was an increase in percentage of foetuses with at least sixteen forelimb phalanges ossified (14%). This value was statistically significant ($p<0.05$) compared to controls (table B.6.6.2.6/7).

At mid and low dose groups, treatment did not show effects on foetal data, nor in external malformations, visceral or skeletal anomalies (table B.6.6.2.6/7-8).

**Table B.6.6.2.6/6. Summary of foetal data (mean ± standard deviation)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **0** | **100** | **250** | **500 (350)** |
| **Mated** | 24 | 24 | 24 | 24 |
| **Pregnant** | | | | |
| *Total* | 22 | 21 | 20 | 18 |
| *Pregnancy index* | 92% | 88% | 83% | 75% |
| **Corpora lutea** | 10.73±1.8 | 11.38±2.6 | 11±2.1 | 11±2.4 |
| **Total nº of live implants** | 9.36±1.7 | 9.86±2.7 | 9.05±2.3 | 8.44±3 |
| **Total nº of implant** | 9.82±1.7 | 10.48±2.6 | 9.85±2.6 | 10.22±2.2 |
| **% male foetuses** | 58.14±10.7 | 60.29±15.5 | 51.16±12.6 | 52.3±20.2 |
| **Embryonic/foetal deaths** | | | | |
| *Early* | 0.14±0.3 | 0.38±0.8 | 0.05±0.2 | 0.33±1.2 |
| *Late* | 0.32±0.8 | 0.29±0.6 | 0.75±1.2 | 1.39±2.7 |
| *Total* | 0.45±0.6 | 0.67±1 | 0.8±1.1 | 1.72±2.8 |
| **Implantation loss %** | | | | |
| *Pre* | 8.34±7.7 | 7.67±8.4 | 11.47±11.2 | 6.88±7.7 |
| *Post* | 4.37±8.3 | 6.3±9.8 | 7.19±9.6 | 16.4±26.4 |
| **Mean foetal weight (g)** | 38.91±4.6 | 40.23±4.6 | 37.56±4.6 | 38.13±3.7 |
| **Total litter weight (g)** | 360.92±63.6 | 390.48±89.6 | 332.67±69.9 | 322.15±104.6 |
| **Mean placental weight (g)** | 5.65± | 5.95±0.7 | 5.48±0.9 | 5.7±0.7 |
| **Total placental weight (g)** | 52.27±9.3 | 57.51±13.4 | 48.48±11.8 | 47.63±15.2 |
| **Gravid uterus weight (g)** | 553.49±83.1 | 595.87±123.1 | 528.75±108.7 | 513.32±100.8 |

*No statistically significant differences*

**Table B.6.6.2.6/7. Summary of foetal external and visceral findings**

| External and visceral findings | Dose level (mg/kg/day) (no. foetuses examined) | | | |
|---|---|---|---|---|
| | **0 (206)** | **100 (207)** | **250 (181)** | **500 (153)** |
| | **% ¥** | **% ¥** | **% ¥** | **% ¥** |
| **Total number affected** | 2.4 | 7.5 | 4.4 | 3.1 |
| Third ventricle dilated | 2.4 | 2.5 | - | 3.1 |
| Small foetus | - | 3.6 | 4.4 | - |
| Small placenta | - | 0.5 | - | - |
| Forelimb flexure | - | 0.4 | - | - |
| Unilateral irridial haemorrhage of eye | 0.5 | 1.1 | - | - |
| Gaseous distension of stomach | 0.6 | - | - | - |
| Pale kidneys | - | 0.4 | - | - |
| Pale lungs | - | 0.4 | - | - |

| | | | | |
|---|---|---|---|---|
| Gastroschisis | 0.4 | - | - | - |

*¥= group mean per litter*
*- = not applicable*
*No statistically significant differences*

**Table B.6.6.2.6/8. Summary of foetal skeletal findings**

| Skeletal development | | Dose level (mg/kg bw/day) (no. of foetuses examined) | | | |
|---|---|---|---|---|---|
| | | **0 (206)** | **100 (207)** | **250 (181)** | **500/350 (153)** |
| | | **%¥** | **%¥** | **%¥** | **%¥** |
| Number of ribs | 12/12 | 29.2 | 24.2 | 28.5 | 26.0 |
| | 12/13 | 14.2 | 13.1 | 9.3 | 17.7 |
| | 13/13 | 56.6 | 62.6 | 61.6 | 56.3 |
| | 13/14 | - | - | 0.5 | - |
| Number of ossified sternebrae | <4 | - | 0.4 | - | - |
| | 4 | 0.9 | - | - | 0.5 |
| | >4 | 99.1 | 99.6 | 100.0 | 99.5 |
| Number of post lumbar vertebral centra | <19 | 9.3 | 9.1 | 18.9 | 12.1 |
| | ≥19 | 90.7 | 90.9 | 81.1 | 87.9 |
| Number of post lumbar vertebral arches | <10 | 74.0 | 72.8 | 79.9 | 67.2 |
| | ≥10 | 26.1 | 27.2 | 20.1 | 32.8 |
| Number of metacarpals | 8 | 100.0 | 100.0 | 100.0 | 100 |
| | >8 | - | - | - | - |
| Number of forelimbs phalanges | <16 | 13.5 | 4.7 | 8.2 | 1.5 |
| | ≥16 | 86.5 | 95.3 | 91.8 | **98.5* (↑14%)** |
| Number of polleces | <4 | 3.2 | 4.1 | 4.3 | 1.0 |
| | ≥4 | 96.8 | 95.9 | 95.8 | 99.0 |
| Number of metatarsals | 8 | 100.0 | 100.0 | 100.0 | 100.0 |
| Number of hindlimbs phalanges | <16 | 0.5 | - | 1.5 | - |
| | ≥16 | 99.5 | 100.0 | 98.5 | 100.0 |
| Number of tarsals | <4 | 0.8 | 0.9 | 1.1 | 0.5 |
| | ≥4 | 99.2 | 99.1 | 98.9 | 99.5 |
| Size of fontanelles | Small | 83.6 | 90.8 | 81.9 | 75.8 |
| | Medium | 16.4 | 9.2 | 16.3 | 24.2 |
| | Large | - | - | 1.8 | - |
| | | **Dose level (mg/kg bw/day) (no of foetuses examined)** | | | |
| | | **0 (206)** | **100 (207)** | **250 (181)** | **500/350 (153)** |
| | | **%¥** | **%¥** | **%¥** | **%¥** |
| **HEAD/NECK** | | | | | |
| Incomplete ossification of one cranial bone | | 2.6 | 2.2 | 1.0 | - |
| Incomplete ossification of more than one cranial bone | | 0.4 | 0.4 | 1.9 | - |
| Irregular ossification of one cranial bone | | - | 0.4 | 0.5 | - |
| Incomplete ossification of one facial bone | | - | - | - | - |

| | | | | |
|---|---|---|---|---|
| **RIBS** | | | | |
| Unilateral/bilateral rudimentary 13th rib(s) | 6.8 | 4.6 | 5.2 | 8.7 |
| Unilateral/bilateral floating 13th ribs(s) | 9.8 | 4.7 | 9.9 | 5.6 |
| Unilateral/bilateral shortened 13th rib(s) | 22.6 | 23.5 | 19.2 | 18.1 |
| Unilateral/bilateral thickening of distal region of one or more rib(s) | 0.5 | 0.7 | - | 0.8 |
| Two or more ribs fused | - | - | - | 0.6 |
| One rib not ossified | 0.6 | - | - | - |
| One rib medially modulated | - | - | - | 0.8 |
| 8th left rib articulates with 8th and 9th thoracic vertebral arch | 0.5 | - | - | - |
| 9th left rib articulates with 9th plus additional thoracic vertebral arch | 0.5 | - | - | - |
| 9th thoracic center associates with 9th left thoracic vertebral arch plus additional vertebral arch | 0.5 | - | - | - |
| **VERTEBRAE** | | | | |
| One thoracic vertebral centrum semi-bipartite | 0.5 | 0.5 | 0.4 | - |
| More than one thoracic vertebral centre semi-bipartite | 0.6 | - | - | 0.6 |
| Asymmetric ossification of one or more thoracic vertebral center | 0.5 | 0.5 | - | - |
| Two thoracic vertebral arches displaced | - | 0.5 | - | - |
| One or more thoracic vertebral centres misaligned | 0.5 | - | - | 1.2 |
| 8th vertebral center hemicentric and asymmetrically ossified | 0.5 | - | - | - |
| Incomplete ossification of one sternebrae | 0.5 | 0.4 | - | - |
| incomplete ossification of more than one sternebrae | 1.4 | - | 1.0 | - |
| Asymmetric ossification of two or more sternebrae | 1.8 | - | - | 0.5 |
| More than one sternebrae mis-aligned | 0.5 | - | - | 0.5 |
| Two or more sternebrae fused | 0.6 | 1.1 | - | 3.5 |
| No ossification or one sternebrae | - | 0.4 | - | - |

*¥= group mean per litter*
*- = not applicable*
** p<0.05*

**<u>Assessment and conclusion by applicant</u>:**

Eugenol was not teratogenic to rabbits at dose levels up to 500 / 350 mg/kg bw/d. The NOAEL for foetotoxicity / development is 250 mg/kg bw/d based on slight increase in post-implantation loss at 500 / 350 mg/kg bw/d. The NOAEL for maternal toxicity is 100 mg/kg bw/d based on reduced food consumption and clinical signs shortly after dosing at 250 mg/kg bw/d and above.

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.
The EU-agreed endpoint maternal NOAEL was 100 mg/kg bw/d (rabbit) and the developmental NOAEL was 250 mg/kg bw/d (rabbit). It was concluded there is no evidence of teratogenicity; target / critical effect: post-implantation loss, maternal clinical signs, and reduced food consumption in rabbits (EFSA Journal 2012;10(11):2914).The EU-agreed ADI and AOEL was derived from the developmental NOAEL obtained in this study, a safety factor of 100 was applied to the NOAEL to give an overall ADI / AOEL of 1.0 mg/kg bw/d (EFSA Journal 2012;10(11):2914).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with applicant conclusion. This study has been conducted according to required guideline, and does not present important deviations. Therefore, this study is considered acceptable.

An increase incidence of post implantation loss in high dose group was observed. This finding was not statistically significant and presented a wide standad desviation, mainly due to two females that showed an increased number of late embryonic deaths. This finding was associated to test substance administration. In addition, increased percentage of foetuses with at least sixteen forelimb phalanges ossified was noted (14%), however this is not indicative of a treatment-related increase in bone ossification as there were no trends with the ossification of other bones of the skeleton.

On the other hand, several clinical signs were described in the female dams at mid and high doses. Additionally reduction of food consumption was described through gestation day 6-12 at high dose group, and through gestation day 9-12 in mid dose group.

**NOAEL** $_{\text{developmental toxicity}}$: 250 mg/kg bw/day based on increased postimplantation loss.

**NOAEL** $_{\text{maternal toxicity}}$: 100 mg/kg bw/day based on clinical signs and decreased food consumption.

## B.6.7. NEUROTOXICITY

No neurotoxicity studies were submitted.

### B.6.7.1. Neurotoxicity studies in rodents

Neurotoxicity studies in rodents with eugenol were not provided.

According to Commission regulation (EU) No 283/2013 such studies shall be performed for active substances with structures that are similar or related to those capable of inducing neurotoxicity, and for active substances which induce specific indications of potential neurotoxicity, neurological signs or neuropathological lesions in toxicity studies at dose levels not associated with marked general toxicity. Performance of such studies shall also be considered for substances with a neurotoxic mode of pesticidal action.

A review of the available information about neurotoxicity linked to eugenol has been carried out and it is presented in Volume 1, chapter 2.6.7.

### B.6.7.2. Delayed polyneuropathy studies

Delayed polyneuropathy studies performed with eugenol were not provided.

According to Commission regulation (EU) No 283/2013, delayed polyneuropathy studies shall be performed for active substances of similar or related structures to those capable of inducing delayed polyneuropathy such as organophosphorus compounds. As eugenol does not fulfil these criteria, further testing is not deemed necessary by the RMS.

## B.6.8. OTHER TOXICOLOGICAL STUDIES

### B.6.8.1. Toxicity studies on metabolites and relevant impurities

*This section includes the assessment of the relevant impurity methyleugenol.*

*Methyleugenol is a relevant impurity of eugenol. No toxicological data on methyleugenol were provided during the first evaluation of eugenol, and EFSA set a critical area of concern and data gap because an acceptable level without toxicological concern could not be established. EFSA requested "further toxicological assessment and reference values in order to conduct the human health risk assessment". To address this point, the applicant has conducted a literature search for methyleugenol. A total of eighteen publications have been submitted to evaluate the toxicological relevance of methyl eugenol for the endpoints that include information to evaluate ADME (2 studies), short-term toxicity (3 studies), genotoxicity ((9 studies), carcinogenicity (3 studies) and other supporting toxicology studies (6 studies). Additionally, the RMS has considered an in vitro metabolism study of methyleugenol (B.6.8.1.1.1-03). These studies have not been previously evaluated under Directive 91/414/EEC or Regulation (EU) 1107/2009 for methyleugenol and are submitted for the purpose of renewal of the active substance, eugenol.*

#### B.6.8.1.1. Toxicity studies on relevant impurities (methyleugenol)

##### B.6.8.1.1.1. ADME

**B.6.8.1.1.1- 01. Toxicokinetics: Rat and mice**

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Hong, S.P., Fuciarelli, A.F., Johnson, J.D., Graves, S.W., Bates, D.J., Waidyanatha, S. and Smith C.S. |
| **Report year** | 2013 |
| **Report title** | Toxicokinetics of methyleugenol in F344 rats and B6C3F$_1$ mice |
| **Report No** | Not applicable, published study (*Xenobiotica*, 43 (3), 293-302) |
| **Document No** | KCA 5.8.1/01 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 417 (2010) |
| **Deviations from current test guideline OECD TG 417 (2010)** | -Test material not characterised (batch and purity not reported).<br>-Dose selection not justified<br>-No individual data were provided (mean values only). |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol (MEG); Purity: not stated ; Lot/Batch No.: not specified |
| **Vehicle** | Corn oil (oral)<br>Cremophor:ethanol:water (1/1/8; v/v/v) (intravenous) |
| **Test system** | F344/N rats and B6C3F1 mice |
| **Route** | Oral (gavage) and intravenous injection |
| **Test method** | This study was conducted as a follow up to the NTP (2000) studies (KCA 5.8.1/3a).<br>Plasma toxicokinetic parameters of methyleugenol in rats and mice have been estimated following intravenous and oral administration of the test substance:<br><u>Dosage intravenous route</u>:<br>Eighteen non-fasted rats/sex and 36 non-fasted mice/sex were given a single IV bolus injection (2 mL/kg for rats; 4mL/kg for mice) of methyleugenol in Cremophor:ethanol:water (1/1/8; v/v/v), at a dose of 37 mg/kg through a Silastic® catheter.<br><u>Dosage oral route</u>:<br>Twenty-one non-fasted rats/sex/dose group and 42 non-fasted mice/sex/dose group were given a single gavage bolus (5 mL/kg for rats; 10 mL/kg for mice) of methyleugenol in corn oil at 37, 75, or 150 mg/kg. |

| | |
|---|---|
| | Animals were anaesthetised for blood collection. Following IV dosing, three rats/sex/time point were bled at each of the first 6 time points (up to 1 h post-dosing) and up to 2 mL of blood was collected. The rats were returned to their cages to recover and randomly re-assigned (3 rats/sex/time point) to the remaining 6 time points for additional sampling. For the second bleeding of each rat which occurred no sooner than 1 h following the initial bleeding, the maximum amount of blood was collected prior to sacrifice (3 rats/sex). Three mice/sex/time point were bled at each time point. Each mouse was bled only once and the maximum amount of blood was collected prior to sacrifice.<br>Following gavage dosing, three rats/sex/ time point/dose group were bled at each of the first 7 time points (up to 2 h post-dosing) and up to 2 mL of blood was collected. The rats were returned to their cages to recover and randomly re-assigned (3 rats/sex/time point/dose group) to the remaining 7 time points for blood collection. Three mice/sex/time point/dose group were bled at each time point. Each mouse was bled only once at 2 (IV 37 mg/kg, gavage 37 and 75 mg/kg only), 5, 10, 15 (gavage 150 mg/kg only), 20, 30 min, and 1, 1.5, 2, 3, 4, 5 (gavage 37 and 75 mg/kg only), 6, 8, 12 (gavage groups only), and 24 h (gavage 150 mg/kg only) post-dose, and the maximum amount of blood was collected prior to termination.<br>Blood samples were collected into glass tubes containing EDTA anticoagulant, mixed gently, and placed on wet ice. Plasma was separated from blood within 60 min of collection by centrifugation at ~1750g for 10 min in a refrigerated (~4°C) table-top centrifuge and stored in polypropylene vials at −70°C.<br>*Toxicokinetics:*<br>Plasma was extracted using an ethyl acetate solution and analysed using gas chromatography (method validated according to FDA guidelines for bioanalytical methods). Toxicokinetic parameter estimates following IV or gavage administration were derived only from plasma methyleugenol measurements that were above the LOQ. |

**Results**

The highest plasma concentrations were observed at the 2-min time point following IV administration in both sexes of rats and mice. The plasma concentration-versus-time profiles following gavage administration to rats demonstrated a rapid absorption phase that occurred within 15 min post dosing in both sexes. In mice, the plasma concentration-versus-time profiles following gavage administration showed a rapid absorption phase that occurred within 30 min post dosing. Absorption following gavage administration was rapid with $T_{max}$ values within 15 min in rats and 30 min in mice. $AUC_T$ values increased with dose in both species but the magnitude of the increases was greater than proportional to dose (Table B.6.8.1.1.1-01/1 and Table B.6.8.1.1.1-01/2).

**Table B.6.8.1.1.1-01/1. Toxicokinetic parameters estimates following IV administration of 37 mg/kg bw methyleugenol in rats and mice**

| Parameter* | Rats | | Mice | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| **$C_0$ (µg/mL)** | 20.0 ± 2.0 | 28.9 ± 2.3 | 10.9 ± 2.8 | 13.6 ± 2.4 |
| **$T_{1/2\alpha}$ (min)** | 17.1 ± 1.2 | 10.9 ± 0.7 | 6.21 ± 0.98 | 7.51 ± 1.16 |
| **$T_{1/2\beta}$ (min)** | 131 ± 19 | 119 ± 9 | 71.4 ± 12.7 | 38.7 ± 3.8 |
| **$AUC_\infty$ (µgmL$^{-1}$ min)** | 593 ± 16 | 624 ± 11 | 136 ± 10 | 203 ± 18 |
| **$AUC_T$ (µgmL$^{-1}$ min)** | 542 ± 15 | 557 ± 10 | 111 ± 8 | 176 ± 18 |
| **$AUC_T$/dose(µgmL$^{-1}$ min)** | 14.6 ± 0.4 | 15.0 ± 0.3 | 2.99 ± 0.23 | 4.76 ± 0.48 |
| **Cl (mL min$^{-1}$ kg)** | 62.3 ± 1.6 | 59.3 ± 1.0 | 271 ± 20 | 182 ± 16 |
| **$V_d$ (L/kg)** | 11.8 ± 1.7 | 10.2 ± 0.8 | 28.0 ± 5.4 | 10.2 ± 1.4 |
| *Estimate ± SE. Parameters estimated using two-compartmental model. | | | | |

**Table B.6.8.1.1.1-01/2. Toxicokinetic parameters estimates following oral administration of 37, 75 and 150 mg/kg bw methyleugenol in rats**

| Parameter* | Species | Male | | | Female | | |
|---|---|---|---|---|---|---|---|
| | | 37 mg/kg | 75 mg/kg | 150 mg/kg | 37 mg/kg | 75 mg/kg | 150 mg/kg |
| **$C_{max}$ (µg/mL)** | Rat | 0.314 ± 0.180 | 2.22 ± 1.27 | 1.93 ± 1.12 | 0.341 ± 0.049 | 0.895 ± 0.067 | 4.77 ± 0.81 |
| | Mouse | 0.153 ± 0.075 | 0.505 ± 0.207 | 3.74 ± 1.34 | 0.132 ± 0.015 | 0.520 ± 0.389 | 3.46 ± 0.42 |
| **$T_{max}$ (min)** | Rat | 5 | 5 | 15 | 10 | 10 | 5 |
| | Mouse | 20 | 30 | 30 | 20 | 20 | 20 |
| **$AUC_T$ (µgmL$^{-1}$ min)** | Rat | 21.0 ± 2.2 | 122 ± 12 | 465 ± 31 | 23.0 ± 3.5 | 82.8 ± 6.1 | 498 ± 32 |
| | Mouse | 10.2 ± 0.8 | 30.2 ± 2.5 | 205 ± 27 | 12.9 ± 0.9 | 24.9 ± 3.2 | 185 ± 25 |
| **$AUC_T$/dose (µgmL$^{-1}$ min)** | Rat | 0.567 ± 0.059 | 1.63 ± 0.16 | 3.10 ± 0.21 | 0.622 ± 0.096 | 1.10 ± 0.08 | 3.32 ± 0.22 |
| | Mouse | 0.275 ± 0.021 | 0.402 ± 0.033 | 1.37 ± 0.18 | 0.348 ± 0.025 | 0.333 ± 0.08 | 1.23 ± 0.16 |
| **$Cl_{app}$ (mL min$^{-1}$ kg)** | Rat | 1650 ± 160 | 603 ± 59 | 321 ± 21 | 1460 ± 210 | 885 ± 64 | 293 ± 19 |
| | Mouse | 3050 ± 360 | 2160 ± 240 | 717 ± 93 | 2550 ± 160 | 2940 ± 370 | 807 ± 110 |
| **Absolute bioavailability (%)** | Rat | 3.9 ± 0.9 | NA[a] | NA[a] | 4.1 ± 1.3 | NA[a] | NA[a] |
| | Mouse | 9.2 ± 6.4 | NA[a] | NA[a] | 7.3 ± 3.7 | NA[a] | NA[a] |

*Estimate ± SE. Parameters estimated using two-compartmental model
[a]Not applicable due to the lack of dose-proportionality

In rats, absolute bioavailability following a gavage dose of 37 mg/kg was similar in both sexes with values of 3.9 ± 0.9% for males and 4.1 ± 1.3% for females. In mice, absolute bioavailability following gavage dose of 37 mg/kg, was 9.2 ± 6.4% for males and 7.3 ± 3.7% for females (Table B.6.8.1.1/2).

Methyleugenol was cleared rapidly in rats of both sexes with apparent clearance (Clapp) decreasing ~5 times with increasing dose. Methyleugenol was cleared rapidly in mice of both sexes with Clapp decreasing ~4-fold (males) and ~3-fold (females) with increasing dose.

There was no sex difference in toxicokinetic parameters for rats or mice.

Methyleugenol was rapidly absorbed following gavage administration with both $C_{max}$ and $AUC_T$ increasing more than proportional to the dose. Methyleugenol was cleared rapidly following both IV and gavage administration in both species and sexes. The absolute bioavailability following gavage administration was low (≤ 9.2%) in rats and mice of both sexes. Thus, the toxicokinetic data are consistent with extensive first-pass metabolism and saturation of metabolism at doses higher than 37 mg/kg bw.

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable with restrictions).

Methyleugenol is rapidly and completely absorbed in rodents following gavage and intravenous administration.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with the notifier's assessment and conclusion and deems the study as supporting information to assess the blood toxicokinetics of methyleugenol in the rat and mouse. This study is a scientific publication from a peer-reviewed journal and deviations from current OECD TG 417 (2010) include limited detail of reporting (no individual results reported), no rationale for dose selection or incomplete characterisation of the test item. For these reasons the study is deemed as supporting information only.

### B.6.8.1.1.1-02. Metabolism *in vitro*: rat liver microsomes

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Cartus, A.T., Herrmann, K., Weishaupt, L.W., Merz, K.H., Engst, W., Glatt, H. and Schrenk, D. |
| **Report year** | 2012 |
| **Report title** | Metabolism of methyleugenol in liver microsomes and primary hepatocytes: pattern of metabolites, cytotoxicity and DNA adduct formation |
| **Report No** | Not applicable, published study (*Toxicol. Sci*, 129 (1), 21-34) |
| **Document No** | KCA 5.8.1/02 |
| **Guidelines followed in study** | No guideline |

| | |
|---|---|
| **Deviations from current test guideline OECD TG 417 (2010)** | -Test material not characterised (batch and purity not reported).<br>-No individual data were provided (mean values only). |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol; Purity: not stated ; Lot/Batch No.: not specified |
| **Test system** | Rat (male Wistar) liver microsomes (Aroclor 1254-induced and not-induced)<br>Human liver microsomes (150 gender-mixed donors)<br>Bovine (female German black pied cattle) liver microsomes |
| **Reference metabolites** | Eugenol, isoeugenol, 2,3,6-hydroxymethyleugenol, (*E*)-3´-oxomethylisoeugenol, (*RS*)-2´,3´-dihydroxy-2´,3´-dihydromethyleugenol, 6-hydroxymethylisoeugenol, (*E*)-3,4-dimethoxycinnamic acid, 3,4-dimethoxypropylbenzene, 1´-oxomethyleugenol, (*R/S*)-methyleugenol-2´,3´-epoxide, chavibetol, and 3,4-dihydroxyallylbenzene.<br>Purity and batch no. not reported for any of the compounds. |
| **Test method** | Microsomal incubation conditions<br>Incubations of microsomes (2mg microsomal protein/mL) were performed with methyleugenol (dissolved in DMSO) at concentrations 0.1, 0.5 or 2.5 mM (final concentration 5%) with an NADPH-regenerating system consisting of 1mM NADPH, 0.5 unit/mL glucose-6-phosphate dehydrogenase, 5mM glucose-6-phosphate, 3mM magnesium chloride and 50mM phosphate buffer (pH 7.4). After preincubation with substrate for 5min at 37ºC, the NADPH-generating system was added and the mixture was incubated for up to 24h. Controls were carried out with heat-inactivated microsomes and with intact microsomes without the NADPH-generating system. Incubations were performed in triplicates.<br>Isolation, culture and incubation of primary rat hepatocytes<br>Livers of male Wistar rats were perfused and isolated hepatocytes were seeded on rat tail collagen-coated Petri dishes or multiwall plates in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 1% antibiotics solution and left for 4h to attach. Cells were treated with methyleugenol or the synthesised metabolites from 1000 x stock solutions in DMSO for 2 and 12 h (isolation of DNA) or 24 h (cytotoxicity assay).<br>Phase I and Phase II metabolism of methyleugenol in rat primary hepatocytes<br>Rat primary hepatocytes ($10^6$ cells in 8.0 mL culture medium) were pre-treated with Aroclor 1254 (25 µM) for 48h. The culture medium was replaced and 200 µM of methyleugenol was added. Samples (2x200 µL of incubation supernatant) were taking hourly over 5h.One of each sample was directly applied to HPLC analysis, the other was post incubated with glucuronidase/sulphatase (400 units, 4µL) and was incubated for 10h at 37ºC. Samples were prepared and analysed using HPLC.<br>Identification and quantification of metabolites<br>Samples were diluted with acetone (-20ºC) containing an internal standard (0.1 or 0.5 mM). After cooling the samples were sonified and the precipitated protein removed by centrifugation. Identity of metabolites was checked by comparison with the synthesised standards and also it was verified using $^1$H NMR. For the quantification of microsomal metabolites the synthesised reference compounds were analysed using HPLC at various concentrations (1, 10, 50, 100, 500 and 1000 µM) in methanol. Injection volumes were 50 µL each. The area under the curve (peaks) were plotted against concentration. Metabolite concentrations were calculated from the slope of linear regression at the most sensitive wavelength. The internal standard was 3,4-dimethoxypropylbenzene (elution time 31 min). |

**Results**

Microsomes from human, rat, and bovine liver are capable of forming a broad spectrum of phase I metabolites from methyleugenol and the major pathway in microsomes from all species tested was the metabolite 1´-hydroxymethyleugenol (compound number 2) (Table B.6.8.1.1.1-02). The metabolite 6-hydroxymethyleugenol (compound number 4) was the second major one detectable in bovine microsomes, and not or hardly detectable

from human and rat microsomes. Human microsomes showed higher amounts of the side-chain dihydrodiol (*RS*)-2´,3´-dihydroxy-2´,3´-dihydromethyleugenol (compound number 7) and the 3´-hydroxylated product 3´-hydroxymethylisoeugenol (compound number 3).

**Table B.6.8.1.1.1-02. Concentrations (µM) of Methyleugenol (at initial concentrations 100 µM or 500 µM) and metabolites detected in microsomal supernatants**

| Compound | ARLM | | RLM | | BLM | | HLM | |
|---|---|---|---|---|---|---|---|---|
| | 100 µM* | 500 µM** | 100 µM** | 500 µM** | 100 µM** | 500 µM** | 100 µM** | 500 µM** |
| **Methyleugenol** | 18.1 ± 0.2 | 71.5 ± 4 5 | 51.2 ± 5.2 | 285.5 ± 24.6 | 22.9 ± 2.1 | 259.5 ± 24.7 | 10.9 ± 3.0 | 257.5 ± 13.4 |
| **1'-Hydroxymethyleugenol (2)** | **39.4 ± 2.9** | **203.5 ±14.4** | **9.2 ± 0.4** | **52.0 ± 2 1** | **29.8 ± 1.2** | **85.0 ± 5.6** | **21.9 ± 1.2** | **64.5 ± 5.4** |
| **3'-Hydroxymethylisoeugenol (3)** | 9.8 ± 3.0 | 38.5 ± 0 5 | 10.4 ± 2.8 | 48.5 ± 1 3 | 12.4 ± 0.9 | 32.0 ± 0.4 | 21.8 ± 1.0 | 64.0 ± 1.0 |
| **6-Hydroxymethyleugenol (4)** | 7.6 ± 3.9 | 61.0 ± 0.4 | ND | NQ | 17.7 ± 0.2 | 19.5 ± 0.04 | ND | 3.0 ± 0.00 |
| **3'-Oxomethylisoeugenol (6)** | ND | 10.5 ± 0 1 | 0.6 ± 0.2 | 6.0 ± 0.02 | 0.5 ± 0.1 | 6.5 ± 0.01 | 1.1 ± 0.1 | 11.5 ± 0.02 |
| **(*RS*)-2',3'-dihydroxy-2',3'-dihydromethyleugenol (7)** | ND | 3.5 ± 0.0 | 1.6 ± 1.4 | 6.5 ± 0.02 | 3.8 ± 0.9 | 7.0 ± 0.01 | 8.9 ± 1.7 | 10.5 ± 0.02 |
| **Eugenol (9)** | ND | 5.0 ± 0.005 | 2.4 ± 0.1 | 1.1 ± 0.03 | 0.5 ± 0.1 | 7.0 ± 0.03 | 1.4 ± 0.1 | 11.5 ± 0.05 |
| **Chavibetol (11)** | ND | 0.5 ± 0.001 | 1.1 ± 0.03 | 0.4 ± 0.01 | NQ | NQ | NQ | 3.0 ± 0.01 |
| **Sum of metabolites** | **56.7 ± 4.7** | **322.5 ± 14.8** | **25.2 ± 5.1** | **114.5 ± 7.8** | **64.6 ± 4.6** | **152.5 ± 15.4** | **55.2 ± 2.0** | **165.0 ± 21.0** |

Data represents mean ± SD from three incubations
ARLM: Aroclor 1264-induced rat liver microsomes; RLM: rat liver microsomes; BLM: bovine liver microsomes; HLM: human liver microsomes
*: 10min after incubation
**: 2h after incubation
ND: Not detectable; NQ: not quantifiable

The metabolic pathways of methyleugenol in liver microsomes are displayed in Figure B.6.8.1.1.1-02.

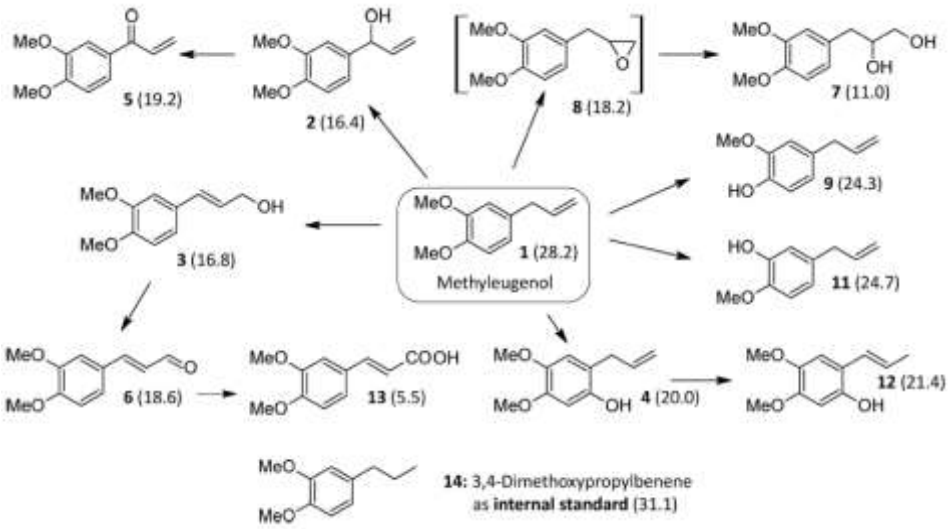



**Figure B.6.8.1.1.1-02: Phase I metabolic pathway of methyleugenol in liver microsomes**

**<u>Assessment and conclusion by applicant</u>:**

This new study is considered to be acceptable as supporting information (reliable with restrictions) on the basis that it does not follow standard methodology but provides some useful information on methyleugenol metabolism *in vitro*.

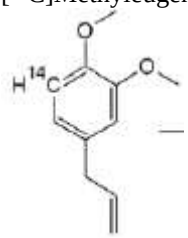
**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:** The RMS agrees with the notifier's assessment and conclusion and deems the study as supporting information to assess the metabolism *in vitro* of methyleugenol using liver microsomes (rat, bovine and human) and primary rat hepatocytes. This study is a scientific publication from a peer-reviewed journal and deviations from current OECD TG 417 (2010) include limited detail of reporting incomplete characterisation of the test item and it does not follow standard methodology. For these reasons the study is deemed as supporting information only.

**B.6.8.1.1.1-03. Metabolism *in vitro*: rat liver microsomes**

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Minet, E.F., Gentile, D., Clive, M. and Massey, E.D. |
| **Report year** | 2012 |
| **Report title** | A comparative *in vitro* kinetic study of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol activation and detoxification in human, mouse and rat liver and lung fractions. |
| **Report No** | Not applicable, published study (*Xenobiotica*, Vol 42 (5), 429-441) |
| **Document No** | KCA 5.1.1/07 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study (*in vitro* comparative metabolism study) |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | <u>Labelled Methyleugenol</u> (test item)<br>[$^{14}$C]Methyleugenol; Lot/Batch No.: not stated, Radiopurity/specific activity: not stated<br>  <br><u>Methyleugenol</u> (reference item); Lot/Batch No: Not stated, Purity: not stated |
| **Test systems** | Microsomes and S9 fractions from human, rat and mouse purchased from commercial suppliers.<br>**<u>Human (non-smokers)</u>**:<br>Liver microsomes: 1 ♂, 2 ♀, batch SHH/JVT<br>Liver S9 : 2 ♂, 1 ♀, batch FRA259048<br>Lung microsomes : : 5 ♂, 5 ♀, batch APT<br>Lung S9 : 2 ♂, batch FRA50406<br>**<u>Mouse (ICR/CD-1)</u>**:<br>Liver microsomes : 150 ♂, batch ZLR<br>Liver S9 : 100 + ♂, batch VLH<br>Lung microsomes : 6 ♂, batch CMM<br>Lung S9 : 6 ♂, batch CMS<br>**<u>Rat (Sprague Dawley)</u>**:<br>Liver microsomes: 1 ♂, batch MIC254015<br>Liver S9 :15 ♂, batch FRA254002<br>Lung microsomes : 10 ♂, MIC501005<br>Lung S9 : 12 ♂, batch FRA501007 |

| | |
|---|---|
| **Test method** | Incubations with [$^{14}$C]-methyleugenol (20 µM, 1.8 µCi) were performed in triplicate in microfuge tubes at 37ºC with hepatic microsomes (1 mg/mL) or S9 fractions (1 mg/mL), reaction buffer (50 mM potassium phosphate, pH 7.4) and relevant cofactors: NADPH (0.6 U isocitrate-dehydrogenase), 250 µM $NADP^+$, 2.5 mM DL isocitric acid and 5 mM $MgCl_2$, UDPGA (2 mM), GSH (500 µM). The incubation time was set to 180 min.<br>Covalent binding to protein:<br>Covalent binding was investigated as an indirect method to measure the formation of the quinone methide intermediate. Control experiments with glutathione (GSH) were performed to assess the protein binding. Samples were stored at -20ºC overnight to aid protein precipitation and centrifuged at 10000 g for 10min. Extraction of the protein pellets was done with one acetonitrile:methanol (2:1 v/v) and two with 70 % methanol washes followed by sonication and centrifugation. The supernatant was combined and analysed by LSC.<br>Glucuronidase treatment:<br>Supernatants from UDPGA incubations were submitted for glucuronidation treatment. Deconjugation reactions were performed overnight at 37ºC with β-glucuronidase (*Helix pomatia*, 10.4 µL of 10 mg/mL).<br>Metabolite identification & quantification:<br>Metabolites were identified using HPLC analyses of post-incubate samples with a radio UV-HPLC. The identity of the 1'-hydroxymetabolite was confirmed by chromatography with synthetic 1'-hydroxyeugenol. The identities of other metabolites in the profile were not investigated for the purpose of this study, which focused on the 1'-hydroxy and quinone methide metabolites.<br>Quantification of peak metabolites was done by integration as a % of the total soluble radioactivity. The total soluble radioactivity (supernatant) is a % of the total dose recovered, which is a percentage of the total dose recovered.<br>Kinetics:<br>Km and Vmax were established using the incubation conditions described above. Incubation time was 10 min for human and mouse and 30 min for rat. The following substrate concentrations were tested: 5, 7.5, 10, 12.5, 15, 20, 25, 30, 40, 50, 75, 100, 125, 150, 200 and 250 µM. Km and Vmax were calculated by non-linear regression with Prism V.4. |

**Results**

The chromatographic profile of [$^{14}$C]-methyleugenol in human hepatic microsomes and S9 fractions is displayed in Figure B.6.8.1.1.1-03/1. The formation of 1'-hydroxy and phenoxy-glucuronide conjugates were identified, whereas the identities of other soluble metabolites were not investigated. These metabolites were identified in all three species following incubation with hepatic microsomes.

Covalent binding of [$^{14}$C]-methyleugenol to liver microsomal proteins after incubations with NADPH was significantly lower in comparison with [$^{14}$C]-eugenol in the presence of glutathione (Figure B.6.8.1.1.1-03/2).

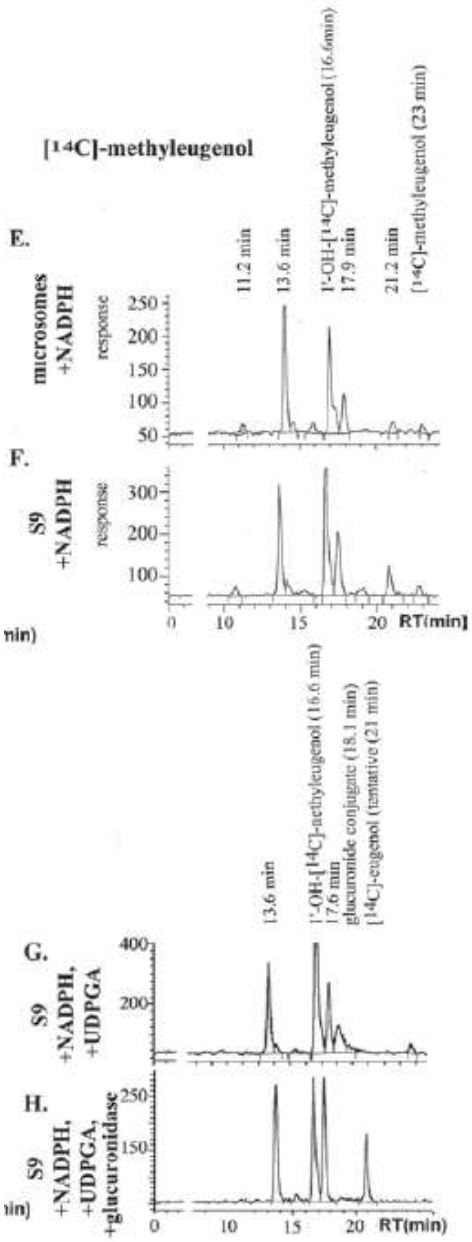



**Figure B.6.8.1.1.1-03/1. Chromatograms of metabolites of [$^{14}$C]-methyleugenol in microsomes with NADPH (E), in S9 fractions supplemented with NADPH (F), in S9 supplemented with NADPH and UDPGA (G) and in S9 fractions supplemented with NADPH and UDPGA.**

The kinetics and quantification of 1'-hydroxy, covalent binding and glucuronidated (phase II) metabolites across the three species in both liver microsomes or S9 fractions are displayed in Table B.6.8.1.1.1-03. The percentage of 1'-hydroxy metabolite formation was highest in the mouse, 52.4 % versus 24.4 % in rat or 22.2 % in human liver microsomes. The *in vitro* clearance of methyleugenol through the formation of this metabolite was highest in the mouse whereas two-fold and four-fold lower in the rat and human, respectively (Table B.6.8.1.1.1-03/1). Covalent binding, that is the formation of the quinone methide metabolite intermediate, was comparable between human and rat, being the mouse the highest percentage of formation. However, covalent binding was considerable lower in contrast to eugenol). No phase II metabolites of [$^{14}$C]-methyleugenol were detected in the absence of NADPH whereas in the presence of NADPH glucuronidation was detected in humans and to a lower extent in mouse and rat, all being considerably lower to glucuronidation detected with [$^{14}$C]-eugenol.

The formation of the 1'-hydroxy and covalent binding of [$^{14}$C]-methyleugenol is none or much lower in the lung compared to liver.

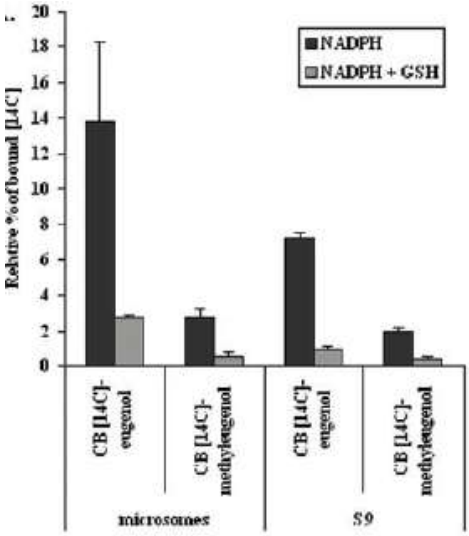



**Figure B.6.8.1.1.1-03/2. Relative percentage of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol covalently bound to human hepatic microsomal/S9 proteins after incubation in the presence of NADPH ± GSH**

**Table B.6.8.1.1.1-03/1. Relative percentage and kinetic values ($K_m$, $V_{max}$, CLint) for 1'-hydroxylation (A), covalent binding (CB) (B) and phenolic glucuronidation (C,D) of [$^{14}$C]-methyleugenol in human, mouse and rat hepatic microsomes + NADPH, and S9 fractions ± NADPH + UDPGA**

| **A** | | **Microsomes + NADPH** | | |
|---|---|---|---|---|
| Species | 1'-hydroxylation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 22.2 % ± 0.2 | 127.8 | 0.95 | 7.4 |
| Mouse | 52.4 % ± 13.3 | 78.49 | 2.49 | 31.8 |
| Rat | 24.4 % ± 5.7 | 67.4 | 1.2 | 17.8 |
| **B** | | **Microsomes + NADPH** | | |
| Species | CB | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 2.7 % ± 0.5 | NC | NC | NC |
| Mouse | 7.8 % ± 3.1 | 241.1 | 0.1 | 0.4 |
| Rat | 2.5 % ± 0.5 | 424.6 | 0.2 | 0.5 |
| **C** | | **S9 + UDPGA** | | |
| Species | Glucuronidation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | No peak | NC | NC | NC |
| Mouse | No peak | NC | NC | NC |
| Rat | No peak | NC | NC | NC |
| **D** | | **S9 + NADPH + UDPGA** | | |
| Species | Glucuronidation | Km (µM) | Vmax (nmol/min/mg prot.) | CLint (µL/min/mg prot.) |
| Human | 12.2 % ± 0.4 | 52.9 | 0.1 | 1.9 |
| Mouse | 4.0 % ± 0.4 | NC | NC | NC |
| Rat | 3.9 % ± 0.8 | NC | NC | NC |

In mouse and human, co-incubation of NADPH with UDPGA considerably reduced the formation of the 1'-hydroxy-[$^{14}$C]-eugenol proximate-carcinogen and the cytotoxic quinone methide (Table B.6.8.1.1.1-03/2). However, the formation of 1'-OHmethyleugenol does not seem to be affected by the co-incubation of NADPH with UDPGA, which could indicate eugenol is further detoxified by glucuronidation.

**Table B.6.8.1.1.1-03/2. Relative percentage of 1'-hydroxy (1'-OH) and covalently bound (CB) [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol metabolites following incubation with human, mouse and rat hepatic S9 fractions with NADPH ± UDPGA.**

| Species | Cofactors | 1'-OH Eugenol | Eugenol CB | 1'-OHmethyleugenol | Methyleugenol CB |
|---|---|---|---|---|---|
| Human | NADPH | 3.7 % ± 0.2 | 7.2 % ± 0.3 | 34.7 % ± 0.6 | 2.0 % ± 0.2 |
| | NADPH + UDPGA | **No peak** | **0.9 % ± 0.01** | 34.3 % ± 0.9 | 1.2 % ± 0.2 |
| Mouse | NADPH | 15.4 % ± 0.7 | 1.2 % ± 0.3 | 54.7 % ± 3.2 | 1.06 % ± 0.1 |
| | NADPH + UDPGA | **No peak** | **0.3 ± 0.04** | 64.1 % ± 1.7 | 0.3 % ± 0.06 |
| Rat | NADPH | 9.8 % ± 0.7 | 3.1 % ± 0.05 | 39.0 % ± 2.0 | 0.5 % ± 0.1 |
| | NADPH + UDPGA | 6.1 % ± 0.4 | 1.9 % ± 0.03 | 40.3 % ± 0.1 | 0.5 % ± 0.2 |

Results for each metabolite are expressed as relative percentage (%) of starting substrate dose ± SD for independent triplicates. Key changes following addition of UDPGA are in bold.

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable with restrictions).

In liver, methyleugenol generates a significant amount of the 1-hydroxy proximate carcinogen while eugenol glucuronidation prevents the formation of both 1-hydroxyeugenol and the quinone methide.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS considers the study as relevant to address a top-level comparison of metabolism of methyleugenol in various species. Two metabolic routes (phase I - covalent binding and 1'-hydroxylation and phase II -glucuronidation) are investigated in both liver and lung. Based on the results of the study, methyleugenol is metabolised to 1'-OHMethyleugenol in the mouse and to a lesser extent in human and rat. No glucuronidation was observed in the absence of UDPGA. The extent of glucuronidation was higher in humans (12.2 %) in comparison to mouse (4.0 %) or rat (3.9 %). Covalent binding was detected, implying the formation of the quinone methide reactive intermediate, which was depleted by the addition of glutathione. Overall, the RMS considers that, under the test conditions of the study, the metabolism *in vitro* of methyleugenol differs from the metabolism of eugenol, i.e. formation of the reactive 1'-OHmethyleugenol metabolite.

### B.6.8.1.1.2. Short term toxicity

**B.6.8.1.1.2-01. Oral 14-week toxicity study in rats with methyleugenol**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 408 (2018). |
| **Deviations from current test guideline OECD TG 408 (2018)** | -Dose schedule administration was 5 days per week.<br>-Food consumption was not measured.<br>-Ophthalmological examination was not performed.<br>-Thyroid gland, ovaries, uterus, brain, adrenals, prostate and pituitary gland were not weighted.<br>- Circulating thyroid hormones (T4, T3, TSH) levels were not measured.<br>-Complete histopathology was only performed on negative controls and top dose groups. |
| **Previous evaluation** | No |

| | |
|---|---|
| **GLP/Officially recognised testing facilities** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Test system** | F344/N rats |
| **Test method** | Groups of 9-10 male and 10 female F344/N rats received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, 5 days per week for 14 weeks; groups of 10 male and 10 female rats received deionised water only. Groups of 10 male and 10 female for a special study rats received the same doses for 22 or 23 days. These animals from the special study were used to obtain samples for haematology and clinical chemistry analysis at day 5 and week 4. Dosing volumes were 5 mL/kg bw. Feed and water were available *ad libitum*. Rats were housed three (males) or five (females) per cage. Cages and racks were rotated every 2 weeks. Clinical findings were recorded and the animals were weighed weekly and at the end of the studies.<br><br>*Necropsy*<br><br>Necropsy was performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.<br><br>*Clinical pathology*<br><br>Blood was collected from the retro-orbital sinus of special study rats on day 5 and at week 4, and from all core study rats surviving to the end of the studies for haematology and clinical chemistry determinations.<br><br>Haematology: haematocrit; haemoglobin concentration, erythrocyte and reticulocyte counts, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, platelet count and morphology, leukocyte count and differentials.<br><br>Clinical chemistry**:** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and total bile acids.<br><br>*Histopathology*<br><br>Complete histopathology was performed on all core study water control, vehicle control, and 1000 mg/kg bw/d dose groups. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, oesophagus, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. In addition, the kidney, liver, salivary gland, and stomach of male and female rats and the adrenal gland and spleen of male rats in the 10, 30, 100, and 300 mg/kg bw/d groups, the testis and uterus of rats in the 100 and 300 mg/kg groups.<br><br>*Sperm motility and vaginal cytology*<br><br>At the end of the studies, sperm samples were collected from vehicle control, 30, 100, and 300 mg/kg bw/d male rats for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda epididymis, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from vehicle control, 30, 100, and 300 mg/kg bw/d female rats for vaginal cytology evaluations. The following parameters were evaluated: the estrous cycle lengths and relative frequency of estrous stages. |

| | |
|---|---|
| **Statistical analysis of results** | *Survival*<br>The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.<br><br>*Histopathology analysis*<br>Lesions detected were analysed using Fisher exact test, a procedure based on the overall proportion of affected animals was used.<br><br>*Analysis of continuous variables*<br>Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and bodyweight data, which have approximately normal distributions, were analysed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Haematology, clinical chemistry, plasma concentration, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analysed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. |

**Results**

**Survival:** All rats survived until the end of the study (Table B.6.8.1.1.2-01/1).

**Table B.6.8.1.1.2-01/1. Survival in 14-week rat study**

| | % Survival | |
|---|---|---|
| **Dose (mg/kg bw/day)** | **Males** | **Females** |
| **water control** | 10/10 (100%) | 10/10 (100%) |
| **Vehicle control** | 10/10 (100%) | 10/10 (100%) |
| **10** | 10/10 (100%) | 10/10 (100%) |
| **30** | 10/10 (100%) | 10/10 (100%) |
| **100** | 10/10 (100%) | 10/10 (100%) |
| **300** | 9/9 (100%) | 10/10 (100%) |
| **1000** | 10/10 (100%) | 10/10 (100%) |

**Clinical signs**: Clinical findings possibly related to test substance administration included emaciation and urine staining in 100, 300, and 1000 mg/kg bw/day female dose rats.

**Bodyweights:** Final mean bodyweights and bodyweight gains of 300 and 1000 mg/kg bw/day dose groups of males, and all dosed groups of females were statistically significantly lower than controls (Table B.6.8.1.1.2-01/2).

**Table B.6.8.1.1.2-01/2. Bodyweight and bodyweight gains in 14-week rat study (mean ± standard error)**

| Dose (mg/kg bw/day) | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | **Initial** | **Final** | **bwg** | **Initial** | **Final** | **bwg** |
| **Water control** | 114 ± 4 | 327±6 | 214 ± 4 | 96 ±2 | 192 ± 3 | 96 ± 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Vehicle control** | 118± 4 | 331±7 | 214 ± 5 | 97 ±2 | 196 ± 4 | 99 ± 5 |
| **10** | 114± 4 | 331±5 | 218 ± 5 | 94 ±2 | 184 ± 1***(↓7%)** | 89 ± 2***(↓10%)** |
| **30** | 117± 5 | 332±8 | 216 ± 4 | 98 ±3 | 187 ± 4***(↓5%)** | 89 ± 3***(↓10%)** |
| **100** | 119± 4 | 322±7 | 203 ± 4 | 97 ±2 | 183 ± 2****(↓7%)** | 86 ± 2****(↓13%)** |
| **300** | 116± 5 | 304±4****(↓8%)** | 188 ± 4****(↓12%)** | 97 ±3 | 180 ± 2****(↓8%)** | 83 ± 3****(↓16%)** |
| **1000** | 112± 4 | 231±4****(↓30%)** | 119 ± 5****(↓45%)** | 95 ±3 | 164 ± 4****(↓16%)** | 69 ± 3****(↓30%)** |

** Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test. ** P≤0.01*

## Haematology

Data of haematology parameters are listed in Tables B.6.8.1.1.2-01/3-4 for males and females, respectively. At week 14, there was a slight decrease in haemoglobin concentrations and haematocrit values in the 300 mg/kg bw/day female dose group and in top dose groups for both sexes. The haematocrit and haemoglobin decreases would be consistent with anaemia, but there was no corresponding decrease in erythrocyte counts. There was, however, an erythrocyte microcytosis demonstrated by decreased mean cell volumes in the 300 mg/kg bw/day male dose group (2% decrease) and in top dose groups for both sexes in almost all measure points. Animals in the 300 and 1000 mg/kg bw/day dose groups also had decreased mean cell haemoglobin values; this would be consistent with the decreases in mean cell volumes. This suggests that, while there were equal numbers of circulating erythrocytes for animals in the 300 and 1000 mg/kg bw/day dose groups at week 14, the erythrocytes were smaller, resulting in lower haematocrit and haemoglobin values. There was evidence of a thrombocytosis at all time points, demonstrated by increased platelet counts in the 100 mg/kg bw/day or greater dose groups at study termination.

**Table B.6.8.1.1.2-01/3. Summary of haematology parameters in males in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Water** | **Vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Day 5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 4 | 10 | 9 | 9 | 10 | 10 | 10 | 10 |
| Week 14 | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| **Haematocrit (%)** | | | | | | | |
| Day 5 | 41.0 ± 0.4 | 40.5 ± 0.4 | 39.5 ± 0.5 | 40.5 ± 0.6 | 39.8 ± 0.5 | 40.7 ± 0.6 | 40.7 ± 0.5 |
| Week 4 | 43.7 ± 0.5 | 43.7 ± 0.5 | 43.8 ± 0.6 | **46.2 ± 0.4* (↑6%)** | 44.9 ± 0.4 | 45.6 ± 0.6 | 41.2 ± 1.1 |
| Week 14 | 45.6 ± 0.5 | 45.8 ± 0.7 | 45.5 ± 0.3 | 45.4 ± 0.3 | 47.0 ± 0.3 | 46.5 ± 0.4 | **40.0 ± 1.4** (↓13%)** |
| **Haemoglobin (g/dL)** | | | | | | | |
| Day 5 | 13.5 ± 0.1 | 13.5 ± 0.2 | 13.1 ± 0.2 | 13.4 ± 0.2 | 13.3 ± 0.1 | 13.4 ± 0.2 | 13.5 ± 0.2 |
| Week 4 | 15.3 ± 0.2 | 15.2 ± 0.2 | 15.2 ± 0.2 | **16.0 ± 0 2* (↑5%)** | 15.5 ± 0.1 | 15.7 ± 0.2 | 14.4 ± 0.4 |
| Week 14 | 14.7 ± 0.1 | 14.9 ± 0.2 | 14.8 ± 0.1 | 14.7 ± 0.1 | 15.3 ± 0.1 | 15.3 ± 0.1 | **13.3 ± 0.4** (↓11%)** |
| **Erythrocytes ($10^6$/µL)** | | | | | | | |
| Day 5 | 6.99 ± 0.13 | 6.94 ± 0.08 | 6.73 ± 0.11 | 6.90 ± 0.13 | 6.82 ± 0.10 | 7.01 ± 0.11 | 7.09 ± 0.12 |
| Week 4 | 7.73 ± 0.14 | 7.75 ± 0.10 | 7.74 ± 0.14 | **8.26 ± 0.08** (↑7%)** | **8.01 ± 0.09* (↑3%)** | **8.24 ± 0.12** (↑6%)** | **8.68 ± 0.07** (↑12%)** |
| Week 14 | 9.14 ± 0.09 | 9.08 ± 0.13 | 9.03 ± 0.07 | 8.96 ± 0.06 | 9.32 ± 0.05 | 9.32 ± 0.08 | 9.22 ± 0.10 |
| **Reticulocytes ($10^6$/µL)** | | | | | | | |
| Day 5 | 0.34 ± 0.02 | 0.34 ± 0.02 | 0.44 ± 0.04 | 0.32 ± 0.03 | 0.33 ± 0.04 | 0.40 ± 0.05 | 0.25 ± 0.04 |
| Week 4 | 0.33 ± 0.06 | 0.24 ± 0.03 | 0.31 ± 0.05 | 0.27 ± 0.04 | 0.25 ± 0.03 | 0.28 ± 0.04 | 0.31 ± 0.03 |
| Week 14 | 0.15 ± 0.02 | 0.12 ± 0.02 | 0.14 ± 0.01 | 0.14 ± 0.02 | 0.15 ± 0.01 | 0.15 ± 0.01 | 0.11 ± 0.02 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Mean cell volume (fL)** | | | | | | | |
| Day 5 | 58.9 ± 0.5 | 58.3 ± 0.4 | 58.9 ± 0.4 | 58.8 ± 0.4 | 58.2 ± 0.3 | 58.0 ± 0.3 | 57.4 ± 0.3 |
| Week 4 | 56.4 ± 0.5 | 56.3 ± 0.2 | 56.7 ± 0.3 | 55.9 ± 0.3 | 56.1 ± 0.2 | **55.3 ± 0.2** (↓2%)** | **47.5 ± 1.2** (↓16%)** |
| Week 14 | 49.9 ± 0.1 | 50.6 ± 0.2 | 50.6 ± 0.2 | 50.7 ± 0.2 | 50.3 ± 0.2 | **49.9 ± 0.1** (↓1%)** | **43.3 ± 1.6** (↓15%)** |
| **Mean cell haemoglobin (pg)** | | | | | | | |
| Day 5 | 19.4 ± 0.2 | 19.5 ± 0.1 | 19.5 ± 0.2 | 19.5 ± 0.1 | 19.5 ± 0.2 | 19.1 ± 0.1 | **19.1 ± 0.1* (↓2%)** |
| Week 4 | 19.8 ± 0.2 | 19.5 ± 0.2 | 19.7 ± 0.2 | 19.3 ± 0.2 | 19.3 ± 0.1 | **19.0 ± 0.1* (↓3%)** | **16.6 ± 0.4** (↓15%)** |
| Week 14 | 16.1 ± 0.1 | 16.4 ± 0.1 | 16.4 ± 0.1 | 16.4 ± 0.1 | 16.4 ± 0.0 | 16.4 ± 0.1 | **14.4 ± 0.5** (↓12%)** |
| **Mean cell haemoglobin concentration (g/dL)** | | | | | | | |
| Day 5 | 32.9 ± 0.2 | 33.4 ± 0.2 | 33.2 ± 0.1 | 33.1 ± 0.1 | 33.4 ± 0.2 | 33.0 ± 0.1 | 33.2 ± 0.2 |
| Week 4 | 35.0 ± 0.2 | 34.7 ± 0.4 | 34.8 ± 0.2 | 34.5 ± 0.3 | 34.5 ± 0.2 | 34.4 ± 0.2 | 35.0 ± 0.2 |
| Week 14 | 32.4 ± 0.1 | 32.6 ± 0.1 | 32.4 ± 0.1 | 32.4 ± 0.2 | 32.5 ± 0.1 | 32.8 ± 0.1 | 33.2 ± 0.2 |
| **Platelets ($10^3$/µL)** | | | | | | | |
| Day 5 | 885.6 ± 11.7 | 903.8 ± 20.8 | 893.2 ± 13.9 | 897.2 ± 26.4 | 889.0 ± 16.9 | 953.8 ± 14.7 | 1030.5 ± 39.2 |
| Week 4 | 783.6 ± 29.3 | 767.9 ± 12.7 | 778.6 ± 18.2 | **817.7 ± 16.3* (↑6%)** | **870.2 ± 14.0** (↑13%)** | **983.3 ± 7.2**b (↑28%)** | **1045.6 ± 56.9** (↑36%)** |
| Week 14 | 635.4 ± 10.2 | 681.9 ± 27.8 | 650.4 ± 8.4 | 663.1 ± 12.7 | **716.8 ± 7.8** (↑5%)** | **781.9 ± 10.9** (↑15%)** | **937.9 ± 78.4** (↑37%)** |
| **Leukocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 5.78 ± 0.67 | 6.91 ± 0.58 | 5.13 ± 0.78 | 5.94 ± 0.52 | 5.13 ± 0.50 | 6.67 ± 0.61 | 5.91 ± 0.70 |
| Week 4 | 8.40 ± 0.45 | 6.13 ± 0.49 | 8.10 ± 0.64 | 8.12 ± 0.48 | 7.53 ± 0.56 | 7.59 ± 0.72 | 7.77 ± 0.79 |
| Week 14 | 8.81 ± 0.58 | 9.14 ± 0.58 | 10.26 ± 0.50 | 9.44 ± 0.63 | **11.99 ± 0.43* (↑31%)** | 8.98 ± 1.10 | 10.13 ± 1.01 |
| **Segmented neutrophils ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.88 ± 0.11 | 1.18 ± 0.11 | 0.91 ± 0.21 | 0.90 ± 0.13 | 0.83 ± 0.11 | 0.98 ± 0.14 | 0.91 ± 0.13 |
| Week 4 | 0.84 ± 0.12 | 0.78 ± 0.11 | 1.07 ± 0.14 | 0.75 ± 0.06 | 0.94 ± 0.15 | 1.01 ± 0.11 | 1.32 ± 0.20 |
| Week 14 | 1.35 ± 0.13 | 1.50 ± 0.22 | 1.51 ± 0.09 | 1.52 ± 0.25 | 2.43 ± 0.32 | 1.87 ± 0.33 | 1.80 ± 0.23 |
| **Lymphocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 4.46 ± 0.52 | 5.30 ± 0.47 | 3.85 ± 0.52 | 4.66 ± 0.43 | 4.01 ± 0.41 | 5.14 ± 0.48 | 4.61 ± 0.56 |
| Week 4 | 7.37 ± 0.43 | 5.18 ± 0.4 | 6.78 ± 0.50 | **7.15 ± 0.47* (↑38%)** | 6.31 ± 0.47 | 6.43 ± 0.63 | 6.20 ± 0.62 |
| Week 14 | 7.04 ± 0.55 | 7.40 ± 0.43 | 8.27 ± 0.50 | 7.49 ± 0.48 | 9.01 ± 0.41 | 6.78 ± 0.77 | 8.14 ± 0.84 |
| **Monocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.42 ± 0.08 | 0.36 ± 0.08 | 0.32 ± 0.09 | 0.35 ± 0.07 | 0.26 ± 0.05 | 0.46 ± 0.11 | 0.41 ± 0.10 |
| Week 4 | 0.17 ± 0.05 | 0.17 ± 0.04 | 0.17 ± 0.08 | 0.19 ± 0.04 | 0.21 ± 0.05 | 0.13 ± 0.04 | 0.21 ± 0.04 |
| Week 14 | 0.31 ± 0.04 | 0.15 ± 0.04 | 0.36 ± 0.07 | 0.30 ± 0.07 | 0.42 ± 0.12 | 0.29 ± 0.10 | 0.14 ± 0.03 |
| **Eosinophils ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.02 ± 0.01 | 0.06 ± 0.03 | 0.03 ± 0.02 | 0.02 ± 0.01 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.01 ± 0.01 |
| Week 4 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.06 ± 0.02 | 0.05 ± 0.02 | 0.05 ± 0.03 | 0.02 ± 0.01 | 0.05 ± 0.03 |
| Week 14 | 0.11 ± 0.03 | 0.09 ± 0.04 | 0.12 ± 0.04 | 0.14 ± 0.06 | 0.09 ± 0.03 | 0.06 ± 0.03 | 0.03 ± 0.02 |

** Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test. ** (P≤0.01).*
*b) n=9*

**Table B.6.8.1.1.2-01/4. Summary of haematology parameters in females in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Water** | **Vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Day 5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 4 | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| Week 14 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Haematocrit (%)** | | | | | | | |
| Day 5 | 41.7 ± 0.6 | 41.2 ± 0.5 | 40.3 ± 0.8 | 41.7 ± 0.6 | 41.6 ± 0.6 | 40.2 ± 0.7 | 39.9 ± 0.4 |
| Week 4 | 44.9 ± 0.4 | 44.7 ± 0.4 | 44.7 ± 0.6 | 45.3 ± 0.5 | 45.4 ± 0.6 | 45.4 ± 0.4 | 44.2 ± 0.9 |
| Week 14 | 45.8 ± 0.5 | 45.7 ± 0.6 | 45.2 ± 0.6 | 45.3 ± 0.3 | 45.0 ± 0.6 | 44.4 ± 0.3 | **42.9 ± 0.6**(↓6%)** |
| **Haemoglobin (g/dL)** | | | | | | | |
| Day 5 | 13.9 ± 0.2 | 13.7 ± 0.1 | 13.4 ± 0.3 | 14.0 ± 0.2 | 13.8 ± 0.2 | 13.3 ± 0.3 | 13.3 ± 0.2 |
| Week 4 | 14.9 ± 0.2 | 14.9 ± 0.1 | 15.0 ± 0.2 | 15.1 ± 0.2 | 15.1 ± 0.2 | 15.1 ± 0.2 | 14.7 ± 0.3 |
| Week 14 | 15.1 ± 0.1 | 15.1 ± 0.1 | 14.9 ± 0.1 | 14.9 ± 0.1 | 14.8 ± 0.1 | **14.5 ± 0.1**(↓4%)** | **14.1 ± 0 2**(↓7%)** |
| **Erythrocytes ($10^6$/µL)** | | | | | | | |
| Day 5 | 7.38 ± 0 12 | 7.27 ± 0 11 | 7.01 ± 0.15 | 7.36 ± 0.15 | 7.32 ± 0.13 | 7.17 ± 0.15 | 7.17 ± 0.08 |
| Week 4 | 7.87 ± 0 10 | 7.81 ± 0.09 | 7.81 ± 0.10 | 7.89 ± 0.08 | 7.85 ± 0.10 | 8.06 ± 0.09 | **8.73 ± 0.10** (↑12%)** |
| Week 14 | 8.60 ± 0 10 | 8.51 ± 0 11 | 8.48 ± 0.10 | 8.45 ± 0.10 | 8.37 ± 0.10 | 8.35 ± 0.08 | 8.45 ± 0.11 |
| **Reticulocytes ($10^6$/µL)** | | | | | | | |
| Day 5 | 0.23 ± 0.03 | 0.24 ± 0.03 | 0.16 ± 0.03 | 0.21 ± 0.02 | 0.20 ± 0.02 | 0.17 ± 0.02 | **0.13 ± 0.01* (↓46%)** |
| Week 4 | 0.09 ± 0.04 | 0.04 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.02 | **0.08 ± 0.02* (↑100%)** | **0.10 ± 0.01** (↑150%)** | **0.10 ± 0.01** (↑150%)** |
| Week 14 | 0.13 ± 0.01 | 0.12 ± 0.01 | 0.14 ± 0.02 | 0.16 ± 0.03 | 0.15 ± 0.02 | 0.17 ± 0.03 | 0.17 ± 0.02 |
| **Mean cell volume (fL)** | | | | | | | |
| Day 5 | 56.5 ± 0.3 | 56.9 ± 0.3 | 57.6 ± 0.3 | 56.7 ± 0.3 | 57.0 ± 0.3 | 56.3 ± 0.3 | **55.4 ± 0.2** (↓3%)** |
| Week 4 | 57.0 ± 0.3 | 57.1 ± 0.2 | 57.1 ± 0.3 | 57.5 ± 0.3 | 57.8 ± 0.4 | 56.4 ± 0.2 | **50.6 ± 0.9** (↓11%)** |
| Week 14 | 53.4 ± 0.2 | 53.7 ± 0.2 | 53.4 ± 0.2 | 53.5 ± 0.2 | 53.7 ± 0.2 | 53.3 ± 0.2 | **50.7 ± 0.2** (↓6%)** |
| **Mean cell haemoglobin (pg)** | | | | | | | |
| Day 5 | 18.8 ± 0.1 | 18.9 ± 0.2 | 19.2 ± 0.1 | 19.0 ± 0.1 | 18.8 ± 0.1 | 18.6 ± 0.1 | 18.6 ± 0.1 |
| Week 4 | 19.0 ± 0.1 | 19.1 ± 0.2 | 19.2 ± 0.1 | 19.2 ± 0.1 | 19.2 ± 0.1 | 18.8 ± 0.2 | **16.8 ± 0.2**(↓12%)** |
| Week 14 | 17.6 ± 0.1 | 17.7 ± 0.1 | 17.6 ± 0.1 | 17.7 ± 0.1 | 17.7 ± 0.1 | **17.3 ± 0 1** (↓2%)** | **16.8 ± 0.1** (↓5%)** |
| **Mean cell haemoglobin concentration (g/dL)** | | | | | | | |
| Day 5 | 33.2 ± 0.1 | 33.3 ± 0.2 | 33.3 ± 0.2 | 33.5 ± 0.1 | 33.1 ± 0.2 | 33.1 ± 0.1 | 33.5 ± 0.2 |
| Week 4 | 33.3 ± 0.1 | 33.3 ± 0.2 | 33.5 ± 0.1 | 33.4 ± 0.2 | 33.2 ± 0.1 | 33.3 ± 0.3 | 33.3 ± 0.3 |
| Week 14 | 33.0 ± 0.1 | 33.1 ± 0.1 | 33.0 ± 0.2 | 32.9 ± 0.1 | 32.9 ± 0.2 | 32.6 ± 0.1 | 33.0 ± 0.2 |
| **Platelets ($10^3$/µL)** | | | | | | | |
| Day 5 | 897.2 ± 36.0 | 837.9 ± 23.1 | **917.1 ± 24.7*(↑9%)** | 865.3 ± 29.0 | **913.8 ± 15.7*(↑9%)** | **1003.3 ± 32.0**(↑20%)** | **1057.4 ± 30.8**(↑26%)** |
| Week 4 | 795.3 ± 24.7 | 764.3 ± 22.6 | 760.5 ± 34.6 | 816.1 ± 37.8 | 852.4 ± 38.8 | 846.3 ± 51.8c | **977.9 ± 42.9**(↑28%)** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Week 14 | 616.2 ± 8.8 | 664.6 ± 27.4 | 631.8 ± 14.1 | 662.2 ± 13.7 | **718.2 ± 28.5*(↑8%)** | **738.5 ± 19.0**(↑11%)** | **842.6 ± 37.6**(↑27%)** |
| **Leukocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 4.58 ± 0.65 | 5.31 ± 0.65 | 4.52 ± 0.66 | 5.92 ± 0.78 | 4.97 ± 0.52 | 5.19 ± 0.92 | 7.03 ± 0.85 |
| Week 4 | 6.26 ± 0 94 | 5.53 ± 0.74 | 6.06 ± 0.78 | 5.15 ± 0.72 | 6.05 ± 0.92 | 7.66 ± 0.60 | 7.19 ± 1.13 |
| Week 14 | 6.43 ± 0.42 | 5.50 ± 0 58 | 5.62 ± 0.65 | 6.33 ± 0.52 | 6.23 ± 0.62 | **7.74 ± 0.72* (↑40%)** | **8.78 ± 0.47**(↑60%)** |
| **Segmented neutrophils ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.58 ± 0.08 | 0.77 ± 0 10 | 0.44 ± 0.07 | 0.68 ± 0.11 | 0.72 ± 0.11 | 0.65 ± 0.11 | 1.10 ± 0.16 |
| Week 4 | 0.76 ± 0 11 | 0.61 ± 0.07 | 0.78 ± 0.08 | 0.60 ± 0.09 | 0.89 ± 0.16 | 0.92 ± 0.10 | 0.84 ± 0.12 |
| Week 14 | 0.87 ± 0.08 | 0.88 ± 0 15 | 1.13 ± 0.23 | 0.96 ± 0.11 | 1.17 ± 0.18 | 1.35 ± 0.34 | 0.91 ± 0.07 |
| **Lymphocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 3.86 ± 0 56 | 4.38 ± 0 56 | 3.99 ± 0.60 | 5.08 ± 0.69 | 4.10 ± 0.45 | 4.35 ± 0.79 | 5.73 ± 0.69 |
| Week 4 | 5.19 ± 0.77 | 4.64 ± 0.63 | 5.06 ± 0.69 | 4.32 ± 0.58 | 4.94 ± 0.75 | 6.48 ± 0.56 | 6.10 ± 0.95 |
| Week 14 | 5.33 ± 0 37 | 4.44 ± 0.45 | 4.30 ± 0.47 | 5.12 ± 0.43 | 4.86 ± 0.50 | **6.12 ± 0.39*(↑38%)** | **7.64 ± 0.43**(↑72%)** |
| **Monocytes ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.10 ± 0.03 | 0.13 ± 0.04 | 0.05 ± 0.03 | 0.14 ± 0.04 | 0.11 ± 0.03 | 0.13 ± 0.08 | 0.17 ± 0.06 |
| Week 4 | 0.22 ± 0.07 | 0.24 ± 0.07 | 0.14 ± 0.04 | 0.15 ± 0.06 | 0.12 ± 0.04 | 0.19 ± 0.08 | 0.17 ± 0.06 |
| Week 14 | 0.17 ± 0.05 | 0.14 ± 0.03 | 0.09 ± 0.02 | 0.17 ± 0.06 | 0.16 ± 0.02 | 0.19 ± 0.07 | 0.17 ± 0.04 |
| **Eosinophils ($10^3$/µL)** | | | | | | | |
| Day 5 | 0.04 ± 0.02 | 0.03 ± 0.02 | 0.00 ± 0.00 | 0.02 ± 0.01 | 0.05 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 |
| Week 4 | 0.07 ± 0.03 | 0.05 ± 0.02 | 0.05 ± 0.03 | 0.06 ± 0.03 | 0.05 ± 0.02 | 0.03 ± 0.02 | 0.05 ± 0.03 |
| Week 14 | 0.07 ± 0.02 | 0.07 ± 0.03 | 0.08 ± 0.02 | 0.05 ± 0.02 | 0.03 ± 0.02 | 0.07 ± 0.03 | 0.04 ± 0.02 |

** Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test. ** (P≤0.01).*
*c=8*

## Clinical chemistry

Data of clinical chemistry parameters are listed in tables B.6.8.1.1.2-01/5-6 for males and females, respectively. The serum activities of alanine aminotransferase and sorbitol dehydrogenase were increased in the 100 mg/kg bw/day or greater male and female groups at various time points and would be consistent with hepatocellular injury or leakage. Additionally, bile acid concentrations were increased in the 300 and 1000 mg/kg bw/day males dose groups at all time points, and 300 and 1000 mg/kg bw/day female dose groups at weeks 4 and 14, and would be consistent with cholestasis or altered hepatic function. However, serum alkaline phosphatase activity, another marker of cholestasis, was either unaffected or decreased in the same animals. A hypoproteinaemia and hypo-albuminemia were evidenced by decreased total protein and albumin concentrations in the 300 and 1000 mg/kg dose groups at the majority of time points. There were increases of creatinine concentrations in the 100 mg/kg bw/day or greater female groups at all time points. However, urea nitrogen, another marker of renal function, was either unaffected or decreased in the same animals.

**Table B.6.8.1.1.2-01/5. Summary of clinical chemistry parameters in males in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Water** | **Vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Day 5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 4 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 14 | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| **Urea nitrogen (mg/dL)** | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Day 5 | 19.3 ± 0.6 | 19.7 ± 0.7 | 20.0 ± 0.7 | 20.6 ± 0.8 | 20.5 ± 0.6 | 21.5 ± 0.8 | 19.4 ± 0.8 |
| Week 4 | 20.2 ± 0.3 | 22.3 ± 0.5 | 22.4 ± 0.3 | 22.7 ± 0.9 | **19.3 ± 0.3** (↓13%)** | **21.6 ± 0.6* (↓3%)** | **19.6 ± 0.7** (↓12%)** |
| Week 14 | 21.1 ± 0.8 | 21.1 ± 0.5 | 20.4 ± 0.4 | 20.2 ± 0.6 | 21.5 ± 0.5 | 19.5 ± 0.7 | 19.3 ± 1.0 |
| **Creatinine (mg/dL)** | | | | | | | |
| Day 5 | 0.50 ± 0.02 | 0.45 ± 0.60b | 0.54 ± 0.02 | 0.49 ± 0.02b | 0.53 ± 0.02 | 0.57 ± 0.03b | **0.61 ± 0.03**b (↑35%)** |
| Week 4 | 0.68 ± 0.02 | 0.68 ± 0.02 | 0.66 ± 0.03 | 0.66 ± 0.02 | 0.69 ± 0.01 | 0.68 ± 0.01 | 0.71 ± 0.01 |
| Week 14 | 0.64 ± 0.04 | 0.66 ± 0.02 | 0.64 ± 0.01 | 0.62 ± 0.03 | 0.66 ± 0.02 | 0.71 ± 0.03 | 0.71 ± 0.02 |
| **Total protein (g/dL)** | | | | | | | |
| Day 5 | 5.71 ± 0.06 | 5.67 ± 0.06 | 5.58 ± 0.08 | 5.33 ± 0.35 | 5.65 ± 0.06 | **5.44 ± 0.06* (↓4%)** | **5.00 ± 0.07** (↓12%)** |
| Week 4 | 5.99 ± 0.19 | 6.30 ± 0.05 | 6.21 ± 0.07 | 6.36 ± 0.07 | 6.03 ± 0.14 | 6.22 ± 0.12 | 6.09 ± 0.12 |
| Week 14 | 6.89 ± 0.10 | 7.18 ± 0.06 | 7.01 ± 0.08 | 6.96 ± 0.04* | 7.01 ± 0.08 | **6.81 ± 0.11** (↓5%)** | **6.36 ± 0.10** (↓12%)** |
| **Albumin (g/dL)** | | | | | | | |
| Day 5 | 3.6 ± 0.1 | 3.5 ± 0.1 | 3.5 ± 0.0 | 3.3 ± 0.2 | 3.4 ± 0.0 | **3.1 ± 0.2* (↓12%)** | **3.1 ± 0.1** (↓12%)** |
| Week 4 | 3.7 ± 0.0 | 3.7 ± 0.1 | 3.6 ± 0.1 | 3.7 ± 0.1 | 3.6 ± 0.1 | 3.7 ± 0.1 | 3.6 ± 0.1 |
| Week 14 | 3.9 ± 0.1 | 4.0 ± 0.0 | 3.8 ± 0.1 | 3.9 ± 0.1 | 3.9 ± 0.1 | 4.0 ± 0.1 | **3.6 ± 0.1** (↓10%)** |
| **Alanine aminotransferase (IU/L)** | | | | | | | |
| Day 5 | 48 ± 2 | 50 ± 2 | 57 ± 3 | 50 ± 2 | 54 ± 2 | **70 ± 4** (↑40%)** | **96 ± 4** (↑92%)** |
| Week 4 | 42 ± 2 | 45 ± 1 | 47 ± 2 | 50 ± 4 | 47 ± 2 | 48 ± 2 | **55 ± 4* (↑22%)** |
| Week 14 | 50 ± 3 | 47 ± 1 | 45 ± 2 | 49 ± 2 | **60 ± 2** (↑28%)** | **69 ± 4** (↑47%)** | **82 ± 5** (↑74%)** |
| **Alkaline phosphatase (IU/L)** | | | | | | | |
| Day 5 | 753 ± 17 | 761 ± 21 | 765 ± 18 | 749 ± 27 | 757 ± 19 | 759 ± 22 | 723 ± 28 |
| Week 4 | 573 ± 14 | 577 ± 20 | 572 ± 12 | 554 ± 21 | 557 ± 18 | 545 ± 18 | **509 ± 13** (↓12%)** |
| Week 14 | 270 ± 6 | 264 ± 6 | 264 ± 7 | 258 ± 7 | **238 ± 7* (↓10%)** | **207 ± 6** (↓22%)** | 263 ± 13 |
| **Creatine kinase (IU/L)** | | | | | | | |
| Day 5 | 492 ± 74 | 498 ± 52b | 540 ± 71 | 462 ± 57 | 570 ± 105 | 643 ± 68 | 611 ± 62 |
| Week 4 | 432 ± 56 | 414 ± 34 | 415 ± 38b | 522 ± 80 | 375 ± 20 | 481 ± 61 | 673 ± 94 |
| Week 14 | 599 ± 130b | 634 ± 155b | 482 ± 105 | 529 ± 79 | 489 ± 77 | 593 ± 85 | 531 ± 68 |
| **Sorbitol dehydrogenase (IU/L)** | | | | | | | |
| Day 5 | 5 ± 1c | 5 ± 1c | 5 ± 1 | 5 ± 1b | 5 ± 1d | 6 ± 1c | 6 ± 1b |
| Week 4 | 8 ± 1 | 7 ± 1 | 7 ± 1 | 8 ± 1 | 7 ± 0 | 7 ± 0 | 11 ± 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Week 14 | 10 ± 1 | 10 ± 1 | 9 ± 0 | 10 ± 0 | 11 ± 1 | **15 ± 1** (↑50%)** | **15 ± 1** (↑50%)** |
| **Bile acids (µmol/L)** | | | | | | | |
| Day 5 | 24.9 ± 2.6 | 21.9 ± 2.9c | 24.9 ± 4.6 | 26.6 ± 2.9b | 21.5 ± 3.6c | **39.4 ± 7.8*b (↑80%)** | **48.0 ± 8.0**c (↑120%)** |
| Week 4 | 13.0 ± 3.0 | 11.2 ± 1.2 | 11.5 ± 0.9 | 12.1 ± 1.9 | 10.4 ± 0.6 | **15.6 ± 1.9* (↑40%)** | **46.2 ± 12.9** (↑312%)** |
| Week 14 | 12.5 ± 2.6 | 9.5 ± 1.2 | 10.4 ± 1.5 | 10.2 ± 1.7 | 8.5 ± 0.7 | **19.7 ± 3.3** (↑107%)** | **89.4 ± 26.6** (↑841%)** |

** Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test. ** (P≤0.01).*
*b=9 ; c=8 ;d=6*

**Table B.6.8.1.1.2.-01/6. Summary of clinical chemistry parameters in females in 14-week rats study (mean ± standard error)**

| | Water | Vehicle | 10 | 30 | 100 | 300 | 1000 |
|---|---|---|---|---|---|---|---|
| Day 5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 4 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Week 14 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Urea nitrogen (mg/dL)** | | | | | | | |
| Day 5 | 19.3 ± 0.9 | 19.5 ± 0.5 | 20.5 ± 0.5 | 19.5 ± 0.4 | 20.0 ± 0.5 | 19.6 ± 0.6 | 19.9 ± 0.6 |
| Week 4 | 22.5 ± 0.8 | 20.6 ± 0.4 | 22.6 ± 0.7 | 21.8 ± 0.7 | 20.8 ± 0.5 | 19.2 ± 0.5 | 19.3 ± 0.9 |
| Week 14 | 23.5 ± 0.5 | 24.6 ± 0.5 | 23.5 ± 0.9 | **21.1 ± 0.6** (↓14%)** | **21.9 ± 0.6** (↓11%)** | **23.5 ± 0.5* (↓5%)** | **20.5 ± 0.8** (↓17%)** |
| **Creatinine (mg/dL)** | | | | | | | |
| Day 5 | 0.51 ± 0.04 | 0.49 ± 0.02b | 0.48 ± 0.02b | 0.47 ± 0.03 | **0.55 ± 0.01* (↑12%)** | **0.57 ± 0.02** (↑16%)** | **0.61 ± 0.01** (↑24%)** |
| Week 4 | 0.59 ± 0.01 | 0.59 ± 0.01 | 0.58 ± 0.02 | 0.62 ± 0.01 | **0.64 ± 0.01** (↑8%)** | **0.67 ± 0.01** (↑14%)** | **0.71 ± 0.01** (↑20%)** |
| Week 14 | 0.59 ± 0.04 | 0.61 ± 0.03 | 0.62 ± 0.02 | 0.67 ± 0.02 | 0.64 ± 0.01 | **0.72 ± 0.03** (↑18%)** | **0.75 ± 0.03** (↑23%)** |
| **Total protein (g/dL)** | | | | | | | |
| Day 5 | 5.8 ± 0.1 | 5.7 ± 0.1 | 5.7 ± 0.1 | 5.7 ± 0.1 | 5.6 ± 0.1 | 5.5 ± 0.1 | **4 9 ± 0.1** (↓14%)** |
| Week 4 | 6.0 ± 0.1 | 6.1 ± 0.1 | 6.1 ± 0.1 | 6.2 ± 0.1 | 5.8 ± 0.2 | **5.7 ± 0.1** (↓7%)** | **5.7 ± 0.1** (↓7%)** |
| Week 14 | 7.4 ± 0.1 | 7.1 ± 0.1 | 6.9 ± 0.1 | 6.9 ± 0.1 | **6.7 ± 0.1* (↓6%)** | **6.4 ± 0.1** (↓10%)** | **6 2 ± 0.1** (↓13%)** |
| **Albumin (g/dL)** | | | | | | | |
| Day 5 | 4.1 ± 0.1 | 4.0 ± 0.1b | 4.0 ± 0.1b | 3.9 ± 0.1 | 4.0 ± 0.1 | 3.8 ± 0.1 | **3 3 ± 0.1** (↓18%)** |
| Week 4 | 3.8 ± 0.1 | 3.8 ± 0.1 | 3.6 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.1 | 3.6 ± 0.1 | **3.5 ± 0.1* (↓8%)** |
| Week 14 | 4.5 ± 0.1 | 4.3 ± 0.1 | 4.1 ± 0.1 | 4.3 ± 0.1 | 4.2 ± 0.1 | **3.9 ± 0.1** (↓9%)** | **3.7 ± 0.1** (↓14%)** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Alanine aminotransferase (IU/L)** | | | | | | | |
| Day 5 | 44 ± 1 | 43 ± 1 | 44 ± 2 | 44 ± 1 | **49 ± 2** (↑14%)** | **61 ± 3** (↑42%)** | **68 ± 7** (↑58%)** |
| Week 4 | 34 ± 1 | 34 ± 1 | 35 ± 1 | 36 ± 1 | **37 ± 1* (↑9%)** | **45 ± 1** (↑32%)** | **62 ± 3** (↑82%)** |
| Week 14 | 45 ± 1 | 49 ± 3 | 44 ± 2 | 45 ± 2 | 46 ± 2 | 54 ± 2 | **77 ± 4** (↑57%)** |
| **Alkaline phosphatase (IU/L)** | | | | | | | |
| Day 5 | 619 ± 21 | 616 ± 13 | 627 ± 22 | 644 ± 25 | 612 ± 24 | 621 ± 17 | **489 ± 20** (↓21%)** |
| Week 4 | 458 ± 16 | 407 ± 11 | 438 ± 11 | 424 ± 11 | 402 ± 11 | **357 ± 12* (↓13%)** | 387 ± 12 |
| Week 14 | 222 ± 10 | 245 ± 10 | 229 ± 6 | 228 ± 6 | 211 ± 7 | **194 ± 10** (↓21%)** | 277 ± 17 |
| **Creatine kinase (IU/L)** | | | | | | | |
| Day 5 | 643 ± 111 | 580 ± 62 | 600 ± 100 | 488 ± 73 | 666 ± 111 | 524 ± 54b | 614 ± 74 |
| Week 4 | 359 ± 35 | 308 ± 40 | 405 ± 39 | 318 ± 38 | 363 ± 49 | **507 ± 65* (↑65%)** | 481 ± 76 |
| Week 14 | 365 ± 50 | 423 ± 65 | 305 ± 31 | 403 ± 58 | 415 ± 64 | 486 ± 83 | 513 ± 67 |
| **Sorbitol dehydrogenase (IU/L)** | | | | | | | |
| Day 5 | 4 ± 0 | 4 ± 0 | 4 ± 0 | 4 ± 0 | 4 ± 0 | **6 ± 0* (↑50%)** | **5 ± 0* (↑25%)** |
| Week 4 | 6 ± 0 | 5 ± 0 | 5 ± 0 | 5 ± 0 | **6 ± 0** (↑20%)** | **6 ± 0** (↑20%)** | **8 ± 0** (↑60%)** |
| Week 14 | 7 ± 0 | 8 ± 0 | 7 ± 0 | 7 ± 0 | 7 ± 0 | 9 ± 1 | 11 ± 1 |
| **Bile acids (µmol/L)** | | | | | | | |
| Day 5 | 24.4 ± 3.7 | 27.1 ± 4.4b | 18.4 ± 1.8b | 23.3 ± 2.4 | 25.0 ± 2.5 | 34.3 ± 3.3b | 44.1 ± 7.3 |
| Week 4 | 16.6 ± 4.6 | 14.2 ± 2.0 | 15.5 ± 2.6 | 14.3 ± 2.3 | 18.7 ± 3.2 | **33.4 ± 5.1** (↑135%)** | **66.7 ± 6.5** (↑370%)** |
| Week 14 | 15.6 ± 3.1 | 15.0 ± 2.5 | 14.3 ± 2.2b | 17.0 ± 2.5 | 24.4 ± 5.8 | **33.3 ± 7.1* (↑122%)** | **180.7 ± 39.4** (↑1105%)** |

** Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test. ** (P≤0.01).*
*b=9*

## Organ weights

Terminal bodyweights were statistically significant reduced in 300 and 1000 mg/kg/bw/day male dose groups (8 and 31%) and in all female dose groups (6-18%). Absolute and/or relative liver weights of 100, 300, and 1000 mg/kg bw/day male and female dose groups were statistically significantly greater than controls. Testis weight (both absolute and relative) of top dose male group was also higher than controls (64% and 137%, respectively). Moreover, absolute and relative thymus weights in all dosed males and in top dose female group were significantly lower than controls. Increased relative kidney weights were recorded in 30 and higher male groups (7-41%), and in all female dose groups (7-26%), however, no histological alterations were found in kidneys of rats tested. Non dose-related increased of spleen weights were noted in mid and top dose male groups, and in all female dose groups (see Tables B.6.8.1.1.2-01/7-8).

**Table B.6.8.1.1.2-01/7. Summary of organ weight data in males in 14-week rats study (mean ± standard error)**

| Dose (mg/kg bw/day) |
|---|

| | Water | Vehicle | 10 | 30 | 100 | 300 | 1000 |
|---|---|---|---|---|---|---|---|
| | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| **Necropsy** | | | | | | | |
| body wt | 340 ± 6 | 343 ± 8 | 343 ± 5 | 345 ± 8 | 336 ± 7 | **314 ± 4** (↓8%)** | **236 ± 5** (↓31%)** |
| **Heart** | | | | | | | |
| Absolute | 0.971 ± 0.020 | 0.981 ± 0.025 | 0.935 ± 0.020 | 1.011 ± 0.017 | 0.944 ± 0.023 | 0.929 ± 0.015 | **0.778 ± 0.021** (↓21%)** |
| Relative | 2.86 ± 0.0 | 2.86 ± 0.04 | 2.73 ± 0.05 | 2.94 ± 0.04 | 2.81 ± 0.05 | 2.96 ± 0.04 | **3.30 ± 0.06** (↑15%)** |
| **R. Kidney** | | | | | | | |
| Absolute | 1.215 ± 0.029 | 1.150 ± 0.030 | 1.186 ± 0.019 | 1.236 ± 0.030 | 1.219 ± 0.026 | 1.258 ± 0.037 | 1.112 ± 0.034 |
| Relative | 3.58 ± 0.06 | 3.35 ± 0.03 | 3.46 ± 0.05 | **3.59 ± 0.05* (↑7%)** | **3.63 ± 0.05** (↑8%)** | **4.00 ± 0.09** (↑19%)** | **4.71 ± 0 12** (↑41%)** |
| **Liver** | | | | | | | |
| Absolute | 13.555 ± 0.471 | 12.872 ± 0.428 | 13.462 ± 0.224 | 14.082 ± 0.430 **(↑9%, ns)** | **14 548 ± 0.453*b (↑13%)** | **15.106 ± 0.495** (↑17%)** | 13.885 ± 0.448 |
| Relative | 39.86 ± 0.92 | 37.42 ± 0.42 | 39.27 ± 0.32 | **40.82 ± 0.47* (↑9%)** | **43.65 ± 0.85**b (↑17%)** | **47.99 ± 1.23** (↑28%)** | **58.83 ± 1 51** (↑57%)** |
| **Lung** | | | | | | | |
| Absolute | 1.315 ± 0.031 | 1.336 ± 0.029 | 1.308 ± 0.046 | 1.401 ± 0.031 | 1.377 ± 0.041 | 1.343 ± 0.029 | **1.122 ± 0.036** (↓16%)** |
| Relative | 3.88 ± 0.09 | 3.90 ± 0.07 | 3.82 ± 0.12 | 4.08 ± 0.08 | 4.10 ± 0.09 | **4.28 ± 0.11* (↑10%)** | **4.76 ± 0.14** (↑22%)** |
| **Spleen** | | | | | | | |
| Absolute | 0.739 ± 0.009 | 0.780 ± 0.025 | 0.758 ± 0.011 | 0.803 ± 0.027 | 0.820 ± 0.016 | 0.780 ± 0.013 | **0.563 ± 0.013** (↓28%)** |
| Relative | 2.18 ± 0.03 | 2.27 ± 0.03 | 2.22 ± 0.04 | 2.33 ± 0.06 | **2.44 ± 0.03** (↑7%)** | **2.48 ± 0.05** (↑9%)** | **2.39 ± 0.04** (↑5%)** |
| **R. Testis** | | | | | | | |
| Absolute | 1.412 ± 0.032 | 1.437 ± 0.031 | 1.411 ± 0.019 | 1.440 ± 0.031 | 1.466 ± 0.028 | 1.419 ± 0.031 | **2.357 ± 0.195** (↑64%)** |
| Relative | 4.16 ± 0.08 | 4.19 ± 0.04 | 4.12 ± 0.03 | 4.18 ± 0.05 | 4.37 ± 0.05 | 4.51 ± 0.09 | **9.94 ± 0.74** (↑137%)** |
| **Thymus** | | | | | | | |
| Absolute | 0.312 ± 0.016 | 0.355 ± 0.016 | **0.297 ± 0.007**(↓16%)** | **0.314 ± 0.012** (↓12%)** | **0.296 ± 0.014** (↓17%)** | **0.264 ± 0.018** (↓26%)** | **0.176 ± 0.007** (↓50%)** |
| Relative | 0.92 ± 0.05 | 1.04 ± 0.04 | **0.87 ± 0.02* (↓16%)** | **0.91 ± 0.03* (↓12%)** | **0.88 ± 0.04** (↓15%)** | **0.84 ± 0.06** (↓19%)** | **0.75 ± 0.03** (↓28%)** |

** Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test. ** (P≤0.01).*
*b=9*

**Table B.6.8.1.1.2-01/8. Summary of organ weight data in females in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Water | Vehicle | 10 | 30 | 100 | 300 | 1000 |
| | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| **Necropsy** | | | | | | | |
| body wt | 195 ± 3 | 200 ± 4 | **185 ± 1** (↓8%)** | **188 ± 3** (↓6%)** | **182 ± 3** (↓9%)** | **185 ± 3** (↓8%)** | **164 ± 4** (↓18%)** |
| **Heart** | | | | | | | |
| Absolute | 0.638 ± 0.013 | 0.634 ± 0.018b | 0.627 ± 0.017 | 0.607 ± 0.012 | 0.584 ± 0.012 | 0.602± 0.012 | **0.565 ± 0.018** (↓11%)** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Relative | 3.28 ± 0.06 | 3.15 ± 0.06b | 3.38 ± 0.09 | 3.23 ± 0.05 | 3.20 ± 0.06 | 3.26 ± 0.08 | **3.44 ± 0.07* (↑9%)** |
| **R. Kidney** | | | | | | | |
| Absolute | 0.690 ± 0.015 | 0.683 ± 0.015 | 0.677 ± 0.025 | 0.699 ± 0.013 | 0.702 ± 0.012 | 0.743 ± 0.013 | 0.705 ± 0.022 |
| Relative | 3.55 ± 0.06 | 3.41 ± 0.05 | **3.65 ± 0.12* (↑7%)** | **3.72 ± 0.05** (↑9%)** | **3.85 ± 0.05** (↑13%)** | **4.03 ± 0.09** (↑18%)** | **4.30 ± 0.10** (↑26%)** |
| **Liver** | | | | | | | |
| Absolute | 6.679 ± 0.117 | 6.585 ± 0.168 | 6 318 ± 0.158 | 6.366 ± 0.163 | 6.701 ± 0 115 | **8.369 ± 0.212** (↑27%)** | **9.517 ± 0.267** (↑45%)** |
| Relative | 34.35 ± 0.45 | 32.85 ± 0.43 | 34.09 ± 0.70 | 33.89 ± 0.70 | **36.73 ± 0.34** (↑12%)** | **45.30 ± 1.03** (↑38%)** | **58.05 ± 1.06** (↑77%)** |
| **Lung** | | | | | | | |
| Absolute | 0.896 ± 0.028 | 0.939 ± 0.029 | 0 923 ± 0.028 | 0.934 ± 0.022 | 0.894 ± 0.013 | 0.947 ± 0.032 | 0.850 ± 0.025 |
| Relative | 4.60 ± 0.10 | 4.68 ± 0.11 | 4.98 ± 0.15 | 4.98 ± 0.13 | 4.91 ± 0.09 | **5.14 ± 0.20* (↑10%)** | **5.18±0.07* (↑11%)** |
| **Spleen** | | | | | | | |
| Absolute | 0.571 ± 0.013 | 0.449 ± 0.017 | **0.574 ± 0.014** (↑28%)** | 0.499 ± 0.015 | 0.506 ± 0.014 | **0.517 ± 0.019* (↑15%)** | 0.494 ± 0.019 |
| Relative | 2.95 ± 0.09 | 2 24 ± 0.08 | **3 10 ± 0.07** (↑38%)** | **2.66 ±0.06** (↑19%)** | **2.78 ± 0.07** (↑24%)** | **2.80 ± 0.11** (↑25%)** | **3.01 ± 0.07** (↑34%)** |
| **Thymus** | | | | | | | |
| Absolute | 0.248 ± 0.014 | 0.268 ± 0.015 | 0 238 ± 0.007 | 0.238 ± 0.012 | 0.244 ± 0.015 | 0.248 ± 0.009 | **0.165 ± 0.009** (↓38%)** |
| Relative | 1.27 ± 0.05 | 1 33 ± 0.06 | 1.29 ± 0.04 | 1.26 ± 0.06 | 1.33 ± 0.07 | 1.34 ±0.04 | **1.01 ± 0.06** (↓24%)** |

** Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test. ** (P≤0.01).b=9*

**Histological findings (see Tables B.6.8.1.1.2-01/9-10)**

*Liver*
Increased incidences of liver lesions occurred in 300 and 1000 mg/kg bw/d males and females dose groups, and the lesions were generally more severe in males than in females. Hepatic lesions with increased incidences included cytologic alteration, cytomegaly, Kupffer cell pigmentation, bile duct hyperplasia, and foci of cellular alteration. Moreover, one hepatocellular adenoma was present in a male rat of top dose group.

*Adrenal cortex*
Incidences of cortical hypertrophy of the adrenal cortex were statistically significantly higher in 100, 300, and 1000 mg/kg bw/day males and top dose female groups, compared with controls.

*Submandibular salivary glands*
The incidences of cytoplasmic alteration of the submandibular salivary glands were statistically significant increased in female rats administered 30 mg/kg or greater doses and for male rates administered 100 mg/kg or greater doses. Cytoplasmic alteration of the submandibular salivary gland consisted of a loss of cytoplasmic zymogen granules with reduction in the size of serous cells and their ducts.

*Sexual organs*
Top dose male group showed increased incidences of moderate dilatation of the seminiferous tubules and testicular degeneration characterized by diffuse loss of spermatogenic cells within the seminiferous tubules. Spermatogonia remaining within the seminiferous and epididymal tubules were morphologically normal.

On the other hand, the incidences of mild uterine atrophy were significantly increased in 300 and 1000 mg/kg bw/day dose groups.

*Stomach*

The incidences of atrophy and chronic inflammation of the mucosa of the glandular stomach were statistically significantly higher in 300 and 1000 mg/kg bw/day dose groups for both sexes. Lesions were generally of minimal to mild severity in the 300 mg/kg bw/day groups and mild to moderate in the top dose groups. Atrophy consisted of a decrease in the thickness of the gastric mucosa due to generalized loss of glandular epithelial parietal and chief cells accompanied by condensation of the lamina propria. Inflammation was of mild severity and consisted of fibrosis and a diffuse infiltration of the lamina propria by lymphocytes, neutrophils, and macrophages.

**Table B.6.8.1.1.2-01/9. Summary of histopathological findings in males in 14-week rats study**

| Males F344/N rats (a) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Doses of methyleugenol (mg/kg bw/d)** | **water** | **vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Number of animals observed | 10 | 10 | 10 | 10 | 10 | 9 | 10 |
| **Liver** | | | | | | | |
| Cytologic Alteration | 0 | 0 | 0 | 0 | 0 | **9** (100%)** | **10** (100%)** |
| Cytomegaly | 0 | 0 | 0 | 0 | 0 | **9** (100%)** | **10** (100%)** |
| Pigment, Kupffer Cell | 0 | 0 | 0 | 0 | 0 | 0 | **10** (100%)** |
| Basophilic focus | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Mixed cell focus | 0 | 0 | 0 | 0 | 0 | 2 | **9** (90%)** |
| Hyperplasia, bile duct | 0 | 0 | 0 | 0 | 0 | **6** (67%)** | **10** (100%)** |
| Hepatocellular adenoma | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| **Glandular stomach** | | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0 | **7** (78%)** | **10** (100%)** |
| Inflammation, chronic | 0 | 0 | 0 | 0 | 0 | **9** (100%)** | **10** (100%)** |
| **Adrenal cortex** | | | | | | | |
| Hypertrophy | 0 | 0 | 0 | 0 | **4** (40%)** | **9** (↑100%)** | **10** (↑100%)** |
| **Submandibular salivary gland** | | | | | | | |
| Cytoplasmic alteration | 0 | 0 | 0 | 3 | **10** (100%)** | **9** (100%)** | **10** (100%)** |
| **Testis** | | | | | | | |
| Dilatation | 0 | 0 | 0 | 0 | 0 | 0 | **10** (100%)** |
| Degeneration | 0 | 0 | 0 | 0 | 0 | 0 | **10** (100%)** |

** Significantly different (P≤0.05) from the vehicle control group by Fisher exact test. **p≤0.01.*
*a) Results expressed as number of animals with lesion*

**Table B.6.8.1.1.2-01/10. Summary of histopathological findings in females in 14-week rats study**

| Females F344/N rats (a) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Doses of methyleugenol (mg/kg bw/d)** | **water** | **vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Number of animals observed | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Liver** | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cytologic Alteration | 0 | 0 | 0 | 0 | 0 | 0 | **10**(100%)** |
| Cytomegaly | 0 | 0 | 0 | 0 | 0 | **9**(90%)** | **10**(100%)** |
| Pigment, Kupffer Cell | 0 | 0 | 0 | 0 | 0 | 0 | **9**(90%)** |
| Basophilic focus | 0 | 0 | - | - | - | - | - |
| Mixed cell focus | 0 | 0 | 0 | 0 | 0 | 2 | **8** (80%)** |
| Hyperplasia, bile duct | 0 | 0 | 0 | 0 | 0 | 0 | **9**(90%)** |
| Hepatocellular adenoma | 0 | 0 | - | - | - | - | - |
| **Glandular stomach** | | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0 | **9** (90%)** | **10** (100%)** |
| Inflammation, chronic | 0 | 0 | 0 | 0 | **6** (60%)** | **10** (100%)** | **10** (100%)** |
| **Adrenal cortex** | | | | | | | |
| Hypertrophy | 0 | 2 | 0 | 0 | 0 | 0 | **10** (100%)** |
| **Submandibular salivary gland** | | | | | | | |
| Cytoplasmic alteration | 0 | 0 | 0 | **7** (70%)** | **10** (100%)** | **10** (100%)** | **10** (100%)** |
| **Uterus** | | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0 | **4* (40%)** | **10** (100%)** |

** Significantly different (P≤0.05) from the vehicle control group by Fisher exact test.**p≤0.01.*
*a) Results expressed as number of animals with lesion*

## Reproductive parameters

No statistically significant differences in sperm motility or in vaginal cytology parameters between dose groups and controls were observed (see Tables B.6.8.1.1.2-01/11-12).

**Table B.6.8.1.1.2-01/11. Summary of reproductive parameters in males in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **Vehicle** | **30** | **100** | **300** |
| | 10 | 10 | 10 | 9 |
| **Weights (g)**<br>**Necropsy body wt**<br>**L. cauda epididymis**<br>**L. epididymis**<br>**L. testis** | 343 ± 8<br>0.1573 ± 0.0050<br>0.4347 ± 0.0084<br>1.5113 ± 0.0279 | 345 ± 8<br>0.1433 ± 0.0093<br>0.4243 ± 0.0115<br>1.5297 ± 0.0366 | 336 ± 7<br>0.1564 ± 0.0027<br>0.4332 ± 0.0054<br>1.5095 ± 0.0348 | **314 ± 4** (↓8%)**<br>0.1532 ± 0.0027<br>0.4214 ± 0.0113<br>1.4970 ± 0.0338 |
| **Spermatid measurements** | | | | |
| **Spermatid heads ($10^7$/g testis)** | 9.35 ± 0.28 | 9.86 ± 0.35 | 9.98 ± 0.38 | 10.10 ± 0.18 |
| **Spermatid heads ($10^7$/testis)** | 14.10 ± 0.37 | 15.06 ± 0.59 | 14.98 ± 0.40 | 15.12 ± 0.46 |
| **Spermatid count (mean/$10^{-4}$ mL suspension)** | 70.48 ± 1.86 | 75.30 ± 2.97 | 74.90 ± 1.99 | 75.61 ± 2.31 |
| **Epididymal spermatozoal measurements** | | | | |
| **Mortality (%)** | 93.39 ± 0.89 | 93.69 ± 0.36 | 94.32 ± 0.56 | 93.22 ± 1.43 |
| **Concentration ($10^6$/g cauda epididymal tissue)** | 538 ± 30 | 638 ± 40 | 513 ± 19 | 580 ± 34 |

*** Significantly different (P≤0.01) from the vehicle control group by Williams' test.*

**Table B.6.8.1.1.2-01/12. Summary of reproductive parameters in females in 14-week rats study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **Vehicle** | **30** | **100** | **300** |
| | 10 | 10 | 10 | 9 |
| **Necropsy body wt (g)** | 200 ± 4 | **188 ± 3** (↓6%)** | **182 ± 3** (↓9%)** | **185 ± 3** (↓8%)** |
| **Estrous cycle length (days)** | | | | |
| Estrous stages (% of cycle) | 5.06 ± 0.11b | 4.95 ± 0.12 | 5.30 ± 0.31 | 5.55 ± 0.30 |
| Diestrus | 38.3 | 40.8 | 37.5 | 43.3 |
| Proestrus | 15 | 15 | 15 | 16.7 |
| Estrus | 32.5 | 30 | 31.7 | 30.8 |
| Metestrus | 10 | 14.2 | 15.8 | 9.2 |
| Uncertain diagnosis | 4.2 | 0 | 0 | 0 |

*** Significantly different (P≤0.01) from the vehicle control group by Williams' test.*

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable without restrictions).

The 90-day oral NOAEL in the rat is 10 mg/kg bw/d, based on mortality, reduced body weight gain, clinical chemistry and histopathology.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study was used as dose range-finding study for long-term and carcinogenicity toxicity studies. The study deviations do not compromise the acceptability.

Reduced bodyweight and bodyweight gain were noted in all female groups. Increased platelet levels (thrombocytosis) were found in 100 mg/kg bw/day dose and higher male and female dose groups, together with an increase of haematocrit, haemoglobin and bile acid parameters in top dose groups of both sexes. Most relevant changes in organ weights were recorded in liver at 30 mg/kg bw/dose and higher dose levels. Liver weights findings were supported by further histopathological alterations, although were only recorded at 300 and 1000 mg/kg bw/dose groups. In addition, cytoplasmic alterations in submandibular salivary gland were noted in 30 and higher dose levels in female rats whereas in male rats this was observed at 100 mg/kg and higher dose levels.

Thus, **NOAEL** could be stablished on **7.1 mg/kg bw/day (5-day/week dose scheme)**, based on increased platelet concentration (thrombocytosis) (males) and cytoplasmic alterations in submandibular salivary gland (females).

**B.6.8.1.1.2-02. Oral 14-week toxicity study in mice with methyleugenol**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 408 (2018). |
| **Deviations from current test guideline OECD TG 408 (2018)** | - -Dose schedule administration was 5 days per week.<br>-Food consumption was not measured.<br>- Ophthalmological examination was not performed.<br>- Haematology and clinical biochemistry analysis were not performed.<br>-Thyroid gland, ovaries, uterus, brain, adrenals, prostate and pituitary gland were not weighted.<br>- Circulating thyroid hormones (T4, T3, TSH) levels were not measured.<br>- Animals died during study were not necropsied (organ weights).<br>- Complete histopathology was only performed on negative controls, 300 and 1000 mg/ kg bw/day dose groups. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Test system** | B6C3F1 mice |
| **Test method** | Groups of 10 male and 10 female B6C3F1 mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, 5 days per week for 14 weeks; groups of 10 male and 10 female mice received deionized water only. Dosing volumes were 10 mL/kg bw. Feed and water were available *ad libitum*. Male mice were housed individually and female mice were housed five per cage. Cages and racks were rotated every 2 weeks. Clinical findings were recorded and the animals were weighed weekly and at the end of the studies.<br><br>*Necropsy*<br><br>Necropsy was performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.<br><br>*Histopathology*<br><br>Complete histopathology was performed on all core study water control, vehicle control, 300 and 1000 mg/kg bw/d dose groups. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, oesophagus, gallbladder (mice only), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. In addition, the stomach of male and female mice in the 10, 30, and 100 mg/kg groups, the liver of 100 mg/kg male mice and 10, 30, and 100 mg/kg female mice, and the nose of 100 mg/kg male and female mice were examined. |

| | |
|---|---|
| | *Sperm motility and vaginal cytology*<br><br>At the end of the studies, sperm samples were collected from vehicle control, 10, 30, and 100 mg/kg male mice for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda epididymis, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from vehicle control, 10, 30, and 100 mg/kg female mice for vaginal cytology evaluations. The following parameters were evaluated: the estrous cycle lengths and relative frequency of estrous stages. |
| **Statistical analysis of results** | *Survival*<br>The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.<br><br>*Histopathology analysis*<br>Lesions detected were analysed using Fisher exact test, a procedure based on the overall proportion of affected animals was used.<br><br>*Analysis of continuous variables*<br>Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and bodyweight data, which have approximately normal distributions, were analysed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Haematology, clinical chemistry, plasma concentration, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analysed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. |

**Results**

**Survival:** All mice of top dose group, except one male, died before the end of the study. Additionally, one male of 300 mg/kg bw/day and one female of 10 mg/kg bw/day dose groups died during week 3 and 12, respectively (table B.6.8.1.1.2-02/1).

**Table B.6.8.1.1.2-02/1. Survival in 14-week mice study**

| | % Survival | |
|---|---|---|
| **Dose (mg/kg bw)** | **Male** | **Females** |
| **water control** | 10/10 (100%) | 10/10 (100%) |
| **Vehicle control** | 10/10 (100%) | 10/10 (100%) |
| **10** | 10/10 (100%) | 9/10 (90%) c |
| **30** | 10/10 (100%) | 10/10 (100%) |
| **100** | 10/10 (100%) | 10/10 (100%) |
| **300** | 9/10 (90%) a | 10/10 (100%) |
| **1000** | 1/10 (10%) b | 0/10 (0%) d |

*a Week of death 3*
*b Weeks of death 1, 1, 1, 1, 1, 5, 8, 9, 12*
*c Week of death 12*
*d Weeks of death 1, 1, 1, 1, 1, 1, 3, 4, 4, 6*

**Clinical signs**: The only clinical finding was toxicity manifested as generalized morbidity in animals of top dose groups.

**Bodyweights:** No differences in bodyweights were found at study termination. Bodyweight gains were statistically significant reduced in the 300 mg/kg bw/day dose groups compared with controls (Table B.6.8.1.1.2-02/2).

**Table B.6.8.1.1.2-02/2. Bodyweight and bodyweight gains in 14-week mice study (mean ± standard error)**

| Dose (mg/kg bw/day) | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Initial | Final (% of controls) | bwg | Initial | Final (% of controls) | bwg |
| **Water control** | 22.7 ± 0.5 | 32.1 ± 1.2 | 9.4 ± 1.1 | 19 ± 0.4 | 30.9 ± 1.1 | 11.8 ± 0.9 |
| **Vehicle control** | 22.3 ± 0.4 | 33.4 ± 0.7 | 11.1 ± 0.5 | 19 ± 0.3 | 29.6 ± 0.6 | 10.7 ± 0.5 |
| **10** | 22.9 ± 0.4 | 33.4 ± 0.8 | 10.5 ± 0.5 | 18.5 ± 0.3 | 29.2 ± 0.7 | 10.7 ± 0.7 |
| **30** | 22.3 ± 0.3 | 32.6 ± 0.8 | 10.3 ± 0.6 | 19.4 ± 0.4 | 29.3 ± 0.7 | 9.9 ± 0.6 |
| **100** | 23.2 ± 0.5 | 33.7 ± 0.9 | 10.5 ± 0.7 | 19.5 ± 0.2 | 29.2 ± 0.5 | 9.6 ± 0.5 |
| **300** | 22.3 ± 0.4 | 30.7 ± 0.6 | 8.4 ± 0.5** **(↓24%)** | 19.4 ± 0.2 | 27.5 ± 0.6 | 8.1 ± 0.4** **(↓24%)** |
| **1000** | 21.1 ± 0.7 | 27.3 **(↓18%)** | 6.2 **(↓44%)** | 18.4 ± 0.3 | - | - |

*** Significantly different (P≤0.01) from the vehicle control group by Williams' or Dunnett's test*

**Organ weights**

Absolute and relative liver weights were statistically significant increased in 30 or greater male dose groups (11-28%) and in the top dose female groups (14% and 23%, respectively). Absolute and relative thymus weights were significantly lower than controls in the 100 and 300 mg/kg bw/day female dose groups, whereas in males, absolute thymus weight was only reduced in top dose group (Tables B.8.1.1.2-02/3-4).

**Table B.6.8.1.1.2-02/3. Summary of organ weight data in males in 14-week mice study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | |
|---|---|---|---|---|---|---|
| | **Water** | **Vehicle** | **10** | **30** | **100** | **300** |
| | 10 | 10 | 10 | 10 | 10 | 9 |
| **Necropsy** | | | | | | |
| body wt | 33.9 ± 1.2 | 34.0 ± 0.7 | 34.1 ± 0.8 | 33.7 ± 0.7 | 34.2 ± 0.9 | 32.0 ± 0.6 |
| **Heart** | | | | | | |
| Absolute | 0.154 ± 0.004 | 0.160 ± 0.006 | 0.158 ± 0.007 | 0.148 ± 0.004 | 0.150 ± 0.007 | 0.146 ± 0.009 |
| Relative | 4.57 ± 0.14 | 4.72 ± 0.20 | 4.63 ± 0.17 | 4.39 ± 0.11 | 4.38 ± 0.15 | 4.56 ± 0.31 |
| **R. Kidney** | | | | | | |
| Absolute | 0.316 ± 0.10 | 0.317 ± 0.007 | 0.312 ± 0.010 | 0.320 ± 0.008 | 0.305 ± 0.012 | 0.288 ± 0.010 |
| Relative | 9.36 ± 0.27 | 9.33 ± 0.20 | 9.14 ± 0.18 | 9.50 ± 0.13 | 8.91 ± 0.22 | 8.99 ± 0.28 |
| **Liver** | | | | | | |
| Absolute | 1.602 ± 0.051 | 1.514 ± 0.057 | 1.651 ± 0.055 **(↑9% ns)** | **1.722 ± 0.038* (↑14%)** | **1.687 ± 0.069* (↑11%)** | **1.818 ± 0.058** (↑20%)** |
| Relative | 47.41 ± 1.17 | 44.51 ± 1.47 | **48.36 ± 1.04* (↑9%)** | **51.15 ± 0.87** (↑15%)** | **49.29 ± 1.54** (↑11%)** | **56.81 ± 1.75** (↑28%)** |
| **Lung** | | | | | | |
| Absolute | 0.195 ± 0.015 | 0.191 ± 0.007 | 0.176 ± 0.007 | 0.180 ± 0.006 | 0.182 ± 0.010 | 0.177 ± 0.011 |
| Relative | 5.73 ± 0.36 | 5.62 ± 0.18 | 5.16 ± 0.15 | 5.37 ± 0.23 | 5.32 ± 0.25 | 5.53 ± 0.36 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Spleen** | | | | | | |
| Absolute | 0.079 ± 0.004 | 0.075 ± 0.003 | 0.079 ± 0.004 | 0.076 ± 0.003 | 0.077 ± 0.005 | 0.073 ± 0.002 |
| Relative | 2.36 ± 0.14 | 2.21 ± 0.11 | 2.31 ± 0.10 | 2.26 ± 0.09 | 2.24 ± 0.11 | 2.29 ± 0.07 |
| **R. Testis** | | | | | | |
| Absolute | 0.118 ± 0.002 | 0.120 ± 0.003 | 0.117 ± 0.002 | 0.118 ± 0.004 | 0.117 ± 0.004 | 0.117 ± 0.001 |
| Relative | 3.51 ± 0.09 | 3.54 ± 0.08 | 3.46 ± 0.09 | 3.49 ± 0.10 | 3.41 ± 0.10 | 3.67 ± 0.07 |
| **Thymus** | | | | | | |
| Absolute | 0.041 ± 0.004 | 0.044 ± 0.002 | 0.042 ± 0.003 | 0.039 ± 0.002 | 0.039 ± 0.004 | **0.036 ± 0.002* (↓18%)** |
| Relative | 1.20 ± 0.11 | 1.30 ± 0.05 | 1.22 ± 0.07 | 1.17 ± 0.08 | 1.13 ± 0.09 | 1.12 ± 0.08 |

** Significantly different (P≤0.05) from the vehicle control group by Williams' test. ** (P≤0.01).*

**Table B.6.8.1.1.2-02/4. Summary of organ weight data in females in 14-week mice study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | | | |
|---|---|---|---|---|---|---|
| | **Water** | **Vehicle** | **10** | **30** | **100** | **300** |
| | 10 | 10 | 9 | 10 | 10 | 10 |
| **Necropsy** | | | | | | |
| body wt | 31.4 ± 1.1 | 30.0 ± 0.7 | 30.7 ± 0.8 | 30.7 ± 0.6 | 29.6 ± 0.6 | **27.6 ± 0.4** (↓8%)** |
| **Heart** | | | | | | |
| Absolute | 0.131 ± 0.005 | 0.136 ± 0.007 | 0.130 ± 0.003 | 0.136 ± 0.007 | 0.123 ± 0.005 | **0.115 ± 0.005* (↓15%)** |
| Relative | 4.20 ± 0.15 | 4.55 ± 0.23 | 4.27 ± 0.19 | 4.43 ± 0.17 | 4.19 ± 0.24 | 4.17 ± 0.21 |
| **R. Kidney** | | | | | | |
| Absolute | 0.232 ± 0.009 | 0.223 ± 0.009 | 0.221 ± 0.007 | 0.226 ± 0.007 | 0.222 ± 0.013 | 0.209 ± 0.005 |
| Relative | 7.41 ± 0.17 | 7.45 ± 0.26 | 7.22 ± 0.18 | 7.37 ± 0.16 | 7.56 ± 0.52 | 7.58 ± 0.16 |
| **Liver** | | | | | | |
| Absolute | 1.425 ± 0.047 | 1.345 ± 0.041 | 1.457 ± 0.062 | 1.514 ± 0.047 | 1.395 ± 0.041 | **1.530 ± 0.058* (↑14%)** |
| Relative | 45.56 ± 1.04 | 44.90 ± 1.10 | 47.59 ± 1 92 | 49.44 ± 1.32 | 47.27 ± 1.42 | **55.35 ± 1.50** (↑23%)** |
| **Lung** | | | | | | |
| Absolute | 0.200 ± 0.023 | 0.192 ± 0.008 | 0.180 ± 0.007 | 0.180 ± 0.005 | 0.173 ± 0.010 | 0.171 ± 0.007 |
| Relative | 6.33 ± 0.56 | 6.43 ± 0.30 | 5.86 ± 0.15 | 5.88 ± 0.16 | 5.87 ± 0.37 | 6.20 ± 0.24 |
| **Spleen** | | | | | | |
| Absolute | 0.094 ± 0.003 | 0.095 ± 0.007 | 0.090 ± 0.003 | 0.088 ± 0.005 | 0.081 ± 0.002 | 0.082 ± 0.002 |
| Relative | 3.02 ± 0.12 | 3.17 ± 0.21 | 2.96 ± 0.17 | 2.87 ± 0.16 | 2.75 ± 0.09 | 2.97 ± 0.08 |
| **Thymus** | | | | | | |
| Absolute | 0.054 ± 0.003 | 0.060 ± 0.004 | 0.056 ± 0.004 | 0.055 ± 0.003 | **0.049 ± 0.003* (↓18%)** | **0.044 ± 0.002** (↓27%)** |
| Relative | 1.73 ± 0.09 | 2.00 ± 0.14 | 1.81 ± 0.11 | 1.77 ± 0.10 | **1.66 ± 0.08* (↓17%)** | **1.58 ± 0.06** (↓21%)** |

** Significantly different (P≤0.05) from the vehicle control group by Williams' test. ** (P≤0.01).*

**Necropsy**

Significant chemical-related gross lesions were observed in the liver of male and female mice in the top dose groups. One male and two females had enlarged livers, one male had a liver nodule, and one female had a pale liver.

**Histopathology**

Histopathological findings are summarised in tables B.6.8.1.1.2-02/5-6 for males and females, respectively.

Hepatic alterations such as cytologic alteration, necrosis, bile duct hyperplasia, and focal subacute inflammation were described in the top dose male group (40-50%) and in the 300 and 1000 mg/kg bw/day female groups (100% and 40%, respectively). Female top dose group had generally lower incidences of lesions due to early mortality. Cytologic alteration was observed primarily in periportal sites and was the term used to describe a variety of hepatocellular alterations that included mild nuclear and cytoplasmic enlargement (hypertrophy) and increased cytoplasmic eosinophilia. Necrosis of scattered individual hepatocytes occurred throughout the hepatic lobules. Bile duct hyperplasia consisted of proliferation of immature biliary cells within portal areas. Inflammation consisted of multiple small foci of primarily mononuclear inflammatory cells randomly scattered throughout the liver.

The incidences of atrophy, degeneration, edema, mitotic alteration, and cystic glands of the fundic region of the glandular stomach were increased in females mice administered 30 mg/kg or greater doses compared to controls. The incidences of atrophy, degeneration, edema, mitotic alteration, and cystic glands were statistically significantly increased in 300 mg/kg bw/day female dose group (100%, 100%, 60%, 100% and 70%, respectively). Lower incidences of degeneration, mitotic alteration and cystic gland were also recorded in the 30 and 100 mg/kg bw/day female groups. On the other hand, in males dose groups, the incidences of cystic glands (60%) in the 30 mg/kg bw/day group, and degeneration and mitotic alteration (90% and 100%, respectively) in the 300 mg/kg bw/day group were statistically significantly increased compared with controls, however, a clear dose-response relationship was not observed.

In the top dose mice groups, early death and subsequent autolysis significantly precluded adequate histopathologic evaluation of gastric lesions. Lesions were generally of minimal to mild severity in the 30, 100, and 300 mg/kg bw/day dose groups and of mild to marked severity in the 1000 mg/kg dose groups. Atrophy consisted of a generalized decrease in the thickness of the mucosal epithelium due to loss of parietal and chief cells and shortening of the mucosal glands. Necrosis in the glandular epithelium was characterised by necrosis of parietal cells primarily, and to a lesser extent, chief cells. Degeneration consisted of dilated glands lined by dysplastic atypical epithelial cells and cellular detritus. Cystic glands were dilated and lined by flattened epithelium. Increased numbers of morphologically normal mitotic figures were present in regenerative areas of the glandular epithelium. Edema was of minimal to mild severity and occurred in the *lamina propria*.

Statistically significant increases of minimal to mild focal degeneration of the olfactory epithelium of the nose were observed in the 30 and 1000 mg/kg bw/day dose males group, and in 30-300 mg/kg females dose groups. The incidences and severities of these lesions in both sexes were not dose-related.

**Table B.6.8.1.1.2-02/5. Summary of histopathological findings in males in 14-week mice study (mean ± standard error)**

| **Male B6C3F1 mice (a)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Doses of methyleugenol (mg/kg bw/d)** | **water** | **vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Number of animals observed | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Liver** | | | | | | | |
| Cytologic Alteration | 0 | 0 | 0 | 0 | 0 | 0 | **5* (50%)** |
| Necrosis | 0 | 0 | 0 | 0 | 0 | 0 | **5* (50%)** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hyperplasia, bile duct | 0 | 0 | 0 | 0 | 0 | 0 | **5* (50%)** |
| Inflammation, subacute | 0 | 0 | 0 | 0 | 0 | 0 | **4* (40%)** |
| **Glandular stomach** | | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0 | | 3 |
| Degeneration | 0 | 0 | 0 | 1 | 1 | **9** (90%)** | 3 |
| Necrosis | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Edema | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Mitotic alteration | 0 | 0 | 0 | 1 | 0 | **10** (100%)** | 0 |
| Cystic glands | 0 | 0 | 0 | **6** (60%)** | 0 | 2 | 1 |
| **Nose** | | | | | | | |
| Epithelial cell, degeneration | 0 | 0 | 0 | **4* (40%)** | 0 | 1 | **5* (50%)** |

** Significantly different (P≤0.05) from the vehicle control group by Fisher exact test. **p≤0.01.*
*a) Results expressed as number of animals with lesion*

**Table B.6.8.1.1.2-02/6. Summary of histopathological findings in females in 14-week mice study (mean ± standard error)**

| **Female B6C3F1 mice (a)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Doses of methyleugenol (mg/kg bw/d)** | **water** | **vehicle** | **10** | **30** | **100** | **300** | **1000** |
| Number of animals observed | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **Liver** | | | | | | | |
| Cytologic Alteration | 0 | 0 | 0 | 1 | 0 | **10** (100%)** | **4* (40%)** |
| Necrosis | 0 | 0 | 0 | 0 | 0 | **10** (100%)** | **4* (40%)** |
| Hyperplasia, bile duct | 0 | 0 | 0 | 0 | 0 | **10** (100%)** | **4* (40%)** |
| Inflammation, subacute | 0 | 0 | 0 | 0 | 0 | **10**(100%)** | **1** |
| **Glandular stomach** | | | | | | | |
| Atrophy | 0 | 0 | 0 | **0** | **0** | **10** (100%)** | **3** |
| Degeneration | 0 | 0 | 0 | **4* (40%)** | **3** | **10** (100%)** | **3** |
| Necrosis | 0 | 0 | 0 | **0** | **0** | **0** | **3** |
| Edema | 0 | 0 | 0 | **0** | **0** | **6** (60%)** | **3** |
| Mitotic alteration | 0 | 0 | 0 | **4* (40%)** | **5* (50%)** | **10** (100%)** | **0** |
| Cystic glands | 0 | 0 | 0 | **4* (40%)** | **0** | **7** (70%)** | **0** |
| **Nose** | | | | | | | |
| Epithelial cell, degeneration | 2 | 0 | 0 | **8** (80%)** | **4* (40%)** | **5* (50%)** | 3 |

** Significantly different (P≤0.05) from the vehicle control group by Fisher exact test. **p≤0.01.*
*a) Results expressed as number of animals with lesion*

## Reproductive parameters

Male mice of the 10 and 30 mg/kg bw/day dose groups had statistically significantly lower cauda epididymis, epididymis, and testis weights compared with controls. As noted, these weights reductions in male reproductive organs were not dose-related. Additionally, 100 mg/kg bw/day dose group showed a statistically significant decrease in spermatozoal concentration. There were no significant differences in vaginal cytology parameters between dosed and vehicle control mice (Tables B.6.8.1.1.2-02/7-8).

**Table B.6.8.1.1.2-02/7. Summary of reproductive parameters in males in 14-week mice study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **Vehicle** | **10** | **30** | **100** |
| | 10 | 10 | 10 | 10 |
| **Weights (g)**<br>**Necropsy body wt**<br>**L. cauda epididymis**<br>**L. epididymis**<br>**L. testis** | 34.0 ± 0.7<br>0.0159 ± 0.0006<br>0.0470 ± 0.0012<br>0.1163 ± 0.0018 | 34.1 ± 0.8<br>**0.0111 ± 0.0009** (↓30%)**<br>**0.0347 ± 0.0028** (↓26%)**<br>**0.1028 ± 0.0022** (↓12%)** | 33.7 ± 0.7<br>**0.0099 ± 0.0012** (↓38%)**<br>**0.0307 ± 0.0032** (↓35%)**<br>**0.1011 ± 0.0025** (↓13%)** | 34.2 ± 0.9<br>0.0159 ± 0.0006<br>0.0452 ± 0.0012<br>0.1161 ± 0.0030 |
| **Spermatid measurements** | | | | |
| **Spermatid heads (107/g testis)** | 20.00 ± 0.97 | 22.99 ± 1.05 | 22.00 ±0.94 | 19.27 ± 0.67 |
| **Spermatid heads (107/testis)** | 2.33 ± 0.12 | 2.36 ± 0.12 | 2.22 ± 0.09 | 2.24 ± 0.12 |
| **Spermatid count (mean/10-4 mL suspension)** | 72.75 ± 3.76 | 73.80 ± 3.60 | 69.28 ± 2.77 | 70.15 ± 3.74 |
| **Epididymal spermatozoal parameters** | | | | |
| **Motility (%)** | 90.40 ± 0.86 | 88.91 ± 0.73 | 90.78 ± 1.10 | 90.10 ± 0.73 |
| **Concentration (106/g cauda epididymal tissue)** | 1466 ± 90 | 2153 ± 268 | 2129 ± 309 | **967 ± 50* (↓34%)** |

** Significantly different (P≤0.05) from the vehicle control group by Dunn's test. ** Significantly different (P≤0.01) from the vehicle control group by Dunnett's test.*

**Table B.6.8.1.1.2-02/8. Summary of reproductive parameters in females in 14-week mice study (mean ± standard error)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **Vehicle** | **10** | **30** | **100** |
| | 10 | 9 | 10 | 10 |
| **Necropsy body wt (g)** | 30.0 ± 0.7 | 30.7 ± 0.8 | 30.7 ± 0.6 | 29.6 ± 0.6 |
| **Estrous cycle length (days)** | | | | |
| Estrous stages (% of cycle) | 4.10 ± 0.10 | 4.19 ± 0.13b | 4.40 ± 0.15 | 4.40 ± 0.15 |
| Diestrus | 30.8 | 30.6 | 36.7 | 28.3 |
| Proestrus | 15.0 | 13.9 | 15.8 | 18.3 |
| Estrus | 35.0 | 38.0 | 37.5 | 37.5 |
| Metestrus | 19.2 | 16.7 | 10.0 | 15.0 |
| Uncertain diagnosis | 0.0 | 0.9 | 0.0 | 0.8 |

*Differences from the vehicle control group are not significant by Williams' test (necropsy body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.*

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable without restrictions).

The 90-day oral NOAEL in the mouse is 10 mg/kg bw/d, based on increased liver weights (males).

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study was used as dose range-finding study for long-term and carcinogenicity toxicity studies. The study deviations not compromise the acceptability.

| |
|---|
| All the animals of the top dose groups, except one male, died before study termination. The only clinical sign observed was morbidity in these deceased animals. Bodyweight gains reductions were only statistically significant in the 300 mg/kg bw/day dose groups. Absolute and relative liver weights were statistically significant increased in 30 or greater male dose groups, and only in the top dose female group. Histopathology alterations of liver were only observed at top dose level. However, glandular stomach of females showed several alteration at 30 or greater dose tested, probably caused by direct chemical administration in the stomach mucosa *via* gavage.<br><br>Thus, **NOAEL** is stablished on **7.1 mg/kg bw/day (5-day/week dose scheme),** based on histopathological alterations in glandular stomach (females). |

**B.6.8.1.1.2-03. 14-week toxicity and cell proliferation study in rats and mice with methyleugenol**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Abdo, K.M., Cunningham, M.L., Snell, M.L., Herbert, R.A., Travlos, G.S., Eldridge, S.R. and Bucher, J.R. |
| **Report year** | 2001 |
| **Report title** | 14-week toxicity and cell proliferation of methyleugenol administered by gavage to F344 rats and B6C3F1 mice*. |
| **Report No** | Not applicable, published study (*Food and Chemical Toxicology, 39:303-316*) |
| **Document No** | KCA 5.8.1/03b |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**This study summarised the oral 14-week studies in rats and mice conducted with methyleugenol and reported by NTP (B.6.8.1.1.2-01 and B.6.8.1.1.2-02 sections). In order to avoid duplications, the results were not assessed again. Only new experiments not present in NTP report are described and assessed in this section by RMS.*

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No: not stated; Purity: 99% |
| **Test system** | Female F344/N rats and male B6C3F1 mice |
| **Test method** | Groups of 10 female rats received methyleugenol in 0.5% methylcellulose by gavage at doses 0, 37, 75, 150, 300 and 1000 mg/kg bw/day for 5 days per week for 30 days or 90 days. Groups of 10 male mice received methyleugenol at doses 0, 9, 18.5, 37, 75, 150 and 300 mg/kg/day on the same dosing schedule.<br><br>At the end of each gavage study the animals were fasted for 24 h with free access to water. This was done because gastrin fluctuates physiologically in relation to meals. A midline abdominal incision was made and blood collected from the dorsal aorta (rats) or cardiac puncture (mice). Individual blood samples were centrifuged at 1500 rpm for 30 min. Sera were separated into 1.5 ml tubes and stored at -80ºC. The serum was shipped on dry ice for gastrin analysis. The analysis was performed by radioimmunoassay.<br><br>After blood collection, animals were euthanized by $CO_2$ asphyxiation and the stomachs removed. A small slit was made in the glandular section of the stomach, an electrode was inserted, and the pH of the gastric contents was determined using a pH meter.<br><br>*Cell proliferation*<br><br>Stomach squamous epithelial cell proliferation was evaluated by the number of cells |

| | |
|---|---|
| | per unit length muscularis stained positive for bromodeoxy-uridine (BrdU). The total number of BrdU positive cells in four 0.25 fields were quantitated. This is the unit length labelling index (ULLI).<br>Two hours prior to termination, animals in the gastrin and gastric pH studies were injected intraperitoneal (i.p.) with bromodeoxy-uridine (100 mg/kg in 0.01 N NaOH), which is incorporated into the DNA of newly replicating cells. Two hours later, the animals were euthanized by $CO_2$ asphyxiation, exsanguination and livers were removed, and a mid-lobe section of the right anterior lobe of the liver was fixed in 10% neutral buffered formalin (NBF) for 24 h. Following measurement of the glandular stomach contents pH, the entire stomach was opened along the lesser curvature, pinned to a piece of cardboard, rinsed clean of stomach contents and fixed in NBF overnight. Serial sections of the stomach were longitudinally trimmed to include fundus and pyloris as well as forestomach on a single section. A cross-section of small intestine was also fixed as a positive control for the staining technique. Tissues were embedded in paraffin and serial sections mounted. Each slide contained liver, stomach and intestine pieces for simultaneous processing and staining. Following de-paraffination and rehydration, one section was stained with hematoxylin and eosin for histological evaluation and another was stained immuno- histochemically for BrdU incorporation by a variation on the method of Sugihara *et al*. (1986). Random areas of the slides were chosen for counting stained and unstained hepatocyte nuclei (>1000 hepatocytes/animal) |
| **Statistical analysis of results** | The Student's t-test (two-sided, unequal variance) was used to compare control and methyleugenol treated groups at each time point. A P value of 0.05 was judged to be statistically significant. Cell proliferation statistics were performed using nonparametric Kruskal-Wallis test. The Mann-Whitney U test was used for pair-wise comparison when a significant F ratio was detected. |

**Results**

**Gastrin and gastric pH:**

*Rats*
Gastric pH showed a statistically significant increase in female rats that received 1000 mg/kg bw/day dose at the end of 30 or 90 days of treatment (191% and 73%, respectively), and in female rats given 300 mg/kg bw/day for 90 days (41%), compared with controls. The increase in pH was accompanied by a statistically significant increase of serum gastrin levels in these groups (Table B.6.8.1.1.2-03/1).

**Table B.6.8.1.1.2-03/1. Serum gastrin levels and gastric stomach pH in female F344 rats treated by gavage with methyleugenol**

| Dose (mg/kg bw/day) | Dose duration (days) | Serum gastrin (pg/ml) (mean±S.E.M.) | Stomach pH (mean±S.E.M.) |
|---|---|---|---|
| **Vehicle control** | 30 | 62±7 | 2.2±0.1 |
| **37** | 30 | **25±4* (↓60%)** | 2.4±0.3 |
| **75** | 30 | **22±2* (↓65%)** | 1.7±0.1 |
| **150** | 30 | 57±14 | 1.9±0.2 |
| **300** | 30 | **27±23* (↓57%)** | **1.6±0.2* (↓27%)** |
| **1000** | 30 | **604±20** (↑874%)** | **6.4±0.1** (↑191%)** |
| | | | |
| **Vehicle control** | 90 | 41±3 | 2.2±0.3 |
| **37** | 90 | 46±3 | 1.8±0.1 |
| **75** | 90 | 60±6 | 2.2±0.2 |
| **150** | 90 | **8±11**(↓80%)** | 2.0±0.2 |

| | | | |
|---|---|---|---|
| **300** | 90 | **409±68**(↑898%)** | **3.1±0.5*(↑41%)** |
| **1000** | 90 | **506±192**(↑1134%)** | **3.8±0.3**(↑73%)** |

** Significantly different by Student s t-test (P≤0.05); ** (p≤0.01)*

*Mice*
Gastric pH was statistically significant higher in 75 mg/kg bw/day male mice dose group for 30 days, whereas serum gastrin was in 150 and 300 mg/kg bw/day dose groups for 30 days. As noted, no dose-relationship was observed in these parameters, and the increase of serum gastrin was not correlated with a pH increase. No differences were recorded in 90-day treatment design (Table B.6.8.1.1.2-03/2).

**Table B.6.8.1.1.2-03/2. Serum gastrin levels and gastric stomach pH in male B6B3F1 mice treated by gavage with methyleugenol**

| Dose (mg/kg bw/day) | Dose duration (days) | Serum gastrin (pg/ml) (mean±S.E.M.) | Stomach pH (mean±S.E.M.) |
|---|---|---|---|
| **Vehicle control** | 30 | 3.5±0.8 | 2 1±0.1 |
| **9** | 30 | 2.9±1 | 2 3±0.1 |
| **18.5** | 30 | 3.5±0.6 | 2 2±0.1 |
| **37** | 30 | 3.8±1 | 2 5±0.1 |
| **75** | 30 | 4.8 ±1.6 | **2.7±0.2* (↑29%)** |
| **150** | 30 | **15±2** (↑329%)** | 2.4±0.1 |
| **300** | 30 | **11±2* (↑214%)** | 2.4±0.2 |
| | | | |
| **Vehicle control** | 90 | 3.1±0.7 | 2.8±0.3 |
| **9** | 90 | 3.5±0.8 | 2.6±0.3 |
| **18.5** | 90 | 1.9±0.4 | 2 3±0.1 |
| **37** | 90 | 2.6 ±0.8 | 3.0±0.4 |
| **75** | 90 | 3.0±0. | 2.7±0.3 |
| **150** | 90 | 6.9 ±2.2 | 2.0±0.1 |
| **300** | 90 | 7.5±2.3 | 2.0±0.1 |

**<u>Cell proliferation:</u>**

*Rats*
Statistically significant increases in cell proliferation indicated by an increase in the BrdU-labelled cells was observed in the fundic glands of the glandular stomach and in the liver of female rats given methyleugenol for 30 or 90 days (Table B.6.8.1.1.2-03/3). None of the statistically significant increases observed in the gastric pits or the pylorus were greater than two-fold the values observed in the controls. Thus, the increases at these two sites were not considered biologically significant by authors. In forestomach, only 30-day top dose group showed greater two-fold values, however a non-clear dose-response was noted.

**Table B.6.8.1.1.2-03/3. Effect of methyleugenol on cell proliferation as indicated by increase in BrdU labelling index in the forestomach, glandular stomach and liver of female rats**

| | | Labelling index (number of BrdU positive cells per mm) | | | | |
|---|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **Dosing duration (days)** | **Forestomach** | **Gastric pits** | **Fundic glands** | **Pylorus** | **Liver** |
| **Vehicle control** | 30 | 14 | 52 | 1 | 94 | 0.086 |
| **37** | 30 | 28 | 58 | **2a (↑100%)** | **143a (↑52%)** | 0 205 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **75** | 30 | **23a (↑64%)** | 67 | 1 | **135a (↑44%)** | 0.086 |
| **150** | 30 | 21 | **85a (↑63%)** | **11a,b (↑1000%)** | **121a (↑29%)** | **0.254a,b (↑195%)** |
| **300** | 30 | **24a (↑71%)** | **88a (↑69%)** | **54a,b (↑5300%)** | **113a (↑20%)** | **0.962a,b (↑1019%)** |
| **1000** | 30 | **54a,b (↑286%)** | **87a (↑67%)** | **61a (↑6000%)** | **116a (↑23%)** | **1.618a,b (↑1781%)** |
| | | | | | | |
| **Vehicle control** | 90 | 24 | 61 | 4 | 149 | 0 116 |
| **37** | 90 | 27 | 73 | **8b (↑100%)** | 130 | 0.428 |
| **75** | 90 | 37 | 55 | **8a,b (↑100%)** | 137 | 0 248 |
| **150** | 90 | 27 | 59 | **25a,b (↑525%)** | 152 | **0.553a,b (↑376%)** |

*a) P≤0.05.*
*b) Biologically significant defined by at least twofold greater than vehicle control, and at least one animal with a value greater than the highest control value, and supported by dose response.*

*Mice*
Statistically significant increases in cell proliferation indicated by the increase in the percent of BrdU-labelled cells occurred in the fundic glands of the glandular stomach of male mice given 150 or 300 mg/kg bw/day methyleugenol for 30 days, and in all dose groups that received methyleugenol for 90 days (Table B.6.8.1.1.2-03/4). None of the statistically significant decreases observed in the forestomach gastric pits or the pylorus of 30-day treatment schedule was greater than twofold the values observed in controls, and as described in the following table, no dose-relationship was noted. Thus, the changes at forestomach, gastric pics and pylorus were not considered biologically significant.

**Table B.6.8.1.1.2-03/4. Effect of methyleugenol on cell proliferation as indicated by increase in BrdU labelling index in the forestomach, glandular stomach and liver of male mice**

| | | Labelling index (number of BrdU positive cells per mm) | | | | |
|---|---|---|---|---|---|---|
| **Dose (mg/kg bw/day)** | **Dosing duration (days)** | **Forestomach** | **Gastric pits** | **Fundic glands** | **Pylorus** | **Liver** |
| **Vehicle control** | 30 | 3 | 80 | 3 | 134 | 0.003 |
| **9** | 30 | 6 | **57a (↓29%)** | 1 | **82a (↓40%)** | 0.002 |
| **18.5** | 30 | **4a (↑33%)** | **57a (↓29%)** | 1 | **99a (↓26%)** | 0.000 |
| **37** | 30 | 5 | **51a (↓29%)** | 1 | **102a (↓24%)** | 0.008 |
| **75** | 30 | 5 | 71 | 3 | **113a (↓16%)** | 0.003 |
| **150** | 30 | 6 | **118a (↑47%)** | **57ab (↑1800%)** | 117 | 0.007 |
| **300** | 30 | 4 | **100a (↑25%)** | **18ab (↑500%)** | **116a (↓13%)** | 0.003 |
| | | | | | | |
| **Vehicle control** | 90 | 10 | 96 | 1 | 124 | 0.005 |
| **9** | 90 | 8 | 115 | **3a (↑200%)** | 130 | 0.007 |
| **18.5** | 90 | **5a (↓50%)** | 103 | **6ab (↑500%)** | 140 | 0.01 |
| **37** | 90 | 7 | 84 | **6ab (↑500%)** | 137 | 0.0014 |
| **75** | 90 | 8 | 113 | **15ab (↑1400%)** | 140 | 0.008 |

**Conclusion**

The resulting elevation of gastric pH was observed in female rats given high doses of methyleugenol for 30 days. Longer exposure up to 90 days demonstrated that this was a sustained response in the presence of methyleugenol and could be observed at 300 mg/kg bw/day. Serum gastrin concentrations increased in response to high pH values due to the absence of acid feedback repression mechanism and were observed to be significantly elevated at doses as low as 150 mg/kg bw/day. Mice appeared to be less sensitive than rats to this effect. Methyleugenol increased cell proliferation of the fundic glands. This effect is probably a result of a feedback reaction to mucosal atrophy and the trophic effect of gastrin (*Johnson et al., Regulation of gastrointestinal mucosal growth. In: Walsh, J.H. (Ed.), Gastrin. Raven Press, New York, pp. 287, 1993*).

Methyleugenol cause the loss of parietal and chief cells of the glandular stomach. Acid conditions and production of protein digesting enzymes are the function of these two types of cells, respectively. Loss of these cells could inhibit proper protein utilization and essentially lead to protein deficiency.

The magnitude of any possible risk to humans, however, has not been estimated. It would appear unlikely that humans would be exposed to sufficient amounts of methyleugenol to produce the gastric lesions resulting in increased stomach pH and elevated serum gastrin, so methyleugenol should not be considered to contribute to stomach cancers in humans.

**Assessment and conclusion by applicant:**

Not provided.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** Rats were more sensitive than mice to methyleugenol treatments. In rats, gastric pH and gastrin levels were statistically significant increased at highest doses both 30 and 90 days treatments schedule. Moreover, stomach squamous epithelial cells of fundic glands and liver cell proliferation was increased in rats administered methyleugenol at 150 mg /kg bw/day or greater doses for 30 days. The findings in fundic glands were also reproduced for all dose stages (37-150 mg/kg bw/day) in the 90-day experiments, whereas liver cell proliferation was only increased in the top dose level (150 mg/kg bw/day).

In mice, statistically significant increases of fundic glands cell proliferation was recorded in highest dose levels (150-300 mg/kg bw/day) for 30 days, and in all treated groups (9-75 mg/kg bw/days) for 90 days.

To sum up, gastric pH and gastrin levels were increased in rats, but no in mice, after methyleugenol 30 or 90 days treatments. For both species, fundic glands were the glandular stomach region in which cell proliferation was much higher compared with other anatomic parts after methyleugenol treatments, whereas liver cell proliferation increases were only recorded in rats. The described stomach effects were caused by direct test substance application to stomach mucosa due to oral gavage administration.

Therefore, and in agreement with author´s conclusions, it seems unlikely that humans would be exposed *via* oral ingestion to sufficient amounts of methyleugenol to generate gastric lesions resulting in increased stomach pH and elevated serum gastrin.

### B.6.8.1.1.3. Genetic Toxicity

#### B.6.8.1.1.3- 01. Bacterial gene mutation *in vitro*

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 471 (1983) |
| **Deviations from current test guideline OECD TG 471 (2020)** | -Only four strains tested<br>-Very limited level of reporting (solvent, negative/positive controls not stated)<br>-An additional indicator of S9-mix efficacy should be used in combination with 2-aminoanthracene<br>-No historical control data (HCD) |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Yes |
| **Reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Solvent** | Not stated |
| **Negative control** | Solvent (not specified) |
| **Positive control** | Sodium azide for TA100 and TA1535(-S9)<br>4-Nitro-o-phenylenediamine for TA98 (-S9)<br>9-Aminoacridine for TA1537 (-S9)<br>2-Aminoanthracene for all strains with metabolic activation |
| **Test system** | *S. typhimurium* TA98, TA100, TA1535 and TA1537 |
| **Metabolic activation** | S9 mix from male Sprague-Dawley rat or Syriam hamster liver induced with Aroclor 1254 |
| **Test method** | Testing was performed as reported by Mortelmans *et al.* (1986).<br>Methyleugenol was incubated at concentrations up to 666 µg/plate with the *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 either in buffer or with S9 mix for 20 min at 37ºC. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C. Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of methyleugenol. The high dose was limited by toxicity. All trials were repeated.<br><br>No statistical analysis was stated/reported. |
| **Criteria for assessment of results** | A positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive. |

**Results**

Methyleugenol, tested up to a maximum concentration of 666 µg/plate did not induce mutations in *S. typhimurium* strains TA98, TA100, TA1535 or TA1537 with or without metabolic activation (Table B.6.8.1.1.3-01/1).

**Table B.6.8.1.1.3-01/1. Average revertants per plate in tester strains TA100, TA98, TA1535 and TA1537**

| Strain | Concentration (µg/plate) | Revertants/plate (mean ± standard error from three plates) | | | | | |
|---|---|---|---|---|---|---|---|
| | | -S9 | | +10% hamster S9 | | +10% rat S9 | |
| | | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| TA100 | 0 | 120 ± 3.2 | 90 ± 6.4 | 106 ± 4.4 | 103 ± 8.7 | 111 ± 7.8 | 98 ± 8.1 |
| | 3 | 101 ± 0.0 | 86 ± 3.5 | | 90 ± 8.0 | | 95 ± 5.3 |
| | 10 | 100 ± 6.4 | 93 ± 4.0 | 106 ± 4.6 | 89 ± 6.1 | 93 ± 3.3 | 94 ± 2.7 |
| | 33 | 114 ± 8.3 | 93 ± 10.7 | 109 ± 4.6 | 90 ± 6.8 | 109 ± 4.4 | 92 ± 4.3 |
| | 100 | 105 ± 11.6 | 96 ± 2.7 | 116 ± 7.0 | 80 ± 14.4 | 110 ± 0.7 | 91 ± 7.6 |
| | 333 | 29 ± 8.2[a] | 16 ± 13.1[a] | 99 ± 4.2 | 78 ± 1.0 | 95 ± 9.4 | 97 ± 2.6 |
| | 666 | | | 38 ± 38.0[a] | | 0 ± 0.0[a] | |
| Test summary | | Negative | Negative | Negative | Negative | Negative | Negative |
| Positive control | | 358 ± 7.0 | 388 ± 4.3 | 1469 ± 61.2 | 1111 ± 49.2 | 534 ± 46.0 | 351 ± 22.9 |
| TA1535 | 0 | 24 ± 2.6 | 20 ± 3.5 | 10 ± 2.4 | 12 ± 2.1 | 10 ± 2.2 | 9 ± 0.6 |
| | 3 | 37 ± 0.6 | 20 ± 2.3 | | 8 ± 0.9 | | 6 ± 0.00 |
| | 10 | 33 ± 2.0 | 21 ± 2.3 | 13 ± 3.5 | 8 ± 2.3 | 8 ± 0.9 | 7 ± 0.3 |
| | 33 | 37 ± 7.5 | 22 ± 3.3 | 14 ± 1.5 | 9 ± 2.8 | 9 ± 0.0 | 9 ± 2.6 |
| | 100 | 32 ± 3.5 | 26 ± 2.7 | 11 ± 2.2 | 10 ± 3.7 | 6 ± 1.5 | 7 ± 1.0 |
| | 333 | 5 ± 5.0[a] | 2 ± 0.7[a] | 10 ± 2.1 | 9 ± 2.3 | 4 ± 0.3 | 8 ± 0.9 |
| | 666 | | | 4 ± 2.6 [a] | | 0 ± 0.0[a] | |
| Test summary | | Negative | Negative | Negative | Negative | Negative | Negative |
| Positive control | | 375 ± 15.5 | 410 ± 15.2 | 381 ± 7.9 | 369 ± 7.9 | 146 ± 2.8 | 168 ± 21.2 |
| TA1537 | 0 | 5 ± 0.6 | 5 ± 0.3 | 6 ± 1.0 | 5 ± 0.3 | 7 ± 0.7 | 6 ± 0.9 |
| | 3 | 6 ± 1.5 | 3 ± 0.9 | | 9 ± 1.5 | | 8 ± 2.1 |
| | 10 | 4 ± 1.2 | 3 ± 0.9 | 5 ± 1.2 | 6 ± 0.9 | 5 ± 0.9 | 4 ± 1.0 |
| | 33 | 5 ± 0.3 | 4 ± 1.2 | 5 ± 1.5 | 5 ± 1.2 | 4 ± 0.3 | 9 ± 1.5 |
| | 100 | 4 ± 1.5 | 4 ± 0.6 | 6 ± 1.5 | 5 ± 1.0 | 7 ± 0.0 | 7 ± 1.2 |
| | 333 | 0 ± 0.0[a] | 3 ± 0.03[a] | 3 ± 1.2 | 4 ± 1.3 | 6 ± 0.3 | 5 ± 2.2 |
| | 666 | | | Toxic | | 0 ± 0.0[a] | |
| Test summary | | Negative | Negative | Negative | Negative | Negative | Negative |
| Positive control | | 184 ± 4.7 | 521 ± 48.1 | 424 ± 75.9 | 426 ± 15.5 | 157 ± 8.7 | 124 ± 11.9 |
| TA 98 | 0 | 15 ± | 16 ± 1.7 | 25 ± 2.2 | 31 ± | 17 ± 3.6 | 20 ± 4.1 |
| | 3 | 15 ± | 13 ± 2.2 | | 31 ± | | 27 ± 0.9 |
| | 10 | 15 ± | 14 ± 0.9 | 27 ± 3.5 | 28 ± | 24 ± 2.7 | 23 ± 2.6 |
| | 33 | 15 ± | 13 ± 1.8 | 31 ± 5.8 | 26 | 29 ± 2.0 | 20 ± 2.3 |
| | 100 | 14 ± | 13 ± 0.9 | 28 ± 0.7 | 29 | 20 ± 3.5 | 29 ± 5.5 |
| | 333 | 0 ± 0.0[a] | 3 ± 3.0[a] | 15 ± 7.7[a] | 21 | 29 ± 5.0 | 19 ± 0.3 |
| | 666 | | | Toxic | | Toxic | |
| Test summary | | Negative | Negative | Negative | Negative | Negative | Negative |
| Positive control | | 626 ± 20.6 | 412 ± 8.2 | 1274 ± 85.7 | 1362 ± 55.5 | 473 ± 34.3 | 444 ± 76.4 |

[a] Slight toxicity

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (category 4, not assignable: secondary source review).

Methyleugenol was not genotoxic *in vitro.*

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion. Under the conditions of the study, eugenol is negative in *S. typhimurium strains* TA100, TA1535, TA1537 and TA98, in the presence and absence of metabolic activation.

Deviations from current OECD TG 471 (2020) include only four instead of five *S. typhimurium* strains tested (no TA102 or WP2*uvr*A were tested) and limited level of detail. For these reasons, the RMS deems the study as supporting information only.

### B.6.8.1.1.3- 02. Bacterial gene mutation *in vitro*

| Data point | CA 5.8.1 (EU data requirement) |
|---|---|

| | |
|---|---|
| **Report author** | Haworth, S., Lawlor, T., Mortelmans, K., Speck, W. and Zeiger, E. |
| **Report year** | 1983 |
| **Report title** | Salmonella mutagenicity test results for 250 chemicals |
| **Report No** | Not applicable, published study (*Environ. Mutagen. Suppl.* 1, 3-142) |
| **Document No** | KCA 5.4.1/02 |
| **Guidelines followed in study** | Modification of pre-incubation procedure test (Yahagi *et al.* 1975*)*. Previous to OECD TG 471 (1983) |
| **Deviations from current test guideline OECD TG 471 (2020)** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Reliability** | Not acceptable |

**Results**

Methyleugenol was not tested in this study.

| |
|---|
| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level for eugenol, but not for methyleugenol. It is acceptable for renewal of the active substance.<br>Eugenol was not mutagenic in *S. typhimurium.* |
| **Assessment and conclusion by RMS:**<br>The RMS could not evaluate this study as the compound is not tested as part of this publication. |

**B.6.8.1.1.3- 03. Single Cell Electrophoresis (Comet assay) *in vitro***

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Groh, I.A.M., Cartus, A.T., Vallicotti, S., Kajzar, J., Merz, K.H., Schrenk, D. and Esslen, M. |
| **Report year** | 2012 |
| **Report title** | Genotoxic potential of methyleugenol and selected methyleugenol metabolites in cultured Chinese hamster V79 cells |
| **Report No** | Not applicable, published study (*Food Funct.*, **3**, 428-436) |
| **Document No** | KCA 5.8.1/07 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | No available guideline |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Reliability** | Supporting information |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol, Purity: not stated; Batch no.: not stated |
| **Solvent** | Dimethyl sulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Menadione |
| **Test system** | Male Chinese hamster V79 lung fibroblasts |

| | |
|---|---|
| **Test method** | The Comet assay was performed according to the method of Gedik *et al.* (1998)[1]. V79 cells ($10^6$ or $5x10^5$) were cultured in 5mL medium containing 10 % FCS and they were allowed to grow for 24 h prior to treatment. V79 cells were treated for 1h or 24 h with the solvent control or the test compound (5, 10, 25, 50, 75 and 100 µM) in medium containing 10 % FCS. A positive control was also used (10 µM menadione). DNA was allowed to unwind (300 mM NaOH, 1mM EDTA, pH 13.5, 20 min at 4ºC) followed by horizontal gel electrophoresis. Slides were washed and stained with ethidium bromide and fluorescence microscopy was performed. The slides were scored (50 images per slide randomly selected from electrophoresis) and the results were parameterised with respect to tail intensity (calculated as percentage of overall DNA intensity). The quantitative data were derived from at least three independent sets of experiments and from the evaluation of 100 individual cells per concentration in each experiment. |

**Results**

Methyleugenol significantly induced DNA strand breaks in V79 cells at concentrations > 10 µM after 1h incubation without an increase in efficacy after 24 h (Figure B.6.8.1.1.3-03/1).

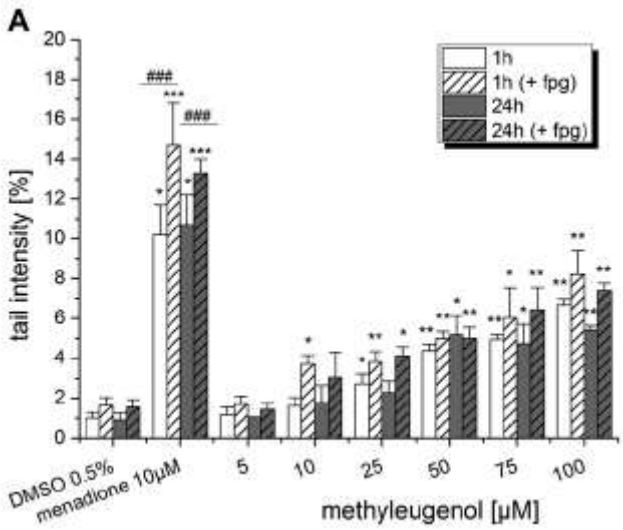



**Figure B.6.8.1.1.3-03/1. DNA-strand breaking properties of methyleugenol in V79 cells. Data presented as mean ± SD [Student's *t*-test* = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001].**

| |
|---|
| **<u>Assessment and conclusion by applicant</u>:**<br>This new study is considered to be acceptable as supporting information as it does not provide enough information on methodology for a full assessment (reliable with restrictions). |
| **<u>Assessment and conclusion by RMS</u>:**<br>The RMS agrees with the notifier's assessment and conclusion. The RMS deems the study as supporting information only as the study is not a validated method to evaluate DNA reactivity |

**B.6.8.1.1.3- 04. Micronucleus test *in vitro***

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |

[1] Gedik, C.M., Wood, S.G. and Collins, A.R. (1998), *Free Radical Res.* **29**, 609-615

| | |
|---|---|
| **Report author** | Groh, I.A.M., Cartus, A.T., Vallicotti, S., Kajzar, J., Merz, K.H., Schrenk, D. and Esslen, M. |
| **Report year** | 2012 |
| **Report title** | Genotoxic potential of methyleugenol and selected methyleugenol metabolites in cultured Chinese hamster V79 cells |
| **Report No** | Not applicable, published study (*Food Funct.*, **3**, 428-436) |
| **Document No** | KCA 5.8.1/07 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 487 (2010) |
| **Deviations from current test guideline OECD TG 487 (2016)** | -No characterisation of the test item<br>-Dose selection not adequately explained<br>-No metabolic activation used<br>-Very limited reporting of experimental and results, no individual data provided,<br>-No historical data provided |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol, Purity: not stated; Batch no.: not stated |
| **Solvent** | Dimethyl sulphoxide (DMSO) |
| **Metabolic activation** | None |
| **Negative control** | Solvent |
| **Positive control (-S9)** | Mitomycin C (MMC) |
| **Test system** | Male Chinese hamster V79 lung fibroblasts |
| **Test method** | V79 cells ($1x10^5$ or $5x10^4$) were seeded into Petri dishes and they were allowed to grow for 24 h in an incubator (37ºC, 5% $CO_2$). Cells were rinsed with 5 mL PBS buffer and were treated for 1h or 24 h with methyleugenol at concentrations 0, 10, 25, 50 and 100 µM. The positive control was MMC (0.6 µM) for 24h. After removing the test item by medium replacement, the cells were incubated with FCS-containing medium for another 20 h and washed with PBS buffer. Micronuclei scoring was performed with at least three independent sets of experiments and the evaluation of 2000 individual cells per concentration in each experiment. |

**Results**

No enhanced micronuclei frequency was observed after treatment of V79 cells with methyleugenol (Table B.6.8.1.1.3-04/1).

**Table B.6.8.1.1.3-04/1. Average micronucleated cells per 1000 cells after treatment with methyleugenol**

| Substance | Micronucleated cells per 1000 cells | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **10 µM** | **25 µM** | **50 µM** | **100 µM** |
| Solvent (DMSO 0.5 %) | 13.7 ± 4.4 | | | | |
| Methyleugenol | | 13.5 ± 4.3 | 13.3 ± 1.8 | 12.5 ± 4.8 | 11.7 ± 4.3 |
| Positive control (MMC) : 90.2 ± 13.0 | | | | | |

Data represent mean ± SD

| |
|---|
| **Assessment and conclusion by applicant:**<br>This new study is considered to be acceptable as supporting information as it does not provide enough information on methodology for a full assessment (reliable with restrictions). |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion. Methyleugenol did not induce micronuclei *in vitro* in the absence of metabolic activation. The study is a scientific publication for a peer-reviewed journal. No guideline was stated in the study and it was compared against the current OECD TG 487 (2016). The RMS deems the study as supporting information based on the deviations from the current guideline, which include: poor level of reporting, no individual data provided, test item was not fully characterised, no metabolic activation used and no historical data were provided. Furthermore, there is no justification for the dose selection and the highest concentration according to the current guidance should be determined at 10mM in the absence of cytotoxicity. |

**B.6.8.1.1.3- 05. Mammalian Gene Mutation assay *in vitro***

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Groh, I.A.M., Cartus, A.T., Vallicotti, S., Kajzar, J., Merz, K.H., Schrenk, D. and Esslen, M. |
| **Report year** | 2012 |
| **Report title** | Genotoxic potential of methyleugenol and selected methyleugenol metabolites in cultured Chinese hamster V79 cells |
| **Report No** | Not applicable, published study (*Food Funct.*, **3**, 428-436) |
| **Document No** | KCA 5.8.1/07 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 476 |
| **Deviations from current test guideline**<br>**OECD TG 476 (2016)** | -No characterisation of the test item<br>-Dose selection not adequately explained<br>-No metabolic activation used<br>-Longer treatment time<br>-Very limited reporting of experimental and results, no individual data provided,<br>-No historical data provided |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Not applicable, published study |
| **Reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol, Purity: not stated; Batch no.: not stated |
| **Solvent** | Dimethyl sulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) |
| **Metabolic activation** | None |
| **Test system** | Male Chinese hamster V79 lung fibroblasts |
| **Test method** | Procedure according to the method of Bradley *et al.*[2], with slight modifications.<br>V79 cells ($10^6$) were seeded with FCS-containing medium (10 %) and allowed to grow. After 24h the cell culture medium was removed and the flasks were rinsed with 5mL PBS buffer. The test item was dissolved in DMSO and diluted with FCS-free medium and 10 mL of this solution was added to each flask. A positive control experiment was carried out with 20 µM MMNG. Cells were treated for 1h or 24h and after this time the medium was replaced and 10 mL of FCS-containing medium was added. V79 cells ($10^6$) |

[2] Bradley, M.O. *et al.*, *Mutat. Res., Rev. Genet. Toxicol.*, 1981, **87**, 81-142

| | |
|---|---|
| | were suspended in 10 mL FCS-containing medium and incubated for 48 h. After this time, medium was removed and the cells were rinsed with 5 mL PBS buffer and treated with Trypsin solution. V79 cells were transferred into the flasks and incubated for another 48h. Cell culture medium was removed, cell layer rinsed with 5 mL PBS and 1mL trypsin solution was added to each flask. V79 cells were suspended in 10 mL FCS-containing medium, counted and 106 cells were transferred with 15 mL thioguanine-containing medium. The viability control (plating efficiency) 240 cells each were suspended in 10 mL FCS-containing medium and transferred to two petri dishes. After 9 days petri dishes and incubation flasks were rinsed with 0.9% saline and 5 mL ethanol. Methylene blue solution (0.5 % in ethanol) was added and the cells were kept for at least 30min at -20ºC. After removal of methyleneblue the cells were washed with tap water and air dried. The colonies per vessel were counted and the mean (number) was calculated for each treatment. The mutation frequency was calculated as: MF = $mean_{flask}$ x 240/$mean_{plate}$ |

**Results**

Methyleugenol (≤ 100 µM) did not induce mutations at both time points (1h or 24h treatment) (Table B.6.8.1.1.3-05/1).

**Table B.6.8.1.1.3-05/1. Mutant frequencies after treatment of V79 with methyleugenol**

| Substance | Mutant Frequency per $10^6$ cells (1h treatment) | | | | |
|---|---|---|---|---|---|
| | **0 µM** | **10 µM** | **25 µM** | **50 µM** | **100 µM** |
| Solvent (DMSO 0.5 %) | 6.4 ± 4.1 | | | | |
| Methyleugenol | | 3.7 ± 0.9 | 6.3 ± 5.1 | 2.5 ± 1.6 | 4.5 ± 3.5 |
| | Mutant Frequency per $10^6$ cells (24h treatment) | | | | |
| Solvent (DMSO 0.5 %) | 8.1 ± 5.8 | 10.1 ± 4.1 | 6.4 ± 4.6 | 3.1 ± 1.9 | - |
| Methyleugenol | | | | | |
| Positive control (MNNG) : 269 ± 45 (1h) ; 258 ± 92 (24h) | | | | | |

**<u>Assessment and conclusion by applicant</u>:**

This new study is considered to be acceptable as supporting information as it does not provide enough information on methodology for a full assessment (reliable with restrictions).

**<u>Assessment and conclusion by RMS</u>:**

The RMS agrees with the notifier's assessment and conclusion. Methyleugenol did not induce mutations in V79 cells at both treatment times (1h or 24h). No guideline was stated in the study and it was compared against the current OECD TG 476 (2016). The RMS deems the study as supporting information based on the deviations from the current guideline: poor level of reporting, no individual data provided, test item was not fully characterised, no metabolic activation used, treatment time exceeded the recommended by guidance and no historical data were provided. Furthermore, there is no justification for the dose selection and the highest concentration according to the current guidance should be determined at 10mM in the absence of cytotoxicity.

**B.6.8.1.1.3- 06. Chromosome aberration *in vitro***

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |

| | |
|---|---|
| **Guidelines followed in study** | Not stated, comparable to OECD TG 473 (1997) |
| **Deviations from current test guideline OECD TG 473 (2016)** | -Treatment times longer (-S9) and shorter (+S9) than the recommended 3-6h<br>-Only 200 metaphases scored instead of 300<br>-No justification provided for the selection of concentrations<br>-Limited level or reporting: no individual data and only percentage of cells with aberrations reported.<br>-No historical data provided |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Yes |
| **Reliability** | Supporting information only (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control (-S9)** | Mitomycin-C (MMC) |
| **Positive control (+S9)** | Cyclophosphamide |
| **Test system** | Chinese Hamster Ovary Cells (CHO) |
| **Metabolic activation** | Sprague-Dawley Rat S9 |
| **Test method** | Testing was performed according to the method described by Galloway *et al.* (1987).<br>In the assay without S9, cells were incubated in McCoy's 5A medium with methyleugenol for 14.7 hours; Colcemid was added and incubation continued for 2h. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa.<br>For the assay with S9, cells were treated with methyleugenol and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 10.5 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9. The harvest time for the Abs test was based on the cell cycle information obtained in the SCE test. Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. Two hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).<br>Chromosomal aberration data were presented as percentage of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. |
| **Criteria for assessment of results** | For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) are considered weak evidence for a positive response; significant differences for two or more doses indicates the trial is positive.<br>A positive trend test in the absence of a statistically significant increase at any one dose results in an equivocal call. Ultimately, the trial calls are based on a consideration of the statistical analyses as well as the biological information available to the NTP reviewers |

**Results**

No significant induction of chromosome aberrations occurred following incubation with methyleugenol in either the presence or the absence of S9 (Table B.6.8.1.1.3-06/1). The doses tested in this assay were similar to those used in the SCE test (B.6.8.1.1.3-07) and were limited by toxicity to 233 µg/mL.

**Table B.6.8.1.1.3-06/1. Induction of chromosomal aberrations in Chinese Hamster Ovary cells by Methyleugenol**

| Compound | Concentration (µg/mL) | Total cells scored | Number of aberrations | Aberrations/Cell | Cells with aberrations (%) |
|---|---|---|---|---|---|
| **-S9**<br>**Exposure time : 16.7 hours**<br>**Overall call : negative** | | | | | |
| Negative control (DMSO) | - | 200 | 1 | 0.01 | 0.5 |
| Positive control (MMC) | 0.4 | 25 | 30 | 1.20 | 56.0 |
| Methyleugenol[a] | 50 | 200 | 2 | 0.01 | 1.0 |
| | 108 | 200 | 2 | 0.01 | 1.0 |
| | 233 | 200 | 0 | 0.00 | 0.0 |
| | 500 | 0 | | | |
| **+S9**<br>**Exposure time : 12.5 hours**<br>**Summary : Negative** | | | | | |
| Negative control (DMSO) | - | 200 | 3 | 0.02 | 1.5 |
| Positive control (Cyclophosphamide) | 20 | 25 | 15 | 0.60 | 40.0 |
| Methyleugenol[b] | 50 | 200 | 3 | 0.02 | 1.5 |
| | 108 | 200 | 8 | 0.04 | 4.0 |
| | 233 | 200 | 10 | 0.05 | 4.5 |
| | 500 | 0 | | | |

[a] Significance of percent cells with aberrations tested by the linear regression trend test versus log of dose : P = 0.726
[b] Significance of percent cells with aberrations tested by the linear regression trend test versus log of dose : P = 0.015

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (category 4, not assignable: secondary source review).

Methyleugenol was not genotoxic in the *in vitro* chromosome aberration assay but did show positive effects in the SCE assay. It is noted that the SCE assay is no longer recommended by the OECD.

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion. Methyleugenol does not induce chromosomal aberrations up to 233 µg/mL following 14.5h and 2h exposure without and with metabolic activation, respectively. Despite a negative call, the RMS is of the opinion that a positive trend is observed in the activation experiment and a longer treatment time would have allowed to confirm/disregard it. The authors noted that toxicity occurred at 233 µg/mL although no estimations such as RICC or RPD are presented. For this reason, the RMS deems the outcome as equivocal (no statistical differences between groups). The study has been checked for compliance against the current OECD TG 473 (2016). Deviations from the current guidance include the number of metaphases scored (200 instead of 300), longer/shorter treatment times in –S9/+S9 experiments, respectively, no individual data provided, data only reported as percentage of aberrations and no historical data provided. Based on the deviations from the current guidance, the RMS deems the study as supporting information only.

**B.6.8.1.1.3- 07. Mammalian DNA damage (Sister Chromatid Exchange)**

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |

| | |
|---|---|
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | Not stated |
| **Deviations from current test guideline** | A comparable guideline is not available. |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Yes |
| **Reliability** | Supportive only, since there is no current guideline available for this test |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control (-S9)** | Mitomycin-C (MMC) |
| **Positive control (+S9)** | Cyclophosphamide |
| **Test system** | Chinese Hamster Ovary Cells (CHO) |
| **Metabolic activation** | Sprague-Dawley Rat liver S9 |
| **Test method** | In the SCE test without S9, CHO cells were incubated for 26 hours with methyleugenol in supplemented McCoy's 5A medium. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing methyleugenol was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with methyleugenol, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no methyleugenol. Incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level. Statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points. |
| **Criteria for assessment of results** | An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more doses resulted in a determination that the trial was positive. A statistically significant trend (P<0.005) in the absence of any responses reaching 20% above background led to a call of equivocal. |

**Results**

Methyleugenol induced SCEs in each of two replicate trials conducted with induced rat liver S9 in cultured CHO cells (B.6.8.1.1.3-07/1). No significant increase in SCEs was observed without S9.

**Table B.6.8.1.1.3-07/1. Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Methyleugenol**

| Compound | Concentration (µg/mL) | Total cells scored | Total chromosomes | Total SCEs | SCEs/Chromosome | SCEs/cell | Hrs in BrdU | Relative change of SCEs/Chromosome (treated cells/untreated) |
|---|---|---|---|---|---|---|---|---|
| **-S9**<br>**Overall call : negative** | | | | | | | | |
| Negative control (DMSO) | - | 50 | 1.044 | 392 | 0.37 | 7.8 | 26.0 | |
| Positive control | 0.001 | 50 | 1.046 | 549 | 0.52 | 11.0 | 26.0 | 39.78 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (MMC) | 0.004 | 10 | 210 | 182 | 0.86 | 18.2 | 26.0 | 130.82 |
| Methyleugenol[a] | 5 | 50 | 1.046 | 373 | 0.35 | 7.5 | 26.0 | -5.03 |
| | 17 | 50 | 1.039 | 405 | 0.38 | 8.1 | 26.0 | 3.81 |
| | 50 | 50 | 1.044 | 448 | 0.42 | 9.0 | 26.0 | 14.29 |
| | 167 | 0 | | | | | | |
| **+S9 Trial 1 Summary : Positive** | | | | | | | | |
| Negative control (DMSO) | - | 50 | 1.047 | 359 | 0.34 | 7.2 | 26.0 | |
| Positive control (Cyclophosphamide) | 0.125 | 50 | 1.048 | 675 | 0.64 | 13.5 | 26.0 | 87.84 |
| | 0.500 | 10 | 208 | 204 | 0.98 | 20.4 | 26.0 | 186.04 |
| Methyleugenol[b] | 17 | 50 | 1.048 | 469 | 0.44 | 9.4 | 26.0 | 30.52* |
| | 50 | 50 | 1.045 | 421 | 0.40 | 8.4 | 26.0 | 17.50* |
| | 167 | 50 | 1.044 | 607 | 0.58 | 12.1 | 26.0 | 69.57* |
| | 500 | 0 | | | | | | |
| **+S9 Trial 2 Summary : Positive** | | | | | | | | |
| Negative control (DMSO) | | 50 | 1.050 | 398 | 0.37 | 8.0 | 26.0 | |
| Positive control (MMC) | 0.125 | 50 | 1.046 | 588 | 0.56 | 11.8 | 26.0 | 48.31 |
| | 0.500 | 10 | 208 | 207 | 0.99 | 20.7 | 26.0 | 162.56 |
| Methyleugenol[c] | 50 | 50 | 1.047 | 430 | 0.41 | 8.6 | 26.0 | 8.35 |
| | 167 | 50 | 1.047 | 477 | 0.45 | 9.5 | 26.0 | 20.19* |
| | 250 | 50 | 1.048 | 548 | 0.52 | 11.0 | 26.0 | 37.95* |

*Positive response P ≥20% increase over solvent control
[a] Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose : P = 0.013
[b] Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose : P = 0.000
[c] Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose : P = 0.000

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (category 4, not assignable: secondary source review).

Methyleugenol was not genotoxic in the *in vitro* chromosome aberration assay but did show positive effects in the SCE assay. It is noted that the SCE assay is no longer recommended by the OECD.

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion. Methyleugenol induces SCE's in the presence of metabolic activation in CHO cells.

The study is only considered as supporting information to evaluate DNA damage (see Reliability).

### B.6.8.1.1.3- 08. Unscheduled DNA synthesis test *in vitro*

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Burkey, J.L, Sauer, J.M., McQueen, C.A. and Sipes, I.G. |
| **Report year** | 2000 |
| **Report title** | Cytotoxicity and genotoxicity of methyleugenol and related congeners – a mechanism of activation for methyleugenol |
| **Report No** | Not applicable, published study (*Mutat. Res.*, **453**, 25-33) |
| **Document No** | KCA 5.8.1/09 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 482 (1986) – deleted |
| **Deviations from current test guideline** | A comparable guideline is not available. |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |

| | |
|---|---|
| **Reliability** | Supportive only, since it is not a mutagenicity study, but rather a DNA damage study that is not required by the EU and there is no current guideline available for this test. |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol, Purity: 99%, batch no.: not stated |
| **Solvent** | Dimethylsulphoxide (DMSO) |
| **Negative control** | Solvent |
| **Positive control** | Fluorene and aminofluorene |
| **Test system** | Rat F344 (male) and B6C3F1 mouse (female) hepatocytes |
| **Test method** | Hepatocytes from F344 male rats and B6C3F1 female mice were incubated for 18-20 h with methyleugenol at a wide range of concentrations between 0 and 5000 µM. Following incubation, the medium was removed and the cell monolayer was scraped into phosphate buffer containing Triton-X 100. The medium and cell scrapings were analysed for lactate dehydrogenase (LDH) activity to assess cytotoxicity. In an unscheduled DNA synthesis (UDS) second assay, isolated hepatocytes were incubated for 18-20 h with methyleugenol at concentrations between 0.1 and 1000 µM. The hepatocytes were simultaneously exposed to [$^3$H] thymidine. Following incubation, cells were treated with sodium citrate to swell the nuclei of the cells and fixed before examination. The number of silver grains per nucleus and three neighbouring areas of cytoplasm in the same cell were counted. Twenty cells per slide, with three slides per dose level were counted when possible. A positive control was not included.<br><br>In hepatocytes obtained from rats and mice, methyleugenol showed a relatively flat cytotoxicity curve. In rat and mouse hepatocytes, methyleugenol caused UDS at concentrations between 5 (10 for mice) and 500 mM with peak UDS at 10 mM. |

**Results**

Methyleugenol is minimally cytotoxic to isolated rat and mouse hepatocytes. Methyleugenol caused UDS in rat and mice hepatocytes (Figure B.6.8.1.1.3-08/1).

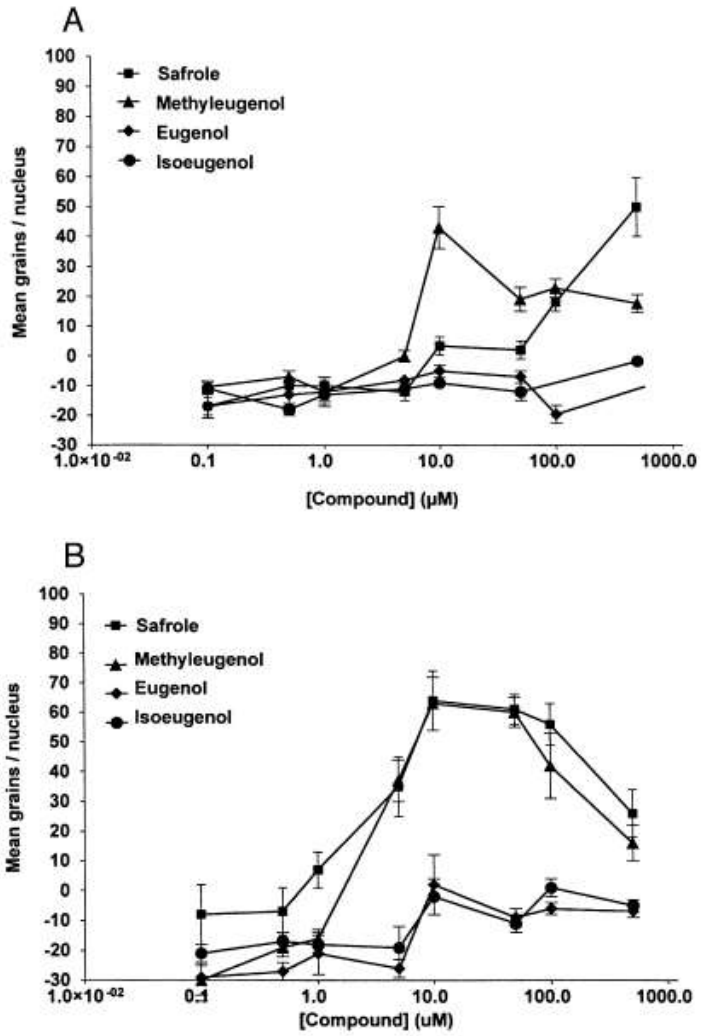



**Figure B.6.8.1.1.3-08/1. UDS in isolated male rat (A) and female mouse (B) hepatocytes following incubation with methyleugenol for 18h.**

| |
|---|
| **Assessment and conclusion by applicant:**<br>This new study is considered to be acceptable as supporting information (not reliable); this test method is no longer accepted for regulatory use however the result (positive) is consistent with the self-classification of methyleugenol as Muta.2. |
| **Assessment and conclusion by RMS:**<br>The RMS agrees with the notifier's assessment and conclusion. The study is only considered as supporting information to evaluate DNA damage (see Reliability). |

**.B.6.8.1.1.3-09. *In vivo* Micronucleus test**

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |

| | |
|---|---|
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 474 (1997) |
| **Deviations from current test guideline OECD TG 474 (2016)** | -No positive control used in the study<br>-No historical control data provided |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | Yes |
| **Reliability** | Study acceptable |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 8334801, Purity: 99% |
| **Solvent** | 0.5 % methylcellulose |
| **Negative control** | Deionised water |
| **Test system** | Male and female B6C3F1 mice |
| **Test method** | At the end of the 14-week toxicity study (ref: KCA 5.8.1/03a), peripheral blood samples were obtained from male and female mice.<br>Groups of 10 male and 10 female mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 10, 30, 100, 300 and 1000 mg/kg bw/day, 5 days per week for 14 weeks; the control group (10 male and 10 female mice) received deionised water only. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with a chromatin-specific fluorescent dye mixture of Hoechst 33258/pyronin Y and coded. Slides were scanned to determine the frequency of micronuclei in 10000 normochromatic erythrocytes (NCEs) in each of ten animals per dose group. The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analysed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group. |
| **Criteria for assessment of results** | In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dose group is less than or equal to 0.025 divided by the number of dose groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects. The percentage of PCEs among total erythrocytes was determined by an analysis of variance on ranks (classed by sex), and individual dosed groups were compared with the concurrent solvent control with a t-test on ranks |

**Results**

Methyleugenol, administered in doses of 10 to 1000 mg/kg by gavage to male and female B6C3F1 mice for 14 weeks, did not increase the frequency of micronucleated NCEs in peripheral blood and did not alter the percentage of PCEs among total erythrocytes (an indication of bone marrow toxicity) (Table B.6.8.1.1.3-09/1).

**Table B.6.8.1.1.3-09/1. Frequency of micronuclei in peripheral blood erythrocytes of mice following gavage administration of methyleugenol for 14 weeks**

| Compound | Dose (mg/kg) | Number of mice with erythrocytes scored | Micronucleated NCEs/1000 NCEs | PECs (%) |
|---|---|---|---|---|
| **Male** | | | | |
| Methyleugenol | 0 | 10 | 0.81 ± 0.10 | 1.40 ± 0.05 |
| | 10 | 10 | 0.70 ± 0.10 | 1.02 ± 0.06 |
| | 30 | 10 | 0.78 ± 0.10 | 1.51 ± 0.08 |
| | 100 | 10 | 0.84 ± 0.05 | 1.61 ± 0.11 |
| | 300 | 10 | 0.63 ± 0.06 | 1.18 ± 0.12 |
| | 1000 | 10 | 0.65 ± 0.02 | 1.16 ± 0.11 |

| | | | | |
|---|---|---|---|---|
| P = 0.915[a] | | | | |
| **Female** | | | | |
| Methyleugenol | 0 | 10 | 0.46 ± 0.09 | 1.27 ± 0.15 |
| | 10 | 10 | 0.45 ± 0.06 | 1.15 ± 0.13 |
| | 30 | 10 | 0.43 ± 0.06 | 1.38 ± 0.13 |
| | 100 | 10 | 0.54 ± 0.08 | 1.41 ± 0.09 |
| | 300 | 10 | 0.45 ± 0.08 | 1.37 ± 0.10 |
| | 1000 | 10 | 0.63 ± 0.08 | 1.21 ± 0.13 |
| P = 0.027 | | | | |

NCE : Normochromatic erythrocyte ; PCE : polychromatic erythrocyte
Results are expressed as mean ± standard error
[a] Significance of micronucleated NCEs/1000 NCEs tested by the one-tailed trend test ; significant at $P \leq 0.025$

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (category 4, not assignable: secondary source review).

Methyl eugenol was not genotoxic *in vivo*.

**Assessment and conclusion by RMS:**

The RMS agrees with the notifier's assessment and conclusion. Methyleugenol did not induce micronuclei in peripheral blood-derived erythrocytes in any dose groups. Furthermore, exposure to bone marrow is demonstrated by a statistically-significant reduction in body weight gain in both male and female mice at 300 mg/kg bw dose group and also mortality observed at 1000 mg/kg bw dose groups where all animals except one male mouse died before the end of the study.

The study was checked for compliance against the current OECD TG 474 (2016) and deviations include no positive control and no historical data provided. Despite these deviations, the RMS is of the opinion that they do not affect the outcome of the study and therefore, the study is deemed relevant to evaluate the *in vivo* cytogenicity of methyleugenol.

### B.6.8.1.1.3-10. *In vivo* Alkaline Elution Assay (Comet)

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Ding, W., Levy, D.D., Bishop, M.E., Lyn-Cook Lascelles, E., Kulkarni, R., Chang, C.W., Aidoo, A. and Manjanatha, M.G. |
| **Report year** | 2011 |
| **Report title** | Methyleugenol genotoxicity in the Fischer 344 rat using the Comet assay and pathway focused gene expression profiling |
| **Report No** | Not applicable, published study (*Toxicol. Sci.*, **123**, 103-112) |
| **Document No** | KCA 5.8.1/11 |
| **Guidelines followed in study** | Not stated, comparable to OECD TG 489 (2016) |
| **Deviations from current test guideline OECD TG 489 (2016)** | -Test item not characterised<br>-Number of animals was lower than recommended by guideline (3 instead of 5 in the main experiment and 4 instead of 5 in the time-course experiment)<br>-Number of total cells per organ per animal is less than 150 recommended by guidance<br>-Limited reporting of results<br>-No historical control data are provided |
| **Previous evaluation** | No, not previously submitted |
| **GLP/Officially recognised testing facilities** | No |
| **Reliability** | Supporting information (see deviations from guideline) |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol, Purity: not stated; batch no.: not stated |
| **Vehicle** | 0.5 % aqueous methylcellulose |
| **Negative control** | 0.5 % aqueous methylcellulose |
| **Positive control** | Methyl methanesulphonate (MMS) |
| **Test system** | Male Fischer 344 rats (liver, lung, bone marrow, bladder and kidney) |
| **Route** | Oral (gavage) |
| **Test method** | Groups of three rats received single doses of 400 or 1000 mg/kg bw of methyleugenol in 0.5% methylcellulose by gavage. The choice of these doses was based on previous studies that evaluated the genotoxicity of estragole, a derivative of methyleugenol, and the limit dose of 2000 mg/kg bw recommended by the OECD. Additional rats were treated by gavage with single doses of 100 mg/kg bw MMS or a 0.5% aqueous solution of methylcellulose to serve as positive and vehicle controls, respectively. Rats were euthanized 3 and 24 h after the treatment. In a second experiment, groups of four rats were administered single doses of 2000 mg/kg bw and sacrificed at 1, 3, 6 and 8h following administration.<br><br>Upon sacrifice, the organs/tissues (liver, kidney, bladder, lung, and bone marrow) were aseptically removed and stored in cold tissue dissociation buffer (Hanks Balanced Salt Solution, 20mM EDTA, and 10% dimethyl sulphoxide) for isolation of single cells. It should be noted that stomach, fore stomach, mammary gland, and subcutaneous skin are among the target organs for methyleugenol carcinogenicity; however, they were excluded from the analysis because in preliminary studies, cells obtained from these tissues often resulted in hedgehog or ghost cells.<br><br>Cell suspensions were mixed with 1% low-melting-point agarose and applied to microscope slides pre-coated with agarose before storage at 4°C for 30 min to solidify the agarose. With the cover slips gently removed, the slides were placed in freshly prepared lysis buffer (2.5M NaCl, 0.1M EDTA, 10mM Tris, with 10% dimethyl sulfoxide and 1% Triton X-100 added just before use) and stored at 4°C overnight. The slides then were transferred into a chilled alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13) and allowed to remain in the solution for 40 min in the dark to unwind DNA. After unwinding, electrophoresis was performed in the same solution at 4°C in the dark for 30 min at 25 V and ~300 mA. The slides then were washed with neutralizing buffer (0.4M Tris, adjusted to pH 7.5 with HCl) 3x for 5 min each to neutralize the remaining alkali and remove detergent; the slides next were fixed with icecold ethanol (100%) and dried overnight. Prior to scoring, the slides were stained with SYBR Gold. Two slides were scored from each tissue/treatment/ sampling time; 100 cells were selected randomly from each slide and scored using a system consisting of a Nikon 501 fluorescent microscope and Comet IV digital imaging software.<br>Percent DNA (% DNA) in tail, defined as the fraction of DNA in the tail divided by the total amount of DNA associated with a cell multiplied by 100, was used as the parameter for DNA damage analysis. |
| **Criteria for assessment of results** | DNA damage measured as % DNA in tail was analysed as a function of dose or time by one-way ANOVA followed by the Holm-Sidak test to evaluate the differences in DNA damage among groups. Because the standard deviations of the DNA damage tended to increase with the magnitude of the response, a logarithmic transformation was performed before conducting the analyses. A P-value of less than 0.05 was considered statistically significant. |

**Results**

Cell viability, as determined by the Trypan Blue dye exclusion test, was >75%.

Rats exposed to 400 or 1000 mg/kg bw had no statistically significant differences in DNA damage compared to vehicle controls (Figure B.6.8.1.1.3-10/1). The DNA damage caused by methyleugenol exposure was either equal to or slightly but not significantly higher than control values, and the responses were not affected appreciably by

the sampling times. By contrast, MMS treatment produced significant increases ($p \leq 0.05$) in DNA damage compared with the vehicle control in all tissues examined, namely liver, lung, kidney, bladder, and bone marrow, with the greatest levels of DNA damage noted in bladder and kidney (Figure B.6.8.1.1.3-10/1). In general, the responses were higher at 3 h after treatment than at 24 h, suggesting that there was a time-dependent reduction in DNA damage.

In the second experiment, treatment with 2000 mg/kg bw methyleugenol the DNA damage was similar to the control at all time points for all tissues examined except for the bone marrow at 8h sampling time that there was a slight but statistically-significant ($p < 0.05$) increase in DNA damage (Figure B.6.8.1.1.3-10/2). Due to the high turnover rate of bone marrow cells, the authors interpret this increase as incidental rather than a real increase in DNA damage.

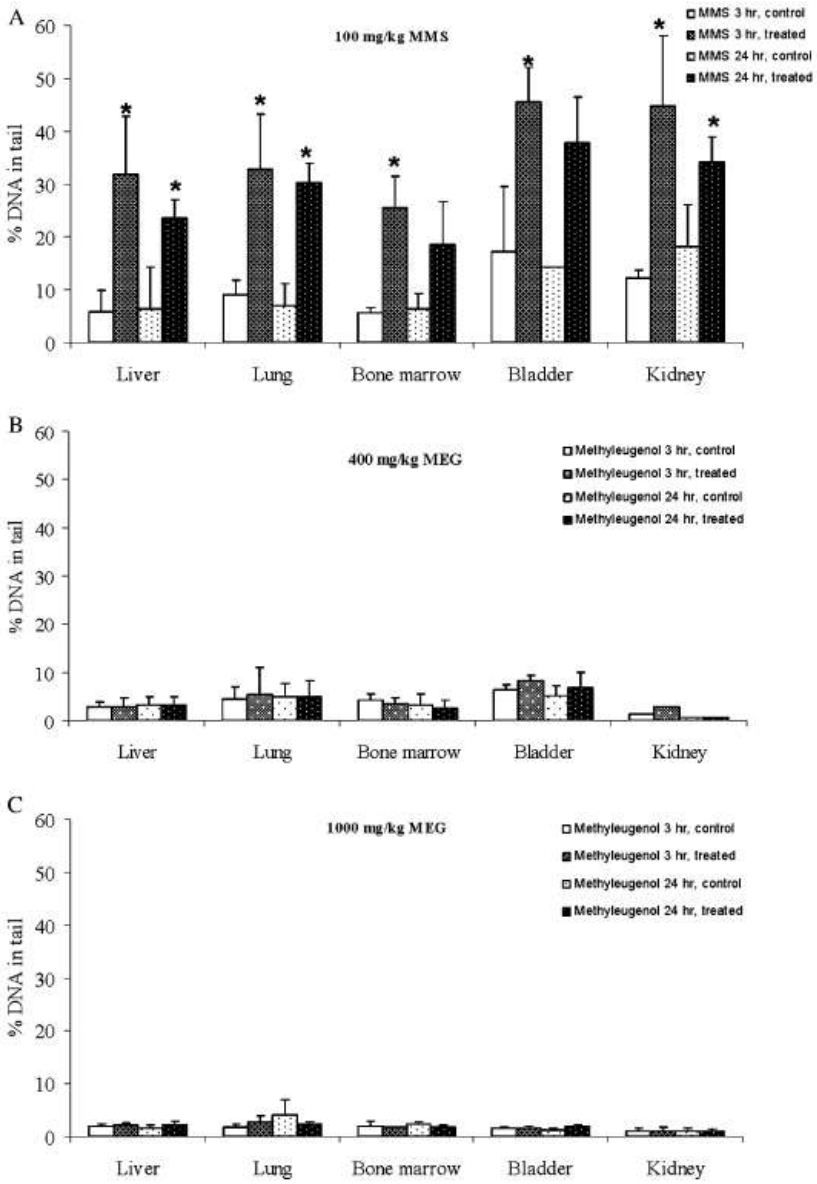



**Figure B.6.8.1.1.3-10/1. Dose-response % DNA in tail following treatment with MMS (A), 400 mg/kg MEG (B) and 1000 mg/kg MEG (C)**

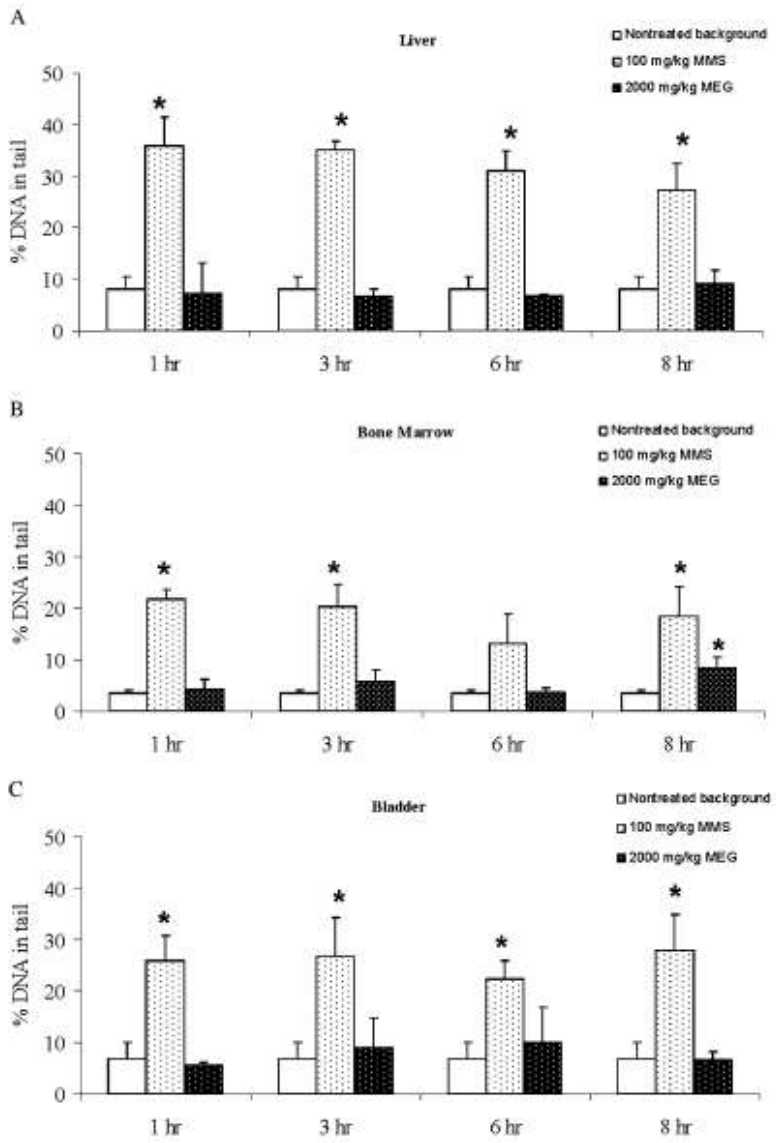



**Figure B.6.8.1.1.3-10/2. Dose-response % DNA in tail following treatment with 2000 mg/kg bw MEG at different time points (1h, 3h, 6h and 8h sampling times)**

| |
|---|
| **<u>Assessment and conclusion by applicant</u>:**<br>This new study is considered to be acceptable (reliable with restrictions).<br>Methyleugenol did not induce DNA damage in rat liver in the *in vivo* alkaline Comet assay but did cause DNA damage in a modified (non-standard) assay. |
| **<u>Assessment and conclusion by RMS</u>:**<br>The RMS agrees with the notifier's assessment and conclusion. Methyleugenol did not induce DNA damage in rat liver, lung, bladder and kidney. The RMS, however, deems the result in the bone marrow at 8h as positive. The study is a scientific publication for a peer-reviewed journal. No guideline was stated in the study and it was compared against the current OECD TG 489. Deviations from the current OECD guidance include no characterisation of the test item, less animals per treatment group than recommended by guidance, limited level of reporting, less than 150 cells scored as recommended by guidance and no historical data provided. For these reasons, the RMS deems this study as supporting information only. |

### B.6.8.1.1.4. Long-term/chronic toxicity

#### B.6.8.1.1.4-01. Two-year toxicity study in rats with methyleugenol

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 453 (2018). |
| **Deviations from current test guideline OECD TG 453 (2018)** | -Food consumption was not measured.<br>-Ophthalmological examination was not performed.<br>-Organ weights were not presented.<br>-Circulating thyroid hormones (T4, T3, TSH) levels were not measured.<br>-No haematology, urinalysis and clinical chemistry were performed.<br>-Statistical analysis not performed in bodyweight measurements and in some non-neoplastic incidences. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 9224705, Purity: 99% |
| **Test system** | F344/N rats |
| **Test method** | Groups of 50 male and 50 female F344/N rats received methyleugenol (MEG) in 0.5% methylcellulose by gavage at doses of 37, 75 and 150 mg/kg bw/day, 5 days per week for 105 weeks. Groups of 60 rats/sex received 0.5% methylcellulose vehicle only as negative control. Stop-exposure groups of 60 male and 60 female rats received 300 mg/kg bw/day methyleugenol in 0.5% methylcellulose by gavage for 52 weeks followed by the 0.5% methylcellulose vehicle only for the remaining 53 weeks of the study.<br><br>From the 60 rat/sex control and 300 mg/kg bw/days groups, five male and female vehicle control rats and five male and five female rats receiving 300 mg/kg were euthanized at 6 and 12 month for histologic evaluation.<br><br>Dosing volumes were 5 mL/kg bw. Feed and water were available *ad libitum*. Rats were housed three (males) or five (females) per cage. Cages and racks were rotated every 2 weeks. Clinical findings were recorded and the animals were weighed weekly and at the end of the studies.<br><br>*Necropsy*<br><br>Necropsy was performed on all animals.<br><br>*Histopathology*<br><br>Complete histopathology was performed on all rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, oesophagus, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach |

| | |
|---|---|
| | (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. |
| **Statistical analysis of results** | *Survival*<br>The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.<br><br>*Analysis of Neoplasm and Non-neoplastic Lesion Incidences*<br>The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and non-neoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.<br><br>This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).<br><br>Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of lesion incidence, and reported P values are one sided. Values of P greater than 0.5 are presented as 1-P with the letter N added to indicate a lower incidence or negative trend in neoplasm occurrence relative to the control group (e.g., P=0.99 is presented as P=0.01N). For neoplasms and non-neoplastic lesions detected at the interim evaluation, the Fisher exact test, a procedure based on the overall proportion of affected animals, was used. |
| **Historical control data** | Neoplasm incidences from Battelle Columbus Laboratories (data as November 2017, range not specified), that conducted the 2-year studies, and from NTP carcinogenesis program from 1990-1997 (Haseman *et al.*, *Toxicol Pathol.*, 1998, 26(3):428-41) period are exposed below: |

| | **Historical Incidence at Battelle Columbus Laboratories** | | | **Historical Incidence at NTP 1990-1997 (Haseman *et al.*)** | | |
|---|---|---|---|---|---|---|
| | **Total** | **Mean ± SD** | **Range** | **Total** | **Rate** | **Range** |
| **Liver** | | | | | | |
| **Males** | | | | | | |
| Adenomas | 12/400 | 3.0 ± 3.0 | 0-8% | 31/1352 | 2.3 | 0-10% |
| Carcinomas | 4/400 | 1.0 ± 1.8 | 0-4% | 9/1352 | 0.7 | 0-6% |
| Adenoma or carcinoma | 16/400 | 4.0 ± 3.5 | 0-10% | 38/1352 | 2.8 | 0-10% |

| | | | | | | |
|---|---|---|---|---|---|---|
| Hepatocholangiocarcinoma | n.a. | n.a. | n.a. | 0 | 0 | 0 |
| **Females** | | | | | | |
| Adenomas | 1/401 | 0.3 ± 0.7 | 0-2% | 8/1351 | 0.6 | 0-6% |
| Carcinomas | 0/401 | 0 | 0 | 1/1351 | 0.1 | 0-2% |
| Adenoma or carcinoma | 1/401 | 0.3 ± 0.7 | 0-2% | 9/1351 | 0.7 | 0-6% |
| Hepatocholangiocarcinoma | n.a. | n.a. | n.a. | 1/1351 | 0.1 | 0-2% |
| **Forestomach** | | | | | | |
| **Males** | | | | | | |
| Squamous cell carcinoma | n.a. | n.a. | n.a. | 0/1354 | 0 | 0 |
| Squamous cell papilloma | n.a. | n.a. | n.a. | 2/1354 | 0.1 | 0-2% |
| Squamous cell carcinoma or papilloma | n.a. | n.a. | n.a. | 2/1354 | 0.1 | 0-2% |
| **Females** | | | | | | |
| Squamous cell carcinoma | 0/401 | 0 | 0 | 0/1351 | 0 | 0 |
| Squamous cell papilloma | 2/401 | 0.5 ± 0.9 | 0-2% | 2/1354 | 0.1 | 0-2% |
| Squamous cell carcinoma or papilloma | 2/401 | 0.5 ± 0.9 | 0-2% | 2/1354 | 0.1 | 0-2% |
| **Kidney** | | | | | | |
| **Males** | | | | | | |
| Renal tubule adenoma | 3/400 | 0.8 ± 1.0 | 0-2% | 10/1352 | 0.7 | 0-6% |
| Renal tubule carcinoma | 2/400 | 0.5 ± 1.4 | 0-4% | 3/1352 | 0.2 | 0-2% |
| Adenoma or carcinoma | 5/400 | 1.3 ± 1.5 | 0-4% | n.a. | n.a. | n.a. |
| **Females** | | | | | | |
| Renal tubule adenoma | n.a. | n.a. | n.a. | 0/1348 | 0 | 0 |
| Renal tubule carcinoma | n.a. | n.a. | n.a. | 1/1348 | 0.1 | 0-2% |
| Adenoma or carcinoma | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| **Skin** | | | | | | |
| **Males** | | | | | | |
| Fibroma | 17/402 | 4.3 ± 3.6 | 0-12% | 69/1354 | 5.1 | 0-12% |
| Fibrosarcoma | 3/402 | 0.8 ± 1.0 | 0-2% | 17/1354 | 1.3 | 0-4% |
| Fibroma or fibrosarcoma | 20/402 | 5.0 ± 3.4 | 0-12% | n.a. | n.a. | n.a. |
| **Females** | | | | | | |
| Renal tubule adenoma | n.a. | n.a. | n.a. | 22/1351 | 1.6 | 0-4% |
| Renal tubule carcinoma | n.a. | n.a. | n.a. | 8/1351 | 0.6 | 0-4% |
| Adenoma or carcinoma | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| **Malignant mesothelioma** | | | | | | |
| **Males** | | | | | | |
| Malignant mesothelioma | 7/402 | 1.7 ± 2.2 | 0-6% | n.a. | n.a. | n.a. |
| **Mammary gland fibroadenoma** | | | | | | |
| **Males** | | | | | | |
| fibroadenoma | n.a. | n.a. | n.a. | 58/1354 | 4.3 | 0-12% |
| **Females** | | | | | | |
| fibroadenoma | n.a. | n.a. | n.a. | 556/1351 | 41.2 | 8-60% |

**Results**

**Survival:** All 150 and 300 mg/kg bw/day male rats died before study termination, the presence of moribund animals and the number of natural deaths was higher than controls. On the other hand, the survival rates (animals surviving at study termination and mean survival days) of the 37 and 75 mg/kg bw/day male dose groups were slightly lower than controls.

Survival at study termination on female 150 and 300 mg/kg bw/day dose groups was slightly lower than controls (22% and 27%, respectively, vs 37% in controls), and no statistical significance was displayed. It is only remarkable that the number of moribund animals was increased in these top dose groups. On the other hand, 37 and 75 mg/kg bw/day dose groups showed survival rates (animals surviving at study termination and mean survival

days) slightly higher than controls. The other parameters were similar between study groups (see table B.6.8.1.1.4-01/1).

**Table B.6.8.1.1.4-01/1. Survival of rats in the 2-year gavage study of methyleugenol**

| | Dose (mg/kg bw/day) | | | | |
|---|---|---|---|---|---|
| | **Control (vehicle)** | **37** | **75** | **150** | **300 (stop exposure)** |
| **Males** | | | | | |
| **Animals initially in study** | **60** | **50** | **50** | **50** | **60** |
| **6-Month interim evaluation (a)** | 5 | 0 | 0 | 0 | 5 |
| **12-Month interim evaluation (a)** | 5 | 0 | 0 | 0 | 5 |
| **Accidental deaths (a)** | 0 | 1 | 3 | 1 | 1 |
| **Moribund** | 15 | 18 | 15 | 20 | 23 |
| **Natural deaths** | 15 | 15 | 17 | 29 | 26 |
| **Animals surviving to study termination** | **20 (33%)** | **16 (32%)** | **15 (30%)** | **0** | **0** |
| **Percent probability of survival at end of study (b)** | 40 | 33 | 32 | 0 | 0 |
| **Mean survival (days) (c)** | 678 | 659 | 646 | 615 | 538 |
| **Survival analysis (d)** | **P<0.001** | **P=0.389** | **P=0.294** | **P<0.001** | **P<0.001** |
| **Females** | | | | | |
| **Animals initially in study** | **60** | **50** | **50** | **50** | **60** |
| **6-Month interim evaluation (a)** | 5 | 0 | 0 | 0 | 5 |
| **12-Month interim evaluation (a)** | 5 | 0 | 0 | 0 | 5 |
| **Accidental deaths (a)** | 0 | 0 | 1 | 1 | 0 |
| **Moribund** | 17 | 16 | 14 | 26 | 25 |
| **Natural deaths** | 11 | 9 | 13 | 12 | 9 |
| **Animals surviving to study termination** | **22 (37%)** | **25 (50%)** | **22 (44%)** | **11 (22%)** | **16 (27%)** |
| **Percent probability of survival at end of study (b)** | 44 | 50 | 45 | 23 | 32 |
| **Mean survival (days) (c)** | 659 | 672 | 675 | 647 | 638 |

| | | | | | |
|---|---|---|---|---|---|
| **Survival analysis (d)** | **P=0.015** | **P=0.640N** | **P=0.807N** | **P=0.053** | **P=0.343** |

*(a) Censored from survival analyses.*
*(b) Kaplan-Meier determinations.*
*(c) Mean of all deaths (uncensored, censored, and terminal sacrifice).*
*(d) The result of the life table trend test (Tarone, 1975) is in the vehicle control column [the 300 mg/kg (stop-exposure) group was excluded from the trend test], and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower mortality in a dose group is indicated by **N**.*

**Clinical signs:** Only moribund clinical signs were recorded and attributed to test substance administration.

**Bodyweight:** Mean bodyweights of all dosed males and females were less than those of the vehicle controls throughout most of the 2-year study. There were no signs of recovery in mean bodyweights in the stop-exposure 300 mg/kg bw/day group in the remaining 53 weeks of the study without methyleugenol. The reductions of bodyweights were >10% from week 37 in top dose male group (11-27%) and from week 29 in top dose female group (13-26%) to study termination. In 150 mg/kg bw/day dose group, reductions of bodyweights were >10% from week 53 in males (11-23%) and from week 33 in females (11-26%) groups to the end of the study. In low dose groups, only females of 75 mg/kg bw/day groups showed a continuous reduction of bodyweight from week 45 to study termination (11-21%) (see Table B.6.8.1.1.4-01/2-3).

**Table B.6.8.1.1.4-01/2. Mean bodyweights of male rats in the 2-year gavage study of methyleugenol**

| | **Dose (mg/kg bw/day)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Control (vehicle)** | **37** | | **75** | | **150** | | **300** | |
| **week** | **Mean bw** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** |
| **1** | 127 | 126 | 100 | 127 | 100 | 124 | 98 | 126 | 100 |
| **5** | 247 | 244 | 99 | 243 | 99 | 245 | 100 | 243 | 99 |
| **9** | 304 | 300 | 99 | 303 | 100 | 298 | 98 | 297 | 98 |
| **13** | 341 | 342 | 100 | 339 | 99 | 332 | 97 | 325 | 95 |
| **17** | 368 | 366 | 99 | 361 | 98 | 355 | 96 | 341 | 93 |
| **21** | 386 | 384 | 99 | 379 | 98 | 373 | 97 | 355 | 92 |
| **25** | 396 | 394 | 100 | 387 | 98 | 375 | 95 | 360 | 91 |
| **29** | 415 | 410 | 99 | 398 | 96 | 385 | 93 | 371 | 90 |
| **33** | 414 | 412 | 99 | 402 | 97 | 389 | 94 | 373 | 90 |
| **37** | 426 | 416 | 98 | 403 | 95 | 394 | 92 | 379 | **89 (↓11%)** |
| **41** | 427 | 418 | 98 | 407 | 95 | 397 | 93 | 376 | **88 (↓12%)** |
| **45** | 441 | 425 | 96 | 416 | 94 | 401 | 91 | 384 | **87 (↓13%)** |
| **49** | 449 | 430 | 96 | 416 | 93 | 409 | 91 | 387 | **86 (↓14%)** |
| **53** | 455 | 431 | 95 | 416 | 92 | 404 | **89 (↓11%)** | 389 | **86 (↓14%)** |
| **57** | 458 | 435 | 95 | 421 | 92 | 401 | **87 (↓13%)** | 392 | **86 (↓14%)** |
| **61** | 462 | 431 | 93 | 420 | 91 | 403 | **87 (↓13%)** | 394 | **85 (↓15%)** |
| **65** | 463 | 434 | 94 | 419 | 91 | 404 | **87 (↓13%)** | 393 | **85 (↓15%)** |
| **69** | 464 | 434 | 94 | 420 | 91 | 398 | **86 (↓14%)** | 384 | **83 (↓17%)** |
| **73** | 465 | 435 | 94 | 425 | 92 | 405 | **87 (↓13%)** | 386 | **83 (↓17%)** |
| **77** | 466 | 435 | 93 | 425 | 91 | 405 | **87 (↓13%)** | 376 | **81 (↓19%)** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **81** | 464 | 431 | 93 | 412 | 89 | 390 | **84 (↓16%)** | 363 | **79 (↓21%)** |
| **85** | 461 | 438 | 95 | 416 | 90 | 382 | **83 (↓17%)** | 337 | **73 (↓27%)** |
| **89** | 448 | 431 | 96 | 409 | 91 | 386 | **86 (↓14%)** | 341 | **76 (↓24%)** |
| **93** | 444 | 422 | 95 | 393 | **89 (↓11%)** | 346 | **78 (↓22%)** | | |
| **97** | 433 | 408 | 94 | 381 | **88 (↓12%)** | 331 | **77 (↓23%)** | | |
| **101** | 414 | 387 | 94 | 397 | 96 | | | | |
| **Total Mean** | 409 | 393 | 96 | 382 | 95 | 365 | 95 | 351 | 94 |
| **Mean per weeks** | | | | | | | | | |
| 1-13 | 255 | 253 | 99 | 253 | 99 | 250 | 98 | 248 | 98 |
| 14-52 | 414 | 406 | 98 | 397 | 96 | 386 | 93 | 370 | **89 (↓11%)** |
| 53-101 | 454 | 427 | 94 | 412 | 91 | 388 | **85 (↓15%)** | 376 | **82 (↓18%)** |

*a) Interim evaluations occurred during weeks 27 and 52 for the vehicle control and 300 mg/kg (stop-exposure) groups.*

**Table B.6.8.1.1.4-01/3. Mean bodyweights of female rats in the 2-year gavage study of methyleugenol**

| | Dose (mg/kg bw/day) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Control (vehicle)** | **37** | | **75** | | **150** | | **300** | |
| **week** | **Mean bw** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** |
| 1 | 108 | 106 | 98 | 107 | 99 | 107 | 99 | 107 | 99 |
| 5 | 162 | 161 | 99 | 160 | 99 | 161 | 99 | 158 | 97 |
| 9 | 186 | 185 | 100 | 183 | 99 | 183 | 98 | 178 | 96 |
| 13 | 203 | 199 | 98 | 199 | 98 | 195 | 96 | 189 | 93 |
| 17 | 211 | 207 | 98 | 206 | 98 | 197 | 93 | 192 | 91 |
| 21 | 219 | 216 | 99 | 210 | 96 | 201 | 92 | 195 | 89 |
| 25 | 226 | 221 | 98 | 214 | 94 | 206 | 91 | 203 | 90 |
| 29 | 236 | 226 | 96 | 219 | 93 | 213 | 90 | 205 | **87 (↓13%)** |
| 33 | 241 | 233 | 97 | 223 | 92 | 215 | **89 (↓11%)** | 208 | **86 (↓14%)** |
| 37 | 252 | 241 | 95 | 231 | 91 | 222 | **88 (↓12%)** | 212 | **84 (↓16%)** |
| 41 | 264 | 249 | 94 | 236 | 90 | 223 | **85 (↓15%)** | 213 | **81 (↓19%)** |
| 45 | 274 | 257 | 94 | 243 | **89 (↓11%)** | 230 | **84 (↓16%)** | 215 | **78 (↓22%)** |
| 49 | 288 | 269 | 94 | 252 | **88 (↓12%)** | 235 | **82 (↓18%)** | 219 | **76 (↓24%)** |
| 53 | 293 | 280 | 96 | 255 | **87 (↓13%)** | 240 | **82 (↓18%)** | 225 | **77 (↓23%)** |
| 57 | 303 | 282 | 93 | 262 | **87 (↓13%)** | 246 | **81 (↓19%)** | 230 | **76 (↓24%)** |
| 61 | 312 | 293 | 94 | 269 | **86 (↓14%)** | 247 | **79 (↓21%)** | 239 | **77 (↓23%)** |
| 65 | 321 | 300 | 94 | 273 | **85 (↓15%)** | 251 | **78 (↓22%)** | 243 | **76 (↓24%)** |
| 69 | 326 | 304 | 93 | 276 | **85 (↓15%)** | 251 | **77 (↓23%)** | 254 | **78 (↓22%)** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 73 | 334 | 307 | 92 | 274 | **82 (↓18%)** | 255 | **77 (↓23%)** | 255 | **76 (↓24%)** |
| 77 | 336 | 314 | 93 | 277 | **83 (↓17%)** | 256 | **76 (↓24%)** | 257 | **77 (↓23%)** |
| 81 | 337 | 315 | 94 | 279 | **83 (↓17%)** | 262 | **78 (↓22%)** | 259 | **77 (↓23%)** |
| 85 | 346 | 320 | 92 | 284 | **82 (↓18%)** | 269 | **78 (↓22%)** | 265 | **77 (↓23%)** |
| 89 | 348 | 317 | 91 | 280 | **81 (↓19%)** | 266 | **76 (↓24%)** | 263 | **76 (↓24%)** |
| 93 | 350 | 316 | 91 | 279 | **80 (↓11%)** | 259 | **74 (↓26%)** | 259 | **74 (↓26%)** |
| 97 | 348 | 314 | 90 | 274 | **79 (↓21%)** | 257 | **74 (↓26%)** | 263 | **76 (↓24%)** |
| 101 | 344 | 316 | 92 | 277 | **80 (↓20%)** | 257 | **75 (↓25%)** | 256 | **74 (↓26%)** |
| **Total Mean** | 276 | 260 | 95 | 240 | 95 | 227 | 95 | 222 | 94 |
| **Mean per weeks** | | | | | | | | | |
| 1-13 | 165 | 163 | 99 | 162 | 99 | 162 | 98 | 158 | 96 |
| 14-52 | 246 | 235 | 96 | 226 | 92 | 216 | **88 (↓12%)** | 207 | **85 (↓15%)** |
| 53-101 | 331 | 306 | 93 | 274 | **83 (↓17%)** | 255 | **77 (↓23%)** | 251 | **76 (↓24%)** |

*a)Interim evaluations occurred during weeks 27 and 52 for the vehicle control and 300 mg/kg (stop-exposure) groups.*

**Necropsy:**

Gross lesions were observed in the liver and stomach of males and females. Focal areas of discoloration, nodules (raised lesions less than 5 mm in diameter), and masses (raised lesions greater than 5 mm in diameter) were observed in the livers of dosed rats. The incidences and multiplicity of focal areas of discoloration, nodules, and masses increased with increasing dose. Grossly, there was diffuse thickening of the entire glandular portion of the stomach with or without the presence of one or more nodules and masses.

**Histopathology:**

**Neoplastic effects**

Summary of main neoplastic effects is exposed in tables B.6.8.1.1.4-01/4-5.

*Liver*

Hepatocellular adenomas were increased in all dose-groups for both sexes compared with controls. At 12-month interim examination, 80% of males developed hepatocellular adenomas.

Incidences of hepatocellular carcinomas were increased compared with controls in 75, 150 and 300 mg/kg bw/day male dose groups (28%, 50% and 72%, respectively), and in 150 and 300 mg/kg bw/day female dose groups (16% and 44%, respectively). The incidences of hepatocellular adenomas and carcinomas were dose related and exceed the overall mean and the range of HCD from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 (Haseman *et al.*, *Toxicol Pathol.*, 1998, 26(3):428-41).

The incidences of hepatocholangioma and hepatocholangiocarcinoma were increased only in top dose male and female dose groups (12% and 14% for males, and 16% and 18% for females, respectively). The incidences of cholangiocarcinomas were higher than mean and out of range of NTP Carcinogenicity studies (1990-1997).

*Stomach*

Occurrence of malignant neuroendocrine tumours was statistically significant in 75 and higher female dose groups (24%, 52% and 72% for 75, 150 and 300 mg/kg bw/day, respectively) compared with controls (0%). The incidence of this type of tumour was present only in 150 mg/kg bw/day male dose groups (8%). Benign neuroendocrine tumours were also found in 75 and higher female dose group, without showing a dose-relation pattern (26%, 18%

and 10% for 75, 150 and 300 mg/kg bw/d, respectively). Incidences of benign or malignant neuroendocrine tumors have not been described in the HCD from Battelle Columbus Laboratories or in the Haseman *et al.* review (NTP 1990-1997 period update, 2-year studies).

In core study female rats, one female of the 150 and 300 mg/kg bw/day dose groups (2%) developed squamous cell carcinoma in the forestomach. This observation was not found in any of the HCD reported by Battelle laboratories and NTP (1990-1997). In addition, one female of the 75 (2%) and two females of the 150 (4%) mg/kg bw/day dose groups developed squamous cell papilloma. Although any of these findings were statistically significantly greater than controls, the occurrence of squamous cell papillomas in the 150 mg/kg bw/day female group exceed the mean and range of both HCD.

*Kidney*

Increased incidences of renal tubule adenoma were described in 75, 150 and 300 mg/kg bw/day male dose groups (34%, 26% and 40%, respectively) compared with controls (8%). The incidences in all groups exceeded the historical control range from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997. On the other hand, no renal tubule adenomas or carcinomas were described in females.

*Skin*

The incidences of fibroma in 37 and 75 mg/kg bw/day males dose groups (18% and 16%), and combined incidences of fibroma or fibrosarcoma in 37, 75, and 150 mg/kg males (24%, 16% and 16%) were significantly increased compared with controls; however, the incidences did not increase with increasing dose. The incidences of these lesions in these groups exceeded the HCD mean and range from Battelle Columbus Laboratories and NTP Carcinogenicity studies (1990-1997). Fibromas were well-demarcated, solid, expansive masses composed of well-differentiated fibrous connective tissue. Fibrosarcomas were expansive, locally invasive masses composed of anaplastic spindle cells.

*Malignant mesothelioma*

Statistically significant incidences of malignant mesothelioma were described in 150 and 300 mg/kg bw/day male dose groups (24% and 10%, respectively), compared with controls (2%). The incidence was higher and out of the range of HCD from Battelle Columbus Laboratories.

*Mammary gland*

Statistically significant incidences of mammary gland fibroadenomas were described in 75 and 150 mg/kg bw/day male dose groups (30% and 26%, respectively), compared with controls (10%). The incidence was higher and out of the range of HCD from the NTP Carcinogenicity (1990-1997). However, no increased occurrence of mammary gland fibroadenomas was recorded in top dose male group and in any of female dose groups.

**Table B.6.8.1.1.4-01/4. Summary of neoplastic findings in males in 2-year rats study**

| | Doses of methyleugenol (mg/kg bw/d) | | | | |
|---|---|---|---|---|---|
| | **0** | **37** | **75** | **150** | **300** |
| **Liver** | | | | | |
| **Neoplastic effects** | | | | | |
| **12-month interim evaluation** | | | | | |
| **Hepatocellular adenoma** | **0** | | | | **4* (80%)** |
| **2-year study** | | | | | |
| **Hepatocellular adenoma** | 5/50 (10%) | **12/50* (24%)** | **23/50** (46%)** | **38/50**(76%)** | **32/50** (64%)** |
| **Hepatocellular carcinoma** | 2/50 (4%) | 3/50 (6%) | **14/50** (28%)** | **25/50**(50%)** | **36/50** (72%)** |
| **Hepatocholangioma** | 0/50 | 0/50 | 0/50 | 1/50 (2%) | **6/50** (12%)** |
| **Hepatocholangiocarcinoma** | 0/50 | 0/50 | 1/50 (2%) | 1/50 (2%) | **7/50** (14%)** |
| **Glandular stomach** | | | | | |
| **Neoplastic effects** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **2-year study** | | | | | |
| **Benign neuroendocrine tumour** | 0/50 | 0/50 | 0/50 | 3/50 (6%) | 2/50 (4%) |
| **Malignant neuroendocrine tumour** | 0/50 | 0/50 | 0/50 | **4/50* (8%)** | 2/50 (4%) |
| **Kidney** | | | | | |
| **Neoplastic effects** | | | | | |
| **Renal tubule adenoma** | 4/50 (8%) | 6/50 (12%) | **17/50** (34%)** | **13/50** (26%)** | **20/50** (40%)** |
| **Skin** | | | | | |
| **Neoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **subcutaneous fibroma** | 1/50 (2%) | **9/50** (18%)** | **8/50* (16%)** | 5/50 (10%) | 4/50 (8%) |
| **subcutaneous fibroma or fibrosarcoma** | 1/50 (2%) | **12/50** (24%)** | **8/50* (16%)** | **8/50** (16%)** | 4/50 (8%) |
| **Other tissues** | | | | | |
| **Neoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **Malignant mesothelioma** | 1/50 (2%) | 3/50 (6%) | 5/50 (10%) | **12/50** (24%)** | **5/50* (10%)** |
| **Mammary gland fibroadenoma** | 5/50 (10%) | 5/50 (10%) | **15/50** (30%)** | **13/50** (26%)** | 6/50 (12%) |

** Significantly different (P≤0.05) from the vehicle control group by Fisher exact test (interim evaluations), or the Poly-3 test (2-year study). ** P≤0.01*

**Table B.6.8.1.1.4-01/5. Summary of neoplastic findings in females in 2-year rats study**

| | Doses of methyleugenol (mg/kg bw/d) | | | | |
|---|---|---|---|---|---|
| | **0** | **37** | **75** | **150** | **300** |
| **Liver** | | | | | |
| **Neoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **Hepatocellular adenoma** | 1/50 (2%) | **8/50** (16%)** | **11/49** (22%)** | **33/49** (67%)** | **43/50** (86%)** |
| **Hepatocellular carcinoma** | 0/50 | 0/50 | 4/49 (8%) | **8/49**(16%)** | **22/50** (44%)** |
| **Hepatocholangioma** | 0/50 | 0/50 | 0/49 | 0/49 | **8/50** (16%)** |
| **Hepatocholangiocarcinoma** | 0/50 | 0/50 | 0/49 | 3/49 (6%) | **9/50** (18%)** |
| **Stomach** | | | | | |
| **Neoplastic effects** | | | | | |
| **glandular stomach** | | | | | |
| **2-year study** | | | | | |
| **Benign neuroendocrine tumour** | 0/50 | 0/50 | **13/50** (26%)** | **9/50** (18%)** | **5/50* (10%)** |
| **Malignant neuroendocrine tumour** | 0/50 | 1/50 (2%) | **12/50** (24%)** | **26/50** (52%)** | **36/50** (72%)** |
| **forestomach** | | | | | |
| **2-year study** | | | | | |
| **squamous cell carcinoma** | 0/50 | 0/49 | 0/50 | 1/50 (2%) | 1/50 (2%) |
| **squamous cell papilloma** | 0/50 | 0/49 | 1/50 (2%) | 2/50 (4%) | 0/50 |
| **combined squamous cell carcinoma or papilloma** | 0/50 | 0/49 | 1/50 (2%) | 3/50 (6%) | 1/50 (2%) |

** Significantly different (P≤0.05) from the vehicle control group by Poly-3 test (2-year study). ** P≤0.01*

**Non-neoplastic effects**

Summary of main neoplastic effects is exposed in tables B.6.8.1.1.4-01/6-7.

*Liver*

Non-neoplastic lesions included eosinophilic, hepatocellular hypertrophy, oval cell hyperplasia, cystic degeneration, and bile duct hyperplasia (only females). These incidences were statistically significant in all the animals of the 6 and 12-month interim evaluation, and in all the dose groups of the 2-year study.

*Stomach*

Increased atrophy incidences were described in the female rats groups treated with methyleugenol. The incidence rate was very high in all female dose groups (66-90%) compared with controls (0%). In males, these incidents were, however, increase with dosage level. In the 6 and 12-month interim examinations, atrophy was found in 100% of top dose animals. On the other hand, incidences of neuroendocrine cell hyperplasia were described in all female dose groups, and no in controls, however the incidence in top dose group was not statistically significant and a dose-related trend was not clearly noted. In rat males, same rate of neuroendocrine cell hyperplasia was recorded in the 150 and 300 mg/kg bw/day dose groups (16% vs 0% in controls).

*Kidney*

Increased incidences of renal tubule hyperplasia were recorded in 75 and higher male dose groups compared with controls (40%, 42% and 44%, respectively vs 26% in controls). The only statistically significant non-neoplastic lesion found in females was nephropathy in top dose group (90% vs 70% in controls).

*Bone marrow*

Increased incidences of bone marrow hyperplasia were recorded in all dose female groups (30%, 22%, 40% and 50% for 37, 75, 150 and 300 mg/kg bw/day dose groups, respectively) compared with controls (8%). Bone marrow hyperplasia consisted of a marked increase in the density of erythroid or myeloid cells or a mixture of both, frequently accompanied by proliferation of megakaryocytes. Bone marrow hyperplasia was considered to be secondary to the increased incidences of necrosis and inflammation associated with the large and multiple liver neoplasms in dosed animals.

*Salivary gland*

Most of the rats of all dose groups treated with methyleugenol during 2-year study and all animals from 6 and 12-month interim examinations showed cytoplasmic alteration of the submandibular salivary gland. Cytoplasmic alteration consisted of a loss of the eosinophilic granules within the striated ducts of the submandibular salivary glands.

The increased eosinophilic granularity (cytoplasmic alteration) in the submandibular salivary gland in dosed rats is considered secondary to the toxic effects of methyleugenol on the glandular stomach. Dietary factors such as protein starvation are known to cause loss of zymogen granules (McBride *et al., J. Dent. Res*., 1987, 66, 1445-1448). Methyleugenol administration in rats may have created such a condition due to loss of parietal and chief cells of the glandular stomach. Acid conditions and production of protein digesting enzymes are the functions of these two types of cells, respectively. Loss of these cells could inhibit proper protein utilization and essentially lead to protein deficiency.

*Spleen*

Increase incidences of splenic fibrosis were found in 150 and 300 mg/kg bw/day female dose groups (24% and 30%, respectively, vs 3% in controls).

**Table B.6.8.1.1.4-01/6. Summary of non-neoplastic findings in males in 2-year rats study**

| | Doses of methyleugenol (mg/kg bw/d) | | | | |
|---|---|---|---|---|---|
| | 0 | 37 | 75 | 150 | 300 |
| **Liver** | | | | | |
| **Non-neoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **Oval cell hyperplasia** | 0 | | | | 5** (100%) |
| **Hepatocyte hypertrophy** | 0 | | | | 5** (100%) |
| **Mixed cell foci** | 0 | | | | 5** (100%) |
| **12-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **Oval cell hyperplasia** | 0 | | | | 5** (100%) |
| **Hepatocyte hypertrophy** | 0 | | | | 5** (100%) |
| **Cystic degeneration** | 0 | | | | 5** (100%) |
| **Eosinophilic foci** | 0 | | | | 5** (100%) |
| **Mixed cell foci** | 0 | | | | 5** (100%) |
| **2-year study** | | | | | |
| **Eosinophilic foci** | 11/50 (22%) | 28/50** (56%) | 43/50** (86%) | 47/50** (94%) | 39/50** (78%) |
| **Mixed cell foci** | 1/50 (2%) | 7/50** (14%) | 14/50** (28%) | 8/50** (16%) | 2/50 (4%) |
| **Hepatocyte hypertrophy** | 0/50 | 13/50** (26%) | 25/50**(50%) | 30/50** (60%) | 26/50** (52%) |
| **Oval cell hyperplasia** | 14/50 (28%) | 17/50 (34%) | 24/50* (48%) | 34/50** (68%) | 27/50** (54%) |
| **Cystic degeneration** | 4/50 (8%) | 2/50 (4%) | 25/50** (50%) | 38/50** (76%) | 41/50** (82%) |
| **Glandular stomach** | | | | | |
| **Non-neoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **Atrophy** | 0 | | | | 5** (100%) |
| **12-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **Atrophy** | 0 | | | | 5** (100%) |
| **2-year study** | | | | | |
| **Neuroendocrine cell hyperplasia** | 0/50 | 0/50 | 1/50 (2%) | 8/50** (16%) | 8/50** (16%) |
| **Atrophy** | 0/50 | 14/50** (28%) | 32/50** (64%) | 37/50** (74%) | 29/50** (58%) |
| **Kidney** | | | | | |
| **Non-neoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **Renal tubule, hyperplasia, focal** | 13/50 (26%) | 13/50 (26%) | 20/50** (40%) | 21/50** (42%) | 22/50** (44%) |
| **Salivary gland** | | | | | |
| **Non-neoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **cytoplasmic alteration** | 0 | | | | 5** (100%) |
| **12-month interim evaluation** | | | | | |
| **Number examined** | 5 | | | | 5 |
| **cytoplasmic alteration** | 0 | | | | 5** (100%) |
| **2-year study** | | | | | |
| **cytoplasmic alteration** | 4/50 (8%) | 50/50 (100%) | 49/50 (98%) | 48/48 (100%) | 48/48 (100%) |

**Significantly different (P≤0.05) from the vehicle control group by Fisher exact test (interim evaluation), or the Poly-3 test (2-year study). ** P≤0.01*

**Table B.6.8.1.1.4-01/7. Summary of non-neoplastic findings in females in 2-year rats study**

| | Doses of methyleugenol (mg/kg bw/d) | | | | |
|---|---|---|---|---|---|
| | **0** | **37** | **75** | **150** | **300** |
| **Liver** | | | | | |
| **Nonneoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **Oval cell hyperplasia** | **0** | | | | **5** (100%)** |
| **Hepatocyte hypertrophy** | **0** | | | | **5** (100%)** |
| | | | | | |
| **12-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **Oval cell hyperplasia** | **0** | | | | **5** (100%)** |
| **Hepatocyte hypertrophy** | **0** | | | | **5** (100%)** |
| | | | | | |
| | | | | | |
| **Mixed cell foci** | **0** | | | | **5** (100%)** |
| **2-year study** | | | | | |
| **Eosinophilic foci** | 10/50 (20%) | **20/50** (40%)** | **27/49** (55%)** | **31/49** (63%)** | **37/50**(74%)** |
| **Mixed cell foci** | 6/50 (12%) | 4/50 (8%) | **19/49** (39%)** | 9/49 (18%) | 7/50 (14%) |
| **Hepatocyte hypertrophy** | 1/50 (2%) | **13/50** (26%)** | **16/49** (33%)** | **26/49** (53%)** | **31/50** (62%)** |
| **Oval cell hyperplasia** | 1/50 (2%) | **15/50** (30%)** | **19/49** (39%)** | **35/49** (71%)** | **34/50** (68%)** |
| **Cystic degeneration** | 0/50 | 0/50 | 1/49 (2%) | 4/49 (8%) | **29/50** (58%)** |
| Bile duct hyperplasia | 11/50 (22%) | 11/50 (22%) | 17/49 (35%) | **22/49** (45%)** | **30/50** (60%)** |
| **Glandular stomach** | | | | | |
| **Nonneoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **Atrophy** | **0** | | | | **5** (100%)** |
| **12-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **Atrophy** | **0** | | | | **5** (100%)** |
| **2-year study** | | | | | |
| **Neuroendocrine cell hyperplasia** | 0/50 | **5/50* (10%)** | **11/50** (22%)** | **9/50** (18%)** | 3/50 (6%) |
| **Atrophy** | 0/50 | **41/50** (82%)** | **45/50** (90%)** | **39/50** (78%)** | **33/50** (66%)** |
| **Kidney** | | | | | |
| **Nonneoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **Nephropathy** | 35/50 (70%) | 42/50 (84%) | 41/50 (82%) | 44/50 (88%) | **45/50* (90%)** |
| **Bone marrow** | | | | | |
| **Nonneoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **Bone marrow hyperplasia** | 4/50 (8%) | 15/50 (30%) | 11/49 (22%) | 20/50 (40%) | 25/50 (50%) |

| Salivary gland | | | | | |
|---|---|---|---|---|---|
| **Nonneoplastic effects** | | | | | |
| **6-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **cytoplasmic alteration** | **0** | | | | **5** (100%)** |
| **12-month interim evaluation** | | | | | |
| **Number examined** | **5** | | | | **5** |
| **cytoplasmic alteration** | **0** | | | | **5** (100%)** |
| **2-year study** | | | | | |
| **cytoplasmic alteration** | 1/50 (2%) | 48/50 (96%) | 49/50 (98%) | 49/49 (100%) | 49/50 (98%) |
| **Spleen** | | | | | |
| **Nonneoplastic effects** | | | | | |
| **2-year study** | | | | | |
| **splenic fibrosis** | 3/50 (6%) | 3/50 (6%) | 5/50 (10%) | 12/49 (24%) | 15/50 (30%) |

**Significantly different (P≤0.05) from the vehicle control group by Fisher exact test (interim evaluations),or the Poly-3 test (2-year study). ** P≤0.01*

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable without restrictions). Methyleugenol can be considered as a multisite carcinogen in rat.

Methyleugenol caused increased incidences of neoplastic and non-neoplastic lesions in the liver, glandular stomach and kidney of rats. There were also significant increases in neoplastic lesions including renal tubule adenoma or carcinoma, malignant mesothelioma, mammary gland fibroadenoma, skin fibroma, and skin fibroma or fibrosarcoma (combined). Effects were seen at all dose levels therefore it is not possible to determine a NOAEL, however it can be assumed that the NOAEL lies below 37 mg/kg bw/d.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is GLP compliant and, although presents several deviations, it is considered acceptable to evaluate the carcinogenicity potential of methyleugenol in rats.

A complete histopathology analysis was performed after test substance administration, and there were unequivocal evidences of the carcinogenicity potential of methyleugenol in both sexes.

Hepatocellular adenomas were recorded in all dose levels tested, whereas hepatocellular carcinomas at ≥75 mg/kg bw/day doses in males and at ≥150 mg/kg bw/day doses in females. Cholangiocarcinoma incidences were only recorded at top dose of both sexes. Malignant and benign neuroendocrine tumours were mainly noted in females stomach at ≥75 mg/kg bw/day doses, whereas renal tubule adenoma incidences were increased in 75 and higher male dose groups.

The neuroendocrine tumors of the glandular stomach induced by this chemical were considered the result of long-term gastrin stimulation of the enterochromaffin-like cells (Thake *et al., Exp. Toxicol. Pathol*., 1995, 47(2-3), 107-16). In the 14-week studies of methyleugenol (Abdo, K.M. *et al.*, B.6.8.1.1.2-03), there was evidence of inflammation, cellular degeneration, and necrosis in addition to gastric mucosal atrophy, suggesting that parietal cell cytotoxicity may have preceded mucosal atrophy. Because fundic mucosa atrophy was observed in the current studies of MEG, it is probable that hypochlorhydria and hypergastrinemia played a role in the neuroendocrine cell proliferative response. While these factors suggest that the trophic action of gastrin may be the driving force behind the neuroendocrine tumors, MEG has some genotoxic activity that should also be considered when attempting to determine the pathogenesis of the neuroendocrine proliferative response.

On the other hand, due to most male rat kidney tumors are generally associated with α2μ-globulin, a possible mechanism that involves globulin formation as a contributing factor of renal tubule adenoma cannot be ruled out (Baetcke *et al.*, α2μ-globulin: Association with chemically induced renal toxicity and neoplasia in the male rat; *Risk Assessment Forum Document; U.S. Environmental Protection Agency: Washington, DC*, 1991).

| |
|---|
| Additionally, equivocal evidences of fibroma/fibrocarcinoma on skin, malignant mesothelioma, and mammary gland fibroadenomas with a non-clear dose response were described in males.<br><br>All the neoplastic incidences exposed above were statistically significant compared with controls and exceed the overall mean and the range of the HCD published by Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 period.<br><br>Non-neoplastic effects were also recorded in most of the organs in which neoplasms were found, and in other organs such as bone marrow and spleen in females and salivary gland in both sexes.<br><br>Therefore, due to adverse effects (neoplastic and non-neoplastic multisite changes) were observed at all dose levels, a NOAEL cannot be established.<br><br>Based on the results of the study and according to the criteria of Regulation (EC) No 1272/2008, classification of methyleugenol as Carcinogen**, Category 2 (Carc. 2; H351)** is proposed. |

**B.6.8.1.1.4-02. Two-year toxicity study in mice with methyleugenol**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | National Toxicology Program |
| **Report year** | 2000 |
| **Report title** | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). |
| **Report No** | NIH Publication No. 00-3950 (CAS No. 93-15-2). NTP TR 491 |
| **Document No** | KCA 5.8.1/03a |
| **Guidelines followed in study** | None. The study was checked for compliance with OECD TG 453 (2018). |
| **Deviations from current test guideline OECD TG 453 (2018)** | -Food consumption was not measured.<br>-Ophthalmological examination was not performed.<br>-Organ weights were not presented.<br>-Circulating thyroid hormones (T4, T3, TSH) levels were not measured.<br>-No haematology, urinalysis and clinical chemistry were performed.<br>-Statistical analysis not performed in bodyweight measurements and in some non-neoplastic incidences. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Yes |
| **Acceptability/ reliability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 9224705, Purity: 99% |
| **Test system** | B6C3F1 mice |
| **Test method** | Groups of 50 male and 50 female B6C3F1 mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 0, 37, 75 and 150 mg/kg bw/day, 5 days per week for 105 weeks.<br><br>Dosing volumes were 10 mL/kg bw. Feed and water were available *ad libitum*. Male mice were housed individually and female mice were housed five per cage. Cages and racks were rotated every 2 weeks. Clinical findings were recorded and the animals were weighed weekly and at the end of the studies.<br><br>*Necropsy*<br><br>Necropsy was performed on all animals.<br><br>*Histopathology*<br><br>Complete histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral |

| | |
|---|---|
| | gland, oesophagus, gallbladder, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (with adjacent skin) (except male mice), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. |
| **Statistical analysis of results** | *Survival*<br>The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.<br><br>*Analysis of Neoplasm and Non-neoplastic Lesion Incidences*<br>The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and non-neoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.<br><br>This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats (Portier *et al.*,1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).<br><br>Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of lesion incidence, and reported P values are one sided. Values of P greater than 0.5 are presented as 1-P with the letter N added to indicate a lower incidence or negative trend in neoplasm occurrence relative to the control group (e.g., P=0.99 is presented as P=0.01N). For neoplasms and non-neoplastic lesions detected at the interim evaluation, the Fisher exact test, a procedure based on the overall proportion of affected animals, was used. |
| **Historical control data** | Neoplasm incidences from Battelle Columbus Laboratories (data as November 2017, range not specified), that conducted the 2-year studies, and from NTP carcinogenesis program from 1990-1997 (Haseman *et al.*, *Toxicol Pathol.*, 1998, 26(3):428-41) period are exposed below: |

| | **Historical Incidence at Battelle Columbus Laboratories** | | | **Historical Incidence at NTP 1990-1997 (Haseman *et al.*)** | | |
|---|---|---|---|---|---|---|
| | **Total** | **Mean ± SD** | **Range (%)** | **Total** | **Rate (%)** | **Range (%)** |
| **Liver** | | | | | | |
| **Males** | | | | | | |
| Adenomas | 201/514 | 39.2 ± 11.0 | 21-58% | 397/1350 | 29.4 | 4-60% |
| Carcinomas | 102/514 | 20.0 ± 8 1 | 8-38% | 241/1350 | 17.9 | 6-29% |

| | | | | | | |
|---|---|---|---|---|---|---|
| Adenoma or carcinoma | 267/514 | 52.1 ± 14 9 | 25-72% | 570/1350 | 42.2 | 10-68% |
| Hepatoblastoma | 2/514 | 0.3 ± 1 | 0-3% | 0 | 0 | 0 |
| Hepatocholangiocarcinoma | n.a. | n.a. | n.a. | 1/1350 | 0.1 | 0-2% |
| **Females** | | | | | | |
| Adenomas | 108/511 | 21.0 ± 9 5 | 6-40% | 234/1350 | 17.3 | 2-50% |
| Carcinomas | 37/511 | 7.2 ± 6.7 | 0-22% | 113/1350 | 8.4 | 0-20% |
| Adenoma or carcinoma | 138/511 | 26.8 ± 14 1 | 8-58% | 319/1350 | 23.6 | 6-56% |
| Hepatoblastoma | 0/511 | 0 | 0 | 1/1350 | 0.1 | 0-2% |
| Hepatocholangiocarcinoma | 1/511 | 0.2 ± 0.6 | 0-2% | 2/1350 | 0.1 | 0-2% |
| **Glandular stomach** | | | | | | |
| **Males** | | | | | | |
| Carcinomas | n.a. | n.a. | n.a. | 0 | 0 | 0 |
| **Females** | | | | | | |
| Carcinomas | n.a. | n.a. | n.a. | 1/1353 | 0.1 | 0-2% |

## Results

**Survival:** The survival rates (animals surviving at study termination and mean survival days) of male treated groups were similar to control group. By contrast, in female dosed groups, the animals surviving at study termination were reduced compared with controls, particularly at top dose group (36%, 36% and 4% for low, mid and high dose groups, respectively), compared with controls (62%). Additionally, an increase of the number of natural deaths, together with a decrease in the mean survival (days), were noted in female dose groups (see Table B.6.8.1.1.4-02/1).

**Table B.6.8.1.1.4-02/1. Survival of mice in the 2-year gavage study of methyleugenol**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| | **Control (vehicle)** | **37** | **75** | **150** |
| **Males** | | | | |
| **Animals initially in study** | **50** | **50** | **50** | **50** |
| **Accidental deaths (a)** | 0 | 0 | 1 | 2 |
| **Missing (a)** | 1 | 0 | 0 | 0 |
| **Moribund** | 5 | 6 | 5 | 9 |
| **Natural deaths** | 6 | 8 | 7 | 4 |
| **Animals surviving to study termination** | **38 (76%)** | **36 (72%)** | **37 (74%)** | **35 (70%)** |
| **Percent probability of survival at end of study (b)** | 78 | 72 | 76 | 73 |
| **Mean survival (days) (c)** | 690 | 705 | 679 | 686 |
| **Survival analysis (d)** | **P=0.815** | **P=0.825** | **P=1.000** | **P=0.829** |

| Females | | | | |
|---|---|---|---|---|
| **Animals initially in study** | **50** | **50** | **50** | **50** |
| **Accidental deaths (a)** | 5 | 0 | 0 | 0 |
| **Missing (a)** | 0 | 0 | 0 | 0 |
| **Moribund** | 5 | 7 | 8 | 13 |
| **Natural deaths** | 14 | 24 | 23 | 35 |
| **Animals surviving to study termination** | **31 (62%)** | **18 (36%)** | **18 (e) (36%)** | **2 (4%)** |
| **Percent probability of survival at end of study (b)** | 62 | 38 | 37 | 4 |
| **Mean survival (days) (c)** | 696 | 662 | 664 | 637 |
| **Survival analysis (d)** | **P<0.001** | **P=0.009** | **P=0..013** | **P<0.001** |

*(a) Censored from survival analyses.*
*(b) Kaplan-Meier determinations.*
*(c) Mean of all deaths (uncensored, censored, and terminal sacrifice).*
*(d) The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns.*

**Clinical signs:** Only moribund clinical signs were recorded and attributed to test substance administration.

**Bodyweight:** Mean bodyweights of 75 and 150 mg/kg bw/day male dose groups were slightly lower than controls. It was only >10% at last two weeks in the 75 mg/kg bw/day group, and at last week in 150 mg/kg bw/day group (Table B.6.8.1.1.4-02/2).

On the other hand, all the females dose groups showed reduced bodyweights compared with controls throughout study. The reductions of bodyweights were >10% from week 17 in top dose group (11-46%), from week 37 in the mid dose female group (12-45%), and from week 65 in the low dose group (11-39%) to study termination (Table B.6.8.1.1.4-02/3).

**Table B.6.8.1.1.4-02/2. Mean bodyweights of male mice in the 2-year gavage study of methyleugenol**

| | Dose (mg/kg bw/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Control (vehicle)** | **37** | | **75** | | **150** | |
| **week** | **Mean bw** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** |
| 1 | 24.4 | 24.2 | 99 | 24.2 | 99 | 24.2 | 99 |
| 5 | 28.9 | 28.9 | 100 | 28.4 | 98 | 28.5 | 99 |
| 9 | 33.2 | 33.6 | 101 | 32.9 | 99 | 32.7 | 99 |
| 13 | 37.4 | 37.6 | 101 | 36.8 | 98 | 36.0 | 96 |
| 17 | 41.4 | 41.8 | 101 | 40.4 | 98 | 39.4 | 95 |
| 21 | 45.4 | 45.0 | 99 | 43.4 | 96 | 42.1 | 93 |
| 25 | 47.0 | 46.5 | 99 | 45.0 | 96 | 43.6 | 93 |
| 29 | 48.0 | 47.8 | 100 | 46.1 | 96 | 44.8 | 93 |
| 33 | 49.8 | 48.8 | 98 | 47.5 | 95 | 46.8 | 94 |
| 37 | 50.6 | 49.8 | 98 | 48.1 | 95 | 47.3 | 94 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 41 | 50.7 | 50.2 | 99 | 48.7 | 96 | 47.8 | 94 |
| 45 | 51.7 | 50.3 | 97 | 48.8 | 94 | 48.7 | 94 |
| 49 | 52.5 | 51.0 | 97 | 49.3 | 94 | 49.3 | 94 |
| 53 | 52.3 | 51.5 | 99 | 49.7 | 95 | 49.4 | 95 |
| 57 | 52.4 | 51.1 | 98 | 48.9 | 93 | 49.4 | 94 |
| 61 | 52.6 | 51.7 | 99 | 49.4 | 94 | 49.1 | 93 |
| 65 | 52.9 | 51.8 | 98 | 49.6 | 94 | 49.9 | 94 |
| 69 | 52.2 | 51.5 | 99 | 49.1 | 94 | 49.7 | 95 |
| 73 | 51.9 | 51.0 | 98 | 49.4 | 95 | 49.2 | 95 |
| 77 | 51.7 | 50.5 | 98 | 48.9 | 95 | 48.5 | 94 |
| 81 | 52.8 | 50.4 | 96 | 48.9 | 93 | 48.6 | 92 |
| 85 | 53.6 | 50.5 | 94 | 49.6 | 93 | 49.9 | 93 |
| 89 | 53.7 | 50.3 | 94 | 49.3 | 92 | 48.8 | 91 |
| 93 | 53.1 | 49.1 | 93 | 48.3 | 91 | 48.2 | 91 |
| 97 | 52.4 | 47.8 | 91 | 46.1 | **88 (↓12%)** | 46.9 | 90 |
| 101 | 52.2 | 47.1 | 90 | 44.0 | **84 (↓16%)** | 45.4 | **87 (↓13%)** |
| **Total Mean** | 48 | 47 | 98 | 45 | 95 | 45 | 94 |
| **Mean per weeks** | | | | | | | |
| 1-13 | 31 | 31.1 | 100 | 30.6 | 99 | 30.4 | 98 |
| 14-52 | 48.6 | 47.9 | 99 | 46.4 | 96 | 45.5 | 94 |

**Table B.6.8.1.1.4-02/3. Mean bodyweights of female mice in the 2-year gavage study of methyleugenol**

| | **Dose (mg/kg bw/day)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Control (vehicle)** | **37** | | **75** | | **150** | |
| **week** | **Mean bw** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** | **Mean bw** | **% bw (referred to controls)** |
| 1 | 19.1 | 19.2 | 101 | 19.2 | 101 | 19.3 | 101 |
| 5 | 23.0 | 22.8 | 99 | 22.6 | 98 | 22.5 | 98 |
| 9 | 26.8 | 25.9 | 97 | 25.6 | 96 | 24.8 | 93 |
| 13 | 29.4 | 29.6 | 101 | 28.8 | 98 | 26.6 | 91 |
| 17 | 34.7 | 34.3 | 99 | 33.0 | 95 | 28.8 | **83 (↓17%)** |
| 21 | 38.6 | 37.6 | 97 | 35.6 | 92 | 31.0 | **80 (↓20%)** |
| 25 | 40.5 | 39.7 | 98 | 37.6 | 93 | 32.9 | **81 (↓19%)** |
| 29 | 43.6 | 42.5 | 98 | 40.1 | 92 | 35.0 | **80 (↓20%)** |
| 33 | 47.1 | 44.7 | 95 | 42.4 | 90 | 37.1 | **79 (↓21%)** |
| 37 | 49.2 | 45.4 | 92 | 43.1 | **88 (↓12%)** | 36.8 | **75 (↓25%)** |
| 41 | 50.1 | 46.6 | 93 | 43.4 | **87 (↓13%)** | 37.9 | **76 (↓24%)** |
| 45 | 51.6 | 47.7 | 92 | 44.6 | **86 (↓14%)** | 39.5 | **77 (↓23%)** |
| 49 | 53.9 | 50.1 | 93 | 46.2 | **86 (↓14%)** | 41.1 | **76 (↓24%)** |
| 53 | 54.0 | 50.0 | 93 | 45.6 | **84 (↓16%)** | 39.7 | **74 (↓26%)** |
| 57 | 56.9 | 51.0 | 90 | 46.6 | **82 (↓18%)** | 40.6 | **71 (↓29%)** |
| 61 | 58.3 | 52.3 | 90 | 47.0 | **81 (↓19%)** | 40.8 | **70 (↓30%)** |
| 65 | 59.0 | 52.4 | **89 (↓11%)** | 45.6 | **77 (↓23%)** | 39.0 | **66 (↓34%)** |
| 69 | 60.1 | 52.2 | **87 (↓13%)** | 44.0 | **73 (↓27%)** | 37.9 | **63 (↓37%)** |
| 73 | 59.0 | 51.7 | **88 (↓12%)** | 42.5 | **72 (↓28%)** | 35.3 | **60 (↓40%)** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 77 | 60.1 | 49.7 | **83 (↓17%)** | 40.8 | **68 (↓32%)** | 36.1 | **60 (↓40%)** |
| 81 | 61.7 | 47.1 | **76 (↓24%)** | 38.5 | **62 (↓38%)** | 35.6 | **58 (↓42%)** |
| 85 | 63.2 | 44.0 | **70 (↓30%)** | 36.8 | **58 (↓42%)** | 34.7 | **55 (↓45%)** |
| 89 | 62.7 | 43.0 | **69 (↓31%)** | 34.6 | **55 (↓45%)** | 34.6 | **55 (↓45%)** |
| 93 | 61.7 | 40.5 | **66 (↓33%)** | 34.1 | **55 (↓45%)** | 33.7 | **55 (↓45%)** |
| 97 | 61.1 | 38.0 | **62 (↓38%)** | 34.8 | **57 (↓43%)** | 32.9 | **54 (↓46%)** |
| 101 | 60.9 | 37.4 | **61 (↓39%)** | 34.2 | **56 (↓44%)** | 33.0 | **54 (↓46%)** |
| **Total Mean** | 49 | 42 | 96 | 38 | 95 | 34 | 96 |
| **Mean per weeks** | | | | | | | |
| 1-13 | 24.6 | 24.4 | 100 | 24.1 | 98 | 23.3 | 96 |
| 14-52 | 45.5 | 43.2 | 95 | 40.7 | 90 | 35.6 | **79 (↓21%)** |
| 53-101 | 59.9 | 46.9 | **79 (↓21%)** | 40.4 | **68 (↓32%)** | 36.5 | **61 (↓39%)** |

**Histopathology:**

**Neoplastic effects**

Summary of main neoplastic effects is exposed in tables B.6.8.1.1.4-02/4-5.

*Liver*

Hepatocellular adenomas were statistically significantly higher in all dosed males (86%, 76% and 78% for low, mid and high dose groups, respectively) and females (96%, 94% and 82% for low, mid and high dose groups, respectively) compared with controls (53% and 40% for male and female control groups).

Hepatocellular carcinomas were also increased in all female dose groups (74%, 96% and 94% for low, mid and high dose groups, respectively) compared with controls (14%). However, only low (40%) and mid (38%) dose male groups showed a statistically significant increase compared with controls (20%). The top male dose groups displayed an incidence similar to control group (18% vs 20% in controls), so a dose-relationship was not observed.

The occurrences of hepatoblastoma were statistically significant increase in all female dose groups (12%, 22% and 30% for low, mid and high dose groups, vs 0% in controls), and slightly increased in top dose male group (6%) without showing statistically significance. Additionally, 2 females of top dose group developed hepatocholangiocarcinoma, whereas this type of tumour was not recorded in mice males.

The incidences of hepatocellular adenomas in both sexes, hepatoblastoma in females and in top dose male group, and hepatocholangiocarcinoma, exceed the overall mean and the range of HCD from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 (Haseman *et al.*, *Toxicol Pathol.*, 1998, 26(3):428-41). On the other hand, incidences of hepatocarcinomas in low (40%) and mid (38%) male dose groups were higher than historical control mean of the Battelle laboratories (20%) and NTP Carcinogenesis studies (17.9%), and exceed the range of NTP (6-29%) report 1990-1997. However, the occurrence in the low and mid dose groups was slightly out and in the limit of historical range revealed by Battelle laboratories (8-38%). By contrast, the percentage of hepatocarcinomas in all female dose groups exceeds the overall mean and the range of both HCD sources.

*Glandular stomach*

Isolated incidences of carcinoma (2%) and malignant neuroendocrine tumours (4%) were noted in top dose males, without showing statistical significance. The incidences of carcinoma in males were higher than mean and exceed the range reported by NTP carcinogenesis studies during 1990-1997. The HCD occurrences in glandular stomachs from Battelle laboratories were not showed. No differences in neoplastic alterations were found between female dose groups and controls.

Malignant neuroendocrine tumors were locally expansive invasive masses which caused moderate to marked thickening of the mucosa. The neoplastic cells formed clusters, cords, or poorly defined glandular-like structures

displacing and/or replacing the normal architecture of the glandular mucosa. The cells were large, polygonal to spindle-shaped with scant to abundant finely granular amphophilic cytoplasm and large pleomorphic, mostly vesicular nuclei that had one or more prominent nucleoli. Invasion into the submucosa was extensive and occurred as nests of neoplastic neuroendocrine cells surrounded by moderate amounts of fibrous tissue. The carcinoma was an ulcerated, locally extensive, invasive mass that obliterated the mucosal architecture extending into and replacing the submucosa and much of the muscle layers of the stomach. The neoplastic cells occurred as sheets, clusters, and poorly defined cords and were anaplastic, polygonal to spindle-shaped cells with scant eosinophilic cytoplasm and large pleomorphic hyper-chromatic nuclei.

**Table B.6.8.1.1.4-02/4. Summary of neoplastic findings in males in 2-year mice study**

| | Doses of methyleugenol (mg/kg bw/d) | | | |
|---|---|---|---|---|
| | **0** | **37** | **75** | **150** |
| **Liver** | | | | |
| **Hepatocellular adenoma** | 26/49 (53%) | **43/50** (86%)** | **38/50** (76%)** | **39/50** (78%)** |
| **Hepatocellular carcinoma** | 10/50 (20%) | **20/50* (40%)** | **19/50* (38%)** | 9/50 (18%) |
| **Hepatoblastoma** | 0/49 | 0/50 | 1/50 (2%) | 3/50 (6%) |
| **Hepatocholangiocarcinoma** | - | - | - | - |
| **Glandular stomach** | | | | |
| **Carcinoma** | 0/49 | 0/48 | 0/49 | 1/50 (2%) |
| **Malignant neuroendocrine tumour** | 0/49 | 0/48 | 0/49 | 2/50 (4%) |

** Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test (2-year study). ** P≤0.01*

**Table B.6.8.1.1.4-02/5. Summary of neoplastic findings in females in 2-year mice study**

| | Doses of methyleugenol (mg/kg bw/d) | | | |
|---|---|---|---|---|
| | **0** | **37** | **75** | **150** |
| **Liver** | | | | |
| **Hepatocellular adenoma** | 20/50 (40%) | **48/50** (96%)** | **46/49** (94%)** | **41/50** (82%)** |
| **Hepatocellular carcinoma** | 7/50 (14%) | **37/50** (74%)** | **47/49** (96%)** | **47/50** (94%)** |
| **Hepatoblastoma** | 0/50 | **6/50** (12%)** | **11/50** (22%)** | **15/50** (30%)** |
| **Hepatocholangiocarcinoma** | 0/50 | 0/50 | 0/49 | 2/50 (4%) |

** Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test (2-year study). ** P≤0.01*

**Non-neoplastic effects**

Summary of main non-neoplastic effects is exposed in tables B.6.8.1.1.4-02/6-7.

*Liver*

The incidences of eosinophilic foci were significantly increased in male dosed groups (40%, 50% and 38% for low, mid and high dose group, respectively, vs 20% in controls). In all dosed groups of males and females, the incidences of oval cell hyperplasia were significantly increased; in males, the incidences were increased with the dose (16%, 54% and 92% for low, mid and high dose, respectively vs 0% in controls), whereas in females, most of animals of low dose group developed oval cell hyperplasia (92%), and the incidence was slightly reduced in the mid and high dose groups (73% and 76%, respectively). The incidences of periportal hypertrophy were significantly greater in mid and top dose male groups (14% and 92%), and in all female dose groups (20%, 14% and 26% in low, mid and high dose groups, respectively), than controls of both sexes (0%).

The incidences of bile duct hyperplasia in 75 and 150 mg/kg female mice (22% and 18%, respectively), and hemosiderin pigmentation (22%, 49% and 38% for low, mid and high dose groups, respectively) in all female dose groups were significantly increased, compared with controls. Moreover, a dose-related trend was recorded in

hepatocyte necrosis incidences for mid and high female dose (33% and 34%), and in hematopoietic cell proliferation incidences in all female dose groups (28%, 47% and 48% for low, mid and high dose groups, respectively).

*Glandular stomach*

Dose-related increases of atrophy, ectasia, and hyperplasia incidences were recorded in all male dose groups, and the differences were statistically significant compared with controls. In female dose groups, ectasia occurrences were statistically significantly higher in dosed groups (67%, 67% and 84% for low, mid and high dose groups, respectively), compared with controls (31%); whereas same atrophy incidence was noted in mid and high dose group (22% vs 0% in controls).

One lesion observed in mice but not in rats consisted of multifocal hyperplasia of the columnar foveolar epithelium of the gastric pits. This hyperplasia often extended toward the base of the mucosa with displacement of parietal and chief cells of the mucosal glands and was often accompanied by minimal to mild chief cell hyperplasia.

Atrophy of the glandular mucosa was histologically similar to that observed in rats. It was characterised by loss of glandular epithelial cells (chief and parietal cells) in the fundic region of the stomach with concomitant reduction in the height of the fundic mucosa.

*Bone marrow*

Dose-related increases of bone marrow hyperplasia were recorded in males (53%, 66% and 70% in low, mid and high dose groups vs 29% in controls). In female dose groups, a high incidence was noted in all the treated groups (94-100% vs 36% in controls). Bone marrow hyperplasia was considered to be secondary to the changes in hepatic and gastric pathology.

**Table B.6.8.1.1.4-02/6. Summary of non-neoplastic findings in males in 2-year mice study**

| | Doses of methyleugenol (mg/kg bw/d) | | | |
|---|---|---|---|---|
| | **0** | **37** | **75** | **150** |
| **Liver** | | | | |
| **Eosinophilic foci** | 10/49 (20%) | **20/50* (40%)** | **25/50** (50%)** | **19/50* (38%)** |
| **Hepatocyte hypertrophy** | 0/49 | 1/50 (2%) | **7/50** (14%)** | **46/50** (92%)** |
| **Oval cell hyperplasia** | 0/49 | **8/50** (16%)** | **27/50** (54%)** | **46/50** (92%)** |
| **Inflammation, chronic** | 19/49 (39%) | 21/50 (42%) | **28/50* (56%)** | **28/50* (56%)** |
| **Hemosiderin pigmentation** | - | - | - | - |
| **Bile duct hyperplasia** | - | - | - | - |
| **Hepatocyte necrosis** | - | - | - | - |
| **Hematopoietic cell proliferation** | - | - | - | - |
| **Glandular stomach** | | | | |
| **Atrophy** | 0/49 | 3/48 (6%) | **35/49** (71%)** | **45/50** (90%)** |
| **Ectasia** | 13/49 (27%) | **25/48* (52%)** | **40/49** (82%)** | **49/50** (98%)** |
| **Hyperplasia** | 0/49 | 1/48 (2%) | **15/49** (31%)** | **20/50** (40%)** |
| **Bone marrow** | | | | |
| **Bone marrow hyperplasia** | 14/49 (29%) | 26/49 (53%) | 33/50 (66%) | 35/50 (70%) |

** Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test (2-year study) ** P≤0.01*

**Table B.6.8.1.1.4-02/7. Summary of non-neoplastic findings in females in 2-year mice study**

| | Doses of methyleugenol (mg/kg bw/d) | | | |
|---|---|---|---|---|
| | **0** | **37** | **75** | **150** |
| **Liver** | | | | |
| **Eosinophilic foci** | - | - | - | - |
| **Hepatocyte hypertrophy** | 0/50 | **10/50** (20%)** | **7/49** (14%)** | **23/50** (26%)** |

| | | | | |
|---|---|---|---|---|
| **Oval cell hyperplasia** | 0/50 | **46/50** (92%)** | **36/49** (73%)** | **38/50** (76%)** |
| **Inflammation, chronic** | - | - | - | - |
| **Hemosiderin pigmentation** | 0/50 | **11/50** (22%)** | **24/49** (49%)** | **19/50** (38%)** |
| **Bile duct hyperplasia** | 1/50 (2%) | 1/50 (2%) | **11/49** (22%)** | **9/50** (18%)** |
| **Hepatocyte necrosis** | 5/50 (10%) | 9/50 (18%) | **16/49** (33%)** | **17/50** (34%)** |
| **Hematopoietic cell proliferation** | 4/50 (8%) | **14/50** (28%)** | **23/49** (47%)** | **24/50** (48%)** |
| **Glandular stomach** | | | | |
| **Atrophy** | 0/45 | 0/49 | **10/46** (22%)** | **10/45** (22%)** |
| **Ectasia** | 14/45 (31%) | **33/49** (67%)** | **31/46** (67%)** | **38/45** (84%)** |
| **Hyperplasia** | - | - | - | - |
| **Bone marrow** | | | | |
| **Bone marrow hyperplasia** | 18/50 (36%) | 47/50 (94%) | 46/48 (92%) | 50/50 (100%) |

** Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test (2-year study). ** P≤0.01*

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable (reliable without restrictions). Methyleugenol can be considered as a multisite carcinogen in mice.

Methyleugenol caused increased incidences of neoplastic lesions in the liver in males and females, and in the glandular stomach in males. Increased incidences of non-neoplastic lesions in the liver and glandular stomach were also observed in both sexes. Effects were seen at all dose levels therefore it is not possible to set a NOAEL, however it can be assumed that the NOAEL lies below 37 mg/kg bw/day.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This study is GLP compliant and, although presents several deviations, it is considered acceptable to evaluate the carcinogenicity potential of methyleugenol in mice.

A complete histopathology analysis in dosed groups revealed the carcinogenicity potential of methyleugenol in female liver, together with other neoplastic and non-neoplastic changes in liver, glandular stomach and bone marrow in both sexes.

The carcinogenicity potential in mice seems to be less than observed previously in rats, and the liver and glandular stomach consolidate as the mainly target organs.

Hepatocellular adenomas were recorded in all dose levels in males and females, whereas increase dose-related incidences of hepatocellular carcinomas and hepatoblastomas were described only in females. It is noteworthy that top dose male group showed a hepatocellular carcinoma incidence similar to control group, whereas the low and mid dose groups displayed a two-fold increase compared with these groups. Thus, the result of liver carcinomas in males was equivocal, particularly in top dose male group, and the cause should have been enough clarified by the authors of the study.

On the other hand, low and non-statistically significant occurrences of hepatocholangiocarcinoma, together with carcinoma and malignant neuroendocrine tumour incidences were described exclusively in top dose female and male groups, respectively.

Non-neoplastic alterations were also recorded in all dose levels in liver, glandular stomach and bone marrow of treated-mice.

Therefore, due to adverse effects (neoplastic and non-neoplastic multisite changes) were observed at all dose levels, a NOAEL cannot be established.

Based on the results of the study and according to the criteria of Regulation (EC) No 1272/2008, classification of methyleugenol as Carcinogen**, Category 2 (Carc. 2; H351)** is proposed.

**B.6.8.1.1.4-03. Two-year toxicity study in rat and mice with methyleugenol**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Johnson, J.D., Ryan, M.J., Toft, J.D., Graves, S.W., Hejtmancik, M.R., Cunningham, M.L., Herbert, R. and Abdo, K.M |
| **Report year** | 2000 |
| **Report title** | Two-year toxicity and carcinogenicity study of methyleugenol in F344/N rats and B6C3F1 mice |
| **Report No** | Not applicable, published study (*Journal of Agricultural and Food Chemistry*, 48:3620-3632) |
| **Document No** | KCA 5.8.1/12b |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

This study summarised the oral 2-year studies in rats and mice conducted with methyleugenol and reported by NTP (B.6.8.1.1.4-01 and B.6.8.1.1.4-02 sections). In order to avoid duplications, the results were not assessed again (please refer to the studies B.6.8.1.1.4-01 and B.6.8.1.1.4-02).

### B.6.8.1.1.5. Other toxicity studies

**B.6.8.1.1.5-01. Identification of methyleugenol-DNA adducts on liver human samples**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Herrmann, K., Schumacher, F., Engst, W., Appel, K.E., Klein, K., Zanger, U.M. and Glatt, H. |
| **Report year** | 2013 |
| **Report title** | Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human liver samples |
| **Report No** | Not applicable, published study (*Carcinogenesis*, 34(5):1025-1030) |
| **Document No** | KCA 5.8.1/14 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol dietary exposure. Dosage and diet source not stated. |
| **Test system** | Human liver samples |
| **Test method** | *Liver samples* |

| | |
|---|---|
| | Methyleugenol DNA adducts were analysed in liver tissue samples collected from 30 (18 male and 12 female) human subjects using isotope-dilution ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). No data on dietary habits of the donors were recorded; therefore dietary methyleugenol exposure was not quantified prior to analysis. All liver samples were histologically examined and only non-tumorous tissue was used. Patient documentation included age, sex, medical diagnosis, pre-surgical medication, and alcohol and smoking habits. Samples from patients with hepatitis, cirrhosis or chronic excessive alcohol use were excluded.<br><br>*Analysis of DNA adducts*<br><br>DNA was isolated from homogenized hepatic tissue by standard phenol–chloroform extraction. Then, samples were extracted triply with an equal volume of water-saturated butanol (1:1). The pooled butanol phases were washed with butanol saturated water, evaporated to dryness under reduced pressure and re-dissolved in 25 µl of 75% methanol for UPLC-MS/MS analysis. Isolated DNA was contaminated with RNA (up to 57% of the total nucleic acids in the samples used). For the calculation of the actual amount of DNA used, authors determined the ratio of 2′-deoxyguanosine and guanosine in the DNA hydrolysates (before butanol extraction) by UPLC-MS/MS using 15N-labeled nucleosides as internal standards. The final amount of DNA analysed was 112.5 µg.<br><br>Sample separation and analytic detection were carried out with an Acquity UPLC system equipped with an Acquity BEH-Phenyl column (1.7 µm, 2.1 100 mm) and a Xevo TQ triple quadrupole mass.<br><br>**$N_2$-MIE-dG (*$N_2$-trans*-methylisoeugenol-3´-yl-2´-deoxyguanosine**) was detected using the transitions 444.1/449.1* → 328.2/333.2* (loss of deoxyribose); 444.1/449.1* → 177.1/177.1* (loss of dG), and 444.1/449.1* → 164.1/169.1* (loss of deoxyribose and phenylethyl group).<br><br>**$N_6$-MIE-dA (*$N_6$-trans*-methylisoeugenol-3´-yl-2´-deoxyadenosin)** was detected using the transitions 428.1/433.1* → 177.2/177.2* (loss of dA, quantifier); 428.1/433.1* → 312.2/ 317.2* (loss of deoxyribose), and 428.1/433.1* → 146.1/146.1* (loss of dA and one methoxy group). |
| **Statistical analysis of results** | Not stated |

**Results**

Detection of adducts

One human sample showed strong signal corresponding to $N_2$-MIE-dG (Figure B.6.8.1.1.5-01/1). The level of $N_2$-MIE-dG exceeded 60-fold the signal of $N_6$-MIE-dA, which is similar to the ratio of these adducts formed by methyleugenol in experimental models. Thus, there was an evidence of hepatic methyleugenol DNA adducts formation. On the other hand, there were also evidences for the presence of $N_2$-MIE-dG adducts, although at lower levels, in the other 28 subjects based on the detection of all three m/z transitions. There was a statistically significant linear correlation between the $N_2$-MIE-dG and $N_6$-MIE-dA levels in the 30 subjects studied ($r2 = 0.39$, $P = 0.0002$). The relatively low correlation coefficient may be due to differential repair of both adducts and/or background signals. In conclusion, the presence of $N_6$-MIE-dA adducts could be demonstrated, although an accurate quantification was not possible due to the low levels. The presence of this minor adduct, in addition to $N_2$-MIE-dG, represents a further verification of the adduct formation by methyleugenol. Methyleugenol-DNA adducts formed by reactive metabolites in target cells from mutagenicity test are shown in figure B.6.8.1.1.5-01/2.

**Conclusion**

The maximal and median levels of both adducts combined were 37 and 13 for $N_2$-MIE-dG and $N_6$-MIE-dA, respectively, per 108 nucleosides (corresponding to 4700 and 1700 adducts, respectively, per diploid genome: 6.6 × $10^9$ base pairs). The authors exposed that these results were the first demonstration of DNA adducts formed by a xenobiotic in human liver using the gold standard method UPLC-MS/MS. It has been estimated for diverse rat

and mouse hepatocarcinogens that 50–5500 adducts per 108 nucleosides are present after repeated treatment at the TD50 (daily dose that halves the probability to stay tumor-free in long-term studies). The study concludes that the exposure to methyleugenol leads to substantial levels of hepatic DNA adducts and, therefore, may present a potential carcinogenic risk.

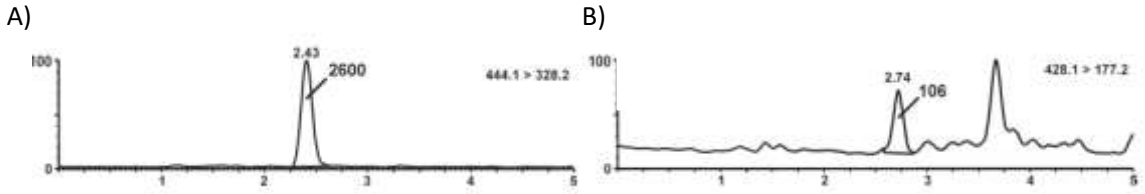
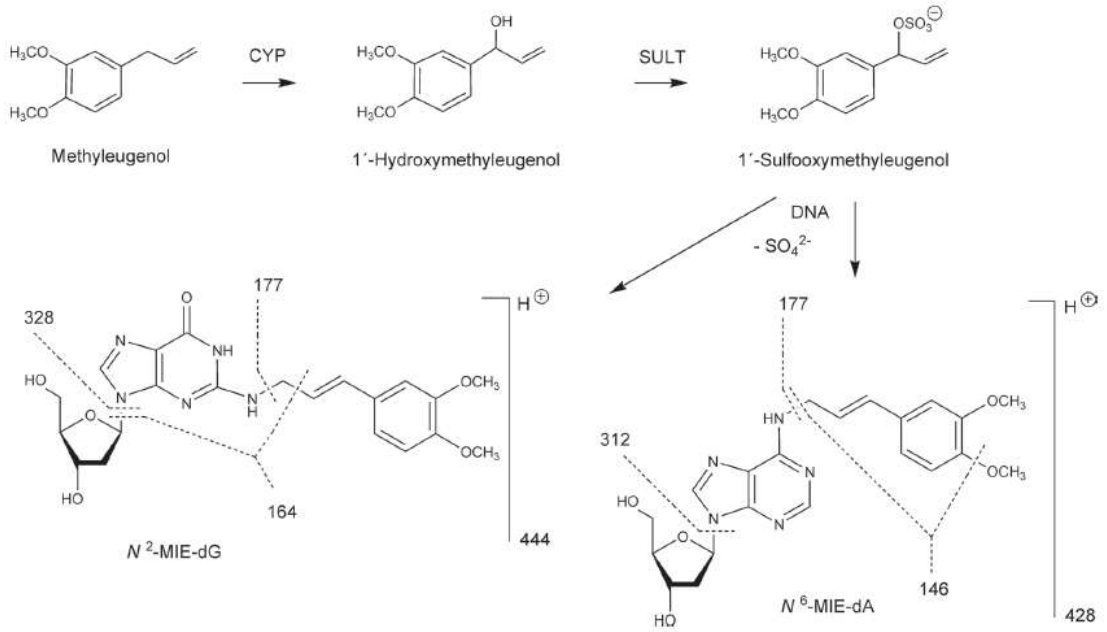



**Figure B.6.8.1.1.5-01/1. m/z chromatogram of $N_2$-MIE-dG (A) and $N_6$-MIE-dA (B) obtained from a human sample.**




*Collision-induced fragmentations used for the mass spectrometric analysis are indicated for the adducts. CYP, cytochrome P450; SULT, sulfotransferase.*

**Figure B.6.8.1.1.5-01/2. Bioactivation pathway of methyleugenol with the structures of the 2′-deoxynucleoside adducts formed**

| |
|---|
| **<u>Assessment and conclusion by applicant</u>:**<br>This new study is considered to be acceptable as supporting information (non-standard methodology; reliable with restrictions).<br>Methyleugenol DNA-adducts are observed in human liver tissues collected from surgical patients, and these adducts may be the result of a dietary exposure, however data of methyleugenol dietary exposure were not recorded. |
| **<u>Assessment and conclusion by RMS</u>:**<br>**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion.<br>The study is a scientific publication from a published journal. This study showed the evidence of the genotoxic potential of methyleugenol in humans and its plausible role in liver tumorigenesis. However, the study lacks of |

| |
|---|
| important information for a proper assessment and conclusion, such a as methyleugenol dietary exposure data, the source, and the clinical information of the subjects used in the study. For these reasons, the RMS deems the study as supporting information only. |

**B.6.8.1.1.5-02. Methyleugenol-DNA adducts on human liver HepG2 cells**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Zhou G.D., Moorthy B., Bi J., Donnelly K.C. and Randerath K. |
| **Report year** | 2007 |
| **Report title** | DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells |
| **Report No** | Not applicable, published study (*Environmental and Molecular Mutagenesis*, 48:715-721) |
| **Document No** | KCA 5.8.1/15 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch no.: not stated; purity: not stated |
| **Test system** | Human liver HepG2 cells |
| **Test method** | *Cell treatments*<br>Human hepatoma cells (HepG2) were treated during 24h with methyleugenol at 0, 50, 150 and 450 µM. Cells were treated in duplicate for all doses and DMSO (test chemical dissolvent) served as negative control. After chemical incubation, cells were harvested and DNA was isolated by using RNase and proteinase K treatments followed by phenol/chloroform extraction.<br><br>*Analysis of DNA adducts*<br>The samples were analysed in duplicate or triplicate by the dinucleotide/monophosphate version of the $^{32}$P-postlabeling assay, which is based on the resistance to cleavage by nuclease P1 of the inter-nucleotide phosphodiester linkage on the 30-side of a bulky adduct (Randerath, *et al.*, *Carcinogenesis*, 1989, 10:1231–1239). $^{32}$P-postlabeled DNA adducts were then separated by thin-layer chromatography on polyethyleneimine (PEI)-cellulose sheets, and radioactivity of fractions from individual cell cultures was determined by scintillation counting. The extent of covalent DNA adduct formation was estimated by calculating relative adduct labelling (RAL) values from sample count rates, the amount of DNA assayed (expressed as picomole DNA monomer units or DNA-P), and the specific activity of [$\gamma$-$^{32}$P]-ATP<br><br>Relative adduct labelling (RAL) = Adduct nucleotides [cpm]/ DNA – P[pmol] x Spec.act.$_{ATP}$ [cpm/pmol]. |
| **Statistical analysis of results** | Not stated. |

**Results**

Detection of adducts

Methyleugenol induced DNA adducts with a chromatographic pattern of two spots (Figure B.6.8.1.1.5-02/1), which were absent from parallel DMSO control maps. The structures of the two main adducts were identified as derivatives of $N_2$-(trans-propenylbenzene-30-yl) deoxyguanosine (spot 1, major adduct) and $N_2$-(allylbenzene-10-yl) deoxyguanosine (spot 2, minor adduct) after exposure of HepG2 cells at 150 µM of methyleugenol.

**Figure B.6.8.1.1.5-02/1. Two-dimensional PEI-cellulose TLC maps of $^{32}$P-postlabeled.**

In addition, the overall binding activity for methyleugenol was not dose-related, and the highest activity was observed at 50 µM, as shown in table B.6.8.1.1.5-02/1.

**Table B.6.8.1.1.5-02/1. RAL 3 $10^9$ values of adducts in HepG2 cells and ratios of adduct 1/adduct 2 in these cells and adult mouse liver**

| | Adduct (mean ± SEM) | | | | Adduct 1/adduct 2 | |
|---|---|---|---|---|---|---|
| | Dose (µM) | Adduct 1 | Adduct 2 | Total | HepG2[a] | Mouse[b] |
| Control | 0 | 0 | 0 | 0 | | |
| Methyleugenol | 50 | 34.35 ± 0.69 | 3.17 ± 0.75 | 37.52 ± 0.06 | 11 | 7.1 |
| | 150 | 29.22 ± 2.54 | 2.54 ± 0.35 | 31.76 ± 2.19 | | |
| | 450 | 34.16 ± 7.18 | 3.19 ± 0.25 | 37.35 ± 6.94 | | |

*a)means from three doses*
*b)means from two doses (Randerath et al. Carcinogenesis, 1984 5 1613–1622).*

According to the authors, these results demonstrate that methyleugenol is capable of altering the DNA in human hepatoma cells and thus may contribute to human carcinogenesis.

CYP1A2 has been identified as the major enzyme catalysing the 1´-hydroxylation of methyleugenol. Since HepG2 cells have been shown to contain very low or undetectable CYP1A2 activities (Wilkening *et al.*, *Drug Metab. Dispos.*, 2003, 31:1035–1042; Majer *et al.*, *Mutat. Res.*, 2004, 551, 2004:153–166), it is unlikely that, in this current study, CYP1A2 contributed significantly to the metabolic activation of methyleugenol. As HepG2 cells also contain sulphotransferases (Majer *et al.*, *Mutat. Res.*, 2004, 551, 2004:153–166), which play important roles in the sulphation of the 1´-hydroxylated derivatives, it is possible that differential affinities of sulphotransferases for this type of compounds may have contributed to the differences in adduct levels induced by methyleugenol. Furthermore, Ioannides and colleagues (Ioannides *et al.*, *Chem. Biol. Interact.*, 1985 53:303–311) have shown that compounds lacking methylenedioxy substituents (such as estragole, methyleugenol, and allylbenzene) are potent inducers of P448.

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (non-standard methodology; reliable with restrictions).

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| Methyleugenol DNA-adducts are observed in human hepatoma cells and these adducts may contribute to human carcinogenesis. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion.<br>The study is a scientific publication from a published journal. This study showed the ability of methyleugenol to bind DNA in HepG2 cells, and provides further findings that could link methyleugenol genotoxicity with its plausible role in liver tumorigenesis. |

**B.6.8.1.1.5-03. Correlation between tumors and DNA adducts in rats**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Waddell, W.J., Crooks, N.H., and Carmichael, P.L. |
| **Report year** | 2004 |
| **Report title** | Correlation of tumors with DNA adducts from methyleugenol and tamoxifen in rats |
| **Report No** | Not applicable, published study (*Toxicological Sciences*, 79:38-40) |
| **Document No** | KCA 5.8.1/16 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

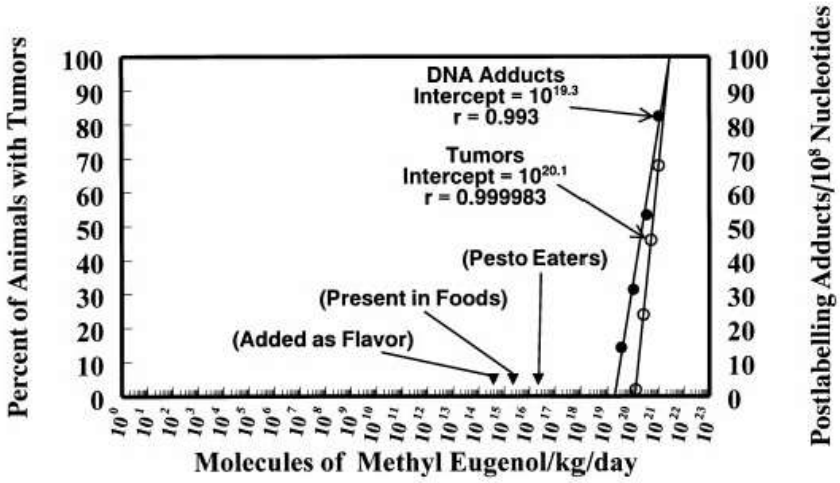
| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: 9224705, Purity: 99% |
| **Test system** | F344/N male rats |
| **Test method** | Data of tumour incidence in F344/N male rats, together with DNA adduct formation were plotted on the same graphs on a logarithmic scale in order to correlate adduct formation and tumour incidence with dose.<br>Data of tumor incidences in F344/N male rats were obtained from the 2-year NTP carcinogenesis study (B.6.8.1.1.4-01). DNA adduct data on $^{32}$P-postlabelling methyleugenol was obtained from a previous study conducted by Carmichael and colleagues (Carmichael *et al. Hum. Exp. Toxicol*, 1999. 18, 55); whereas human exposure data on methyleugenol, with details of consumption by categories of eaters, were taken from a previous publication (Wadell *et al. Toxicol. Sci.*, 2002, 68, 275–279) and were added to the correlation graph. |
| **Statistical analysis of results** | The regression statistics were calculated by the SlideWrite software. Justifications for calculating thresholds based on a log-linear plot using the Rozman scale (Rozman *et al.*, *Drug Metab. Rev.*, 1996, 28**,** 29–52) were provided by Waddell *et al.* (*Toxicol. Sci.*, 2002, 68, 275–279). |

**Results**

The resulting graph for methyleugenol showed a linear response for both adduct formation and tumour incidence (Figure B.6.8.1.1.5-03/1). The threshold dose of administered methyleugenol for adduct formation (zero adducts) was $10^{19\ 3}$ molecules of methyleugenol/kg/day, whereas a threshold of $10^{20\ 1}$ molecules of methyleugenol/kg/day was calculated for tumour formation. Moreover, the data also revealed that tumor formation did not begin until there were about 30 adducts/$10^8$ nucleotides. This threshold for tumor formation is lower than obtained for B6C3F1 mice of 30 adducts/$10^6$ nucleotides reported by Phillips *et al. (Carcinogenesis*, 1984, *5, 1623–1628).* However,

the data must to be taken with caution because they have been obtained from different species and different experiments.

On the other hand, according to the human exposure data, amount of methyleugenol is between $10^{14}$ (added as flavour) and $10^{17}$ (pesto eaters) methyleugenol/kg/day. The authors concluded the safety factor for methyleugenol is several orders of magnitude; therefore, there should be no cause for concern for humans at current levels of exposure.




*Open circles are percentage of F344/N male rats with hepatocellular carcinomas after 2-year methyleugenol administration plotted against molecules of methyleugenol/kg/day. Closed circles are DNA adducts in liver from methyleugenol plotted against dose of methyleugenol. Also it is shown consumptions of this flavour by various categories of eaters.*

**Figure B.6.8.1.1.5-03/1. Correlation plot of hepatocarcinoma incidences on F344/N rats against methyleugenol-$^{32}$P-DNA adducts and dose.**

**Conclusion**

The liver tumours generation is correlated with DNA-adducts formation after methyleugenol treatment, being 30 adducts/$10^8$ nucleotides the threshold for tumorigenesis initiation in F344/N male rats.

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| **Assessment and conclusion by applicant:**<br>This new study is considered to be acceptable as supporting information (non-standard methodology; reliable with restrictions).<br>Methyleugenol DNA-adducts are observed in human hepatoma cells and these adducts may contribute to human carcinogenesis. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion.<br>The study is a scientific publication from a published journal. This study showed the correlation between the development of hepatocellular carcinomas and methyleugenol-DNA adducts formation with the increasing dose in F344/N male rats. This analysis supports the findings that link the genotoxic potential of methyleugenol with liver neoplasm development. |

**B.6.8.1.1.5-04. Hepatocellular cancer and methyleugenol induction of DNA-adducts in rats.**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Williams, G.M., Iatropoulos, J., Jeffrey, A.M. and Duan, J-D |
| **Report year** | 2012 |
| **Report title** | Methyleugenol hepatocellular cancer initiating effects in rat liver |

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|---|---|
| **Report No** | Not applicable, published study (*Food and Chemical Toxicology*, 53:187-196) |
| **Document No** | KCA 5.8.1/17 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: TI959A, Purity: >98% |
| **Test system** | F344/N male rats |
| **Test method** | -Groups of 12 male F344/N rats received methyleugenol at 0, 27, 54 and 107 mg/kg bw/day *via* oral gavage 3 days per week for 16 weeks (initiation phase), followed by control diet (0.5% aqueous methylcellulose) for other 24 weeks (promotion phase).<br><br>-Groups of 20 male F344/N rats received methyleugenol at 0, 27, 54 and 107 mg/kg/bw/day *via* oral gavage 3 days per week for 16 weeks (initiation phase), followed by phenobarbital (PB) 500 ppm treatment in the diet for other 24 weeks (promotion phase). At 8 week and 16 week, 3/rats/group were necropsied.<br><br>At 40 weeks, the previous night the necropsy, rats were fasted, and on the following day, all the remaining animals were euthanized. Rats were exsanguinated under isofluorane anaesthesia.<br><br>Morbidity, mortality and clinical observations were conducted twice daily. Bodyweight was taken the day prior to study initiation, thereafter weekly to determine gavage volumes and before necropsy.<br><br>For clinical chemistries, at 16 weeks, prior to necropsy, blood samples were taken in non-heparinized tubes, and sera was collected and shipped frozen overnight for an external company for liver enzyme analysis of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, sorbitol dehydrogenase, and alkaline phosphatase.<br><br>At all necropsy time points (8, 16 and 40 weeks), bodyweights were measured and the livers removed and weighed. Slices of all liver lobes were used to DNA extraction for $^{32}$P nucleotide post-labelling. Two standard liver slices were taken and immersed in buffered formalin for routine H&E and immunohistochemistry microscopic evaluation (Proliferating cell nuclear antigen-PCNA at 8 and 16 weeks, and placental type-glutathione S-transferase, GST-P at 8, 16 and 40 weeks).<br><br>Only at 16 weeks, white blood cells (WBC) were isolated from whole blood.<br><br><u>*Nucleotide post-labelling analysis for DNA adducts*</u><br>DNA was isolated using QIAGEN columns and processed for $^{32}$P nucleotide post-labelling following the previous protocol (Williams *et al.*, *Toxicol. Sci.*, 1998, 45, 152–161; Jeffrey, A.M. *et al. Drug Chem. Toxicol*. 25, 93–107). The labelled modified bases were then resolved using a two-directional thin-layer chromatography system (TLC). Briefly, adducts were detected by placing the TLC plates against sensitized screens in BioRad Sample Loading Docks for 5 min (normal nucleotides) or 10 min or longer (adducts). The signals were detected by transferring the sensitised screens to |

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|---|---|
| | a BioRad Molecular Imager model and scanning at 200 mm resolution. After this, the areas corresponding to radioactivity on the TLC plates were quantitated using the Windows based software for Bio-Rad's Image Analysis Systems, and these values were copied into a spreadsheet for calculations of the adduct levels.<br><br>The evaluations of the chromatograms were done by comparison of the maps of samples from exposed rats with maps from negative controls. A positive result is obtained when chromatograms from exposed animals exhibit additional radioactive areas (spots) not present in the controls.<br><br>*Quantitation of hepatocellular proliferation*<br>Cell proliferation was assessed at 8 and 16 weeks, by scoring of PCNA (Proliferating cell nuclear antigen) positive cells. 805-1660 hepatocellular nuclei were counted, and the replicating fraction of PCNA-positive hepatocellular nuclei was obtained to establish the rate of proliferation.<br><br>*Quantitation of hepatocellular altered foci (HAF)*<br>Quantification of HAF was used to assess initiation of hepatocarcinogenesis. Foci were identified by immunohistochemical staining for GST-P (placental-type glutathione S-transferase) together with H&E staining. In two standard sections, the number of foci displaying positive immunohistochemical staining in clusters of 3 or more cells over the entire microscopic glass slide surface area was obtained and expressed as GST-P positive (GST-P+) foci per liver sample. |
| **Statistical analysis of results** | Statistical analysis for the data sets included the Holm–Sidak pairwise multiple comparison procedures, the Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks, and multiple comparisons and a one or two-way ANOVA |

**Results**

**Survival:** No compound-related deaths occurred in the study. Three deaths occurred immediately post dosing and showed evidence of being due to the gavage procedure. The first happened during the second day of dosing in the mid dose group, and the animal was then replaced. A second occurred at low dose group during the week 9, and a third, during week 11 in the high dose group. The latter two rats were not replaced.

**Clinical signs:** No clinical signs were attributed to test substance administration.

**Bodyweight:** A reduction in bodyweight was observed in all methyleugenol treated groups compared to controls during the initiation phase, displaying statistical significance only at 8 weeks (3%, 3% and 5% for low, mid and high dose group, respectively). During the 24-week post-initiation, no differences in bodyweights were found between treated groups and controls (Table B.6.8.1.1.5-04/1).

**Table B.6.8.1.1.5-04/1. Bodyweights at selected weekly time points for all rats (mean ± standard deviation)**

| Dose groups (mg/kg bw/day) | Initiation phase→weeks 1 - 16 (number of animals) | | | Promotion/maintenance phase →weeks 17 – 40 (number of animals) | |
|---|---|---|---|---|---|
| | 0 | 8 | 16 | 40 | |
| | | | | PB- | PB+ |
| **Control** | 139 ± 11 (32) | 278 ± 11.2 (32) | 328 ± 15.8 (29) | 394 ± 18.9 (12) | 363 ± 19.3 (14) |
| **27** | 139 ± 11 (32) | **270 ± 11.2 (32)* (↓3%)** | 323 ± 15.9 (28) | 389 ± 30.5 (12) | 404 ± 23.6 (13) |
| **54** | 139 ± 11 (32) | **269 ± 10.1 (32)* (↓3%)** | 317 ± 17.2 (29) | 388 ± 25.6 (12) | 400 ± 20.2 (14) |
| **107** | 139 ± 11 (32) | **263 ± 11.0 (32)* (↓5%)** | 305 ± 143.7 (28) | 384 ± 20.4 (12) | 382 ± 27.8 (13) |

**p < 0.05 compared to controls (Holm–Sidak method)*

## Liver weights

A dose-related increase in relative liver weights in both mid and high dose groups compared to control was observed at 8 weeks (12% and 15%, respectively). In the 24 weeks promotion phase, no differences in both absolute and relative liver weights with or without phenobarbital (PB) were detected between treated groups and controls. However, these differences were statistically significantly higher within all groups when compared the animals that received PB and the ones that received the vehicle (Table B.6.8.1.1.5-04/2).

**Table B.6.8.1.1.5-04/2. Liver weights at selected weekly time points for all rats (mean ± standard deviation)**

| Dose groups (mg/kg bw/day) | | Initiation phase | | | | Promotion/maintenance phase | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 8 weeks | N | 16 weeks | N | 40 weeks | | | |
| | | | | | | PB- | N | PB+ | N |
| Control | abs liver wt | 11.23 ± 0.41 | 3 | 8.80 ± 0 30 | 3 | 12.40 ± 2.99 | 12 | 14.63 ± 2.51 | 14 |
| | rel liver wt | 3.95 ± 0.13 | 3 | 2.89 ± 0.05 | 3 | 3.14 ± 0.69 | 12 | **3.68 ± 0.49** (↑17%)** | 14 |
| 27 | abs liver wt | 11.05 ± 1.24 | 3 | 9.55 ± 1.47 | 3 | 12.02 ± 3.25 | 12 | 14.89 ± 2.13 | 13 |
| | rel liver wt | 4.06 ± 0.28 | 3 | 3.28 ± 0.61 | 3 | 3.07 ± 0.69 | 12 | **3.68 ± 0.43** (↑20%)** | 13 |
| 54 | abs liver wt | 11.68 ± 0.53 | 3 | 9.08 ± 0.45 | 3 | 12.70 ± 3.85 | 12 | 15.49 ± 2.57 | 14 |
| | rel liver wt | **4.43 ± 0.14* (↑12%)** | 3 | 3.04 ± 0.08 | 3 | 3.20 ± 0.80 | 12 | **3.86 ± 0.52** (↑21%)** | 14 |
| 107 | abs liver wt | 11.32 ± 0.55 | 3 | 10.88 ± 1.64 | 3 | 12.28 ± 3.52 | 12 | 14.08 ± 2.26 | 13 |
| | rel liver wt | **4.54 ± 0.14* (↑15%)** | 3 | 3.58 ± 0 58 | 3 | 3.18 ± 0.78 | 12 | **3.68 ± 0.50** (↑16%)** | 13 |

** Statistically significant at p < 0.05 compared to control group (Holm–Sidak method).*
*** Significantly (p < 0.05) greater than PB- within the same dose group.*

## Clinical chemistry

No changes were present between methyleugenol treated groups and controls in serum levels of any of the five liver enzymes monitored at the 16 week time point (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, sorbitol dehydrogenase, and alkaline phosphatase).

## Immunochemistry

The hepatocellular replicating fraction values, expressed in percent of PCNA-positive cells over total hepatocytes counted, were statistically significantly higher in all methyleugenol treated groups at week 8 (105%, 111% and 180% for low, mid and high dose group, respectively) and 16 (74%, 83% and 148% for low, mid and high dose group, respectively), than controls. Comparing mean replicating fraction values of all groups at the two interim terminations, the 16 week values were still elevated compared to control but were slightly reduced from those at 8 weeks (Table B.6.8.1.1.5-04/3).

**Table B.6.8.1.1.5-04/3. Percent proliferating cell nuclear antigen (PCNA) replicating fraction (RF) from hepatocytes.**

| 8 Week termination | | 16 Week termination | |
|---|---|---|---|
| **Dose groups (mg/kg bw/day)** | **Replicating fraction mean ± SD** | **Dose groups (mg/kg bw/day)** | **Replicating fraction mean ± SD** |
| **Control** | 4.32 ± 0.81 | **Control** | 3.35 ± 0.22 |
| **27** | **8.85 ± 2.34a (↑105%)** | **27** | **5.82 ± 0.65a (↑74%)** |
| **54** | **9.10 ± 0.40a (↑111%)** | **54** | **6.12 ± 0.18a (↑83%)** |

| 8 Week termination | | 16 Week termination | |
|---|---|---|---|
| **Dose groups (mg/kg bw/day)** | **Replicating fraction mean ± SD** | **Dose groups (mg/kg bw/day)** | **Replicating fraction mean ± SD** |
| **107** | **12.11 ± 1.53a (↑180%)** | **107** | **8.31 ± 1.26a (↑148%)** |

** Statistically significant at p < 0.05 compared to control group (Holm–Sidak method).*

**DNA-adduct analysis**

The mean adduct values in all methyleugenol-treated groups of all measure points were statistically significantly higher than controls (see Table B.6.8.1.1.5-04/4). Two-thin-layer chromatography (TLC) analysis revealed the presence of two major adducts and one minor DNA adduct in the livers of all methyleugenol treated groups at 8, 16 and 40 week time points, whereas one major adduct was detected in control group. During the promotion/maintenance phase without methyleugenol treatment (but with or without PB), a reduction of liver DNA adducts compared to levels at 8 and 16 weeks were observed in all treated groups, including controls. The DNA adducts formation after methyleugenol administration seems to be reversible as noted at the end of promotion phase (40 week termination), in which rats did not receive methyleugenol. According to the authors, the reduction in adduct levels is probably due to DNA repair mechanisms in hepatic cells.

**Table B.6.8.1.1.5-04/4. Nucleotide $^{32}$P-postlabeling results from liver**

| | **DNA adducts expressed per $10^6$ normal nucleotides (n=3)** |
|---|---|
| **Dose groups (mg/kg bw/day)** | **Liver mean ± SD** |
| **8 Week termination** | |
| **Control** | 0.04 ± 0.03 |
| **27** | **13.70 ± 2 12* (↑34150%)** |
| **54** | **36.17 ± 2 10* (↑90325%)** |
| **107** | **48.65 ± 12.65* (↑121525%)** |
| **16 Week termination** | |
| **Control** | 0.10 ± 0.06 |
| **27** | **28.14 ± 13.18* (↑28040%)** |
| **54** | **41.55 ± 17.25* (↑41450%)** |
| **107** | **63.47 ± 15.10* (↑63370%)** |
| **40 Week termination without PB** | |
| **Control** | 0.03 ± 0.00 |
| **27** | **7.59 ± 2.88* (↑25200%)** |
| **54** | **8.79 ± 2.59* (↑29200%)** |
| **107** | **17.53 ± 3 37* (↑58333%)** |
| **40 Week termination with PB** | |
| **Control** | 0.01 ± 0.00 |
| **27** | **4.17 ± 0.28* (↑41600%)** |

| | |
|---|---|
| **54** | **6.50 ± 0.35* (↑64900%)** |
| **107** | **14.83 ± 2 35* (↑148200%)** |

**p<0.05*

**Histopathology**

During the initiation phase, methyleugenol (MEG) treated groups showed dose and time related increases in liver morphologic findings compared to control group. At 8 weeks, methyleugenol produced a diffuse hepatocellular hypertrophy, with sporadic karyomegaly and binucleation, and also activation of Kupffer cells with pigment. In periportal regions, there was an enlargement of the cholangiole plexus expanding the canals of Herring, which give rise to cholangiocyte-lined cholangioles. At 16 weeks, these morphologic findings were marginally greater.

At the 40 week termination, in both control groups (control/PB- and control/PB+), histopathology findings included centrilobular hepatocellular hypertrophy, sporadic enlargement of the periportal cholangiole plexus expanding the canals of Herring, midzonal (mainly) hepatocellular macrovesicular fat phanerosis, which occurred in all PB+ groups (including the control group), periportal infiltrate of mononuclear cells, which occurred only in the control/PB- group, and multiple foci of hepatocellular necrosis with a minor contiguous small haemorrhage, which occurred mainly in the control/PB+ group. According to the authors these changes can reflect an adaptive compensatory response to increased hepatobiliary metabolic demands.

Moreover, promotion/maintenance phase led to the development of hepatocellular adenomas in the high dose for PB- and PB+ groups (100% and 77%, respectively). In the control and the low dose groups no adenomas were found. In the mid dose group only one adenoma (8.3%) was observed in the PB- group and three adenomas (21.4%) were observed in the PB+ group (Table B.6.8.1.1.5-04/5). No liver carcinomas were noted in any of the treated animals with or without PB.

**Table B.6.8.1.1.5-04/5. Summary of liver histopathology findings**

| Dose groups (mg/kg bw/day) | Hepatocellular altered foci | | Hepatocellular adenoma (c) | |
|---|---|---|---|---|
| | Incidence (%) (a) | Multiplicity/size in µm (b) | Incidence (%) | Multiplicity/size in µm (d) |
| **8 Week MEG** | | | | |
| **Control** | 0/3 | 0 | 0/3 | 0 |
| **27** | 0/3 | 0 | 0/3 | 0 |
| **54** | 0/3 | 0 | 0/3 | 0 |
| **107** | 3/3 (100) | 1.7/204 | 0/3 | 0 |
| **16 Week MEG** | | | | |
| **Control** | 0/3 | 0 | 0/3 | 0 |
| **27** | 2/3 (66.7) | 0.7/204 | 0/3 | 0 |
| **54** | 2/3 (66.7) | 1.0/204 | 0/3 | 0 |
| **107** | 3/3 (100) | 3.7/918 | 0/3 | 0 |
| **16 Week MEG plus 24 weeks without PB** | | | | |
| **Control** | 0/12 | 0 | 0/12 | 0 |
| **27** | 1/12 (8.3) | 0.1/200 | 0/12 | 0 |
| **54** | 11/12 (91.7) | 2.8/300 | 1/12 (8.3) | <1/510 |
| **107** | 12/12 (100) | 9.7/260 | 12/12 (100) | 1.1/1000 (e) |
| **16 Week MEG plus 24 weeks with PB** | | | | |
| **Control** | 1/14 (7.1) | 0.1/200 | 0/14 | 0 |
| **27** | 2/13 (15.4) | 0.2/200 | 0/13 | 0 |
| **54** | 12/14 (85.7) | 6.9/400 | 3/14 (21.4) | <1/700 |
| **107** | 13/13 (100) | 29.6/350 | 10/13 (77) | 2.8/1000 (e) |

*a) GST-P+ HAF in percent incidence of rats with at least one HAF per liver sample (2 slides).*
*b) GST-P+ HAF multiplicity (number) value per group, expressed as HAF/liver sample and the greatest diameter (size) in lm.*
*c) Hepatocellular adenoma(s) in absolute and percent incidence with at least one adenoma per rat, no statistical significance was evident comparing mid and high PB- and PB+ groups.*
*d) Multiplicity of adenoma(s) per group and greatest diameters in µm.*

*e) High PB+ greater than high PB- dose groups at p < 0.005.*

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| **Assessment and conclusion by applicant:**<br>This new study is considered to be acceptable as supporting information. The study is well conducted, however investigated parameters are limited compared to those recommended in OECD test guidelines (reliable with restrictions).<br>Methyleugenol induce rapid initiating activity in male rat liver with DNA adducts formation and possibly cell proliferation which could contribute to its hepatocarcinogenicity. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion. This study showed how methyleugenol promotes liver neoplasm and histopathology alterations through DNA-adduct formation as first impairment event. The authors reported in same *in vivo* study the relation between DNA alteration and the further development of liver neoplasm. Therefore, these results support previous findings that link the genotoxic potential of methyleugenol with liver neoplasm development.<br>Therefore, due to adverse effects (mainly DNA-adducts and altered liver foci), were observed at all dose levels, a NOAEL cannot be established. |

**B.6.8.1.1.5-05. Bioactivation of methyleugenol**

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|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Gardner, I., Wakazono, H., Bergin, P., de Waziers, I., Beaune, P., Kenna, J.G., Caldwell, J. |
| **Report year** | 1997 |
| **Report title** | Cytochrome P450 mediated bioactivation of methyleugenol to 1'-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes |
| **Report No** | Not applicable, published study (*Carcinogenesis*, 18,9:1775-1783) |
| **Document No** | KCA 5.8.1/18 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch no.: not stated; purity: not stated |
| **Test system** | F344/N rats |
| **Test method** | <u>*Animal treatment with P450 inductors*</u><br>Inducers of cytochromes P450 were administered intraperitoneally (i.p.): sodium phenobarbital (100 mg/kg bw/day for 3 days; *n*=3) and isoniazid (100 mg/kg bw/day for 10 days; *n*=6) were administered in saline, dexamethasone (80 mg/kg bw/day for 3 days; *n*=3) and isosafrole (150 mg/kg bw/day for 3 days; *n*=3) were administered in tricaprylin. Control animals received equivalent volumes (< 1 ml/kg per rat) of the saline or tricaprylin dose vehicles alone.<br><br><u>*In vivo administration of methyleugenol*</u><br>5 male F344/N rats/group were treated with methyleugenol dissolved in tricaprylin, at 10, 30, 100 and 300 mg/kg bw/day for five days *via* i.p injection. |

| | |
|---|---|
| | *Preparation of hepatic microsomes*<br>Animals were killed by cervical dislocation and the livers were removed and placed in ice cold sucrose buffer. Livers were blotted to remove excess buffer, weighed, and minced thoroughly with scissors. Five volumes of fresh sucrose buffer were added and homogenates were prepared using eight strokes of a Potter homogenizer, at 1000 rpm. The homogenates were strained through muslin and centrifuged at 10000xg for 20 min., the pellets were resuspended in fresh sucrose buffer and re-centrifuged. The combined 10000xg supernatants were centrifuged at 100000xg for 60 min to sediment the microsomal fraction. Microsomal pellets were resuspended in TRIS buffer, then snap frozen in liquid nitrogen and stored at -80°C until use.<br><br>*Determination of microsomal cytochrome P450 and protein content*<br>Microsomal cytochrome P450 content was determined by the reduced carbon monoxide difference spectrum method of Omura and Sato (Omura, T., *et al. J. Biol.Chem.,*1964*;* 239: 2370-2378) using the reported extinction coefficient of 91 mM/cm. Protein content was determined using a bicinchonic acid assay method.<br><br>*Measurement of 1 '-hydroxymethyleugenol formation*<br>Methyleugenol (1-2000µM) was incubated with microsomal suspensions (0.5 mg protein/ml) from untreated rats in the presence of an NADPH generating system (1 mM NADP+, 10 mM glucose-6-phosphate and 0.5 units glucose-6-phosphate dehydrogenase). Control experiments consisted of incubations performed with (i) methyleugenol and microsomes in the absence of the NADPH generating system. (ii) methyleugenol and the generating system without microsomes, and (iii) microsomes and generating system without methyleugenol.<br><br>Methyleugenol transformation was analysed by gas chromatography coupled to mass (GC-MS). Samples were dissolved in 100 µl acetonitrile and derivatised by heating at 60°C for 10 min with *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). Monitoring was performed in the single ion mode at m/z 250 (for 1' -hydroxysafrole-Trimethylsilyl) and m/z 266 (for 1'-hydroxymethyleugenol-Trimethylsilyl). 1'–hydroxymethyleugenol was quantitated using a calibration curve prepared by adding known amounts of 1'-hydroxymethyleugenol to boiled microsomes. Initial experiments verified that reaction rates were linear with respect to protein concentration and time.<br><br>*Inhibition studies*<br>The experiments were performed using 20 µM methyleugenol, and the inhibitors: Troleandomycin (200 µM), furafylline (25 and 100 µM), cimetidine (50 µM), p-nitrophenol (50 and 200 µM), diallylsulfide (200 µM and 1 mM), α-naphthoflavone (1 and 10 µM), quinine (1 and 10 µM) and tolbutamide (200 µM, 500 µM and 1mM).<br><br>*Human liver microsomes*<br>13 human liver samples were obtained from deceased humans kidney donors. Liver samples were obtained within 1 h of circulatory arrest and small portions (~1 $cm^3$) were cut, snap-frozen by immersion in liquid nitrogen, and stored at -80°C. Subsequently, liver samples were thawed and microsomal fractions were isolated as described above. |
| **Statistical analysis of results** | Kinetic analysis was performed using PC-Non Lin software with a weighting of $1/y^2$. Ki was determined mathematically and graphically from Dixon plots. Statistical significance was assessed either by two-way analysis of variance followed by the least significant difference test or by the non-parametric Mann-Whitney U-test, as appropriate. The level of statistical significance was set at $P < 0.05$. |

## Results

### Kinetics of formation of 1 '-hydroxymethyleugenol

Analysis of the kinetics of 1 '-hydroxylation of methyleugenol formation in control male F344 rat liver microsomes yielded a non-linear Eadie-Hofstee plot (Figure B.6.8.1.1.5-05/1), indicating the existence of distinct high and low affinity enzymatic reactions. The high affinity activity exhibited a *Km* of 74.9±9.0 µM and a Vmax of 1.42±0.17 nmol/min/mol P450 (mean±SD of three experiments undertaken with different microsomal preparations). Due to the low solubility of methyleugenol it was not possible to define the kinetics of the low affinity reaction accurately, but it had an apparent *Km* > 2 mM.

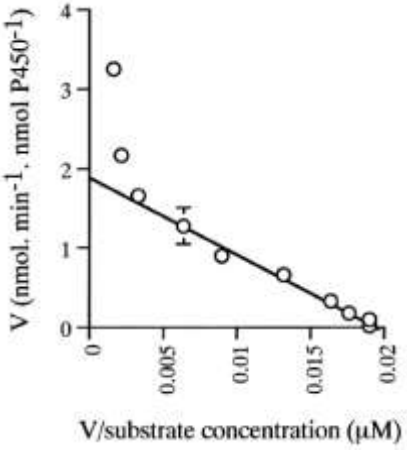


**Figure B.6.8.1.1.5-05/1. Kinetics of l '-hydroxylation of methyleugenol in control rat liver microsomes.**

### Methyleugenol metabolism

*Induction of 1-hydroxymethyleugenol in vivo*

Rats treated i.p. with methyleugenol for 5 days exhibited statistically significant increase and dose-related formation of l '-hydroxymethyleugenol at 30 (38%), 100 (45%) and 300 (66%) mg/kg bw/day. (Table B.6.8.1.1.5-05/1).

**Table B.6.8.1.1.5-05/1. Auto-induction of bioactivation of methyleugenol in rats treated with the compound *in vivo* for 5 days**

| | Methyleugenol treatment (mg/kg bw/day) | | | | |
|---|---|---|---|---|---|
| | **0** | **10** | **30** | **100** | **300** |
| **Rate of 1 '-hydroxylation of methyleugenol (nmol/min/nmol P450)** | 0.42±0.03 | 0.43 ± 0.04 | **0.58 ± 0.07** (↑38%)** | **0.61 ± 0.09** (↑45%)** | **0.70 ± 0.13** (↑66%)** |

***p<0.01*

*P450 isozymes induction and 1´- hydroxymethyleugenol formation*

Microsomal suspension obtained from rats previously treated with P450 inducers were incubated with different doses of methyleugenol (1-2000µM). At 20 µM of methyleugenol, formation of 1 '-hydroxymethyleugenol was increased significantly compared with vehicle treated animals (Table B.6.8.1.1.5-05/2). Phenobarbitone, isoniazid, dexamethasone and isofrole induced P450 protein expression and higher formation of 1-hydroxymethyleugenol than controls. Phenobarbital has been shown to induce CYP 2B1/2, **CYP 2C** and CYP 3A isozymes, dexamethasone is an inducer of CYP 3A and 2B isozymes, isosafrole induces CYP 1A1 and CYP 1A2 isozymes, whereas isoniazid is a selective **CYP 2E1** inducer.

**Table B.6.8.1.1.5-05/2. Induction of liver microsomal 1' -hydroxylation of methyleugenol**

| | | 1 'hydroxymethyleugenol formation | |
|---|---|---|---|
| **Inducer** | **Cytochrome P450 content (nmol/mg protein)** | **(nmol/min/mg protein)** | **(nmol/min/nmol P450)** |
| **Saline control** | 0.70 ± 0.05 | 0.36 ± 0.03 | 0 51 ± 0.04 |
| **Tricaprylin control** | 0.73 ± 0.09 | 0.31 ± 0.02 | 0.42 ± 0.03 |
| **Phenobarbitone** | **1.82 ± 0.09* (↑160%)** | **2.11 ± 0.14* (↑486%)** | **1.16 ± 0.08* (↑127%)** |
| **Isoniazid** | **0.86 ± 0.07* (↑22%)** | **0.73 ± 0.09* (↑102%)** | **0.85 ± 0.10* (↑66%)** |
| **Dexamethasone** | **1.33 ± 0.15* (↑90%)** | **2.03 ± 0.10* (↑463%)** | **1.52 ± 0.08* (↑198%)** |
| **Isosafrole** | **1.16 ± 0.04* (↑65%)** | **1.91 ± 0.26* (↑430%)** | **1.65 ± 0.22* (↑223%)** |

**p<0.05*

*P450 isozymes inhibition and 1´- hydroxymethyleugenol formation*

Assays undertaken at a low methyleugenol concentration (20 µM) revealed that formation of 1´hydroxymethyleugenol was inhibited by p-Nitrophenol **(**PNP), diallylsulfide, tolbutamide and α-naphthoflavone, but not by troleandomycin, furafylline, quinine or cimetidine (Table B.6.8.1.1.5-05/3). PNP was the most potent inhibitor, causing 54% inhibition, followed by tolbutamide (40%), diallylsulphide (37%) and a-naphthoflavone (24%).

**Table B.6.8.1.1.5-05/3. Influence of isozyme-selective inhibitors of cytochromes P450 on bioactivation of methyleugenol in microsomes from livers of untreated rats**

| **P450 inhibitors** | **l '-hydroxymethyleugenol formation (percent of control activity)** |
|---|---|
| **p-Nitrophenol (50 µM)** | **78 ± 5* (↓22%)** |
| **p-Nitrophenol (200 µM)** | **46 ± 12* (↓54%)** |
| **Diallylsulfide (200 µM)** | 88 ± 10 |
| **Diallysulfide (1 mM)** | **63 ± 0* (↓37%)** |
| **Tolbutamide (200 µM)** | 83 ± 10 |
| **Tolbutamide (500 µM)** | **73 ± 7* (↓27%)** |
| **Tolbutamide (1 mM)** | **60 ± 7* (↓40%)** |
| **α-naphthoflavone (1 µM)** | 88 ± 7 |
| **α-naphthoflavone (10 µM)** | **76 ± 2.5* (↓24%)** |
| **Troleandomycin (50 µM)** | 111 l± 9 |
| **Troleandomycin (200 µM)** | 89 ± 10 |
| **Furafylline (25 µM)** | 100 ± 3 |
| **Furafylline (100 µM)** | 89 ± 5 |
| **Quinine (1 µM)** | 100 ± 0.2 |
| **Quinine (10 µM)** | 98 ± 1 |
| **Cimetidine (50 µM)** | 90 ± 9.8 |

**p<0.05*

*1' -Hydroxylation of methyleugenol in human liver microsomes*

1' -hydroxylation of methyleugenol in human liver microsomal fractions was conducted using a concentration of substrate (200 µM) approximately equivalent to the Vmax of the high affinity enzymatic component identified in rat liver microsomes. These studies revealed a 37-fold variation in the rate of the reaction. The rate of 1'–hydroxylation observed in the most active human liver sample (1328±100 pmol/min/mg protein) was similar to

those seen in control male rat liver microsomes, when assayed at 200 µM substrate (1.28±0.23 nmol/min/mg protein).

**Conclusions**

Isozyme-selective chemical inhibitors experiments revealed that, when assayed at low substrate concentration (20 µM), 1'-hydroxylation of methyleugenol in liver microsomes from untreated rats is catalysed predominantly by **CYP 2E1,** and by one or more additional isozymes (most probably **CYP 2C6**).

Involvement of **CYP 2E1** in 1'-hydroxymethyleugenol formation is consistent with the inhibition of the reaction seen in the presence of diallylsulfide and PNP (37% and 54% inhibition, respectively), which selectively inhibit this isozyme. This interpretation is further supported by the observation that methyleugenol competitively inhibited hydroxylation of PNP to 4-nitrocatechol (data not shown), which is a reaction catalysed by CYP 2E1. Moreover, CYP 2El has been implicated previously in the bioactivation and metabolism of a number of other hepatotoxins and carcinogens, such as paracetamol, benzene, nitrosamines and carbon tetrachloride (Guengerich, F.P., *et al., Chem. Res. Toxicol.,* 1991, 4, 168-179; Patten, C.J., *et al., Chem. Res. Toxicol.,* 1993, 6, 511-518).

Involvement of **CYP 2C6** in 1'-hydroxylation of methyleugenol seems likely in view of the significant (40%) inhibition of the reaction by tolbutamide, which has been shown to inhibit CYP 2C6 mediated reactions and by α-naphthoflavone (24% inhibition). α-naphthoflavone has been shown to inhibit tolbutamide hydroxylation (Veronese, M., *et al., Drug. Met. Disp.,* 1990, 18, 356-361), and to inhibit the $N_4$- hydroxylation of sulfamethoxazole, which is catalysed by CYP 2C6 (Cribb, A.E., *et al., Drug. Met. Disp.,* 1995, 23, 406-414). Although α-naphthoflavone is known to be a potent inhibitor of CYP 1A isozymes (Shimada, T. *et al., Biochem. Pharmacol.,* 1988, 37, 459-464), 1'-hydroxylation of methyleugenol was not inhibited significantly by the potent CYP 1A2 inhibitor furafylline (Kunze, K.L. *et al., Chem. Res. Toxicol.,* 1992, 6, 649-656; Sesardic, B. *et al., Br. J. Clin. Pharmacol.,* 1990, 29, 651-653). This excludes the possibility that CYP 1A2 makes a significant contribution to catalysis of the high affinity component of 1'–hydroxymethyleugenol formation in livers of untreated rats. Moreover, the relatively modest induction of 1 -hydroxymethyleugenol formation evident in microsomes from livers of rats treated with phenobarbital (2.3-fold) is also consistent with induction of CYP 2C6.

Since l '-hydroxylation is the first step in metabolic bioactivation of methyleugenol, any factor increasing the fraction of a dose of methyleugenol metabolized by this pathway is likely to increase the amount of electrophilic metabolite bound to cellular DNA and protein, and thus increase the potency of methyleugenol as a carcinogen and hepatotoxic.

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable as supporting information (reliable with restrictions; non-standard study design).

Methyleugenol caused a dose-dependent auto-induction of the 1'-hydroxylation of methyleugenol reaction in rats. Hydroxylation of methyleugenol activities varied considerably (37-fold) in the human liver samples tested; the highest hydroxylation activity was similar to that of the liver microsomes of control rats. These results suggest that the risk to humans ingesting methyleugenol is subject to marked inter-individual variability

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion. This study revealed the P450 isozymes involved in the first step of methyleugenol metabolism. The results showed that hydroxylation of the chemical is catalysed predominantly by **CYP 2E1** and **CYP 2C6** isozymes.

Moreover, the 37-fold variation in the rate of 1'-hydroxymethyleugenol formation observed in the 13 human liver preparations raises the possibility that the toxic potential of the compound is subject to metabolic activities and expression levels of the enzymes involved, which imply an inter-individual variability in the human population.

**B.6.8.1.1.5-06. Gene expression changes in liver mice after methyleugenol treatment**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Iida, M., Anna, C.H., Holliday, W.M., Collins, J.B., Cunningham, M.L., Sills, R.C. and Devereux, T.R. |
| **Report year** | 2005 |
| **Report title** | Unique patterns of gene expression changes in liver after treatment of mice for 2 weeks with different known carcinogens and non-carcinogens. |
| **Report No** | Not applicable, published study (*Carcinogenesis*, 26,3: 689-699) |
| **Document No** | KCA 5.8.2/04 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Methyleugenol. Lot/Batch No.: not stated, Purity: not stated |
| **Test system** | B6C3F1 female mice |
| **Test method** | Four female B6C3F1 mice per group were administered by gavage a single dose of methyleugenol at 75 mg/kg bw/day for 2 weeks, among other chemicals known to be or not liver carcinogens.<br>At the end of the treatment period, mice were killed and the livers were collected and early gene expression changes associated with liver carcinogenesis were identified. First, expression of 12 genes found previously to be altered in liver after 2 weeks treatment with oxazepam and Wyeth-14643 was examined in livers from the various chemical treatment groups (Iida, M., *et al.*, *Carcinogenesis*, 2003, 24: 757-770). These gene expression changes were not good early markers for eugenol in this study. In addition, expression of 20842 genes was assessed by oligonucleotide microarray.<br><br><u>*RNA isolation, direct label protocol and feature extraction*</u><br>Total liver RNA was isolated with RNeasy Midi Kit according to the manufacturer's protocols. The quality of the RNA was evaluated by measuring the 260:280 nm absorbance ratio. Gene expression analysis was conducted using the Agilent Mouse Oligo array of 20842 genes. Ten micrograms of RNA from each sample was labelled using the Agilent Fluorescent Direct Label Kit protocol. Hybridizations were performed for 16 h in a rotating hybridization oven using the Agilent 60mer oligo microarray processing protocol. Two hybridizations with fluor reversals were performed for each liver RNA sample, and four individual livers were used from each chemical treatment group. The control samples were pools of RNA from 10 individual livers, and the RNA was isolated from each individual liver and tested for quality prior to mixing. Females in treated groups were compared with female controls.<br><br>Microarray expression data were analysed using Rosetta Resolver System and GeneSpring software.<br><br>*Reverse transcription*<br>To remove genomic DNA, RNA samples were incubated with 1 U/mg RNA of RNase-free DNase. The DNase I was then inactivated by addition of 2.5 mM EDTA and heating. Reverse transcription of RNA (1 mg) was carried out with MuLV enzyme. A 1:10 dilution of this cDNA with DNase-free water was used for real-time PCR (RT- |

| | |
|---|---|
| | PCR) analysis in 96-well plates.<br><br>*Real-time PCR analysis*<br>Quantitative gene expression levels were determined using real-time PCR and SYBR Green I dye or TaqMan MGB probes. Samples were analysed in duplicate and a sample without reverse transcriptase was included with each plate to detect contamination by genomic DNA. Amplification was carried out as follows: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of 95ºC for 15s and 60ºC for 30s. For SYBR Green I dye reactions dissociation curves were also created by adding the following steps to the end of the amplification reaction: 95ºC for 15s and 60ºC for 20s, then gradually increasing to 95ºC over 20 min, with a final hold at 95ºC for 15s. Fold increases or decreases in gene expression were determined by quantitation of cDNA from target (treated) samples relative to a calibrator sample (untreated). The β-actin gene was used as the endogenous control for normalization of initial RNA levels. To determine this normalized value, $2^{-\Delta\Delta CT}$ values were compared between target and calibrator samples, where the change in crossing threshold ($\Delta Ct$)= $\Delta Ct_{target\ gene}$ -$\Delta Ct_{\beta\text{-actin}}$ , and $\Delta\Delta Ct$= $\Delta Ctcontrol$-$\Delta Ct_{treatment}$. |
| **Statistical analysis of results** | Gene expression values in the microarrays analysis that were increased or decreased ≥ 1.5-fold with a p value ≤0.05 by t-Student test, in three or four out of four animals (using two chips per animal) were selected. |

**Results**

Expression analysis of 12 genes by Q-PCR

Authors analysed the expression of 12 genes that were previously found altered in mice liver after oxazepam and Wyeth 14643 treatments. The results were statistically significant only for 4 genes: *GADD45β, TSC-22* and *EST2* were up-regulated, whereas *LZTS2* was down-regulated (Table B.6.8.1.1.5-06/1).

**Up-regulated genes:**
-*GADD45β* (Growth arrest and DNA damage inducible beta) is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents.

-*TSC-22* (Transforming growth factor b stimulated clone 22) is a putative tumor suppression gene and a transcription factor that plays a role in tumor suppression through the induction of cancer cell apoptosis.

-*EST2* (telomerase-reverse-transcriptase) encodes a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis. Studies in mouse suggest that telomerase also participates in chromosomal repair, since *de novo* synthesis of telomere repeats may occur at double-stranded breaks.

**Down-regulated gene:**
-*LZTS2* (Leucine Zipper Tumor Suppressor 2) encodes a protein which belongs to the leucine zipper tumor suppressor family of proteins, involved in transcription regulation and cell cycle control.

**Table B.6.8.1.1.5-06/1. Up and down-regulated genes after methyleugenol treatments**

| Gene | Methyleugenol |
|---|---|
| *GADD45β* | 1.56 ± 0.32 |
| *TSC-22* | 1.55 ± 0.51 |
| *LZTS2* | 0.59 ± 0.04 |
| *EST2* | 1.54 ± 0.74 |

Microarrays analysis

Changes in the expression of the following genes were found in the microarray analysis (± 1.5 fold-change): *CDK1A*, *CCNG1*, *EGR 1* and *DNASEα* were up-regulated, whereas *FHIT*, *WWOX*, *INHBA* and *CISH* were down-regulated. The results were further confirmed by the gold standard methodology QRT-PCR (see Table B.6.8.1.1.5-06/2).

**Up-regulated genes:**
*CDKN1A*: This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin/cyclin-dependent kinase 2 or cyclin/cyclin-dependent kinase 4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen, a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair.

*CCNG1*: The protein encoded by this gene is a member of the cyclin family and contains the cyclin box. This protein is involved in eukaryotic cell cycle regulations.

*EGR1*: It is a transcriptional regulator that binds to DNA and regulates the transcription of numerous target genes, and thereby plays an important role in regulating the response to growth factors, DNA damage, and ischemia. It plays a role in the regulation of cell survival, proliferation and cell death. Activates expression of p53/TP53 and TGFB1, and thereby helps prevent tumor formation.

*DNASE2a:* This gene encodes a member of the DNase family. The protein, located in the lysosome, hydrolyses DNA under acidic conditions and mediates the breakdown of DNA during erythropoiesis and apoptosis.

**Down-regulated genes:**
*FHIT:* The protein encoded by this gene is a P1-P3-bis(5'-adenosyl) triphosphate hydrolase involved in purine metabolism. This gene encompasses the common fragile site *FRA3B* on chromosome 3, where carcinogen-induced damage can lead to translocations and aberrant transcripts. In fact, aberrant transcripts from this gene have been found in about half of all esophageal, stomach, and colon carcinomas. The encoded protein is also a tumor suppressor, as loss of its activity results in replication stress and DNA damage.

*WWOX*: This gene encodes a member of the short-chain dehydrogenases/reductases (SDR) protein family. This gene spans the FRA16D common chromosomal fragile site and appears to function as a tumor suppressor gene. Expression of the encoded protein is able to induce apoptosis, while defects in this gene are associated with multiple types of cancer.

*INHBA:* This gene encodes a member of the TGF-beta (transforming growth factor-beta) superfamily of proteins. The encoded preproprotein is proteolytically processed to generate a subunit of the dimeric activin and inhibin protein complexes. These complexes are involved in reproduction and development.

*CISH*: CIS family members are known to be cytokine-inducible negative regulators of cytokine signalling, so they are important negative regulators for inflammatory signalling.

**Table B.6.8.1.1.5-06/2. Up and down-regulated genes by oligo array and QRT-PCR after treatment for 2 weeks**

| | | |
|---|---|---|
| *CDKN1A* [Cyclin-dependent 1A (P21)] | Oligo array | 1.737 |
| | RT-PCR | 2.81±1.03 |
| *CCNG1* (Cyclin G1) | Oligo array | 1.55 |
| | RT-PCR | 3.54±1 |
| *EGR 1* (Early growth) | Oligo array | 2.072 |
| | RT-PCR | 4.30±2.37 |
| *FHIT* (Fragile histidine triad gene) | Oligo array | 0.387 |
| | RT-PCR | 0.004±0.001 |
| *WWOX* (WW domain-containing oxidoreductase) | Oligo array | 0.285 |
| | RT-PCR | 0.76±0.02 |

| | | |
|---|---|---|
| *INHBA* (Inhibin b-A) | Oligo array | 0.527 |
| | RT-PCR | 0 32±0.14 |
| *CISH* ( Cytokine inducible SH2-containing protein) | Oligo array | 0.123 |
| | RT-PCR | 0.32±0.14 |
| *DNASE2α* (Deoxyribonuclease IIα) | Oligo array | 1.927 |
| | RT-PCR | 0.82±0.07 |

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable for the renewal of eugenol and is only supportive (reliable with restrictions as the study is not GLP, only dose rate is tested, do not inform on the hazard assessment).

The analyses revealed that several cancer-related genes, including Fhit, Wwox, Tsc-22 and Gadd45b, were induced or repressed in unique patterns for specific carcinogens and not altered by the non-carcinogens such a eugenol. The data indicate that even if the tumour response, including molecular alterations, is similar, such as for oxazepam and methyleugenol, early gene expression changes appear to be carcinogen specific and seem to involve apoptosis and cell cycle related genes.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The study is a scientific publication from a published journal.

This study shows the gene expression changes occurred in liver as consequence of methyleugenol treatment. It is noteworthy the increased or decreased expression levels in genes involved in key cellular processes:

| Cell process | *Gene* |
|---|---|
| **Cell cycle** | ↑*GADD45*β<br>↑*CDKN1A*<br>↑*CCNG1*<br>↑*EGR1*<br>↓*LZTS2* |
| **Apoptosis** | ↑*TSC-22*<br>↑*EGR1*<br>↑*DNASEα*<br>↓*FHIT*<br>↓*WWOX* |
| **DNA repair** | ↑*EST2*<br>↑*EGR1*<br>↓*FHIT*<br>↓*WWOX* |
| **Tumor suppressors** | ↑*CDKN1A*<br>↑*TSC-22*<br>↓*FHIT*<br>↓*WWOX*<br>↓*LZTS2* |
| **Inflammation** | ↓*CISH* |

Relevant genes involved in DNA repair (*EST2* and *EGR1*), cell cycle regulation (*CDKN1A*, *CCNG1*, *EGR1* and *GADD45β)* and apoptosis (*TSC-22*, *EGR1* and *DNASEα*) are up-regulated. These findings are signs that cell presents DNA damage, and the DNA repair mechanisms are thereupon activated. Consequently, due to this potential DNA damage, cell activates cycle arrest and apoptosis pathway. The down-regulated expression in *CISH* revealed that an inflammatory process is occurring in liver tissue. Moreover, relevant tumor suppressors such as *FHIT*, *WWOX* and *LZTS2* are down-regulated, and these are common features in tumorigenesis.

## B.6.8.2. Supplementary studies on the active substance

*Four supplementary published studies have been provided to support the renewal of the active substance eugenol. Three of these studies were included in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC (Yokota et al., 1988, B.6.8.2.1, Rompelberg, et al. 1993, B.6.8.2.2 and Thompson et al., 1998, B.6.8.2.3). Thus, one new study (Iida et al., 2005, B.6.8.2.4), which analyses gene expression changes induced after eugenol treatment, has been submitted in the renewal of active substance.*

### B.6.8.2.1. Liver enzymatic activity in rats

| | |
|---|---|
| **Data point** | CA 5.8 (No EU data requirement) |
| **Report author** | Yokota, H., Hashimoto, H., Motoya, M., Yuasa A. |
| **Report year** | 1988 |
| **Report title** | Enhancement of UDP-Glucuronyltransferase, UDP-Glucose dehydrogenase, and glutathione S-transferase activities in rat liver by dietary administration of Eugenol. |
| **Report No** | Not applicable, published study *(Biochemical Pharmacology*, 1988; 37, 5: 799-802). |
| **Document No** | KCA 5.8.2/02 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supportive only (not a relevant endpoint or test system). |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: not specified |
| **Test system** | Male Fisher F344/N rats |
| **Test method** | Male Fisher F344/N rats were fed a diet containing eugenol to observe its effect on liver drug-detoxifying enzymes such as UDP-Glucuronyltransferase (GT), UDP-glucose dehydrogenase (DH) and glutathione S-transferase (GST). For that purpose, the following experiment was performed.<br><br>-Three groups of Fisher F344/N rats (3 animals/group) of 6-7 weeks of age were tested with eugenol *via* diet at 0, 1%, 3% and 5% (equivalent to 0, 1000 and 3000 mg/kg bw/day) during 23 days.<br><br>-Another experiment was carried out during 13-week, in which eugenol was tested *via* diet with the following schedule:<br>Week 1: 1%<br>Week 2: 5%<br>Week 3-8: 3%<br>Week 9-10: 1%<br>Week 11-12: 3%<br>Week 13: 0 %<br>A group with a control diet was used.<br>3 male Fisher rats were included in both control and dose groups.<br><br>Rat´s livers were weighted after necropsy and prior of microsome extraction. However, histopathological examinations of livers were not performed. |

| | |
|---|---|
| | *Preparation of liver microsomes from rats.*<br>Liver microsomes were prepared from eugenol-treated rats as described by Imai and Sato (1974) as follows. Rat livers, after being perfused with 0.15 M KCl, were minced and homogenized with 4 vol. of the same solution. The homogenate was centrifuged for 15 min at 9000g. Then, this fraction was centrifuged at 105000 g for 60 min to obtain the microsomes.<br><br>*Assay and other methods.*<br>UDP-Glucuronyl-transferase activities toward various substrates were assayed at the following concentrations: l-naphthol (0.5 mM); 4-nitrophenol (0.5 mM); 4-hydroxybiphenyl (0.5 mM); 4-methylumbelliferone (1 mM); and bilirubin (0.1 mM). UDP-Glucose dehydrogenase activity was assayed by the method of Strominger and Maxwell (1957). Glutathione S-transferase activity was assayed by spectrophotometric methods. Cytochrome P-450 was determined by the method of Omura and Sato (1964). Protein was determined by the method of Lowry.<br><br>Histopathological examinations of liver were not performed prior microsome extraction. |
| **Statistical analysis of results** | Not stated. |

**Results**

**Liver weights:** Liver weights were not affected significantly by a diet containing 3 % eugenol for 13 weeks.

**Enzymatic assays:** The activities of GT of liver microsomes towards various xenobiotic substances such as 4-nitrophenol, 1-naphthol, 4-hydorybiphenyl and 4-methylumbelliferone were enhanced by dietary administration of eugenol, but the activity of GT toward its endogenous substrate, bilirubin, was not changed. Dose-response relationships between the enhancement GT activities toward these xenobiotics and the dose of eugenol were observed. The induced higher activities of GT toward these xenobiotics were maintained during 13 weeks of eugenol treatment. Similar results on DH and GST activities in the liver cytosol were obtained by dietary administration of eugenol, while no effect on cytochrome P-450 content in the liver microsomes from the rats fed eugenol diet was observed during 13 weeks.

**Conclusion:**
These results suggest that the intracellular content of the active intermediates of various drugs or carcinogens would be reduced by this specific enhancement of drug-detoxifying enzymes in the liver of rats given a diet containing eugenol.

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| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. Dietary administration of eugenol induced a dose dependent elevation of activity of detoxifying enzymes in rat liver suggesting the intracellular content of the active intermediates of various drugs or carcinogens would be reduced. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This published study suggests that eugenol, in certain concentrations might induce the activity of important detoxifying enzymes and exerts a protective role in drugs detoxification, controlling antioxidant defences and cell survival. |

**B.6.8.2.2. Enzymatic activity of phase I and phase II enzymes in rats**

| | |
|---|---|
| **Data point** | CA 5.8 (No EU data requirement) |
| **Report author** | Rompelberg, C.J.M., Verhagen, H., Van Bladeren, P.J., |
| **Report year** | 1993 |

| | |
|---|---|
| **Report title** | Effects of the naturally occurring alkenylbenzenes Eugenol and trans-anethole on drug-metabolizing enzymes in the rat liver. |
| **Report No** | Not applicable, published study (*Fd. Chem. Toxic*., 31, 9: 637-645). |
| **Document No** | KCA 5.8.2/03 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supportive only (not a relevant endpoint or test system). |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol; Lot/Batch No.: not specified, Purity: 99% |
| **Test system** | Male Wistar rats |
| **Test method** | In order to study the effects of eugenol on drug-metabolizing enzyme activities *in vivo*, male Wistar rats (3 animals/group) were treated *via* gavage with eugenol at 0, 250, 500 and 1000 mg/kg bw/day for 10 days. The control group was given corn oil. In all instances doses were administered in a volume of 10 ml/kg body weight. 24 hr after the last dose the rats were killed under ether anaesthesia and blood was collected from the aorta into tubes containing EDTA or heparin.<br>Livers were perfused *in situ* by way of the portal vein with 1% heparin in phosphate buffered and stored.<br><br>Haematological parameters were determined in EDTA-blood samples: numbers of erythrocytes, leucocytes and platelets, haemoglobin content and haematocrit.<br><br>*Preparation of microsomes and cytosols.*<br>Frozen livers were thawed on ice and homogenized in four volumes of ice-cold 1.15% KCI using a Potter- Elvehjem glass-teflon homogenizer. The cell debris, nuclei and mitochondria were removed by centrifugation. The supernatant was centrifuged at 100000g for 60min at 4°C. The supernatant representing the cytosolic fraction was separated and the pellet containing the microsomal fraction was resuspended in ice-cold sodium phosphate buffer.<br><br>*Enzyme assays.*<br>Cytosolic and microsomal protein content was quantified by the method of Lowry. Total microsomal cytochrome P-450 content was determined according to the method of Rutten (1987). Microsomal dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) was assayed by the fluorimetric method of Rutten *et al.* (1992).<br>Cytosolic GT activity was determined using 4- chlorophenol and 4-hydroxybiphenyl as substrates as described by Mulder and van Doom (1975). GST activity was determined using l-chloro-2,4-dinitrobenzene as substrate as described by Habig (1974).<br><br>DTD activity was assayed by the method of Emster (1967) with some modifications. The methods for determination of EROD, PROD, GT, GST and DTD activities were performed on a Cobas-Bio centrifugal analyser (Wortelboer. 1992). Levels of L-aspartate aminotransferase (ASAT) and L-alanine aminotransferase (ALAT) were assessed in plasma using a Cobas-Bio centrifugal analyser as described by the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974).<br><br>*HPLC of GST subunits.* |

| | |
|---|---|
| | Isolation of GST and separation of GST subunits were performed as described by Bogaards (1989). GST subunits were detected with a 2140 Rapid Spectral Detector at 214 nm. |
| **Statistical analysis of results** | Data are presented as means ± standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test and an orthogonal polynomial test (Dixon, 1988). A probability of $P \leq 0.05$ was considered significant. |

**Results**

**Survival:** No mortality or morbidity signs were observed during the study

**Clinical signs:** No clinical signs were detected during experiment in eugenol dose groups.

**Bodyweights:** A decrease in bodyweight gain was observed at study termination in high dose group (12%) compared with controls, although differences were not statistically significant, most likely due to high standard deviations (table B.6.8.2.2/2).

**Table B.6.8.2.2/1. Summary of bodyweight (g)**

| | Dose (mg/kg bw/day) (Mean ± SD) | | | |
|---|---|---|---|---|
| **Day** | **0** | **250** | **500** | **1000** |
| **0** | 246.0 ± 12.1 | 244.7 + 10.2 | 242.0 ± 10.1 | 250.0 ± 22.3 |
| **6** | 266.7 ± 16.3 | 258.0 ± 21.9 | 264.3 ± 5.9 | 249.3 ± 33.7 |
| **10** | 283.0± 18.1 | 275.0 ± 19.3 | 270.7 ± 6.4 | 257.7 ± 40.4 |

**Table B.6.8.2.2/2. Summary of bodyweight gain (g) (%)**

| | Dose (mg/kg bw/day) | | | |
|---|---|---|---|---|
| **Day** | **0** | **250** | **500** | **1000** |
| **0** | 0 | 0 | 0 | 0 |
| **6** | 20.7 (8%) | 13.3 (5%) **(↓3%)** | 22.3 (9%) **(↑1%)** | -0.7 (0) **(↓8%)** |
| **10** | 37 (15%) | 30.3 (12%) **(↓3%)** | 28.7 (12%) **(↓3%)** | 7.7 (3%) **(↓12%)** |

*(% bodyweight gain referred to day 0)*

**Clinical chemistry:** Administration of eugenol did not display statistically significant differences between treated groups and controls. However, several changes (>10% or <10%) were recorded in some clinical chemistry parameters (see table B.6.8.2.2/3):
-Decrease of WBC levels in mid and high dose groups (13% and 12%, respectively).
-Increase in PLT and ASAT levels in high dose group (12% and 13%, respectively).
-Increase of ALAT levels in all dose groups (22%, 38% and 60%, for low, mid and high dose group, respectively).

**Table B.6.8.2.2/3. Summary of clinical chemistry parameters (mean ±SD)**

| | Dose (mg/kg bw) | WBC (X $10^9$/litre) | RBC (X $10^{12}$/litre) | PLT (X $10^9$/litre) | HGB (mM) | HCT (litre/litre) | ASAT (U/litre plasma) | ALAT (U/litre plasma) |
|---|---|---|---|---|---|---|---|---|
| **Controls** | **0** | 7.8 ± 1.9 | 6.9 ± 0.1 | 913 ± 91 | 8.9 ± 0.2 | 0.4 ±0.0 | 56.0 ± 3.6 | 31.3 ± 9.5 |
| **Eugenol (doses)** | **250** | 7.2 ± 3.8 | 7.1 ±0.4 | 906 ± 82 | 9.0 ± 0.4 | 0.4 ±0.0 | 58.3 ± 2.9 | 38.3 ± 10.0 **(↑22%)** |
| | **500** | 6.8 ± 3.9 **(↓13%)** | 7.3 ± 0.3 | 908 ± 92 | 9.1 ±0.4 | 0.4 ±0.0 | 59.7± 10.6 | 43.3 ± 6.5 **(↑38%)** |
| | **1000** | 6.9 ± 2.0 **(↓12%)** | 7.2 ± 0.3 | 1020 ± *55* **(↑12%)** | 9.3 ± 0.4 | 0.4 ±0.0 | 63.3 ± 8.0 **(↑13%)** | 50.0 ± 9.5 **(↑60%)** |

*WBC white blood cells (leucocytes)*

*RBC red blood cells (erythrocytes)*
*PLT platelets*
*HGB haemoglobin*
*HCT haematocrit*

**Liver weight:** No differences in liver weights were observed between treated groups and controls (table B.6.8.2.2/4).

**Table B.6.8.2.2/4. Summary of relative liver weight (mean ±SD)**

| Dose (mg/kg bw/day) | Relative liver weight (%) |
|---|---|
| **0** | 3.9 ± 0.1 |
| **250** | 4.1±0.1 |
| **500** | 4.1 ±0.0 |
| **1000** | 4.2 ± 0.4 |

**Effects of eugenol on phase-I enzyme activities:** An increase, not statistically significant, of total cytochrome P-450 content was recorded in low and high dose groups compared with controls (15% and 19%, respectively). An induction not dose-related of P-450 levels were detected in all treated groups (table B.6.8.2.2/5).

**Table B.6.8.2.2/5. Summary of total amount of P-450 (mean ±SD)**

| Dose (mg/kg bw/day) | Total P450 (pmol/mg protein) |
|---|---|
| **0** | 493 ± 128 |
| **250** | 568 ± 23 **(↑15%)** |
| **500** | 523 ± 38 **(↑6%)** |
| **1000** | 585 ± 122 **(↑19%)** |

**Effects of eugenol on phase-II enzyme activities:**

At high dose group, statistically significant increase in cytochrome P-450-dependent activity for 7-ethoxy-resorufin O-deethylation (EROD) and 7-pentoxyresorufin O-depentylation (PROD) were recorded (2.5 and 3.4 fold compared with controls, respectively). UDP-glucuronyl transferase (GT) activity towards the substrate 4-chlorophenol was statistically significant increased in all treated rats (1.6-3.1 fold compared with controls), and activity towards 4-hydroxybiphenyl as substrate was statistically significantly higher in mid and high dose groups (2.3 and 3.2 fold compared with controls, respectively).

On the other hand, statistically significant increase in cytosolic glutathione S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene was found for in mid and high dose groups (1.3and 1.5 fold compared with controls). In addition, significantly increased levels of GST subunit 2 were measured by HPLC in the liver cytosol of rats treated with 500 and 1000 mg/kg bw/day of eugenol.

DTD activities were elevated 2.2-, 1.5- and 2.4-fold in the low, mid and high dose groups, respectively, although none of these increases was statistically significant.

**Assessment and conclusion by applicant:**

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. Administration of eugenol induces phase-II biotransformation enzymes in rat liver *in vivo*.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This published study shows that eugenol increases the levels and activity of phase I and II liver enzymes, exerting a protective role in drugs detoxification, controlling antioxidant defences and cell survival. Moreover, an increase of ASAT enzyme was recorded in high dose groups, whereas and increase of ALAT was recorded in all dose groups. A NOAEL < 250 mg/kg bw/day could be established based on increased levels of ALAT in all dose groups.

### B.6.8.2.3. Toxicity *in vitro* of eugenol and its quinone methide metabolite

| | |
|---|---|
| **Data point** | CA 5.8.1 (EU data requirement) |
| **Report author** | Thompson, D.C., Barhoumi, R. and Burghardt, R.C. |
| **Report year** | 1998 |
| **Report title** | Comparative toxicity of eugenol and its quinone methide metabolite in cultured liver cells using kinetic fluorescence bioassays. |
| **Report No** | Not applicable, published study (*Toxicol. Appl. Pharm.*, Vol 149, 55-63) |
| **Document No** | KCA 5.8.2/01 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated and accepted in DAR (2011) |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/Reliability** | Supporting only |

**Materials and Methods**

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|---|---|
| **Test chemical** | Eugenol (2-methoxy-4-(2-propenylphenol); Lot/Batch No.: not specified, Purity: not specified<br>Eugenol Quinone Methide (EQM): prepared in situ |
| **Test system** | Rat liver cells (Clone 9, ATCC) |
| **Test method** | *Cell culture*:<br>The rat liver cell line Clone 9 was grown in Ham's nutrient mixture F-12 containing 10% foetal bovine serum. Cells were seeded at a density of 25000 cells/cm$^2$ and were cultured 48h before laser cytometry analyses.<br>*Fluorescent probes*:<br>Cellular GSH levels were evaluated with the cell-permeant monochlobimane (mBCl) probe, which is non-fluorescent until forms a fluorescent adduct with GSH in a reaction catalysed by glutathione S-transferase.<br>The laser cytometer was used at an excitation wavelength of 351-363 nm, and the emitted fluorescence (461 nm) was detected with a barrier filter (BP 485/45).<br>The calcium-sensitive fluorophore, fluo-3, which exhibits about a 40-fold enhancement of fluorescence intensity with $Ca^{2+}$ binding, was used to monitor changes in $Ca^2$.<br>DCFDA was used to evaluate the rate of toxicant-induced formation of hydroperoxides.<br>The potentiometric probe rhodamine 123 was used to provide a relative measure and to monitor dynamic changes in mitochondrial membrane potential (MMP) in the kinetic studies.<br>*Experimental*:<br>For the kinetic analysis, cells were scanned once to identify background fluorescence prior to the addition of either 50 µM mBCl eugenol (1-1000) µM) or 50 µM mBCl plus EQM (1-1000 µM) or 50 µM mBCl plus EQM (1-1000) and then fluorescence intensity was recorded as above.<br>For ($Ca^{2+}$), measurements, cells were loaded with fluo-3, AM (3µM) for 1 h at 37°C, whereas for DCFDA (3 µM) rhodamine 123 (5 µg/mL), DiBAC4 (3) (5 µM), and SNARF (5µM), cells were loaded for 20 min at 37°C. Cells loaded with DiBAC4 (3) were scanned in the continued presence of extracellular DiBAC4(3), whereas cells loaded with fluo-3, |

| | |
|---|---|
| | DCFDA, rhodamine 123 and SNARF were washed four times with serum-free media prior analysis.<br>For analysis of gap junction-mediated intercellular communication (GJIC), cells were loaded with 10 µg/ml CFDA, a membrane-permeable, no fluorescent precursor of carboxyfluorescein, in medium for 15 min at 37°C, washed three times, and maintained in serum-free medium prior to FRAP analysis.<br>For the cytotoxicity assays, cells were incubated with test compounds for 2 h. At this time point, cells were loaded with indicator dyes from LIVE/DEAD. |

**Results**

The most potent effects of eugenol were seen on GJIC and GSH levels.

Suppression of GJIC occurred very early and at low concentrations of eugenol. Within 5 min of addition, the rate constant for GJIC decreased by approximately 27 and 88 % by the 10 and 100 µM eugenol concentrations, respectively. By 10 min, 10 µM eugenol further suppressed GJIC (approximately 52 %), whereas the effect of the higher concentration already appeared to be maximal.

A significant depletion of GSH was seen as early as 3 min following exposure to 10 µM eugenol. By 10 min, this concentration of eugenol resulted in a 25 % loss of the mBCl-GSH fluorescence intensity. The 1 µM concentration had no significant effect on GSH levels compared with control cells, while higher concentrations of eugenol did not further lower GSH levels beyond that observed with the 10-µM dose. In addition to its effects on GJIC and GSH, eugenol inhibited basal cellular ROS generation at low concentrations of 10 and 100 µM, respectively. No significant changes in MMP, [$Ca^{2}$+], PMP or intracellular pH were detected at the 10-µM concentration.

In order to correlate the initial changes observed in the kinetic experiments with cell death in this cell line, long-term experiments were carried out. The $IC_{50}$ determined after 48 h of exposure of the two compounds were 35 and 17.8 µM for eugenol and EQM, respectively. In a second set of experiments, the ability of eugenol and EQM to induce cell death was assessed following 2-h incubations with various concentrations of each compound. As shown in the following table, a concentration of 100 µM EQM produced greater than 90 % cell death at this time point. A similar concentration of eugenol did not produce significant cell death. However, 1000 µM eugenol caused greater than 90 % cell death within 2 h. In the presence of GSHEt, cell death from both 100 µM EQM and 1000 µM eugenol was completely prevented.

**Table B.6.8.2.3. Effect of GSHEt on cytotoxicity of eugenol and EQM**

| Treatment group | Cytotoxicity (% live cells) | |
|---|---|---|
| | (-)GSHEt | (+)GSHEt (1 mM) |
| **Control** | 100 | 100 |
| **Eugenol (100 µM)** | > 90 | > 90 |
| **Eugenol (1000 µM)** | < 10 | > 90 |
| **EQM (1000 µM)** | < 10 | > 90 |

**Conclusion:**

Eugenol depleted intracellular GSH, inhibited GJIC and generation of ROS and had a modest effect on MMP at concentration of 10 to 100 µM. At high concentrations (1000 µM), eugenol also affected [$Ca^{2+}$], PMP, and pH.

Effects of EQM were seen at lower concentrations (1 to 10 µM). The earliest and most potent effects of either eugenol or EQM were seen on GSH levels and GJIC. Co administration of glutathione ethyl ester enhanced intracellular GSH levels by almost 100 % and completely protected cells from cell death caused by eugenol and EQM.

These results suggest that eugenol mediates its hepatotoxic effects primarily through depletion of cytoprotective thiols-dependent processes such as GJIC. Furthermore, these results support the hypothesis that the toxic effects of eugenol are mediated through its quinone methide metabolite. The effects of EQM closely mimicked those of eugenol, with the major difference being that EQM elicited the same effects at lower concentrations. EQM is more potent than eugenol, and this is consistent with the hypothesis that EQM is the toxic metabolite of eugenol and it is directly responsible for the hepatotoxic effects of the parent compound.

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| **Assessment and conclusion by applicant:**<br>The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.<br>The primary effects of eugenol on Clone 9 cells were the depletion of reduced GSH pools (significant at concentrations as low as 10 µM) and interference with GJIC. Also, eugenol depleted generation of ROS and had a low effect on MMP at concentration of 10 to 100 µM. At high concentrations (1000 µM), eugenol also affected $[Ca^{2+}]i$, PMP, and pH. Effects of EQM were seen at lower concentrations.<br>The authors suggested eugenol mediates its hepatotoxic effect primarily through depletion of cytoprotective thiols and interference in thiol-dependent processes such as GJIC. Furthermore, the results support the hypothesis that the toxic effects of eugenol are mediated through its quinone methide metabolite. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** The RMS agrees with the notifier's assessment and conclusion. This study is a scientific publication from a peer-reviewed journal. The RMS deems the study as supporting information to evaluate the potential mechanism of toxicity of eugenol and its reactive metabolite EQM in liver cells. The postulated mechanism of toxicity involves the depletion of cytoprotective thiols and interference in thiol-dependent processes such as GJIC. |

**B.6.8.2.4. Gene expression changes in liver mice after eugenol treatment**

| | |
|---|---|
| **Data point** | CA 5.8 (EU data requirement) |
| **Report author** | Iida, M., Anna, C.H., Holliday, W.M., Collins, J.B., Cunningham, M.L., Sills, R.C. and Devereux, T.R. |
| **Report year** | 2005 |
| **Report title** | Unique patterns of gene expression changes in liver after treatment of mice for 2 weeks with different known carcinogens and non-carcinogens. |
| **Report No** | Not applicable, published study (*Carcinogenesis*, 26,3: 689-699) |
| **Document No** | KCA 5.8.2/04 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting only (not a relevant endpoint or test system). |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol. Lot/Batch No.: not stated, Purity: not stated |
| **Test system** | B6C3F1 female mice |
| **Test method** | 4 female B6C3F1 mice per group were administered by gavage a single dose of eugenol at 75 mg/kg bw/day for 2 weeks, among other chemicals known to be or not liver carcinogens.<br>At the end of the treatment period, mice were killed and the livers were collected and early gene expression changes associated with liver carcinogenesis were identified. First, expression of 12 genes found previously to be altered in liver after 2 weeks treatment with oxazepam and Wyeth-14643 was examined in livers from the various chemical treatment groups (Iida, M., *et al.*, *Carcinogenesis*, 2003, 24: 757-770). These gene expression changes were not good early markers for eugenol in this study. In addition, expression of 20842 genes was assessed by oligonucleotide microarray. |

| | |
|---|---|
| | *RNA isolation, direct label protocol and feature extraction*<br>Total liver RNA was isolated with RNeasy Midi Kit according to the manufacturer's protocols. The quality of the RNA was evaluated by measuring the 260:280 nm absorbance ratio. Gene expression analysis was conducted using the Agilent Mouse Oligo array of 20842 genes. Ten micrograms of RNA from each sample was labelled using the Agilent Fluorescent Direct Label Kit protocol. Hybridizations were performed for 16 h in a rotating hybridization oven using the Agilent 60mer oligo microarray processing protocol. Two hybridizations with fluor reversals were performed for each liver RNA sample, and four individual livers were used from each chemical treatment group. The control samples were pools of RNA from 10 individual livers, and the RNA was isolated from each individual liver and tested for quality prior to mixing. Females in treated groups were compared with female controls.<br><br>Microarray expression data were analysed using Rosetta Resolver System and GeneSpring software.<br><br>*Reverse transcription*<br>To remove genomic DNA, RNA samples were incubated with 1 U/mg RNA of RNase-free DNase. The DNase I was then inactivated by addition of 2.5 mM EDTA and heating. Reverse transcription of RNA (1 mg) was carried out with MuLV enzyme. A 1:10 dilution of this cDNA with DNase-free water was used for real-time PCR (RT-PCR) analysis in 96-well plates.<br><br>*Real-time PCR analysis*<br>Quantitative gene expression levels were determined using real-time PCR and SYBR Green I dye or TaqMan MGB probes. Samples were analysed in duplicate and a sample without reverse transcriptase was included with each plate to detect contamination by genomic DNA. Amplification was carried out as follows: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of 95ºC for 15s and 60ºC for 30s. For SYBR Green I dye reactions dissociation curves were also created by adding the following steps to the end of the amplification reaction: 95ºC for 15s and 60ºC for 20s, then gradually increasing to 95ºC over 20 min, with a final hold at 95ºC for 15s. Fold increases or decreases in gene expression were determined by quantitation of cDNA from target (treated) samples relative to a calibrator sample (untreated). The β-actin gene was used as the endogenous control for normalization of initial RNA levels. To determine this normalized value, $2^{-\Delta\Delta CT}$ values were compared between target and calibrator samples, where the change in crossing threshold (ΔCt)= $\Delta Ct_{target\ gene}$ -$\Delta Ct_{\beta\text{-actin}}$ , and ΔΔCt= ΔCtcontrol-$\Delta Ct_{treatment}$. |
| **Statistical analysis of results** | Gene expression values in the microarrays analysis that were increased or decreased ≥ 1.5-fold with a p value ≤0.05 by t-Student test, in three or four out of four animals (using two chips per animal) were selected. |

**Results**

Expression analysis of 12 genes by Q-PCR

Authors analysed the expression of 12 genes that were previously found altered in mice liver after oxazepam and Wyeth 14643 treatments. The results were statistically significant only for 3 genes: *GADD45β and EST2* were up-regulated, whereas *IGFBP5* was down-regulated (table B.6.8.2.4/1).

**Up-regulated genes:**
-*GADD45β* (Growth arrest and DNA damage inducible beta) is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents.

-*EST2* (telomerase-reverse-transcriptase) encodes a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase

activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis. Studies in mouse suggest that telomerase also participates in chromosomal repair, since *de novo* synthesis of telomere repeats may occur at double-stranded breaks.

**Down-regulated gene:**
- *IGFBP5* (Insulin Like Growth Factor Binding Protein 5) encodes a protein involved in insulin pathway and metabolism of proteins.

**Table B.6.8.2.4/1. Up and down-regulated genes after eugenol treatments**

| Gene | Eugenol |
|---|---|
| *GADD45β* | 2.21 ± 0.21 |
| *IGFBP5* | 0.4 ± 0.09 |
| *EST2* | 1.98 ± 0.37 |

Microarrays analysis

Changes in the expression of the following genes were found in the microarray analysis (± 1.5 fold-change): Only *DNASEα* was up-regulated, whereas *INHBA* and *CISH* were down-regulated. The results were further confirmed by the gold standard methodology QRT-PCR (see table B.6.8.2.4/2).

**Up-regulated genes:**
*DNASE2a:* This gene encodes a member of the DNase family. The protein, located in the lysosome, hydrolyses DNA under acidic conditions and mediates the breakdown of DNA during erythropoiesis and apoptosis.

**Down-regulated genes:**
*INHBA:* This gene encodes a member of the TGF-beta (transforming growth factor-beta) superfamily of proteins. The encoded preproprotein is proteolytically processed to generate a subunit of the dimeric activin and inhibin protein complexes. These complexes are involved in reproduction and development.

*CISH*: CIS family members are known to be cytokine-inducible negative regulators of cytokine signalling, so they are important negative regulators for inflammatory signalling.

**Table B.6.8.2.4/2. Up and down-regulated genes by oligo array and QRT-PCR after treatment for 2 weeks**

| | | |
|---|---|---|
| *INBHA* (Inhibin b-A) | Oligo array | 0.534 |
| | RT-PCR | 0.1±0.02 |
| *CISH* ( Cytokine inducible SH2-containing protein) | Oligo array | 0.105 |
| | RT-PCR | 0.1±0.02 |
| *DNASE2a* (Deoxyribonuclease IIα) | Oligo array | 1.792 |
| | RT-PCR | 0.98±0.03 |

**Assessment and conclusion by applicant:**

This new study is considered to be acceptable for the renewal of eugenol and is only supportive (reliable with restrictions as the study is not GLP, only dose rate is tested, do not inform on the hazard assessment).

The analyses revealed that several cancer-related genes, including Fhit, Wwox, Tsc-22 and Gadd45b, were induced or repressed in unique patterns for specific carcinogens and not altered by the non-carcinogens such a eugenol. The data indicate that even if the tumour response, including molecular alterations, is similar, such as for oxazepam and methyleugenol, early gene expression changes appear to be carcinogen specific and seem to involve apoptosis and cell cycle related genes.

**Assessment and conclusion by RMS:**

| |
|---|
| **Outcome and conclusion of the study:** The study is a scientific publication from a published journal.<br><br>This study shows the gene expression changes occurred in liver as consequence of eugenol treatment. In the QRT-PCR analysis *GADD45β* and *EST2* were up-regulated. These genes are involved in DNA repair, cell cycle regulation and cellular senescence. Only *CISH* down-regulation was the most relevant finding in the microarray approach.<br><br>In contrast to expression changes observed as a result of methyleugenol treatment (B.6.8.1.12), no relevant changes compared with control livers were found after eugenol dosage; no differences were noted in expression genes involved in key cellular processes such as DNA repair, cell cycle, apoptosis or tumor suppression. |

## B.6.8.3. Studies on endocrine disruption

*The ED criteria according to Points 3.6.5 and 3.8.2 of Annex II of Regulation (EC) No 1107/2009, as amended by Commission Regulation (EU) 2018/605, and subsequently the ECHA/EFSA guidance document (2018), should be applied for all substances which have a pending decision on approval or renewal of approval.*

*The dataset for assessing the ED properties of eugenol comprises a number of mammalian toxicology studies that include: sub-chronic and short-term studies (section B.6.3), carcinogenicity/long-term studies (section B.6.5), and prenatal developmental toxicology studies (section B.6.6.2).*

*In vitro mechanistic data available from the US EPA CompTox Chemicals Dashboard (v.4) for the thyroid, estrogenic, androgenic and steroidogenic pathways has been evaluated as part of the ED assessment (sections B.6.8.3.1, B.6.8.3.2, B.6.8.3.3 and B.6.8.3.4, respectively). In silico QSAR predictions from Danish database were also included (B.6.8.3.7).*

*Additionally, the data set also includes 2 studies provided by the applicant (B.6.8.3.5, and B.6.8.3.6).*

### B.6.8.3.1. Thyroid activity in ToxCast model

| | |
|---|---|
| **Data point** | CA 5.8.3 (EU data requirement) |
| **Report author** | US EPA ToxCast: https://comptox.epa.gov/dashboard |
| **Report year** | Data extracted on March 2021 |
| **Report title** | Thyroid activity in ToxCast model |
| **Report No** | Not applicable |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP** | Not applicable |
| **Acceptability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol |
| **Test system** | US EPA ToxCast program |
| **Test method** | In the ToxCast program, thousands of chemicals, including eugenol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities.<br>The US EPA ToxCast program has been consulted for the eugenol https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data in relation to the thyroid pathway. |

**Results:**

The EDSP21 tab in the CompTox Chemicals Dashboard v4 includes 13 thyroid receptor bioassays for eugenol (table B.6.8.3.1, Study ID Matrix No: 1-13). Negative results were obtained in 12 of them. A positive result was obtained for eugenol in the thyroid peroxidase assay NCCT_TPO_AUR_dn; Study ID Matrix No. 5). The $AC_{50}$ value was 5.57 µM (Hill Model). The limit of cytotoxicity for this assay was reported to be 1000 µM (Figure B.6.8.3.1).

**Table B.6.8.3.1. Summary of US EPA ToxCast EDSP21- thyroid bioactivity assays for eugenol**

| Assay endpoint | Study ID Matrix | Assay type | Organism | Result | AC50 # | Flag |
|---|---|---|---|---|---|---|
| ATG_THRa1_TRANS_up | 1 | mRNA induction | human | Inactive | - | - |
| ATG_THRa1_TRANS_dn | 2 | mRNA induction | human | Inactive | - | - |
| LTEA_HepaRG_THRSP_dn | 3 | mRNA induction | human | inactive | - | - |
| LTEA_HepaRG_THRSP_up | 4 | mRNA induction | human | inactive | - | - |
| **NCCT_TPO_AUR_dn** | **5** | **Thyroid peroxidase activity** | **rat** | **Active** | **5.57** | **-** |
| NCCT_HEK293T_CellTiterGLO | 6 | ATP content | human | Inactive | - | - |
| NCCT_QuantiLum_inhib_2_dn | 7 | Enzyme (monooxygenase) activity | n/a (cell component source: E. coli) | Inactive | - | - |
| Tox21_TSHR_Agonist_ratio | 8 | cAMP measurement | human | Inactive | - | - |
| Tox21_TSHR_Antagonist ratio | 9 | cAMP measurement | human | Inactive | - | - |
| Tox21_TSHR_wt_ratio | 10 | cAMP measurement | human | Inactive | - | - |
| Tox21_TR_LUC_GH3_Agonist | 11 | Luciferase induction | Rat | Inactive | - | - |
| Tox21_TR_LUC_GH3_Antagonist | 12 | Luciferase induction | Rat | Inactive | - | - |
| Tox21_TR_LUC_GH3_Antagonist _viability | 13 | ATP content | Rat | Inactive | - | - |

*# Chemical concentration (in µM) at which 50% of the maximum response is achieved.*

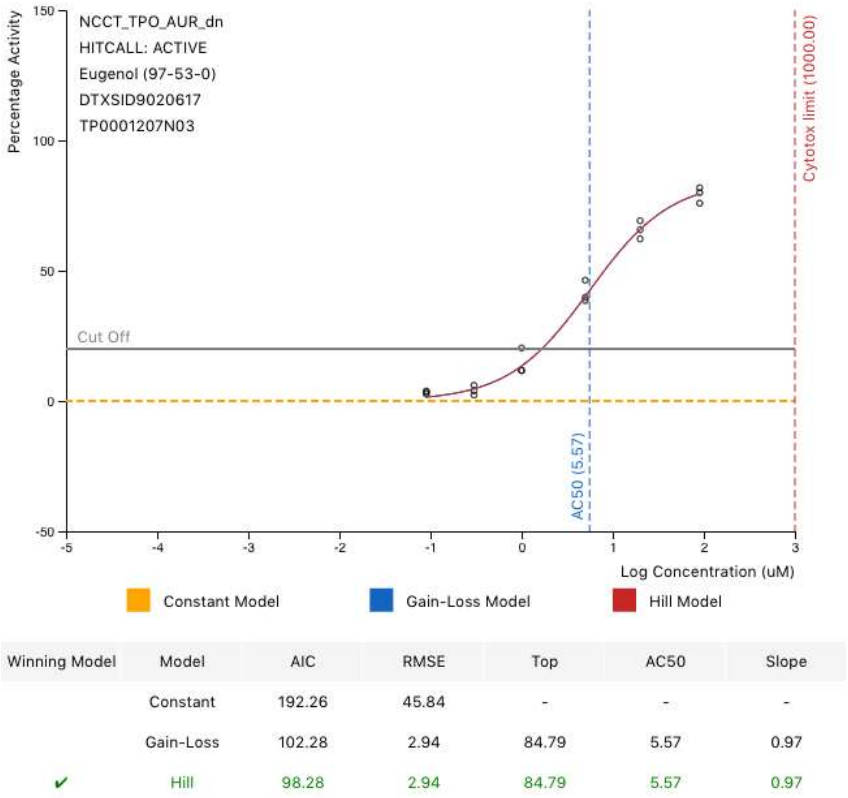



**Figure B.6.8.3.1: Dose-response curve of the active thyroid ToxCast bioassay (ID Matrix 5)**

| |
|---|
| **Assessment and conclusion by Applicant:**<br>Three *in vitro* ToxCast™ thyroid assays (ATG_THRa1_TRANS_up, Tox21_TR_LUC_GH3_Agonist and Tox21_TR_LUC_GH3_Antagonist) were listed for eugenol under the endocrine disrupter screening programme assays (EDSP21) on the CompTox™ Dashboard. Data were available for the thyroid receptor in human liver cell lines and in rat pituitary gland cell lines. Eugenol was inactive in these assays; there is no evidence for T-mediated activity.<br>Thyroid changes were not observed across the available *in vivo* studies assessing T-parameters, and there was no evidence for receptor transactivation in the *in vitro* ToxCast™ assays. Therefore, following assessment of the integrated lines of evidence for the T-modality, it is concluded that there is no evidence for T-mediated activity at tolerable doses. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study**: As of the date of this report, there were 13 assays on thyroid activity associated with the EDSP21 tab in the CompTox Chemicals Dashboard.<br>Based on this battery of *in vitro* assays, eugenol is active (assay NCCT_TPO_AUR_dn) for thyroid peroxidase inhibition (TPO). This somehow is in agreement with the alerts structural data raised by the models QSAR1 and QSAR 2 which predicted that eugenol has potential inhibit Thyroperoxidase (TPO) activity. |

### B.6.8.3.2. Estrogenic activity in ToxCast model

| | |
|---|---|
| **Data point** | CA 5.8.3 (EU data requirement) |
| **Report author** | US EPA ToxCast: https://comptox.epa.gov/dashboard |
| **Report year** | Data extracted on March 2021. |
| **Report title** | Estrogenic activity in ToxCast model |
| **Report No** | Not applicable |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP** | Not applicable |
| **Acceptability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol |
| **Test system** | US EPA ToxCast program |
| **Test method** | In the ToxCast program, thousands of chemicals, including eugenol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities.<br>The US EPA ToxCast program has been consulted for the eugenol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the estrogenic pathway. |

**Results**

Eugenol was tested in 19 assays included in the ToxCast ER Bioactivity Model component. (Table B.6.8.3.2/1). The results were positive/active for only 2 assays (ATG_ERa_TRANS_up and ATG_ERE_CIS_up) and negative for 17 assays.

**Table B.6.8.3.2/1. Summary of US EPA ToxCast EDSP21- estrogenic bioactivity assays for eugenol**

| Assay endpoint | Study ID Matrix | Assay type | Organism | Result | AC50 # | Flag |
|---|---|---|---|---|---|---|
| ACEA_ER_80hr | 14 | real-time cell-growth kinetics | human | Inactive | - | - |
| ACEA_ER_AUC_viability | 15 | real-time cell-growth kinetics | human | Inactive | - | - |
| **ATG_ERa_TRANS_up** | **16** | **mRNA induction** | **human** | **Active** | **43.26** | **-** |
| **ATG_ERE_CIS_up** | **17** | **mRNA induction** | **human** | **Active** | **24.58** | **Borderline active. Less than 50% efficacy** |
| NVS_NR_hER | 18 | radiolligand binding | human | Inactive | - | - |
| OT_ER_ERaERa_0480 | 19 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ER_ERaERa_1440 | 20 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ER_ERaERb_0480 | 21 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ER_ERaERb_1440 | 22 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ER_ERbERb_0480 | 23 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ER_ERbERb_1440 | 24 | protein fragment complementation assay | human | Inactive | - | - |
| OT_ERa_EREGFP_0120 | 25 | fluorescent protein induction | human | Inactive | - | - |
| OT_ERa_EREGFP_0480 | 26 | fluorescent protein induction | human | Inactive | - | - |
| TOX21_ERa_BLA_Agonist_ratio | 27 | beta lactamase induction | human | Inactive | - | - |
| TOX21_ERa_BLA_Antagonist_ratio | 28 | beta lactamase induction | human | Inactive | - | - |
| TOX21_ERa_BLA_Antagonist_viability | 29 | ATP content | human | Inactive | | |
| TOX21_ERa_LUC_VM7_Agonist | 30 | luciferase induction | human | Inactive | - | - |
| TOX21_ERa_LUC_VM7_Antagonist_specificity | 31 | luciferase induction | human | Active | - | - |
| TOX21_ERa_LUC_VM7_Antagonist_specificity_viability | 32 | ATP content | human | Inactive | - | - |

# *Chemical concentration (in µM) at which 50% of the maximum response is achieved.*

Both this positives/actives are cell-based, multiplexed-readout assays that use HepG2 liver cell line with measurements taken at 24 hours after chemical dosing in a 24-well plate. The ATG_ERE_CIS_up assay presented the flags:"borderline active" and "less than 50% efficacy". However, active result from ATG_ERa_TRANS_up was not flagged and more reliable. The dose-response curves from these active assays are shown in figure B.6.8.3.2.

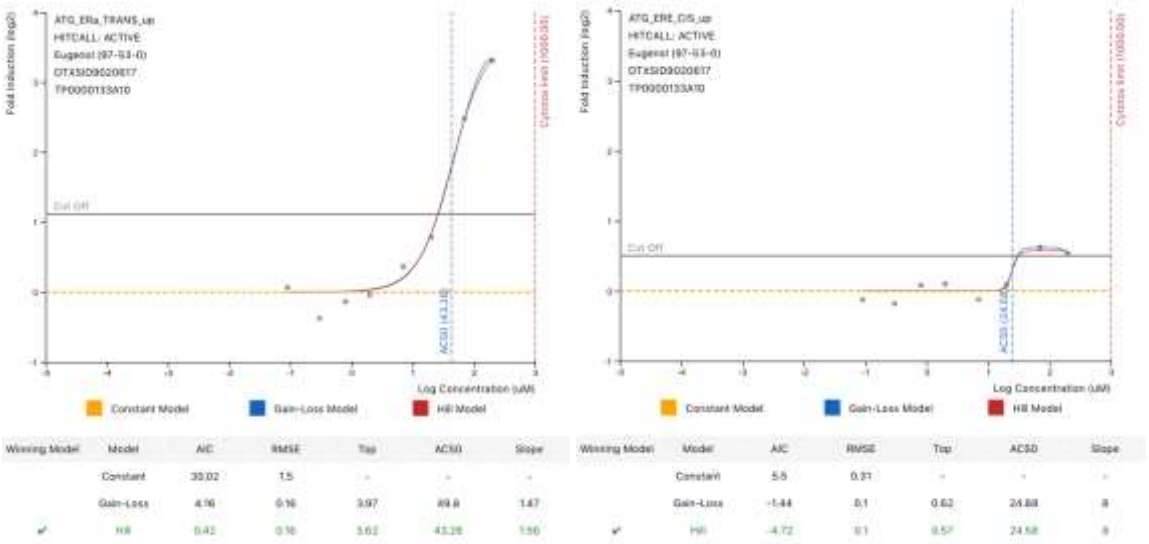



**Figure B.6.8.3.2: Dose-response curves of the active estrogenic ToxCast bioassays (ID Matrix 16 y 17)**

The CERAPP (from consensus) model indicates that eugenol is active as an ER agonist, ER antagonist and as receptor binding. On the other hand, the scores from the ER ToxCast model predictions shows that eugenol is inactive as an ER agonist or antagonist with AUC (area under the curve) values of 0 (Table B.6.8.3.2/2).

**Table B.6.8.3.2/2. ER ToxCast model predictions**

| Model | Agonist | Antagonist | Binding |
|---|---|---|---|
| ToxCast Pathway Model (AUC) | 0.00 | 0.00 | - |
| CERAPP* Potency Level (From Literature) | - | Inactive (Inactive) | - |
| CERAPP* Potency Level (Consensus) | Active (weak) | Active (weak) | Active (weak) |

**CERAPP: Collaborative Estrogen Receptor Activity Prediction Project (CERAPP; Mansouri et al., CERAPP: Collaborative Estrogen Receptor Activity Prediction Project. Environ Health Perspect. 2016 Jul;124 (7):1023-33). The CERAPP is a large-scale modelling project which has investigated the efficacy of using predictive computational models trained on high-throughput screening data (e.g. from the EDSP21 initiative) to evaluate the ER-related activity of thousands of chemicals, and identify priorities for further testing.*

The ToxCast Model Dashboard includes predictions of the estrogen receptor activity of eugenol, based on the Collaborative Estrogen Receptor Activity Prediction Project.

The 19 assays associated with the ToxCast ER prediction model are collectively included in the ED Excel spreadsheet/lines of evidence under Study ID Matrix No. 14-32

**Assessment and conclusion by Applicant:**

Two of the ToxCast ER Model assays proposed in the excel file sheet gave positive results:

- ATG_ERa_TRANS_up: the AC50 was determined to be 43.26 µM with a limit of cytotoxicity reported to be 1000 µM.

- ATG_ERE_CIS_up: the AC50 was determined to be 24.58 µM with a limit of cytotoxicity reported to be 1000 µM. The assay was flagged as having less than 50% efficacy and being “borderline” in respect of the Active outcome.

| |
|---|
| Overall, negative results are reported for estrogen receptor agonist and antagonist activity. Therefore, eugenol is not predicted to be an estrogen receptor agonist or antagonist. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study**: As of the date of this report, there were 19 assays associated with the ToxCast ER Bioactivity prediction model. Based on this battery of *in vitro* assays, eugenol is considered to have weak bioactivity in the ER pathway.<br>The positive result in ATG_ERE_CIS_up is of low relevance due to the aforementioned flags, but the active result from CERAPP (consensus) and the positive in ATG_ERa_TRANS_up should be considered more carefully. Although it should be mentioned that the activity detected is not supported by the other *in vitro* experiments in this battery. |

#### B.6.8.3.3. Androgenic activity in ToxCast model

| | |
|---|---|
| **Data point** | CA 5.8.3 (EU data requirement) |
| **Report author** | US EPA ToxCast: https://comptox.epa.gov/dashboard |
| **Report year** | Data extracted on March 2021. |
| **Report title** | Androgenic activity in ToxCast model |
| **Report No** | Not applicable |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP** | Not applicable |
| **Acceptability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol |
| **Test system** | US EPA ToxCast program |
| **Test method** | In the ToxCast program, thousands of chemicals, including eugenol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities.<br>The US EPA ToxCast program has been consulted for the eugenol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the androgenic pathway. |

**Results**

Eugenol was tested in 15 assays included in the ToxCast AR Bioactivity Model (Table B.6.8.3.3/1). The results were inactive for 14 assays and active for 1 assay.

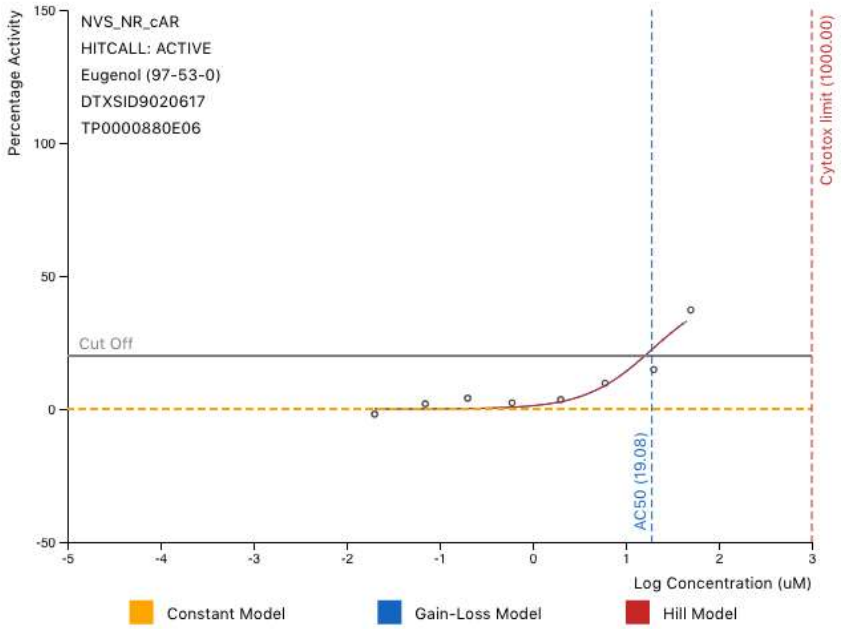
NVS_NR_cAR was the assay with positive results. The $AC_{50}$ was determined to be 19.08 µM (Hill Model). The limit of cytotoxicity was reported to be 1000 µM (see Figure B.6.8.3.3). However, a flag of "less than 50% efficacy (Table B.6.8.3.3/1) was displayed. The dose-response curves from these active assays are shown in Figure B.6.8.3.3.

**Table B.6.8.3.3/1. Summary of US EPA ToxCast EDSP21-androgenic bioactivity assays for eugenol**

| Assay endpoint | Study ID Matrix | Assay type | Organism | Result | AC50 # | Flag |
|---|---|---|---|---|---|---|
| ATG_AR_TRANS_up | 33 | mRNA induction | human | Inactive | - | - |

| Assay endpoint | Study ID Matrix | Assay type | Organism | Result | AC50 # | Flag |
|---|---|---|---|---|---|---|
| **NVS_NR_cAR** | **34** | **radiolligand binding** | **chimpanzee** | **Active** | **19.08** | **Less than 50% efficacy** |
| OT_AR_ARELUC_AG_1440 | 35 | luciferase induction | chinese hamster | Inactive | - | - |
| OT_AR_ARSRC1_0480 | 36 | protein fragment complementation assay | human | Inactive | - | - |
| OT_AR_ARSRC1_0960 | 37 | protein fragment complementation assay | human | Inactive | - | - |
| TOX21_AR_BLA_Agonist_ratio | 38 | beta lactamase induction | human | Inactive | - | - |
| TOX21_AR_BLA_Antagonist_ratio | 39 | beta lactamase induction | human | Inactive | - | - |
| TOX21_AR_BLA_Antagonist_viability | 40 | ATP content | human | Inactive | - | - |
| TOX21_AR_LUC_MDAKB2_Agonist | 41 | luciferase induction | human | Inactive | - | - |
| TOX21_AR_LUC_MDAKB2_Antagonist | 42 | luciferase induction | human | Inactive | - | - |
| TOX21_AR_LUC_MDAKB2_Antagonist__viability | 43 | ATP content | human | Inactive | - | - |
| TOX21_AR_LUC_MDAKB2_Antagonist_Specificity | 44 | luciferase induction | human | Inactive | - | - |
| TOX21_AR_LUC_MDAKB2_Antagonist_Specificity_viability | 45 | ATP content | human | Inactive | - | - |
| UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist | 46 | Protein-protein binding | human | Inactive | - | - |
| UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist | 47 | Protein-protein binding | human | Inactive | - | - |

*# Chemical concentration (in µM) at which 50% of the maximum response is achieved.*




| Winning Model | Model | AIC | RMSE | Top | AC50 | Slope |
|---|---|---|---|---|---|---|
| | Constant | 65.65 | 14.74 | - | - | - |
| | Gain-Loss | 54.96 | 3.59 | 44.65 | 19.08 | 1.21 |
| ✔ | Hill | 50.96 | 3.59 | 44.65 | 19.08 | 1.21 |

**Figure B.6.8.3.3: Dose-response curves of the active androgenic ToxCast bioassay (ID Matrix 34).**

The scores from the AR ToxCast model predictions show that eugenol is inactive as an AR agonist or antagonist with AUC (area under the curve) values of 0. The ToxCast Models Dashboard includes predictions of the androgen receptor activity of eugenol based on the COMPARA. COMPARA (Consensus) prediction model shows that eugenol is inactive as an AR agonist, AR antagonist and as AR binding (Table B.6.8.3.3/2).

**Table B.6.8.3.3/2. AR ToxCast model predictions**

| Model | Agonist | Antagonist | Binding |
|---|---|---|---|
| ToxCast Pathway Model (AUC) | 0.00 | 0.00 | - |
| COMPARA (Consensus) [#] | Inactive | Inactive | Inactive |

*[#]COMPARA is a larger scale collaboration between 35 international groups, using QSAR models to predict AR activity using a common training set of 1746 compounds provided by U.S. EPA. A key result is a consensus model of AR agonist and antagonist activity that is run against the DSSTox chemical library.*

The 15 assays associated with the ToxCast AR prediction model are collectively included in the ED Excel spread sheet/lines of evidence under Study ID Matrix No. 33-47.

**Assessment and conclusion by Applicant:**

One of the ToxCast AR Model assays proposed in the excel file sheet gave positive result :

- NVS_NR_cAR: the AC50 was determined to be 19.08 µM with a limit of cytotoxicity reported to be 1000 µM. The assay was flagged as having less than 50% efficacy.

| |
|---|
| Overall, negative results are reported for androgen receptor agonist and antagonist activity. Therefore, eugenol is not predicted to be an androgen receptor agonist or antagonist. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** As of the date of this report, there were 15 assays associated with the ToxCast AR Bioactivity prediction model.<br>All assays in the battery were negative for AR activity. The only exception being **NVS_NR_cAR**. However, the fact that this assay was flagged as having less than 50% efficacy; makes the results of this experiment on their own insufficient for concluding that eugenol has any potential to interact with the AR.<br>Moreover, according to the AR ToxCast model predictions, eugenol is inactive for AR agonism, antagonism, and AR binding. |

#### B.6.8.3.4. Steroidogenic activity in ToxCast model

| | |
|---|---|
| **Data point** | CA 5.8.3 (EU data requirement) |
| **Report author** | US EPA ToxCast: https://comptox.epa.gov/dashboard |
| **Report year** | Data extracted on March 2021. |
| **Report title** | Steroidogenic activity in ToxCast model |
| **Report No** | Not applicable |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP** | Not applicable |
| **Acceptability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol |
| **Test system** | US EPA ToxCast program |
| **Test method** | In the ToxCast program, thousands of chemicals, including eugenol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities.<br>The US EPA ToxCast program has been consulted for the eugenol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the steroidogenic pathway. |

**Results:**

Eugenol was tested in 26 assays included in the ToxCast Steroidogenesis Bioactivity Model (Table B.6.8.3.4, Study ID Matrix No.: 48-73).

Of these assays, 24 consist of a series of *in vitro* mechanistic bioassays in H295R human adrenocortical carcinoma cells which assess the potential for eugenol to modulate steroid hormone biosynthesis (estrogen, androgen, progestogens and glucocorticoids). The results were inactive for 23 assays and active for the 3 following assays:

- CEETOX_H295R_CORTIC_up. The $AC_{50}$ was determined to be 43.56 µM. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 57)

- CEETOX_H295R_ESTRADIOL_up. The $AC_{50}$ was determined to be 32.56 µM. The bioassay has a "flag" indicating that eugenol has borderline activity in this assay. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 63)
- CEETOX_H295R_ESTRONE_up. The $AC_{50}$ was determined to be 36.26 µM. The bioassay has two "flags" indicating that: eugenol has borderline activity in this assay, and that "only highest concentration above baseline, active". Thus, only the highest concentration in the assay was above the baseline. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 65)

The dose-response curves from these assays are shown in Figure B.6.8.3.4/2.

These three CEETOX assays are performed using H295R human adrenocortical cells and measure the up-regulation of the hormones: corticosterone, estradiol and estrone, respectively, associated with the steroidogenesis pathway for the metabolism of cholesterol for steroid hormones in the adrenal glands. The steroidogenesis pathway is shown in Figure B.6.8.3.4/1.

**Table B.6.8.3.4. Summary of US EPA ToxCast EDSP21- steroidogenesis bioactivity assays for eugenol**

| Assay endpoint | Study ID Matrix | Assay type | Organism | Result | AC50 # | Flag |
|---|---|---|---|---|---|---|
| CEETOX_H295R_11DCORT_dn | 48 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_11DCORT_up | 49 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_OHPREG_dn | 50 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_OHPREG_up | 51 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_OHPROG_dn | 52 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_OHPROG_up | 53 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_ANDR_dn | 54 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_ANDR_up | 55 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_CORTIC_dn | 56 | Hormone induction | human | Inactive | - | - |
| **CEETOX_H295R_CORTIC_up** | **57** | **Hormone induction** | **human** | **Active** | **43.56** | **-** |
| CEETOX_H295R_CORTISOL_dn | 58 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_CORTISOL_up | 59 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_DOC_dn | 60 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_DOC_up | 61 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_ESTRADIOL_dn | 62 | Hormone induction | human | Inactive | - | - |
| **CEETOX_H295R_ESTRADIOL_up** | **63** | **Hormone induction** | **human** | **Active** | **32.56** | **Borderline active** |
| CEETOX_H295R_ESTRONE_dn | 64 | Hormone induction | human | Inactive | - | - |
| **CEETOX_H295R_ESTRONE_up** | **65** | **Hormone induction** | **human** | **Active** | **36.26** | **Borderline active Only highest concentration above baseline active** |
| CEETOX_H295R_PROG_dn | 66 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_PROG_up | 67 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_TESTO_dn | 68 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_TESTO_up | 69 | Hormone induction | human | Inactive | - | - |
| TOX21_Aromatase_Inhibition | 70 | Luciferase induction | human | Inactive | - | - |
| CEETOX_H295R_MTT_cell_viability_up | 71 | Hormone induction | human | Inactive | - | - |
| CEETOX_H295R_MTT_cell_viability_dn | 72 | Hormone induction | human | Inactive | - | - |
| TOX21_Aromatase_inhibition_viability | 73 | ATP content | human | Inactive | - | - |

*# Chemical concentration (in µM) at which 50% of the maximum response is achieved.*

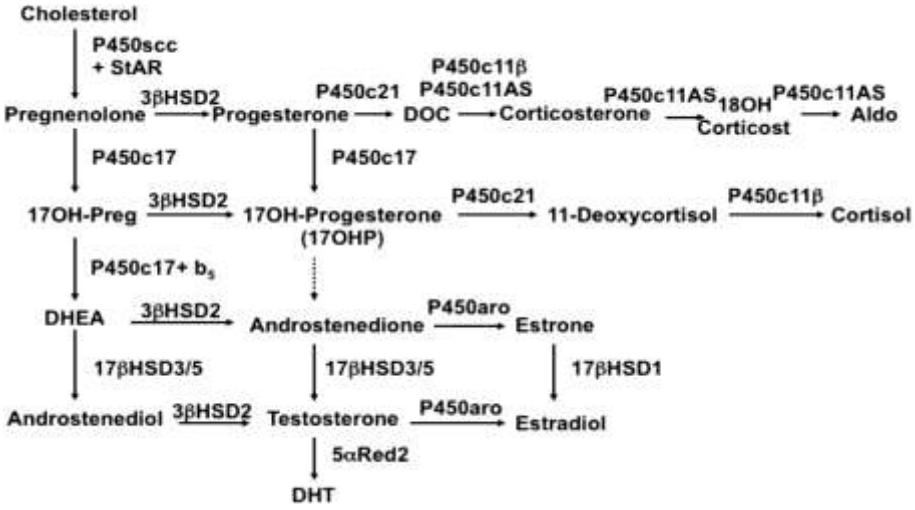



**Figure B.6.8.3.4/1: Steroidogenesis pathways.**

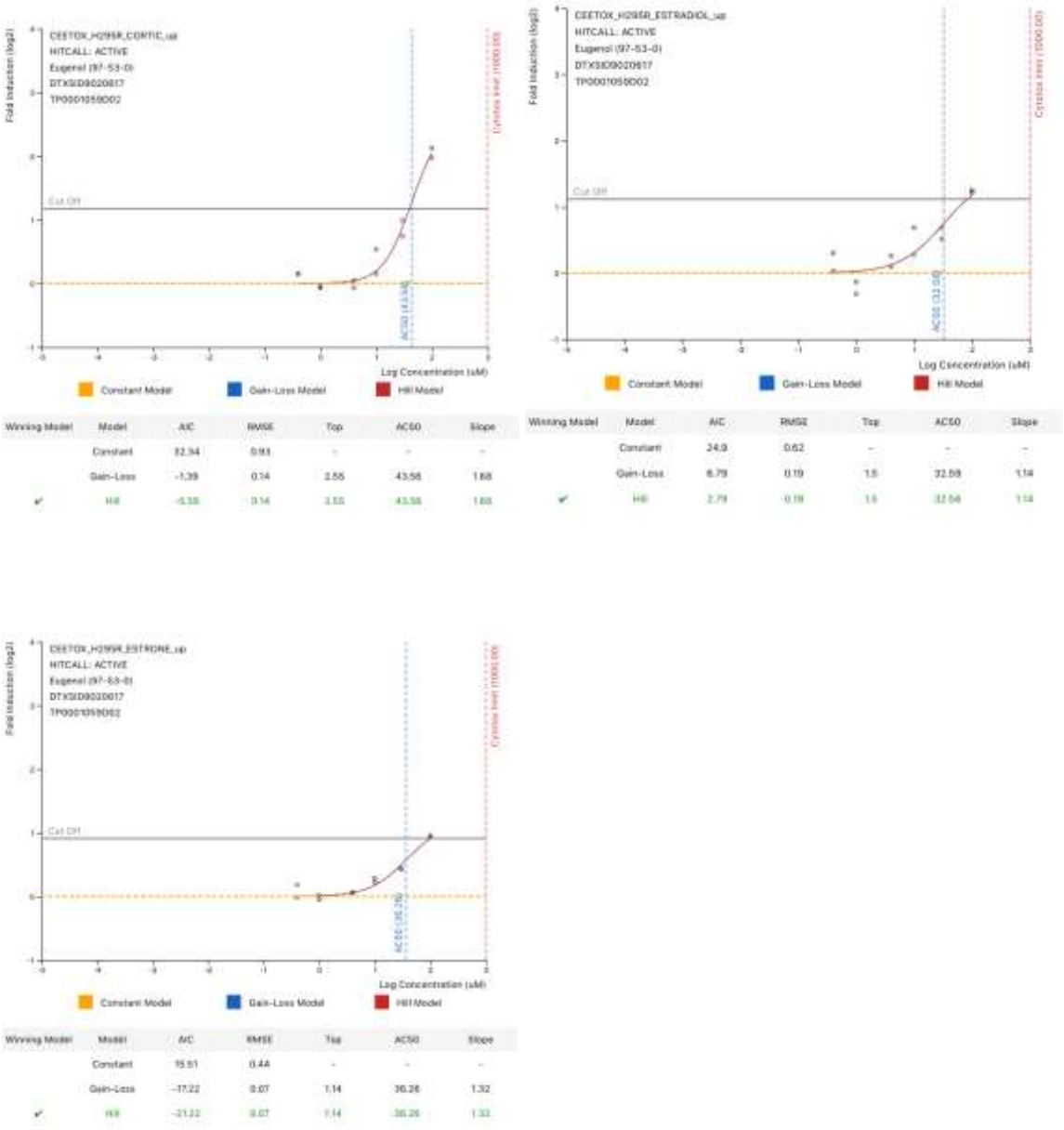



**Figure B.6.8.3.4/2: Dose-response curves of the active steroidogenesis ToxCast bioassays (ID Matrix 57, 63 and 65)**

The 26 assays associated with the ToxCast steroidogenesis prediction model are collectively included in the ED Excel spreadsheet/lines of evidence under Study ID Matrix No. 48-73.

**<u>Assessment and conclusion by Applicant:</u>**

Thirteen *in vitro* ToxCast™ steroidogenesis assays proposed in the excel file sheet were listed for eugenol under the endocrine disrupter screening programme assays (EDSP21) on the CompTox™. Negative results are reported for all these assays. Therefore, eugenol is not predicted to have activity on steroidogenesis.

A published paper is also available that investigated the potential estrogenic and androgenic activity of eugenol *in vitro.* Eugenol showed no evidence for (anti)-androgenic activity in androgen-inducible yeast screen and no

| |
|---|
| estrogenic activity in the estrogen responsive yeast screen, however there was some evidence for an anti-estrogenic effect. Eugenol was able to compete with 3H-estradiol for binding to ERα and ERß but required concentrations in the order of $10^4$ to $10^5$ times higher than 17ß-estradiol. Due to limited reporting, it is not possible to determine the biological relevance of the positive result, and it is considered that this result is not sufficient evidence for EAS-mediated activity.<br>Overall, there was no evidence for EAS-mediated activity in a range of *in vitro* assays. Following assessment of the integrated lines of evidence for the EAS-modalities, it is concluded that there is no evidence for EAS-mediated adversity in the available data. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study**: As of the date of this report, there were 26 assays associated with the ToxCast Steroidogenesis Bioactivity prediction model. Three assays displayed positive results.<br>Overall, the available *in vitro* mechanistic bioassay data shows that eugenol may have some potential to interact with components of the steroidogenesis pathway *in vitro,* potentially affecting the production of estradiol, estrone and glucocorticoids. However, it must be considered that the estradiol and estrone induction assays are both "flagged", so reliability of results should be taken cautiously. |

**B.6.8.3.5. Estrogenic/androgenic/anti-estrogenic/anti-androgenic activity assessment.**

| | |
|---|---|
| **Data point** | CA 5.8.3 (No EU data requirement) |
| **Report author(s)** | Howes, M.J., Houghton, P.J., Barlow, D.J., Pocock, V.J., Milligan, S.R. |
| **Report year** | 2002 |
| **Report title** | Assessment of estrogenic activity in some common essential oil constituents |
| **Report No** | Not applicable, published study (*J. Pharm. Pharmacol*. 54 (11), 1521–1528.) |
| **Document No** | KCA 5.8.3/02 |
| **Guidelines followed in study** | No test method available |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/ Officially recognised testing facilities** | Not applicable, published study |
| **Reliability** | Supportive only |

**Materials and Methods**

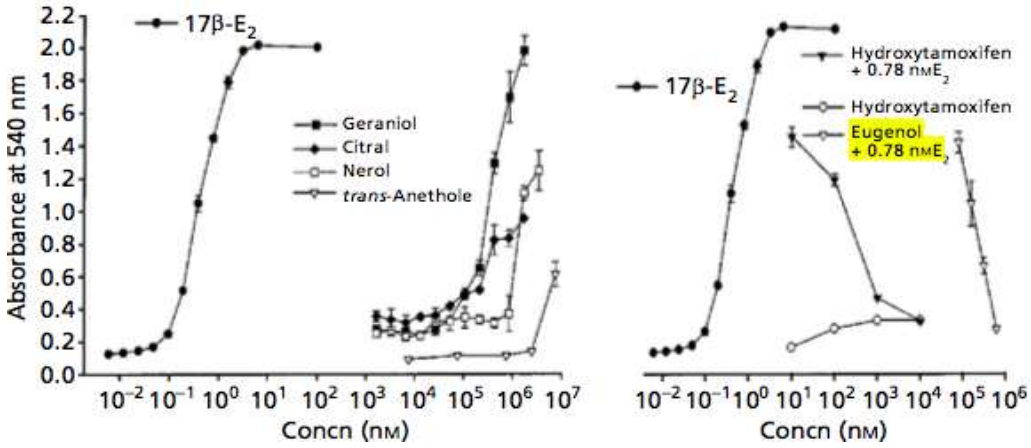
| | |
|---|---|
| **Test chemical** | Eugenol (99% purity from Aldrich Chemical Company) was among the essential oil constituents assessed in this study. |
| **Controls** | Positive: 17β-estradiol is used as the standard for ER activity against which all other chemicals are assesses. |
| **Test system** | *Recombinant yeast assay* → Yeast cells (*Saccharomyces cerevisiae* Y190)<br>*Endometrial cell assay* →Ishikawa Var I human cells.<br>*Assessment of estrogen receptor binding* → recombinant human ERα and $ER_B$ |
| **Test method** | The study aims to investigate the potential estrogenic activity of a number of essential oil constituents (among which is eugenol).<br>*A recombinant yeast assay*/screen was conducted using 96-well plates to assess estrogenic activity (as described by Routledge *et al.*, *Environ. Toxicol. Chem*., 1996, 15: 241- 258) and anti-estrogenic activity (as described by Sohoni *et al.*, *J. Endocrinol*., 1998, 158: 327-339). An MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd, West Sussex, UK) was used to monitor the absorbance of the assay solutions. Cell counts to determine gross cytotoxicity were undertaken when assay results appeared to show anti-estrogenic activity. Cell counts were conducted by diluting 10 µL assay solution from each well per plate with phosphate-buffered saline, following the assay incubation period (10 cell counts per concentration of test substance).<br>The *Ishikawa Var I human endometrial cell assay* was conducted as described by Markiewicz *et al.*, (*J. Steroid Biochem. Mol*., 1993, 45: 399-405) to assess estrogenic activity of compounds. To assess anti-estrogenic activity of compounds the assay was performed as described for |

| | |
|---|---|
| | analysis of estrogenic activity, but with the prior addition to the test wells of 17β-estradiol at a concentration that produced a sub-maximal response ($7.8x10^{-10}$ M). The anti-estrogenic activity of test compounds was compared with the activity of a standard range of concentrations of hydroxytamoxifen ($10^{-8}$ to $10^{-5}$ M). A microtitre plate reader plus the necessary Software were used to monitor the absorbance of the assay solutions. When assay results appeared to show anti-estrogenic activity, total protein assays were undertaken to determine potential cytotoxicity, as described by Skehan *et al.*, (*J. Natl Cancer Inst*., 1990, 82: 1107-1112).<br><br>Additional *screening for androgenic and anti-androgenic activity*, based on an androgen-inducible yeast assay described by Sohoni *et al., (J. Endocrinol*., 1998, 158: 327-339) was carried out for eugenol and the other essential oil constituents.<br><br>*Assessment of estrogen receptor binding:* The potential estrogen receptor binding activity eugenol was studied using recombinant human ERα and $ER_B$ and conducted as described by Milligan *et al., Clin. Endocrinol. Metab.,* 2000, 85: 4912-4915). Radioactivity of the assay samples was determined by recording counts with a scintillation counter.<br><br>*Molecular graphics*: Assessment of the structure-activity relationship between ER and receptor ligands such as eugenol was conducted using the computer program HyperChem. The search results generated give the energy and torsion angles for each conformation of the molecule. Following inspection of the data, similar conformations of the molecule were grouped together. The conformation of lowest energy representative of each group was selected for further investigation. The conformers selected were compared with the structure of the known hER ligands 17β-estradiol or raloxifene. For ER antagonist molecule such as eugenol the ER**α** structure in the presence of raloxifene was selected.<br><br>Once the molecule being assessed was positioned in the ligand-binding domain, then its position and conformation were further adjusted, by refinement of the potential energy of the system. This was achieved by conducting a molecular mechanics optimization using an MM+ force field and a conjugate gradient least squares refinement (Polak Ribiere) with termination criteria of 4000 maximum cycles or a potential energy gradient of 0.01 kcal $(Å\ mol)^{-1}$. This process lowers the energy of the molecular system by adjusting its geometry. The final torsion angles of each molecule positioned within the ligand-binding domain were determined, and an assessment was made of the potential interactions (H-bond, electrostatic interactions and van der Waals forces) between the ligand and surrounding residues of the ligand-binding domain. Once completed, the model complex was assessed using the computer program WebLab ViewerLite (MSI Inc., USA). The atomic van der Waals surfaces of the molecular system were displayed to assess steric complementarity. |
| **Data analysis** | For assessment of anti-estrogenic activity and receptor binding, results are expressed as mean ± s.e., and analysed by analysis of variance. All data points consist of 4 replicates (n=4) |

**Results:**

*Assessment of estrogenic and anti-estrogenic activity in the yeast assay*

The estrogenic potency ($EC_{50}$) of 17β-estradiol was at least $10^5$ to $10^6$ times that of any of the essential oil constituents (Figure B.6.8.3.5/1). When these compounds were tested in the same assay for their ability to inhibit 17β-estradiol mediated stimulation of β-galactosidase activity, only eugenol showed a dose-dependent anti-estrogenic activity without any evidence of cytotoxicity (Figure B.6.8.3.5/1).




*Results are mean values ± s.e.m.; n=4 wells per point. Where no error bars are visible, the errors were smaller than the symbols.*

**Figure B.6.8.3.5/1: Relative estrogenic activity of 17 β-estradiol (17 β-$E_2$) and relative anti-estrogenic activity of hydroxytamoxifen and eugenol (highlighted) with 0.78 nm 17 β-estradiol ($E_2$) in a yeast screen bearing the human estrogen receptor.**

*Assessment of estrogenic and anti-estrogenic activity in Ishikawa Var-I cells*

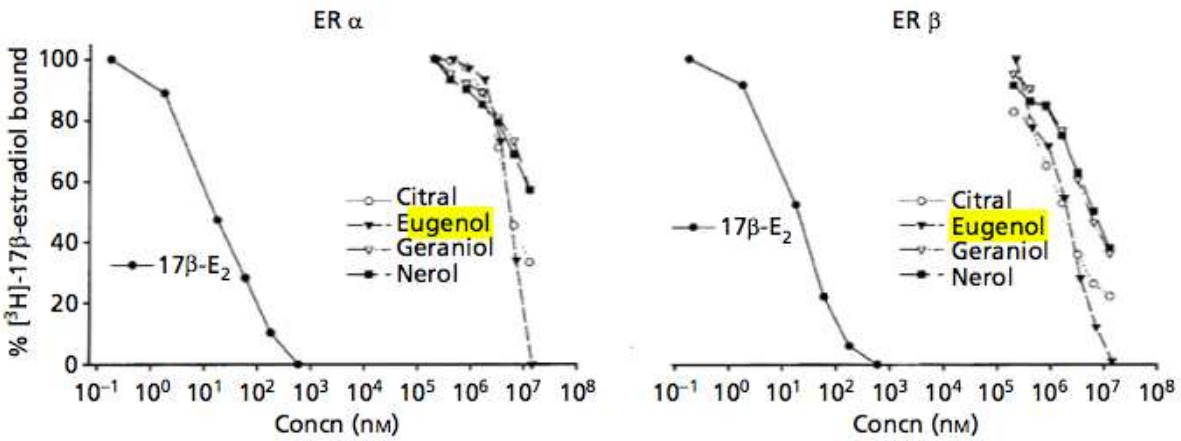
Neither eugenol, nor any other compound tested showed any ability to stimulate alkaline phosphatase in the Ishikawa cells (data not shown). In view of the volatility of the compounds and therefore their possible rapid loss from the assay wells, the assay was repeated using daily additions of the test compounds. However, there was still no evidence of any estrogenic activity.

*Assessment of androgenic and anti-androgenic activity.*

Neither eugenol nor any of the other compounds tested, showed evidence of stimulation of β-galactosidase activity, nor evidence of anti-androgenic activity.

*Receptor binding*

Eugenol (as well as citral, geraniol, nerol) was able to compete with $^3$H-estradiol for binding to both the ERα and ERβ, but required concentrations in the order of $10^4$ to $10^5$ times higher than 17β-estradiol did (Figure B.6.8.3.5/2).




*Results are mean values±s.e.m; n=4 wells per point. Where no error bars are visible, the errors were smaller than the symbols.*

**Figure B.6.8.3.5/2: Competitive displacement of [2,4,6,7-$^3$H]17β-estradiol from isolated ERα and ERβ by 17β-estradiol (17 β-$E_2$), citral, geraniol, nerol and eugenol (highlighted).**

*Molecular graphics:*

Results are expressed as the shortest bond distances (Å) between the potential hER ligands and the residues on the ERα (Figure B.6.8.3.5/3 and Table B.6.8.3.5).

**Table B.6.8.3.5. Potential interactions of compounds with the residues of the ligand-binding domain of the ERα (shortest bond distances determined).**

| Ligand | Arginine (ARG) residue 394 | Glutamic acid (GLU) residue 353 | Histidine (HIS) residue 524 | Water molecule |
|---|---|---|---|---|
| Citral a (**4**) (aldehyde substituent) | 2.83 Å | – | – | – |
| Citral b (**5**) (aldehyde substituent) | 2.98 Å | – | – | – |
| Geraniol (**2**) (hydroxyl substituent) | 2.06 Å | 2.54 Å | – | – |
| Nerol (**6**) (hydroxyl substituent) | 2.11 Å | 2.80 Å | – | – |
| Eugenol (**7**) (hydroxyl substituent) | 2.05 Å | 2.25 Å | – | 3.15 Å |
| 17*β*-Estradiol (**1**) (hydroxyl substituents) | 2.25 Å | 2.11 Å | 2.44 Å | 3.15 Å |
| Raloxifene (hydroxyl substituent and imidazole ring) | 2.15 Å | 2.97 Å | 1.93 Å | 2.20 Å |

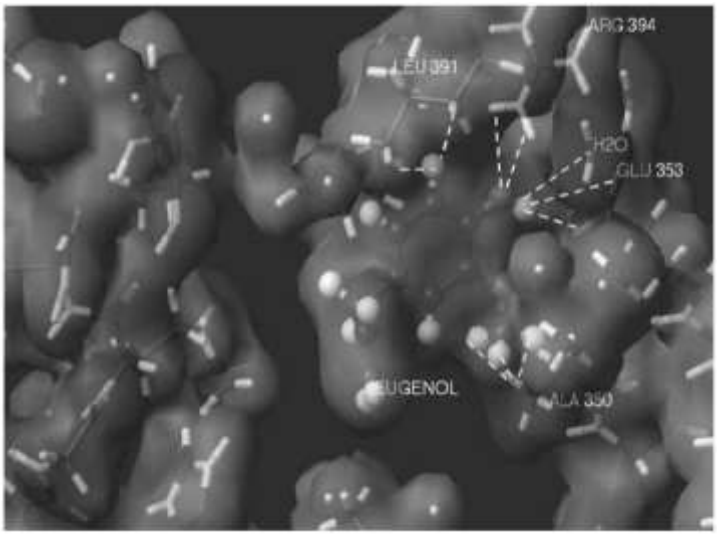



**Figure B.6.8.3.5/3: Potential interactions of eugenol with the surrounding residues of the ligand-binding domain of the ER , and potential displacement of the ligand-binding domain residues by eugenol** .

**<u>Assessment and conclusion by Applicant:</u>**

Eugenol showed evidence of anti-estrogenic activity in the yeast screen and was able to compete with labelled estradiol for binding to both ERα and ERβ. However, eugenol was approximately $10^6$ times less effective than hydroxytamoxifen. No androgenic activity was demonstrated. According to the authors, there was no evidence that eugenol possessed any hER agonist activity and the biological significance of the result was uncertain.

This new study is considered to be acceptable for the renewal of eugenol as supporting information (not reliable: the study is not GLP and reporting is limited therefore methodology cannot be fully evaluated). In conclusion, eugenol showed some activity in this *in vitro* assay but is not expected to have ED properties regarding EA-mediated effects.

**<u>Assessment and conclusion by RMS</u>:**

**Outcome and conclusion of the study:**

The yeast assays conducted by Howes *et al.*, used the methodology developed by Routledge & Sumpter in 1996, and Sohoni & Sumpter in 1998, for estrogenic/anti-estrogenic/androgenic/anti-androgenic assessments. Both methods were considered one of the first versions of ER STTA assay (OECD TG 455) and AR STTA assay (OECD TG 458), respectively, that nowadays use mammalian stably-transfected cell lines.

There was no evidence that eugenol possessed hER agonist activity. However, eugenol showed dose-dependent anti-estrogenic activity in the yeast screen assay without any evidence of cytotoxicity, and was able to compete with labelled $^{3}$H-17βestradiol for binding to both isolated recombinant ER α and ERβ.

On the other hand, no androgenic/anti-androgenic activity was detected for eugenol.

Therefore, RMS deems that the potential anti-estrogenic activity of eugenol might be further investigated.

### B.6.8.3.6. Antifertility effects of eugenol in rats.

| | |
|---|---|
| **Data point** | CA 5.8.3 (No EU data requirement) |
| **Report author(s)** | Poli, V. and Challa, C. |
| **Report year** | 2019 |
| **Report title** | A comparative study of eugenol and *Ocimum sanctum* Linn. leaf extract on the antifertility effect in female albino rats. |
| **Report No** | Not applicable, published study (*Journal of Chinese Medical Association*, 82: 231-234) |
| **Document No** | KCA 5.8.3/03 |
| **Guidelines followed in study** | No detailed test guidelines are currently available to conduct this type of study. |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/ Officially recognised testing facilities** | No |
| **Reliability** | Supportive only |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol. Lot/Batch No.: not stated, Purity: not stated |
| **Test system** | Wistar albino female rats |
| **Test method** | Six Wistar female rats received eugenol pure compound (99%) at a dosage of 0.4 ml/day for 15 days by intramuscular injection (i.m). Six female rats were administered with 1 ml of saline (vehicle).<br>Twenty four hours after the last dose, the animals were autopsied and the reproductive tissues like ovary, uterus, and vagina were excised at 4ºC and used for biochemical analysis. The blood was collected by puncturing the heart for hormone determination (FSH, LH, testosterone, prolactin, estradiol, progesterone). The biochemical parameters such as total proteins, total carbohydrates and total lipids were also measured.<br><u>Determination of estrous cycle</u><br>Vaginal smear using saline solution was taken twice daily every day during the entire treatment period, and the cell type obtained in vaginal smear was observed. The duration of estrus cycle together with that of various phases was determined. All the four phases of the estrus cycle are determined according to the presence of cell types in the vaginal smear:<br>Proestrus phase –The presence of nucleated or nucleated plus cornified cells.<br>Estrus phase –The presence of cornified cells only.<br>Metestrus –The presence of mixture of leukocytes and epithelial cells.<br>Diestrus phase –Mainly leukocytes with few epithelial cells. |
| **Data analysis** | The data were expressed as a mean value with their standard deviation (SD), and different groups were compared using one-way ANOVA analysis with a Dunnetts multiple comparison test. Differences were considered statistically significant: $p < 0.05$. |

**Results:**

Effect of eugenol on estrous cycle and sex hormones

Eugenol enhanced the duration of proestrus, metestrus, diestrus phases, and total duration of cycles (29%, 22%, 16% and 17%, respectively) compared with controls. On the other hand, eugenol had no effect on hormones such as FSH, LH, and PRL. However, serum estradiol and progesterone levels were statistically significant increased (87% and 48%) compared with control, whereas testosterone levels were reduced (78%) (see table B.6.8.3.6/1).

**Table B.6.8.3.6/1. Effect of eugenol on estrus cycle and sex hormones in rats.**

| Parameters | Control | Eugenol |
|---|---|---|
| **Estrus cycle (in hours)** | | |
| **Proestrus** | 14 ± 1.01 | **18 ± 1.26* (↑29%)** |
| **Estrus** | 16 ± 1.28 | 16 ± 1.21 |
| **Metestrus** | 27 ± 1.98 | **33 ± 2.39* (↑22%)** |
| **Diestrus** | 50 ± 3.82 | **58 ± 4.69* (↑16%)** |
| **Total duration** | 107 ± 6.42 | **125 ± 8.46* (↑17%)** |
| **Sex Hormones Level** | | |
| **FSH (U/mL)** | 0.10 ± 0.01 | 0.10 ± 0.01 |
| **LH (U/mL)** | 0.10 ± 0.07 | 0.10 ± 0.07 |
| **Prolactin (ng/ml)** | 1.00 ± 0.09 | 1.00 ± 0.09 |
| **Testosterone (ng/ml)** | 0.18 ± 0.012 | **0.04 ± 0.002* (↓78%)** |
| **Estradiol (pg/ml)** | 11.36 ± 0.82 | **21.19 ± 1.75* (↑87%)** |
| **Progesterone (ng/ml)** | 85.42 ± 5.09 | **126.72 ± 9.16* (↑48%)** |

*$*p<0.001$*

Effect of eugenol on tissue biochemistry

The tissue somatic index (100 g body weight) of the ovary displayed statistically significant increase (32%), while no significant effect was observed on sex accessory tissues like the uterus and vagina following the administration of eugenol. On the other hand, a statistically significant increase of ovarian and uterine proteins (68% and 66%, respectively), and a reduction in vaginal proteins were observed (18%). The ovarian carbohydrates were lower than controls (21%), whereas the uterine and vaginal carbohydrates were enhanced (19% and 39%, respectively). The total lipids were increased significantly in ovary and uterus (92% and 59%, respectively), whereas in vagina the values were reduced (14%), compared with controls (table B.6.8.3.6/2).

**Table B.6.8.3.6/2. Effect of eugenol on tissue somatic index, total proteins, total carbohydrates, and total lipids in ovary, uterus, and vagina.**

| Parameters | Ovary | | Uterus | | Vagina | |
|---|---|---|---|---|---|---|
| | Control | Eugenol | Control | Eugenol | Control | Eugenol |
| **Tissue somatic index (w/w%)** | 0.082 ± 0.005 | **0.108 ± 0.006* (↑32%)** | 0.312 ± 0.023 | 0.306 ± 0.021 | 0.050 ± 0.003 | 0.049 ± 0.003 |
| **Total proteins (mg/g)** | 213.65 ± 16.81 | **359.38 ± 28.64* (↑68%)** | 168.69 ± 11.08 | **279.81 ± 20.87* (↑66%)** | 230.70 ± 18.68 | **190.03 ± 12.51* (↓18%)** |
| **Total carbohydrates (mg/g)** | 6.89 ± 4.01 | **5.45 ± 3.15* (↓21%)** | 5.75 ± 3.26 | **6.85 ± 3.98* (↑19%)** | 6.01 ± 3.32 | **8.38 ± 4.96* (↑39%)** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Total lipids (mg/g)** | 38.60 ± 2.95 | **74.17 ± 6.31* (↑92%)** | 59.43 ± 4.80 | **94.58 ± 8.40* (↑59%)** | 44.19 ± 3.79 | **37.82 ± 2.71* (↓14%)** |

**Assessment and conclusion by applicant:**

Eugenol showed evidence of antifertility activity under the conditions of the study. This new study is considered as supportive only for the renewal of eugenol due to significant limitations with the study design (not reliable: the study is not GLP, authors only used a single dose of eugenol, the dose route is not compliant with OECD TG or data requirements (i.m.) and it's not a standard study design. Therefore, no conclusion on ED properties are made for this study, and it is not included in the ED assessment.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The RMS agrees with the applicant's assessment and conclusion.

This study presents important deviations such as is not GLP, authors only used a single dose of eugenol or the dose route is not compliant with OECD TG or data requirements. However, the anti-fertility effects displayed after eugenol treatment (increased oestrus cycle length, and increase estradiol and progesterone serum levels) could be compatible with the potential anti-estrogenic role of eugenol observed in the "*in vitro*" assays.

### B.6.8.3.7. *In silico* QSAR predictions.

| | |
|---|---|
| **Data point** | CA 5.8.3 (EU data requirement) |
| **Report author** | Danish QSA. https://qsar food.dtu.dk/ |
| **Report year** | Data extracted on October 2021 |
| **Report title** | Not applicable |
| **Report No** | Not applicable |
| **Document No** | Not applicable |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No, not previously submitted |
| **GLP** | Not applicable |
| **Acceptability** | Yes |

**Materials and Methods**

| | |
|---|---|
| **Test chemical** | Eugenol |
| **Test system** | Danish QSAR database |
| **Test method** | Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database and the OECD QSAR Toolbox v.4.5. The Danish (Q)SAR Database ( https://qsar food.dtu.dk/) was released in November 2015 and has since then been expanded and updated a number of times. It currently holds many predictions for around 650000 substances and is a tool that allows industry, research, authorities and others to search for hazard information on chemical substances, especially those with little or no testing data. While the Danish (Q)SAR Database contains around 650000 substances, there may still be substances of interest to some users which are not contained in the database. Or even when contained, there may in some cases be interest to obtain more detailed predictions in the QSAR Prediction Reporting Format, which includes information on prediction probability, possible alerts, nearest analogs in the training set etc. The Danish (Q)SAR Models website offers users to make on-the-fly predictions for user-defined chemical structures by use of >30 models developed by DTU in the Leadscope software. The use of the commercial Leadscope® Enterprise Server software as a back-end to this website was made possible based on a |

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|---|---|
| | collaboration agreement between Leadscope Inc. and the National Food Institute at the Technical University of Denmark. |

**Results:**

**T-modality**

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, T-related non-test information was gathered for eugenol. Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database.

The results of the Danish QSAR database for eugenol in respect of T-mediated endocrine endpoints are summarised below.

**Table B.6.8.3.8/01: The results of Danish QSAR database for eugenol regarding T-modality**

| | Exp | Battery | CASE Ultra | Leadscope | SciQSAR |
|---|---|---|---|---|---|
| **Thyroperoxidase (TPO) inhibition QSAR1 (Rat *in vitro*)** | POS | NA | NA | POS_IN | NA |
| **Thyroperoxidase (TPO) inhibition QSAR2 (Rat *in vitro*)** | POS | NA | NA | POS_IN | NA |
| **Thyroid Receptor α Binding (Human *in vitro*)** | | | | | |
| mg/L | | | 110.7643 | 208.6025 | 100.1151 |
| µM | | | 674.528 | 1270.34 | 609.6722 |
| Positive for IC50 ≤ 10 µM | | | | | |
| Positive for IC50 ≤ 100 µM | | | | | |
| Domain | | OUT | OUT | OUT | OUT |
| **Thyroid Receptor β Binding (Human *in vitro*)** | | | | | |
| mg/L | | 2843.621 | 5313.734 | 13.48465 | 373.5077 |
| µM | | 17316.98 | 32359.38 | 82.11832 | 2274.573 |
| Positive for IC50 ≤ 10 µM | | | | | |
| Positive for IC50 ≤ 100 µM | | | | | |
| Domain | | IN | IN | OUT | OUT |
| *Key: POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model* | | | | | |

The Danish QSAR Database reports that eugenol lacks the potential to interact with the thyroid receptor.

The Danish QSAR Database reports that eugenol gave positive results in respect of thyroid peroxidase (TPO) inhibition in two respective Leadscope global, binary composite QSAR models: QSAR1 and QSAR2 (and was within the applicability domain of these models). These models were developed to predict the potential of substances to inhibit thyroperoxidase using the US EPA ToxCast data sets (training set for QSAR1 = 877 chemicals; training set for QSAR2 = 1519 chemicals) and the commercial software Leadscope® Predictive Data Miner. The highest ranking structural features associated with activity were versions of phenols, anisole and aniline whereas the most frequent structural features associated with inactivity included ethers, esters, aryl halides and a tertiary amine [3]. The structure of eugenol includes both a phenol and an anisole group, and the substance therefore has the features associated with TPO inhibition activity. Moreover, positive results were obtained in the experimental training data set (from EpiSuite experimental databases or DK DTU QSAR models training sets).

Overall, OECD Conceptual Framework Level 1 (non-experimental) data for eugenol indicated that the substance has the potential to inhibit thyroperoxidase.

**EAS-modality**

[3] Rosenberg SA, Watt ED, Judson RS, Simmons SO, Paul Friedman K, Dybdahl M, Nikolov NG, Wedebye EB. QSAR models for thyroperoxidase inhibition and screening of U.S. and EU chemical inventories. *Computational Toxicology*. 2017, Vol 4,pp 11-21. https://doi.org/10.1016/j.comtox.2017.07.006.

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, EAS-related non-test information was gathered for eugenol. Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database, and results are summarised below.

**Table B.6.8.3.8/02: Results of Danish QSAR database for eugenol regarding EAS-modality.**

| | Exp. | Battery | CASE Ultra | Leadscope | SciQSAR |
|---|---|---|---|---|---|
| Estrogen Receptor α Binding, Full training set (Human *in vitro*) | | NEG_IN | NEG_IN | NEG_IN | NEG_IN |
| Estrogen Receptor α Binding, Balanced Training Set (Human *in vitro*) | | NEG_OUT | INC_OUT | NEG_IN | INC_OUT |
| Estrogen Receptor α Activation (Human *in vitro*) | | NEG_IN | NEG_IN | NEG_IN | INC_OUT |
| Estrogen Receptor Activation, CERAPP data (*in vitro*) | | NA | NA | POS_OUT | NA |
| Androgen Receptor Inhibition (Human *in vitro*) | | NEG_IN | NEG_IN | NEG_IN | NEG_IN |
| Androgen Receptor Binding, CoMPARA data (*in vitro*) | NEG | NA | NA | INC OUT | NA |
| Androgen Receptor Inhibition, CoMPARA data (*in vitro*) | NEG | NA | NA | INC OUT | NA |
| Androgen Receptor Activation, CoMPARA data (*in vitro*) | NEG | NA | NA | NEG_IN | NA |
| *Key: POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model* | | | | | |

The Danish QSAR Database predictions report that eugenol lacks the potential to interact with estrogen and androgen receptors.

OECD ToolBox

The results of the OECD QSAR Toolbox v.4.2 profilers for eugenol in respect of E-related endpoints are shown below:

**Table B.6.8.3.8/03. Results of the OECD QSAR Toolbox v.4.2 for eugenol**

| **[1] Estrogen Receptor Binding, alerts in:** | |
|---|---|
| parent only | Weak binder, OH group |
| metabolites from *in vivo* Rat metabolism simulator only | Moderate binder, OH group; Weak binder, OH group |
| metabolites from Rat liver S9 metabolism simulator only | Moderate binder, OH group; Weak binder, OH group |
| **[2] rtER Expert System - USEPA, alerts in** | |
| parent only | Alkoxyphenols |
| metabolites from *in vivo* Rat metabolism simulator only | No alert found |
| metabolites from Rat liver S9 metabolism simulator only | No alert found |
| OECD QSAR Toolbox v.4.2 profilers | |
| *Profiler predictions are supporting information to be used together with the relevant QSAR predictions* | |

[1] Estrogen receptor binding: Weak binder, OH group: The OECD Toolbox v.4.2 predicts that eugenol is not a potential ER binder (moderate/weak binder).

[2] rtER Expert System USEPA: Alkoxyphenols: USEPA profiler predicts that eugenol meets the criteria of chemical classes that are potential ER binders, on the basis that is an alkoxyphenol substance.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** The *in silico* QSAR predictions displayed that eugenol has the potential to inhibit thyroperoxidase (TPO), but it is not a potential ER or AR binder.

## B.6.9. MEDICAL DATA AND INFORMATION

### B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | Volpe M.J. |
| **Report year** | 2021 |
| **Report title** | Manufacturer Medical Report |
| **Report No** | n.a. |
| **Document No** | KCA 5.9.1/01 |
| **Guidelines followed in study** | n.a. |
| **Deviations from current test guideline** | n.a. |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | No |
| **Acceptability/ reliability** | Supporting information |

The manufacturing plant conducts regular medical monitoring of workers involved in the production, packaging or handling of eugenol. There have been no reports of reactions or ill health in any workers.

### B.6.9.2. Data collected on humans

#### B.6.9.2.1. Maximization test in humans volunteers

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | Greif N. |
| **Report year** | 1967 |
| **Report title** | Cutaneous safety of fragrance material as measured by the Maximization test |
| **Report No** | Not applicable, published study (*American Perfumer and Cosmetics*, Vol.82). |
| **Document No** | KCA 5.9.2/01 |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supporting information |

| | |
|---|---|
| **Test chemicals** | Eugenol extra (undefined); Lot/Batch No.: non stated, Purity: 8% |
| **Test system** | Human |
| **Test method** | Eugenol extra (undefined) was tested in 25 human volunteers using a maximisation test protocol. After provoking an irritant reaction with 1 ml of 5% sodium lauryl sulphate (SLS), and render the skin more permeable to the test agent, a patch test was performed using 8% eugenol extra for 48 hours using an occlusive patch. The tested concentration was an arbitrary decision to use twice the concentration of materials than is conventionally found in a product applied to the skin. The experimental design would be biased against the test materials to elicit cogent and realistic data. Treatment with SLS and the test substances was repeated and alternated four times to give five exposures in total over a period of 15 days. Ten days after the final application a challenge application |

| | |
|---|---|
| | with the same concentration was performed for 49 hours and the sensitisation reaction was assessed on two successive days after patch removal. The test area is examined immediately and again on each of two successive days. The reactions are then scored for each subject on a scale of 0 to 4+. A summation of the scores establishes a sensitization rate. Then, a sensitization grade from l (weak) to 5 (extreme) for each test substance can be established (see table below). Even though Kligman classifies 0-2 reactions in a panel of 25 subjects as a weak sensitizer, for the purpose of this study, scoring of 0/25 reactions is classified as "non-sensitizing”. |

| Sensitisation rates/subjects | Grade | Classification |
|---|---|---|
| 0-2/25 | 1 | Weak |
| 3-7/25 | 2 | Mild |
| 8-13/25 | 3 | Moderate |
| 14-20/25 | 4 | Strong |
| 21-25/25 | 5 | Extreme |

**Result**

Eugenol displayed a total score of 0/25; there were no positive reactions in any of the volunteers. Therefore, eugenol was classified as non-sensitizer.

**Assessment and conclusion by applicant:**

This study has been previously reviewed at EU level and is acceptable for renewal of the active substance. EFSA (2012) concluded that eugenol is a sensitizer.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This is a published study that classified test substance eugenol as non-sensitizer in a maximization test performed in human volunteers following Kligman methodology (Kligman, A. M. *et al. J. lnvest. Derm.* 1966, 47: 369). This study used an arbitrary concentration of 8% eugenol extra (undefined), based on the decision to use twice the concentration of materials than is conventionally found in a product applied to the skin.

By contrast, maximization test carried out in guinea pigs with >95% eugenol following OECD TG 406 guideline elicited eugenol as sensitizer. Overall, an assessment of skin sensitization studies provided (see section B.6.2.6), and based on criteria of Regulation (EC) 1272/2008, a classification as **Skin Sensitisation–Category 1 (Skin Sens. 1; H317)** is proposed for eugenol by RMS.

### B.6.9.2.2. Human skin sensitisation data from patch tests.

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | Rothenstein, A.S., Booman, K.A., Dorsky, J., Kohrman, K.A., Schwoeppe, E.A., Sedlak, R.I., Steltenkamp, R.J., Thompson, G.R. |
| **Report year** | 1987 |
| **Report title** | Eugenol and clove leaf oil: A survey of consumer patch sensitisation. |
| **Report No** | Not applicable, published study (*Food and Chemical Toxicology*, 21(6):727-733). |
| **Document No** | KCA 5.9.2/02 |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |

| | |
|---|---|
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supporting information |

| | |
|---|---|
| **Test chemicals** | Eugenol: Lot/Batch No.: non stated, Purity: 100%<br>Clove leaf: Lot/Batch No.: non stated, Purity: 84-88%<br>Clove stern: Lot/Batch No.: non stated, Purity: 82-95%<br>Clove bud: Lot/Batch No.: non stated, Purity: 70-84%<br>Cinnamon leaf Ceylon: Lot/Batch No.: non stated, Purity: 80-88%<br>Bay leaf: Lot/Batch No.: non stated, Purity: 50-65%<br>Pimenta berry (allspice): Lot/Batch No.: non stated, Purity: 65-90% |
| **Test system** | Human |
| **Test method** | A survey of human skin sensitisation data from patch tests on materials containing eugenol and clove leaf oil was analysed to define the potential of eugenol and clove oil leaf to induce delayed skin hypersensitivity or to elicit reactions. Results from a total of 11632 patch tests on eugenol itself, or various consumer products containing eugenol and/or clove leaf oil, and on fragrance blends containing eugenol and/or clove leaf oil were collected from fragrance and formulation companies. |

**Result**

One instance of induced hypersensitivity and one instance of pre-existing sensitisation were observed at eugenol patch-test concentrations of 0.05 and 0.09% in consumer products (table B.6.9.2.2/1) and fragrance blends B.6.9.2.2/2), respectively. In both cases, subsequent patch testing showed skin responses to mixtures of eugenol and isoeugenol, but no further attempt was made to define the causative agent. The survey indicates that, at the concentration present in consumer products eugenol alone or as part of clove leaf oil has a very low potential either to elicit pre-existing sensitisation or to induce hypersensitivity.

**Table B.6.9.2.2/1. Human sensitization survey: eugenol and clove leaf oil in consumer products**

| Product type | Total eugenol concn in patch test mixture (%) | Concn of eugenol added as eugenol in product (%) | Clove leaf oil concn in product (%) | Product concn in patch test vehicle (%) | Test method | No. of tests | No. of sensitization reactions: Elicited | No. of sensitization reactions: Induced |
|---|---|---|---|---|---|---|---|---|
| Personal care | $6 \times 10^{-2}$ | 0 | $7 \times 10^{-2}$ | 100 | HRIPT | 50 | 0 | 0 |
| | $1 \times 10^{-2}$–$4 \times 10^{-2}$ | $1 \times 10^{-2}$–$4 \times 10^{-2}$ | 0 | 100 | Varied* | 499 | 0 | 0 |
| | $7 \times 10^{-3}$ | $7 \times 10^{-2}$ | $3 \times 10^{-3}$ | 10 | HRIPT | 50 | 0 | 0 |
| | $3 \times 10^{-4}$–$8 \times 10^{-3}$ | $1 \times 10^{-3}$–$4 \times 10^{-1}$ | 0 | 0.2–100 | Varied* | 1835 | 0 | 0 |
| | $3 \times 10^{-4}$ | 0 | $4 \times 10^{-4}$ | 100 | HRIPT | 50 | 0 | 0 |
| | $4 \times 10^{-5}$–$2 \times 10^{-4}$ | $4 \times 10^{-3}$–$4 \times 10^{-2}$ | 0 | 0.5–2 | HRIPT | 546 | 0 | 0 |
| | $3 \times 10^{-5}$ | 0 | $3 \times 10^{-4}$ | 10 | HRIPT | 51 | 0 | 0 |
| | $1 \times 10^{-5}$–$2 \times 10^{-5}$ | $1 \times 10^{-4}$–$1 \times 10^{-3}$ | 0 | 1–10 | HRIPT | 408 | 0 | 0 |
| | $1 \times 10^{-5}$ | 0 | $1 \times 10^{-5}$ | 100 | HRIPT | 50 | 0 | 0 |
| | $1 \times 10^{-6}$–$3 \times 10^{-6}$ | $6 \times 10^{-5}$–$4 \times 10^{-4}$ | 0 | 0.25–5 | HRIPT | 272 | 0 | 0 |
| | $2 \times 10^{-7}$ | 0 | $2 \times 10^{-6}$ | 10 | HRIPT | 53 | 0 | 0 |
| Household | $5 \times 10^{-2}$ | — | 0 | — | HRIPT | 23 | 0 | 1† |
| | $8 \times 10^{-4}$ | $1 \times 10^{-2}$ | 0 | 8 | HRIPT | 209 | 0 | 0 |
| | $6 \times 10^{-5}$ | $4 \times 10^{-3}$ | $4 \times 10^{-5}$ | 2 | Varied* | 400 | 0 | 0 |
| | $5 \times 10^{-5}$–$6 \times 10^{-5}$ | $3 \times 10^{-3}$–$4 \times 10^{-3}$ | 0 | 2 | Varied* | 900 | 0 | 0 |
| | $5 \times 10^{-5}$ | $3 \times 10^{-3}$ | $4 \times 10^{-4}$ | 2 | PPT | 500 | 0 | 0 |
| | $4 \times 10^{-5}$ | $4 \times 10^{-3}$ | 0 | 1 | HRIPT | 190 | 0 | 0 |
| | $3 \times 10^{-5}$ | 0 | $3 \times 10^{-5}$ | 100 | Varied* | 350 | 0 | 0 |
| | $3 \times 10^{-9}$–$1 \times 10^{-5}$ | $6 \times 10^{-7}$–$5 \times 10^{-3}$ | 0 | 0.1–0.5 | HRIPT | 427 | 0 | 0 |

HRIPT = Human repeat insult patch test PPT = Prophetic patch test

*HRIPT and PPT procedures were used.

†Resolved to eugenol-isoeugenol mixture; see Table 3 for the reaction grades.

**Table B.6.9.2.2/2. Human sensitization survey: eugenol and clove leaf oil in fragrance blends**

| Total concn of eugenol in patch test mixture (%) | Eugenol concn added as eugenol in fragrance blend (%) | Clove leaf oil concn in fragrance blend (%) | Fragrance blend concn in patch test vehicle (%) | Test method | No. of tests | No. of sensitization reactions: Elicited | No. of sensitization reactions: Induced |
|---|---|---|---|---|---|---|---|
| $1 \times 10^{-1}$–$2 \times 10^{-1}$ | $5 \times 10^{-1}$–6 | 0 | 2–30 | HRIPT | 561 | 0 | 0 |
| $9 \times 10^{-2}$ | 3 | 0 | 3 | HRIPT | 83 | 1* | 0 |
| $5 \times 10^{-2}$–$8 \times 10^{-2}$ | $7 \times 10^{-1}$–10 | 0 | 0.5–7 | HRIPT | 581 | 0 | 0 |
| $2 \times 10^{-2}$–$4 \times 10^{-2}$ | $3 \times 10^{-2}$–4 | 0 | 1–50 | HRIPT | 1110 | 0 | 0 |
| $2 \times 10^{-2}$ | 2 | $2 \times 10^{-2}$ | 1 | HRIPT | 55 | 0 | 0 |
| $1 \times 10^{-2}$ | $4 \times 10^{-2}$–0.3 | 0 | 0.3–3 | HRIPT | 129 | 0 | 0 |
| $1 \times 10^{-2}$ | $4 \times 10^{-2}$ | $5 \times 10^{-1}$ | 2 | HRIPT | 54 | 0 | 0 |
| $1 \times 10^{-2}$ | 0 | $7 \times 10^{-1}$ | 2 | HRIPT | 71 | 0 | 0 |
| $1 \times 10^{-3}$–$6 \times 10^{-3}$ | $4 \times 10^{-1}$–$5 \times 10^{-1}$ | 0 | 1–5 | HRIPT | 914 | 0 | 0 |
| $9 \times 10^{-4}$ | $7 \times 10^{-3}$ | 0 | 12 | OCPT | 10 | 0 | |
| $7 \times 10^{-4}$–$8 \times 10^{-4}$ | $3 \times 10^{-2}$–$4 \times 10^{-2}$ | 0 | 2–3 | HRIPT | 208 | 0 | 0 |
| $6 \times 10^{-4}$ | $8 \times 10^{-3}$ | 0 | 8 | OCPT | 51 | 0 | |
| $3 \times 10^{-3}$–$5 \times 10^{-4}$ | $1 \times 10^{-2}$–$2 \times 10^{-2}$ | 0 | 1–5 | HRIPT | 705 | 0 | 0 |
| $5 \times 10^{-7}$ | $4 \times 10^{-4}$ | 0 | 12 | OCPT | 10 | 0 | |
| $1 \times 10^{-7}$ | $6 \times 10^{-5}$ | 0 | 2 | PPT | 100 | 0 | 0 |
| $7 \times 10^{-8}$ | $3 \times 10^{-5}$ | 0 | 2 | HRIPT | 53 | 0 | 0 |

HRIPT = Human repeat insult patch test OCPT = 48-hr Occluded patch test PPT = Prophetic patch test

*Resolved to eugenol–isoeugenol mixture: see Table 3 (subject no. 2) for the grading of this reaction.

**Assessment and conclusion by applicant:**

This study has been previously reviewed at EU level and is acceptable for renewal of the active substance. EFSA (2012) concluded that eugenol is a sensitizer.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** No cases of delayed hypersensitivity that were directly attributable to eugenol or clove leaf oil were induced in the 11487 patch tests on consumer products and fragrance blends containing these ingredients, or in the 74 patch tests on neat eugenol. In the survey the highest reported test concentration of eugenol, from either clove leaf oil or eugenol, was 2.5%. In one instance a household product tested at eugenol concentration of 0.05% induced hypersensitivity. Evidence of pre-existing sensitization directly attributable to eugenol or clove leaf oil was not observed in the 11632 patch tests included in this survey. In one instance, an elicited reaction to a fragrance blend at a eugenol patch test concentration of 0.09% was observed, however the cause was only traced to an isoeugenol-eugenol mixture.

Based on criteria of Regulation (EC) 1272/2008, and after an overall assessment of the skin sensitization studies presented for the renewal process of the active substance eugenol, the RMS proposes a classification as **Skin Sensitisation–Category 1 (Skin Sens. 1; H317)** for eugenol.

### B.6.9.2.3. Clinical case report I.

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | De Frutos, F.J.O., Vergara, A., Isarria, M.J., Prado-Sanchez, M., Vanaclocha, F. |
| **Report year** | 2004 |
| **Report title** | Eczema alérgico de contacto profesional en auxiliar de odontología (Occupational allergic contact eczema in a dental assistant). |
| **Report No** | Not applicable, published study (*Actas dermo-sifiliogrficas*, 96, 1:56-8). |
| **Document No** | KCA 5.9.2/03 |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supporting information |

| | |
|---|---|
| **Test chemicals** | Nickel sulphate, cobalt chloride, potassium dichromate, gold, sodium thiosulfate, the liquid used in endodontics (containing eugenol at unknown concentration), fragrance mix and colophony. |
| **Test system** | Human |
| **Test method** | Skin tests were carried out applying compounds in the upper back area for 48 h following the GEIDC (Spanish Contact Dermatitis Research Group) standard battery, the Chemotechnique dental screening series and her own products. Observation and positivity was checked at 72 and 168 hours. |

**Summary**

The study presents the case of a 46-year-old woman with pruritic, flaky, erythematous-edematous lesions, with indistinct borders, on the backs and sides of the fingers, which had been developing for several month. The skin tests performed with the GEIDC (Spanish Contact Dermatitis Research Group) standard battery, the Chemotechnique dental screening series and her own products showed positivity to nickel sulphate, cobalt chloride, potassium dichromate, gold sodium thiosulfate, the liquid used in endodontics known as eugenol, fragrance mix and colophony. Eugenol is a perfumed product that is used in cements and sealants for endodontics and periodontology. The authors report that there are 5 articles regarding cases of occupational allergic contact eczema from this product in dentists or nurses and assistants who work in dental clinics.

**Table B.6.9.2.3. Positivity of the skin test.**

| | 72h | 168h |
|---|---|---|
| **Nickel sulphate** | + + | + + |
| **Cobalt chloride** | + + | + + |
| **Gold sodium thiosulfate** | - | + + |
| **Potassium dichromate** | + + | + + |
| **Eugenol (2% vaseline)** | + + | + + |
| **Endodontics liquid** | + + | + + |

**Assessment and conclusion by applicant:**

This study has been previously reviewed at EU level and is acceptable for renewal of the active substance. EFSA (2012) concluded that eugenol is a sensitizer.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This is a clinical case report of a 46-year-old woman employed as dental clinic assistant. She was preparing endodontics mixtures and handling all the material. She developed pruritic, flaky, erythematous-edematous lesions, with indistinct borders, on the backs and sides of the fingers, which had been developing for several month. The woman was subjected to skin tests to ascertain the compound that causes the skin sensitization and allergy process. Positivity was observed in the compound test with eugenol 2%, endodontics liquid, fragrance mix and colophony. The eugenol concentration and source of the endodontics liquid, fragrance mix and colophony was unknown.

Based on criteria of Regulation (EC) 1272/2008, and after an overall assessment of the skin sensitization studies presented for the renewal process of the active substance eugenol, the RMS proposes a classification as **Skin Sensitisation–Category 1 (Skin Sens. 1; H317)** for eugenol.

### B.6.9.2.4. Clinical case report II.

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | Sanchez-Perez, J., Garcia-Diez, A. |
| **Report year** | 1999 |
| **Report title** | Occupational allergic contact dermatitis from eugenol, oil of cinnamon and oil of cloves in a physiotherapist. |

| | |
|---|---|
| **Report No** | Not applicable, published study (*Contact Dermatitis*, 41:346). |
| **Document No** | KCA 5.9.2/04 |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | No |
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supporting information |

| | |
|---|---|
| **Test chemicals** | Clove oil and cinnamon oil |
| **Test system** | Human |
| **Test method** | Patch testing with the GEIDC (Spanish Contact Dermatitis Research Group) standard battery, fragrance essential oils series. Reactions were measured at 2 and 4 days. |

**Summary**

The study presents the case of a 32-year-old man, a physiotherapist for 3 ½ years, who had a history for the past 7 years of recurrent vesicular eczema of the sides of the fingers and fingertips. 7 month previously, he had developed pruriginous, vesicular, erythematosquamous plaques and fissures on all fingers and both palms, which had persisted despite topical corticosteroid treatment. A biopsy from the palm showed subacute spongiotic dermatitis. In his work, the patient massaged with topical products, including ‘‘Balsam from ash extract’’ cream. Stopping the use of this cream led to improvement within 2 days. Patch testing with the GEIDC standard series, fragrance series, essential oils series, ‘‘Balsam from ash extract’’ cream and its components revealed reactions at 2 and 4 days to fragrance mix, eugenol, ‘‘Balsam from ash extract’’ cream, and 2 of its components, cinnamon oil 0.5% pet and clove oil 1% pet. Patch tests with cinnamon oil and clove oil were negative in 39 controls.

**Assessment and conclusion by applicant:**

This new study is considered as supportive information for the renewal of eugenol as it provides information on human sensitisation consistent with classification, however it is rated as not reliable due to limited reporting, the patient was allergic to a number of other substances and previous eugenol exposure was not fully documented.

EFSA (2012) concluded that eugenol is a sensitizer.

**Assessment and conclusion by RMS:**

**Outcome and conclusion of the study:** This is a clinical case report of a 32-year-old man employed as physiotherapist. He performed patient massaged with topical products, including ‘‘Balsam from ash extract’’ cream. The man was subjected to skin tests to ascertain the compound that causes the skin sensitization and allergy process. Positivity was observed in the compound test with eugenol and clove oil 1%. However, the eugenol and clove oil concentration in massage products such as balsam and creams was unknown.

Based on criteria of Regulation (EC) 1272/2008, and after an overall assessment of the skin sensitization studies presented for the renewal process of the active substance eugenol, the RMS proposes a classification as **Skin Sensitisation–Category 1 (Skin Sens. 1; H317)** for eugenol.

## B.6.9.3. Direct observation

### B.6.9.3.1. Clinical case report

| | |
|---|---|
| **Data point** | CA 5.9 (EU data requirement) |
| **Report author** | Hartnoll, G., Moore, D., Douek, D. |
| **Report year** | 1993 |
| **Report title** | Near fatal ingestion of oil of cloves. |

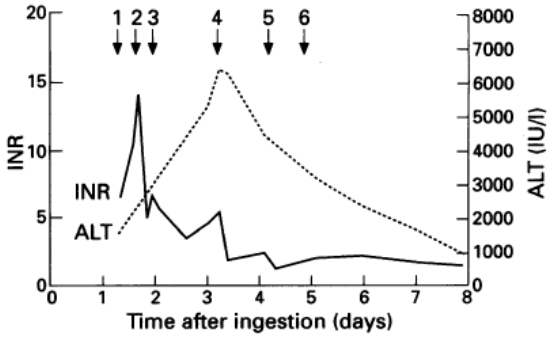
| | |
|---|---|
| **Report No** | Not applicable, published study *(Archives of Disease in Childhood*, 69: 392-393). |
| **Document No** | KCA 5.9.3/01 |
| **Guidelines followed in study** | Not applicable |
| **Deviations from current test guideline** | Not applicable |
| **Previous evaluation** | Yes, evaluated in DAR (2011) |
| **GLP/Officially recognised testing facilities** | Not applicable, published study. |
| **Acceptability/ reliability** | Supporting information |

| | |
|---|---|
| **Test chemicals** | Clove oil |
| **Test system** | Human |

**Summary**

A 2-year-old male consumed 5-10 mL of clove oil. Within 3 hours the child deteriorated into a coma with a marked acidosis and 8.5 hours after ingestion had a generalised seizure which was treated with diazepam. Also at this time, an intravenous bolus dose of dextrose was also given at this time because blood glucose could not be detected. Twenty-four hours after ingestion the patient was still unconscious and clinical tests showed evidence of liver toxicity. His international normalized ratio (INR) was 6.51 and alanine aminotransferase was 1549 IU/l (normal range <34 IU/l). At this point his haemoglobin concentration was 131 g/l with a platelet count of 56x $10^9$/l. A repeat INR after vitamin K was 10.48 and fibrin degradation products were >250 IU/l (normal <250). Fresh blood was aspirated from his nasogastric tube and so a primary diagnosis of disseminated intravascular coagulopathy (DIC) was made, although the deranged clotting could have been solely due to hepatic dysfunction. In order to treat the presumed DIC, the patient was started on heparin 200 IU/hour, fresh frozen plasma, and fibrinogen. Treatment for disseminated intravascular coagulation was continued from the third to fourth days after ingestion by administration of fresh frozen plasma, heparin, antithrombin III, protein C and factor VII, guided by the INR and alanine aminotransferase results. At this time the patient's right optic disc margin was blurred and his level of consciousness and liver function test deteriorated further. This coincided with a further deterioration in his conscious level, corresponding to the continuing deterioration of his liver function tests. There were no other signs of raised intracranial pressure. The child's condition improved during the fifth day, he was fully conscious on day 6 and subsequently made a full recovery (see figure 6.9.3).

**Figure B.6.9.3: Parameters evolution over time.**




*INR and alanine aminotransferase (ALT) and treatments given. (1) Vitamin K 5 mg intravenous; (2) fresh frozen plasma, heparin, and fibrinogen (1 g); (3) antithrombin III 1050 U, protein C 880 U; (4) antithrombin III 525 U, protein C 880 U, factor VII 550 U, neomycin, and lactulose; (5) antithrombin III 525 U, protein C 880 U, factor VII 500 U; (6) vitamin K 5 mg intravenous.*

| |
|---|
| **Assessment and conclusion by applicant:**<br>This study has been previously reviewed at EU level and is acceptable for renewal of the active substance. |
| **Assessment and conclusion by RMS:**<br>**Outcome and conclusion of the study:** This is a clinical case report of an accidental clove oil ingestion in a 2-year-old male. He fell into a coma within 3 hours post ingestion. Twenty-four hours after ingestion the patient was still unconscious, clinical tests showed evidence of liver toxicity, and disseminated intravascular coagulation was diagnosed. Patient was treated with fresh frozen plasma, heparin, antithrombin III, protein C and factor VII. Full recovery was observed on day 6.<br><br>Eugenol is metabolised to glucuronide and sulphate conjugates by the liver, and has been shown to be hepatotoxic in rats with cell death occurring in >85% of rat hepatocytes exposed to eugenol after five hours; this effect is completely prevented by the administration of acetylcysteine (B.6.1.1.6; Thompson, *et al.*, 1991).<br><br>N-acetyl-cysteine (NAC), a precursor of GSH synthesis, is nowadays widely used as primary curative treatment for accidental clove oil or eugenol containing products ingestion. |

### B.6.9.4. Epidemiological studies

No epidemiological studies have been presented for the renewal of the active substance eugenol.

### B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

Information on signs of poisoning is available in accidental ingestion cases described in B.6.9.3 section.

### B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

| |
|---|
| **Assessment and conclusion by applicant:**<br>First aid measures include removal of patient to fresh air if inhalation exposure is suspected. It is recommended any contaminated clothing is immediately removed and that any exposed skin is washed with soap and water. In the case of ocular exposure, severe or lasting irritation may occur, affected eyes should be rinsed with water for at least 15 minutes. Following oral exposure, induce vomiting in patient and contact medical services. A doctor should be called if any symptoms persist or the patient feels unwell. |
| **Assessment and conclusion by RMS:**<br>N-acetylcysteine might be beneficial in cases of acute poisoning. |

## B.6.10. REFERENCES RELIED ON

### B.6.10.1. Review of the scientific open literature.

#### B.6.10.1.1. Summary

A literature review of eugenol in accordance with the EFSA guidance document published in the EFSA Journal 2011; 9(2):2092, together with the Appendix to this guidance; and AGES report (EFSA supporting publication 2013:EN-511), has been conducted, and the results are summarized below.

In this literature search, the aim was to find scientific peer-reviewed open literature on eugenol dealing with toxicological and toxicokinetic studies, operator exposure, residues, fate and behaviour in the environment and ecotoxicological studies which were published between 1/1/2005 and 16/02/2021 for eugenol and between 1/1/1995 and 16/02/2021 for methyleugenol.

To achieve this goal, a wide range of reference collections was consulted utilising the online various databases.

The following initial search strategy was used::

- The search terms were defined, initially using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.

- Specific search terms were then used relating to the specific data requirements in question (i.e. for toxicology, human exposure) to refine the search.

- The search of publications was conducted, using the two-stage approach outlined above. A list of titles, authors and dates were extracted. Any duplicate summary records were removed, before proceeding.

The remaining summary records were then assessed for relevance in a two-step process:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records which were obviously irrelevant to the specific data requirements in question.

- Secondly, a detailed assessment of full-text documents was conducted to identify potentially relevant, irrelevant or relevant and reliable records that were not excluded in the first step.

All literature papers that were considered relevant to the risk assessment following this process are summarised in the corresponding sections of the dossier, where an assessment of their reliability is also presented. The results of this search were as follows:

| **Study selection process** | **Eugenol** | | | |
|---|---|---|---|---|
| | **Toxicology (including human exposure)** | **Residues** | **Environmental fate and behaviour** | **Ecotoxicology** |
| Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance) | 28270 | | | |
| Total number of documents retrieved (without duplicates) after focused search strategy (combining terms for active substance and specific data requirements) | 1191 | 487 | 733 | 1865 |
| Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature) | 1166 | 473 | 730 | 1756 |
| Number of publications for which detailed assessment of full-text required after rapid assessment for relevance | 25 | 13 | 3 | 109 |

| | | | | |
|---|---|---|---|---|
| Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature) | 13 | 3 | 1 | 66 |
| **Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)** | **12** | **10** | **2** | **43** |

| **Study selection process** | **Methyleugenol** | | | |
|---|---|---|---|---|
| | **Toxicology (including human exposure)** | **Residues** | **Environmental fate and behaviour** | **Ecotoxicology** |
| Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance) | 4 133 | | | |
| Total number of documents retrieved (without duplicates) after focused search strategy (combining terms for active substance and specific data requirements) | 191 | 102 | 147 | 329 |
| Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature) | 176 | 98 | 147 | 275 |
| Number of publications for which detailed assessment of full-text required after rapid assessment for relevance | 15 | 4* | 0 | 54 |
| Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature) | 1 | 1* | 0 | 50 |
| **Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)** | **14** | **3*** | **0** | **4** |

**The same papers were also identified in the eugenol literature search.*

The search strategy captured scientific peer-reviewed literature published during the last fifteen years from a wide range of reference collections consulted through online wide and various databases.

Details on these various reference collections are provided hereafter (see B.6.10.1.2) fulfilling requirements from EFSA and AGES on the bibliographic database. The search strategy leading to the inclusion or exclusion of publications is described below (see B.6.10.1.3 and B.6.10.4).

### B.6.10.1.2. Search strategy

The following initial search strategy was used:

- The search terms were defined using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.

- Specific search terms relating to the data requirements in question (i.e. for toxicology, human exposure, residues, environmental fate and behaviour and ecotoxicology) were then used to refine the search.

- These search terms are listed in Section B.6.10.1.2.4 below.

#### B.6.10.1.2.1. Date of the literature search.

The literature search was conducted on 2nd July 2020 and 16th February 2021.

#### B.6.10.1.2.2. Time period covered in the literature search.

The search identified scientific peer-reviewed open literature published in the last 15 years (from 1/1/2005 to 16/02/2021) for eugenol and in the last 25 years (from 1/1/1995 to 16/02/2021) for methyleugenol.

#### B.6.10.1.2.3. Bibliographic databases used in the literature search.

In line with EFSA guidance, a number of different sources were used in the literature search with the aim to locate all relevant scientific peer-reviewed open literature. The reference collections searched were AGRICOLA, AGRIS (FAO), PASCAL, PubMed (MEDLINE) inc. TOXNET, Science Direct, Springer, and Wiley. All sources and the rationale for their inclusion in the search are presented in the table below.

**Table B.6.10.1.2.3. Databases used in the literature search by applicant**

| Database | Description/Rationale for Inclusion |
|---|---|
| 1. AGRICOLA | Coverage of the database includes agricultural economics and rural sociology, agricultural production, animal sciences, chemistry, entomology, food and human nutrition, forestry, natural resources, pesticides, plant science, soils and fertilizers, and water resources. More than 6.7 million records (09/2019). 1970-present. |
| 2. AGRIS (FAO) | AGRIS is one of the most comprehensive search engines in food and agricultural scientific literature providing free access to millions of bibliographic records in 90 different languages. Up to hundreds of organisations worldwide contribute to knowledge and data to the AGRIS platform, resulting in a multilingual bibliographic collection of food and agricultural scientific research with special attention to scientific information produced in the global south. Therefore, AGRIS is used by whoever is inclined to find literature on any of FAO's areas of interest. |
| 3. PASCAL | The site is an archive of the PASCAL and FRANCIS bibliographic databases in exact, human and social sciences, produced by the Inist-CNRS from 1972 to 2015. It provides access to more than 20 millions references (articles, patents, maps, conferences, books, reports and thesis) in exact sciences and technology, in biological and medical sciences, in art, archaeology, economics, ethnology, geography, history of science and technology, literature, linguistics, administrative and legal sciences, education and religion sciences. |
| 4. PubMed MEDLINE (including TOXNET) | MEDLINE contains information on every area of medicine. More than 30 million records (08/2019). 1946-present. |
| 5. Science Direct | ScienceDirect provides access to more than 16 million articles, 2500 journals, 370 full open access journals, 39000 books and 330000 topic pages to help researchers discover more insights, achieve more breakthroughs and move their research forward. |
| 6. Springer | Springer is a leading global scientific, technical and medical portfolio, providing researchers in academia, scientific institutions and corporate R&D departments with quality content through innovative information, products and services. It handles more than 2900 journals and 300000 book. |
| 7. Wiley | Wiley is one of the largest and most authoritative collections of online journals, books, and research resources, covering life, health, social, and physical sciences. |

### B.6.10.1.2.4. Input parameters (search items) for the literature search

Any documents relating to the essential oils, their synonyms and other common names were collected. The reference collections were queried by name e.g. "eugenol" in addition to common variants such as eugenic acid, and "clove oil". The tables below present the general search terms used:

**Table B.6.10.1.2.4/1. List search terms used to identify all literature related to the active substance (eugenol), its metabolites and plant protection products containing the active substance**

| | |
|---|---|
| **Common name** | Eugenol<br>Eugenic acid<br>Eugenol methyl ether Allylguaiacol<br>Eugenic acid Caryophyllic acid<br>p-Allylguaiacol<br>p-Eugenol 1,3,4-Eugenol<br>allylveratrol<br>Caryophyllic acid |

| | |
|---|---|
| | Clove oil<br>Nutmeg oil<br>Cinnamon oil<br>Basil oil<br>Bay leaf<br>Phenol, 2-methoxy-4-(2-propenyl)- |
| **Chemical name (IUPAC and CA)** | 2-methoxy-4-prop-2-enylphenol<br>2-methoxy-4-(2-propen-1-yl)phenol<br>2-Methoxy-4-(2-propenyl)phenol<br>2-Methoxy-4-allylphenol<br>4-Allyl-2-methoxyphenol<br>4-Allylguaiacol<br>1-Hydroxy-2-methoxy-4-allylbenzene<br>1-Hydroxy-2-methoxy-4-prop-2-enylbenzene<br>1-Hydroxy-2-methoxy-4-propenylbenzene<br>2-Methoxy-1-hydroxy-4-allylbenzene<br>2-Methoxy-4-(2-propen-1-yl)phenol<br>2-Methoxy-4-allylphenol<br>2-methoxy-4-(2-propenyl)phenol<br>2-Methoxy-4-prop-2-enylphenol<br>2-Metoksy-4-allilofenol<br>4-Allyl-1-hydroxy-2-methoxybenzene<br>4-Allyl-2-methoxyphenol<br>4-Allylcatechol 2-methyl ether<br>4-Allylcatechol-2-methyl ether<br>4-Allylguaiacol<br>4-Hydroxy-3-methoxy-1-allylbenzene<br>4-Hydroxy-3-methoxyallylbenzene<br>5-Allylguaiacol<br>1-Allyl-3-methoxy-4-hydroxybenzene |
| **Metabolite(s)** | - |
| **Product name** | Mevalone, Andromeda, 3Logy, ES-00108, Trigemol, Cagenoleta, Eugeti |
| **CAS No.** | 97-53-0 |
| **EC No.** | 202-589-1 |

**Table B.6.10.1.2.4/2. List search terms used to identify all literature related to the compound methyleugenol**

| | |
|---|---|
| **Common name** | Methyl Eugenol<br>Methyl eugenol<br>Methyleugenol<br>methyl-eugenol<br>ME<br>Benzene, 1,2-dimethoxy-4-(2-propenyl)-<br>O-Methyl eugenol<br>O-Methyleugenol |
| **Chemical name (IUPAC and CA)** | 1,2-dimethoxy-4-prop-2-enylbenzene 1,2-Dimethoxy-4-(2-propen-1-yl)benzene 1,2-Dimethoxy-4-(2-propenyl)benzene<br>1,2-Dimethoxy-4-allylbenzene<br>1,3,4-Eugenol methyl ether<br>1-(3,4-Dimethoxyphenyl)-2-propene<br>1-Allyl-3,4-dimethoxybenzene<br>3,4-Dimethoxyallylbenzene<br>4-Allyl-1,2-dimethoxybenzene<br>4-Allylveratrole<br>Benzene, 1,2-dimethoxy-4-(2-propenyl)-<br>Benzene, 4-allyl-1,2-dimethoxy-<br>4-Allyl-1,2-dimethoxybenzene |
| **Metabolite(s)** | - |
| **Product name** | Mevalone |
| **CAS No.** | 93-15-2 |
| **EC No.** | 202-223-0 |

The specific search terms for toxicology were:

**Table B.6.10.1.2.4/3. Subjects search list used for toxicology**

| Toxicology (including human exposure) (N = 36) | | |
|---|---|---|
| bystander | mice | resident |
| bystanders | mouse | residents |
| dog | monkey | skin |
| dogs | monkeys | workers |
| epidermal | occupation | human |
| epidermis | occupational | humans |
| eye | operator | adult |
| eyes | operators | adults |
| guinea pig | rabbit | child |
| guinea pigs | rabbits | children |
| hamster | rat | neonate |
| hamsters | rats | neonates |

**Table B.6.10.1.2.4/4. Effects search list used for toxicology**

| Toxicology effect list (N = 286) | | | |
|---|---|---|---|
| absorption | EC10 | hormone disruptor | NOAER |
| acute | EC20 | hormone disruptors | NOEC |
| adrenal | EC50 | hormones | NOED |
| adsorption | ecotoxic | immunotoxic | NOEL |
| adverse | ecotoxicity | immunotoxicity | NOER |
| allergen | ecotoxicological | immunotoxin | oestradiol |
| allergenic | ecotoxicology | immunotoxins | oestrogen |
| allergens | ED10 | *in vitro* | oestrogenic |
| allergic | ED20 | *in vivo* | oestrogenicity |
| androgen | ED50 | inhalation | oral |
| androgenic | effect | inhale | orally |
| aneugen | effects | intersex | ovaries |
| aneugens | egg production | irritant | ovary |
| aneugenic | eliminate | irritants | ovum |
| anomalous | elimination | irritating | PBPK |
| anomalies | eliminations | irritation | phototoxicity |
| anomaly | embryo | kinetic | phytotoxic |
| aromatase | embryos | LC10 | phytotoxicity |
| behavior | emergence | LC20 | phytotoxin |
| behavioral | endocrin | LC50 | phytotoxins |
| behaviors | endocrine | LD10 | pituitary |
| behaviour | endocrine disrupter | LD20 | poison |
| behavioural | endocrine disrupters | LD50 | poisoning |
| behaviours | endocrine disruption | lethal | poisonings |
| bioaccumulate | endocrine disruptor | LOAED | poisonous |
| bioaccumulation | endocrine disruptors | LOAEL | poisons |
| bioconcentrate | endocrinology | LOAER | population |
| bioconcentration | enzymatic | LOED | populations |
| biomagnification | enzyme | LOEL | pregnancies |
| birth | enzymes | LOER | pregnancy |
| births | enzymic | LR10 | pregnant |
| BMD | epidemiological | LR20 | reproduce |
| body weight | epidemiology | LR50 | reproduction |
| body weights | ER10 | malformation | reproductive |
| bodyweight | ER20 | malformations | sensitised |
| bodyweights | ER50 | malformities | sensitiser |
| cancer | estradiol | malformity | sensitisers |
| cancers | estrogen | mammary | sensitising |

| | | | |
|---|---|---|---|
| carcinogen | estrogenic | maternal | sensitize |
| carcinogenic | estrogenicity | mechanism | sensitizer |
| carcinogenicity | excretion | mechanisms | sensitizers |
| chromosomal | exposure | mechanistic | sensitizing |
| chromosome | fecundity | medical | sex ratio |
| chromosomes | fertile | metabolic | sex ratios |
| chronic | fertility | metabolise | sex shift |
| clastogens | fetal | metabolism | sex shifts |
| clastogen | fetus | metabolite | sexual maturation |
| clastogenic | fetuses | metabolites | sexual maturity |
| clastogenicity | foetal | metamorphose | spawning |
| corticosteroid | foetus | metamorphosis | sperm |
| corticosteroids | foetuses | mixture | spiggin |
| cortisol | gavage | mixtures | steroid |
| CYP | gene | mortalities | steroidogenesis |
| cytochrome | genes | mortality | steroidogenic |
| cytochromes | genetic | mutant | steroids |
| deformation | genotoxic | mutants | subacute |
| deformations | genotoxicity | mutate | subchronic |
| deformities | gestation | mutation | testes |
| deformity | gonad | mutations | testis |
| dermal | gonads | neurobehavior | testosterone |
| dermally | growth | neurobehavioral | thyroid |
| developed | hatch | neurobehaviors | toxic |
| developing | hatching | neurobehaviour | toxicities |
| development | hazard | neurobehavioural | toxicity |
| developmental | hazardous | neurobehaviours | toxicological |
| developments | hazards | neurotoxic | toxicology |
| diet | health | neurotoxicity | toxin |
| dietary | hormonal | neurotoxin | toxins |
| diets | hormone | neurotoxins | utero |
| distribution | hormone disrupter | NOAEC | uterus |
| distributions | hormone disrupters | NOAED | vigour |
| DNA | hormone disruption | NOAEL | vitellogenin |

### B.6.10.1.3. Search results

The total numbers of records (before removing duplicates) for the search are summarised in the table below. The peer-reviewed literature search strategy highlighted 28270 documents of potential interest for eugenol and 4133 documents of potential interest for methyleugenol to this review before the removal of duplicates occurring as a result of searching the individual reference collections separately.

**Table B.6.10.1.3/1. Numbers of records (before removing duplicates) for the eugenol search**

| **Databases (through online STN)** | **Number of hits Eugenol First research** | **Number of hits Eugenol Second research** | **Number of hits Eugenol Total** |
|---|---|---|---|
| 1. AGRICOLA | 7 | 90 | 97 |
| 2. AGRIS (FAO) | 409 | 14 | 423 |
| 3. PASCAL | 508 | - | 508 |
| 4. PubMed MEDLINE (including TOXNET) | 4202 | 307 | 4509 |
| 5. Science Direct | 10224 | 288 | 10512 |
| 6. Springer | 6888 | 913 | 7801 |
| 7. Wiley | 3920 | 500 | 4420 |
| Total number of documents retrieved (with duplicates) (global search results related to | **26158** | **2112** | **28270** |

| | | | |
|---|---|---|---|
| the active substance, its metabolites and plant protection products containing the active substance) | | | |

**Table B.6.10.1.3/2. Numbers of records (before removing duplicates) for the methyleugenol search**

| **Databases (through online STN)** | **Number of hits Methyleugenol First search** | **Number of hits Methyleugenol Second search** | **Number of hits Methyleugenol Total** |
|---|---|---|---|
| 1. AGRICOLA | - | 18 | 18 |
| 2. AGRIS (FAO) | 114 | 14 | 128 |
| 3. PASCAL | - | - | - |
| 4. PubMed MEDLINE (including TOXNET) | 463 | 37 | 500 |
| 5. Science Direct | 1575 | 24 | 1599 |
| 6. Springer | 789 | 111 | 900 |
| 7. Wiley | 904 | 84 | 988 |
| Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance) | **3845** | **288** | **4133** |

To identify which of these records mentioned the aspects of interest to this particular project, a text mining application was built and applied to each document in turn and only those which specifically mentioned: identify individual words and features, split the documents; use the gazetteer lists to identify any important key words and phrases, identify the title and abstract of the document, look within the title and abstract for patterns matching the natural language expressions, and index the results.

After removing duplicates and text mining process, the total number of hits potentially relevant is presented below:

**Table B.6.10.1.3/3. Number of documents identified as potentially relevant after text mining and removal of duplicates**

| **All databases** | **Number of hits Eugenol** | **Number of hits Methyleugenol** |
|---|---|---|
| Toxicology | **1191** | **191** |

### B.6.10.1.4. Study selection

The remaining summary records were assessed for relevance in a two-step process:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records which were considered irrelevant and the justification was recorded.

- Secondly, a detailed assessment relevance and reliability of full-text documents was conducted for any remaining summary records that were not excluded in the first step.

#### B.6.10.1.4.1. Rapid assessment

The total number of publications excluded after rapid assessment for relevance and the number of publications for which a detailed assessment of the full-text was required are summarised in the table below.

**Table B.6.10.1.4.1/1. Numbers of publications excluded/identified after rapid assessment for relevance for eugenol**

| Study selection process | Eugenol |
|---|---|
| | Toxicology (including human exposure) |
| Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature) | 1166 |
| Number of publications for which detailed assessment of full-text required after rapid assessment for relevance | 25 |

**Table B.6.10.1.4.1/2. Numbers of publications excluded/identified after rapid assessment for relevance for methyleugenol**

| Study selection process | Methyleugenol |
|---|---|
| | Toxicology (including human exposure) |
| Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature) | 176 |
| Number of publications for which detailed assessment of full-text required after rapid assessment for relevance | 15 |

#### B.6.10.1.4.2. Detailed assessment

The total number of publications excluded and the final number of relevant papers identified after detailed assessment of full-texts for relevance are summarised in the tables below.

**Table B.6.10.1.4.2/1. Criteria for Relevance – Detailed Screening**

| Data requirement | Justification for Search Strategy |
|---|---|
| **Toxicological and metabolism studies (OECD IIA 5.1 to 5.10 and OECD IIIA 7.1 and 7.6)** | |
| ADME (OECD IIA 5.1) | 1.Defined test material<br>2.*In vivo* tests in relevant test species for toxicological testing (*i.e* rat, mouse, rabbit, dog)<br>3.*In vitro* tests<br>4.PBPK modelling<br>5.Specific endpoint can be clearly related to this data requirement |
| Acute toxicity, irritation, sensitisation (a.s.) (OECD IIA 5.2) | 1.Defined test material<br>2.Relevant test species for toxicological testing (*i.e* rat, mouse, rabbit, dog, guinea pig)<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |
| Short-term toxicity<br>(OECD IIA 5.3) | 1.Defined test material<br>2.Relevant test species for toxicological testing<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |
| Genotoxicity<br>(OECD IIA 5.4) | 1.Defined test material<br>2.*In vitro* tests<br>3.*In vivo* tests in relevant test species for toxicological testing<br>4.Specific endpoint can be clearly related to this data requirement |
| Long-term toxicity and carcinogenicity<br>(OECD IIA 5.5) | 1.Defined test material<br>2.Relevant test species for toxicological testing (*i.e.* rat, mouse)<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |
| Reproductive toxicity<br>(OECD IIA 5.6) | 1.Defined test material<br>2.Relevant test species for toxicological testing<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |

| Data requirement | Justification for Search Strategy |
|---|---|
| Neurotoxicity<br>(OECD IIA 5.7) | 1.Defined test material<br>2.*In vivo* tests in relevant test species for toxicological testing<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |
| Studies on metabolites and impurities<br>(OECD IIA 5.8) | 1.Defined test material<br>2.*In vitro* tests<br>3.*In vivo* tests in relevant test species for toxicological testing<br>4.Relevant (physiologic) route of exposure<br>5.Specific endpoint can be clearly related to this data requirement |
| Supplementary studies on active substance (e.g. immunotoxicity studies on mixture effects) (OECD IIA 5.10) | 1.Defined test material<br>2.Relevant test species for toxicological testing<br>3.Specific endpoint can be clearly related to this data requirement |
| Endocrine disrupting properties<br>(OECD IIA 5.10) | 1.Defined test material<br>2.*In vitro* tests<br>3.*In vivo* tests in relevant test species for toxicological testing<br>(*i.e* rat, mouse, rabbit, dog)<br>4.Relevant (physiologic) route of exposure<br>5.Specific endpoint can be clearly related to this data requirement |
| Medical data<br>(OECD IIA 5.9) | 1.Defined test material<br>2.Epidemiological studies<br>3.Poisonings, clinical cases<br>4.Relevant (physiologic) route of exposure |
| Acute toxicity, irritation, sensitisation (PPP) (OECD IIIA 7.1) | 1.Defined test material<br>2.Relevant test species for toxicological testing (*i.e* rat, mouse, rabbit, dog, guinea pig)<br>3.Relevant (physiologic) route of exposure<br>4.Specific endpoint can be clearly related to this data requirement |
| Exposure assessment (PPP)<br>(OECD IIIA 7.3-7.5) | 1.Defined test material<br>2.Field studies<br>3.Calculations<br>4.Specific endpoint can be clearly related to this data requirement |
| Dermal absorption (PPP)<br>(OECD IIIA 7.6) | 1.Defined test material<br>2.*In vitro* tests<br>3.*In vivo* tests in relevant test species for toxicological testing (*i.e.* rat)<br>4.Specific endpoint can be clearly related to this data requirement |

**Table B.6.10.1.4.2/2. Numbers of publications excluded/identified after detailed assessment for relevance for eugenol**

| Study selection process | Eugenol |
|---|---|
| | Toxicology (including human exposure) |
| Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature) | 13 |
| **Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)** | **12** |

**Table B.6.10.1.4.2/3. Numbers of publications excluded/identified after detailed assessment for relevance for methyleugenol**

| Study selection process | Methyleugenol |
|---|---|
| | Toxicology (including human exposure) |

| | |
|---|---|
| Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature) | 1 |
| **Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)** | **14** |

All publications excluded regarding toxicology and human exposure studies following the detailed assessment are presented in the tables below, with a reason for not including in the dossier.

**Table B.6.10.1.4.2/4. Publications excluded from the risk assessment after detailed assessment of full-text documents – results for eugenol.**

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| **TOXICOLOGY AND HUMAN EXPOSURE** | | | | | |
| Sukhomlinova E.A., Shormanov V.K., Kvachakhiia L.L., Elizarova M.K. (N/A article in Russian) | 2009 | [Study of eugenol distribution in the body of warm-blooded animals after lethal poisoning] | Sud Med Ekspert. 52(2):35-8 | Article in Russian, full text could not be obtained | Agree with the applicant decision |
| Jaganathan S.K. | 2012 | Antiproliferative and molecular mechanism of eugenol-induced apoptosis in cancer cells | Molecules 17(6)6290-304 | General review paper with no information relevant to the toxicological assessment of eugenol | Agree with the applicant decision |
| Dibazar S.P, Fateh S., Daneshmandi S. | 2014 | Clove (Syzygium aromaticum) ingredients affect lymphocyte subtypes expansion and cytokine profile responses: An *in vitro* evaluation | Journal of Food and Drug Analysis 22(4):448-454 | Non-standard assay type using a clove extract. Results are not relevant to the toxicological assessment of eugenol. | Agree with the applicant decision |

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| Said M.M., Ogawa K., Pitchakarn P., Takahashi S., Asamoto M., Shirai T. | 2009 | Cyclooxygenase 2 and prostaglandin E2 are not involved in N-nitrosodiethylamine-initiated early rat hepatocarcinogenesis | Journal of Toxicologic Pathology 22:263-271 | Study considered not relevant and not reliable based on non-standard study design. Main treated groups pre-treated with N-nitrosodiethylamine (DEN) prior to eugenol exposure. Only one dose of eugenol was used. Saline / eugenol controls limited to 3 animals per group so not sufficient for statistical power. Relevant parameters not included. | Agree with the applicant decision |
| Ellis J.K, Carmichael P.L., Gooderham N.J. | 2006 | DNA adduct levels in the liver of the F344 rat treated with the natural flavour methyl eugenol | Toxicology 226(1):73-74 | Study investigates methyleugenol, not eugenol, therefore not relevant to the eugenol literature search. Conference abstract only – insufficient details available on methodology. | Agree with the applicant decision |
| Lienou L.L., Telefo P.B., Rodrigues G.Q., Donfack J.N., Araújo R.A., Bruno J.B., Njimou J.R., Mbemya T.G., Santos R.R., Souza J.F., Figueiredo J.R., Rodrigues A.P.R. | 2020 | Effect of different extracts and fractions of Senecio biafrae (Oliv. &Hiern) J. Moore on *in vivo* and *in vitro* parameters of folliculogenesis in experimental animals | Journal of Ethnopharmacology 6;251:112571 | Test material is a plant extract containing multiple components; results cannot be attributed to eugenol alone and therefore study is not relevant to the toxicological assessment of eugenol. | Agree with the applicant decision |

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| Ogawa Y., Akamatsu M., Hotta Y., Hosoda A., Tamura H. | 2010 | Effect of essential oils, such as raspberry ketone and its derivatives, on antiandrogenic activity based on *in vitro* reporter gene assay | Bioorganic & Medicinal Chemistry Letters 20(7):2111-4 | Study partially relevant but not reliable due to limited reporting on methodology and test material information. | Agree with the applicant decision |
| Bolaji S.M., Austin O.I., Adebayo A.B., Abel N.A., Olalekan J.O., Rachael H., Andrew E. | 2019 | Effect of Eugenol on Aluminium Chloride-Induced Neurotoxicity in the Hippocampus and Dendate Gyrus of Wistar Rats | IBRO Reports, Volume 7 Supplement, Page 9 | Conference abstract only – insufficient details available on methodology and study design not relevant to toxicological assessment (investigation of eugenol treatment on neurotoxicity induced by another substance). | Agree with the applicant decision |
| Khaki Amir Afshin ; Khaki Arash ; Moghimian Maryam ; Soltani Malihe ; Barghi Bita ; Shokoohi Majid (No 3b in CA 9.4/02a) | 2021 | Eugenol improves tissue damage and oxidative stress in adult female rats after ovarian torsion/detorsion. | J Obstet Gynaecol. 2021 Feb 4:1-6. doi: 10.1080/01443615.2020.1816938 | Methodology not compliant with regulatory requirements; study investigated effects of intraperitoneally administered eugenol on experimentally-injured rat ovaries. Investigations and reporting are limited compared to a regulatory study, and only low-dose acute exposure was used for control purposes. Study not considered relevant for regulatory submission. | Agree with the applicant decision |

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| Sellamuthu, R., | 2014 | Eugenol | Encylopedia of Toxicology (Third Edition), Pages 539-541 | General review of the toxicological properties of eugenol, with no reference to original sources of information. Does not provide any additional information, and original sources cannot be assessed for quality. Not relevant for the dossier. | Agree with the applicant decision |
| Melles D., Vielhaber T., Baumann A., Zazzeroni R., Karst U. | 2013 | In chemico evaluation of skin metabolism: Investigation of eugenol and isoeugenol by electrochemistry coupled to liquid chromatography and mass spectrometry | Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 15;913-914:106-12 | Non-standard study type not relevant to the toxicological assessment of eugenol. | Agree with the applicant decision |
| Rahimullah Siddiqui a; Fakhrul Islam a; Saeed Alshahrani a; Mohammad Ashafaq a; Sohail Hussain a; Manal Mohammed b; Muhammad Sultan c; Abdulmajeed M. Jali a (No 4b in CA 9.4/02a) | 2021 | Renoprotective effects of cinnamon oil against APAP-Induced nephrotoxicity by ameliorating oxidative stress apoptosis and inflammation in rats. | Saudi Pharmaceutical Journal Available online 23 January 2021 In Press Corrected Proof What are Corrected Proof articles? | Methodology not compliant with regulatory requirements; study investigated ameliorating effects of cinnamon oil against acetaminophen induced nephrotoxicity. Investigations are limited compared to a regulatory study, and only one dose of cinammon oil alone was used for control purposes. Eugenol content of test material not reported. Study not considered relevant for regulatory submission. | Agree with the applicant decision |

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| Anita Y., Radifar M., Kardono L.B.S., Hanafi M., Istyastono E.P. | 2012 | Structure-based design of eugenol analogs as potential estrogen receptor antagonists | Bioinformation 8(19):901-6 | Development of structure-based virtual screening protocol; not relevant to regulatory dossier | Partially agree with the applicant decision. This is study suggests through *in silico* approach that eugenol may exert some degree antagonistic/binding activity at the ER receptor. This study supports Howes *et al.* (B.6.8.3.5) findings, but not adds new information. Partially relevant. |

**Table B.6.10.1.4.2/5. Publications excluded from the risk assessment after detailed assessment of full-text documents – results for methyleugenol.**

| Author(s) | Year | Title | Source | Reason for not including in dossier (based on relevance criteria) | RMS conclusion |
|---|---|---|---|---|---|
| **TOXICOLOGY AND HUMAN EXPOSURE** | | | | | |
| Guo, K-F., Dai M., Liu Y-M., Zhang J-C., Chen Y-M., Ye H., Li M-B., Mao R-R., Cao J. | 2020 | Acute Administration of Methyleugenol Impairs Hippocampus-Dependent Contextual Fear Memory and Increases Anxiety-like Behavior in Mice | Journal of Agricultural and Food Chemistry 15;68(28):7490-7497 | Study not relevant; does not investigate any relevant parameters | Agree with the applicant decision |

All relevant publications for toxicology identified following the detailed assessment are presented in the tables below:

**Table B.6.10.1.4.2/6. Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance: sorted by author for eugenol.**

| Author(s) | Data requirement (indicated by the corresponding CA and CP data point | Year | Title | Source |
|---|---|---|---|---|
| **TOXICOLOGY AND HUMAN EXPOSURE** | | | | |
| De Frutos F.J.O., Vergara A., Isarria M.J., Prado-Sanchez M., Vanaclocha F. | CA 5.9.2/03 | 2005 | Eczema alérgico de contacto profesional en auxiliar de odontología (Occupational allergic contact eczema in a dental assistant) | Actas Dermosifiliogr 96(1):56-8 |
| Guénette S.A., Ross A., Marier J-F., Beaudry F., Vachon P. | CA 5.1.1/08 | 2007 | Pharmacokinetics of eugenol and its effects on thermal hypersensitivity in rats. | European Journal of Pharmacology 562:60–67 |
| Hikiba H., Watanabe E., Barrett J.C., Tsutsui T. | CA 5.4.1/10<br><br>Previously evaluated in DAR 2011 | 2005 | Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells | Journal of Pharmacological Science 97(1):146-152 |
| Howes M-J. R., Houghton P.J., Barlow D.J., Pocock V.J., Milligan S.R. | CA 5.8.3/02 | 2002 | Assessment of estrogenic activity in some common essential oil constituents | Journal of Pharmacy and Pharmacology 54(11):1521-1528 |
| Iida M., Anna C.H., Holliday W.M., Collins J.B., Cunningham M.L., Sills R.C., Devereux T.R. | CA 5.8.2/04 | 2005 | Unique patterns of gene expression changes in liver after treatment of mice for 2 weeks with different known carcinogens and non-carcinogens. | Carcinogenesis 26(3):689-699 |
| Maeda Y., Takeyoshi M., Chuma T., Iwata H. | CA 5.2.6/04 | 2020 | α-Sens: The improved ARE-Nrf2-based sensitization screening assay using normalized transcriptional activity | Toxicology 439:152476 |
| Martins C., Dorand C., Laires A., Rueff J., Rodrigues A.S. | CA 5.4.1/15 | 2011 | Genotoxic and apoptotic activities of the food flavourings myristicin and | Food and Chemical Toxicology 49:385-392 |

| Author(s) | Data requirement (indicated by the corresponding CA and CP data point | Year | Title | Source |
|---|---|---|---|---|
| | | | eugenol in AA8 and XRCC1 deficient EM9 cells. | |
| Minet E.F., Daniela G., Meredith C., Massey E.D. | CA 5.1.1/07 | 2012 | A comparative *in vitro* kinetic study of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol activation and detoxification in human, mouse, and rat liver and lung fractions. | Xenobiotica 42(5):429-441 |
| Poli V., Challa C. | CA 5.8.3/03 | 2019 | A comparative study of eugenol and Ocimum sanctum Linn. leaf extract on the antifertility effect in female albino rats. | Journal of Chinese Medical Association 82:231-234 |
| Sanchez-Perez J., Garcia-Diez A. | CA 5.9.2/04 | 1999 | Occupational allergic contact dermatitis from eugenol, oil of cinnamon and oil of cloves in a physiotherapist | Contact Dermatitis 41:346 |
| Someya H., Higo Y., Ohno M., Tsutsui T.W., Tsutsui T. | CA 5.4.1/11 | 2008 | Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells | Mutation Research 650:39-47 |

**Table B.6.10.1.4.2/7. Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance: sorted by author for methyleugenol.**

| Author(s) | Data requirement (indicated by the corresponding CA and CP data point | Year | Title | Source |
|---|---|---|---|---|
| **TOXICOLOGY AND HUMAN EXPOSURE** | | | | |
| Abdo K.M., Cunningham M.L., Snell M.L., Herbert R.A., Travlos G.S., Eldridge S.R., Bucher J.R. | CA 5.8.1/03b<br>CA 5.8.1/04b | 2001 | 14-week toxicity and cell proliferation of methyleugenol administered by gavage to F344 rats and B6C3F1 mice | Food and Chemical Toxicology 39:303-316 |
| Burkey J.L., Sauer J-M, McQuenn C.A., Sipes I.G. | CA 5.8.1/09 | 2000 | Cytotoxicity and genotoxicity of methyleugenol and related | Mutation Research 453:25-33 |

| Author(s) | Data requirement (indicated by the corresponding CA and CP data point | Year | Title | Source |
|---|---|---|---|---|
| | | | congeners – a mechanism of activation for methyleugenol | |
| Cartus A.T., Herrmann K., Weishaupt L.W., Merz K-H., Engst W., Glatt H., Schrenk D. | CA 5.8.1/02 | 2012 | Metabolism of methyleugenol in liver microsomes and primary hepatocytes: pattern of metabolites, cytotoxicity and DNA-adduct formation | Toxicological Sciences 129(1):21-34 |
| Ding W., Levy D.D., Bishop M.E., Lascelles E.L-C, Chang C-W, Aidoo A., Manjanatha M.G. | CA 5.8.1/11 | 2011 | Methyleugenol genotoxicity in the Fischer 344 rat using the comet assay and pathway-focused gene expression profiling. | Toxicological Sciences 123(1):103-112 |
| Gardner I., Wakazono H., Bergin P., de Waziers I., Beaune P., Kenna J.G., Caldwell J. | CA 5.8.1/18 | 1997 | Cytochrome P450 mediated bioactivation of methyleugenol to 1'-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes | Carcinogenesis 18(9):1775-1783 |
| Groh I.A.M., Cartus A.T., Vallicotti S., Kajzar J., Merz K-H, Schrenk D., Esselen M. | CA 5.8.1/07 | 2012 | Genotoxic potential of methyleugenol and selected methyleugenol metabolites in cultured Chinese hamster V79 cells | Food & Function 3:428-436 |
| Herrmann K., Schumacher F., Engst W., Appel K.E., Klein K., Zanger U.M., Glatt H. | CA 5.8.1/14 | 2013 | Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human liver samples. | Carcinogenesis 34(5):1025-1030 |
| Hong S.P., Fuciarelli A.F., Johnson J.D., Graves S.W., Bates D.J., Waidyanatha S., Smith C.S. | CA 5.8.1/01 | 2013 | Toxicokinetics of methyleugenol in F344 rats and B6C3F1 mice | Xenobiotica 43(3):293-302 |
| Johnson J.D., Ryan M.J., Toft J.D., Graves S.W., Hejtmancik M.R., Cunningham M.L., Herbert R., Abdo K.M. | CA 5.8.1/12b<br>CA 5.8.1/13b | 2000 | Two-year toxicity and carcinogenicity study of methyleugenol in F344/N rats and B6C3F1 mice | Journal of Agricultural and Food Chemistry 48:3620-3632 |

| **Author(s)** | **Data requirement (indicated by the corresponding CA and CP data point** | **Year** | **Title** | **Source** |
|---|---|---|---|---|
| Minet E.F., Daniela G., Meredith C., Massey E.D. | CA 5.1.1/07 | 2012 | A comparative *in vitro* kinetic study of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol activation and detoxification in human, mouse, and rat liver and lung fractions. | Xenobiotica 42(5):429-441 |
| National Toxicology Program | CA 5.8.1/03a<br>CA 5.8.1/04a<br>CA 5.8.1/05<br>CA 5.8.1/08<br>CA 5.8.1/10<br>CA 5.8.1/12a<br>CA 5.8.1/13a | 2000 | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies) | Technical report No. 491, NIH publication No. 00-3950 |
| Waddell W.J., Crooks N.H., Carmichael P.L. | CA 5.8.1/16 | 2004 | Correlation of tumors with DNA adducts from methyleugenol and tamoxifen in rats | Toxicological Sciences 79:38-40 |
| Williams G.M., Iatropoulos J., Jeffrey A.M., Duan J-D. | CA 5.8.1/17 | 2012 | Methyleugenol hepatocellular cancer initiating effects in rat liver | Food and Chemical Toxicology 53:187-196 |
| Zhou G-D, Moorthy B., Bi J., Donnelly K.C., Randerath K. | CA 5.8.1/15 | 2007 | DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. | Environmental and Molecular Mutagenesis 48:715-721 |

#### B.6.10.1.5. Conclusion

In the literature search for the active substance eugenol and methyleugenol, 26158 and 3845 references for eugenol and methyleugenol, respectively, were identified and evaluated for their potential relevance for data requirements "toxicological, toxicokinetic studies and human exposure".

After rapid and detailed assessments for relevance, the following publications were considered to be relevant:

| | **Number of publications considered to be relevant Eugenol** | **Number of publications considered to be relevant Methyleugenol** |
|---|---|---|
| Toxicology, including human exposure | 11 | 14 |

**Assessment and conclusion by RMS:**

The RMS has checked the review of literature data submitted by the applicant and considers that it is acceptable.

The RMS agrees to the applicant in considering the studies selection as relevant, since it provides supporting information for the toxicology assessement. The RMS also agrees to the applicant regarding to the publications excluded of the toxicology section.

Additionally, according to the EFSA Guidance Document published in EFSA Journal 2011; 9(2): 2092, the RMS has noticed the following consideration:

-The list of publications retrieved after removing duplicates, as well as the number of potentially relevant and excluded publications after rapid and detailed assessment, has not been reported for each database.

## B.6.10.2. LIST OF REFERENCES

| Data Point | Author(s) | Year | Title<br>Report No.<br>Document No.<br>Source (where different from company)<br>GLP/ Officially recognised testing facilities[2,3]<br>Published or not | Vertebrate study Y/N | Data protection claimed Y/N | Justification if data protection is claimed | Owner | Previously used[1]<br><br>Y/N<br><br>If yes, for which data point? |
|---|---|---|---|---|---|---|---|---|
| CA 5.1.1/01 | Fischer, I.U., von Unruh, G.E. and Dengler, H.J. | 1990 | The metabolism of eugenol in man.<br>*Xenobiotica*, **20** (2), 209-222<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.1.1 & B.6.1.2 |
| CA 5.1.1/02 | Sutton J.D., Sangster S.A. and Caldwell J. | 1990 | Dose-dependent variation in the disposition of eugenol in the rat.<br>*Biochem. Pharmacol*, **34** (3), 465-466<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.1.1 & B.6.1.2 |
| CA 5.1.1/03 | Swanson A.B., Miller E.C. and Miller J.A. | 1981 | The side-chain epoxidation and hydroxylation of the hepatocarcinogens safrole and estragole and some related compounds by rat and mouse liver microsomes.<br>*Biochim. Biophys. Acta*, **673**, 504-516<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.1.2 |
| CA 5.1.1/04 | Delaforge M., Janiaud P., Levi P. and Morizot J.P. | 1980 | Biotransformation of allylbenzene analogues *in vivo* and *in vitro* through the | Y | N | n/a | Publication | Y<br>B.6.1.2 |

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|---|---|---|---|---|---|---|---|---|
| | | | epoxide-diol pathway, *Xenobiotica*,, **10** (10), 737-744<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.1.1/05 | Thompson, D., Constantin-Teodosiu, D., Egestad, B., Mickos, H. and Moldeus P. | 1990 | Formation of glutathione conjugates during oxidation of eugenol by microsomal fractions of rat liver and lung, *Biochem. Pharmacol*, **39** (10), 1587-1595<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.1.2 |
| CA 5.1.1/06 | Thompson, D.C, Constantin-Teodosiu, D. and Moldeus, P. | 1991 | Metabolism and cytotoxicity of eugenol in isolated rat hepatocytes, *Chem. Biol. Interactions*, **77**, 137-147<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.1.2 |
| CA 5.1.1/07 | Minet, E.F., Gentile, D., Clive, D. and Massey, E.D. | 2012 | A comparative *in vitro* kinetic study of [$^{14}$C]-eugenol and [$^{14}$C]-methyleugenol activation and detoxification in human, mouse, and rat liver and lung fractions, *Xenobiotica*, **42**, (5), 429-441<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.1.1/08 | Guénette, S.A., Ross, A., Marier, J.F., Beaudry, F. and Vachon, P. | 2007 | Pharmacokinetics of eugenol and its effects on thermal hypersensitivity in rats, | Y | N | n/a | Publication | N |

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| | | | *Eur. J. Pharmacol*, **562**, 60–67<br><br>GLP: No<br>Published | | | | | |
| CA 5.2.1/01 | Jenner P.M., Hagan E.C., Taylor J.M., Cook E. L. and Fitzhugh O.G. | 1964 | Food Flavourings and Compounds of related Structure I. Acute Oral Toxicity<br>*Fd Cosmet. Toxicol*, **2**, 327-343<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.2.1 |
| CA 5.2.1/02 | Sober H.A., Hollander F. and Sober E.K. | 1950 | Toxicity of Eugenol: Determination of $LD_{50}$ on rats<br>*Proc. Soc. Exp. Biol. Med.*, **73** (1), 148-151<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.2.1 |
| CA 5.2.1/03 | National Toxicology Program (NTP) | 1983 | Carcinogenesis studies of eugenol in F344/N rats and B6C3F1 mice (feed studies) Technical report series No. 223 NIH Publication no.84-1779<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.2.1 |
| CA 5.2.1/04<br>5.3.1/03 | Lauber F.U. and Hollander F. | 1950 | Toxicity of the Mucigogue, Eugenol, Administered by Stomach Tube to Dogs;<br>*Gastroenterology*, **15** (5), 481-486<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.2.1 |

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| CA 5.2.3/01 | Clark C.G. | 1988 | Acute inhalation toxicity of eugenol in rats *Arch. Toxicol.*, **62**, 381-386<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.2.3 |
| CA 5.2.4/01 | Motoyoshi K., Toyoshima Y., Sato M. and Yoshimura M | 1979 | Comparative Studies on the irritancy of oils and synthetic perfumes to the skin of rabbit, guinea pig, rat, miniature swine and man *Cosmetics & Toiletries*, **94** (8):41-48<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.2.4 |
| CA 5.2.6/01 | Takeyoshi M., Noda S., Yamazaki S., Kakishima H., Yamasaki K. and Kimber, I. | 2004 | Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the Local Lymph Node Assay *J. Appl. Toxicol.*, **24**, 77-81<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.2.6 |
| CA 5.2.6/02 | Lalko J., Isola D. and Api A.M | 2004 | Ethanol and diethyl phthalate: vehicle effects in the Local Lymph Node Assay *Int. J. Toxicol.*, **23**, 171-177<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.2.6 |
| CA 5.2.6/03 | Hilton J., Dearman R.J., Fieding | 1996 | Evaluation of the sensitising potential of | Y | N | n/a | Publication | Y B.6.2.6 |

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| | I., Basketter D.A. and Kimber I. | | Eugenol and Isoeugenol in mice and guinea pigs<br>*J. Appl. Toxicol.*, **16** (5), 459-464<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.2.6/04 | Maeda Y., Takeyoshi M., Chuma T. and Iwata H. | 2020 | α-Sens: The improved ARE-Nrf2-based sensitization screening assay using normalized transcriptional activity<br>*Toxicology*, **439**, 152476<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.3.1/01<br>5.3.1/02<br>5.3.2/01<br>5.3.2/02<br>5.5/01<br>5.5/02 | National Toxicology Program (NTP) | 1983 | Carcinogenesis studies of eugenol in F344/N rats and B6C3F1 mice (feed studies) Technical report series No. 223 NIH Publication no.84-1779<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.3.1 &<br>B.6.3.2<br><br>B.6.5.1 &<br>B.6.5.2 |
| CA 5/05 | Sipes I.G., Mattia A. | 2006 | Eugenol and related hydroxyallylbenzene derivatives. WHO Food Additive Series: 56. Safety Evaluation of certain food additives and contaminants. Prepared by the Sixty-fifth meeting of the Joint FAO/WHO Expert Committee on | N | N | n/a | Publication | Y<br>B6.5.1 |

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| | | | Food Additives (JECFA). World Health Organisation, Geneva (2006).<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.3.2/03 | Hagan, E.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A. and Brouwer, J.B. | 1967 | Food flavourings and compounds of related structure. II: Subacute and chronic toxicity. *Fd Cosmet. Toxicol.*, **5**, 141-157<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.3.1 |
| CA 5.4.1/01 5.4.1/06 | Ishidate M. Sofuni T. Yoshikawa K. Hayashi M. Noshmi T. Sawada M. And Matsuoka A. | 1984 | Primary mutagenicity screening of food additives currently used in Japan. *Food Chem. Toxic*., **22** (8), 623–636<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.1 & B.6.4.1.2 |
| CA 5.4.1/02 5.8.1/06 | Haworth S. Lawlor T. Mortelmans K., Speck W. and Zeiger A. | 1983 | Salmonella mutagenicity test results for 250 chemicals. *Environ. Mutagen. Suppl.* **1**, 3-142<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.1 |
| CA 5.4.1/03 5.4.2/11 | Green, N.R. and Savage, J.R. | 1978 | Screening of safrole, eugenol, and their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. | N | N | n/a | Publication | Y B.6.4.1.1 |

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| | | | *Mutat. Res.*, **57**, 115-121<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.4.1/04 | Florin I., Rutberg L., Curvall M. and Enzell C.R. | 1980 | Screening of tobacco smoke constituents for mutagenicity using the Ames test *Toxicology*, **15** (3), 219-232<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.1 |
| CA 5.4.1/05 | Azizan A. and Blevins R.D | 1995 | Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames Salmonella/Microsomal assay. *Arch. Environ.Contam.Toxicol.*, **2**8, 248-258<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.1 |
| CA 5.4.1/07 | Stich H.F. Rosin M.P. Wu C.H. and Powrie W.D. | 1981 | The action of transition metals on the genotoxicity of simple phenols, phenolic acids and cinnamic acids; *Cancer Lett.*, **14**, 251-260<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.2 |
| CA 5.4.1/08 | Galloway, S.M. Armstrong, M.J. | 1987 | Chromosome aberrations and sister chromatid exchanges in | N | N | n/a | Publication | Y B.6.4.1.2 |

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| | Reuben, C. Colman, S. Brown, B. Cannon, C. Bloom, A.D. Nakamura, F. Ahmed, M. Duk, S. Rimpo, J. Margolin, B.H. Resnick, M.A. Anderson, B. and Zeiger, E. | | Chinese hamster ovary cells: evaluations of 108 chemicals; *Environ. Mol. Mutagen*. **10** (Suppl 10), 1-175<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.4.1/09 | Maralhas A. Monteiro A. Martins C. Kranendonk M. Laires A. Rueff J. and Rodrigues A.S. | 2006 | Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol; *Mutagenesis*, **21** (3), 199-204<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.2 |
| CA 5.4.1/10 | Hikiba, H. Watanabe, E. Barrett, J.C. and Tsutsui, T.. | 2005 | Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syriam hamster embryo cells; *J. Pharmacol. Sci*, **97**, 146-152<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.2 |
| CA 5.4.1/11 | Someya, H. Higo, Y. Ohno, M. Tutsui, T.W. and Tsutsui, T. | 2008 | Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells; *Mutat. Res.*, **650**, 39-47<br><br>GLP: No | N | N | n/a | Publication | N |

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| | | | Published | | | | | |
| CA 5.4.1/12 | Jansson, T. Curvall, M. Hedin, A. and Enzell, C.R | 1986 | *In vitro* studies of biological effects of cigarette smoke condensate. II. Induction of sister-chromatid exchanges in human lymphocytes by weakly acidic, semivolatile constituents; *Mutat. Res.*, **169**, 129-139<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.2 |
| CA 5.4.1/13 | Myhr, B.C. and Caspary, W.J. | 1991 | Chemical mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells: results for 31 coded compounds in the National Toxicology Program; *Environ.Mol. Mutagen.*, **18**, 51-83<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.3 |
| CA 5.4.1/14 | Bodell, W.J. Ye, Q. Pathak, D.N. and Pongracz, K | 1998 | Oxidation of eugenol to form DNA adducts and 8-hydroxy-2'-deoxyguanosine: Role of quinone methide derivative in DNA adduct formation; *Carcinogenesis* **19** (3), 437-443<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y B.6.4.1.4 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CA 5.4.1/15 | Martins, C., Doran, C., Laires, A., Rueff, J. and Rodrigues, A.S. | 2015 | Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells; *Food and Chemical Toxicology*, **49**, 385-392<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.4.2/01 | Hayashi M., Kishi M., Sofuni T. and Ishidate M. | 1988 | Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals *Fd. Chem. Toxic*., **26**, 487–500<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/02 | Woolverton C., Fotos P., Mokas M. and Mermigas M. | 1986 | Evaluation of eugenol for mutagenicity by the mouse micronucleus test *J. Oral Pathol*., **15**, 450-453<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/03 | Rompelberg C.J.M., Stenhuis W.H., de Vogel N., Van Osenbrugge n W.A., Schouten A.and Verhagen H. | 1995 | Antimutagenicit y of eugenol in the rodent bone marrow micronucleus test *Mutat. Res*, **346**, 69–75<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CA 5.4.2/04 | Shelby M.D., Erexson G.L., Hook G.J. and Tice R. | 1993 | Evaluation of a three-exposure mouse bone marrow micronucleus protocol: Results with 49 chemicals *Environ. Mol. Mutagen*, **21**, 160-179<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/05 | Ellahueña M.F., Pérez-Alzola L.P., Orellana-Valdebenito M., Muñoz C. and Lafuente-Indo N. | 1994 | Genotoxic evaluation of eugenol using the bone marrow micronucleus assay *Mutat. Res*, **320**, 175-180<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/06 | Rompelberg C.J.M., Evertz S.J.C.J., Bruijntjes-Rozier G.C.D.M, van den Heuvel P.D. and Verhagen H. | 1996a | Effect of eugenol on the genotoxicity of established mutagens in the liver *Fd. Chem. Toxic.*, **34** (1), 33-42<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/07 | Maura A., Pino A. and Ricci R. | 1989 | Negative evidence *in vivo* of DNA-damaging, mutagenic and chromosomal effects of eugenol *Mutat. Res*, **227**, 125–129<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CA 5.4.2/08 | Rompelberg C.J.M., Steenwinkel M-J.S.T., van Asten J.G., van Delft J.H.M., Baan R.A. and Verhagen H. | 1996 b | Effect of Eugenol on the mutagenicity of Benzo[a]Pyrene and the formation of Benzo[*a*]Pyrene -DNA adducts in the λ-lacZ-transgenic mouse *Mutat. Res*, **369**, 87-96<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/09 | Rompelberg C.J.M., Vogels J.T.W.E., de Vogel N., Bruijntjes-Rozier G.C.D.M, Stenhuis W.H., Bogaards J.J.P. and Verhagen H. | 1996c | Effect of short-term dietary administration of eugenol in humans *Hum. Exp. Toxicol*, **15**, 129-135<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/10 | Philips D.H. | 1990 | Further evidence that eugenol does not bind to DNA *in vivo* *Mutat. Res*, **245**, 23-26<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.4.2 |
| CA 5.4.2/12 | Chen, Y., Huo,J., Liu, Y., Zeng, Z., Zhu, X., Chen, X., Wu, R., Zhang, L. and Chen, J. | 2021 | Development of a novel flow cytometry-based approach for reticulocytes micronucleus test in rat peripheral blood *J. Appl. Toxicol*., **45**, 595-606<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.5/03 | Miller, E.C., Swanson, | 1983 | Structure-activity studies | Y | N | n/a | Publication | Y |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A.B., Phillips, D.H., Fletcher, T.L., Liem, A., Miller, J.A. | | of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivates related to safrole and estragole *Cancer Research*, 43:1124-1134<br><br>GLP : No<br><br>Published | | | | | B.6.5.1 & B.6.5.2 |
| n/a (included by RMS) | National Toxicology Program | 2002 | Isoeugenol; Reproductive assessment by continuous breeding when administered to Sprague-Dawley rats by gavage. The Immune Research Corporation Study Nº. 7244-203. NTP-RACB-97-004. GLP : Yes Published | N | N | n/a | Publication | N |
| CA 5.6.2/01 | Morgareidge, K. | 1973a | Teratologic evaluation of FDA 71-30 (Oil of clove) in rats Food and Drug Research Laboratories Study No 1574c<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y B.6.6.2 |
| CA 5.6.2/02 | Morgareidge, K. | 1973 b | Teratologic evaluation of FDA 71-30 (Oil of clove) in rabbits. Food and Drug Research Laboratories Study No 1576c<br><br>GLP: No | Y | N | n/a | Publication | Y B.6.6.3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Published | | | | | |
| CA 5.6.2/03 | Morgareidge, K. | 1973c | Teratologic evaluation of FDA 71-30 (Oil of clove) in mice.<br>Food and Drug Research Laboratories Study No 1573c<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.6.4 |
| CA 5.6.2/04 | Morgareidge, K. | 1973 d | Teratologic evaluation of FDA 71-30 (Oil of clove) in hamsters<br>Food and Drug Research Laboratories Study No 1575c<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.6.4 |
| CA 5.6.2/05 | [redacted] | 2004 | Eugenol: oral gavage pre-natal developmental toxicity study Report no. 1620/034<br><br>GLP: Yes<br><br>Published | Y | N | n/a | Xeda (agreement) | Y<br>Conclusion on the peer review of eugenol (EFSA Journal 2012;10(11):2914) |
| CA 5.6.2/06 | [redacted] | 2004 | Eugenol: oral gavage pre-natal developmental toxicity study Report no. 1620/035<br><br>GLP: Yes<br><br>Published | Y | N | n/a | Xeda (agreement) | Y<br>Conclusion on the peer review of eugenol (EFSA Journal 2012;10(11):2914) |
| CA 5.8.1/01 | Hong, S.P., Fuciarelli, A.F., Johnson, J.D., Graves, S.W., Bates, D.J., Waidyanatha S., Smith, C.S | 2013 | Toxicokinetics of methyleugenol in F344 rats and B6C3F1 mice. *Xenobiotica*, 43(3):293-302<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CA 5.8.1/02 | Cartus, A.T., Herrmann, K., Weishaupt, L.W., Merz, K-H., Engst, W., Glatt, H., Schrenk, D. | 2012 | Metabolism of methyleugenol in liver microsomes and primary hepatocytes: pattern of metabolites, cytotoxicity and DNA-adduct formation. *Toxicological sciences*, 129(1):21-34<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.8.1/03a 5.8.1/04a 5.8.1/05 5.8.1/08 5.8.1/10 5.8.1/12a 5.8.1/13a | National Toxicology Program | 2000 | Toxicology and carcinogenesis studies of methyleugenol in F344/N rats and B6C3F1 mice (gavage studies). Technical report No. 491 NIH publication No. 00-3950<br><br>GLP: Yes<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.1/03b 5.8.1/04b | Abdo, K.M., Cunningham, M.L., Snell, M.L., Herbert, R.A., Travlos, G.S., Eldridge, S.R., Bucher, J.R. | 2001 | 14-week toxicity and cell proliferation of methyleugenol administered by gavage to F344 rats and B6C3F1 mice. *Food and Chemical Toxicology*, 39:303-316<br><br>GLP: Yes<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.1/07 | Groh, I.A.M., Cartus, A.T., Vallicotti, S., Kajzar, J., Merz, K-H, Schrenk, D., Esselen, M. | 2012 | Genotoxic potential of methyleugenol and selected methyleugenol metabolites in cultured Chinese hamster V79 cells. | N | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Food and Function*, 3:428<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.8.1/09 | Burkey, J.L., Sauer, J-M, McQuenn, C.A., Sipes, I.G. | 2000 | Cytotoxicity and genotoxicity of methyleugenol and related congeners: a mechanism of activation for methyleugenol. *Mutation Research*, 453:25-33<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.8.1/11 | Ding W., Levy D.D., Bishop M.E., Lascelles E.L-C, Chang C-W, Aidoo A., Manjanatha M.G. | 2011 | Methyleugenol genotoxicity in the Fischer 344 rat using the comet assay and pathway-focused gene expression profiling. *Toxicological Sciences*, 123(1):103-112<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.1/12b 5.8.1/13b | Johnson, J.D., Ryan, M.J., Toft, J.D., Graves, S.W., Hejtmancik, M.R., Cunningham, M.L., Herbert, R., Abdo, K.M. | 2000 | Two-year toxicity and carcinogenicity study of methyleugenol in F344/N rats and B6C3F1 mice. *Journal of Agricultural and Food Chemistry*, 48:3620-3632.<br><br>GLP: Yes<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.1/14 | Herrmann, K., Schumacher, F., Engst, W., Appel, | 2013 | Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinoge | N | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | K.E., Klein, K., Zanger, U.M., Glatt, H. | | n, in human liver samples. *Carcinogenesis*, 34(5):1025-1030<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.8.1/15 | Zhou, G-D, Moorthy, B., Bi J., Donnelly, K.C., Randerath ,K. | 2007 | DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. *Environmental and molecular mutagenesis*, 48:715-721<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.8.1/16 | Waddell, W.J., Crooks, N.H., Carmichael, P.L. | 2004 | Correlation of tumors with DNA adducts from methyleugenol and tamoxifen in rats. *Toxicological Sciences*, 79:38-40<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.8.1/17 | Williams, G.M., Iatropoulos, J., Jeffrey, A.M., Duan, J-D | 2012 | Methyleugenol hepatocellular cancer initiating effects in rat liver. *Food and Chemical Toxicology*, 53:187-196<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.1/18 | Gardner, I., Wakazono, H., Bergin, P., de Waziers, I., Beaune, P., | 1997 | Cytochrome P450 mediated bioactivation of methyleugenol to 1'-hydroxymethyle | Y | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Kenna, J.G., Caldwell, J. | | ugenol in Fischer 344 rat and human liver microsomes. *Carcinogenesis*, 18(9):1775-1783<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.8.2/01 | Thompson, D. C., Barhoumi, R., Burghardt, R.C. | 1998 | Comparative Toxicity of Eugenol and Its Quinone Methide Metabolite in Cultured Liver cells Using Kinetic Fluorescence Bioassays. *Toxicology and Applied Pharmacology*, 149:55–63<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.8 |
| CA 5.8.2/02 | Yokota, H., Hashimoto, H., Motoya, M., Yuasa, A. | 1988 | Enhancement of UDP-Glucuronyltransferase, UDP-Glucose dehydrogenase, and glutathione S-transferase activities in rat liver by dietary administration of Eugenol. *Biochemical Pharmacology*, 37(5):799-802<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | Y<br>B.6.8 |
| CA 5.8.2/03 | Rompelberg, C.J.M., Verhagen, H., Van Bladeren, P.J. | 1993 | Effects of the naturally occurring alkenylbenzenes Eugenol and trans-anethole on drug-metabolizing enzymes in the rat liver | Y | N | n/a | Publication | Y<br>B.6.8 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Food and Chemical Toxicology*, 31 (9):637-645<br><br>GLP: No<br><br>Published | | | | | |
| CA 5.8.2/04 | Iida, M., Anna, C.H., Holliday, W.M., Collins, J.B., Cunningham, M.L., Sills, R.C., Devereux, T.R. | 2005 | Unique patterns of gene expression changes in liver after treatment of mice for 2 weeks with different known carcinogens and non-carcinogens *Carcinogenesis*, 26(3):689-699<br><br>GLP: No<br><br>Published | Y | N | n/a | Publication | N |
| CA 5.8.3/01a, b | Staphyt Ltd, Eden Research plc | 2021 | Eugenol: Assessment of endocrine disrupting properties Report no. 21/10<br><br>GLP : No<br><br>Not published | N | N | n/a | Eden research plc | N |
| CA 5.8.3/02 | Howes, M.J., Houghton, P.J., Barlow, D.J., Pocock, V.J., Milligan, S.R. | 2002 | Assessment of estrogenic activity in some common essential oil constituents. *J. Pharm. Pharmacol*. 54 (11), 1521–1528.<br><br>GLP: No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.8.3/03 | Poli, V., Challa, C. | 2019 | A comparative study of eugenol and Ocimum sanctum Linn. leaf extract on the antifertility effect in female albino rats | N | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Journal of Chinese Medical Association, 82: 231-234.<br><br>GLP : No<br><br>Published | | | | | |
| CA 5.9.1/01 Filed in confidential Doc K | Volpe, M.J. | 2021 | Manufacturer Medical Report Not GLP Not published | N | N | n/a | Eden Research plc | N |
| CA 5.9.2/01 | Greif, N. | 1967 | Cutaneous safety of fragrance material as measured by the Maximization test American Perfumer and Cosmetics, Vol.82<br><br>GLP : No<br><br>Published | N | N | n/a | Publication | Y B.5.9.2 |
| CA 5.9.2/02 | Rothenstein, A.S., Booman ,K.A., Dorsky, J., Kohrman, K.A., Schwoeppe, E.A., Sedlak, R.I., Steltenkamp ,R.J., Thompson ,G.R. | 1983 | Eugenol and Clove leaf oil: A survey of consumer patch sensitisation Food and Chemical Toxicology, 21(6):727-733<br><br>GLP : No<br><br>Published | N | N | n/a | Publication | Y B.6.9.2 |
| CA 5.9.2/03 | De Frutos, F.J.O., Vergara, A., Isarria, M.J., Prado-Sanchez, M., Vanaclocha F. | 2004 | Eczema alérgico de contacto profesional en auxiliar de odontología (Occupational allergic contact eczema in a dental assistant) Actas Dermosifiliogr; 96(1):56-8 | N | N | n/a | Publication | N |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | GLP : No<br><br>Published | | | | | |
| CA 5.9.2/04 | Sanchez-Perez, J., Garcia-Diez, A | 1999 | Occupational allergic contact dermatitis from eugenol, oil of cinnamon and oil of cloves in a physiotherapist Contact Dermatitis, 41:346<br><br>GLP : No<br><br>Published | N | N | n/a | Publication | N |
| CA 5.9.3/01 | Hartnoll, G., Moore, D., Douek, D. | 1993 | Near fatal ingestion of oil of cloves Archives of Disease in Childhood, 69: 392-393<br><br>GLP : No<br><br>Published | N | N | n/a | Publication | Y<br>B.6.9.2 |