Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

1-phenylethan-1-one (1-phenylethylidene)hydrazone

EC Number: **211-979-0**

CAS Number: **729-43-1**

Index Number: **-**

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Note on confidential information

Please be aware that this report is intended to be made publicly available. Therefore it should not contain any confidential information. Such information should be provided in a separate confidential Annex to this report, clearly marked as such.

CONTENTS

1 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

1.1.1 [Anonymous 2017]

Study 1 reference:

Anonymous 2017. STUDY OF ACETOPHENONE AZINE HYDROLYSIS IN ARTIFICAL SWEAT (non GLP). Date: 14. August 2017.

Test type

The purpose of this study is to establish if Acetophenone Azine undergoes hydrolysis in artificial sweat and to identify the hydrolysis products if there are any.

Detailed study summary and results:

The stability of acetophenone-azine was examined in artificial sweat for 5 days at 37°C. Two detection modes were: UV-photometry at 245 nm and mass spectrometry with APCI ionization. In the first 8 hours no major changes were detected. After 24 hours 30-40% of the initial acetophenone-azine amount was hydrolysed. After 72 hours approximately 95% of the test item is reacted and after 120 hours only traces can be detected in the 2nd and the 3rd sample while in sample 1 no more acetophenone-azine is present. Based on this it can be stated that the test item completely hydrolyses within 5 days. The hydrolysis product is identified as acetophenone.

Material and methods

TEST ITEM

Test substance name:	Acetophenone Azine
Chemical name:	1-phenyl-, (1-phenylethylidene)hydrazone
CAS number:	729-43-1
Batch number:	Confidential
Purity:	Confidential
Appearance:	Yellow powder
Expiry date:	n.a.
Storage condition:	Room temperature (15-25^{o}C, below 70 RH%)
Safety Precautions:	Routine safety precautions for unknown materials (lab coat, mask, gloves and safety glasses) were applied to assure personnel health and safety.

CONDITIONS:

In the publication by Nadia Raison Peyron *et al.,* in 2016 using patch tests of allergies the results are positive at 0.001% and negative for controls at 0.01%. However, it is not possible to make the link between hydrolysis and allergy studies because in hydrolysis, the phase must be aqueous (at least for the most part, and in the allergy test the substance is dissolved in 100% acetone). Also, if the substance is soluble (i) and quantifiable (ii) at concentrations of 0.01 or 0.005%, it is preferable to conduct the hydrolysis at these concentrations. However, some attention was paid relative to the problem of analytical limit. If the parent substance is measured, it should be ensured

that the substance is quantifiable at least 10-15% of the initial concentration so that the measurement of the hydrolysis is accurate constant. If these are the products of hydrolysis which are measured, and as they are in smaller quantity, it is necessary that they have to be quantifiable at 0.001-0.0015% (if you work at 0.01%). This was normally possible because the quantification limits in water are often close to the ppt. the labo was able to go down in concentrations to reach such concentrations of 0.001-0.0015%. in principle the desired detection limit will probably not be a problem even if they start from the 0.05%

An amount was included in the protocol for the determination of the degradation products from hydrolysis. Moreover, after having examined the spectra, any peaks (other than Acetophenone azine) that have been detected, have to be identified and concentration reported.

DETAILED PROTOCOL:

- A description of the hydrolysis test protocol is as following:
- Artificial sweat was used int the protocol. Based on the standard EN 1811, the composition of the artificial sweat is: 5 g NaCl, 1 g lactic acid and 1 g urea in 1 L of deionized water adjusted to a value of pH of 6.47 with ammonia.
- A single concentration of acetophenone azine was diluted at a concentration of 0.1% w/w in an artificial sweat solution (publication N.Raison Payron) (addition of solvent for solubility may be required).
- pH was adjusted and measured at the beginning and at the end of the test. Samples were collected at diffenrent times in 3 replicates.
- The Sampling time were 0h – 30min - 1h - 3h - 8h - 24h (3h relative to standard sweat/ cuir and 8h : relative to the wearing of clthe during one day and 3 days, 5 days).
- Incubation of samples in plastic tubes (10-20 mL) at 37°C under mechanical mixing for the sampling times in 3 replicates (3 tubes of the same sample, run in the same time).
- Filtration was at 0.45 microns only if required.
- Analysis of samples (plus controls of a time zero sample and incubated samples without test item) by LC/QTOF MS for any degradation products of Acetophenone Azine. Approximate quantification of Hydrazine was based on standard for each sample:

  A) Examination of spectra to look for other chemicals that may have been produced, with rapid evaluation of probable identity of peaks other than Acetophenone Azine or Hydrazine.

  B) For any peaks, report the probable ID and order of magnitude of concentration (if possible). Including evaluation of available data. Literature search and evaluation as required. Provide recommendation for course of action for the steps below

INSTRUMENTATION AND SAMPLE HANDLING

Method

Chromatograph:	Waters 2695 Separation mode
Column:	Synergi Hydro-RP 80A
	3 mm ID x 150 mm L, 4 □m
	S/N: 411335-4
Column temperature:	45 °C
Mobile phase:	Eluent A: MeOH

Eluent B: Water

Gradient table:

Time (min.)	Eluent A (%)	Eluent B (%)
0.00	20	80
15.00	40	60
26.00	60	40
30.00	85	15
35.00	85	15
35.20	90	10
42.00	90	10
42.10	20	80
47.00	20	80

Flow rate:	0.7 ml/min
Detector1:	Bruker MicrOTOFQ mass spectrometer
Ionization mode:	Atmospheric Pressure Chemical Ionization (APCI) in positive mode, drying temperature: 350 °C
Detector2:	Waters 2996 Photodiode array detector, 245 nm
Time of run and data acquisition:	47 min
Sample temperature:	5.0 °C
Sample volume:	20 µL

Sample handling:

The artificial sweat was made according to EN 1811: 5 g NaCl (Spektrum 3D, LOT: MC3/12/03/99), 1 g lactic acid (Szkarabeusz Kft., LOT: 17.0273) and 1 g urea (Szkarabeusz Kft., LOT: 17.0111) were dissolved in 1 litre of deionized water and the pH was adjusted to a value of 6.47 with ammonia. For the three parallel measurements, three different artificial sweat solution were prepared. 10 ml acetophenone-azine stock solution was prepared at 1 mg/ml nominal concentration level. The actual concentrations of the stock and the reference solutions are shown in Table 1.

Table 1: Actual concentration of the sample and reference solutions

Solution	Concentration of acetophenone azine (mg/ml)
1. sample	0.95
2. sample	0.95
3. sample	0.98

Ref1	0.98
Ref2	0.97
Ref3	0.93
Ref4	1.05
Ref5	0.98
Ref6	1.08
Ref7	1.07
Ref8	1.06
Ref9	1.00
Ref10	0.93
Ref11	1.01
Ref12	1.08
Ref13	1.00
Ref14	0.97
Ref15	0.93

1 ml of this stock solution was further diluted with the artificial sweat to 200 ml. This was the t_0 starting point of the hydrolysis. The nominal concentration of the test item in these solutions was 0.005 mg/ml. The solutions were transferred to Erlenmeyer flasks with stopper closed agitated and thermostated at 37°C. Samples (1 ml) were taken for mass spectrometric analysis right at mixing, at 0.5, 1, 3, 5, 8 and 24 hours, 3 and 5 days to HPLC vials. After sampling 200 µl THF was added to each vials (to avoid precipitation) and they were placed to the sample compartment of the HPLC cooled to 5 °C.

The reference solutions were prepared the same way, the difference was that the final volume of those solutions was 100 ml and they were immediately placed to the 5°C compartment, where the hydrolysis was considered to be so slow, that practically no reaction took place.

The degradation degree of the test item in the samples was calculated on the basis of the peak areas (both in UV and the extracted ion chromatograms) in the samples taken by comparison to the areas measured in the reference solution. The injection sequence followed the "*sample-reference-sample*-sample-reference-sample" block order. The peak areas were compared always to the reference value between two samples. This way if despite keeping them at 5°C any degradation took place in the autosampler, the reference solution undergo the same changes, thus this error was corrected.

Results

The chromatogram of the test item and the corresponding mass spectrum are shown in Figures 1 and 2 respectively. The smaller peak marked with "2" is the peak of the test item. The more intensive signal approximately 1.5 minutes later is a "system" peak and originates from a residual PEG contamination in the ion source (see Figure 3).

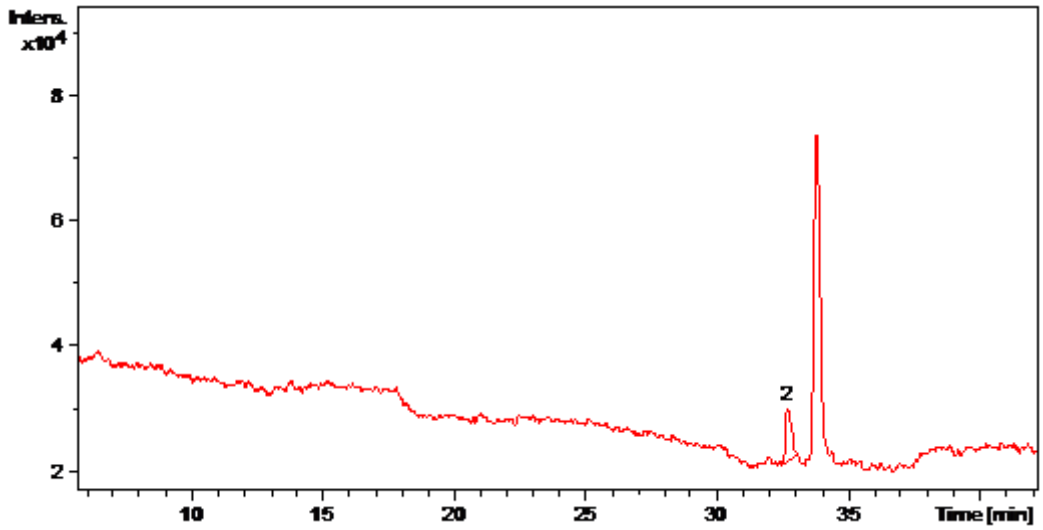


Figure 1: Total ion chromatogram of the test item solution at initial t_0 time point

Based on the test item structure, an intensive signal was expected in the UV region, therefore the reaction was followed by the UV trace as well. Figure 4 shows the UV and the TIC traces. The use of UV chromatogram has another advantage as well: if there are degradation aromatic products that can not be ionized in the MS system, then they will be still detectable.

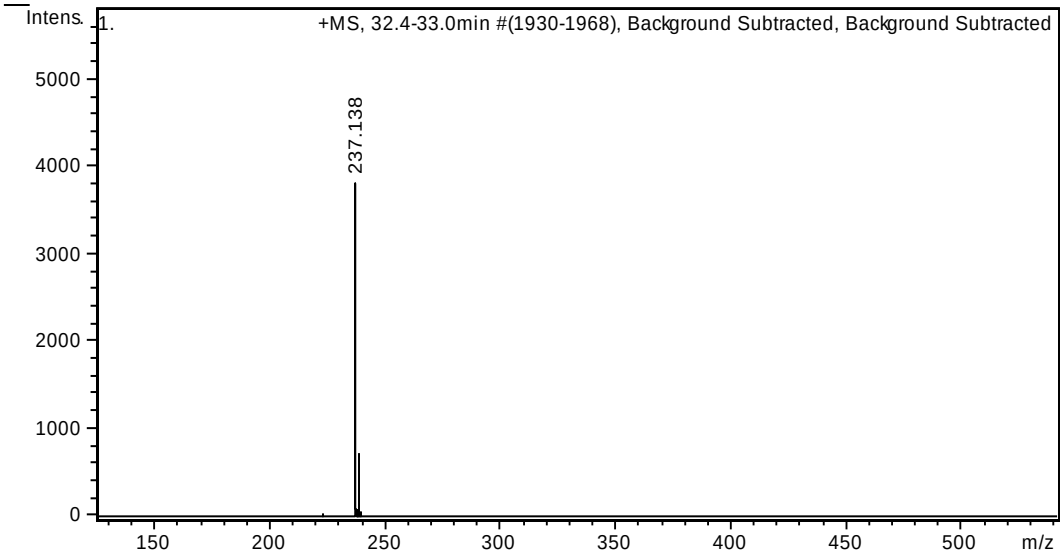


Figure 2: The mass spectrum of peak “2” at initial t_0 time point

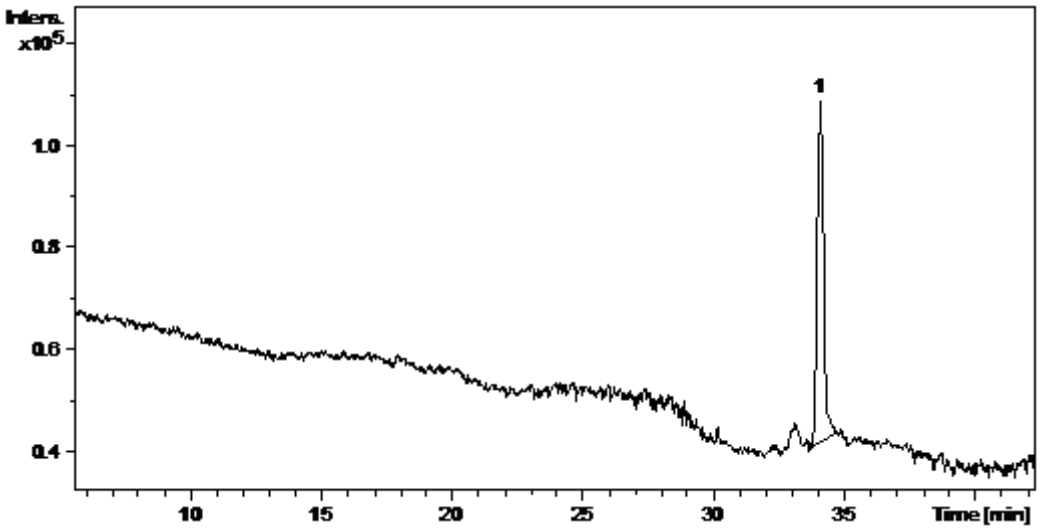


Figure 3: Total ion chromatogram of the blank solution

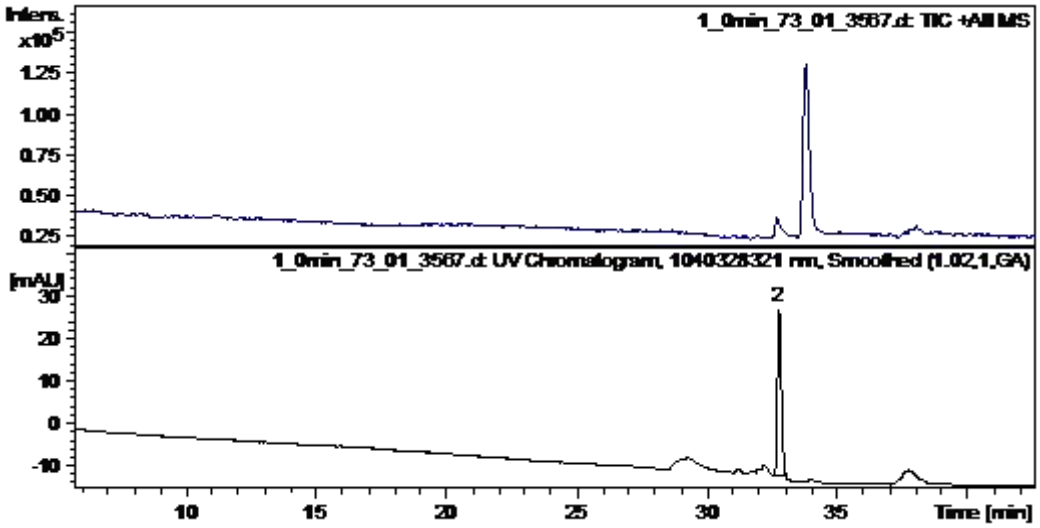


Figure 4: The UV trace (bellow) and the total ion chromatogram of the test item solution

TEST ITEM STABILITY IN THE AUTOSAMPLER

Since the solutions were not analysed directly after sampling, the autosampler stability of the samples was checked for 2 days. The peak areas in the UV chromatogram are shown in Table 2.

Table 2: Autosampler stability results

Sampling time	UV peak area
t0	376.7
24 h	386.4
48 h	384.6

It can be stated that the area did not change within the experimental error in the period examined. The test item does not hydrolyse at 5°C.

REPEATABILITY OF THE INJECTIONS

The uncertainty of the sample preparation and the lab operations, thus repeatability of the results was checked by the parallel preparation and analysis of the reference solution Ref 12 five times. The peak areas for the UV and the TIC chromatograms are presented in Table 3.

Table 3: Repeatability results

Injection	UV peak area	EIC peak area
1.	409.7	615178.3
2.	423.4	631341.5
3.	430.0	611827.2
4.	427.7	548652.6
5.	430.6	588356.3
SD	8.7	32095.1
RSD %	2.0	5.4

As Table 3 shows the average relative standard deviation is found 2% for the UV analysis while for mass spectrometry 5.4 %.

HYDROLYSIS OF ACETOPHENONE-AZINE

Table 4 shows the peak areas measured by both detection methods. For better comparison the original values are corrected with the exact concentration of the stock and reference solutions (normalized to 1 mg/ml stock solution concentration). The differences are expressed as area % of the reference peak area.

Table 4 : Peak area changes of the test item's peak

	Area		Area corr.		Difference (%)		Corr. fact.
sample	EIC	UV	EIC corr	UV corr	EIC	UV	
1_0min	91040	386	95832	407	6	4	0.95
ref1	88346	384	90149	392			0.98
2_0min	78663	389	82804	409	-8	5	0.95
3_0min	78888	407	80498	415	-6	7	0.98
ref2	83428	377	86008	388			0.97
1_30min	91124	386	95920	406	12	5	0.95
2_30min	85583	377	90088	397	-5	-2	0.95
ref3	88124	376	94757	404			0.93
3_30min	98249	401	100254	409	6	1	0.98
1_1h	86957	390	91534	411	16	6	0.95
ref4	82841	408	78897	389			1.05
2_1h	83394	377	87783	397	11	2	0.95
3_1h	105948	400	108110	409	-2	7	0.98

ref5	107992	375	110196	383			0.98
1_3h	116394	351	122520	370	11	-3	0.95
2_3h	103281	378	108717	398	-5	4	0.95
ref6	123741	413	114575	382			1.08
3_3h	112885	387	115189	395	1	3	0.98
1_5h	106702	354	112318	372	16	2	0.95
ref7	103473	391	96703	365			1.07
2_5h	102437	362	107828	381	12	4	0.95
3_5h	91891	360	93766	368	15	-2	0.98
ref8	86092	396	81219	373			1.06
1_8h	78925	348	83079	366	2	-2	0.95
2_8h	556927	348	586239	367	2	-4	0.95
ref9	577825	383	577825	383			1
3_8h	521541	374	532185	381	-8	-1	0.98
1_24h	347457	217	365744	229	-38	-40	0.95
ref10	546193	355	587305	381			0.93
2_24h	332224	237	349710	250	-41	-35	0.95
ref11	429360	272	425109	269			1.01
3_24h*	362098	244	369488	249	-37	-35	0.98
1_72h**	24065	18	25332	19	-95	-95	0.95

	Area		Area corr.		Difference (%)		Corr. fact.
sample	EIC	UV	EIC corr	UV corr	EIC	UV	
ref12	615178	410	569610	379			1.08
2_72h**	28263	23	29751	25	-95	-94	0.95
ref13	573086	385	573086	385			1
3_72h**	34712	26	35420	26	-93	-93	0.98
ref13_10X	54265	40	542647	396			0.1
1_120h***	0	0	0	0	-100	-100	0.95
ref14_10X	55313	38	570238	389			0.097
2_120h***	2028	0	2134	0	-100	-100	0.95
ref15_10X	48875	34	525536	368			0.093
3_120h***	1404	0	1433	0	-100	-100	0.98
ref14	524819	375	541050	387			
ref15	467702	353	502906	380			

* The sample 3_24h is compared to the reference peak area of solution ref10, because due to a possible injection fault, the peak area of solution ref11 is unexpectedly low.

** The samples taken at 72 hours are compared to the 10x dilution of solution ref13 (ref13_10x in the table) because due to the hydrolysis the test item concentration is found more than a magnitude of order lower.

*** The samples taken at 120 hours are compared to the 10x dilution of solution ref14 and ref15 (ref14_10x and ref15_10x in the table) because due to the hydrolysis the test item concentration is found more than a magnitude of order lower

IDENTIFICATION OF THE HYDROLYSIS PRODUCTS

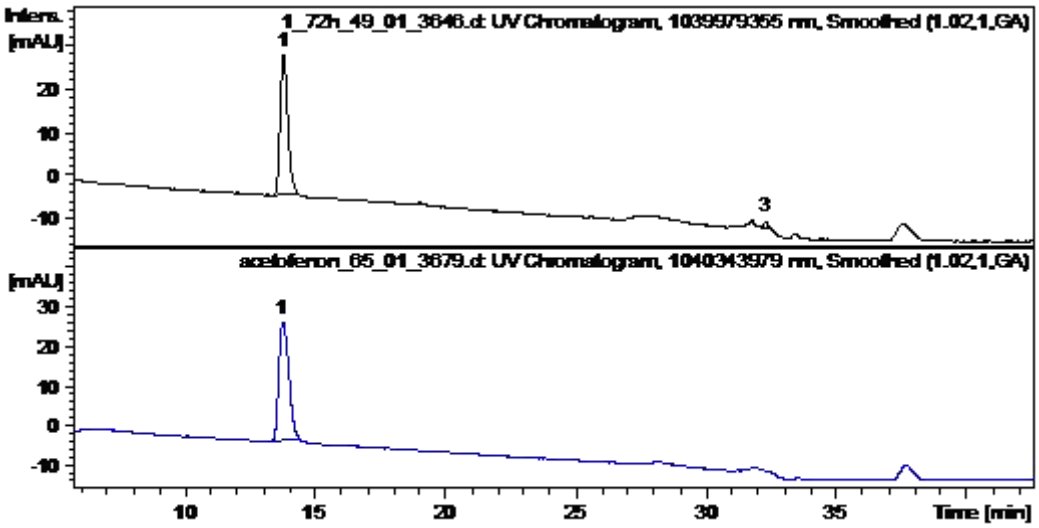


Figure 5: The UV trace of acetophenone (below) and the 72h sample of the test item solution

Figure 5 shows the chromatograms used for the identification of the hydrolysis product. The only peak detected appeared in the UV chromatogram at 13.8 minutes. Based on the retention times it can be identified as acetophenone. The reason why this peak does not appear neither in the total ion nor in the extracted ion chromatograms is that acetophenone can not be ionized by LC/APCI-MS techniques.

CONCLUSION

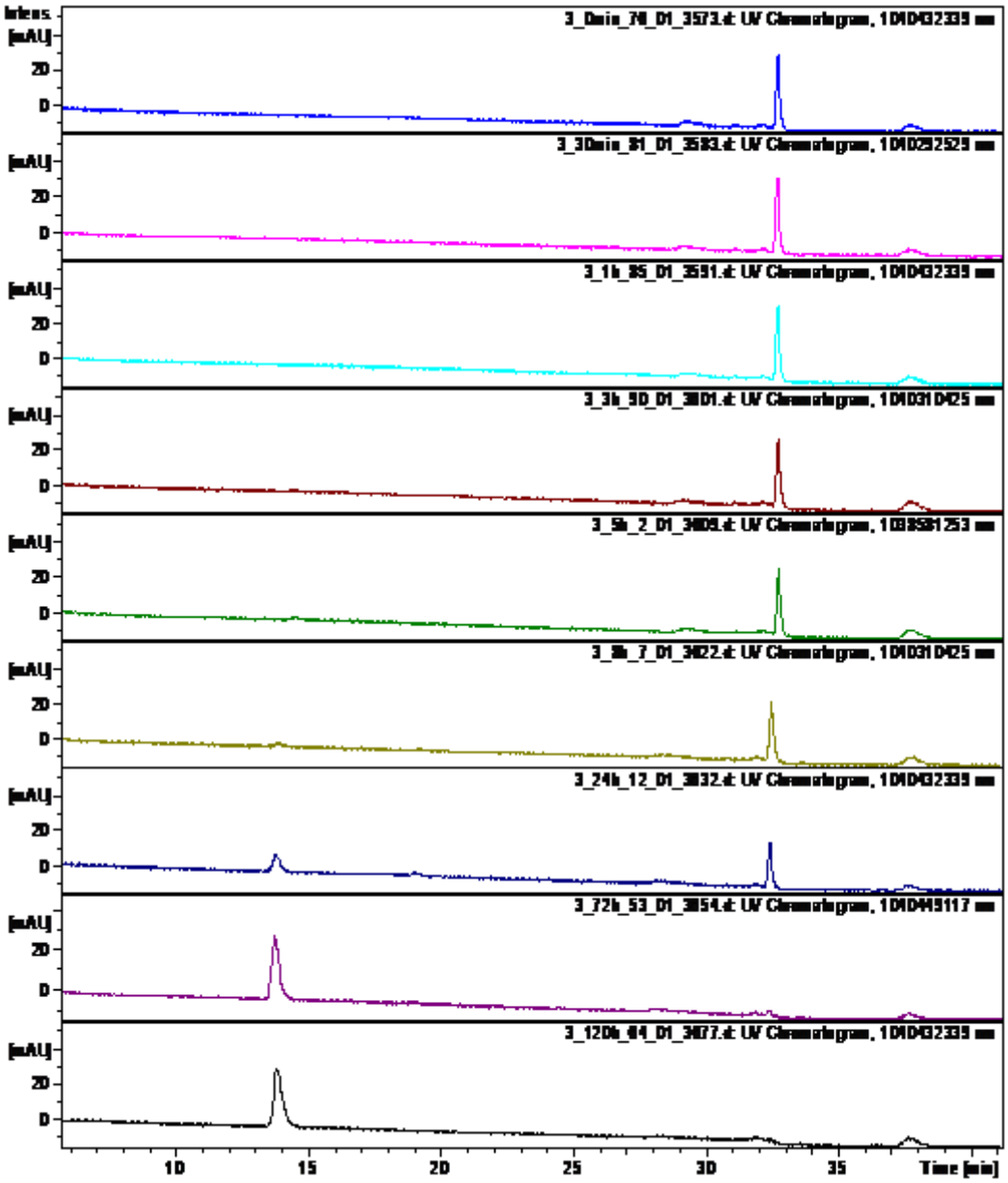


Figure 6: The UV traces of samples from 0 hours to 72 hours

The stability of acetophenone-azine was examined in artificial sweat for 5 days at 37°C. Two detection modes were: UV-photometry at 245 nm and mass spectrometry with APCI ionization. In the first 8 hours no major changes were detected. After 24 hours 30-40% of the initial acetophenone-azine amount was hydrolysed. The decrease was detectable both in the UV and the EIC chromatograms. After 72 hours approximately 95% of the test item is reacted and after 120 hours only traces can be detected in the 2nd and the 3rd sample while in sample 1 no more acetophenone-azine is present. Based on this it can be stated that the test item completely hydrolyses within 5 days. The hydrolysis product could not be identified by mass spectrometry, but according to the basic organic chemical principles the production of hydrazine and acetophenone were expected. The hydrazine has too low mass - and no chromophores - for detection while acetophenone cannot be ionized by APCI. But the injection of acetophenone standard at the same concentration level resulted in the appearance of peak at the same retention

time showing approximately the intensity. So the hydrolysis product is considered to be acetophenone.

The pH of the test solutions became a bit more acidic compared to the starting state as shown in Table 5

Table 5: pH of the samples

pH	Sample 1	Sample 2	Sample 3
t0	6.61	6.64	6.67
120 hours	5.46	5.45	5.45

2 HEALTH HAZARDS

2.1 Skin sensitisation

2.1.1 Human data

2.1.1.1 [Human case 1]

***Study reference 1* : [Nadia Raison-Peyron *et al.*, 2016]**

[Nadia Raison-Peyron, Ola Bergendorff, Jean Luc Bourrain and Magnus Bruze. Acetophenone azine: a new allergen responsible for severe contact dermatitis from shin pads. 2016 Aug. *Contact Dermatitis*. 75 (2), 106-110].

Detailed study summary and results:

Contact dermatitis resulting from the use of shin pads is usually caused by rubber components, dyes, benzoyl peroxide, or formaldehyde resins. To investigate and identify a new allergen in shin pads that was responsible for severe contact dermatitis in a young football player. High-performance liquid chromatography (HPLC) of samples of shin padswasperformed. The boy was patch tested with pieces of shin pads and with acetophenone azine, a chemical substance identified by HPLC in the foam of the shin pads. HPLC identified acetophenone azine at concentrations of approximately 20 μg/g of shin pad samples. Patch tests gave strongly positive reactions to pieces of shin pads and to acetophenone azine down to 0.001% in acetone, whereas acetophenone and hydrazine sulfate were both negative. Twenty controls were negative for acetophenone azine 0.01% in acetone. Acetophenone azine is a new, strong allergen of shin pads, and more generally of other sport equipment based on ethylene vinyl acetate. It may be used as a biocide, but this has to be confirmed. Further investigations are needed to understand factors such as exposure, cross-reaction patterns, metabolism, and the optimal patch test preparation.

Test type

Case history

A 13-year-old boy with no history of atopy or contact dermatitis presented with acute, vesicular dermatitis on his shins 1month after wearing shin pads for playing football as a goalkeeper (Fig. 1). This eruption became generalized 1week later, and resulted in hospitalization. Hypereosinophilia was noted (1120/mm^3; normal, <700/mm^3). A skin biopsy confirmed the diagnosis of eczema. Three months after complete resolution of the eruption, the patient was referred to us for investigation.

Patch tests and protocol
Because of the high number of patch tests, the patient was patch tested over several sessions:

first, with the European baseline series (Trolab, Stallergènes, Antony, France) and plastics/glues and rubber series (Chemotechnique, Vellinge, Sweden), and

at a second time with dyes and preservative series (Chemotechnique),with dimethylfumarate 0.1% and 0.01% wt/wt in petrolatum and with all of the topical medicaments used.

Large pieces of the black shin pad foam in close contact with the skin were also tested 'as is', simply moisturized with acetone, water, and ethanol.

Test item and administration

a. Chemicals tested in patch test:

Most cases of contact dermatitis caused by shin or knee pads seem to be irritant reactions resulting from friction and sweating (2), but allergic contact dermatitis may be under-reported. The main allergens are rubber additives that are included in the baseline series (mercapto and thiuram derivatives, and *N*-isopropyl-*N*′-phenyl-*p*-phenylenediamine) and in rubber series (thioureas and carbamates) (3, 4) or dyes (3), benzoyl peroxide (3), and urea formaldehyde or phenol formaldehyde resins (5, 6). When allergic contact dermatitis caused by protective equipment is suspected, it is always important to test a large piece of the equipment, as is was done with the shin pads. Occasionally, patch testing with ultrasonic bath extracts of the shin pads may help to detect a contact allergy (7). Topical ointments and lotions used in the same skin area should also be tested.

Results from other chemicals: Our patient suffered from allergic contact dermatitis caused by his shin pads, but he did not react to any of the allergens mentioned above.

However, acetophenone azine was identified in the shin pads.
When acetophenone azine was detected in the black foam of the shin pads, the patient was patch tested withthis substance (Sigma-Aldrich, Saint-Louis, MO, USA)

b. Acetophenone azine

Name: Acetophenone azine
Origin: Sigma-Aldrich
CAS: 729-43-1
Vehicles: acetone and water, at 1.0% wt/vol stock solutions, which were further diluted to 0.1%, 0.01%, 0.001% and 0.0001% wt/vol

Negative and positive controls:

Number of subjects tested: 20 control subjects (dermatitis patients) with acetophenone azine at 0.01% wt/vol in acetone.

Dilutions tested: 0.1%, 0.03%, 0.01%, 0.003%, 0.001%, 0.0003%, 0.0001% and 0.00003% wt/wt in pet.

c. Hydrazine

Name : Hydrazine sulfate 2% in pet as hydrazine was not available
Origin: Chemotechnique

Topical application : Tests were applied on the upper back, in IQ Chambers® (Chemotechnique). Readings were performed on day (D) 2 and D3, according to ICDRG guidelines.

Results :

At the first patch test session, all patch tests gave negative results, except for a positive reaction to abitol (1+ on D2 and D3) with no apparent relevance.
We observed strong reactions to pieces of the black foam moisturized with ethanol, acetone, and water (2+ on D2; 3+ on D3).

Testing with acetophenone azine resulted in positive reactions to acetone dilutions at 1%, 0.1%, 0.01%, and 0.001%, and to aqueous solutions at 1% and 0.1% (Fig. 2). All other tests based on acetone and water solutions gave negative results.
Patch testswith acetophenone and hydrazine sulfate all gave negative results. The results are summarized in Table 6.
Table 6 : Results of patchs tests with acetophenone, hydrazine sulfate and acetophenone azine

Table 1. Results of patch tests with acetophenone, hydrazine sulfate, and acetophenone azine

Concentration (%)	Acetophenone (pet.) D2/D3	Hydrazine sulfate (pet.) D2/D3	Acetophenone azine (aq.) D2/D3	Acetophenone azine (acetone) D2/D3
2	NT	−/−	NT	NT
1	NT	NT	++/++	++/++
0.1	−/−	NT	+/+	++/++
0.03	−/−	NT	NT	NT
0.01	−/−	NT	−	+?/+
0.003	−/−	NT	NT	NT
0.001	−/−	NT	−/−	−/+?
0.0003	−/−	NT	NT	NT
0.0001	−/−	NT	−/−	−/−
0.00003	−/−	NT	NT	NT

NT, not tested.

Twenty control subjects did not react to acetophenone azine at 0.01% wt/vol in acetone (1 of 1 versus 0 of 20; $p<0.05$, Fisher's exact test, two-sided).

Chemical analysis

Chromatographic analyses were performed with a high-performance liquid chromatography (HPLC) method suitable for identifying allergens in rubber items (1).
Each sample (0.5 g) of the shin pad was measured in order to estimate the area in contact with the skin, and then cut into small pieces with a pair of scissors. The samples were placed in 10-ml test tubes with Teflon-lined screw caps containing 5 ml of acetone (Scantec Nordic AB, Partille, Sweden). The test tubes were placed on a shaker and, after 10min of extraction at room temperature, the extracts were pipetted into round-bottomed flasks and evaporated under vacuum. The extracts were then dissolved in 1ml of acetonitrile, which was filtered before injection onto the HPLC column. A reversed-phase column [Alltima C18, 4 mm, 150 × 4.6 mm, polyether ether ketone (PEEK)-lined; Alltech Associates, Deerfield, IL, USA] was eluted with acetonitrile and aqueous zinc sulfate (10−5 mol/l) at a ratio of 50:50 for 5 min, and then a linear gradient to 100% acetonitrile for 35min. The eluent was pumped with an Agilent1260 (Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 1 ml/min, and monitored at 280nmwith an Agilent 1100 Series diode-array detector (Agilent Technologies). All devices in contact with the mobile phase after the injector were made of PEEK.

Acetophenone azine was identified after comparison of retention times and ultraviolet (UV) spectra recorded by the diode-array detector, and the concentration in the shin pads was determined after comparison of areas of sample peaks with the area of a sample of acetophenone azine with a known concentration. Duplicate analyses were performed for each sample.

Results

Acetophenone azine was identified in the shin pad by an external chemical laboratory (Service commun des laboratoires, Massy, France). Further analyses were performed by our own laboratory with HPLC to determine the amount of acetophenone azine in the product and to search for thiourea derivatives and other rubber allergens.
The chromatograms showed a few dominant peaks with absorption at 280nm (Figure 7). Comparison of retention time and the UV spectrum with a reference sample of acetophenone azine established the identification of the peak eluting at ~20 min. The spectrum had a characteristic shape that was very different from those of other known rubber additives normally screened with this HPLC method. No other peaks in the chromatograms could be identified. The concentrations were found to be 19,24 and 23 µg/g, respectively, in the three samples from the shin pad. These concentrations correspond to 1.2, 1.0 and 1.7 µg/cm2, respectively.

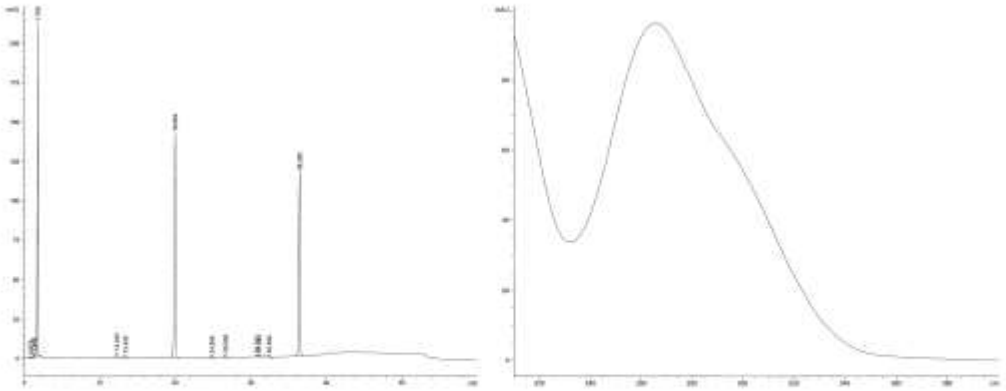


Fig. 3. Left: high-performance liquid chromatography chromatogram of an extract of the shin pad monitored at 280 nm. Right: ultraviolet spectrum obtained from the peak eluting at 19.952 min in the chromatogram. Analysis of a reference sample of acetophenone azine showed an identical retention time as the reference sample. The peak at 36.392 min could not be identified.

Figure 7: HPLC results

Conclusion

At patch testing, the patient reacted positively to acetophenone azine in acetone down to 0.001%, whereas 20 controls did not react to a 10-fold higher concentration (0.01%).

Discusssion:

Acetophenone azine was found as a component of EVA of the black foam of the patient's shin pads, but we do not know why and how it is incorporated in EVA. It is perhaps used as a catalyst during the process of polymerization of EVA. It is also known from older publications to have a broad spectrum of antimicrobial activity, and in particular antihelminthic activity. More generally, ketazines are used as industrial microbiocides with bactericidal and fungicidal properties.

Acetophenone azine, also called bis(1-phenylethy lidene)hydrazine or methylphenylketazine (CAS no.729-43-1), has the molecular formula C16H16N2 and a molecular weight of 236.31. It is marketed as a white powder.
Acetophenone azine has been produced by reacting hydrazine hydrate with acetophenone.
It is mentioned in the Safety Data Sheet that acetophenone azine may cause allergic reactions in certain sensitive individuals after prolonged or repeated exposure.
Acetophenone azine itself could be the sole allergen responsible for this severe contact dermatitis.

Under conditions with increased sweating, such as playing football, acetophenone azine could be hydrolysed to acetophenone and hydrazine. Hydrazine and some of its derivatives are known irritants and contact sensitizers. Severe contact dermatitis has been reported in chemistry students after handling of hydrazine derivatives. Patch test reactions were strongly positive with the suspected product called *N*-(□-chlorobenzylidene) phenylhydrazine at 0.01% in acetone, and cross-sensitization was observed between various hydrazine derivatives.
Five of 6 students who were exposed to these chemicals felt ill, and 3 further students became ill a few months later. A biphasic course, as in our case, was described: circumscribed lesions developed quickly in directly contaminated areas, and this was followed by widespread erythema and oedema with papular, vesicular and urticaria-like lesions after 4–12 days.
Recently, when the local lymph node assay was used in combination with examination of the structural and physicochemical properties to identify dermal sensitizers, haloalkanes and hydrazines were among the most potent ones.
However, benzophenone azine, an azine derived from another cyclic ketone, did not show thermal decomposition even at temperatures as high as 230°C when analysed by gas chromatography, and homogeneous hydrolysis of benzophenone azine was reported only in the presence of various strong acids in a previous article. We therefore think that hydrazine is not the allergen concerned in our case. Furthermore, our patient did not react to the two possible hydrolysis substances, hydrazine (tested as hydrazine sulfate) and acetophenone.
In conclusion, acetophenone azine is a strong sensitizer that is possibly used as a biocide in the plastic industry. Further investigations are needed to investigate factors such as exposure, cross-reaction patterns, metabolism, and the optimal patch test preparation.
It is important to know why this product is used by the manufacturers of EVA copolymers; these thermoplastic copolymers are widely used all over the world by industries and consumers.

2.1.1.2 [Human cases 2 and 3]

Study reference 2 : **[Nadia Raison-Peyron et al., 2017]**

[Nadia Raison-Peyron , Ola Bergendorff, Aurélie Du-Thanh, Jean-Luc Bourrain, and Magnus Bruze. Two new cases of severe allergic contact dermatitis caused by acetophenone azine. 2017 June. *Contact Dermatitis*, 76 (6), 357–381.]

Clinical Case 2

Patient and clinical observations
An 11-year-old non-atopic football player experienced an itchy, erythematous and vesicular eruption, initially localized to both shins, in close contactwith football shin pads, after having used these two or three times a week during a 3-month period. In the following 2months, the eruption spread all over the body, including the face, when he continued to play football with a jersey garment under the shin pads (Fig. 1). He also had erythematous, vesicular and scaly lesions on both soles 3 days after starting to wear new flip-flops without socks, 8 months after the beginning of the dermatitis on the shins. The eczematous eruption resolved slowly with residual depigmentation under treatment with a corticosteroid cream.

Patch test
Test items

Commercial allergens:
After the patient had recovered from the eczematous eruption, patch testing with IQ Ultra® chambers (Chemotechnique, Vellinge, Sweden) was performed on the back with the European

baseline series, a plastic and glues series, and a rubber series (Chemotechnique). The patches were removed from the back after 48 h.

Patch tests with pieces of shin pads and flip-flop soles moistened with acetone, ethanol, and water were performed.

Acetophenone azine:

Origin: Sigma-Aldrich, Saint-Louis, MO, USA
Doses tested : 0.1% and 0.01% wt/vol in acetone

Hydrazine sulfate :
Doses tested: 1% pet.
Origin : Chemotechnique

Results :

Patch tests with commercial allergens all gave negative results on day (D) 2 and D4.

Patch tests with pieces of shin pads and flip-flop soles moistened with acetone, ethanol, and water gave strong reactions (++/++, D2 and D3) that persisted 12 days later.

Patch tests with acetophenone azine (0.1% and 0.01% wt/vol in acetone gave positive results (++/++, D2 and D3) (Fig. 2), while results were negative for hydrazine sulfate 1% pet. (Chemotechnique).
Analysis of samples of the inner foam of the shin pads and of the sole of the flip-flops by high-performance liquid chromatography (HPLC) coupled with a diode array detector, as described previously (1), identified acetophenone azine, at 69 and 21 μg/g, respectively, in the two samples.

Clinical Case 3

Patient and clinical observations
A 12-year-old non-atopic boy presented with acute itchy, vesicular dermatitis of both soles soon after wearing new sneakers. Four months later, he also experienced a severe and diffuse eczematous eruption with secondary depigmentation, mainly on his back and upper limbs, and also involving the cheeks. The dermatitis of the soles relapsed when he bought and used sneakers of another sports brand.

Patch test
Test items

Commercial allergens:
Patch testing was performed 3 months later with the European baseline series and a shoe series (Chemotechnique),

Patch tests with pieces of the soles of the sneakers in water, ethanol and acetone were performed.

Acetophenone azine:

Origin: Sigma-Aldrich, Saint-Louis, MO, USA
Doses tested : 0.1% and 0.01% wt/vol in acetone

Hydrazine sulfate :
Doses tested: 1% pet.
Origin : Chemotechnique

Results :

Patch tests with commercial allergens with negative results on D2 and D3.
Patch tests with pieces of the soles of the sneakers in water, ethanol and acetone gave ++ positive reactions to the samples in water on D2 and D3, and + positive reactions to the samples in acetone on D2 and D3, but negative results with thematerialmoistened with ethanol.

Acetophenone azine diluted as above gave a strong reaction (++ on D2 and D3), whereas hydrazine sulfate 1% pet. gave a negative result. Acetophenone azine was detected by HPLC in the foamof sneaker soles from both sports brands: 15 μg/g for the first brand, and <0.5 μg/g for the second brand.

Discussion:

Acetophenone azine or methylphenylketazine (CAS no. 729-43-1) is a new allergen in ethylene vinyl acetate (EVA) copolymers, which are widely used in sports equipment, but also in shoes such as sneakers and flip-flops. A few months ago, we reported the first case of severe allergic contact dermatitis caused by acetophenone azine in the foam of the shin pads used by a young football player (1).
Clinically, the 3 young boys presented with the same type of eruption: first, localized eczema on the skin in close contact with EVA foam; and second, a severe and diffuse eczematous rash on the whole body, including the face. Post-inflammatory depigmentation was observed in all of them.

In the second case, the patient had acute dermatitis on both soles, soon afterwearing new sneakers.We do not know whether the patient had been sensitized by another item of sports equipment or by previous shoes, or whether sensitization coincided with elicitation, which is possible, because acetophenone azine is a strong allergen.
Acetophenone azine is, to our knowledge, not intentionally added during manufacture of the EVA copolymers that we have studied. Instead, we believe that acetophenone azine is formed *in situ* during the manufacturing process as a byproduct of reactions between compounds originating from other additives.
The acetophenone azine molecule comprises two different entities: two acetophenone substructures connected with a central hydrazine moiety. We hypothesize that acetophenone is formed from decomposition of the radical initiator dicumylperoxide, and that hydrazine is formed from decomposition of the foaming agent azodicarbonamide. Further studies are needed to prove this explanation, which would illustrate the fact that new allergens can be formed during the production of polymers, similarly to what occurs during rubber vulcanization (2).

Conclusion:
These 2 new cases of severe allergic contact dermatitis caused by acetophenone azine confirm that this substance is a strong sensitizer. If the number of cases of allergic contact dermatitis caused by this chemical in young people increases, it will be important to regulate this substance in the EU.

2.1.1.3 [Human case 4]

***Study reference 3 : [*De Fré Charlotte *et al.,* 2017]**

[Charlotte De Fré, Ola Bergendorff, Nadia Raison-Peyron, Karen van de Voorde, Elien Romaen, Julien Lambert, Christina Persson and Olivier Aerts. Acetophenone azine: a new shoe allergen causing severe foot Dermatitis. 2017 Dec. *Contact Dermatitis*. 77 (6), 406–429].

The first adult case with allergic contact dermatitis of the legs, caused by AA present in shin pads, inwhom, additionally, AA-containing sport shoes was reported and was shown to be the cause of recalcitrant foot dermatitis.

Patient and clinical observations
A 29-year-old non-atopicmalehockey playerwas referred to us for the evaluation of dermatitis on both legs, which had commenced shortly after thewearing of a new pair of shin pads, lined with a grey foam). Dermatitis had started on his shins, and had rapidly spread to his trunk and arms. Previously, dermatitis had also occurred after the wearing of another (older) brand of shin pads, with a similar, blue inner foam. More recently, the patient had experienced severe dermatitis on the soles of both feet, which he related to the wearing of new sports shoes with a grey foam insole. Occasionally, generalized skin lesions would appear on top of the foot dermatitis.

Patch test
Test items

Commercial allergens:
Patch testing was performed with the Belgian baseline series andwith additional series (cosmetics, rubbers, plastics and glues, shoe allergens, and textile colourants), all from Chemotechnique (Vellinge, Sweden), mounted on Allergeaze® patch test chambers (SmartPractice, Calgary, Canada), and occluded for 2 days with Fixomull® stretch (BSN Medical, Hamburg, Germany).

Both patch tests with pieces of the internal grey foam of the patient's newest shin pads, and of the similar grey foam of the sport shoe insoles, were performed 'as is', moistened with acetone.
The older shin pads were not brought in by the patient, and could therefore not be patch tested separately.

Acetophenone azine:
Origin: N.R.-P
Doses tested : 0.1% and 0.01% in acetone.
All tests were removed on day (D) 2 and read on D2, D4, and D7, according to ESCD guidelines [1].

Results :

Positive reactions to pieces of the grey foam, contained in the shin pads and in the soles of the sport shoes, were seen on D2 and on D4 (+ and ++, respectively) (Figure 8).
Moreover, ++ and + reactions were observed to AA 0.1% and 0.01%, respectively, on D2 and D4 (Figure 8). No later-occurring reactions were observed.

Chemical analyses, performed in Malmö as detailed in the report by Raison-Peyron *et a*l, 2016 described above, of the foam layers of both brands of shin pads, and of the grey foam soles of the sport shoes, confirmed the presence of acetophenone azine in all three items.
The highest concentration of acetophenone azine, namely 88 µg/g, was found in the most recent brand of shin pads (grey foam), to which a positive patch test reaction was also obtained. In the older pair of shin pads (blue foam), possibly accounting for initial sensitization, acetophenone azine was detected at 60 µg/g, whereas the concentration in the shoe soles was only 8 µg/g. The patient was successfully managed with topical corticoids, and, following avoidance of the culprit shin pads and shoes, his dermatitis did not recur.

[1] Johansen J D, Aalto-Korte K, Agner T et al. European Society of Contact Dermatitis guideline for diagnostic patch testing – recommendations on best practice. *Contact Dermatitis* 2015: **73**: 195–221.

Fig. 2. Positive reactions on day 4 to the grey foam present in the shin pads (++; upper pieces) and in the soles of the sport shoes (++; lower pieces).

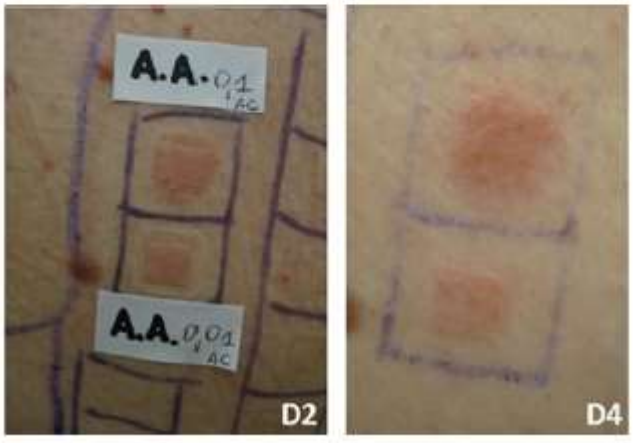


Fig. 3. Positive reactions on day (D) 2 and D4 to acetophenone azine (AA) 0.1% (++) and 0.01% (+) in acetone (AC).

Figure 8 : Results of patch tests in the grey foam and in the soles of he sport shoes and on days 2 and 4 to acetophenone azine at 0.1% and 0.011% in acetone.

Discussion:

Shin pad dermatitis hasmainly been reported in children, and common allergens include mercapto and thiuram derivatives, *N*-isopropyl-*N*-phenyl-*p*-phenylenediamine, thioureas, dithiocarbamates, (disperse) dyes, benzoyl peroxide, and urea formaldehyde and phenol formaldehyde resins . Contact irritant reactions, especially in atopic children, are a differential diagnosis. For allergic foot dermatitis, compounds contained in footwear, socks or stockings may be the causal agents, and reported allergens include chromium, rubber accelerators, and dyes. Recently, octylisothiazolinone, which is used as an antifungal during the processing of leather, and dimethylthiocarbamyl benzothiazole, which is a strongly sensitizing rubber compound, have also gained attention as new shoe allergens. Our case adds to the evidence that AA (syn. methylphenylketazine, CAS no. 729-43-1), owing to its presence in shin pads, is indeed a problematic sensitizer for children, also for adults. Moreover, the presence of this strong sensitizer in even more widely used consumer products, such

as (sport) shoes, might warrant the inclusion of this allergen in a shoe series, or as an addition to the baseline series, for example 0.1% in acetone .

Conclusion:

This new case of severe allergic contact dermatitis caused by acetophenone azine confirm that this substance is also a strong sensitizer in adult.

2.1.2 Experimental data

2.1.2.1 [Anonymous 2018a]

Study reference:

Anonymous 2018a. Keratinosens Test An In Vitro Skin Sensitisation Assay.

PRINCIPLE:

The ARE-Nrf2 luciferase test method is proposed to investigate the key event 2 of the skin sensitisation pathway involved in adverse effects, that is to say the inflammatory response as well as the expression of the genes associated with the cell activation pathway of the keratinocytes.
The test follows OECD guideline 442D. Testing were conducted in accordance with this guideline.

At present, the only *in vitro* ARE-Nrf2 luciferase assay method covered by OECD 442D Guideline is the KeratinoSens™ method. The KeratinoSensTM test method was considered scientifically valid to be used as part of an Integrated Approach to Testing and Assessment (IATA), to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling.

The cell line used contains the luciferase gene under the transcriptional control of a promoter fused to a gene known to intensify its expression under the effect of skin sensitizers. The signal of the luciferase thus reflects the activation by the sensitizers of endogenous genes dependent on the factor Nrf2.

Dilution tests are conducted prior to conducting the test in sterile saline or sterile culture medium as a first option, or in DMSO as a second option if the product is not soluble or does not form a dispersion stable. The negative control used is (dimethylsulfoxide (DMSO)) and the positive control used is cinnamic aldehyde). For each test chemical and positive control substance, one experiment is needed to derive a prediction (positive or negative), consisting of at least two independent repetitions containing each three replicates (n = 6). The cells are grown for 24 hours in microplates. After exposure of the treated plates for 48 h in the KeratinoSens^{TM} test method, a quantitative measurement by luminescence detection of the induction of the luciferase gene is thus carried out.
The following parameters are calculated in the test method: the mean maximum value of the induction of the luciferase activity (I_{max}) for each tested test and positive control; the value of $CE_{1.5}$ representing the concentration for which an induction of luciferase activity is above the threshold of 1.5 times (i.e. an activity increased by 50%); and the IC_{50} and IC_{30} concentrations corresponding to a 50% and 30% reduction in cell viability.

DETAILED STUDY SUMMARY AND RESULTS:

Test type

The objective of the KeratinoSens assay is to evaluate the potential of the test item to activate the Nrf2 transcription factor. This test is part of a tiered strategy for the evaluation of skin sensitisation potential. Thus, data generated with the present Test Guideline should be used to support the discrimination between skin sensitizers and non-sensitizers in the context of an integrated approach to testing and assessment.

This *in vitro* test uses Human adherent HaCaT keratinocytes, an immortalized cell line. The KeratinoSens is a stably transfected cell line with a plasmid containing a luciferase gene under the transcriptional control of the SV40 origin of replication promoter. This promoter is fused with an ARE sequence. Sensitizers with electrophilic properties provoke the dissociation of Keap-1 from the transcription factor Nrf2. The free Nrf2 binds to the ARE sequence contained in the plasmid and therefore induces transcription of firefly luciferase.

Potential skin sensitizers are applied to the cells at 12 different concentrations and for a period of 48 hours. Sensitizers with electrophilic properties will provoke the dissociation of Keap-1 from the transcription factor Nrf2. The free Nrf2 will then bind to the ARE sequence contained in the plasmid and will therefore induce transcription of firefly luciferase. The luciferase reporter gene is under control of a single copy of the ARE-element of the human AKR1C2. The luciferase production will then be measured by flash luminescence.

In parallel, cytotoxicity is measured by a MTT reduction and is taken into consideration in the interpretation of the sensitisation results. This evaluation is performed in at least two independent runs.

The test method is applicable to:

. soluble test items or those that form a stable dispersion in an appropriate vehicle (*e.g.* DMSO, water or treatment culture medium).

. test items with a log P $\leq$5.

Assay limitations may be experienced with substances with log P between 5 and 7 which tend to produce false negative results. Therefore, negative results obtained from the testing of such substances will be considered inconclusive. Substances with a log P >7 fall out of the applicability domain of the assay and cannot be tested.

In the absence of a log P value or if the log P is not applicable, a visual inspection under microscope will be performed to each well to evaluate the presence or absence of emulsion/precipitate at the end of treatment. In case of the presence of emulsion/precipitate, the conclusion on the lack of activity cannot be drawn with sufficient confidence and will be considered inconclusive.

Furthermore, because of the limited metabolic capability of the cell line used and because of the experimental conditions, pro-haptens (i.e. test items requiring enzymatic activation) and pre-haptens (i.e. test items activated by auto-oxidation) in particular with a slow oxidation rate may also provide negative results in the KeratinoSens.

Methods

The KeratinoSens cells were first plated on 96-well plates and grown for 24 hours at 37°C. Then the medium was removed and the cells were exposed to the vehicle control or to different concentrations of test item and of positive controls. The treated plates were then incubated for 48 hours at 37°C. At the end of the treatment, cells were washed and the luciferase production was measured by flash luminescence. In parallel, the cytotoxicity was measured by a MTT reduction test and was taken into consideration in the interpretation of the sensitisation results. Two independent validated runs were performed as part of this study.

Results

All acceptance criteria were met for the positive and negative controls in each run; both runs were therefore considered as validated.

Both runs were performed using the following concentrations: 0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µM in culture medium containing 1% DMSO.

At these tested concentrations:

slight to strong test item precipitate were observed in treated wells at concentrations ≥ 62.5 µM in the first run and ≥ 31.3 in the second run,

a high decrease in cell viability (*i.e.* cell viability < 70%) was noted at concentrations ≥ 125 µM in the first run and ≥ 250 µM in the second run,

the corresponding IC_{30} and IC_{50} were calculated to be 97.68 and 163.11µM and 152.77 and 238.11 µM, in the first and second runs respectively,

statistically significant gene-fold inductions above the threshold of 1.5 were noted in comparison to the negative control at several successive concentrations in both runs (from 0.98 to 15.6 µM in the first run and from 0.49 to 31.3 µM in the second run). Moreover, an apparent dose response relationship was also noted, followed by a decrease of induction related to the appearance of cytotoxicity (*i.e*. from 62.5 µM in both runs),

the I_{max} values were 2.14 and 3.31 and the calculated $EC_{1.5}$ were 0.63 and estimated < 0.49 µM in the first and second runs, respectively.

The geometric means IC_{30} and IC_{50} of the two validated runs were calculated to be 122.16 and 197.07 µM, for the first and second runs, respectively.

The evaluation criteria for a positive response are met in both runs, the final outcome is therefore positive. This positive result can be used to support the discrimination between skin sensitizers and non-sensitizers in the context of an integrated approach to testing and assessment.

***Conclusion*Under the experimental conditions of this study, the test item, Acetophenone azine, was positive in the KeratinoSens assay and therefore was considered to activate the Nrf2 transcription factor.**

DETAILED STUDY

Designed study:
based on the OECD guideline 442D: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, adopted on February 2015.

The study will be performed in compliance with laboratory standard operating procedures and the following OECD principles of Good Laboratory Practice:

- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17 and all subsequent OECD consensus documents,
  Conformance to these GLP standards satisfies the Mutual Acceptance of Data (MAD) between members of OECD including the United States and Japan,
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L50 of 20.2.2004),
- Article Annexe 2 à l'article D523-8 du code concernant les principes de l'OCDE des Bonnes Pratiques de Laboratoire (BPL).

The study will be performed in a Test facility certified by the French National Authorities for Good Laboratory Practice compliance but GLP status will not be claimed. The study will follow established practices and standard operating procedures of the laboratory.

This draft study plan will be superseded by the final study plan when an agreement has been reached between the two parties and when all the information required by the Good Laboratory Practice regulations is known.

Test substance

Test item

Name:	Acetophenone azine
Synonyms:	Acetophenone azine, 97 % L03924 All denominations correspond to the same test item.
CAS No.:	729-43-1
Batch No.:	Confidential
Description:	Yellow powder
Storage conditions:	At room temperature.
Molecular weight:	236.32 g/mol
Log P (See § Assay limitations):	3.7
Specific test item requirements (handling conditions):	None.
Purity:	Confidential
Correction factor:	No correction factor

The identity, including batch number, purity, composition, concentrations and any other characteristics which appropriately define each batch of test item, as well as stability and storage conditions, should be provided by the Sponsor. The origins of data and information regarding the test item characterization will be documented in the study report.

Vehicle and negative control
Based on solubility results, the selected vehicle was dimethylsulfoxide (DMSO).

Negative control
The negative control was DMSO, and it was applied to cells in culture medium at a final concentration of 1%.
If several test items were assayed concurrently, the results of the negative control item were shared.

Positive control

Name:	Cinnamic aldehyde (CA)
Synonym:	trans-Cinnamaldehyde
CAS No.:	14371-10-9
Storage conditions:	At +4°C and under nitrogen gas

Since several test items were assayed concurrently, the results of the positive control were shared.
For each run, the positive control item was dissolved in DMSO to a final concentration of 200 mM. This solution was then further diluted to a final concentration of 6.4 mM. It was diluted in DMSO by serial dilutions in the Master plate 100x, using a dilution factor of 2, to obtain a total of 5 concentrations. Subsequently, each formulation of the Master plate 100x was diluted 25-fold in treatment medium in another 96-well plate called "Master plate 4x". The final tested concentrations ranged from 4 to 64 µM. All these formulations were prepared within 4 hours before use, then kept at room temperature and protected from light until use.

Test item formulations
On the basis of solubility results, the test item was solubilized in DMSO at 100 mM. Vortex for at least 10 minutes, then sonication for 10 minutes and finally vortex for at least 10 minutes were used in order to help solubilize the test item in the vehicle.

One formulation was prepared for each run. It was then diluted in DMSO by serial dilutions, using a dilution factor of 2 to obtain a total of 12 concentrations in a 96-well plate; this 96-well plate was called "Master plate 100x". Subsequently, each formulation of the Master plate 100x was 25-fold diluted in treatment medium in another 96-well plate called "Master plate 4x" taking care to adjust all wells to the same DMSO level.
All formulations were prepared within 4 hours before use, and kept at room temperature and protected from light until use.
Chemical analysis of the test item formulations
Since no chemical analysis of the test item formulations is performed, this will be specified as deviation to the principles of Good Laboratory Practice in the final report.

TEST SYSTEM

- KeratinoSens cells: the cell line KeratinoSens is stably transfected with a modified plasmid. This plasmid contains an ARE sequence from the AKR1C2 gene and a SV40 promotor which are inserted upstream of a luciferase gene. The resulting plasmid was transfected into HaCaT keratinocytes and clones with a stable insertion selected in the presence of Geneticin / G-418. Induction of luciferase gene is the endpoint evaluated and reflects the activation by the test item of the Nrf2 transcription factor in this test.
- Supplier: this cell line was provided by Givaudan.
- Batch: the original batch was validated by the supplier.
- Storage conditions: at – 80°C
- Mycoplasm: absence of mycoplasm was confirmed.

SPECIFIC EQUIPMENT, MEDIA AND COMPUTER SYSTEM (INDICATIVE LIST)
Specific equipment
96 well plate Luminometer with injectors and optical density reader (Varioskan Flash).
Media
Maintenance medium No. 1: DMEM containing GlutaMAX™, 1000 mg/L D-Glucose, Sodium Pyruvate and supplemented with 9.1% Fetal calf serum (FCS), and 500 µg/mL G-418,
Maintenance medium No. 2: DMEM with 9.1% FCS without G-418,
Treatment medium: DMEM with 1% FCS without G-418,
Freezing medium: DMEM with 20% FCS and 10% DMSO.
Computer system
The laboratory's computer systems used in the study are detailed in the following table:

Software	Version	Application function
CITPharma (CITAC)	3	Test item receipt and inventories, reagents, matrix
CITAC-CITEquipment	1	CIT Application Center: Web business portal Management of the equipments
PANORAMA E2	2.60.0000	Acquisition of temperature and humidity in study rooms (study and laboratory rooms, cold chambers)
SkanIt DDE	2.4.3	Pilot and acquire data from VarioSkan Flash
CITAC-CITMaster	3	CIT Application Center: Web business portal Master schedule sheet (including Study Note) Master schedule sheet - Study event

a: version number of the applications will be specified in the study report.

STUDY DESIGN
The test item was tested in two independent validated runs using cells from a different passage number. The plates were processed as described below in the paragraph Method.

Solubility assay

A solubility assay was performed prior the first treatment in order to select the vehicle (among DMSO, water for injections or treatment culture medium). Vortex for 10 minutes, then sonication for 10 minutes and finally vortex for 10 minutes were used in order to improve the solubility of the test item.
Since the test item was found soluble in DMSO at 100 mM, this stock formulation was diluted in treatment culture medium to the final concentration of 1000 µM. Then, a visual inspection of the sample was performed to evaluate the presence or absence of precipitate/emulsion.

Method for a run of KeratinoSens assay
Cell seeding for testing
Cells were grown using general culture procedures up to 80-90% confluence
the day prior to treatment, cells were washed twice with D-PBS containing 0.05% EDTA, harvested, re-suspended in Maintenance medium No. 2 and counted using Trypan Blue dye. Cell concentration was adjusted to a density of 8 x 104 cells/mL,
cells were then distributed into four 96-well plates (three white plates and one transparent plate), by adding 125 µL (representing 1 x 104 cells) per well taking care to avoid sedimentation of the cells during seeding,
after seeding, the cells were grown for 24 (± 1) hours in the 96-well microtiter plates prior to test item addition.

Treatment
After the 24-hour growing period, the medium was removed by aspiration and replaced by 150 µL of treatment medium,
from the Master plate 4x, a volume of 50 µL was added to each well of the three white assay plates and 50 µL to the transparent plate for the cytotoxicity evaluation,
all plates were covered by a sealing membrane to avoid evaporation of volatile test items and to avoid cross-contamination between wells,
the plates were then incubated for 48 (± 2) hours at 37°C, 5% CO2, 90% humidity.

Endpoint measurements
Microscopic observation to evaluate the presence or absence of precipitates -transparent plate
After the 48 (± 2) hours incubation period, the presence or absence of precipitate/emulsion was determined in each well by microscopic inspection.

Luminescence flash signal to evaluate induction signal – white plates
After incubation, the supernatants from the white assay plates were discarded,
the cells were washed once with D-PBS,
a volume of 20 µL of passive lysis buffer was added to each well and the cells were incubated for 20 (± 2) minutes at room temperature and under orbital shaking, the plates containing the passive lysis buffer were then placed in the luminometer for reading using the following program:
50 µL of the luciferase substrate was added to each well,
1 second after this addition, the luciferase signal was integrated for 2 seconds.

Absorbance signal to evaluate the cytotoxicity – transparent plate
For the cell viability assay plate, the medium was replaced by 200 µL of treatment medium,
a volume of 27 µL of a MTT solution at 5 mg/mL in D-PBS was then added to each well of the transparent 96-well plate,
the plates were covered with a sealing membrane and returned at 37°C in the incubator in humidified atmosphere for 4 hours (± 10 minutes),
at the end of the incubation period, the medium was removed and a volume of 200 µL of a 10% SDS solution was added to each well,
the plates were covered with a sealing membrane and placed at 37°C in the incubator in humidified atmosphere for an overnight period to extract the formazan from cells,
after the overnight incubation, the absorption of each well was determined at 600 nm using the plate reader.

Results analysis

Data evaluation was performed using a validated Excel sheet. The generated raw data (luminescence data for the luciferase activity and absorbance data for the MTT test) were pasted into an Excel template, and all data processing was performed automatically.

For the MTT and the luciferase data, the background value recorded in the empty well without cells (blank) was subtracted.
For the MTT data, the % viability was calculated for each well in the test plate in relation to average of the six negative control wells.
For the luciferase data, the average value of the six negative control wells was set to 1, and for each well in the plate, the fold induction was calculated in relation to this value.

For wells in which a statistically significant gene-induction (using a student test, also called T-test) over the 1.5 threshold was found, the following parameters were calculated from the processed raw data:
Imax: maximal induction factor of luciferase activity compared to the negative control over the complete dose-response range measured,
EC1.5: concentration at which a 1.5-fold luciferase gene induction is obtained,
IC50 and IC30: concentrations effecting a reduction of cellular viability by 50% and 30%,
indication whether significant 1.5-fold gene induction occurred below the IC30.

The data were plotted in graphs and the Imax and the EC1.5 values were visually checked since uneven dose-response curves or large variation may lead to wrong extrapolations.

Also, the individual and overall geometric means IC50 and IC30 were calculated, when applicable.

ACCEPTANCE AND EVALUATION CRITERIA

Acceptance criteria
Each run was considered valid if the following criteria were met:
the positive control results should be positive, thus the gene induction should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations,
the average EC1.5 value for the positive control should be within two standard deviations of the historical mean. In addition, the average induction (Imax) in the three replicate plates for the positive control at 64 µM should be between 2 and 8. If the latter criterion was not fulfilled, the dose-response of Cinnamic Aldehyde was carefully checked, and the run was accepted if there was a clear dose-response with increasing luciferase activity at increasing concentrations for the positive control,
the average coefficient of variation of the luminescence reading in the negative control wells of the triplicate plates should be < 20%.

Evaluation criteria of the test item
The results of each run are analyzed individually and if the test item is classified as positive in two runs, the final outcome is considered positive. If the test item is classified as negative in two runs, the final outcome is negative. In case, the first two runs were not concordant, a third run was performed and the final outcome was that of the two concordant runs.

The test item is considered as positive if the following four conditions are all met in two of two or in two of three runs, otherwise the KeratinoSens prediction is considered as negative:
the Imax is > 1.5-fold and statistically significantly different as compared to the negative control (as determined by a two-tailed, unpaired Student's T-test),
at the lowest concentration with a gene induction > 1.5-fold (i.e. at the EC1.5 determining value), the cell viability is > 70%,
the EC1.5 value is < 1000 µM (or < 200 µg/mL for test item without MW),

there is an apparent overall dose-response for luciferase induction (or a reproducible biphasic response).

Raw data specific to the study were retained in the study files. These study files contain all data related to the solubility of the test item, its formulation preparations and results analysis. Data not specific to the study and data including but not limited to, cell plating, treatment, luminescence and absorbance readings of test item, negative and positive controls were recorded in the laboratory files.

The following study materials are retained in the archives of the laboratoryfor 10 years after the signature of the study report by the Study Director:
study plan,raw data, test item sample,correspondence, final report and any amendments.

The total duration of archiving (depending on regulations) is the responsibility of the Sponsor.

In addition, the data not specific to the study are also archived at the laboratoryfor a period specified in internal procedures.

The study was performed in accordance with the study plan No. 45854 TIK. There were no deviations from the agreed study plan.

RESULTS

SOLUBILITY TEST

In the solubility test, the test item was found soluble in DMSO at 100 mM, following vortex for 10 minutes, then sonication for 10 minutes and finally vortex for 10 minutes. Therefore, this vehicle was selected for the preparation of the test item stock formulations.

Strong precipitate was observed once the test item stock formulation was diluted in the treatment culture medium to a final concentration of 1000 µM.

KERATINOSENS RUN (Figures 9 and 10)

The Imax, IC30, IC50, EC1.5 and viability values obtained for cells treated with test item in each validated run as well as the mean and SD values are presented in Appendix 1. The viability values (%), induction values, Imax, IC30, IC50 and EC1.5 values obtained with the positive control are presented in Appendix 2. In addition the luminescence values of all negative control wells and the %CV between these values for each validated run are also presented in Appendix 2.

All acceptance criteria were met for the positive and negative controls in each run; both runs were therefore considered as validated.

Both runs were performed using the following concentrations: 0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µM in culture medium containing 1% DMSO.
At these tested concentrations:
slight to strong test item precipitate were observed in treated wells at concentrations ≥ 62.5 µM in the first run and ≥ 31.3 in the second run, a high decrease in cell viability (i.e. cell viability < 70%) was noted at concentrations ≥ 125 µM in the first run and ≥ 250 µM in the second run, the corresponding IC30 and IC50 were calculated to be 97.68 and 163.11 µM and 152.77 and 238.11 µM, in the first and second runs, respectively,
statistically significant gene-fold inductions above the threshold of 1.5 were noted in comparison to the negative control at several successive concentrations in both runs (from 0.98 to 15.6 µM in the

first run and from 0.49 to 31.3 µM in the second run). Moreover, an apparent dose response relationship was also noted, followed by a decrease of induction related to the appearance of cytotoxicity (i.e. from 62.5 µM), the Imax values were 2.14 and 3.31 and the calculated EC1.5 were 0.63 and estimated < 0.49 µM in the first and second runs, respectively.

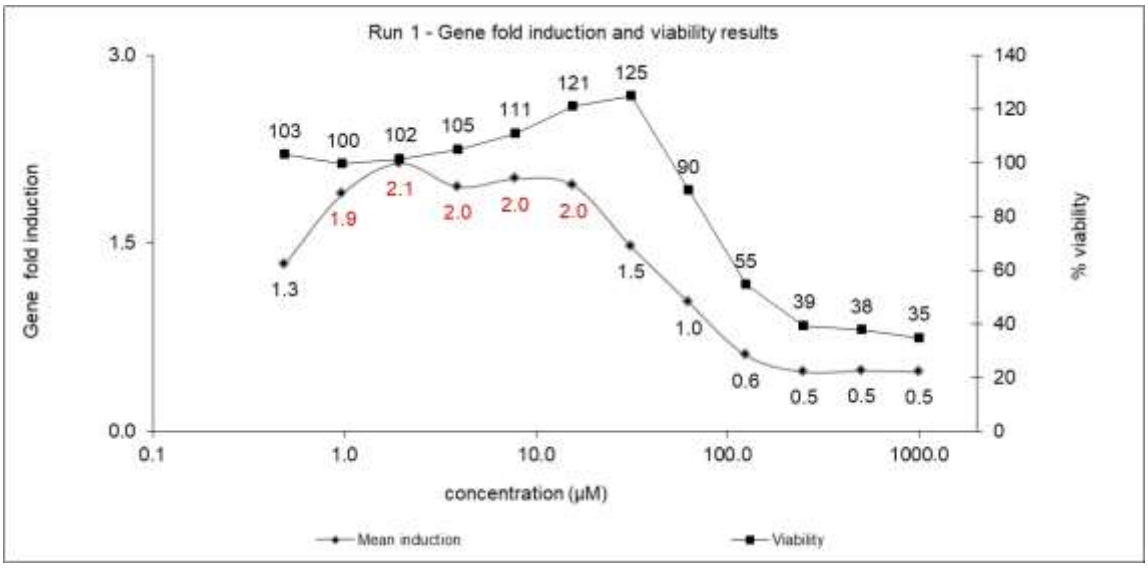


Figure 9 : Mean gene-induction and mean viability dose-response curves for cells treated with the test item in the first validated run

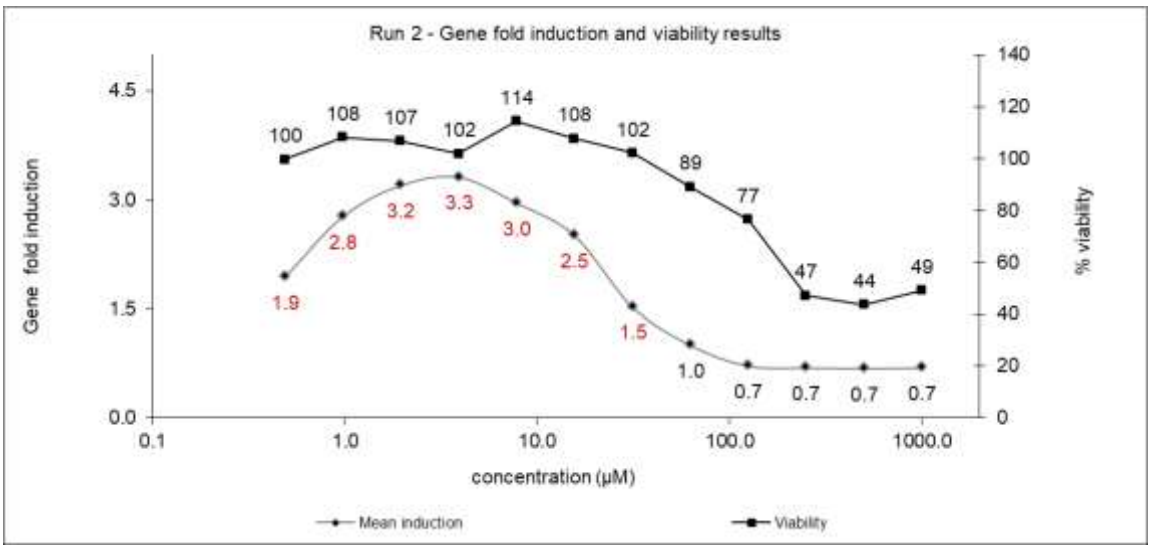


Figure 10: Mean gene-induction and mean viability dose-response curves for cells treated with the test item in the second validated run.

The geometric means IC30 and IC50 of the two validated runs were calculated to be 122.16 and 197.07 µM, respectively.

The evaluation criteria for a positive response are met in both runs, the final outcome is therefore positive. This positive result can be used to support the discrimination between skin sensitizers and non-sensitizers in the context of an integrated approach to testing and assessment.

CONCLUSION

Under the experimental conditions of this study, the test item, Acetophenone azine, was positive in the KeratinoSens assay and therefore was considered to activate the Nrf2 transcription factor.

2.1.2.2 [Anonymous 2018b]

Study reference:

Anonymous 2018b. Assessment Of The Skin Sensitization Potential Using The Human-Cell Line Activation Test.

PRINCIPLE:

The *h-CLAT* test allows to investigate the key event 3 of the skin sensitization pathway by quantifying changes in the expression of cell surface markers associated with the process of activation of monocytes and dendritic cells (i.e. CD86 and CD54), The measured expression levels of CD86 and CD54 cell surface markers are then used for supporting the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency when used in integrated approaches such as IATA.

This is a recognized test benefiting from a positive opinion from ECVAM and it follows OECD Guideline 442E. Testing has been conducted in accordance with this guideline.

The h-CLAT method quantifies changes of cell surface marker expression (i.e. CD86 and CD54) using a human monocytic leukemia cell line, THP-1 cells, following 24 hours exposure to the test chemical. These surface molecules are typical markers of monocytic THP-1 activation and may mimic DC activation, which plays a critical role in T-cell priming. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorochrome-tagged antibodies. Cytotoxicity measurement is also conducted concurrently to assess whether upregulation of surface marker expression occurs at sub-cytotoxic concentrations.

To perform this test, the substance must be soluble in water and have a log p < 3.5. The Log Kow value of the test item is slightly > 3.5 (i.e. 3.7). However, this slightly high Log Kow value is not considered to be a limitation for the applicability of this test since the positive outcome obtained in two validated runs guaranted the test system exposure to the substance.

A dose finding assay is performed to determine the CV75, being the test chemical concentration that results in 75% cell viability (CV) compared to the solvent/vehicle control (DMSO). The CV75 value is used to determine the concentration of test chemicals for the CD86/CD54 expression measurement.

Dilution tests are done prior to conducting the test in sterile saline or sterile culture medium as a first option, or in DMSO as a second option if the product is not soluble or does not form a stable dispersion in the previous two solvents/vehicles.

Starting form the 100 mg/mL (saline medium) or 500 mg/mL (in DMSO) stock solutions of the chemicals, eight concentrations are prepared, by two-fold serial dilutions using the corresponding solvent/vehicle. The final range of concentrations in the plate is 7.81-1000 µg/mL.
For each test chemical and control substance, one experiment is needed to obtain a prediction. Each experiment consisting of at least two independent repetitions are carried out during the test. A negative control (lactic acid) and a positive control (2,4-dinitrochlorobenzene –DNCB) are used.

The relative fluorescence intensity of surface markers compared to solvent/vehicle control are calculated and used in the prediction model, to support the discrimination between sensitisers and non-sensitisers.

DETAILED STUDY SUMMARY AND RESULTS:

Test type

This draft GLP compliance statement will be superseded by the final GLP compliance statement when all the information required by the Good Laboratory Practice regulations is known.

The study was performed in compliance with the laboratorystandard operating procedures and the following principles of Good Laboratory Practice:

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17 and all subsequent OECD consensus documents,

Conformance to these GLP standards satisfies the Mutual Acceptance of Data (MAD) between members of OECD including the United States and Japan,

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L50 of 20.2.2004),

Article Annexe 2 à l'article D523-8 du code de l'environnement concernant les principes de l'OCDE des Bonnes Pratiques de Laboratoire (BPL).

The objective of the study was to determine the ability of the test item to induce an increase in cell surface markers expression in THP-1 cells using the h-CLAT test method.

Methods

A solubility assessment was first performed in 0.9% NaCl and DMSO to select the vehicle and highest concentration to be used for test item formulation preparations.

Following the solubility assays, the cytotoxic potential was assessed in a Dose-Range Finding assay in order to select sub-toxic concentrations for testing in the main test.

The skin sensitizing potential of the test item was then evaluated in the main test, in three validated runs (Runs A, C and D).

In each run, the test item formulations were applied to THP-1 cells and cultured in a 24-well plate for 24h ± 30 minutes at 37°C, 5% CO_2 in a humidified incubator. A set of control wells was also added in each plate to guarantee the validity of each run. At the end of the incubation period, cells from each well were distributed to three wells of 96-well plate: the first well was labeled with IgG1-FITC antibodies, the second one was labeled with CD86-FITC antibodies and the third one was labelled with CD54-FITC antibodies. Then, just before flow cytometry analysis of CD86 and CD54 expression, all cells were dyed with Propidium Iodide for viability discrimination.

For each run, the Mean Fluorescence Intensity (MFI) obtained for each test sample was corrected by the isotype control IgG1 MFI value to obtain the corrected MFI. Corrected MFI value from the corresponding vehicle control was set to 100% CD54 and CD86 expression by default. Then, corrected MFI values from each test sample were compared to the corresponding vehicle control to obtain the Relative Fluorescence Index for CD86 and CD54 expression for each tested concentration (RFI CD86 and RFI CD54).

Results

Solubility assessment

The test item was found soluble in DMSO at 250 mg/mL.

Dose-Range Finding

During both DRF assays, no decrease in cell viability (*i.e.* cell viability < 75%) was noted in test item treated wells. No mean CV75 value was therefore calculated, and the highest tested concentration retained for the main test was 500 µg/mL.

Summary results

Summary results of all runs and conclusion

Study No. 45853 TIH

Test item Name	Conc. (µg/mL)	RFI for CD86				RFI for CD54				Viability (%)				Run conclusion				General conclusion
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
ACETOPHENONE AZINE	139.5	87	89	104	98	144	154	**350**	193	94.5	91.6	94.3	94.9	N	I	P2	P2	**Positive**
	167.5	89	74	87	98	193	**206**	**258**	**210**	92.3	89.9	95.0	94.7					
	200.9	81	81	86	99	179	181	**356**	169	92.6	91.4	95.2	94.9					
	241.1	92	78	116	97	103	154	**219**	**200**	92.5	91.2	93.8	94.2					
	289.4	86	88	104	90	149	**215**	156	**214**	93.3	90.9	94.4	93.7					
	347.2	83	69	82	88	184	152	175	**200**	91.2	89.7	94.4	92.4					
	416.7	42	73	96	108	154	156	161	190	92.8	91.2	93.6	94.9					
	500.0	75	79	82	89	120	**254**	144	**241**	90.9	78.8	95.1	99.1					

N = run with negative outcome
P_1 = run with positive outcomefor CD86
P_2 = run with positive outcome for CD54
P_{12} = run with positive outcome for CD86 and CD54

I = Invalidated run
Inc = Inconclusive run

Conc. = concentration
RFI = Relative Fluorescence Index
I = Invalidated run

Discussion

The Log K_{ow} value of the test item is slightly > 3.5 (*i.e.* 3.7). However, this slightly high Log K_{ow} value is not considered to be a limitation for the applicability of this test since the positive outcome obtained in two validated runs guaranted the test system exposure to the test item.

Conclusion

Under the experimental conditions of this study, the test item, Acetophenone azine, was found to be positive in the h-CLAT method.

DETAILED STUDY

Objective

The objective of the study was to determine the ability of the test item to induce an increase in cell surface markers expression in THP-1 cells using the h-CLAT test method.

The h-CLAT method is an in vitro assay that quantifies changes of cell surface marker expression (i.e. CD86 and CD54) on a human monocytic leukemia cell line, THP-1 cells, following 24 hours exposure to the test chemical. These surface molecules are typical markers of monocytic THP-1 activation and may mimic DC activation, which plays a critical role in T-cell priming. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorochrome-tagged antibodies. Cytotoxicity measurement is also conducted concurrently to assess whether upregulation of surface marker expression occurs at sub-cytotoxic concentrations. The Relative Fluorescence Intensity of surface markers compared to solvent/vehicle control are calculated and used in the prediction model, to support the discrimination between sensitizers and non-sensitizers.

The design of this study was based on:

DB-ALM Protocol No. 158: human Cell Line Activation Test (h-CLAT),

OECD guideline No. 442E: "In vitro skin sensitization: human Cell Line Activation Test (h-CLAT)", 29 July 2016.

With the following exceptions:

reactivity check was performed for each ATCC batch of cells and each working cell bank, and not each time frozen cells are thawed. Validation of cells reactivity was guaranteed in each run by running concurrently both positive controls (NiSO4 and DNCB) instead of only one (DNCB),

according to the OECD guideline No. 442E, the first Dose Range Finding (DRF) assay should be performed at the maximum concentration of 1000 µg/mL before running another assay with a maximum concentration of 5000 µg/mL if no cytotoxicity is noted in the first assay. In the present study design, the first DRF assay was performed at 5000 µg/mL if allowed by solubility. A second DRF assay was performed in case no concentration leading to viability > 75% was obtained in the first DRF assay,

when preparing cells for treatment, they were seeded between 0.1 and 0.2 x 106 cells/mL before incubating them for 48h to 72 hours (as noted in the DB-ALM protocol), instead of at 0.2 x 106 cells/mL for 48h incubation, or 0.1 x 106 cells/mL for 72h as noted in the OECD guideline.

MATERIALS AND METHODS

Test substance

Name:	Acetophenone azine
Synonyms:	Acetophenone azine, 97 % L03924

All denominations correspond to the same test item and the denomination retained in the study report is Acetophenone azine.

CAS No.:	729-43-1
Batch No.:	Confidential
Description:	Yellow powder
Storage condition:	At room temperature
Specific test item requirements	
(handling conditions):	None
Purity:	Confidential
Correction factor:	No correction factor
Log K_{ow}:	3.7
Surfactant:	No
Expiry (or re-test) date:	Will be specified in the study report

The molecular weight and the Log P value were confirmed by the Sponsor Representative in emails dated 09 and 10 January 2018.

Negative and positive controls

Vehicle control

Based on the results of the solubility assay, the selected vehicle was dimethylsulfoxide (DMSO, Sigma-Aldrich).

Positive control

DNCB

Name: 2,4-Dinitrochlorobenzene (DNCB)

Supplier: Sigma-Aldrich

CAS No. 97-00-7

Purity: Confidential

Classification: Extreme sensitizer

As several test items were assayed concurrently, the DNCB positive control was shared.

NiSO4

Name: Nickel Sulfate ($NiSO_4$)

Supplier: Merck

CAS No. 10101-97-0

Purity: Confidential

Classification: Moderate sensitizer

As several test items were assayed concurrently, the $NiSO_4$ positive control was shared.

Solubility assessment

The solubility of the test item was assessed visually for each preparation (particles, drops, cloudiness, non-miscible phases, etc) and recorded in the study files. A preparation was deemed appropriate for cell treatment as long as it was qualified as a solution or stable dispersion (homogenous emulsion/suspension).

Saline (0.9% NaCl) and DMSO are the only vehicles allowed in the assay. The vehicle was chosen between these two in the order of preference, and in accordance with the steps described below.

First, the test item was dissolved in saline at 100 mg/mL.

As the test item was not soluble neither in saline at the concentration of 100 mg/mL, nor in DMSO at 500 mg/mL, it was then dissolved at 250 mg/mL in DMSO.

Vortex for 2 minutes, then sonication for 5 minutes and finally vortex for 5 minutes were used in order to help solubilize the test item in the vehicle.

Test item and controls preparation

Positive controls preparation

The positive control DNCB was prepared at the concentration of 8 µg/mL in DMSO as follows:

on the treatment day, the required quantity of DNCB was mixed with DMSO at the concentration of 2 mg/mL,

this solution was then 250-fold diluted in cRPMI in order to obtain a 8 µg/mL DNCB stock solution.
The positive control $NiSO_4$ was prepared at the concentration of 200 µg/mL in 0.9% NaCl as follows:

on the treatment day, the required quantity of $NiSO_4$ was mixed with 0.9% NaCl at the concentration of 10 mg/mL,
this solution was then 50-fold diluted in cRPMI in order to obtain a 200 µg/mL $NiSO_4$ stock solution.
Both positive control stock solutions were prepared within 4 hours before use, and kept at room temperature and protected from light until use.

Vehicle control preparation

As DMSO was the vehicle selected at completion of the solubility assay, DMSO control formulation was included as vehicle control, and consisted in DMSO dissolved at 0.2% in cRPMI.

Test item preparation

All test item preparations were prepared in glass vials only. Test concentrations prepared and vehicle used were indicated by the Study Director in the study files, and no study plan amendment was issued for these purposes.

Fresh stock formulations of the test item were prepared for each run, using the vehicle and concentration identified in the § Solubility assessment. These concentrations were the same for all runs.

Test item formulations prepared in DMSO were 500 x concentrated; then 2 x concentrated formulations were prepared by 1:250 dilution in cRPMI. A DMSO vehicle control was also prepared (0.4% DMSO in cRPMI). The above mentioned dilutions of the test item and vehicle controls were performed to insure a constant percentage of the vehicle in the final volume of cell suspension in the well (*i.e.* 0.2% for DMSO).

The aspect of the stock formulations was evaluated and recorded in the study files.

The precipitation in the treatment conditions (*i.e.* when diluted in cRPMI) was checked and any observation was reported in the study files.

The test item formulations were kept at room temperature and protected from light until use, *i.e.* within 4 hours after preparation of the stock formulations. No control of concentration was performed during the study.

TEST SYSTEM

Cells

The THP-1 is an immortalized human monocytic leukemia cell line derived from an acute monocytic leukemia patient. The THP-1 cell line is obtained from ATCC (Ref: TIB-202, American Type Culture Collection, Manassas, USA) by the intermediate of LGC Standards (Molsheim, France).

The THP-1 cells are stored in a cryoprotective medium in a liquid nitrogen container.

Cells were grown using general culture procedures. They were cultured in cRPMI medium and maintained in a humidified incubator set at 37°C, 5% CO_2 and were not allowed to exceed a cell density of 1 x 10^6 cells/mL or more than 30 passages.

The culture medium (cRPMI) was composed of RPMI 1640 with 10% FBS, 0.05 mM 2-mercaptoethanol and with penicillin and streptomycin.

During cell culturing, cell viability was checked using trypan blue.

Cell culture for testing

For testing, THP-1 cells were seeded at a density between 0.1 x 10^6 cells/mL and 0.2 x 10^6 cells/mL, and pre-cultured in culture flasks for 48 hours to 72 hours, respectively. Cell did not exceed density of 1 x 10^6 cells/mL. On the day of testing, cells harvested from culture flasks were resuspended with fresh culture medium at 2 x 10^6 cells/mL. Then, 500 µL of cells suspension were distributed into a 24-well flat-bottom plate (*i.e.* 1 x 10^6 cells/well).

Reactivity check

Two weeks after thawing, a reactivity check was performed to qualify the cells of each working cell bank before testing. A reactivity check assay was performed by testing cell response after contact with Lactic Acid, DNCB and $NiSO_4$.

REAGENTS, EQUIPMENT AND COMPUTER SYSTEMS

Reagents

2-mercaptoethanol,
Blocking solution (0.01% globulin in FACS buffer),
BSA (Bovine Serum Albumin),
Dimethylsulfoxide (DMSO),
Dulbecco's Phosphate Buffered Saline (D-PBS) without Ca^{2+}/Mg^{2+},
FACS buffer (D-PBS with 0.1% (w/v) BSA),
FITC labeled mouse IgG1 antibody (Dako, Ref: X0927) - isotype control,
FITC labeled mouse anti-human CD86 antibody (BD Pharmingen, Ref: 555657),
FITC labeled mouse anti-human CD54 antibody (Dako, Ref: F7143 Clone: 6.5B5),
Inactivated Fetal Bovine Serum (FBS),
Penicillin and streptomycin,
Propidium Iodide (PI),
RPMI 1640 culture medium containing L-glutamine and HEPES (RPMI),
Saline (0.9% NaCl),
Trypan blue,
Water for injections.

All reagents used during this study were recorded in the study files.

Equipment

Precision scales, humidified incubator, laminar flow hood, centrifuge, fridge, water bath, flow cytometer, pipettes, ;timer, vortex, glassworks and tools, microscope.

Computer systems

The laboratory's computer systems used in the study are detailed in the following table:

Software	Version number	Application function
CITPharma	3	Test item receipt and inventories, reagent, matrix
CITAC-CITMaster	3	CIT Application Center: Web business portal Master schedule sheet (including Study Note) Master schedule sheet - Study event
CITAC-	1	CIT Application Center : Web business portal

CITEquipment		Management of the equipments
MACSQuantify	2.8	Cytometer
PANORAMA E^2	2.60.0000	Acquisition of temperature and humidity in study rooms (study and laboratory rooms, cold chambers)

STUDY DESIGN

The study was divided in two successive phases. First, a Dose-Range Finding assay (DRF) was performed to assess test item toxicity. Secondly, based on cytotoxicity data obtained from the DRF, a concentration series was tested in successive runs in the main test.

At each phase, all information relating to test item concentrations and run identification were given by the Study Director in the study files and no study plan amendment was issued for that purpose.

Dose-Range Finding assay (DRF)

The DRF consisted in two separated assays.

Treatments of DRF assays were performed at the following concentrations: 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 µg/mL,

Each assay was performed as described here below.

Test item stock solutions were prepared at 8 different concentrations by 2-fold dilutions using the selected vehicle. These stock formulations were then diluted 250-fold (as DMSO is the selected vehicle) into cRPMI to obtain working solutions.

The working solutions were finally used for exposure by adding 500 µL of working solutions to the volume of THP-1 cell suspension in the plate (500 µL) to achieve a further 2-fold dilution. In order to avoid evaporation of volatile chemicals and cross-contamination between wells, a sealer was placed on each 24-well plate just after treatment, before putting the plastic lids back on each plate.

The treated plates were then incubated for 24 hours ± 30 minutes in a humidified incubator set at 37°C and 5% CO_2.

At the end of the treatment phase, cells were transferred into sample tubes and collected by centrifugation. The supernatants were discarded and the remaining cells were resuspended with 600 µL of FACS buffer. Finally, cells were resuspended in 200 µL FACS buffer and the plate was positioned into the plate-reader of the flow cytometer. A volume of 10 µL of Propidium Iodide (PI) solution at 12.5 µg/mL was added automatically by the flow cytometer before acquisition of a sample to obtain a final PI concentration of 0.625 µg/mL per well.

Main test

The main test consisted in three validated runs (Runs A, C and D) and one invalidated run (Run B) being performed as described here below.

Test item stock solutions were prepared at 8 different concentrations by 1.2-fold dilutions using the selected vehicle. The highest concentration corresponded to the highest achievable non-cytotoxic concentration as no CV75 was obtained. The maximum concentration in the plates was 500 µg/mL.

All stock formulations were then 250-fold diluted into cRPMI to obtain working solutions.

In parallel, the working solutions of positive controls DNCB and $NiSO_4$ and vehicle control were prepared as noted in § Test item and controls preparation.

All working solutions were finally used for exposure by adding 500 µL of working solutions to the volume of THP-1 cell suspension in the plate (500 µL) to achieve a further 2-fold dilution. In order

to avoid evaporation of volatile chemicals and cross-contamination between wells, a sealer was placed on each 24-well plate just after treatment, before putting the plastic lids back on each plate.

The treated plates were then incubated for 24 hours ± 30 minutes in a humidified incubator set at 37°C and 5% CO_2.

During the main test, treatments were performed at the following final concentrations: 139.54, 167.45, 200.94, 241.13, 289.35, 347.22, 416.67 and 500 µg/mL.

At the end of the treatment phase, cells were transferred into sample tubes and collected by centrifugation, washed twice with 1 mL FACS buffer and blocked with 600 µL of blocking solution and incubated at 4°C for 15 minutes (± 1 minute). After blocking, cells were split in three aliquots of 180 µL into a 96-well round bottom plate and centrifuged before staining with antibodies. A volume of 50 µL of FITC-labelled anti-CD86, anti-CD54 or mouse IgG1 (isotype) antibodies prepared in FACS buffer was added to each aliquot before incubation for 30 minutes (± 2 minutes) at 4°C.

Finally, cells were washed with 150 µL FACS buffer 2 times and re-suspended in 200 µL FACS buffer. The plate was then positioned into the plate-reader of the flow cytometer. A volume of 10 µL of PI solution at 12.5 µg/mL was added automatically by the flow cytometer before acquisition of a sample to obtain a final PI concentration of 0.625 µg/mL per well.

Flow cytometry analysis

DRF assays

The PI uptake is analyzed using flow cytometry with the acquisition channel B3. A total of 10 000 living cells (PI negative) are acquired. In case of low viability which does not allow obtaining 10 000 living cells, a total of 30 000 events is acquired. Alternatively, cells were acquired for a maximum of 1 minute after the initiation of the acquisition.

Main test

The non-specific binding of IgG1 and the expression CD86 and CD54 was analyzed by flow cytometry with the acquisition channel B1 in order to obtain the Mean Fluorescence Intensity (MFI); whereas the viability (PI uptake) was analyzed with the acquisition channel B3. A total of 10 000 living cells (PI negative) were acquired. When the viability was low and did not allow obtaining 10 000 living cells, a total of 30 000 events was acquired. Alternatively, cells were acquired for a maximum of 1 minute after the initiation of the acquisition.

In case cell viability is less than 50%, no MFI is presented in the study report and the corresponding test item concentration are considered too high for interpretation because of the diffuse labelling cytoplasmic structures that are generated following cell membrane destruction.

Calculations

Estimation of the CV75 value (when applicable)

The percentage of living cells (PI negative cells) is used as the value for cell viability.

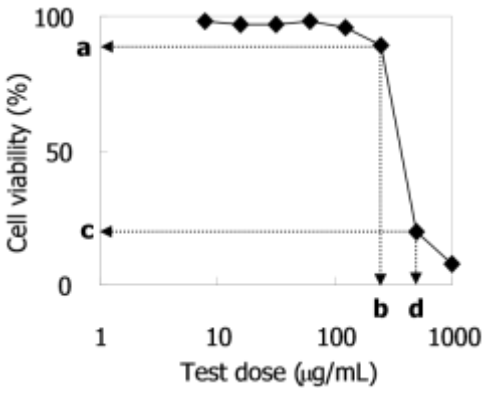


Figure 11: Example of results from a DRF assay

The CV75 value is derived from the dose-response curve as shown in Figure 11 (75% of cell viability, lying between a and c). CV75 is defined as the estimated concentration that is required to elicit 75% cell viability. The CV75 value is calculated by log-linear interpolation utilizing the following equation:

$$\text{Log CV75} = \frac{(75 - c) \times \text{Log } b - (75 - a) \times \text{Log } d}{a - c}$$

Main test

Based on the Mean Fluorescence Intensity (MFI), the Relative Fluorescence Intensity (RFI) of CD86 and CD54 were calculated according to the following equation:

$$\text{RFI} = \frac{\text{MFI of test item-treated (CD86 or CD54) - MFI of test item-treated IgG1}}{\text{MFI of control-treated (CD86 or CD54) - MFI of control-treated IgG1}} \times 100$$

RFI = Relative Fluorescence Intensity

MFI = Mean Fluorescence Intensity

Acceptance criteria

DRF

Viability of control cells treated with cRPMI (and DMSO if applicable) should be ≥ 90%,
viability of control cells treated with 0.2% DMSO should be ≥ 90%, if applicable.

Main test

The following applies for each run.

Controls acceptance criteria

Viability of cells treated with cRPMI and DMSO controls should be ≥ 90%,
in cRPMI and DMSO control wells, MFI ratio of both CD86 and CD54 to isotype control should be > 105%,
in the DMSO control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI > 150% and CD54 RFI ≥ 200%),
in the positive controls (DNCB and $NiSO_4$), RFI values of both CD86 and CD54 should meet positive criteria (CD86 RFI ≥ 150 and CD54 RFI ≥ 200) and cell viability should be more than 50%.

Test item acceptance criteria

For a test item noted as cytotoxic in the DRF phase, and resulting in a negative outcome in the main test, cell viability at 1.2 x CV75 should be < 90% in each run,
cell viability of at least 4 out of 8 concentrations should be > 50%.

Main test interpretation

Individual run interpretation

A run conclusion is positive if at least one of the conditions below is met:

RFI of CD86 is ≥ 150 at any concentration leading to ≥ 50% viability,
RFI of CD54 is ≥ 200 at any concentration leading to ≥ 50% viability.

In other circumstances, the run is considered as negative.

Prediction model

Based on the individual run conclusions, a final prediction is made as follows (see Figure 12):

if the first two runs are both positive for CD86 and/or are both positive for CD54, the h-CLAT prediction is considered POSITIVE and a third run does not need to be conducted,

if the first two runs are negative for both markers, the h-CLAT prediction is considered NEGATIVE (with due consideration of the highest-tested dose conditions) without the need for a third run,

if however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be based on the majority result of the three individual runs (*i.e.* 2 out of 3). In this respect, it should be noted that if two independent runs are conducted and one is only positive for CD86 (hereinafter referred to as P_1) and the other is only positive for CD54 (hereinafter referred to as P_2), a third run is required. If this third run is negative for both markers (hereinafter referred to as N), the h-CLAT prediction is considered NEGATIVE. On the other hand, if the third run is positive for either marker (P_1 or P_2) or for both markers (hereinafter referred to as P_{12}), the h-CLAT prediction is considered POSITIVE.

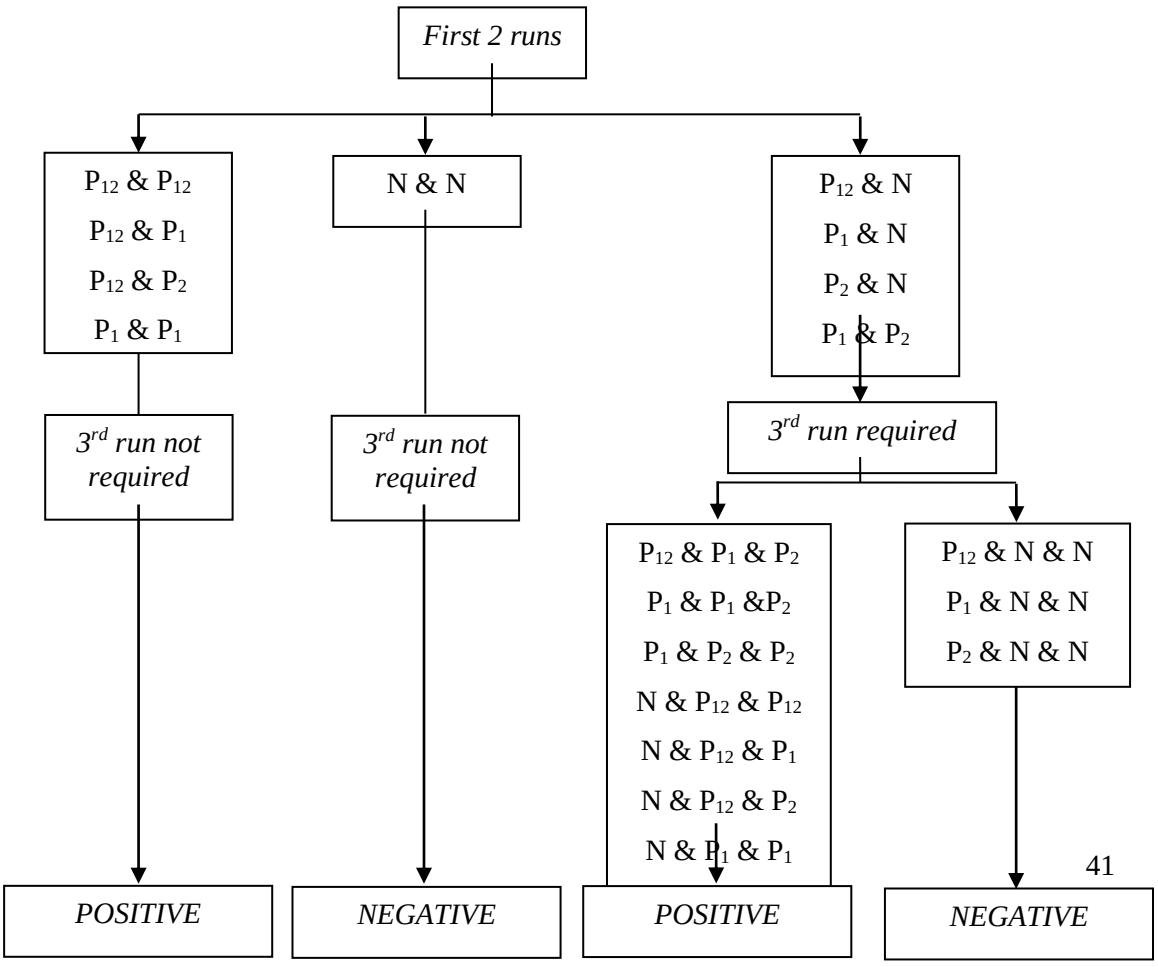


Figure 12: Prediction model used in the h-CLAT test method. An h-CLAT prediction should be considered in the framework of an IATA, considering the AOP on skin sensitization published by OECD, as well as the applicability domain of the h-CLAT method. P_1: run with only CD86 positive; P_2: run with only CD54 positive; P_{12}: run with both CD86 and CD54 positive; N: run with neither CD86 nor CD54 positive. The boxes show the relevant combinations of results from the two/three successive runs, but do not reflect the order in which they may be obtained.

Classification

Results from the present study can be used to support the discrimination between skin sensitizers (i.e. UN GHS Category 1) and non-sensitizers in the context of Integrated Approaches to Testing and Assessment (IATA). However, results obtained at completion of the study are not usable on their own, neither to sub-categorize skin sensitizers into sub-categories 1A and 1B as defined by UN GHS, for authorities implementing these two optional sub-categories, nor to predict potency for safety assessment decisions.

RAW DATA

Raw data specific to the study are retained in the study files. These study files contain all data related to the solubility of the test item, its formulation preparations and results analysis. Data not specific to the study and data including but not limited to, cell plating, cell treatment with test item, vehicle, negative and positive controls, cell staining, flow cytometry data from positive, vehicle and negative controls were recorded in the laboratory files.

ARCHIVING

The following study materials are retained in the archives of the laboratory for 10 years after the signature of the study report by the Study Director:

study plan,
raw data,
a sample of the test item,
correspondence,
final report and any amendments.

The total duration of archiving (depending on regulations) is the responsibility of the Sponsor.

In addition, data not specific to the study are also archived at the laboratory for a period specified in internal procedures.

Deviation

on 08 February 2018, the density of the cells maintained for use in the main test exceeded the limit of 1 x 106 cells/mL (i.e. 1.06 x 106 cells/mL). In view of the very limited deviation to the cells density limit mentioned in the study plan, and considering that main test runs were validated based on acceptable results obtained with both positive controls, the reactivity of the cells was demonstrated and this deviation is considered not to have compromised the validity of the study.

RESULTS

SOLUBILITY ASSESSMENT

Results obtained from the solubility assay are summarized in the table below:

Vehicle	Concentration (mg/mL)	Aspect	Retained vehicle and maximum stock concentration?
0.9% NaCl	100	Powder not soluble	No
DMSO	500	Not soluble (*i.e*. suspension not harvestable using a micropipette, due to a large amount of non-solubilized powder)	No
DMSO	250	Homogeneous yellow suspension	Yes

Therefore, DMSO was the selected vehicle, and the following test item concentrations were tested in the DRF phases: 3.91, 7.81, 15.63, 31.25, 62.50, 125, 250 and 500 µg/mL.

DRF RESULTS (Appendix 1)

Results from each DRF assay are presented in Appendix 1.

The following results were obtained in the first DRF assay (*i.e.* DRF 1):

at post-treatment observation, slight to strong test item precipitate was observed at concentrations ≥ 62.50 µg/mL,

flow cytometry measurement after Propidium Iodide staining revealed no cell viability decrease below 75% at any tested concentration. Therefore, no CV75 value was calculated.

The following results were obtained in the second DRF assay (*i.e.* DRF 2):

at post-treatment observation, slight to strong test item precipitate was observed from the lowest concentration of 3.91 µg/mL,

flow cytometry measurement after Propidium Iodide staining revealed no cell viability decrease below 75% at any tested concentration. Therefore, no CV75 value was calculated.

Based on the results from both DRF assays, no mean CV75 was calculated, and the maximum concentration tested in the main test was therefore 500 µg/mL.

MAIN TEST: individual run results (table 7)

Results from each run are presented in table 7.

All acceptance criteria were reached in each run except for the Run B, where the cell viability of the positive control $NiSO_4$ was < 50% (i.e. 45.3%). Therefore, this run was invalidated.

Run A:

Strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 µg/mL,

RFI CD86 and RFI CD54 did not exceed the positivity thresholds at any tested concentration.
The run A was therefore considered negative.

Run C:

Moderate to strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 µg/mL,
RFI CD86 did not exceed the positivity thresholds at any tested concentration. RFI CD54 exceeded the positivity threshold from 139.54 µg/mL to 241.13 µg/mL.
The run C was therefore considered positive for RFI CD54.

Run D:

Moderate to strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 µg/mL,
RFI CD86 did not exceed the positivity thresholds at any tested concentration. RFI CD54 reached or exceeded the positivity threshold at the concentrations of 167.45; 241.13; 289.35; 347.22 and 500.00 µg/mL (*i.e.* 210; 200; 214; 200 and 241, respectively).
The run D was therefore considered positive for RFI CD54.

MAIN TEST: summary results in table 7

Study No. 45853 TIH

Test item Name	Conc. (µg/mL)	RFI for CD86				RFI for CD54				Viability (%)				Run conclusion				General conclusion
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
ACETOPHENONE AZINE	139.5	87	89	104	98	144	154	**350**	193	94.5	91.6	94.3	94.9	N	I	P2	P2	**Positive**
	167.5	89	74	87	98	193	**206**	**258**	**210**	92.3	89.9	95.0	94.7					
	200.9	81	81	86	99	179	181	**356**	169	92.6	91.4	95.2	94.9					
	241.1	92	78	116	97	103	154	**219**	**200**	92.5	91.2	93.8	94.2					
	289.4	86	88	104	90	149	**215**	156	**214**	93.3	90.9	94.4	93.7					
	347.2	83	69	82	88	184	152	175	**200**	91.2	89.7	94.4	92.4					
	416.7	42	73	96	108	154	156	161	190	92.8	91.2	93.6	94.9					
	500.0	75	79	82	89	120	**254**	144	**241**	90.9	78.8	95.1	99.1					

N = run with negative outcome
P_1 = run with positive outcomefor CD86
P_2 = run with positive outcome for CD54
P_{12} = run with positive outcome for CD86 and CD54
I = Invalidated run
Inc = Inconclusive run
Conc. = concentration
RFI = Relative Fluorescence Index
I = Invalidated run

Discussion

The Log K_{ow} value of the test item is slightly > 3.5 (*i.e.* 3.7). However, this slightly high Log K_{ow} value is not considered to be a limitation for the applicability of this test since the positive outcome obtained in two validated runs guaranted the test system exposure to the test item.

CONCLUSION

Under the experimental conditions of this study, the test item, Acetophenone azine, was found to be positive in the h-CLAT.

2.1.2.3 [Anonymous 2018c]

Study reference:

Anonymous 2018c. Acetophenone Azine: Skin Sensitization Test (Local Lymph Node Assay). Date: 24 August 2018.

Test type: Local Lymph Node Assay, OECD 429. GLP

Principle:
The basic principle of LLNA test is that a sensitizer induces a proliferation of the lymphocytes in the lymph nodes draining the site of test substance application in mice. A radioactive marker incorporated into the DNA of dividing lymphocytes is used to measure cell proliferation. This proliferation is proportional to the dose applied and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization..
The method follows OECD guideline 429. Testing will be conducted in accordance with this guideline.

A dilution of the test substance is recommended in appropriate vehicle before being applied to the ear of the mice. Individual data should be provided.

The mouse is the species of choice for this test. A minimum of 4 animals per dose group with a minimum of three concentrations of the test substance are used plus a negative control receiving only the vehicle and a positive control. A pre-validation is carried out. In the absence of information to determine the highest dose to be tested, a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA.

Preliminary summary test

Test substance

Formulation
The solubility of the test items was examined in a short Preliminary Compatibility Test. The following standard OECD vehicles were assessed: Acetone: Olive oil 4:1 (v/v) mixture, *N*,*N*-dimethylformamide (abbreviated as DMF), Methyl ethyl ketone, Propylene glycol, Dimethyl sulfoxide, and 1% aqueous Pluronic® PE9200 solution. The best vehicle taking into account the test item characteristics and the requirements of the relevant OECD guideline was considered to be DMF. The 5% (w/v, i.e. 0.05 g per ml with added vehicle) dilution was the highest concentration which was suitable for the test. All the formulations used in the Preliminary Irritation / Toxicity Test appeared to be solutions by visual examination.

The data of the chemicals (at least the supplier, batch number, expiry date, storage conditions) used for formulation of the vehicle will be documented in the Final Report.

Test animals

- *Species and strain:* CBA/CaOlaHsd mice
- *Source:* Envigo , San Pietro al Natisone (UD), Zona Industriale Azzida, 57, 33049 Italy
- *Hygienic level:* SPF at arrival; standard housing conditions during the study
- *Justification of strain:* On the basis of OECD Guideline, mice of CBA/Ca or CBA/J strain can be used. Females are used because the existing database is predominantly based on females.
- *Number of animals:* Main Assay: 4 animals / dose group
- Preliminary Test: 1 animal / dose group

- *Sex:* Female, nulliparous, non-pregnant
- Age of animals at starting: 9 weeks old (age-matched, within one week)
- Body weight range at starting: 17.4–17.6grams
  (The weight variation in animals in the study did not exceed ± 20 % of the mean weight.)
- *Acclimatisation time:* 28 days

Administration/exposure

Dose Selection (Preliminary Irritation / Toxicity Test)

The Preliminary Irritation/Toxicity Test was started according to the Study Plan on CBA/CaOlaHsd mice using four doses (1 animal/dose) with the concentrations of 5, 0.5, 0.05 and 0.005% (w/v) in DMF. The preliminary experiment was conducted in a similar experimental manner to the main study, but it was terminated on Day 6 and the radioactive proliferation assay was not performed.

Clinical observations

During the Preliminary Irritation / Toxicity Test no mortality or clinical signs were observed. Clinical observations are summarized in Table 8.

No body weight loss was observed in any animals during the observation period. Individual body weight values are summarized in Table 9.

Measurements of the ears

Ear thickness of the animals was measured by using a thickness gauge on Days 1, 3 and 6, and by ear punch weight determination after the euthanasia of the experimental animals on Day 6. The ear thickness values and ear punch weights were within the acceptable range. The ear thickness values and the weights of the ear punches (2 per animal) are summarized in Table 10.

Lymph nodes

The draining auricular lymph nodes of the animals were visually examined: they were considered normal for all animals (subjective judgement by analogy with observations of former experiments).

Based on these results, 5% (w/v) dose is selected as top dose for the main test.

No ear thickness measurements will be taken in the main test.
The summarized Clinical Observations are indicated in table 11.

Table 8 : Summary of Preliminary Study Data

Acetophenone azine						
Prelim Concentrations	**Physical Formulation**	**Clinical Observations**	**Body Weigh t**	**Erythema**	**Ear Thickness**	**Ear Biopsy weight**
100%(w/v)	U	-	-	-	-	-
50%(w/v)	U	-	-	-	-	-
25%(w/v)	U	-	-	-	-	-
10%(w/v)	U	-	-	-	-	-

5%(w/v)	A	A	A	A	A	A

Notes: U=Unacceptable; A=Acceptable; E=Equivocal; NM=Not measured

Treatments in the main assay will be performed as follows:

Groups	Test item concentration	No. of animals
Negative (vehicle) control (DMF)	-	4
Acetophenone azine 5% (w/v) in DMF	5% (w/v)	4
Acetophenone azine 2.5% (w/v) in DMF	2.5% (w/v)	4
Acetophenone azine 1% (w/v) in DMF	1% (w/v)	4
Positive control (25 % HCA in DMF)	-	4

Note: To minimise animal use, the positive controls may be part of a concurrent study (performed in the same experimental period) using the same vehicle and same batch of animals.

Results and discussion

RESULTS OF THE PRELIMINARY IRRITATION / TOXICITY TEST

Table 9**:** Individual Body Weights for all Animals with Group Means

Animal Number	Identity Number	Test Group Name	Initial Body Weight (g)	Terminal Body Weight* (g)	Change# (%)
6671	1	5% (w/v)	17.6	17.7	0.6
6672	2	0.5% (w/v)	17.4	17.9	2.9
6670	3	0.05% (w/v)	17.4	17.6	1.1
6673	4	0.005% (w/v)	17.6	19.2	9.1

Notes:
1. *: Terminal body weights were measured on Day 6.
2. #: = (Terminal Body Weight – Initial Body Weight) x 100 / Initial Body Weight

Table 10: Individual Ear Thickness for all Animals

Animal Number	Identity Number	Test Group Name	Ear Thickness on Day 1 (mm)		Ear Thickness on Day 3 (mm)		Ear Thickness on Day 6 (mm)		Biopsy weight* on Day 6 (mg)
			Right	Left	Right	Left	Right	Left	

6671	1	5%	0.22	0.22	0.25	0.25	0.22	0.22	12.60
6672	2	0.5%	0.22	0.22	0.23	0.23	0.23	0.22	14.16
6670	3	0.05%	0.22	0.21	0.23	0.23	0.22	0.22	13.26
6673	4	0.005%	0.22	0.23	0.24	0.24	0.23	0.23	13.61

Note:
1. *: Historical control range: 12.50-21.30 mg. Positive response is over 26.63 mg (≥25%).

Table 11: Summarized Clinical Observations

Period	Group	Identity No.	Animal No.	Clinical observations
DAY 1	5%	1	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5%	2	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05%	3	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005%	4	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 2	5%	1	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5%	2	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05%	3	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005%	4	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 3	5%	1	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5%	2	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05%	3	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005%	4	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 4	5%	1	6671	Symptom-free, ES: 0
	0.5%	2	6672	Symptom-free, ES: 0
	0.05%	3	6670	Symptom-free, ES: 0
	0.005%	4	6673	Symptom-free, ES: 0
DAY 5	5%	1	6671	Symptom-free, ES: 0
	0.5%	2	6672	Symptom-free, ES: 0
	0.05%	3	6670	Symptom-free, ES: 0
	0.005%	4	6673	Symptom-free, ES: 0

DAY 6	5%	1	6671	Symptom-free, ES: 0
	0.5%	2	6672	Symptom-free, ES: 0
	0.05%	3	6670	Symptom-free, ES: 0
	0.005%	4	6673	Symptom-free, ES: 0

Notes:

1. The clinical observation of animals on the first day was performed simultaneously with the body weight measurements.
2. ES: Erythema score

Main LLNA study

Detailed study summary and results:

The object of this study was to determine the skin sensitisation potential of Acetophenone azine following dermal exposure in mice. The study was being performed with vertebrate animals as the applied regulatory *in vitro* alternative tests indicated a positive result, but did not allow full regulatory classification. Therefore, an *in vivo* study was being run to provide reliable information about the skin sensitisation potential of the test item for regulatory acceptance.

Before the start of this *in vivo* study, the Sponsor confirmed that existing data was not sufficient for the labelling or for the specific regulatory purpose for Skin Sensitisation.

Based on the results of the Preliminary Compatibility Test, the test item characteristics and on the recommendations of the OECD Guideline, the best vehicle for the test item was *N*,*N*-dimethylformamide (abbreviated as DMF). The 5% (w/v) formulation was the highest concentration suitable for the test. The 5% (w/v) formulation and all the diluted formulations appeared to be solutions by visual examination.

A Preliminary Irritation/Toxicity Test was performed in CBA/CaOlaHsd mice using four doses (1 animal/dose): 5, 0.5, 0.05 and 0.005% (w/v) in DMF and based on the results, 5% (w/v) was selected as top dose for the Main Assay.

In the Main Assay, twenty female CBA/CaOlaHsd mice were allocated to five groups, each group comprised four animals:

- groups (three) of animals received Acetophenone azine (formulated in DMF) at either 5, 2.5 or 1% (w/v),
- a negative control group received the vehicle (DMF) only,
- a positive control group received 25 % (w/v) HCA (dissolved in DMF).

The test item solutions were applied to the dorsal surface of the ears of the experimental animals (25 µL/ear) for three consecutive days (Days 1, 2 and 3) and then maintained on study for an additional 3 days. Cell proliferation in the (local) lymph nodes was assessed by measuring disintegrations per minutes after the incorporation of tritiated methyl thymidine (^{3}HTdR) into the lymph nodes and the values obtained were used to calculate stimulation indices (SI) in comparison with the control group.

There was no mortality or signs of systemic toxicity observed during the study. No test item residue was noted on the ears of the animals in any groups. No marked body weight losses (≥5%) were observed in any groups.

The SI values were 0.7, 0.4 and 0.5 at concentrations of 5, 2.5 and 1% (w/v), respectively. The SI value for the positive control substance α-Hexylcinnamaldehyde (HCA) dissolved in the same vehicle was 3.7 therefore demonstrating the appropriate performance of the assay. The

lymphoproliferative response of the HCA was in line with historical control data for the positive control, therefore confirming the validity of the assay.

In conclusion, under the conditions of the present assay, Acetophenone azine, tested in *N,N*-dimethylformamide, did not show any sensitisation potential (non-sensitizer) in the Local Lymph Node Assay.

No classification/labelling is triggered according to Regulation (EC) No 1272/2008 (CLP) / GHS (rev. 7) 2017.

Main LLNA study:

***Test type* :**
Local Lymph Node Assay, OECD Guidelines for Testing of Chemicals No. 429, Skin Sensitisation: Local Lymph Node Assay (22 July 2010); Commission Regulation (EC) No. 440/2008 of 30 May 2008, B.42., Skin Sensitisation: Local Lymph Node Assay (Official Journal L142, 31/05/2008) amended by Commission Regulation (EU) No 640/2012 of 6 July 2012 and the Principles of Good Laboratory Practice (Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17).

The basic principle underlying the Local Lymph Node Assay (LLNA) is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application.

Generally, under appropriate test conditions, this proliferation is proportional to the concentration applied, and provides a means of obtaining an objective, quantitative measurement of sensitisation potential. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in the vehicle treated control. The ratio of the proliferation in test groups to that in the control, termed Stimulation Index (SI), is determined and must be at least equal or greater than three, for a test substance to be classified as a skin sensitizer

The purpose of this study was to determine the skin sensitisation potential of Acetophenone azine following dermal exposure in the Local Lymph Node Assay.

Test substance

- *Name:* Acetophenone azine
- *Batch Number:* Confidential
- *CAS number:* 729-43-1
- *Description:* Yellow powder
- *Purity:* Confidential
- *Storage conditions:* Room temperature (15-25 °C, ≤ 70 RH%)
- *Safety precautions:* Enhanced safety precautions (nitrile gloves) were applied considering the supplied safety datasheet to assure personnel health and safety.

Identification, Receipt

The test item of a suitable chemical purity was provided by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor and will be archived with

the raw data. Identification of the test item was performed on the basis of information provided by the Sponsor.

Formulation

The solubility of the test item was examined in a short Preliminary Compatibility Test. The following standard OECD vehicles were assessed: Acetone: Olive oil 4:1 (v/v) mixture, *N*,*N*-dimethylformamide, Methyl ethyl ketone, Propylene glycol, Dimethyl sulfoxide and 1% aqueous Pluronic® PE9200. The best vehicle taking into account the test item characteristics and the requirements of the relevant OECD guideline was considered to be DMF. The highest achievable concentration based on the regulatory requirements of the OECD guideline and the physical characteristics of the test item was 5 % (w/v). The 5% (w/v) formulation in DMF and all diluted formulations appeared to be solutions by visual examination.

The test item was weighed and formulations prepared daily on a weight:volume basis (as % (w/v)).

Analytical determination of the test item concentration, stability and homogeneity was not performed because of the character and the short period of study.

Test animals

- *Species and strain:* CBA/CaOlaHsd mice
- *Source:* Envigo , San Pietro al Natisone (UD), Zona Industriale Azzida, 57, 33049 Italy
- *Hygienic level:* SPF at arrival; standard housing conditions during the study
- *Justification of strain:* On the basis of OECD Guideline, mice of CBA/Ca or CBA/J strain can be used. Females are used because the existing database is predominantly based on females.
- *Number of animals:* Main Assay: 4 animals / dose group
  Preliminary Test: 1 animal / dose group
- *Sex:* Female, nulliparous, non-pregnant
- Age of animals at starting: 9 weeks old (age-matched, within one week)
- Body weight range at starting: 19.1–21.7grams
  (The weight variation in animals in the study did not exceed ± 20 % of the mean weight.)
- *Acclimatisation time:* 28 days

Husbandry

Animal health:	Only healthy animals were used for the study. Health status was certified by the veterinarian.
Housing:	Group caging (Main Assay) Individual caging (Preliminary Test)
Enrichment:	Mice were provided with glass tunnel-tubes
Cage type:	Type II. polypropylene / polycarbonate
Bedding / Nesting:	Bedding and certified nest building material was available to animals during the study
Light:	12 hours daily, from 6.00 a.m. to 6.00 p.m.
Temperature:	17.0 – 24.9°C
Relative humidity:	31 - 80 %
Ventilation:	15-20 air exchanges/hour

The temperature and relative humidity were recorded twice every day during the acclimatisation and experimental phases.

Food and feeding

Animals received ssniff® SM Rat/Mouse – Breeding and Maintenance, 15 mm, autoclavable "Complete feed for Rats and Mice – Breeding and Maintenance" (Batch numbers: 883 29966 and 840 33675, Expiry dates: 31 October 2018 and 31 January 2018, respectively; produced by ssniff Spezialdiäten GmbH (Ferdinand-Gabriel-Weg 16, D-59494 Soest, Germany), and Gel diet Transport (Batch Numbers: 60181770010101 and 60181080030101, Expiry dates: 26 December 2018 and 19 April 2019, respectively; produced by Scientific Animal Food & Engineering, Route de Saint Bris, 89290 Augy, France) *ad libitum*. The food was considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study.

Water supply

Animals received tap water from the municipal supply from 500 mL bottles, *ad libitum*. Water quality control analysis was performed at least once every three months and microbiological assessment was performed monthly by Veszprém County Institute of State Public Health and Medical Officer Service (ÁNTSZ, H-8201 Veszprém, József Attila u. 36., Hungary). Copies of the relevant Certificates of Analysis are retained in the Archive.

Bedding and nesting

Bedding of certified wood chips especially designed to keep animals in the best natural environment was provided for animals during the study. Lignocel® 3/4-S Hygienic Animal Bedding produced by J. Rettenmaier & Söhne GmbH + Co.KG (D-73494 Rosenberg, Germany) was available to animals during the study. Certified nest building material was also provided for animals (ARBOCEL crinklets natural produced by J. Rettenmaier & Söhne GmbH + Co.KG).

Identification and randomisation

A unique number written on the tail with a permanent marker identified each animal. The animal number was assigned on the basis of the laboratory Master File. The cages were marked with identity cards with information including study code, cage number, and dose group, sex and individual animal number. The animals were randomised and allocated to the experimental groups. The randomisation was checked by computer software using the body weight to verify the homogeneity and variability between the groups.

Administration/exposure

Control group and treatment

Negative Control

Animals assigned to the negative control group were treated with the vehicle only concurrent to the test item treated groups. Based on the result of the Preliminary Compatibility Test, DMF was selected for vehicle of the study. Data of the chemical used for vehicle in the study are listed below:

Name:	*N*,*N*-dimethylformamide
Synonym:	DMF
Batch No.:	Confidential
Manufacturer:	VWR
Storage condition:	Room temperature

Positive Control

Animals assigned to the positive control group were treated with 25 % (w/v) α-Hexylcinnamaldehyde solution (dissolved in DMF) concurrent to the test item treated groups. The relevant data of the positive control substance are listed below:

Name:	α-Hexylcinnamaldehyde
Synonym:	α-Hexyl cinnamic aldehyde
Abbreviation:	HCA
CAS No.:	101-86-0
Batch No. :	Confidential
Manufacturer:	Sigma-Aldrich Co.
Appearance:	Faint yellow liquid
Nominal purity:	Confidential
Purity:	Confidential
Storage condition:	Roomtemperature

Other chemicals used in the study

The data of the chemicals used in the study are summarised in Table 12.

Table 12: Chemicals Used in the Experiments

Chemical	Manufacturer / Supplier	Batch Number	Expiry date
Distilled water (Aqua Purificata)	Magilab Kft.	Confidential	11 December 2018
Phosphate buffered saline	Sigma-Aldrich Co.	Confidential	28 February 2019
Trichloroacetic acid (Abbreviation: TCA)	Sigma-Aldrich Co.	Confidential	31 January 2020
[Methyl-3H]-Thymidine	American Radiolabeled Chemicals Inc.	Confidential	-
OptiPhase HiSafe 3	PerkinElmer	152-Confidential	01 May 2019

Instrument system

Name:Tri-Carb 2810 Liquid Scintillation Analyze. Manufacturer PerkinElmer. Serial *Number:* DG10084483. IQ / OQ Protocol #: 1593646-1

ADMINISTRATION OF THE TEST ITEM

Dose Selection and Justification of Dose Selection

The Preliminary Irritation/Toxicity Test was started according to the Study Plan on CBA/CaOlaHsd mice using four doses (1 animal/dose) at test item concentrations of 5, 0.5, 0.05 and 0.005% (w/v) in DMF. The preliminary experiment was conducted in a similar experimental manner to the main study, but it was terminated on Day 6 and the radioactive proliferation assay was not performed.

The maximum concentration of test item in an acceptable solvent was established according to OECD guideline 429. Based on the observation of the solubility test, the maximum available concentration was 5% (w/v).

In the Preliminary Irritation / Toxicity Test, all mice were observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Both ears of each mouse were observed for erythema and scored using Table 13. Ear thickness was also measured using a thickness gauge on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose) and Day 6. Additional quantification of the ear thickness was performed by ear punch weight determination after the euthanasia of the experimental animals.

Table 13: Erythema Scoring

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2

Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Note: Excessive local skin irritation is indicated by an erythema score ≥ 3 and/or an increase in ear thickness of ≥ 25 % on any day of measurement.

During the Preliminary Irritation / Toxicity Test no mortality or clinical signs were observed. No test item residue was noted on the ears of the animals in any groups. Clinical observations are summarised in Table 11 in appendice 3.

No body weight loss was observed in any animals during the observation period (Table 8 in appendice 3).

The ear thickness values and ear punch weights were within the acceptable range (Table 9 in appendice 3).

The draining auricular lymph nodes of the animals were visually examined: they were normal in both dose groups (subjective judgement by analogy with observations of former experiments).

Based on these observations, 5% (w/v) dose was selected as top dose for the Main Assay. The experimental groups and dose levels for the main experiment are summarised in Table 14.

Table 14: Experimental Groups and Treatments

Groups	**Test item concentration** (% w/v)	**No. of animals**
Negative (vehicle) control (DMF)	-	4
Acetophenone azine 5% (w/v) in DMF	5	4
Acetophenone azine 2.5% (w/v) in DMF	2.5	4
Acetophenone azine 1% (w/v) in DMF	1	4
Positive control (25% HCA in DMF)	-	4

Topical application

During the study, animals were topically dosed with 25 µL of the appropriate formulation using a pipette on the dorsal surface of each ear. Each animal was dosed once a day for three consecutive days (Days 1, 2 and 3). There was no treatment on Days 4, 5 and 6.

OBSERVATIONS

Clinical Observations

During the study (Day 1 to Day 6) each animal was observed daily for any clinical signs, including local irritation and systemic toxicity. Clinical observations were performed twice a day (before and after treatments) on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Individual records were maintained.

Measurement of Body Weight

Individual body weights were recorded on Day 1 (beginning of the test) and on Day 6 (prior to ^{3}HTdR injection) with a precision of ± 0.1 g.

PROLIFERATION ASSAY

Injection of Tritiated Thymidine (3HTdR)

On Day 6, animals were taken to the radioactive suite and each mouse was intravenously injected via the tail vein with 250 µL of sterile PBS (phosphate buffered saline) containing approximately 20 µCi of ^{3}HTdR using a gauge 25G x 1" hypodermic needle with 1 mL sterile syringe. Once injected, the mice were left for 5 hours (± 30 minutes).

Removal and Preparation of Draining Auricular Lymph Nodes

Five hours (± 30 minutes) after intravenous injection the mice were euthanized by asphyxiation with ascending doses of carbon dioxide (deep anaesthesia was confirmed before making incision, death was confirmed before discarding carcasses).

The draining auricular lymph nodes were excised by making a small incision on the skin between the jaw and sternum, pulling the skin gently back towards the ears and exposing the lymph nodes. The nodes were then removed using forceps. The carcasses were discarded after cervical dislocation or after cutting through major cervical blood vessels.

Once removed, the nodes of mice from each test group was pooled and collected in separate Petri dishes containing a small amount (1-2 mL) of PBS to keep the nodes wet before processing.

Preparation of Single Cell Suspension of Lymph Node Cells

A single cell suspension (SCS) of pooled lymph node cells (LNCs) was prepared and collected in disposable tubes by gentle mechanical disaggregating of the lymph nodes through a cell strainer using the plunger of a disposable syringe. The cell strainer was washed with PBS (up to 10 mL). Pooled LNCs were pelleted with a relative centrifugal force (RCF) of 190 x g (approximately) for 10 minutes at 4 °C. After centrifugation supernatants were discarded. Pellets were gently resuspended and 10 mL of PBS was added to the tubes. The washing step was repeated twice. This procedure was repeated for each group of pooled lymph nodes.

Determination of Incorporated 3HTdR

After the final washing step, supernatants were removed. Pellets were gently agitated resuspended and 3 mL of 5 % (w/v) TCA solution was added to the tubes for precipitation of macromolecules.

After overnight (approximately 18 hours) incubation at 2-8 °C, precipitates were centrifuged (approximately 190 x g for 10 minutes at 4ºC), and supernatants were removed. Pellets were resuspended in 1 mL of 5 % (w/v) TCA solution and dispersed by using an ultrasonic bath. Samples were transferred into a suitable sized scintillation vial filled with 10 mL of scintillation liquid and thoroughly mixed. The vials were loaded into a β-scintillation counter and ^{3}HTdR incorporation was measured (10-minute measurement).

The β-counter expresses the ^{3}HTdR incorporation as the number of radioactive disintegrations per minute (DPM). Background level was also measured in duplicates by adding 1 mL of 5 % (w/v) TCA solution into a scintillation vial filled with 10 mL of scintillation liquid.

EVALUATION OF THE RESULTS

DPM was measured for each pooled group of nodes. The measured DPM values were corrected with the background DPM value ("DPM"). The average of the two measured DPM values of 5% (w/v) TCA solutions was used as background DPM value. The results were expressed as "DPN" (DPM divided by the number of lymph nodes) following the industry standard for data presentation.

Stimulation index (SI = DPN value of a treated group divided by the DPN value of the negative control group) for each treatment group was also calculated. A stimulation index of 3 or greater is an indication of a positive result.

Interpretation of Results

The test item is regarded as a sensitizer if both of the following criteria are fulfilled:

- That exposure to at least one concentration of the test item resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than recorded in control mice, as indicated by the stimulation index.
- The data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

Acceptability of the test

The Local Lymph Node Assay is considered valid if it meets the following criteria:

- the DPN value of the negative (vehicle) control group falls within the range of historical laboratory control data,
- the positive control substance produces a significant lymphoproliferative response increases (SI>3),
- each treated and control group includes at least 4 animals,
- the test item does not cause serious systemic or local toxicity.

Use of radioactive materials was recorded in the appropriate register. Regular decontamination of the working area with a verification of decontamination was carried out. Radioactive waste materials were processed according to normal laboratory standards.
The conduct of the study was permitted by Institutional Animal Care and Use Committee (IACUC) on 15 August 2018.

Deviations of the study

Due to technical reasons, temperature values (minimum of 17.0°C) outside the expected range of 19-25°C and relative humidity values (maximum of 80%) outside the expected range of 30-70% were recorded occasionally during the study.

These differences of the environmental parameters were considered not to adversely affect the results or integrity of the study as confirmed by the clinical Veterinarian.

Since a strong sensitizing effect was expected, only one animal per dose group was treated with the test item in the Preliminary Irritation / Toxicity Test for animal welfare reasons. Therefore, group caging was not possible and animals were housed individually.

This deviation is considered to have no impact on the outcome of the study and interpretation of the results.

Results and discussion

- *Clinical observation*

No mortality or signs of systemic toxicity were observed during the study. No test item residue was noted on the ears of the animals in any groups.

- *Body weight measurement*

No marked body weight losses (≥5%) were observed in any groups. Individual and mean body weights are given in Table 15.

Table 15: Individual Body Weights for all Animals with Group Means

Animal Number	Test Group Name	Initial Body Weight (g)	Terminal Body Weight* (g)	Change# (%)
6720	Negative (vehicle) control	20.8	19.8	-4.8
6722	in DMF	20.6	21.6	4.9
6730		21.2	22.4	5.7
6711		19.1	19.8	3.7
	Mean	**20.4**	**20.9**	**2.3**
6721	Acetophenone azine	21.4	22.1	3.3
6724	5% (w/v)	20.0	20.8	4.0
6713	in DMF	19.8	19.9	0.5
6729		19.3	19.5	1.0
	Mean	**20.1**	**20.6**	**2.2**
6718	Acetophenone azine	21.0	21.1	0.5
6712	2.5% (w/v)	20.2	21.2	5.0
6723	in DMF	19.8	20.2	2.0
6727		19.6	18.8	-4.1
	Mean	**20.2**	**20.3**	**0.9**
6717	Acetophenone azine	21.0	22.8	8.6
6728	1% (w/v)	20.2	20.2	0.0
6719	in DMF	20.8	22.1	6.3
6714		19.3	19.8	2.6
	Mean	**20.3**	**21.2**	**4.4**
6715	Positive control	21.7	21.6	-0.5
6725	25 % (w/v) HCA	20.5	20.2	-1.5
6726	in DMF	19.4	20.1	3.6
6716		19.4	20.6	6.2
	Mean	**20.3**	**20.6**	**1.9**

Notes:
*: Terminal body weights were measured on Day 6.

#: = (Terminal Body Weight – Initial Body Weight) / Initial Body Weight x 100

- *Proliferation assay*

The results of the proliferation assay are summarised in Table 16 and figure 12. The appearance of the lymph nodes was normal in the negative control group and in the 5, 2.5 and 1% (w/v) test item treated dose groups. Larger than normal lymph nodes were observed in the positive control group.

Table 16: DPM, DPN and Stimulation Index Values for all Groups

Test Group Name	**Measured DPM / group**	**DPM**	**Number of lymph nodes**	**DPN**	**Stimulation Index**
Background (5 % (w/v) TCA)	34	-	-	-	-
Negative control (DMF)	3743	3709.0	8	463.6	1.0
Acetophenone azine 5% (w/v) in DMF	2534	2500.0	8	312.5	0.7
Acetophenone azine 2.5% (w/v) in DMF	1475	1441.0	8	180.1	0.4
Acetophenone azine 1% (w/v) in DMF	2032	1998.0	8	249.8	0.5
Positive control (25 % (w/v) HCA in DMF)	13608	13574.0	8	1696.8	3.7

The stimulation index values were 0.7, 0.4 and 0.5 at concentrations of 5, 2.5 and 1% (w/v), respectively.

- *Interpretation of observations*

The test item was powder, which was formulated in DMF. Since there were no confounding effects of irritation or systemic toxicity at the applied concentrations, the proliferation values obtained are considered to reflect the real potential of the test item to cause lymphoproliferation in the Local Lymph Node Assay. The resulting stimulation indices observed under these exaggerated test conditions was considered to be good evidence that Acetophenone azine is a non-sensitizer in this specific study design (Figure 12). The size of lymph nodes was in good correlation with this conclusion. Based on the observed results, the test item does not need classification according to the GHS or CLP.

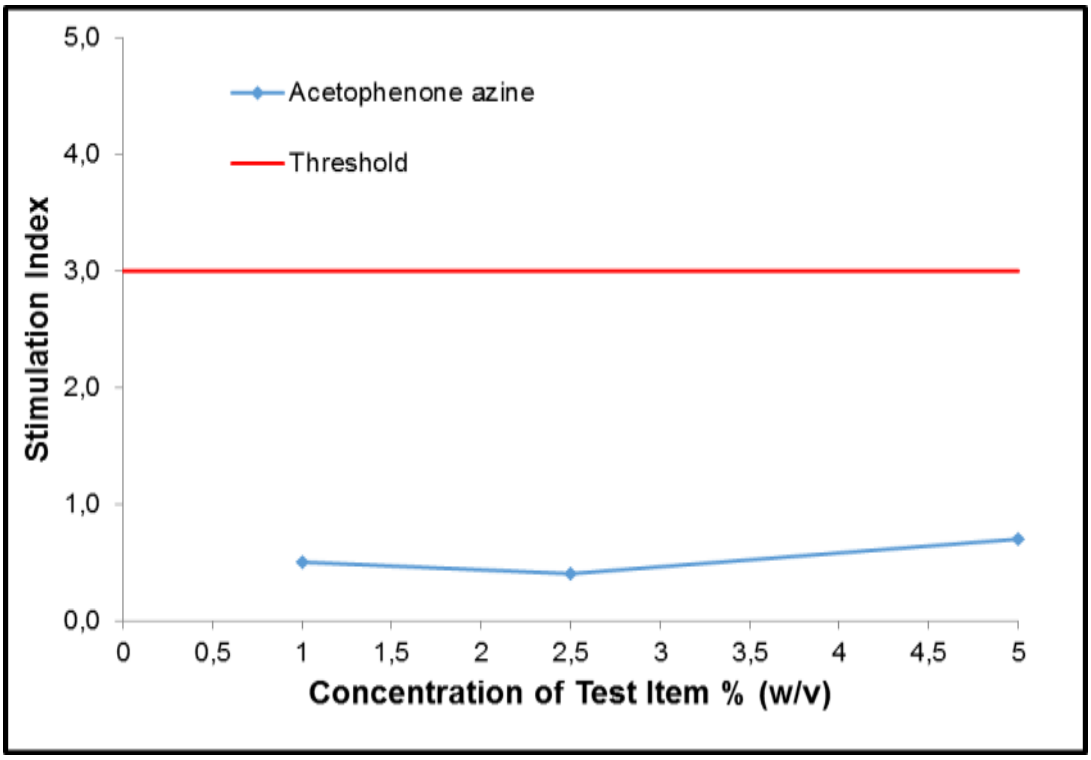


Figure 12: Test Item Stimulation Index Values

- *Reliability of the test*

The result of the positive control substance α-Hexylcinnamaldehyde (HCA) dissolved in the same vehicle was used to demonstrate the appropriate performance of the assay [1]. The positive control substance was examined at a concentration of 25 % (w/v) in the relevant vehicle (DMF) using CBA/CaOlaHsd mice.

No mortality, cutaneous reactions or signs of toxicity were observed for the positive control substance in the study. A lymphoproliferative response in line with historical positive control data (stimulation index value of 3.7) was noted for HCA in the Main Assay. This value was considered to confirm the appropriate performance of the assay.

Furthermore, the DPN values observed for the vehicle and positive control substance in this experiment were in within the historical control range. Each treated and control group included 4 animals.

- *CONCLUSION*

In conclusion, under the conditions of the present assay, Acetophenone azine, tested in *N,N*-dimethylformamide, did not show a sensitisation potential (non-sensitizer) in the Local Lymph Node Assay.

No classification/labelling is triggered according to Regulation (EC) No 1272/2008 (CLP) / GHS (rev. 7) 2017.

APPENDICE 1 : VIABILITY VALUES, INDUCTION VALUES, IMAX, IC30, IC50 AND EC1.5 VALUES OBTAINED AFTER TREATMENT WITH THE TEST ITEM IN EACH RUN AS WELL AS THE MEAN AND SD VALUES

Evaluation of the viability (%) of cultures treated with the test item for each run

Concentrations (µM)

Acetophenone azine	*0.49*	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.3*	*62.5*	*125*	*250*	*500*	*1000*
Viability (%) in Run 1	103	100	102	105	111	121	125	90	55	39	38	35
Viability (%) in Run 2	100	108	107	102	114	108	102	89	77	47	44	49
Mean viability (%)	**101**	**104**	**104**	**103**	**113**	**114**	**113**	**89**	**66**	**43**	**41**	**42**
Geometric Mean (%)	**101**	**104**	**104**	**103**	**113**	**114**	**113**	**89**	**65**	**43**	**41**	**41**
SD	**2**	**6**	**4**	**2**	**2**	**9**	**16**	**0**	**15**	**5**	**4**	**10**

Gene induction values, I_{max}, IC_{30}, IC_{50} and $EC_{1.5}$ values, mean and SD values obtained after treatment with the test item in each run

Concentrations (µM)

Acetophenone azine	*0.49*	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.3*	*62.5*	*125*	*250*	*500*	*1000*
Induction values in Run 1	1.3	1.9	2.1	2.0	2.0	2.0	1.5	1.0	0.6	0.5	0.5	0.5
Induction values in Run 2	1.9	2.8	3.2	3.3	3.0	2.5	1.5	1.0	0.7	0.7	0.7	0.7
Mean induction	**1.6**	**2.3**	**2.7**	**2.6**	**2.5**	**2.2**	**1.5**	**1.0**	**0.7**	**0.6**	**0.6**	**0.6**
SD	**0.4**	**0.6**	**0.8**	**1.0**	**0.7**	**0.4**	**0.0**	**0.0**	**0.1**	**0.1**	**0.1**	**0.1**

Imax and $EC_{1.5}$ results

Acetophenone azine	Imax	$EC_{1.5}$ (µM)	IC_{50} (µM)	IC_{30} (µM)
Run 1	2.14	0.63	163.11	97.68
Run 2	3.31	< 0.49	238.11	152.77
Mean	**2.72**	**n.c.**	**n.r.**	**n.r.**
Geometric Mean	**n.r.**	**n.c.**	**197.07**	**122.16**
SD	**0.83**	**n.c.**	**53.03**	**38.95**

n.c.: not calculated

n.r.: not requested by the OECD Guideline

APPENDICE 2 : VIABILITY (%), INDUCTION VALUES, IMAX, IC30, IC50 AND EC1.5 OBTAINED WITH THE POSITIVE CONTROL AS WELL AS LUMINESCENCE VALUES OF NEGATIVE CONTROL WELLS AND THE %CV BETWEEN THESE VALUES FOR EACH RUN

Evaluation of the viability (%) of cultures treated with the positive control for each run

Concentrations (µM)

cinnamic aldehyde	*4*	*8*	*16*	*32*	*64*
Viability (%) in Run 1	105	113	115	126	120
Viability (%) in Run 2	104	110	113	120	126
Mean viability (%)	**105**	**112**	**114**	**123**	**123**
Geometric Mean (%)	**105**	**112**	**114**	**123**	**123**
SD	**1**	**2**	**2**	**4**	**4**

Gene induction values, I_{max}, IC_{30}, IC_{50} and $EC_{1.5}$ values obtained with the positive control for each run

Concentrations (µM)

cinnamic aldehyde	*4*	*8*	*16*	*32*	*64*	**Imax**	**$EC_{1.5}$ (µM)**	**IC_{50} (µM)**	**IC_{30} (µM)**
Run 1	1.2	1.4	1.7	2.4	8.0	7.98	10.92	-	-
Run 2	1.2	1.2	1.5	2.2	4.8	4.77	15.97	-	-
Mean	**1.2**	**1.3**	**1.6**	**2.3**	**6.4**	**6.37**	**n.r.**	**n.r.**	**n.r.**
Geometric Mean	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**13.21**	**-**	**-**
SD	**0.0**	**0.1**	**0.2**	**0.2**	**2.3**	**2.27**	**3.57**	**-**	**-**

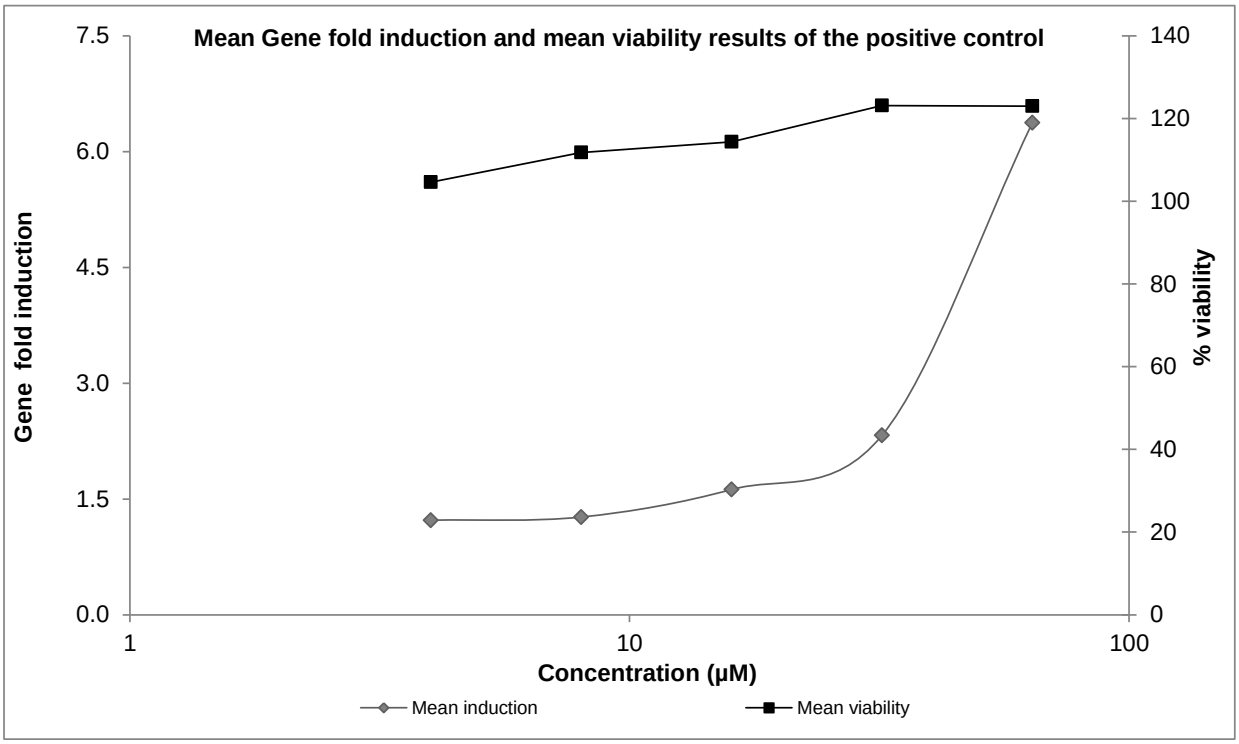
- : no data available

n.r.: not requested by the OECD Guideline

Luminescence values for the negative control wells and the %CV between these values for each run

Negative control	**Luminescence reading**							**Mean**	**% CV**
Run 1	Replicate 1	580854	748665	808683	700497	689826	668489	671367	19.39
	Replicate 2	447463	539466	572362	526764	530916	507480		
	Replicate 3	761509	781486	899105	788003	816934	716095		
Run 2	Replicate 1	296099	443522	415290	349674	337270	414803	341332	14.48
	Replicate 2	313322	325023	351939	330480	357768	371592		
	Replicate 3	249926	313081	290699	286485	328644	368362		

Graphical representation of the mean gene-induction and mean viability dose-response curves for cells treated with positive control in both validated runs



APPENDICE 3 : RESULTS OF THE PRELIMINARY IRRITATION / TOXICITY TEST

Table 8: Individual Body Weights for all Animals (Preliminary Irritation/Toxicity Test)

Animal Number	Test Group Name	Initial Body Weight (g)	Terminal Body Weight* (g)	Change# (%)
6671	5% (w/v)	17.6	17.7	0.6
6672	0.5% (w/v)	17.4	17.9	2.9
6670	0.05% (w/v)	17.4	17.6	1.1
6673	0.005% (w/v)	17.6	19.2	9.1

Notes:
2. *: Terminal body weights were measured on Day 6.
2. #: = (Terminal Body Weight – Initial Body Weight) / Initial Body Weight x 100

Table 9: Individual Ear Thickness for all Animals (Preliminary Irritation/Toxicity Test)

Animal Number	Test Group Name	Ear Thickness on Day 1 (mm)		Ear Thickness on Day 3 (mm)		Ear Thickness on Day 6 (mm)		Biopsy weight* on Day 6 (mg)
		Right	Left	Right	Left	Right	Left	
6671	5% (w/v)	0.22	0.22	0.25	0.25	0.22	0.22	12.60
6672	0.5% (w/v)	0.22	0.22	0.23	0.23	0.23	0.22	14.16
6670	0.05% (w/v)	0.22	0.21	0.23	0.23	0.22	0.22	13.26
6673	0.005% (w/v)	0.22	0.23	0.24	0.24	0.23	0.23	13.61

Note:
1. *: Historical control range: 12.50-21.30 mg. Positive response is over 26.63 mg (≥25%).

Table 10: Summarised Clinical Observations (Preliminary Irritation/Toxicity Test)

Period	Test Group Name	Animal No.	Clinical observations
DAY 1	5% (w/v)	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5% (w/v)	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05% (w/v)	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005% (w/v)	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 2	5% (w/v)	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5% (w/v)	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05% (w/v)	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005% (w/v)	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 3	5% (w/v)	6671	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.5% (w/v)	6672	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.05% (w/v)	6670	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
	0.005% (w/v)	6673	Before treatment: symptom-free, ES: 0 After treatment: symptom-free, ES: 0
DAY 4	5% (w/v)	6671	Symptom-free, ES: 0
	0.5% (w/v)	6672	Symptom-free, ES: 0
	0.05% (w/v)	6670	Symptom-free, ES: 0
	0.005% (w/v)	6673	Symptom-free, ES: 0
DAY 5	5% (w/v)	6671	Symptom-free, ES: 0
	0.5% (w/v)	6672	Symptom-free, ES: 0
	0.05% (w/v)	6670	Symptom-free, ES: 0
	0.005% (w/v)	6673	Symptom-free, ES: 0
DAY 6	5% (w/v)	6671	Symptom-free, ES: 0
	0.5% (w/v)	6672	Symptom-free, ES: 0
	0.05% (w/v)	6670	Symptom-free, ES: 0
	0.005% (w/v)	6673	Symptom-free, ES: 0

Notes:

1. The clinical observation of animals on the first day was performed simultaneously with the body weight measurements.
2. ES: Erythema score (for details see Table 2)

APPENDICE 4 : SUMMARISED CLINICAL OBSERVATIONS

Table 11: Summarised Clinical Observations

Group	Animal No.	CLINICAL OBSERVATIONS					
		DAY 1	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**	**DAY 6**
Negative control (DMF)	6720	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6722	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6730	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6711	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
Acetophenone azine 5% (w/v) in DMF	6721	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6724	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6713	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6729	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
Acetophenone azine 2.5% (w/v) in DMF	6718	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6712	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6723	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6727	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free

Note:

1. BT: before treatment, AT: after treatment

Table 12: Summarised Clinical Observations (continued)

Group	Animal No.	CLINICAL OBSERVATIONS					
		DAY 1	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**	**DAY 6**
Acetophenone azine 1% (w/v) in DMF	6717	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6728	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6719	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6714	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
Positive control (25 % (w/v) HCA in DMF)	6715	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6725	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6726	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free
	6716	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	BT: symptom-free AT: symptom-free	Symptom-free	Symptom-free	Symptom-free

Note:

1. BT: before treatment, AT: after treatment

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