Section A6.4 Annex Point IIA VI.6.4		**SUBCHRONIC TOXICITY**	
Section A6.4.3 Annex Point IIA VI.6.4		**Subchronic toxicity inhalation (14 weeks)**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Monsanto co: Three-month inhalation toxicity of acetone cyanohydrin in male and female Sprague-Dawley rats. Report 1984 **(DOC IV_46).**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	/	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No	
2.2	**GLP**	Yes	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Acetone cyanohydrin	
3.1.1	Lot/Batch number	T501-3	
3.1.2	Specification		
3.1.2.1	**Description**	Light amber liquid; concentration of saturated vapours at 20 °C = 3660 mg/m^3	
3.1.2.2	**Purity**	98.5 %	
3.1.2.3	**Stability**	No significant decomposition during the course of the study	
3.2	**Test Animals**		
3.2.1	Species	Albino rats	
3.2.2	Strain	Sprague-Dawley	
3.2.3	Source	Charles River	
3.2.4	Sex	Male (M) and female (F)	
3.2.5	Age/weight at study initiation	M 49 days, 215-235 g F 54 days, 170-189g	
3.2.6	Number of animals per group	15 rats per treatment and sex group (total of 60M and 60F)	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Inhalation	
3.3.1	Duration of treatment	14 weeks (at least 69 exposures)	

3.3.2	Frequency of exposure	6 hr/day, 5 days/week.		
3.3.3	Post exposure period	No (all animals were necropsied next day after the last exposure)		
3.3.4	**Inhalation**			
3.3.4.1	**Concentrations**	Nominal concentrations	0 ppm;10 ppm; 30 ppm ; 60 ppm	
		Analytical concentrations	/ ; 10.1 ± 0.91 ; 28.6 ± 1.8 ; 57.7 ± 2.9 ppm	
3.3.4.2	**Particle size**	/		
3.3.4.3	**Type or preparation of particles**	/		
3.3.4.4	**Type of exposure**	Whole body; rats were exposed in their individual wire mesh cages.		
3.3.4.5	**Vehicle**	Air		
3.3.4.6	**Concentration in vehicle**	See 3.3.4.1		
3.3.4.7	**Duration of exposure**	6hr/day		
3.3.4.8	**Controls**	Control rats remained in their individual wire mesh cages.		
3.4	**Examinations**	Clinical observations, Haematology, Clinical Chemistry, Pathology (gross necropsy and microscopic examination)		
3.4.1	**Observations**			
3.4.1.1	**Clinical signs**	Daily, before exposure, between the second and fifth hour of each exposure, and immediately after exposure percentage estimates of animals exhibiting any gross signs of toxicity were recorded. Thorough examination: weekly.		
3.4.1.2	**Behavioural testing**	No		
3.4.1.3	**Mortality**	Yes/no		
3.4.2	**Body weight**	Prior to the start of the study, prior to sacrifice, and weekly during exposures.		
3.4.3	Food consumption	No		
3.4.4	Water consumption	No		
3.4.5	Ophthalmoscopic examination	No		
3.4.6	**Haematology**	Yes, blood sampling at the time of necropsy. Parameters: Red Blood Cell Count, White Blood Cell Count (WBC), haematocrit, haemoglobin concentration., mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, reticulocyte count, differential WBC.		
3.4.7	**Clinical Chemistry**	Yes, blood sampling at the time of necropsy. Parameters: urea nitrogen, glucose, total protein, globulin, albumin, total bilirubin; alkaline phosphatase, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, lactic dehydrogenase; thiocyanate, T3 and T4; serum protein electrophoresis.		

3.4.8	**Urinalysis**	Yes, urine collection overnight prior to necropsy day. Parameters: thiocyanate, urinary volume.	
3.5	**Pathology**		
3.5.1	Organ Weights	Yes ; organs: adrenals, testes with epididymides, heart, kidneys, liver, and spleen.	
3.5.2	Gross and histopathology	Yes ; Histopathology in control and high exposure group. Organs and tissues in addition to weighted organs: abdominal aorta, bone and bone marrow, brain, esophagus, eyes, ovaries, intestine, lung, lymph nodes, mammary gland, nasal turbinates, pancreas, pituitary, prostate, salivary gland, sciatic nerve, skeletal muscle, skin, spinal cord, stomach, thymus, thyroid, trachea, urinary bladder, uterus.	
3.5.3	Other examinations		
3.5.4	Statistics	ANOVA (Dunnett's test), Mann-Whitney a fisher exact tests with Bonferroni inequality procedure	
3.6	**Further remarks**	No	
		4 RESULTS AND DISCUSSION	
4.1	**Observations**		
4.1.1	Clinical signs	The incidence of toxic signs (salivation, discharges about nose) immediately after exposure time was the same in ACH exposed and control animals.	
4.1.2	Mortality	None	
4.2	**Body weight**	No significant decreases in body weight were observed.	
4.3	**Food consumption, compound intake**	Not reported and not relevant	
4.4	**Ophtalmoscopic examination**	Not reported	
4.5	**Blood analysis**		
4.5.1	Haematology	Slight changes in RBC count – all within physiological range - were observed in low and mid exposure animals.	
4.5.2	Clinical chemistry	Slight decrease in blood glucose was observed in mid and high exposure females, and in plasma globulin in mid and low exposure females. All values remained in normal ranges. No changes were found in T3 and T4 levels and in protein fractions	
4.5.3	Urinalysis	Thiocyanate levels were increased in exposed animals, significantly in mid and high exposure groups.	
4.6	**Sacrifice and pathology**		
4.6.1	Organ weights	No differences in absolute organ weights or organ-to-body weight ratios were noted.	
4.6.2	Gross and histopathology	No gross or microscopic lesions were detected which could be attributed to acetone cyanhydrin exposure.	
4.7	**Discussion and conclusions**	No signs of toxicity were observed which could be attributed to acetone cyanohydrin exposure. No treatment related differences in laboratory findings were observed except higher levels of thiocyanate in serum and urine. Changes in other clinical chemistry measurements were within	

		biological variation for the rat and were not considered compound-related. There were no exposure- related gross or microscopic pathology changes observed. In conclusion, NOAEL "no adverse-effect" exposure level was ≥ 57.7 ppm.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Sprague- Dawley male and female rats were exposed to acetone cyanohydrin (ACH) vapours, 6 hours per day, 5 days per week for 14 weeks. Four groups (15 males and 15 females per group) were exposed to mean concentrations 0, 10.1, 28.6 or 57.7 ppm (0, 36, 101, or 204 mg ACH/$m^{3)}$ equivalent to 0, 11, 32, and 65 mg hydrogen cyanide/m^3.	
5.2	**Results and discussion**	There were no treatment-related deaths or significant changes in body weight gain or haematology. Irritation of the nose and eyes was observed, but no more in exposed than in non-exposed animals. A decrease in blood glucose was recorded in high- and mid-exposure females, and a decrease in total serum protein and globulin concentrations was noted in the mid- and low-dose females. A comprehensive microscopic evaluation of tissues revealed no abnormalities, and no changes in serum T3 or T4 levels were observed.	
5.3	**Conclusion**	The NOAEL reported from the study was 204 mg ACH/m^3. *This can be estimated to correspond to a daily dose of 15 mg cyanide/kg body weight (calculated with a minute volume of 175 ml/min and an average body weight of 275 g and assuming 100% absorption).*	
5.3.1	LO(A)EL		
5.3.2	NO(A)EL	≥ 57.7 ppm acetone cyanohydrin	
5.3.3	Other		
5.3.4	Reliability	2	
5.3.5	Deficiencies	The study deviates from standard subchronic toxicity testing procedure, but no serious deficiencies in methods, statistical processing and conclusions were identified.	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.5 Annex Point IIA VI.6.5	**CHRONIC TOXICITY**	
Justification:	The biocidal product Uragan D2 is the same as the technical grade active substance hydrogen cyanide. Hydrogen cyanide is a highly toxic substance by inhalatory exposure for humans and for all species of laboratory organisms. The effects on humans and epidemiological studies are of primary significance for the assessment of chronic effects. **Summary see section III_6.12.** No chronic toxicity study of hydrogen cyanide administered to laboratory animals by means of inhalation has been published.	
References:	1. NTP (1993): Sodium Cyanide Administered In Drinking Water to F344/N Rats and B6c3f1 Mice. Toxicology Report Series No. 37. (NIH Publication 94-3386) **(DOC IV_40)** 2. NTP (1996): Toxicology and carcinogenesis studies of acetonitrile in F344/N rats and B6C3F$_1$ mice (Inhalation studies). NIH publication No 94 – 3363. **(DOC IV_49)** 3. J. W. Howard, R. F. Hanzal, Chronic Toxicity for Rats of Food Treated with Hydrogen Cyanide, Hazleton Laboratories, Falls Church, Va., Agricultural and Food Chemistry, Volume 3, April 1955, No.4 **(DOC IV_42)**	
Summaries:	The results of NTP (**2**) combined chronic inhalation toxicity – carcinogenicity study of acetonitrile in rats and mice **(for summary see section DOC III_ 6.5.1a)** offer an adequate substitute. There was no evidence of significant exposure-related clinical effects or non-neoplastic lesions in rats and mice exposed for 2 years to acetonitrile concentrations of up to 670 (rats) and 335 (mice) mg/m^3 for 6h/d, 5d/w. Chronic studies with oral exposure of animals are discussed together with subchronic oral administration of inorganic cyanides in drinking water or in diet (**1**) and **(3) (for summaries see section DOC III 6.4.1a and 6.7a*).*** As prolongation of exposure times had not modified or increased the toxic effects, special chronic (non-carcinogenic) toxicity studies in laboratory animals are not warranted.	
Undertaking of intended data submission	No studies are planned.	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.5 Annex Point IIA VI.6.5		**CHRONIC TOXICITY**	
Section A6.5 Annex Point IIA VI.6.5		**Carcinogenicity (inhalation) study (with acetonitrile)**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	National toxicology program (1996): Toxicology and carcinogenesis studies of acetonitrile in F344/N rats and B6C3F$_1$ mice (Inhalation studies). NIH publication No 94 – 3363. **DOC IV_49**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OCDE guideline 1996	
2.2	**GLP**	Yes (NTP, 1996)	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Acetonitrile vapours	
3.1.1	Lot/Batch number		
3.1.2	Specification	Not reported	
3.1.2.1	**Description**	Volatile liquid with boiling temperature of 82 °C	
3.1.2.2	**Purity**	≥ 99 %	
3.1.2.3	**Stability**		
3.2	**Test Animals**		
3.2.1	Species	Albino rat Mice	
3.2.2	Strain	Fischer 344 rats B6C3F1 mice	
3.2.3	Source		
3.2.4	Sex	Males and females	
3.2.5	Age/weight at study initiation		
3.2.6	Number of animals per group	12 male and 12 female rats per group 15 male and 15 female mice per group (one control and three experimental groups were used in the study)	
3.2.6.1	**At interim sacrifice**	4 male and 4 female rats and 5 male and 5 female mice from each dose group for the 15-months interim evaluation	

3.2.6.2	**At terminal sacrifice**	2x8 rats of the control group 2x8 rats of the 100 ppm group 2x8 rats of the 200 ppm group 2x8 rats of the 400 ppm group 2x10 mice of the control group 2x10 mice of the 50 ppm group 2x10 mice of the 100 ppm group 2x10 mice of the 200 ppm group	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Inhalation	
3.3.1	Duration of treatment	103 weeks	
3.3.2	Interim examination and sacrifice(s)	15 months: haematology, weight of liver, lungs and kidney	
3.3.3	Final sacrifice	After 103 weeks	
3.3.4	**Inhalation**		
3.3.4.1 Concentrations		Rats: 0, 100, 200, 400 ppm = 0, 168, 335, 670 mg/m^3 Mice: 0, 50, 100, 200 ppm = 0, 84, 168, 335 mg/m^3 The doses selected for the 2-year study of acetonitrile in rats were based on reduced survival of 800 ppm males and 1,600 ppm males and females in the 13-week study. The exposure concentrations selected for the 2-year study in mice were based on reduced survival and gross and histopathologic lesions in 400, 800, and 1,600 ppm groups of male and female mice in the 13-week study.	
3.3.4.2 Type of exposure		Whole body	
3.3.4.3 Duration of exposure		6 h/day, 5 d/week, 103 weeks	
3.4	**Examinations**		
3.4.1	Body weight	Weekly for the first 13 weeks, thereafter at 4-week- intervals, at two-week- intervals for the last 13 weeks.	
3.4.2	Food consumption	Yes	
3.4.3	Water consumption	No	
3.4.4	Clinical signs	Yes, twice daily	
3.4.5	Macroscopic investigations	Yes	
3.4.6	Ophthalmoscopic examination	No	
3.4.7	Haematology	Yes: haematocrit, haemoglobin, erythrocyte count and volume	
3.4.8	Clinical Chemistry	No	
3.4.9	Urinalysis	No	
3.4.10	Gross pathology	Yes	

3.4.11	Organ Weights	Yes	
3.4.12	Histopathology	Complete histopathological examination in all animals.	
		Organs examined: Tissue masses with regional lymph nodes, bones, skin, thyroid, adrenals, parathyroid, pituitary, thymus, bone marrow, salivary gland, oesophagus, stomach, small and large intestines, liver, kidneys, spleen, heart, nose, larynx, lungs, trachea, uterus, mammary gland, ovary, testes, prostate, seminal vesicle, urinary bladder, brain	
		4 RESULTS AND DISCUSSION	
4.1	**Body weight**	No treatment related effects	
4.2	**Clinical signs and survival**	The survival of male mice of the highest exposure group had significantly higher survival than controls. No other treatment related effects.	
4.3	**Macroscopic investigations**	No treatment related effects	
4.4	**Ophthalmoscopic examination**	Not performed	
4.5	**Haematology**	Haematological examination in rats has shown slightly lower haemoglobin levels and erythrocyte volume in the highest exposure groups.	
4.6	**Clinical Chemistry**	Not performed	
4.7	**Urinalysis**	Not performed	
4.8	**Gross Pathology**	No treatment related effects	
4.9	**Organ Weights**	No treatment related effects	
4.10	**Histopathology**	There was no evidence of significant exposure-related non-neoplastic lesions in rats or mice. In **rats**, there were non-significantly higher incidences of hepatocellular adenoma (0 - 2 - 2 - 6%, range of historical controls 0 - 8%) and hepatocellular carcinoma (2 - 0 - 0 - 6%, historical controls 0 - 4%) in the highest exposure group of males. In addition, male rats exhibited an increased incidence of basophilic foci in liver that was statistically significant in the 335 and 670 mg/m^3 groups. The foci were not atypical in appearance, as are those considered to be preneoplastic. NTP concluded that "a causal relationship between acetonitrile exposure and liver neoplasia in male rats is uncertain." The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) in male **mice** of the highest exposure group (42%) was significantly higher than in the control group, and at the upper limit of the range of historical controls (10 - 42%). In contrast, in females the incidence was inversely related to concentration, and incidence in controls was at the upper limit of the range of historical controls. The incidence of hepatocellular adenoma or carcinoma (combined) was significantly higher in male mice exposed to a concentration of 168 mg/m^3 (61%) compared to the control group, but in male mice exposed to a concentration of 335 mg/m^3 it was even lower than incidence in the control group. Focal hyperplasia of for stomach was observed in male and female mice exposed to acetonitrile, the incidence being significantly higher compared to controls in males exposed to 335 mg/m^3 and females	

		exposed to 168 or 335 mg/m^3. The severity of lesions ranged from minimal thickening of the stratum spinosum accompanied by increased number of basal cells, to marked epithelial thickening and folding with focal ulceration. The incidence of squamous cell papillomas was not significantly different compared to controls and remained in the range of historical controls.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	**2-year study in rats**: The doses selected for the 2-year study of acetonitrile were based on reduced survival of 800 ppm males and 1,600 ppm males and females in the 13-week study. Groups of up to 56 male and 56 female rats were exposed to 0, 100, 200, or 400 ppm (equivalent to 0, 168, 335, or 670 mg/m^3) acetonitrile by inhalation for 6 hours per day, 5 days per week for 2 years. Four male and four female rats from each exposure group were evaluated at 15 months for histopathology and haematology parameters. **2-year study in mice:** The exposure concentrations selected for the 2-year study were based on reduced survival and gross and histopathologic lesions in 400, 800, and 1,600 ppm groups of male and female mice in the 13-week study. Groups of 60 male and 60 female mice were exposed to 0, 50, 100, or 200 ppm (equivalent to 0, 84, 168, or 335 mg/m^3) acetonitrile by inhalation for 6 hours per day, 5 days per week for 2 years. Five male and five female mice from each exposure group were evaluated at 15 months for histopathology.	
5.2	**Results and discussion**	**2-year study in rats** Survival, Body Weights, Clinical Findings, and Haematology: Two-year survival, mean body weights, organ weights, behaviour, general health, and appearance of exposed male and female rats were similar to those of the controls. The hematologic effects observed were minor and of no biological significance: slightly lower haemoglobin levels and erythrocyte volume in the highest exposure groups. Pathology Findings: The incidences of hepatocellular adenoma (3/48), hepatocellular carcinoma (3/48), and hepatocellular adenoma or carcinoma (combined; 5/48) were greater in male rats exposed to 400 ppm than in the controls (one carcinoma). The incidences of hepatocellular adenoma and hepatocellular carcinoma were within the range of historical controls. However, the incidence of hepatocellular adenoma or carcinoma (combined) slightly exceeded the range of historical controls. In addition, the incidences of basophilic, eosinophilic, and mixed cell foci in 400 ppm males were marginally greater than in controls, suggesting hepatotoxicity of acetonitrile. There were no exposure-related liver lesions in female rats. **2-year study in mice** Survival, Body Weights, and Clinical Findings: Two-year survival of exposed male and female mice was similar to that of the controls, except that the survival of male mice in the 200 ppm group was significantly greater than that of the controls. Mean body weights and organ weights of exposed groups of male and female mice were similar to those of the controls, and no clinical observations in any group were clearly related to acetonitrile exposure. Pathology Findings: There were no increases in the incidences of neoplasms that were considered related to acetonitrile exposure in mice. The incidence of squamous hyperplasia of the epithelium of the forestomach was significantly increased at 15 months in 200 ppm females. At 2 years, the increased incidence of this lesion was dose	

		related in all exposed groups of males and females. The relevance of higher incidence of lung tumours in male mice is dubious with respect to increased survival in the highest exposure group of males, and to extremely wide range of historical controls (10 – 42%). In contrast, in females the incidence was inversely related to concentration, and incidence in controls was at the upper limit of the range of historical controls. The incidence of hepatocellular adenoma or carcinoma in male mice was not a monotonous function of exposure concentration.	
5.3	**Conclusion**	There was only equivocal evidence of carcinogenic activity of acetonitrile in male F344/N rats based on marginally increased incidences of hepatocellular adenoma and carcinoma. There was no evidence of carcinogenic activity of acetonitrile in female F344/N rats exposed to 100, 200, or 400 ppm. There was no evidence of carcinogenic activity of acetonitrile in male or female B6C3F1 mice exposed to 50, 100, or 200 ppm. Exposure to acetonitrile by inhalation resulted in increased incidences of hepatic basophilic foci in male rats and of squamous hyperplasia of the forestomach in male and female mice. Assuming that at least 12 % of acetonitrile is converted to cyanide, six hour exposure of rats to acetonitrile concentration of 670 mg/m^3 (the highest concentration used in the 2-year study) corresponds to a daily dose ≥ 10 mg cyanide per kg bw. As the conversion is slow enough to supply body tissues with cyanide for whole 24 hours, this exposure is equivalent to a continuous (24-hour) inhalation exposure to hydrogen cyanide in a concentration of 20 mg/m^3 or to 8-hour exposure to hydrogen cyanide in a concentration of 60 mg/m^3.	
5.3.1	Reliability	*1*	
5.3.2	Deficiencies	No relevant identified.	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.5 Annex Point IIA VI.6.5		**CHRONIC TOXICITY**	
Section A6.5 Annex Point IIA VI.6.5		**Chronic toxicity (oral)**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	J. W. Howard, R. F. Hanzal, Chronic Toxicity for Rats of Food Treated with Hydrogen Cyanide, Hazleton Laboratories, Falls Church, Va., Agricultural and Food Chemistry, Volume 3, April 1955, No.4 **(DOC IV_42)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No guidelines available	
2.2	**GLP**	No (GLP was not compulsory at the time the study was performed)	
2.3	**Deviations**		
		3 MATERIALS AND METHODS	
3.1	**Test material**	HCN	
3.1.1	Lot/Batch number	Not reported	
3.1.2	Specification	Not reported	
3.1.2.1	**Description**	Hydrogen cyanide was generated by the action of sulphuric acid on sodium cyanide aeroids (supplied by the American Cyanamid Co.)	
3.1.2.2	**Purity**	Not reported	
3.1.2.3	**Stability**		
3.2	**Test Animals**		
3.2.1	Species	Albino rat	
3.2.2	Strain		
3.2.3	Source	Carworth Farm	
3.2.4	Sex	Males and females	
3.2.5	Age/weight at study initiation	Male: 58g Female: 55g	
3.2.6	Number of animals per group	10 males and 10 females per group (one control and two experimental groups were used in the study)	

3.2.6.1	At interim sacrifice	No	
3.2.6.2	**At terminal sacrifice**	12 rats of the control group 12 rats at the 100 ppm group 15 rats at the 300 ppm group	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral in food	
3.3.1	Duration of treatment	104 weeks	
3.3.2	Interim sacrifice(s)	No	
3.3.3	Final sacrifice	After 104 weeks	
3.3.4	Frequency of exposure	Daily	
3.3.5	Post exposure period	No	
3.3.6	Type	**Oral** In food – commercially available dog meal treated with HCN vapours. The food was prepared fresh every 2 days and analysed for its initial hydrogen cyanide content. The content of HNC in food markedly dropped after 48 hours (to about one third). Food ad libitum	
3.3.7	Concentration		
3.3.8	Vehicle	Food	
3.3.9	Concentration in vehicle	Target concentration in food was 100 and 300 ppm, average initial content 106 and 301 ppm.	
3.3.10	Total volume applied	Food ad libitum.	
3.3.11	Controls	Plain diet	
3.4	**Examinations**		
3.4.1	Number of animals	Initially – number of animals not reported At the end of study - all surviving animals	
3.4.2	Time points	Initially and at termination of the study	
3.4.3	Parameters	Not reported	
3.4.4	Body weight	Yes	
3.4.5	Food consumption	Yes	
3.4.6	Water consumption	No	
3.4.7	Clinical signs	Yes	
3.4.8	Macroscopic investigations	Yes	
3.4.9	Ophthalmoscopic examination	No	
3.4.10	Haematology	Yes	

		Cyanide and thiocyanate content in plasma and red blood cells	
3.4.11	Clinical Chemistry	No	
3.4.12	Urinalysis	No	
3.4.13	**Pathology**	Yes	
3.4.13.1	**Organ Weights**	Yes; all surviving animal Liver, kidneys, adrenals, spleen, brain, heart, ovaries, testes,	
3.4.14	**Histopathology**	Yes; all surviving animals Thyroid, stomach, small and large intestines, liver, kidneys, adrenals, spleen, heart, lungs, uterus, testes, ovary, cerebrum, cerebellum of the brain	
3.4.15	Other examinations	Cyanide and thiocyanide content in liver and kidneys	
3.5	**Statistics**	Not reported	
3.6	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Body weight**	The growth curves were almost identical for the three groups of males throughout the 104-week feeding period. The females showed considerable variation. The control group exhibited an abnormal rise after 91 weeks of feeding. The variation at the dose 100 ppm was due to tumour development in one rat (died after 78 weeks) and general senility with weight loss of two rats. The 300 ppm level appeared to be of normal nature.	
4.2	**Food consumption**	The food consumption data indicate that the intake of the experimental rats was comparable to that of the control rats.	
4.3	**Water consumption**	Not performed	
4.4	**Clinical signs**	During the 2 years of feeding no gross signs of cyanide toxicity were observed.	
4.5	**Macroscopic investigations**	Autopsies revealed the same general abnormalities and signs of senility in the control and experimental rats: pale, granular, and thickened livers, congestion of medulla of the kidney, abnormally small spleens, enlarged adrenals, atrophies, encysted and inflamed genital organs, and enlarged, hemorrhagic pituitaries. Many nodes and tumours were found throughout the viscera. Infection of the ears was also evidenced.	
4.6	**Ophthalmoscopic examination**	Not performed	
4.7	**Haematology**	No effect	
4.8	**Clinical Chemistry**	Not performed	
4.9	**Urinalysis**	Not performed	
4.10	**Pathology**	No effect	
4.11	**Organ Weights**	No effect	
4.12	**Histopathology**	No effect	

4.13	**Other examinations**	Content of cyanide and thiocyanate in tissue (in γ per 100 grams or ml): **Control group:** Cyanide was absent in all tissue. Thiocyanate – average values: plasma – 361 red blood cells– 73 liver – 566 kidneys – 577. **100 ppm group:** Cyanide was absent in plasma, liver, kidneys. Red blood cells – 5.40 Thiocyanate – average values: plasma – 936 liver – 719 kidney – 1023 **300 ppm group:** Cyanide was absent in plasma, kidneys. Liver – only in one rat Red blood cells (50% rats) – 1.97 Thiocyanate – average values: plasma – 1123 red blood cells - 246 liver – 665 kidney – 1188	
4.14	**Time to tumours**	Not recorded	
4.15	**Other**		
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Non-guideline study The study of the chronic toxicity to rats of food fumigated with hydrogen cyanide.	
5.2	**Results and discussion**	Food containing 100 and 300 ppm of HCN produced no noticeable signs of cyanide toxicity. Haematological values determined initially and at the termination of the study, were within normal limits. Neither gross nor microscopic examination of the tissues revealed evidence of pathology due to the HCN feeding. No free cyanide was found in the plasma, liver or kidneys of the rats sacrificed in the 100 ppm group. In most instances cyanide was found in the red blood cells. These four tissues showed a definite rise in thiocyanate content over those of the control. Cyanide was not found in the plasma or kidneys of rat at the 300 ppm level. It was found in the liver of one rat and in the red blood cells of less than 50% of the rats in this group. Definite rises in thiocyanate were found in all four tissues. This study showed that a diet containing 100 or 300 ppm of HCN as a result of fumigation is non-toxic to male and female albino rats over a 2-year period.	
5.3	**Conclusion**	Feeding of food containing 100 and 300 ppm of hydrogen cyanide produced no signs of cyanide toxicity during a 2-year- feeding period. At termination haematological values were within normal limits and neither gross nor microscopic examination of tissues revealed evidence of pathology due to hydrogen cyanide feeding. At mean food consumption of 15 – 18g per day the average daily dose of HCN in 100ppm and 300ppm group animals was about 3 and 10 mg/kg bw, respectively.	
5.3.1	Reliability	3	
5.3.2	Deficiencies	Detailed findings and type and frequency of tumours not reported. No statistical evaluation.	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 Annex Point IIA VI.6.6	**GENOTOXICITY STUDIES**	

Section A6.6.1 Annex Point IIA VI.6.6.1	**In Vitro Gene Mutation**	
Justification: Literature data:	Data on in vitro mutagenity have been found in literature. Since only a small number of data for HCN is available, data for cyanides are used as substitute material. Genotoxicity test for sodium cyanide described in the NIH report on NTP study (1993) is used as **a key in vitro study of genotoxicity in bacteria (DOC IV_40). Summary see section 6.6.1a.**	
References:	Summaries and evaluations in this section are based mostly on exhaustive and reliably peer reviewed documents: ATSDR (2004, Toxicological profile of cyanide) (**DOC IV_1**) and IPCS (2004, WHO, CICAD 61: Hydrogen cyanide and cyanides: human health aspects). (**DOC IV_5**) and Hazardous Substance Data Bank (HSDB), National Library of Medicine´s TOXNET system: Hydrogen cyanide *Peer reviewed* (**DOC IV_2**). **1.** Kushi A. Matsumoto T, Yoshida D. 1983. Mutagen from the gaseous phase of protein pyrolyzate. Agric Biol Chem 47: 1979-1982 **(DOC IV_50). Summary see section 6.6.1b.** 2. Yamamoto H, Mohanan PV. 2002. Melatonin attenuates brain mitochondria DNA damage induced by potassium cyanide in vivo and in vitro. Toxicology 179:29-36. **(DOC IV_51) Summary see section 6.6.2a.** 3. Monsanto Co.: CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay. Acetone Cyanohydrin **(DOC IV_52) Summary see section 6.6.2b.** 4. De Flora S, Camoirano A, Zanacchi P, et al. 1984. Mutagenicity testing with TA97 and TA102 of 30 DNA-damaging compouds, negative with other Salmonella strains. Mutat Res 134:159-165. **(DOC IV_53).** 5. Henderson L, Wolfreys A, Fedyk J, et al. 1998. The ability of the Comet assay to discriminate between genotoxins and cytotoxins. Mutagenesis 13:89-94 **(DOC IV_54).** 6. Friedman MA, Staub J. 1976. Inhibition of mouse testicular DNA synthesis by mutagens and carcinogens as a potential simple mammalian assay for mutagenesis. Mutat Res 37: 67-76 **(DOC IV_55)** 7. Kubo T, Urano K, Utsumi H. 2002. Mutagenicity characteristics of 255 environmental chemicals. J Health Sci 48(6):545-554. **(DOC IV_56).** 8. Bhattacharya R, Lakshmana Rao PV. 1997. Cyanide induced DNA fragmentation in mammalian cell cultures. Toxicology 123:207-215	
Findings:	**Bacterial tests** An only positive mutagenic response was reported for hydrogen cyanide in strain TA100 without metabolic activation. Adding S-9 mix to the culture decreased the induction of reverse mutations by cyanide to 40% of the no activated reaction. Cyanide in the form of potassium cyanide tested negative in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100, TA97, and TA102. Negative results were also obtained in the DNA repair test in *Escherichia coli* WP67, CM871, and WP2 with potassium cyanide. Cyanide in the form of sodium cyanide tested negative in *S. typhimurium* strains TA97, TA98, TA100, and TA1535, with and	

	without metabolic activation. Potassium cyanide failed to induce reverse mutations in *S. typhimurium* strains TA97 or TA102 with or without metabolic activation; KCN was one of 30 compounds that induced direct nonreparable damage in repair-deficient *E. coli* but failed in reverting strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 of *S. typhimurium* **(4).** Also in other studies, sodium cyanide was not mutagenic in any of several strains of *Salmonella typhimurium* with or without S9 activation. In cultured A549 human epithelial-like lung carcinoma cells, ≥1 mM potassium cyanide induced dose related reductions in cell viability by 8 hours and dose-dependent increases in electrophoretically detectable double-strand DNA breaks by 24 hour). Based on this temporal relationship and the fact that the induced DNA fragments were smaller than 0.5 Mbp, the authors concluded that the genotoxic effect of cyanide was indirect and based on the activation of endonucleases by calcium entering the damaged cells. **Mammalian cells** Dose-related increases in DNA breaks were induced in rat thymocytes treated for 6 hours with ≥1.25 mM potassium cyanide or in baby hamster kidney (BHK-21) cells at 5 mM. Incubation of cells in calcium-free medium significantly reduced the level of DNA damage, supporting the hypothesis that a cytotoxic-related calcium-influx contributes to this fragmentation of DNA **(8).** 81 chemicals evaluated for genotoxicity in an *in vitro* alkaline elution assay for DNA strand breaks in primary cultures of rat hepatocytes. The study included a battery of assays for cytotoxicity (including tetrazolium dye reduction, trypan blue dye exclusion after 3 hours of recovery, ATP content, K+ content, and cell blebbing) to distinguish between genotoxicity and false-positive results resulting from the loss of membrane integrity in damaged cells. DNA strand breakage following treatment with ≥6 mM potassium cyanide was determined to be associated with the induction of endonucleolytic DNA degradation caused by cytotoxicity (ATP content ≤5% of control, increased cell blebbing). electrophoretically detected significant DNA breakage (DNA migration) in TK6 human lymphoblastoma cells treated with potassium cyanide at 2 mg CN–/mL a concentration reducing cell survival (as measured by trypan blue exclusion) by 30%. Dose-related increases in electrophoretically detectable DNA breaks were induced by potassium cyanide (0.1–2 mM) in a crude mitochondrial fraction isolated from the brains of male ddy mice **(2).** Sodium cyanide did not induce DNA-strand breaks in cultured mouse lymphoma cells. In (**5**) there is electrophoretically detected significant DNA breakage (DNA migration) in TK6 human lymphoblastoma cells treated with potassium cyanide at 2 mg CN–/mL a concentration reducing cell survival (as measured by trypan blue exclusion) by 30%.	
	Genetic toxicity *in vitro* testing of cyanide has yielded conflicting results, but overall, cyanide does not appear to have significant mutagenic activity. Sodium cyanide was not mutagenic in any of several strains of *Salmonella typhimurium* with or without S9 activation, and did not induce DNA-strand breaks in cultured mouse lymphoma cells. Potassium cyanide was not mutagenic in five strains of *S. typhimurium* with or without S9 at any dose level tested. Positive result with HCN is an isolated finding: in a study of products of pyrolysis of proteins, hydrogen cyanide was reported to induce mutations in *S. typhimurium* strain TA100 but only in the absence of S9	

	activation, other studies using the same strain (**see e.g. the key study**) did not confirm this finding.	
Conclusion	Cyanide ion does not appear to have significant in vitro mutagenic activity in bacteria. Minimum concentrations of CN reported to cause injury of DNA in mammalian cells (> 0.1 – 2 mM of KCN) are on the level of lethal concentrations in organs and blood observed in human accidental acute poisonings (0.08 - 0.4 mM CN, **DOC IIIA 6.12a,c)**. DNA breaks at these concentrations are attributed to fatal blockade of mitochondrial function and resulting accumulation of reactive oxygen species Occupational exposures to HCN lead to concentrations by two to three orders of magnitude lower.	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 **Annex Point IIA VI.6.6**		**GENOTOXICITY STUDIES**	
Section A6.6.1 **Annex Point IIA VI.6.6.1**		**In vitro gene mutation study in bacteria**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	NTP. 1993. Sodium cyanide administered in drinking water to F344/N rats and B6C3F$_1$ mice. NTP, Toxicology Report Series No. 37. (NIH Publication 94-3386) **(DOC IV_40)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2		/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No reported. Method and procedure essentially identical with standardised test B14.	
2.2	**GLP**	US FDA GLP regulation	
2.3	**Deviations**	Only 4 strains of S. typhimurium were used.	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Sodium cyanide	
3.1.1	Lot/Batch number		
3.1.2	Specification		
3.1.2.1	**Description**		
3.1.2.2	**Purity**	98%	
3.1.2.3	**Stability**	Not known	
3.2	**Study Type**	Bacterial reverse mutation test	
3.2.1	Organism/cell type	Salmonella typhimurium strains TA100, TA1535, TA97, and TA98,	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	S9 mix 10 or 30% rats and hamster liver, Aroclor induced	
3.2.4	Positive control	Yes	
3.3	**Administration / Exposure; Application of test substance**		
3.3.1	Concentrations	0.3 to 333 µg per plate	
3.3.2	Way of application		

3.3.3	Pre-incubation time	Sodium cyanide was incubated with the S. typhimurium tester strains either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37^0C	
3.3.4	Other modifications	Top agar supplemented with l-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates	
3.4	**Examinations**	Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C. Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of sodium cyanide.	
3.4.1	Number of cells evaluated		
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**		
4.1.1	Without metabolic activation	Not genotoxic	
4.1.2	With metabolic activation	Not genotoxic	
4.2	**Cytotoxicity**	At doses ≥ 100µg per plate.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Sodium cyanide (0.3 to 333 micrograms per plate) was tested for mutagenicity in Salmonella typhimurium strains TA100, TA1535, TA97, and TA98, with and without Aroclor-induced rat and hamster S9 at concentrations of 10% and 30%. Sodium cyanide was incubated with the S. typhimurium tester strains either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37°C. Top agar supplemented with l-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C. Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of sodium cyanide.	
5.2	**Results and discussion**	**See table below.**	
5.3	**Conclusion**	Sodium cyanide in doses 0.3 to 333 micrograms per plate was not mutagenic in any of several strains of Salmonella typhimurium with or without S9 activation. The testing with the strain T100 was completely negative for doses per plate up to the level toxic for bacteria (100 or 333 micrograms per plate); positive controls indicated full sensitivity of the tes*t*	
5.3.1	Reliability	*2*	
5.3.2	Deficiencies	Only 4 strains of S. typhimurium were used.	

Table: Mutagenicity of Sodium Cyanide in Salmonella typhimurium 1

		Revertants/plate[2]						
Strain / Dose		**S9**			**+ hamster S9**		**+rat S9**	
(µg/ plate)		**Trial 1**	**Trial 2**	**Trial 3**	**10%**	**30%**	**10%**	**30%**
TA100	0.0	103± 1.5	89± 6.2	92± 3.5	107± 9.3	108± 1.2	86± 6.9	116± 3.5
	1.0	89± 9.8		104± 4.5		108± 5.5		121± 4.5
	3.3	108± 1.2	83± 9.4	89± 4.6	104± 7.0	111± 3.8	94± 1.7	120± 6.0
	10.0	101± 1.5	82± 9.0	108± 3.7	114± 6.1	117± 10.0	116± 4.6	133± 10.3
	33.0	103± 6.6	91± 8.6	85± 1.2	99± 11.0	108± 4.3	107± 12.6	121± 6.0
	100.0	101± 7.8	3± 2.5	96± 2.9	79± 9.5	115± 6.0	80± 6.6	122± 12.4
	333.0		0± 0.0		1± 0.7		12± 4.4	
Trial summary		**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**
Positive control[3]		415± 31.5	348± 15.5	644± 59.0	719± 25.7	448± 41.5	1,125± 21.5	706± 45.9
TA1535	0.0	14± 2.3	10± 1.5		13± 0.3	16± 2.1	11± 0.7	13± 2.8
	1.0	14± 0.3				21± 2.7		14± 2.5
	3.3	14± 0.6	9± 0.7		9± 2.1	16± 3.0	10± 2.1	14± 1.2
	10.0	9± 1.2	7± 1.8		13± 2.6	13± 1.8	8± 1.5	14± 2.4
	33.0	12± 0.9	4± 0.7		12± 2.7	19± 3.8	7± 2.6	16± 1.0
	100.0	12± 2.3	3± 0.7		11± 2.8	21± 1.2	8± 1.2	18± 1.2
	333.0		0± 0.0		4± 1.7		3± 1.5	
Trial summary		**Negative**	**Negative**		**Negative**	**Negative**	**Negative**	**Negative**
Positive control		516± 8.2	200±6.5		80± 0. 9	92± 6.4	141± 6.1	178± 6.6

Strain / Dose		**S9**			**+ hamster S9**		**+rat S9**	
(µg/ plate)		**Trial 1**	**Trial 2**	**Trial 3**	**10%**	**30%**	**10%**	**30%**
TA97	0.0	143± 3.2	115± 2.3		142± 4.7	191± 11.9	134± 8.5	206± 9.2
	1.0	119± 4.5				167± 12.7		226± 9.5
	3.3	133± 3.5	123± 5.9		154± 6.0	167± 9.6	144± 4.4	217± 8.0
	10.0	119± 6.2	100± 4.5		149± 9.4	179± 9.4	134± 4.3	192± 3.8
	33.0	130± 4.6	106± 5.9		156± 7.5	173± 9.1	143± 9.1	219± 3.9
	100.0	163± 3.0	109± 4.9		163± 6.6	179± 5.0	128± 8-9	229± 7.8
	333.0		76± 15.6		130± 21.5		116± 7.7	
Trial summary		**Negative**	**Negative**		**Negative**	**Negative**	**Negative**	**Negative**
Positive control		721± 103.8	355± 32.1		1,222± 40.0	1,900±122.0	1,533±62.5	1,097± 43.9

Table:Mutagenicity of Sodium Cyanide in Salmonella typhimurium (continued)

		Revertants/plate[2]					
Strain / Dose			**S9**			**+ hamster S9**	
(µg/ plate)		**Trial 1**	**Trial 2**	**Trial 3**	**10%**	**10%**	**30%**
TA98	0.0	19± 1.5	10± 2.3	15± 2.1	18±2.4	23± 2.5	18± 2.4
	0.3			14± 1.5		16± 2.2	
	1.0	12± 1.5		13±.3.6		20± 2.0	20± 2.0
	3.3	14± 1.5	5± 2.0	16± 3.3	13± 2.6	16± 3.8	21± 3.2
	10.0	14± 1.8	0± 0.0	13± 1.7	14± 2.9	22± 3.3	24± 1.2
	33.0	11± 1.0	1±1.0	12± 0.7	8± 0.6	16± 1.5	13± 3.2
	100.0	0± 0.0	0± 0.0		3± 0.6		7± 2.2
	333.0		0± 0.0		0± 0.3		
Trial summary		**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**
Positive control		321± 10.5	253± 67.0	423±9.7	706± 24.1	406± 26.5	387±44.5

		+rat S9	
TA98 (continued)	10%	10%	30%
0.0	18± 3.0	19± 2.3	22±0.0
0.3		19± 3.2	
1.0		23± 2.2	24±2.2
3.3	21± 2.6	17± 1.7	24±2.6
10.0	12± 2.4	20± 1.5	23±1.8
33.0	9± 0.7	17± 1.5	22±2.1
100.0	3± 0.0		7±1.5
333.0	0± 0.0		
Trial summary	**Negative**	**Negative**	**Negative**
Positive control	433±24.2	199± 8.0	407± 46.6

[1] Study was performed at Microbiological Associates, Inc. The detailed protocol can be found through original article; 0 µg/plate is the solvent control.

[2] Revertants are presented as mean ± standard error from three plates.

[3] The positive controls in the absence of metabolic activation were 4-nitro-o-phenylenediamine (TA98), sodium azide (TAl 00 and Al535), and 9-aminoacridine (TA97). The positive control for metabolic activation with all strains was 2-aminoanthracene.

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 **Annex Point IIA VI.6.6**		**GENOTOXICITY STUDIES**	
Section A6.6.1 **Annex Point IIA VI.6.6.1**		**In vitro gene mutation study in bacteria**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Kushi A., Matsumoto T., Yoshida D. (1983): Mutagen from the Gaseous Phase of Protein Pyrolyzate; Agric. Biol. Chem. 47(9) **(DOC IV_50)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No	
2.2	**GLP**	No, GLP was not compulsory at the time the study was performed	
2.3	**Deviations**	No Guideline/No deviation	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Processed Gaseous Phase of Protein Pyrolyzate (see 3.1.2).	
3.1.1	Lot/Batch number		
3.1.2	Specification	Various amounts of albumin were heated at 500 °C with nitrogen flow. The gas phase was obtained by filtering with a glass fibre filter and material was collected in a dry ice – acetone cold trap. The trapped material was removed with methanol. Solution of the trapped material was subjected to gas chromatography. Mutagenic activity of each of resulting 6 peaks was determined by bubbling of the gas at the outlet of GC to a test tube containing 0.1 ml of bacterial solution and 0.4 ml of P-buffer.	
3.1.2.1	**Description**		
3.1.2.2	**Purity**		
3.1.2.3	**Stability**	Not known	
3.2	**Study Type**	Bacterial reverse mutation test	
3.2.1	Organism/cell type	S. typhimurium: TA 98, TA 100	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	S9 mix Rats liver, PCB induced	
3.2.4	Positive control	Not used	

3.3	**Administration / Exposure; Application of test substance**		
3.3.1	Concentrations	Calculated doses cc 6.5-40 µg per plate	
3.3.2	Way of application	Methanol solution of the trapped material was subjected to gas chromatography. Mutagenic activity of each of resulting 6 peaks was determined by bubbling of the gas at the outlet of GC to a test tube containing 0.1 ml of bacterial solution and 0.4 ml of P-buffer.	
3.3.3	Pre-incubation time	No pre-incubation	
3.3.4	Other modifications	The same procedure of trapping was applied to HCN evolved by adding HCl to KCN solution.	
3.4	**Examinations**		
3.4.1	Number of plates evaluated	Not reported.	
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**		
4.1.1	Without metabolic activation	Mutagenicity was observed in TA 100 for peak No 2 (coinciding almost completely with HCN standard) for doses of cc 27 and 40 µg per plate (**see Fig.3**). Similar result was obtained with HCN evolved from KCN.	
4.1.2	With metabolic activation	No	
4.2	**Cytotoxicity**	No observed.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**		
5.2	**Results and discussion**	A fraction of the gaseous phase of albumin pyrolisate, corresponding best to HCN on GC, was mutagenic for TA 100 without metabolic activation with an S-shaped albumine dose-response relationship. Maximum number of colonies per plate was about 180. Results for HCN evolved from KCN **see table.**	
5.3	**Conclusion**	Mutagenic for TA 100 without metabolic activation.	
5.3.1	Reliability	3	
5.3.2	Deficiencies	Vague quantification of doses and results.	

Table for Gene Mutation Assay: HCN evolved from KCN.

Concentration * [µg/plate]	Number of mutant cells*		Comments
TA 100	— S9	+ S9	
0	110	127	
6,5	110	127	
13	125	132	
27	255	147	
40	220	157	Not cytotoxic

Concentration * [µg/plate]	Number of mutant cells*		Comments
TA 98	— S9	+ S9	
0	67	67	
6,5	67	67	
13	69	71	
27	70	72	Not cytotoxic
40	-	-	

* values are estimated from the plot

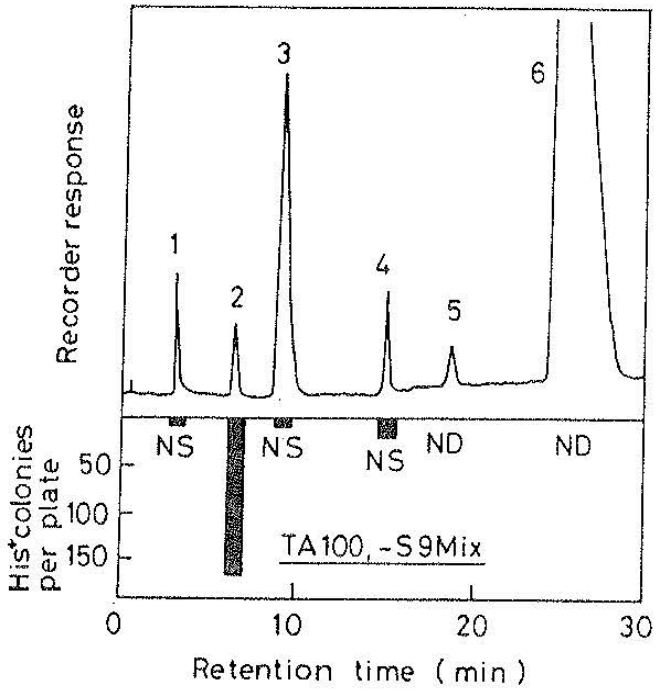

FIG. 3. Gas Chromatogram of the Gaseous Phase and the Mutagenic Activity of Each Peak.

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 Annex Point IIA VI.6.6		**GENOTOXICITY STUDIES**	
Section A6.6.2 Annex Point IIA VI.6.6.2		**In vitro cytogenicity study in mammalian cells**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Hiro-aki Yamamoto, P.V. Mohanan: Melatonin attenuates brain mitochondria DNA damage induced by potassium cyanide in vivo and in vitro Toxicology 179 (2002): 29 - 36 **(DOC IV_51)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No guideline reported. Procedure used is specifically designed to study primary toxic effects of cyanides.	
2.2	**GLP**	No	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Potassium cyanide	
3.1.1	Lot/Batch number	/	
3.1.2	Specification	/	
3.1.2.1	**Description**		
3.1.2.2	**Purity**		
3.1.2.3	**Stability**	Not reported	
3.2	**Study Type**	Brain mitochondria DNA damage	
3.2.1	Organism/cell type	Forty eight male ddy mice (SLC, Shizuoka); crude brain mitochondria fraction (1000 g supernatant of homogenised brains).	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	/	
3.2.4	Positive control	Incubation with 1.5 mM H_2O_2 for 15 min, in the presence of Fe^{2+} (0.003 mM).	
3.3	**Administration / Exposure; Application of test substance**		
3.3.1	Concentrations	0, 0.1, 1.0 or 2.0 mM potassium cyanide	
3.3.2	Way of application	1ml of supernatant was incubated with KCN at 37 °C for 60 min.	

3.3.3	Pre-incubation time	100mg of fresh brains was homogenised with ice cold buffer supplied along with DNA extractor kit; the homogenate was centrifuged at 1,000 G for 1 min at 4 °C.	
3.3.4	Other modifications	Incubation in the presence of melatonin (1.5 mM)	
3.4	**Examinations**	Agarose gel electrophoresis of EcoR1 or Hind II digested mitochondrial DNA.	
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**	Potassium cyanide inflicted damage to mitochondrial DNA in a concentration dependent manner. The effect was similar to the effect of incubation with hydrogen peroxide. Both effects were abolished in the presence of melatonin, potent scavenger of hydroxyl radicals.	
4.2	**Cytotoxicity**	Incubation of crude mitochondria fraction with 0.1 or 1.0 mM KCN increased lipid peroxidation from 0.48 (nmol/mg protein) to 0.75 and 1.09, respectively. This effect was also abolished by melatonin. Discussion: The results suggest that reactive oxygen species may play a cardinal role for mitochondrial DNA damage induced by cyanide.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	see Summary 6.6.4a	
5.2	**Results and discussion**	see Summary 6.6.4a	
5.3	**Conclusion**	see Summary 6.6.4a	
5.3.1	Reliability	see Summary 6.6.4a	
5.3.2	Deficiencies	see Summary 6.6.4a	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 Annex Point IIA VI.6.6		**GENOTOXICITY STUDIES**	
Section A6.6.2 Annex Point IIA VI.6.6.2		**In vitro cytogenicity study in mammalian cells**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Monsanto Co.: CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay. Acetone Cyanohydrin **(DOC IV_52)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	/	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No	
2.2	**GLP**	Yes	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Acetone Cyanohydrin	
3.1.1	Lot/Batch number	/	
3.1.2	Specification	/	
3.1.2.1	**Description**	Clear amber liquid	
3.1.2.2	**Purity**		
3.1.2.3	**Stability**		
3.2	**Study Type**	Mammalian Cell Forward Gene Mutation Assay	
3.2.1	Organism/cell type	Chinese hamster ovary cells (CHO-Kl-BH4 cell line)	
3.2.2	Deficiencies / Proficiencies	Thorough preliminary studies of cytotoxicity and mutagenicity both without metabolic activation and with various concentrations of S-9.	
3.2.3	Metabolic activation system	2% S-9 metabolic activation preparation	
3.2.4	Positive controls	Ethyl methane sulphonate (200mg/L), dimethylnitrosamine (100 mg/L): The former is a direct acting mutagen, while the latter requires metabolic (S-9) activation.	
3.3	**Administration / Exposure; Application of test substance**	All treatment levels and controls were done in duplicate.	
3.3.1	Concentrations	100, 500, 700, 850 and 950 mg/L	

3.3.2	Way of application	Solution (0.05 ml) of tested or control substance was added to flask containing cell culture in 5 ml of F12 medium. 0 or 1 ml of the S-9 activation preparation was added.	
3.3.3	Pre-incubation time	Cells for testing were obtained from frozen stock aliquots, and incubated for 16 – 24 hours in F12FCM5 medium.	
3.3.4	Other modifications		
3.4	**Examinations**	Cultures were incubated for 5 hours, washed and incubated in F12FCM5 for additional 19 hours, diluted to 1000cells /ml, plated for initial survival assessment and incubated for 7 days. After selection of mutant CHO cells (i.e., 6-TG resistant) the plates were incubated for further 7 days to allow for colony formation. The colonies of selected mutant CHO cells were fixed, stained and counted.	
3.4.1	Number of cells evaluated	The total number of clones on the five plates was determined.	
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**		
4.1.1	Without metabolic activation	Negative for ACH concentrations 100 – 950 mg/L	
4.1.2	With metabolic activation	Negative for ACH concentrations 100 – 950 mg/L	
4.2	**Cytotoxicity**	Relative post-treatment survival decreased with ACH concentrations from 100% at 100mg/L to 50% at 700 mg/L to 0% at 1000 mg/L.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Acetone Cyanohydrin was evaluated for cytotoxicity in the CHO cell line at doses of >0.3, 1.0, 3.33, 10, 33.3, 100, 333 and 1000 ug/ml of treatment volume both without a metabolic activation (S-9) preparation and with 1, 2, 5 and 10% concentrations (of the total treatment volume) of a metabolic activation (S-9) preparation. Acetone Cyanohydrin was then evaluated in a Preliminary Mutagenicity Assay at dose levels of 100, 500 and 900 ug/ml of treatment volume both without a metabolic activation and with 1, 2, 5 and 10% concentrations of the metabolic activation (S-9) preparation. Acetone Cyanohydrin was then evaluated in the CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay at dose levels of 100, 500, 700, 850 and 950 ug/ml of treatment volume both without metabolic activation and with a 2% concentration of the metabolic activation preparation. Cells for testing were obtained from frozen stock aliquots, and incubated for 16 – 24 hours in F12FCM5 medium. Solution (0.05 ml) of tested or control substance was added to flask containing cell culture in 5 ml of F12 medium. 0 or 1 ml of the S-9 activation preparation was added. Cultures were incubated for 5 hours, washed and incubated in F12FCM5 for additional 19 hours, diluted to 1000cells /ml, plated for initial survival assessment and incubated for 7 days. After selection of mutant CHO cells (i.e., 6-TG resistant) the plates (5 plates per dose) were incubated for further 7 days to allow for colony formation. The colonies of selected mutant CHO cells were fixed, stained and counted. The total number of clones on the five plates was determined.	

		The mutant frequency was calculated by correcting the total number of mutant clones by the cloning efficiency of the cells at the time of mutant selection.	
5.2	**Results and discussion**	Relative post-treatment survival decreased with ACH concentrations: 0, 100, 500, 700, 850, 950, 1000 mg/L: 98, 99, 78, 58, 46, 37, 0 without activation 100, 98, 77, 58, 53, 49, 1 with 2% S-9 The results of the Preliminary Mutagenicity Assay, after critical review, indicated that the 2% concentration of S-9 demonstrated the most pronounced effect of all the concentrations employed, and would be used as the optimum S-9 level in the mutagenicity assay. No significant difference in the frequency of mutations between acetone cyanohydrin treated cells and solvent controls were detected CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay (**see Table**): the dose range included clearly cytotoxic concentrations. The effect of positive controls proves validity of the testing procedure used.	
5.3	**Conclusion**	The results were negative in the CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay according to the criteria of the test protocol.	
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

Table: Mammalian cell forward gene mutation assay of Acetone Cyanohydrin (number of mutants per 10^6 "expression survivors")

Treatment	Concentration mg/L	S-9 0	S-9 1%	S-9 2%	S-9 5%	S-9 10%
Solvent		**3.4**	**5.5**	**3.3**	**3.3**	**5.8**
ACH	**100**	**3.4**	**3.9**	**1.6**	**1.5**	**5.0**
	500	**4.1**	**3.3**	**8.2**	**3.4**	**2.5**
	900	**5.5**	**2.2**	**12.8**	**1.5**	**5.8**
EMS	**200**	**313**				
DMN	**100**			**299**		
Solvent		**11.1**		**4.0**		
ACH	**100**	**2.7**		**6.1**		
	500	**3.4**		**5.8**		
	700	**8.3**		**1.7**		
	850	**6.8**		**5.9**		
	950	**0.5**		**0.4**		

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 Annex Point IIA VI.6.6		**GENOTOXICITY STUDIES**	
Section A6.6.2 Annex Point IIA VI.6.6.2		**In vitro cytogenicity study in mammalian cells**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Hiro-aki Yamamoto, P.V. Mohanan: Melatonin attenuates brain mitochondria DNA damage induced by potassium cyanide in vivo and in vitro Toxicology 179 (2002): 29 - 36 **(DOC IV_51)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No guideline reported. Procedure used is specifically designed to study primary toxic effects of cyanides.	
2.2	**GLP**	No	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Potassium cyanide	
3.1.1	Lot/Batch number	/	
3.1.2	Specification	/	
3.1.2.1	**Description**		
3.1.2.2	**Purity**		
3.1.2.3	**Stability**	Not reported	
3.2	**Study Type**	Brain mitochondria DNA damage	
3.2.1	Organism/cell type	Forty eight male ddy mice (SLC, Shizuoka); crude brain mitochondria fraction (1000 g supernatant of homogenised brains).	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	/	
3.2.4	Positive control	Incubation with 1.5 mM H_2O_2 for 15 min, in the presence of Fe^{2+} (0.003 mM).	
3.3	**Administration / Exposure; Application of test substance**		
3.3.1	Concentrations	0, 0.1, 1.0 or 2.0 mM potassium cyanide	
3.3.2	Way of application	1ml of supernatant was incubated with KCN at 37 °C for 60 min.	

3.3.3	Pre-incubation time	100mg of fresh brains was homogenised with ice cold buffer supplied along with DNA extractor kit; the homogenate was centrifuged at 1,000 G for 1 min at 4 °C.	
3.3.4	Other modifications	Incubation in the presence of melatonin (1.5 mM)	
3.4	**Examinations**	Agarose gel electrophoresis of EcoR1 or Hind II digested mitochondrial DNA.	
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**	Potassium cyanide inflicted damage to mitochondrial DNA in a concentration dependent manner. The effect was similar to the effect of incubation with hydrogen peroxide. Both effects were abolished in the presence of melatonin, potent scavenger of hydroxyl radicals.	
4.2	**Cytotoxicity**	Incubation of crude mitochondria fraction with 0.1 or 1.0 mM KCN increased lipid peroxidation from 0.48 (nmol/mg protein) to 0.75 and 1.09, respectively. This effect was also abolished by melatonin. Discussion: The results suggest that reactive oxygen species may play a cardinal role for mitochondrial DNA damage induced by cyanide.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	see Summary 6.6.4a	
5.2	**Results and discussion**	see Summary 6.6.4a	
5.3	**Conclusion**	see Summary 6.6.4a	
5.3.1	Reliability	see Summary 6.6.4a	
5.3.2	Deficiencies	see Summary 6.6.4a	

	Evaluation by Competent Authorities
Date	
Evaluation of applicant's justification	
Conclusion	
Remarks	

Section A6.6 Annex Point IIA VI.6.6		**GENOTOXICITY STUDIES**	
Section A6.6.3 Annex Point IIA VI.6.6.3		**In vitro cytogenicity study in mammalian cells**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Monsanto Co.: CHO/HGPRT Mammalian Cell Forward Gene Mutation Assay. Acetone Cyanohydrin **(DOC IV_52)**	
1.2	**Data protection**	No	
1.2.1	Data owner	/	
1.2.2	Companies with letter of access	/	
1.2.3	Criteria for data protection	/	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No	
2.2	**GLP**	Yes	
2.3	**Deviations**	/	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Acetone Cyanohydrin	
3.1.1	Lot/Batch number	/	
3.1.2	Specification	/	
3.1.2.1	**Description**	Clear amber liquid	
3.1.2.2	**Purity**		
3.1.2.3	**Stability**		
3.2	**Study Type**	Mammalian Cell Forward Gene Mutation Assay	
3.2.1	Organism/cell type	Chinese hamster ovary cells (CHO-Kl-BH4 cell line)	
3.2.2	Deficiencies / Proficiencies	Thorough preliminary studies of cytotoxicity and mutagenicity both without metabolic activation and with various concentrations of S-9.	
3.2.3	Metabolic activation system	2% S-9 metabolic activation preparation	
3.2.4	Positive controls	Ethyl methane sulphonate (200mg/L), dimethylnitrosamine (100 mg/L): The former is a direct acting mutagen, while the latter requires metabolic (S-9) activation.	
3.3	**Administration / Exposure; Application of test substance**	All treatment levels and controls were done in duplicate.	
3.3.1	Concentrations	100, 500, 700, 850 and 950 mg/L	