8EHQ1102-15224

8EHQ-02-15224

American Chemistry Council Good Chemistry Makes It Possible

November 25, 2002

88030000019

2002 NOV 25 PM 2:21 RECEIVED OPPT CBIC

BY COURIER

OPPT Document Control Office
EPA East Bldg, Room 6428
1201 Constitution Ave NW
Washington DC 20460

Contain NO CBI

Attention: TSCA Section 8(e) Coordinator

RE: Pyrolysis C5 - Mouse Micronucleus Range Finding Study in Mice

Dear Sir or Madam:

The American Chemistry Council Olefins Panel submits this letter on behalf of certain of its members (listed below) pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA) to inform EPA of results of the range-finding portion of a mouse micronucleus test that was conducted for Pyrolysis C5s. The Panel has not made a determination as to whether a significant risk of injury to health or the environment is presented by these findings.

The Pyrolysis C5s stream was tested pursuant to the Olefins Panel's testing plan for the C5 non-Cyclics Category under the High Production Volume Chemical Challenge Program (available at http://www.epa.gov/chemrtk/olefins/olefintp.pdf). The Pyrolysis C5s stream is a hydrocarbon distillate fraction separated from pyrolysis gasoline that consists primarily of C5 dienes and other C5 hydrocarbons and low levels of higher boiling C4 hydrocarbons and volatile C6 hydrocarbons. CAS Registry numbers that are used by Panel members to identify Pyrolysis C5s streams include: 68476-55-1 (Hydrocarbons, C5-rich), 68476-43-7 (Hydrocarbons, C4-6, C5 rich), 68527-19-5 (Hydrocarbon, C1-4, debutanizer fraction), 68603-00-9 (Distillates, petroleum, thermal cracked naphtha and gas oil) and 68956-55-8 (Hydrocarbons,C5-unsatd).

2002 DEC -9 AM 11:15 RECEIVED OPPT NCIC

A preliminary toxicity study was conducted to select appropriate dose levels for the micronucleus test. One or two male and two female CD-1 mice received up to two 6-hour exposures of 0, 500, 1,000, 2,000 or 3,000 ppm of the Pyolysis C5s stream. Animals were then observed for a 48 hour period. The purpose of the study was to determine the highest dose that would produce signs of toxicity without producing extreme clinical signs or having a significant effect on survival. At the highest two exposures, all animals died. At the 1,000

64052

ppm exposure, one animal died and the two surviving animals exhibited clinical signs including hunched posture, piloerection and unsteady gait. Hunched posture and piloerection was also noted at the lowest exposure level, 500 ppm.

Attached is the draft report for the micronucleus study that includes the information from the range-finding study. A final report is not yet available, but will be forwarded when received from the laboratory.

The following members of the Panel produce one or more of the streams included in the C5 non-cyclics Category and are sponsors of the testing for the category: BP Amoco Chemical Company, Chevron Phillips Chemical Company LP, The Dow Chemical Company, Equistar Chemicals, LP, ExxonMobil Chemical Company, The Goodyear Tire & Rubber Company, Huntsman Corporation, NOVA Chemicals Inc., and Shell Chemical Company LP.

If you have any questions, please contact me by phone at 301 924 2006, by email at Elizabeth_Moran@americanchemistry.com, or write to me at the address given at the bottom of the first page of this letter.

Yours truly,

Elizabeth J. Moran
Manager, Olefins Panel

Attachment: draft report: Pyrolysis C5s Mouse Micronucleus Test dated 16 October 2002

cc: Richard H. Hefter (MC 7403)

NOTE

This report is considered by the Study Director to be the 'final draft'. It has been **audited** by the Huntingdon Life Sciences Quality Assurance Department.

The sponsor is requested to review this document and communicate any comments to the Study Director as soon as possible. When these comments have been received, the FINAL REPORT containing Study Director and QA Statements will be issued.

PLEASE NOTE

In compliance with GLP any changes to the final report after the date of issue will be in the form of a separate amendment to the report.

Date: 16 October 2002 **V. 1.**

PYROLYSIS C5s
MOUSE MICRONUCLEUS TEST

Data requirement:	EC Directive 2000/32/EC, 4C – B.12. OECD Guideline 474 OPPTS Method 870.5395
Huntingdon Life Sciences Limited Project Identity:	CSS/010
Study completed on:	*To be advised*

Sponsor

American Chemistry Council
1300 Wilson Boulevard
Arlington
VA 22209
USA

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Author: C. E. Mason

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A), (B) or (C).

Submitter/Agent .....

Name ..... Signature .....

Title ..... Date .....

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L77/8).

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Draft

....

Christine Mason, B.Sc., Date
Study Director,
Department of Genetic Toxicology

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study.

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	20 May 2002	20 May 2002
Study Based Inspections		
Exposure	12 June 2002	18 June 2002
Dosing	13 June 2002	18 June 2002
Test item control and disposition	12 June 2002	18 June 2002
Process Based Inspections		
Slide scoring	3 July 2002	5 July 2002
Report Audit	1 October 2002	1 October 2002

Draft

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Study Based Inspections: Inspections and audits of phases of this study were conducted and reported to the Study Director and Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of other routine and repetitive procedures employed on this type of study were carried out. These were promptly reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results of this study to reflect the raw data.

....

Neal Jolly, B.Sc., M.R.Q.A., Date
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

CONTRIBUTING SCIENTISTS

Christine Mason, B.Sc.,
Study Director,
Department of Genetic Toxicology

Julie Connell, F.I.A.T.,
Chief Technician,
Department of Genetic Toxicology

Paul Morris, B.Sc., Ph.D.,
Senior Study Analyst,
Department of Toxicology

Chris Davison, B.Sc.,
Statistician,
Department of Statistics

CONTENTS

	Page
STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS	2
COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS	3
QUALITY ASSURANCE STATEMENT	4
CONTRIBUTING SCIENTISTS	5
SUMMARY	8
INTRODUCTION	9
TEST SUBSTANCE	11
EXPERIMENTAL PROCEDURE	12
ASSESSMENT OF RESULTS	15
MAINTENANCE OF RECORDS	16
RESULTS	17
CONCLUSION	18
REFERENCES	19

CONTENTS - continued

TABLES

		Page
1.	Summary of results and statistical analysis	20
2.	Results for individual animals - 24 hour sampling time after 2nd exposure	21

APPENDICES

1.	Mortality data	22
2.	Animal bodyweights	23
3.	Historical negative and positive control values	24
4.	GLP Compliance Statements, 2001	26
5.	Robust summary	28
6.	Composition of the Test Substance	29
7.	Certificate of Analysis	30
8.	Protocol	31
9.	ADMINISTRATION OF PYROLYSIS C5s BY INHALATION TO MICE	53

SUMMARY

This study was designed to assess the potential induction of micronuclei by Pyrolysis C5s in bone marrow cells of mice. Mice were treated with two 6-hour exposure periods of inhalation administration of the test substance, approximately 24 hours apart, at exposure levels of 40, 125 and 500 parts per million (ppm). A preliminary toxicity test had previously shown that an exposure level of 500 ppm was expected to be approximately the maximum tolerated; this level was therefore selected as an appropriate maximum for use in the micronucleus test.

The test substance and negative control were administered by whole body inhalation exposure. The negative control group received clean air. The positive control group was dosed orally with mitomycin C at 12 mg/kg bodyweight on one occasion, approximately 24 hours before termination.

Following the preliminary toxicity test, no substantial differences in toxicity were observed between the sexes, in line with current guidelines, the main test was performed using male animals only.

Bone marrow smears were obtained from seven male animals in the negative control, each of the test substance groups and five male animals in the positive control group 24 hours after the final dose. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. A record of the incidence of micronucleated mature erythrocytes was also kept.

The target vapour concentrations of Pyrolysis C5s were 40, 125 and 500 ppm for the low, intermediate and high dose groups. Analysed mean chamber concentrations for the two exposure periods were 44, 130 and 490 ppm, indicating that the test atmospheres were within the acceptable ranges of 10, 4 and 2% respectively, from target.

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decreases in the proportion of immature erythrocytes were observed in mice treated with Pyrolysis C5s and killed 24 hours after two 6-hour periods of whole body inhalation exposure, compared to negative control values ($P>0.01$ in each case).

The positive control compound, mitomycin C, produced significant increases in the frequency of micronucleated immature erythrocytes ($P<0.01$).

It is concluded that Pyrolysis C5s did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered by whole body inhalation exposure in this *in vivo* test procedure.

INTRODUCTION

The purpose of this study was to assess the potential of Pyrolysis C5s to induce mutagenic effects in mice following whole body inhalation exposure in an *in vivo* cytogenetic system (Boller and Schmid 1970, MacGregor *et al* 1987, Mavournin *et al* 1990). The inhalation route was chosen for this particular study as the most likely route of human exposure.

The performance of this study was in compliance with the following Guidelines:

> OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test, Guideline 474.
>
> EC Commission Directive 2000/32/EC Annex 4C – B.12. Mutagenicity – *In vivo* mammalian erythrocyte micronucleus test. No. L 136/50.
>
> US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5395 Mammalian erythrocyte micronucleus test. EPA 712-C-98-226.

The bone marrow micronucleus test is a short-term assay for identification of genotoxic effects associated with mutagens and carcinogens (Mavournin *et al* 1990). Young adult mice are chosen for use because of the high rate of cell division in the bone marrow, because of the wealth of background data on this species, and because of their general suitability for toxicological investigations.

In mitotic cells in which chromosomal breakage has been caused by the test substance or its metabolites, acentric fragments of the chromosomes do not separate at the anaphase stage of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and hence will form single or multiple micronuclei in the cytoplasm of these cells. Micronuclei are seen in a wide variety of cells, but erythrocytes are chosen for examination since micronuclei are not obscured by the main nucleus and are therefore easily detected in this cell type (Boller and Schmid 1970).

A few hours after the last mitosis is completed, erythroblasts expel their nucleus. Young erythrocytes, less than 24 hours old, stain blue with Giemsa due to the presence of ribonucleic acid and are termed immature erythrocytes (polychromatic erythrocytes). This ribonucleic acid gradually disappears so that more mature erythrocytes stain orange/pink (*ie* they show normochromatic staining). Mature erythrocytes may also be examined for the presence of micronuclei.

Substances which interfere with the mitotic spindle apparatus will cause non-disjunction or lagging chromosomes at anaphase which may not be incorporated into the daughter nuclei. These lagging chromosomes are not excluded from the erythroblast with the main nucleus and hence also give rise to micronuclei.

Any toxic effects of the test substance on the nucleated cells may lead either to a reduction in cell division or to cell death. These effects in turn lead to a reduction in the number of nucleated cells and immature erythrocytes. If the proportion of immature erythrocytes is found to be significantly less than the control value, this is taken as being indicative of toxicity.

The protocol was approved by Huntingdon Life Sciences Management on 2 April 2002, by the Sponsor on 9 April 2002 and by the Study Director on 16 May 2002.

The in-life phase of the study was performed at the Department of Inhalation Toxicology at Huntingdon Life Sciences, Huntingdon, Cambridgeshire, PE28 4HS, England. Slide scoring and reporting were performed by the Department of Genetic Toxicology and statistical analysis by the Department of Statistics at Huntingdon Life Sciences, Eye, Suffolk, IP23 7PX, England.

The experimental start and completion dates of the study were 22 May 2002 and 26 July 2002 respectively.

TEST SUBSTANCE

Identity:	Pyrolysis C5s
Alternative name:	Isoprene, Pentadiene
Composition:	Complex mixture of hydrocarbons (Appendix 6)
Lot number:	QA1001A100
Expiry date:	*to be advised on completion of CSS/017 and included in final report*
Appearance:	Clear, colourless liquid
Storage conditions:	*ca* 10°C in the dark under nitrogen
Source:	Dow Chemical, PO Box 150, Highway 1 South, Plaquemine, LA 70765-0150, USA
Date received:	21 January 2002

EXPERIMENTAL PROCEDURE

ANIMALS

All animals used on this study were CD-1 mice. Males weighed between 28 and 30 grams and females weighed between 22 and 24 grams on despatch from Charles River UK Limited, Margate, Kent, England.

On arrival the weight of the animals was checked and found to be acceptable. The animals were randomly assigned to groups and tail marked. Each group was kept, with the sexes separated, in cages and maintained in a controlled environment. Temperature and relative humidity recordings were within the target ranges of 21±2°C and 55±10% respectively throughout the study. The room was illuminated by artificial light for 12 hours per day. All animals were allowed free access to pelleted expanded rat and mouse No.1 maintenance diet (SQC grade obtained from Special Diets Services Ltd, Witham, Essex, UK) and tap water *ad libitum*, except during exposure periods when food and water were withdrawn. Food and tap water are routinely analysed for quality at source. All animals were acclimatised for a minimum of 6 days, examined daily and weighed prior to dosing.

TREATMENT PROCEDURE AND ATMOSPHERE ANALYSIS

Full details of the treatment procedure and atmosphere analysis are presented in Appendix 8 (Administration of Pyrolysis C5s by Inhalation to Mice).

POSITIVE CONTROL FORMULATION AND ADMINISTRATION

Mitomycin C, obtained from Sigma Chemical Company, batch number 31K2502, was used as the positive control compound. It was prepared as a solution in purified water, at a concentration of 1.2 mg/ml, just prior to administration and was dosed orally by gavage at 10 ml/kg bodyweight.

DATES OF DOSING

Preliminary toxicity test:	29 and 30 May 2002
Micronucleus test:	12 and 13 June 2002

PRELIMINARY TOXICITY TEST

The purpose of this test was to determine a suitable dose level for use in the micronucleus test. The dosages employed were used to give an approximate indication of the maximum tolerated dose, *ie* the highest dosage which would be expected to elicit signs of toxicity without producing extreme clinical signs or having a significant effect on survival. The experimental design is shown below:

Group	Treatment	Two 6-hour exposures (ppm)	Number of mice	
			Male	Female
1	Pyrolysis C5s	500	2	2
2	Pyrolysis C5s	1000	1[a]	2
3	Pyrolysis C5s	2000	2	2
4	Pyrolysis C5s	3000	2	2

a One male animal was found dead before the first exposure period. Death was considered to be due to fighting. No spare animals were available to replace it.

ppm Parts per million

Following dosing, the animals were observed regularly during the working day for a period of 48 hours and any mortalities or clinical signs of reaction during the experiment were recorded. At the end of this observation period, surviving animals were killed and discarded.

MICRONUCLEUS TEST

From the results obtained in the preliminary toxicity test, no substantial differences in toxicity were observed between the sexes and in line with current guidelines, the main test was performed using males only. Exposure levels of 40, 125 and 500 ppm were selected for the micronucleus test. The experimental design is shown below:

Group	Treatment	Two 6-hour exposures (ppm)	Number of mice
			Male
1	Negative control	-	14
2	Pyrolysis C5s	40	7
3	Pyrolysis C5s	125	7
4	Pyrolysis C5s	500	14
5	Mitomycin C	12 (mg/kg)	5

Following dosing, the animals were examined regularly and any mortalities or clinical signs of reaction were recorded. Seven males from the negative control, each of the test substance groups and five males from the positive control group were sacrificed 24 hours after the 2nd exposure period.

The animals were killed by cervical dislocation following carbon dioxide inhalation and both femurs dissected out from each animal. The femurs were cleared of tissue and the proximal epiphysis removed from each bone. The bone marrow of both femurs from each animal was flushed out and pooled in a total volume of 2 ml of pre-filtered foetal calf serum. The cells were sedimented by centrifugation, the supernatant was discarded and the cells were resuspended in a small volume of fresh serum. A small drop of the cell suspension was transferred to a glass microscope slide and a smear was prepared in the conventional manner (Schmid 1976). Three smears were made from each animal. The prepared smears were fixed in methanol (> 10 minutes). After air-drying the smears were stained for 10 minutes in 10% Giemsa (prepared by 1 : 9 dilution of Giemsa with purified water). Following rinsing in purified water and differentiation in buffered purified water, the smears were rinsed in purified water, air-dried and mounted with coverslips using DPX.

The stained smears were examined (under code) by light microscopy to determine the incidence of micronucleated cells per 2000 polychromatic erythrocytes per animal. One smear per animal was examined and the remaining smears were held temporarily in reserve in case of technical problems with the first smear.

Micronuclei are identified by the following criteria:

- Large enough to discern morphological characteristics
- Should possess a generally rounded shape with a clearly defined outline
- Should be deeply stained and similar in colour to the nuclei of other cells - not black
- Should lie in the same focal plane as the cell
- Lack internal structure, *ie* they are pyknotic
- There should be no micronucleus-like debris in the area surrounding the cell.

The proportion of immature erythrocytes for each animal was assessed by examination of at least 1000 erythrocytes. A record of the number of micronucleated mature erythrocytes observed during assessment of this proportion was also kept as recommended by Schmid (1976).

ASSESSMENT OF RESULTS

The results for each treatment group were compared with the results for the concurrent control group using non-parametric statistics. Non-parametric statistical methods were chosen for analysis of results because:

- They are suited to analysis of data consisting of discrete/integer values with ties such as the incidence of micronucleated immature erythrocytes
- The methods make few assumptions about the underlying distribution of data and therefore the values do not require transformation to fit a theoretical distribution (where data can be approximately fitted to a normal distribution, the results of non-parametric analysis and classical analysis of variance are very similar)
- 'Outliers' are frequently found in the proportion of immature erythrocytes for both control and treated animals; non-parametric analysis based on rank does not give these values an undue weighting.

For incidences of micronucleated immature erythrocytes, exact one-sided P-values are calculated by permutation (StatXact, CYTEL Software Corporation, Cambridge, Massachussetts). Comparison of several dose levels are made with the concurrent control using the Linear by Linear Association test for trend in a step-down fashion if significance is detected (Agresti *et al.* 1990); for individual inter-group comparisons (*ie* the positive control group) this procedure simplifies to a straightforward permutation test (Gibbons 1985). For assessment of effects on the proportion of immature erythrocytes, equivalent permutation tests based on rank scores are used, *ie* exact versions of Wilcoxon's sum of ranks test and Jonckheere's test for trend.

A positive response is normally indicated by a statistically significant dose-related increase in the incidence of micronucleated immature erythrocytes for the treatment group compared with the concurrent control group ($P<0.01$); individual and/or group mean values should exceed the laboratory historical control range (Morrison and Ashby 1995). A negative result is indicated where individual and group mean incidences of micronucleated immature erythrocytes for the group treated with the test substance are not significantly greater than incidences for the concurrent control group ($P>0.01$) and where these values fall within the historical control range. An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant dose-related decrease in the proportion of immature erythrocytes ($P<0.01$).

MAINTENANCE OF RECORDS

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen that are unsuitable, by reason of instability, for long term retention and archiving may be disposed of.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of ten years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

PRELIMINARY TOXICITY TEST

Mortality data for the study are presented in Appendix 1.

To determine a suitable dose level for use in the micronucleus test, four groups of animals were dosed with Pyrolysis C5s at concentrations of 500, 1000, 2000 and 3000 ppm. At 3000 ppm, all animals (two male and two female) died following the first six hour exposure period.

At 2000 ppm, one male and one female animal died after the first exposure and the remaining male and female died after the second exposure.

At 1000 ppm, one male animal died before the start of exposures. This death was considered to be due to fighting and was not treatment related. As a result only one male and two female animals were available for dosing. One female died after the first exposure period. The remaining male and female animal survived to scheduled termination. Clinical signs observed in these animals included hunched posture, piloerection and unsteady gait.

At 500 ppm, Clinical signs included hunched posture and piloerection during the second exposure period. No other clinical signs were recorded and all four animals survived to scheduled termination.

On the basis of these results and in consultation with the Study Sponsor, 500 ppm was considered to be the maximum tolerated dose. Dose levels of 40, 125 and 500 ppm were selected for use in the micronucleus test.

MICRONUCLEUS TEST

No substantial differences in toxicity between the sexes, as evidenced by clinical signs, were observed in the preliminary toxicity test, therefore, in line with current guidelines the main micronucleus test was performed using male animals only.

Table 1 gives a summary of the results of the micronucleus test and the results of statistical analysis. The results for individual animals are presented in Table 2. Animal bodyweight data is presented in Appendix 2. Appendix 3 summarises the historical vehicle and positive control data for micronucleated polychromatic erythrocytes.

Clinical signs and mortalities

No mortalities occurred in this test, and no clinical signs were observed for the negative control, test substance treated and positive control group animals over the duration of the test.

Bodyweights recorded throughout the test showed some weight loss in all groups but these were small and were not considered to be treatment related.

Micronucleated immature erythrocyte counts (mie)

The test substance did not cause any statistically significant increases in the number of micronucleated immature erythrocytes at either sampling time (P>0.01).

Mitomycin C caused significant increases (P<0.01) in the frequency of micronucleated immature erythrocytes.

Micronucleated mature erythrocytes (mme)

The test substance did not cause any substantial increases in the incidence of micronucleated mature erythrocytes at either sampling time.

Proportion of immature erythrocytes (% ie/(ie + me)

The test substance failed to cause any significant decreases in the proportion of immature erythrocytes (P>0.01).

Mitomycin C did not cause any statistically significant decreases in the proportion of immature erythrocytes (P>0.01).

VAPOUR CONCENTRATION

Control of the delivery of Pyrolysis C5s vapour to the exposure chambers was good. The target concentrations were 40, 125 and 500 ppm for Groups 2, 3 and 4 (low, intermediate and high dose groups). Analysed mean chamber concentrations for the two exposure periods were 44, 130 and 490 ppm, indicating that the test atmospheres were within the acceptable ranges of 10, 4 and 2% respectively, from target.

CONCLUSION

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decreases in the proportion of immature erythrocytes were observed in mice treated with Pyrolysis C5s and killed 24 hours after two 6-hour periods of whole body inhalation exposure, compared to negative control values (P>0.01 in each case).

It is concluded that Pyrolysis C5s did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered by whole body inhalation exposure in this *in vivo* test procedure.

REFERENCES

AGRESTI, A., MEHTA, C.R. and PATEL, N.R. (1990) Exact inference for contingency tables with ordered categories. *Journal of the American Statistical Association*, **85**, 453.

BOLLER, K. and SCHMID, W. (1970) Chemical mutagenesis in mammals. The bone marrow of the Chinese hamster as an *in vivo* test system. Haematological findings after treatment with Trenimon (translation). *Humangenetik*, **11**, 34.

CYTEL (1995) *StatXact 3 for Windows: Statistical Software for Exact Nonparametric Inference.* Cytel Software Corporation, NC, USA.

GIBBONS, J.D. (1985) Nonparametric Statistical Inference, 2nd edition, Marcel Dekker, New York.

JONCKHEERE, A.R. (1954) A distribution-free k-sample test against ordered alternatives. *Biometrics*, **41**, 133-145.

MacGREGOR, J.T., HEDDLE, J.A., HITE, M., MARGOLIN, B.H., RAMEL, C., SALAMONE, M.F., TICE, R.R. and WILD, D. (1987) Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutation Research*, **189**, 103.

MAVOURNIN, K.H., BLAKEY, D.H., CIMINO, M.C., SALAMONE, M.F. and HEDDLE, J.A. (1990) The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the US Environmental Protection Agency Gene-Tox Program. *Mutation Research*, **239**, 29.

MORRISON, V. and ASHBY, J. (1995) High resolution rodent bone marrow micronucleus assays of 1,2-dimethylhydrazine: implication of systemic toxicity and individual responders. *Mutagenesis*, **10**, 129.

SAS INSTITUTE (1999) SAS OnlineDoc® Version Eight. SAS Institute Inc., Cary, NC, USA.

SCHMID, W. (1976) The micronucleus test for cytogenetic analysis. In: HOLLANDER, A. (ed.) *Chemical Mutagens, Principles and Methods for their Detection*, **4**, 31. Published by Plenum Press, New York.

WILCOXON, F. (1945). Individual comparisons by ranking methods. *Biometrics Bulletin*, **1**, 80-83.

TABLE 1

Summary of results and statistical analysis

Sampling time after 2nd exposure	Treatment	Exposure (ppm)	% ie/(ie+me) † (mean)	Incidence mie (mean)	Incidence mme[a] (group mean)
24 Hours	Negative control	-	49	1.9	2.7
	Pyrolysis C5s	40	47	2.0	2.7
	Pyrolysis C5s	125	49	1.4	1.1
	Pyrolysis C5s	500	40	1.4	3.3
	Mitomycin C#	12 (mg/kg)	52	49.8**	0.8

Negative control	Clean air
ppm	Parts per million
#	Dosed on one occasion approximately 24 hours before termination
% ie/(ie+me)	Proportion of immature erythrocytes
mie	Number of micronucleated cells observed per 2000 immature erythrocytes examined
mme	Number of micronucleated cells calculated per 2000 mature erythrocytes

Results of statistical analysis using the appropriate nonparametric method of analysis based on permutation (one-sided probabilities):

** $P < 0.01$ (significant)
otherwise $P > 0.01$ (not significant)

[a] Formula for calculation of incidence mme (group mean):

$$\frac{\text{Sum of group incidence } \textbf{mme} \text{ scored x 2000}}{\text{Sum of group } \textbf{me} \text{ scored}}$$

† Occasional apparent errors of ± 1% may occur due to rounding of values for presentation in the table

TABLE 2

Results for individual animals - 24 hour sampling time after 2nd exposure

Treatment	Exposure (ppm)	Animal number	ie	me	% ie/(ie+me)	Incidence mie	Incidence mme
Negative control	-	M 201	543	470	54	1	1
		M 202	475	543	47	2	0
		M 203	365	703	34	0	1
		M 204	670	346	66	0	0
		M 205	379	690	35	5	1
		M 206	412	590	41	3	1
		M 207	660	354	65	2	1
Pyrolysis C5s	40	M 211	467	547	46	2	0
		M 212	528	474	53	1	0
		M 213	531	472	53	2	2
		M 214	467	565	45	3	2
		M 215	475	542	47	3	1
		M 216	387	630	38	0	0
		M 217	473	534	47	3	0
Pyrolysis C5s	125	M 221	584	461	56	2	0
		M 222	543	492	52	1	0
		M 223	499	524	49	1	0
		M 224	516	504	51	2	1
		M 225	520	480	52	1	0
		M 226	423	591	42	1	0
		M 227	451	557	45	2	1
Pyrolysis C5s	500	M 231	465	595	44	0	0
		M 232	380	658	37	4	2
		M 233	480	540	47	3	0
		M 234	378	637	37	1	2
		M 235	406	620	40	0	1
		M 236	341	661	34	1	1
		M 237	416	585	42	1	1
Mitomycin C#	12 (mg/kg)	M 241	504	504	50	119	0
		M 242	613	400	61	17	0
		M 243	526	511	51	58	1
		M 244	463	555	45	26	0
		M 245	553	471	54	29	0

Negative control	Clean air
ppm	Parts per million
#	Dosed on one occasion approximately 24 hours before termination
% ie/(ie+me)	Proportion of immature
mie	Number of micronucleated cells observed per 2000 immature
me	Total number of mature erythrocytes examined for
mme	Number of micronucleated mature erythrocytes observed

APPENDIX 1

Mortality data

Phase	Group	Treatment	Exposure (ppm)	Mortality ratio = No. of deaths / No. dosed		
				Males	Females	Combined
Preliminary toxicity test	1	Pyrolysis C5s	500	0/2	0/2	0/4
	2	Pyrolysis C5s	1000	0/1	1/2	1/3
	3	Pyrolysis C5s	2000	2/2	2/2	4/4
	4	Pyrolysis C5s	3000	2/2	2/2	4/4
Micronucleus test	1	Negative control	-	0/7	0/0	0/7
	2	Pyrolysis C5s	40	0/7	0/0	0/7
	3	Pyrolysis C5s	125	0/7	0/0	0/7
	4	Pyrolysis C5s	500	0/7	0/0	0/7
	5	Mitomycin C	12 (mg/kg)	0/5	0/0	0/5

ppm Parts per million

APPENDIX 2

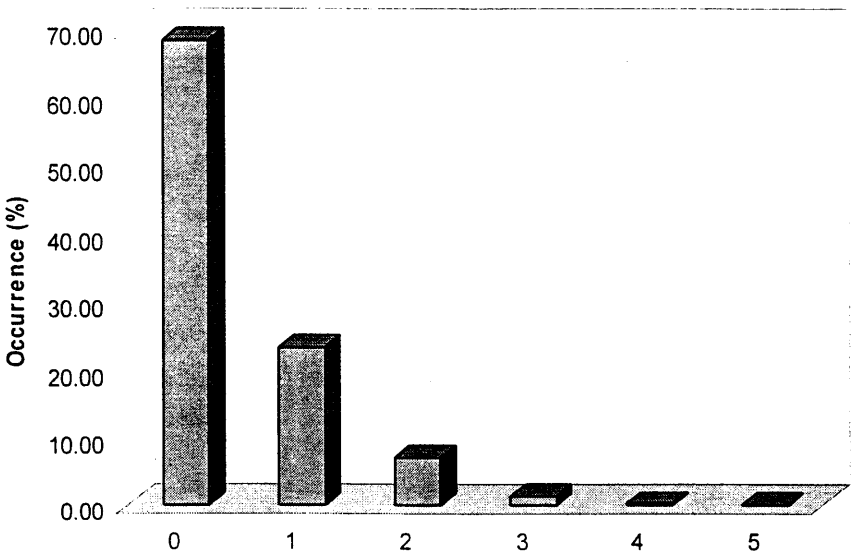
Animal bodyweights

Animal number		Treatment (ppm)	Bodyweight (g) Arrival Individual	Arrival Mean	At start of 1st exposure period Individual	At start of 1st exposure period Mean	At start of 2nd exposure period Individual	At start of 2nd exposure period Mean	Termination Individual	Termination Mean
201	M	Negative control	28.4	27.0	31.5	32.1	31.9	32.4	31.6 †	32.3
202	M	(-)	25.2		33.6		34.4		34.5	
203	M		25.3		29.4		29.9		29.8 †	
204	M		28.6		33.3		33.7		33.5 †	
205	M		27.0		31.3		31.5		30.9 †	
206	M		25.8		32.7		32.6 †		32.9	
207	M		28.5		32.8		32.9		32.6 †	
211	M	Pyrolysis C5s	26.8	27.0	34.0	32.3	33.8 †	32.2	32.9 †	31.7
212	M	(40 ppm)	27.2		33.3		32.9 †		32.9	
213	M		25.8		30.9		30.6 †		30.5 †	
214	M		28.4		32.2		32.3		31.5 †	
215	M		26.3		32.0		32.6		32.0 †	
216	M		28.1		31.9		31.4 †		31.0 †	
217	M		26.5		31.9		32.0		31.4 †	
221	M	Pyrolysis C5s	29.0	27.4	33.3	32.8	33.5	32.7	33.2 †	32.3
222	M	(125 ppm)	25.3		32.5		31.7 †		30.9 †	
223	M		28.0		33.2		32.7 †		33.3	
224	M		27.7		34.0		33.2 †		33.6	
225	M		27.3		32.0		32.1		31.8 †	
226	M		27.1		31.9		32.1		31.0 †	
227	M		27.1		32.9		33.4		32.2 †	
231	M	Pyrolysis C5s	24.9	26.4	32.0	32.3	31.8 †	32.1	31.3 †	31.6
232	M	(500 ppm)	26.0		30.4		30.1 †		30.0 †	
233	M		27.4		32.3		32.2 †		31.1 †	
234	M		26.5		32.6		32.3 †		31.0 †	
235	M		26.0		32.7		32.3 †		32.0 †	
336	M		26.0		30.8		31.0		30.9 †	
237	M		27.7		35.2		35.3		34.9 †	
241	M	Mitomycin C	25.5	26.7	31.8	32.8	32.0	33.2	32.0	32.9
242	M	(12 mg/kg)	26.9		33.5		34.2		33.9 †	
243	M		26.9		32.7		32.9		32.9	
244	M		27.3		33.6		33.9		32.9 †	
245	M		26.8		32.4		33.1		32.6 †	

† Denotes weight loss from previous weighing
ppm Parts per million

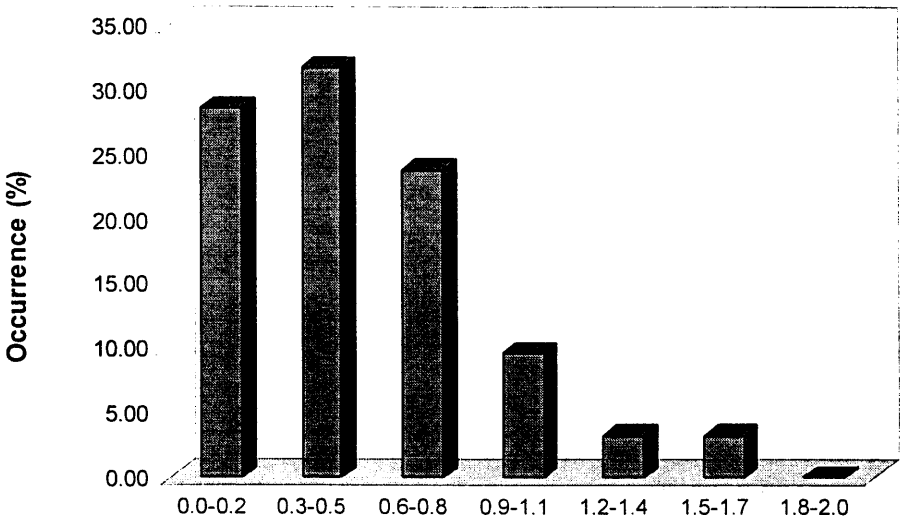
APPENDIX 3

Historical negative control values (1 July 2000 - 30 June 2002)
Frequency of micronucleated immature erythrocytes (Individual animals)



Number of micronucleated immature erythrocytes
(MIE per 2000 cells)
Individual mean 0.42

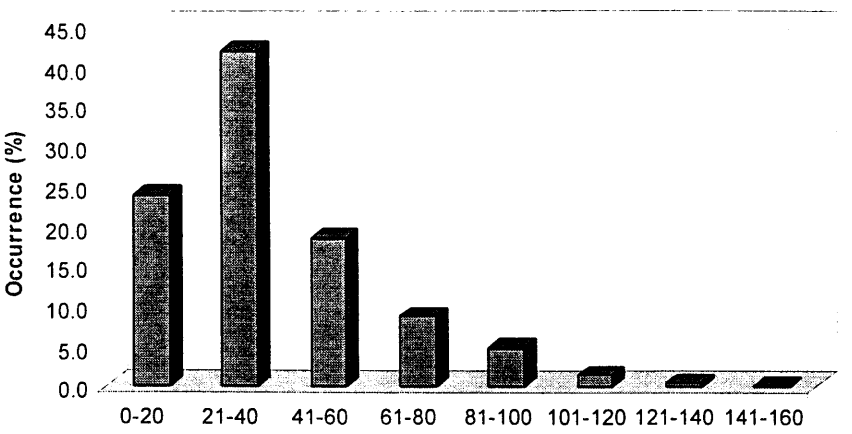
Historical negative control values (1 July 2000 - 30 June 2002)
Frequency of miconucleated immature erythrocytes (Group mean)



Number of micronucleated immature erythrocytes
(MIE per 2000 cells)
Group mean 0.41

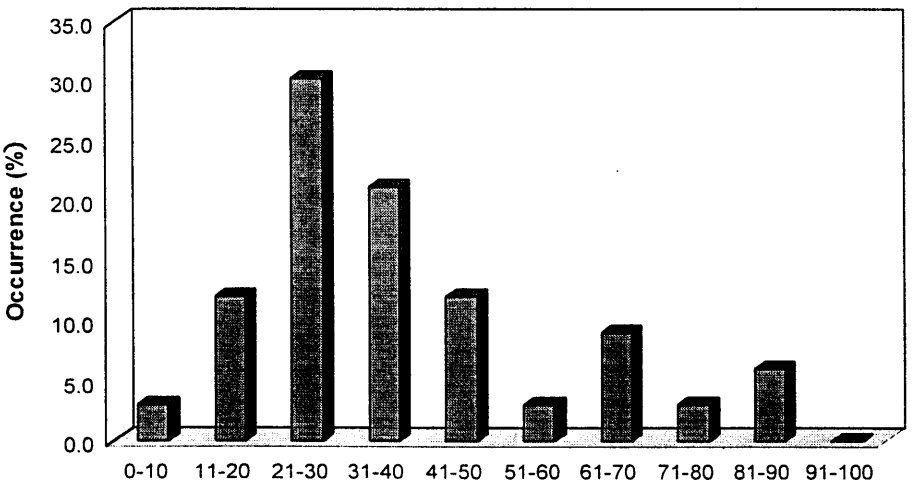
APPENDIX 3 - continued

Historical positive control values (1 July 2000 - 30 June 2002)
Frequency of micronucleated immature erythrocytes (Individual animals)
Mitomycin C at 12 mg/kg



Individual mean of micronucleated immature erythrocytes
(MIE per 2000 cells)
Individual mean 37.63

Historical positive control values (1July 2000 - 30 June 2002)
Frequency of micronucleated immature erythrocytes (Group mean)
Mitomycin C at 12 mg/kg



Group mean number of micronucleated immature erythrocytes
(MIE) per 2000 cells
Group mean 37.92

APPENDIX 4
Eye Research Centre
GLP Compliance Statement 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

29th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

APPENDIX 4 - continued

Huntingdon Research Centre
GLP Compliance Statement 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Wooley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION
15th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

APPENDIX 5

Robust summary

5.6 GENETIC TOXICITY 'IN VIVO'

Type	:	Micronucleus assay
Species	:	mouse
Sex	:	male
Strain	:	CD-1
Route of admin.	:	inhalation
Exposure period	:	2 x 6 hours approximately 24 hours apart
Doses	:	40, 125, and 500 parts per million.
Result	:	negative
Method	:	OECD Guide-line 474 "Genetic Toxicology: Micronucleus Test"
Year	:	2002
GLP	:	yes
Test substance	:	See below

Test substance	:	Identity:	Pyrolysis C5s
		Alternative name:	Isoprene, Pentadiene
		Composition:	Complex mixture of hydrocarbons
		Lot number:	QA1001A100
		Expiry date:	To be advised
		Appearance:	Clear, colourless liquid
		Storage conditions:	ca 10°C in the dark under nitrogen
		Source:	Dow Chemical, PO Box 150, Highway 1 South, Plaquemine, LA 70765-0150, USA
		Date received:	21 January 2002

Reliability	:	(1) valid without restriction

Reference: Huntingdon Life Sciences Ltd. 2002. Mouse Micronucleus Test. Project ID CSS 010. Huntingdon Life Sciences Ltd., Cambridgeshire, England

APPENDIX 6

Composition of the Test Substance

Pyrolysis C5s

Component identity	*Empirical formula*	*Typical composition*
1,4-Pentadiene	C_5H_8	2.6402%
Butyne-2	C_4H_6	0.9455%
Pentene-1	C_5H_{10}	6.3361%
2-Methyl-butene-1	C_5H_{10}	3.5895%
Isoprene	C_5H_8	17.8389%
trans-Pentene-2	C_5H_{10}	2.8318%
cis-Pentene-2	C_5H_{10}	1.776%
2-Methyl-butene-2	C_5H_{10}	2.5095%
trans-1,3-Pentadiene	C_5H_8	10.0028%
Cyclopentadiene	C_5H_6	7.7%
cis-1,3-Pentadiene	C_5H_8	6.122%
Cyclopentene	C_5H_8	7.0142%
Cyclopentane	C_5H_{10}	1.3326%
Butene	C_4H_8	0.0032%
1,3-Butadiene	C_4H_6	0.0403%
N-Butane	C_4H_{10}	0.0193%
trans-Butene-2	C_4H_8	0.1625%
cis-Butene-2	C_4H_8	0.4682%
1,2-Butadiene	C_4H_6	0.3055%
3-Methyl-butene-1	C_5H_{10}	0.5599%
Iso-pentane	C_5H_{12}	8.0069%
3,3-Dimethyl, 1-butene	C_6H_{12}	0.0714%
4-Methyl-1-pentene	C_6H_{12}	0.161%
2-Methyl-pentane	C_6H_{14}	1.7335%
1,5-Hexadiene	C_6H_{10}	0.1675%
3-Methyl-pentane	C_6H_{14}	0.3081%
Hexane	C_6H_{14}	0.041%
Dicyclopentadiene	$C_{10}H_{12}$	5.8744%
Ethylacetylene	C_4H_6	0.0102%
N-Pentane	C_5H_{12}	9.5163%
2,3-Dimethyl butane	C_6H_{14}	0.1772%
3-Methyl-1-Pentane	C_6H_{12}	0.3278%

APPENDIX 7

Certificate of Analysis

To be included

APPENDIX 8

Study number: CSS/010

Huntingdon Life Sciences

CONFIDENTIAL

PROTOCOL

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Sponsor

American Chemical Council
1300 Wilson Boulevard
Arlington
VA 22201
USA

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 16

Huntingdon Life Sciences Ltd., registered in England No: 1815730

Study number: CSS/010

Huntingdon Life Sciences

STUDY DETAILS PAGE

Study number:	CSS/010
Study title:	Mouse micronucleus test
Test substance:	
Identity:	Pyrolysis C5s
CAS No:	68476-55-1
Batch number:	QA1001A100
Expiry:	Assumed to be stable for the duration of the test. Verification to be conducted after the completion of all testing and results to be included in the report.
Appearance:	Clear, colourless liquid
Storage conditions:	*ca* 10°C in the dark under nitrogen
Purity/Assay:	Not advised, Sponsor's responsibility
Route of administration:	Inhalation exposure
Dosages for the preliminary toxicity test:	500, 1000, 2000, 3000 ppm
Stability of test substance:	Not assessed in this study
Analysis of achieved concentration:	To be assessed during exposure
Sponsor's representative for American Chemistry Council:	Dr Elizabeth J. Moran, Ph.D.
Head of Department, Genetic Toxicology:	Dr M. Gillian Clare
Study Director:	Mrs Christine E. Mason
Person acting in the temporary absence of the Study Director:	Dr Zahid Mehmood
Location of study:	
Inhalation exposure and statistical analysis:	Departments of Inhalation and Statistics, Huntingdon Life Sciences Ltd., Huntingdon, Cambridgeshire, PE28 4HS, England.
Slide scoring and reporting:	Department of Genetic Toxicology, Huntingdon Life Sciences Ltd., Eye, Suffolk, IP23 7PX, England.
Proposed study dates	
Experimental start:	*ca* 22 May 2002
Experimental completion:	*ca* 19 July 2002
Draft report:	30 August 2002

STUDY DIRECTOR APPROVAL OF PROTOCOL

C.E. Mason — 16th May 2002

Christine E. Mason
Study Director
Huntingdon Life Sciences Ltd

Date

Study number: CSS/010

Huntingdon Life Sciences

PROTOCOL APPROVAL

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Gill Clare
Dr M. Gillian Clare
Management
Huntingdon Life Sciences Ltd

2nd April 2002
Date

American Chemical Council
Sponsor

April 9 2002
Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Director approval of the protocol is given on the study details page of the protocol once such details have been established and agreed. The completed page will be issued prior to the start of the study.

Study number: CSS/010

CONTENTS

Page

PROTOCOL APPROVAL iii

1 INTRODUCTION 2

2. BACKGROUND 2

3. EXPERIMENTAL PROCEDURE 3

4. ASSESSMENT OF RESULTS 9

5. REPORTING 10

6. MAINTENANCE OF RECORDS 11

7 GOOD LABORATORY PRACTICE 11

8 QUALITY ASSURANCE 11

9 HEALTH & SAFETY 12

10. REFERENCES 12

Study number: CSS/010

1. INTRODUCTION

The object of this study is to assess the mutagenic potential of the test substance in an *in vivo* cytogenetic test system which complies with the following guidelines:

OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test. Guideline 474.

EC Commission Directive 2000/32/EC Annex 4C – B.12. Mutagenicity – *In vivo* mammalian erythrocyte micronucleus test. No. L 136/50.

US EPA (1998) Health Effects Test Guidelines: OPPTS 870.5395 Mammalian erythrocyte micronucleus test. EPA 712-C-98-226.

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

2. BACKGROUND

The bone marrow micronucleus test, originally developed by Matter and Schmid (1971), is a widely employed and internationally accepted short-term assay for identification of genotoxic effects (chromosome damage and aneuploidy) associated with mutagens and carcinogens (Mavournin *et al* 1990). This *in vivo* system allows consideration of various factors including pharmacokinetics, metabolism and DNA repair which cannot be accurately modelled in an *in vitro* system. Young adult mice are chosen for use because of the high rate of cell division in the bone marrow, because of the wealth of background data on this species, and because of their general suitability for toxicological investigations.

In mitotic cells in which chromosomal breakage has been caused by the test substance or its metabolites, acentric fragments of the chromosomes do not separate at the anaphase stage of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and hence will form single or multiple micronuclei (Howell-Jolly bodies) in the cytoplasm of these cells. Micronuclei are seen in a wide variety of cells, but erythrocytes are chosen for examination since micronuclei are not obscured by the main nucleus and are therefore easily detected in this cell type (Boller and Schmid 1970).

A few hours after the last mitosis is completed, erythroblasts expel their nucleus. Young erythrocytes, less than 24 hours old, stain blue with Giemsa due to the presence of ribonucleic acid and are termed polychromatic. This ribonucleic acid gradually disappears so that more mature erythrocytes stain orange/pink (*ie* they show normochromatic staining). Virtually all the chromosome damage detected in immature erythrocytes will have been caused during the recent exposure to the test substance. Mature erythrocytes may also be examined for the presence of micronuclei. No substantial increases in the incidence of micronuclei in mature erythrocytes would usually be expected 24 hours after administration of a chromosome-damaging agent; any micronucleus-like artifacts (which could otherwise possibly give a false positive result) are therefore readily distinguishable in this cell type (Schmid 1976).

Substances which interfere with the mitotic spindle apparatus will cause non-disjunction (unequal separation of the chromosomes at anaphase resulting in aneuploidy) or lagging chromosomes at anaphase which may not be incorporated into the daughter nuclei. These lagging chromosomes are not excluded from the erythroblast with the main nucleus and hence also give rise to micronuclei.

Any toxic effects of the test substance on the nucleated cells may lead either to a reduction in cell division or to cell death. These effects in turn lead to a reduction in the number of nucleated cells and immature erythrocytes; to compensate for this, peripheral blood is shunted into the bone marrow (von Ledebur and Schmid 1973). If the proportion of immature erythrocytes is found to be significantly less than the control value, this is taken as being indicative of toxicity. A very large decrease in the proportion would be indicative of a cytostatic or cytotoxic effect.

3. EXPERIMENTAL PROCEDURE

Animal management

Species	Mouse
Strain	CD-1 albino outbred of Swiss origin (Specific Pathogen Free)
Source	Charles River UK Limited, Margate, England.
Age	*ca* 35 days old (on despatch)
Weight range (male)	28 - 30 g (on despatch); within ±20% of mean at treatment
Weight range (female)	22 - 24 g (on despatch); within ±20% of mean at treatment
Acclimatisation	On receipt, animals will be randomly assigned to groups, identified by unique tailmark and weighed. Animals will be acclimatised for at least seven days before the experiment is performed.

Study number: CSS/010

Housing	The animals will be housed and dosed within one room. Room temperature will be controlled within the limits 21 ± 2°C and relative humidity will be controlled to 55 ± 10% RH. These parameters will be continuously monitored using a Kent Clearspan chart recorder. Lighting will be controlled to provide 12 hours artificial light (0630-1830 hours) in each 24-hour period. Environmental controls provide at least 12 room-air changes per hour. An auxiliary power supply is available to ensure conditions are maintained in the event of main supply failure. Animals will be housed in groups, with the sexes separated, in suspended polypropylene, solid bottom cages (North Kent Plastic Cages Ltd) equipped with stainless steel, grided lids. Each cage measures 30 cm long, 10.5 cm wide and 11.5 cm high. Each cage will be identified by label (colour-coded according to group) which will display the study number, cage number, sex and individual animal numbers. The cages will be suspended on moveable batteries. Animals in the positive control group will be housed in the same conditions. The animals within each dosage group will be kept in a separate ventilated cabinet within the dosing room to prevent cross-contamination between groups. Room air is exhausted through the holding cabinets, therefore, the cabinets draw their air supply from the main room. Environmental control sensors are mounted in the exhaust ducting ensuring that the main room and holding cabinet temperature and relative humidity are uniform.
Diet	Animals will be provided with pelleted expanded rat and mouse No. 1 maintenance diet (SQC grade obtained from Special Diets Services Ltd, Witham, Essex, UK) and tap water *ad libitum* except diet and water will be withheld during the period of inhalation exposure. Food and water are routinely analysed for quality at source and a copy of the relevant certificates will be maintained in the study data file. Dietary contaminants, known or reasonably anticipated, are not expected to be present at levels that interfere with study objectives.

Positive control

Identity	Mitomycin C
Supplier	Acros Chemica or other suitable supplier
Appearance	Blue Powder
Vehicle	Purified water
Dosage preparation	Solution prepared just prior to use

Study number: CSS/010

Preliminary toxicity test

Animals will be dosed via the inhalation route as detailed below and will be examined for toxic signs and mortalities during exposure and at least once daily after each exposure period. Surviving animals will be killed and discarded 24 hours after the second exposure.

The design of the preliminary toxicity test will depend on available information on the acute toxicity of the test substance. Typically (in the first instance) four groups of two male and two female mice will be treated with the test substance for two six hour exposure periods, at the appropriate dose levels and sacrificed 24 hours after the second exposure, to accurately determine an estimated maximum tolerated dosage (MTD) for use in the main micronucleus test. The MTD is defined as a dose producing signs of toxicity such that a slightly higher dose would be expected to cause (or has already been shown to cause) lethality or unacceptably severe clinical signs. Dosing concentrations will be based on data for the test substance but will not exceed 50 % of the Lower Explosive Limit (LEL).

Main test

Where no substantial difference in toxicity between sexes is evident from the preliminary study, the main study will be performed using male animals only. Where a substantial difference in toxicity is apparent, the main test will be performed using male and female animals. Treatment levels will be selected for use in the main test in consultation with the Study Sponsor.

The study designs are shown below. Animals in negative and test substance groups will be treated by inhalation exposure. Animals in the positive control group will be treated once orally by intragastric gavage using a dosage volume of 10 ml per kg.

Study design - main test (single sex)

Group/ colour code	Material	Exposure (ppm)	Sampling time[a] (hours)	No. of animals M	Animal numbers M
1 : White	Clean air	-	24	7	201-207
2 : Yellow	Pyrolysis C5s	*x*/4	24	7	211-217
3 : Blue		*x*/2	24	7	221-227
4 : Pink		*x*	24	7	231-237
5 : Orange	Mitomycin C	12 (mg/kg)	24	5	241-245

a Sampling time from completion of 2nd exposure period

x The estimated maximum tolerated dosage or 50% of the LEL.

ppm parts per million

Study number. CSS/010

Study design - main test (both sexes)

Group/ colour code	Material	Exposure (ppm)	Sampling time [a] (hours)	No. of animals		Animal numbers	
				M	F	M	F
1 : White	Clean air	-	24	5	5	201-205	206-210
2 : Yellow	Pyrolysis C5s	*x*/4	24	5	5	211-215	216-220
3 : Blue	Pyrolysis C5s	*x*/2	24	5	5	221-225	226-230
4 : Pink	Pyrolysis C5s	*x*	24	5*	5*	231-235	236-240
5 : Orange	Mitomycin C	12 (mg/kg)	24	5	5	241-245	246-250

a Sampling time from completion of 2nd exposure period

* If the test substance has shown toxicity in the preliminary toxicity test, additional animals may be dosed with the test substance to ensure an adequate group size for analysis of micronucleus induction. Any animals not used to prepare bone marrow smears will be killed and discarded at the 24 hour sampling time.

x The estimated maximum tolerated dosage or 50% of the Lower Explosive Limit (LEL).

Animals will be examined during each exposure period and at least once daily after exposure for adverse clinical signs until the time of sacrifice.

Route of administration

The inhalation route is selected since this is a possible route of exposure in man.

Exposure levels: The exposure levels will be selected in consultation with the Sponsor taking account of all available data, including the results of preliminary studies, any acute inhalation and repeat dose inhalation toxicity study in rats and mice.

Groups and exposure levels: The allocation of animals to the experimental groups, group numbers and exposure levels are shown above.

Exposure: The test atmospheres for Groups 2-4 will be administered by inhalation as described below.

Exposure duration: All animals will receive two six-hour exposures on consecutive days with a single sampling time 24 hours after the final exposure. The actual dose regime will be determined following consultation with the Sponsor and will be confirmed by protocol amendment.

Study number: CSS/010

Route	:	Inhalation by whole-body exposure in 0.75 m^3 exposure chambers. Animals will be housed singly in stainless steel mesh compartments during exposure.
Chamber conditions	:	Chamber temperature, humidity and air flow will be monitored and recorded at intervals during exposure. The exposure chamber will be maintained 1-10 mm H_2O below ambient pressure.
Treated at	:	Constant air borne concentration.
Controls (Group 1)	:	Air.
Frequency	:	Once daily for 6 hours per day, for 2 consecutive days
Generator	:	All glass vaporiser.
Method	:	The test substance (liquid) will be metered to the vaporiser (through which air is passed) from a reservoir pressurised with inert gas. The air supply is provided from a compressor and is filtered, temperature and humidity controlled. Air will be introduced into each exposure chamber at a total rate of 150 litres per minute. The vapour/air mixture produced passes directly into the exposure chamber. The exposure system will be fully detailed in the report.
Concentrations	:	To be determined.
Sampling	:	Samples of the chamber atmospheres will be drawn through a manifold by a diaphragm pump. At intervals, an automated valve system will take a sample from each chamber to a suitable gas IR Spectrophotometer. The sampling frequency will be at least once hourly from each chamber (i.e four chambers per hour). Periodically, the concentration of certain components of the test atmosphere will be detected by capillary GC.
Concentration	:	Determined as nominal from the total amount of test substance used during generation and the total air volume during exposure. Certain components will be analysed periodically by capillary GC using a method to be advised by Protocol Amendment. An estimate of total hydrocarbon concentration will be obtained by IR absorption at 3-4 µm from samples taken on-line from 4 positions within each chamber such that spatial and temporal variations are established.

Study number: CSS/010

Pre-study system characterisation : Before commencement of treatment the system will be characterised at the target exposure vapour concentrations without animals in order to:

- demonstrate reproducibility of vapour concentration.
- demonstrate homogeneity of vapour concentration and distribution between levels in the chamber.

Procedure

The animals are removed from their cages and placed into the chamber appropriate to the treatment group. A separate chamber is used for each group. The air supplies to the generator and chambers are turned on.

With the animals in position in the chamber, exposure commences from the moment when generation commences. Operation of the exposure system is monitored continuously during the period of exposure with generation system checks recorded at 30-minute intervals.

During exposure the chamber atmosphere is sampled to determine the concentration of test vapour on at least 3 occasions during each exposure.

On completion of the 6-hour exposure period, the generation system is switched off, and the air supplies disconnected. The system is allowed to clear for approximately 25 minutes before the animals are removed from chamber and returned to their holding cages.

The nominal rate of vapour production is calculated by recording the amount of test substance delivered to the generation system during the exposure. The test substance usage over the six hours exposure is divided by the total airflow through the system. Prior to the entry of the vapour/air mixture into the exposure chamber, losses of test vapour from the airstream may occur due to condensation or impaction on surfaces within the system. Any such losses may be quantified and included in calculation of the nominal chamber concentration. Nominal concentration is compared with the actual chamber concentration determined from analysis of chamber air samples to provide a measure of generation efficiency.

Preparation of bone marrow smears

At the appropriate time after completion of exposure or administration of the positive control, each group of animals will be killed by exposure to rising levels of CO_2, followed by cervical dislocation. The femurs will be dissected from each animal and the proximal heads removed. Using a 2 ml syringe fitted with a 21 g needle, the contents of both femurs from each animal will be flushed out and pooled in a total volume of 2 ml of pre-filtered foetal calf serum. The cell suspension will be sedimented by centrifugation, the supernatant will be discarded and the cells will be resuspended in a small volume of fresh foetal calf serum. A small drop of the suspension will be transferred to a glass microscope slide and a smear prepared in the conventional manner (Schmid 1976). At least three smears will be prepared from each animal.

Fixation and staining of slides

The prepared slides will be fixed in methanol and air-dried. At least one smear from each animal will be stained in Giemsa's stain, differentiated in pH 6.8 buffered purified water, air-dried, and finally mounted with a coverslip using DPX.

Microscopic examination

Slides will be scored blind to eliminate any bias. Coded slides will be examined by light microscopy and 2000 immature erythrocytes per animal will be examined for the presence of micronuclei. Usually only one smear is examined per animal, the remaining smears being held temporarily in reserve in case of technical problems with the first smear.

Micronuclei are identified by the following criteria:

Large enough to discern morphological characteristics

Should possess a generally rounded shape with a clearly defined outline

Should be deeply stained and similar in colour to the nuclei of other cells - not black

Should lie in the same focal plane as the cell

Lack internal structure, *ie* they are pyknotic

There should be no micronucleus-like debris in the area surrounding the cell

The proportion of immature erythrocytes will be assessed by examination of a total of at least 1000 erythrocytes per animal.

4. ASSESSMENT OF RESULTS

The results obtained for each treatment group will be compared with the results obtained for the concurrent vehicle control group using non-parametric statistical methods. Non-parametric statistical methods are chosen for analysis of results because:

They are suited to analysis of data consisting of discrete/integer values with ties such as the incidence of micronucleated immature erythrocytes.

The methods make few assumptions about the underlying distribution of data and therefore the values do not require transformation to fit a theoretical distribution (where data can be approximately fitted to a normal distribution, the results of non-parametric analysis and classical analysis of variance are very similar).

"Outliers" are frequently found in the proportion of immature erythrocytes for both control and treated animals; non-parametric analysis based on rank does not give these values an undue weighting.

Analysis will be performed for the single sex study using data from all animals that survive to scheduled termination to maximise the power of statistical analysis. If less than five animals per group survive to scheduled termination the study will be repeated.

For incidences of micronucleated immature erythrocytes, exact one-sided p-values are calculated by permutation (StatXact, CYTEL Software Corporation, Cambridge, Massachussetts). Comparison of several dose levels is made with the concurrent control using the Linear by Linear Association test for trend in a step-down fashion. If significance is detected for individual comparisons this procedure simplifies to a permutation test (Agresti *et al.* 1990; Gibbons 1985).

For assessment of effects on the proportion of immature erythrocytes, exact versions of Wilcoxon's sum of ranks test and Jonckheere's test for trend are used.

A positive response is normally indicated by a statistically significant dose-related increase in the incidence of micronucleated immature erythrocytes for the treatment group compared with the concurrent control group ($P<0.01$); group mean values should exceed the laboratory historical control range (Morrison and Ashby 1995). A negative result is indicated where group mean incidences of micronucleated immature erythrocytes for the group treated with the test substance are not significantly greater than incidences for the concurrent control group ($P>0.01$) and where these values fall within the historical control range. An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

Bone marrow cell toxicity is normally indicated by a substantial and statistically significant decrease in the proportion of immature erythrocytes ($P<0.01$).

5. REPORTING

The report will contain details of the test substance, methodology, results and the interpretation of the data. Negative and positive control data, the protocol and any subsequent amendments will also be included as an appendix. Tabulated results will show individual animal results, and group mean results. GLP Compliance and Quality Assurance statements will be included.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

Upon study completion, two types of report are issued:

Draft report: Following QA audit, for review by the Sponsor

Final report: After approval by the Sponsor

Reports will be supplied on quarto paper and the following number of copies will be supplied:

Draft report: 1 unbound (double sided)

Final report: 1 bound (double sided with original signatures)

4 bound (double sided)

Any additions or corrections to an authorised final report will be documented as a formal amendment to the report.

Study number: CSS/010

6. MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of ten years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

7. GOOD LABORATORY PRACTICE

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

8. QUALITY ASSURANCE

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Study protocol and subsequent amendments.
Study based inspections	:	At least one phase of this study will be inspected.
Process based inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections which will be reported to appropriate Company Management only.

9. HEALTH & SAFETY

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

10. REFERENCES

AGRESTI, A., MEHTA, C.R. and PATEL, N.R. (1990) Exact inference for contingency tables with ordered categories. *Journal of the American Statistical Association*, **85**, 453.

BOLLER, K. and SCHMID, W. (1970) Chemical mutagenesis in mammals. The bone marrow of the Chinese hamster as an *in vivo* test system. Haematological findings after treatment with Trenimon (translation). *Humangenetik*, **11**, 34.

GIBBONS, J.D. (1985) *Nonparametric Statistical Inference, 2nd edition*, Marcel Dekker, New York.

MacGREGOR, J.T. *et al.* (1987) Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutation Research*, **189**, 103.

MATTER, B. and SCHMID, W. (1971) Trenimon-induced chromosomal damage in bone marrow cells of six mammalian species, evaluated by the micronucleus test. *Mutation Research*, **12**, 417.

MAVOURNIN, K.H., BLAKEY, D.H., CIMINO, M.C., SALAMONE, M.F. and HEDDLE, J.A. (1990) The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the US Environmental Protection Agency Gene-tox Program. *Mutation Research*, **239**, 29.

MORRISON, V. and ASHBY, J. (1995) High resolution rodent bone marrow micronucleus assays of 1,2-dimethylhydrazine : implication of systemic toxicity and individual responders. *Mutagenesis*, **10**, 129.

Study number: CSS/010

SCHMID, W. (1976) The micronucleus test for cytogenetic analysis. In: Hollander, A. (ed.) *Chemical mutagens, principles and methods for their detection*, Vol. 4. Plenum Press, New York.

von LEDEBUR, and SCHMID, W. (1973) The micronucleus test. Methodological aspects. *Mutation Research*, **19**, 109.

Study Number : CSS/010
Protocol Amendment Number : 1

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : Christine E. Mason

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: C. E. Mason Date: 22nd May 2002
(Study Director)

For the Sponsor

Approved by: [signature] Date: May 31, 2002

Study Number : CSS/010
Protocol Amendment Number : 1

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Reasons for amendments : Amendment to method of sampling of the test atmospheres and determination of nominal concentration.

Amendments

3. EXPERIMENTAL PROCEDURE

Sampling : ~~Samples of the chamber atmospheres will be drawn through a manifold by a diaphragm pump. At intervals, an automated valve system will take a sample from each chamber to a suitable gas IR Spectrophotometer. The sampling frequency will be at least once hourly from each chamber (i.e four chambers per hour). Periodically, the concentration of certain components of the test atmosphere will be detected by capillary GC.~~

Samples of the test atmosphere from each exposure chamber will be drawn through a manifold by a diaphragm pump. At intervals, an automated valve system will divert a sample from each of the chambers in turn to a gas chromatograph. The samples will be injected onto a gas chromatograph (GC) by a motorised valve. The sampling frequency will be at least once per hour from each chamber of test atmosphere.

Concentration : ~~Determined as nominal from the total amount of test substance used during generation and the total air volume during exposure.~~

~~Certain components will be analysed periodically by capillary GC using a method to be advised by Protocol Amendment. An estimate of total hydrocarbon concentration will be obtained by IR absorption at 3-4 µm from samples taken on line from 4 positions within each chamber such that spatial and temporal variations are established.~~

The analysed concentration of significant components of the test atmospheres will be determined by capillary GC analysis with external standardisation. Spatial and temporal variations of concentrations in the chamber will be characterised during preliminary work without animals. Details of the analytical system will be confirmed in the report.

The nominal concentration will be determined from the amount of test material used and the airflow through the chamber.

Study Number : CSS/010
Protocol Amendment Number : 2

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : Christine E. Mason

The signature of the Study Director authorises the implementation of this amendment to protocol. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: C.E. Mason Date: 7th June 2002
(Study Director)

For the Sponsor

Approved by: [signature] Date: June 18, 2002

Study Number : CSS/010
Protocol Amendment Number : 2

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Reasons for amendments : Selection of exposure levels for the micronucleus test and the addition of 2 satellite animals in the high exposure group, following consultation with the Study Sponsor.

Change to the number of days animals will be acclimatised for as a result of a change to the supplier delivery date.

Amendments

3. EXPERIMENTAL PROCEDURE

Main test

Exposure levels for the preliminary toxicity test were 500, 1000, 2000 and 3000 ppm. Animals died during exposure at levels of 1000, 2000 and 3000 ppm. All animals at 500 ppm survived to scheduled termination. On the basis of these results levels selected for the micronucleus test are 125, 250 and 500 ppm.

In order to ensure an adequate group size for analysis of micronucleus induction, two additional animals will exposed at the highest level (500 ppm) to replace any animals that may die during exposure (group size 7+2). These satellite animals will be killed at the appropriate time after exposure and will not be used to prepare bone marrow smears unless used as replacements.

As a result of a change in the delivery date of the animals from 5th June 2002 to 6th June 2002, animals on the micronucleus test will be acclimatised for six days.

Study Number : CSS/010
Protocol Amendment Number : 3

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director Christine E. Mason

The signature of the Study Director authorises the implementation of this amendment to protocol. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: C. E. Mason Date: 11th June 2002
(Study Director)

For the Sponsor

Approved by: [signature] Date: June 29, 2002

Study Number : CSS/010
Protocol Amendment Number : 3

Huntingdon Life Sciences

PYROLYSIS C5s

MOUSE MICRONUCLEUS TEST

Reasons for amendments : Dose levels previously selected for the micronucleus test in consultation with the Study Sponsor were 125, 250 and 500 ppm. In order to ensure a NOEL the Study Sponsor has requested dose levels of 40, 125 and 500 ppm.

Amendments

3. EXPERIMENTAL PROCEDURE

Main test

Dose levels selected for the micronucleus test are 40, 125 and 500 ppm.

APPENDIX 9

ADMINISTRATION OF PYROLYSIS C5s BY INHALATION TO MICE

Author

Paul Morris

CONTENTS

Page

TEST SUBSTANCE AND ADMINISTRATION

Test substance 55
Administration 55
Test Atmosphere Generation 56
Exposure Chambers 57
Procedure 58
Test Atmosphere Analysis 59
Chamber Monitoring System 59
Target Concentrations 60
Exposure Chamber Conditions 61

RESULTS

Vapour concentration 62
Nominal concentration 62
Test material composition 63
Chamber Temperature and Relative Humidity 63
Discussion 63
Calculations 64

FIGURES

A. Schematic of the vapour generation system used in the main test 65
B. Schematic of an inhalation chamber used to expose mice 66

TABLES

A. Operating conditions for the rodent inhalation exposure system during the main test 67
B. Chamber concentrations of Pyrolysis C5s (ppm) during the main test - daily mean and individual values 68
C. Nominal concentrations of Pyrolysis C5s (ppm) during the main test - individual exposure values 69
D. Pyrolysis C5s test atmosphere composition (%) - study mean values 70
E. Chamber temperature and relative humidity during the main test- exposure mean values 71

APPENDIX

A. Methods of sample collection and analysis for Pyrolysis C5s 72

ADDENDUM 83

TEST SUBSTANCE AND ADMINISTRATION

TEST SUBSTANCE

The test substance, Pyrolysis C5s, is a volatile liquid mixture with the boiling points of the majority of components below approximately 50°C.

A consignment, comprising fifteen cylinders, each with a stated net content of 45 lbs (Batch number QA1001A100), was received from Dow Chemical on 21 January 2002. The test substance was stored securely in the original containers in a large water tank contained in a refrigerated store with the temperature regulated at approximately 10°C until it was transferred to the atmosphere generation system.

Information provided by the Sponsor indicated that the test substance was stable for the intended duration of use on the study. The composition was defined in Huntingdon Life Sciences report number CSS017/022791. Information regarding the purity and stability of the test substance is the responsibility of the Sponsor.

ADMINISTRATION

For the main micronucleus test, the test material was administered to the mice by inhalation in whole-body exposure chambers as described below. For the preliminary toxicity test, the test material was also administered to the mice by inhalation in whole-body exposure chambers. A description of the preliminary toxicity test is presented in Addendum A.

The chamber atmospheres were produced by metering the liquid test substance into glass vapour generators through which dried air was passed at a group dependent flow rate ranging from 50 to 150 l/minute. The atmosphere produced by the generation system was (except for Group 4) further diluted with air to give a total flowrate of 150 l/minute and the final chamber concentrations of test aerosol.

The in-line airflow to the vapour generation apparatus was verified using a dry type gas meter and a calibrated flow meter during the preliminary phase of the study. During the study, the airflow to the atmosphere generation system was monitored throughout each of the exposures using calibrated in-line tapered tube gas flowmeters

The settings of the test substance metering system required to obtain the target chamber concentrations were determined, where possible, during preliminary generation trials without animals present and based on the gas chromatographic (GC) analysis of chamber atmosphere samples. Minor adjustments were made to the test material delivery rates in order to maintain chamber concentrations close to target.

Animals assigned to Group 1 (Air control) received an exposure to compressed air only, from the same source as used for the generation of the test atmospheres.

The duration of administration was two 6-hour exposures, over consecutive days for all Groups.

The usage of Pyrolysis C5s was determined, for each day of treatment, for each test group.

TEST ATMOSPHERE GENERATION (FIGURE A)

The vapour for all Groups was generated using a glass vapouriser supplied by a Precidor 5003 syringe pump. This was achieved by metering the test substance from a 50 ml (Fortuna®) polypropylene syringe for Group 2 and a 140 ml (Tyco Instruments®) polypropylene syringe with a rubber plunger for Groups 3 and 4. The syringe was mounted on an infusion pump (Precidor® type 5003) to a vapour generator, which comprised a glass frit contained in a glass vessel. Air was passed through the vapouriser at a rate of 50 l/minute for Group 2, 90 l/minute for Group 3 and 150 l/minute for Group 4. The vapour/air mixture passed out of the vapouriser into the chamber inlet ducting.

The weight of the syringe was measured at the start and end of each exposure on a top pan balance to calculate test substance usage. Prior to the start of each exposure, the pumps were turned on briefly to prime the length of the liquid delivery line from the syringe to almost up to the glass frit in the vapouriser thus removing air from the delivery line. Additionally, a damp cloth, wetted with cold water, was placed over the syringe in each infusion pump. This was to reduce the temperature and vapour pressure of the test article within the syringe and hence the build up of vapour, which would prevent consistent generation of the test aerosol.

Different chamber concentrations were achieved by varying the syringe pump infusion rate.

For all groups exposed to Pyrolysis C5s, the vapour/air mixture produced in the vapour generators was passed through fibre reinforced PVC tubing (10 mm internal diameter) into the base of the secondary dilution vessel. A further supply of clean and dry air was supplied to Groups 2 and 3 to ensure a total chamber airflow of approximately 150 l/minute. The air supply for Group 4 was provided solely by the vapour generation system (to ensure that the lower explosive limit was not exceeded).

Diluent air flow was measured using a tapered tube flow meter situated at the front of a purpose-built stainless steel trolley on which the secondary dilution vessel was mounted. Generation air was measured on a similar flowmeter mounted on the vapour generation trolley.

The test atmosphere was then passed through flexible ducting to a tangential inlet mounted at the apex of the appropriate exposure chamber.

A schematic of the vapour generation system is presented in Figure A.

The control group was exposed using a similar system to that used for the test groups, but received compressed air only at a rate of approximately 150 l/minute.

The air supplied to the vapour generators and secondary dilution vessels was filtered to remove any residual particulate and was dried (dew point ~2°C).

EXPOSURE CHAMBERS (FIGURE B)

The exposure chambers were of stainless steel and glass construction and consisted of a cuboidal body fitted with a pyramidal base and top. The internal volume of each chamber was approximately 0.75 m^3. At the apex of the upper pyramidal figure was the tangentially mounted air duct. Immediately below this was a perforated canister, which ensured equal distribution of the test atmosphere within the chamber.

Access to the chamber was through the front of the box section *via* a hinged door with a glass panel and stainless steel frame. The door was sealed using moulded rubber sealing strip.

Exposure cages constructed of stainless steel mesh were suspended on a framework arranged on 4 levels. Each level was able to hold four cages, with each cage used to house up to four mice in separate compartments. This gave a potential animal exposure capacity of 16 mice per chamber level.

Projecting through the rear wall of each chamber was one 0.6-cm diameter stainless steel tube protruding between levels 2 and 3 of the chamber. This was used for collection of chamber atmosphere samples. Spatial distribution studies were conducted during preliminary trials of previous studies conducted using Pyrolysis C5s (Huntingdon Life Sciences Reports CSS011/020072 and CSS012/023478).

The pyramidal base of each chamber was fitted with a 2-inch drain. The drain connected with a common drainage system *via* a ball valve.

A square tubular exhaust plenum, 3 inches in diameter and perforated along the ventral surface, was situated in the pyramidal base. This connected to the main extract system.

A wet and dry alcohol bulb thermohygrometer was suspended in the chamber. This was visible through the glass-panelled door and was used to monitor chamber temperature and relative humidity.

A Magnehelic pressure gauge (0-25mm water gauge) was connected with each chamber by a nylon tube. This was mounted on the secondary dilution vessel trolley and was used to monitor the atmospheric pressure inside the chamber, relative to the exposure room. The internal pressure within each chamber was maintained in the range -2 to -4mm water below ambient pressure when operational.

Extract flow was adjusted using gate valves mounted in the extract ducting between the chamber and filters.

Extraction of the chambers was accomplished by means of a single fan mounted on the outside wall of the building withdrawing air through a manifold to which all chambers were connected. The chamber air extract was vented to atmosphere *via* an exhaust stack.

PROCEDURE

A separate exposure chamber was used for each group. The Control animals were exposed using an identical exposure chamber to that used for the test groups.

Prior to the start of each exposure, the syringes used for all Groups were filled with the appropriate amount of the test material for the exposure.

The mice were transferred from the holding cages and placed into the individual compartments of the exposure cages.

The diluent and generator airflows were turned on and the exposure chamber doors were checked to ensure they were secured. The chamber pressure, relative to the exposure room was checked using each of the associated Magnehelic gauges to ensure that operation of the chamber took place at a slightly negative pressure.

The infusion pumps were switched on; the exposure start time was noted and simultaneously the chamber environmental monitoring system was activated (see below). At intervals of 30 minutes, any clinical signs in the mice were recorded together with checks of generation and chamber operational parameters.

The wet and dry bulb temperatures of a thermohygrometer placed in each chamber were also recorded at approximately 30-minute intervals throughout each exposure. Relative humidity was found using a look-up table. The flow rate of air to the exposure chambers was measured using calibrated flow meters and also checked approximately every 30 minutes.

Results of the determination of Pyrolysis C5s in the chamber atmosphere were automatically recorded for each chamber at 30-minute intervals throughout each exposure.

At the end of six hours generation, the infusion pumps were switched off and the syringes carefully removed from the inlet line and the contents weighed. The vapour in the test chambers was allowed to clear for at least 15 minutes before the animals were removed.

At the end of this time, the mice were unloaded from the chambers and returned to their respective holding cages.

The chambers were washed with hot water.

A summary of the operating conditions is presented in Table A.

TEST ATMOSPHERE ANALYSIS

A gas chromatograph was used to measure the concentrations of Pyrolysis C5s in the test atmospheres within the four inhalation chambers. Operating details of the Gas Chromatography system, its standardisation and validation are given in Appendix A.

The Gas Chromatograph was located adjacent to the exposure chambers.

The instrument was connected to each selected sampling port by programmed switching of valves under the control of the CEMS-2 program. Gas sampling lines were 0.6 cm diameter stainless steel tubing. A further set of automated valves admitted standards from gas sampling bags for calibration of the Gas Chromatograph and for daily checking of the standard response. To minimise the opportunity for carry over of the test substance within the sample lines, the conduit in which the sample stream passed to the Gas Chromatograph was purged with clean air for 60 seconds and further flushed with sample between analyses.

Before the start of each exposure, the operating conditions for the Gas Chromatograph were identified and the instrument response checked using prepared standard gas mixtures. An automatic warning message was generated in response to any deviation from the accepted response range for the standards.

Linear regression analysis of the Gas Chromatograph response to standards was incorporated into the system program to enable concentrations to be calculated from the signals provided by the chromatograph. The accumulated calibration data were reviewed at intervals during the study and, if necessary, the regression data incorporated into the program were revised. Details of such reviews are retained with the raw data.

CHAMBER MONITORING SYSTEM

A personal computer (PC) running the Chamber Environmental Monitoring System (CEMS-2) software was used to monitor and record the system performance during each exposure. The data collection sequence and display were controlled by a PC and all information collected was displayed on a monitor. Simultaneously, the data was stored electronically. This program was composed of three basic stages of operation: an initial setting up (pre-exposure) phase, an exposure monitoring phase and the post exposure data collation and presentation phase. A study data-protocol containing study specific design and detail drive the program. The CEMS-2 system holds a certificate of validation in compliance with GLP. All information collected was printed as a hard copy.

Setting-up phase

In the initial phase, prompted by the program screen display, study identification, dates, times and other relevant study details were entered and stored together with barometric pressure and gas chromatograph calibration data sets.

A pre-monitor calibration check using valid gasbag standards was conducted prior to the start of the exposure.

Exposure monitoring phase

This phase was started coincident with the commencement of generation. The concentration of Pyrolysis C5s in the chamber atmosphere of each test Group was analysed and recorded during a 30-minute cycle. The data were displayed on screen, printed and all downloaded to the database. This cycle of monitoring took place in the following sequence: High dose, Intermediate dose, Low dose and Air control and was repeated throughout the six-hour exposure period. A total of twelve cycles were recorded.

Post exposure phase

At the end of 6 hours, the data collected during exposure were collated into separate groups. Any data that required interpolation using the calibration data were calculated. The mean values, together with standard deviation were calculated for each parameter recorded. This data were printed and stored to the database. The first set of data was excluded from calculation of the mean because chamber concentrations did not stabilise until approximately 15 - 20 minutes from the start of exposure (equilibration time, t_{99} was 23 minutes).

TARGET CONCENTRATIONS

The target concentrations of Pyrolysis C5s were:

Group	Designation	Concentration (ppm)
2	Low dose	40
3	Intermediate dose	125
4	High dose	500

The target concentrations were selected in consultation with the Sponsor, following the review of the preliminary toxicity test (see Addendum A).

EXPOSURE CHAMBER CONDITIONS

Chamber analysed concentration of Pyrolysis C5s

Chamber atmosphere was sampled in sequence from each of the four exposure chambers (Chambers 4 - 1 sampled sequentially) and from one point within each chamber. Air from each chamber was continually drawn through a transfer line, which was therefore equilibrated with the mean concentration from each chamber. When not being sampled, these transfer lines were pumped to waste (Figure B).

Every seven minutes, air from the transfer lines was switched to the injection loop of the gas chromatograph for automated analysis and data processing.

The analytical methodology is presented in Appendix A.

Nominal concentration of chamber atmospheres

Each chamber nominal concentration was calculated from the mass of liquid used over the six-hour exposure period and the exposure mean airflow. The ideal gas equation was used with the molecular weight of the liquid and the measured chamber conditions to compute the volume of vapour produced from the mass of liquid used. The calculation is detailed in Table C.

Airflow, temperature and relative humidity

These parameters were recorded manually, as described above under the Test Atmosphere generation and Procedure sections.

RESULTS

VAPOUR CONCENTRATION

Analysed concentration

The data are presented as follows:

Daily mean and Individual values Table B

The study mean concentrations (the means of daily mean values) for the groups exposed to Pyrolysis C5s are presented below:

Group	Chamber concentration (ppm)	
	Target	Analysed [a]
2 (Low dose)	40	44
3 (Intermediate dose)	125	130
4 (High dose)	500	490

[a] Calculation standardised using isoprene as the major component

The mean chamber concentrations were 10.0, 4.0 and 2.0% from target for Groups 2, 3 and 4 (Low, Intermediate and High doses) respectively. Analysed concentrations were in excellent agreement with the target concentrations. The coefficients of variation of the daily means were 3.7, 5.8 and 2.9% for Groups 2, 3 and 4 respectively. These results demonstrate that the test atmospheres were generated consistently well over both days of the study.

NOMINAL CONCENTRATION

The data are presented in Table C and are summarised below:

Group	Nominal concentration (ppm)	A/N ratio (%)
2 (Low dose)	52	83.5
3 (Intermediate dose)	182	75.2
4 (High dose)	512	95.8

$$A/N = \left(\frac{\text{Analysed concentration}}{\text{Nominal concentration}}\right) \times 100$$

For each Group, the nominal concentration for each exposure was calculated from the equations used for the calculation of the nominal concentrations that are detailed in Table C.

The expected mean ratios of analysed to nominal concentration (A/N), expressed as a percentage for the study, should be approximately 100% for all dose groups. The A/N ratio for Groups 2 and 3 are lower than expected. However, this discrepancy can be accounted for by the loss of test material during

priming of the inlet lines to the vapouriser prior to the start of the exposure. Otherwise, differences from the ideal A/N of 100% maybe due to inaccuracies in the measurements of weight, airflow, temperature and analysed concentrations. The coefficients of variations for the mean nominal concentrations are 3.6, 35.4 and 4.9% for Groups 2, 3 and 4 respectively. These values are acceptable for a two day study.

TEST MATERIAL COMPOSITION

A compositional analysis of the test aerosol was performed once during the preliminary toxicity study and once during the main study. Samples were taken manually from the left upper middle manual sampling port of the chamber containing a target aerosol concentration of 500 ppm (Group 1 for the preliminary test; Group 4 for the main study, see Figure B) and analysed on a separate cryogenically cooled GC system. Only components that comprised >1% of the total composition were quantified. These were analysed as a percentage of the total response of a flame ionisation detector. The components were tentatively identified by comparison of their response and order of elution with those provided by the sponsor for the test material. Details of the sampling and analysis are presented in Appendix A. The results of the composition analysis for both phases of the study are presented in Table D.

Overall, the composition of the test atmosphere remained fairly constant over the duration of the study and remained similar to the composition supplied by the sponsor. Changes in the percentage composition during the study of the total contents of Pyrolysis C5s for each component were in general no more than ± 1%.

CHAMBER TEMPERATURE AND RELATIVE HUMIDITY

The daily mean chamber temperatures and relative humidities are presented in Table E.

The chamber temperatures were similar for all groups for most days of the study.

For Groups 2, 3 and 4 (Low, Intermediate and High doses), the recorded relative humidity (RH) in the range, 30 - 35%, was lower than the target range of 40 - 60%.

The low values of RH for Groups 2 and 3 probably arise from generation and dilution of the chamber atmospheres with air that was supplied from a compressor system incorporating a refrigerant drier. This deviation from the target conditions had no discernible effect upon the animals and is not considered to have affected the outcome of the study.

DISCUSSION

Control of the delivery of Pyrolysis C5s vapour to the exposure chambers was good, as reflected in the study mean concentrations, which were within 10% of the target values for all Groups.

All individual samples in all exposures, collected from the Group 1 (Air control) chamber showed traces of Pyrolysis C5s. This was considered to be "carryover" from the preceding sample in the common

sampling line rather than the presence of Pyrolysis C5s within the exposure chamber. The mean amount present in terms of the peak area response was equivalent to less than 2% of the area response of the preceding Group 2 (Low dose) sample (target concentration 40 ppm). Due to the trace nature of Pyrolysis C5s in the Group 1 samples, no quantification was attempted.

The agreement between the analysed and the nominal chamber concentration values for Group 4 (High dose) were acceptable. The low A/N ratio for Group 2 can be attributed to the low volume of test material required and the necessity to prime the inlet lines of the vaporiser before commencement of the exposure. The low A/N ratio for Group 3 was only apparent during the first day of exposure and may be as a result of a weighing error.

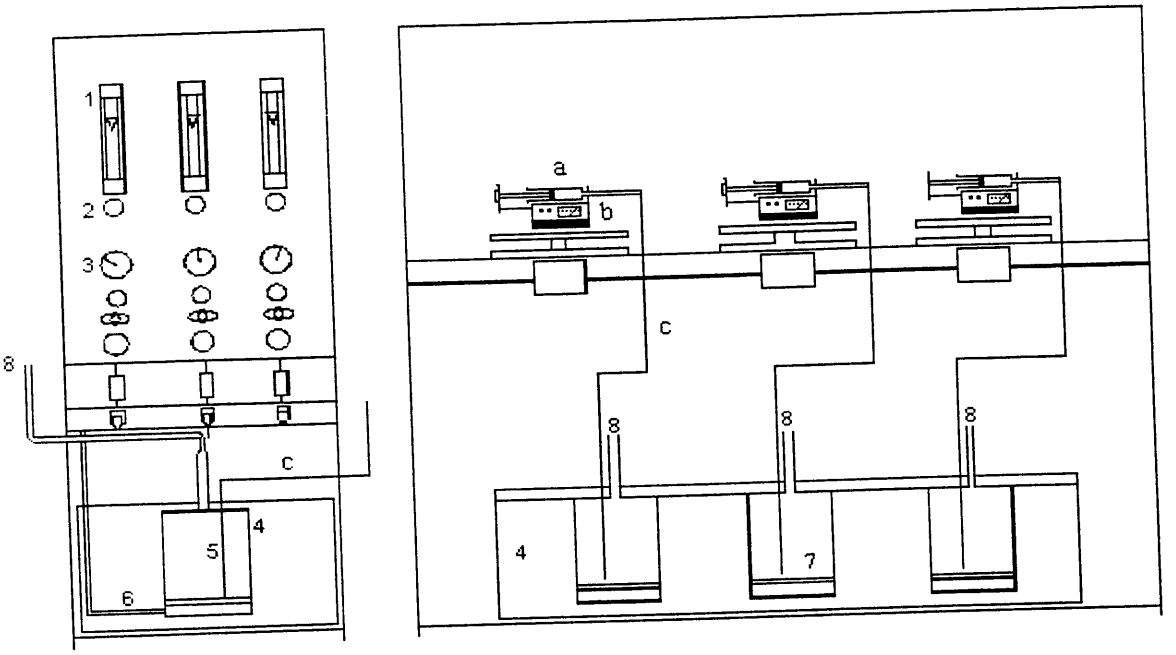
The compositional analysis of the Pyrolysis C5s test atmosphere remained consistent over the duration of the study.

CALCULATIONS

In order to minimise the cumulative errors that result from repeated rounding of numbers, much of the data in this report has been calculated continuously using unrounded numbers and only rounded for printing. Consequently, any further calculations using these rounded numbers may include rounding errors in the last significant figure, possibly leading to small apparent discrepancies with other data in the report.

FIGURE A

Schematic of the vapour generation system used in the main test

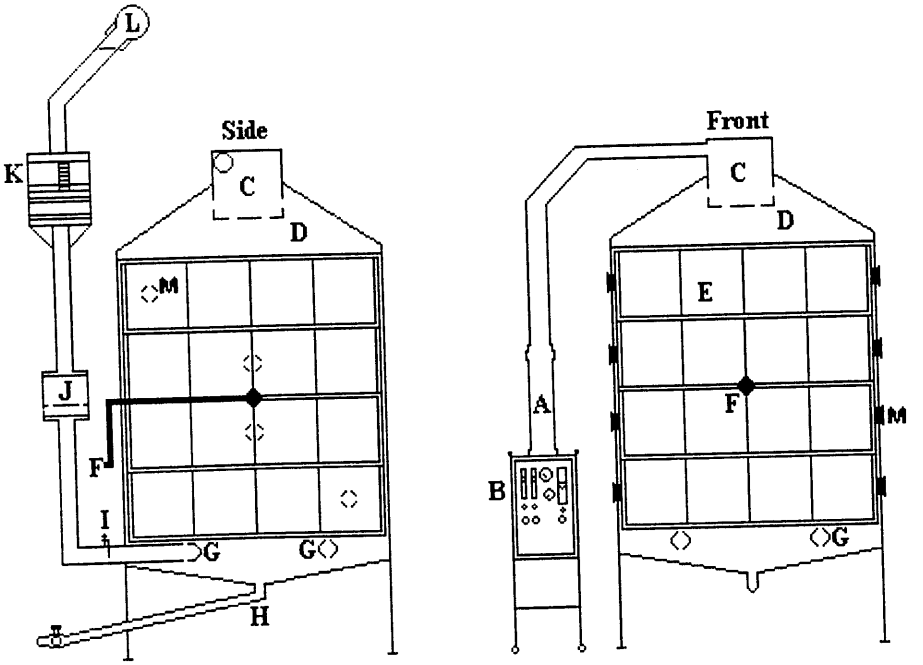


Key

1	Diluent air rotameters	a	Polypropylene syringe
2	Rotameter control valve	b	Syringe Driver (Precidor 5003)
3	Reservoir pressure display (not	c	Test compound feed line to vapour generator
4	Water bath		
5	Vapour generator		
6	Diluent air		
7	Sinter Diameter (130 mm)		
8	Vapour/air mixture (to chamber)		

FIGURE B

Schematic of an inhalation chamber used to expose mice



Key

A	Vapour inlet pipe	G	Exhaust plenum
B	Air flow control and chamber monitoring	H	Drain
		I	Gate valve
C	Dispersion device	J	Pre-filter
D	Exposure chamber (0.75 m^3)	K	Powered extract filter
E	Animal exposure cages	L	Main exhaust
F	CEMS sampling port	M	Manual Sampling ports

TABLE A

Operating conditions for the rodent inhalation exposure system during the main test

Parameter	Group			
	1 (Air control)	2 (Low dose)	3 (Intermediate dose)	4 (High dose)
Target concentration of Pyrolysis C5s (ppm)	0	40	125	500
Chamber airflows (l/minute)				
Target air flow to vapour generator	0	50	90	150
Target supplementary airflow	150	100	60	0
Vapour generator settings				
Test material feed	N/A	Precidor 5003 syringe pump		
Syringe size (ml)	N/A	50	140	140
Sinter diameter (mm)	130	130	130	130
Chamber negative pressure (mm/H_2O)	2 - 4	2 - 4	2 - 4	2 - 4

N/A Not applicable

TABLE B

Chamber concentrations of Pyrolysis C5s (ppm) during the main test - daily mean and individual values

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Air control)	Group 2 (Low dose)	Group 3 (Inter. dose)	Group 4 (High dose)
1	1	TA	BLQ	ASR	150
	2	TA	50	159	402
	3	TA	44	168	458
	4	TA	46	158	472
	5	TA	45	126	500
	6	TA	44	129	508
	7	TA	45	122	494
	8	TA	40	127	498
	9	TA	45	129	502
	10	TA	31	122	474
	11	TA	39	123	476
	12	TA	37	123	498
	Mean [b]	TA	42	135	480
	sd		5.2	17.5	30.3
2	13	TA	BLQ	132	245
	14	TA	53	105	485
	15	TA	45	117	529
	16	TA	38	117	483
	17	TA	44	121	477
	18	TA	44	124	508
	19	TA	46	134	461
	20	TA	48	126	531
	21	TA	52	137	507
	22	TA	45	124	508
	23	TA	38	132	508
	24	TA	38	132	507
	Mean [b]	TA	45	124	500
	sd		5.2	9.3	21.6
Overall mean		TA	44	130	490
sd			1.6	7.5	14.2
CV(%)			3.7	5.8	2.9

[b] The initial concentration measurement of each exposure was excluded from all calculations.

TA Trace amount of Pyrolysis C5s detected due to carryover in sampling lines.
Peak not quantified

BLQ Below Limit of Quantification

ASR Above Standard Range

sd Standard deviation

CV (%) Coefficient of Variation (sd × 100/mean)

TABLE C

Nominal concentrations of Pyrolysis C5s (ppm) during the main test - individual exposure values

Group	Exposure		Barometric pressure (mmHg)	Pyrolysis C5s usage (kg)	Chamber Airflow [C] (l/minute)	Chamber concentration		A/N ratio (%)
	No.	Duration (minute)				Analysed (ppm)	Nominal [D] (ppm)	
2	1	360	759	0.00836	150	42	53	78.5
	2	360	759	0.00797	150	45	51	88.6
Mean			759	0.00817	150	44	52	83.5
sd			0.0	0.000276	0.0	2.1	1.9	7.09
CV (%)			0.0	3.4	0.0	4.9	3.6	8.5
3	1	360	759	0.03553	150	135	227	59.4
	2	360	759	0.02136	150	124	136	91.0
Mean			759	0.02845	150	130	182	75.2
sd			0.0	0.010020	0.0	7.8	64.2	22.31
CV (%)			0.0	35.2	0.0	6.0	35.4	29.7
4	1	360	759	0.07707	150	480	494	97.2
	2	360	759	0.08283	150	500	529	94.5
Mean			759	0.07995	150	490	512	95.8
sd			0.0	0.004073	0.0	14.1	24.8	1.89
CV (%)			0.0	5.1	0.0	2.9	4.9	2.0

C Includes 50 l/minute through vaporiser for Group 2 and 90 l/minute through vaporiser for Group 3

D Calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760 \text{ mm Hg}}{\text{Atm}}$$

where V = gaseous volume of Pyrolysis C5s (litres)
W = mass of Pyrolysis C5s (kg)
M = approximate molecular weight of Pyrolysis C5s (70 g/mole)
R = gas constant (0.08206 l atm mol^{-1} K^{-1})
T = temperature (K), = temperature (°C, see Table E) + 273)
Atm = atmospheric pressure (mmHg)
V_a = volume of air (litres) passing through the chamber during the exposure

A/N Analysed/nominal concentration ratio expressed as a percentage
sd Standard deviation
CV (%) Coefficient of Variation (sd × 100/mean)

TABLE D

Pyrolysis C5s Test atmosphere composition (%) - study mean values

Component [e]	Prelim Toxicity Test			Main Study			Shipped Composition
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	
isopentane	8.86	8.60	8.73	7.34	7.58	7.46	8.01
1,4-pentadiene	3.48	3.63	3.56	3.39	3.55	3.47	2.64
1-pentene	7.61	7.63	7.62	7.11	7.45	7.28	6.34
2-methyl-1-butene	3.97	3.98	3.98	3.79	3.92	3.86	3.59
n-pentane	11.69	11.40	11.55	10.85	11.16	11.01	9.52
isoprene	19.33	19.46	19.40	19.02	19.53	19.28	17.84
trans-2-pentene	3.23	3.24	3.24	3.42	3.50	3.46	2.83
cis-2-pentene	1.72	1.70	1.71	1.69	1.76	1.73	1.78
2-methyl-2-butene	2.51	2.60	2.56	2.47	2.55	2.51	2.51
trans-1,3-pentadiene	12.76	12.73	12.75	13.29	13.56	13.43	10.00
cyclopentadiene	2.30	2.30	2.30	1.87	1.85	1.86	7.70
cis-1,3-pentadiene	7.65	7.71	7.68	8.07	8.22	8.15	6.12
cyclopentene	8.64	8.78	8.71	9.23	9.40	9.32	7.01
cyclopentane	1.78	1.70	1.74	2.27	1.78	2.03	1.33
2-methylpentane	1.58	1.60	1.59	2.36	1.72	2.04	1.73
dicyclopentadiene	1.15	1.54	1.35	1.16	0.96	1.06	5.87
Total identified	98.26	98.60	98.43	97.33	98.49	97.91	94.82

[e] Identification of each component was tentatively made by comparison of their normalised response given by a GC-FID and elution order with the shipped composition provided by the sponsor for Pyrolysis C5s prior to shipment to Huntingdon Life Sciences

TABLE E

Chamber temperature and relative humidity during the Main Test- exposure mean values

Exposure No.	Mean chamber temperatures (°C) and relative humidity (%RH)							
	Group 1 (Air control)		Group 2 (Low dose)		Group 3 (Intermediate dose)		Group 4 (High dose)	
	Temp	RH	Temp	RH	Temp	RH	Temp	RH
1	21.0	50.9	21.2	33.0	21.0	29.5	21.9	28.1
2	20.3	49.7	20.2	33.6	20.4	28.8	20.9	31.2
Mean	20.7	50.3	20.7	33.3	20.7	29.2	21.4	29.7
sd	0.49	0.85	0.71	0.42	0.42	0.49	0.71	2.19
CV (%)	2.4	1.7	3.4	1.3	2.0	1.7	3.3	7.4

sd standard deviation
CV Coefficient of variation (sd × 100/mean)

APPENDIX A

Methods of sample collection and analysis for Pyrolysis C5s

SAMPLE COLLECTION

The raw data for the method validation is located in study CSS/011.

Chamber concentration

Samples of chamber air were collected in sequence from each of the four exposure chambers (Chambers 4 - 1 sampled sequentially) from one sampling point within each chamber. Air from each chamber was continually drawn through a transfer line, which was therefore equilibrated with the mean concentration from each chamber. When not being sampled, the air from the transfer lines was pumped to waste.

At approximately seven-minute intervals, the air from the transfer lines was switched to the injection loop of the Gas Chromatograph for automated analysis and data processing.

METHOD OF ANALYSIS

Chamber atmosphere samples were analysed by gas chromatography. The method of sample analysis is detailed, together with a summary of the method validation, in the Inhalation Analytical Procedure at the end of this Appendix.

CALCULATIONS

GC analysis

The samples of chamber atmosphere were injected into a gas chromatograph, which was calibrated using vapour standards prepared in gas sampling bags. The method for calculating the concentration of Pyrolysis C5s from the mass used to prepare each vapour standard is given below in equations 1 and 2.

$$\text{Concentration} = \frac{V}{V_a + V} \times 1{,}000{,}000 \text{ ppm} \qquad (1)$$

$$V = \frac{W \times R \times T}{M} \times \frac{760}{Atm} \qquad (2)$$

where V = gaseous volume of Pyrolysis C5s (litres)
W = mass of Pyrolysis C5s (kg)
M = approximate molecular weight of Pyrolysis C5s (70 g/mole)
R = Gas constant (0.08206 ml atm $mmol^{-1}$ K^{-1})
T = temperature (K)
Atm = atmospheric pressure (mmHg)
V_a = volume of air (litres)

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

COMPOUND SPECIFIC INHALATION ANALYTICAL PROCEDURE FOR ISOPRENE IN PYROLYSIS C5s VOLATILES

The analysis of isoprene in Pyrolysis C5s volatiles in air

The method outlined in this document has been validated and is considered fit for the purpose of monitoring test atmospheres and blood headspace in an Inhalation Toxicology study.

This document details the basic procedures for the analysis of isoprene in Pyrolysis C5s sampled by an automated on-line sampling system directly from the inhalation chambers. The resulting samples, of approximate concentration 700 to 7000 ppm, are analysed by GC. Study specific amendments and additions will be detailed within a supplementary document.

EFFECTIVE DATE:	3 March 2002

Test substance

Pyrolysis C5s is a mixture of mostly C5 hydrocarbons with some higher and lower homologues and has an approximate molecular weight of 70. The major component is isoprene (2-methyl-1,3-butadiene) and standardisation is calculated using the area response for this peak. The two other major components are n- pentane and *trans*-1,3-pentadiene. The composition of Pyrolysis C5s is analysed through the course of the exposure by measurement of the peak area of these components in comparison to that of isoprene. Identification of these three components was achieved qualitatively against pure standards of each component.

Appearance	Colourless liquid
Storage	A pressure vessel in a cold storage facility at 10°C

Equipment

Balance and data printer	Sartorius	R200D with YDP-02
Syringes	Hamilton	500 series gas-tight (500 ml)
Gas sample bags	SKC INC	Tedlar® 232-series (1 and 3 dm^3 capacity)
Syringe valve	Mininert	Push button valve
Vacuum pump	AEG	ADEB 56 (or equivalent)
Flow meter	J & W Scientific	ADM1000 (acoustic displacement)
Wet gas meter	Zeal	DM3B (1 l)

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Preparation of samples for analysis

Gas samples

Vapour samples are collected using an automated system fitted with electronically controlled valves, which are manipulated using the Chamber Environment Monitoring System (CEMS-2) software.

The test atmosphere is drawn directly from the inhalation chamber through the sample line to the gas-sampling valve located on the GC.

Initially, the gas-sampling valve of the GC is set to the "load" position and the valve is automatically switched to the "inject" position after 60 seconds. Simultaneously, the GC activates the start of the run sequence.

Preparation of calibration standards

Gas standards

Standards are prepared using the following method, the actual standard concentration ranges used are as detailed in the study specific supplement.

Collect the liquid sample directly from the storage cylinder into an empty glass bottle. Some of this liquid sample is then transferred to a glass vial fitted with a sealing valve. The glass vial is then stored at 4°C.

Evacuate gas sample bags of appropriate volumes and introduce measured volumes of air using a wet gas meter. Use a gas tight syringe fitted with a sealing valve to accurately dispense an aliquot of Pyrolysis C5s into the top standard gas bag via the injection port. Using a gas tight syringe, accurately dispense measured volumes of the Pyrolysis C5s vapour from the top standard gas bag into the gas sample bags via the injection port to produce standards covering the concentration range described in the study specific supplement.

Storage of standards and samples

The maximum storage periods for the various sample types are detailed below:

Sample type	Storage conditions	Storage period
Gas standards	Room temp., dark	3500-700ppm: 8 days 7000ppm: 7 days

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Calibration and quantification

Gas analysis

Calibrate by injecting duplicates of each calibration standard gas bag, as detailed in the study specific supplement, at the beginning of each analytical sequence. Measure the peak area response of the major component peak (isoprene) in each injection of the calibration standard gas bag and derive the line of best fit using a 1/concentration^2 weighted least squares method.

For each injection of the sample measure the peak area response and determine the amount present in the sample using the equation below:

$$\text{Amount}(\text{ppm}) = \frac{(A - I)}{S}$$

Where A = Peak area response of isoprene in Pyrolysis C5s in the sample chromatogram
S = Slope of calibration line derived from calibration data
I = Intercept of calibration line derived from calibration data

At the beginning of each analytical sequence, conduct a "Pre-Monitor Calibration check" to ensure the gas bags are within accepted tolerance limits of the calibration model i.e. run four QC standards covering the nominal range (including the LOQ) prior to the start of sample analysis.

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Chromatographic conditions

Analytical column	PE-5 (100% dimethyl polysiloxane), 25m x 0.25mm i.d. 0.25μm film
Carrier gas	Helium (1.0 ml/min)
Split vent	Helium (49 ml/min)
Septum purge	Helium (2.6 ml/min)
Split ratio	1:50
Make up	Helium (30 ml/min)
Oxidant	Air (470 ml/min)
Fuel	Hydrogen (50 ml/min)
Injection volume	250 μl *via* an automated gas valve
Injector temperature	100°C
Detector temperature	250°C
Column temperature	Isothermal at 0°C for 5 minutes. Temperature controlled by liquid CO_2 delivered using a cryogenic GC unit
Retention time in Pyrolysis C5s mixture	n-pentane peak of approximately 2.95 minutes; isoprene peak approximately 3.15 minutes, trans-1,3-pentadiene approximately 3.50 minutes

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Quality assurance measures

Gas analysis

When the method is established on a chromatographic system six injections of a standard will be used to verify performance of the system. The parameters and acceptance criteria are set out below;

Parameter	Acceptance criteria
Repeatability (CV, n=6)	<5%
Repeatability at LOQ (CV, n=6)	<10%
QC tolerance	< ±5%
QC tolerance at LOQ	< ±10%

The highest calibration standard will be compared against a standard of similar concentration prepared independently. The ratio of response factors will be acceptable if within the range 0.95 to 1.05.

A Pre-monitor calibration check, must precede every exposure for the analysis to be regarded as valid. The results of the quality check standards must lie within the QC tolerance limits.

A quality check standard of low concentration will be run to verify the LOQ for the run. The LOQ for the run will be regarded as the concentration of the lowest acceptable quality check standard.

APPENDIX A
(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Chromatographs

System No.	Components of gas chromatography system		
	Manufacturer	Model No.	Description
1	Hewlett Packard	5890A	Chromatograph with capillary inlets, heated automatic gas sampling valve, ECD and FID.
	Hewlett Packard	G1513A	Autoinjector }
	Hewlett Packard	18596CX	Controller }6890 Series Autosampler
	Hewlett Packard	G1512AX	Turntable }
	Thermo Finnegan [i]	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
2	Pye Unicam	PU4550	Chromatograph with gas valve and FID.
	Pye Unicam	PU4700	Autosampler
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
3	Shimadzu	GC-14A	Chromatograph with FID.
	Shimadzu	AOC-1400	Autosampler
	Shimadzu	AOC-14	Autoinjector
	Shimadzu		Split injection system
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
6	Shimadzu	GC-14A	Chromatograph with FID.
	Shimadzu	MGS-4	Automated gas valve
	Shimadzu	SPL-14A	Split injection system
	Shimadzu	CR4-A	Integrator
7	Shimadzu	GC-14A	Chromatograph with FID.
	Shimadzu	MGS-4	Automated gas valve
	Shimadzu	SPL-14A	Split injection system
	Shimadzu	CR4-A	Integrator
8	Hewlett Packard	5890A	Chromatograph with capillary inlets, heated automated gas sampling valve and FID.
	Hewlett Packard	18593B	Autoinjector }
	Hewlett Packard	18596CX	Controller }7673 Autosampler
	Hewlett Packard	G1512AX	Turntable }
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
9	Perkin Elmer	Autosystem XL	Automatic Chromatograph with programmable split/less capillary injector, heated automatic gas sampling valve and FID.

[i] Thermo Finnegan has previously traded as ThermoQuest, Thermo Separation Products (TSP) and Spectra Physics. Individual equipment items and manuals may be identified with these trade-names.

ECD Electron capture detector

FID Flame ionisation detector

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

CSS/010 - STUDY SPECIFIC SUPPLEMENT TO THE INHALATION ANALYTICAL PROCEDURE FOR ISOPRENE IN PYROLYSIS C5s

This supplement details additions and amendments to the procedure to be used for the GC assay of isoprene in Pyrolysis C5s obtained from air samples collected on the above study.

The assay, incorporating the additions and amendments, is suitable for the analysis of isoprene in Pyrolysis C5s, in solution, at concentrations within the range of 400 to 3500 ppm.

Details given in this supplement supersede those in the compound specific IAP.

EFFECTIVE DATE :	**16 May 2002**

Analytical standard

Name	Isoprene pentadiene
Batch number	QA1001A100
Purity	See below
Expiry date	None supplied
Supplier	Dow Chemical

Composition of Pyrolysis C5s

Component name	Weight %
Isopentane	8.01
1,4-pentadiene	2.64
2-butyne	0.95
1-pentene	6.34
2-methyl-1-butene	3.59
n-pentane	9.52
Isoprene	17.84
Trans-2-pentene	2.83
Cis-2-pentene	1.78
2-methyl-2-butene	2.51
Trans-1,3-pentadiene	10.00
Cyclopentadiene	7.70
Cis-1,3,-pentadiene	6.12
Cyclopentene	7.01
Cyclopentane	1.33
2-Methylpentane	1.73
Dicyclopentadiene	5.87

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Preparation of standard solutions

Prepare standard gas bags in the nominal range 400 to 3500 ppm.

Calibration and Quantification

Calibration of the instrument is performed using standard gas bags in the nominal range 400 to 3500 ppm.

Chromatographs

The analysis is performed using chromatograph 6.

Repeatability and Linearity of analysis

Precision data showed coefficients of variation for isoprene in Pyrolysis C5s of less than 5% with solutions in the range of 90 to 1350 ppm.

Least squares regression analysis with a 1/concentration^2 weighting of the peak area response against concentration of standard (90 to 1350 ppm) produced a correlation coefficient of 0.99996 and relative errors less than 5% in the range 1350 to 90 ppm. The Limit of Quantification (LOQ) for isoprene in Pyrolysis C5s will be set by the lowest acceptable check standard, however, the LOQ and Limit of Detection (LOD) are potentially as low as 7.94 and 2.62 ppm respectively (calculated statistically using the standard deviation obtained for a solution of concentration 90 ppm).

Stability

Standards of isoprene in Pyrolysis C5s at 90ppm (approaching the limit of quantification) stored at room temperature for 7 days and subsequently analysed against fresh standards showed concentrations within 20% of their nominal concentrations.

Pyrolysis C5s Total composition analysis

Preparation of Samples for Analysis

Two samples were collected during the preliminary exposures and during the main study exposures from the relevant chamber at a dose level of 500ppm and analysed off line by cryogenically cooled GC. The aim was to monitor any change in composition of Pyrolysis C5s throughout the course of the study. Only components of greater than 1% of the total composition were monitored.

The chamber was sampled from its left-upper middle side-port, which provides access for manual sampling of the chamber atmosphere. The atmosphere samples were pumped through silicone rubber tubing and a connected glass vessel for one minute. The glass vessel was comprised of a main chamber with inlet and outlet ports opened and closed by rotating gas-tight valves. A rubber septum was positioned in the wall of the chamber. After the minute had elapsed, the valves on the glass vessel were closed.

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Following collection, the samples were injected onto the GC via a gas sample valve. The sample entered the sample valve *via.* polythene tubing attached to a needle which was inserted into to rubber septum of the glass vessel. The sample was then drawn into the sample valve using a polythene syringe connected to the back side of the sample valve.

The GC sampling was controlled by a switching valve powered by nitrogen. When this valve was in the OFF position, liquid CO_2 was allowed to pass through to the oven of the GC and cool the column. At a pre-determined time, this valve was switched to the ON position, halting the flow of the liquid CO_2. Simultaneously, the gas sample valve was opened and the sample allowed to the enter the GC.

Chromatographic conditions (off-line analysis)

Analytical column	DB-1 (100% dimethyl polysiloxane), 20m x 0.1mm i.d. 0.4μm film
Carrier gas	Helium (0.4 ml/min)
Split vent	Helium (8 ml/min)
Septum purge	Helium (2.6 ml/min)
Split ratio	1:20
Make up	Helium (30 ml/min)
Oxidant	Air (470 ml/min)
Fuel	Hydrogen (50 ml/min)
Injection volume	250 μl *via* an automated gas valve
Injector temperature	40°C
Detector temperature	200°C
Column temperature program	0°C increasing at 6°C/minute to 126°C.
Gas sample valve	Off at start of run. On at 0.2min. Off at end of run.
Retention time	Major component peaks of Pyrolysis C5s mixture: n-pentane approximately 4.3 minutes; isoprene approximately 4.4 minutes, trans-1,3-pentadiene approximately 4.8 minutes

APPENDIX A

(Methods of sample collection and analysis for Pyrolysis C5s - continued)

Off-line Chromatograph

The off-line analysis is performed using chromatograph 9.

Retention time (on line analysis)

Retention time	Major component peaks of Pyrolysis C5s mixture: n-pentane approximately 3.2 minutes; isoprene approximately 3.4 minutes, trans-1,3-pentadiene approximately 3.8 minutes

EFFECTIVE DATE :	**16 May 2002**

Repeatability and Linearity analysis

Precision data showed coefficients of variation for isoprene in Pyrolysis C5s of less than 6% with solutions in the range of 30 to 550 ppm.

Least squares regression analysis with a 1/concentration^2 weighting of the peak area response against concentration of standard (30 to 550 ppm) produced a correlation coefficient of 0.99995 and relative errors less than 5% in the range 550 to 30 ppm. The Limit of Quantification (LOQ) for isoprene in Pyrolysis C5s will be set by the lowest acceptable check standard, however, the LOQ and Limit of Detection (LOD) are potentially as low as 15.1 and 4.98 ppm respectively (calculated statistically using the standard deviation obtained for a solution of concentration 30 ppm).

Stability

Standards of isoprene in Pyrolysis C5s at 30 ppm (approaching the limit of quantification) stored at room temperature for 3 days and subsequently analysed against fresh standards showed concentrations within 5% of their nominal concentrations.

ADDENDUM CONTENTS

Page

TEST SUBSTANCE AND ADMINISTRATION

Administration....84
Test Atmosphere Generation....84
Exposure Chambers....86
Procedure....86
Test Atmosphere Analysis and Chamber Monitoring System....87
Target Concentrations....87
Exposure Chamber Conditions....87

RESULTS

Vapour concentration....89
Nominal concentration....89
Chamber Temperature and Relative Humidity....90
Discussion....90

FIGURE

1A. Schematic of the vapour generation system used in the preliminary toxicity test....91

TABLES

1A. Operating conditions for the rodent inhalation exposure system during the preliminary toxicity test....92
2A. Chamber concentrations of Pyrolysis C5s (ppm) during preliminary toxicity test – daily mean and individual values....93
3A. Nominal concentrations of Pyrolysis C5s (ppm) during preliminary toxicity test – individual exposure values....94
4A. Chamber temperature and relative humidity during preliminary toxicity test - exposure mean values....95

TEST SUBSTANCE AND ADMINISTRATION

ADMINISTRATION

For the preliminary toxicity test, the test material was administered to the mice by inhalation in whole-body exposure chambers as described below:

The chamber atmospheres were produced by metering the liquid test substance into glass vapour generators through which dried air was passed at a group dependent flow rate ranging from 50 to 150 l/minute. The atmosphere produced by the generation system was (except for Group 4) further diluted with air to give a total flowrate of 150 l/minute and the final chamber concentrations of test aerosol.

The in-line airflow to the vapour generation apparatus was verified using a dry type gas meter and a calibrated flow meter during the preliminary phase of the study. During the study, the airflow to the atmosphere generation system was monitored throughout each of the exposures using calibrated in-line tapered tube gas flowmeters

The settings of the test substance metering system required to obtain the target chamber concentrations were determined, where possible, during preliminary generation trials without animals present and based on the gas chromatographic (GC) analysis of chamber atmosphere samples. Minor adjustments were made to the test material delivery rates in order to maintain chamber concentrations close to target.

The duration of administration was two 6-hour exposures, over consecutive days for all Groups.

The usage of Pyrolysis C5s was determined, for each day of treatment, for each test group.

TEST ATMOSPHERE GENERATION (FIGURE 1)

The vapour for Groups 2, 3 and 4 (Low Intermediate, High Intermediate and High Dose) were supplied from individual reservoirs of liquid Pyrolysis C5s maintained at pressure. The top of each reservoir[1] was fitted with a central, "O" - ring sealed filler cap, a system to allow pressurisation and release of the helium head pressure and a safety pressure release valve set to operate at above the study operating pressure. The reservoirs were mounted on electronic load cells [2] and the weight of each reservoir and contents could be displayed continuously. Each load cell was set to read zero weight with the empty reservoir in place before the first occasion of filling with the test substance and the minimum permissible start weight for the study exposures was calculated. Except during filling, the Pyrolysis C5s in the reservoirs was maintained under a helium pressure of 10 psi for all Groups.

---

[1] Newson Gale Ltd, 51 Norsey Road, Billericay, Essex, CM11 1BG, England

[2] Huntleigh Industrial Controls Ltd, Load Cell Division, Portman Moor Industrial Estate, East Moors, Cardiff, South Glamorgan, CF22 2HB

The pressurised reservoirs supplied the test substance to vapour generators which comprised a glass frit contained in a glass vessel. For each test group, the liquid delivery line to the glass vessel was fitted with a toggle valve to allow isolation of the test substance supply. Downstream of this, a particulate filter (stainless steel, 0.5 µm pore size [3]) was fitted, to protect the subsequent metering valve from any entrained particulate. The metering valve (Nupro S Series Needle Valve [3]) controlled the test substance delivery rate. Fluid passing through each metering valve was delivered onto the glass frit surface of the vaporiser, at ambient temperature. The air supply (50 to 150 l/minute) for the generator was first passed through a separate copper coil, at ambient temperature. The test substance vapour in air mixture was passed through fibre reinforced PVC tubing (10 mm internal diameter).

The vapour for Group 1 (Low Dose) was generated using glass vapouriser supplied by a Precidor 5003 syringe pump. This was achieved by metering the test substance from a 140 ml (Tyco Instruments®) polypropylene syringe with a rubber plunger. The syringe was mounted on an infusion pump (Precidor® type 5003) to a vapour generator, which comprised a glass frit contained in a glass vessel. Air was passed through the vapouriser at a rate of 60 l/minute. The vapour/air mixture passed out of the vapouriser into the chamber inlet ducting.

The weight of the syringe was measured at the start and end of each exposure on a top pan balance to calculate test substance usage. Prior to the start of each exposure, the pump was turned on briefly to prime the length of the liquid delivery line from the syringe to almost up to the glass frit in the vapouriser thus removing air from the delivery line. Additionally, a damp cloth, wetted with cold water, was placed over the infusion pump. This was to reduce the temperature and vapour pressure of the test article within the syringe and hence the build up of vapour which would prevent consistent generation of the test aerosol.

Different chamber concentrations were achieved by varying the syringe pump infusion rate.

For all groups exposed to Pyrolysis C5s, the vapour/air mixture produced in the vapour generators was passed through fibre reinforced PVC tubing (10 mm internal diameter) into the base of the secondary dilution vessel. A further supply of clean and dry air was supplied to Groups 1, 2 and 3 to ensure a total chamber airflow of approximately 150 l/minute. The air supply for Group 4 was provided solely by the vapour generation system (to ensure that the lower explosive limit was not exceeded).

Diluent air flow was measured using a tapered tube flow meter situated at the front of a purpose-built stainless steel trolley on which the secondary dilution vessel was mounted. Generation air was measured on a similar flowmeter mounted on the vapour generation trolley.

The test atmosphere was then passed through flexible ducting to a tangential inlet mounted at the apex of the appropriate exposure chamber.

A schematic of the vapour generation system is presented in Figure A.

No control group was present during the preliminary toxicity test.

---

[3] Nupro Co, Willoughby, Ohio 44094, USA

The air supplied to the vapour generators and secondary dilution vessels was filtered to remove any residual particulate and was dried (dew point ~2°C).

EXPOSURE CHAMBERS (FIGURE B)

The exposure chambers were identical to those used in the main micronucleus test and have been described in the main section of this report.

Spatial distribution studies were conducted during preliminary trials of previous studies conducted using Pyrolysis C5s (Huntingdon Life Sciences Reports CSS011/020072 and CSS012/023478).

PROCEDURE

A separate exposure chamber was used for each group.

Prior to the start of each exposure, the mass of test substance in the pressure vessels used for Groups 2, 3 and 4 was checked to ensure there was sufficient material for the scheduled duration of generation and the syringe used for Group 1 was filled with the appropriate amount of the test material for the exposure.

The mice were transferred from the holding cages and placed into the individual compartments of the exposure cages.

The diluent and generator airflows were turned on and the exposure chamber doors were checked to ensure they were secured. The chamber pressure, relative to the exposure room was checked using each of the associated Magnehelic gauges to ensure that operation of the chamber took place at a slightly negative pressure.

For Group 1, the infusion pump was switched on and for Groups 2, 3 and 4, the test substance supply toggle valves between the pressure vessel and the metering valves were opened; the exposure start time was noted and simultaneously the chamber environmental monitoring system was activated (see below). At intervals of 30 minutes, any clinical signs in the mice were recorded together with checks of generation and chamber operational parameters.

The wet and dry bulb temperatures of a thermohygrometer placed in each chamber were also recorded at approximately 30-minute intervals throughout each exposure. Relative humidity was found using a look-up table. The flow rate of air to the exposure chambers was measured using calibrated flow meters and also checked approximately every 30 minutes.

Results of the determination of Pyrolysis C5s in the chamber atmosphere were automatically recorded for each chamber at 30-minute intervals throughout each exposure.

At the end of six hours generation, the infusion pumps were switched off and the syringes carefully removed from the inlet line and the contents weighed. The vapour in the test chambers was allowed to clear for at least 15 minutes before the animals were removed.

At the end of this time, the mice were unloaded from the chambers and returned to their respective holding cages.

The chambers were washed with hot water.

A summary of the operating conditions is presented in Table 1A.

TEST ATMOSPHERE ANALYSIS AND CHAMBER MONITORING SYSTEM

As for the main micronucleus test, a gas chromatograph was used to measure the concentrations of Pyrolysis C5s in the test atmospheres within the four-inhalation chambers during the preliminary toxicity test. The cycle of monitoring took place in the following sequence: High dose, High Intermediate dose, Low Intermediate dose and Low dose and was repeated throughout the six-hour exposure period. A total of twelve cycles were recorded. Operating details of the Gas Chromatography system, its standardisation and validation are given in Appendix A. Remaining details concerning test atmosphere analysis and the chamber monitoring system can be found in the test atmosphere analysis section of the main micronucleus test study section of this report.

TARGET CONCENTRATIONS

The target concentrations for the preliminary toxicity test of Pyrolysis C5s were:

Group	Designation	Concentration (ppm)
1	Low dose	500
2	Low Inter. dose	1000
3	High Inter. dose	2000
4	High dose	3000

The target concentrations were selected in consultation with the Sponsor, following the review of available data and Huntingdon Life Sciences report numbers CSS011/020072 and CSS012/023478.

EXPOSURE CHAMBER CONDITIONS

Chamber analysed concentration of Pyrolysis C5s

Chamber atmosphere was sampled in sequence from each of the four exposure chambers (Chambers 4 - 1 sampled sequentially) and from one point within each chamber. Air from each chamber was continually drawn through a transfer line, which was therefore equilibrated with the mean concentration from each chamber. When not being sampled, these transfer lines were pumped to waste (Figure B).

Every seven minutes, air from the transfer lines was switched to the injection loop of the gas chromatograph for automated analysis and data processing.

The analytical methodology is presented in Appendix A.

Nominal concentration of chamber atmospheres

Each chamber nominal concentration was calculated from the mass of liquid used over the six-hour exposure period and the exposure mean airflow. The ideal gas equation was used with the molecular weight of the liquid and the measured chamber conditions to compute the volume of vapour produced from the mass of liquid used. The calculation is detailed in Table 3A.

Airflow, temperature and relative humidity

These parameters were recorded manually, as described above under the Test Atmosphere generation and Procedure sections.

RESULTS

VAPOUR CONCENTRATION

Analysed concentration

The data are presented as follows:

Daily mean and Individual values Table 2

The study mean concentrations (the means of daily mean values) for the groups exposed to Pyrolysis C5s are presented below:

Group	Chamber concentration (ppm)	
	Target	Analysed [f]
1 (Low dose)	500	461
2 (Low Inter. dose)	1000	946
3 (High Inter. dose)	2000	2128
4 (High dose) [f]	3000	2990

[a] Calculation standardised using isoprene as the major component
[f] Group 4 mice only exposed on Day 1 of preliminary toxicity test.

The mean chamber concentrations were 8.5, 5.7, 6.4 and 0.3% from target for Groups 1, 2, 3 and 4 (Low, Low Intermediate, High Intermediate and High doses) respectively. Analysed concentrations were in excellent agreement with the target concentrations. The coefficients of variation of the daily means were 17.0, 9.5, and 1.1% for Groups 1, 2 and 3 respectively. These results demonstrate that the test atmospheres were generated consistently well over both days of the study.

NOMINAL CONCENTRATION

The data are presented in Table 3A and are summarised below:

Group	Nominal concentration (ppm)	A/N ratio (%)
1 (Low dose)	510	90.1
2 (Low Inter. dose)	929	101.7
3 (High Inter. dose)	1944	109.5
4 (High dose) [f]	2820	106.0

[f] Group 4 mice only exposed on Day 1 of preliminary toxicity test.

$$A/N = \left(\frac{\text{Analysed concentration}}{\text{Nominal concentration}}\right) \times 100$$

For each Group, the nominal concentration for each exposure was calculated from the equations used for the calculation of the nominal concentration that are detailed in Table 3A.

The expected mean ratios of analysed to nominal concentration (A/N), expressed as a percentage for the study, should be approximately 100% for all dose groups. All A/N ratios are acceptably close to 100%. Differences from the ideal A/N of 100% maybe due to inaccuracies in the measurements of weight, airflow, temperature and analysed concentrations. The coefficients of variations for the mean nominal concentrations are 10.5, 5.3, and 1.4% for Groups 1, 2, and 3 respectively. This indicates consistency with vapourisation of the test material and little likelihood of residue being retained anywhere throughout the whole of the generation system.

CHAMBER TEMPERATURE AND RELATIVE HUMIDITY

The daily mean chamber temperatures and relative humidities are presented in Table 4A.

The chamber temperatures were similar for all groups for most days of the study.

For Groups 2, and 3 (Low Intermediate and High Intermediate doses), the recorded relative humidity (RH) in the range 30 - 35%, lower than the target range of 40 - 60%.

The low values of RH for Groups 2 and 3 probably arise from generation and dilution of the chamber atmospheres with air that was supplied from a compressor system incorporating a refrigerant drier. This deviation from the target conditions had no discernible effect upon the animals and is not considered to have affected the outcome of the study.

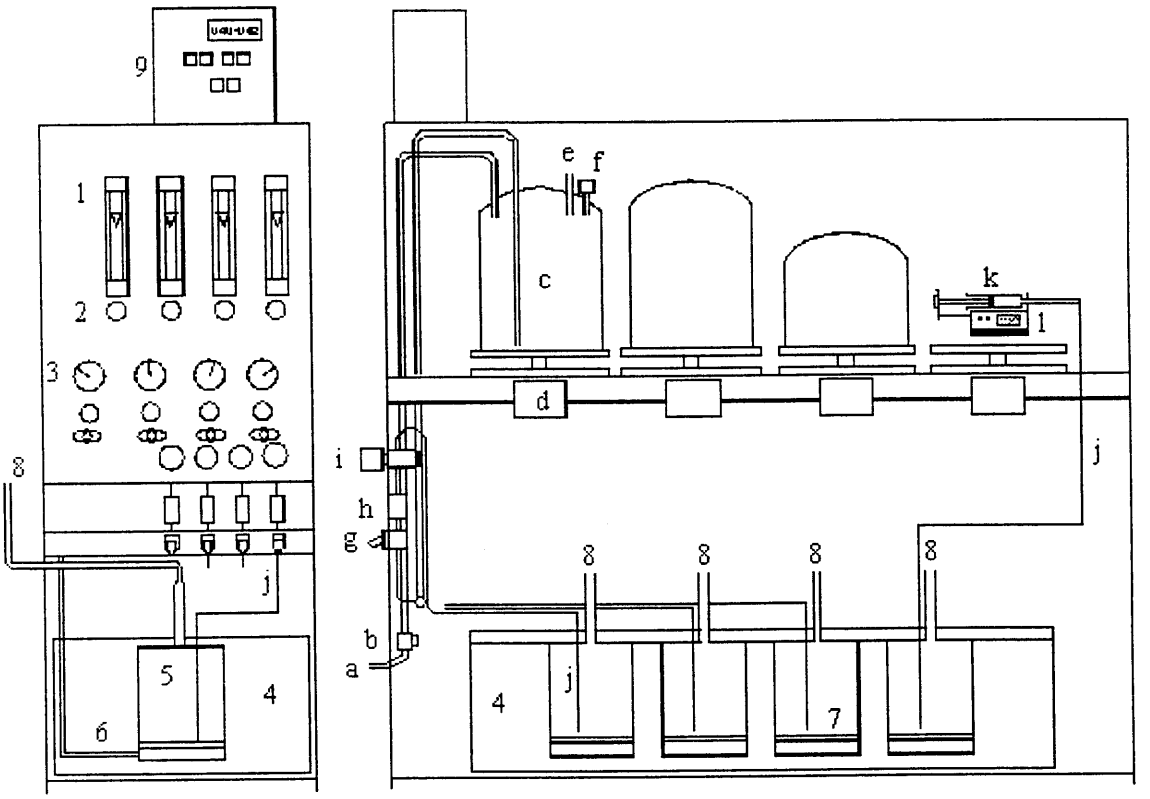
DISCUSSION

Control of the delivery of Pyrolysis C5s vapour to the exposure chambers was good, as reflected in the study mean concentrations, which were within 9% of the target values for all Groups.

The agreement between the analysed and the nominal chamber concentration values for Group 4 (High dose) were acceptable. This indicates consistency with vapourisation of the test material.

FIGURE 1A

Schematic of the vapour generation system used in the preliminary toxicity test



Key

1	Diluent air rotameters	a	Test substance inlet from bulk source
2	Rotameter control valve	b	Test substance feed line filters
3	Reservoir pressure display	c	Liquid reservoir
4	Water bath	d	Load cell
5	Vapour generator	e	Helium (pressure) inlet
6	Diluent air	f	Safety valve
7	Sinter Diameter (130 mm)	g	Toggle valve
8	Vapour/air mixture (to chamber)	h	0.5 µm stainless steel filter
9	Load cell display	i	Micro-metering needle valve
		j	Test compound feed line to vapour generator
		k	Polypropylene syringe
		l	Syringe Driver (Precidor 5003)

TABLE 1A

Operating conditions for the rodent inhalation exposure system during the preliminary toxicity test

Parameter	Group			
	1 (Low dose)	2 (Low Inter. dose)	3 (High Inter. dose)	4 (High dose)
Target concentration of Pyrolysis C5s (ppm)	500	1000	2000	3000
Chamber airflows (l/minute)				
Target air flow to vapour generator	60	50	90	150
Target supplementary airflow	90	100	60	0
Vapour generator settings				
Test material feed	Precidor 5003 syringe pump	Generation Unit		
Syringe size (ml)	140	N/A	N/A	N/A
Reservoir pressure (Helium, psi)	N/A	10	10	10
Sinter diameter (mm)	130	130	130	130
Chamber negative pressure (mm/H_2O)	2 - 4	2 - 4	2 - 4	2 - 4

N/A Not applicable

TABLE 2A

Chamber concentrations of Pyrolysis C5s (ppm) during preliminary toxicity test - daily mean and individual values

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Low dose)	Group 2 (Low Inter. dose)	Group 3 (High Inter. dose)	Group 4 (High dose)
1	1	400	390	1368	717
	2	458	674	2075	3010
	3	490	1026	2040	2922
	4	531	1047	2051	2969
	5	622	1075	2172	2965
	6	558	1151	2101	3086
	7	498	1150	2164	2959
	8	483	1159	2115	3076
	9	500	953	2157	2981
	10	515	947	2176	2993
	11	520	969	2063	2988
	12	498	949	2102	2944
	Mean [b]	516	1009	2111	2990
	sd	44.0	138.9	50.4	50.9
2 [g]	13	310	859	1479	893
	14	325	882	2064	2935
	15	340	873	2149	3093
	16	461	889	2200	2974
	17	375	852	2145	2986
	18	393	886	2242	2938
	19	396	877	2094	3079
	20	451	871	2143	3013
	21	460	891	2143	3039
	22	387	891	2167	3108
	23	425	915	2127	3059
	24	445	878	2109	2981
	Mean [b]	405	882	2144	3019
	sd	47.2	15.5	48.8	61.0
Overall mean		461	946	2128	2990 [g]
sd		78.5	89.8	23.3	N/A
CV(%)		17.0	9.5	1.1	N/A

[b] The initial concentration measurement of each exposure was excluded from all calculations.

[g] Group 4 test atmosphere was generated in the absence of mice. Exclude exposure 2 Group 4 mean from overall mean

N/A Not Applicable

sd Standard deviation

CV (%) Coefficient of Variation (sd × 100/mean)

TABLE 3A

Nominal concentrations of Pyrolysis C5s (ppm) during preliminary toxicity test - individual exposure values

Group	Exposure		Barometric pressure (mmHg)	Pyrolysis C5s usage (kg)	Chamber Airflow [c] (l/minute)	Chamber concentration		A/N ratio (%)
	No.	Duration (minute)				Analysed (ppm)	Nominal [d] (ppm)	
1	1	360	755	0.0852	150	516	548	94.2
	2	360	765	0.0744	150	405	472	85.9
Mean			760	0.0798	150	461	510	90.1
sd			7.1	0.00768	0.0	78.5	53.7	5.91
CV (%)			0.9	9.6	0.0	17.0	10.5	6.6
2	1	360	755	0.150	150	1009	963	104.8
	2	360	765	0.141	150	882	894	98.6
Mean			760	0.146	150	946	929	101.7
sd			7.1	0.0064	0.0	89.8	48.8	4.33
CV (%)			0.9	4.4	0.0	9.5	5.3	4.3
3	1	360	755	0.300	150	2111	1924	109.7
	2	360	765	0.310	150	2144	1963	109.2
Mean			760	0.305	150	2128	1944	109.5
sd			7.1	0.0071	0.0	23.3	27.4	0.34
CV (%)			0.9	2.3	0.0	1.1	1.4	0.3
4	1	360	755	0.440	150	2990	2820	106.0
	2 [g]	360	765	0.450	150	3019	2851	105.9
Mean			760	0.445	150	2990 [g]	2820 [g]	106.0 [g]
sd			7.1	0.0071	0.0	N/A	N/A	N/A
CV (%)			0.9	1.6	0.0	N/A	N/A	N/A

[c] Includes 60 l/minute through vaporiser for Group 1, 50l/minute through vaporiser for Group 2 and 90 l/minute through vaporiser for Group 3

[d] Calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\ \text{mm Hg}}{\text{Atm}}$$

where V = gaseous volume of Pyrolysis C5s (litres)
W = mass of Pyrolysis C5s (kg)
M = approximate molecular weight of Pyrolysis C5s (70 g/mole)
R = gas constant (0.08206 l atm mol^{-1} K^{-1})
T = temperature (K), = temperature (°C, see Table 4 + 273)
Atm = atmospheric pressure (mmHg)
V_a = volume of air (litres) passing through the chamber during the exposure

[g] Group 4 test atmosphere was generated in the absence of mice. Exclude exposure 2 Group 4 mean from overall mean

A/N Analysed/nominal concentration ratio expressed as a percentage
N/A Not Applicable
sd Standard deviation
CV (%) Coefficient of Variation (sd × 100/mean)

TABLE 4A

Chamber temperature and relative humidity during preliminary toxicity test - exposure mean values

Exposure No.	Mean chamber temperatures (°C) and relative humidity (%RH)							
	Group 1 (Low dose)		Group 2 (Low Inter. dose)		Group 3 (High Inter. dose)		Group 4 (High dose)	
	Temp	RH	Temp	RH	Temp	RH	Temp	RH
1	21.0	62.5	21.0	35.8	21.0	30.2	21.0	37.4
2	21.0	64.7	21.2	35.9	21.1	30.5	[g]	[g]
Mean	21.0	63.6	21.1	35.9	21.1	30.4	21.0	37.4
sd	0.00	1.56	0.14	0.07	0.07	0.21	N/A	N/A
CV (%)	0.0	2.4	0.7	0.2	0.3	0.7	N/A	N/A

[g] Group 4 test atmosphere was generated in the absence of mice. No temperature or RH data recorded

sd standard deviation

CV(%) Coefficient of variation (sd × 100/mean)