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MUTAGENICITY TEST ON

T-5711

IN AN *IN VIVO* RAT MICRONUCLEUS ASSAY

DATA REQUIREMENT

U.S. EPA FIFRA Guideline 84-2

FINAL REPORT

AUTHOR

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PERFORMING LABORATORY

Hazleton Washington, Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

HWA Study No.: 15515-0-454

SUBMITTED TO

3M Corporation
3M Center
St. Paul, Minnesota 55144

STUDY COMPLETION DATE

April 30, 1993

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

Information claimed confidential on the basis of its falling within the scope of FIFRA § 10(d)(1)(A),(B), or (C) has been removed to a confidential appendix, and is cited by cross-reference number in the body of the study.

Company: _____

Company Agent: _____
(Typed Name)

_____
(Title)

_____ _____
Signature Date

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the EPA FIFRA Good Laboratory Practice Standards as set forth in Title 40 of the U.S. Code of Federal Regulations Part 160. Any significant deviations from the protocol and/or GLP are attached as an appendix to this report. There were no deviations from the aforementioned regulation which affected the quality or integrity of the study or the interpretation of the results in this report.

Study Director:

Hemalatha Murli

4/30/93
Date

Hemalatha Murli, Ph.D.
Mammalian Cytogenetics
Department of Molecular and Cellular Toxicology

Study Monitor:

_____

_____
Date

Applicant/Submitter:

_____

_____
Date

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QUALITY ASSURANCE STATEMENT

PROJECT TITLE: *IN VIVO* RAT MICRONUCLEUS ASSAY

PROJECT NO.: 20996 ASSAY NO.: 15515

PROTOCOL NO.: 454 EDITION NO.: 5

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspection/Date	Findings Reported	Auditor
Weighing of Test Article-3/2/1993	3/02/1993	K. Newland
Draft Report Review-4/16,19/1993	4/23/1993	B. Mullett
Final Report Review-4/30/1993	4/30/1993	C. Lee

Quality Assurance Unit 4/30/93 Date Released

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STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160); the Organization for Economic Cooperation and Development Principles of Good Laboratory Practice C(81)30 (Final) Annex 2, issued 1979-1980 (effective 1981). To the best of the signers' knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director. All raw data, documentation, records, protocols, and a copy of the final report generated as a result of this study will be archived in the storage facilities of Hazleton Washington, Inc., for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Hazleton Washington, Inc., for an additional period of time, or sent to a storage facility designated by the Sponsor.

SUBMITTED BY:

José Arriaga

4/30/93
Date

José Arriaga, B.S.
Research Associate

Study Director:

Hemalatha Murli

4/30/93
Study Completion Date

Hemalatha Murli, Ph.D.
Mammalian Cytogenetics
Department of Molecular and Cellular Toxicology

15515-0-454

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SUMMARY

Mutagenicity Test on T-5711 in an *in vivo* Rat Micronucleus Assay

The objective of this *in vivo* assay was to evaluate the ability of the test article, T-5711, to induce micronuclei in bone marrow polychromatic erythrocytes of Sprague-Dawley rats. The test article was suspended in 0.5% carboxymethylcellulose (high viscosity).

For the dose selection study, 3 males and 3 females per dose level were dosed by oral gavage at 500, 1625, 2750, 3875, and 5000 mg/kg. Mortality was observed with 1 male at 5000 mg/kg and 1 female at 3875 mg/kg.

Based upon these results, rats were dosed for the micronucleus assay by oral gavage at 1250, 2500, and 5000 mg/kg. Ten animals (five males and five females) were randomly assigned to each dose/harvest time group. Vehicle and positive control groups euthanatized approximately 24 hours after dosing were included in the assay. The animals were dosed with the test article and were euthanatized approximately 24, 48 and 72 hours after dosing for extraction of the bone marrow.

The test material, T-5711, did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the rat bone marrow micronucleus test.

Mutagenicity Test on T-5711 in an in vivo Micronucleus Assay

I. SPONSOR: 3M Corporation

II. MATERIAL (TEST ARTICLE):

A. Client's Identification: L-1276 (T-5711)

B. Dates Received: February 24, 1993; March 12, 1993

C. Physical Description: Cream-colored granular material

D. Genetics Assay No.: 15515

III. TYPE OF ASSAY: *In Vivo* Rat Micronucleus Assay

IV. PROTOCOL NO.: 454, Edition 5

V. STUDY DATES:

A. Initiation Date: February 24, 1993

B. Experimental Start Date: March 2, 1993

C. Experimental Termination Date: March 23, 1993

VI. SUPERVISORY PERSONNEL:

A. Study Director: Hemalatha Murli, Ph.D.

B. Laboratory Supervisor: José Arriaga, B.S.

VII. OBJECTIVE:

The objective of this *in vivo* assay was to evaluate the ability of the test article, T-5711, to induce micronuclei in bone marrow polychromatic erythrocytes of Sprague-Dawley rats. This study was conducted using modifications of the procedures suggested by Heddle et al. (1983).

VIII. MATERIALS:

Adult male and female rats, strain Sprague-Dawley, were purchased from Charles River Laboratories, Raleigh, NC. This healthy, random bred strain was selected to maximize genetic heterogeneity and at the same time assure access to a common source.

Animals were isolated by sex. Animals were housed two per cage during quarantine, and housed individually at randomization. The temperature and humidity were maintained at 74.1 to 80.4°F and 50±20%, respectively for the dose selection study and at 72±6°F and 50±20%, respectively for the micronucleus study. A 12-hour light/12-hour dark cycle was maintained. A commercial diet (Purina® Certified Laboratory Chow® #5002) and water were available ad libitum for the duration of the study. The feed was analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. The water was analyzed on a retrospective basis for specified microorganisms, pesticides, alkalinity, heavy metals, and halogens. Sanitary, stainless steel, hanging wire cages were used. Personnel handling animals or working within the animal facilities were required to wear suitable protective garments and equipment.

Animals were quarantined for seven days before being placed on study. Animals were randomly assigned to study groups and were individually weighed prior to dosing. All animals were dosed based upon the individual body weights. Animals were uniquely identified by ear tag. Dose or treatment groups were identified by cage card.

At the termination of the study all surviving animals were euthanatized by CO_2 inhalation, followed by penetration of the thorax. Any extra animals not used for the study were reassigned for personnel training purposes.

IX. SOLUBILITY AND STABILITY:

The test article, T-5711, was supplied as a cream-colored granular material. The solubility of the test article was evaluated for the dose range finding assay. In that study, solubility was evaluated in corn oil and 0.5% high viscosity carboxymethylcellu-lose (CMC). Uneven and unsuitable suspensions were obtained in corn oil. An opaque, slightly viscous suspension was obtained when 0.48 ml of CMC was added to 594.5 mg of T-5711, resulting in a final volume of about 1.2 ml, and a final concentration of about 495.4 mg/ml. This suspension was homogenized in a Tissuemizer® for about 30 seconds, resulting in a more uniform suspension that passed easily through an 18G gavage needle. Thus, the vehicle used to solubilize the test article for the bone marrow micronucleus assay was CMC. The stability of the test article

under the preparation and dosing conditions of the assay is the responsibility of the sponsor.

X. DOSE SELECTION STUDY

A. Dose Selection:

No toxicity information was available on T-5711 and dose levels of 500, 1625, 2750, 3875, and 5000 mg/kg body weight were administered by oral gavage for a dose selection study.

B. Dosing Information:

The animals used in the dose selection assay were dosed on Tuesday, March 2, 1993. The weight range of the animals used in the dose range finding assay was 278.6 - 319.8 grams and 199.8 - 244.9 grams for the males and females, respectively. Dosing suspensions were prepared just prior to dosing and were prepared by making a 500 mg/ml stock for the high dose (5000 mg/kg). This was prepared by adding 41.4 ml of CMC to 30.0012 g of T-5711, resulting in a final volume of 60.0 ml and a final concentration of 500 mg/ml. This opaque, cream-colored suspension was mixed for about 2 minutes in a Tissuemizer® to ensure homogeneity. Dilutions of this stock were prepared for the 3875, 2750, 1625, and 500 mg/kg dose levels.

Dosing was achieved using a 10 ml/kg dosing volume. All animals were about 9 weeks old at the time of dosing. An outline of the dosing scheme is found in the following table.

A total of 30 animals was used in this assay.

		DOSE GROUPS	
	TREATMENT	M	F
T-5711	500 mg/kg	3	3
	1625 mg/kg	3	3
	2750 mg/kg	3	3
	3875 mg/kg	3	3
	5000 mg/kg	3	3

All doses given are on an acute (one-time only) basis.
Animals and were ≈9 weeks old at the time of dosing.

C. Results and Interpretation:

All animals were examined after dosing and daily throughout the duration of the study (three days) for toxic effects and/or mortalities.

No toxic effects were noted in any of the animals observed immediately and about 3 hours after dosing.

Approximately 21.5 hours after dosing, 1 male (#6160) from the 5000 mg/kg dose group was experiencing convulsions and lacrimation of the eyes, and appeared languid. All other animals appeared normal and healthy at this time.

Approximately 47 hours after dosing, this male (#6160) from the 5000 mg/kg dose group was found dead. All other animals appeared normal and healthy at this time.

Approximately 69 hours after dosing, 1 female (#6184) from the 3875 mg/kg dose group was found dead. All other animals appeared normal and healthy at this time and remained so until the end of the observation period. The mortality data for this assay are summarized in the following table:

Summary of Mortalities Within 3 Days in Rats Dosed Acutely with T-5711

Treatment	Observations	
	Male	Female
500 mg/kg	0/3	0/3
1625 mg/kg	0/3	0/3
2750 mg/kg	0/3	0/3
3875 mg/kg	0/3	1/3
5000 mg/kg	1/3	0/3

D. Conclusion:

Based upon the mortality and toxic signs in the animals in this study the maximum tolerated dose (MTD) was estimated to be about 5000 mg/kg. The results of this range finding assay were sufficient to select doses for a rat micronucleus assay.

XI. MICRONUCLEUS STUDY:

A. Dose Selection:

The dose levels used in this assay were based on the dose selection study and animals were dosed at 1250, 2500, and 5000 mg/kg body weight, administered by oral gavage.

B. Micronucleus Assay Dosing Information:

The animals used in the micronucleus assay were dosed on Tuesday, March 16, 1993. Cyclophosphamide (CP, Sigma, Lot #70H0948), the positive control, was solubilized in sterile deionized water (Lot#13, prepared at HWA) and was administered by oral gavage at 60 mg/kg. The vehicle control consisted of CMC (Sigma, Lot#121F-0544, prepared on 1/20/1993; expiration date 1/20/1994) and was administered concurrently with the test article at a volume of 10 ml/kg. The weight range of the animals used in the micronucleus assay was 236.1 - 287.3 grams and 175.5 - 274.4 grams for the males and females, respectively. The dosing suspensions for the assay were prepared by making a 500 mg/ml stock for the high dose (5000 mg/kg). This was prepared by adding 133.5 ml of CMC to 90.0002g of T-5711, resulting in a final volume of 180 ml and a final concentration of 500 mg/ml. This opaque, cream-colored suspension was mixed for about 5 minutes in a Tissuemizer® to ensure homogeneity. Dilutions of this stock were prepared for the 2500 and 1250 mg/kg dose levels. A second group of animals (designated Secondary Dose Group) was also assigned to the study and was dosed with the high dose of the test article. These animals were only used in the assay as replacements for any which died in the primary dose group.

The test article dosed animals were euthanatized approximately 24, 48 and 72 hours after administration of the test article. The positive and vehicle control animals were euthanatized approximately 24 hours after the administration of the control articles. An outline of the dosing scheme is found in the following table:

Dosing Scheme for Micronucleus Assay

A total of 120 animals was used in this assay.

Number of Animals Assigned

Treatment	Primary Dose Groups						Secondary Dose Groups[a]	
	24 Hr		48 Hr		72 Hr			
	M	F	M	F	M	F	Male	Female
T-5711								
1250 mg/kg	5	5	5	5	5	5	-	-
2500 mg/kg	5	5	5	5	5	5	-	-
5000 mg/kg	5	5	5	5	5	5	5	5
Vehicle Control, 0.5% high viscosity carboxymethylcellulose								
10 ml/kg	5	5	-	-	-	-	-	-
Positive Control, Cyclophosphamide								
60 mg/kg	5	5	-	-	-	-	-	-

[a] The animals assigned to the secondary dose groups were dosed and were only used to replace animals which died in the primary dose group at the high dose level. All extra animals not used as replacements were euthanatized at the completion of the trial.

The age of the animals at the time of dosing was ≈8 weeks.

Volumes dosed were 10 ml/kg and were based upon individual animal weights.

XII. BONE MARROW HARVEST, SLIDE PREPARATION AND ANALYSIS:

At the appropriate harvest time, the animals were euthanatized with CO_2 and the adhering soft tissue and epiphyses of both tibiae were removed. The marrow was aspirated from the bone and mixed in a syringe with ~0.1 ml fetal calf serum to form a suspension. The cells were then placed on slides and air-dried, fixed in methanol, and stained in May-Grunwald solution followed by Giemsa (Schmid, 1975). The air-dried slides were coverslipped using Depex® mounting medium.

The coded slides were then scored for micronuclei and the polychromatic (PCE) to normochromatic (NCE) cell ratio. Standard forms were used to record these data. One thousand PCEs per animal were scored. The frequency of micronucleated cells was expressed as percent micronucleated cells based on the total PCEs present in the scored optic field. The normal frequency of micronuclei in this rat strain is about 0.0-0.4%.

The frequency of PCEs versus NCEs was determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring the first 1000 erythrocytes.

XIII. EVALUATION CRITERIA:

A. General:

The criteria for the identification of micronuclei were those of Schmid (1976). Micronuclei were darkly stained and generally round, although almond and ring-shaped micronuclei occasionally occur. Micronuclei had sharp borders and were generally between 1/20 and 1/5 the size of the PCE. The unit of scoring was the micronucleated cell, not the micronucleus; thus the occasional cell with more than one micronucleus was counted as one micronucleated PCE, not two (or more) micronuclei. The staining procedure permitted the differentiation by color of PCEs and NCEs (bluish-grey and red, respectively).

B. Data Presentation and Interpretation

Data are summarized by sex and dose groups for the different time points. Individual animal data are also presented. The analysis of the data was performed using an Analysis of Variance on the square root arcsine transformation which was performed on the proportion of cells with micronuclei per animal (square root arcsine proportion). Once the Analysis of Variance had been performed, Tukey's Studentized range test (HSD) with adjustment for multiple comparisons (Sokal and Rohlf, 1981) was used at each harvest time to determine which dose groups, if any, were significantly different ($p<0.05$) from the vehicle control. Analyses were performed separately for each harvest time and sex combination, and also at each harvest time for the sexes combined.

The criteria for determining a positive response involved a statistically significant dose-related increase in micronucleated PCEs, or the detection of a reproducible and statistically significant positive response for at least one dose level. A test

article that induced neither a statistically significant dose response nor a statistically significant and reproducible increase at one dose level was considered negative. In either case, the final decision was based on scientific judgment.

XIV. RESULTS AND INTERPRETATION:

All animals were observed immediately after dosing and periodically throughout the duration of the assay for toxic symptoms and/or mortalities. All animals in the vehicle and positive control groups appeared normal after dosing and remained healthy until the appropriate harvest times. All test article dosed groups appeared normal immediately after dosing, and approximately 19 and 23 hours after dosing.

Approximately 47 hours after dosing, 1 male (#6333) from the secondary dose group and 1 female (#6387) from the 2500 mg/kg and 48 hour harvest group were found dead. All remaining animals from 5000 mg/kg dose group appeared languid. All animals in the 1250 mg/kg dose group and all surviving animals in the 2500 mg/kg dose group appeared normal and healthy at this time.

Approximately 71.5 hours after dosing, 2 males (#'s6314, 6379) and 1 female (#6360) from the 5000 mg/kg and 72 hour harvest group, and 1 male (#6421) and 1 female (#6386) from the secondary dose group were found dead. All remaining animals from the 5000 mg/kg dose group and the secondary dose group appeared languid. All animals from the 1250 and 2500 mg/kg dose groups appeared normal and healthy at this time.

The test article, T-5711, induced no significant increases in micronucleated polychromatic erythrocytes over the levels observed in the vehicle controls in either sex or at any of the harvest times. Possibly due to toxicity, a statistically significant reduction in the PCE/NCE ratio was observed in the males from the positive control group. The positive control, CP, induced significant increases in micronucleated PCEs in both sexes, with means and standard errors of 2.24% ± 0.46% and 2.22% ± 0.33% for the males and females, respectively. The data summarized by dose group are presented in Table 1 and individual animal data are found in Tables 2 through 4.

XV. CONCLUSION:

The test material, T-5711, did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the rat bone marrow micronucleus test.

XVI. REFERENCES:

Heddle, J.A., Hite, M., Kirkhart, B., Larsen, K., MacGregor, J.T., Newell, G.W., and Salamone, M.F.: The induction of micronuclei as a measure of genotoxicity. Mutation Res., 123:61-118, 1983.

Schmid, W.: The micronucleus test. Mutation Res., 31:9-15, 1975.

Schmid, W.: The micronucleus test for cytogenetic analysis. In, Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4 (A. Hollaender, ed.). Plenum, pp. 31-53, 1976.

Sokal, R.R. and Rohlf, J.J.: Biometry, Freeman, 1981.

XVII. DEVIATION FROM THE SIGNED PROTOCOL:

The following deviation was made from the protocol.

The temperature was maintained at 74.1 to 80.4°F for the dose selection study and not maintained at 72±6°F. This was due to technical difficulties at the animal facility. This slight deviation did not affect the animals in any way.

XVIII. EXPERIMENT DATA TABLES

TABLE 1

MICRONUCLEUS DATA SUMMARY TABLE

SPONSOR: 3M Corporation

TEST ARTICLE: T-5711

ASSAY: 15515

TREATMENT	DOSE	HARVEST TIME (HR)	% MICRONUCLEATED PCEs MEAN OF 1000 PER ANIMAL ± S.E. MALES	FEMALES	TOTAL	RATIO PCE:NCE MEAN ± S.E. MALES	FEMALES
VEHICLE CONTROL 0.5% CARBOXYMETHYLCELLULOSE	10 ml/kg	24	0.12 ± 0.05	0.02 ± 0.02	0.07 ± 0.03	0.66 ± 0.04	0.58 ± 0.13
POSITIVE CONTROL CYCLOPHOSPHAMIDE	60 mg/kg	24	2.24 ± 0.46*	2.22 ± 0.33*	2.23 ± 0.27	0.34 ± 0.07*	0.51 ± 0.13
TEST ARTICLE T-5711	1250 mg/kg	24	0.04 ± 0.02	0.16 ± 0.06*	0.10 ± 0.04	0.84 ± 0.19	0.40 ± 0.02
		48	0.10 ± 0.04	0.12 ± 0.04	0.11 ± 0.03	0.63 ± 0.04	0.54 ± 0.09
		72	0.06 ± 0.04	0.10 ± 0.03	0.08 ± 0.02	0.46 ± 0.05	0.43 ± 0.16
	2500 mg/kg	24	0.14 ± 0.04	0.14 ± 0.02*	0.14 ± 0.02	0.63 ± 0.09	0.52 ± 0.14
		48	0.10 ± 0.04	0.08 ± 0.03	0.09 ± 0.03	0.79 ± 0.15	0.47 ± 0.11
		72	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.02	0.49 ± 0.11	0.53 ± 0.13
	5000 mg/kg	24	0.10 ± 0.05	0.08 ± 0.02	0.09 ± 0.03	0.82 ± 0.10	0.79 ± 0.10
		48	0.16 ± 0.02	0.08 ± 0.04	0.12 ± 0.02	0.63 ± 0.14	0.52 ± 0.16
		72	0.12 ± 0.04	0.12 ± 0.05	0.12 ± 0.03	0.74 ± 0.19	0.39 ± 0.11

* Significantly different from the corresponding vehicle control, $p<0.05$.

TABLE 2

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M Corporation

TEST ARTICLE: T-5711

ASSAY NO.: 15515

TREATMENT	MALES			FEMALES		
	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE
Vehicle Control (24 hour harvest)	6357	3	0.61	6317	0	0.95
	6367	0	0.69	6355	0	0.51
	6376	1	0.63	6390	0	0.72
	6438	1	0.56	6407	4	0.15
	6442	1	0.80	6434	0	0.55
Positive Control (24 hour harvest)	6312	23	0.37	6318	13	0.35
	6375	18	0.60	6346	30	0.92
	6396	16	0.33	6359	16	0.37
	6419	15	0.17	6373	25	0.72
	6432	40	0.25	6415	27	0.20
Test Article (24 hour harvest) 1250 mg/kg	6324	1	0.82	6322	1	0.41
	6335	1	0.37	6351	3	0.32
	6363	0	0.54	6364	0	0.46
	6406	0	1.04	6399	1	0.37
	6408	0	1.43	6410	3	0.43
2500 mg/kg	6321	2	0.31	6347	2	0.33
	6337	2	0.82	6378	1	0.72
	6384	1	0.67	6397	2	0.63
	6431	2	0.60	6402	1	0.84
	6439	0	0.77	6444	1	0.08
5000 mg/kg	6332	0	1.07	6330	1	0.73
	6343	1	0.52	6331	1	0.61
	6372	0	0.82	6349	1	0.70
	6418	1	0.66	6382	1	1.19
	6424	3	1.02	6420	0	0.71

MN = Micronucleus
PCE = Polychromatic erythrocyte
No. MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

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TABLE 3

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M Corporation

TEST ARTICLE: T-5711

ASSAY NO.: 15515

TREATMENT	MALES			FEMALES		
	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE
Test Article (48 hour harvest) 1250 mg/kg	6325	0	0.59	6328	2	0.43
	6427	2	0.79	6374	0	0.86
	6428	2	0.60	6394	1	0.39
	6443	1	0.58	6426	2	0.46
	6447	0	0.60	6430	1	0.55
2500 mg/kg	6319	0	0.39	6313	1	0.38
	6326	0	0.54	6338	1	0.45
	6327	2	1.10	6352	0	0.25
	6380	1	1.11	6362	1	0.78
	6401	2	0.80	6387*		
5000 mg/kg	6329	1	0.44	6341	1	0.25
	6348	2	0.93	6358	0	0.35
	6377	2	0.19	6361	2	1.07
	6413	2	0.89	6412	1	0.27
	6422	1	0.69	6414	0	0.64

MN = Micronucleus
PCE = Polychromatic erythrocyte
No. MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

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TABLE 4

MICRONUCLEUS TEST - INDIVIDUAL ANIMAL DATA

SPONSOR: 3M Corporation TEST ARTICLE: T-5711

ASSAY NO.: 15515

TREATMENT	MALES			FEMALES		
	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE	ANIMAL NUMBER	NO. MN PCEs (1000)	RATIO PCE:NCE
Test Article (72 hour harvest) 1250 mg/kg	6354	0	0.45	6345	0	0.46
	6370	2	0.46	6350	1	0.13
	6425	0	0.63	6393	1	0.36
	6429	0	0.43	6433	2	0.15
	6441	1	0.32	6435	1	1.04
2500 mg/kg	6320	0	0.57	6334	2	0.30
	6398	2	0.25	6366	0	0.97
	6404	1	0.20	6403	0	0.33
	6417	0	0.72	6405	1	0.36
	6437	1	0.69	6445	1	0.71
5000 mg/kg	6342	2	0.20	6315	3	0.53
	6383	1	0.90	6323	1	0.16
	6391	2	1.29	6385	1	0.16
	6392	0	0.81	6411	1	0.33
	6446	1	0.48	6436	0	0.75

MN = Micronucleus
PCE = Polychromatic erythrocyte
No. MN PCEs = Micronucleated PCEs
NCE = Normochromatic erythrocyte

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HWA STUDY NO. _____
PROTOCOL NO. 454
EDITION 5

IN VIVO RAT MICRONUCLEUS ASSAY

Hazleton Washington, Inc. (HWA) will conduct this study in compliance with EPA and FDA Good Laboratory Practice (GLP) Guidelines. This protocol, critical phases of the work in progress and the final report will be subject to audit by Quality Assurance in accordance with SOPs at Hazleton Washington, Inc. The study will be conducted by HWA at 9200 Leesburg Pike, Vienna, Virginia 22182.

PART 1. SPONSOR INFORMATION AND APPROVALS

I. SPONSOR IDENTIFICATION

Company Name: _____

Address: _____

II. TEST ARTICLE IDENTIFICATION: _____

_____

III. TEST ARTICLE ANALYSIS

Determination of the test article stability and the test article characteristics as defined in the GLP regulations of FDA (21 CFR 58.105), EPA-TSCA (40 CFR 792.105), and EPA-FIFRA (40 CFR 160.105) is the responsibility of the Sponsor.

IV. NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with U.S. federal regulation codes (FDA, 21 CFR 58.10; EPA-TSCA, 40 CFR 792.10; EPA-FIFRA, 40 CFR 160.10) and certain foreign agencies, consulting laboratories must be notified if all or part of a study is to be submitted to the agency. HWA maintains a master schedule of studies which fall under regulatory review. Please indicate which agency, if any, might receive the results of this study:

☐ Undetermined ☐ FDA ☐ EPA-TSCA ☐ EPA-FIFRA

☐ MAFF ☐ MOHW ☐ OECD ☐ OTHER _____

V. STUDY DATES

Proposed Experimental Start Date: _____

Proposed Experimental Termination Date: _____

VI. APPROVAL OF STUDY PROTOCOL

Study Director: _____ Date: _____
Hemalatha Murli, Ph.D.

Sponsor: _____ Date: _____

PART 2 - STUDY PROTOCOL

IN VIVO RAT MICRONUCLEUS ASSAY

I. OBJECTIVE

The objective of this study is to evaluate a test article for clastogenic activity and disruption of the mitotic apparatus in polychromatic erythrocyte stem cells in rat bone marrow in vivo.

II. DEFINITIONS

Micronucleus: a small chromatin body, consisting of entire chromosome(s) and/or of acentric chromosome fragment(s), which lags behind at mitotic anaphase. After telophase, these chromosome(s) and fragment(s) may not be included in the daughter nuclei, and may form single or multiple micronuclei in the cytoplasm.

III. RATIONALE

The micronucleus test can serve as a rapid screen for clastogenic agents and test articles which interfere with normal mitotic cell division (Schmid, 1975; Heddle et al., 1983). Micronuclei are formed from chromosomes or chromosome fragments left behind during anaphase and can be scored during interphase because they persist (Schmid, 1975). In this assay, polychromatic erythrocytes (PCEs) in the bone marrow are scored for the presence of micronuclei. During maturation from erythroblast to erythrocyte the nucleus is extruded, while micronuclei, if present, remain in the cytoplasm. Detection of micronuclei in non-nucleated cells is thus facilitated, and time involved in searching for metaphase spreads in treated cell populations is eliminated. Test articles affecting spindle-fiber function or formation as well as clastogenic agents can be detected through micronucleus induction (Schmid, 1975).

IV. MATERIALS

A. Animals

Young adult male and female rats of the Sprague-Dawley strain, 8-10 weeks old at the time of dosing, will be purchased from Charles River Laboratories, Inc., or

Harlan Sprague-Dawley, Inc. This strain has been selected to maximize genetic heterogeneity and at the same time ensure access to a common source.

B. Control Articles

Cyclophosphamide (CP, 60 mg/kg) will be used as the positive control article and will be administered by oral gavage. The vehicle control article will consist of the solvent or vehicle used for the test article and will be administered by the same route as, and concurrently with, the test article and in amounts equal to the maximum volumes administered to the experimental animals. The maximum dosing volume will not exceed 20 ml/kg. The vehicles generally used in the assay are water, 0.9% saline, 0.5% aqueous carboxymethylcellulose solution, or corn oil. Other vehicles may be requested by the sponsor.

V. EXPERIMENTAL DESIGN

A. Animal Husbandry

Animals will be isolated by sex. Animals will be housed two per cage during quarantine, but will be housed singly prior to experiment initiation. Animals are housed under the following climatic conditions: temperature, 72° ± 6°F; humidity, 50% ± 20%; light cycle, 12 hours light/dark. A commercial diet (Purina Certified® Laboratory Chow #5002) and tap water will be available ad libitum unless contraindicated by the particular experimental design. The feed is analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. The water is analyzed biannually on a retrospective basis for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens. Animals will be quarantined for at least 7 days before being placed on study.

Animals will be assigned to study groups at random according to Hazleton Standard Operating Procedures. Animals will be weighed prior to dosing. They will be dosed based upon the individual animal weights. Animals will be uniquely identified by ear tag. Treatment groups will be identified by cage label.

Sanitary cages and bedding will be used. Personnel handling animals or working within the animal facilities will be required to wear suitable protective garments and equipment.

B. Dose Selection

The high dose generally will be selected as 80% of the maximum tolerated dose. The high dose should produce some indication of toxicity (e.g., death, depression of ratio of PCEs to normochromatic erythrocytes (NCEs). One-half and one-quarter of this high dose will normally be used as the intermediate and low dose levels, respectively. Use of a high dose increases the likelihood that a weak clastogen will be detected, and is therefore recommended.

If no appropriate range finding data are available, a range finding study can be performed.

DOSE RANGEFINDING STUDY

The dose-rangefinding study will be conducted using five treatment groups. Each of the five groups will consist of 3 male and 3 female rats.

Group Designation and Treatment Regimens

Group No.	Number of Rats Male	Female	Route	Duration (Days)
1	3	3	PO	3
2	3	3	PO	3
3	3	3	PO	3
4	3	3	PO	3
5	3	3	PO	3

The route of administration will be oral gavage. In the event that test article characteristics preclude oral gavage, IP injection will be employed. These routes of administration have

been selected because they are the most common routes of administration for this test procedure. Other routes of administration may be used as indicated by scientific evidence. The test material will generally be solubilized in one of the following solvents: water, 0.9% saline, 0.5% aqueous carboxymethylcellulose solution, or corn oil. Other vehicles may be requested by the sponsor. The dosing volume will not exceed 20 ml/kg. All animals will be dosed based upon individual body weights. Dose levels will be assigned by a protocol amendment

Body weights will be taken prior to dosing. Dosing formulation will be prepared just prior to dosing. Dosing solutions will be prepared and held at ambient temperatures until dosing (approximately 1-2 hours). All animals will be euthanized 3 days after receiving a single dose.

Daily observations for toxic signs and mortality for the duration of the study. Animals will be euthanized by CO_2 inhalation followed by penetration of the thorax.

The daily observations toxic symptoms and/or mortalities data will be used to estimate the Maximum Tolerated Dose (MTD). Doses will then be assigned for the subsequent cytogenetics assay.

MICRONUCLEUS STUDY

C. Dosing Schedule and Route of Administration

Normally an acute dosing regimen (single administration) will be used (see Table below). Harvest will be approximately 24, 48, 72 hours after administration of the test article, and at approximately 24 hours after administration of the control articles. A total of 110 animals will be used. Equal numbers of males and females will be used at each treatment group. An additional group of animals consisting of at least five males and five females may be dosed as a secondary dose group with the high dose of the test material. This group will be dosed if toxicity is expected at the high dose and the animals in this group will only be used as replacements for any which die prior to euthanasia. The use of the secondary dose group will be determined by the study director. Freshly prepared solutions will be employed.

NUMBER OF ANIMALS USED FOR MICRONUCLEUS ASSAY

Treatment	Harvest Times After Treatment (Males and Females) 24 Hours	48 Hours	72 Hours	Total
Negative Control	5 + 5	---	---	5 + 5
Positive Control	5 + 5	---	---	5 + 5
Low Dose	5 + 5	5 + 5	5 + 5	15 + 15
Medium Dose	5 + 5	5 + 5	5 + 5	15 + 15
High Dose	5 + 5	5 + 5	5 + 5	15 + 15
TOTAL	25 + 25	15 + 15	15 + 15	55 + 55

The route of administration will be oral gavage. In the event that test article characteristics preclude oral gavage, IP injection will be employed. These routes of administration have been selected because they are the most common routes of administration for this test procedure. Other routes of administration may be used as indicated by scientific evidence.

D. Extraction of Bone Marrow and Preparation of Slides

Animals will be killed with CO_2, followed by penetration of the thorax, and hind limb bones will be removed for marrow extraction. The marrow will be flushed from the bone and transferred to centrifuge tubes containing 3 ml fetal calf serum (one tube for each animal). Following centrifugation to pellet the tissue, some of the supernatant will be drawn off, the cells resuspended, and the suspension spread on slides and air-dried.

The slides will then be fixed in methanol, stained in acridine orange, and analyzed under fluorescent microscopy. For control of bias, all slides are coded for analysis.

E. Scoring the Slides

An attempt will be made to score one-thousand PCEs per animal. The frequency of micronucleated cells will be expressed as percent micronucleated cells based on the total PCEs percent. The normal background frequency of micronuclei in the rat strain is around 0.0-0.4%.

The frequency of PCEs versus mature erythrocytes (NCEs) will be determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring the first 1000 erythrocytes on the slide.

VI. DATA

The criteria for the identification of micronuclei are those of Schmid (1976). Micronuclei are bright yellow, usually round bodies, with sharp borders and are generally between 1/20 and 1/5 the size of the PCE, which will stain bright orange. The unit of scoring is the micronucleated cell, not the micronucleus; thus the occasional cell with more than one micronucleus is counted as one micronucleated PCE, not two (or more) micronuclei.

The staining procedure permits the differentiation by color of polychromatic and normochromatic erythrocytes (bright orange and ghost-like, dark green respectively).

Data Presentation

The data reported will include the number of PCEs scored, the number of micronucleated PCEs, the percentage of micronucleated PCEs, and the ratio of polychromatic to normochromatic erythrocytes for each experimental animal.

Evaluation Criteria

The criteria for a positive response is a statistically significant dose-related increase in micronucleated PCEs, or the detection of a reproducible and statistically significant positive response for at least one dose level. A test article that induces neither a statistically significant dose response nor a statistically significant and reproducible increase at one dose level is considered negative. In either case, the final decision is based upon scientific judgement.

VII. TEST INTERPRETATION

The analysis of this data will be performed using an analysis of variance on the arcsine transformations of the proportion of cells with micronuclei per animal. If the analysis of variance is significant ($p<0.05$), a t test with multiple comparisons (Sokal and Rohlf, 1981) will be used to determine which dose groups, if any are significantly different from the negative control. Analyses are performed separately for each harvest time and sex combination.

VIII. REFERENCES

1. Heddle, J.A., Hite, M., Kirkhart, B., Larsen, K., MacGregor, J.T., Newell, G.W. and Salamone, M.F.: The induction of micronuclei as a measure of genotoxicity. Mutation Res., 123:61-118, 1983.

2. Schmid, W.: The micronucleus test. Mutation Res., 31:9-15, 1975.

3. Schmid, W.: The micronucleus test for cytogenetic analysis. In, Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4 (A. Hollaender, ed.). Plenum, pp. 31-53, 1976.

4. Sokal, R.R. and Rohlf, J.J.: Biometry, Freeman, 1981.

IX. REPORT FORMAT

HWA employs a standard report format for each assay design. Reports will be issued individually by type of test and by test article. Each final report will provide the following information.

- Sponsor identification.
- Quality Assurance statement.
- Statement of GLP Compliance.
- Signatures of study director and senior lab technician.
- Test article identification and HWA Study Number. A physical description of the test article and date of receipt will be included in this section.
- Type of assay and protocol number.
- Dates of study initiation and completion.
- Study director and senior technician.
- Methods.
- Evaluation criteria.
- Interpretation of results.
- Conclusions.
- References.
- Test results presented in tabular form.

X. CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated, and maintained with this protocol. The Sponsor will be notified of any change or revision.

XI. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived in the storage facilities of Hazleton for at least one year following submission of the final report to the sponsor. After the one year period, the sponsor may elect to have the aforementioned materials retained in the storage facilities of Hazleton for an additional period of time or sent to a storage facility designated by the sponsor.

3M Internal Correspondence

cc: R. G. Perkins - 220-2E-10

To: **B. C. Copley - 53-3S-02**

From: **R. D. Howells - 53-3S-02**

Subject: **Key to FC Alcohol Tox Samples**

Date: **September 14, 1995**

T5877	Wide Range EtFOSE FM 3924 Lot 547 Retain from 2 year feeding study	Analytical Request 41220 L-13203
T5710	Narrow Range EtFOSE Lot 884 Typical Raw Material for FC-807	Analytical Request 41220 L-10059
T5711	Wide Range MeFOSE Lot 555 Typical Raw Material for FX-845	Analytical Request 41220 L-1276
T5794	Narrow Range MeFOSE Notebook 97900-107-2 Lab Prepared Sample	Analytical Request 41343 L-13097
T5795	Wide Range EtFOSE Notebook 97900-112-2 Lab Prepared Sample	Analytical Request 41343 L-13098
T5878	Wide Range MeFOSE Lab Prepared from Washed POSF	Analytical Request 42607

RDH/jld R. D. Howells

Attachments

REQ 41220

R. Howells

GC/MS analyses of these samples were accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the samples into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of10 degrees per minute. The results of these analyses combined with their corresponding GC/FID area percents show the following:

Component I.D.	L-10059 N.R. N-EtFOSE Lot	W.R. N-EtFOSE Lot 547
N-Ethyl-carboxamides (Rf-C(O)N(Et)H)	0.22 %	1.76 %
N-EtFOS Amide ($C_8F_{17}SO_2N(Et)H$)	0.04 %	0.13 %
$C_2F_5SO_2N(Et)CH_2CH_2OH$	0.01 %	0.12 %
$C_3F_7SO_2N(Et)CH_2CH_2OH$	0.13 %	1.17 %
$C_4F_9SO_2N(Et)CH_2CH_2OH$	0.11 %	1.52 %
$C_8F_{17}SO_2N(Et)_2$	0.03 %	0.04 %
$C_5F_{11}SO_2N(Et)CH_2CH_2OH$	0.03 %	1.34 %
$C_6F_{13}SO_2N(Et)CH_2CH_2OH$	0.51 %	3.52 %
$C_8F_{17}SO_2N(Et)CH_2CH_2Cl$	0.08 %	0.16 %
$C_7F_{15}SO_2N(Et)CH_2CH_2OH$	0.82 %	1.40 %
N-EtFOSE $C_8F_{17}SO_2N(Et)CH_2CH_2OH$	96.09 %	87.16 %
$C_8F_{15}SO_2N(Et)CH_2CH_2OH$	0.78 %	0.50 %
$C_8F_{17}SO_2N(Et)(CH_2CH_2O)_2H$	0.17 %	-----
$C_8H_{17}SO_2N(Et)H$	0.16 %	0.37 %
$C_8F_{17}SO_2N(Et)CH_2CH_2OCO_2CH_2CH_3$ (or similar)	0.21 %	0.22 %
Other High Boilers	0.28 %	0.59 %

Component I.D.	L-1276 N-MeFOSE Lot
N-Methyl-carboxamides (R_f-C(O)N(Me)H)	1.46 %
$C_2F_5SO_2N(Me)CH_2CH_2OH$	0.24 %
$C_8F_{17}SO_2N(Me)_2$	trace
$C_3F_7SO_2N(Me)CH_2CH_2OH$	1.15 %
$C_8F_{17}SO_2N(Me)H$	trace
$C_4F_9SO_2N(Me)CH_2CH_2OH$	1.62 %
$C_5F_{11}SO_2N(Me)CH_2CH_2OH$	1.34 %
$C_6F_{13}SO_2N(Me)CH_2CH_2OH$	5.05 %
$C_8F_{17}SO_2N(Me)CH_2CH_2Cl$	0.13 %
$C_7F_{15}SO_2N(Me)CH_2CH_2OH$	1.69 %
N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$	83.88 %
$C_9F_{19}SO_2N(Me)CH_2CH_2OH$	0.87 %
$C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$	0.34 %
$C_8H_{17}SO_2N(Me)H$	0.54 %
Other High Boilers	1.55 %

Further work has been done by GC on these samples which involves derivatization of the alcohols with trifluoroacetic anhydride (TFAA) and again with BSA (to give the trimethylsilyl ethers). This work was designed to investigate the potential problems that could be overlooked by any one method of analysis. Preliminary results show that analyzing the EtFOSE underivatized could hide a significant amount of N-EtFOS Amide under the C-3 alcohol peak in wide range material. However, analyzing the same material that has been derivatized with TFAA shows that any EtFOSE-chloride that is present in the sample is now completely masked by the derivatized C-8 alcohol. The BSA derivative has not been evaluated yet, but similar problems are expected because of the number of different components in the sample. The same sort of problems will most likely exist with MeFOSE and will be even more complicated in the analysis of MeFOSEA.

7/2/93

REQ 41343 J. Grant

GC/MS analyses of these samples were accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the samples into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of10 degrees per minute. The results of these analyses combined with their corresponding GC/FID area percents show the following:

Component I.D.	W.R. N-EtFOSE Precut 97900-112-1	W.R. N-EtFOSE main cut 97900-112-2
N-Ethyl-carboxamides (Rf-C(O)N(Et)H)	68.34 %	1.27 %
$C_6F_{13}SO_2N(Et)H$	1.28 %	0.32 %
$C_2F_5SO_2N(Et)CH_2CH_2OH$	trace	trace
$C_7F_{15}SO_2N(Et)H$	trace	trace
N-EtFOS Amide ($C_8F_{17}SO_2N(Et)H$)	0.41 %	trace
$C_6F_{13}SO_2N(Et)_2$	1.41 %	trace
$C_3F_7SO_2N(Et)CH_2CH_2OH$	1.53 %	0.51 %
$C_4F_9SO_2N(Et)CH_2CH_2OH$	0.62 %	0.42 %
$C_5F_{11}SO_2N(Et)CH_2CH_2OH$	1.48 %	2.16 %
$C_6F_{13}SO_2N(Et)CH_2CH_2OH$	20.16 %	60.86 %
$C_7F_{15}SO_2N(Et)CH_2CH_2OH$	3.52 %	22.55 %
N-EtFOSE $C_8F_{17}SO_2N(Et)CH_2CH_2OH$	1.14 %	11.92 %

Component I.D.	N.R. N-MeFOSE B.P. 132 97900-107-2

mw 137 possibly -$SO_2N(Me)CH_2CH_2O$-	0.58 %
$C_7F_{15}SO_2N(Me)CH_2CH_2OH$	0.41 %
N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$	98.19 %
$C_9F_{19}SO_2N(Me)CH_2CH_2OH$	0.59 %
$C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$	trace
$C_8H_{17}SO_2N(Me)H$	trace
Other High Boilers	0.23 %

Component I.D.	W.R. N-EtFOS Amide 97900-111
N-Ethyl-carboxamides (R_f-C(O)N(Et)H)	10.23 %
$C_3F_7SO_2N(Et)H$	trace
$C_4F_9SO_2N(Et)H$	0.71 %
$C_5F_{11}SO_2N(Et)H$	3.58 %
$C_6F_{13}SO_2N(Et)H$	52.50 %
$C_7F_{15}SO_2N(Et)H$	20.06 %
N-EtFOS Amide ($C_8F_{17}SO_2N(Et)H$)	12.93 %
$C_8F_{15}SO_2N(Et)H$	trace
numerous other impurities of most homologs that include hydrides, chlorine in the backbone, and unidentified high boilers	trace

To: I. Muggli 53-6S-02

From: R. M. Payfer 236-2B-11 (612)733-4212

Subject: SA&C Analytical Request No. **42607**

Date: Dec. 21, 1993

GC/MS analysis of this sample was accomplished using a 25 m X 0.32 mm HP-1 GC column to introduce the sample into the Finnigan SSQ-70 mass spectrometer. The sample components were ionized using chemical ionization with methane as the reagent gas. The GC column was operated from 40 to 300 C at a rate of 10 degrees per minute. GC analysis with flame ionization detection was also done, and the area percent values from this work were applied to the peak identities from the mass spec work. The results of these analyses (which are not necessarily quantitative) show the following:

Mol. Weight	Component I.D.	L-13202 N-MeFOSE
427	N-Methyl-carboxamides C_7F_{15}-C(O)N(Me)H	0.06 %
527	$C_8F_{17}SO_2N(Me)_2$	0.12 %
513	$C_8F_{17}SO_2N(Me)H$	0.25 %
357	$C_4F_9SO_2N(Me)CH_2CH_2OH$	0.03 %
407	$C_5F_{11}SO_2N(Me)CH_2CH_2OH$	0.52 %
457	$C_6F_{13}SO_2N(Me)CH_2CH_2OH$	3.38 %
507	$C_7F_{15}SO_2N(Me)CH_2CH_2OH$	2.16 %
557	N-MeFOSE $C_8F_{17}SO_2N(Me)CH_2CH_2OH$	89.48 %
519	$C_8F_{15}SO_2N(Me)CH_2CH_2OH$	0.84 %
607	$C_9F_{19}SO_2N(Me)CH_2CH_2OH$	0.55 %
573	$C_8F_{16}ClSO_2N(Me)CH_2CH_2OH$	0.67 %
665	$C_8F_{16}SF_5$-$SO_2N(Me)CH_2CH_2OH$	0.29 %

601	$C_8F_{17}SO_2N(Me)(CH_2CH_2O)_2H$	0.54 %
	Other High Boilers	1.05 %