AR226_3315

TRADE SECRET

Study Title

H-25425: Subchronic Toxicity
90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations

Supplement 1

Laboratory Project ID: DuPont-11418

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines
OPPTS 870.3100 (AUG-1998)

AUTHOR: Susan A. MacKenzie, V.M.D., Ph.D., D.A.B.T.

STUDY COMPLETED ON: August 1, 2003

SUPPLEMENT 1 COMPLETED ON: March 19, 2004

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

WORK REQUEST NUMBER: [redacted]

SERVICE CODE NUMBER: [redacted]

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This supplement was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan Number 3850).

Applicant / Sponsor: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Director: Susan A. MacKenzie 19-MAR-2004
Susan A MacKenzie, V.M.D., Ph.D., D.A.B.T. Date
Senior Research Toxicologist

Applicant / Sponsor: _____ _____
DuPont Representative Date

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

25425

Dates of Inspections:

Supplement No. 1: March 1-2, 2004

Dates Findings Reported to:

Study Director: March 5, 2004

Management: March 5, 2004

Reported by: Kimberly B. Brebner 19-March-2004

Kimberly B. Brebner
Staff Quality Assurance Auditor

Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Approved by: _____ 19 MAR 2004
Scott E. Loveless, Ph.D.
Management
Date

Issued by Study Director: Susan A. MacKenzie 19-MAR-2004
Susan A. MacKenzie, V.M.D., Ph.D., D.A.B.T.
Senior Research Toxicologist
Date

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STUDY INFORMATION

9th Collective Nomenclature: [redacted]

Synonyms/Codes: [redacted]
- H-25425

Haskell Number: 25425

CAS Registry Number: [redacted]

Purity: [redacted]

[redacted]

Known Impurities: [redacted]

Physical Characteristics: Amber-yellow-brown solid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: October 3, 2002 / (see report cover page)

In-Life Initiated/Completed: October 15, 2002 / April 15, 2003

REASON FOR SUPPLEMENT 1

As described in protocol amendments 1, 6, 8, and 9, the sponsor requested analysis of plasma samples collected at the time of necropsy for concentration of perfluorooctanoic acid (PFOA), and analysis of selected whole blood samples, collected prior to and during the dosing phase of the study, for total fluorine. This supplement contains the results of the analyses which were conducted by Emily R. Decker of Exygen Research, State College, PA (PFOA concentration) under the supervision of John M. Flaherty, and Ward R. Gibson of Jackson Laboratory, Deepwater, NJ (total fluorine concentration), under the supervision of Robert M. Good.

SUMMARY

Four groups of young adult male and female Crl:CD®(SD)IGS BR rats were administered H-25425 in 0.05% methylcellulose in deionized water by gavage for approximately 90 days (92 days in males and 93 days in females), at dosages of 0, 10, 100, or 1000 mg/kg/day. Selected animals from each dosage group were designated for evaluations of subchronic toxicity, recovery from subchronic toxicity, or reproductive toxicity. Under the conditions of this study, the NOEL for subchronic toxicity endpoints was 100 mg/kg/day in male and female rats, based on reversible, minimal to mild thyroid follicular hypertrophy, observed in the 1000 mg/kg/day groups. Under the conditions of this study, the NOEL for reproductive endpoints was 1000 mg/kg/day, based on a lack of effects on any reproductive endpoint in P_1 or F_1 generation animals exposed to the highest dosage tested.

Blood was collected at necropsy, at the end of dosing and after a one-month recovery period (control and high-dose rats only), for analysis of perfluorooctanoic acid (PFOA). PFOA was extracted from plasma by protein precipitation in acetonitrile. The compound was quantified by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis using selected reaction monitoring (SRM). Quantification was performed using extracted calibration standards containing an internal standard. The lower limit of quantitation (LLOQ) for this method is 10 ppb.

Blood was collected at selected timepoints throughout the dosing phase of the study for analysis of total fluorine. Samples were analyzed from males and females in all dose groups on test day 22, from high-dose males and females on test day 89, and from high-dose males on test days -4 (pretest), 3, and 9. Whole blood was analyzed for total fluorine by the Wickbold torch combustion method and an ion selective electrode. The limit of detection for the method is 0.5 ppm.

Results of both of these analyses are presented in this supplemental report.

The results of the PFOA and total fluorine analyses are summarized in Tables 1 and 2, respectively. The details of the analyses of PFOA, which were conducted by Exygen Research, are reported in Appendix A. Individual animal total fluorine data are reported in Appendix B.

PFOA concentrations, measured at the end of the dosing period, were below the LLOQ in all control males and in all females dosed with up to 100 mg/kg/day. At the end of the dosing period, the mean concentration in male rats was 51.7 ppb in the 10 mg/kg/day group, 147 ppb in the 100 mg/kg/day group, and 3621 ppb in the 1000 mg/kg/day group. After a one-month recovery period, the mean concentration in the 1000 mg/kg/day male group was 400 ppb. Females dosed with 1000 mg/kg/day had a mean concentration of 51.7 ppb at the end of dosing. All females in this dose group had concentrations below the LLOQ at the end of the recovery period. Concentrations in control males and females were below the LLOQ at the end of the recovery period.

Low, but measurable, levels of total fluorine were present in blood from all but one control males and all control females on test day 22. Mean values were 1.18 ppm in males and 1.38 ppm in females. A dose-related increase in concentration was observed in both sexes on test day 22. Male mean values were 1.31 ppm in 10 mg/kg/day males, 1.86 ppm in 100 mg/kg/day males, and 4.74 ppm in 1000 mg/kg/day males. Mean female values were 1.54 ppm in 10 mg/kg/day females, 1.91 in 100 mg/kg/day females, and 3.32 ppm in 1000 mg/kg/day females. In male rats dosed with 1000 mg/kg/day, concentrations were below the limit of detection for all rats prior to dosing, but demonstrated increasing concentrations over time, with no plateau evident near the end of the dosing period (test day 89). In female rats dosed with 1000 mg/kg/day, total fluorine levels did not change from test day 22 to 89, and blood concentrations were lower than in males on both test days.

TABLE 1

PLASMA PFOA CONCENTRATIONS

DESIGN DOSAGE (mg/kg/day)	MEASURED CONCENTRATION (ppb)					
	MALE			FEMALE		
	MEAN	S.D.	n	MEAN	S.D.	n
End of Exposure[a]						
0	<10.0*	0	10	<10.0*	0	10
10	51.7	19.4	10	<10.0*	0	10
100	147	172	10	<10.0*	0	10
1000	3621	846	10	51.7	25.1	10
End of 1-Month Recovery[b]						
0	<10.0*	0	10	<10.0*	0	10
1000	400	305	10	<10.0*	0	10

* All values below the lower limit of quantitation (10.0 ppb).
n = number of samples
S.D. = standard deviation
a test day 92 (males) or 93 (females)
b test day 126 (males and females)

TABLE 2

SUMMARY OF BLOOD TOTAL FLUORINE ANALYSIS

	BLOOD FLUORINE (ppm) [a]			
DAYS ON TEST	Group I / II 0 mg/kg/day	Group III / IV 10 mg/kg/day	Group V / VI 100 mg/kg/day	Group VII / VIII 1000 mg/kg/day
MALES				
-4	b	b	b	<0.50* 0 (5)
3	b	b	b	1.65 0.16(5)
9	b	b	b	3.59 0.26(5)
22	1.18 0.44(5)	1.31 0.36(5)	1.86 0.31(5)	4.74 0.45(5)
89	b	b	b	9.86 0.66(5)
FEMALES				
22	1.38 0.40(5)	1.54 0.22(5)	1.91 0.39(5)	3.32 0.34(5)
89	b	b	b	3.30 0.62(5)

Data arranged as: Mean
Standard deviation (Number of values included in calculation)

a Where individual value was <0.5, 0.5 was used to calculate the mean and standard deviation.
b Samples not evaluated for this time point.
* All values below the limit of detection (0.50 ppm).

APPENDIX A

ANALYTICAL REPORT

STUDY TITLE

H-25425: Subchronic Toxicity
90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations

DATA REQUIREMENTS

Analytical Method Requirements

STUDY DIRECTOR

Susan A. MacKenzie

ANALYTICAL PHASE COMPLETED ON

November 19, 2003

PERFORMING LABORATORY / TESTING FACILITY

Exygen Research
3058 Research Drive
State College, PA 16801
Phone: 814-272-1039

STUDY SPONSOR

E. I. du Pont de Nemours and Company (DuPont)
P.O. Box 50
Newark, DE 19714-0050 USA

PROJECT

Exygen Study Number: 008-342
Sponsor Study No.: DuPont-11418

Total Pages: 81

Exygen Study No.: 008-342

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Exygen Study Number 008-342, entitled "H-25425: Subchronic Toxicity 90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations," conducted for DuPont, was performed in compliance with US EPA TSCA (40 CFR part 792) by Exygen Research.

Emily R. Decker
Principal Investigator
Exygen Research

11/19/03
Date

Susan A. MacKenzie
Study Director
DuPont

14-JAN-2004
Date

S. Mark Kennedy
Sponsor Representative
DuPont

24-NOV-2003
Date

Exygen Study No.: 008-342

QUALITY ASSURANCE STATEMENT

Exygen Research's Quality Assurance Unit reviewed Exygen Study Number 008-342, entitled, "H-25425: Subchronic Toxicity 90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations". All phases were reviewed for conduct according to Exygen Research's Standard Operating Procedures, the Study Protocol, and all applicable Good Laboratory Practice Standards. All findings were reported to the Study Director and to management.

Phase	Date Inspected	Date Reported to Principal Investigator	Date Reported to Exygen Management	Date Reported to Study Director and Sponsor Management
1. Protocol Review	10/08/03	10/08/03	10/23/03	11/13/03
2. Standard Preparation, Fortification, and Extraction	10/08/03	10/10/03	10/23/03	11/13/03
3. Raw Data & Draft Analytical Report Review	10/27,28,29, 30/03	11/06/03	11/12/03	11/13/03
4. Raw Data and Final Analytical Report Review	11/18/03	11/18/03	11/19/03	11/19/03

Lydia J. Shaffer
Quality Assurance Auditor

11/19/03
Date

Exygen Study No.: 008-342

CERTIFICATION OF AUTHENTICITY

This report, for Exgyen Study Number 008-342, is a true and complete representation of the raw data for the study.

Submitted by: Exygen Research
3058 Research Drive
State College, PA 16801
(814) 272-1039

Principal Investigator, Exygen:

Emily R. Decker — 11/19/03
Scientist/Principal Investigator — Date
Exygen Research

Exygen Research Facility Management:

John M. Flaherty — 11/19/03
Vice President — Date
Exygen Research

Study Director, DuPont:

Susan A. MacKenzie — 14-JAN-2004
DuPont — Date

Sponsor Representative, DuPont:

S. Mark Kennedy — 24-NOV-2003
DuPont — Date

Exygen Study No.: 008-342

STUDY IDENTIFICATION

H-25425: Subchronic Toxicity
90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations

SPONSOR STUDY NUMBER:	DuPont-11418
EXYGEN STUDY NUMBER:	008-342
TYPE OF STUDY:	Residue
SAMPLE MATRIX:	Rat Plasma
TEST SUBSTANCE:	Pentadecafluorooctanoic acid (PFOA)
SPONSOR:	E. I. du Pont de Nemours and Company P.O. Box 50 Newark, DE 19714-0050 Phone: (302) 366-5255
STUDY DIRECTOR:	Susan A. MacKenzie DuPont
TESTING FACILITY:	Exygen Research 3058 Research Drive State College, PA 16801
ANALYTICAL PHASE TIMETABLE:	Study Initiation Date: 10/03/02 Analytical Start Date: 10/03/03 Analytical Termination Date: 10/09/03 Analytical Report Completion Date: 11/19/03

Exygen Study No.: 008-342

PROJECT PERSONNEL

The Study Director for this project at DuPont was Susan A. MacKenzie. The following personnel from Exygen Research were associated with various phases of the study:

Name	Title
Paul Connolly	Technical Lead-LC/MS
Emily Decker	Scientist
Karen Risha	Scientist
Amy Sheehan	Associate Scientist
Shawn Robb	Sample Custodian

Exygen Study No.: 008-342

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Exygen Study No.: 008-342

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Exygen Study No.: 008-342

1.0 SUMMARY

Exygen Research (Exygen) analyzed samples of rat plasma for residues of PFOA in according to protocol DuPont-11418 (**Appendix A**) using the analytical method ExM-008-276 Revision 1 (**Appendix B**).

The limit of quantitation for this method is 10 ppb, which was established at Exygen in the method validation study 008-277.

Residues of PFOA in the rat plasma samples ranged from < 10.0 ppb to 4800 ppb.

The average recovery ± standard deviation for PFOA in fortified rat plasma samples was 100% ± 11.2%.

2.0 OBJECTIVE

The objective of this analytical phase was to analyze rat plasma samples received at Exygen using the method ExM-008-276 Revision 1 entitled "Method of Analysis for the Determination of Pentadecafluorooctanoic Acid (PFOA) in Serum by LC/MS/MS".

3.0 INTRODUCTION

This report details the results of the analysis of rat plasma samples for PFOA using the analytical method ExM-008-276 Revision 1.

The study DuPont-11418 was initiated on October 03, 2002, when the study director signed protocol number DuPont-11418. The analytical start date was October 3, 2003, and the analytical termination date was October 9, 2003.

4.0 TEST SYSTEM

The control rat plasma used for control samples and laboratory control fortified samples was received from DuPont on 10/01/03. One hundred nineteen rat plasma samples were received at Exygen on September 23, 2003. All samples were logged in by Exygen personnel upon receipt and placed in frozen storage (≤ -20°C).

Sample login and chain of custody information can be found in the raw data package associated with this study. Storage records will be kept at Exygen Research and a true copy of the storage records will be furnished upon request.

Exygen Study No.: 008-342

5.0 REFERENCE MATERIAL

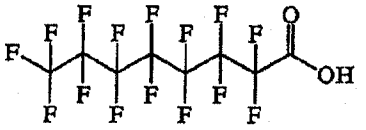
The analytical standard pentadecafluorooctanoic acid (PFOA) was received at Exygen on March 05, 2003 from Critical Path Services. The control article (internal standard) n-perfluorooctanoic acid (1,2 di-^{13}C) was received at Exygen on February 20, 2003 from DuPont.

The available information for the reference material is listed below. The analytical standard was stored in a refrigerator and the control article was stored at room temperature.

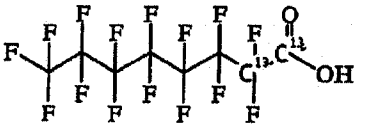
Compound	Exygen Inventory No.	Lot No.	Purity (%)	Expiration Date
PFOA	SP2516	GVS-Nr.6V 06687	97	03/05/04
^{13}C-PFOA	SP2492	NA	96.35	01/31/05

The structure of perfluorooctanoic acid (PFOA) and the internal standard are given below.

PFOA
Chemical Name = perfluorooctanoic acid
Molecular weight = 414



^{13}C – PFOA
Chemical Name = perfluorooctanoic acid, [1,2-di-^{13}C]
Molecular weight = 416



6.0 DESCRIPTION OF ANALYTICAL METHOD

6.1 Extraction Procedure

1. Measure 0.1 mL of sample into 1 mL eppendorf centrifuge tubes (fortify with analyte as needed, close lid, and vortex ~ 10 seconds).
2. Add enough acetonitrile containing internal standard at 0.005 µg/mL (accounting for fortification volume) to make extraction volume 500 µL and vortex for ~ 10 seconds.
3. Centrifuge for ~ 10 minutes at ~ 14,000 rpm.
4. Analyze samples using electrospray LC/MS/MS.

6.2 Preparation of Standards and Fortification Solutions

The stock standard solution of PFOA was prepared on September 17, 2003, at a concentration of 100 µg/mL by dissolving approximately 10 mg of the standard (corrected for purity) in methanol. The stock standard solution of ^{13}C-PFOA was prepared on September 17, 2003, at a concentration of 100 µg/mL by dissolving approximately 10 mg of the standard (corrected for purity) in methanol. A fortification solution of PFOA at 10 µg/mL was prepared by taking 10 mL of the stock standard solution and bringing the volume up to 100 in acetonitrile. A 1.0 µg/mL fortification solution of ^{13}C-PFOA was prepared by taking 1.0 mL of the stock and bringing the volume up to 100 mL with acetonitrile.

A set of standards containing PFOA with internal standards was prepared by dilution of the 10.0 µg/mL PFOA solution and the 1.0 µg/mL ^{13}C-PFOA solution in the following manner. These solutions were used to fortify the samples and the extracted calibration standards.

Initial Conc. (µg/mL)[1]	Volume (mL)	Diluted to (mL)	Final Conc. (µg/mL)[1]
10.0	10.0	100[2]	1.0
10.0	1.0	100[2]	0.1
10.0	0.1	100[2]	0.01

[1] of PFOA only
[2] 0.5 mL of the 1.0 µg/mL ^{13}C-PFOA solution was added prior to making final volume to give a concentration of 0.005 µg/mL of ^{13}C-PFOA in each solution.

The calibration standards are processed through the extraction procedure, identical to the samples. The extracted standards are assigned a two-week expiration date from the date of extraction based on refrigerator stability data obtained during the method validation study (Exygen study: 008-277). The fortification of the standards before extraction is done according to the following table:

Conc. Of PFOA Fortification Solution (µg/mL)	Fortification Volume (µL)	Volume of Control Sample (mL)	Conc. of Extracted Calibration Standard (ppb)
0.01	100	0.1	10
0.01	200	0.1	20
0.1	50	0.1	50
0.1	100	0.1	100
0.1	200	0.1	200

The extraction solution was prepared by taking 0.5 mL of the 1.0 µg/mL ^{13}C-PFOA solution and bringing the volume up to 100 mL with acetonitrile. The dilution solution was prepared by taking 0.5 mL of the 1.0 µg/mL ^{13}C-PFOA solution and bringing the volume up to 100 mL with 50:50 methanol:water.

The stock standard solution and all fortification/calibration standard solutions were stored in a refrigerator (4° ± 2°C) when not in use. Documentation of standard preparation can be found in the raw data associated with this report.

6.3 Chromatography

Quantification of PFOA was accomplished by analysis using electrospray LC-MS/MS. The retention time of PFOA was ~ 2.0 min., with no significant interfering peaks (< 20% of the LOQ standard) in the control samples corresponding to the analyte retention times.

6.4 Instrument Sensitivity

The smallest amount of PFOA injected during the chromatographic run was equivalent to 10 ppb.

6.5 Description of Instrument and Operating Conditions

A PE Sciex LC-MS/MS coupled to a Hewlett Packard HPLC system was used. Data acquisition and processing were performed using Analyst 1.2 software.
Detailed operating conditions are listed below:

Mass Spec:	PE SCIEX API 4000 Biomolecular Mass Analyzer
Interface:	SCIEX Turbo Ion Spray Liquid Introduction Interface Turbo Ion Spray temperature = 350° C with N2 at ~7 L/min
Computer:	Dell Optiplex GX110
Software:	PE Sciex Analyst: Version 1.2
HPLC:	Hewlett Packard (HP) Series 1100

HP Quat Pump
HP Vacuum Degasser
HP Autoinjector
HP Column Compartment

Filter Cartridge(s): 2 Keystone Hypercarbs in tandem, exiting pump
HPLC Column: Genesis C_8 (Jones Chromatograhpy), 2.1 mm x 50 mm, 4µ
Column Temp.: 30° C
Mobile Phase (A): 2 mM Ammonium Acetate in water
Mobile Phase (B): Methanol

Time	% A	% B	Flow (mL/min)
0	40	60	0.3
3.0	40	60	0.3
3.5	0	100	0.3
3.7	0	100	0.5
7.0	0	100	0.5
7.5	40	60	0.5
9.0	40	60	0.5
9.5	40	60	0.3
12.0	40	60	0.3

Injection Vol.: 5 µL

Ions monitored:

Analyte	Mode	Transition Monitored
PFOA	Negative	413 → 369
^{13}C-PFOA	Negative	415 → 370

6.6 Quantitation and Example Calculation

Five microliters of sample or calibration standard were injected into the LC-MS/MS. The peak area was measured and the standard curve was generated by linear regression using 1/x weighting of the ratio analyte peak area/internal standard peak area versus the ratio of the concentration of analyte/concentration of internal standard using Analyst 1.2 (or equivalent) software system. The residue concentration for PFOA was determined from the following equations:

Equation 1 was used to calculate the amount of analyte found (in ppb or ng/mL, based on ratio of analyte peak area to internal standard peak area) using the standard curve (linear regression parameters) generated by the Analyst software program.

Equation 1:

$$\text{Analyte found (ppb)} = \frac{((\text{Analyte Peak area/IS peak area}) - \text{intercept}))}{\text{slope}} \times \text{IS conc. (ng/mL)} \times \text{DF}$$

For samples fortified with known amounts of analyte prior to extraction, Equation 2 was used to calculate the percent recovery.

Equation 2:
Recovery (%) =

$$\frac{\text{Analyte found (ppb)} - \text{avg. Analyte found in control (ppb)}}{\text{Amount Analyte added (ppb)}} \times 100$$

For field samples fortified with known amounts of analyte prior to extraction, Equation 3 was used to calculate the percent recovery.

Equation 3:
Recovery (%) =

$$\frac{\text{Analyte found (ppb)} - \text{avg. Analyte found in sample (ppb)}}{\text{Amount Analyte added (ppb)}} \times 100$$

An example of a calculation using an actual sample follows: Rat plasma sample Exygen ID 0302239 Spk A (Set: 100303A), fortified at 10 ppb with PFOA.
Where:

analyte peak area	=	60581
IS peak area	=	143204
IS conc. (ng/mL)	=	4.167 ng/mL
intercept	=	-0.0104953
slope	=	0.176029
dilution factor (DF)	=	1
ppb added (fort level)	=	10
amt found in control	=	ND

From equation 1:

$$\text{Analyte found (ppb)} = \frac{[(60581/143204) - -0.0104953] \times 4.167 \times 1}{0.176029}$$

$$= 10.3 \text{ ppb}$$

From equation 2:

$$\text{\% Recovery} = \frac{(10.3 \text{ ppb} - 0)}{10 \text{ ppb}} \times 100$$

$$= 103\%$$

Note: This example calculation was done using rounded numbers, and therefore may be slightly different from the values shown in the RAW DATA.

7.0 EXPERIMENTAL DESIGN

Each analytical set was extracted according to method ExM-008-276 Revision 1 and consisted of a reagent blank, one matrix control, two laboratory matrix controls fortified at known concentrations, one field sample fortified at a known concentration, 19-20 field samples, and one field sample extracted in duplicate.

8.0 RESULTS

The PFOA found in the control rat plasma samples are listed in **Table I.**

Individual recoveries for PFOA in the laboratory fortified rat plasma samples are detailed in **Table II.** The average percent recoveries ± standard deviation for PFOA in the rat plasma samples was 100% ± 11.2%.

Individual results for the PFOA in the rat plasma samples are given in **Table III.** Residues of PFOA in the rat plasma samples ranged from < 10.0 ppb to 4800 ppb.

A typical calibration curve for PFOA and representative chromatograms are given in **Figures 1-5.**

9.0 CIRCUMSTANCES THAT MAY HAVE AFFECTED THE QUALITY OR INTEGRITY OF THE DATA

There are no known circumstances that have affected the quality or integrity of the data.

10.0 CONCLUSIONS

The rat plasma samples were successfully analyzed according to method ExM-008-276 Revision 1.

11.0 RETENTION OF DATA AND SAMPLES

When the final report is complete, all original paper data generated by Exygen Research will be shipped to the sponsor. This does not include facility-specific raw data such as instrument logs. Exact copies of all raw data, as well as a signed copy of the final analytical report and all original facility-specific raw data, will be retained in the Exygen Research's archives for the period of time specified in 40 CFR Part 792. Retained samples of reference substances are archived by the sponsor.

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TABLES

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Table I. Summary of PFOA (ppb) in Rat Plasma Control Samples

Sponsor ID	PFOA (ng/mL)
669160 Control A 10/03/03	ND
669160 Control A2 10/03/03	ND
669160 Control A 10/07/03	ND
669160 Control A2 10/07/03	ND
669180 Control A 10/08/03	ND
669180 Control A2 10/08/03	ND

Table II. Summary of Recoveries of PFOA (%) in Rat Plasma Samples

Sponsor ID	PFOA (ppb)	Amount Fortified (ppb)	Recovery (%)
669160 (LCS A) 10/03/03	10.3	10.0	103
669160 (LCS B)10/03/03	951	1000	95
665047 (Matrix Spk) 10/03/03	1030	1000	103
669180 (LCS A2) 10/03/03	10.2	10.0	102
669160 (LCS B2)10/03/03	987	1000	99
665074 (Matrix Spk) 10/03/03	1090	1000	107
669160 (LCS A) 10/07/03	10.1	10.0	101
669160 (LCS B)10/07/03	1040	1000	104
665096 (Matrix Spk) 10/07/03	5570	1000	121
669160 (LCS A2) 10/07/03	10.3	10.0	103
669160 (LCS B2)10/07/03	633	1000	63
665096 (Matrix Spk) 10/07/03	907	1000	91
669180 (LCS A2) 10/08/03	9.72	10.0	97
669180 (LCS B2)10/08/03	1060	1000	106
665215 (Matrix Spk) 10/08/03	1060	1000	106
669180 (LCS A2) 10/08/03	9.72	10.0	97
669180 (LCS B2)10/08/03	1020	1000	102
665242 (Matrix Spk) 10/08/03	1070	1000	103
		AVERAGE:	100
		STD DEV:	11.2
		% RSD:	11.1

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Table III. Summary of Residue Found for PFOA (ppb) in Rat Plasma Samples

Sponsor ID	PFOA (ppb)
665047	< 10.0
665047 Dup	< 10.0
665049	< 10.0
665070	< 10.0
665130	< 10.0
665076	< 10.0
665015	< 10.0
665068	< 10.0
665148	< 10.0
665016	< 10.0
665086	< 10.0
665098	< 10.0
665149	< 10.0
665081	< 10.0
665159	< 10.0
665043	< 10.0
665064	< 10.0
665121	< 10.0
665106	< 10.0
665008	< 10.0
665053	51.8
665074	20.2
665074 Dup	20.0
665128	52.5
665080	87.7
664993	39.6
665131	58.1
665060	54.9
664995	74.3
665136	43.0
665033	34.5
665122	731

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Table III (cont'). Summary of Residue Found for PFOA (ppb) in Rat Plasma Samples

Sponsor ID	PFOA (ppb)
665155	592
665073	549
665089	547
665119	636
665137	1010
665103	845
665143	500
665054	840
665035	868
665160	4170
665096	4380
665096 Dup	4340
665082	3640
665107	2790
665152	4800
665120	3620
664999	4090
665026	4000
665162	2270
665027	2470
665158	966
665002	109
665067	82.2
665095	771
665031	85.0
665146	257
665062	236
665138	579
665112	525
665007	387
665211	ND

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Table III (cont'). Summary of Residue Found for PFOA (ppb) in Rat Plasma Samples

Sponsor ID	PFOA (ppb)
665271	ND
665271 Dup	ND
665178	ND
665317	ND
665230	ND
665225	ND
665174	ND
665319	ND
665221	ND
665278	ND
665201	ND
665197	ND
665256	ND
665323	ND
665276	ND
665228	ND
665260	ND
665189	ND
665204	ND
665327	ND
665308	< 10.0
665215	< 10.0
665215 Dup	< 10.0
665277	< 10.0
665241	ND
665236	< 10.0
665324	< 10.0
665332	< 10.0
665239	< 10.0
665182	< 10.0
665268	< 10.0
665295	< 10.0

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Table III (cont'). Summary of Residue Found for PFOA (ppb) in Rat Plasma Samples

Sponsor ID	PFOA (ppb)
665251	< 10.0
665300	< 10.0
665340	< 10.0
665259	< 10.0
665341	< 10.0
665199	< 10.0
665238	< 10.0
665250	< 10.0
665170	< 10.0
665234	63.4
665242	36.9
665242 Dup	35.8
665183	37.1
665261	66.0
665291	35.3
665337	43.8
665333	20.8
665200	33.4
665310	101
665326	80.2
665288	< 10.0
665282	< 10.0
665275	ND
665229	< 10.0
665237	ND
665209	< 10.0
665335	< 10.0
665303	< 10.0
665328	ND
665299	< 10.0

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FIGURES

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Figure 1. Typical Calibration Curve for PFOA

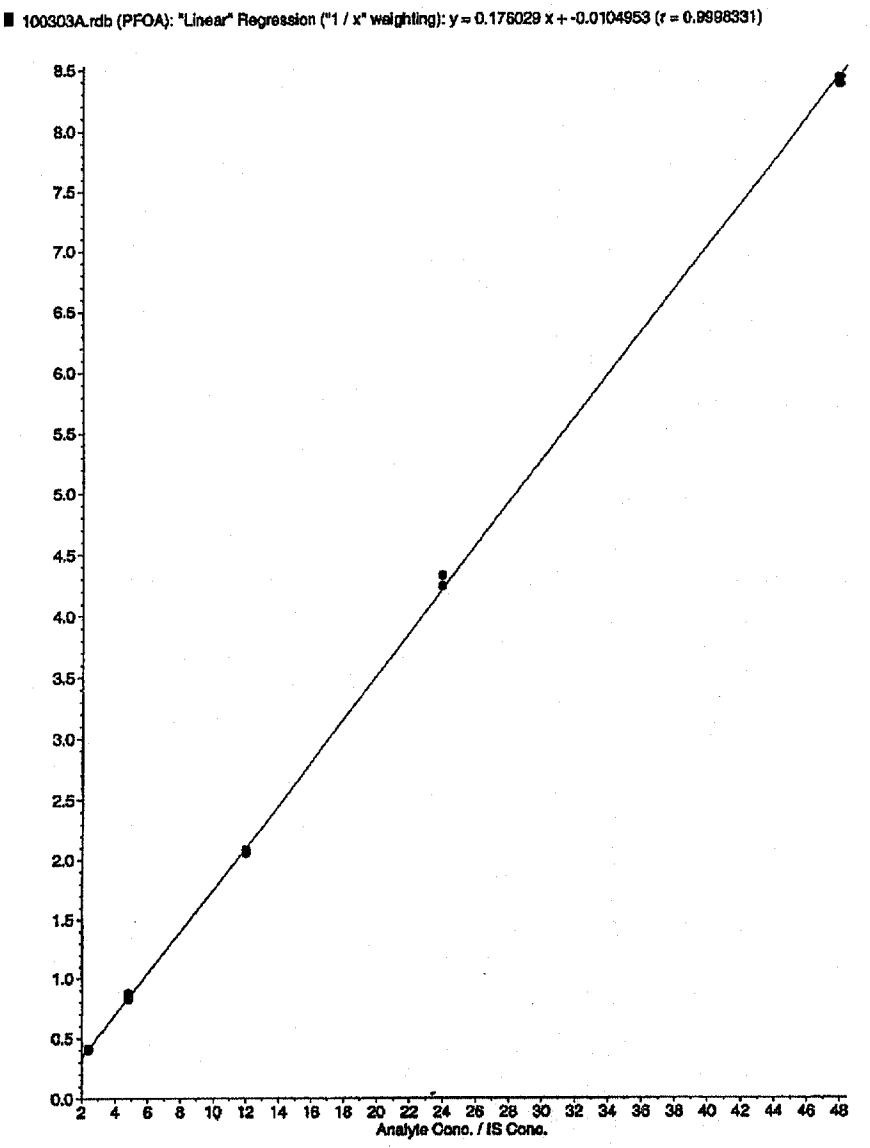

Figure 2. Chromatogram Representing a Calibration Standard for PFOA at 10 ppb with ~ 5 ng/mL of ^{13}C-PFOA

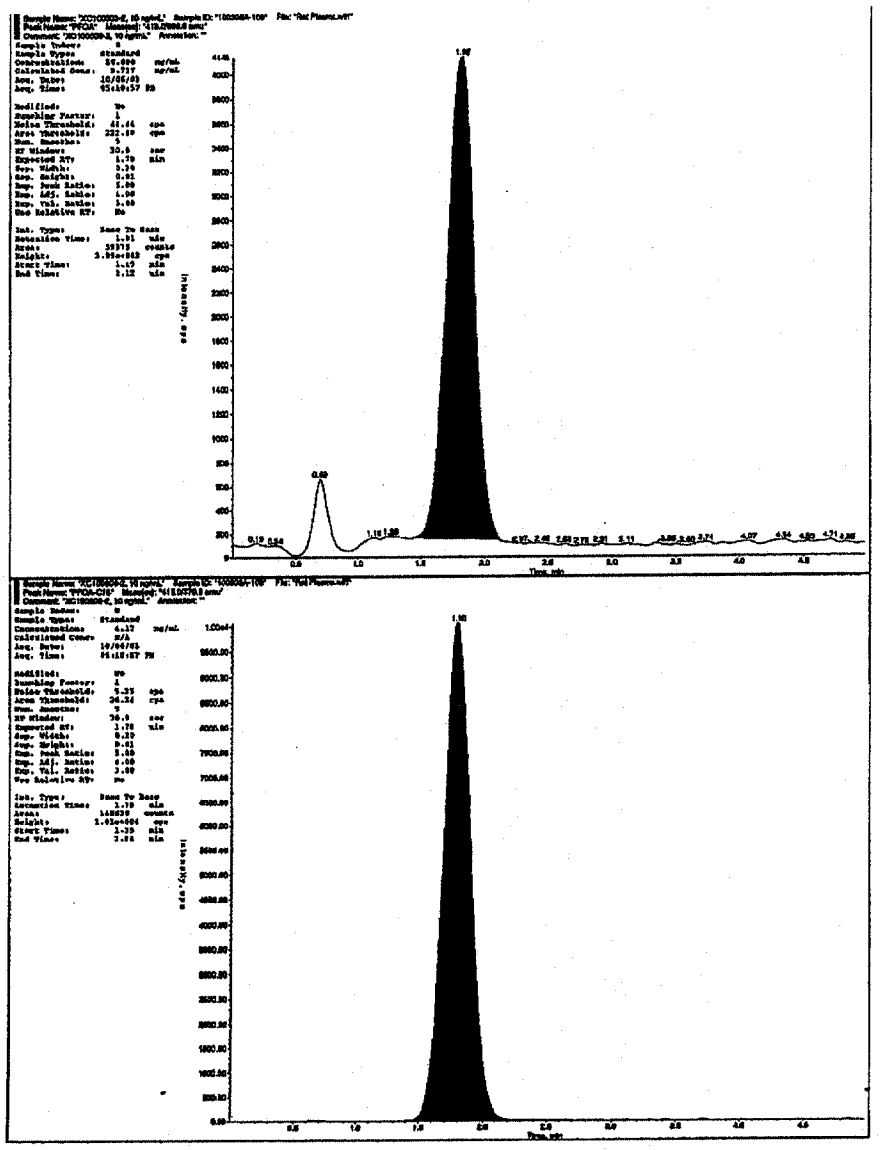

Figure 3. Chromatogram Representing Control Rat Plasma for PFOA with ~ 5 ng/mL of ^{13}C-PFOA (Exygen ID: 0302239 Control A, Set: 100303A)

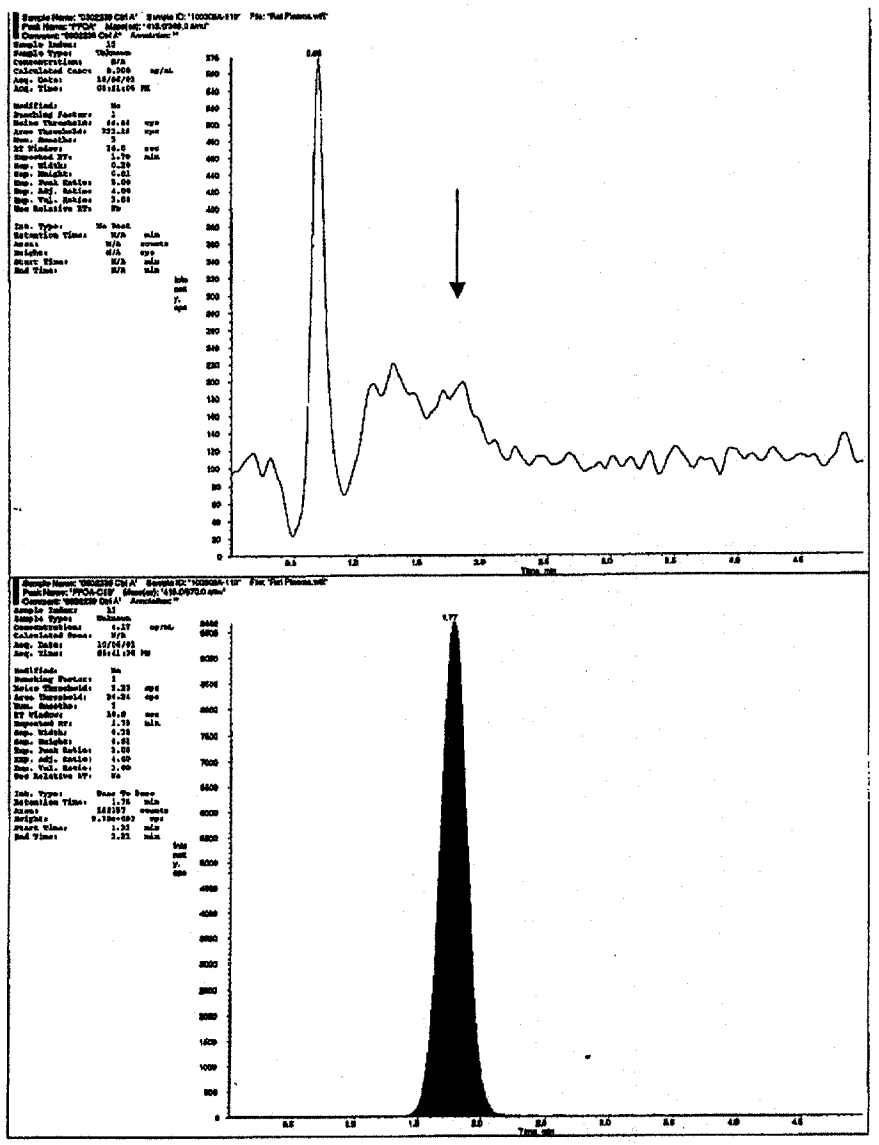

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Figure 4. Chromatogram Representing Control Rat Plasma Fortified at 10 ppb with PFOA and ~ 5 ng/mL of ^{13}C-PFOA (Exygen ID: 0302239 Spk A, Set: 100303A)

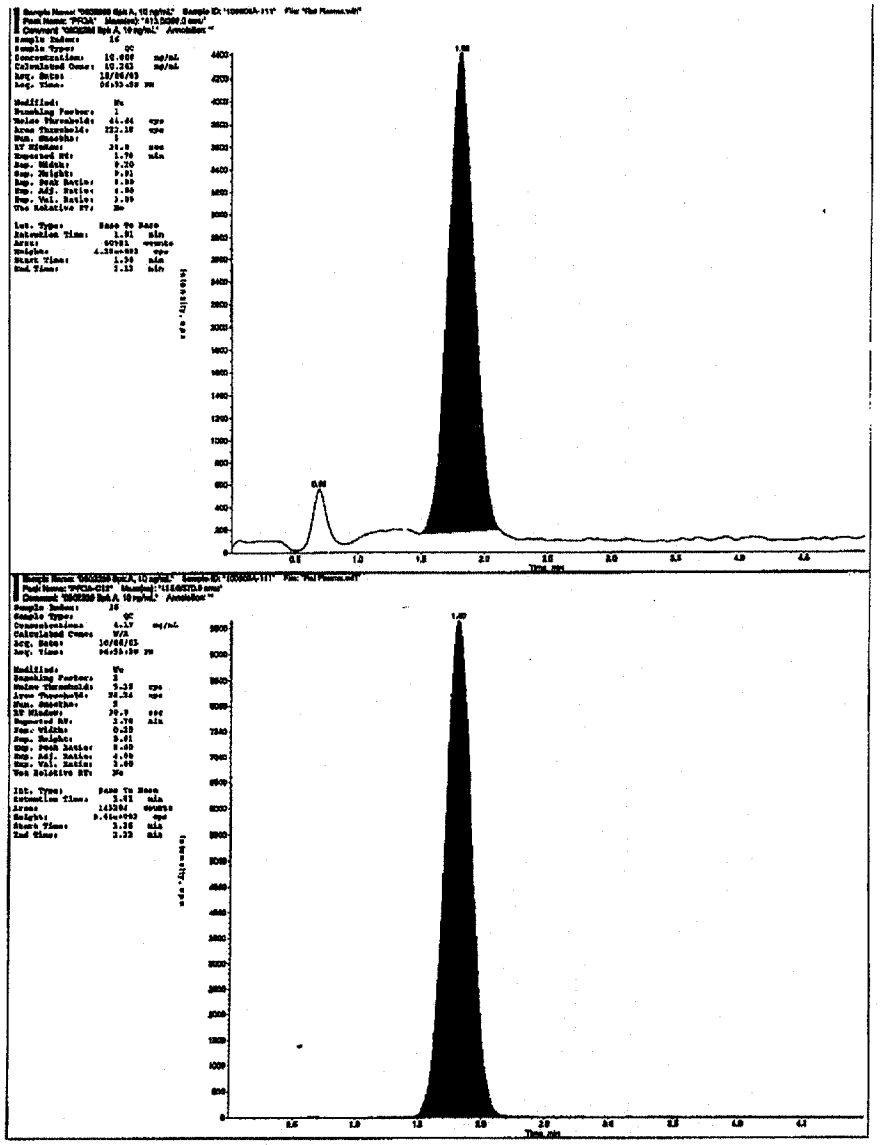
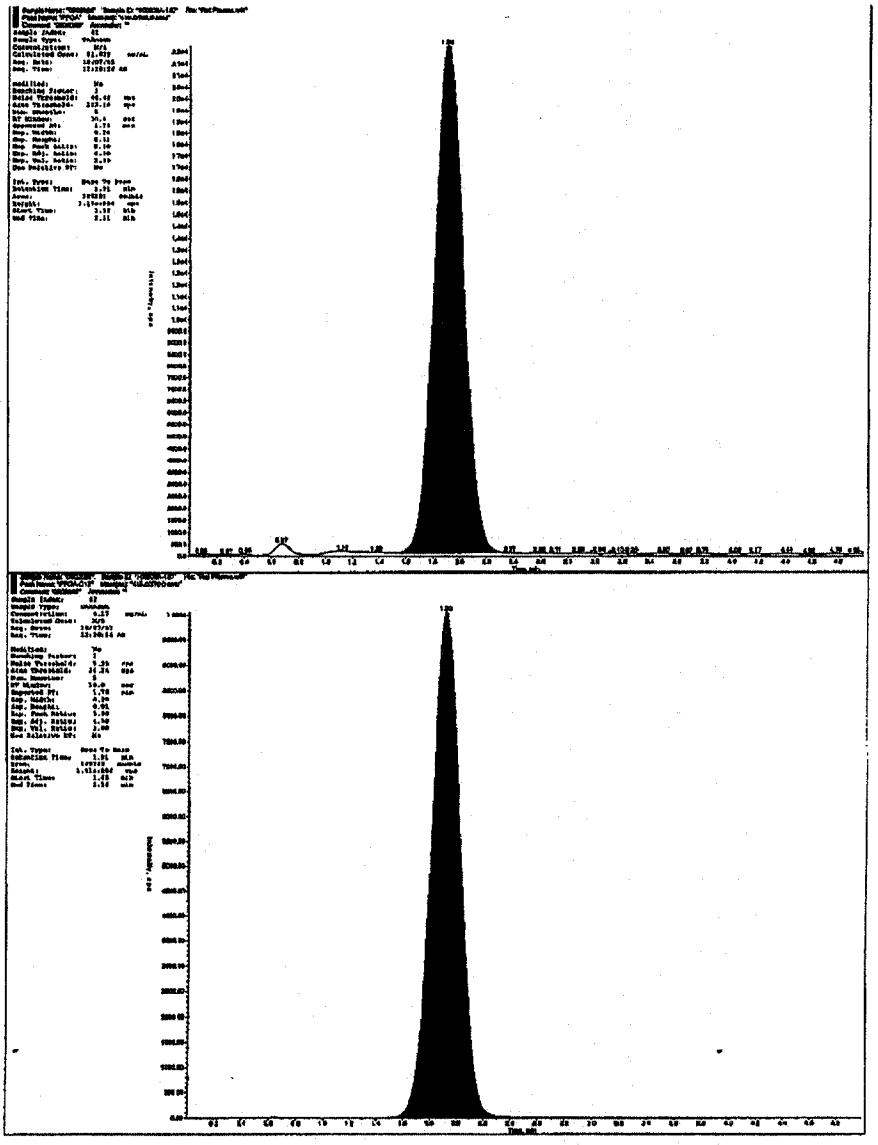


Figure 5. Chromatogram Representing Rat Plasma Sample for PFOA with ~ 5 ng/mL of ^{13}C-PFOA (Exygen ID: 0302026, Sponsor ID: 665053, Set: 100303A)



APPENDIX A

Study Protocol
DuPont-11418
(Exygen Study No. 008-342)
and
Amendments and Deviation

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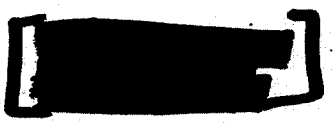
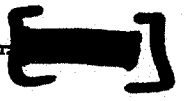
DuPont-11418

H-25425: Subchronic Toxicity
90-Day Gavage Study in Rats with One-Generation Reproduction Evaluations



Protocol

Haskell Animal Welfare Committee Number: 

1

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INTRODUCTION

The test substance, H-25425 is a [redacted] used as a fabric protector. The dose levels for this study were selected based on the toxicity and an analysis of blood fluorine concentrations from a range-finding study in which rats were dosed by oral gavage with 1, 10, 100, or 1000 mg/kg/day for approximately 45 days.

No compound-related effects on body weight, clinical signs of toxicity, organ weights, or gross pathology lesions were observed in rats in any dose group. Blood fluorine levels appeared to have reached a plateau within 1-2 weeks of dosing with 1000 mg/kg/day, and levels remained very low throughout the entire exposure period.

Therefore, the limit dosage of 1000 mg/kg/day may be a no-observed-effect level (NOEL) or may produce mild toxicity. The 10 and 100 mg/kg/day dosages are expected to be NOELs.

OBJECTIVE

The objective of this study is to evaluate the potential subchronic and reproductive toxicity of H-25425 when administered by gavage to male and female rats. A recovery group will also be included to investigate the reversibility of any observed toxicological effects. The oral route of administration was selected as the most efficient way to deliver an accurate dosage.

SPONSOR AND TEST FACILITY

This study is sponsored by the DuPont Chemical Solutions Enterprise (DCSE), E. I. du Pont de Nemours and Company, Wilmington, Delaware. The sponsor's approval was effective the date the sponsor authorized the Work Authorization Form. The study will be conducted at Haskell Laboratory for Health and Environmental Sciences, E.I. du Pont de Nemours and Company, Newark, Delaware. The test substance characterization was performed at Regional Analytical Services (RAS) at DuPont Jackson Laboratories, Deepwater, NJ.

REGULATORY COMPLIANCE

The study will be conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850).[1,2,3] Areas of noncompliance will be documented in the final report. The 90-day study design complies with the U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides, and Toxic Substances (OPPTS) 870.3100 test guidelines.[4]

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STUDY DESIGN

The study design is as follows:

Group		Number/Group			
Male	Female	Male	Female	Daily Dosage[a] (mg/kg)	Concentration[b] (mg/mL)
I	II	50	50	0 (Control)	0
III	IV	40	40	10	2
V	VI	40	40	100	20
VII	VIII	50	50	1000	200

a. Weight of test substance/kg animal body weight.
b. Suspensions will be adjusted for purity [redacted]

Five of the 40 or 50 rats/sex/dose will be started on study approximately 70 days after study start for the rest of the rats, and will be designated for 10-day hepatic biochemical analysis.

Study Parameters	Frequency
Body Weight	Day 0 and weekly thereafter
Food Consumption	Day 0 and weekly thereafter
Detailed Clinical Observations	Day 0 and weekly thereafter
Mortality/Moribundity Checks	Twice daily
Clinical Pathology*	Week 7 and Week 14
Ophthalmoscopic Evaluations*	Pretest and Week 13
Neurobehavioral Evaluations*	Pretest and Week 13
Biochemical Analysis	
Subchronic	Week 14
Recovery	Week 17
Satellite (day 10)	After 10 days of dosing
Reproductive Assessments	
P_1 adults	Week 11-18
F_1 weanlings	Week 13-18
Vaginal Smears (P_1 Females)	Week 7-9
Developmental Landmarks (F_1 adults)	Week 19-23
Necropsy	
Subchronic	Week 14
P_1 adults	Week 12-18
Weanlings	Week 17-18
Recovery	Week 18
F_1 adults	Week 23-25
Blood fluorine animals	Week 26

* Clinical pathology, ophthalmoscopic, and neurobehavioral evaluations may also be conducted on recovery animals near the end of the recovery phase, if necessary.

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MATERIALS AND METHODS

A. Test Substance

The test substance was supplied by the sponsor and was assigned Haskell Laboratory Number 25425. The sponsor reported purity was [redacted] The sponsor is responsible for characterization of the test substance. The test substance characterization was performed at DuPont Regional Analytical Services, a non-GLP laboratory. The characterization was conducted in compliance with ISO9002 regulations and considered valid and sufficient for the purposes of this study.

Available information on the purity, composition, contaminants, synonyms, CAS registry number, basic physical properties, hazards, and hazardous material classification(s) will be provided by the sponsor on the Haskell Laboratory Sample Evaluation form. Stability of the test substance will be confirmed by analyses near the beginning and end of the study.

B. Test Species

Male and female Crl:CD®(SD)IGS BR rats will be obtained from Charles River Laboratories, Inc. The location of the supplier (city/state) will be documented in the study records and final report. The Crl:CD®(SD)IGS BR rat has been selected on the bases of extensive experience with this strain at Haskell Laboratory, and its suitability with respect to longevity, hardiness, and low incidence of spontaneous diseases.

Approximately 352 rats (176 males and 176 females) will be received approximately 2 weeks prior to study start. An additional 40 rats (20 males and 20 females) will be received as a separate shipment approximately 59 days after study start, and will be added to the test groups (5 rats/sex/group) and designated for the 10-day hepatic biochemical analysis.

C. Animal Husbandry

1. Housing

a. Subchronic Study, Premating Dosing Period, Postweaning Period

All rats will be housed individually in stainless steel, wire-mesh cages suspended above cage boards.

H-25425: Subchronic Toxicity
90-Day Gavage Study in Rats with One-Generation Reproductive Evaluations DuPont-11418

b. Reproductive Study

All male rats will be housed individually during non-mating periods in stainless steel, wire-mesh cages.

i Cohabitation Period

All rats will be housed as breeding pairs in stainless steel, wire-mesh cages. At the end of the cohabitation period, females without evidence of copulation will be housed individually in polycarbonate pans.

ii Gestation Period

- Days 0-20: Females will be housed individually in stainless steel, wire-mesh cages.
- Day 20-Delivery: Females will be housed individually in polycarbonate pans.

iii Lactation Period

Adult females will be housed with their litters in polycarbonate pans.

2. Cage Rack Positioning

Cage racks with wire mesh cages will be relocated within the animal room and cages on the racks will be repositioned at least every other week:

- for animals designated for subchronic evaluation,
- up to approximately 70 days for animals designated for reproductive assessment, and
- up to sacrifice (approximately postnatal day 60) for F1 generation rats.

3. Environmental Conditions

Animal rooms will be maintained at a temperature of 22 ± 3°C and a relative humidity of 50% ± 20%. Animal rooms will be artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle.

4. Water and Food

All rats, except when fasted, will be provided tap water and pelleted PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum*.

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5. Animal Health Monitoring

As specified in the Haskell Laboratory animal health and environmental monitoring program, the following procedures are performed periodically to assure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Feed samples are analyzed for total bacterial, spore and fungal counts.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Data are maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at Haskell Laboratory, all rats will be housed one per cage, sexes separate, in quarantine. The rats will be:

- quarantined for at least 5 days.
- assigned a unique 6-digit Haskell number, the last 3 digits of which will be tattooed on the animal tail.
- weighed at least 3 times during quarantine.
- observed with respect to weight gain and any gross signs of disease or injury.
- given an ophthalmology examination.

The rats will be released from quarantine by the laboratory animal veterinarian or designee on the basis of acceptable body weights and clinical signs.

Rats that are accidentally killed or removed from study during the pretest period will be discarded without necropsy. Rats that are found dead or sacrificed *in extremis* during the pretest period will be sent to Pathology and given a gross examination to check for the presence of disease. Dependent upon these findings, further diagnostic procedures may be employed at the

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discretion of the study director, a pathologist, or the laboratory animal veterinarian. The results will not be reported in the final report unless considered significant to the evaluation of the study.

E. Assignment to Groups

Rats of each sex will be selected for use on study on the basis of adequate body weight gain and freedom from any ophthalmological abnormalities or clinical signs of disease or injury. They will be distributed by computerized, stratified randomization into study groups as designated in the Study Design, so that there are no statistically significant differences among group body weight means within a sex. The weight variation of selected rats will not exceed ± 20% of the mean weight for each sex.

Each group of study animals, assigned prior to study start, will consist of three or four subgroups: the first 10 rats/sex/dose will be designated for evaluation of subchronic toxicity; the next 5 rats/sex/dose will be designated for collection of blood for possible analysis of fluorine; the next 20 rats/sex/dose will be designated for reproductive assessment; the remaining 10 rats/sex/dose (control and high-dose groups only) will be designated for recovery assessment. An additional subgroup (5 rats/sex/dose) will be received as a separate shipment and will be added to each dose group approximately 70 days after study start, for evaluation of hepatic biochemical analysis following a 10-day exposure. The rats designated for possible fluorine analysis may receive other evaluations, at the discretion of the study director, if considered valuable in interpretation of reversibility of effects.

Each animal will be assigned a unique 6-digit Haskell animal number and an individual cage identification number. The last 3 digits of the Haskell animal number will be tattooed on the tail of each rat. The Haskell animal number and cage identification number will both be included on the cage label. Pups from the reproductive assessment will be tattooed when they are 21 days old. A list of corresponding numbers for the pups will be identified in study records.

At study start (test day 0) the rats will be between 6 and 8 weeks of age. On test day 0, when possible, rats with body weights that are not within ± 20% of the mean within a sex will be removed from study and replaced with rats having body weights within that range (subject to the same selection criteria as the original rats). The rats that are added to the study for 10-day biochemical evaluation will be the same approximate age at the start of their exposure phase as the other rats were at study start.

Rats that have not been assigned to a test group or which have been removed from study on test day 0 for out-of-range body weights, will be released for other laboratory purposes or be sacrificed by carbon dioxide asphyxiation and discarded without pathological evaluation, at the discretion of the study director.

The rats designated for the 10-day biochemical analysis will arrive separately, approximately 59 days after study start. They will be handled the same as the other rats during the quarantine and pre-test periods, except they will not receive an ophthalmological or neurobehavioral examination. They will be distributed by computerized, stratified randomization into study

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groups (5/sex/dose), so that there are no statistically significant differences among group body weight means within a sex when this subset of rats is compared among themselves.

F. Test Substance Preparation and Administration

H-25425 will be suspended in 0.5% methylcellulose in deionized water. Dosing suspensions of the test substance will be prepared daily and stored at room temperature until used. The method of mixing the test substance with the vehicle will be documented in the study records. Animals designated for evaluation of subchronic toxicity, recovery, and possible analysis of blood for fluorine will be dosed daily by gavage for at least 90 days. Animals designated for 10-day biochemical evaluation will be dosed daily by gavage for 10 days, starting approximately 70 days after study start. Animals designated for reproductive evaluations will be dosed daily by gavage for approximately 70 days, and then daily during the 14-day mating period. The P_1 male rats will continue to be dosed following the cohabitation period until sacrifice. Pregnant females will be dosed during the three-week gestation period. Pregnant females in the process of delivery or showing signs of delivery will not be dosed. From gestation day 18 until delivery, dose volumes will be based on the gestation day 18 body weights. Lactating females and females with no evidence of copulation will be dosed until pups are weaned on postpartum day 21. The volume of H-25425 given to each rat (5 mL/kg) will be based on the most recently recorded body weight. Male and female control animals will be similarly treated with 0.5% methylcellulose at the same dose volume as used in the other groups.

Samples of each test dosing suspension will be collected three times during the study: near the beginning, near the middle (approximately test day 45) and near the end of the exposure phase of the study. If necessary, additional samples may be collected at the discretion of the study director or designee. Analysis of the first sampling will verify homogeneity, concentration (average of homogeneity samples), and room temperature stability. The subsequent samplings will address concentration. Samples will be submitted, shortly after preparation, to the Analytical Group of Environmental Sciences (ES) at Haskell Laboratory. On days samples are collected, the suspensions remaining after dosing will be refrigerated for additional analysis if required. Refrigerated suspensions will be discarded if no additional analysis is necessary.

G. Body Weights

During the study, all rats will be weighed once each week unless experimental findings or special scheduling situations warrant a change in the weighing schedule. In addition, rats designated for neurobehavioral evaluations, undergoing functional observational battery and motor activity assessments, will be weighed on the days of those observations. Female rats designated for reproduction assessment will be weighed on gestation days 0, 7, 14, 18, and 21 and lactation days 0, 7, 14, and 21. Weaned F_1 rats will be weighed weekly until approximately postnatal day 60.

H. Food Consumption and Food Efficiency

During the study, the amount of food consumed by each rat over the weighing interval will be determined by weighing each feeder at the beginning and end of the interval and subtracting the

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final weight and the amount of spillage from the feeder during the interval from the initial weight. From these measurements, mean daily food consumption over the interval will be determined. From the food consumption and body weight data, the mean daily food efficiency will be calculated. Food consumption for the rats being sampled for blood fluorine will only be collected during the exposure period.

During the reproductive assessment, food consumption will be determined as follows for designated rats:

- Premating Dosing period – individual food consumption will be determined weekly, ending approximately test day 70.
- Cohabitation period, beginning on approximately test day 70 and for males following cohabitation period - food consumption will not be determined.
- Gestation period – individual food consumption will be determined on gestation days 0, 7, 14, and 21.
- Lactation period – food consumption will not be determined.
- Post-weaning F1 rats - individual food consumption will be determined weekly, ending on approximately postnatal day 60.

I. Detailed Clinical Observations and Mortality

During the test period, cage-site examinations to detect moribund or dead rats and abnormal behavior and/or appearance among rats will be conducted at least twice daily throughout the study. Moribund rats will be sacrificed. At every weighing, each rat will be individually handled and examined for abnormal behavior and appearance. Detailed clinical observations in a standardized arena will also be evaluated on rats designated for subchronic toxicity and recovery. The detailed clinical observations will include (but are not limited to) evaluation of fur, skin, eyes, mucous membranes, occurrence of secretions and excretions, autonomic nervous system activity (lacrimation, piloerection, and unusual respiratory pattern), changes in gait, posture, response to handling, presence of clonic, tonic, stereotypical, or bizarre behavior. Any abnormal clinical signs noted will be recorded.

Prior to sacrifice of moribund rats, if possible, and at the discretion of the clinical pathologist and/or study director, blood samples for hematology and clinical chemistry measurements will be collected for clinical pathology evaluations.

J. Ophthalmology

Two ophthalmological examinations will be conducted by a veterinary ophthalmologist. The pretest examination will be performed on all rats received for the study (except those designated for 10-day hepatic biochemical evaluation), prior to assignment to groups. Any rats with preexisting ophthalmological abnormalities may be eliminated from consideration for use in the study. Surviving rats designated for subchronic toxicity (10 rats/sex/dose) and recovery (10 rats/sex in control and high dose groups) evaluations will be examined prior to the final

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sacrifice. Both eyes of each rat will be examined by focal illumination and indirect ophthalmoscopy. The eyes will be examined in subdued light after mydriasis has been produced.

A third ophthalmological examination may be performed on rats designated for recovery prior to sacrifice if compound-related eye lesions are observed in animals sacrificed at the end of the test period.

A report of findings from each ophthalmology examination will be submitted by the examining veterinarian.

K. Neurobehavioral Evaluation

For all the following assessments, the experimenter will be unaware of the group designation of the animal. Prior to initiation of test substance administration and then during week 13 of test substance administration, sensory motor function and motor activity will be evaluated on animals designated for recovery (control and high dose). Sensory motor function and motor activity will also be evaluated on animals designated for the subchronic toxicity evaluation subset (10 animals/sex/dose) in the low- and mid-dose groups. Sensory motor function and motor activity assessments may also be performed on recovery animals at the end of the one-month recovery period. Spare rats will also be evaluated for the functional observational battery (FOB) and motor activity prior to study start.

1. Sensory Motor Function

Simple assessments of sensory motor function (grip strength, response to approach/touch, tail pinch, and sharp auditory stimulus) will be made. Fore- and hind-limb grip strength will be measured by a strain gauge device (Chatillon® Digital Force gauge). Due to technical difficulty of evaluating pupil size in albino rats under ambient lighting conditions, pupil size will be evaluated by assessment of pupillary response. Pupillary response to a beam of light will be measured immediately prior to removing the rats from the motor activity chambers.

2. Motor Activity

Motor activity (MA) will be assessed following the sensory motor function evaluation on the same day. Rats will be individually tested in one of 30 nominally identical, automated activity monitors (Coulbourn®). Groups will be counterbalanced across the monitors and time of day to the fullest extent possible. The infrared monitoring device enables measurement of 2 dependent variables, duration of movement and number of movements. A continuous movement is counted as one movement regardless of duration. Each test session will be 60 minutes in duration, and the results will be expressed for the total session as well as for 6 successive 10-minute blocks. Presence of defecation and urination on the cageboards below the motor activity cages will also be evaluated following each motor activity session.

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L. Clinical Pathology Evaluation

A clinical pathology evaluation will be conducted on rats designated for subchronic toxicity during week 7 and week 14 (prior to necropsy). A clinical pathology evaluation will be conducted on rats designated for recovery near the end of the recovery phase. Coagulation parameters and plasma and urine fluoride will not be determined during week 7. The day before collection of blood samples for the clinical pathology evaluation, the rats will be placed in metabolism cages. These rats will be fasted overnight (at least 15 hours) and urine will be collected from each rat. Blood samples for hematology and clinical chemistry measurements will then be collected from the orbital sinus of each rat while the rat is under light carbon dioxide anesthesia. Blood samples for coagulation parameters and plasma fluoride will be collected from each rat at sacrifice from the abdominal *vena cava*, while the rat is under carbon dioxide anesthesia. Blood samples will be evaluated for quality by visual examination prior to analysis.

Additional blood, collected from the *vena cava* at the final sacrifice, will be placed in a serum tube, processed to serum, and frozen at -70 to -80°C. Serum may be used for additional testing as documented by protocol amendment, or will be discarded when the final report issues. Bone marrow smears will be prepared at the final sacrifice from all rats sacrificed at that time point, and will be evaluated if warranted by experimental findings.

At the discretion of the study director or clinical pathologist, blood samples for clinical pathology tests may be collected from animals showing clinical evidence of toxicity or sacrificed *in extremis*.

1. Hematology

The following hematology parameters will be determined:

Red blood cell count	Red cell distribution width
Hemoglobin	Absolute reticulocyte count
Hematocrit	White blood cell count
Mean corpuscular volume	Differential white blood cell count
Mean corpuscular hemoglobin	Platelet count
Mean corpuscular hemoglobin concentration	Microscopic blood smear examination

New methylene-blue-stained blood smears will be prepared from all hematology samples and will be evaluated, if required, to substantiate or clarify the results of hematology findings.

2. Coagulation Parameters

The following coagulation parameters will be determined at sacrifice only:

Prothrombin time
Activated partial thromboplastin time

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3. Serum Chemistry

The following serum chemistry parameters will be determined:

Aspartate aminotransferase	Total protein
Alanine aminotransferase	Albumin
Sorbitol dehydrogenase	Globulin
Alkaline phosphatase	Calcium
Total bilirubin	Inorganic phosphorus
Urea nitrogen	Sodium
Creatinine	Potassium
Cholesterol	Chloride
Triglycerides	Plasma fluoride (sacrifice only)
Glucose	

4. Urinalysis

The following urinalysis parameters will be determined:

Quality	Blood
Color	Glucose
Clarity	Protein
Volume	Bilirubin
Osmolality	Ketone
pH	Microscopic urine sediment examination
Urobilinogen	Urine fluoride (sacrifice only**)
	**EDTA will be placed in urine vials prior to collection of this urine specimen

M. Total Fluorine Level Evaluation

On test day -4 (pre-bleed), and test days 3, 9, 22, 34, 55, 76, and 89, blood (approximately 0.6 mL) will be collected from the orbital sinus of designated animals (5 rats/sex/dose), while the animals are under light carbon dioxide anesthesia, for possible total blood fluorine analysis. On the day of blood collection (except test day -4), blood will be collected from the animals 2 hours (±30 minutes) after dosing. The blood will be collected in plastic tubes containing EDTA while on ice and stored frozen. During the last week of the approximately 90-day exposure period the animals will be placed in metabolism cages for collection of feces and urine. Urine and feces will be collected daily at 24-hour intervals from each animal. The exact time period of collection of urine and feces will be documented along with the total volume of urine obtained. Urine and feces will be stored frozen until analyzed. The decision to analyze for blood fluorine and the method for determining blood fluorine will be documented in the study records. The type of kinetic analysis to be used on blood fluorine data will be determined after study start and will be addressed in a protocol amendment.

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Blood will also be collected for possible total blood fluorine analysis at 3, 7, 11, 18, 25, 36, 50, 71, and 92 days postdosing (after the last dose) for both male and female animals of each dose group. Animals will be sacrificed at the end of the approximately 90-day recovery period. The blood, urine, and fecal samples collected postdosing may be analyzed as needed to answer questions regarding distribution and metabolism of the test substance.

N. Reproductive Assessment

1. Breeding

a. Start of cohabitation

After approximately 10 weeks of exposure to the test substance.

b. Duration of cohabitation period

Until evidence of copulation is observed (designated as day 0 of gestation), or until 2 weeks have elapsed.

c. Evidence of copulation

Once daily, each female will be examined for the presence of an intravaginal copulation plug or sperm in the vaginal lavage sample. The day evidence of copulation is observed is designated as day 0 of gestation.

d. Cohabitation

Each female will be continually housed on a 1:1 basis with a randomly selected male of the same dosage level, in the male's cage. On the day copulation is confirmed, the female will be transferred back to individual cage housing.

Based on the outcome of the first mating and at the discretion of the reproductive toxicologist, the females may be cohoused again in the manner outlined above, except with different males, to produce a second set of litters.

2. Estrous Cycle Evaluation

Vaginal smears will be collected daily from all P_1 female rats in order to determine the stages of the estrous cycle. Vaginal smears will be collected beginning three weeks prior to mating, and continuing until copulation is confirmed, or the cohabitation period has ended.

Vaginal smears will also be collected from all P_1 parental female rats at the time of sacrifice to determine the stage of estrous cycle.

3. Gestation Procedures

After being transferred into polycarbonate pans (on day 20 of gestation for mated females, or at the end of the cohabitation period for females without evidence of copulation), female rats will be observed at least twice daily for signs of delivery and pups.

4. Lactation Procedures

The day when delivery is complete is designated day 0 postpartum. At each examination period, pups will be individually handled and examined for abnormal behavior and appearance; any dead, missing, or abnormal pups will be recorded.

a. Day 0 postpartum

Live and dead pups in each litter will be counted as soon as possible after delivery is completed. Live pups in each litter will be individually weighed.

b. Day 4 postpartum

Litters will be culled randomly to 8 (4/sex when possible). Extra pups will be euthanatized (by decapitation) and discarded without pathological examination. Litters of eight pups or fewer will not be reduced. Litter counts will be determined prior to and after culling and individual pup weights will be determined prior to culling.

c. Days 7 and 14 postpartum

Pups in each litter will be counted by sex and individually weighed.

d. Day 21 postpartum (Weaning)

Pups in each litter will be counted by sex and individually weighed (3 of 4 rats/sex/litter will then be sacrificed and grossly examined on postnatal day 21). Randomly selected weanlings (one/sex/litter) will be placed in individual cages, monitored for attainment of developmental landmarks.

5. Post Weaning and Developmental Landmarks

Developmental landmarks in the F_1 generation male and female rats (one/sex/litter) will be monitored on a daily basis until criterion is achieved. Body weight and food consumption will be determined weekly until termination (approximately postnatal day 60). Animals will also be weighed on the day the developmental landmark is achieved.

a. Body Weight and Food Consumption

Body weight and food consumption will be determined weekly until termination (approximately postnatal day 60).

b. Vaginal Patency

Female rats will be examined beginning on test day 0 (postnatal day 21) until achievement, or postnatal day 43, whichever comes first. Body weight will be recorded on the day of achievement.

c. Preputial Separation

Male rats will be examined beginning on test day 14 (postnatal day 35) until achievement, or postnatal day 55, whichever comes first. Body weight will be recorded on the day of achievement.

O. Anatomic Pathology

1. Pretest

See Section D. Pretest Period.

2. Rats Designated for Subchronic Toxicity Evaluation

All rats found dead, accidentally killed, sacrificed *in extremis*, or sacrificed by design will undergo a gross and microscopic evaluation. All rats removed from study (except for out-of-range body weight on test day 0) will be sent to Pathology for gross evaluation and collection of tissues. Rats will be euthanized by carbon dioxide anesthesia and exsanguination. Rats sacrificed by design will be fasted after 3 p.m. on the afternoon before their scheduled sacrifice (at least 15 hours). A final sacrifice will be performed on surviving rats following the final clinical pathology evaluation. The order of sacrifice for scheduled deaths will be random among all treatment groups within a sex. Blood samples for coagulation parameters and plasma fluoride evaluation will be collected from the abdominal *vena cava* of each rat at sacrifice, while the rat is under carbon dioxide anesthesia.

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The following tissues will be collected from rats which are found dead or accidentally killed (tissue integrity permitting), sacrificed *in extremis*, removed from study (except out-of-range body weight on test day 0), or sacrificed by design.

Digestive System	Cardiovascular System	Musculoskeletal System
liver	heart	skeletal muscle
esophagus	aorta	femur/knee joint
stomach		sternum
duodenum	Hematopoietic System	
jejunum	spleen	Reproductive System
ileum	thymus	Male
cecum	mandibular lymph node	testes
colon	mesenteric lymph node	epididymides
rectum	bone marrow[a]	prostate
salivary glands		seminal vesicles
pancreas	Endocrine System	Female
	pituitary gland	ovaries
Urinary System	thyroid gland	uterus
kidneys	parathyroid glands	mammary glands
urinary bladder	adrenal glands	
		Miscellaneous
Respiratory System	Nervous System	skin
lungs	brain (including cerebrum,	eyes (including retina and optic nerve)
trachea	cerebellum, medulla/pons)	gross observations[b]
nose	spinal cord (cervical,	
larynx	mid-thoracic, lumber)	
pharynx	sciatic nerve	

a Bone marrow will be collected with the femur and sternum.
b Gross observations made at necropsy for which histopathology is not appropriate (e.g., fluid, ruffled fur, and missing anatomic parts) will generally not be collected.

All tissues will be placed in the appropriate fixative.

Rats sacrificed by design will have the following organs weighed: liver, kidneys, adrenal glands, thymus, brain, spleen, heart, thyroid (weighed after fixation) and ovaries and uterus (female) or testes and epididymides (male). Relative organ weights (percent of final body weight; ratio to brain weight) will be calculated. Final body weights determined just prior to necropsy will be used in the assessment of organ weight changes. Organs from rats found dead, sacrificed *in extremis*, or accidentally killed may be weighed at the discretion of the pathologist or study director.

Bone marrow smears will be prepared at the final sacrifice from all surviving rats and will be evaluated if warranted by experimental findings.

All tissues collected from rats designated for subchronic toxicity evaluation in the high-concentration and control groups, and from rats that are found dead or accidentally killed (tissue integrity permitting), or are sacrificed *in extremis*, will be further processed to slides, stained with hematoxylin and eosin, and examined microscopically. Gross lesions and any target organs from

rats in the low- and intermediate-concentration groups will be evaluated microscopically. Selected gross observations for which a microscopic diagnosis would not be additive (e.g., osteoarthritis, pododermatitis, tail chronic dermatitis, calculus, and deformities of the teeth, toe, tail, or ear pinna) will be saved, but will generally not be processed for microscopic evaluation. Tissues from rats removed from study will not be processed for microscopic evaluation unless considered necessary by the study director or pathologist.

Additional procedures to identify and/or clarify histologic features of lesions may be performed at the discretion of the pathologist and will be documented in the final report.

3. Rats Designated for Possible Analysis of Total Blood Fluorine Levels

Any rats designated for possible analysis of blood fluorine that are found dead, accidentally killed, or are sacrificed *in extremis* will be discarded without pathological evaluation. At the end of the approximately 90-day post-dosing period, these rats (5 rats/sex/dose) will be sacrificed by carbon dioxide anesthesia and exsanguination and the following tissues will be collected and weighed: liver, kidney, testes, and fat (approximately 1 gram). These tissues will be placed in plastic freezer bags and stored in the freezer until analysis of total fluorine levels, if needed.

4. Rats Designated for Recovery

Any rats designated for recovery evaluations that are found dead, accidentally killed, or are sacrificed *in extremis* will be given a gross pathological evaluation. The same tissues collected for the subchronic animals at the approximately test day 90 sacrifice will be collected for all recovery animals and saved in the appropriate fixative. One month after the final dose, rats designated for recovery (10 rats/sex in control and high dose groups) will be sacrificed and undergo a gross examination. Tissues will be collected and gross lesions and target organs, identified in animals sacrificed at the end of the approximately 90-day exposure period, will be examined microscopically.

5. Rats Designated for Reproductive Assessment

Sacrifice Schedule

Animals	Schedule
Adult Males	After airing litters (at discretion of reproductive toxicologist)
Pregnant Females	On day of weaning litters
Nonpregnant Females	With pregnant females
Weanlings	3 of 4 rats/sex/litter will be sacrificed on postnatal day 21.
F_1 Adults	At postnatal day 60

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a. P_1 Adults

All P_1 parental rats will be sacrificed by carbon dioxide anesthesia and exsanguination, and will receive gross pathological examination, including the females that die or are sacrificed *in extremis* prior to the end of lactation and those for which mating did not result in production of offspring. The uteri of all cohabited females will be examined for the presence and number of implantation sites.

Sperm parameters for the first 10 surviving P_1 male animals sacrificed by design in each treatment group will be evaluated. The right epididymis will be removed, and the right cauda epididymis will be weighed. Sperm will be collected from the right cauda epididymis and percent motility and morphology will be determined. The left epididymis and testis will be frozen in liquid nitrogen and stored between -65°C and -85°C for sperm and spermatid counts, respectively.

The following tissues and all gross lesions will be collected from all P_1 adults (including those that die anytime prior to the scheduled sacrifice) and preserved in appropriate fixative for possible future histopathological examination.

Tissues Collected from P_1 Adults

Male	Female	Both Sexes
Testis[a]	Ovaries	Thyroid
Epididymis[a]	Uterus (with oviducts)	Gross lesions[b]
Prostate	Vagina	Pituitary
Seminal Vesicles		
Coagulating Gland		

a Testes and epididymides collected from male rats will be placed in Bouin's solution. All other tissues (reproductive and non-reproductive) collected from male and female rats will be placed in formalin.
b Gross lesions observed at necropsy for which histopathology is not appropriate or would not be additive will generally not be collected.

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Reproductive organs of animals with impaired reproductive performance (e.g., failure to conceive or deliver healthy offspring, or effects on estrous cyclicity or sperm parameters) will be evaluated microscopically. Gross lesions will also be evaluated microscopically at the discretion of the study director or pathologist.

b. Offspring

Offspring that are found dead during the lactation period will undergo a gross pathological evaluation.

c. F_1 Weanlings

Three F_1 weanlings/sex/litter (randomly selected), litter size permitting, will be sacrificed by carbon dioxide asphyxiation and undergo a gross pathological evaluation on postnatal day 21. All gross lesions will be preserved for possible future histopathological examination.

d. F_1 Adults

All F_1 generation rats will be sacrificed by carbon dioxide asphyxiation and exsanguination, and will receive a gross pathological examination, including F_1 rats that die or are sacrificed *in extremis* prior to postnatal day 60. Selected tissues and all gross lesions will be preserved in appropriate fixative for possible future histopathological examination.

The following table lists tissues to be collected and/or weighed (tissue integrity permitting):

Organ Weights from F_1 Adults

Male	Female	Both Sexes
Testes	Uterus (with oviducts and cervix)	Thyroid (after fixation)
Epididymides		Liver
Seminal Vesicles (with coagulating glands)		Brain
Prostate		

Tissues Collected from F_1 Adults

Male	Female	Both Sexes
Testis[a]	Ovaries	Thyroid
Epididymes[a]	Uterus (with oviducts)	Gross lesions[b]
Prostate	Vagina	Pituitary
Seminal Vesicles		
Coagulating Gland		

a Testes and epididymides collected from male rats will be placed in Bouin's solution. All other tissues (reproductive and non-reproductive) collected from male and female rats will be placed in formalin.
b Gross lesions observed at necropsy for which histopathology is not appropriate or would not be additive will generally not be collected.

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Histopathology will be evaluated in reproductive organs, at the discretion of the study director or reproductive toxicologist.

P. Biochemical Analysis

1. Rats designated for Biochemical Analysis

Biochemical analysis (hepatic peroxisome proliferation) will be evaluated in rats sacrificed after 10 days of dosing (10-dose biochemical subgroup), at the approximately 90-day sacrifice (subchronic toxicity subgroup), and at the end of the recovery phase of the study (recovery subgroup). Samples for biochemical evaluation will be taken from the first 5 rats/sex/dose designated for subchronic toxicity evaluation and from the first 5 rats/sex/dose designated for recovery.

An additional 40 Crl:CD®(SD)IGS BR rats (5 rats/sex/dose) will be obtained from Charles River Laboratories, Inc. approximately 59 days after study start for the rest of the rats, and designated for 10-day biochemical analysis. These rats will be dosed daily with the test substance by oral gavage for 10 days, starting soon after the reproduction subgroup has been removed for mating. Rats will be fasted after 3 p.m. on the afternoon before their scheduled sacrifice (at least 15 hours). On the 11th day, surviving rats will be euthanatized by carbon dioxide anesthesia and exsanguination.

Procedures for animal husbandry, environmental conditions, water and food, pretest period (no ophthalmology examination), assignment to groups, body weights, food consumption, and food efficiency in the rats designated for 10-day biochemical analysis will be the same as for rats designated for subchronic toxicity evaluation. No ophthalmological or neurobehavioral evaluations or detailed clinical observations will be conducted. These rats will be considered part of the same dose groups as the rats started at study start; however, data from these animals will be reported separately from data from the rest of the rats in each group. Livers will be collected and weighed, and relative organ weights (percent of final body weight) will be calculated. Final body weights determined just prior to necropsy will be used in the assessment of liver weight changes.

2. Biochemical Analysis

At necropsy, a portion of the liver will be placed in liver homogenization buffer and maintained on ice for preparation of hepatic peroxisomes.

Hepatic peroxisomes will be prepared in accordance with DuPont Haskell Standard Operating Procedure (SOP) BT015-P. Peroxisomal β-oxidation activity will be determined using [14C]palmitoyl CoA as the substrate according to SOP [redacted] The protein content of the peroxisomes will be measured before and after analyses by the Biorad method.[5] Final calculations will be based on the post-assay protein determination.

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STATISTICAL ANALYSES

Significance will be judged at $p < 0.05$. Males and females will be analyzed separately. The method for kinetic analysis will be determined after study start and documented in study records. In-life data from the 10-day biochemical analysis subgroup will only be compared among these subgroups.

Statistical Methods for Subchronic Toxicity Assessments

		Method of Statistical Analysis	
Parameter	Preliminary Test	If preliminary test is not significant	If preliminary test is significant
Body Weight Body Weight Gain Food Consumption Food Efficiency β-Oxidation Organ Weight	Test for lack of trend[6]	Sequential application[7] of the Jonckheere-Terpstra trend test[8]	Preliminary tests for pairwise comparison
	OR[a]		
	Levene's test for homogeneity[9] and Shapiro-Wilk test[10] for normality[b]	One-way analysis of variance[11] followed with Dunnett's test[12]	Kruskal-Wallis test[13] followed with Dunn's test[14]
Clinical Pathology[c]	Levene's test for homogeneity[9] and Shapiro-Wilk test[10] for normality[b]	One-way analysis of variance[11] followed with Dunnett's test[12]	Kruskal-Wallis test[13] followed with Dunn's test[14]
Motor Activity[c]	Levene's test for homogeneity[9] and Shapiro-Wilk test[10] for normality[b]	Repeated measures analysis of variance followed by contrasts[15]	Sequential application[7] of the Jonckheere-Terpstra trend test[8]
Grip Strength Foot splay	Bartlett's test[16] for homogeneity of variances	One-way analysis of variance[11] followed with Dunnett's test[12]	Kruskal-Wallis test[13] followed with Dunn's test[14]
Survival Incidence of Clinical Observations Incidence of Ophthalmological Observations Incidence of FOB Descriptive Parameters	None	Cochran-Armitage test for trend[21][d]	
Incidence of Microscopic Lesions	None	None	

a Pairwise comparisons and associated preliminary tests are only conducted if the test for lack of trend is significant.
b If the Shapiro-Wilk test is not significant but Levene's test is significant, a robust version of Dunnett's test will be used.
c Test day and block (10-minute intervals) will be used as repeated-measure factors.
d When an individual observation is recorded as being less than a certain value, calculations are performed on half the recorded value. For example, if bilirubin is reported as <0.1, 0.05 is used for any calculations performed with that bilirubin data.
e If the incidence is not significant, but a significant lack of fit occurs, then Fisher's exact test[17] with a Bonferroni correction is used.

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The following table lists the indices of reproductive function that will be calculated for the P_1 and F_1 adults.

Statistical Methods for Reproductive Assessments

Parameter	Preliminary Test	Method of Statistical Analysis: If preliminary test is not significant	Method of Statistical Analysis: If preliminary test is significant
Body Weight Body Weight Gain Food Consumption Gestation Length Organ Weight Implantation Site Numbers Implantation Efficiency Mean Number of Pups Per Litter Percent Born Alive Precoital Interval Estrous Cycle Length Sperm Parameters Vaginal Patency Preputial Separation	Test for lack of trend[6]	Sequential application[7] of the Jonckheere-Terpstra trend test[8]	Preliminary tests for pairwise comparison
		OR[a]	
	Levene's test for homogeneity[9] and Shapiro-Wilk test[10] for normality[b]	One-way analysis of variance[11] followed with Dunnett's test[12]	Kruskal-Wallis test[13] followed with Dunn's test[14]
Incidence of Clinical Observations Number of Females Cycling Normally Mating Index Fecundity Index Fertility Index Gestation Index Viability Index Lactation Index	None	Cochran-Armitage test for trend[11][c]	
Mean Pup Weights (Covariates: litter size, sex ratio) Anogenital Distance (Covariate: pup weight) Sex Ratio	None	Linear contrast of least squares means[15]	Dunn's test[14]

a Pairwise comparisons and associated preliminary tests are only conducted if the test for lack of trend is significant.
b If the Shapiro-Wilk test is not significant but Levene's test is significant, a robust version of Dunnett's test will be used.
c If the incidence is not significant, but a significant lack of fit occurs, then Fisher's exact test[17] with a Bonferroni correction is used.

For each parameter analyzed with a trend test, the test will be applied to the data sequentially. If a significant dose-response is detected, data from the top dose group will be excluded and the test repeated until no significant trend is detected.[7] For litter parameters, the proportion of affected fetuses per litter or the litter mean will be used as the experimental unit for statistical evaluation.[11] Where the data are tied and the standard large sample version of Jonckheere's test is not applicable, exact p values will be calculated using permutation methodology.[19]

Additional statistical tests will be used, and other parameters analyzed, if deemed necessary. The statistical methods used for analysis will be documented in the final report.

SAFETY AND HOUSEKEEPING

Good housekeeping procedures will be practiced to avoid contamination of the neat dosing material and potential health hazards. To avoid skin contact, gloves will be worn when handling the test substance. Animal carcasses, feces, and unused diet will be incinerated.

RECORDS AND SAMPLE RETENTION

All original records or true copies of original records (sponsor-supplied characterization of test substance) will be retained at Haskell Laboratory, E.I. du Pont de Nemours and Company, Newark, Delaware or at Iron Mountain Records Management, 200 Todds Lane, Wilmington, Delaware. Laboratory-specific or site-specific records such as personnel files and equipment records will be retained at the facility where the work was done. Preserved wet tissues, embedded tissues, histological slides, blood smears and bone marrow smears will be retained at Haskell Laboratory. A sample of the test substance will be collected for archive purposes and retained at Haskell Laboratory.

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PROTOCOL APPENDIX

Study Function	Study Personnel
Study Director:	Susan A. MacKenzie Senior Research Toxicologist
Analytical Assessment	Janet C. Maslanka Analytical Chemist
Neurobehavioral Evaluation:	Linda A. Malley Neurobehavioral Toxicologist
Biochemical Analyses:	John C. O'Connor Biochemical Toxicologist
Clinical Pathology Evaluation:	Nancy E. Everds Clinical Pathologist
Anatomic Pathology Evaluation:	John F. Hansen Anatomical Pathologist
Reproductive Assessments:	Robert M. Parker Reproduction Toxicologist

Proposed Study Dates	
Initiation of Test Substance Administration	October 15, 2002
Scheduled Sacrifice (final)	April, 2003

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SIGNATURES

Approved by: _____ 04-OCT-2002
Robert B. Wyatt, Jr.
Primary Technician
Date

_____ 04-Oct-2002
Janet C. Maslanka
Analytical Chemist
Date

_____ 03-Oct-2002
Linda A. Malley
Neurobehavioral Toxicologist
Date

_____ 04-OCT-2002
Robert M. Parker
Reproduction Toxicologist
Date

_____ 03-Oct-2002
Nancy E. Ewers
Clinical Pathologist
Date

_____ 03-Oct-2002
John E. Hansen
Anatomic Pathologist
Date

_____ 03-Oct-2002
John C. O'Connor
Biochemical Toxicologist
Date

_____ 03-Oct-2002
Scott E. Lovelace
Management
Date

_____ 03-OCT-2002
Susan A. MacKenzie
Study Director
Date

cc: [redacted]

Exygen Study No.: 008-342

[redacted]

Protocol Amendment No. 1

The protocol is amended as follows:

1. Rats designated for evaluation of 10-day hepatic biochemical analysis will be received approximately 79 days after study start and dosing will begin approximately 90 days after study start. This changes references to timing of receipt and dosing of these rats on:
   - Page 5, Materials and Methods, B. Test Species, paragraph 2.
   - Page 8, Materials and Methods, E. Assignment to Groups, paragraphs 2 and 6.
   - Page 9, Materials and Methods, F. Test Substance Preparation and Administration, paragraph 1.
   - Page 21, Materials and Methods, P. Biochemical Analysis, 1. Rats Designated for Biochemical Analysis, paragraph 2.

   Rationale: The timing of this analysis was changed to accommodate work schedules during the holidays.

2. Page 13, Materials and Methods, M. Total Fluorine Level Evaluation,
   - Change header to read Total Fluorine and Perfluorooctanoic Acid Level Evaluations.
   - Add the following paragraphs at the end of the section:

   "Blood will be collected from the *vena cava* at necropsy, from all rats designated for subchronic toxicity evaluation and all rats designated for recovery evaluation. Plasma will be prepared and stored frozen at –80 to –20°C until analyzed for concentration of perfluorooctanoic acid (PFOA). Plasma will be evaluated from control and high-dose rats. The decision to evaluate low- and intermediate-dose groups will be based on results in the high-dose group.

   Plasma samples will be extracted by organic solvent protein precipitation using a protein precipitation column, and then analyzed for PFOA by LC-MS-MS."

Rationale: A business decision was made to evaluate PFOA plasma levels at the end of the dosing period and after recovery.

Approved by: _____ 7-Nov-2002
John C. O'Connor
Biochemical Toxicologist
Date

_____ 7-NOV-2002
Susan A. MacKenzie
Study Director
Date

[redacted]

Exygen Study No.: 008-342

[redacted]

Protocol Amendment No. 6

Protocol amendment No. 1, item 2, is amended as follows (changes bolded):

Page 13, Materials and Methods, M. Total Fluorine Level Evaluation,

- Change header to read Total Fluorine and Perfluorooctanoic Acid Level Evaluations.
- Add the following paragraphs at the end of the section:

"Blood will be collected from the *vena cava* at necropsy, from all rats designated for subchronic toxicity evaluation and all rats designated for recovery evaluation. Plasma will be prepared and stored frozen at –80 to –20°C until analyzed for concentration of perfluorooctanoic acid (PFOA). Plasma will be evaluated from control and high-dose rats. The decision to evaluate low- and intermediate-dose groups will be based on results in the high-dose group.

Plasma samples will be extracted by organic solvent protein precipitation using a protein precipitation column, and then analyzed for PFOA by LC-MS-MS. Results of analysis will be reported in a supplementary report."

Rationale: Due to timing on analytical methods development, the results of these analyses will not be available when the main report issues, but will issue later, as a supplement.

Approved by: Susan A. MacKenzie 10-Jul-2003
Susan A. MacKenzie
Study Director
Date

[redacted]

Exygen Study No.: 008-342

[redacted]

Protocol Amendment No. 8

Protocol amendment No. 6, is amended as follows (changes bolded):

Page 13, Materials and Methods, M. Total Fluorine Level Evaluation. Change header to read Total Fluorine and Perfluorooctanoic Acid Level Evaluations.
Add the following paragraphs at the end of the section:

"Blood will be collected from the *vena cava* at necropsy, from all rats designated for subchronic toxicity evaluation and all rats designated for recovery evaluation. Plasma will be prepared and stored frozen at –80 to –20°C until analyzed for concentration of perfluorooctanoic acid (PFOA). **Plasma samples will be extracted by organic solvent protein precipitation and then analyzed for PFOA by an appropriate method at Exygen Research (3058 Research Drive, State College, PA 16801).** Results of analysis will be reported in a supplementary report."

Reason: To change the evaluation to all subchronic toxicity and recovery group rats, change the method of extraction and analysis, and add the name and address of the laboratory performing the PFOA analysis.

Approved by: Susan A. MacKenzie 23-SEP-2003
Susan A. MacKenzie, Study Director — Date

[redacted]

Exygen Study No.: 008-342

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Page 1 of 1

PROTOCOL DEVIATION
Deviation Number: 1
Date of Occurrence: 10/07-08/03,10/08/03

Exygen Study Number: 008-342 Protocol Number: DuPont-11418

DESCRIPTION OF DEVIATION
1. Amendment 8, Analytical Method Section 4.5.4.e--accepted recovery of 63% for Exygen sample ID 0302239 Spk B2 at 1000 ppb.
2. Amendment 8, Analytical Method Section 4.5.4.e--accepted recovery of 119% for Exygen sample ID 0302047 Spk C at 1000 ppb.

ACTIONS TAKEN
for example - deviation issued, SOP revision, etc.
1-2. Protocol Deviation issued.

Recorded By: Emily R Decker Date: 11/7/03

IMPACT ON STUDY
1. No negative impact because the LLOQ fortification and the laboratory fortified field sample were within the acceptable range for this analytical set.

2. No negative impact because both laboratory control fortified samples were within the acceptable range for this analytical set.

Emily R Decker — Principal Investigator Signature — Date 11/7/03
Susan A. MacKenzie — Study Director Signature — Date 11/13/03
Management Signature — Date 11/18/03
(Sponsor) — Date —
Exygen QAU Review — Date 11/18/03

U:\Forms\PROTOCOL_DEVIATION.doc 9/25/2002/6

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Exygen Study No.: 008-342

APPENDIX B

Analytical Method ExM-008-276, Revision 1 "Method of Analysis for the Determination of Pentadecafluorooctanoic Acid (PFOA) in Serum by LC/MS/MS"

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TITLE

Method of Analysis for the Determination of Perfluorooctanoic Acid (PFOA) in Serum by LC/MS/MS - Revision 1

AUTHOR

Emily Decker

DATE ISSUED

September 24, 2003

SPONSOR

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PERFORMING LABORATORY

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METHOD NUMBER

ExM-008-276 Revision 1

TOTAL NUMBER OF PAGES

20

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Exygen Study No.: 008-342

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1. SUMMARY

This report details a method of analysis for perfluorooctanoic acid (PFOA) in serum.

PFOA is extracted from serum by protein precipitation in acetonitrile. Quantification of PFOA is accomplished by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis using selected reaction monitoring (SRM). The chemical formula of PFOA is given in section 2 of this method.

The lower limit of quantitation (LLOQ) for this method is 10 ppb for PFOA in serum.

Quantification is performed using extracted calibration standards containing an internal standard.

This method was developed using monkey serum. The overall percent recovery ± standard deviation for PFOA in monkey serum at 10, 100, and 1000 ppb was 94% ± 6.9% (Table I).

A representative calibration curve for PFOA in monkey serum is shown in Figure 1. Representative chromatograms for PFOA in monkey serum are shown in Figures 2 to 4.

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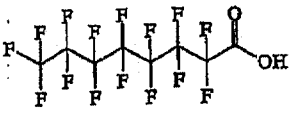
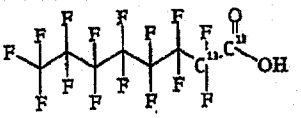
2. EXPERIMENTAL COMPOUNDS

The structure of perfluorooctanoic acid (PFOA) and the internal standard are given below.

PFOA
Chemical Name = perfluorooctanoic acid
Molecular weight = 414



^{13}C – PFOA
Chemical Name = perfluorooctanoic acid, [1,2-di-^{13}C]
Molecular weight = 416



3. CHEMICALS AND SUPPLIES

3.1. CHEMICALS

Chemical	Grade	Source	Catalog No.
Methanol (MeOH)	HPLC	EM Science	JT9093-2
Acetonitrile (ACN)	HPLC	EM Science	AX0145-1
Ammonium Acetate	Reagent	Sigma-Aldrich	A-7330
OmniSolv Water	HPLC	EM Science	WX0004-1

3.2. STANDARDS

Standard	Lot Number	Purity (%)	Source
perfluorooctanoic acid (PFOA)	21002	[redacted]	Oakwood Products
perfluorooctanoic acid, [1,2-di-^{13}C]	NA		DuPont

3.3. Equipment and Supplies

Equipment	Supplier
Balance, analytical (display at least 0.0001 g)	Mettler
Centrifuge	Eppendorf
2 mL eppendorf centrifuge tubes	VWR
Disposable micropipets (50-100uL, 100-200uL)	Drummond (VWR)
Class A pipets and volumetric flasks	various suppliers
Hypercarb drop-in guard column (4 mm) (part # 844017-400)	Keystone
Stand-alone drop-in guard cartridge holder	Keystone
125-mL LDPE narrow-mouth bottles	Nalgene
2 mL clear HPLC vial kit (cat # 5181-3400)	HP
Standard lab equipment (graduated cylinders, disposable tubes etc.)	various suppliers
LC/MS/MS and HPLC systems	As described in section 4.5.

Note: Equivalent materials may be substituted for those specified in this method if they can be shown to produce satisfactory results.

Notes:

1. In order to avoid contamination, the use of disposable labware is highly recommended (tubes, pipets, etc.).
2. PTFE or PTFE lined containers or equipment, including PTFE-lined HPLC vials for the HPLC autosampler must not be used.
3. It is necessary to check the solvents (methanol) for the presence of contaminants by LC/MS/MS before use. Certain lot numbers have been found to be unsuitable for use.
4. Use disposable micropipets or pipets to aliquot standard solutions to make calibration standards and sample fortifications.
5. The hypercarb cartridges should be changed when the system contaminants (elevated baseline) move to within 1 minute from the elution of PFOA.

3.4. Solutions

(1) 2 mM ammonium acetate solution is prepared by weighing 0.15 g of ammonium acetate and dissolving in 1 L of OmniSolv Water.

Note: The aforementioned example is provided for guidance, alternative volumes may be prepared as long as the ratios of the solvent to solute are maintained.

3.5. Preparation of Standards and Fortification Solutions

Analytical standards are used for three purposes:

1. Calibration Standards – These standards are prepared in control monkey serum and are used to calibrate the response of the detector used in the analysis.
2. Laboratory Control Spikes – These fortifications are usually prepared at concentrations corresponding to the LLOQ and 10x LLOQ and are used to determine analytical recovery. Laboratory control spikes are prepared in control monkey serum.
3. Matrix Spikes – These fortifications are prepared by spiking into the field samples at known concentrations. Matrix spikes are used to evaluate the effect of the sample matrix on analytical recovery.

The absolute volumes of the standards may be varied by the analyst as long as the correct proportions of solute to solvent are maintained. Alternate concentrations may be prepared as long as the preparation and concentration are accurately recorded in the raw data. Note also that additional concentrations may be prepared if necessary.

3.5.1. Stock solution

Prepare individual stock solutions of ~ 100 µg/mL of PFOA and ^{13}C-PFOA by weighing out ~ 10 mg of analytical standard (corrected for purity) and dilute to 100 mL with methanol in a 100-mL volumetric flask. Each stock solution (in 125-mL LDPE bottles) is to be stored in a refrigerator at 2°C to 6°C and is stable for a maximum period of 1 year from the date of preparation.

3.5.2. Fortification Solutions

a. Prepare a fortification standard of 10.0 µg/mL for PFOA by diluting 10.0 mL of the PFOA solution described in 3.5.1 to 100 mL with acetonitrile in a volumetric flask.
b. Prepare a fortification standard of 1.0 µg/mL for ^{13}C-PFOA by diluting 1.0 mL of the ^{13}C-PFOA solution described in 3.5.1 to 100 mL with acetonitrile in a volumetric flask.
c. Prepare a fortification standard of 1.0 µg/mL for PFOA containing 0.005 µg/mL of ^{13}C-PFOA by diluting 10.0 mL of the PFOA solution described in 3.5.2.a and 0.5 mL of the ^{13}C-PFOA solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.
d. Prepare a fortification standard of 0.1 µg/mL for PFOA containing 0.005 µg/mL of ^{13}C-PFOA by diluting 1.0 mL of the PFOA solution described in 3.5.2.a and 0.5 mL of the ^{13}C-PFOA solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.
e. Prepare a fortification standard of 0.01 µg/mL for PFOA containing 0.005 µg/mL of ^{13}C-PFOA by diluting 0.1 mL of the PFOA solution

described in 3.5.2.a and 0.5 mL of the ^{13}C-PFOA solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.

Store all fortification standard solutions in a refrigerator (in 125-mL LDPE bottles) at 2°C to 6°C for a maximum period of 1 year from the date of preparation, after which time it is necessary to make new standards using the stock solution.

3.5.3. Calibration Standards

The calibration standards are processed through the extraction procedure, identical to the samples. The fortification of the standards before extraction is done according to the following table:

Conc. Of Mixed Fortification Solution (µg/mL)	Fortification Volume (µL)	Volume of Control Sample (mL)	Conc. of Extracted Calibration Standard (ppb)
0.01	100	0.1	10
0.01	200	0.1	20
0.1	50	0.1	50
0.1	100	0.1	100
0.1	200	0.1	200

4. METHOD

4.1. FLOW DIAGRAM

The flow diagram of the method is given below, followed by a detailed description of each step.

Method Flow Diagram

Measure 0.1 mL of sample (using disposable micropipette)
(fortify samples when designated)
↓
↓
Protein precipitation in acetonitrile
↓
↓
LC/MS/MS analysis

4.2. SAMPLE PROCESSING

No sample processing is needed for serum samples. However, frozen samples must be allowed to completely thaw, un-aided, at room temperature. Samples

stored refrigerated should also be allowed to equilibrate to room temperature. All samples must be thoroughly mixed before being sampled for extraction.

4.3. Sample Preparation

a. Each batch of samples extracted (typically 30 or less) must include at least one reagent control (acetonitrile blank), one matrix control (method blank), and two matrix controls fortified at known concentrations to verify procedural recovery for the batch.
b. At least one sample per batch should be extracted in duplicate
c. At least one sample extracted should be separately fortified at a known concentration and carried through the procedure to verify recovery. Additional matrix spikes may be performed at the sponsor's request.

4.4. Extraction

1. Measure 0.1 mL of sample into 2 mL eppendorf centrifuge tubes (fortify with analyte as needed, close lid, and vortex ~ 10 seconds).
2. Add enough acetonitrile containing internal standard at 0.005 µg/mL (accounting for fortification volume) to make extraction volume 500 µL and vortex for ~ 10 seconds.
3. Centrifuge for ~ 10 minutes at ~ 14,000 rpm.
4. Analyze samples using electrospray LC/MS/MS.

4.5. Quantitation

4.5.1. LC/MS/MS System and Operating Conditions

Instrument:	PE SCIEX API 4000 Biomolecular Mass Analyzer SCIEX Turbo Ion Spray Liquid Introduction Interface
Computer:	Dell OptiPlex GX 110
Software:	PE Sciex Analyst 1.2
HPLC Equipment:	Hewlett Packard (HP) Series 1100 Quatpump G1311A Vacuum Degasser G1322A Autoinjector G1313A Column Compartment G1316A

Note: Two 4 × 10 mm hypercarb drop-in guard cartridges (Keystone, part # 844017-400) are attached in-line after the purge valve and before the sample injector port to trap any residue contaminants that may be in the mobile phase and/or HPLC system.

HPLC Column: Genesis C_8 (Jones Chromatography), 2.1 mm x 50 mm, 4µ
Column Temperature: 30° C
Injection Volume: 5 µL
Mobile Phase (A): 2 mM Ammonium Acetate in OmniSolv Water
Mobile Phase (B): Methanol

Time	% A	% B	Flow (mL/min)
0.0	40	60	0.3
3	40	60	0.3
3.5	0	100	0.3
3.7	0	100	0.5
7	0	100	0.5
7.5	40	60	0.5
9	40	60	0.5
9.5	40	60	0.3
12	40	60	0.3

It may be necessary to adjust the HPLC gradient in order to optimize instrument performance.

Ions monitored:

Analyte	Mode	Transition Monitored	Approximate Retention Time
PFOA	Negative	413 → 369	~2.8 min.
^{13}C-PFOA	Negative	415 → 370	~2.8 min.

The retention times may vary, on a day-to-day basis, depending on the batch of mobile phase etc. Drift in retention times is acceptable within an analytical run, as long as the drift continues through the entire analysis and the standards are interspersed throughout the analytical run.

Note: An alternative LC/MS/MS system may be used once demonstrated to be equivalent.

4.5.2. Tune File Parameters

The mass spectrometer is tuned for the analyte by infusing a ~ 1 µg/mL standard solution of PFOA (at 10 µL/min, using an infusion pump) via a "T" into a stream of mobile phase containing 60% methanol and 40% 2mM ammonium acetate at 0.3 mL/min flow rate. The analyte is initially tuned for the parent ion and then tuned for the product ion. Once the instrument is tuned, the optimized parameters are saved as a tune file. This tune file is then used during routine analysis.

4.5.3. Calibration Procedures

a. Inject the same aliquot (5 µL) of each calibration standard into the LC/MS/MS.

b. Use linear standard curves for quantitation. Linear standard curves are generated for each analyte by linear regression using 1/x weighting of the ratio analyte peak area/internal standard peak area versus the ratio of the concentration of analyte/concentration of internal standard using Analyst 1.2 (or equivalent) software system. Any calibration standard found to be a statistical outlier by using the appropriate outlier test (e.g. Huge Outlier Test), may be excluded from the calibration curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected and at least one calibration standard at the LLOQ must be retained.

c. The correlation coefficient (r) for calibration curves generated must be ≥0.9925 (r^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

4.5.4. Sample Analysis

a. Inject the same aliquot (5 µL) of each standard, sample, recovery, control, etc. into the LC/MS/MS system.

b. Standards corresponding to at least five or more concentration levels must be included in an analytical set.

c. An entire set of calibration standards should be injected at the beginning of a set followed by calibration standards interspersed every 5-10 samples (to account for a second set of standards). As an alternative, an entire set of calibration standards may be included at the beginning and at the end of a sample set. In either case, calibration standards must be the first and last injection in a sample set.

d. The concentration of each sample/fortification/control is determined from the standard curve, based on the peak area of each analyte. The standard responses should bracket responses of the residue found in each sample set. If necessary, dilute the samples in 50:50 methanol:water to give a response within the standard curve range.

e. Fortification recoveries falling within 85 to 115% (80 to 120% for levels at the LLOQ) are considered acceptable.

f. Extracted samples must be stored refrigerated between 2°C to 6°C until analysis.

g. Samples in which no peaks are detected (i.e. signal : noise ratio < 3:1) at the corresponding analyte retention times will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention

times but are less than the lowest concentration of the calibration standards (10 ppb where LLOQ = 10 ppb) will be reported as <10 ppb.

4.6. ACCEPTANCE CRITERIA

The following criteria must be met to ensure the presence of PFOA and ^{13}C-PFOA:

1. The chromatograms must show the following peaks:
   PFOA: a daughter ion at 369 amu from a parent of 413 amu
   ^{13}C-PFOA: a daughter ion at 370 amu from a parent of 415 amu

2. Any analyte present in method blanks must be at least 5-fold lower than the LLOQ. Any analyte present in the reagent blank must be at least 5-fold lower than the LLOQ.

3. Recoveries of lab control spikes and matrix spikes must be between 85-115% (80-120% for levels at the LLOQ) of their known values. Any method fortification (lab control spike) falling outside the acceptable limits warrants re-extraction of the entire analytical set. Any matrix spike outside the acceptable range should be evaluated by the analyst to determine if re-extraction is warranted.

4. Any calibration standard found to be a statistical outlier by using an appropriate outlier test, may be excluded from the curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected and at least one calibration standard at the LLOQ must be retained.

5. The correlation coefficient (r) for calibration curves generated must be ≥0.9925 (r^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

4.7. PERFORMANCE CRITERIA

The following two criteria must be performed as a system suitability test, before the commencement of analysis when using an instrumentation set-up that has not been used for this method.

First Criterion:
Run a standard solution on LC/MS/MS corresponding to the estimated LLOQ and obtain a signal-to-noise ratio of at least 9:1, compared to a reagent blank. If this criterion cannot be met, optimize and change instrument operating parameters.

Second Criterion:
Run a set of standards of five or more concentration levels, spanning a range starting at or below the LLOQ up to the highest concentration level to be included in the analysis. Generate a calibration curve for each analyte and obtain a linear regression with a coefficient of determination (r^2) of at least 0.985. Once this criterion is met, samples may be analyzed with standards interspersed.

4.8. Time Required for Analysis

A set of 35 samples (1 reagent control, 1 matrix control, 1 laboratory spike, 2 laboratory control spikes, and 30 samples) can be taken through the extraction procedure in approximately 8 hours by one person. The LC/MS/MS analysis (standards and 35 samples) will take approximately 9 hours.

5. CALCULATIONS

a. Use Equation 1 to calculate the amount of analyte found (in ng/mL, based on peak area) using the standard curve (linear regression parameters) generated by the Mass Lynx software program.

Equation 1:

$$\text{Analyte found (ppb)} = \frac{(\text{Analyte Peak area/IS peak area} - \text{Intercept}) \times \text{IS conc. (ng/mL)}}{\text{slope}}$$

b. For samples fortified with known amounts of analyte prior to extraction, use Equation 2 to calculate the percent recovery.

Equation 2:

$$\text{Recovery (\%)} = \frac{\text{Analyte found (ppb)} - \text{avg. Analyte found in control (ppb)}}{\text{Amount Analyte added (ppb)}} \times 100\%$$

6. SAFETY

The analyst should read the material safety data sheets for all standards and reagents before performing this method. Use universal precautions when handling standards and reagents, including working in fume hoods and wearing laboratory coats, safety glasses, and gloves. Use blood-borne pathogen handling precautions when handling serum.

Exygen Study No.: 008-342

Table I. Recovery of PFOA from Fortifications In Monkey Serum

Summary of PFOA Recoveries for 10 ppb Fortification in Monkey Serum

Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk A	Lot 40K7400	05/30/03	05/30/03	10	95
0300808 Spk B	Lot 40K7400	05/30/03	05/30/03	10	94
0300808 Spk C	Lot 40K7400	05/30/03	05/30/03	10	98
				AVERAGE:	96
				STANDARD DEVIATION:	2.1
				RELATIVE STANDARD DEVIATION:	2.2

Summary of PFOA Recoveries for 100 ppb Fortification in Monkey Serum

Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk D	Lot 40K7400	05/30/03	05/30/03	100	92
0300808 Spk E	Lot 40K7400	05/30/03	05/30/03	100	90
0300808 Spk F	Lot 40K7400	05/30/03	05/30/03	100	93
				AVERAGE:	92
				STANDARD DEVIATION:	1.5
				RELATIVE STANDARD DEVIATION:	1.7

Summary of PFOA Recoveries for 1000 ppb Fortification in Monkey Serum

Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk G	Lot 40K7400	05/30/03	05/30/03	1000	107
0300808 Spk H	Lot 40K7400	05/30/03	05/30/03	1000	95
0300808 Spk I	Lot 40K7400	05/30/03	05/30/03	1000	81
				AVERAGE:	94
				STANDARD DEVIATION:	13.0
				RELATIVE STANDARD DEVIATION:	13.8
				OVERALL AVERAGE:	94
				OVERALL STANDARD DEVIATION:	6.9
				OVERALL RELATIVE STANDARD DEVIATION:	7.3

Exygen Study No.: 008-342

Figure 1. Calibration curve for PFOA in Control Monkey Serum

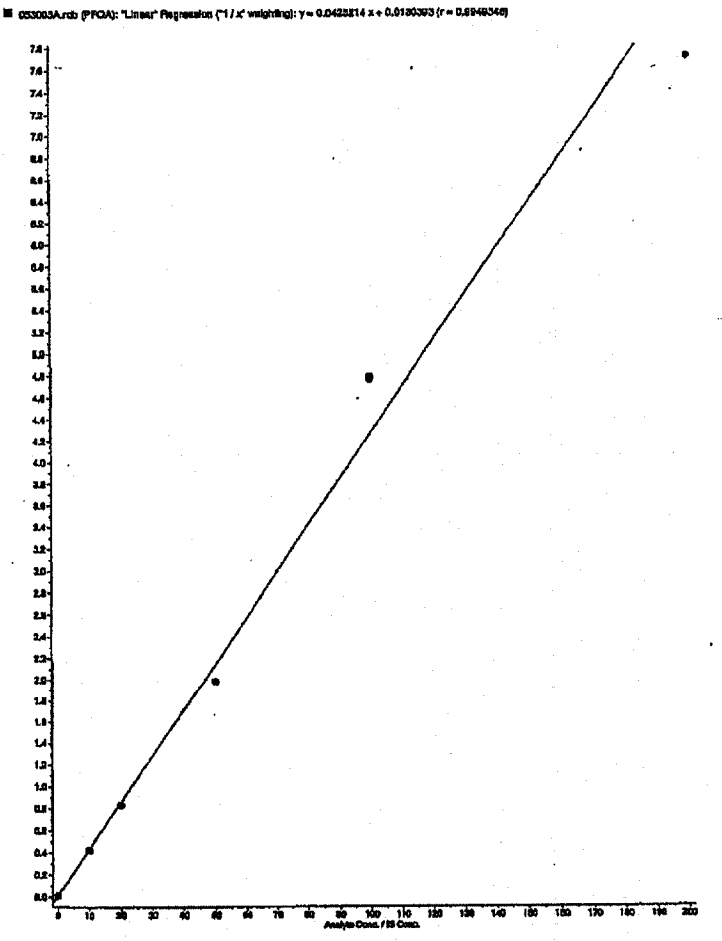


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Figure 2. Representative Chromatogram of a Control Monkey Serum Sample for PFOA with ~ 5 ng/mL of ^{13}C-PFOA

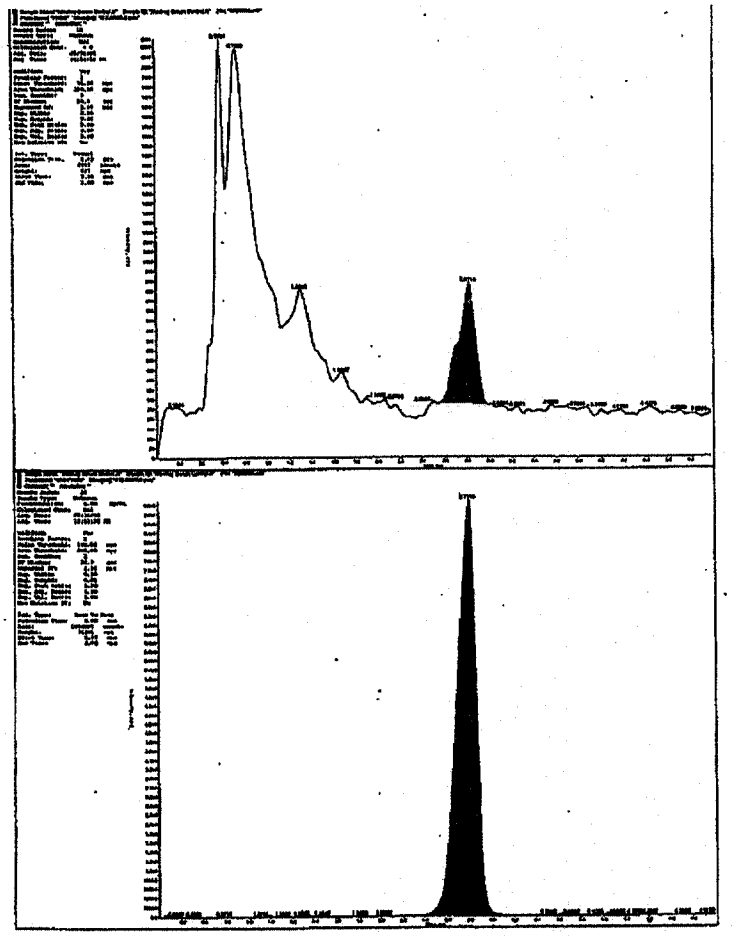

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Figure 3. Representative Chromatogram of a 10 ppb Standard for PFOA in Control Monkey Serum with ~ 5 ng/mL of ^{13}C-PFOA

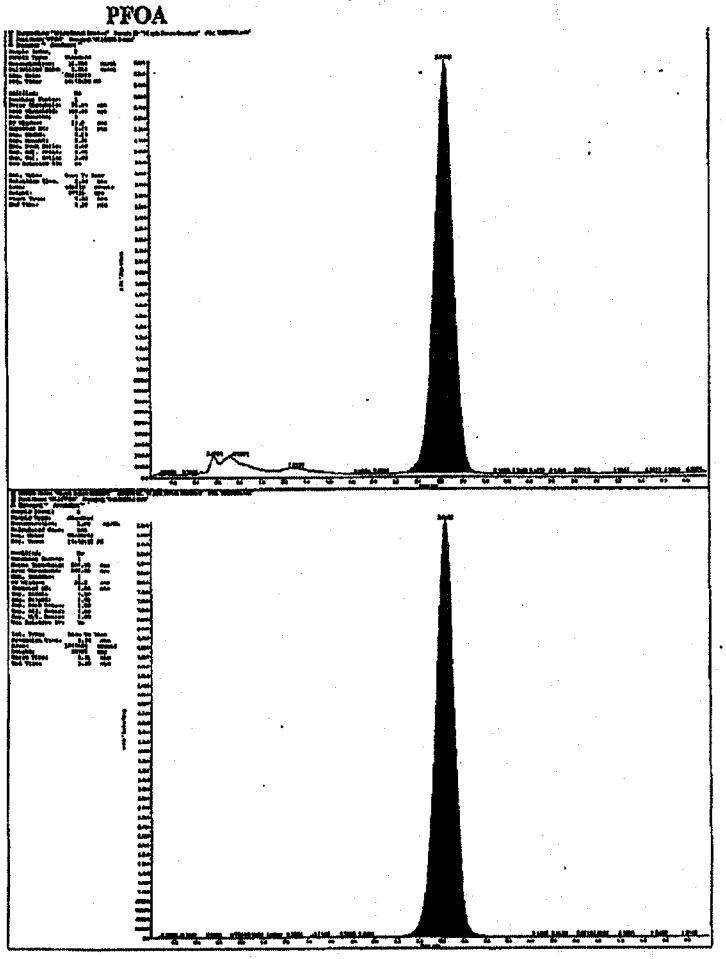

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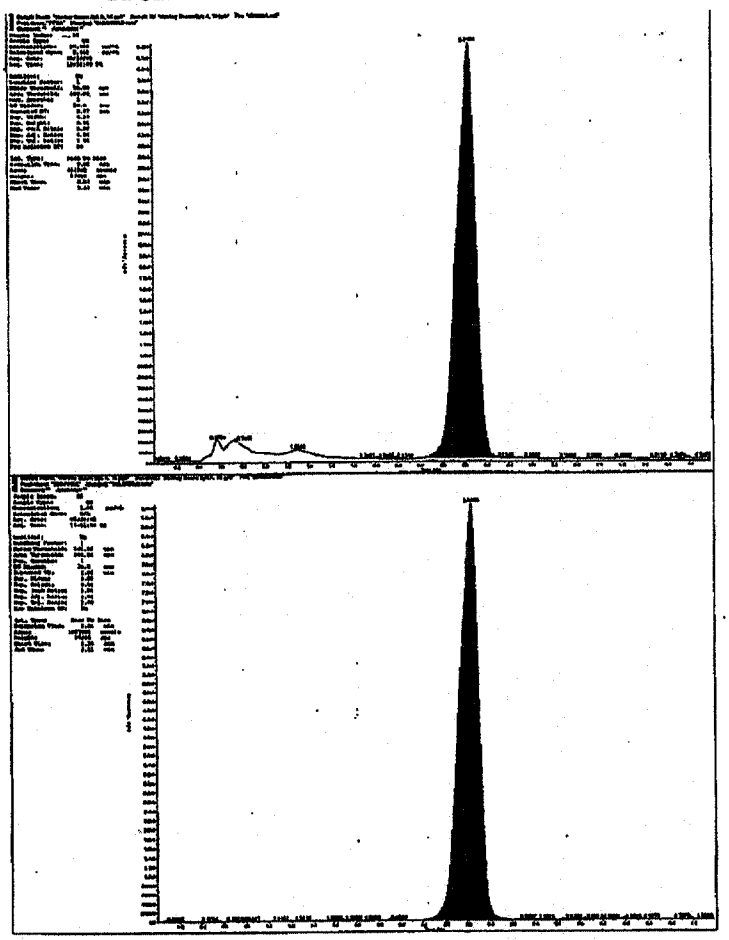
Figure 4. Representative Chromatogram of Control Monkey Serum Fortified at 10 ppb with PFOA and with ~ 5 ng/mL of ^{13}C-PFOA



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APPENDIX B

INDIVIDUAL ANIMAL BLOOD FLUORINE

Individual Animal Blood Fluorine

	Blood Fluorine µg Day -4	Blood Fluorine ppm Day -4	Blood Fluorine µg Day 3	Blood Fluorine ppm Day 3	Blood Fluorine µg Day 9	Blood Fluorine ppm Day 9	Blood Fluorine µg Day 22	Blood Fluorine ppm Day 22	Blood Fluorine µg Day 89	Blood Fluorine ppm Day 89
Male, Group I, 0 mg/kg/day										
665023	-[a]	-	-	-	-	-	<0.05	<0.50	-	-
665072	-	-	-	-	-	-	0.77	1.51	-	-
665151	-	-	-	-	-	-	0.59	1.47	-	-
665164	-	-	-	-	-	-	0.71	1.43	-	-
665154	-	-	-	-	-	-	0.31	0.97	-	-
Male, Group III, 10 mg/kg/day										
665132	-	-	-	-	-	-	0.72	1.43	-	-
665156	-	-	-	-	-	-	0.65	1.38	-	-
665061	-	-	-	-	-	-	0.44	0.77	-	-
665145	-	-	-	-	-	-	0.91	1.77	-	-
665019	-	-	-	-	-	-	0.58	1.22	-	-
Male, Group V, 100 mg/kg/day										
665048	-	-	-	-	-	-	0.94	1.85	-	-
665030	-	-	-	-	-	-	0.54	1.62	-	-
665028	-	-	-	-	-	-	1.31	2.38	-	-
665052	-	-	-	-	-	-	0.81	1.67	-	-
665150	-	-	-	-	-	-	0.87	1.77	-	-
Male, Group VII, 1000 mg/kg/day										
665084	<0.05	<0.5	0.89	1.87	1.59	3.87	1.68	5.26	5.00	10.96
665142	0.05	<0.5	0.84	1.56	1.47	3.56	2.05	4.36	3.43	9.96
665118	0.16	<0.5	0.82	1.48	1.53	3.20	1.78	5.15	4.37	9.59
665108	0.19	<0.5	0.75	1.60	2.03	3.56	2.26	4.26	4.62	9.50
665024	<0.05	<0.5	0.95	1.75	1.07	3.76	2.18	4.68	3.27	9.28

[a] A dash indicates that samples were not evaluated for the respective time point

Individual Animal Blood Fluorine

	Blood Fluorine µg Day -4	Blood Fluorine ppm Day -4	Blood Fluorine µg Day 3	Blood Fluorine ppm Day 3	Blood Fluorine µg Day 9	Blood Fluorine ppm Day 9	Blood Fluorine µg Day 22	Blood Fluorine ppm Day 22	Blood Fluorine µg Day 89	Blood Fluorine ppm Day 89
Female, Group II, 0 mg/kg/day										
665176	-[a]	-	-	-	-	-	0.99	2.07	-	-
665283	-	-	-	-	-	-	0.68	1.27	-	-
665297	-	-	-	-	-	-	0.42	1.16	-	-
665301	-	-	-	-	-	-	0.42	1.07	-	-
665269	-	-	-	-	-	-	0.57	1.34	-	-
Female, Group IV, 10 mg/kg/day										
665218	-	-	-	-	-	-	0.92	1.86	-	-
665309	-	-	-	-	-	-	0.72	1.65	-	-
665177	-	-	-	-	-	-	0.66	1.45	-	-
665202	-	-	-	-	-	-	0.66	1.30	-	-
665235	-	-	-	-	-	-	0.63	1.44	-	-
Female, Group VI, 100 mg/kg/day										
665313	-	-	-	-	-	-	0.68	1.50	-	-
665233	-	-	-	-	-	-	1.07	2.43	-	-
665216	-	-	-	-	-	-	0.85	2.00	-	-
665266	-	-	-	-	-	-	1.07	2.07	-	-
665270	-	-	-	-	-	-	0.69	1.54	-	-
Female, Group VIII, 1000 mg/kg/day										
665302	-	-	-	-	-	-	1.58	3.38	1.23	2.93
665179	-	-	-	-	-	-	1.07	3.34	1.34	3.03
665287	-	-	-	-	-	-	1.73	3.82	1.10	2.64
665172	-	-	-	-	-	-	1.60	3.19	1.83	4.13
665194	-	-	-	-	-	-	1.25	2.88	1.23	3.75

[a] A dash indicates that samples were not evaluated for the respective time point.