PERFLUOROOCTANESULFONATE, POTASSIUM SALT (PFOS): A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-145

ENVIRONMENTAL LABORATORY PROJECT NUMBER: U2723

U. S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1075
and
OECD Guideline 203

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STUDY INITIATION DATE: May 16, 2001

STUDY COMPLETION DATE: January 7, 2002

Submitted to

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-145

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This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

STUDY DIRECTOR:

Susan J. Palmer
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07 January 2002
Date

SPONSOR APPROVAL:

Sponsor

1/10/02
Date

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	June 8, 2001	June 8, 2001	June 13, 2001
Matrix Fortification, Analytical Sampling and Test Initiation	June 11, 2001	June 11, 2001	June 14, 2001
Analytical Data and Draft Report	July 11 & 12, 2001	July 12, 2001	July 13, 2001
Biological Data and Draft Report	July 12, 2001	July 12, 2001	July 13, 2001
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Marshall T. Hynson
Quality Assurance Program Supervisor

1/7/2002
Date

REPORT APPROVAL

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WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-145

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Ms. Rochelle Robideau
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	454A-145
TEST SUBSTANCE:	Perfluorooctanesulfonate, Potassium Salt (PFOS)
STUDY:	Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 3.0, 6.3, 13, 25 and 50 mg a.i./L
TEST DATES:	Experimental Start (OECD) – June 10, 2001 Experimental Start (EPA) – June 11, 2001 Biological Termination – June 15, 2001 Experimental Termination – June 18, 2001
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Rainbow Trout (*Oncorhynchus mykiss*)	
SOURCE OF TEST ORGANISMS:	Thomas Fish Company Anderson, California 96007	
AGE OF TEST ORGANISMS:	Juveniles	
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:		
WET WEIGHT:	Mean = 0.34 g	Range = 0.25 to 0.47 g
STANDARD LENGTH:	Mean = 3.1 cm	Range = 2.8 to 3.4 cm
TOTAL LENGTH:	Mean = 3.6 cm	Range = 3.4 to 4.0 cm

96-HOUR LC50:	22 mg a.i./L
95% CONFIDENCE INTERVAL:	18 to 27 mg a.i./L
NO MORTALITY CONCENTRATION:	6.3 mg a.i./L
NO-OBSERVED-EFFECT-CONCENTRATION:	6.3 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for 3M Corporation at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from June 11, 2001 to June 15, 2001. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454A-145 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the acute effects of Perfluorooctanesulfonate, Potassium Salt (PFOS) to the rainbow trout, *Oncorhynchus mykiss*, during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Rainbow trout were exposed to a geometric series of five test concentrations and a negative (dilution water) control. Two replicate test chambers were maintained in each treatment and control group, with 10 rainbow trout in each test chamber for a total of 20 rainbow trout per test concentration. Two abiotic replicate test chambers also were maintained at the highest concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 3.1, 6.3, 13, 25 and 50 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and the control group at the beginning of the test, at approximately 48 hours, and at test termination.

Rainbow trout were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality and other clinical signs of toxicity were made at approximately 4, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was used to calculate or estimate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and clinical observation data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Perfluoro-octanesulfonate, Potassium Salt (PFOS): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)". The protocol was based on procedures outlined in the U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine* (1); OECD Guideline for Testing of Chemicals, 203: *Fish, Acute Toxicity Test* (2); and ASTM Standard E729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International, Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 86.9% and an expiration date of August 31, 2001. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 3.1, 6.3, 13, 25 and 50 mg a.i./L, based on a test substance purity of 86.9%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A 40-L primary stock solution was prepared in dilution water at a concentration of 150 mg a.i./L. The primary stock solution was mixed with an electric mixer and sonicated for approximately 23 hours to aid in the solubilization of the test substance. Two replicates of each test solution were prepared at concentrations of 3.1, 6.3, 13, 25 and 50 mg a.i./L by adding the appropriate volume of primary stock to dilution water in the test aquaria to achieve a final volume of 15 L. Each solution was stirred with a stainless steel whisk for approximately one minute. All test solutions appeared clear and colorless.

Test Organism

The rainbow trout, *Oncorhynchus mykiss*, was selected as the test species for this study. The rainbow trout is representative of an important group of aquatic vertebrates and was selected for use in the test based upon past history of use in the laboratory. Rainbow trout used in the test were obtained from Thomas Fish Company, Anderson, California.

The fish were held for approximately five weeks prior to the test in water from the same source and at approximately the same temperature as used during the test. During the 14-day holding period preceding the test, water temperatures ranged from 11.9 to 12.8°C. The pH of the water ranged from 8.2 to 8.4 and dissolved oxygen ranged from 9.2 to 10.3 mg/L (≥85% of saturation). Instrumentation and procedures used for water measurements are described in the *Environmental Conditions* section of this report. The fish were acclimated to test conditions for approximately 51 hours prior to test initiation. During the acclimation period, no mortalities occurred and the fish showed no signs of disease or stress. At test initiation, the rainbow trout were collected from the acclimation tank and indiscriminately distributed two at a time to the test chambers until each contained 10 fish.

During the holding period, the fish were fed a commercially-prepared diet (Zeigler Brothers, Inc., Gardners, PA). The fish were not fed during the acclimation period (at least 48 hours prior to the test) or during the test.

All fish used in the test were from the same source and year class, and the length of the longest fish was no more than twice the length of the shortest. The average standard length of 10 negative control fish measured at the end of the test was 3.1 cm with a range of 2.8 to 3.4 cm, with an average total length of 3.6 cm with a range of 3.4 to 4.0 cm. The average wet weight (blotted dry) of 10 negative control fish at the end of the test was 0.34 g with a range of 0.25 to 0.47 g. Loading was 0.23 g fish/L of test water.

Test Apparatus

Test chambers were 25-L polyethylene aquaria containing 15 L of test solution. The depth of water in a representative test chamber was approximately 17.5 cm. Test chambers were indiscriminately positioned in an environmental chamber set to maintain the desired temperature throughout the test. The test chambers were labeled with the project number, test concentration and replicate.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 1.

The well water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water are presented in Appendix 2.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 126 lux at the surface of the water of one representative test chamber.

Temperature was measured in each test chamber at the beginning of the test and at approximately 24-hour intervals thereafter using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder, which was verified prior to test initiation with a liquid-in-glass thermometer. The target test temperature during the study was 12 ± 1°C. Dissolved oxygen and pH measurements were made on water samples collected from all replicate test chambers of each treatment and control at test initiation and at approximately 24-hour intervals thereafter. Hardness, alkalinity and specific conductance were measured in the dilution water at test initiation.

Light intensity was measured using a SPER Scientific Ltd. Model 840006C light meter. Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4).

Observations

All organisms were observed periodically to determine the number of mortalities in each control and treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The mortality data were analyzed, when possible, using the computer program of C. E. Stephan (5). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (6, 7, 8). In this study, there was less than 50% mortality in any treatment group prior to the 96-hour observation period. Therefore, the 24, 48 and 72-hour LC50 values were estimated to be greater than the highest concentration tested. The 96-hour LC50 value was calculated using probit analysis. The no mortality concentration and NOEC were determined by visual interpretation of the mortality and observation data.

Analytical Chemistry

Water samples were collected at mid-depth from each biotic test chamber of each treatment and control group at the beginning of the test, at approximately 48 hours and at test termination to measure concentrations of the test substance. Additional samples were collected on Day 1 of the test from the negative control replicates to confirm the results of the Day 0 analysis. Samples also were collected for analysis at approximately 48 and 96 hours from the abiotic test chambers prepared at a concentration of 50 mg a.i./L. All samples were collected in plastic vials and analyzed as soon as possible without storage. Analytical procedures used in the analysis of the samples are provided in Appendix 3.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix 3). Nominal concentrations selected for use in this study were 3.1, 6.3, 13, 25 and 50 mg a.i./L. Samples collected at test initiation had measured concentrations that ranged from 93 to 103% of nominal concentrations. Measured concentrations for biotic samples taken at 48 hours ranged from 94 to 103% of nominal, while the abiotic samples ranged from 105 to 106% of nominal. Measured concentrations for biotic samples taken at 96 hours ranged from 91 to 105% of

nominal, while the abiotic samples were 102% of nominal. When measured concentrations of the biotic samples analyzed at test initiation, approximately 48 hours and at test termination were averaged, the mean measured concentrations for this study were 3.0, 6.3, 13, 25 and 50 mg a.i./L, representing 97, 100, 100, 100 and 100% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the 12 ± 2°C range established for the test. Dissolved oxygen concentrations remained ≥9.2 mg/L (85% of saturation) throughout the test. Measurements of pH ranged from 8.1 to 8.5 during the test. Measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 2).

Daily observations of mortality and other signs of toxicity observed during the test are presented in Table 3. Rainbow trout in the negative control group appeared normal and healthy throughout the test period. Rainbow trout in the 3.0 and 6.3 mg a.i./L treatment groups also appeared normal and healthy during the test, with no mortalities or overt signs of toxicity observed. After 96-hours of exposure, mortality in the 13, 25 and 50 mg a.i./L treatment groups was 20, 50 and 100%, respectively. LC50 values and 95% confidence intervals, estimated or calculated from the mortality data at 24, 48, 72 and 96 hours, are shown in Table 4. A graph of the concentration-response curve is presented in Figure 1.

CONCLUSIONS

The 96-hour LC50 value for rainbow trout (*Oncorhynchus mykiss*) exposed to Perfluoro-octanesulfonate, Potassium Salt (PFOS) was 22 mg a.i./L with a 95% confidence interval of 18 to 27 mg a.i./L. The 96-hour no mortality concentration and the NOEC were 6.3 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine.*

2 **Organisation for Economic Cooperation and Development.** 1993. OECD Guidelines for Testing of Chemicals. *Guideline 203: Fish, Acute Toxicity Test.* Adopted by the Council on 12 July 1992.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition. American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

5 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

6 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50", *Aquatic Toxicology and Hazard Evaluations*, American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

Table 1

Summary of Analytical Chemistry Data

Nominal Test Concentration[1] (mg a.i./L)	Sampling Time (Hours)	PFOS Measured Concentration[2] (mg a.i./L)	Percent of Nominal[2]	Mean Measured Test Concentration (mg a.i./L)	Mean Measured Percent of Nominal
0.0 (Negative Control)	0	< LOQ[1,3]	–	< LOQ	–
	0	< LOQ[3]	–		
	48	< LOQ	–		
	48	< LOQ	–		
	96	< LOQ	–		
	96	< LOQ	–		
3.1	0	3.15	102	3.0	97
	0	3.02	97.5		
	48	2.90	93.6		
	48	3.01	97.1		
	96	2.83	91.4		
	96	2.97	95.9		
6.3	0	6.22	98.7	6.3	100
	0	6.21	98.6		
	48	6.16	97.8		
	48	6.43	102		
	96	6.15	97.6		
	96	6.60	105		
13	0	13.2	102	13	100
	0	12.1	93.2		
	48	12.7	97.4		
	48	12.3	94.9		
	96	13.1	101		
	96	12.6	96.7		
25	0	25.0	99.9	25	100
	0	25.7	103		
	48	24.3	97.2		
	48	25.7	103		
	96	25.7	103		
	96	26.2	105		
50	0	49.7	99.4	50	100
	0	49.8	99.5		
	48	51.1	102		
	48	51.5	103		
	96	49.6	99.1		
	96	50.8	102		
50 (Abiotic)	48	53.1	106	52	104
	48	52.6	105		
	96	50.9	102		
	96	51.0	102		

[1] The limit of quantitation (LOQ) was 0.200 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (400).

[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since nominal and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

[3] Results are mean of duplicate re-preparations. Original Negative Controls were contaminated during analytical sampling.

Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Concentration (mg a.i./L)	Rep.	0 Hour[1]			24 Hours			48 Hours			72 Hours			96 Hours		
		Temp.[2] (°C)	DO[3] (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH
Negative Control	A	12.5	10.4	8.3	12.1	9.6	8.3	12.1	9.4	8.2	12.1	9.4	8.2	12.1	9.4	8.1
	B	12.6	10.7	8.4	12.1	9.6	8.3	12.2	9.4	8.2	12.3	9.4	8.2	12.2	9.4	8.1
3.0	A	12.5	10.6	8.4	12.2	9.4	8.3	12.3	9.4	8.2	12.5	9.4	8.2	12.5	9.4	8.1
	B	12.4	10.8	8.4	12.0	9.8	8.3	12.0	9.4	8.2	12.2	9.4	8.2	12.2	9.4	8.1
6.3	A	12.1	10.6	8.4	11.8	9.6	8.4	11.7	9.4	8.2	11.8	9.4	8.2	11.8	9.4	8.1
	B	12.1	10.8	8.4	11.7	9.8	8.4	11.7	9.3	8.2	11.7	9.4	8.2	11.7	9.4	8.1
13	A	11.7	10.8	8.4	11.4	9.8	8.4	11.3	9.3	8.2	11.4	9.2	8.3	11.3	9.2	8.2
	B	11.9	10.8	8.4	11.6	9.8	8.4	11.6	9.2	8.2	11.6	9.3	8.3	11.6	9.3	8.2
25	A	12.3	10.8	8.4	11.7	9.8	8.4	11.6	9.3	8.2	11.7	9.2	8.3	11.7	9.2	8.2
	B	12.5	10.8	8.4	11.8	9.8	8.5	11.7	9.4	8.2	11.7	9.3	8.3	11.7	9.2	8.2
50	A	12.9	10.8	8.4	11.8	9.8	8.4	11.8	9.4	8.3	11.9	9.4	8.3	11.9	9.4	8.2
	B	12.9	10.8	8.4	11.9	9.6	8.4	11.9	9.2	8.3	11.9	9.2	8.3	11.9	9.2	8.2
	C[4]	13.8	10.0	8.3	12.0	10.0	8.4	12.0	9.8	8.4	12.0	9.8	8.4	12.0	9.8	8.4
	D[4]	13.7	10.2	8.3	12.0	10.0	8.5	12.0	9.8	8.4	12.0	9.8	8.4	12.0	9.8	8.4

[1] The 0-hour dilution water measurements for hardness, alkalinity and specific conductance were 132 mg/L as $CaCO_3$, 184 mg/L as $CaCO_3$ and 270 µmhos/cm, respectively.
[2] Temperature measured continuously during the test ranged from approximately 12.0 to 12.5°C.
[3] A dissolved oxygen concentration of 6.5 mg/L represents 60% saturation at 12°C in freshwater.
[4] Abiotic replicate.

Table 3

Cumulative Percent Mortality and Treatment-Related Effects

Mean Measured Concentration (mg a.i./L)	Rep.	No. Exposed	4 Hours		24 Hours		48 Hours		72 Hours		96 Hours		Cumulative Percent Mortality
			No. Dead [1]	Observed Effects [2]	No. Dead	Observed Effects	No. Dead	Observed Effects	No. Dead	Observed Effects	No. Dead	Observed Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
3.0	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
6.3	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
13	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	1	9 AN	20
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	3	7 AN	
25	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	6	4 AN	50
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	4	6 AN	
50	A	10	0	10 AN	0	10 AN	1	9 AN	3	7 AN	10	--	100
	B	10	0	10 AN	0	10 AN	0	10 AN	4	6 AN	10	--	

[1] Cumulative number of dead fish.
[2] Observed Effects: AN = appear normal.

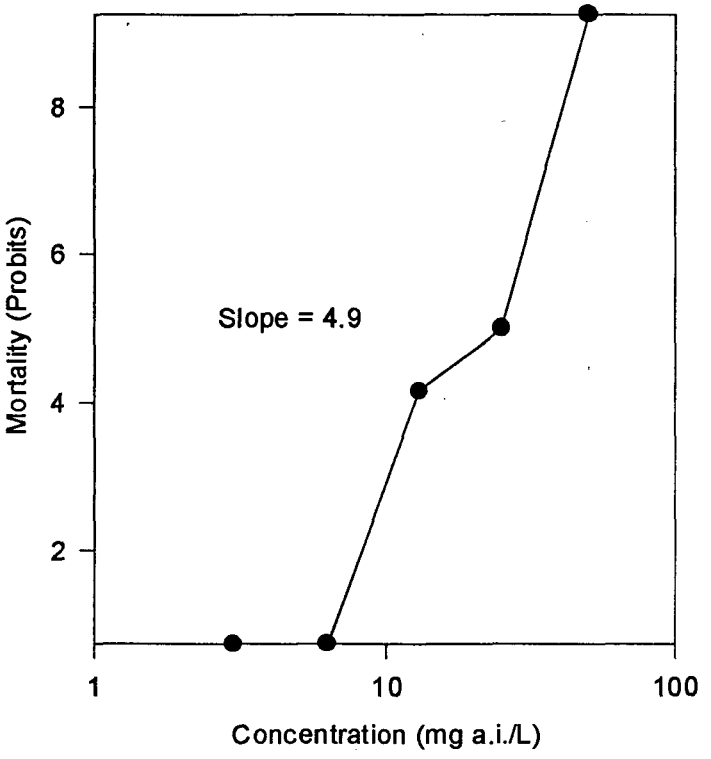
Table 4

LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	>50	--[1]	NA[1]
48 Hours	>50	--[1]	NA[1]
72 Hours	>50	--[1]	NA[1]
96 Hours	22	18 – 27	Probit Analysis

[1] The LC50 value and 95% confidence interval could not be statistically calculated. The LC50 value was estimated to be greater than the highest concentration tested.

Figure 1. Concentration-Response Curve (96-Hour Data)



Appendix 1

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

	Mean	Range
Specific Conductance (µmhos/cm)	311 (N = 4)	310 – 315
Hardness (mg/L as $CaC0_3$)	130 (N = 4)	128 – 132
Alkalinity (mg/L as $CaC0_3$)	177 (N = 4)	176 – 178
pH	8.3 (N = 4)	8.2 – 8.4

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Pesticides and Organics			
Component	Measured Concentration	Component	Measured Concentration
Aclonifen	<0.03 µg/L	Dichlorvos	<0.01 µg/L
Alachlor	<0.01 µg/L	Dicofol	<0.25 µg/L
Ametryn	<0.01 µg/L	Diethyltoluamide	<0.02 µg/L
Atrazine	<0.01 µg/L	Difenoconazole	<0.03 µg/L
Azinphos-ethyl	<0.04 µg/L	Dimethoate	<0.02 µg/L
Azinphos-methyl	<0.08 µg/L	Dimethomorph	<0.05 µg/L
Azoxystrobin	<0.25 µg/L	Disulfoton	<0.02 µg/L
Bifenthrin	<0.05 µg/L	DMST	<0.05 µg/L
Bioallethrin	<0.05 µg/L	Dodemorph	<0.01 µg/L
Bitertanol	<0.05 µg/L	Endosulfan-α	<0.01 µg/L
Bromacil	<0.05 µg/L	Endosulfan-β	<0.01 µg/L
Bromophos	<0.02 µg/L	Endosulfan-sulfate	<0.02 µg/L
Bromophos-ethyl	<0.02 µg/L	Epoxiconazole	<0.05 µg/L
Bromopropylate	<0.02 µg/L	Eptam	<0.02 µg/L
Bupirimate	<0.05 µg/L	Esfenvalerate	<0.02 µg/L
Carbaryl	<0.05 µg/L	Ethion	<0.05 µg/L
Carbofuran	<0.03 µg/L	Ethofumesate	<0.02 µg/L
Carboxin	<0.02 µg/L	Ethoprophos	<0.01 µg/L
Chlorfenvinphos	<0.02 µg/L	Etridiazole	<0.02 µg/L
Chloridazon	<0.05 µg/L	Etrimfos	<0.05 µg/L
Chlorpropham	<0.02 µg/L	Fenarimol	<0.05 µg/L
Chlorpyrifos	<0.01 µg/L	Fenchlorphos	<0.01 µg/L
Chlorpyrifos-methyl	<0.01 µg/L	Fenitrothion	<0.03 µg/L
Chlorothalonil	<0.04 µg/L	Fenoxycarb	<0.03 µg/L
Coumaphos	<0.02 µg/L	Fenpiclonil	<0.05 µg/L
Cyanazine	<0.05 µg/L	Fenpropathrin	<0.25 µg/L
Cyfluthrin	<0.05 µg/L	Fenpropimorph	<0.01 µg/L
Cypermethrin	<0.25 µg/L	Fenthion	<0.01 µg/L
Cyproconazole	<0.05 µg/L	Fenvalerate	<0.02 µg/L
Deltamethrin	<0.02 µg/L	Fluazifop-butyl	<0.02 µg/L
Demeton	<0.02 µg/L	Fluoroglycofen-ethyl	<0.02 µg/L
Demeton-O	<0.02 µg/L	Fluroxypyr-meptyl	<0.05 µg/L
Desethylatrazine	<0.01 µg/L	Flutolanil	<0.02 µg/L
Desisopropylatrazine	<0.02 µg/L	Fonophos	<0.01 µg/L
Desmetryn	<0.01 µg/L	Furalaxyl	<0.02 µg/L
Diazinon	<0.01 µg/L	Heptenophos	<0.02 µg/L
Dichlobenil	<0.01 µg/L	Imazalil	<0.01 µg/L
Dichloran	<0.03 µg/L	Iprodion	<0.05 µg/L
Dichlorbenzamide	<0.02 µg/L	Kresoxim-methyl	<0.02 µg/L
Dichlorfenthion	<0.01 µg/L	Lenacil	<0.05 µg/L
Dichlorfluanid	<0.03 µg/L	Lindane	<0.02 µg/L

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on November 15, 2000.

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Pesticides And Organics (Page 2)			
Component	Measured Concentration	Component	Measured Concentration
Malathion	<0.02 µg/L	Methoxychlor	<0.01 µg/L
Metalaxyl	<0.05 µg/L	Metolachlor	<0.01 µg/L
Metamitron	<0.05 µg/L	Metribuzin	<0.02 µg/L
Metazachlor	<0.02 µg/L	Mevinphos	<0.01 µg/L
Methidathion	<0.02 µg/L	Nitrothal-Isopropyl	<0.05 µg/L
Paclobutazole	<0.05 µg/L	Pyrifenox-1	<0.01 µg/L
Parathion	<0.01 µg/L	Pyrifenox-2	<0.01 µg/L
Parathion-methyl	<0.01 µg/L	Pyrimethanil	<0.01 µg/L
Penconazole	<0.05 µg/L	Quizalofop-ethyl	<0.02 µg/L
Pendimethalin	<0.03 µg/L	Simazine	<0.01 µg/L
Permethrin-cis	<0.01 µg/L	Sulfotep	<0.02 µg/L
Permethrin-trans	<0.01 µg/L	Tebuconazole	<0.05 µg/L
Phosalone	<0.05 µg/L	Tebufenpyrad	<0.05 µg/L
Phosmet	<0.02 µg/L	Terbutryn	<0.01 µg/L
Phosphamidon-cis	<0.05 µg/L	Terbuthylazine	<0.01 µg/L
Pirimicarb	<0.01 µg/L	Tetrachlorvinphos	<0.01 µg/L
Pirimiphos-ethyl	<0.01 µg/L	Tetrahydroftalimide	<0.05 µg/L
Pirimiphos-methyl	<0.01 µg/L	Tetramethrin	<0.01 µg/L
Prochloraz	<0.02 µg/L	Thiabendazole	<0.05 µg/L
Procymidon	<0.01 µg/L	Thiometon	<0.04 µg/L
Prometryn	<0.01 µg/L	Tolclofos-methyl	<0.01 µg/L
Propachlor	<0.01 µg/L	Tolylfluanid	<0.04 µg/L
Propazine	<0.01 µg/L	Triadimefon	<0.05 µg/L
Propham	<0.02 µg/L	Triadimenol	<0.05 µg/L
Propiconazole	<0.05 µg/L	Triallate	<0.02 µg/L
Propoxur	<0.03 µg/L	Triazophos	<0.02 µg/L
Propyzamide	<0.02 µg/L	Trifluralin	<0.02 µg/L
Prosulfocarb	<0.02 µg/L	Vamidothion	<0.01 µg/L
Pyrazophos	<0.03 µg/L	Vinclozolin	<0.01 µg/L
Metals			
Magnesium	13.2 mg/L	Nickel	<1.4 µg/L
Sodium	21 mg/L	Copper	<1.0 µg/L
Calcium	35 mg/L	Zinc	<2.3 µg/L
Iron	<0.02 mg/L	Molybdenum	<0.7 µg/L
Potassium	6.2 mg/L	Silver	<0.3 µg/L
Aluminum	<0.09 mg/L	Cadmium	<0.3 µg/L
Manganese	0.72 µg/L	Arsenic	<0.25 µg/L
Beryllium	<0.3 µg/L	Mercury	<0.025 µg/L
Chromium	<0.6 µg/L	Selenium	<1 µg/L
Cobalt	<0.4 µg/L		

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on November 15, 2000.

Appendix 3

THE ANALYSIS OF PFOS IN FRESHWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-145

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-145

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

01/07/02
DATE

MANAGEMENT:

Willard B. Nixon, Ph.D.
Director, Analytical Chemistry

1/7/02
DATE

Introduction

Freshwater samples were collected from a 96-hour toxicity test designed to determine the effects of PFOS (Perfluorooctanesulfonate, Potassium Salt) to the rainbow trout (*Oncorhynchus mykiss*). This study was conducted by Wildlife International, Ltd. and identified as Project No.: 454A-145. The analyses of these water samples were performed at Wildlife International, Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on June 11, 13, and 15, 2001 and were analyzed between June 11 and June 18, 2001.

Test Substance and Internal Standard

The test substance used for this study was Wildlife International, Ltd. identification number 4675. The test substance, referred to hereafter as PFOS, was used to prepare calibration and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International, Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard, referred to hereafter as 4H PFOS, was stored under ambient conditions.

Analytical Method

The method used for the analysis of the freshwater samples was developed at Wildlife International, Ltd. and entitled "Analytical Method for the Determination of PFOS in Freshwater, Saltwater, and Algal Medium". This methodology was included as Appendix II of Wildlife International, Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation. Several modifications from the validated methodology were implemented for the present study. First, the concentration of the internal standard was changed from 100 µg/L to 10.0 µg/L to better match the calibration and test sample instrumental concentrations. Second, a guard cartridge was used in conjunction with a shorter (100 to 50 mm) analytical column and warmer oven temperature (30 to 40°C) for long-term protection of the analytical column and faster run times.

Samples were diluted in a 50% methanol: 50% NANOpure® water solution containing 10.0 µg 4H PFOS /L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (50 mm x 2 mm I.D., 3-µm particle size) fitted with a Keystone Javelin C_{18} guard cartridge (20 x 2 mm). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 10.0 µg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.500 to 5.00 µg a.i./L, were analyzed with the samples. The same and most prominent peak response for PFOS was utilized to monitor PFOS in all calibration, quality control, and study samples. No attempt was made to quantify PFOS on the basis of individual isomeric components. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentration ratios (PFOS : internal standard) of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.200 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (400).

Matrix Blank and Fortification Samples

Three matrix blank samples were analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). A representative ion chromatogram of a matrix blank is presented in Figure 5.

Freshwater was fortified at 1.00, 10.0 and 100 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected for the mean procedural recovery of 101%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-145-18, nominal concentration of 6.3 mg a.i./L in freshwater.

Peak Area Ratio = Analyte Peak Area/Internal Standard Peak Area

Concentration Ratio = Concentration of Analyte/Concentration of Internal Standard

Internal Standard Concentration: 0.0100 mg/L

First Initial Volume: 0.500 mL

First Final Volume: 10.0 mL

Second Initial Volume: 0.100

Second Final Volume: 10.0

Dilution Factor: 2000

PFOS Peak Area: 349928

Internal Standard Peak Area: 319027

Peak Area Ratio: 1.0969

Calibration curve equation.

Slope: 3.3385

Intercept: 0.0235

Curve is weighted (1/x)

$$\text{PFOS (mg a.i./L) at instrument} = \frac{\text{Peak area ratio - (Y-intercept)}}{\text{Slope}} \times \text{Internal Standard Concentration}$$

$$= \frac{1.0969 - 0.0235}{3.3385} \times 0.0100$$

$$= 0.003215$$

PFOS (mg a.i./L) in sample = PFOS (mg a.i./L) at instrument × Dilution Factor

$$= 0.003215 \times 2000$$

$$= 6.43$$

$$\text{Percent of Nominal Concentration} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

$$= \frac{6.43}{6.3} \times 100$$

$$= 102\%$$

Quantitation software for recoveries: MacQuan, version 1.6.

RESULTS

Sample Analysis

Freshwater samples were collected from the 96-hour toxicity test with the rainbow trout (*Oncorhynchus mykiss*) at test initiation, June 11, 2001 (Day 0), on June 13, 2001 (Day 2) and at test termination, June 15, 2001 (Day 4). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 93.2 to 103% of the nominal concentrations. Samples collected at Day 2 had a measured concentration range of 93.6 to 103% of nominal values. Samples collected at test termination (Day 4) had a measured concentration range of 91.4 to 105% of nominal values (Table 4). Samples from the abiotic 50 mg a.i./L treatment group were comparable to samples from the 50 mg a.i./L treatment group with the fish present (Table 4). A representative ion chromatogram of a test sample is shown in Figure 7.

Table 1

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C_{18} column (50 mm x 2 mm I.D., 3-µm particle size)
GUARD COLUMN:	Keystone Javelin C_{18} column (20 x 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	70.0% Methanol : 30.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	10.0 µL
PFOS RETENTION TIME:	Approximately 4.4 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 3.0 minutes
PFOS MONITORED MASS:	499 amu
INTERNAL STANDARD MONITORED MASS:	427 amu

Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of PFOS[1] (mg a.i./L)
Number (454A-145-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3[2]	Matrix Blank	< LOQ

[1] The limit of quantitation (LOQ) was 0.200 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (400).

[2] Result is mean of duplicate re-preparations. Original matrix blank was contaminated during analytical sample preparation.

Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-145-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered[1]
	Fortified[1]	Measured[1]	
MAS-1	1.00	1.15	115
MAS-4	1.00	0.972	97.2
MAS-7	1.00	1.04	104
MAS-2	10.0	9.61	96.1
MAS-5	10.0	9.69	96.9
MAS-8	10.0	10.4	104
MAS-3	100	91.9	91.9
MAS-6	100	102	102
MAS-9	100	101	101
		Mean =	101
		Standard Deviation =	6.64
		CV =	6.58%
		N =	9

[1] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

Table 4

Measured Concentrations of PFOS in Freshwater Samples
from a Rainbow Trout (*Oncorhynchus mykiss*) 96-Hour Toxicity Test

Nominal Test Concentration[1] (mg a.i./L)	Sample Number (454A-145-)	Sampling Time (Day)	PFOS Measured Concentration[2] (mg a.i./L)	Percent of Nominal[2]
0.0	1A	0	< LOQ[1,3]	--
(Negative Control)	2A	0	< LOQ[3]	--
	13	2	< LOQ	--
	14	2	< LOQ	--
	27	4	< LOQ	--
	28	4	< LOQ	--
3.1	3	0	3.15	102
	4	0	3.02	97.5
	15	2	2.90	93.6
	16	2	3.01	97.1
	29	4	2.83	91.4
	30	4	2.97	95.9
6.3	5	0	6.22	98.7
	6	0	6.21	98.6
	17	2	6.16	97.8
	18	2	6.43	102
	31	4	6.15	97.6
	32	4	6.60	105
13	7	0	13.2	102
	8	0	12.1	93.2
	19	2	12.7	97.4
	20	2	12.3	94.9
	33	4	13.1	101
	34	4	12.6	96.7

[1] The limit of quantitation (LOQ) was 0.200 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (400).

[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since nominal and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

[3] Results are mean of duplicate re-preparations. Original Negative Controls were contaminated during analytical sampling.

Table 4 (Continued)

Measured Concentrations of PFOS in Freshwater Samples
from a Rainbow Trout (*Oncorhynchus mykiss*)96-Hour Toxicity Test

Nominal Test Concentration[1] (mg a.i./L)	Sample Number (454A-145-)	Sampling Time (Day)	PFOS Measured Concentration[2] (mg a.i./L)	Percent of Nominal[2]
25	9	0	25.0	99.9
	10	0	25.7	103
	21	2	24.3	97.2
	22	2	25.7	103
	35	4	25.7	103
	36	4	26.2	105
50	11	0	49.7	99.4
	12	0	49.8	99.5
	23	2	51.1	102
	24	2	51.5	103
	37	4	49.6	99.1
	38	4	50.8	102
50 (Abiotic)	25	2	53.1	106
	26	2	52.6	105
	39	4	50.9	102
	40	4	51.0	102

[1] The limit of quantitation (LOQ) was 0.200 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (400).

[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since nominal and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

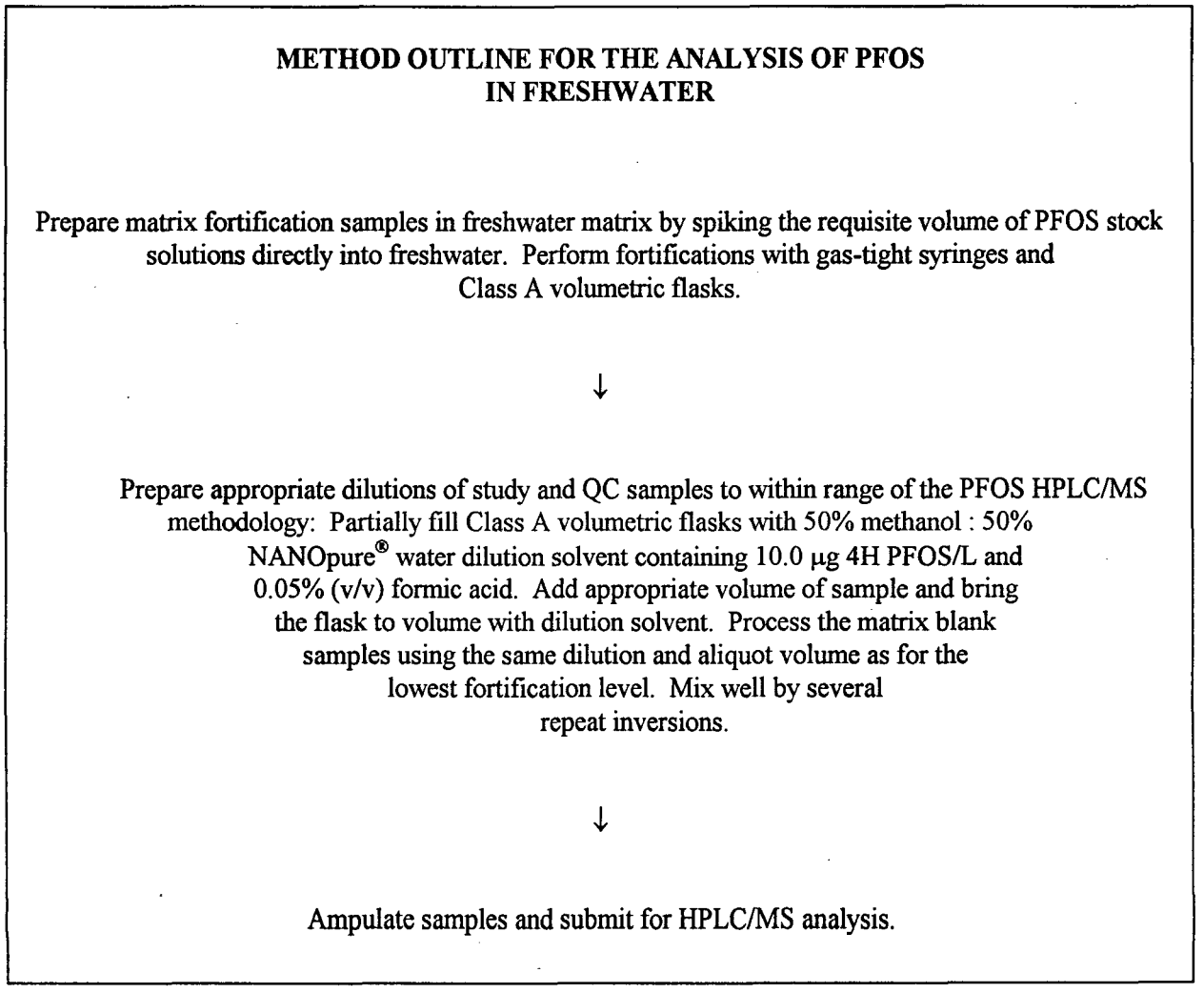


Figure 1. Analytical method flowchart for the analysis of PFOS in freshwater.

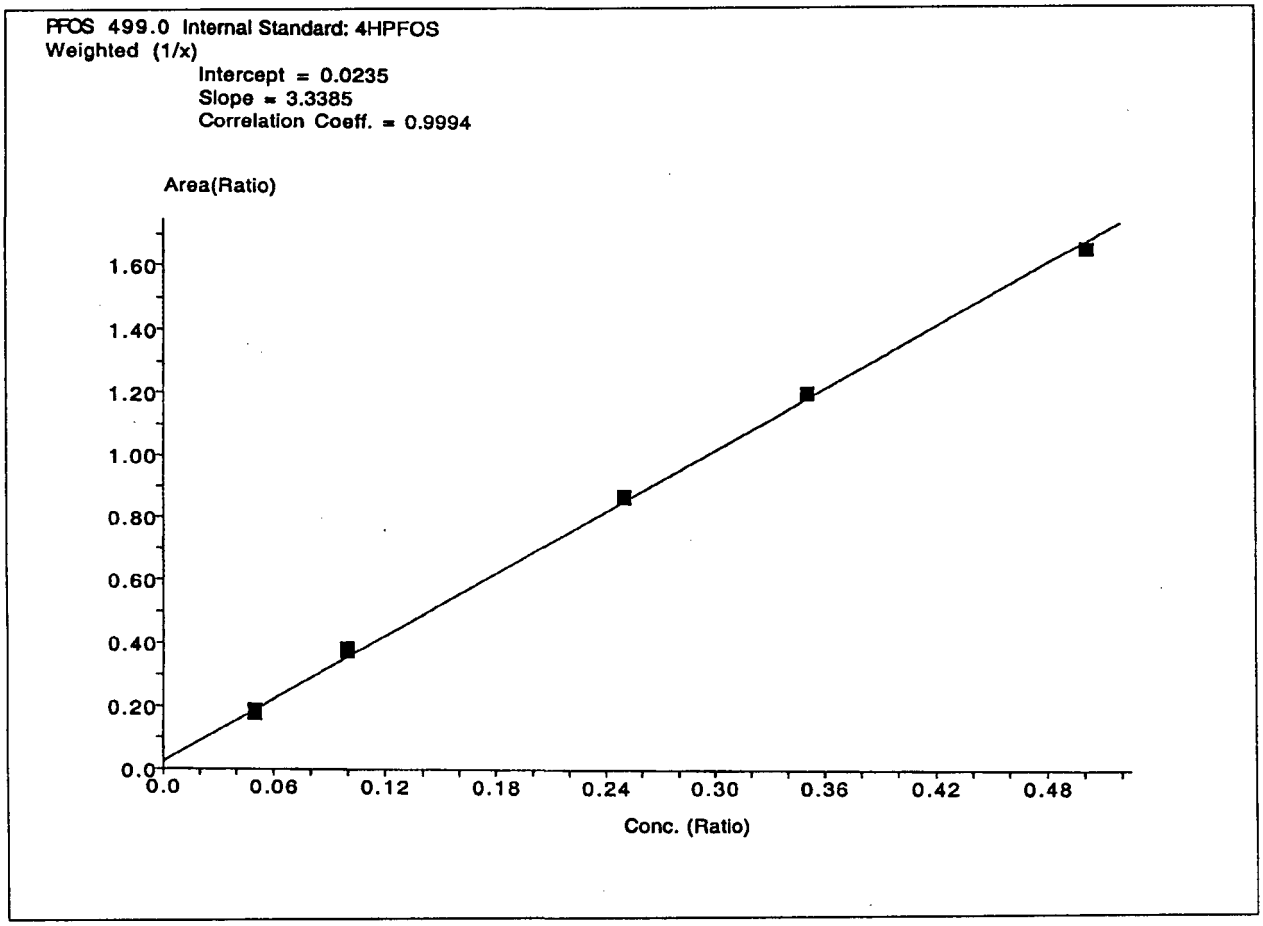


Figure 2. A typical calibration curve for PFOS.

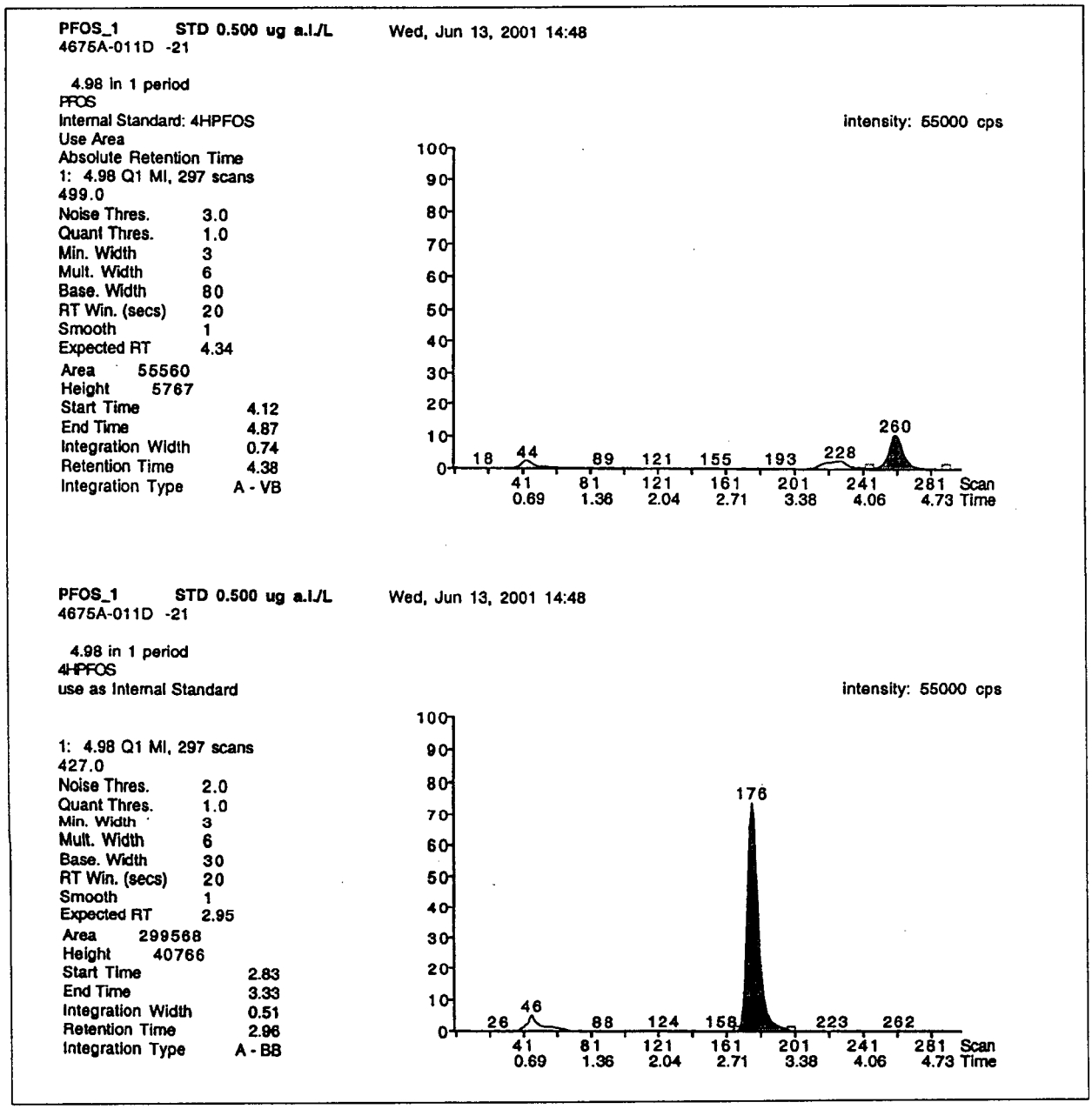


Figure 3. A representative ion chromatogram of a low-level (0.500 µg a.i./L) PFOS standard. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

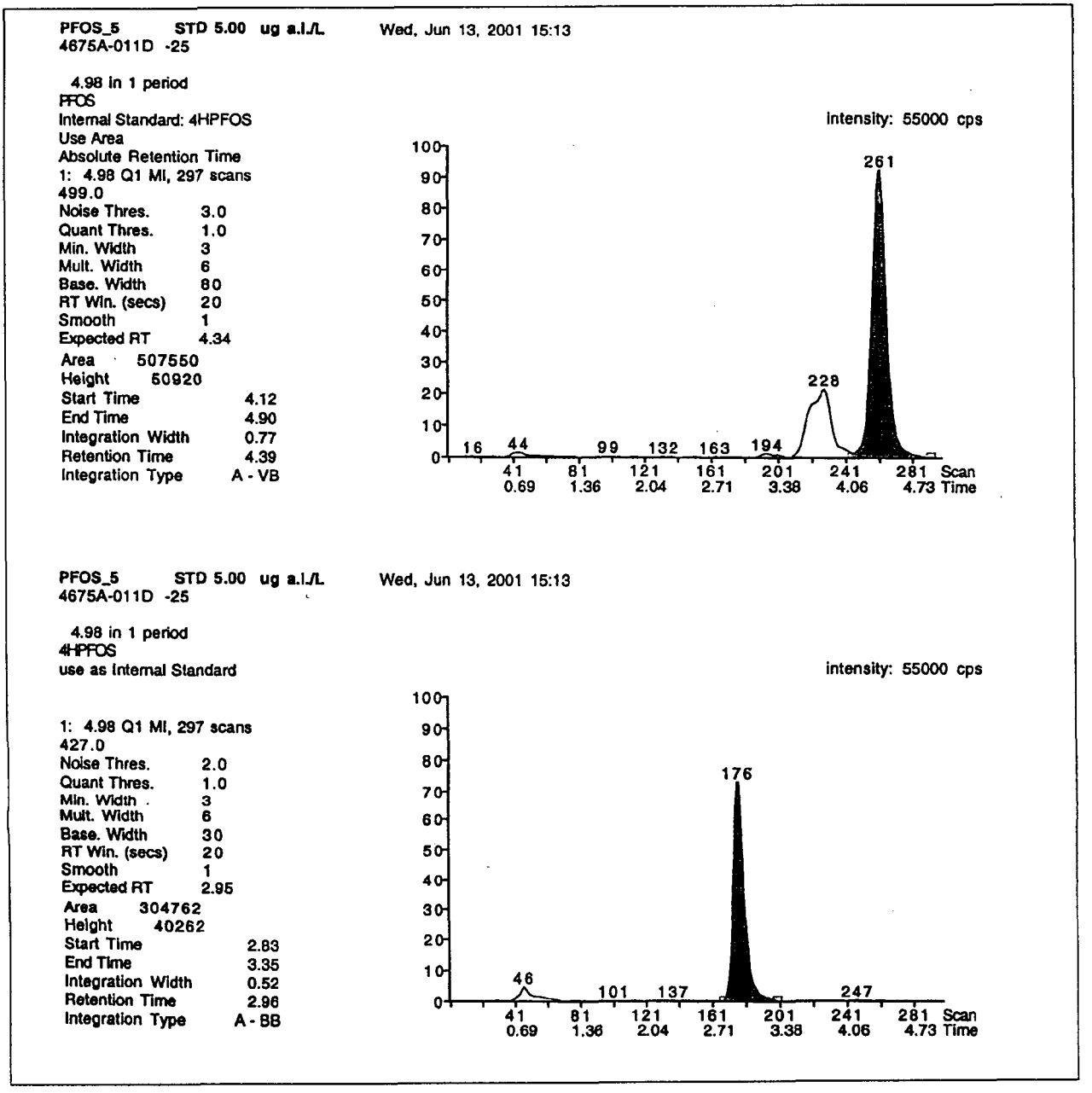


Figure 4. A representative ion chromatogram of a high-level (5.00 µg a.i./L) PFOS standard. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

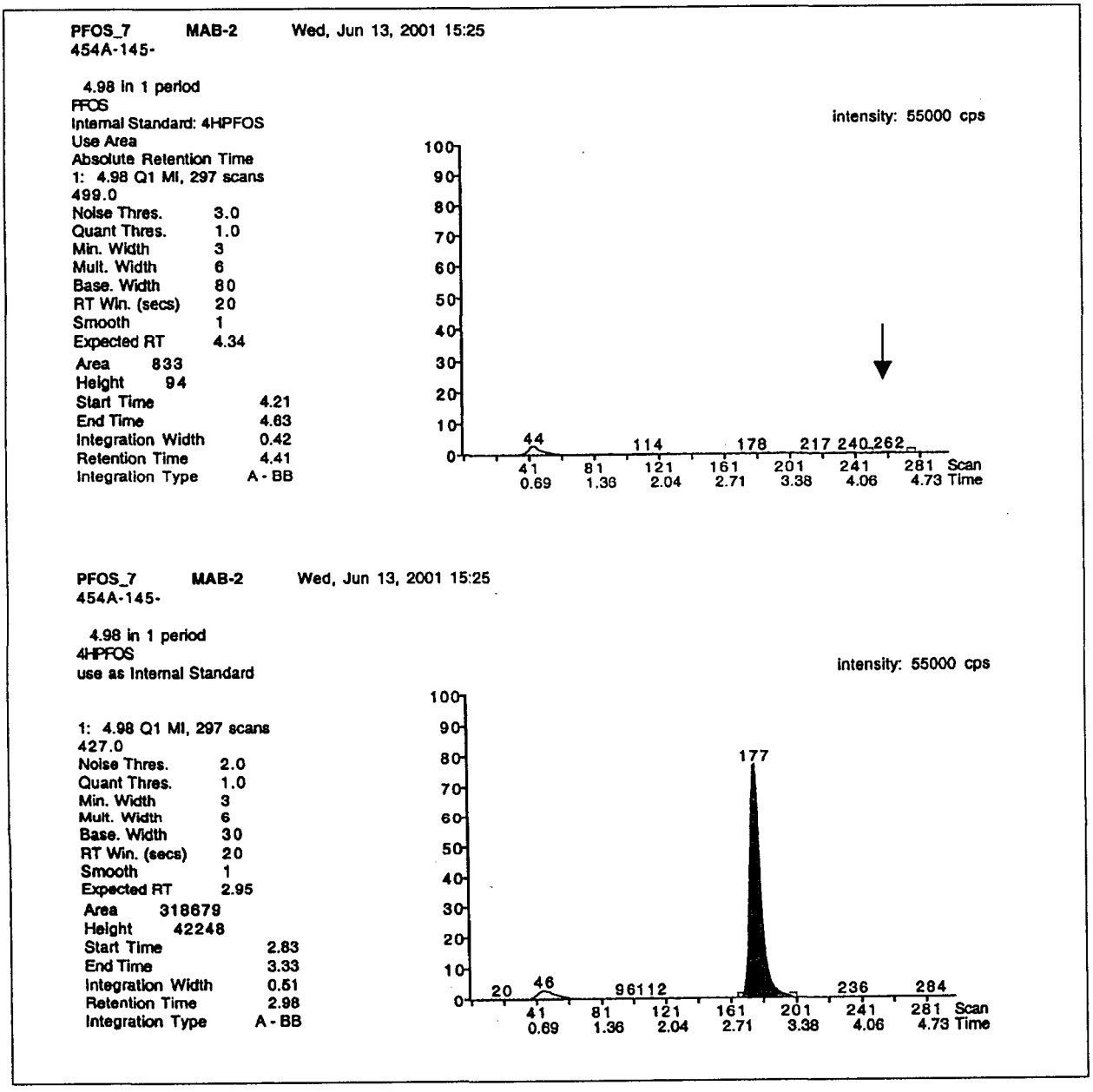


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-145-MAB-2). Dilution factor = 400x. The arrow indicates the retention time of PFOS. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

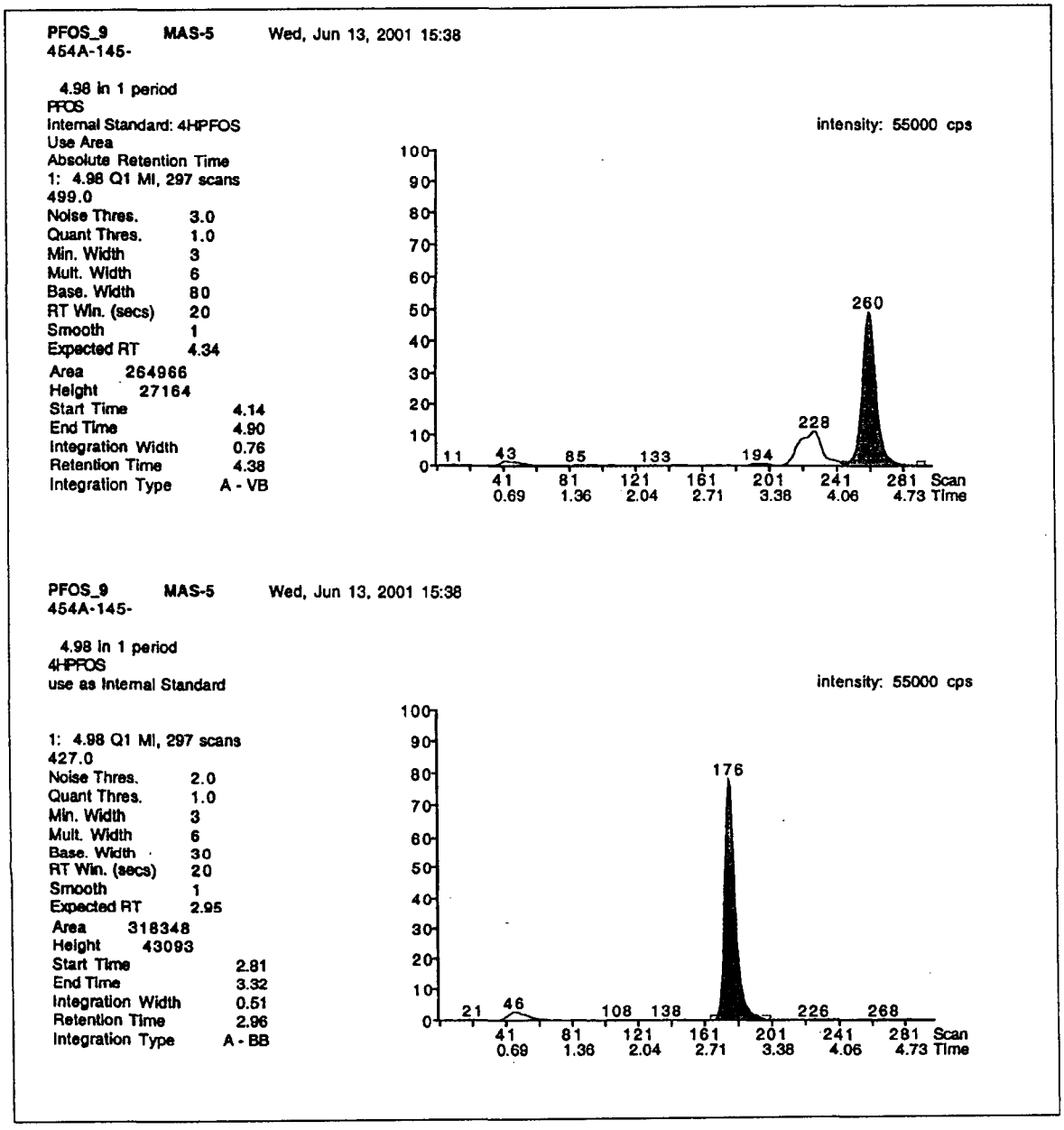


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-145-MAS-5). Nominal Concentration = 10.0 mg a.i./L, Dilution factor = 4000x. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

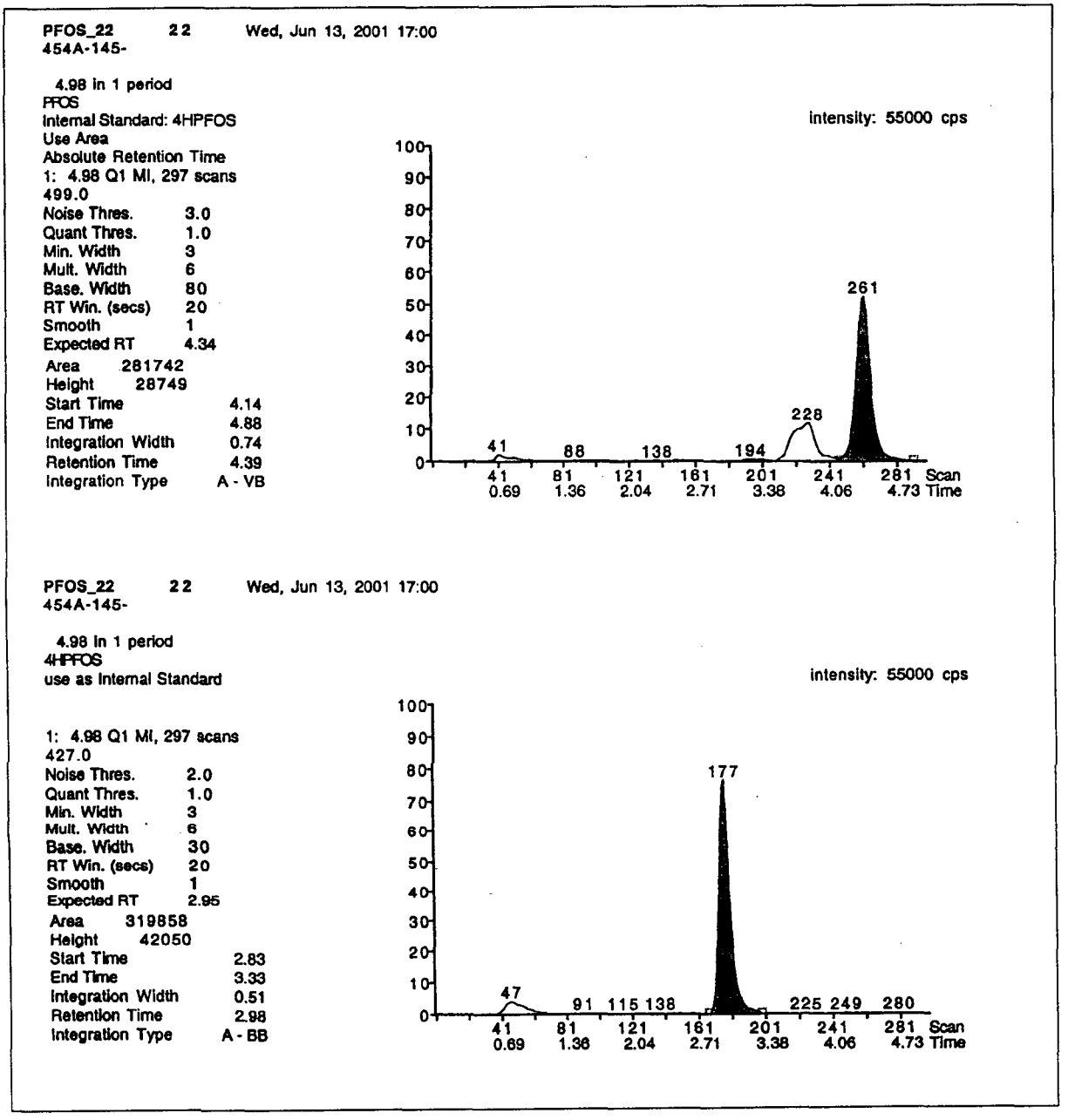


Figure 7. A representative ion chromatogram of a test sample (454A-145-22). Nominal Concentration = 25 mg a.i./L, Dilution factor = 10000x. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

Appendix 4

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to add the proposed experimental start and termination dates, test concentrations, and test and reference substance identification.

2. The test chambers used in the study were polyethylene, but were not Teflon®-lined. The Teflon®-liners were included in the protocol in error, so this change to the protocol had no impact upon the study.

Appendix 5

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Director, Analytical Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Raymond L. Van Hoven, Ph.D., Scientist
5. Susan J. Palmer, Senior Biologist
6. Molly McCoy, Biologist
7. Frank J. Lezotte, Chemist