17) OECD 203-OPPTS 850.1075, 96-Hour static acute toxicity test with the (fathead minnow), 454A-115

PERFLUORO BUTANE SULFONATE, POTASSIUM SALT (PFBS):
A 96-HOUR STATIC ACUTE TOXICITY TEST
WITH THE FATHEAD MINNOW (*Pimephales promelas*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-115

3M LAB REQUEST NO. E00-1429

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

AUTHORS:

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Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: April 4, 2000

STUDY COMPLETION DATE: March 20, 2001

Submitted to

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935 Bush Avenue
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Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: Perfluoro Butane Sulfonate, Potassium Salt (PFBS): A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-115

STUDY COMPLETION: March 20, 2001

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 with the following exceptions:

The test substance was not characterized in compliance with Good Laboratory Practices prior to its use in the study. However, subsequent GLP compliant characterization resulted in a purity similar to the original characterization purity.

The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Kurt R. Drottar
Senior Biologist

3/20/01
DATE

SPONSOR APPROVAL:

3/23/01
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	December 18, 2000	December 18, 2000	December 20, 2000
Matrix Fortification	December 18, 2000	December 18, 2000	December 20, 2000
Observations	December 21, 2000	December 21, 2000	January 4, 2001
Analytical Data and Draft Report	January 16 and 17, 2001	January 17, 2001	January 18, 2001
Biological Data and Draft Report	January 18 and 19, 2001	January 19, 2001	January 23, 2001
Final Report	March 16, 2001	March 16, 2001	March 16, 2001

James H. Coleman

James H. Coleman
Quality Assurance Representative

3-20-01

DATE

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: Perfluoro Butane Sulfonate, Potassium Salt (PFBS): A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-115

STUDY DIRECTOR:

Kurt R. Drottar
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3/20/01
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Non-Target Plants

3/20/01
DATE

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SUMMARY

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-115
TEST SUBSTANCE:	Perfluoro Butane Sulfonate, Potassium Salt (PFBS)
STUDY:	Perfluoro Butane Sulfonate, Potassium Salt (PFBS): A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 220, 437, 888, 1655 and 3341 mg a.i./L
TEST DATES:	Experimental Start (OECD) – December 11, 2000 Experimental Start (EPA) – December 18, 2000 Biological Termination – December 22, 2000 Experimental Termination – December 22, 2000
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Fathead Minnow (*Pimephales promelas*)	
SOURCE OF TEST ORGANISMS:	Wildlife International, Ltd. cultures Easton, Maryland 21601	
AGE OF TEST ORGANISMS:	Juveniles	
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:		
WEIGHT (g):	Mean = 0.32;	Range = 0.22 to 0.47
TOTAL LENGTH (mm):	Mean = 35;	Range = 30 to 40

96-HOUR LC50:	1938 mg a.i./L
95% CONFIDENCE LIMITS:	888 and 3341 mg a.i./L
NO MORTALITY CONCENTRATION:	888 mg a.i./L
NO-OBSERVED-EFFECT-CONCENTRATION:	888 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 December 18, 2000 to December 22, 2000. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-115 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of Perfluoro Butane Sulfonate, Potassium Salt (PFBS) to the fathead minnow, *Pimephales promelas*, during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Fathead minnows 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 fathead minnows in each test chamber for a total of 20 fathead minnows per test 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 204, 408, 816, 1632 and 3263 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.

Fathead minnows were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality and other clinical signs of toxicity were made at approximately 2, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was used to calculate 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 Butane Sulfonate, Potassium Salt (PFBS): A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1075 (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 June 28, 2000 and was assigned Wildlife International Ltd. identification number 5292. The test substance was described as a white powder. It was identified as Potassium Perfluoro butane sulfonate, AKA Developmental Product, AKA PFBS, from lot 2. Information provided by the Sponsor indicated a purity of 97.9%. A subsequent revision of the certificate of analysis indicated a purity of 97.3% and an Expiration/Reassessment Date of January 17, 2002. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 204, 408, 816, 1632 and 3263 mg a.i./L. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A 60-L primary stock solution was prepared in dilution water at a concentration of 3263 mg a.i./L. The primary stock solution was mixed with an electric mixer to aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with dilution water to prepare the four additional test concentrations. The appropriate amount of primary stock was mixed with dilution water in the test chambers. All test solutions appeared clear and colorless. Test concentrations were corrected for the original purity of the active ingredient in the test substance (97.9%).

Test Organism

The fathead minnow, *Pimephales promelas*, was selected as the test species for this study. The fathead minnow 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. Fathead minnows used in the test were obtained from cultures maintained at Wildlife International, Ltd.

Fathead minnows were held at approximately the same temperature as used during the test. The fish were held for approximately 8 months prior to testing. The fish were acclimated to test conditions for approximately 52 hours prior to test initiation. During the holding and acclimation periods, the fish showed no signs of disease or stress. During the 14-day holding period preceding the test, water temperatures ranged from 19.3 to 22.6 °C. The pH of the water ranged from 8.2 to 8.5 and dissolved oxygen ranged from 7.5 to 8.2 mg/L. Instrumentation and methods used for water measurements are described in the *Environmental Conditions* section of this report. At test initiation, the fathead minnows were collected from the acclimation tank and transferred to the test chambers.

During the holding period, fathead minnows 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 total length of the longest fish was no more than twice the length of the shortest. The average total length of 10 negative control fish measured at the end of the test was 35 mm with a range of 30 to 40 mm. The average wet weight (blotted dry) of 10 negative control fish at the end of the test was 0.32 grams with a range of 0.22 to 0.47 grams. Loading was 0.21 g fish/L of test water present in the test chambers at any given time.

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 16.5 cm. Test chambers were impartially positioned in an environmental chamber set to maintain a temperature of 22±2°C. 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 and 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 well water used by Wildlife International, Ltd. 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 222 lux at the surface of the water. Light intensity was measured using a SPER Scientific Ltd. light meter.

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. The target test temperature during the study was 22±2°C. Dissolved oxygen and pH measurements were made on water samples 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.

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

Observations were made to determine the number of mortalities. The number of individuals exhibiting clinical signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 2, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The 24, 48, 72 and 96-hour LC50 values and the 95% confidence intervals were calculated when possible by probit analysis, the moving average method or binomial probability with non-linear interpolation (5, 6, 7) using the computer software of C.E. Stephan (8). In this study, the binomial method was used to calculate all LC50 values. The no mortality concentration and NOEC were determined by visual interpretation of the mortality and clinical observation data.

Analytical Chemistry

Water samples were collected at mid-depth from each replicate test chamber of each treatment and control group at the beginning of the test, at 48 hours and at test termination to measure concentrations of the test substance. The 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 PFBS 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 204, 408, 816, 1632 and 3263 mg a.i./L. Samples collected at test initiation had measured values that ranged from 100 to 105% of nominal values. Measured values for samples taken at 48 hours ranged from 99 to 109% of nominal. Measured values for samples taken at 96 hours ranged from 99 to 123% of nominal. When measured concentrations of the samples analyzed at test initiation, approximately 48 hours and at test termination were averaged, the mean measured concentrations for this study were 220, 437, 888, 1655 and 3341 mg a.i./L. Mean measured concentrations were used in the calculation of LC50 values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the 22±2°C range established for the test. Dissolved oxygen concentrations remained ≥7.5 mg/L (86% of saturation) throughout the test. Measurements of pH ranged from 8.3 to 8.5 during the test.

Daily observations of mortality and other clinical signs of toxicity observed during the test are shown in Table 3. Fathead minnows in the negative control, 220 and 888 mg a.i./L treatment groups appeared normal and

healthy during the test. One fish in the 437 mg a.i./L treatment group was observed to be lying on the bottom after 2 hours of exposure. However, throughout the remainder of the test all fish in the 437 mg a.i./L treatment group appeared normal. After 96-hours of exposure, mortality in the 1655 and 3341 mg a.i./L treatment groups was 30 and 100%, respectively. LC50 values and 95% confidence limits at 24, 48, 72 and 96 hours were calculated from the mortality data, and are shown in Table 4. A graph of the concentration-response curve is presented in Figure 1.

CONCLUSIONS

The 96-hour LC50 value for fathead minnows (*Pimephales promelas*) exposed to Perfluoro Butane Sulfonate, Potassium Salt (PFBS) was 1938 mg a.i./L with 95% confidence limits of 888 and 3341 mg a.i./L. The 96-hour no-mortality concentration and the NOEC were 888 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.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th 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

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	A	0	<LOQ[1]	<LOQ	--
	B	0	<LOQ		
	A	48	<LOQ		
	B	48	<LOQ		
	A	96	<LOQ		
	B	96	<LOQ		
204	A	0	205	220	108
	B	0	209		
	A	48	211		
	B	48	213		
	A	96	250		
	B	96	232		
408	A	0	426	437	107
	B	0	407		
	A	48	432		
	B	48	415		
	A	96	498		
	B	96	446		
816	A	0	824	888	109
	B	0	851		
	A	48	888		
	B	48	876		
	A	96	962		
	B	96	926		
1632	A	0	1692	1655	101
	B	0	1674		
	A	48	1665		
	B	48	1617		
	A	96	1673		
	B	96	1611		
3263	A	0	3354	3341	102
	B	0	3417		
	A	48	3327		
	B	48	3264		
	A	96	--[2]		
	B	96	--[2]		

[1]The limit of quantitation (LOQ) was 50.0 mg a.i./L.
[2]Samples not collected due to 100% mortality.

Table 2

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

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Fathead Minnow, *Pimephales promelas*
Dilution Water: Well Water

Mean Measured Test Concentration (mg a.i./L)	Replicate	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	21.5	8.4	8.4	21.1	8.1	8.4	20.7	8.3	8.3	20.8	7.8	8.3	21.1	8.0	8.4
	B	22.1	8.4	8.3	22.6	8.0	8.4	22.5	8.1	8.4	22.5	7.7	8.3	22.7	7.8	8.4
220	A	22.0	8.3	8.4	22.3	8.0	8.4	22.1	8.2	8.4	22.2	7.8	8.4	22.4	8.0	8.5
	B	22.0	8.3	8.4	22.4	7.9	8.4	22.3	8.2	8.4	22.3	7.8	8.4	22.6	7.7	8.5
437	A	22.2	8.3	8.4	22.4	7.7	8.4	22.1	7.8	8.4	22.2	7.5	8.4	22.8	7.5	8.4
	B	22.1	8.3	8.4	22.2	7.8	8.4	22.3	7.9	8.4	22.1	7.6	8.4	22.4	7.5	8.5
888	A	22.0	8.3	8.4	21.1	8.1	8.5	20.7	8.0	8.4	20.8	7.9	8.4	21.6	7.8	8.5
	B	21.8	8.4	8.4	21.8	7.8	8.4	21.6	7.9	8.4	21.8	7.6	8.4	22.0	7.6	8.5
1655	A	22.0	8.4	8.4	22.1	7.8	8.4	21.8	8.1	8.4	22.1	8.0	8.5	22.3	7.9	8.5
	B	22.1	8.4	8.4	22.5	7.6	8.4	22.4	7.6	8.4	22.4	7.5	8.4	22.5	7.5	8.5
3341	A	22.1	8.4	8.4	22.6	7.9	8.4	22.4	–[4]	–	–	–	–	–	–	–
	B	22.0	8.5	8.4	22.5	8.0	8.4	22.4	–[4]	–	–	–	–	–	–	–

[1] The 0-hour dilution water measurements for hardness, alkalinity and specific conductance were 128 mg/L as $CaCO_3$, 182 mg/L as $CaCO_3$ and 340 μmhos/cm, respectively.
[2] Temperature measured continuously during the test ranged from approximately 21.0 to 22.0°C.
[3] A dissolved oxygen concentration of 5.2 mg/L represents 60% saturation at 22°C in freshwater.
[4] Measurements discontinued due to 100% mortality.

Table 3

Cumulative Percent Mortality and Treatment-Related Effects

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Fathead Minnow, *Pimephales promelas*
Dilution Water: Well Water

Mean Measured Test Concentration (mg a.i./L)	Replicate	No. Exposed	2 Hours		24 Hours		48 Hours		72 Hours		Cumulative Percent Mortality	96 Hours		Cumulative Percent Mortality
			No. Dead[1]	Effects[2]	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects		No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
220	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
437	A	10	0	9AN, 1R	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
888	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
1655	A	10	0	10 AN	3	7 AN	5	5 AN	5	5 AN	30	5	5 AN	30
	B	10	0	10 AN	0	10 AN	0	10 AN	1	9 AN		1	9 AN	
3341	A	10	0	10 AN	10	--	10	--	10	--	100	10	--	100
	B	10	0	10 AN	10	--	10	--	10	--		10	--	

[1] Cumulative number of dead fish.

[2] Observed Effects: AN = Appears Normal, R = Lying on Bottom

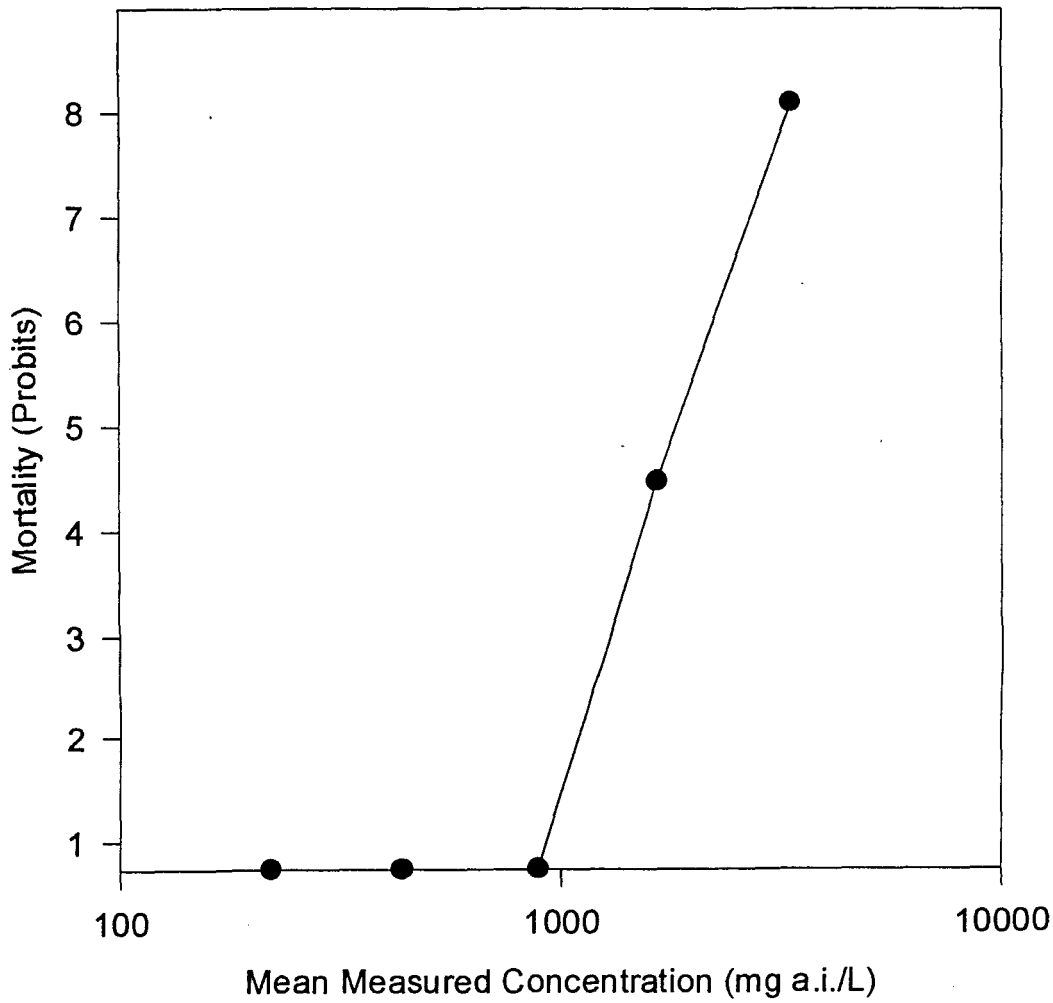
Table 4

LC50 Values

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

Time	LC50 (mg a.i./L)	Lower 95% Confidence Limits (mg a.i./L)	Upper 95% Confidence Limits (mg a.i./L)	Statistical Method
24 Hours	2118	1655	3341	Binomial
48 Hours	1999	1655	3341	Binomial
72 Hours	1938	888	3341	Binomial
96 Hours	1938	888	3341	Binomial

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

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

	Mean	Range
Specific Conductance (μmhos/cm)	313 (N = 4)	310 – 315
Hardness (mg/L as $CaCO_3$)	131 (N = 4)	128 – 132
Alkalinity (mg/L as $CaCO_3$)	178 (N = 4)	176 – 178
pH	8.0 (N = 4)	8.0 – 8.1

Appendix 2

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

Component	Measured Concentration	Component	Measured Concentration
Pesticides and Organics			
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-sulfte	<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 October 14 and 15, 1999.

Continued

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]
Page 2

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	11.0 mg/L	Nickel	<1.1 µg/L
Sodium	18.0 mg/L	Copper	<0.7 µg/L
Calcium	29 mg/L	Zinc	<0.25 µg/L
Iron	<0.015 mg/L	Molybdenum	<0.3 µg/L
Potassium	1.1 mg/L	Silver	<0.2 µg/L
Aluminum	<0.02 mg/L	Cadmium	<0.1 µg/L
Manganese	<0.1 µg/L	Arsenic	<0.5 µg/L
Beryllium	<0.2 µg/L	Mercury	<0.025 µg/L
Chromium	<0.5 µg/L	Selenium	<0.5 µg/L
Cobalt	<0.2 µg/L		

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on October 14 and 15, 1999.

Appendix 3

THE ANALYSIS OF PFBS IN FRESHWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-115

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PERFLUORO BUTANE SULFONATE, POTASSIUM SALT (PFBS): A 96-HOUR STATIC ACUTE TOXICITY TEST with the FATHEAD MINNOW (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-115

3M ENVIRONMENTAL LAB PROJECT NUMBER: E00-1429

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

03-30-01
DATE

MANAGEMENT:

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

3/20/01
DATE

Introduction

Freshwater samples were collected from a static acute aquatic toxicity study designed to determine the effects of PFBS (Perfluoro Butane Sulfonate, Potassium Salt) to the fathead minnow (*Pimephales promelas*). This study was conducted by Wildlife International, Ltd. and identified as Project No.: 454A- 115. 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 December 18, 20 and 22, 2000 and were analyzed on each sample receipt day.

Analytical Standard

The analytical standard was received from 3M Environmental Technology and Safety Services on March 27, 2000, assigned Wildlife International, Ltd. Identification number 5216, and stored under ambient conditions. The analytical standard, a white powder, was identified as: Potassium Perfluorobutane Sulfonate (L-7038, Lot 2), expiration date: March 2010. The analytical standard was further identified with the 3M Environmental Laboratory test control and reference number TCR #

The test substance had a reported purity of 97.90%. A subsequent revision of the certificate of analysis indicated a purity of 97.3% and an Expiration/Reassessment Date of January 17, 2002. The analytical standard was the same material and lot number as the test substance (Wildlife International, Ltd. Identification number The analytical standard was used to prepare calibration and matrix fortification samples.

Analytical Method

Water samples were analyzed according to the method entitled "Analytical Method Validation for the Determination of Perfluorobutane Sulfonate, Potassium Salt (PFBS) in Freshwater" (Wildlife International, Ltd. Project No. 454C-115). Samples were diluted in a 50% methanol : 50% NANOpure® water solution so that they fell within the calibration range of the PFBS methodology. Aliquots of the dilutions were transferred to autosampler vials and submitted for analysis by direct injection. Concentrations of PFBS in freshwater samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) interfaced with a Perkin-Elmer API 100LC mass spectrometer (single quadrupole) operated in selective ion monitoring (SIM) detection mode. The mass spectrometer was equipped with a Perkin-Elmer TurboIonSpray ion source. Chromatographic separations were achieved using a Keystone PRISM RP column (30 mm × 1.5 mm, 3-µm

particle size) fitted with a Keystone Javelin C_{18} Guard Cartridge (20 mm × 2 mm). The instrument parameters are summarized in Table 1 and a method flowchart is provided in Figure 1.

Primary and Secondary Stock Solutions.

All primary and secondary stock preparations were adjusted for the purity of the analytical standard (97.90%). A 10.0 mg a.i./mL primary stock solution of PFBS in methanol was prepared by weighing 1.024 g of the analytical standard and bringing to a final volume of 100 mL with methanol. Secondary stock solutions (1000, 100, 10.0, 1.00, and 0.100 mg a.i./L) of PFBS in methanol were prepared by serial volumetric dilution from the primary stock.

Calibration Standards and Calibration Curves

Calibration standards were prepared in 50:50 methanol: NANOpure® water by appropriate dilutions of the 10.0 mg a.i./L stock solution of PFBS in methanol. The calibration standards of PFBS, ranging in concentration from 0.0100 to 0.0500 mg a.i./L, were analyzed with each sample set. Five calibration standards (different concentrations) were analyzed with the samples. The calibration standard series was injected at the beginning and end of each run, and one standard was injected, at a minimum, after every five samples. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFBS in the samples was determined by substituting the peak area responses into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

Limit of Quantitation

The method limit of quantitation (LOQ) for these analyses was set at 50.0 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (5000).

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 directly fortified (*i.e.* without use of carrier solvent) with PFBS at 100, 1500 and 12000 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 2). Sample concentrations were not corrected for the mean procedural recovery of 103%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-115-4, nominal concentration of 204 mg a.i./L (208 mg/L) in freshwater.

First Initial Volume: 0.200 mL
First Final Volume: 10.0 mL
Second Initial Volume: 0.250 mL
Second Final Volume: 25.0 mL
Dilution Factor: 5000
PFBS Peak Area: 1835872

Calibration curve equation:
Slope: 41637464
Intercept: 95950.78906
Curve regression weighted 1/x

$$\text{PFBS (mg a.i./L) measured at instrument} = \frac{\text{peak area - (y-intercept)}}{\text{slope}}$$

PFBS (mg a.i./L) in sample = PFBS measured at instrument (mg a.i./L) × dilution factor

$$= \frac{1835872 - 95950.78906}{41637464} \times 5000$$

$$= 209$$

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

$$= \frac{209}{204} \times 100 = 102\%$$

Calculated with HPLC/MS instrument software: MacQuan, version 1.6.

RESULTS

Sample Analysis

Freshwater samples were collected from an acute toxicity study with the fathead minnow (*Pimephales promelas*) at test initiation, December 18, 2000 (Day 0), on December 20, 2000 (Day 2), and at test termination, December 22, 2000 (Day 4). The measured concentrations of PFBS in the samples collected at initiation of exposure of the test organisms (Day 0) ranged from 99.7 to 105% of the nominal concentrations. Samples collected at Day 2 had a measured concentration range of 99.1 to 109% of nominal values. Samples collected at test termination (Day 4) had a measured concentration range of 98.7% to 123% of nominal values (Table 3). 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 operated in Selective Ion Monitoring (SIM) Mode
ION SOURCE:	Perkin-Elmer TurboIonSpray
ANALYTICAL COLUMN:	Keystone PRISM RP (30 mm × 1.5 mm, 3-µm particle size)
GUARD COLUMN:	Keystone Javelin C_{18} cartridge (20 mm × 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	3.00 min
FLOW RATE:	200 µL/min
MOBILE PHASE:	25% NANOpure® Water with 0.1% Ammonium Formate: 75% Methanol
INJECTION VOLUME:	5.0 µL
PFBS PEAK RETENTION TIME:	Approximately 2.3 minutes
PFBS MONITORED MASS:	299.0 amu

Table 2

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-115-)	Sample Type	Concentrations of PFBS (mg a..i./L)		
		Fortified	Measured[1]	Recovered[1]
MAB-1	Matrix Blank	0.00	<LOQ[2]	--
MAB-2	Matrix Blank	0.00	<LOQ	--
MAB-3	Matrix Blank	0.00	<LOQ	--
MAS-1	Matrix Fortification	100	108	108
MAS-4	Matrix Fortification	100	91.3	91.3
MAS-7	Matrix Fortification	100	101	101
MAS-2	Matrix Fortification	1500	1480	98.7
MAS-5	Matrix Fortification	1500	1650	110
MAS-8	Matrix Fortification	1500	1480	98.4
MAS-3	Matrix Fortification	12000	12800	106
MAS-6	Matrix Fortification	12000	13200	110
MAS-9	Matrix Fortification	12000	11900	99.1
				Mean = 103
				Standard Deviation = 6.38
				CV = 6.22%
				N = 9

[1] Measured and Percent Recovered values were calculated using MacQuan, version 1.6 software. Manual calculations may vary slightly.

[2] The limit of quantitation (LOQ) was 50.0 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (5000).

Table 3

Measured Concentrations of PFBS in Freshwater Samples from a Fathead Minnow Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-115-)	Sampling Time (Day)	PFBS Measured Concentration[1] (mg a.i./L)	Percent of Nominal[1]
0.0	1	0	< LOQ[2]	--
	2	0	< LOQ	--
	13	2	< LOQ	--
	14	2	< LOQ	--
	25	4	< LOQ	--
	26	4	< LOQ	--
204	3	0	205	101
	4	0	209	102
	15	2	211	103
	16	2	213	105
	27	4	250	123
	28	4	232	114
408	5	0	426	105
	6	0	407	99.7
	17	2	432	106
	18	2	415	102
	29	4	498	122
	30	4	446	109
816	7	0	824	101
	8	0	851	104
	19	2	888	109
	20	2	876	107
	31	4	962	118
	32	4	926	114

[1] Measured and Percent of Nominal values were calculated using MacQuan, version 1.6 software. Manual calculations may vary slightly.

[2] The limit of quantitation (LOQ) was 50.0 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (5000).

Table 3 (Continued)

Measured Concentrations of PFBS in Freshwater Samples from a Fathead Minnow Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-115-)	Sampling Time (Day)	PFBS Measured Concentration[1] (mg a.i./L)	Percent of Nominal[1]
1632	9	0	1692	104
	10	0	1674	103
	21	2	1665	102
	22	2	1617	99.1
	33	4	1673	103
	34	4	1611	98.7
3263[3]	11	0	3354	103
	12	0	3417	105
	23	2	3327	102
	24	2	3264	100
	--	4	--	--
	--	4	--	--

[1] Measured and Percent of Nominal values were calculated using MacQuan, version 1.6 software. Manual calculations may vary slightly.

[2] The limit of quantitation (LOQ) was 50.0 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (5000).

[3] This treatment group was not sampled on Day 4 due to 100% mortality on Day 1. Fish were not in the test chambers during Day 2 sampling.

METHOD OUTLINE FOR THE ANALYSIS OF PFBS IN FRESHWATER

Prepare each matrix fortification sample by weighing the requisite amount of PFBS test substance on an analytical balance and transferring directly into a Class A volumetric flask partially filled with freshwater. Rinse weighing paper and the sides of the flask with repeat freshwater rinses. Swirl the flask to dissolve the test substance and then bring to final volume with freshwater. Sonicate, as appropriate, and mix with several repeat inversions. The matrix blank is unfortified freshwater.

↓

Prepare appropriate dilutions of study and QC samples to within the calibration range of the PFBS methodology: Partially fill Class A volumetric flasks with 50% methanol : 50% NANOpure® water dilution solvent. Add the appropriate volume of sample and bring to volume with dilution solvent. Perform secondary dilutions as necessary. Process matrix blank samples using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.

↓

Ampulate samples and submit for LCMS analysis.

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

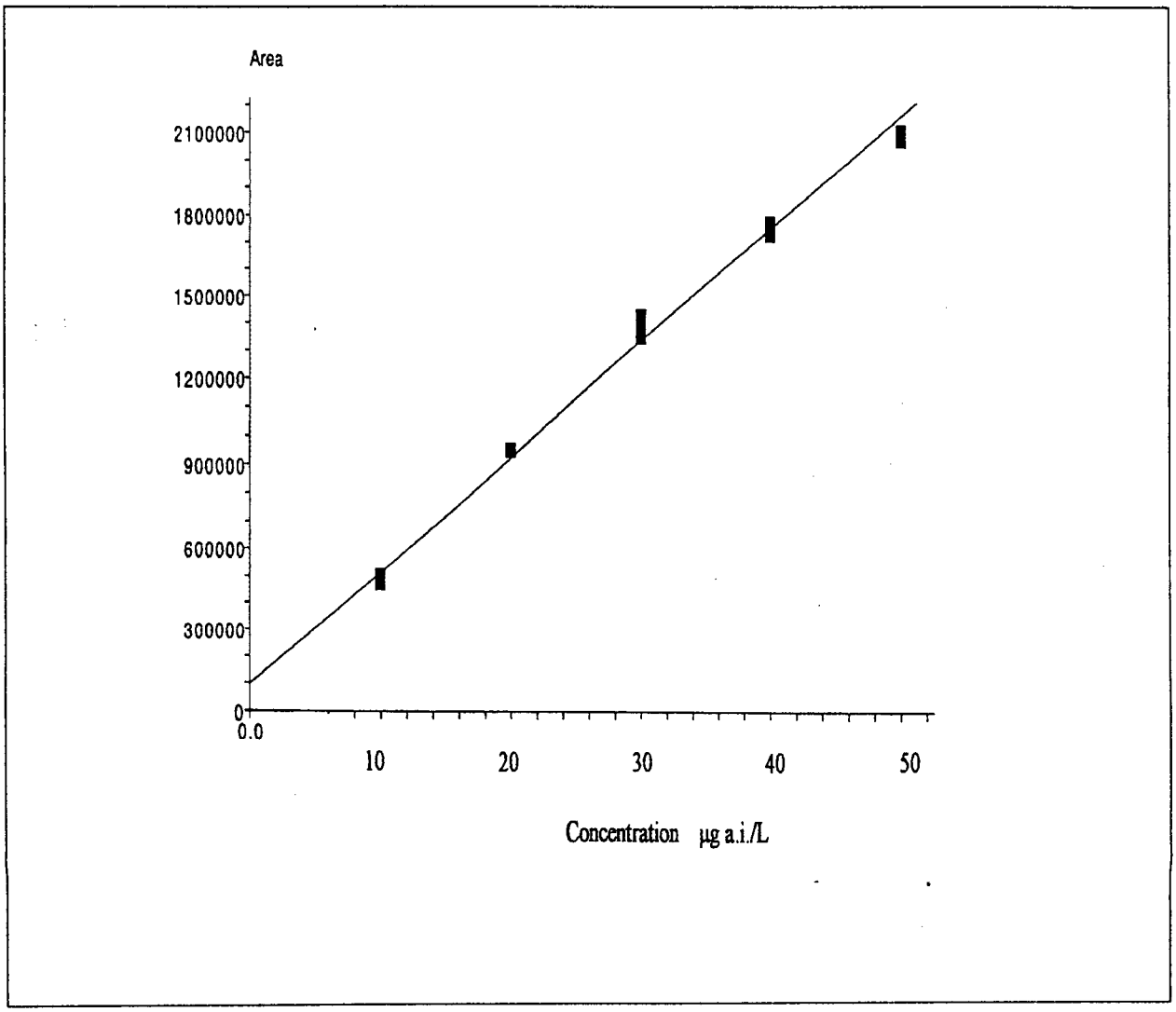


Figure 2. A typical calibration curve for PFBS. Slope = 41637464; Intercept = 95950.78906; r = 0.9969.

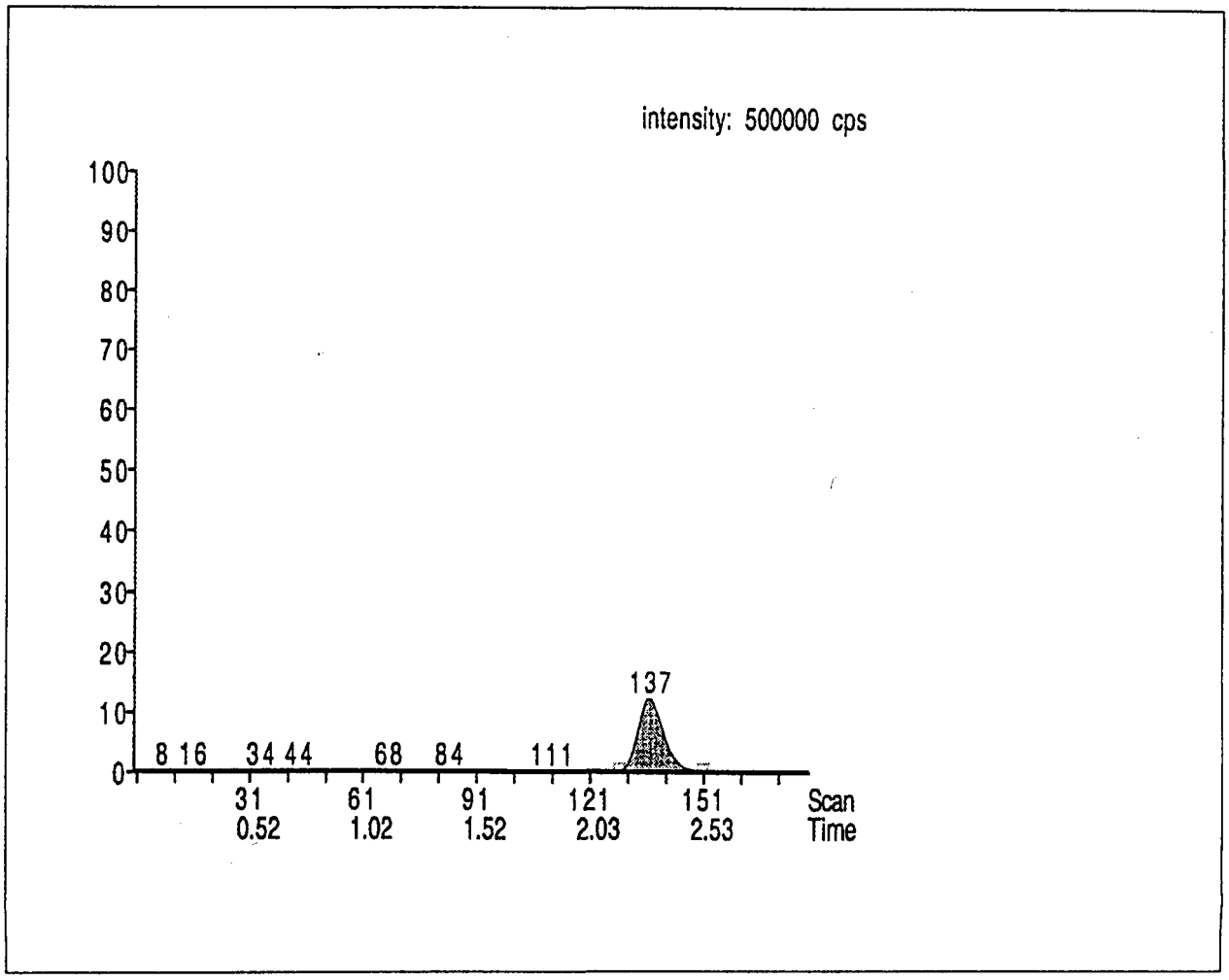


Figure 3. A representative ion chromatogram of a low-level (0.0100 mg a.i./L) PFBS standard.

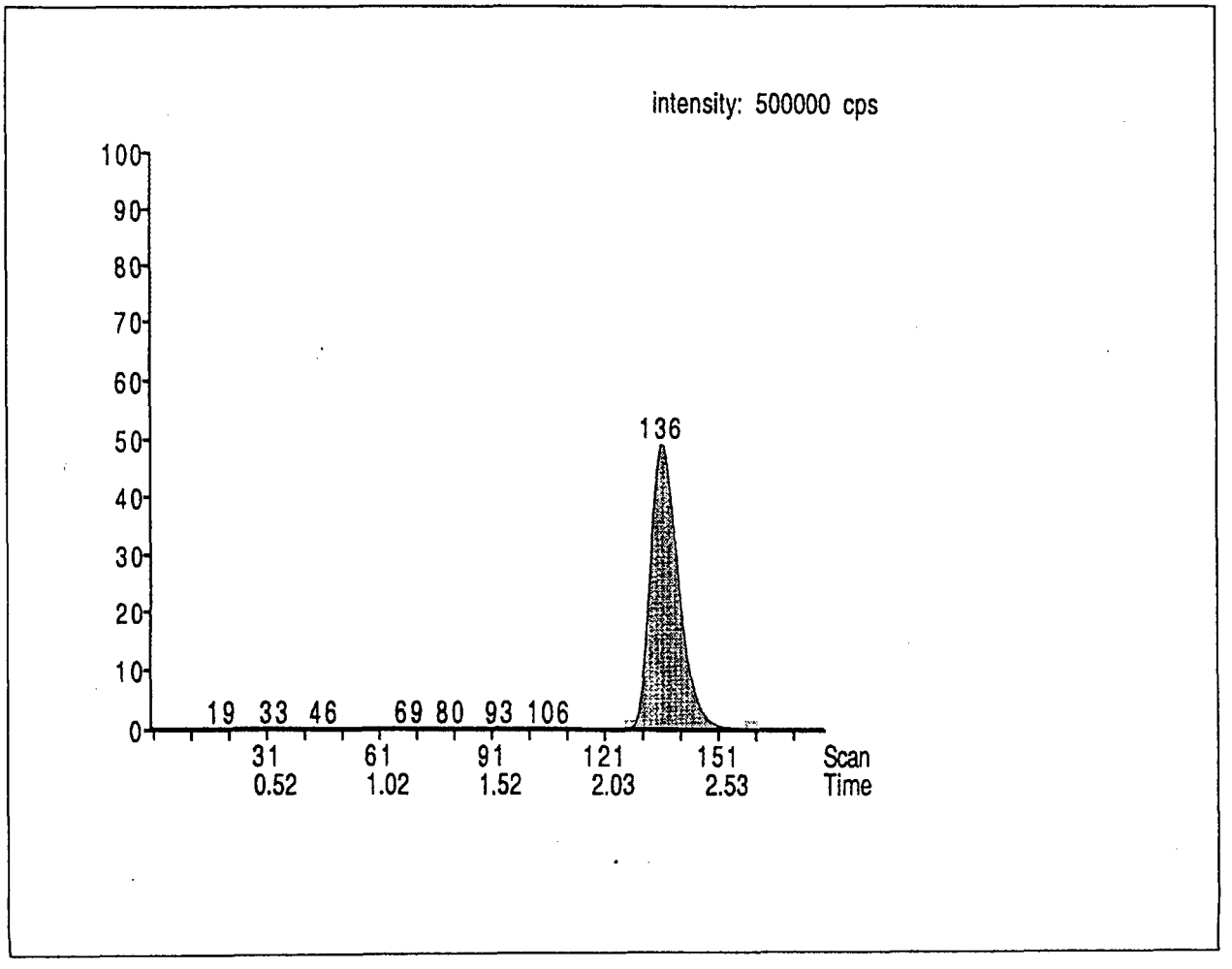


Figure 4. A representative ion chromatogram of a high-level (0.0500 mg a.i./L) PFBS standard.

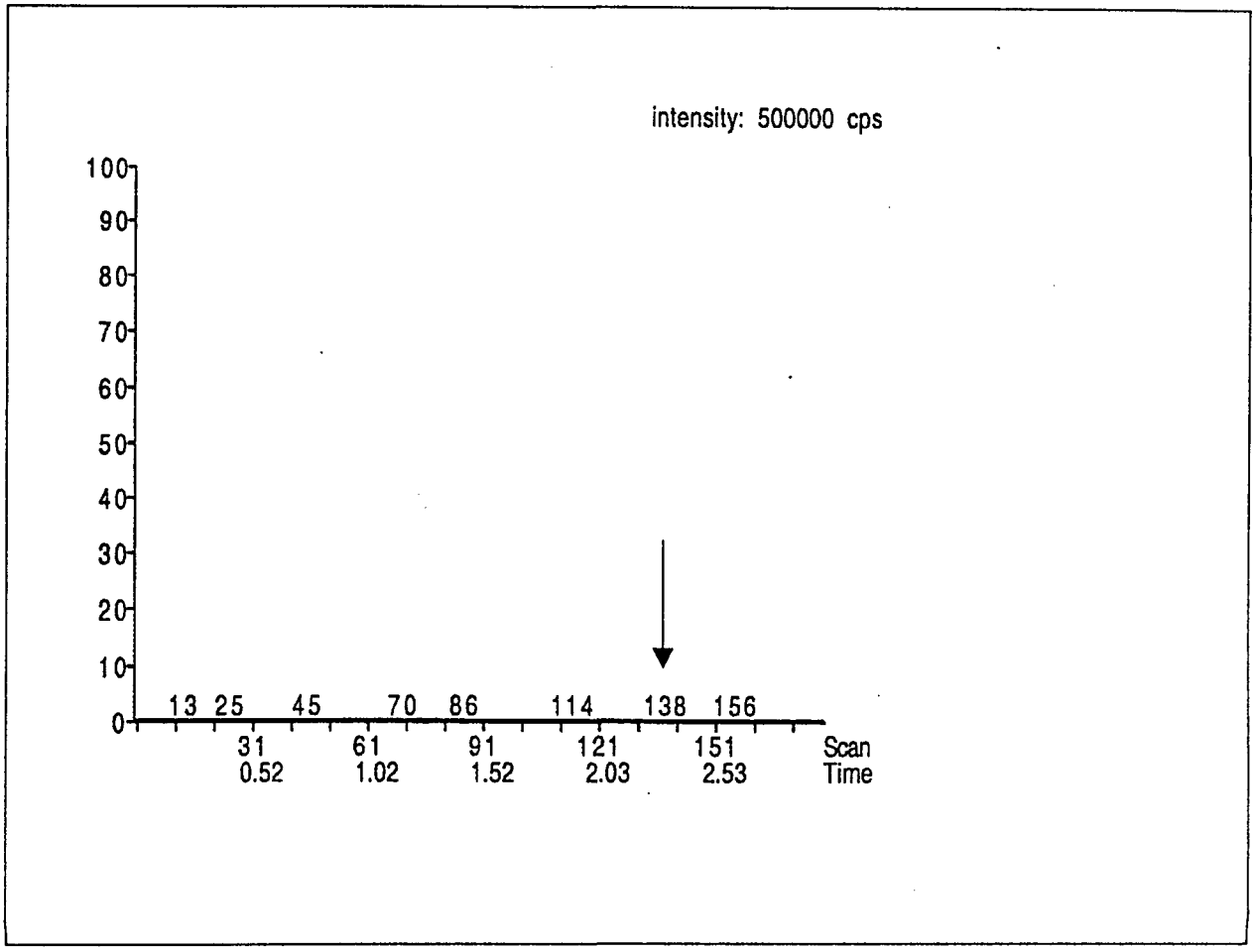


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-115-MAB-1). The arrow indicates the retention time of PFBS.

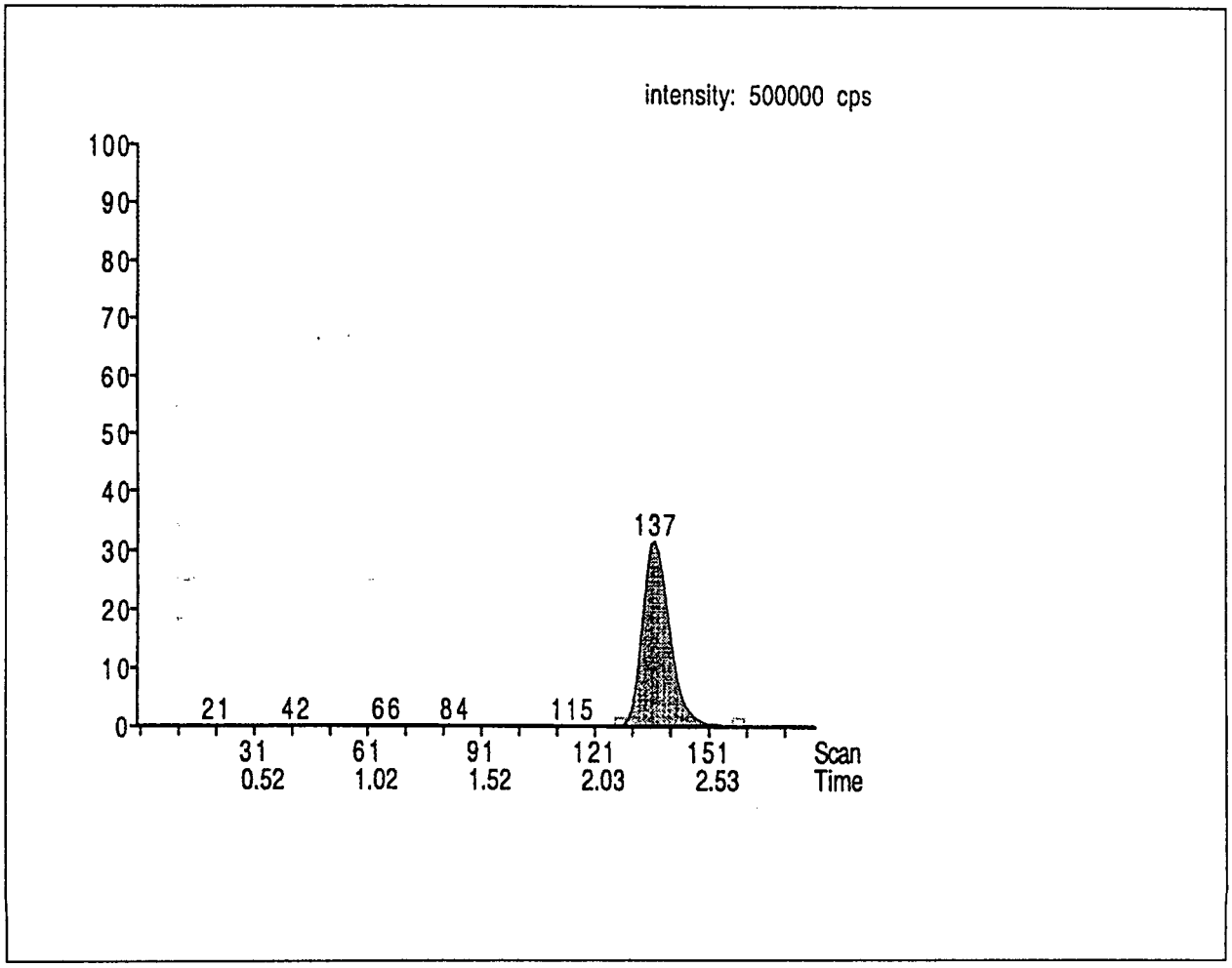


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-115-MAS-2, nominal PFBS concentration of 1500 mg a.i./L, dilution factor = 50000x).

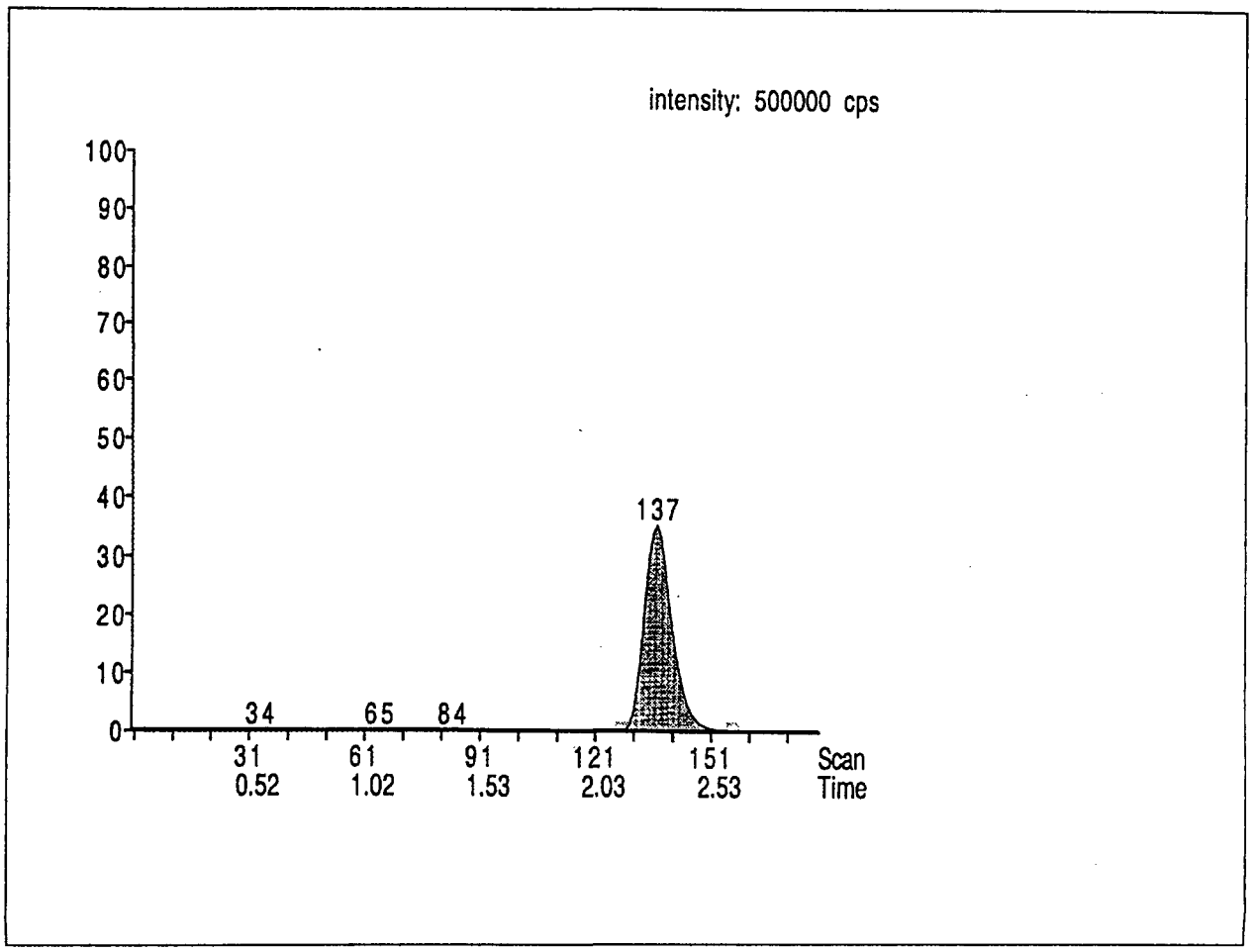


Figure 7. A representative ion chromatogram of a test sample (454A-115-7, nominal PFBS concentration of 816 mg a.i./L, dilution factor = 25000x).

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 and test concentrations.
2. Unnecessary temperature measurements were made in the 3341 mg a.i./L treatment group on Day 2 of the test.
3. Feed provided to the fish was not analyzed for contaminants

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. Kurt R. Drottar, Senior Biologist
6. Susan T. Platania, Biologist