NON-GLP STUDY: PFOS BACKGROUND SCREEN IN SELECTED WILDLIFE INTERNATIONAL, LTD. AVIAN AND AQUATIC FEED MATRICES

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454C-101-2

AUTHORS:

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Submitted to:

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REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: Non-GLP Study: PFOS Background Screen in Selected Wildlife International, Ltd. Avian and Aquatic Feed Matrices

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454C-101-2

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SUMMARY

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

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	454C-101-2
TEST SUBSTANCE:	PFOS
STUDY:	Non-GLP Study: PFOS Background Screen in Selected Wildlife International, Ltd. Avian and Aquatic Feed Matrices
TEST DATES:	Experimental Start – January 18, 1999 Experimental Termination – June 9, 1999

TEST SYSTEMS:	Avian Feed:	WLI Basal Ration (low-calcium diet) WLI Basal Ration + Limestone (high-calcium diet)
	Aquatic Feed:	Artemia Cysts Yeast, Cerophyll®, and Trout Chow (YCT) Green Algae (*Selenastrum capricornutum*)

SUMMARY: PFOS concentrations were significantly below limits of quantitation in avian feed used by Wildlife International, Ltd. in avian toxicology studies. Low (ppb-level) concentrations of PFOS were measured in dry artemia cysts used as starting feed material in selected aquatic toxicology tests. The concentration levels anticipated in *applied* artemia (decapsulated and diluted in saltwater) are not expected to be of consequence in associated chronic effects testing. PFOS was not detected in YCT and green algae feed suspensions used by Wildlife International, Ltd. in chronic aquatic toxicology studies.

INTRODUCTION

The integrity of environmental toxicology studies can be compromised if measurable quantities of the test substance under investigation are present in components of the test system. This is especially important in chronic or reproductive studies. In these studies, the potential for toxicological effects from a non-controlled source of the test substance introduced into the test system is greater than those expected in relatively shorter-term acute studies. Random, or non-controlled input sources, may bias the interpretation of toxicological effects from the introduction of controlled doses of the test substance into the test system. The types of feed used to sustain test species in chronic studies are a significant potential source of non-controlled input. Consequently, the screening of the feed to be used in anticipated toxicological effects testing is useful to ensure the success of the studies.

In this investigation, selected feed used by Wildlife International, Ltd. for avian and aquatic toxicology studies were screened for PFOS (perfluorooctane sulfonic acid, potassium salt) content. This study was conducted by Wildlife International, Ltd. and identified as Project Number 454C-101-2. The analyses of samples were performed at Wildlife International Ltd. analytical laboratory using high performance liquid chromatography with mass spectrometric detection (LCMS). Blank and fortified avian feed samples were prepared and analyzed between January 18 and February 3, 1999. Blank aquatic feed samples tests were prepared and analyzed between June 8 and 9, 1999. The raw data and a copy of the final report generated by Wildlife International, Ltd. are filed under Project Number 454C-101-2 in archives located on the Wildlife International, Ltd. site.

PURPOSE

The purpose of this study was to determine background concentrations of PFOS, if detectable, in selected avian and aquatic feed to be used in future environmental effect studies.

EXPERIMENTAL DESIGN

The testing consisted of either extraction (avian feed and artemia cysts) or dilution (YCT and green algae) of blank and/or fortified feed materials followed by PFOS analysis by high performance liquid chromatography with mass spectrometric detection (LCMS).

Quantitation was performed with external standards of PFOS using concentrations that bracketed the expected instrumental concentrations of the samples. Internal standardization was used for avian feed screens only. For each sample set, one calibration curve was generated from the analyses of PFOS standard solutions. For avian feed screens, all calibration standards and samples contained the same nominal concentration (0.100 mg/L) of the internal standard.

MATERIALS AND METHODS

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International, Ltd. identification number 4675 upon receipt. The test substance, a white powder, was identified as: FC-95, Lot Number: 217, Expiration Date: 2008. The test substance had a reported purity of 98.9% and was stored under ambient conditions.

Internal Standard

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 was stored under ambient conditions. The internal standard is referred to hereafter as 4HPFOS.

Reagents and Solvents

All solvents used were HPLC grade or equivalent.

Test Systems

Avian Feed

Two avian feed (game-bird ration) types were investigated. The feed types differ only in the omission or addition of supplemental calcium. Wildlife International, Ltd. (WLI) Basal Ration is a relatively low-calcium diet employed in acute toxicology studies. WLI Basal Ration + Limestone is a high-calcium diet used in pilot and reproductive studies. A calcium-enriched diet for female birds enhances egg production. The specifications for the ingredients common to these types of feed are presented in Appendix I.

Aquatic Feed

The feed used at Wildlife International, Ltd. for early life-stage studies with the fathead minnow (*Pimephales promelas*) and life-cycle studies with the saltwater mysid (*Mysidopsis bahia*) consists of decapsulated artemia cysts (brine shrimp eggs) suspended in saltwater. Dry artemia cysts (Bonneville Artemia International, Salt Lake City, UT), prior to decapsulation and suspension in freshwater, were selected for the PFOS screen. Life-cycle studies with the cladoceran (*Daphnia magna*) employ freshwater suspensions of YCT (yeast, Cerophyll®, and trout chow) and green algae (*Selenastrum capricornutum*). These freshwater feed suspensions were taken for PFOS analysis.

Test Procedures

The analytical method for the analysis of PFOS in avian diet involved an extraction with methanol (HPLC grade, 99.9+%) and analysis by liquid chromatography mass spectrometry. Each type of avian diet was fortified at 1000 mg a.i./kg PFOS (target nominal concentration) by dry mixing the feed with the test substance, and diluting the fortification to 1.00 mg a.i./kg PFOS (target nominal concentration) with the appropriate unfortified (blank) feed. For each avian feed type, duplicate 10-gram sub-samples of fortified (1.00 mg a.i./kg) and blank avian diets were transferred into 16-ounce French square bottles. One hundred milliliters of methanol was added to each bottle. The bottles were sealed with screw caps, placed on a tabletop shaker and shaken for a minimum of 30 minutes. The samples were vacuum filtered using qualitative filter paper. The avian diet was rinsed three times with methanol into the filtrate. The filtrates were then transferred into 200-mL volumetric flasks and brought to volume with methanol. Final dilutions were performed by transferring five milliliters of each sample into 10-mL volumetric flasks partially-filled with NANOpure® water. The flasks were fortified with 100 µL of a 10 µg a.i./mL solution of 4HPFOS (internal standard) in methanol. The solutions were brought to volume with NANOpure® water, mixed and filtered through a 13-mm, 0.2-µm Nylon® filter (Gelman Acrodisc®, lot 1572) into HPLC autosampler vials. The vials were capped and submitted for LCMS analysis.

The analytical method for the analysis of PFOS in artemia cysts consisted of extraction of replicate 10-gram samples of unfortified feed with 25 milliliters of methanol (HPLC grade, 99.9+%). Approximately three milliliters of each extract was removed from the methanol-saturated feed. A sub-sample of each 3-mL extract was ampulated and submitted for LCMS analysis. The analytical method for the analysis of PFOS in freshwater suspensions of YCT and green algae consisted of dilution prior to

LCMS analysis. Replicate one-milliliter sub-samples of each unfortified aqueous suspension were diluted to 10-mL final volumes with a solution of 50% methanol and 50% NANOpure® water containing 0.05% v/v formic acid and 0.100 mg/L of the internal standard. A sub-sample of each dilution was ampulated and submitted for LCMS analysis.

Concentrations of PFOS 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. Chromatographic separations were achieved using one of two Keystone Betasil C_{18} columns (2-mm I.D., 3-µm particle size). A 100–mm column was used for avian feed screens and a 50-mm column was used for the aquatic feed screens. The instrument parameters are summarized in Table 1.

Calibration Curves

Calibration standards of PFOS, prepared in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4HPFOS (internal standard)/L and 0.05% formic acid (v/v), were analyzed with the samples. For avian feed samples, the calibration standards ranged in concentration from 0.005 to 0.040 mg a.i./L. For aquatic feed samples, the calibration standards ranged in concentration from 0.001 to 0.010 mg a.i./L. A minimum of three calibration standards was analyzed with each set of samples. The set of standards was injected at the beginning and end of each run, and one standard was injected, at a minimum, after every four samples. For avian feed samples, weighted (1/x) 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. For aquatic feed samples, weighted (1/x), linear regression equations were generated using peak area response versus PFOS concentration (internal standard not applied). A representative calibration curve is presented in Figure 1. The concentration of PFOS in the samples was determined by substituting the peak area response or peak area response ratio (as appropriate) into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 2 and 3, respectively.

Limits of Quantitation

The method limit of quantitation (LOQ) for analyses of avian feed samples was set at 0.20 mg a.i./kg. The LOQ was calculated as the product of the lowest calibration standard analyzed

(0.005 mg a.i./L), the extraction volume-to-sample weight ratio (0.1 L/0.01 kg), and dilution factor of the matrix blank samples (4).

The LOQ for analyses of artemia cyst samples was set at 0.0003 mg a.i./kg, calculated as the product of the lowest calibration standard analyzed (0.001 mg a.i./L), the extraction volume to sample weight ratio (0.025 L/0.01 kg), and dilution factor of the samples (0.12).

The LOQ for analyses of freshwater suspensions of YCT and green algae samples was set at 0.010 mg a.i./L, calculated as the product of the lowest calibration standard analyzed (0.001 mg a.i./L), and the dilution factor of the samples (10).

Reagent Blanks

Reagent blanks were analyzed to assess the presence of potential interferences. No chromatographic interferences were observed in the blanks at or above the limit of quantitation. For the avian feed screen, the reagent blank consisted of an aliquot of methanol diluted with NANOpure® water, fortified with internal standard, and filtered as for the study samples. For the aquatic feed screen, the reagent blank consisted of dilution solvent (a solution of 50% methanol and 50% NANOpure® water containing 0.05% v/v formic acid and 0.100 mg/L of the internal standard). A representative ion chromatogram of a reagent blank sample from the avian feed screen is presented in Figure 4.

RESULTS AND DISCUSSION

Avian Feed

The results of the avian feed screen are presented in Table 2. The PFOS concentration of all blank avian feed samples was significantly below the LOQ for the methodology (0.2 mg a.i./kg). The feed fortified at 1.00 mg a.i./kg had a mean PFOS recovery of 83.3 ± 10%. No difference in analytical behavior was observed between the low- and high-calcium diet matrices. A representative ion chromatogram of an unfortified high-calcium diet sample is presented in Figure 5. A corresponding representative ion chromatogram of a fortified sample is presented in Figure 6.

Aquatic Feed

The results of the aquatic feed screen are presented in Table 3. Concentrations of PFOS ranging from 0.0009 to 0.001 mg a.i./kg were determined in dry artemia cysts. PFOS was not detected in either the YCT or green algae freshwater suspensions. Representative ion chromatograms of each aquatic feed matrix are presented in Figures 7-9.

CONCLUSIONS

PFOS concentrations were significantly below limits of quantitation in avian feed used by Wildlife International, Ltd. in avian toxicology studies. Low (ppb-level) concentrations of PFOS were measured in dry artemia cysts used as starting feed material in selected aquatic toxicology tests. The concentration levels anticipated in *applied* artemia (decapsulated and diluted in saltwater) are not expected to be of consequence in associated chronic effects testing. PFOS was not detected in YCT and green algae feed suspensions used by Wildlife International, Ltd. in aquatic toxicology studies.

Table 1

Typical LCMS 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 (100 mm or 50 mm x 2 mm I.D., 3-µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	10 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72% Methanol : 28% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	50.0 µL: avian feed screen 25.0 µL: aquatic feed screen
PFOS RETENTION TIME:	Approximately 7.5 minutes (100-mm column) Approximately 3.6 minutes (50-mm column)
INTERNAL STANDARD RETENTION TIME:	Approximately 4.9 minutes (100-mm column)
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

Table 2

Determination of PFOS in Avian Feed

Sample Number (454C-101-2-)	Concentration of PFOS (mg a.i./Kg)		Percent Recovery[4]
	Fortified	Measured	
FS-MAB-1[1]	NA	<LOQ[3]	NA
FS-MAB-2[1]	NA	<LOQ	NA
FS-MAS-1[1]	1.00	0.783	78.3
FS-MAS-2[1]	1.00	0.724	72.4
FS-MAB-3[2]	NA	<LOQ	NA
FS-MAB-4[2]	NA	<LOQ	NA
FS-MAS-3[2]	1.00	0.873	87.3
FS-MAS-4[2]	1.00	0.951	95.1
		Mean =	83.3%
		Standard Deviation =	10.0%
		N =	4

[1]Wildlife International, Ltd. Basal Ration (low-calcium diet).

[2]Wildlife International, Ltd. Basal Ration + Limestone (high-calcium diet).

[3]The limit of quantitation (LOQ) was 0.20 mg a.i./kg calculated as the product of the lowest standard concentration (0.005 mg a.i./L), the extraction volume to sample weight ratio (0.1 L/0.01 kg), and the dilution factor of the matrix blanks (4).

[4]Percent recovery = [measured result (mg a.i./kg)/nominal concentration (mg a.i./kg)] x 100.

Table 3

Determination of PFOS in Aquatic Feed

Sample Number (454C-101-2-)	Concentration of PFOS	
	Measured (mg a.i./kg)	Measured (mg a.i./L)
ART-1[1]	0.0010	NA[4]
ART-2[1]	0.0009	NA
YCT-1[2]	NA	ND[5]
YCT-2[2]	NA	ND
GALG-1[3]	NA	ND
GALG-2[3]	NA	ND

[1]ART = Dry artemia cysts. Bonneville Artemia, Bonneville Artemia International, Salt Lake City, UT.
[2]YCT = Yeast, Cerophyll®, and Trout Chow. Freshwater suspension. Wildlife International, Ltd. Lot No. 99-9.
[3]GALG = Green algae (*Selenastrum capricornutum*). Freshwater suspension. Wildlife International, Ltd. Lot No. 99-22.
[4]NA = Not applicable.
[5]ND = Not detected. Estimated instrument detection limit was 0.0001 mg a.i./L.

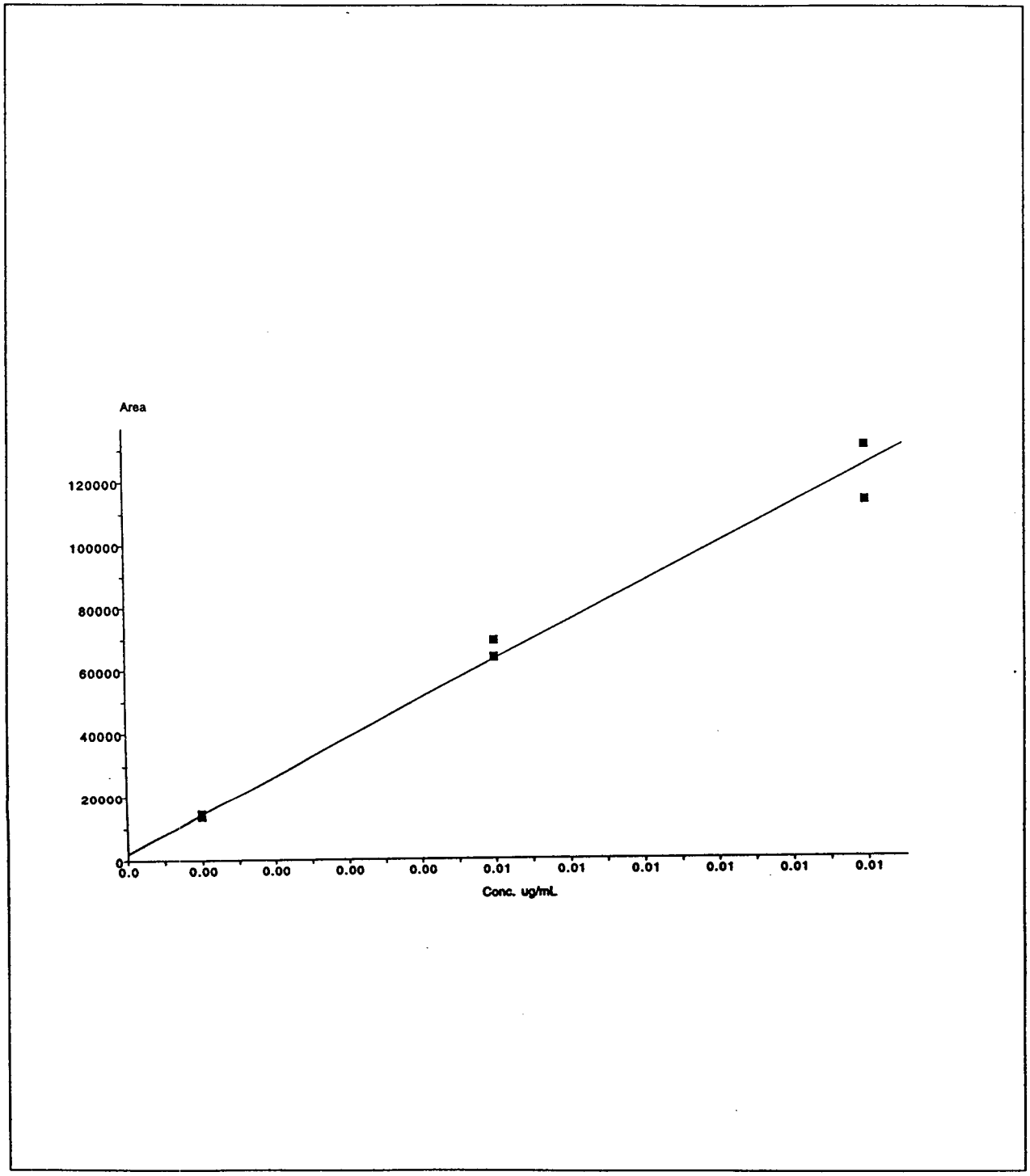

Figure 1. A representative calibration curve for PFOS. Intercept = 1964.6; Slope = 12284942; r^2 = 0.9920,weighted (1/x).

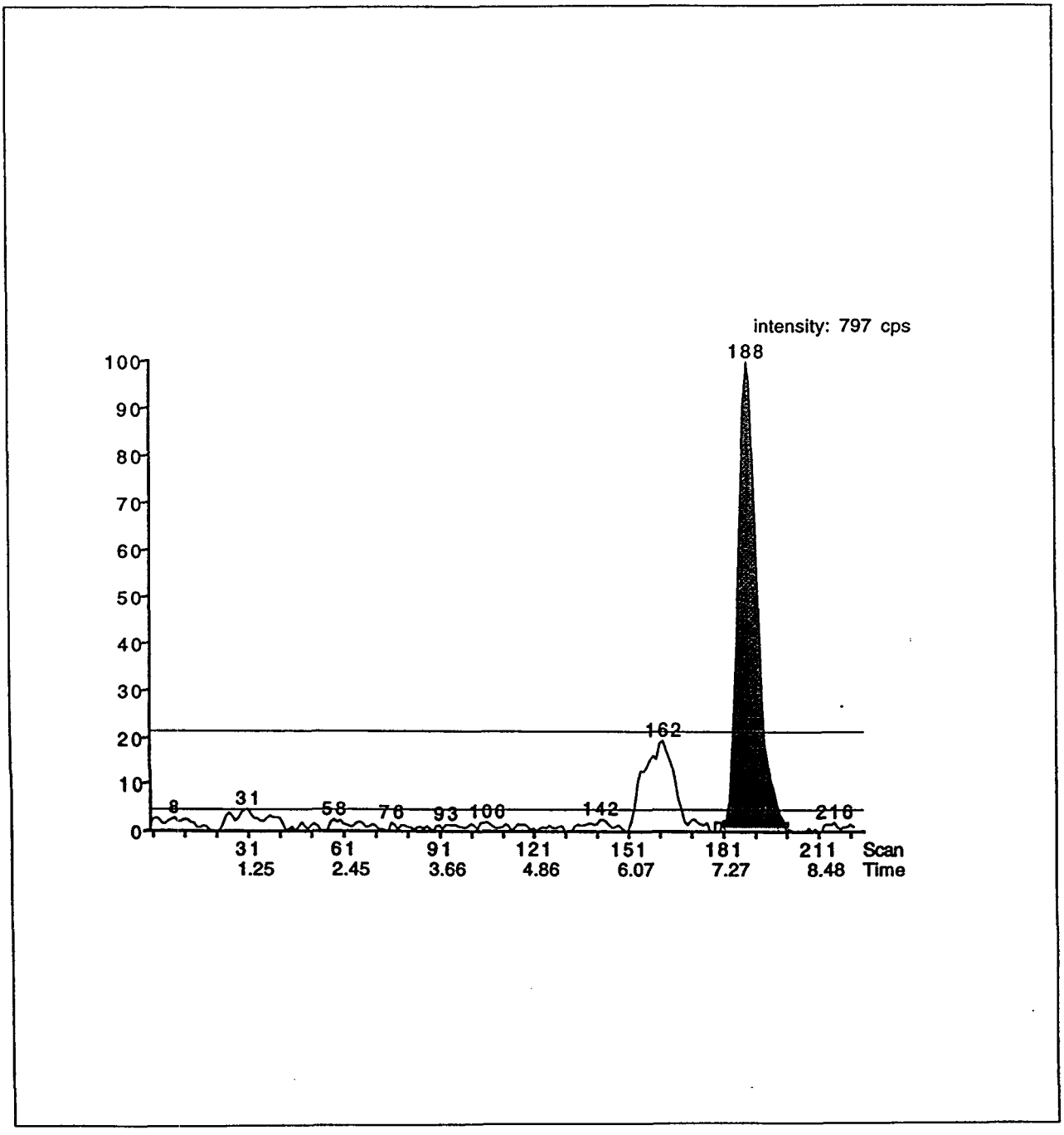

Figure 2. A representative ion chromatogram of a low-level (0.005 mg a.i./L) calibration standard.

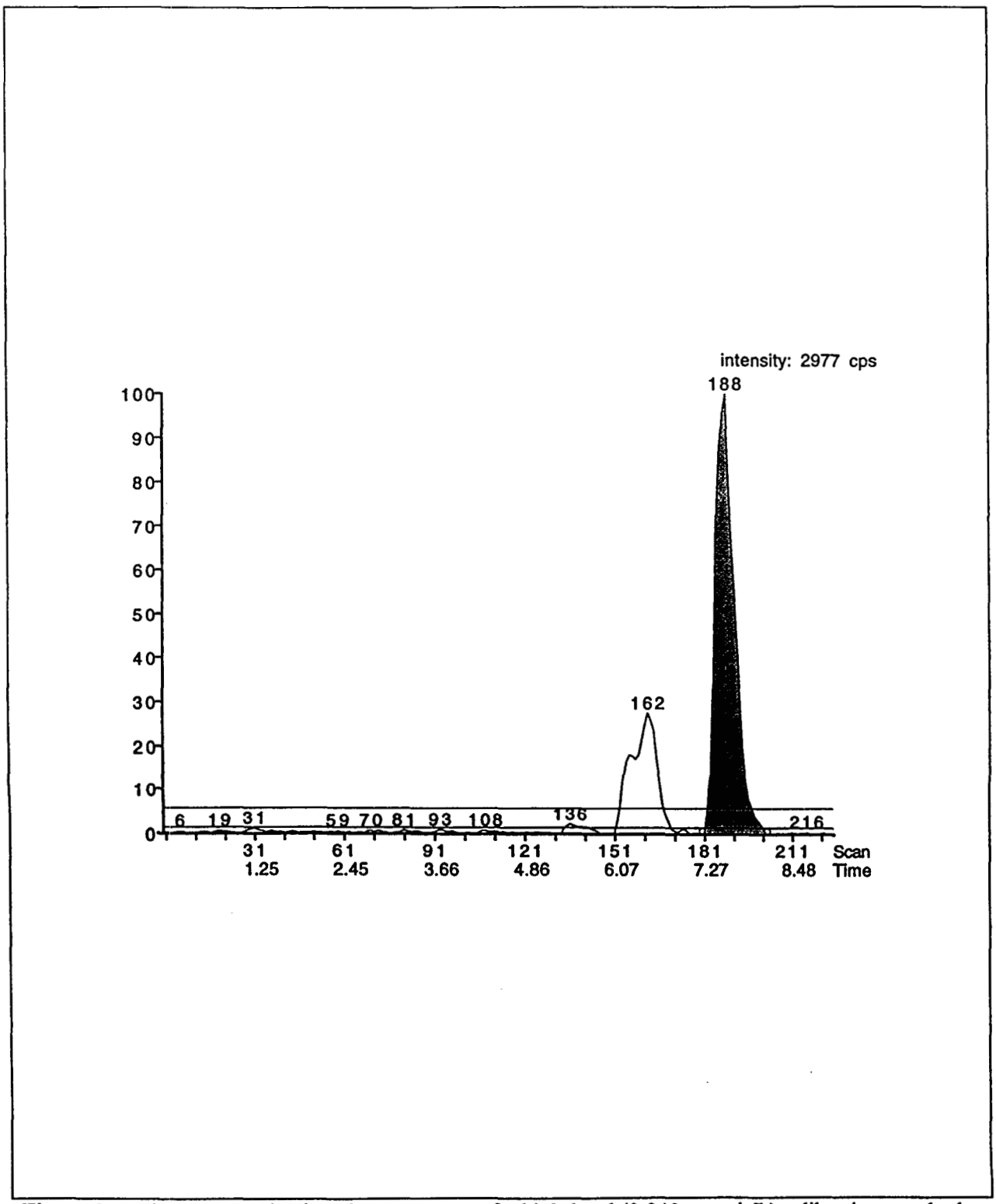

Figure 3. A representative ion chromatogram of a high-level (0.040 mg a.i./L) calibration standard.

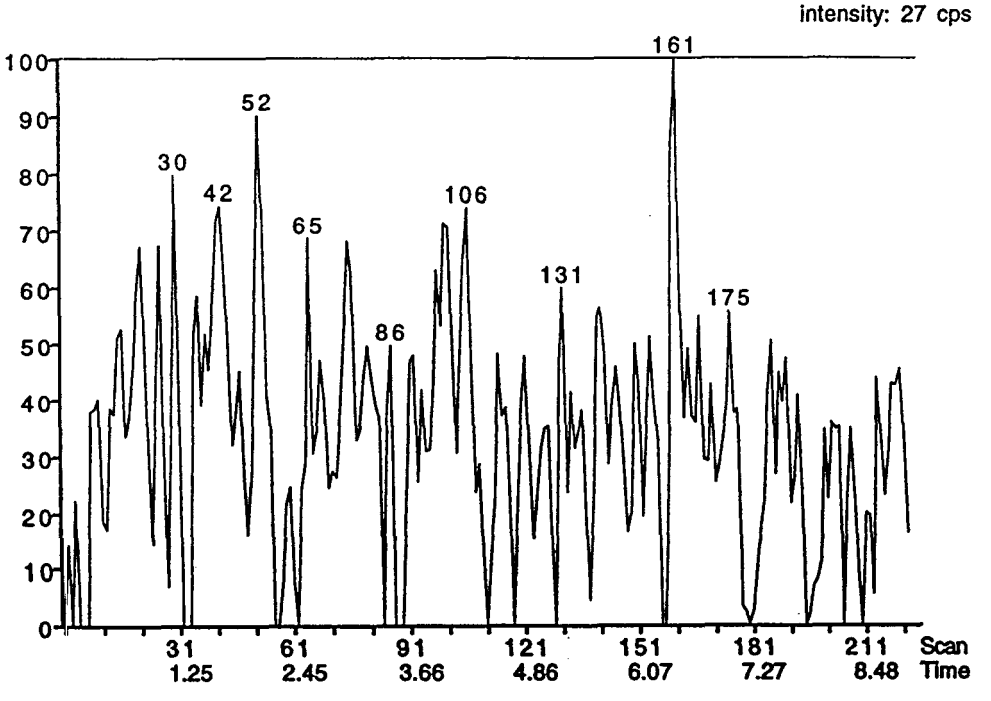

Figure 4. A representative ion chromatogram of a reagent blank sample from avian feed screen (methanol extraction blank).

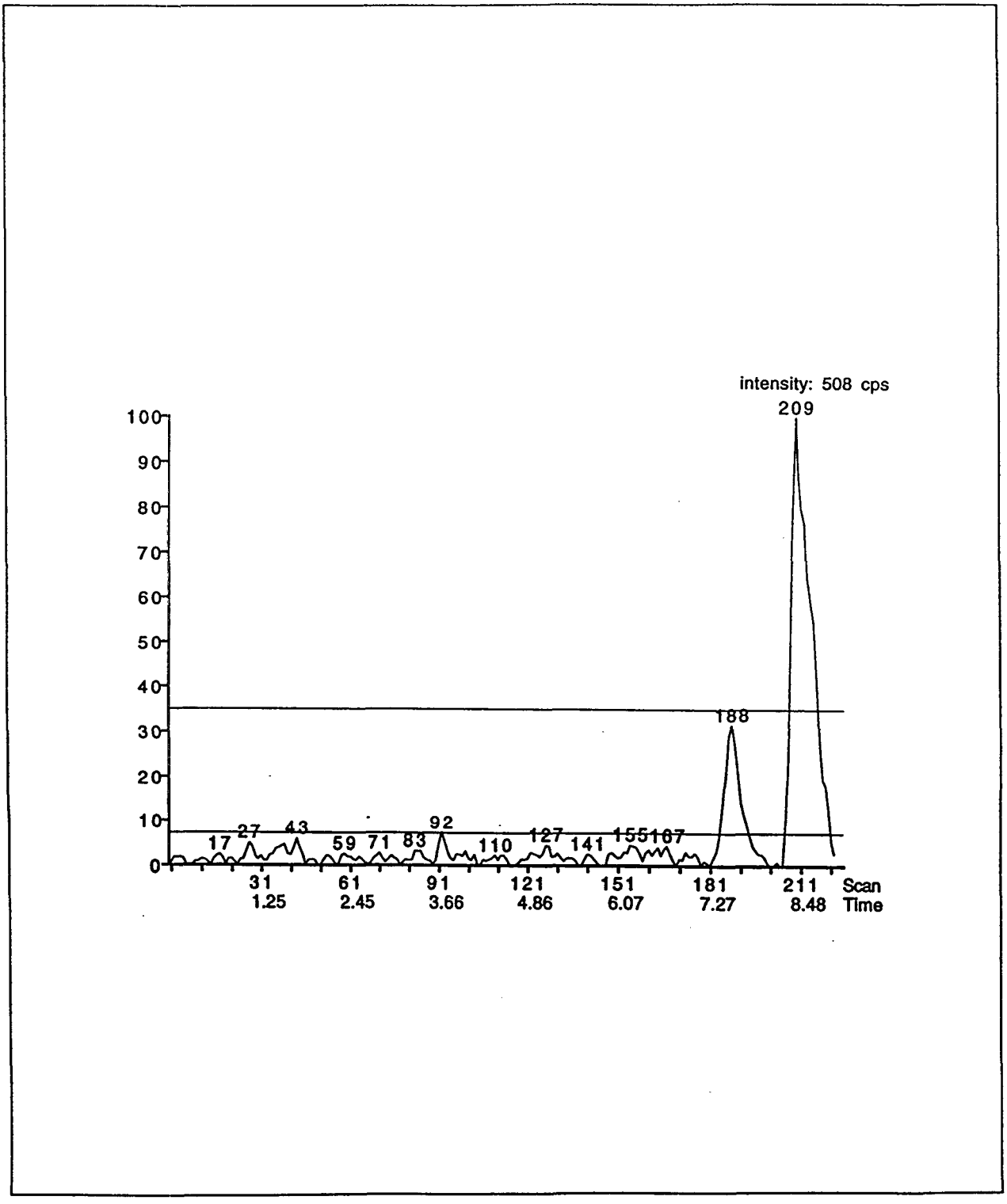

Figure 5. A representative ion chromatogram of an avian feed matrix blank sample (454C-101-2-FS-MAB-4).

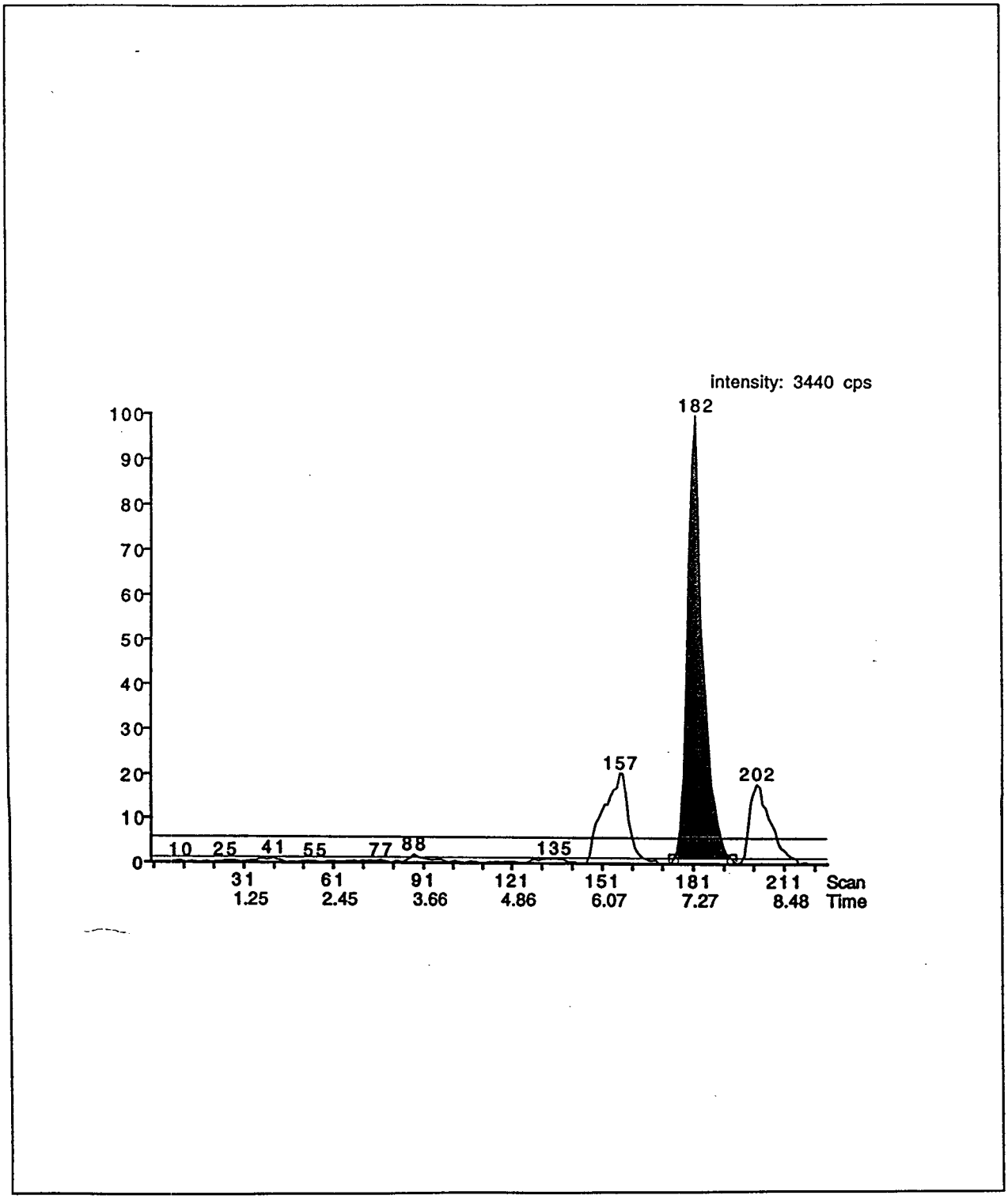

Figure 6. A representative ion chromatogram of an avian feed matrix fortification sample (454C-101-2-FS-MAS-4, 1.00 mg a.i./kg nominal concentration).

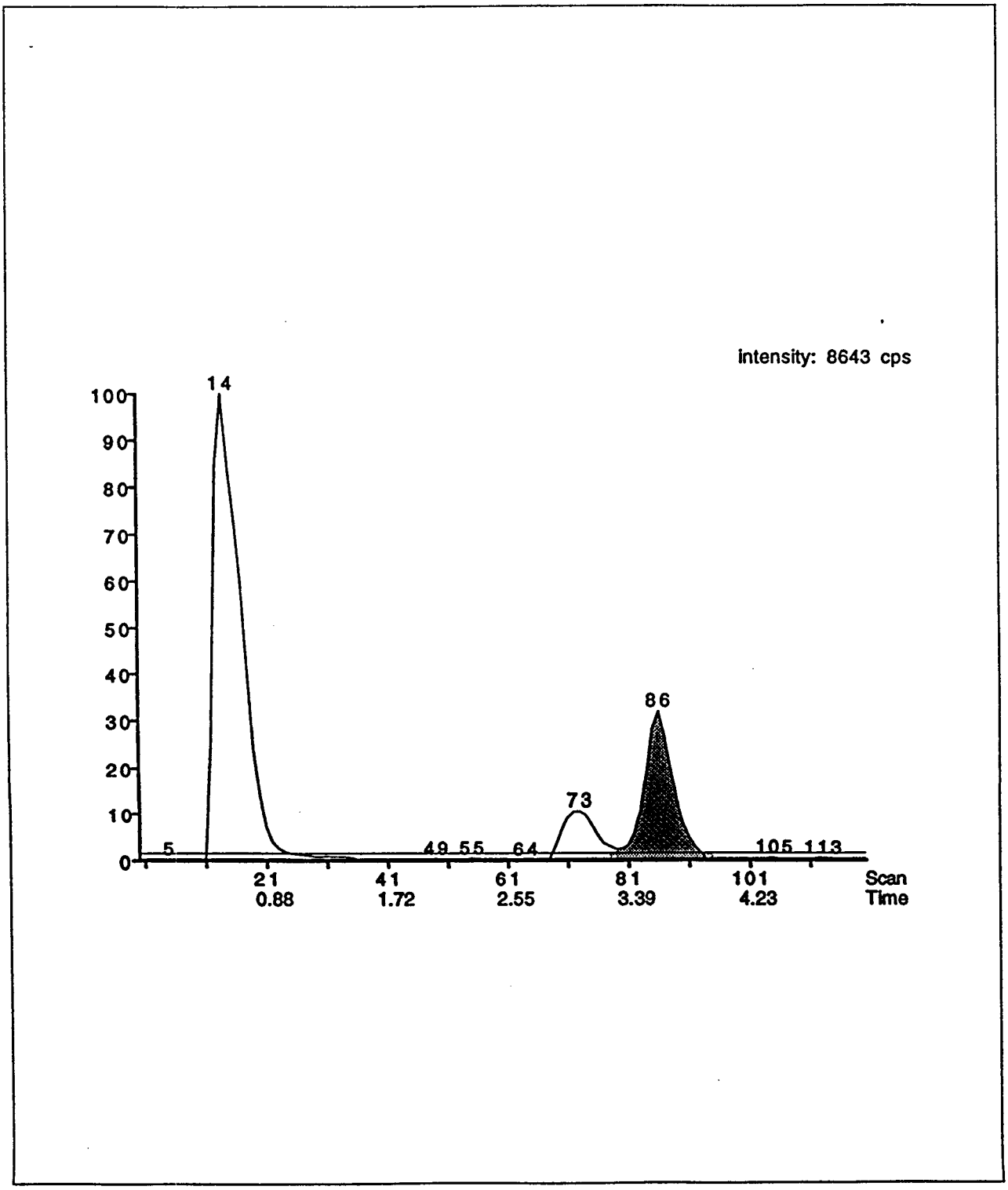

Figure 7. A representative ion chromatogram of an artemia cyst aquatic feed sample (454C-101-1-ART-1).

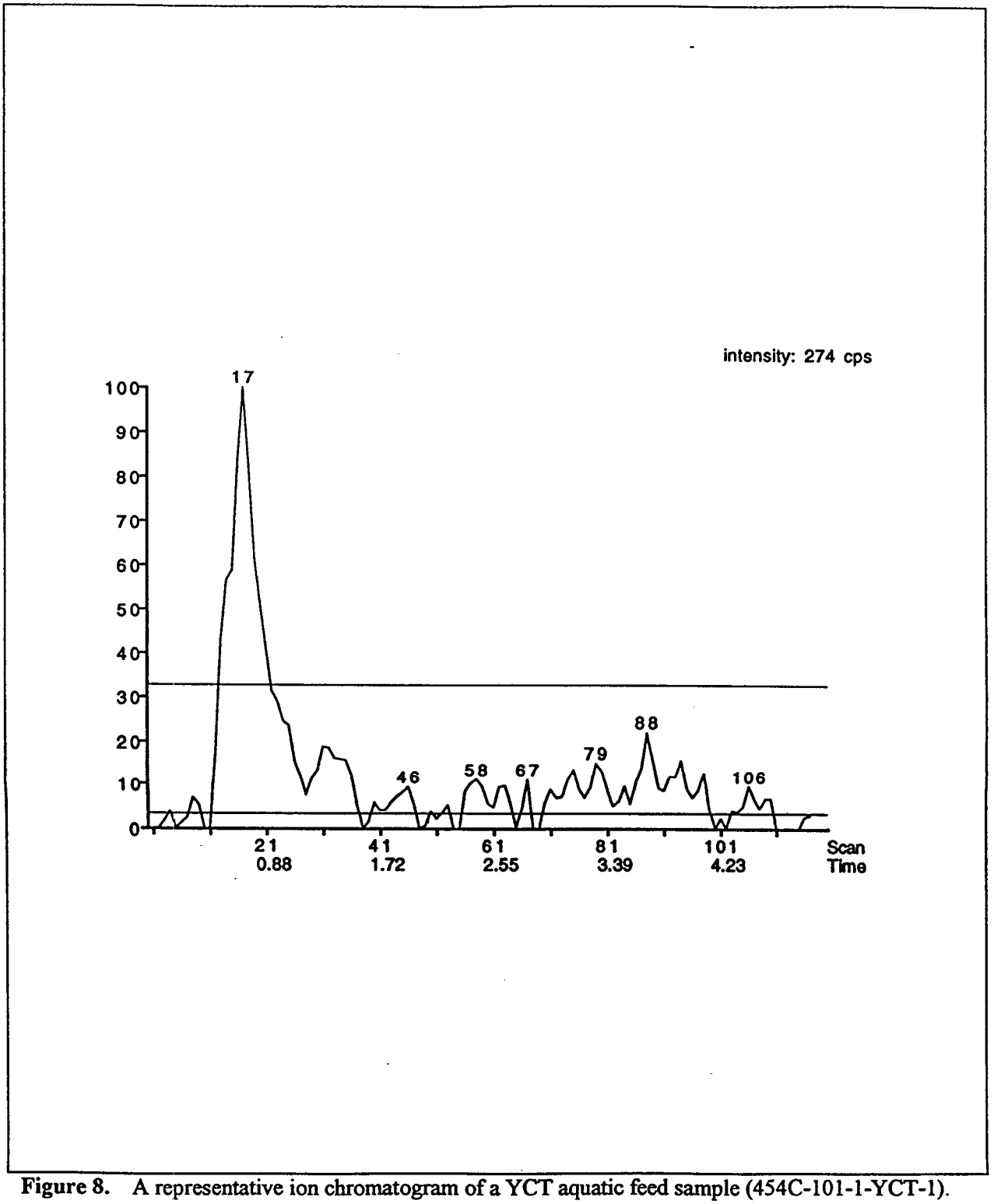

Figure 8. A representative ion chromatogram of a YCT aquatic feed sample (454C-101-1-YCT-1).

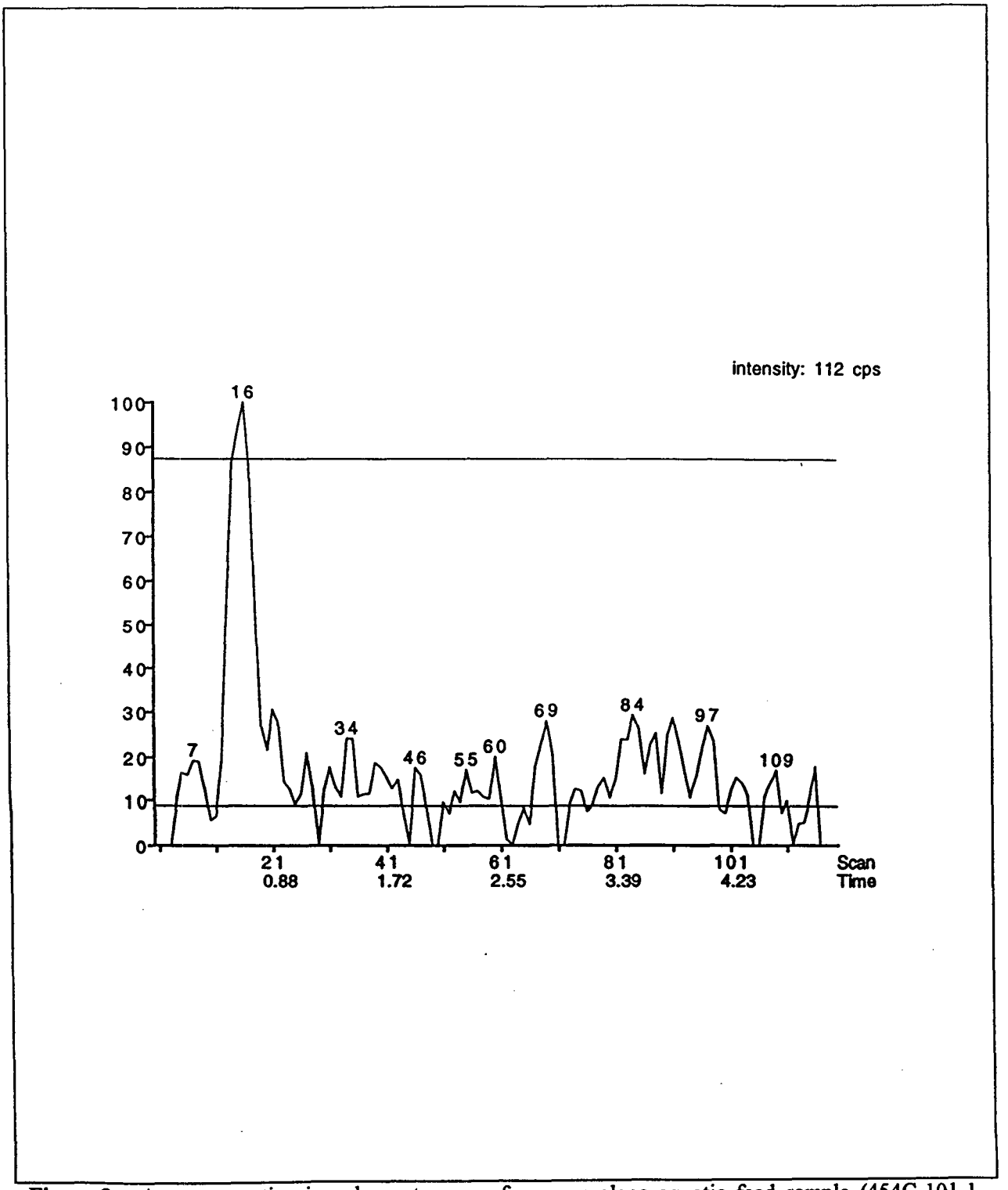

Figure 9. A representative ion chromatogram of a green algae aquatic feed sample (454C-101-1-GALG-1).

APPENDIX I

Diet Formulation
Wildlife International, Ltd. Game Bird Ration[1]

INGREDIENTS	PERCENT (%)
Fine Corn Meal	44.83
Soy Bean Meal, 48% Protein	30.65
Wheat Midds	6.50
Protein Base	6.00
Agway Special, 60% Protein	4.00
Alfalfa Meal, 20% Protein	3.00
Dried Whey	2.50
Ground Limestone	0.90
Eastman CalPhos	0.60
Methionine Premix + Liquid	0.35
Vitamin and Mineral Premix (see below)	0.32
GL Ferm (Fermatco)[2]	0.25
Salt Iodized	0.10
Total	100.00

VITAMIN AND MINERAL PREMIX	AMOUNT ADDED PER TON
Vitamin D_3	2,000,000 I.C.U.
Vitamin A	7,000,000 I.U.
Riboflavin	6 grams
Niacin	40 grams
Pantothenic Acid	10 grams
Vitamin B_{12}	8 mgs
Folic Acid	600 mgs
Biotin	64 mgs
Pyridoxine	1.2 grams
Thiamine	1.2 grams
Vitamin E	20,000 I.U.
Vitamin K (Menadione Dimethylpyrimidinol Bisulfite)	5.8 grams
Manganese	102 grams
Zinc	47 grams
Copper	6.8 grams
Iodine	1.5 grams
Iron	51 grams
Selenium	182 mgs

[1] The guaranteed analysis is a minimum of 27% protein, a minimum of 2.5% crude fat and a maximum of 5% crude fiber.
[2] Fermentation By-Products

APPENDIX II

Personnel Involved in the Study

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

1. Raymond L. Van Hoven, Ph.D., Scientist
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Jon A. MacGregor, B.S., Scientist
4. Norman A. Glassbrook, M.S., Senior Chemist
5. Keri B. Leach, B.S., Chemist