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TITLE

Method of Analysis for the Determination of Perfluorooctanoic Acid (PFOA) in Serum by LC/MS/MS - Revision 2

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1. SUMMARY

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

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

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

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

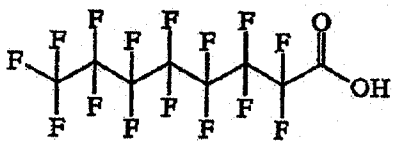
This method was developed using monkey serum. The overall percent recovery ± standard deviation for PFOA in monkey serum at 10, 100, and 1000 ppb was 94% ± 6.9% (Table I).

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

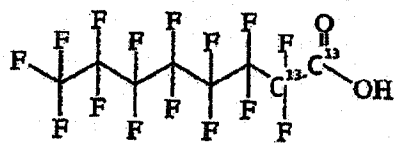
2. EXPERIMENTAL COMPOUNDS

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

PFOA
Chemical Name = perfluorooctanoic acid
Molecular weight = 414



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



3. CHEMICALS AND SUPPLIES

3.1. CHEMICALS

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

3.2. STANDARDS

Standard	Lot Number	Purity (%)	Source
perfluorooctanoic acid (PFOA)	21002	97	Oakwood Products
perfluorooctanoic acid, [1,2-di-^{13}C]	NA	96.35	DuPont

3.3. EQUIPMENT AND SUPPLIES

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

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

Notes:

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

3.4. SOLUTIONS

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

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

3.5. Preparation of Standards and Fortification Solutions

Analytical standards are used for three purposes:

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

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

3.5.1. Stock solution

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

3.5.2. Fortification Solutions

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

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

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

3.5.3. Calibration Standards

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

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

4. METHOD

4.1. FLOW DIAGRAM

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

Method Flow Diagram

Measure 0.1 mL of sample (using disposable micropipette)
(fortify samples when designated)

↓

Protein precipitation in acetonitrile

↓

LC/MS/MS analysis

4.2. SAMPLE PROCESSING

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

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

4.3. Sample Preparation

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

4.4. Extraction

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

4.5. Quantitation

4.5.1. LC/MS/MS System and Operating Conditions

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

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

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

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

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

Ions monitored:

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

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

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

4.5.2. Tune File Parameters

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

4.5.3. Calibration Procedures

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

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

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

4.5.4. Sample Analysis

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

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

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

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

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

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

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

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

4.6. Acceptance Criteria

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

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

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

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

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

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

4.7. Performance Criteria

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

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

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

4.8. TIME REQUIRED FOR ANALYSIS

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

5. CALCULATIONS

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

Equation 1:

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

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

Equation 2:

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

6. SAFETY

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

Table I. Recovery of PFOA from Fortifications In Monkey Serum

Summary of PFOA Recoveries for 10 ppb Fortification in Monkey Serum

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

Summary of PFOA Recoveries for 100 ppb Fortification in Monkey Serum

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

Summary of PFOA Recoveries for 1000 ppb Fortification in Monkey Serum

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

Figure 1. Calibration curve for PFOA in Control Monkey Serum

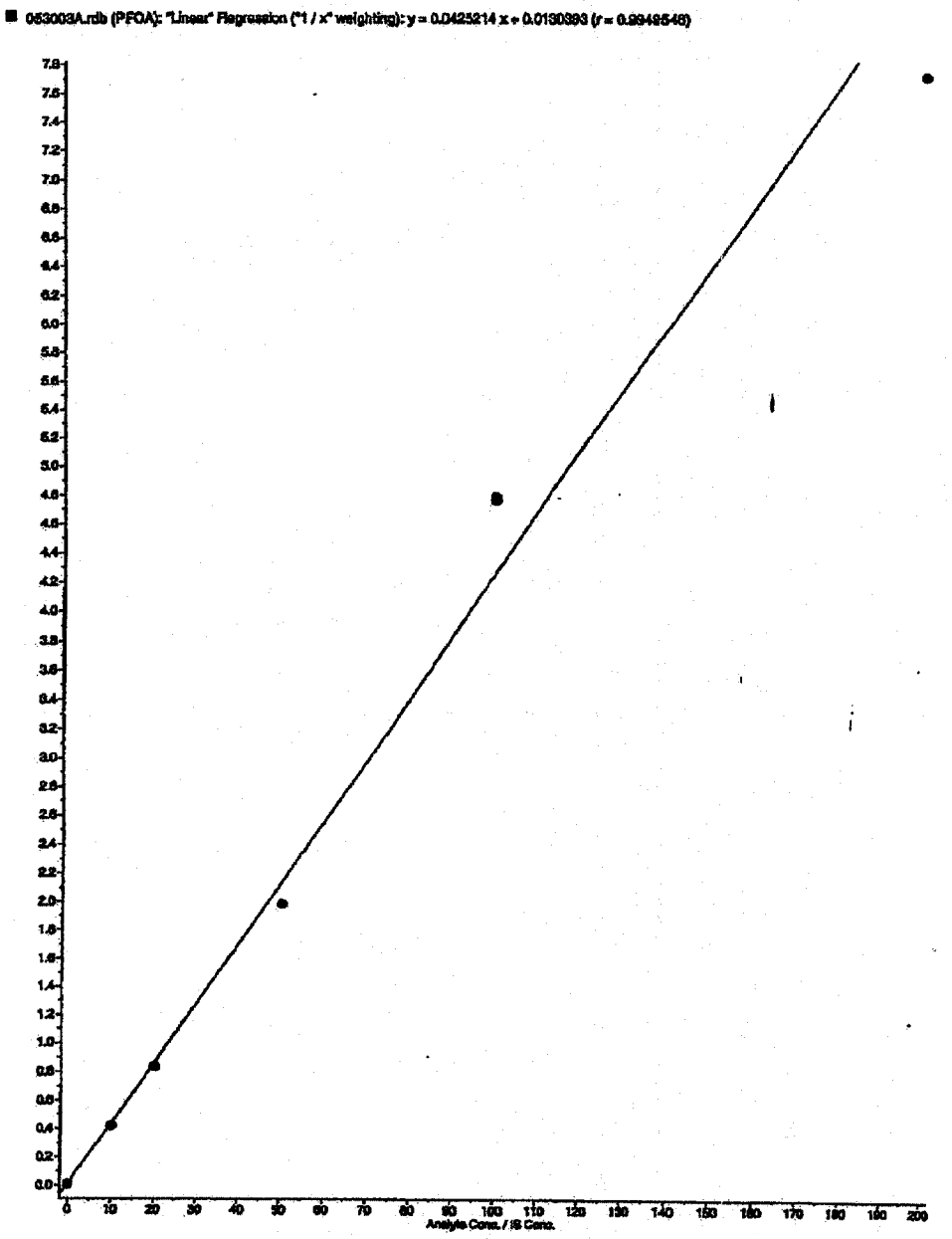


Figure 2. Representative Chromatogram of a Control Monkey Serum Sample for PFOA with ~ 5 ng/mL of ^{13}C-PFOA

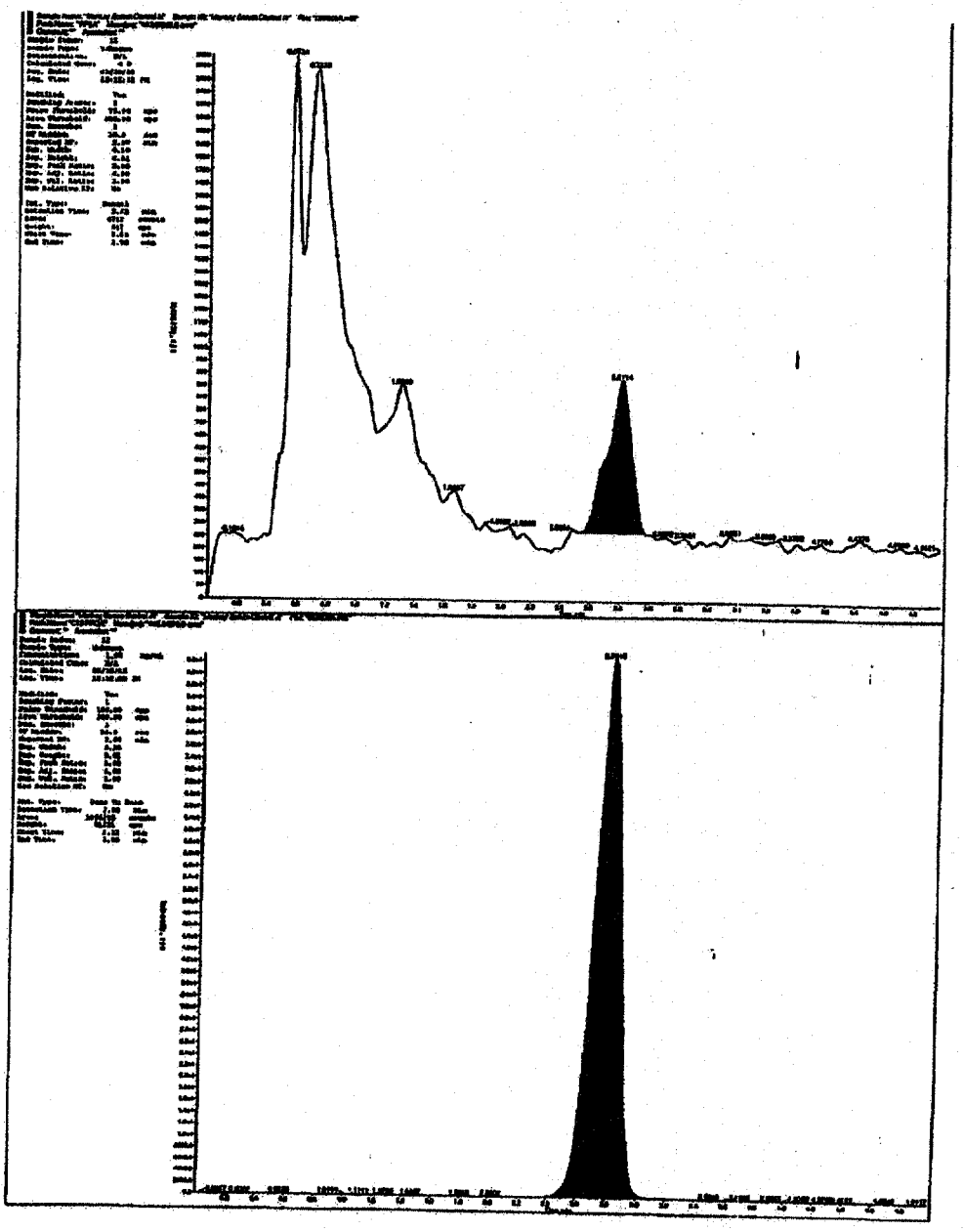


Figure 3. Representative Chromatogram of a 10 ppb Standard for PFOA in Control Monkey Serum with ~ 5 ng/mL of ^{13}C-PFOA

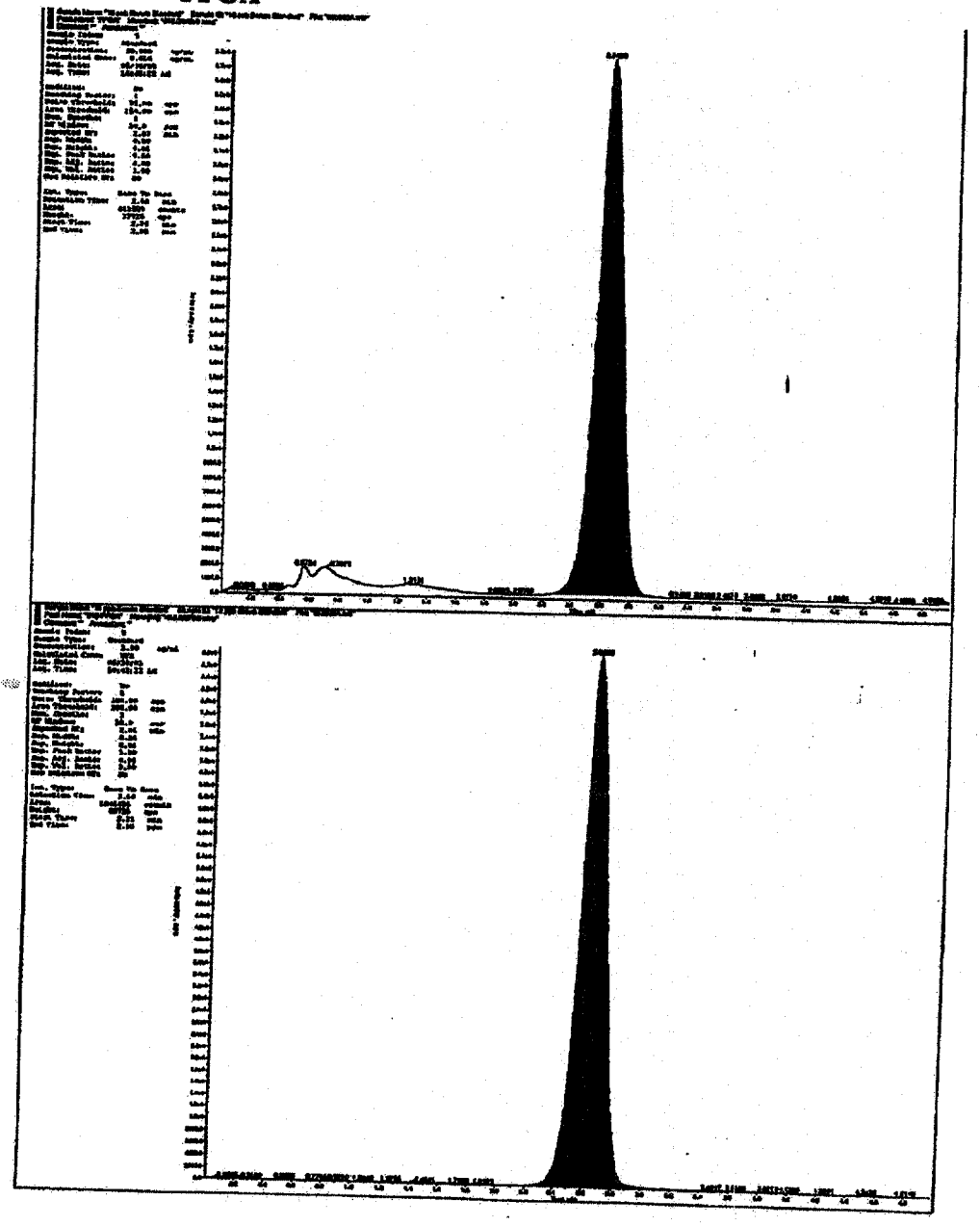


Figure 4. Representative Chromatogram of Control Monkey Serum Fortified at 10 ppb with PFOA and with ~ 5 ng/mL of ^{13}C-PFOA