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METHOD VALIDATION REPORT

TITLE: Method Validation for the Quantitation of Ammonium Perfluorooctanoate (APFO), Measured as the Perfluorooctanoate Anion (PFOA, 0.05 to 10 µg/mL Concentration Range), in Human Serum by Turbo Ion Spray LC/MS/MS

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REPORT: 02120VDJA_DU.DOC

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ABSTRACT

A sensitive, selective, accurate, and reproducible analytical method was developed by Advion BioSciences, Inc., Ithaca, New York to quantitate ammonium perfluorooctanoate (APFO), measured as the perfluorooctanoate anion (PFOA), in human serum samples. PFOA and its internal standard (IS) were isolated from human serum samples (25 µL) by a liquid-liquid extraction procedure. An aliquot of the organic layer from each sample was evaporated to dryness, reconstituted and analyzed by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the negative ion mode. The assay demonstrated a lower limit of quantitation of 0.05 µg/mL for PFOA. The calibration curve range was quadratic from 0.05 µg/mL to 10 µg/mL for PFOA. The coefficients of determination (r^2) of the calibration curves were ≥0.9950 for PFOA.

Precision and accuracy quality control (QC) samples were prepared at concentrations of 0.15, 3, and 8 µg/mL PFOA. The intra- and inter-assay precision (CV) results calculated from QC samples ranged from 2.07 to 7.70% for PFOA. The intra- and inter-assay accuracy (RE) calculated from QC samples ranged from –12.3 to 9.00% for PFOA.

The results for PFOA were reproducible upon reinjection of prepared samples after storage for up to 65 hours at room temperature. The extraction recovery of PFOA and the IS from human serum were 81.8 and 90.9%, respectively. The matrix effect

on the ionization of PFOA and the IS was an enhancement of 1.62 and 8.07%, respectively.

SIGNATURE PAGE

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Report Number: 02120VDJA_DU.DOC

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QAU STATEMENT

Periodic audits of the method validation for the quantitation of APFO, measured as PFOA, in human serum were conducted by the Quality Assurance Unit of Advion BioSciences, Inc. (Advion).

The study was inspected on the following dates:

Phase of Study Inspected	Date of Inspection	Date Reported to Management
Data Review	6, 7, 11 June 2002	6, 7, 11 June 2002
Report	20 June 2002	20 June 2002

Based on the inspections and the data reviewed, this report is a complete and accurate representation of the data.

George C. Amedro

George C. Amedro, B.S.
Quality Assurance Auditor

12 July 02
Date

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1. INTRODUCTION

The purpose of this report is to describe the method validation for the quantitative determination of ammonium perfluorooctanoate (APFO), measured as the perfluorooctanoate anion (PFOA), in human serum samples. The objectives of the method validation were to validate a sample preparation procedure, determine the lower limit of quantitation (LLQ) for routine analysis, demonstrate accuracy of dilution, determine recovery and matrix effect, and to provide selective, accurate, and reproducible quantitative results by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS). This work was conducted in accordance with Advion BioSciences, Inc. (Advion) Standard Operating Procedures (SOP).

2. EXPERIMENTAL

2.1. CHEMICALS AND MATERIALS

Pentadecafluorooctanoic acid ammonium salt (Lot# 421207/1) was obtained from Sigma-Aldrich, St. Louis, MO. The internal standard (IS), 9H-hexadecafluorononanoic acid (9H-HDFNA, No Lot# available), was obtained from Sigma-Aldrich, St. Louis, MO. A detailed list of chemicals and materials is found in Appendix A.

Stock and working solutions used to prepare the calibration curves and quality control (QC) samples for PFOA were prepared as described in Appendix A.

2.2. LC/MS/MS INSTRUMENTATION

The liquid chromatography/mass spectrometry system consisted of two LC-10AD vp pumps (Shimadzu, Columbia, MD), an SCL-10A vp pump controller (Shimadzu, Columbia, MD), a Perkin-Elmer Series 200 autosampler (Perkin-Elmer Corp., Norwalk, CT), a Betasil C_{18} column (2 x 50 mm, 5 µm particle size, ThermoHypersil-Keystone, Bellefonte, PA), a model 7970 column heater (Jones Chromatography USA Inc., Lakewood, CO), and a SCIEX API 3000 mass

spectrometer (Applied BioSystems, Concord, Ontario). A detailed list of the instrumentation and instrument conditions is found in Appendix A.

2.3. Sample Preparation and Extraction Procedure

For each analytical run, duplicate 25-µL aliquots of the calibration curve samples were prepared as described in Appendix A. The calibration curve concentrations were 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 7.5, and 10 µg/mL for PFOA.

Human serum QC samples were prepared in advance of the validation study at concentrations of 0.15, 3, and 8 µg/mL PFOA for QC1, QC2, and QC3, respectively, as detailed in Appendix A. QC4 (50-µg/mL dilution QC) was prepared at a concentration exceeding the calibration curve range, and was assayed using a 10-fold dilution.

2.4. Human Serum Validation Data

The data were collected using selected reaction monitoring (SRM) turbo ion spray LC/MS/MS in the negative ion mode. Peak areas were integrated by the SCIEX program MacQuan, version 1.6, residing on a Macintosh computer. Background subtraction was performed using Excel 98 to correct for the presence of PFOA in the control serum lots as detailed in Appendix A (A.14.0). Following peak area integration and background subtraction, the results tables from MacQuan were saved as text files and uploaded to the Advion BioSciences, Inc. (Advion) file server where a weighted ($1/x^2$) quadratic regression was performed using the software package Watson v 6.1.0.03 (InnaPhase, Inc., Philadelphia, PA). All concentration data were downloaded from Watson to Excel 97 with three decimal places for this report, with the exception of QC LLQ and Std 1 replicates, which were downloaded with four decimal places. Means, CV values, and RE values were calculated in Excel prior to rounding. Concentration data, concentration means, CV values, and RE values were rounded to three significant figures in Excel prior to reporting. The data for the human serum validation are stored in Advion Notebook 02120.

All calculations were based on the peak area ratio of PFOA to the internal standard. Concentrations of PFOA in QC samples were determined by inverse prediction from the calibration curve.

2.5. ASSAY EVALUATION

2.5.1. Intra- and Inter-Assay Precision

The intra- and inter-assay precision of the method was assessed by determining the coefficient of variation (CV) observed in the analysis of QC samples. The CV was determined by dividing the standard deviation by the mean concentration for each QC level and expressing the result as a percentage. The intra-assay precision was expressed as the CV of the replicate analyses of a QC level during a single validation run. The inter-assay precision was expressed as the CV of the overall mean of the individual replicate concentrations of the QC samples for three validation runs. QC1, QC2, and QC3 were assayed in replicates of six. In addition, the inter-assay CV was also reported for calibration standards at all levels.

2.5.2. Intra- and Inter-Assay Accuracy

The intra- and inter-assay accuracy of the method was assessed by determining the relative error (RE) observed in the analysis of QC samples. The RE was determined by subtracting the nominal concentration from the mean concentration for each QC level, dividing the result by the nominal value, and then expressing the result as a percentage. The intra-assay accuracy was expressed as the RE of the replicate analyses of QC samples during a single validation run. Inter-assay accuracy was expressed as the RE of the overall mean of the individual replicate concentrations of the QC samples for three validation runs. QC1, QC2, and QC3 were assayed in replicates of six. In addition, the inter-assay RE was also reported for calibration standards at all levels.

2.5.3. Sensitivity: Lower Limit of Quantitation (LLQ)

A human serum QC was prepared at a concentration of 0.05 µg/mL PFOA and assayed in replicates of six. The LLQ was determined by obtaining the back-calculated concentrations from these QC samples. The mean concentration, CV, and RE were also calculated.

2.5.4. Selectivity

The selectivity of the assay was determined by LC/MS/MS. To monitor for interference from the biological matrix within runs, unfortified control blank human serum samples were assayed with all experiments except recovery and matrix effect.

In addition, six replicate zero samples (control blank human serum samples fortified with IS) were extracted in each of the two different lots of control serum used for the standard curves and QC samples. These zero samples were used for the background subtraction due to the presence of PFOA in the control serum lots (Section A.14.0.).

To monitor for lot-specific matrix interference, six separate lots of human serum were spiked with PFOA at the LLQ and upper limit of quantitation (ULQ) concentration levels and were analyzed along with a control blank and zero sample prepared from each lot.

2.5.5. Chromatographic Carryover

The carryover of analyte from the injection of one sample to another was assessed in each run by placing control blanks after each high standard (STD9) and monitoring for analyte and IS response.

2.5.6. Accuracy and Precision of Dilution

The accuracy and precision of dilution in the analysis of PFOA in human serum was assessed. QC4 samples (50 µg/mL) were analyzed at a 10-fold dilution in replicates of six. The mean concentration, CV, and RE were calculated.

2.6. Stability of PFOA in Human Serum

The stability of PFOA in human serum during sample storage, preparation and analysis will be assessed and reported in the 1 to 100 ng/mL-concentration range human serum method validation.[1]

2.7. Reproducibility of Reinjecting Prepared Samples

The reproducibility of reinjecting extracted and reconstituted serum samples was investigated by reinjecting a set of previously-assayed standards and QC samples that had been stored after injection at room temperature for 65 hours. The CV and RE of the QC samples were used to assess processed sample stability in reconstitution solution. All QC samples were assayed in replicates of six.

2.8. Extraction Recovery

The determination of extraction recovery was initially performed in Runs 6 and 7, but the data obtained from those experiments are believed to be inaccurate, mainly due to the higher than expected absolute recoveries seen (100% maximum expected from procedure after correction for 1/10 aliquot removed from organic layer during extraction). It is suspected that because of the large differences (10-fold) in concentration between the samples spiked before extraction (pre-extract) and the samples spiked after extraction (post-extract) in those experiments, coupled with the non-linearity seen in detector responses, the predicted recoveries were biased. The experiment was repeated in Run 8 in which the post-extract samples were diluted 10-fold to compensate for the 1/10 aliquot. Accordingly, the concentrations of the

pre- and post-extract samples would now be much closer to each other and thus be less biased by the non-linearity.

The extraction recovery of PFOA from human serum was determined in MacQuan by performing separate weighted ($1/x^2$) regressions for the pre-extract and post-extract samples which were prepared at the nominal concentrations 0.15, 3, and 8 µg/mL in replicates of five. The extraction recovery was calculated as the slope ratio of the pre-extract curve over the post-extract curve. The IS was spiked post-extraction for PFOA recovery determination.

The extraction recovery of the IS from human serum was determined by comparing the peak area ratios (PAR) of pre-extract and post-extract samples (5 µg/mL 9H-HDFNA) assayed in replicates of five. In this experiment only, the PFOA was used as internal standard and was spiked post-extraction for the 9H-HDFNA recovery determination. The extraction recovery (% Recovery) was determined by dividing the pre-extract PAR by the post-extract PAR and expressing the result as a percentage.

2.9. MATRIX EFFECT ON IONIZATION OF PFOA AND 9H-HDFNA IN HUMAN SERUM

The matrix effect on the ionization of PFOA in human serum extract was determined by comparing the peak area of samples (0.15, 3, and 8 µg/mL for PFOA) spiked in blank human serum extract (post-extract) with the peak area of an unextracted solution (neat spike). The matrix effect was determined by subtracting the neat spike peak area from the post-extract peak area, dividing the result by the neat peak spike peak area, and expressing the result as a percentage. Five replicates were used at each concentration.

The matrix effect on the ionization of 9H-HDFNA in human serum extract was similarly determined by comparing the peak area of blank human serum extract spiked with 5 µg/mL 9H-HDFNA after extraction with the peak area of an

unextracted solution (neat spike). The post-extract and neat solution samples were prepared in replicates of five.

3. RESULTS AND DISCUSSION

A quantitative analytical procedure using turbo ion spray LC/MS/MS in the negative ion mode was developed to meet the high sensitivity, selectivity, and reproducibility requirements for the determination of PFOA in human serum. A list of the validation runs is presented in Table 1.

The full-scan turbo ion spray mass spectrum of PFOA showed the base peak ion at m/z = 413 which is attributed to the deprotonated molecule (Figure 1). The full-scan product ion spectrum of PFOA showed the base peak product ion at 169 (Figure 2).

The full-scan turbo ion spray mass spectrum of 9H-HDFNA showed the base peak ion at m/z = 445 which is attributed to the deprotonated molecule (Figure 3). The full-scan product ion spectrum of 9H-HDFNA showed the base peak product ion at 381 (Figure 4).

The following respective $[M-H]^-$ ions were used in data acquisition for PFOA and the IS in human serum (nominal):

PFOA	$m/z = 413 \rightarrow 169$
9H-HDFNA	$m/z = 445 \rightarrow 381$

The SRM chromatograms from a representative control blank human serum extract are shown in Figure 5. SRM chromatograms from a representative control human serum extract containing only the internal standard (zero sample), are shown in Figure 6. Both Figures 5 and 6 show the presence of PFOA in the control human serum.

Figures 7 and 8 are SRM chromatograms from representative extracts from calibration standards 1 (LLQ) and 9 (ULQ), respectively.

3.1. ASSAY EVALUATION RESULTS

3.1.1. Intra- and Inter-Assay Precision

Table 2 presents the precision data for the PFOA QC samples from the three validation runs. The intra- and inter-assay precision (CV) was ≤7.70% for PFOA at QC1, QC2, and QC3 concentration levels. Table 3 presents the precision data for calibration standards. The CV for calibration standards was ≤10.0% for PFOA. These data indicate acceptable intra- and inter-assay precision for the determination of PFOA in human serum.

Only five replicates of QC1 were successfully prepared in Run 3 due to a broken test tube. Also, results for only five replicates of QC3 in Run 5 were calculated due to a probable preparation error. This is in compliance with Advion SOPs.

3.1.2. Intra- and Inter-Assay Accuracy

Table 2 presents the accuracy data for the PFOA QC samples from the three validation runs. The intra- and inter-assay accuracy (RE) ranged from –12.3 to 9.00% for PFOA. Table 3 presents the accuracy data for calibration standards. The RE for calibration standards ranged from –3.99 to 6.92% for PFOA in human serum. These data indicate acceptable intra- and inter-assay accuracy for the determination of PFOA in human serum.

3.1.3. Calibration Curve Fit

The calibration curves were fit by a weighted ($1/x^2$) quadratic regression. Coefficients of determination (r^2) were ≥0.9950 for PFOA in human serum. The calibration curve statistics are shown in Table 4.

3.1.4. Sensitivity: Lower Limit of Quantitation (LLQ)

Tables 5 shows the results from the QC at the LLQ for PFOA. The experiment demonstrated that 0.05 µg/mL is an acceptable LLQ level for PFOA. The CV was 2.95%, and the RE was 9.73% for the LLQ QC.

3.1.5. Selectivity

The assay was highly selective for PFOA. No chromatographic interferences were observed at the retention time of PFOA or the IS in the control serum samples analyzed (Figure 5). The control blanks did show the presence of PFOA in the control human serum.

The selectivity between matrix lots produced acceptable results after background subtraction (Table 6). The precision (CV) for the PFOA samples was 7.18 and 5.21% for the LLQ and ULQ samples, respectively. The corresponding accuracy was 0.133 and 11.8% for the LLQ and ULQ samples.

3.1.6. Chromatographic Carryover

A chromatographic peak was observed at the retention time for PFOA in the human serum control blanks following high standards. The responses were comparable to those of the control blanks injected before the standard curves. This indicates that carryover was negligible for PFOA. Carryover for the internal standard was insignificant.

3.1.7. Accuracy and Precision of Dilution

The results from QC4 samples diluted 10-fold are shown in Table 7. The CV was 4.25% and the RE value was –10.6% for PFOA. These data demonstrate acceptable results for the dilution of samples.

3.2. Reproducibility of Reinjecting Extracted Samples

The results of reinjecting extracted and reconstituted samples containing PFOA after storage for 65 hours at room temperature in reconstitution solution are shown in Table 8. The CV values for samples injected initially and those reinjected after 65 hours in reconstitution solution ranged from 2.68 to 8.12%, and the RE values range from –12.3 to –1.41% for PFOA. These data indicate that reinjecting samples after storage for 65 hours in reconstitution solution at room temperature is acceptable.

3.3. Extraction Recovery

The extraction recoveries of PFOA and the IS, 9H-HDFNA, from human serum are shown in Tables 9 and 10, respectively. The mean recovery values were 81.8% for PFOA and 90.9% for 9H-HDFNA.

3.4. Matrix Effect on Ionization of PFOA and 9H-HDFNA in Human Serum

The results of the matrix effect of human serum extract on the ionization of PFOA and the IS are shown in Tables 11 and 12, respectively. The overall mean matrix effect was 1.62% for PFOA and 8.07% for 9H-HDFNA, indicating slight enhancement of ionization.

4. SOP DEVIATION

According to Advion SOP DOCU0015.02, the volume of non-matrix solvent used for spiking QC samples must be ≤5% of the total volume. QC4 (dilution QC) was prepared by spiking a volume of stock solution that was 10% of the total volume so that new stock solution did not have to be prepared. This deviation did not affect the quality of the data.

There were no other known SOP deviations during the validation.

5. DATA RETRIEVAL

The data for the human serum validation are stored in Advion Notebook 02120 and in the Advion archives.

6. CONCLUSIONS

The turbo ion spray LC/MS/MS assay procedure for PFOA in human serum has proven to be sensitive, selective, accurate, and reproducible. Its high sensitivity allows reliable and reproducible quantitation of PFOA down to a level of 0.05 µg/mL in human serum based on 25-µL samples.

7. REFERENCE

1. Advion Report No. 02086VDJA_DU.DOC, "Method Validation for the Quantitation of Ammonium Perfluorooctanoate (APFO), Measured as the Perfluorooctanoate Anion (PFOA, 1 to 100 ng/mL Concentration Range) in Human Serum by Turbo Ion Spray LC/MS/MS," to be issued.

8. TABLES

Table 1: Validation Run Descriptions

Run No.	Assay Date	Run Description	Status
1	6-May-02	Accuracy and Precision; Dilution	Accepted
2	7-May-02	Accuracy and Precision; LLQ	Rejected
3	8-May-02	Accuracy and Precision; LLQ	Accepted
4	9-May-02	Reinjection of Run 1	Accepted
5	10-May-02	Accuracy and Precision; Selectivity	Accepted
6	22-May-02	Recovery and Matrix Effect	Rejected
7	23-May-02	Recovery and Matrix Effect	Accepted (recovery rejected)
8	5-June-02	Recovery	Accepted

Table 2: Accuracy and Precision of the PFOA Assay in Human Serum for Quality Control Samples from Three Validation Runs

	PFOA Concentration (µg/mL)		
	QC1	QC2	QC3
Run Number	0.15	3	8
1	0.147	2.73	8.47
	0.138	2.55	8.35
	0.139	2.56	7.54
	0.156	2.59	7.45
	0.144	2.67	7.37
	0.125	2.68	7.57
Mean	0.142	2.63	7.79
CV (%)	7.33	2.68	6.26
RE (%)	-5.67	-12.3	-2.61
3	0.164	2.82	7.62
	0.159	2.78	7.97
	0.158	2.74	7.48
	0.152	2.85	7.33
	0.153	2.77	7.77
	NS	2.69	7.83
Mean	0.157	2.77	7.67
CV (%)	3.10	2.07	3.06
RE (%)	4.80	-7.56	-4.16
5	0.169	2.96	7.83
	0.166	2.88	8.17
	0.157	2.78	8.06
	0.162	2.88	EQB
	0.165	2.81	7.75
	0.162	2.92	8.65
Mean	0.164	2.87	8.09
CV (%)	2.53	2.32	4.38
RE (%)	9.00	-4.24	1.12
Overall Mean	0.154	2.76	7.84
Overall CV (%)	7.70	4.33	5.02
Overall RE (%)	2.59	-8.04	-2.06

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

NS = No sample. Only 5 replicates were prepared for QC1 in Run 3.

EQB = Exceeds quadratic bounds

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Table 3: **Accuracy and Precision of the PFOA Assay in Human Serum for Calibration Standards from Three Validation Runs**

	PFOA Concentration (µg/mL)								
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9
Run Number	0.05	0.1	0.25	0.5	1	2	5	7.5	10
1	0.0534	0.103	0.255	0.574	1.01	2.06	4.80	7.75	9.84
	0.0463	0.0967	0.246	0.469	0.932	1.95	4.83	6.97	11.6
3	0.0513	0.112	0.243	0.539	1.02	2.02	4.90	7.34	10.5
	0.0457	0.102	0.231	0.492	1.01	1.90	4.66	7.20	11.0
5	0.0562	0.106	0.252	0.505	1.05	2.11	4.95	7.26	11.0
	0.0436	0.0950	0.244	0.488	0.996	1.95	4.66	7.26	10.1
Mean	0.0494	0.102	0.245	0.511	1.00	2.00	4.80	7.30	10.7
CV (%)	10.0	6.06	3.42	7.55	3.82	3.88	2.49	3.49	6.08
RE (%)	-1.17	2.45	-1.93	2.23	0.233	-0.125	-3.99	-2.71	6.92

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

Table 4: Calibration Curve Parameters for PFOA in Human Serum from Three Validation Runs

Run Date	Run Number	A	B	C	Coefficient of Determination (r^2)
6-May-02	1	-0.011789	0.360080	0.000634	0.9950
8-May-02	3	-0.009249	0.317953	-0.000595	0.9955
10-May-02	5	-0.008130	0.289836	-0.000240	0.9951
Mean		-0.009723	0.322623	-0.000067	0.9952

$y = Ax^2 + Bx + C$, weighted $1/x^2$

Table 5: Lower Limit of Quantitation of PFOA in Human Serum

	PFOA Conc. (µg/mL)
	QC LLQ
Run 3	0.05
	0.0562
	0.0537
	0.0574
	0.0540
	0.0547
	0.0532
Mean	0.0549
CV (%)	2.95
RE (%)	9.73

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

Table 6: **Lower Limit of Quantitation of PFOA and Selectivity in Six Separate Lots of Human Serum**

	PFOA Concentration (µg/mL)	
Run 5	LLQ	ULQ
Serum lot	0.05	10
22-48635	0.0544	10.6
55-03231	0.0467	10.6
55-03232	0.0508	10.8
55-03681	0.0531	11.4
55-03624	0.0503	11.6
55-03707	0.0451	12.0
Mean	0.0501	11.2
CV (%)	7.18	5.21
RE (%)	0.133	11.8

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

Table 7: Accuracy and Precision of the PFOA Assay in Human Serum Dilution QC Samples from One Validation Run

	PFOA Conc. (µg/mL)
	QC4 (1 in 10 dilution)
Run 1	50
	47.5
	42.7
	45.3
	43.9
	42.9
	46.0
Mean	44.7
CV (%)	4.25
RE (%)	-10.6

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

Table 8: **Reinjection Reproducibility of QC Samples Containing PFOA After Storage in Reconstitution Solution for 65 Hours at Room Temperature**

	PFOA Concentration (µg/mL)			
	QC1	QC2	QC3	QC4 (1 in 10 dilution)
	0.15	3	8	50
Run 1	0.147	2.73	8.47	47.5
t=0	0.138	2.55	8.35	42.7
	0.139	2.56	7.54	45.3
	0.156	2.59	7.45	43.9
	0.144	2.67	7.37	42.9
	0.125	2.68	7.57	46.0
Mean	0.142	2.63	7.79	44.7
CV (%)	7.33	2.68	6.26	4.25
RE (%)	-5.67	-12.3	-2.61	-10.6
Run 4	0.150	2.78	8.10	47.6
t=65 hours	0.146	2.88	8.81	45.2
	0.140	2.62	7.76	47.4
	0.145	2.79	7.52	45.8
	0.141	2.65	7.63	46.0
	0.118	2.57	7.50	46.3
Mean	0.140	2.72	7.89	46.4
CV (%)	8.12	4.43	6.37	2.05
RE (%)	-6.67	-9.48	-1.41	-7.27

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

Table 9: Extraction Recovery of PFOA from Human Serum

Run 8	PFOA Concentration (µg/mL)	
	Pre-Extract	Post-Extract
QC1	0.162	0.155
0.15 µg/mL	0.141	0.152
	0.155	0.147
	0.145	0.149
	0.146	0.144
Mean	0.150	0.149
RE (%)	-0.133	-0.400
QC2	3.21	3.40
3 µg/mL	3.07	3.27
	2.95	3.35
	3.05	3.36
	3.50	3.20
Mean	3.16	3.32
RE (%)	5.22	10.5
QC3	8.15	7.25
8 µg/mL	7.40	7.15
	7.51	7.12
	7.53	7.18
	7.39	7.22
Mean	7.60	7.18
RE (%)	-5.05	-10.2

Regression	Pre-Extract	Post-Extract	Recovery (%)
Coefficient of Determination (r^2)	0.9930	0.9878	
Intercept	0.01125	-0.01220	
Slope	0.23325	0.28522	81.8

Pre-Extract = Matrix spiked with PFOA prior to extraction
Post-Extract = Matrix spiked with PFOA after extraction
RE = [(Mean-Nominal)/Nominal]*100
%Recovery = ($Slope_{Pre\text{-}extract}$/$Slope_{Post\text{-}extract}$)*100

Table 10: Extraction Recovery of the Internal Standard, 9H-HDFNA, from Human Serum

Run 8	Peak Area Ratio (IS/PFOA)		Recovery (%)
	Pre-Extract	Post-Extract	
IS	0.43745	0.49204	
5 µg/mL	0.45424	0.49670	
	0.44736	0.49028	
	0.46195	0.50136	
	0.44684	0.49258	
Mean	0.44957	0.49459	90.9
CV (%)	2.03	0.901	

Pre-Extract = Matrix spiked with 9H-HDFNA prior to extraction

Post-Extract = Matrix spiked with 9H-HDFNA after extraction

CV = (SD/Mean)*100

%Recovery = $(Mean_{Pre\text{-}extract}/Mean_{Post\text{-}extract})*100$

Table 11: Matrix Effect on Ionization of PFOA in Human Serum

Run 7	PFOA Area		Matrix Effect (%)
	Post-Extract	Neat Spike	
QC1	19775	19573	
0.15 µg/mL	19835	18856	
	20826	20522	
	19880	18616	
	20597	18333	
Mean	20183	19180	5.23
CV (%)	2.43	4.59	
QC2	426092	399353	
3 µg/mL	418407	408972	
	408036	419660	
	425636	400556	
	430791	401508	
Mean	421792	406010	3.89
CV (%)	2.10	2.10	
QC3	772284	772628	
8 µg/mL	747001	794112	
	757322	805450	
	769807	817268	
	776010	803311	
Mean	764485	798554	-4.27
CV (%)	1.57	2.09	
Overall Mean			1.62

Post-Extract = Matrix spiked with PFOA after extraction

Neat Spike = Solution of PFOA without matrix

CV = (SD/Mean)*100

Matrix Effect = $[(Mean_{Post\ Extract} - Mean_{Neat\ Spike})/Mean_{Neat\ Spike}]*100$, where a negative number indicates ion suppression and a positive number indicates enhancement

Table 12: Matrix Effect on Ionization of 9H-HDFNA in Human Serum

Run 7	9H-HDFNA Area		Matrix Effect (%)
	Post-Extract	Neat Spike	
IS	349304	306352	
5 µg/mL	336593	330146	
	351612	310681	
	335236	322237	
	335737	311522	
Mean	341696	316188	8.07
CV (%)	2.36	3.08	

Post-Extract = Matrix spiked with 9H-HDFNA after extraction

Neat Spike = Solution of 9H-HDFNA without matrix

CV = (SD/Mean)*100

Matrix Effect = $[(Mean_{Post\ Extract} - Mean_{Neat\ Spike})/Mean_{Neat\ Spike}]*100$, where a negative number indicates ion suppression and a positive number indicates enhancement

9. FIGURES

Figure 1: Full-Scan Turbo Ion Spray Mass Spectrum of PFOA

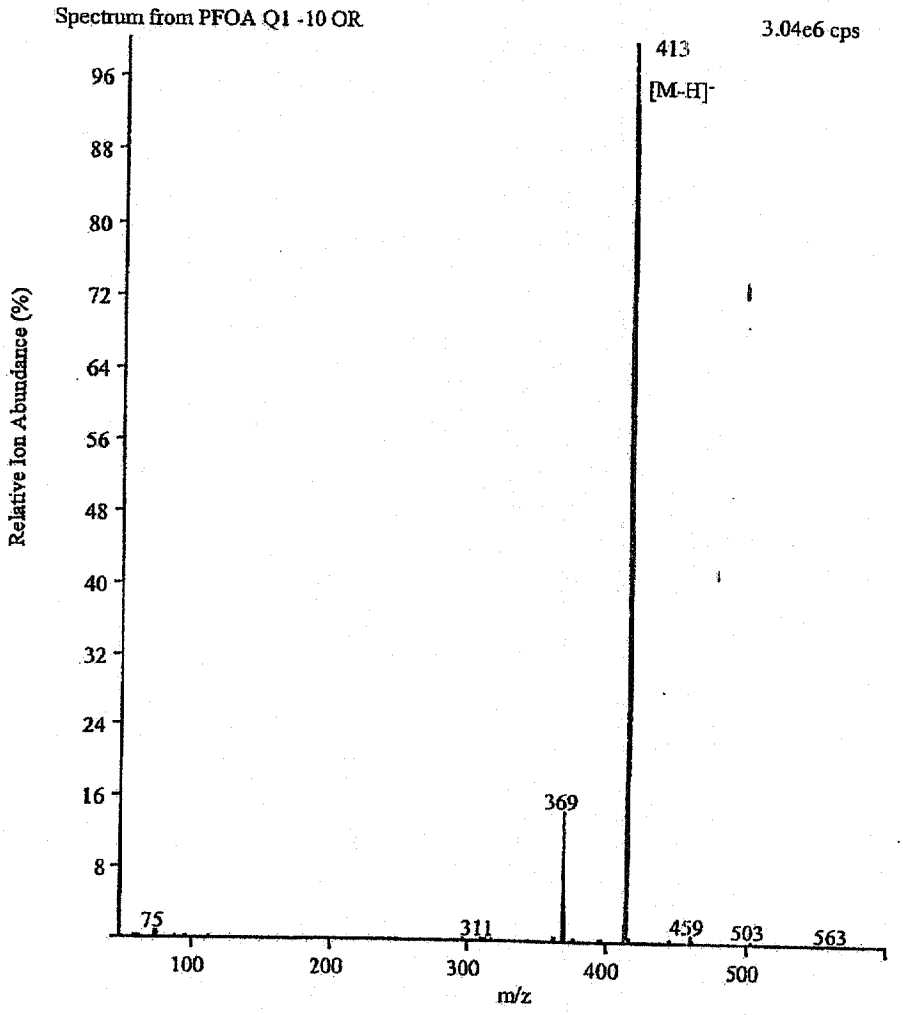


Figure 2: Full-Scan Product Ion Mass Spectrum of PFOA

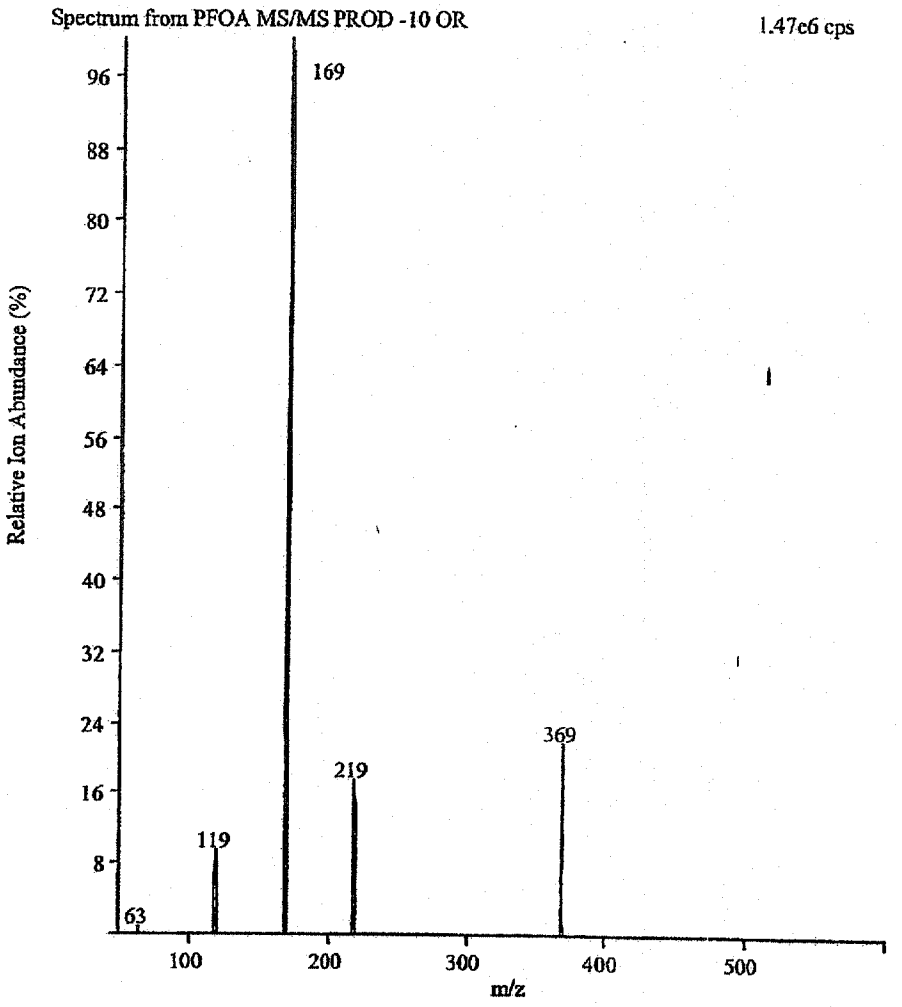


Figure 3: Full-Scan Turbo Ion Spray Mass Spectrum of 9H-HDFNA

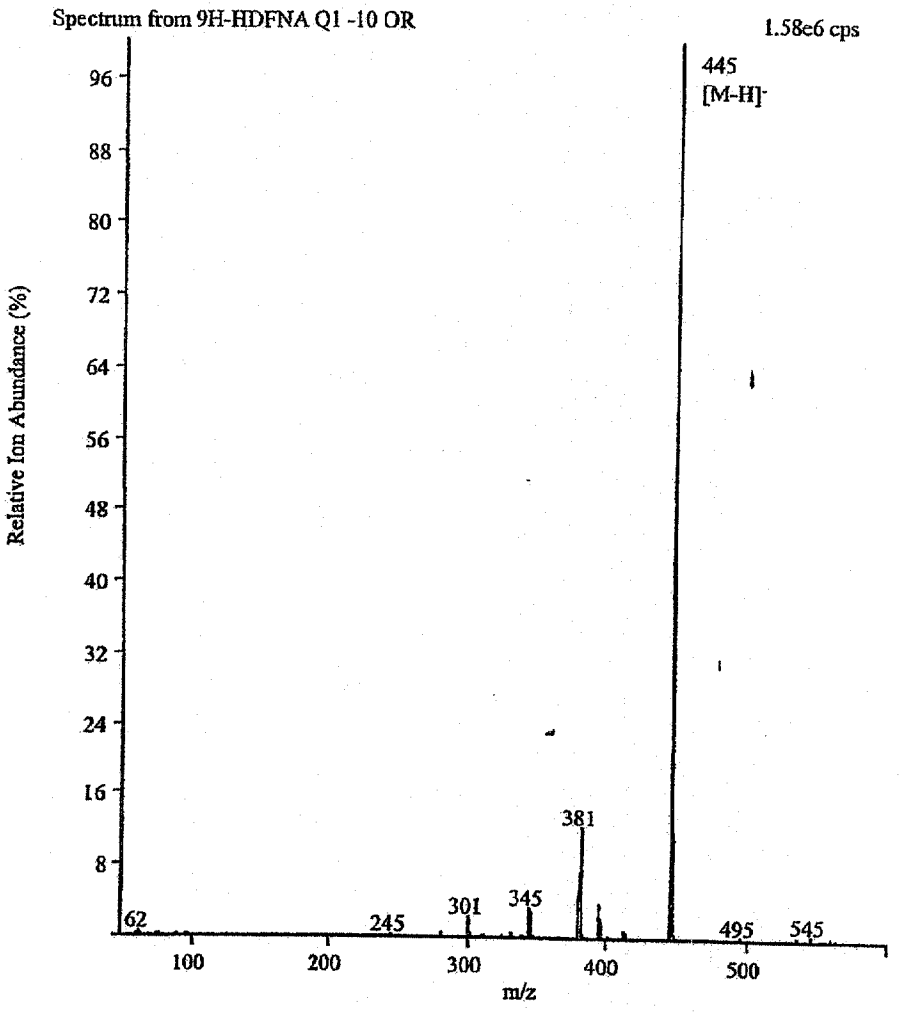


Figure 4: Full-Scan Product Ion Mass Spectrum of 9H-HDFNA

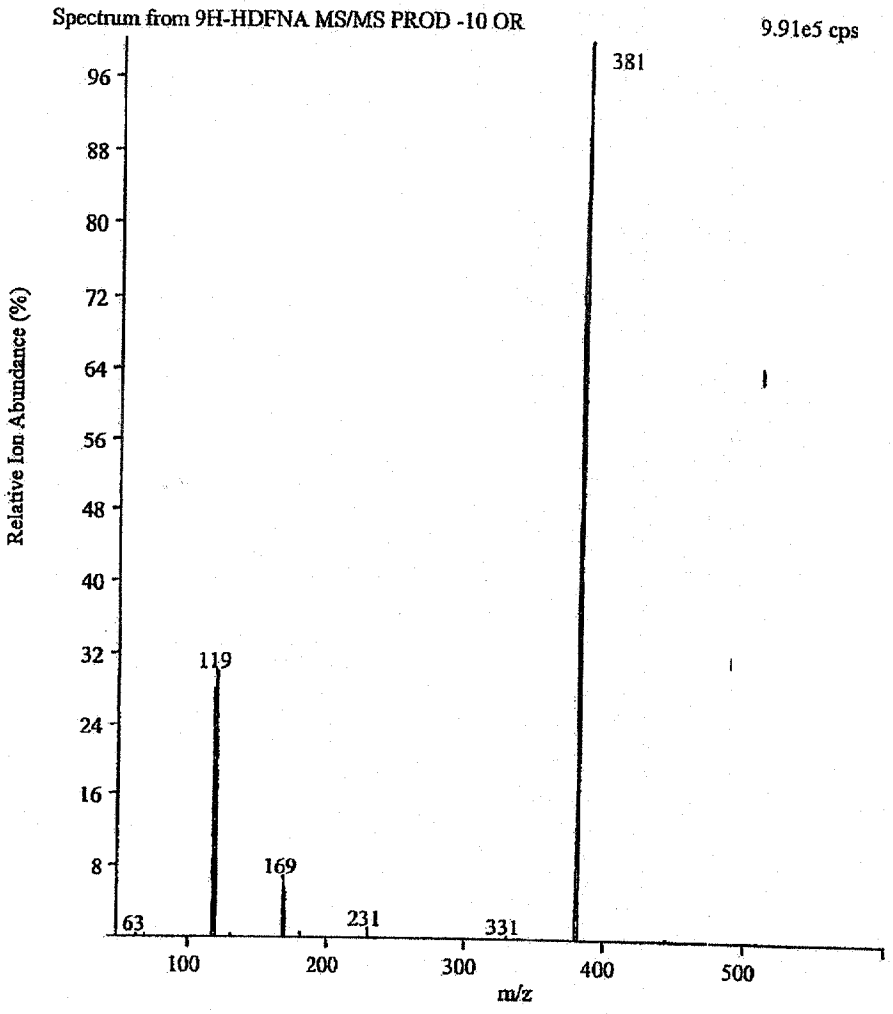
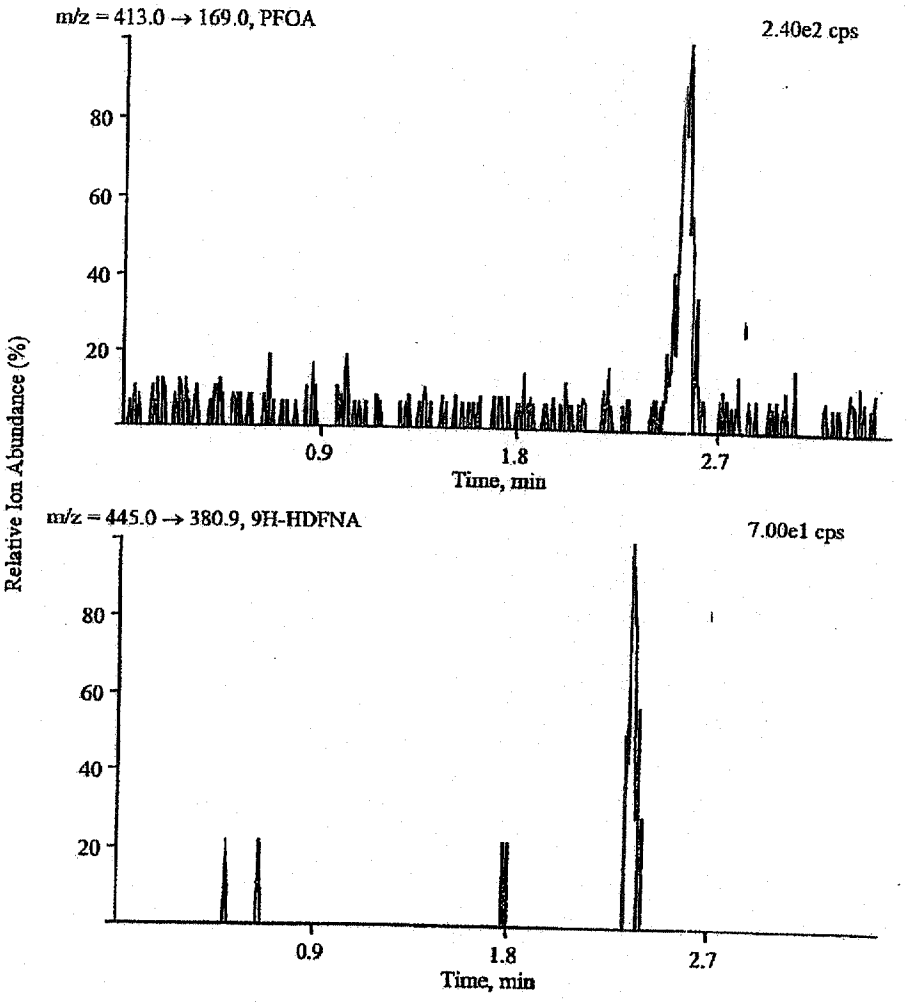
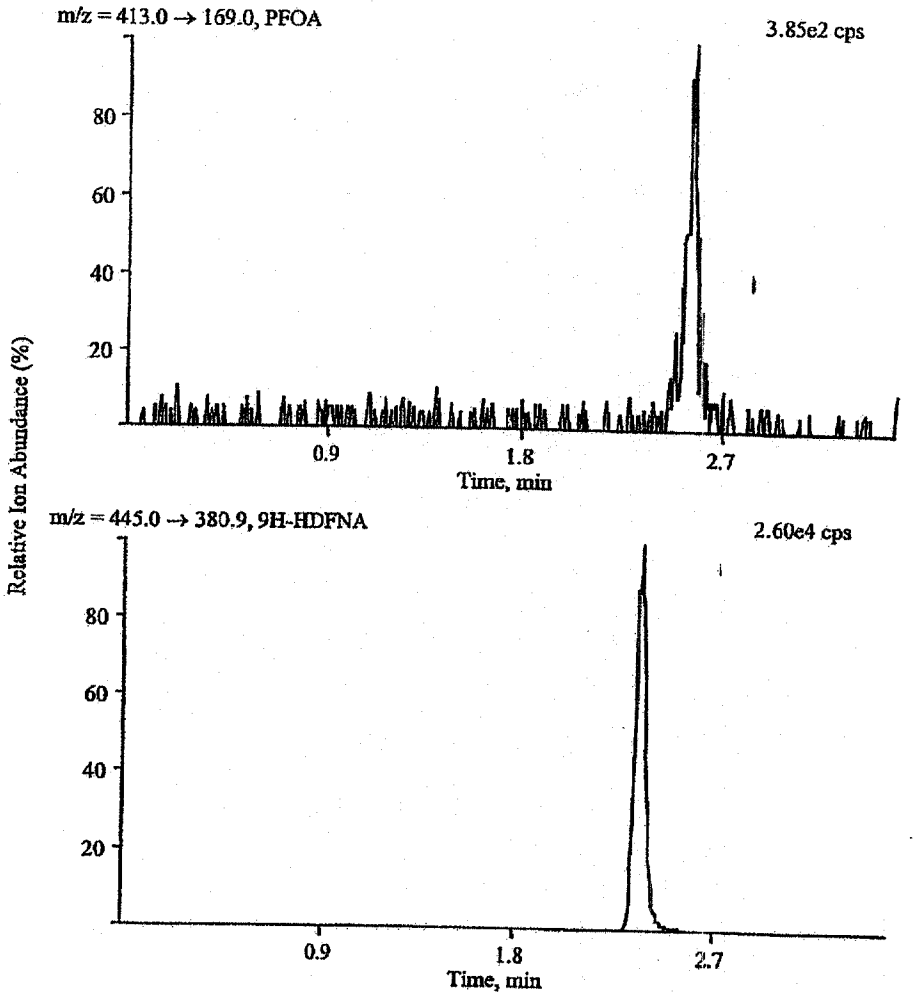


Figure 5: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard in Control Blank Human Serum Extract**



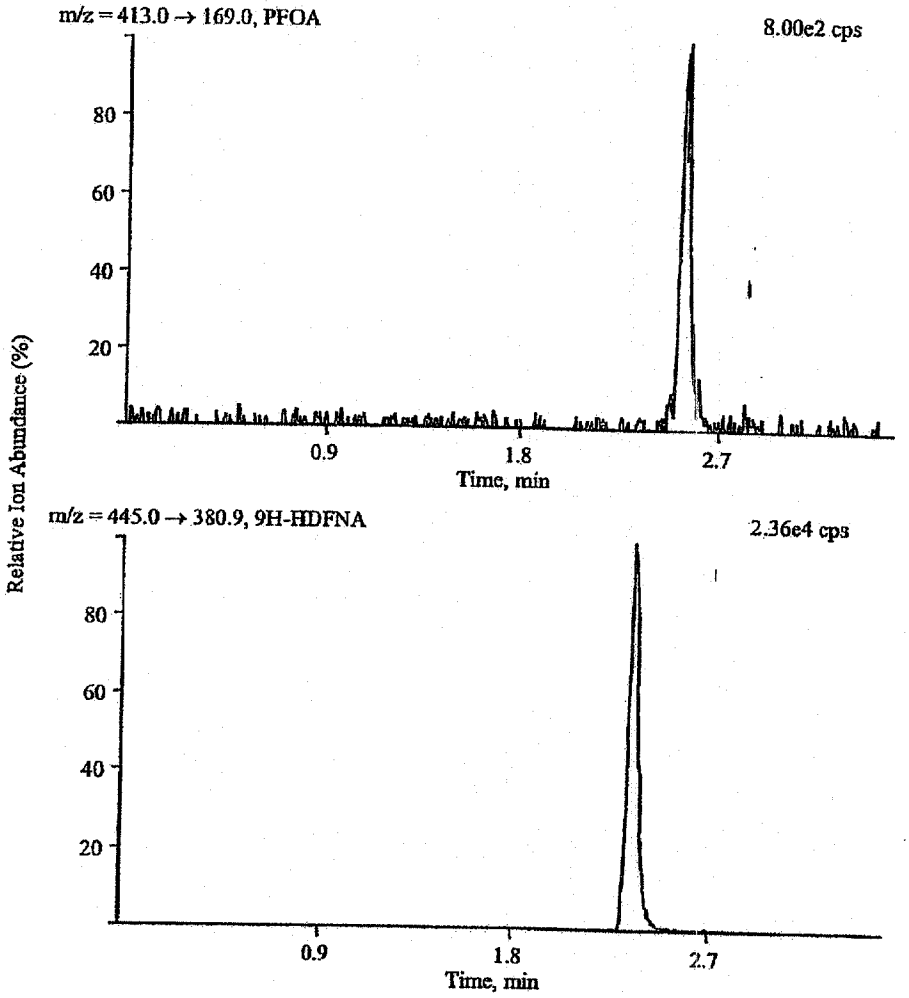
Control Blank, Run 1, Injection 7

Figure 6: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard in a Human Serum Extract Containing Internal Standard Only (Zero Sample)**



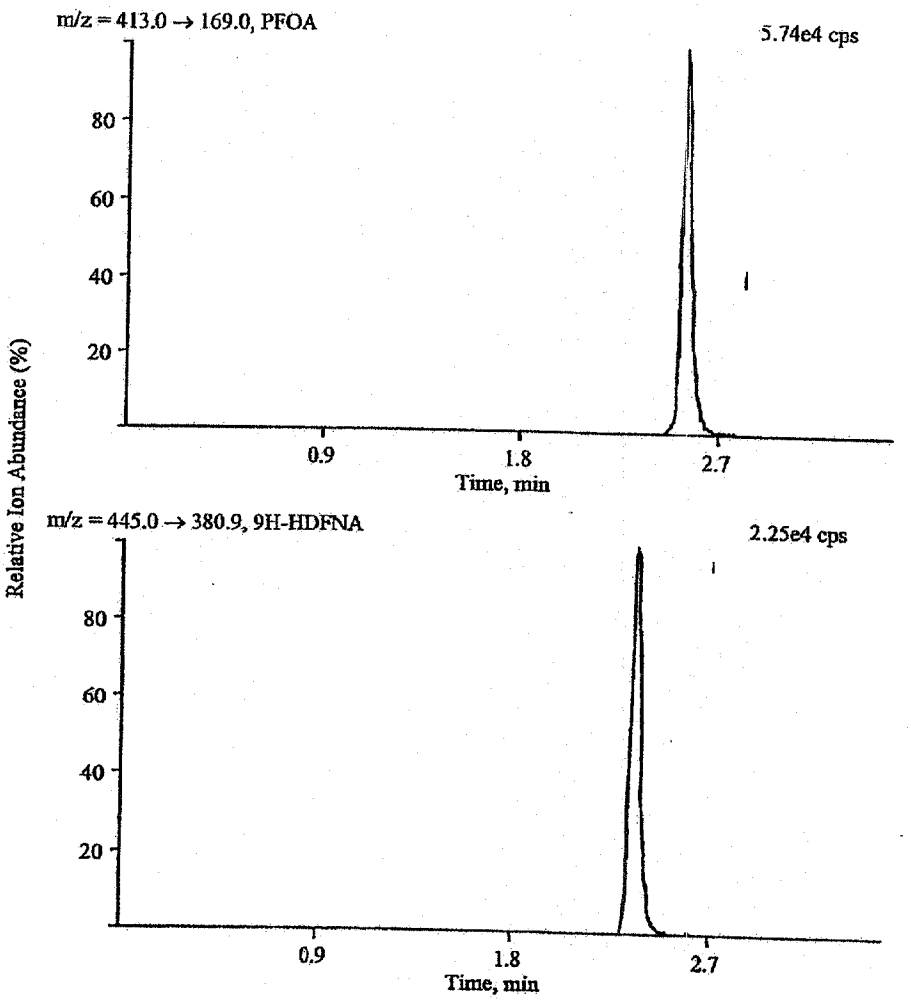
Zero Sample, Run 1, Injection 1

Figure 7: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard from a Standard 1 Human Serum Extract (0.05 µg/mL)**



Standard 1, Run 1, Injection 8

Figure 8: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard from a Standard 9 Human Serum Extract (10 µg/mL)**



Standard 9, Run 1, Injection 16

10. APPENDIX A: QUANTITATION OF AMMONIUM PERFLUOROOCTANOATE (APFO), MEASURED AS THE PERFLUOROOCTANOATE ANION (PFOA, 0.05 TO 10 µg/mL CONCENTRATION RANGE), IN HUMAN SERUM BY TURBO ION SPRAY LC/MS/MS

AUTHORS: David J. Anderson, M.S.
Kimberly L. Norwood, B.A.
Kathy Jo Palmer, B.S.
Jennie L. Romney, M.P.S.

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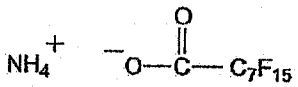
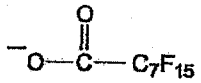
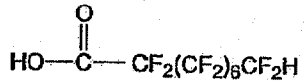
A.1.0 CHEMICAL STRUCTURE(S)



Ammonium Perfluorooctanoate (APFO) $C_8H_4F_{15}NO_2$ Monoisotopic Mass = 431.0



Perfluorooctanoate Anion (PFOA) $C_8F_{15}O_2$ Monoisotopic Mass = 413.0



9H-Hexadecafluorononanoic Acid (9H-HDFNA) $C_9H_2F_{16}O_2$ Monoisotopic Mass = 446.0

A.2.0 SPECIMEN(S)

This assay uses 25-µL aliquots of human serum. Human serum samples should be stored at –20 °C.

A.3.0 ASSAY PRINCIPLE

Perfluorooctanoate anion (PFOA) and its IS, 9H-hexadecafluorononanoic acid (9H-HDFNA), were extracted from human serum samples (25 µL) using a liquid-liquid extraction procedure. An aliquot of the organic layer of each sample was evaporated to dryness, reconstituted, and then analyzed by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the negative ion mode.

A.4.0 COMPOUNDS

Pentadecafluorooctanoic acid ammonium salt (also known as ammonium perfluorooctanoate, APFO): Lot# 421207/1, 100% purity, 0.9582 salt factor, Sigma-Aldrich, St. Louis, MO.

Salt Factor: $\frac{C_8F_{15}O_2}{C_8H_4F_{15}NO_2} = \frac{413.064}{431.10258} = 0.9582$

9H-HDFNA: No Lot# available, 100% purity assumed, Sigma-Aldrich, St. Louis, MO.

A.5.0 CHEMICALS

All chemicals may be substituted with that of an equivalent manufacturer and grade of chemical.

Tetrabutylammonium Hydrogen Sulfate	HPLC Grade, Cat# J360-07, J.T. Baker, Phillipsburg, NJ
	HPLC Grade, Cat# 32503-27-8, Aldrich, Milwaukee, WI
Sodium Carbonate, Annhydrous	Cat# 3604-01, J.T. Baker, Phillipsburg, NJ

Sodium Bicarbonate	Cat# 3509-01, J.T. Baker, Phillipsburg, NJ
Ethyl Acetate	Cat# 100-4, Burdick & Jackson, Muskegon, MI
Ammonium Acetate	Cat# 24,019-2, Aldrich, Milwaukee, WI
Ammonium Hydroxide	Cat# AX1303-13, EM Science, Gibbstown, NJ
Water	Cat# 365-4, Burdick & Jackson, Muskegon, MI
Methanol	Cat# 230-4, Burdick & Jackson, Muskegon, MI
Acetonitrile	Cat# BJ015-4, Burdick & Jackson, Muskegon, MI
Human Serum	Biological Specialty Corporation, Colmar, PA

A.6.0 MATERIALS AND EQUIPMENT

Mass Spectrometer	SCIEX API 3000 atmospheric pressure ionization tandem triple quadrupole mass spectrometer equipped with TurboIonSpray™ interface, Applied BioSystems, Concord, Ontario
Data Acquisition System	API Standard Software, MacQuan v 1.6, LC2Tune v 1.4, MacDAD v 1.4.1, Bundler v 1.4, Multiview v 1.4, and Sample Control v 1.4, on a Power Macintosh G3/450, Applied BioSystems, Concord, Ontario
HPLC Pumps	Shimadzu LC-10AD vp pumps, Shimadzu Co., Columbia, MD
LC Pump Controller	Shimadzu SCL-10A vp, Shimadzu Co., Columbia, MD
Autosampler	Perkin-Elmer Series 200, Perkin-Elmer Corp., Norwalk, CT
HPLC Column	Betasil C_{18}, 5 µm particle size, 2 x 50 mm, Cat# 852055-701, ThermoHypersil-Keystone, Bellefonte, PA

Column Heater	Model 7970, Jones Chromatography USA Inc., Lakewood, CO
Harvard Syringe Pump 11	Harvard Apparatus Inc., South Natick, MA
Polypropylene 96-Well Blocks	Cat# 267006, Beckman Instruments, Inc., Fullerton, CA
Screw-capped Polypropylene Vials	16.5 x 101 mm, Cat# 60.541, Sarstedt, Inc., Newton, SC 28658
Screw-capped Polypropylene Vials	16.5 x 57 mm, Cat# 60.542, Sarstedt, Inc., Newton, SC
Siliconized Graduates Microtubes	1.5 mL, Cat# MH-815S, Phenix Research Products, Hayward, CA 94545
Polypropylene Centrifuge Tubes	50 mL with screw caps, Cat# 21008-178, VWR Scientific, Bridgeport, NJ
Polypropylene Bottles	175 mL Nalgene, Cat# 16120-953, VWR Scientific, Bridgeport, NJ
Polypropylene Bottles	1 L Nalgene, Cat# 16120-962, VWR Scientific, Bridgeport, NJ
TurboVap 96 Evaporator	Cat# 71000, Zymark Instruments, Hopkinton, MA 01748
Beckman GS6KR Refrigerated Centrifuge	Beckman, Palo Alto, CA 94304
Multi-tube Vortexer	Cat# 58816-115, VWR Scientific, West Chester, PA
Mechanical Shaker	Cat# 6000, Eberbach Corp., Ann Arbor, MI
Watson	Laboratory Information Management System v 6.1.0.03, InnaPhase Corp., Philadelphia, PA

Other general laboratory glassware and supplies were used.

A.7.0 PREPARATION OF CALIBRATION STANDARDS

Mix all solutions well. Unless otherwise noted, store solutions at 4 °C and bring to ambient temperature before use. Unless otherwise noted, prepare fresh solutions every three months or as needed.

A.7.1 Preparation of Analytical Standard Solutions

Standard Stock Solution, PFOA (500 µg/mL): Weigh approximately 2 mg pentadecafluoroactanoic acid ammonium salt and transfer to a 16.5 x 57 polypropylene vial. Dilute with appropriate volume of methanol to yield a 500-µg/mL solution after correction for salt factor. Vortex for one minute.

A.7.2 Preparation of Standard Curve

Prepare Standards 1 through 9 by dilution of Standard Standard Stock Solution or the appropriate standard into control serum according to the following table. Standard 9 is prepared in a 5-mL volumetric flask. Standard 1 through Standard 8 are prepared in 1.5-mL siliconized polypropylene microtubes.

Analytical Standard Dilution Table:

Standard No.	Final Conc. (µg/mL)	Solution to Use	µL of Solution	µL Control Serum	mL Total Volume
9	10	Std Stock	100	Add serum to 5-mL mark	5
8	7.5	STD9	375	125	0.5
7	5	STD9	250	250	0.5
6	2	STD9	100	400	0.5
5	1	STD9	50	450	0.5
4	0.5	STD7	50	450	0.5
3	0.25	STD7	25	475	0.5
2	0.1	STD5	50	450	0.5
1	0.05	STD5	25	475	0.5

A.8.0 PREPARATION OF INTERNAL STANDARD SOLUTIONS

Mix all solutions well. Unless otherwise noted, store solutions at 4 °C and bring to ambient temperature before use. Unless otherwise noted, prepare fresh solutions every three months or as needed.

Internal Standard Stock Solution, 9H-HDFNA (500 µg/mL): Weigh approximately 2 mg 9H-HDFNA and transfer to a 16.5 x 57 polypropylene vial. Dilute with appropriate volume of methanol to yield a 500-µg/mL solution. Vortex for one minute.

Internal Standard Working Solution, 9H-HDFNA (5 µg/mL): Transfer 250 µL of Internal Standard Stock Solution into a 25-mL class "A" volumetric flask containing approximately 20 mL of water. Dilute to volume with water. Vortex and transfer to a 50-mL polypropylene centrifuge tube.

A.9.0 PREPARATION OF QUALITY CONTROL SAMPLES

A.9.1 Preparation of Serum QC Samples

Prepare a 500-µg/mL QC Stock Solution in the same manner as the Standard Stock Solution in A.7.1. Prepare QC samples in a 5-mL volumetric flask by dilution of the QC Stock Solution, QC3, or QC2 into approximately 3 mL of control human serum (from a different lot than is used to prepare the standard curve) and diluting to volume according to the following table:

QC #	Analyte Conc. (µg/mL)	Solution to Use	µL of Solution	Dilute to Final Volume (mL)
QC4	50	QC Stock	500	5
QC3	8	QC Stock	80	5
QC2	3	QC3	1875	5
QC1	0.15	QC3	93.75	5
QC LLQ	0.05	QC2	83.1	5

Transfer 0.2-mL aliquots of each QC into microcentrifuge tubes and store frozen at -20 °C. Note that the QC LLQ was a one-time preparation and was not stored.

A.10.0 PREPARATION OF OTHER SOLUTIONS

HPLC-grade bottled water or equivalent should be used wherever water is called for. Mix all solutions well. Different volumes of the solutions in this section may be prepared if necessary. Unless otherwise noted, store at room temperature. Prepare fresh solutions every three months or as needed, unless otherwise noted.

0.5 M Tetrabutylammonium Hydrogen Sulfate, pH 10: Dissolve approximately 17 g of tetrabutylammonium hydrogen sulfate in 40 mL water in a 175-mL polypropylene bottle. Adjust the pH to 10 with 10 M and 1 M sodium hydroxide. Transfer to a 100-mL class "A" volumetric flask and dilute to the mark with water. Transfer to a 175-mL polypropylene bottle.

10 M Sodium Hydroxide: Dilute approximately 40 g sodium hydroxide to the mark with water in a 100-mL class "A" volumetric flask. Mix and transfer to a 175-mL polypropylene bottle.

1 M Sodium Hydroxide: Dilute 10 mL 10 M sodium hydroxide to the mark with water in a 100-mL class "A" volumetric flask. Mix and transfer to a 175-mL polypropylene bottle.

0.25 M Sodium Carbonate:0.25 M Sodium Bicarbonate: Dissolve 26.5 g sodium carbonate and 21 g sodium bicarbonate in approximately 500 mL water. Transfer to a 1-L polypropylene graduated cylinder and dilute to the mark with water. Mix and transfer to a 1-L polypropylene bottle.

1 M Ammonium Acetate: Dissolve 7.7 g ammonium acetate to the mark with water in a 100-mL class "A" volumetric flask. Mix and transfer to a 175-mL polypropylene bottle.

2 mM Ammonium Acetate: Add 2 mL of 1 M ammonium acetate to approximately 900 mL water in a 1000-mL polypropylene graduated cylinder and dilute to volume with water. Mix and transfer to a 1-L polypropylene bottle.

10:90 Methanol:2 mM Ammonium Acetate: Combine 100 mL methanol and 900 mL 2 mM ammonium acetate in a 1000-mL polypropylene graduated cylinder. Transfer to a 1-L polypropylene bottle.

90:10 Methanol:2 mM Ammonium Acetate: Combine 900 mL methanol and 100 mL 2 mM ammonium acetate in a 1000-mL polypropylene graduated cylinder. Transfer to a 1-L polypropylene bottle.

1% Ammonium Hydroxide: Dilute 3.6 mL ammonium hydroxide to the mark with water in a 100-mL class "A" volumetric flask. Mix and transfer to a 100-mL glass reagent bottle.

0.01% Ammonium Hydroxide: Dilute 2.5 mL 1% ammonium hydroxide to the mark with water in a 250-mL class "A" volumetric flask. Mix and transfer to a 250-mL glass reagent bottle.

80:20 Acetonitrile:0.01% Ammonium Hydroxide: Combine 800 mL acetonitrile and 200 mL 0.01% ammonium hydroxide in a 1-L glass reagent bottle.

A.11.0 LIQUID-LIQUID EXTRACTION PROCEDURE

1. Thaw samples and QCs.
2. Prepare the standards as outlined in Section A.7.2.
3. Label 16.5 x 101 mm screw-capped polypropylene tubes for each sample, standard, QC, control blank, and zero sample. Standards, carryover control blanks, and selectivity control blanks are analyzed in duplicate. QCs are analyzed in replicates of six. Six replicate zero samples are prepared in each of the two different lots of control serum used for the human serum standard curves and human serum QC samples.
4. Aliquot 25 µL of each standard, QC, zero sample, control blank, and sample into the appropriate tube.
5. Add 25 µL of Internal Standard Working Solution (5 µg/mL 9H-HDFNA) to all tubes except for the control blank samples. Add 25 µL of water to each control blank.
6. Add 1 mL of 0.5 M tetrabutylammonium hydrogen sulfate, pH 10 to each tube.
7. Add 2 mL of 0.25 M sodium carbonate:0.25 M sodium bicarbonate to each tube.
8. Add 3 mL ethyl acetate to each tube.
9. Shake on reciprocal shaker for 20 minutes at medium speed.
10. Centrifuge at 3200 rpm for 20 minutes
11. Transfer a 300-µL aliquot of the organic layer to a 96-well block.
12. Evaporate the eluate to dryness under nitrogen at 35 °C for approximately 30 minutes at a maximum pressure of 50 psi.
13. Reconstitue the dried extracts in two steps: Add 400 µL acetonitrile and vortex for 60 seconds. Add 400 µL purified water and vortex for 60 seconds.
14. Apply a cover film to the 96-well block.

A.12.0 TYPICAL INSTRUMENT CONDITIONS

HPLC Conditions:

Mobile Phase (gradient): A: 10:90 methanol:2 mM ammonium acetate

B: 90:10 methanol:2 mM ammonium acetate

Mobile Phase Gradient Conditions:

Time (min)	Function/Value
0.0	45% B
0.5	45% B
2.3	100% B
3.0	100% B
3.5	45% B
5.00	STOP

Flow Rate	300 µL/min
Autosampler	PE Series 200 (with 50-µL stainless steel loop)
Autosampler Temperature	Ambient
Autosampler Needle Wash	80:20 acetonitrile:0.01% ammonium hydroxide
Injection Volume	10 to 15 µL
HPLC Column	Betasil-C_{18}, 5 µm particle size, 2 mm x 50 mm
Typical Initial Column Pressure	73 bar (variable)
Autosampler Run Time	4.3 minutes

Analyte	Representative Retention Time (minutes)
PFOA*	2.5
9H-HDFNA	2.3

*Note: The PFOA chromatographic peak may show a small peak on its leading edge, which is most likely an isomeric component in the PFOA chemical standard.

Mass Spectrometer Conditions:

Curtain Gas	UHP Nitrogen
Nebulizer Gas	UHP Nitrogen
Collision Gas	UHP Nitrogen
TurboIonSpray™ Temperature	350 °C
Auxiliary Gas	UHP Nitrogen at 8 L/min

Selected Reaction Monitoring:

Analyte	Transition Monitored (±0.2)	Dwell Time
PFOA	m/z = 413 → 169	200 ms
9H-HDFNA	m/z = 445 → 381	200 ms

Ionization Mode	Negative Ion
Ion Spray Voltage:	3000 V
Declustering Potential	10 V
Collision Energy	25.5 eV
MS Acquisition Time	3.0 minutes
Pause Time	2 ms

Calibrate the mass axis of the instrument by infusion of Rhodapex mass calibration solution at a flow rate of 10 µL/min. Optimize the sensitivity of the instrument using an infusion of a 0.1 µg/mL mixed solution of PFOA and 9H-HDFNA at 10 µL/min into a flow of 190 µL/min of mobile phase using the initial gradient condition (45% eluent B). Mass spectrum peak widths should be approximately 0.6 amu at half-height in both single MS and MS/MS modes.

One set of calibration standards was injected at the beginning and one set of calibration standards was injected at the end of each analytical run. Additional control blank and zero samples may be used to monitor possible carryover or

contamination. Three replicates of each background subtraction zero sample are placed before each standard curve.

All polytetrafluoroethylene (PTFE)-containing materials are removed from the LC pumps and replaced with either stainless steel or PEEK. This is done to remove these items as potential sources of PFOA.

A.13.0 SYSTEM SUITABILITY

System suitability criteria were established during the validation based upon the response of the 0.05 µg/mL PFOA calibration standards in human serum. The system suitability samples for sample analysis are prepared for each analytical run, and injections are made into the system prior to sample analysis and subject to the following criteria. The peak shape and retention times for the analyte and internal standard should be comparable to that observed during the validation. The signal (peak height) for the 0.05-µg/mL system suitability samples should be a minimum of 400 cps in order to proceed with sample analysis.

A.14.0 CALCULATIONS

Calculated concentrations are based on peak area ratios of analyte to IS. The peak area for the analyte transition is divided by the peak area for the corresponding internal standard transition. The calibration curves are fit using a $1/x^2$ quadratic regression.

Background Subtraction procedure:

1. Background subtraction zero samples prepared with the control human serum lot used to prepare the standard curves are identified as ZERO_HSTD, and those prepared with the control human serum lot used to prepare the QC samples are identified as ZERO_HQC. Three replicates are placed before each standard curve.
2. After analysis, the data are processed in MacQuan, and the mean area ratio (area PFOA/area IS) from the six zero samples in each of the two control serum lots is

calculated in Excel from the data in the MacQuan results table (.DAT file). This yields two mean area ratios. If the coefficient of variation (CV) of a mean ratio is greater than 20%, then outlier values are removed and the mean is re-calculated. The Excel data are saved electronically (_ZERO file), and a hardcopy of the spreadsheet, showing the mean area ratios, is included in the analytical run documentation.

3. The applicable mean area ratios are subtracted from the raw area ratios for the standards and QC samples. Background subtraction calculations are performed in Excel using four significant figures for the mean area ratios, and again using the data in the MacQuan results table (.DAT file). The Excel data are saved electronically (_SUB file), and a hardcopy of the spreadsheet, showing the original and the background-subtracted data, is included in the analytical run documentation.
4. The background-subtracted data are copied to the original MacQuan results table (.DAT file) in Excel with the background-subtracted area ratios in the "Area" column of the .DAT file. The Excel feature of "paste values" is used. A value of 1 is placed in the "IS Area" column in the .DAT file. This will provide the correct background-subtracted area ratios for the Watson LIMS regression. The label "n/a" is entered in the analyte area column for control blanks. The Excel data are saved electronically (_SUB.DAT file) but not printed.
5. The background-subtracted data (_SUB.DAT file) are uploaded to Watson LIMS. These data will appear in the hardcopy printout from Watson LIMS (Import Information: Peak Areas).
6. The electronic files are placed into the appropriate folder for the analytical run on the file server in the LIMFILES directory.

Data are acquired and integrated using SCIEX software applications Sample Control (v. 1.4) and MacQuan (v. 1.6). The data are formatted in the Advion laboratory information management system (Watson, v. 6.1.0.03).

PFOA concentrations can be expressed as APFO by multiplying the PFOA values by 1/salt factor.