Analytical Report: LIMS E02-1053

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Analytical Laboratory Report Title

Comparative Analysis of Fluorochemicals in Human Serum Samples Obtained Commercially

Data Requirement

Not Applicable

Author

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Study Completion Date

11/13/02

Performing Laboratory

Extractions and Analyses

3M Environmental Laboratory
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Project Identification

Analytical Report: E02-1053

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52

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Compliance Statement

Analytical Laboratory Report Title: Comparative Analysis of Fluorochemicals in Human Serum Samples Obtained Commercially

Study Identification Number: E02-1053

This study was not conducted under Good Laboratory Practices.

William Reagen, Ph.D., *Laboratory Management, Sponsor Representative* — Date 11/13/02

Lisa A Stevenson

Lisa Stevenson, *Principal Analytical Investigator* — Date 11/13/02

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Quality Assurance Statement

Analytical Laboratory Report Title: Comparative Analysis of Fluorochemicals in Human Serum Samples Obtained Commercially

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This study has been inspected by the 3M Environmental Laboratory Quality Assurance Unit (QAU) as indicated in the following table. The findings were reported to the Principal Analytical Investigator (PAI) and laboratory management.

Inspection Dates	Phase	Date Reported to	
		Management	PAI
10/17/02	Sample Spiking	10/18/02	10/18/02
10/22/02	Analysis	10/23/02	10/23/02
10/30/02, 10/31/02, 11/07-08/02	Data	11/01/02, 11/08/02	11/01/02, 11/08/02
11/01/02, 11/07-08/02	Draft report	11/01/02, 11/08/02	11/01/02, 11/08/02

Matthew A. Mawhinney 11/13/02

QAU Representative Date

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Study Initiation: 10/16/02
Study Completion: 11/13/02

Location of Archives

All original raw data and analytical report have been archived at the 3M Environmental Laboratory according to 3M Standard Operating Procedures. The analytical reference standard reserve samples are archived at the 3M Environmental Laboratory according to 3M Standard Operating Procedures. Remaining specimens pertaining to the analytical phase of this study will be archived at 3M Environmental Laboratory for as long as the quality of the preparation affords evaluation.

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Executive Summary

A screening study was undertaken to compare branched and linear isomers of perfluorooctanoate (PFOA - $C_7F_{15}COO^-$) in 4 lots of commercial pooled human sera with concentrations ranging from 0.65 – 5.6 ng/mL PFOA.

Results from this study showed a wide distribution of the percentage of branched isomers of PFOA compared to the linear isomer of PFOA in the commercial pooled populations (Table 1). Since this is a preliminary screening study a larger sample size would be needed to determine if a significant statistical difference exists between pooled human sera samples.

As shown in Table 1, two of the lots of commercial pooled sera showed the presence of branched isomers of PFOA while the other two lots showed a much lower percentage (by at least 40x) of PFOA branched isomers.

Table 1. PFOA Data Summary of Pooled Serum

Sample	Identification	Branched Isomer Area	Linear Isomer Area	% Branched/ Sum Branched + Linear
Pooled Human Serum	TCR-687-Bioresource Lot 020821	<361*	114603	<0.31
	TCR-688-Lampire Lot X324B	8059	41466	16
	TCR-689-Sigma Lot 022K0965	8824	55100	14
	TCR-690-Golden West Lot G01406042	<361*	168154	<0.21

* Area threshold found as 361 in the extracted PFOA-Acid 0.534 ng/mL standard.

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Introduction and Purpose

The purpose of the study is to determine the relative isomer ratios of PFOA fluorochemical in 4 lots of commercial pooled human sera with concentrations ranging from 0.65 – 5.6 ng/mL PFOA. The PFOA concentrations were determined in study E02-1039. Analyses of sera extracts for determining the relative isomer ratios of PFOA were completed by the 3M Environmental Laboratory under study number E02-1053, and the results of these analyses are presented in this report. The analytical portion of this study was initiated on 16 October 2002.

Specimen Receipt and Maintenance

The 3M Environmental Laboratory received pooled human sera samples collected from various commercial vendors in July and August, 2002. All specimens were received frozen in good condition on dry ice. All specimens were immediately transferred to storage at -20°C ± 10°C, and maintained at that temperature except when removed for extraction and analysis as described in the method. The samples were kept isolated from the test materials (analytical standards) during storage.

Table 2. Pooled human serum samples received from commercial vendors in July and August, 2002

Samples	Identification	Lot #
Pooled Human Serum	TCR-687-Bioresource	020821
	TCR-688-Lampire	X324B
	TCR-689-Sigma	022K0965
	TCR-690-Golden West	G01406042

The control matrix used in sera analyses performed during E02-1053 was obtained from a commercial source and is presented in Appendix A. Samples analyzed at the 3M Environmental Laboratory will be stored and maintained following 3M Standard Operating Procedures.

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Chemical Characterization of the Reference Substances

Perfluorooctanoate Ammonium Salt (PFOA-NH4)
CAS Number: 3825-26-1

Chemical Formula: $C_7F_{15}CO_2^-NH_4^+$ Molecular Weight: 431

This chemical is a 3M electrochemical fluorination production lot and contains, as determined by NMR, approximately 20% branched:80% linear isomers by weight.

Perfluorooctanoate-Acid (PFOA-Acid)
CAS Number: 335-67-1

Chemical Formula: $C_7F_{15}CO_2H$ Molecular Weight: 414

This chemical is a commercial product obtained from Oakwood Products and contains approximately 1% branched:99% linear isomers.

The molecular ion 413 was selected as the primary ion for PFOA. This ion was fragmented further during analysis to produce ions 119, 169, 219, and 369. The total ion current (TIC) was monitored for analysis.

Chemical characterization information on the reference substances used in this study is presented in tabular form below.

Table 3. Characterization of the Analytical Reference Substances in Study E02-1053

Location	3M Lab	
Substance	PFOA-NH4 TCR-99131-037	PFOA-Acid TCR-617
Source	3M	Oakwood Products
Expiration Date	12/15/2006	NA
Storage Conditions	-20°C ± 10°C	Room Temperature
Chemical Lot Number	332	210002
Physical Description	White powder	White crystal
Purity	95.2%*	99.51%**

NA—Not available
*See Certificate of Analysis from Centre Analytical Laboratories in Appendix F.
**See Certificate of Analysis from 3M.

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Sample Preparation and Analysis

Human serum samples were analyzed in this study. Sera samples were extracted beginning on 17 October 2002 using a solid phase extraction (SPE) procedure. Sample extracts were analyzed using high-performance liquid chromatography-electrospray/tandem mass spectrometry (HPLC-ES/MS/MS) in the multiple reaction mode versus extracted rabbit sera standards.

Qualitative analysis of branched and linear isomers of PFOA was accomplished using NMR certified standards of a linear isomer PFOA standard (PFOA-Acid) and a mixed branched and linear isomer PFOA standard (PFOA-NH_4). It was determined that the branched PFOA isomers elute within an approximate 0.2 minute retention time window from the linear PFOA isomer.

Method Summaries

Following is a brief description of the method used during this analytical study by the 3M Environmental Laboratory. A detailed description of the method used in this study is located in Appendix B.

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PREPARATORY AND ANALYTICAL METHOD

- **ETS-8-231.1,** "Solid Phase Extraction and Analysis of Fluorochemical Compounds from Biological Matrices"
- Human sera was prepared using 2.0 mL of serum then diluted to 10 mL with reagent grade water. The diluted serum/water was spiked with the appropriate analyte mixture. Acetonitrile (ACN) was added as an extraction solvent, which also served to precipitate the proteins. The sample was capped, mixed, and put on the centrifuge to clarify the supernatant. The supernatant was transferred to a clean tube, diluted with water, and passed through a pre-conditioned C_{18} SPE cartridge.

The analyte(s) of interest were eluted from the SPE cartridge with 2.0 mL of methanol and analyzed. Analyses were performed by monitoring two or more product ions selected from a single primary ion characteristic of PFOA using HPLC-ES/MS/MS. For example, the molecular anion 413 ($C_7F_{15}COO^-$), selected as the primary ion for analysis, was fragmented further to produce characteristic daughter ions 119, 169, 219, and 369. The total ion current (TIC) peak areas are the sum of the signal for each daughter ion at specific retentions times for each target analyte isomer that were monitored for analysis. Daughter ions may also be referred to as product ions.

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ANALYTICAL EQUIPMENT

The following is representative of the settings used during the analytical phase of this study.

Liquid Chromatograph: Hewlett-Packard® Series 1100 Liquid Chromatograph system
Analytical column: Keystone® Betasil™ C_{18} 2x100 mm (5 µm)
Column temperature: 40°C
Mobile phase components:
Component A: 2mM ammonium acetate
Component B: methanol
Flow rate: 300 µL/min
Injection volume: 1-30 µL
Solvent Gradient: 16.0 minutes

Time (minutes)	*%B*
0.0	40%
10.0	90%
11.0	90%
11.5	100%
12.5	100%
13.0	40%
16.0	40%

Mass Spectrometer: Micromass® API/Mass Spectrometer Quattro Ultima Triple Quadrupole system
Software: Mass Lynx™ 3.5
Cone Voltage: 20–60 V
Collision Gas Energy: 20–50 eV
Mode: Electrospray Negative
Source Block Temperature: 150°C ±10°C
Electrode: Z-spray
Analysis Type: Multiple Reaction Monitoring (MRM)

Table 4. Target Ions Monitored in 3M Laboratory Analyses and Observed Retention Times

Target Analyte	Primary Ion (AMU)	Product Ion (AMU)	Isomer retention time	
			Branched	Linear
PFOA	413.0	119, 169, 219, 369	~7.9 min.	~8.1 min.

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Data Quality Objectives and Data Integrity

The following data quality objectives (DQOs) were indicated for this study:

Calibration: Calibration curves were not a component of this study. Isomer ratios of extracted rabbit matrix standards (labeled as RBS-date of extraction-concentration) were evaluated to determine instrument response.

Limits of Peak Area Threshold: The limit of peak area threshold was the lowest standard point that had a signal to noise ratio of at least 2 times that of the baseline noise.

Acceptance Criteria: The isomer ratio of branched:linear PFOA in the extracted continuing verification (QC) sample (labeled as RBS-date of extraction-QC-concentration) is required to meet ±30% agreement versus the extracted initial standard at the same concentration.

Confirmatory Methods: No confirmatory method will be used.

Demonstration of Specificity: Isomer identification will be substantiated by chromatographic retention times of the total ion current (TIC).

Data Summary, Analyses, and Results

Data quality objectives for the analytical phase of this study outlined above were met with the exceptions noted in this report.

Summary of Data Results

Table 5. PFOA Data Summary of Pooled Serum

Sample	Identification	Branched Isomer Area	Linear Isomer Area	% Branched/ Sum Branched + Linear
Pooled Human Serum	TCR-687-Bioresource Lot 020821	<361*	114603	<0.31
	TCR-688-Lampire Lot X324B	8059	41466	16
	TCR-689-Sigma Lot 022K0965	8824	55100	14
	TCR-690-Golden West Lot G01406042	<361*	168154	<0.21

* Area threshold found as 361 in the extracted PFOA-Acid 0.534 ng/mL standard

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Summary of Quality Control Analyses Results

Calibration: Quantitation was not a component of this study.

Comparison of branched:linear ratio in the extracted rabbit matrix standard was based on the total ion current (TIC) of peak areas at retention times consistent for the target analyte (i.e. 7.9 and 8.1 minutes for PFOA-NH4, and 7.9 and 8.1 minutes for PFOA-Acid).

Limits of Peak Area Threshold: The peak area threshold was determined based on the extracted PFOA-Acid 0.534 ng/mL standard, using the branched isomer TIC peak area at ~2 times the baseline noise.

Table 6. Limit of Peak Area Threshold in the Analyses of Sera Extracts

Analyte	Concentration ng/mL	Branched Isomer TIC Peak Area	Instrument
PFOA-Acid	0.534	361*	Quattro Ultima

* Based on the extracted PFOA-Acid 0.534 ng/mL standard, branched isomer area at retention time 7.9 minutes, with a TIC peak area ~2 times the baseline noise.

PFOA-Acid – standard spiked with PFOA-Acid standard mix

Blanks: All blanks were below the limit of peak area threshold for the compounds of interest.

Acceptance Criteria: The isomer ratio of branched:linear PFOA in the extracted continuing verification (QC) sample was within +/- 10% for all QC data. Refer to Appendix C for detailed information regarding QC data.

Precision: Precision was not a component of this study.

Matrix Spikes: Matrix spike data were not a component of this study.

Spike Recoveries: Spike recoveries were not determined for the continuing verifications (QCs).

Surrogates: Surrogates were not a component of this study.

Statement of Data Quality

The ratio of isomers observed in the standards throughout this study were stable and a reliable identifier of the source product.

Statistical Methods and Calculations

Statistical methods were limited to the calculation of means, standard deviations, and percent difference. See Appendix E for example calculations used in E02-1053.

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Statement of Conclusion

Results from this study showed a wide distribution of the percentage of branched isomers of PFOA compared to the linear isomer of PFOA in the commercial pooled populations (Table 1). Since this is a preliminary screening study a larger sample size would be needed to determine if a significant statistical difference exists between pooled human sera samples.

As shown in Table 1, two of the lots of commercial pooled sera showed the presence of branched isomers of PFOA while the other two lots showed a much lower percentage (by at least 40x) of PFOA branched isomers.

References

3M Environmental Laboratory Study # E02-1039, November 2002

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Appendix A: Characterization of the Control Matrix

Table 7. Characterization of the Control Matrix Used for Analyses in Study E02-1053

Control Matrix	Rabbit Serum TN-A-4511
Source	Sigma
Expiration Date	09/26/2005
Storage Conditions	-20C ± 10C
Chemical Lot #	99H8400
Physical Description	Rabbit Serum

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Appendix B: Extraction and Analytical Method

This appendix includes the following method:

ETS-8-231.1, Solid Phase Extraction and Analysis of Fluorochemical Compounds from Biological Matrices, (19 pages)

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Method

Solid Phase Extraction and Analysis of Fluorochemical Compounds from Biological Matrices

Method Number: ETS-8-231.1

Adoption Date: 11/13/01

Revision Date: 2/18/02

Effective Date: 2/18/02

Approved By:

02/18/02

Willliam K. Reagen
Laboratory Manager

Date

Exact Copy of Original

LAS 11/07/02
Initial Date

1 Scope and Application

This method describes the extraction of target analytes from fish, rat liver, rat sera, mouse liver, and mouse sera using solid phase extraction (SPE). This method may also be extended to other biological matrices provided that the data quality objectives are met.

2 Method Summary

An amount of biological material, determined by the analyst, is prepared (fluids diluted and tissues homogenized) at a 1/6 dilution, or other dilution as determined by the analyst using reagent grade water. An aliquot of the dilution/homogenate is spiked with the appropriate surrogate or analyte mixture. Acetonitrile (ACN) is added as an extraction solvent and also serves to precipitate the proteins. The sample is capped, mixed, and put on the centrifuge to clarify the supernatant. The supernatant is transferred to a clean tube, diluted with water, and passed through a pre-conditioned C_{18} SPE cartridge. Finally, the analytes of interest are eluted from the SPE cartridge and analyzed by high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ES/MS/MS).

3 Definitions

3.1 Dilution

A dilution expressed as 1:5 or 1/6 is defined as: 1 mL of sample + 5 mLs of diluent for a total of 6 mLs combined, unless otherwise noted.

3.2 SPE cartridge

A column containing an open solvent reservoir at one end and packed with bonded silica sorbents at the other end. It is designed to retain the compounds of interest under some solvent conditions and elute them under others. A separation is thus achieved; compounds can be removed from difficult biological matrices and introduced into appropriate solvents for analysis.

3.3 Reagent grade water

Water with no detectable concentration(s) of the target analyte(s).

3.4 Quality control sample

Sample used to monitor the extraction efficiency (as a matrix spike) and to verify the continued accuracy of the initial calibration curve (as a continuing calibration verification).

4 Warnings and Cautions

4.1 Health and Safety Warnings

Always wear appropriate gloves, eyewear, and clothing when working with solvents, samples and/or equipment.

Use caution with the voltage cables for the probe. When engaged, the probe employs a voltage of approximately 5000 volts.

4.2 Cautions

Take care not to allow the SPE column to run to dryness after the methanol and water washes. After washing is complete, add sample then allow all of the liquid to pass through the SPE column to dryness.

Do not operate solvent pumps above capacity of 400 bar (5800 psi) back pressure. If the back pressure exceeds 400 bar, the HPLC will initiate automatic shutdown.

Do not run solvent pumps to dryness.

5 Interferences

To minimize interferences, Teflon should not be used for sample storage or any part of instrumentation that comes in contact with the sample or extract.

6 Instrumentation, Supplies, and Materials

The following instrumentation, supplies, and materials are used while performing this method. Equivalent instrumentation, supplies, and materials may be used in place of those listed.

6.1 Instrumentation

Vortex mixer, VWR, Vortex Genie 2

Ultra-Turrax T25 tissue homogenizer

Vacuum Pump

SPE Extraction Manifold

Centrifuge, Mistral 1000 or IEC

Shaker, Eberbach or VWR

Balance (+/- 0.1000 g)

Micromass, Quattro II or Ultima triple quadrupole Mass Spectrometer equipped with an electrospray ionization source

HP1100 or Agilent low pulse solvent pumping system, solvent degasser, column compartment, and autosampler

6.2 Supplies and Materials

Eppendorf or disposable pipettes, plastic or glass

Dissecting scalpels

Polypropylene bottles, capable of holding 50 mL to 1 L (Nalgene)

Volumetric flasks, glass, type A

40 mL glass vials (ICHEM)

Plastic sampule vials, Wheaton, 6 mL (or other appropriate size)

Centrifuge tubes, polypropylene, 15 mL and 50 mL

Labels

Graduated pipettes, glass

Syringes, capable of measuring 5 µL to 1000 µL

Bottle-Top Dispenser (capable of dispensing 5mL of solvent)

SPE extraction cartridge, 1 g, Sep-Pak 6 cc tri-functional C_{18} (Waters)

75 mL sample reservoir (or other appropriate size)

Crimp cap glass autovials and caps

Crimpers

HPLC analytical column, specifics to be determined by the analyst and documented in the raw data.

7 Reagents and Standards

Reagent grade water, Milli-Q™, Nanopure II, or equivalent

Acetonitrile, HPLC grade or equivalent

Methanol, HPLC grade or equivalent

Ammonium acetate, reagent grade or equivalent

Biological fluids or tissues, frozen from supplier

7.1 Reagents preparation

2.0 mM ammonium acetate solution: Weigh approximately 0.300 g ammonium acetate. Pour into a 2000 mL volumetric container containing reagent grade water, mix until all solids are dissolved, bring to volume using reagent grade water. Store at room temperature.

Note: When preparing different volumes than those listed in reagents preparation, target analyte standard preparation, and surrogate standard preparation, adjust accordingly.

7.2 Target analyte standard preparation

Prepare target analyte standard(s) for the standard curve. Multicomponent analyte standards are acceptable. The following is an example only and may or may not be appropriate for all standard preparations.

Weigh approximately 100 mg of target analyte into a 100 mL volumetric flask and record the actual weight in the standard logbook or other appropriate location.

Bring to volume with methanol for a stock standard of approximately 1000 ppm (µg/mL).

Dilute the stock solution with methanol for a working standard 1 solution of approximately 50 ppm. Example calculation: 1000 µg/mL x 5 mL/100 mL = 50 µg/mL.

Dilute working standard 1 with methanol to produce a working standard 2 solution of approx. 5.0 ppm. Example calculation: 50 µg/mL x 10 mL/100mL = 5.0 µg/mL.

Dilute working standard 1 with methanol to produce a working standard 3 solution of approx. 0.50 ppm. Example calculation: 50 µg/mL x 1.0 mL/100 mL = 0.5 µg/mL.

7.3 Surrogate standard preparation

Prepare surrogate standard(s). The following is an example only and may or may not be appropriate for all surrogate standard preparations.

Weigh approximately 90-110 mg of surrogate standard into a 100-mL volumetric flask and record the actual weight.

Bring to volume with methanol for a surrogate standard stock of approximately 900 - 1100 ppm.

Prepare a surrogate standard working standard. Transfer approximately 1 mL of surrogate standard stock to a 10-mL volumetric flask and bring to volume with methanol for a working standard of 90-110ppm. Record the actual volume transferred and standard concentrations in the standards logbook or other appropriate location.

7.4 Internal standard preparation

Prepare internal standard(s). The following is an example only and may or may not be appropriate for all internal standard preparations.

Weigh approximately 90-110 mg of internal standard into a 100-mL volumetric flask and record the actual weight.

Bring to volume with methanol for an internal standard stock of approximately 900 - 1100 ppm.

Prepare an internal standard working standard. Transfer approximately 1 mL of internal standard stock to a 10-mL volumetric flask and bring to volume with methanol for a working standard of 90-110ppm. Record the actual volume transferred and standard concentrations in the standards logbook or other appropriate location.

8 Sample Handling

All samples are received frozen and must be kept frozen until the extraction is performed.

Allow samples to thaw to room temperature prior to extraction.

Typically fresh matrix standards are prepared with each analysis. Extracted standards and samples are stored in capped autovials until analysis.

If analysis will be delayed, extracted standards and samples may be refrigerated at approximately 4°C indefinitely or may be stored at room temperature until analysis can be performed.

9 Quality Control

9.1 Blanks

9.1.1 Solvent Blank

An aliquot of methanol is used as a solvent blank. Solvent blanks are not extracted.

9.1.2 Method Blank

An aliquot of 1.0 mL of water, or other appropriate amount, is used as a method blank. Four method blanks are extracted and analyzed with each set following this procedure (two are spiked with surrogate and two are not spiked).

9.1.3 Matrix Blank

An aliquot of 1.0 mL or 1.0 g of matrix (diluted or homogenized) is used as a matrix blank. Other amounts may be used, as appropriate. Matrix blanks are prepared from one of three sources: 1) a study control matrix from a study control animal received with a sample set; 2) a commercially obtained sample of the same species as the study animals; or 3) a surrogate matrix, also obtained commercially, but of a different species than the study animal. (eg. if rat is used to generate standard curves and CCVs for a mouse study). The matrix to use is dependent on the matrix used for the curve.

9.1.3.1 Study control matrix curve - if the study control matrix is used for the curve, prepare four (4) matrix blanks using the study control matrix (two spiked with surrogate and two not spiked).

9.1.3.2 Commercially obtained (same species) matrix curve – if the commercially obtained matrix is used for the curve, prepare four (4) matrix blanks using the same commercially available matrix (two spiked with surrogate and two not spiked).

9.1.3.3 Surrogate matrix curve – if a surrogate matrix is used for the curve, prepare four (4) matrix blanks using the same commercially available matrix and prepare four (4) matrix blanks using a commercially available matrix of the same species as the study animals (two spiked with surrogate and two not spiked).

9.1.3.4 If limited matrix is available, the number of method and matrix blanks may be adjusted and will be noted in the study protocol or in the raw data.

9.2 Sample Replicate

Samples replicates are prepared according to each study protocol or project outline.

9.3 Surrogate standard

If surrogate standard is a component of the study, all samples are spiked with surrogate standard prior to extraction to obtain a concentration in the mid-range of the calibration curve, with the exception of blank samples as described above.

Typically surrogate standard is spiked into the 1.0 mL diluted/homogenized sample removed for extraction. However, surrogate may be spiked directly into the matrix prior to diluting with water, into the diluted/homogenized sample prior to removing the 1.0 mL sample, or into the 1.0 mL diluted/homogenized sample removed for extraction.

9.4 Internal standard

If internal standard is a component of the study, all samples are spiked with internal standard after extraction to obtain a concentration in the mid-range of the calibration curve.

Typically internal standard is spiked into the 2.0 mL of extract in the 15 mL centrifuge tube, before transferring to the autovial.

9.5 Lab Control Sample

Lab control samples are not a component of this method.

9.6 Quality Control (QC) Sample

Prepare quality control (QC) samples to monitor extraction efficiency and to verify the continued accuracy of the initial calibration curve. Typically 1.0 mL, or other appropriate amount, of the same matrix used to prepare the initial calibration curve is used for each QC sample.

Twelve (12) quality control samples (QC) will be prepared for each matrix during the course of a study. A minimum of 3 QC samples must be prepared (one at each level) on each day of sample extraction. (e.g. If the study is such that samples will be extracted on three different days then four QC samples must be prepared on each day of extraction for a total of twelve.)

QC samples will consist of four samples at each of three levels of analyte. The levels listed below may be used and may represent sample concentrations diluted into the range of the calibration curve:

Low level: 3X to 5X the LLOQ,

Mid-level: equivalent to a point near the middle of the calibration curve,

High level: 80% of the ULOQ

Two QC sample levels are analyzed after every tenth sample injection starting after the last calibration standard injection, with a minimum of three QC per analysis. Solvent blanks are not considered samples but may be included as such for determining when QC samples will be analyzed.

QC samples extracted with a particular sample set must be analyzed in the same analytical run. Any QC samples extracted during the course of the study may be included in subsequent analyses.

If samples from multiple extraction dates are analyzed in one analytical run, then QC samples from the same sample extraction dates must be included in that analysis.

Each QC is expected to show an accuracy of 75-125% of expected. A minimum of 2/3 of all QC samples must meet this criteria, and a minimum of 1/2 of the QC samples at each level must meet this criteria. If not, the set must either be re-analyzed or re-extracted.

9.7 Sample Dilution

Any sample with an area greater than that of the highest acceptable standard will need to be diluted into the range of the calibration curve. If samples are diluted into the range of the curve during analyses and enough sample remains, a post-run dilution validation will be performed to verify sample values.

To perform the dilution validation, one sample will be separated into two representative samples (i.e. two 1.0 mL aliquots for fluid samples or two 1.0 gram amounts for tissue samples, or other amount as determined by the analyst

and documented in a note to file) then diluted using two procedures. The first procedure consists of diluting the sample with additional matrix prior to extraction (fluid adding fluid), while the second procedure consists of diluting the extract with solvent post-extraction (methanol extract adding additional methanol solvent.)

If the relative percent difference is not within 15% for these two samples; additional testing will be required to determine which value is a correct representation of the sample concentration.

10 Calibration and Standardization

10.1 Instrument Calibration

One calibration curve will be prepared from extracted matrix standards, in the same matrix as the samples, per study. It will consist of a minimum of nine (9) levels. Additional calibration curves may be extracted on separate sample extraction dates, as determined by the analyst and documented in a note to file.

Transfer 1.0 mL, or other appropriate amount, of diluted control fluid or homogenized control tissue to a 15 mL centrifuge tube using a disposable plastic pipette. This will be repeated while preparing aliquots for the standard curve. Be sure to mix or shake the control matrix container between aliquots to ensure a homogenous sample is removed.

Record each standard volume on the weight/volumes sheet or extraction worksheet, as appropriate.

Four 1.0 mL aliquots, or other appropriate amount, of control matrix serve as matrix blanks.

The standard concentrations and spiking amounts listed in Table 1 may be used, when appropriate, to spike one standard curve. A total of 9 standards, four matrix blanks, and four method blanks are prepared in addition to the QC samples and test samples. The number of standards and blanks may be adjusted as determined by the analyst and documented in a note to file.

Use Attachment C, or other appropriate form, as an aid in calculating the concentrations of the working standards. Refer to section 12 to calculate the actual concentration of analyte in each calibration standard and QC sample.

Typically the target analyte standard is spiked into the 1.0 mL diluted/homogenized sample removed for extraction. However, it may be spiked directly into the matrix prior to diluting with water, into the diluted/homogenized sample prior to removing the 1.0 mL sample, or into the 1.0 mL diluted/homogenized sample removed for extraction.

Analyze the extracted matrix standard curve prior to each set of extracts. The curve equation will be determined by regression analysis using the peak areas of the target analyte(s) using MassLynx or other suitable software.

Any level outside 75% - 125% of nominal must be deactivated, and regression re-calculated, except the LLOQ which must be within 30% of nominal. All levels must show a response greater than twice that of the blank. A maximum of three (3) levels may be deactivated in any one set, or the set will be re-analyzed.

TABLE 1 APPROXIMATE SPIKING AMOUNTS FOR STANDARDS AND SPIKES USING 1.0 mL OF MATRIX			
Working standard (approximate concentration)	µL	Approximate final concentration of analyte **in Matrix diluted 1:5**	Approximate final concentration of analyte **in Final 2.0 mL volume**
-	-	Blank	Blank
0.500 ug/mL	1.5	5.00 ng/g or ng/mL	0.375 ng/mL
0.500 ug/mL	3.0	10.0 ng/g or ng/mL	0.750 ng/mL
0.500 ug/mL	8.0	25.0 ng/g or ng/mL	2.00 ng/mL
0.500 ug/mL	16	50.0 ng/g or ng/mL	4.00 ng/mL
0.500 ug/mL	32	100 ng/g or ng/mL	8.00 ng/mL
5.00 ug/mL	5.6	175 ng/g or ng/mL	14.0 ng/mL
5.00 ug/mL	8.0	250 ng/g or ng/mL	20.0 ng/mL
5.00 ug/mL	16	500 ng/g or ng/mL	40.0 ng/mL
5.00 ug/mL	24	750 ng/g or ng/mL	60.0 ng/mL
5.00 ug/mL	32	1000 ng/g or ng/mL	80.0 ng/mL
5.00 ug/mL	40	1250 ng/g or ng/mL	100 ng/mL
50.0 ug/mL	5.0	1500 ng/g or ng/mL	125 ng/mL
50.0 ug/mL	6.0	1750 ng/g or ng/mL	150 ng/mL
Surrogate Std 100 ug/mL	10	6500 ng/g or ng/mL	500 ng/mL

11 Procedures

11.1 Tissue Sample Preparation

Obtain frozen tissue samples

Cut approximately 1.0000 g of tissue (+/- 0.1000 g), or other appropriate amount, using a dissecting scalpel. This part of the procedure is best performed quickly, not allowing the tissue to thaw.

Weigh the tissue directly into a tared plastic sampule vial.

Record the weight on the weight/volume sheet, extraction worksheet, or other appropriate location.

Return unused tissue to the freezer after extraction amounts have been removed.

Add 2.5 mL of reagent water to sampule vial, or other volume as determined by the analyst and documented in a note to file.

Homogenize the sample. Put the Ultra-Turrax grinder probe in the sample and grind for approximately 2 minutes, or until the sample is homogeneous.

Rinse the probe into the tube containing the sample with 2.5 mL of reagent grade water, or other volume as determined by the analyst and documented in a note to file, using a pipette.

Take the grinder apart and clean it with methanol after each sample. Refer to ETS-9-52 for more information.

If an amount other than 1.0000 g (not within +/- 0.1000 g) is removed for an initial weight, adjust the water volume accordingly to maintain a 1/6 dilution. (e.g. if 0.5 g is removed for extraction, add a total of 2.5 mL of water.), or other ratio as determined by the analyst and documented in a note to file.

11.2 Fluid Sample Preparation

Obtain frozen fluid sample and allow it to thaw at room temperature or in lukewarm water.

Label a 15 mL polypropylene centrifuge tube with the study number, sample ID, extraction date and analyst initials. See attached worksheet (Attachment A or similar worksheet) for documenting the remaining steps.

Vortex mix the fluid sample for approximately 15 seconds, then transfer 1.0 mL of fluid, or other appropriate amount to a plastic sampule vial, or other appropriate container.

Return unused samples to freezer after extraction amounts have been removed.

Add 5.0 mL of reagent water to the 1.0 mL of fluid for a 1/6 dilution, or other dilution as determined by the analyst and documented in a note to file.

If a volume other than 1.0 mL is removed for an initial volume, adjust the water volume accordingly to maintain the same dilution as above.

11.3 Tissue and Fluid Sample Extraction

After tissue or fluid samples have been prepared according to sections 11.1 and 11.2, vortex mix or shake by hand the diluted/homogenized sample for approximately 15 seconds then transfer 1.0 mL, or other appropriate volume, to a clean 15 mL polypropylene centrifuge tube.

Return unused diluted/homogenized portions to the freezer after extraction amounts have been removed.

Record the volume removed on the extraction worksheet, (Attachment A or similar worksheet).

Spike blanks, samples, and standards, ready for extraction with surrogate standard as described in this method.

Spike each calibration standard matrix with the appropriate amount of standard as described in this method for the calibration curve standards and each QC sample.

Vortex mix the standard curve samples and QC samples for approximately 5 seconds.

To each sample and standard, add 5.0 mL of acetonitrile, cap, and vortex mix or shake by hand approximately 15 seconds.

Place all samples on the shaker at an appropriate speed for 20 minutes to adequately mix (a setting of approximately 300 rpm on the models listed in section 6.1).

Remove from the shaker and centrifuge at an appropriate speed for 10 minutes to adequately pellet the precipitate (a setting of approximately 2000 rpm on the models listed in section 6.1).

Add 40.0 mL of reagent grade water to a clean 50 mL centrifuge tube. Remove samples from the centrifuge and decant the supernatant into the water in the 50 mL tube, taking care not to introduce any of the matrix solids into the solution. Cap and mix by inverting several times. In this step the order of addition may be changed (i.e. the sample may be put into the centrifuge tube and then the water added).

Attach the reservoir to the SPE cartridge and attach this reservoir/cartridge unit to a vacuum manifold.

NOTE: When running the vacuum, set the vacuum chamber at approximately 15 kPA - to give an approximate elution flow of 5-7 mL/min. Flows may vary through cartridges and the kPA may be raised for slow tubes and drying after most have been drawn down.

Prepare the SPE cartridge by washing twice with approximately 5.0 mL of methanol, followed by approximately two 5.0 mL aliquots of water, **taking care not to allow the column to run to dryness after each wash**.

After washing is complete, pour the sample into the reservoir/cartridge unit and **allow all of the liquid to pass through the column to dryness.**

Run the vacuum on high for approximately 5 minutes to adequately dry each SPE cartridge.

Place a collection 15 mL polypropylene centrifuge tube under each cartridge and elute with 2.0 mL of methanol.

Spike extracted blanks, samples, and standards with internal standard as described in this method.

Label each glass autovial, as appropriate, with the study number, vial file archive number, animal number/gender/timepoint or LIMS number, matrix, final solvent, analyte components (if needed), extraction type, extraction date, and analyst(s) performing the extraction.

Transfer each eluant to a glass autovial and cap.

11.4 Extract Analysis

11.4.1 Software set-up

On the MassLynx main page, set up a sample list name. Save the list as instrument designator letter, last 2 digits of test year-month-day, and a letter that will increase through the alphabet with each additional list for that day.

Example Sample List: IYYMMDDa or A020204a

I = Initial of the instrument name (A = "Amelia")

YY = Test year (02)

MM = Test month (02)

DD = Test day (04)

a = First sample list (run) of the day (the next sample list will end with 'b', the next 'c',

and so on.)

Assign a filename using the instrument designator letter, the last 2 digits of the test year-month-day, and a 3-digit sequential file number that starts with 1 and increases by one for each filename.

Example filename: IYYMMDD### or A020204001

I = Initial of instrument name

YY = Test year

MM = Test month

DD = Test day

= 3-digit sequential file number starting with 1 through 999 (001)

Also, as part of the samplelist, assign a method (MS) for acquiring, an inlet file, a bottle number, an injection volume, and sample descriptions.

To create a method, click on Method Editor button in the MS Status Pane and select SIR (Single Ion Recording) or MRM (Multiple Reaction Monitoring). Set Ionization Mode as appropriate and mass to 499 or other appropriate mass(es). Also set the acquisition start and stop times. Save acquisition method. If MS/MS instruments are employed, additional product ion fragmentation information may be collected. See Micromass MassLynx "Guide to Data Acquisition" for additional information on MRM.

Typically the analytical batch run sequence begins with system suitability, solvent blanks, and a set of extracted matrix standards.

Sample extracts are analyzed with two QC samples injected after every tenth sample injection. Solvent blanks should be analyzed periodically to monitor possible analyte carryover and are not considered sample extracts but may be included as such.

11.4.2 HPLC set-up

Set up sample tray according to the sample list prepared above.

Set up the HPLC to the following conditions or at conditions the analyst considers appropriate for optimal response. Record actual conditions in the instrument logbook, or other appropriate location:

Sample size = 10 µL injection

Inject/sample = 1

Cycle time = 10.0 minutes

Flow rate = 300 µL/min

Mobile phase: Solvent A = 2 mM Ammonium Acetate, Solvent B = Methanol

Solvent gradient program:

Time	% Solvent B
0.00	10%
1.00	10%
5.50	95%
7.50	95%
8.00	10%

11.4.3 Instrument set-up

Refer to ETS-9-24, "Operation and Maintenance of the Micromass Quattro II Triple Quadrupole Mass Spectrometer Fitted with an Atmospheric Pressure Ionization Source," for details.

Check the solvent level in HPLC reservoirs and refill if necessary.

Check the stainless steel capillary at the end of the probe. Use an eyepiece to check the tip. The tip should be flat with no jagged edges. If the tip is found to be unsatisfactory, disassemble the probe and replace the stainless steel capillary.

Turn on the nitrogen.

Open the tune page. Click on operate to initiate source block and desolvation heaters.

Open the Inlet Editor.

Download the HPLC method and initiate solvent flow to begin system equilibrium.

Set the flow to 10–500uL/min or as appropriate

Set HPLC pump to "On"

Observe droplets or mist coming out of the tip of the probe. A fine mist should be expelled with no nitrogen leaking around the tip of the probe. Readjust the tip of the probe if no mist is observed

Allow to equilibrate for approximately 10 minutes.

Typical instrument parameters include:

Drying gas 250–400 liters/hour

ES nebulizing gas 10–15 liters/hour

HPLC constant flow mode, flow rate 10–500 µL/min

Pressure <400 bar (this parameter is not set, it is a guide to ensure the HPLC is operating correctly.)

Source block temperature approximately 150°C

Desolvation temperature approximately 250°C

These settings may change in order to optimize the response

Print the tune page, sample list, and acquisition method from MassLynx and store it in the study binder with a copy taped into the instrument log.

Click on start button in the Acquisition Control Panel (the location of the start button may vary among MassLynx versions, refer to appropriate MassLynx User's Guide).

12 Data Analysis and Calculations

12.1 Calculations

If other calculations are used than those listed, they will be documented in the raw data.

Calculate the matrix amount contained in the initial dilution using the following equation:

$$\text{Matrix Amount (g/mL or mL/mL)} = \frac{\text{IW (g) (or IV (mL))}}{\text{(IW(g) (or IV(mL))+ DV (mL)}}$$

Calculate actual concentrations of analyte in calibration standards using the following equation:

$$\text{Concentration (ng/g or ng/mL)} = \frac{\text{Spike Concentration (ug/mL)} \times \text{ Spiked Amount (mL)}}{\text{SV (mL)} \times \text{Matrix Amount (g/mL or mL/mL)}} \times \frac{1000\ \text{ng}}{1\ \text{ug}}$$

IW = Initial weight (where 1.0 g = 1.0 mL)

IV = Initial volume

DV = Diluent volume (reagent grade water)

SV = Sample volume removed for extraction (typically 1.0 mL)

AR = Analytical result from MassLynx summary

DF = Dilution factor

FV = Final volume

MA = Matrix amount

∂ curve = MA of tissue/fluid standard curve, assumed to be 1 g or 1 mL/5 mL water

∂ sample = MA of tissue/fluid sample (___g or mL of sample/5 mL water)

Calculate spike percent recoveries using the following equation:

$$\text{\% Recovery} = \frac{\text{Observed Result} - \text{Matrix Blank Result}}{\text{Spiking Level}} \times 100$$

Calculate relative standard deviation using the following equation:

$$\text{Relative Standard Deviation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

Calculate percent deviation using the following equation:

$$\%\ \text{Deviation} = \frac{\text{Expected Conc.} - \text{Calculated Conc.}}{\text{Expected Conc.}} \times 100$$

Calculate actual concentration of analyte in fluid (µg/mL):

$$\text{AR (ng/mL)} \times \text{DF} \times \frac{\partial \text{ curve (mL/mL)}}{\partial \text{ sample (mL/mL)}} \times \frac{\text{FV (mL) in Curve}}{\text{FV (mL) in Matrix}} \times \frac{1.0\ \mu g}{1000\ ng} = (\mu g/g)$$

Calculate actual concentration of analyte in tissue (µg/g):

$$\text{AR (ng/g)} \times \text{DF} \times \frac{\partial \text{ curve (g/mL)}}{\partial \text{ sample (g/mL)}} \times \frac{\text{FV (mL) in Curve}}{\text{FV (mL) in Matrix}} \times \frac{1.0\ \mu g}{1000\ ng} = (\mu g/g)$$

13 Method Performance

13.1 System Suitability

System suitability will be determined prior to the start and at the completion of each analytical run. Prior to the calibration curve and after the last sample of the run three (3) mid-level unextracted calibration standards will be analyzed. As applicable, the peak area precision, retention time precision, resolution, and peak asymmetry will be monitored at the beginning and the end of the run separately. The peak area precision must be equal to or less than 5.0% RSD, the precision of the retention time must be equal to or less than 2.5% RSD, the resolution must be > 2.0, and the peak asymmetry (fronting or tailing) must be $0.5<AF<2.0$, where AF is the asymmetry factor.

If any item of the system suitability fails, system maintenance must be completed prior to running a second set of system suitability samples and the system suitability must pass before starting the calibration. If system suitability fails at the completion of a run, the sample set must be reanalyzed.

13.2 Quantitation

The coefficient of determination value for the calibration curve, plotted by regression using the peak areas of the analyte(s), must be 0.990 or better.

All active calibration curve points must be within 25% of the theoretical value with the exception of the LOQ point, which may deviate up to 30%.

Calibration standards with peak areas less than two times the curve matrix blank will be deactivated to disqualify a data range that may be affected by background levels of the analyte.

A valid calibration curve must contain at least 6 active points above and including the LOQ.

If the curve cannot meet these criteria, the sample set must be reanalyzed or reextracted.

13.3 Accuracy

Two thirds of all quality control samples and 1/2 of each quality control sample at each level are expected to show an accuracy of 75-125%.

Surrogates and internal standards must have a percent deviation < 50%. Deviations outside this range will be reanalyzed to confirm. If the second analysis confirms the original, the deviation will be documented in the raw data. If the second analysis is within 50%, then the second value will replace the original value.

14 Pollution Prevention and Waste Management

Sample waste is disposed of in noninfectious biohazard waste containers.

Flammable solvent waste is disposed of in high BTU containers.

Glass pipette waste is disposed of in broken glass containers located in the laboratory.

15 Records

Complete the extraction worksheet attached to this method, or other applicable worksheet, and store with the study raw data.

Each page generated for a study must contain the following information (if applicable): study/project or instrument number, acquisition method, integration method, sample name, extraction date, dilution factor (if applicable), and analyst. Other information may be added if applicable to the study.

Print the tune page, sample list, and acquisition method from MassLynx to include with the study raw data. Copy these pages and tape into the instrument runlog.

Plot the calibration curve by the appropriate regression. Print these graphs and store with the study raw data.

Print data integration summary, integration method, and chromatograms from MassLynx, and store with the study raw data.

Summarize data using suitable software (Excel 7.0 or LIMS) and store in the study folder.

Back up electronic data to appropriate medium. Record in study notebook the file name and location of backup electronic data.

16 Attachments

Attachment A: Extraction Worksheet

Attachment B: Sample Weight/Volume Worksheet

Attachment C, Calibration Standard Concentration Worksheet

Attachment D, Dilutions Summary Worksheet

17 References

ETS-9-24, "Operation and Maintenance of the Micromass Quattro II Triple Quadrupole Mass Spectrometer Fitted with an Atmospheric Pressure Ionization Source"

ETS-9-52, "Operation and Maintenance of a Tissue Grinder"

18 Affected Documents

None

19 Revisions

Revision Number	Revision Description	Revision Date
1	Minor formatting changes. Added detailed information to all sections concerning the extraction procedure, analytical procedure, and calculations. Added attachments and references.	02/18/02

Attachment A – Extraction Worksheet

Study Number:
Prep Date:
Analysts initials:
Box#:

Method Revision: ETS-8-231.1
Matrix:
Sample Timepoint:

Sample Number	Sample Number or description	Volume of diluted sample removed	Amount and spike mix used	Amount and surrogate spike mix used	Type of column used and lot	Elution solvent and volume	Comments

Blank matrix _____ TN-A-_____; Amount weighed/aliquoted: _____ g/mL

1. Homogenize sample
2. Aliquot 1 mL of diluted matrix into 15 mL polypropylene tube
3. Spike samples accordingly
4. Add ____ mL of ACN (TN-A-_____) to each diluted sample and shake or vortex mix
5. Shake sample for 20 min @ _____ rpm (Shaker _____)
6. Centrifuge sample for 10 min @ _____ rpm (Centrifuge _____)
7. Add 40 mL of _____ water to 50 mL polypropelene centrifuge tube.
8. Decant extract into centrifuge tubes with water
9. Shake sample slightly to ensure proper mixing
10. Attach 6 mL C18 SPE cartridges and 75 mL reservoirs to vacuum manifold
11. Condition column with two washes of ~5 mL MeOH (TN-A-_____) - do not allow column to go to dryness
12. Wash column with two washes of ~5 mL _____ water - do not allow column to go to dryness
13. Filter sample through conditioned column, discarding filtrate
14. Allow column to go to dryness. After dripping stops, draw a high vacuum through column for at least 5 minutes.
15. Elute column with solvent (_____TN-A-_____) into appropriate 15 mL centrifuge tube
16. Spike samples with _____ uL of internal standard # _____, conc._____
17. Transfer sample into appropriately labeled autovial and cap

Note: In vacuum steps above set the vacuum chamber at approximately 15 kPA - this should give approximately 5-7 mL/min elution flow
Flows may vary through cartridges - kPA may be raised for slow tubes and drying after most have been drawn down and shut off.

Attachment B - Sample Weight/Volume Worksheet

Prep Date(s):
Analyst(s):
Sample Matrix:
Method/Revision:

Study Number:
Equipment Number:
Final Solvent & TN Number:

Sample ID	Initial Wt./Vol. g/mL/L	Water Volume added (mL)	Volume Removed (mL)		Comments

Form Completion Verified By:_____

Attachment C: Calibration Standard Concentration Worksheet

Prep date(s):
Analyte(s):
Sample matrix:
Method/revision:

Standard number:
Equipment number:
Final solvent and TN:
Blank Tissue or Fluid/identifier:

Analyte mix std approx. 0.500 ug/mL:
Analyte mix std approx. 5.00 ug/mL:
Analyte mix std approx. 50.0 ug/mL:
Surrogate std approx. 100 ug/mL:

Actual concentrations of standards in the analyte mix

Analyte Std conc ug/mL		**All** Am't spiked mL	**All** Final Volume: mL	**All** Initial Fluid Dilution mL/mL	**All** Initial Tissue Density g/mL
0.500		0.0015	2.00	0.1667	0.1600
0.500		0.0030	2.00	0.1667	0.1600
0.500		0.0080	2.00	0.1667	0.1600
0.500		0.0160	2.00	0.1667	0.1600
0.500		0.0320	2.00	0.1667	0.1600
5.00		0.0056	2.00	0.1667	0.1600
5.00		0.0080	2.00	0.1667	0.1600
5.00		0.0160	2.00	0.1667	0.1600
5.00		0.0240	2.00	0.1667	0.1600
5.00		0.0320	2.00	0.1667	0.1600
5.00		0.0400	2.00	0.1667	0.1600
50.0		0.005	2.00	0.1667	0.1600
50.0		0.006	2.00	0.1667	0.1600

Calculated concentrations of standards in relation to the final 2.0 mL solvent and initial matrix

2.0 mL Final Volume		**Fluid Matrix**		**Tissue Matrix**	
Analyte Final conc. ng/mL	**Surrogate** Std conc ng/mL	**Analyte** Final conc. ng/mL	**Surrogate** Std conc ng/mL	**Analyte** Final conc. ng/g	**Surrogate** Std conc ng/mL
0.375	100	5.00	100	5.00	100
0.750		10.0		10.0	
2.00	**Surrogate**	25.0	**Surrogate**	25.0	**Surrogate**
4.00	Final conc	50.0	Final conc	50.0	Final conc
8.00	ng/mL	100	ng/mL	100	ng/g
14.0	0.500	175	6500	175	6500
20.0		250		250	
40.0		500		500	
60.0		750		750	
80.0		1000		1000	
100		1250		1250	
125		1500		1500	
150		1750		1750	

Attachment D: Dilutions Summary Worksheet

Study:
Dilution Date/Analyst:
Box Number:

Solvent/TN Number:
Extraction Date/Analyst:
Matrix/Timepoint:

Sample Number or Description	Dilutions								
	1/	1/	1/	1/	1/	1/	1/	Comments	Verified By:

Notes:
1/10 dilution = _____ of sample + _____ of solvent

Form Completion Verified By: _____

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Appendix C: QC Data Summary Tables

Table 8. Acceptance Criteria Summary of PFOA-NH4 QC samples Analyzed 11/01/02

Sample Identification	PFOA-NH4 % Branched/ Sum Branched + Linear	% Difference
RBS101702-10 ng/mL-1	26.02	NA
RBS101702-QC-10 ng/mL-1-1	25.35	3
RBS101702-QC-10 ng/mL-1-2	24.93	4

% Difference: The difference between the total ion current peak area ratios of the branched:linear isomers for PFOA in the extracted standard (labeled RBS-date of extraction-concentration) at the same level as the extracted QC (labeled RBS-date of extraction-QC-concentration).

Table 9. Acceptance Criteria Summary of PFOA-Acid QC samples Analyzed 11/01/02

Sample Identification	PFOA-Acid % Branched/ Sum Branched + Linear	% Difference
RBS101702-10 ng/mL-1	2.22	NA
RBS101702-QC-10 ng/mL-1-1	2.24	-1
RBS101702-QC-10 ng/mL-1-2	2.04	8

% Difference: The difference between the total ion current peak area ratios of the branched:linear isomers for PFOA in the extracted standard (labeled RBS-date of extraction-concentration) at the same level as the extracted QC (labeled RBS-date of extraction-QC-concentration).

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Appendix D: Data Spreadsheets

3M Environmental Laboratory

ANALYSIS 11/01/02

TIC

Identification	Branched Peak Ret Time 7.6 **Branched PFOA-NH4 Area****	Branched Peak Ret Time 7.9 **Branched PFOA-NH4 Area**	Linear Peak Ret Time 8.1 **Linear PFOA-NH4 Area**	Filename D021101a	Branched Ret Time 7.9/ Sum of Branched:Linear * 100 **Branched/Linear**
Standards					
RBS101702-10 ng/mL-1	0	93029	264494	D021101003	26.02
RBS101702-QC-10 ng/mL-1-1	0	81108	238850	D021101027	25.35
RBS102802-QC-10 ng/mL-1-1	0	65454	195485	D021101029	25.08
RBS101702-QC-10 ng/mL-1-2	0	59384	178832	D021101055	24.93
RBS102802-QC-10 ng/mL-1-2	0	56858	168536	D021101057	25.23
WB102802-H2O Blk-1	0	355	2873	D021101043	11.00
RBS102802-Sera Blank-1*	0	0	3846	D021101045	0.00
0.5 ng/mL-1	0	1566	6752	D021101063	18.83
Pooled					
TCR-687-Bioresource*	0	361	114603	D021101007	0.314
TCR-688-Lampire	0	8059	41466	D021101009	16.27
TCR-689-Sigma	0	8824	55100	D021101011	13.80
TCR-690-Golden West*	0	361	168154	D021101013	0.214

* Below Limit of Peak Area Threshold

** Branched peak at 7.6 minutes observed only in PFOA-NH4 standards at concentrations > 25 ng/mL. Not included in any calculations.

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PFOA-Acid Pooled data were not included in the scope of the study and were not reported

TIC

Identification	Branched Peak Ret Time 7.9 **Branched PFOA-Acid Area**	Linear Peak Ret Time 8.1 **Linear PFOA-Acid Area**	Filename D021101a2	Branched Ret Time 7.9/ Sum of Branched:Linear * 100 **Branched/Linear**
Standards				
RBS101702-10 ng/mL-2	7971	351306	D021101005	2.22
RBS101702-QC-10 ng/mL-2-1	5022	219508	D021101031	2.24
RBS102802-QC-10 ng/mL-2-1	2733	225367	D021101033	1.20
RBS101702-QC-10 ng/mL-2-2	4416	211715	D021101059	2.04
RBS102802-QC-10 ng/mL-2-2	1909	198898	D021101061	0.951
WB102802-H2O Blk-1	260	2873	D021101043	8.30
RBS102802-Sera Blank-1	545	3787	D021101045	12.58
0.5 ng/mL-2	361	6473	D021101065	5.28
Pooled				
TCR-687-Bioresource*	361	113845	D021101007	0.316
TCR-688-Lampire	8207	41469	D021101009	16.52
TCR-689-Sigma	8823	55095	D021101011	13.80
TCR-690-Golden West*	361	168119	D021101013	0.214

* Below Limit of Peak Area Threshold

11/01/02 Analysis Identification	TIC Branched Peak Ret Time 7.6 PFOA-NH4 Area**	Branched Peak Ret Time 7.9 PFOA-NH4 Area	Linear Peak Ret Time 8.1 PFOA-NH4 Area	Filename	Ret Time 7.9/ Sum of Branched:Linear * 100	% Diff
Standards & CCVs						
RBS101702-10 ng/mL-1	0	93029	264494	D021101003	26.02	NA
RBS101702-QC-10 ng/mL-1-1	0	81108	238850	D021101027	25.35	3
RBS102802-QC-10 ng/mL-1-1*	0	65454	195485	D021101029	25.08	4
RBS101702-QC-10 ng/mL-1-2	0	59384	178832	D021101055	24.93	4
RBS102802-QC-10 ng/mL-1-2*	0	56858	168536	D021101057	25.23	3

* Data not used/reported. Not extracted on the same date as the samples and therefore not included in tables 8 and 9.

** Branched peak at 7.6 minutes observed only in PFOA-NH4 standards at concentrations > 25 ng/mL. Not included in any calculations.

TIC

11/01/02 Analysis Identification	Branched Peak Ret Time 7.9 PFOA-Acid Area	Linear Peak Ret Time 8.1 PFOA-Acid Area	Filename	Ret Time 7.9/ Sum of Branched:Linear * 100	% Diff
Standards & CCVs					
RBS101702-10 ng/mL-2	7971	351306	D021101005	2.22	NA
RBS101702-QC-10 ng/mL-2-1	5022	219508	D021101031	2.24	-1
RBS102802-QC-10 ng/mL-2-1*	2733	225367	D021101033	1.20	46
RBS101702-QC-10 ng/mL-2-2	4416	211715	D021101059	2.04	8
RBS102802-QC-10 ng/mL-2-2*	1909	198898	D021101061	0.951	57

* Data not used/reported. Not extracted on the same date as the samples and therefore not included in tables 8 and 9.

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Appendix E: Example Calculations

Calculations used for Analyses in Study E02-1053

Percentage of Branched:Linear Isomer (also referred to as ratio in the report)

$$\text{Percentage} = \frac{\text{Branched TIC Peak Area}}{(\text{Branched TIC Peak Area} + \text{Linear TIC Peak Area})} * 100$$

Sample TCR-688 PFOA Percentage: (8059/(8059+41466)) * 100 = 16%

$$\%\ \text{Difference} = \frac{(\text{Expected TIC \% Branched/Sum Initial Std} - \text{Observed TIC \% Branched/Sum QC})}{\text{Expected TIC \% Branched/Sum Initial Std}} * 100$$

Sum = Branched peak area + Linear peak area

% Difference of PFOA RBS102202-QC-10 ng/mL-1-1 sample analyzed 10/22/02

Initial Std = RBS101702-10 ng/mL-1 TIC % Branched/Sum = 22
QC = RBS102202-QC-10 ng/mL-1-1 TIC % Branched/Sum = 24

% Difference = |(22 – 24) / 24| = 9

3M Environmental Laboratory

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Analytical Report: LIMS E02-1053

Appendix F: Interim Certificate(s) of Analysis

3M Environmental Laboratory

Centre Analytical Laboratories, Inc.
3048 Research Drive State College, PA 16801 www.centrelab.com
Phone: (814) 231-8032 Fax: (814) 231-1253 or (814) 231-1580

INTERIM CERTIFICATE OF ANALYSIS

Centre Analytical Laboratories COA Reference #: 023-033 (Revision 1)
3M Product: Ammonium Perfluorooctanoate
Test Control Reference #: TCR-99131-37, Lot #: 332
Purity: 95.2%

Test Name	Specifications	Result
Purity[1]		95.2%
Appearance	White, crystalline solid	Conforms
Identification NMR		Positive
Metals (ICP/MS) 1. Calcium 2. Magnesium 3. Sodium 4. Potassium 5. Nickel 6. Iron 7. Manganese		1. 0.001 wt./wt.% 2. <0.001 wt./wt.% 3. 0.005 wt./wt.% 4. <0.001 wt./wt.% 5. <0.001 wt./wt.% 6. <0.001 wt./wt.% 7. <0.001 wt./wt.%
Total % Impurity (NMR)		0.34 wt./wt.%
Total % Impurity (LC/MS)		4.49 wt./wt.%
Total % Impurity (GC/MS)		None Quantified
Residual Solvents (TGA)		None Detected
Purity by DSC		99.7%
Inorganic Anions (IC) 1. Chloride 2. Fluoride 3. Bromide 4. Nitrate 5. Nitrite 6. Phosphate 7. Sulfate		1. <0.015 wt./wt.% 2. <0.005 wt./wt.% 3. <0.040 wt./wt.% 4. <0.009 wt./wt.% 5. <0.006 wt./wt.% 6. <0.006 wt./wt.% 7. <0.040 wt./wt.%
Organic Acids[2] (IC) 1. TFA 2. PFPA 3. HFBA 4. NFPA		1. <0.1 wt./wt.% 2. <0.1 wt./wt.% 3. <0.1 wt./wt.% 4. <0.25 wt./wt.%
Elemental Analysis[3]: 1. Carbon 2. Hydrogen 3. Nitrogen 4. Sulfur 5. Fluorine	Theoretical Value = 22.3% Theoretical Value = 0.935% Theoretical Value = 3.25% Theoretical Value = 0% Theoretical Value = 66.1%	1. 18.9 wt./wt.% 2. 1.31 wt./wt.% 3. 3.75 wt./wt.% 4. 4.34 wt./wt.% 5. 63.2 wt./wt.%
Ammonium Analysis[3] Ion Selective Electrode	Theoretical Value = 4.18%	3.49 wt./wt. %

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INTERIM CERTIFICATE OF ANALYSIS

Centre Analytical Laboratories COA Reference #: 023-033 (Revision 1)
3M Product: Ammonium Perfluorooctanoate
Test Control Reference #: TCR-99131-37, Lot #: 332

Date of Last Analysis: 12/15/00

Expiration Date: 12/15/06

Storage Conditions: < -10 °C

Re-assessment Date: 12/15/06

[1]Purity = 100% - (Total Metal impurities, 0.006% + Total NMR impurities, 0.34% + Total LC/MS impurities, 4.49%)

Total impurity from all tests = 4.84%
Purity = 100% - 4.84% = 95.2%

[2] TFA Trifluoroacetic acid
HFBA Heptafluorobutyric acid
NFPA Nonafluoropentanoic acid
PFPA Pentafluoropropanoic acid

[3]Theoretical value calculations based on the empirical formula, $C_8F_{15}O_2^{(-)}NH_4^{(+)}$ (MW=431.1)

LC/MS Purity Profile:

Peak #	Retention Time (min)	Mass(s)	Identity	Area	% Area
1	12.140	269	C_6	167596	0.73
2	13.504	331, 319	C_7 homologue/F_{13}	860991	3.76
3	14.099	369	PFOA	21861700	-
Total	-	-	-	22890287	4.49

This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 160).

Prepared By: Charles Simons — 10/9/01 Date
Scientist, Centre Analytical Laboratories

Reviewed By: John M Flaherty — 10/9/01 Date
John Flaherty
Laboratory Manager, Centre Analytical Laboratories

3M ENVIRONMENTAL LABORATORY

Note to File

Project or Study Number: FACT-TCR-005
Associated Study Number: LIMS # E00-1762

The expiration dates for PFOA lots HU 14401DU and 332 (TCR-99131-018 and TCR-99131-037) may be extended 5 years (12/15/06) as stability was demonstrated by studies E00-1851 (hydrolysis), E00-2192 (photolysis), and E01-0415 (biodegradation).

UAC 10/04/01

Recorded By:	Date
Lisa Clemen Lisa A Clemen	10/04/01 10/04/01

Exact Copy of Original
UAC 10/05/01
Initial Date

Exact copy of copy
UAC 10/19/01

Form ETS-4-15.0

3M SPECIALTY MATERIALS & MANUFACTURING DIVISION ANALYTICAL LABORATORY

Request No. **62631**
Study No. **FACT-TCR-005**

To: William Reagen - (8-6565) – Environmental Lab- 2-3E-09

From: Tom Kestner - (3-5633) - SMMD Analytical Lab - 236-2B-11

Subject: **Chemical Characterization of PFOA, Lot 332, TCR-99131-37 by ^{1}H-NMR, ^{19}F-NMR, and IR Spectroscopy**

Date: December 11, 2000

SAMPLE DESCRIPTION:

- PFOA, lot 332, TCR-99131-37; Nominal product =C_7F_{15}-$CO_2^{(-)}$ $NH_4^{(+)}$ (white powder). The sample was stored in a –20 °C freezer at all times except when aliquots were removed for analysis.

OBJECTIVE:

This sample was subjected to ^{1}H-NMR and ^{19}F-NMR spectral analyses to determine the purity of the nominal product and to characterize as many impurity components as possible. An FT-IR spectrum was also acquired for the purpose of confirming the nominal perfluorinated carboxylate salt functional group.

EXPERIMENTAL:

FT-NMR

A portion of the thawed sample was accurately weighed, spiked with a known amount of 1,4-bis(trifluoromethyl)benzene (p-HFX), and then totally dissolved in acetone-d_6 for subsequent analysis by NMR. An initial 400 MHz ^{1}H-NMR spectrum (# h62631.401) and a 376 MHz ^{19}F-NMR spectrum (# f62631.401) were acquired using a Varian UNITYplus 400 FT-NMR spectrometer. A second portion of the sample was also accurately weighed, spiked with a known amount of p-HFX, and then totally dissolved in deuterated trifluoroacetic acid (CF_3CO_2D = TFAD). An additional 400 MHz ^{1}H-NMR spectrum (#h62631.402) and a 376 MHz ^{19}F-NMR spectrum (#f62631.402) were collected in the TFAD solvent. The primary purpose of using the TFAD solvent was to help minimize the interferences associated with broad $NH_4^{(+)}$ resonance that had been observed in ^{1}H-NMR spectrum of the acetone-d_6 solution. The sample preparation method described above was intended to permit the use of the p-HFX as an internal standard for absolute weight percent measurements in either solution.

FT-IR

A portion of the sample was prepared for FT-IR spectral analysis using the KBr disk technique. A transmission FT-IR spectrum (#A62631) was then acquired using a Digilab FTS-40 FT-IR spectrophotometer.

RESULTS:

The combined NMR spectral data indicated the sample of PFOA, lot 332, TCR-99131-37 consisted of a high purity form of the nominal isomeric product mixture, C_nF_{2n+1}-$CO_2^{(-)}$ $NH_4^{(+)}$, where 'n' was mainly 7. A few trace-level impurity components were also observed, tentatively assigned, and quantified from the NMR spectral data.

December 11, 2000

3M SMMD Analytical Lab Request # 62631
3M SMMD Analytical Lab: Bldg. 236-2B-11

RESULTS (cont.):

The qualitative and quantitative compositional results that were derived from the single trial ^{1}H-NMR and ^{19}F-NMR spectral analyses are summarized in **TABLE-1**. Any water that may have been present in the sample was ignored for calculation purposes. The ^{19}F-NMR relative weight percent concentrations shown in TABLE-1 should be very close to their respective absolute weight percent values with the stated assumptions. In order to perform the relative weight percent calculations, I assumed all of the fluorocarbon chains contained 8 carbon atoms except where noted. In general, the ^{19}F-NMR technique is not particularly well suited for characterizing small amounts of potential fluorochemical homolog impurity components unless the chains are very short. A more complete characterization of any other impurity homologs would require analysis by electrospray MS or a similar technique. Trace amounts of other unidentified impurities are also detected in the NMR spectra, but additional work would be required in an effort to identify or quantify these other materials.

The FT-IR spectrum was used to verify the nominal perfluorinated carboxylate salt functional group.

Copies of the NMR and IR spectra are attached for your reference. If you have any questions about these results, or if any further work is needed, please let me know.

Tom Kestner

c: Lisa Clemen – ET&S – 2-3E-09
Tanya Rude - QAI
Rick Payfer – SA&C Analytical Lab – 236-2C-11

File Reference: wr62631.studyFACTTCR005.doc/78

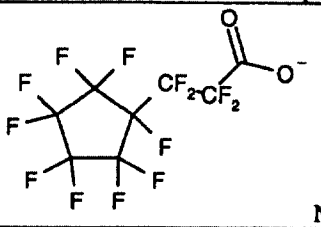
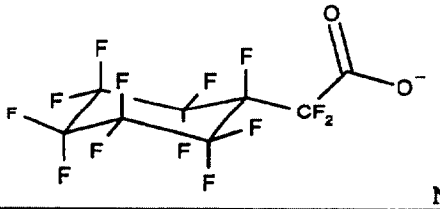
December 11, 2000

3M SMMD Analytical Lab Request # 62631
3M SMMD Analytical Lab: Bldg. 236-2B-11

TABLE-1

Sample: PFOA, lot 332, TCR-99131-37

Overall Compositional Results by ^{19}F-NMR Analysis & ^{1}H-NMR Internal Standardization Analysis

Structural Assignments [1]	^{19}F-NMR Relative Wt. % Concentrations
Normal chain isomer $CF_3(CF_2)_x\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x assumed to be 6 for calculation purposes)	≈77.7%
Internal monomethyl branched isomers $CF_3(CF_2)_x\text{-}CF(CF_3)\text{-}(CF_2)_y\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x+y assumed to be 4 for calculation purpose, and $x \neq 0$, $y \neq 0$)	≈12.5%
Isopropyl branch isomer $(CF_3)_2CF\text{-}(CF_2)_x\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x assumed to be 4 for calculation purposes)	≈9.0%
t-butyl branch isomer $(CF_3)_3C\text{-}(CF_2)_x\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x assumed to be 3 for calculation purposes)	≈0.24%
 Possible $NH_4^{(+)}$	≈0.19%
Internal gem-dimethyl branch isomers $CF_3\text{-}(CF_2)_x\text{-}C(CF_3)_2\text{-}(CF_2)_y\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x+y assumed to be 3 for calculation purposes: $x \neq 0$)	≈0.13%
 Possible $NH_4^{(+)}$	≈0.11%
Possible alpha branch isomer $C_xF_{2x+1}\text{-}CF(CF_3)\text{-}CO_2^{(-)}\ NH_4^{(+)}$ (where x assumed to be 5 for calculation purposes)	≈0.085%
Probable F-SF_4-R_f , possibly as F-SF_4-C_nF_{2n}-$CO_2^{(-)}\ NH_4^{(+)}$ (where n assumed to be 7 for calculation purposes)	≈0.029%
Possible $CF_3CF_2CO_2^{(-)}\ NH_4^{(+)}$	≈0.009%
$CF_3\text{-}O\text{-}CF_2\text{-}R_f$, where $-R_f$ is undefined	Trace
	^{1}H-NMR Absolute Wt.% Concentration
Total unassigned aliphatic materials	≈0.002-0.003%

1) Trace amounts of other unidentified components are also detected in the NMR spectra.

Certificate of Analysis

Nominal Product: $CF_3(CF_2)_n$-CO_2H, where average n ≈ 6

Pentadecafluorooctanoic acid

Product Code: TCR-617, Lot 210002

October 28, 2002

Tom Kestner and Joel Miller

The sample of **TCR-617, lot 210002** was analyzed using a combination of ^{19}F-NMR, ^{1}H-NMR, and LC/MS analysis techniques. The overall qualitative and quantitative compositional results that were derived from these combined analyses are summarized below in TABLE-1.

TABLE-1
Sample: TCR-617, Lot 210002
Quantitative and Qualitative Compositional Results by Combined ^{19}F/^{1}H-NMR and LC/MS Analyses

Component Structures [1]	^{1}H/^{19}F-NMR Relative **Weight%** Concentrations (single trial analysis)
$CF_3(CF_2)_n$-CO_2H where average n = 6.02 by ^{19}F-NMR. LC/MS showed n=6 (major), n=5, n=4 (minors).	≤99.51% **Purity**
Probable $(CF_3)_2$-CF-$(CF_2)_n$-CO_2H assume n=4 for calculation purposes	0.39%
Probable $(C_6F_{13}O)$-CO_2H acyclic ether acid as possible CF_3CF_2-O-$CF(CF_3)$-CF_2CF_2-CO_2H	0.057%
Possible $CF_3(CF_2)_x$-$CF(CF_3)$-$(CF_2)_y$-CO_2H where x≠0, y≠0 and assume x+y = 4 for calculation purposes	0.019%
Possible $(CF_3)_3$-C-$(CF_2)_n$-CO_2H assume n=3 for calculation purposes	0.013%
Possible C_nH_{2n+2} saturated aliphatic hydrocarbons	0.0079%

1. Trace amounts of other unassigned components were also detected in the NMR spectra.

Thomas C. Kestner
10-28-02

10/28/02

Analytical Report: LIMS E02-1053

Appendix G: Report Signature Page

11/13/02

William Reagen, Ph.D., *Laboratory Management, Sponsor Representative* Date

Lisa A. Stevenson 11/13/02

Lisa Stevenson, *Principal Analytical Investigator* Date

3M Environmental Laboratory