Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 819 (2005) 329–338
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative determination of perfluorooctanoic acid in serum and plasma by liquid chromatography tandem mass spectrometry

John M. Flaherty [a], Paul D. Connolly [a], Emily R. Decker [a], S. Mark Kennedy [b], Mark E. Ellefson [c], William K. Reagen [c], Bogdan Szostek [b,*]

[a] *Exygen Research, Inc. (Exygen) 3058 Research Drive State College, PA 16801, USA*
[b] *DuPont Haskell Laboratory for Health and Environmental Sciences, 1090 Elkton Rd., Newark, DE 19714-0050, USA*
[c] *3M Environmental Laboratory, 935 Bush Ave., Bldg 2-3E-09, St. Paul, MN 55106, USA*

Received 11 October 2004; accepted 1 March 2005

Abstract

A selective and sensitive method for analysis of perfluorooctanoic acid (PFOA) in human serum and plasma, utilizing liquid chromatography tandem mass spectrometry (LC–MS/MS), has been developed and thoroughly validated to satisfy strict FDA guidelines for bioanalytical methods. A simple, automated sample preparation procedure, involving extraction of the target analyte with acetonitrile on protein precipitation media in a 96-well plate format was developed, allowing efficient handling of large numbers of samples. The proposed method uses the calibration standards prepared in a surrogate matrix (rabbit serum or plasma) and ^{13}C-labeled PFOA as the internal standard to account for matrix effects, instrument drift, and extraction efficiency. Human serum and plasma could not be used for matrix matching of calibration standards as endogenous levels of PFOA observed in the control human serum and plasma significantly exceeded the targeted lower limit of quantitation (LLOQ) of the method. Precision and accuracy of the method were demonstrated by analysis of rabbit serum and plasma control samples fortified at 0.5, 5, and 40 ng/mL PFOA and human serum and plasma fortified at 1.0, 5.0, 40 ng/mL PFOA. The LLOQ of 0.5 ng/mL PFOA was experimentally demonstrated for rabbit and human serum and plasma. Within-day precision and accuracy, short-term stability, freeze–thaw stability, equivalence of response between PFOA and APFO (the ammonium salt of PFOA), and dilution of concentrated samples were also investigated. The results of the validation experiments comply with the precision and accuracy limits defined by the FDA guidance document: "Guidance for Industry, Bioanalytical Method Validation", May 2001.

Keywords: Perfluorooctanoic acid; LC–MS/MS; Serum; Plasma

1. Introduction

The availability of validated analytical methodology for analysis of perfluorooctanoic acid (PFOA) and its ammonium salt (APFO, C8) in various environmental and biological matrices has recently gathered significant attention. PFOA and other fluoroorganic compounds have been detected in human serum [1,2] and various biota species [3,4] creating concerns about the sources and potential health and environmental impact of these compounds. As the origins of PFOA in human blood and the environment, as well as its actual levels in human blood for the general population and different environmental compartments are investigated, improved and rigorously validated analytical methods need to be established. In addition, anticipating analysis of large numbers of samples, a simple and automated sample preparation technique is desirable.

PFOA is used as a polymerization aid in making PTFE (polytetrafluoroethylene) and other fluoropolymers. PTFE is present in the component parts of analytical instrumentation, some sample preparation products such as SPE cartridges, and some general-use labware. PFOA may be present at trace levels in these products and has been detected in common

* Corresponding author. Tel.: +1 302 366 5331; fax: +1 302 366 5003.
E-mail address: bogdan.szostek@usa.dupont.com (B. Szostek).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.03.002

HPLC solvents. All this creates significant "blank" problems for analysis at trace levels, requiring diligent screening of all reagents, labware, sample collection implements, and instrumentation used for the measurement.

Before the use of LC–MS techniques became routine, PFOA analysis in biological matrices had been dominated by gas chromatographic techniques (GC). PFOA requires derivatization in order to be efficiently analyzed by GC. Belisle and Hagen [5] derivatized PFOA as the methyl ester using diazomethane and developed protocols for analysis of PFOA in human plasma and urine as well as rat and monkey liver tissue. A similar derivatization procedure with detection by GC-ECD has been utilized by Kudo et al. [6] for determination of PFOA in rat tissues. Other researchers, experiencing problems with losses of methyl ester during sample preparation, developed derivatization with larger chemical moieties. Ylinen et al. [7] established a GC-ECD method for determination of PFOA as the benzyl ester derivative in animal urine and plasma. The GC-based methods, though in general useful for analysis of PFOA in biological matrices, require complex and tedious sample preparation that is not easily automated and generally lack the sensitivity and selectivity necessary for determination of PFOA in human blood. A more sensitive method for determination of PFOA in rat liver was developed by Ohya et al. [8] utilizing derivatization and HPLC-fluorescence detection.

Introduction of LC–MS/MS technology to PFOA analysis allowed determination of PFOA at trace levels in biological matrices after relatively simple sample preparation. Sottani and Minoia [9] showed method validation data for analysis of PFOA in human serum with an LLOQ of 25 ng/mL by LC–MS/MS with negative ion electrospray. The sample preparation procedure was analogous to the 5 ng/mL LLOQ LC–MS/MS method used by Hansen et al. [1] involving MTBE extraction of the buffered sample with addition of the ion pairing agent tetrabutyl ammonium hydrogen sulfate, subsequent evaporation of MTBE, and dissolution of the sample in methanol. A slightly lower limit of quantitation for determination of PFOA (3.9 ng/mL) in human serum, using methodology developed by Hansen et al. [1] was reported by Kubwabo et al. [10]. Kuklenyik et al. [11] reported an LC/MS/MS method for determination of various perfluorinated organic compounds, including PFOA in human serum and milk. The method involves automated solid-phase extraction of samples treated with 0.1 M formic acid on 60 mg/3 mL Oasis-HLB columns, eluted with 1% NH_4OH/acetonitrile. Even though the authors report problems with reagent blanks for PFOA at ~1 ng/mL, they demonstrated acceptable method accuracy for quantitation of PFOA at 1 ng/mL. Inoue at al. [12] described an LC/MS method for determination of three perfluorinated organic compounds, including PFOA in human plasma using a column-switching system for on-line sample extraction and subsequent analysis. Human plasma samples were pretreated with an equivalent volume of acetonitrile, centrifuged, and the resulting supernatant was loaded onto the extraction column and subsequently back-flushed into the analytical column. The reported limit of quantitation of 0.5 ng/mL for plasma was estimated based on signal-to-noise (S/N) ratio, rather than derived experimentally by testing the proposed method with matrix spiked at the stated LOQ. A similar approach, utilizing large volume injections of acetonitrile-extracted human plasma onto a capillary column-switching system for determination of perfluorooctane sulfonate and PFOA, was proposed by Holm et al. [13]. Using 250 μL injection of plasma extract, a limit of detection of 0.5 ng/mL (S/N ratio of 3) was achieved.

This work was directed towards developing and rigorously validating a method in 96-well plate format that would allow fast and accurate quantitation of PFOA at 500 pg/mL in human serum or plasma. The goal of the method development was to simplify the sample preparation procedure as compared with methods proposed in the literature, minimize the number of operations performed on a sample and make the method easily adaptable to high-throughput processing on robotic stations. Similarly, the LC/MS/MS was optimized to handle large numbers of samples without the need for complex systems for on-line sample treatment in order to be easily adaptable to commercial laboratories. As many published methods often lack thorough method validation data that would be acceptable to regulatory agencies, this work was designed to provide comprehensive validation data. The validation protocol was developed based on a set of guidelines developed by FDA for bioanalytical analysis [14]. The major complication encountered in the validation process at an LLOQ of 500 pg/mL was the difficulty in obtaining blank human serum or plasma with sufficiently low endogenous levels of PFOA. A number of animal plasma and serum matrices were screened in order to find appropriate surrogate matrices. The method validation and LLOQ demonstration was performed for both human and rabbit plasma and serum matrices using ^{13}C-labeled PFOA to account for matrix effects, instrument drift, and extraction efficiency.

2. Experimental

2.1. Standards and chemicals

The standard of perfluorooctanoic acid (99.2%) was obtained from Oakwood Products, Inc. (West Columbia, SC, USA) and characterized by DuPont (Newark, DE, USA). Analysis by ^{19}F NMR confirmed that the PFOA standard essentially contained 98.7% straight chain PFOA and 0.53% branched PFOA isomers. The internal standard: ^{13}C-perfluorooctanoic acid ($C_6F_{13}{}^{13}CF_2{}^{13}CO_2H$, ^{13}C-PFOA) (96.4%) was provided by DuPont (Newark, DE, USA). Ammonium perfluorooctanoic acid (APFO) (95.2%) containing a mixture of straight chain and branched forms (78.0% linear, 22.0% branched PFOA isomers) was provided by the 3 M Environmental Laboratory (St Paul, MN, USA).

Chemicals and reagents used in the extraction procedures or in the mobile phase were of reagent grade and were obtained from VWR Scientific (Bridgeport, NJ, USA) and Sigma–Aldrich (St Louis, MO, USA). Solvents used for the mobile phase (acetonitrile, water) were of HPLC grade and were obtained from EM Science (Gibbstown, NJ, USA).

The control rabbit serum and plasma as well as the control human serum and plasma were purchased from Lampire Biological Laboratories, Inc. (Pipersville, PA, USA). Six different lots of rabbit serum and plasma, and six different lots of human serum and plasma were purchased and stored frozen ($\leq -20\,^{\circ}C$).

2.2. Chromatographic conditions

An Agilent 1100 series (Palo Alto, CA) HPLC system consisting of a quaternary pump, vacuum degasser, autosampler, and controlled-temperature column compartment was used. Two 4 mm × 10 mm Hypercarb drop-in guard cartridges (Keystone, Bellefonte, PA, USA) were attached inline after the purge valve and before the sample injector port to trap any residue contaminants that may be in the mobile phase and/or HPLC system. Chromatographic separation was achieved using a Genesis C8 column (50 mm × 2.1 mm i.d., 4 μm particle size; originally supplied by Argonaut, Foster City, CA and currently supplied by Grace/Vydac, Hesperia, CA, USA) operated at a temperature of 30 °C. The mobile-phase consisted of a 2 mM ammonium acetate solution in water (A) and methanol (B). The following gradient elution was employed: 60% B (isocratic) to 3.0 min. (0.3 mL/min), to 100% B (linear) at 3.5 min (0.3 mL/min), 100% B (isocratic) at 3.7–7 min (0.5 mL/min) at 7 min (0.5 mL/min), to 60% B (linear) at 7.5 min (0.5 mL/min), 60% B (isocratic) at 9 min (0.5 mL/min), 60% B (isocratic) at 9.5 min (0.3 mL/min), 60% B (isocratic) at 12 min (0.3 mL/min). Retention times under these conditions were similar for both PFOA and ^{13}C-PFOA (∼2.0 min).

2.3. Mass spectrometric conditions

A PE SCIEX API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA), equipped with a SCIEX Turbo V ion-spray interface, operated in the negative ion MS/MS mode, and using selected reaction monitoring (SRM) was employed. Instrument tuning was conducted for each analyte by direct-infusion of a ∼1 μg/mL standard solution of PFOA at a flow rate of 10 μL/min introduced via a "T" into a stream of mobile phase containing 60% methanol and 40% 2 mM ammonium acetate in water at a 0.3 mL/min flow rate. The analyte was initially tuned for the parent ion and then subsequently tuned for the product ion. Typically, the following tune parameters were used: source temperature 350 °C; desolvation temperature (500 °C), desolvation (curtain) gas flow 20 L/h; nebulizer gas flow 30 L/h; ion spray voltage −4000 V; declustering potential −40 V; entrance potential −6 V; dwell time 500 ms. The optimal settings for collision energy, corresponding to a nearly 100% fragmentation of the molecular (or precursor) ions, were similar for each analyte, −14 eV.

For quantitative purposes, the instrument was operated in SRM mode, monitoring the transition: PFOA, *m/z* 413 → 369 *m/z*, and ^{13}C-PFOA, *m/z* 415 → 370 *m/z*. Quantitation using these transitions was performed using the Analyst 1.2 software provided by PE (Applied Bioscience, Foster City, CA).

2.4. Standards and sample preparation

Stock solutions of PFOA and ^{13}C-PFOA were prepared at a concentration of 100 μg/mL in methanol. Fortification solutions of PFOA at 1.0, 0.1, and 1.0 μg/mL of ^{13}C-PFOA were prepared by appropriate dilutions of the stock solutions in acetonitrile.

Typically, a set of calibration standards containing from 0.05 to 5 ng/mL PFOA and 1 ng/mL ^{13}C-PFOA was prepared by dilution of the 0.1 μg/mL PFOA and the 1.0 μg/mL ^{13}C-PFOA stock solutions in acetonitrile. The matrix-matched calibration standards were made by processing 0.5 mL of an acetonitrile standard and 50 μL of appropriate matrix using the protein precipitation procedure. Separate standards were made for plasma and serum work. The fortifications of samples were done in a similar manner. First, an acetonitrile stock solution containing appropriate concentration of PFOA and ^{13}C-PFOA was made and 0.5 mL of the acetonitrile fortification solution and 50 μL of appropriate matrix were processed using the protein precipitation procedure. For freeze/thaw and storage stability, approximately 0.5 mL of appropriate matrix was spiked with PFOA and then 50 μL aliquots were used for extraction with acetonitrile containing ^{13}C-PFOA internal standard. The stock standard solution and all fortification/calibration standard solutions were stored in a refrigerator ($4 \pm 2\,^{\circ}C$) when not in use.

The protein precipitation procedure involved manually loading a 50 μL aliquot of each specimen (serum or plasma) onto an Argonaut protein precipitation column, arrayed in a 96-well plate format (Isolute, Argonaut, Foster City, CA) and a 500 μL aliquot of acetonitrile containing an appropriate concentration of analyte and/or internal standard was then added to each column. A Packard MultiProbe II sample-handling robot (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA) was used to automate repetitive dispensing of the acetonitrile. Repeated vacuum cycles were applied to the extraction plate manifold until all acetonitrile had been drawn through the column. Four hundred microliter aliquots of the eluate from each column were then transferred to autosampler vials for subsequent analysis. A 5 μL aliquot was injected onto the HPLC.

Frozen samples of serum or plasma were allowed to completely thaw, un-aided, at room temperature. Samples that had been stored refrigerated were allowed to equilibrate to room temperature. All samples were thoroughly mixed before being sampled for extraction.

2.5. Method validation protocol

The method validation protocol was derived from the US FDA's Guidance for Industry for Bioanalytical Method Validation [14]. The challenge in designing the validation protocol was the difficulty finding human serum or plasma with at most 0.1 ng/mL endogenous PFOA (0.1 ng/mL constitutes 20 % of desired method LLOQ). A similar but lesser challenge was encountered when plasma and serum of various animals were screened. The rabbit serum and plasma met the blank criteria for 0.5 ng/mL LLOQ and were used as surrogate matrices for method validation and for preparation of matrix-matched calibration standards for validation of the method with human serum and plasma. The evaluation of method specificity, linearity, accuracy and precision, LLOQ, and storage stability was done with rabbit serum and rabbit plasma. Method accuracy, precision, and LLOQ were evaluated for human serum and plasma.

The specificity of the method was determined by analyzing six control rabbit serum and plasma samples from six different lots. These six control samples were evaluated for the presence of interfering signal and compared to a 0.5 ng/mL standard of PFOA prepared in 50:50 methanol:water to determine the level of inherent PFOA present in the control matrix. The linearity of the method was established by the triplicate analysis of extracted calibration standards ranging from 0.5 to 40 ng PFOA/mL of plasma or serum. The LLOQ of the method for rabbit serum and rabbit plasma was determined by analyzing three replicates of a control sample and five replicates of fortified samples at 0.5 ng/mL.

The within-day and between-day precision and accuracy of the method were determined by extracting and analyzing two sets of fortified rabbit serum and plasma samples on two separate days by two separate analysts. One set contained six control samples fortified at 0.5 ng/mL, six control samples fortified at 5.0 ng/mL, and six control samples fortified at 40 ng/mL. The second set contained six control samples fortified at 5.0 ng/mL. The method accuracy and precision for samples outside the calibration range was examined by the analysis of three fortified samples prepared at 10 μg/mL, extracted, and the extracts subsequently diluted in 50:50 methanol:water into the range of calibration standards.

To demonstrate the method applicability to the determination of APFO, six control samples of rabbit serum and plasma were fortified with 5.0 ng/mL of APFO and analyzed against a set of PFOA calibration standards.

The short-term stability of samples and extracts was determined by fortifying six control samples at 5.0 ng/mL and six at 40 ng/mL. Three replicates at each level were immediately analyzed, whereas the other fortified samples were allowed to remain at room temperature for approximately 24 h before analyzing. After analysis, the initial extract and standard vials were allowed to remain at room temperature for ∼16 h and then re-injected. A fresh set of calibration standards was prepared and compared to those that sat at room temperature for approximately 24 h.

The stability of the analyte during the freeze/thaw cycle was evaluated by fortifying three control samples at 5.0 ng/mL and three at 40 ng/mL. These samples were kept frozen for approximately 24 h and then allowed to thaw at room temperature. This process was repeated three more times, and the samples were analyzed after the fourth thawing process.

Validation of the method for human serum and plasma was conducted using six different lots of control human serum and plasma. Each lot was used to prepare one control sample and samples fortified with PFOA at 1.0, 5.0, and 40 ng/mL (three replicates per fortification level). These were analyzed using extracted standards prepared in control rabbit serum and plasma. The recoveries were calculated by subtracting the amount of endogenous PFOA determined in the control matrix from the amount obtained for the fortified sample and comparing that with the spiked amount. Initially, the human serum and plasma were fortified at 0.5 ng/mL in order to establish the LLOQ at this level. However, endogenous levels of PFOA present in commercially available human serum and plasma prohibited accurate recoveries at this level. Therefore, a validation of LLOQ at 0.5 ng/mL was conducted with ^{13}C-PFOA in human serum and plasma. Single lots of human serum and plasma were tested by preparing one control and three replicate samples fortified at 0.5, 0.75, and 1 ng/mL with ^{13}C-PFOA and analyzed with a set of ^{13}C-PFOA standards prepared in human serum and plasma.

3. Results and discussion

3.1. Chromatographic and mass spectrometric conditions

The selection of an analytical column for the analysis of PFOA in serum or plasma was based on previous experience gathered from methods developed for other biological and environmental matrices. The Genesis C8 column showed suitable retention and peak shape for PFOA in a variety of matrices. It offers relatively short analytical runtimes (∼12 min) with good separation of the PFOA signal originating from the injected sample and the PFOA signal originating from the instrument ("system PFOA"). Since various parts of HPLC systems are made of PTFE and since APFO is used as the polymerization aid in making PTFE, the background PFOA originating from the HPLC system has to be eliminated or distinguished from the PFOA originating from the injected sample. Instrument background can be minimized only to a certain degree by extensive flushing of the HPLC system or replacement of PTFE parts with those made of other materials. However, there is a limit to that approach, as not all the parts can be replaced. For example, in the HP1100 HPLC the outlet check valve of the pump passes all of the mobile phase

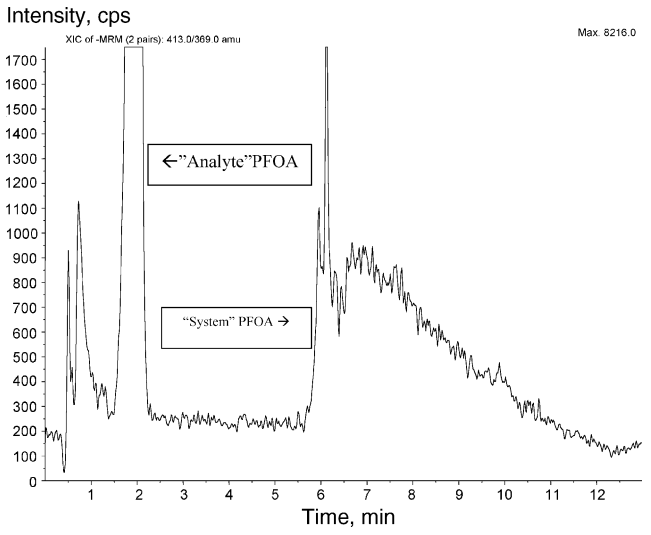

Fig. 1. SRM chromatogram demonstrating the elution of "analyte" PFOA and "system" PFOA.

through a solid PTFE frit (Part Number: 01018-22707). In order to assist the analytical column in separating this "system PFOA" from the sample PFOA, Hypercarb filters (Keystone Scientific) were placed at the exit of the pump prior to the autosampler. The appropriate gradient design allows elution of the analyte PFOA at essentially isocratic conditions (40% 2 mM ammonium acetate/60% methanol) without interference from the "system PFOA". Immediately following elution of the analyte PFOA, the HPLC, Hypercarb filters, and analytical column are flushed with 100% methanol at an increased flow rate to elute the "system PFOA". Fig. 1 illustrates this principle with the analyte PFOA being eluted at 2.0 min and the "system PFOA" eluting as a broad peak starting at approximately 6 min and returning to the baseline value by 12 min.

The 5 μL injection volume was determined to be optimal considering the required sensitivity of the method and chromatographic performance. Larger injection volumes have been shown to cause excessive peak fronting or splitting. Acceptable peak shapes are obtained for these extracts with 5 μL injections. The composition of the extracts from serum:acetonitrile or plasma:acetonitrile extraction also appears to be critical to the chromatographic performance. Injections of samples in 100% organic solvent (acetonitrile or methanol) resulted in broad, fronting peaks for this chromatographic system. Samples requiring dilution were initially diluted in 100% acetonitrile; however, the chromatography was unacceptable. Samples diluted in 50:50 methanol:water showed excellent peak shape.

The mass transition selected to monitor for PFOA was (negative ions) $413 \rightarrow 369$. This represents the major fragment of the parent molecule with the loss of the terminal carboxyl group. The transition $415 \rightarrow 370$ is the equivalent transition for the dual ^{13}C-labeled internal standard.

3.2. Method validation

Specificity of the method for PFOA was determined by analyzing control rabbit plasma and serum samples from different lots along with a calibration standard at 0.5 ng/mL. For all tested lots a signal for PFOA was detected (signal to noise ratio larger than 3:1) originating from endogenous levels of PFOA present in the tested matrices. No PFOA signal was detected for either instrumental or reagent blanks supporting the claim that the detected PFOA originated from the biological matrix. Typically, the PFOA signal (peak area) obtained for control rabbit serum or plasma lots did not exceed 1/3 the peak area obtained for the 0.5 ng/mL standard prepared in the examined matrix. Occasionally, a result not meeting this criterion was obtained, but re-analysis after extensive vortexing of rabbit plasma or serum to remove inhomogeneity in the sampled aliquots of the matrix brought the results to acceptable range.

The linearity of the method was established by analyzing calibration standards ranging from 0.5 to 40 ng/mL in triplicate, separately, in rabbit plasma and rabbit serum. For each set of standards, the coefficients of determination (r^2) were greater than 0.985 using a linear model of the calibration curve with $1/X$ weighing using peak areas for PFOA. All calibration standards deviated less than 20% from the nominal concentration of the LLOQ and less than 15% for the other levels. Injection precision was tested for the 0.5 ng/mL calibration standard by re-injecting it three times. Less than 5% RSD was demonstrated with experimentally determined RSDs of 4.25% for rabbit serum and 3.47% for rabbit plasma.

Table 1 summarizes spike recovery data obtained from the method validation in rabbit serum and rabbit plasma. The Table presents the average recovery for a given sample fortification level and standard deviation of the recovery for the number of replicates indicated. The accuracy and precision (within-day) of the method is expressed as the recovery and standard deviation of recovery. All three fortification levels for rabbit plasma and rabbit serum (Table 1) demonstrated recoveries in the range of ±15% of nominal 100% recovery and standard deviations not exceeding 15%. The between-day accuracy and precision of the method was tested by repeating the fortification for the 5 ng/mL level by a different analyst on another day. The data obtained for rabbit plasma and serum (Table 1) met the criteria outlined for within-day and between-day method precision and accuracy.

The method performance was also tested for rabbit serum and rabbit plasma samples fortified at 10 μg/mL PFOA, extracted, and diluted with 50:50 methanol:water to bring the concentration into the range of calibration. Satisfactory recovery ($100 \pm 15\%$) and precision were obtained for both rabbit serum and plasma (Table 1). Initially, the samples were diluted in acetonitrile prior to analysis. It was discovered that this resulted in poor chromatography for PFOA and the internal standard though the recoveries were comparable to results using methanol:water dilution, which improved the chromatography.

Table 1
Percent recoveries for PFOA fortified rabbit serum and plasma samples

Sample matrix	Experiment	Fortification level (ng/mL)			
		0.5	5	40	10000
Rabbit serum	Day 1, analyst 1; six replicates	101 ± 12	108 ± 1.5	94 ± 1.2	–
	Day 2, analyst 2; six replicates	–	108 ± 2.1	–	–
	LLOQ validation; five replicates	91 ± 8.0	–	–	–
	Dilution; three replicates	–	–	–	88 ± 0.6
	APFO validation; six replicates	–	101 ± 2.6	–	–
Rabbit plasma	Day 1, analyst 1; six replicates	98 ± 13	111 ± 4.1	96 ± 2.6	–
	Day 2, analyst 2; six replicates	–	106 ± 0.8	–	–
	LLOQ validation; five replicates	88 ± 17	–	–	–
	Dilution; three replicates	–	–	–	87 ± 1.5
	APFO validation; six replicates	–	101 ± 4.4	–	–

Method validation for APFO in rabbit serum and plasma was conducted at 5.0 ng/mL fortification level by analyzing APFO fortified samples with a set of PFOA calibration standards. The accuracy and precision of the method to measure APFO in rabbit serum and plasma was demonstrated (Table 1).

The LLOQ of the method was determined by analyzing three replicates of a control sample and five replicates of fortified samples at 0.5 ng/mL PFOA. The LLOQ at 0.5 ng/mL is justified as the average recoveries are between 80 and 120% (Table 1) with an acceptable level of precision. Additionally, the response expressed as the peak area obtained for 0.5 ng/mL fortifications was at least three times that of the control samples for both rabbit plasma and rabbit serum.

Table 2 summarizes the recovery and standard deviation data obtained for short-term storage stability and freeze–thaw stability. The stability experiments were conducted using rabbit serum and plasma at 5 and 40 ng/mL PFOA fortification as described in Section 2. The calibration standards (data not shown) were shown to be stable (±15% of original value) after remaining at room temperature for approximately 24 h (except for the 0.5 ng/mL rabbit serum calibration standard). This standard differed by 18% as compared to a standard that was prepared and analyzed 24 h earlier. The fortified samples were shown to be stable (±15% of original value) after remaining at room temperature for approximately 24 h before being extracted. (Table 2). The rabbit plasma and serum samples were shown to be stable throughout freeze/thaw with acceptable recoveries and precision for both fortification levels (Table 2).

In order to demonstrate the method applicability to analysis of PFOA in human serum or plasma, a partial validation involving demonstration of method precision and accuracy was done using three different lots of human serum and plasma with calibration standards prepared with rabbit serum or plasma, respectively. The fortifications of human serum and plasma samples were done at three different levels: 1, 5, and 40 ng/mL PFOA. The endogenous levels of PFOA were determined for each lot of human plasma and serum. The spike recoveries were calculated by subtracting the endogenous levels of PFOA from the results obtained for different levels of fortifications for each lot of human plasma and serum and comparing that with the spiked amount. The results of this validation are presented in Table 3. All samples met the validation criteria of accuracy and precision set by the FDA validation guidelines [14].

According to the FDA validation guidelines [14] the method lower limit of quantitation needs to be demonstrated experimentally by fortifying sample matrix at the level of LLOQ and meeting the criteria of accuracy and precision. The initial intent was to fortify the human controls at 0.5 ng/mL; however, endogenous levels of PFOA in commercially available controls (Table 3) prohibited accurate recoveries at this level. Therefore, a validation of LLOQ was conducted using ^{13}C-PFOA in human serum and plasma. A set consisting of one matrix control and three samples fortified at 0.5, 0.75,

Table 2
Percent recoveries for storage stability of PFOA fortified rabbit serum and plasma samples

Sample matrix	Experiment	Fortification level (ng/mL)	
		5.0	40
Rabbit serum	Fortified, extracted, analyzed immediately	91 ± 6.7	91 ± 1.2
	Fortified, stored for 24 h, extracted, analyzed	91 ± 3.6	98 ± 6.1
	Fortified, extracted, stored for 16 h, analyzed	90 ± 3.1	91 ± 2.5
	Freeze/thaw stability	90 ± 14	91 ± 0.6
Rabbit plasma	Fortified, extracted, analyzed immediately	86 ± 5.8	90 ± 0.0
	Fortified, stored for 24 h, extracted, analyzed	96 ± 5.6	96 ± 2.5
	Fortified, extracted, stored for 24 h, analyzed	85 ± 4.9	90 ± 1.0
	Freeze/thaw stability	86 ± 1.5	88 ± 4.7

Table 3
Percent recoveries for PFOA fortified human serum and plasma samples

Sample matrix	Lot (PFOA, ng/mL)	Lot (PFOA, ng/mL)	Fortification level (ng/mL)		
			1.0	5	40
Human serum	A[a] (5.5)[b]	D (6.0)[c]	101 ± 5.2	104 ± 6.8	92 ± 1.5
	B (7.0)	E (3.5)	89 ± 3.5	102 ± 3.6	99 ± 11
	C (5.2)	F (3.2)	95 ± 13	112 ± 6.2	95 ± 1.0
Human plasma	A1[d] (1.5)	A1[e] (2.2)	108 ± 6.1	107 ± 4.0	94 ± 1.0
	B1 (3.2)	B1 (2.9)	118 ± 1.5	95 ± 4.6	95 ± 0.6
	C1 (3.4)	C1 (3.9)	114 ± 6.0	102 ± 3.0	95 ± 0.6

Fortification with ^{13}C-PFOA		0.5 ng/mL	0.75 ng/mL	1.0 ng/mL
Human serum	C	101 ± 2.7	109 ± 2.5	103 ± 2.3
Human plasma	A1	104 ± 3.1	106 ± 2.1	102 ± 1.0

[a] Lots A, B, C were used for 1 ng/mL fortifications.
[b] Background level of PFOA present in a particular matrix lot.
[c] Lots D, E, F were used for 5 and 40 ng/mL fortifications.
[d] Lots A1, B1, C1 analyzed for 1 ng/mL fortifications.
[e] Lots A1, B1, C1 analyzed for 5 and 40 ng/mL fortifications.

and 1 ng/mL ^{13}C-PFOA was prepared in human serum and plasma and analyzed with a set of ^{13}C-PFOA calibration standards prepared in human serum. The results of this experiment are presented in Table 3. This data clearly demonstrates that the LLOQ of 0.5 ng/mL is justified for human serum and plasma.

Figs. 2 and 3 present representative chromatograms obtained during the method validation for serum and plasma samples, respectively. These chromatograms demonstrate that the contribution of instrument blank to the analyte peak was practically eliminated by the use of the guard column system. The contribution of PFOA originating from the rabbit plasma and serum used to prepare calibration standards is less than 30% of the projected LLOQ of the method (0.5 ng/mL). Human control plasma and serum are shown to contain endogenous PFOA at levels above the projected LLOQ.

3.3. Sequence design and quality criteria for routine runs

The method validation data led to the establishment of guidelines for the sample sequence on the instrument and the number and type of QC samples that are included in a routine run of the proposed method. It is recommended that the entire set of calibration standards should be injected at the beginning of a sample set followed by calibration standards interspersed every 5–10 samples (to account for a second set of standards). As an alternative, an entire set of calibration standards may be included at the beginning and at the end of a sample set. However, it is still recommended that calibration standards be interspersed every 5–10 samples. In either case, calibration standards must be the first and last injection in a sample set. Each batch of samples analyzed (typically 35 or less) should include at least one reagent control (acetonitrile blank), one matrix control (method blank), and two matrix controls fortified at known concentrations to verify procedural recovery for the batch. At least one sample per batch should be extracted in duplicate. At least one sample extracted should be separately fortified at a known concentration and carried through the procedure to verify recovery.

The following data quality criteria were set for this method: the correlation coefficient (R) for calibration curves, linear, $1/x$, generated must be $\geq$0.9925 ($R^2 \geq 0.985$), any analyte present in the method blanks must be at least three-fold lower than the LLOQ, any analyte present in the reagent blank must be at least five-fold lower than the LLOQ.

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

The stated quality criteria were applied to analysis of authentic human serum samples originating from 255 individuals employed at three different production sites that use perfluorooctanoic acid or ammonium perfluorooctanoate. To evaluate the precision of the method every fifth sample was processed and analyzed in duplicate. These samples were grouped by ranges of PFOA found, percent relative standard deviation (%RSD) for each duplicate analysis was calculated, and then average %RSD and standard deviation of the %RSD was calculated for each group. The following data were obtained: for samples containing 0–10 ng/mL PFOA (n = 4 samples) the %RSD was 5.7 ± 6.2%, for 10–100 ng/mL (n = 16) the %RSD was 8.1 ± 5.1%, for 100–1000 ng/mL (n = 19) the %RSD was 4.7 ± 4.9%, and for >1000 ng/mL (n = 11) the %RSD was 3.6 ± 2.5%. This data demonstrates good method precision for a wide range of concentrations of PFOA found in authentic samples. In addition to duplicate analysis of selected samples, fortifications of rabbit serum and control human serum as well as fortifications of authentic samples

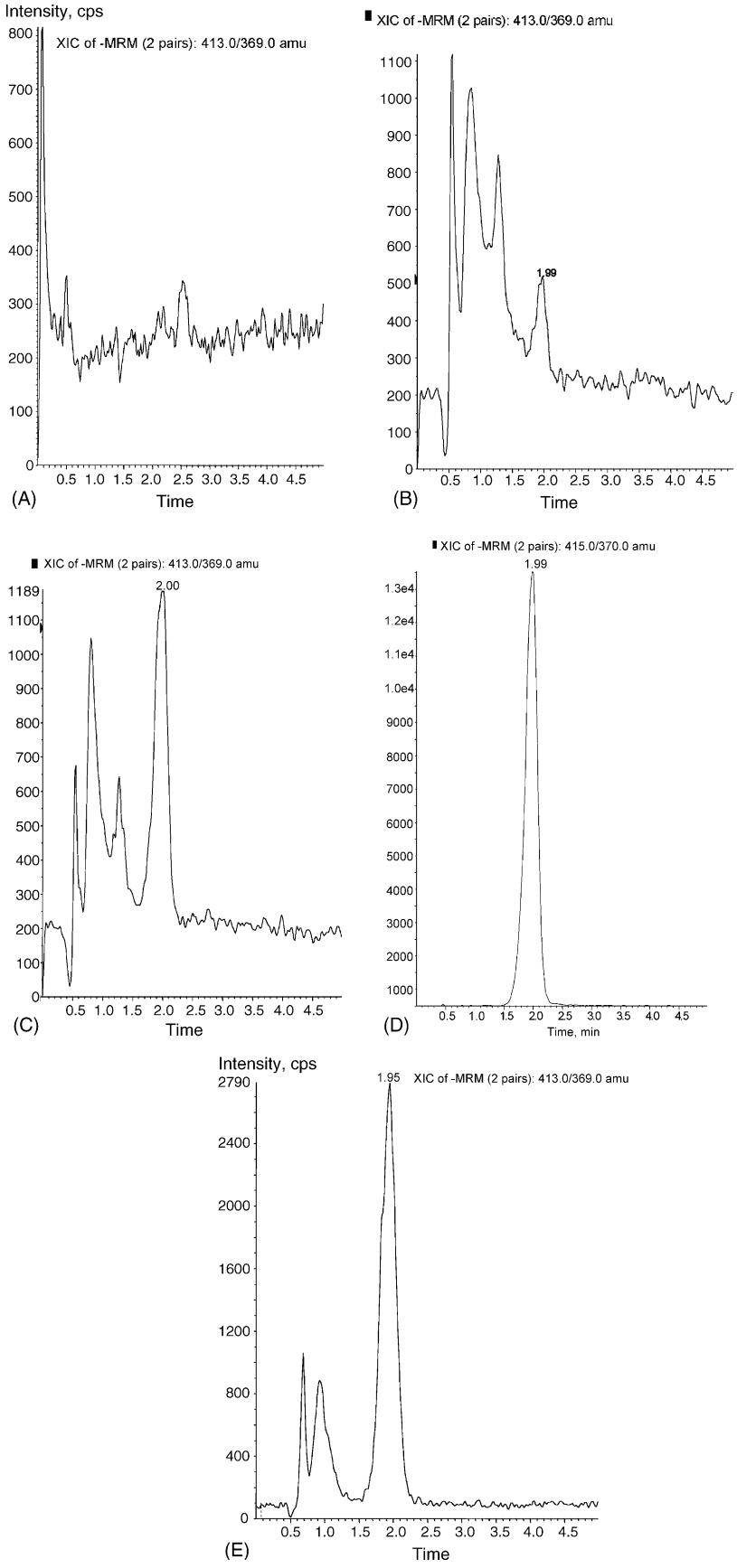

Fig. 2. Chromatograms obtained for SRM transitions 413 → 369 of instrument blank (A), control rabbit serum (B), 0.5 ng/mL PFOA rabbit serum calibration standard (C), human serum control (E), and SRM transition 415 → 370 for ^{13}C-PFOA internal standard (D).

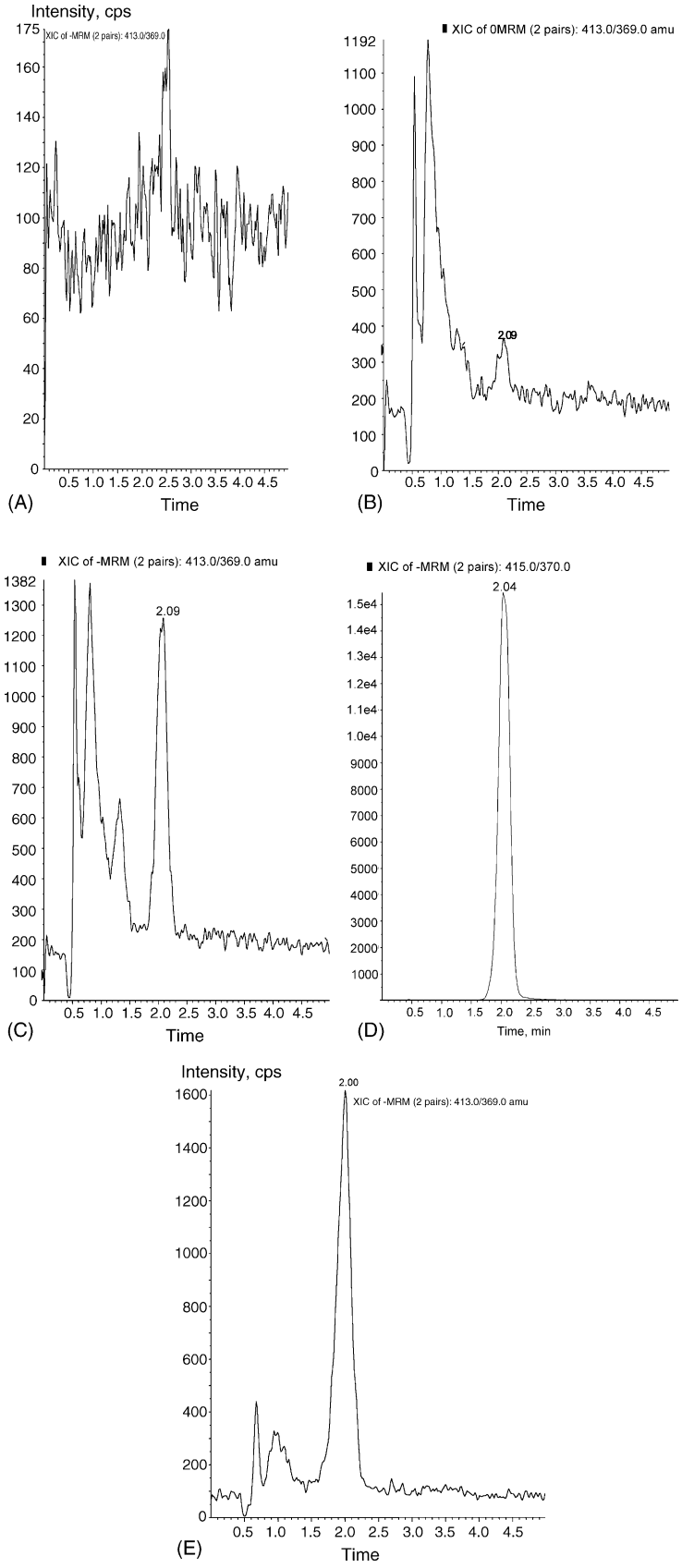

Fig. 3. Chromatograms obtained for SRM transitions 413 → 369 of instrument blank (A), control rabbit plasma (B), 0.5 ng/mL PFOA rabbit plasma calibration standard (C), human plasma control (E), and SRM transition 415 → 370 for ^{13}C-PFOA internal standard (D).

were analyzed. The percent recovery for each fortification was determined. The typical method performance observed for analysis of authentic samples is represented by data obtained for analysis of a batch of 116 authentic samples. For this batch of samples processed and run in smaller sets at different days, a total of six samples of control rabbit serum were fortified at 5 ng/mL PFOA and six at 50 ng/mL, six each of control human serum samples were fortified at 20 and 1000 ng/mL PFOA, and twenty six authentic samples were fortified at 1000 ng/mL PFOA. The following average percent recoveries and standard deviations were obtained for this batch of samples: $101 \pm 4.9\%$ (rabbit serum fortifications, $n = 12$), $109 \pm 4.6\%$ (control human serum fortifications at 20 ng/mL, $n = 6$), $91 \pm 8.8\%$ (control human serum fortifications at 1000 ng/mL, $n = 6$), and $91 \pm 9.4\%$ (authentic human serum samples fortifications at 1000 ng/mL, $n = 26$). This data demonstrates the method performance on authentic samples consistent with data obtained during method validation.

4. Conclusions

A selective and sensitive method for analysis of perfluorooctanoic acid in human serum and plasma using a 96-well-plate format has been developed and rigorously validated under strict FDA guidelines for bioanalytical method validation. The LLOQ of the method is 0.5 ng/mL, and, therefore, the method is adequate to measure PFOA serum or plasma levels in the general human population. The LLOQ was established using the ^{13}C-PFOA as a surrogate to circumvent complications arising from endogenous PFOA in human serum and plasma. The criteria established for method accuracy and precision (recoveries in the range of $\pm 15\%$ of nominal 100% recovery and standard deviations not exceeding 15%; $\pm 20\%$ at LLOQ) were met for rabbit serum and plasma control samples fortified at 0.5, 5, and 40 ng/mL PFOA and human serum and plasma fortified at 1.0, 5.0, 40 ng/mL PFOA. Within-day precision and accuracy, short-term stability, freeze–thaw stability, equivalence of response between PFOA and APFO (the ammonium salt of PFOA), and dilution of concentrated samples were demonstrated to meet the above criteria. Quality criteria were established to demonstrate the day-to-day performance of this method in routine runs.

References

[1] K.J. Hansen, L.A. Clemen, M.E. Ellefson, H.O. Johnson, Environ. Sci. Technol. 35 (2001) 766.
[2] G.W. Olsen, K.J. Hansen, L.A. Stevenson, J.M. Burris, J.H. Mandel, Environ. Sci. Technol. 37 (2003) 888.
[3] K. Kannan, J.W. Choi, N. Iseki, K. Senthilkumar, D.H. Kim, S. Masunaga, J.P. Giesy, Chemosphere 49 (2002) 225.
[4] K. Kannan, S. Corsolini, J. Falandysz, G. Oehme, S. Focardi, J.P. Giesy, Environ. Sci. Technol. 36 (2002) 3210.
[5] J. Belisle, D.F. Hagen, Biochem Anal. 101 (1980) 369.
[6] N. Kudo, N. Bandai, Y. Kawashima, Toxicol. Lett. 99 (1998) 183.
[7] M. Ylinen, H. Hanhijarvi, P. Peura, O. Ramo, Arch. Environ. Contam. Toxicol. 14 (1985) 713.
[8] T. Ohya, N. Kudo, E. Suzuki, Y. Kawashima, J. Chromatog. B 720 (1998) 1.
[9] C. Sottani, C. Minoia, Rapid Commun. Mass Spectrom. 16 (2002) 650.
[10] C. Kubwabo, N. Vais, F.M. Benoit, J. Environ. Monit. 6 (2004) 540.
[11] Z. Kuklenyik, J.A. Reich, J.S. Tully, L.L. Needham, A.M. Calafat, Environ. Sci. Technol. 38 (2004) 3698.
[12] K. Inoue, F. Okada, R. Ito, M. Kawaguchi, N. Okanouchi, H. Nakazawa, J. Chromatogr. B 810 (2004) 49.
[13] A. Holm, S.R. Wilson, P. Molander, E. Lundanes, T. Greibrokk, J. Sep. Sci. 27 (2004) 1071.
[14] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001.