A NEW ANALYTICAL METHOD FOR PERFLUORINATED COMPOUNDS USING ENZYMATIC DIGESTION COUPLED WITH SOLID PHASE EXTRACTION

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Introduction

Perfluorinated compounds (PFCs) are a group of chemicals that have attracted increasing attention in recent years. PFCs have been used for more than 50 years, and are widely used in industry, particularly in the manufacture of electronic and textile products. Recent studies on the global distribution of PFCs have detected perfluorooctane sulfonate (PFOS) in the tissues of human and wildlife, including fish, birds, and marine mammals[1]. Overall, the information available to date indicates that PFOS is persistent and bioaccumulative in various food chains.

There are several chemical extraction methods for PFC analyses of biological tissues, such as ion pairing extraction, methanol-acetonitrile extraction, as well as formic acid digestion and alkaline digestion. However, these extraction methods have their limitations. For example, the ion pairing method is a liquid-liquid extraction of homogenized proteins without strong digestion, and thus particulates in tissue homogenates might affect the recoveries for some longer chain compounds (see results and discussion). There are no good chemical digestion methods for PFCs. An alkaline digestion followed by solid-phase extraction (SPE) worked well with small amounts of soft tissues such as in mussels and oysters[2], but ion suppression occurred during the analysis and the method is suitable only for small sample volumes. Recoveries for most compounds using these methods (i.e. ion pairing, alkaline digestion) were acceptable, but recoveries for shorter chain (C4-C5) and longer chain (C11-C18) compounds were poor, ranging from 10-40%.

An enzymatic digestion method represents a possible improvement over previous methods because large proteins can be digested into smaller, water-soluble amino acids without strong chemical treatment. This digestion method has been used for other analytical chemical work, such as heavy metals in mussel and hair samples[3,4], drugs in rat brain[5], as well as drugs in chicken liver and muscle[6]. The methods used previously involved enzymatic digestion followed by ultrasonication, acid digestion or SPE. The most commonly used enzymes were proteases such as proteinase K, among others. Proteinase K is stable and has high activity. It is able to digest native proteins with a broad range of substrate specificity. Because of these advantages, in the present study proteinase K was chosen for sample pretreatment before extraction. However, the use of an enzyme for PFC analysis has not been reported previously because of several limitations, such as the presence of background levels of PFCs in commercially available enzyme preparations. Here we report an improved enzyme digestion method for trace level PFC analysis in biological samples. This new digestion method was coupled with SPE and tested for a series of PFCs.

Materials and Methods

A. Analytical chemicals. Potassium salts of PFOS, perfluorohexanesulfonate (PFHxS), perfluorooctanesulfonamide (PFOSA), perfluorononanoic acid (PFNA), and PFOA were purchased from Wellington Laboratories Inc. (ON, Canada). Perfluorohexanoic acid (PFHxA) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Perfluoroheptanoic acid (PFHpA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), and perfluorododecanoic acid (PFDoDA) were purchased from Fluorochem Ltd (Derbyshire, UK). Perfluorobutanesulfonate (PFBS) was purchased from Chiron (Trondheim, Norway). $^{13}C_4$-PFOS, $^{13}C_2$-PFDA, $^{13}C_5$-PFNA, and $^{13}C_4$-PFOA were purchased from Wellington Laboratories. The purities of the analytical standards were all ≥ 95%. Oasis® WAX (6 cc, 150 mg, 30 μm) solid phase extraction (SPE) cartridges were purchased from Waters (Milford, MA). Milli-Q water was used throughout the whole experiment. Methanol (residual pesticide and PCB analytical grade), ammonium acetate (97%), ammonium solution (25%), and acetic acid (99.9%) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

B. Chemicals for enzymatic digestion. Two types of proteinase k were purchased from two suppliers (Cat. No. 25530015, Invitrogen Corporation, and Cat. No. 82452, Sigma-Aldrich). Three types of calcium chloride ($CaCl_2$)

were also purchased from two companies (Cat. No. 030-00525 from Wako Pure Chemical Industries Ltd, Osaka, Japan and Cat. Nos. 239224-500G and 383147-100G from Sigma-Aldrich). Five types of glycerol were purchased from two companies (Cat. Nos. 070-04941, 079-00614 and 072-00626 from Wako Pure Chemical Industries Ltd, Osaka, Japan, and Cat. Nos. G7757-500ML and G7893-500ML from Sigma Aldrich). Tris-HCl solution (Cat. No. 316-90221) was purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan.

C. Test and environmental samples, and sample preparation. Chicken eggs and edible tissues of fish and chicken (without skin) were purchased from a local food market in Tsukuba city, Japan in 2007. Stock test samples were prepared by homogenization using a blender or a hand-held homogenizer. To enhance the surface area of the tissue for better enzymatic digestion efficiency, further homogenization of the samples was carried out using a T-homogenizer. The samples were homogenized with the addition of Milli-Q water due to the stickiness of the sample after homogenization.

D. Enzymatic digestion working solution. Enzymatic digestion working solutions were freshly prepared according to the manufacturers' recommendations. Briefly, the buffer solution (10mL) was prepared as follows: Milli-Q: glycerol: 1 M Tris HCl in 4:5:1 v/v, with the addition of 22 mg calcium chloride ($CaCl_2$. And finally, the amount of enzyme was added as 0.5 mg/mL buffer.

E. Enzymatic digestion procedure. One g of the sample homogenate was transferred to a 15 mL polypropylene (PP) tube. Different amounts (0.5, 1, 2 mg) of enzyme in buffer solution were added to the sample homogenates to test for the amount of enzyme that was required for the experiment. The mixtures were then gently mixed and were then incubated at 60^{o}C (the manufacturers' recommendations ranged from 60-65 ^{o}C) with gentle shaking, for different incubation periods (0.5, 1, 2, 4, 8 hrs) to determine the optimal incubation period for the digestion (see Section F.)

F. Sample pass-through cartridge experiment. A sample pass-through cartridge experiment was performed to determine the optimal digestion parameters (see Section E). This experiment was a qualitative experiment that indicated whether the amount of enzyme used and the incubation time were sufficient to produce digested homogenate that could pass through the SPE cartridge. The results of this experiment governed the optimization of the enzymatic digestion procedure.

G. Solid Phase Extraction (SPE) After enzymatic digestion, the digested homogenates were diluted into 100 mL Milli-Q water for SPE. The SPE procedure of the digested samples was similar to that described previously[7]. The cartridges were first preconditioned by passage of 4 mL 0.1%NH_4OH/MeOH, and then by 4 mL MeOH and 4 mL Milli-Q water. The diluted homogenates were passed through the pre-conditioned cartridges at a rate of 1 drop/sec. The cartridges were then washed (4 mL of 25 mM ammonium acetate buffer at pH 4) and the target analytes eluted with 4 mL of MeOH and 4 mL of 0.1% NH_4OH in methanol.

H. Instrumental analysis. Analysis of the target analytes was performed by using a high performance liquid chromatography-tandem mass spectrometer (HPLC-MS/MS), comprising an Agilent HP1100 liquid chromatography interfaced with a Micromass (Beverly, MA, USA) Quattro Ultima Pt mass spectrometer operated in negative electrospray ionization (ESI) mode. A 10 µL aliquot of the sample extract was injected onto a guard column (XDB-C8, 2.1 mm i.d. x 12.5 mm, 5 µm; Agilent Technologies, Palo Alto, CA) connected sequentially to a Betasil C18 column (2.1 mm i.d. x 50 mm length; Thermo Hypersil-Keystone, Bellefonte, PA), and RSpak JJ-50 2D ion exchange column (2.0 mm i.d. x 150 mm length, 5 µm; Shodex, Showa Denko K.K., Kawasaki, Japan), separately to confirm the results with the mobile phases ammonium acetate and methanol[7,8].

I. Qualify assurance and quality control (QA/QC). To achieve lower detection limits, all accessible polytetrafluorethylene (PTFE) materials were removed from the instruments and apparatus such as the blender to minimize the background signal due to contamination[9]. All of the chemicals used in the enzymatic digestion were tested for PFC contamination (see Results and Discussion below). Procedural blanks and recoveries were conducted with every batch of samples for extraction. All of the sample extractions, procedural blanks and recoveries were conducted in duplicates.

Results and Discussion

The first difficulty with the enzymatic digestion procedure was to find PFC-free reagent. Several reagents were

tested and some were found to be unacceptable because of high background PFC levels, showing that commercially available reagents are manufactured without sufficient attention to PFC contamination. Trace amounts of PFCs were found in two of the five glycerol samples (PFUnDA: 4-6 pg/mL, PFOA: 5.1 pg/mL), two of the enzyme samples (PFUnDA: 9.5 pg/mg, PFOA 7 pg/mg; PFPeA: 7.7pg/mg; PFBA: 18.3/mg), and one sample of $CaCl_2$ (PFOA: 2.3 pg/g). Methanol and ethanol were used to remove the PFCs in the enzyme solutions, but methanol was found to inhibit the activity of the enzyme, whereas ethanol had no effect. However, in order to avoid further contamination or deactivation of the enzyme or the other chemicals, the purchased reagents with PFC levels below detection limits were used in further experiments. After these reagents were chosen, a series of experiments was conducted to evaluate 1) the effect of chemicals (enzyme, buffer) and chemical parameters (temperature and incubation time) on PFC standards; 2) PFC levels in procedural blanks and recoveries; and 3) the effectiveness of the enzyme digestion. The test parameters for the enzyme digestion are listed in Table 1.

Table 1 A summary of the test parameters and results of the pass through test

	Egg (mL)	Enzyme (mg)	Buffer (mL)	PFC standard	Incubation Time (h)	Speed	Pass though	Volume (mL) can pass through
B1-1	0	0.5	1	×	1	*****	√	100
B1-2	0	0.5	1	×	2	*****	√	100
C1-1	0	0	1	√	1	*****	√	100
C1-2	0	0	1	√	2	*****	√	100
C2-1	0	0.5	1	√	1	*****	√	100
C2-2	0	0.5	1	√	2	*****	√	100
S1-1	0.5	0	1	×	1	*	√	100
S1-2	0.5	0	1	×	2	*	×	90
S2-1	0.5	0.5	1	×	1	****	√	100
S2-2	0.5	0.5	1	×	2	****	√	100
S3-1	2	0.5	1	×	1		×	10
S3-2	2	0.5	1	×	2		×	10

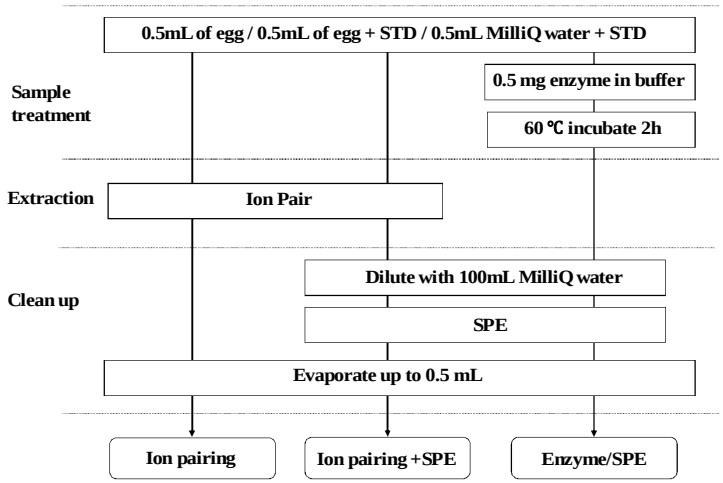
B: Blank, C: Control: S: Sample; *: Slow, ***: Fast; √: can pass through; ×: cannot pass through**

Briefly, procedural blanks were all below the corresponding limits of quantification (LOQs). Recoveries from those of control (C) and normal SPE (without any treatment) were almost the same, which indicated that the presence of the enzyme at prolonged high temperature (60°C) did not affect procedural recoveries. In addition, a preliminary experiment using 0.5 mL egg sample with and without the enzyme showed that the SPE cartridge was blocked when samples (0.5 and 2 mL egg) without enzyme were loaded. Since PFC concentrations in the tested food items were low, 2 mL egg homogenates were used for better sensitivity. Another set of experiments in which the 2 mL egg samples were digested for different periods of time and with different amounts of enzyme were carried out.

The activity of the enzyme was tested in the presence of MeOH at different concentrations in the enzyme cocktail before matrix-spike recovery tests were conducted because the PFC standards were prepared in MeOH. The results showed that the addition of 0.1 mL Milli-Q water containing 0.01% MeOH did not affect the passage of the egg samples through the cartridge using the optimal digestion conditions.

A comparison of the recoveries produced by two different extraction methods (ion pairing with SPE and enzymatic digestion with SPE) are shown in Figure 1 and Table 2. Most of the procedural recoveries of PFCs using enzymatic digestion were comparable to those of ion paring extraction and ion pairing + SPE except for some of the longer chain compounds, such asPFDoDA, and PFUnDA. Matrix-spike recoveries using 0.5 mL egg, ion pairing + SPE and enzymatic digestion + SPE, much lower recoveries were found for PFDoDA and PFUnDA using the enzymatic method. For the rest of the compounds, the matrix recoveries were similar. However, when 2 mL egg samples were used for enzymatic digestion, much lower recoveries were observed, even for PFOA, PFNA and PFDA (Table 2).

Figure 1.Experimental flow chart



To conclude, although some uncertainty remains regarding the enzyme digestion parameters and limitations may apply for large sample volumes, the concentrations of PFOS detected using enzymatic digestion and ion pairing methods were comparable. PFHxS, PFPeA and PFBA could be detected using the enzymatic digestion method, but could not be detected using the ion pairing extraction method. This result indicated that the enzymatic digestion method was efficacious and increased sample size using PFC-free enzymes will provide more information on other PFCs. Further improvement and optimization steps after enzymatic digestion are needed, and a range of other food samples and matrices will be tested using this method.

Table 2. A comparison of procedural and matrix-spike recoveries using ion pair+SPE and enzymatic digestion+SPE methods

		Procedural recoveries			Matrix spike recoveries		
		Ion pairing	Ion pairing +	Enzyme + SPE	Ion pairing	Enzyme + SPE	Enzyme + SPE
Spiked STD	[ng]	1	1	1	1	1	1
Egg	[mL]	0	0	0	0.5	0.5	2
Final vol	[mL]	0.5	0.5	0.5	0.5	0.5	0.5
Sulfonates							
PFOS	C8	101	103	82	n/a	n/a	n/a
Carboxylates							
PFDA	C10	110	95	72	113	113	63
PFNA	C9	100	100	87	94	107	14
PFOA	C8	93	87	94	100	102	54
13C-standards							
$^{13}C_4$-PFOS		n/a	n/a	n/a	99	82	34
$^{13}C_2$-PFDA		n/a	n/a	n/a	97	90	61
$^{13}C_5$-PFNA		n/a	n/a	n/a	101	96	16
$^{13}C_4$-PFOA		n/a	n/a	n/a	96	100	59

n/a: not applicable

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