Journal of Chromatography A, 1237 (2012) 64–71

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A matrix effect-free method for reliable quantification of perfluoroalkyl carboxylic acids and perfluoroalkane sulfonic acids at low parts per trillion levels in dietary samples

Robin Vestergren[1], Shahid Ullah[2], Ian T. Cousins[3], Urs Berger*

Department of Applied Environmental Science (ITM), Stockholm University, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 5 March 2012
Accepted 7 March 2012
Available online 14 March 2012

Keywords:
Food
Diet
Human exposure
Perfluoroalkyl acids
PFOA
PFOS

ABSTRACT

In recent exposure modeling studies diet has been identified as the dominant pathway of human exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). However, the paucity of highly sensitive and accurate analytical data to support these studies means that their conclusions are open to question. Here a novel matrix effect-free method is described for ultra-trace analysis of perfluoroalkyl carboxylic acids and perfluoroalkane sulfonic acids in dietary samples of varied composition. The method employs ion pair extraction of the analytes into methyl *tert*-butyl ether and subsequent solid phase extraction clean-up on Florisil and graphitized carbon. The target compounds are separated and detected using ultra performance liquid chromatography coupled to tandem mass spectrometry. Special care was taken to avoid procedural blank contamination and potential contamination sources were elucidated. The performance of the method was validated for five different food test matrices including a duplicate diet sample. Method detection limits in the low to sub pg g^{-1} range were obtained for all target analytes, which is 5–100 times more sensitive than previously reported for duplicate diet samples. Total method recoveries were consistently between 50 and 80% for all analytes in all tested food matrices and effects of co-extracted matrix constituents on ionization of the target compounds were found to be negligible. The precision of the method (defined as percentage relative standard deviation) at concentrations close to the respective method limits of quantification was <15% for all analytes. Accurate quantification at ultra-trace levels was demonstrated by laboratory control spike experiments. For the first time the presence of long-chain PFCAs in duplicate diet samples is reported. The method presented here can thus support an improved assessment of human exposure from dietary intake for a range of PFCA and PFSA homologues. Re-analysis of duplicate diet samples, which had been analyzed earlier using another analytical methodology, indicated that dietary intake of PFOA and PFOS may previously have been overestimated.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs) have been manufactured and used commercially in a wide range of applications during the last six decades. Concerns regarding the ubiquitous presence of PFCAs and PFSAs in human serum [1], their biopersistence [2], and potential toxicity [3] have prompted researchers to investigate the major pathways of human exposure. For the general population in industrialized countries, exposure modeling studies have unanimously concluded that for adult humans direct exposure via the diet is the major ongoing exposure pathway for PFOS and PFOA with a minor contribution from drinking water and ingestion of dust [4–9]. However, the empirical data to support these conclusions are few and there are inconsistent findings in reported concentration data of PFCAs and PFSAs in food [10]. Furthermore, a vast majority of the analyzed food samples have been reported below the respective limits of detection [11–14]. Thus, in order to derive reliable dietary exposure estimates there is a need for highly sensitive and accurate analytical techniques that can be applied to a series of PFCA and PFSA homologues in a wide range of food samples.

The primary challenge in analyzing these compounds in food samples is posed by the very low concentrations encountered (typically low parts per trillion or pg g^{-1}) in most food samples

* Corresponding author. Tel.: +46 8 674 7099; fax: +46 8 674 7636.
E-mail addresses: robin.vestergren@itm.su.se (R. Vestergren), shahid.ullah@itm.su.se (S. Ullah), ian.cousins@itm.su.se (I.T. Cousins), urs.berger@itm.su.se (U. Berger).
[1] Tel.: +46 8 16 40 32; fax: +46 8 674 7636.
[2] Tel.: +46 8 674 7340; fax: +46 8 674 7636.
[3] Tel.: +46 8 16 40 12; fax: +46 8 674 7636.

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.03.023

of non-animal origin [11–17]. Quantitative analysis of PFCAs and PFSAs at low pg g^{-1} concentrations in complex matrices requires a high sample to extract concentration factor, which further requires a rigorous clean-up in order to eliminate matrix effects, and good control of procedural blank contamination. In addition, it is highly challenging to develop simple extraction and clean-up methods that can span the physical–chemical property range of PFCAs and PFSAs (i.e. from hydrophilic short-chain to hydrophobic long-chain compounds) for a range of complex and heterogeneous matrices. The majority of recent studies that attempted to analyze PFCAs and PFSAs in food items have employed extraction with medium polar organic solvents (methanol or acetonitrile) [7,11–15]. As the high water content of many food samples has previously been shown to affect the extraction and clean-up performance, many studies have employed freeze-drying prior to sample work up [12,13,15–18]. Extraction is usually followed by weak anion exchange solid phase extraction (SPE) [19] and/or dispersive graphitized carbon clean-up [20] to reduce effects of co-extracted matrix components. Although some food samples have been analyzed successfully with this approach, recoveries are strongly matrix dependent and may display a large variation for the different homologues. For example, absolute recoveries between 0 and 20% were observed for long-chain PFCAs in multiple food items in some recent studies [13,15,16]. Stable isotope labeled internal standards and matrix matched calibration can to some extent compensate for recovery losses and ionization suppression/enhancement [21–23]. However, poor total method recovery will ultimately constrain the method performance for detection of ultra-trace concentrations. In addition to these difficulties, procedural blank contamination has often been found to prevent detection in the low pg g^{-1} range [7,12,13]. Although procedural blank contamination has been recognized as a problem in the analysis of perfluoroalkyl acids at trace levels for many years [24], it is seldom explicitly investigated and described in the published literature.

The objective of this study was to develop and validate an analytical method for a range of PFCA and PFSA homologues, which can be broadly applied to a wide range of dietary samples of varied composition. To allow extraction from fresh samples of varying water content, a previously described ion-pair extraction method into a non-polar solvent [25] was modified and combined with a Florisil/graphitized carbon SPE clean-up. The method was optimized with respect to applicability range, sensitivity (sample concentration factor), as well as avoiding matrix effects and procedural blank contamination in order to achieve method detection and quantification limits in the low pg g^{-1} range. The performance of the newly developed method and its potential application in estimating human dietary exposure are discussed.

2. Experimental

2.1. Standards and reagents

All native and isotope labeled PFCA and PFSA standard compounds were purchased from Wellington Laboratories (Guelph, ON, Canada) in 2 μg mL^{-1} solution mixtures. The 9 target analytes were perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorohexane sulfonic acid (PFHxS) and perfluorooctane sulfonic acid (PFOS). Internal standards were $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnDA, $^{13}C_2$-PFDoDA, $^{18}O_2$-PFHxS and $^{13}C_4$-PFOS. Perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA) and perfluorobutane sulfonic acid (PFBS) were only qualitatively included in the method, due to the lack of authentic isotope labeled standards for these compounds. $^{13}C_8$-PFOA and $^{13}C_8$-PFOS were used as volumetric standards in the calculation of total method recovery of the internal standards. All isotope labeled standards were certified to contain <0.5% of their native analogues.

All reagents were analytical reagent grade. Tetrabutyl ammonium hydrogen sulfate (TBA) was purchased from MERCK, sodium hydroxide (NaOH) from Akzo Nobel, sodium carbonate (Na_2CO_3) from Riedel-de Haën, sodium hydrogen carbonate ($NaHCO_3$) and ammonium acetate (CH_3COONH_4) from KEBO, and anhydrous granulated sodium sulfate (Na_2SO_4) from Scharlau. Florisil sorbent (60/100 mesh) and Supelclean graphitized carbon (ENVI-carb) were obtained from SUPELCO. The water used in the method was HPLC grade (PROLABO Chromanorm) and was passed through a mixed mode C8 plus quaternary amine (CUQAX) SPE cartridge supplied by UCT to remove residual PFCAs and PFSAs. Methyl *tert*-butyl ether (MTBE) was purchased from Rathburn chemicals and was pre-cleaned by passing through a column manually packed with Florisil sorbent (see Section 2.4). Methanol (MeOH, LiChrosolv grade) was supplied by MERCK and was used without further purification. All laboratory disposables (polypropylene or glass) were rinsed with methanol before usage.

2.2. Food samples

Five different dietary test matrices were included in this study for method development purposes, namely; total daily intake homogenate (hereafter referred to as duplicate diet), baby food composite, fish composite, meat composite and vegetable composite. The choice of test matrices was made to reflect differences in water, protein and fat content, but also with respect to their potential importance in dietary exposure studies. The duplicate diet was prepared by taking duplicate portions of all the food and drinks consumed by one individual male adult during one day. Food items included in the duplicate diet sample were prepared or cooked as consumed, whereas the vegetable, meat and fish composites were prepared from raw food items. A full description of the test matrices is provided in the Supplementary Data including Tables S1–S4. All food composite samples were homogenized using a kitchen blender, divided into aliquots and stored at −18 °C in polypropylene containers until analysis.

For the evaluation of inter-method comparability, a fish fillet sample from a worldwide interlaboratory comparison study (ILC) [17] and a set of 10 duplicate diet samples were analyzed. The duplicate diet samples originated from a previously published study by Fromme et al., who quantified PFHxA, PFOA, PFHxS and PFOS [7]. The 10 samples were randomly selected among the samples containing levels of PFOA and/or PFOS quantified above the reported limits of detection (50–100 pg g^{-1}) on wet weight (w.wt.) basis in the Fromme study [7]. All samples were obtained coded, i.e. they could not be linked to published concentrations of PFCAs and PFSAs. The samples were additionally analyzed using another recently developed method, which is published elsewhere [26].

2.3. Extraction procedure

The extraction method was based on the ion-pair extraction method developed by Ylinen et al. [25] with some modifications. Isotope labeled internal standards were spiked (200 pg of each standard in 20 μL of methanol) to 5 g w.wt. of duplicate diet, baby food or vegetable sample or 2.5 g w.wt. of fish or meat sample in a 50 mL polypropylene tube. The spiked standards were left to equilibrate with the sample material at room temperature overnight. To release analytes from the sample matrix, 1.5 mL of aqueous NaOH (0.4 mol L^{-1}) was added and the samples were vortex mixed for 30 s and left for 30 min. After addition of 2 mL of 0.25 mol L^{-1}

$Na_2CO_3/NaHCO_3$ buffer (adjusted to pH 10 with NaOH) and 1 mL of 0.5 mol L^{-1} TBA-solution (both in pre-cleaned HPLC water) the samples were vortex mixed. A volume of 5 mL of MTBE was added and the mixture was vortex mixed again for 30 s. The samples were extracted in an ultrasonic bath at room temperature for 10 min after which the organic phase was separated by centrifugation for 10 min at 3500 rpm. The top MTBE layer was transferred to a 15 mL polypropylene tube and the extraction was repeated twice with 5 mL MTBE. The combined extracts were evaporated to a final volume of approximately 3 mL under a gentle stream of dry nitrogen gas.

2.4. Solid phase extraction clean-up

SPE was performed using a Florisil and ENVI-carb sorbent mixture. The Florisil sorbent was dried at 450 °C overnight and deactivated with 0.5% (w/w) HPLC water prior to use. To prepare SPE columns, 1.5 g of Florisil was mixed with 25 mg of ENVI-carb and used to fill a 10 mL disposable glass pipette with a plug of pre-cleaned (MeOH) glass wool in the bottom. To remove remaining moisture from the extracts, 1 g of anhydrous granular Na_2SO_4 was applied on top of the column. The cartridge was rinsed with 5 mL of MeOH and conditioned with 5 mL of MTBE before the sample extract (3 mL) was loaded. The cartridge was then washed with 10 mL of MTBE that was discarded. The target compounds were eluted with 6 mL of a 30/70 MeOH/MTBE mixture (vol/vol). The MeOH/MTBE extract was evaporated to ~100 μL under nitrogen and the final volume was adjusted to ~250 μL by addition of the volumetric standards (100 pg of each standard in 50 μL of MeOH) and 100 μL aqueous ammonium acetate (4 mmol L^{-1}). Prior to analysis, the extracts were cooled overnight to −18 °C and subsequently centrifuged at 10,000 rpm for 10 min. An aliquot of 100 μL of the clear supernatant was transferred to an auto-injector vial.

2.5. Instrumental analysis and quantification

The purified sample extracts were analyzed using an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo TQS tandem mass spectrometer (MS/MS) with an electrospray ionization (ESI) interface (all from Waters Corp.). A “PFC isolator column” obtained from Waters (“PFC kit”) was inserted in the UPLC system prior to the injector to trap and delay contamination originating from the UPLC instrument and solvents. The analytical separation column was a BEH C18 (1.7 μm particles, 50 mm × 2.1 mm, Waters). The column temperature was set to 65 °C. An injection volume of 5 μL in the partial loop injection mode was used in all experiments. The mobile phase consisted of solvent (A) 10% MeOH in water and solvent (B) MeOH, both containing 2 mmol L^{-1} ammonium acetate. A binary gradient elution program was applied with a constant flow rate of 0.4 mL min^{-1}. Initial conditions of 90% A and 10% B were held until 2 min, then the percentage of B was linearly increased to 20% until 5 min. At 5 min, a step increase to 100% B occurred, which was held until 8 min to complete the elution. The column was reconditioned for 2 min at the starting composition of 90% A prior to the next injection.

The ESI source was operated in the negative ion mode with a capillary voltage of 3.6 kV, a source temperature of 150 °C, and a nitrogen desolvation gas flow rate of 650 L h^{-1}. The argon collision gas pressure was 5.9 μbar. Table S5 in the Supplementary Data lists the MS/MS transitions, cone voltages and collision energies applied for the different target analytes and isotope labeled standards. The MassLynx Software v. 4.1 (Waters) was used for instrument control, data acquisition and processing.

Quantification was performed using the internal standard method (isotopic dilution) for all target analytes. For PFHpA, which did not have a corresponding isotope labeled standard, $^{13}C_2$-PFHxA was used for quantification. A six point calibration curve ranging for all compounds from 0.018 to 9 pg μL^{-1} in MeOH/water (50/50, containing 2 mmol L^{-1} ammonium acetate) was used to calculate relative response factors and to control the linear range of the instrumental response. All quantified concentrations given in this paper are on a sample w.wt. basis and were not blank corrected.

2.6. Method validation and applicability

A series of nine procedural blank experiments were performed for assessment of the method detection limits (MDLs) and the method limits of quantification (MLQs) of the different target analytes. In order to fully reflect the variability in instrumental noise and method extraction efficiency, procedural blanks were performed in triplicate on three different days. Procedural blank chromatograms contained detectable signals for all analytes. The MDL was derived from the arithmetic mean plus three times the standard deviation of the analyte signal in the procedural blanks. Analogously, the MLQ was derived from the mean blank concentration plus ten times the standard deviation.

Total method recoveries (calculated against external calibration after volume correction using the volumetric standards) were evaluated by spiking 100 pg g^{-1} of the internal standards to the five different test matrices in triplicate. In addition, the duplicate diet sample matrix was spiked with the internal standards at four different concentrations (1, 10, 100, and 1000 pg g^{-1}) to test if total method recoveries were concentration dependent. All spiking concentrations in this paper are given on a sample w.wt. basis. Possible matrix effects on the ionization in ESI-MS/MS determination were evaluated by comparison of response factors for internal standards spiked to purified matrix extracts (immediately before instrumental analysis) and internal standards dissolved in the corresponding pure solvent (MeOH/water with 2 mmol L^{-1} ammonium acetate).

Precision and accuracy were tested according to a guidance document for bioanalytical method validation [27]. Triplicate analyses of laboratory control spikes of the duplicate diet sample matrix with native PFCAs and PFSAs at 20 pg g^{-1} were performed. Precision is expressed as the percentage relative standard deviation (%RSD) of the three replicate analyses. The accuracy of quantified concentrations was evaluated by comparison with theoretical levels in the laboratory control spike experiments after subtraction of endogenously present concentrations. Furthermore, the fish fillet sample from the ILC study was analyzed in triplicate and quantified concentrations were compared to the indicative values from the ILC study [17]. Inter-method comparability at low pg g^{-1} concentrations in non-fortified samples was evaluated by analyzing the set of 10 duplicate diet samples both using the method presented here and another recently developed method [26]. In short, the method of comparison employed a different set of internal standards, acetonitrile extraction, clean-up on a mixed mode copolymeric (C8 + quaternary amine) SPE phase, and HPLC coupled to high resolution MS analysis [26]. The 10 duplicate diet samples had previously also been analyzed by another laboratory using yet another methodology [7]. However, this method [7] only comprised four analytes and reported relatively high limits of detection compared to the more recently developed methods.

3. Results and discussion

3.1. Design of the method

It is worth elaborating on the design of the method before discussing the method performance and analytical results. Extraction and enrichment of the target analytes was achieved by exploiting the amphiphilic properties of PFCAs and PFSAs. In order to make

the method broadly applicable without freeze-drying the samples, ion-pair extraction into an organic solvent (MTBE) was identified as a suitable technique that would prevent co-extraction of water from the food samples. Previous studies of PFCAs and PFSAs in soil and sediment samples have shown that aged residues may display a different extraction behavior compared to fortified standards [28]. In the method presented here, an equilibration time of 16 h for internal standards and the release of analytes from the food matrix with the help of NaOH helped ensure that the internal standards correctly mimicked the behavior of naturally embedded target analytes. In order to achieve MDLs in the low pg g^{-1} range a high concentration factor is needed between sample and extract. As ion-pair extraction of PFCAs and PFSAs from complex matrices (e.g. fish muscle and fish liver samples) has previously been associated with matrix effects on ionization due to the co-extraction of lipids [29], a rigorous clean-up procedure was developed. The polar–polar interactions between the target analytes and a magnesium silicate sorbent (Florisil) were exploited to separate analytes from co-extracted lipids and hydrophobic matrix constituents. Additionally, graphitized carbon was mixed into the sorbent to selectively retain aromatic compounds [20]. Freezing overnight and subsequent centrifugation was finally employed to achieve an extract clear of any type of sample matrix.

3.2. Blank contamination

A widely reported challenge in ultra-trace analysis of PFCAs and PFSAs is the background contamination of the LC system [21,30]. To overcome this problem a “PFC isolator column” was inserted prior to the injector to trap and delay any analytes originating from the mobile phase solvents and LC system. Less frequently reported, but potentially more problematic, is the contamination of procedural blank extracts. All laboratory disposable equipments included in this method were sonicated with MeOH and analyzed to determine potential sources of contamination. None of the labware articles were found to contribute to background levels of any analyte, with the exception of commercially available Florisil SPE cartridges, which were excluded from the method. The purity of solvents was tested by evaporating 50 mL of solvent to dryness followed by reconstitution in 200 μL of MeOH. Artifacts originating from the evaporation procedure were investigated by testing both nitrogen gas and vacuum evaporation, but comparable results were obtained with both methods. All solvents tested, namely MTBE, MeOH, and water from various suppliers, were found to contain low, yet detectable amounts of target analytes. PFHxA, PFOA and PFDA were generally found to be present at the highest concentrations around 0.1–0.4 pg mL^{-1}, while PFNA (0.01–0.11 pg mL^{-1}), PFUnDA (0.06–0.1 pg mL^{-1}), PFDoDA (0.02–0.04 pg mL^{-1}), PFHxS (0.03–0.04 pg mL^{-1}), and PFOS (0.01–0.03 pg mL^{-1}) were present at lower concentrations. The solvents from the suppliers listed in the experimental section were used as they displayed the lowest levels of contamination. However, further purification of MTBE and water was achieved using Florisil and CUQAX SPE clean-up, respectively. For MeOH no efficient purification procedure was found because rapid breakthrough of PFCAs and PFSAs was observed on all SPE cartridges tested. By analyzing extraction blanks, SPE blanks and solvent blanks separately, it was concluded that the small remaining procedural blank contamination originated primarily from residuals in MeOH and water, which could not be eliminated entirely.

3.3. Investigation of matrix effects and interferences

MS/MS detection with an ESI interface demonstrates excellent selectivity and sensitivity for the target analytes, however, method recovery calculations and MDLs may be adversely affected by ionization suppression through co-eluting matrix constituents [31,32]. The presence (or absence) of matrix effects in the method was examined by fortification of purified food sample extracts with internal standards immediately prior to injection into the UPLC/MS/MS system (Table 1). Internal standards were chosen in this experiment in order to avoid misinterpretations due to the presence of native PFCAs and PFSAs in all investigated food matrices (see Section 3.7) and in procedural blank extracts (see Section 3.2). The majority of food extracts displayed only a minor ionization enhancement or suppression of the internal standards (<12% deviation from solvent based standards), which was in the range of the quantification uncertainty. A slight matrix ionization suppression (−14%) was observed for $^{13}C_2$-PFUnDA in the vegetable extract whereas a small enhancement (up to 22%) of the signal was observed for $^{13}C_2$-PFUnDA and $^{13}C_2$-PFDoDA in the fish and duplicate diet extract. The small deviation in signal area response in food extracts compared to solvent based standard solutions demonstrates the efficiency of the clean-up procedure to remove a wide variety of matrix components despite the high concentration factor between sample and extract.

Potential misidentification of PFHxS and PFOS due to interferences in the m/z 399–80 and 499–80 transitions, respectively, have been reported in samples of animal origin [33]. Therefore, to confirm the identity of PFHxS and PFOS in the test matrices, the qualifier transitions m/z 399–99 and 499–99, respectively, were additionally measured for quality assurance. The ratio of peak areas of the transitions to m/z 80 and 99 in all food extracts did not differ significantly from that in the standard solution, which again demonstrates the efficient clean-up and indicates that accurate quantification can be achieved using the more sensitive transition to m/z 80.

3.4. Total method recoveries

Total method recoveries were determined by fortification experiments with the internal standards as described in Section 2.6. Internal standards were used in these experiments due to the ubiquitous presence of native PFCAs and PFSAs in all food sample matrices (see Section 3.7) and in procedural blank extracts (see Section 3.2). Using native analytes would have led to overestimation of recoveries at low spiking levels and thus to an apparent concentration dependence of analyte recoveries. A drawback of this approach

Table 1
Ionization suppression and/or enhancement for the internal standards fortified at 200 pg to purified food sample extracts (250 μL). Matrix effects are expressed as deviation (%) in signal area response from that of a solvent-based internal standard solution.

	Mean matrix effect (%) ± 1 standard deviation (n = 3)							
	$^{13}C_2$-PFHxA	$^{13}C_4$-PFOA	$^{13}C_5$-PFNA	$^{13}C_2$-PFDA	$^{13}C_2$-PFUnDA	$^{13}C_2$-PFDoDA	$^{18}O_2$-PFHxS	$^{13}C_4$-PFOS
Duplicate diet	12 ± 5	2 ± 6	8 ± 7	13 ± 6	13 ± 6	19 ± 6	19 ± 7	20 ± 6
Vegetable	7 ± 3	−7 ± 2	−3 ± 2	−7 ± 4	−14 ± 4	1 ± 5	14 ± 2	14 ± 4
Baby food	3 ± 10	−13 ± 12	−3 ± 11	−2 ± 14	1 ± 13	3 ± 13	6 ± 10	4 ± 10
Fish	3 ± 1	7 ± 3	20 ± 3	14 ± 6	19 ± 7	22 ± 6	9 ± 4	21 ± 5
Meat	−8 ± 3	−12 ± 12	5 ± 14	10 ± 11	12 ± 2	12 ± 4	−4 ± 12	9 ± 11

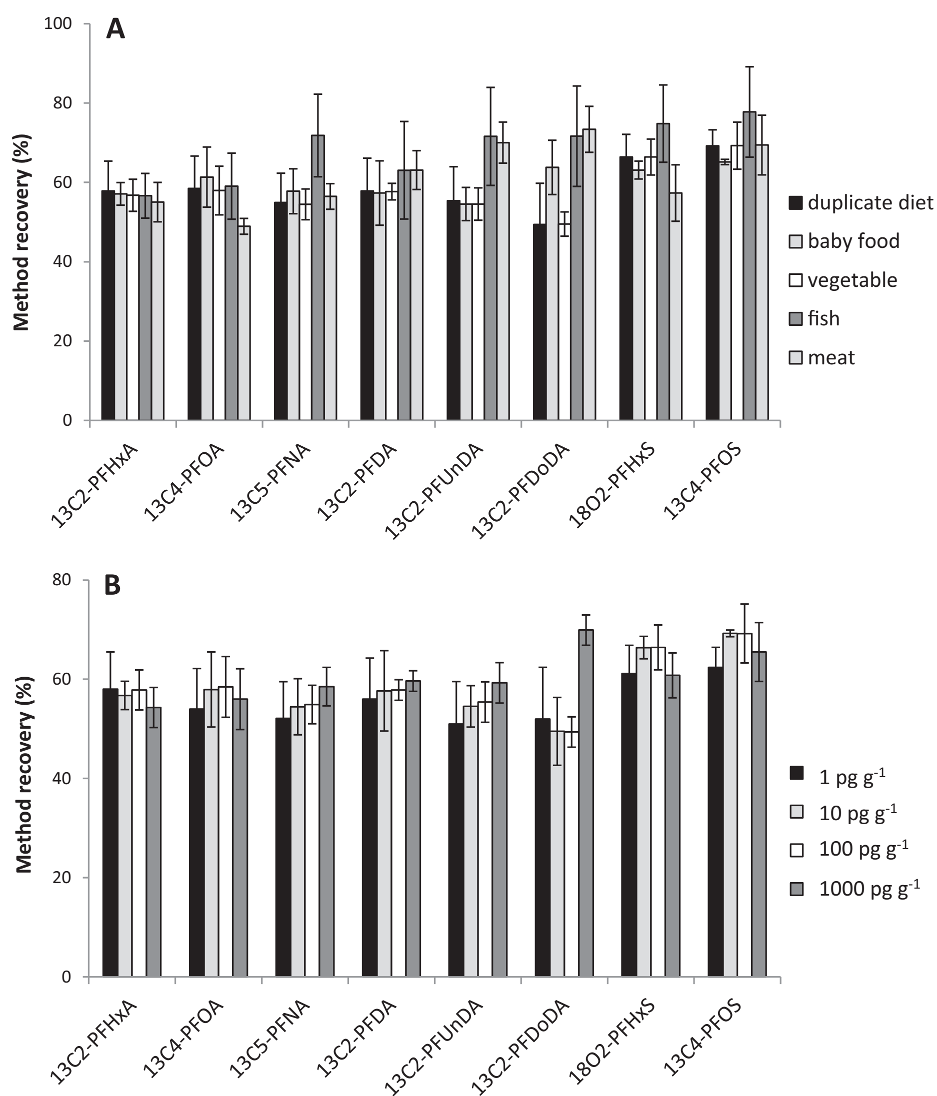

Fig. 1. Total method recoveries (%) for the internal standards (A) spiked at 100 pg g^{-1} to the different food matrices (see legend) and (B) spiked at different concentrations (see legend) to the duplicate diet homogenate. Error bars depict the standard deviations ($n = 3$).

is, however, that potential analytes, which lack corresponding isotope labeled analogues (e.g. PFBS, PFTrDA and PFTeDA), could only be included qualitatively in the method. Since $^{13}C_2$-PFHxA and $^{13}C_4$-PFOA showed comparable recoveries (Fig. 1), PFHpA was included in the validated method with the assumption that quantification using either of these two internal standards would lead to accurate results also for PFHpA.

Fig. 1 displays total method recoveries for the internal standards spiked at 100 pg g^{-1} to the five sample matrices investigated (Fig. 1A) and spiked at four different concentrations to the duplicate diet homogenate (Fig. 1B). Recoveries of 60–80% were observed for most of the internal standards and food matrices. Slightly lower recoveries were occasionally observed for some PFCAs. However, all calculated recoveries were consistently above 50% (Fig. 1A and 1B) and were therefore acceptable for quantitative purposes when using isotope labeled standards. In line with previous observations [1,25] it was crucial to maintain an alkaline pH (~10) and to use a freshly prepared TBA solution to achieve reproducible extraction recoveries.

The total method recoveries at different concentrations spanning the range of expected levels in food samples were measured by fortifying the duplicate diet homogenate in triplicate at 1, 10, 100 and 1000 pg g^{-1} with the isotope labeled standards prior to extraction (Fig. 1B). Comparable recoveries regardless of spike level demonstrated that total method recoveries were not concentration dependent. Chromatograms of the extracts of duplicate diet samples spiked with 10 pg g^{-1} and 1 pg g^{-1} of internal standards are shown in Figures S1 and S2, respectively, in the Supplementary Data.

3.5. *Method detection and quantification limits and linearity of instrumental response*

The linearity of the instrumental response was assessed with standard calibration curves with native analyte concentrations ranging from 0.018 to 9 pg μL^{-1} (5 μL injection volume). Excellent linearity ($R^2 > 0.99$ and residuals deviating <10% from the calculated linear trend line) was obtained for all target analytes, showing that instrumental limits of quantification below 100 fg on column could be achieved. Figures of merit for the calibration curves are displayed in Table S6 in the Supplementary Data.

Table 2 lists the calculated MDLs and MLQs for all analytes as determined from 9 replicate procedural blank extractions (see Section 2.6). The MDLs and MLQs reported here are among the lowest reported so far (compare e.g. [16,22,34]). When comparing method sensitivity it should also be noted that previous studies [15,16,34]

Table 2
Method detection limits (MDLs) and method limits of quantification (MLQs) in pg g^{-1} for PFCAs and PFSAs in the different food matrices derived from nine procedural blank extractions.

		PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFHxS	PFOS
Duplicate diet, baby food and vegetable composite (5 g sample intake)	MDL	2.4	0.9	3.3	0.9	1.5	1.3	0.5	0.3	0.8
	MLQ	5.4	2.4	6.7	2.5	3.1	3.5	1.5	0.6	2.2
Fish and meat composite (2.5 g sample intake)	MDL	4.7	1.7	6.6	1.8	2.9	2.6	1.0	0.6	1.7
	MLQ	11	4.9	13	5.1	6.2	7.0	3.0	1.2	4.5

have estimated the MDL from the instrumental signal to noise ratios. Although frequently used, the estimation of method detection limits from solvent calibration curves ignores recovery losses from sample preparation, variability in blank levels and matrix effects, and will therefore probably underestimate the true MDL. In this study, we chose a more conservative approach to define the MDL (and MLQ) that incorporates recovery losses as well as the variability in procedural blank levels [35].

3.6. Accuracy and precision

The accuracy and precision of the method were evaluated by triplicate analyses of laboratory control spikes of the duplicate diet fortified with all analytes at 20 pg g^{-1} (see also Section 2.6). The quantified mean concentrations obtained in the laboratory control spikes after subtraction of the endogenous concentrations (see Section 3.7 and Table 3) compared favorably with the theoretical spike values. The percentage agreement for each analyte was 95% (PFHxA), 111% (PFHpA), 99% (PFOA), 92% (PFNA), 102% (PFDA), 94% (PFUnDA), 89% (PFDoDA), 68% (PFHxS) and 89% (PFOS) of the theoretical value. A significant deviation from the theoretical concentration was only observed for PFHxS. This can be attributed to larger uncertainties in the calculation method due to the relatively high endogenous concentrations of 22 pg g^{-1} found in the duplicate diet sample. The precision (%RSD) of the triplicate analyses was between 4 and 15% for all analytes. Quantified mean concentrations in the ILC fish samples were 17.1 ng g^{-1} (PFOA), 17.6 ng g^{-1} (PFNA), 18.1 ng g^{-1} (PFDA), 14.0 ng g^{-1} (PFUnDA), 14.8 ng g^{-1} (PFDoDA) and 144 ng g^{-1} (PFOS). These results were all within 14% deviation from the indicative mean values reported in the ILC study [17]. Precision in this experiment was 5 to 9% for all analytes with the exception of PFOA (14%).

3.7. Analysis of food samples including inter-method comparability

Table 3 displays detected and quantified concentrations of PFCAs and PFSAs in the five test matrices. Extracted MS/MS chromatograms for all detected analytes in the baby food sample are shown in Fig. 2. Further example chromatograms are given in Figures S3 and S4 in the Supplementary Data. A total detection frequency of 73% was observed for all analytes in the five food matrices and 60% of the analyte concentrations were above the respective MLQs. The concentrations of PFOS and long-chain PFCAs measured in the fish composite were within the range of recently reported concentrations in various lean and fatty fish from the Netherlands [18,34] and marine fish samples from the Baltic Sea [36]. In the present study, PFHpA and PFOA were found in all samples close to their MLQs, confirming the ubiquitous presence of these compounds in food samples of different origins [18].

To evaluate inter-method comparability for non-fortified, low contaminated samples, a separate set of duplicate diet samples (n = 10) were analyzed using the method presented here and the results were compared to concentrations obtained by another method recently developed by Ullah et al. [26]. The method by Ullah et al. is completely independent from the present approach

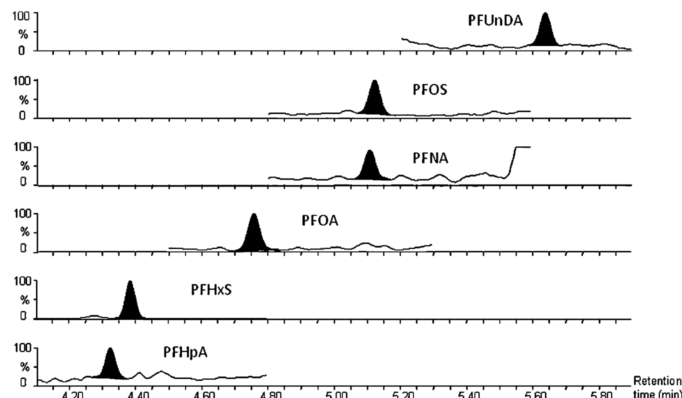

Fig. 2. Extracted MS/MS chromatograms of PFCAs and PFSAs in the baby food sample. For quantified concentrations see Table 3.

(see Section 2.6). An overview of the analytical characteristics (method recoveries, MDLs and MLQs) of the present method and the method of comparison [26] is presented in Table S7 in the Supplementary Data. Detailed results for the two methods are given in Tables S8 and S9 in the Supplementary Data. All target analytes could be detected and quantified in at least one of the samples. However, only PFOS and PFOA were repeatedly above MLQ for both methods. A comparison of concentrations of six analytes that were determined above MLQ using both methods showed an agreement of results within a factor of 2 (Fig. 3). This is the first method inter-comparison for PFCAs and PFSAs at low parts per trillion levels in food. Considering the facts that the two methods are completely independent from each other, that the MDLs are pushing the boundaries of what is possible today, and that there remain substantial challenges in producing accurate results in PFCA and PFSA analysis [17], the observed agreement between the method presented here and that of Ullah et al. [26] represents a significant step forward towards reliable quantification of PFCAs and PFSAs at ultra-trace levels.

Although good agreement was observed between these two recently developed methods, the previously reported concentrations of PFOA (70–210 pg g^{-1}) and PFOS (60–270 pg g^{-1}) in the same samples [7] could not be reproduced (see also Tables S8–S10 in the Supplementary Data). As only one sample analyzed with the present method was found to contain PFOS > 50 pg g^{-1}, and PFOA concentrations were consistently <25 pg g^{-1}, there appears to be a systematic difference between the two sets of results. Analytical characteristics of the present method and the original method [7] are compared in Table S7 in the Supplementary Data.

Both PFCAs and PFSAs are highly persistent [37] and involatile, thus are not expected to degrade or evaporate during sample storage [21]. It may be possible that the analytes have become sorbed to sample containers during storage, but we believe this is unlikely because the contact area between the sample and container walls was small compared to the total sample volume. The analytes could theoretically have become more strongly sorbed to the matrices during the long-term storage of the samples. However, the two recently developed methods (the one presented here and in [26])

Table 3
Estimated (between MDL and MLQ) and quantified (**bold > MLQ**) concentrations in tested food homogenates. All samples were analyzed in triplicate.

	Mean concentration (pg g^{-1} w.wt.) ± 1 standard deviation (n = 3)								
	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFHxS	PFOS
Duplicate diet	**11.2 ± 3.4**	**5.1 ± 2.2**	6.0 ± 0.9	<MDL	<MDL	<MDL	<MDL	**21.8 ± 7.1**	**10.8 ± 4.2**
Baby food	<MDL	**8.5 ± 2.4**	**7.6 ± 1.7**	2.3 ± 0.6	<MDL	1.4 ± 0.1	<MDL	**36.6 ± 11.4**	**8.4 ± 3.5**
Vegetable	**13.1 ± 6.7**	**8.8 ± 2.6**	**8.2 ± 1.4**	<MDL	<MDL	<MDL	<MDL	**9.2 ± 4.9**	**5.9 ± 1.6**
Fish[a]	<MDL	**14.4 ± 4.7**	**17.5 ± 2.6**	**19.8 ± 1.3**	**18.6 ± 2.0**	**52.9 ± 2.8**	**15.3 ± 1.1**	**39.3 ± 7.8**	**177 ± 18**
Meat	9.9 ± 3.0	**19.1 ± 6.1**	**14.3 ± 1.9**	**7.4 ± 3.9**	**7.9 ± 3.2**	2.6 ± 0.4	2.9 ± 0.4	**80.6 ± 23.1**	**24.6 ± 7.2**

[a] PFTrDA and PFTeDA were additionally detected in the fish homogenate at estimated concentrations of 47 ± 7 and 7 ± 2 pg g^{-1}, respectively.

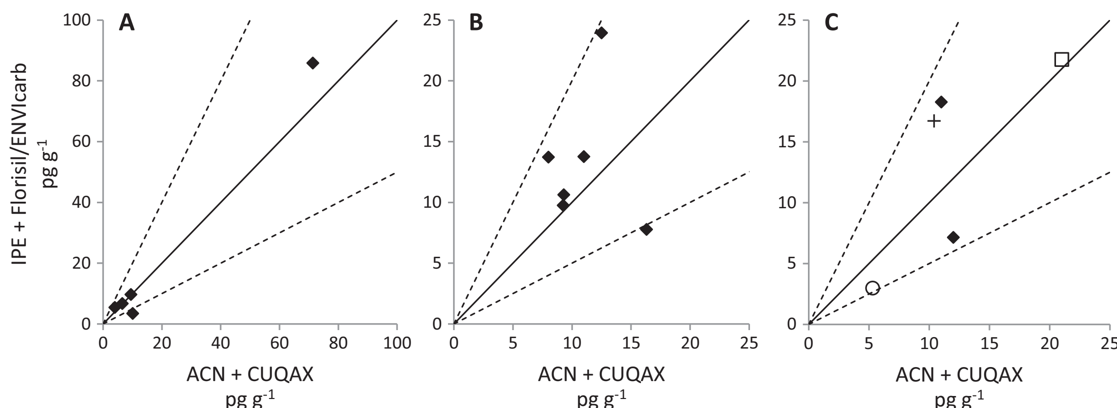

Fig. 3. Quantified concentrations of (A) PFOS, (B) PFOA and (C) PFHxA (diamonds), PFHpA (square), PFNA (cross), and PFHxS (circle) in duplicate diet samples using two independent analytical methodologies IPE + Florisil/ENVI-carb (presented in this paper) and ACN + CUQAX [26]. The solid line represents 1:1 agreement and dashed lines represent a 1:2 and 2:1 deviation between results obtained by the two methods.

use very different extraction approaches but achieved comparable results for the stored food samples, making incomplete extraction rather unlikely. The method used for subsampling of the stored food samples could also have introduced a bias in the results. Before subsampling the samples were thawed and during this thawing some phase separation may have occurred with water rising to the top of the sample containers. As the whole samples were not re-homogenized, it is thus feasible that the subsamples were not entirely homogeneous and that lower levels were present in the recently analyzed subsamples compared to the whole samples. Although we cannot entirely rule out the possible artifacts arising from sample storage and subsampling, a more plausible explanation, in our opinion, is that the modern methods have superior sensitivity and quality control, and thus the previous method has overestimated concentrations of PFOA and PFOS in these dietary samples.

4. Conclusions

A novel analytical method employing ion-pair extraction and Florisil/ENVI-carb clean-up that allows efficient and matrix effect-free extraction and enrichment of a range of PFCA and PFSA homologues from food samples has been developed and validated. Combined with state-of-the-art UPLC/MS/MS, the method achieves MDLs in the low to sub pg g^{-1} (parts per trillion) concentration range for all target analytes in a wide range of dietary matrices. Compared to previously described methods for food analysis, the method presented here represents a significant step forward with respect to:

- *Sensitivity*. MDLs are a factor of 5–100 (depending on the homologue) lower than in earlier studies [7,15] and at least equal to MDLs of recently reported methods [16,22,34]. This improvement in method performance enabled the first detection and quantification of PFNA, PFDA and PFUnDA in duplicate diet samples.
- *Reliability of results*. This is the first study to demonstrate comparable results of two independent methods for low pg g^{-1} concentrations of four PFCAs and two PFSAs in complex matrices using non-fortified samples.
- *Applicability*. The rigorous clean-up approach exploiting the amphiphilic properties of PFCAs and PFSAs makes the method applicable to a wide range of (food) matrices, irrespective of the water or fat content. Solids and liquids can be analyzed alike.

Furthermore, the method has the potential to include further (shorter- and longer-chain) homologues of PFCAs and PFSAs as well as other fluorinated surfactants. This has been shown by the successful analysis of PFTrDA and PFTeDA in the fish homogenate.

The method inter-comparison study revealed systematically lower concentrations of PFOA and PFOS in duplicate diet samples using recent methods compared to previously reported data. Although the reasons for this discrepancy could not be entirely elucidated, we hypothesize that earlier methods may have overestimated concentrations of PFOA and PFOS in dietary samples due to less rigorous quality control measures compared to state-of-the-art methods today. This leads to the further hypothesis that dietary exposure to PFOA and PFOS has been previously overestimated. However, this hypothesis needs to be confirmed by a wider monitoring of representative dietary samples using analytical techniques such as the one presented here.

Acknowledgements

We thank E.I. DuPont de Nemours & Co., Inc. (unrestricted research grant) and the European Commission Seventh Framework Project PERFOOD (KBBE-227525) for jointly funding the Ph.D. studies of R.V. and PERFOOD alone for funding the Ph.D. studies of S.U. We are also grateful to Dr. Hermann Fromme (Bavarian Health and Food Safety Authority, Munich, Germany) and Dr. Martin Schlummer (Fraunhofer IVV, Freising, Germany) for providing

the duplicate diet samples used in the method inter-comparison described in this paper. We thank Dr. Michael P. Mawn, Dr. Pim de Voogt, Dr. Martin Schlummer and the anonymous reviewers for valuable comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.03.023.

References

[1] K.J. Hansen, L.A. Clemen, M.E. Ellefson, H.O. Johnson, Environ. Sci. Technol. 35 (2001) 766.
[2] G.W. Olsen, J.M. Burris, D.J. Ehresman, J.W. Froehlich, A.M. Seacat, J.L. Butenhoff, L.R. Zobel, Environ. Health Perspect. 115 (2007) 1298.
[3] C. Lau, K. Anitole, C. Hodes, D. Lai, A. Pfahles-Hutchens, J. Seed, Toxicol. Sci. 99 (2007) 366.
[4] R. Vestergren, I.T. Cousins, D. Trudel, M. Vormuth, M. Scheringer, Chemosphere 73 (2008) 1617.
[5] R. Vestergren, I.T. Cousins, Environ. Sci. Technol. 43 (2009) 5565.
[6] D. Trudel, L. Horowitz, M. Vormuth, M. Scheringer, I.T. Cousins, K. Hungerbuhler, Risk Anal. 28 (2008) 251.
[7] H. Fromme, M. Schlummer, A. Möller, L. Gruber, G. Wolz, J. Ungewiss, S. Bohmer, W. Dekant, R. Mayer, B. Liebl, D. Twardella, Environ. Sci. Technol. 41 (2007) 7928.
[8] M. Lorber, P. Egeghy, Environ. Sci. Technol. 45 (2011) 8006.
[9] P. Egeghy, M. Lorber, J. Expo. Sci. Environ. Epidemiol. 21 (2011) 150.
[10] W. D'Hollander, P. De Voogt, W. De Coen, L. Bervoets, Rev. Environ. Contam. Toxicol. 208 (2010) 179.
[11] S.A. Tittlemier, K. Pepper, C. Seymour, J. Moisey, R. Bronson, X.L. Cao, R.W. Dabeka, J. Agric. Food Chem. 55 (2007) 3203.
[12] I. Ericson, R. Marti-Cid, M. Nadal, B. Van Bavel, G. Lindstrom, J.L. Domingo, Agric. Food Chem. 56 (2008) 1787.
[13] I. Ericson-Jogsten, G. Perello, X. Llebaria, E. Bigas, R. Marti-Cid, A. Kärrman, J.L. Domingo, Food Chem. Toxicol. 47 (2009) 1577.
[14] S.K. Ostertag, H.M. Chan, J. Moisey, R. Dabeka, S.A. Tittlemier, J. Agric. Food Chem. 57 (2009) 8534.
[15] A. Kärrman, K.H. Harada, K. Inoue, T. Takasuga, E. Ohi, A.A. Koizumi, Environ. Int. 35 (2009) 712.
[16] L.S. Haug, S. Salihovic, I. Ericson Jogsten, C. Thomsen, B. Van Bavel, G. Lindstrom, G. Becher, Chemosphere 80 (2010) 1137.
[17] S.P.J. Van Leeuwen, C.P. Swart, I. Van der Veen, J. De Boer, J. Chromatogr. A 1216 (2009) 401.
[18] C.W. Noorlander, S.P.J. Van Leeuwen, J.D. De Biesebek, M.J.B. Menglers, M.J. Zeilmaker, J. Agric. Food Chem. 59 (2011) 7496.
[19] S. Taniyasu, K. Kannan, M.K. So, A. Gulkowska, E. Sinclair, T. Okazawa, N. Yamashita, J. Chromatogr. A 1093 (2005) 89.
[20] C. Powley, S.W. George, T.W. Ryan, R.C. Buck, Anal. Chem. 77 (2005) 6353.
[21] A.S. Lloyd, V.A. Bailey, S.J. Hird, A. Routledge, D.B. Clarke, Rapid Commun. Mass Spectrom. 23 (2009) 2923.
[22] O. Lacina, P. Hradkova, J. Pulkrabova, J. Hajslova, J. Chromatogr. A 1218 (2011) 4312.
[23] M.D. Malinsky, C.D. Jacoby, W.K. Reagen, Anal. Chim. Acta 683 (2011) 248.
[24] J.W. Martin, K. Kannan, U. Berger, P. De Voogt, J. Field, J. Franklin, J.P. Giesy, T. Harner, D.C.G. Muir, B. Scott, M. Kaiser, U. Järnberg, K.C. Jones, S.A. Mabury, H. Schroeder, M. Simcik, C. Sottani, B. Van Bavel, A. Kärrman, G. Lindstrom, S. Van Leeuwen, Environ. Sci. Technol. 38 (2004) 248A.
[25] M. Ylinen, H. Hahnhijärvi, P. Peura, O. Rämö, Arch. Environ. Contam. Toxicol. 14 (1985) 713.
[26] S. Ullah, R. Vestergren, T. Alsberg, U. Berger, Presentation at the 31st International Symposium on Halogenated Persistent Pollutants - Dioxin 2011, Brussels, Belgium, 21–25 August, 2011.
[27] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J. 1 (2007) E30.
[28] N. Wang, B. Szostek, P.W. Folsom, L.M. Sulecki, V. Capka, W.R. Berti, J.T. Gannon, Environ. Sci. Technol. 39 (2005) 531.
[29] S.P.J. Van Leeuwen, A. Kärrman, B. Van Bavel, J. De Boer, G. Lindstrom, Environ. Sci. Technol. 40 (2006) 7854.
[30] B.S. Larsen, M.A. Kaiser, Anal. Chem. 79 (2007) 3966.
[31] C.R. Mallet, Z. Lu, J.R. Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.
[32] U. Berger, M. Haukås, J. Chromatogr. A 76 (2005) 210.
[33] J.P Benskin, M. Bataineh, J.W. Martin, Anal. Chem. 79 (2007) 6455.
[34] A. Ballesteros-Gómez, S. Rubio, S. Van Leeuwen, J. Chromatogr. A 1217 (2010) 5913.
[35] J. Corley, Handbook of Residue Analytical Methods for Agrochemicals, John Wiley & Sons Ltd., 2003, p. 1.
[36] U. Berger, A. Glynn, K.E. Holmström, M. Berglund, E. Halldin Ankarberg, A. Törnkvist, Chemosphere 76 (2009) 799.
[37] D.M. Lemal, J. Org. Chem. 69 (2004) 1.