Significant improvements in the analysis of perfluorinated compounds in water and fish

Results from an interlaboratory method evaluation study

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Abstract

This international method evaluation study on perfluorinated compounds (PFCs) in environmental samples was organized to assess the performance of 21 North American and European laboratories on the analysis of PFCs in water and fish. A study protocol was provided to assess accuracy, precision, matrix effects and to study the use of in-house standards. The participants used common native and mass labelled standards provided for this study to quantify the PFC levels in the samples. Matrix effects in the determination of PFCs can be considerable and can decrease the sensitivity, the accuracy and internal standard recoveries. Therefore, two quantification methods were evaluated: standard addition quantification (SAQ) and quantification against solvent based calibration curve quantification (SBCCQ; using mass labelled internal standards (IS)).

The between laboratory reproducibility (i.e. coefficient of variance) was smaller for the SBCCQ results (except for PFBS and PFHxS that had no mass labelled analogues as IS) compared to those obtained by the SAQ method. The precision of individual laboratories is good (mean for all PFCs in water 12% and 6.8% in fish). The good performance is partially attributable to the use of well-characterized common standards and mass labelled internal standards. Therefore, the SBCCQ method is recommended. However, the SAQ method is recommended when matrix effects are encountered and no mass labelled analogues are available to account for those effects.

The results show that analytical methods for PFCs in water and fish have improved considerably. Critical steps are i) the use of well defined native standards for quantification, ii) the use of mass labelled internal standards (preferably one for each target compound) and iii) minimization of matrix effects by a better clean up or matrix matched calibration curve

Definitions

Common standard: standard stock solutions originating from the same source and distributed among the participants. Every participant used these standards (instead of their in-house standards) for quantification.

In-house standards: The standards in a participant's laboratory that they use for quantification of PFCs (not being the common standards mentioned above).

Mass labeled internal standards: ^{13}C, ^{18}O or deuterated internal standards.

Native compound; native analogue: The compound that is not mass labelled.

Solvent based calibration curve quantification (SBCCQ): A calibration curve constructed in solvent only (e.g. MeOH). No matrix is involved in this type of calibration.

Standard addition quantification (SAQ): Quantification method used when detector response changes are suspected due to matrix constituents.

1. Introduction

Perfluorinated compounds (PFCs) are omnipresent in the environment [1-3]. To study the distribution of these chemicals in the environment and to assess the environmental and human exposure, many laboratories have developed methods for analysis of PFCs in environmental matrices. For several years, the quality of data obtained was a major issue of concern [4]. Identified problems in the quantification were the limited availability of high quality standards and mass labelled standards, severe matrix effects and interferences, the occurrence of branched isomers and blank problems due to contamination from labware and instrumentation. This was reflected in the 1st interlaboratory study (ILS) conducted in 2004/2005 and organized within the framework of the European Perforce project. The between laboratory coefficients of variation for environmental samples amounted to 95% for PFOS in water and 125% for PFOS in a fish sample [5]. This illustrated that improvement of method performance was required in order to obtain reliable analytical results.

Meanwhile, a large number of high quality standards has become commercially available, and the PFC list continues to expand. Furthermore, a wide range of mass labelled standards is available for use as internal standards. Earlier on, many laboratories used ion-pair extraction for biota often leading to inaccurate results. Nowadays, more diverse extraction, clean-up and quantification approaches exist [6] with good performance characteristics. Yamashita et al. reported on a method evaluation study of perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) in water (performed in the framework of an ISO technical working group) [7]. They reported on good performance (23-32% RSDs for PFOS and 27-30% RSDs for PFOA) in seawater. This showed that nowadays good performance can be achieved, although it was only demonstrated for PFOS and PFOA. The present study was initiated and aimed at evaluation of the following analytical aspects:

- Analysis of 11 perfluorinated carboxylates, 4 perfluorinated sulfonates and perfluorinated sulfonamide (PFOSA);
- Comparison of results obtained by standard addition quantification (SAQ) and solvent based calibration curve quantification (SBCCQ);
- Determination the precision of individual laboratories;
- Evaluating the influence of in-house standards on the quantification;
- Quantification of the matrix effect.

This work was performed on a fish sample and a freshwater sample.

2. Design of the study

This study was designed to evaluate the performance of current state of the art methods in terms of quantification principles, use of standards, accuracy, precision and matrix effects. The participants were invited to a workshop to stimulate exchange of expertise and receive instructions on how to conduct the experiments laid down in an exercise protocol. This is discussed in more detail below.

2.1 Starting and evaluation workshops

The start of the study was marked by a 2-days workshop (18/19 March 2007, IVM, Amsterdam) to which the participants attended. In this workshop, specialists from industries and research institutes provided their technical insights in the extraction, clean up and analysis of PFCs in water and biota samples. Based on these discussions, critical issues were determined that should be controlled in order to obtain accurate and precise analytical data. This included for example the use of well-characterized native and mass labeled standards, blank problems and matrix effects. Based on these discussions, a protocol was developed (see below) and distributed to the participants together with the sample materials. After analysis of the distributed samples and collection of the data, the results were statistically evaluated and critically assessed at another workshop (18/19 October 2007, IVM, Amsterdam). All participants contributed to a thorough technical discussion on their data. Based on these discussions, data was maintained, withdrawn (in case of non-confidence) or adopted and re-submitted.

2.2 Study protocol

A protocol was developed for discussion during the starting workshop. The aim of the protocol was to provide guidance to the participants for performing the study. The protocol included directions on how to deal with these critical aspects and directions on how to carry out the experimental work to obtain data on e.g. accuracy, precision, and matrix effects. During the starting workshop, discussions led to adaptation of the protocol and finally participants agreed at performing the experiments mentioned in Table 1.

This design enabled the determination of performance characteristics of the two quantification methods used. Solvent-based calibration curve quantification (SBCCQ) was chosen as this is a commonly applied (routine) method in most laboratories. The standard addition quantification (SAQ) method was chosen as this method is very suitable for unknown matrices (and matrix effects) as it intrinsically takes matrix effects into account. The SAQ method was derived from an FDA protocol (Guidance for Industry, Bioanalytical Method Validation, Food and Drug Administration [8]). Furthermore, the design of the study enabled the determination of factors contributing to method accuracy and precision (e.g. use of in-house standards and matrix interferences).

Table 1 Study design. In-house methods and instruments were used for all experiments.

Study aspect	Experimental execution
Evaluation of variance caused by in-house standard	Quantification of a 50 ng/mL in-house standard against the common standard (50 ng/mL). No mass labelled standards were used.
Accuracy by two different quantification methods: quantification by solvent based calibration curves (SBCCQ) and quantification by standard addition (SAQ)	SBCCQ: analysis of the sample by in-house method, but using common native and mass labelled standards for quantification. SAQ: analysis of the sample by standard addition of 1, 2 and 4 times the PFC levels already present in the sample. No mass labelled standards were used.
Precision of the analytical procedures applied	Triplicate analysis of the sample using in-house methods and by SBCCQ quantification (using common native and mass labelled standards for quantification).
Matrix interferences in the final determination by ESI-MS(/MS)	Preparation of a sample extract and fortification by 50 ng/mL of the common standard. Peak areas are compared to those of a 50 ng/mL standard. No mass labelled standards were used.

After the starting workshop, the protocol (describing above experiments in detail) was sent for comments to Dr. B. Reagen (3M Environmental Laboratory), Dr. F. Morandi (Solvay-Solexis), Dr. C. Powley (Dupont Haskell laboratories) and Dr. U. Berger (ITM, Stockholm University) for evaluation. Their remarks were taken into account in the final protocol. The study matrices concerned a fish sample and a freshwater sample. Details on (the preparation of) these samples are provided below. The PFCs included in this study are mentioned in Table 3.

2.3 Material preparation

2.3.1 Water sample

The water sample was taken in April 2007 from the North Sea Canal (which connects the Amsterdam city and harbours with the North Sea) close to the Assendelft-Spaarndam ferry. The water here is mainly freshwater, possibly with a little elevated salinity due to the inflow of seawater from the IJmuiden locks. Five 30 liter high density polyethylene (HDPE) tanks were filled with water (Figure 1) and after transport to the laboratory they were stored at 4°C. Residuals were allowed to settle and after 1 week, the water was slowly decanted in a large 150 liter tank while filtering over 3 stainless steel sieves with (top to bottom) 1.0, 0.53 and 0.22 µm pore sizes for removal of residual particles. The large tank containing ca 150 liter of water sample was maintained at 4°C under continues mixing using a stainless steel stirring device. All materials that came in contact with the water sample rinsed 3 times with ultra pure MeOH prior to use. The containers for sampling and water storage were tested for blank contributions, as will be discussed later.

Figure 1 Freshwater sample location overview and sampling of bulk freshwater sample at North Sea Canal.

The water sample was characterized (Omegam Laboratories, Amsterdam, The Netherlands) and the results (Table 2) show a typical freshwater composition.

Table 2 Characterization of the water sample.

Parameter	Value
PH	6.4
Conductivity	1529 mS/m
Calcium	160 mg/L
Magnesium	320 mg/L
Dissolved Organic Carbon (DOC)	14 mg C/L
Total Organic Carbon (TOC)	14 mg C/L
Hardness	35 meq/L

A preliminary analysis of the water sample was carried out, and based on the low PFCs levels detected, it was decided to spike the water sample with relevant PFCs mentioned in Table 3. This was done so as to facilitate the detection of the target compounds by all laboratories.

Table 3 Full names and abbreviations of PFCs covered in this study. Spiked additions of PFCs made to the samples during preparation of the water and fish sample, including the source of standards used for spiking the samples. The spike concentrations for the sulfonates are based on the anion.

Full name	Abbreviation	Water Spike (ng/L)	Supplier	Fish Spike (ng/g ww)	Supplier
Perfluorinated acids	PFCAs				
Perfluorobutanoic acid	PFBA	25	Wellington	-	-
Perfluoropentanoic acid	PFPeA	5	Wellington	-	-
Perfluorohexanoic acid	PFHxA	5	Wellington	-	-
Perfluoroheptanoic acid	PFHpA	5	Wellington	-	-
Perfluorooctanoic acid	PFOA	25	Wellington	22.6	Acros
Perfluorononanoic acid	PFNA	5	Wellington	17.2	Aldrich
Perfluorodecanoic acid	PFDA	5	Wellington	21.9	ABCR
Perfluoroundecanoic acid	PFUnA	5	Wellington	17.8	Aldrich
Perfluorododecanoic acid	PFDoA	5	Wellington	20.1	Sigma
Perfluorotridecanoic acid	PFTrA	-	-	-	-
Perfluorotetradecanoic acid	PFTeA	-	-	-	-
Perfluorinated sulfonates	PFSAs				
Perfluorobutane sulfonate	PFBS	17.7	Wellington	-	-
Perfluorohexane sulfonate	PFHxS	9.5	Wellington	-	-
Perfluoroheptane sulfonate	PFHpS	-	-	-	-
Perfluorooctane sulfonate	PFOS	23.2	Wellington	145	Fluka
Perfluorodecane sulfonate	PFDS	-	-	-	-
Other					
Perfluorooctanesulfonamide	PFOSA	5	Wellington	3.2	Wellington

2.3.2 Fish sample

The preparation of the fish sample was subcontracted to IMARES (IJmuiden, The Netherlands). The sample was prepared from fillets of flounder (*Pleuronectes Platessa*) from the North Sea (52°55’N – 03°30’E (52.916667, 3.500000)), which was purchased (ship TX43) on June 15, 2007. The fish was transported to IMARES and filleted. Approx. 25 kg of fillets were minced thoroughly homogenized in a Stephan cutter (after addition of 0.02% butylhydroxytoluene as an antioxidant) for 1 hour. Details on the preparation process of similar materials can be found elsewhere [9]. About 55 g of homogenate was packed in a glass jar tightly closed to prevent leakage. Approx. 250 lots were produced. The jars were sterilized at 121°C and 3 bar for 45 minutes. Because of the very low levels of some of the target compounds in the fish material, a selection of PFCs were spiked (from a solution in methanol, see Table 4 for concentration information) to the fish sample during the homogenization

step. The between-jar and within-jar homogeneity was tested to ensure that all jars contained a homogeneous material and to determine the variety due to the production of the material. These tests were carried out by duplicate analysis of 9 lots out of the complete batch. The compounds determined were PFOS and PFOA, using a method according to Powley et al. [10;11]. The relative standard deviations (RSD) were 5.9% and 3.6% (n=18 determinations), respectively. The data was analysed by ANOVA to assess homogeneity between the lots and within each lot using SoftCRM software (http://www.eie.gr/iopc/softcrm/index.html). The differences between the different lots were not significantly different, and therefore it was concluded that the material was homogeneous and suitable for this study.

2.3.3 Common native and mass labeled standards

At the starting workshop (IVM, Amsterdam, The Netherlands, 18-19 March, 2003) the use of standards for quantification was discussed. As it was anticipated that the use of diverse standards would significantly influence the results, it was decided to use analytical standards from the same source (so called "common standards") so as to rule out this source of analytical variance. Wellington Laboratories kindly supplied these analytical standards free of charge. Furthermore, they supplied mixtures of mass labeled standards. The standards supplied are mentioned in Table 4. These standards were used in several experiments (see Appendix 3 for details).

Table 4 Analytical standards of the PFCs and mass labeled analogues that were provided for quantification purposes. The ampoule ID appeared at the label of the ampoule.

Compound	Concentration in the ampoule (µg/mL)	Ampoule ID
PFCAs		
PFBA	2.0	NS-1*
PFPA	2.0	NS-1
PFHxA	2.0	NS-1
PFHpA	2.0	NS-1
PFOA	2.0	NS-1
PFNA	2.0	NS-1
PFDA	2.0	NS-1
PFUnA	2.0	NS-1
PFDoA	2.0	NS-1
PFTrA	2.0	NS-1
PFTeA	2.0	NS-1
PFSAs		
PFBS	2.0	NS-2
PFHxS	2.0	NS-2
PFOS	20.0	NS-2
PFDS	2.0	NS-2
Other		
PFOSA	2.0	NS-2
Mass labeled internal standards		
$^{13}C_4$-PFBA	2.0	MS-A**
$^{13}C_2$-PFHxA	2.0	MS-A
$^{13}C_4$-PFOA	2.0	MS-A
$^{13}C_5$-PFNA	2.0	MS-A
$^{13}C_2$-PFDA	2.0	MS-A
$^{13}C_2$-PFUnA	2.0	MS-A
$^{13}C_2$-PFDoA	2.0	MS-A
$^{13}C_4$-PFOS	2.0	MS-B
d_3-N-MePFOSA	2.0	MS-B

* NS= Native standard; **MS= Mass labeled standard

2.3.4 Blank tests of sampling tanks and transport bottles

Blank tests were carried out on all sample tanks in order to rule out possible contributions from the sampling, storage and shipment tanks. For the water sample, the sampling tanks (5 equal tanks of 30 liter each, 1 storage tank of 150 L and a valve, all made of HDPE) were rinsed and tumbled with 500 mL MeOH. The MeOH was concentrated to a final volume of approx. 0.7 mL and the PFCs were quantified by HPLC-ESI-MS/MS. The transportation bottles (4 different types of 0.5 L each, all

HDPE) were rinsed with 100 ml MeOH. The MeOH was subsequently concentrated and analysed. A blank of 500 mL MeOH was concentrated and analysed to account for possible contributions from the solvent and the procedure.

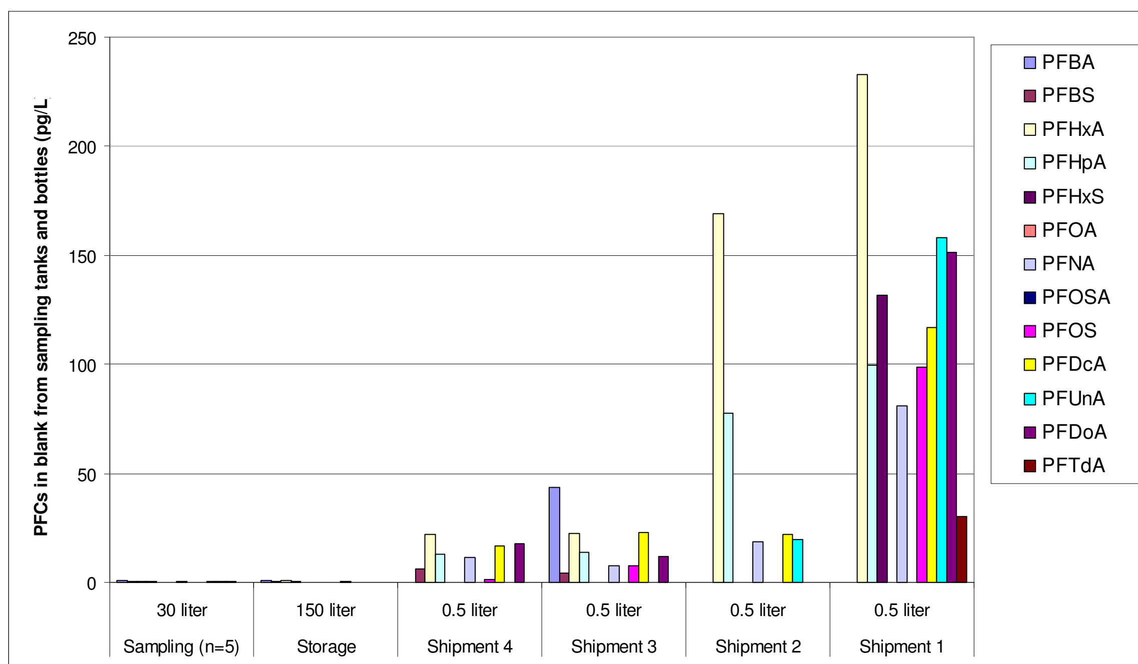

Figure 2 Overview of blanks from the water sampling tanks (30 L, mean of 5 identical tanks, all HDPE), storage tank (150 L, HDPE) and 4 different types of shipment tanks (500 mL, HDPE and PP containers). The results are reported in pg/L, meaning the absolute amounts in the blank were divided by the volume of the tank (e.g. 500 mL) to arrive at the blank contribution to the sample. Shipment tank nr 4 showed the lowest contribution and was selected for shipment of the samples.

The results of the tank blanks in Figure 2 show that PFCs were present in all sample, storage and shipment tanks. Although absolute levels in the 30 and 150 L tanks were higher than in the 0.5 L tanks, they were lower when expressed on a pg/L basis, taking into account the volume of the tanks. Shipment tank 1 showed elevated levels compared to the other types. It was found out after the experiment that the lid of that specific bottle contained an inlay lined with PTFE. Presumably, this has caused the elevated levels of some perfluorinated acids, but it the presence of the perfluorinated sulfonates cannot be explained.

For the fish sample, all equipment used for preparation of the sample were made of stainless steel and cleaned thoroughly prior to use. The transportation jars were made of glass (volume 70 mL) and a steel lid (lined with a synthetic coating). A blank test was performed on the jars and lids. Four jars (4 replicates) were filled with MeOH, closed with the lids and tumbled for 24 hours. The MeOH was concentrated and analysed by LC-ESI-Ion Trap MS(/MS). Single MS was used for detection of PFBS, PFHxS and PFOS, whereas MS/MS was used for detection of the PFCAs and PFOSA. The

MeOH concentrates appeared slightly clouded. The results showed no presence of PFCs was found in the jars and lids (<2.5 ng absolute per jar and lid).

2.3.5 Shipment of materials

The materials for the study provided to the participants included: the protocol; the water sample (11 bottles of 500 mL); the fish sample (4 jars of approx. 55 g each); ampoules of native and mass labeled standards (NS-1, NS-2, MS-A and MS-B) and report forms. The laboratory analysis took place over summer 2007.

2.4 Methods used by the participants

In this study, a variety of methods were used by the participants. For the water study, most laboratories concentrated the sample by solid phase extraction (SPE) using Oasis HLB, Oasis WAX or C18 columns. Liquid-liquid extraction with methyl-*tert*-butyl ether (MTBE) was applied by 3 laboratories (see Table 5). Each technique has a specific working area in terms of target analytes [6]. For example, PFBA cannot be extracted from water efficiently by C18 and LLE, but may be extracted using Oasis WAX and HLB. SPE methods limit to the dissolved fraction of a water sample, whereas the LLE method allows extracting the dissolved and particle-associated fraction of a water sample. It should be noted that in this study, the particles >0.22 µm have been removed from the sample in the production phase. Clean-up of SPE cartridges consists of a simple wash step after loading the sample on the cartridge. The laboratories applying LLE have not used any clean-up step.

For extraction of biota, many laboratories adopted the liquid-solid extraction (LSE) method by Powley et al. [10;11] using a medium polar solvent (MeOH or AcN), whereas in the 1st ILS, most laboratories used the ion pair extraction (IPE), initially published by Hansen et al. [12]. The latter method was used by 1 laboratory only. Clean-up of fish extracts is often performed by suspending with Envicarb (or active) and glacial acetic acid. Other clean-up methodologies used include freezing out matrix constituents, clean-up of the fish extract by SPE (Oasis HLB) and centrifugation or filtration for removal of solids.

The analysis is often performed by LC-ESI-MS/MS using a triple quadrupole MS systems. Other systems include LC-ESI-(single quadruple)MS, LC-ESI-time of flight (TOF)MS/MS, LC-ESI-ion trap (IT)MS(/MS) and flow injection analysis (FIA)-ESI-MS/MS. In the latter case, the sample is injected ‘at once’ without chromatographic separation.

Table 5 Analytical methods used by participants in this study.

	Fish		Water		Instrumental determination
Lab	Extraction	Clean-up	Extraction (SPE)	Clean-up	
1	IPE (TBA*)	Filtration (Nylon)	C_{18}	Wash: water	LC-ESI-QQQMS
2	-	-	Oasis-HLB	No clean-up	LC-ESI-QQQMS
3	LSE (acetonitrile)	Envicarb	-	-	LC-ESI-QQQMS
4	LSE (methanol)	Active carbon	C_{18}	No clean-up	LC-ESI-QQQMS
5	LSE (methanol)	SPE (washing unkn, methanol-type elution)	SPE (type confidential)	Confidential	LC-ESI-QQQMS
6	-	-	Oasis-HLB	Wash: water	LC-ESI-QQQMS
8	LSE (acetonitrile)	Envicarb	Oasis-WAX	Wash: water	LC-ESI-QQQMS
9	LSE (acetonitrile), freezing out, centrifugation, decant. in 2% H_3PO_4, SPE (Oasis-HLB, wash with 5% methanol), elution with methanol (+5% NH_4OH)	SPE (see left)	C_{18}	No clean-up	LC-ESI-QQQMS
10	LSE (acetonitrile)	Envicarb	-	-	FIA**-ESI-QQQMS
11	LSE (acetonitrile)	Envicarb	Oasis HLB	Wash: methanol:H_2O (60:40)	LC-ESI-QTOFMS
12	LSE (methanol)	Reconstitution in H_2O	Styrene-dyvinylbenzene SPE, use of IPA***	Wash: methanol:H_2O (20:80)	LC-ESI-QMS
13	LSE (acetonitrile)	Envicarb	-	-	LC-ESI-ITMS(MS)
14	LSE (acetonitrile)	Envicarb	Oasis HLB	Wash: methanol:H_2O (40:60)	LC-ESI-QQQMS
15	LSE (acetonitrile)	Envicarb	Oasis HLB	Wash: acetonitrile:H_2O (40:60)	LC-ESI-QQQMS
17	LSE (acetonitrile)	Envicarb	LLE (MTBE****)	No clean-up	LC-ESI-QQQMS
19	LSE (acetonitrile)	Envicarb, filtration 0.2 µm	Oasis HLB	Wash: acetate buffer	LC-ESI-QQQMS
20	-	-	n.a.*****	n.a.	LC-ESI-QQQMS
21	LSE (methanol)	Freezing, centrifugation	SPE (Chromabond HR-P)	Wash: water	LC-ESI-QQQMS
22	-	-	Oasis-HLB	No clean-up	LC-ESI-QQQMS
23	-	-	LLE (MTBE)	No clean-up	LC-ESI-QQQMS
24	-	-	LLE (MTBE)	No clean-up	LC-ESI-QQQMS
	n=15		n=17		

* TBA tetrabutylammonium hydrogen sulfate
** FIA flow Injection Analysis
*** IPA ion pairing agent
****MTBE methyl-*tert*-butyl ether
***** Not applicable, because 1 mL water was directly injected (large volume injection with preconcentration on the analytical column)

2.5 Collection of data and statistical evaluation

The data was collected using report forms specially designed for this study. Participants were asked to provide details on their extraction and clean-up methods, the chromatographic and mass spectrometric conditions and the results of the individual experiments.

3. Results and discussion

After submission by the laboratories, the results were collected and basic statistical analysis was performed. At the evaluation meeting, the submitted results of individual laboratories were evaluated with specific criteria in mind. These criteria included (for the SBCCQ) the linearity and range of the calibration curve and whether or not the sample was within the calibration curve and for the SAQ method criteria for the spiking levels and the linearity of the standard addition curve were applied. A similar approach was followed for assessing precision and matrix effects. As a result, some submitted data was removed from the dataset because of lacking confidence. This resulted in a revised dataset, on which below discussion is based.

3.1 In-house standards vs. common standards

The in-house standards from a variety of (commercial) suppliers were tested against the common standards as supplied by Wellington Laboratories. The participants analysed their in house standard (approx. 50 ng/mL) and the common standard (dilution, approx. 50 ng/mL). No mass labeled standards were used (so variety of the ESI-MS(/MS) may have slightly influenced the results). On the other hand, the standards were analysed on the same day under repeatability conditions. The common standards consisted of linear isomer only (for each compound), whereas many standards from other suppliers contain both linear and branched isomers.

The average comparability (see Figure 1) was in the range of 95-105%. However, large differences were found for individual observations and laboratories. The Acros, Dr. Ehrenstorfer and Fluorochem standards were on the low side (72-85%), whereas several suppliers were on the high side (average, up to 116%). Some observations are listed below see Appendix 4 for details):

- Large variety was found in results from individual laboratories going from 46% (PFOS, lab 12) to 185% (PFUnA, lab 24). The low PFOS value (a standard from Fluka) was confirmed by Laboratories 19 and 24, but not by 11, 13, 14 and 17). The influence of different batches of standards may play a role here (but was not investigated). The high value for PFUnA (Sigma) was not confirmed by the other laboratories using Sigma standards (5 and 23, both 102%);
- Oakwood standards (lab 8/9) slightly over estimated the common standards (99-111%, except PFTeA for lab 9). The same holds for the Lancaster standards (lab 8, 95-118%). It should be noted that only a small number of observations of standards originating from these suppliers were available;
- Interchim PFHxS standards were used by 4 laboratories and resulted in values of 92-98%, being close to 100%;
- Wellington Laboratories standards ranged from 75-117%. The exception to this was PFDoA (66/143% for lab 3/4) and 62% for PFOA and 68% PFOS, both lab 10. It is somewhat surprising that some of these observations showed large deviation from the common standards as both originated from the same supplier. It is not known if different lots of standards were involved that may explain the variance, but it is clear that this may reflect the variance in instrument calibration between laboratories (when not using mass labelled standards).

It should be noted that experimental factors may have contributed to the variance (e.g. ESI variability, dilution errors, different response of branched and linear isomers), and these should not be neglected when interpreting these results. Nevertheless, it can be concluded that the use of standards from different (commercial) suppliers can have a considerable impact on instrument calibration and therefore the quantification of PFC levels in samples.

For reasons of comparability, it is very useful to agree on the use of well defined (common) standards (in this study >99% linear isomers), as this will allow easier exchange and interpretation of results generated by different laboratories. However, isomer profiles may be different in environmental samples and it may be desirable to match the profile observed in the sample with a similar standard. Therefore, the use of linear only or isomeric profile standards should be judged case by case.

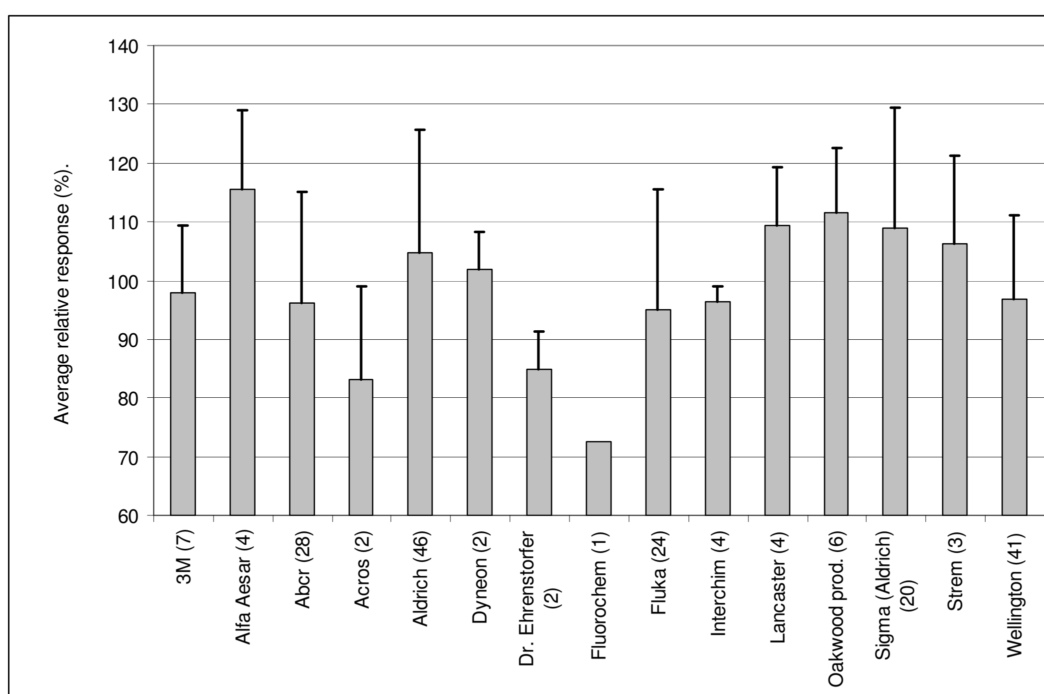

Figure 1 *The variability of commercially available in-house PFC standards. Variability was assessed by analysis of a 50 ng/mL in-house and common standard and comparison of peak areas, resulting in a relative response. No mass labelled internal standards were used for correction. In above graph, all observations for all PFCs (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFBS, PFHxS, PFOS, PFDS, PFOSA) were grouped per supplier (neglecting different lot-numbers, purities and isomeric composition). The number of observations per supplier are given in between brackets. The authors do by no means recommend products from specific suppliers indicated in the graph.*

3.2 Accuracy evaluation: SAQ versus SBCCQ

Participants were asked to quantify the PFC levels in the samples by 2 methods: SBCCQ and SAQ. The first method is common practice (routine) in many laboratories, whereas the latter method is less often applied. The SBCCQ is a simpler approach than the SAQ, but matrix effects may occur. The use of a mass labeled analogue for each target compound allows accounting for these effects (assuming that the mass labeled analogue behaves similarly as the native analogue does). The SAQ method is especially suitable to quantify levels in samples with unknown matrix effects as it accounts for these effects. The SAQ also accounts for possible losses that may occur at several stages of analysis (extraction, clean-up, electrospray ionization).

The SAQ method used in this study was derived from US-FDA guidelines [8]. Participants were asked to analyse the samples after spiking the target compounds at 0, 1, 2 and 4 times the concentrations already present in the sample (the 0-level). Mass labeled standards were not used in this case. For the solvent calibration method, the participants were asked to spike the mass labeled standards prior to analysis (or, in the case of the water sample, directly after receipt of the samples) and to equilibrate the spike in the sample overnight. Details on the execution of these experiments can be found in Appendix 3.

The data shows that the SAQ results are always more variable than those obtained by the SBCCQ method (Figure 2 and Table 1 and Table 2). This is concluded from the higher ranges for nearly all compounds (this is most pronounced in the water sample). Exceptions are PFBS, PFHxS and PFOSA in water. For PFBS and PFHxS, no mass labelled internal standards were available and the results of these have been corrected using the mass labelled PFOS internal standard. It is clear that in these cases the SAQ method provides a better between-laboratory reproducibility than the SBCCQ method. For PFOSA, the D3-N-MeFOSA internal standard is not suitable, as will be discussed below. Furthermore, in case of the SAQ results, the average is often higher than the median value, indicating a non-normal distribution of the dataset (which in turn is an indication for poorer between laboratory comparison). Finally, the average SAQ results are (and often also the median) higher than the SBCCQ results (except PFOSA and PFHxS in water and PFDoA in fish). For PFOS in fish, the SAQ result was 33% higher, whereas for other results, this was less pronounced. Although this suggests an overestimation by the SAQ method, it should be noted that the SBCCQ method was in some cases (PFUnA, PFDoA and PFOSA in water and PFOA, PFUnA, PFDoA) not able to recover the spiked amount. Underlying reasons for the overestimation and the larger variance in the SAQ dataset could be:

- The intrinsic uncertainty of the SAQ method contributes to the variance of the SAQ dataset. This uncertainty is due to the fact that the concentration is derived by extrapolation of a regression curve beyond the actual quantified range. This can partly be solved by performing replicate spikes at each level, which then narrows down the 95% confidence interval of the standard addition curve. However, in this study, no replicate spikings were performed;
- R^2 values of the resulting SAQ addition curves of individual laboratory datasets was often far below $R^2 > 0.99$, whereas this value was met in most cases with the SBCCQ (due to the use of mass labeled standards for many compounds);

- The SAQ spiking levels did in several cases not match the 1, 2 and 4x level as mentioned in the protocol. At the evaluation meeting, these protocol deviations were assessed and extreme deviations have been removed from the dataset;
- SAQ is not employed on a routine basis in laboratories, resulting in a higher chance of errors being made in performing the analytical work and interpretation and calculation of results.

As compared to the 1st ww ILS, the performance of the group has improved considerably. RSD values in this study are 16-69% and 22-47% for water and fish (SBCCQ), whereas they were 47-250% and 65-235%, respectively in the 1st ww ILS (excluding values close to the LOQ) [5]. The underlying reasons for this is the improved knowledge on the behaviour of PFCs (and therefore better design and control of the methods) and the use of well defined (mass labeled) standards, as discussed below.

D3-N-MeFOSA was used as the internal standard for PFOSA, but turned out not to be suitable. Several laboratories have observed considerable losses of this internal standard, and therefore, some laboratories decided not to report the PFOSA value (e.g lab 19), whereas others decided to use ^{13}C-PFOS is internal standard. The uncertainty is also reflected in the higher RSD values compared to other compounds that were corrected by a ^{13}C labeled analogue. It was hypothesized that d3-N-MeFOSA degrades when in contact with water, or that losses due to low solubility may occur. An additional experiment showed that losses of >80% are observed over a 46 hours period when spiked to ultrapure water. After 46 hours, the remaining water was replaced by methanol. After vigorous homogenization, the d3-N-MeFOSA was partially recovered again, showing that adsorption to the LC-vial surface caused the concentration decrease. This shows that d3-N-MeFOSA is not a suitable internal standard for correction of results of PFOSA.

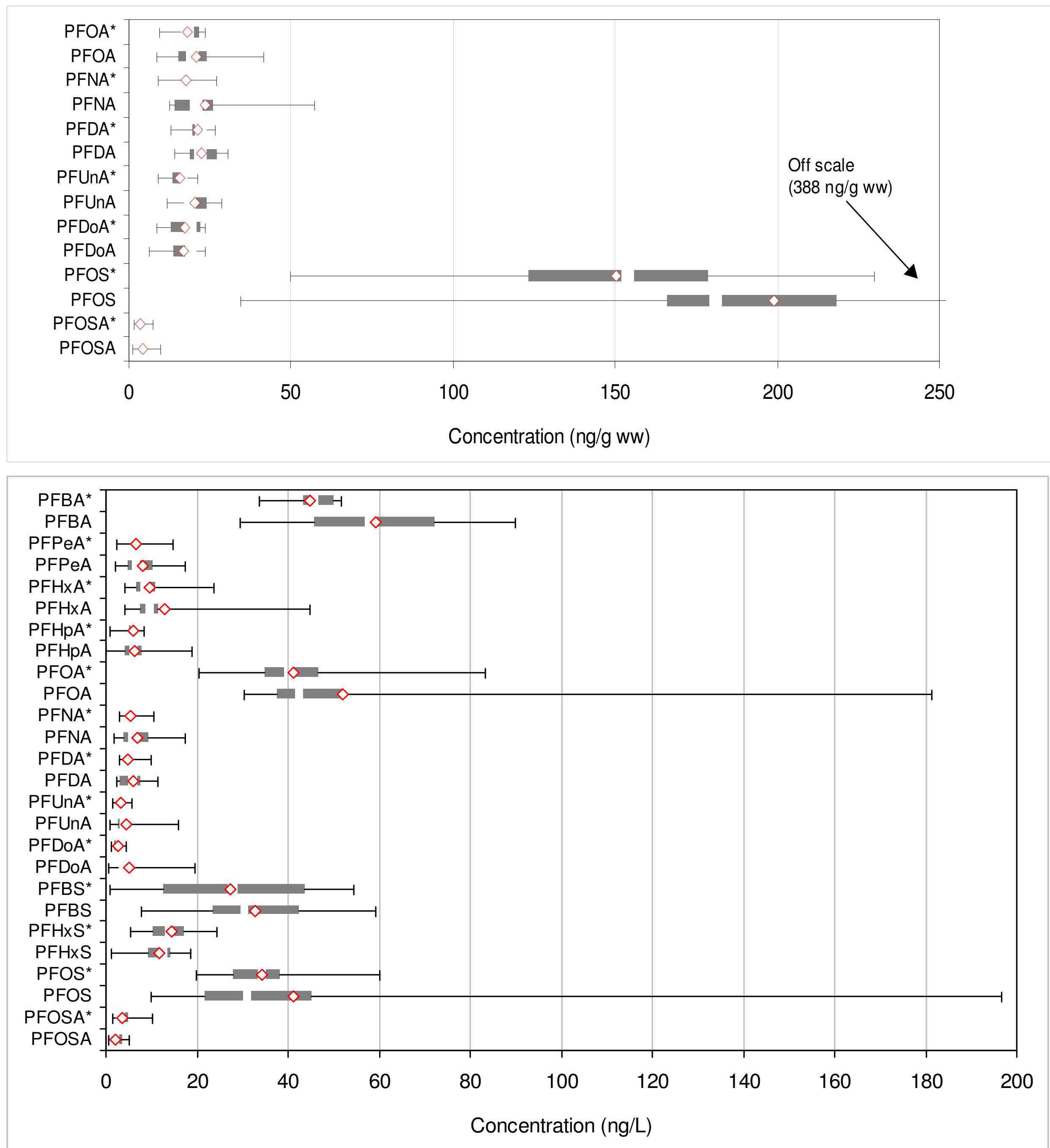

*Figure 2 Data distribution for the SBCCQ data (marked by *) and the SAQ data. The top figure shows the data for the fish sample and the bottom figure shows the data for the water sample. The number of data points is indicated in Table 3 and 4. Bars represent lower quartile, median (blank interruption of bar) and upper quartile. The diamond represents the average of the dataset.*

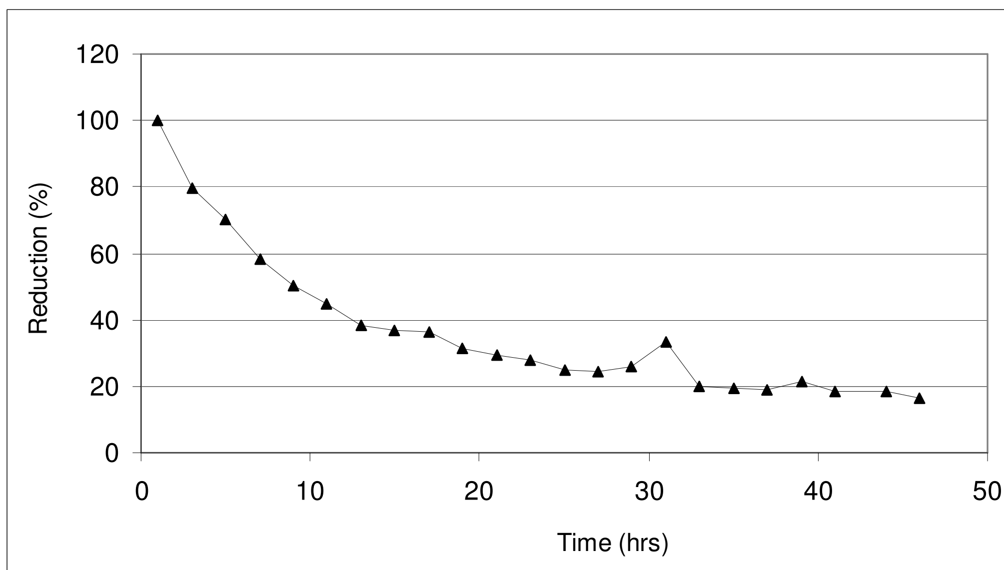

Figure 3 Losses of D3-N-MeFOSA in water over time due to low water solubility. ^{13}C-PFOS was used as internal standard.

Table 1 Water sample: summary statistics of SBCCQ and SAQ quantification methods.

	Spike	SBCCQ							SAQ						
		Average	STDEV	RSD*	MIN	MAX	N **		Average	STDEV	RSD	MIN	MAX	N **	
	(ng/L)	(ng/L)	(ng/L)	%	(ng/L)	(ng/L)	>LOQ	<LOQ	(ng/L)	(ng/L)	%	(ng/L)	>LOQ	>LOQ	<LOQ
PFBA	25	44.8	7.0	16	33.7	51.6	5		59.1	23.3	39	29.6	89.9	5	
PFPeA	5	6.8	3.7	55	2.3	14.7	9	1	8.0	4.7	59	2.0	17.3	10	1
PFHxA	5	9.5	4.8	50	4.2	23.8	13		13.5	11.3	83	4.1	44.7	13	
PFHpA	5	5.9	2.0	34	0.8	8.4	12		6.5	5.0	77	0.1	19.1	12	
PFOA	25	41.1	13.1	32	20.5	83.2	18		52.9	35.1	66	30.3	181.4	18	
PFNA	5	5.3	2.0	37	2.9	10.4	15	1	7.1	4.4	63	1.9	17.4	14	
PFDA	5	4.8	1.8	38	2.9	9.9	13	2	6.1	3.1	52	2.3	11.3	13	
PFUnA	5	3.3	1.3	39	1.5	5.7	12	1	4.6	4.1	87	0.9	15.9	11	1
PFDoA	5	2.8	1.2	44	1.2	4.5	10	1	5.0	5.7	114	0.7	19.6	9	2
PFTrA	-							2	10.3			0.6	19.9	2	4
PFTeA	-														6
PFBS	17.7	27.5	18.2	66	0.9	54.4	12		33.7	14.7	43	7.8	59.3	11	
PFHxS	9.5	14.4	5.5	38	5.5	24.3	14		11.4	4.5	40	1.2	18.6	13	
PFOS	23.2	34.4	9.9	29	19.9	60.3	18		42.3	43.1	102	10.0	196.8	18	
PFDS	-	0.5			0.2	0.7	2	3	2.0			0.5	3.5	2	5
PFOSA	5	3.5	2.4	69	1.6	10.1	11	2	2.2	1.6	72	0.5	5.2	11	

* Underlined: the compounds for which no mass labeled internal standard was available in this study.

** N number of observations submitted, and if these were reported above or below the LOQ

Table 2 Fish sample: summary statistics of SBCCQ and SAQ quantification methods.

		SBCCQ							SAQ						
	Spike (ng/g)	Average (ng/g)	STDEV (ng/g)	RSD %	MIN (ng/g)	MAX (ng/g)	N* >LOQ	N* <LOQ	Average (ng/g)	STDEV (ng/g)	RSD %	MIN (ng/g)	MAX (ng/g)	N* >LOQ	N* <LOQ
PFBA	Na							2	0.0			0.0	0.0	1	
PFPeA	Na	0.1			0.1	0.1	1	2	0.1			0.1	0.1	1	1
PFHxA	Na	0.0			0.0	0.0	1	2	2.1			0.1	4.1	2	1
PFHpA	Na	0.1			0.0	0.2	3	3	1.7			0.1	3.3	2	3
PFOA	22.6	18.0	4.1	23	9.2	23.6	14		21.5	8.3	39	8.6	41.5	13	
PFNA	17.2	17.5	4.6	26	8.9	27.3	12		23.9	13.2	55	12.6	57.2	12	
PFDA	21.9	21.1	4.6	22	12.9	26.7	12		22.9	5.3	23	14.2	30.6	12	
PFUnA	17.8	15.9	4.1	26	9.0	21.0	11		20.2	5.3	26	11.9	28.6	11	
PFDoA	20.1	17.3	5.2	30	8.5	23.6	11		16.8	5.2	31	6.2	23.4	11	
PFTrA	Na	30.6			0.2	60.9	2	3	7.9			0.1	23.2	3	1
PFTeA	Na							3	1.0			0.1	1.9	2	
PFBS	Na	0.0			0.0	0.0	1	3	3.5			3.5	3.5	1	1
PFHxS	Na	0.0			0.0	0.1	2	3	1.6			0.1	4.6	3	
PFOS	145	150	44.0	29	49.9	230	14		200	93.1	47	34.5	388	11	
PFDS	Na	2.9			2.9	2.9	1	3	1.4			0.0	2.8	2	3
PFOSA	3.2	3.6	1.7	47	1.5	7.5	10		4.3	2.5	57	1.2	9.8	9	1

*N number of observations submitted, and if these were reported above or below the LOQ.

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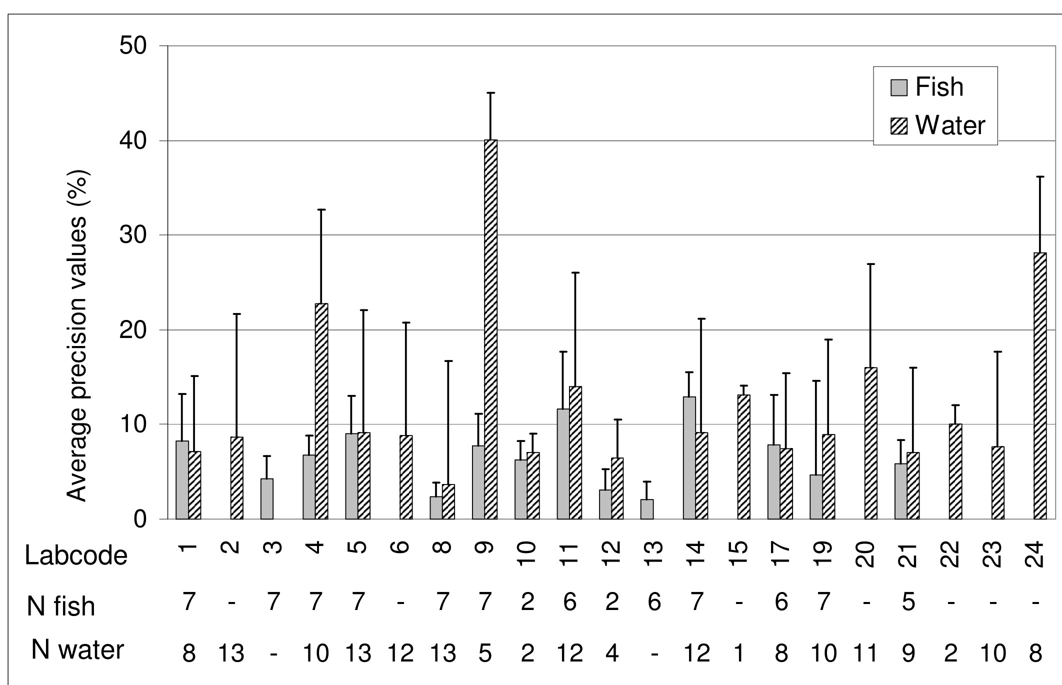
3.3 Precision evaluation

The study included an evaluation of the precision of individual laboratories. The laboratories were asked to perform the SBCCQ in triplicate ('experiment 2' and 'experiment 5'). Details on individual laboratories can be found in Appendix 4.

The precision of individual laboratories is good. The precision criterium in the FDA guideline is 15% for at least 5 replicates [8]. When taking the average precision (average of all compounds) values per lab into account, most laboratories meet this criterium for the water sample (except Lab 4, 9, 20 and 24 which had higher values) and for the fish sample all laboratories meet this criterium (see Figure 4 top). Limited precision is either caused by very low concentrations (e.g. PFTeA in water and PFPeA and PFTrA in fish – see Figure 4 bottom) or, in the case of PFOSA, a poor performance of the d3-N-MeFOSA (as explained earlier). The data in Figure 4 (bottom) shows that precision data for the fish sample was better (<7.5% for PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS) than for the water sample, suggesting that the analysis of fish is better controlled than the water analysis. The most likely explanation for the overall good precision data is the use of a broad range of mass labeled internal standards.



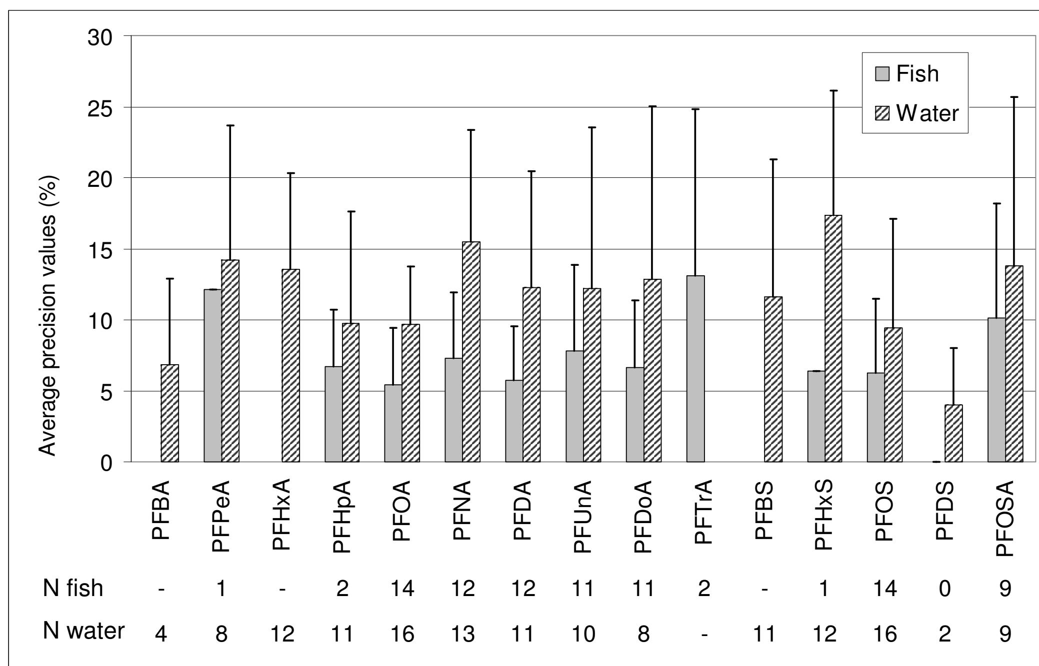


Figure 4 Average precision (as RSD, or %) of the different laboratories for water and fish (solvent based calibration). Top plot: the average of all precision values (of all PFCs) reported by a single laboratory for the PFCs they had analysed. Bottom plot: the average of each PFC of all precision values reported by all laboratories for a specific PFC. Error bars indicate the standard deviation of the average. N indicates the number of submitted precision data values).

3.4 Matrix effect

Ion suppression or enhancement in the electrospray ("matrix effects") were tested in order to determine to what extent clean-up strategies remove potential interferences from a sample extract (experiment 4 for fish and experiment 7 for water). For the water sample, no specific clean-up was applied. Most laboratories concentrated the sample by SPE and after sample loading a simple wash step (water, water/MeoH or water/AcN mixture) was applied for removal of salts and other interferences. For the fish sample, the majority of the laboratories applied an Envirocarb clean-up step as originally published by Powley et al. [10;11]. Other methods included freezing-out matrix components (e.g. lipids) and reconstitution of the extract in water and subsequent concentration and clean-up by SPE (Oasis HLB). For method information, see Table 5. The experiments were carried out by spiking a sample extract (just before injection: "pre-injection") with (in most cases) 50 ng/mL PFCs. The resulting response was compared to the response of a 50 ng/mL standard solution (after correction for the response already present in the sample without the pre-injection spike). The average matrix effect per PFC and matrix is plotted in Figure 5. Data of individual laboratories can be found in Appendix 4.

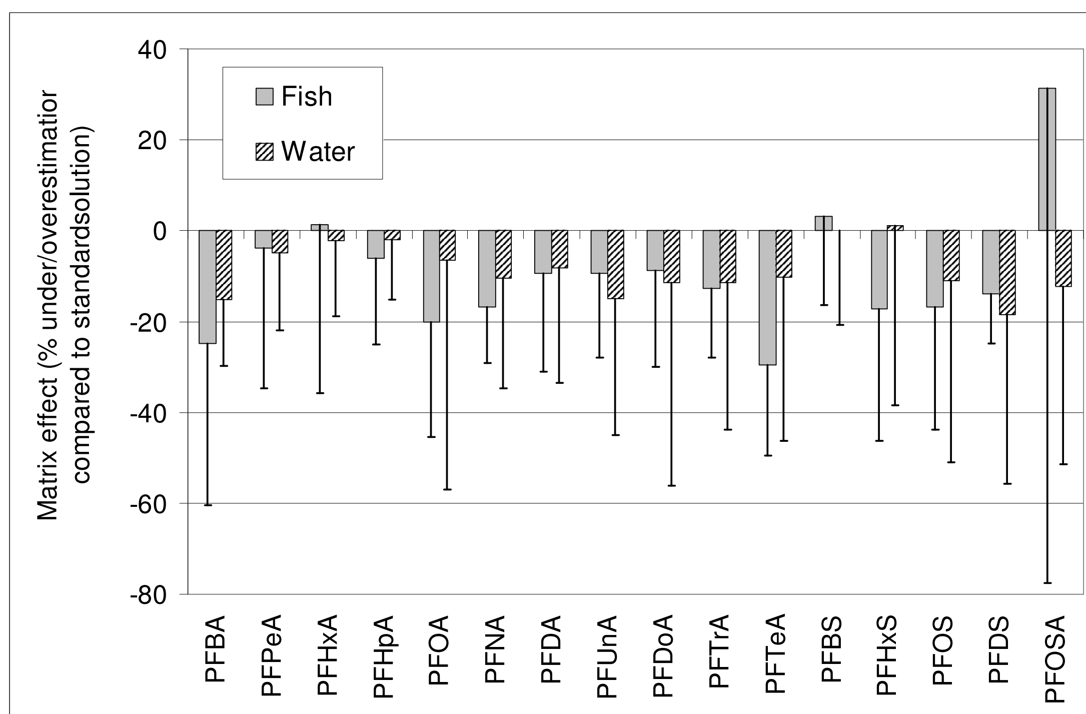


Figure 5 LC-ESI-MS(/MS) matrix effects determined by comparing the response of an extract spiked prior to instrumental analysis and comparison of response with a standard. Y-axis indicates the deviation from the standard solution response (%). Average values (of all laboratories for a specific PFC) are plotted together with standard deviation. One laboratory used flow injection analysis instead of LC (Table 2), but was maintained in above dataset .No mass labelled standards were used.

On average, predominantly matrix suppression was found (negative values). However, enhancement was also observed by several laboratories on individual compounds (as indicated by the positive values and the error bars). For the fish sample, on average a matrix effect of +31% (PFOSA) to -30% (PFTeA) was found. Individual lab data showed larger variations from (suppression) -83% (lab 10, PFOS) to (enhancement) +330% (Lab 1, PFOSA). The latter value explains the high average PFOSA value mentioned before. Lab 10 used flow injection analysis (instead of HPLC), and therefore the complete extract is injected at once without chromatographic separation. They found a suppression of approx. -80%. For the water sample, the matrix effect was on average –18% to +1%, but in this matrix extreme values were also observed (-99%, Lab 15 for PFOS and +142%, lab 24 for PFOA). In the water sample, some laboratories (5, 6 and 14) found a suppression for the PFBA to PFHpA (decreasing with increasing chain length). Possibly, co-extracted and early eluting organic acid complexes (e.g. humic acid) have suppressed their responses, but this was not confirmed by other laboratories.

It should be noted that the results here depend on the design and execution of the experiment. For example, the matrix effect as it is defined here is PFC concentration dependant. The amount spiked prior to injection was in most cases 50 ng/ml per PFC, which is relatively high compared to concentrations often encountered in the environment (e.g. seawater). The matrix effect becomes more pronounced when lower concentrations are spiked (e.g. 5 ng/mL). Therefore, in low contaminated envi-

ronmental samples, matrix effects are likely to be larger. In principle, mass labeled analogues correct for these effects as it is assumed that they behave similarly as the native compounds, and therefore enable an accurate determination of their native analogues. However, for PFHpA, PFTrA, PFHxS, PFBS and PFOSA no ^{13}C analogues were available (at the time of the study) and therefore no correction for matrix effects could be made. For PFOSA, we used d3-N-MeFOSA as the mass labeled analogue. Because this compound elutes at a different retention time than PFOSA, this compound can in principle not correct accurately for matrix effects occurring in the electrospray at the time PFOSA elutes.

4. Conclusions

This study has shown that the accurate and precise analysis of PFCs in freshwater and fish samples is feasible if several critical steps in the analysis are addressed appropriately. Identified critical steps are i) the use of well defined native standards, ii) the use of mass labeled internal standards (preferably one for each target compound) and iii) minimization of matrix effects (e.g. by clean-up of sample extracts). Apart from these, it's important to control and minimize the background contamination (blanks). Standard addition quantification is a useful technique for analysis of matrices with unknown matrix effects or for compounds for which no mass labeled standard is available. Solvent based calibration curve quantification combined with mass labeled standards facilitating the analysis of PFCs and is very suitable for analysis under routine conditions. Standards from different (commercial) suppliers can have a considerable influence on instrument calibration (and therefore quantificantion). For reasons of comparability among laboratories, it is advisable to agree beforehand on the use of a selection of common native, and internal standards.

5. Recommendations

- *Lower concentration ranges* - The samples in this study were spiked with relevant perfluorinated carboxylates (PFCAs), perfluorinated sulfonates (PFSAs) and PFOSA at levels that facilitated detection. In the environment, however, lower concentration levels are often encountered (e.g. marine water and fish, sub-ng/g and sub-ng/L levels). In this study, we've seen that performance (e.g. precision) decreased at lower concentrations close to the LOQ. It will therefore be challenging to maintain the same level of performance at these environmentally relevant low concentrations. It is therefore recommended to conduct such interlaboratory study;
- *Other matrices* – This and other studies focused so far on water and fish sample matrices. However, several other matrices are important from environmental fate or human exposure point of view (e.g. food, air, and sewage treatment plant (STP) samples). Within the framework of the EU Perforce project, an intercomparison on STP samples showed large variety between results of the 2 to 3 participating laboratories. This is due to the complexity of the matrix and the presence of a wide range of potential interfering compounds. Such matrices call for method optimization and comparison studies, including workshops for discussing the pitfalls on the analysis of STP matrices;
- *Other compounds* – So far, PFCAs, PFSAs and PFOSA were the study compounds. Other challenging compounds are the fluorotelomer alcohols (FTOHs), telomer acids and telomer sulfonates;

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Acknowledgements

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Appendix I. Invitation letter for the method evaluation study

Announcement third international interlaboratory study on perfluorinated compounds – method evaluation for water and fish

10 January 2007

Dear colleague,

We are pleased to announce the 3rd International Interlaboratory Study on perfluorinated compounds (PFCs). This ILS will focus on the matrices surface water and fish tissue. The 1st PFC ILS showed that intercomparability of analytical results for environmental matrices was relatively poor for fish and water[1], showing the need for further development of the analytical methodologies. Considerable analytical developments have taken place since then. Recently published studies showed that methods are now available for accurate measurement of a wide range of PFCs in water and biota. It's the right time now to test these methods groupwise by averages of an ILS. This ILS is designed to obtain valuable information on method performance should result in guidelines for methods that deliver accurate analytical data for regulatory, routine and/or research purposes. This study will be carried out according to international standards on method performance studies (ie. ISO-5725). To obtain maximum benefit from the analytical progress in the field, a technical analytical workshop is organised prior to the actual study.

Workshop

Participants are asked to attend a workshop which aims at discussion of the ins and outs of PFC analysis in water and fish. We will aim at an interactive workshop where experts in the field (from industries and academia) share their insights with you. Participants are also invited to bring in their experiences with method development. Pro's and con's of current protocols will be discussed and the requirements for accurate measurements.

The workshop will be organised at the Institute for Environmental Studies, Vrije Universiteit, Amsterdam, The Netherlands. The date is set at 19-20 March 2007. The program of the workshop is under construction, but will be available soon.

Study design

Participants are asked to perform replicate analyses of the samples, to carry out a recovery experiment and to perform a blank test. Furthermore, the participants are asked to provide detailed information, such as extraction and clean-up conditions and chromatograms. This study will target at PFBS, PFHxS, PFOS, PFDS, PFBA, PFHxA, PFOA, PFNA, PFDA, PFUnA, PFDoA and other PFCs may be added to the list during the workshop discussions.

Matrices

- *Water sample*. A surface water sample will be provided;
- *Fish tissue sample.* A fish tissue will be prepared from naturally contaminated fish fillets. The fillets will be packed in glass jars, which will be sterilised. The homogeneity of the material will be determined;
- *Study standard.* A study standard with a selection of PFCs in undisclosed concentrations will be provided for evaluating the instrument calibration.

Planning

19-20 March	Workshop participants to discuss methods for accurate analysis and analytes
March	Dispatch of test materials to participants
March to May	Laboratory measurements
15 June	Deadline submission test results
Summer	Evaluation (statistical) of results / preparation of summary report
September	Workshop participants to discuss and evaluate results
31 October	Report of method evaluation

The results will be discussed in detail at a workshop organised in conjunction with Dioxin 2007, Tokyo, Japan. Based on the results and discussions at this workshop, a final method performance evaluation report will be produced. We will aim on producing guidelines for analysis. The guidelines and results will be disseminated and will be available for the scientific community.

Participation

The participation in this study is free of charge thanks to a generous sponsoring of Plastics Europe. Furthermore, costs can be kept low due to kind support of Wellington Laboratories who will provide standards.

The travel and subsistence costs for the workshop are on the participant´s own account. There will be no fee for attending the workshop. The number of participants for this study is limited to 25-30 and the registration may be closed after the maximum has been reached. Please provide your contact and address details in the provided Excel sheet and return this sheet to

---

[1] Stefan P. J. van Leeuwen, Anna Kärrman, Bert van Bavel, Jacob de Boer, and Gunilla Lindström Environ. Sci. Technol.; 2006; 40(24) pp 7854 - 7860

Gerda.Hopman@ivm.falw.vu.nl. For questions and remarks, please contact Stefan van Leeuwen (see details below).

Human matrices ILS

A new ILS for human matrices is expected to be announced in 2007 by Prof. Dr. Gunilla Lindstrom, Örebro University. For further details you may wish to contact Prof. Dr. Gunilla Lindstrom at Gunilla.Lindstrom@nat.oru.se.

We're looking forward to your participation in this study. Should you have any question, please don't hesitate to ask.

With kind regards,

Stefan van Leeuwen and Jacob de Boer

Institute for Environmental Studies

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1081 HV Amsterdam

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Appendix II. Participating laboratories

Laboratory name*	Name	Address	Postal code	City	Country	Phone	E-mail address
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Ecole Nationale Veterinaire de Nantes – LABERCA	Bruno Veyrand	Atlanpôle La Chantrerie - BP 50707	44307	Nantes	France	+33 2 40 68 78 80	veyrand@vet-nantes.fr
TestAmerica	Gail DeRuzzo	4955 Yarrow St.	80002	Arvada, CO	USA	+1 303-736-0116	mdymerski@stl-inc.com
AXYS Analytical Services Ltd	Dale Hoover	2045 Mills Rd. West	V8L 5X2	Sidney, BC	Canada	250 655-5800	pe_study@axys.com
VITO	Ab Borburgh	Boeretang 200	2400	Mol	Belgium	+32 14 33 50 18	guido.vanermen@vito.be
Cefas Burnham Laboratory	Paul Roberts	Remembrance Avenue	CM0 8HA	Burnham-on-Crouch	United Kingdom	+44(0)1621 787212	paul.roberts@cefas.co.uk
GKSS Research Centre Geesthacht GmbH	Lutz Ahrens	Max-Planck-Straße 1	21502	Geesthacht	Germany	+49 4152 872353	lutz.ahrens@gkss.de
3M Environmental Laboratory	William Reagen	3M Center, Building 260-05-N-17	55144-1000	St. Paul, Minnesota	USA	+1 651-733-9739	wkreagen@mmm.com
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* Laboratories in italic font have submitted data.

Appendix III. Protocol PFC method evaluation study

Version 19-07-2007

Introduction and aim

The distinct nature of perfluorinated compounds (PFCs) results in specific behaviour. This has challenged analytical chemists over the past 5-7 years. Early inventories showed that difficulties arose with accuracy of the methods applied. This was also confirmed by the 1st worldwide interlaboratory study (ILS), which showed that comparability of data was poor for the environmental matrices. Since then, knowledge on physicochemical properties improved and considerable developments took place in the fields (internal) standards, method development and clean-up. This resulted in improved accuracies as showed in some recent studies.

In order to assess the current status of method comparability, a new international study was initiated. The aim of this study is to asses the possibilities to produce precise and accurate data for a range of PFCs in a fish and water sample. In order to obtain information on the accuracy and precision of the data, it's important that replicate analysis are carried out, as well as an accuracy test. Below, the different aspects of the experimental part of the study are covered.

Important note:

Please carefully read this protocol. Working according to protocols other than your in-house routine protocols may be a source of errors (e.g. due to unclarities), as we've learned from previous studies involving protocols. Also, the distinct behaviour of these compounds requires more attention compared to the classical persistent organic pollutants.

It is therefore very important to understand the aims of the study, the experimental design and the way of reporting. Please discuss this thoroughly with your colleagues if they will be performing the practical work.

Should you have any question, please don't hesitate to contact me at Stefan.van.Leeuwen@ivm.falw.vu.nl or tel. +31 20 5959 545.

Please not that the deadline for data submission is set at 31 August 2007. This leaves only limited time to perform the analytical work. It is therefore important to plan the work accurately in order to start your experiments in time.

Good luck with performing the work.

Stefan van Leeuwen

Matrices covered and materials provided

The matrices covered in this study are a fish sample and a water sample. The water sample is taken from a local freshwater canal, which is connected to the Amsterdam harbour area. After the suspended particulate matter (SPM) is allowed to settle, the water is filtrated to remove small (<0.20 µm) particles. Characterization of the sample (pH, salinity etc) will be carried out by the coordinator. Analysis of the water material revealed very low levels and therefore, the water has been spiked with relevant PFCs and homogenized prior to preparing individual bottles (ca 500 mL of water). For each analysis, a separate bottle will be provided.

The fish (flounder) sample is taken from the Western Scheldt. The muscle tissue is collected by filleting and the material is ground. Relevant PFCs were spiked to the ground muscle tissue prior to thorough homogenization. Jars were filled with approx. 65 grams of muscle homogenate. The jars are sterilized at 3 bar, 120°C. The homogeneity of the jars is tested and confirmed. Several jars of fish material will be provided that should be sufficient to carry out sub-sampling.

The box sent to you contains the following materials:

- Water sample: 11 bottles of approx 500 mL each;
- Fish sample (if applicable): 4 jars of approx 65 g each;
- Standards: 2 boxes containing 4 glass ampoules. The contents of the ampoules are mentioned in Table 1 and 2 and exact concentrations can be found at the Wellington sheets provided with the ampoules;
- This protocol.

PFCs covered in the study

Table 1 shows the PFCs covered in this study. It is anticipated that not all mentioned PFCs are relevant for each matrix. The short chain compounds may predominantly be found in the water sample, whereas the longer chains may be found in the fish.

Table 1 Full names, abbreviations and chemical formulas of PFCs covered in this study and provided. The ampoule number appears at the label the common standard ampoule.

Full name	Abbreviation	Ampoule
Perfluorinated acids	PFCAs	
Perfluorobutanoic acid	PFBA	NS-1*
Perfluoropentanoic acid	PFPA	NS-1
Perfluorohexanoic acid	PFHxA	NS-1
Perfluoroheptanoic acid	PFHpA	NS-1
Perfluorooctanoic acid	PFOA	NS-1
Perfluorononanoic acid	PFNA	NS-1
Perfluorodecanoic acid	PFDA	NS-1
Perfluoroundecanoic acid	PFUnA	NS-1
Perfluorododecanoic acid	PFDoA	NS-1
Perfluorotridecanoic acid	PFTrA	NS-1
Perfluorotetradecanoic acid	PFTeA	NS-1
Poly and perfluorinated sulfonates	PFSAs	
Perfluorobutane sulfonate	PFBS	NS-2
Perfluorohexane sulfonate	PFHxS	NS-2
Perfluorooctane sulfonate	PFOS	NS-2
Perfluorodecane sulfonate	PFDS	NS-2
Other		
Perfluorooctanesulfonamide	PFOSA	NS-2

* NS= Native standard

Table 2 Full names, abbreviations and chemical formulas of the mass labeled PFCs provided in this study. The ampoule number appears at the label the common standard ampoule.

Full name	Abbreviation	Suggested internal standard-native standard pairs	Ampoule
$^{13}C_4$-perfluorobutanoic acid	$^{13}C_4$-PFBA	PFBA	MS-A*
$^{13}C_2$-perfluorohexanoic acid	$^{13}C_2$-PFHxA	PFPA, PFHxA	MS-A
$^{13}C_2$-perfluorooctanoic acid	$^{13}C_4$-PFOA	PFHpA, PFOA	MS-A
$^{13}C_5$-perfluorononanoic acid	$^{13}C_5$-PFNA	PFNA	MS-A
$^{13}C_2$-perfluorodecanoic acid	$^{13}C_2$-PFDA	PFDA	MS-A
$^{13}C_2$-perfluoroundecanoic acid	$^{13}C_2$-PFUnA	PFUnA	MS-A
$^{13}C_2$-perfluorododecanoic acid	$^{13}C_2$-PFDoA	PFDoA, PFTrA, PFTeA	MS-A
$^{13}C_4$-perfluorooctane sulfonate	$^{13}C_4$-PFOS	PFBS, PFHxS, PFOS, PFDS	MS-B
N-methyl-d_3-perfluorooctanesulfonamide	d_3-N-MePFOSA	PFOSA	MS-B

* MS= Mass labelled standard

Protocol

In order to avoid confusion of definitions used in analytical chemistry, we have defined some terms. The *italic, blue printed* terms are defined at the end of the protocol.

1. PFCs in this study

The minimal requirement for PFCs to be determined are PFOA and PFOS. It's recommended also to analyse (a selection of) the other compounds mentioned in Table 1.

2. Use of in-house methods in combination with this protocol

Participants should use their own, in house validated methods for the analytical work in this study. These methods should be fit-for-purpose (as supported by in-house validation studies).

3. Design of the study

This study is designed to obtain information of different aspects of method validation and quality assurance. We aim at obtaining information on:

1. Variety of *in-house standards* used by the participants and originating from different (commercial) sources;
2. Accuracy by two different quantification methods: (2a) quantification by *solvent based calibration curves* and (2b) quantification by *standard addition;*
3. Matrix interferences in the final determination by ESI-MS(/MS);
4. Precision of the analytical procedures applied.

To obtain this information, a series of experiments were developed and agreed upon at the Amsterdam Workshop meeting (18/19 March 2007). Participants should conduct these experiments in order to evaluate their performance as well as potential sources of bias at different stages of the analytical procedure. It is very important to stick to the protocol as this will enable a thorough evaluation of the different aspects of PFC analysis and also this will help the comparison of data obtained by different laboratories. Details on the experiments are mentioned in Appendix 1.

Addendum 1.

A *common standard* with all *native compounds* will be provided (1 ampoule with mixed PFCAs and 1 ampoule with PFSAs and PFOSA, see Table 1 for the compounds). You should analyse both the common standard and an in-house standard of approx. 50 ng/mL, in triplicate. The response of your in-house standard will be compared to the response of the common standard (see report forms). Please note that the concentration of your in-house standard should be expressed on the anion (see 9).

Relative response = $\text{response}_{\text{in-house standard}} / \text{response}_{\text{common standard}}$

Addendum 2a.

The levels in the samples should be quantified using the solvent based calibration curve. The provided *mass labelled internal standards* (see Table 2) should be employed for correction of the total analytical procedure. An estimate of the recovery of the internal standards should be given in the report forms. Three replicates of the samples should be analysed to obtain information on the precision of the methods.

Addendum 2b.

In order to determine the accuracy of the analytical procedures, you are also asked to determine the levels in the sample by standard addition. For this purpose you should fortify 3 samples with the native compounds (using the common standards provided) at approx. 1, 2 and 4x the PFC concentrations already present in the sample. To get to know these original concentrations, you should analyse a fish and water sample prior to this experiment, using the solvent based calibration curve ("preliminary analysis"). One additional jar and bottle are provided for this purpose.

For example, if the fish sample already contains 20 ng/g PFOS by natural contamination, then 3 additional samples should be fortified with 20, 40 and 80 ng/g, thereby arriving at PFOS concentrations of 40, 60 and 100 ng/g. These fortified samples should be analysed (together with a non-fortified sample). See Appendix 2 for more information. After analysis of the samples, the PFC concentration can then be determined according to the standard addition calculation method.

Please note that different concentration levels already in the sample may require spiking at different levels (for an indication of concentrations you can refer to above section "Matrices covered an materials provided"). The indicative concentrations in the samples are

water: <LOQ to 25 ng/L

fish: <LOQ to 150 ng/g (III.1)

Addendum 3.

Matrix effects in the final determination (ESI-MS(/MS)) can be determined by analysis of an extract fortified with the native compounds. For this purpose, 1 sample should be extracted and half the extract volume should be fortified with the native compounds at a level of approx. 50 ng/mL extract. The other half should not be fortified. The net response of the extract fortification should then be compared with the response of the solvent based standard at approx. 50 ng/mL level.

$$\text{Matrix effect} = (\text{response}_{\text{fortified extract}} - \text{response}_{\text{non-fortified extract}})/\text{response}_{\text{solvent based standard}}$$

Addendum 4.

This is covered under 2a.

4. Planning of the analysis.

The analysis should be carried out preferably rapidly after receipt of the materials. Please carefully read this protocol prior to analysis and forward any question you may have to Stefan van Leeuwen (Stefan.van.Leeuwen@ivm.falw.vu.nl). It is advised to analyse (if feasible) all fish samples in one analytical batch and all water samples in another analytical batch. Concerning the water samples, the mass labelled internal standards should be spiked to samples upon receipt!! Please see the sample table (Appendix 1) which samples require spiking.

5. Treatment of jars and bottles prior to subsampling

The outside surface of the lid of the jar may show some white residiues due to the sterilisation process. You can simply wipe these off using a tissue or similar. The jars with fish material should be opened carefully, without causing losses of the contents (some separation of liquid and lipids from the tissue might have taken place during preparation). Immediately before subsampling the complete contents of the jars should be thoroughly re-homogenised. Rehomogenisation within the jar is not recommended, as space is limited. It's recommended to rehomogenise the material in a pre-cleaned beaker or similar.

The water sample should be homogenised prior to extraction, e.g. by sonication for 5 minutes.

6. Notes for common standards supplied and spiking

Spiking of the native or mass labeled compounds should be performed using the mixes MS-A, MS-B, NS-1 and NS-2 as provided by Wellington. These mixture standards are dissolved in methanol. Sufficient material is provided to carry out this study and a little extra. The amounts are not sufficient to cover a lot of other experiments outside the scope of this study. After spiking, the compounds should be allowed to incubate with the sample material for e.g. 16 hours. This is especially recommended for the flounder sample.

Note: The common standards provided show concentrations concerning the salt. However, in this study, we will report the concentrations as the anion only (see 'reporting' also). Therefore, the concentrations on the 'certificates of analysis' should be adjusted. A sheet has been added to the 'certificates of analysis' showing the anion-concentrations.

7. Blanks

The determination of blanks is very important to monitor a possible contamination throughout the various stages of analysis. You should perform multiple blank tests to allow for correction of results if required.

8. Quantification of branched and linear isomers

The water and fish samples may contain branched isomers of certain PFCs. The sum of branched and linear isomers should be quantified as linear only. The provided common standards consists of linear isomers only.

9. Reporting.

Report forms are provided

The concentrations of PFCs determined in the samples should be reported on the basis of the anion only (and not as the salt or protonated acid). The common standards will de provided in concentration units based on the anion only, in order to facilitate the reporting on anion basis. The only exception to this is the non-ionic PFOSA.

Chromatograms of the following measurements should be submitted: a procedure blank, a calibration standard at approx. 10 ng/mL level, a sample from the precision test. The chromatograms should give a clear impression of the separation and mass transitions (if applicable) should be shown. All PFCs determined should be indicated in these chromatograms by their PFC abbreviation (see table) and the chromatograms should be clearly marked with your laboratory code and the analysed matrix or solution. Chromatograms should preferably be submitted electronically (pasted in a MS Word file).

The report forms and chromatograms should be emailed to Stefan.van.Leeuwen@ivm.falw.vu.nl. If a confidentiality issue rises (e.g. sharing methodological information among the participants), please contact Stefan van Leeuwen.

Evaluation of the data

During September 2007 all information will be evaluated. The submitted data will be evaluated statistically in order to find out what factors in the analytical approach are determinant for accu-

rate data. The purpose of the methodological information supplied is to determine if the methods used are fit-for-purpose. This will be evaluated by looking at:

- Methodological information i.e. if the methods for extraction and clean-up, separation and detection are fit-for-purpose;
- Is the method under control, judged from:
    - Peak separation in chromatograms and separation from matrix constituents;
    - Blank contamination;
    - Recovery of the mass labelled internal standards (70%<recovery<120%);
    - Repetitions of sample determination (RSD <15%).
- The quantification method (solvent based calibration combined with mass labelled internal standards vs. standard addition).

Evaluation meeting

An evaluation meeting will is planned at 18 and 19 October 2007 at IVM in Amsterdam. Prior to the meeting, a draft-report will be sent to all participants.

Final report

The final report will be produced after the evaluation meeting.

Definitions:

Common standard: The standard to be used for quantification of all samples is a common standard, consisting of 3 ampoules. Every participant should use these standards for quantification instead of their in-house standards.

In-house standards: The standards in your laboratory that you use for quantification of PFCs (not being the common standards provided in this study).

Mass labelled internal standards: 13C or deuterated internal standards. In this study, several 13C-PFCAs are provided, as well as 13C-PFOS and deuterated Me-PFOSA (see Table 2)

Native compound: The compound that is not mass labelled

Solvent based calibration curve: A calibration curve constructed in solvent only (e.g. MeOH). No matrix is involved in this type of calibration.

Standard addition: Quantification method used when detector response changes are suspected due to matrix constituents. In this study, the standard addition method will be compared to the solvent based quantification curve method.

Appendix 1 (of protocol). Sample table and directions for analysis.

	In-house standards evaluation		
	Sample type	**Addition of native compounds**	**Add ^{13}C-PFC internal standards**
Experiment 1. Evaluation of different sources of in-house standards			
	Your in-house standard at 50 ng/mL, replicate 1	Na	Na
	Your in-house standard at 50 ng/mL, replicate 2	Na	Na
	Your in-house standard at 50 ng/mL, replicate 3	Na	Na

	Fish		
Jar nr	**Sample type**	**Addition of native compounds**	**Add ^{13}C-PFC internal standards**
Experiment 2. Quantification by solvent based calibration curve and determination of precision			
	Fish repetition 1	Na	13C, directly after receipt
	Fish repetition 2	Na	13C, directly after receipt
J1	Fish repetition 3	Na	13C, directly after receipt
*Experiment 3. Quantification by standard addition**			
	Fish	Prior to extraction, spike native PFCs, level 0	na
	Fish	level 1x	na
	Fish	level 2x	na
J2	Fish	level 4x	na

Experiment 4. Matrix effect in ESI-MS(MS) determination			
J3	Extract of fish sample	No additional spiking	na

	Extract of fish sample	After extraction, spike native PFCs at the level of 50 ng/ml	na

* Please consider the following: when the original concentration for PFUnA is e.g 15 ng/g, you should spike the other samples with 15, 30 and 80 ng/g (in addition to the already present PFUnA). However, when PFOS is present at e.g. 100 ng/g, you should spike 100, 200 and 400 ng/g. An example for conducting spiking is shown (for water) in Appendix 2. In case this is unclear, please contact the coordinator.

Water			
Bottle	**Sample type**	**Addition of native compounds**	**Add ^{13}C-PFC internal standards**
Experiment 5. Quantification by solvent based calibration curve and determination of precision			
B1	Water repetition 1	Na	directly after receipt
B2	Water repetition 2	Na	directly after receipt
B3	Water repetition 3	Na	directly after receipt
*Experiment 6. Quantification by standard addition**			
B4	Water	Prior to extraction, spike native PFCs, level 0	na
B5	Water	level 1x	na
B6	Water	level 2x	na
B7	Water	level 4x	na
Experiment 7. Matrix effect in ESI-MS(MS) determination			
B8	Extract of water sample	No additional spiking	na
B8	Extract of water sample	After extraction, spike native PFCs at the level of 50 ng/ml	na

* Please consider the following: when the original concentration for PFBA is e.g 5 ng/L, you should spike the other samples with 5, 10 and 20 ng/L (in addition to the already present PFBA). However, when PFOS is present at e.g. 20 ng/L, you should spike 20, 40 and 80 ng/L.
To perform accurate spiking, we have supplied additional bottles of sample material. An example for conducting spiking is shown (for water) in Appendix 2. In case this is unclear, please contact the coordinator.

Appendix 2 (of protocol). Spiking information of native PFCs (addendum 2b).

In addendum 2b, it is asked to spike 1, 2 and 4x the amount determined in the original sample by preliminary analysis. As you will presumably anticipate, the PFC concentrations in the water sample will vary. On the other hand, the concentrations in the native standards are all equal (2 µg/mL for all PFCs except PFOS (20 µg/mL).

So how can I meet the 1,2 and 4x spike factors while the concentrations in the samples vary?

A suggestion is to carry out the preliminary analysis and then create a spiking matrix. In below example, five samples were spiked at concentrations chosen such that most PFCs are close to the 1, 2 and 4x criterion (as indicated in blue in below table). This is a reasonable compromise between workload and trying to meet the criterium the best you can.

Table 1 Example op spiking matrix.

		Spiking levels	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	Concentration obtained from preliminary analysis*	All PFCs except PFOS** (ng/L)	5	10	20	50	100
	(ng/L)	PFOS only** (ng/L).	50	100	200	500	1000
			Resulting spiking factors				
PFBA	5		1	2	4	10	20
PFPeA	10		0.5	1	2	5	10
PFHxA	5		1	2	4	10	20
PFHpA	10		0.5	1	2	5	10
PFOA	25		0.2	0.4	0.8	2	4
PFNA	5		1	2	4	10	20
PFDA	5		1	2	4	10	20
PFUnA	5		1	2	4	10	20
PFDoA	5		1	2	4	10	20
PFBS	20		0.25	0.5	1	2.5	5
PFHxS	10		0.5	1	2	5	10
PFOS	20		2.5	5	10	25	100
PFOSA	5		1	2	4	10	20

* Concentration as determined by **your** preliminary analysis of 1 sample, see addendum 2b, page 4 and 5. The figures here are virtual numbers only, used for explaining the approach.

** Note that PFOS concentration in spiking solution is 10 times higher than for the other PFCs.

Appendix IV. Submitted data per participant

Submitted data on the comparison of common and in-house standards (experiment 1). Data was submitted prior to the evaluation workshop (18/19 October 2007), but underlined information was delivered or updated after the workshop.

Lab nr.	1		2		3		4		5		8		9		10		11		12		13		14		17		19		20		21		22		23		24	
	R*	S**	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
PFBA							93	W	108	Si	116	Ab	94	Al			121	Al			60	Ab			100	W	56	Ab	96	?								
PFPeA					80	W	90	W	103	Si	113	Aa	103	Aa											91	W			105	Ab							115	Al
PFHxA					94	W	89	W	104	Si	118	Fl	97	3M			117	Ab			78	Ab	87	Fl	87	Fl	58	Ab	95	Ab	93	Fl					100	Fl
PFHpA			117	Al	104	W	98	W	101	Si	112	L	99	Al			123	?					115	Al	99	Al	72	Ac	95	Ab	99	St					116	Al
PFOA	120	Fl	116	Al	75	W	95	W	96	Si	113	L	110	3M	62	W	109	Ab	89	Al	72	Fc	91	Al	88	Fl	94	Ac	105	Ab	96	St	89	E	111	Si	96	Al
PFNA	78	Al	120	Al	117	W	97	W	103	Si	95	L	108	O			87	Al	118	Al	88	Al	84	Al	71	Al	53	Al	114	Ab	123	St			96	Si	123	Al
PFDA	129	Al	95	Al	90	W	87	W	105	Si	118	L	110	O			120	Fl			133	Ab	102	Fl	91	Al	72	Ab	100	Ab	104	Fl			104	Si	141	Al
PFUnA	74	Al	129	Al	108	W	100	W	102	Si	122	Ab	109	O			106	Al			145	Al	106	Al	94	Al	86	Al	99	Ab					102	Si	185	Si
PFDoA	103	Al	134	Al	66	W	143	W	87	Si	112	Aa	111	O			123	Al				Ac	101	Al	79	Al	64	Al							111	Si	132	Al
PFTrA	109	Al					97	W			108	W	118	Al											96	W												
PFTeA							103	W			134	Aa	132	O			112	Al					124	Al	93	W	74	Ab										
PFBS							102	W	120	Si	109	Fl	90	3M			97	D			137	S	106	D			103	S	122	W	94	Fl						
PFHxS	97	I			94	W	102	W	95	Si	116	Fl	82	3M			98	I	108	Fl	114	Fl	97	I			74	Fl	101	W	92	I					105	Fl
PFOS	100	Ab	133	Al	91	W	101	W	99	O	95	W	92	3M	68	W	104	Fl	46	Fl	86	Fl	104	Fl	94	Fl	54	Fl	97	Ab	107	Fl/W	81	E	109	Si	58	Fl
PFDS					111	W	105	W			99	W	115	3M			100	Ab					98	Al	107	Al												
PFOSA	97	Ab			95	W	NA		93	W	108	Ab	100	3M			92	Ab					102	Ab	85	Ab	98	W	105	Ab	101	Ab						

Comments
Lab 9: For PFBA, PFPeA, PFHxA, PFHpA, PFBS and PFHxS; n=1 for the result common standard.
Lab 15: No data provided
*R: result, defined as the overestimation or underestimation compared to the common standards (%)
**S: Supplier. See right for supplier codes

3M: 3M Company; Aa: Alfa Aesar; Ab: ABCR; Ac: Acros; Al: Aldrich; D: Dyneon; E: Dr. Ehrenstorfer; Fl: Fluka: Fc: Fluorochem; I: Interchim; L: Lancaster; O: Oakwood Products; S: Sigma (Aldrich); St: Strem; W: Wellington

Summary statistics of the submitted data on the comparison of common and in-house standards (experiment 1).

PFC	Average	STDEV	RSD	MIN	MAX	N
PFBA	94	22	24	56	121	9
PFPeA	100	12	12	80	115	8
PFHxA	94	15	17	58	118	13
PFHpA	104	13	13	72	123	13
PFOA	96	15	16	62	120	19
PFNA	98	20	20	53	123	17
PFDA	106	18	17	72	141	16
PFUnA	111	26	24	74	185	15
PFDoA	105	25	24	64	143	13
PFTrA	106	9	9	96	118	5
PFTeA	111	22	20	74	134	7
PFBS	108	14	13	90	137	10
PFHxS	98	11	12	74	116	14
PFOS	90	21	23	46	133	19
PFDS	105	6	6	98	115	7

Submitted accuracy and precision data for the water matrix. Data was submitted prior to the evaluation workshop (18/19 October 2007), but information in underlined font was delivered or updated after the workshop.

Exp. 5. Solvent base curve results (average of n=3) (ng/L)

Lab nr.	1	2	4	5	6	8	9	10	11	12	14	15	17	19	20	21	22	23	24
PFBA		49.8		51.6		45.582	33.7		nd					43.31					
PFPeA		10.1	ND	6.6	6.2	5.356	<10		2.3		5.68			6.39	3.4				15
PFHxA		10.0	6.0	8.0	11.2	8.773	23.8		4.2		10.9		8.6	8.1	6.7	6.6			11
PFHpA		7.6	8.4	6.6	7.0	7.682	-		0.8		6.30		5.2	6.6	4.7	5.2			5
PFOA	47.6	40.0	40.1	32.8	36.5	46.671	20.5	83.23	23.8	47.0	41.7		34	39.9	34.4	37.2	46	38.2	50
PFNA	6.6	4.9	2.9	4.1	4.7	4.178	<2.0	_	3.2	8.00	5.48		6.1	5.8	5.3	3.2		5.2	10
PFDA	9.9	5.0	3.0	3.4	4.9	5.456	<4.0	_	2.9	_	5.50		4.4	5.8	3.5	4.1		4.0	< 5
PFUnA	5.7	3.7	5.3	2.3	1.9	3.833	<4.0	_	1.5	_	3.52		3.5	2.5	3.0			2.3	
PFDoA	3.6	4.3	ND	2.1	4.5	2.943	<10	_	1.2	_	3.79		3.0	1				1.5	
PFTrA	nd		ND	nd	_	n.d.	<2.0	_	nd	_	<0.15		n.n.					<0.2	
PFTeA			ND	nd	3.5	n.d.	<4.0	_	nd	_	3.65		n.n.					<0.2	
PFBS		42.9	54.4	21.4	46.0	30.668	50.5	_	9.7	_	28.9				13.7	26.7		0.9	4
PFHxS	19.2	15.2	17.4	9.5	24.0	13.198	5.50	_	9.2	16.1	11.9				14.6	12.1		9.0	24
PFOS	27.7	37.2	33.1	22.0	44.2	35.605	<93	31.77	20	29.01	36.1	46	28	42.6	28.5	35.9	60	22.7	39
PFDS					0.7	n.d.	<1.9				<0.07					_		0.2	< 5
PFOSA	4.5	10.1	4.4	1.6	2.6	4.009	-		1.7		2.46		<2.1		2.9	1.94		2.4	< 5

Notes

Lab 20 Use of large volume injection (LVI). Calibration performed by spiking ultrapure water and LVI rather than use of solvent based calibration

Exp 5. Precision results (n=3) (ng/L)

Lab nr.	1	2	4	5	6	8	9	10	11	12	14	15	17	19	20	21	22	23	24
PFBA		3.0		1.5		1.296	4.8							3.2				na	
PFPeA		1.2	na	0.3	0.59	0.109	NA		0.2		1.40			0.36	1.17			na	4
PFHxA		0.5	0.74	0.2	2.4	0.202	11		0.9		0.33		0.54	0.55	0.46	0.42		na	3
PFHpA		0.5	1.93	0.7	0.67	0.296	-		0.1		0.32		0.37	0.2	0.40	0.33		na	1
PFOA	0.49	1.7	5.85	1.8	0.39	1.217	11	8.78	0.9	3.11	0.33		0.59	3.43	1.21	0.11	5.97	1.11	13
PFNA	0.45	0.3	1.12	0.0	0.14	0.327	NA		0.3	0.63	0.44		0.43	0.66	0.96	0.28		0.23	8
PFDA	0.81	0.5	1.41	0.6	0.20	0.168	NA		0.4		0.35		0.17	0.65	0.54	0.32		0.20	
PFUnA	0.25	0.4	0.88	0.4	0.23	0.198	NA		0.1		0.69		0.30	0.35	0.55			0.17	
PFDoA	0.46	0.5	na	0.3	0.35	0.176	NA		0.2		0.87		0.61	0.13				0.03	
PFTrA	nd		na			n.a.	NA											0.00	
PFTeA			na		0.14	n.a.	NA				5.48							0.00	
PFBS		5.2	8.43	1.1	4.9	0.538	11		0.6		2.42				4.43	1.82		0.09	0
PFHxS	1.13	1.4	1.44	1.6	1.6	0.720	3.6		2.4	0.56	0.73				3.61	0.77		1.73	5
PFOS	1.63	2.7	3.85	2.2	8.5	1.335	NA	1.12	2.7	2.33	0.89	6	1.3	4.89	2.05	3.12	4	0.50	10
PFDS			na		0.03	n.a.	NA											0.01	
PFOSA	0.55	1.2	1.75	0.2	0.04	0.037	-		0.5		0.07				0.18	0.23		0.46	

Exp. 5 Precision (n=3) (RSD, %)

Lab nr.	1	2	4	5	6	8	9	10	11	12	14	15	17	19	20	21	22	23	24
PFBA		6.0		2.9		2.8	14.3							7.4					
PFPeA		11.9		4.5	9	2.0			8.7		24.6			5.6	34.4				24.5
PFHxA		5.0	12.3	2.1	21	2.3	45.0		21.4		3.0		6.3	6.8	6.9	6.3			29.1
PFHpA		6.6	23.0	10.6	9	3.9			12.5		5.1		7.2	3.1	8.5	6.4			17.3
PFOA	1.0	4.3	14.6	5.4	1	2.6	53.7	10.6	3.9	6.6	0.8		1.7	8.6	3.5	0.3	12.9	2.9	26.0
PFNA	6.8	6.1	38.7	0.3	3	7.8			10.2	7.9	7.9		7.1	11.5	18.1	8.8		4.5	75.0
PFDA	8.2	10.0	47.2	16.2	4	3.1			14.6		6.3		3.9	11.1	15.4	7.8		5.1	
PFUnA	4.4	10.8	16.6	15.8	12	5.2			4.4		19.7		8.4	14.2	18.3			7.3	
PFDoA	12.8	11.6		16.2	8	6.0			17.4		22.8		20.2	10.0				2.1	
PFTrA																			
PFTeA											150.0								
PFBS		12.1	15.5	5.0	11	1.8	21.8		6.2		8.4				32.3	6.8		10.3	9.1
PFHxS	5.9	9.2	8.3	16.5	7	5.5	65.5		26.1	3.5	6.1				24.7	6.4		19.2	19.8
PFOS	5.9	7.3	11.6	10.1	19	3.7		3.5	13.6	8.0	2.5	13.1	4.6	11.5	7.2	8.7	7.2	2.2	24.6
PFDS					4													4.1	
PFOSA	12.2	11.9	39.7	12.7	2	0.9			29.4		2.9				6.2	11.7		19.2	
Average	7.1	8.7	23	9.1	8.5	3.7	40	7	14	6.5	20	13	7.4	9.0	16	7.0	10	7.7	28

Exp. 6. Standard addition results (ng/L)

Lab nr.	1	2	4	5	6	8	9	10	11	12	14	15	17	19	20	21	22	23	24
PFBA		89.9	na	57.9		29.62	72.3							45.74					
PFPeA		5.7	< 12	10.8	9.0	4.59	13.8		17.3		6.76			6.48	4.0				2
PFHxA		11.4	11.5	8.3	13.3	4.08	44.7		26.4		10.0		8.3	7.66	7.2	6.10			10
PFHpA		6.9	0.1	9.0	6.4	2.24	-		19.1		8.27		5.7	4.93	7.6	5.15			2
PFOA	31.9	46.7	32.4	37.5	44.0	40.91	68	181.37	69.6	52.9	49.7	38	33	44.69	30.30	37.9	58	40.7	
PFNA	7.0	6.1	1.9	3.7	3.5	4.32	11.6		5.6	17.4	10.2		3.9		4.8	5.70		11.8	
PFDA	10.6	6.3	11.3	5.9	2.7	2.31	2.3		7.5		8.42		4.7		3.1	5.10		7.6	
PFUnA	15.9	4.2	3.3	4.2	4.0	0.93	<LOQ		3.7		7.03		3.9		2.2			1.8	
PFDoA	19.6	3.6	< 4.7	2.8	1.5	0.71	<LOQ		3.5		6.06		2.9					4.3	
PFTrA	19.9		< 23.9	-0.1		<0	0.6						n.n.					<0.2	
PFTeA			< 6.8	0.0	<LOQ	<0	<LOQ						n.n.					<0.2	
PFBS		43.7	39.3	21.9	44.0	26.66	41.1		59.3		23.1			30.41		24.1		7.8	
PFHxS	18.6	14.1	1.2	12.4	11.0	8.03			15.7	12.4	12.6				14.1	13.6		7.4	9.5
PFOS	71.4	29.7	46.0	48.7	21.0	50.02	32.2	196.84	11.0	40.2	42.0	12	26		23.3	23.9	42	16.4	10.0
PFDS			< 9.6	-0.1	<LOQ	<0	3.5						<5,6					0.5	
PFOSA	1.6	1.5	ND	3.2	0.5	0.80	-		1.3		5.21		2.4		4.4	1.24		1.1	

Submitted accuracy and precision data for the fish matrix. Data was submitted prior to the evaluation workshop (18/19 October 2007), but underlined data was delivered or updated after the workshop.

Experiment 2. Solvent base curve results (average of n=3) (ng/g ww)

Lab nr.	1	3	4	5	8	9	10	11	12	13	14	15	17	19	21
PFBA				<0.1		<									
PFPeA				<0.1	0.067	<					<0.23				
PFHxA				<0.1	(0,038)	<					<0.54				0
PFHpA		0.21		<0.2	0.120	<					<0.36				0
PFOA	9.2	12.91	14.7	16.8	23.314	17.3	18.04	20	23.6	19.3	22.3		22	16.71	15.7
PFNA	8.9	17.88	17.4	15.2	27.265	13.0		20.1		16.8	22.9		17	17.10	17.0
PFDA	13.7	12.90	22.5	23.3	23.918	26.2		15.9		21.3	26.7		24	21.12	21.9
PFUnA	9.0	11.70	14.8	17.7	18.304	17.9		10		17.6	20.9		21	16.20	
PFDoA	8.5	12.76	18.9	23.6	22.955	16.5		12.8		11.9	22.9		21	18.80	
PFTrA				<0.2	0.179	<		60.9			<0.18				
PFTeA				<0.2	(0,036)	<					<0.15				
PFBS				<0.2		<					<0.1				0
PFHxS				<0.2	0.089	<					<0.09				0
PFOS	49.9	118.63	180.0	149	174.667	149	184.36	104	158.7	115.8	170		230	137.24	184
PFDS				<0.2		<				2.9	<0.06				
PFOSA	1.5	3.07	2.7	3.4	5.191	2.61				2.5	3.59			7.48	3.45

Notes

Lab 9 None of these results were obtained using background subtraction.

Lab 14 All: One point calibration (5 ng/mL); PFOSA also corrected with 13C-PFOS

Lab 21 IS-conc. In calibr.solution is 50 ng/ml, IS-conc. in sample is 10 ng/ml, Fish is 1g in 5 ml final extract

Experiment 2. Precision results (n=3)(ng/g ww)

Lab nr.	1	3	4	5	8	9	10	11	12	13	14	15	17	19	21
PFBA															
PFPeA					0.008										
PFHxA					0.002										
PFHpA		0.02			0.005										
PFOA	0.4	1.03	1.4	0.9	0.422	0.391	1.38	2	0.38	0	3.1		0.60	0.22	
PFNA	0.8	1.31	1.3	0.8	0.235	1.01		2.7		0.2	3.5		1.8	0.11	
PFDA	0.7	0.36	1.7	2.0	0.808	2.83		1.2		0.5	3.5		0.66	0.17	
PFUnA	0.8	0.38	0.8	1.9	0.880	1.17		2		0	3.2		1.8	0.23	
PFDoA	0.4	0.31	1.2	1.6	0.246	2.05		1.7		0.7	3.2		1.2	0.07	
PFTrA					0.009			13.0							
PFTeA					0.002										
PFBS															
PFHxS					0.006										
PFOS	3.9	2.59	14.1	25	2.199	13.7	8.90	3	7.37	1	14		38	0.79	
PFDS										2					
PFOSA	0.3	0.13	0.1	0.3	0.178	0.137				0.8	0.37			2.04	

Experiment 2. Precision results (n=3) (RSD, %)

Lab nr.	1	3	4	5	8	9	10	11	12	13	14	15	17	19	21
PFBA															
PFPeA					12.1										
PFHxA															
PFHpA		9.5			3.9										
PFOA	4.4	8.0	9.3	5.4	1.8	2.3	7.7	11.2	1.6	1.9	14.0		2.7	1.3	4.1
PFNA	8.5	7.3	7.7	5.3	0.9	7.7		13.4		1.3	15.3		10.6	0.6	8.5
PFDA	4.8	2.8	7.6	8.6	3.4	10.8		7.5		2.2	13.1		2.8	0.8	4.3
PFUnA	8.3	3.2	5.2	10.6	4.8	6.5		21.1		0.7	15.2		8.6	1.4	
PFDoA	4.9	2.4	6.5	6.8	1.1	12.4		13.2		5.8	13.9		5.7	0.4	
PFTrA					4.7			21.4							
PFTeA															
PFBS**															
PFHxS**					6.4										
PFOS**	7.8	2.2	7.9	16.8	1.3	9.2	4.8	3.1	4.6	0.8	8.2		16.5	0.6	3.6
PFDS**															
PFOSA	18.8	4.2	3.1	9.7	3.4	5.2					10.4			27.3	8.7
Average	8.2	5.0	6.8	9.0	4.0	7.7	6.2	13.0	3.1	2.1	12.9		7.8	4.6	5.8

Experiment.3. Standard addition results (ng/g)

Lab nr.	1	3	4	5	8	9	10	11	12	13	14	15	17	19	21
PFBA					0.01										
PFPeA			< 2.4		0.12										
PFHxA			< 2.2		0.06					4					1.8
PFHpA			3.3		0.13										1.3
PFOA	8.6	16.2	23.4	22.7	30.47	14.6	24.58	41.5		17	19.6		24	15	15.2
PFNA	14.3	18.9	12.6	20.3	36.59	12.9		33.8		12.7	21.0		23	57.2	21.0
PFDA	20.9	20.9	24.7	26.5	28.07	14.5		30.6		14.2	18.9		29	23.2	21.9
PFUnA	17.1	18.9	13.4	24.2	22.57	23.5		28.6		12	16.7		19	27	
PFDoA	6.2	14.8	19.1	21.3	23.36	11.6		21.2		13.4	14.3		20	19.9	
PFTrA	0.3		< 1.5		0.11			23.2							
PFTeA					0.05					1.9					
PFBS					<0					3.5					1.6
PFHxS	0.2				0.11					4.6					1.7
PFOS	173.2	129.7	173.6	159	388.24	230	180.94				207	35		320	192
PFDS					0.0034					3					-
PFOSA	3.1	4.1	< 2.0	2.5	5.92	1.18				9.8	4.00			4.0	3.45

Notes

Lab 17 PFOS: sub-optimal addition

Lab 19 PFOSA: one more spikelevel, 78.30 ng/g

Lab 21 Correcteion for 13C internal standards applied. Therefore not included in statistics

Submitted matrix effect data for the water matrix. Data was submitted prior to the evaluation workshop (18/19 October 2007), but underlined data was delivered or updated after the workshop.

Exp. 7. Matrix effect (ESI-MS/MS)

Lab	1	2	4	5	6	8	9	10	11	12	14	15	17	19	20	21	22	23	24
PFBA		1.03	0.79	0.69			1.00		0.83		0.66			0.94					
PFPeA		1.00	0.82	0.94	0.75	0.83	0.92		1.28		0.78			1.07					1.13
PFHxA		1.00	0.89	1.01	0.91	0.86	0.87		1.29		0.79		0.75	1.20		1.04			1.15
PFHpA		1.04	0.79	1.02	0.98	0.81			1.10		0.97		0.78	1.14		1.13			1.04
PFOA	0.34	1.05	0.77	0.99	1.12	0.83	0.91	0.09	1.07	0.92	1.19	0.04	0.87	1.06		1.05	1.12	0.97	2.42
PFNA	0.34	1.03	0.72	1.18	1.42	0.93	0.76		0.96	0.80	1.02		0.96	0.99		0.78		0.77	0.76
PFDA	0.43	1.01	0.79	1.10	1.16	1.00	0.76		1.01		1.08		0.92	0.39		1.18		1.18	0.87
PFUnA	0.42	0.97	0.72	1.10	0.59	1.24	0.70		0.96		1.17		0.92	0.31				1.10	
PFDoA	0.28	0.99	0.75	1.05	0.59	1.95	0.66		0.91		1.09		0.92	0.27				1.16	
PFTrA		1.15	0.60	1.08		1.43	0.77		0.88		0.88		0.87	0.30					
PFTeA			0.49	1.13	1.46	1.27	0.69		1.00		0.80		0.87	0.36					
PFBS		0.93	0.91	0.98	1.21	0.83	0.90		1.27		1.07			1.10		1.30		0.56	0.96
PFHxS	0.34	0.94	0.76	0.93	2.03	0.77	0.86		1.11		0.86			0.87		1.28		1.30	1.08
PFOS	0.29	0.92	0.77	1.04	1.26	0.87	0.93	0.20	1.09	1.15	1.01	0.01	1.09	0.44		1.07	1.46	1.15	1.25
PFDS	-	0.92	0.67	1.04	0.50	1.34	0.94		1.03		0.98		0.70	0.22				1.27	0.18
PFOSA	0.96	1.08	1.02	0.97	0.87	0.99			0.97		1.05		0.92	0.20		1.58		0.78	0.02
Average	0.42	1.00	0.77	1.01	1.06	1.04	0.83	0.15	1.05	0.96	0.96	0.02	0.88	0.68		1.16	1.29	1.05	0.99
RSD	52	6	16	11	39	31	13	54	13	19	16	99	12	58		19	19	24	63

Comments:

Lab 20 Not Applicable

Table. Submitted matrix effect data for the fish matrix. Data was submitted prior to the evaluation workshop (18/19 October 2007), but underlined data was delivered or updated after the workshop.

Exp. 4. Matrix effect (ESI-MS/MS)

Lab nr.	1	3	4	5	8	9	10	12	14	15	17	19	21
PFBA		0.67	0.90	0.89		0.20			1.26		0.59		
PFPeA		0.99	0.83	0.72	1.55	0.69			1.16		0.79		
PFHxA		0.97	0.85	0.77	1.81	0.63			1.14		0.78		1.15
PFHpA		1.00	0.91	0.79	1.17	0.72			1.08		0.79	0.76	1.23
PFOA	0.87	1.09	0.83	0.88	0.70	0.90	0.18		1.05		0.67	0.63	0.98
PFNA	0.98	0.91	0.78	0.81	0.68	0.98			0.96		0.70	0.66	0.86
PFDA	0.79	1.18	0.85	0.76	0.69	0.80			1.13		0.71	0.86	1.30
PFUnA	1.14	0.99	0.82	0.78	0.65	0.86			1.19		0.72	1.01	
PFDoA	1.12	1.00	0.84	0.76	0.70	0.87			1.35		0.70	0.87	
PFTrA	1.03	1.14	0.84	0.89	0.65	0.83			0.96		0.77	0.75	
PFTeA		0.93	0.83	0.86	0.66	0.80			0.37		0.74	0.44	
PFBS			1.22	0.84	1.18	0.74			1.07				1.13
PFHxS	0.19	0.93	0.87	0.83	0.96	0.68			1.08				1.08
PFOS	0.86	0.86	0.82	1.22	0.63	0.91	0.17		0.89	1.15	0.79	0.69	0.99
PFDS		0.77	0.91	0.75	0.79	0.93			1.06		0.82		
PFOSA	4.31	0.74	1.23	0.83	0.9	0.80			1.00		0.81	0.87	1.67
Average	1.25	0.94	0.90	0.84	0.91	0.77	0.17	N.a.	1.05	1.15	0.74	0.75	1.16

Comments:

Lab 9 Na+/K+ correction applied after analysis (sulfonates)

Lab 21 Modified calculation

Appendix V. Protocol for the analysis of PFCs in water and fish

Protocols are very useful for providing guidance to analytical laboratories. It will help them to analyse samples. Protocols are also useful for the users of the resulting data. If a protocol has been followed exactly by the lab, than the user can rely on the accuracy and precision of the data. Generally, there are two ways for establishing a protocol:

1. By standardization *a method of analysis*, typically standardizing a detailed method using single extraction, clean-up or LC-MS technology;
2. By standardization of the *performance criteria* of methods of analysis.

Standardization bodies such as the European Committee for Standardization (CEN) and the International Organization for Standardization (ISO) sometimes work along the line of standardization of single technologies for specific analytical questions. In that case, very extensive method validation and evaluation studies are the basis of such standards. Typically, these standards set very strict guidelines for 1) the sample matrices, 2) the target analytes and 3) the analytical methodology. On the other hand, standards like ISO-17025 (ISO, 2005) and NEN-7777 (NEN, 2003) set strict guidelines on *the performance* of methods (in a single laboratory) rather than on the methods themselves. The benefit of the latter is the fact that it will maintain flexibility towards future developments on improvements in terms speed, efficiency, accuracy, LOQs and precision. Also, this allows the introduction of additional target analytes and matrices.

In this study, we have focused on specific performance characteristics (accuracy, precision, selectivity, method of quantification) by means of a method evaluation study, whereas others were not included (e.g. establishment of limit of quantification, reproducibility, robustness). It was a prerequisite that participating laboratories were competent and participated with fit-for-purpose and (in-house) validated methods. This protocol will focus on the issues covered in this study.

This protocol enables routine analysis of the PFCs in this study in fish muscle tissue and surface water samples. Adoption of these recommendations should enable the delivery of accurate and precise data. Other compounds or matrices are outside the scope of this protocol. The information in this protocol is based on the opening workshop (with specific contributions to the discussions by dr. C. Powley, dr. F. Morandi and dr. U. Berger), the FDA Guidance for industry document (US FDA, 2001) and other documents on performance of laboratories and methods (ISO, 2005; NEN, 2003).

Within this study, several extraction methods were used including solid phase extraction (SPE) using Oasis HLB, Oasis WAX, C18 and liquid-liquid extraction with methyl-*tert*-butyl ether (MTBE). Each technique has a specific working area in terms of target analytes (van Leeuwen and de Boer, 2007). For example, PFBA cannot be extracted from water efficiently by C18 and LLE, but may be extracted using Oasis Wax and HLB. SPE methods limit to the dissolved fraction of a water sample, whereas the LLE method allows extracting the dissolved and particle-associated fraction of a water sample. Clean-up of SPE cartridges consists of a simple wash step after loading the sample on the cartridge. Extraction of target compounds from fish can be performed by liquid-solid extraction (LSE) using a medium polar solvent (acetonitrile or methanol) or using MTBE in combination with an ion pairing reagent (TBA). Clean-up of fish extracts is often performed by mixing with Envicarb (or active) and

glacial acetic acid. Other clean-up methodologies include freezing out matrix constituents, clean-up of the fish extract by Oasis HLB SPE and centrifugation or filtration for removal of solids.

The analysis is often performed by LC-ESI-MS/MS using a triple quad MS systems. Other systems include LC-ESI-MS, LC-ESI-time of flight (TOF)MS/MS, flow injection analysis (FIA)-ESI-MS/MS and LC-ESI-MS(/MS).

Solvent based vs standard addition quantification

Solvent based quantification is a suitable quantification technique if combined with mass labeled internal standards that account for losses and matrix effects. When no mass labeled analogues of the target analytes are available, one should quantify the levels in the sample with measures to ensure a good accuracy (ie. absence of matrix effects and good recoveries of spikes). Standard addition quantification is a viable approach that considers both matrix effects and recovery of analytes in the analytical method.

Blanks

Blank problems are a very relevant issue in the analysis of PFCs. In the production of PTFE (often used in laboratory equipment), PFCs are used (commonly PFHxA, PFHpA and PFOA).

Therefore, blanks should be reduced and omitted as much as possible to allow trace analysis of PFCs in environmental samples.

Blanks can originate from various sources including:

- Instrumental (PTFE) parts, resulting in an instrument blank;
- Mobile phases, resulting in a solvent blank;
- Contamination from the analytical procedure (reagents, lab ware), resulting in a procedure blank.

Criterium: Blank responses ≤20% of lowest calibration standard (US FDA, 2001).

Use of (internal) standards

The standards of the target analytes should be of sufficient quality (>99% if available) and should be well defined in terms of isomeric composition. Whether or not quantification should be based on the linear isomer only, or on an electrochemical fluorination-type of isomer profile depends on the research question to be answered.

Within this study, it was shown that mass labeled internal standards enable correction of matrix effects and losses during the analysis. These effects can vary for every individual compound. Therefore, as much as (commercially) available mass labeled analogues of the native compounds should be used (preferably ^{13}C or ^{18}O). These standards should be well defined and pure (>99% pure). D3-N-MeFOSA is not a suitable internal standard due to decomposition in an aqueous environment.

Criterium: Standards of target analyses should be of sufficient quality (>99% if available) and should be well defined in terms of isomeric composition.

> Criterium: As much as (commercially) available mass labeled analogues of the native target analytes should be used (preferably ^{13}C or ^{18}O). These standards should be well defined and pure (>99% pure).

The mass labeled internal standards should be added and homogenized into the sample and it should be left to equilibrate with the matrix for sufficient time. The use of ^{13}C standards is convenient for correction for losses and matrix effects occurring during extraction, clean-up, concentration of the extract and determination by LC-MS based methods. Analysis of samples without using mass labeled internal standards is not recommended (unless accuracy, precision, selectivity etc can be demonstrated for each sample type).

Calibration curve

The calibration curves should describe response-to-analyte amount in a coherent manner. The generally applied correlation coefficient is a suitable descriptor allowing the evaluation the quality of the calibration curve.

> Criterium: Correlation coefficient (R) of calibration curve ≥ 0.992 ($R^2 >= 0.985$)

In this study the use of mass labelled standards has proven to produce high quality calibration curves with R^2 values generally >0.95 and in some cases 1.00.

Accuracy of the method

The ISO-17025 standard and the NEN 7777 allow for accuracy determination by several means. This includes in-house experimental determination of recovery of an added spike of native compounds, use of a laboratory reference material (LRM). In case that mass labeled standards are used, there's no need to check recoveries of spiked *native* compounds on a routine basis as it is assumed that the mass labeled compounds correct accurately. It's therefore sufficient to check recoveries of the *mass labeled* compounds compared to the responses of the standard solutions.

Accuracy can also be determined by external sources such as use of certified reference materials (CRMs, but not available for PFCs), successful participation in interlaboratory comparisons (i.e. comparisons with external laboratories, methods, instruments and analysts).

> Criterium for compounds with no mass labeled compounds: net recovery of a spiked sample should be 80 to 120%.

> Criterium for mass labeled compounds: recovery (defined as response in sample) should be +/-50% of the response in the standard.

Mass labeled standard recoveries can also be used as an indication for robustness of the methods applied. When small changes in methodology or matrix composition do not lead to dramatic changes of the recoveries, then the analytical method is robust.

It should be noted that matrix effects can alter or decrease the response of the mass labeled compounds and thereby absolute recovery figures may be decreased of elevated. This can negatively effect the determination of the method recoveries and give false positive or negative impression if the method is controlled.

Precision of the method

Precision is a measure for the agreement of subsequent analysis of the same sample under repeatable conditions. Within this project, three replicate analysis were carried out by each participant to investigate the precision of their methods.

Criterium: RSD of replicate analyses should be ≤15% (minimum 5 replicates) (US FDA, 2001)

The precision of the methods within this study in most laboratories met above criterium (even with only 3 replicates analysed).

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