Improved analytical methods for perfluoroalkyl acids (PFAAs) and their precursors – a focus on human dietary exposure

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Doctoral Thesis

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To my Parents.

Abstract

Per- and polyfluoroalkyl substances (PFASs) are a large group of emerging anthropogenic environmental contaminants. They can be divided into two sub-groups, 1) perfluoroalkyl acids (PFAAs) and 2) so called precursors, i.e. PFASs that can potentially be transformed in the environment or in humans to form PFAAs. Long-chain PFAAs are of specific concern because they are persistent, bioaccumulative and toxic in animal studies. PFASs have been used worldwide in a wide variety of industrial and consumer product applications. They are today spread in the global environment including wildlife and humans. Dietary intake (food and drinking water) is assumed to be the main human exposure pathway for PFAAs. Improved analytical methods for quantification of PFASs in food and drinking water were needed in order to understand and quantify dietary human exposure to these compounds.

The main aim of this doctoral thesis was to develop sensitive, precise, accurate and fully validated analytical methods for the determination of a range of PFAAs and selected precursor compounds in dietary samples including different kinds of food, food packaging materials and drinking water. The methods were based on liquid chromatography (LC) coupled to different mass spectrometric (MS) techniques. Dietary samples were extracted by solvent extraction followed by a cleanup step employing solid phase extraction (SPE). The SPE cleanup could at the same time be used as a fractionation step for ionic and neutral PFASs in multi-chemical methods encompassing PFAAs and their precursors.

Paper I describes the development of a method for simultaneous quantification of perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and perfluoroalkyl phosphonic acids (PFPAs) in drinking water at sub ng/L levels. Methyl piperidine was used in the eluent of the SPE cleanup and in the mobile phase of the LC/MS system. This proved to be a crucial parameter for achieving satisfactory method recoveries, excellent chromatographic resolution and good detector sensitivity for PFPAs. In **paper II** a novel method is presented for quantitative determination of the same three groups of PFAAs at low pg/g levels in a broad range of food matrices. It was demonstrated that PFPAs and other long-chain PFAAs show a strong tendency to sorb to solid surfaces. A quantification procedure based on matrix-matched and extracted calibration standards is suggested for accurate quantification of PFPAs despite matrix and sorption effects. A first screening of tap water samples and different food items in **paper I and II** revealed that human dietary exposure to PFPAs in Europe is currently not of concern.

A method for simultaneous analysis of PFCAs and polyfluoroalkyl phosphate esters (PAPs, potential precursors of PFCAs) in food and food packaging materials was developed as described in **paper III**. The method was superior to existing methods in terms of chromatographic resolution and sensitivity for PAPs. Method detection limits for food samples were in the sub to single digit pg/g range. A screening of targeted food samples and their packaging showed that PAPs could potentially contribute to the total human exposure to PFCAs. In **paper IV** temporal trends of PFSAs (including perfluorooctane sulfonic acid (PFOS)) and their sulfonamide-based precursors in herring muscle 1991-2011 were investigated. A multi-chemical analytical method was developed including PFSAs and three classes of acidic and neutral precursors. Rapidly decreasing time trends were observed for PFOS precursors, whereas PFOS concentrations did not show a significant change over time. This means that precursors in edible fish may have played an important role for human exposure to PFOS in the 1990s, but are probably negligible today. Investigation of isomer profiles of PFOS and precursors in herring raised further questions about the relative importance of different human exposure pathways for PFOS (**paper IV**).

Svensk sammanfattning

Per- och polyfluorerade alkylsubstanser (PFAS) utgör en stor grupp av organiska antropogena miljöföroreningar. Dessa kan delas upp i två undergrupper, 1) perfluoralkylsyror (PFAA) och 2) så kallade prekursorer, dvs sådana PFAS som potentiellt kan omvandlas till PFAA i miljön, eller i människan. Av särskild angelägenhet är långkedjiga PFAA, på grund av deras persistens, bioackumulerbarhet och toxicitet. PFAS har använts över hela världen i ett brett fält av industriella applikationer och de ingår i många konsumentprodukter. Idag är de globalt spridda i miljön och deras förekomst har påvisats i såväl vilda djur som människa. Födointaget (mat och dricksvatten) anses vara den dominerande källan för PFAS i människa. För kvantifiering av PFAS i mat och dricksvatten, med avsikten att förstå människans exponering av dessa ämnen, krävdes förbättrade analytiska tekniker.

Huvudmålet för detta doktorandarbete var att utveckla känsliga, exakta och validerade analytiska metoder för bestämning av ett antal PFAA och utvalda prekursorer i matprover, förpackningsmaterial och dricksvatten. Metoderna bygger på vätskekromatografi (LC) kopplad till olika masspektrometriska (MS) tekniker. Födoämnesprover extraherades med lösningsmedelsextraktion följt av ett fastfasextraktionssteg (SPE) för upprening. Uppreningen med SPE utnyttjades även som ett fraktioneringssteg för att separera joniserade och neutrala PFAS i multi-komponentmetoder omfattande PFAA och deras prekursorer.

Artikel I beskriver utvecklingen av en metod för kvantifiering av perfluoralkylkarboxylsyror (PFCA), perfluoralkansulfonsyror (PFSA) och perfluoralkylfosfonsyror (PFPA) i dricksvatten vid pg/l-nivåer. Elueringsmedlen som användes vid SPE-upprening och LC/MS-analys innehöll metylpiperidin som tillsats. Detta visade sig vara nödvändigt för att uppnå tillfredsställande metodutbyte, utmärkt kromatografisk separation samt god detektorkänslighet för PFPA. I **artikel II** presenteras en ny metod för kvantitativ bestämning av samma tre grupper av PFAA, vid låga pg/g-nivåer i ett brett spann av matmatriser. Här visades att PFPA och andra långkedjiga PFAA har en stark benägenhet att binda till fasta ytor. En kvantifieringsmetod baserad på matrisanpassade och extraherade kalibreringsstandarder föreslås för korrekt kvantifiering av PFPA, matris- och adsorptionseffekter till trots. En första kontroll av olika kranvatten och matprover (**artikel I och II**) visade att human exponering i Europa för PFPA inte är oroväckande hög.

En metod för samtidig analys av PFCA och polyfluoralkylfosfatestrar (PAP, potentiella prekursorer till PFCA) i mat och förpackningsmaterial utvecklades enligt beskrivningen i **artikel III**. Metoden var överlägsen existerande metoder vad gäller kromatografisk upplösning och känslighet för PAP. Metodens detektionsgräns för matprover var entals pg/g eller lägre. En kontroll av utvalda födoämnen och deras förpackningar visade att PAP potentiellt kan svara för den totala humanexponeringen vad gäller PFCA. Tidstrender för PFSA (inklusive perfluoroktansulfonsyra, PFOS) och deras sulfonamidbaserade prekursorer i strömmingsmuskel under perioden 1991-2011 studerades i **artikel IV.** En analytisk multikomponentmetod utvecklades för PFSA samt tre grupper av sura och neutrala prekursorer. Hastigt sjunkande tidstrender observerades för PFOS-prekursorer, medan koncentrationen av PFOS inte förändrades signifikant över tiden. Detta innebär att prekursorer i matfisk kan ha spelat en viktig roll för human exponering för PFOS på 1990-talet, men att de idag förmodligen är försumbara. Studiet av isomerprofiler för PFOS och prekursorer i strömming aktualiserade vidare frågeställningar rörande den relativa vikten av olika humana exponeringsvägar för PFOS (**artikel IV**).

Abbreviations

diPAP = Polyfluoroalkyl phosphate diester

ECF = Electrochemical fluorination

ESI = Electrospray ionization

FASAA = Perfluoroalkane sulfonamido acetic acid

FASA = Perfluoroalkane sulfonamide

FASE = Perfluoroalkane sulfonamido ethanol

FOSA = Perfluorooctane sulfonamide

GC = Gas chromatography

HPLC = High performance liquid chromatography

monoPAP = Polyfluoroalkyl phosphate monoester

MS = Mass spectrometry

MS/MS = Tandem mass spectrometry

PAP = Polyfluoroalkyl phosphate ester

PFAA = Perfluoroalkyl acid

PFAS = Per- and polyfluoroalkyl substance

PFBA = Perfluorobutanoic acid

PFBS = Perfluorobutane sulfonic acid

PFCA = Perfluoroalkyl carboxylic acid

PFOA = Perfluorooctanoic acid

PFOPA = Perfluorooctyl phosphonic acid

PFOS = Perfluorooctane sulfonic acid

PFPA = Perfluoroalkyl phosphonic acid

PFSA = Perfluoroalkane sulfonic acid

PreFOS = PFOS precursor

QToF-HRMS = Quadrupole time-of-flight high resolution mass spectrometry

SPE = Solid phase extraction

triPAP = Polyfluoroalkyl phosphate triester

UHPLC = Ultra high performance liquid chromatography

(U)HPLC = UHPLC and/or HPLC

List of papers

This thesis is based on four individual scientific research papers.

Paper I

Simultaneous determination of perfluoroalkyl phosphonates, carboxylates, and sulfonates in drinking water. Ullah S, Alsberg T, Berger U. *J. Chromatogr. A.* **2011**, 1218, 6388-6395.

Paper II

Determination of perfluoroalkyl carboxylic, sulfonic, and phosphonic acids in food. Ullah S, Alsberg T, Vestergren R, Berger U. *Anal. Bioanal. Chem.* **2012,** 404, 2193-2201.

Paper III

Polyfluoroalkyl phosphate esters and perfluoroalkyl carboxylic acids in target food samples and packaging – method development and screening. Gebbink WA, Ullah S, Sandblom O, Berger U. *Environ. Sci. Pollut. Res.* **2013**, DOI: 10.1007/s11356-013-1596-y.

Paper IV

Temporal trends of perfluoroalkane sulfonic acids and their sulfonamide-based precursors in herring from the Swedish west coast 1991–2011 including isomer-specific considerations. Ullah S, Bignert A, Huber S, Berger U. Manuscript.

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Statement of responsibility

My contributions to the four papers included in this thesis were:

Paper I and II

I have performed all analytical lab work regarding method development and validation, sample collection, extraction and analysis. I was also responsible for data acquisition, interpretation and took the lead in writing the paper.

Paper III

I contributed to this study by developing the analytical instrumental method. I was further involved in data interpretation and in writing the paper.

Paper IV

I contributed to the planning of this study. I developed and optimized the analytical method and was responsible for the chemical analysis of all samples. I also interpreted the data and took the lead in writing the paper.

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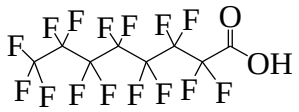
1. Introduction

1.1. Terminology and properties of per- and polyfluoroalkyl substances (PFASs)

Per- and polyfluoroalkyl substances (PFASs) is a generic name for all highly fluorinated aliphatic substances that contain one or more fully fluorinated carbon atoms, in such a manner that they contain the perfluoroalkyl moiety C_nF_{2n+1}- (Buck et al. 2011). The terminology 'perfluorinated' comprises compounds where all H atoms attached to C atoms in the non-fluorinated analogues have been replaced by F atoms, and 'polyfluorinated' means that all H atoms attached to one or several (but not all) C atoms have been replaced by F atoms. This structural replacement makes many of these substances immiscible with aqueous or hydrocarbon solvents (i.e. they are both hydrophobic and oleophobic). Perfluoroalkyl substances are considered more hydrophobic than hydrocarbons. For instance, perfluorooctane is not miscible with the more polar octane. The C-F bond is the most stable single bond known in organic chemistry. As a result, many perfluorinated PFASs are inert against hydrolysis, photolysis, microbial degradation and metabolism, even at relatively high temperatures. This extraordinary chemical and thermal stability is favorable in industrial applications, but it also makes some PFASs very persistent in the global environment. Because of the oleophobic and hydrophobic nature of the fluorinated carbon chain, PFASs provide highly useful and durable properties as monomer surfactants as well as incorporated into polymers (Kissa 2001). The two main subsets of PFASs investigated in this thesis are the highly persistent perfluoroalkyl acids (PFAAs) and so called precursor compounds, i.e. PFASs that can potentially be transformed in the environment or in humans to form PFAAs. Compound names, acronyms and molecular formulas of the PFASs selected for this thesis are listed in Table 1 and a hierarchical overview is given in Figure 1.

PFAAs are surfactants with unique physical-chemical properties such as an extraordinary high surface tension. They consist of a fully fluorinated carbon chain of typically three to fifteen carbon atoms and an acidic functional group. Perfluoroalkyl carboxylic acids (PFCAs) are a subgroup of PFAAs (**paper I-III**). PFCAs have the general chemical formula $C_nF_{2n+1}COOH$. The most frequently studied PFCA is perfluorooctanoic acid (PFOA) with its chemical structure given below as example.



Perfluoroalkane sulfonic acids (PFSAs) are a second major subgroup of PFAAs (**paper I, II and IV**). PFSAs have the general chemical formula $C_nF_{2n+1}SO_3H$. The most frequently studied PFSA is perfluorooctane sulfonic acid (PFOS). PFOS has recently been classified as a persistent, bioaccumulative and toxic substance that undergoes long-range transport. It has thus been included in the Stockholm Convention on Persistent Organic Pollutants under Annex B (Wang et al. 2009). Its chemical structure is given below.

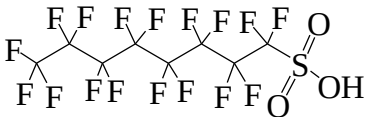


Table 1: Compound names, acronyms and molecular formulas of PFASs included in this study.

Compound name	Acronym	Molecular formula
Perfluoroalkyl carboxylic acids (PFCAs)		
Perfluorobutanoic acid	PFBA	$CF_3(CF_2)_2COOH$
Perfluoropentanoic acid	PFPeA	$CF_3(CF_2)_3COOH$
Perfluorohexanoic acid	PFHxA	$CF_3(CF_2)_4COOH$
Perfluoroheptanoic acid	PFHpA	$CF_3(CF_2)_5COOH$
Perfluorooctanoic acid	PFOA	$CF_3(CF_2)_6COOH$
Perfluorononanoic acid	PFNA	$CF_3(CF_2)_7COOH$
Perfluorodecanoic acid	PFDA	$CF_3(CF_2)_8COOH$
Perfluoroundecanoic acid	PFUnDA	$CF_3(CF_2)_9COOH$
Perfluorododecanoic acid	PFDoDA	$CF_3(CF_2)_{10}COOH$
Perfluoroalkane sulfonic acids (PFSAs)		
Perfluorobutane sulfonic acid	PFBS	$CF_3(CF_2)_3SO_3H$
Perfluorohexane sulfonic acid	PFHxS	$CF_3(CF_2)_5SO_3H$
Perfluorooctane sulfonic acid	PFOS	$CF_3(CF_2)_7SO_3H$
Perfluorodecane sulfonic acid	PFDS	$CF_3(CF_2)_9SO_3H$
Perfluoroalkyl phosphonic acids (PFPAs)		
Perfluorohexyl phosphonic acid	PFHxPA	$CF_3(CF_2)_5PO_3H_2$
Perfluorooctyl phosphonic acid	PFOPA	$CF_3(CF_2)_7PO_3H_2$
Perfluorodecyl phosphonic acid	PFDPA	$CF_3(CF_2)_9PO_3H_2$
Perfluoroalkane sulfonamido acetic acids (FASAAs)		
Perfluorooctane sulfonamido acetic acid	FOSAA	$CF_3(CF_2)_7SO_2NHCH_2COOH$
N-Methyl perfluorooctane sulfonamido acetic acid	MeFOSAA	$CF_3(CF_2)_7SO_2N(CH_3)CH_2COOH$
N-Ethyl perfluorooctane sulfonamido acetic acid	EtFOSAA	$CF_3(CF_2)_7SO_2N(C_2H_5)CH_2COOH$
Perfluoroalkane sulfonamides (FASAs) and sulfonamido ethanols (FASEs)		
Perfluorooctane sulfonamide	FOSA	$CF_3(CF_2)_7SO_2NH_2$
N-Methyl perfluorobutane sulfonamide	MeFBSA	$CF_3(CF_2)_3SO_2NHCH_3$
N-Methyl perflurooctane sulfonamide	MeFOSA	$CF_3(CF_2)_7SO_2NHCH_3$
N-Ethyl perfluorooctane sulfonamide	EtFOSA	$CF_3(CF_2)_7SO_2NHC_2H_5$
N-Methyl perfluorobutane sulfonamido ethanol	MeFBSE	$CF_3(CF_2)_3SO_2N(CH_3)CH_2CH_2OH$
N-Methyl perfluorooctane sulfonamido ethanol	MeFOSE	$CF_3(CF_2)_7SO_2N(CH_3)CH_2CH_2OH$
N-Ethyl perfluorooctane sulfonamido ethanol	EtFOSE	$CF_3(CF_2)_7SO_2N(C_2H_5)CH_2CH_2OH$
Polyfluoroalkyl phosphate mono-, di- and tri-esters (mono-, di- and triPAPs)		
6:2 Fluorotelomer phosphate monoester	6:2 monoPAP	$(O)P(OH)_2(OCH_2CH_2C_6F_{13})$
8:2 Fluorotelomer phosphate monoester	8:2 monoPAP	$(O)P(OH)_2(OCH_2CH_2C_8F_{17})$
10:2 Fluorotelomer phosphate monoester	10:2 monoPAP	$(O)P(OH)_2(OCH_2CH_2C_{10}F_{21})$
6:2/6:2 Fluorotelomer phosphate diester	6:2/6:2 diPAP	$(O)P(OH)(OCH_2CH_2C_6F_{13})_2$
8:2/8:2 Fluorotelomer phosphate diester	8:2/8:2 diPAP	$(O)P(OH)(OCH_2CH_2C_8F_{17})_2$
10:2/10:2 Fluorotelomer phosphate diester	10:2/10:2 diPAP	$(O)P(OH)(OCH_2CH_2C_{10}F_{21})_2$
6:2/6:2/6:2 Fluorotelomer phosphate triester	6:2/6:2/6:2 triPAP	$(O)P(OCH_2CH_2C_6F_{13})_3$
Additional mono-, di- and triPAPs were included in the study, see **paper III** and Figure 1.		

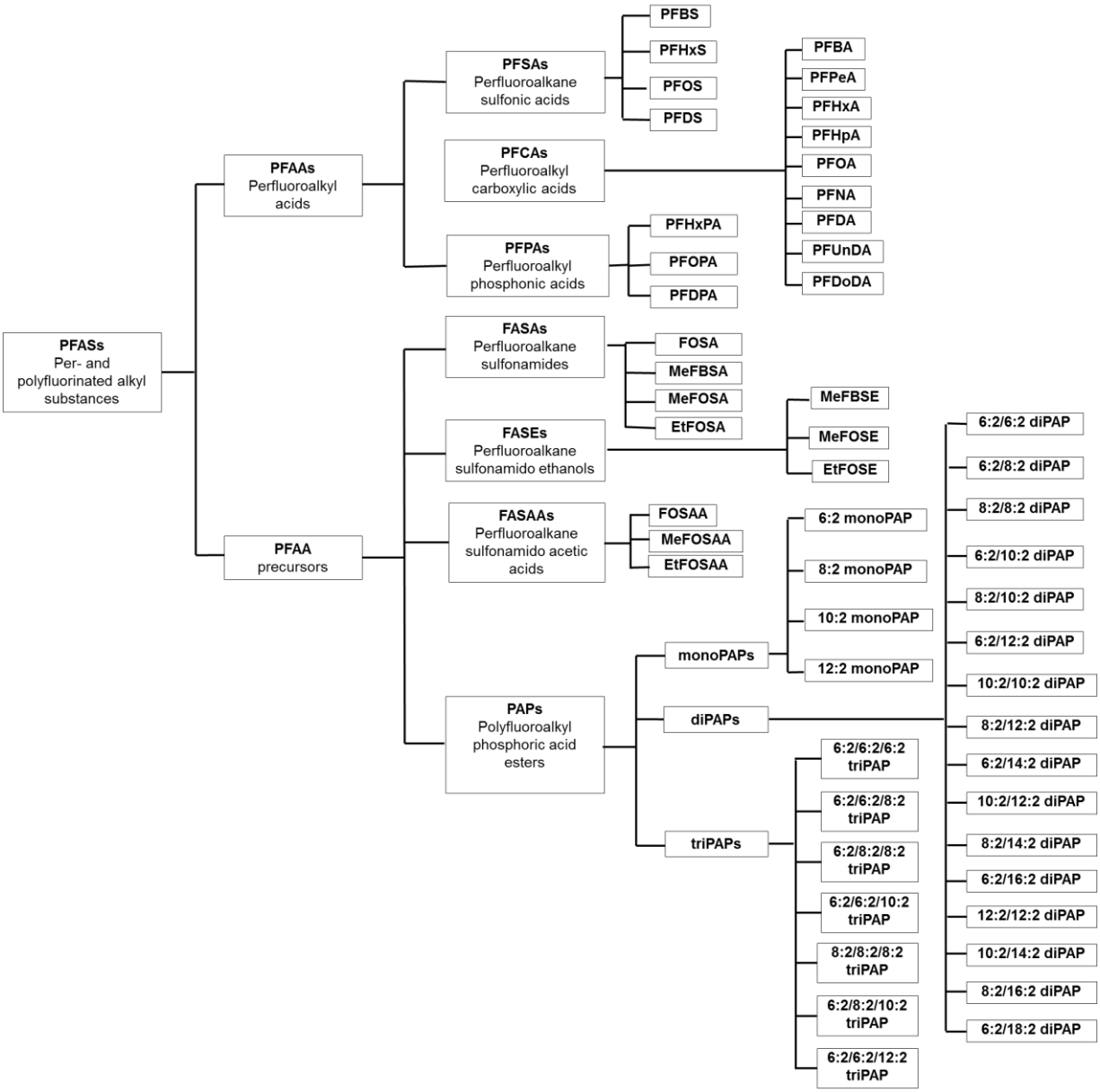
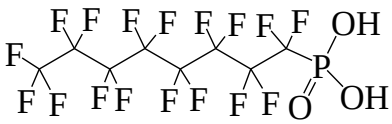


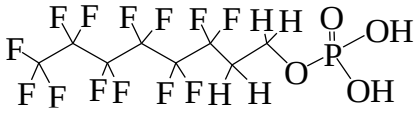
Figure 1: Hierarchy within the PFAS family including individual compounds investigated in this thesis. Acronyms are according to Buck et al. (2011).

Perfluoroalkyl phosphonic acids (PFPAs) are another subgroup of PFAAs that recently gained scientific interest around the world (**paper I and II**). The chemical formula of PFPAs is $C_nF_{2n+1}PO_3H_2$, containing two hydroxyl groups in the functional group. One example of a PFPA is perfluorooctyl phosphonic acid (PFOPA) with the structure shown below.

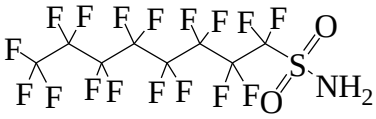


Along with direct emissions of PFAAs, also emissions of precursors of these compounds may play an important role for accumulation of PFAAs in the environment and in wildlife including humans, due to their potential to be (bio)transformed to PFAAs (also called indirect sources of PFAAs). Polyfluoroalkyl phosphate mono-, di- and tri-esters (mono-, di- and triPAPs, **paper III**) belong to the precursor compounds due to their potential to degrade to

PFCAs (D'Eon et al. 2011, Lee et al. 2010). PAPs are commercially used as surfactants. The chemical structure of e.g. the monoPAPs is $C_nF_{2n+1}CH_2CH_2OPO_3H_2$. As an example the structure of 6:2 monoPAP is given below.



Perfluoroalkane sulfonamides (FASAs) are another group of precursor compounds that can potentially be transformed to PFSAs (**paper IV**). The common chemical formula of FASAs is $C_nF_{2n+1}SO_2NHR$ with R being a H or an alkyl rest. The most studied FASA is perfluorooctane sulfonamide (FOSA) with its structure given below.



Several further groups of precursor compounds are derivatives of FASAs with the general formula $C_nF_{2n+1}SO_2NR^1R^2$. These include perfluoroalkane sulfonamido ethanols (FASEs) and perfluoroalkane sulfonamido acetic acids (FASAAs) (**paper IV**).

1.2. Production and use

PFASs have mainly been manufactured by two distinct industrial processes, i.e. electrochemical fluorination (ECF), a method for the preparation of fluorocarbon-based organofluorine compounds, and telomerization, a polymerization reaction to create longer straight-chain substances (Kissa 2001).

ECF (Figure 2), invented by Joseph Simons in 1940, is a process in which a hydrocarbon feedstock chemical (here octane sulfonyl fluoride) is dissolved in liquid anhydrous hydrogen fluoride (HF) within a copper electrochemical cell, leading to the replacement of all H atoms by F atoms. This process yields perfluorooctane sulfonyl fluoride (POSF), a major raw material for production of PFOS and other POSF derivatives such as FOSAs and FOSEs (Lehmler 2005; Martin et al. 2010).

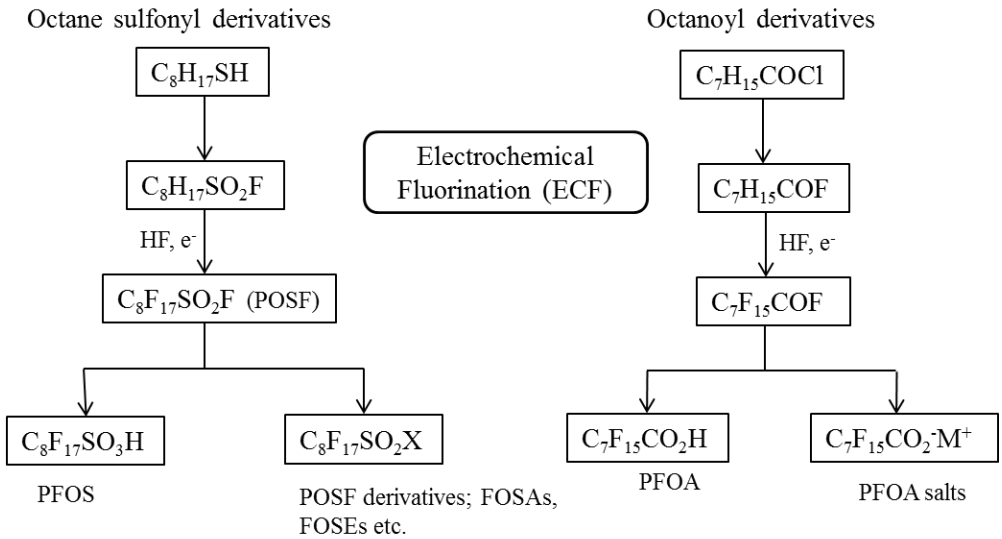


Figure 2: General synthesis process of ECF to produce PFOS, POSF derivatives, PFOA and its salts. Adopted from Buck et al. (2011).

The ECF process induces C-C bond breaking leading to a mixture of compounds including shorter chain homologues and branched isomers. The 3M Company was the major historical global manufacturer of perfluorooctane chemicals using ECF. 3M later voluntarily phased out the production during the years 2000-2002 and replaced it by an alternative shorter chain product line using perfluorobutane chemistry (Olsen et al. 2009).

Telomerization is the second important manufacturing process for PFASs (Figure 3). This technology has been used to manufacture fluorotelomer substances with high purity and PFOA as a side product. Telomerization yields straight-chain products (no branched isomers) with an even number of fluorinated carbons. In the first step pentafluoroethyl iodide (telogen) is reacted with tetrafluoroethylene (taxogen) to produce a mixture of longer chain perfluoroalkyl iodides (telomer A). In the second step the telomer A is further reacted with ethylene to form fluorotelomer iodide (telomer B). The resulting products of telomer A and telomer B are the main building blocks for further reactions, which produce fluorotelomer based surfactants and polymers. Additionally, telomer B can undergo hydrolysis to form perfluoroalkyl-2-ethanols, also known as fluorotelomer alcohols (FTOHs). The chemical structure of FTOHs is $F(CF_2)_n(CH_2)_2OH$ and they are often abbreviated as n:2 FTOH. FTOHs are also hydrolysis products of PAPs.

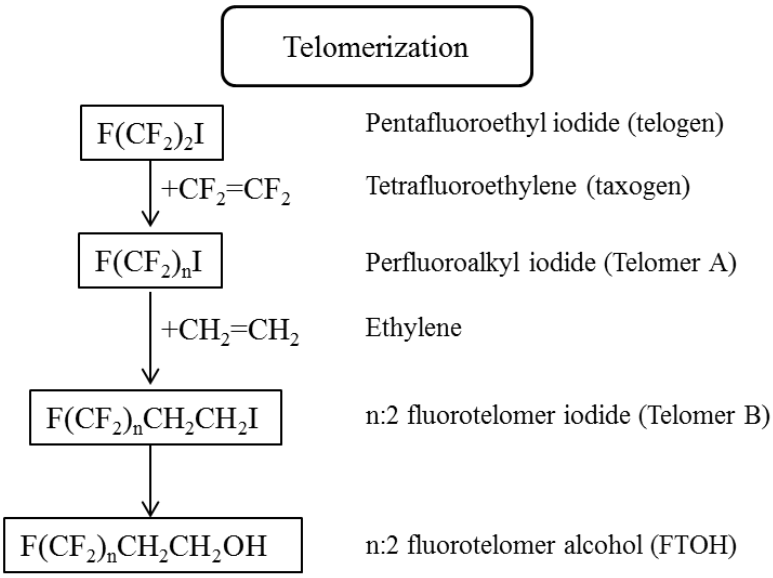


Figure 3: General process of telomerization to produce FTOHs. Adopted from Buck et al. (2011).

PFASs have been produced and widely used in numerous industrial and commercial applications since the 1950s (Buck et al. 2011, Kissa 2001, Prevedouros et al. 2006). Applications included paints, polishes, packaging materials, fire-fighting foams, insecticides, cook-ware, lubricants, cosmetics and surface treatment for paper, carpets, textile and leather products (water, oil and stain repellents often in the form of side-chain fluorinated polymers) as well as industrial processes such as the production of fluoropolymers (Kissa 2001). For a more detailed discussion of the application areas of individual PFASs see Buck et al. (2011) and the introductions of **paper I-IV**.

1.3. Emissions and global occurrence

The cumulative historical production volume of POSF was estimated to 96 000 t worldwide between 1970 and 2002 (Paul et al. 2009). Paul and coworkers further estimated that direct emissions from POSF-derived products have been the major source to the environment and resulted in releases of 450 to 2700 t PFOS into wastewater streams. After 3M's phase-out was completed in 2002, manufacturing of POSF related chemicals was taken up in other parts of the world such as in China (Wang et al. 2009, Xie et al. 2013), where PFOS and its higher-weight derivatives are still produced today. On the other hand, the cumulative global historical emissions of PFCAs between 1951 and 2004 were estimated to 3200 to 7300 t from direct and indirect sources, whereby the majority (~80%) of PFCAs have been discharged to the environment during manufacturing and subsequent use of fluoropolymer materials (Prevedouros et al. 2006). Today, environmental emissions of PFASs to air, water or soil still occur via a variety of pathways and diffuse sources such as from production sites, from downstream industry using PFAS formulations (e.g. textile industry), use of fire-fighting foams, via wastewater effluents or via consumer product use and disposal (Ahrens et al. 2011, Bossi et al. 2008, Kim et al. 2013, Müller et al. 2011, Perkola and Sainio 2013).

The global environmental occurrence of PFASs in wildlife was first discovered in 2001 with the help of a newly developed analytical method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) (Giesy and Kannan 2001, Hansen et al. 2001). This triggered a lot of research in method improvement and environmental monitoring of PFASs over the last dozen years. Due to the high-volume production and widespread emissions as well as the persistence, bioaccumulation potential and potential for long-range environmental transport of some PFASs (Conder et al. 2008, Paul et al. 2009, Prevedouros et al. 2006), these compounds have since been found to be ubiquitous in the global abiotic and biotic environment including humans (Glynn et al. 2012, Houde et al. 2006 and 2011, Kannan et al. 2004, Kärrman et al. 2006, Lindh et al. 2012). Many PFASs have also been found in remote locations far from human activities (Butt et al. 2010, Houde et al. 2006, Zhao et al. 2012). Among all PFASs, PFOA and PFOS are the compounds which have been studied most extensively. PFOA and PFOS have both been found to be persistent, toxic and bioaccumulative in the environment and in wildlife (Andersen et al. 2008, Conder et al. 2008, Lau et al. 2004, OECD hazard assessment 2002).

1.4. PFASs in food and food contact materials

Food items can potentially be contaminated with PFASs through environmental accumulation, through food processing or via migration from surface-treated food contact materials (i.e. packaging materials in direct contact with food). Wastewater treatment plant effluents, landfill effluents, atmospheric deposition and sewage sludge used as fertilizer are sources of PFAS contamination to soil and environmental water bodies. As a result, these compounds can enter into cultivated plants (Felizeter et al. 2012, Stahl et al. 2009) and farm animals (Vestergren et al. 2013) in the terrestrial environment or they can accumulate through the marine or limnic food web into edible fish species (Berger et al. 2009). PFAS formulations are further utilized in food packaging to form grease- and water-repellent coatings for food contact materials made from paper and paperboard (Trier et al. 2011). Thus, migration of PFASs from food packaging materials is another potential source of PFAS contamination in food (Begley et al. 2008). PFASs have been quantified over an extensive concentration range in food items from various countries around the globe (Ericson et al. 2008a, Ji et al. 2012, Ostertag et al. 2009, Tittlemier et al. 2007), though some of the earlier findings may have been flawed by a lack of rigorous quality assurance (Vestergren et al. 2012a).

1.5. Human exposure and toxicology

Since the first identification of PFASs in human blood (Olsen et al.1999), the internal human exposure to these compounds (usually measured and expressed as concentrations in serum) has been investigated in many studies around the globe (Haug et al. 2011, Hölzer et al. 2011, Kannan et al. 2004, Vestergren et al. 2009). Temporal trend analyses showed a recent decrease in PFOS and PFOA concentrations in human serum (Glynn et al. 2012, Olsen et al. 2012, Yeung et al. 2013), which is likely due to the 3M phase-out of the production of these compounds in 2000-2002. On the other hand, longer chain PFCAs showed a recent increase in humans in several studies (Glynn et al. 2012, Haug et al. 2009, Wang et al. 2011, Yeung et al. 2013). The assessment of sources and pathways of human exposure to PFASs is a critically important research topic, which is still under debate despite a decade of intense research.

Human exposure to PFASs can occur through a number of routes, such as food consumption, drinking water, breast milk, ingestion of non-food materials, dermal contact, inhalation of house dust and inhalation of air. Among all of these, dietary intake has been identified as a major human exposure pathway to PFASs for the general population (Ericson et al. 2008a, Fromme et al. 2009, Vestergren et al. 2008 and 2012b, Yamaguchi et al. 2013). However, the exposure pattern appears to vary depending on the type of PFAS, geographical location, types of food and food consumption pattern. For example, fish consumption is considered to be an important exposure pathway for PFOS (Berger et al. 2009, Falandysz et al. 2006, Haug et al. 2010, Vestergren et al. 2012b, Yamaguchi et al. 2013), while drinking water was identified as a significant route of exposure for short chain PFCA and PFSA homologues (Vestergren et al. 2012b). The contribution of drinking water to the total intake of PFOA is estimated to be higher than for PFOS but still quite small (<10%) (Ericson et al. 2008b, Vestergren et al. 2009). Some geographical variations have also been reported. Kärrman et al. (2009) studied human blood levels in relation to dietary intake in two cities in Japan based on a steady state one compartment toxicokinetic model. According to the model, dietary intake of PFOS and PFOA accounted for 22.4 and 23.7%, respectively, of the serum levels of these two compounds in Osaka females, compared to 92.5 and 110.6%, respectively, in Miyagi females. These findings suggest that the relative importance of other exposure routes than dietary intake varies between different locations.

Internal human exposure to PFAAs is probably the result of a combination of direct exposure to PFAAs and exposure to precursors with subsequent biotransformation to PFAAs (D'Eon et al. 2011). Vestergren et al. (2008) estimated the relative importance of precursor compound degradation for human PFOS and PFOA levels of the general population to 2-5 and 2-8%, respectively. However, these results are still under debate (Vestergren et al. 2012b). In **paper III and IV** we investigated the potential role of precursors for internal human PFAA exposure in two case studies.

A series of laboratory studies on PFAS toxicity to animals (mostly rodents or mammals) have been conducted during the last decade. Most of the (earlier) studies focused on PFOA and PFOS and reported toxicity with endpoints such as decreased body weight, increased liver weight and hormonal disruption (Lau et al. 2006). It was reported that e.g. subchronic exposure of rats and monkeys to PFOS may lead to significant weight loss, increased liver weight and reduction of serum cholesterol and thyroid hormone (Seacat et al. 2002 and 2003). Additionally, PFOS has been shown to be toxic to aquatic organisms (Shi et al. 2008). Exposure to PFOA was associated with alteration of the fatty acid metabolism, production of hepatotoxicity, reduction of circulating thyroid hormones and androgen, and hypothermia in the rat (Lau et al. 2004).

Such findings together with the widespread occurrence and exceptional stability of PFOA and PFOS led to concern about their potential threat to human health. It is extremely difficult to extrapolate the observed effects to potential human health effects due to differences in toxic response to PFAAs between animal species and even between different genders within a given species. However, human epidemiological studies found a negative association between combined serum levels of PFOA and PFOS and sperm count in young men (Joensen et al. 2009), evidence for disruption of thyroid function by both PFOA and PFOS (Knox et al. 2011) and an association between PFOA levels and elevated cholesterol (Nelson et al. 2010). Recently, a review about epidemiological evidence on human health effects of PFOA was published (Steenland et al. 2010). Moreover, it was reported that the birth weight and size of newborns were negatively correlated with PFOA concentrations in serum (Fei et al. 2007). Epidemiological studies have raised a concern about chronic exposure effects of PFAAs, mainly in relation to cholesterol metabolism (Fletcher et al. 2013). It has also been shown that concentrations of PFOS and PFOA in blood positively correlated with thyroid disease in the US general population (Melzer et al. 2010).

1.6. State-of-the-art in PFAS analysis prior to this thesis

Prior to this PhD thesis only few studies of PFASs in food had been published (Ericson et al. 2008a, Fromme et al. 2007, Tittlemier et al. 2006 and 2007). These studies generally did not take precursors to PFAAs into account and did not analyze a range of homologues, but rather focused only on PFOA and PFOS. Furthermore, these early studies either lacked the analytical sensitivity necessary for reliable quantification of PFASs in food or they only investigated composite samples, not distinguishing between different food items. Available data on dietary intake of PFAAs differed substantially. The main reason for this was the lack of adequate analytical methods with rigorous quality control to measure a range of PFASs in dietary samples (Vestergren et al. 2012a). Insufficient sensitivity and accuracy were due to the combination of the very low (yet still relevant) levels of PFASs in foodstuffs and the analytical challenges inherent to PFAS trace analysis, such as matrix effects, background contamination, varying isomer patterns of analytes and lack of isotopically labeled standards for most relevant analytes. As a result, agreement of quantified concentrations between laboratories was often poor (Berger et al. 2011).

Prior to this thesis, method detection limits in analysis of PFASs (mostly PFAAs) in food or biota were typically in the range of 0.1 to 1 ng/g wet weight (ww). This was by far not low enough for reliable quantification of a suite of PFAAs in different food items, which is a prerequisite for exposure assessment and source apportionment. Typical levels of PFAAs in common high-consumption food items are in the low pg/g ww or even fg/g ww range (Vestergren et al. 2012b). Therefore, there was a need for developing methods with detection limits that were about two orders of magnitude lower than what could be achieved before. Such methods should preferably be multi-chemical methods comprising PFCAs, PFSAs and PFPAs, they should be quick and easy to use and they should be applicable to a wide range of food matrices and beverages. In addition to sensitivity, a development of methods suitable for routine analysis was also timely. Furthermore, methods should be developed for sensitive analysis of PFAA precursors in food enabling a first screening of these compounds in dietary items.

2. Aim and objectives

The overarching aim of this PhD thesis was to develop sensitive, precise, accurate and fully validated analytical methods for the determination of a range of PFAAs and selected precursor compounds in dietary samples including different kinds of food, food contact materials and drinking water.

Due to the protein binding capacity and fairly good water solubility of several of the PFASs, both solid foods and beverages may be pathways of human exposure. Thus, the target matrices for method development included protein- and lipid-rich food items (e.g. fish, meat, dairy), fiber- and water-rich food items (e.g. vegetables) and food composites/food duplicates as well as drinking water (tap water). The specific objectives of the different papers were to:

Paper I and II

Develop and validate trace analytical methods for the quantification of PFCAs, PFSAs and PFPAs in drinking water and in a wide range of food items. A first screening of PFPAs in dietary samples from Europe should reveal if this class of PFAAs is of potential concern for human exposure.

Paper III

Develop a method for determination of mono-, di- and triPAPs as well as PFCAs in food and food contact materials. Through analysis of targeted food samples and their packaging a first assessment of the potential importance of PAPs used in food contact materials for internal human exposure to PFCAs should be made.

Paper IV

Investigate the time trends of PFSAs and their sulfonamide-based precursors in fish with the aim to explore temporal changes in the relative importance of human exposure to precursors for total human exposure to PFSAs through fish consumption.

3. General approaches in method development

3.1. Selection of matrices

Earlier studies on human dietary exposure to PFASs (mostly PFOS and PFOA) have often been performed with food composite samples. This approach does not lead to information about the relative importance of different food items for dietary exposure to PFASs in general or to compound-specific differences in this relative importance. In the present study robust methods were developed that can be applied to a wide range of dietary samples. The following matrices were chosen in method development and sample analysis: i) drinking (tap) water (**paper I**), ii) solid food items including fish, meat and a variety of vegetables (**paper II and IV**), iii) food cauldrons/duplicates and composites (**paper II and III**), iv) food contact materials (**paper III**).

3.2. Extraction and cleanup

3.2.1. Solvent extraction

In solvent extraction (sometimes also referred to as solid-liquid extraction) a solvent or solvent mixture with selected properties (polarity, aromatic or aliphatic etc.) is added to a solid sample matrix to extract the soluble material including the target chemicals, while insoluble material can be separated by centrifugation. In complex extraction problems or for multi-chemical methods also a sequence of solvents with varying polarity or pH can be used. The extract can be subjected to additional treatment, i.e. further cleanup with e.g. solid phase extraction (SPE, see section 3.2.2.) or directly injected into a HPLC or gas chromatography (GC) system. Solvent extraction was applied in the present study to extract the PFASs (including precursors) from all solid samples (**paper II-IV**). The samples were homogenized before extraction and acetonitrile or methanol was used as solvent. In **paper II and III** an aliquot of 5 g of homogenized food sample was extracted with acetonitrile. Additionally, in **paper III** methanol was used to extract PFCAs and PAPs from food packaging materials. In **paper IV** PFSAs and their sulfonamide-based precursors were simultaneously extracted from 2.4 g homogenized herring muscle using acetonitrile as solvent.

3.2.2. Solid phase extraction (SPE)

Matrix effects often play an important role in trace analysis of PFASs (**paper I-III**). In order to achieve low detection limits and accurate results, disturbing matrix constituents need to be removed from the extract prior to instrumental analysis (Vestergren et al. 2012a). SPE cleanup can be used to simultaneously remove (endogenous) interfering compounds from a complex sample matrix and enrich the analytes of interest in the extract. In this process separation occurs via a variety of property differences of the analytes and co-extracted compounds resulting in different interactions with the solid sorbent and elution solvent. Understanding the specific physical-chemical properties of the analytes of interest helps in selecting a suitable SPE sorbent.

In the present thesis SPE was employed for extract cleanup and enrichment of the target compounds in all four sub-studies (**paper I-IV**). The analytes included in **paper I and II** were PFCAs, PFSAs and PFPAs and the matrices comprised drinking water and a variety of food items including food homogenates. Various SPE columns with different stationary phases were evaluated in **paper I and II**. Finally, the analytes were enriched on a mixed-mode C8+quaternary amine SPE column. The optimization of sample extraction and cleanup is described in detail in **paper I and II**. For the analysis of PAPs and PFCAs (mixture of neutral and anionic analytes) in **paper III** a mixed-mode weak anion exchange+reversed phase column was found to be the best in terms of analyte recoveries and efficient extract

cleanup. In **paper IV** PFSAs and their precursors (mixture of neutral and anionic analytes) were analyzed in herring muscle and liver samples. The best analyte enrichment and purification of the fish extracts was achieved using a mixed-mode C8+aminopropyl SPE cartridge followed by dispersive cleanup with graphitized carbon.

3.3. Instrumental analysis

3.3.1. Choice of instrumentation and ionization technique

The majority of studies published so far on PFAS (mainly PFAA) analysis employed liquid chromatography coupled to triple quadrupole tandem mass spectrometry (MS/MS) as detection method due to its broad availability and high sensitivity. Beside this, quadrupole time of flight (QToF) MS has also been used to analyze PFASs in environmental samples. Although today QToF analyzers usually show lower sensitivity in PFAS analysis than triple quadrupole MS/MS systems, they possess a range of other advantages (**paper I**, Berger et al. 2004, Martin et al. 2004). The final extracts from all four sub-studies of the present thesis (**paper I-IV**) were instrumentally analyzed on an Acquity ultra performance liquid chromatography (UPLC, registered trademark of Waters, Milford, MA, USA) system coupled to either a QToF Premier high resolution mass spectrometer (HRMS, Micromass, Manchester, UK) or, alternatively, a triple quadrupole tandem mass spectrometer (Xevo TQ-S, Waters). The QToF was utilized to analyze several groups of PFAAs (PFCAs, PFSAs and PFPAs) in drinking water and food samples as described in **paper I and II**. QToF-HRMS was used because of its high mass accuracy and high acquisition speed that allows for data acquisition in full scan mode resulting in chromatograms that contain information on co-extracted matrix constituents. The high mass accuracy allows for obtaining highly specific extracted mass chromatograms. Furthermore, response factors between different isomers of e.g. PFOS are similar in single stage MS, whereas they vary considerably in MS/MS (Riddell et al. 2009). Thus, quantification of the sum of all isomers of a given PFAA using a linear calibration standard is possible when using QToF-MS. The MS was operated with full scan data acquisition performed in three parallel scan functions for PFCAs, PFSAs and PFPAs in **paper I and II**. Additionally, Enhanced Duty Cycle (EDC) at a center mass of *m/z* 499 (PFOPA) was used in the function for PFPAs to increase the sensitivity in QToF-MS. In contrary, in **paper III and IV** the MS/MS system was used to analyze PAPs and PFCAs in food and food contact materials as well as PFSAs and their precursors in fish muscle and liver. The triple quadrupole instrument in multiple reaction monitoring (MRM) mode was chosen for these sub-studies due to the superior sensitivity compared to the QToF instrument, especially for some precursor compounds. E.g. the PAPs could not be detected in a preliminary test on the QToF instrument, while they showed very low instrumental detection limits in MRM acquisition (**paper III**).

The most frequently used ionization technique to analyze PFASs by mass spectrometry is electrospray ionization in negative ion mode (ESI^-). In ESI^- PFASs (particularly the acidic PFAAs) dissociate to create the pseudo-molecular ion $(M-H)^-$. This technique was used to analyze all target PFASs in all four of the present sub-studies, with the exception of FASEs as described in **paper IV**. In **paper IV** also a GC based method was used for analysis of alkyl-FASAs and FASEs. However, none of these analytes were detected in the investigated fish samples using the GC method. It is therefore not discussed here any further.

3.3.2. Liquid chromatography

Numerous instrumental separation methods for PFASs have been published, most of them based on liquid chromatography. For semi-volatile precursor compounds gas chromatography is often the method of choice (Jahnke and Berger 2009). In liquid chromatography a reversed

phase column and a mobile phase gradient of ammonium acetate buffered water and methanol (or, alternatively, acetonitrile (ACN)) are typically employed. In this study, high (or ultra-high) performance liquid chromatography ((U)HPLC) coupled to the mass spectrometric detector was used for analysis of all target analytes in all sub-studies. However, the (U)HPLC parameters were carefully optimized in each study as described in **paper I-IV**, in order to optimize the resolution (e.g. branched and linear isomers, signal shape of di-anionic compounds etc.), sensitivity and analysis time for each set of analytes. A detailed comparison of the methods used in **paper I-IV** including type of separation column and mobile phase composition is given in Table 2. For the first time 1-methyl piperidine (1-MP) as an ion-pairing agent was used in the mobile phase to increase the chromatographic resolution and detection sensitivity for di-anionic PFASs in the methods described in **paper I-III**. A detailed discussion of the advantages of using 1-MP is given in **paper I** as well as in section 4.1.3.

Furthermore, instrumental blank contamination is a challenge in trace analysis of PFAAs. Possible sources of instrumental blank contamination as well as techniques for reducing the contamination have been described (LIoyd et al. 2009, Yamashita et al. 2004). In the present study a trapping column was installed between the eluent mixer and the injector to trap and delay the PFAA contamination from the UHPLC system. In **paper I** a Zorbax Extend C18, 50 × 2.1 mm, 3.5 µm particles column (Waters) was used, while in **paper II-IV** a "PFC isolator column" (Waters "PFC kit") was used.

3.3.3. Quantitative analysis of branched isomers

It is worth to note that for most target analytes only the linear isomer was quantified in all sub-studies. This was due to the very low levels in the investigated sample matrices that prevented detection or quantification of branched isomers. However, in some studies branched isomers were quantifiable for PFOA, PFOS and/or FOSA. In these cases the linear isomer (lin) and sum of branched isomers (br) were quantified separately. Lin-PFOS and br-PFOS were quantified using the relative response factors (relative to the internal standard) of lin-PFOS and br-PFOS, respectively, obtained from a calibration standard consisting of a mixture of 78.8% lin-PFOS and 21.2% br-PFOS. The relative response factor of lin-PFOA or lin-FOSA (purely linear reference standards) was applied for quantification of both lin-PFOA and br-PFOA or lin-FOSA and br-FOSA, respectively. This may have imparted some uncertainty on the results for br-PFOA and br-FOSA. However, separate quantification of lin- and br-PFOA was only possible in **paper I** on PFAAs in drinking water using QToF-MS detection. As discussed in section 3.3.1. response factors of linear and branched isomers are expected to be similar in single stage MS (Riddell et al. 2009). On the other hand, lin-FOSA and br-FOSA were quantified separately in **paper IV** using MS/MS. Since the response factor of br-FOSA relatively to lin-FOSA in MS/MS is not known, concentration values of br-FOSA have to be considered semi-quantitative.

3.4. Method validation

Method validation is an important part of method development in chemical analysis. Method validation qualitatively and quantitatively evaluates a method's performance and sets boundaries to the method's application range. The developed methods in this study (**paper I-IV**) were validated by determining the procedural blank contamination, method detection limits (MDLs) and method limits of quantification (MLQs), analyte recoveries, method linearity, precision, accuracy, inter-method comparability and method applicability ranges. In the absence of blank contamination MDLs and MLQs were determined from analyte signals in chromatograms obtained from low-level contaminated or low-level spiked samples. MDLs and MLQs were defined at a signal-to-noise ratio of 3 and 10, respectively. Whole method

linearity was calculated for all analytes spiked to a non-contaminated sample matrix over a concentration range from the individual MLQs up to the highest expected concentrations in the investigated samples with 6 data points. This procedure integrates both detector linearity and potential concentration dependence of analyte recoveries. Additionally, compound specific recoveries at two different spike levels were determined in triplicate experiments performed at three different days. The coefficients of variation (CVs) from these triplicate experiments were used as a measure of method precision. As certified reference materials were not available for the selected sample matrices and analytes, accuracy was evaluated by analyzing samples that have previously been analyzed in worldwide inter-laboratory comparison studies or by controlled laboratory spike experiments (for PFPAs in **paper II**). Inter-method comparability was additionally assessed by analyzing the same set of samples using the developed method in **paper II** and another completely independent method recently developed in our lab (Vestergren et al. 2012a). All method validation experiments and their results are described in detail in **paper I-IV** and the respective Supplementary Material documents.

Table 2: Overview of the four developed methods.

		Method I (**Paper I**)	Method II (**Paper II**)	Method III (**Paper III**)	Method IV (**Paper IV**)
Extraction and cleanup	Analytes	PFCAs (C5-C12), PFSAs (C4, C6, C8, C10) and PFPAs (C6, C8, C10)	PFCAs (C4-C12), PFSAs (C4, C6, C8, C10) and PFPAs (C6, C8, C10)	Mono-, di- and triPAPs, PFCAs (C4-C15)	PFSAs (C4, C6, C8, C10) and selected precursors
	Matrix	Tap water (500 mL)	Diverse food matrices (5 g) Baby food was used as test matrix	Diverse food matrices (5g), packaging materials	Fish muscle and liver
	Extraction	CUQAX (C8 + quaternary amine)	ACN:water (90:10) and ACN	ACN or MeOH	ACN
	SPE column		CUQAX (C8 + quaternary amine)	Oasis WAX (mixed mode weak anion exchange)	CUNAX2 (C8+aminopropyl)
	Washing solvent	MeOH	MeOH:MTBE (95:5) containing 2% HCOOH	2% aqueous HCOOH and water	2% aqueous HCOOH and water
	Eluent	Warm MeOH:ACN (80:20) with 2% 1-MP	MeOH:ACN (60:40) with 2% 1-MP	MeOH, and 1% NH4OH in MeOH	MeOH, and 2% NH4OH in MeOH
	Recoveries	40-56%, 56-97% and 55-77% for PFPAs, PFCAs and PFSAs extracted from HPLC grade water	59-98% for all analytes extracted from baby food	72 – 110% for all detected analytes	50 -115% and 150% only for FASEs
Chromatography	Analytical column	Zorbax Extend C18	UPLC BEH C18	UPLC BEH C18	UPLC HSS T3
	Dimensions	150 × 1.0 mm	50 × 2.1 mm	50 × 2.1 mm	100 × 2.1 mm
	Particle size	3.5 µm	1.7 µm	1.7 µm	1.8 µm
	Injection volume	5 µL	5 µL	5 µL	5 µL
	Mobile phase	MeOH, ACN, water, 2 mM ammonium acetate, 5 mM 1-MP	MeOH, ACN, water, 2 mM ammonium acetate, 5 mM 1-MP	MeOH, ACN, water, 2 mM ammonium acetate, 5 mM 1-MP	MeOH, ACN, water, 2 mM ammonium acetate
	Flow rate	50 µL/min	120 µL/min	300 µL/min	250 µL/min
MS detection	Instrument	QToF-HRMS	QToF-HRMS	Xevo TQ-S-MS/MS	Xevo TQ-S-MS/MS
	Quantitative detection	ESI^-, full scan	ESI^-, full scan	ESI^-, MRM	ESI^-, MRM
	Method detection limits	0.095-0.17 ng/L, 0.027-0.17 ng/L and 0.014-0.052 ng/L for PFPAs, PFCAs and PFSAs	5.5-17 pg/g, 1.8-20 pg/g and 2.2-4.5 pg/g for PFPAs, PFCAs and PFSAs	As low as pg/g range	0.05 – 7 pg/g for all except for alkyl-FOSAs and FASEs (18 to 80 pg/g)
	Whole method linearity (r^2)[a]	≥0.99	≥0.99	≥0.99	≥0.99

[a] The concentration ranges used in the determination of method linearity are given in **paper I-IV**

4. Results and discussion

4.1. Analytical methods for PFCAs, PFSAs and PFPAs (paper I and II)

4.1.1. Development of extraction and cleanup methods

Quantitative analysis of PFAAs at low or sub pg/g levels in food and drinking water requires a relatively large sample intake in order to achieve a high sample to extract concentration factor. To avoid matrix effects that could jeopardize accurate quantification an efficient cleanup method is then needed that is able to eliminate the co-extracted matrix constituents (Vestergren et al. 2012a). One of the major challenges in the development of the methods described in **paper I and II** was to develop an efficient and quick sample cleanup that allowed quantitative enrichment of PFCAs, PFSAs and PFPAs in the extract, while any potentially disturbing matrix constituents should be removed from drinking water and a broad range of food matrices.

Different SPE sorbents were tested for enrichment of all analytes and removal of matrix constituents (see **paper I**). Only one commercial SPE cartridge, the CUQAX256 (C8 + quaternary amine, 500 mg – 6 mL; United Chemical Technologies, Bristol, PA, USA), was found to efficiently trap and release all target compounds including the PFPAs. The CUQAX256 column was consequently used in method I (for drinking water, **paper I**) and method II (for food samples, **paper II**). It contains a sorbent that provides hydrophobic interactions (C8) as well as anion exchange capability (quaternary amine). The structure is schematically drawn in Figure 4.

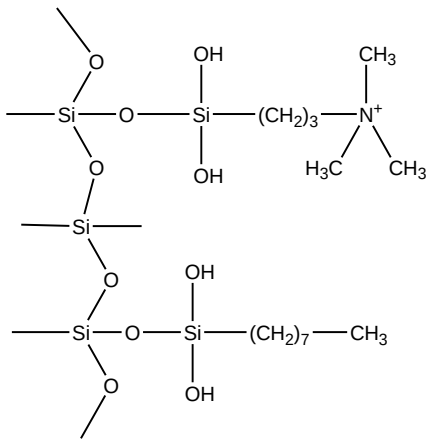


Figure 4: Structure of the sorbent of the applied SPE cartridge in **paper I and II** (CUQAX256).

In method I 500 mL tap water (drinking water) were directly applied to the SPE cartridge applying nitrogen gas pressure to extract and enrich the target analytes (**paper I**). In method II 5 g food samples were extracted using solvent extraction with acetonitrile:water (90:10) and pure acetonitrile (see Table 2). Pure acetonitrile efficiently extracted most of the PFCAs and PFSAs but addition of 10% water was necessary for quantitative extraction of PFPAs as well as the short chain PFBA and PFBS. The raw extract was concentrated and applied to the CUQAX256 cartridge (**paper II**). In both methods the cartridge was then washed and the analytes were subsequently eluted with a mixture of methanol and acetonitrile containing 2% 1-methyl piperidine (1-MP). In method I, however, the elution solvent had to be warmed to approximately 60 °C to achieve good recoveries for PFPAs. This was not necessary for food extracts and was omitted in method II to minimize co-elution of matrix constituents. The cleanup procedure proved to be very efficient in removing matrix constituents from a wide

range of sample matrices such as baby food, fish, meat, liver, food cauldrons, vegetables and tap water. After concentration, the extracts from the SPE cleanup could directly be injected into the instrumental system. Strong matrix effects were only observed for PFPAs. These were, however, a result of the tendency of PFPAs to sorb to solid surfaces in the absence of matrix (see **paper II** and section 4.1.4. and 4.4.).

4.1.2. Development of an instrumental separation and detection method

Although numerous methods have been described in the literature for the analysis of PFCAs and PFSAs, these methods did not result in satisfactory chromatographic resolution of the PFPAs. Variation of the mobile phase pH between 4 and 10, adjusted with formic acid and ammonium hydroxide, respectively, did not improve the signal shape for PFPAs. However, addition of 1-MP as a strong ion-pairing agent to the mobile phase, resulting in a pH ≥10, improved the chromatographic resolution and the instrumental response for PFPAs significantly (Figure 5). Additionally, also the response for the PFCAs and PFSAs increased (**paper I**). The role of 1-MP in the chromatography of PFPAs is discussed in detail in section 4.1.3. The same mobile phase composition and gradient were finally used in method I and method II, but the column was changed in method II to obtain shorter run times.

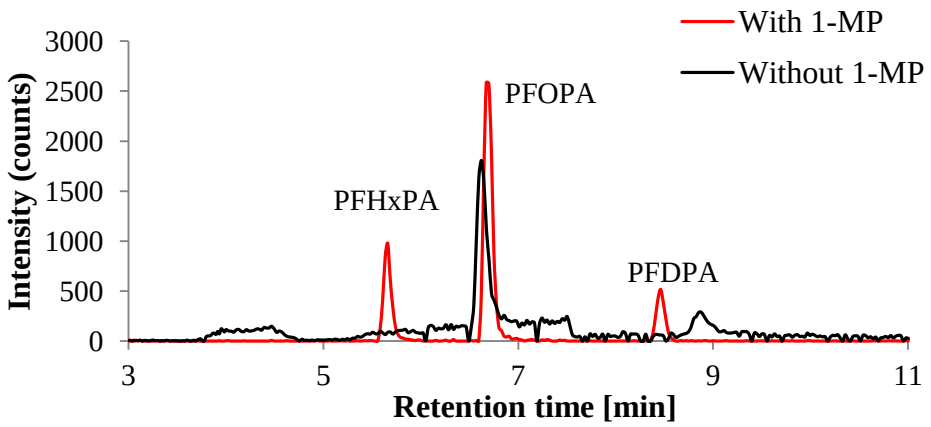


Figure 5: Extracted HPLC/HRMS mass chromatograms of PFPAs (25 pg on column) with and without 1-MP in the mobile phase.

Enhanced Duty Cycle (EDC) in QToF-MS was used in the scan function for PFPAs to increase the sensitivity for PFPAs, which otherwise suffer from a relatively low MS response compared to e.g. PFOS. By choosing *m/z* 499 (pseudo-molecular ion of PFOPA) as center mass for EDC, an increase of sensitivity was observed not only for PFOPA but also for PFHxPA and PFDPA, which led to the low method detection limits for PFPAs described in **paper I and II**.

4.1.3. The role of 1-methyl piperidine in cleanup, chromatography and ionization

In method I and II, the use of 1-MP in the SPE eluent and in the HPLC mobile phase was the key to successful and sensitive analysis of PFPAs (as well as PFCAs and PFSAs) in complex matrices. We assume that the interaction of the PFPAs with the SPE sorbent of the CUQAX256 cartridge is primarily an ionic interaction between the di-anionic phosphonate group and the quaternary amine of the sorbent (Figure 4). This interaction is very strong and allows washing out matrix constituents from the cartridge with methanol or acidified MeOH:MTBE (95:5) (Table 2) without eluting the PFAAs. 1-MP is then needed in the eluent

to undergo ion-pairing with the functional groups of the PFAAs and thus displace them from the quaternary amine moieties.

The increase in sensitivity especially for PFPAs in the instrumental method when using 1-MP in the mobile phase (Figure 5) was based on the combination of three effects. Firstly, better chromatographic resolution resulting in sharper peaks; secondly, suppressed baseline noise; and thirdly reduced sorption to surfaces in the autoinjector vial (see **paper II**). 1-MP presumably acted in two ways in HPLC. As an ion-pairing agent it masked the negative charges of the PFAAs leading to an increase in the retention on a C18 stationary phase through hydrophobic interactions. Furthermore, the (protonated) amine group of 1-MP may have sorbed to negative charges on the silica surface, thus shielding the remaining active sites of the silica.

A high pH value of the HPLC mobile phase favors the formation of negatively charged ions in MS detection, leading to better sensitivity for acidic analytes. The superior effect of 1-MP on the MS response for the PFAAs (compared to e.g. ammonium hydroxide at the same pH) is probably attributable to the low escaping force of the (protonated) 1-MP in the shrinking electrospray droplets. The ammonium ion would be depleted from the droplets through volatilization of ammonia, therewith driving the deprotonation of ammonium even further. In contrast, the concentration of 1-MP in the shrinking droplet remains unaltered or even increases. Hence, the pH increasing effect of 1-MP likely remains up to the point when the droplets burst due to electrostatic repulsion.

4.1.4. Method recoveries and matrix effects

Good method recoveries were obtained in both method I and II for all PFCAs and PFSAs when calculated versus a solvent based external calibration standard. Unusually high recoveries of up to several hundred percent were observed for PFPAs in both methods (see **paper I and II**). On the other hand, in method I the PFPA recoveries from spiked HPLC quality water were reasonable (Table 2). In **paper I** it was therefore hypothesized that the calculated high recoveries might have been due to an ionization enhancement effect of PFPAs in the mass spectrometer by co-eluting matrix constituents from the drinking water. However, when the effect was further investigated in **paper II**, it was shown that significant sorption of PFPAs to the polypropylene vial occurred in the solvent based calibration standard, i.e. in the absence of 1-MP and matrix residuals. This sorption effect led to injection of lower concentrations of PFPAs than anticipated in the calibration standard, which was probably the major reason for the overestimation of absolute recoveries in sample extracts, both in method I and II. Isotope labeled internal standards that would correct for such effects (as well as for recovery losses) are so far not available for PFPAs. Therefore, a matrix matched external calibration standard was used for calculation of true sample processing recoveries for method II. Results for all investigated PFAAs are given in Figure 6 and Table 2. Recoveries for PFCAs and PFSAs were not significantly different from calculations versus the solvent based standard. On the other hand, reasonable and satisfactory recoveries were now obtained for the PFPAs (Figure 6). As long as stable isotope labeled standards for PFPAs are not available, accurate quantification of these compounds can thus only be achieved by using a matrix matched and extracted calibration standard for each individual sample.

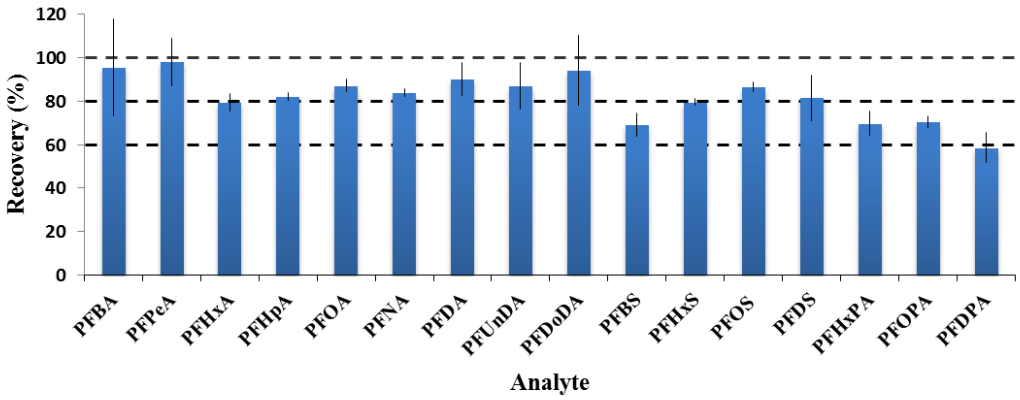


Figure 6: Whole method recoveries (*n*=3, standard deviation shown) of PFAAs spiked at 0.1 ng/g to a baby food sample. Recovery calculation was done versus a matrix matched external calibration standard.

4.2. Method development and analysis of PAPs (paper III)

The two main goals of the study described in **paper III** were to develop a sensitive method for simultaneous analysis of mono-, di- and triPAPs as well as PFCAs in complex samples and to use the method in a first screening of food and food packaging materials for the presence of PAPs and PFCAs.

4.2.1. Method development and sensitivity for PAPs and PFCAs

The analytical methods for PAPs published so far in the literature often lack a proper cleanup step in sample preparation, making them especially vulnerable for matrix effects. Therefore, a cleanup step was introduced based on Oasis WAX SPE. After loading of the sample extract, the target compounds were eluted in two separate fractions. In fraction 1 neutral triPAPs were eluted with pure methanol and in fraction 2 ionic compounds (mono-, diPAPs and PFCAs) were eluted with basic methanol. In **paper III**, both fractions were then analyzed with the same instrumental method. However, the neutral triPAPs could potentially be analyzed with higher sensitivity using another ionization method, e.g. in positive ion mode. This should be explored in the future and would make use of the fractionation potential of the SPE cleanup step.

Typical chromatographic methods used for PAPs so far employ a reversed phase column and a mobile phase consisting of ammonium acetate buffered methanol and water. In our lab these conditions led to good chromatography for di- and triPAPs. However, poor chromatographic resolution was observed for monoPAPs, likely due to their di-anionic properties. The same problem was earlier observed for the di-anionic PFPAs in **paper I** and successfully solved by using 1-MP in the mobile phase (see sections 4.1.2. and 4.1.3.). Also for monoPAPs addition of 1-MP led to a significant improvement of the chromatographic resolution as well as detection sensitivity in ESI^--MS/MS (**paper III**). Structural isomers of di- and triPAPs tend to co-elute from the (U)HPLC column. A slow gradient was therefore developed that allowed (partial) separation of most of the relevant di- and triPAPs. With the developed chromatographic method a total of 27 mono-, di- and triPAPs could be identified in a technical PAP mixture. The majority of these PAPs were also successfully separated from each other (Figure 7).

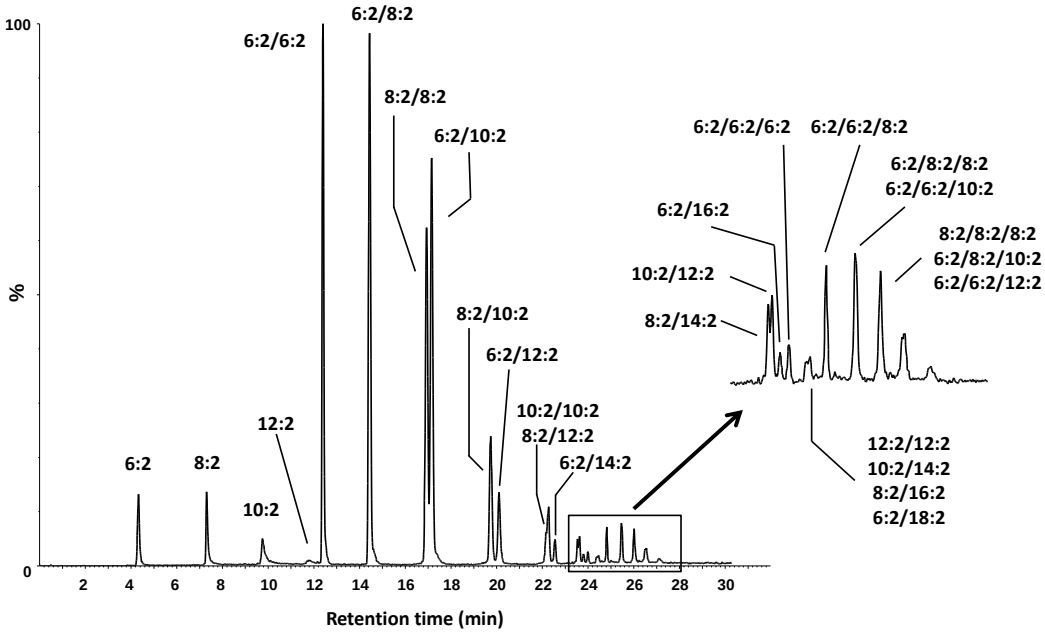


Figure 7: Total ion chromatogram of separated and identified mono-, di- and triPAPs in the technical PAP mixture Zonyl®-RP on a BEH C18 analytical column.

The achieved method detection and quantification limits for mono- and diPAPs were one order of magnitude lower than for methods published earlier (D'Eon et al. 2009 and 2011). This was the result of the combination of a highly sensitive MS/MS instrument and the use of 1-MP in the mobile phase, which significantly enhanced the ionization of the PAPs as well as other fluorinated surfactants as outlined in **paper I**.

4.2.2. PAPs and PFCAs in food and its packaging material

The developed method (method III) was applied to a variety of targeted food matrices as well as their packaging materials (suspected to contain PAPs) obtained in a local supermarket and a fast food restaurant in Stockholm in 2012. PAPs have previously been identified in food contact materials (Trier et al. 2011) and have been shown to migrate into food simulants (Begley et al. 2008). In the packaging materials analyzed in the present study (**paper III**), several mono-, di- and triPAPs were identified. DiPAPs were detected in all the materials with 6:2/6:2 and 6:2/8:2 diPAPs as the most prominent compounds. However, an estimation of extractable ΣdiPAPs in the presently analyzed packaging materials revealed about three to four orders of magnitude lower values than in food packaging materials obtained from the Danish, Swedish and Canadian market in 2009 (Trier et al. 2011). This could be an indication of recent changes in the production of food contact materials.

DiPAPs were also detected in all analyzed food samples, whereas mono- and triPAPs were not detected in any of them. The most dominant compounds were 6:2/6:2 and 6:2/8:2 diPAPs identified in food samples. Generally the highest detection frequency and concentrations were found for 6:2/6:2 diPAP ranging from 0.9 to 13 pg/g wet weight followed by 8:2/8:2 diPAP detected in six food samples at levels ranging from 1.0 to 3.6 pg/g wet weight. Additionally, twelve PFCAs (PFBA to PFPeDA) as possible degradation products of PAPs (D'Eon et al. 2011, Lee et al. 2010) were analyzed in the food samples and packaging materials. PFHxA to PFPeDA were detected in 12 of the 14 packaging materials and PFPeA to PFUnDA and PFTrDA were detected in all but one of the food samples at low pg/g wet weight levels. Comparison of levels and patterns between PAPs in packaging materials and corresponding

food samples did not give a clear picture on the potential migration behavior. Neither did comparisons between PAPs and PFCAs show a clear correlation between these two groups of PFASs (**paper III**). From this first screening of food samples it is thus not possible to draw conclusions on migration or degradation of PAPs used in food contact materials. However, from the concentrations of PAPs found in the targeted food samples it cannot be ruled out that PAPs do significantly contribute to human PFCA exposure, especially for individuals with a high intake of food packed in PAPs treated packaging materials (**paper III**). Furthermore, PAPs may have played an important role for PFCA exposure in Sweden only a few years ago (Trier et al. 2011) and/or may still play an important role in other parts of the world.

4.3. Temporal trends of PFSAs and their sulfonamide-based precursors in fish muscle (paper IV)

Several methods have been described for analysis of PFSAs in fish tissues including some recent studies achieving low pg/g detection limits (**paper II**, Guo et al. 2012, Vestergren et al. 2012a), but none of them is able to analyze a range of sulfonamide-based PFSA precursors. Some earlier studies have been published for simultaneous analysis of PFSAs and their precursors in fish or fish-like matrices (Ahrens et al. 2009, Asher et al. 2012, Lacina et al. 2011, Tittlemier et al. 2006), but they either did not include a wide range of neutral as well as acidic PFSA precursors or they lack the analytical sensitivity necessary to quantify precursors in fish muscle. Therefore, in order to investigate the temporal trends of PFSAs and their sulfonamide-based precursors in herring muscle between 1991 and 2011, a highly sensitive method had to be developed for simultaneous analysis of PFSAs and their precursors in fish tissues.

4.3.1. Method development and performance

When developing a multi-chemical method for trace analysis in complex matrices such as fish muscle and liver, the chemical properties of all analytes as well as of the matrix must be considered. In method IV (**paper IV**) both neutral and ionic compounds should be analyzed. Therefore, extraction and cleanup techniques were developed that encompassed both groups of substances. Solvent extraction with acetonitrile worked well for all analytes, however, also lipids from the fish matrix were co-extracted, posing a challenge for the subsequent cleanup step. Several SPE sorbents were tested for an efficient cleanup of the extract and enrichment of all target analytes. A CUNAX22Z SPE cartridge (mixed mode C8+aminopropyl; United Chemical Technologies) showed the best performance and was employed. In a similar way as in method III (**paper III**, see section 4.2.1.) neutral and ionic compounds were fractionated during elution. Details of the method are described in **paper IV**. Both fractions were initially analyzed by UHPLC/ESI$^-$-MS/MS. However, FASEs showed relatively high instrumental detection limits using this method. Therefore, the fraction with the neutral precursors was additionally analyzed by a previously developed GC/MS method. Despite method detection limits in the low pg/g wet weight range using the GC-based method, the FASEs were not detected in any sample. On the other hand, the UHPLC/MS/MS method achieved detection limits for PFSAs, FOSA and FOSAAs in the single digit to sub pg/g wet weight range and temporal trends for these compounds could be established, including individual quantification of branched and linear isomers of PFOS and FOSA.

4.3.2. Time trends of PFSAs and their precursors in herring muscle and implications for human exposure

Significantly decreasing or unchanged temporal trends were observed for all detected analytes in herring muscle from the Swedish west coast (Kattegat). Decreasing trends were found for

FOSA, MeFOSAA and EtFOSAA (PFOS precursors, also called PreFOS according to Martin et al. 2010) and for PFDS, whereas no significant changes over the whole study period were detected for PFHxS and PFOS (Figure 8). The PreFOS showed relatively short disappearance half-lives (compared to PFDS) of 4.6-6.6 yr. This rapid decrease of PreFOS concentrations in herring may reflect 3M's phase-out of the POSF product line together with the possibility of environmental transformation of PreFOS to PFOS or other transformation products. In combination with the unchanged levels of PFOS over the whole study period, the rapid declines of PreFOS levels led to a decreasing relative contribution of PreFOS to ΣPFOS+PreFOS over time, from more than 60% in the early 1990s to roughly 25% in recent years (Figure 3 in **paper IV**).

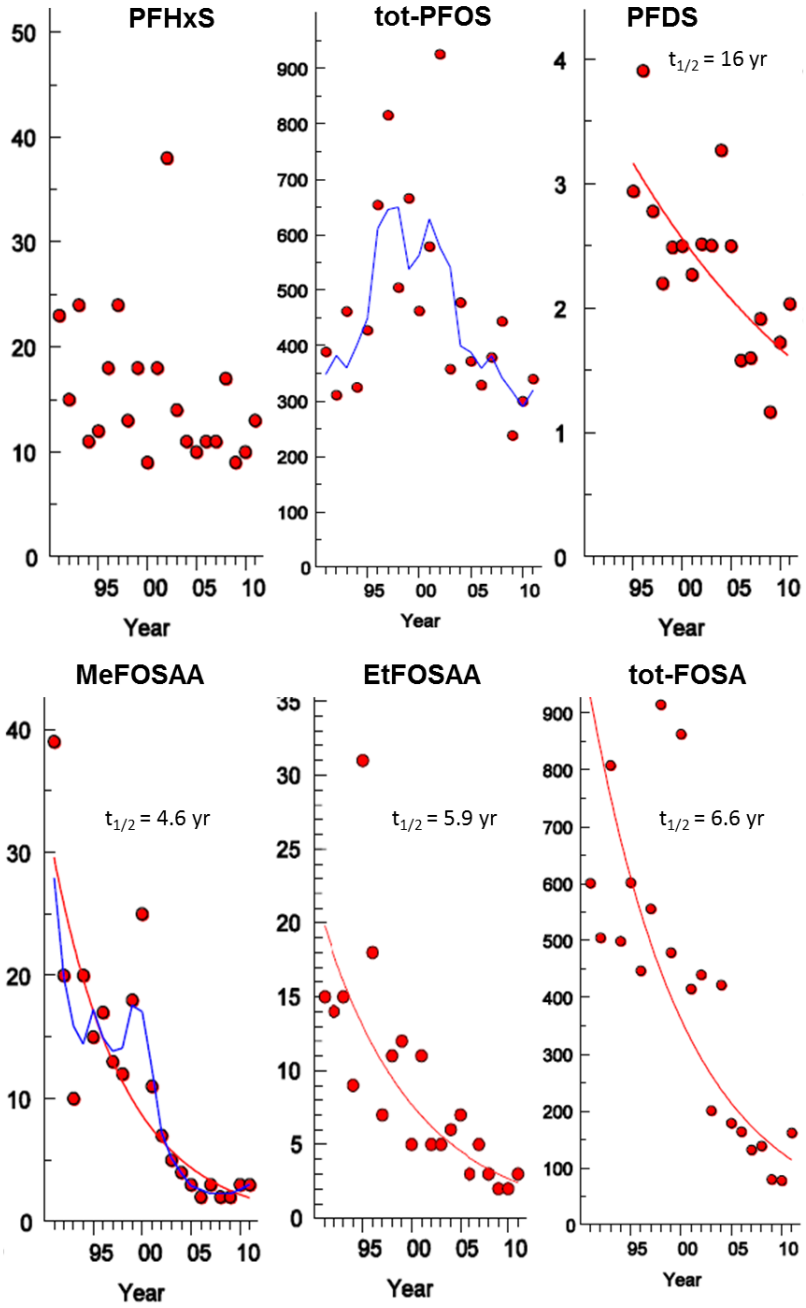


Figure 8: Time trends of PFSA and PreFOS concentrations in herring muscle (pg/g ww) 1991-2011. The red line depicts the log-linear regression of a significant decrease with $t_{1/2}$ as disappearance half-life. The blue line is a 3-point running mean smoother and is shown where significant.

Isomer specific analysis of PFOS and FOSA in herring muscle revealed that the branched isomers of PFOS and FOSA contributed with less than 10% to total PFOS and FOSA, respectively, throughout the investigated time period. This profile is different from the isomer profile of technical POSF derivatives produced by ECF (roughly 70% linear and 30% branched), which is assumed to constitute the major part of the cumulative environmental PFOS and PreFOS emissions. The pattern differences most likely reflect toxicokinetic discrimination within the herring or in lower trophic pray species, whereby branched PFOS isomers are eliminated more rapidly than linear PFOS (**paper IV**, Sharpe et al. 2010).

In contrary, PFOS isomer profiles in human serum or plasma usually show a relative enrichment of branched PFOS (30-55% of total PFOS, e.g. Glynn et al. 2012) compared to technical POSF products and a strong enrichment compared to fish. Nevertheless, there is strong evidence in the literature that fish consumption is an important current pathway of human exposure to PFOS (Vestergren et al. 2012b). Under the assumption that the patterns and trends of PFOS and FOSA observed in herring muscle in the present study are representative for fish and fish products consumed by the general Swedish population, we can conclude that fish consumption alone cannot explain the PFOS profiles observed in Swedish human blood (see detailed discussion in **paper IV**). Further investigations of compound-specific and species-specific toxicokinetics and of the patterns and concentrations of PFOS and all relevant PreFOS compounds in all human exposure media are necessary to fully understand human exposure pathways and processes for PFOS and eventually also for other PFSAs.

4.4. Sorption / matrix effects

As strong surfactants PFAAs are highly sorptive and may stick to any kind of surface (Higgins et al. 2006, Sepulvado et al. 2011, Tang et al. 2010). The sorption behavior of PFAAs, especially PFOS and PFOA, on activated carbon, anion exchange resins and polymers has been examined and described earlier (Deng et al. 2010, Senevirathna et al. 2011, Yu et al. 2009). The authors of all these studies assumed that electrostatic interactions, hydrophobic interactions and ion exchange interactions were involved in the sorption process. These interactions and resulting strong sorption or enrichment at phase interfaces affect not only the fate, transport and accumulation behavior of PFAAs in aqueous environments, but also their quantitative analytical determination.

In the present study the sorption behavior of ionic PFASs was investigated in **paper I-III**. In **paper I and II** we hypothesized that abnormally high method recoveries for PFPAs were due to matrix effects in ionization (ionization enhancement) and/or due to sorption of PFAAs to the polypropylene injection vials in the solvent based calibration standards (see section 4.1.4.). Therefore, in **paper II** we examined sorption to different types of injection vials for all investigated PFAAs (see Electronic Supplementary Material of **paper II**). Solutions of PFAAs in methanol or in methanol:water (1:1) all containing 5% 1-MP were prepared and filled in polypropylene (PP), polyethylene (PE) and glass autoinjector vials. The vials were allowed to equilibrate overnight and the PFAAs in the vials were subsequently quantified by HPLC/QToF-HRMS analysis. Normalized signal areas (relative to the highest area observed and set to 100% for each PFAA) are shown in Figure 9. The strongest sorption was observed for all analytes to the glass vials, while sorption of short chain PFAAs and of PFPAs from a pure methanol solution to the PP vial was considerably stronger than from a methanol:water (1:1) solution. Best overall results were obtained for the methanol:water (1:1) solution in the PE vial.

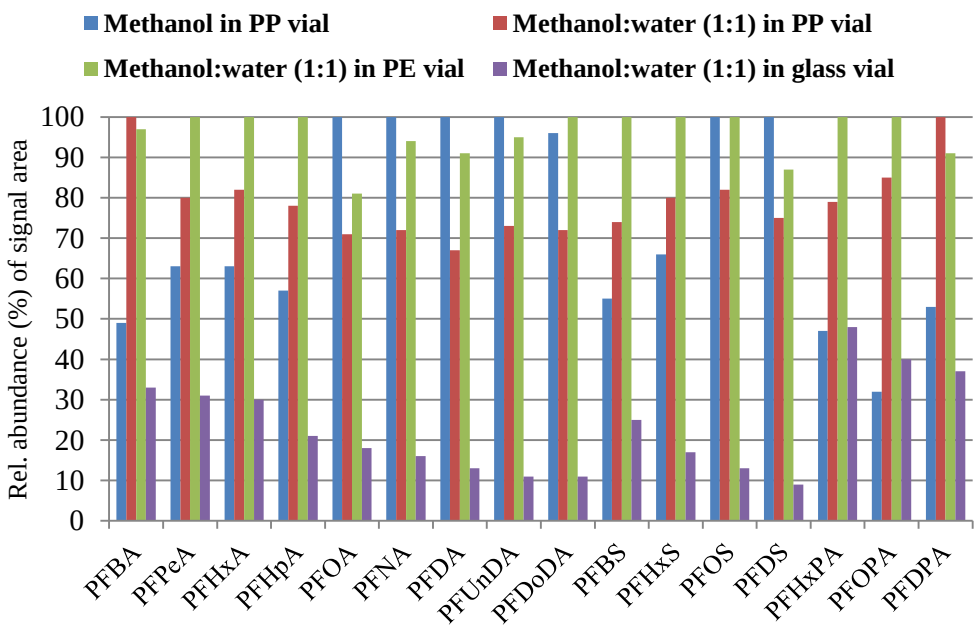


Figure 9: Sorption effects for PFCAs, PFSAs and PFPAs tested in different solvents in polypropylene (PP), polyethylene (PE) and glass vials.

However, different results for the sorption behavior of PAPs to injection vial walls were obtained in **paper III**. PAP standards dissolved in methanol resulted in higher signal intensities compared to PAPs dissolved in methanol:water or acetonitrile:water (1:1). These differences in sorption effects between different groups of ionic PFASs are most probably related to differences in solubility in different solvent compositions.

4.5. Improved methodologies

Highly sensitive and fully validated analytical methods for PFPAs in complex matrices did not exist prior to the present study. Only one study was available on analysis of PFPAs in environmental samples (surface water, D'Eon et al. 2009). However, the method described by D'Eon and coworkers is not applicable to other matrices and does not include a wide range of PFCAs and PFSAs. Thus, the methods described in **paper I and II** are the first multi-chemical methods including three different classes of PFAAs and at the same time enabling detection and quantification of PFPAs at pg/L levels in water or at low pg/g levels in solid matrices. Representative chromatograms of PFPAs spiked at low levels to drinking water and to a baby food sample are shown in Figure 10.

A common feature of the developed methods in this thesis is that they are easy to use and therefore easily transferable to other laboratories. All methods are in principle just based on a single cleanup step by SPE applying commercial cartridges. By carefully optimizing the SPE procedure (see e.g. flow-chart in Figure 11) a highly efficient extract cleanup can be achieved. In case of multi-chemical methods including chemical groups with different physical-chemical properties, even an extract fractionation can be achieved at the same time (**paper III and IV**, Figure 11). This is an advantage because analytes with different properties often require different instrumental techniques for detection at trace levels (**paper IV**).

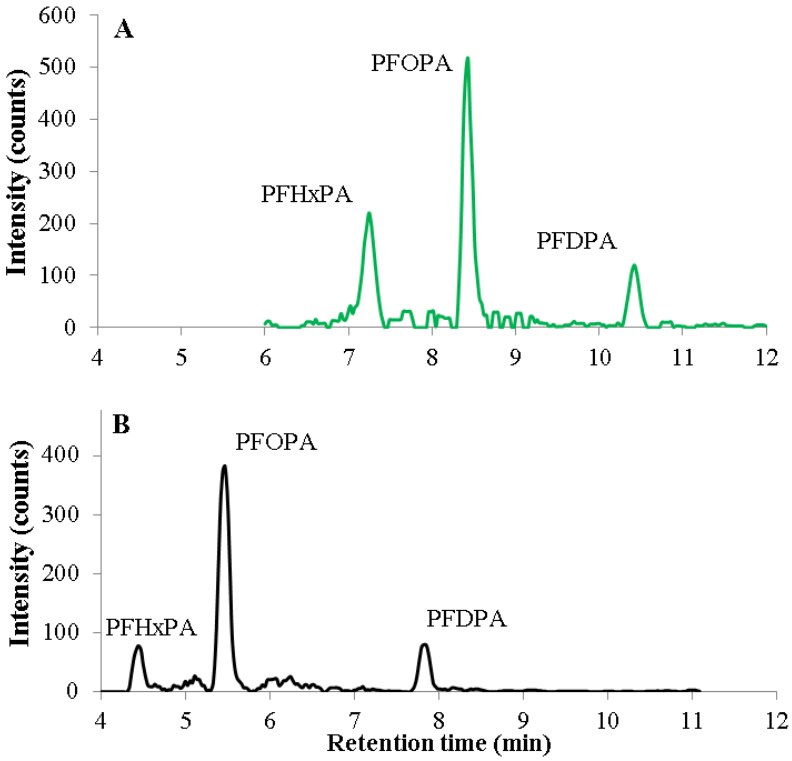


Figure 10: Extracted HPLC/HRMS mass chromatograms of PFPAs (A) spiked at 0.5 ng/L to tap water and (B) spiked at 60 pg/g to baby food. The differences in retention times between A and B are a result of different chromatographic conditions used in method I and II (Table 2).

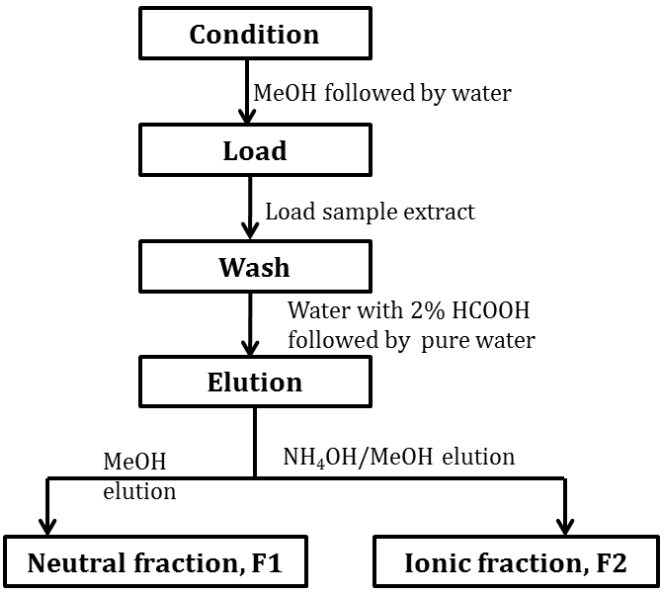


Figure 11: Cleanup and fractionation procedure by SPE for fish and food extracts described in **paper III and IV**.

Only few laboratories around the world are capable of analysing PFASs at single digit pg/g levels in complex matrices. Interlaboratory comparison studies with such low-contaminated samples have not yet been performed. However, to assess the inter-method comparability of method II described in **paper II** at ultra-trace levels, we analyzed a carp muscle sample using

method II as well as a completely independent method developed in our laboratory (Vestergren et al. 2012a). A total of 9 PFAAs (PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFHxS and PFOS) were quantified by both methods (**paper II**, Figure 12). Excellent agreement between the two sets of results was obtained. This was the first proof of inter-method comparability of quantitative results at low pg/g levels for a broad range of PFAA homologues.

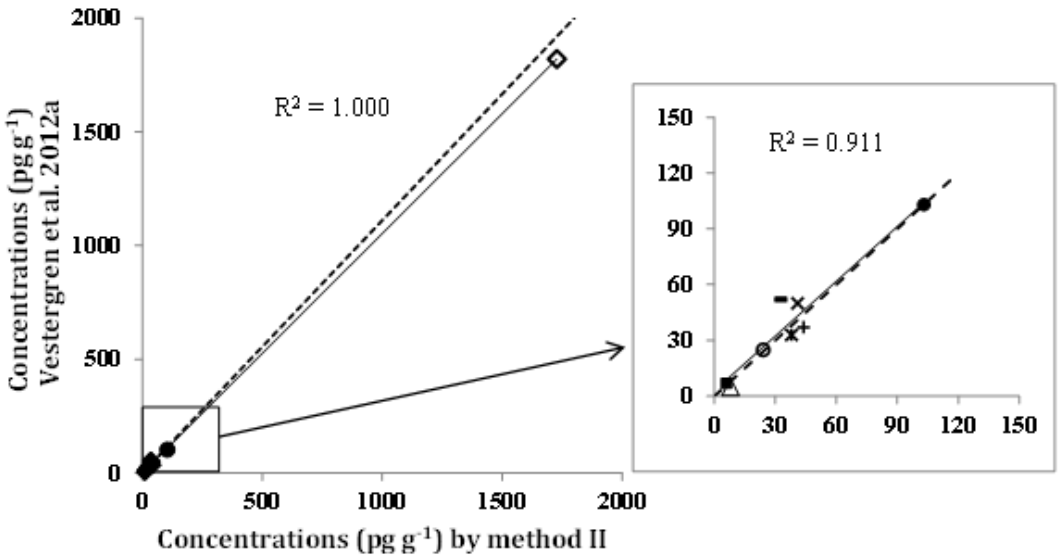


Figure 12: Comparison of quantified concentrations for 9 PFAAs in a fish muscle sample using two independent analytical methods. The following analytes were quantified: PFHxA (Δ), PFHpA (■), PFOA (-), PFNA (○), PFDA (●), PFUnDA (×), PFDoDA (∗), PFHxS (+), PFOS (✧). The solid lines represent the linear regressions for all data points (left) or all except PFOS (right) and the dashed lines represent a 1:1 agreement.

4.6. Implications of analytical results obtained by the developed methods

4.6.1. PFAAs in food and drinking water

In **paper I** seven drinking water (tap water) samples from six European countries, i.e. Italy, Germany, Belgium, The Netherlands, Norway and Sweden were analyzed. PFPAs were not found above their MDLs in the drinking water except for PFOPA in the two samples collected in Amsterdam, The Netherlands, where PFOPA was detected close to its MDL of 0.095 ng/L. Among all PFAAs highest levels were found for PFBS (18.8 ng/L) and PFOA (8.56 ng/L) in samples from Amsterdam and for PFOS (8.81 ng/L) in the sample collected in Stockholm, Sweden. In **paper II** food samples of diet duplicates, five different vegetables, meat and fish collected from Sweden, Germany, Belgium, Check Republic, Norway, The Netherlands and Bangladesh were analyzed. PFPAs were not identified in any of the food samples. The detection frequencies for PFCAs and PFSAs were 46%, 49%, 64% and 77% in duplicate diet, vegetables, meat and fish samples, respectively. Taken together, these results preliminarily suggest that human dietary intake of PFPAs in Europe is currently not of concern. However, the elevated levels of PFBS, a replacement chemical for PFOS, found in drinking water from Amsterdam call for better emission controls for this chemical and further research into drinking water purification techniques for PFAAs.

4.6.2. PFAAs and their precursors in food and packaging materials

In **paper III** targeted food samples were purchased at a Swedish grocery store and a fast food restaurant in Stockholm and analyzed together with their packaging materials for PFCAs and PAPs. Mono-, di- and/or triPAPs were detected in all food packaging materials and up to nine diPAPs (ranging from 0.9 to 36 pg/g) were detected in the food items along with a number of PFCAs. These preliminary results suggest that PAPs used in packaging materials may contribute to human PFCA exposure but are currently not likely to be a significant source of PFCAs for the general Swedish population. However, to better understand the potential importance of PAPs used in food contact materials for human exposure to PFCAs, migrations studies from packaging materials into the packed food matrices should be performed. In **paper IV** we demonstrated that the relative importance of sulfonamide-based PFOS precursors present in edible fish to human PFOS exposure has likely decreased over the last 20 years. Whereas dietary intake of PreFOS may have played a significant role for human serum PFOS levels in the 1990s, today the role of PreFOS is probably negligible. Specific changes in isomer patterns of PFOS and FOSA were investigated but are difficult to interpret, as differences in toxicokinetics of PFAS isomers exist and are not yet well understood (Benskin et al. 2010). Taken together, the results from the analyses performed in **paper III and IV** in combination with literature data on PAPs extracted from food contact materials (Trier et al. 2011) suggest that the relative importance of indirect exposure to the investigated precursor PFASs (mainly precursors to C6 and C8 PFAAs) has decreased during the last decade compared to the relative importance of direct PFAA exposure. However, this may be different for the longer chain C9-C11 PFCAs, which currently show increasing levels in human serum (Glynn et al. 2012).

5. Conclusions and outlook

In **paper I and II** two novel and highly sensitive methods are described for simultaneous determination of PFPAs, PFCAs and PFSAs in drinking water and food, respectively. These are the first published methods that are applicable to the analysis of three groups of PFAAs in virtually all types of food and beverage samples. The methods are thus an important tool for future dietary intake estimation and risk assessment of PFAAs. Due to the ease-of-use of these methods, they are even suitable for monitoring or screening studies involving large numbers of samples. The methods can potentially also be applied to environmental samples like waste water, surface water, sea water, sediment, soil or biota and may thus assist in future studies targeted at a better understanding of environmental processes of PFAAs.

The methods developed in **paper III and IV** are among the first highly sensitive methods that combine the analysis of PFAAs and selected precursor compounds in single samples. The role of PFAA precursors for the environmental transport and fate of PFAAs, including bioaccumulation and human exposure, is not well understood and a current research topic. Multi-chemical methods like the ones described in **paper III and IV** are the key for a better understanding of the relative importance of precursor compounds for the presence of PFAAs in different environmental media and in humans, today, in the past and in the future. The methods described in **paper III and IV** are not only the first multi-chemical methods for analysis of PFAAs and their precursors in food, but they are also expected to be applicable to virtually any type of solid matrix and can thus be used for environmental fate studies. In this regard, the methods could for example be used in biomagnification studies investigating the role of precursors in food chain transfer and magnification of PFAAs.

In terms of analytical method development, it can generally be concluded that methods for PFAAs have today reached a very high level of reliability (accuracy and precision) at ultra-trace levels, whereas methods for precursor compounds are usually less sensitive and less reliable. For many potential precursor compounds analytical methods are still lacking. The main focus in future method development should thus be on improving the quality and application range (number of analytes as well as variety of matrices) of analytical methods for precursors.

A critical factor for future improvements of the quality of analytical methods for PFASs in general is the availability of pure analytical standards (e.g. for PAPs) and isotopically mass-labeled standards (e.g. for PFBS, PFDS, C13-C15 PFCAs, PFPAs and PAPs). Furthermore, in **paper IV** we demonstrated the power of isomer specific analysis (branched vs. linear) as a tool to shed light on environmental processes of PFASs. Future method development should target at a better separation and individual quantification of isomers, not only for PFOS, but also for PFCAs and a variety of precursor compounds.

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