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Memòria presentada per optar al grau de
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Marta Llorca-Casamayor

Amb el treball:
"Analysis of perfluoroalkyl substances in food and evironmental matrices"

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Als meus éssers estimats

Si no conec una cosa, la investigaré
(Louis Pasteur, 1822-1895)

Un científic s'ha de prendre la llibertat de plantejar qualsevol
qüestió, dubtar de qualsevol afirmació,
de corregir els errors
(Julius Robert Oppenheimer, 1904-1967)

El dia que l'home se n'adoni de les seves profundes equivocacions,
s'haurà acabat el progrés de la ciencia
(Charles Chaplin, 1889-1977)

La investigació duta a terme en aquesta memòria ha estat possible gràcies al projecte finançats per la Unió Europea, CONffIDENCE ((211326–CP Collaborative Project) i la beca de curta estada ECO-ITN Marie Curie (PITN-GA-2009-238701)), així com també al projecte del Ministerio de Economía y Competitividad, SCARCE (CSD-2009-00065) i el projecte bilateral entre el Ministerio i el Marroc “Evaluación de la calidad de aguas residuales en Marruecos mediante biosensores” (2009MA0021)

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AGRAÏMENTS

Un cop escrita (i reescrita) la tesi, només em queda la redacció més difícil: la dels agraïments. Se'm fa molt difícil plasmar amb paraules els meus sentiments, i encara més els que són de gratitud. No obstant, potser si començo pel principi, tot plegat és més senzill.

En primer lloc m'agradaria agrair a la meva directora de tesi, la Dra. Marinella Farré, el que un dia em proposés de començar aquesta empresa. Sense tu no hauria arribat on sóc. Et vull agrair tot el que m'has ensenyat, i encara m'ensenyes, tant del mon de la investigació com de la vida, perquè m'has deixat prendre partit en aquest gran objectiu que et vas proposar, el qual avui sento tant meu com teu. Perquè m'has mostrat que si encares aquesta vida amb esforç i tenacitat (i una mica de sort) es pot arribar a on et proposis, fins i tot a la Fi del Mon. Com una vegada va dir algú que admiro molt: "tornaria a començar només per tornar a viure aquests moments...".

En segon lloc, vull agrair al meu director de tesi, el Prof. Damià Barceló, haver-me obert sempre les portes de la investigació i confiar en mi per realitzar aquest projecte. Gràcies per haver supervisat sempre els meus treballs, inclosa la present tesi, així com pels valuosos consells.

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To carry on, I want to thank Prof. Dr. Thomas P. Knepper to give me the opportunity to work in his laboratory with his wonderful team: Jutta, Sacha, Ian, Marcos, Toby, Vanesa and Heike. Thanks for the great time I spent in the laboratory.

Vull agrair també a la Sílvia Díaz l'haver-me donat la primera oportunitat de treballar en la investigació.

No puc oblidar tampoc el temps passat amb els company i amics que encara esteu al laboratori, estudiants en pràctiques, màsters, doctorands, post-doctorands, tècnics i secretàries, als que m'heu precedit en la marxa del CSIC, així com als que he tingut la sort de conèixer durant el breu període de la vostra estança. Podria dir molts noms però no pararia d'omplir pàgines i pàgines i ja us dic ara que quan començo a inspirar-me no hi ha comes que m'aturin ni per respirar. Em sento contenta d'haver passat tants bons moments amb tots vosaltres. També vull fer una especial menció als meus "hermanitos" per estar

sempre allí, els que encara hi sou o als que ja heu marxat (Linaki), així com també a la nostre “mare adoptiva”. Gràcies també als meus nous companys, heu fet que la transició sigui d’allò més agradable. A Girona em sento com a casa.

No puc oblidar tampoc als meus amics de fora, als que hi sou i els que no hi podeu ser. Gràcies per haver estat capaços de suportar les meves conductes curioses per “punxar” i no haver dubtat mai que aquest mot no era una cosa il·legal.

Al meu estimat Josep per haver estat sempre amb mi. Tot això no hagués començat si tu no m’haguessis donat el teu suport i, el que és més important, comprensió.

I per últim, però no menys important, a la meva família, tant la nova (Mª Josep, Miquel i Rocio) i la futura (família Cobos i companyia), com la de tota la vida (Mama, Anna, Nico, avis i yayes). En especial vull agrair a la meva mare tot el suport donat en tots aquests anys, per haver-me fet costat sempre, i per haver-me ensenyat que quan tens una fita no hi ha ningú que s’hi pugui interposar.

Gràcies a tots!

SUMMARY AND THESIS LAYOUT

Per- and poly- fluoroalkyl substances (PFASs) are a group of manmade substances synthesised for more than 60 years. Due to their specific properties, PFASs have been widely used for industrial applications including: electronics, textiles, food packaging, flame retardant formulae and laboratory tools, among others. However, it was during the last fifteen years ago that interest for this group of compounds as environmental pollutants was initiated. Due to their high release levels into the environment, stability and accumulation, PFASs have been found ubiquitous in the environment and in biota.

In this context, the main goal of this doctoral PhD thesis was the study of the occurrence, fate and behaviour of a wide range of PFASs (13 perfluoroalkyl acids, 4 perfluoroalkyl sulphonamides, 1 perfluoroalkyl sulphonamide and 3 perfluoroalkyl phosphonic acids), in the environment, in food and, finally, in some human matrices. In addition, the physicochemical properties studies and the aerobic biodegradation experiments of 3 perfluoroalkyl phosphonic acids were also evaluated.

Therefore, the first specific objective was the development of different analytical methods to study a wide range of PFASs in selected matrices. The analytical approaches have been developed according the specific requirements of each matrix, and the limitations presented in their analysis. The analytical methods have been based on the use of on-line turbulent flow chromatography, online pre-concentration and off-line solid phase extraction, followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Different mass analysers have been used and compared.

These methods have been employed to assess the presence of PFASs in different type of waters from different step along the water cycle: treated wastewater, river water, ground water and drinking water. The occurrence of PFASs has been also studied in fish and other food commodities. And finally, the PFAS accumulation in human samples was studied in cord blood and breast milk.

This work is divided into six chapters. The first chapter is the general introduction and the objectives of this PhD Thesis. In the introduction, different aspects are revised, such as synthesis and global production, environmental fate and occurrence, human accumulation, toxicology, regulations and future trends.

Chapters 2, 3, 4 and 5 present the experimental work undertaken during this PhD thesis research. Each chapter has been divided into a specific introduction, results (presented through the corresponding publications) and discussion. Part of the results contained in Chapters 2 and 5 correspond to the experimental work carried out in the laboratory of Prof. Dr. Thomas P. Knepper (Hochschule Fresenius, Institute for Analytical Research, Idstein, Germany) during a four month research internship under the framework of the Marie Curie ITN programme - Environmental ChemOinformatics (ECO), a part of the European Doctoral Programme.

Chapter 6 contains the general conclusions. Finally, a small summary in Catalan is presented.

The cited literature and indexes for tables and figures can be found at the end of this work, as well as the list of publications produced along the duration of the pre-doctoral studies, but which are not presented in this thesis.

The distribution of the publications included in this Thesis is the following:

Chapter 2: Environmental occurrence

Publication 1: "Analysis of perfluoroalkyl substances in waters from Germany and Spain." Llorca, M., Farré, M., Picó, Y., Müller, J., Knepper, T. P. and Barceló, D. (2012). Science of The Total Environment, 431(0): 139-150.

Publication 2: "Analysis of perfluorinated compounds in sewage sludge by pressurized solvent extraction followed by liquid chromatography–mass spectrometry." Llorca, M; Farré, M; Picó, Y; Barceló,D; (2011). Journal of Chromatography A, 1218 (30) 4840-4846**.**

Publication 3: "Fate of a broad spectrum of perfluorinated compounds in soils and biota from Tierra del Fuego and Antarctica." Llorca, M., Farré, M., Tavano, M. S., Alonso, B., Koremblit, G. and Barceló, D. (2012). Environmental Pollution 163(0): 158-166.

Chapter 3: PFASs in food

Publication 4: "Development and validation of a pressurized liquid extraction liquid chromatography-tandem mass spectrometry method for perfluorinated compounds determination in fish." Llorca, M., Farré, M., Picó, Y., and Barceló, D. (2009). Journal of Chromatography A 1216(43): 7195-7204.

Publication 5: "Study of the performance of three LC-MS/MS platforms for analysis of perfluorinated compounds." Llorca, M., Farré, M., Picó, Y., and Barceló, D. (2010) Analytical and Bioanalytical Chemistry 398(3): 1145-1159.

Publication 6: "Infant exposure of perfluorinated compounds: Levels in breast milk and commercial baby food." Llorca, M., Farré, M., Picó, Y., Teijón, M. L., Álvarez, J. G. and Barceló, D. (2010). Environment International 36(6): 584-592.

Chapter 4: PFASs accumulation in humans

Publication 7: "Analysis of perfluoroalkyl substances in cord blood by turbulent flow chromatography coupled to tandem mass spectrometry." Llorca, M., Pérez, F., Farré, M., Agramunt, S., Kogevinas, M. and Barceló, D. (2012). Science of The Total Environment 433(0): 151-160.

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LIST OF ABBREVIATIONS

General Acronyms

AcH: Acetic acid
APCI: Atmospheric pressure chemical ionisation
ASE: Accelerated solvent extractor
bisPFOPIA: Bis(perfluorooctyl) phosphinic acid
EC_{50}: Effective concentration to 50% of test organisms
ECF: Electrochemical fluorination
EI: Electron impact
EPA: Environmental Protection Agency
EPI: Enhanced product ion
ESI: Electrospray ionisation source
ESR: Electron Spin Resonance
FEP: Fluorinated ethylene propylene
FID: Flame ionization detection
GC: Gas chromatography
HLB-SPE: Hydrophilic-lipophilic-balanced solid phase extraction
HPLC: High performance liquid chromatography
IT: Ion Trap
K_{ow}: Distribution constant octanol-water
LC_{50}: Lethal concentration to 50% of test organisms
LC: Liquid chromatography
LC-MS: Liquid chromatography-mass spectrometry
LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry
LLE: Liquid-liquid extraction
LOEC: Lowest observed effect concentration
log K_D: Logarithm of the distribution coefficient
LRET: Long-range environmental transport
LSE: Liquid solid extraction
MALDI: Matrix-assisted laser desorption ionisation
MeOH: Methanol
MLOD: Method limit of detection
MOE: Margin of exposure
MS: Mass spectrometry
MTBE: Methyl tertiary butyl ether
NCI: Negative chemical ionisation
NMR: Nuclear Magnetic Resonance
NOEC: No-observable-effect-concentration
NOEL: No-observable-effect-level
NR-LETH: 100% mortality or 0% survival of organism
NR-ZERO: 0% mortality or 100% survival of organisms
OECD: Organisation for Economic Co-operation and Development
OW: Office of Water

PBT-chemical: Persistent, bioaccumulative and toxic
PCI: Positive chemical ionisation
PE: High density polyethylene
Peek: Polyether ether ketone
PFASs: Per- and Poly- fluorinated alkyl substances
PHA: Provisional Health Advisories
PLE: Pressurised liquid extraction
POPs: Persistent Organic Pollutants
PP: Polypropylene
PPAR-α: Peroxisome proliferator-activated receptor - alpha
PTFE: Poly(tetrafluoroethylene)
PUF: Polyurethane foam plugs SPE
PVDF: Poly(vinylidene fluoride)
PVF: Polyvinyl fluoride
Q: Quadrupole
QqLIT or QTrap: Hybrid quadrupole-linear ion trap
QqQ: Triple quadrupole
QqTOF: Hybrid quadrupole-time of flight
QTrap or QqLIT: Hybrid quadrupole-linear ion trap
QSAR: Quantitative Structure Activity Relationship
RP: Reversed phase chromatography
SIM: Selected ion monitoring
SPE: Solid phase extraction
SRM: Selected reaction monitoring
TBA: Tetrabutyl amonium
TBAS: Tetrabutyl ammonium hydrogen sulfate
TDCA: Taurodeoxycholic bile acid
Teflon: Tetrafluoroethylene polymers
TIS: Turbo ion spray
UAE: Ultrasound assisted extraction
UPLC: Ultra performance liquid chromatography
USEPA: United States Environmental Protection Agency
UV: Ultra Violet
WAX-SPE: Weak anion-exchange solid phase extraction
WF: Water Framework Directive
WWTPs: Waste water treatment plant
XAD: Resin-based SPE

Compounds

FOSA: Perfluoroctane sulfonamide
FOSE: Perfluorooctane sulfonamidoethanol
FSAs: Perfluorinated sulfonamides
FTAC: Fluorotelomer acrylate
FTAL: Fluorotelomer aldehyde
FTCAs: Fluorotelomer carboxylates

FTI: Fluorotelomer iodide
FTMAC: Fluorotelomer methacrylate
FTO: Fluorotelomer olefin
FTOHs: Fluorotelomer Alcohols
FTSs: Fluorotelomer sulphonates
FTSA: Fluorotelomer sulfonic acid
FTUAL: Fluorotelomer unsaturated aldehyde
FTUCAs: Fluorotelomer unsatured carboxylate
ip-PFNA: isopropyl perfluorononanoic acid
NBuFOSAs: *N*-butyl perfluorooctane sulphonamide
NEtFBSA: *N*-ethyl perfluorobutane sulphonamide
NEtFBSE: *N*-ethyl perfluorobutane sulphonamidoethanol
NEtFOSA: *N*-ethyl perfluooctane sulphonamide
NEtFOSAA: *N*-Ethyl perfluorooctane sulphonamidoacetic acid
NEtFOSE: *N*-ethyl perfluooctane sulphonamidoethanol
NEtFOS(M)AC: *N*-Ethyl perfluorooctane sulphonamidoethyl acrylate
NMeFBSE: *N*-methyl perfluorobutane sulphonamidoethanol
NMeFOSA: *N*-methyl perfluooctane sulphonamide
NMeFOSE: *N*-methyl perfluooctane sulphonamidoethanol
PAPs: Polyfluoroalkyl phosphate surfactants
PBSF: Perfluorobutane sulphonyl fluoride
PFBA: Perfluorobutanoic acid
PFBS: Perfluorobutane sulphonate
PFCAs: Perfluorocarboxylic acids
PFDA: Perfluorodecanoic acid
PFDoA: Perfluorododecanoic acid
PFDPA: Perfluorodeca phosphonic acid
PFDS: Perfluorodecane sulphonate
PFHpA: Perfluoroheptanoic acid
PFHxA: Perfluorohexanoic acid
PFHxDA: Perfluorohexadecanoic acid
PFHxI: Perfluorohexyl iodide
PFHxPA: Perfluorohexa phosphonic acid
PFHxS: Perfluorohexane sulphonate
PFNA: Perfluorononanoic acid
PFNL·H_2O: Perfluorononanal
PFOA: Perfluorooctanoic acid
PFODA: Perfluorooctadecanoic acid
PFOPA: Perfluoroocta phosphonic acid
PFOS: Perfluorooctane sulphonate
PFOSI: Perfluorooctanesulphinic acid
PFP: Pentafluorophenyl
PFPAs: Perfluoroalkyl phosphonic acids
PFPeA: Perfluoropentanoic acid
PFPIAs: Perfluoroalkyl phosphinic acids
PFSAs: Perfluorosulphonates

PFTeA: Perfluorotetradecanoic acid
PFTrA: Perfluorotridecanoic acid
PFUnA: Perfluoroundecanoic acid
POF: Perflurooctanoyl fluoride
POSF: Perfluorooctane sulphonyl fluoride
SFHx-HxDA: Perfluorohexylhexadecane
THPFOS: 1*H*,1*H*,2*H*,2*H*-perfluorooctane sulphonate
UAcid: Unsaturated acid

CHAPTER 1

Introduction

1.1. Definitions

Per- and Poly- fluorinated alkyl substances (PFASs) are manmade compounds synthesised for more than 60 years. According to the definition by Buck *et al.* (2011) perfluoroalkyl substances are “aliphatic substances for which all of the H atoms attached to C atoms in the nonfluorinated substance, from which they are notionally derived, have been replaced by F atoms, except those H atoms whose substitution would modify the nature of any functional groups” while polyfluoroalkyl substances are “aliphatic substances for which all H atoms attached to at least one (but, not all) C atoms have been replaced by F atoms, in such a manner that they contain the perfluoroalkyl moiety $C_nF_{2n+1}^-$”.

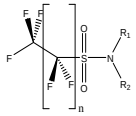
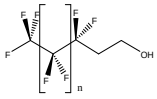
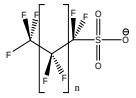
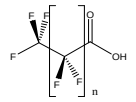
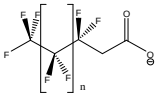
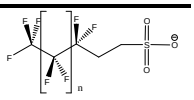
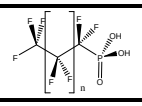
Per- and poly- fluoroalkyl substances are used as a backbone in some polymers. The fluoropolymers are “carbon-only polymer backbone with fluorines directly attached” while the side-chain fluorinated polymers are classified as “variable composition non-fluorinated polymer backbone, usually with polyfluorinated side chains” (Johansson *et al.* 2012).

PFASs are a wide group of compounds varying in their structure and, thus, exhibit different properties, environmental fate and toxicity, but their common trend is a general high stability by the carbon-chain bond (one of the strongest in nature). The atomic structure of fluorine has a Van der Waals radius of 1.35 Å, lower than the other halogens, and the highest electronegativity of the periodic table, being 3.98 in Pauling scale. As a consequence of the high electronegativity of fluorine, the carbon-fluorine bond is very strong (~110 kcal/mol) (Rayner-Canham 2003) and stable, making some of these compounds Persistent Organic Pollutants (POPs).

On the other hand, the high ionisation potentials of fluorine (1st: 1681 kJ/mol, 2nd: 3374 kJ/mol and 3rd: 6147 kJ/mol (Dean 1999) and its low polarisability leads to weak inter- and intramolecular interactions (3M 2000b). Perfluoroalkanes present a double character, hydrophobic and oleophobic, and when they are mixed with hydrocarbons and water, form three immiscible phases. However, these compounds are more commonly used with a charged moiety, such as carboxylic acid, sulphonic acid, phosphate or quaternary ammonium group, which decreases their intrinsically hydrophobic character. These functionalised chemicals present surfactant properties and make them suitable to be used as emulsifiers during fluoropolymerisation synthesis (3M 2000b) among other applications.

Most common PFASs, under the industrial point of view, are summarised in Table 1.1.

Table 1.1: Perfluoroalkyl chemicals

Class	Compound	Abbreviation	Formula	Chemical structure
Perfluorinated sulphonamides (FSAs)	*N*-methyl perfluorobutane sulfonamidoethanol	NMeFBSE	$F(CF_2)_4SO_2N(CH_3)CH_2CH_2OH$	
	N-ethyl perfluorobutane sulfonamidoethanol	NEtFBSE	$F(CF_2)_4SO_2N(CH_2CH_3)CH_2CH_2OH$	
	Perfluoroctane sulphonamide	FOSA	$F(CF_2)_8SO_2NH_2$	
	N-methyl perfluorooctane sulfonamide	NMeFOSA	$F(CF_2)_8SO_2N(CH_3)H$	
	N-ethyl perfluorooctane sulfonamide	NEtFOSA	$F(CF_2)_8SO_2N(CH_2CH_3)$	
	N-methyl perfluorooctane sulfonamidoethanol	NMeFOSE	$F(CF_2)_8SO_2N(CH_3)CH_2CH_2OH$	
	N-ethyl perfluorooctane sulfonamidoethanol	NEtFOSE	$F(CF_2)_8SO_2N(CH_2CH_3)CH_2CH_2OH$	
Fluorotelomer Alcohols (FTOHs)	4:2 fluorotelomer alcohol	4:2 FTOH	$F(CF_2)_4CH_2CH_2OH$	
	6:2 fluorotelomer alcohol	6:2 FTOH	$F(CF_2)_6CH_2CH_2OH$	
	8:2 fluorotelomer alcohol	8:2 FTOH	$F(CF_2)_8CH_2CH_2OH$	
	10:2 fluorotelomer alcohol	10:2 FTOH	$F(CF_2)_{10}CH_2CH_2OH$	
	12:2 fluorotelomer alcohol	12:2 FTOH	$F(CF_2)_{12}CH_2CH_2OH$	
Perfluorosulphona -tes (PFSAs)	Perfluorobutane sulphonate	PFBS	$F(CF_2)_4SO_3^-$	
	Perfluorohexane sulphonate	PFHxS	$F(CF_2)_6SO_3^-$	
	Perfluorooctane sulphonate	PFOS	$F(CF_2)_8SO_3^-$	
	Perfluorodecane sulphonate	PFDS	$F(CF_2)_{10}SO_3^-$	
Perfluorocarboxyli -c acids (PFCAs)	Perfluorobutanoic acid	PFBA	$F(CF_2)_4COOH$	
	Perfluoropentanoic acid	PFPeA	$F(CF_2)_5COOH$	
	Perfluorohexanoic acid	PFHxA	$F(CF_2)_6COOH$	
	Perfluoroheptanoic acid	PFHpA	$F(CF_2)_7COOH$	
	Perfluorooctanoic acid	PFOA	$F(CF_2)_8COOH$	
	Perfluorononanoic acid	PFNA	$F(CF_2)_9COOH$	
	Perfluorodecanoic acid	PFDA	$F(CF_2)_{10}COOH$	
	Perfluoroundecanoic acid	PFUnA	$F(CF_2)_{11}COOH$	
	Perfluorododecanoic acid	PFDoA	$F(CF_2)_{12}COOH$	
	Perfluorotridecanoic acid	PFTrA	$F(CF_2)_{13}COOH$	
	Perfluorotetradecanoic acid	PFTeA	$F(CF_2)_{14}COOH$	
	Perfluorohexadecanoic acid	PFHxDA	$F(CF_2)_{16}COOH$	
	Perfluorooctadecanoic acid	PFODA	$F(CF_2)_{18}COOH$	
Fluorotelomer carboxylates (FTCAs, FTUCAs)	6:2 fluorotelomer carboxylate	6:2 FTCA	$F(CF_2)_6CH_2CO_2^-$	
	6:2 fluorotelomer unsatured carboxylate	6:2 FTUCA	$F(CF_2)_6CHCO_2^-$	
	8:2 fluorotelomer carboxylate	8:2 FTCA	$F(CF_2)_8CH_2CO_2^-$	
	8:2 fluorotelomer unsatured carboxylate	8:2 FTUCA	$F(CF_2)_8CHCO_2^-$	
	10:2 fluorotelomer carboxylate	10:2 FTCA	$F(CF_2)_{10}CH_2CO_2^-$	
	10:2 fluorotelomer unsatured carboxylate	10:2 FTUCA	$F(CF_2)_{10}CHCO_2^-$	
Fluorotelomer sulphonates (FTSs)	6:2 fluorotelomer sulphonate	6:2 FTS THPFOS	$F(CF_2)_6CH_2CH_2SO_3^-$	
	8:2 fluorotelomer sulphonate	8:2 FTS	$F(CF_2)_8CH_2CH_2SO_3^-$	
	10:2 fluorotelomer sulphonate	10:2 FTS	$F(CF_2)_{10}CH_2CH_2SO_3^-$	
Perfluoro phosphonic acids (PFPAs)	Perfluorohexa phosphonic acid	PFHxPA	$F(CF_2)_6PO_3H_2$	
	Perfluoroocta phosphonic acid	PFOPA	$F(CF_2)_8PO_3H_2$	
	Perfluorodeca phosphonic acid	PFDPA	$F(CF_2)_{10}PO_3H_2$	

1.2 Production and industrial applications

PFASs were synthetized for first time during the 1940s. The Manhattan project was a research program led by the United States, with the United Kingdom and Canada, that produced the first atomic bomb during World War II. The project considered the need to replace some materials in order to reduce the radioactive contamination generated in the industrial-scale nuclear reactors. Then, DuPont engineers developed fluoro-chemicals, and Teflon was launched as a gasket material in the modular cell of the nuclear reactors (Sanger *et al.* 1989). After this first application, Teflon gained widespread use (Fall 2010).

1.2.1 Synthesis

The first synthesis process that allowed the large scale manufacture of fluorocarbons was the *Fowler Process* (Fowler *et al.* 1947). This process is based on two steps:

1) Oxidation:

$$2\ CoF_2 + F_2 \rightarrow 2\ CoF_3$$

2) High temperature fluorination

$$C_6H_{14} + 28\ CoF_3 \rightarrow C_6F_{14} + 14\ HF + 28\ CoF_2$$

During the same period, 3M company discovered the *electrochemical fluorination* (ECF): Simon's process (Simons *et al.* 1949). This process is based on the electrolysis of a solution of an organic compound in a solution of hydrogen fluoride, as follows:

$$R_3C\text{-}H + HF \rightarrow R_3C\text{-}F + H_2$$

However, different processes have been introduced in the industrial synthesis in order to: 1) improve reaction yields and, 2) introduce different functional groups for decreasing their hydrophobic character and to make them adequate to be used as emulsifiers, among others.

The main synthesis processes for perfluorosulphonic acids, sulphonamides and perfluorocarboxylic acids are summarised in Figures 1.1 and 1.2.

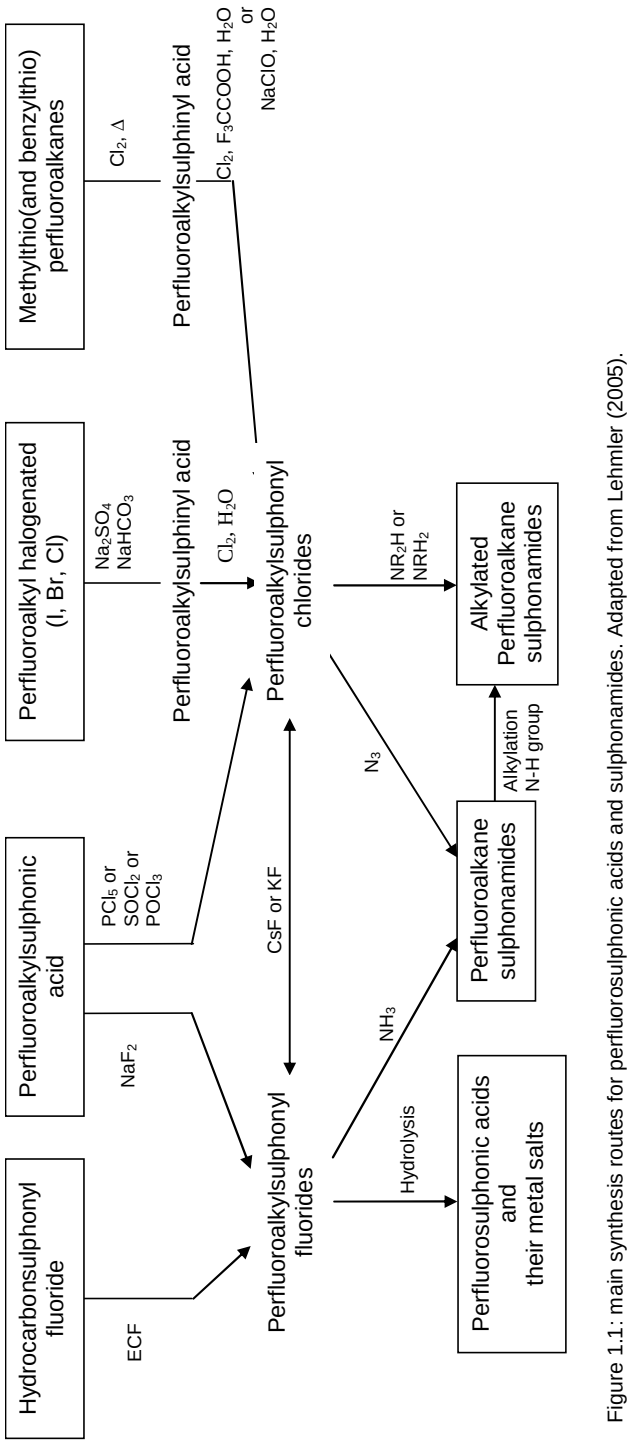


Figure 1.1: main synthesis routes for perfluorosulphonic acids and sulphonamides. Adapted from Lehmler (2005).

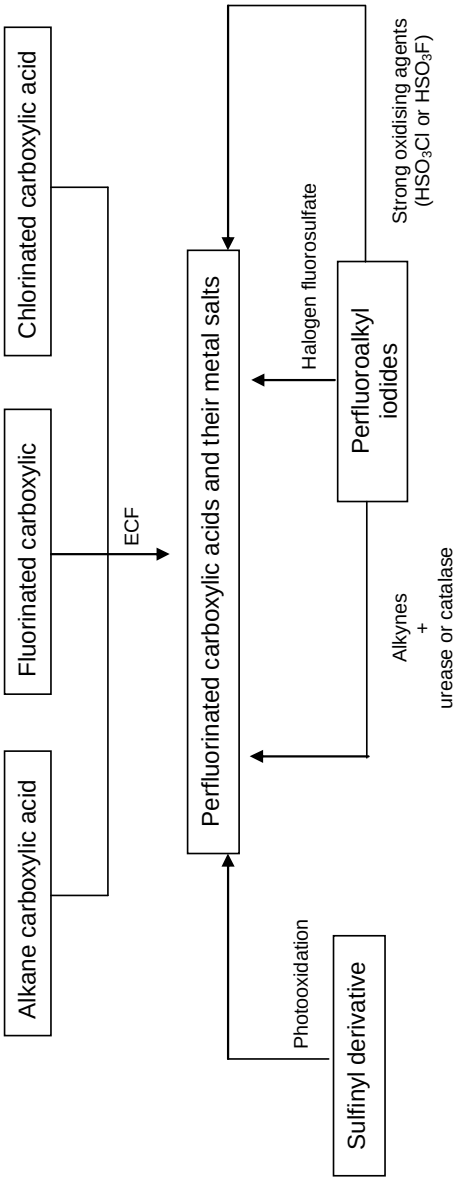


Figure 1.2: main synthesis routes for perfluorinated carboxylic acids. Adapted from Lehmler (2005).

As can be seen in Figure 1.1, the main routes of synthesis for sulphonyl fluorides are by the reaction with metal fluorides and by the ECF. These fluorides can be hydrolysed to perfluorooctanesulphonic acid, being one of the main synthesis processes. On the other hand, the sulphonyl fluoride or chloride can react in ammonia medium (Roesky *et al.* 1970) or via azide formation for the synthesis of perfluoroalkane sulphonamides (Lehmler 2005). These primary perfluoroalkanesulphonamides can be used during the synthesis of *N*-substituted perfluoroalkanesulphonamides and *N,N*-dialkyl perfluoroalkane-sulphonamides via alkylation of the N-H group. Another route for the synthesis of the N-substituted sulphonamides is by the reaction of the corresponding sulphonyl halide with the primary or secondary amine in the presence of a base (Lehmler 2005).

On the other hand, for pephluorinated carboxylic acids and their metal salts, the main synthesis routes are summarised in Figure 1.2. The first synthesis method was by ECF of the corresponding alkane carboxylic acid, but the yield of this reaction is really low (10-20%) (Kissa 2001; Lehmler 2005). Due to the lack in yield obtained by these ECF, fluorinated or chlorinated carboxylic acids were employed as a starting material by ECF reaction with most successful yields (~76%) (Kissa 2001; Lehmler 2005). Other synthesis methodologies include the photooxidation of the corresponding sulphinyl derivatives (Hu *et al.* 1989), the reaction of perfluoroalkyl iodides with alkynes in the presence of urease or catalase (Kitazume *et al.* 1988), with the presence of strong oxidising agents such as chlorosulphonic acid or fluorosulphonic acid (Hauptschein *et al.* 1961a; Hauptschein *et al.* 1961b), or a halogen fluorosulphate (Shack *et al.* 1980).

For industrial synthesis of fluoroalkenes such as vinylidene fluoride, tetrafluoroethylene, chlorotrifluoroethylene, trifluoroethylene and hexafluoropropene, the telomerisation process is commonly used instead of the ECF (Améduri *et al.* 1997; Kissa 2001). A simplified schedule of industrial telomerisation is shown in Figure 1.3.

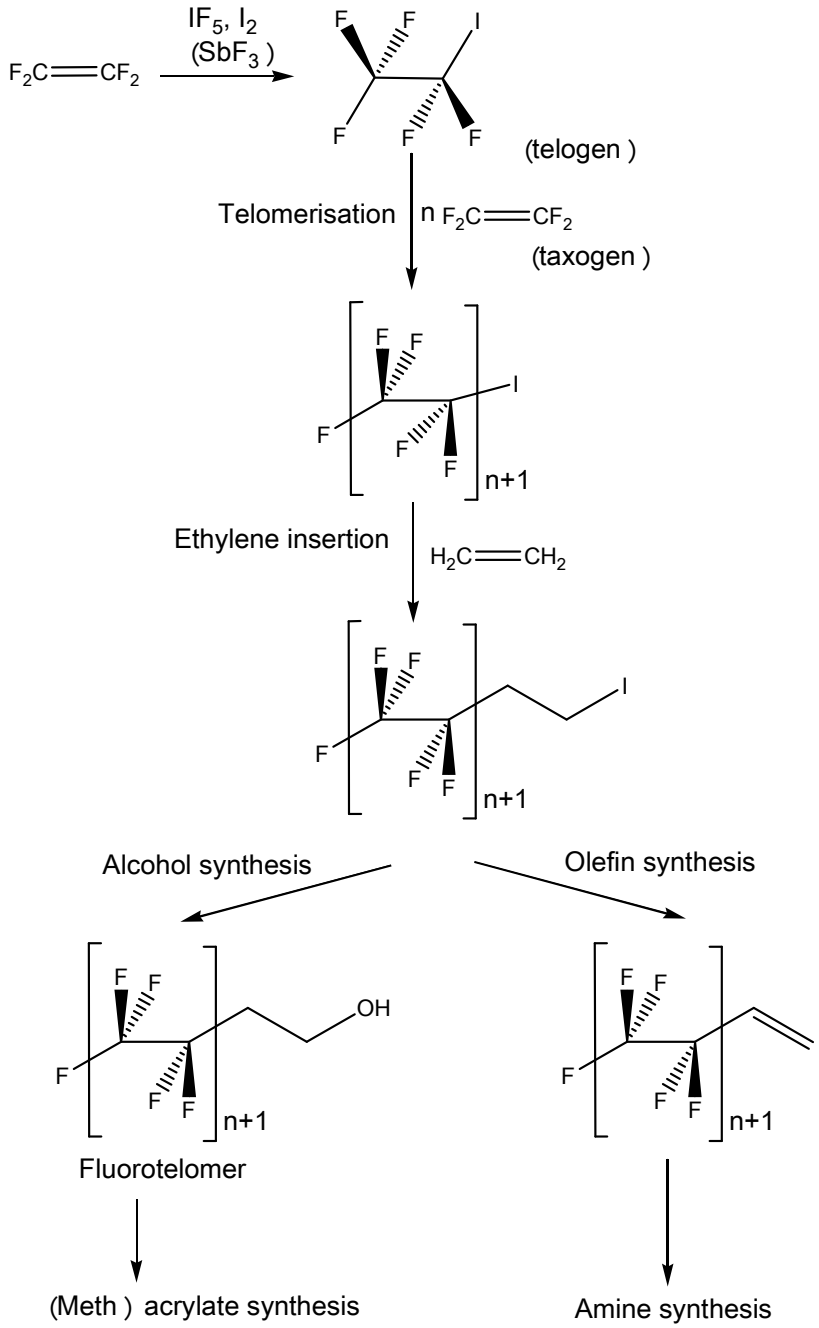


Figure 1.3: Industrial synthesis of fluorotelomer alcohols and related compounds by telomerisation of tetrafluoroethylene (taxogen) with perfluoroethyl iodide (telogen); adapted from Lehmler (2005).

1.2.2 Industrial applications

Since the 1940s, PFASs have been manufactured for a wide number of industrial and consumer products. These compounds are employed as fire resistant additives and oil, stain, grease and water repellents. As components of products, they repel water and oil, reduce surface tension much lower than other surfactants, act like catalysts for oligomerisation and polymerisation, and function where other compounds would rapidly degrade (3M 2000b). They are used to provide non-stick surfaces on cookware and waterproof, breathable membranes for clothing, and in many industry segments including the aerospace, automotive, building/construction, chemical processing, electronics, semiconductors, and textile industries (USEPA 2010). The most relevant applications are described in the "Organofluorine Chemistry: Principles and Commercial Applications" (Banks *et al.* 1994) and include:

- Textile repellent finishes (GoreTex®)
- Fluorosurfactants providing a predictable wetting, levelling and surface tension reduction properties for use in floor finishes and coatings, sealers/caulks, specially cleaners and personal care products (Masurf®)
- Fluoroplastics as poly(tetrafluoroethylene) (PTFE), perfluorinated compolymers, amorphous perlfuoroplastics, poly(chlorotrifluoroethylene), partially fluorinated plastics as poly(vinylidene fluoride) (PVDF) and poly(vinyl fluoride), increasing their resistance and being used in laboratory materials, due also to their chemical and physical inert properties.
- Fluoroelastomers as copolymers of hexafluoropropylene and vinylidene fluoride, terpolymers of tetrafluoroethylene, vinylidene fluoride, hexafluoropropylene and the perlfuoromethylvinylether. This synthetic rubber has wide chemical resistance and superior performance due to the high temperature application in different media.
- Fluoropolymer coatings as tetrafluoroethylene polymers (Teflon) or PVDF used in non-adherent surfaces, such as frying pans or food packaging materials.
- During the 1980s and 1990s, PFASs were also used for biomedical applications such as in blood diseases treatments, cancer therapy or ophthalmology, among others.

Figure 1.4 summarises the main applications of different PFASs.

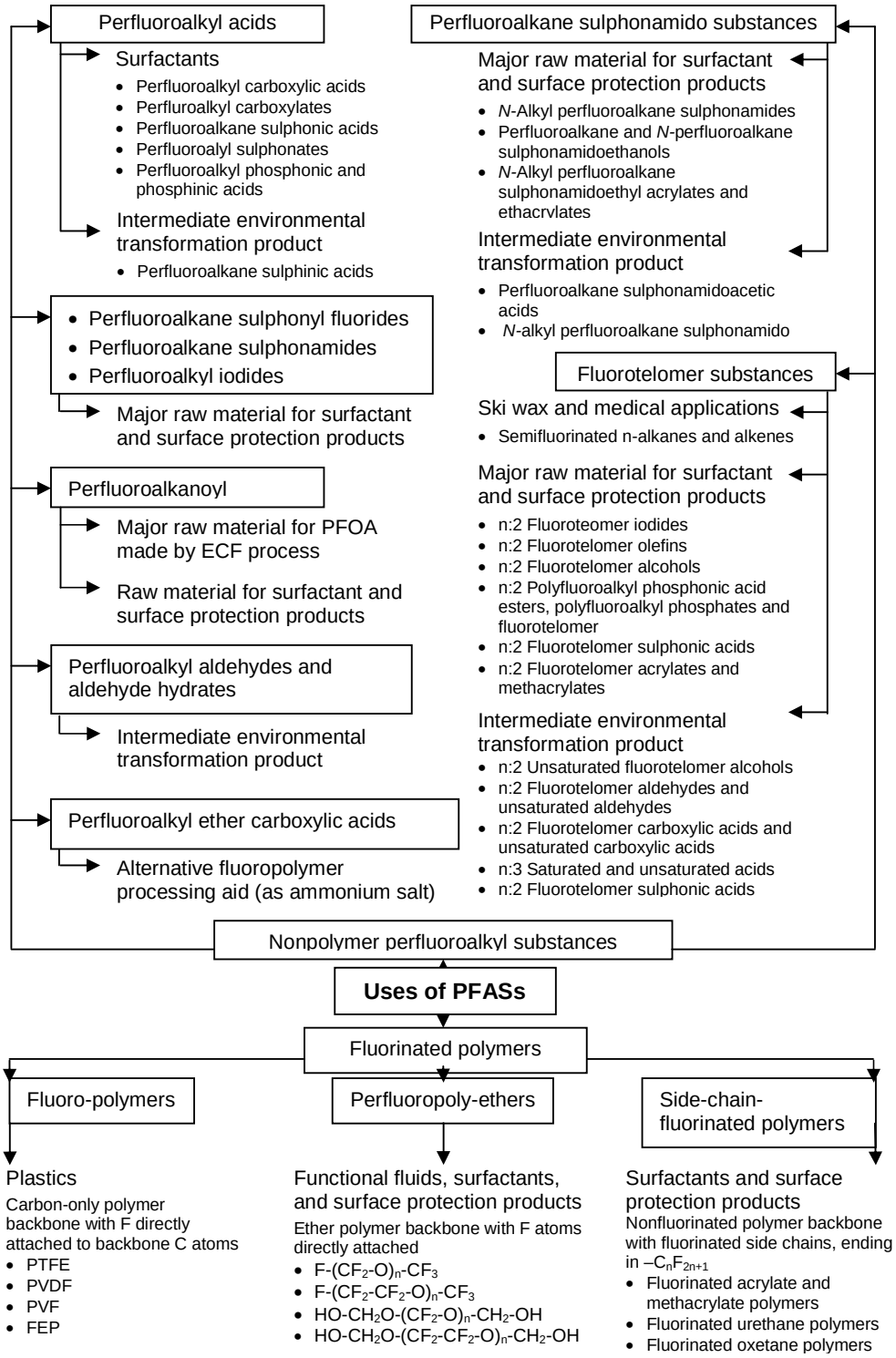


Figure 1.4: Uses of alkylfluorinated substances, adapted from Buck *et al.* (2011).

1.2.3 Global production

Perfluoroalkyl carboxylic acids (PFCAs). The commercial production of PFASs began with perfluorooctanoic acid (PFOA) in 1947, by electrochemical fluorination synthesis (3M Company Technical Bulletin 1995). After a few years, this compound was used in the manufacturing of fluoropolymers (Kissa 2001). The production increased in the 1960s and 1970s when they were included in fire-fighting foam formulations. During the 1970s the total worldwide emissions of acidic perfluorinated substances, and especially PFOA, have been estimated to be between 50 and 100 tonnes (Prevedouros *et al.* 2006). On the other hand, between 1951 and 2004 the total of PFASs and PFOA emitted to the environment varied between 2610 to 5720 tonnes, with peak of production during year 2000 (Stock *et al.* 2010).

Perfluorosulfonic acids. The first perfluorosulphonic acid synthesised was perfluorooctane sulphonate (PFOS) in 1949; 3M began producing PFOS-based compounds by electrochemical fluorination, resulting in the synthetic precursor perfluorooctane sulphonyl fluoride (Paul *et al.* 2009). From 1949 to 2002, 3M produced approximately 3665 tonnes, 78% of the estimated global production of perfluorosulphonic acids using this process (OECD 2002) in the United States (Decatur, Alabama) and Belgium (Antwerp) (Paul *et al.* 2009). Linear PFOS was used as the main perfluorosulphonic acid, although there are 89 linear and branched congeners with different physical, chemical, and toxicological properties (Rayne *et al.* 2008; Rayne *et al.* 2009). The main industrial applications are stain repellents, and, together with PFOA, it has also been used to obtain components of fire-fighting foams. PFOS is also widely employed in some impregnation agents for textiles, paper, and leather; in wax, polishes, paints, varnishes, and cleaning products for general use; in metal surfaces, and carpets and it is used in multiple photolithographic chemicals including: photoacid generators and anti-reflective coatings in the semiconductor industry; and, finally, as component of a hydraulic fluid used in commercial aviation (Skydrol) (FOEN 2009). However, in spite of their wide range of different applications, the most important emission sources of PFOS are metal plating and through fire-fighting foams (FOEN 2009).

In the late 1990s, the Environmental Protection Agency (EPA) received information indicating that PFOS was widespread in the blood of the general population and it started the concern about its persistence, bioaccumulation and toxicity. Following discussions between EPA and 3M company, the latter terminated production of these chemicals, starting with the phase-out of the perfluorooctyl chemistry in 2000 (3M 2000a). According to 3M Company (3M 2003), the global production of PFOS in 2000 was estimated at approximately 3535 metric tonnes, and decreased to an estimated 175 metric tonnes worldwide in 2001 (Stock *et al.* 2010). Currently, 3M Company is producing perfluorobutyl-based products, such as perfluorobutane sulfonate (PFBS), although production volumes are unknown (Stock *et al.* 2010). In 2002, the US-EPA identified 350 fluorinated compounds as Toxic Substances Control Act (US Environmental Protection Agency, 2002), where many of them are PFASs and, at least 80 of them are produced in large amounts (more than 4.5 tonnes in 2002) (USEPA 2002b; Stock *et al.* 2010).

Fluorotelomers. Another important group of PFASs are fluorotelomers. These compounds have been manufactured since the 1970s, with an estimated global production between 2000 and 2002 of 5000 – 6000 tonnes/year (Prevedouros *et al.* 2006). These compounds are employed as raw materials of fluorotelomer iodides, olefins, alcohols, and acrylate monomer. Residual telomer raw materials are potential indirect sources of perfluoro carboxylic acids by, for example, oxidation (Prevedouros *et al.* 2006). Fluorotelomer based products have been used in surface treatment products providing high performance surfactants in products that must flow evenly, such as paints, coatings, and cleaning products, fire-fighting foams for use on liquid fuel fires, or the engineering coatings used in semiconductor manufacture.

The production of these compounds was estimated at 12000 tonnes/year in 2004 and current production is assumed to be comparable or greater than this amount (Stock *et al.* 2010).

1.3 Environmental Fate

Most of the PFASs are physical, chemical and biologically stable and have been found to be widely spread in the environment (Armitage *et al.* 2006; Prevedouros *et al.* 2006; Kovářová 2008). Because of this wide distribution, some of these analytes are considered as persistent organic pollutants (POPs). PFOS have been included under the Stockholm Convention (UNEP 2010).

Most of the studies carried out during the last 15 years have been focussed on assessing the environmental fate of PFOS and PFOA, because of their extended use during the past decades, environmental distribution and persistency. In the next sections, the main physical and chemical properties, degradation processes and, in general, environmental fate are summarised. In addition, their environmental occurrence and possible effects on human health are also summarised.

1.3.1 Physical/Chemical Properties

Perfluoroalkyl sulphonates and perfluoroalkyl carboxylic acids are strong acids that exist in equilibrium between the neutral and anionic forms. In general, both the anionic and neutral forms are soluble in water. In regard to the more studied compounds, PFOS is water soluble, strongly acid and thermally stable. The potassium salt of PFOS has a mean solubility of 680 mg/L in pure water. PFOS anion can form strong ion pairs with many cations, which results in salting out in natural waters that contain relatively great amounts of dissolved solids. The solubility of PFOS is inversely proportional to the salt contents; therefore, the PFOS solubility in seawater is approximately 12.4 mg/L. On the other hand, the mean solubility in pure octanol was reported at 56 mg/L. However, due to the surface active properties, when the standard test system to measure the distribution constant octanol-water (K_{ow}) is performed, three different layers are obtained. Thus, an

octanol/water partition coefficient cannot be directly measured. Furthermore, other physiochemical properties, such as the bio-accumulation factor and the soil adsorption coefficient, cannot be estimated with conventional Quantitative Structure Activity Relationship (QSAR) models for PFOS. In addition, K_{ow} is not suitable to predict other properties, because PFOS is bound to the proteins instead, to be accumulated into the lipids (Jones *et al.* 2003).

PFOA is a perfluorinated organic acid (pK_a: 2.5 (USEPA 2005; Gilliland 1992)). The typical structure has a linear chain of eight carbon atoms. Most of the reported physical chemical properties are for the free acid. The free acid is expected to be completely dissociated in water, leaving the anionic carboxylate in the water and the perfluoroalkyl chain on the surface. In aqueous solutions, individual molecules of PFOA anion loosely associate on the water surface and partition between the air / water interface. Several reports note that PFOA salts self-associate at the surface, but with agitation they disperse and micelles form at higher concentrations (Calfours *et al.* 1985; Simister *et al.* 1992; Edwards *et al.* 1997; USEPA 2005). Water solubility has been reported for PFOA, but it is unclear whether these values are for a micro-dispersion of micelles, rather than true solubility. Due to these same surface-active properties of PFOA, and the test protocol for the OECD (Organisation for Economic Co-operation and Development) shake flask method, PFOA is anticipated to form multiple layers in octanol/water, much like those observed for PFOS. Therefore, an octanol/water partition coefficient cannot be determined.

Although Henry's law constant for perfluorosulphonic acids and perfluorocarboxylic acid values suggests partitioning into air for neutral forms, the prediction of the amount that partitions into air for ionic forms is rather complex, because of the uncertainty over the degree to which carboxylic and sulphonic acids partition from water to the atmosphere. The uncertainty arises with regard to the value of the acid dissociation constant (i.e., pK_a), or the fraction of the acid form present at environmentally relevant pH. Both acids have been detected in air, water, and soil samples collected throughout the world. The oceans are the final sink and the route of transport for sulphonic and carboxylic acids, being detected on the surface and at depths of over 1,000 metres (Yamashita *et al.* 2005). Some sulphonic/carboxylic acids have the potential for long-range environmental transport (LRET) by a combination of dissolved-phase Ocean and gas-phase atmospheric transport; however, determining which is the predominant transport pathway is complicated by the uncertainty concerning water to atmosphere partitioning. Furthermore, there is evidence that transport and subsequent oxidation of volatile alcohol sulphonic and carboxylic acid precursors may contribute to the concentration levels in the environment, including remote areas such as the Antarctic or Arctic continents. The global fate of POPs is associated with different biogeochemical cycles and geophysical drivers. The occurrence of PFASs into remote areas such as the Antarctica, could be partially explained by the theory of cold condensation, concerning the chemical movements or chemical transformations from sources under the impact of environmental forces, such as temperature, and interaction with other environmental compartments (soil, oceans, etc.) (Lohmann *et al.* 2007).

1.3.2 Degradation reactions

1.3.2.1 Photolysis

The indirect photolytic half-life for PFOS, was estimated to be greater than 3.7 years at 25 ºC, whereas, in the case of PFOA, the half-life was around 349 days, using an iron oxide photo-initiator model (ATSDR ; USEPA 2002a; Brook. D. *et al.* 2004; Cheng *et al.* 2008; EFSA 2008). Yamamoto *et al.* (2007) studied, for the first time, the photodegradation of PFOS in water and alkaline 2-propanol under a low-pressure mercury lamp at 254 nm and 32 W, for 10 days. The authors observed that degradation rate of PFOS was around 8% after 1 day and 68% after 10 days of irradiation, while in propanol the degraded yields were of 76 and 92% after 1 and 10 days irradiation, respectively (Yamamoto *et al.* 2007).

Currently, new groups of compounds are in use in replacement of PFOS and PFOA, which also should be considered. Plumlee *et al.* (2009) irradiated different perfluorochemical surfactants in aqueous hydrogen peroxide solutions using artificial sunlight to study transformation under aquatic environmental conditions. In these experiments indirect photolysis was observed for the N-ethyl perfluorooctane sulphonamidoethanol (N-EtFOSE), N-ethyl perfluorooctane sulphonamido acetate (N-EtFOSAA), N-ethyl perfluorooctane sulphonamide (N-EtFOSA), and perfluorooctane sulphonamide acetate (FOSAA). The degradation of N-EtFOSE, gave other perfluoroalkanesulphonamides and PFOA. Perfluorooctane sulphonamide (FOSA) and PFOA were the final degradation products. UV-visible absorption spectra for the perfluorochemicals, showing absorbance in the UV region below the range of natural sunlight, were also reported. In order to elucidate the environmental fate of perfluoroalkyl sulphonamides, the indirect photolysis is, therefore, an important aspect to be studied. The photolytic transformation of these analytes into perfluorinated acids, such as PFOA, could mean that a significant fraction of these compounds can be accumulated in the ocean.

On the other hand, perfluoroalkyl sulphonamides are not the only photolytic precursors of carboxylic acids. Ellis *et al.* (2004) studied the atmospheric degradation of fluorotelomers, showing how these analytes are oxidised in the atmosphere to fluorinated aldehydes (which absorb strongly in the region 300-350 nm). The authors postulated that the atmospheric photolysis of the aldehydes will proceed via C-C bond scission with a final sink of perfluorocarboxylic acids (Ellis *et al.* 2004). A similar process was observed by Gauthier *et al.* (2005) for the 8:2 fluorotelomer alcohol in aqueous hydrogen peroxide solutions, with PFOA and PFNA as final end-products of the photolysis.

In spite of these studies, there is still a wide lack of knowledge, in terms of PFASs photolysis. Further research is needed in order to elucidate any possible degradation pathway through the photolysis of the most recalcitrant PFASs.

1.3.2.2 Hydrolysis

In general, PFASs can be considered highly resistant to degradation and, concretely, long hydrolytic half-life has been reported for both, PFOS and PFOA.

Under experimental conditions of 50 ºC and different pHs (1.5, 5, 7, 9, or 11) no hydrolytic losses of PFOS were measured after a 49 days study (Hatfield 2001). Based on mean values and the precision of the different measures, the hydrolytic half-life of PFOS was estimated to be higher than 41 years at 25 ºC.

In the case of PFOA, the study on the hydrolysis reactions performed by 3M Environmental Laboratory (Report No. E00-1851, (USEPA 2002a)) showed that the half-life estimated for PFOA at 25 °C, at different pH values between 5 and 9, was greater than 92 years, with the most likely value of 235 years. However, there are controversial studies regarding the half-life of PFOA (Environment Canada 2010). In this sense, it has been reported that PFOA is the end-product in the troposphere of other acidic fluorides, as a consequence this process is another source of PFOA in the environment (Ellis *et al.* 2004).

In spite of the high resistance to hydrolysis shown by some PFASs, other groups can show different trends. For example, whereas some polyfluoroalkyl phosphate surfactants (PAPs) are relatively labile toward hydrolysis, as happens with trimesters (Schwarzenbach *et al.* 2003), phosphate monoesters and diesters are stable, with lifetimes on the order of several years under environmental conditions (Wolfenden 1998). The hydrolytic stability of both 8:2 monoPAPS and 8:2 diPAPS was investigated under aggressive conditions of pH 9 and 50 °C by D'Eon *et al.* (2007), and a minimum lifetime of 26 years was reported (Wolfenden 1998).

Perfluoroalkyl phosphate esters, perfluoroalkyl telomer acrylates and fluorotelomer iodides were also studied by Rayne *et al.* (2010). In these studies, the main conclusions were that the hydrolysis of perfluoroalkyl phosphate esters (half-lives of several years in marine systems and as low as several days in some landfills) of monomeric acrylates could occur, and this process may contribute to current environmental loadings of fluorotelomer alcohols and perfluoroalkyl carboxylic acids. In addition, the same process applies for fluorotelomer iodides. On the other hand, the hydrolysis of the polymeric acrylates are expected to be slower, with half-lives in soil and natural waters ranging between several centuries to several millennia (Rayne *et al.* 2010).

The hydrolysis represents an important route of generation of recalcitrant PFASs, such as PFOA and PFOS or the polymeric perfluoroalkyl acrylates. In contrast, this process may not suppose an important degradation pathway for these compounds, since their hydrolytic half-life is in the order of 41, 92 – 235 years and several centuries, respectively.

1.3.2.3 Biodegradation

Biodegradation studies have been carried out in a wide variety of microbial sources and exposure regimes (Gledhill *et al.* 2000b; Gledhill *et al.* 2000a; Gledhill *et al.* 2000c; Lange 2001; Beach *et al.* 2006). This is the most important mechanism to remove organic contaminants in wastewater treatment plants (WWTPs) (and also in the environment) under aerobic or anaerobic conditions (Parsons *et al.* 2008). The carbon-fluorine bond is one of the most stable in nature and it limits the biodegradability of PFASs and, consequently, these cannot be mineralised. The substantial energy yield from defluorination indicates that, from a thermodynamic point of view, there is no reason why microorganisms should not be able to obtain energy for growth from reductive defluorination although such organisms have not been described to date (Vargas C 2000). These organisms should be evolved in order to benefit from this potential source of energy and use the enzymatic machinery necessary to catalyse this reaction and harness the energy produced, as in the case of chlorinated aliphatic compounds (Dolfing J. 2000; Parsons *et al.* 2008).

The high concentration found for short chain PFASs led to the hypothesis that these compounds could be the biodegradation products of longer related PFASs, such as fluorotelomers (Kannan 2011). Under both aerobic and anaerobic conditions, Schröder *et al.* (2003) reported the biodegradation of PFOS, PFOA and non-ionic surfactants, including partially fluorinated alkyl ethoxylates, perfluorooctanesulphonyl-amidopolyethoxylate and perfluorooctanesulphonyl-amido–polyethoxylate methyl ether in spiked wastewater samples. Fluorinated compounds were partly degraded under aerobic conditions to form carboxylic acids. Regarding the non-substituted PFASs, such as PFOS, they were found to be rapidly removed (within 2 days) under anaerobic conditions, in contrast to the slower removal of PFOA. Among the non-ionic PFASs, only the sulphonyl compounds were removed. The same authors proceed with an extensive study of the biodegradation of PFOS and PFOA in aerobic and anaerobic reactors containing sludge from WWTPs (Meesters *et al.* 2004; Parsons *et al.* 2008). No biodegradation was observed under aerobic conditions, although under anaerobic conditions it was observed that the removal of PFOS was followed by PFOA. Nevertheless, neither compound could be further detected after 26 days, nor any metabolites, nor increases in fluoride ion concentration were detected (Meesters *et al.* 2004; Parsons *et al.* 2008).

The ability of sludge systems to transform *N*-EtFOSE has been studied by Boulanger *et al.,* (2005). In their work the degradation of *N*-EtFOSE (a primary monomer used in 3M's polymer surface protection products) to lesser-substituted perfluorooctane compounds in bioreactors amended with aerobic and anaerobic sludge was followed during 96 h. *N*-EtFOSAA and PFOSulfinate were detected as main metabolites from the aerobic experiment, but no biodegradation was achieved under anaerobic conditions.

The degradation of fluorotelomers has been studied under a wide variety of conditions and organisms and, in this case, it should be noted that the results are highly dependant on conditions and organisms used in each case (Key *et al.* 1997). For example, Wang *et al.* (2005) studied the biodegradation in a simulated water treatment

process. Different metabolites were identified, indicating that α-oxidation did not take place. The results demonstrated that perfluorinated fluorotelomer carbon bonds are defluorinated and mineralised by microorganisms under conditions which may occur in a WWTP, forming shorter fluorinated carbon metabolites (Wang *et al.* 2005). Later, Myers *et al.* (2010) studied the degradation of fluorotelomer carboxylic acids, and the rapid degradation has indicated that the sorption to the sediment was the greater effect for longer-chain carbon compounds. PFOA and PFDA were the degradation products, but in general terms, it was found that degradation products were dependent on initial carbon length as described by Dinglasan *et al.* (2004). In parallel, Frömel *et al.* (2010) performed the aerobic biodegradation of a commercial mixture of fluorotelomers ethoxylates with an unfiltered effluent from a municipal WWTP. Fluorotelomer ethoxylates were rapidly transformed with approximately 1-day half-lives, due to the ω-oxidation of the terminal hydroxyl group to the respective carboxylic acid, followed by sequential shortening of ethoxylate units which led to fluorotelomer carboxylates. The transformation rates suggested the long half-lives of some fluorotelomer ethoxylate congeners in the environment (Frömel *et al.* 2010). In addition, the degradation of fluorotelomer alcohols into PFHxA and PFOA was also studied (Frömel *et al.* 2010).

Recently, polyfloroalkyl phosphates have been studied. For example, ingested mono- and di- polyfluoroalkyl phosphates can be degraded to PFOA and other carboxylic acids in rats (D'Eon *et al.* 2007), and these compounds were also degraded to perfluorinated acids by microbials present in soil and wastewater (D'Eon *et al.* 2007).

Table 1.2 summarises different PFAS biodegradation experiments reported in the literature. As can be seen, some experiments on polyfluoroalkyl substances have been carried out, but there is still a lack of knowledge about the organism's ability to degrade perfluoroalkyl substances.

Table 1.2: Biodegradation experiments conducted for PFASs with different organisms

Studied PFASs	Biodegradation Process	Results	Reference
2(*N* -ethyl perfluorooctane sulphonamide)ethanol	Aerobic waste water treatment sludge	• Degraded to PFOS and other metabolites within 25 days	(3M 2000b)
PFOS, PFOA, fluorinated alkyl ethoxylates, fluorosulphonyl ethoxylates,	Sewage sludge from WWTP under aerobic and anaerobic conditions	• Partialy degraded under aerobic conditions to form carboxylic acids • PFOS removed within 2 days under anaerobic conditions. PFOA removal slower than PFOS • Partialy removal of sulphonyl compounds	(Schröder 2003)
8:2 FTOH	Aerobic biodegradation in a mixed microbial system	• Half-life of the 8:2 FTOH ~0.2 days/mg • Fluorotelomer acids and PFOA as degradation products	(Dinglasan *et al.* 2004)
PFOA and PFOS	Aerobic and anaerobic reactors containing sludge from WWTPs	• Removal of PFOS followed by PFOA under anaerobic conditions	(Meesters *et al.* 2004)
N- alkylperfluoroocta sulphonamidoethanol	Bioreactors amended with aerobic and anaerobic sludge	• *N*-Ethyl sulphonamide and PFOS sulphinate as metabolites from aerobic biotransformation • No transformation of *N*- alkylperfluoroocta sulphonamidoethanol under anaerobic conditions	(Boulanger *et al.* 2005)
FTOHs	Simulated water treatment process microorganism	• Defluorination and mineralisation of fluorotelomer carbon bonds • Formation of shorter fluorinated carbon metabolites	(Wang *et al.* 2005).
FTOHr-based polymer	aerobic incubation of soil	• Half-life of 8:2 fluorotelomer alcohol ~28 days • PFOA as a degradation product	(Koch *et al.* 2007)
N-ethyl perfluorooctane sulphonamidoethanol	Activated sludge from a WWTP	• Half-life ~ 0.25 days • *N*-Ethyl sulphonamide, FOSA, PFOS and PFOA as a degradation products	(Rhoads *et al.* 2008)
fluorotelomer carboxylic acids	Soil-water microorganism	• Rapid degradation • PFOA and PFDA as degradation products	(Myers *et al.* 2010)
fluorotelomers ethoxylates commercial mixture	aerobic biodegradation with effluent water from WWTP	• Half-life ~ 1 day • PFHxA and PFOA	(Frömel *et al.* 2010)
6-2 Fluorotelomer alcohol	biodegradation in soil and mixed bacterial culture	• Half-life < 2 days (both medias) • PFHxA, PFPeA, PFBA and 4-3 acid as degradation products • 5-3 ketone aldehyde degradation product in mixed bacterial culture	(Liu *et al.* 2010)

1.3.2.4 Thermal degradation

Although the thermal degradation of PFASs is expected to occur at very low rates, different studies suggested that PFOS would have lower thermal stability than other PFASs, due to the carbon-sulphur bond. The energy of this bond is weaker than carbon-carbon or carbon-fluorine and under incineration conditions could be broken (Dixon 2001; Yamada et al. 2003; Giesy et al. 2010).

In addition, PFASs can be generated by thermolysis in high temperature processess. For example, the thermolysis of fluoropolymers has been identified as a potential environmental source of PFOA (Ellis *et al.* 2001; 2003; Environment Canada 2010). However, due to the high temperatures required for this process (365 ºC), the unlikely contribution to the PFOA long-range transport was pointed out by the authors (Ellis et al. 2001; Ellis 2003; Environment Canada 2010). Nevertheless, the thermolysis of fluoropolymers could be a significant source of trifluoroacetate (Ellis *et al.* 2001), which is a greenhouse gas (Ellis *et al.* 2001).

1.3.3 Adsorption/Desorption

Although the behaviour of PFASs in the environment is related in a great manner to adsorption and desorption processes in soil, few data are available. It is noteworthy that PFOS appears to adsorb strongly to soil, sediment, and sludge with an average distribution coefficient (Kd) greater than 1 mL/g, and Koc greater than 10,000 mL/g (Ellefson 2001; Giesy *et al.* 2010). In addition, soil adsorption of PFOS can be achieved in less than 24 h and, in some cases, could be a faster process occurring at around 1 min. This rapid equilibrium, combined with the fact that desorption does not take place even with organic solvents (Kdes < 1 mL/g), makes PFOS not mobile in soil, sediment and sludge (Giesy *et al.* 2010). Since PFOS is a strong acid, it probably forms strong bonds in soils, sediments, and sludge via a chemisorption mechanism (Giesy *et al.* 2010). The findings of Ellefson are supported by the study carried out by Johnson *et al.* (2007) in which the isotherms and solid/solution distribution ratio for PFOS in different types of soil were studied. PFOS adsorption is due to the organic carbon present in soil, or to the electrostatic attractions when organic carbon is not present (Johnson *et al.* 2007). However, it should also be mentioned that the adsorption rate of PFOS was smaller than their analog, hydrocarbon. Although, PFOS cannot be mobilised from soil, other PFASs that have long soil half lives of 6 months (Clarke *et al.* 2011) can be mobilised by rainfall (Gottschall *et al.* 2010) being therefore, a problem for groundwater and plants.

Zhou *et al.* (2010) have studied the sorption of PFASs on activated sludge, and the different sorption kinetics according to their carbon chain length and different functional groups were considered. The calculated distribution coefficients indicate that PFOS had a higher sorption tendency to activated sludge than PFOA (Zhou *et al.* 2009).

1.3.4 Bioaccumulation and biomagnification

Due to the low tendency to be degraded by any physical, chemical or biological process, these compounds are persistent in the environment. In addition, PFASs present the tendency to bioaccumulate in living organisms and biomagnify through the food chain. For example, some studies have been focussed on assessing the bioaccumulation factors of PFOS and FOSA, indicating their increase with the trophic level in bioaccumulation factors higher than the unit in both cases (Tomy *et al.* 2004; Tomy *et al.* 2009).

PFASs are widely distributed in the environment, and a high number of studies have been carried out during the last decade in order to assess the presence and distribution of PFASs in biota, particularly in the aquatic food chain. Figure 1.5 summarises some studies reporting the accumulation of PFASs along the marine food web. It should be noted that PFOS and PFOA have been the most studied compounds, and also those with the highest accumulative trends. However, as a result of the phase-out of production of perfluorooctanesulphonyl-based compounds by a major producer, concentrations of PFOS in the aquatic environment have declined since the early 2000s. Whereas, for other PFASs the accumulation behaviour continues being poorly studied, in particular for the new compounds that were introduced in the market as replacement of PFOS and PFOA.

In most of the studies, especially in those before 2008, PFOS was the prevalent compound. PFOS has been classified as persistent and bioaccumulative, and similarities in chemical structure and environmental behaviour of PFOS and the PFCAs that have been detected in wildlife have generated concerns about the bioaccumulation potential of PFCAs. Differences between partitioning behaviour of the acidic compounds and persistent lipophilic compounds make difficult the understanding of PFCAs bioaccumulation and the subsequent classification of the bioaccumulation potential of PFCAs, according to existing regulatory criteria. Based on available research, five key points should be considered, in order to evalute the bioaccumulation potential of perfluoro acidic compounds, including perfluorocarboxilic acids (Conder *et al.* 2008). The key points are

- Bioconcentration and bioaccumulation of perfluorinated acids are directly related to the length of each compound's fluorinated carbon chain;
- PFASs are more bioaccumulative than PFCAs of the same fluorinated carbon chain length;
- PFCAs with seven fluorinated carbons or less are not considered bioaccumulative, according to the range of promulgated bioaccumulation,"B", regulatory criteria of 1000–5000 L/kg;
- PFCAs with seven fluorinated carbons or less have low biomagnification potential in food webs, and;
- more research is needed to understand the bioaccumulation potential of PFCAs with longer fluorinated carbon chains (>7 fluorinated carbons), as PFCAs with longer

fluorinated carbon chains may exhibit partitioning behaviour similar to or greater than PFOS.

The bioaccumulation potential of perfluorinated acids with seven fluorinated carbons or less appears to be several orders of magnitude lower than classic lipophilic POPs classified as bioaccumulative.

Another relevant trend in the accumulation of PFASs is that the environmental behaviour of this class of chemicals differs from other known POPs. Due to their hydrophylic nature, PFASs do not accumulate in lipids, but are found at the greatest concentrations in blood and liver (Giesy et al. 2001). The physico-chemical structure of PFASs, as with PFOS, suggests the possibility of interactions of either the sulphonic acid group or the hydrophobic alkyl chain with serum proteins. One of the possible sites of action might be to interact with those serum proteins that are involved in endocrine and immunological functions. It has been proposed that interaction of chemicals with certain serum proteins might disturb normal endocrine function.

Since PFASs enter in the food chain, this is one of the routes of human exposure to this group of contaminants. Also, during the last decade, an important research has been paid to elucidate the human accumulation of PFASs (Ericson *et al.* 2008a; Pico *et al.* 2010). Main sources of PFASs for humans are presented in Figure 1.6.

PFASs have been found to accumulate in the blood of exposed organisms, as well as in other target organs, such as the liver. The potential for these surfactant molecules to interfere with hormone/protein interactions in blood is of concern, given the importance of these interactions (Jones *et al.* 2003). Different authors have studied the elimination half-life of some PFASs in different mammals, being controversial in some cases. Table 1.3 summarises some of the published works.

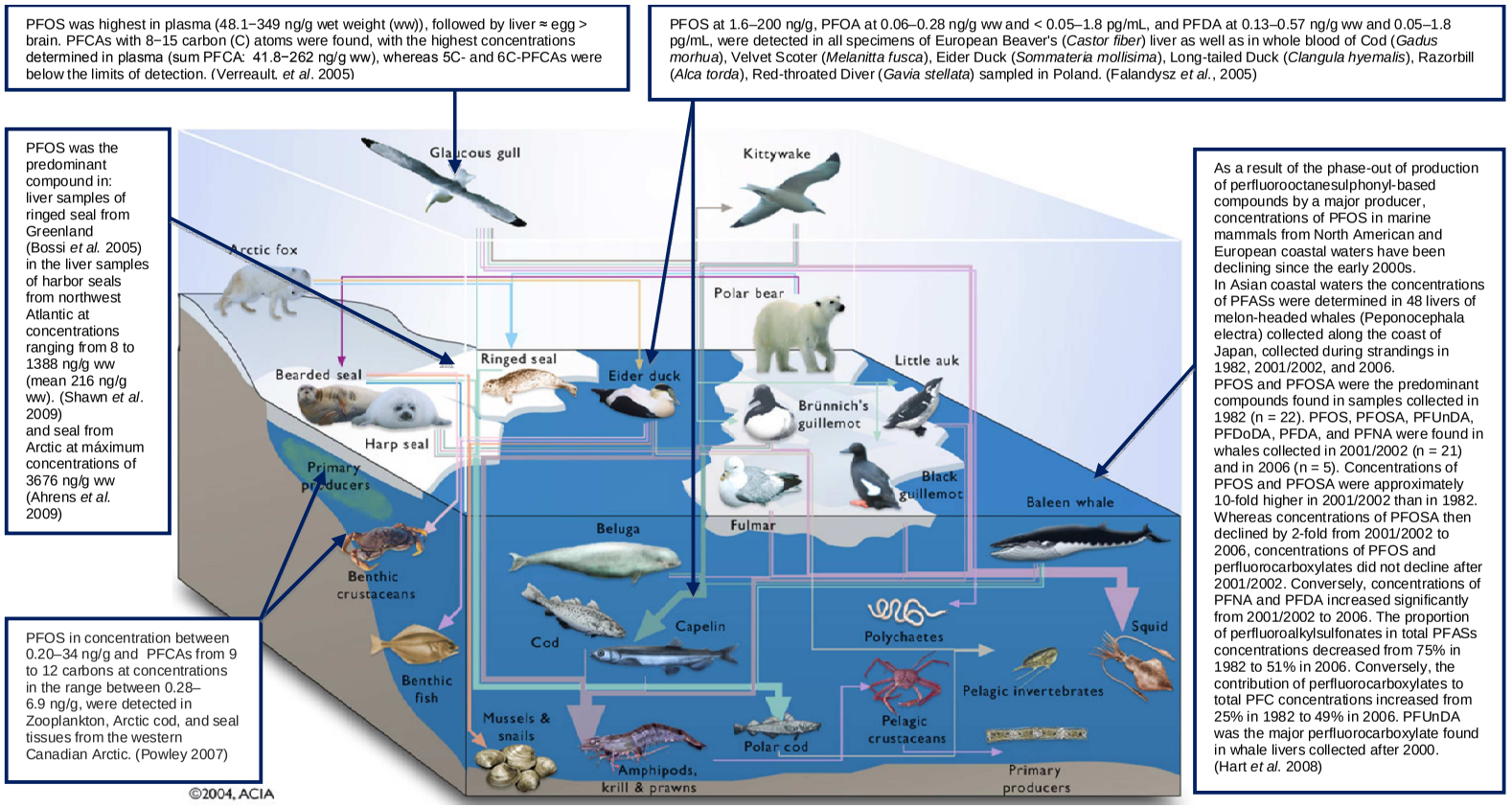


Figure 1.5: Summary of different studies of the accumulation of PFASs in different organisms along the aquatic food chain.

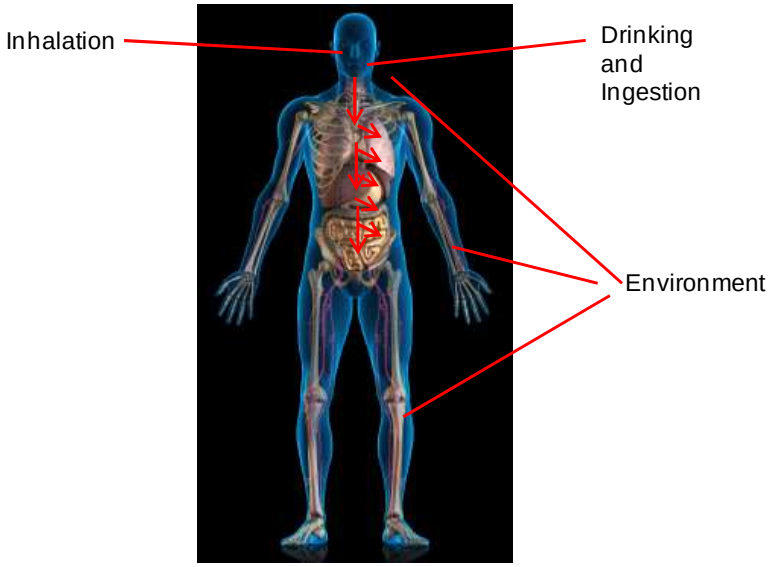


Figure 1.6: scheme of the main input sources into the human body (Figure 1.6)

Table 1.3: Half-life elimination of different PFASs from serum/plasma reported in different studies. Table adapted from Lau *et al.* (2007).

Specie	PFBS	PFHxS		PFOS	PFBA		PFOA		Reference
		F	M		F	M	F	M	
Rat				100 d	1.6-1.8 h	7-9 h	2-4 h	4-6 d	(Johnson *et al.* 1979; Kemper *et al.* 2003; Chang *et al.* 2007)
Mouse					3 h	17 h	17 d	19 d	(Lau *et al.* 2005; Chang *et al.* 2007)
Rabbit							7 h	5.5 h	(Hundley *et al.* 2006)
Dog							8-13 d	20-30 d	(Hanhijarvi *et al.* 1982)
Monkey	3.5-4 d	87 d	141 d	150 d	1.7 d		30 d	21 d	(Noker *et al.* 2003; Butenhoff *et al.* 2004; Lieder *et al.* 2006; Chang *et al.* 2007)
Human			8.5 y	5.4 y				3.8 y	(Olsen *et al.* 2007)

List of acronyms: Female (F); Male (M); hours (h); days (d); years (y).

The transport and accumulation of PFASs in different organisms do not follow common pathways as occur with other POPs. Luebker *et al.* (2002) performed an *in vitro* assay in order to determine if there was any interaction between the liver-fatty acid binding protein (intracellular lipid-carrier protein) and some PFASs including PFOS, PFOA, and different sulphonamides (Luebker *et al.* 2002). In this way, the interference of PFASs between the binding of the liver-fatty acid protein and the 11-(5-dimethylaminonapthalenesulphonyl)-undecanoic acid was studied. The results showed

that PFOS exhibited the highest level of inhibition of the binding in the competitive binding assays, followed by sulphonamides and PFOA (Luebker *et al.* 2002). This work provides evidence that PFASs are bioaccumulated through the binding with endogenous organism ligands, with a possible contribution to toxicity (Luebker *et al.* 2002). Another example is the study carried out by Han *et al.* (2003). The authors studied the PFOA binding to rat and human plasma proteins. The results showed that most PFOA was in protein-bound form in rat plasma, being the primary PFOA binding protein in plasma serum albumin. Similar results were found for human serum albumin, but in that case the transport pathway was different (Han *et al.* 2003). Another work performed by Jones *et al.* (2003) has been focussed on the study of the ability of PFOS and other related longer carbon chain analytes to displace a variety of steroid hormones from specific binding proteins (among them albumin) in fish and bird serums. The results concluded that PFOS can weakly displace estrogen or testosterone in fish and cortisone in birds. An increase was observed of the displacement for the corticosterone with the chain length and, in general, perfluoroalkyl sulfonic acids were more potent than perfluoroalkyl carboxylic acids for concentrations higher than 160 mg/L in serum (Jones *et al.* 2003).

In section 1.7 human accumulation, fate and occurrence in humans will be presented and discussed.

1.4 Environmental sources of PFASs

Direct and indirect sources of PFASs contamination in the environment have been identified. Direct sources are the discharges or emmissions at production sites, fluorotelomer manufacturing and processing, fluortelomer dispersions, aqueous fire-fighting foams and consumer and industrial products. Indirect sources are mainly the degradation of perfluoroalkyl based products and different industrial point sources ch as galvanising industry, paper industry and WWTPs. Another important source related to WWTPs is the contaminated sludge when applied on agricultural land (Clarke et al., 2011). In this case, the analytes present in the amended soil can be mobilised by rainfall reaching groundwater (Table 1.4) (Gottschall et al., 2010). Other indirect sources show different patterns of emission. For example, these releases are dependent on the lifespan, use and disposal of the products. While secondary sources like textiles and carpets have a long lifespan in apartments, others, like paper and packaging, have a short lifespan and are carried out to waste disposal sites (Paul *et al.* 2009). In addition, the disposal of treated products or products contain theirein may lead to new point sources in form of landfill sites (Schultz et al. 2003, Boulanger *et al.* 2005b), even though used precursor substances might have to be degraded in situ before they attain any leachate (van Zelm *et al.* 2008)

1.5 Occurrence of PFASs

1.5.1 Occurrence in wastewater and sludge of wastewater treatment plants

1.5.1.1 Wastewater

Due to the high production and consumption of fluoropolymer and telomer-alcohols, industrial discharge is considered to be one of the major sources of PFASs in the environment. It is known that PFOS and PFOA were ubiqutous compounds due to their high production and use. For example, Tang *et al.* (2006) reported that wastewater from the semiconductor industry contained PFOS around 1,650 mg/L. Currently, a stop in production of PFOS influenced the decrease of PFOS concentration in WWTPs, however currently, other PFASs used in substitution are found.

Another important source in PFASs in the influents of WWTPs is domestic wastewater. The presence of PFASs in domestic effluents is due to the cleaning of these compounds during washing, as well as use of products containing these compounds, such as shampoos.

In Chapter 2, fate and behaviour of PFASs in wastewater and WWTPs will be discussed in depth, but it should be mentioned that removal rates in conventional WWTPs are not complete. In addition, also during wastewater treatment processes the atmospheric emmission of more volatile compounds can take place.

Some studies of the occurrence of PFASs in wastewater are summarised in Table 1.4. Becker *et al.* (2010) calculated the mass flow of PFOA and PFOS in a WWTP facility. The authors found that while PFOA was fully discharged into the river, about half of PFOS was retained in the sewage sludge, supporting the adsorption/desorption experiment performed by Zhou *et al.* (2010). The lack of total removal of PFASs in wastewater treatments has been demonstrated, and many works have reported high concentrations of PFASs in treated effluents (Loganathan *et al.* 2007; Ahrens *et al.* 2009).

In Spain, PFASs have been studied in effluent water from different WWTPs, located in Catalonia, by Sánchez-Avila *et al*. (2010). The sampling sites were located near to surface river water, which was also investigated by the same authors. The results for WWTP effluents showed values below 1 ng/L, with the exception of PFOS (72.1 ng/L) and PFOA (61.9 ng/L). These results suggest that PFASs are discharged into the river through WWTPs effluents, and then PFASs can reach the drinking water.

Table 1.4: PFASs in influent and effluent waters from WWTPs, published results

Analytes	Origin	Matrices	Results		Reference
PFOS, PFDS, PFOA, PFNA, PFDA	Municipal WWTP	Influent and effluent water	**Influent**: PFOS = 15.7 ng/L; PFDS = 6.3 ng/L; PFOA = 15.0 ng/L; PFNA = 1.1 ng/L; PFDA = 5.6 ng/L;	**Effluent**: PFOS = 24 ng/L; PFDS = 8.2 ng/L; PFOA = 11 ng/L; PFNA = 3.4 ng/L; PFDA = 2.3 ng/L;	(Schultz *et al.* 2006b)
PFOS PFHS PFOA PFNA PFDA PFUnA 8:2 FTCA 8:2 FTUCA	6 WWTPs in New York State	Effluent water	**Effluent:** PFOS= 3 - 68 ng/L; PFHxS= 2 - 39 ng/L; PFOA= 58 - 1050 ng/L; PFNA= 4 - 376 ng/L;	PFDA= 2.5 - 47 ng/L; PFUnA= 2.5 - 10 ng/L; 8:2 FTCA= 2.5 - 7 ng/L; 8:2 FTUCA= 2.5 - 29 ng/L;	(Sinclair *et al.* 2006)
PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA, PFDoA	2 WWTPs: • A: rural area • B: urban area	Influent and effluent water	**Influent:** PFHxS = 2.6 - 6.1 ng/L; PFNA = 2.6 - 6.2 ng/L; PFOA = 22 - 184 ng/L; PFOS = 7 - 16 ng/L; FOSA = 0.29 - 1.9 ng/L; PFDA = 0.17 - 1.4 ng/L; PFUnA = < 0.5 - 1.9 ng/L; PFDoA = < 0.5 ng/L;	**Effluent:** PFHxS = 6.3 - 9.5 ng/L; PFNA = 2.4 - 9.5 ng/L; PFOA = 122 - 183 ng/L; PFOS = 8 - 28 ng/L; FOSA = 1.7 - 2.5 ng/L; PFDA = 0.64 - 7.9 ng/L; PFUnA = < 0.5 ng/L; PFDoA = < 0.5 ng/L;	(Loganathan *et al.* 2007)
PFOA, PFOS	4 WWTPs from Germany	Influent and effluent water	**Influent:** PFOA= 1.8 - 40 ng/L; PFOS= <1 - 85 ng/L;	**Effluent:** PFOA= 5.7 - 250 ng/L; PFOS= <1 - 85 ng/L;	(Becker *et al.* 2008a)
PFHxA, PFOPA, PFDPA, PFOA, PFNA, PFDA, PFUnA, PFOS	WWTPs from Canada	Effluent water	**Effluent:** PFHxPA= 0.33 – 6.5 ng/L; PFOPA= 0.76 – 2.5 ng/L;	PFDPA= 0.38 – 0.46 ng/L; PFOA= 5.8 – 180 ng/L;	(D'Eon *et al.* 2009b)
PFOA, PFOS	From sewage treatment plants: • A: 95% domestic wastewater • B: 60% industrial wastewater and 40% domestic wastewater	influent, primary effluent, aeration tank effluent and final effluent	**Influent**: PFOS = 7.9 - 374.5 ng/L; PFOA = 14.1 - 638.2 ng/L; **Primary Effluent:** PFOS = 9.9 - 338.2 ng/L; PFOA = 11.1 - 531.7 ng/L;	**Aeration Tank Effluent**: PFOS = 11.9 - 560.9 ng/L; PFOA = 22.6 - 939.6 ng/L; **Secondary Effluent**: PFOS = 7.3 - 461.7 ng/L; PFOA = 15.8 - 1057.1 ng/L;	(Yu *et al.* 2009)

(Table 1.4)

PFHxA PFHpA PFOA PFNA PFDA PFUdA PFDoA PFHxS PFHpS PFOS	15 municipal WWTPs, 4 livestock WWTPs and 3 industrial WWTPs	Influent and effluent water	**Influent:** PFHxA= 2.1 - 13.4 ng/L; PFHpA= 1.2 - 11.8 ng/L; PFOA= 2.3 - 615 ng/L; PFNA= 0.7 - 19.4 ng/L; PFDA= 0.6 - 5.1 ng/L; PFUdA= < 0.5 ng/L; PFDoA= nd; PFHxS= 1.2 - 30.2 ng/L; PFHpS= 1.2 - 7.9 ng/L; PFOS= 0.9 - 68.1 ng/L;	**Effluent**: PFHxA= 1.1 - 16.1 ng/L; PFHpA= 1.2 - 16.1 ng/L; PFOA= 3.4 - 591 ng/L; PFNA= 0.7 - 15.8 ng/L; PFDA= 0.6 - 4.2 ng/L; PFUdA= 0.5 - 0.6 ng/L; PFDoA= nd; PFHxS= 0.8 - 14.5 ng/L; PFHpS= 0.3 - 1.5 ng/L; PFOS= 0.9 - 5.7 ng/L;	(Guo *et al.* 2010)
PFBS, PFHpS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA	3 WWTPs from Hong Kong	Influent and effluent water	**Influent**: PFBS = 1.1 - 2.8 ng/L; PFHpS = nd; PFOS = 29.4 - 49.9 ng/L; PFBA = nd; PFPeA = 6.3 - 8.7 ng/L; PFHxA = 1 ng/L; PFHpA = < MLOD; PFOA = < MLOD; PFNA = < MLOD; PFDA = nd; PFUnDA = nd; PFDoA = nd; PFTrA = nd; PFTeA = < MLOD;	**Effluent:** PFBS = 1.3 - 1.5 ng/L; PFHpS = nd; PFOS = 19 - 28.8 ng/L; PFBA = nd; PFPeA = < MLOD; PFHxA = 0.7 - 1.2 ng/L; PFHpA = < MLOD; PFOA = 4.1 ng/L; PFNA = 0.6 ng/L; PFDA = nd; PFUnDA = nd; PFDoA = nd; PFTrA = nd; PFTeA = < MLOD	(Ma *et al.* 2010)
PFOA, PFOS	Water from river and WWTP in Bavaria, Germany	Effluent water	**Effluent:** PFOA= 20 - 3900 ng/L;	PFOS= 106 - 336 ng/L;	(Becker *et al.* 2010)
PFBS, PFHxS, PFOS, PFOA, PFNA	Different WWTPs from Catalonia, Spain	Surface river water and effluent water from WWTP	**Effluent:** PFBS = 0.07 – 2.03 ng/L PFHxS = 0.03 – 25.3 ng/L	PFOS = 0.03 – 72.1 ng/L PFOA = 3.47 – 61.9 ng/L PFNA = 0.06 – 14.1 ng/L	(Sánchez-Avila *et al.* 2010)

(Table 1.4)

PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS	Central WWTP in two industrial zones in Thailand	Influent and effluent water	**Influent:** PFPeA= 0.5 - 14.4 ng/L; PFHxA= 0.1 - 70 ng/L; PFHpA= 0.8 - 32.2 ng/L; PFOA= 6.6 - 142.1 ng/L; PFNA= 15.3 - 174.5 ng/L; PFDA= 1.2 - 63.1 ng/L; PFUnA= 3.1 - 81.9 ng/L; PFDoA= 0.5 - 10 ng/L; PFHxS= 25.8 - 31.7 ng/L; PFOS= 381.3 - 465.4 ng/L;	**Effluent**: PFPeA= 7.9 - 32.4 ng/L; PFHxA= 1.0 - 77.4 ng/L; PFHpA= 1.8 - 46.8 ng/L; PFOA= 16.9 - 149.8 ng/L; PFNA= 24 - 353.2 ng/L; PFDA= 1.8 - 118.8 ng/L; PFUnA= 5.8 - 157.6 ng/L; PFDoA= 7.9 ng/L; PFHxS= 8.7 - 50.4 ng/L; PFOS= 190.1 - 552.8 ng/L;	(Kunacheva *et al.* 2011)

1.5.1.2 WWTPs-Sludge

Higgins *et al.* (2005) reported, for first time, the occurrence of PFASs in sediments and sludge from a WWTP in San Francisco. This study compared the period between 1998 till 2004. Concentrations ranging from 1.2 to 2610 ng/g dw were reported, being PFOS of the compound at higher concentrations. During recent years, some other studies were carried out to assess PFASs in sludge (Table 1.5). PFOS is a compound with an exceptional stability and, due to its higher partition coefficient (in comparison with other PFASs as carboxylic acids), occurred at high concentrations in sewage sludge. Furthermore, PFOS is the end-point of the degradation of different fluorochemicals used in a variety of industrial applications, such as 2-(*N*-ethyl perfluorooctane sulphonamide) acetic acid and 2-(*N*-methyl perfluorooctane sulphonamide) acetic acid, among others, as noted in the previous sections. These compounds have been also identified, in general, in WWTPs sludge at higher concentrations than PFOS. This could indicate that part of the PFOS is directly generated in the degradation process of related products.

Table 1.5: PFASs in sludge samples, published results

Analytes	Origin	Matrices	Results		Reference
PFHxS, PFOS, PFDS, FOSAA, N-MeFOSAA, N-EtFOSAA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	8 WWTPs from different parts of USA, at least 50% domestic waste (1998 – 2004)	Digested sludge (n=8)	PFOA = 5.20-29.4 ng/g dw PFNA = 3.25-10.3 ng/g dw PFDA = 1.16-72.6 ng/g dw PFUnA = 5.34-8.58 ng/g dw PFDoA = 3.49-32.7 ng/g dw PFTeA = <3-77.7 ng/g dw	PFHxS = n.d-3.18ng/g dw PFOS = 14.4-2610 ng/g dw PFDS = 11.0-426 ng/g dw FOSAA = 3.32-26.9 ng/g dw N-MeFOSAA = 30.9-154 ng/g dw N-EtFOSAA = 21.3-335 ng/g dw	(Higgins *et al.* 2005)
PFBS, PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTeA, PFHxDA, PFODA	6 WWTPs from New York State (2005)	Combined sludge liquors	PFOA = 18-241 ng/g dw PFNA = < 25 ng/g dw PFDA = < 25-91 ng/g dw PFUdA = < 25-115 ng/g dw PFDoA = < 25 ng/g dw PFTeA = < 25 ng/g dw	PFHxDA = < 25 ng/g dw PFODA = < 25 ng/g dw PFBS = na PFHxS = < 10-18 ng/g dw PFOS = < 10-65 ng/g dw FOSA = < 25 ng/g dw	(Sinclair E 2006)
PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA, PFDoA	WWTPs from: • (A) rural area (Kentucky) • (B) urban area (Georgia) (2005)	Sludge	PFOS = 8.2–990 ng/g dw (A) and 2.5–77 ng/g dw (B) PFOA = 8.3–219 ng/g dw (A) and 7.0–130 ng/g dw (B)	PFHxS, PFNA = <2.5-67 ng/g dw PFDA = 12-201 ng/g dw FOSA = <2.5-117 ng/g dw PFUnDA = 5.9-37 ng/g dw PFDoDA = 7.2-48 ng/g dw	(Loganathan *et al.* 2007)
PFOA, PFNA, PFDA, PFUnA, PFHxS, PFOS, FOSA	WWTPs from Denmark	Sludge	PFOA = 0.7-19.7 ng/g dw PFNA = 0.4-8.0 ng/g dw PFDA = 1.2-32 ng/g dw PFUnA = 0.5-4.4 ng/g dw	PFHxS = 0.4-10.7 ng/g dw PFOS = 4.8-74.1 ng/g dw FOSA = 0.5-3.6 ng/g dw	(Bossi *et al.* 2008)
PFOA, PFOS	16 municipal WWTPs (2006)	Sewage sludge	PFOS = 236-5383 ng/g dw PFOA = 454-4780 ng/g dw		(Guo R. 2008)
PFOA, PFNA, PFDA, PFUnA, PFOS, (4:2, 6:2, 8:2, 10:2) diPAP	6 WWTPs from Ontario, Canada (2002)	Sludge	PFOS = 100±70 ng/g dw PFCAs = 1.6±0.6 to 0.17±0.10 ng/g dw	4:2 diPAP = nd 6:2 diPAP = 55-590 ng/g dw 8:2 diPAP = 12-860 ng/g dw 10:2 diPAP = 28-220 ng/g dw	(Eon J. 2009)

(table 1.5)

PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrA, PFTeA, PFHxS, PFOS	WWTPs from Decatur and New York city (2007)	Sludge	PFHxA = 16.9 ng/g dw PFHpA = 8.2 ng/g dw PFOA = 31.0 ng/g dw PFNA = 3.5 ng/g dw PFDA = 6.1 ng/g dw PFUdA = <2 ng/g dw	PFDoA = <2 ng/g dw PFTrA = <2 ng/g dw PFTeA = <3.5 ng/g dw PFHxS = 110.2 ng/g dw PFOS = 372.8 ng/g dw	(Yoo H. 2009)
PFOA, PFOS	Sewage treatment plants (STPs) in Singapore • (A) 95% domestic WW • (B) 60% industrial WW and 40% domestic WW (2006 – 2007)	Grab samples of: • Primary sludge • Activated sludge • Secondary sludge • Thickened sludge • Anaerobically digested sludge	**Primary sludge:** PFOA = 8-26.9 ng/g dw PFOS = 13.1-317.9 ng/g dw **Activated sludge**: PFOA = 8.3-54.4 ng/g dw PFOS = 21-617.7 ng/g dw **Secondary sludge**: PFOA = 6.5-45 ng/g dw PFOS = 14-377.1 ng/g dw	**Thickened sludge**: PFOA = 17.5-39.9 ng/g dw PFOS = 28.2-390.1 ng/g dw **Anaerobically digested sludge:** PFOA = 17.4-69.0 ng/g dw PFOS = 30.7-702.2 ng/g dw	(Yu 2009)
TFA, PFPrA, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA, PFBS, PFHxS, PFOS	8 WWTPs from Shangai, China (2008)	Samples of: • Waste activated sludge • Chemical sludge • Activated sludge of aeration tank • Primary sludge	TFA = 253-352 ng/g dw PFPrA = 7.15-241 ng/g dw PFBA = 4.90-131 ng/g dw PFPeA = 1.65-22.9 ng/g dw PFHxA = 0.35-118 ng/g dw PFHpA = 0.489-137 ng/g dw PFOA = 9.21-75.5 ng/g dw PFNA = 0.801-4.63 ng/g dw	PFDA = 0.33-6.21 ng/g dw PFUnA = 0.305-23.4 ng/g dw PFDoA = 0.426-4.48 ng/g dw PFTeA = 0.257-0.730 ng/g dw PFBS = 1.17-2.61 ng/g dw PFHxS = 0.915-3.06 ng/g dw PFOS = 28.1-135 ng/g	(Li *et al.* 2010)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	15 municipal WWTPs, 4 livestock WWTPs and 3 industrial WWTPs from Korea (2008)	Sludge	PFHxA = nd PFHpA = nd PFOA = <MLOQ-5.3 ng/g dw PFNA = <MLOQ PFDA = 2.2-11.8 ng/g dw	PFUnA = 2.8-10.8 ng/g dw PFDoA = <MLOQ-8.7 ng/g dw PFHxS = nd PFHpS = nd-8.6 ng/g dw PFOS = 3.3-54.1 ng/g dw	(Guo R. 2010)

(table 1.5)

PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, FOSA, N-MeFOSA, N-EtFOSA	WWTPs from Hong Kong • Plant (A) and (B): secondary treatment by activated sludge method • Plant (C): chemically enhanced primary treatment (2008)	Sludge	PFBA = 3.1-111.4 ng/g PFPeA = 0.5-10.1 ng/g PFHxA = 0.3-27.8 ng/g PFHpA = 0.4-4 ng/g PFOA = 1.3-15.7 ng/g PFNA = 0.5-23 ng/g PFDA = 0.3-15.2 ng/g PFUnA = 0.4-7.8 ng/g PFDoA = 0.6-8.6 ng/g PFTrA = 0.2-19 ng/g	PFTeA = 0.2-46 ng/g FOSA = nd N-MeFOSA = nd N-EtFOSA = nd PFBS = 0.6-6.4 ng/g PFHxS = nd PFHpS = 106.6 ng/g (one sample) PFOS = 3.1-7304.9 ng/g PFDS = nd	(Ma R. 2010)
PFPeA, PHxA, PFHpA, PFOA, PFNA, PFDoA, PFTeA, 6:2FTUCA, 8:2FTUCA, PFBS, PFHxS, PFOS, FOSA, n-MeFOSA, n-EtFOSA, n-MeFOSAA, n-EtFOSAA	WWTPs from Zürich, Switzerland (2008)	Digested sewage sludge	∑PFCAs = 16.9-21.6 ng/g dw (PFOA = 5.0-9.1 ng/g dw) (PFOA>PFDoA >PFHxA>PFNA>PFHpA) PFOS = 117-670 ng/g dw 6:2 FTUCA = 2.1-3.4 ng/g dw 8:2 FTUCA = 5.4-14.8 ng/g dw FOSA = 2–5 ng/g dw		(Zhang T. *et al.* 2010)
PFBS, PFHxS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, FOSA, N-MeFOSA, NEtFOSA	20 WWTPs from Spain (2006)	Sewage sludge	PFPeA = <0.05-4.69 ng/g dw PFHxA = <0.03-2.60 ng/g dw PFHpA = <0.01-2.04 ng/g dw PFOA = <0.03-7.94 ng/g dw	PFNA = <0.01-10.23 ng/g dw PFDA = <0.04-24.29 ng/g dw PFHxS = <0.01-18.20 ng/g dw PFOS = <0.01-286.81 ng/g dw	(Navarro *et al.* 2011)
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFHxS, PFOS, PFDS, FOSA	WWTPs from Catalonia, Spain (2010)	Sewage sludge (n=5)	PFBA = <MLOD-22.6 ng/g dw PFPeA = <MLOQ-17.2 ng/g dw PFHxA = <MLOD-4.8 ng/g dw PFHpA = <MLOQ-4.5 ng/g dw PFOA = 7.0-30.3 ng/g dw PFNA = 1.0-2.4 ng/g dw PFDA = 6.1-23.5 ng/g dw PFUnA = <MLOQ-12.2 ng/g dw PFDoA = 2.7-11.3 ng/g dw	PFTrA = < MLOQ PFTeA = <MLOQ-5.0 ng/g dw PFHxDA = <MLOQ-4.9 ng/g dw PFODA = <MLOD-0.9 ng/g dw PFBS = <MLOD-7.6 ng/g dw PFHxS = <MLOQ PFOS = 53-121.1 ng/g dw PFDS = <MLOD-7.5 ng/g dw FOSA = <MLOD-10.7 ng/g dw	(Llorca *et al.* 2011a)

(table 1.5)

PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS	WWTPs from industrial zones in Thailand	Samples from: • Activated sludge • Sludge	Activated Sludge Concentrations: PFPeA = 7.6-29.4 ng/L PFHxA = 0.5-79.4 ng/L PFHpA = 1.7-43.3 ng/L PFOA = 13.7-142.0 ng/L PFNA = 12.1-308.4 ng/L PFDA = 4.8-81.3 ng/L PFUnA = 11.9-338.2 ng/L PFDoA = 7.6-48.4 ng/L PFHxS = 26.8-27.5 ng/L PFOS = 348.7-672.9 ng/L	Sludge Concentrations: PFPeA = 2.9-3.3 ng/g PFHxA = 0.3-99.9 ng/g PFHpA = 1.6-52.6 ng/g PFOA = 11.3-136.0 ng/g PFNA = 5.1-512.8 ng/g PFDA = 3.8-327.7 ng/g PFUnA = 45.2-78.2 ng/g PFDoA= ND-310.6 ng/g PFHxS = 36.6-157.7 ng/g PFOS = 396.9-552.6 ng/g	(Kunacheva *et al.* 2011)

1.5.2 Surface river water and sediments

1.5.2.1 Surface river water

The presence of PFASs in surface river waters has been widely reported during recent years (Table 1.6). Most of these data rely on the concentrations of PFOA and PFOS. In general, concentration gradients can vary, up to several orders of magnitude, between different areas along the same river, or lake, reflecting the proximity to industrial sources and WWTPs. In several Japanese studies, PFOS and PFOA were found in river samples with concentrations in the range from 0.30 to 157 ng/L for PFOS, and from 1.6 to 104 ng/L, also for PFOS and 3.8 – 311 ng/L for PFOA (Saito *et al.* 2003). In another study, carried out by Sinclair *et al.* (2006), 51 surface river water samples from New York State (USA) were analysed. The authors found PFOS, PFOA and PFHxS, ranging from 1.6 to 756 ng/L, 15 – 49 ng/L and 0.9 – 7.4 ng/L, respectively. In Europe, noteworthy is the study performed by Skutlared *et al.* (2006) in the Ruhr River. In this study, the occurrence of 12 PFASs, including PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFBS and PFOS were assessed. The results showed extremely high concentrations of some compounds as, for example, PFOA, which was present at concentrations up to 33900 ng/L in an effluent of the Ruhr River, the Moehne River. The authors found that the main source of contamination was located in an agricultural area near Brilon-Scharfenberg. In addition, it was demonstrated that this source leads to the consecutive pollution of Lake Moehn, the Ruhr River and the corresponding drinking waters. In another example in China, Wang *et al.* (2010) studied the environment around a manufacturing facility. The authors found a decreasing trend of the PFOS, PFOA and PFHxS concentrations in soils, water, and chicken eggs, with the increased distance from the production factory. This study indicates that the production site was the primary source of PFASs in that region.

On 31 January 2012, the Commission launched a Proposal for a DIRECTIVE OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL amending the Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. In this proposal, the maximum annual average of PFOS and its derivatives in surface waters was recommended to vary between 0.13 and 0.65 ng/L and the maximum allowable concentration was set in the range of 7.2 and 36 µg/L (European Commission 2012).

Table 1.6: PFASs in waters from different published works

Analytes	Origin	Matrices	Results		Reference
Spain					
PFBS, PFHxS, PFOS, PFOA, PFNA	Rivers: Muga, Fluvià, Ter, Besós, Llobregat, Ebro Different WWTPs (2009) Catalonia (Spain)	Surface river water and effluent water from WWTP	(Surface river:) PFBS = 0.07 – 0.88 ng/L PFHxS = 0.03 – 0.64 ng/L PFOS = 1.09 – 9.56 ng/L PFOA = 0.79 – 9.63 ng/L PFNA = 0.06 – 1.62 ng/L	(Effluent WWTP:) PFBS = 0.07 – 2.03 ng/L PFHxS = 0.03 – 25.3 ng/L PFOS = 0.03 – 72.1 ng/L PFOA = 3.47 – 61.9 ng/L PFNA = 0.06 – 14.1 ng/L	(Sánchez-Avila *et al.* 2010)
PFOS, PFHxS, PFBS, PFOA, PFNA	Northern Spain: Asturias, Cantabria and Basque Country (Spain)	Sea emissaries (n=3) Ports (n=5) Sewage treatmen plant effluent (n=3) Industrial WW effluent (n=1)	PFOS = 0.01 – 5.11 ng/L PFHxS = < MLOD – 0.31 ng/L PFBS = < MLOD – 5.08 ng/L	PFOA = 0.03 – 3.53 ng/L PFNA = 0.01 – 1.40 ng/L	(Gómez *et al.* 2011)
PFBS, PFHxA, PFHpA, PFHxS, THPFOS, PFOA, PFNA, PFOS, FOSA, PFDA, PFUnA, PFDS, PFDoA, PFTrA	Ebro River (Garcia and Mora) Francolí River Cortiella River (Spain)	Surface river water	PFBS = < MLOQ PFHxA = < MLOQ PFHpA = MLOQ – 3.38 ng/L PFHxS = MLOQ – 0.78 ng/L THPFOS = < MLOQ PFOA = MLOQ – 24.9 ng/L PFNA = MLOQ – 0.64 ng/L	PFOS = MLOQ – 5.88 ng/L FOSA = MLOQ – 0.20 ng/L PFDA = MLOQ – 0.82 ng/L PFUnA = < MLOQ PFDS = < MLOQ PFDoA = < MLOQ PFTrA = < MLOQ	(Ericson *et al.* 2008b)
PFBS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, ip-PFNA, PFOS, PFDA, PFDS	L'Albufera de Valencia (Spain)	Surface water	PFBS = < MLOD – 5.50 ng/L PFPeA = < MLOD – 5.40 ng/L PFHxA = < MLOQ – 6.90 ng/L PFHpA = < MLOQ – 18.4 ng/L PFOA = 0.99 – 120.2 ng/L	PFNA = 0.02 – 18.5 ng/L ip-PFNA = < MLOD – 5.44 ng/L PFOS = 0.94 – 58.1 ng/L PFDA = < MLOD – 10 ng/L PFDS = < MLOD – 1.29 ng/L	(Pico *et al.* 2011)

(table 1.6)

Other countries					
PFOA, PFNA, PFOS, PFDA, PFUdA, FOSA	Conasauga River, Altamaha River and streams and ponds of Dalton (Georgia, USA)	Surface river	PFOA = 2.6 – 1280 ng/L PFNA = 0.6 – 456 ng/L PFOS = 0.2 – 368 ng/L	PFDA = 0.1 – 160 ng/L PFUdA = 0.1 – 117 ng/L FOSA = 10.7 – 420 ng/L	(Konwick *et al.* 2008)
PFOS	Rivers: Tone, Arakawa, Tama (Tokyo, Japan)	Surface river water	PFOS = 0.5 – 58 ng/L		(Takazawa *et al.* 2009)
PFOS, PFOA, PFHpA, PFNA, PFDA, PFDoA, PFHxS	Rivers from Northern of China	Surface river water	PFOS = < MLOD – 31 ng/L PFOA = 0.43 – 82 ng/L PFHpA = < MLOD – 35 ng/L PFNA = < MLOD – 4.9 ng/L	PFDA = < MLOD – 5.7 ng/L PFDoA = < MLOD – 0.29 ng/L PFHxS = < MLOD – 5.8 ng/L	(Wang *et al.* 2011)
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFBS, PFOS	Rhine river, Mohene river and tributaries, Ruhr area	Surface river and lake water	(Rhine:) PFBA = 2 – 3 ng/L PFPeA = 2 – 42 ng/L PFHxA = 2 – 77 ng/L PFHpA = 2 – 11 ng/L PFOA = 2 – 48 ng/L PFBS = 2 – 46 ng/L PFOS = 2 – 26 ng/L (Ruhr area:) PFBA = 2 – 143 ng/L PFPeA = 2 – 1638 ng/L PFHxA = 2 – 1248 ng/L PFHpA = 25 – 148 ng/L PFOA = 9 – 3640 ng/L PFBS = 3 – 71 ng/L PFOS = 5 – 193 ng/L	(Mohene river and tributaries:) PFBA = 9 – 200 ng/L PFPeA = 25 – 2670 ng/L PFHxA = 73 – 3040 ng/L PFHpA = 46 – 989 ng/L PFOA = 11 – 33900 ng/L PFBS = 17 – 1450 ng/L PFOS = 2 – 5900 ng/L	(Skutlarek *et al.* 2006)

1.5.2.2 Sediment

There are a few works reporting concentration of PFASs in sediment samples from the Iberian Peninsula. An example was published by Picó *et al.* (2011). The authors investigated different points from l'Albufera de Valencia (Spain), assessing the presence of PFASs between the method limit of detection (MLOD) and 10.9 ng/g, where the highest concentrations corresponded to PFOA and PFOS. In the same study, the presence of PFASs in surface waters was assessed, showing the distribution of these compounds between water and sediments. The sediment–water distribution coefficient (log K_D) was calculated for every sampling point. The authors found the log K_D in the range 2.31–4.51 and positively correlated this with perfluoroalkyl chain length. In another study, Gómez *et al.,* (2011) have analysed different sediments from the Cantabrian Sea area (at the southern edge of Bay of Biscay, Northwest of Spain). The results, in most of the cases, were below the MLOD in sediment river samples. In another earlier work, Alzaga *et al.* (2005) detected low ng/g concentrations of PFOA and PFDA in harbour sediments. On the other hand, several studies have been carried out in other European countries. Becker *et al.* (2008) reported the presence of PFOA and PFOS in the sediment of the Roter Main River (Bayreuth, Germany). The concentrations were between 0.02 and 0.31 ng/g, being PFOS at higher amounts. Austria and France have also studied sediment samples, finding a different profile of PFASs, but at similar concentrations as the ones reported for Iberian Peninsula (Clara *et al.* 2009; Labadie *et al.* 2011).

The presence of these analytes has been assessed in different countries from North America (Higgins *et al.* 2005; Senthil Kumar *et al.* 2009) and Asia (Nakata *et al.* 2006; Senthilkumar *et al.* 2007; Bao *et al.* 2009; Naile *et al.* 2010; Bao *et al.* 2011; Yang *et al.* 2011). The distribution pattern of PFASs is similar in all the cases, with levels between low pg/g and low ng/g, being the sulphonates at higher concentrations, in most of the cases.

1.5.3 Biological samples

The new proposal for Directive amending the list of priority substances (European Commission 2012), recommended the maximum annual average of PFOS and its derivatives in surface waters was recommended as maximum concentration in biota 9.1 µg/Kg of wet weight.

PFASs have been detected in wildlife samples collected from around the world.

Fluorinated analytes have been detected in arctic mammals, ocean-going birds, fur seals and penguin eggs from the Antarctic Peninsula and other remote location, far from human settlements (Kannan et al. 2001; Tao et al. 2006; Dietz et al. 2008; Sonne et al. 2008; Schiavone et al. 2009). The effects of the accumulation of PFOS, long chain perfluoroalkyl carboxylic acids, and other POPs in Arctic top predators were studied by Sonne et al. (2010). In this study, the presence of PFASs was related to chemical stress, which also seemed to impair immunity and promote decreased fecundity.

Higher concentrations of PFASs were, in general, found when biota from industrialised areas were studied, as was expected. In this case, fish and birds are the most commonly studied animals (Taniyasu *et al.* 2003; Sinclair *et al.* 2006; Naile *et al.* 2010). Fish have been the main biological matrix studied, as common for POPs, in general, because their possible toxicological effects on humans through their consumption. Among PFASs, PFOS is the most crucial and prominent compound. Reports suggest no considerable differences in PFAS concentrations among fresh water and marine fish species. PFOA is the second most frequently detected compound in fish, but at much lower concentrations than PFOS. The difference between the concentrations of PFOS and PFOA in fish suggests a lower potential of PFOA in bioaccumulation. This observation was further confirmed by laboratory experiments, which revealed a 1,000-fold lower bioconcentration factor for PFOA compared with PFOS (Martin *et al.* 2003; Gruber *et al.* 2007). The other PFASs are, generally, detected at lower concentrations than PFOS (Martin *et al.* 2003). The marine food webs have been identified as the most representative example of PFAS biomagnification (Tomy *et al.* 2004; Houde *et al.* 2006; Kelly *et al.* 2009; Butt *et al.* 2010).

Few publications have reported the accumulation of PFASs in bivalves. But, similarly PFOS was the more prevalent accumulated in the aquatic invertebrates, such as shrimp, mussels, clams and oysters (Gulkowska et al. 2006). The concentrations ranging from 1 to 6.0 ng/g (wet weight) in oysters from the Ariake Sea (Nakata et al. 2006) and China (Gulkowska et al. 2006) were reported, respectively. Cunha et al. (2005) measured high concentrations of PFOS in mussels from several estuaries in the North of Portugal. Nania et al. (2009) found higher concentrations of PFOA in comparison to PFOS in clams, although mussels were at comparable concentrations in the North area of Portugal. These differences have been attributed to the habitats and the feeding behaviours of these species.

1.5.4 Drinking water

In order to investigate the possible source of PFASs in drinking water, some authors have compared the concentrations of PFASs found in catchment sites and in tap water. This reveals that the total removal of PFASs by purification processes is not achieved (Skutlarek *et al.* 2006; Loos 2007). In addition, possible degradation products generated in the environment or during water treatment processes should be considered (Dinglasan *et al.* 2004; Wang *et al.* 2005; Frömel *et al.* 2010; Lee *et al.* 2010). For example, Skutlarek *et al.* (2006) showed extremely high concentrations of PFOA in tap water in the zone of Ruhr area, which are in agreement with the concentrations found in environmental surface waters and the evidence of the ineffective removal of PFASs by water treatment steps during, in this case, bank filtration and artificial recharge. In another example, PFOS and PFOA were measured in Lake Maggiore (Switzerland) and in drinking water at concentrations around 9 ng/L and 3 ng/L, respectively. The analysis of drinking water produced from the lake gave almost identical results, revealing the poor

performance of sand filtration and chlorination applied by the local waterworks (Loos 2007).

Similar conclusions can be extracted from the work carried out by Takagi *et al.* (2008), which studied the occurrence of PFASs in different waters, including raw water and drinking water from Japan. In raw water, the results for PFOS and PFOA varied between 0.26–22 ng/L and 5.2–92 ng/L, respectively, and in tap water similar results were also obtained in the ranges between 0.16–22 ng/L and 2.3–84 ng/L for PFOS and PFOA, respectively. The authors found positive correlations between PFAS concentrations in raw water and tap water samples, showing how the potable water treatments were not efficient enough in removing these compounds. In addition, other perfluoroalkylated and polyfluoroalkylated substances that, in general, are not assessed, but are generated during the water treatment processes, should be considered (Dinglasan *et al.* 2004; Wang *et al.* 2005; Frömel *et al.* 2010; Lee *et al.* 2010).

Regarding the occurrence of PFASs in Spanish tap water, Ericson *et al.* (2008b; 2009) studied the presence of these contaminants in drinking water from different areas of Catalonia. The concentrations of PFASs were in the range of 0.02 and 69 ng/L in tap waters, below the Provisional Health Advisories (PHA) concentration levels established by the Environmental Protection Agency's Office of Water (EPA – OW) in 2009 (0.4 µg/L for PFOA and 0.2 µg/L for PFOS) to protect against potential risk from exposure to these chemicals through drinking water (USEPA 2009).

1.5.5 Human matrices

The presence of organic fluorine in humans was first reported by Taves (1968) using RMN techniques. Later, due to the advances in analytical techniques, PFASs have been assessed in different human matrices, such as breast milk at low ng/L (So *et al.* 2006b; Tao *et al.* 2008; Völkel *et al.* 2009), saliva (Tao 2009), urine (Ylinen *et al.* 1985; Tao 2009), seminal plasma (Guruge *et al.* 2005), blood (Inoue et al. 2004; Kubwabo *et al.* 2004; Calafat *et al.* 2006a; Ericson *et al.* 2007; Monroy *et al.* 2008) and human liver (Kärrman *et al.* 2009). As regards to the levels of PFASs, generally PFOS is the compound detected at higher concentrations in males, rather than in females (Yeung *et al.* 2005). Calafat et al. (2006a), found different patterns of accumulation in different ethnic groups (Calafat *et al.* 2006a). However, studies of workers, occupationally exposed to perfluoroalkyl, showed that levels of both PFOA and PFOS are approximately one order of magnitude higher than those reported in the general population (Lau *et al.* 2007), with mean concentrations of 5 mg/g and 1 to 2 mg/g respectively (Olsen *et al.* 2001). A study performed by Olsen *et al.* (2007) have suggested that the mean biological half-life of PFOS, PFHxS and PFOA in human serum in retired perfluoroalkyl-exposed workers is 5.4, 8.5 and 3.8 years, respectively.

1.6 Ecotoxicology

Accumulation and biomagnification of PFASs have been shown to be especially relevant in aquatic ecosystems. In the next two sections, some studies reporting the acute and the chronic toxicity of PFASs for aquatic and some terrestrial species are explained.

1.6.1 Acute toxicity

Different acute toxicity assays have been performed during recent years, in order to elucidate different endpoints, such as the effective concentration to 50% of test organisms (EC50), no-observable-effect-concentration (NOEC), lethal concentration to 50% of test organisms (LC50) and the lowest observed effect concentration (LOEC). Table 1.7 summarises some of the available toxicity results in the published literature. The acute toxicity of PFASs can be assumed to be negligible due to the high concentration levels required for toxicity responses (in the range of ppm) which are much higher than environmental concentrations.

In the case of the same acute toxicity test for PFOA and PFOS, it is noteworthy that the effective concentration of the latter is lower than for the former, indicating more toxic effects for PFOS. An example can be seen in Table 1.7 for *Daphnia magna* and *Moina macrocopa,* where PFOS is approximately 10 times more toxic than PFOA in these organisms (Ji *et al.* 2008). The same pattern can be observed for aquatic vertebrates (Hagenaars *et al.* 2011).

On the other hand, the toxicity tested in algae species showed a distinct relationship between hydrophobicity and toxicity of PFHxA, PFOA and PFNA (Latala *et al.* 2009). The toxicity increases with the number of carbon atoms in the chain. These results are supported by the toxicity assays performed by Hagenaars *et al.* (2011) in vertebrate Zebra Danio fish with PFBA, PFOA, PFBS, PFOS and PFNA. In the case of algae, differences can be noted in the responses, depending on the species: blue-green algae and diatoms are more sensitive to PFASs than green algae. The authors attributed these differences to differences in the cell wall structure (Latala *et al.* 2009).

Considering other toxicological effects, PFASs resulted in malformations of the tail and of an uninflated swim bladder in Zebra Danio fish (Hagenaars *et al.* 2011). Another differentiate effect is that C-8 PFASs can cause oedemas and effects on length while head malformations were a more specific action of the sulphonated PFASs (Hagenaars *et al.* 2011).

Table 1.7: Acute toxicities available in the literature, from EPA ECOTOX database (EPA).

	Species Common Name	Species	End-point	Measurement	Exposure time (days)	PFBA (µg/L)	PFHxA (µg/L)	PFOA (µg/L)	PFOS (µg/L)	PFNA (µg/L)	Reference
Aquatic organisms	**Algae**										
	Green Algae	*Chlorella vulgaris*	EC_{50}	Population growth rate	3		4032402	977205		496565	(Latala *et al.* 2009)
	Blue-green Algae	*Geitlerinema amphibium*	EC_{50}	Population growth rate	3		12823038	248442		139038	(Latala *et al.* 2009)
	Diatom	*Skeletonema marinoi*	EC_{50}	Population growth rate	3		60524741	368522		58396	(Latala *et al.* 2009)
	Invertebrates										
	Microcrustacean	*Daphnia magna*	EC_{50}	Immobilisation	1			675050	193000		(Ji *et al.* 2008; Li 2009)
	Microcrustacean	*Daphnia magna*	EC_{50}	Immobilisation	2			476520	63000		(Ji *et al.* 2008)
	Microcrustacean	*Daphnia magna*	NOEC	Immobilisation	1			500000	100000		(Ji *et al.* 2008; Li 2009)
	Microcrustacean	*Moina macrocopa*	EC_{50}	Immobilisation	2			199510			(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	LOEC	Immobilisation	1			250000			(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	NOEC	Immobilisation	1			125000			(Ji *et al.* 2008)
	Flatworm	*Dugesia japonica*	LC_{50}	Mortality	4			337000	23000		(Li 2009)
	Flatworm	*Dugesia japonica*	NOEC	Mortality	4			150000	10000		(Li 2009)
	Vertebrates										
	Zebra Danio	*Danio rerio*	EC_{50}	Multiple or delayed effects	4	1900780		205720	16740	3000000	(Hagenaars *et al.* 2011)
	Zebra Danio	*Danio rerio*	LC_{50}	Mortality	5	3000000		500000	28210	3000000	(Hagenaars *et al.* 2011)
	Zebra Danio	*Danio rerio*	LOEC	Multiple or delayed effects	5	500000		75000	100000	1000000	(Hagenaars *et al.* 2011)
	Zebra Danio	*Danio rerio*	NOEC	Multiple or delayed effects	5	250000		50000	< 100000	500000	(Hagenaars *et al.* 2011)
	Common Carp	*Cyprinus carpio*	LOEC	Vitellogenin	4			6582	5395		(Kim *et al.* 2010)

Acronyms in order of appearance: effective concentration to 50% of test organisms (EC_{50}), no-observable-effect-concentration (NOEC), lethal concentration to 50% of test organisms (LC_{50}), lowest observed effect concentration (LOEC)

1.6.2 Chronic toxicity

Several toxicological studies have been conducted to assess the effects of PFASs, and in particular PFOA and PFOS. In these studies, subchronic effects, developmental / reproductive toxicity, and the carcinogenicity of PFASs were evaluated in different animal species (Lau *et al.* 2004). Table 1.8 summarises different works reporting toxicity assays using as test species aquatic and terrestrial organisms.

For example, the Interactome Analysis Workflow, GeneGo pathway maps and GO processes pinpointed peroxisome proliferator-activated receptor - alpha (PPAR-α) as a key mechanistic target for PFOA-induced toxicity in rats including: hepatotoxic effects, steatsis development with a perturbed fatty acid homeostasis and the induction of peroxisome proliferation (GeneGo Incorporated 2009). In another study by Cui *et al.* (2010) the elimination of PFOA and PFOS in rats was studied. The experiments were conducted after subchronic exposure by oral doses. In this study, it was shown that the elimination in urine is faster compared with feces. It indicates that urinary excretion is the primary body elimination route in rats. On the other hand, the higher elimination rate of PFOA compared to PFOS through excretion indicates the lower accumulation of acids in rats, and this could induce lower toxicities compared to PFOS (Table 1.8). In general, in subacute and chronic studies, the liver was identified as the target organ for these two compounds, inducing liver tumours in rats (EFSA 2008). Other works have demonstrated interaction with thyroid hormones, high-density lipoprotein, cholesterol and triglycerides (Lau *et al.* 2007; Peden-Adams *et al.* 2008). The studies performed in animals during pregnancy have demonstrated that some PFASs can pass through the placental barrier. For example, Grasty et al. (2005) studied the transplacental exposure to PFOS in rodents. The results have shown that PFOS induced neonatal mortality and the lung problems of born-alive pups. However, little is known about PFAS concentration levels and potential effects on development of children.

Table 1.8: Chronic toxicities available in the literature. Values from the EPA ECOTOX database (EPA).

	Species Common Name	Species	End-point	Measurement	Exposure time (days)	PFOA (µg/L)	PFOS (µg/L)	Reference
Aquatic organisms	**Plantae**							
	Eurasian Watermilfoil	*Myriophyllum spicatum*	NOEC	Morphology - lenght	21	23900		(Hanson *et al.* 2005)
	Invertebrates							
	Microcrustacean	*Daphnia magna*	LC_{50}	Mortality	14	> 100000	12500	(Li 2010)
	Microcrustacean	*Daphnia magna*	LC_{50}	Mortality	21	> 100000	9100	(Li 2010)
	Microcrustacean	*Daphnia magna*	LC_{50}	Mortality	7	> 100000	20000	(Li 2010)
	Microcrustacean	*Daphnia magna*	LOEC	Mortality	14		10000	(Li 2010)
	Microcrustacean	*Daphnia magna*	LOEC	Immobilization	21		50000	(Sanderson *et al.* 2004)
	Microcrustacean	*Daphnia magna*	LOEC	Mortality	21		10000	(Li 2010)
	Microcrustacean	*Daphnia magna*	LOEC	Progeny counts/numbers	21	32000	5000	(Li 2010)
	Microcrustacean	*Daphnia magna*	NOEC	Immobilisation	21	> 88600	25000	(Sanderson *et al.* 2004; Colombo *et al.* 2008)
	Microcrustacean	*Daphnia magna*	NOEC	Length	21	25000		(Ji *et al.* 2008)
	Microcrustacean	*Daphnia magna*	NOEC	Population growth rate	21	50000		(Ji *et al.* 2008)
	Microcrustacean	*Daphnia magna*	NOEC	Progeny counts/numbers	21	12500	10000	(Ji *et al.* 2008; Li 2010)
	Microcrustacean	*Daphnia magna*	NR-ZERO	Mortality	21	6250		(Ji *et al.* 2008)
	Microcrustacean	*Daphnia magna*	EC_{50}	Survival	21	31250		(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	NOEC	Progeny counts/numbers	7	6250		(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	NOEC	Time to first progeny	7	25000		(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	NOEC	Survival	7	12500		(Ji *et al.* 2008)
	Microcrustacean	*Moina macrocopa*	NR-LETH	Mortality	7	50000		(Ji *et al.* 2008)
	Pacific Oyster	*Crassostrea gigas*	NR-ZERO	Mortality	56	6.5	6.6	(Jeon *et al.* 2010)
	Vertebrates							
	Rainbow Trout	*Oncorhynchus mykiss*	LOEC	Gene expression (Liver) or Organ weight in relationship to body weight (liver)	14	1800000	1000	(Oakes *et al.* 2005; Tilton *et al.* 2008)

(Table 1.8)

	Species Common Name	Species	End-point	Measurement	Exposure time (days)	PFOA (µg/L)	PFOS (µg/L)	Reference
Terrestrial organisms	**Plantae**							
	Lettuce	*Lactuca sativa*	EC_{50}	Weight - Growth and length	5	170000	99	(Li 2009)
	Lettuce	*Lactuca sativa*	LC_{50}	Germination	5	1734000	> 200000	(Li 2009)
	Lettuce	*Lactuca sativa*	LOEC	Lenght	5	2000000	100000	(Li 2009)
	Lettuce	*Lactuca sativa*	NOEC	Germination	5	1000000	> 200000	(Li 2009)
	Cucumber	*Cucumis sativus*	EC_{50}	Weight - Growth and length	5	1254000	> 200000	(Li 2009)
	Cucumber	*Cucumis sativus*	LC_{50}	Germination	5	2000000	> 200000	(Li 2009)
	Cucumber	*Cucumis sativus*	NOEC	Germination	5	2000000	> 200000	(Li 2009)
	Pak Choi	*Brassica rapa ssp. chinensis*	EC_{50}	Weight - Growth and length	7	278000	130000	(Li 2009)
	Pak Choi	*Brassica rapa ssp. chinensis*	LC_{50}	Germination	7	579000	200000	(Li 2009)
	Pak Choi	*Brassica rapa ssp. chinensis*	LOEC	length	7	250000	100000	(Li 2009)
	Pak Choi	*Brassica rapa ssp. chinensis*	NOEC	Germination	7	250000	200000	(Li 2009)
	Vertebrates							
	Norway Rat	*Rattus norvegicus*	NOEL	Weight - Growth	182.64	0.015%		(Abdellatif *et al.* 1990)
	Norway Rat	*Rattus norvegicus*	LOEL	Physiology, general	10	100 mg/Kg	20 mg/Kg	(Dupont-Haskell-Laboratory 2000)
	Guinea Pig	*Cavia porcellus*	LD_{50}	Mortality	14	217000 µg/Kg		(Dupont-Haskell-Laboratory 2000)
	Domestic Dog	*Canis familiaris*	NR-LETH	Mortality	48	450000 µg/Kg		(Dupont-Haskell-Laboratory 2000)

Acronyms in order of appearance: no-observable-effect-concentration (NOEC), lethal concentration to 50% of test organisms (LC_{50}), lowest observed effect concentration (LOEC), 0% mortality or 100% survival of organisms (NR-ZERO), effective concentration to 50% of test organisms (EC_{50}), 100% mortality or 0% survival of organism (NR-LETH), no-observable-effect-level (NOEL), lethal dose to 50% of test organisms (LD_{50})

1.7 Human exposure and bioaccumulation

The main routes of human exposure have been identified as inhalation, ingestion, and drinking.

These compounds are bioaccumulated in organisms, which, in addition, can result in a biomagnification factor through the food chain (Tomy *et al.* 2004; Kelly *et al.* 2009). Once in the food chain, increased levels of PFASs in animal-derived foods will be detected. Fish has been shown to be the main influence of PFASs in dietary exposure. Food preparation is another source of contamination (Kantiani *et al.* 2010), but preliminary data on the influence of domestic cookware on levels of PFASs in the preparation of food indicates no elevated levels for a limited number of experiments (Jogsten *et al.* 2009). Packaging may also introduce PFASs, used in greaseproof packaging for fast foods and special packages. In these situations PFASs enter into food via migration from food package.

In 2008 EFSA established the tolerable daily intake for PFOA and PFOS (EFSA 2008). There can be identified two different sources of food contamination:

(i) Direct environmental exposure of plants and animals
(ii) Indirect contamination by cooking, food packaging and food processes

1.7.1 Direct contamination: PFASs through diet

Fish consumption, daily products as milk, meat or vegetables were identified as main entrances of PFASs through diet (Carabias-Martínez *et al.* 2005; Tittlemier *et al.* 2006; EFSA 2008; Ericson *et al.* 2008a; Kantiani *et al.* 2010; Pico *et al.* 2010; Lacina *et al.* 2011). Food items can become contaminated with PFASs in a variety of ways (Tittlemier *et al.* 2007; Clarke *et al.* 2011; Loewen *et al.* 2005; Kim *et al.* 2007):

- Bioaccumulation through the aquatic food chain
- Vegetal uptake from contaminated soil and water
- Animal uptake from contaminated pastures, feed and water
- Animal production inhalation

A relatively low number of studies were published regarding the analysis of PFASs in food samples. Fish have been the most studied matrix and, in general, the highest levels correspond to big fish (swordfish, hake) (Pico *et al.* 2010). Other published works have been focused in the study of the presence of these compounds in different food stuffs. One example is the study of accumulation in juvenile chickens, performed by Yeung *et al.* (2009a). The authors observed that PFOS and PFDA were accumulated at much higher concentrations than PFOA and liver was the main target organ during exposure. Another Japanese study of chicken eggs was performed by Wang *et al.* (2008). The authors observed the presence of PFOS in the 100% of analysed eggs at concentration ranging from 45 to 87 ng/g ww. Gurugue *et al.* (2008) carried out an investigation of PFASs in

farm and pet animals. The researchers concluded that PFOS was present at higher concentrations in liver samples of chicken, pig and cattle (low ng/g). A similar work, was carried out by Ericson *et al.* (2008a) in Catalonia. The authors found PFOS in pork, chicken, veal, lamb and eggs at low ng/g. Other edible market products have been analysed by Jogsten *et al.* (2009). The results in veal steak, pork loin and chicken breast (raw, grilled and fried), black pudding and other edible products from supermarkets showed the presence of PFOS in 8 of the 20 food items analysed at levels varying between 0.330 to <0.001 ng/g of fresh weight. In addition, PFHxA in raw veal, chicken nuggets, frankfurter, sausages and packaged lettuce. However, it should be noted that these concentration levels are lower than reported levels for fish, but they are a source of PFASs in human diet and contribute to an increase in human exposure.

1.7.2 Indirect contamination

PFASs are used in grease- and water-repellent coatings for food packaging which can be an indirect source of food contamination by cooking, food packaging and food processes.

Tittlemeier *et al.* (2007) appointed food preparation as one possible source of contamination. However, preliminary data concerning this source of PFASs through domestic cookware during preparation of food indicated no elevated levels for a limited number of experiments (Powley *et al.* 2005). As a complementary work, Del Gobbo *et al.* (2008) reported that cooking decreases PFAS concentrations in fish.

Migration should also be considered, from food packaging to food due to the use of greaseproof packaging in, for example, fast foods and other specially packaged products (Tittlemier *et al.* 2007). Fluorochemical-treated paper has been tested to determine the migration amount that occurs into food and food-simulating liquids as well as the characteristics of the migration (Begley *et al.* 2005; Begley *et al.* 2008). Microwave popcorn and chocolate spread were used to investigate migration. Results indicate that fluorochemical paper additives migrate to food during actual package use.

Another indirect source that has been studied is the exposure to PFOA in a variety of consumer products including clothing, upholstery, sealants, waxes, paints and cleaners. It was concluded that the exposure to PFAS through these treated products is not a significant source of contamination to the general public (Washburn *et al.* 2005).

1.7.3 Human accumulation and possible consequences

The presence of PFASs in human matrices has been investigated during recent decades, because there is a wide uncertainty about the accumulation processes as well as the acute and chronic toxicity effects. PFASs have been assessed in different human matrices, as described in the section concerning the occurrence in human matrices.

Regarding the presence in human blood, these analytes have been reported in adult donors from different countries (Kubwabo *et al.* 2004; Calafat *et al.* 2007a; Calafat *et al.* 2007b; Ericson *et al.* 2007), as well as in cord blood and maternal blood because breastfeeding and placental transfer during pregnancy are pointed to as the main transferability routes (Inoue *et al.* 2004; Monroy *et al.* 2008; Fromme *et al.* 2010; Gützkow *et al.* 2011; Lien *et al.* 2011). The presence of PFASs in cord blood, human breast milk and seminal plasma indicates their presence in the human reproductive system and, consequently, in human accumulation.

A risk assessment conducted by Tittlemeier *et al.* (2007), through the study of dietary exposure of Canadians to PFASs via consumption of meat, fish, fast foods and food items prepared in their packaging, pointed to the likelihood that dietary exposure could cause adverse human health effects. The daily exposure to PFASs from food was used to calculate the margin of exposure (MOE) estimate. This value, compared with toxicological reference points established in feeding studies involving non-human primates and rodents, was 10^4 and 10^5 greater for PFOS and PFOA, respectively, indicating that there is a difference between the toxicological effects observed and the average Canadian's dietary exposure (Tittlemier *et al.* 2007). Another study focused on the indirect exposure of these analytes through the ingestion of chemicals, applied to food-contact paper packaging, in rats was studied for PAPs (D'Eon *et al.* 2007). Increased levels of PFOA were observed in the dosed animals, linking ingestion of PAPS with in vivo production of perfluoroalkyl acids.

Other types of human exposure have been investigated by different authors. Stock *et al.* (2010) summarise some of the obtained results. For example, PFAS-treated consumer goods, such as carpets and apparel, accounted for an estimated exposure of 120 ng/day and daily intakes of PFASs via water, dust and air were estimated to be 0.3, 28 and 12 ng/day, respectively (Tittlemier *et al.* 2007). It has been observed that the linear PFASs are correlated between human diet and blood by De Silva *et al.* (2006). Although the authors found that the presence of linear isomers in human blood is the predominant conformation for PFASs, it suggests that the organisms are more likely to be exposed to a linear form. It could be that linear isomers are preferentially absorbed and/or branched isomers are more readily eliminated (Loveless *et al.* 2006).

1.8 Legislation

Because of their bioaccumulation (Tomy *et al.* 2004; Kelly *et al.* 2009; Jeon *et al.* 2010) and potential health concerns, including toxicity (Kudo *et al.* 2003; Newsted *et al.* 2005; Ji *et al.* 2008; Yanai *et al.* 2008; Bhhatarai *et al.* 2011), and their possible contribution to cancer promotion, non-governmental organisations, national and international authorities have addressed the PFASs issue and legislative actions were proposed. One of the major fire-fighting foam manufacturers, 3M, started in 2000 the voluntary phasing out of the production of PFOS. In Europe, the hazard assessment of the OECD from the year 2002 identified PFOS as a PBT-chemical (persistent, bioaccumulative and toxic).

In 2006, the EPA and the eight major PFASs producer companies (Arkema, Asahi, BASF Corporation (successor to Ciba), Clariant, Daikin, 3M/Dyneon, DuPont, Solvay Solexis) in the industry launched the "PFOA Stewardship Program". The companies committed to phasing out global facility emissions and product content of PFASs by 95% by 2010 and to work toward eliminating emissions and product content by 2015 (USEPA 2006)**.** During the same year, the OECD investigated production of PFSAs, PFCAs and products or mixtures containing PFSAs and PFCAs (OECD 2006). The reported results were lower than previous studies in 2003 with a decrease from 3000 to 175 tonnes of PFOS and PFOS containing products manufactured and/or imported. The values are in agreement with the phasing out of PFOS-based products by the 3M Company and the use of related products, and certain products for which no substitutes are available (Stock *et al.* 2010). PFASs are also prime candidates for chemicals that will need authorisation within the REACH regulation (European Commission 2002).

More recently, as a result of PFOS PBT-chemical classification, it has been included as a persistent organic pollutant (POP) under the Stockholm Convention for global regulation of production and use (UNEP 2010). In addition, a draft proposal to integrate PFOS in the WFD has been proposed in January 2012.

PFASs are now included in different research programs in EEUU, Canada and Europe. The EU-VII European Research Framework Program has funded research projects to assess the distribution, toxicity and persistence of these compounds PFASs. On the other hand, currently, the production of shorter PFASs has increased, because of their use in the industry as a replacement of longer PFSA and PFCA products. An example of the used shorter PFASs is the PFBS-based products (OECD 2006).

Table 1.9 summarises some of the current World legislations.

Table 1.9: Summary of some of the World current legislation regarding PFASs

Year	Organism	Legislation	Act	Reference
European Union				
2002	Organisation for Economic Co-operation and Development (OECD)	34th OECD Chemical Committee meeting, December 2002	PFOS classified as a PBT-chemical (persistent, bioaccumulative and toxic)	(OECD 2002)
2004	European Commission	Persistent Organic Pollutants Regulation (EC) 850/2004	Controls in the use and disposal of PFOS in fire fighting foams	
2006	European Commission	DIRECTIVE 2006/122/ECOF, amending for the 30th time Council Directive 76/769/EEC	Restrictions on the marketing and use of PFOS	
2006	OSPAR Commission	OSPAR Commission	To encourage any activities to reduce the risks associated with PFOS, including the substitution of PFOS with safer substitutes which pose less risk	(OSPAR_Commission 2006)
2008	European Food Safety Authority (EFSA)	Tolerable Daily Intake (TDI)	TDI established for: PFOS = 150 ng/Kg b.w per day, PFOA = 1500 ng/Kg b.w per day	(EFSA 2008; EFSA 2012)
2010	Stockholm Convention	the Stockholm Convention for global regulation of production and use	PFOS identified as a persistent organic pollutant (POP)	(UNEP 2010a)
2010	United Nations Economic Commission for Europe (UNECE)	Convention on Long-Range Transboundary Air Pollution (LRTAP)	PFOS proposed as a POP	(UNECE 2010)
2012	European Commission	"Water Framework Directive: Proposal for a Directive of the European Parliament and of the Council amending Directives 200/60/EC and 2008/150/EC as regards priority substances in the field of water policy", January 2012	PFOS and its derivative salts maximum permission levels*: Annual average values Inland surface waters = $6.5 \cdot 10^{-4}$ μg/L, Other surface waters = $1.3 \cdot 10^{-4}$ μg/L, Maximum allowable concentrations in Inland surface waters = 36 μg/L, and Other surface waters = 7.2 μg/L, Biota = 9.1 μg/Kg, in fish	(European_Commission 2012)
European Countries				
2012	Spanish Ministry, Spain		Controls use and disposal of PFOS in fire fighting foams	(CNRCOP 2012)
United States				
1988 - 2003	United States Environmental Protection Agency	Chemicals nominated for in-depth toxicological evaluation for carcinogenesis testing in fiscal years 1988–2003; National Toxicology Program, US EPA	Evaluation of PFBS, PFDA, PFHxS, PFOS and PFOA	(NTP 2005)

(table 1.9)

2007	United States Environmental Protection Agency	Consent Orders and Significant New Use Rules (SNURS)	To require companies to inform them before manufacturing or importing any listed PFOS chemicals and the prohibition of some of them	
2009	United States Environmental Protection Agency	Drinking water standards and helath advisories, provisional levels; Office of Water	PFOA = 400 ng/L PFOS = 200 ng/L	(EPA 2009)
2012 and will occur during 2013-2015	United States Environmental Protection Agency	Third Unregulated Contaminant Monitoring Rule (UCMR 3)	minimum reporting level in drinking water PFOS = 40 ng/L PFOA = 20 ng/L PFNA = 20 ng/L PFHxS = 30 ng/L PFHpA = 10 ng/L PFBS = 90 ng/L	(USEPA 2012)
States				
2006	Washington	PBT rule (WAC 173-333-320)	PFOS classified as a PBT-chemical (persistent, bioaccumulative and toxic)	(Washington State Department of Ecology 2006)
2007	New Jersey	Drinking water guidance values	Maximum allowable concentration of PFOA = 40 ng/L	(New Jersey Department of Environmental Protection *et al.* 2007)
2007	Minnesota	Groundwater regulations	Health risk limits for PFOA = 300 ng/L, PFOS = 300 ng/L	(Minnesota Pollution Control Agency 2007)
Canada				
2008	Department of Justice, Canada	Perfluorooctane Sulphonate Virtual Elimination Act, 2008	Prohibition of the manufacture, use, sale, offer for sale, and imort of PFOS or manufactured products containing these substances	(Department of Justice Canada 2008)
Japan				
2009	Japan	Regulation of production and use under the Chemical Substances Control Law	PFOS: Class I Specified Chemical: Its production or use is prohibited except for essential uses	(Carloni 2009)

*Inland surface waters: Rivers, lakes, artificial water bodies or heavily modified water bodies.

1.9 Temporary trends

The occurrence of PFASs in the environment, food and human is proportional with their production and use along the years. Figure 1.7 summarises the timeline of the most important events and regulations for PFASs and Figure 1.8 summarises the global production and emission to the environment since the first industrial synthesis process.

The timelines show the correlation between PFAS production and emission to the environment. It can be seen how, after PFAS regulation, the emissions to the media decrease over the following years in Europe and in North America. It happened with PFOS in 2000, when the maximum production of PFASs was reached and the global production of PFOSs was estimated at 3535 metric tonnes. During the same year, 3M Company, the major producer of C8 based PFASs, announced their phasing out. The consequence of this phasing out was the decrease in production of approximately 20 times less than the 175 metric tonnes produced during 2001. The emission of PFASs to the media has been estimated to be between 2610 and 5720 tonnes during the period 1951 and 2004. In 2006, after the 8 major producer companies launched the "2010/2015 PFOA Stewardship Program", the production of these compounds decreased and, in order to follow with the production of PFASs based products, the producers started the substitution of longer carbon chains (C8) for shorter ones such as PFBS (Quinete *et al.* 2010). As a consequence of this substitution, the studies during recent years detected the small increase in the presence of short carbon chain PFASs in water samples (Ahrens *et al.* 2009; Eschauzier *et al.* 2010; Ullah *et al.* 2011). However, no clear evidence exist on the persistence of these compounds into the media (Quinete *et al.* 2010).

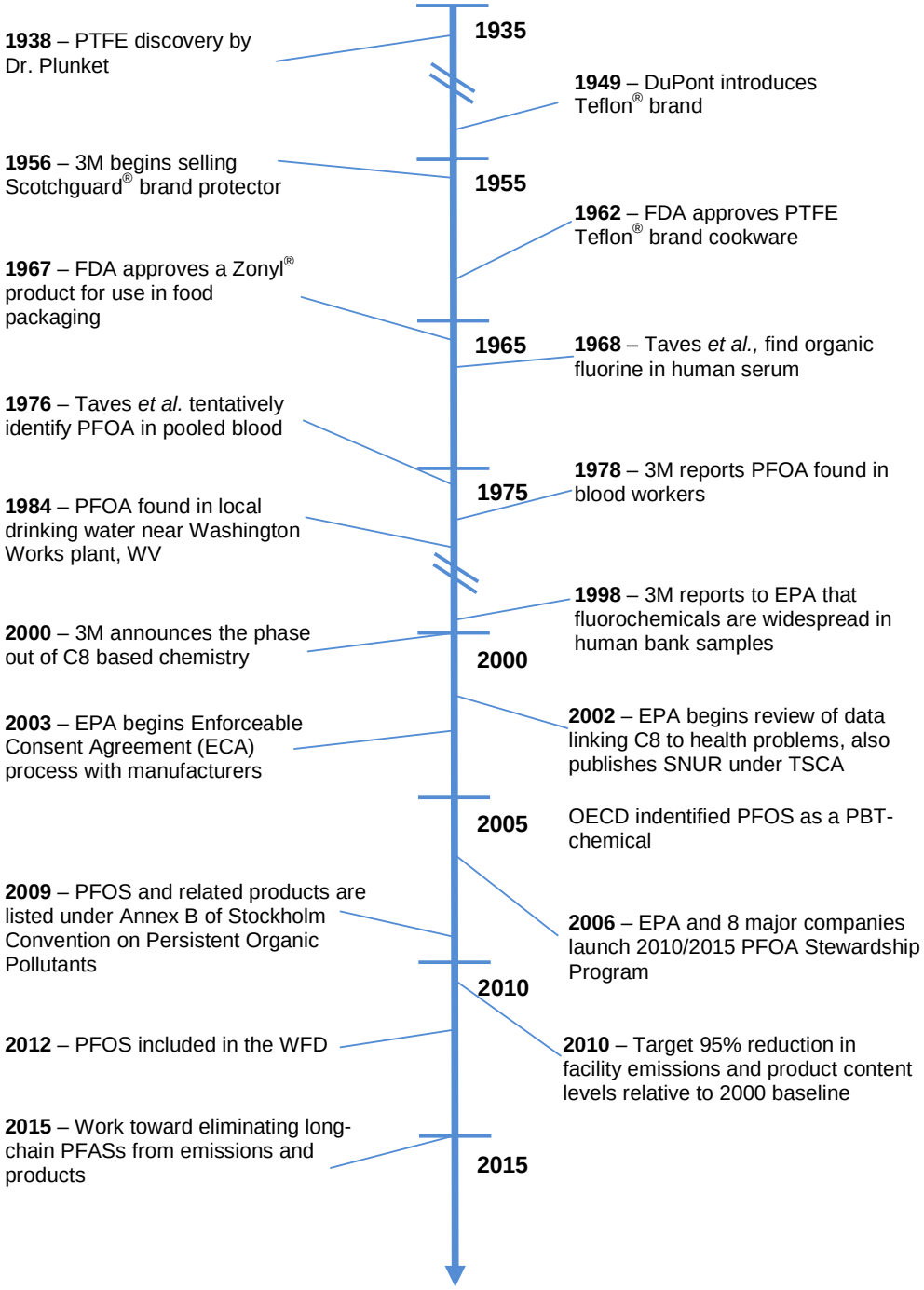


Figure 1.7: Timeline of the most important events for PFASs. Adapted from Lindstrom *et al.* (2011).

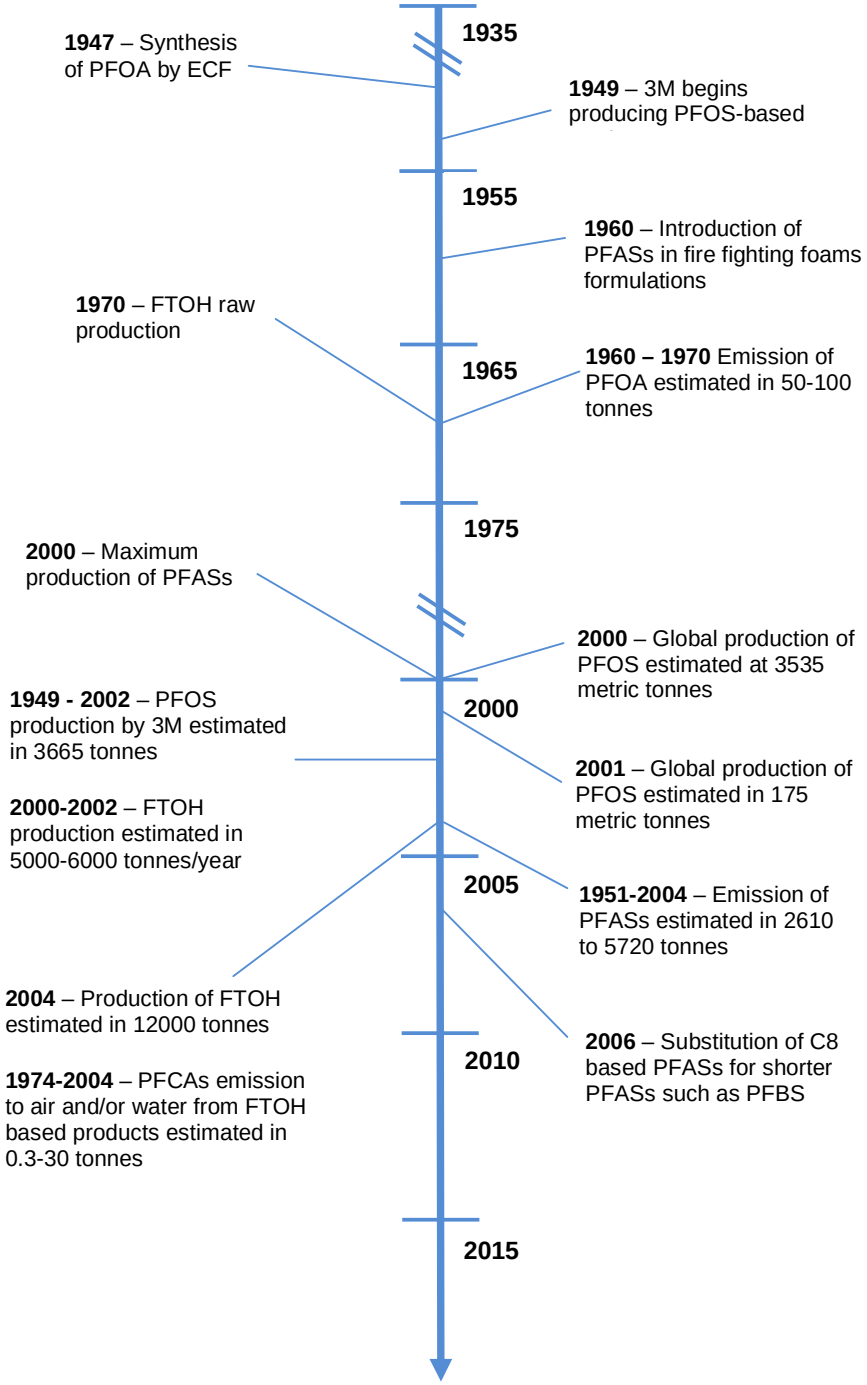


Figure 1.8: Timeline of the temporary trends of PFAS production and emission.

1.10 Analysis

Different drawbacks that can hamper the quantification of PFASs need to be commented on:

- Contamination
- Blanks availability
- Losses during storage and preservation
- Chemical standards variability and
- Matrix effects

Contamination. A wide variety of laboratory materials are made of, or contain, fluoropolymers such as polytetrafluoroethylene or perfluoroalkoxy compounds (Taniyasu *et al.* 2005). Therefore, since the start of the analytical process, any contact with possible sources of contamination should be avoided. In this sense, polypropylene (PP) containers or bottles (Sinclair *et al.* 2006; Guo *et al.* 2010; Navarro *et al.* 2011) and high-density polyethylene (PE) bottles (Yu *et al.* 2009; Hu *et al.* 2010; Sun *et al.* 2011) were recommended for the sampling and storage of liquid samples. For solid samples, foil containers are commonly employed (Llorca *et al.* 2011a). In addition, in order to remove any trace of contaminants from containers, they need to be rinsed before their use with deionised water and acetone (Skutlarek *et al.* 2006).

On the other hand, analytical instrumentation has been identified as one of the main contamination sources. For example, in liquid chromatography-mass spectrometry (LC-MS) systems, the contamination from solvents employed for the mobile phases, or from instrument tubing, injection and degasser valves (commonly made of PVDF or Teflon), have been identified as the main sources of contamination. These instrumental sources can be assessed by the use of blank materials, and the following possibilities can be considered, in order to decrease or avoid the contamination:

- The use of an extra chromatographic column, or filter, before the injection valve in order to increase the retention time of the analytes from the mobile phase tubes and degasser valves and to be able to differentiate between PFASs from the system and the sample (Kuklenyik et al. 2004; Kuklenyik *et al.* 2005; Gledhill *et al.* 2007)
- Blanks subtraction or
- Metalising the whole system and replacing all the instrumental system made of PVDF, or Teflon, by polyether ether ketone (Peek) or metal tubing.

Blanks. Another important drawback in PFASs is the lack of blanks during the analysis because of the contamination from labware and instrumentation (van Leeuwen *et al.* 2009). It represents the most difficult problem to deal with, since the lowest concentrations can be overestimated in these cases. In order to elucidate the most outstanding method for the analysis of PFASs, different inter-laboratory studies have been performed, as well as the most difficult analytical parts. The studies performed with

non-complex environmental samples, fish, milk, blood and human serum showed that there is an implicit blank contamination during blank sample analysis (Longnecker *et al.* 2008; Lindström *et al.* 2009; van Leeuwen *et al.* 2009; Keller *et al.* 2010). It is recommended, in these cases, to perform, in parallel to the sample extraction, the extraction of the method solvents as well as a known blank matrix, in order to rule out any contamination source. Nevertheless, sometimes, it is difficult to find a matrix blank and, up to now, there are only available serum and milk certified reference materials (NIST), which can be used as blanks, as well as for validation purposes.

Sampling, losses and sample preservation. Losses can be produced by the adsorption to sample containers such as glass due to the hydrophobic head (Martin *et al.* 2004a), or polymeric containers such as PP and PE container surfaces when short chain PFASs are in water solutions.

For solid samples, the losses for possible sorption to PE or PP bottles can be assumed to be negligible (Sun *et al.* 2011), although the adsorption of long carbon chain PFASs in the recipients make take place (Loveless *et al.* 2006; Frömel *et al.* 2010). In the case of water samples stored in glass vessels, there is a considerable controversy since the partial adsorption has been reported for high concentrations (Holm *et al.* 2004), but this is not expected in real samples with a complex matrix (Kärrman *et al.* 2006). Another important factor is losses by volatilisation in the case of volatile compounds, such as FTOHs and short carbon chain perfluoroalkyl substances such as PFBA. For this reason, it is recommended to avoid headspace in sampling bottles (Liu *et al.* 2005).

Sample transportation is performed, in general, by keeping the sample under cool conditions (Guo *et al.* 2008; Yu *et al.* 2009; Hu *et al.* 2010; Llorca *et al.* 2011b). During sample storage, biodegradation, biotransformation or sample matrices alterations should be prevented. In general, a sample is conserved in the freezer or just using combinations of solvents, such as acetonitrile, among others (Wang *et al.* 2005). Once in the laboratory, the samples should be kept in optimal conditions in order to assure their traceability and to avoid any degradation or damage. However, it has been shown that when pH decreases, PFASs become increasingly associated with the available protons, and then PFASs can be more easily adsorbed into the container's surface (Dinglasan *et al.* 2004). Szostek *et al.* (2006) studied the stability of the volatile FTOHs in water preserving samples by freezing, refrigerating, solvent addition or combined with refrigeration to preserve the samples. The authors concluded that aqueous samples can be safely stored in the freezer in a glass vial, sealed with a septum lined with alumina foil and no biodegradation nor biotransformation were observed under these conditions. On the other hand, the use of biological inhibitors, such as formalin, can suppress the analytical response of the LC-MS system during the analysis, being not requested for the preservation of most PFASs, due to their biological stablility (Schultz *et al.* 2006a).

In the case of solid matrices, drying procedures are usually applied. There are different drying processes which have been applied for PFAS analysis, including air-drying at room temperature until a constant weight is reached (Sinclair *et al.* 2006; Li *et al.* 2010), using soft temperature (40 ºC) along 3 – 4 days in porcelain bowls or PP

containers (Navarro *et al.* 2011; Sun *et al.* 2011), or in an oven at 103 ºC overnight (Guo *et al.* 2008). Other, more specific procedures, consist of direct freezing (- 20 ºC) prior to any treatment, in order to perform the lyophilisation (Guo *et al.* 2008; Llorca *et al.* 2011a) or previous centrifugation to remove supernatant in sediment and soil samples (Bossi *et al.* 2008) and lyophilisation (Yu *et al.* 2009). Then, the samples are homogenised and kept frozen until analysis in PP containers (Sinclair *et al.* 2006; Llorca *et al.* 2011a) or high density PE bottles (Yu *et al.* 2009; Sun *et al.* 2011).

Standards purity. Standards purity represents a problem, since there are a variety of available PFASs suppliers, which have different purities and isomeric composition. The differences in isomeric composition can drive to a non-optimal quantification of the PFASs, because the percentage of branched and linear isomers, in sample and standard, is not necessarily the same. For example, there are different branched isomers of the linear PFHxS and PFOS, as well as PFOA in blood or breast milk samples, and misbehaviour has been observed during quantification in different interlaboratories (Lindström *et al.* 2009; Keller *et al.* 2010). It is necessary to use a high purity standard, linear or branched, depending on the chosen analyte, in order to perform a complete identification. On the other hand, different interlaboratories regard the necessity to unify quantification methods, in order to generate comparable and usefull data between laboratories (Lindström et al. 2009; van Leeuwen et al. 2009; Keller *et al.* 2010).

Matrix effects. Due to the matrix complexity, sometimes it is necessary to perform an extra cleaning step during sample treatment in order to avoid, or at least decrease, any undesirable matrix effect. The most common matrix effect known in LC-MS/MS analysis is ion suppression, or ion enhancement, when electrospray ionisation source (ESI) is used as an interface during sample ionisation. Depending on the matrix type, an ion suppression (or ion enhancement) can be observed, due to the facility (or difficulty) of the sample to be ionised in front of the PFASs (Zhang *et al.* 2010). The use of labelled internal standards, added to the sample just before the analytical approach, can be useful in terms of signal normalisation and it can decrease the matrix effect (van Leeuwen *et al.* 2009). However, the most important factor for reducing matrix effect is the clean-up process (van Leeuwen *et al.* 2009). An example of ion enhancement has been observed during the analysis of PFOS in biological matrices, due to an interference in the MS transition 499 > 80 in with the taurodeoxycholic bile acid (TDCA) (Hansen *et al.* 2001; Benskin *et al.* 2007; Keller *et al.* 2010). In order to discriminate between both analytes, it is important to monitor two different MS transitions when the chromatographic separation with C18 based columns does not enable separation between compounds. However, these separations can be achieved by many chromatographic columns available on the market, such as pentafluorophenyl (PFP) columns for reversed phase chromatography.

1.10.1 Sample treatments

Sample treatment aims to extract the analyte from the matrix, to enrich the analyte of interest, as well as to reduce the matrix effect by purifying the extracted sample. Different strategies are commonly used, depending on the matrix type: solid, liquid or gaseous

phase (Figure 1.9). This section will summarise most common strategies for sample pre-treatment in the analysis of PFASs.

1.10.1.1 Solid and biological matrices

Main sample preparation and extraction procedures for the analysis of PFASs in solid matrices have been based on solid-liquid extraction.

Sample treatment is usually applied for complex matrices such as food, sludge or sediments in order to avoid or decrease matrix effects. It is important to assure no alteration of the composition of PFASs by hydrolysis or other possible processes. For example, the hydrolysis of fluorotelomer compounds to fluorotelomer alcohol can take place during the solvent extraction from soils, as reported by Dasu *et al.* (2010).

Ionic-pair extraction. This method by Ylinen *et al.* (1985), was employed for the first time for the extraction PFOA from plasma and urine. The procedure employs tetrabutyl amonium (TBA) as counter ions, and it was used in combination with gas chromatography (GC) coupled to flame ionisation detection (FID). Later on, Hansen *et al.* (2001) adapted this procedure in order to make it amenable for liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The approach developed by Hansen *et al.* is based on the use of methyl tertiary butyl ether (MTBE) and a filtration step was incorporated in order to remove solids in suspension. The ion pair extraction procedure has been the basis of several procedures for the analysis of biota samples (Giesy *et al.* 2001; Van De Vijver *et al.* 2003; Yeung *et al.* 2009), food samples (Taniyasu *et al.* 2003; Yeung *et al.* 2009), as well as in soils and sediments (D'Eon *et al.* 2009a; Ellington *et al.* 2009; Zhang *et al.* 2010). However, main limitations are: i) the co-extraction of lipids and other matrix constituents and the absence of a clean-up step to overcome the effects of matrix compounds and (ii) the wide variety of recoveries observed, typically ranging from <50% to >200%.

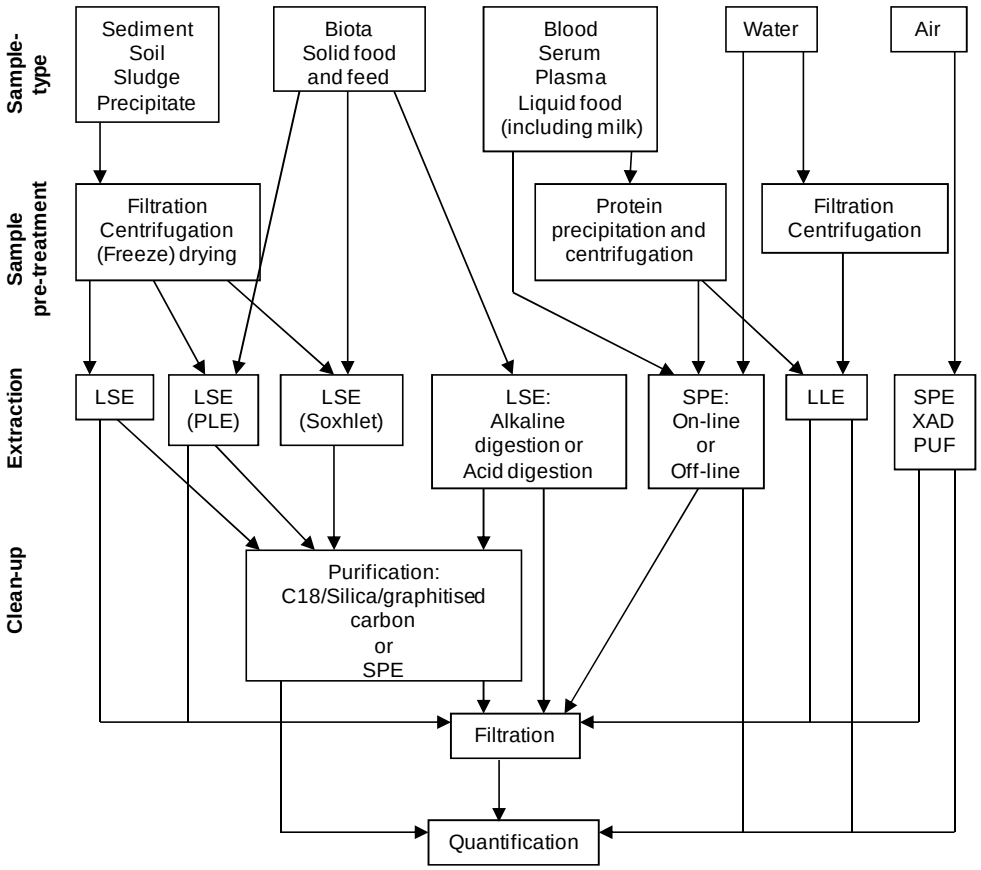


Figure 1.9: Scheme of the extraction methods used for the analysis of PFASs.
Scheme adapted from van Leewen *et al.* (2007) and *Picó et al.* (2010).
List of acronyms: liquid solid extraction (LSE), pressurised liquid extraction (PLE), solid phase extraction (SPE), liquid-liquid extraction (LLE), resin based SPE (XAD), polyurethane foam plugs SPE (PUF).

Solid-liquid extraction. This is the most commonly used methodology for the extraction of non-volatile PFASs from solid matrices, due to the facility and simplicity of the extraction and the solvents required. However, this method is suitable for not too complex solids. For the analysis of soils and sediments, Powley *et al.* (2005) performed a methanol or acetonitrile extraction with a shaker. Bossi et al. (2008) used a similar approach based on ultrasound assisted extraction (UAE) with temperatures between 40 – 60 ºC along 20 – 30 min (Bossi *et al.* 2008; Rhoads *et al.* 2008; Li *et al.* 2010; Navarro *et al.* 2011; Sun *et al.* 2011). Liu *et al.* (2010) also carried out a solvent extraction, assisted by an ultrasonic bath at 50 ºC for 2 – 7 days, depending on the target compounds.

For biological matrices, the ultrasonic extraction using mixtures of hexane and acetone or pure methanol has been commonly employed. The same solvent mixtures have also been employed for the extraction of PFASs from biological matrices by stirring. In these cases, the milled samples are covered by the solvent and stirred at room temperature for a few minutes, followed by a clean-up step, in general with anionic exchange SPE (Lau *et al.* 2004; Newsted *et al.* 2005; Guruge *et al.* 2008; Fromme *et al.* 2009; Haug *et al.* 2009). In general, the extraction is followed by a clean-up step using sodium sulphate and acidic attack in order to remove the lipid content and the extracts are passed through a silica gel column (Tittlemier *et al.* 2007; 2011).

During recent years, the use of techniques based on pressurised liquid extraction (PLE) for soil, sediments and biological samples has been increased (Schröder 2003; Carabias-Martínez *et al.* 2005; Björklund *et al.* 2006; Kunacheva *et al.* 2011). The use of PLE instead of Soxhlet or hot vapour extraction allows decreasing the extraction time due to the use of high pressure. The most frequent solvent extractor for PFASs is pure methanol. However, after this first extraction a clean-up process is necessary in order to eliminate or decrease the matrix interferences (Zhang *et al.* 2010). The clean-up it is carried out by SPE or dispersed extraction. This methodology allows limits of detection in the range of pg/g and low ng/g with good recovery yields.

Solid-acidic liquid extraction. Acid liquid extraction is widely applied, and the current methodologies are based on the procedure presented by Higgins *et al.* (2005). In general, the method is based on ultrasonic extraction using formic acid or acetic acid as extracting solvent. After the digestion step, the mixture is centrifuged and the supernatant is collected. Then, the whole procedure is repeated once or twice. In general, the combined extracts need an extra clean-up step, which is performed generally using SPE and filtration (Loganathan *et al.* 2007; Guo *et al.* 2008; Yu 2009). In the case of some biological matrices such as blood, the supernatant is directly analysed after protein precipitation in acidic conditions and centrifugation (Calafat *et al.* 2007; Lien *et al.* 2011). The use of this procedure allows achieving recovery rates of between 40 and 119% and confers better analytical parameters than those obtained by ionic-pair extraction.

Solid-alkaline liquid extraction. This procedure is based on the use of alkaline methanol (with sodium hydroxide or ammonium hydroxide) as extracting solvent. This methodology has been used for the analysis of acids, sulphates and fluorinated sulphonamide compounds in foodstuffs and biological matrices by different authors.

Sometimes a neutralisation step could be required after alkaline treatment (Yoo *et al.* 2009; Ma *et al*. 2010). Several authors have used alkaline digestion for protein precipitation and extraction of PFASs from fish (So *et al.* 2006; Naile *et al.* 2010), or different foodstuffs such as vegetables, milk-based dairy products, bread, jam, different meats and fish (Haug *et al.* 2010) and raw and cooked meats (Jogsten *et al.* 2009). As a last clean-up step, anionic exchange SPE or dispersed solvent (EnviCarb) are commonly used. The alkaline extraction permitted better recoveries than solid-liquid extraction and, in general, better sensitivity is achieved.

Examples of the different extraction procedures are summarised in Table 1.10.

Table 1.10: sample pre-treatments for solid matrices from different published works

Matrices	Pre-treatment	References
	Ionic Pair extraction	
Sludge and sediments, plasma and urine, biota and food	1) 0.5 - 2 g sample; 2) $NaCO_3$ 0.25 M + TBAS 0.5 M, pH 10; 3) extraction with 2 x 5 mL aliquots of MTBE; 4) MTBE aliquots combined and dryed under N_2 current; 5) reconstituted in 0.5 mL of MeOH; 6) Optional filtration by 0.2 µm nylon filter or clean-up by Envicarb cartridges	Sludge and sediments: (D'Eon et al. 2009a; Ellington et al. 2009; Zhang et al. 2010) Plasma and urine: (Ylinen et al. 1985; Hansen et al. 2001)
	Solid-liquid extraction	Biota: (Giesy et al. 2001; Van De Vijver et al. 2003; Yeung et al. 2009b)
Sludge or soil	1) dry sludge + surrogate internal standards; ***i)*** 2) Soxhlet or hot vapour extraction, 6 h with MeOH; 3) Purification and reconstitution in initial conditions of mobile phase ***ii)*** 2) PLE by ASE with MeOH 3) Purification and reconstitution in initial conditions of mobile phase ***iii)*** 2) MeOH, wrist-action shaker, mixed or ultrasonic bath; 3) settled or centrifugated; 4) Purification by 25 mg Envi-Carb graphitised carbon adsorbent or SPE by C18 5) 500 µL supernatant + 500 µL water for Envi-Carb extract and internal standards addition 6) Evaporation to dryness the SPE extract, reconstitution in initial conditions mobile phase, internal standard addition ***iv)*** 2) ACN (2-7d) at 50ºC; 2) centrifugation and supernatant separation; 3) Addition of HCl 5 M and centrifugation; 4) Clean-up by C18 cartridges in some cases	Food: (Taniyasu et al. 2003; Yeung et al. 2009b)

(table 1.10)

Biological matrices	1) sample + surrogate internal standards; 2) hexane:acetone or MeOH; 2) ultrasonic bath or shaking at room temperature; 3) clean-up by anionic exchange SPE; ***i)*** 4) evaporation under N_2; 5) reconstitution in initial condition mobile phase and internal standard addition; ***ii)*** 4) clean-up by Na_2SO_4 and acidic attack; 5) purification through silica gel column 6) reconstitution in initial condition mobile phase and internal standard addition;	***i)*** (Lau *et al.* 2004; Newsted *et al.* 2005; Guruge *et al.* 2008; Fromme *et al.* 2009; Haug *et al.* 2009) ***ii)*** (Tittlemier *et al.* 2007; Tittlemier *et al.* 2011)
	Solid-acid liquid extraction	
Biological matrices	1) homogenised sample + surrogate; 2) solvent at 1% AcH; 3) shaken and centrifugation; 4) supernatant separation 5) internal standard addition and direct analysis	(Calafat *et al.* 2007a; Lien *et al.* 2011)

(table 1.10)

Sludge and sediment	1) homogenised sample + surrogate; 2) solvent at 1% AcH; ***i)*** 3) shaking, sonication at 60 ºC and centrifugation; 4) supernatant separation and repeat steps 2-4 twice; 5) supernatants combination; 6) SPE by C18 or Oasis HLB 7) concentrated under N_2 and filtration or clean-up by silica cartridge and concentrated; 8) reconstitution in mobile phase initial conditions 9) internal standard addition ***ii)*** 3) Supernatant + 10 mL 1% AcH 4) Combined extracts and volume adjusted to 200 mL with water 5) SPE by Oasis WAX; 6) Evaporated under N_2 7) Reconstituted in MeOH ***iii)*** 3) Centrifuged and supernatant evaporated under N_2 4) Reconstitution in 1 mL of acetonitrile and incubated in an ultrasonic bath 5) Purification by activated charcoal + 50 µL of AcH, mixed 1 min 6) Centrifuged and 0.15 mL filtered by .2 µm GHP Accrodisc 7) Addition of in 0.35 mL water	***i)*** (Higgins *et al.* 2005; Sinclair E 2006; Loganathan *et al.* 2007; Guo R. 2008; Yu 2009; Guo R. 2010) ***ii)*** (Pico *et al.* 2011) ***iii)***(Gómez *et al.* 2011)
	Solid-alkaline liquid extraction	
Biological matrices and food stuff	1) sample + surrogate internal standard addition 2) MeOH at 1% NH_4OH or 10 mM of NaOH 3) vortexed and sonication; alkaline digestion between 30 min and 3 h 4) SPE 5) extract dryed under N_2; 6) reconstitution in initial conditions mobile phase and internal standard addition	(So *et al.* 2006a; Jogsten *et al.* 2009; Haug *et al.* 2010; Naile *et al.* 2010)

(table 1.10)

Sludge and sediment	1) sample + surrogate internal standard addition 2) MeOH at 1% NH_4OH or 10 mM of NaOH 3) vortexed and sonicated; alkaline digestion between 30 min and 3 h ***i)*** 4) neutralisation (w/HCl); 5) 10 mL ACN:MeOH(1/1, v/v), shaken 1 h; supernatant separation (repeat steps 4-5 one more time); 6) 2 mL of combined extracts (20ml) + 98 mL water at pH4; 7) sonicated 30 min; 8) SPE by HLB cartridges 9) extract dryed under N_2; 10) reconstitution in initial conditions mobile phase and internal standard addition ***ii)*** 4) supernatant + water (1% AcH); 5) preconcentration under N_2 to 5ml; 6) purification by dispersed solvent (EnviCarb) and rinse with 2.5 mL MeOH (1% NH_4OH,1% AcH); 7) concentrate to 1ml under N_2.	***i)*** (Yoo H. 2009) ***ii)*** (Ma R. 2010)
	Other extraction procedures	
Activated sludge from WWTP	1) PFAS were extracted from supernatant of the sludge experiments; 2) SPE by C18 cartridges (conditioning: 10 mL MeOH, 10 mL water; elution: 10 mL MeOH); 3) reduced under a gentle stream of N_2 and filtered before analysis.	(Saez *et al.* 2008)
Suspended soil and mixed liquor suspended solid from WWTPs	1) suspended soil and mixed liquor suspended soil filtered by GF/B filter; 2) PLE (ASE200, Dionex,USA) of GF/B filter (3 static cycles, MeOH solvent, 100 ºC, 2000 psi, 10 min); 3) extract dried to reduce < 10 mL (60 ºC, water bath); 4) reduced extract + 1 L water; 5) SPE by Oasis HLB plus and PrepSepC-Agri (short) in tandem; 6) elution with 4 mL of MeOH under gravity conditions; 7) dryness under N_2; 8) reconstitution in 1-2 mL of water:ACN (6:4) and vortexed	(Shivakoti *et al.* 2010)

List of acronyms in order of appearance: tetrabutyl ammonium hydrogen sulphate (TBAS), methyl tert-butyl ether (MTBE), methanol (MeOH), accelerated solvent extractor (ASE), acetic acid (AcH)

1.10.1.2 Water matrices

SPE is the technique of choice for the clean-up step in the analytical procedures used for the analysis of PFAS in water. SPE is performed, in general, after a previous filtration step to separate solids from the liquid phase as indicated in Figure 1.9 (Saito *et al.* 2003; Schultz *et al.* 2006a, 2006b). However, the filtration step can result in analyte losses, due to the adsorption of PFASs on the filters or, the contamination of the samples from the filters. Schultz *et al.* (2006a, 2006b) observed different losses when nylon, cellulose acetate, and polyethersulphone filters were employed. An alternative method presented by these authors is the centrifugation to separate the liquid from the solids.

Solid phase extraction has been carried out using both off-line and on-line methodologies.

Off-line methods. Different extraction SPE cartridges have been explored according to the different polarities of PFASs. Broadly, good recoveries were reported using anionic exchange cartridges, such as Oasis WAX-SPE (Weak Anion-eXchange). Oasis WAX-SPE cartridges have shown good recoveries, including with short-chain compounds (between four and six carbons), and have been applied in many monitoring studies (Taniyasu *et al.* 2005; Ericson *et al.* 2008; D'Eon *et al.* 2009b; Ericson *et al.* 2009; Llorca *et al.* 2011). For longer chains PFASs, less polar phases have been used such as C18 and Oasis HLB (Hydrophilic-lipophilic-balanced) (Takazawa *et al.* 2009; Sánchez-Avila *et al.* 2010; Gómez *et al.* 2011). When an ion-pairing agent is used for decreasing the polarity of the ion pair complex, a non-polar solvent such as MTBE is usually selected. Non-ionic PFASs may be extracted from the matrix by the non-polar media (C18 SPE as has been described before or hexane). Moderate polar media (Oasis HLB and OasisWAX SPE, a hexane–acetone mixture or acetonitrile) have also been applied for extraction of non-ionic PFASs. However, one of the critical points in PFASs analysis is background contamination in the analytical blanks (Taniyasu *et al.* 2003; Villagrasa *et al.* 2006; Suja *et al.* 2009).

On-line methods. During recent years, the development of on-line methodologies have increased, due to their advantages, such as the reduction of the sample manipulation, the increase of the analytical robustness, and the reduction of the time of analysis. Nevertheless, these approaches are slowly implemented, in spite of their excellent analytical performance characteristics for routine analysis. Different on-line approaches are available but, on-line solid phase extraction and turbulent flow chromatography will be highlighted.

- On-line solid phase extraction. This method coupled to LC-MS/MS has been employed for the pre-concentration and extraction of PFASs from water samples (Wilson *et al.* 2007; Gosetti *et al.* 2010). The columns used include Kromasil C18 and a Poros HQ column. However, due to the low sample required for on-line approaches, as well as the increase of the robustness for the low sample manipulation, this method has also been used in the analysis

of biological matrices such as blood and milk by different authors (Kuklenyik *et al.* 2005; Apelberg *et al.* 2007; Calafat *et al.* 2007; Wilson *et al.* 2007; Haug *et al.* 2009).

- Turbulent flow chromatography. The main difference with the SPE is that they employ a laminar flow instead of the turbulent flow. The turbulent flow allows generating turbulence inside the extraction column working at high flows (1.5 – 3.0 mL/min). This turbulent flow uses stationary phase columns with a poor size > 5 µm, allowing a good removal of high molecular weight compounds such as proteins, whereas the small molecules are trapped by the column pores, being a mixed mechanism of sorption and size exclusion chromatography. The retention of the analytes in activated pore sites is due to the difference between diffusion of large and small compounds. This technique has been used in the analysis of water samples (Takino *et al.* 2003) although it is more suitable for biological matrices with high matrix effect, such as urine or hair (Perez *et al.* 2012). Other important factors to take into consideration are the small sample volumes required (between 10 and 20 µL), since this is an extraction methodology and not a concentration method. Finally, it should be mentioned that the reuse of the turbulent flow columns is for up to 500 extractions.

Examples of the different extraction procedures are summarised in Table 1.11, and detailed and more specific information is given in the different chapters of this Thesis.

Table 1.11: sample pre-treatments for water matrices and last clean-up step for some biological matrices from different published works

Matrices	Pre-treatment	Reference
	Off-line	
Surface river water, deep water, precipitation water	1) Sample + surrogate internal standard ***i)*** 2) Filtration (glass microfibre membrane 0.7 µm) 2) anionic exchange SPE 3) Evaporated under N_2 4) Reconstituted in initial conditions of mobile phase and internal standard addition ***ii)*** 2) SPE by Oasis HLB , or other C18 3) Extract reduced to dryness under N_2 4) Reconstitution in initial conditions of mobile phase and filtered (0.2 µm) ***iii)*** 2) XAD resins	***i)*** (Pico *et al.* 2011) ***ii)*** (Scott *et al.* 2006; Konwick *et al.* 2008; Eschauzier *et al.* 2010; Sánchez-Avila *et al.* 2010; Gómez *et al.* 2011) ***iii)*** (Scott *et al.* 2006)
Drinking water	1) Sample + surrogate internal standard 2) SPE with different stationary phases 3) Extract reduced to dryness under N_2 4) Reconstitution in initial conditions of mobile and internal standard addition	
	On-line	
Water	1) Sample + surrogate internal standard 2) Filtration ***i)*** 3) Kromasil C18 or Poros HQ as SPE extraction columns ***ii)*** 3) Turbulent flow chromatography by C18 column	***i)*** (Wilson *et al.* 2007; Gosetti *et al.* 2010) ***ii)*** (Takino *et al.* 2003)
Blood, serum and other biological matrices clean-up after SLE extraction	1) Sample extraction by SLE ***i)*** 2) on-line SPE with C18 based columns ***ii)*** 2) Turbulent flow chromatography clean-up with C18 based columns	***i)*** (Kuklenyik *et al.* 2005; Apelberg *et al.* 2007; Calafat *et al.* 2007; Wilson *et al.* 2007; Haug *et al.* 2009) ***ii)*** (Perez *et al.* 2012)

List of acronyms in order of appearance: solid phase extraction (SPE), Hydrophilic-lipophilic-balanced (HLB), resin-based SPE (XAD), solid-liquid extraction (SLE)

1.10.2 Instrumental analysis

The first analytical method for the analysis of organic fluorine in serum samples was based on the use of Nuclear Magnetic Resonance (NMR) (Taves 1968). Later, GC coupled to different detectors such as a microwave plasma detector for PFOS (Hagen *et al.* 1981) or mass spectrometers (Pankow *et al.* 1998) were also employed. In addition, different analytical techniques have been applied as complementary techniques, such as NMR for the analysis of fluorinated compounds in water analysis (Moody *et al.* 2001), the Electron Spin Resonance (ESR) in the study of different cellular medias with fluorinated

compounds (Romanelli *et al.* 1994), or the total fluorine analysis of water and blood by combustion ion chromatography (Miyake *et al.* 2007a; Miyake *et al.* 2007b). However, due to the physicochemical nature of PFASs the technique is more employed for the separation of this analytes is liquid chromatography, used in general coupled with mass spectrometry.

1.10.2.1 Separation

GC-MS was widely used for the analysis of volatile and short carbon chain PFASs, such as trifluoroacetic acid, perfluorobutanoic acid (PFBA) (Martin *et al.* 2002; Alzaga *et al.* 2004; Scott *et al.* 2006; Ellington *et al.* 2009). Different examples are summarised in Table 1.12. But, nowadays LC coupled to different MS detectors is the technique of choice, since this can be applied for non-volatile compounds, due to its high sensitivity, robustness and accuracy. LC separation has been mainly carried out with reversed phase (RP) C18 and C8 columns. RP columns with shorter alkyl chain bonded phases (e.g., C8, C6, phenyl, phenylhexyl) have been used in the separation of branch isomers for other analytes, but they are not commonly employed in routine analysis. It is more common to use C18 columns, where the discrimination between isomers can be achieved by increasing the LC column temperature to 35 °C or 40 °C (Kuklenyik *et al.* 2004; Calafat *et al.* 2006a; Calafat *et al.* 2006b). However, the use of C18 columns presents a chromatographic problem with short PFASs, such as trifluoroacetic acid, perfluoropropyl acid or perfluoroethyl sulphonates. These compounds are not easily retained in the column and can be eluted with the died volume or, if they are retained, present broad peaks and are not adequately resolved, as has been reported by Taniyasu *et al.* (2008). The authors explored the chromatographic properties and separation of short-chain PFSAs on RP-C18 and ion-exchange columns. The results showed that RP columns are not suitable for the analysis of short-chain PFSAs, especially trifluoroacetic acid. As a proper alternative, ion-exchange columns have superior retention properties for more hydrophilic substances enabling the analysis of short-chain. Another possibility for increasing the retention of shorter PFASs in RP-C18 columns is the use of an ionic pair between mobile phase and the most hydrophilic fluorinated compounds. Nevertheless, this latter technique is not applied, due to the problems associated with the use of some ionic pair reagents, which usually are trifluoroacetic acid, heptafluorobutanoic acid or perfluoroheptanoic acid. It could drive to an overestimation during the quantification, as well as ion suppression if the LC is coupled to a mass spectrometer analyser (Gustavsson *et al.* 2001).

Using RP chromatography the mobile phases most commonly employed are water and methanol, acetonitrile or a combination of both as organic phase (Table 1.12). In order to increase the chromatographic peak resolution, as well as, to improve the efficiency of the analyte ionisation in the ionisation source some modifiers are used to increase the conductivity of the solvent phase. The most common modifier employed is ammonium acetate at concentrations between 2 and 20 mM. The injected volume in the LC systems is, in general, between 10 and 20 µL.

Table 1.12: examples of the instrumental separation, based on gas and liquid chromatography, for some of the most studied PFASs

Analytes	Instrumental Separation	Analyzer	References
	Gas chromatography		
Sulphonamides, FTOH	**GC** • Injection volume: 1 µL • Column: 30m DB-Wax column (0.25 mm i.d., 250-µm film thickness) • Carrier gas: Helium	**MS(Q)** • Ionisation source: NCI and PCI • MS mode: SIM	(Martin *et al.* 2002)
PFCA anilides	**GC** • Injection volume: 1 µL • Column: ZB-5 Zebron fused silica capillary column (30 m x 0.25 mm i.d.) • Carrier gas: Helium	**MS (Q)** • MS mode: SIM	(Scott *et al.* 2006)
FTOH	**GC** • Injection volume: 1 µL • Column: Restek Rtx-1701 capillary column, 40m x 0.25mm I.D., 0.25µm film thickness with a 10-m deactivated Integra-Guard guard column • Carrier gas: Helium	**MS(Q)** • Ionisation source: EI, NCI and PCI • MS mode: SIM	(Ellington *et al.* 2009)
PFCAs	**GC** • Injection volume: 1 µL • Column: 60m x 0.25mm i.d. (cyanopropylphenyl-methylpolysiloxane, 1.4 µm film thickness) ZB-624 column • Carrier gas: Helium	**MS(Q)** • Ionisation source: NCI • MS mode: SIM	(Alzaga *et al.* 2004)
	Liquid Chromatography		
PFHxS, PFOS, PFDS, PFOA, N-Ethylfluorosulphonils, perfluorosulphonamidos, and metabolites of partly fluorinated alkylethoxylates	**HPLC** • Injection volume: 10 µL • Column: Multospher 100 RP 5-5 (C8, 5 µm, spherical; 250 × 4.6 mm I.D.) or using a PF-C8 column (150 × 4.6 mm I.D.) filled with spherical perfluorinated RP-C8 material (5 µm) • Mobile phase: MeOH:water	**MS/MS(QqQ)** • Ionisation source: ESI or APCI in positive and negative mode • MS mode: SRM	(Schröder 2003)
Perfluorosulphonamides, PFHxS, PFOS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	**HPLC** • Injection volume: 12 µL • Column: Betasil C8 column (3 x 50 mm, 5 μm), preceded by a Betasil C8 precolumn (3 x 10 mm), heated at 40 °C for branched isomers separation • Mobile Phase: water (20mM NH_4Ac, pH 4): MeOH	**MS/MS(QqQ)** • Ionisation source: TIS • MS mode: SRM	(Kuklenyik *et al.* 2004)

(Table 1.12)

PFHxS, PFOS, PFDS, FOSAA, N-MeFOSAA, N-EtFOSAA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	**HPLC** • Injection volume: 20 µL • Column: 40mm x 2.1 mm TargaSprite C18 (5 µm pore size) and C18 guard column • Mobile phase: water (2mM NH_4Ac):MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Higgins *et al.* 2005)
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	**HPLC** • Injection volume: 100-200 µL • Column: Zorbax Rx-C8 (15 cm x 2.1 mm I.D, 5 µm) analytical column and Luna C18(2) (3 cm x 4.6 mm I.D, 3 µm) inserted in the HPLC between the pump and injector to delay any fluorochemicals originating from PTFE instrument components • Mobile phase: Acetonitrile (0.15% AcH)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Powley *et al.* 2005)
PFBS, PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTeA, PFHxDA, PFODA	**HPLC** • Injection volume: 10 µL • Column: Keystone Betasil C18 (50 x 2 mm; 5 µm) • Mobile Phase: water (2 mM NH_4Ac):MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Sinclair *et al.* 2006)
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFBS, PFOS	**HPLC** • Injection volume: 10 µL • Column: C8 Phenomenex guard column C8 Luna (3 µm, C8, 50 x 2 mm) • Mobile Phase: water (10mM NH_4Ac): MeOH (10mM NH_4Ac)	**MS/MS(QqLIT)** • Ionisation source: ESI • MS mode: SRM	(Scott *et al.* 2006)
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFBS, PFOS	**HPLC** • Injection volume: 50 µL • Column: NUCLEODUR SPHINX-RP (2.0 x 150 mm, 3 µm) • Mobile Phase: water/ MeOH, 75/25 (10mM NH_4Ac): acetonitrile/ MeOH, 75/25 (10mM NH_4Ac)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Skutlarek *et al.* 2006)
PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA, PFDoA	**HPLC** • Injection volume: 10 µL • Column: Keystone Betasil C18 (50 x 2 mm; 5 µm) • Mobile Phase: water (2 mM NH_4Ac):MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Loganathan *et al.* 2007)
PFOA, PFNA, PFOS, PFDA, PFUnA, FOSA	**HPLC** • Injection volume: 3 µL • Column: C18 column (5 µm particle size, 50 × 2.1 mm) • Mobile Phase: water : MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Konwick *et al.* 2008)

(table 1.12)

PFOA, PFOS	**HPLC** Injection volume: 10 µL • Column: C18 reversed phase • Mobile phase: water (NH_4Ac 5 mM):MeOH	**MS/MS(QqTOF)** • Ionization source: ESI • MS mode: SRM	(Guo *et al.* 2008)
PFOA, PFNA, PFDA, PFUnA, PFOS, FOSA	**HPLC** • Injection volume: 20 µL • Column: C18 Betasil column (2.1×50 mm) • Mobile phase: Water (2 mM NH_4Ac):MeOH(2 mM NH_4Ac)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Bossi *et al.* 2008)
N-EtFOSE, N-EtFOSAA, FOSAA, FOSA, N-EtFOSA, PFOSI (perfluorooctane sulfinate), PFOA, PFOS	**HPLC** • Injection volume: 30 µL • Column: Targa Sprite C18 (5-µm pore size equipped with a C18 guard column • Mobile phase: water (2 mM NH_4Ac):MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Rhoads *et al.* 2008)
PFHxA, PFOA, PFNA, PFOS, PFBS, PSUnA, FOSA, 6:2 FTS, 6:2 FTOH, 8:2 FTOH	**HPLC** • Mobile phase: water (5 mM NH_4Ac):MeOH • FTOHs detected as acetate adducts	**MS (Q)** • Ionisation source: ESI • MS mode: SIM	(Saez *et al.* 2008)
PFOA, PFOS	**HPLC** • Injection volume: 10 uL • Column: Zobax Extend C18 (150 x 2.1 mm ; 5 µm) and guard column XDB-C8 (2.1 mm i.d. x 2.5 mm; 5 µm) at 30ºC • Mobile phase: water (2 mM NH_4Ac):MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Yu *et al.* 2009)
PFOA, PFNA, PFDA, PFUnA, PFOS, 4:2, 6:2, 8:2, 10:2 diPAP	**HPLC** • Injection volume: no data • Column: Gemini C18 (50 mm × 4.6 mm, 3 µm) and Ascentis Express C18 (50 mm × 4.6 mm, 2.7 µm) • Mobile Phase: water(10mM NH_4Ac):MeOH(10mM NH_4Ac)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Eon *et al.* 2009)
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrA, PFTeA, PFHxS, PFOS	**UPLC** • Injection volume: 20 µL • Column: Waters BEH C18 (100 × 2.1 mm; 2.1 µm) at 35ºC and a Waters BEH C18 trapping cartridge • Mobile phase: water:Acetonitrile (pH4, AcH)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Yoo *et al.* 2009)
PFBS, PFHxS, PFOS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	**HPLC** • Injection volume: 10 µL • Column: Agilent Eclipse XDB-C18 (2.1 x 100 mm; 5 µm) at 40ºC • Mobile phase: water (5 mM NH_4Ac):acetonitrile	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Shivakoti *et al.* 2010)

(table 1.12)

TFA, PFPrA, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA, PFBS, PFHxS, PFOS	**HPLC** • Injection volume: 10 µL • Column: Hypersil Gold C18 (150 mm × 2.1 mm; 3 µm pore size) at 30 ºC • Mobile phase: water (2 mM NH_4Ac) : MeOH. HPLC tubing made up of PTFE replaced with PEEK tubing	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Li *et al.* 2010)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, FOSA, N-MeFOSA, N-EtFOSA	**UPLC** • Injection volume: 10 µL • Column: Waters BEH C18 (50 mm x 2.1 mm; 1.7 µm particle size). • Mobile phase: water/MeOH (2 mM NH_4Ac, 95/5) : MeOH (2 mM NH_4Ac).UPLC system were replaced by PEEK	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Ma *et al.* 2010)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	**HPLC** • Injection: 10 µL • Column: Betasil C18 (100 x 2.1 mm, 5 µm) and guard column 12.5 x 2.1 mm Narrow Bore C18 • Mobile phase: water (NH_4Ac 2 mM):MeOH	**MS/MS(QqQ)** • MS mode: SRM • Ionisation source: ESI	(Guo *et al.* 2010)
PFPeA, PHxA, PFHpA, PFOA, PFNA, PFDoA, PFTeA, 6:2 FTUCA, 8:2 FTUCA, PFBS, PFHxS, PFOS, FOSA, n-MeFOSA, n-EtFOSA, n-MeFOSAA, n-EtFOSAA	**HPLC** • Injection volume: 20 µL • Column: 70mm×2mm×3µm Nucleodur C18 gravity • Mobile Phases: 2.5 mM NH_4Ac MeOH:Water (95:5) and 2.5 mM NH_4Ac Water:MeOH (95:5)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM with collision induced fragmentation	(Zhang *et al.* 2010)
PFBS, PFPA, PFHxA, PFHpA, PFOA, PFNA, ip-PFNA, PFOS, PFDA, PFDS	**HPLC** • Injection volume: 20 µL • Column: LiChroCARTLiChrospher 100 RP-18 (250×4 mm; 5 µm) • Mobile Phase: water(20mM NH_4Ac): MeOH (20mM NH_4Ac)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Pico *et al.* 2011)
PFOS, PFOA, PFNA, PFHxS, PFBS	**UPLC** • Injection volume: no data • Column: Acquity UPLC BEH C_{18} column (1.7 µm particle size, 50 × 2.1 mm) before injection LiChroCART HPLC RP-18e column (125 × 2 × 5 µm) • Mobile Phase: water(2mM NH_4Ac):Acetonitrile	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Sánchez-Avila *et al.* 2010; Gómez *et al.* 2011)

(table 1.12)

PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA	**HPLC** • Injection volume: 20 µL • Column: Nucleodur C18 gravity column (70 mm × 2 mm; 3 µm) • Mobile phase: 2.5 mM NH_4Ac MeOH:water (95:5) and 2.5 mM NH_4Ac water:MeOH (95:5)	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM with collision induced fragmentation	(Sun *et al.* 2011)
PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS	**HPLC** • Injection volume: 10 µL • Column: Agilent Eclipse XDB-C18 (2.1 x 100 mm; 5 µm) • Mobile phase: • water (5 mM NH_4Ac) : acetonitrile	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Kunacheva *et al.* 2011)
PFBS, PFHxS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, FOSA, N-MeFOSA, NEtFOSA	**HPLC** • Injection volume: 20 µL • Column: Varian Polaris C18 analytical column (50 mm × 2.0 mm; 3 µm particle diameter) at 40°C • Mobile phase: water (2 mM NH_4Ac) : MeOH	**MS/MS(QqQ)** • Ionisation source: ESI • MS mode: SRM	(Navarro *et al.* 2011)

List of acronyms in order of appearance: gas chromatography (GC), mass spectrometry (MS), quadrupole (Q), negative chemical ionization (NCI), positive chemical ionisation (PCI), selected ion monitoring (SIM), electon impact (EI), liquid chromatography (LC), methanol (MeOH), electrospray ionisation (ESI), atmospheric pressure chemical ionization (APCI), selected reaction monitoring (SRM), triple quadrupole (QqQ), turbo ion spray (TIS), high performance liquid chromatography (HPLC), acetic acid (AcH), hybrid quadrupole-linear ion trap (QqLIT), hybrid quadrupole-time of flight (QqTOF), polytetrafluoroethylene (PTFE), polyether ether ketone (PEEK), ultra performance liquid chromatography (UPLC)

1.10.2.2 Interfaces: ionisation sources

The main pre-requisite for analysing ions by MS is the successful converting neutral compounds into molecular ions or ionised fragments in the gaseous state in the ion source. An ideal source for mass-spectrometric analysis should provide high ionisation efficiency, high stability with minimum kinetic energy distribution and minimum angular dispersion of the ions. On the other side, when molecular ion is of interest, the ion sources should produce intact molecular ions, fragments for structural information and it should be possible to couple it with various chromatographs (Cooks *et al*., 1997).

The most commonly coupled technique is LC where the interface between LC and MS is electrospray ionisation (ESI), used in negative ionisation mode, due to its characteristics. However, atmospheric-pressure chemical ionisation (APCI) and ESI in positive mode have been employed for specific PFASs analysis (Table 1.12). Nevertheless, GC is also applied for the analysis of volatile and semi-volatile PFASs such as FTOHs and the most commonly used interface is negative chemical ionisation (NCI) or electron impact (EI) (Table 1.12).

The main ionization sources are summarised in Table 1.13.

1.10.2.3 Detection

Although there are different techniques available for PFASs analysis, the most commonly used are based on mass spectrometry. The use of mass spectrometry (MS) coupled to gas chromatography, liquid chromatography, or as a solely analytical technique, has increased since the start of the last century. Up till now, different types of MS analysers were developed. In this terminology, MS was definied by the 2002 Nobel Laureate in Chemistry, John B. Fenn who discovered electrospray ionisation:

"Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields.

Clearly, the sine qua non of such a method is the conversion of neutral analyte molecules into ions. For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions. In recent years, the efforts of many investigators have led to new techniques for producing ions of species too large and complex to be vaporized without substantial, even catastrophic, decomposition."

Table 1.13: summary of main ionisation methods. Adapted from Cooks *et al*. (1997)

Ionisation source	Acronym	Ionisation agent	Characteristics	Application
Volatile molecules				
Electron Ionisation or Electron Impact	EI	Electrons (~70 eV)	• Reproducible spectra • Extensive fragmentation • Cationic ions	For structural elucidation, coupled to GC
Chemical Ionisation	CI	Gaseous ions (reagent gas)	• Soft ionisation source • Molecular ions • Cationic and anionic ions	For MW determination, coupled to GC
No-volatile molecules				
Field Ionisation	FI	Intense electric field	• Intact molecular ions from high MW compounds • Solid samples • Cations	Condensed phase, MW and structure determination
Field Desorption	FD	Energetic particles, photons, electric field	• Intact molecular ions from high MW compounds • Solid samples • Cations	Condensed phase, MW and structure determination
Thermospray	TSP	Soft thermal energy, electric field	• Temperature dependant spectra • Liquid samples • Anions and cations	Interfase for LC-MS
Electrospray ionisation	ESI	Intense electric field	• Multiple charged ions for high MW; soft ionisation soruce • Liquid samples • Ionization in liquid phase • Anions and cations	Interfase for LC-MS
Atmospheric-pressure chemical ionisation	APCI	Intense electric field	• Multiple charged ions for high MW; not as soft as ESI • Liquid samples • Ionisation in gas phase • Anions and cations	Interfase for LC-MS
Atmospheric pressure photoionisation	APPI	Energetic particles, photons, electromagnetic field	• Liquid samples • Ionisation in gas phase • Anions and cations	Interfase for LC-MS, nonpolar or low-polarity compounds not efficiently ionised by other ionisation sources
Desorption electrospray	DESI	Particles impact	• Ambient ionisation • Solid samples • Anions and cations	Direct analysis, low sample preparation
Direct Analysis in Real Time	DART	Particles impact	• Ambient ionization • Solid samples • Anions and cations	Direct analysis, low sample preparation
Fast atom/ion bombardment	FAB/FIB	Particles impact	• Soft energy ions • Liquid samples • Anions and cations	Direct analysis. It is being replaced by MALDI
Matrix-assisted laser desorption ionisation	MALDI	Laser desorption	• Multiple charged ions for high MW; not as soft as ESI • Solid samples • Anions and cations	Direct analysis. Coupled to time of flight instruments.

However, before starting an analysis with MS, there should be different drawbacks considered, of these analysers with PFAS studies. One of the main drawbacks is the complex matrices. They produce matrix effects, produced in the ionisation source: ion suppression or enhancement. Therefore, in order to assess and normalise instrumental signal, it is required to use labelled internal standards. Some current limitations associated to the labelled standards purity have been presented before. Another important effect during PFASs analysis is the interference of PFOS transition 499 > 80 in biological matrices with TDCA as has been explained in the drawbacks section. Nevertheless, the drawbacks can be minimised and, currently, the analysis of PFASs is performed, in general, by MS^2 with different analysers. Both techniques, GC and LC, can be coupled to different MS analysers.

There are several different types of mass analysers, depending on ion movement or storage. The first one is based on ion transport and includes: electrostatic and magnetic sectors, quadrupoles (Q), sime of flight (TOF) and hybrids combinations of these ones. The second type of analysers are based on ion storage such as ion traps (IT), Fourier-transform ion cyclotron resonance (FT-ICR). The properties of mass analysers are evaluated according to mass range, mass resolution, ion transmission efficiency, mass accuracy, dynamic linear range, scan rate and sensitivity. Table 1.14 summarises some of these parameters. Nonetheless, the selection of the analysers is also based on economic costs as well as their flexibility. In this sence, the most commonly spectrometers used in the analysis of PFASs in all the matrix types are the triple quadrupoles (QqQ) due to their versatility, sensibility and robustness according to its maintaince cost. In the following section more detailed information about the analysers used in the analysis of PFASs is given: ion trap, triple quadrupole and hybrid quadrupole – linear ion trap as well as some iformation about the hybrid quadrupole – time of flight and the orbitrap.

Table 1.14: comparative parameters of the most common mass spectrometer analysers (Leonards *et al*., 2011)

Mass spectrometer	**Dynamic linear range**	**Mass accuracy**	**Mass resolution**
Sector magnet	10,000	1-2 ppm	100,000
Single quadrupole	10,000	100,000 ppm	4,000
Triple quadrupole	10,000	100,000 ppm	5,000
Hybrid quadrupole - ion trap	1,000	100,000 ppm	7,000
Linear ion trap	10,000	50-200 ppm	1,000
Time of flight	100	5 ppm (lock mass)	15,000
FT-ICR	> 5,000	> 1 ppm	500,000
Orbitrap	> 5,000	5 ppm 1-2 ppm (lock mass)	200,000

Ion Trap. Between the 1950s and 1960s, the Ion Trap (IT) mass spectrometer was developed by physicists, Wolfgang Paul and Hans Georg Dehmelt. It consists of two end-cap electrodes of hyperbolic cross section that normally are operated at ground potential. Located at the end caps is a ring electrode to which a radiofrequency voltage is applied (Figure 1.10). IT confines ions spatially using electric and magnetic fields alone or in combination. All the experiments are performed in the same space, but at different times. The scan is performed by changing the radio frequency voltage amplitude (Cooks *et al.* 1997). It could work in different modes: in scan mode with high efficiency because all the ions are trapped in the electrodes, in selected ion monitoring mode (SIM) allowing the destabilisation of undesirable ions and the enhancement of the sensitivity of the selected ions, in tandem performing MS^n experiments. This last mode allows to perform multiple fragmentations in sequence and to elucidate the structures of unknown analytes. However, this analyser has low resolution (unit) and the possibility of undesired ion-molecule reactions inside the analyser. In order to decrease these reactions is necessary to introduce helium as a damper gas. The main advantages are that this technique is simple and easy to maintain, it can be coupled to other analysers such as quadrupole and it can be coupled to GC and LC. Few authors have used this technology for PFAS studies (Tseng *et al.* 2006).

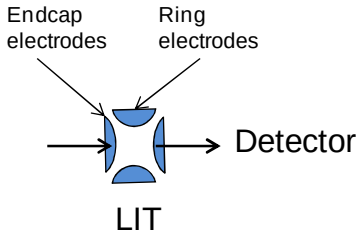


Figure 1.10: IT structure

Triple Quadrupole. The introduction of the quadrupole (Q) as an analyser was in the middle of the last century. Nowadays, although the use of single Q is still common in routine analysis (more in GC than in LC), the possibility of using three quadrupoles in tandem, named triple quadrupole (QqQ) mass spectrometer, in the analysis of different substances has allowed better sensitivity, simplicity of the operations and the possibility to determinate a target compound in different matrices duo to its selectivity. Quadrupoles work as a mass filter and are based on transport ions. The quadrupole mass analyser consists of four hyperbolic, or cylindrical rods placed parallel in a radial array, operating by applying ac potentials. The movement of the ions is in two dimensions and only ions which undergo stable motion in both directions will remain within the device and be detected by an ion detector as they emerge from the quadrupole (Cooks *et al.* 1997). It is highly sensitive and sensitivity can be increased for working with hyperbolic rods like TSQ Quantum (Thermo Fisher). This instrument is selective working in SRM mode. It allows to work at low resolution (unit, in general) and at medium-low resolution. The Q could be coupled to other analysers such as TOF, IT or another Q and combined with GC and LC. The QqQ allows performing multiple scan modes, for example, with scan in the first quadrupole and SIM in the second one, SRM

with collision-induced diossociation (CID) which increases the selectivity and makes them useful for quantification purposes or scan in first and second quadrupoles after a neutral loss. Figure 1.11 shows an example of a triple quadrupole structure. The triple quadrupole is the technique of choice for environmental and biological analysis of PFASs due to its versatility, sensibility, robustness and simplicity. Different examples are reported in Table 1.12.

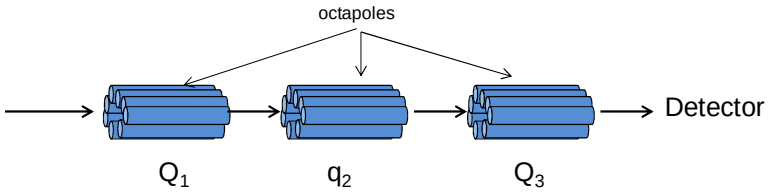


Figure 1.11: QqQ structure

Hybrid quadrupole-linear ion trap. The sensitivity and selectivity of the Q and the high efficiency of IT make the hybrid quadrupole-linear ion trap (QqLIT or QTrap) one of the most interesting tools in environmental and biological studies, always in terms of low resolution. It can be coupled to a LC system. In an IT, the ions are trapped in a radio frequency quadrupole field in contrast to a Q, where the ions pass through a quadrupole analyser with a superimposed radio frequency field. It allows to perform MS^3 experiments, which is a useful tool for structure elucidation during the identification of different compounds in degradation studies and, due to the conformation of ion trap, the screening in scan mode is more efficient than in a quadrupole analyser, which is more selective. The QTrap, typically, consists of two octapoles (Q_1 and q_2) and a LIT with a ring electrode and two hyperbolic endcap electrodes (Figure 1.12). The operation modes are, for example, scanning in both analysers, SIM in Q and scan in LIT, which is named enhanced product ion (EPI), data dependant scan with collision induced dissociation, scans with a neutral loss, SRM and MS^3. Different authors have used this mass spectrometer for the analysis of different PFASs with quantification and screening purposes (Scott *et al.* 2006; D'Eon *et al.* 2010; Frömel *et al.* 2010).

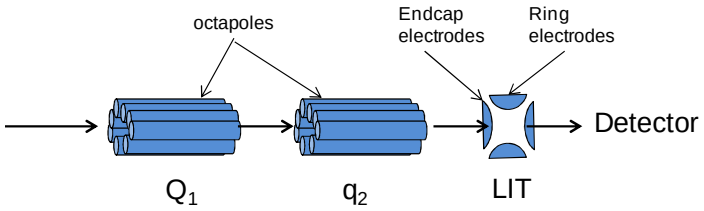


Figure 1.12: hybrid QqLIT structure

Hybrid quadrupole time-of-flight. The hybrid analyser quadrupole time-of-flight (QqTOF) provides an excellent selectivity of the Q and a med-to-high mass resolution of the TOF due to its structure (Figure 1.13) and high sensitivity, which is used for the analysis of big molecules such as proteins. The basis of the instrument is the measure of the flight time of accelerated ions. The movement is generated by the application of a potential and it is dependant of the ration m/z. This instrument could be coupled to LC, GC, and capillary electrophoresis or by pulse ionization source sample introduction like MALDI or plasma desorption. It allows the accurate ionic mass identification of product, since it can perform exact mass experiments and it represents an important tool in degradation studies, too (Jones *et al.* 2003; Martin *et al.* 2004; Guo *et al.* 2008).

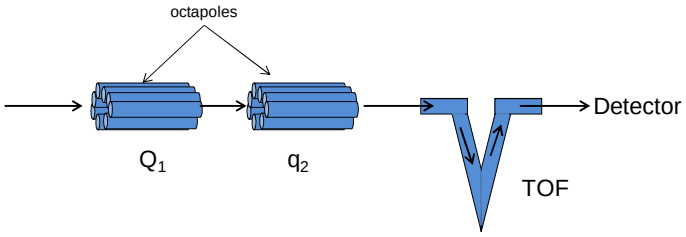


Figure 1.13: hybrid QqTOF in one of the TOF structures

Orbitrap. The Orbitrap analyser was discovered by Makarov (2000) and works with the same principles as Fourier Transform Ion Cyclotron Resonance mass spectrometer: the ions are trapped in an electrostatic field between the electrodes and the inner electrode, and are balanced by centrifugal forces (Figure 1.14). The Orbitrap works as analyser and detector and, in general, it is coupled to a LC system. The ion cycles around the central electrode are a function of the electric field, as well as of the oscillation, due to the ratio mass/charge. In addition, the ions also move back and forth along the axis of the central electrode (Makarov *et al.* 2006). This technique has started to be used in the analysis of PFASs in human breast milk and fish samples (Kadar *et al.* 2011a; Kadar *et al.* 2011b).

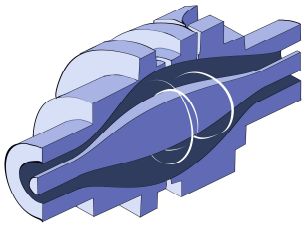


Figure 1.14: Orbitrap structure

Other instrumental tools have been employed for more specific studies, such as radioactivity and liquid chromatography / accurate radioisotope counting (LC/ARC) in the study of aerobic biodegradation PFASs in a flow-through soil incubation system (Liu *et al.* 2010).

1.11 Objectives

Under this context, the overall objective of the present Doctoral Thesis was **to assess the occurrence of a wide range of PFASs in environmental samples, drinking water, food, and to study the possible sources of human exposure with special enphasis on: food and drinking water**.

Therefore, the specific objectives were:

1. To develop and validate analytical methods based on off-line and on-line approaches, using different mass analysers for the analysis of PFASs in different type of matrices, including:

    - *Food* (fish, daily products, infant food and drinking water),
    - *Environmental samples* (biota, biota and soils from remote areas, sediments, surface and ground water),
    - *Wastewater*
    - *Sludge,*
    - and *human biological matrices* (human breast milk and blood).

2. To study the presence of PFASs in the environment, food, drinking water, human samples of cord blood and breast milk, and wastewater.
3. To assess PFASs human routes of exposure through the diet
4. To assess the possible accumulation of PFAS in cord blood and human breast milk.

CHAPTER 2

Environmental Occurrence

2.1 Introduction

In this chapter, we will deal to discuss the fate and behavior of PFASs in the water cycle. In addition, the accumulation of PFASs in biota samples from remote areas will be reported and discussed. Finally, the presence of PFASs in marine plastic pellets will be also reported.

Figure 2.1 are summarizes the main sources of PFASs in the environment and their relation between the different environmental compartments.

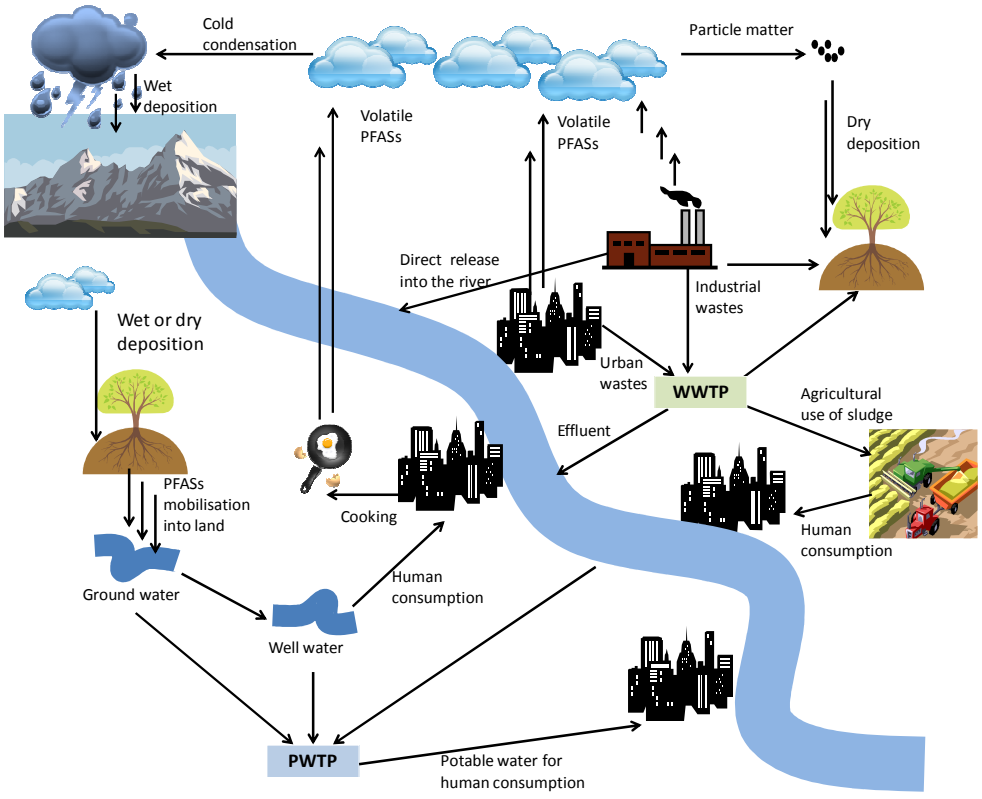


Figure 2.1: Environmental sources of PFASs.
WWTP: wastewater treatment plant; **PWTP:** potable water treatment plant.

As it has been presented in the previous chapter, the main sources of PFASs in the environment can be classified as:

- **Direct sources**
  - Direct discharges and emissions at industrial sources and direct discharges by WWTPs

- **Indirect sources**
  - Degradation processes produced in other industrial point sources.
  - WWTPs discharges, atmospheric emissions and the use of sludge in soil amendment for agriculture
  - Degradation during lifespan of materials containing PFASs or their polymers
  - Disposal in landfills of products containing or based on PFASs

The principal sources of PFASs to the environment are direct emissions at industrial sites. The industrial residues of PFASs can be generated by different processes:

1. During the synthesis of fluoroalkyl substances (Prevedouros *et al.* 2006),
2. As indirect product generated during the synthesis of PFASs (Prevedouros *et al.* 2006),
3. During combustion processes of some materials (the most volatile PFASs) and,
4. As residual compounds in the manufacture of products made of PFASs (Prevedouros *et al.* 2006) among others.

However, the diffuse contamination it is also very important. The uses of products containing PFASs as raw materials are an indirect source of PFASs to the environment. For example, by heating cookware and ovens materials that contain PFASs (Begley *et al.* 2005). Another example is their release through domestic wastewaters, for example during the washing of fabrics or by the use of shampoos or other consumables that contain PFASs.

Conventional wastewater treatments at WWTPs present low removal rates for some PFASs. For this reason, WWTPs constitute an emission source of PFASs. In addition, most volatile compounds can be emitted to the atmosphere during the water treatment processes. In addition, the use of sewage sludge in agricultural lands could be another indirect source of PFASs in soils and groundwater (Clarke *et al.* 2011). Once in soils, PFASs can enter into human diet via consumption of crops from contaminated soils or they can be mobilised by air-borne transport, or pass to surface waters and ground waters by draining (Navarro et al. 2011; Yoo et al. 2011). According to their potential significance for agricultural utilisation and persistence in soils, different groups of organic contaminants commonly found in sewage sludge were recently scored (Clarke et al. 2011). In this classification, PFASs obtained 10 scores over 11 based on their persistence in soil (more than 6 months), their potential accumulation in human food chain, their potential bioaccumulation, as well as their possible soil ecotoxicity. On the

other hand, it should be considered that PFASs in sludge amended soil can be mobilised by rainfall reaching phreatic waters (Gottschall et al. 2010). In general, the occurrence of PFASs is at higher ng/g concentrations (Guo et al. 2010; Li et al. 2010; Ma et al. 2010; Zhang et al. 2010). In spite of the current limitation on the use of PFOA, this is the only long chain acidic detected in sludge. Its occurrence can be associated to the biodegradation of other longer chain congeners currently in use is suggested (Frömel et al. 2010; Lee et al. 2010). The predominance of shorter C chains is supported by Ma *et al.* (2010). The authors found a dominance of even-chain length perfluoroalkyl carboxylic acid in all of the WWTP sludge samples investigated. A strong aerobic degradation of fluorotelomer alcohols in WWTPs is suggested. In addition, currently long chain compounds are replaced by short chain PFASs, which should be further studied in the future. In this sense, considerable amounts of these products can also reach WWTP sludge, partially contributing to these concentrations. In spite of the lack of data reporting the profile of PFASs present in sewage sludge during the past, available data seems to show a strong decrease in the presence of long chain PFASs and, at the same time, an increase of short C-chain compounds.

In general, high concentrations of PFASs in surface waters can be directly related to heavy industrialised and urban areas. For example, up to 1371 ng/L of PFOS were measured in the river Krka in Slovenia. The river Scheldt shows concentrations up to 153 ng/L, the Seine 97 ng/L and the Rhine 32 ng/L. High concentrations of PFOA were measured in Danube (25 ng/L), Scheldt (88 ng/L), and Rhone 116 (ng/L) (Loos et al. 2009). Also high concentrations of PFOA have been reported in different river waters as, for example, in Japan. The river concentrations were found in the range between 54 and 192 ng/L (Murakami et al. 2008). Other studies reported the presence of PFAS in lakes from industrial areas. For example in Lake Ontario concentrations were in the range of 21 – 70 ng/L for PFOS and between 27 and 50 ng/L for PFOA (Boulanger et al. 2005).

Regarding their presence in Spanish surface waters, Ericsson et al. (2008) reported the concentrations of PFASs in different Catalan rivers in an area near to Tarragona city (Ebro River in Garcia and Mora, and the Francolí and Cortiella Rivers). In these locations the concentrations were between 0.19 and 25 ng/L (including PFOA and PFOS). In another study, Sánchez-Avila *et al.* (2010) investigated the concentrations of five compounds (PFBS, PFHxS, PFOS, PFOA and PFNA) in different surface waters, sea water and wastewaters from Catalonia, including a sample from a high industrialised area settled near to the Llobregat river. In this sampling site PFOS and PFOA were the compounds found at higher concentrations but it should be mentioned that all the investigated compounds were also found at quantifiable concentrations. The concentrations reported in this study were comparable with those obtained for other river waters from other industrialised areas in Europe. However, the profile of compounds was quite different than in other European countries such as Germany, where the occurrence of PFOA in surface water is generally found at higher concentrations than PFOS (Skutlarek et al. 2006). A recent work by Picó *et al*. (2011), reported the spatial distributions of PFASs in water and sediments from the L'Albufera Natural Park (València, Spain). The results of this study showed that the most frequently found compounds were PFOS and PFOA, with concentrations ranging from 0.94 to 58.1 ng/L

and from 0.99 to 120 ng/L for PFOS and PFOA, respectively. However, in the sediments, the profile was the opposite; the concentrations of PFOS were higher than PFOA, attributed to their physicochemical properties. The presence of these compounds showed an important spatial distribution being widespread along all sampling sites, which is in agreement with data reported in other European rivers. However, the Mediterranean Rivers are affected in a great manner by climate episodes, such as first-flush, which can re suspend contaminants contained in the sediments. As expected, the higher concentrations were found near the mouth corresponding generally to a heavily populated and industrialised area. The compound found at higher concentration was PFHpA, around 30 ng/L. Recently, the concentration patterns have shown that, in general, more frequent compounds, and also those at higher concentration, were short chain PFASs. This indicating a tendency to replace more persistent long chain PFASs by new short chain ones.

Few studies reported the concentration in coastal waters and open sea, as for example the concentrations in coastal waters from the Pacific and Atlantic Ocean were in the range of 0.23 – 57.5 ng/L for PFOS and 0.24 – 192 ng/L for PFOA. In the open Ocean the concentrations vary between the limits of detection and 0.1 ng/L and 0.15 – 0.5 ng/L for PFOS and PFOA, respectively (Yamashita et al. 2005).

Due to their widespread, PFASs are present in remote areas such as the Arctic [atmosphere (Shoeib et al. 2006), Arctic Ocean (Wania 2007), biological samples (Martin et al. 2004; Butt et al. 2010; Sonne 2010; Stemmler et al. 2010)] or Antarctic [biological samples as penguins or seals (Tao et al. 2006; Schiavone et al. 2009)]. T he occurrence of PFASs into remote areas is associated to biogeochemical cycles and geophysical drivers. Their presence could be explained by the theory of “cold condensation”, concerning the chemical movements or chemical transformations from sources under the impact of environmental forcing, such as temperature, and interactions with other environmental compartments (vegetation, soil and oceans) (Lohmann et al. 2007). Some PFASs exhibit the potential for long-range environmental transport by a combination of dissolved-phase water and gas-phase atmospheric transport. The predominant transport pathway is complicated due to the uncertainty over water and atmosphere partitioning. Furthermore, there is evidence that transport and subsequent oxidation of volatile alcohol PFASs precursors may contribute to the levels of these analytes in the environment.

On the other hand, the distribution of PFASs for example in oceans, can be related to another contamination problem. In 2010, the European plastics production was about 57 million tons (Mt). The 57.9% of the production is recovered by recycling (6 Mt) and alternative energy source (8.3 Mt) but the other 42.1% is not recovered. This disposal plastic is an increasing cause of water pollution since industrial products may become marine debris if they are improperly disposed on land or if they are lost during transport or loading/unloading at port facilities (US EPA 2002). Research has shown that hazardous pollutants as persistent organic pollutants (POPs) are adsorbed and concentrated onto the surface of plastic pellets being the source the surrounding seawater (Mato et al. 2000; Hirai et al. 2011). Because contaminated pellets may be ingested by animals they could be a source of hydrophobic contaminants in the marine food chain as POPs (Gregory

2009). Partitioning and reactivity properties are important to understand and model the environmental behaviour of PFASs. According to their octanol-water partitioning coefficient (Wania 2007), PFASs can be adsorbed onto marine plastic debris surface and this can be another source of these compounds into the environment as well as another type of long-range transport. A well known example of marine debris are small plastic resin pellets made of PP or PE, with about 2-6 mm in diameter, which are the raw material for the manufacture of plastic products (Derraik 2002). These pellets have been released into the marine environment from accidental spillage during production and processing, transport and handling. Some are buoyant whilst others become suspended or sink and can arrive to constitute a contamination source in the Oceans (Coe et al. 1997; Redford et al. 1997; Gregory 2009).

However, it should be pointed out that the systematic study of PFASs in environmental waters that has started around one decade ago, being focussed only in to study a few number of compounds, such as PFOS and PFOA. Therefore, the occurrence, fate and behaviour, as well as the routes of human exposure, for most of the compounds currently in use continue being poorly known and understood.

It is known that, drinking water is one of the primary routes of human exposure (D'Hollander et al. 2010; Pico et al. 2010). At the same time, one of the main sources of PFASs in drinking waters is the contamination of the natural water bodies. This fact has been one of the reasons that has driven the study of the occurrence of PFASs in natural waters during the last years. For example, Ericson *et al.* (2008; 2009) studied PFASs in 4 mineral bottled waters and tap waters from Catalonia. The results confirmed the presence of these analytes between 0.13 and 0.40 ng/L in bottled waters and 0.02 – 69 ng/L in tap waters. An earlier example by Skutlarek *et al. (2006)* it showed the occurrence of PFASs at concentrations between 2 and 519 ng/L in Ruhr area tap water. The higher concentrations corresponded to PFOA. The analysis of surface river waters from the same zones of Ruhr area disclosed similar levels thus indicating that the total removal of these compounds was not achieved during the water treatment processes. In addition, higher contamination levels were related to certain materials in agricultural fields. In another example, PFOS and PFOA were found at concentrations of 9 ng/L and 3 ng/L, respectively, in surface water from the Lake Maggiore (Switzerland). The analysis of drinking water produced from the lake presented very similar trends of contamination, revealing the poor performance of sand filtration and chlorination applied by the local waterworks (Loos 2007). The study of tap water from Brazil, Japan, China, India, USA and Canada confirmed the occurrence of PFASs in all analysed sites (Takagi et al. 2008; Mak 2009; Quinete et al. 2009).

The occurrence and the toxicity of these compounds have driven to their regulation. For example, in January 2009 the USEPA (EPA 2009) set the short-term exposure scenario (acute toxicity) for PFOS and PFOA establishing the Provisional Health Advisory (PHA) at 200 ng/L and 400 ng/L, respectively. However, the current regulations are focused in a low number of the compounds, and only acute exposures are under regulation, whereas the chronic exposure was not considered.

Recently, in April 2012, in United States launched the third Unregulated Contaminant Monitoring Rule (UCMR-3) that will require the monitoring of 30 contaminants using EPA and/or consensus organization analytical methods during 2013-2015 (USEPA 2012). Among the 30 selected contaminants for the assessment of their entry points to the distribution system, 6 PFASs were selected: PFOS (40 ng/L), PFOA (20 ng/L), PFNA (20 ng/L), PFHxS (30 ng/L), PFHpA (10 ng/L) and PFBS (90 ng/L). Together with the EPA, States laboratories and public water systems (PWSs) will participate in the UCMR 3.

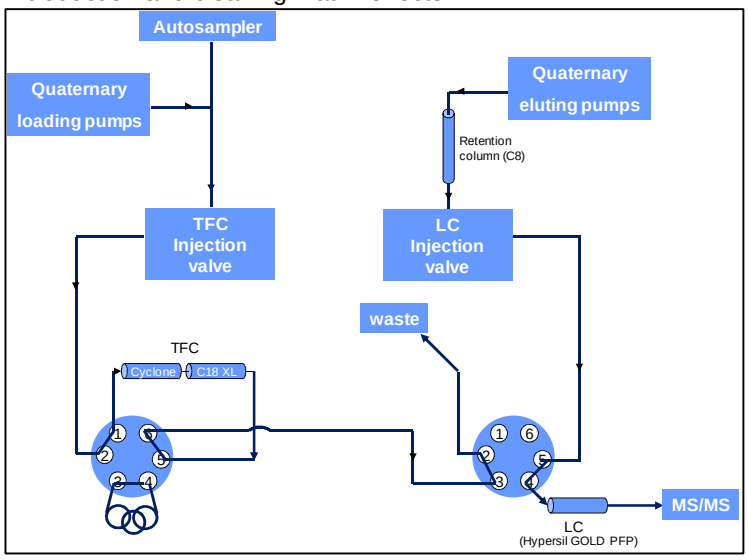
2.1.1. Analysis of PFASs in Waters

2.1.1.1 Sample extraction

Most of the analytical methods used for the determination of PFASs in water are based in off-line SPE. However, on-line extraction/clean-up procedures present a series of advantages, in terms of robustness and shorter time of analysis. Different on-line options we should highlighted:

- **Turbulent flow chromatography (TFC)** the basic principle for TFC is that the target analytes are retained in the active pores of a chromatographic column whereas interferences are eliminated by the turbulent flow that is produced when high flow passes through a restricted access material (RAM) column. Due to the poor size of the columns (> 5 µm), the generated turbulent flow (between 1.5 – 3 mL/min) allows the retention of small analytes into the activated pore sites due to the difference between diffusion of big and small compounds).
  The use of TFC coupled to LC/MS shows a great potential for the rapid, direct analysis of PFASs in complex matrices. TFC-LC/MS avoids the need for any time-consuming sample preparation, such as SPE, and it allowed a total sample analysis in few minutes. Coupling this technology with LC-MS/MS offers high sensitivity and specificity, as well. For example, MLOD and MLOQ of 5.4 and 19 ng/L, respectively, for PFOS were reported using this technology (Takino et al. 2003). Figure 2.2 (a) and (b) shows an example of system configuration in TFC-LC-MS/MS which has been used in this Thesis for the analysis of complex matrices such as blood (see chapter 4). In this case, two different columns in tandem were used.

A) Sample introduction and cleaning matrix effects



B) Transfer step

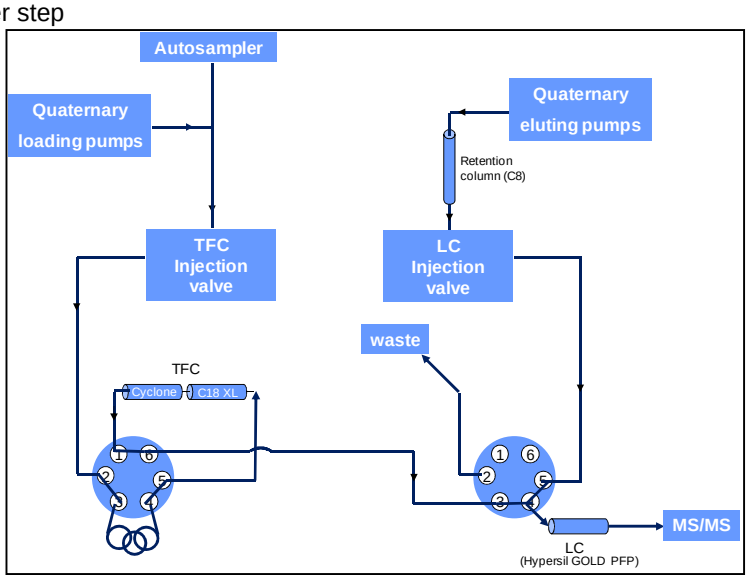
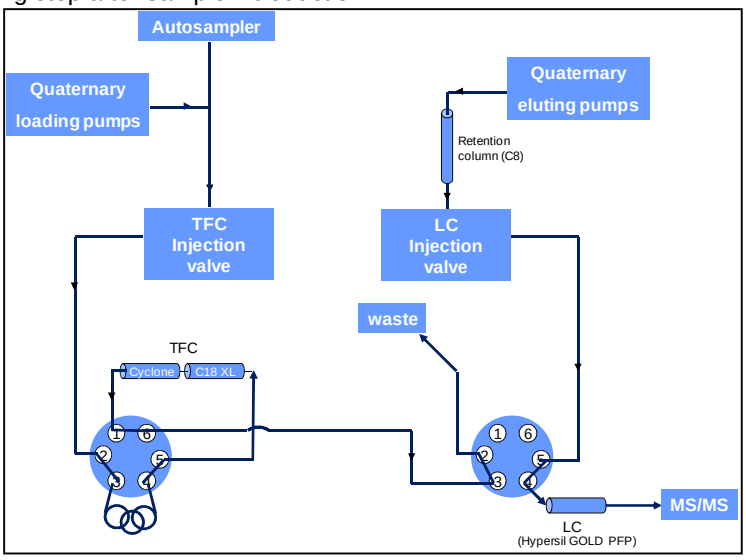


Figure 2.2 (a): Example of TFC system connections in Aria TLX-1 Thermo Fisher equipment

C) Cleaning step after sample introduction



D) Last cleaning and returning to initial conditions

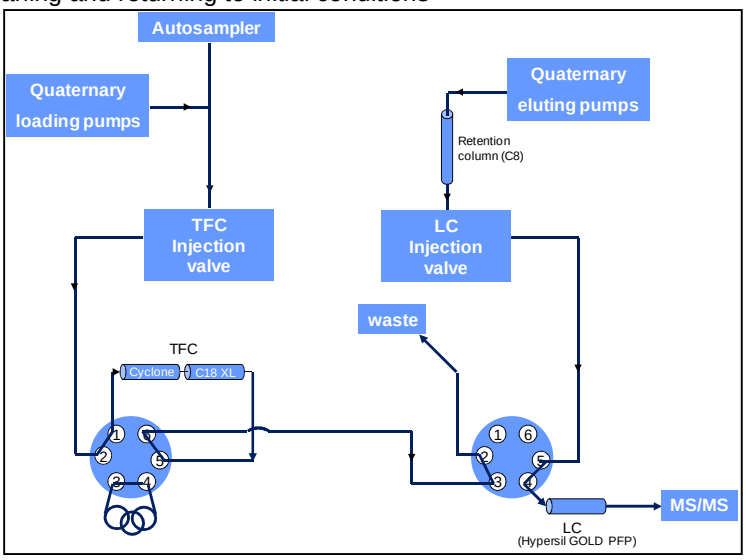
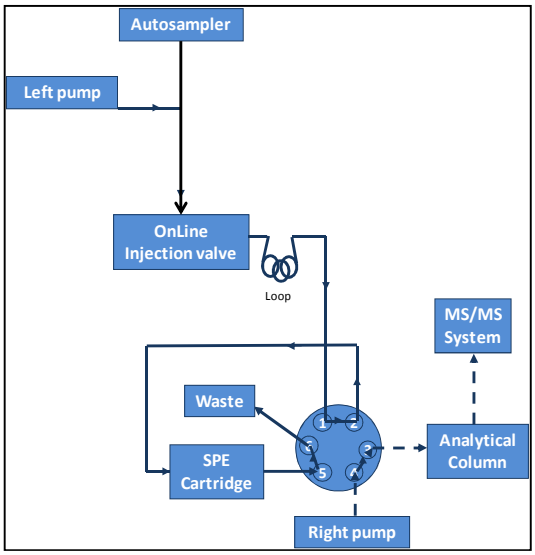


Figure 2.2 (b): Example of TFC system connections in Aria TLX-1 Thermo Fisher equipment

- **On-line solid phase extraction**. The on-line SPE is based on extraction and clean up of the sample coupled to an LC system. In general, the sample is loaded onto the cartridges, which have been previously conditioned with the optimum solvents. Then, the sample is loaded at flow-rates higher than 0.5 mL/min, being the analytes trapped into the activate sites of the cartridge. While the analytes are retained on the SPE column, the matrix is flushed to waste and the analytical column is equilibrated with the chromatographic pump (Gosetti et al. 2010). Then, the valve is switched to injection position (see Figure 2.3), and using the mobile phase the analytes are transferred to the LC column. These analytes are separated into the LC and finally detected by MS (/MS). Once the elution of the compounds has been completed, the valve is switched again into the loading position to equilibrate the on-line SPE cartridge with the loading phase into initial conditions (Gosetti et al. 2010). Figure 2.3 shows an example of on-line solid phase extraction positions.
  The advantages of this technique *vs*. off-line SPE are faster analysis and low sample manipulation as well as the higher robustness of the method. In general, these methods allow extracting up to 5 mL but it can change depending on the automated on-line system. The main disadvantage of this technique in front of the newer TFC, and another type of on-line extractions based on E-Quan systems, is that the cartridge can only be used one time. SPE cartridges, with different stationary phases can be used for PFASs analysis including: both ionic exchanges, C18 based phases or combining hydrophilic and lipophilic characters (i.e: Waters).
  In the case PFASs analyzed in water samples by on-line methods, there are few works published in the literature. For example, Wilson *et al.* (2007) developed a method for the extraction of 1 mL of surface water, for the analysis of PFOA and PFOS, with a Kromasil C18 enrichment column coupled to a nano-liquid chromatography and nano-spray mass spectrometry. The MLOD and MLOQ of PFOA and PFOS were 0.5 and 1 ng/L, respectively. Another SPE on-line extraction and purification method was developed by Gosetti *et al.* (2010) where 0.35 mL of water was pre-concentrated in a Poros HQ column and then the 9 analytes were separated by LC coupled to a mass spectrometer in tandem. The MLOD varied between 9 and 49 ng/L.

A) Loading position



B) Inject position

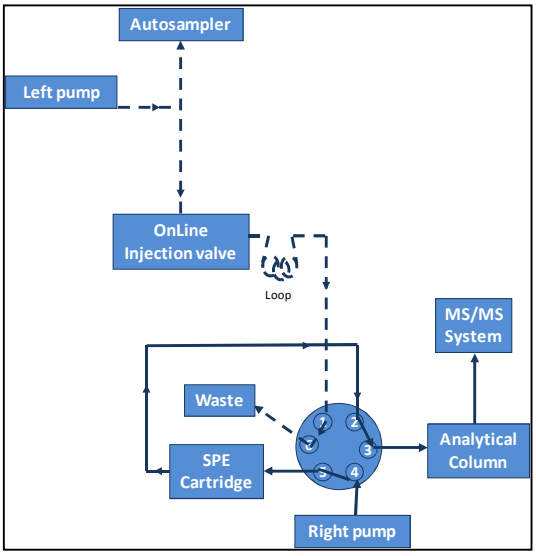


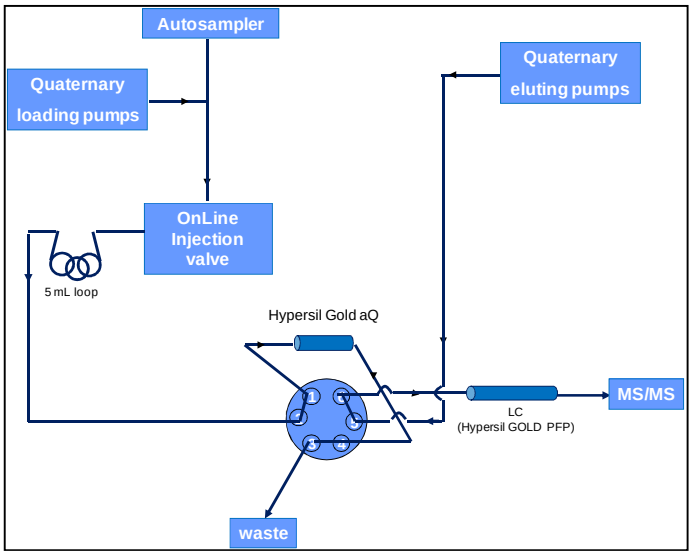
Figure 2.3: on-line solid phase extraction positions adapted from Gosetti *et al.* (2010).

In addition to the different on-line clean-up approaches, for the analysis of clean water samples another option are **on-line pre-concentration systems** as the ***E-Quan™ technology developed by Thermo*** (Thermo Fisher Scientific, Franklin, MA).

The *E-Quan™ technology* is based on the use of higher diameter columns with the same stationary phases used for separation. These columns allow the retention of polar compounds from aqueous samples and it can be used for more than 1000 runs, being therefore cheaper as compared to the common on-line-SPE cartridges (Gosetti et al. 2010). The basis of this technology is an on-line extraction method involving column switching. The main advantages are the same as in the case of on-line SPE: improved throughput and robustness due to the low sample manipulation and significant reduction of the time of analysis, as well as, enhanced resolution and high sensitivity under MS/MS (Thermo Fisher Scientific 2006-2007).

The EQuan system consists of two HPLC pumps with a pre-concentration column (Hypersil GOLD™ 12 µm, 20 × 2.1 mm), an analytical column (Hypersil GOLD 3 µm, 50 × 2.1 mm) coupled to a TSQ Quantum™ triple quadrupole mass spectrometer. The configuration of these quadrupoles allows high analytical sensitivity. With this of equipment and direct injection using volumes between 0.1 to 5 mL of the samples a reduction of the time of sample preparation is achieved (Thermo Fisher Scientific 2006-2007). As in the case of on-line SPE, the sample is loaded into the pre-concentration column. After enrichment, the analytes are transferred to the analytical column for their separation by switching the valve into loading mode (Figure 2.4).

A) Loading mode



B) Eluting mode

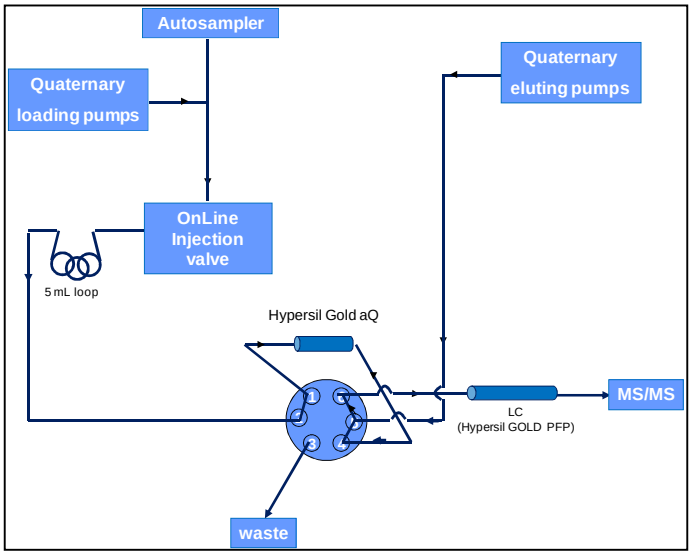


Figure 2.4: Scheme of on-line system based in EQuan technology.

2.1.1.2 Sample detection

According to the matrix type, which will be analysed, different analysers must be considered depending on their sensitivity and mass accuracy. The main aim has been to study the occurrence of PFASs in waters, sludge, sediment, biota and plastic pellets. Table 2.1 summarizes the main advantages and disadvantages of different mass spectrometers.

Table 2.1: Main advantages and disadvantages of different mass spectrometers

MS	Advantages	Disadvantages	Optimum
Sector magnet	• High resolution • High sensitive • Enough confirmation points	• Too expensive	No
Q	• Low cost • High sensitive	• Lack in confirmation points • Low resolution	No
QqQ	• Low cost • High sensitive • SRM	• Low resolution	Yes
LIT	• Low cost • High efficiency in scan • MS^n experiments	• SRM with low sensitivity	No
QqLIT	• High efficiency in scan • SRM • High sensitive • MS^n experiments	• Mid cost	Yes
TOF	• High resolution	• Lack in confirmation points • Mid cost	No
Q-TOF	• High resolution • SRM	• Mid cost • Low sensitivity	No
FT-ICR	• High resolution • Enough confirmation points • MS^n experiments	• Too expensive	No
Orbitrap	• High resolution • Enough confirmation points • MS^n experiments	• Too expensive	No

SRM: selected reaction monitoring

2.2 Experimental work

In the next sections PFASs were investigated in different types of matrices including:

- Waters – wastewater effluents, surface river waters, groundwater, waters samples from different steps in a PWTP, tap water and bottled drinking water
- Sewage sludge,
- Biota and soil samples from Tierra de Fuego and the Antarctica, and
- Marine plastic pellets and marine sediments

The experimental results are reported in the following publications.

2.2.1 PFASs in the water cycle

Scientific publication 1:

Llorca, M., Farré, M., Picó, Y., Müller, J., Knepper, T. P. and Barceló, D. (2012).

"Analysis of perfluoroalkyl substances in waters from Germany and Spain"

431: 139-150 Science of The Total Environment

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Analysis of perfluoroalkyl substances in waters from Germany and Spain

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ABSTRACT

Water has been identified as one of the main routes of human exposure to perfluoroalkyl substances (PFASs). This work assessed the presence of 21 PFASs along the whole water cycle using a new fast and cost effective analytical method based on an online sample enrichment followed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The method was validated for different types of matrices (ultrapure water, tap water and treated wastewater). The quality parameters for the 21 selected compounds presented good limits of detection (LOD) and quantification (LOQ) ranging, in general, from 0.83–10 ng/L to 2.8–50 ng/L, respectively. The method was applied to assess the occurrence of PFASs in 148 water samples of different steps along the whole water cycle, including: mineral bottled water, tap water, river water and treated effluent wastewater, from Germany to Spain. In addition, in order to prove the good performance of the online analytical method, the analysis of PFASs was carried out in parallel using a method based on offline anionic solid phase extraction (SPE) followed by LC–MS/MS. Consistent results were obtained using both approaches.

The more frequently found compounds were perfluoroalkyl acids, such as the perfluorobutanoic acid which was in the 54% of the tap water samples investigated with concentrations in the range between 2.4 and 27 ng/L, the perfluoroheptanoic acid (0.23–53 ng/L) and perfluorooctanoic acid (0.16–35 ng/L), and the sulphonate perfluorooctanesulfonate (0.04–258 ng/L) which was the second more frequent compound and also the compound found in with the higher concentration. It should be remarked that the 88% of the samples analyzed presented at least one of the compounds at quantifiable concentrations. In addition, PFASs including short chain compounds were proved to be prevalent in drinking water, and the 50% of the drinking water samples showed quantifiable concentrations of PFASs. It should be said that the great majority of the samples may not pose an immediate health risk to consumers, and just 6 of the drinking water samples presented concentrations of PFOS exceeding the Provisional Health Advisory (PHA) level established by the Office of Water from the USEPA for PFOS, which was set in 200 ng/L.

1. Introduction

Perfluoroalkyl substances (PFASs) are a large group of chemicals used since the 1950s in different industrial and commercial applications, due to their inertia and their high physical, chemical, and biological stability. However, PFASs are persistent in the environment, they can be accumulated and be biomagnified through the trophic chain (Tomy et al., 2004). Animal studies have demonstrated different chronic and subchronic effects from PFASs including, hepatotoxicity, developmental toxicity, immunotoxicity, and carcinogenicity as well as hormonal effects from PFASs (Lau et al., 2007). In-utero exposure experiments to PFOS conducted in mice and rats, have demonstrated that the presence of PFOS produced the reduction of body weight and developmental delay in neuromotor maturation in the offspring (Fuentes et al., 2007; Lau et al., 2006; Luebker et al., 2005). The interaction of PFASs with thyroid hormones, high density lipoprotein, cholesterol and triglycerides has been also assessed, indicating the disruption of PFAS onto different metabolic routes (Lau et al., 2007; Peden-Adams et al., 2008). The elimination of perfluorophosphonic acids (PFPAs) has been assessed in rats concluding that the mono-PFPAs and di-PFPAs may also have significant lifetimes in the body of rats and it was demonstrated that the mono-PFPAs may bind to blood cells underestimating their concentration in plasma and sera samples (D'Eon and Mabury, 2010). The biological fate of the mono-PFPAs and di-PFPAs determined in the study suggested that there was a potential human exposure and if the exposure does occur they may be long-lived in the body. In the human body, PFASs tend to be associated with fatty acid binding proteins in the liver or albumin proteins in blood (Han et al., 2003) and have been detected in human serum, cord blood and breast milk in several studies (Haug et al., 2009; Sundstrom et al., 2011; Apelberg et al., 2007; Fromme et al., 2010).

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In 2006 the Environmental Protection Agency (US Environmental Protection Agency, 2011b) and the eight major PFASs producer companies (Arkema, Asahi, BASF Corporation (successor to Ciba), Clariant, Daikin, 3M/Dyneon, DuPont, Solvay Solexis) in the industry launched the "PFOA Stewardship Program". The companies committed to phase out global facility emissions and product content of by 95% by 2010 and to work toward eliminating emissions and product content by 2015 (US Environmental Protection Agency, 2006). In 2008, the European Food Safety Authority (EFSA) carried out a compilation and evaluation of the results of different toxicological studies in order to set the no observable adverse effect levels (NOAELs), the lowest observed adverse effect levels (LOAELs), and the critical endpoints (EFSA, 2008) for a selected group of PFASs. With this information it was established that the tolerable daily intake (TDI) for perfluorooctane sulfonic acid (PFOS) was at 0.150 ng/g and perfluorooctanoic acid (PFOA) at 1.50 ng/g. Recently, PFOS has been included as a persistent organic pollutant (POP) under the Stockholm Convention for global regulation of production and use (United Nations Environment Programme, 2010). PFASs are also prime candidates for chemicals that will need authorization within the REACH regulation (European Commission, 2002). In 2009 the Office of Water (OW) from the EPA established the Provisional Health Advisory for PFOA and PFOS as being 400 ng/L and 200 ng/L (US Environmental Protection Agency, 2009), respectively, and they are susceptible to be introduced into the Safe Drinking Water Act (SDWA) (US Environmental Protection Agency). However, these values are calculated for a short-life exposure, and long-life exposures may be more suitable for drinking water. On the other hand, in addition to the individual concentrations the total sum of compounds should also be considered. In addition, drinking water has been identified as one of the major routes of human exposure (Emmett et al., 2006; Skutlarek et al., 2006; Pico et al., 2010). During recent years several works have been carried out to assess the presence of PFASs in drinking water. Ericson et al. studied the presence of PFASs including perfluoroheptanoic acid, perfluorohexane sulfonate, PFOA, perfluorononanoic acid, PFOS, perfluorooctane sulfonamide and perfluorodecanoic acid in bottled mineral water and tap water in Catalonia (NE, Spain). The concentrations reported by Ericson et al., in tap water were between 0.02 and 69 ng/L (Ericson et al., 2009; Ericson et al., 2008). In another example, Skutlarek et al. assessed the occurrence of PFASs in tap water from the Ruhr area in Germany. In this study, high concentrations of PFASs were found with values between 2 and 519 ng/L, and the compounds found with higher concentrations were PFOA and PFOS (Skutlarek et al., 2006). The most concentrated samples came from the application of hazardous waste materials to agricultural fields. During the past years, several studies have assessed the concentrations of PFAS in tap water, for example in Brazil (Quinete et al., 2009), the reported concentrations of PFAS were from 0.09 to 6.7 ng/L, in Japan concentrations were from 0.16 to 84 ng/L (Takagi et al., 2008), and in China, India, Japan, Canada and USA between the limits of quantification and 130 ng/L (Mak et al., 2009). In order to elucidate the possible sources of PFASs in tap waters, the occurrence of PFAS has been assessed in surface water, and in these works the presence in tap water was partially related to the ineffective removal during water treatments of surface waters (Loos et al., 2007; Skutlarek et al., 2006). Currently, there is an emerging use of new short chain PFASs in replacement of the more persistent and toxic ones, such as PFOS and PFOA, but there is still a lack of information about their presence, as well as their possible occurrence, fate and behavior in the environment as well the bioaccumulation and biomagnifications through the food chain. In addition, the possible degradation of products generated in the environment or during water treatment processes should be taken into consideration (Dinglasan et al., 2004; Frömel and Knepper, 2010; Lee et al., 2010; Wang et al., 2005). Therefore, there is an urgent need for the continuous assessment of PFASs in drinking water, especially due to their possible negative implications in human health. Different studies have proved the accumulation of PFAS in human tissues and fluids, such as, breast milk (Kärrman et al., 2009; Llorca et al., 2010; Tao, 2009; Völkel et al., 2009), urine (Perez et al., 2012; Tao, 2009), saliva (Tao, 2009), blood (Guruge et al., 2005; Tao, 2009; Yeung et al., 2005), seminal plasma (Guruge et al., 2005) and liver (Kärrman et al., 2009).

On the other hand, rapid analytical approaches continue to be required, but online analytical methods, in spite of its excellent analytical performance characteristics (low solvent consumption, low amounts of sample required, reduction of analysis times and sample manipulation and robustness) and advantages for routine analysis, continue to be implemented less, and few authors have reported their use (Takino et al., 2003; Wilson et al., 2007; Apelberg et al., 2007; Calafat et al., 2007; Gosetti et al., 2010; Haug et al., 2009; Kuklenyik et al., 2005), in comparison to the off-line methodologies.

In this context, the main objectives of this work were (i) to develop and validate a new online approach based in a retention and preconcentration chromatographic column which can be reused for the rapid screening of PFASs in water; (ii) to assess the occurrence of 21 PFASs in water along the whole drinking water cycle; and (iii) to assess the human risk associated to PFASs ingestion through drinking water. A total number of 148 samples were analyzed from the whole water cycle of two European countries: Spain and Germany. These results give an integrative response about the occurrence of PFASs in water, as well as, an indication about the possible sources of contamination of whole water cycle including drinking water.

The analytical methodology presented here is an advance for the rapid screening of PFASs in water, being of special relevance for routine analysis of PFASs in drinking waters. The occurrence in waters can help during the implementation of the maximum limits, for some of these compounds, claimed for different agencies and organizations for health protection.

2. Materials and methods

2.1. Chemicals and standards

Perfluoroalkyl substances included in this study were supplied from Wellington Laboratories Inc. (Canada) including: i) a mixture of PFASs [MXB; >98%] containing: perfluorobutanoic (PFBA), perfluoro pentanoic (PFPeA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), per fluorodecanoic (PFDA), perfluoroundecanoic (PFUdA), perfluorododecanoic (PFDoA), perfluorotridecanoic (PFTrA), perfluorotetradecanoic (PFTeA), perfluorohexadecanoic (PFHxDA) and perfluorooctadecanoic (PFODA) acids, perfluorobutanesulfonate (PFBS), perfluorohexasulfonate (PFHxS), perfluorooctanesulfonate (PFOS), perfluorodecanesulfonate (PFDS); ii) the perfluorooctanesulfonamide (PFOSA), >99%; iii) the perfluorophosphonic acids: perfluorohexylphosphonic acid (PFHxPA), perlfuorooctylphosphonic acid (PFOPA) and perfluorodecylphosphonic acid (PFDPA) [chemical purity>98%; methanol]. Perfluoroalkyl substances used as surrogate internal standards (added before extraction procedure in order to normalize all the analytical process) were purchased from Wellington Laboratories Inc. (Canada) including: i) a mixture of labeled PFASs [MXA; >98%] containing: [$^{13}C_4$]-perfluorobutanoic acid (MPFBA ($^{13}C_4$)), Ion [$^{18}O_2$]-perfluorohexanesulfonate (MPFHxS ($^{18}O_2$)), [$^{13}C_2$]-perfluorohexanoic acid (MPFHxA ($^{13}C_2$)), Ion [$^{13}C_4$]-perfluorooctanesulfonate (MPFOS ($^{13}C_4$)), [$^{13}C_4$]-perfluorooctanoic acid (MPFOA ($^{13}C_4$)), [$^{13}C_5$]-perfluorononanoic acid (MPFNA ($^{13}C_5$)), [$^{13}C_2$]-perfluorodecanoic acid (MPFDA ($^{13}C_2$)), [$^{13}C_2$]-perfluoroundecanoic acid (MPFUdA ($^{13}C_2$)), [$^{13}C_2$]-perfluorododecanoic acid (MPFDoA ($^{13}C_2$)); ii) 6-chloroperfluorohexylphosphonic acid (ClPFHxPA) [chemical purity>98%; methanol]. Internal standards (added before analysis in order to assess and compensate possible losses during sample manipulation and extraction) were purchased from Wellington Laboratories Inc. (Canada) including: Ion [$^{13}C_8$]-perfluorooctanesulfonate (M8PFOS), [$^{13}C_2$]-perfluorooctanoic acid (M2PFOA).

During the analysis of German samples, ultrapure Milli-Q water was prepared by a Millipore-Q-system (Millipore, Milford, MA, USA). Methanol (MeOH) suprasolv, ammonium acetate salt (NH_4Ac, MW, 77.08; >98%), ammonia, n-hexane and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany). During the analysis of Spanish samples, water and methanol CHROMASOLV®Plus, for HPLC grade, ammonium acetate salt (NH_4Ac: MW, 77.08; ≥98%), ammonium hydroxide (NH_4OH: MW, 35.05; ≥98%) and formic acid (CHOOH: MW, 46.03; ≥95% in water; 1.22 g/mL at 25 °C (lit.)) were obtained from Sigma-Aldrich, Steinheim, Germany.

2.2. Sample collection

A total number of 148 samples were collected from 32 cities in Germany and Spain between 2010 and 2012, including: 6 mineral bottled waters (the most popular brand), 84 tap waters, 2 well waters, 48 surface river waters (24 from Spain and 24 from Germany), the effluents from 5 wastewater treatment plants (WWTPs), and 3 samples collected at different steps of the purification processes in a potable water treatment plant (PWTP) located at the federal state of Hesse (Germany). The PWTP provides drinking water for 300,000 inhabitants. This plant extracts surface water from the Rhine River, and the treatment is based on different steps including sand trap and active charcoal treatments followed by injection wells for a six-week soil passage.

Details regarding sampling locations are given in Tables S1 and S2 of the Supporting information section. Fig. 1 shows the sampling locations of tap water and river waters collected in both countries.

The samples were collected in polypropylene (PP) or glass bottles pre-cleaned with methanol and acetone and kept in a refrigerator at 4 °C until analysis (extracted within the next 15 days with the exception of samples G-S3, G-S4 and G-S5 (see Table S2 from the Supporting information)).

The sample containers and the storage procedure were chosen after discarding the possible contamination or adsorbance of selected compounds onto plastic or glass surfaces. To discard the possible cross-contamination from the containers procedural blanks were carried out, using ultrapure-water stored at room temperature during 2 weeks. After this period, the ultra-pure water was extracted using the same protocol as is used for the samples. Prior to the start of the sample enrichment procedure, the blanks and the water samples were allowed to reach room temperature into their initial containers, and then the bottles with the samples were ultra-sonicated for 3 min in order to resolve any possible adsorption onto the surfaces of containers. Second, in order to assess the possible adsorption onto the surfaces of containers, different types of containers (PP and glass) were filled with ultrapure water fortified with the mixture of PFASs at 50 ng/L. These prepared solutions were stored at 4 °C during the first 2 weeks. Then, the concentration of each compound in solution was assessed. After this first experiment, the samples were frozen and the same experiment was repeated after 1, 2 and 3 months, without significant differences in the resulting concentrations.

2.3. Online sample enrichment and instrumental analysis

5 mL of unfiltered water samples was spiked with an internal standard mixture in methanol to obtain a final concentration of 10 ng/L (2 μL at 25 μg/L) and then was directly processed using the Thermo Scientific Aria TLX-1 system equipped with the EQuan™ technology. This system consists of a PAL auto sampler (CTC Analytics, Zwingen, Switzerland), one mixing quaternary pump and one switching device unit. The entire system was controlled via Xcalibur software, version 2.1.

The online enrichment was achieved using a Hypersil GOLD aQ column (2.1×20 mm, 12 μm particle size from Thermo Fisher Scientific, Franklin, MA), which allows the retention of polar compound from aqueous samples. One of the main advantages of this system in comparison to the common online-SPE cartridges is that the enrichment column can be used for more than 1000 samples, being an important reduction in the analysis expenses (Gosetti et al., 2010). After enrichment, the analytes are transferred to the analytical column for their separation by switching the MS valve into loading mode. The analytical column in this method was a Hypersil GOLD PFP (50×3), 3 μm particle offering an alternative selectivity between fluorinated isomers in reverse phase applications (Thermo Fisher Scientific).

Briefly, the whole procedure consisted first with, loading the sample into the enrichment column using ultrapure water acidified at pH 4.5 with formic acid (adjusting the pH drop by drop with a pH meter symphony VWR®). After the enrichment step, the analytes were transferred to the analytical column for separation. The last step was the equilibration in which the initial conditions were set for the next run while the MS connector changes to waste. An illustrated schedule of the operative modes can be seen in Fig. 2. The gradient used is illustrated in Table 1. As can be seen in Table 1, the chromatographic mobile phases consisted of (D) water 20 mM NH_4Ac, and (E) methanol 20 mM NH_4Ac.

After separation, detection was carried out using a triple quadrupole mass spectrometer Thermo Scientific TSQ Vantage (Thermo Fisher Scientific, San Jose, CA), equipped with a Turbo Ion Spray source. All the analyses were performed operating in the negative electrospray ionization (ESI (−)) mode. Acquisition was performed in selected reaction monitoring mode (SRM) to obtain enough identification points (IP) for confirmation of each analyte according to Commission Decision 2002/657/EC. The main m/z transitions are summarized in Table S3 of the Supporting information.

For identification purposes the following conditions were accomplished: i) analyte retention time in the sample compared to analyte retention time in the calibration curve should be in agreement; ii) two m/z transitions were confirmed for every analyte; iii) ratio between the two transitions in the sample compared to ratio in the calibration curve should be in agreement to [calibration curve average ± SD (calibration curve)].

The quantification of the samples by the off-line enrichment was performed by external calibration curve using surrogate normalization: the plot ratio of the most intensive transition peak area divided with the surrogate standard area against the concentration.

For the assessment of matrix interference in the analysis matrix-matched calibration curves and blank samples were introduced in each run of analysis.

Fig. 3 shows an example of a real sample chromatogram, and another example of a real sample using the offline procedure can be shown in Figure S1 of the Supporting information.

2.4. Off-line solid phase extraction and instrumental analysis

The offline clean-up and enrichment was carried out using a previous protocol for PFASs extraction from different matrices (Llorca et al., 2010) consisting of solid phase extraction (SPE) with anionic exchange cartridges (Oasis WAX 3 cm^3, 60 mg, from Waters), but in this case, the method was modified in order to improve the retention efficiency of PFPAs during the SPE step. A brief description is presented in Section S1 of the Supporting information.

2.5. Quality assurance and quality control

In order to eliminate the sources of contamination from the analytical system all the polytetrafluoroethylene (PTFE) tubing was changed for polyether ether ketone (PEEK) connections. In addition, to minimize the background signal and inter-run variability of all analytes, an extra analytical column (C8 50×3 Thermo Scientific)

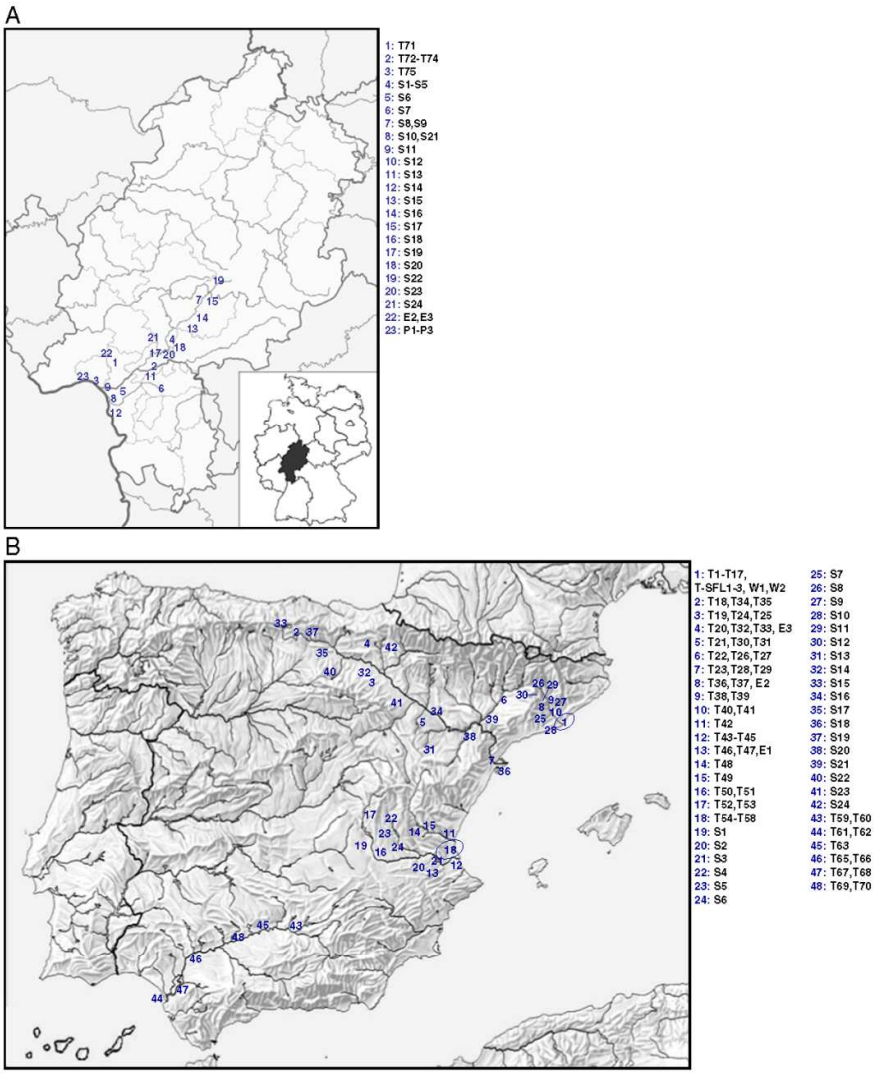

Fig. 1. Sampling sites A) Germany and B) Spain.

was placed directly upstream of the injector to trap the instrumental sources of analytes, as adapted from Flaherty et al. (2005).

In order to rule out any possible source of system contamination, blanks consisted in initial conditions of mobile phase were analyzed every 5 sample injections.

Ultrapure water was used as blank sample for optimization and validation purposes. Spiking experiments were performed with ultrapure water, tap water and a wastewater effluent fortified at three different concentration levels (6, 12 and 24 ng/L). These samples were analyzed prior to fortification in order to assess the initial concentrations of PFASs, being in all cases below the method of limits of detection.

For validation purposes of the online analytical approach, the instrumental limits of detection (ILOD) and quantification (ILOQ), the method limits of detection (MLOD) and quantification (MLOQ), as well as, decision limits (CCα) and detection capability (CCβ), linearity, recoveries and precision according to the 2002/657/EC Decision (2002/657/EC) were calculated. Details about the calculation of the

analytical parameters of the method are reported in Section S2 of the Supporting information., and a summary of the main analytical parameters are presented in Table 2.

3. Results and discussion

3.1. Optimization and analytical performance of the online enrichment

To optimize the enrichment of target compounds into the Hypersil GOLD aQ column different solvents, flow rates and loading times were studied. Based on previous experience, solvents tested were: water, water acidified at different pHs (3.4, 4.5, and 9) and combinations of water:methanol and water:water acidified in different proportions (95:5, 90:10, 80:20, 70:30, 60:40 and 50:50), at different flow rates (1.5, 1.75 and 2 mL/min) and during different loading times (1.5, 2, 3, 3.15 and 3.5 min). The best enrichment rates for most of the compounds were achieved using water acidified with formic acid at pH 4.5 and water at neutral pH (50:50) with a flow rate of 1.75 mL/min during 3.15 min. Elution was accomplished using the chromatographic composition of the mobile phase at initial

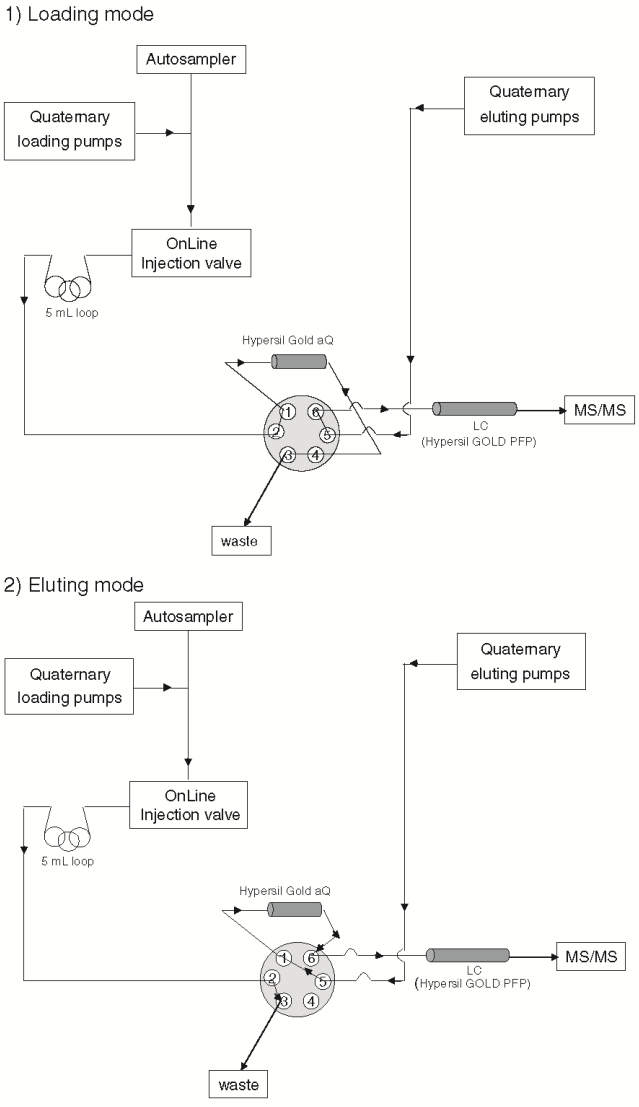

Fig. 2. Scheme of online system: 1) loading mode and 2) eluting mode.

Table 1
Chromatographic mobile phases used for turbulent flow chromatography and liquid chromatography separation.

Time (min:s)	Loading pump				MS valve	Eluting pump			
	Flow (mL/min)	%				Flow (mL/min)	Grad	%	
		(A)	(B)	(C)				(D)	(E)
0:00	1.75	50		50	Loading mode	0.4	Step	90	10
3:25	0.01		100		Eluting mode	0.4	Ramp	90	10
4:55	0.01		100		Eluting mode	0.4	Ramp	20	80
9:55	0.50		100		Loading mode	0.4	Step	10	90
15:25	0.50		100		Loading mode	0.4	Ramp	10	90
16:25	0.50	50		50	Loading mode	0.4	Step	90	10

Loading pump solvents
(A) Water pH 4.5 acidified with formic acid
(B) Methanol
(C) Water.
Eluting pump solvents
(D) Water (20 mM NH_4Ac)
(E) Methanol (20 mM NH_4Ac).

conditions consisting of methanol and ammonium acetate at 20 mM. In order to avoid the possible carry over between samples, the extraction column was cleaned with methanol after every injection. The total run time was 17 min.

The analytical parameters achieved using the online enrichment procedure are reported in Table 2. As can be seen MLOQ were between 2.8 and 50 ng/L for acids, between 0.9 and 82 ng/L for sulfonates and sulfonamides and between 1.2 and 3.3 ng/L for perfluorophosphonic acids. In general, these values were much higher than the limits obtained by the offline approach because of the pre-concentration factor (see Table S4 of the Supporting information). However, the MLOQs achieved by the online procedure were good enough to be used as rapid screening method according to the most restrictive recommendations, and the maximum premised levels established by the Office of Water (OW) from the USEPA for PFOA and PFOS (400 ng/L and 200 ng/L (US Environmental Protection Agency, 2009, 2011a), respectively) and most of the other Health Based Values and/or advisory guidelines for other PFASs: PFBA (1000 ng/L), PFHxS (600 ng/L), PFBS (600 ng/L), PFHxA (1000 ng/L), and PFPeA (1000 ng/L) (Mak et al., 2009; Minnesota Pollution Control Agency, 2007; US Environmental Protection Agency, 2009). In addition, this new method has presented, in general, better MLOQ than those reported by other online SPE approaches (Gosetti et al., 2010). In addition, the new method was proved to be suitable for the analysis of low contaminated water, such as, drinking water. Good level of recoveries and precision was obtained (Table 2). The precision of the online method expressed as intraday and interday reproducibility was, in both cases, below 24% and 28%, respectively.

3.2. Occurrence of PFASs in water

One of the sources of PFASs in drinking water is the contamination of natural waters, however the contamination events that can be occurred during tap water production, the contamination during bottling of mineral water or contamination from bottled materials should be considered. To evaluate the possible contamination of surface waters, which are susceptible to be used for drinking water production by potabilization, different surface samples from Germany to Spain were assessed.

3.2.1. Occurrence of PFASs in German surface waters, water from PWTP and WWTP

The analysis of surface waters from Germany was presented as main compounds PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFDA, PFBS, PFHxS, PFOS, PFHxPA, PFOPA and PFDPA. Table 3 presents a summary of these results. The concentrations of PFASs were in the range between 0.04 and 63 ng/L where the higher concentrations were corresponding to PFBA, PFHpA and PFHxPA. In addition, it should be mentioned that PFHxPA and PFDPA were compounds detected for the first time in surface waters, and PFHpA, the most recalcitrant, was found in the range between 4.0 and 23 ng/L. The presence of PFASs in surface waters was consistent with the concentrations and the profiles found in the WWTP effluents. For example, the effluents from the WWTPs located in Beuerbach, showed a profile of compounds very much similar to those found in surface waters, containing PFBA, PFPeA, PFHxA, PFHpA and PFOA. PFHxPA was confirmed in treated effluents with concentrations ranging between 15 and 35 ng/L, exhibiting the inefficiency on the total removal of PFASs by conventional wastewater treatment processes. Since PFASs are in surface water their occurrence was also expected in PWTPs. Therefore, the presence of the 21 selected compounds through different stages of the process at a German PWTP (samples P1, P2 and P3) was assessed. The first treatment, consisting on sand filtration, showed that it did not remove some compounds (sample P3), such as PFHpA, PFHxPA and PFDPA and in addition the concentrations found were much higher than those in surface water with concentrations of 1589, 53 and 19 ng/L, respectively. This fact indicates a contamination event during the treatment may be due to some pipes into the plant although it was not possible to compare with the concentrations in the influent water to PWTP. After the physical treatment, carbon filtration is applied and a high diminution of the concentration of some compounds was observed (sample P2). After carbon filtration, the concentrations were: PFHpA at 14 ng/L, PFHxPA at 46 ng/L and PFDPA was completely removed. The final treatment is a biological treatment consisting of the residence in wells for 6 weeks. After this treatment (sample P1) and according to PFAS concentrations, the quality of the water was adequate for human consumption, but still with some residual concentrations such as PFHpA (17 ng/L), PFOA (0.62 ng/L) and PFHxPA (27 ng/L). The occurrence of PFHxPA was showed in all the PWTP samples at low ng/L concentration level, being the more frequent compound. In contrast, PFOA was just found in one of the samples at low concentration. Fig. 4 shows the sum of concentrations in German surface water samples. As it was expected the higher concentration of PFASs was found in two of the most industrialized German streams (samples G-S13 and G-S21).

3.2.2. Occurrence of PFASs in Spanish water

Samples from different Spanish locations were assessed. The analysis showed the presence of PFBA (70% of the samples), PFOA (63%) and PFOS (46%), which were the more frequent compounds followed by PFPeA, PFHxA, and PFHpA. Just one of the 24 Spanish surface water samples was found to be free of all the studied PFASs. One of the samples showed an extremely high concentration of PFOS (2709 ng/L). However, this concentration was still 10 times lower than the proposed maximum allowable concentration for PFOS and its derivative salts in inland surfaces (32,000 ng/L), established this year by the European Commission (European Commission, 2012). Fig. 5 presents the accumulate concentrations of PFASs in Spanish surface waters. As can be seen for of the samples, the sum of PFAS concentrations were below 100 ng/L, although in some sites this value was much higher. These sites were corresponding to highly industrialized areas. The results show that the profile of compounds in surface waters was in agreement with those found in the effluents of the WWTP. In surface water, PFBA, PFOS and PFOA were the most frequently found compounds and also those present at higher concentrations. However, it should be remarked that the higher concentrations measured in samples from heavily industrialized areas indicated that the origin of this contamination can be more related with industrial activity, than with urban pollution.

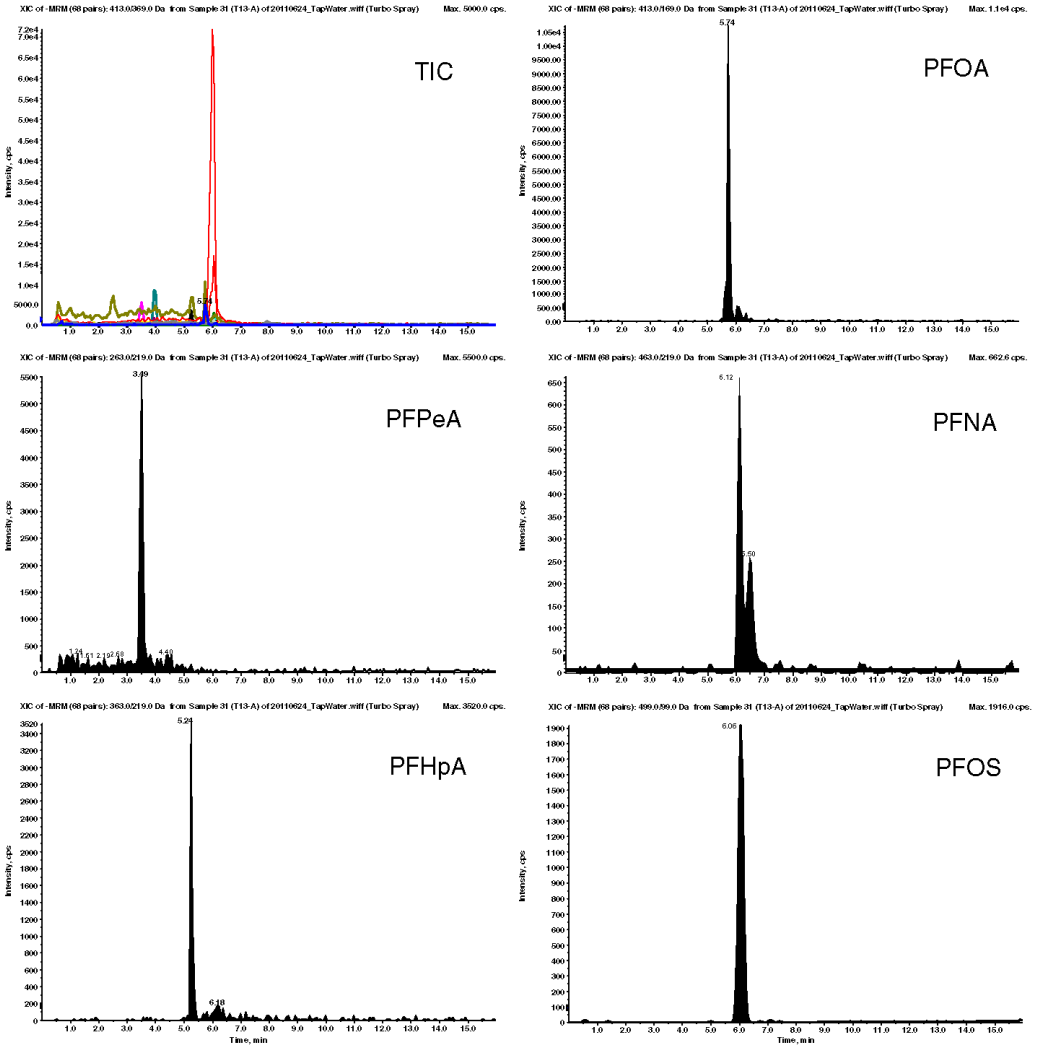

Fig. 3. Chromatogram of drinking tap water sample (T13).

Comparing these results with those from Germany, a different profile was shown. Whereas, in Germany the more frequent analytes were those with a short carbon chain, in Spain the prevalence of C8 compounds (PFOA in 63% and PFOS in 46% of analyzed samples) was shown, as can be seen in Table 3. In contrast to the surface waters from Germany, in Spain PFNA and PFDA were also found (with a maximum concentration of 213 ng/L), and in general trends were more polluted. The main compounds in Spanish surface water were also in agreement with those found into the effluents of WWTPs such as PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFTrA, PFHxS, and PFOS (with a maximum concentration at 501 ng/L). Compared to German effluent waters from WWTP, these last ones presented PFBA, PFPeA, PFHxA, PFHpA, PFOA and FOSA at quantifiable levels but below the Spanish ranges in all the cases, with the exception of PFHpA (Table 3).

3.2.3. Occurrence of PFASs in tap water

In order to assess the presence of PFASs in tap waters, a total number of 75 tap water samples were analyzed, 70 from Spain and 5 from Germany. As can be seen in Table 4, the profiles of compounds found in Spain were somewhat different from those found in German tap water. In Spain a larger number of compounds were found in the samples where the most frequent ones and those at higher concentrations were PFBA (52%), PFPeA (38%), PFOA (37%), PFBS (35%), PFHxS (36%) and PFOS (51%). In the case of German tap waters,

Table 2
Validation parameters.

	MLOD (ng/L)	MLOQ (ng/L)	CCα (ng/L)	CCβ (ng/L)	%Recovery			Precision (%RSD)	
					10 ng/L	50 ng/L	100 ng/L	Intraday (n = 5)	Interday (n = 5)
PFBA	7.8	26	9.4	620	–	83	34	16	25
PFPeA	11	38	13	720	–	42	39	23	25
PFHxA	7.4	50	31	690	–	52	67	21	25
PFHpA	5.1	17	6.8	230	98	104	108	0	22
PFOA	0.83	2.8	0.95	3.3	68	92	104	1	8
PFNA	1.9	6.3	2.0	3.7	51	77	104	5	20
PFDA	2.4	8.0	4.3	28	61	98	109	24	25
PFUdA	1.2	3.9	3.8	9.7	78	64	74	22	25
PFDoA	3.5	12	4.1	16	57	44	51	15	22
PFTrA	10	33	17	18	–	61	57	12	20
PFTeA	3.2	11	16	18	–	46	68	16	26
PFHxDA	1.0	50	25	140	–	68	57	18	26
PFODA	1.0	50	20	40	–	42	61	20	21
PFBS	2.5	8.2	16	27.	73	70	81	9	22
PFHxS	0.27	0.90	0.22	93	61	51	62	5	14
PFOS	0.39	1.3	0.81	2.7	70	111	103	10	26
PFDS	0.35	1.2	0.29	4.5	122	88	105	9	15
FOSA	0.50	1.7	1.5	5.4	101	126	104	12	24
PFHxPA	5.3	18	12	210	–	46	58	16	27
PFOPA	10	33	53	200	–	55	51	14	16
PFDPA	3.5	12	4.0	5.8	–	48	60	23	28

CCα: calculated according to 2002/657/EC guidelines when no reference material exists by the analysis of 20 tap water blanks.
CCβ at 5%.

PFHxA (80%), PFHpA (100%) and PFOA (40%) were the more frequent compounds. PFHpA was found in all the samples (Table 3).

In general, as in the case of surface river water, Spanish concentrations were higher than those found in German samples, being PFOS the most relevant compound (0.19–258 ng/L) with the presence of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFBS and PFHxS (see Table S5). However, PFHpA was more concentrated in German samples (0.23–24 ng/L). T12 and T13 were the most polluted samples with 258 ng/L and 158 ng/L of PFOS, respectively. These samples were from two sites located in the same Spanish municipality. Due to the high concentration levels of PFOS in samples T12 and T13, it was decided to analyze more samples from this municipality on February 2012. The sample codes are facilitated in Table S1 from the Supporting information. Along one week, three different points were analyzed every two days. The results showed the same profile as in samples T12 and T13 (see Table S5 from the Supporting Information). The water is collected in its majority from the Llobregat River, the Ter River and the aquifer. Since the water supplied in Sant Feliu de Llobregat has the same origin than other locations included in this study, showing lower concentrations of PFASs, the contamination in this case can be associated to pipes or the containers used during water transport and storage. Another important factor is the distance from the village. Sample T-SFL 3 was collected from the outside of the city and, even though the samples showed the same profile as the other two sampling sites, the concentration levels were much lower but still higher compared with the rest of Barcelona metropolitan area.

On the other hand, some tap samples from Spain showed PFOPA (1.1–25 ng/L) and PFDPA (8.2–23 ng/L) with these compounds being identified for the first time in tap water. PFDPA was also found in German tap waters as can be seen in Table 4. In general, it was shown that samples from the same area presented similar profile and concentrations, indicating a possible source of contamination during the tap water production into the plant. Detailed information on the concentrations of the samples is given in Table S5 of the Supporting information section.

Two different well waters were also included in this study, from two different sites from the North of Barcelona metropolitan area. PFOA was the only compound found in both samples, at 1.3 and 22 ng/L. The presence of this PFAS could be associated to their occurrence in precipitation waters and the post filtration in soil, in agreement with previous works (De Silva et al., 2009; Gellrich et al., 2011).

3.2.4. Occurrence of PFASs in bottled water

Finally, 6 different commercial brands of bottled mineral water were analyzed. The occurrence of PFASs was found in two of the six brands. The concentration of PFHpA in these samples was 6.6 and 12 ng/L, and one of them contained also PFOS at 1.0 ng/L (Table 4). The positive samples corresponded to the two German mineral waters and could be hypothesized that the origin of PFHpA is the plastic of the bottles, although there is no available data to confirm it. These results were a little bit higher than those reported by a previous publication by Ericson et al. (2008).

3.3. Human exposure associated to drinking water

In January, 2009 the United States Environmental Protection Agency (US Environmental Protection Agency, 2011b) set short-term provisional health advisory-based concentration for PFOA and PFOS of 400 and 200 ng/L (US Environmental Protection Agency, 2009, 2011a), respectively which was determined to be protective in a short-term exposure scenario (acute toxicity). However, there is no available data regarding the continuous exposure to PFAS (chronic toxicity). Later, other health-based values were suggested as for example: for PFHxS at 600 ng/L, for PFBS at 600 ng/L, for PFHxA at 1000 ng/L, and for PFPeA at 1000 ng/L (Minnesota Pollution Control Agency, 2007; US Environmental Protection Agency, 2009). It is also important to note that PHA and HA levels are recommendations only and not regulations and concentrations cannot be enforced by US law. According to these values the majority of the samples may not pose an immediate health risk to consumers, with an exception of 2 of the samples (T12 and T13) where concentrations of PFOS exceeded the Provisional Health Advisory level. However, the long time exposure, which is especially relevant for drinking water should be considered. For example, Post et al. (2009) using a risk assessment approach have set the health-based drinking water concentration protective for lifetime exposure of PFOA at 40 ng/L. This value is 10 times lower than the one for short-term exposure. In a rough approximation, if we consider that long-life exposure values are around 10 times inferior to those for short-time exposure, then for PFOS the value for long-time exposure would be set around

Table 3
Concentration of PFASs in surface river waters and effluent waters from WWTPs from Germany to Spain.

Surface river water															
		PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxS	PFOS	PFOSA
German surface water (n = 24)	Average ng/L	9.2	3.8	5.4	1.5	2.1	<MLOQ	<MLOQ					1.9		2.1
	SD ng/L	7.2	4.0	3.8	6.8	2.2							2.3		2.3
	Median ng/L	7.6	2.5	4.0	1.6	1.3							0.5		1.4
	Maximum ng/L	23	9.4	13	24	6.5							5.6		4.5
	Minimum (MLOQ) ng/L	2.4	0.76	0.23	0.23	0.16	0.03	0.19					0.06		0.04
	% of positive samples	33	17	29	38	29	0	0					21		13
Spanish surface water (n = 24)	Average ng/L	30	3.9	14	12	13	26	46					16		264
	SD ng/L	39	4.9	9.5	8.8	20	24	93					17		812
	Median ng/L	10	2.3	9.7	14	5.3	20	5.1					5.8		5.1
	Maximum ng/L	125	13	31	27	58	52	213					37		2769
	Minimum (MLOQ) ng/L	2.4	0.76	0.23	0.23	0.16	0.03	0.19					0.06		0.04
	% of positive samples	71	21	25	38	83	13	21					21		46

Effluent water from WWTP																
		PFBA	PFPeA	PFOSA	FOSA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxS	PFOS	EOSA	PFHxDA	PFODA	PFDoA
German effluent water (n = 2)	Average ng/L	8.7	0.9	<MLOQ	<MLOQ	1.8	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	5.3	5.3	13
	SD ng/L	1.6														4.8	4.8	10
	Median ng/L	8.7	0.9	0.3	0.3	1.8									0.3	2.9	6.6	13
	Maximum ng/L	9.8	0.9	0.3	0.3	1.8									0.3	6.3	9.4	14
	Minimum (MLOQ) ng/L	3.8	1.8	0.06	0.06	0.04	0.69	1.3	2.4	2.5	2.5	0.13	0.22	0.06	0.06	2.1	1.2	1.7
	% of positive samples	100	50	0	0	50	0	0	0	0	5.1	0	0	0	0	29	8	13
Spanish effluent water (n = 3)	Average ng/L	15	7.1	0.3	0.3	1.7	11	5.3	5.3	5.1	5.1	10	2.14	0.3	0.3	na	na	na
	SD ng/L	14	2.3			0.4	9.4					7.0	252					
	Median ng/L	15	7.1	0.3	0.3	1.7	11	5.3	5.3	5.1	5.1	10	1.15	0.3	0.3			
	Maximum ng/L	25	8.7	0.3	0.3	1.7	18	5.3	5.3	2.4	2.5	15	5.01	0.3	0.3			
	Minimum (MLOQ) ng/L	3.8	1.8	0.06	0.06	0.04	0.69	1.3	2.4	2.5	2.5	0.13	0.22	0.06				
	% of positive samples	67	67	33	67	67	67	33	33	33	33	67	100	33				

na: not analyzed.

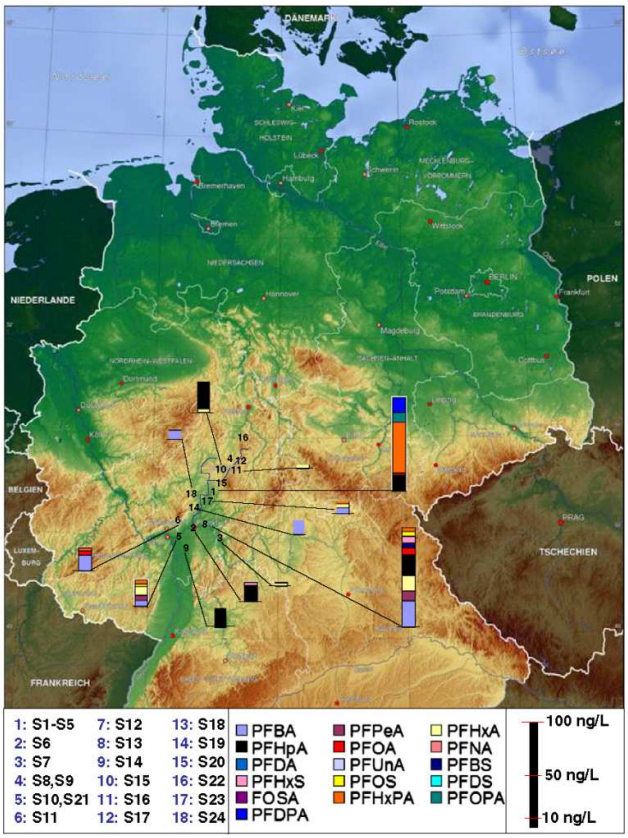

Fig. 4. Results of PFASs in German surface waters. Sum of concentration levels of PFASs at ng/L. In the case of codes 1, 4 and 5 the results corresponded to the average of the sum of concentration levels of PFASs in the samples of these areas.

20 ng/L, and in this study 16 of the samples presented higher concentrations of PFOS. On the other hand it is important to consider the total sum of compounds, the German Drinking Water Commission established the provisional health advisory a value sum of PFOS and PFOA for short time exposure at 300 ng/L, but currently a maximum value of the total sum of PFASs is required for both, long and short-term exposures. Again if we extrapolated the short life exposure values as 10 times lower than those set for long time exposure then the sum of PFOS and PFOA in long life exposure should be set at 30 ng/L and then 11 samples of this study would be considered under risk. Due to the differences in health-based concentrations for humans, further research is highly required for complete risk assessment of human exposure to perfluoroalkyl substances.

4. Conclusions

An online LC–MS/MS method for the analysis of 21 PFASs in different waters has been developed allowing good quality parameters and it can be applied with screening purposes due to the low MLOQ for the wide range of compounds.

Different water matrices including mineral bottled water, tap water, surface river water, and effluent water from WWTP and water from different steps of PWTP have been screened by online LC–MS/MS and then extracted by anionic SPE and analyzed by off-line LC–MS/MS.

The assessment of PFASs in real samples showed that perfluoroalkyl acids in all water types, especially PFBA, PFHpA, and PFOA as well as the sulfonate PFOS in almost all analyzed matrices were the most frequent. The highest concentration levels were found in surface river waters and water from effluent WWTPs with the exceptions of drinking tap water samples from Sant Feliu de Llobregat (T12, T13 and T-SFL 1–3), which presented values higher than 150 ng/L of PFOS. Another interesting result has been found in the effluent of the sand trap (P3) from the PWTP which contained incredible higher levels of PFHpA (1589 ng/L). The presence of PFPAs has been assessed for the first time in Germany and Spain.

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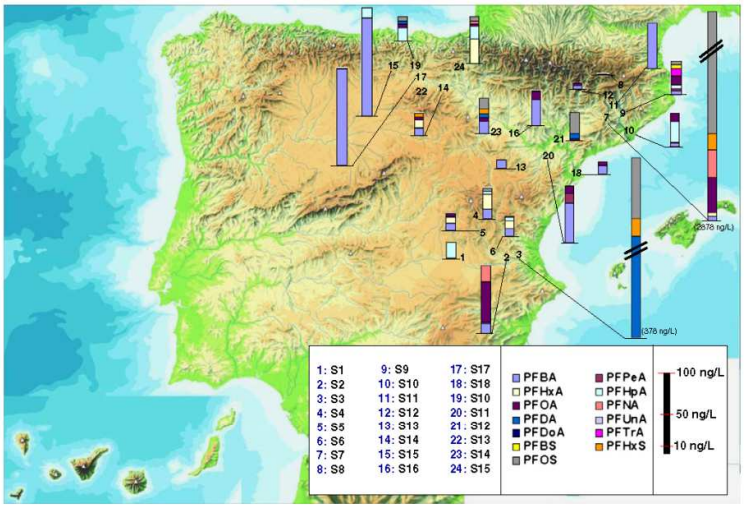

Fig. 5. Results of PFASs in Spanish surface waters. Sum of concentration levels of PFASs at ng/L

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Table 4
Concentration of PFASs in mineral bottled waters and tap waters from Germany to Spain.

Mineral bottled water				
		PFHxA	PFHpA	PFOS
German mineral bottled water (n = 2)	Average ng/L	0.17	12	1.0
	SD ng/L		7.4	
	Median ng/L	0.17	12	1.0
	Maximum ng/L	0.17	17	1.0
	Minimum (MLOQ) ng/L	0.23	0.23	0.04
	% of positive samples	50	100	50
Spanish mineral bottled water (n = 4)	Average ng/L	<MLOQ	<MLOQ	<MLOQ
	SD ng/L			
	Median ng/L			
	Maximum ng/L			
	Minimum (MLOQ) ng/L	0.23	0.23	0.04
	% of positive samples	0	0	0

Tap water													
		PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFBS	PFHxS	PFOS	PFOPA	PFDPA
German tap water (n = 5)	Average ng/L	0.4	1.2	0.9	9.2	1.3	<MLOQ	<MLOQ	<MLOQ	<MLOQ	0.4	<MLOQ	10
	SD ng/L			0.6	8.8	0.9							
	Median ng/L			0.7	7.6	1.3							
	Maximum ng/L	0.4	1.2	1.8	24	1.9					0.4		10
	Minimum (MLOQ) ng/L	2.4	0.76	0.23	0.23	0.16	0.03	0.19	0.11	0.06	0.04	1.2	1.7
	% of positive samples	20	20	80	100	40	0	0	0	0	20	0	20
Spanish tap water (n = 84)	Average ng/L	10	3.8	4.7	8.1	6.7	4.4	2.2	8.3	3.8	46	8	14
	SD ng/L	6.7	4.7	3.8	5.5	8.3	6.5	1.5	9.7	7.2	82	10	6.3
	Median ng/L	10	1.7	3.0	10.8	2.9	0.8	2.3	2.4	0.4	7.0	4.5	11
	Maximum ng/L	27	17	11	16	35	22	4.7	36	28	258	25	23
	Minimum (MLOQ) ng/L	2.4	0.76	0.23	0.23	0.16	0.03	0.19	0.11	0.06	0.04	1.2	1.7
	% of positive samples	52	38	18	13	37	32	8	35	36	51	6	6

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.05.011.

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Supporting Information

Analysis of perfluoroalkyl substances in waters from Germany and Spain

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S1. Off-line solid phase extraction

Off-line sample extraction procedure was based on solid phase extraction (SPE) followed by LC-MS/MS analysis. SPE procedure was based on the adaption of a previous published method by Llorca et al. [34] with anionic exchange cartridges (Oasis WAX 3cc, 60 mg, from Waters). In this case, the method was modified in order to improve the efficiency of PFPAs during SPE. The optimized conditions were: i) conditioning: 2 x 2 mL of methanol, 2 x 2 mL of miliQ water; ii) loading: 150 mL of surface river water and water from WWTP and PWTP (250 mL of bottled water, tap water and well water) at 1 mL/min and dried under N_2; iii) elution: 2 x 2 mL of methanol (1% NH_3) under gravity conditions in a 15 mL PP tube.

Triplicates of a blank of miliQ water (250 mL) were extracted in parallel in order to discriminate any possible cross contamination. All the solvents used during extraction and clean-up procedure were also analyzed.

Extracts were analyzed by LC-(QqQ)-MS/MS. In Spain, the analysis of the extracts was performed with the same LC-MS/MS system exposed before coupled to the on-line enrichment module, but in this case operating off-line. Therefore, the same analytical column and instrumental conditions. For the analysis performed in Germany small modifications were introduced, due to instrumental differences. In the analysis conducted in Germany the chromatographic separation was achieved using an MZ-Aqua Perfect C18 (5µm, 50 x 2.1 mm) column in a HP HPLC chromatograph (Norwalk, CT, USA). The mobile phases composition consisted in (A) Water: Methanol (95:5) 5 mM Ammonium acetate and (B) Water: Methanol (10:90) 5 mM Ammonium acetate. The elution gradient conditions for the LC mobile phase started with 70% of A and it was maintained isocratic during 0.5 min, then it was decreased to 20% during 1.5 min. and raising to 10% in 4 min. more holding for 0.5 min. more. Initial conditions were reached in 1.5 min. and re-equilibration was 6 min. The flow rate was kept at 0.4 mL/min throughout the total chromatographic run of 15 min. The sample injection volume was set at 5 µl. The chromatographic system was coupled to a QqQ mass spectrometer API 2000 (Applied Biosystems, Foster City, CA, USA) using a Turbo Ionspray interface in electrospray negative mode. The whole system was controled by Analyst 1.5 software.

Using the off-line approaches acquisition was performed also in SRM mode according to the Decision 2002/657/EC. The same m/z transitions summarized on table S3 from the Supporting Information were also used for the off-line methods.

When off-line samples are analyzed, an an extra C8 analytical column before injection was used in order to delay PFAS from the system. An example of blank spiked with a mixture of surrogates internal standards can be seen in figure S1.

S2. Quality Assurance and Quality Control

Selectivity For identification purposes, retention times of PFASs in the standards and in the samples were compared at a tolerance of ±2.5%. Moreover, in accordance with the 2002/657EC Decision, the relative ion intensities (each product ion area signal versus the base product ion area signal) of the spiked samples were compared with the relative ion intensities of PFASs standard solutions, at the same concentration levels as used for the construction of the calibration curve.

Limits of detection, limits of quantification and decision limits The values of instrumental limits of detection (ILOD) and the instrumental limits of quantification (ILOQ) were obtained by injection of standard solutions in a mixture water and methanol with the same pH and composition of the mobile phase at initial conditions of the chromatographic separation. ILOD were determined at the minimum detectable amount of each compound with a signal-to-noise ratio of 3. Criteria for ILOQ was established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable, and (4) signal-to-noise ratio at least 8.

Recoveries, linearity range, precision, accuracy, method limit of detection (MLOD) and method limits of quantification (MLOQ) were performed by spiking experiments using fortified blank samples at 6, 12 and 24 ng/L consisting in ultrapure water, tap water and treated wastewater. MLOD were calculated as the minimum concentration of target compounds that can be measured according to previous criteria for ILOQ, analysing the blank samples fortified in decreasing concentrations.

Decision limit (CCα) and detection capability (CCβ) were evaluated in drinking water by off line and on line methods according to the 2002/657/EC Decision (2002/657/EC). CCα was calculated by analysing 20 blank samples and measuring the signal to noise ratio at the time window in which the analyte would be expected in case to be present. Three times the signal to noise ratio was used as the decision limit with a 5% certainty. CCβ values were obtained from the analysis of 20 blank samples fortified at 6 ng/L in off line methodology and at 10 ng/L in on line methodology (near to CCα concentration for all the analytes). The corresponding concentration at the CCα value plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability (β = 5 %).

Linearity The range of linearity was defined by plotting the peak area ratio of the PFASs to the IS versus PFC concentration. The following criteria for linearity range were applied: linear regression with a correlation coefficient better than 0.990, and RSD of four replicates less than 25%. In the present approach, calibration curves presented good linearity ranges and correlation coefficients (R^2) higher than 0.9994 in all cases, and were used for confirmatory purposes. Quantification was performed by internal standard addition.

According to the 2002/657/EC Decision, since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified samples was measured as an alternative to trueness. Each type of blank sample was spiked in quintuplicate as previously described with the PFASs at three levels of concentration.

Recoveries and Precision Recoveries and precision were calculated according to the 2002/657/EC Decision (2002/657/EC). MiliQ water (250 mL), tap water (250 mL) and effluent water (150 mL) were spiked in quintuplicate with the PFASs at three levels of concentration (6, 12 and 24 ng/L) with the mixture of standards in methanol and mixed in an orbital digester 30 min in order to ensure the appropriate distribution in the matrix for the off line extraction. Then, the samples were processed as has been reported before. For the assessment of all the mentioned parameters, the analyte response was always related to the internal standard response (labelled PFASs and Cl-PFHxPA added just before injection at 5 μg/L (final concentration)) to compensate undesirable matrix effects. In the case of on-line enrichment, recoveries were calculated by spiking 5 mL of

blank tap water at three different levels (10, 50 and 100 ng/L); the extraction was carried out as has been described before. Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets at the same spiking levels during the same day (intraday repeatability) and different consecutive days (interday repeatability).

Table S1: Sampling sites and codes of mineral bottled water and tap water.

Water Type	Origin		Code
Mineral Bottled water	Spain	(2011)	B 1
		(2011)	B 2
		(2011)	B 3
		(2011)	B 4
	Germany	(2011)	B 5
		(2011)	B 6
Potable Tap Water	Barcelona metropolitan area (Spain)	Barcelona city (2011)	T 1
		Barcelona city (2011)	T 2
		Barcelona city (2011)	T 3
		Barcelona city (2011)	T 4
		Barcelona city (2011)	T 5
		Barcelona city (2011)	T 6
		Barcelona city (2011)	T 7
		Barcelona city (2011)	T 8
		Barcelona city (2011)	T 9
		Canyelles (2011)	T 10
		Hostalets de Pierola (2011)	T 11
		Sant Feliu de Llobregat 1 (2011)*	T 12
		Sant Feliu de Llobregat 2 (2011)*	T 13
		Badalona 1 (2011)	T 14
		Mataró 1 (2011)	T 15
		Mataró 2 (2011)	T 16
		Badalona 2 (2011)	T 17
	Ebro River Basin (Spain)	Miranda de Ebro (2010)	T 18
		Logroño (2010)	T 19
		Pamplona (2010)	T 20
		Zaragoza (2010)	T 21
		Lleida (2010)	T 22
		Tortosa (2010)	T 23
		Logroño 1 (2011)	T 24
		Logroño 2 (2011)	T 25
		Lleida 1 (2011)	T 26
		Lleida 2 (2011)	T 27
		Tortosa 1 (2011)	T 28
		Tortosa 2 (2011)	T 29
		Zaragoza 1 (2011)	T 30
		Zaragoza 2 (2011)	T 31
		Pamplona 1 (2011)	T 32
		Pamplona 2 (2011)	T 33
		Miranda de Ebro 1 (2011)	T 34
		Miranda de Ebro 2 (2011)	T 35
	Llobregat River Basin (Spain)	Igualada 1 (2011)	T 36
		Igualada 2 (2011)	T 37
		Manresa 1 (2011)	T 38
		Manresa 2 (2011)	T 39
		Martorell 1 (2011)	T 40
		Martorell 2 (2011)	T 41

Water Type	Origin		Code
Potable Tap Water	Xúquer River Basin (Spain)	Port de Sagunt (2011)	T 42
		Sueca 1 (2011)	T 43
		Sueca 2 (2011)	T 44
		Sueca Sales (2011)	T 45
		Alcira 1 (2011)	T 46
		Alcira 2 (2011)	T 47
		Naquera Moncada (2011)	T 48
		Gilet (2011)	T 49
		Requena Norte (2011)	T 50
		Requena Sur (2011)	T 51
		Cuenca Sur (2011)	T 52
		Cuenca Norte (2011)	T 53
	Valencia Metropolitan area (Spain)	Valencia city (2011)	T 54
		Valencia city (2011)	T 55
		Valencia city (2011)	T 56
		Valencia city (2011)	T 57
		Valencia city (2011)	T 58
	Guadalquivir River Basin (Spain)	Linares 1 (2011)	T 59
		Linares 2 (2011)	T 60
		Sanlúcar de Barrameda 1 (2011)	T 61
		Sanlúcar de Barrameda 2 (2011)	T 62
		Andújar 1 (2011)	T 63
		Andújar 2 (2011)	T 64
		Écija 1 (2011)	T 65
		Écija 2 (2011)	T 66
		Sevilla 1 (2011)	T 67
		Sevilla 2 (2011)	T 68
		Córdoba 1 (2011)	T 69
		Córdoba 2 (2011)	T 70
	Hesse (Germany)	Idstein (2011)	T 71
		Frankfurt 1 (2011)	T 72
		Frankfurt 2 (2011)	T 73
		Frankfurt 3 (2011)	T 74
		Wiesbaden (2011)	T 75

Water Type and Origin		Code
Tap water from Sant Feliu de Llobregat (Spain) *	Sant Feliu de Llobregat 1 (2/6/2012)	T-SFL 1A
	Sant Feliu de Llobregat 1 (2/8/2012)	T-SFL 1B
	Sant Feliu de Llobregat 1 (2/10/2012)	T-SFL 1C
	Sant Feliu de Llobregat 2 (2/6/2012)	T-SFL 2A
	Sant Feliu de Llobregat 2 (2/8/2012)	T-SFL 2B
	Sant Feliu de Llobregat 2 (2/10/2012)	T-SFL 2C
	Sant Feliu de Llobregat 3 (2/6/2012)	T-SFL 3A
	Sant Feliu de Llobregat 3 (2/8/2012)	T-SFL 3B
	Sant Feliu de Llobregat 3 (2/10/2012)	T-SFL 3C

* Tap water from Sant Feliu de Llobregat was analyzed from three different points every two days along one week. Sample codes are summarized in the table above, on the right side.

Table S2: sampling sites and codes

Water Type	Origin		Code
Water from PWTP	Wiesbaden, Hesse (Germany)	potable effluent water (2011)	P 1
		charcoal treatment effluent (2011)	P 2
		sand trap effluent (2011)	P 3
Effluent Water from WWTP	Hesse (Germany)	Beuerbach 1 (2011)	G-E 1
		Beuerbach 2 (2011)	G-E 2
German Surface River Water (Hesse, Germany) *	Small stream, agricultural contribution	27.12.2010 - 03.01.2011	G-S1
		13.12.2010 - 20.12.2010	G-S2
		21.11.2010 - 29.11.2010	G-S3
		01.11.2010 - 08.11.2010	G-S4
		06.09.2010 - 13.09.2010	G-S5
	Agricultural influenced stream (2011)		G-S6
	Agricultural influenced stream (2011)		G-S7
	Agricultural influenced stream (2011)		G-S8
	Agricultural influenced stream (2011)		G-S9
	Agricultural influenced stream (2011)		G-S10
	Agricultural influenced stream (2011)		G-S11
	Agricultural influenced stream (2011)		G-S12
	Industrial influenced stream (2011)		G-S13
	Agricultural influenced stream (2011)		G-S14
	Agricultural influenced stream (2011)		G-S15
	Agricultural influenced stream (2011)		G-S16
	Agricultural influenced stream (2011)		G-S17
	Agricultural influenced stream (2011)		G-S18
	Agricultural influenced stream (2011)		G-S19
	Agricultural influenced stream (2011)		G-S20
	Industrial influenced stream (2011)		G-S21
	Agricultural influenced stream (2011)		G-S22
	Agricultural influenced stream (2011)		G-S23
	Agricultural influenced stream (2011)		G-S24

Water Type	Origin		Code
Effluent Water from WWTP	Valencia (Spain)	Alberique (Alcira) (2011)	S-E 1
	Catalonia (Spain)	Igualada (2011)	S-E 2
	Navarra (Spain)	Pamplona (2011)	S-E 3
Well Water	Barcelona metropolitan area (Spain)	Mataró 1 (2011)	W 1
		Mataró 2 (2011)	W 2
Spanish Surface River Water	Xúquer River Basin (Spain)	Xúquer river 1 (2011)	S-S1
		Xúquer river 2 (2011)	S-S2
		Xúquer river 3 (2011)	S-S3
		Cabriel river 1 (2011)	S-S4
		Cabriel river 2 (2011)	S-S5
		Magro river (2011)	S-S6
	Llobregat River Basin (Spain)	Anoia river (2011)	S-S7
		Llobregat river 1 (2011)	S-S8
		Llobregat river 2 (2011)	S-S9
		Llobregat river 3 (2011)	S-S10
		Cardener river 1 (2011)	S-S11
		Cardener river 2 (2011)	S-S12
		Martin river (2011)	S-S13
	Ebro River Basin (Spain)	Ebro river 1 (2011)	S-S14
		Ebro river 2 (2011)	S-S15
		Ebro river 3 (2011)	S-S16
		Ebro river 4 (2011)	S-S17
		Ebro river 5 (2011)	S-S18
		Zadorra river (2011)	S-S19
		Matarranya river (2011)	S-S20
		Segre river (2011)	S-S21
		Najerilla river (2011)	S-S22
		Huerva river (2011)	S-S23
		Arga river (2011)	S-S24

* The sampling points can be seen in figure 1A

Table S3: Mass / charge transitions

Analyte	m/z		
	Precursor ion	1st transition	2nd transition
PFBA	213	169	119
PFPeA	263	219	69
PFHxA	313	269	169
PFHpA	363	319	169
PFOA	413	369	169
PFNA	463	219	169
PFDA	513	469	169
PFUdA	563	519	169
PFDoA	613	569	169
PFTeDA	713	669	169
PFHxDA	813	769	169
PFODA	913	869	169
PFBS	299	80	99
PFHxS	399	80	99
PFOS	499	80	99
PFDS	599	80	99
PFOSA	498	78	498
PFHxPA	399	79	399
PFOPA	499	79	499
PFDPA	599	79	599

1st transition: quantification ion
2nd transition: confirmation ion

Table S4: Off-line quality parameters

	MilliQ water									Tap water									Effluent water						
	MLOD (ng/L)	MLOQ (ng/L)	CCα (ng/L)	CCβ* (ng/L)	% Recoveries			Precision (%RSD)		MLOD (ng/L)	MLOQ (ng/L)	CCα (ng/L)	CCβ* (ng/L)	% Recoveries			Precision (%RSD)		MLOD (ng/L)	MLOQ (ng/L)	% Recoveries			Precision (%RSD)	
					6 ng/L	12 ng/L	24 ng/L	Intraday (n=5)	Interday (n=5)					6 ng/L	12 ng/L	24 ng/L	Intraday (n=5)	Interday (n=5)			6 ng/L	12 ng/L	24 ng/L	Intraday (n=5)	Interday (n=5)
PFBA	0.5	1.7	1.3	1.37	57	82	93	5	17	0.7	2.4	2.6	3.4	50	76	119	3	34	1.1	3.8	148	65	123	25	28
PFPeA	0.21	0.7	0.6	0.66	59	64	70	10	15	0.29	0.76	1.1	1.5	63	70	61	17	21	0.55	1.8	97	110	100	14	17
PFHxA	0.07	0.2	0.14	0.15	90	91	89	7	10	0.07	0.23	0.23	0.31	87	113	98	9	4	1.2	4.1	158	97	83	10	15
PFHpA	0.05	0.2	0.17	0.19	97	90	100	12	14	0.07	0.23	0.23	0.26	105	102	121	14	22	0.21	0.71	99	80	86	17	22
PFOA	0.03	0.1	0.11	0.11	103	97	94	3	7	0.05	0.16	0.21	0.3	108	114	119	4	12	0.01	0.04	77	93	118	7	23
PFNA	0.01	0.03	0.05	0.06	98	95	91	10	12	0.01	0.03	0.12	0.13	110	122	120	8	26	0.03	0.09	81	76	96	13	20
PFDA	0.04	0.13	0.1	0.11	90	93	118	11	18	0.16	0.19	0.19	0.21	88	108	103	17	9	0.4	1.3	87	53	77	8	26
PFUnA	0.28	0.9	0.87	1.02	53	64	79	17	20	0.3	1	1.4	1.7	50	76	84	15	25	0.71	2.4	99	88	88	13	30
PFDoA	0.3	1.0	0.51	0.55	105	113	110	7	11	0.47	1.6	0.51	0.54	120	85	90	4	17	0.99	3.3	82	67	68	24	35
PFTrA	0.6	2.0	1.8	1.89	81	94	99	5	9	0.64	2.1	2.2	3.2	85	66	87	3	12	0.75	2.5	58	55	57	27	30
PFTeA	0.5	1.7	2	2.16	95	89	97	8	13	0.8	2.7	3.8	5.8	116	101	117	2	15	1.1	3.5	55	44	44	15	28
PFHxDA	1.5	5.0	4.2	4.37	59	61	73	4	10	1.7	5.7	4.9	5.3	52	45	80	5	7	1.8	6	57	44	47	14	23
PFODA	1.4	4.7	4	4.52	51	55	59	13	15	1	3.3	4.8	4.9	54	43	62	2	7	1.4	4.6	43	58	49	25	36
PFBS	0.02	0.1	0.81	0.88	99	104	96	9	14	0.03	0.11	1	1.1	115	98	115	15	17	2.7	8.9	117	78	86	6	25
PFHxS	0.02	0.1	0.02	0.02	91	92	87	2	8	0.02	0.06	0.02	0.04	117	115	109	9	10	0.04	0.13	115	79	80	16	17
PFOS	0.005	0.017	0.13	0.14	101	99	91	4	6	0.01	0.05	0.79	0.82	112	124	122	7	13	0.07	0.22	107	68	88	5	8
PFDS	0.34	1.1	1.5	1.61	83	76	86	7	12	0.4	1.3	1.9	2.9	88	88	99	12	25	0.57	2.2	65	56	59	18	31
PFOSA	0.017	0.057	0.73	0.78	50	95	90	7	16	0.02	0.07	0.81	0.82	64	89	86	9	24	0.02	0.06	116	75	94	17	21
PFHxPA	0.51	1.7	0.66	0.69	79	74	84	5	7	0.64	2.1	0.58	1.4	82	63	81	3	8	0.46	1.5	106	110	82	3	13
PFOPA	0.29	1.0	0.54	0.59	54	83	79	9	14	0.35	1.2	0.54	1.1	76	80	88	6	12	0.44	1.5	76	65	61	11	22
PFDPA	0.49	1.6	0.79	0.81	56	70	80	3	8	0.5	1.7	0.7	1.1	124	58	100	1	4	0.83	2.8	107	76	56	5	12

* CCβ by spiking 20 blanks at 6 ng/L

Table S5: Results in all the samples analysed in this study.

	Concentration, ng/L (%RSD [n=3])																					ΣPFASs (ng/L)
Code	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA	PFHxPA	PFOPA	PFDPA	
Blank	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
B 1	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
B 2	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
B 3	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
B 4	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
B 5	<MLOQ	<MLOQ	<MLOQ	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOE	<MLOD	<MLOQ	<MLOD	<MLOQ	<MLOQ	1.02 (10)	<MLOQ	<MLOQ	<MLOD	<MLOE	<MLOQ	1.8
B 6	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOE	<MLOE	<MLOQ	6.8
T 1	<MLOQ	<MLOQ	<MLOQ	<MLOC	<MLOQ	0.5 (9)	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOD	12 (10)	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	13
T 2	<MLOQ	<MLOQ	<MLOQ	<MLOE	5.1 (2)	0.3 (3)	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	14 (6)	<MLOD	<MLOD	<MLOE	4.5 (5)	8.5 (2)	32
T 3	18 (8)	2.4 (2)	<MLOQ	<MLOE	4.6 (11)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	7.8 (14)	<MLOQ	18 (5)	<MLOD	<MLOD	<MLOD	<MLOQ	8.2 (4)	59
T 4	<MLOQ	<MLOD	3.0 (17)	1.6 (7)	2.9 (6)	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	7.5
T 5	6.7 (9)	<MLOQ	<MLOQ	<MLOE	2.7 (4)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	11 (3)	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	20
T 6	9.8 (6)	<MLOQ	<MLOQ	<MLOC	5.4 (14)	1.0 (16)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	8.8 (6)	0.10 (5)	25 (2)	<MLOD	<MLOD	<MLOD	9.0 (4)	<MLOQ	59
T 7	<MLOQ	<MLOD	<MLOQ	<MLOE	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOE	<MLOQ	0.0
T 8	16 (2)	0.7 (2)	<MLOD	<MLOD	3.9 (8)	0.3 (2)	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	3.1 (11)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	1.1 (3)	<MLOQ	25
T 9	1.7 (1)	3.8 (7)	<MLOQ	<MLOQ	5.4 (8)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	24 (3)	0.80 (11)	7.5 (13)	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	43
T 10	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	0.0
T 11	5.0 (8)	<MLOD	<MLOD	<MLOE	0.3 (6)	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	5.3
T 12	9.4 (13)	<MLOQ	<MLOQ	<MLOC	29 (12)	7.3 (6)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	36 (10)	<MLOQ	258 (8)	<MLOD	<MLOD	<MLOD	25 (8)	23 (7)	338
T 13	<MLOQ	17 (28)	<MLOQ	10 (10)	35 (14)	7.7 (8)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	158 (9)	<MLOD	<MLOD	<MLOD	<MLOQ	1e (3)	243
T-SFL 1A	12 (5)	12 (3)	6.0 (5)	11 (7)	14 (5)	11 (5)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	20 (2)	3.1 (13)	190 (7)	<MLOD	1.6 (7)	<MLOD	<MLOE	<MLOD	231
T-SFL 1B	15 (4)	7.3 (12)	10 (15)	16 (15)	14 (21)	13 (10)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	19 (4)	4.1 (4)	219 (12)	<MLOD	2.4 (15)	<MLOD	<MLOD	<MLOD	319
T-SFL 1C	16 (13)	8.1 (14)	9.3 (3)	12 (12)	15 (15)	17 (6)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	18 (6)	4.0 (12)	234 (6)	<MLOD	1.2 (16)	<MLOD	<MLOD	<MLOD	334
T-SFL 2A	16 (7)	12 (13)	8.2 (10)	12 (8)	15 (5)	18 (3)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	22 (13)	4.4 (15)	199 (10)	<MLOD	4.0 (9)	<MLOD	<MLOE	<MLOD	310
T-SFL 2B	14 (16)	12 (10)	11 (14)	11 (10)	12 (10)	11 (9)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	20 (4)	4.0 (7)	209 (10)	<MLOD	3.5 (4)	<MLOE	<MLOE	<MLOD	338
T-SFL 2C	18 (13)	16 (13)	8.9 (13)	12 (8)	12 (15)	22 (12)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	20 (8)	4.4 (15)	240 (10)	<MLOD	2.7 (15)	<MLOD	<MLOD	<MLOD	356
T-SFL 3A	4.8 (7)	1.9 (8)	2.2 (16)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	8.9 (3)	0.23 (10)	23 (12)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	41
T-SFL 3B	4.3 (11)	1.5 (7)	2.0 (3)	1.2 (12)	1.4 (9)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	10 (5)	0.58 (7)	27 (3)	<MLOD	<MLOQ	<MLOE	<MLOD	<MLOD	48
T-SFL 3C	3.6 (5)	2.6 (10)	2.7 (11)	2.2 (14)	1.1 (5)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	11 (10)	0.63 (9)	27 (15)	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	50
T 14	16 (12)	<MLOD	<MLOQ	<MLOQ	1.1 (1)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	0.20 (7)	20 (7)	<MLOD	<MLOE	<MLOE	<MLOQ	11 (1)	49
T 15	19 (7)	<MLOD	<MLOQ	<MLOQ	1.4 (7)	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	1.4 (12)	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	22
T 16	<MLOQ	<MLOD	<MLOQ	<MLOQ	2.9 (4)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	0.10 (1)	2.2 (4)	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	5.2
T 17	<MLOD	<MLOQ	<MLOQ	<MLOE	1.8 (11)	<MLOD	4.7 (14)	3.9 (3)	<MLOD	<MLOE	<MLOD	<MLOQ	<MLOQ	1.5 (3)	<MLOQ	3.7 (7)	<MLOD	0.20 (5)	na	na	na	16
T 18	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	0.46 (9)	2.6 (4)	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOD	0.43 (5)	0.05 (5)	0.45 (14)	<MLOD	<MLOQ	na	na	na	4.0
T 19	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	0.18 (11)	0.17 (12)	0.49 (5)	<MLOD	<MLOQ	na	na	na	0.8
T 20	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	3.0 (7)	2.7 (18)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	0.13 (3)	0.12 (8)	0.26 (3)	<MLOD	0.45 (12)	na	na	na	6.7

		Concentration, ng/L (%RSD [n=3])																				ΣPFASs (ng/L)
Code	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA	PFHxPA	PFOPA	PFDPA	
T 21	< MLOD	< MLOD	2.9 (9)	< MLOD	< MLOD	0.37 (5)	< MLOQ	1.1 (6)	2.1 (12)	< MLOQ	< MLOD	< MLOD	< MLOD	0.20 (15)	0.18 (11)	0.30 (19)	< MLOQ	< MLOQ	na	na	na	7.4
T 22	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	2.3 (6)	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	0.13 (8)	0.07 (14)	0.20 (10)	< MLOD	< MLOQ	na	na	na	2.8
T 23	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	0.43 (7)	1.7 (9)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	0.24 (4)	0.56 (11)	1.3 (2)	< MLOD	< MLOQ	na	na	na	4.3
T 24	6.2 (13)	0.37 (24)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	6.8
T 25	5.8 (3)	0.46 (16)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	6.5
T 26	2.8 (4)	0.91 (28)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	3.7
T 27	< MLOQ	0.54 (30)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	0.5
T 28	< MLOQ	0.63 (28)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.16 (9)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	0.6
T 29	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	0.0
T 30	8.9 (13)	1.5 (11)	< MLOQ	< MLOQ	0.49 (13)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	1.1
T 31	10 (3)	1.5 (16)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	1.2
T 32	3.4 (10)	0.51 (5)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	3.9
T 33	3.3 (6)	0.67 (15)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	4.0
T 34	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.0
T 35	< MLOQ	0.53 (19)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	3.5 (6)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	4.0
T 36	0.94 (5)	1.9 (10)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	2.8
T 37	1.1 (6)	2.1 (18)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	3.9 (6)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	7.1
T 38	12 (7)	3.2 (5)	< MLOQ	< MLOQ	0.48 (16)	0.45 (1)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.19 (5)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	16
T 39	27 (3)	2.5 (17)	< MLOQ	< MLOQ	< MLOQ	0.60 (4)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	4.2 (3)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	34
T 40	6.4 (5)	1.3 (18)	0.46 (14)	< MLOQ	1.9 (3)	0.54 (7)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	1.0 (5)	0.09 (15)	0.24 (6)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	12
T 41	4.6 (14)	2.0 (13)	< MLOQ	< MLOQ	< MLOQ	0.75 (8)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	5.6 (15)	< MLOQ	< MLOD	0.97 (6)	0.16 (3)	0.20 (10)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	14
T 42	10 (15)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.36 (12)	0.36 (13)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.07 (4)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	11
T 43	1.3 (12)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	1.4 (13)	11 (7)	4.7 (3)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	18
T 44	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	1.3 (5)	9.1 (8)	6.6 (4)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	17
T 45	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	1.2 (13)	8.2 (15)	7.0 (8)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	16
T 46	1.9 (10)	1.0 (19)	1.5 (4)	< MLOQ	7.6 (6)	0.70 (12)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	2.4 (9)	28 (8)	22 (11)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	65
T 47	11 (7)	1.1 (13)	1.7 (11)	0.55 (8)	6.8 (8)	0.62 (5)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	2.1 (10)	28 (1)	23 (10)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	79
T 48	0.80 (8)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.72 (12)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	1.5
T 49	15 (13)	0.56 (6)	0.58 (9)	< MLOQ	2.0 (3)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	1.3 (4)	0.20 (13)	7.9 (3)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	28
T 50	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.0
T 51	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.0
T 52	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	0.0
T 53	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	0.0
T 54	15 (5)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	1.5
T 55	25 (16)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.72 (14)	0.72 (14)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	2.6
T 56	22 (13)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	2.2
T 57	16 (3)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	1.6
T 58	12 (5)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	1.2

	Concentration, ng/L (%RSD [n=3])																				ΣPFASs (ng/L)
Code	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA	PFHxPA	PFOPA	PFDPA	
T 59	12 (7)	< MLOQ	< MLOQ	< MLOQ	0.78 (8)	1.1 (16)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	0.25 (2)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	1.4
T 60	11 (9)	< MLOQ	< MLOQ	< MLOQ	0.21 (4)	0.36 (9)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	0.24 (10)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	1.2
T 61	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.0
T 62	< MLOQ	< MLOQ	< MLOQ	< MLOQ	2.1 (12)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	2.1
T 63	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	1.2 (6)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	1.1 (3)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	2.3
T 64	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	1.1 (13)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	1.5 (7)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	2.6
T 65	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	0.22 (10)	< MLOQ	< MLOQ	< MLOQ	2.4 (7)	< MLOQ	2.6
T 66	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.72 (15)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.72
T 67	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.70 (8)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.70
T 68	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.0
T 69	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.0
T 70	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.0
T 71	0.40 (8)	1.8 (1	24 (9)	1.9 (8)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	10 (3)
T 72	< MLOQ	< MLOQ	0.62 (13)	5.7 (15)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	6.3
T 73	< MLOQ	< MLOQ	0.71 (3)	8.1 (9)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	8.8
T 74	< MLOQ	< MLOQ	0.35 (10)	7.6 (12)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	8.0
T 75	< MLOQ	< MLOQ	< MLOQ	0.62 (12)	0.64 (2)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	0.44 (10)	< MLOQ	< MLOQ	na	na	na	1.7

Code	Concentration, ng/L (%RSD [n=3])																				EPF ASs (ng/L)
	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA	PFHxPA	PFOPA	PFDPA	
Blank	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOE	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOE	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOC	< MLOC	0.0
W1	< MLOD	< MLOQ	< MLOQ	< MLOD	1.3 (1)	< MLOE	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOE	0.50 (11)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOC	< MLOC	1.8
W2	1.8 (5)	< MLOQ	< MLOQ	< MLOQ	22 (18)	< MLOE	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOE	< MLOQ	< MLOQ	9.0 (12)	< MLOD	< MLOD	< MLOC	< MLOC	33
S-S1	<MLOD	<MLOD	<MLOD	2.0 (12)	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	90
S-S2	12 (10)	2.3 (24)	<MLOD	<MLOD	2.0 (8)	2.0 (16)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	87
S-S3	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	2.3 (7)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	37 (12)	128 (15)	<MLOD	na	na	na	378
S-S4	13 (8)	<MLOQ	19 (23)	4.1 (15)	<MLOQ	<MLOQ	<MLOD	0.6 (19)	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	3.9 (9)	<MLOD	<MLOD	na	na	na	40
S-S5	9.5 (5)	<MLOQ	7.2 (18)	2.4 (14)	2.7 (15)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	23
S-S6	9.7 (11)	<MLOQ	9.8 (14)	4.2 (12)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	2.0 (11)	<MLOD	<MLOD	na	na	na	26
S-S7	8.7 (10)	<MLOQ	7.2 (16)	<MLOD	68 (7)	52 (6)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	33 (12)	2708 (6)	<MLOD	na	na	na	2678
S-S8	<MLOD	<MLOD	<MLOD	<MLOD	1.2 (13)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	12
S-S9	2.5 (22)	<MLOD	5.1 (18)	1.0 (15)	<MLOE	1.3 (18)	<MLOD	<MLOD	9.8 (10)	<MLOD	<MLOD	4.3 (19)	<MLOD	4 : (21)	<MLOD	5.1 (17)	<MLOD	<MLOD	na	na	na	44
S-S10	5.1 (16)	1.0 (18)	<MLOD	27 (9)	10 (12)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	44
S-S11	59 (18)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOQ	<MLOD	na	na	na	59
S-S12	5.3 (17)	<MLOQ	<MLOD	<MLOD	2.5 (20)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOQ	<MLOD	na	na	na	7.8
S-S13	10 (10)	<MLOQ	<MLOD	<MLOD	4.1 (17)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	4.5 (17)	<MLOD	<MLOD	na	na	na	11
S-S14	9.8 (11)	1.0 (14)	<MLOD	<MLOD	14 (12)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	29
S-S15	125 (12)	<MLOD	<MLOD	<MLOD	14 (12)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	139
S-S16	24 (29)	<MLOD	<MLOD	<MLOD	11 (14)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	1.1 (22)	<MLOD	<MLOD	na	na	na	46
S-S17	125 (10)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	0.6 (29)	<MLOD	na	na	na	126
S-S18	10 (12)	<MLOQ	<MLOD	<MLOD	5.3 (12)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	15
S-S19	<MLOD	<MLOD	<MLOD	17 (11)	5.1 (16)	<MLOD	4.5 (17)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	4.5 (18)	<MLOD	na	na	na	31
S-S20	51 (25)	13 (12)	<MLOD	<MLOD	11 (14)	<MLOE	6.5 (16)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	74
S-S21	<MLOD	<MLOD	<MLOD	<MLOD	2.0 (16)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	27 (14)	<MLOD	na	na	na	36
S-S22	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	na	na	na	0.0
S-S23	16 (12)	<MLOQ	<MLOD	<MLOD	5.3 (41)	<MLOE	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	5.8 (17)	13 (16)	<MLOD	na	na	na	45
S-S24	<MLOD	<MLOQ	31 (11)	17 (15)	2.5 (12)	4.8 (14)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOD	<MLOD	6.1 (24)	<MLOD	na	na	na	62
G-S1	2.0 (22)	<MLOQ	<MLOQ	7.0 (20)	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	63 (21)	<MLOD	14 (25)	86
G-S2	<MLOQ	<MLOQ	<MLOQ	24 (4)	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	6.6 (5)	<MLOC	31
G-S3	<MLOQ	<MLOQ	<MLOQ	4.0 (5)	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	51 (8)	<MLOD	<MLOC	55
G-S4	<MLOQ	0.67 (24)	<MLOQ	15 (35)	0.49 (30)	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	9.4 (6)	12 (21)	39
G-S5	0.89 (17)	1.3 (28)	<MLOQ	<MLOQ	2.0 (18)	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOQ	0.26 (6)	<MLOQ	23 (8)	<MLOD	13 (2)	40
G-S6	<MLOD	<MLOQ	<MLOQ	13 (13)	0.74 (23)	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOE	<MLOQ	2.9 (4)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOC	17
G-S7	<MLOQ	<MLOQ	2.9 (15)	<MLOQ	1.3 (15)	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOE	<MLOQ	0.22 (10)	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOC	4.5
G-S8	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOC	0.0
G-S9	<MLOQ	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOD	0.0
G-S10	<MLOD	<MLOQ	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOE	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOD	<MLOC	0.0
G-S11	34 (10)	<MLOQ	<MLOQ	<MLOQ	3.4 (10)	<MLOQ	<MLOQ	<MLOD	<MLOD	<MLOQ	<MLOD	<MLOD	<MLOD	<MLOE	<MLOQ	0.36 (29)	<MLOQ	<MLOQ	2.5 (20)	<MLOD	<MLOC	20

	Concentration, ng/L, (%RSD) (n=3)																					ΣPFASs (ng/L)
Code	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA	PFHxPA	PFOPA	PFDPA	
G-S12	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	<MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	<MLOQ	< MLOD	< MLOQ	0.0
G-S13	23 (13)	9.4 (25)	13 (6)	18 (19)	6.5 (5)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	4.7 (20)	5.6 (9)	46 (28)	< MLOQ	< MLOQ	2.8 (25)	< MLOD	< MLOQ	88
G-S14	< MLOQ	< MLOD	< MLOD	17 (2)	0.46 (19)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	17
G-S15	< MLOQ	< MLOD	4.2 (26)	23 (29)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	27
G-S16	< MLOQ	< MLOD	3.2 (7)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	3.2
G-S17	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	0.0
G-S18	< MLOQ	< MLOQ	2.7 (13)	9.7 (1)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	12
G-S19	13 (4)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	13
G-S20	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	0.0
G-S21	6.8 (1)	3.7 (7)	7.7 (7)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	1.4 (21)	< MLOQ	< MLOQ	3.0 (16)	< MLOD	< MLOD	23
G-S22	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	0.0
G-S23	6.0 (29)	< MLOD	4.0 (1)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	2.6 (5)	< MLOD	< MLOQ	13
G-S24	8.3 (17)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	1.5 (.3)	< MLOD	< MLOD	< MLOD	≤ MLOD	< MLOD	< MLOQ	< MLOD	≤ MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	10
S-E1	25 (18)	8.7 (31)	3.5 (10)	< MLOQ	17 (6)	4.6 (23)	5.3 (2)	5.3 (2)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	3.6 (24)	15 (19)	27 (25)	< MLOQ	0.27 (10)	< MLOD	< MLOQ	< MLOQ	.12
S-E2	5.3 (15)	5.4 (10)	<MLOD	3.6 (9)	16.4 (12)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	5.1 (7;	115 (18)	<MLOD	<MLOD	na	na	na	.51
S-E3	<MLOD	<MLOD	10 (11)	28 (9)	<MLOD	18 (13)	<MLOD	<MLOD	<MLOD	5.1 (17)	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	501 (12)	<MLOD	<MLOD	na	na	na	563
G-E1	9.8 (17)	0.93 (28)	1.1 (6)	15 (1)	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	3.9 (7)	< MLOQ	31
G-E2	7.6 (4)	< MLOD	< MLOQ	53 (18)	1.8 (18)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	62
P 1	< MLOQ	< MLOQ	< MLOQ	17 (16)	0.52	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	27 (11)	< MLOD	< MLOD	45
P 2	< MLOQ	< MLOQ	< MLOQ	14 (19)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	46 (13)	< MLOD	< MLOQ	60
P 3	< MLOQ	< MLOQ	< MLOQ	1289 (30)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	53 (16)	< MLOD	19 (17)	1661

na: not analyzed

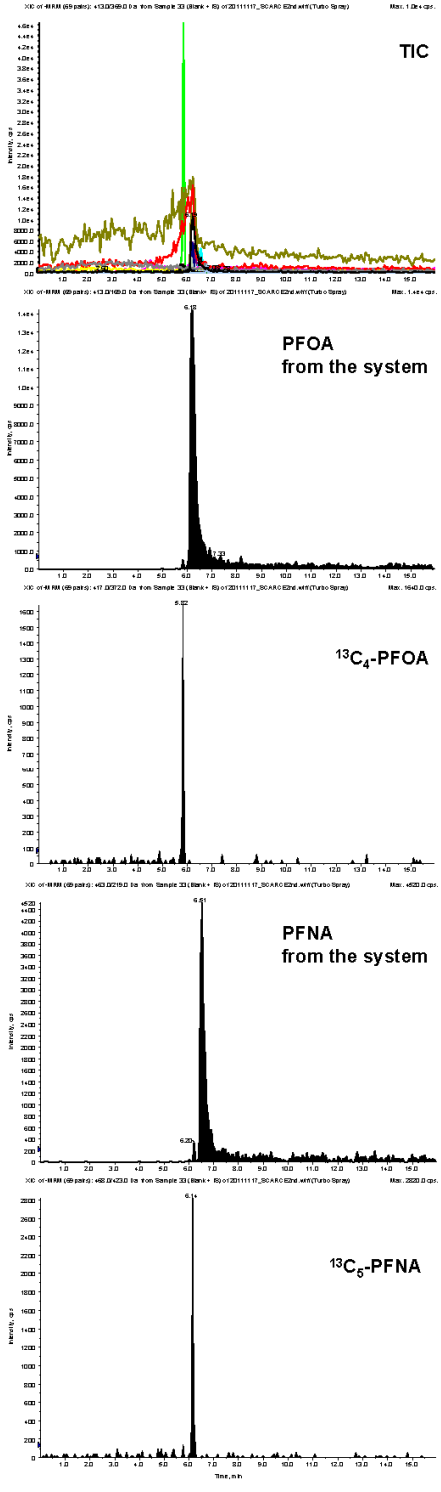

Figure S1: Chromatogram of blank sample spiked with internal standards

2.2.2 PFASs in sewage sludge

Scientific publication 2:

Llorca, M; Farré, M; Picó, Y; Barceló,D. (2011).

"Analysis of perfluorinated compounds in sewage sludge by pressurized solvent extraction followed by liquid chromatography–mass spectrometry"

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Analysis of perfluorinated compounds in sewage sludge by pressurized solvent extraction followed by liquid chromatography–mass spectrometry

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LC–MS/MS

ABSTRACT

Perfluorinated compounds (PFCs) are widely used in everyday life and one of the main recipients of these compounds is waste water treatment plants (WWTPs). Due to the structure and physicochemical properties of PFCs, these compounds could be redistributed from influent water to sludge. This work reports a new validated protocol for the analysis of 13 perfluorinated acids, 4 perfluorosulfonates and the perfluorooctanesulfonamide. The present work has been focused to develop a sensitive and robust method for the analysis of 18 PFCs in sewage sludge, based on pressurized solvent extraction (PSE) followed by solid phase extraction (SPE) clean-up, analytes separation by liquid chromatography and analysis in a hybrid quadrupole-linear ion trap mass spectrometer (LC–QLiT-MS/MS) working in single reaction monitoring (SRM) mode. The final methodology was validated using a blank sewage sludge fortified at different concentration levels. The method limits of detection were ranging in general from 15 to 79 ng/kg. These values were comparable to the decision limit (CCα) and the detection capability (CCβ), which were 17–1134 ng/kg and 18–1347 ng/kg, respectively. The percentage of recovery was from 79 to 111% in the most cases at different spiked levels. Finally, the repeatability of the method was in the range 4% (PFOS and PFOA) to 25% (RSD %). In order to evaluate the applicability of the method, 5 sludge samples were analyzed. The results showed that the 18 PFCs were present in all samples. However, the concentrations for most of them were below the limits of quantification. The compound present at higher concentrations was perfluorooctanesulfonate (PFOS), which was in concentrations from 53.0 to 121.1 μg/kg. The other PFCs were at concentrations between 0.3 and 30.3 μg/kg.

1. Introduction

Perfluorinated compounds (PFCs) comprise a large group of compounds widely used in industrial applications that are characterized by a fully fluorinated hydrophobic linear carbon chain attached to one or more hydrophilic head. They have unique properties to make materials stain, oil, and water resistant, and are widely used in several applications such as stain and water resistant textiles, food packaging, in fire extinguishing formulations, pesticides, paints, personal care products and surfactant agents [1], among others.

PFCs are resistant to breakdown, ubiquitous environmental contaminants, which persist and may be accumulated attached to proteins and biomagnified through the food chain. In recent years, an increasing scientific interest raised due to their widespread distribution. The main direct routes of exposure of PFCs to humans are in their diet and drinking water. PFCs have been found in environment studies of water (at levels of pg/l in lakes [2], ng/l in rivers [3], precipitation water [2]), soils and sediments (at levels of ng/g [4–7]) and biota samples (at levels of μg/kg in fish samples from Germany [8], Spain [9] and North America [2]). Among PFCs, perfluorooctanoic acid (PFOA) and PFOS are regarded as being the terminal degradation end-products, and these are the chemicals that have frequently been detected in environmental samples and often occur at high concentrations. Studies have shown that PFOA and PFOS have potential toxicity to cause liver cancer, affect the lipid metabolism and disturb the immunity system of living organisms [10,11] and human infertility [12] as well. PFCs enter the environment through direct (directly from manufacture wastes or direct application) and indirect sources (due to their decomposition or disposal through products life cycle) [13]. Wastewater treatment plants (WWTPs) have been identified as relevant pathway of PFCs releases into the environment [4,14]. However, few studies have reported the levels of PFCs in sewage sludge. In addition, the routes of introduction of PFCs in sewage sludge remain unclear, but possible ones include the washing residues from treated tex-

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tiles and cooking ware, direct and indirect residues of industrial production and application [5]. Regarding human exposure, these findings are of concern because partially sewage sludge can be used in rural lands. Therefore, these could be an indirect source of PFCs via consumption of crops, air-borne transport, surface waters and ground waters draining from these sites. The concentration levels reported in previous works showed concentrations from 50 to thousands μg/kg for perflurooctanesulfonate (PFOS) [15–20] and ng/kg to hundreds μg/kg for the other PFCs [15–21].

From the analytical point of view the determination of PFCs in sludge and sediments presents a series of limitation in addition to those inherent to their analysis in general such as cross-contamination. The main extra limitations found in the case of sludge and sediment analysis, are the difficulties in their extraction and clean-up steps, because these steps are labor intensive and time consuming, and the high percentage of matrix effects problems (ion-enhancement or ion suppression) which makes practically impossible the quantitative analysis of some compounds. Most previous works were based on extraction procedures using a methanol extraction and alkaline digestion followed by liquid extraction using methanol and acetonitrile [21]. Other procedures that have been applied were ion pair extraction [15,22]. In addition, usually a clean-up step is used in general by solid phase extraction (SPE) with a different retention phase: C_{18}, Oasis HLB or Oasis WAX. However, very few works have reported the use of pressurized liquid extraction for multianalyte analysis of PFCs [7,23].

Under this context, the main objectives of the present study were: (I) to develop an efficient extraction methodology for the analysis of 18 PFCs in sewage sludge based on pressurized solvent, (II) to validate the new developed analytical method extraction followed by analysis by LC–ESI-QqLIT (MS/MS), the most sensitive instrument in our group [24] and (III) to test the good performance of this analytical method by its application in the analysis of real samples.

2. Materials and methods

2.1. Standards and reagents

A mixture of PFCs [MXB; >98%] containing: perfluorobutanoic (PFBA), perfluoropentanoic (PFPA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), perfluoroundecanoic (PFUdA), perfluorododecanoic (PFDoA), perfluorotridecanoic (PFTrA), perfluorotetradecanoic (PFTeA), perfluorohexadecanoic (PFHxDA) and perfluorooctadecanoic (PFODA) acids, perfluorobutanesulfonate (PFBS), perfluorohexasulfonate (PFHxS), perfluorooctanesulfonate (PFOS), perfluorodecanesulfonate (PFDS) and the perfluorooctanesulfonamide (PFOSA), >99%. A mixture of labeled PFCs [MXA: >98%] containing: [$^{13}C_4$]-perfluorobutanoic acid (MPFBA ($^{13}C_4$)), ion [$^{18}O_2$]-perfluorohexanesulfonate (MPFHxS ($^{18}O_2$)), [$^{13}C_2$]-perfluorohexanoic acid (MPFHxA ($^{13}C_2$)), ion [$^{13}C_4$]-perfluorooctanesulfonate (MPFOS ($^{13}C_4$)), [$^{13}C_4$]-perfluorooctanoic acid (MPFOA ($^{13}C_4$)), [$^{13}C_5$]-perfluorononanoic acid (MPFNA ($^{13}C_5$)), [$^{13}C_2$]-perfluorododecanoic acid (MPFDoA ($^{13}C_2$)), [$^{13}C_2$]-perfluorodecanoic acid (MPFDA ($^{13}C_2$)), [$^{13}C_2$]-perfluoroundecanoic acid (MPFUdA ($^{13}C_2$)), added before the extraction procedure, was used as a surrogate in order to normalize all the analytical process. Labeled PFCs: [1,2-$^{13}C_2$]-perfluorooctanoic acid (M2PFOA ($^{13}C_2$); >98%) and ion [$^{13}C_8$]-perfluorooctanesulfonate (M8PFOS ($^{13}C_8$); >98%), added just before LC injection, were used as internal standards in order to normalize the instrumental analysis response. All analytical and labeled standards were purchased from Wellington Laboratories Inc., Canada. Water and Methanol (MeOH) CHROMASOLV® Plus, for HPLC grade, ammonium acetate salt ($AcNH_4$: MW, 77.08; ≥98%) and Ammonium hydroxide (NH_4OH: MW, 35.05; ≥98%) were obtained from Sigma–Aldrich, Steinheim, Germany. Sodium hydroxide base (NaOH: MW, 39.10; >97%) was purchased from Merck. Ottawa Sand from Applied Separations, Allentown.

2.2. Samples

In order to test the good performance of the developed approach, 5 different sewage sludge samples (sludges 1–5) were collected during April 2010 in a domestic WWTP in Catalonia, Spain. In order to avoid contamination of the samples during sampling and transport those were collected using foil containers. Sludge samples were frozen to −20° C prior to any treatment.

Blank sludge samples were used during optimization process and to assess the non-cross contamination along the analytical process.

2.3. Extraction procedure

The pressurized solvent extraction was carried out in a PSE 240 V (Applied Separations, Allentown).

Sludge samples were frozen at −20° C, lyophilized and homogenized. Approximately 0.5 g of sample was spiked with a surrogate mixture at 3 μg/kg and left to rest for 20 min. The spiked material was homogenized with sand and introduced in a 22 ml extraction cell. The cell was extracted during two consecutive cycles with methanol at 70 °C, 100 bar of pressure. Extracts were dried under a gentle stream of nitrogen and reconstituted in 50 ml of water. Solid phase extraction (SPE, Oasis WAX 3cc) was used as a clean-up step, based on an earlier published method [9]. Very briefly, the conditioning was carried out with 2× 2 ml of MeOH (0.1% NH_4OH). 2× 2 ml of MeOH and 2× 2 ml of water. The reconstituted extract was loaded under gravity conditions and dried under vacuum 20 min. Analytes were eluted in a 2× 2 ml MeOH (0.1% NH_4OH), in PP tubes, and dried under a gentle stream of nitrogen. Extracts were transferred using MeOH in a PP insert LC vial, dried under nitrogen conditions and reconstituted in LC initial conditions (Water/MeOH; 90:10). Internal standards were added at 5 μg/l level, in vial. Samples were analyzed by LC–MS/MS.

2.4. Instrumental analysis

The analysis of selected PFCs was performed by LC–ESI–MS/MS. The chromatographic separation was performed using a Symbiosis ™-Pico (Spark Holland, Emmen, The Netherlands) equipped with a LiChroCART® 125-2, Pusopher® STAR, RP-18e (5 μm) analytical column, from Merck, at room temperature. The mobile phase used for the chromatographic separation consisted of aqueous ammonium acetate 20 mM (A) and MeOH (B) and was delivered at flow rate of 0.4 ml/min. The elution gradient condition started at 10% B and rose to 50% B in 2 min, and then it was linearly increased to 70% B in 4 min, and finally increased to 90% B in 8 min. This percentage was maintained for 1 min more. Finally, the mobile phase was returned to initial conditions in 1 min. Initial conditions were maintained for 1 min more. Injection volume was 10 μl.

The LC system was coupled to a quadrupole-linear ion trap mass spectrometer (QqLIT-MS/MS) 4000 QTRAP (Applied Biosystems), equipped with a Turbo Ion Spray source operated in the negative electrospray ionization mode (ESI (−)). The use of this analyzer in the study of PFCs in sludge was decided due to the versatility of the instrument evidences: conventional SRM provides excellent sensitivity and selectivity in the quantitation. Comparing to conventional triple quadrupole (QqQ), the QqLIT system achieved at least 20-fold higher sensitivity than the QqQ system disposed

Table 1
Parent and fragment ions, declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell entrance potential (CEP) optimal conditions for each compound.

Target compounds	Retention times (min)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*) SRM1 SRM2	DP (V)	CE (V)	EP (V)	CXP (V)	SRM ratio (SRM1/SRM2)
PFBA	4.2	213	**169**	−25	−25	−10	−10	210
			119		−25			
PFPeA	5.3	263	**219**	−25	−15	−10	−10	–
PFHxA	5.8	313	**269**	−25	−25	−10	−10	98.4
			169		−25			
PFHpA	6.2	363	**319**	−25	−25	−10	−10	22.3
			169		−25			
PFOA	6.2	413	**169**	−25	−35	−10	−10	2.6
			369		−25			
PFNA	6.5	463	**219**	−25	−25	−10	−10	5.0
			169		−15			
PFDA	7.5	513	**119**	−25	−35	−10	−10	1.2
			469		−35			
PFUdA	8.0	563	**519**	−25	−35	−10	−10	15.8
			219		−35			
PFDoA	8.7	613	**569**	−25	−35	−10	−10	53.0
			269		−35			
PFTrA	9.4	663	**619**	−25	−35	−10	−10	30.0
			219		−35			
PFTeDA	10.1	713	**669**	−25	−35	−10	−10	36.8
			269		−35			
PFHxDA	11.5	813	**769**	−25	−35	−10	−10	28.8
			269		−35			
PFODA	13.0	913	**869**	−25	−35	−10	−10	26.3
			269		−35			
PFBS	5.4	299	**80**	−25	−80	−10	−10	1.7
			99		−80			
PFHxS	6.2	399	**99**	−25	−80	−10	−10	1.8
			80		−80			
PFOS	6.5	499	**80**	−25	−100	−10	−10	1.2
			99		−100			
PFDS	8.0	599	**80**	−25	−100	−10	−10	0.99
			99		−100			
PFOSA	7.1	498	**78**	−25	−50	−10	−10	64.6
			119		−100			

Bold means "Transitions used for quantification".

in the laboratory as was reported in previous work [24]. Acquisition was performed in single reaction monitoring mode (SRM) to obtain sufficient identification points (IP) for confirming each analyte according to Decision 2002/657/EC [25]. The identification of target analytes was carried out using relation between the highest relative abundances of two *m*/*z* transitions and retention times.

The quantification of each compound was carried out using the most intensive *m*/*z*$^-$ transition which is indicated in Table 1. Optimized parameters were as follows: curtain gas (CUR), 30 (arbitrary units); ion source gas 1 (GS1), 25 (arbitrary units); ion source gas 2 (GS2), 60 (arbitrary units); source temperature (TEM), 350 °C; ion spray (IS), −4500 V; entrance potential (EP), −10 V, collision cell exit potential (CXP) −10 V and declustering potential (DP) −25 V. The dwell time of each MRM transition was 50 ms.

2.5. Method validation

Validation experiments were performed by spiking blank sludge samples with all selected analytes at three different levels 9, 50, 100 μg/kg (six replicates at each concentration level, $n = 6$). After homogenization the spiked samples were left to balance during 20 min. After this period, the samples were processed as reported in Sections 2.3 and 2.4. For the assessment of all mentioned parameters, the analyte response was always related to the surrogate internal standard responses to compensate for undesirable matrix effects and looses during the extraction step.

The developed method was validated using an "in-house" procedure according to ISO 11843 [26] using spiked materials because no reference material was available. In accordance with the criteria, performance characteristics of a conventional method include recovery, repeatability, with-in-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ), calibration curves and specificity. In addition for comparative purposes, limits of detection (LODs) and limit of quantification (LOQ) were also determined. Positive identification was considered when a ±2.5% retention time agreement was achieved between the analytes in the samples and standards and a 25% relative abundances margin was achieved between the two selected ion transitions for each analyte.

Selectivity was assured by obtaining four identification points for each analyte through the monitoring of two transitions of each precursor ion corresponding to each target analyte and the retention time of each analyte. Linearity was assessed by constructing seven point calibration curves in triplicate at concentration levels ranging from low ng/kg to 150 μg/kg as they are summarized in Table 2. Least-square linear regression analysis was performed by plotting the peak area of the analyte over the analyte concentration and correlation coefficients (R^2) higher than 0.9900 for all compounds.

LOD and LOQ were calculated for each analyte at a signal-to-noise (S/N) ratio of 3 and 10, respectively. LODs were determined using the most intense transition (higher S/N) for each analyte, while for LOQ the second transition was confirmed visible in the chromatogram. The method limits of quantification (MLOQs) were established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 1.5%, (2) relative standard deviation of four replicates below 19%, (3) peak

Table 2
MLOD, MLOQ, error CCα and error CCβ by calibration curve, according to ISO 11843 when no MPRL is established and matrix effect expressed as % of surrogates used in the extraction method.

	MLOD (ng/kg)	MLOQ (ng/kg)	CCα (ng/kg)	CCβ (ng/kg)	% RSD interday	% of Recoveries			Surrogate (10 μg/l in vial)	Matrix effects (%)
						9 μg/kg	50 μg/kg	100 μg/kg		
PFBA	831	2772	1134	1347	29	102	90	65	MPFBA	−71
PFPeA	69	232	92	108	30	91	80	74		
PFHxA	161	538	184	201	13	110	108	90	MPFHxA	−48
PFHpA	79	264	93	103	15	98	102	80		
PFOA	22	73	31	38	4	114	96	80	MPFOA	−47
PFNA	15	50	17	18	14	106	99	108	MPFNA	−41
PFDA	40	133	61	76	25	102	103	111	MPFDA	−28
PFUdA	57	189	62	66	13	111	99	95	MPFUdA	22
PFDoA	55	183	70	81	16	91	105	86		
PFTrA	65	218	114	149	21	101	107	91		
PFTeDA	69	231	119	154	19	65	109	90	MPFDoA	−46
PFHxDA	67	223	180	260	16	62	110	85		
PFODA	53	176	123	172	21	60	76	70		
PFBS	219	729	262	293	24	105	110	65	MPFHxS	−46
PFHxS	31	102	36	40	24	104	106	93		
PFOS	25	84	34	41	4	99	110	98		
PFDS	45	151	57	65	8	90	97	67	MPFOS	−35
PFOSA	68	228	84	95	8	78	91	89		

Results obtained in spiked sediment at 9 μg/kg level. $n = 6$. Matrix effect (%) = [100 × surrogate peak in extracts ($n = 3$)/surrogate peak in mobile phase ($n = 8$)] − 100. Matrix effects > 0 → ion enhancement. Matrix effects < 0 → ion suppression.

shapes acceptable, and (4) signal-to-noise ratio higher than 10 in sludge spiked material.

CCα and CCβ were calculated according to the ISO 11843 [26] by the calibration curve procedure when no method permitted reference limit (MPRL) is established. CCα was calculated using the sludge blank materials fortified above the minimum required performance level (in this case, 9 μg/kg) in equidistant steps. After analysis of the fortified materials, CCα was calculated as the concentration which after plotting the signal obtained against the added concentration corresponds to the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility. On the other hand, CCβ was calculated as minimum detectable value plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability ($\beta = 5\%$).

Recovery was assessed for each analyte using fortified blank sludge samples at three levels of concentration 9, 50, 100 μg/kg. Analyte recoveries were calculated from the peak areas obtained for each analyte (average of six replicates for each sample) as percentages of the peak areas obtained from the replicate ($n = 6$) analysis of equivalent standard solutions.

Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets for calculating interday repeatability.

Table 2 summarizes MLOD, MLOQ, CCα, CCβ, the percentage of recoveries at different levels of fortification and the interday precision.

In order to establish the possible ion enhancement or ion suppression in the matrix, the percentage of matrix effects was calculated in all matrices according to peak areas relation of surrogate added to samples before extraction vs. surrogate in mobile phase, at 10 μg/l level in vial.

3. Results

3.1. Optimization of the PLE procedure

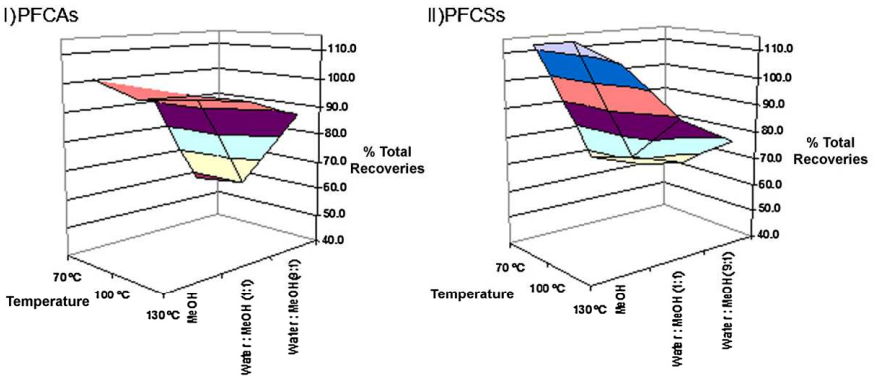
One of the parameters showing the strongest effect on the PSE extraction efficiency is the composition of the extracting solvent, temperature, number of extraction cycles and the cell size. For the selection of the extraction solvent and its composition the following combinations were tested: (1) [water:MeOH (9:1)], (2) [water:MeOH (1:1)] and (3) [MeOH (100%)]. For this series of experiments blank sludge samples were fortified at a 9, 50, 100 μg/kg ($n = 6$). In addition, temperature has played a key role in the extraction procedure development. A series of temperature 70, 100 and 130 °C were evaluated. The minimum temperature was chosen at 5 °C over the MeOH boiling point. In addition, the performance of the extraction was tested using 1–3 extraction cycles. Fig. 1 summarizes these results. As it can be seen, for acids (PFCAs) and sulfate compounds (PFCSs), the percentage of recoveries was increased according to the following the order: [water:MeOH (9:1)] < [water:MeOH (1:1)] < [MeOH (100%)]. Therefore, MeOH was selected as optimum extraction solvent. No apparent differences were found at the different temperatures tested, using MeOH as extraction solvent. A percentage of recoveries near 100% was obtained for all PFCs. Due to this reason, the minimum temperature was selected as the optimum one. No significant differences were found using 1, 2 or 3 cycles of extraction. However due to the variety of sludge matrices and in order to assure the better extraction of all compounds in different sewage sludges, 2 cycles of extraction were set for the final procedure.

The cell volume was also evaluated for the best performance. For this experiment, 0.5 g of fortified blank sludge was introduced in 11 ml and 22 ml cells, in both cases filling the void space with sand. It was observed that the smaller cell, although saved time in the process as well as material and solvent consumption, often presented difficulty in processing the sample. From this series of experiments, extraction using the 22 ml cells proved more efficient for most analytes PFAs and PFSs. As a result of this experiment, the method was validated using the 22 ml volume extraction cells.

Summarizing, the following parameters were set in the final method; Cell extraction volume 22 ml, MeOH 100% as a solvent extraction, pressure of 100 bar, temperature of extraction 70 °C and 2 cycles of extraction with 1 min of static time.

3.2. Validation

The validation procedure for the developed method was carried out taking into account the EU requirements. The most significant parameters considered are described in the following points.



	% Recoveries		
PFCAs	70 °C	100 °C	130 °C
Water : MeOH(9:1)	57.5	60.3	88.5
Water : MeOH (1:1)	94.6	94.7	95.2
MeOH	100.3	95.9	98.3

	% Recoveries		
PFC Ss	70 °C	100 °C	130 °C
Water : MeOH(9:1)	59.8	65.7	78.4
Water : MeOH (1:1)	66.9	71.7	89.6
MeOH	111.9	118.6	110.1

Fig. 1. 3D graphic surface combining: temperature, solvent mixture and percentage of total recoveries with the corresponding data table. (I) PFCAs and (II) PFCSs.

After PLE extraction a purification step was carried out using Oasis WAX 3cc cartridges. The final recoveries of the whole extraction and purification procedure were assessed using the blank sludge fortified at a 9, 50, 100 μg/kg (n = 6), Table 2 summarizes the recovery percentage obtained for each PFC at the different concentration levels. For the lowest level of fortification recoveries were ranging from 92 to 111% for PFCAs [excepting PFTeDA (56%), PFHxDA (63%) and PFODA (54%)]. Apart from PFOSA (80%), for PFCSs the percentages of recoveries were 100–105%. At concentrations of 50 μg/kg, the percentages of recovery were between 97 and 111% for PFCAs and PFCSs, the only exception was PFOSA, the recovery of which was 78%. Finally, for the highest fortification level, the percentage of recovery was 80–107% for PFCAs, 76–98% for PFCSs and 91% for PFOSA. Summarizing, in all cases recoveries were between 70 and 110% for the three levels of fortification and the repeatability was lower than 25% for all analytes.

Table 2 reports main values of MLOQ, MLOQ, CCα and CCβ calculated according to ISO 11843 [26] when no MPRL is established, and the percentage of matrix effects on blanks fortified at the 9 μg/kg (n = 6), the lowest spiked level near to MLOQ of PFBA (2.772 μg/kg). The results revealed that MLOQ for acidic compounds were ranging from 50 to 538 ng/kg for most of compounds. PFCSs, in general, presented low MLOQ, with an exception of PFBS (729 ng/kg). In parallel, CCα values were between 17 and 1134 ng/kg and CCβ between 18 and 1347 ng/kg. On the other hand, a strong matrix effect was measured for each compound. Matrix effects produced ion suppression, with exception of MPFUdA for which ion enhancement was observed. In an attempt to compensate for undesirable matrix effects, quantification was carried out using surrogate internal standards added before the extraction.

3.3. Applicability of the method

The method applicability was assessed by analyzing 5 sewage sludge samples, which were collected in an urban WWTP.

Table 3 shows analytical results of the study and Fig. 2 shows an example of chromatogram obtained in a sludge sample. In general, the higher values were found in the sample no. 4.

The concentration levels of PFCAs were ranging from 0.4 to 30.3 μg/kg, in agreement with previous studies by Zhang et al. [15], Guo et al. [17], Li et al. [18] or Ma et al. [19]. PFOA, PFNA, PFDA and PFDoA were present in all the samples at concentrations higher than 1.0 μg/kg. Long chain acidic compounds were not detected in general, as can be expected due to the biodegradation processes, and the high concentration of PFOA can be associated with the biodegradation of other long chain PFCs congeners currently in use [27,28].

PFOSA was detected in three over the five samples analyzed, being one of the more frequently detected compound, with concentrations ranging from 0.3 to 10.7 μg/kg.

It should be pointed out, that the number of PFCs analyzed in sludge or sediments in previous studies is in general 5–6 compounds including PFOS and PFOA, and for other congeners currently in use no previous data was available for comparison purposes.

Table 3
PFCs concentration in sludge samples.

	μg/kg dw (% RSD)				
	Sludge 1	Sludge 2	Sludge 3	Sludge 4	Sludge 5
PFBA	<MLOD	<MLOD	<MLOD	22.6 (16)	14.9 (2)
PFPeA	17.2 (20)	15.6 (14)	2.6 (15)	<MLOQ	<MLOQ
PFHxA	<MLOD	<MLOD	<MLOD	<MLOQ	4.8 (22)
PFHpA	<MLOQ	0.4 (2)	4.5 (7)	<MLOQ	2.0 (25)
PFOA	9.5 (1)	7.0 (12)	9.5 (12)	30.3 (18)	29.7 (6)
PFNA	1.0 (6)	1.2 (19)	1.1 (24)	2.0 (8)	2.4 (21)
PFDA	8.6 (7)	6.1 (16)	7.2 (19)	23.5 (11)	8.2 (25)
PFUdA	3.7 (21)	<MLOQ	<MLOQ	12.2 (25)	7.8 (24)
PFDoA	6.3 (9)	2.7 (13)	3.0 (18)	11.3 (16)	4.0 (25)
PFTrA	<MLOD	<MLOQ	<MLOQ	<MLOD	<MLOD
PFTeDA	5.0 (18)	2.0 (7)	2.0 (19)	<MLOQ	<MLOQ
PFHxDA	<MLOD	<MLOQ	0.4 (18)	4.9 (9)	<MLOQ
PFODA	0.9 (12)	<MLOD	<MLOD	<MLOD	<MLOD
PFBS	<MLOD	0.9 (17)	<MLOD	<MLOQ	7.6 (18)
PFHxS	<MLOQ	<MLOQ	<MLOQ	<MLOQ	<MLOQ
PFOS	101.0 (13)	72.3 (11)	53.0 (19)	121.1 (8)	73.5 (8)
PFDS	<MLOD	<MLOD	<MLOD	7.5 (23)	<MLOQ
PFOSA	0.3 (6)	1.1 (11)	10.7 (16)	<MLOD	<MLOD

n = 3, dw, dry weight; RSD, relative standard deviation.

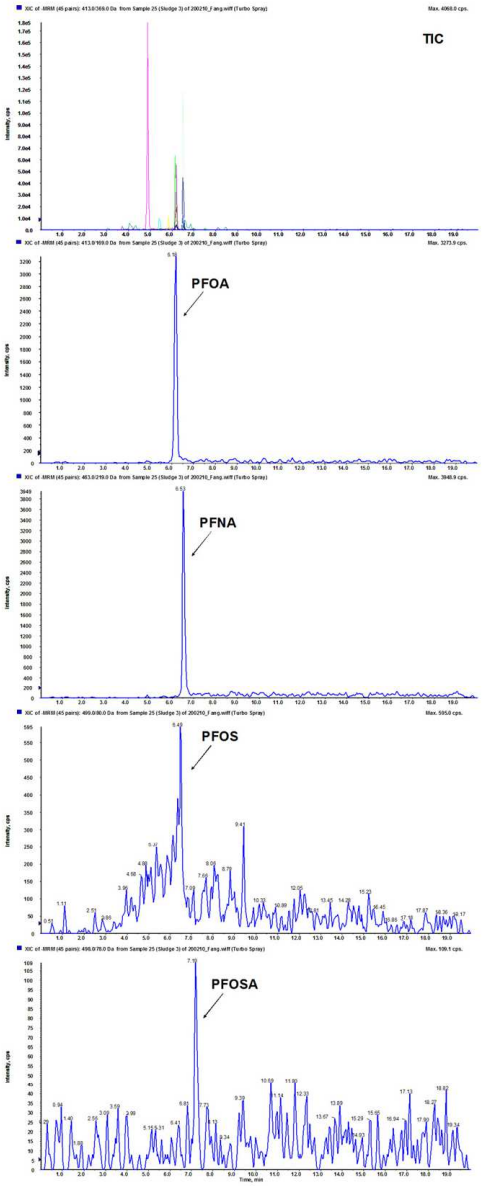


Fig. 2. Total ion chromatogram (TIC) of sludge 3 and extracted ion chromatograms of 4 positive analytes.

In the present study, with exception of PFOS, all compounds belonging to PFCSs were below MLOQ. PFOS was detected in concentrations levels ranging from 53 to 121 μg/kg, being the compound that was found in higher concentrations, in agreement with previous studies [15,17,18,20,22]. Zhou et al. [29] reported the favorable sorption of PFCs on the heterogeneous protein composition of activated sludge, and the different sorption kinetics according to their carbon chain length and different functional groups [30], which could explain the high concentrations of PFOS found in this work, as well as in the previous ones. In addition, in this work the concentration levels of PFOS were between 3 and 10 times higher than those for PFOA. This difference could be associated with the different sorption kinetics in function the different functional groups, in agreement with Zhou et al. [30]. The calculated distribution coefficients indicate that PFOS had a higher sorption tendency to activated sludge than PFOA. On the other hand, Becker et al. [31] showed that in WWTP, the calculated mass flow of PFOA is discharged in a high percentage through the WWTP final effluents while about fifty percent of PFOS is retained in the sewage sludge, fact that also supports the finding of the present work.

4. Conclusions

In the present work a multianalyte method was developed for 18 PFCs in sewage sludge to fulfill requirements for routine analysis. The new robust and sensitive analytical method is based on a PLE step with methanol as solvent followed by SPE (Oasis WAX) clean-up and analysis by LC–MS/MS. The validation showed high recovery rates range between 76 and 111%. The MLOQ were established at ng/kg levels for most compounds. However, for some compounds a high percentage of matrix effect was present, and therefore surrogates internal standards should be used in order to compensate these undesirable effects and to perform a correct quantification.

The applicability of the method was proved by analysis of 5 sewage sludge samples. The results of the analysis of real sewage sludge samples showed and concluded that PFOS was the compound encountered in higher concentrations, and PFOSA and PFCAs were found at levels of μg/kg.

Further studies about the presence and the fate of PFCs into sludge are required, because of the lack of data about some currently in use compounds and in order to elucidate transformation and biodegradation processes, because sewage sludge can be a direct source of PFCs in the environment through their application in soil restoration and agricultural soil, and also an indirect source human exposure through food and groundwater contamination.

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2.2.3 Study of PFASs in biota from remote samples

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Fate of a broad spectrum of perfluorinated compounds in soils and biota from Tierra del Fuego and Antarctica

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ABSTRACT

In this study, the presence of 18 perfluorinated compounds was investigated in biota and environmental samples from the Antarctica and Tierra de Fuego, which were collected during a sampling campaign carried out along February and March 2010.

61 samples were analysed including fish, superficial soils, guano, algae, dung and tissues of Papua penguin by liquid chromatography coupled to tandem mass spectrometry.

The concentrations of PFCs were ranging from 0.10 to 240 ng/g for most of the samples except for penguin dung, which presented levels between 95 and 603 ng/g for perfluorooctane sulfonate, and guano samples from Ushuaia, with concentration levels of 1190–2480 ng/g of perfluorohexanoic acid. PFCs acids presented, in general, the highest levels of concentration and perfluorooctanesulfonate was the most frequently found compound.

The present study provides a significant amount of results, which globally supports the previous studies, related to the transport, deposition, biodegradation and bioaccumulation patterns of PFCs.

1. Introduction

Large-scale production and usage of perfluorinated compounds (PFCs) began in the 1950's because of their excellent surfactant capabilities, stability and amphiphilic properties. Their highly chemical and biological stability is conferred by the carbon-fluor bound. Major applications have included water and grease repelling coatings, fire fighting foams, textiles, prints and adhesives. During the recent years, PFCs have lately received an increasing attention because of their widespread presence in the environment, in human beings and wildlife as well (Houde et al., 2006; Lau et al., 2007). PFCs are persistent organic pollutants (POPs) and can be found bioconcentrated, bioaccumulated and biomagnified throughout the food chain. These compounds can be bioaccumulated because of their binding to proteins (Kennedy et al., 2004; Lau et al., 2007; Peden-Adams et al., 2008). Different studies have revealed that PFCs are toxic to mammals (Trudel et al., 2008). In addition, their elimination by the human organism takes some time (Olsen et al., 2007).

It should be pointed out that the global industrial emissions of PFCs from direct (manufacture, use, consumer products) and indirect (PFCs impurities and/or precursors) sources were estimated to be 3200–7300 tonnes in 2006 (Prevedouros et al., 2006). Throughout the last years, the occurrence of PFCs has been reported in environmental samples, especially these of chain lengths less than ten carbon atoms. These compounds have been globally detected in rivers and oceans and are considered to be significantly transported via aquatic ecosystems. PFCs have been reported to be present in lakes (Boulanger et al., 2004; De Silva et al., 2009), rivers (D'Eon et al., 2009; Takazawa et al., 2009) and even in precipitation water (Scott et al., 2006; Kwok et al., 2010; Young et al., 2007) at ng/L. They have also been found at pg/g levels in sediment from the deep sea (Harino et al., 2009; Higgins et al., 2005; Nakata et al., 2006). It is estimated that a great proportion of PFCs is emitted directly to the aquatic ecosystems during their use and production (Nakata et al., 2006). The long-range transport of these compounds dissolved in water has been modelled through different works (Young et al., 2007; Armitage et al., 2006; Bengtson Nash et al.,

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2010; Wania, 2007). Volatile PFCs dissolved in marine aerosols may be long-range transported to remote regions and may contribute to their contamination with persistent PFCs (Dreyer et al., 2009; Prevedouros et al., 2006).

In addition, PFCs are biomagnified through the food webs, since several studies have reported their occurrence in wildlife (Olivero-Verbel et al., 2006). Concentrations at hundreds ng/g in fish have been reported (Houde et al., 2006b) and the assessment of these compounds in different countries as the North of Germany (Schuetze et al., 2010), Spain (Llorca et al., 2009) and in different regions of North America (De Silva et al., 2009). Different studies have revealed that marine mammals and animals in a high trophic position can accumulate high concentrations of PFCs. Their presence in the Arctic fauna (polar bears Dietz et al., 2008) and in the Arctic atmosphere at levels of pg/m^3 (Shoeib et al., 2006) has been well established. However, fewer studies have reported results from the Antarctic continent to date (Ahrens and Ebinghaus, 2010; Dreyer et al., 2009; Schiavone et al., 2009). The evaluation of PFCs in remote areas such as the Antarctica peninsula is one of the very few forms of evaluation of long-range environmental transport (LRET). The global fate of POPs is associated to different biogeochemical cycles and geophysical drivers. The occurrence of PFCs into remote areas such as the Antarctica could be partially explained by the theory of *cold condensation*, concerning the chemical movements or chemical transformations from sources under the impact of environmental forces, such as temperature, and interaction with other environmental compartments (soil, oceans, etc.) (Lohmann et al., 2007). In addition, the physical-chemical characteristics of PFCs should also be considered, since these properties dictate their environmental behaviour (Wania, 2007).

Just four previous studies have reported the presence of PFCs in different biota samples from the Antarctica continent (Giesy and Kannan, 2001; Tao et al., 2006; Schiavone et al., 2009; Bengtson Nash et al., 2010), whereas this information could be of importance to establish Global PFCs distribution and also the basis of LRET of these compounds.

The main objectives of this study were 1) to determine the presence of 18 PFCs pertaining to different groups as perfluorinated acids (PFCAs), perfluorinated sulphates (PFSs) and perfluorosulfonamides in biota and soil samples from Tierra del Fuego and the Antarctica; 2) establish relationships among remote areas contamination and main contribution pathways, as well as, to contribute to understanding the long-range transport in the southern hemisphere.

2. Material and methods

2.1. Standards and reagents

PFCs mixture [MXB; >98%] containing: perfluorobutanoic (PFBA), perfluoropentanoic (PFPA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), perfluoroundecanoic (PFUdA), perfluorododecanoic (PFDoA), perfluorotridecanoic (PFTrA), perfluorotetradecanoic (PFTeA), perfluorohexadecanoic (PFHxDA) and perfluorooctadecanoic (PFODA) acids, perfluorobutanesulfonate (PFBS), perfluorohexasulfonate (PFHxS), perfluorooctanesulfonate (PFOS) and perfluorodecanesulfonate (PFDS).

A sulfonamide: Perfluorooctanesulfonamide (PFOSA), >99%. A mixture of labelled PFCs [MXA; >98%] containing: [$^{13}C_4$]-Perfluorobutanoic acid (MPFBA ($^{13}C_4$)), Ion [$^{18}O_2$]-Perfluorohexanesulfonate (MPFHxS ($^{18}O_2$)), [$^{13}C_2$]-Perfluorohexanoic acid (MPFHxA ($^{13}C_2$)), Ion [$^{13}C_4$]-Perfluorooctanesulfonate (MPFOS ($^{13}C_4$)), [$^{13}C_4$]-Perfluorooctanoic acid (MPFOA ($^{13}C_4$)), [$^{13}C_5$]-Perfluorononanoic acid (MPFNA ($^{13}C_5$)), [$^{13}C_2$]-Perfluorododecanoic acid (MPFDoA ($^{13}C_2$)), [$^{13}C_2$]-Perfluorodecanoic acid (MPFDA ($^{13}C_2$)), [$^{13}C_2$]-Perfluoroundecanoic acid (MPFUdA ($^{13}C_2$)), added before the extraction procedure, was used as a surrogate internal standard in order to normalize the results of the whole analytical process. Labelled PFCs: [1,2-$^{13}C_2$]-Perfluorooctanoic acid (M2PFOA ($^{13}C_2$); >98%), ion [$^{13}C_8$]-Perfluorooctanesulfonate (M8PFOS ($^{13}C_8$); >98%), added just before LC injection, were used as internal standards in order to correct the instrumental response. All analytical and labelled standards were purchased from *Wellington Laboratories Inc., Canada*. Water and Methanol (MeOH) CHROMASOLV®Plus, for HPLC grade, ammonium acetate salt ($AcNH_4$: MW, 77.08; >98%) and Ammonium hydroxide (NH_4OH: MW, 35.05; >98%) were obtained from *Sigma–Aldrich, Steinheim, Germany*. Sodium hydroxide base (NaOH: MW, 39.10; >97%) was purchased from *Merck*.

2.2. Sample collection

The sampling campaign was supported by *Premios Antárticos de Ciencia, Tecnología y Medio Ambiente 2010*, throughout February and March of 2010, and under the support of Departamento Nacional Antártico Argentino and the Comando Antártico Argentino. The samples were from two different sampling areas I) Tierra del Fuego and II) Antarctica continent. Fig. 1 presents sampling locations.

I) Tierra del Fuego Samples were collected in the Ushuaia region, located on the southern coast of Isla Grande de Tierra del Fuego, bounded on the north by the Martial mountain range and on the south by the Beagle Channel. This region has an area of 9390 km^2 with a sub polar oceanic climate, and the Ushuaia city is the only municipality with 2000 inhabitants.

An amount of 30 composite samples of soils were taken a random strategy. For each composite sample, five samples were collected in quantitatively similar subsamples (~500 g), 2 m away from each one from *Bahía Encerrada* (*Bahía Ushuaia*) and *Playa Larga* (*Bahía Ushuaia*). Besides, 9 algae leave (*Macrocystis pyrifera*) samples were taken from *Playa Larga* (*Bahía Ushuaia*) and *Mary Ann Island* (*Beagle Channel*), 6 guano samples were collected from *Bahía Encerrada* (*Bahía Ushuaia*) and *Mary Ann Island* (*Beagle Channel*), 4 fishes (Rainbow trout: *Oncorhynchus mykiss*) were extracted from *Oliva River* (*Beagle Channel*).

The samples were pre-processed in a central laboratory facility in Ushuaia. Superficial and guano samples were dried and conserved in polypropylene (PP) sterilized vials. Fishes and algae leaves were cut (in the case of fish samples, the skin, livers, muscle and roes were separated), then were homogenized and transferred into PP sterilized vials and finally were frozen and lyophilized. Empty PP sterilized vials were used as blanks during all the sampling and shipping process along with the real samples in order to rule out possible sources of contamination.

II) Antarctica An amount of 3 samples of superficial soil was collected from the *Aitcho Islands*. A total of 6 dung and 6 muscle tissues samples of Papua penguins were collected from the *Ardley Island*, *Neko Bay* and *Winter Island*, whereas 3 algae samples were taken from *Ardley Island*. The preservation of the samples was accomplished by freezing whilst shipping to the central Laboratory facility in Ushuaia. Then the samples were frozen-dried, homogenized and transferred to PP sterilized vials. Empty PP sterilized vials were also used as blanks during the whole process.

Finally, the samples from Tierra de Fuego and Antarctica were transported to the IDAEA-CSIC laboratory for their analysis. Frozen-dry samples were kept under cool conditions (between −2 and 4 °C) during the flight.

Refer to Table 1 for the summarized sampling sites and their GPS coordinates and Fig. 1 for sampling sites map.

2.3. Sample pre-treatments and extraction procedures

Biota Samples were analysed in triplicates according to the method described by Llorca et al. (2009) Very briefly; 2 g of the sample were introduced into a 50 ml PP centrifuge tube, and fortified with 10 μl of a surrogate internal standard mixture at 500 pg/μl. After fortification, the samples were left to rest for 20 min for equilibrating. Sample pre-treatment was consisted of alkaline digestion with 10 ml of NaOH 10 mM in MeOH for 3 h in an orbital shaker table at room temperature, followed by a centrifugation step at 4000 rpm for 10 min. Then, 3 ml of the supernatant were diluted in 30 ml of water and then extracted using Oasis® WAX 3cc cartridges previously conditioned with 2 × 2 ml of MeOH (0.1% NH_4OH), 2 × 2 ml of MeOH and 2 × 2 ml of water. Elution was accomplished with 2 × 2 ml of MeOH (0.1% NH_4OH). During the next step the extracts were dried under N_2 atmosphere and were reconstituted with Water/MeOH (90:10). Finally, an internal standard mixture was added in order to obtain a final concentration of 5 μg/l in the vial.

Soil The samples were extracted and analysed working in triplicates according to the following protocol: 2 g of dry weight sample were introduced in a 50 ml PP centrifuge tube. Then, the surrogates were added (MXA: 10 μl at 500 pg/μl) and left to rest for 20 min at room temperature. After the equilibration time, the ultrasonic extraction was carried out with 10 ml of MeOH for 1 h and then the extract was centrifuged at 4000 rpm during 20 min. Finally, 3 ml of the supernatant were diluted into 30 ml of water and a purification step was carried out by solid phase extraction (SPE), with Oasis® WAX 3cc, according to the previously summarized procedure. The final extracts were dried under a N_2 atmosphere and reconstituted into initial conditions of the mobile phase Water/MeOH (90:10) in a LC vial equipped with PP insert. As a last step, each extract was fortified with a mixture of labelled PFCs internal standards to obtain a final concentration of 5 μg/l in each vial. The analysis was performed by LC-MS/MS.

Blanks were analysed by passing water and reagents through the entire analytical procedure. Procedural blanks consisting in empty sterilized PP sampling containers, transported with those used for sampling during the entire process and shipment as well, were also extracted and analysed in triplicate.

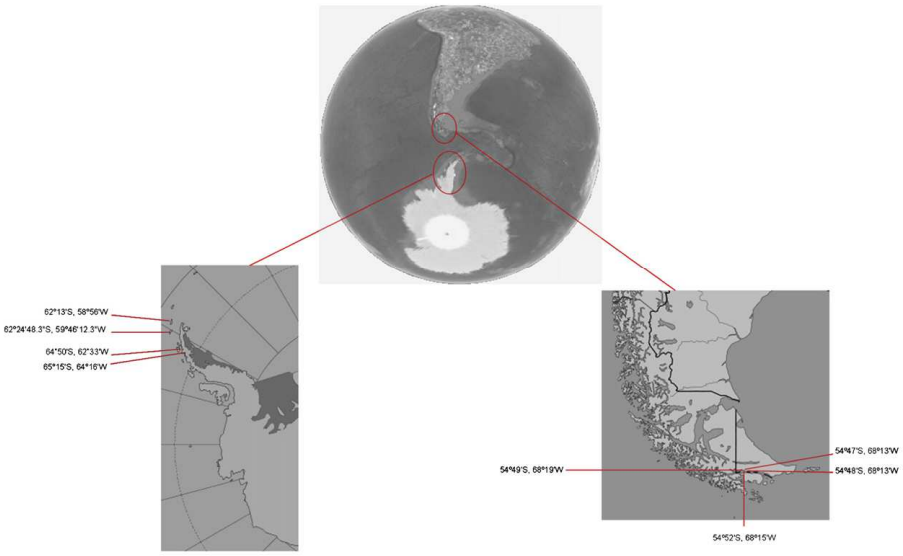


Fig. 1. Map of sampling collection sites. Antarctica sampling points on the left side and Tierra del Fuego points on the right.

2.4. Instrumental analysis

Instrumental analysis, according to previous analytical methods performed by our group (Llorca et al., 2009), was carried out based on LC-ESI-MS/MS. Very briefly, the chromatographic separation was accomplished using a Symbiosis™-Pico (Spark Holland, Emmen, The Netherlands) equipped with a LiChroCART® 125-2, Purospher® STAR, RP-18e (5 μm) analytical column, from Merck, at room temperature. The mobile phase consisted of aqueous ammonium acetate 20 mM (A) and MeOH (B) and was delivered at a flow rate of 0.4 ml/min. The elution gradient condition started at 10% B and rose to 50% B in 2 min, then being linearly increased to 70% B in 4 min, and finally increased to 90% B in 8 min. This percentage was maintained for 1 min more. Finally, the mobile phase was returned to the initial conditions in 1 min, which were maintained for 1 min more. The injection volume was 10 μl.

The LC system was coupled to a quadrupole-linear ion trap mass spectrometer (QqLIT-MS/MS) 4000 QTRAP (Applied Biosystems), equipped with a Turbo Ion Spray source operated in the negative electrospray ionization mode (ESI(−)). Acquisition was performed in single reaction monitoring mode (SRM) to obtain sufficient identification points (IP) for confirming each analyte according to Decision 2002/657/EC. The identification of target analytes was carried out using relation between the highest relative abundances of two m/z transitions and their respective retention times.

For identification purposes different points were accomplished: i) analyte retention time in the sample compared to analyte retention time in the calibration curve should be in agreement; ii) two m/z transition were confirmed for every analyte; iii) ratio between the two transitions in the sample compared to ratio in the calibration curve should be in agreement. These identification points were carefully accomplished for sulfates compounds since it is known that transitions X99 > 80 in biological species can be an interference. The quantification of each compound was carried out using the most intense m/z^- transition, which is indicated in Table S1 in the supporting information section. The optimized parameters were as follows: curtain gas (CUR), 30 (arbitrary units); ion source gas 1 (GS1), 25 (arbitrary units); ion source gas 2 (GS2), 60 (arbitrary units); source temperature (TEM), 350 °C; ion spray (IS), −4500 V; entrance potential (EP), −10 V, collision cell exit potential (CXP) −10 V and declustering potential (DP) −25 V. The dwell time of each MRM transition was 50 ms. Table S1, summarizes the LC-MS/MS optimal parameters. Quantification was carried out by subrogate internal standard additions.

The method limits of quantification (MLOQ) were calculated according to concentration corresponding to a response 10 times equal to signal to noise relation of the analyte in blank biota matrixes, which were in the range of 0.10–2.17 ng/g for PFCAs, 0.08–3.13 ng/g for PFSs, 0.06–0.70 ng/g for PFOSA. MLOQs for guano and penguin dung in the range of 0.80–6.36 ng/g for PFCAs, 57.81 ng/g for PFHxA, 2.60–23.94 ng/g for PFSs, 0.70–1.20 ng/g for PFOSA, and for soils in the range of 0.02–0.27 ng/g for PFCAs, 0.03–0.21 ng/g for PFSs, 0.10–0.12 ng/g for PFOSA. The relative standard deviation or variation coefficient for all positive samples, expressed by %, was below 25%.

In order to avoid any source of instrumental contamination through the analytical process, tubbing of the LC-MS system was replaced by metal, a second analytical column was add prior the injector to delay possible cross contamination from the system or solvents, and through the whole analytical process all sample container, connectors etc., were free of PFCs.

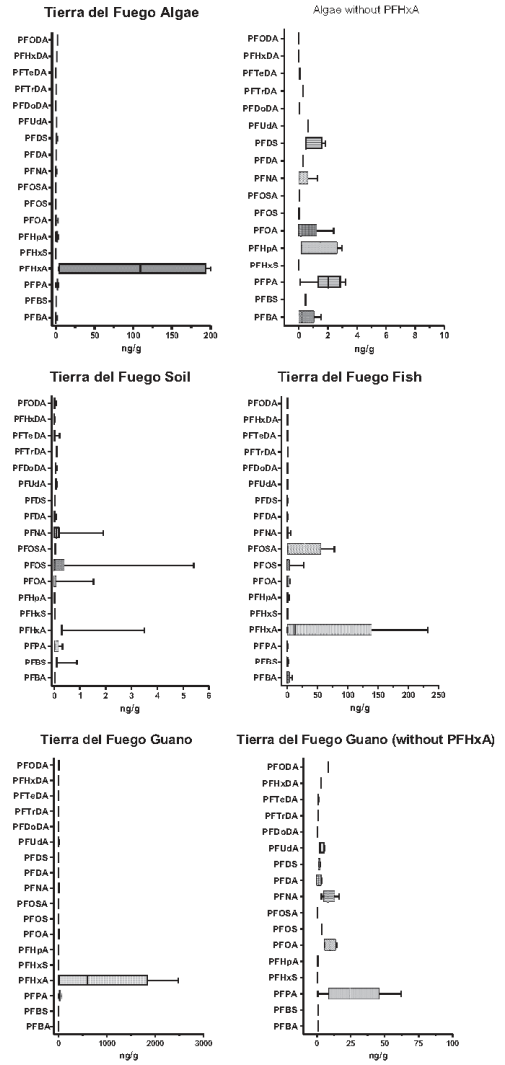
Table 1
Sampling sites and GPS coordinates for the different type of samples.

	GPS	Samples
Tierra del Fuego		
Río Olivia	54°47′S, 68°13′W	Fish
Bahía Encerrada	54°49′S, 68°19′W	Soil
(*Bahía Ushuaia*)		Guano
Playa Larga	54°48′S, 68°13′W	Soil
(*Bahía Ushuaia*)		Algae
Mary Ann Island	54°52′S, 68°15′W	Algae
(*Beagle Channel*)		Guano
Antarctica		
Aitcho Islands	62°24′48.3″S, 59°46′12.3″W	Superficial soil
Ardley Island	62°13′S, 58°56′W	Penguin dung
		Penguin tissues
		Algae
Neko Bay	64°50′S, 62°33′W	Penguin dung
Winter Island	65°15′S, 64°16′W	Penguin dung

3. Results

Figs. 2 and 3 summarize the results of this study and an example of sample chromatograms is presented in Fig. 4. Results for each compound in analysed samples are presented in Tables S2 and S3 of the supporting information section.

Comparing results from Tierra del Fuego and Antarctic region, higher concentrations were detected in Tierra del Fuego, as it was



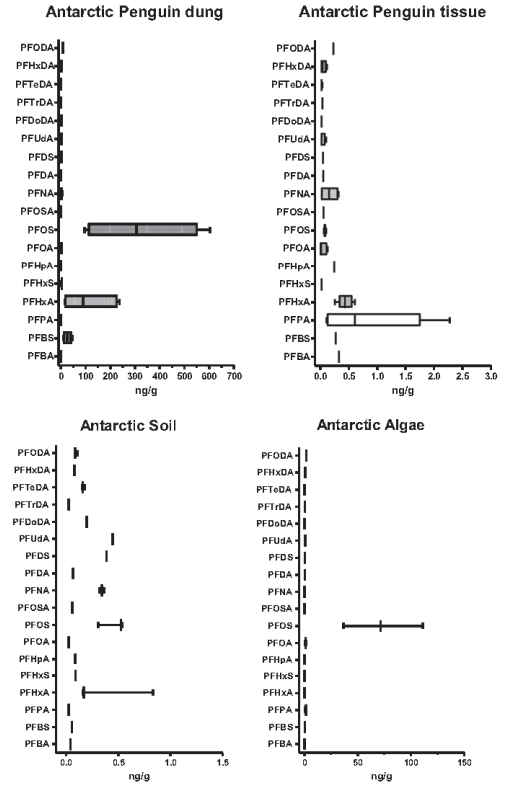


Fig. 3. Box & whisker diagram of *Antarctic* analysed samples; <MLOQ = 1/2MLOQ, <MLOD = 1/2MLOD.

Fig. 2. Box & whisker diagram of *Tierra del Fuego* analysed samples; <MLOQ = 1/2MLOQ, <MLOD = 1/2MLOD.

expected because of the influence of population and activity in the Ushuaia city. Concentrations of PFCs in different types of samples from Tierra del Fuego were ranging from 0.17 to 248 ng/g, being algae, fish and guano samples those presenting the maximum levels of contamination. Guano samples presented extremely high concentrations of PFHxA (1190–2480 ng/g) and PFOSA. PFHxA was present in algae from Tierra del Fuego at concentrations from 4.1 to 200 ng/g, whereas in the Antarctic region PFOS was the predominant compound in algae, with concentrations ranging from 36.6 to 111 ng/g.

Tierra del Fuego soil samples had traces of PFCAs (PFBA, PFPeA, PFHxA, PFOA, PFNA and PFOS) at concentrations ranging between 0.08 and 5.41 ng/g. On the other hand, Antarctic soils contained traces of PFHxA and PFOS at concentrations of 0.16–0.83 ng/g. Other analytes were found below MLOQ in the Antarctic soils.

Due to the sampling permit limitations, all fish and guano samples were collected in Tierra del Fuego. In fish samples, the most representative compounds were PFBA, with concentrations between 7.16 and 8.40 ng/g in liver; PFHxA, exhibiting the higher values, especially in liver samples, were it was in the range between 207 and 232 ng/g; and PFOSA, present in all type of fish samples in the range between 0.57 and 77.80 ng/g and with maximum values in liver again. PFOS and PFOA were also present at concentrations ranging from MLOQ-27.3 ng/g and MLOQ-4.70 ng/g, respectively. PFOS and PFOA were present in skin, muscle and roe, whereas liver samples did not present quantifiable amounts of these compounds.

Guano samples were from 2 different sampling sites both in Tierra del Fuego. Important differences in PFCs concentrations and profiles were found between the different sampling sites. Guano samples from Ushuaia (guano samples 1, 2 and 3 in Table S2 of the supporting information section) presented the highest concentrations for PFCAs, and the highest concentrations were for PFHxA in the range of 1190–2480 ng/g. PFPA was the second most abundant

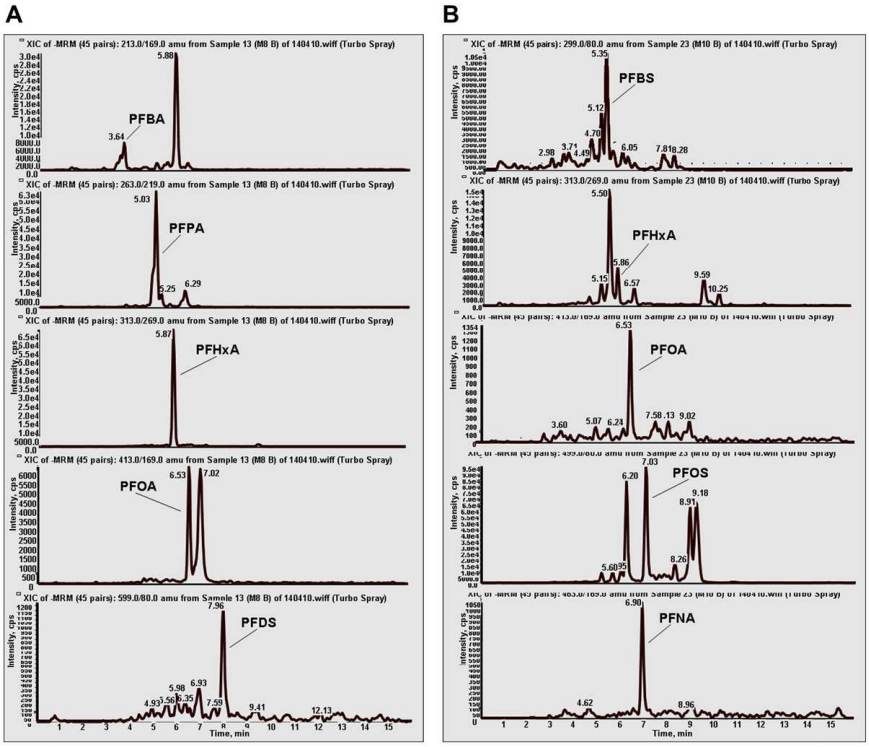


Fig. 4. Extracted ion chromatograms of real positive samples: A) algae samples from Tierra del Fuego; B) penguin tissue samples from Antarctica.

compound with concentrations between 25.6 and 62.2 ng/g. PFOA was in the range of 5.73–13.3 ng/g, PFNA was between 8.89 and 16.2 ng/g and PFDS was around 2 ng/g. Whereas guano samples from the Mary Ann Island, in Beagle Channel, the concentration of PFHxA and PFDS were under the MLOQ. Concentrations of PFPeA were similar to guano samples from Ushuaia, and PFOA was at similar concentrations or slight higher than Ushuaia. PFDA was present in samples from Mary Ann Island in the range of 2.51–3.45 ng/g whereas it was inferior to MLOQ in Ushuaia.

6 penguin samples were collected in the Antarctic region and results of muscle tissues presented trace concentrations between the MLOQ and 2.28 ng/g for all PFCs included in this study. The compounds found at higher concentrations were PFPeA and PFHxA.

Penguin dung concentrations were between 19.9 and 237 ng/g for PFHxA, 0.63 and 3.98 ng/g for PFOA, 0.78 and 4.33 ng/g for PFNA, 10.9 and 45.9 ng/g for PFBS and 95.2 and 603 ng/g for PFOS.

4. Discussion

Algae and soil results are especially interesting because both areas, Tierra del Fuego and the Antarctica can be compared. The predominance in Tierra del Fuego of compounds with carbon chains length inferior to 9 carbons, especially in algae, is of significance because this fact could be associated to the direct introduction of PFCs in water and their assimilation during algae breathing. However, other inputs should also be considered. PFOA is the breakdown product of many polymers used in coatings, showing high persistence in the environment (USEPA, 2010). The biodegradation and arise of oxidation products of fluorotelomer acids and acrylates, alcohols and alkylphosphonic acids are other possible sources of these compounds which are present in algae (Prevedouros et al., 2006; Armitage et al., 2009a, 2009b; Butt et al., 2010). On the other hand, in the Antarctic region, PFOS and PFOA which are the most recalcitrant compounds, were predominant.

Two mechanisms should be considered to explain the LRET capabilities of PFCs. The first suggests atmospheric distribution of neutral, volatile compounds (*flyers*), such as fluorotelomer alcohols and perfluorinated sulfonamido alcohols. *Flyer* compounds are susceptible to suffer atmospheric long-range transport because of their partitioning properties (log *K*aw values estimated between 0 and 1 and log *K*ow around 5), which indicate that these classes of chemicals can be classified as *flyers* according to the Globo-POP model (Lohmann et al., 2007; Wania, 2007). This is also in agreement with the findings of Dreyer et al. (2009). Then, after their transport and cold condensation, these *flyer* compounds can biodegrade as it has already been indicated in previous studies (Rhoads et al., 2008; Wang et al., 2005a, 2005b) or suffer *in-situ* oxidation to form ionic PFCs (Ellis et al., 2004; Wallington et al., 2006). Table 2 presents the partitioning coefficients estimated for selected compounds in the present work, as well as, their classification according to the Globo-POP model. As it can be seen in Table 2, all the selected PFCs were *swimmer* compounds according

Table 2
Partitioning coefficients air/water and octanol/water and the classification of the compounds according to Globo-POP model (Lohmann et al., 2007). Reported values for ionic forms of PFCs.

Compound	log *K*aw	log *K*ow[a]	Classification
PFBA	<<−5	3−7	*Swimmer*
PFPA	<<−5	3−7	*Swimmer*
PFHxA	<<−5	3−7	*Swimmer*
PFHpA	<<−5	3−7	*Swimmer*
PFOA	<<−5	3−7	*Swimmer*
PFNA	<<−5	3−7	*Swimmer*
PFDA	<<−5	3−7	*Swimmer*
PFUdA	<<−5	3−7	*Swimmer*
PFDoDA	<<−5	3−7	*Swimmer*
PFTrDA	<<−5	3−7	*Swimmer*
PFTeDA	<<−5	3−7	*Swimmer*
PFHxDA	<<−5	3−7	*Swimmer*
PFODA	<<−5	3−7	*Swimmer*
PFBS	<<−5	3−7	*Swimmer*
PFHxS	<<−5	3−7	*Swimmer*
PFOS	<<−5	3−7	*Swimmer*
PFDS	<<−5	3−7	*Swimmer*
PFOSA	0−1	3−7	*Flyer*

[a] The log *K*ow is not measurable since these substances are expected to form multiple layers in an octanol–water mixture (U.S. EPA, 2005).

to the mentioned model, excepting PFOSA which is classified as a *flyer*.

The second mechanism is related to the properties of ionic PFCs (negligible vapour pressure, water solubility and moderate sorption to solids), which predicts their accumulation in surface waters (*swimmers*) (Yamashita et al., 2008; Prevedouros et al., 2006; Wei et al., 2007; Ahrens et al., 2010). Recent studies have evaluated the influence of these mechanisms and have been revealed that the dominant phenomenon is the hydrospheric transport for PFOS, PFOA and PFNA (Armitage et al., 2006, 2009a, 2009b). For example, fluorotelomer alcohols have short atmospheric lifetimes in the order of 10–20 days (Ellis et al., 2003). The geographical isolation of Antarctica combined with both, short atmospheric lifetimes of fluorotelomer alcohols and the low yield of the oxidation pathway, significantly reduces the potential for effective atmospheric delivery to the Antarctic continent. In addition, two studies conducted ship-based transect measurements of atmospheric fluorotelomer alcohols levels in the Atlantic Ocean sector from Europe to South America (Jahnke et al., 2007) and from Europe to the Antarctica (Dreyer et al., 2009) showed a clear declining gradient of atmospheric concentrations in these transects. Therefore, atmospheric input of flyer perfluorinated compounds to the Antarctica is principally a function of rapid and direct delivery of contaminated wind masses. The finding of the present work corroborates these previous hypotheses since concentrations obtained for algae, which is an aquatic organism in the low trophic level of the aquatic food chain, were much higher than results obtained for soils, being predominant compounds PFOS and PFOA. Values obtained in soil samples from Tierra del Fuego could indicate multiple sources of PFCs in this area: firstly, directly associated to human activity, followed by atmospheric LRET phenomena and cold condensation. On the other hand, PFCs concentrations in soil from Antarctic region were most likely from atmospheric LRET.

Trace concentrations of PFCs were present in penguin muscle samples, being PFPeA and PFHxA the predominant compounds. Levels of PFOA in penguin muscle were found to be comparable to those reported in previous studies in Arctic fish (Tomy et al., 2004), fur seal pup muscle and penguin eggs analysis (Adélie and Gentoo) from South Shetland islands, Antarctica, with levels between <0.1 and 2.5 ng/g and the hypothesis of oviparous and viviparous transfer of PFCs to eggs and off springs (Schiavone et al., 2009). Evidences of PFCs in penguin dung and penguin tissue could indicate that these compounds are directly introduced into the penguin organism through diet, being possible more influenced by the increasing human activity in the Antarctic peninsula, as has been previously reported for other POPs in this area (Bengtson Nash et al., 2008; Hale et al., 2008).

Concentrations in fish from Tierra del Fuego showed similar PFCs distribution profiles to those encountered in previous studies carried out in other geographic areas. However, concentration levels were lower than those pertaining to more populated and industrialized areas (Llorca et al., 2009), as well as, lower than those found in fish pertaining to higher aquatic food chain levels (Hart et al., 2008). In this case, the most representative compounds were PFBA, PFHxA and PFOSA exhibiting the higher values and especially this last one in liver. The presence of PFOS and PFOA was more representative in other fish tissues as skin, muscle and roe.

Results for guano samples showed great differences according to their sampling site. Guano samples from Ushuaia (guano samples 1, 2 and 3 in Table S2 of the supporting information section) presented the highest concentrations of PFCAs, being PFHxA the most concentrated one (concentrations between 1190 and 2480 ng/g). In this case, the presence of PFHxA could be associated to metabolic degradation of the longest PFCs and other congeners. The source of PFCs can be associated to the diet, because birds from Ushuaia have the habit of going fishing in the port area and in addition, they have included other habits in their diet as the ingestion of human food wastes, whereas guano samples from the Mary Ann Island in Beagle Channel, where birds eat small sized fishes from the deep sea, the concentration and profiles were different. These extremely different profiles can be attributed to the birds' diet.

To date, few authors have studied the presence of PFCs in the Antarctica (Bengtson Nash et al., 2010; Culbertson et al., 2004; Dreyer et al., 2009; Schiavone et al., 2009; Tao et al., 2006; Wei et al., 2007; Ahrens et al., 2009, 2010). Therefore, it is difficult to establish a comparison with previous studies because the here studied matrices are different (see Table 3). However, coincident trends can be established with previous Antarctic studies.

Comparing results for soils, algae, penguin tissues and penguin dung from Antarctica, soil samples exhibited lower values of PFCs. It indicates that, in this case, the contamination can be just associated to atmospheric LRET. Nevertheless, more studies are needed in order to elucidate the atmospheric circulation movement patterns for PFCs. Aquatic organisms and fauna in remote regions as the Antarctica are affected by other influences in addition to the atmospheric LRET: by oceanic transport (Armitage et al., 2006; Prevedouros et al., 2006; Wania, 2007; Bengtson Nash et al., 2010), and the biomagnification through the food chain. Prevedouros et al., estimated that 2–12 tones/year of PFCs are transported to the Artic by oceanic transport, which is greater than the amount estimated to result from atmospheric transport and degradation of precursors (Prevedouros et al., 2006). Although Prevedouros et al. (2006) has pointed water as the dominant exposure medium for Arctic biota for some congeners, this is a debated topic that has been examined in subsequent studies. Measurements of PFCs in seals by Butt et al. (2007, 2008) indicate that exposure to volatile precursors or PFAs resulting from atmospheric oxidation may be more important for Arctic biota. Of course, biotic exposure for some congeners (i.e. anything other than PFOA and PFOS) cannot be explained exclusively by ocean transport, as discussed above. In addition, water was suggested as the primary exposure medium for biota. Yamashita et al. (2008) made reference to the thermohaline circulation system as a transport of PFCs contaminants. Work reported by Ahrens et al. (2010) found levels between < 11 and 51 pg/L of PFOS in Antarctic Circumpolar Current zone and supports the

Table 3
Antarctic concentrations reported in previous works, minimum–maximum concentrations in most of the cases.

	Location	Sample type	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFBS	PFHxS	PFOS	PFDS	FOSA	Volatile FTOH, FTA, FASA and FASE
Ahrens et al., 2009	South Atlantic Ocean	Surface water ($n = 10$); pg/L			<5.7	<5.9	<4.0	<5.1			nd	<1.6	nd	<10		<17–53	
Ahrens et al., 2010	Southern Ocean	Surface water from station number 33 to 39; pg/L	<150	<14	<3.0	<3.0	<5.2	<3.0	<5.5	<11	<5.9	<4.4	<6.3	<11–51		<3.0	
Wei et al., 2007	Between Asia and Antarctica	Surface water ($n = 19$); pg/L			<1–59.5	<1–64.4	<1–44.6	<5–35.5	<1–8.7		<1–3.1	<1–70.2	<1–10.2	<5–71.7			
Tao et al., 2006	Antarctic 2004–2005	Blood of elephant seal ($n = 59$); ng/ml												<0.08–3.52			
	Southern Ocean 2004–2005	Albatrosses liver ($n = 102$); ng/g												<0.5–20.7			
	Antarctica 2001, 1995/1996	Adélie penguin blood ($n = 8$); ng/ml												<0.1			
		Adélie penguin egg ($n = 6$); ng/g												<0.1			
	Antarctica 1998/1999	South polar skua blood ($n = 3$); ng/ml												<0.24–1.36			
		South polar skua egg ($n = 3$); ng/g												2.08–3.12			
Schiavone et al., 2009	Antarctica	Fur seal pup, muscle; ng/g				0.2–0.8	<0.8–1.6	nd	nd	nd	nd		nd	0.6–2.0	nd	<0.4	
		Fur seal pup, liver; ng/g				<1–2.9	<0.4	1.6–4.0	0.1–1.1	<0.9–1.8	<0.4		<0.4	6.2–12.6	<0.4	<0.4	
		Gentoo penguin egg; ng/g				<0.5	<0.2	nd	<0.1–0.3	0.1–1.1	<0.3–1.1		nd	0.2–0.4	na	nd	
		Adelie penguin egg; ng/g				<2.5–8.0	<0.2	<0.1	<1.3–4.1	<2.3–8.8	0.1–0.9		nd	0.2–0.6	na	<0.2–0.5	
Bengtson Nash et al., 2010	Antarctic region	Antarctic Fur Seal liver; ng/g												2.0			
		White-chinned Petrel pectoral muscle; ng/g												1.2			
Dreyer et al., 2009	Southern hemisphere	Gas phase concentrations ($n = 39$); pg/m^3															0.0–7.8
		Particulate phase concentrations ($n = 34$); pg/m^3															0.0–0.6

na: not analyzed.
nd: not detected.

global transport models. This last report could explain the levels of PFOS found in Antarctic algae samples. Finally, aquatic fauna is also influenced by the bioconcentration, bioaccumulation and biomagnification through the food webs, in agreement with the results obtained in the present study for penguin dung and tissues.

This study provides a significant amount of results which globally supports previous models and empirical observations related to the transport, deposition, biodegradation and bioaccumulation patterns of PFCs. However, further efforts are required to establish global circulation patterns and movements of these fluorinated compounds.

5. Conclusions

The present work has studied the presence of 18 PFCs in Tierra del Fuego and the Antarctic region in a total number of 61 samples of different matrices pertaining to different environmental compartments including algae, soils, guano, fish, penguin tissues and penguin dung. The presence of 11 over 18 investigated compounds was detected in Tierra del Fuego (to the authors' knowledge, this is the first work reporting the evaluation of PFCs in this area) and Antarctica.

This study reports the quantifiable presence of PFBA, PFPA, PFHxA, PFOA, PFNA, PFDA, PFBS, PFHxS, PFOS, PFDS and PFOSA, although the most frequent ones were PFHxA, PFOA, PFOS and PFNA. PFCs with carbon chains longer than 10 C have been found below MLOQ. Concentration levels ranged between 0.10 and 240 ng/g in general, but extremely high concentrations of PFOS in guano and PFHxA in penguin dung were detected indicating their exposure through the diet. In most of the cases, results from Tierra del Fuego have shown higher PFCs levels than those from the Antarctica as it was expected.

The presence of PFCs in the Antarctic region can be associated according to the nature of the sample. The absence of long-chain PFCAs from Antarctic samples is a powerful indicator that atmospheric oxidation is not a major source of PFCs in that region. In this context, different introduction routes can be identified: atmospheric LRET (soils); atmospheric LRET and ocean transport (algae) and atmospheric LRET; ocean transport and bioaccumulation and biomagnification (penguin samples). These results support previous studies.

Nevertheless, much more work is still required to evaluate LRET phenomena, the atmospheric circulation of PFCs congeners and their atmospheric oxidation leading to PFCAs, PFSs and low molecular weight products, and how other factors such as the emerging tourism in these areas can influence POPs contamination of remote regions.

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Appendix. Supporting information

Supporting information associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2011.10.027.

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Supporting Information

Fate of a broad spectrum of perfluorinated compounds in soils and biota from Tierra del Fuego and Antarctica

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S1. For identification purposes different points were accomplished: i) analyte retention time in the sample compared to analyte retention time in the calibration curve should be in agreement; ii) two m/z transition were confirmed for every analyte; iii) ratio between the two transitions in the sample compared to ratio in the calibration curve should be in agreement.

Table S1: Analytical and instrumental parameters (LC-QqLit-MS/MS)

Analyte	t_R / min	m/z		CE (V)
		Parent	Daughter	
PFBA	4.2	213	169 *	-25
			119	-25
PFPeA	5.3	263	219 *	-15
PFHxA	5.8	313	269 *	-25
			169	-25
PFHpA	6.2	363	319 *	-25
			169	-25
PFOA	6.5	413	369	-35
			169 *	-25
PFNA	6.9	463	219 *	-25
			169	-15
PFDA	7.5	513	469	-35
			119 *	-35
PFUdA	8.0	563	519 *	-35
			219	-35
PFDoA	8.7	613	569 *	-35
			269	-35
PFTrA	9.4	663	619 *	-35
			219	-35
PFTeDA	10.1	713	669 *	-35
			269	-35
PFHxDA	11.5	813	769 *	-35
			269	-35
PFODA	13.0	913	869 *	-35
			269	-35
PFBS	5.4	299	99	-80
			80 *	-80
PFHxS	6.2	399	99 *	-80
			80	-80
PFOS	6.9	499	99	-100
			80 *	-100
PFDS	8.0	599	99	-100
			80 *	-100
PFOSA	7.7	498	119	-50
			78 *	-100

CE: Collision Energy
* : Quantification transition
t_R: retention time

Table S2 *Tierra del Fuego* samples, concentration of PFCs

		Concentration (SD) (ng/g); (n=3)																
		PFBA	PFBS	PFPA	PFHxA	PFHxS	PFHpA	PFOA	PFOS	PFOSA	PFNA	PFDA	PFDS	PFUdA	PFDoDA	PFTrDA	PFTeDA	PFHxDA	PFODA
Fish skin	1	< MLOD	< MLOQ	0.28 (0.09)	< MLOQ	< MLOD	< MLOQ	0.28 (0.01)	3.07 (0.05)	0.74 (0.11)	0.36 (0.03)	< MLOQ	0.044 (0.009)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ
	2	< MLOD	< MLOQ	0.37 (0.03)	< MLOD	< MLOD	< MLOQ	0.26 (0.05)	4.36 (1.37)	0.90 (0.14)	0.59 (0.16)	< MLOQ	0.08 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ
	3	< MLOD	< MLOQ	0.42 (0.03)	10.90 (0.53)	< MLOD	2.09 (0.13)	3.85 (0.19)	< MLOQ	34.80 (1.67)	5.68 (0.10)	0.19 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ
	4	< MLOD	< MLOQ	0.47 (0.03)	12.30 (0.87)	< MLOD	3.19 (0.40)	4.70 (0.37)	< MLOQ	65.50 (5.87)	4.63 (0.28)	0.81 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ
Liver fish	1	8.13 (0.65)	0.51 (0.09)	< MLOD	232.00 (12.58)	< MLOD	< MLOD	< MLOD	< MLOQ	47.70 (2.41)	< MLOQ	0.15 (0.04)	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
	2	7.16 (0.30)	0.68 (0.07)	< MLOD	207.00 (10.57)	< MLOD	< MLOD	< MLOD	< MLOQ	77.80 (9.47)	< MLOQ	0.29 (0.05)	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
	3	8.40 (0.51)	0.66 (0.05)	< MLOD	222.00 (13.06)	< MLOD	< MLOD	< MLOD	< MLOQ	64.40 (1.33)	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
Fish muscle	1	< MLOD	< MLOQ	< MLOD	42.20 (1.63)	< MLOD	< MLOD	< MLOD	< MLOQ	2.33 (0.36)	0.11 (0.02)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ
	2	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	2.27 (0.14)	0.09 (0.01)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ
	3	< MLOD	1.29 (0.01)	< MLOD	71.70 (8.63)	< MLOD	< MLOD	2.89 (0.57)	< MLOQ	38.00 (1.37)	0.98 (0.03)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
	4	< MLOD	1.28 (0.08)	< MLOD	61.80 (5.68)	< MLOD	< MLOD	3.69 (0.17)	< MLOQ	28.30 (2.45)	0.30 (0.03)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
Roe fish	1	< MLOQ	< MLOD	< MLOD	1.44 (0.62)	< MLOD	< MLOQ	0.72 (0.03)	25.70 (2.72)	0.58 (0.01)	0.99 (0.08)	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ
	2	< MLOQ	< MLOD	< MLOD	2.31 (0.35)	< MLOD	< MLOQ	1.09 (0.29)	27.30 (1.09)	0.57 (0.01)	2.35 (0.10)	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ
Algae	1[a]	0.17 (0.03)	< MLOD	< MLOD	4.81 (0.70)	< MLOD	< MLOQ	0.12 (0.02)	< MLOQ	< MLOQ	0.12 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ
	2[a]	0.10 (0.02)	< MLOD	2.25 (0.30)	3.42 (0.17)	< MLOD	< MLOQ	0.17 (0.01)	< MLOQ	< MLOQ	0.10 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ

	3[a]	0.19 (0.03)	< MLOD	1.82 (0.12)	4.25 (0.54)	< MLOD	< MLOD	0.15 (0.02)	< MLOQ	< MLOQ	0.17 (0.03)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
	4[a]	< MLOQ	< MLOQ	3.22 (0.39)	202.00 (34.61)	< MLOD	2.37 (0.19)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
	5[a]	< MLOQ	< MLOQ	2.67 (0.29)	106.00 (20.33)	< MLOD	1.48 (0.23)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
	6[a]	< MLOQ	< MLOQ	1.49 (0.20)	81.66 (19.37)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
	7[b]	1.10 (0.27)	< MLOQ	2.02 (0.60)	183.00 (2.82)	< MLOD	2.61 (0.19)	0.68 (0.02)	< MLOQ	< MLOQ	0.17 (0.01)	< MLOQ	1.48 (0.19)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
	8[b]	1.03 (0.12)	< MLOQ	3.16 (0.63)	240.00 (1.53)	< MLOD	2.78 (0.07)	2.40 (0.42)	< MLOQ	< MLOQ	1.30 (0.13)	< MLOQ	1.84 (0.11)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
	9[b]	1.52 (0.23)	< MLOQ	1.18 (0.05)	187.00 (6.84)	< MLOD	2.98 (0.26)	1.81 (0.20)	< MLOQ	< MLOQ	1.11 (0.11)	< MLOQ	1.78 (0.21)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
Guano	1[c]	< MLOD	< MLOD	25.60 (2.83)	1190.00 (14.14)	< MLOD	< MLOD	6.08 (0.25)	< MLOD	< MLOQ	9.94 (0.74)	< MLOQ	2.18 (0.28)	5.52 (0.13)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD
	2[c]	< MLOD	< MLOD	29.60 (3.49)	1210.00 (11.18)	< MLOD	< MLOD	5.73 (0.42)	< MLOD	< MLOQ	8.89 (0.54)	< MLOQ	2.11 (0.11)	5.70 (0.02)	< MLOD	< MLOD	< MLOD	1.44 (0.32)	< MLOD	< MLOD	< MLOD
	3[c]	< MLOD	< MLOD	62.20 (5.68)	2480.00 (30.59)	< MLOD	< MLOD	13.30 (0.94)	< MLOD	< MLOQ	16.20 (1.15)	< MLOQ	2.62 (0.07)	< MLOQ	< MLOD	< MLOD	< MLOD	0.99 (0.02)	< MLOD	< MLOD	< MLOD
	4[b]	< MLOD	< MLOD	22.90 (4.10)	< MLOD	< MLOD	< MLOD	6.09 (0.92)	< MLOD	< MLOQ	3.30 (0.30)	3.07 (0.47)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
	5[b]	< MLOD	< MLOD	17.10 (2.63)	< MLOD	< MLOD	< MLOD	14.50 (0.66)	< MLOD	< MLOQ	7.13 (0.41)	2.51 (0.16)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
	6[b]	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	13.20 (0.82)	< MLOD	< MLOQ	7.56 (0.72)	3.45 (0.13)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
Soil	1[c]	< MLOD	< MLOD	0.65 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOD	5.41 (0.17)	< MLOQ	< MLOQ	< MLOQ	< MLOD	0.08 (0.01)	< MLOQ	< MLOD	0.22 (0.01)	< MLOQ	0.039 (0.001)		
	2[c]	< MLOD	< MLOQ	0.06 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOD	0.12 (0.04)	< MLOQ	< MLOQ	< MLOQ	< MLOD	0.11 (0.02)	< MLOQ	< MLOD	0.07 (0.01)	< MLOQ	0.037 (0.002)		
	3[c]	< MLOD	< MLOQ	0.08 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.17 (0.03)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	0.20 (0.04)	< MLOQ	0.079 (0.001)		
	4[c]	< MLOD	< MLOD	0.06 (0.02)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.21 (0.02)	< MLOQ	0.13 (0.01)	< MLOD	< MLOQ	< MLOQ	0.15 (0.01)	< MLOD	< MLOQ	0.020 (0.001)	0.052 (0.002)		
	5[c]	< MLOD	< MLOD	0.15 (0.04)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.42 (0.05)	< MLOQ	0.29 (0.04)	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.019 (0.003)	0.052 (0.003)		

6[c]	< MLOD	< MLOD	0.09 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.39 (0.02)	< MLOQ	0.15 (0.02)	< MLOD	< MLOQ	< MLOD	0.11 (0.01)	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.055 (0.002)
7[c]	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	3.95 (0.67)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
8[c]	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
9[c]	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	3.01 (0.19)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
10[c]	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	0.43 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	0.033 (0.004)
11[a]	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	0.41 (0.02)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.026 (0.002)
12[a]	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	0.99 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.033 (0.001)
13[a]	< MLOD	< MLOQ	0.31 (0.02)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
14[a]	< MLOQ	< MLOQ	0.20 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD
15[a]	< MLOQ	< MLOQ	0.23 (0.04)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD
16[a]	< MLOQ	< MLOQ	0.27 (0.07)	< MLOQ	< MLOD	< MLOQ	0.02 (0.003)	< MLOQ	< MLOQ	0.03 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
17[a]	< MLOQ	< MLOQ	0.08 (0.01)	< MLOQ	< MLOD	< MLOQ	0.07 (0.01)	< MLOQ	< MLOQ	0.07 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
18[a]	< MLOQ	< MLOQ	0.18 (0.02)	< MLOQ	< MLOD	< MLOQ	0.024 (0.001)	< MLOQ	< MLOQ	0.015 (0.003)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD
19[a]	< MLOQ	< MLOQ	0.04 (0.01)	0.91 (0.07)	< MLOQ	< MLOD	0.08 (0.01)	< MLOQ	< MLOQ	0.12 (0.01)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
20[a]	< MLOQ	< MLOQ	0.32 (0.04)	1.01 (0.06)	< MLOQ	< MLOD	0.26 (0.01)	< MLOQ	< MLOQ	0.48 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
21[a]	< MLOQ	< MLOQ	0.10 (0.01)	1.05 (0.10)	< MLOQ	< MLOD	0.07 (0.01)	< MLOQ	< MLOQ	0.14 (0.03)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD
22[a]	< MLOQ	< MLOQ	0.18 (0.03)	3.50 (0.19)	< MLOD	< MLOQ	0.08 (0.01)	< MLOQ	< MLOQ	0.07 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
23[a]	< MLOQ	< MLOQ	0.04 (0.01)	3.32 (0.06)	< MLOD	< MLOD	0.12 (0.03)	< MLOQ	< MLOQ	0.15 (0.04)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD

24[a]	< MLOQ	< MLOQ	0.29 (0.05)	3.12 (0.16)	< MLOD	< MLOQ	0.27 (0.03)	< MLOQ	< MLOQ	0.21 (0.02)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD
25[a]	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	1.53 (0.30)	< MLOQ	< MLOQ	0.85 (0.03)	0.07 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ
26[b]	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	1.11 (0.23)	< MLOQ	< MLOQ	1.44 (0.20)	0.09 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ
27[b]	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.67 (0.04)	< MLOQ	< MLOQ	1.90 (0.05)	0.09 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ
28[b]	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.21 (0.01)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
29[b]	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.18 (0.02)	0.04 (0.01)	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ
30[b]	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.24 (0.01)	0.06 (0.01)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD

a: 54°48'S, 68°13'W; **b**: 54°52'S, 68°15'W; **c**: 54°49'S, 68°19'W

Table S3 *Antarctica* samples, concentration of PFCs

		Concentration (SD) (ng/g); (n=3)																
		PFBA	PFBS	PFPA	PFHxA	PFHxS	PFHpA	PFOA	PFOS	PFOSA	PFNA	PFDA	PFDS	PFUdA	PFDoDA	PFTrDA	PFTeDA	PFHxDA	PFODA
Algae	1	< MLOD	< MLOQ	1.69 (0.20)	< MLOQ	< MLOD	< MLOD	1.80 (0.39)	56.30 (7.29)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD
	2	< MLOD	< MLOQ	1.00 (0.13)	< MLOQ	< MLOD	< MLOD	1.25 (0.25)	111.00 (30.12)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD
	3	< MLOD	< MLOQ	1.41 (0.08)	< MLOQ	< MLOD	< MLOD	0.30 (0.05)	71.50 (5.24)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD
Penguin dung	1[a]	< MLOD	35.70 (5.17)	< MLOQ	19.90 (1.84)	3.77 (0.41)	< MLOD	2.77 (0.65)	460.00 (70.71)	< MLOQ	1.39 (0.27))	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
	2[a]	< MLOD	42.30 (1.25)	< MLOQ	17.30 (1.00)	3.02 (0.12)	< MLOD	3.98 (0.19)	503.00 (79.63)	< MLOQ	4.33 (0.38)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
	3[a]	< MLOD	45.90 (6.47)	< MLOQ	28.60 (1.57)	3.67 (0.61)	< MLOD	2.98 (0.09)	603.00 (12.60)	< MLOQ	2.43 (0.06)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
	4[b]	< MLOD	16.70 (2.96)	< MLOD	237.00 (12.02)	3.51 (0.80)	< MLOD	0.88 (0.10)	153.00 (14.97)	< MLOQ	1.96 (0.06)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
	5[b]	< MLOD	14.80 (2.09)	< MLOD	220.00 (5.26)	3.61 (0.25)	< MLOD	1.02 (0.11)	132.00 (10.42)	< MLOQ	1.88 (0.03)	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
	6[c]	< MLOD	10.90 (0.52)	< MLOD	151.00 (10.47)	2.17 (0.10)	< MLOD	0.63 (0.06)	95.20 (5.15)	< MLOQ	0.778 (0.01)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD
Penguin tissue	1	< MLOD	< MLOQ	1.05 (0.14)	0.61 (0.09)	< MLOQ	< MLOQ	< MLOQ	0.087 (0.009)	< MLOQ	0.31 (0.02)	< MLOD	< MLOQ	0.10 (0.02)	< MLOQ	< MLOQ	< MLOQ	0.10 (0.01)	0.021 (0.003)
	2	< MLOD	< MLOD	2.27 (0.12)	0.44 (0.02)	< MLOD	< MLOQ	0.11 (0.01)	0.101 (0.012)	< MLOQ	0.34 (0.01)	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.12 (0.01)	0.026 (0.001)
	3	< MLOD	< MLOD	1.25 (0.17)	0.52 (0.05)	< MLOD	< MLOQ	0.13 (0.01)	0.103 (0.009)	< MLOQ	0.28 (0.04)	< MLOD	< MLOQ	0.08 (0.01)	< MLOQ	< MLOQ	< MLOQ	0.09 (0.02)	0.028 (0.002)
	4	< MLOD	< MLOQ	0.17 (0.01)	0.25 (0.01)	< MLOD	< MLOD	< MLOQ	0.080 (0.009)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.027 (0.006)	< MLOQ	0.026 (0.001)
	5	< MLOD	< MLOQ	0.15 (0.01)	0.42 (0.01)	< MLOD	< MLOD	< MLOQ	0.076 (0.013)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.028 (0.002)	< MLOQ	0.024 (0.005)
	6	< MLOD	< MLOQ	0.11 (0.02)	0.43 (0.02)	< MLOD	< MLOD	< MLOQ	0.063 (0.017)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	0.038 (0.009)	< MLOQ	0.026 (0.004)
Soil	1	< MLOD	< MLOD	< MLOQ	0.83 (0.03)	< MLOQ	< MLOQ	< MLOQ	0.31 (0.01)	< MLOQ	0.36 (0.02)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	0.18 (0.01)	< MLOQ	0.08 (0.01)
	2	< MLOD	< MLOD	< MLOQ	0.16 (0.01)	< MLOQ	< MLOQ	< MLOQ	0.54 (0.02)	< MLOQ	0.32 (0.01)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.16 (0.03)	< MLOQ	0.11 (0.02)
	3	< MLOD	< MLOD	< MLOQ	0.17 (0.01)	< MLOQ	< MLOQ	< MLOQ	0.52 (0.01)	< MLOQ	0.34 (0.06)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	0.15 (0.01)	< MLOQ	0.08 (0.01)

a: 62°13′S, 58°56′W; b: 64°50'S, 62°33'W; c: 65°15'S, 64°16'W

2.2.4 Assessment of PFASs in beached marine debris and sediments form the Mediterranean area

Plastics have been used during 21st century, playing a key role in the unsustainable development of our world. Plastics are used for a wide range of applications as in packaging, construction, automotive industry, electronic equipments or in sport, leisure and agriculture (The Plastics Portal 2011b).

The European plastics production in 2010 was about 57 million tons (Mt) including: polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET). The most used resin types are polyolefins (PE and PP), which account for around 50% of all plastics demand. The European demand of plastics was distributed, in 2010, in packaging as the largest segment, representing 40.1% of overall demand, followed by Building and Construction (20.4%), Automotive (7%) and Electrical and Electronic equipment (5.6%). Others include different small segments like sport, leisure, agriculture, machinery engineering etc. (Plastics Europe 2011). In 2010 their residues were approximately 24.7 Mt with the 57.9% recovered by recycling (6 Mt) and alternative energy source (8.3 Mt). The other 42.1% was not recovered. This disposal plastic is an increasing cause of water pollution. Industrial products may become marine debris if they are improperly disposed on land or if they are lost during transport or loading/unloading at port facilities (USEPA 2002c). The European plastics industry brought, together, 47 plastics industry organizations from around the world to sign up to "Joint Declaration for Solutions on Marine Litter", which was announced at the 5th International Marine Debris Conference in Hawaii in March 2011 (The Plastics Portal 2011a). Well known examples of marine debris are small plastic resin pellets made of PP or PE, about 2-6 mm in diameter, which are the raw material for the manufacture of plastic products (Derraik 2002). These pellets have been released into the marine environment from accidental spillage during production and processing, transport and handling. Some are buoyant whilst others become suspended or sink (Coe *et al.* 1997; Redford *et al.* 1997; Gregory 2009). Their presence has been reported in most of the world's oceans (USEPA and Gregory 2009).

Hazardous pollutants such as POPs adsorbed and concentrated onto the surface of plastic pellets, where the source is likely being in the surrounding seawater (Mato *et al.* 2000; Hirai *et al.* 2011). Because contaminated pellets may be ingested by animals they could be a source of hydrophobic contaminants in the marine food chain (Gregory 2009). Seabirds are also affected by these types of contaminants. For example, Colabuono *et al.* (2010) assessed the presence of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in plastics ingested by seabirds from the southern Brazil. PCBs were detected in plastic pellets (491 ng/g) and plastic fragments (243 – 418 ng/g) and OCPs in plastic pellets and fragments (68 – 99 ng/g).

In this context, this study aims to assess the presence of 18 PFASs including PFOA and PFOS in different plastic pellet beach samples as well as in sediment beach samples collected from Greece. The sampling points were geographically distributed. Due to the high industrial use of these compounds, these compounds are globally distributed

reaching remote areas as the Antarctic and the Arctic continents (Shoeib *et al.* 2006; Dietz *et al.* 2008). PFASs have been found in water and sediments (Suja *et al.* 2009; Clarke *et al.* 2011; Gómez *et al.* 2011; Houtman 2011; Pico *et al.* 2011; Yang *et al.* 2011) as well in the marine food web due the bioaccumulation and consequent biomagnification (Kelly *et al.* 2009; Kantiani *et al.* 2010; Pico *et al.* 2010). These compounds are considered as emerging organic pollutants since they have not been regulated yet. However, during last years, there are some of them which are proposed to be under regulation (European Commission 2002).

Materials

A mixture of PFASs [MXB; > 98 %] in methanol containing: i) perfluorobutanoic (PFBA), perfluoropentanoic (PFPeA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), perfluoroundecanoic (PFUdA), perfluorododecanoic (PFDoA), perfluorotridecanoic (PFTrA), perfluorotetradecanoic (PFTeA), perfluorohexadecanoic (PFHxDA) and perfluorooctadecanoic (PFODA) acids; ii) perfluorobutanesulfonate (PFBS), perfluorohexasulfonate (PFHxS), perfluorooctanesulfonate (PFOS) and perfluorodecanesulfonate (PFDS) was mixed with the sulfonamide perfluorooctanesulfonamide (PFOSA), > 99 %. This mixture was used for recovery purposes and calibration curves.

A mixture of labeled PFASs [MXA; > 98 %] in methanol containing: [$^{13}C_4$]-perfluorobutanoic acid (MPFBA (13C4)), ion [$^{18}O_2$]-perfluorohexanesulfonate (MPFHxS (18O2)), [$^{13}C_2$]-Perfluorohexanoic acid (MPFHxA (13C2)), ion [$^{13}C_4$]-perfluorooctanesulfonate (MPFOS (13C4)), , [$^{13}C_4$]-perfluorooctanoic acid (MPFOA (13C4)), [$^{13}C_5$]-perfluorononanoic acid (MPFNA (13C5)), [$^{13}C_2$]-perfluorododecanoic acid (MPFDoA (13C2)), [$^{13}C_2$]-perfluorodecanoic acid (MPFDA (13C2)), [$^{13}C_2$]-perfluoroundecanoic acid (MPFUdA (13C2)), added before extraction procedure, was used as a surrogate in order to normalize all the analytical process.

Labeled PFASs: [1,2-$^{13}C_2$]-perfluorooctanoic acid (M2PFOA (13C2); > 98 %) and ion [$^{13}C_8$]-perfluorooctanesulfonate (M8PFOS (13C8); > 98 %), added just before LC injection, were used as internal standards in order to normalize the instrumental analysis response.

All analytical standards, and labeled standards, were supplied by Wellington Laboratories Inc., Canada. Water and Methanol CHROMASOLV®Plus, for HPLC grade and ammonium acetate salt (AcNH4: MW, 77.08; 98 %) were obtained from Sigma-Aldrich, Steinheim, Germany.

Sample collection

Different beached plastic pellets were collected from the beach surface of the Greek Mediterranean Sea. Samples were taken from the high tide line as well as from the berm of the beach from: Corfu Island, Lavrio beach, Kato Achaia beach, Leros Island and Loutropyrgos beach. Plastic pellets were then separated from the sand and stored in aluminum foil at 4ºC. They were shipped to IDAEA-CSIC laboratories (Barcelona) and preserved frozen at -4ºC.

Sediment was sampled as follows: i) the beach face using glass jars from Corfu Island, Kato Achaia beach, Leros Island, Pagasitikos Gulf, and Lavrio, ii) the near shore zone from Loutropyrgos using a shovel, and iii) the bottom of the sea using a core sampler from a sampling boat from Amvrakikos Gulf and Aliveri. The samples were preserved in aluminum foil and shipped to IDAEA-CSIC laboratories. Samples were stored at -4ºC before analysis.

The related samples are summarized in Table 2.2.

Table 2.2: Sample information regarding sampling time, sampling site and possible sources of contamination

Code	Sampling point	Matrix*	Sampling time	Sampling site	Possible contamination sources
1	Corfu Island	P	Spring 2011	beach surface	International Airport and harbour
		S	Spring 2011	beach shore	
2	Lavrio beach	P	Spring 2011	beach surface	Mazut and natural gas power plant; DOW chemicals plant; near to Athens city
		S	December 2010	beach shore	
3	Kato Achaia beach	P	Spring 2011	beach surface	Harbour
		S	Spring 2011	beach shore	
4	Leros Island	P	Spring 2011	beach surface	Airport and harbour
		S	Spring 2011	beach shore	
5	Loutropyrgos	P	December 2010	beach surface	Oil refineries (Aspropyrgos and Elefsina); near to Athens city
		S	December 2010	near shore zone	
6	Pagasitikos Gulf	S	Spring 2011	beach shore	Central Greece International Airport and Cement Industry (Aget Heracles Industry)
7 A	Amvrakikos Gulf	S	Spring 2011	bottom of the sea	National Airport of Preveza-Lefkada and Air force base (Mazona lagoon)
7 B					
8	Aliveri	S	December 2010	bottom of the sea	Cement plant (Heracles); near to Athens city

*Matrix: plastic (P) or sediment (S)

In order to avoid any possible cross contamination, 3 blanks consisting in virgin plastic pellets (2 made of polypropylene (PP) and 1 made of polyethylene (PE)) were obtained from a local manufacturer. Seasand sediment sample was a blank sample obtained through a commercial provider (Sea Sand (SiO2), from Panreac Quimica SA, washed and thin grain; 0.25-0.30 mm of particle size preserved in a PE bottle). The pellet material was verified by Dr. H. Takada's group from the Laboratory of Organic Geochemistry, Tokyo University of Agriculture and Technology, Japan using near-infrared

spectroscopy (Pla- Scan-SH, OPT Research Inc. Tokyo, Japan). Blank samples were shipped, kept and analyzed in parallel with the rest of the samples.

The analysis was carried out along 2010 and 2011.

Sample pre-treatments and extraction procedures

The sample pre-treatment for sediment samples was based on a previous published work by Llorca *et al.* (2011). All the samples were analyzed in triplicate. Very brief, 1 g of dry weight sediment sample was introduced in a 50 mL PP centrifuge tube. Then, the labeled surrogates internal standards were added (10 µL at 50 pg/µL, in methanol) and left to rest for 20 min at room temperature. After the equilibration time, the ultrasonic extraction was carried out with 10 mL of methanol for 1 h. The extract was centrifuged at 4000 rpm during 20 min at 25 ºC, the solvent transferred into a 15 mL PP centrifuge tube and dried under N_2 atmosphere near to dryness. The purification was performed by SPE, with Oasis® WAX 3cc, after reconstitution in 30 mL water. The SPE steps were as follow: i) conditioning: 2 x 2 mL of methanol (0.1% Ammonium Hydroxide), 2 x 2 mL of methanol and 2 x 2 mL of water; ii) loading: samples were loaded under gravity conditions and dried with vacuum for 20 min; iii) eluting: 2 x 2 mL of methanol (0.1% Ammonium Hydroxide). The eluted extract was reduced near to dryness under N_2 current and transferred to a LC vial equipped with a PP insert. The extract was reduced to dryness under N_2 atmosphere and reconstituted into initial conditions of mobile phase: Water/Methanol (80:20).

In the case of plastic pellets, 1 g sample was introduced in a 50 mL PP centrifuge tube, fortified with surrogate internal standards (10 µL at 50 pg/µL, in methanol) and left to rest for 20 min at room temperature. Then, the ultrasonic extraction was carried out following the same procedure as described before for sediment samples. The centrifuged extract was transferred into a 15 mL PP centrifuge tube and dried under N_2 atmosphere near to dryness and directly introduced to a LC vial equipped with a PP insert. Then, the solvent was reduced to dryness under N_2 current and reconstituted into initial conditions of mobile phase Water/Methanol (80:20).

As last step, each extract was fortified with a mixture of labeled PFASs internal standards (M8PFOS and M2PFOA) to obtain a final concentration of 5 µg/L, in vial, in order to normalize the instrumental results.

Solvent blanks were analyzed in parallel by passing methanol through the entire analytical procedure. The plastic pellet blanks, made of PP and PE, were analyzed in parallel in order to avoid the presence of PFASs in the raw material.

Instrumental analysis

The instrumental analysis consisted on liquid chromatography separation coupled to mass spectrometry (LC-MS/MS).

LC was performed using a Symbiosis ™-Pico (Spark Holland, Emmen, The Netherlands), equipped with a LC-column Hypersil GOLD PFP (50 x 3) (Thermo Scientific) suitable for PFASs isomers separation. An extra column BDS Hypersil C8 (50 x 3) (Thermo Scientific) was used after LC pumps, before the injection system, in order to delay the contamination from the system pumps, at room temperature. The mobile phase consisted of (A) aqueous ammonium acetate 20 mM and (B) methanol ammonium acetate 20 mM. The elution gradient conditions started at 20% B and rose to 80 % B in 5 min, and then it was linearly increased to 90 % B in 5 min. This percentage was maintained for 4 more minutes. Finally, the mobile phase returned to the initial conditions in 1 min. These conditions were maintained for 1 more min. The total run time for each injection was 16 min, with the flow rate kept at 0.5 mL/min throughout the run. The injection volume was 10 µL.

The LC system was coupled to a QqLIT-MS/MS, 4000 QTRAP (Applied Biosystems)), equipped with a Turbo Ion Spray source operated in the negative electrospray ionization mode (ESI(-)). Acquisition was performed in SRM mode to obtain enough identification points (IP) for confirmation of each analyte, according to the Commission Decision 2002/657/EC.

The identification of target analytes was performed according to: i) analyte retention time in the sample compared to analyte retention time in the calibration curve should be in agreement; ii) two m/z transition were confirmed for every analyte; iii) ratio between the two transitions in the sample compared to ratio in the calibration curve should be in agreement. Quantification was carried out by surrogate internal standard addition.

Quality parameters

In order to assure the specificity and applicability of the method, different quality parameters were assessed according to the Commission Decision 2002/657/EC.

MLOD were defined as the lowest concentration which the peak area was, at least, 3 times S/N ratio in spiked sample at the lowest level. MLOQ were calculated at 10 times S/N ratio in spiked blank samples at the lowest level and confirmed experimentally.

Recoveries were calculated by spiking, at three different concentrations (10, 25 and 50 µg/Kg for PP plastic pellet blank and 10, 50 and 100 µg/Kg for sediment blank) 1 g of matrix with a mixture of PFASs. The blanks were previously analyzed and confirmed that they can be used as blank materials. The experiments were carried out in quintuplicate. The precision was expressed as intraday repeatability by the analysis of 5 spiked samples, at the lowest level, in the same day. Interday repeatability was calculated by the analysis of 5 spiked samples, at the lowest level, along 5 consecutive days.

2.3 Discussion

2.3.1 PFASs in the Water Cycle

The first step of this work was the development of a multi-analyte analytical method for the rapid analysis of PFASs in water. The main aim was to obtain a robust throughput method, easily to be implemented in water companies for routine analysis of PFASs. In this sense, a method based on online pre-concentration was developed.

After optimization, the method was validated with different types of fortified matrices and the quality parameters were evaluated according to the criteria described in the Commission Decision 2002/657/EC. These results were compared with those using a previously validated method by off-line SPE, for sample purification and pre-concentration. In Figure 2.5 a comparison of MLOQ using the off-line and the on-line approach is presented. This comparison shows that better MLOQ were obtained by the off-line method.

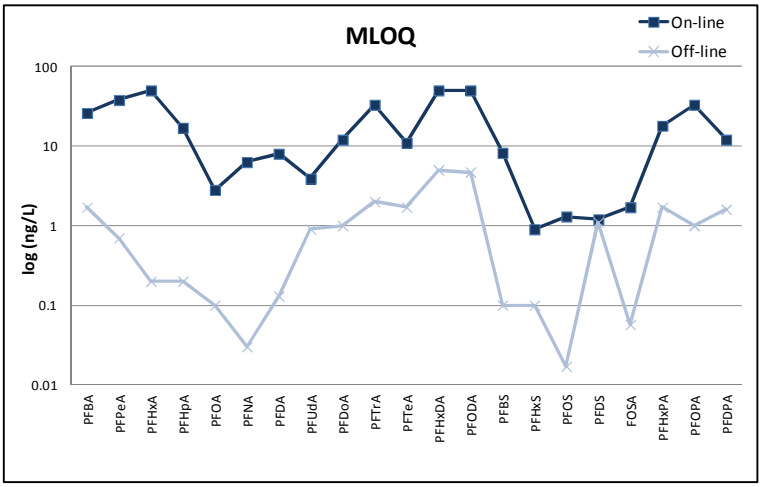

Figure 2.5: MLOQ for off-line and on-line methods developed in this thesis

Although the MLOQ are better with *off-line* approaches, the sample volume required for *on-line* (5 mL) is much lower than for *off-line* (150 mL of surface river water, water from WWTP and PWTP and 250 mL of bottled water, tap water and well water). The time consuming is also reduced and the robustness increases due to the low sample manipulation in the *on-line* approach. All these things make the developed methodology's suitable for the analysis of low-contaminated waters.

Finally, the method was applied to investigate the occurrence of 21 PFASs in water cycle, including: 5 effluents from 5 different WWTPs, 48 river water samples (24 from Spain and 24 from Germany), 2 well water samples, 3 samples along different steps of the purification processes in a PWTP located at the federal state of Hessen (Germany), 84 tap water samples and 6 mineral bottled water samples.

The results showed that the more frequently detected analytes were perfluoroalkyl acids, in particular, PFBA, PFHpA, PFOA and PFOS. The highest concentrations were found in surface waters and effluents from WWTPs (in the range of ng/L and µg/L), as it was expected.

In Figure 2.6 is presented a graph with the main quantified compounds in the effluents of the studied WWTPs. As can be seen, PFASs were detected in all wastewaters. However, each WWTPs exhibited unique distributions of PFASs. In addition, some similar trends were observed between WWTPs in Spain (bars in blue tones) and WWTPs in Germany (bars in orange tones).

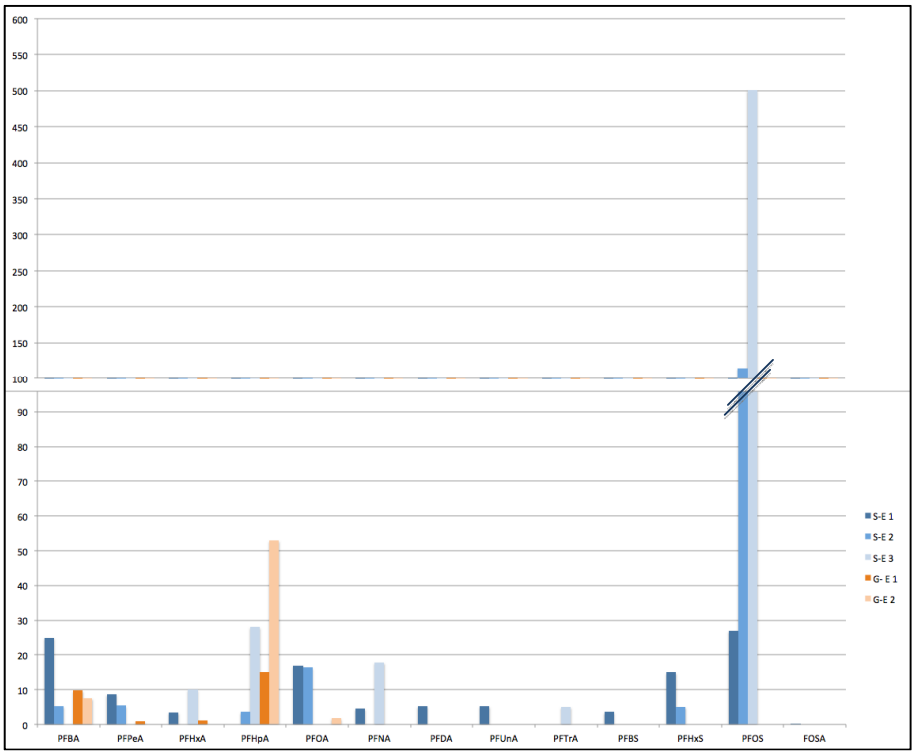

Figure 2.6: main compounds quantified in the effluents of the 5 WWTPs. Blue tones correspond to Spanish WWTPs and orange ones to German WWTPs. The results are expressed in ng/L.

Shorter carbon chain compounds, such as PFBA, PFHxA and PFHpA, were found in all the samples from both countries, reflecting the replacement of PFOS and PFOA. However, PFOS and PFOA were found in surface waters from Germany and Spain indicating their long stability and poor degradability. On the other hand, in Germany samples can be observed that short chain PFASs were the prevalent compounds. In contrast, in Spain PFOS and PFOA were the most frequent analytes. In Spain PFOS was detected in 46% of surface waters samples and PFOA in 63%. Figure 2.7 shows the PFASs profile in river water samples from both countries.

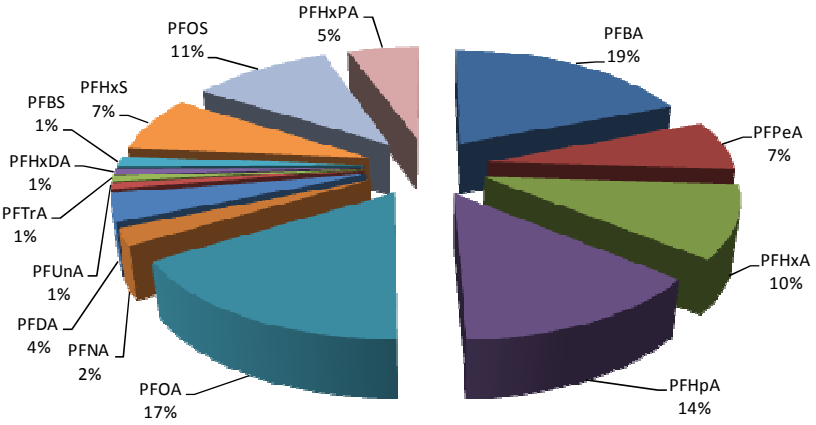

Figure 2.7: contribution percentage of PFASs, for concentrations higher than MLOQ, in river waters from Germany and Spain.

These results are in agreement with other recent studies indicating that shorter chain PFASs are predominant in urban/industrial areas, while longer chain PFASs are, in general, predominant in the fine-grained sediments from major depositional basins (Myers *et al.* 2012). In addition, some authors have encountered that there is a progressive increase of the contamination of short chain PFASs during the last years (graph A from Figure 2.8). However, the study performed by Skutlarek *et al*. (2006) presented extremely high concentrations of these analytes because of a contamination episode. The substitution of 8 carbon chain PFASs by shorter ones can also be observed in the graph B from Figure 2.8 although, it should be considered that the sampled areas belonged to different countries with different climates and the rivers are altered by different water regimes. These differences make impossible to strictly compare the results and just general tendencies can be considered. Even though, the rough approximation represented in graph bars in Figure 2.8 evidences the changes in production of PFASs.

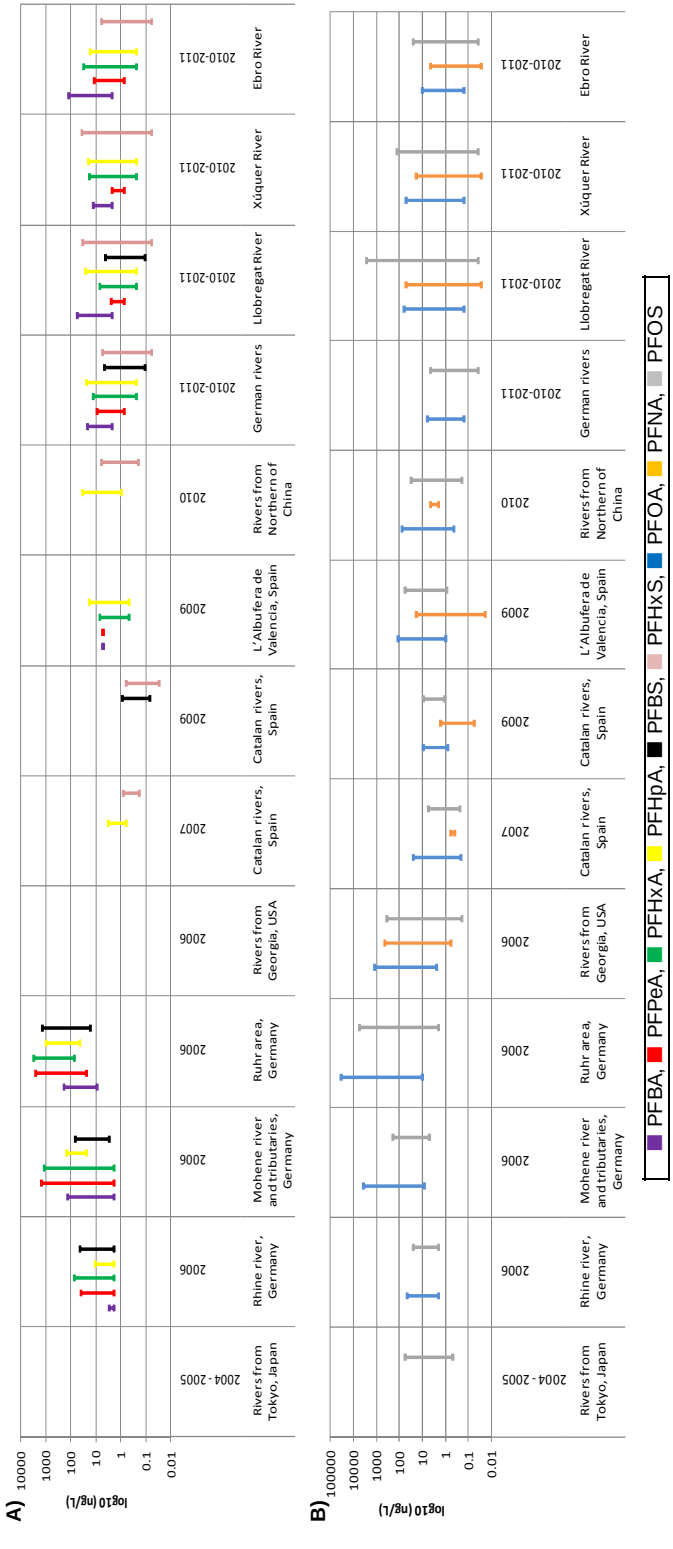

Figure 2.8: Concentration ranges of PFASs in surface river waters, expressed in $\log_{10}$(ng/L), from different published works. Rivers from Tokyo from Takazawa *et al.* (2009), Rhine River, Mohene River and Rhur area River from Skutlarek *et al.* (2006), rivers from Georgia from Konwick *et al.* (2008), 2007 Catalan rivers from Ericson *et al.* (2008b), 2009 Catalan rivers from Sánchez-Avila *et al.* (2010), l'Albufera de València from Picó *et al.* (2011), rivers from Northern of China from Wang *et al.* (2012)(2012), Llobregat River, Xúquer River and Ebro River from this thesis.

On the other hand, as PWTPs are one of the main introductions of PFASs in tap waters, water samples from different steps in a German PWTP were investigated. The results showed a redistribution of PFASs along the water treatments. In this case, the water cached from river for potabilization purposes contained PFHpA, PFHxPA and PFOPA. After the potable treatment, the water from the PWTP effluent (which arrives to the consumers through the tap water) contained PFHpA and PFHxPA at lower concentrations due to the redistribution of these analytes.

Regarding the analysis of different tap waters, German tap water contained PFHpA which was also detected in effluent water from PWTP. In general, the most detected compounds in Germany and Spain have been the shorter carbon chain PFBA, PFPeA and PFHxS as well as the C8 based compounds: PFOA and PFOS (Figure 2.9).

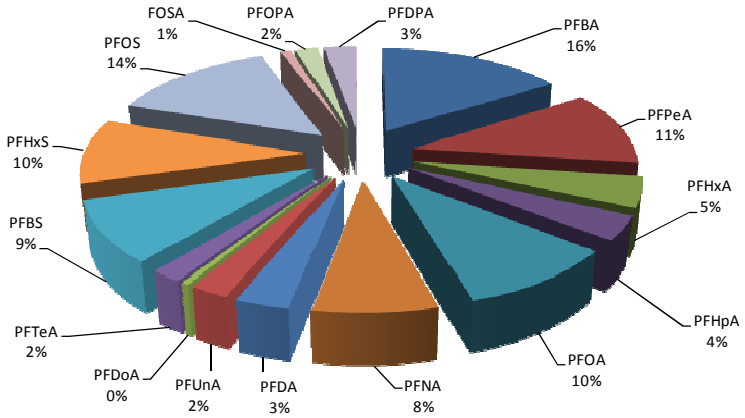

Figure 2.9: Contribution percentage of PFASs, at concentration higher than MLOQ, in tap waters from Germany and Spain.

In the case of Spanish tap waters, the most ubiquitous compound was PFBA followed by PFOS. This pattern is a little bit different than reported before in tap waters from Catalonia by Ericson *et al.* (2008b; 2009). The authors detected PFOA and PFOS as the most ubiquitous compounds. This difference is an indicative of the substitution of longer carbon chain based PFASs in the industry for shorter ones between 2008 and 2011. Although the general concentrations of PFASs were at low ng/L, some tap water samples in Spain showed extremely high contents of PFASs. In particular, the concentrations of PFOS in some cases exceeded 100 ng/L. some tap water samples in Spain showed extremely high contents of PFASs. In particular, in some samples from the same village (near to Barcelona city), the concentrations of PFOS exceeded in some cases 100 ng/L. The origin of this compound could be related to the direct contamination (from water containers or tubing) or from the potable water.

To compare the PFASs in drinking water from this Thesis with previous works, all the results for shorter carbon chain (PFBA, PFPeA, PFHxA, PFHpA, PFBS and PFHxS) and for longer carbon chain (PFOA, PFNA and PFOS) are reported in Figure 2.10.

According with the provisional health advisory levels established by the USEPA in 2009 for PFOA and PFOS (EPA 2009), the majority of the samples may not pose an immediate health risk to consumers, with the exception of 2 samples from Sant Feliu de Llobregat, with PFOS concentrations exceeding these values. Other health-based values for PFHxS (600 ng/L), PFBS (600 ng/L), PFHxA (1000 ng/L), and PFPeA (1000 ng/L) did not exceed the reported concentrations in this thesis (Minnesota Pollution Control Agency 2007; EPA 2009).

After the publication of this work the third Unregulated Contaminant Monitoring Rule for PFOS (40 ng/L), PFOA (20 ng/L), PFNA (20 ng/L), PFHxS (30 ng/L), PFHpA (10 ng/L) and PFBS (90 ng/L) was established (USEPA 2012). Based on these concentrations, the PFASs in tap water samples analyzed were higher for most of the compounds. However, there is no data regarding the continuous exposure, which is especially relevant for drinking water. On the other hand it is important to consider the total sum of compounds. Due to the differences in health-based concentrations for humans, further research is highly required for complete risk assessment of human exposure to PFASs.

As observed in surface river waters, levels in drinking waters along the years showed a similar profile. In general terms, the presence of shorter carbon chain in tap waters has increased (graph A from Figure 2.10). This is another indication of the substitution of C8 based PFASs for shorter ones such as PFBA, PFPeA, PFHxA, PFHpA, PFBS and PFHxS. This hypothesis is also being confirmed with the study along the years of PFOA and PFOS (graph B from Figure 2.10). Even though the phase out of C8 PFASs started in 2000, these compounds are still present in surface river waters (Figure 2.8) and, consequently, in tap waters.

Finally, the results of different brands of mineral water were consistent with previous works (Ericson *et al.* 2008b). In general, concentrations were below de MLOQ with the exception of few German brands, whose contained PFHxA, PFHpA and PFOS at trace concentrations of ng/L.

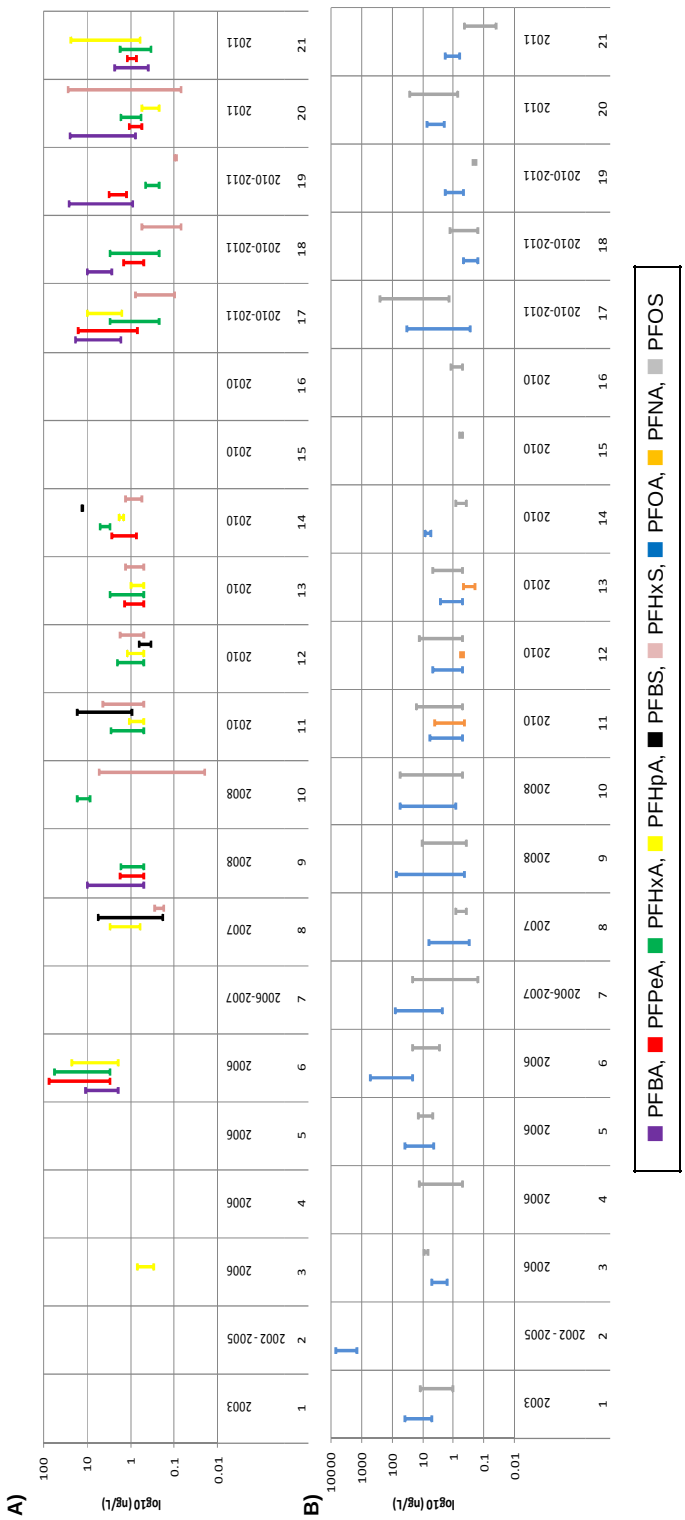

Figure 2.10: Concentration ranges of PFASs in drinking tap waters, expressed in $\log_{10}$(ng/L), from different published works. A) PFBA, PFPeA, PFHxA, PFHpA, PFBS and PFHxS; B) PFOA, PFNA and PFOS. 1) Osaka, Japan, from Saito *et al.* (2004); 2) USA from Emmet *et al.* (2006); 3) Lake Maggiore area, Italy, from Loos *et al.* (2007); 4) Japan from Pham-Hong *et al.* (2006); 5) New Jersey, USA, from New Jersey Department of Environmental Protection (2007); 6) Ruhr area, Germany, from Skutlared *et al.* (2006); 7) Osaka, Japan, from Takagi *et al.* (2008); 8) Catalonia from Ericson *et al.* (2008b); 9) China from Mak *et al.* (2009); 10) Catalonia from Ericson *et al.* (2009); 11) Sweden, 12) Barcelona Italy, 13) Belgium, 14) The Netherlands, 15) Norway, and 16) Germany from Ullah *et al.* (2011); 17) province, Catalonia, 18) Ebro River basin, 19) Llobregat River basin, 20) Xúquer River basin and Valencia city and 21) Germany from this thesis.

2.3.2 PFASs in sewage sludge

Main limitations in the analysis of PFASs in complex matrices are the matrix effects, as matrix enhancement or ion suppression. For example, Dasu *et al.* (2010) reported the hydrolysis of fluorotelomer compounds to fluorotelomer alcohol during the solvent extraction of soils.

In Figure 2.11 are presented the percentage of matrix enhancement and ion suppression obtained in fortified samples.

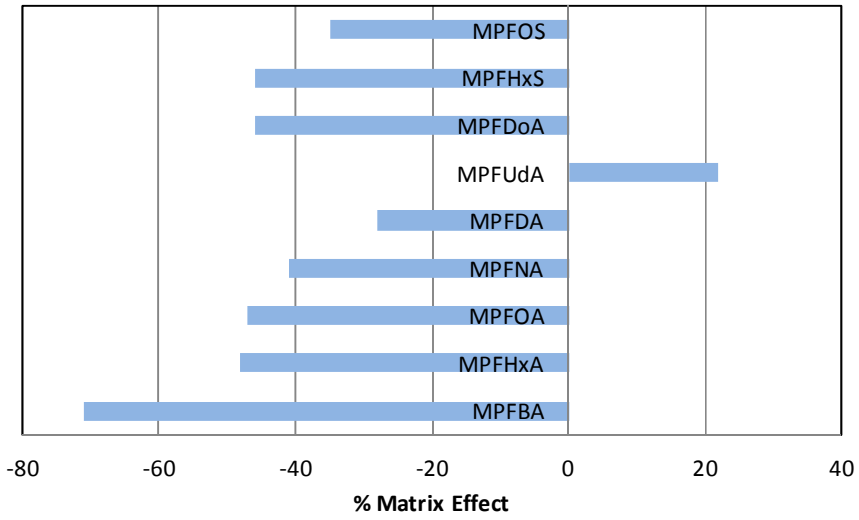

Figure 2.11: Percentage of matrix effect observed in the present work for different spiked samples, with a mixture of PFASs labelled standards at 10 µg/L. The matrix effect was calculated according to:
Matrix effect (%) =
[100 × Surrogate Peak in extracts (n=3)/ Surrogate Peak in mobile phase (n=8)] – 100

Where: matrix effect > 0 = ion enhancement and matrix effects < 0 = ion suppression.

Most of the selected analytes exhibit ion suppression. Therefore, one of the main objectives of this work was the development and the optimization of an analytical approach based on an extraction and detection method able to eliminate most of matrix interferences, but minimizing possible looses of target compounds. In this case we have developed a PLE extraction to improve the extraction efficiency, sample manipulation and reduce solvents consumption.

The methodology allows, in general, a MLOQ between 0.05 – 2.77 ng/g of PFASs, recoveries ranging from 57 to 120 %, in most of the cases, and 2 – 30% of RSD. Comparing these results with other published methods for this type of matrix, good quality

parameters are obtained by PLE extraction (Table 2.3). The extraction by this method, followed by a clean-up based on SPE, decreases the matrix effect of the sample. However, as can be seen in Figure 2.11, the matrix effects were still present due to the problems during the ionization of the samples.

The most relevant results in the sludge samples from 5 different WWTPs are the presence of PFOS and PFOA. In general, PFOS was at much higher amounts than PFOA. Compared to the previous reported results in the effluents of WWTPs from Spain and Germany, the rough mass balance is not concluding since PFOS has been found at extremely high concentrations in sludge and effluents. Once PFASs reach the WWTPs, the treatments resulted ineffective for these highly stable compounds. However, there is a redistribution of the most hydrophilic and the most hydrophobic compounds: while perfluoroalkyl acids are discharged to the media through effluents, perfluoroalkyl sulfonates are more likely retained in the sludge.

Figure 2.12 present the results from previous published works in order to compare them with the results from this thesis.

According to the results pointed out in Figure 2.12, temporary trends related to the industrial uses of PFASs cannot be distinguished. As an example, the presence of the most recalcitrant and ubiquitous in sludge, PFOS, is incredible higher and it has not decreased along the years. However, the represented results correspond to different WWTPs, from different cities, receiving industrial and/or urban wastes. It is impossible to conclude a clear temporary trend in the use of these analytes.

Nevertheless, it is confirmed that sludge from WWTPs contains high concentrations of the most recalcitrant PFASs, PFOA and PFOS. Their presence is related to its initial use or as degradation products from related compounds. The application of this sludge as nutrient source in agricultural lands can introduce PFASs into the food as indirect source.

On the other hand, in recent years the research has been focused on the study of degradation mechanisms of fluorochemicals because an optimum removal treatment for the most recalcitrant compounds such as PFOS or PFOA is required.

Table 2.3: sample extraction procedures for sludge samples from different published works

Analytes	Extraction procedure	Quality Parameters	Reference
	Ionic Pair extraction		
PFOA, PFNA, PFDA, PFUnA, PFOS, 4:2, 6:2, 8:2, 10:2 diPAP	1) 2 g sludge; 2) 4 mL 0.25 M $NaCO_3$ + 1 mL 0.5 M TBAS, pH 10; 3) extraction with 5 mL aliquots of MTBE; 4) MTBE aliquots combined; 5) dryness under N_2; 6) reconstituted in 0.5 mL of MeOH and filtered by 0.2 µm nylon filter.	• MLOQ: 0.0625 ng/g dw (PFCs) 0.375 ng/g dw (diPAPs) • Recoveries: 17-105% (PFCs) 38-53% (diPAPs)	(Eon *et al.* 2009)
PFPeA, PHxA, PFHpA, PFOA, PFNA, PFDoA, PFTeA, 6:2 FTUCA, 8:2 FTUCA, PFBS, PFHxS, PFOS, FOSA, n-MeFOSA, n-EtFOSA, n-MeFOSAA, n-EtFOSAA	1) 0.5 g sludge; 2) (0.5 mL TBAS 0.5 M+ 4 mL 0.25 M $NaCO_3$, pH 10) + 2 x 5 mL MTBE; 3) evaporated under N_2; 4) reconstitution in 5 mL MeOH; 5) clean-up by Envicarb cartridges	• MLOQ: 0.6-30 ng/g • Recoveries: 70-169% • RSD: 2-20%	(Zhang *et al.* 2010)
	Solid-acid liquid extraction		
PFHxS, PFOS, PFDS, FOSAA, N-MeFOSAA, N-EtFOSAA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	1) 0.1 g sludge; 2) 7.5 mL 1% AcH; 3) vortexed, sonicated at 60 °C (15 min) and centrifugated at 3000 rpm, 10 min; 4) supernatant separation and repeat steps 2-4 twice; solid part + 1.7 mL MeOH:water (1% AcH) (9:1, v:v) and vortexed, sonicated, and centrifugated; 5) supernatants combination centrifugued 1 h; 6) SPE by 500 mg C18 (conditioning:10 mL MeOH, 10 mL 1% AcH; rinsed: 10 mL water and 2 h vacuum; elution: 4 mL MeOH); 7) concentrated under N_2 to 2ml; 8) 800 µL MeOH + 1200 µL 0.01% NH_4OHaq + 6 mL MeOH:0.01% NH_4OHaq (70:30) addition; 9) stored at 4 °C and before analysis: 500 µL extract + 50 µL IS	• MLOD: 0.7-2.2 ng/g dw (sludge) • Recoveries: 56-89% (aged sediment), 41-91% (digested sludge), 37-98% (primary sludge)	(Higgins *et al.* 2005)
PFBS, PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTeA, PFHxDA, PFODA	1) 0.1 g sludge + surrogate; 2) 7.5 mL (1% AcH); 3) sonication 20 min at 60 °C; 4) centrifugation at 3500 rpm, 10 min; 5) supernatant separation and pellet with 1.7 mL MeOH (1% AcH, 90:10), sonication 20 min at 60 °C and centrifugation; 6) repeat steps 2-5 twice and combine extracts; 7) 7.5 mL 1% AcH addition; 8) SPE by Oasis HLB (conditioning: MeOH, 1% AcH; washing: 20% MeOH; elution: 5mL MeOH); 9) concentration to 1ml and filtration by 0.2 µm nylon filter.	• Recoveries: 70-130% 37-65% (PFCAs > 11C) • LOQ < 25 ng/g dw	(Sinclair *et al.* 2006)
PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA, PFDoA	1) 0.1 g sludge + surrogate; 2) 7.5 mL (1% AcH); 3) sonication 20 min at 60 °C; 4) centrifugation at 3500 rpm, 10 min; 5) supernatant separation and pellet with 1.7 mL MeOH (1% AcH, 90:10), sonication 20 min at 60 °C and centrifugation; 6) repeat steps 2-5 twice and combine extracts; 7) 7.5 mL 1% AcH addition; 8) SPE by Oasis HLB (conditioning: MeOH, 1% AcH; washing: 20% MeOH; elution: 5mL MeOH); 9) concentration to 1ml and filtration by 0.2 µm nylon filter.	• Recoveries: 59-107% (sludge)	(Loganathan *et al.* 2007)

(Table 2.3)

PFOA, PFOS	1) 0.1 g sludge; 2) 7.5 mL 1% AcH; 3) vortexed, sonicated at 60 ºC, 15 min and centrifugation at 3000 rpm, 10 min; 4) supernatant separation and repeat steps 2-4 twice; 5) solid separated part + 1.7 mL MeOH:water 1% AcH (9:1), vortexed, sonicated and centrifugated; 6) combined supernatants centrifugued 1 h; SPE by 500 mg C18 cartridges (conditioning: 10 mL MeOH,10 mL 1% AcH; rinsed: 10 mL water and 2 h vacuum; elution: 4 mL MeOH); 7) concentrated under N_2 to 2 mL; 8) addition of: 800 µL of MeOH + 1200 µL of 0.01% NH_4OHaq + 6 mL of MeOH:0.01% NH_4OHaq (70:30); 9) stored at 4 ºC; 10) prior analysis, 500 uL extract + 50 uL IS (20-50 ng/mL)	• MLOD: 0.5 ng/g (PFOS) 0.8 ng/g (PFOA) • Recoveries: 85–114% (PFOS) 71–98% (PFOA)	(Guo *et al.* 2008)
PFOA, PFOS	1) 0.1 g freeze dried sludge in PP tube; 2) 7.5 mL 1% AcH; 3) vortexed, sonicated (60ºC) 15 min and centrifugation at 3000 rpm 10 min; 4) supernatant collection (repeat steps 2-4 twice); 5) solid part + 1.7 mL [MeOH:water 1% AcH (9:1)], vortexed, sonicated and centrifugated; 6) supernatants centrifugued 1 h; 7) combined extracts (35.1 mL) loaded onto SPE cartridges (Oasis HLB 500 mg) and rinsed with 10 mL water (conditioning: 5 mL MeOH, 10 mL water(1% AcH)); 8) elution: 2x2 mL MeOH; 9) extracts diluted with 6 mL Cl_2CH_2 and loaded onto the silica cartridge (conditioning: 5 mL Cl_2CH_2/MeOH (60:40, v/v)); 10) eluent concentrated to dryness under N_2; 11) reconstitution in 1 mL MeOH:water (0.01% NH_4OH) (70:30; v:v) and filtration by 0.2 µm nylon syringe filter.	• MLOQ: 1 ng/g dw (PFOS) 5 ng/g dw (PFOA) • Recoveries: 84% (PFOS) 70% (PFOA)	(Yu *et al.* 2009)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	1) 0.1 g sludge + IS + 7.5 mL 1% AcH; 2) sonication at 60 ºC, 20 min and centrifugation at 3500 rpm, 5min; 3) supernatant separation and solid with 1.7 mL MeOH:1% AcH (9:1), sonication and centrifugation; 4) repeat step 3 for solid part twice and combine all the extracts; 5) SPE by Oasis HLB; 6) extract evaporated under N_2 and reconstitution in 1 ml MeOH.	• MLOQ: 1-5 ng/g • Recoveries: 21%(PFDS) 69-119% the rest of PFCs	(Guo *et al.* 2010)
	Solid-alkaline liquid extraction		
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrA, PFTeA, PFHxS, PFOS	1) 0.5 g dw + 0.5 mL NaOH 1 M; 2) 30 min sonication/heat and overnight incubation; 3) neutralization (w/HCl); 4) 10 mL ACN:MeOH(1/1, v/v), shaked 1 h; 5) supernatant separation (repeat steps 4-5 one more time); 6) 2 mL of combined extracts (20ml) + 98 mL water at pH4; 7) sonicated 30 min; 8) SPE by HLB cartridges (conditioning: 5 mL MeOH, 5mL water; wash step: 5 mL 25% MeOH; elution: 5 mL MeOH); 9) extract dryed under N_2; 10) reconstitution in 1ml ACN:water (60:40) + IS additon	• MLOQ: 1.8-6.8 ng/g dw • Recoveries: 82-104%	(Yoo *et al.* 2009)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, FOSA, N-MeFOSA, N-EtFOSA	1) 1 g sludge (5 g Sediment) + 10 mL MeOH (1% NH_4OH); 2) vortexed 30 s and sonicated 10 min at 60 ºC; 3) supernatant + water (1% AcH); 4) preconcentration under N_2 to 5ml; 5) purification by dispersed solvent (EnviCarb) and rinse with 2.5 mL MeOH (1% NH_4OH,1% AcH); 6) concentrate to 1ml under N_2.	• MLOD: 0.14-1.43 ng/g (sludge) • Recoveries: 62-94% (sludge) • RSD: 2-7% (sludge)	(Ma *et al.* 2010)

(Table 2.3)

	Solid-liquid extraction		
	Ultrasonic extraction		
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	1) 5 g liquid sludge + surrogates (into PP tube) 2) 20 mL MeOH, wrist-action shaker, mixed 30 min 3) settled 30 min or centrifugated at 3000 rpm, 20 min 4) 1 mL supernatant + 25 mg Envi-Carb graphitized carbon adsorbent + 50 µL AcH, vortexed and centrifugated 10000 rpm, 30 min 5) 500 µL supernatant + 500 µL water, mixed 6) Internal standard addition	• MLOQ: 1 ng/g • Recoveries: 70-120% • RSD: < 20%	(Powley *et al.* 2005)
PFBS, PFHxS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, FOSA, N-MeFOSA, NEtFOSA	1) 1 g sludge + surrogates + 10 mL MeOH in a PP tube; 2) shaked 10 min, ultrasonic bath at 40 ºC for 30 min and centrifugation at 3000 rpm (15 min); 3) supernatant separated and solid part with 9 mL MeOH in an ultrasonic bath and centrifugation; 4) repeated step 3 one more time and combine all the extracts; 5) purification by disperse solvent (EnviCarb); 6) eluted extract + 1 L water; 7) SPE by Oasis WAX (conditioning: 12 mL MeOH (0.1% NH_4OH),12 mL water; cleaning: 12 mL 25 mM NaAc (pH4); elution: 8 mL MeOH (0.1% NH_4OH)); 8) aliquot + IS	• MLOQ: 0.02-0.71ng/g • Recoveries: 64-129% • RSD: 3-22%	(Navarro *et al.* 2011)
PFOA, PFNA, PFDA, PFUnA, PFOS, FOSA	1) 7 g sludge (dw) + surrogates (into PP tube) 2) 2 x (10 mL MeOH, ultrasonic bath) 3) extracts pooled + 1 L water 4) SPE by C18 (conditioning: MeOH, water; eluting: 10 mL MeOH) 5) evaporated to dryness 6) reconstitution in 1 mL MeOH:Water(2 mM NH_4Ac) (1:1)	• MLOD: 0.4-1.7 ng/g (dw) • Recoveries: 83-105% (FOSA) 26% • RSD: 4-8%	(Bossi *et al.* 2008)
N-EtFOSE, N-EtFOSAA, FOSAA, FOSA, N-EtFOSA, PFOSI (perfluorooctane sulfinate), PFOA, PFOS	1) lyophilized solids + MeOH; 2) sonication 20 min, 60 ºC, and centrifugation at 3600 rpm,15min; 3) extract poured into a new tube + 20 mL MeOH; 4) purification by dispersed solvent (ENVI-Carb); 5) extracts diluted 1/10 with MeOH and again diluted with 3/5 water (0.01% NH_4OH); 6) 0.5 mL in glass autosampler vial; 7) (in parallel) SPE from headspace sampling eluted with 10 mL of MeOH and diluted 3/5 with water (0.01% NH_4OH).	• MLOQ: 0.1 µg/L(vial) 46-49 pmol (microcosm) • Recoveries: 83-119%	(Rhoad *et al.* 2008)
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA	1) 0.5 g sludge (dw) in a 15 mL PP tube + 0.5 mL water + IS; 2) sequentially extraction by 2.5, 1.5 and 1.0 mL of MeOH (shaking the slurry 10 min and sonication 20 min at 40ºC); 3) centrifugation at 3500 rpm, 8 min; 4) combined extracts + Envicarb graphitized carbon adsorbent (300 mg); 5) shaked 20 min, centrifugation at 3500 rpm along 30 min; 6) 4 mL supernatant + IS + 1 mL water (0.01% NH_4OH); 7) stored at – 4 ºC before analysis.	• MLOQ: 0.5-17 ng/g • Recoveries: 94-115% (PFSAs) 21-109% (PFCAs)	(Sun *et al.* 2011)

(table 2.3)

	Solid-liquid extraction		
	PLE extraction		
PFHxS, PFOS, PFDS, PFOA, 9,9,10,10,11,11,12,12,13,13,13-Undecylfluorotredecane-1-sulfonate, N-Ethyl-N-(heptadecafluorooctane)-sulfonyl-glycinic acid, 5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,12-Heptadecafluorodecane sulfonylamido polyethocylate and polyethocylate methyl ether, parly fluorinated alkyl-ethoxylated (2-perfluoroalkylethanol polyglycol ether), metabolites of partly fluorinated alkylethoxylates	1) 2 g sludge (dw); 2) Soxhlet or hot vapour extraction, 6 h [solvent optimization: MeOH, EtOAc, MeOH-HCl] 3) (in parallel to Soxhlet) PLE by ASE [optimization extraction steps: 1) EtOAc-DMF (8:2), 2) MeOH-H_3PO_4 (95:5), 3) MeOH-H_3PO_4 (99:1), 4) MeOH-H_3PO_4 (99:1)].	**Soxhlet and hot vapour** • Recoveries: 48-52% **PLE** • MLOQ 10 µg/g (anionic) 20 µg/g (non ionic) • Recoveries: 105% (ionic) 119% (non ionic)	(Schröder *et al.* 2003)
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFHxS, PFOS, PFDS, FOSA	1) 0.5 g sludge (dw) + surrogates; 2) PLE (MeOH solvent at 70 ºC, 2 cycles,1 min static time, 100 bar); 3) evaporation to 1 mL + 30 mL water; 4) SPE by Oasis WAX (conditioning: 4 mL MeOH (NH_4OH), 4 mL water; loading: under gravity conditions; eluting: 4 mL MeOH (NH_4OH)); 5) dryed under N_2; 6) reconstitution in initial mobile phase conditions + IS	• MLOQ: 50-2772 pg/g • Recoveries: 65-111% • RSD: 4-30%	(This Thesis)
	Other extraction procedures		
PFHxA, PFOA, PFNA, PFOS, PFBS, PSUnA, FOSA, 6:2 FTS, 6:2 FTOH, 8:2 FTOH	1) PFAS were extracted from supernatant of the sludge experiments; 2) SPE by C18 cartridges (conditioning: 10 mL MeOH, 10 mL water; elution: 10 mL MeOH); 3) reduced under a gentle stream of N_2 and filtered before analysis.	• Recoveries: ($^{13}C_2$-PFOA) **>** 60%	(Saez *et al.* 2008)
PFBS, PFHxS, PFOS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	1) suspended soil and mixed liquor suspended soil filtered by GF/B filter; 2) PLE (ASE200, Dionex,USA) of GF/B filter (3 static cycles, MeOH solvent, 100 ºC, 2000 psi, 10 min); 3) extract dried to reduce < 10 mL (60 ºC, water bath); 4) reduced extract + 1 L water; 5) SPE by Oasis HLB plus and PrepSepC-Agri (short) in tandem; 6) elution with 4 mL of MeOH under gravity conditions; 7) dryness under N_2; 8) reconstitution in 1-2 mL of water:ACN (6:4) and vortexed	• MLOQ: 0.02-0.22 ng/g • Recoveries: 64-112% • CV <20%	(Shivakoti *et al.* 2010)

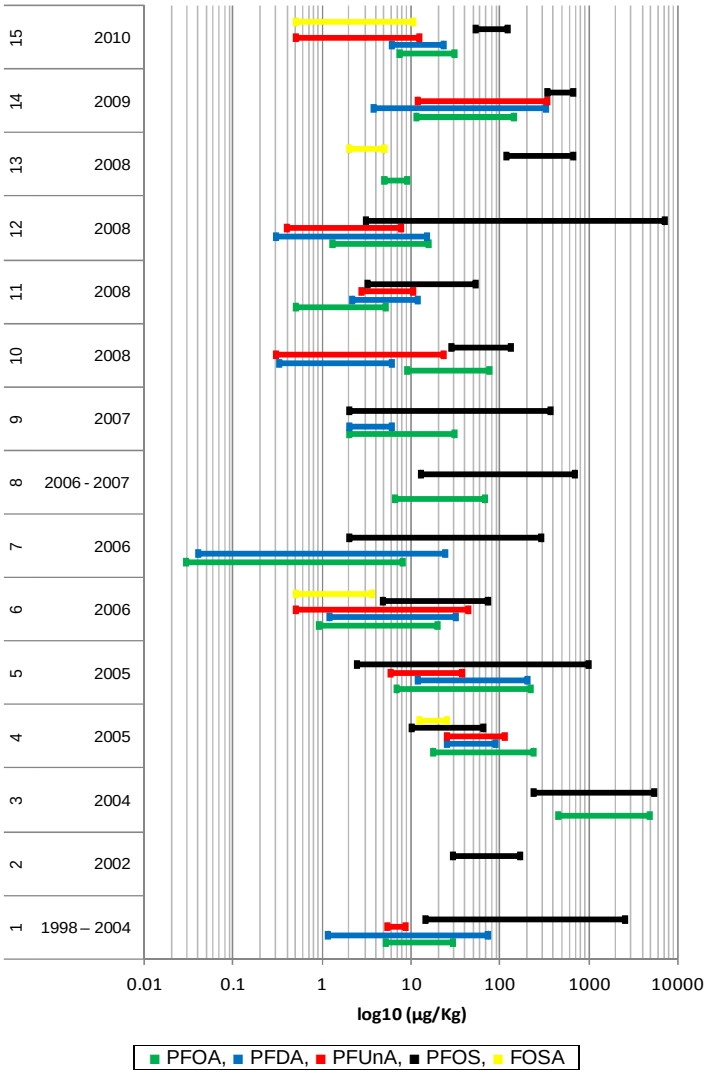

Figure 2.12: PFASs ranges in sludge samples from WWTPs, from previous published works compared with the present thesis, for the most detected analytes in this matrix, expressed in $\log_{10}$ (µg/Kg). 1) digested sludge from USA ; 2) sludge from Ontario, Canada (D'Eon *et al*. 2009b); 3) sewage sludge from China (Guo *et al.* 2008); 4) combined sludge liquors from New York State, USA (Sinclair *et al.* 2006); ; 5) sludge from Kentucky and Georgia, USA (Loganathan *et al.* 2007); 6) sludge from Denmark (Bossi *et al.* 2008); 7) sewage sludge from Spain (Navarro *et al.* 2011); 8) sewage from Singapore (Yu *et al.* 2009); 9) sludge from Decatur and New York city, USA (Yoo *et al.* 2009); 10) sludge from Shangai, China (Li *et al.* 2010); 11) sludge from Korea (Guo *et al*. 2010); 12) sludge from Hong Kong, China (Ma *et al.* 2010); 13) digested sewage sludge from Zürich, Switzerland (Zhang *et al.* 2010); 14) sludge and activated sludge from Thailand (Kunacheva *et al.* 2011); and 15) sewage sludge from Catalonia, Spain (this thesis).

2.3.3 Study of PFASs in biota from remote samples

This study represented valuable data since not many works have reported the presence of PFASs in South Hemisphere. The major difficulties associated to the work were the sampling strategy that needed to be dependent to climatological changes and the sample availability. Other problems were associated to samples preservation, sample pre-treatment (in case that it was necessary) and sample shipment. The main problem with PFASs is the cross contamination during these steps. In order to rule out any possible contamination, empty containers were used as blanks being exactly the same batch of PP inert recipients used for sampling. These containers were carried on, in parallel, with all the samples during sampling process, sample pre-treatment and shipment.

Regarding the occurrence of PFASs in the South Hemisphere, results from Tierra del Fuego (Ushuaia) have shown, in most of the cases, higher PFASs amounts than those from the Antarctica as it was expected. A different distribution pattern depending on the matrix type can be identified. In this context, the PFASs found in superficial soils in Antarctic region can be associated to atmospheric transport according to Dreyer *et al.* (2009). The compounds detected were those with shorter carbon chain such as PFHxA, at low ng/g. The study performed by Dreyer *et al.* revealed that PFASs were susceptible to be transported by the atmosphere because the authors found these analytes in all air samples, predominantly in the gas phase. The concentrations decreased from continental regions toward marine regions and from central Europe towards the Arctic and Antarctica. The atmospheric oxidation of longer fluoroalquil analytes such as fluorotelomers is also an introduction source of PFASs in remote areas. The higher concentrations of PFASs in soil from Ushuaia compared with the Antarctica support such hypothesis. The study also showed evidences that southern hemispheric concentrations of PFASs in atmospheric samples were significantly lower than those for northern hemisphere. In this case, no evidences were found comparing inhabited southern area (Ushuaia) with previous works about fish samples (Ericson *et al.* 2008a; Hart *et al.* 2008) and soil samples (Alzaga *et al.* 2005; Nakata *et al.* 2006; Washington *et al.* 2007) from northern hemisphere.

However, the atmospheric transport cannot explain the higher levels of PFASs in the Antarctica. Yamashita *et al.* (2008) referenced to thermohaline circulation system as a major transport of “swimmer” PFASs. Ahrens *et al.* (2010) reported concentrations of PFASs between < 11-51 pg/L of PFOS in Antarctic Circumpolar Current zone and they supported the global transport models. Later on, Cai *et al.* (2012) reported similar concentration levels for PFOA and PFOS in coastal seawater in Fildes Peninsula and King George Island (Antarctica), confirming the oceanic transport for “swimmer” PFASs. This way of transport was in agreement with concentrations of these compounds detected in algae samples from Ushuaia and from Antarctica (PFOS between 66.3 – 111 µg/Kg and PFOA and PFPeA ranging 0.30 – 1.80 µg/Kg in Antarctic samples, and PFHxA at 3.42 – 240 µg/Kg in samples from Ushuaia). The perfluorocarboxylic acids found in algae samples from Tierra del Fuego had comparable concentrations to other works studying PFASs in commercial harbour areas such as Catalonia coast (Alzaga *et al.* 2005). The absence of harbour activity could explain the differences between algae levels

in Antarctic samples and Tierra del Fuego. Although the introduction of these compounds in algae organisms could be explained by the assimilation during algae respiration, more studies are necessary in order to confirm it.

In the case of PFASs in biological samples such as fish, penguin tissue or penguin dung can be explained by water transport and the introduction into the lower levels of marine food web through the bioaccumulation and consequent biomagnifications.

In order to compare with previous works, Table 2.4 contains the concentration ranges for different samples from the Antarctic region.

The present study reports a significant amount of results which globally supports the previous models and empirical observations related to the transport, deposition and bioaccumulation patterns of PFASs. Further efforts shall be done in order to minimize the presence and emission of POPs in general, and PFASs in particular, for better preservation of remote areas.

Table 2.4: concentration levels of PFASs in Antarctic samples from other published works

	Location	Sample type	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFBS	PFOS	FOSA
(Tao *et al.* 2006)	Antarctic, 2004-2005	Blood of elephant seal; ng/ml							<0.08-3.52	
	Southern Ocean, 2004-2005	Albatrosses liver; ng/g							<0.5-20.7	
	Antarctica, 1995-1996 and 2001	Adélie penguin blood; ng/ml							<0.1	
		Adélie penguin egg; ng/g							<0.1	
	Antarctica 1998-1999	South polar skua blood; ng/ml							<0.24-1.36	
		South polar skua egg; ng/g							2.08-3.12	
(Schiavone *et al.* 2009)	Antarctica	Fur seal pup, muscle; ng/g			0.2-0.8	<0.8-1.6	Nd		0.6-2.0	<0.4
		Fur seal pup, liver; ng/g			<1-2.9	<0.4	1.6-4.0		6.2-12.6	<0.4
		Gentoo penguin egg; ng/g			<0.5	<0.2	Nd		0.2-0.4	Nd
		Adelie penguin egg; ng/g			<2.5-8.0	<0.2	<0.1		0.2-0.6	<0.2-0.5
(Bengtson Nash *et al.* 2010)	Antarctic region	Antarctic Fur Seal liver; ng/g							2	
		Whitechinned Petrel pectoral muscle; ng/g							1.2	
This work	Antarctica	Superficial soil; ng/g	Nd	0.16-0.83	Nd	Nd	Nd	Nd	0.32-0.36	Nd
		Papua Penguin dung; ng/g	Nd	19.9-237	Nd	0.63-3.98	0.78-4.33	10.9-45.9	95.2-603	Nd
		Papua Penguin tissues; ng/g	0.11-2.27	0.26-0.61	Nd	Nd	0.28-0.31	Nd	0.063-0.103	Nd
		Algae; ng/g	1.00-1.69	Nd	Nd	0.30-1.80	Nd	Nd	66.3-111	Nd

Nd: not detected

2.3.4 Assessment of PFASs in beached marine debris and sediments

The study of PFASs in beached plastic pellets and beach sediment samples is a recent approach. The degradation of plastic could contribute to PFASs contamination in marine environments but, residues of these plastics, constitutes a reservoir of some less polar PFASs.

The samples were selected due to their proximity with plastic sources such as airports, harbours, mazut (heavy and low quality fuel oil), oil refineries (Aspropyrgos and Elefsina), natural gas power plant, DOW chemical plant, near to cities (like Athens), cement industry (Aget Heracles Industry) and Air force base (Mazona lagoon). Sampling areas of this study are presented in the Figure 2.13.

Due to the problems associated to the cross contamination during the analysis of PFASs, it was necessary to rule out any possible source of contamination from the virgins plastic pellets as it has been detailed in the corresponding experimental section. The analysis of plastic blanks showed levels below MLOQ, or even below MLOD, in all the cases. These results indicate that PFASs found in the samples are not coming from the raw material.

Regarding the concentrations detected in real samples, the main results are summarized in Figure 2.13. In the case of sediment samples, at least one PFAS was detected. The concentrations of PFASs range from 8.2 to 146 ng/Kg, being PFOA the compound with highest levels. The other quantifiable compounds were PFDA, the sulphonates PFOS and PFDS, in agreement with previous works, and some short chain compounds such as PFBA, PFHxA and PFHpA.

The global fate of PFASs, as in the case of POPs, is associated to different biogeochemical cycles and geophysical drivers. Due to the physicochemical properties of PFASs, these are distributed between different environmental compartments. Based on their specific partitioning property combinations, these compounds have been classified as flyers, multi- or single hoppers or swimmers according to chemical transport behavior at global scale ((Wania 2003, Lohmann *et al.* 2007). Shorter carbon chain could have an atmospheric transport and be deposited by wet, or dry, deposition, on beach sediments because of the typical Mediterranean climate in the Greek region: warm to hot, dry summers and mild to cool with wet winters. The presence of PFPeA, PFOA, PFDA, PFOS and PFDS in the samples should be linked to the water transport and finally redistribution to beach sediment, from the coast through the waves. In addition, other processes such as the degradation of longer related PFASs (for example fluorotelomers to PFOA) or other stable fluorinated compounds in the atmosphere must be considered.

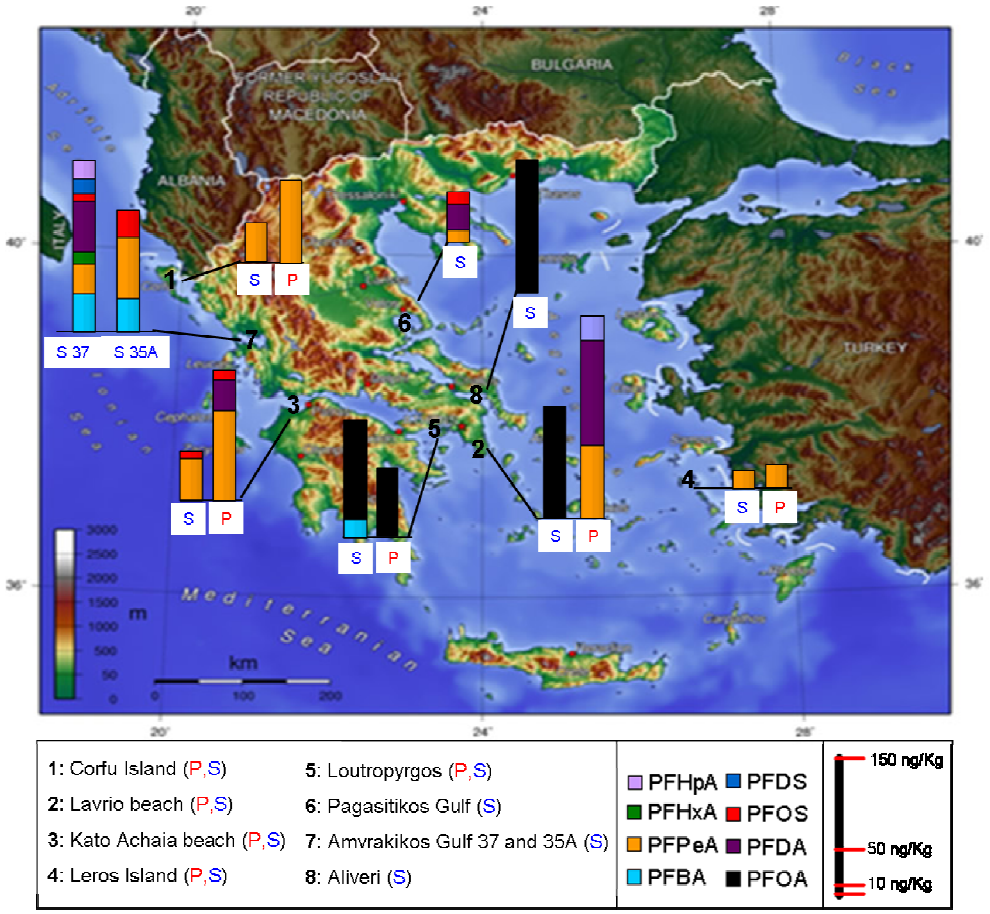

Figure 2.13: sample sites and accumulated concentration of PFASs in sediments (S) and plastic pellets (P); expressed in ng/Kg.

Another contamination source could be from airports, harbors, mazut and natural power plants, oil refineries, cement industries as well as the proximity of the most populated and polluted areas in Greece (Athens or Patras). For example, PFOS was detected as the predominant compound in sampling sites near to Athens city (2, 5 and 8 from Figure 2.13). On the other hand, some waste materials are used in cement kilns as a fuel supplement including car and truck tires (steel belts are easily tolerated in the kilns), paint sludge from automobile industries, waste solvents and lubricants, meat and bone meal (slaughterhouse waste), waste plastics, sewage sludge, rice hulls, sugarcane waste, used wooden railroad ties (railway sleepers) and spent cell liner (SCL) from the aluminum smelting industry (also called Spent Pot Liner or SPL) (Boyd 2001). These sources of fuel supplements can easily contain PFASs and, during the combustion, these compounds would be released into the environment arriving to reach the beach face in, for example, Pagasitikos Gulf (6) and the bottom of the sea in Aliveri (8).

PFASs were detected in all the plastic pellet samples. In general, concentrations of PFAS range from 11 to 116 ng/Kg, despite that most of the compounds were below MLOD or MLOQ. Major compounds were PFPeA with concentrations between 24 and 98 ng/Kg, PFHpA at 28 ng/Kg, PFOA at 76 ng/Kg, PFDA at 35 and 116 ng/Kg and PFOS at 11 ng/Kg. The highest concentrations were found in Lavrio beach and Kato Achaia beach.

Previous research has shown that POPs such as DDE and PCBs become adsorbed and concentrated onto the surface of plastic pellets, where the source is likely to be from the surrounding seawater (Mato *et al.* 2000), similarly to PFASs. In addition, this is in agreement with Berger *et al.* (2011), who studied the recoveries of perfluorinated acids between 4 and 12 C chains, perfluorosulphonates with 4 and 6 C chain and FOSA preserved with ultra pure water in a PP container with PP caps over a period of 3 months. For compounds with low recoveries, the water was removed after the experiment and the PP container extracted with methanol. The results demonstrated an adsorption between 20 and 60% onto PP container when the analytes are preserved in water, and that the percentage of adsorption increased with the number of C atoms. Another study performed by Loveless *et al.* (2006) reported that polymeric container (such PP and high HDPE) can also partially adsorb long chain compounds, such as FOSA, PFOS and PFOA. These studies supported the levels of PFPeA, PFOA, PFDA, PFOS and PFDS found in the surface of analyzed plastic pellet samples since they are made of PP or PE.

The possibility that PFASs can also reach the terrestrial food chain after ingestion of these plastic pellets by fishes (Gregory 2009) and seabirds (Colabuono *et al.* 2010) should be considered. On the other hand, sunken marine debris of all kinds settling to the sea floor at all depths for example in Mediterranean Sea has been reported (Galgani *et al.* 2000).

The relationship between sediment and plastic pellets from the same origin confirms a similar distribution pattern for the analytes (Figure 2.14). This is the case of Corfu Island, Kato Achaia beach, Leros Island and Loutropyrgos although not all the same analytes are present in both matrices. These differences can indicate a different origin of PFASs. For example, Loutropyrgos shows PFBA and PFOA in sediment from near shore zone, while plastic pellets only presented PFOA. The presence of PFBA and PFOA can be explained by an origin in a near area such as the oil refinery. Once produced, they can be transported through short-range transport.

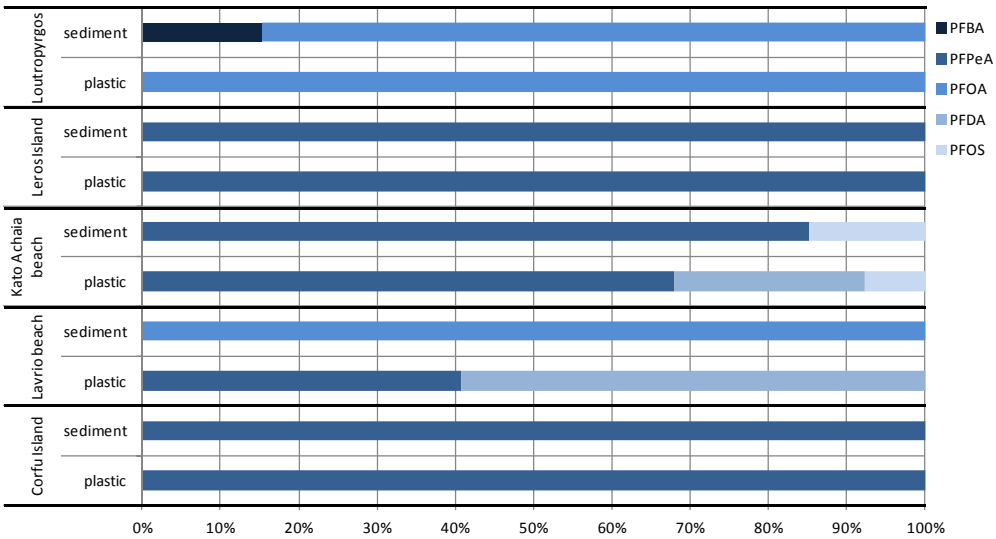

Figure 2.14: percentage contribution of PFASs (higher than MLOQ) in the corresponding sample

In the case of samples from Corfu Island, Kato Achaia beach and Leros Island, the sediment samples corresponded to beach shore while the plastic pellets were collected from the beach surface. In these samples, PFASs in plastic pellets were at higher concentration levels than in sediments. A marine origin of PFASs (for example harbors) is feasible. They can reach the beach shore by water transport through the waves.

On the other hand, the similar distribution pattern for PFASs in both matrixes indicates that the residence time of plastic pellets in the corresponded sampled area was high enough for accumulating these compounds from the water. The levels of plastic pellets and sediments were incredible similar, with the same compounds, indicating that PFASs did not come from longer distances. However, an exception can be observed for Lavrio beach samples (Figure 2.14). The plastic pellets from this sampling point can have a far away source or the difference can be attributed to the different sampling period between plastic pellets and sediment samples. Nevertheless, it is necessary to analyze more samples in order to obtain enough information for more conjectures about PFASs origin and plastic pellets concentrations.

CHAPTER 3

PFASs in food

3.1 Introduction

Sources of food contamination can be classified in:

- Direct environmental exposure of plants and animals and/or bioaccumulation through the food chain
- Indirect contamination: Cooking, food packaging and food processes

Direct environmental contamination. As it has been explained in the introduction, and in chapter no. 2, following their release into the environment, PFASs can be accumulated in plants and animals at the bottom of the food chain that are then consumed by higher animals within the food chain. In particular, contamination of the water cycle has been identified as one of major causes of PFASs in food. On the other hand, the use of sewage sludge as fertiliser, and subsequent run-off, was also found to contribute significantly to food and water contamination (Skutlarek *et al*. 2006). In addition, bioaccumulation in food chains will lead to increased levels of PFASs in animal-derived foods. Bioaccumulation of fish has been shown to be one of the main sources of PFASs in human diet.

In spite, the high number of studies carried out during the last decade, evaluating the accumulation of PFASs in aquatic biota, few extensive monitoring studies have studied the occurrence of PFASs in human diet (Pérez *et al.* 2012b). In a market basket study, in Sweden, Berger *et al.* (2009) found that PFOS and PFOA concentrations were below the quantification limits in composite samples of foods from animal origin. However, predatory fish from the largest lake in Sweden had substantially elevated levels of several PFASs. In another work, Ericson *et al.* (2008) studied the dietary exposure to PFASs in Spain. In this study, the dietary intake of PFASs for different age and gender groups was estimated and it was found to be on average between 0.9 and 1.1 ng/kg bw/day for adult male population. Fish, followed by dairy products and meats, were the main contributors to PFOS intake due to their bioaccumulation and biomagnification through the food chain. Similar conclusions were reported by Berger *et al* (2009). In this work, fish consumption was identified as one of the main sources of human exposure in Sweden. Ostertag *et al.* (2009) estimated the dietary exposure to PFASs from traditional food among Inuit in northern Canada. In this study the bioaccumulation of PFASs through the food chain and their contribution to the Inuit dietary exposure was revealed.

Indirect contamination. Main indirect sources of PFASs contamination in food are during cooking processes using certain types of materials, food packaging and food processes (Pérez *et al.* 2012b). Food preparation is a source of contamination (Tittlemier *et al.* 2007), but preliminary data on the influence of domestic cookware on levels of PFASs in the preparation of food indicated no elevated levels for a limited number of experiments (Powley *et al.* 2005) and, Del Gobbo *et al.* reported that the cooking decreases of PFASs concentrations in fish.

Packaging may also introduce chemicals into food, e.g. PFASs used in greaseproof packaging for fast foods and special packaging. In these situations, PFASs entry into food via migration from food package (Tittlemier *et al.* 2007).

In 2000, the European Commission published a White Paper on Food Safety, which underlined the importance of ensuring the highest possible standards of food safety and proposed a new approach to achieve them. However, it has been recently when PFASs have gained increasing scientific and socio-economic interest as food contaminants due to the unique combination of persistence, toxicity and environmental prevalence. In 2008, PFASs were recognised as emerging contaminants in the food chain by the European Food Safety Authority (EFSA). EFSA has established the Tolerable Daily Intakes (TDI) for PFOS and PFOA at 150 ng/kg body weight/day and 1500 ng/kg body weight/day, respectively (EFSA 2008). PFASs are now included in different health programs in the United States to provide a better assessment of the distribution, toxicity, and persistence of these compounds in humans (Richardson 2008) and are the target of several projects of the VII European Research Framework Programme (Pico *et al.* 2010), such as PERFOOD or *Conffidence*.

However, the risk assessment of the dietary exposure to PFASs is hampered by the lack of sufficient data about the occurrence of these contaminants in food.

Therefore, a growing number of studies have been reported during last few years the occurrence of PFASs in food. The outcome of these studies has been related with the potential dietary intake and the exposure levels (mainly by the estimation of the daily intake). Some selected examples from the literature are summarized in Table 3.1.

Table 3.1: Levels of PFASs in food in ng/g. Adapted from Farré *et al.* (2011).

Food	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTDA	PFBS	PFHxS	PFOS	PFDS	∑PFOSA	Ref.
Bread	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<20	NR	NA	[1]
Miscellaneous cereals	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<10	NR	NA	[1]
Carcase meats	NR	NR	<2	NR	NR	NR	NR	NR	NR	NR	<10	NR	NA	[1]
Offal	NR	NR	<2	NR	NR	NR	NR	NR	NR	NR	<20	NR	NA	[1]
Meat products	NR	NR	<2	NR	NR	NR	NR	NR	NR	NR	<10	NR	NA	[1]
Poultry	NR	NR	<2	NR	NR	NR	NR	NR	NR	NR	<10	NR	NA	[1]
Fish	NR	NR	<3	NR	NR	NR	NR	NR	NR	NR	<5	NR	NA	[1]
Oil and fats	NR	NR	<1	NR	NR	NR	NR	NR	NR	NR	<0.5	NR	NA	[1]
Eggs	NR	NR	<1	NR	NR	NR	NR	NR	NR	NR	1	NR	NA	[1]
Sugars & preserves	NR	NR	<1	NR	NR	NR	NR	NR	NR	NR	1	NR	NA	[1]
Green vegetables	NR	NR	<1	NR	NR	NR	NR	NR	NR	NR	<3	NR	NA	[1]
Potatoes	NR	NR	1	NR	NR	NR	NR	NR	NR	NR	10	NR	NA	[1]
Other vegetables	NR	NR	<10	NR	NR	NR	NR	NR	NR	NR	<3	NR	NA	[1]
Canned vegetables	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	2	NR	NA	[1]
Fresh fruits	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<2	NR	NA	[1]
Fruit products	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<1	NR	NA	[1]
Beverages	NR	NR	<0.5	NR	NR	NR	NR	NR	NR	NR	<0.5	NR	NA	[1]
Milk	NR	NR	<0.5	NR	NR	NR	NR	NR	NR	NR	<0.5	NR	NA	[1]
Dairy products	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<2	NR	NA	[1]
Nuts	NR	NR	<5	NR	NR	NR	NR	NR	NR	NR	<5	NR	NA	[1]
Beef stick	NA	<0.6	<0.5	4.5	<1	<1	<1	<3	NA	NA	2.7	NA	<LOD[a]	[2,3]
Roast beef	NA	<0.6	2.6	<1	<2	<2	<1	<3	NA	NA	<0.6	NA	<LOD[a]	[2,3]
Ground beef	NA	<0.5	<0.4	<1	<1	<1	<1	<3	NA	NA	2.1	NA	<LOD[a]	[2,3]
Luncheon meats, cold cuts	NA	<0.4	<0.4	<1	<1	<1	<1	<3	NA	NA	0.5	NA	<LOD[a]	[2,3]
Fish, marine	NA	<0.4	<0.5	<1	<1	<1	<0.8	<4	NA	NA	2.6	NA	<LOD[a]	[2,3]
Fish, freswater	NA	<0.4-1	<0.5-2	<1	<1-2	<1-2	<0.9-2	<2-5	NA	NA	1.5-2.0	NA	<LOD[a]	[2,3]

(Table 3.1)

Food	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTDA	PFBS	PFHxS	PFOS	PFDS	∑PFOSA	Ref.
Pizza	NA	2.0	0.74	<1	<1	<1	<1	<1	NA	NA	<1	NA	27.3[a]	[2,3]
Microwave pop corn	NA	1.5	3.6	<1	<1	<0.9	<1	<1	NA	NA	0.98	NA	15.3-18.9[a]	[2,3]
Egg breakfast sandwich	NA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NA	NA	<LOD	NA	11.9[a]	[2,3]
French fries	NA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NA	NA	<LOD	NA	4.11-9.72[a]	[2,3]
Chicken nuggets	NA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NA	NA	<LOD	NA	5.87[a]	[2,3]
Fish burger	NA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NA	NA	<LOD	NA	3.82[a]	[2,3]
Vegetables	<LOD	<0.004	<0.027	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.022	<LOD	<LOD	[4]
Cereals	<LOD	<0.009	<0.045	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.027	<LOD	<LOD	[4]
Pulse	<LOD	<0.008	<0.080	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.069	<LOD	<LOD	[4]
White fish	<LOD	<0.004	<0.065	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.407	<LOD	<LOD	[4]
Sea food	<LOD	<0.002	<0.029	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.148	<LOD	<LOD	[4]
Tinned fish	<LOD	<0.007	<0.126	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.271	<LOD	<LOD	[4]
Blue fish	<LOD	<0.010	<0.132	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.654	<LOD	<LOD	[4]
Pork	<LOD	<0.006	<0.053	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.045	<LOD	<LOD	[4]
Chicken	<LOD	<0.004	<0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.021	<LOD	<LOD	[4]
Veal	<LOD	<0.003	<0.003	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.028	<LOD	<LOD	[4]
Lamb	<LOD	<0.012	<0.012	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.040	<LOD	<LOD	[4]
Eggs	<LOD	<0.005	<0.005	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.082	<LOD	<LOD	[4]
Dairy products	<LOD	<0.007	<0.007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.121	<LOD	<LOD	[4]
Whole milk	<LOD	<0.015	<0.015	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.014	<LOD	<LOD	[4]
Semiskimed milk	<LOD	<0.004	<0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.019	<LOD	<LOD	[4]
Fruits	<LOD	<0.004	<0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.017	<LOD	<LOD	[4]
Margarine	<LOD	<0.014	<0.014	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.034	<LOD	<LOD	[4]
Oil	<LOD	<0.035	<0.035	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<0.099	<LOD	<LOD	[4]
Infant formula	NA	<1.2	<48.3	<2.20	NA	NA	NA	NA	<0.7	<1.34-3.59	<11.0-11.3	NA	NA	[5]
Dairy milk	NA	<1.2	<48.3	<2.20	NA	NA	NA	NA	<0.7	<1.34-3.82	<11.0	NA	NA	[5]
Lake trout	NA	NA	0.9-6.0	0.4-7.2	NA	NA	NA	NA	NA	NA	4.4-213	NA	0.9-6	[6,7]
Rainbow	NA	NA	0.9-3.1	1.7-11.9	NA	NA	NA	NA	NA	NA	65-165	NA	49-95	[7]

(Table 3.1)

Food	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTDA	PFBS	PFHxS	PFOS	PFDS	∑PFOSA	Ref.
European eel	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	8-143	NA	NA	[8,9]
Powdered milk	NA	NA	0.37-0.72	0.12-0.22	LOQ-1.3	NA	NA	NA	NA	NA	0.23-1.1	0.055-0.72	NA	thesis
Infant cereals	NA	NA	0.17-0.44	0.044-0.14	<LOQ	NA	NA	NA	NA	NA	0.16-0.46	0.056-0.091	NA	thesis
Market fish	LOQ-54	LOQ-10.2	LOQ-7.95	LOQ-13.1	LOQ-2.22	LOQ-0.12	ND	ND	LOQ-0.07	ND	LOQ-42	LOQ-0.25	LOQ-5.30	thesis

<LOQ: not detected; NA: not analyzed; NR: not reported; ND: not detected
[a]Sum of N-ethyl-PFOSA, PFOSA, N,N-diethyl-PFOSA, N-methyl-PFOSA and N,N'-dimethyl-PFOSA

[1]: reported by (FSA 2006),
[2]: reported by (Tittlemier *et al.* 2007),
[3]: reported by (Tittlemier *et al.* 2006),
[4]: reported by (Ericson *et al.* 2008),
[5]: reported by (Loewen *et al*. 2005),
[6]: reported by (Furdui *et al.* 2007),
[7]: reported by (Martin *et al*. 2004b),
[8]: reported by (Van den Heuvel-Greve *et al.* 2006),
[9]: reported by (Schrap *et al.* 2004)

3.2 Experimental work

It should be mentioned that, when this work has started, most of the data about PFASs were limited to the environmental occurrence and accumulation in biota, but not in the food basket. For example, fish data was available in some species, but in most of the cases was mainly related to the aquatic food chain, and species of large consume were not previously evaluated. In this sense, it was suspected that fish was one of the main contributors of exposure in human diet, but comparative results were not available. About breast milk, some more data was available, especially as a marker of human exposure. In addition, most of the data was related to a very limited number of compounds and, in general, PFOS and PFOA.

Therefore, series of lacks were identified, and series of studies raised which, the main goal was the study of PFASs in human diet. Taken into account that the analytical methods available in that time were not designed for the analysis of food, the first objectives were the analytical development, optimization and validation of analytical approaches specifically designed for:

- Fish – because it was suspected to be one of the main contributors of PFASs in human diet
- Breast milk, milk formulas and baby food – because children are suspected to be a sensitive population in front the PFASs exposure

Then, different studies were carried out in order to contribute to the PFASs occurrence knowledge's in selected commodities, and to evaluate the risk associated to PFOS and PFOA contents in some particular cases.

In addition, the results of a step-by-step validation approach carried out under the frame of the work in the Conffidence EU project will be presented. In this case, we have collaborated in the development and validation of a rapid and cost effective analytical approach for the analysis of PFASs in fish and dairy products, and we have organised a European interlaboratory study to test the good transferability and performance.

Finally, the results of a European survey to study the occurrence of PFASs in European fish markets will be presented. This study was carried out under the frame of the Conffidence project in order to supply and collaborate with the current European data base organised by the EFSA.

The experimental results are provided in the following publications.

3.2.1 Scientific publication 4:

Llorca, M., Farré, M., Picó, Y., and Barceló, D. (2009)

"Development and validation of a pressurised liquid extraction liquid chromatography-tandem mass spectrometry method for perfluorinated compounds determination in fish"

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Development and validation of a pressurized liquid extraction liquid chromatography–tandem mass spectrometry method for perfluorinated compounds determination in fish

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ABSTRACT

This paper describes the development and validation of an analytical methodology to determine eight perfluorinated compounds (PFCs) in edible fish using pressurized liquid extraction (PLE) with water and solid-phase extraction (SPE) with an ion-exchanger as extraction and pre-concentration procedures, followed by liquid chromatography–quadrupole-linear ion trap mass spectrometry (LC–QqLIT–MS). The rapidity and effectiveness of the proposed extraction procedure were compared with those most commonly used to isolate PFCs from fish (ion-pairing and alkaline digestion). The average recoveries of the different fish samples, spiked with the eight PFCs at three levels (the LOQ, 10 and 100 μg kg^{-1} of each PFC), were always higher than 85% with relative standard deviation (RSD) lower than 17%. A good linearity was established for the eight PFCs in the range from 0.003–0.05 to 100 μg kg^{-1}, with $r > 0.9994$. The limits of quantification (LOQs) were between 0.003 and 0.05 μg kg^{-1}, which are well below those previously reported for this type of samples. Compared with previous methods, sample preparation time and/or LOQs are reduced. The method demonstrated its successful application for the analysis of different parts of several fish species. Most of the samples tested positive, mainly for perfluoropentanoic acid (PFPA), perfluorobutane sulfonate (PFBS) and perfluorooctanoic acid (PFOA) but other of the eight studied PFCs were also present.

1. Introduction

Perfluorinated compounds (PFCs) have been and are used in a wide variety of industrial applications, such as stain repellents, textile, paints, waxes, polishes, electronics, adhesives and food packaging [1,2]. They have been manufactured for more than 50 years, having been estimated that from 1951 to 2004 up to 7300 tons were released into the environment following production and use [1]. As a consequence, these compounds show a global distribution all over the world and have been detected not only in environmental samples but also in human blood and liver. PFCs show persistence in the environment and some of them are related to different carcinogenic actions, for example perfluorooctanoic acid (PFOA) has been identified as a potent hepatocarcinogen in rodents [3,4]. Meanwhile PFCs have been recognized as emerging contaminants in the food chain by the European Food Safety Authority (EFSA), which have recently finalized its opinion on perfluorooctane sulfonate (PFOS), PFOA and its salts establishing tolerable daily intakes (TDI) of 150 ng kg^{-1} b.w. day^{-1} for PFOS and 1500 ng kg^{-1} b.w. day^{-1} for PFOA [5]. The opinion of the EFSA on these compounds also highlights that concentration levels, contamination pathways, and toxicological potency should be assessed in the food chain and expresses its concern by the lack of available data [5].

A growing but still insufficient number of studies report on the occurrence of PFCs in food and drink [6–9]. In these papers, bioaccumulation in fish has been shown to be the main influence of PFCs in dietary exposure [10]. Some reports have also found a positive correlation between PFCs concentrations in plasma and consumption of fish, corroborating the importance of this exposure route [11]. Accordingly, these compounds have been widely analyzed in blood, bile and liver [12–16] but not so often in the edible part (muscle) of fish [17,18]. Levels of PFOS and PFOA have been reported in mussels, oysters, shrimp and fish from different countries [19–22]. However, it is often impossible to give details of the other PFCs homologues present in this matrix.

So far, most of the analysis methods to determine PFCs are based on liquid chromatography coupled to mass spectrometry

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or tandem mass spectrometry approaches (LC–MS or LC–MS/MS) [1,2]. Among them, triple quadrupole (QqQ) MS is the most widely employed analyzer because of their high dynamic range and good performance when working in selected reaction monitoring (SRM) mode [1]. In the recent LC–MS/MS methods, ion paired, potassium hydroxide or solvent extractions were applied, for which the reported limits of quantification (LOQ) for PFOA and PFOS were as low as 1 μg kg^{-1} [2]. However, many challenges still remain for either LC–MS/MS or the sample preparation protocols. Hybrid MS instruments have proved to be powerful tools to achieve high sensitivity, specificity and selectivity, as they combine the main advantages of the two analyzers (i.e. quadrupole and time of flight in case of QqTOF or quadrupole and liner ion trap in case of QqLIT) [23,24]. The main advantage of the hybrid QqLIT over other LC–tandem MS equipments relies on that it achieves unequivocal identification and confirmation of target compounds at highly sensitive levels [23,25]. Its unique feature is that the second mass analyzer, Q3, can be run in two different modes, retaining the classical QqQ scan functions such as SRM, product ion, neutral loss, and precursor ion while providing access to sensitive ion trap scans. This allows very powerful scan combinations when performing information-dependent data acquisition (IDA), enhanced product ion (EPI) or MS^3 experiments obtaining concomitantly both quantitative and qualitative information. Simultaneously, modern extraction and clean-up techniques, such as pressurized liquid extraction (PLE), microwave assisted extraction (MAE) or solid-phase microextraction (SPME), have not been applied to the determination of PFCs yet. These techniques provide rapidly and accurately clean extracts for sensitive analysis [24].

Consequently, the aim of this study was the development and validation of a simple, sensitive and selective analytical methodology to determine eight PFCs, using PLE with water and SPE on ion-exchanger for the extraction and pre-concentration of target compounds from various fish samples including liver, muscle and roe. To our knowledge, this work is the first example of the application of PLE for the determination of PFCs from food. Target compounds were perfluorobutanesulfonate (PFBS), perfluoropentanoic acid (PFPA), PFOA, PFOS, perfluoro-7-methyloctanoic acid (i,p-PFNA), perfluorononaoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoro-1-decanesulfonate (L-PFDS). Validation comprised the assessment of linearity, limit of quantification, recovery and precision. To prove the potential of this method, a comparison with ion-pairing and alkaline digestion extractions, – the two most widely employed procedures to extract PFCs from fish – was also included in this study. The ion-pairing forms neutral species of the anionic surface-active FFCs making them extractable from food samples by organic solvents. The use of alkaline digestion helps to extract bound PFC residues by removing lipids and proteins before extraction. Analyte identification and confirmation was performed using a LC–QqLIT–MS/MS in compliance with the EU regulations (EU Commission Decision 2002/657/EC). Finally, PFC residues were determined in different fishes taken in several markets of Valencia and Barcelona cities.

2. Experimental

2.1. Chemicals

The isotope-labelled internal standards (ISs) perfluoro-n-[1,2,3,4^{13}C$_4$]octanoic acid (MPFOA), perfluoro-1-[1,2,3,4^{13}C$_4$] octanesulfonate potassium salt (MPFOS), and perfluoro-n-[1,2^{13}C$_2$]decanoic acid (MPFDA) as well as sodium L-PFDS, PFNA and i,p-PFNA were purchased from Wellington Laboratories (Guelph, Ontario, Canada) as 50 μg ml^{-1} methanolic solutions (1.2 ml). Tetrabutylammonium PFBS (purum ≥ 98%), PFOS sodium salt (98%), PFPA (97%) PFOA (96%), PFDA (97%), were purchased from Aldrich (Steinheim, Germany). Separate stock solutions of the analytes were prepared in methanol at a concentration of 1.0 mg ml^{-1} of free compound or salt. A standard mixture containing the 8 analytes was made from the stock solutions (commercial or laboratory made) to provide different concentrations of the analytes depending on their expected concentrations in fish and on the sensitivity of the method. Concentrations of the analytes in the standard mixture were calculated as free compounds. Working mixtures were diluted from the standard mixture in methanol/water both 20 mM ammonium acetate (10/90, v/v). Solutions of ISs were diluted to a concentration of 2 μg ml^{-1} with methanol/water both 20 mM ammonium acetate (10/90, v/v), and appropriate volumes of the ISs were added to fish samples so as to obtain concentrations of 1.5 μg kg^{-1} in the sample material.

LC-grade 'suprasolv' water, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use. Analytical grade reagent sodium sulfate anhydrous and glacial acetic acid were also from Scharlau. Ammonium acetate (99%, pa for HPLC) and sea sand were from (Sigma–Aldrich, Steinheim, Germany). Ammonium hydroxide (25% in water), sodium hydroxide (analytical grade), tetrabutyl ammonium hydrogen sulfate and methyl-ter-butyl ether were from Merck (Darmstadt, Germany).

Oasis Wax cartridges of 60 mg (3 cm^3), particle size 30 μg and pore size 80 Å used were from Waters (Milford, MA, USA). Oasis WAX is a polymeric reversed-phase, Weak Anion Exchange mixed-mode sorbent that allows for the retention and release of strong acidic compounds (e.g. such as sulfonates).

2.2. Sampling

The following fish species were purchased in retail fish markets and supermarkets as a whole fish: young hake (*Merluccius bilinearis*, $n = 5$), anchovy (*Engraulis encrasicolus*, $n = 5$) and striped mullet (*Mujil cephalus*, $n = 3$). Each sample of young hake and anchovy weighted around 2 kg (ca. 16 specimens/sample and 100 specimens/sample, respectively) and each sample of striped mullet consisted of only one specimen (weights between 180 and 520 g). Furthermore, hake roe ($n = 2$) and swordfish fillets (*Xiphias gladius*, $n = 3$) were also taken in these markets. All the samples were sent in fresh conditions (on ice) to the laboratory. Whole fishes were dissected, taken the liver and the entire right dorsal lateral fillet with the skin. The liver was completely and carefully separated. The livers corresponding to each sample were homogenized together. The right dorsal lateral fillets, swordfish fillets and hake roe were cut in small pieces. Subsamples of 200 g were homogenized using a bapitaurus food chopped (Taurus, Berlin, Germany), placed into polyethylene (PP) bags and stored at −80 °C prior to analysis.

2.3. Sample preparation

2.3.1. Pressurized liquid extraction

The muscle and liver samples (ca. 2 g, fresh weight) were weighted into a porcelain mortar, added with the ISs and homogenized with approximately 25 g of sea sand using a pestle. The advantages of homogenizing the tissue with sea sand were to disrupt the cell membranes (the great pore and particle sizes of this solid support in comparison with others helps to gridding the sample) and to disperse the sample over a large surface area to obtain better extraction. This mixture was put into a 22 ml extraction cell then, this cell was filled up with washed sea sand. Whatman glass fiber filters were placed at the bottom and top of the extraction cell

to avoid the obstruction of metal filters by solid particles. Samples were extracted by PLE using an ASE 200 system (Dionex, Sunnyvale, CA, USA). The sample was heated to 110 °C with a static period of 7 min and extracted by a flush volume of 100% in 3 cycles using water. Pressure was set to 1500 psi and purge time to 1 min. The final extracts had a volume of 42 ml.

The process SPE/clean-up used in this work was based on that reported by Ye et al. [26] for the analysis of perfluorinated compounds in carp fillets. Briefly, Oasis WAX 3 cartridges were conditioned by passing 4 ml of 0.1% ammonium hydroxide in methanol, 4 ml of methanol, and 4 ml of deionized water through the cartridge. The PLE extract was passed through the cartridge, that was then washed with 4 ml of 25 mM acetate buffer (pH 4) followed by 4 ml of methanol. The PFCs were eluted with 4 ml of 0.1% ammonium hydroxide in methanol. SPE extracts were concentrated to 0.5 ml under nitrogen (60 °C) using a Zymark TurboVap concentrator.

2.3.2. Alkaline digestion

Samples were analyzed using a modification according to Ye et al. [26] of a method described by Taniyasu et al. [27]. About 1 g of liver and muscle (fresh weight) was homogenized with 5 ml of water using Ultraturrax T-25 digital homogenizer and added with the IS. The homogenate was combined with 8 ml of 10 mM sodium hydroxide in methanol. Each sample was digested by shaking on an orbital shaker table at room temperature for 16 h. After digestion, samples were centrifuged at 2000 × *g* for 5 min, and 3 ml of the supernatant was diluted with 27 ml of deionized water prior to solid phase extraction (SPE) cleanup as described for the previous procedure.

2.3.3. Ionic-pair extraction

The sample pre-treatment procedure was similar to that described by Hansen et al. [28] except for some modifications. Briefly, 5 ml of distilled water were added to the homogenized tissue sample (about 1 g, fresh weight) and spiked with the ISs. After homogenization on an Ultra Turrax homogenizer, 1 ml of TBA (0.5 M, pH 10) and 2 ml of sodium carbonate solution (0.25 M) were added to the homogenized tissue sample. The sample solution was agitated on a vortex mixer for 20 s and 5 ml MTBE was added. After agitation on an orbital shaker for 20 min, the sample solution was centrifuged at 3000 rpm for 10 min (at 25 °C). The organic and aqueous layers were separated by centrifugation, and an exact volume of MTBE (4.0 ml) was removed from the solution. The aqueous phase was again extracted twice with two fresh portions of MTBE solution (4 ml); all rinses were combined in a second polypropylene tube. The solvent was allowed to evaporate under nitrogen using the Zymark TurboVap before being reconstituted in 0.5 ml of methanol–water both 20 mM ammonium acetate (10/90, v/v).

2.4. Liquid chromatography–quadrupole-linear ion-trap mass spectrometry (QTRAP)

Perfluorinated compounds were analyzed on a 4000 Q TRAP™ MS/MS system from Applied Biosystems/MDS Sciex (Concord, Ontario, Canada) coupled to a Symbiosis™ Pico system (Spark Holland, Emmen, The Netherlands). The latter integrated HPLC and Online SPE system but only the HPLC system was used that basically consist of an Alias™ autosampler and two high pressure gradient LC pumps with a 4-channel solvent selector for each pump. Separations were accomplished on a LiChroCART-LiChrospher 100 RP-18 analytical column of 250 mm × 4 mm and 5 μm particle diameter from Merck at room temperature. The mobile phase consisted of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B) and was delivered at a flow rate of 0.5 ml min^{-1}. The linear gradient elution program was as follows: 10–80% B over 5 min, then 80–90% B over other 5 min followed by an isocratic hold at 90% B for 8 min. At 18 min, B was returned to 10% in 2 min. The total run time for each injection was 20 min and the injection volume 20 μl. The mass spectrometer was operated in the negative ion mode with a TurboIonSpray source. The selected reaction monitoring (SRM) conditions and the retention time of each analyte are listed in Table 1. The other ionization parameters were as follows: curtain gas (CUR), 30 (arbitrary units); ion source gas 1 (GS1), 25 (arbitrary units); ion source gas 2 (GS2), 25 (arbitrary units); source temperature (TEM), 350 °C; ionspray (IS), −4500 V; entrance potential (EP), −10 V, collision cell exit potential (CXP) −10 C and declustering potential (DP) −25 V.

The dwell time of each MRM transition was 150 ms. The mass spectrometer was controlled by Analyst 1.4.2 software from Applied Biosystems/MDS Sciex and the Symbiosis from the Symbiosis Pico for Analyst software.

2.5. Quality assurance

Validation of the method included determination of linearity range, intra-assay precision, accuracy, matrix effects, limit of detection (LOD) and LOQ. With the exception of linearity, the validation experiments were performed by spiking muscle and liver samples of young hake and anchovy with all 8 compounds. For spiking the sample, 1 or 2 g portions of chopped fish were placed in the appropriate container according to the further extraction method and spiked with the PFCs standard solution, taken care to uniformly spread them on the sample. The spiked sample was left for 10 min at room temperature to ensure the appropriate distribution in the matrix. Then, the sample was processed, as reported in Section 2.3, depending on the extraction procedure. Five replicates of sample preparation and analysis were performed at each level. For the assessment of all the mentioned parameters, the analyte response was always related to the IS response (1.5 μg kg^{-1} of each, MPFOA, MPFOS and MPFDA) to compensate for undesirable matrix effects and losses during the extraction procedure. The ISs were selected because at the beginning of this study they were the only available ones.

Procedural blanks were carried out by the three extraction procedures and they did not show contamination by PFCs for the entire method. Blank tests were also carried out on all the sample containers to rule out possible contamination from the sampling, storage and shipment contained. Of the 18 fish samples analyzed, only two samples, one of young hake and other of anchovy did not show PFCs contamination. They were used for the matrix effect and recovery studies as well as, for LOD and LOQ assessment.

Finally, in order to comply with internal quality control (IQC) procedures, two control samples (spiked materials), two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis is in a state of statistical control or not.

2.5.1. Selectivity

For identification purposes, retention times of PFCs in the standards and in the samples were compared at a tolerance of ±2.5%. Moreover, in accordance with the 2002/657/EC Decision [29], the relative ion intensities (each product ion area signal versus the base product ion area signal) of the spiked samples were compared with the relative ion intensities of PFCs standard solutions, at the same concentration levels as used for the construction of the calibration curve.

Selectivity in fish samples was demonstrated by analyzing 10 young hake and 10 anchovy extracts. These test samples, were analyzed by the three methods, being negative for PFCs.

Table 1
IS deprotonated molecules $[M\text{-}H]^-$, monitored transitions (including the tentative identification of the product ion), retention times (RT), collision energies (CE) of the PFCs in the order of retention time.

Analyte	IS	t_R (min)	Transitions m/z	Tentative ion identification	Transitions ratio	CE (V)
PFPA	MPFOA	9.36	263 > 219	$[M\text{-}H\text{-}CO_2]^-$	1.00	−15
PFBS	MPFOS		299 > 80	$[SO_3]^-$	1.00	−80
			299 > 99	$[FSO_3]^-$	0.11	−80
PFOA	MPFOA	11.02	413 > 169	$[C_3F_7]^-$	1.00	−25
			413 > 369	$[M\text{-}H\text{-}CO_2]^-$	0.42	−25
			413 > 219	$[C_4F_9]^-$	0.33	−25
MPFOA		11.02	417 > 372	$[M\text{-}H\text{-}CO_2]^-$		−25
i,p-PFNA	MPFDA	11.54	463 > 169	$[C_3F_7]^-$	1.00	−15
			463 > 219	$[C_4F_9]^-$	0.80	−15
PFNA	MPFDA	11.83	463 > 219	$[C_4F_9]^-$	1.00	−15
			463 > 169	$[C_3F_7]^-$	0.90	−15
PFOS	MPFOS	11.77	499 > 80	$[C_3F_7]^-$	1.00	−15
			499 > 99	$[FSO_3]^-$	0.20	−100
			499 > 280		0.01	−100
MPFOS		11.77	503 > 80	$[C_3F_7]^-$		−100
PFDA	MPFDA	12.85	513 > 119	$[C_2F_5]^-$	1.00	−100
			513 > 469	$[M\text{-}H\text{-}CO_2]^-$	0.40	−35
			513 > 268		0.02	−35
MPFDA		12.85	515 > 471	$[M\text{-}H\text{-}CO_2]^-$		−35
L-PFDS	MPFOS	13.61	599 > 80	$[SO_3]^-$	1.00	−100
			599 > 99	$[FSO_3]^-$	0.20	−100

A majority of the PFCs were separated chromatographically from each other during the LC run, as demonstrated in Table 1. Selectivity was assured by utilizing a QqLIT system in MS/MS mode, in which single chromatographic peaks were observed for all SRM transitions, except for i,p-PFNA and PFNA.

2.5.2. Limits of detection and quantification

The LOD was defined as the lowest concentration for which the peak area was at least three times larger than the background noise. Criteria for the LOQ were established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable, and (4) signal-to-noise ratio at least 10. The LOQs obtained served as the lower limits of the linear range.

2.5.3. Linearity

Linearity range was defined by plotting the peak area ratio of the PFC to the IS versus PFC concentration. The following criteria for linearity range were applied: linear regression through zero with a correlation coefficient better than 0.990, bias from the calibration line less than 25% for all individual calibration points, and RSD of four replicates less than 25%. The lower limit of the linear range was at LOQ.

2.5.4. Matrix effect

The matrix effects were assessed by comparing the response of the analytes at 10 $\mu g\,kg^{-1}$ concentration in 20 mM ammonium acetate methanol/water (10/90, v/v) solution to the response of the analytes spiked at the same concentration into an extract of a blank matrix sample extract (young hake or anchovy) obtained through the sample preparation process.

2.5.5. Recovery and precision

According to the 2002/657/EC Decision [29], since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. Briefly, negative samples of tissue and liver of anchovy and young hake (previously analyzed and found to be not contaminated) were spiked in quintuplicate as previously described with the eight PFCs at three different levels (LOQ, 10.0, 100.0 $\mu g\,kg^{-1}$). Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets as used for recovery tests, with the only difference that independent samples were re-extracted and analyzed on two other occasions for calculating inter-day repeatability.

3. Results and discussion

3.1. LC–MS/MS optimization

Many earlier studies have reported the use of conventional C_{18} LC columns for the separation of PFCs [7,9,10,30]. Under the mobile phase flow rates and gradient described above, PFCs were well resolved at retention times in the range of 9–14 min. The presence of a volatile salt (ammonium acetate) in the mobile phase is essential to obtain a proper peak shape. This salt could cause a suppressing effect on the analyte signal. Several ammonium acetate concentrations (5, 10, 20 and 30 mM) were evaluated to determine the mobile phase that offers short retention time and sufficient resolution for the PFCs with little or no suppression in signal-to-noise ration of the analytes. Up to 20 mM the possible reduction of the MS response of the analyte is compensated for by the improvement in peak shape providing a negligible reduction in the intensity of the signal observed.

Identification of the compounds was based on (1) precursor ion (deprotonated molecule), (2) two or three (when possible) selective product ions, and (3) retention time (Table 1). The most intense product ion of each compound was used for quantification. The only exception was PFPA, for which only one product ion was obtained at reasonable intensity. For the isotope-labelled ISs, only one fragment ion was monitored.

SRM transitions were chosen after optimization of the conditions considering both, sensitivity and selectivity. The precursor → product ion transitions reported in Table 1 are the same reported in earlier studies using LC–QqQ–MS/MS [17,19–21,31–34].

Table 2
Instrumental parameters of the LC–QqLIT–MS method developed for the analysis of PFCs (values obtained by injection of standard solutions in methanol–water 20 mM ammonium acetate (10:90, v/v)).

Compound	Calibration range (ng l^{-1})	R^2	ILOD (pg)	ILOQ (pg)	Repeatability (RSD 50 pg l^{-1})
PFPA	0.05–1000	0.9994	0.0003	0.001	10
PFBS	0.05–1000	0.9996	0.0003	0.001	12
PFOA	0.005–50	0.9994	0.00003	0.0001	13
PFNA	0.01–100	0.9994	0.0006	0.002	15
i,p-PFNA	0.01–100	0.9999	0.0006	0.002	9
PFOS	0.003–300	0.9996	0.0002	0.0006	7
PFDA	0.05–500	0.9996	0.0003	0.001	11
PFDS	0.05–500	0.9999	0.0003	0.001	12

However, those studies have used different precursor → product ion transition for quantification of the perfluorinated carboxylic acids (PFCAs) [19,31–33], e.g. product ions *m/z* 369, 419, 446 instead of the *m/z* 169, 169 and 119 used here for PFOA, PFNA and PFDA, respectively. Apparently, the lower mass fragments are more intense using the QqLIT mass analyzer than the QqQ, at least, in the chosen conditions. For perfluorinated sulfonates (PFSs) the product ions *m/z* 80 and 99, were used previously [19,31–33].

The ion ratios were calculated from calibration samples at five concentration levels between LOQ and 150 LOQ, and did not show dependence on the concentration. The variation of the ion ratios was below 10%, except for PFPS that shows higher variation up to 15%. The evaluated stability of the ion ratios was thereby in agreement with previous studies, in which the ion ratio tolerance between 20% and 50% was used.

The two stereo-isomers, PFNA and i,p-PFNA, are not separable by precursor or product ions, but with the LC-conditions used they were separated to baseline with a 0.2 min difference. Because of the high sensitivity of the QqLIT system, all transitions were acquired simultaneously at a relatively high flow rate for mass spectrometry without loss sensitivity.

The calibration curves obtained for both the quantification and the confirmation SRMs were linear for all compounds in a wide range of concentrations, typically from LOQ to 100 ng ml^{-1} with correlation coefficients (R^2) higher than 0.9994 for all compounds (see Table 2). It has been recently demonstrated that a quite wide dynamic measuring range of the analytical method is needed in order to quantify levels of PFCs in fish because they highly vary between samples [17,19].

In this study, the QqLIT instrument was used to perform the LC–MS/MS analyses in the SRM mode. As an additional feature, in this instrument, the SRM mode can be combined with attractive working modes (EPI and MS^3 modes) for the unambiguous confirmation of compounds. However, these modes have a limitation because the isolation and fragmentation steps are both occurring in the LIT, only fragment ions produced with *m/z* values of 30% of the parent mass and higher are stable in the ion trap. This drawback is difficult to overcome for perfluorinated sulfonates. On the contrary, a shortcoming of the use of the SRM mode is that at low concentrations of analyte, the second SRM transition is not detected, which is solved because of the higher sensitivity of this system.

3.2. Optimization of the PLE procedure

All parameters affecting the PLE extraction efficiency, such as temperature, pressure, static time, cell size, number of extraction cycles and flush volume, were carefully evaluated by the absolute recovery obtained by external standard calibration (without adding the IS). The optimum conditions were those reported in Section 2.3.1. The parameters with stronger influence on the recovery were the temperature, number of cycles and flush volume. The reported values were considered optimum because lower values provided considerable low recoveries whereas higher ones did not provide an increase in the recovery that justifies the longer time required. PLE using water as a solvent has already been reported to determine other contaminants and residues in food [35,36].

These reports [35,36] also check different dispersing agent and solvents. In this study, the sea sand was directly selected because it has thicker particle diameter that the other sorbents favoring the dispersion of the sample [35]. Some procedures treat the sand with EDTA to deactivated metal impurities present in the sorbent surface and, probably, chelates also present in the matrix facilitating decomplexation of analytes. PFCs recoveries does not show differences between EDTA treated and non-treated sand probably because they do not strongly bind to metals.

Methanol, water and methanol–water (50:50, v/v) were tested as extracts at different temperatures, to establish the better conditions. The best results were obtained with water at 110 °C and the coupling of the extraction with the clean-up by SPE is much easier with water. The mixtures of methanol required an additional step to evaporate the sample or to dilute the sample, which is detrimental for the LOD.

3.3. Validation

Selectivity of the method may be deteriorated by presence of endogenous species in biological extracts. It was difficult to find fishes without PFCs, especially when the LOQs are as low as those reported in the present study. However analysis of two blank sam-

Table 3
Recovery and RSD obtained at three concentration levels in liver and muscle fish using the PLE method.

Compound	Liver						Muscle					
	Conc.[a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc.[a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. μg kg^{-1}	Rec., % ($x \pm$ RSD)
PFPA	0.05	85 ± 17	10	86 ± 9	100	85 ± 9	0.025	89 ± 9	10	88 ± 8	100	89 ± 7
PFBS	0.05	87 ± 12	10	88 ± 10	100	89 ± 15	0.025	90 ± 15	10	89 ± 11	100	89 ± 8
PFOA	0.005	92 ± 15	10	99 ± 11	100	101 ± 12	0.0025	97 ± 12	10	93 ± 10	100	93 ± 7
i,p-PFNA	0.01	93 ± 11	10	90 ± 10	100	95 ± 10	0.005	99 ± 10	10	100 ± 7	100	100 ± 9
PFNA	0.01	92 ± 10	10	95 ± 9	100	97 ± 9	0.005	100 ± 9	10	102 ± 10	100	92 ± 6
PFOS	0.003	101 ± 8	10	94 ± 8	100	100 ± 10	0.0015	102 ± 10	10	99 ± 9	100	99 ± 8
PFDA	0.05	101 ± 9	10	102 ± 9	100	93 ± 9	0.025	101 ± 9	10	95 ± 7	100	95 ± 6
PFDS	0.05	101 ± 7	10	96 ± 8	100	97 ± 9	0.025	99 ± 9	10	102 ± 8	100	102 ± 5

[a] Concentration corresponding to the LOQ.

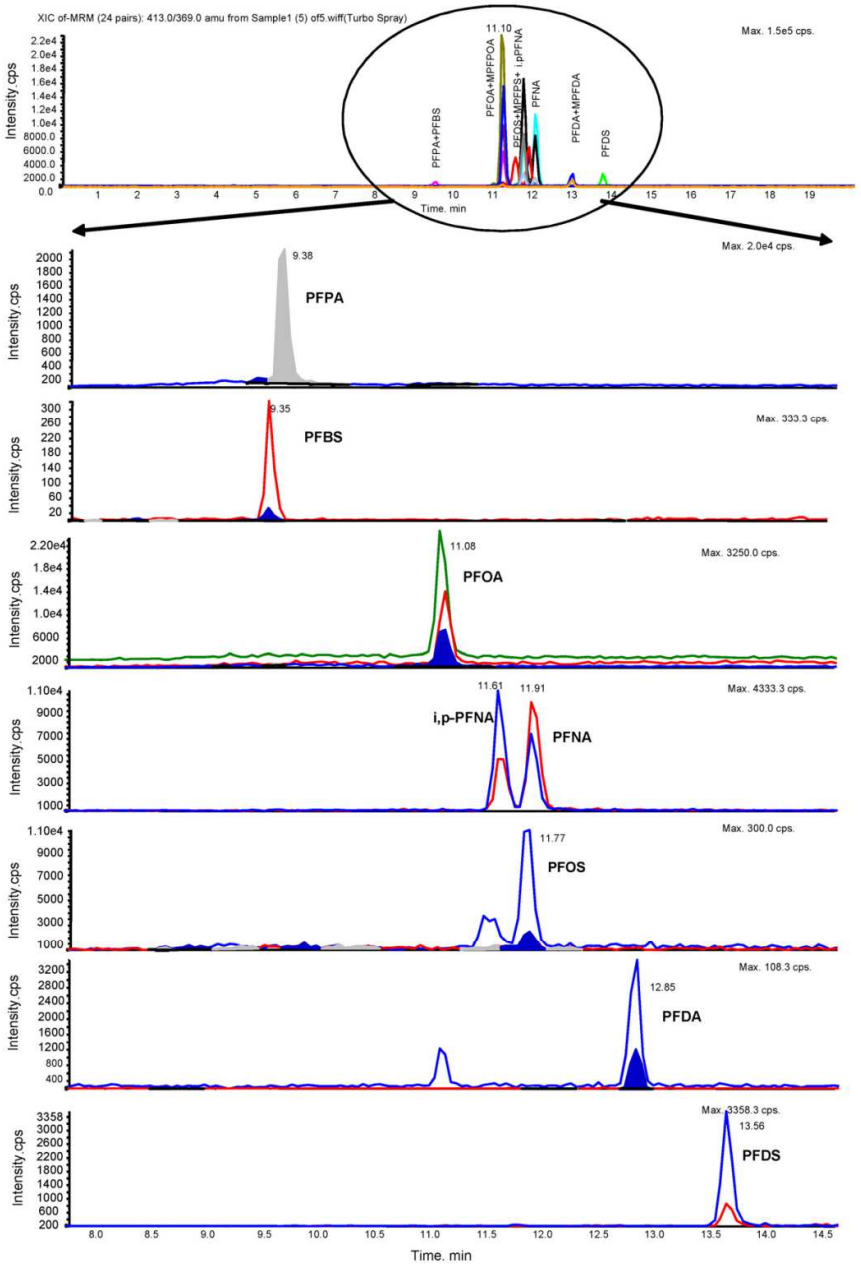

Fig. 1. Extracted ion chromatograms showing the monitored SRM transitions for the studied PFCs in a spiked anchovy sample at 0.05 μg kg^{-1}. The most intense is the one used for quantification, the other for confirmation of the compound.

ples, one of young hake and other of anchovy showed that no peaks were detected at the retention time of none of the 8 PFCs.

The calibration lines showed good linearity over the concentration range selected for the majority of the compounds (Table 2). Quantification was performed on standard in solvent, since matrix-matched standards are not very viable due to the considerable number of different fishes analyzed that have a great different fat content (3–33%). The differences in samples composition can raise recovery problems. For example, the extraction recoveries were >82% for fish muscle but they dropped to approximately 75% for liver samples. The use of isotopically labelled ISs normalized the recovery to an acceptable level, i.e. >85% in liver samples (see Table 3) achieving an improvement in recovery when it was below an acceptable level.

Even through matrix-matched standards were not used, matrix effects on LC–MS/MS were estimated comparing the analytical response given by a neat standard at 10 μg kg^{-1} and the same solution added to a fish extract. These effects did not contribute seriously to dismiss the process efficiency values because they were comprised between 80% and 96% with repeatability RSD < 15%. The use of ISs compensated for the matrix effects totally, in the case of PFOA, i,p-PFNA, PFNA, PFOS, PFDA and PFDS, and partially for PFPA and PFBS.

The LOQs obtained in muscle and liver (Table 3) were far below the concentrations of PFCs reported in fish. For PFOA, i,p-PFNA, PFNA and PFOS, the LOQ was lower than 0.01 μg kg^{-1} for both muscle and liver samples. Markedly higher LOQs were obtained for PFPA, PFBS, PFDA and PFDS (0.05 μg kg^{-1}). Nevertheless, these LOQs were below the reported concentrations in fish [17,19,34] and were thereby low enough to allow use of this method in routine screening and quantitation of PFCs in marketed samples. Comparison of this method to earlier LC–MS/MS methods revealed that the LOQs obtained here for most of the compounds were essentially better [6,7,20,21]. This improvement is mainly because of the application of LC–QqLIT–MS instrument, which allows an increase in sensitivity of more than 100 times over those using conventional QqQ instruments. However, the PLE procedure has also a minor influence in the better sensitivity because it provides high concentration factor and appropriate recoveries. Fig. 1 displays typical extracted ion chromatogram of the PFCs from an extract of spiked anchovy muscle at 0.05 μg kg^{-1} of each compound.

Precision and accuracy are summarized in Table 3. The RSD in liver and fish was lower than 15% for PCFs in fish and lower than (17%) in liver. In fish, the recovery was for the majority of the compounds higher than 88%. In liver samples, recovery was usually higher than 85%. It becomes clear that with the ISs acceptable relative PFCs recoveries (>85–89%) were obtained even in situations when the differences in sample matrix provided lower absolute PFCs recoveries. Precision and recovery were essentially at the equal level as

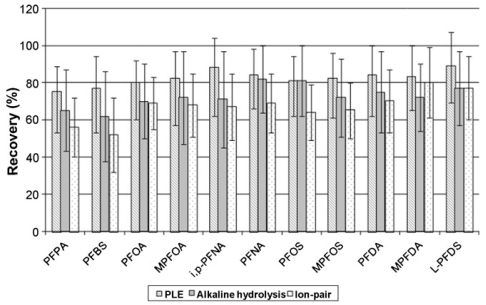

Fig. 2. Histogram of the absolute recoveries (calculated by the external standard method) and RSDs obtained from young hake spiked at 1 μg kg^{-1}.

in other PFCs LC–MS/MS methods, which also employs ISs [19–21].

3.4. Comparison to other methods

Results obtained by the present method were compared to those obtained by the commonly used ion-pair and alkaline hydrolysis methods described in Sections 2.3.2 and 2.3.3. The summarized results are presented in Table 4, in which the average percentage of recovery at the LOQ level is shown. No systematic difference existed between the results, except for the LOQs that are higher using the alkaline digestion than the ion-pair and those are higher than those obtained by the proposed PLE procedure. The accurate determination of PFCs was achieved by employing commercial isotopically labelled ISs, which compensated for target analyte losses and enhanced or suppression matrix effects.

Then, external standard calibration (the analyte response was not related to the IS) was evaluated. The rationale for evaluating external calibration is that neither recovery nor matrix effects, e.g. ion suppression, was accounted for quantitation. The recoveries and the RSDs of the method showed that PLE provides better recoveries and lower RSDs (Fig. 2). Furthermore, the PLE method is much more rapid than the alkaline digestion and provides cleaned extracts than that based on ion pairing. PLE allows to process up to 24 samples and extract them automatically, which saves time and personnel.

3.5. Application to fish samples taken from the market

The applicability of the method was assessed through the analysis of the selected PFCs in several fish samples. Table 5 shows the mean values of PFCs for each type of sample. The highest PFCs con-

Table 4
Recovery and RSD obtained using ion-pair and alkaline hydrolysis in samples of anchovy liver and fish spiked at the LOQ.

Compound	Alkaline hydrolysis				Ion-pairing			
	Liver		Muscle		Liver		Muscle	
	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)
PFPA	0.15	80 ± 17	0.15	85 ± 17	0.07	79 ± 19	0.05	75 ± 18
PFBS	0.15	85 ± 12	0.15	87 ± 12	0.08	75 ± 17	0.05	82 ± 17
PFOA	0.015	92 ± 15	0.015	92 ± 15	0.001	95 ± 14	0.005	96 ± 15
i,p-PFNA	0.03	90 ± 11	0.03	93 ± 11	0.02	92 ± 16	0.015	95 ± 14
PFNA	0.03	95 ± 10	0.03	92 ± 10	0.02	97 ± 15	0.015	99 ± 16
PFOS	0.01	101 ± 8	0.01	93 ± 8	0.004	102 ± 13	0.005	93 ± 12
PFDA	0.15	101 ± 9	0.15	100 ± 9	0.06	93 ± 15	0.05	102 ± 10
PFDS	0.15	99 ± 7	0.15	99 ± 7	0.06	92 ± 17	0.05	94 ± 9

[a] Concentration corresponding to the LOQ.

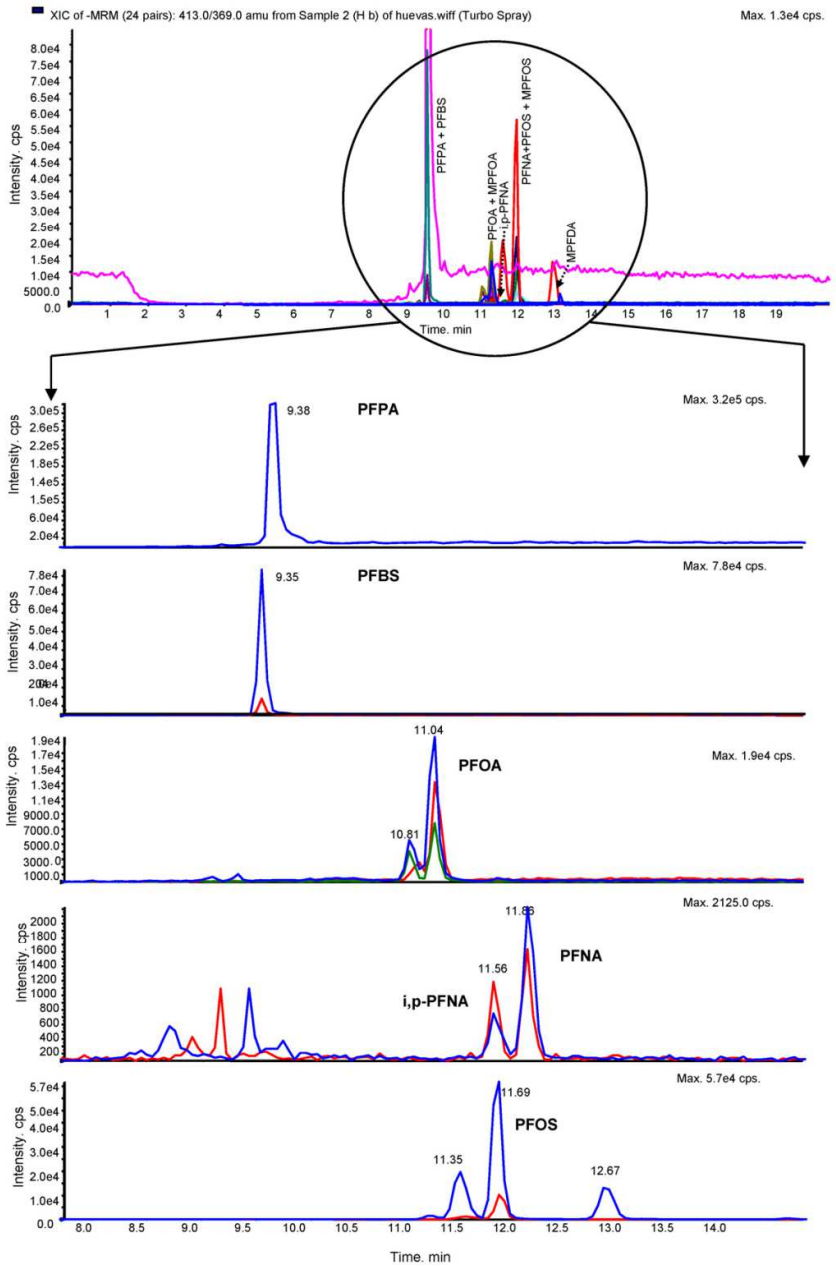

Fig. 3. Extracted ion chromatograms corresponding to the PLE extraction and LC–QqLIT–MS analysis of one hake roe sample. Concentrations calculated were: PFPA, 46.82 μg kg^{-1}; PFBS, 12.82 μg kg^{-1}; PFOA, 1.75 μg kg^{-1}; i,p-PFNA, 0.41 μg kg^{-1}; PFNA, 0.63 μg kg^{-1}; PFOS, 24.35 μg kg^{-1}.

Table 5
Mean PFCs concentrations ($\mu g\ kg^{-1}$) detected in the fish samples analyzed.

Compound	Hake Roe	Swordfish	Stripped Mullet		Young Hake		Anchovy	
			Muscle	Liver	Muscle	Liver	Muscle	Liver
PFPA	50.00	12.84	42.03	12.32	0.52	0.71	0.09	0.12
PFBS	10.00	13.45	<LOQ	2.04	<LOQ	1.24	0.83	2.23
PFOA	2.50	1.25	2.43	2.83	3.25	5.21	0.21	1.03
i,p-PFNA	0.44	3.24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFNA	0.58	1.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFOS	23.04	8.24	<LOQ	<LOQ	1.25	3.54	0.23	0.94
PFDA	<LOQ	0.24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFDS	<LOQ	1.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

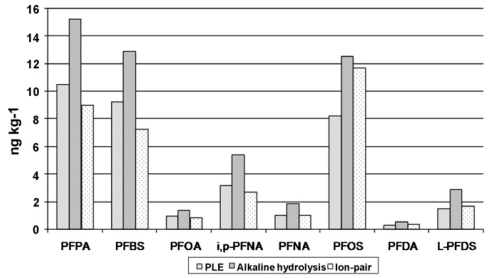

Fig. 4. Histogram of PFCs average concentrations in swordfish muscle by the three extraction methods.

centrations were those corresponding to hake roe. Fig. 3 shows the chromatograms corresponding to one of the three replicates of the hake roe sample, in which PFPA, PFBS, PFOA, PFNA and PFOS were detected. It should be noted that the transition for quantification corresponding to PFOA (413 → 219) shows two additional peaks that cannot be fully confirmed by the second and third confirmatory transitions. These peaks are probably caused by the presence of branched isomers in natural contaminated samples.

The sample of swordfish, which contains all the studied PFCs, was analyzed by the three extraction methods, the results are summarized in Fig. 4. According to Taniyasu et al. [27], the alkaline digestion provided three-to-five higher concentration levels of several PFCs in liver samples than the ion pairing. These authors attributed the greater concentrations of PFCs obtained by the alkaline digestion method to the effective digestion of the matrix and the release of these compounds from the sample. In our study, slightly higher levels in the sample obtained by alkaline digestion were also observed but not so markedly higher as those reported by Taniyasu et al. [27]. Statistical comparison by one-way analysis of variance showed that the differences were not significative, and cannot be considered conclusive.

Analyzing the monitoring results presented in this study, a general conclusion is that PFOA and PFOS levels were in the same range as those found in previous studies carried out in different geographic areas [19–22].

4. Conclusions

The present study demonstrates that LC–MS/MS using a QqLIT mass analyzer was applicable to the simultaneous analysis of 8 PFCs in liver, roe and muscle fish. PLE extraction was chosen for the pretreatment because it was more suitable than par ionic and alkaline digestion for liver and fish samples. It was more rapid and automatic achieving the simultaneous process of up to 24 samples. The proposed method demonstrates to improve LOQs, marginally enhance method recoveries, and decrease analysis times, which will be likely of high value to industry and research laboratories interested in quantitation of PFCs in aquatic organisms.

The high sensitivity of the method provided by the use of QqLIT and the optimized fragmentation conditions, attained reliable quantification at trace level in muscle and liver samples. Separation of i,p-PFNA and PFNA was possible with the LC gradient that combined proper resolution and not too long chromatographic run. The method has shown its feasibility in a study of several edible fish samples from the market. Since the method was developed and validated, it has been routinely used in both laboratories for the screening, quantification and confirmation of PFCs in food as part of a monitoring program.

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3.2.2 Scientific publication 5:

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ORIGINAL PAPER

Study of the performance of three LC-MS/MS platforms for analysis of perfluorinated compounds

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Abstract The analytical suitabilities of three different liquid chromatography–tandem mass spectrometry (LC-MS/MS) systems, (1) triple quadrupole (QqQ), (2) conventional 3D ion trap (IT), and (3) quadrupole–linear IT (QqLIT), to determine trace levels of perfluorinated compounds (PFCs) in fish and shellfish were compared. Sample preparation was performed using alkaline extraction and solid-phase-extraction cleanup. This evaluation was focused on both quantitative (sensitivity, precision, and accuracy) and qualitative (identification capabilities) aspects. In the three instruments, the former facet was evaluated using selected reaction monitoring (SRM), which is the standard mode for quantitative LC-MS/MS analysis. Accuracy was similar in the three systems, with recoveries always over 70 %. Precision was better for the QqLIT and QqQ systems (7–15%) than for the IT system (10–17%). The QqLIT (working in SRM mode) and QqQ systems offered a linear dynamic range of at least 3 orders of magnitude, whereas that of the IT system was 2 orders of magnitude. The QqLIT system achieved at least 20-fold higher sensitivity than the QqQ system, and this was at least tenfold higher sensitivity than for the IT system. In the IT system, identification was based on sensitive full mass range acquisition and MS^n fragmentation and in the QqLIT system, it was based on the use of an information-dependent-acquisition scan function, which allows the combination of an SRM or MS full scan acting as the survey scan and an enhanced product ion scan followed by MS^3 as the dependent scan in the same analysis. Three instruments were applied to monitor the content in fish and shellfish (anchovies, swordfish, tuna, mussels, and oysters) obtained from Valencia and Barcelona markets (Spain). The eight target PFCs were detected at mean concentrations in the range from 10 ng kg^{-1} (perfluoro-7-methyloctanoic acid and perfluoro-1-decanesulfonate) to 4,200 ng kg^{-1} (perfluoropentanoic acid). Furthermore, perfluoroheptanoic and perfluoroundecanoic acids (not covered as target analytes) were identified in some samples.

Keywords Perfluorinated acids · Perfluorinated sulfonates · Liquid chromatography–tandem mass spectrometry · Triple quadrupole · Ion trap · Linear ion trap · Fish

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Introduction

In the early 2000s, preliminary research revealed the wide distribution of perfluorinated sulfonates (PFSAs) and perfluorinated carboxylic acids (PFCAs) in the environment, biota, and humans through the world [1–3]. Further studies evidenced that the main sources of perfluorinated compounds (PFCs) are releases from manufacturing sites, leakage of residual PFCs from fluorotelomer polymers, and breakdown of these precursors themselves [4–6]. Since then, considerable effort has been made to determine the levels of PFCs, the importance of perfluorooctanesulfonate

(PFOS) and other PFCs present in fish and the marine food web having been established, and bioconcentration factors of 4,000 or more have been reported for several PFCs [7–10]. In animal studies, it has been demonstrated that PFCs bind blood proteins, accumulate in the liver, and affect hormone balance, ion channels, and transmission of intercellular signals owing to interaction with lipid cell membranes, and in case of rats, some cancerogenic effects were documented [11–13]. Recently, the European Food Safety Authority established tolerable daily intakes for PFOS and perfluoroctanoic acid (PFOA) [14]. PFOS has been included as a persistent organic pollutant under the Stockholm Convention for global regulation of production and use [15]. PFCs are also prime candidates for chemicals that will need authorization within the REACH regulation [16]. From a human exposure perspective, the analysis of fish fillets and other edible fish products should present an accurate assessment of potential human dietary exposure to PFCs, because these fish products are commonly consumed by humans [17, 18].

Several reviews published on the analysis [19–22], potential sources [23], environmental distribution [1, 6, 24], food occurrence, toxicity [11, 25], and risk assessment [8, 26] remind us of the severity of the analytical challenges facing chemists related to the combined issues of trace concentrations demanding analytical detection limits, complex chemical matrices, unique analytes' molecular properties, and a mandatory high confidence in reported values. These reviews also pointed out that liquid chromatography (LC)–(electrospray ionization)–tandem mass spectrometry (MS/MS) is the technique of choice for the analysis of PFCs in food and the environment because it achieves limits of detection (LODs) in the nanogram per gram to picogram per gram range. LC-MS/MS performed using a triple quadrupole (QqQ) mass spectrometer combined with selected reaction monitoring (SRM) is one of the more widely applied detection systems, as well as being one of those better suited for quantification of PFCs. Nowadays, the performances of 3D ion trap (IT) [27], quadrupole–linear IT (QqLIT) [7, 28], and time-of-flight [27] systems have also been considered for trace quantification of PFCs. However, only one study, performed by Washington et al. [29], compared two QqQ instruments, which showed differences in sensitivity. To our knowledge, there has been no study comparing the best conditions for optimal sensitivity and selectivity of different mass analyzers to determine PFCs in food or environmental samples, and that establishes the strong and weak points of each instrument that can lead to more sensitive and selective determination. The aim of this study was to compare and evaluate three different LC-MS/MS platforms to determine eight relevant PFCs in fish and shellfish: perfluorobutanesulfonate (PFBS), perfluoropentanoic acid (PFPA), PFOA, PFOS, perfluoro-7-methyloctanoic acid (i,p-PFNA), perfluorononaoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoro-1-decanesulfonate (L-PFDS). PFCs were extracted by alkaline digestion, cleaned up by solid-phase extraction (SPE), and further analyzed using the QqQ, IT, and QqLIT systems. The aim was to obtain a fairly generic method that can be extended, in the future, to cope a wide range of foods. The advantages and drawbacks of the IT and QqLIT systems for identifying nontarget PFCs were evaluated using several samples from markets. This identification was conducted by combining alternative full-scan MS to generate MS/MS and MS/MS/MS (MS^3) dependent scans. The application to monitor the content in different fish and shellfish species (anchovies, swordfish, tuna, mussels, and oysters) obtained from the markets is also reported.

Experimental

Chemicals and standards

LC-grade suprasolv water and methanol were purchased from Merck (Darmstadt, Germany). Deionized water (less than 18 MΩ cm resistivity) was from a Milli-Q SP reagent water system (Millipore, Bedford, MA, USA). All the solvents were passed through a 0.45-μm cellulose filter from Scharlau (Barcelona, Spain) before use. Ammonium acetate [puriss. p.a., for high-performance LC (HPLC), purity 99% or higher] was from Sigma-Aldrich (Steinheim, Germany). Ammonium hydroxide (25% in water) and sodium hydroxide (analytical grade) were from Merck. Oasis® WAX cartridges of 60 mg (3 cm^3), particle size 30 μm, and pore size 80 Å were from Waters (Milford, MA, USA). Oasis WAX is a polymeric reversed-phase, weak-anion-exchange mixed-mode sorbent that exhibits retention for acidic, neutral, and, more importantly, strongly acidic compounds (as sulfonates) that are irreversibly retained on strong-anion-exchange resins.

The isotope-labeled internal standards perfluoro-*n*-[1,2,3,4$^{13}C_4$]octanoic acid (MPFOA), perfluoro-1-[1,2,3,4$^{13}C_4$]octanesulfonate potassium salt (MPFOS), and perfluoro-*n*-[1,2$^{13}C_2$]decanoic acid (MPFDA), as well as sodium L-PFDS, PFNA and i,p-PFNA were purchased from Wellington Laboratories (Guelph, ON, Canada) as 50 μg mL^{-1} methanolic solutions (1.2 mL). Tetrabutylammonium PFBS (purum, purity 98% or higher), PFOS sodium salt (98%), PFPA (97%) PFOA (96%), and PFDA (97%), were purchased from Aldrich (Steinheim, Germany). Separate stock solutions of the analytes were prepared in methanol at a concentration of 1.0 mg mL^{-1} of free compound or salt. A standard mixture containing the eight analytes was made from the stock solutions (com-

mercial or laboratory-made) to provide different concentrations of the analytes depending on their expected concentrations in fish and on the sensitivity of the method. The concentrations of the analytes in the standard mixture were calculated as the concentrations of free compounds. Working mixtures were diluted from the standard mixture in methanol/water and 20 mM ammonium acetate (10:90, v/v). Solutions of internal standards were diluted to a concentration of 2 μg mL^{-1} with methanol/water and 20 mM ammonium acetate (10:90, v/v), and appropriate volumes of the internal standards were added to fish and shellfish samples to obtain concentrations of 1.5 μg kg^{-1} in the sample material.

Sample collection

Anchovies (*Engraulis encrasicolus*, *n*=3), swordfish (*Xiphias gladius*, *n*=3), tuna (*Thunnus thynnus*, *n*=3), mussels (*Mytilus galloprovincialis*, *n*=3), and oysters (*Crassostrea gigas*, n=3) were purchased in retail fish markets and supermarkets. Each sample of anchovies, mussels, and oysters weighed around 2 kg and consisted of approximately 100 specimens for fish and 50 for shellfish. Samples of tuna and swordfish were taken as fillets (three fillets per sample). All the samples were sent in fresh conditions (on ice) to the laboratory. Anchovies were dissected, and the entire right dorsal lateral fillet with the skin was taken. The mussels and oysters were washed and scrubbed thoroughly in running water and opened. Whole mussel and oyster soft tissues were removed from a large number of animals and pooled. The right dorsal lateral fillets and swordfish and tuna fillets were cut into small pieces. Subsamples of 200 g were homogenized using a Bapitaurus food chopper (Taurus, Berlin, Germany), placed into polyethylene bags, and stored at −80 °C prior to analysis.

Sample preparation

After the samples were thawed at room temperature, extraction and analysis of PFCs were carried out according to a protocol described elsewhere [5, 30] with some modifications. About 2 g of fish muscle, exactly weighed (fresh weight), was placed in a 15-mL polypropylene tube, homogenized for 2 min with 5 mL of water at 9,700/9,800 rpm using an Ultraturrax T-25 digital homogenizer (Stauffen, Germany), spiked with 1.5 μL of the internal standards, and added to 8 mL of 10 mM sodium hydroxide in methanol. Each sample was digested by shaking it on an orbital shaker table at room temperature for 16 h. After digestion, samples were centrifuged at 2,000*g* for 5 min, and 3 mL of the supernatant was diluted with 27 mL of deionized water prior to SPE cleanup.

These extracts were cleaned up using an Oasis WAX cartridge. The cartridge was first preconditioned by passing 4 mL of 0.1% (v/v) ammonium hydroxide in methanol, 4 mL of methanol, and 4 mL of deionized water at a rate of 2 mL min^{-1} through it. Then, the sample (30 mL) was passed through the preconditioned cartridge at a rate of 2 mL min^{-1}, taking the precaution of not letting the cartridge dry out at any time during the sample loading. The cartridge was then washed with 4 mL of 25 mM acetate buffer (pH 4) followed by 4 mL of methanol and dried completely under a vacuum. The PFCs were eluted with 4 mL of 0.1% ammonium hydroxide in methanol. SPE extracts were evaporated to dryness under nitrogen (60 °C) using a Zymark (Hopkinton, MA, USA) TurboVap LV II because ammonium hydroxide is volatile. Then, 0.5 mL of methanol/water and 20 mM ammonium acetate (10:90, v/v) was used to dissolve the analytes before analysis.

Liquid chromatography–mass spectrometry

Liquid chromatography

Separation was always accomplished on a LiChroCART-LiChrospher 100 RP-18 analytical column of 250 mm× 4 mm and 5-μm particle diameter from Merck at room temperature. The mobile phase consisted of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B) and was delivered at a flow rate of 0.5 mL min^{-1}. The linear gradient elution program was as follows: 10–80% solvent B over 5 min, then 80–90% solvent B over other 5 min, followed by an isocratic hold at 90% solvent B for 8 min. At 18 min, solvent B was returned to 10% in 2 min. The total run time for each injection was 20 min and the injection volume was 20 μL.

Triple quadrupole tandem mass spectrometry

A Quattro LC QqQ mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters) consisting of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and Mass Lynx NT version 4.1 were used. Analysis was performed in negative ion mode. The electrospray ionization source values were as follows: capillary voltage, 3.00 kV; extractor voltage, 1 V; RF lens voltage, 0.5 V; source temperature, 120 °C; desolvation temperature, 350 °C; and desolvation and cone gas (nitrogen 99.99% purity) flows, 600 and 60 L h^{-1}, respectively. The analyzer settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, -1 and 1; multiplier, 650; collision gas (argon,

99.995 %) pressure 2.79×10^{-3} mbar; interchannel delay, 0.02 s; and total scan time, 1.0 s. The mass spectrometer was operated in scan and product ion scan modes to optimize the conditions and select the transitions, and in SRM mode to confirm and quantify the analytes in the samples.

Ion trap tandem mass spectrometry

The LC-IT/MS system consisted of an Esquire 3000 IT LC/MS^n system (Bruker Daltonik, Germany), an Agilent HP1100 LC system (Agilent, Torrance, CA, USA), a computer, and a Daltonic Esquire data acquisition/processing system. The conditions for the source were as follows: temperature, 300 °C; capillary voltage, 4,000 V; end plate offset fixed at −500 V; nebulizer pressure, 10 psi; and drying gas flow 7.00 L min^{-1} at 300 °C. The Esquire 3000 was tuned for each compound, optimizing the voltages on the lenses in the ExpertTune mode of the Daltonic Esquire control software while infusing a standard solution (10 μg mL^{-1}) by a syringe pump at a flow rate of 0.004 mL min^{-1}, which was mixed with the mobile phase at 0.2 mL min^{-1} by means of a T piece. The optimized tuning parameters were set for each compound via time-segment definition.

The mass spectrometer was operated in full scan and multiple reaction monitoring modes. The trap parameters were optimized in ion charge control mode using rolling averaging set at 2. Full scan mode was performed with a target of 50,000 and a maximum accumulation time of 50 ms in an *m/z* range from 50 to 650 u. Multiple reaction monitoring was carried out by setting the target at 200,000 and the maximum accumulation time at 200 ms for both MS and MS^n experiments. Negative ions were detected at unit resolution (scan speed 10,300 u s^{-1}). Four scans were summarized for each spectrum, resulting in a spectral rate of 0.4 Hz. Collision-induced dissociation was performed on the ion of interest by collisions with the helium background gas present in the trap for 40 ms. In these experiments, the deprotonated PFC was subjected to collision-induced dissociation to produce a first set of fragment ions, MS/MS. Subsequently, one of the fragment ions from $[M - H]^-$ was isolated and fragmented to give the next set of fragment ions, MS^3. The fragmentation steps for each compound were optimized by visualizing the changes in the intensities of the fragments ions, whereas the fragmentation cut-off and the fragmentation amplitude were varied manually.

Quadrupole–linear ion trap mass spectrometry

PFCs were analyzed using a 4000 Q TRAP™ MS/MS system from Applied Biosystems/MDS Sciex (Concord, ON, Canada) coupled to a Symbiosis™ Pico system (Spark Holland, Emmen, The Netherlands). The latter integrated an HPLC and online SPE system but only the HPLC system was used, and basically consisted of an Alias™ autosampler and two high-pressure-gradient LC pumps with a four-channel solvent selector for each pump. The mass spectrometer was operated in SRM mode with the resolution set to low (resolution offset drop of 0.1 that provides a mass window 1–4 amu wide) and unit (mass window 1 amu wide) for the first and third quadrupoles, respectively. The other ionization parameters were as follows: curtain gas, 30 (arbitrary units); ion source gas 1, 25 (arbitrary units); ion source gas 2, 25 (arbitrary units); source temperature, 350 °C; ion spray, −4,500 V; entrance potential, −10 V, collision cell exit potential, −10 V; declustering potential −25 V; and dwell time (time used for monitoring each ion transition) 100 ms.

An information-dependent acquisition (IDA) method was programmed combining SRM or enhanced MS as the survey scan and an enhanced product ion (EPI) scan followed by MS^3 as the dependent scan, in the same injection. The SRM parameters used in the survey scan were those previously optimized. The IDA parameters included the acquisition of two ions whose peak height exceeded 500 counts per second and exclusion for 3 s after the acquisition of the same ion. The EPI scan was performed with the first quadrupole set at low resolution and the linear IT scanning from 80 to 362 amu at a rate of 4,000 amu s^{-1}. The dynamic fill-time option was selected on the IT, with a step size of 0.12 amu. Two EPI scans at two different collision energies (−10 and −40 eV) were monitored in each SRM-IDA-EPI cycle. Other operating conditions were as follows: declustering potential, −30 V; collision energy spread, 10 arbitrary units. The complete SRM-IDA-EPI cycle time was 0.97 s.

Method validation

Validation studies were performed using anchovy-spiked muscle. Anchovy blanks were from a sample previously analyzed in the laboratory, in which PFCs were not detected above their LODs. According to several studies [9, 31], in environmental samples, low PFC concentrations [many times at levels below their limits of quantification (LOQs) and/or LODs] were frequently found. This non-detected-PFC sample was used to evaluate the matrix effect, recovery, LOQs, and LODs. Portions of chopped fish (2 g) were placed in the appropriate container and spiked with the PFC standard solution, care was taken to uniformly spread them on the sample. The spiked sample was left for 10 min at room temperature to ensure the appropriate distribution in the matrix. Then, the sample was processed. Five replicates of sample preparation and analysis were performed at each level. The analyte response was always

Table 1 Comparison of the precursor ion to product ion transitions obtained by liquid chromatography (*LC*)–triple quadrupole (*QqQ*)–tandem mass spectrometry (*MS*) and LC–quadrupole–linear ion trap (*QqLIT*)–MS/MS working in selected reaction monitoring (*SRM*) mode

	SRM1[a]	Product ion identification	CE (V)	SRM2[b]	Product ion identification	CE (V)	Additional SRM	SRM2/SRM1 (% RSD, n=21)[c]
LC-QqQ-MS/MS								
PFPA	263→219	$[M\text{-}H\text{-}CO_2]^-$	-10	–	–	–	–	–
PFBS	299→80	$[SO_3]^-$	-30	299→99	$[FSO_3]^-$	-25	–	0.40 (8)
PFOA	413→169	$[C_3F_7]^-$	-15	413→369	$[M\text{-}H\text{-}CO_2]^-$	- 10	–	0.30 (10)
MPFOA	417→172	$[C_3F_7]^-$	-15	417→372	$[M\text{-}H\text{-}CO_2]^-$	-10	–	0.30 (12)
PFOS	499→80	$[SO_3]^-$	-45	499→98.5	$[FSO_3]^-$	-45	–	0.60 (16)
MPFOS	503→80	$[SO_3]^-$	-45	503→98.5	$[FSO_3]^-$	-45	–	0.65 (14)
PFNA	463→419	$[M\text{-}H\text{-}CO_2]^-$	-10	463→219	$[C_4F_9]^-$	-15	–	0.50 (13)
i,p-PFNA	463→419	$[M\text{-}H\text{-}CO_2]^-$	-10	463→219	$[C_4F_9]^-$	-10	–	0.42 (17)
PFDA	513→469	$[M\text{-}H\text{-}CO_2]^-$	-10	513→169	$[C_3F_7]^-$	-10	–	0.30 (15)
MPFDA	515→471	$[M\text{-}H\text{-}CO_2]^-$	-15	515→171	$[FSO_3]^-$	-10	–	0.30 (15)
L-PFDS	599→80	$[SO_3]^-$	-45	463→219	$[C_4F_9]^-$	-45	–	0.25 (10)
LC-QqLIT-MS/MS								
PFPA	263→219	$[M\text{-}H\text{-}CO_2]^-$	-15	–	–	–	–	–
PFBS	299→80	$[SO_3]^-$	-80	299→99	$[FSO_3]^-$	-80	–	0.11 (8)
PFOA	413→169	$[C_3F_7]^-$	-25	413→369	$[M\text{-}H\text{-}CO_2]^-$	-25	413→216	0.43 (11)
MPFOA	417→172	$[C_3F_7]^-$	-25	417→372	$[M\text{-}H\text{-}CO_2]^-$	-25	–	0.45 (9)
PFOS	499→80	$[SO_3]^-$	-100	499→99	$[FSO_3]^-$	-100	–	0.20 (18)
MPFOS	503→80	$[SO_3]^-$	-100	503→99	$[FSO_3]^-$	-100	–	0.25 (16)
PFNA	463→169	$[C_3F_7]^-$	-20	463→219	$[C_4F_9]^-$	-20	469→419	0.80 (10)
i,p-PFNA	463→219	$[C_4F_9]^-$	-20	463→169	$[C_3F_7]^-$	-20	469→419	0.90 (15)
PFDA	513→119	$[C_2F_5]^-$	-35	513→469	$[M\text{-}H\text{-}CO_2]^-$	-35	513→269	0.40 (17)
MPFDA	515→119	$[C_2F_5]^-$	-35	515→471	$[M\text{-}H\text{-}CO_2]^-$	-10	515→271	0.42 (14)
L-PFDS	599→80	$[SO_3]^-$	-100	599→469	$[FSO_3]^-$	-100	–	0.20 (13)

CE collision energy, *RSD* relative standard deviation, *PFPA* perfluoropentanoic acid, *PFBS* perfluorobutanesulfonate, *PFOA* perfluorooctanoic acid, *MPFOA* perfluoro-*n*-[1,2,3,4^{13}C$_4$]octanoic acid, *PFOS* perfluorooctanesulfonate, *MPFOS* perfluoro-1-[1,2,3,4^{13}C$_4$]octanesulfonate potassium salt, *PFNA* perfluorononaoic acid, *i,p-PFNA* perfluoro-7-methyloctanoic acid, *PFDA* perfluorodecanoic acid, *MPFDA* perfluoro-*n*-[1,2^{13}C$_2$]decanoic acid, *L-PFDS* perfluoro-1-decanesulfonate

[a] Quantifier precursor ion to product ion transition

[b] Qualifier precursor ion to product ion transition

[c] RSDs were calculated from mean values obtained from the matrix-matched calibration curves, so as to consider how they varied depending on concentration (seven different concentrations from the limit of quantification (LOQ) of each compound to 3 orders of magnitude higher injected in triplicate).

related to the internal standard response (1.5 μgkg^{-1} of each of MPFOA, MPFOS, and MPFDA) to compensate for undesirable matrix effects and losses during the extraction procedure. The internal standards were selected because, at the beginning of this study, they were the only ones available.

According to Commission Decision 2002/657/EC [32], the criteria for PFC identity confirmation are as follows: (1) the retention time (the relative retention time of the PFC shall correspond to that of the calibration solution at a tolerance of ±2.5%), (2) the presence of MS ions (when mass-spectrometric determination is performed by the recording of full scan spectra, the presence of all measured diagnostic ions with a relative intensity of more than 10% and when SRM is used, the presence of two precursor ion to product ion transitions for each PFC); and (3) the relative intensities of the detected ions (percentage of the intensity of the most intense ion or transition) shall correspond to those of the calibration standard at comparable concentrations within tolerances ranging from 20 to 50% depending on the relative intensity of the base peak.

The major obstacle to an accurate and reliable analysis of PFCs was the problems of procedural blanks because of their presence in various items of equipment, sample containers, polypropylene material, or SPE cartridges. Blank tests were performed with all these items and the corresponding modifications to eliminate PFC contamina-

Table 2 Relative abundances of the MS^n ions by LC–ion trap (*IT*)–MS^n and LC-QqLIT-MS^3 obtained in spiked anchovy extracts at a concentration of 50 μg kg^{-1}

PFCs	LC-IT-MS^n[a]			LC-QqLIT-MS^3[b]		
	MS	MS/MS	MS^3	Precursor ion	EPI	MS^3
PFPA	263 (100)	219 (100)	–	263 (100)	219 (100)	–
PFBS	299 (100)	–	–	299 (100)	99 (50) 80 (100)	–
PFOA	413 (100)	369 (100)	319 (15), 269 (100), 219 (50), 169 (15)	413 (100)	369 (100) 219 (40) 169 (80)	219 (100) 169 (20)
MPFOA	417 (100)	372 (100)	322 (15), 272 (100), 222 (50), 172 (15)	417 (100)	372 (100) 222 (45) 172 (20)	222 (100) 172 (25)
PFOS	499 (100)	–	–	499 (100)	99 (40) 80 (100)	– –
MPFOS	503 (100)	–	–	503 (100)	99 (30) 80 (100)	– –
PFNA	463 (100)	419 (100)	319 (22), 269 (100), 219 (40), 169 (15)	463 (100)	419 (100) 219 (30) 169 (30)	219 (100) 169 (15)
i,p-PFNA	463 (100)	419 (100)	319 (20), 269 (100), 219 (20), 169 (15)	463 (100)	419 (100) 219 (50) 169 (60)	219 (100) 169 (25)
PFDA	513 (100)	513 (20), 469 (100)	319 (20), 269 (100), 219 (40)	513 (100)	469 (100) 219 (40) 169 (50)	219 (100) 169 (25)
MPFDA	515 (100)	515 (20), 471 (100)	471 (20), 271 (100), 221 (30)	515 (100)	471 (100) 271 (10) 119 (5)	221 (100) 171 (45)
L-PFDS	599 (100)	–	–	599 (100)	99 (40) 80 (100)	– –

The ion selected in the enhanced product ion (*EPI*) mode as a precursor ion for MS^3 is given in *italics*.

[a] MS/MS and MS^3 were carried out by isolating the most abundant precursor ion with a width of 4.0 *m/z*, a cutoff of 200 for L-PFDS, MPFDA, and PFDA and a cuttoff of 150 for the other perfluorinated compounds (PFCs), and collisions with the helium background gas present in the trap for 40 ms at an amplitude ranging from 1.0 to 2.0 V.

[b] EPI data were obtained at a CE of -40 V for PFSAs and of -10 V for PFCAs. MS^3 data were obtained at excitation energies of 50 V and CEs of -40 V.

tion, if present, were made. The QqQ, the IT, the sample containers, or the polypropylene material used did not show background signals of the PFCs. However, background signals for PFOA and PFOS were always observed at concentrations near their LOQs in the QqLIT and in the SPE cartridge, after preconditioning with water/methanol and elution with 0.1 % ammonium hydroxide in methanol. After all the accessible polytetrafluorethylene materials in the LC-QqLIT system were replaced, the background signal disappeared. In the case of SPE cartridges, the background signal disappeared after a conditioning step consisting of passing 5 mL of the elution solution prior to water/methanol conditioning. Different controversial studies support the cross-contamination of samples during analysis of PFCs. Yamashita et al. [33] examined the contamination in Oasis HLB and Sep-Pak (C_{18}) SPE cartridges. Both SPE cartridges were a cause of PFC contamination by PFOS and PFOA. On the other hand, Taniyasu et al. [5] evaluated Oasis HLB and Oasis WAX columns and detected a few target PFCs at a few picograms per liter, including PFOA, PFDA, and perfluoroundecanoic acid (PFUnDA). Taking all the reported precautions, the procedural blanks did not show contamination by PFCs for the entire method. Recent studies have also reported the absence of PFC contamination in procedural blanks [31].

The LOD was defined as the lowest concentration for which the peak area was at least 3 times larger than the background noise. The criteria for the LOQ were established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less

Table 3 Limit of detection (*LOD*) and limit of quantification (*LOQ*), both in micrograms per kilogram, obtained with the different mass analyzers tested

	QqQ in SRM		QqLIT in SRM		QqLIT (EPI)		IT-MS		IT-MS/MS		IT-MS^3	
PFCs	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
PFPA	0.3	1	0.01	0.05	0.05	0.25	0.3	1	5	15	5	15
PFBS	0.3	1	0.01	0.05	0.10	0.50	5	15	5	15	5	15
PFOA	0.1	0.5	0.001	0.005	0.005	0.025	0.06	0.2	2	15	8	24
MPFOA	0.1	0.5	0.001	0.005	0.005	0.025	0.06	0.2	2	6	8	24
PFOS	0.3	1	0.0005	0.003	0.0025	0.010	1	3	3	10	3	10
MPFOS	0.3	1	0.0005	0.003	0.0050	0.030	1	3	3	10	3	10
PFNA	0.1	0.5	0.003	0.01	0.020	0.060	0.2	0.7	2	6	2	6
i,p-PFNA	0.1	0.5	0.003	0.01	0.020	0.060	0.2	0.7	2	6	3	10
PFDA	0.1	0.5	0.01	0.05	0.05	0.25	0.1	0.3	3	10	6	18
MPDA	0.1	0.5	0.01	0.05	0.05	0.25	0.1	0.3	3	10	6	18
PFDS	0.1	0.5	0.01	0.05	0.10	0.50	3	10	3	10	3	10

than 25%; (2) relative standard deviation (RSD) of four replicates below 19%; (3) peak shapes acceptable; and (4) signal-to-noise ratio at least 10. The LOQs obtained served as the lower limits of the linear range.

The linear range was defined by plotting the peak area ratio of the PFC to the internal standard versus PFC concentration. The following criteria for the linear range were applied: linear regression through zero with a correlation coefficient better than 0.990; bias from the calibration line less than 25% for all individual calibration points; and RSD of four replicates less than 19%. The lower limit of the linear range was at the LOQ.

The matrix effects were assessed by comparing the response of the analytes at 10 μg kg^{-1} concentration in 20 mM ammonium acetate and methanol/water (10:90, v/v) solution to the response of the analytes spiked at the same concentration into an extract of a blank matrix sample extract (young hake or anchovy) obtained through the sample preparation process.

As no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. Briefly, five negative samples of tissue of anchovy were independently spiked as previously described with the eight PFCs at the LOQ of the instrument and analyzed. The precision, determined as the RSD, was obtained from the spiked samples (replicate extraction and analysis) on the same day (intraday precision, $n=5$) and on different days (interday precision, $n=5$).

Finally, to comply with internal quality control procedures, two control samples (spiked materials), two solvent injections, and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behavior chart during the entire duration of the study to establish if the analysis was in a state of statistical control or not.

Results and discussion

Comparison of the three instruments

The three instruments checked have different abilities to determine PFCs. The QqLIT and QqQ systems are quadrupole-based instruments and both show appropriate sensitivity for monitoring specific precursor ion to product ion transitions. Besides, working as a QqQ, the QqLIT provides access to sensitive IT scans. This allows very powerful scan combinations when performing IDA, EPI, or MS^3 experiments obtaining concomitantly both quantitative and qualitative information. The 3D IT is also capable of performing full mass range acquisitions with high sensitivity allowing nontarget screening.

Although very specific LC-MS systems are used, an appropriate separation is still required, In this case, the two stereoisomers PFNA and i,p-PFNA are not separable by precursor or product ions [7]. In addition to the LiChrospher 100 RP-18 column, three other C_{18} reversed-phase columns were tested: a XTerra MS C_{18} column (100 mm× 2.1-mm inner diameter, 3.5-μm particle size) (Waters), a Luna C^{18} column (150 mm×4.6-mm inner diameter, 3-μm particle size) (Phenomenex, Paris, France), and a Luna C_{18} column (250 mm × 4.6-mm inner diameter, 5-μm particle size). The LiChrospher column gave the best resolution, especially between the PFNA and its branched isomer, i,p-PFNA, in a similar time of analysis (chromatographic run of less than 20 min).

First, LC-QqQ-MS/MS and LC-QqLIT-MS/MS were compared in SRM mode. When operating in SRM mode, the Q TRAP instrument works like a QqQ instrument; thus, different performances with respect to other QqQ instruments may be related to the source and ion transport efficiency or the collision cell efficiency and setting. This is

Table 4 Analytical performance of the proposed LC-QqQ-MS/MS, IT-MS/MS, and LC-QqLIT-MS/MS methods (the analyte response was always related to the internal standard response, PFBS, PFOS and L-PFDS to $^{13}C^4$-MPFOS, PFPA, PFOA, PFNA, and i,p-PFNA to $^{13}C_4$-MPFOA, and PFDA to $^{13}C_2$-PFDA, to compensate for undesirable matrix effects and losses during the extraction procedure)

PFCs	LC-QqQ-MS/MS				IT-MS/MS				LC-QqLIT-MS/MS			
	Linearity (r^2)[a]	Precision (% RSD) (n =5)[b]		Recovery (n=5)[b]	Linearity (r^2)[a]	Precision (% RSD) (n =5)[b]		Recovery (n=5)[b]	Linearity (r)[a]	Precision (% RSD) (n =5)[b]		Recovery (n=5)[b]
		Intraday	Interday			Intraday	Interday			Intraday	Interday	
PFPA	0.991	8	12	75	0.981	14	17	74	0.993	8	12	78
PFBS	0.990	13	16	80	0.979	15	17	82	0.992	12	14	84
PFOA	0.997	7	10	97	0.981	14	16	98	0.998	9	13	99
PFOS	0.999	8	11	96	0.978	12	16	96	0.997	10	15	95
PFNA	0.999	7	13	81	0.988	13	15	83	0.998	9	12	80
i,p-PFNA	0.998	9	12	85	0.986	10	14	84	0.999	7	10	88
PFDA	0.999	7	14	101	0.985	11	15	100	0.997	7	9	102
L-PFDS	0.997	8	15	93	0.986	12	17	90	0.996	8	11	95

[a] Calculated in pure solvent

[b] Calculated from spiked anchovy samples at 5 times the LOQ of each instrument

particularly important for instruments of different generations, such as those compared in this study. To judge the sensitivity of both instruments, SRM transitions were chosen after optimization of the conditions, considering both sensitivity and selectivity. Two transitions per compound were selected to comply with EU requirements for confirmatory analysis [32]. MS/MS parameters were optimized as shown in Table 1. The precursor ion to product ion transitions were almost the same using LC-QqQ-MS/MS and LC-QqLIT-MS/MS. For PFCAs, typical fragment ions in the MS/MS spectra corresponded to losses of 44 amu, assigned to $[M–H–CO_2]$ ions, characteristic of the presence of carboxylic acid functionalities in the molecule, as well as to the fragment ions at m/z 169 and 219 corresponding to $[C_3F_7]^-$ and $[C_4F_9]^-$. However, different precursor ion to product ion transitions for quantification of the PFCAs were used depending on the mass analyzers (e.g., product ions m/z 369, 419, and 446 by QqQ-MS/MS and m/z 169, 169, and 119 by QqLIT-MS/MS for PFOA, PFNA, and PFDA, respectively). Apparently, the lower-mass fragments are more intense using the QqLIT than the QqQ, at least in the conditions chosen. For PFSAs, the distinctive product ions m/z 80 and 99 corresponding to $[SO_3]^-$ and $[FSO_3]^-$ were obtained by both mass analyzers.

The ratio between the two optimized SRM transitions (SRM2/SRM1) was calculated for use as an identification criterion, in addition to the retention time and the presence of both transitions. The ratio of the two transitions of all PFCs studied as a function of concentration remained relatively constant except at very low concentrations and never exceeded the tolerable value (20% for PFOS and MPFOS and 25% for the others by QqQ and 20% for PFNA and i,p-PFNA, 30% for PFBS and L-PFDS, and 25% for the others by QqLIT). From the values shown in Table 1, it can be seen that PFPA presents only one useful transition, which does not allow the appropriate confirmation of the identity of the compound. In the case of PFBS determined by LC-QqLIT, there was difference in intensity of up to 8 times between the two transitions. This is a limitation when identifying this compound in samples at low concentrations, because the presence of the less intense transition (SRM2) should be present with a signal-to-noise ratio of 3. Although both LC-QqQ-MS/MS and LC-QqLIT-MS/MS in SRM mode from a quantitative point of view are the preferred techniques, they do not always achieve the criteria for the identify confirmation of a compound. The new mass analyzers with distinct scanner advantages can help to identify PFCs correctly.

IT and QqLIT instruments working in full mass range acquisition modes (MS, MS/MS, or MS^3) can improve the identification and confirmation capabilities. Table 2 shows the fragmentation obtained for the PFCs. Multiple-stage

Table 5 Mean concentration and the respective standard deviation obtained for the eight target PFCs and other PFCs identified in fish and shellfish samples taken from the market

	Mean±standard deviation (μg kg^{-1}, n=3)								
Sample	PFPA	PFBA	PFOA	i,p-PFNA	PFNA	PFOS	PFDA	PFDS	PFCs identified
Anchovies 1	0.52±0.06	0.33±0.03	0.78±0.08	ND	0.052±0.007	1.05±0.09	0.95±0.08	0.013±0.001	–
Anchovies 2	1.26±0.11	0.25±0.02	1.15±0.12	ND	0.12±0.01	0.88±0.09	0.23±0.03	ND	–
Anchovies 3	0.98±0.07	0.42±0.05	2.54±0.03	ND	0.085±0.009	1.25±0.13	0.083±0.009	ND	–
Swordfish 1	42.05±3.92	10.29±0.99	12.18±1.15	0.52±0.05	0.92±0.11	15.36±1.47	0.085±0.007	0.062±0.006	PFHpA
Swordfish 2	2.03±0.18	8.75±0.87	8.84±0.80	ND	0.88±0.09	9.72±0.88	1.26±0.11	0.12±0.02	–
Sword fish 3	13.05±1.52	4.32±0.56	7.74±0.73	0.025±0.003	0.94±0.08	8.25±0.74	4.18±0.37	0.045±0.005	–
Tuna 1	5.00±0.45	6.24±0.71	0.59±0.06	0.33±0.03	0.25±0.03	0.033±0.004	25.10±2.07	ND	PFUnDA
Tuna 2	25.00±2.73	10.52±1.24	8.05±0.85	0.038±0.005	2.52±0.25	12.04±1.11	0.64±0.05	0.022±0.003	–
Tuna 3	1.25±0.09	3.52±0.38	1.48±0.12	1.25±0.09	0.32±0.03	2.25±0.21	0.29±0.04	ND	–
Mussels 1	8.25±0.74	0.12±0.01	6.52±0.62	0.017±0.002	0.12±0.01	15.02 ±1.35	0.32±0.03	0.043±0.005	PFHpA
									PFUnDA
Mussels 2	ND	0.54±0.06	2.84±0.25	0.035±0.004	0.53±0.04	12.82±1.11	1.25±0.10	0.014±0.002	–
Mussels 3	10.25±1.04	0.29±0.03	5.45±0.49	0.012±0.002	0.082±0.009	17.98±1.25	0.32±0.03	ND	–
Oysters 1	12.01±1.13	0.48±0.04	1.08±0.10	ND	ND	8.25±0.75	0.15±0.01	ND	–
Oysters 2	ND	0.36±0.04	2.34±0.21	0.014±0.002	0.12±0.02	4.02±0.33	0.45±0.03	ND	PFUnDA
Oysters 2	9.58±0.89	0.48±0.05	5.82±0.54	ND	0.051±0.006	1.25±0.11	0.25±0.02	ND	–

ND none detected, *PFHpA* perfluoroheptanoic acid

fragmentation using ^{3}D IT has a limitation because the isolation and fragmentation steps both occur in the IT (3D or linear), only product ions with *m/z* values higher than 30% of the precursor mass are stable in the IT. That is the reason why an IT cannot be used for MS/MS analysis of PFSAs, because of the large mass difference between the precursor ions (*m/z* 299, 499, and 599) and the product ions (*m/z* 99 and 80). However, IT technology was useful for monitoring of PFCAs because fragmentation resulted in decarboxylation as well as characteristic $[C_3F_7]^-$ and $[C_4F_9]^-$ product ions. MS^3 gave, in addition to these characteristic product ions, $[C_6F_{13}]^-$ and $[C_5F_{11}]^-$.

The IDA software permitted the collection of SRM transitions for each compound or "enhanced MS," the acquisition of the EPI spectrum of each substance found in the chromatogram fulfilling the selection criteria for the acquisition of the dependent scan. In the EPI mode, the precursor ions above a predefined intensity threshold are instantaneously transmitted to the collision cell and fragmented; the fragments are trapped in the third quadrupole before filtration. The MS^3 mode selects EPI fragments of interest to fragment them again. One advantage is that the MS/MS spectrum obtained is most similar to the QqQ fragmentation using SRM mode (Table 2). The product ions of PFSAs, the *m/z* of which are lower than one third of those of the parent ion, can be properly confirmed. One drawback of the IDA procedure might be due to the impossibility of optimizing the fragmentation parameters for each different precursor during the product ion scan. The use of a collision energy common to all compounds might preclude all chances of achieving a good fragmentation, especially if very different chemical structures are involved (e.g., PFCAs and PFSAs).

The LODs and LOQs in Table 3 indicate that LC-QqLIT-MS/MS working in SRM mode can measure low PFCs concentrations in the normal ranges for fish (LODs ranged from 0.001 μg kg^{-1} for PFOS to 0.01 μg kg^{-1} for L-PFDS). It achieved at least 20-fold higher sensitivity than LC-QqQ-MS/MS. LC-QqLIT-MS/MS working in IDA mode is also more sensitive than LC-QqQ-MS/MS. For example, for PFOS a LOD of 0.001 μg kg^{-1} was obtained compared with 0.3 μg kg^{-1} by QqQ-MS/MS or 3 μg kg^{-1} by IT-MS/MS. The IT provides the worst LODs and LOQs; these LODs are only comparable to those obtained by LC-QqQ-MS/MS when the IT worked in full scan single MS acquisition mode, but this mode is not selective enough to work with routine samples. Each MS fragmentation step added to the method made the sensitivity worse. According to a recent study by Luque et al. [31], ITs achieved the best sensitivity by full scan or ion isolation, which indicated that the latter can be used for sensitive monitoring of PFCs in biological samples. However, as pointed out in most studies [31, 34], the applicability of the full scan or ion isolation for analyzing complex matrices remains dubious because of the lower selectivity, particularly for PFOS analysis, where known mass interferences from the presence of bile salt in biota exist.

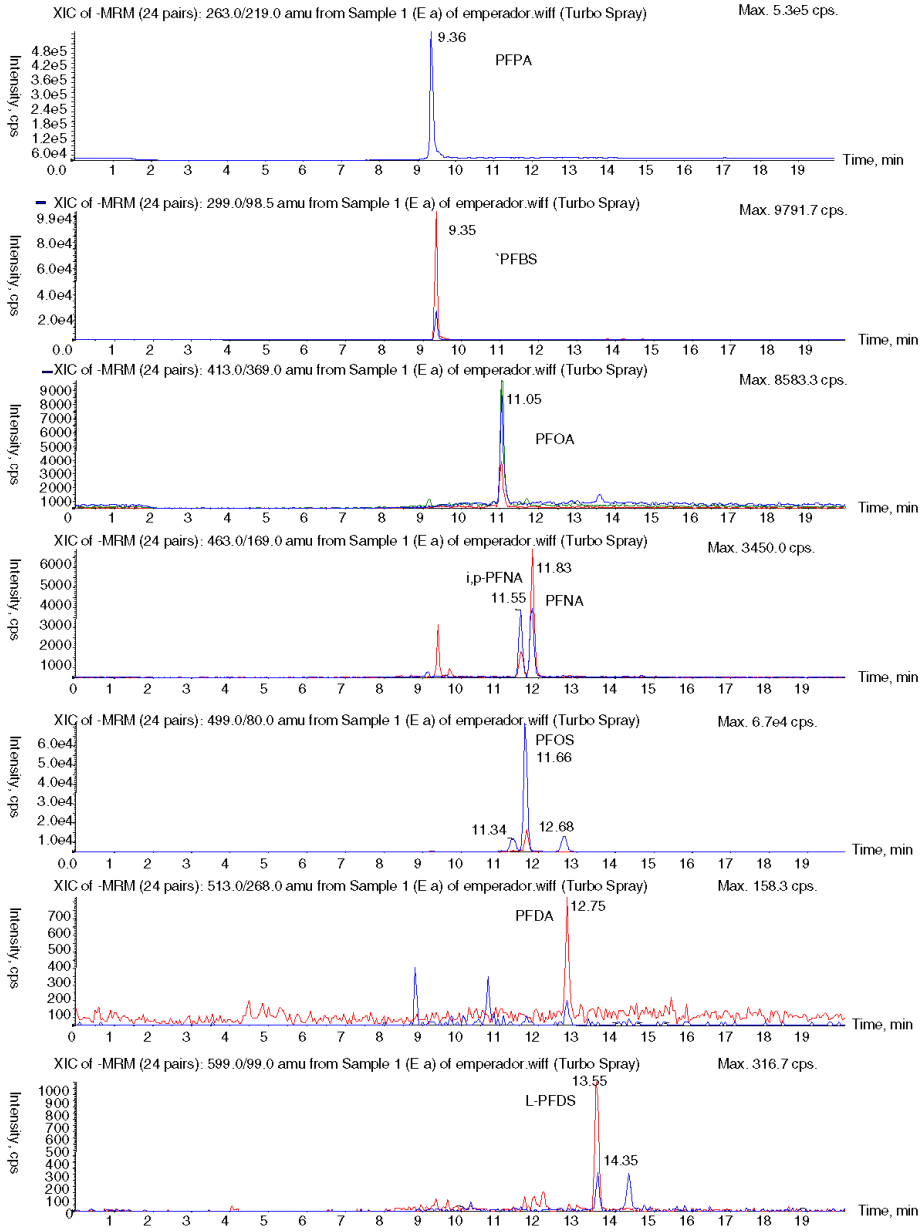

Fig. 1 Extracted ion chromatograms (*XIC*) obtained from swordfish sample 1 using quadrupole–linear ion trap tandem mass spectrometry (MS) in selected reaction monitoring (SRM) mode. *MRM* multiple reaction monitoring, *PFPA* perfluoropentanoic acid, *PFBS* perfluorobutanesulfonate, *PFOA* perfluorooctanoic acid, *PFNA* perfluorononaoic acid, *i,p-PFNA* perfluoro-7-methyloctanoic acid, *PFOS* perfluorooctanesulfonate, *PFDA* perfluorodecanoic acid, *L-PFDS* perfluoro-1-decanesulfonate

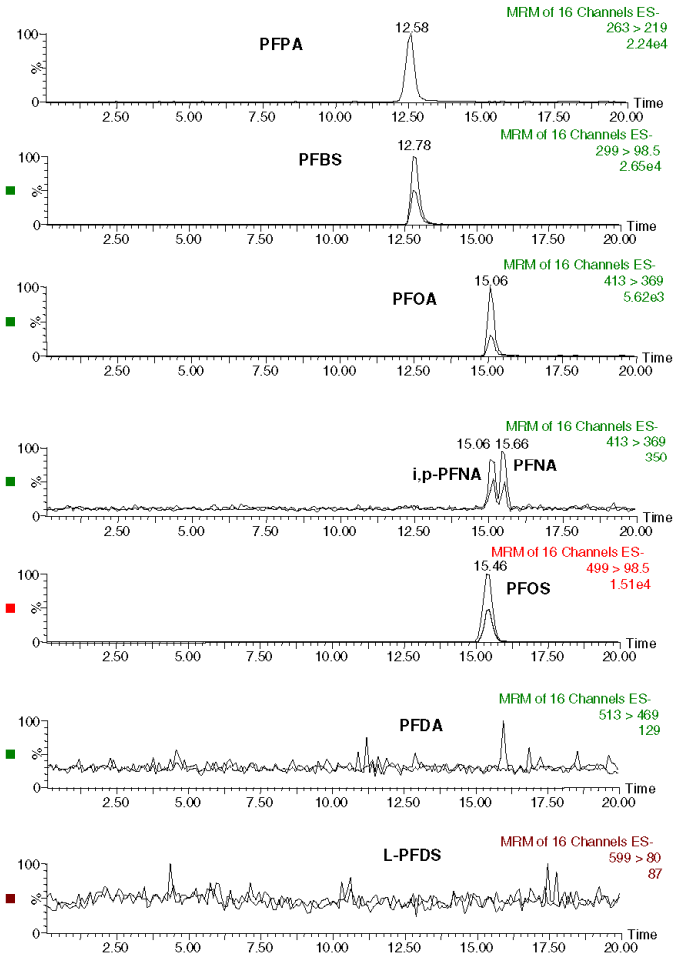

Fig. 2 XIC obtained from swordfish sample 1 using triple quadrupole MS/MS in SRM mode

The three instruments can be applied to monitor fish and shellfish samples. If measurements of lower PFC concentrations are required, the final extract volume could be reduced, the sample volume injected could be increased, or the mass-spectrometric sensitivity has to be upgraded.

For quantitative purposes, peak area data of the most abundant transition (SRM1) were considered. The results of the validation study are shown in Table 4. They demonstrated better interday and intraday precision for the QqQ and QqLIT, with RSD≤14%, than for the IT, with RSD ≤ 17, and good linear dynamic range, over 3 orders of magnitude for the QqQ and QqLIT, with r^2>0.99 for most compounds, but not for the IT, with r^2≤0.98. The linear dynamic range for the IT was acceptable only for 2 orders of magnitude, with r^2>0.99. Obviously, the recovery was almost the same with the three systems (between 70 and 100%) because it is a parameter that mainly depends on the extraction procedure. Evaluation of the ionic suppression or enhancement due to coeluted compounds of the matrix was performed by comparing the regression curves obtained with standards added to the matrix extract and those prepared in pure solvents. Only ion suppression was observed. This result agrees with those reported in the second worldwide interlaboratory study, in which predominantly ion suppression was found [9]. However, in that interlaboratory study, ion enhancement was also observed for individual compounds in the same matrix. Perfluorinated sulfonamide showed the largest enhancement. This is a neutral PFC that was not included in our study. In our study, the use of mass-labeled analogues as internal stand-

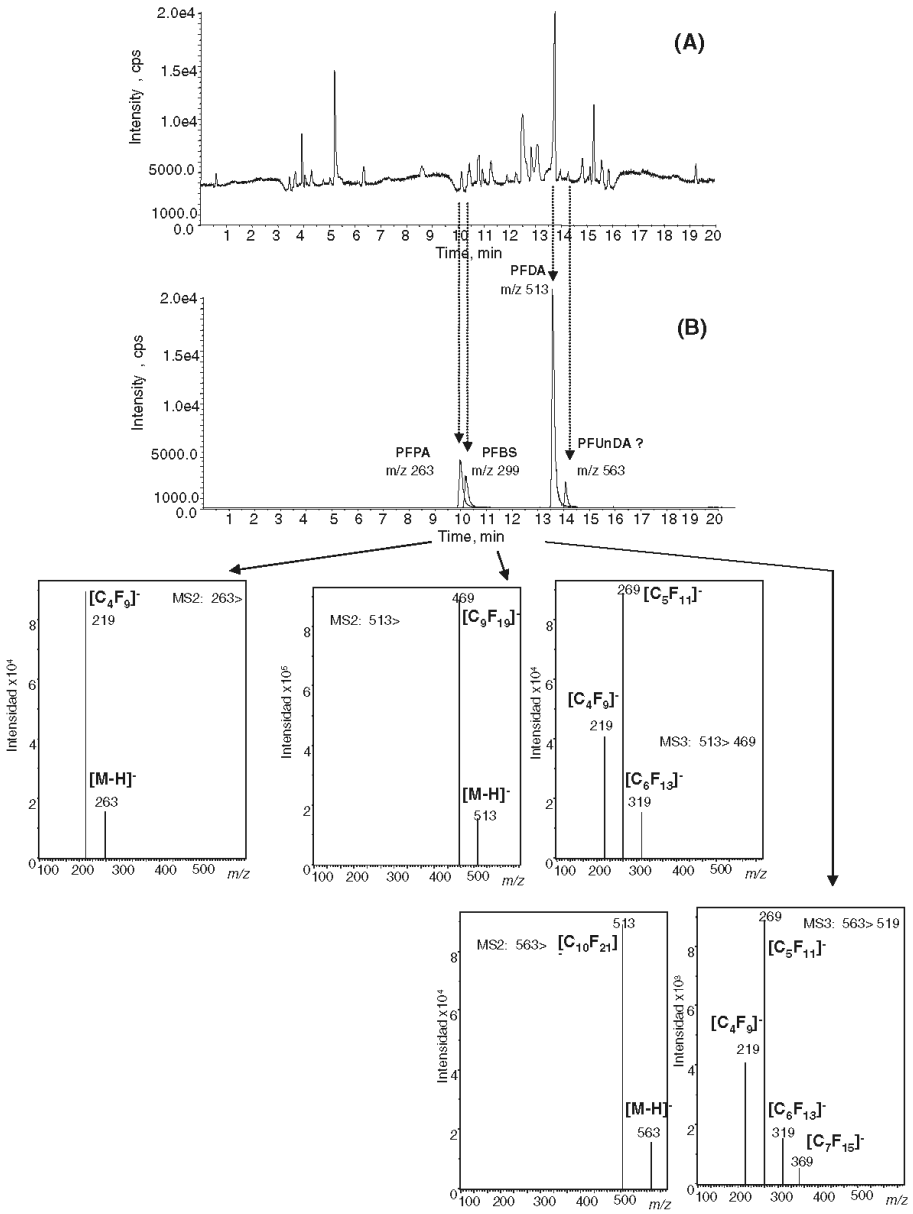

Fig. 3 MS/MS and MS/MS/MS chromatograms in the ^{3}D ion trap obtained from an extract of tuna sample 1. Mass spectra of the main peaks are shown as an *insert* and the *vertical axes* are linked to appreciate the differences in sensitivity. *PFUnDA* perfluoroundecanoic acid

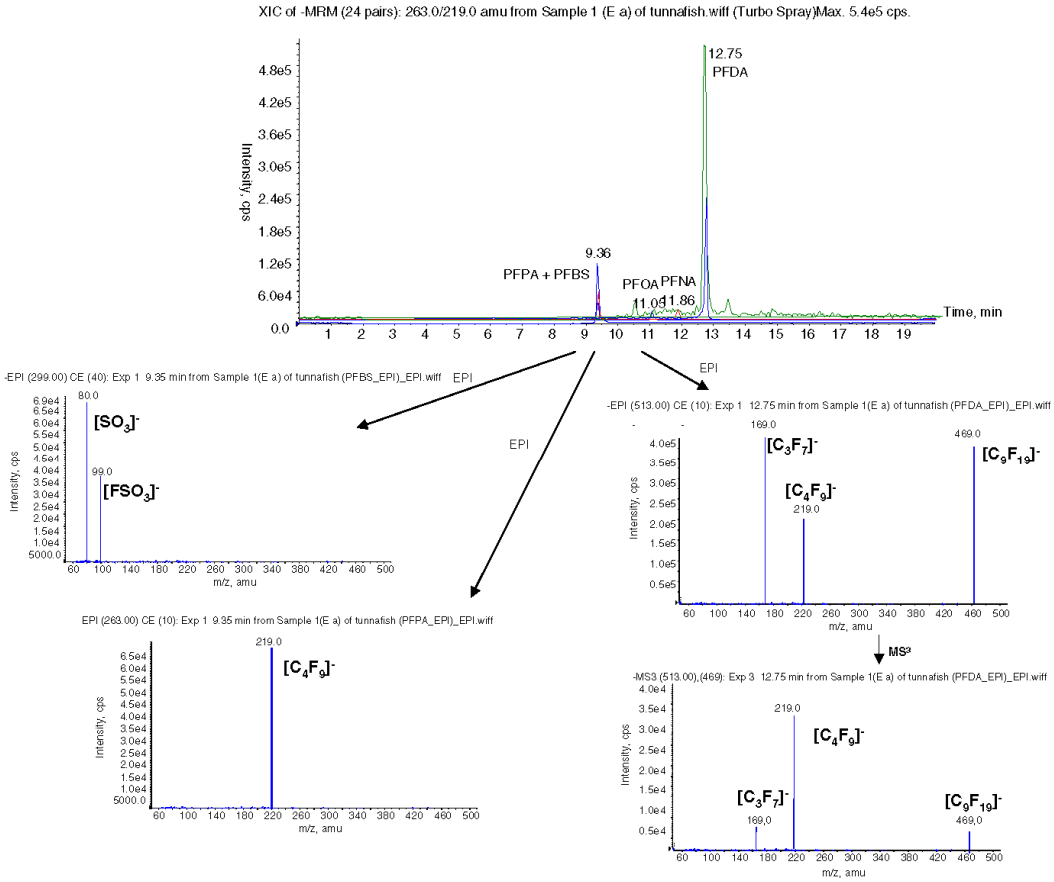

Fig. 4 Example of total ion chromatogram, MS/MS spectra using enhanced product ion (*EPI*) mode, and MS/MS/MS spectra of PFPA, PFBS and PFDA obtained for tuna sample 1. *CE* collision energy

ards corrected for most of the matrix effect, even for those compounds without mass-labeled analogues available (the suppression effect was lower than 10 %). Matrix-matched calibration curves were not used for quantitative determinations because the suppression effect is low and the differences in the composition of the different types of samples are also important.

Application to the analysis of fish and shellfish from the market

The three instruments in their different working modes were also applied to the analysis of fish and shellfish samples taken randomly from markets in Valencia and Barcelona to evaluate the presence of the PFCs and compare the efficiency and confirmation capability of the three instruments. The samples were analyzed in triplicate to establish the accuracy of the measurements. Table 5 shows the results (mean value plus standard deviation) obtained for quantification with LC-QqLIT-MS/MS working in the SRM mode. As compared with the second worldwide interlaboratory study on PFCs [9], the RSDs in this study are 7–17%, whereas those of the interlaboratory study were 22–47%. The data in this study are in good agreement and even better than the between-laboratory variances reported and within the interday precision obtained using QqLIT-MS/MS. The underlying reasons for this could be the use of mass-labeled standards. All the compounds included in the study were present in fish and shellfish at concentrations ranging from 10 ng kg^{-1} (i,p-PFNA and L-PFDS) to 4,200 ng kg^{-1} (PFPA). The high sensitivity of LC-QqLIT-MS/MS is experimentally con-

firmed in Figs. 1 and 2, which show the chromatograms of swordfish extract 1 determined in SRM mode by LC-QqLIT-MS/MS and LC-QqQ-MS/MS, respectively. PFDA and L-PFDS were not identified using LC-QqQ-MS/MS .

Figure 3 illustrates the MS/MS and MS^3 chromatograms obtained in a 3D IT of tuna sample 1 (the mass spectra obtained are also shown as an insert). The MS fragments obtained were the same as reported for the other mass analyzers. The PFSAs do not give product ion mass spectra in the 3D IT. However, the lack of fragmentation of these compounds at high amplitudes can be considered additional confirmation. Figure 3 also shows in the same tuna sample 1 the identification of PFUnDA from the MS spectrum obtained in the IT and the subsequent fragmentations of the peak. With use of single MS, the PFCs at relatively low concentration were missed owing to the high background encountered, and for this reason PFCs were screened against a nominal mass database, which contained data on 30 compounds out of the compound classes of PFCAs, PFSAs, and perfluorosulfonamides. Perfluoroheptanoic acid and PFUnDA may have been detected in several samples.

Figure 4 refers to the sample extract of tuna sample 1 analyzed by LC-QqLIT in the EPI mode and by MS^3. The MS^3 mass spectra could not be obtained for PFPA and PFBS. However, as the fragmentation in the linear IT is more similar to that of the QqQ, PFSAs can be properly confirmed. The presence in the sample of PFPA, PFBS, and PFDA was inferred from both the SRM peaks (at the corresponding retention times) and the EPI spectra.

Conclusions

The data presented show the capabilities of modern MS for detection and identification of analytes such as PFCs in complex matrices. From the different instruments tested, the QqLIT working in SRM modes provides the best LOQs; the sensitivity of the IDA methods (EPI and EPI-MS^3) is approximately 20 times lower compared with that of SRM methods. The other mass analyzers, QqQ and 3D IT, can determine most of these compounds in the samples analyzed. The QqLIT and 3D IT present additional features, such as the MS^3 fragmentation and the possibility to search for other nontarget PFCs that help to confirm the identity or to identify other nontarget related compounds.

Therefore, combination of the information obtained in several systems according to the performance characteristics of the compounds is still the best option. The versatility of the QqLIT instrument evidences an interesting combination: conventional SRM provides excellent sensitivity and selectivity in the quantitation and the IDA mode, combining an SRM or MS scan as the survey scan and an EPI scan as the dependent scan within the same run, conserves the quantitative performance of SRM methods, and provides additional product ion spectra at low concentrations. Despite the advantages of the IT scanning possibilities, some limitations have also been evidenced for compounds that present scarce MS/MS fragmentation under the conditions selected (PFPA) or that provided fragments at low *m/z* ratios (PFSAs).

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3.2.3 PFASs in infant daily intake

The experimental work and results are presented in the following publications:

Scientific publication 6:

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"Infant exposure of perfluorinated compounds: Levels in breast milk and commercial baby food"

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Infant exposure of perfluorinated compounds: Levels in breast milk and commercial baby food

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ABSTRACT

In this study, an analytical method to determine six perfluorinated compounds (PFCs) based on alkaline digestion and solid phase extraction (SPE) followed by liquid chromatography-quadrupole-linear ion trap mass spectrometry (LC-QqLIT-MS) was validated for the analysis of human breast milk, milk infant formulas and cereals baby food. The average recoveries of the different matrices were in general higher than 70% with a relative standard deviation (RSD) lower than 21% and method limits of detection (MLOD) ranging from 1.2 to 362 ng/L for the different compounds and matrices.

The method was applied to investigate the occurrence of PFCs in 20 samples of human breast milk, and 5 samples of infant formulas and cereal baby food (3 brands of commercial milk infant formulas and 2 brands of cereals baby food). Breast milk samples were collected in 2008 from donors living in Barcelona city (Spain) on the 40 days postpartum. Perfluorooctanesulfonate (PFOS) and perfluoro-7-methyloctanoic acid (i, p-PFNA) were predominant being present in the 95% of breast milk samples. Perfluorooctanoic acid (PFOA) was quantified in 8 of the 20 breast milk samples at concentrations in the range of 21–907 ng/L. Commercial formulas and food were purchased also in 2009 from a retail store. The six PFCs were detected in all brands of milk infant formulas and cereals baby food analyzed, being perfluorodecanoic acid (PFDA), PFOS, PFOA and i, p-PFNA the compounds detected in higher concentrations (up to 1289 ng/kg). PFCs presence can be associated to possible migration from packaging and containers during production processes.

Finally, based on estimated body weight and newborn intake, PFOS and PFOA daily intakes and risk indexes (RI) were estimated for the firsts 6 month of life. We found that ingestion rates of PFOS and PFOA, with exception of one breast milk sample did not exceed the tolerable daily intake (TDI) recommended by the EFSA. However, more research is needed in order to assess possible risk associated to PFCs contamination during early stages of life.

1. Introduction

Perfluorinated compounds (PFCs) have been manufactured since the 1960s for a wide range of industrial and consumer applications. The strong carbon fluorine (C–F) bonds of PFCs give them a high thermal, chemical and biological stability. These compounds have been employed in textiles and food packaging due to their unique properties as repellents of water and oils. PFCs have been also used as surfactants and lubricants in fire-fighting foams, pesticides and personal care products (Voogt and Saez, 2006). Until the last years the environmental occurrence of PFCs has received little attention, mainly because of their low acute toxicity and difficulties in their chemical analysis. However, their manufacture, use and disposal have led to their widespread distribution in the environment. PFCs have been detected in different water matrices (as rivers (Kannan et al., 2002), lakes (Furdui et al., 2007), rainwater (Kim and Kannan, 2007), and wastewater (Bossi et al., 2008)), in the air (Kim and Kannan, 2007), wildlife (Giesy and Kannan, 2001; Kelly et al., 2009) and humans (Kärrman et al., 2009; Tao et al., 2008a,b). These compounds are biomagnified in the aquatic food chains (Kannan et al., 2005; Kelly et al., 2009) and are highly persistent (Olsen et al., 2007). A study performed with serum of retired production workers showed that half-life elimination of PFOS, perfluoro hexanesulfonate (PFHxS) and PFOA appears to occur over a long period of time. Differences in species-specific pharmacokinetics may be due, in part, to a saturable renal resorption process (Olsen et al., 2007). On the other hand,

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results obtained in epidemiological studies in PFOS and PFOA exposed workers have not shown concluding evidences of increased cancer risk (Olsen et al., 2003). However, different studies have revealed toxicological effects of PFCs, such as the suppression of humoral immunity in mice (Peden-Adams et al., 2008). The toxicology of PFCs has been reviewed (Kudo and Kawashima, 2003; Lau et al., 2007). One of the more relevant observations is the decrease of the thyroid hormone levels in the serum of monkeys and rodents after PFC exposure (Benvenga et al., 2002; Lau et al., 2007; Luebker et al., 2002; Weiss et al., 2009). PFCs are structurally homologous to free fatty acids (Holmes et al., 2009; Luebker et al., 2002), and as such they bind to liver fatty acids-binding protein and albumin which is mainly in blood and liver (Jones et al., 2003). By altering thyroid hormone levels, PFCs may affect fetal and neonatal development (Larsen and Delallo, 1989).

During the last years different studies have assessed the levels of PFCs in human breast milk (Kärrman et al., 2009, 2007; Kishikawa and Kuroda, 2009; Völkel et al., 2009), reporting levels of concentration in the range of ng/mL. Different investigations have studied possible relations between different factors as mother ages, birth weight, infant sex, or the levels of thyroid hormone in infant's blood (Inoue et al., 2004; Tao et al., 2008a,b), but not correlations were established. All these results indicate that further studies are needed to determine how a long exposure in humans can result in reproductive impairments.

In this context, the aims of the current study were: 1) To validate an analytical method based on solid phase extraction followed by liquid chromatography coupled to tandem mass spectrometry (SPE-LC-MS/MS) for the analysis of PFCs in breast milk samples and baby food; 2) to analyze the PFCs in different types of childbirth food (human breast milk, milk infant formulas and cereals baby food); and 3) to evaluate the Risk Index (RI) for daily childhood intake based on the guidelines of the European Food Safety Authority (EFSA).

2. Materials and methods

2.1. Chemicals and standards

Perfluoro-n-octanoic acid (PFOA) [MW: 414; >99%], perfluoro-n-nonanoic acid (PFNA) [MW: 464; >99%], perfluoro-7-methyl octanoic acid (i,p-PFNA) [MW: 464], perfluoro-n-decanoic acid (PFDA) [MW: 514; >99%], potassium perfluoro octanesulfonate (PFOS) [MW: 538.22], sodium perfluoro-1-decanesulfonate (PFDS) [MW: 622.13; >99%] were purchased from Wellington Laboratories Inc., Canada. Internal standard sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octane sulfonate ($^{13}C_4$-PFOS) [MW: 526.08; >99%] and perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid ($^{13}C_4$-PFOA) [MW: 418; >99%] and the surrogate perfluoro-n-[1,2-$^{13}C_2$] decanoic acid ($^{13}C_2$-PFDA) [MW: 516; >99%] were also purchased from Wellington Laboratories Inc. Water and Methanol (MeOH) were of HPLC grade and they were from Merck (Darmstadt, Germany). Ammonium acetate salt (AcNH4: MW, 77.08; ≥98%) was obtained from Sigma-Aldrich, Steinheim, Germany. Sodium hydroxide base (NaOH: MW, 39.997; >97%) was from Merck.

2.2. Sample collection and sample preparation

Following institutional review board approval, 20 samples from women residing in Barcelona city (Spain) were included in this study. The experimental protocol was approved by a local ethical committee in accordance with the Spanish regulation, and the informed consent was obtained from all participating subjects. After signing the informed consent, the mothers were asked to complete a questionnaire for information about residence, age, number of infants previously breast fed, newborn weight and newborn sex, mother habits, type of work and diet.

Breast milk samples were collected either using a breast pump or by hand expressing the milk into the pre-washed polypropylene (PP) tubes containers on the 40 days postpartum at the hospital. Aliquots of 25–30 ml of breast milk were collected into 50 mL PP tubes, stored at −20 °C. Before extraction, samples were lyophilized, homogenized and stored at −36 °C.

3 brands of powdered milk based infant formulas and 2 brands of dry cereals baby food from retail store were included in this study. Powdered milk infant formulas were supplied in 400 g tin packing, and the composition in proteins, fat and carbohydrates were in the range of 10.5–11%, 27.5–29% and 55–56.9%, respectively. Dry cereals baby food were supplied in 250 g plastic bag packages with composition in proteins, fat and carbohydrates in the range of 6–6.5%, 1–1.2% and 87.4–88%. The composition of the studied infant formulas as given on the label was:

> Baby Cereal (A) — Made with cereal grains (rice) that have been enzymatically hydrolyzed to be easy to digest without gluten. This cereal contains specially adapted milk, made with skim milk and a blend of vegetable oils, to suit babies' nutritional needs.
>
> Baby Cereal (B) — Made with wheat based infant cereal grain (rice and corn) enzymatically hydrolyzed without milk, lactose and gluten.

Sample pre-treatment and extraction procedure was based on an alkaline digestion according to a protocols described before (Llorca et al., 2009; Ye et al., 2008, Taniyasu et al., 2005) followed by a clean-up using solid phase extraction (SPE) with C_{18} Sep-Pack cartridges.

Briefly, approximately 1 g of each lyophilized sample was weighted and transferred into a 15 mL PP tube, then 2 mL of ultra-pure water were added and shaken. In order to evaluate the recoveries sample homogenates were fortified with the surrogate internal standards and digested with 8 mL of NaOH (10 mM in MeOH) during 3 h at 125 u/min on an orbital shaker table at room temperature. After the orbital digestion the samples were centrifuged during 10 min. at 4000 rpm and 3 mL of supernatant was taken and diluted with 27 mL of water in a 50 mL PP tube and vortexed during 5 min. A SPE was performed using Sep-Pak cartridges preconditioned with 5 mL of MeOH and 5 mL of water. Then, pre-treated samples were loaded onto the cartridge, under gravity conditions, and dried under vacuum in a J.T. Baker (Phillipsburg, NJ EEUU). The elution was carried out with 5 mL of MeOH in a 15 mL of PP tube and reduced to dryness under a gentle stream of nitrogen. The extracts were reconstituted with 150 µL of mobile phase at its initial gradient conditions, and in order to account the matrix effects during the analysis the instrument performance internal standard was introduced (at 1.5 ng/mL level in vial). The extraction procedures were carried out working in triplicate for all samples.

2.3. Instrumental analysis

The analysis of PFCs was performed by LC-ESI-MS/MS. LC was performed using a Symbiosis ™-Pico (Spark Holland, Emmen, The Netherlands) with a C18 LiChroCART® Purosphere Star-18e analytical column (125 mm × 4 mm i.d., 5 µm) from Merck (Darmstadt, Germany) at room temperature. The mobile phase consisted of (A) aqueous ammonium acetate 20 mM (B) methanol. The elution gradient conditions for the LC mobile phase were as follows: 10–80% B over 5 min, then 80–90% B over other 5 min followed by an isocratic hold at 90% B for 8 min. At 18 min, B was returned to 10% in 2 min. The total run time for each injection was 20 min. The flow rate was kept at 0.5 mL/min throughout the run, and the sample volume injected was 20 µL. The LC system was coupled to a quadrupole-linear ion trap mass spectrometer (QLIT-MS/MS) 4000 QTRAP (Applied Biosystems), equipped with a Turbo Ion Spray source employed in the negative electrospray ionization mode (ESI(−)). Acquisition was performed in multiple reaction monitoring (MRM) mode to obtain sufficient quantification points for confirmation of each analyte.

Table 1
Molecular weight of the PFCs analyzed in this study. Retention times, main transitions, internal standards (i.s.), declustering potential, collision energies and entrance potential (EP).

	Molecular weight	Retention time (min)	(*m/z*) Transitions	i.s	Declustering Potential (V)	Collision Energy (V)	EP (V)
PFOA	414.07	11.05	413>369 413>219 413>169 *	MPFOA	−25	−25	−10
PFOS	538.22	11.69	499>280 499>99 499>80 *	MPFOS		−100	
i.p-PFNA	464.08	11.54	463>219 463>169 *	MPFOA		−15	
PFNA	464.08	11.84	463>219 * 463>169	MPFOA		−25	
PFDA	514.08	12.8	513>468 513>268 513>119 *	MPFDA		−35	
PFDS	622.00	13.5	599>99 599>82 599>80 *	MPFOS		−100	

* *m/z* quantification transitions.

Identification and quantification of target analytes were carried out using *m/z* transitions and retention times (Table 1). Optimized parameters were as follows: curtain gas (CUR), 30 (arbitrary units); ion source gas 1 (GS1), 25 (arbitrary units); ion source gas 2 (GS2), 25 (arbitrary units); source temperature (TEM), 350 °C; ion spray (IS), −4500 V; entrance potential (EP), −10 V, collision cell exit potential (CXP) −10 °C and declustering potential (DP) −25 V. The dwell time of each MRM transition was 150 ms.

Table 2
Instrumental parameters of the LC-QqLIT-MS method developed for the analysis of PFCs (values obtained by injection of standard solutions).

	linear range	linearity	ILOD	ILOQ	Precision (RSD %)	
	(ng/L)	(r^2)	(fg)	(fg)	Intraday	Interday
PFOA	0.005–50	0.9995	0.03	0.1	7.77	10.00
PFOS	0.003–300	0.9996	0.2	0.6	8.69	10.20
ip-PFNA	0.01–100	0.9994	0.6	2.0	9.58	10.40
PFNA	0.01–100	0.9994	0.6	2.0	13.10	16.10
PFDA	0.5–500	0.9996	0.3	1.0	14.60	18.70
PFDS	0.05–500	0.9997	0.3	1.0	8.20	8.52

Linear range in standard solutions. *ILOD* and *ILOQ:* instrumental limit of detection and quantification respectively.

2.4. Quality assurance and quality control

Validation of the method included determination of linearity range, intra-assay precision, accuracy, matrix effects, limit of detection (LOD) and limits of quantification (LOQ). Matrix-spike recoveries of individual PFCs through the analytical procedure were determined by spiking of six selected compounds in ultra-pure water, blank breast milk, and milk infant formula and cereals baby food. Five replicates of each type of sample spiked at 3 levels of concentration were performed (50, 750 and 1500 ng/L).

For spiking experiments the samples, approximately 1 g of lyophilized samples (milk or baby food) and 1 mL of ultra-pure water were placed in a PP container and were spiked with the mixture

Table 3
Internal standards, main MS^2 transitions and collision for MS^2 and EPI experiments, excitation energies (AF2), collision energies (CE) and main product ions in MS^2, EPI and MS^3 experiments.

Compound	Internal Standard	MS^2		EPI					MS^3	
		Transitions	CE (V)	*m/z* (% ratio)	Assignment	CE (V)	AF2	CE	*m/z* (% ration)	Assignment
PFOA	MPFOA	413>169	−25	169 (100)	$[C_3F_7]^-$	−25	100	−25		
		413>369	−25	369 (88)	$[C_7F_{15}]^-$				219 (100)	$[C_4F_9]^-$
									169 (24)	$[C_3F_7]^-$
									369 (20)	$[C_7F_{15}]^-$
		413>219	−25	219 (50)	$[C_4F_9]^-$				169 (100)	$[C_3F_7]^-$
									219 (13)	$[C_4F_9]^-$
PFOS	MPFOS	499>80	−100	80 (100)	$[SO_3]^-$	−20	200	−40		
		499>99	−100	99 (50)	$[SO_3F]^-$					
		499>280	−100	280 (35)	$[C_4F_8SO_3]^-$				130 (100)	$[CF_2SO_3]^-$
									280 (10)	$[C_4F_8SO_3]^-$
ip-PFNA	MPFOA	463>169	−15	419 (100)	$[C_8F_{17}]^-$	−25	150	−25	219 (100)	
		463>219	−15	219 (82)	$[C_4F_9]^-$					$[C_4F_9]^-$
									419 (10)	$[C_8F_{17}]^-$
				169 (55)	$[C_3F_7]^-$				169 (100)	$[C_3F_7]^-$
				269 (36)	$[C_5F_{11}]^-$				219 (15)	$[C_4F_9]^-$
PFNA	MPFOA	463>219	−15	219 (100)	$[C_4F_9]^-$	−25	150	−25	169 (100)	$[C_3F_7]^-$
									219 (20)	$[C_4F_9]^-$
		463>169	−15	169 (75)	$[C_3F_7]^-$					
PFDA	MPFDA	513>119	−35	119 (100)	$[C_2F_5]^-$	−25	100	−25		
		513>469	−35	469 (88)	$[C_9F_{19}]^-$				219 (100)	$[C_4F_9]^-$
									269 (60)	$[C_5F_{11}]^-$
									469 (5)	$[C_9F_{19}]^-$
		513>169	−35	169 (85)	169 (85)					
PFDS	MPFOS	599>80	−100	80 (100)	$[SO_3]^-$	−25	−100	−100		
		599>99	−100	99 (90)	$[SO_3F]^-$					

Table 4
Method limits of detection (MLOD) and quantification (MLOQ) and recoveries in spiked samples (n = 5) at different spiking levels.

Analyte	Ultra-pure water				Human Breast Milk				Milk infant formulas				Cereals Baby Food			
	MLOD ng/L	MLOQ ng/L	Recovery (%)		MLOD ng/L	MLOQ ng/L	Recovery (%)		MLOD ng/kg	MLOQ ng/kg	Recovery (%)		MLOD ng/kg	MLOQ ng/kg	Recovery (%)	
			50 ng/L	750 ng/L			50 ng/L	750 ng/L			50 ng/L	750 ng/L			50 ng/L	750 ng/L
PFOA	2	5	95 ± 12	98 ± 7	4.5	15.2	75 ± 8	80 ± 10	29.7	99	81 ± 14	81 ± 14	9.5	31.6	89 ± 4	94 ± 11
PFOS	0.4	1.2	78 ± 1	81 ± 8	3.5	11.7	90 ± 12	87 ± 11	1.8	6.2	86 ± 13	86 ± 13	5	16.4	97 ± 9	95 ± 7
PFNA	0.4	1	95 ± 12	96 ± 18	3.5	11.5	76 ± 13	75 ± 9	12	39	96 ± 3	96 ± 3	17	57.5	99 ± 8	96 ± 7
i.p-PFNA	2	13	93 ± 6	97 ± 6	3.1	10.5	69 ± 5	70 ± 8	3.09	10.3	84 ± 6	84 ± 6	12	39.9	105 ± 8	96 ± 3
PFDA	39	130	105 ± 9	98 ± 8	25.6	85.3	93 ± 7	90 ± 11	363	1212	110 ± 13	110 ± 13	167	557	98 ± 8	99 ± 10
L-PFDS	0.6	2.1	97 ± 14	101 ± 8	1.2	4.0	95 ± 6	100 ± 2	11	35	53 ± 1	53 ± 1	5	15.2	99 ± 1	107 ± 6

Analyte	Ultra-pure water				Human Breast Milk				Milk infant formulas				Cereals Baby Food			
	MLOD ng/L	MLOQ ng/L	Recovery (%)		MLOD ng/L	MLOQ ng/L	Recovery (%)		MLOD ng/kg	MLOQ ng/kg	Recovery (%)		MLOD ng/kg	MLOQ ng/kg	Recovery (%)	
			50 ng/L	750 ng/L			50 ng/L	750 ng/L			50 ng/L	750 ng/L			50 ng/L	750 ng/L
PFOA	2	5	95 ± 12	98 ± 7	4.5	15.2	75 ± 8	80 ± 10	29.7	99	81 ± 14	81 ± 14	9.5	31.6	89 ± 4	94 ± 11
PFOS	0.4	1.2	78 ± 1	81 ± 8	3.5	11.7	90 ± 12	87 ± 11	1.8	6.2	86 ± 13	86 ± 13	5	16.4	97 ± 9	95 ± 7
PFNA	0.4	1	95 ± 12	96 ± 18	3.5	11.5	76 ± 13	75 ± 9	12	39	96 ± 3	96 ± 3	17	57.5	99 ± 8	96 ± 7
i.p-PFNA	2	13	93 ± 6	97 ± 6	3.1	10.5	69 ± 5	70 ± 8	3.09	10.3	84 ± 6	84 ± 6	12	39.9	105 ± 8	96 ± 3
PFDA	39	130	105 ± 9	98 ± 8	25.6	85.3	93 ± 7	90 ± 11	363	1212	110 ± 13	110 ± 13	167	557	98 ± 8	99 ± 10
L-PFDS	0.6	2.1	97 ± 14	101 ± 8	1.2	4.0	95 ± 6	100 ± 2	11	35	53 ± 1	53 ± 1	5	15.2	99 ± 1	107 ± 6
																106 ± 1

of standards, taken care to uniformly spread them on the sample. The spiked samples were left for 15 min at room temperature to ensure the appropriate distribution in the matrix. Then, the samples were processed, as reported before. For the assessment of all the mentioned parameters, the analyte response was always related to the IS response (1.5 µg/L in vial of each, $^{13}C_4$-PFOS and $^{13}C_4$-PFOA to compensate for undesirable matrix effects and losses during the extraction procedure).

Procedural blanks were carried out in order to assure the not contamination by PFCs for the entire method. Blank tests were also carried out on all the sample containers to rule out possible contamination from the sampling, storage and shipment containers.

Blank tests were carried out with ultra-pure water and a breast milk sample which did not showed any contamination. This breast milk sample was not included in the present study because it was selected in a special manner. The sample was from a donor accomplishing the following requirements: Living in a non-industrial area, never having been a smoker, less than 33 years, having previously breastfed other babies and the milk samples were collected after 1 year of lactation. This breast milk was also used to prepare the spikes materials used as control samples, and also it was used in order to calculate the MLOD in breast milk.

Finally, in order to comply with internal quality control (IQC) procedures, two control samples (spiked materials) two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis is in a state of statistical control or not.

2.4.1. Selectivity

For identification purposes, retention times of PFCs in the standards and in the samples were compared at a tolerance of ±2.5%. Moreover, in accordance with the 2002/657/EC Decision, the relative ion intensities (each product ion area signal versus the base product ion area signal) of the spiked samples were compared with the relative ion intensities of PFCs standard solutions, at the same concentration levels as used for the construction of the calibration curve.

A majority of the PFCs except for i,p-PFNA and PFNA were separated chromatographically from each other during the LC run. Selectivity was assured by utilizing a QqLIT system in MS/MS mode, in which single chromatographic peaks were observed for all SRM transitions.

2.4.2. Limits of detection and quantification

Instrumental limits of detection (ILOD) for PFCs were determined at the minimum detectable amount of each compound with a signal-to-noise ratio of 3. Criteria for the limits of quantification (LOQ) were established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable, and (4) signal-to-noise ratio at least 8.

Method limits of detections (MLOD) were calculated as the minimum concentration of target compounds that can be measured according to previous criteria, analysing the different matrices fortified in decreasing concentrations.

Table 2 summarizes instrumental parameters of the LC-QqLIT-MS method for the analysis of PFCs. The values of ILOD and ILOQ were obtained by injection of standard solutions in methanol-water 20 mM ammonium acetate (10/90, v/v). Quantification was performed using the internal standard method. The calibration curves obtained for both the quantification and the confirmation SRMs were linear for all compounds in a wide range of concentrations with correlation coefficients (R^2) higher than 0.9994 for all compounds.

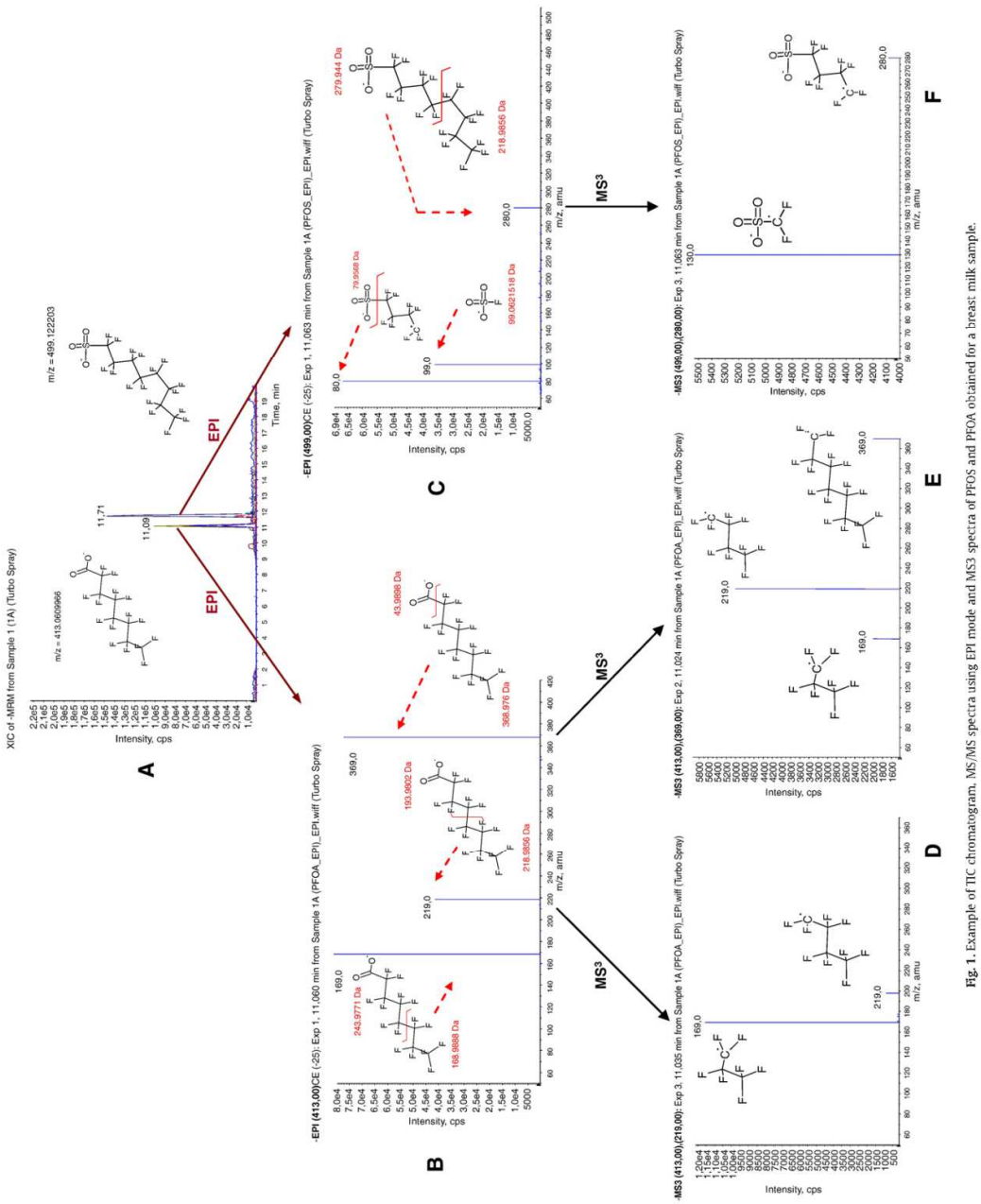

Fig. 1. Example of TIC chromatogram, MS/MS spectra using EPI mode and MS3 spectra of PFOS and PFOA obtained for a breast milk sample.

Table 5
Human Breast Milk results.

Sample	Human Breast Milk (ng/L)					
	PFOA	T-PFOS	i,p-PFNA	PFNA	PFDA	L-PFDS
1	78 ±	52	<LOQ	<LOQ	<LOQ	<LOQ
2	176	60	34	<LOQ	<LOQ	43
3	<LOQ	28	95	<LOQ	<LOQ	<LOQ
4	907	111	260	<LOQ	<LOQ	<LOQ
5	609	52	35	<LOQ	<LOQ	<LOQ
6	289	84	83	<LOQ	237	<LOQ
7	604	865	40	<LOQ	<LOQ	<LOQ
8	21	185	27	<LOQ	<LOQ	<LOQ
9	<LOQ	89	134	<LOQ	<LOQ	<LOQ
10	291	101	72	<LOQ	<LOQ	<LOQ
11	<LOQ	41	93	<LOQ	1095	58
12	15	32	21	<LOQ	<LOQ	<LOQ
13	<LOQ	84	57	<LOQ	<LOQ	<LOQ
14	<LOQ	99	59	<LOQ	<LOQ	<LOQ
15	<LOQ	28	53	<LOQ	<LOQ	59
16	<LOQ	56	52	<LOQ	<LOQ	<LOQ
17	<LOQ	99	46	<LOQ	<LOQ	40
18	<LOQ	<LOQ	34	<LOQ	<LOQ	<LOQ
19	<LOQ	97	52	<LOQ	<LOQ	54
20	<LOQ	156	178	<LOQ	<LOQ	70

<LOQ: lower than limit of quantification.

2.4.3. Linearity

The range of linearity was defined by plotting the peak area ratio of the PFC to the IS versus PFC concentration. The following criteria for linearity range were applied: linear regression with a correlation coefficient better than 0.990, and RSD of four replicates less than 25%.

2.4.4. Recovery and precision

According to the 2002/657/EC Decision, since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified samples was measured as an alternative to trueness. Each type of sample was spiked in quintuplicate as previously described with the six PFCs at three levels of concentration (50, 750 and 1500 ng/L).

Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets for calculating interday repeatability.

3. Results and discussion

3.1. Analytical method

The QqLIT instrument was used to perform the LC–MS/MS analyses in the SRM mode. As an additional feature, in this instrument, the SRM mode can be combined with attractive working modes the Enhanced Product Ion Scan (EPI) and MS^3 modes, for the unambiguous confirmation of compounds. Operating with the EPI mode, Q1 filters the desired parent ion which is fragmented in the Q2 region. Fragment ions are trapped in the Q3 region for a specified time prior to being scanned out. The main limitation is the low stability of fragment ions because the isolation and fragmentation steps are both occurring in the LIT, only fragment ions produced with m/z values of 30% of the parent mass and higher are stable in the ion trap. However, the EPI and MS^3 modes were used for confirmatory purposes. Table 3 summarizes optimized conditions in MS^2 and MS^3 and the main product ions obtained for selected compounds.

In Table 4 are reported the method limits of detection (MLOD) and method limits of quantification (MLOQ) determined for the different type of matrices included in this study. MLODs were in the range of 0.4 to 39 ng/L in ultra-pure water, between 1.2 and 26 ng/L in human breast milk, 1.8–363 ng/kg in milk infant formulas and 5–167 ng/kg in cereals baby food. Recoveries for spiked samples were in the range of 80–110% for most compounds and matrices and RSD% was always below 21% (Table 4). Therefore, the applicability of the method for PFCs in milk and childbirth food was proved.

PFCs chromatograms showed that the transition corresponding to PFOA has a unique and well-defined peak whereas the extracted chromatogram corresponding to PFOS showed smaller additional peaks. These additional peaks have also been reported by other authors (Llorca et al., 2009) and tentatively assigned to the presence of branched isomers in real samples. EPI and MS^3 modes were used to reconfirm the identity of detected compounds. Fig. 1 shows an example of PFOS and PFOA confirmation in a real breast milk sample and the main fragmentation pathways of these compounds.

3.2. Concentrations measured

Table 5 summarizes the results obtained for breast milk samples. PFOS, i,p-PFNA and PFOA were the compounds more frequently found. PFOS and i,p-PFNA were detected and quantified in 95% of the 20 samples analyzed. PFOA was quantified in 45% of samples. Concentrations measured were in the range of 28–865 ng/L and 21 to 260 ng/L for PFOS and i,p-PFNA, respectively. As can be seen in the histograms presented in Fig. 2, for the majority of breast milk samples PFOS was ranging between 100 and 200 ng/L and i,p-PFNA was below 100 ng/L in agreement with previously reported studies (Kärrman et al., 2009; So et al., 2006). PFOA was present in less number of samples than PFOS

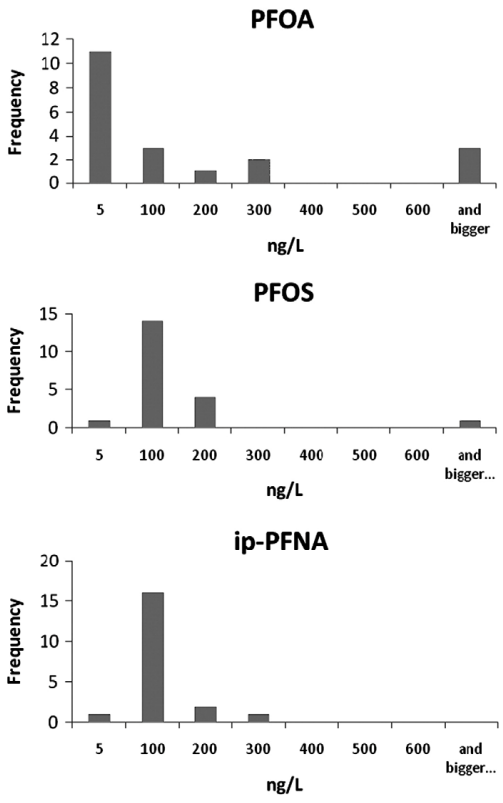

Fig. 2. Histograms of no. of samples grouped according their concentrations in ng/L of the 3 PFCs detected in higher concentrations in breast milk samples ($n=20$).

Table 6
Concentrations of the 6 PFCs analyzed in this study in cereals baby food and milk infant formulas.

	Sample	Concentration (ng/kg)					
		PFOA	T-PFOS	ip-PFNA	PFNA	PFDA	L-PFDS
Cereals baby food	**A**	438	458	438	138	266	90.6
	B	166	162	166	44.0	236	56.1
Milk infant formulas	**1**	723	1098	723	219	1289	719
	2	488	403	488	162	959	93.6
	3	374	229	374	118	693	55.1

and i,p-PFNA, but some of the samples with PFOA were in high concentrations. In most of the samples (Table 5) PFNA, PFDA and L-PFDS were also detected but in concentrations below the MLOQ.

No significant correlations were found between PFCs concentration in breast milk and mother´s age, birth's sex or birth's weight in agreement with previous works (Tao et al., 2008a,b).

PFCs contents, expressed as ng/kg sample (powdered) and ng/L (reconstituted), in milk-based infant formulas and cereal baby food, are reported in Table 6. The six PFCs included in this work were detected in all of the infant milk formula samples and baby food analyzed. For milk infant formulas the compound detected in higher concentrations was PFDA with concentration ranging from 693 to 1289 ng/kg followed by PFOS, PFOA and i,p-PFNA which concentration were ranging from 229 to 1098 ng/kg, 374–723 ng/kg and 374–723 ng/kg, respectively. In Fig. 3 are presented the average contribution of the total PFCs in the milk-based infant formula analyzed. The presence of PFCs in the milk could be associated to possible a migration/contamination from packaging and production processes. This conjecture is supported by the fact that the pattern of PFCs present in these products is different of that present in the human milk. The presence of PFDA indicates dissimilar sources of contamination. In the case of cereals baby food concentrations in general were lower, but again all compounds were quantified. Predominant compounds were PFOS, PFOA and i,p-PFNA with concentrations ranging from 300 to 430 ng/kg. The average contributions of PFCs in cereals baby food are presented in Fig. 3. It is noted that there is a general predominance of acid forms (PFOA, i,p-PFNA and PFDA). Sulphonated forms, although being present, represent approximately 30% of the total PFCs measured. The levels of PFOS and PFOA were similar in milk infant formulas and in cereals baby food. A previous study by Tao et al. (2008b) reports the only available data on infant formulas. According to it, PFC concentrations in infant formula from the United States were 10-fold lower than the concentrations found in the Asian breast milk samples (in the range of those found in this study). PFOS was detected in one formula sample at a concentration of 11.3 pg/mL and perfluorohexanesulfonate in two formula samples at concentrations of 1.36 and 3.59 pg/mL. On the contrary, our results present higher levels and a wide profile of PFCs in milk-based infant formulas. Because of the scarce data available, it is difficult to explain the reason of the differences found between our results and those of Tao et al. (2008b).

This data are one of the first evidences that infant and follow-up formulas as well as other baby food can be consider as one of the possible sources of PFCs intake. Several studies recommend not to use the drinking water from contaminated areas for preparation of baby food, considering this water as the major source of PFCs intake (Kannan et al., 2002). However, in non-particular contaminated areas the levels of PFCs in drinking water are almost negligible.

3.3. Infant exposure to selected PFCs

In order to evaluate possible risks to infant health associated to PFCs intake, the Risk Index (RI) was calculated for human breast milk, cereals baby food and milk infant formulas according to the EFSA guidelines (European Food Safety Authority, 2008). The Daily Intake (DI) was calculated according to:

$$\text{DI (ng of PFC / Kg of body weight / day)} = \text{ng of PFCs} = \left(\text{Consumption}^{a} \times \text{PFC concentration}^{b}\right)$$

[a] Consumption expressed in mL of milk or g of milk based infant formula or baby food per day and [b] PFCs concentration in ng/mL or ng/g.

DI of PFOS and PFOA through mother's breast were calculated based on the general infant's milk ingestion rate during the first 6 months of life. Infant daily consumption of breast milk can vary depending on child's age and its solid food intake. We calculate the daily milk consumption rate assuming 800 mL/day per infant from month 0 to 6. Usual consumptions were considered for milk infant formulas. For cereals baby food 50 g of dry food per day was considered between the third and six month of life, as a normal rate.

The risk index (RI) was calculated according to the expression:

$$\text{RI} = \text{DI} / \text{TDI}$$

where, TDI is the tolerable daily intake.

According to the EFSA guideline (European Food Safety Authority, 2008), TDI for PFOS was 150 ng/kg of b.w. and for PFOA 1500 ng/kg of b.w.

In Table 7 are summarized the results of RI estimation for breast milk samples during the first 6 month, the results for milk infant formulas and cereals baby food. As can be seen in Table 7 all results were below 1, with exception of one breast milk sample. Therefore, according to the criteria used for this estimation just in one case a certain degree of toxicological risk can be considered. However, reference doses for PFOA and PFOS are not well established and there is a lack of consensus about TDI values for these compounds between different organizations. Since RI is based on TDI sources should be interpreted with caution. In addition, the risk associated with PFCs exposure via breastfeeding should be compared with their benefits.

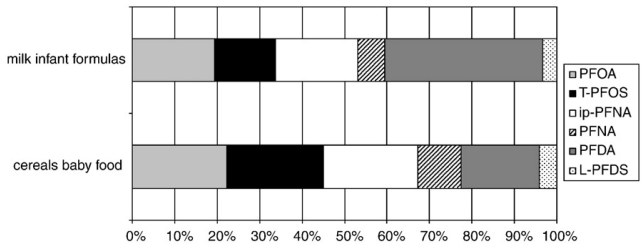

Fig. 3. Composition of PFOA, T-PFOS, i,p-PFNA, PFNA, PFDA and L-PFDS to sum of total concentrations in milk infant formula and cereals baby food.

Table 7
Risk indexes calculated for PFOA and PFOS.

		PFOA TDIs (1500 ng/kg)					
		Day 40	2 month	3 month	4 month	5 month	6 month
Average baby mass weight (kg)		4.4	5.4	6.2	6.9	7.6	8
Intake of breast milk V (mL)		800	800	800	800	800	800
Intake of milk based infant formula (g)		110	120	130	140	150	150
Intake of cereal baby food (g)					40	50	60
Breast milk	**1**	0.0094	0.0067	0.0058	0.0052	0.0048	0.0045
	2	0.0213	0.0174	0.0151	0.0136	0.0123	0.0117
	3	<0.0018	0.0015	0.0000	0.0000	0.0000	0.0000
	4	0.1100	0.0896	0.0781	0.0701	0.0637	0.0605
	5	0.0739	0.0602	0.0524	0.0471	0.0428	0.0406
	6	0.0350	0.0285	0.0248	0.0223	0.0203	0.0192
	7	0.0732	0.0596	0.0519	0.0467	0.0424	0.0402
	8	0.0026	0.0021	0.0018	0.0016	0.0015	0.0014
	9	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	10	0.0353	0.0287	0.0250	0.0225	0.0204	0.0194
	11	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	12	0.0019	0.0015	0.0013	0.0012	0.0011	0.0010
	13	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	14	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	15	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	16	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	17	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	18	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	19	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
	20	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018
Milk infant formula	**1**	0.0121	0.0107	0.0101	0.0098	0.0095	0.0090
	2	0.0081	0.0072	0.0068	0.0066	0.0064	0.0061
	3	0.0062	0.0055	0.0052	0.0051	0.0049	0.0047
Cereals baby food	**A**				0.0017	0.0019	0.0022
	B				0.0006	0.0007	0.0008
		PFOS TDIs (150 ng/kg)					
		Day 40	2 month	3 month	4 month	5 month	6 month
Average baby mass weight (kg)		4.4	5.4	6.2	6.9	7.6	8
Intake of breast milk V (mL)		800	800	800	800	800	800
Intake of milk based infant formula (g/day)		110	120	130	140	150	150
Intake Mass (g)					40	50	60
Breast milk	**1**	0.0631	0.0514	0.0448	0.0402	0.0365	0.0347
	2	0.0727	0.0592	0.0516	0.0463	0.0421	0.0400
	3	0.0336	0.0273	0.0238	0.0214	0.0194	0.0185
	4	0.1344	0.1095	0.0954	0.0857	0.0778	0.0739
	5	0.0631	0.0514	0.0448	0.0402	0.0365	0.0347
	6	0.1016	0.0828	0.0721	0.0648	0.0588	0.0559
	7	1.0490	0.8548	0.7445	0.6689	0.6073	0.5770
	8	0.2247	0.1831	0.1595	0.1433	0.1301	0.1236
	9	0.1077	0.0878	0.0764	0.0687	0.0624	0.0592
	10	0.1224	0.0997	0.0869	0.0781	0.0709	0.0673
	11	0.0496	0.0404	0.0352	0.0316	0.0287	0.0273
	12	0.0387	0.0315	0.0274	0.0246	0.0224	0.0213
	13	0.1023	0.0834	0.0726	0.0653	0.0592	0.0563
	14	0.1199	0.0977	0.0851	0.0764	0.0694	0.0659
	15	0.0339	0.0276	0.0240	0.0216	0.0196	0.0186
	16	0.0682	0.0555	0.0484	0.0435	0.0395	0.0375
	17	0.1196	0.0974	0.0848	0.0762	0.0692	0.0658
	18	<0.014	<0.014	<0.014	<0.014	<0.014	<0.014
	19	0.1173	0.0956	0.0833	0.0748	0.0679	0.0645
	20	0.1886	0.1537	0.1339	0.1203	0.1092	0.1037
Milk infant formula	**1**	0.1830	0.1627	0.1535	0.1485	0.1445	0.1373
	2	0.0672	0.0597	0.0563	0.0545	0.0530	0.0504
	3	0.0382	0.0339	0.0320	0.0310	0.0301	0.0286
Cereals baby food	**A**				0.0177	0.0201	0.0229
	B				0.0063	0.0071	0.0081

In summary, a robust and sensitive analytical method for the analysis of 6 PFCs was validated for their application in breast milk, infant milk formulas and baby food based on SPE-LC-QqLIT-MS, with MLOQ in the low ng/L range for most of compounds and matrices. The application of the method to breast milk samples of donors living in Barcelona city (Spain) showed comparable results to other studies performed in USA, Germany, China, and Spain. PFOS, PFOA and i,p-PFNA were predominant compounds. Different brand samples of

commercial baby food were also analyzed and the 6 PFCs were quantified in all the samples. There is only a previous study that reported much lower levels of PFCs in infant formulas than this one. Because of the scarce data available, it is difficult to outline an explanation. Finally, RIs calculated for breast milk samples and baby food did not exceed maximum limit according to the EFSA recommendations with exception of 1 sample of breast milk. Lactation is a considerable source of PFCs exposure for infants, and according to data obtained from this study approximately 300 ng of PFCs per day may transferred from lactating mother to infants. These results justify further investigation on human monitoring of PFCs and their possible toxicological effects.

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3.2.4 Development and validation step-by-step of a rapid analytical method for the determination of PFASs in fish and dairy products

Most of the methods for the analysis of PFASs in fish and dairy products have been based on ion-pair extraction followed by liquid extraction or alkaline digestion and, finally, solid phase extraction (Farré *et al.* 2011).

Under the frame of the EU project Conffidence, the development of a simplified method for the rapid and cost-effective analysis of 3 compounds (PFOS, PFOA and PFOSA) in milk and fish was proposed.

In terms of time of extraction, and cost reduction, the method was based on methanol extraction and active charcoal clean-up followed by LC-MS/MS analysis. In order to assess the good performance of the method and their possible transferability, an in-house validation and transferability test were carried out by 2 participants: the group of VCH-ICT Prague and our group at IDAEA-CSIC.

The method was as follows:

Fish extraction. 2 g of homogenised fish sample is transferred into 15 mL PP tube and mixed with 6 mL of methanol using homogenizer. Isotopically labelled standards $^{13}C_4$-PFOS, $^{13}C_4$-PFOA and $^{13}C_8$-FOSA (final volume at level 1 µg/L corresponding to 3 µg/kg spiked level in 2 g of sample) were added with the exception of the sample SC which is used for the preparation of the calibration curve. Then, 340 mg of activated charcoal is added to the suspension. After 1 min vortexing, the sample is centrifuged (10000 rpm, 5 min, 20ºC). The supernatant is transferred to the centrifuge filter and filtered (0.2 µm) through centrifuge filter tube (5000 rpm, 2 min, 20ºC). Then, 500 µL of filtrate is transferred into a PP LC-vial prior to an instrumental analysis.

Milk extraction. 2 mL of milk is transferred into 50 mL PP centrifuge tube and mixed with 2 mL of 0.1 M formic acid in methanol solution (to precipitate proteins) and 6 mL of methanol using homogenizer (minishaker). Isotopically labelled standards $^{13}C_4$-PFOS, $^{13}C_4$-PFOA, and $^{13}C_8$-FOSA (final volume at level 1 µg/L corresponding to 3 µg/L spiked level in 2 mL of sample) is added with the exception of the sample MC which is used for the preparation of the calibration curve. Then, 100 mg of activated charcoal is added to the suspension. After 1 min vortexing, the sample is centrifuged (10000 rpm, 5 min, 20ºC) and the supernatant transferred to the centrifuge filter (5000 rpm, 2 min, 20ºC). Finally, 500 µL of the filtrate is transferred into a PP LC-vial prior to an instrumental analysis.

Instrumental analysis. The instrumental analysis performed for every participant was based on liquid chromatography separation coupled to a triple quadrupole mass analyzer (LC-(QqQ)-MS/MS). All chromatographic separations were conducted using a C18 analytical column with mobile phases consisting of (A) methanol and (B) 2 - 20 mM ammonium acetate in water at a flow rate of 0.3 mL/min. The linear gradient elution program was the optimum for every laboratory. In all the cases, an electrospray ionization source (ESI) operating in the negative ion mode was used as ionization source. The

samples were analyzed by conventional triple quadrupole (QqQ), with the exception of two laboratories which employed a hybrid triple quadrupole – linear ion trap.

3.2.4.1 Method validation

The tested parameters included linearity range, intra-assay precision, accuracy, matrix effects, method limit of detection and quantification (MLOD and MLOQ, respectively) based on IUPAC Technical Report (Thompson *et al.* 2002), decision limit (CCα) and detection capability (CCβ) as well as the recovery yields. With the exception of linearity, the validation experiments were performed by spiking six replicates of blank fish muscle or milk with the 3 selected compounds (PFOS, PFOA and FOSA) at three different concentration levels (0.1; 0.5; 1; 2.5 and 5 µg/kg or µg/L). Then, the sample was processed as reported before. The method validation for fish and fish feed has been previously published by Hrádková *et al.* (2010).

For the assessment of all the mentioned parameters, the analyte response was always related to the IS response to compensate for undesirable matrix effects and losses during the extraction procedure. The matrix effects were assessed by comparing the response of the analytes in the initial mobile phase at the same concentration into an extract of a blank matrix sample extract (young hake or anchovy) obtained through the sample preparation process.

According to the 2002/657/EC Decision, since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. Negative samples of tissue of anchovy and young hake (previously analyzed and found to be not contaminated) were spiked in six replicates as previously described with the PFASs at different levels as mentioned above. Precision, expressed as repeatability, was calculated by repeated analyses on the same sample set as used for recovery tests, with the only difference that independent samples were extracted and analyzed on two other occasions for calculating interday repeatability.

Table 3.2 shows some validated parameters for fish tissue and milk, respectively.

Table 3.2: Performance characteristics for fish tissue (spiked level for recovery: 1 µg/kg ww for fish and 1 µg/L ww for milk)

Analyte	Performance characteristics						
	Recovery (n=6; %)	RSD (n=6; %)	MLOD (µg/kg or µg/L)	MLOQ (µg/kg or µg/L)	Linearity range (µg/kg or µg/L)	CCα (µg/kg or µg/L)	CCβ (µg/kg or µg/L)
Fish							
PFOS	107	9	0.075	0.15	0.15 – 15	0.015	0.21
PFOA	90	3	0.15	0.3	0.3 – 15	0.14	0.47
FOSA	90	4	0.15	0.3	0.3 – 15	0.18	0.49
Milk							
PFOS	92	6	0.3	0.75	0.75 – 75	0.21	0.87
PFOA	93	7	0.15	0.3	0.3 – 75	0.41	0.65
FOSA	81	12	0.15	0.3	0.3 – 75	0.55	0.77

According to the quality parameters, both methodologies (for fish and milk) are suitable for the analysis of PFASs in the matrices of interest being reproducible, robust,

sensitive and fast. Once the methods were validated by the same laboratory that developed the procedures, there was evaluated the method transferability in IDAEA-CSIC laboratories.

3.2.4.2 Method transfer validation

The method transfer validation was carried out according to 2002/657/EC Decision. For transferability purposes, salmon and bottled milk were purchased form supermarket and analyzed with the described methods with few variations. An extra pre-concentration step after centrifuge filtration was applied: 1 mL of filtrate was transferred into a PP LC-vial prior to an instrumental analysis and reduced to dryness under N_2 atmosphere conditions. The residue was reconstituted in a 500 µL of initial mobile phase conditions (water: methanol, 90:10). Once it was assured the no sample contamination with the selected PFASs, the purchased samples were spiked, in six replicates, with the three selected compounds at three different levels (fish: 1, 15, 20 µg/Kg; milk: 1, 20, 40 µg/L), left to rest 1 h and finally homogenised prior extraction. The analysis was carried out in a different LC-MS/MS instrument: Waters Alliance 2690 liquid chromatograph (Waters, Milford, MA, USA) provided with a XTerra® MS C_{18} 3.5µm [(2.1 x 100mm), Waters (*Milford, Massachusetts, USA*)] column, coupled to a Quattro LC triple quadrupole (TQD) mass spectrometer (Micromass, Manchester, UK).

The transfer validation parameters include selectivity, MLOD, MLOQ, linearity, trueness, the uncertainty of the recoveries, the decision limit and the detection capability. In order to establish the trueness of the method, since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified samples was measured as an alternative to trueness. Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets but changing: person who makes the analysis and the centrifugation parameters changed: 10 min at 4000 rpm. Finally, the uncertainty of the method was calculated by recoveries uncertainties, and the decision limits (CCα) and detection capability (CCβ) were calculated by ISO 11843 by the analysis of 20 blank materials. Table 3.3 shows some of the transfer parameters.

Table 3.3: method transfer parameters

	Fish			Milk		
	PFOS	**PFOA**	**FOSA**	**PFOS**	**PFOA**	**FOSA**
Recovery (n=6; %)	150 / 90 / 87[a]	120 / 91 / 80[a]	150 / 104 / 90[a]	107 /110 / 86[b]	125 /82 / 83[b]	125 / 112 / 80[b]
RSD (n=6; %)	19 / 4 / 4[a]	26 / 11 / 10[a]	13 / 11 / 5[a]	10 / 9 / 16[b]	13 / 6 / 14[b]	13 / 8 / 18[b]
LOD (µg/Kg)[c]	0.29	0.15	0.28	0.20	0.05	0.19
LOQ (µg/Kg)[c]	0.52	0.51	0.84	0.68	0.17	0.65
Linearity range (µg/Kg)	0.15 - 150	0.3 - 150	0.3 - 150	0.75 - 150	0.30 - 150	0.30 - 150
CCα (1 %) (µg/Kg)[c]	0.87	0.85	0.72	0.55	0.38	0.77
CCβ (5 %) (µg/Kg)[c]	1.29	1.35	1.09	0.61	0.80	1.19

[a] Spiked level: 1 µg/Kg (ww) / 15 µg/Kg (ww) / 20 µg/Kg (ww)
[b] Spiked level: 1 µg/L / 20 µg/L / 40 µg/L
[c] Spiked level: 1 µg/Kg (ww) for fish and 1 µg/L for milk

In general, the method transferability presented higher recoveries at low spiked levels (>100%), but these results are in agreement with 2002/657/EC. Higher MLOD, MLOQ, CCα and CCβ were obtained but, as these parameters can be affected by the capabilities

of the instrument used in the analysis, the method transferability was considered successful in this point. The transferability study presented a low variability which is inherent in the analytical laboratory and technician error. In this context, both methodologies were suitable for the analysis of fish and milk samples.

3.2.4.3 Interlaboratory study

In order to evaluate the applicability of the methodology to other laboratories, the following and step-by-step approach interlaboratory study was organised.

A total number of 8 expert laboratories in the analysis of PFASs, from 6 different European countries, were invited to take part in this study. 88 samples were prepared for the exercise including 5 salmon fish tissue samples *(salmo salar)* for every participant (1 blank and 2 x 2 sample at 2 different fortified batches) and 5 cow milk samples with 3% of fat for every participant (1 blank and 2 x 2 sample at 2 different fortified batches). Additionally, 500 µL of an undisclosed mixture of the three selected analytes was prepared in methanol and delivered to the participants in order to determine the uncertainty of the instrumental analysis. 1 mL of concentrated standard solution and ~50 g of activated charcoal were distributed to each participant, in order to diminish any possible discrepancy from the origin of the standards. Table 3.4 contains the sample list delivered to every participant. In order to minimize additional sources of variation, all the samples were collected, transported, prepared and delivered to the participants at the same time from a central laboratory (Environmental Chemistry Department, IDAEA-CSIC, Barcelona). All the material used during sample preparation was free of PFASs. Before the shipment, all the samples were pasteurised to assure the good quality of the samples.

Table 3.4: List of the samples and material (code, number of samples)

Type of sample	Packaging and volume	Code	No. of samples	Concentration (µg/L)		
				PFOA	L-PFOS (anion)	FOSA
Standard mixture in methanol	PP eppendorf, 1 mL	**PFOA, FOSA: 1000 µg/L L-PFOS(anion): 960 µg/L**	1	1000	960	1000
Fish sample (salmon)	PP bag, 20 g	**SA**	2	0.5–50 µg/kg Concentration range of positive target compounds		
		SB	2			
		SC	1	Blank		
Milk sample	PP bag, 20 mL	**MA**	2	0.5–50 µg/L Concentration range of positive target compounds		
		MB	2			
		MC	1	Blank		
Unknown MIX	PP eppendorf, 0.5 mL	**MIX**	1	1–20 µg/L		
Activated charcoal	PP centrifuge tube, 50 mL	**Activated charcoal**	1	–	–	–

The sample preparation was performed in IDAEA-CSIC and consisted in:

Fish spiked samples: i) fresh salmon was purchased from the market (1 Kg of fresh weight, approximately); ii) the sample was transported to laboratory under cool conditions where fish muscle was cut and homogenised in a shaker followed by blender homogenisation until fish tissue was reduced to a doughy mass (approximately 30 min); iii) 3 x 2 g of sample were analyzed in order to determine the blank level. The results showed that the 3 selected PFASs were below MLOQ and the material was ready to be used for spiking purposes; iv) 2 x 350 g of sample was weighted onto a silver foil and it was spread lengthways and width of the paper, leaving only a thin layer of sample; v) in parallel, 2 mL of a mixture of 3 PFASs at optimum spiking level was prepared in methanol solution; vi) fish was spiked with the mixture, drop to drop, along the sample in order to obtain the maximum homogeneity; vii) the spiked sample was left to rest for 30 min and, after this time, it was introduced in the blender one more time (approximately 30 min); viii) the homogenised sample was transferred into a PP 50 mL centrifuge tubes (~10 g) and preserved on a freezer (-20ºC) during 12 h; ix) the samples were transported under cool conditions (-4 ºC) to pasteurization laboratory; x) defrosted samples were transferred into PP vacuum bags and pasteurised under thermal conditions by High Temperature methodology using a plate heat exchanger, in an oven, along 30 min at 68 ºC.

Milk spiked material: i) pasteurised milk was purchased from the supermarket; ii) 3 x 2 mL of sample were analyzed in order to determine the blank level. The results showed that PFASs were below MLOQ and the material was ready to be used for spiking purposes; iii) 2 x 500 mL of blank milk was introduced in volumetric flasks and spiked with the optimum level of 3 selected compounds; iv) the spiked sample was left to rest for 30 min at room temperature to ensure the appropriate distribution in the matrix and, after this time, it was homogenised 3 h into an orbital digester; v) homogenised samples were divided in 50 sub samples in 50 mL PP centrifuge tubes (~10 mL) and preserved on a freezer (-20ºC) during 12 h; vi) the samples were transported under cool conditions (-4 ºC) to pasteurization laboratory; vii) defrosted samples were transferred into PP vacuum bags and pasteurised under thermal conditions by High Temperature methodology using a plate heat exchanger, in an oven, along 30 min at 68 ºC.

In order to ensure the sample homogenization for every matrix and every spiked concentration, 10 samples of every type were randomly selected. 5 of them were analyzed in IDAEA-CSIC and the other 5 were analyzed in another group in Prague,to test the homogeneity of the samples. A criterion for homogeneity was based on F-test. According to this criteria, when $F < F_{crit}$ the material is homogeneous. All delivered samples did pass the homogeneity test for High Temperature pasteurization type.

Chemicals The standards were supplied by Wellington Laboratories (Ontario, Canada) separately and mixed in the central laboratory. Prepared mixture was used for spiking experiments, for mix unknown preparation and for standards delivered to each participant: PFOA, FOSA and linear PFOS. The mixture prepared for calibration purposes and spiked experiments was the same in order to minimize differences between laboratories.

Analytical methods Fish and milk extraction procedures were based on in-house validated method. Interlaboratory participants were requested to follow series of precautions and procedures summarized below before to analytical process:

- *Storage conditions.* Samples were stored in material free of PFASs in order to avoid the cross contamination. PP containers could be used in the storage. The samples should be stored in the freezer at -20ºC before analysis (If the sample was frozen before analysis, it must be defrosted and homogenised at environment temperature).
- *Applicability of analytical method.* The analytical method was evaluated in the range of temperatures between 15 and 25ºC and under common laboratory conditions (pressure of 1 atm). The minimum weight of sample for analysis purposes was 10 g (fish) and 10 mL (milk).
- *Sample manipulation.* The sample should not be in contact with material as PTFE, PVDF or other fluorinated polymers which contains traces of some PFASs.
- *Verifications before analysis.* Before analytical procedure, it must be assessed the good performance of all required instrumentation parameters including the calibration of the LC-MS/MS instrument, the calibration and the good stability of all target analytes.
- *Number of replicates.* Triplicates in order to assess the repeatability (intraday) have to be analysed for each container delivered for fish tissue, milk sample and blank matrix.

The participant laboratories were requested to follow a specific program for the analysis by LC-MS/MS in order to rule out any possible cross contamination or carry over between samples.

The identification of the target analytes in the samples were confirmed by two points:

- Comparison of the obtained t_R in the samples with the [average ± 2.5% RSD] of t_R of standards in the calibration curve according to 2002/657/EC Decision.
- For PFOA and PFOS, comparison of the ratio between the m/z of the most intensive transition and the second one in the samples with the [average ± 2.5% RSD] of the ratio for the standards in the calibration curve. For the quantification of target analytes, the external calibration curve by matrix-matched standards in blank materials (SC and MC) was used; a minimum of five points in the concentration range 0.05–50 µg/L for fish and 0.1–25 µg/L for milk, concentration in vial. Plot ratio of area peak A/A_{is} (axis Y), against concentrations C (axis X) was employed.

Statistical parameters The results were reported to the organizers (IDAEA-CSIC) in a supplied excel datasheet indicating laboratory information as participant code, name of contact person, organization, address and email address. The samples and standards background were also requested including sample type, packaging and volume, code, number of delivered samples and concentration ranging. The checklist for fish and milk included: delivering of standards and samples (damage packaging, condition of sample, weight of standards, and weight of unknown standard mixture and storage conditions), homogenization and extraction details, and detection and quantification information. The results for fish, milk and unknown mixture were reported including: sample code, analyte, results of different measurements (triplicates, in µg/L or µg/Kg), mean value (µg/L or µg/Kg), standard deviation (SD) and relative standard deviation (RSD, %). Performance

character in fish and milk were expressed with limits of detection and quantification for individual analytes in every matrix and how these values were calculated (low calibration curve or others), recoveries of the method for spiking blank material at 1 µg/L or µg/Kg, levels of individual calibration points, instrumental parameters and selected reaction monitoring (SRM) transitions for identification, quantification and qualification.

The statistic analysis was carried out according to the Association of Official Analytical Chemists guidelines (AOAC 2002) and IUPAC (2006), following the next schedule:

1) ***Initial data review.*** This procedure was performed in order to rule out any unusual value (average, repetitivity within laboratory: RSD_r (%), reproducibility: RSD_R (%)).
2) ***Outliers study.*** Outliers were studied for determining the probability that the apparent aberrant value(s) was a part of the main group of values considered as a normal population. The outlier study was performed by applying the following tests (in order): (i) Cochran test for removal of extreme individual values from a set of laboratory value laboratories (within-laboratory). The method was performed as a 1-tail test at a probability value of 2.5%. The Cochran test statistic was computed as the within-laboratory variance for each laboratory and divided the largest value by the sum of all variances. The resulting quotient was compared with tabulated Cochran values for the same probability and the number of laboratories and, (ii) Grubbs test in order to remove laboratories with extreme averages. The statistic applied was the single value test followed by pair value test.
3) ***Bias.*** It was estimated according to the systematic deviation from the real spiked level of individual results. In addition, it was calculated the error of a single value, the marginal percentage of recovery for every laboratory and the total percentage of recovery.
4) ***Precision.*** The precision was calculated as the replication within-laboratory (repeatability expressed as standard deviation $S_r \rightarrow RSD_r$, %) and among laboratories (reproducibility standard deviation among all laboratories SR $\rightarrow RSD_R$, %) according to guidelines.
5) ***HORRAT values.*** The HORRAT value is the ratio of the reproducibility relative standard deviation (RSD_R, %) in front to the predicted reproducibility relative standard deviation ($PRSD_R$, %). The $\%PRSD_R$ value was calculated based on Horwitz curve as $2 \cdot C^{-0.1505}$, where *C* was the estimated mean concentration.

Criteria of good performance

Blanks control. In order to rule out any possible contamination during extraction and analytical procedures, every participant laboratory assessed, in parallel with the sample extraction, the same procedure in blank solvent and blank sample (samples with codes SC and MC).

Matrix effect. The matrix effects were studied by comparison of the response obtained for the respective analytes in: (*i*) a mixture of analytes prepared in an initial conditions of mobile phase and (*ii*) a mixture of analytes spiked in a blank matrix (code SC and MC) extract obtained through the sample preparation process. The matrix effect was expressed as:

% Matrix Effect = 100 x [(A/Ais matrix standard) / (A/Ais solvent standard)]

Where *A* was the area of the interested analyte, *Ais* was the area of the corresponding isotopically labelled analogue. Matrix and solvent standard were at the same concentration level.

Linear regression. Linear regression through zero, with a correlation coefficient higher than 0.990, bias from the calibration line less than 25% for all individual calibration points, and RSD of four replicates less than 25% was requested. The linearity range was tested using a plot of isotope corrected responses factor against concentration levels.

Recoveries. Recoveries of the method were assessed at the minimum level (1 µg/L in milk and 1 µg/kg in fish). Generally, the acceptable percentage of recovery in spiked samples according to 2002/657/EC Decision should be in the range of – 50% to + 20% for level of 1 µg/kg. Specifically, for the purpose of this study based on realised transfer validation study, the recoveries were expected to be in the range of 80–120% for each analyte expressed as:

% Recovery = [experimental concentration / theoretical concentration] x 100

Limits of detection and quantification. The method limit of detection was defined as the lowest concentration for which the peak area was at least three times larger than the background noise in spiked sample at the lowest concentration. In this context, the LOD and method limit of quantification were calculated according to:

LOD = 3 x Concentration / (S/N ratio) and

LOQ = 10 x Concentration / (S/N ratio), or experimentally by LOQ = lowest calibration curve point (LCL).

3.2.5 Survey study

In order to assess the presence of PFASs in real food samples and approximate the total daily intake, as well as the risk index associated, different edible fish has been analyzed from four different European markets with different diet habits.

The main fishery areas were the Mediterranean Sea (Spanish coast), the Cantabric Sea, the North East of Atlantic Ocean and the Baltic Sea. The samples included wild and aquaculture origin as well as 18 different species of different fish families (bivalves, whiting, cod, hake, herring, salmon, trout, canned tuna from European waters, as well as pangasius fish from Vietnam). More detailed information can be seen in table 3.5.

Table 3.5: Samples origin from different European markets

Fish	Species	Fishing area	Samples
Bivalves	*Cerastoderma edule, ruditapes decussatus, mytilus edulis*	Denmark, NE Atlantic (Spain) and Mediterranean Sea (Catalan coast)	22
Cod	*Pollachius virens, gadus morhula, theragra chalcogramma*	NE Atlantic Spain and Denmark, China, Netherlands and Pacific Ocean	12
Salmon	*Salmo salar*	Norway, France and Scotland	9
Trout	*Oncorhynchus mykiss, argentina silus*	Denamrk, Czech Republic, Spain, NE Atlantic Ocean, Turkey, Netherlands, Argentine, Peru, Germany	19
Tuna (canned in brine in most of the cases)	*Thunnus albacaras, skipjack, yellowfin tuna, katsuwonus pelamis*	NE Atlantic Ocean (Spain), East Pacific Ocean, India Ocean, North Atlantic Ocean, Thailand, Italy, Philippines, Indic Ocean and Ivory Coast	10
Whiting	*Merluccius merluccius, micromesistius poutassou*	Mediterranean Spanish coast	8
Hake	*Merluccius capensis*	NE Atlantic (Spain)	3
Herring		North Sea, Baltic Sea and Denmark	18
Pangasius	*Pangasius hypothalamus*	Vietnam	9

The samples were processed by solid-liquid extraction, prior alkaline digestion along 3 h, followed by SPE clean-up with Oasis WAX 3cc cartridges and finally analyzed by LC-MS/MS-QqLIT.

3.3 Discussion

One of the main issues in the analysis of PFASs in food is to obtain clean extracts without big looses in recovery rates. In this sense, one of the main efforts was devoted to the improvement of the extraction and the clean up steps, to decrease matrix effect associated to selected food matrices. The methodologies were validated according to 2002/657/EC guideline.

On the other hand, the instrumental analysis of PFASs presents one of the major limitations due to the possible sources of cross contamination (laboratory materials, instruments and solvents). In this sense, series of precautions were taken along the development, sampling and analysis.

In general terms:

- Avoid the contact with polymers containing PFASs,
- Liquid samples were collected in glass or polypropylene (PP) bottles, and PIREX and similar materials were avoided,
- Solid samples were collected in aluminium foil, and all the processes for their grind and store were in stain steel recipients,
- The instruments used to performed all the analysis were metalised, as much as, it was possible in order to avoid possible contamination from tubing
- All the solvents were from the maximum purity
- A second C18 analytical column was inserted exactly before the injector in order to delay the possible residual contamination from the degasser pumps and seals in the system, as well as, the contamination from solvents.

Finally, in order to assure the identification of the analytes in the samples, a great part of the work was performed by mass spectrometry. In this context, different analyzers have been studied including: a triple quadrupole (QqQ), a hybrid quadrupole – linear ion trap (QqLIT) and an ion trap (IT). Depending on the type of the analysis, it should be decided the use of one or another. For example, the use of IT for screening and structural elucidation purposes is usual. This instrument has a high efficiency because all the ions are retained in the trap until the moment of their destabilization and it is possible to detect more m/z fragments than with QqQ. The trap also allows to perform MS^n experiments and to get wide molecule structural information. If we are focused in quantitative analysis, it is better the use of a QqQ in order to have enough identification points for every analyte. This analyzer is more selective than IT due to the application of a specific energy which is only stable for the selected ion. However, if screening, structural elucidation and a quantitative analysis are performed simultaneously, a hybrid analyzer QqLIT could be used. This instrument has high selectivity due to the quadrupole which allows the quantification, high efficiency due to the IT for the screening purposes, and MS^3 experiments with the QqLIT combination for structural elucidations. Detailed information was reported in the second publication presented in this chapter. The analytical part of this section of the thesis has been performed in a hybrid QqLIT mass spectrometer analyzer due to the capabilities for identification, quantification and structural information of this instrument.

For the extraction fish samples, different methods were assessed for the extraction of 8 selected compounds. Later on, this method was a little bit modified in order to improve the efficiency in the extraction step for 21 PFASs.

Three different methodologies were compared including: alkaline digestion, PLE and the ionic-pair extraction. In order to decide the optimum technique, different blank samples of young hake were spiked at 1 µg/kg concentration, homogenised and extracted by the three different methods. The experiments were performed in triplicates and the recovery results are shown in Figure 3.1.

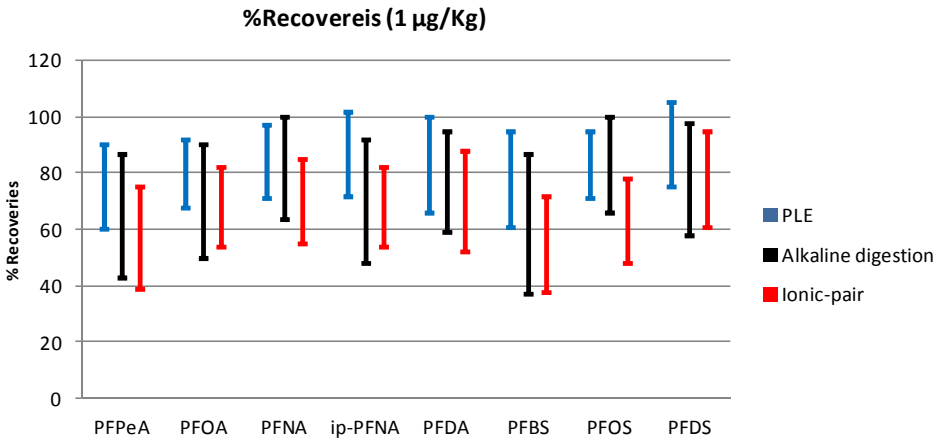

Figure 3.1: Bars of the absolute recoveries amplitude (calculated by the external standard method) obtained from young hake spiked at 1µg/kg.

The recoveries and the RSDs of the method showed that PLE provides slightly better recoveries and lower RSDs. It should also be considered that, the PLE method is much more rapid than the alkaline digestion (3 h) and provides cleaner extracts than that based on ion pairing. This methodology also allows good MLOQ settled between 3 and 50 ng/Kg in liver and 1.5 and 25 ng/Kg in muscle. Nevertheless, in order to extend the method to 21 PFASs, the alkaline digestion was selected as the optimum methodology. This method can cover a wide range of PFASs properties which includes: alkaline acids (4-18 carbon chain), alkaline sulphonates (4-10 carbon chain), alkaline sulfonamides (8 carbon chain) and alkaline phosphonic acids (6-10 carbon chain). The Figure 3.2 shows the graph of the MLOD and MLOQ for the multiresidual method based on alkaline digestion. Figure 3.3 shows the percentage of recoveries obtained by spiking blank salmon with 5 µg/Kg of PFASs. The methodology allows good quality parameters for these analytes, covering a wide range of PFASs.

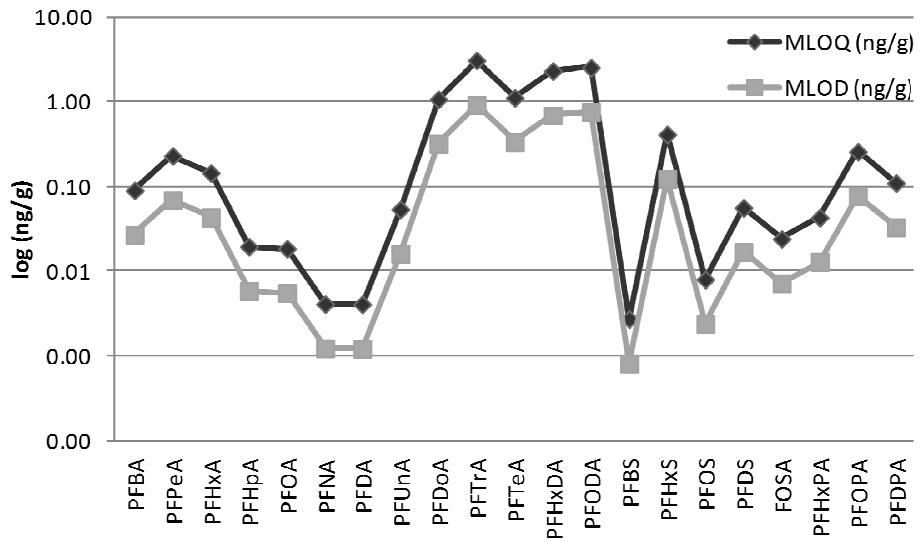

Figure 3.2: logarithm (10) of the MLOD and MLOQ for the alkaline digestion method with 21 PFASs.

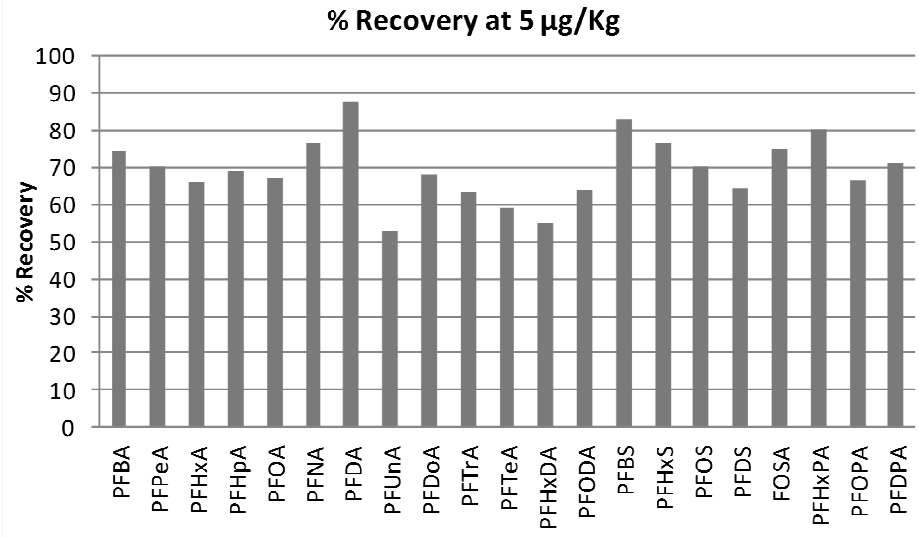

Figure 3.3: Percentage of recovery by alkaline digestion method for 21 PFASs. Results obtained by spiking blank salmon at 5 µg/Kg concentration level (n=3).

For milk and cereal samples, the first step was the alkaline digestion in order to precipitate lipids and proteins present in the matrix. After 3 hours of digestion, the samples are centrifuged and the supernatant purified by anionic SPE. For assessing the applicability of the method, different quality parameters were calculated according to 2002/657/EC guideline. The method allowed good MLOD and MLOQ (Figure 3.4), percentage of recoveries (in the range of 80 to 110%) and repeatitibility below 24% in all the cases.

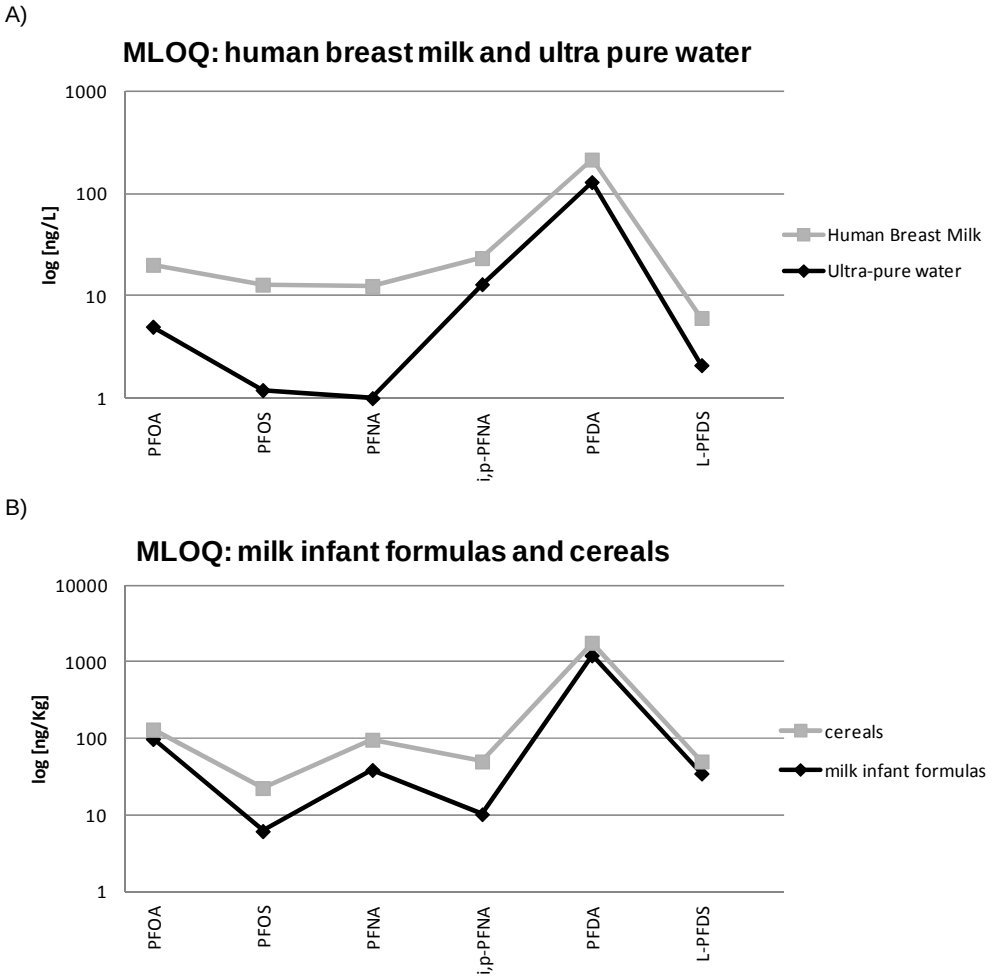

Figure 3.4: logarithm of MLOQ for **A)** ultra pure water and human breast milk and **B)** cereals and milk infant formulas.

The instrumental analysis in this study was performed by LC coupled to a hybrid QqLIT mass spectrometer. The analysis for quantification purposes was performed in SRM mode. Additionally, the hybrid QqLIT allows working, in parallel, with other acquisition modes such as EPI and MS^3 as it has been mentioned before, although there is a limitation in sensitivity due to the poor stability of fragment ions in LIT. In our case, the EPI and MS^3 modes were used for confirmatory purposes in the case of unambiguous confirmation compounds in SRM mode. In the case of fluorinated compounds, there is a clear fragmentation pathway due to the loss of $-CF_2-$ neutral fragments (m=50) for acids and sulphonates, and the loss of ionic SO_3^- or FSO_3^- (m=80 and 99, respectively) for sulphonates. These losses can be observed as transitions during SRM experiments as well as during MS^3, depending on the initial PFASs, and they can be applied for isomer identification, a normal practice in biological matrices. These types of confirmation tools are also useful for sulphonates identification because of the usual interferences in the transition of [m/z of molecular ion] > 80, due to the presence of some bile acids such as TDCA (Hansen *et al.* 2001; Benskin *et al*. 2007; Keller *et al.* 2010).

Another part of the work was devoted to validate a cost effective analytical method developed under the Conffidence project aiming to provide methods with easy implementation in routine analysis.

The results of the interlaboratory study were assessed different statistical tests based on the "*2002 AOAC INTERNATIONAL, Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method Analysis*" guidelines.

The statistical studies, included: initial review data (example of the undisclosed mixture in methanol can be seen in Figure 3.5), outliers studies (Table 3.6), bias, precision (repeatability and reproducibility), and the HORRAT value. The results are summarized in Table 3.7. The Z-Scores were also calculated to assure the homogeneity of the results. They were calculated according to $Z=(x-\mu)/\sigma$, where $\underline{x}$ was a raw score to be standardised; $\underline{\mu}$ was the real spiked concentration and $\underline{\sigma}$ was the standard deviation of 12.5% from spiked concentration. Results were normalised and Z-Scores graphs were build in order to visually compare the results. Figures 3.6, 3.7 and 3.8 represent the Z-scores obtained in the analysis of undisclosed mixture (MIX), fish A and milk B, respectively.

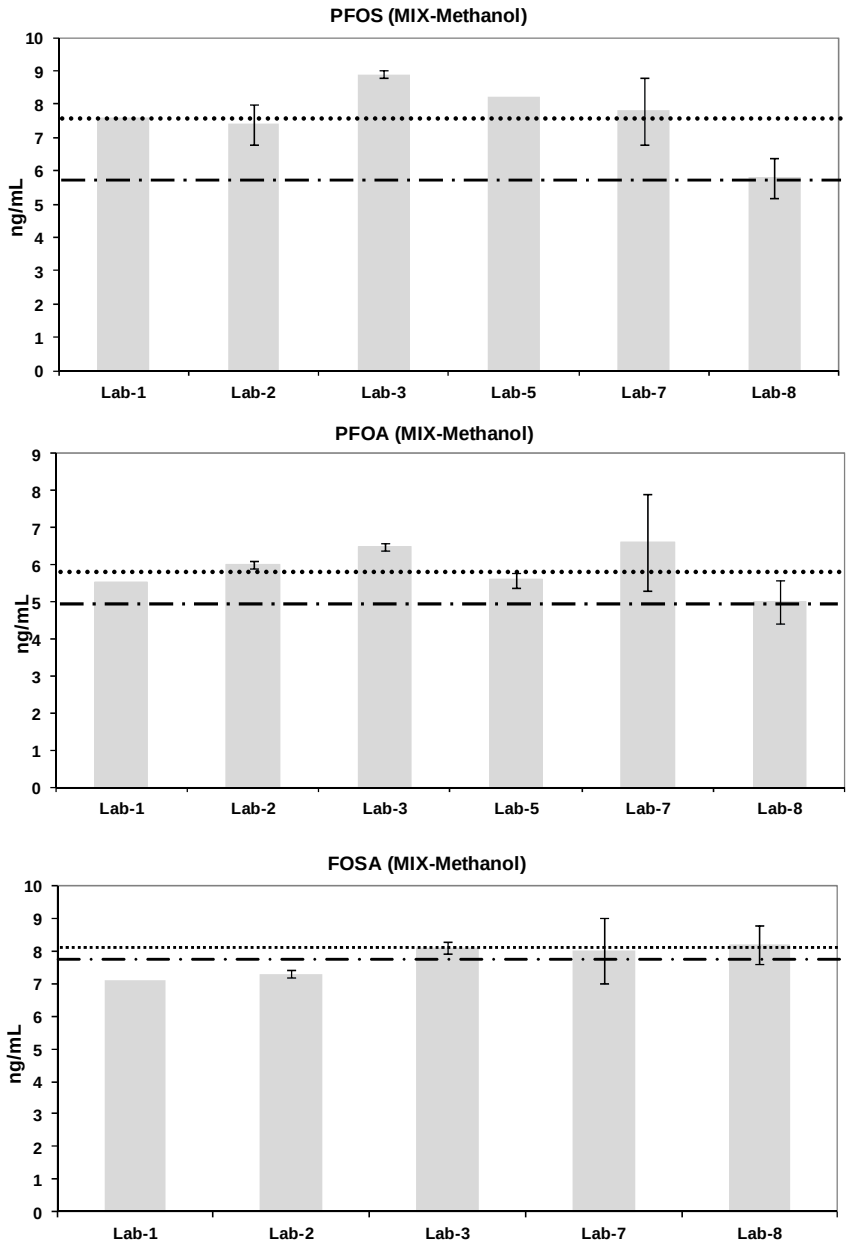

Figure 3.5: Results reported by the different participants for undisclosed mixture in methanol solution. Line (- - -) represents the real spiked concentration for every analyte and line (- · - · -) the average concentration for all the laboratories.

Table 3.6: Outlier statistical tests for PFASs in different matrices analyzed during 1st Interlaboratory

Material / Matrix	Spiked level	Analyte	No. of labs	Samples delivered for lab	Total values	Replicates	Cochran test (P=2.5%; 1-tail)		Single Grubbs test (P=2.5%; 2-tail)		Paired Grubbs test (P=2.5%; 2-tail)		Number of outliers
							Tabulated value	Calculated value	Tabulated value	Calculated value	Tabulated Value	Calculated value	
MIX-Methanol	5.76 µg/L	PFOS	6	1	6	3	65.8	0.62	64.0	13.9	81.3	13.2	0
	5.00 µg/L	PFOA	6	1	6	3	65.8	0.81	64.0	17.3	81.3	18.7	0
	8.00 µg/L	FOSA	5	1	5	3	72.6	0.72	73.5	3.0	90.3	9.7	0
Milk (MA)	0.81 µg/L	PFOS	7	2	12	3	43.1	0.39	36.1	2.2	48.5	5.1	0
	1.38 µg/L	PFOA	7	2	12	3	43.1	0.40	36.1	16.9	48.5	45.9	0
	1.00 µg/L	FOSA	7	2	10	3	48.6	0.74	42.8	0.8	56.4	3.4	0
Milk (MB)	32 µg/L	PFOS	7	2	14	3	38.3	0.36	31.7	6.4	43.5	16.3	0
	29 µg/L	PFOA	7	2	14	3	38.3	0.33	31.7	8.1	43.5	17.9	0
	36 µg/L	FOSA	7	2	12	3	43.1	0.52	36.1	2.0	48.5	-10.5	0
Fish (SA)	0.65 µg/Kg	PFOS	7	2	14	3	38.3	0.58	31.7	7.8	43.5	10.9	0
	0.74 µg/Kg	PFOA	7	2	14	3	38.3	0.78	31.7	6.8	43.5	6.8	0
	0.86 µg/Kg	FOSA	6	2	12	3	43.1	0.27	36.1	5.3	48.5	14.3	0
Fish (SB)	38 µg/Kg	PFOS	7	2	14	3	38.3	0.36	31.7	1.8	43.5	4.0	0
	41 µg/Kg	PFOA	7	2	14	3	38.3	0.70	31.7	7.0	43.5	15.5	0
	30 µg/Kg	FOSA	6	2	12	3	43.1	0.34	36.1	2.6	48.5	7.2	0

Table 3.7: Interlaboratory results for PFASs. Method based on active charcoal clean-up and LC-MS/MS analysis

Material	Spiked level	Analyte	No. of labs a[(b)]	Samples delivered for lab *	Total values	Mean	Bias	Repeatability	Reproducibility	HORRAT	
Matrix								Mean RSDr, %	RSD_R, %		
MIX-Methanol	5.76 µg/L	PFOS	7^0	1	7	7.61 µg/L	1.85	5	17	0.49	HORRAT ≤ 0.5
	5.00 µg/L	PFOA	7^0	1	7	5.86 µg/L	0.86	13	16	0.47	HORRAT ≤ 0.5
	8.00 µg/L	FOSA	7^0	1	6	7.73 µg/L	-0.27	13	12	0.36	HORRAT ≤ 0.5
Milk (MA)	0.81 µg/L	PFOS	7^0	2	12	0.92 µg/L	0.11	11	46	0.77	0.5 < HORRAT ≤ 1.5
	1.38 µg/L	PFOA	7^0	2	12	1.60 µg/L	0.22	5	87	2.04	HORRAT > 1.5
	1.00 µg/L	FOSA	7^0	2	10	0.63 µg/L	-0.37	13	61	1.36	0.5 < HORRAT ≤ 1.5
Milk (MB)	32 µg/L	PFOS	7^0	2	14	29 µg/L	-3.16	6	20	0.76	0.5 < HORRAT ≤ 1.5
	29 µg/L	PFOA	7^0	2	14	29 µg/L	-0.32	4	33	1.22	0.5 < HORRAT ≤ 1.6
	36 µg/L	FOSA	7^0	2	12	32 µg/L	-3.43	3	31	1.20	0.5 < HORRAT ≤ 1.5
Fish (SA)	0.65 µg/Kg	PFOS	7^0	2	14	0.61 µg/Kg	-0.04	8	34	0.71	0.5 < HORRAT ≤ 1.5
	0.74 µg/Kg	PFOA	7^0	2	14	0.82 µg/Kg	0.08	11	45	0.97	0.5 < HORRAT ≤ 1.6
	0.86 µg/Kg	FOSA	6^0	2	12	0.80 µg/Kg	-0.06	11	39	0.86	0.5 < HORRAT ≤ 1.5
Fish (SB)	38 µg/Kg	PFOS	7^0	2	14	28 µg/Kg	-10.1	8	31	1.18	0.5 < HORRAT ≤ 1.5
	41 µg/Kg	PFOA	7^0	2	14	31 µg/Kg	-10.31	5	32	1.25	0.5 < HORRAT ≤ 1.6
	30 µg/Kg	FOSA	6^0	2	12	21 µg/Kg	-9.06	7	33	1.22	0.5 < HORRAT ≤ 1.5

$a^{(b)}$; a=number of laboratories remaining after removal the number of outliers indicated by (b). Results obtained by outlier statistical tests are reported on table 3.6.
* Samples delivered for laboratory: number of delivered samples to every laboratory. More detailed information is reported on table 1.
HORRAT = ($\%RSD_R$ / $\%PRSD_R$);
$\%PRSD_R = 2C^{-0.1505}$; C calculated by Horwitz curve
HORRAT value meaning:

HORRAT ≤ 0.5	bad reproducibility due to lack of study independence
0.5 < HORRAT ≤ 1.5	normal method reproducibility, the expected one
HORRAT > 1.5	reproducibility higgher than expected
HORRAT > 2	reproducibility problem; rejection of the method due the "weakness"

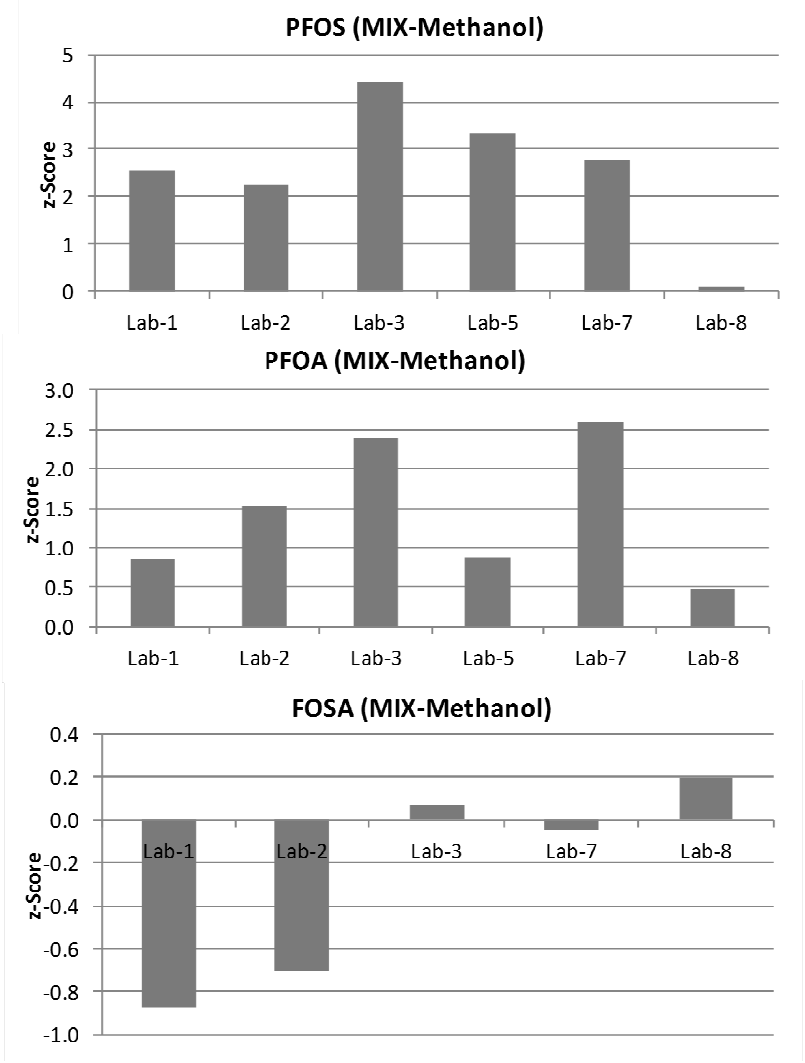

Figure 3.6: Z-Scores graphs for undisclosed mixture of PFASs in methanol

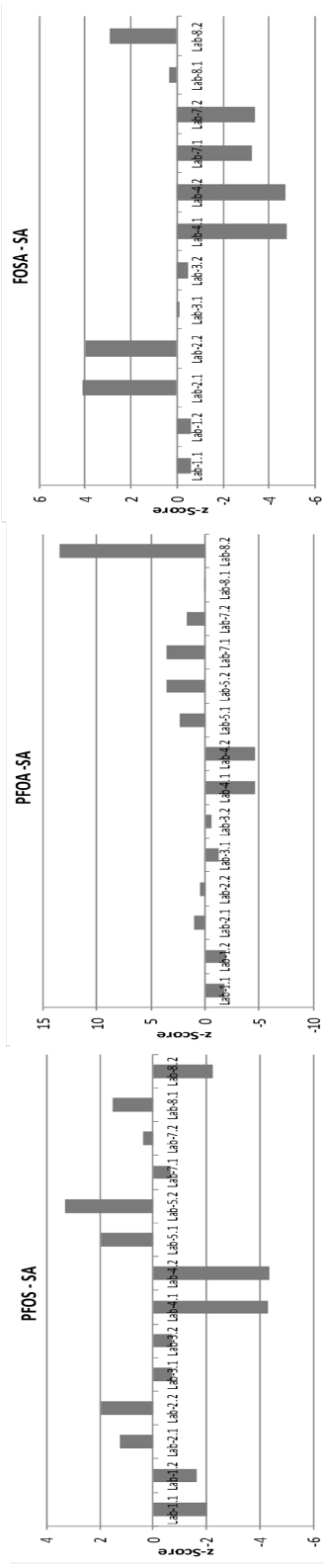

Figure 3.7: Z-Scores graphs for fish spiked at low concentration level (SA)

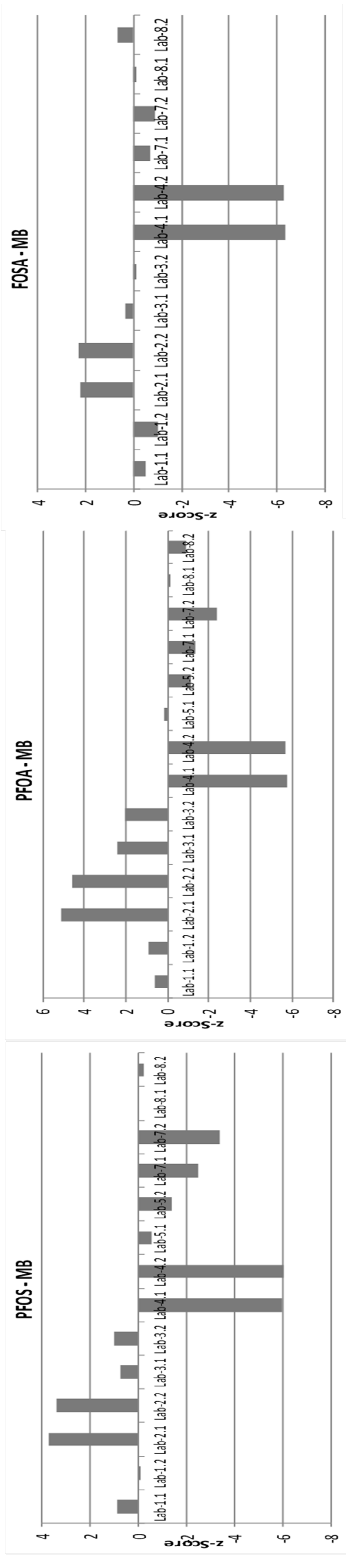

Figure 3.8: Z-Scores graphs for milk spiked at high concentration level (MB)

According to the statistical tests, no-outliers were present on reported data. However, it should be mentioned that values supplied by laboratory no 4 presented a systematic error compared to other participants but, as they passed the outlier tests, they were not eliminated for the statistical evaluation.

Good repeatability among participants was obtained, with RSDr values for all participants below 14%. In contrast, more controversial results have been found for reproducibility (RSDR). The unknown mixture of standards presented a good reproducibility being below 18% for all analytes while, the RSDR reported for low spiked materials were ranging from 46 to 87% for milk spiked near to MLOQ concentration (MA) and between 34 – 45% for salmon spiked near to MLOQ (SA). In contrast, values for higher concentrations were below 34% in all the cases. However, the conclusion based on the HORRAT parameter shows that the reproducibility was normal for all spiked materials. Based on repeatability experiments, the method could not be applied at low levels in fish and milk since there was found a mild RSDR%. A possible explanation for this mild repeatability at low concentrations are the higher levels found in milk blank (MC) in some participants, and the background found in some others as, for example, for PFOA.

Values reported for different participants presented different bias type (over estimation or under estimation) depending on analyzed matrix. In contrast, FOSA seemed to be under estimated in most of the cases presenting a negative mean bias. The under estimation observed for milk and salmon at higher spiked levels (MB and SB, respectively) induced to hypothesize a possible matrix effect also known as ion suppression, which was supported by matrix effect percentage calculated by every laboratory.

The hierarchical cluster analysis was performed in order to obtain more information about the distribution of the reported results by participant laboratories. All the results for each type of matrix, and analyte, were grouped. The resulting data was grouped according to the Nearest Neighbour Cluster method and the measured interval between results was the squared Euclidean distance. The graphical representation was through dendograms (Figures 3.9 and 3.10 for fish (SA and SB) and milk (MA and MB) samples, respectively). The hierarchical clusters confirmed the observed distribution of the results in Z-Scores graphs. Laboratory 4 seems to have a systematic bias during the analysis of the samples at high levels as can be seen for SB – PFOS and PFOA (Figure 3.9) as well as MB – PFOS, PFOA and FOSA (Figure 3.10). However, dissimilarity between this laboratory and the rest of the participants in the samples SA – PFOS and PFOA was also detected (Figure 3.9). In contrast, laboratory 7 presented a mild accuracy in the results reported for the samples MA – PFOS and PFOA. This fact was also confirmed in Z-Scores and dendograms (Figure 3.10). These results indicated a cross contamination during instrumental analysis. This cross contamination derives in a low accuracy in the lower spiked sample MA. This fact was not observed in fish samples.

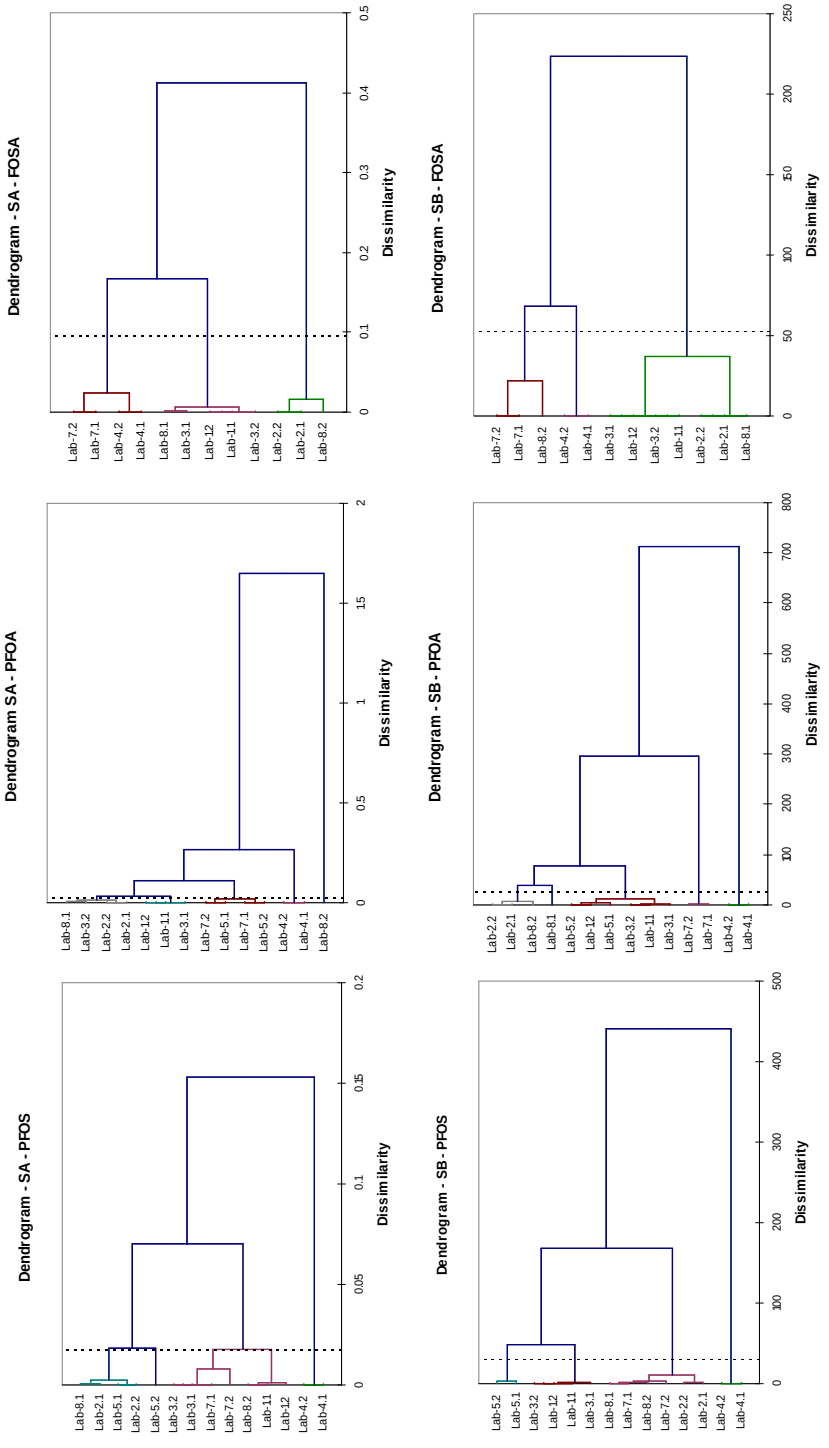

Figure 3.9: Dendograms based on Squared Euclidean Distance for fish samples SA and SB

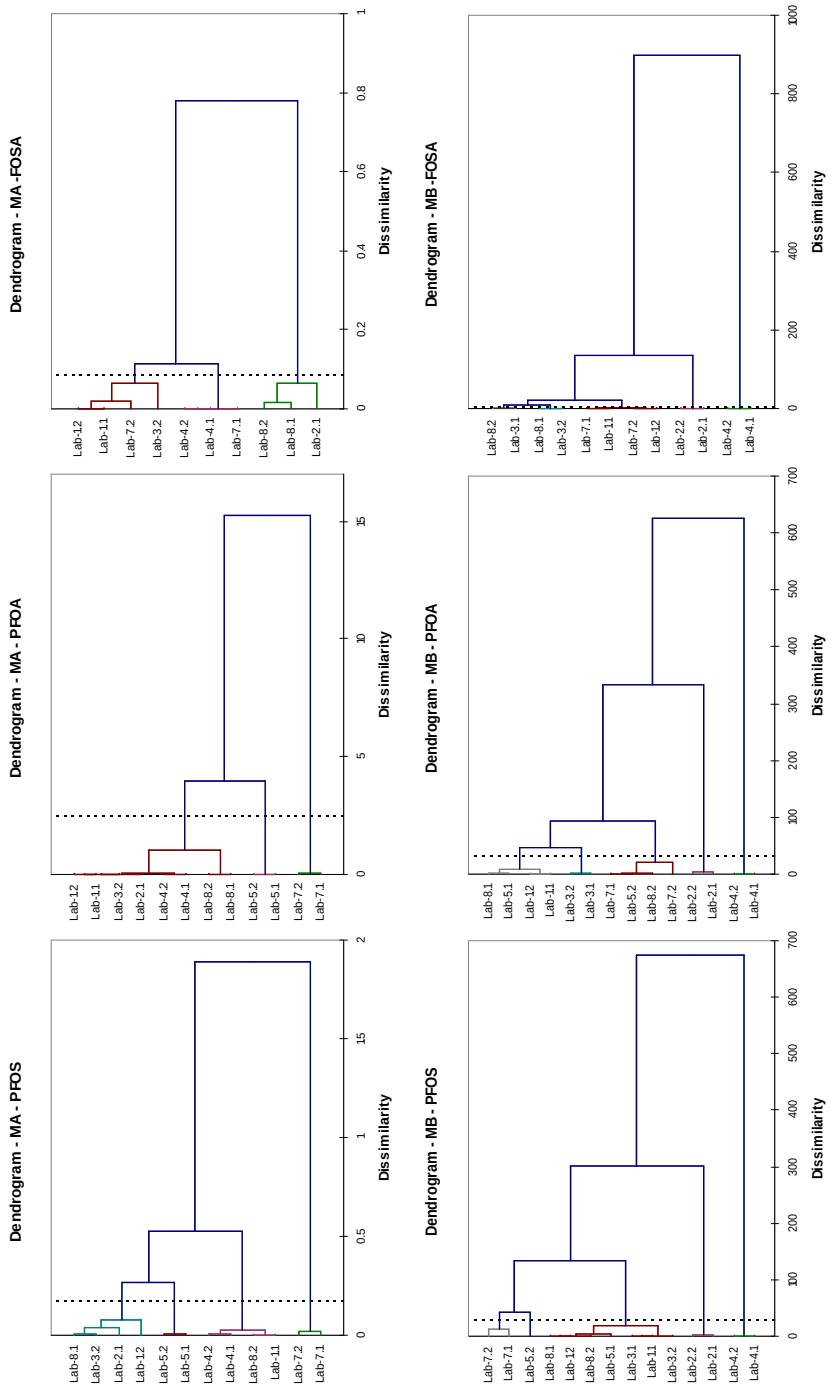

Figure 3.10: Dendograms based on Squared Euclidean Distance for milk samples MA and MB

It could be concluded that, the tested methodology, can be used in routine analysis at concentration levels higher than 1 µg/L or 1 µg/Kg for milk and fish, respectively. However, a new proficiency test with more participants is needed in order to identify if the methodology can be applied at low µg/L or µg/Kg in routine analysis.

About the study of the occurrence of PFASs in different matrices, fish samples were confirmed as an important contributor to human diet. In both studies that we have devoted to fish, PFASs were detected in all the samples. In the first study, PFPeA, PFOA and PFOS were the more frequent compounds.

Due to the concentration showed by the first study that we performed in fish, later on was decided to perform a more extended monitoring of these compounds in different market fish from different European countries, with different diets. The sample strategy was focused in different countries including: Spain, The Netherlands, Denmark and Czech Republic. In some cases, in order to distinguish any possible relationship between concentrations and sample origin or breeding, Principal Components Analysis (PCA) was carried out with the software XLSTAT (Version 2008.7.03). The new graphs were performed with the principal components 1 and 2 (F1 and F2).

The samples showed the presence of PFASs in almost all of them at quantifiable concentration levels. In the case of 19 trout samples (Figure 3.11), the most ubiquitous compound was PFHxA (black), being present in 6 over 19 analyzed samples and FOSA (orange; in 6 over 19). The concentrations ranged from low ng/g to 3.5 ng/g. However, it cannot be established any type of tendency with this information and some statistical studies with PCA were performed. The results for the statistics, regarding the relationship between PFASs concentrations and fishing area, are reported in Figure 3.12. The results showed a slightly tendency of the samples from Czech Republic with more concentration of FOSA, although more samples should be necessary in order to establish a clear distribution of PFASs.

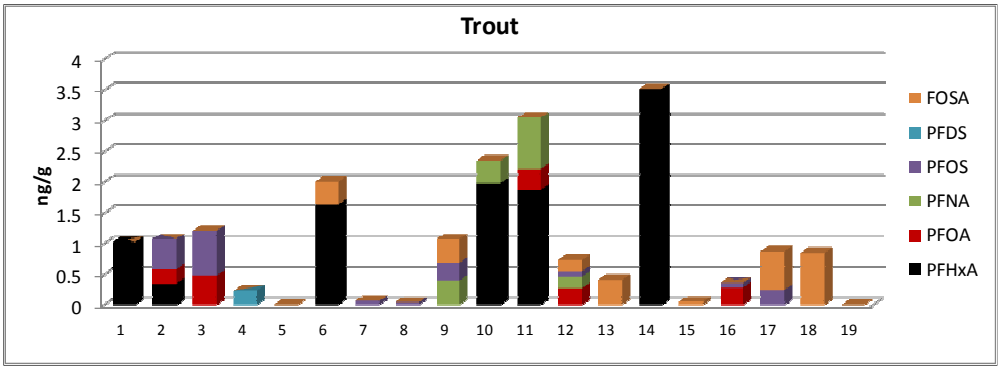

Figure 3.11: PFASs concentrations in trout samples

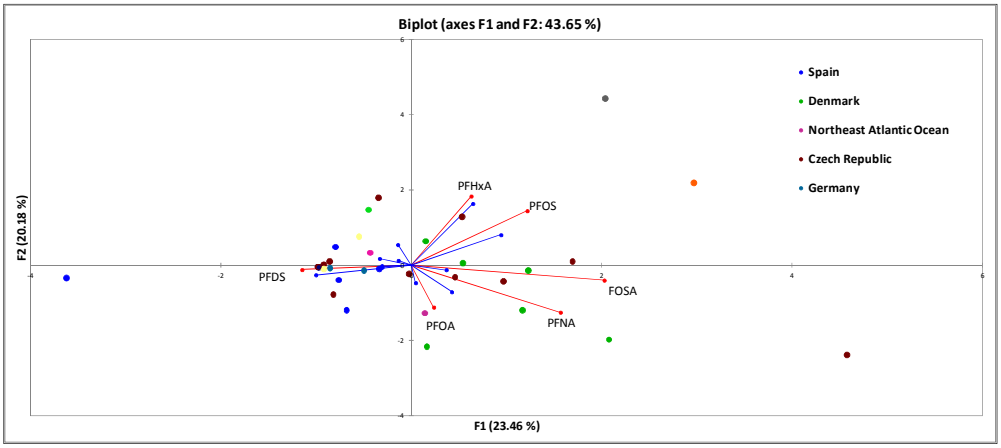

Figure 3.12: PCA corresponding to trout samples vs sample fishing area

Another interesting result was found in canned tuna. The main results can be seen in the histogram representation in Figure 3.13. PFASs have been found widespread in this type of sample, being in 9 over 10 analyzed samples above MLOQ. The distribution profile shows that PFOS was in almost all the samples (80%), followed by PFHxA (50%). The highest concentrations were for PFOS, although it was started its phase out in 2000 (3M 2000; USEPA 2006). The statistical studies based on PCA of PFASs vs fishing area showed that there is no relationship between concentration and origin. As it can be appreciated in the histograms of Figure 3.13, the distribution pattern of these compounds is very similar between all the samples. Nevertheless, the study of the influence of fish species and the concentration of PFASs revealed that *thunnus albacaras* specie presented more PFNA while *kastuwonus pelamis* contained more PFOS and PFHxA (Figure 3.14). However, there are few samples for confirming this fact.

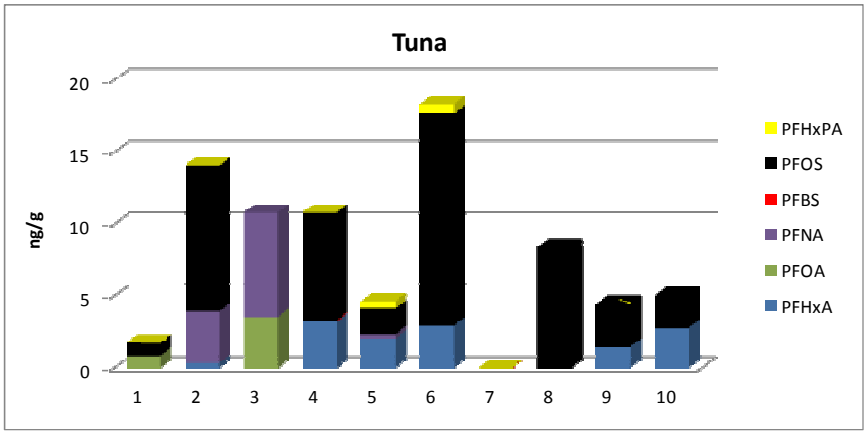

Figure 3.13: PFASs concentrations in canned tuna samples

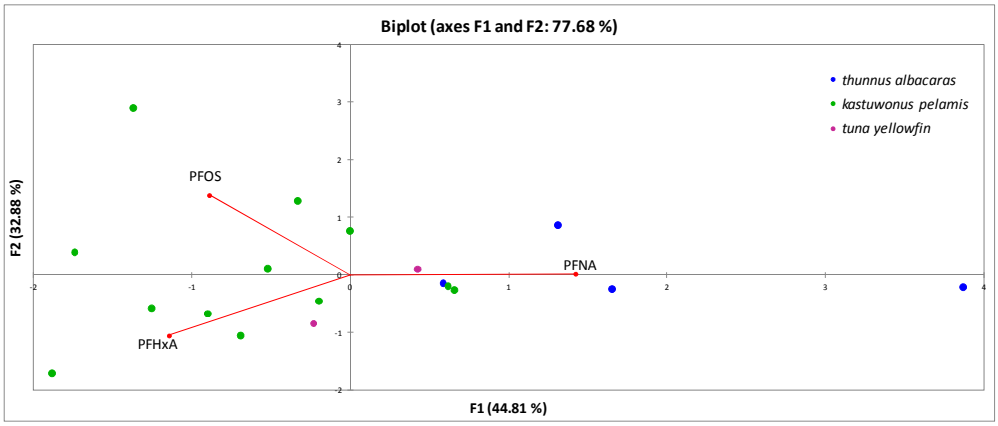

Figure 3.14: PCA corresponding to tuna samples vs fish specie

On the other side, salmon samples presented, at least, one PFASs at quantifiable concentrations, between 0.07 and 3.1 ng/g. The highest concentration levels corresponded to PFHxA. The distribution pattern does not conclude any accumulative pattern. In the case of hake samples, 3 specimens from the Cantabria Sea were analyzed. The concentrations were between 0.74 and 0.82 ng/g. It should be necessary the analysis of more samples in order to have a wide spectrum of PFASs in this specie.

The analysis of different whiting samples, from the Mediterranean Sea, all of them from wild origin, showed the presence of PFASs in the 88% of the samples. Despite of being the shorter analyzed fishes in this study and, maybe, they have less tendency to accumulate PFASs, it has been reported a concentration range between 0.5 – 10 ng/g, as a sum of total PFASs. It looks like a slightly tendency of the blue whiting to accumulate PFHxA and the whiting to accumulate FOSA and PFOS. However, these are only conjectures since much more samples are necessary in order to establish a clear pattern. In contrast, the study of 18 specimens of herring fish concluded that 17 over the 18 samples contained, at least, one PFAS above MLOQ. The most ubiquitous compounds were PFHxA followed by PFOS (Figure 3.15). The PCA study of the concentration levels in herring samples vs fishing area can be found in Figure 3.16. A distribution of PFASs according to the highest concentrations of PFHxA and PFBA in samples from the North Sea and the highest concentrations of FOSA, PFOS, PFOA, PFNA and PFBS in the samples from the Baltic Sea can be hypothesised.

In the case of cod samples, the sum of total PFASs ranged between 0.5 and 12 ng/g. The most ubiquitous analyte was PFOS followed by FOSA, although the compound which was found at higher concentration levels was PFHxA.

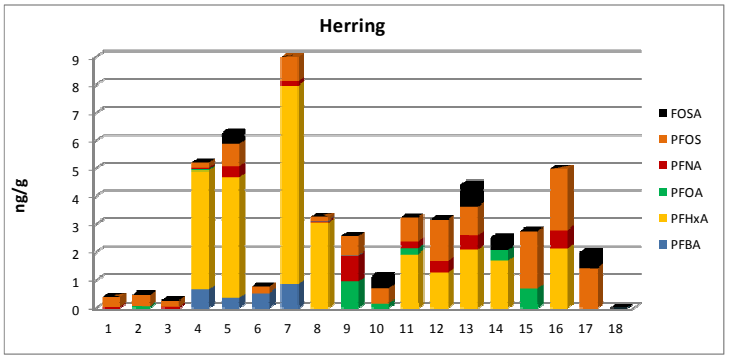

Figure 3.15: sum of the total concentration of PFASs in herring samples.

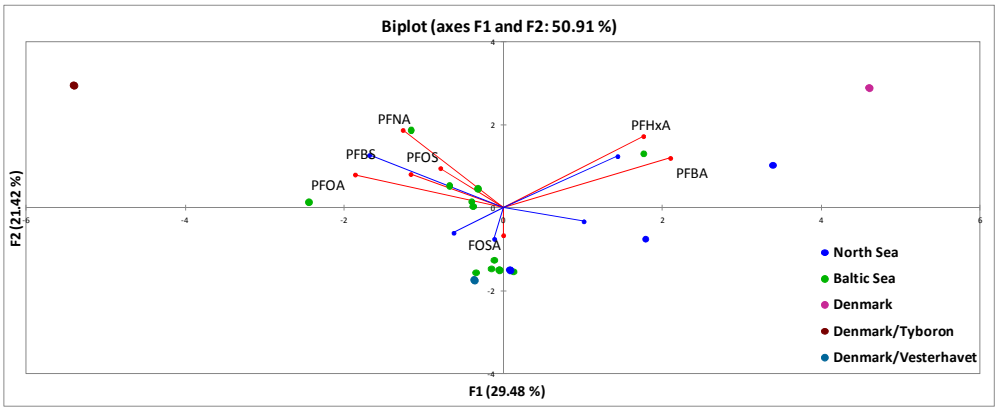

Figure 3.16: PCA statistical studies of PFASs concentration levels in herring samples vs fishing area.

On the other hand, bivalves are an interesting sample since they are filter-feeding organisms and, consequently, they tend to accumulate some soluble contaminants. The results concluded a different pattern for the cockle samples and mussels ones. While cockle contained traces of PFBA, PFOA and PFNA between 0.5 and 15 ng/g in most of the cases (samples from 1 to 4 in Figure 3.17), mussel samples showed an ubiquity distribution of PFHxA arriving to 25 ng/g and followed by FOSA at lower concentration levels. In order to elucidate any PFASs distribution depending on the fishing area, PCA statistics were performed and are summarized in Figure 3.18. According to PCA statistics, different distribution of PFASs depending on fishing area could be identified: while samples coming from Mediterranean Sea region presented higher concentrations of PFPeA, PFNA and PFOA (blue colour in Figure 3.18), the samples from Denmark showed higher concentrations for PFHxA, PFOS and FOSA or, at least, these compounds were most ubiquitous in this area. A distribution according PFASs concentrations and analyte vs sample origin (wild or aquaculture) was not identified.

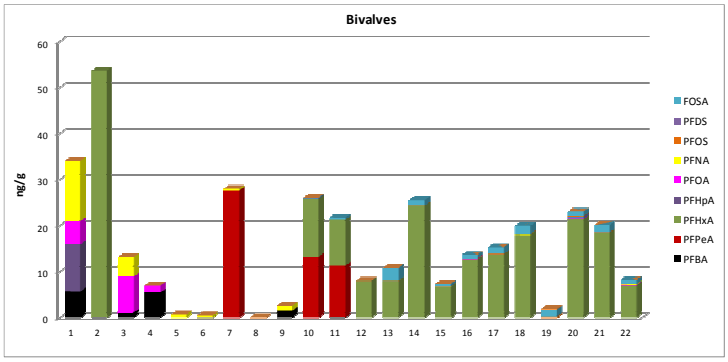

Figure 3.17: concentration levels of PFASs in bivalve samples.

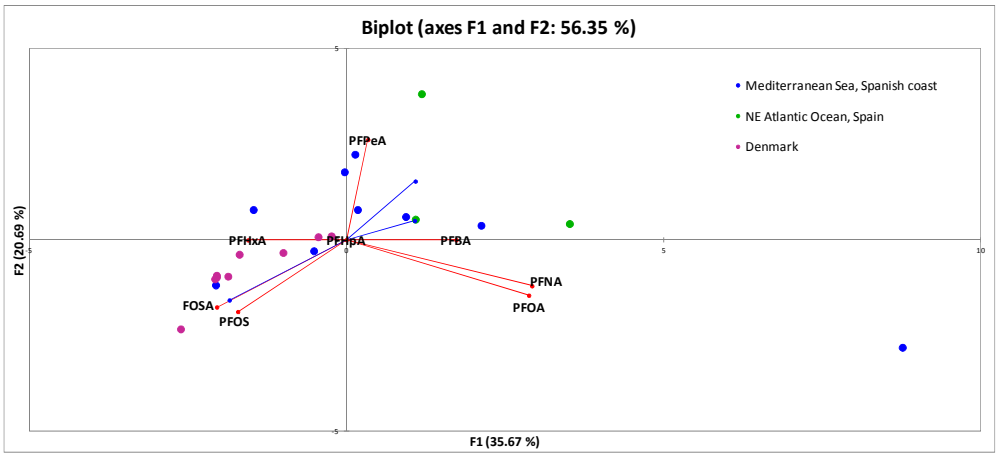

Figure 3.18: PCA statistics of PFASs concentration levels vs fishing area.

The last analyzed samples were from pangasius fish. Although this specie has an Asiatic or American origin, it is increasing their consumption in Europe due to its low cost. In the case of this study, the 9 analyzed samples were from Vietnam, although they were purchased in different European markets. Despite of the same fishing area, these samples presented different concentrations of PFASs, being PFHxA the most ubiquitous and also the most concentrated compound.

In order to approximate the concentration levels found to a real European diet through the ingestion of fish, it was calculated the Daily Intake according to EFSA guidelines (2008) for PFOA and PFOS.

The Daily Intake was calculated for a man and woman between 25 and 40 years with a medium weight (70 and 60 Kg, respectively). The proposed diet, among other food than can be taken, was the punctual consumption of 300 g, approximately, of fish fillet. The Daily Intake was calculated according to:

Daily Intake (ng /Kg day) = [(Consumption x PFC concentration) / body weight]

The Risk Index was calculated as:

Risk Index = [Daily Intake / Tolerable Daily Intake],

where Total Daily Intake was established by the EFSA in 2008 at 150 ng/Kg day for PFOS and 1500 ng/Kg day for PFOA. The calculated Risk Index concluded that these samples may not pose an immediate risk for human health through the consumption of fish although it should be considered the sum of all perfluoroalkyl substances as well as the long-time exposure.

Infant daily products

Due to human exposure and their bioaccumulation, PFASs have been identified in humans. In this context, human breast milk can be one of first routes of exposure. For this reason, different studies have reported the presence of PFASs in breast milk. Figure 3.19 shows the concentrations of PFOS and PFOA reported in different works.

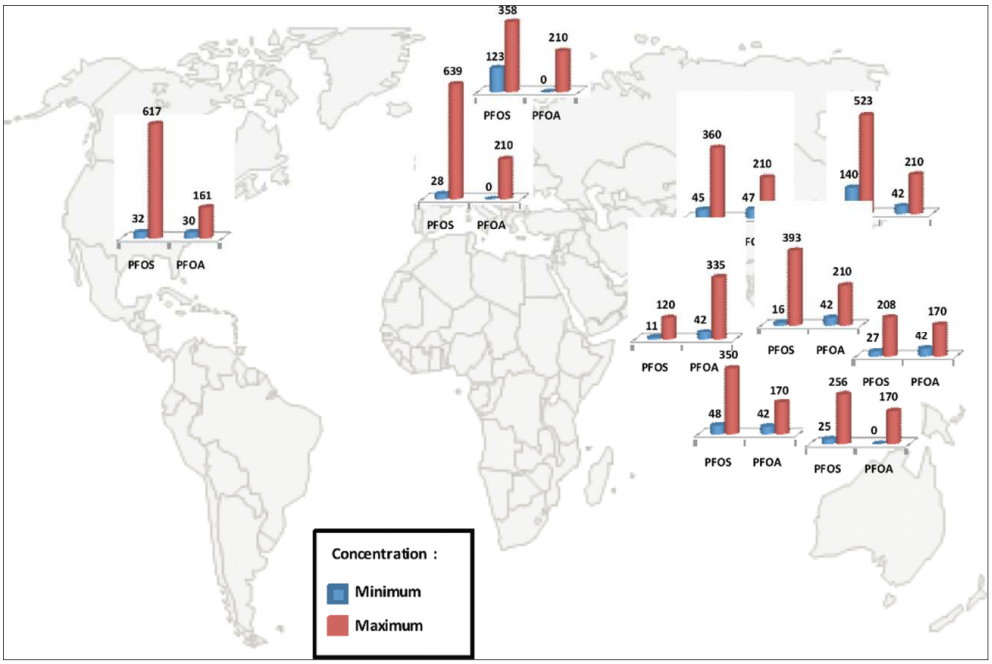

Figure 3.19: Maximum and minimum concentrations (µg/mL) of PFOS and PFOA in breast milk from several countries (Pico *et al*. 2010).

In our study, PFOS and ip-PFNA were the predominant PFASs, being detected in the 95% of the samples. In contrast, PFOA was only quantified in 8 over 20 analyzed samples. The concentrations ranged from 28 to 865 ng/L for PFOS and from 15 to 907 ng/L for PFOA. In general, the maximum concentrations reported in this study were higher than those reported in previous works. However, in terms of medium concentration the results were comparable. On the other hand, comparing the profile of the most common investigated compounds and their concentrations, no significant differences can

be attributed to different countries although the concentrations were higher in more developed and industrialised countries, such as EEUU, Canada and Japan.

In addition, during the recent years there is decay on the concentrations found accumulated in human breast milk, reflecting the stop in production of PFOS (Figure 3.20) and the limitations in PFOA emissions and production.

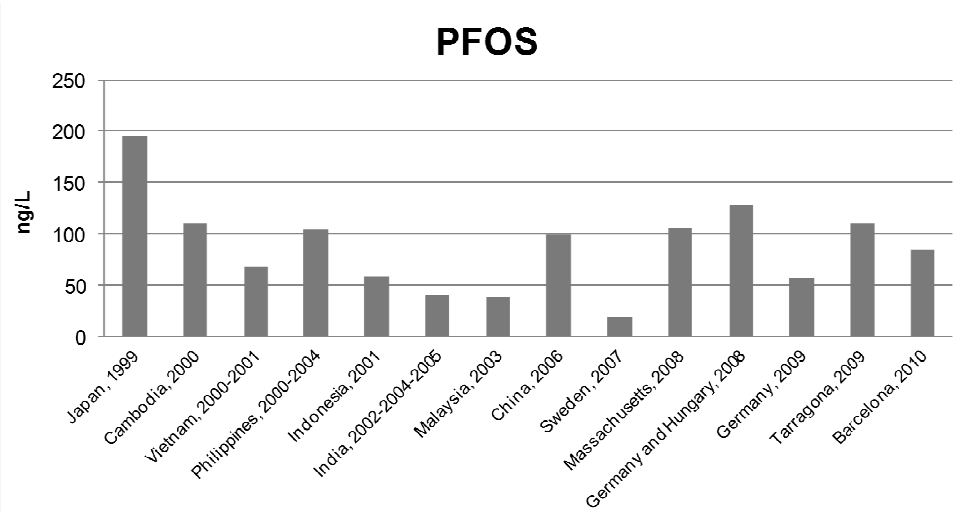

Figure 3.20: Median of concentration levels of PFOSs in different human breast milk samples from different countries ordered according the published year. Values from Japan, Malaysia, Philippines, Indonesia, Vietnam, Cambodia and India are from Tao *et al.* (2008), Chinese are from So *et al.* (2006b), Massachusetts from Tao *et al.* (2008b), Swedish from Kärrman *et al.* (2007), Germany and Hungary from Völkel *et al.* (2008), German from Völkel *et al*. (2009), Tarragona from Kärrman *et al.* (2009) and Barcelona from this thesis (Llorca *et al.* 2010).

Continuing with children intake, it was studied the occurrence of PFASs in different brands of milk infant formulas and baby food from the Spanish market. The results of this work cannot be compared with previous data because it was the first work investigating commercial baby food. In this case, predominant compounds in both matrices were PFOS, PFOA as well as the ip-PFNA. The concentration ranged for milk infant formulas between 55 and 1298 ng/Kg, where the highest concentration levels corresponded to PFDA. In the case of cereals, the concentrations were little bit lower ranging from 300 to 430 ng/Kg. It is believed that the presence of PFASs in milk could be associated to a possible migration/contamination from packaging and production processes.

Due to the presence and the concentration levels of PFASs in infant daily products, the possible health risk associated to the intake of this food was evaluated. The Risk Index was calculated according to the EFSA guidelines (2008) as in the case of market fish samples:

a) The Daily intake was calculated according to:

Daily Intake (ng/Kg of body weight /day) = [Consumption/PFAS concentration],

where consumption is expressed in mL of milk, or g of baby food, per day and PFASs concentration in ng/mL. Daily Intake of PFOS and PFOA through mother´s breast was calculated with a generalised infant's milk ingestion rate during the firsts 6 month of life. In the case of cereals, a general consumption between the 3rd and the 6th month of life as a normal rate was estimated.

b) The Risk Index was calculated according to the expression:

Risk Index = Daily Intake / Tolerable Daily Intake,

where the Tolerable Daily Intake according to EFSA guideline was: 150 ng/L for PFOS and 1500 ng/L for PFOA.

The calculated Risk Index showed that all the samples were below 1, with the exception of one breast milk sample. This sample presents a certain degree of toxicological risk for PFOS. However, reference doses for PFOA and PFOS are not well established since it should be considered the total sum of PFASs. On the other hand, there is a lack of consensus about the Tolerable Daily Intake values for these compounds between different organizations.

CHAPTER 4

PFAS accumulation in humans

4.1 Introduction

The presence of fluoride concentrations in human serum was reported for the first time in the 1960s by Taves (1968). The first PFAS detected in serum was PFOA, and thanks to the advances in analytical instrumentation, PFOS was also found in blood serum. In 1976, the company 3M started a medical monitoring of employees involved with PFOA production, and mean concentrations of up to 10 µg/ml of PFOA were detected (3M, 1999). In 1997, 3M also reported the presence of PFOS in sera (3M, 1999). In the beginning of year 2000, the monitoring of PFOS was extended to blood with respect to the general population.

Due to the extensive production of PFOS and PFOA during almost three decades, and their high stability and accumulation, these two compounds were found in blood studies during the 2000s (Kannan *et al*. 2004; Kärrman *et al.* 2005). For example, Kannan *et al.* (2004) measured the concentrations of four compounds, PFOS, PFHxS, PFOA, and PFOSA in 473 human blood/serum/plasma samples collected from the United States, Colombia, Brazil, Belgium, Italy, Poland, India, Malaysia, and Korea. PFOS was the predominant compound in blood, with the higher concentrations (reaching 30 ng/mL) in the samples from the United States and Poland.

Since then, several studies have been carried out to assess the accumulation of PFASs in human fluids. In Table 4.1 different studies reporting the concentrations of PFASs in human samples are summarised. As can be seen in Table 4.1, during the last decade, breast milk has been evaluated in many studies. However, it should be pointed out that the pathway leading to human exposure is still not well established. Direct exposure via indoor air, through the use of clothes and carpet protection products may be a relevant route of exposure (Guo *et al.* 2009), but as reviewed in previous chapters, other important human exposure pathways are food (Tittlemier *et al.* 2006; Begley *et al.* 2005), drinking water (Ericson *et al.* 2008; Wilhelm *et al.* 2008), and during recent years, trans-placental exposure is also suspected to be an important exposure pathway (Fromme *et al.* 2010). If PFASs can cross the placenta, the developing foetus is exposed to these contaminants *in utero*. It should be remarked that the exposures of fetuses, infants, and children are of the greatest concern as these are the most sensitive stages of human development.

As can be seen in Table 4.1, the levels of PFOS and PFOA in human serum have slightly declined in North America and Europe over the past decade, but the levels of PFNA have increased, reflecting the phasing out of production in these geographical areas. Whereas, over the past few years, the concentrations of PFOS in serum have increased exponentially in some areas of China likely reflecting increased PFOS production in China.

Table 4.1: concentration of PFASs in different human samples reported in the literature. Ordered according to Continent and sampling year

Year	Matrix	Studied compounds	Most frequent compounds	Maximum concentrations	Reference
	High exposed population				
1998	Serum plasma of retired workers from 3M company in Decatur, USA	PFOA, PFOS, PFHxS	• PFOS, PFOA, PFHxS (100%)	PFOS = 1740 ng/mL PFOA = 2435 ng/mL PFHxS = 791 ng/mL	Olsen et al. 2007
	Susceptible population				
	Maternal blood and cord blood				
Asia					
2003	Maternal and cord blood, from Japan	PFOS, PFOA, FOSA	Maternal blood • PFOS (100%) • PFOA (20%) Cord blood • PFOS (100%)	Maternal blood PFOS = 17.6 ng/mL PFOA = 2.3 ng/mL Cord blood PFOS = 5.3 ng/mL	Inoue et al. 2004
Canada					
2004 – 2005	Maternal blood and cord blood, from Canada	PFOS, PFOA, PFHxS, PFHpA, PFNA, and PFDeA	Maternal blood • PFOS, PFOA and PFNA (100%) • PFHxS (45.5%) Cord blood • PFOS, PFOA (100%) • PFNA (26%) • PFHxS (20%)	Maternal blood PFOS = 20 ng/mL PFOA = 2.64 ng/mL PFHxS = 2.67 ng/mL PFNA = 0.96 ng/mL Cord blood PFOS = 9.11 ng/mL PFOA = 2.374 ng/mL PFHxS = 2.77 ng/mL PFNA = 0.80 ng/mL	Monroy et al. 2008
Europe					
2007 – 2008	Maternal and cord blood, from Norway	PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA, PFTrA among others	• PFOS > PFOA were the most frequent ones	Maternal blood PFOS = 17.7 ng/mL PFOA = 4.24 ng/Ml PFHxS = 1.64 ng/mL Cord blood PFOS = 6.49 ng/mL PFOA = 3.23 ng/mL PFHxS = 1.13 ng/mL	Gutzkow et al. 2012
2007 – 2009	Maternal and cord blood, from Germany	PFBS, PFOS, PFNA, PFDA, PFDoA, PFOA, PFHxS	Maternal blood • PFOS, PFOA (100%) • PFHxS (97%) • PFNA (83%) • PFDA (21%) Cord blood • PFOS, PFOA (100%) • PFHxS (73%) • PFNA (30%)	Maternal blood PFOS = 9.4 ng/mL PFOA = 8.7 ng/mL Cord blood PFOS = 2.8 ng/mL PFOA = 4.2 ng/mL	Fromme et al. 2010

(Table 4.1)

Year	Matrix	Studied compounds	Most frequent compounds	Maximum concentrations	Reference
2010	Cord blood, from Catalonia and Crete	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFHxS, PFOS, PFDS, FOSA	• PFOS (85%) • PFOA (68%) • PFHxS (57%)	PFOS = 23.4 ng/mL PFOA = 9.18 ng/mL PFHxS = 82.2 ng/mL	This Thesis
	Human breast milk				
Asia					
2004	Breast milk, from China	PFBS, PFHxS, PFOS, PFHxA, PFHpA,PFOA, PFNA, PFDA, PFUnA, 8:2 FTCA, 8:2FTUCA	• PFOS, PFHxS, PFOA, PFNA, PFDA and PFUnA (100%) • PFHpA (37%)	PFHxS= 0.10 ng/mL PFOS = 0.36 ng/mL PFHpA = 0.006 ng/mL PFOA = 0.21 ng/mL PFNA = 0.028 ng/mL PFDA = 0.015 ng/mL PFUnA = 0.056 ng/mL	So et al. 2006
USA					
2008	Breast milk, from USA	PFOS, PFOA, PFHxS, PFNA, PFHpA, PFDA, PFUnA, PFDoA, PFBS	• PFOS (96%) • PFOA (89%) • PFHxS (51%) • PFNA (64%)	PFOS = 0.62 ng/mL PFOA = 0.16 ng/mL PFHxS = 0.064 ng/mL PFNA = 0.018 ng/mL	Tao et al. 2008
Europe					
1996 – 2004	Breast milk, from Sweden	PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA	• PFOS and PFHxS (100%) • FOSA (67%) • PFOA (8%) • PFNA (17%)	PFHxS = 0.17 ng/mL PFOS = 0.47 ng/mL FOSA = 0.03 ng/mL PFOA = 0.49 ng/mL PFNA = 0.02 ng/mL	Karrman et al. 2007
2007 – 2009	Breast milk, Germany	PFBS, PFOS, PFNA, PFDA, PFDoA, PFOA, PFHxS	• PFOS (72%) • PFOA (2%) • PFHxS (3%)	PFOS = 0.11 ng/mL PFOA = 0.25 ng/mL PFHxS = 0.03 ng/mL	Fromme et al. 2010
2008	Breast milk, from Germany	PFOS, PFOA	• PFOS (100%) • PFOA (16%)	PFOS = 0.64 ng/mL PFOA = 0.46 ng/mL	Volkel et al. 2008
2008	Breast milk, from Catalonia	PFBS, PFOS, PFDA, PFHxA, PFHpA, PFNA, PFOA, PFUnA, PFDoA, PFTeA,PFHxS, THPFOS	• PFHxS and PFOS (100%)	PFHxS = 0.11 ng/mL PFOS = 0.22 ng/mL	Karrman et al. 2009
2010	Breast milk, from Catalonia	PFOA, PFOS, i,p-PFNA, PFNA, PFDA and PFDS	• PFOS and ip-PFNA (95%) • PFOA (45%)	PFOA = 0.91 ng/mL PFOS = 0.87 ng/mL ip-PFNA = 0.26 ng/mL	This thesis

(Table 4.1)

Year	Matrix	Studied compounds	Most frequent compounds	Maximum concentrations	Reference
	General population				
	Blood				
Asia					
2003	Male blood serum (correlated with seminal plasma study), from Sri Lanka	THPFOS, FOSA, PFOS, PFHxS, PFBS, PFDoA, PFUnA, PFDA, PFNA, PFOA, PFHpA, PFHxA, PFPeA	• PFOS, PFHxS, PFUnA, PFNA, PFOA, PFDA (100%)	PFOS = 18.2 ng/mL PFHxS = 2.05 ng/mL PFUnA = 0.244 ng/mL PFNA = 1.3 ng/mL PFOA = 23.5 ng/mL PFDA = 0.201 ng/mL	Gurugue et al. 2005
Canada and USA					
2001-2002	Blood of general population, from USA	FOSA, Et-FOSA-AcOH, Me-FOSA-AcOH, PFHxS, PFOS, PFOA, PFHxA, PFNA, PFDA, PFUnA, PFDoA	• PFOS, PFOA PFHxS. PFOS, PFOA, PFHxS, and PFOSA (100%) • Et-FOSA-AcOH (81.5%) • Me-FOSA-AcOH (98.1%) • PFNA (92.6%)	PFOS = 43.05 ng/mL PFOA = 7.43 ng/mL PFHxS = 4.85 ng/mL PFNA = 1.20 ng/mL Et-FOSA-AcOH = 0.49 ng/mL Me-FOSA-AcOH = 0.88 ng/mL FOSA = 0.21 ng/mL	Calafat et al. 2006
2003 – 2004	Blood of general population, from USA	FOSA, Me-FOSA-AcOH, Et-FOSA-AcOH, PFBS, PFHxS, PFOS, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	• PFOS (99.9%) • PFOA (99.7%) • PFHxS (98.3%) • PFNA (98.8%)	PFOS = 435 mg/mL PFOA = 77.2 mg/mL PFHxS = 82.0 mg/mL PFNA = 11.5 mg/mL	Calafat et al. 2007
2004	Blood of general population, from Canada	PFOS, PFOA and FOSA	• PFOS (100%) • PFOA (29%)	PFOS = 65.1 ng/mL PFOA = 7.2 ng/mL	Kubwabo et al. 2004
Australia					
1996 – 2004	Human serum, from Australia	PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA	• PFHxS, PFOS, PFOA, PFNA, PFDA and PFUnA (100%) • FOSA (75%)	PFHxS = 11.8 ng/mL PFOS = 48 ng/mL FOSA = 0.49 ng/mL PFOA = 5.3 ng/mL PFNA = 2.5 ng/mL PFDA = 1.8 ng/mL PFUnA= 1.5 ng/mL	Karrman et al. 2007
2002 – 2003	Blood of general population, from Australia	PFBS,PFHxS, PFOS, PFDS, PFHxA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, FOSA, PFHpA	PFOS, PFHxS, PFOA, PFNA, and FOSA (100%)	PFOS = 29.5 ng/mL PFOA = 9.9 ng/mL PFHxS = 19 ng/mL FOSA= 2.4 ng/mL PFNA = 2 ng/mL	Karrman et al. 2006

(Table 4.1)

Year	Matrix	Studied compounds	Most frequent compounds	Maximum concentrations	Reference
Europe					
1993 – 1997	Blood of general population, from Denmark	PFOS and PFOA	• PFOS (100%) • PFOA (99.7%)	PFOS = 62.1 ng/mL PFOA = 13.3 ng/mL	Eriksen et al. 2011
2006	Blood of general population, from Catalonia	PFBS, PFHxS, PFOS, THPFOS, PFDS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFTrA, FOSA	• PFOA, PFOS and PFHxS (100%) • PFNA (54.1%) • FOSA (87.5%) • PFDA (16.6%) • PFUnA (39.6%)	PFOS = 16.2 ng/mL PFOA = 3.13 ng/mL PFHxS = 20 ng/mL PFNA = 1.49 ng/mL FOSA = 1.35 ng/mL PFDA = 0.71 ng/mL PFUnA = 0.84 ng/mL	Ericson et al. 2007
2009	Blood of general population, from Norway	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA,PFBS, PFHxS, PFHpS, PFOS, PFDS, FODA, MeFOSA, EtFOSA	• PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFHxS, PFOS (100%) • PFHpS (80%) • PFBS (60%) • FOSA (50%)	PFOS = 51 ng/mL PFOA = 6.5 ng/mL PFHxS = 2.3 ng/mL PFUnA = 4.3 ng/mL PFDA = 2.6 ng/mL PFNA = 3.6 ng/mL PFTrA = 1.7 ng/mL	Haug et al. 2009
	Organs				
Europe					
2008	Liver, from Catalonia	PFBS, PFOS, PFDA, PFHxA, PFHpA, PFNA, PFOA, PFUnA, PFDoA, PFTeA,PFHxS, THPFOS	• PFHxS, PFOS, PFNA, PFDA, PFUnA (100%) • PFOA (50%)	PFHxS = 1.64 ng/g PFOS = 52.13 ng/g PFOA = 1.73 ng/g PFNA = 1.38 ng/g PFDA = 2.06 ng/g PFUnA = 3.49 ng/g	Karrman et al. 2009
	Non-invasive matrices				
Asia					
2003	Seminal plasma (correlated with male blood serum), from Sri Lanka	THPFOS, FOSA, PFOS, PFHxS, PFBS, PFDoA, PFUnA, PFDA, PFNA, PFOA, PFHpA, PFHxA, PFPeA	• PFOS and PFHxS (100%) • PFOA (70%)	PFOS = 0.529 ng/mL PFHxS = 0.166 ng/mL PFOA = 2.13 ng/mL	Gurugue et al. 2005
Europe					
2011	Urine, from Catalonia	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFOS, PFDS, FOSA,	• PFBA (100%) • PFOA (57%) • PFHxS (50%)	PFBA = 1496 ng/mL PFOA = 2.89 ng/mL PFHxS = 39.3 ng/mL	Perez et al. 2012
2011	Hair, from Catalonia	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFOS, PFDS, FOSA, FHEA, FOEA, FDEA	• PFOS (46%) • PFOA (33%) • PFBA (21%)	PFOA = 6 ng/g PFOS = 7 ng/g PFBA = 39 ng/g	Perez et al. 2012

Potential health effects of PFASs have been studied in animal experiments, and different effects have been related to their exposure. For example, liver toxicity by the increasing of neonatal and adult mortality by liver deformation and liver cancer (Lau *et al*. 2003), decrease of neonatal body weight (Seacat *et al.* 2002), developmental delays (Lau *et al.* 2003), behavioural changes (Johansson *et al.* 2008), and testosterone deficiencies (Bookstaff *et al.* 1990), among others. In spite of that, the mechanisms of PFASs toxicity are not very well understood, PFOS and PFOA have both been related to fatty acid metabolism alterations, lipid transport, cholesterol synthesis and inflammation processes in rodent studies.

However, to extrapolate these results to humans is difficult, partly because of differences in body half-lives in rats and humans (days for rats *vs.* years for humans), and because certain modes of action, such as peroxisome proliferation, are also thought to be less important in humans than in rodents (Begley *et al*. 2005). In addition, it should be mentioned that PFASs have been linked to many health effects in animal studies, but often at higher exposure levels than are found in people.

Few human health studies of PFASs have been conducted in the general population. To date, associations have been found between PFOS or PFOA levels in the general population, such as reduction of female fertility and sperm quality, reduction of birth weight, attention deficit hyperactivity disorder (ADHD), increasing total and non-HDL (bad) cholesterol levels, and changes in thyroid hormone levels. However, some results are inconsistent across the current studies, and further work is needed to confirm these initial findings. The accumulation of PFASs in umbilical cord blood of the general population has been related to lower birth weight, but the findings have not always been consistent across the different studies (Apelberg *et al.* 2007; Washino et al. 2009; Fei *et al.* 2007). Small reductions in infant mass/height and head circumference have also been found in US new born. Higher odds of attention deficit hyperactivity disorder (ADHD) were recently found in 12 to 15-year- old American children with higher levels of PFOS, PFOA, PFHxS, and PFNA in their blood (Hoffman *et al.* 2009). It has been suggest that PFASs may reduce human fertility. In a Danish study of women, it has been found that higher PFOS and PFOA concentrations were associated with a longer time to pregnancy, and with having irregular menstrual cycles (Fei *et al.* 2009). However, young Danish men with high combined PFOS and PFOA levels also had half the number of normal sperm compared to men with lower levels. Participants of both studies had PFC levels similar to those found in the US population (Joensen *et al.* 2009).

Finally, few studies have examined the effects of PFASs on thyroid hormone levels in humans; existing results are somewhat inconsistent and difficult to interpret. Thyroid disruption is of particular concern during pregnancy, because thyroid hormones play a critical role in foetal brain development.

More consistent data have been obtained from highly exposed community living near a chemical plant, indicated that PFOS and PFOA have been associated with preeclampsia (pregnancy-induced hypertension), birth defects, and increased uric acid levels, which is a marker of heart disease. However, in these cases concentrations of

exposure were much higher that in general population, and these studies were focussed on PFOS and PFOA, but data on other compounds are unknown.

In this context, the study of PFAS health effects in humans is still initial and a series of uncertainties remains concerning:

- The main sources and pathways of human exposure;
- The information about human health effects at population exposure levels, especially for exposures in utero, in infancy, and in childhood;
- The assessment of new PFASs occurrence in human;
- The assessment about PFASs accumulation in different organs and tissues, and better explain their behaviour in human body, and elimination pathways
- The Mechanisms of action on human health;
- The Information about safety of new shorter chain compounds;

Under this context, first there is a need to enlarge the data reporting PFAS accumulation in humans. In particular, the information on a wide range of PFASs, including new short chain compounds is needed. However, the analysis of PFASs in complex samples presents a series of limitations such as, for example, availability of labelled standards for all the compounds. In order to solve some analytical problems in complex matrices about robustness and sensitivity, some interlaboratory studies have been carried out in order to obtain the optimum method (van Leeuwen *et al*. 2005; Longnecker *et al*. 2008; Lindström *et al*. 2009).

The analytical methods typically applied for PFASs determination in human fluids consisted on *off-line* SPE extraction (Kärrman *et al*. 2005). However, this technique presents the limitation of the sample volume, because sometimes it is not possible to obtain large volumes of blood (Kuklenyik *et al*. 2005; van Leeuwen *et al*. 2007). In recent years there has been an increasing number of techniques, based on on-line SPE, in order to solve the limitation of sample volume (Kuklenyik *et al.* 2005; Apelberg *et al.* 2007; Calafat *et al*. 2007; Haug *et al*. 2009; Pérez *et al*. 2012).

For these reasons, an analytical approach has been developed for the analysis of 18 PFASs in blood. In this case, the advantages of turbulent flow chromatography, in terms of low sample volume requirements, purity of the extracts directly injected into the chromatography column, reduction of sample manipulation and, therefore, robustness were explored. Additionally, a pilot study about PFAS content in cord blood from two Mediterranean areas was carried out.

4.2 Experimental work

The experimental work and results are presented in the following publication.

Scientific publication 7:

Llorca, M., Pérez, F., Farré, M., Agramunt, S., Kogevinas, M. and Barceló, D. (2012)

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Analysis of perfluoroalkyl substances in cord blood by turbulent flow chromatography coupled to tandem mass spectrometry

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ABSTRACT

A fast on-line analytical method based on turbulent flow chromatography (TFC) in combination with tandem mass spectrometry has been applied for the first time for the analysis of eighteen perfluoroalkyl substances (PFASs), in cord blood. A simple and rapid sample pre-treatment was optimised consisting on protein precipitation of 100 μL of sample with acetonitrile (1:1) followed by centrifugation during 10 min. The method was adapted to be sensitive enough and robust with minimum sample injection volume requirements (20 μL). The optimised methodology presented method limits of detection (MLOD) between 0.031 and 0.76 μg/L, detection capabilities (CCα) in the range between 0.005 and 0.99 μg/L and decision limits (CCβ) ranging from 0.006 to 1.16 μg/L. The recoveries in blank blood were calculated by spiking experiments with a mixture of 18 PFASs and established between 70 and 126% for most of compounds. Isotopic dilution was carried out for quantification of selected analytes. In-house validation of this new approach was carried out according to the requirements in the 2002/657/EC Decision. Finally the good applicability of this new approach was proved by the analysis of 60 cord blood samples from two different Mediterranean cities, Barcelona (Spain) and Heraklion (Greece). Ions perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS) were found at highest concentration and the more frequently compounds were PFHxS, PFOS and perfluorooctanoic acid (PFOA). The newly developed method proved to be suitable for large-scale epidemiologic studies, and to the data on PFASs exposure during pregnancy.

1. Introduction

Perfluoroalkyl substances (PFASs) are synthetic chemicals that have been widely used in the manufacturing of industrial and consumer products during the last 60 years (Lehmler, 2005).

Due to their high stability and industrial use their presence has been identified to be widespread in the environment, and many studies have detected PFASs in surface waters (Konwick et al., 2008; Quinete et al., 2009; Skutlarek et al., 2006), aquatic environments (Konwick et al., 2008; Yeung et al., 2009), sediments (Nakata et al., 2006), sludge (Llorca et al., 2011) and soils worldwide (Washington et al., 2008). Once into the environment these compounds can be accumulated (Yeung et al., 2009) by living organisms attached to proteins (Jones et al., 2003), and can be bio-magnified through the food chain (Houde et al., 2006; Kannan et al., 2001). Fish has been identified as one of main contributors of PFASs to the human diet (Falandysz et al., 2006). However, whereas some PFASs such as perfluorooctanoic acid (PFOA) and perfluorooctyl sulfonate (PFOS) have been widely studied, the industry has replaced these recalcitrant compounds with new ones, for which information about their occurrence and behaviour in the environment is lacking (Quinete et al., 2010). Humans can be exposed to PFASs through diet, by drinking water, through dermal contact and by inhalation (D'Hollander et al., 2010; Fromme et al., 2009)). In 2008 the EFSA (European Food Safety Authority) Journal reported that the data available allow only indicative values, later in January 2009 US EPA established the Provisional Health Advisories (PHA) limits for PFOS (0.2 μg/L) and for PFOA (0.4 μg/L) in drinking water (EPA, 2009), and in March 2010 the European Commission in order to define exposure levels suggested to the Member States to monitor along 2010 and 2011 the presence of PFASs in food (EFSA, 2011).

During the last decade different studies have been carried out to measure the presence of these compounds in human tissues and

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fluids, as for example, human breast milk (Llorca et al., 2010b; Tao et al., 2008), blood (Guruge et al., 2005; Yeung et al., 2005) and human liver (Kärrman et al., 2009).

Because of their persistence and their potential to accumulate PFASs are of toxicological concern. Several studies have been conducted to assess the potential toxicological behaviour of PFOA and PFOS, including sub chronic, developmental/reproductive, and chronic toxicity/carcinogenicity studies in several animal species. Recently, the European Food Safety Authority (EFSA) has performed a wide revision of these toxicological studies in order to establish the no-observed-adverse-effect level (NOAEL), the lowest-observed-adverse-effect level (LOAEL), and the critical endpoints for several PFASs (EFSA, 2008), and the liver has been identified as one of main target organs in sub acute and chronic studies (EFSA, 2008). Other authors have observed the interaction between PFASs with the thyroid hormones, high density lipoprotein, cholesterol and triglycerides (Lau et al., 2007; Peden-Adams et al., 2008).

During the last decade different studies have been performed to evaluate the possible association between PFASs to endocrine disrupting effects and potential risks to human health and the environment. For example, PFOA has been related to endocrine disruption effects (Kennedy et al., 2004). However, the mechanism and scope of the endocrine disruption of PFASs on human health continue to be poorly understood, and continue to lack large epidemiologic studies correlating the presence of PFASs with physiological effects, such as, anogenital distance studies. Human studies have correlated shorter anogenital distance (AGD) with exposure to endocrine disruptors in the environment, because this distance is regulated by the dihydrotestosterone. This measurement represents a non-invasive method for neonatal to determine male feminisation and thereby predict neonatal and adult reproductive disorders (Welsh et al., 2008). In addition, trans-placental exposure of PFASs, such as, PFOS, PFOA and PFNA (perfluorononanoic acid) has been proved in different studies. Monroy et al. (2008) showed that the median levels of PFOA, PFOS, perfluorononanoic acid (PFNA), and PFHxS (perfluorohexanesulfonate) in the ppb range in cord blood serum, which was supported by Fromme et al. (2010). Gützkow et al. (2011) found the same profile of PFASs in maternal and cord blood. Similar concentrations were reported for blood serum of the population in EEUU (Calafat et al., 2006), Japan (Inoue et al., 2004a) and South Africa (Ruan et al., 2010), and even higher in Germany (Brede et al., 2010). However, reports on the profile of other PFASs in cord blood are limited, and little is known about their levels and potential effects on children development.

Analytical methods applied in these studies are in general based on protein precipitation to reduce matrix interference and to reduce sample viscosity and clots formation followed by on-line and off-line solid phase extraction (SPE) and analytes separation and detection by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The group of Calafat, presented a method for the analysis of eighteen PFASs in human serum consisting in dilution with 0.1 M formic acid and 20 min sonication followed by SPE-LC–MS/MS showing LOD in the range between 0.050 and 0.80 μg/L (Kuklenyik et al., 2005). More recently, the work developed by Haug et al. (2009) uses only 150 μL of serum sample with protein precipitation by methanol in on-line analysis. The method allows LODs between 0.002 and 0.050 μg/L for 18 PFASs. In 2010 Gosetti et al. presented another on-line SPE method. In this approach 1 mL of blood is required, the method consisted on the blood centrifugation for 10 min, then dilution of the supernatant in acetonitrile, centrifugation 10 min more, dilution of the supernatant with water and methanol acidified with formic acid followed by on-line SPE and ultra high performance liquid chromatography (UHPLC)–MS/MS analysis. This method has presented good LOD for 8 PFASs, in the range between 0.003 and 0.015 μg/L. Another method based on UPLC–MS/MS was presented by Lien et al. (2011), in this case the samples were processed with protein precipitation with formic acid and methanol, followed by sonication and centrifugation, and the supernatants were analysed by UPLC–MS/MS. The method has shown LOD between 0.05 and 1 μg/L for the twelve PFASs. Although these approaches presented in general adequate LOD and limits of quantification (LOQ), the more sensitive approaches require, in general, the use of volumes of samples around 1 mL for each measure, which is a limited factor for large cohort studies. In addition, with exception of Kuklenyik et al. (2005) and Haug et al. (2009), the methodologies were focused on the study of just 8 to 12 compounds, and the shorter alkyl-chain compounds were not studied. In this context, the aim of the present study was the development of a sensitive and robust analytical method for the analysis of eighteen PFASs in cord blood, using low volumes of sample and a simplified sample pre-treatment. Therefore, specific objectives were (i) the development of an analytical method using a simplified sample pre-treatment followed by online turbulent flow chromatography (TFC)-LC–MS/MS; (ii) the validation of the method according to the 2002/657/EC Decision; (iii) to assess the applicability of the method by the analysis sixty cord blood samples from two Mediterranean cities, Barcelona and Heraklion, and present the preliminary results about the concentrations of selected compounds in these two cities (iv) to study the possible correlation between PFASs concentration and anogenital distances of new born.

TFC-LC–MS/MS was the technique of choice in this work because it eliminates time-consuming sample clean up, increases productivity and reduces solvent consumption without sacrificing sensitivity. This technique is based on the use of high flow rates in 0.5 or 1.0 mm internal diameter columns packed with large particles in the size range of 30–60 μm. Samples are applied to the column using an aqueous mobile phase. Small molecules diffuse more extensively than macromolecules (e.g., proteins and lipids) and are driven into the pores of the sorbent. Due to the high flow rate, the larger molecules and matrix constituents are flushed to waste and do not have an opportunity to diffuse into the particle pores. The trapped analytes are desorbed from the TFC and transferred onto the analytical LC–MS/MS system for further separation and detection. Another advantage in front of the on-line procedures based on SPE cartridges is that TFC columns can be reused for almost 500 extractions, therefore reducing the analytical cost. Although TFC is a promising technique for rapid sample preparation, very few uses in residue analysis have been reported in the literature, and to the author's knowledge is for the first time applied to the analysis of PFASs in complex matrices.

2. Materials and methods

2.1. Chemicals and materials

A mixture of PFASs standard solution in methanol (2 mg/L) [MXB; >98%] containing: perfluorobutanoic (PFBA), perfluoropentanoic (PFPA), perfluorohexanoic (PFHxA), perfluoroheptanoic (PFHpA), perfluorooctanoic (PFOA), perfluorononanoic (PFNA), perfluorodecanoic (PFDA), perfluoroundecanoic (PFUdA), perfluorododecanoic (PFDoA), perfluorotridecanoic (PFTrA), perfluorotetradecanoic (PFTeA), perfluorohexadecanoic (PFHxDA) and perfluorooctadecanoic (PFODA) acids, perfluorobutanesulfonate (PFBS), perfluorohexasulfonate (PFHxS), perfluorooctanesulfonate (PFOS) and perfluorodecanesulfonate (PFDS), a standard solution of perfluorooctanesulfonamide (PFOSA) in methanol (50 mg L^{-1}) > 99%; a mixture of labelled standards in methanol (2 mg/L)[MXA; >98%] containing: [$^{13}C_4$]-Perfluorobutanoic acid (MPFBA), Ion [$^{18}O_2$]-Perfluorohexanesulfonate (MPFHxS), [$^{13}C_2$]-Perfluorohexanoic acid (MPFHxA), Ion [$^{13}C_4$]-Perfluorooctanesulfonate (MPFOS), [$^{13}C_4$]-Perfluorooctanoic acid (MPFOA), [$^{13}C_5$]-Perfluorononanoic acid (MPFNA), [$^{13}C_2$]-Perfluorododecanoic acid (MPFDoA), [$^{13}C_2$]-Perfluorodecanoic acid (MPFDA), [$^{13}C_2$]-Perfluoroundecanoic acid (MPFUdA), and a solution of [$^{13}C_8$]-Perluorooctanesulfonamide (MPFOSA) in methanol (50 mg/L), were purchased from Wellington

Laboratories Inc., Canada. HPLC grade methanol, acetonitrile, and water were purchased from CHROMASOLV®Plus from Sigma-Aldrich, Steinheim, Germany. Ammonium acetate salt ($AcNH_4$: MW, 77.08; >98%) and formic acid were also from Sigma-Aldrich.

Polypropylene (PP) insert vials and inert taps used were from Agilent Technologies. Plastic lithium heparin tubes (10 mL; Vacutainer Blood Collection System) used to collect and store cord blood samples were from BD, NJ, USA.

2.2. Preparation of standard solutions

Working standard solutions containing all analytes were prepared by serial dilutions in methanol. All stock solutions and standards were stored in polypropylene vials at or below −20 °C.

2.3. Blanks

A blank blood sample was collected in a plastic lithium heparin tube from a lamb in a slaughterhouse from Mercabarna, Barcelona (Spain). The sample was selected as blank after screening and proved to be free on selected compounds.

2.4. Sample collection

Cord blood samples were obtained at the time of delivery from the NewGeneris (www.newgeneris.org) mother–child cohorts at Barcelona (Hospital del Mar) and the RHEA study at Heraklion (Greece). 40 mL of cord blood was collected when the placenta was still intra-uterus in order to prevent the umbilical cord from collapsing. Anogenital distance was measured at the newborns enrolled in the study within the 2 first days of life. 30 samples from Spain and 30 samples from Greece were selected, corresponding to the 15 babies with the highest and the lowest anogenital distance measurements respectively from each cohort, in order to explore its relationship with PFOS/PFOA concentrations. These compounds were initially selected because the majority of previous studies have indicated that these compounds can be related to endocrine disruptor effects and reproductive toxicity.

2.5. AGD measurement

Before each measurement, the new-born needed to be calm to avoid altering the evaluation. The digital Calliper was switched on and set to zero and then the baby was held on abduction of his legs with the left hand. On all male infants anogenital distance (AGD), anoscrotal distance (ASD) and penis width (PW) were estimated. AGD is the distance between the upper basis of penis and anus centre. ASD is the distance between the lowest point of the scrotum and the anus centre, while PW is the diameter in the basis of penis. Respectively, on all female infants anoclitoral distance (ACD) and anofourchetal distance (AFD) were measured. ACD is the distance between the clitoris and anus center, whereas AFD is the distance between the fourchette and anus center.

2.6. Sample pretreatment

100 μL aliquot of each cord blood sample was placed in PP centrifugation tube and 10 μL of labelled internal standards mixture in methanol 100 μg/L was added before subsequent addition of 100 μL of acetonitrile. This mixture was centrifuged at 4000 rpm during 10 min to induce the protein precipitation.

80 μL of the supernatant was transferred into auto sampler vials, which were stored at 10 °C until injection. The injection volume was 20 μL.

2.7. Turbulent flow chromatography

Thermo Scientific Aria TLX-1 system utilising TurboFlow™ technology (Thermo Fisher Scientific, Franklin, MA) was used for purification and separation purposes. This system comprised a PAL auto sampler (CTC Analytics, Zwingen, Switzerland), two mixing quaternary pump (eluting pump and loading pump), and a three-valve switching device unit with six-port valve. A schematic representation of this system is illustrated in Fig. S1. The entire system was controlled via Aria software, version 1.6.

TFC was carried out using two extraction columns connected in tandem for the clean-up step: Cyclone, 50×0.5 mm, 60 μm particle size, 60 Å pore size and C18 XL 50×0.5 mm, 60 μm particle size, 60 Å pore size (Thermo Fisher Scientific, Franklin, MA); and the separation of target analytes was achieved using a Hypersil GOLD PFP (50×3) analytical column, 3 μm (Thermo Fisher Scientific).

The procedure was carried out using the focus operation mode consisting on first the sample loading into the TFC columns, retention of the analytes and washing the interfering substances with water, followed by a transfer step in which the analytes of interest were desorbed from the TFC column onto the analytical column. Water acidified with formic acid at pH 3.4 with methanol (7:3) proved to be the most suitable solvent for complete recovery of the analytes from the TFC column. The third step is the actual chromatographic separation on the analytical LC column using an elution gradient. The fourth and last step was the equilibration in which the initial conditions were set for the next run. Note that equilibration and loading of the TFC column for the next run can already start before the end of the analytical separation. To avoid carry-over, the composition of the conditioning solution for the TFC column was also tested.

The gradient used is illustrated in Table 1. The solvent system used was as follows:

Loading pump A: 0.1% formic acid in water; B: Acetone:Isopropanol:Acetonitrile (10:45:45) C: Water; D: Methanol.
Eluting pump E: Ammonium acetate 20 mM in water F: Ammonium acetate 20 mM in methanol.

The total run time for each injection was 16 min.

2.8. Detection

The triple quadrupole mass spectrometer Thermo Scientific TSQ Vantage (Thermo Fisher Scientific, San Jose, CA), equipped with a Turbo Ion Spray source was used for analytical purposes. All analysis was performed operating in the negative electrospray ionisation (ESI (−)) mode. Acquisition was performed in selected reaction monitoring mode (SRM) to obtain enough identification points (IP) for confirmation of each analyte according to Commission Decision 2002/657/EC. Table S1 contains MS parameters. Fig. S2 shows the chromatogram of a blank blood lamb fortified at 100 ng/L and Fig. S3 shows the chromatograms of a blank blood lamb fortified at 250 ng/L compared with a real sample.

2.9. Quality control

In order to eliminate sources of contamination from the analytical system all the polytetrafluoroethylene (PTFE) tubing was changed for polyether ether ketone (PEEK) connections. In addition, to minimise the background signal and inter-run variability of all analytes, an extra analytical column (C8 50×3 Thermo Scientific) was placed directly upstream of the injector to trap the instrumental sources of analytes, as adapted from Flaherty et al. (2005). However, no contamination from the system was observed.

For cord blood analysis, procedural blanks were processed identical to samples to monitor contamination during the extraction

Table 1
Gradient used on the loading pump and eluting pump for the TFC-LC (ESI)–MS/MS analysis.

Time (Min:sec)	Loading pump					Step	Eluting pump			
	Flow (ml/min)	%					Flow (ml/min)	Grad	%	
		(A)	(B)	(C)	(D)				(E)	(F)
0:00	1.50	100				Loading sample	0.4	Step	90	10
0:20	0.20			100		Cleaning matrix effects	0.4	Ramp	90	10
0:30	0.20	70			30	Transfer step	0.2	Ramp	90	10
2:30	1.50				100	Cleaning column I	0.4	Ramp	20	80
7:30	1.00		100			Cleaning column II	0.4	Ramp	10	90
14:00	1.50	20			80	Loading loop step	0.4	Step	10	90
15:30	1.50	100				Cleaning column III	0.4	Step	90	10

Loading pump
(A) Water pH 3.4 acidified with formic acid
(B) Acetone:Isopropanol: (10:45:45)
(C) Water
(D) Methanol
Eluting pump
(E) Water (20 mM NH_4Ac)
(F) Methanol (20 mM NH_4Ac)

method. Instrumental blanks (of blank blood) were injected every 3–5 samples to monitor carry-over, and standards were run before and after serum samples to monitor sensitivity drift.

The values of instrumental limits of detection (ILOD) and the instrumental limits of quantification (ILOQ) were obtained by injection of standard solutions in methanol–water 20 mM ammonium acetate (10/90, v/v). ILOD were determined experimentally at the minimum detectable amount of each compound with a signal-to-noise ratio of 3, by the injection of standard solutions in mobile phase in decreasing concentrations. Criteria for ILOQ established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve calculated concentration and the real standard concentration less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable, and (4) signal-to-noise ratio at least 8.

Recoveries, linearity range, precision, accuracy, method limit of detection (MLOD) and method limits of quantification (MLOQ) were performed by spiking experiments using fortified blank blood samples.

MLOD were calculated as the minimum concentration of target compounds that can be measured according to previous criteria for ILOQ, analysing the blank blood samples fortified in decreasing concentrations. The experiments started at 1 μg/L and followed in the decreasing order of 0.75, 0.5, 0.25, 0.10, 0.075, 0.050, 0.025 and 0.010 μg/L of spiked concentration level.

For identification purposes, retention times of PFASs in the standards and in the samples were compared at a tolerance of ±2.5%. Moreover, in accordance with the 2002/657EC Decision, the relative ion intensities (each product ion area signal versus the base product ion area signal) of the spiked samples were compared with the relative ion intensities of PFASs standard solutions, at the same concentration levels as used for the construction of the calibration curve.

Table 2 summarises instrumental quality parameters of the LC–QqQ–MS method for the analysis of PFASs. The range of linearity was defined by plotting the peak area ratio of the PFASs to the IS versus PFAS concentration. The linearity was calculated with the analysis of the following standard mixture concentrations: 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, 10, 25, 50, 75, 100, 150 and 250 μg/L. The lowest calibration concentration was established at the corresponding MLOQ for every analyte (Table 2). All the standards were fortified with internal standards at 5 μg/L. The criterion for linearity range was as follows: linear regression with a correlation coefficient better than 0.990, and RSD of four replicates less than 25%. In the present approach, calibration curves presented good linearity ranges and correlation coefficients (R^2) higher than 0.990 in all the cases, and were used for confirmatory purposes. Quantification was performed by internal standard addition.

According to the 2002/657/EC Decision, since no certified reference materials were available for all the analytes in the matrix of interest, the recovery from fortified samples was measured as an alternative to trueness. Each type of blank sample was spiked in quintuplicate as previously described with the PFASs at three concentration levels (5, 10 and 50 μg/L). 100 μL of blank lamb blood was spiked with 5 μL of selected concentration of PFASs mixture, in water, in order to obtain a final concentration of 5, 10 or 50 μg/L. The samples were homogenised in an orbital digester during 1 h and preserved at −4 °C for 24 h prior to analysis. Then, the samples were extracted as has been described before without the addition of internal standard. A procedural blank was always carried out in parallel to spiked experiments in order to rule out any possible contamination source. The accuracy of the method was calculated as follows: 1) the percentage of relative recovery by comparing the response of the extract *vs.* the response of the standards in mobile phase solution analysed by off-line method and, 2) the bias of the spiked experiments from the spiked amount by the difference of [real concentration − spiked concentration].

Decision limit (CCα) was evaluated by analysing 20 blank samples and measuring the signal to noise ratio at the time window in which the analyte would be expected in case to be present. Three times the signal to noise ratio was used as the decision limit with a 5% certainty. Detection capability (CCβ) values were obtained from the analysis of 20 blank samples, this time fortified at as close as possible to the CCα concentrations. Three different concentrations were spiked in order to cover the CCα's wide range: 0.05, 0.5 and 1.0 μg/L and analysed 20 times. The fortification of blank samples was performed as has been described for recovery experiments. The corresponding concentration at the CCα value plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability (β = 5%).

The precision of the method was calculated by repeatability (intra-day precision) and reproducibility (inter-day precision) experiments. They were calculated as the average percentage relative standard deviation (RSD%) of fortifying 5 whole blood blank samples at 10 μg/L. Surrogate labelled standards were always added at a concentration of 5 μg/L. For intra-day, the 5 replicates were analysed at different times during the same day. For inter-day, the different replicates of fortified aliquots were analysed during ten consecutive days (one per day and every replicate analysed randomly twice along the ten days).

Table 2
Validation parameters (injection volume: 20 μL).

	Off-line		On-line whole blood													
	ILOD (pg)	ILOQ (pg)	MLOD (μg/L)	MLOQ (μg/L)	Linearity (μg/L)	R^2	[a,b] Recovery (%)			[c,b] Bias			CCα (μg/L)	CCβ (μg/L)	[d] Precision (%RSD)	
							5 μg/L	10 μg/L	50 μg/L	5 μg/L	10 μg/L	50 μg/L			Intraday	Interday
PFBA	0.35	1.2	0.05	0.15	MLOQ–250	0.9962	55	50	57	−2.3	−5.0	−22	0.08	0.11	15	23
PFPeA	0.55	1.8	0.76	2.54	MLOQ–250	0.9959	94	103	50	−0.3	0.3	−25	0.99	1.16	12	16
PFHxA	0.20	0.67	0.14	0.48	MLOQ–250	0.9995	120	105	114	1.0	0.5	7.0	0.27	0.37	12	16
PFHpA	0.59	2.0	0.11	0.37	MLOQ–250	0.9996	126	72	120	1.3	−2.8	10	0.34	0.5	13	14
PFOA	0.20	0.67	0.11	0.35	MLOQ–250	0.9997	83	104	120	−0.85	0.4	10	0.12	0.13	5	14
PFNA	0.02	0.08	0.20	0.66	MLOQ–250	0.9990	83	109	116	−0.85	0.9	8.0	0.24	0.27	5	12
PFDA	0.04	0.12	0.087	0.29	MLOQ–250	0.9998	116	124	100	0.80	2.4	0.00	0.12	0.15	4	10
PFUdA	1.0	3.3	0.50	1.67	MLOQ–250	0.9942	63	57	115	−1.9	−4.3	7.5	0.88	1.14	11	13
PFDoA	2.6	8.6	0.22	0.74	MLOQ–250	0.9947	60	50	96	−2.0	−5.0	−2.0	0.40	0.53	13	17
PFTrA	2.0	6.7	0.32	1.06	MLOQ–250	0.9956	56	57	66	−2.2	−4.3	−17	0.34	0.36	12	23
PFTeA	4.0	13	0.22	0.74	MLOQ–250	0.9930	50	60	50	−2.5	−4.0	−25	0.32	0.38	20	20
PFHxDA	5.0	17	0.53	1.77	MLOQ–250	0.9933	57	66	60	−2.2	−3.4	−20	0.99	1.3	10	21
PFODA	8.0	27	0.42	1.41	MLOQ–250	0.9930	54	50	58	−2.3	−5.0	−21	0.82	1.1	19	21
PFBS	0.17	0.56	0.064	0.21	MLOQ–250	0.9997	104	58	86	0.20	−4.2	−7.0	0.09	0.11	8	8
PFHxS	0.15	0.49	0.0031	0.010	MLOQ–250	0.9999	97	111	125	−0.15	1.1	12.5	0.005	0.0063	6	6
PFOS	0.15	0.49	0.018	0.06	MLOQ–250	0.9999	100	120	85	0.00	2.0	−7.5	0.038	0.053	8	10
PFDS	0.03	0.11	0.094	0.31	MLOQ–250	0.9941	119	102	111	0.95	0.2	5.5	0.17	0.23	9	9
PFOSA	0.10	0.33	0.026	0.087	MLOQ–250	0.9999	112	77	81	0.60	−2.3	−9.5	0.045	0.059	5	8

[CCα]: decision limit was calculated according Decision 2002/657/EC when there is no established permitted limit (according to ISO 11843) by 20 analysed blanks.
[CCβ]: detection capability for substances which there is no permitted limit according to Decision 2002/657/EC ($CC\beta = CC\alpha + 1.64\ SD_{CC\alpha}$)
Relative Recovery (%) = [(On-line analyte response in spiked matrix) / (Off-line analyte response in standard solution)]
[a] *Recovery*: expressed as relative % of recovery:
[b] *Recovery and bias*: these two parameters are used to express the accuracy
[c] *Bias*: expressed as the difference between calculated concentration and real spiked concentration
[d] *Precision*: calculated at 10 μg/L of spiked blank blood. *Intraday*: n = 10; *Interday*: n = 5.

3. Results and discussion

3.1. Sample pretreatment

In order to optimise the results of the method performance first, the need of sample pretreatment was assessed in front of two different protein precipitation procedures consisting on a) dilution of 100 μL whole blood sample in acidified water (1:1) (water pH 3.4, formic acid) as described by different authors (Kärrman et al., 2005; Kuklenyik et al., 2004, 2005), followed by 1 min. agitation and centrifugation at 4000 rpm during 10 min more to induce protein precipitation; and b) dilution with acetonitrile (1:1) followed by the same procedure as before which has been described in different works (Inoue et al., 2004b). In case of direct injection, 20 μL blood was introduced in the TFC system, whereas using the protein precipitation procedures 20 μL of supernatants were analysed. The protein precipitation using acetonitrile was chosen as the optimum approach, since direct injection resulted in strong ion suppression effects, and with acidified water brought loses of sensitivity, especially in the case of acidic analytes.

3.2. Development and optimisation of the turbo flow chromatography–tandem mass spectrometry method

The investigated PFASs were subjected to an MS/MS characterisation using the information gained from our previous methods (Llorca et al., 2010a, 2010b). MS/MS parameters were optimised by continuous infusion of a mixture of 200 μg/L of standard solution of each analyte in methanol:water (1:1) solvent. The optimal ESI source conditions and fragment ions for each analyte are shown in Table S1 of the Supporting Information Section.

TFC parameters were optimised by direct injection to the MS/MS system. The turbulent flow system was operated in focus mode in order to obtain the better peak symmetry, chromatographic resolution and sensitivity. The total run time was 5 min. Different types of turbulent flow extraction columns were tested, including Cyclone, Cyclone P, Cyclone MAX, C18, C18 XL, C8 XL and Fluoro, as well as, different columns combinations, solvents, solvents pH (3.4, 4.4, and 8), loading times (10, 20, 30s at 1.5 mL/min), elution solvents, loop volume (100 and 250 μL), and time of transfer (1, 1.5, 2 and 2.5 min). Fig. S4 of the Supporting Information Section shows an example of turbulent flow chromatograms with the different columns at pH 3.4, and Fig. 1A presents the percentage of recoveries using solvent loading at different pH. Cyclone MAX presented too high affinity for most of the selected compounds, and then the elution from the stationary phase was mostly achieved during the second cleaning cycle with the mixture of Acetone:Isopropanol:Acetonitrile (10:45:45). With C8 XL column, the 50% of the analytes were just eluted from the TFC during the wash cycle. Good results were obtained using Cyclone P and Cyclone columns, but slight better recoveries and transfer of the acidic compounds were obtained with the Cyclone phase, whereas with the C18 XL TFC showed the highest recoveries for perfluoroalkyl sulfonates. Finally, a combination of Cyclone and C18 XL columns disposed in tandem was selected. In Fig. 1B, are presented the obtained recoveries with different transfer solvents, as can be seen although in general better recoveries were obtained with water:methanol (5:95) or with water:methanol: magic mix (5:85:10), then the short chain PFASs were loosed. Therefore, the mixture of acidified water and methanol was selected as the optimal one. Comparing the transference using different loop volumes, the best results were achieved using the loop of 250 μL. The optimal time of elution was set at 2 min.

It should be mentioned that the method could distinguish between linear and branched isomers of PFOS and PFHxS. Although it is not complete, it is clear that the branched isomers are present (Fig. S3 II).

3.3. Quality parameters of the analytical method

The validation procedure for the developed method was carried out according to the EU requirements, as stated in the commission decision 2002/657/EC.

The MLOD ranged from 0.003 to 0.11 μg/L for most of the compounds. However, it should be pointed out that perfluoro alkyl acids presented higher MLODs between 0.22 and 0.76 μg/L. The decision

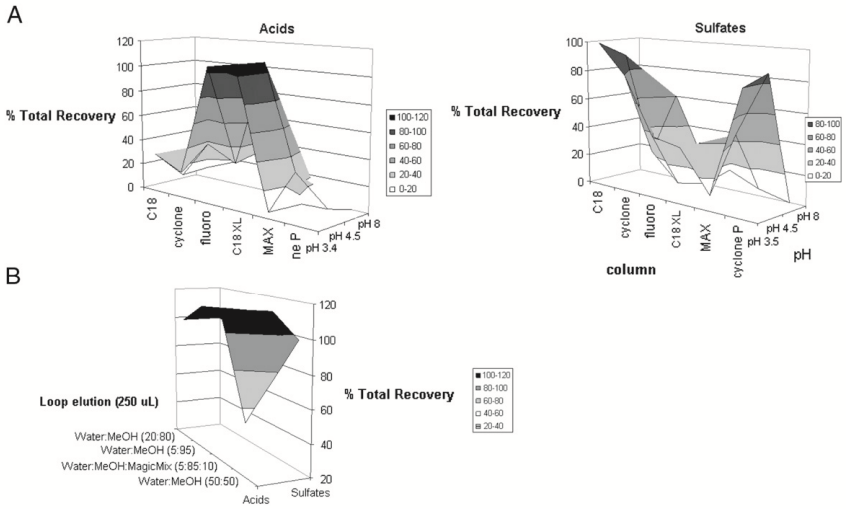

Fig. 1. A: The total percentage of recoveries using water at different pHs as a loading solvent with different turbulent flow extraction columns. B: The recoveries obtained with different transfer solvents.

limit (CCα) and the detection capability (CCβ) values were calculated according to the EU regulation (European Commission, 2002). In Table 2 are presented CCα and CCβ values, as can be seen were ranging from 0.005 to 0.990 μg/L for CCα, and between 0.006 and 1.160 μg/L for CCβ.

According to the European Commission (2002), when no minimum required performance limits (MRPLs) have been established, it is recommended to calculate the recoveries percentages by spiking concentrations at 1, 1.5 and 2 times CCβ. Accordingly, spiking experiments were carried out at 5, 10 and 50 μg/L. The lowest value was established at 5 μg/L because all the analytes were quantifiable at this concentration. On the other hand, instead of spiking at 1.5 times the CCβ value, it was decided to spike at 50 ng/L in order to obtain a wide spectrum of recoveries. As can be seen in Table 2 the recovery values were between 50 and 126% for the acidic compounds, between 58 and 125% for the sulphonate, and between 77 and 112% for the sulphonamide.

Repeatability (intra-day precision) and reproducibility (inter-day precision) were calculated as the average percentage relative standard deviation (RSD%) of fortified blanks. The results obtained indicate repeatability ranges of 5 and 20%, whereas reproducibility was generally higher with 8 and 23%, and in both cases always was lower than 25%. This methodology allows good quality parameters and the analysis of blood samples in just 26 min which is an advantage in front of the off-line methodologies based in ionic pair extraction (Kubwabo et al., 2004; Sottani and Minoia, 2002), liquid–liquid extraction (Szostek and Prickett, 2004) or solid phase extraction (Kärrman et al., 2005; Kuklenyik et al., 2004). Other advantages are the reduction of the sample manipulation and, consequently, improved analytical robustness.

Comparing the analytical procedure presented in this work with previous on-line approaches (Calafat et al., 2006; Holm et al., 2004; Inoue et al., 2004a; Kuklenyik et al., 2005), one of the advantages is that the extraction columns are use to performed a high number of analytical cycles (more than 500 in general), reducing the cost of the analytical procedure. On the other hand, turbulent flow chromatography improves the sample clean-up, reducing matrix interferents and, as a consequence, improving the method sensitivity.

3.4. Application in cord blood samples

Sixty cord blood samples were analysed. Thirty samples were obtained from newborns of the Hospital del Mar mother–child cohort in Barcelona city (Spain), and the other thirty samples were collected from the RHEA mother–child cohort at Heraklion city (Greece). The results of this study are summarised in Tables S2 and S3 from the Supporting Information and Fig. 2 shows the levels of PFASs higher than MLOQ. As can be seen all the samples were found to contain at least one of studied compounds. In general, perfluoroalkyl sulfonates were found in higher concentrations than perfluoroalkyl acids. The more frequently found compounds were PFHxS and PFOS followed by PFOA in agreement with previous studies from Calafat et al. (2007), and Ericson et al. (2007) regarding PFASs in serum samples. Other studies of cord blood have reported PFOA, PFOS, PFNA at μg/L (Fromme et al., 2010; Gützkow et al., 2011). PFHxS was found in all the samples collected from Barcelona donors in concentrations in the range between 26 and 83 μg/L followed by PFOS present in the 87% of the samples. In this study an unexpected concentration of PFHxS was obtained in comparison to other studies regarding PFASs in cord blood (Gützkow et al., 2011). However the co-elution of PFHxS with endogenous steroid sulphates which can cause the over estimation of this compound (Benskin et al., 2007) was discarded, because a Hypersil GOLD PFP analytical column was employed. This column from Thermo Fisher Scientific offers an alternative selectivity between fluorinated isomers in reverse phase. In addition, retention times and the ratio between the two optimised SRM transitions with the respective standard in calibration curve were carefully checked for each sample. In contrast, for the samples from Heraklion, PFOS was the most frequently found. It was quantified in 21 samples of the 30 from Greece (70%), being the concentrations in the range between 2 and 23 μg/L in quantifiable samples. Comparing the median concentrations of these two compounds were 44 μg/L for PFHxS and 4 μg/L for PFOS and 4 μg/L for PFHxS and 11 μg/L for PFOS for the samples collected in Barcelona and Heraklion, respectively. The levels of PFOA were, in general, higher in samples from Greece with a median concentration of 4.45 μg/L. The percentage of samples with quantifiable concentrations of PFOA was 83% for the samples

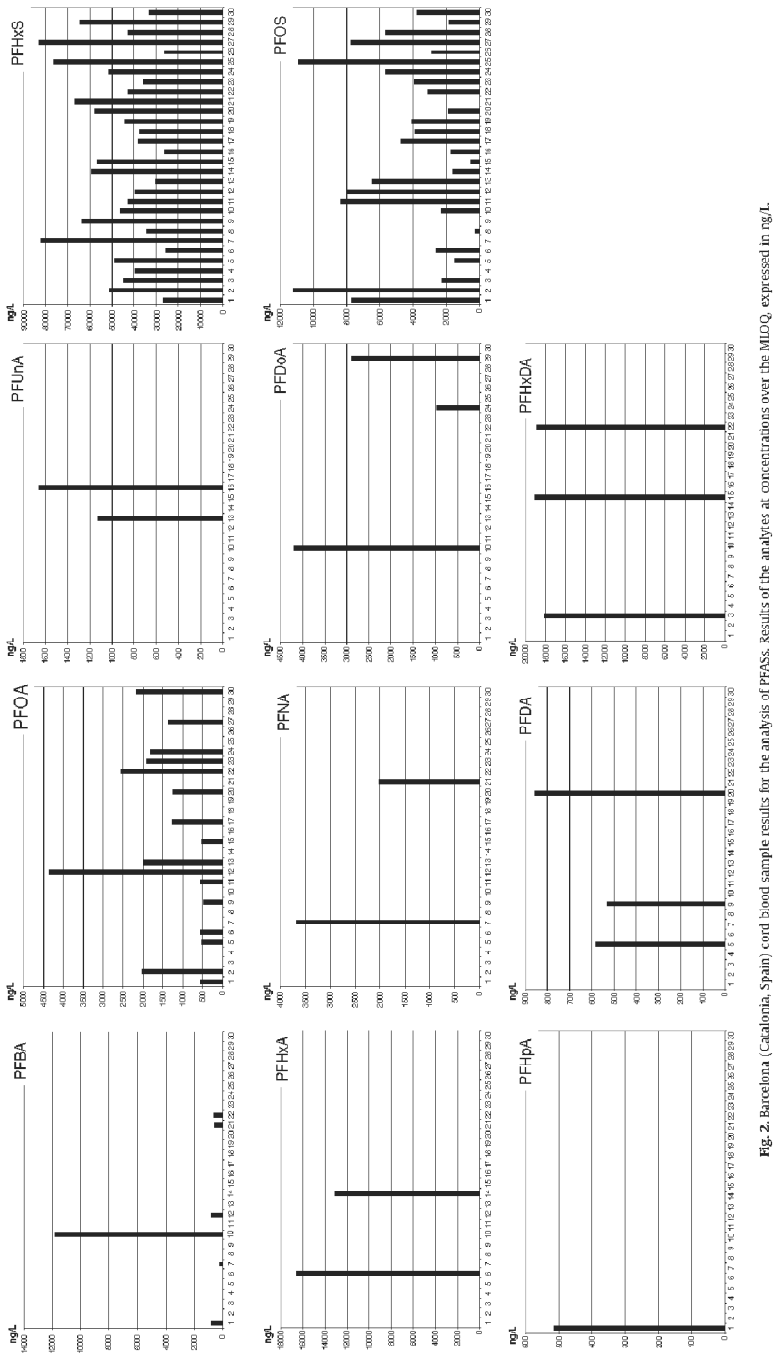

Fig. 2. Barcelona (Catalonia, Spain) cord blood sample results for the analysis of PFASs. Results of the analytes at concentrations over the MDQ, expressed in ng/L.

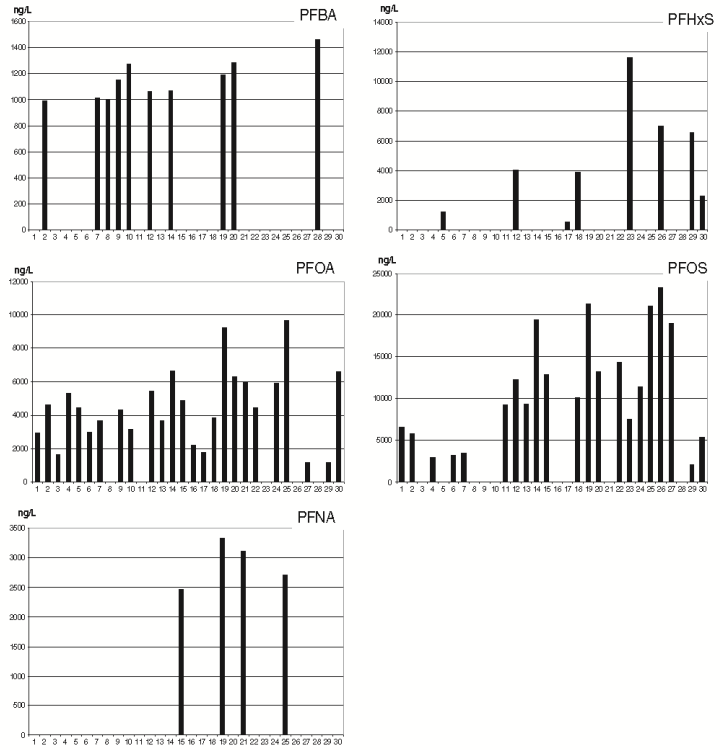

Fig. 3. Heraklion (Greece) cord blood sample results for the analysis of PFASs. Results of the analytes at concentrations over the MLOQ, expressed in ng/L.

from Greece, while only the 53% of the samples from Barcelona contained PFOA (Fig. 3).

In spite that the number of analysed samples was too low to obtain any conclusive results, comparing the median concentrations of the values of PFASs higher than MLOQ in cord blood the samples from Barcelona presented slight high concentrations, whereas a higher number of samples from Greece presented at least one of studied compounds. The results obtained here highlight the need to carry out a more extensive study to establish if there are significant differences between both cities, and in the positive case to establish causes of the difference, such as, diet or lifestyle. On the other hand, in the present study, no correlation was found between the concentrations of PFASs measured in the samples and other characteristics as the new born sex, mother habits (as diet or smoking) or mother weight among others, when different statistical methods were applied, such as, the one-way analysis of variance (ANOVA) with a 95% of confidence interval, and the principal components analysis (PCA). However, the main limitation in this case was the low number of samples. In future works, in order to study possible correlation between PFASs and ambient parameters, mother habits or age, the number of cord blood samples have to be much increased and should be included the analysis mother's blood.

When AGD was divided into "lowest" (36.58 ± 4.68 mm) and "highest" (48.58 ± 4.33 mm) categories—with 15 newborns in each one, the lowest AGD group had higher levels of PFOS (2464.3 ± 1579.2 μg/L versus 1651.4 ± 1300 μg/L in the highest AGD group, $p = 0.07$). An opposite pattern was observed for PFOA in the lowest (117.5 ± 49.5 μg/L) versus the highest AGD group (1194.3 ± 4019.3 μg/L, p ns).

These preliminary results suggest that PFOS/PFOA levels could be differently distributed depending on the anogenital distance. To confirm our hypothesis of PFOS/PFOA as an etiological factor for reduced anogenital distance it would be necessary to perform a further analysis, taking into account potential confounders and involving a larger sample. As these emergent contaminants are usually found in food, it would be very useful to explore its levels relationship with the food frequency questionnaire information.

4. Conclusions

A new method based on TFC-LC–MS/MS was developed for the analysis of 18 PFASs in cord blood. Sample pretreatment consisted in a rapid protein precipitation by dilution with acetonitrile followed by 10 minutes of centrifugation. The results of the in-house validation showed good linearity ranges, for most of the compounds between 0.010 and 250 μg/L ($R^2 > 0.9900$), with exception of the shorter alkyl-chain compounds (PFBA and PFPeA) and longer alkyl-chain compounds with more than 12 carbons (PFTrA, PFHxDA and PFODA), which had shorter linearity range. In these cases, the linearity ranges were shorter. The percentage of recoveries calculated at different spiking levels (5, 10 and 50 μg/L) was between 50 and 126% for perfluoroalkyl acids, 58 and 125% for perfluoroalkyl sulfonates and between 77 and 112% for FOSA. The MLODs were between 0.003 and 0.76 μg/L. The CCα and CCβ values were established between 0.005

and 1 μg/L for CCα, and between 0.006 and 1.3 μg/L for CCβ, being at similar levels to the MLOQ.

In order to assess the applicability of the method, 60 cord blood samples from donors in Barcelona and Heraklion were analysed. The results concluded that PFHxS and PFOS were the PFASs at highest concentration levels. The last two perfluoroalkyl sulfonates and PFOA were detected as the most ubiquitous compounds. In general, the levels of PFOA and PFOS were at highest levels in Greek samples than in Barcelona samples. In contrast, the reported values of PFHxS were higher in Barcelona samples than in Greek ones. In spite just a few numbers of samples showing the presence of shorter chain compounds such as PFBA and PFPeA, these results indicate the need to continue the investigation of shorter chain compounds and their bioaccumulation.

Finally, in this preliminary study no correlation was found between the profile of compounds in the samples and other characteristics of the donors, such as, mother habits or mother weight. However, the number of analysed samples was insufficient in order to obtain conclusive statistical values and wide studies should be carried out in order to elucidate bioaccumulation of PFASs, with special attention to new and shorter alkyl-chain compounds, and their behaviour and effects in human health.

Preliminary results suggest that PFOS/PFOA levels could be differently distributed depending on the anogenital distance and underpinning the necessity to perform epidemiologic studies to better assess the potential reproductive toxicity of PFASs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.05.080.

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Supporting Information

Analysis of perfluoroalkyl substances in cord blood by turbulent flow chromatography coupled to tandem mass spectrometry

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Table S1: MS parameters

Analyte	m/z		Relative Intensity	CE (V)
	Parent	Daughter		
PFBA	213	169	100	-10
		119	0.1	-8
PFPeA	263	69	100	-15
		219	0.02	-8
PFHxA	313	269	100	-10
		169	0.02	-10
PFHpA	363	319	100	-10
		169	13	-15
PFOA	412.79	369	100	-15
		169	32	-15
PFNA	462.93	419	100	-10
		219	19	-15
PFDA	512.75	469	100	-14
		169	10	-17
PFUdA	563	519	100	-15
		169	6	-15
PFDoA	612.67	569	100	-15
		169	4	-15
PFTeDA	712.51	669	100	-15
		169	6	-15
PFHxDA	812.51	769	100	-15
		169	5	-15
PFODA	912.77	869	100	-15
		169	7	-15
PFBS	298.82	99	52	-43
		80	100	-27
PFHxS	398.72	99	58	-50
		80	100	-50
PFOS	498.72	99	92	-41
		80	100	-49
PFDS	598.7	99	81	-42
		80	100	-54
PFOSA	498	78	100	-40
		478	10	-18

Capillary temperature : 270 ºC; Vaporizer temperatura: 300 ºC; Aux Gas Pressure (Arb): 15; Sheath Gas (Arb): 30; Ion Sweep Gas Pressure (Arb): 0.5; Spray Voltage: 2000 V; Declustering Voltage: 5 V; Q1 Peak Width (FWHM): 0.7; Scan time: 0.02 s; MS Acquire time: 16 min.

Table S2:Concentrations of PFASs in cord blood samples (Barcelona)

	Concentration (%RSD); n=3 (ng/L)																	
	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	PFOSA
1	813 (20)	< MLOD	< MLOD	516 (17)	553 (24)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	26749 (7)	7696 (1)	< MLOD	< MLOQ
2	< MLOQ	< MLOD	< MLOD	< MLOD	2019 (9)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	51141 (9)	11212 (2)	< MLOQ	< MLOQ
3	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	18099 (19)	< MLOQ	< MLOQ	44825 (12)	2253 (5)	< MLOD	< MLOD
4	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	39418 (11)	< MLOQ	< MLOD	< MLOQ
5	< MLOQ	< MLOQ	< MLOD	< MLOQ	511 (19)	< MLOQ	583 (14)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	48790 (7)	1483 (25)	< MLOQ	< MLOQ
6	< MLOQ	< MLOD	16538 (15)	< MLOQ	545 (25)	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	25681 (12)	2616 (14)	< MLOQ	< MLOQ
7	182 (7)	< MLOD	< MLOD	< MLOQ	< MLOQ	3679 (16)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	82205 (14)	< MLOQ	< MLOD	< MLOQ
8	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	34292 (17)	279 (16)	< MLOD	< MLOQ
9	< MLOQ	< MLOD	< MLOD	< MLOD	473 (11)	< MLOQ	532 (26)	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	63636 (11)	< MLOQ	< MLOD	< MLOQ
10	11791 (14)	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	4190 (26)	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	46158 (10)	2310 (5)	< MLOQ	< MLOQ
11	< MLOQ	< MLOD	< MLOD	< MLOD	547 (25)	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	42475 (9)	8329 (5)	< MLOQ	< MLOQ
12	833 (24)	< MLOD	< MLOD	< MLOD	4344 (15)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	39333 (19)	7999 (11)	< MLOD	< MLOQ
13	< MLOQ	< MLOD	< MLOD	< MLOD	1573 (9)	< MLOQ	< MLOQ	1126 (8)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	30299 (15)	6499 (10)	< MLOD	< MLOQ
14	< MLOQ	< MLOD	13086 (20)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	59529 (7)	1595 (21)	< MLOQ	< MLOQ
15	< MLOQ	< MLOD	< MLOD	< MLOQ	525 (18)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	19036 (17)	< MLOQ	< MLOQ	56535 (8)	518 (16)	< MLOD	< MLOD
16	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	1651 (11)	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	26114 (3)	1773 (7)	< MLOD	< MLOQ
17	< MLOQ	< MLOD	< MLOD	< MLOQ	1275 (14)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	38264 (3)	4723 (8)	< MLOD	< MLOD
18	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	37346 (10)	3889 (6)	< MLOD	< MLOQ
19	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	44246 (6)	4095 (5)	< MLOQ	< MLOD
20	< MLOQ	< MLOD	< MLOD	< MLOD	1247 (23)	< MLOQ	856 (23)	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	57892 (9)	1875 (10)	< MLOQ	< MLOQ
21	568 (25)	< MLOD	< MLOD	< MLOD	< MLOQ	2006 (15)	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	66725 (11)	< MLOQ	< MLOQ	< MLOQ
22	611 (25)	< MLOD	< MLOD	< MLOQ	2549 (18)	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	18921 (23)	< MLOQ	< MLOQ	42580 (7)	3138 (15)	< MLOQ	< MLOD
23	< MLOQ	< MLOD	< MLOD	< MLOD	1904 (17)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	35745 (8)	3900 (3)	< MLOD	< MLOD
24	< MLOQ	< MLOD	< MLOD	< MLOQ	1814 (12)	< MLOQ	< MLOD	< MLOQ	970 (18)	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	51513 (3)	5631 (10)	< MLOD	< MLOD
25	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	76209 (12)	10917 (13)	< MLOD	< MLOD
26	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	26294 (14)	2881 (25)	< MLOD	< MLOQ
27	< MLOQ	< MLOD	< MLOD	< MLOQ	1358 (25)	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	83132 (8)	7724 (15)	< MLOQ	< MLOD
28	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	42591 (4)	5641 (17)	< MLOD	< MLOQ
29	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	2887 (25)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	64687 (11)	1834 (9)	< MLOD	< MLOD
30	< MLOQ	< MLOD	< MLOD	< MLOD	2165 (18)	< MLOQ	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	33169 (5)	3789 (3)	< MLOQ	< MLOD
Range	MLOQ - 11791	-	MLOD - 16538	MLOD - 516	MLOQ - 4344	MLOQ - 3679	MLOQ - 856	MLOD - 1661	MLOD - 4190	-	-	MLOD - 19036	-	-	26114 - 82205	MLOQ - 11212	-	-
Geometric mean	2466	< MLOQ	14862	516	1488	2842	657	1394	2582	< MLOQ	< MLOQ	18685	< MLOQ	< MLOQ	47262	4408	< MLOQ	< MLOQ
SD	4574		2512		1040	1183	174	379	1520			511			16219	3047		
Median*	712	< MLOQ	14862	516	1275	2842	583	1394	2887	< MLOQ	< MLOQ	18921	< MLOQ	< MLOQ	44246	3889	< MLOQ	< MLOQ

*Median of values higher than MLOQ

Table S3: Concentrations of PFASs in cord blood samples (Greece)

	Concentration (%RSD); n=3 (ng/L)																	
	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFTrA	PFTeA	PFHxDA	PFODA	PFBS	PFHxS	PFOS	PFDS	FOSA
1	< MLOD	< MLOQ	< MLOQ	< MLOD	2965 (24)	<MLOD	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	6545 (14)	< MLOD	< MLOQ
2	990 (30)	< MLOQ	< MLOQ	< MLOQ	4635 (29)	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	5785 (26)	< MLOQ	< MLOQ
3	< MLOD	< MLOQ	< MLOD	< MLOD	1650 (21)	<MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ
4	< MLOD	< MLOQ	< MLOQ	< MLOQ	5285 (14)	<MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	2905 (19)	< MLOQ	< MLOQ
5	< MLOQ	< MLOQ	< MLOQ	< MLOQ	4435 (26)	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	1255 (10)	< MLOQ	< MLOQ	< MLOQ
6	< MLOQ	< MLOQ	< MLOQ	< MLOD	2975 (1)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	3185 (31)	< MLOD	< MLOQ
7	1015 (2)	< MLOQ	< MLOD	< MLOD	3690 (1)	<MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	3500 (27)	< MLOQ	< MLOQ
8	1090 (4)	< MLOQ	< MLOD	< MLOD	< MLOQ	<MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ
9	1148 (24)	< MLOQ	< MLOQ	< MLOD	4290 (10)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ
10	1271 (30)	< MLOQ	< MLOQ	< MLOQ	3155 (24)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ
11	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	9150 (17)	< MLOQ	< MLOQ
12	1063 (24)	< MLOQ	< MLOQ	< MLOD	5420 (14)	<MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	4070 (21)	12280 (23)	< MLOQ	< MLOQ
13	< MLOD	< MLOQ	< MLOQ	< MLOD	3665 (20)	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOQ	9250 (1)	< MLOQ	< MLOQ
14	1069 (29)	< MLOQ	< MLOD	< MLOQ	6625 (30)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	19350 (29)	< MLOD	< MLOQ
15	< MLOD	< MLOQ	< MLOQ	< MLOD	4875 (6)	2465 (30)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	12820 (21)	< MLOD	< MLOQ
16	< MLOQ	< MLOQ	< MLOD	< MLOD	2205 (23)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ
17	< MLOQ	< MLOQ	< MLOD	< MLOQ	1770 (10)	<MLOQ	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	566 (27)	< MLOQ	< MLOD	< MLOQ
18	< MLOQ	< MLOQ	< MLOQ	< MLOQ	3840 (4)	<MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	3875 (25)	10075 (20)	< MLOD	< MLOQ
19	1187 (33)	< MLOQ	< MLOD	< MLOQ	9195 (20)	3330 (27)	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	21200 (25)	< MLOQ	< MLOQ
20	1284 (23)	< MLOQ	< MLOD	< MLOD	6295 (30)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	13180 (30)	< MLOQ	< MLOQ
21	< MLOD	< MLOQ	< MLOQ	< MLOQ	5980 (28)	3105 (30)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ
22	< MLOD	< MLOQ	< MLOQ	< MLOD	4450 (31)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	14300 (10)	< MLOD	< MLOQ
23	< MLOD	< MLOQ	< MLOD	< MLOQ	< MLOQ	<MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	11600 (12)	7500 (22)	< MLOQ	< MLOQ
24	< MLOD	< MLOQ	< MLOD	< MLOD	5920 (6)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	11410 (25)	< MLOD	< MLOD
25	< MLOD	< MLOQ	< MLOQ	< MLOQ	9645 (17)	2705 (9)	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	21000 (29)	< MLOQ	< MLOQ
26	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOQ	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	6980 (1)	23350 (26)	< MLOD	< MLOQ
27	< MLOD	< MLOQ	< MLOQ	< MLOQ	1176 (25)	<MLOQ	< MLOD	< MLOD	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	18950 (8)	< MLOD	< MLOD
28	1460 (25)	< MLOQ	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	< MLOQ	< MLOQ	< MLOD	< MLOD
29	< MLOD	< MLOQ	< MLOQ	< MLOD	1170 (2)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOQ	6585 (24)	2050 (21)	< MLOD	< MLOQ
30	< MLOD	< MLOQ	< MLOQ	< MLOD	6585 (24)	<MLOQ	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	2305 (3)	5355 (10)	< MLOD	< MLOQ
Range	MLOD - 1460	-	-	-	MLOQ - 9195	MLOQ - 3330	-	-	-	-	-	-	-	-	MLOQ - 11600	MLOQ - 23350	-	-
Geometric mean	1051	< MLOQ	< MLOQ	< MLOQ	4508	2901	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	4654	11106	< MLOQ	< MLOQ
SD	355				2136	389									3628	6598		
Median*	1069	< MLOQ	< MLOQ	< MLOQ	4450	2905	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	< MLOQ	4070	10743	< MLOQ	< MLOQ

*Median of values higher than MLOQ

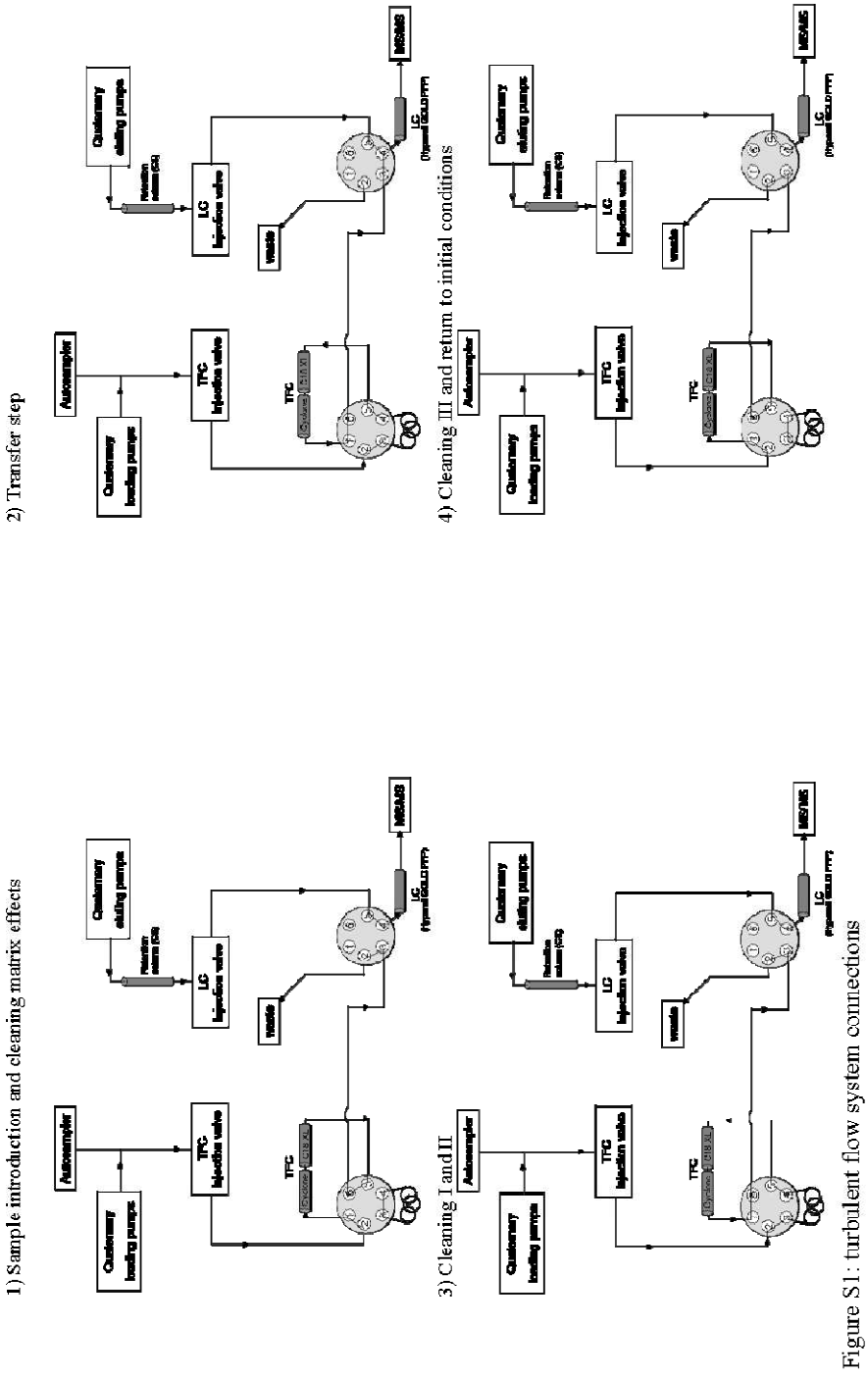


Figure S1: turbulent flow system connections

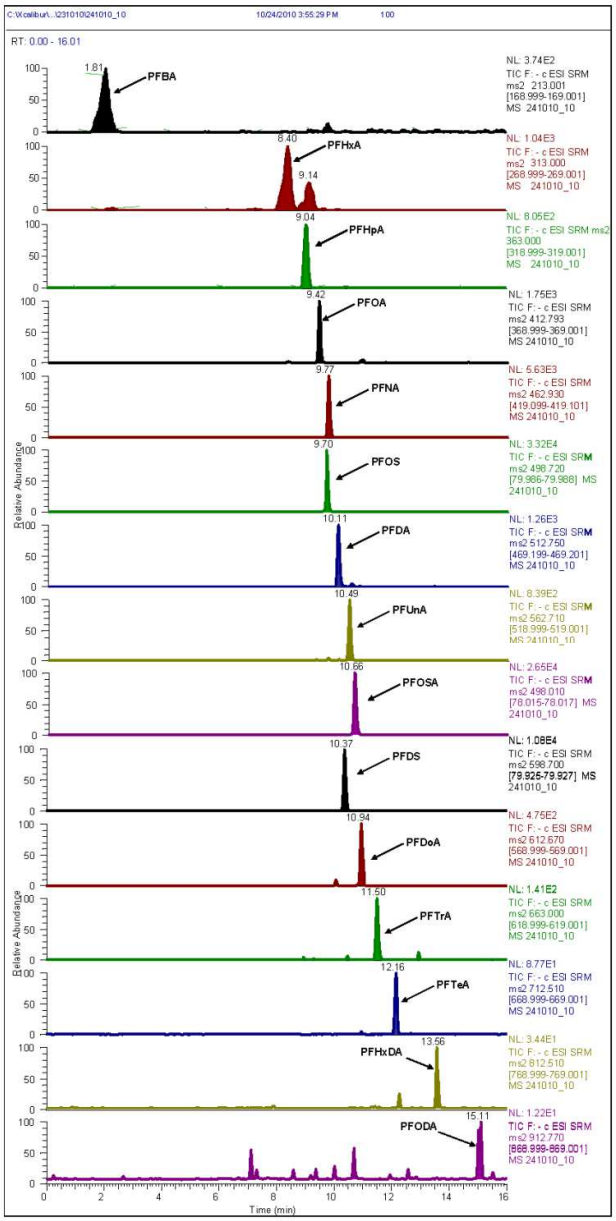


Figure S2: Example of extracted ion chromatogram of a fortified blank at 100 µg/L of lamb blood

I) II)

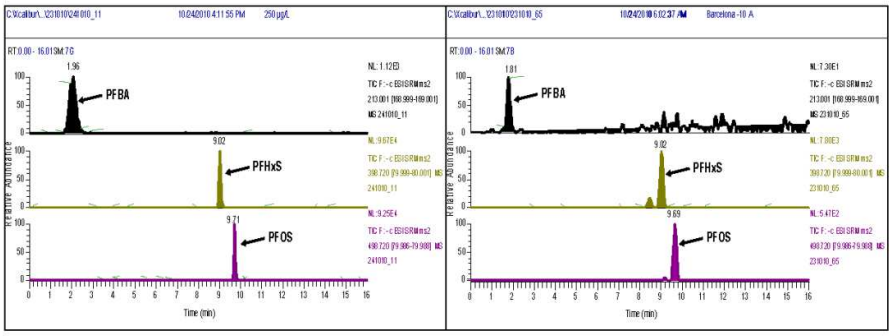
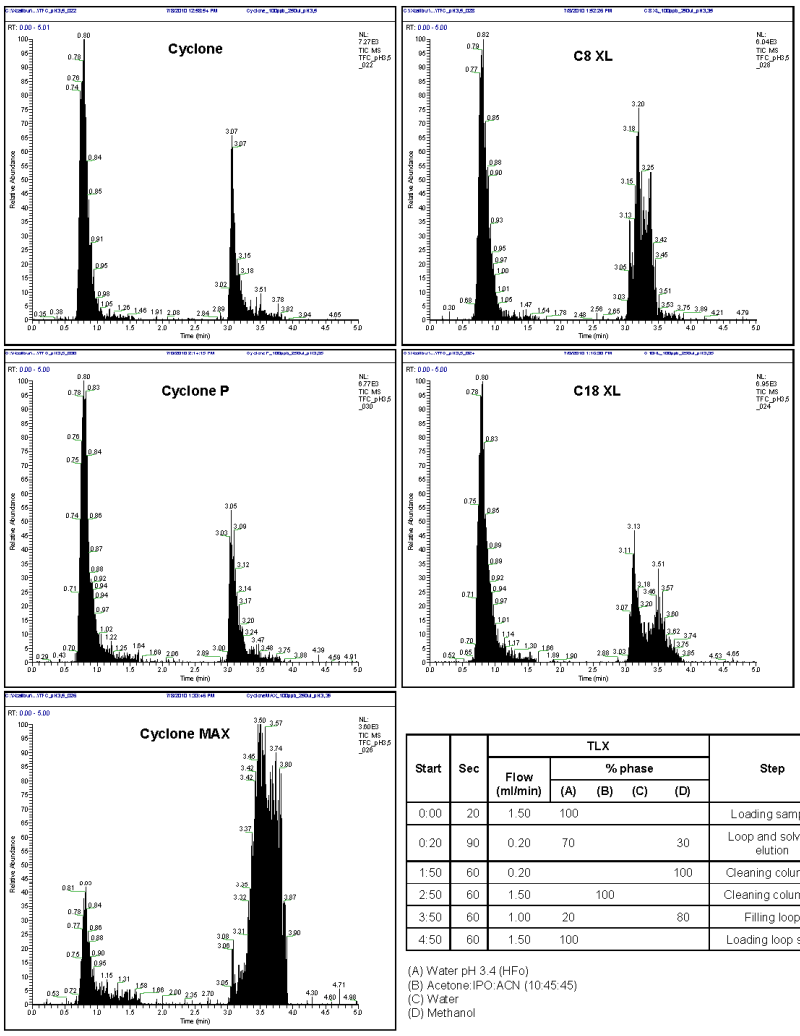


Figure S3: Example of extracted ion chromatogram for spiked lamb blood at 250 µg/L (I) compared with a real sample from Barcelona donor (sample 10) with PFBA, PFHxS and PFOS at quantifiable concentrations (II).



Start	Sec	TLX					Step
		Flow (ml/min)	% phase				
			(A)	(B)	(C)	(D)	
0:00	20	1.50	100				Loading sample
0:20	90	0.20	70			30	Loop and solvent elution
1:50	60	0.20				100	Cleaning column I
2:50	60	1.50		100			Cleaning column II
3:50	60	1.00	20			80	Filling loop
4:50	60	1.50	100				Loading loop step

(A) Water pH 3.4 (HFo)
(B) Acetone:IPO:ACN (10:45:45)
(C) Water
(D) Methanol

Figure S4: Total ion chromatograms of 100 µg/L standard mixture at pH ~ 3.4 during TLX test with different columns.

4.3 Discussion

4.3.1 Analytical method optimisation

In the case of *on-line* extraction, the method optimisation involves different steps. The first one deals with the cross contamination from the system. The other two steps comprise the selection of the optimum extraction columns, the optimum solvents and the optimum time events and flows.

In order to decrease possible sources of contamination from the chromatographic system, the first step was to assess the contamination from the turbulent flow chromatograph coupled to MS. The results of this evaluation have indicated the need to reduce contaminant parts, and it was required to change all the PTFE tubing for polyether ether ketone (PEEK) connections. In order to assure the no-overestimation of PFASs in the samples, due to cross-contamination from solvent or some seals or pumps in the system, an extra chromatographic column (BDS Hypersil C8 (50 x 3) (Thermo Scientific)) was installed after the LC eluting pumps, in order to delay any possible contamination from the degasser pumps, which are known to contain PVDF valves. A detailed scheme is presented in Figure 4.1.

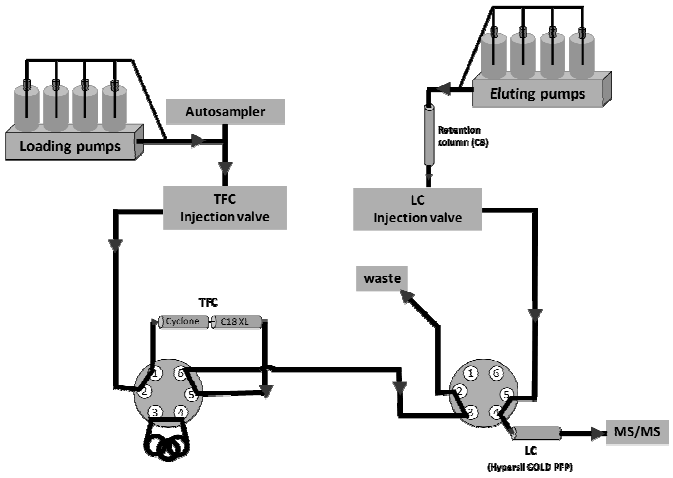


Figure 4.1: Thermo Scientific Aria TLX-1 system configuration in loading mode

The *on-line* turbulent flow chromatography is based on three consequential steps:

1) Sample loading, which is performed at turbulent flows. In this step, the sample is loaded into the affinity to the stationary phase, the analytes are retained and interferences eliminated to waste. In this step the loading time, the flow and if necessary, a cleaning step should all be optimised. In this part were tested different turbulent flow columns, with different stationary phases, in combination with different loading solvents (water at different pH) and different loading times. The maximum retention was obtained with an anionic exchange column and cyclone for acids and C18XL for sulphonates, with water at pH of 3.4 and loading flow at 1.5 mL/min during 20 sec.

2) Transfer step, the valve switches the position and the analytes are eluted and transferred from the extraction column to the chromatographic column by the solvents that are in the loop, in this case. In this step we require the maximum affinity of the analytes with the elution solvent and the minimum with the stationary phase from turbulent flow columns. In this part, optimisation involves the elution solvent which can be a mixture depending on the requirements, as well as the transfer time, the loop volume and, if it is necessary, an extra elution solvent. In the case of PFASs selected in this study, the optimum elution volume was 250 µL loop filled with water (pH 3.4): methanol [2:8], with an extra elution solvent with water (pH 3.4) : methanol [7:3], flowing during 2 min at 0.2 mL/min. Optimum results were obtained using two extraction columns in tandem (cyclone and C18XL), because the anionic exchange column retained the analytes with high efficiency and it was not possible to elute them.

3) Washing and equilibration to initial conditions. In this step, the chromatographic separation is performed in parallel, because the connection valve has changed to initial conditions again. The washing process is important, in order to rule out any possible memory effect in the turbulent flow extraction columns. In the case of this study, the cleaning solvents were methanol, a mixture of acetonitrile: isopropanol: acetone. Then, the system was equilibrated into initial conditions.

After validation in water, the extraction methodology was transferred to blood samples. The tests were made in blank lamb blood, obtained from the slaughterhouse. In order to obtain the maximum efficiency during extraction step, it was necessary to precipitate the proteins from the blood with acetonitrile (1:1) and obtain the serum before turbulent flow extraction. Another important change was the addition of a cleaning step with water at 0.2 mL/min for 20 sec. after sample loading, because, although serum was cleaner than whole blood, it was still dirtier than water.

When the developed methodology was transferred from water to blood samples, a problem with the quantification and confirmation of sulphonates was found. Also observed was a co-elution of PFA sulphonates with endogenous steroid sulphates, such as taurodeoxycholic bile acid (TDCA) that can cause a 10-20 fold over-reporting for these compounds. This fact was also reported by Benskin *et al.* (2007). It was decided to use a Hypersil GOLD PFP (pentafluorophenyl) column, offering an alternative selectivity between fluorinated isomers, in order to rule out the possible co-elution. On the other

hand, the quantification transition for PFA sulfonates was the X99 > 99, because the TDCA bile acid has a similar transition at 499 > 80 (in negative ionisation mode).

4.3.2 Cord blood results

A total number of sixty cord blood samples were analysed, thirty from newborns of the Hospital del Mar mother-child cohort in Barcelona city (Catalonia, Spain) and thirty form RHEA mother-child cohort at Heraklion city (Crete, Greece). The analysis reported the presence of PFASs in cord blood although most of studied analytes were below MLOQ or, in some cases, below MLOD. Perfluoroalkyl acids were, in general, at lower concentration levels than perfluoroalkyl sulphonates with the highest values for PFHxS and PFOS. The distribution of PFASs in these samples has been similar to other reported works (Figure 4.2). The concentrations for PFOS and PFOA were lower than for the general population from other countries (black line in Figure 4.2). However, compared with previous works focused on cord blood samples, the concentration levels for this study were higher, indicating the increasing of PFASs in humans, especially in maternal blood once it was ruled out any possible source of contamination during sampling, sample pre-treatment and analysis.

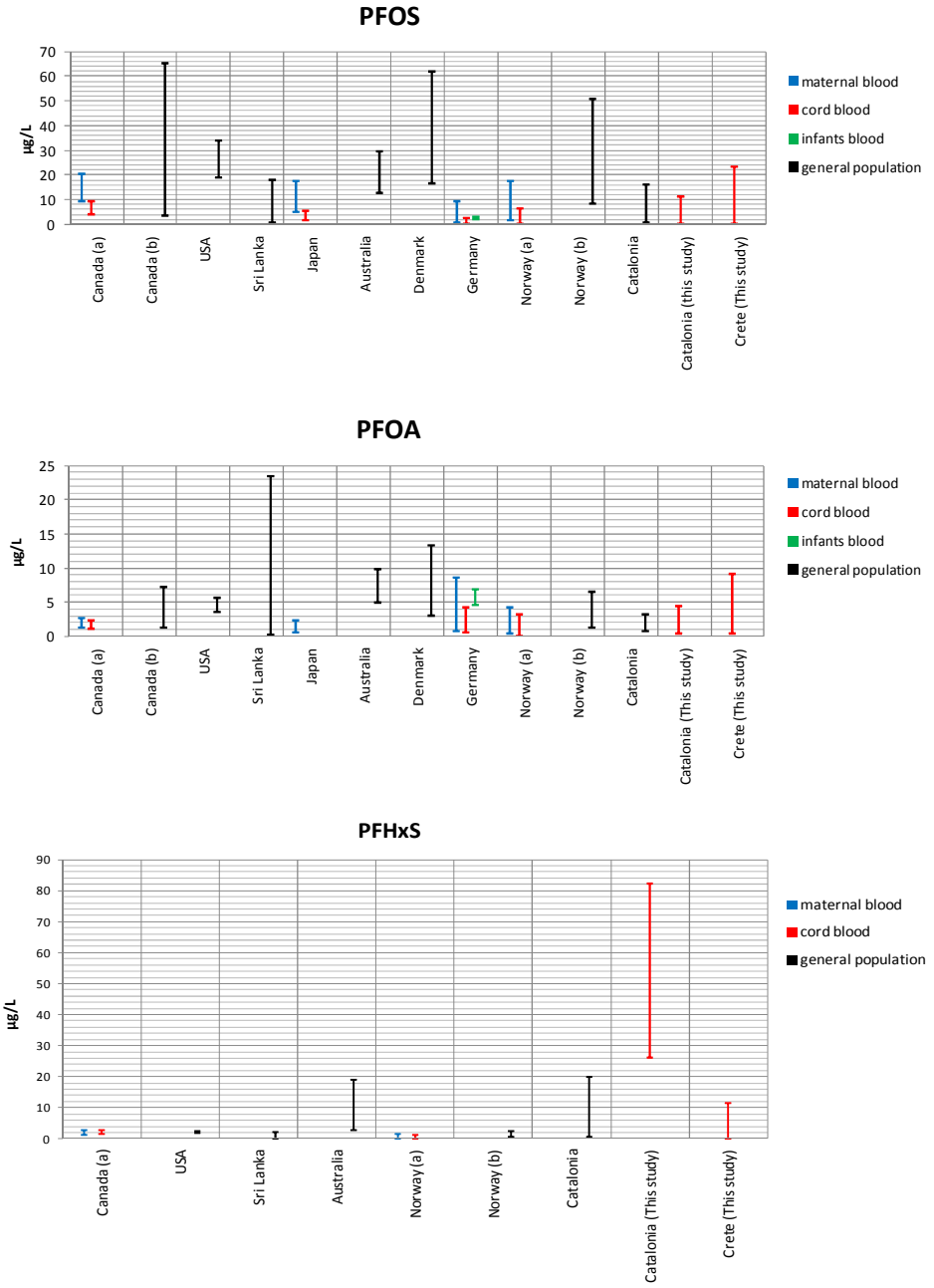


Figure 4.2: Concentration range of PFASs in previous reported works for maternal blood (pregnant at delivery time), cord blood, infant blood and general population blood. The studies from the other countries correspond to: Canada (a) from (Monroy *et al*. 2008), Canada (b) from (Kubwabo *et al*. 2004), USA from (Calafat *et* al. 2007), Sri Lanka from (Guruge *et al.* 2005), Japan from (Inoue *et al*. 2004), Australia from (Kärrman *et al.* 2006), Denmark from (Eriksen *et al.* 2011), Germany from (Fromme *et al*. 2010), Norway (a) from (Gützkow *et al*. 2011), Norway (b) from (Haug *et al*. 2009), Catalonia from (Ericson *et al*. 2007) and Catalonia and Crete from this study.

In this thesis, the highest concentrations were found for PFHxS, PFOS, PFOA and PFNA. De Silva *et al.* (2006) studied the possible isomers correlation in blood samples as well as the presence of even and odd PFASs. The authors concluded that even acids are at higher concentrations than odd acids. This hypothesis is supported by the higher levels for even PFASs in blood samples from the other reported works as well as the work reported in this section (Figure 4.2).

Another important point in this work has been that Barcelona and Heraklion samples showed similar distribution of PFASs. It rules out the hypothesis of different distribution pattern in function of the country. However, it can be observed that, in general, PFHxS was at higher levels in samples from Barcelona than from Heraklion. In contrast, PFOA and PFOS maximum levels in Greek samples were approximately twice those of Barcelona, but the average from Catalan samples was higher for these two analytes.

Despite the low number of samples, it was decided to apply different statistical tests for comparing PFASs concentrations and qualitative mother and child characteristics, such as new born sex, mother habits (diet, smoking) and weights, among others. The one-way analysis of variance (ANOVA) with a 95% of confidence interval with different qualitative characteristics concluded that no-correlation exists. The study of the results by PCAs concluded that no evidence distribution pattern can be extracted. In order to obtain more reliable results, the number of samples should be increased as well as the fact that the mother and new born questionnaire should be more comprehensive.

On the other hand, human studies have correlated shorter anogenital distance (AGD) with exposure to endocrine disruptors in the environment, because this distance is regulated by the dihydrotestosterone. Due to the toxicity effects of these analytes observed in different animals, it was studied the correlation between new born AGD and PFASs concentration levels. The preliminary results suggested that PFOS and PFOA levels could be distributed depending on the AGD in different way. However, this is only a preliminary result and it would be necessary to carry out further studies in order to confirm the hypothesis of etiological factor for reduced AGD depending on PFOS and PFOA levels.

4.3.3 Human sources and relationship with the environment

In order to find a possible relationship between the concentrations of PFASs in human blood and food and drinking water, PCA studies have been performed with XLSTAT software (Version 2008.7.03). The new graphs contained the principal components 1 and 2 (F1 and F2). Figure 4.3 shows the PCA of PFASs contributions into the human diet through the food and drinking water samples analysed during this thesis research.

In the graph can be seen the high contribution of shorter PFASs, such as PFHxS, PFBA, PFPeA, PFHxA and PFBS associated with the consumption of drinking water, fish

and bivalve. In the case of water, it could be extrapolated that the presence of the same compounds in blood is directly related to the drinkable water.

In the case of food samples analysed in this thesis research, there was not found a direct relationship between these food samples and blood concentration levels. Nevertheless, this study supports the contribution of PFASs into the human body through fish and milk consumption. However, a wide number of blood samples should be studied, considering different average ages, as well as different non-invasive human matrices such as nails, saliva or hair and combine them with the dietary habits, in order to have a better knowledge about PFASs' origin and human presence.

The humans' relationship with the environment has been studied through the presence of PFASs in WWTPs, and some environmental samples such as surface river water or biota analyzed in this thesis. Figure 4.4 shows the PCA graph for environmental and WWTP samples. The results suggest a human source, because of their industrial production, the use of different products made of PFASs or from human body wastes. This latter observation is supported by the presence of some of these compounds in human urine (Tao 2009; Perez *et al.* 2012) such as, for example, PFBA. Nonetheless, in order to have a wide spectrum of human wastes and environmental contributions, deeper studies focused on the human body should be performed.

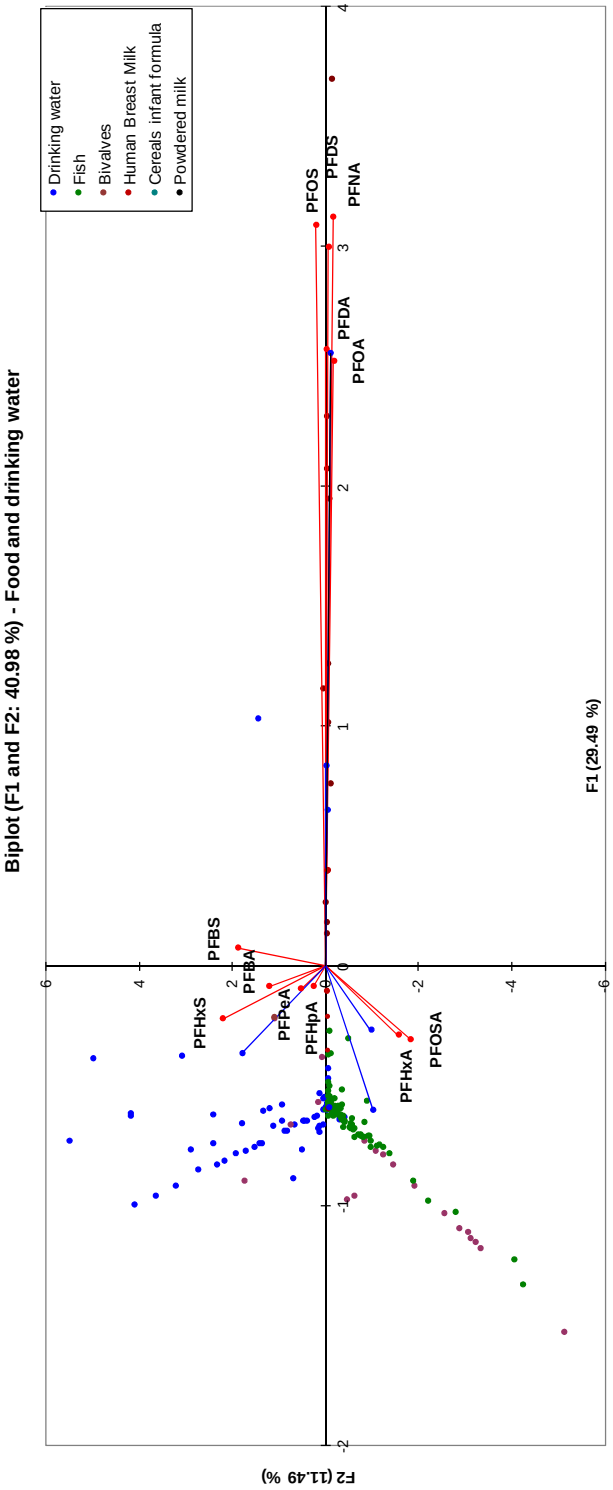


Figure 4.3: PCA graph for food and drinking water samples from all analysed matrices in this thesis, contribution of PFASs to human diet

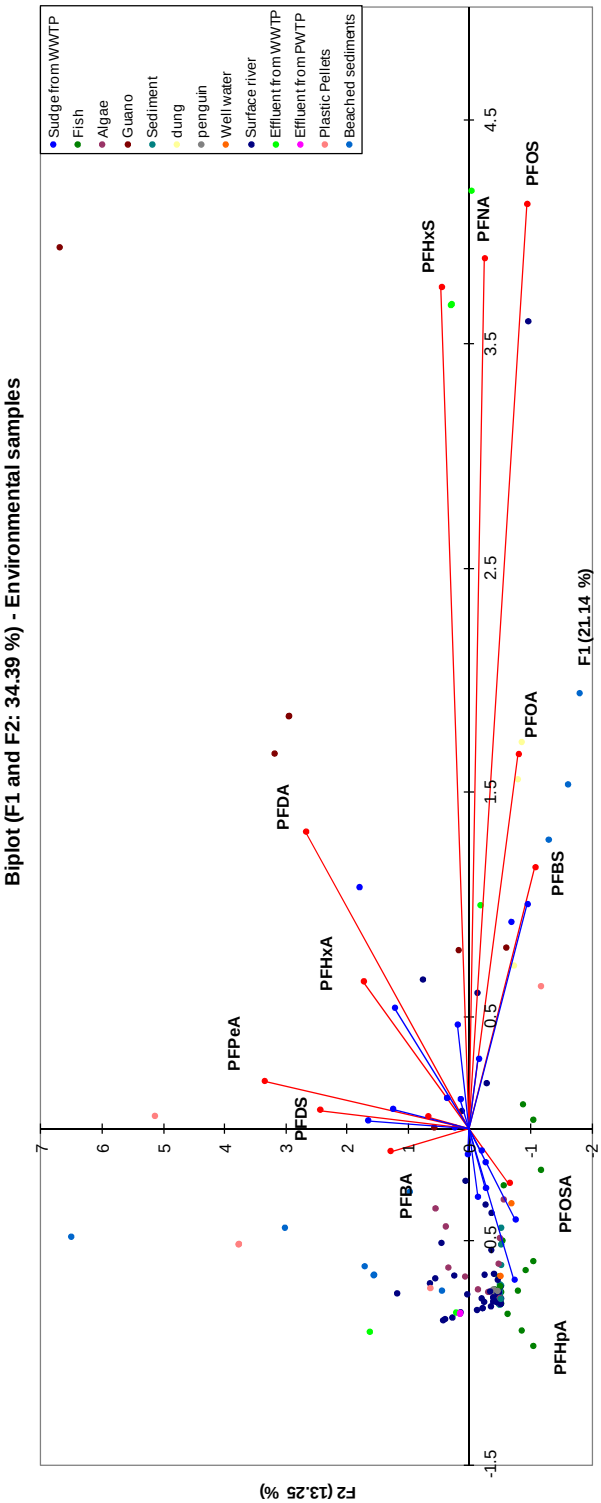


Figure 4.4: PCA graph for environmental and WWTP samples from all analysed matrices in this thesis

CHAPTER 5

Physicochemical properties and aerobic biodegradation of perfluoroalkyl phosphonic acids

5.1 Introduction

The objectives of the last study of this Thesis were to establish some physicochemical properties (partitioning constants water/n-hexane and water/octanol and adsorption behaviour to soils and sediments) and to assess the possible biodegradation under aerobic conditions in WWTPs of three perfluoroalkyl phosphonic acids (PFPAs). In this study, the selected compounds were perfluorohexaphosphonic acid (PFHxPA), perfluorooctaphosphonic acid (PFOPA) and perfluorodecaphosphonic acid (PFDPA). Part of this study was carried out in Hoschule Fresenius, Idstein, Germany, in the group of Prof. Dr. Thomas P. Knepper, from January to May 2011, as a short stage research during the PhD thesis

PFPAs are used in food contact papers as leveling and wetting agents and as defoaming additives in pesticide applications (MASONSURFACTANTS ; D'Eon *et al.* 2009b). In spite of the high number of industrial applications of perfluoroalkyl phosphonic acids as surfactants (e.g., PFOPA was listed as a high production-volume chemical in 1998 and 2002, with 4,500–230,000 kg produced annually (Howard *et al.* 2007; D'Eon *et al.* 2009b)), few works have studied their fate and behavior in the environment. As well, few works have reported their environmental occurrence. D'Eon *et al.*, (2009b) detected PFPAs in different treated wastewater effluents in Canada. In addition, PFPAs lack hydrogen atoms, suggesting they may be similarly resistant to degradation as perfluorinated carboxylic acids and perfluoro sulfonates (D'Eon *et al.* 2009). More recently, the same authors reported de accumulation of PFPAs in rat experiments, and it was also demonstrated that the mono-PFPAs may bind to blood cells underestimating their concentration in plasma and sera samples (D'Eon *et al.* 2010).

5.2 Experimental work

This section provides the experimental work performed under the frame of the physicochemical assessment and biological studies of the three perfluoroalkyl phosphonic compounds.

Chemicals and reagents

Perfluorohexylphosphonic acid (PFHxPA), perlfuorooctylphosphonic acid (PFOPA) and perfluorodecylphosphonic acid (PFDPA) [chemical purity > 98 %; methanol] supplied for Wellington Laboratories Inc. (Canada).

6-Chloroperfluorohexylphosphonic acid (ClPFHxPA) [chemical purity > 98 %; methanol] was used as a surrogate and was purchased from Wellington Laboratories Inc. (Canada).

Ultra pure Milli-Q water was prepared by a Millipore-Q-system (Millipore, Milford, MA, USA). Methanol Suprasolv, ammonium acetate salt ($AcNH_4$: MW, 77.08; >98 %), ammonia, 1-octanol (98%), n-hexane and acetonitrile were obtained from Merck (Darmstadt, Germany).

Water used for DNA analysis was supplied by Sigma. Polymerase chain reaction (PCR) *buffer*, dNTPs, Q solution, *Taq* Polimerase and $MgCl_2$ were purchased from Qiagen. The primers Eub357F and Eub907R were supplied by Roche.

Physicochemical properties

***Partitioning coefficients*.** The partitioning coefficients hexane/water and 1-octanol/water were calculated according to OECD guideline 123.

Very briefly, 10 mL of a mixture n-hexane:miliQ water (1:1) for distribution and 1-octanol:miliQ water (1:9) for partition coefficient experiments were prepared in a 15 mL PP tube with a mixture of PFPAs at 100 µg/L of final concentration. The mixture was prepared in 5 mL of n-hexane for distribution experiments and in 1 mL of 1-octanol for partition experiments. Then, the corresponding water volume was added and the PP tube was mixed during 1 min, and then the mixture reached the equilibrium in an orbital digester at 100 rpm. In order to follow the process, an aliquot of 0.100 mL of hexane and an aliquot of 0.100 mL of water were taken at different times: 0, 1, 2, 24, 48 and 96 hours. All the experiments were carried out in triplicate and, in parallel, triplicate blanks were used in order to rule out any possible source of contamination.

0.100 mL of water aliquot was diluted with 0.050 mL of methanol containing surrogate-internal standard (IS) at 30 µg/L in order to obtain initial conditions of mobile phase (water:methanol, 7:3) and IS at 9 µg/L.

0.100 mL of hexane aliquot was dried under N_2 (g) and reconstituted in a 0.100 mL of water. Then, 0.050 mL of methanol at 300 µg/L of IS was added in order to obtain a final concentration of 9 µg/L of Cl-PFHxPA.

Octanol phase was not analyzed.

***Adsorption experiments in sludge*.** The experiments were carried out according to the procedure described by the OECD guideline 106, by the indirect method.

These experiments were performed using two types of sewage sludge from Beuerbach WWTP (Hesse, Germany): 1) activated sludge and 2) final sludge.

Very briefly, the procedure was as follows: the samples were dried under ambient conditions and homogenized. Approximately 2 g of dried sample was introduced into a PP tube and 10 mL of milliQ water with $CaCl_2$ (0.01 M) was added, according to ISO 10390-1, in order to minimize the cation exchange. This first part of the experiment was

carried out in quadruplicates and two procedural blanks were included consisting in milliQ water $CaCl_2$ (0.01 M). The mixture was mixed 1 min and stirred overnight (~12 h) in an orbital digester at 100 rpm. The first aliquot of water (200 µL) was taken as a blank before start the spiked experiments. Then, three of the PP tubes were spiked with PFPAs at optimum concentration in order to achieve 100 µg/L (10 µL of PFPAs at 1 mg/L in methanol). The other PP tube with sludge was used as a blank. The prepared tubes were mixed 1 min and stirred in an orbital digester at 100 rpm along all experimental process. During the sampling process, 200 µL of water was taken after the following times: 0, 2.5, 4, 7.5, 24 and 48 hours.

Water aliquots were introduced into a PP tube and centrifuged at 4000 rpm, at room temperature, for 20 min. 0.140 mL of the supernatant was filtered by 0.45 µm Nylon filter and filled up with 0.06 mL of methanol with 30 µg/L of IS to obtain a final concentration of 9 µg/L for Cl-PFHxPA.

***Algae adsorption experiments*.** *Desmodesmus subspicatus* (pond scum, green weed) was used in algae adsorption experiments in order to evaluate the bioavailability of the organism to PFAPs. The algae medium was constituted with different nutrients ($NaHCO_3$, $CaCl_2$, NH_4Cl, $MgSO_4$, $MgCl_2$ and KH_2PO_4 at mg/L levels) and trace elements (Na_2EDTA, $FeCl_2$, $MnCl_2$, H_3BO_3, Na_2MoO_4, $ZnCl_2$, $CoCl_2$, $CuCl_2$) at pH of 8. For this experiment, the algae culture was spiked with 6.75, 12.5, 25, 50 and 100 µg/L of the corresponding PFPA (in algae medium). The experiments were carried out in quartz glass bottles. The bottles were exposed to natural light and stirred in an orbital digester at 100 rpm along all the experiment time. Aliquots of 500 µL were taken after 0, 0.5, 1, 5, 24, 144 and 288 hours of exposure. In order to control any possible adsorption of the analytes to the glass bottles, another glass bottle containing just the algae medium was spiked with PFPAs at 100 µg/L. Two blank experiments containing: a) the algae medium and b) algae, were carried out in parallel in order to avoid any possible cross contamination during experiments or analysis. The pH of the samples was continuously controlled.

All the aliquots were immediately filtered through 0.45 µm cellulose filter. Then, 0.28 mL of the filtered aliquot was transferred into a PP vial and filled up with 0.12 mL of methanol at 30 µg/L of Cl-PFHxPA. The rest of the aliquot was diluted with methanol in order to stop any possible biodegradation and stored at -4 ºC in a PP tube.

Aerobic degradation experiments. Degradation experiments were carried out in order to assess the biodegradation under aerobic conditions in WWTPs. The experiments were performed according to OECD guideline 309: Aerobic Mineralization in Surface Water – Simulation Biodegradation Test.

For experiment purposes, wastewater effluent was taken from Beuerbach WWTP (Hesse, Germany). The wastewater effluent was distributed in seven amber glass bottles (250 mL). 5 bottles were spiked with a mixture of PFPAs to obtain a final concentration of 500 µg/L (50 mg/L of PFPAs mixture in methanol was evaporated under N_2(g) conditions and reconstituted in the same volume of water; 2.5 mL of water mixture at 50 mg/L was

used for spiking experiments). Three of the bottles were used to follow the biotransformation processes. The other two spiked bottles were mixed with 3.75 g of NaN_3 (15% of NaN_3 in the bottles) in order to stop the biological activity and to assess any other possible physicochemical processes such as hydrolysis or glass adsorption. In addition, two non spiked wastewater effluents were used for blank purposes.

Along 3 months, the bottles were stirred 24 h/day in an orbital digester at 100 rpm and the pH was controlled. Dark conditions were used in order to minimize the algae growth. Aerobic conditions were maintained by aeration 30 min/day.

Every two days 0.5 mL was sampled from each bottle during the first week and once per week during the rest of the experiment. The samples were filtered through 0.45 µm cellulose filter. Then, 0.28 mL of the aliquot was transferred into a PP vial and filled up with 0.12 mL of methanol at 30 µg/L of ClPFHxPA. The rest of the aliquot was diluted with methanol (1:1) and stored at -4ºC.

In order to adapt the organisms to phosphonic degradation, one more experiment was carried out in parallel. The blank effluent was spiked with 10 µg/L of triphenylphosphinoxid (TPPO). The experiment was carried out according to an earlier work by Knepper *et al.* (2006) to adapt the organisms from the WWTP effluent to the phosphonic degradation. The authors found that in a biologically active fixed-bed bioreactor (FBBR) wastewater organisms were able to degrade TPPO in 21 days.

Instrumental analysis

Degradation and algae experiments were analyzed by LC-QqLIT working in electrospray negative mode. The chromatographic separation was achieved using a Synergi 4u Fusion-RP 80A (50 x 2.0 mm) column in a Perkin Elmer Series 200 HPLC chromatograph (Norwalk, CT, USA). The chromatograph was coupled to a QqLIT mass spectrometer Q Trap 3200 (Applied Biosystems, Foster City, CA, USA) using a Turbo Ionspray interface. Acquisition was performed using the selected reaction monitoring mode (SRM) mode with a dwell time of 200 msec. Other mass spectrometer parameters were (arbitrary units): entrance potential at -11 V, curtain gas at 25, collision gas 6, ion spray voltage at -4500 V, temperature at 450 ºC, ion source gas 1 at 55 and ion source gas 2 at 65, collision cell entrance potential at -20 V and cell exit potential at -10 V. The study of the possible degradation products was performed using the Scan mode in the first quadrupole working in negative and positive ionization conditions. The injection volume was set at 5 µL for the SRM mode and 30 µL for the Scan mode, and the flow rate was kept at 300 µL/min.

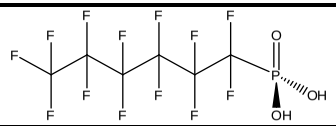
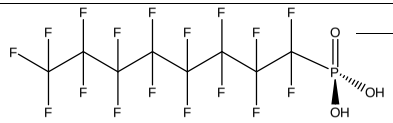
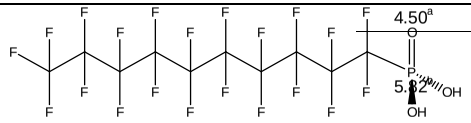
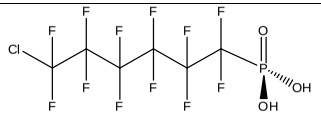
On the other hand, the analysis of the adsorption experiments was carried out by LC-QqQ/MS. The separation was achieved using a MZ-Aqua Perfect C18 (5µm, 50 x 2.1 mm) column in a HP HPLC chromatograph (Norwalk, CT, USA). The chromatograph was

coupled to a QqQ mass spectrometer API 2000 (Applied Biosystems, Foster City, CA, USA) using a Turbo Ionspray interface working in electrospray negative mode. The acquisition of the samples was done by SRM with a dwell time of 150 msec. The other mass spectrometer parameters were (arbitrary units): entrance potential at -10 V, curtain gas at 25, collision gas at 6, ion spray voltage at -4500 V, temperature at 450 ºC, ion source gas 1 at 55 and ion source gas 2 at 65, collision cell entrance potential at -30 V and cell exit potential at -10 V. The injection volume was set at 5 µL and the flow rate kept 400 µL/min.

In both cases, mobile phases consisted in (A) water:MeOH (95:5) 5 mM $AcNH_4$ and (B) water:MeOH (10:90) 5 mM $AcNH_4$ with the gradient as follows: starting at 70% of A and at 0.5 min decreased to 20% in 1.5 min. From 2 to 6 min the %A decreased until 10% and was maintained along 0.5 min. After this time, the %A returned to 70% in 1.5 min and was maintained for 6 more min. The total run time was 12 min.

Main mass/charge transitions, retention times and other instrumental characteristics are summarized in Table 5.1. Figure 5.1 shows an example of a chromatogram of PFPAs standards at 50 µg/L. The identification was carried out by the comparison of the retention times in the samples with the calibration curve standards and, by the comparison of the ratio between the most intensive transition and the pseudo transition with the same m/z in the sample vs. standards ratio in the calibration curve. The quantification was performed using matrix-matched standards, the plot ratio of the most intensive transition peak area is divided with the internal standard area against the concentration.

Table 5.1: Main transitions of PFPAs

	MW (amu)	Structure	Retention time (min)	Precursor Ion	Quantification transition	DP (V)	CE (V)	Identification pseudo-transition [c]	Ratio [d]
PFHxPA	400		3.33[a] 4.05[b]	399	79 ($-PO_3^-$)	-38 [a] -30	-60 [a] -107	399	1.5
PFOPA	500		3.91[a] 5.12[b]	499	79 ($-PO_3^-$)	-50 [a] -33	-79 [a] -86	499	1.2
PFDPA	600		4.50[a] 5.82[b]	599	79 ($-PO_3^-$)	-58 [a] -30	-87 [a] -77	599	1.7
Cl-PFHxPA	416		3.41[a] 4.35[b]	415	79 ($-PO_3^-$)	-44 [a] -32	-58 [a] -80	-	-

[a] LC-QqLIT
[b] LC-QqQ
[c] The DP for pseudo-transitions was set at the same voltage than for quantification transitions and the CE was set at -5 V for LC-QqLIT and at -10 V for LC-QqQ
[d] Ratio = [Quantification transition / pseudo transition]
MW : Molecular weight
DP : Declustering potential
CE : Collision energy

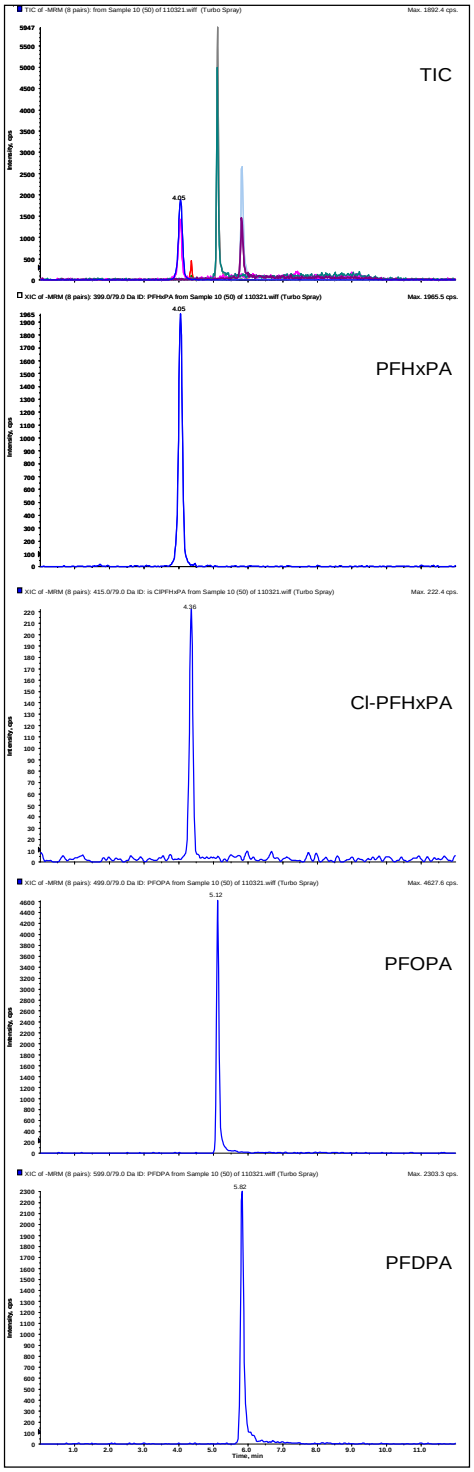


Figure 5.1: total ion chromatogram (TIC) and extracted ion chromatograms for PFPAs in standard mixture at 50 µg/L analyzed by LC-QqQ.

PCR amplification, DGGE separation and sequencing analysis

In order to identify different bacterium species present in the effluent water used for degradation experiments, the Polymerase Chain Reaction (PCR) amplification and separation of the different bacterium species was performed by denaturing gradient gel electrophoresis (DGGE). These analysis have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities (Boon *et al.* 2002). The analysis was carried out in effluent water before degradation experiments, and during these ones, at different times: 0, 15, 30, 45 and 60 days. PCR was performed for the amplification of the gene 16S from ribosomal DNA. The rDNA was extracted from 1 mL of sample by the comercial kit FAST DNA spin kit (MP Biomedicals) with a 50 µL of MiliQ water as a final extract. The extraction quality and quantity was assured using the Nanodrop spectrophotometer (ND-2000) by measuring the reflectance between 260/280 nm and 260/230 nm. The rDNA concentration was enough to precede with the PCR experiments. It was preceded, in parallel, with Agarose Gel Electrophoresis for the separation of rDNA fragments. The analytical conditions were: 0.8% of agarose concentration (MinSubCell, Bio-rad), 100V, 30 min, 4µL of sample with 1 µL of *loading buffer*. This electrophoresis was performed in order to visually assure the DNA state. The results showed similar levels of rDNA by both methods. The PCR experiments were carried out with Eub357FGC (modified with guanine and citosine queues for separation purposes) and Eub907R as primers. PCR reagents used for 1 mL of sample included: water (30 mL), buffer (3 mL), dNTPs (5 mL), Q solution (5 mL), Eub357F (10 µM ; 1 mL), Eub907R (10 µM; 1 mL), *Taq* Polimerase (0.2 mL) and $MgCl_2$ (3 mL).

The conditions were adapted from Muyzer *et al.* (1993) (Table 5.2). The rDNA amplification was controlled by Agarose Gel Electrophoresis (1.5% of agarose concentration, 100 V, 30 min, 4 µL of sample and 1 µL of loading buffer). The results concluded that the ciclation was successful with these primers and the amplified rDNA can be separated by DGGE. Prior to separation, the samples were concentrated at 25 µL in a rotary evaporator (Concentrator plus, Eppendorf).

DGGE was performed with a UV trans illuminator with a denaturing gradient of 30-70% along 16 h, with a sample volume of 25 µL. Using DGGE it was obtained a specific pattern of bands for every sample as can be seen in Figure 5.2. 50 over all the visual bands were selected for sequencing purposes due to their high intensity in the DGGE. These 50 bands were stored in 1.5 mL tube with 50 µL of molecular grade water in order to benefit the DNA pass from the DGGE band to the water. The DGGE bands were re-amplified for sequencing (Table 5.2).

The sequencing analysis was performed in Macrogen (www.macrogen.com).

Table 5.2: PCR reagents and conditions

PCR program*		
Temperature (ºC)	**Time**	**Cicles**
96	5 min	-
94	30 sec	5
56	1 min	
72	2 min	
94	30 sec	30
54	1 min	
72	2 min	
72	5 min	-
4	∞	

* Muyzer *et al.* (1993)

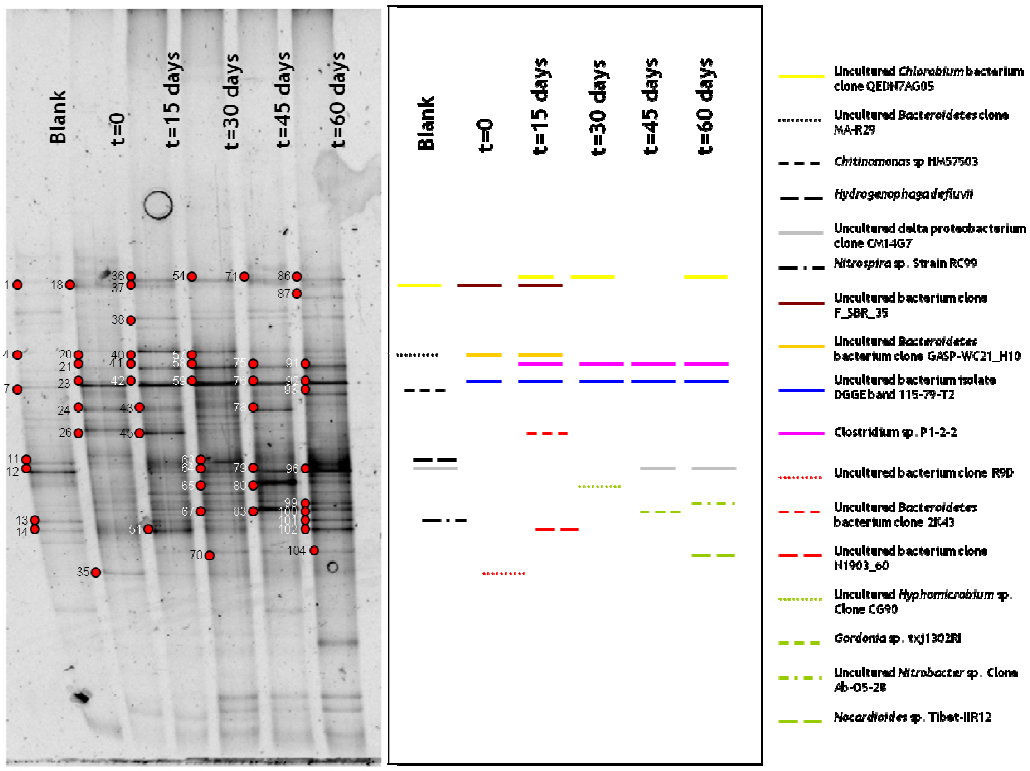


Figure 5.2: on the left side there is the DGGE analysis of the 6 effluent samples before to start degradation experiments (Blank) and at times 0, 15, 30, 45 and 60 days. In red can be seen the sequenced bands. On the right can be seen the corresponding sequenced band with the organism identified.

5.3 Discussion

5.3.1 Physicochemical experiments

5.3.1.1 Distribution and partition coefficients

In this case, the use of chemo informatics tools to predict these coefficients is very difficult because the controversial data on these compounds available in the literature. In table 5.3 can be seen the comparison between the experimental results obtained in this work and theoretical values.

Table 5.3: experimental and theoretical logP values for PFAPs

	PFHxPA	PFOPA	PFDPA
Experimental results			
Experimental LogP (average of 24 and 48 h)	0.68	1.87	2.06
Theoretical values*			
ALOGPs	3.62	4.04	4.34
AC logP	0.64	1.88	3.12
miLogP	3.17	4.45	5.73
ALOGPs	2.64	3.62	4.6
MLOGP	2.96	3.99	4.92
KOWWIN	4.55	6.48	8.41
XLOGP2	3.06	4.55	6.03
XLOGP3	2.97	4.31	5.65
Average logP	2.95	4.16	6.35

*Theory values calculated according to “Virtual Computational Chemistry Laboratory” (VCCLAB_Partners 2007)

The empirical distribution constants (Distribution constant (D) and the partition coefficient octanol-water (logP)), were also difficult to be established due to the three phases that are formed following the experimental process described by the OECD guideline 123.

According to this procedure PFHxPA tends to be in water while the longer PFPAs can be found in water and sediments/soils or biota.

5.3.1.2 Adsorption experiments in sludge

The logP obtained in the previous experiments was confirmed by the adsorption experiments performed in sludge and activated sludge. The experiments were carried out following the indirect method described in the OECD guideline 106. In Figure 5.3 the results of these experiments are summarized. During the whole experiment shaken was required in order to avoid loses by adsorption to the inner walls of the container.

As can be seen in Figure 5.3, long chain compounds were adsorbed to sludge whereas, for the shortest one, the compound can be found in both phases. With these experiments, the percentages distributed in both phases when the equilibrium was achieved, after 10 h of contact, were calculated. In the case of PFDPA, this percentage of adsorption in sludge was around 80% of exposure and 70% for the activated sludge. In the case of PFOPA, the percentage of adsorption was similar to PFDPA adsorption in sludge but in activated sludge the percentage was lower (45%).

The distribution coefficient (K_d), according OECD guideline 106, was calculated based on the next equation:

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} (cm^3 g^{-1})$$

where A_{eq} is the percentage of adsorption at equilibrium (%), V_0 is the initial volume of aqueous phase and m_{soil} is the mass of the soil used in the experiment.

The K_d for sludge was settled at: [11.9 ± 1.7] cm^3/g for PFOPA and [14.7 ± 5.6] cm^3/g for PFDPA. For activated sludge the results of the K_d were: [12.4 ± 5.2] cm^3/g for PFOPA and [42.7 ± 5.9] cm^3/g for PFDPA. As can be seen in both cases, the K_d of PFDPA was higher than PFOPA values. This result was expected since PFDPA has a higher C chain than PFOPA and, consequently, the molecule is likely to be more lipophylic.

In the case of PFHxPA, the equilibrium was not achieved.

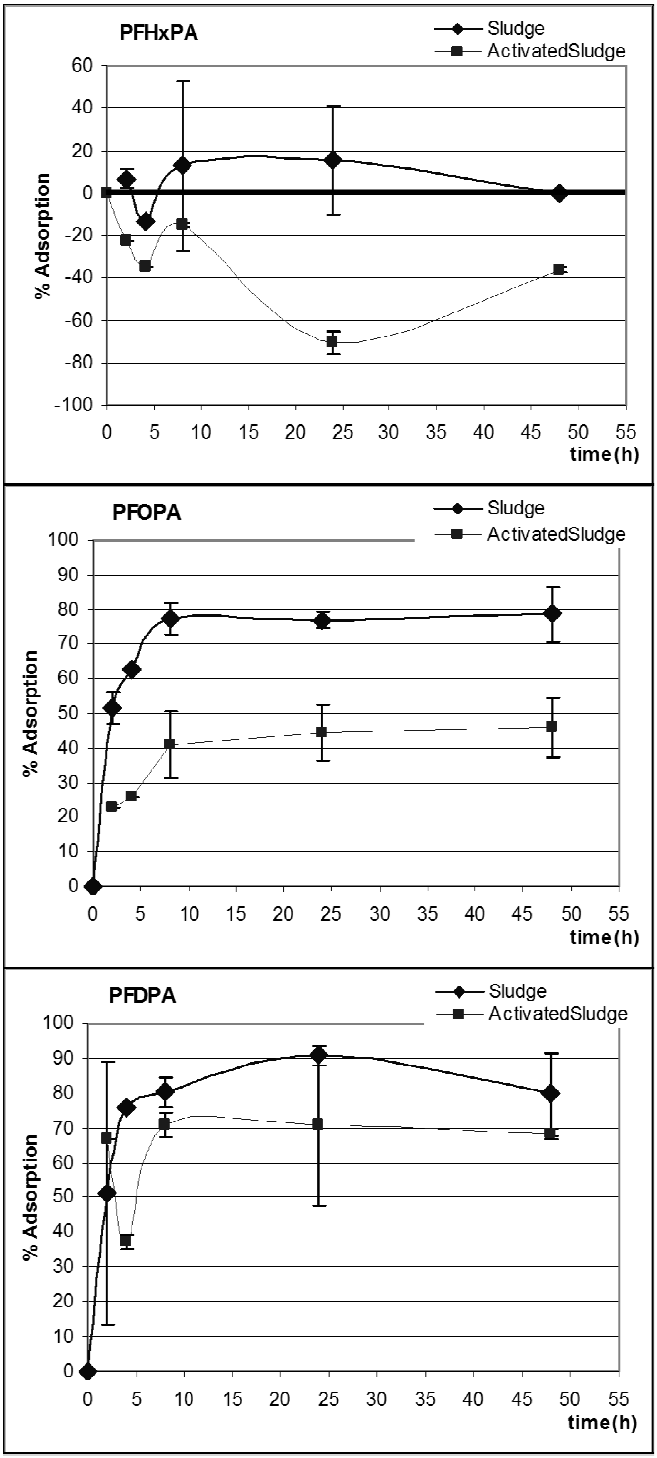


Figure 5.3: percentage of adsorption *vs.* time of exposure for PFHxPA, PFOPA and PFDPA in sludge and activated sludge.

5.3.2 Algae adsorption

Algae adsorption experiments were carried out using *Desmodesmus subspicatus* alga as test specie.

The main result of these experiments in the lack of adsoption in algae, however an interesting result is the inhibition of photosynthetic activity. In the bottles with PFDPA the green colour disappeared, whereas algae colour in the blank bottles was constant. In addition, when absorbance at 340 nm was measured it was shown that in presence of PFDPA the absorbance was 4 times lowed that in absence of this compound (Table 5.4).

Table 5.4: algae absorbance at 340 nm of experiments at 100 ng/mL

Analyte	Absorbance (λ = 340 nm)
Medium	0
PFHxPA	0.847
PFOPA	1.084
PFDPA	0.257

5.3.3 Biodegradation experiments

The molecular structure of selected compounds makes them difficult to be biodegraded. In order to confirm that the biodegradation processes under aerobic conditions cannot efficiently removed these compounds, in this work it was carried out this process under controlled aerobic conditions.

The results concluded that, for PFHxPA and PFOPA, no degradation process occurred. The disappearance of C6 and C8 PFAPs was similar to the experiments with NaN_3 (Figure 5.4). It indicates that the analytes are adsorbed onto experimental bottle walls.

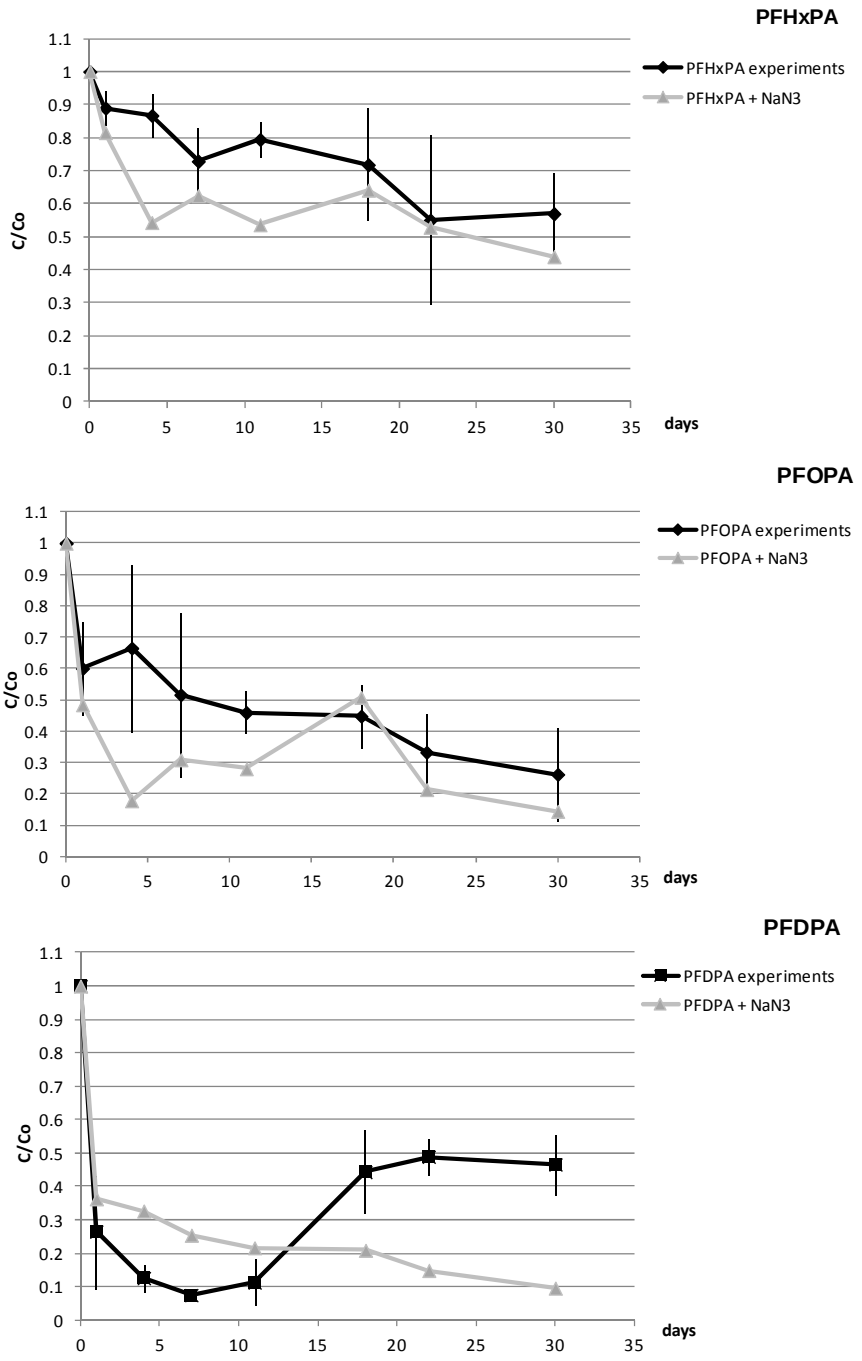


Figure 5.4: Concentration of PFPAs along the experimental degradation time in front of the initial concentration. It is represented the average of the concentration in experimental bottles and the standard plus NaN_3.

In contrast, the results for the experimental bottles with PFDPA showed a different profile as it can be appreciated in Figure 5.4. Between the 2nd and the 10th day of exposure, the concentration of PFDPA was lower than the concentration in the controls with NaN_3. After 10 days of exposure, the PFDPA appeared again at concentrations higher than reported ones for NaN_3 controls. It could indicate that this analyte is degraded, metabolized or adsorbed by the organisms. The degradation or metabolization was ruled out since no degradation compounds were found during LC-MS/MS studies. However, the disappearance of PFDPA coincided with the generation of a biofilm (or membrane) after two days of exposure (Figure 5.5). The second hypothesis pointed that the decrease of the PFDPA is related to the generation of this membrane.

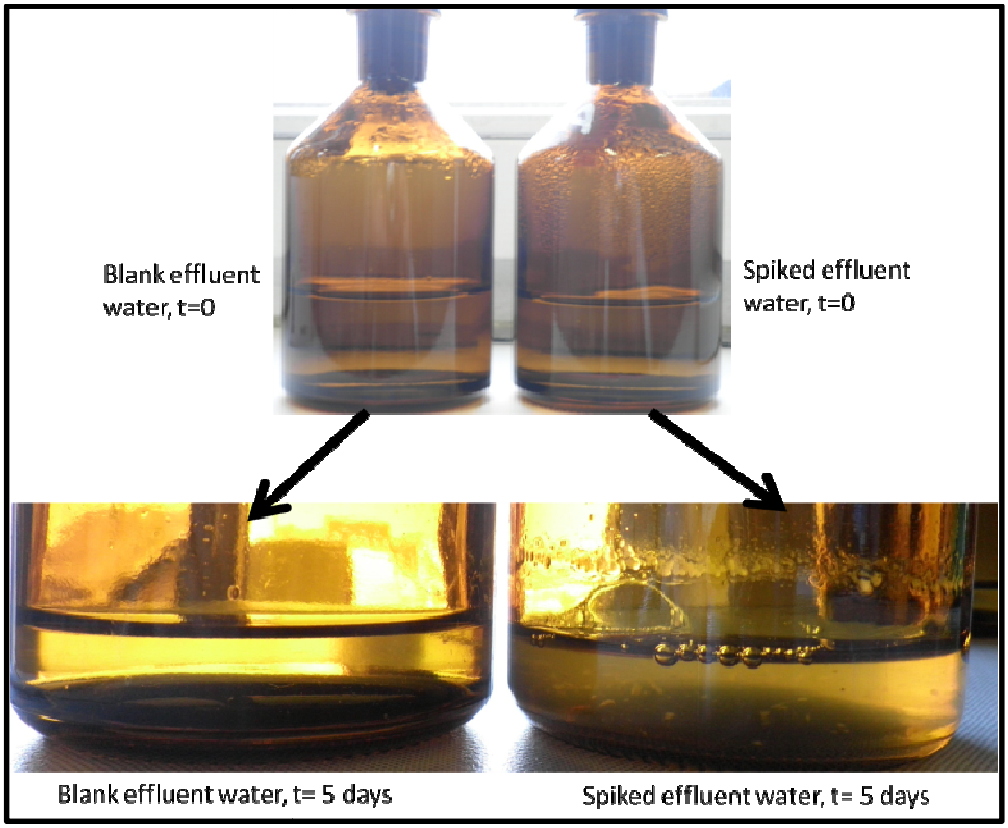


Figure 5.5: degradation experiments of blank and spiked experimental bottles 0 h and after 5 days.

In order to disclose where the PFDPA was, different aliquots without filtration of the experiments were extracted: 1:1, with water, acetonitrile, n-hexane and sodium dodecyl sulphate at 1% (SDS). The supernatants were analyzed by LC-MS/MS in SRM and Scan modes. The intensities of the extracted ion chromatogram for the transition 599 > 79, corresponding to PFDPA retention time, are summarized in Table 5.5.

Table 5.5: PFDPA intensities depending on the extracting solvent

Extracting solvent	Intensity of the transition 599 > 79 (cps)
water	676
acetonitrile	1420
n-hexane	551
SDS	8452

The results showed that, using a soft cellular disruptor, such as ACN, it is possible to precipitate the external proteins of the biofilm and recover some PFDPA, which was up take in the extracellular part of the membrane cell. On the other side, using SDS as strong disruptor agent, a higher amount of PFDPA was recovered. The results indicates that could be uptake by the adapted organisms as a source of phosphate (energy), or due to the similarity between this compound and one of the phospholipids from the extracellular membrane, as it has been suggested by Pearsons *et al.* (2008). The other possibility could be that the organisms included PFDPA in cells and excrete them after a short period of time. The excreted material could generate a membrane complex arising from the outer membrane of the cell envelope (Rothfield *et al.* 1969). The increasing amount of PFDPA after the 10^{th} day of experiment suggests that this compound could be excreted by the cells or the death of the responsible organism that has been absorbing it. The experiment was repeated two more times, separated in time, in order to be sure that no errors were committed during the experimental processes. The generation of the biofilm was observed in both repeated experiments.

In order to try to disclose which the responsible organism was, it was decided to have a microscope view. The observed organisms are in Figure 5.6.

A) B)

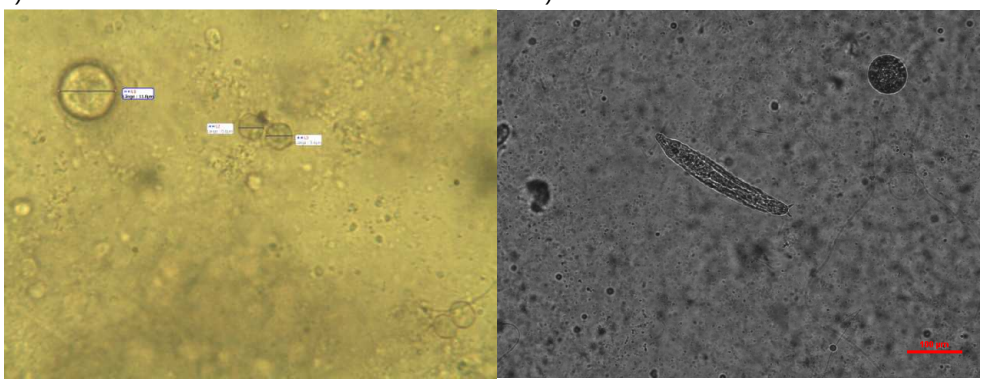


Figure 5.6: microscope views of the generated biofilm or membrane.

However, due to the complexity of the matrix, the identification of the organisms was not feasible by microscope and it was necessary to perform the DNA identification by PCR technique. Therefore, the experiments were repeated and the experimental time

was enlarged. However, it was possible to rule out alga as a responsible organism because the experiments were performed in dark conditions.

The separation of the rDNA by denaturing gradient gel electrophoresis (DGGE) showed different pattern bands as can be seen in Figure 5.7. Considering that the bands at the same height correspond to the same bacteria specie, it can be observed different species that have been present along all the experimental degradation time. These species can be candidates for the generation of the membrane. The red points (Figure 5.2) are the bands that have been sequenced.

The sequences were compared with those at the National Center for Biotechnology Information (NCBI). However, a conclusive identification of the specie or species that can uptake this compound was not achieved, but some groups were identified such as *Clostridium*. This specie is associated to anaerobic conditions. Although, it could be generated if the aeration of the samples was not enough, the other possibility is that, inside the biofilm (membrane), there were anaerobic or microaerophilic conditions. On the other hand, the last samples presented *Gordonia* and *Nocardioides* species which can be related to bioremediation studies (Militon *et al.* 2010).

There were also identified different fertilizers that can disturb the abundance of bacterial groups. PFDPA can act as a phosphate fertilizer inducing the proliferation of *Nitrospira* specie which is associated to agricultural soils conservation (Miller *et al.* 2007; Jangid *et al.* 2008) and it is present in the experiments.

It should be necessary to elucidate the phylogenetic affiliation of every band from the DGGE in order to get more information about the results. Most of the sequences obtained from the NCBI on line web page had a high homology with non cultured bacterium and it is necessary to characterize the bacterium aggregations.

CHAPTER 6

Conclusions

According to the objectives of this thesis, the research carried out and the results described in the previous chapters, the following conclusions can be drawn.

1. During recent years, considerable public and scientific attention was spent on the research related to the occurrence of perfluoroalkyl substances in the environment and food, as well as their possible implications in human health. However, this research was focussed on a limited number of compounds: PFOA and PFOS. Since perfluoroalkyl substances are a wide group of compounds, and new compounds are being introduced in the industry, to investigate the presence of PFASs in the environment and food, new multi-analyte, sensitive and robust analytical methods are requested.

   Different analytical methods have been developed and validated for the analysis of up to 21 PFASs in waters, sediments and sludge, sewage sludge, food (including fish, meat, dairy products, baby food and prepared foods), biota, sea plastic pellets, and human biological matrices (including breast milk and cord blood). In each case the methods were proved to be suitable for the trace-level determination of these compounds in the different types of samples.

2. The transferability and the analytical performance of a new analytical method developed by the ICT-Prague under the framework of the Confidence EU-project, for the analysis of PFASs in fish and milk, has been evaluated. The validation of this analytical method was carried out by a step-by-step approach. First, an in-house feasibility study was carried out, showing the good performance of the method when it was performed by expert laboratories. Second, an international interlaboratory exercise was organised. The results of this exercise showed that although, according to the recommendations of the EFSA (1 µg/Kg or L), the method was sensitive enough, the method was not suitable in terms of robustness.

3. An on-line SPE-LC-MS/MS method has been developed and validated to assess the occurrence of PFASs in the whole water system. In the majority of the samples PFBA and PFHxA were detected, indicating the industrial substitution of longer for shorter PFASs. In addition, the analysis of drinking water reveals the recalcitrant character of PFASs since 50% of the samples contained, at least, one of the investigated compounds. Although 6 samples exceeded the health level for PFOS (PFOS 200 ng/L) according to the EFSA, most of the drinking samples presented concentrations much lower than those considered to pose any acute risk for human health. However, the Provisional Health Advisory levels established by the EFSA and the USEPA

are established solely for PFOS and PFOA under acute exposure conditions. Since drinking water is one of the main routes of human exposure to PFASs, these recommendations should be extended to other compounds and chronic exposures should be also considered. The results of this work underpin the need to establish new regulations about PFAS contamination in continental waters.

4. The investigation of sewage sludge showed the presence of high concentrations of PFAS ranging from 53 to 121 µg/Kg, for PFOS, the most recalcitrant analyte. Therefore, use of sewage sludge in amended soils for agricultural lands should be considered as a possible source to PFASs in the environment, ground waters and food.

5. The presence of PFASs in penguin tissues, guano and soils from the Southern Hemisphere and the Antarctic continent was proved. These results support previous hypotheses, based on long-range environmental transport which combine the atmospheric transport and cold condensation process for the most volatile PFASs, and the oceanic transport (by thermohaline circulation among other geophysical processes) for the non-volatile compounds.

6. The occurrence of PFASs in food contributes to human exposure through diet. Considering this fact, different foods including human breast milk, infant daily products (powdered milk and cereals) and market fish from different European countries were assessed. The results of these studies have indicated the generalised occurrence in food of shorter PFASs and the more recalcitrant compounds: PFOA and PFOS. With the exception of one sample of breast milk, the concentrations of these compounds in food did not show any immediate risk for human health according to the Provisional Health Advisory levels established EFSA and USEPA. However, chronic exposure should be considered in future recommendations and food legislations.

7. Human accumulation has been also studied. The presence of PFASs in breast milk and cord blood has been proved in both cases. In cord blood 18 compounds were investigated in sixty samples, and the presence of these compounds was confirmed in all the samples. PFBA, PFHxS, PFOA and PFOS were the more frequent compounds. The results of this study supported previous studies indicating the transplacental transport of PFASs. On the other hand, 20 breast milk samples were investigated, and the presence of PFASs was also confirmed in all the samples.

8. The use of PFASs in plastics polymers can result in a new source of PFASs in the environment. PFASs have been studied in plastic pellets collected from different coastal areas in Greece. The results have shown that PFASs profile in beach plastic pellets and in sediments collected in the same areas were similar items, suggesting a common contamination source. In addition, plastic degradation combined with adsorption of PFASs onto plastic pellet surfaces has opened another contamination source, as well as another source of environmental transport.

9. Finally, the physicochemical properties and the biodegradation of perfluoroalkyl phosphonic acids under aerobic conditions, during the treatments in the WWTPs, were studied. The perfluoalkyl phosphonic acids are a group of PFASs that has been little studied. In this work the partitioning coefficients were established according to the OECD guideline 123 (OECD_123). In addition, aerobic biodegradation experiments were carried out, confirming the lack of biodegradation of these compounds. However, the bacterial uptake of PFDPA was found. But, more research will be required in order to identify the microorganisms involved in the uptake of this compound, as well as the mechanisms that are involved.

Resum en català

CAPÍTOL 1: Introducció

1. Definicions

Les substàncies per- i poli- fluoro alquíliques (PFASs) són compostos de caràcter alifàtic sintetitzats per l'home des dels anys 40. En els compostos perfluoro alquílics tots els àtoms d'hidrogen han estat substituïts per àtoms de fluor mentre que, els compostos polifluoro alquílics són aquells en els què s'ha substituït com a mínim un àtom d'hidrogen (però no tots) per àtoms de fluor (Buck *et al.* 2011).

Els compostos PFAS formen un gran grup de compostos els quals varien en la seva estructura i, per tant, tenen diferents toxicitats i destí en el medi ambient. No obstant, tots són altament estables degut a l'enllaç carboni – fluor, un dels més estables en la natura. Aquesta elevada estabilitat ha fet que alguns d'ells hagin estat classificats com a compostos orgànics persistents (POPs). D'altra banda, els alts potencials d'ionització i la baixa polaritzabilitat indueixen a interaccions inter- i intramoleculars dèbils (Dean 1999; 3M 2000b). La cadena fluoroalquílica i el grup funcionalitzat donen a aquests compostos un doble caràcter hidrofòbic i hidrofílic que indueix la formació de tres fases immiscibles quan es mesclen amb hidrocarburs i aigua. Degut a aquest doble caràcter i a l'elevada estabilitat, una de les principals aplicacions d'aquests compostos és com a surfactants en la síntesi de fluoropolímersv(3M 2000b).

La Taula 1.1 del capítol 1 mostra les estructures més comunes d'aquests anàlits.

2. Producció i usos industrials

Els compostos PFASs van ser sintetitzats per primera vegada com a material del mòdul de contenció de reactors nuclears, anomenat Tefló, durant la II Guerra Mundial (Sanger *et al.* 1989). El Procés Fowler (Fowler *et al.* 1947) representa la primera ruta de síntesi d'aquests anàlits mitjançant l'oxidació del fluor i la posterior fluoració a elevades temperatures. Durant el mateix període es va desenvolupar la Fluoració Electroquímica (ECF) o Procés Simon (Simons *et al.* 1949), el qual es basa en l'electròlisi d'un compost alquílic en presència d'àcid fluorhídric. S'han desenvolupat altres processos de síntesi en els últims anys per millorar els percentatges de recuperació de les reaccions i la introducció de diferents grups funcionals. Les Figures 1.1 i 1.2 de la secció 1.2 del capítol 1 mostren un esquema de les principals rutes de síntesi. D'altra banda, la síntesi industrial de fluoroalquens com el vinilidè fluorat, entre d'altres, es fa mitjançant la telomerització ja que té majors recuperacions que l'ECF (Améduri *et al.* 1997; Kissa 2001).

Les principals aplicacions industrials els inclouen com a additius en productes resistents al foc, en repel·lents d'oli, de taques, de greix i d'aigua, redueixen la tensió superficial en major grau que d'altres surfactants i actuen com a catalitzadors en processos d'oligomerització i polimerització (3M 2000b). En usos més quotidians, es troben com additius en superfícies antiadherents d'estris de cuina, en teixits de roba que permeten la transpiració i en diferents sectors industrials incloent la indústria

aeroespacial, automobilística, construcció, processos químics, i materials electrònics i semiconductors (USEPA 2010).

La producció global d'aquests compostos va augmentar exponencialment des dels anys 40 amb la seva introducció en nous productes industrials. El màxim de producció correspon a l'any 2000 amb l'emissió de 5720 tones d'un dels anàlits més utilitzat en la indústria, l'àcid perfluorooctanòic (PFOA), 3535 tones del ió perfluorooctasulfat (PFOS) i la producció entre 5000 i 6000 tones anuals de fluorotelomers (3M 2003; Prevedouros *et al.* 2006; Stock *et al.* 2010). A finals dels any 90, l'EPA va rebre informació relativa a la presència de PFOS en sang d'humans que no estaven relacionats amb la producció d'aquests compostos. Degut als resultats de l'informe, es va començar a focalitzar l'estudi en la persistència, bioacumulació i toxicitat d'aquests anàlits. L'any 2000, l'empresa 3M, la major productora de PFASs, va començar a reduir voluntàriament la manufacturació de productes que contenien compostos perfluorats de cadena octa carbonada (3M 2000). A partir d'aquest moment l'emissió global de PFOA i PFOS en el medi ambient va començar a disminuir. No obstant, en paral·lel a la reducció de l'emissió de PFOA i PFOS, s'ha observat un increment dels compostos utilitzats com a perfluorats alternatius de cadena més curta com per exemple el ió perfluorobutà sulfat (PFBS) (Stock *et al.* 2010). D'altra banda, tot i que la producció de PFOA i PFOS ha estat limitada a molts pocs productes, l'ús de fluorotelomers en la indústria ha seguit augmentat arribant a 12000 tones/anuals l'any 2004 i majors actualment (Stock *et al.* 2010).

3. Destí ambiental

La presència d'aquests compostos en el medi ambient ha estat detectada de forma ubiqua degut a la seva estabilitat física, química i biològica. Per aquest motiu, alguns dels PFASs són considerats com a POPs i el ió PFOS ha esta inclòs en la Convenció d'Estocolm per a la regulació global de producció i usos (UNEP 2010b). El destí d'aquests anàlits ve determinat per diferents propietats fisicoquímiques així com biològiques que es resumeixen a continuació.

- ***Propietats fisicoquímiques.*** Els sulfats i els àcids carboxílics perfluorats són àcids forts que estan en equilibri entre la forma neutre i la iònica. En general, són compostos solubles en aigua en els que la constant octanol-aigua depèn directament de la llargada de la cadena alifàtica. No obstant, degut al doble caràcter amfòter i a la capacitat de formar tres fases en presència d'alcans i aigua, no és possible de determinar les constants d'equilibri octanol-aigua ni altres factors com el de bioacumulació per models de predicció de propietats. D'altra banda, aquestes constants tampoc poden ser utilitzades per predir el comportament dels PFASs ja que, per exemple, s'ha vist que no s'acumulen als lípids en teixits biològics com és el cas d'altres POPs.

  La constant de la Llei de Henry per predir la presència dels anàlits en l'atmosfera és també un punt de controvèrsia en el cas dels compostos PFASs degut al desconeixement que es té de la constant d'acidesa o de la forma àcida

dominant al pH ambiental. No obstant s'ha observat que, tot i tenir constants desfavorables, el PFOA i el PFOS es troben en l'aire, aigua i sediments sent els oceans considerats el seu destí final. La combinació entre la fase dissolta de PFASs i el transport atmosfèric han estat postulats com les principals vies de transport ambiental de llarga distància (LRET). En el cas del transport atmosfèric, s'ha explicat parcialment per la teoria de la condensació freda en la qual intervenen moviments químics i transformacions químiques degut al impacte de les forces ambientals (temperatura i interacció amb altres compartiments com sòls, oceans, etc.) arribant a zones remotes com l'Antàrtica (Lohmann *et al.* 2007). Per exemple, s'ha evidenciat que els compostos més volàtils com els fluorotelòmers són transportats per l'atmosfera i oxidats a àcids carboxílics abans de ser dipositats al sediment.

- ***Fotòlisi.*** Els estudis realitzats en aquest àmbit experimental postulen que la fotòlisi indirecta de PFOS té un temps de vida mitjana superior a 3,7 anys, a 25ºC, i 349 dies per al PFOA (ATSDR ; USEPA 2002; Brook. *et al.* 2004; Cheng *et al.* 2008; EFSA 2008). Tot i aquesta possible degradació via fotòlisi de dos dels compostos més recalcitrants, s'ha de tenir present que altres PFASs han estat identificats com a precursors fotolítics dels àcids perflorocarboxílics com per exemple les sulfonamides (Plumlee *et al.* 2009).

- ***Hidròlisi.*** Els estudis realitzats fins al moment donen suport als resultats ambientals trobats. Mentre el PFOS i el PFOA tenen un temps de vida hidrolític mitjà de 41 i 92 anys, respectivament, a 25ºC (USEPA 2002; Hatfield 2001), d'altres PFASs com els àcids fosfònics s'aproxima a 26 anys (D'Eon *et al.* 2007). D'altra banda, la hidròlisi representa una ruta important de síntesi dels compostos més recalcitrants com són el PFOA i el PFOS en comparació a la seva eliminació.

- ***Biodegradació.*** La biodegradació dels compostos perfluoroalquílics està limitada degut a l'enllaç carboni – fluor. Des d'un punt de vista termodinàmic aquests anàlits podrien ser utilitzats per diferents organismes com a font d'energia. No obstant, aquests organismes encara no han estat trobats en la natura (Vargas 2000). Els estudis realitzats fins al moment mostren com el PFOA i el PFOS són productes finals de degradació de compostos de cadena alifàtica fluoro hidrocarbonada més llarga en diferents condicions aeròbiques. Per exemple, simulant els efectes dels microorganismes presents en l'aigua de sortida d'una depuradora o bé en el fang activat utilitzat com a tractament biològic en les estacions depuradores d'aigua residual, s'ha detectat la presència d'ambdós anàlits a partir de la degradació de fluorotelòmers, sulfonamides o àcids fosfònics hidrocarbonats (Boulanger *et al.* 2005; Wang *et al.* 2005; D'Eon *et al.* 2007; Frömel *et al.* 2010).

- ***Degradació tèrmica.*** Degut a l'alta estabilitat dels PFASs, no s'espera degradació tèrmica tot i que alguns autors hipotetitzen la possible degradació per incineració del PFOS degut a l'enllaç carboni-sofre (Dixon 2001; Yamada *et al.*

2003; Giesy *et al.* 2010). D'altra banda, s'ha observat la generació de PFOA per termòlisi de fluoropolímers (Ellis *et al.* 2001; Ellis 2003; Environment Canada 2010).

- ***Adsorció/Desorció.*** Aquest tipus d'equilibri s'ha estudiat en diferents sòls per diferents PFASs. El més estudiat ha estat el PFOS amb el que s'ha detectat una clara tendència a adsorbir-se en els centres actius del sòl o fang en menys de 24 h. Aquesta ràpida adsorció combinada amb la baixa desorció, inclús amb solvents orgànics, fa que sigui molt poc mòbil (Giesy *et al.* 2010). L'adsorció s'ha trobat directament relacionada al grup funcional (Zhou *et al.* 2009) i, tot i la poca mobilitat del PFOS en sòls, altres PFASs en presenten més arribant a ser considerats un problema per les aigües subterrànies i les plantes que s'alimenten del sòl.

- ***Bioacumulació.*** Diferents estudis constaten la bioacumulació de PFASs en la sang d'organismes exposats així com en òrgans com el fetge (Jones *et al.* 2003). El mecanisme de transport a través de la sang és mitjançant l'enllaç amb proteïnes de baix pes molecular contribuint a una major toxicitat (Luebker *et al.* 2002). Els processos d'eliminació també s'han estudiat en diferents organismes tot i que no s'ha pogut establir un patró clar del temps d'eliminació mitjà depenent de l'organisme i l'anàlit (Taula 1.3 del capítol 1).

Degut a la persistència en el medi ambient i la tendència a ser acumulats, els compostos perfluorats són subjecte d'estudis toxicològics.

4. Contaminació del medi ambient

Tal com s'ha comentat anteriorment, els PFASs són distribuïts globalment degut a les seves característiques fisicoquímiques i processos de degradació. La presència d'aquests compostos en el medi ambient ha de ser considerada d'interès públic ja que sòls, fangs i diferents cossos d'aigua estan implícits en processos amb finalitats humanes com, per exemple, l'agricultura i la ramaderia. D'aquesta manera poden arribar a la dieta mitjançant els aliments i l'aigua de beguda.

Una de les principals vies d'entrada de PFASs en el medi ambient són les estacions d'aigua depuradora (EDAR). En un estudi realitzat a Catalunya en el qual es compara les concentracions en aigües superficials de riu abans i després de la desembocadura de diferents EDARs s'observa com, la concentració d'aquests anàlits, augmenta en l'aigua provinent de la sortida d'EDARs (Sánchez-Avila *et al.* 2010).

Degut a la utilització dels compostos PFASs, aquests arriben en forma de residu urbà, industrial o bé de l'agricultura a les EDARs a nivells de centenars de ng/L en les aigües d'entrada de depuradora (Becker *et al.* 2010). Tot i els tractaments de depuració de les aigües aplicats actualment l'estabilitat dels PFASs fa que, en comptes de ser eliminats o degradats, hi hagi una tendència clara a la redistribució entre fangs i aigües finals del tractament de depuració. Aquest és el cas dels compostos més recalcitrants com el PFOS o el PFOA. Mentre que el PFOS tendeix a ser adsorbit en els centres

actius dels fangs, el PFOA tendeix a estar solubilitzat en les aigües (Becker *et al.* 2010; Zhou *et al.* 2010). D'altra banda, s'ha observat la degradació a PFASs més estables dels compostos polifluoro alquílics de cadena més llarga en els diferents passos dels tractaments (Frömel *et al.* 2010; Lee *et al.* 2010).

Finalitzat el tractament de les EDARs, les aigües depurades poden ser utilitzades en regs de camps de cultiu. Aquesta aplicació pot conduir a una contaminació dels cultius amb PFASs arribant fins i tot a contaminar les aigües subterrànies. En cas de no utilitzar les aigües de sortida de depuradora per regs, aquestes són abocades directament als rius, exposant flora i fauna a una possible contaminació per PFASs, així com també comprometent els usos que es derivin de la utilització de l'aigua del riu com, per exemple, la captació per plantes de tractament d'aigua potable (ETAPs) (Loganathan *et al.* 2007; Ahrens *et al.* 2009; Sánchez-Avila *et al.* 2010).

En el cas dels fangs, aquests poden ser utilitzats com a fertilitzant en l'agricultura degut a l'alt contingut en nutrients. La presència de PFASs pot comportar una contaminació dels cultius o bé de les aigües subterrànies amb els compostos més mòbils (Gottschall *et al.* 2010; Clarke *et al.* 2011).

5. Contaminació per PFASs

5.1. Aigües superficials i sediments

Aigua superficial. Els compostos més estudiats són el PFOS i el PFOA degut a la seva ubiqüitat. En general, les concentracions varien diferents ordres de magnitud al llarg del recorregut del mateix riu sent un reflexa de la proximitat o llunyania del focus de contaminació com zones industrials o EDARs. Per exemple, un estudi realitzat en la conca del riu Ruhr, Alemanya, mostren una contaminació per PFOA provinent de l'agricultura arribant a concentracions de 33900 ng/L en el riu Moehne. Aquesta contaminació va provocar la conseqüent contaminació del riu Ruhr, el llac Moehne i l'aigua potable de la zona (Skutlarek *et al.* 2006).

A nivell català, s'ha estudiat la presència de PFASs en diferents rius trobant concentracions entre 0,19 i 25 ng/L (Ericson *et al.* 2008b; Sánchez-Avila *et al.* 2010). Aquests anàlits també s'han trobat en un estudi més recent en l'Albufera de València tot constatant la distribució en les aigües de la Península Ibèrica (Pico *et al.* 2011). En general, en els estudis realitzats durant els últims anys s'està trobant més presència de PFASs de cadena més curta. Aquest fet remarca la tendència de substituir a nivell industrial els compostos de cadena més llarga.

Actualment s'ha proposat incloure el PFOS i els seus derivats dins la Directiva Europea Marc de l'Aigua. Els valors de mitjana anual són entre 0,13 i 0,65 ng/L mentre que el màxim de concentració permesa és entre 7,2 i 36 µg/L. Aquests nivells representen el màxim de concentració acceptable.

Sediments. L'estudi de diferents sediments mostra la distribució global d'aquests compostos en aquesta matriu. En general, les concentracions oscil·len entre pocs pg/g i

ng/g. Tal com succeeix en les EDARs, els compostos sulfats mostren una clara tendència a quedar adsorbits en la superfície del sediment tot i que també es troben en grans concentracions en les aigües, com és el cas del PFOS (Pico *et al.* 2011).

5.2. Mostres biològiques

Els compostos per- i poli-fluoroalquílics han estat detectats en mostres de biota arreu del Món.

Degut al impacte humà, les àrees industrialitzades són les que han estat més investigades. Els principals estudis han estat enfocats en anàlitzar peixos degut a la implicació per al consum humà (Taniyasu *et al.* 2003; Sinclair *et al.* 2006; Naile *et al.* 2010). S'observa una major presència de PFOS en comparació a altres PFASs, incloent PFOA. Aquesta diferència confirma els experiments realitzats en els quals la bioacumulació del PFOS és fins a 1000 vegades major que la del PFOA (Martin *et al.* 2003; Gruber *et al.* 2007). Fruit d'aquesta bioacumulació se'n deriva la seva biomagnificació dins la cadena tròfica. En aquest cas, la cadena tròfica marina ha estat identificada com una de les principals afectades per PFASs (Tomy *et al.* 2004; *Houde et al.* 2006; Kelly *et al.* 2009; Butt *et al.* 2010). D'altra banda, els estudis en peixos de riu i de mar no conclouen una major assimilació de PFASs en funció de l'origen.

Dins la Directiva Europea Marc de l'Aigua s'ha establert el màxim de concentració en biota en 9,1 µg/Kg (European Commission 2012).

5.3. Aigua de beguda

Els compostos fluoroats s'han trobat en aigües de beguda de diferents estudis. Degut a que la presència d'aquests anàlits en l'aigua potable està directament relacionada amb les estacions potabilitzadores d'aquesta o bé amb el seu mecanisme de transport, diferents autors han investigat la possible relació entre ambdues fonts. Els resultats conclouen una relació directa entre la presència de PFAS en la font de subministrament d'aigua a la planta potabilitzadora (per exemple riu) i la presència d'aquests compostos en l'aigua d'aixeta (Skutlarek *et al.* 2006; Loos 2007; Takagi *et al.* 2008). En general, els compostos més detectats són el PFOA i el PFOS, a pocs ng/L, amb l'excepció d'alguns episodis de contaminació de les aigües de captació amb PFAS (Skutlarek *et al.* 2006). Els tractaments de potabilització actuals no només no són prou eficients per la completa eliminació i/o redistribució dels PFASs en les aigües de beguda sinó que poden arribar a ser font de generació d'alguns compostos de degradació fluorats en algun dels tractaments de potabilització.

Degut a la presència en les aigües de beguda, aquests anàlits han estat també objecte de regulació provisional en aquesta matriu. Els nivells provisionals de salut segons l'Oficina de l'Aigua de l'Agència de Protecció Medi Ambiental dels Estats Units són de 0,4 µg/L per al PFOA i 0,2 µg/L per al PFOS (EPA 2009).

5.4. Matrius humanes

La presència de compostos fluorats en matrius humanes ha estat estudiada per diferents autors des de l'any 1968. La matriu més estudiada fins al moment és la llet materna degut a la simplicitat de la presa de mostra i la implicació tant en el sistema reproductiu com en la salut dels infants (So *et al.* 2006; Tao *et al.* 2008a; Tao *et al.* 2008b; Völkel *et al.* 2009). No obstant, també s'han estudiat altres matrius humanes com la saliva (Tao 2009), l'orina (Ylinen *et al.* 1985; Tao 2009), el semen (Guruge *et al.* 2005), la sang (Inoue *et al.* 2004; Kubwabo *et al.* 2004; Guruge *et al.* 2005; Calafat *et al.* 2006; Ericson *et al.* 2007; Monroy *et al.* 2008) i el fetge (Kärrman *et al.* 2009).

En estudis realitzats diferenciant per sexes, les concentracions més elevades corresponen al PFOS i, en general, són més freqüents en mascles que en femelles (Yeung *et al.* 2005). També s'han identificat algunes diferències ètniques a nivell de concentració (Calafat *et al.* 2006).

D'altra banda, persones exposades a aquests anàlits mostren una concentració de fins a un ordre de magnitud superior en sang a la de la població en general amb un baix índex d'eliminació (Lau *et al.* 2007).

6. Ecotoxicologia

L'ecotoxicologia ha estat estudiada en diferents organismes, en la seva majoria aquàtics degut a la rellevància de la presència de PFASs en aquest medi.

Toxicitat aguda

Durant els últims anys s'han efectuat diferents assajos de toxicitat aguda per tal de determinar diferents efectes de toxicitat com la dosi efectiva per al 50% d'organismes (EC50), la concentració d'efecte no observat (NOEC), la dosi letal per al 50% d'organismes (LC50) i la concentració mínima de l'efecte observat (LOEC). En la Taula 1.7 del capítol 1 es troben resumits alguns dels estudis realitzats fins al moment. La toxicitat aguda dels PFASs es pot considerar negligible degut a què la concentració necessària per a les respostes toxicològiques, 1 ppm, és molt més altea que els nivells ambientals en què es troben.

En general, per als tests realitzats amb PFOA i PFOS, s'observa que és necessària una menor concentració d'aquest últim per obtenir la mateixa resposta tant en organismes invertebrats com la *Daphnia magna* com en organismes vertebrats (Ji *et al.* 2008; Hagenaars *et al.* 2011) denotant una major toxicitat del PFOS.

Toxicitat crònica

Els assajos de toxicitat crònica han estat duts a terme, en la seva majoria, per a PFOA i PFOS incloent estudis de toxicitat subcrònica, desenvolupament / reproducció, i toxicitat crònica / carcinogènesi tant en organismes aquàtics com terrestres (Taula 1.8 de la secció del capítol 1).

Alguns estudis mostren efectes hepatotòxics i la inducció de la proliferació dels peroxisomes en rates (GeneGo Incorporated 2009). En general, el fetge és l'òrgan diana amb l'aparició de tumors en rates (EFSA 2008) així com la interacció amb hormones tiroïdals, lipoproteïnes d'alta densitat, colesterol i triglicèrids (Lau *et al.* 2007; Peden-Adams *et al.* 2008).

D'altra banda, els estudis sobre els efectes de PFASs durant l'embaràs en rosegadors conclouen l'exposició trans-placental del PFOS (Grasty *et al.* 2005).

7. Exposició humana i bioacumulació

Les principals vies d'exposició humana han estat identificades via inhalació, ingesta d'aliments i begudes.

Els compostos fluorats en aliments poden arribar mitjançant la bioacumulació, inclús arribant a derivar en una biomagnificació dins la cadena tròfica (Tomy *et al.* 2004; Kelly *et al.* 2009). La preparació d'aliments és una altra font de contaminació d'aquesta matriu tot i que, per exemple, la cocció s'ha vist que no té una gran aportació de PFASs (Jogsten *et al.* 2009). D'altra banda, l'empaquetat d'aliments pot introduir aquests compostos via migració des de l'embolcall cap a l'aliment. Hi ha dues vies principals d'entrada de PFASs en els aliments:

(i) Contaminació directa per exposició de plantes i animals

(ii) Contaminació indirecta mitjançant la cuina d'aliments, l'empaquetat i els processos de preparació d'aliments

Degut a la presència d'aquests compostos en els aliments, l'any 2008 l'EFSA va establir els límits tolerables d'ingesta diària per al PFOS i el PFOA.

La publicació número 1 recull un resum exhaustiu de la introducció de PFASs a través de la dieta així com de l'exposició humana i els mètodes analítics més utilitzats.

Contaminació directa: PFASs a través de la dieta

Les principals vies d'entrada a través de la dieta són per el consum de peix, productes diaris com la llet, i pel consum de carn o vegetals (EFSA 2008; Kantiani *et al.* 2010; Pico *et al.* 2010).

La contaminació dels aliments pot ser deguda a l'exposició dels animals a PFASs (Tittlemier *et al.* 2007). En el cas dels vegetals, aquests poden ser contaminats degut a l'ús de fertilitzants provinents de fangs de depuradora que contenen aquests anàlits (Clarke *et al.* 2011) o bé als compostos presents en l'aigua de pluja (Loewen *et al.* 2005; Kim *et al.* 2007).

Els estudis realitzats fins al moment mostren una major aportació de PFASs via el consum de peix ja que contenen concentracions més elevades (µg/g – ng/g),

corresponent les màximes concentracions als peixos més grans, comparat amb altres aliments com ous, pollastre, formatge, etc. (Ericson *et al.* 2008; Wang *et al.* 2008; Jogsten *et al.* 2009; Pico *et al.* 2010).

Contaminació indirecta

Degut a l'ús de PFASs com a repel·lents d'oli i aigua en alguns estris de cuina i embolcalls d'aliments, aquests són una font indirecta de contaminació mitjançant el cuinat, l'empaquetat i els processos de preparació d'aliments.

Els estudis referents al traspàs de PFASs via el cuinat d'aliments no conclouen una font gaire elevada de contaminació i, fins i tot, s'ha vist com la preparació de peix disminueix la concentració d'aquests compostos en la mostra (Powley *et al.* 2005; Del Gobbo *et al.* 2008).

Per contra, la migració de l'embolcall a l'aliment s'ha demostrat ser una font important de PFASs, sobretot en menjar com les crispetes i altres preparats alimentaris com els anomenats "menjar ràpid" (Begley *et al.* 2005; Tittlemier *et al.* 2007; Begley *et al.* 2008).

Una altra font indirecta és l'ús que se'n deriva dels PFASs en roba, tapisseries, impermeables, ceres, pintures i productes de neteja tot i que s'ha demostrat que no tenen una contribució significativa d'aquests anàlits (Washburn *et al.* 2005).

Acumulació humana i possibles conseqüències

La incertesa sobre els processos d'acumulació i els possibles efectes de toxicitat aguda en humans ha donat pas a tota una sèrie d'investigacions relacionades amb la presència d'aquests anàlits en matrius humanes.

La matriu més estudiada ha estat la sang. En aquest cas, els PFASs han estat trobats en voluntaris de diferents països així com també en sang de cordó umbilical i sang materna (Kubwabo *et al.* 2004; Calafat *et al.* 2007; Ericson *et al.* 2007). La transferència de PFASs via placenta durant l'embaràs és una de les principals rutes d'aquests anàlits juntament amb la lactància (Fromme *et al.* 2010; Gützkow *et al.* 2011). La presència d'aquests compostos en el sistema reproductiu ha quedat confirmada no només amb la detecció dels PFASs en el cordó umbilical i en la llet materna sinó també amb els nivells trobats en el semen. Aquests estudis confirmen l'acumulació humana.

Degut als efectes ecotoxicològics en diferents organismes, així com l'exposició humana i l'acumulació observada, els PFASs són objecte de regulacions restrictives.

8. Legislació

Tal com s'ha esmentat anteriorment, degut als efectes tòxics observats fins al moment, així com els nivells i l'exposició en humans, els PFASs han estat objecte

d'accions legislatives per entitats no governamentals i autoritats nacionals i internacionals. Algunes d'elles estan resumides a la Taula 1.9 del capítol 1 de la tesi.

La primera empresa en regular voluntàriament l'ús de PFAS en els seus productes va ser la 3M, la major fabricant de productes d'extinció d'incendis, l'any 2000 disminuint la presència de PFOS en les seves formulacions. Aquest mateix compost va ser identificat l'any 2002 per la OECD com a persistent, bioacumulatiu i tòxic (PBT).

L'any 2006, l'EPA i les 8 major productores industrials de PFAS (Arkema, Asahi, BASF Corporation (successor to Ciba), Clariant, Daikin, 3M/Dyneon, DuPont, Solvay Solexis) van fundar el "Programa d'Administració de PFOA" ("PFOA Stewardship Program"). El programa preveia la disminució global en l'emissió de PFASs en un 95% per al 2010 i la completa eliminació del compost en les formulacions per l'any 2015 (USEPA 2006). Els estudis realitzats posteriorment a l'esmentat programa evidencien la disminució de productes que contenen PFOS i alguns dels seus derivats de 3000 a 175 tones entre l'any 2003 i el 2006 (Stock *et al.* 2010). Aquests compostos han estat també proposats com a candidats en la regulació de compostos químics que necessiten autorització dins la regulació "REACH" (European Commission 2002).

Posteriorment, degut a la seva classificació química com a PBTs, han estat inclosos com a contaminants persistents orgànics en la Convenció d'Estocolm per la regulació global de producció i ús (UNEP 2010) i dins la Directiva Marc de l'Aigua Europea tal com s'ha comentat anteriorment (European Commission 2012).

Actualment els PFASs han estat inclosos en diferents programes de salut als Estats Units, Canadà i Europa. Un exemple són els diferents projectes dins el marc del VII Programa Marc Europeu de Recerca enfocats al desenvolupament de diferents mètodes analítics per a PFASs en diferents matrius i l'estudi de la seva presència. D'altra banda, s'estan produint PFASs de cadena més curta com per exemple el ió perfluorobutà sulfat com a substitució dels compostos de més llargs en les formulacions (OECD 2006).

9. Tendències temporals

La presència de PFASs en el medi ambient, aliments i humans està directament relacionat amb la seva producció i, conseqüentment, amb el seu ús al llarg dels anys.

El màxim d'emissió de PFASs va ser assolit durant l'any 2000 estimant una producció de PFOS en 3535 tones mètriques. Durant aquest mateix any l'empresa 3M, la major productora de PFOS, va començar voluntàriament la disminució de producció d'aquest compost i, conseqüentment, l'emissió global d'aquest anàlit s'ha vist disminuïda al llarg dels anys. D'altra banda s'ha pogut observar un lleuger augment en la presència de compostos de cadena més curta en mostres d'aigua (Ahrens *et al.* 2009; Eschauzier *et al.* 2010; Ullah *et al.* 2011). No obstant, es desconeixen els efectes persistents en el medi així com els possibles efectes de degradació dels PFASs de cadena més curta tot i que són utilitzats com a substituts dels més persistents (Quinete *et al.* 2010).

Les figures 1.7 i 1.8 del capítol 1, resumeixen els esdeveniments més importants i regulacions així com la producció global i l'emissió al medi ambient des de la primera producció industrial, respectivament.

10. Anàlisi

Degut a l'estructura i a l'ús dels PFASs, abans de començar amb el procés analític cal tenir en consideració alguns inconvenients que dificulten la seva quantificació. Els principals inconvenients són:

- contaminació,
- disponibilitat de blancs,
- pèrdues durant l'emmagatzematge i la conservació,
- variabilitat d'estàndards i
- els efectes de matriu.

Contaminació. La major part del material de laboratori està fet, o conté, fluoropolímers (Taniyasu *et al.* 2005). És imprescindible evitar l'ús d'aquests estris. És recomanable utilitzar en substitució material de polipropilè (Sinclair *et al.* 2006; Guo *et al.* 2010; Navarro *et al.* 2011) o polietilè d'alta densitat (Yu *et al.* 2009; Hu *et al.* 2010; Sun *et al.* 2011) per la presa de mostra i emmagatzematge de mostres líquides. Per matrius sòlides es tendeix a utilitzar el paper d'alumini. Per assegurar que aquests materials no contenen PFASs es procedeix també a la seva neteja abans de ser utilitzats amb aigua desionitzada i acetona (Skutlarek *et al.* 2006).

D'altra banda, la instrumentació analítica ha estat identificada com una de les principals fonts de contaminació durant el procés analític. Un clar exemple és el sistema de vàlvules desgasificadores en els cromatògrafs de líquids els quals estan fets de tefló o PVDF. És important portar un rigorós control del nivell de contaminació a partir de l'anàlisi de blancs juntament amb altres estratègies tals com:

- l'ús d'una columna cromatogràfica, o filtre, abans de la vàlvula d'injecció per tal d'incrementar el temps de retenció dels PFASs de la fase mòbil, o provinents del sistema desgasificador, i poder diferenciar entre els compostos del sistema i els de la mostra (Kuklenyik *et al.* 2004; Kuklenyik *et al.* 2005; Gledhill *et al.* 2007)
- sostracció de blancs o
- canviar tots els capil·lars del sistema fets de PVDF o Tefló per tubs de polièter èter cetona (Peek) o tubs metàl·lics.

Blancs. Degut a la contaminació implícita durant el procés analític, els blancs analítics són molt difícils d'obtenir (van Leeuwen *et al.* 2009). Aquest problema pot donar lloc a la sobreestimació de la concentració en mostres reals tal com s'ha pogut comprovar en diferents estudis interlaboratori duts a terme amb diferents tipus de matrius incloent peix, llet, sang i sèrum humà (Longnecker *et al.* 2008; Lindström *et al.* 2009; van Leeuwen *et al.* 2009; Keller *et al.* 2010). Es recomana, en aquests casos, dur a terme l'anàlisi dels solvents d'extracció i blancs de mostra en paral·lel a l'anàlisi de les mostres reals. No obstant, és difícil trobar una matriu blanca i, fins al moment, només hi ha material de referència certificat per llet i sèrum el qual pot ser utilitzat tant com a blanc com per a processos de validació (NIST).

Presa de mostra, pèrdues i conservació. La pèrdua d'anàlit per adsorció en les parets dels recipients de vidre ha estat observada per diferents autors (Holm *et al.* 2004; Martin *et al.* 2004) tot i que no s'espera en mostres reals amb matrius complexes (Kärrman *et al.* 2006). També s'ha observat adsorció en els recipients de polipropilè i polietilè per a PFASs de cadena curta en aigua tot i que són negligibles en mostres sòlides (Loveless *et al.* 2006; Frömel *et al.* 2010; Sun *et al.* 2011). D'altra banda, per evitar la pèrdua per volatilització de compostos com els fluorotelòmers o àcids carboxílics de cadena més curta (C4) es recomana evitar l'espai d'aire en els recipients de presa de mostra (Liu *et al.* 2005).

El transport de les mostres es duu a terme, en general, mantenint-les refrigerades. Durant l'emmagatzematge s'ha de prevenir qualsevol reacció de biodegradació, biotransformació o altres alteracions de la matriu encara que els PFASs siguin extremadament estables. Les mostres són conservades en el congelador o tan sols utilitzant solvents orgànics com acetonitril per aturar qualsevol procés biològic (Wang *et al.* 2005). No obstant, l'ús d'inhibidors biològics, com el formaldehid, poden suprimir la resposta de l'espectròmetre de masses (Schultz *et al.* 2006). D'altra banda, també s'ha observat que la disminució del pH augmenta l'associació de PFAS amb protons fent que siguin més fàcilment adsorbits en la superfície dels recipients (Dinglasan *et al.* 2004).

En el cas de mostres sòlides, el més usual és la completa eliminació de l'aigua continguda ja que depèn de la naturalesa de la mostra i podria interferir en els resultats. L'eliminació d'aigua es fa mitjançant diferents tècniques incloent l'assecat a temperatura ambient (Sinclair *et al.* 2006; Li *et al.* 2010), l'aplicació de temperatures suaus (40 ºC) o en el forn a 103 ºC (Guo *et al.* 2008; Navarro *et al.* 2011; Sun *et al.* 2011), així com la liofilització després de la congelació de la mostra (Guo *et al.* 2008) o la centrifugació per eliminar el líquid sobrenedant abans de la liofilització (Yu *et al.* 2009). Després de l'eliminació del contingut d'aigua les mostres s'homogeneïtzen i es guarden congelades en recipients de polipropilè i/o polietilè (Sinclair *et al.* 2006; Yu *et al.* 2009; Sun *et al.* 2011).

Puresa dels estàndards. La puresa dels estàndards representa un problema analític ja que hi ha diferents proveïdors que ofereixen diferents pureses i diferent composició isomèrica. Aquesta mescla isomèrica representa un problema durant la quantificació ja que tant mostra com estàndard contenen diferents isòmers. És important

l'ús d'estàndards purs, lineals o ramificats, per tal de confirmar la completa identificació així com la unificació de mètodes analítics per generar dades útils (Lindström *et al.* 2009; van Leeuwen *et al.* 2009; Keller *et al.* 2010).

Efecte matriu. Degut a la complexitat d'algunes matrius, moltes vegades és necessari una purificació exhaustiva per tal d'eliminar, o com a mínim disminuir, qualsevol efecte de la matriu. Un dels problemes més comunament associats a aquest efecte són la supressió i l'augment iònic de la senyal en l'espectròmetre de masses degut a la ionització feta amb electroesprai com a interfase durant la ionització de la mostra degut a la facilitat, o dificultat, de la mostra de ser ionitzada en comparació amb els PFASs (Zhang *et al.* 2010). Per tal de disminuir aquest efecte instrumental es pot addicionar la molècula en qüestió marcada isotòpicament (patró intern) per normalitzar el senyal (van Leeuwen *et al.* 2009). Per exemple, en el cas de l'anàlisi de PFOS en matrius biològiques s'ha observat un augment del senyal instrumental degut a una interferència amb la transició 499 > 80 amb l'àcid biliar taurodeoxicoholic (Hansen *et al.* 2001; Benskin *et al.* 2007; Keller *et al.* 2010). En aquests casos es procedeix a millorar la separació cromatogràfica entre ambdós compostos amb columnes cromatogràfiques més llargues o bé adequades per a una bona separació isomèrica tals com les recobertes amb pentafluorofenil.

10.1 Tractament de la mostra

Es poden diferenciar diferents tipus de tractament en funció de l'estat físic de la mostra (sòlid, líquid o gas). La Figura 1.9 del capítol 1 mostra l'esquema general dels diferents tipus de tractaments analítics.

10.1.1 Matrius sòlides i biològiques

Els principals mètodes es resumeixen a continuació i la Taula 1.10 del capítol 1 recull alguns exemples bibliogràfics.

Extracció per parell iònic. Aquest ha estat el primer mètode utilitzat per l'extracció de PFOA en plasma i orina (Ylinen *et al.* 1985). S'ha utilitzat per l'extracció de PFASs de matrius biològiques i ambientals. Actualment l'ús del parell iònic amb tetrabutil amoni o metil tert-butil amoni ha quedat limitat a matrius molt complexes degut a certes limitacions com la co-extracció de lípids i altres constituents de la matriu fent necessari un altre procés de purificació així com el baix interval de percentatges de recuperació (<50 - >200%).

Extracció sòlid-líquid. Aquest és el mètode més utilitzat per extraure PFASs poc volàtils de sòlids i matrius biològiques per la facilitat i simplicitat de l'extracció així com dels solvents necessaris. En general s'utilitza metanol, acetonitril o una mescla d'ambdós mitjançant agitació o bé extracció assistida per ultrasons a diferents temperatures per sòlids (Powley *et al.* 2005; Bossi *et al.* 2008). En el cas de mostres biològiques s'utilitza metanol o una mescla d'hexà i acetona en ultrasons o agitant. Posteriorment es procedeix a fer una purificació per extracció en fase sòlida (SPE) (Lau *et al.* 2004; Haug

et al. 2009). Sovint s'utilitza un procés extra de purificació amb sulfat de sodi o atac suau àcid per eliminar el contingut de lípids (Tittlemier *et al.* 2011).

En els últims anys s'han desenvolupat tècniques d'extracció més sofisticades com l'extracció per líquids pressuritzats per comptes de la tècnica Soxhlet ja que l'alta pressió permet disminuir el temps d'extracció (Schröder 2003; Kunacheva *et al.* 2011). En general l'extracció es fa per metanol en el cas dels PFASs i és necessari una posterior purificació per SPE. Aquesta tècnica permet obtenir bons percentatges de recuperació dels anàlits.

***Extracció sòlid-líquid acidificat*.** En general aquesta extracció es fa mitjançant àcid fòrmic o acètic en ultrasons. Després de la digestió el sobrenedant es centrifuga i es purifica per SPE i filtració (Calafat *et al.* 2007; Loganathan *et al.* 2007). Aquesta tècnica s'utilitza per precipitar les proteïnes de les matrius biològiques i, molt sovint, després de la precipitació la mostra és centrifugada i s'anàlitza directament el sobrenedant (Calafat *et al.* 2007). S'obtenen uns bons percentatges de recuperació i millors resultats que l'extracció per formació del parell iònic.

Extracció sòlid-líquid alcalí. En aquest tipus d'extracció s'utilitza metanol amb hidròxid de sodi o d'amoni per procedir a fer la precipitació de proteïnes en mostres de llet i peix entre d'altres. En general, després de la precipitació cal fer una purificació per SPE (Pico *et al.* 2010). S'obtenen millors percentatges de recuperació que amb l'extracció sòlid-líquid sense modificadors.

10.1.2 Matrius aquoses

Les principals tècniques d'extracció es basen en SPE prèvia filtració de la mostra. Sovint la filtració suposa la pèrdua d'anàlit per adsorció en els filtres i/o la contaminació de la mostra. Com a alternativa es fa la separació dels sòlids per centrifugació (Schultz *et al.* 2006). Actualment existeixen dues metodologies per SPE que s'utilitzen per a PFASs: SPE fora de línia i SPE en línia. La Taula 1.11 del capítol 1 recull un resum de les tècniques trobades en la bibliografia.

Mètodes fora de línia. En funció de la polaritat dels PFASs s'utilitzen diferents tipus de cartutxos d'extracció incloent intercanvi iònic (per general aniònic) com els Oasis WAX amb bons percentatges de recuperació per cadenes llargues i curtes o cartutxos amb característiques hidrofíliques i lipofíliques com els Oasis HLB per a PFASs menys polars de cadena llarga. Per a PFASs de mitjana polaritat o baixa es procedeix a fer l'extracció per SPE amb solvents apolars com l'hexà o l'acetona (Pérez *et al.* 2012).

Mètodes en línia. Durant els últims anys s'han desenvolupat diferents mètodes en línia degut als avantatges que suposa: reducció de la manipulació de mostra i, per tant, la robustesa del mètode així com la disminució del temps d'anàlisis i del volum de mostra. Hi ha dues metodologies diferents per a l'extracció en línia: SPE i cromatografia de flux turbulent.

- SPE en línia acoblada al LC-MS/MS mitjançant un flux laminar. S'utilitza per la pre-concentració i l'extracció de mostres aquoses (Wilson *et al.* 2007; Gosetti *et al.* 2010) i biològiques com sang i llet després d'haver fet una precipitació de les proteïnes (Kuklenyik *et al.* 2005; Apelberg *et al.* 2007; Calafat *et al.* 2007; Wilson *et al.* 2007; Haug *et al.* 2009). Les fases estacionàries inclouen C18 i modificacions per poder rebre grans volums d'aigua com les Poros HQ. Entre aquests mètodes d'extracció s'ha d'incloure la tecnologia EQuanTM de Thermo Fisher la qual permet l'extracció de grans percentatges d'aigua i la reutilització de la columna fins a 1000 vegades.

- Cromatografia de flux turbulent acoblada al LC-MS/MS. El flux turbulent genera turbulències amb flux entre 1,5 i 3 mL/min que es tradueixen en un seguit de moviments aleatoris de l'extracte dins la columna. Els anàlits queden retinguts als centres actius de la columna degut a la diferència en la difusió dels compostos grans com proteïnes i els més petits. Aquesta tècnica s'ha utilitzat per aigües (Takino *et al.* 2003) tot i que és més eficient per mostres biològiques com orina (Perez *et al.* 2012). Per aquest procés es requereix poc volum de mostra (10-20 µL) ja que és una tècnica d'extracció, no de pre-concentració. D'altra banda, les columnes poden ser utilitzades fins a 500 vegades presentant avantatges econòmics.

10.2 Anàlisi instrumental

Els primers estudis de PFASs es van realitzar per Ressonància Magnètica Nuclear (Taves 1968). Més tard es va començar a estudiar la possibilitat d'anàlitzar aquests compostos per cromatografia de gasos (GC) acoblada a detectors d'emissió atòmica per al PFOS (Hagen *et al.* 1981) i l'espectrometria de masses (Pankow *et al.* 1998), Ressonància d'Espí Electrònic (Romanelli *et al.* 1994) o per cromatografia iònica de combustió (Miyake *et al.* 2007a; Miyake *et al.* 2007b). No obstant, les tècniques instrumentals més utilitzades es basen en GC i LC acoblades a diferents espectròmetres de masses, tècniques que es descriuen a continuació.

10.2.1 Separació

La cromatografia per gasos ha estat utilitzada per l'anàlisi de PFASs àcids de cadena curta (C3 – C5), els compostos més volàtils i, en un primer moment, per al PFOA. La Taula 1.12 del capítol 1 recull un resum de la bibliografia. No obstant, donat la poca volatilitat dels PFASs més estudiats (PFOA i PFOS), l'ús de LC-MS/MS és el més comú oferint alta sensibilitat, robustesa, precisió i exactitud.

La separació dels anàlits en LC es fa, en general, mitjançant columnes de fase reversa amb fase estacionària de C18 i C8. En la separació d'isòmers estructurals es tendeix a augmentar la temperatura de la columna per modificar les retencions en comptes d'utilitzar columnes més específiques (Kuklenyik *et al.* 2004). Per la separació de PFASs de cadena més curta (C2-C3) l'ús de C18 és problemàtic ja que aquests compostos no queden retinguts en la columna i són eluïts amb el volum mort o, en cas de quedar retinguts, presenten pics deformats i poc resolts. Alguns autors han provat de

millorar la retenció formant el parell iònic entre la fase mòbil i els compostos fluorats més hidrofílics. Tot i així, l'ús d'aquesta tècnica dóna sobreestimació en la quantificació dels compostos similars als utilitzats per formar el parell iònic (àcid trifluoroacètic o perfluoroheptanòic) i queda totalment descartada en LC-MS degut a la supressió iònica (Gustavsson *et al.* 2001).

Les fases mòbils més utilitzades són aigua i metanol o acetonitril, o una combinació d'ambdós, com a fase orgànica. Sovint s'addicionen modificadors per augmentar la resolució cromatogràfica dels pics i millorar l'eficiència d'ionització en les fonts d'ionització en incrementar la conductivitat del solvent.

10.2.2 Interfases: fonts d'ionització

El pre-requisit més important per l'anàlisi d'ions per MS és l'èxit en convertir les molècules en molècules carregades (o fragments carregats) en estat gasós dins la font d'ionització. Per tant, és necessari discernir abans de l'estudi quina és la interfase òptima per a l'anàlisi en concret buscant la màxima eficiència en la ionització, elevada estabilitat amb la mínima energia cinètica possible i la mínima dispersió angular dels ions així com la possibilitat de poder ser acoblada a diferents espectròmetres de masses (Cooks *et al.*, 1997).

Les principals fonts d'ionització estan resumides en la Taula 1.13 del capítol 1. En l'anàlisi de PFASs tant en matrius ambientals com biològiques, la tècnica més utilitzada és la LC amb una interfase d'electroesprai (ESI) operant en mode negatiu. No obstant, per a estudis més específics, també s'ha utilitzat la ionització química a pressió atmosfèrica (APCI) i l'ESI en mode positiu. Per als estudis dels PFASs més volàtils o semi-volàtils, com els fluorotelòmers, la tècnica cromatogràfica utilitzada és la GC amb una interfase de ionització química negativa (NCI) o d'impacte electrònic (EI).

10.2.3 Detecció

En l'estudi de PFASs la tècnica més utilitzada per la detecció és l'espectrometria de masses amb diferents tipus d'anàlitzadors. No obstant, cal considerar els efectes matriu en l'estudi de mostres complexes (supressió i increment de la senyal iònica) així com de possibles interferències amb alguns àcids biliars en el cas de mostres biològiques tal com s'ha descrit en la secció dels majors problemes analítics.

Actualment l'estudi de PFASs es fa per MS^2 per tal de complir amb els criteris mínims de confirmació analítica. Aquesta tècnica està acoblada a LC amb una interfase d'electroesprai operant en mode negatiu en la majoria dels casos. No obstant, per a casos més concrets de PFASs s'ha utilitzat el mode positiu així com la ionització química a pressió atmosfèrica. En el cas de la GC, les interfases més utilitzades són la ionització química negativa o el impacte electrònic. La Taula 1.13 del capítol 1 resum alguns dels treballs bibliogràfics. Ambdues tècniques cromatogràfiques permeten l'acoblament a diferents anàlitzadors incloent:

Trampa Iònica (IT). La IT va ser el primer anàlitzador per MS que es va desenvolupar. La IT té una alta eficiència analítica degut a l'estructura de la trampa la qual permet també fer experiments MS^n per múltiples fragmentacions donant informació estructural. No obstant, aquest anàlitzador és poc utilitzat per la baixa resolució i la possibilitat de que es donin reaccions ió-molècula dins la trampa. La IT pot ser acoblada a GC i LC.

Triple Quadrupol (QqQ). L'anàlitzador QqQ millora la sensibilitat en front de la IT. Té un mode operatiu senzill i permet determinar diferents compostos en diferents matrius ja que és molt selectiu. Els anàlisis es fan en general per mode de monitorització de reaccions seleccionades (SRM) i permet ser acoblat tant a LC com a GC.

Híbrid Quadrupol-Trampa d'ions ***Lineal (QqLIT o QTrap).*** L'alta sensibilitat i selectivitat del quadrupol i l'alta eficiència de la IT fa que la QqLIT sigui un dels instruments més sensibles per estudis ambientals i biològics, sempre parlant en termes de baixa resolució. Generalment està acoblat a LC. L'ús de la IT permet realitzar experiments de MS^3 donant informació estructural dels ions prèviament seleccionats en el quadrupol. Aquesta tècnica s'utilitza molt en estudis d'elucidació estructural i per a una primera identificació de compostos desconeguts gràcies als modes de crivatge que ofereixen informacions complementaries si es fa en el quadrupol o en la trampa d'ions lineal.

Híbrid Quadrupol-Temps de Vol (QqTOF). La combinació de l'alta selectivitat dels quadrupols i la mitjana-alta resolució del temps de vol permet anàlisis acurats de massa exacta. Aquest anàlitzador representa una eina imprescindible en estudis d'identificació de molècules com per exemples en estudis de degradació.

Orbitrap-Transformada de Fourier. Aquest anàlitzador es fonamenta en els principis de ressonància del Sincrotró i la Transformada de Fourier. Els ions són atrapats en un camp electrostàtic entre els elèctrodes externs i l'intern i es mouen per forces centrífugues. L'Orbitrap funciona com a anàlitzador i detector i, en general, està acoblat a LC. Els ions es mouen en cicles al voltant de l'elèctrode central per el camp elèctric i per oscil•lació (Makarov *et al.* 2006). Aquesta tècnica permet realitzar estudis d'alta resolució en la identificació de compostos.

S'han utilitzat altres tècniques instrumentals s'han utilitzat per a estudis més específics com la radioquímica (Liu *et al.* 2010).

11. Objectius

En el context actual, els principals objectius d'aquesta tesi doctoral han estat l'estudi de compostos perfluorats en el medi ambient i en aliments així com avaluar la presència en mostres humanes. Amb aquestes finalitats, el primer objectiu va ser el desenvolupament i validació de diferents mètodes analítics per a l'estudi de compostos perfluoroalquílics en diferents matrius ambientals, aliments i matrius humanes així com l'avaluació de la presència d'aquests compostos en el medi ambient, diferents aliments i mostres humanes i, finalment, l'impacte humà que se'n deriva.

Els objectius específics van ser:

1. Desenvolupar i validar mètodes analítics basats en extraccions en línia i fora de línia amb l'anàlisi per LC acoblat a diferents espectròmetres de masses per l'estudi de PFASs en aliments (peix, aliments infantils i aigua de beguda), matrius ambientals (sediments i aigua superficial) i matrius biològiques (llet materna i sang).

2. Estudiar la presència de PFASs en el medi ambient.

3. Estudiar els PFASs en diferents rutes d'exposició humana.

4. Estudiar la possible acumulació de PFASs en mostres biològiques humanes.

CAPÍTOL 2: Presència de PFASs en el medi ambient i el cicle de l'aigua

S'han identificat diferents fonts i modes de distribució de PFASs que afecten el medi ambient incloent el cicle de l'aigua, aire i terra: producció, ús, plantes depuradores i la distribució global. La figura 2.1 del capítol 2 mostra els diferents processos entre els quals inclouen: producció de PFASs i productes que els contenen, usos dels materials que contenen com estris de cuina, teixits de roba o xampús, residus urbans, agrícoles i industrials, el seu abocament i els usos que se'n fa com en l'agricultura, reutilització en aigües potables o d'altres i, finalment, la distribució global un cop introduïts en el medi tal com s'ha descrit anteriorment.

1. Cicle de l'aigua

Degut al desconeixement actual sobre l'impacte humà que es deriva de la presència dels PFASs en l'aigua, és necessari l'estudi de la distribució d'aquests anàlits en tot el seu cicle per tal de contribuir en el coneixement dels efectes adversos associats als nivells trobats.

Per aquest motiu, dins el marc de la tesi es va fixar com a objectiu estudiar la presència de 21 PFASs en 148 mostres provinents de tot el cicle de l'aigua incloent: aigua de sortida de depuradora, aigua de riu superficial el qual rep l'abocament de les depuradores, aigües de pou, aigua dins el tractament de potabilització des del qual es capta aigua del riu, aigua potable d'aixeta i diferents aigües minerals embotellades. Les mostres corresponen a diferents punts d'Espanya i Alemanya. Es van escollir aquests dos països ja que tenen diferent clima i, per tant, diferent règim fluvial per tal de discernir diferents patrons de comportament dels PFASs. Part d'aquest treball ha estat realitzat al Hoschule Fresenius, al laboratori del professor Thomas P. Knepper, durant una estada de 4 mesos.

La metodologia aplicada ha estat desenvolupada dins el marc de la tesi i es basa en l'extracció en fase sòlida en línia mitjançant la tecnologia E-QuanTM en un sistema

Thermo Scientific Aria TLX-1 equipat amb una columna Hypersil GOLD aQ (2,1 x 20 mm, 12 µm ; Thermo Fisher Scientific). L'extracció de 5 mL d'aigua es fa a un flux de 1,75 mL/min durant 3,15 min. Aquest tipus de columna reté els compostos polars de mostres amb grans volums d'aigua i pot ser utilitzada per més de 1000 extraccions. Un cop retinguts els anàlits, aquests són eluïts a la columna cromatogràfica amb la fase mòbil consistent en aigua (20 mM acetat d'amoni) i metanol (20 mM acetat d'amoni) en condicions de gradient i de flux normal. El mètode es va comparar amb l'extracció en fase sòlida fora de línia mitjançant cartutxos Oasis WAX i validat seguint la Decisió de la Comissió Europea 2002/657/EC (Commission Decision 2002/657/EC). El mètode té uns límits de detecció entre 2,7-11 ng/L, percentatge de recuperació entre 50-120%, i desviació estàndard relativa (RSD) entre 1-25% (publicació número 2).

Els resultats més destacats són:

- Els compostos més freqüents són els àcids, especialment els de cadena curta (C4 i C7) i el PFOA així com el ió PFOS. L'augment de la concentració de PFASs de cadena més curta al llarg dels anys és una conseqüència de la substitució industrial que s'està fent dels PFASs de cadena més llarga (PFOA i el PFOS).

- Les concentracions més elevades s'han trobat en aigua de sortida d'EDAR i de riu el qual rep l'abocament dels efluents de les EDARs. Els nivells són de l'ordre de ng/L - µg/L.

- En el cas de l'estació potabilitzadora, els PFASs s'han detectat en totes les mostres posant de manifest la captació d'aquests anàlits des del riu i la difícil eliminació d'aquests compostos de l'aigua captada.

- L'aigua de sortida de la potabilitzadora arriba al consumidor via l'aigua d'aixeta. Aquesta presenta nivells de PFASs entre 0,03 – 258 ng/L essent el PFOS l'anàlit a concentracions més elevades arribant a superar en 6 casos en concret els valors provisionals de salut establerts per la USEPA (2002; 2005). Aquestes mostres provenen de la mateixa població propera a l'àrea metropolitana de Barcelona. Tot i que els nivells no són molt elevats, s'ha de considerar l'exposició diària per la qual encara no hi ha establert cap límit.

- Referent a l'aigua embotellada, tan sols s'ha detectat àcid perfluoroheptanòic en una mostra d'Alemanya. La resta de mostres estaven per sota el límit de quantificació.

2. Estacions d'aigua depuradora (EDAR)

Els compostos PFASs són recalcitrants en les EDARs tal com s'ha vist en el treball anterior. Per aquest motiu es va optar per estudiar la presència d'aquests compostos en mostres de fang de depuradora de diferents EDARs catalanes degut a l'ús futur que se'n pot fer en aplicar-lo, per exemple, com a font de nutrients en camps de cultiu arribant a ser una font de contaminació dels aliments.

Els objectius concrets han estat l'estudi de 18 PFASs en 5 fangs de depuradora mitjançant extracció per líquids pressuritzats i anàlisi per LC-MS/MS amb un mètode desenvolupat, optimitzat i validat dins el marc de la tesi. Els límits de detecció oscil·len entre 15 i 79 ng/Kg, la recuperació entre 79 i 111%, i la RSD entre 4 i 25% i que està descrit en la publicació 3.

Els resultats més destacats són:

- Els PFASs estan presents en totes les mostres. La seva presència en els fangs i en les aigües de sortida de depuradora (treball anterior) remarquen el caràcter recalcitrant d'aquests anàlits. No hi ha una eliminació sinó una redistribució en les aigües i els fangs.

- El PFOA i el PFOS són els compostos més recalcitrants.

- El PFOS és l'anàlit que es troba a concentracions més elevades (53,0-121 µg/Kg).

- L'aplicació d'aquests fangs en terres d'agricultura pot donar pas a una contaminació dels aliments i inclús de les aigües freàtiques per una mobilització dels anàlits.

3. Presència en el medi ambient

La distribució global dels PFASs degut a les seves característiques fisicoquímiques ha fet que arribin en zones remotes com ara l'Àrtic i l'Antàrtic, així com que també estiguin presents en oceans.

En aquest context, i dins el marc de la tesi, es va proposar l'estudi d'aquests anàlits en mostres provinents d'una zona poblada de la Patagonia Argentina (Ushuaia) i d'una àrea remota com l'Antàrtida. La presa de mostra es va dur a terme entre febrer i març del 2010 en el marc dels "Premios Antárticos de Ciencia, Tecnología y Meidoambiente" organitzats per l'empresa Àrea Tàctica i en els quals es va participar amb un projecte titulat "Anàlisis de compostos perfluorats en mostres mediambientals i de biota de Terra del Foc i l'Antàrtida". Entre les mostres es van incloure sediments, peixos, algues, guano de diferents aus i mostres de pingüí. Els resultats es recullen en la publicació número 4.

D'altra banda, en aquests últims anys ha esta creixent la preocupació pels residus de plàstics flotants que s'han trobat en els oceans degut a l'impacte en la flora i la fauna marina. En aquest cas l'objectiu era fixat va ser l'estudi de 21 PFASs en mostres de sediment marí i plàstics flotants pertanyents a la mateixa zona del Mar Mediterrani (part de Grècia) per determinar-ne la concentració i qualsevol possible relació entre les concentracions.

Els resultats més destacats són els següents:

<u>PFASs en mostres provinents de la Patagonia Argentina i la península Antàrtica</u>:

- Els PFASs estan presents tant en zones urbanitzades (Ushuaia) com en zones remotes (Antàrtida). No obstant, els nivells en les zones urbanitzades per a mostres similars és major (centenars de ng/Kg) que en mostres de zones remotes (pocs ng/Kg).

- La bioacumulació d'aquests anàlits es confirma a través dels nivells trobats en les mostres de biota (peix, guano, pingüí).

- Els resultats donen suport a les hipòtesis desenvolupades sobre el transport d'aquests anàlits via oceànica a través de la corrent termohalina i altres fenòmens físics i el transport atmosfèric a via la "cold condensation".

PFASs en sediments marins i plàstics flotants de la costa grega:

- Els resultats en les mostres de plàstics flotants i sediments marins mostren un mateix perfil de PFASs. Aquest fet constata la mateixa procedència de PFASs. Els plàstics adsorbeixen els PFASs de l'aigua i poden ser un altre medi de transport d'aquests compostos.

- Les hipòtesis apunten la indústria i zones molt transitades (cimenteres, ports, aeroports, etc) com a principal font de contaminació.

CAPÍTOL 3: PFASs a través dels aliments

La ingesta d'aliments és considerada una de les principals vies d'entrada de PFASs en l'organisme humà juntament amb el consum de begudes i la inhalació. És important conèixer les principals vies de contaminació i el potencial toxicològic associat degut al risc humà que implica. Per aquest motiu és necessari conèixer les concentracions d'aquests compostos en aliments així com el desenvolupament de mètodes analítics òptims, ràpids i eficients per al seu estudi.

En aquest context, els objectius fixats per aquesta part de la tesi han estat el desenvolupament d'un mètode analític per peixos, la comparació interlaboratori d'un mètode d'extracció de PFASs en peix i llet basat en extracció per carbó actiu i anàlisi per LC-MS/MS i, finalment, l'estudi de diferents aliments.

1. Desenvolupament del mètode analític

1.1 Desenvolupament d'un mètode per l'extracció de peix

El principal objectiu va ser el desenvolupament d'un mètode analític basat en l'extracció de peix per líquids pressuritzats ja que aquest aliment és una de les principals vies d'introducció de PFASs en la dieta. En un primer moment el mètode va ser

desenvolupat per a l'estudi de 8 compostos (els resultats es recullen en la publicació número 5). Posteriorment, aquest va ser ampliat a 21 compostos i va haver de ser modificat per tal d'obtenir l'extracció òptima de tots els anàlits amb uns límits de quantificació entre 3 i 50 ng/Kg, recuperacions entre 85 i 102% i desviació estàndard relativa interdia de 7 – 15%. Aquest últim es basa en la digestió alcalina seguit d'extracció en fase sòlida.

1.2 Anàlisi instrumental

Emmarcat en aquest objectiu s'ha procedit a la comparació de diferents instruments analítics basats en LC-MS(/MS) per a l'anàlisi de PFASs en aliments. S'han comparat tres anàlitzadors: trampa d'ions, triple quadrupol i un híbrid quadrupol-trampa d'ions lineal. Els resultats obtinguts es mostren en a publicació número 6.

Basant-nos en els resultats obtinguts, la trampa iònica és una eina molt útil en l'estudi de compostos desconeguts per poder fer cribatges en el mode operatiu "full scan". També dóna molta informació estructural ja que es poden realitzar diferents fragmentacions de massa, arribant a MS^n, ja sigui per reaccions de massa seleccionada (SIM) o bé per "data dependent scan". No obstant, és un instrument poc sensible i poc selectiu en el cas de voler quantificar un compost però molt útil per fer elucidacions estructurals. D'altra banda, el triple quadrupol permet fer també estudis de cribatge operant en mode "full scan" donant l'opció de treballar amb aquest mode operatiu en ambdós quadrupols obtenint també un espectre complert dels ions producte. La informació espectral en aquest cas és complementària a l'obtinguda amb la trampa d'ions, degut al diferent mode operatiu. No obstant, no dóna gaire informació estructural tot i que permet treballar en mode "selected reaction monitoring" (SRM) i quantificar els compostos gràcies a la seva selectivitat. En general, aquests instruments són més sensibles que les trampes d'ions.

La combinació d'ambdós anàlitzadors la trobem amb l'híbrid quadrupol-trampa d'ions lineal. Aquest espectròmetre de masses permet fer estudis de cribatge en mode "full scan" en el quadrupol i en la trampa d'ions donant així informació complementària. També permet operar en mode SRM i, per tant, quantificar els anàlits i, finalment, també existeix la possibilitat de fer MS^3 gràcies a la trampa d'ions. Aquesta última característica fa que sigui un instrument molt útil per a l'elucidació estructural.

2. Validació d'un mètode ràpid i eficient per l'extracció de mostres de peixos i de productes làctics

En aquest cas es va marcar com a objectiu l'organització, preparació de les mostres i avaluació interlaboratori d'un mètode d'extracció i purificació de PFOA, PFOS i perfluorooctasulfonamida (FOSA) en mostres de llet i peix. El procediment es basa en l'extracció amb metanol, purificació amb carbó actiu i posterior determinació per LC-MS/MS. Aquest procediment va ser desenvolupat i validat en el marc del projecte europeu CONffIDENCE a nivell intralaboratori (Institute of Chemical Technology, Prague) i per un laboratori extern (IDAEA-CSIC). A continuació, per tal d'avaluar el procediment

analític a major escala, es va procedir a fer un exercici interlaboratori en el qual el laboratori de l'IDAEA-CSIC n'era el centre operatiu.

Les mostres de llet i peix es van preparar fortificant les matrius a dues concentracions diferents: nivell proper al límit de quantificació del mètode i nivell sobre els 20 ng/mL en peix i 20 ng/g en peix. També es van preparar en paral·lel mostres de blanc. Totes les mostres es van pasteuritzar i enviar a -20ºC als 8 participants, incloent instituts de recerca, universitats i laboratoris d'anàlisi.

Els resultats s'han avaluat mitjançant diferents paràmetres estadístics segons les directrius de l'apèndix D de la 2002 Association of Analytical Communities International (2002 AOAC): mitjana, biaix, repetibilitat i reproductibilitat, valor de HORRAT i Z-Scores. Els resultats mostren una repetibilitat inferior al 14%, reproductibilitat bona per a la mescla incògnita i per a les mostres fortificades a nivells alts, però molt baixa per a les mostres fortificades a nivells baixos. D'altra banda, els paràmetres HORRAT mostren que el mètode és reproduïble, tot i que el càlcul més restrictiu dels Z-Scores mostra una sobreestimació i subestimació de les concentracions a nivells de fortificació baixos, mentre que els valors són entre 2 i -2 per a les mostres fortificades a nivells alts.

La principal conclusió és que el mètode pot ser aplicat en l'estudi de PFOA, PFOS i FOSA en mostres de peix i llet que tinguin una concentració superior a 1 ng/g o ng/mL, respectivament.

3. PFASs a través de la dieta

Donat que la ingesta d'aliments és una de les principals vies d'exposició de PFASs per als humans, els principal objectiu va ser la valoració de la concentració d'aquests compostos en mostres de consum diari. Les matrius escollides van ser el peix, ja que ha estat descrit com un dels principals contribuïdors als nivells de PFASs en la dieta, i aliments infantils (llet materna, llet en pols i cereals) ja que els infants són població sensible.

Per a l'estudi de mostres de peix es van anàlitzar 21 PFASs en 139 mostres provinents de diferents mercats Europeus i amb diferent origen (salvatge o piscifactoria), incloent 18 espècies diferents i diferents famílies (bivalves, llucet, lluç, bacallà, arengada, salmó, truita, tonyina enllaunada i pangasius del Vietnam). Els resultats mostren concentracions entre 0,12 i 54 ng/g, on les més elevades corresponen a l'àcid perfluorohexanòic en unes mostres d'escopinyes de piscifactoria del Mar Cantàbric i Mediterrani, així com de PFOS en truites congelades del mercat de Dinamarca. L'avaluació dels resultats mitjançant l'anàlisi per components principals denota que no hi ha diferència en funció de l'origen (salvatge o piscifactoria) tot i que s'observa una lleugera tendència en la concentració de PFASs i el tipus d'anàlit en funció de la regió europea de pesca i de la família.

En el cas d'aliments infantils, s'han anàlitzat 8 PFASs en 20 mostres de llet materna, tres de llet en pols i dues de cereals infantils. Els resultats en llet materna constaten la bioacumulació d'aquests anàlits, ja que s'han trobat en totes les mostres a nivells entre

15 i 907 ng/L. Els compostos a concentracions més elevades han estat el PFOA, l'àcid isopropil-perfluorononanòic i el PFOS. Un dels motius de la seva presència en aquest tipus de matriu és que aquests compostos s'associen a proteïnes de baix pes molecular. En el cas dels cereals i llet en pols, aquests compostos també estan a nivells per damunt el límit de quantificació en totes les mostres (44 - 1100 ng/Kg) i, en general, la concentració en llet en pols és superior a la concentració en cereals. En aquest cas, la procedència de la contaminació poden ser per migració des dels recipients d'emmagatzematge i/o el procés de producció de l'aliment. Aquest treball està compilat en la publicació número 7.

Per tal d'avaluar el risc associat a la ingesta d'aquests aliments, tant peixos com aliments infantils, s'ha calculat l'índex de risc per al PFOA i el PFOS segons els barems de l'EFSA (EFSA 2008). Els valors són inferiors a 1 en tots els casos amb 2 excepcions, que corresponen a mostres de llet materna. Tot i que el risc associat no és molt elevat, s'ha de considerar la ingesta continua d'aquests aliments i, per tant, l'exposició crònica, igual que en el cas de l'aigua de beguda.

CAPÍTOL 4: Acumulació en humans

Els capítols 2 i 3 posen de manifest els diferents tipus d'exposició humana als PFASs. En el capítol 4 els principals objectius van ser avaluar la presència en l'ésser humà per poder estudiar l'acumulació. Es va escollir l'estudi de sang de cordó umbilical per dos motius: la transferència de PFASs transplacenta és una de les principals vies d'exposició d'aquests compostos en nadons i pertany al sistema reproductiu humà. D'altra banda, també es va procedir a avaluar l'acumulació humana a partir dels resultats de l'anàlisi de mostres de llet materna obtinguts en la publicació número 7, pertanyent al capítol 3 de la tesi.

1. Presència de PFASs en sang de cordó umbilical

Dins el marc de l'estudi de 60 mostres de sang de cordó umbilical provinent de diferents donants de Barcelona (Catalunya) i Heraklion (Creta) es va proposar el desenvolupament d'un mètode analític ràpid i robust en el qual fos necessari poc volum de mostra mitjançant l'extracció en línia per cromatografia de flux turbulent en l'equip Thermo Scientific Aria TLX-1, acoblat a LC-MS/MS per 18 PFASs. El mètode va ser validat segons la Decisió 2002/657/EC, obtenint uns límits de detecció entre 3 i 760 ng/L, recuperació de 50 – 126%, i desviació estàndard relativa interdia de 6 – 23%.

Els resultats del treball de l'estudi en sang de cordó umbilical es recullen en la publicació científica número 8. En general, les concentracions més elevades corresponen a l'ió perfluorohexasulfat i al PFOS (µg/L), essent ambdós els més ubiqües juntament amb el PFOA. Aquests resultats constaten el pas de PFASs durant l'embaràs via transplacenta tot i que no s'ha pogut establir una relació directa entre els hàbits materns i els nivells de PFASs. La presència d'aquests anàlits en el sistema reproductor ha quedat palès també per els nivells de PFASs en les mostres de llet materna. D'altra

banda també s'ha avaluat la possible relació entre l'escurçament de la distància ano-genital (AGD) amb l'exposició de disruptors endocrins ja que la distància està regulada per la dihidrotestosterona. La relació estudiada entre la AGD i les concentracions de PFOA i PFOS en les mostres de sang suggereix una lleugera tendència a l'escurçament de la distància en funció dels nivells d'aquests dos anàlits.

2. Bioacumulació humana i relació entre les fonts d'exposició i el medi ambient

Per tal d'avaluar la relació entre els nivells de PFASs trobats en les mostres humanes i les diferents fonts d'exposició estudiades en aquesta tesi es va procedir a fer l'anàlisi per components principals amb tots els resultats trobats.

Els resultats mostren l'alta contribució de PFASs de cadena més curta (C4 – C5), tant àcids com sulfats, està directament relacionada amb el consum d'aigua, peix i bivalves. En el cas concret de l'aigua es pot extrapolar que els PFASs en sang venen del seu consum.

La relació entre els humans i el medi ambient s'ha avaluat a partir de la presència de PFASs en depuradores, mostres d'aigua de riu i biota. Tot i que els PFAS en aquestes matrius estan directament relacionats amb la seva producció ambiental, l'ús de productes fets de PFASs o dels residus humans, caldria fer un estudi més exhaustiu sobre els residus generats pels humans i la presència d'aquests compostos en aquestes matrius ambientals i de residus, com per exemple orina entre d'altres.

CAPÍTOL 5: Propietats fisicoquímiques i biodegradació aeròbica dels àcids perfluorofosfònics

Els compostos PFASs són un problema actual degut a la seva àmplia presència en el medi, animals i humans, la poca degradabilitat i el poc coneixement toxicològic que se'n té, així com de les seves propietats fisicoquímiques experimentals. Per aquest motiu és necessari encarar els estudis futurs en aquest sentit.

Dins el marc de la tesis s'ha proposat estudiar diferents propietats fisicoquímiques i la possible biodegradació de tres compostos perfluorofosfònics: àcid perfluorohexafosfònic (PFHxPA), àcid perfluorooctafosfònic (PFOPA) i àcid perfluorodecafosfònic (PFDPA). Part d'aquest treball ha estat realitzat al Hoschule Fresenius, al laboratori del professor Thomas P. Knepper, durant una estada de 4 mesos i l'Institut Català per la Recerca de l'Aigua.

Els compostos perfluorofosfònics han estat escollits ja que s'utilitzen com a additius en papers de contacte amb aliments i en pesticides. Aquests compostos poden migrar del paper als aliments i entrar al cos humà. Els estudis de bioacumulació realitzats en rates mostren la seva poca eliminació. És per aquest motiu que és important caracteritzar aquests compostos.

En aquest context, els principals objectius han estat: i) determinar les propietats fisicoquímiques com la constant de distribució aigua/hexà i aigua/octanol segons el procediment OECD 123, i el percentatge d'adsorció en fang i fang activat de depuradora d'EDAR segons el procediment OECD 106; ii) estudiar l'adsorció en l'alga *Desmodesmus subspicatus* per estudis de biodisponibilitat; iii) estudiar la biodegradació i possible formació de productes de transformació per els organismes de les aigües de sortida d'EDAR segons el procediment OECD 309.

Els paràmetres fisicoquímics s'han determinat experimentalment. Quan es comparen amb els paràmetres calculats amb alguns programes d'estimació de propietats (VCCLAB Partners 2007) són molt diferents, posant de manifest la dificultat en estimar les propietats d'aquests compostos i la necessitat del desenvolupament de programes més restrictius per a aquests. En el cas de l'adsorció en fangs, s'ha pogut veure que les cadenes més llargues assoleixen abans l'equilibri i que en el cas del PFHxPA aquest no s'assoleix mai. D'acord amb els resultats experimentals trobats, el PFHxPA es trobaria en major proporció a les aigües mentre que el PFOPA i el PFDPA es trobarien majoritàriament en els fangs i sediments.

Pel que fa als estudis de biodisponibilitat, després d'un dia d'exposició, les algues perden el color verd en presència de PFDPA, el que implica una disminució en l'absorbància. Aquest fet pot ser degut a la inhibició de la síntesi de clorofil·la per part del PFDPA o bé la mort de les algues.

Els estudis de biodegradació mostren uns resultats interessants des del punt de vista del PFDPA. En presència d'aquest anàlit, el segon dia d'exposició es comença a generar un biofilm, el qual està relacionat amb la desaparició d'aquest compost a l'aigua, el qual

només es pot recuperar amb la destrucció completa del biofilm. S'ha provat d'identificar l'organisme responsable de la generació del biofilm mitjançant la Reacció en Cadena de Polimerasa. Els resultats no assenyalen a un únic organisme que es pugui relacionar amb la presència de PFDPA, però sí que es pot descartar una alga, ja que els experiments es van dur a terme en la foscor. S'han trobat alguns possibles candidats en els què el PFDPA, degut a la seva similitud amb els fosfolípids de les membranes cel·lulars, pot ser assimilat per formar aquesta paret. El capítol 5 de la tesi recull tots els resultats d'aquests estudis.

CAPÍTOL 6: Conclusions

D'acord amb els objectius d'aquesta tesi, la investigació que s'ha dut a terme i els resultats que s'han descrit anteriorment donen pas a les següents conclusions.

1. Durant els últims anys, part de la recerca científica i el públic en general, s'han centrat en l'estudi de la presència de substàncies perfluorades en el medi ambient i aliments, així com les possibles implicacions per a la salut humana. No obstant, la recerca s'ha centrat en dos compostos en concret: PFOA i PFOS. Donat que els PFASs són un grup ampli de substàncies, i contínuament s'introdueixen nous compostos en la indústria, es requereixen mètodes nous, multianalítics, sensibles i robustos per investigar la seva presència en el medi i els aliments.
   En el marc de la tesi s'han desenvolupat i validat diferents mètodes analítics per a l'anàlisi de fins a 21 compostos perfluorats en aigües, sediments i fans, fangs de depuradora, aliments (peix, carn, productes làctics, aliments infantils i preparats alimentaris), biota, plàstics marins, i matrius biològiques humanes (llet materna i sang de cordó umbilical). S'ha provat que els mètodes desenvolupats són adequats per a la determinació a nivell traça d'aquests anàlits en diferents mostres.

2. S'ha procedit a la preparació i avaluació interlaboratori d'un mètode analític per a l'anàlisi de PFASs en peix i llet. Aquest mètode va ser desenvolupat i validat en els laboratoris del ICT-Praga, en el marc del projecte Europeu Conffidence, i validat posteriorment als laboratoris de l'IDAEA-CSIC. La validació es va dur a terme "step-by-step". Els resultats de l'exercici interlaboratori mostren que tot i que, d'acord amb les recomanacions de l'EFSA (1 µg/Kg o L), el mètode es prou sensible, no és prou robust per ser aplicat en anàlisis de rutina en laboratoris analítics.

3. S'ha desenvolupat i validat un mètode en línia SPE-LC-MS/MS per a l'anàlisi de mostres del cicle de l'aigua. La majoria de les mostres contenen PFBA i PFHxA a concentracions quantificables. Aquest fet indica la substitució industrial dels PFASs de cadena més llarga per els de més curta. D'altra banda, el 50% de les aigües de beguda analitzades contenen, com a mínim, un PFAS posant de manifest el caràcter

recalcitrant dels PFASs. Tot i que la concentració de PFOS de 6 mostres d'aigua de beguda excedeix el nivell de salut (200 ng/L), la majoria de les mostres contenen concentracions inferiors a les considerades com a límit d'una exposició aguda per a la salut. No obstant, els nivells de seguretat establerts per l'EFSA i la USEPA només s'han decretat per al PFOS i el PFOA per a exposicions agudes. Seria necessari estendre les recomanacions a altres compostos PFASs i estudiar-ne els efectes de l'exposició crònica ja que l'aigua de beguda és considerada una de les principals rutes d'exposició humana. Els resultats d'aquest treball posen de manifest la necessitat d'aquestes noves regulacions per a PFAS en aigües continentals.

4. La investigació de fangs de depuradora mostra les elevades concentracions d'aquests anàlits, arribant a concentracions entre 53 i 121 µg/Kg per al més recalcitrant, el PFOS. D'aquesta manera, l'ús d'aquests fangs en sòls d'agricultura s'ha de considerar com una possible font de PFASs en el medi ambient, aigües subterrànies i aliments.

5. S'han detectat diferents PFASs en mostres de pingüí, guano i sediments provinents de l'Hemisferi Sud i el continent Antàrtic. Aquests resultats confirmen les hipòtesis prèvies, referents al transport de llarga distància d'aquests anàlits mitjançant el transport atmosfèric i la "condensació freda" per als més volàtils, i el transport oceànic (circulació termohalina entre altres processos geofísics) dels compostos menys volàtils.

6. La presència de PFASs en aliments contribueix a l'exposició humana a través de la dieta. Per aquest motiu s'han analitzat diferents aliments incloent llet materna, productes infantils (llet en pols i cereals) i peix de diferents mercats europeus. Els resultats indiquen la presència dels compostos de cadena més curta i dels més recalcitrants (PFOA i PFOS) en els aliments. Amb l'excepció d'una mostra de llet materna, les concentracions d'aquests anàlits no representen un risc immediat per la salut d'acord amb els nivell de seguretat establerts per l'EFSA i la USEPA. No obstant, s'hauria de considerar l'exposició crònica per procedir amb futures recomanacions i legislacions referents a aliments.

7. L'acumulació en humans s'ha estudiat a través de l'anàlisi de diferents mostres de llet materna i sang de cordó umbilical. Les mostres de cordó umbilical contenen concentracions quantificables de PFASs essent el PFBA, PFHxS, PFOA i PFOS els més freqüents. Aquests resultats recolzen estudis anteriors indicant el transport via transplacenta d'aquests anàlits. D'altra banda, les 20 mostres de llet analitzades contenen també PFASs a nivells quantificables.

8. L'ús de PFASs en polímers plàstics pot donar pas a una nova font d'aquests anàlits en el medi ambient. L'estudi en diferents mostres de plàstics marins i sediments

provinents de la costa grega confirma la presència de PFASs. El perfil que s'observa en ambdós tipus de mostres és molt similar. Aquest fet suggereix una mateixa font de contaminació. S'ha de considerar que la degradació dels plàstics combinada amb l'adsorció de PFASs en la superfície del plàstic dóna pas a una altra font de contaminació i de transport ambiental.

9. Finalment s'ha dut a terme l'estudi de diferents propietats fisicoquímiques i la biodegradació dels àcids perfluorofosfònics en condicions aeròbiques en tractaments de depuradora. Aquests anàlits pertanyen a un grup molt poc estudiat. Els coeficients de partició entre diferents fases s'han determinat seguint la directriu 123 de la OECD. D'altra banda, la biodegradació aeròbica confirma la poca biodegradació d'aquests anàlits. No obstant, s'ha detectat l'assimilació de PFDPA per part dels bacteris. Seria necessari dur a terme més experiments per tal d'identificar els organismes involucrats en aquesta assimilació així com els mecanismes.

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INDEX III: Additional publications

During the past two years several collaborations, with researchers within the CSIC group and other groups abroad, have been achieved. These collaborations yielded the following scientific publications that are not included in the Thesis:

Journal papers:

1. Pico, Y., Farré, M., Llorca, M. and Barceló, D. (2010). "Perfluorinated Compounds in Food: A Global Perspective." Critical Reviews in Food Science and Nutrition.

2. Kantiani, L., Llorca, M., Sanchís, J., Farré, M., Picó, Y., Barceló, D. (2010). "New analytical approaches to emerging contaminants in food". Analytical and Bioanalytical Chemistry, 398: 2413-2427.

3. Rodriguez-Rodriguez, C.E., Jelic, A., Llorca, M., Farré, M., Caminal, G., Petrovic, M., Barceló, D., Vicent, T. (2011) "Solid-phase treatment with the fungus Trametes versicolor substantially reduces pharmaceutical concentrations and toxicity from sewage sludge". Bioresource Technology, 102: 5602–5608.

4. Sanchís, J., Kantiani, L., Llorca, M., Rubio, F., Ginebreda, A., Fraile, J., Garrido, T., Farré, M. (2011) "Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry". Analytical and Bioanalytical Chemistry, 402(7): 2335-45.

5. Pérez, F., Llorca, M., Farré, M., Barceló, D. (2012). "Automated analysis of perfluorinated compounds in human hair and urine samples by turbulent flow chromatography coupled to tandem mass spectrometry". Analytical and Bioanalytical Chemistry, 402(7):2369-78.

6. Díaz-Cruz, M. S., Gago-Ferrero, P., Llorca, M. and Barceló, D. (2012). "Analysis of UV filters in tap water and other clean waters in Spain." Analytical and Bioanalytical Chemistry **402**(7): 2325-2333.

7. Rodríguez-Rodríguez, C.E, Barón, E., Gago-Ferrero, P., Jelić, A., Llorca, M., Farré, M., Díaz-Cruz, M.S., Eljarrat, E., Petrović, M., Caminal, G., Barceló, D., Vicent, T., (2012). "Removal of pharmaceuticals, polybrominated flame retardants 1 and UV-filters from sludge by the fungus Trametes versicolor in bioslurry reactor". Journal of Hazardous Materials (*accepted*).

Book Chapters:

1. Farré, M., Llorca, M., Pérez, S., Barceló, D. (2011). "Perfluorinated Compounds in food". T.P. Knepper and F.T. Lange (eds.), Polyfluorinated Chemicals and Transformation Products, The Handbook of Environmental Chemistry, 17, DOI 10.1007/978-3-642-21872-9_7 Springer-Verlag Berlin Heidelberg.

2. Llorca, M., Pérez, F., Farré, M., Picó, Y., Barceló, D. (2012). "Perfluorinated Compounds' Analysis, Environmental Fate and Occurrence: The Llobregat River as Case Study". S. Sabater et al. (eds.), The Llobregat: The Story of a Polluted Mediterranean River, The Handbook of Environmental Chemistry, DOI 10.1007/698_2012_147, Springer-Verlag Berlin Heidelberg.

3. Pérez, F., Llorca, M., Farré, M., Picó, Y., Barceló, D. (2012). "Perfluorinated Compounds in Drinking Water, Food and Human Samples". D. Barceló (ed.), Emerging Organic Contaminants and Human Health, Handbook of Environmental Chemistry, DOI 10.1007/698_2011_136, Springer-Verlag Berlin Heidelberg.