Analytical method development for the occurrence, distribution and transformation assessment of fluorinated compounds in biota and packaging materials

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eman ta zabal zazu

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A thesis submitted for the international degree of Doctor of Philosophy

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Azkenean amaitu da orain dala 4 urte hasitako bidaia. Momentu oso onak, lagun ikaragarriak eta bidaia ahaztezinak egin arren, ezin da ukatu bidea ez dala horren erreza izan. Hori dela eta, bidaia honetan zehar modu batean edo bestean lagundu nauzuen guztioi eskerrak emon nahi deutsuedaz:

Lehenik eta behin, eskerrak Olatz eta Ailetteri. Olatz, eskerrik asko orain dela 6 urte ikerketaren mundu hau erakusteagaitik. Soluzio posiblerik ez dagoela pentsatu dodan bakoitzean zure bulegotik esperantza txiki batekin irteten laguntzeagaitik eta urte guzti honetan zugandik ikasi dodan guztiagaitik. Ailette, gracias por toda la ayuda durante estos años, eres una Superwoman que llega a todo (el huracán Irma)! La verdad es qué no sé como lo haces! Ahora que está Arhane ya no hay excusas para irse a casa eh! =). Eskerrak bioi tesi hau posible egiteagaitik!

Zelan ez, eskerrak ere departamentuko gainontzeko irakasleoi (Nestor, Maitane, Are…), behar izan dodanean emondako laguntzagaitik. Eta eskerrak Urtzi eta Mareneri nire arrainak bizirik mantentzen laguntzeagaitik.

Arantza eta Ekhine, zuekin hasi zan nire abentura. Arantza, mila esker zure laguntza eta irakaspenengaitik, eta nire hasierako ibilbidea errezagoa egiteagaitik. Ekhine, zuri eskerrak orain dala 6 urte laborategira sartu nintzen lehenengo momentutik nigaz amaierara arte egotearren. Behar izan dodan guztietan nirekin egon zarelako laguntzeko prest (zenbat skype ordu??). Eta nola ez…. Manolito, zuri eskerrak emon nahi deutsudaz hiruron partez, gehienetan zugaz hasarratu, gure onetik atera eta desesperatu garen arren, zuri esker dakigu LC-MS/MS-aren inguruan ikasi dogun guztia!!! Plazerra izan da, neskak, zuekin bidaia hau egitea.

Mintegiko jendeari zer esan; orain dala 6 urte sartu nintzenetik zenbat bazkari, juerga, popcorn party eta istorio elkarrekin. Asko joanda zagozie iada: Arantza, Ekhine, Azibar

(edo Don Azibar esan behar da orain??? =), 17:15 direnean iada ez dogu musika delika-rik entzuten… zenbat bota zaitudan faltan azken urte honetan!), Oihana (Next stop: Salamanca!!), Oier, Oscar, Julen, Joana, Jone, Sandra…. beste batzuk doktore izan eta mintegitik beste pasillora emigratu zenduen: Josean (mila esker urte honeetan zehar nire informatikoa izateagaitik; ez dot errez ahaztuko master lan guztia galdu nebanean eta azken momentuan berreskuratzea lortu zendunean, eskerrik asko!!), Olaia, Ainara eta Olivia, eta beste batzuk, berriz, Plentzira: Mire (eskerrik asko zure laguntzagaitik, batez ere ordenagailu pantailan dana gorriz dagoenean ere azkenean lortu daitekeela erakusteagaitik!) eta Naiara (eskerrik asko beti laguntzeko prest egoteagaitik eta arrainak hain ondo zaintzeagaitik!!). Pati zuri eskerrak ere QTOF-agaz nire lehen pausuak emoten laguntzearren. Eskerrik asko danori urte guzti honeetan hor egoteagaitik!! Ezin aipatu barik itzi mintegian oraindik bizirauten dozuenak: Laura, Leire K., Leire M. eta Haizea. Aupa neskak, ez da askorik falta eta!!! Eskerrik asko laurori batez ere nire azken txanpa honetan emondako animo eta aholkuengaitik. Animo zuri ere Janire, hurrengoa zu zara eta! Eskerrik asko ere Zamudioko jendeari eta zorte on tesia egiten hasi zarienori: Denis eta Bastian.

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Eta zientzia mundu honetatik aparte dagozen eta zertan ondo nabilen ulertzen ez dozuenoi bebai eskerrik asko. Familia, Gontzal eta kuadrillakoak, batez ere azken

txanpa honetan aguantatu behar izan dozuen guztiagaitik!! Laster betikoa izango naz barriro, edo hori espero dot behintzat =)!!!

Ispasterren, 2017ko urriaren 22an

Amumari

1-MP	1-methyl piperidine
4:2 FTSA	4:2 fluorotelomer sulfonic acid
5:3 FTCA	5:3 fluorotelomer carboxylic acid
6:2 FTAB	6:2 fluorotelomer sulfonamidoalkyl betaine
6:2 FTCA	6:2 fluorotelomer carboxylic acid
6:2 FTNO	6:2 fluorotelomer sulfonamidoalkyl amine oxide
6:2 FTSA	6:2 fluorotelomer sulfonic acid
6:2 FTUCA	6:2 fluorotelomer unsaturated carboxylic acid
6:2 monoPAP	6:2 polyfluoroalkyl phosphate monoester
6:2 diPAP	6:2 polyfluoroalkyl phosphate diester
6:2 triPAP	6:2 polyfluoroalkyl phosphate triester
6:6 PFPIA	6:6 perfluoroalkyl phosphinic acid
6:8 PFPIA	6:8 perfluoroalkyl phosphinic acid
7:3 FTCA	7:3 fluorotelomer carboxylic acid
8:2 FTAC	8:2 fluorotelomer acrylate
8:2 FTCA	8:2 fluorotelomer carboxylic acid
8:2 FTSA	8:2 fluorotelomer sulfonic acid
8:2 FTUCA	8:2 fluorotelomer unsaturated carboxylic acid
8:2 FTOH	8:2 fluorotelomer alcohol
8:2 FTSA	8:2 fluorotelomer sulfonic acid
8:2 monoPAP	8.2 polyfluoroalkyl phosphate monoester
8:2 diPAP	8:2 polyfluoroalkyl phosphate diester
8:8 PFPIA	8:8 perfluoroalkyl phosphinic acid
10:2 FTCA	10:2 fluorotelomer carboxylic acid
10:2 FTUCA	10:2 fluorotelomer unsaturated carboxylic acid
10:2 monoPAP	10:2 polyfluoroalkyl phosphate monoester
10:2 diPAP	10:2 polyfluoroalkyl phosphate diester

A

ACN	acetonitrile
ANOVA	analysis of variance
Anvisa	Brazilian Health and Regulatory Agency
AP	alkaline phosphatase
APCI	atmospheric pressure chemical ionisation
APPI	atmospheric pressure photoionization
au	arbitrary units
auto-MS/MS	data-dependent acquisition mode

B

BBB	blood-brain barrier

BCFs	bioconcentration factors
BPA	bisphenol-A
Br-	branched isomer

C

CCD	central composite design
CH_3COOH	acetic acid
Cl-PFHxPA	6-chloroperfluorohexyl phosphonic acid
CRM	certified reference material
C18	

D

diPAPs	polyfluoroalkyl phosphate diesters
dSPE	dispersive solid phase extraction

E

ECF	electrochemical fluorination
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EIC	extracted ion current
EI-MS	electron ionisation-mass spectrometry
ESI	electrospray ionisation
EtFOA	*N*-ethylperfluorooctanamide
EtFOSA	*N*-ethyl perfluorooctane sulfonamide
EtFOSE	*N*-ethyl perfluorooctane sulfonamidoethanol
EtOAc	ethyl acetate
EtOH	ethanol
EQS	environmental quality standard
EU	European Union

F

FASAs	perfluoroalkane sulfonamides
FASAAs	perfluoroalcane sulfonamidoacetic acids
FASEs	perfluoroalcane sulfonamidoethanols
Fluorisil	magnesium silicate sorbent
FOSA	perfluorooctane sulfonamide
FOSAA	perfluorooctane sulfonamidoacetic acid
FOSE	perfluorooctane sulfonamidoethanol
FTAB	betaine-based PFAS
FTACs	fluorotelomer acrylates

FTCAs	fluorotelomer carboxylic acids
FTSAS	fluorotelomer sulfonic acids
FTUCAs	fluorotelomer unsaturated carboxylic acids
FTOH	fluorotelomer alcohol
Full MS-ddMS2	full scan–data dependent MS2
FUSLE	focused ultrasound solid liquid extraction
FWHM	full width at half maximum

G

GC	gas chromatography

H

HAMS	high accurate mass spectrometry
HLB	hydrophilic-lipophilic balanced sorbents
HRMS	high-resolution mass spectrometry
HOAc	acetic acid

I

IPE	ion-pair extraction
IT	ion trap

J

JetStream ESI	heated-electrospray ionisation source

K

K	condition factor
K_{ow}	octanol-water partition coefficient

L

L-	linear isomer
LBR	liver-to-blood ratio
LC	liquid chromatography
LC-ESI-MS/MS	liquid chromatography-electrospray ionisation-tandem mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-QqQ-MS/MS	liquid chromatography-triple quadrupole-tandem mass spectrometry
LC-QToF-MS	liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

LOD	limit of detection
LOQ	limits of quantification
LSI	liver somatic index

M

M10:2 FTCA	2-perfluorodecyl-[1,2-$^{13}C_2$]-ethanoic acid
M6:2 FTCA	2-perfluorohexyl-[1,2-$^{13}C_2$]-ethanoic acid
M8:2diPAP	(1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl) phosphate
M8:2 FTCA	2-perfluorooctyl-[1,2-$^{13}C_2$]-ethanoic acid
M8:2 FTUCA	2H-perfluoro-[1,2-$^{13}C_2$]-2-decenoic acid
MAX	mix-mode strong anion exchange
MDL	method detection limit
MeFOA	N-methylperfluorooctanamide
MeOH	methanol
monoPAPs	polyfluoroalkyl phosphate monoesters
MPFBA	perfluoro-n-[$^{13}C_4$] butanoic acid
MPFDA	perfluoro-n-[1,2-$^{13}C_2$] decanoic acid
MPFDoDA	perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid
MPFHxA	perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid
MPFHxS	perfluoro-1-hexane [$^{18}O_2$] sulfonate
MPFNA	perfluoro-n-[1,2,3,4,5-$^{13}C_5$] nonanoic acid
MPFOA	perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid
MPFOS	perfluoro-1-[1,2,3,4-$^{13}C_4$] octanesulfonate
MPFUnDA	perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl tert-butyl ether
MQL	method quantification limit

N

NCI-MS	negative chemical ionisation-mass spectrometry
NESI	electrospray ionisation in the negative mode
N-EtFOSA	N-ethyl perfluorooctane sulfonamide
NMR	nuclear magnetic resonance
nPFASs	neutral polyfluoroalkyl substances

O

OCPs	organochlorine pesticides

P

PAPs	polyfluoroalkyl phosphates
PBDEs	polybrominated diphenyl ethers
PFAAs	perfluoroalkyl acids
PFASs	per- and polyfluoroalkyl substances
PFBA	perfluorobutanoic acid
PFBS	perfluorobutane sulfonic acid
PFCs	perfluorinated compounds
PFCAs	perfluoroalkyl carboxylic acids
PFDA	perfluorodecanoic acid
PFDoDA	perfluorododecanoic acid
PFDPA	perfluorodecane phosphonic acid
PFDS	perfluorodecane sulfonic acid
PFEtS	perfluoroethane sulfonate
PFHpA	perfluoroheptanoic acid
PFHpS	perfluoroheptane sulfonic acid
PFHxA	perfluorohexanoic acid
PFHxDA	perfluorohexadecanoic acid
PFHxPA	perfluorohexane phosphonic acid
PFHxS	perfluorohexane sulfonic acid
PFNA	perfluorononanoic acid
PFNS	perfluorononane sulfonic acid
PFOA	perfluorooctanoic acid
PFOAAmS	perfluorooctane amidoalkyl ammonium salt
PFOAB	perfluorooctane amidoalkyl betaine
PFOANO	perfluorooctane alkylamido amine oxide
PFODA	perfluorooctadecanoic acid
PFOPA	perfluorooctane phosphonic acid
PFOS	perfluorooctane sulfonic acid
PFOSAm	perfluorooctane sulfonamidoalkyl amine
PFOSAmS	perfluorooctane sulfonamidoalkyl ammonium salt
PFOSB	perfluorooctane sulfonamidoalkyl betaine
PFOSi	perfluorooctane sulfinate
PFOSNO	perfluorooctane sulfonamidoalkyl amine oxide
PFSAs	perfluoroalkyl sulfonic acids
PFPAs	perfluoroalkyl phosphonic acids
PFPeA	perfluoropentanoic acid
PFPeDA	perfluoropentadecanoic acid
PFPiAs	perfloroalkyl phosphinates
PFPrA	perfluoropropionic acid
PFTeDA	perfluorotetradecanoic acid
PFTrDA	perfluorotridecanoic acid

PFUnDA	perfluoroundecanoic acid
PIE	Plentzia Marine Station
PLE	pressurized liquid extraction
POPs	persistent organic pollutants
POSF	perfluorooctane sulfonyl fluoride

Q

QqLIT	quadrupole-linear ion trap
QqQ	triple-quadrupole mass spectrometer
QTOF	quadrupole time of flight
QTRAP	quadrupole ion-trap
QuEChERS	quick, easy, cheap, rugged and safe

R

RP	reverse phase
RSD	relative standard deviation

S

SAmPAPs	perfluorooctane sulfonamido ethanol-based phosphate esters
SD	standard deviation
SLE	solid liquid extraction
SPE	solid phase extraction
SRM	selected reaction-monitoring
s/n	signal to noise ratio

T

$t_{1/2}$	half-life
TBA	tetrabutylammonium
TDI	tolerable daily intakes
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TM	telomerisation
TOF	time of flight
t_R	retention time
Tricaine	ethyl 3-aminobenzoate methanesulfonate
triPAPs	polyfluoroalkyl phosphate triesters

U

UHPLC	ultra high performance liquid chromatography
UPV/EHU	University of the Basque Country

USE	ultrasound assisted extraction
US EPA	United States Environmental Protection Agency

W

WAX	weak anion exchanger
WCX	mix-mode weak cation exchanger
WWTPs	wastewater treatment plants

Chapter 1

Introduction

Per- and polyfluoroalkyl substances (PFASs) were first introduced between the 1940s and 1950s as surface protectors [1] and during the last decades, industry exploited advances in organofluorine chemistry to continuously bring new formulations to the market. Currently, at least 3000 PFASs are in use on the global market [2]. Although the growing production of these compounds has not ceased, by 1968 Taves presented evidence of the presence of a fluorocarbon molecule in human serum [3]. Moreover, in 1976, Taves and co-workers used nuclear magnetic resonance (NMR) to tentatively identify perfluorooctanoic acid (PFOA) or a related compound in human serum [4]. These outcomes caused a global concern among the scientific and regulatory communities. Starting in the late 1990s, and particularly in recent years, there has been an explosion of publications and monitoring studies describing the presence of fluorinated compounds in environmental systems and in humans [5]; but, what we really know about this fluorinated compound family?

1.1 Terminology, properties, manufacture and classification of PFASs

Organofluorine substances comprise a large number of anthropogenic organic compounds that contain a carbon-fluorine bond [6]. A subset of this wide family are the highly fluorinated aliphatic substances referred to as "per- and polyfluoroalkyl substances", denoted by the acronym PFASs [7]. PFASs consist of a hydrophobic alkyl chain of varying length (typically C_4 to C_{16}) and a hydrophilic end group. The hydrophobic chain can be partially or fully fluorinated. The term perfluoro- denotes substitution of all hydrogen atoms attached to carbon atoms, except those whose substitution would affect the nature of the functional groups present; likewise, the term polyfluoro- denotes partially substitution of hydrogen atoms by fluorine atoms. For the partially fluorinated compounds, the position and the number of fluorinated compounds determines the properties of the substance. For instance, commonly described polyfluorinated compounds contain a $–CH_2-CH_2–$ moiety between the hydrophilic part

and the fully fluorinated remaining carbon chain, $F(CF_2)_n$-CH_2-CH_2-X. These compounds are named with the X:Y designation, where X is the number of perfluorinated C atoms and Y is the number of non-fluorinated C atoms (e.g. 8:2 fluorotelomer alcohol (8:2 FTOH), see ***Figure 1.1***).

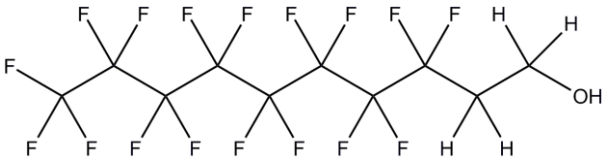


Figure 1.1: *Structure of 8:2 FTOH ($F(CF_2)_8$-CH_2-CH_2-OH).*

The hydrophilic end group can be neutral or positively or negatively charged [8]. Therefore, the resulting compounds are non-ionic (e.g. $–CH_2$-CH_2OH, $–SO_3NH_2$), cationic (e.g. fluorinated hydrophobic chain attached to a quaternary ammonium group) or anionic (e.g. $–COO^–$, $–SO_3^–$, and $–PO_3^–$) surface active agents due to their amphiphilic character.

PFASs present unique physicochemical properties due to their chemical structure [9,10]. On the one hand, the carbon-fluorine bond (one of the strongest found in organic chemistry) conferred them high chemical and biological stability. This resistance provides them rigidity, low chemical reactivity and environmentally persistence. On the other hand, PFASs are chemically unusual, since they are both hydrophobic and lipophobic, being able to repeal both water and grease.

PFASs have been produced via two major manufacturing processes: electrochemical fluorination (ECF) and telomerisation (TM) [8]. The historically major global fluorochemical manufacturer (The 3M Co.) began producing fluorochemicals in 1949, using the ECF process [11]. ECF replaces hydrocarbon hydrogens with fluorines via electrolysis in hydrogen fluoride [8]. This is a relatively crude process, producing

fluorinated molecules of various carbon chain lengths and a mixture of linear, branched and cyclic isomers. Perfluorooctane sulfonyl fluoride (POSF) has been the major target compound produced in this manner [1]. The 3M Company was the major producer of POSF, with the total cumulative production estimated to be approximately 96,000 t between 1970 and 2002 [11]. The two largest production sites were in the Unites States (Decatur, Alabama) and Belgium (Antwerpen). POSF was itself a commercially viable product, and in 1997 was sold for use as an industrial raw material (mainly outside the US). However, the primary use of POSF was to be an intermediate in the synthesis of functionally derivatised fluorochemicals and high molecular weight polymeric products [12]. The other major production process, TM, has been used commercially since 1970s [13]. TM produces fluorinated chemicals by iterative reaction of perfluoroethyl iodide (a telogen, CF_3-CF_2I) with perfluoroethylene (a taxogen, CF_2=CF_2), producing even, straight-chain alcohols ($F(CF_2CF_2)_nCH_2CH_2OH$, FTOH) that differ in length by CF_2CF_2 and can be converted into different fluorinated congeners [8]. The major difference between these processes is that ECF chemistry generates a characteristic distribution of 20-30 % structural isomers, whereas TM produces only the straight chain isomer with an even number of carbons.

There are numerous families of PFASs classified relying on their particular structure. *Figure 1.2* summarises the most common families of fluorinated compounds that have been detected in environmental and human matrices.

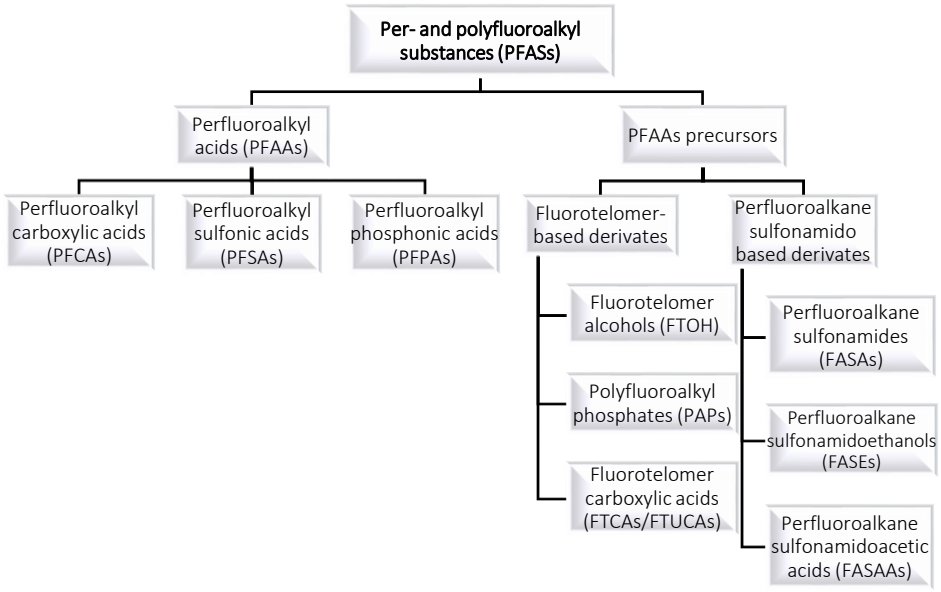


Figure 1.2: *Classification of environmentally relevant per- and polyfluoroalkyl substances (PFASs).*

As previously mentioned, the PFASs acronym stands for the broad family of per- and polyfluorinated alkyl substances. Among the former family, perfluoroalkyl acids (PFAAs), including perfluoroalkyl carboxylic (PFCAs), sulfonic (PFSAs) and phosphonic (PFPAs) acids, are predominantly monitored. PFAAs are strong acids compared to their hydrocarbon counterparts and have low pKa values (e.g. 2.80 for PFOA) [8]; consequently, the anionic form is dominant with little propensity to escape via volatilisation. They are emitted directly to the environment throughout their product life cycle from manufacture to use and disposal. However, since they are the end product of a variety of polyfluorinated substances, they can form indirectly from environmental degradation or metabolism [14]. Among them, perfluorooctane sulfonic acid (PFOS) and PFOA are of greatest concern as they are present in almost all environmental samples and are generally detected at the highest concentrations [15]. PFAAs-precursors comprise of fluorotelomer and perfluoroalkane sulfonamido-based products. Fluorotelomer-based products are synthesised by TM process, where

different length FTOHs are the main products [7]. FTOHs are typically used as precursor compounds in the production of other fluorinated derivates, such as polyfluoroalkyl phosphates (PAPs). Within perfluoroalkane sulfonamido based products, perfluoroalkane sulfonamides (FASAs), perfluoroalkane sulfonamidoethanols (FASEs) and perfluoroalkane sulfonamidoacetic acids (FASAAs) can be found. Those of 8 perfluorinated C atoms are, in general, much more abundant than those with other chain lengths [7]. Examples of each family are shown in ***Table 1.1***.

Table 1.1: *Overview of per- and polyfluoroalkyl substances: chemical formulas, family names and examples.*

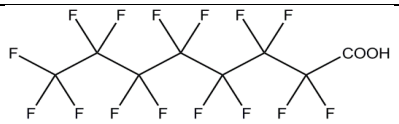
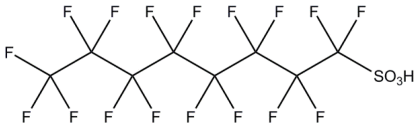
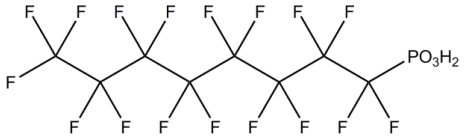
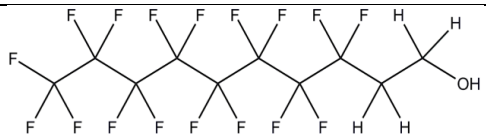
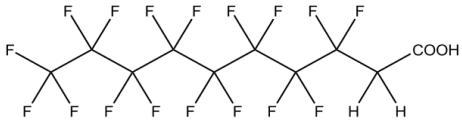
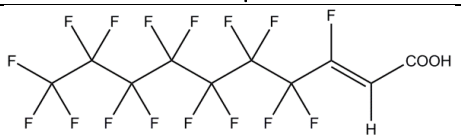
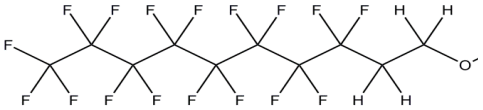
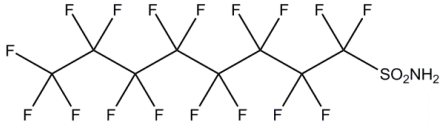
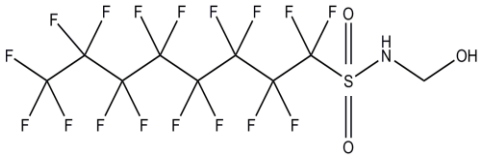
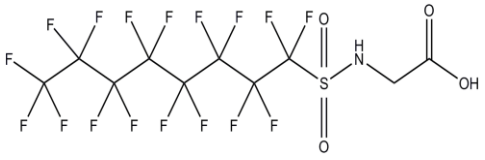
Formula	Family name	Example
PERFLUOROALKYL ACIDS (PFAAS)		
CF_3-$(CF_2)_x$-COOH	Perfluoroalkyl carboxylic acids (PFCAs)	 Perfluorooctanoic acid (PFOA)
CF_3-$(CF_2)_x$-SO_3H	Perfluoroalkyl sulfonic acids (PFSAs)	 Perfluorooctane sulfonic acid (PFOS)
CF_3-$(CF_2)_x$-PO_3H_2	Perfluoroalkyl phosphonic acids (PFPAs)	 Perfluorooctane phosphonic acid (PFOPA)
FLUOROTELOMER BASED DERIVATES		
CF_3-$(CF_2)_x$-CH_2CH_2-OH	Fluorotelomer alcohols (FTOHs)	 8:2 fluorotelomer alcohol (8:2 FTOH)
CF_3-$(CF_2)_x$-CH_2-COOH	Fluorotelomer carboxylic acids (FTCAs)	 8:2 fluorotelomer carboxylic acids (8:2 FTCA)

Table 1.1: *(Continuation).*

Formula	Family name	Example
CF_3-$(CF_2)_x$-CF=CH-COOH	Fluorotelomer unsaturated carboxylic acids (FTUCAs)	 8:2 fluorotelomer unsaturated carboxylic acids (8:2 FTUCA)
$(CF_3$-$(CF_2)_x$-CH_2CH_2-$O)_xP(=O)OH_{3-x}$ where x= 1, 2 or 3	Polyfluoroalkyl phosphates (PAPs)	 8:2 polyfluoroalkyl phosphate monoester (8:2 monoPAP)
PERFLUOROALKYL SULFONAMIDO BASED DERIVATES		
CF_3-$(CF_2)_x$-$SO_2NH(R')$ where R´= C_mH_{2m+1} (m= 0,1,2,4)	Perfluoroalkane sulfonamides (FASAs)	 Perfluorooctane sulfonamide (FOSA)
CF_3-$(CF_2)_x$-$SO_2N(R')CH_2CH_2OH$ where R´= C_mH_{2m+1} (m= 0,1,2,4)	Perfluoroalkane sulfonamidoethanols (FASEs)	 Perfluorooctane sulfonamidoethanol (FOSE)
CF_3-$(CF_2)_x$-$SO_2N(R')CH_2COOH$ where R´= C_mH_{2m+1} (m= 0,1,2,4)	Perfluoroalkane sulfonamidoacetic acids (FASAAs)	 Perfluorooctane sulfonamidoacetic acid (FOSAA)

1.2 Sources of exposure to PFASs

PFASs are widely used due to their special properties, such as chemical and thermal stability, acid resistance and water, dirt and grease repellency [5]. Among their principal applications, they can be used as surface protectors in carpets, leather, cookware,

sports clothing, paper, food containers, fabric and upholstery, and as performance chemicals in products such as fire-fighting foams, floor polishes, shampoos, paints, inks or pesticides [12,16]. Furthermore, PFASs are also used in industrial applications as surfactants, emulsifiers, wetting agents, additives and coatings [17].

Among PFASs exposure sources, food has consistently been implicated as the major human exposure pathway [18,19]. In fact, the use of PFASs in the food packaging industry is currently receiving considerable attention from scientists and policymakers since food packaging can contribute to the indirect human dietary exposure via migration into food [20]. PAPs and high molecular weight polymers are the principal PFASs used in packaging materials [12]. However, although there are no reports published on PAP degradation during microwave heating, attention should also be paid to PFAAs, since PAPs are known precursors of PFCAs [14,21]. To date, the presence of PFCAs and PFSAs has been reported in food packing materials from Australia, Spain, China, Greece, Thailand, Poland and the United States [22–32]. For instance, Poothong and co-workers analysed 34 food packaging items from the Thai market in order to ensure PFOS and PFOA presence [28]. PFOS and PFOA were detected in almost all food-packaging items, and the highest concentrations were found in fried-chicken box for PFOS (92 ng/dm^2) and in ice cream cup for PFOA (17 ng/dm^2). Moreover, Zafeiraki et al. [27] analysed 42 food packaging items from the Greek market, with the highest levels detected in microwave popcorn bags, reporting concentrations up to 276 ng/g for perfluorobutanoic acid (PFBA), 341 ng/g for perfluorohexanoic acid (PFHxA) and 5 ng/g for perfluoroheptanoic acid (PFHpA). Indeed, high concentrations in popcorn bags were also reported in other studies. For instance, Moreta and Tena reported concentrations up to 280 ng/g for PFBA, 37 ng/g for perfluoropentanoic acid (PFPeA), 405 ng/g for PFHxA and 7.5 ng/g for PFHpA in Spanish popcorn bags [23]. Relative abundances of PFASs vary among product types and manufacturing countries, reflecting differences in production patterns. Although PAPs are known to be used as coating agents for food-

contact materials of paper and board, few works have been focused on the monitorisation of these PFAA-precursors [33–36]. In fact, only one work reported quantifiable results for PAPs [36]. For example, Trier et al. [33], together to Gebbink et al. [35], detected qualitatively PAPs in food packaging items from the Danish and Swedish market, respectively. Moreover, Shoeib and co-workers quantified polyfluoroalkyl phosphate monoesters (monoPAPs) (138-282 ng/g) for the first time in food packaging materials from an Egyptian market [36].

Another important source that could contribute to the presence of PFASs in food is the use of these compounds in pesticide formulations. PFASs can be used in plant protection agents, both as active ingredients (the pesticide) and as additives (adjuvants). In some pesticide formulations, wetting agents are used to lower the surface tension in the spray solution and provide uniform wetting and spreading when the spray is in contact with leaf surfaces. However, these wetting agents often produce a high level of foaming in the spray tank, leading to a worker exposure problem [37]. To prevent foaming, several major pesticide manufacturers have tested and patented the use of PFPAs and perfluoroalkyl phosphinates (PFPiAs) as anti-foaming agents in various pesticide formulations and adjuvants [38–44]. However, a lack of quantitative information on these formulation production and use has been reported. In this sense, Posner et al. [45] claimed that there is a lack of information from manufacturers about pesticide components and that it is unclear whether and to what extent they are used on the Nordic and European markets. Moreover, there is no restrict regulation about the use of PFASs in pesticides; in the case of PAPs, although they have been used as defoaming adjuvant in pesticide formulations, the approval for this use was rescinded in 2006 [46]. However, PFPAs and PFPiAs are still known to be used in pesticides in Sweden (e.g. in a fungicide intended to prevent the occurrence of leaf fungus in potatoes) [47]. Moreover, commercial mixtures based on fluorinated substances, such

as Masurf FS-780 and Fluowet PL-80, are still permitted for use in pesticide formulations in countries such as Germany [48] and Canada [49].

As mentioned above, PFASs can also be the active ingredient of pesticides; this is the case of the Sulfluramid pesticide, whose active ingredient is N-ethyl perfluorooctane sulfonamide, EtFOSA. This pesticide, which is now banned under the Stockholm Convention on Persistent Organic Pollutants (POPs), was firstly registered in 1989 as an alternative to Mirex [50]. Brazil has an exemption from the Stockholm Convention to produce and use Sulfluramid [51], placing the country among the top 3 contemporary producers and consumers of PFOS-related substances globally. Sulfluramid is used in Latin America as active ingredient in the manufacturing of ant baits, for the control of leaf-cutting ants from the genus *Atta spp.* and *Acromyrmex spp.*, which are the insects that cause more injuries to national agriculture [52]. Apart from the agricultural uses, Sulfluramid has also been used in domestic medium (e.g. to control termites, cockroaches, household ants) [53]. However, The Brazilian Health and Regulatory Agency (Anvisa) proceeded to re-evaluate the register of Sulfluramid-based household pesticides, setting a period of 1 year for companies to sell their stocks and remove products as provided for in Anvisa Resolution, RE No. 41 of 8 January 2015 [54]. Moreover, the report pointed out that substitutes to Sulfluramid for combating *Atta spp.* and *Acromyrmex spp.* have not yet been identified. Thus, it appears that the use of Sulfluramid for agricultural purposes in Brazil and elsewhere in Latin America will continue indefinitely for some time [53]. Concern over the use of Sulfluramid arises from the tendency of its active ingredient, EtFOSA, to transform to PFOS, a highly persistent and globally distributed environmental contaminant [55].

1.3 PFASs occurrence in the environment and humans

PFASs are ubiquitously distributed in the abiotic and biotic environment, as well as in humans, primarily resulting from anthropogenic sources.

1.3.1 Abiotic environmental occurrence

PFASs have been reported in a huge variety of environmental compartments and ecosystems, such as aquatic ecosystems [56–59], soil [60–64] and air [65,66].

Several investigations around fluoropolymer facilities have demonstrated the damaging consequences of their industrial discharges on the quality of the aquatic ecosystems [67–69]. Moreover, wastewater treatment plants (WWTPs) are known to be a significant sources of PFASs to water, due to the incomplete removal of PFASs from wastewater influent and potential degradation of precursors during the wastewater treatment process [70,71]. For instance, Bach and co-workers [67] studied a river located in southern France, which receives wastewater from an industrial site where two facilities produce fluoropolymers. Based on the average concentrations detected in the river, 4295 kg PFHxA, 1487 kg 6:2 fluorotelomer sulfonic acid (6:2 FTSA), 965 kg perfluorononanoic acid (PFNA), 307 kg perfluoroundecanoic acid (PFUnDA), and 14 kg PFOA were estimated to be discharged in the river by the two facilities over a year. Moreover, Pan and co-workers [70] studied the removal efficiency of PFASs in Southern China WWTPs, where the average of total PFASs concentrations detected were 20-232 ng/L in influents, 16-234 ng/L in effluents and 32-49 ng/g in sludge.

Rivers drain some of the most populated and industrialised areas and represent major sources of PFASs to marine waters. For instance, González-Gaya et al. [56] reported the PFAS distribution along the Atlantic, Pacific and Indian oceans. The Atlantic Ocean presented the broadest range in concentrations of total PFASs (131−10,900 pg/L) compared to the other oceanic basins. Total concentrations in the Pacific Ocean ranged from 344 to 2,500 pg/L and from 176 to 1,976 pg/L in the Indian Ocean. PFOS was the most abundant compound, accounting globally for the 33 % of the total PFASs. Moreover, concentrations ranges between 246-515 pg/L were observed in the Western Mediterranean Sea, being PFHxA, PFHpA, PFOA, perfluorohexane sulfonic acid (PFHxS)

and PFOS the predominant detected PFASs [57]. In the Baltic Sea, concentrations from 1.2 to 14 ng/L have been reported, with PFNA contributing to the 34 %, followed by a 19 % for PFOS and a 13 % for PFOA [59]. Finally, PFASs concentrations up to 118 ng/L were reported in the Bohai Sea, where PFOA was the predominant compound, accounting for the 51-90 % of PFASs [58].

Despite their low pKa values, which render them relatively non-volatile [13], PFAAs are widespread in the environment. The presence of PFAAs in remote locations such as the Arctic [72–75] has raised the question about the transport of these compounds from their application areas. Two main pathways have been studied; on the one hand, PFAAs have been found to be the end-products of the transformation of neutral precursors, such as FTOHs or FASEs [76]. The first pathway involves the atmospheric transport of volatile precursors to remote areas. During atmospheric transport, the neutral precursors may be oxidised to produce the ionic compounds [65,76,77]. On the other hand, the second pathway involves long-range aqueous transport in their ionic form directly by the oceanic currents [72,78] or associated to particle and/or sea-spray [79,80].

Although oceans are known to be the dominant global PFAAs reservoirs [13], soil could also play an important role as PFAAs sink [60]. Up to now, most of the studies have focused on soil contamination due to the discharge of fluorochemical facilities [61,62] or due to the application of wastewater treatment biosolids to agricultural fields [63,64]. However, Rankin and co-workers [60] analysed apparently not contaminated (distant from obvious human activity) 62 soils representing all continents and obtained PFCAs and PFSAs concentrations ranging from 29-14,300 pg/g and <LOD-3,270 pg/g, respectively. These results confirmed the global distribution of PFASs in terrestrial settings and, given the remote location of many of the soil sources (e.g. Antarctica), the

ubiquitous detection of PFCAs and PFSAs confirmed that long rate transport plays an important role in the fate of PFASs.

Finally, a wide range of neutral polyfluoroalkyl substances (nPFASs) have been found in the global atmosphere (e.g. FTOHs, FASAs or FASEs) [65,66]. Although urban sites showed the highest levels of nPFASs, long rate transport was the responsible of the occurrence of these substances in rural and remote sites [66].

1.3.2 Occurrence in biota

The first report of the global distribution of PFASs in wildlife was published by Giesy and Kannan in 2001 [81]. These authors reported PFOS concentrations in the tissues of wildlife, including, fish, birds, and marine mammals. Since then, many studies have examined PFASs, mainly PFAAs, in all different types of wildlife (e.g. invertebrates, reptiles, fish, birds, mammals) along the world [82], including remote regions such as the Arctic [75]. Since PFASs are generally hydrophobic but lipophobic, they will not accumulate in fatty tissues as it is usually the case for other persistent halogenated compounds (e.g. organochlorine pesticides (OCPs) or polybrominated diphenyl ethers (PBDEs)), and they are primarily retained in protein-rich compartments (blood, liver and kidneys) [83]. Moreover, bioconcentration and bioaccumulation of PFASs in animals increase with the carbon chain length [83–85]. For instance, bioconcentration factors (BCFs) calculated for fish were low for C_8-C_{11} PFCAs (4-11,000 L/Kg), while BCFs of longer chain PFCAs (C_{12}-C_{14} PFCAs) were higher (18,000-40,000 L/Kg) [86]. Furthermore, studies have reported that given equal perfluoroalkyl chain length, sulfonates bioconcentrate to a greater extent than carboxylates, possibly because of tighter binding to proteins [87]. Biomagnification of PFASs (increased pollutant concentration in predator *versus* prey) is observed when moving up within the food chain and the trophic levels, including PFOS and long chain PFCAs [75,88], in top predators such as bear [75] or wild mink [89]. Trophic magnification has been illustrated by studies of

PFOS and long chain PFCAs in the lichen-caribou-wolf food chain [90] or plankton-fish-egret food chain [91].

Information of the environmental fate of several PFAA precursors, e.g. PAPs, is limited. In fact, an unique study reported the presence of PAPs and PFPiAs in the Great Lakes region trout [92]. Moreover, concern has arisen about alternative PFAS replacement chemicals [93] and, recently, they have been included in monitoring studies. For instance, long-chain PFAS replacement, perfluorobutane sulfonic acid (PFBS), has been reported in flounder (*Platichthys flesus*) muscle sample from the Western Scheldt (The Netherlands) at 80.12 ng/g wet weight [94]. Furthermore, apart from PFBS, a PFOS replacement chemical, F-53B (a chlorinated polyfluorinated ether sulfonic acid) [95], was detected in Greenland marine mammals by Gebbink and co-workers [96].

To date, most of the efforts have focused on aquatic biota, since, among the different foodstuffs, fish and shellfish seem to make the highest contribution to dietary PFAS exposure [97,98] (see ***Figure 1.3***).

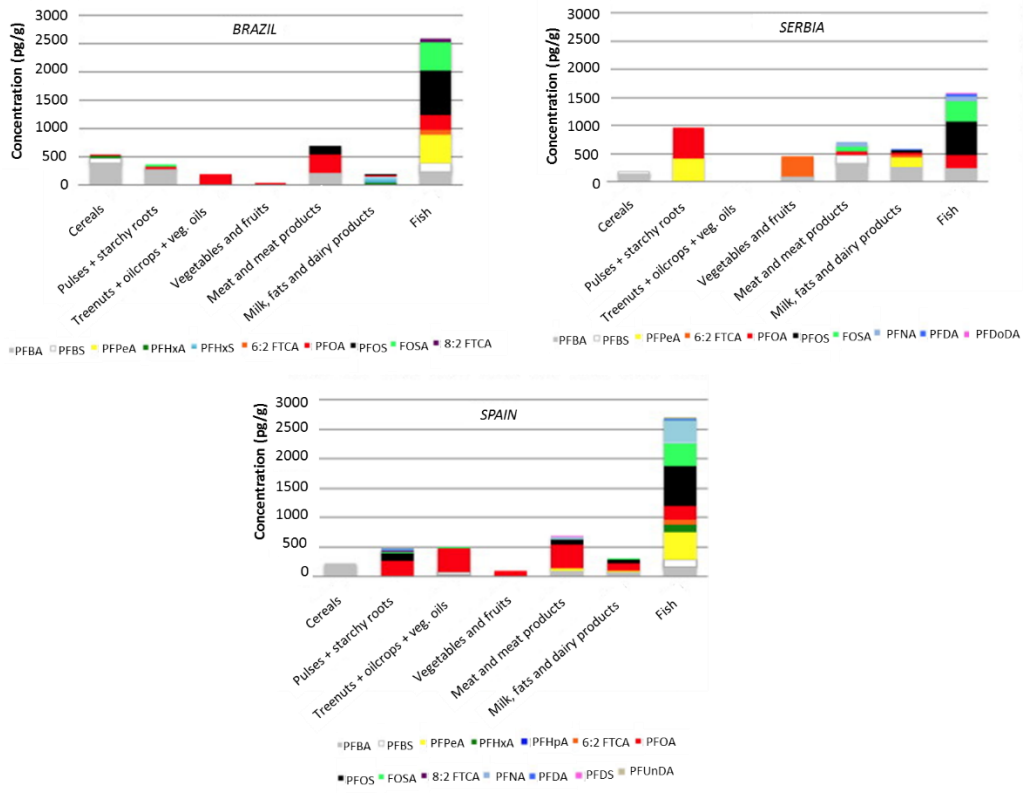


Figure 1.3: *Concentration in pg/g for the compounds detected in the different food groups from Brazil, Serbia and Spain* [97].

Moreover, PFOS is the PFAS showing the highest concentration in fish and shellfish. For instance, Hong et al. reported PFAAs concentrations ranging from 3.2 to 180 ng/g in South Korean fish, being PFOS the predominant PFAA [85]. Habibullah-Al-Mamun et al. [99] reported ΣPFAAs concentrations in finfish and shellfish from Bangladesh ranging from 0.32 to 14.58 ng/g and from 1.31 to 8.34 ng/g, respectively. PFOS was the predominant PFAA in finfish (0.1-3.86 ng/g), whereas PFOA was the most abundant in shellfish (0.07-2.39 ng/g).

The metabolism of PFAA-precursors is another subject that is nowadays being studied. Metabolism in animals involves many of the pathways and metabolites

identified from microbial degradation by sludge, soil, or microbial cultures [100]. Up to now, biodegradation of FTOHs and perfluorooctane sulfonamide (FOSA) [101], EtFOSA [102], PFPiAs [103], fluorotelomer saturated and unsaturated carboxylic acids (FTCAs/FTUCAs)) [104] and 8:2 fluorotelomer acrylate (8:2 FTAC) [105,106] have been studied in fish. The results of these studies have underlined the rapid biotransformation of precursors and the persistence of the PFCA and PFSA terminal metabolites.

Apart from animals, plants also play an important role in PFASs occurrence. For instance, field crops can contain PFASs on their surfaces or in their tissues after uptake from environmentally contaminated irrigation water [107] and from soil amended with sewage sludge [108,109]. In this sense, the bioaccumulation of PFAAs in different plants or crops has been studied in the recent years [107–112]. For instance, it has been found that, while long chain PFAAs tend to accumulate in roots, translocation from roots to edible parts is restricted and highly dependent on the hydrophobicity of the compounds; the higher water solubility, the higher translocation through the plant [110–112]. Moreover, biodegradation and further plant uptake of various PFAA-precursors and metabolites have also been reported recently [113–115].

1.3.3 Occurrence in humans

Different pathways have been considered to assess human exposure to PFASs [116]. For the general population the major source arises from food intake [116–118], especially fish consumption [97,98,119]. Moreover, a lack of an efficient PFAS removal process in drinking water treatment turn drinking water into a source of exposure [17,120]. Human exposure also arises from indoor and ambient air and house dust. Previous studies have shown that indoor air concentrations of PFASs were 1 to 2 orders of magnitude higher than outdoor values [121–123]. Moreover, the exposure from indoor air differs from that of house dust since exposure to ionic PFASs is higher for house dust [124–127], while that of neutral PFASs is higher for indoor air [128–130],

reflecting the lower volatility of ionic PFASs. The neutral precursor 8:2 FTOH was the most frequently PFAS detected in indoor air [128–130]. For instance, neutral precursors FTOHs, fluorotelomer acrylates (FTACs), perfluorooctane sulfonamidoethanols (FOSEs), and FOSAs were quantified with median levels of 11,783 pg/m^3, 737 pg/m^3, 130 pg/m^3 and 243 pg/m^3, respectively, in German schools indoor air [130]. It has to be highlighted that toddlers have higher intakes from dust ingestion than adults in all scenarios because they ingest larger quantities of dust through increased hand-to-mouth contact and related behaviour (see ***Figure 1.4***) [131,132].

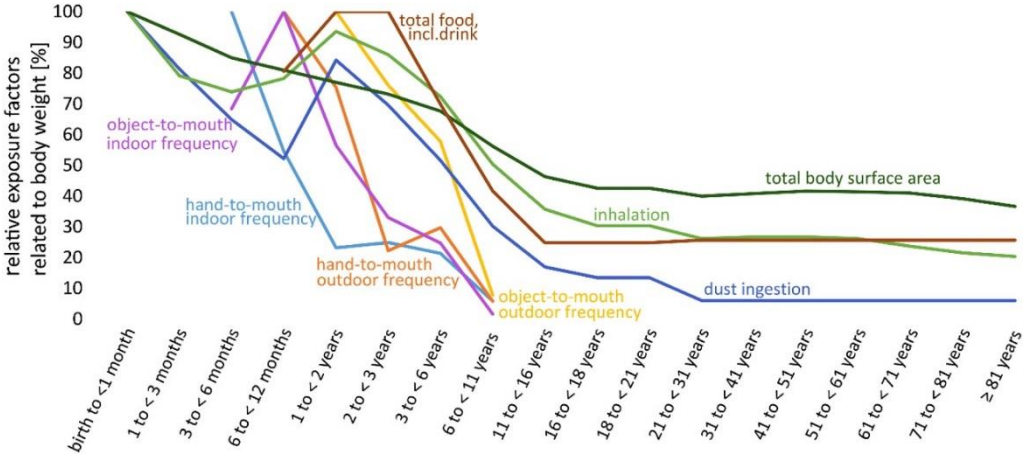


Figure 1.4: *Relative exposure factors related to body weight, compared over lifetime, for different age classes* [133].

The above-mentioned sources lead to a high-risk PFAS exposure for humans. Indeed, PFASs and potential precursors have been detected in human blood samples all around the world [134–139]. Furthermore, a higher exposed population has been recently identified; occupational exposure of fluorochemical plant workers can show 2-3 orders of magnitude larger PFAS concentrations in serum than the general population [140]. Moreover, ski waxing technicians and firefighters constitute other occupationally exposed populations [141–143]. For instance, Norwegian ski waxers had around 10-40 times higher median concentrations of PFCAs in serum than the general population,

except for PFUnDA and perfluorotridecanoic acid (PFTrDA), which were seven and three times higher, respectively [141].

Breast-milk is the natural and essential food for infants. However, questions have been raised for some time whether environmental contaminants in breast milk could adversely affect infant development and health [144,145]. Since several studies have reported PFASs and potential precursors presence in breast-milk [146–148], investigations have been carried out in order to determine if breastfeeding could be a PFAS excretion route for lactating mothers and exposure route for nursing infants [144,145,149]. For instance, in an Norwegian toddlers study, every month of breastfeeding was associated with an increase of 3.3 % PFOS, 4.7 % of PFOA and 6.1 % perfluoroheptane sulfonic acid (PFHpS) in toddlers plasma [149]. Moreover, Mondal and co-workers [145] reported that each month of breastfeeding was associated with lower maternal serum concentrations of PFOA (–3 %), PFOS (–3 %), PFNA (–2 %), and PFHxS (–1 %) and that the infant PFOA and PFOS serum concentrations were 6 % and 4 % higher per month of breastfeeding, respectively. Furthermore, Thomsen and co-workers concluded that after one year of breastfeeding, concentrations of PFOS and PFOA were reduced by 37 and 94 %, respectively, concluding that lactation was an important route of excretion for mothers [150].

1.4 Toxicity and regulation

PFASs have received an increasing attention during the recent years because of their toxicity. PFASs have been found to be peroxisome proliferators, developmental and endocrine disruptors, and tumour promoters [15,151–153]. Moreover, recent studies have reported that PFAA-precursors are more toxic that PFAAs themselves; what is more, the longer carbon chain, the more toxic they are [154,155].

Based on the risks associated with these chemicals, the major fluorochemical manufacturer in North America (The 3M Co.) phased out POSF-based products in 2002 [156], and returned to the market with perflurobutyl-based materials, considering that shorter chain PFASs are less persistent and toxic. Moreover, several major North American PFAS manufacturers entered in 2006 into a voluntary stewardship agreement to phase out the use and production of long-chain PFAAs by 2015 [157,158]. In the case of Europe, the European Union (EU) issued a Directive that regulated from June 2008 the general use of PFOS and derivates [159] and, one year later, PFOS was added to the United Nations Stockholm Convention on POPs [160]. Due to the growing concern about this class of chemicals, PFOS and its derivatives have also been listed as priority hazardous substances in the field of water policy under the Directive 2013/39/EU [161]. Moreover, an environmental quality standard (EQS) value was established for PFOS in biota (9.1 μg/kg) [161]. PFASs have also been announced as emerging contaminants in the food chain by the European Food Safety Authority (EFSA), which have recently established the tolerable daily intakes (TDI) of 150 ng/kg/day for PFOS and 1,500 ng/kg/day for PFOA [162]. Furthermore, EFSA recommended that an additional monitoring focused on PFASs is needed. On this account, Commission Recommendation 2010/161/EU invited the Member States to monitor the presence of PFOS and PFOA, different chain length (C_4-C_{15}) PFAAs similar to PFOS and PFOA, and their precursors, in order to estimate the relevance of their presence in food [163].

1.5 Analysis of PFASs in solid matrices

The need for worldwide assessment of the risks associated with exposure to this class of chemicals requires highly sensitive and accurate analytical methods. *Table 1.2* shows a summary of the analytical methods developed in the last 5 years (2012-2017) concerning packaging materials, soil, vegetables and fish matrices.

Table 1.2: Overview of method development studies for solid matrices including packaging material, soil, fish and vegetables during 2012-2017.

Sample	Compounds	Extraction	Clean-up	Stationary phase	Mobile phase	Detection	Detection limits	Ref.
Microwave popcorn bags	PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFOS	PLE (MeOH)	-	Acquity BEH C18	0.1 % HCOOH in ACN and water	LC-ESI-MS/MS (QTOF)	0.7-18 ng/g (LOD) 2-53 ng/g (LOQ)	[24]
Paper and board packaging material	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFTeDA, 6:2 monoPAP, 8:2 monoPAP, 10:2 monoPAP, 6:2 diPAP, 8:2 diPAP, 10:2 diPAP, 6:2 triPAP	SLE (MeOH)	-	BEH C18	Water:MeOH (95:5) and MeOH:ACN:water (75:20:5) both containing 2mM NH_4OAc and 5mM 1-MP	LC-ESI-MS/MS (QqQ)	26-660 fg (LOD) 0.2-1.5 pg/g (MDL)	[35]
Paper and board packaging material	PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFOS	FUSLE (EtOH)	-	Acquity BEH C18	0.1 % HCOOH in ACN and 0.1 % HCOOH in water	LC-ESI-MS/MS (QTOF)	0.5-2.2 ng/g (LOD) 1.4-7 ng/g (LOQ)	[25]
Paper packaging material	PFOS, PFOA	PLE (MeOH)	-	Eclipse XDB C18 and Eclipse Plus C18	10 mM NH_4OAc in water and ACN	LC-ESI-MS/MS (QqQ)	Not reported	[29]
Microwave popcorn bags	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFOS	FUSLE (EtOH)	-	Acquity BEH C18	0.8 % HCOOH in ACN and water	LC-ESI-MS/MS (QTOF)	0.2-0.5 ng/g (LOD) 0.4-1.6 ng/g (LOQ)	[23]
Soil	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFTrDA, PFTeDA, PFHxDA, PFODA, PFBS, PFHxS, PFHpS, PFOS, PFNS, PFDS, 8:2 FTCA	SLE (MeOH)	SPE (C18)	Kinetex C18	10 mM NH_4COOH in water and 10 mM NH_4COOH in MeOH	LC-ESI-MS/MS (QqQ)	0.01-6 ng/g (LOQ)	[164]
Soil	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFTrDA, PFTeDA, PFBS, PFHxS, PFHpS, PFOS, PFDS, FOSA, 4:2 FTSA, 6:2 FTSA, 8:2 FTSA, 6:2 FTCA, 8:2 FTCA, 10:2 FTCA, 5:3 FTCA, 6:2 FTUCA, 8:2 FTUCA, 10:2 FTUCA	SLE 1) 1 % CH_3COOH in water 2) MeOH:1% CH_3COOH in water (90:10)	SPE (WAX)	Acquity BEH C18	2 mM NH_4OAc in water:MeOH (9:1) and MeOH	LC-ESI-MS/MS (QqQ)	2 ng/g (LOQ)	[165]
Soil	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFTrDA, PFTeDA, PFHxDA, PFBS, PFHxS, PFHpS, PFOS, PFDS, 6:2 FTUCA, 8:2 FTUCA, 4:2 FTSA, 6:2 FTSA, 8:2 FTSA, FOSA, FOSAA, EtFOSA, 5:3 FTCA, 7:3 FTCA, PFOSAm, PFOSAmS, PFOAAmS, PFOSNO, PFOANO, PFOSB, PFOAB, 6:2 FTAB, 6:2 FTNO	SLE (0.1 % NH_4OH)	Graphitised carbon	Zorbax SB-C8	0.15 % CH_3COOH in water and 0.15 % CH_3COOH in ACN	LC-ESI-MS/MS (QTRAP)	0.005-2 mg/mL (LOD)	[166]

Table 1.2: Continuation.

Sample	Compounds	Extraction	Clean-up	Stationary phase	Mobile phase	Detection	Detection limits	Ref.
Vegetables	PFHxS, PFOS	IPE (TBA/MTBE)	SPE (Graphitised carbon + WAX)	C18	10 mM NH_4OAc in MeOH	LC-ESI-MS/MS (QTRAP)	3-130 pg/g (MDL)	[167]
Vegetables and fish	PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFBS, PFHxS, PFOS, PFDS, PFHxPA, PFOPA, PFDPA	SLE (ACN:water (9:1))	SPE (MAX)	BEH C18	Water:ACN (95:2) and MeOH:ACN:water (70:20:5) with 2 mM NH_4OAc and 5mM 1-MP in both	LC-ESI-MS/MS (QTOF)	1.8-20 pg/g (MDL) 6.0-66 pg/g (MQL)	[168]
Vegetables and fish	PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFHxS, PFOS	IPE (TBA/MTBE)	SPE (Fluorisil + Graphitised carbon)	BEH C18	2 mM NH_4OAc in water:MeOH (90:10) and 2 mM NH_4OAc in MeOH	LC-ESI-MS/MS (QqQ)	Vegetables: 0.6-6.6 pg/g (MDL) 1.2-13 pg/g (MQL) Fish: 0.3-2.4 pg/g (MDL) 0.6-5.4 pg/g (MQL)	[169]
Vegetables and fish	PFOS, PFOA	SLE (MeOH)	SPE (WAX)	Synergi Fusion C18	20 mM NH_4OAc in water and 20 mM NH_4OAc in MeOH	LC-ESI-MS/MS (QqQ)	0.20-0.47 µg/Kg (LOD) 0.50-0.70 µg/Kg (LOQ)	[170]
Fish	PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDa, PFDoDA, PFTrDA, PFHxS, PFOS, PFDS, PFHxPA, PFOPA, PFDPA, 6:6 PFPIA, 8:8 PFPIA, 6:8 PFPIA, 6:2 diPAP, 8:2 diPAP, 10:2 diPAP	IPE (TBA/MTBE)	-	Restek Ultra C18	10 mM NH_4OAc in water and MeOH	LC-ESI-MS/MS (QqQ)	1-125 pg/g (MDL)	[92]

1-MP: 1-methylpiperidine; 4:2 FTSA: 4:2 fluorotelomer sulfonic acid; 5:3 FTCA: 5:3 fluorotelomer carboxylic acid; 6:2 FTAB: 6:2 fluorotelomer sulfonamidoalkyl betaine; 6:2 FTCA: 6:2 fluorotelomer carboxylic acid; 6:2 FTNO: fluorotelomer sulfonamidoalkyl amine oxide; 6:2 FTSA: 6:2 fluorotelomer sulfonic acid; 6:2 FTUCA: 6:2 fluorotelomer unsaturated carboxylic acid; 6:2 monoPAP: 6:2 polyfluoroalkyl phosphate monoester; 6:2 diPAP: polyfluoroalkyl phosphate diester; 6:2 triPAP: 6:2 polyfluoroalkyl phosphate triester; 6:6 PFPIA: 6:6 perfluoroalkyl phosphinic acid; 6:8 PFPIA: 6:8 perfluoroalkyl phosphinic acid; 7:3 FTCA: 7:3 fluorotelomer carboxylic acid; 8:2 FTCA: 8:2 fluorotelomer carboxylic acid; 8:2 FTSA: 8:2 fluorotelomer sulfonic acid; 8:2 FTUCA: 8:2 fluorotelomer unsaturated carboxylic acid; 8:2 monoPAP: 8:2 polyfluoroalkyl phosphate monoester; 8:2 diPAP: 8:2 polyfluoroalkyl phosphate diester; 8:8 PFPIA: 8:8 perfluoroalkyl phosphinic acid; 10:2 FTCA: 10:2 fluorotelomer carboxylic acid; 10:2 FTUCA: 10:2 fluorotelomer unsaturated carboxylic acid; 10:2 monoPAP: 10:2 polyfluoroalkyl phosphate monoester; 10:2 diPAP: 10:2 polyfluoroalkyl phosphate diester; ACN: acetonitrile; CH_3COOH: acetic acid; EtFOSA: ethyl perfluorooctane sulfonamide; EtOH: ethanol; FOSA: perfluorooctane sulfonamide; FOSAA: perfluorooctane sulfonamidoacetic acid; FUSLE: focused ultrasonic solid-liquid extraction; HCOOH: formic acid; IPE: ion-pair extraction; LC-ESI-MS/MS: liquid chromatography-electrospray ionization-tandem mass spectrometry; LOD: limit of detection; LOQ: limit of quantification; MAX: mix-mode strong anion exchanger; MDL: method detection limit; MeOH: methanol; MQL: method quantification limit; MTBE: methyl tertbutyl ether; NH_4COOH: ammonium formate; NH_4OAc: ammonium acetate; NH_4OH: ammonium hydroxide; PFBA: perfluorobutanoic acid; PFBS: perfluorobutane sulfonic acid; PFDA: perfluorodecanoic acid; PFDS: perfluorodecane sulfonic acid; PFDoDA: perfluorododecanoic acid; PFDPA: perfluorodecane phosphonic acid; PFHpA: perfluoroheptanoic acid; PFHpS: perfluoroheptane sulfonic acid; PFHxA: perfluorohexanoic acid; PFHxDA: perfluorohexadecanoic acid; PFHxPA: perfluorohexane phosphonic acid; PFHxS: perfluorohexane sulfonic acid; PFNA: perfluorononanoic acid; PFNS: perfluorononane sulfonic acid; PFOA: perfluorooctanoic acid; PFOAAmS: perfluorooctane amidoalkyl ammonium salt; PFOAB: perfluorooctane amidoalkyl betaine; PFOANO: perfluorooctane alkylamido amine oxide; PFODA: perfluorooctadecanoic acid; PFOPA: perfluorooctane phosphonic acid; PFOS: perfluorooctane sulfonic acid; PFOSAm: perfluorooctane sulfonamidoalkyl amine; PFOSAmS: perfluorooctane sulfonamidoalkyl ammonium salt; PFOSB: perfluorooctane sulfonamidoalkyl betaine; PFOSNO: perfluorooctane sulfonamidoalkyl amine oxide; PFPeA: perfluoropentanoic acid; PFTeDA: perfluorotetradecanoic acid; PFTrDA: perfluorotridecanoic acid; PFUnDA: perfluoroundecanoic acid; PLE: pressurised liquid extraction; QqQ: triple quadrupole; QTOF: quadrupole time of flight; QTRAP: quadrupole ion-trap; SLE: solid-liquid extraction; SPE: solid phase extraction; TBA: tetrabutylammonium; WAX: weak anion exchanger.

1.5.1 Extraction

Since the early 2000s, three main extraction approaches have been used for the extraction of PFASs from biota: (i) ion-pair extraction (IPE), (ii) alkaline digestion and (iii) the use of an organic solvent combined with an energy source (solid-liquid extraction, SLE).

The IPE method, developed by Hansen and co-workers [171], has been widely applied in the past. In this extraction method, tetrabutylammonium (TBA) is used as ion-pair reagent, while the neutral forms generated are extracted into methyl tert-butyl ether (MTBE). This method is flexible and has been used for the extraction of a selection of PFASs in biota, such as fish, shellfish and mammals [92,172,173] and vegetables [174,175]. However, the method has shown several disadvantages [176]; for instance, co-extraction of lipids and other (disturbing) matrix constituents in the absence of a clean-up step to overcome the effects of matrix compounds. Recently, the efficiency of the IPE method has been improved by digesting the samples with an alkaline solution before extraction in order to release analytes from the sample matrix [167,169].

Another extraction strategy widely used is the alkaline digestion using potassium hydroxide (KOH):methanol (MeOH) or sodium hydroxide (NaOH):MeOH mixtures. Because of the specific protein-binding properties of PFAS, alkaline digestion of lipids and proteins before extraction has often been used to achieve accurate and reliable measurement of PFAS in biological samples [177–180]. Taniyasu and co-workers [177] presented a comparison of the two above mentioned extraction methods (IPE and alkaline digestion) for biota samples and reported that the alkaline digestion provided three-to-five higher concentration levels of several PFASs in liver samples than ion pairing. They attributed these differences in concentrations to the effective digestion of the matrix and the release of these compounds from the sample.

An alternative to the use of IPE and alkaline digestion consists on the use of different mixtures of organic solvents by simple shaking (e.g. vortex mixing, probe homogeniser) or assisted by sonication. Protein precipitation using acetonitrile (ACN) is a well-established and common SLE method for analysis of PFAS in biota samples [181,182] because of its easy handling and good recovery. A drawback of the SLE procedures described so far is the limited efficiency of extraction of a wide range of PFAS, including, water-soluble short chain or non-polar long chain compounds. Recently, Ullah and co-workers [168] reported that addition of 10 % water to the ACN in the first extraction step increased the extraction recoveries of the short chain PFAAs from water-free matrices, while using pure ACN in the second step ensured efficient extraction of the long chain analytes. These authors employed extraction with ACN:water combined with ultra-sonication for vegetables, meat and fish samples and recoveries between 59-98 % for all the analytes were obtained. Moreover, QuEChERS (quick, easy, cheap, rugged and safe) procedure, based on extraction of target analytes with ACN and their transfer (supported by inorganic salts and acidification) into the organic phase has also be used for fish [183] and vegetables [110]. Although ACN has been the most used organic solvent for the extraction of PFASs from biota samples, analytical methods using different solvents (e.g. water [184], MeOH [170], water:tetrahydrofuran (THF) [185,186]) have also been developed. For instance, Llorca and co-workers [184] used water and pressurized liquid extraction (PLE), obtaining recoveries higher than 85-89 % for liver and muscle samples. This extraction method provided better recoveries than alkaline digestion and IPE. What is more, PLE was much more rapid than the alkaline digestion and provided cleaner extracts than that based on IPE. Moreover, Luque et al. [186] developed a new method for the simultaneous monitoring of PFCAs and PFSAs (recoveries ranged from 85 to 111 %) in fish and marine birds by microextraction with THF:H_2O (75:25) mixture. The benefit of this mixture was the different types of interactions that could be established with the polar groups of PFASs (e.g. ion-dipole

and hydrogen bonding). These properties allowed the extraction of ionic and nonionic PFASs with carbon chain lengths between C_4 and C_{14} using a low solvent volume and avoiding, therefore, the subsequent evaporation step.

In the case of environmental abiotic matrices, common extraction procedures are based on four different methods: (i) acetic acid and MeOH SLE, (ii) pure MeOH SLE, (iii) NaOH digestion and (iv) IPE. Methods using acetic acid and/or MeOH were developed for application in environmental abiotic samples, whereas those applying NaOH digestion and the IPE were initially designed for biological matrices and later adapted for the abiotic ones. Nowadays, all these extraction methods have been widely used for soil samples (acidified MeOH SLE [165], MeOH SLE [180,187–190], NaOH digestion [191,192] and IPE [61,193–195]).

A recent study compared the above mentioned extraction methods for soil and sediment in order to select the one that provided the best recoveries and the highest sensitivity [164]. While extraction using MeOH with or without acetic acid yielded the highest recoveries, extraction using only MeOH was the most sensitive. IPE was the least sensitive extraction method and the lowest number of compounds was detected using the NaOH digestion which can be explained because the basic pH can promote the binding between PFSAs and soil cations, preventing their extraction. Moreover, PFAS recovery performance was evaluated for two SLE methods using MeOH:NaOH and MeOH:ammonium hydroxide (NH_4OH) for different soil types [166]. While both methods yielded satisfactory results overall, especially for PFAAs or fluorotelomer sulfonic acids (FTSAs), the extraction approach using a milder solvent (NH_4OH) provided excellent limits of detection and moderate matrix effects. Meanwhile, while the strongest extraction method (NaOH) yielded better recovery rates for novel PFAS (e.g. betaine-based PFAS (FTAB, quaternary ammonium PFAS, or fluorotelomer thioether derivatives), yet led to higher limits of detection and lower instrumental accuracy.

In the case of packaging materials, extraction by PLE has been mostly applied [24,26–29]. Moreover, a conventional SLE by means of MeOH has also been widely applied [35,196,197]. Recently, a new extraction method based on focused ultrasound solid-liquid extraction (FUSLE) was developed [25] and comparable results with those obtained with PLE were achieved for PFAAs.

1.5.2 Clean-up

When we are dealing with complex matrices, a clean-up step is usually necessary. This is the case of alkaline digestion or most extractions performed using an organic solvent combined with an energy source. The most usual clean-up process is solid phase extraction (SPE), which represents the option for isolation and/or pre-concentration of PFASs. In recent years, widely used cartridges include WAX (mix-mode weak anion exchanger) and HLB (hydrophilic-lipophilic balanced) sorbents (see ***Figure 1.5***). However, HLB sorbent phase has shown some drawbacks [177], such as low recoveries (< 30 %) of the most polar, short chain (C_4-C_6) ionic PFAAs. In order to improve the recovery of short-chain PFAAs and to separate neutral PFASs and FTOHs from other fluorinated compounds, a WAX sorbent was found to be an appropriate approach. In fact, WAX clean-up has been widely used after alkaline digestion or SLE in soil [165,180,187,191,192] and biota samples [170,179,180]. Moreover, Ullah and co-workers [168] suggested that for the extraction of PFPAs a mix-mode strong anion exchange (MAX, see ***Figure 1.5***) yielded better results than WAX sorbents, while for PFCAs and PFSAs both sorbents provided satisfactory results.

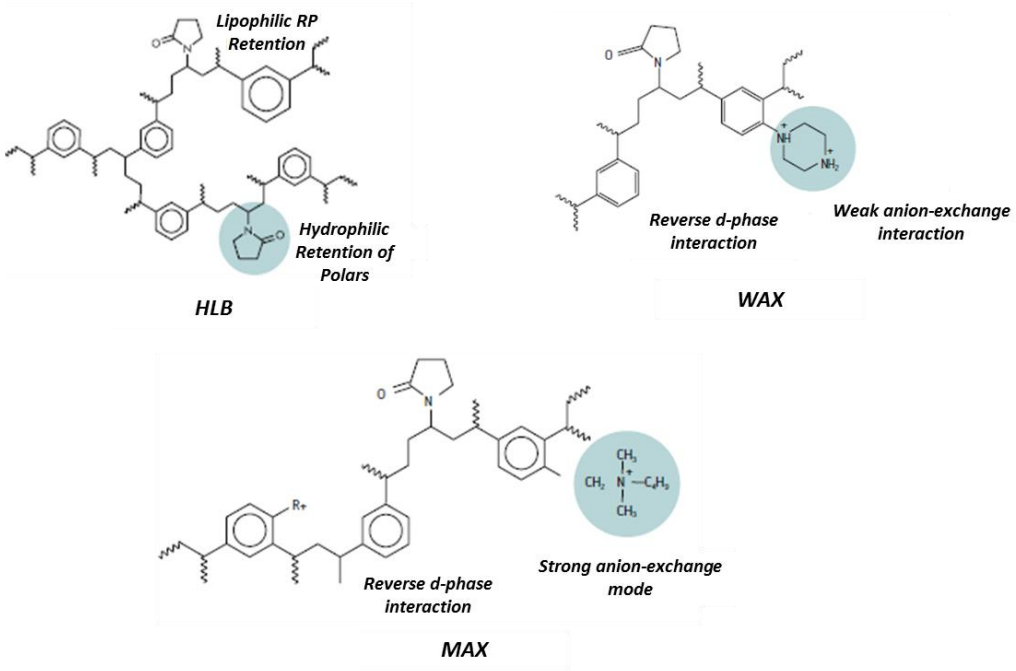


Figure 1.5: Examples of different SPE sorbent structures.

Graphitised carbon is another clean-up approach used in the last years. This sorbent adsorbs compounds via dispersive interaction with π electrons. π electrons in PFASs are strongly associated with the highly electronegative fluorine atoms and therefore do not interact effectively with the sorbent, even in the presence of a weak eluting solvent such as MeOH. However, most nonperfluorinated species with any degree of aromaticity are strongly associated with the graphitised carbon, resulting in a very effective purification of PFASs containing extracts [198]. This clean-up approach has been widely applied in soil [61,166,188,193,198,199], vegetables [188–190,200] and fish samples [182], e.g. to remove pigments. Furthermore, with the development of more efficient extraction procedures, more rigorous clean-up procedures are necessary to limit the effects of the matrix on the ionisation efficiency. It is for that reason that some works combine WAX and graphitised carbon sorbents in order to increase the efficiency of the cleaning step [167,185,189,201].

Moreover, as IPE extraction has been previously associate with matrix effects on ionisation due to co-extraction of lipids, a rigorous clean-up step is suggested [167,169]. Apart from the clean-up sorbents that are usually used, Vestergren and co-workers [169] developed an analytical method using a combination of a magnesium silicate sorbent (Florisil) and graphitised carbon. The polar-polar interaction between the target analytes and Florisil was exploited to separate PFASs from co-extracted lipids and hydrophobic matrix constitutes. Additionally, graphitised carbon was mixed with the sorbent to selectively retain aromatic compounds.

In the case of the packaging materials, although most of the methods published do not use a clean-up step, some works used WAX [36,196] or Florisil/alumina [27] with cleaning purposes.

1.5.3 Analysis

Methods based on liquid chromatography (LC) are the most commonly used for the determination of PFASs. They can be employed with different detection methods, but mass spectrometry (MS), with different configurations of MS analysers, is commonly considered as the reference detector [202]. The most common MS instrumental set-up used for PFASs analysis is the triple-quadrupole mass spectrometer (QqQ), which is one of the best suited for quantification of PFASs. Nowadays, the performance of ion trap (IT), quadrupole-linear ion trap (QqLIT), and time of flight (TOF) have also been exploited for trace quantification of PFASs [203]. For instance, Llorca et al. [204] reported a comparison between QqQ, QqLIT and IT instruments to determine trace levels of PFASs in fish and shellfish. The three instruments checked showed different abilities to determine PFASs. The QqLIT and QqQ systems are quadrupole-based instruments and both show appropriate sensitivity for monitoring specific precursor ion to product transitions. Accuracy was similar in the three systems and precision was better for the QqLIT and QqQ systems (7-15 %) than for the IT system (10-17 %). The

QqLIT and QqQ offered a linear dynamic range of at least 3 orders of magnitude, whereas the IT showed only 2 orders of magnitude. The QqLIT system achieved at least 20-fold higher sensitivity than the QqQ system, and this was at least 10-fold times more sensitive than the IT analyser. Moreover, high-resolution mass spectrometry (HRMS) is regarded as an excellent option, because of its sensitivity, resolving power, and quantification capabilities [205]. TOF and Orbitrap-based technologies are nowadays the most popular analysers used in LC-HRMS. In some cases, however, for unequivocal identification of the compounds of interest it is necessary to combine the information provided by the HRMS with that obtained by use of tandem mass spectrometry (MS/MS).

Although the method of choice for the determination of ionic PFAS is LC-MS, neutral and volatile PFASs, such as FTOHs, have also been analysed by gas chromatography (GC) [206–208]. Moreover, some works analyse ionic PFAAs, such as PFCAs, by GC after derivatisation (mainly consisting in the formation of the methyl ester derivates) [209]. After separation, various detectors have been applied but it appears that electron ionisation-mass spectrometry (EI-MS) is used most frequently, whereas negative chemical ionisation-mass spectrometry (NCI-MS) with ammonia as reagent gas is the most sensitive detector for the determination of PFCAs by GC [209]. The drawbacks of the use of GC-MS (need for derivatisation) have made LC coupled to MS the most widely used technique for the analysis of PFASs.

The interfaces most often used for LC-MS or LC-MS/MS determination of organic environmental contaminants are the atmospheric pressure photoionisation (APPI) sources, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Today, it is widely accepted that APCI is less susceptible to matrix effects than ESI because ionisation takes place in the gas phase [210]. However, APCI has found fewer applications in environmental analysis than ESI because the range of compounds

that (due to their polarity and/or molecular weight) can be analysed by APCI is shorter than in ESI. This is the case of ionic compounds such as PFOS whose analysis by APCI is not suitable. ESI operating in the negative ion mode has been the interface most widely used for the analysis of anionic PFASs in environmental samples (e.g. see ***Table 1.2***). The use of APPI was explored by Takino et al. [211]. The authors found as the main advantages of this technology, the absence of matrix effects, but the limits of detection were considerably higher than those obtained by LC-ESI-MS/MS.

According to the literature reviewed, LC separation of PFASs has been mainly carried out with C18 and C8 columns [212]. However, Taniyasu and co-workers [213] reported that when reverse phase (RP)-C18 columns were used, peaks of very short chain PFASs, such as perfluoropropionic acid (PFPrA) and perfluoroethane sulfonate (PFEtS), were broad and not adequately resolved, whereas trifluoroacetic acid (TFA) was not retained. This suggested that RP columns were not suitable for the analysis of short-chain PFASs, especially TFA. As a proper alternative, ion-exchange columns showed superior retention properties for more hydrophilic substances, enabling the analysis of short-chain PFASs.

Regarding mobile phases, mixtures of ACN-water and MeOH-water, often modified with ammonium acetate (from 1 to 20 mmol/L) to improve LC separation and MS sensitivity, have been usually used. Inoue et al. [214] investigated the effect of the mobile phase ammonium acetate concentration on the peak responses of PFOS, PFOA and FOSA. Maximum responses were obtained at a concentration of 1 mmol/L ammonium acetate. Moreover, Ullah and co-workers [215] tested different mobile phases containing MeOH, ACN, and water at pH values between 3 and 11 in the presence of 2 mmol/L ammonium acetate, but the results were not satisfactory for PFPAs. However, the addition of 1-MP (1-methyl piperidine) as an ion-pairing agent to the mobile phase, resulting in a pH between 10 and 11, considerably improved both the

chromatographic resolution and the instrumental response of PFPAs, and suppressed baseline noise. This agent acts as an ion-pairing agent; it masks the negative charges of the phosphonate group, leading to an increase in the retention of PFPAs on a C18 stationary phase through hydrophobic interactions. Furthermore, the protonated amine group of 1-MP may sorb to negative charges on the silica surface, thus shielding the remaining active sites of the silica. In addition, a high pH value of the mobile phase generally favours the formation of negatively charged ions in MS detection, leading to a better sensitivity for acidic analytes. Additionally, also the PFCAs and PFSAs showed a distinctive sensibility increase in the presence of 1-MP, which was especially pronounced for short chain compounds.

1.6 References

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Chapter 2

Aims and objectives

Per- and polyfluoroalkyl substances (PFASs) have attracted increasing attention as emerging environmental contaminants during the recent years. Their widespread occurrence, together with their toxicity, have caused a global concern among scientific and regulatory communities. Although new fluorinated alternatives have continuously been brought to the market, recent studies have evidenced that some of these alternatives can be potential precursors of PFASs. Therefore, their use has become a new source of PFASs to the environment and humans. To date, although potential PFASs precursors are being included in monitoring studies, there are few developed analytical methods for the simultaneous determination of PFASs and their potential precursors in different environmental and source matrices.

On the other side, within the different human PFASs exposure sources, food intake seems to be the principal, being fish and shellfish the highest dietary PFAS contributors. Moreover, attention should also be paid on their use as pesticides or as oil repellents in packaging materials in order to assess the possible transfer of PFASs into the food chain.

Within this context, the objectives of the present work were established:

i) Optimisation of different robust and reliable analytical methods for the determination of PFASs and their potential precursors in biotic (vegetables, fish and mussels) and abiotic (soil) environmental samples, as well as, in different packaging materials. The analytes selected comprised a wide range of PFAS families, including perfluoroalkyl carboxylic (PFCAs), sulfonic (PFSAs) and phosphonic (PFPAs) acids, as well as, perfluorooctane sulfonamide (FOSA). Moreover, 10 potential PFASs precursors were also selected

comprising polyfluoroalkyl phosphates (PAPs), fluorotelomer saturated acids (FTCAs) and fluorotelomer unsaturated acids (FTUCAs).

ii) Biodegradation, leaching, plant uptake and distribution of Sulfluramid pesticide and its transformation products in soil-carrot mesocosm.

iii) Determination of PFASs and potential precursors in grey mullet (*Chelon labrosus*) liver and oysters from the north coast of Spain, France and Portugal.

iv) Uptake, tissue distribution and biotransformation of 8:2 diPAP in gilt-head bream (*Sparus aurata*).

v) Determination of PFASs in microwave popcorn bags from different countries around the world.

Chapter 3

Focused ultrasound solid–liquid extraction for the determination of perfluorinated compounds in fish, vegetables and amended soil

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3.1 Introduction

An emerging contaminant is a chemical or a material that is characterised by a perceived, potential or real threat to human health or the environment. Among the different emerging compounds defined in the recent years (pharmaceuticals, certain hormones…), perfluorinated compounds (PFCs) have become of emerging concern due to their potential toxicity, persistence and bioaccumulation [1]. PFCs represent a large group of organic compounds that are characterised by a fully or partially fluorinated hydrophobic and lipophilic carbon chain attached to one or more different hydrophilic functional groups [1]. The hydrophilic end group can be neutral, or positively or negatively charged. The resulting compounds are non-ionic, cationic or anionic surface active agents due to their amphiphilic character [2]. The highly chemical and biological stability of PFCs is conferred by the carbon-fluorine bond. This covalent bond (one of the strongest found in organic chemistry) is resistant to hydrolysis, photolysis, metabolism and biodegradation [3]. This resistance confers to PFCs rigidity, low chemical reactivity and environmentally persistence; therefore, they have the potential to be bioaccumulative.

PFCs are widely used due to their special properties, such as chemical and thermal stability, acid resistance and water, dirt and grease repellency [4]. Among the principal applications, they can be used as surface protectors in carpets, leather, cookware, sports clothing, paper, food containers, fabric and upholstery and as performance chemicals in products such as fire-fighting foams, floor polishes, shampoos, paints and inks [5-6]. Furthermore, PFCs are also used in industrial applications as surfactants, emulsifiers, wetting agents, additives and coatings [7].

Due to the growing concern about this class of chemicals, in the year 2000 the largest producer of PFCs, the 3M Company, announced the phase out of the production of

perfluorooctane sulfonic acid (PFOS). Since then, new shorter-chained PFCs (C_4-C_7) and their precursors are being introduced as replacements considering that these are less persistent or toxic in humans [8]. However, continued manufacturing of PFC precursors may result in further accumulation of PFOS and other PFC residues to the environment, wildlife and humans [4, 9]. In 2004, Environment Canada initiated a temporary ban on fluoropolymers containing fluorotelomer alcohols (FTOHs) [9] and Norway banned the use of PFOS in firefighting foams, textiles and impregnation agents (max. content 0.005 %) [6]. Moreover, in 2006 the US Environmental protection Agency (US EPA) announced a voluntary stewardship program to reduce by 95 % perfluorooctanoic acid (PFOA) and related chemicals in the environment by 2010 and to eliminate all of them by 2015 [7]. Furthermore, the European Union (EU) issued a Directive that prohibited from June 2008 the general use of PFOS and derivates [10]. In May 2009, PFOS was listed as "restricted use" compounds under the Stockholm Convention on persistent organic pollutants (POPs) [11]. However, PFOA and the homologous chemicals of PFOS, which may degrade to PFOS, are not regulated yet [12]. Finally, PFCs have been announced as emerging contaminants in the food chain by the European Food Safety Authority (EFSA), which have recently established the tolerable daily intakes (TDI) of 150 ng/kg/day for PFOS and 1500 ng/kg/day for PFOA [13]. Furthermore EFSA recommended that an additional monitoring focused on PFCs is needed. On this account, Commission Recommendation 2010/161/EU invited the Member States to monitor the presence of PFOS and PFOA, different chain length (C_4-C_{15}) PFCs similar to PFOS and PFOA, and their precursors, in order to estimate the relevance of their presence in food [14].

Due to the concern on exposure to PFCs, a special interest has grown to develop robust analytical methods in the last years [15].

As previously mentioned in the general introduction and according to the literature [3, 13-27], three main approaches are used for the extraction of PFCs from solid samples: (i) ion-pairing, (ii) alkaline digestion and (iii) the use of an organic solvent combined with an energy source. Moreover, the extraction methods described above usually need a clean-up step. This is the case of alkaline digestion or most extraction performed by an organic solvent combined with an energy source. The most usual clean-up process is solid phase extraction (SPE), which represents the option for isolation and/or pre-concentration of PFCs in biotic samples. Widely used cartridges are WAX (mix-mode weak anion exchanger), MAX (mix-mode strong anion exchanger) and HLB (hydrophilic-lipophilic balanced sorbents).

The drawbacks of the use of GC-MS (need for derivatisation) have made LC coupled to MS the most widely used technique for the analysis of PFCs. The most common MS instrumental set-up used for PFC analysis is the triple-quadrupole mass spectrometer (QqQ), which is one of the best suited for quantification of PFCs. Nowadays, the performance of ion trap (IT), quadrupole-linear ion trap (QqLIT), and time of flight (TOF) have also been exploited for trace quantification of PFCs [28-29].

Within this context, the aim of the present work was to develop a method for the accurate and precise determination of four families of PFCs (PFCAs, PFSAs, PFPAs and perfluorooctane sulfonamide (FOSA)) in food samples including vegetables (lettuce, pepper and carrot) and fish, as well as in amended-soil used for the growing of different crops. Focused ultrasound solid-liquid extraction (FUSLE) was tested for the extraction step, while different clean-up approaches of the extracts using SPE cartridges (reverse and mix-mode) were evaluated. Matrix effect was thoroughly studied both in the clean-up and LC-MS/MS (triple quadrupole) analysis steps.

3.2 Experimental section

3.2.1 Reagents and materials

The names of the target analytes, the abbreviations, the chemical structure, the supplier of the standards, the purity of the standards, the octanol-water partition coefficient (as log K_{ow}) and pKa values are included in ***Table 3.1***. In the case of the surrogate standards, the information has been included in ***Table 3.2***.

Table 3.1: *Structures, suppliers, purity, log K_{ow} and pKa values of the target analytes.*

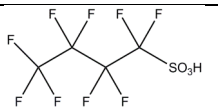
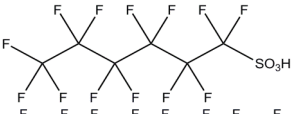
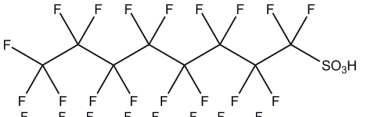
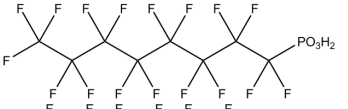
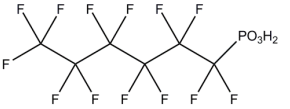
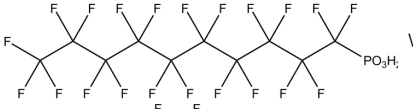
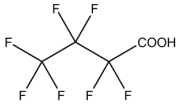
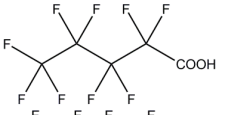
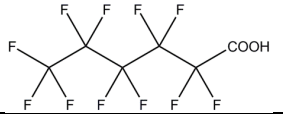
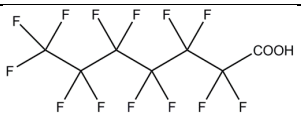
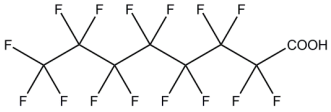
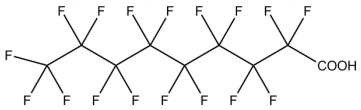
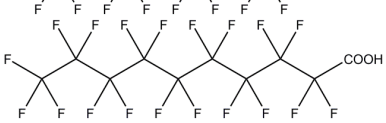
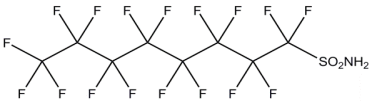
Analyte	Abbreviation	Structure	Supplier	Purity %	Log K_{ow}	pKa
Perfluorobutane sulfonic acid	PFBS		Wellington (Canada)	>98	2.4[d]	0.1[b]
Perfluorohexane sulfonic acid	PFHxS		Wellington (Canada)	>98	4.3[d]	0.1[b]
Perfluorooctane sulfonic acid	PFOS		Wellington (Canada)	>98	6.3[d]	0.1[b]
Perfluorooctane phosphonic acid	PFOPA		Wellington (Canada)	>98	5.8[c]	2.4/4.5[c]
Perfluorohexane phosphonic acid	PFHxPA		Wellington (Canada)	>98	3.6[c]	2.1/4.4[c]
Perfluorodecane phosphonic acid	PFDPA		Wellington (Canada)	>98	8.3[c]	3.4/5.6[c]
Perfluorobutanoic acid	PFBA		Wellington (Canada)	>98	2.4[d]	0.2-0.4[a]
Perfluoropentanoic acid	PFPeA		Wellington (Canada)	>98	3.4[d]	0.5[a]
Perfluorohexanoic acid	PFHxA		Wellington (Canada)	>98	4.4[d]	0.9[a]

Table 3.1: *Continuation.*

Analyte	Abbreviation	Structure	Supplier	Purity %	Log K_{ow}	pKa
Perfluoroheptanoic acid	PFHpA		Wellington (Canada)	>98	5.3[d]	-[f]
Perfluorooctanoic acid	PFOA		Wellington (Canada)	>98	6.3[d]	2.8[a]
Perfluorononanoic acid	PFNA		Wellington (Canada)	>98	7.3[d]	2.6[e]
Perfluorodecanoic acid	PFDA		Wellington (Canada)	>98	7.9[d]	2.6[a]
Perfluorooctane sulfonamide	FOSA		Dr. Ehrenstorfer (Germany)	97.5	7.6[d]	6.5[b]

[a] *[30]*
[b] *[31]*
[c] *[32]*
[d] *[33]*
[e] *[34]*
[f] *not reported*

Table 3.2: *Supplier, abbreviations and purities for surrogates standards, as well as which target analyte is corrected with each isotopic analogue.*

Surrogate	Abbreviation	Corrected compounds	Purity %	Supplier
Perfluoro-1-hexane [$^{18}O_2$] sulfonate	MPFHxS	L-PFHxS, L-PFBS	>98	Wellington (Canada)
Perfluoro-1-[1,2,3,4-$^{13}C_4$] octane sulfonate	MPFOS	L-PFOS	>98	Wellington (Canada)
Perfluoro-n-[$^{13}C_4$] butanoic acid	MPFBA	PFBA	>98	Wellington (Canada)
Perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid	MPFHxA	PFPeA, PFHxA, PFHpA, PFHxPA	>98	Wellington (Canada)
Perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid	MPFOA	PFOA	>98	Wellington (Canada)
Perfluoro-n-[1,2,3,4,5-$^{13}C_5$] nonanoic acid	MPFNA	PFNA, FOSA	>98	Wellington (Canada)
Perfluoro-n-[1,2-$^{13}C_2$] decanoic acid	MPFDA	PFDA	>98	Wellington (Canada)
Perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid	MPFUdA	-	>98	Wellington (Canada)
Perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid	MPFDoA	-	>98	Wellington (Canada)

Stock solution for PFOS, PFOA and FOSA were dissolved individually in MeOH in order to prepare approximately 5000 mg/L solutions. 100 mg/L dilutions were prepared in MeOH every month and dilutions at lower concentrations were prepared daily. Perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), potassium perfluoro-1-butane sulfonate (L-PFBS), sodium perfluoro-1-hexane sulfonate (L-PFHxS) and potassium perfluoro-1-octane sulfonate (L-PFOS) were obtained at 5 mg/L in MeOH and the surrogate mixture (sodium perfluoro-1-hexane [$^{18}O_2$] sulfonate, MPFHxS, sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octane sulfonate, MPFOS, perfluoro-n-[$^{13}C_4$] butanoic acid, MPFBA, perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid, MPFHxA, perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid, MPFOA, perfluoro-n-[1,2,3,4,5-$^{13}C_5$] nonanoic acid, MPFNA, perfluoro-n-[1,2-$^{13}C_2$] decanoic acid, MPFDA, perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid, MPFUnDA, perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid, MPFDoDA) was obtained at 2 mg/L in MeOH. Perfluorooctane phosphonic acid (PFOPA), perfluorohexane phosphonic acid (PFHxPA) and perfluorodecane phosphonic acid (PFDPA) were obtained individually in MeOH at 50 mg/L. All the chemicals standards were stored at 4 °C in the dark and the stock solutions were stored at -20 °C.

MeOH (HPLC grade, 99.9 %) and acetone (HPLC grade, 99.8 %) were supplied by LabScan (Dublin, Ireland), ACN (HPLC grade, 99.9 %) by Sigma Aldrich (Steinheim, Germany), acetic acid (HOAc, 100 %), hydrochloric acid (HCl, 36 %), sodium hydrogen carbonate ($NaHCO_3$, 99.5 %) and potassium hydroxide (KOH, 85 %) by Merck (Darmstadt, Germany), formic acid (HCOOH, 98-100 %) by Scharlau (Barcelona, Spain) and ammonium hydroxide (NH_4OH, 25 %) and sodium carbonate (Na_2CO_3, 99.8 %) by Panreac (Barcelona, Spain). Ultra-pure water was obtained using a Milli-Q water purification system (< 0.05 µS/cm, Milli-Q model 185, Millipore, Bedford, MA, USA).

Waters Oasis-HLB (poly(divinylbenzene-co-*N*-vinylpirrilidone polymer, 200 mg), Waters Oasis-MAX (poly(divinylbenzene-co-*N*-vinylpirrilidone + quaternary amine polymer, 150 mg) and Waters Oasis-WAX (poly(divinylbenzene-co-*N*-vinylpirrilidone + secondary amine polymer, 150 mg) SPE cartridges were purchased from Waters Corporation (Milford, USA).

For the mobile phase composition, MeOH and ACN (Romil-UpS, Waterbeach, Cambridge, UK) were used. 1-methyl piperidine (1-MP, $\geq$ 98 %) was obtained from Merck and ammonium acetate (NH_4OAc) was purchased from Sigma Aldrich.

A Cryodos-50 laboratory freeze-dryer from Telstar Instrument (Sant Cugat del Valles, Barcelona, Spain) was used to freeze-dry the samples. For extraction, a Bandelin Sonoplus HD 3100 sonifier ultrasonic cell disruptor/homogeniser (100 W, 20 kHz; Bandelin Electronic, Berlin, Germany) equipped with a 3-mm titanium microtip was used. Fractions were evaporated in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) using a gentle stream of nitrogen. After the extraction step, the supernatant was filtered through polyamide filters (0.45 µm, 25 mm, Macherey-Nagel, Germany) and polypropylene microfilters (0.2 µm, 13 mm, Pall, USA) were used to filter extracts before LC-MS/MS analysis.

Fish (hake, prawn and tuna) samples and vegetables (lettuce, carrot and pepper) were obtained from a local market.

3.2.2 Sample treatment and FUSLE

Vegetable and fish samples were frozen and freeze-dried before the extraction step. For optimisation experiments, a known amount of matrix was weighted, covered with acetone, spiked with target analytes and stirred during 24 hours. After that, acetone was evaporated and the sample was aged for one week.

Under optimal conditions 0.5 g of sample were placed together with 7 mL of an ACN: Milli Q water (9:1) mixture in a 40 mL vessel and surrogate standards (MPFHxS, MPFOS, MPFBA, MPFHxA, MPFOA, MPFNA, MPFDA, MPFUdA, MPFDoA) were added (25 µL of a 0.5 ng/µL solution). The FUSLE step was performed in the pulsed mode for 2.5 min in duplicate, with a pulsed time on of 0.8 s and pulsed time off of 0.2 s and at 10 % of ampitude. Extractions were carried out at 0 °C in an ice-water bath. After the extraction step, the supernatant was filtered through a polyamide filter and FUSLE extract was evaporated to ~ 1 mL under a nitrogen stream using a Turbovap LV Evaporator depending on the clean-up selected.

3.2.3 Clean-up

3.2.3.1 Oasis-HLB

This clean-up approach was a modification performed to the method published by Loos et al. [35]. Briefly, the extract evaporated to ~ 1 mL was diluted in 6 mL of Milli-Q water previously adjusted at pH 1 with HCl. The 200-mg Waters Oasis-HLB cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water previously adjusted at pH 1. After the sample was loaded (pH=1), 5 mL of a (95:5) Milli-Q water: MeOH mixture was added with cleaning purposes and the cartridges were dried for 1 h under vacuum. Then, the analytes were eluted using 8 mL of MeOH and collected in a single vial. The eluate was concentrated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 250 µL of LC-MS grade MeOH. Finally, the reconstituted extract was filtered through a 0.2 µm polypropylene filter before LC-MS/MS analysis.

3.2.3.2 Oasis-WAX

This clean-up approach was a modification performed to the method published by Chu et al. [36]. Briefly, the extract evaporated to ~ 1 mL was diluted in 6 mL of Milli-Q water at pH 7. The 200-mg Waters Oasis-WAX cartridges were conditioned with 5 mL

of MeOH and 5 mL of Milli-Q water at pH 7. After the sample was loaded, 1 mL of formic acid (2 %) and 1 mL of Milli-Q water: MeOH (95:5, v/v) mixture were added with cleaning purposes and the cartridges were dried for 1 h under vacuum. Then, the analytes were eluted using 4 mL of acetone with 2.5 % NH_4OH and collected in a single vial. After elution, the extract was concentrated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 250 μL of LC–MS grade MeOH. Finally, the reconstituted extract was filtered through a 0.2 μm polypropylene filter before the LC-MS/MS analysis.

3.2.3.3 Oasis-MAX

This clean-up approach was performed according to the standardised method published by Waters [37]. Briefly, the extract evaporated to ~ 1 mL was diluted in 6 mL of Milli-Q water. The 150-mg Waters Oasis-MAX cartridge was conditioned with 5 mL MeOH and 5 mL water. The concentrated sample extract was loaded, and the cartridge was rinsed with 2 mL of 5 mol/L NH_4OH in 5 % MeOH followed by 4 mL of MeOH. The analytes were subsequently eluted with 8 mL of 2 % formic acid in MeOH. The extract was evaporated to dryness under nitrogen at 35 °C and reconstituted in 250 μL of LC-MS grade MeOH. Finally, the reconstituted extract was filtered through a 0.2 μm polypropylene filter before LC-MS/MS analysis.

3.2.4 LC-MS/MS analysis

An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, autosampler and column oven coupled to an Agilent 6430 triple quadrupole (QqQ) mass spectrometer equipped with both ESI and APCI sources (Agilent Technologies, Palo Alto, CA, USA) was employed for the separation and quantification of PFCs. Under optimised conditions, mobile phase A consisted of water:MeOH (95:5, v/v) mixture and mobile phase B of MeOH:water (95:5, v/v), and both contained 2 mmol/L NH_4OAc and

5 mmol/L 1-MP. The gradient profile started with 90 % A (hold time 0.3 min) and continued with a linear change to 80 % A up to 1 min, to 50 % A up to 1.5 min and to 20 % A up to 5 min (hold time 5 min) followed with a linear change to 0 % A up to 13 min and a hold time until 16 min. Initial conditions were regained at 17 min followed by equilibration until 26 min. The flow rate was set at 0.2 mL/min and the volume injected was 5 µL.

Two chromatographic columns were tested for analyte separation. An ultra high performance liquid chromatographic (UHPLC) Agilent Zorbax Extend-C18 (2.1 mm, 50 mm, 1.8 µm) column (pH range 2.0-11.5) and an Agilent Zorbax SB-C18 (2.1 mm, 50 mm, 1.8 µm) column (pH range 1-8). In all the cases an UHPLC Zorbax Eclipse XDB-C18 pre-column (2.1 mm, 5 mm, 1.8 µm) was used. The column temperature was set to 35 °C for Agilent Zorbax Extend-C18 column and at 40 °C in the case of Agilent Zorbax SB-C18 column.

Quantification was performed in the selective reaction monitoring (SRM) acquisition mode. Nitrogen was used as nebuliser, drying and collision gas. ESI in negative mode was carried out using a capillary voltage of 3000 V, a drying flow rate of 10 L/min, a nebuliser pressure of 50 psi (1 psi=6.8948 kPa) and drying gas temperature of 350 °C.

Fragmentor electric voltage and collision energy were optimised for ESI in the 60-220 V and 5-45 eV ranges, respectively, by injection of individual compounds. Optimised values are included in ***Table 3.4*** (Results and Discussion section).

Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

3.3 Results and discussion

3.3.1 Optimisation of LC-MS/MS

3.3.1.1 Optimisation of the chromatographic column and the mobile phase

In a first approach, Zorbax SB C-18 column was tested for the separation of up to 14 analytes, including carboxylic, sulfonate, phosphonate and sulfonamide derivatives of PFCs using a mobile phase A consisting of 95:5 water: MeOH and a mobile phase B consisting of 95:5 MeOH: water, with 5 mmol/L ammonium acetate in both A and B. However, the chromatographic signal, especially of phosphonated PFCs, was very poor. According to the results obtained by Ullah et al. [38], 1-MP can improve the chromatographic behaviour of PFCs since 1-MP behaves as an ion-pairing agent that masks the negative charges of the phosphonate group, leading to an increase in the retention on a C-18 stationary phase through hydrophobic interactions. In order to test the use of 1-MP in the mobile phase, the chromatographic column had to be changed since a chromatographic column able to support pHs up to 11 was necessary. In this sense, Zorbax Extend-C18 column which stands pHs up to 11.5 was chosen. As can be observed in ***Table 3.3*** for the calibrations curves (see calibration ranges in ***Table 3.4***) for PFOPA, PFOS, FOSA and PFOA, the addition of 1-MP significantly improved the slope of the calibration curve for PFOPA and PFOA and, in a less extent, of PFOS. No improve was observed for FOSA.

Table 3.3: *Comparison of calibration slopes to study the influence of 1-MP in the mobile phase.*

Analyte	With 1-MP Slope ± s (ng/mL)	Without 1-MP Slope ± s (ng/mL)
PFOS	182 ± 19	159 ± 2
PFOA	26 ± 1	8.5 ± 0.1
FOSA	47 ± 4	66.2 ± 0.7
PFOPA	4.96 ± 0.09	1.33 ± 0.06

Furthermore, different compositions of the mobile phase containing MeOH, ACN and water were tested. Mobile phase A, consisting of 95:5 water: MeOH, and mobile phase B, consisting of 95: 5 MeOH: water, with 2 mmol/L ammonium acetate and 5 mmol/L 1-MP in both A and B was selected since, when ACN was added, the sensibility obtained was worse. ***Figure 3.1*** shows a chromatogram for a fortified carrot sample (25 ng/g) obtained under optimised conditions.

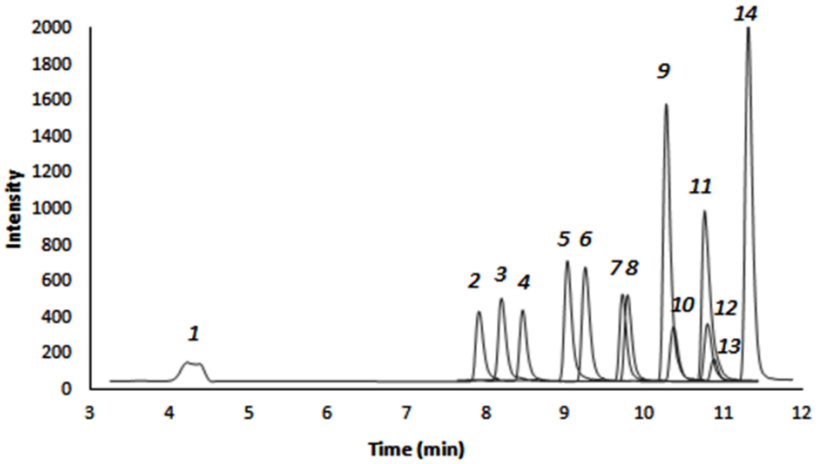


Figure 3.1: *Chromatogram of a 25 ng/g fortified carrot sample extracted by FUSLE and Oasis WAX clean-up. (1) PFBA, (2) PFHxPA, (3) PFPeA, (4) PFBS, (5) PFHxA, (6) PFOPA, (7) PFHpA, (8) PFHxS, (9) PFOA, (10) PFDPA, (11) PFNA, (12) PFOS, (13) FOSA, (14) PFDA.*

3.3.1.2 Optimisation of the electrospray ionisation

According to the literature [15], ESI has been mostly used for the determination of PFCs using LC-MS. Only in the case of Esparza et al. [32], APCI showed better sensitivity when PFPAs and PFOS were investigated, but since the simultaneous determination of up to 14 PFCs was aimed in the present work, only ESI was optimised. During optimisation of ESI PFOS, PFOA, FOSA and PFOPA were studied. Three variables were

studied: the capillary voltage (3-6 kV), the nebuliser pressure (30-50 psi) and the drying gas nitrogen flow (8-12 L/min). Drying gas temperature was fixed at 350 °C according to the manufacturer.

A central composite design (CCD) was built using the Statgraphics program (Statgraphics centurion XV). The CCD consisted of a 2^3 factorial design with a six star points located at $\pm\alpha$ from the center of the experimental domain and three replicates of the central point. An axial distance α of 1.68 was selected in order to guarantee the rotatability.

Figures 3.2 (a-d) show the response surfaces obtained using only the significant ($p < 0.05$) parameters.

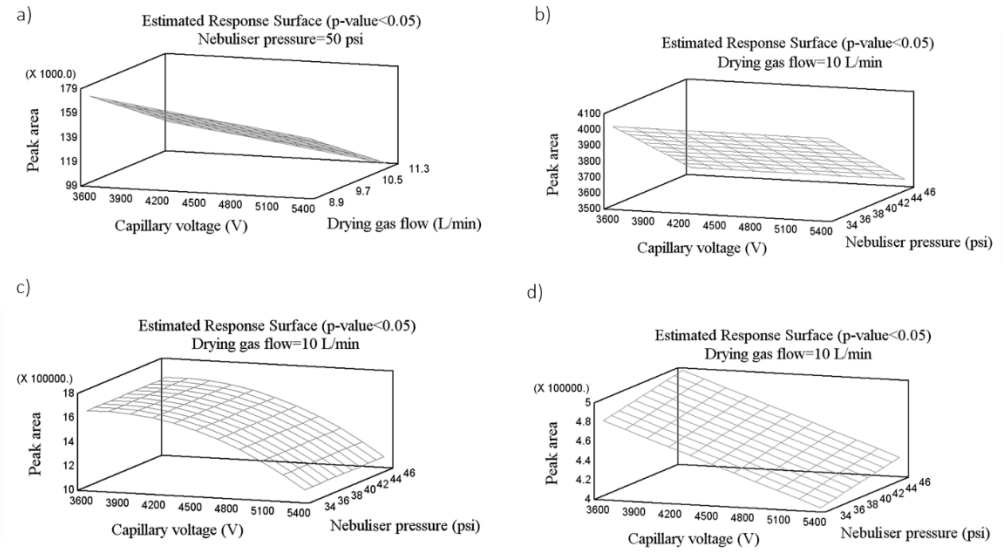


Figure 3.2: *Response surfaces for (a) PFOA when the nebuliser pressure was fixed at 50 psi, (b) PFOPA when the drying gas flow was fixed at 10 L/min, (c) PFOS when the drying gas flow was fixed at 10 L/min and (d) FOSA when the drying gas flow was fixed at 10 L/min.*

As it can be observed in ***Figure 3.2 (a)*** for PFOA, capillary voltage had a negative effect and a similar behaviour was observed for PFOS and FOSA (***Figures 3.2 (c)*** and

3.2 (d), respectively), except for PFOPA, which showed no effect for this parameter (*Figure 3.2 (b)*). According to these results, the capillary voltage was fixed at 3000 V for the rest of the experiments.

The drying gas flow was significant only for PFOA and FOSA (see *Figures 3.2 (a)* and *3.2 (d)*, respectively). While PFOA showed the highest responses at a low value of this parameter, 8 L/min, FOSA showed the highest responses at a high value of this parameter. An intermediate value, 10 L/min, was fixed for drying gas flow.

Finally, the nebuliser pressure was significant for FOSA and PFOPA (see *Figures 3.2 (d)* and *3.2 (b)*, respectively). While PFOPA showed the highest signals at a low value of this parameter, 30 psi, FOSA showed the highest signals at a high value of this parameter, 50 psi. A high value, 50 psi, was fixed for drying gas flow.

In summary, the optimised parameters were fixed as follows: capillary voltage at 3000 V, drying gas flow at 10 L/min and nebuliser pressure at 50 psi.

Parameters related to the mass spectrometry were also studied; thus, fragmentor voltage and collision energy were optimised considering all the target analytes and surrogates. The fragmentor voltage (60, 100, 150, 220 and 240 V) was optimised in order to obtain the highest signal of the precursor ion, while minimising its fragmentation. Optimisation was performed in the MS2 Scan mode and *Table 3.4* summarises optimum fragmentor values for each target analyte and surrogates.

In order to obtain the best signals for the product ions, the collision energy was studied in the 5-45 eV range at 5 eV increments. The most intense product ions were selected as the quantifiers and, when possible, qualifier ions were also selected. *Table 3.4* summarises optimum collision energies, as well as, the precursor and product ions for each target analyte and surrogates.

Table 3.4: Precursor and product ions (first ion was used as quantifier and the second as qualifier) at optimum fragmentor (V) and collision energy (eV) values, as well as the calibration ranges, the correlation coefficients, the instrumental LODs and LOQs for target analytes.

Analytes	Precursor ion	Product ion	Fragmentor (V)	Collision Energy (eV)	Calibration range (ng/mL)	Determination coefficient	LOD (ng/mL)	LOQ (ng/mL)
PFBA	213	169	60	5	3.7-207	0.993	2.29	3.73
PFHxPA	399	79	100	10	1.7-207	0.995	1.28	1.73
PFPeA	263	219/175	60	5	4.9-207	0.995	2.32	4.92
PFBS	299	99/80	100	30	4.0-207	0.995	2.01	3.99
PFHxA	313	269/119	60	5	3.3-207	0.996	1.97	3.33
PFOPA	499	79	150	20	2.6-207	0.996	1.54	2.58
PFHpA	363	319/169	60	10	3.5-207	0.994	1.94	3.47
PFHxS	399	99/80	150	20	2.7-207	0.993	1.47	2.73
PFOA	413	369/169	60	5	4.2-207	0.995	2.47	4.22
PFDPA	599	79	100	5	2.5-207	0.992	1.41	2.46
PFNA	463	419/169	60	5	5.7-179	0.992	2.47	5.65
PFOS	499	99/80	150	45	0.7-194	0.994	0.46	0.73
FOSA	498	78	220	5	4.1-179	0.994	1.91	4.09
PFDA	513	469/269	100	5	3.6-179	0.978	1.81	3.61
MPFBA	217	172	60	5				
MPFHxA	315	270	60	5				
MPFHxS	403	103	150	30				
MPFOA	417	372	60	5				
MPFOS	503	99	60	45				
MPFNA	468	423	60	5				
MPFDA	515	470	100	5				
MPFUdDA	565	520	60	5				
MPFDoDA	615	570	100	5				

3.3.1.3 Calibration ranges, determination coefficients and instrumental limits of detection

Under optimised chromatographic and mass spectrometric values, calibration curves were built with standard solutions (in MeOH) from 1 ng/mL to 150 ng/mL range and at 8 concentration levels. As it can be seen in *Table 3.4*, determination coefficients, without correction with the corresponding internal standard, in the range of 0.992-0.996 were obtained, except for PFDPA, in which case the coefficient value obtained was 0.978. Instrumental limits of detection (LODs) and quantification (LOQs) were estimated and defined as the average response (n=3) of the low concentration level

(1 ng/mL) of the calibration curve plus three and ten times the standard deviation, respectively [39]. As can be observed in ***Table 3.4***, the LODs and LOQs obtained were below 2.47 ng/mL and 5.65 ng/mL, respectively.

3.3.2 Optimisation of FUSLE

For the optimisation of FUSLE PFOS, PFOA and FOSA were chosen as target analytes. In addition, hake and carrot samples were used during the optimisation.

Six extraction solvents were tested according to the literature: MeOH, acetone, acetic acid, 9:1 MeOH: acetic acid, 9:1 ACN: Milli-Q water and 10 mmol/L KOH in MeOH. The experiments were performed in triplicate. Aliquots of 0.5 g (dry weight) of spiked hake and carrot were extracted with 7 mL of the different solvents mentioned above for 2.5 min. ***Figures 3.3 (a-b)*** show the responses obtained (normalised to the highest signal) for hake and carrot, respectively.

In the case of hake (see ***Figure 3.3 (a)***), the responses obtained were significantly higher when 9:1 ACN: Milli-Q water mixture was used for all target analytes. However, in the case of carrot samples (***Figure 3.3 (b)***), this evidence was not so clear. Although 9:1 ACN: Milli-Q water mixture was also the best extractant for FOSA, the same results were not obtained for PFOS and PFOA. In the case of PFOS, acetone, MeOH, 10 mM KOH in MeOH and 9:1 ACN Milli-Q water provided similar recoveries. In the case of PFOA 10 mM KOH in MeOH provided the best results, but statistically no difference was found if compared with 9:1 ACN:Milli-Q water (95 % of confidence level). According to the results mentioned above, 9:1 ACN: Milli-Q water was chosen as extraction solvent for further experiments. Similar results were obtained by Ullah et al. [19] for food samples. Furthermore, Martínez-Moral et al. [24] reported that ACN was the best extraction solvent for sewage sludge samples, while Moreta and Tena [26] used ethanol for the extraction of six perfluorocarboxylic acids and PFOS from packaging material.

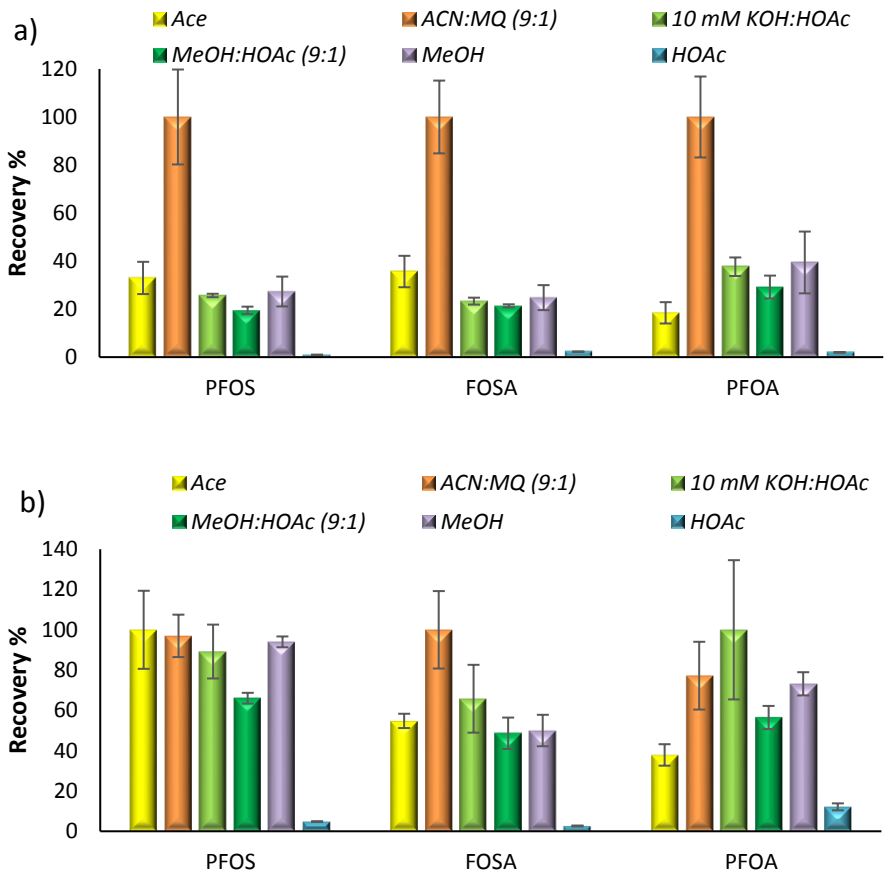


Figure 3.3: *Influence of solvent type during FUSLE extraction in (a) hake and (b) carrot samples. Signals were normalised to the highest chromatographic response. Average responses (n=3) and standard deviations were represented.*

In order to improve FUSLE extraction efficiency three extraction solvent volumes were tested: 4, 7 and 10 mL. The experiments were performed in triplicate. 7 mL (see ***Figure 3.4*** for carrot) provided the highest recoveries, as well as the lowest relative standard deviations. Similar results in terms of extraction volumes were obtained by Martínez-Moral et al. [24] for sewage sludge.

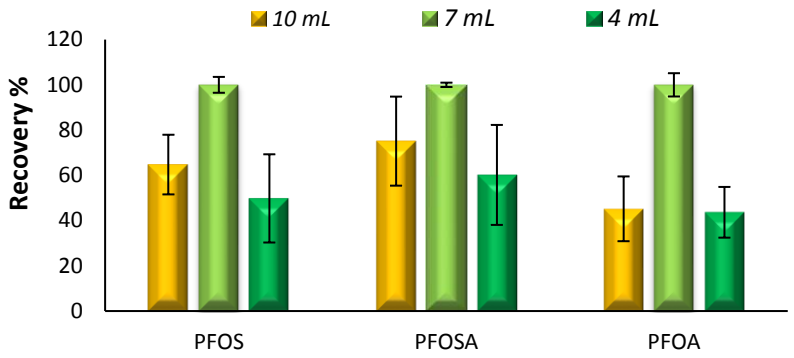


Figure 3.4: *Influence of solvent volume during FUSLE extraction in carrot samples. Signals were normalised to the highest chromatographic response. Average responses (n=3) and standard deviations were used.*

Extraction efficiency was also tested at room temperature and at 0 °C, but no significant differences were observed (see ***Figure 3.5*** for carrot).

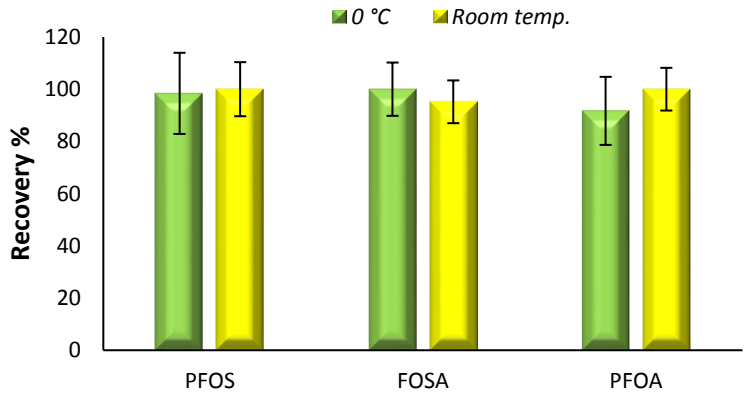


Figure 3.5: *Influence of extraction temperature in carrot. Signals were normalised to the highest chromatographic response. Average responses (n=3) and standard deviations were used.*

A CCD was carried out using Statgraphics in order to optimise extraction time (0.5-5 min), pulsed time on or sonication time (0.2-0.8 s) and amplitude (10-56 %). In pulsed sonication, extraction time is divided in different cycles. A cycle is a sum of the period of time that pulsed time is on (sonication time) and the period of time that pulse is off. In this work cycles of 1 s were used. The CCD consisted of a 2^3 factorial design with six

star points located at ± α from the center of the experimental domain and three replicates of the central point. An axial distance α of 1.68 was selected in order to guarantee the rotatability. The responses obtained were scaled in the logarithmic form. ***Figure 3.6*** shows the response surfaces obtained using only the significant (p < 0.1) parameters.

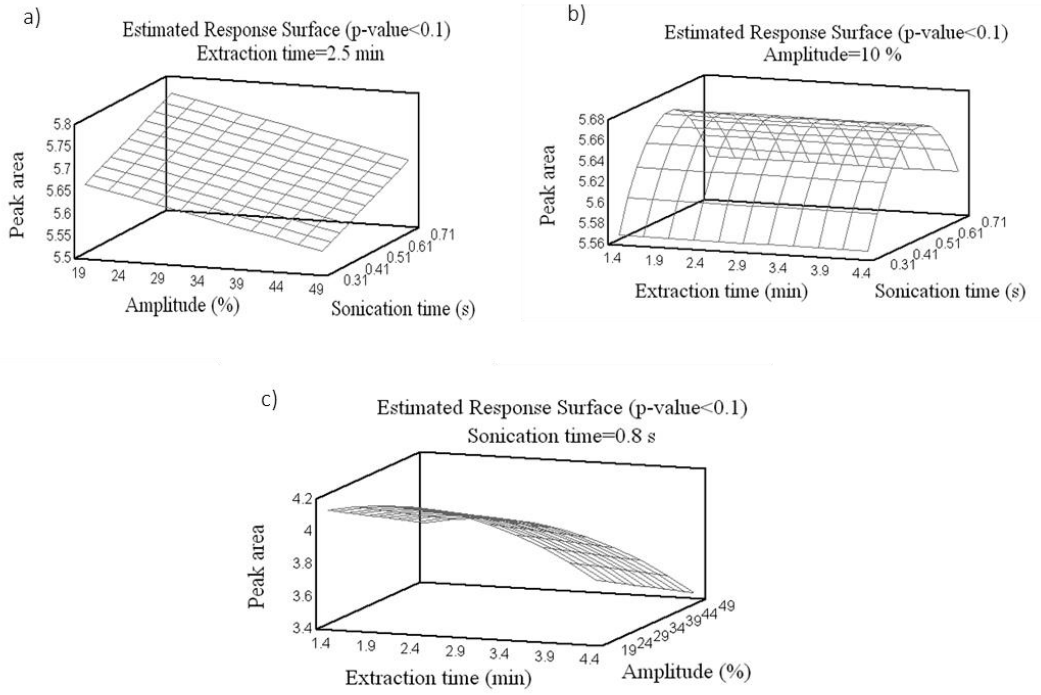


Figure 3.6: *Response surfaces obtained for carrot during the FUSLE optimisation for (a) PFOS when extraction time was fixed at 2.5 min, (b) FOSA when amplitude was fixed at 10 % and (c) PFOA when sonication time was fixed at 0.8 s.*

As can be observed, the sonication time had a positive effect for PFOS (see ***Figure 3.6 (a)***), showing the highest values at the highest value of this parameter, 0.8 s. In the case of FOSA (see ***Figure 3.6 (b)***) the highest response was obtained at an intermediate value of this parameter, 0.5 s. The sonication time was fixed at 0.8 s for the rest of the experiments.

The amplitude was significant for PFOS and PFOA (see ***Figures 3.6 (a)*** and ***(c)***, respectively) and both analytes showed the highest responses at a low value of this parameter, 10 %. Therefore, the lowest value was chosen for amplitude, 10 %. Besides, low amplitudes increase the life of the titanium tips.

Finally, the extraction time was significant for PFOS and PFOA and the highest yields were obtained at an intermediate value of this parameter (see ***Figure 3.6 (c)*** for PFOA). According to this result, an intermediate value (2.5 min) was fixed for extraction time.

In summary, optimum extraction conditions were fitted as follows: extraction time at 2.5 min, sonication time at 0.8 s and amplitude at 10 %.

In the absence of a certified reference material (CRM) and in order to determinate whether exhaustive extraction was carried under optimised condition, repeated extractions were performed. Up to three successive extractions were performed on the same samples. Each experiment was carried out in triplicate. Results are included in ***Figures 3.7 (a)*** and ***(b)*** for carrot and hake, respectively.

In the case of hake samples, a unique extraction was sufficient for quantitative extraction. In the case of carrot samples, two successive extractions were necessary for quantitative extraction, while recoveries lower than 20 % were obtained in the third extraction. A third extraction was not considered in order to avoid increasing the solvent volume (7 mL x 3) submitted to the evaporation step. Similar results were obtained by Martínez-Moral et al. [24] for the determination of these target analytes in sewage sludge where a second FUSLE step was necessary. In the case of the extraction of PFCs from packaging material a single FUSLE extraction was necessary according to Moreta and Tena [26].

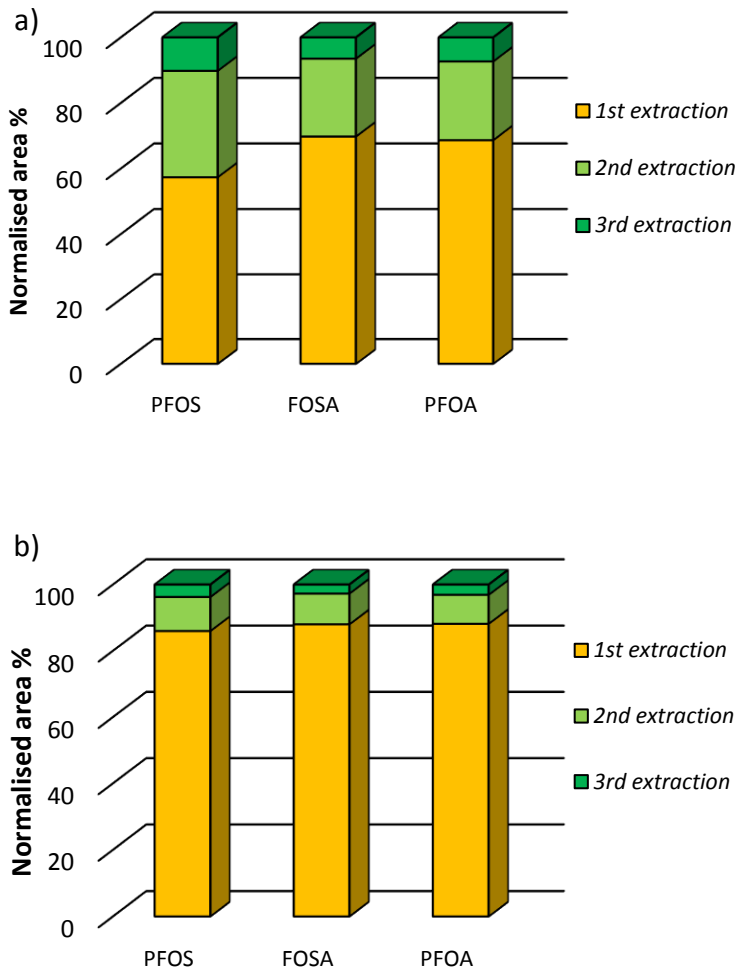


Figure 3.7: *Influence of the number of repeated extractions in (a) carrot and (b) hake samples.*

Although the CCD provided the highest responses when an intermediate value of extraction time was used (2.5 min), since successive extractions showed that a second extraction was needed for a quantitative extraction, 5 min extraction was tested. For this reason, two consecutives extractions of 2.5 min were compared with a unique extraction of 5 min for carrot samples. As it can be observed in ***Figure 3.8*** and in concordance with the results obtained in the CCD, a single 5 min extraction did not guarantee quantitative extraction and, finally, 2 x 2.5 min extraction was chosen.

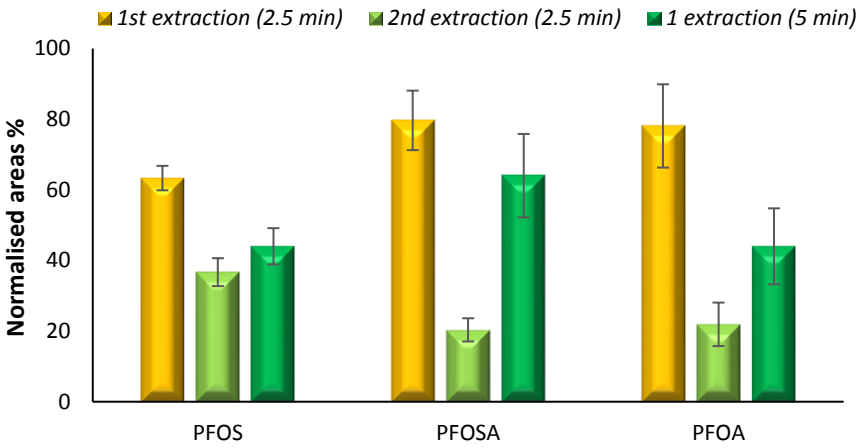


Figure 3.8: *Comparison of extraction yield at different extraction time values: (a) 1st extraction of 2.5 min, (b) 2nd extraction of 2.5 min and (c) 5 min extraction.*

3.3.3 Optimisation of the clean-up step

3.3.3.1 Extraction efficiency of the different clean-up procedures

As mentioned in the experimental section, different clean-up approaches were performed in order to determine the suitability of each of them. The extraction efficiency was calculated by comparing the responses obtained when the sample was spiked at 1.5 ng/µL before and after clean-up step (see ***Table 3.5***). Waters Oasis MAX was only tested for carrot samples.

As shown in ***Table 3.5*** Waters Oasis HLB and Waters Oasis WAX showed the best efficiencies for all the target analytes. In the case of Waters Oasis HLB a modification of the method published by Loos et al. [35] was performed. In order to increase extraction efficiencies the analytes must be in their non-ionic form. Since PFCs are very acidic analytes acidification of the sample (pH=1) was carried out in our work compared to pH=7 used in the referenced work.

***Table 3.5**: Efficiencies (%) for different clean-up approaches for carrot and hake samples.*

Carrot			
Analyte	**Oasis HLB**	**Oasis WAX**	**Oasis MAX**
PFOS	75	90	3
PFOA	98	93	3
FOSA	59	77	33
PFOPA	63	82	1
Hake			
Analyte	**Oasis HLB**	**Oasis WAX**	**Oasis MAX**
PFOS	88	91	-[a]
PFOA	92	83	-
FOSA	87	88	-
PFOPA	54	98	-

[a] *Not performed for hake samples*

Waters Oasis WAX approach was a modification performed to the method published by Chu et al. [36]. The retention mechanism was mixed mode (both ion exchange and reverse phase), which improves retention for strong acidic compounds. While Chu et al. loaded the sample at pH=4, different pHs values (4 and 7) were tested in the present work. While comparable results were obtained for PFOS, PFOA and FOSA, higher extraction efficiencies were obtained for PFOPA at pH 7 (***see Figure 3.9***).

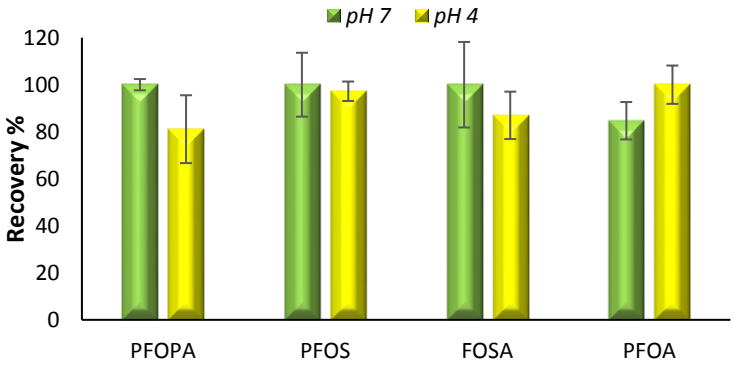


***Figure 3.9:** Influence of sample pH in the clean-up step using Oasis WAX. Signals were normalised to the highest chromatographic response. Average responses (n=3) and standard deviations were used.*

In the case of Waters Oasis MAX the clean-up approach was performed according to the standardised method published by Waters [37], where the retention mechanism was also mixed mode. Recoveries lower than 3 % were obtained for all the target analytes except for FOSA, 33 % (***Table 3.5***). Thus, this clean-up approach was discarded. Similar results were obtained by Liu et al. [27] for the determination of PFPAs in sewage sludge. However, Ullah et al. [19] obtained satisfactory results when a similar cartridge, CUQAX256 (C18 + quaternary amine, United Chemical Technologies, UCT, Bristol, PA), was used to determinate perfluoroalkyl carboxylic, sulfonic and phosphonic acids in food. In this sense, further studies should be carried out in order to improve the results obtained with Waters Oasis-MAX cartridge.

3.3.3.2 Matrix effect for the different clean-up approaches

The extraction efficiency can be affected by the composition of the sample matrix since high levels of matrix compounds may compete with the sorptive material or can lead to matrix effects during LC-MS/MS determination due to changes of the ESI ionisation efficiency.

Therefore, matrix effects occurring at LC-MS/MS detection were evaluated by comparing the responses obtained for carrot and hake samples which were spiked with 1.5 ng/µL after the clean-up step and a standard solution in MeOH at the same concentration. Non-spiked blank samples were also analysed and their response was considered in matrix effect calculations. The results are included in ***Figures 3.10 (a)*** and ***(b)*** for carrot and hake, respectively, where values close to 100 % indicate a lack of matrix effect.

As shown in ***Figures 3.10 (a)*** and ***(b)***, only extracts cleaned up using Waters Oasis-HLB showed significant matrix effect during the detection step (signal enhancement for PFOPA and signal suppression for PFOA). Therefore, Waters Oasis-HLB clean-up was discarded from method validation.

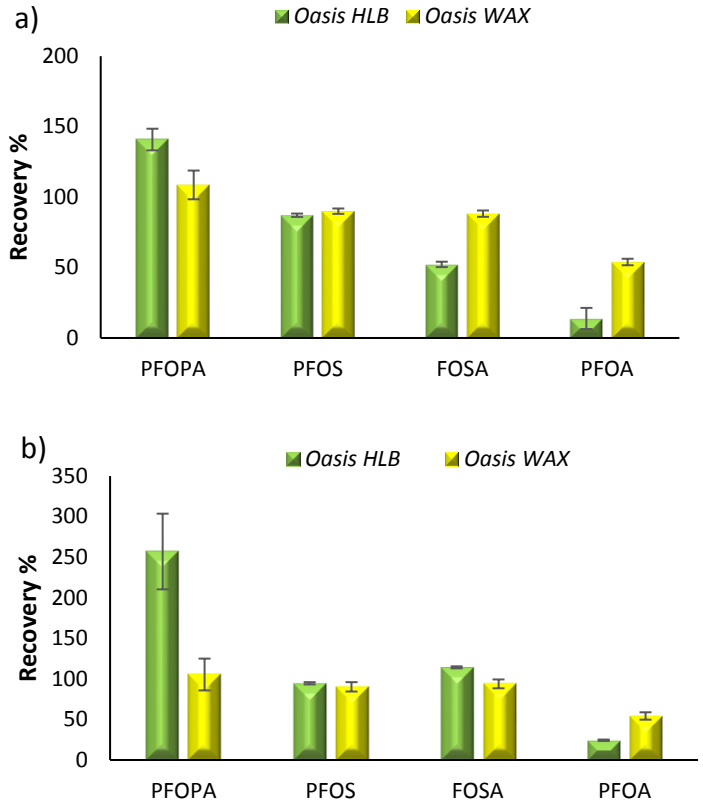


Figure 3.10: Matrix effect in the detection for (a) carrot and (b) hake samples.

3.3.4 Method validation and application to real samples

Method validation was only performed for FUSLE extraction with a posterior clean-up with Waters Oasis-WAX. Apparent recovery, defined as the recovery obtained after correction with the corresponding surrogate, was calculated using carrot and hake samples spiked at 12.5 ng/g and 25 ng/g and at 25 ng/g and 50 ng/g for pepper, lettuce and amended soil. Furthermore, matrix-matched calibration was also performed for carrot samples with samples spiked at the same concentrations [19]. Recoveries obtained are included in *Table 3.6*.

Table 3.6: *Apparent recoveries at two different levels for carrot (12.5 ng/g and 25 ng/g), pepper (25 ng/g and 50 ng/g), lettuce (25 ng/g and 50 ng/g), hake (12.5 ng/g and 25 ng/g) and amended soil (25 ng/g and 50 ng/g). In the case of carrot samples, apparent recoveries were calculated by means of external and matrix-matched calibration approaches. For the rest of the matrices external calibration was only used. Method detection limits (MDLs in ng/g) are also included.*

	Carrot				
Analyte	Apparent recovery with external calibration 12.5 ng/g	Apparent recovery with external calibration 25 ng/g	Recovery with matrix-matched calibration 12.5 ng/g	Recovery with matrix-matched calibration 25 ng/g	MDL (ng/g)
PFBA	113	118	169	94	3.2
PFPeA	80	94	76	93	1.9
PFHxA	75	81	87	94	0.9
PFHpA	79	86	83	92	0.5
PFOA	69	74	78	85	0.7
PFNA	65	69	81	93	0.3
PFDA	65	70	77	85	0.5
PFBS	92	100	86	98	0.9
PFHxS	68	73	81	86	0.8
PFOS	65	69	78	86	1.0
FOSA	116	106	81	76	1.3
PFHxPA	101	104	36	126	0.8
PFOPA	125	134	88	90	1.6
PFDPA	129	136	84	89	1.5

	Pepper			Lettuce		
	25 ng/g	50 ng/g	MDL (ng/g)	25 ng/g	50 ng/g	MDL (ng/g)
PFBA	91	74	6.9	94	87	8.7
PFPeA	92	73	12.0	71	64	7.8
PFHxA	90	74	8.2	75	75	6.8
PFHpA	77	68	5.6	84	84	7.3
PFOA	93	66	7.5	77	76	5.3
PFNA	88	70	6.4	75	77	5.3
PFDA	86	67	6.7	78	78	6.6
PFBS	94	68	10.1	58	76	8.7
PFHxS	87	62	9.3	75	76	2.4
PFOS	90	69	6.3	83	85	8.3
FOSA	97	77	8.5	98	95	12.4
PFHxPA	96	86	10.1	96	86	11.1
PFOPA	95	96	2.1	85	111	3.2
PFDPA	80	111	11.5	105	111	8.2

	Hake			Amended soil		
	12.5 ng/g	25 ng/g	MDL (ng/g)	25 ng/g	50 ng/g	MDL (ng/g)
PFBA	-[a]	117	12.5	101	98	1.0
PFPeA	75	77	1.2	88	90	2.1
PFHxA	103	102	0.5	91	91	2.4
PFHpA	79	93	0.4	88	98	3.7

Table 3.6: Continuation.

	Hake			Amended soil		
	12.5 ng/g	25 ng/g	MDL (ng/g)	25 ng/g	50 ng/g	MDL (ng/g)
PFOA	85	96	0.4	83	93	3.2
PFNA	86	85	0.4	92	89	2.6
PFDA	82	86	0.2	92	89	3.4
PFBS	105	94	0.4	98	100	1.8
PFHxS	84	94	0.6	78	77	1.2
PFOS	83	94	0.8	90	90	1.5
FOSA	104	88	0.4	55	56	7.0
PFHxPA	96	96	0.5	123	105	7.0
PFOPA	29	87	1.7	111	119	14.0
PFDPA	80	99	1.9	112	103	22.0

[a]*: not detected*

As can be observed for the results obtained for carrot samples, matrix-matched calibration was unnecessary and good apparent recoveries were obtained after correction with the corresponding surrogate.

Apparent recoveries in the 80-120 % range were obtained in most of the cases. In the case of hake samples, PFBA was not detected at the lowest concentration. It should be mentioned that RP columns are not suitable for the analysis of short-chain PFCAs since broad peaks are obtained. Better results might be obtained for PFBA using and ion exchange column [40]. FOSA showed the lowest recoveries (approx. 55 %) for amended soil samples.

Method detection limit (MDL) of each analyte was determined by spiking five replicates of each blank matrix with each analyte at the lowest concentration used in the validation (see ***Table 3.6***). The lowest MDL values were obtained for hake and carrot samples, always lower than 1.89 ng/g (except for PFBA). Similar MDL values were reported by Naile et al. (MDL 0.1-2 ng/g) [3] when alkaline digestion with a posterior clean-up by Waters Oasis HLB was performed or by Moreta and Tena (LOD 0.5-2.2 ng/g) for packaging material using FUSLE. Furthermore, similar MDL values were reported by Bossi et al. [2] when ion-pair extraction was performed (MDL 3-7 ng/g). However, better

MDL values were also reported; for instance, Ullah et al. [19] reported MDL values between 0.002-0.02 ng/g when extraction with ACN/water and clean-up on a mixed-mode co-polymeric sorbent (C8 + quaternary amine) were used in food samples. For the rest of the matrices, MDL values were in the 1-12 ng/g level.

The precision of the method, expressed as relative standard deviation (RSD), was evaluated at the two concentration levels mentioned above and five replicates were performed at each level. Similar RSD values were obtained after correction with the corresponding surrogate for both fortification levels, in the 2-15 %, except for PFPeA and PFOPA in hake (23 % and 38 %, respectively). Similar results were reported when SPE clean-up approaches were used. For instance, Liu et al. [27] obtained RSD values between 1 - 14 % when Waters Oasis WAX approach was used. Moreover, Llorca et al. [13], who optimised PLE extraction with a posterior Waters Oasis WAX clean-up approach, obtained RSD values between 5 - 17 %.

Finally, the optimised and validated method was applied to the analysis of several food samples bought in a local supermarket (fresh hake, fresh tuna, frozen prawn, lettuce, pepper and carrot). FOSA (2.8 ng/g) was the only PCF detected in fresh hake, while FOSA (1.1 ng/g) and PFOS (3.7 ng/g) were detected in the case of tuna. In the case of frozen prawn, as well as in the case of vegetables, concentrations lower than the MDL values were obtained.

3.4 Conclusions

Different steps for the analysis of up to 14 PFCs, including carboxylic, sulfonate, phosphonic and sulfonamide derivatives, were successfully optimised in the present work. A thorough optimisation of the LC-MS/MS analysis of the target compounds was carried out, including the chromatographic column, the mobile phase, the ionisation conditions and the mass spectrometric variables. It should be underlined that mobile

phase using 1-MP as ion-pair reagent increased the sensitivity of carboxylic, sulfonate and phosphonic PFCs. FUSLE extraction rendered quantitative extraction of the target analytes in two successive 2.5 min extractions using 7 mL of a (9:1) ACN: Milli-Q mixture. For SPE clean-up Waters Oasis-HLB, Waters Oasis-WAX and Waters Oasis-MAX cartridges were evaluated. The low extraction efficiency obtained with the Waters Oasis-MAX cartridges and the strong matrix effect observed for Waters Oasis-HLB discarded them from further validation and finally FUSLE coupled to Waters Oasis-WAX clean-up was chosen for method validation of the four families of PFCs studied in the present work.

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Chapter 4

Biodegradation and uptake of the pesticide Sulfluramid in a soil/carrot mesocosm

Environmental Science and Technology, under revision (2017)

4.1 Introduction

Perfluorooctane sulfonate (PFOS; $C_8F_{17}SO_3^-$) has attracted considerable international regulatory and scientific attention due to its widespread occurrence and links to adverse health effects in humans and wildlife [1]. On account of these risks, PFOS and its precursors were added to *Annex B* of the United Nations Stockholm Convention on Persistent Organic Pollutants in 2009 [2], and to the list of priority hazardous substances in the EU water policy Directive 2013/39/EU in 2013 [3]. Presently, manufacturing of PFOS and PFOS-precursors continues in some countries under Stockholm Convention production and use exemptions. These contemporary sources of PFOS are poorly characterised and may pose a considerable ongoing risk to humans and wildlife [4–6].

Brazil is currently among the main global producers of the PFOS-precursor *N*-ethyl perfluorooctane sulfonamide (EtFOSA; $C_8F_{17}SO_2NHC_2H_5$), which is the active ingredient in Sulfluramid, a commercial pesticide. EtFOSA is produced from the starting material perfluorooctane sulfonyl fluoride (POSF; $C_8F_{17}SO_2F$), which is imported into Brazil from China. Brazil holds an exemption under *Annex B* to manufacture and use Sulfluramid to manage leaf-cutting ants from the genus *Atta ssp*. and *Acromyrmex spp.*, which jeopardise agricultural activities in parts of Latin America [7]. Alternatives to Sulfluramid are not currently available; and while the country is phasing out production and use of baits for domestic use, commercial applications in agriculture are expected to continue into the foreseeable future [8].

The manufacture and use of Sulfluramid in Brazil from 2004 to 2015 is expected to produce between 167 and 603 tonnes of PFOS [9,10]. However, there are considerable uncertainties surrounding these estimates, owing to an absence of manufacturing data but also a lack of information surrounding PFOS yields in the environment. For example, the only study to investigate soil biodegradation of EtFOSA reported very low (4 %)

yields of PFOS following incubation of a pure standard of EtFOSA over 182 days [11]. Studies involving other perfluorooctane sulfonamides have demonstrated considerably higher PFOS yields (and in some cases formation of perfluoroalkyl carboxylic acids) under biological [12–15] and abiotic [16,17] conditions (reviewed elsewhere [18]). Among these studies, a soil-vegetable mesocosm study involving perfluorooctane sulfonamide (FOSA), the N-dealkylation product of EtFOSA, demonstrated that FOSA was totally degraded to PFOS in presence of carrot while no degradation was observed in absence of vegetable [12]. Collectively these data suggest that in the natural environment (and in particular in the presence of a vegetable crop), yields of PFOS from EtFOSA may be considerably higher than 4 %. However, to date there are no soil-vegetable mesocosm studies involving EtFOSA or commercial Sulfluramid formulations.

Data on the environmental occurrence of EtFOSA in South and Central America are also scarce [9,10]. Nevertheless, one study reported low but detectable levels of EtFOSA in air samples from Costa Rica [19] and others have observed elevated concentrations of potential EtFOSA transformation products in both South American surface waters [9,20,21] and biota [22]. The unusually high ratio of FOSA:PFOS in Brazilian surface water is hypothesised to be a marker of Sulfluramid use, but this requires further investigation. To date, there are no studies which have examined the occurrence of EtFOSA or its transformation products around agricultural regions where Sulfluramid is deployed. Such data, together with improved estimates of EtFOSA production and PFOS degradation yields, are clearly needed in order to determine the importance of Sulfluramid as a source of environmental PFOS.

Despite some recent work involving leaching and plant uptake [12,23–25] of PFAAs, only a single study has investigated the fate of a PFOS-precursor (FOSA) in a soil-vegetable mesocosm [12]. There are no peer-reviewed studies investigating the fate and behaviour of EtFOSA or commercial Sulfluramid baits in soil-vegetable mesocosm.

Considering the use pattern of Sulfluramid, this information is urgently needed in order to characterise the likelihood of environmental contamination arising from the use of this commercial pesticide. The purpose of this study was to investigate biodegradation, leaching, plant uptake, and distribution of EtFOSA and its transformation products in soil-carrot mesocosms. Experiments were performed with both technical standards and a commercially available, characterised Sulfluramid bait, providing new estimates for EtFOSA-derived PFOS formation under environmentally-relevant conditions. Furthermore, since commercial EtFOSA is manufactured as an isomeric mixture, we studied the fate and behaviour of individual isomers using isomer-specific analytical methodologies. To our knowledge, this is the first isomer-specific study of any PFAS in a soil and/or soil-vegetable mesocosm. Collectively, these data provide valuable new insight on the importance of EtFOSA as a contemporary source of PFOS.

4.2 Experimental section

4.2.1 Reagents and materials

Technical EtFOSA (95 %) originated from Lancaster Synthesis (Wyndham, NH) [26]. Isomeric purity could not be determined due to a lack of purified branched isomer standard. Grão Forte, a commercial Sulfluramid formulation (determined to contain 0.0024 % EtFOSA (∑branched+linear isomers)) was obtained from Insetimax Industrial Chemicals (Brazil). L-EtFOSA, L-FOSA, perfluorooctane sulfonamido acetate (L-FOSAA), perfluorodecanoate (PFDA), characterised isomeric mixtures of PFOS and perfluorooctanoate (PFOA), and the isotopically labeled standards of EtFOSA, FOSA, PFOS, PFOA and PFDA were purchased from Wellington Laboratories (Guelph, ON, Canada) (see ***Table 4.1***).

Table 4.1: List of compounds quantified in the present work, their acronyms, chemical formula, and optimum LC-MS/MS conditions.

Compound (acronym)	Chemical formula	Precursor ion (m/z)	Product ions (m/z)	Cone voltage(V)	Collision energy (V)
N-ethylperfluorooctane sulfonamide (EtFOSA)	$C_8F_{17}SO_2NH(CH_2CH_3)$	526	169 (L-, 6-), 219 (5-), 269 (4-), 319 (3-), 419 (1-)	32	25 (169), 25 (269), 25 (319), 15 (419)
Perfluorooctane sulfonamido acetate (FOSAA)	$C_8F_{17}SO_2NH(CH_2COOH)$	556	498, 419	8	25
Perfluorooctane sulfonamide (FOSA)	$C_8F_{17}SO_2NH_2$	498	78, 169, 478	55	30 (78), 25 (169), 25 (478)
Perfluorooctane sulfonate (PFOS)	$C_8F_{17}SO_3^-$	499	80 (L-,3,4,5-,dm-), 169 (6-), 419 (1-)	65	40 (80), 35 (99). 40 (130), 25 (419)
Perfluorooctanoic acid (PFOA)	$C_7F_{15}CO_2^-$	413	119 (4-), 219 (5-), 169 (6-), 369 (L-)	22	20 (119), 15 (219), 20 (169), 10 (369)
Perfluorodecanoic acid (L-PFDA)	$C_9F_{19}CO_2^-$	513	469, 269, 219	26	10 (469), 15 (269), 20 (219)
N-ethyl-d_5-perfluoro-1-octanesulfonamide ([d_5]-N-EtFOSA)	$C_8F_{17}SO_2NH(CD_2CD_3)$	531	169	32	25
Perfluoro-1-[$^{13}C_8$]octanesulfonamide (MFOSA)	[1,2,3,4,5,6,7,8-$^{13}C_8$]$F_{17}SO_2NH_2$	506	78	55	25
Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate (MPFOS)	C_4F_9[1,2,3,4-$^{13}C_4$]$F_8SO_3^-$	503	80	65	40
Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid (MPFOA)	C_3F_7[2,3,4,5-$^{13}C_4$]$F_8{}^{13}CO_2^-$	417	372	22	10
Perfluoro-n-[1,2,-$^{13}C_2$]decanoic acid (MPFDA)	C_8F_{17}[2-$^{13}C_1$]$F_2{}^{13}CO_2^-$	515	470	26	10

LiChrosolv methanol (MeOH) and formic acid were purchased from Merck (Darmstadt, Germany), Chromasolv acetonitrile (ACN), 25 % ammonium hydroxide (NH_4OH) solution and ammonium formate salts were provided by Sigma-Aldrich (Steinheim, Germany). Evolute WAX solid-phase extraction (SPE) cartridges were obtained from Biotage (Uppsala, Sweden). Finally, water was purified with a Millipore water purification system (Milli-Q water) and had a resistance of 18,2 MΩ cm^{-1}.

All the reagents used for the Hoagland nutritive solution preparation, potassium nitrate (KNO_3, 99.0 %), calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, 98.0 %), ammonium phosphate monobasic ($(NH_4)H_2PO_4$, 96.0-102.0 %), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$, 99.0-100.5 %), manganese chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$, 98.0-102.0 %), boric acid (H_3BO_3, 99.8 %), zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$, 99.0-104.0 %), copper sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$, 99.0-100.0 %) and sodium molybdate dehydrate ($Na_2MoO_4{\cdot}2H_2O$, 98.0-100.0 %) were purchased from Panreac (Castellar del Vallès, Spain).

4.2.2 *Experimental design and soil fortification*

A total of six, 81 day mesocosm experiments were carried out concurrently (***Table 4.2***). Experiments 1 and 2 were conducted in duplicate and involved incubation of technical ∑EtFOSA (3.8 mg/kg; ∑branched+linear isomers) in microbially-active soil (referred to herein as `active´ soil), with and without carrot (*Daucus carota ssp sativus*), respectively. Experiments 3, 4, and 5, were designed as control incubations: Experiment 3 was conducted in duplicate and involved fortifying soil autoclaved at 112 °C under vacuum for 4 h (referred to herein as `inactive´ soil) with technical ∑EtFOSA (3.8 mg/kg) to monitor leaching and abiotic losses. Experiments 4 and 5 were single blank experiments which contained unfortified active soils without and with carrot, respectively, to monitor contamination introduced from water and air.

Table 4.2: Biodegradation and uptake experiments performed for the pesticide Sulfluramid in a soil/carrot mesocosm, half-life of the L-EtFOSA and mole mass-balance.

	Soil type	Fortification	Fortified soil concentration (mg/kg)[1]	Crop	$t_{1/2}$ L-EtFOSA (days)	L-PFOS % yield on day 81	Mole balance of linear isomers relative to T = 0 (%)				
							Day 0	Day 14	Day 28	Day 56	Day 81
Exp 1	Live	Technical EtFOSA	3.8 (1.9)	Carrot	35.8 ± 3.7	34	100 ± 4	119 ± 5	113 ± 9	105 ± 12	98 ± 5
Exp 2	Live	Technical EtFOSA	3.8 (1.9)	none	33.6 ± 9.0	24	100 ± 17	99 ± 1	100 ± 9	129 ± 1	110 ± 1
Exp 3	Sterile	Technical EtFOSA	3.8 (1.9)	none	40.0 ± 7.8	12	100 ± 13	104 ± 2	87 ± 0.5	104 ± 4	83 ± 3
Exp 4	Live	Unfortified	-	none	-	-	-	-	-	-	-
Exp 5	Live	Unfortified	-	Carrot	-	-	-	-	-	-	-
Exp 6	Live	Commercial Sulfluramid	0.12 (0.08)	Carrot	11.5 ± 2.1	277	100 ± 4	112 ± 0.5	151 ± 6	174 ± 3	175 ± 11

[1] Nominal soil concentration of ∑EtFOSA isomers are based on concentrations in the standard/commercial formulation measured using a linear standard. Linear isomer concentrations are provided in parentheses. For experiment 6, baits were added to the surface of the soil but for this calculation a homogenous distribution in soil was assumed.

Finally, Experiment 6 was carried out in duplicate and involved fortifying active soil containing carrot with the commercial Sulfluramid formulation Grão Forte (0.0024 % ∑EtFOSA). All experiments contained PFDA (100 ng/g) which functioned as an internal negative control, as previously described [15].

An acidic sandy loam soil (pH = 5.7 ± 0.2), which is common to regions of Brazil [27] was used in the present work. Soil chemistry parameters are provided in ***Table 4.3***. In Experiments 1-3 (***Table 4.2***), soils were weighed, covered with acetone and fortified with technical EtFOSA in order to achieve a 3.8 mg/kg nominal concentration. After stirring for 24 h, the soil-acetone mixture was placed under a fume hood in order to let the solvent evaporate. Soil was then aged for one week. For Experiment 6, 10 g of Grão Forte was added to the surface of each pot containing 2 kg of soil.

Table 4.3*: Soil characteristics.*

Parameter	Universal substrate
TOC %	53 ± 9
N %	0.35 ± 0.05
pH	5.7 ± 0.2
Cation Exchange Capacity (meq/100 g)	48 ± 4
Particles < 0.002 mm	4.5 ± 0.5
0.002 - 0.05 mm	27 ± 3
0.05 - 2 mm	53 ± 6
> 2mm	16 ± 2
soil type	sandy loam

4.2.3 Plant cultivation and sampling

All experiments were performed in a climate-controlled greenhouse with interior conditions set to 25 °C / 50 % humidity during the day (14 h) and 18 °C / 60 % humidity at night (10 h). Prior to germination, seeds were soaked in Milli-Q water. The washed seeds were distributed randomly on dampened filter paper in a Petri dish and covered with moistened filter paper. Upon germination (12 - 14 days), 4 seedlings were transplanted to each pot containing 2 kg of soil (fortified or non-fortified). Each pot

represented a single time point, and a total of 5 time points were sampled over the course of the experiment. Pots were arranged randomly and regularly watered with distilled water and Hoagland nutritive solution. The Hoagland solution was prepared monthly according to Epstein and Bloom's work [28]. Leachate from each pot was collected at the same time as soil sampling on days 14, 28, 56 and 81, resulting in 14 day, 28 day, 56 day, and 81 day composite leachates samples. Exact volumes collected for each pot are provided in ***Table 4.4***. Blanks, consisting of Milli-Q water stored in the same PE bottles, were also analysed in parallel to assess background contamination. On the last two time points, carrots were collected and divided into peel, core and leaf compartments. Soil was air-dried and carrots were freeze-dried. All samples were stored at -80 °C prior to extraction and analysis.

Table 4.4: *Amount of leachate water in all the experiments performed (Exp1-6).*

	Leachate water (mL)			
	0-14 days	0-28 days	0-56 days	0-81 days
Exp 1 (Live soil + carrot)	200 (Pot 1)	435 (Pot 1)	400 (Pot 1)	970 (Pot 1)
	185 (Pot 2)	450 (Pot 2)	1117 (Pot 2)	1075 (Pot 2)
Exp 2 (Live soil-only)	590 (Pot 1)	1045 (Pot 1)	2444 (Pot 1)	3664 (Pot 1)
	595 (Pot 2)	1135 (Pot 2)	2620 (Pot 2)	3962 (Pot 2)
Exp 3 (Sterile soil-only)	559 (Pot 1)	1015 (Pot 1)	2195 (Pot 1)	3030 (Pot 1)
	600 (Pot 2)	1030 (Pot 2)	2231 (Pot 2)	2877 (Pot 2)
Exp 4 (Live soil-only)	200 (Pot 2)	500 (Pot 2)	1465 (Pot 2)	2838 (Pot 2)
Exp 5 (Live soil + carrot)	450 (Pot 1)	230 (Pot 1)	440 (Pot 1)	350 (Pot 1)
Exp 6 (Live soil + carrot)	200 (Pot 1)	433 (Pot 1)	350 (Pot 1)	227 (Pot 1)
	228 (Pot 2)	385 (Pot 2)	305 (Pot 2)	351 (Pot 2)

4.2.4 *Extraction and clean-up*

4.2.4.1 *Baits extraction procedure*

A detailed description of the bait extraction procedure, including method validation, can be found elsewhere [29]. Briefly, baits (0.1 g) were fortified with 5 ng of isotopically-labeled standards, 8 mL ACN and 20 stainless steel beads (3.2 mm diameter). The mixture was placed into a bead blender (1600 MiniG®, SPEX SamplePrep, USA) for

10 min at 1500 rpm followed by centrifugation at 2700 rpm for 5 min and the ACN was transferred into a clean 15 mL polypropylene test tube. The procedure was repeated using ACN with 25 mM sodium hydroxide and the supernatants were combined. The extracts were placed in a Turvobap LV evaporator and reduced to 1 mL under a gentle stream of nitrogen. The extracts were cleaned using dispersive solid phase extraction (dSPE) approach. For that purpose, 25 mg of graphitised carbon (Supelclean ENVI-Carb 120/240) and 50 µL of glacial acetic acid were added in the Eppendorf and the samples were vortexed and centrifuged for 10 min at 10 000 rpm. 100 µL of the eluate were transferred to a vial, and 100 µL of 20 mM ammonium formate and 20 mM formic acid in water were added. All extractions were carried out in triplicate along with procedural blanks.

4.2.4.2 *Soil and carrot extraction*

Soil and carrot extractions were performed according to Avendaño and Liu [11], with slight modifications. Briefly, 0.5 g of dried sample was fortified with 2 ng of isotopically-labeled standards and 8 mL ACN, and then sonicated for 20 min. After sonication, the mixture was placed in an angular shaker for 40 min, centrifuged at 2900 rpm for 20 min and the ACN was transferred into a clean 15 mL polypropylene test tube. The procedure was repeated using ACN with 25 mM sodium hydroxide and the supernatants were combined. The extracts were placed in a Turvobap LV evaporator and reduced to dryness (soil extracts) or to approx. 1 mL (carrot extracts). Soil extracts were reconstituted in 400 µL MeOH: Milli-Q water (1:1, v/v) with 20 mM formic acid and 20 mM ammonium formate, while a portion (200 µL) of the carrot extract was mixed with 200 µL of Milli-Q water containing 20 mM formic acid and 20 mM ammonium formate. Extracts were transferred to microvials prior to instrumental analysis.

4.2.4.3 Leachate extraction

All the leachate was filtered through 0.7 µm borosilicate glass fiber filters, the pH was adjusted to approximately 7.0 and 2 ng of isotopically labeled compounds were added and agitated for 24 h prior to extraction. Afterwards, samples were extracted using the procedure reported by Gilljam et al. [9]. Briefly, 500 mL aliquot of leachate was passed through a 200 mg Evolute-WAX cartridge, which had been previously conditioned with 4 mL of 0.3 % NH_4OH in MeOH, followed by 4 mL of 0.1 M formic acid in Milli-Q water. After the sample was loaded, 5 mL of 20 % MeOH in 0.1 M formic acid followed by 2 mL 0.3 % NH_4OH in Milli-Q water were added with cleaning purposes and the cartridges were dried for 5 min under vacuum. Finally, the analytes were eluted using 4 mL of 0.3 % NH_4OH in MeOH. Multiple cartridges were used when the leachate amount was higher than 500 mL and the eluates were mixed, evaporated to 1 mL, and diluted to 2 mL with 20 mM formic acid and 20 mM ammonium formate prior to analysis.

The filters were extracted separately to assess potential sorption of target analytes, as previously described for solid matrices.

4.2.5 Instrumental analysis

Quantitative analysis of EtFOSA and its transformation products was carried out by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a Waters Acquity UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters). The method, which has been previously described [30], facilitates chromatographic separation and quantification of individual PFAS isomers (see example chromatograms in ***Figures 1-4***).

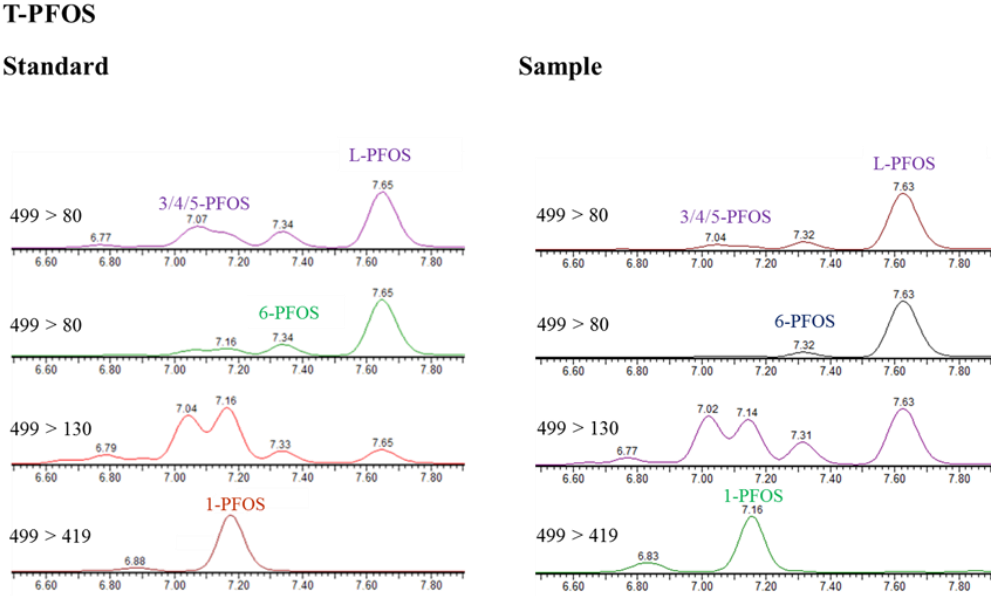


Figure 4.1: PFOS isomer chromatograms in a technical standard and in soil sample (Exp 3, t = 81 days).

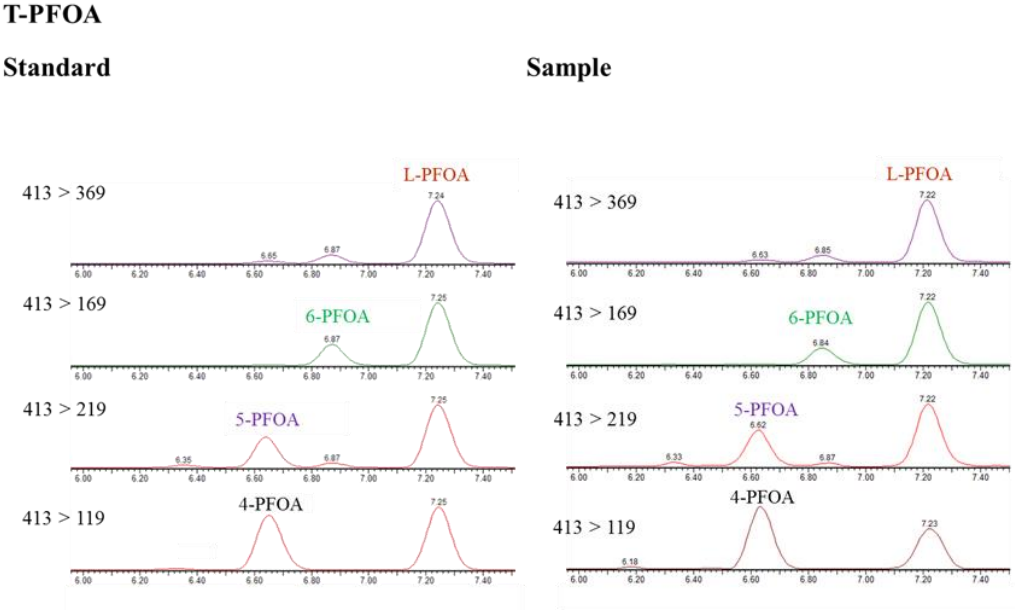


Figure 4.2: PFOA isomer chromatograms in a technical standard and in soil sample (Exp 3, t = 81 days).

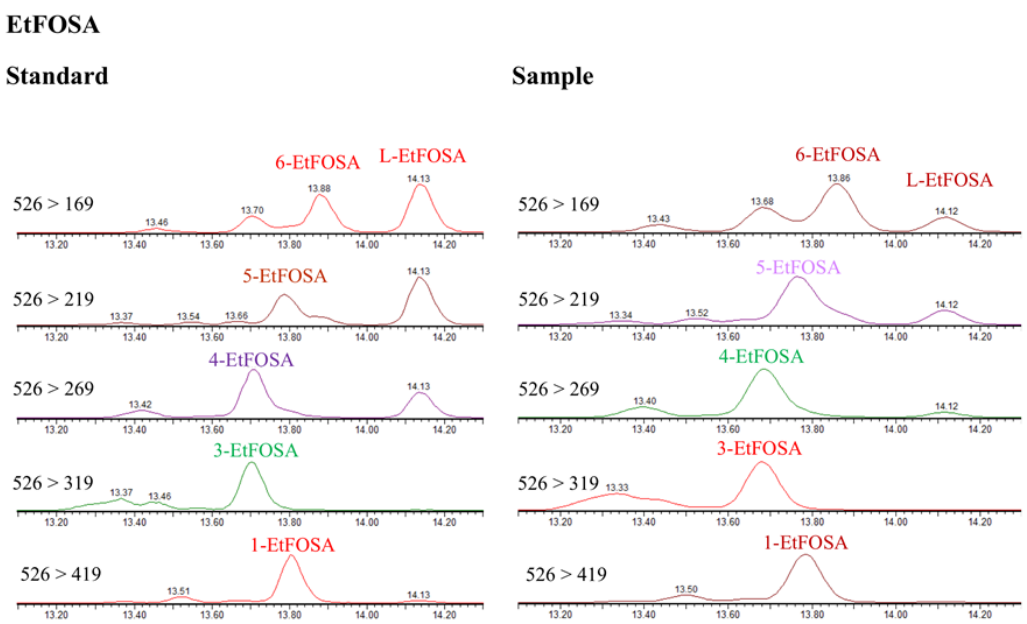


Figure 4.3: EtFOSA isomer chromatograms in a technical standard and in soil sample (Exp 3, t = 81 days).

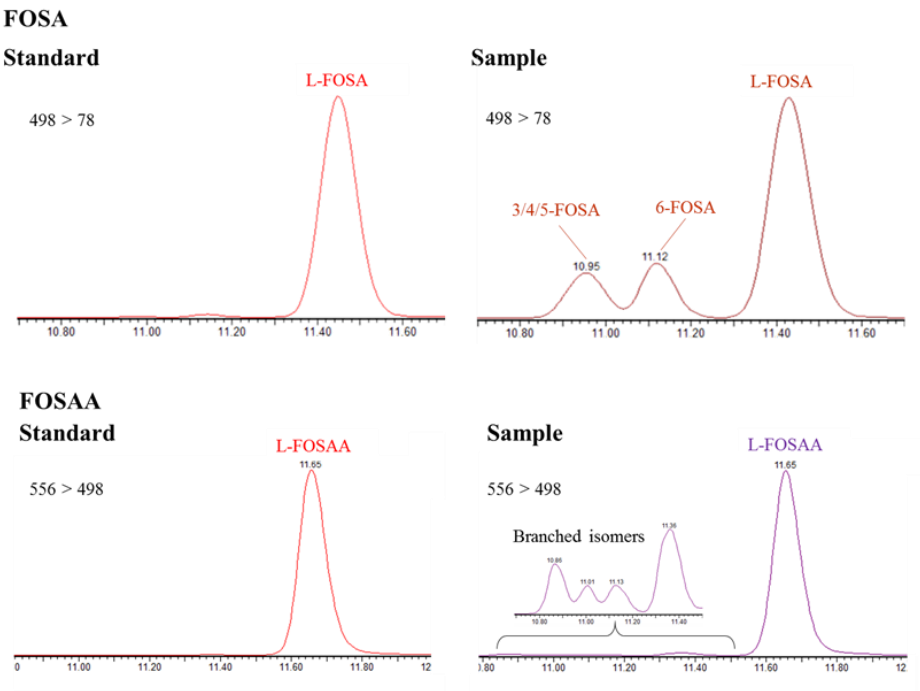


Figure 4.4: FOSA and FOSAA isomer chromatograms in a technical standard and in soil sample (Exp 3, t = 81 days).

Extracts (10 µL) were injected onto an Ascentis Express F5 guard column (2.7 µm, 2.1 mm × 0.5 cm) coupled to an Ascentis Express F5 (2.7 µm, 2.1 mm × 10 cm) analytical column maintained at 30 °C. The mobile phase consisted of 20 mM formic acid and 20 mM ammonium formate in Milli-Q water (mobile phase A) and 100 % MeOH (mobile phase B). The flow rate was maintained at 0.25 mL/min. The gradient profile started at 90 % A (hold time 1 min), followed by a linear decrease to 40 % A by 3 min, then to 12 % A by 14 min and finally 0 % by 14.5 min (hold time 1 min). The mobile phase composition was returned to initial conditions by 16.5 min and then equilibrated by 21.5 min. The mass spectrometer was operated under selected reaction monitoring (SRM) mode, with 2 to 5 transitions per analyte (see ***Table 4.1***).

4.2.6 Quality control

Prior to analysis of samples, spike/recovery experiments were performed in soil (*n* = 4), carrot (*n* = 4) and glass filters (*n* = 4) at a fortification level of 30 ng/g, and in water (*n* = 4) at a fortification level of 10 ng/mL. Limits of detection (LODs) and quantification (LOQs) were estimated as the concentration producing a signal-to-noise ratio of 3 and 10, respectively (***Table 4.5***). We also evaluated potential losses from freeze-drying by analysing soil fortified with target analytes with and without a freeze-drying step. Following method validation, ongoing assessment of method performance was carried out through the inclusion of blanks and spiked samples in every batch.

4.2.7 Isomer nomenclature and identification

In all cases, isomers were denoted by either 'L-' (linear isomer), 'Br-' (∑branched isomers), or a number denoting the location of the perfluoromethyl branching point (1-, 2-, 3-, etc.). Individual PFOS and PFOA isomers could be identified in chromatograms (***Figure 4.1-4.2***) by matching their relative retention times and MS/MS product ions to those reported previously [30,31]. In the case of EtFOSA (***Figure 4.3***), tentative structural

assignments were made by comparing MS/MS product ions and retention times to that of PFOS. For example, 6-EtFOSA and 6-PFOS both produced *m/z* 169 product ion and eluted closest to their respective linear isomers, while 1-EtFOSA and 1- PFOS both eluted between 5- and 6-isomers and produced a unique *m/z* 419 product ion.

4.2.8 Data handling and statistical analysis

Concentrations for a single time point in each experimental replicate were based on analysis of $n = 3$ soil or carrot samples or $n = 1$ sample of composite leachate. Quantification of target analytes was performed using an isotope dilution approach, with the exception of FOSAA, where matrix-matched calibration approach was performed due to the lack of a homologous isotopically labeled standard. Calibration curves (1/x weighting) were prepared from around the limit of quantification (LOQ) to 250 ng/mL and determination coefficients, R^2, were always in the range of 0.994-0.998. Individual PFOS and PFOA isomers were determined using isomer-specific calibration curves prepared from characterised technical standards (see standards and reagents section). For targets where characterised isomeric mixtures were unavailable (i.e. EtFOSA, FOSA and FOSAA), Σ branched isomers were quantified separately from the linear isomer using a linear isomer calibration curve. In this case, the concentration of branched isomers should be considered semi-quantitative, owing to differences in response factors between branched and linear isomers.

EtFOSA rate constants were determined by fitting soil concentrations (C_{soil}) to the equation $\ln(C_{soil}) = a - k_d t$, where k_d is the apparent depletion rate constant, t is time, and a is a constant (see ***Figure 4.5***). The apparent half-life ($t_{1/2}$) was calculated by dividing ln(2) by the k_d. Since the concentrations in replicate pots for a given experiment were not significantly different ($p > 0.05$; Analysis of Variance (ANOVA) test), $t_{1/2}$ was determined for each of the replicates, and these were used to calculate an average half-life and pooled standard deviation.

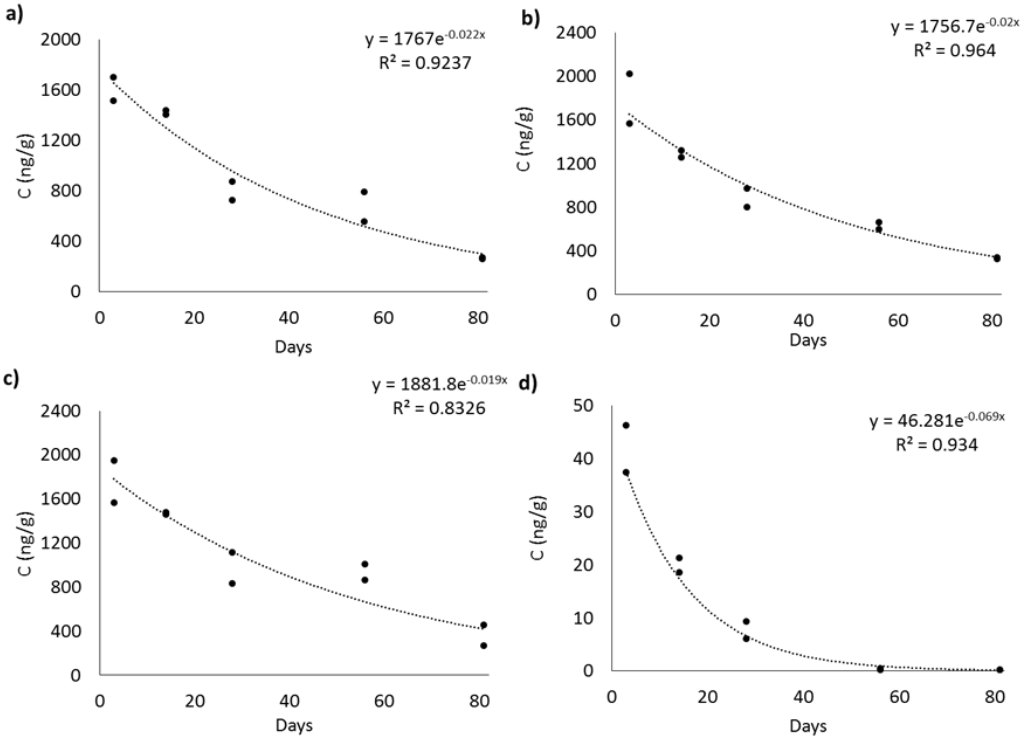


Figure 4.5. Concentration profiles of L-EtFOSA and fitted first order degradation curves for a) Experiment 1, b) Experiment 2, c) Experiment 3, d) Experiment 6.

Losses not accounted for by the internal negative control (e.g. from volatilisation or irreversible sorption) as well as the potential for novel product formation were monitored by calculating the total number of moles in the system at each time point and comparing this to the total number of moles at t = 0. Finally, bioconcentration factors (BCFs) were determined in carrot peel, core and leaf as a ratio between the concentration determined in each of the carrot compartment (d.w.) and the concentration detected in soil (d.w.).

4.3 Results and discussion

4.3.1 Quality control

Spike/recovery experiments involving L-FOSAA, L-FOSA, and isomeric mixtures of EtFOSA, PFOS, and PFOA resulted in internal standard-corrected percent recoveries

ranging from 76-109 % for soil, 49-120 % for carrot, 76-130 % for leaching water and 65-130 % for filters (see ***Table 4.5***), indicating good accuracy of the method.

Table 4.5: *Apparent recoveries (%) ± standard deviation (n=4) for target analytes in soil, carrot, leachate and filter samples and instrumental limits of detection (LOD) and quantification (LOQ).*

Analyte	Soil	Carrot	Leachate	Filter	LOD (ng/L)	LOQ (ng/L)
L-PFOA	103 ± 13	77 ± 14	78 ± 6	117 ± 13	12.0	39.9
6-PFOA	94 ± 15	62 ± 6	122 ± 16	80 ± 13	20.9	69.7
5-PFOA	106 ± 17	80 ±17	99 ± 33	85 ± 15	15.7	52.5
4-PFOA	86 ± 14	67 ± 5	99 ± 31	90 ± 17	27.8	92.7
dm-PFOA	88 ± 15	92 ± 23	123 ± 23	117 ± 20	13.9	46.5
L-PFOS	97 ± 8	88 ± 18	89 ± 6	130 ± 14	8.1	27.1
6-PFOS	92 ± 11	70 ± 6	106 ± 19	91 ± 8	15.0	50.0
3,4,5-PFOS	79 ± 7	74 ± 8	106 ± 24	88 ± 7	5.8	19.4
1-PFOS	101 ± 19	120 ± 5	98 ± 15	75 ± 12	27.9	92.9
dm-PFOS	76 ± 7	49 ± 2	106 ± 13	65 ± 4	34.8	115.8
L-FOSA	109 ± 9	91 ± 19	129 ± 7	105 ± 19	2.0	6.7
Br-FOSA	[a]	[a]	[a]	[a]	2.0[b]	6.7[b]
L-EtFOSA	86 ± 7	89 ± 7	130[c] ± 10	116 ± 12	1.3	4.4
6-EtFOSA	0.61[c] ± 0.03	0.45[c] ± 0.02	0.45[c] ± 0.02	0.62[c] ± 0.05	1.3[b]	4.4[b]
5-EtFOSA	0.42[c] ± 0.04	0.36[c] ± 0.06	0.31[c] ± 0.04	0.54[c] ± 0.05	1.3[b]	4.4[b]
4-EtFOSA	0.42[c] ± 0.02	0.27[c] ± 0.02	0.28[c] ± 0.01	0.38[c] ± 0.03	1.3[b]	4.4[b]
3-EtFOSA	0.10[c] ± 0.01	0.06[c] ± 0.01	0.068[c] ± 0.004	0.088[c] ± 0.006	1.3[b]	4.4[b]
1-EtFOSA	0.33[c] ± 0.03	0.25[c] ± 0.04	0.28[c] ± 0.01	0.27[c] ± 0.05	1.3[b]	4.4[b]
Br-EtFOSA	92 ± 14	68 ± 6	117[c] ± 12	80 ± 16	1.3[b]	4.4[b]
L-PFDA	107 ± 24	100 ± 4	76 ± 4	88 ± 8	11.2	37.4
L-FOSAA	103 ± 17	86 ± 12	98 ± 6	99 ± 12	3.6	12.0
Br-FOSAA	[a]	[a]	[a]	[a]	3.6[b]	12.0[b]

[a] *Apparent recoveries were not determined due to the lack of branched isomer standards.*
[b] *Estimated from the linear isomer.*
[c] *Ratios between the individual branched and linear isomers.*

No significant differences ($p > 0.05$) were observed between freeze-dried and non-freeze dried soils, indicating that losses during the freeze-drying step were negligible. The internal negative control PFDA, which was incubated in all the experiments to monitor losses in situ, was recovered quantitatively from all pots and displayed no significant change in concentration over the course of the experiments (***Figure 4.6***). Monitoring of unfortified soil (Experiment 5) and soil-carrot (Experiment 4) mesocosms revealed the occurrence of PFOS, PFOA, and FOSA in both soils and leachate. For PFOS

and FOSA, soil and leachate concentrations in unfortified experiments were always < 4.4 % of fortified experiments. For PFOA, exclusively linear isomer was observed in soil and leachates from unspiked experiments, but these were usually much lower than dosed experiments. A detailed discussion surrounding the observation of PFOA, including potential sources, is included in sub-section *Observation of PFOA*.

4.3.2 Biodegradation of technical EtFOSA in soil and soil-carrot mesocosms

Incubations of technical EtFOSA with active soil + carrot (Experiment 1) or active soil (Experiment 2), resulted in over 81 % depletion of L-EtFOSA after 81 days. L-EtFOSA half-lives were 35.8 ± 3.7 days (Experiment 1) and 33.6 ± 9.0 days (Experiment 2). These half-lives are nearly 2.5-fold higher than the 13.9 ± 2.1 days estimated by Avendaño and Liu [11] for EtFOSA (assumed to be the sum of branched and linear isomers) in aerobic soil (no vegetable). The higher half-lives observed here may be due to differences in experimental setup, soil bioactivities, or soil chemistry. Notably, total organic carbon was considerably higher in the present work compared to Avendaño and Liu (53 % versus 5.9 %, respectively) [11], which may be reflected in increased sorption and decreased biodegradation in the present work. Mole balance from the present work (*Table 4.2*) ranged from 100-119 % and 99-130 % for Experiments 1 and 2, respectively, indicating minimal losses due to volatilisation, consistent with Avendaño and Liu [11]. Notably, PFAS concentrations in leachate and carrot were low relative to soil, and did not significantly alter the mole balance.

Product formation curves are provided in *Figure 4.6* (see *Tables 4.6-4.10* for raw data). By day 81 in Experiments 1 and 2, L-FOSA and L-PFOS were the principal metabolites (37 – 59 % and 24 – 34 % yield, respectively), followed by L-FOSAA (5 – 8 % yield; *Table 4.11*). These results somewhat contrast with previous observations by

Avendaño and Liu [11] in which FOSA and FOSAA were the main metabolites (30.3 and 34.2 % yields, respectively), followed by PFOS (< 4 %) [11].

The combination of higher L-PFOS and lower L-FOSA yields in the soil-carrot mesocosms compared to soil-only mesocosms is notable as it suggests that conversion of L-EtFOSA to L-PFOS is enhanced in the presence of carrot. This result is consistent with our prior experiments involving incubations of FOSA, where conversion to PFOS was significantly enhanced in the presence of carrot, compared to without [12]. Further work is needed to assess product yields in other crops, in particular, those relevant to Sulfluramid application in Brazil (e.g. eucalyptus). The presence of a crop could lead to considerably higher yields of PFOS than expected from soil biodegradation experiments.

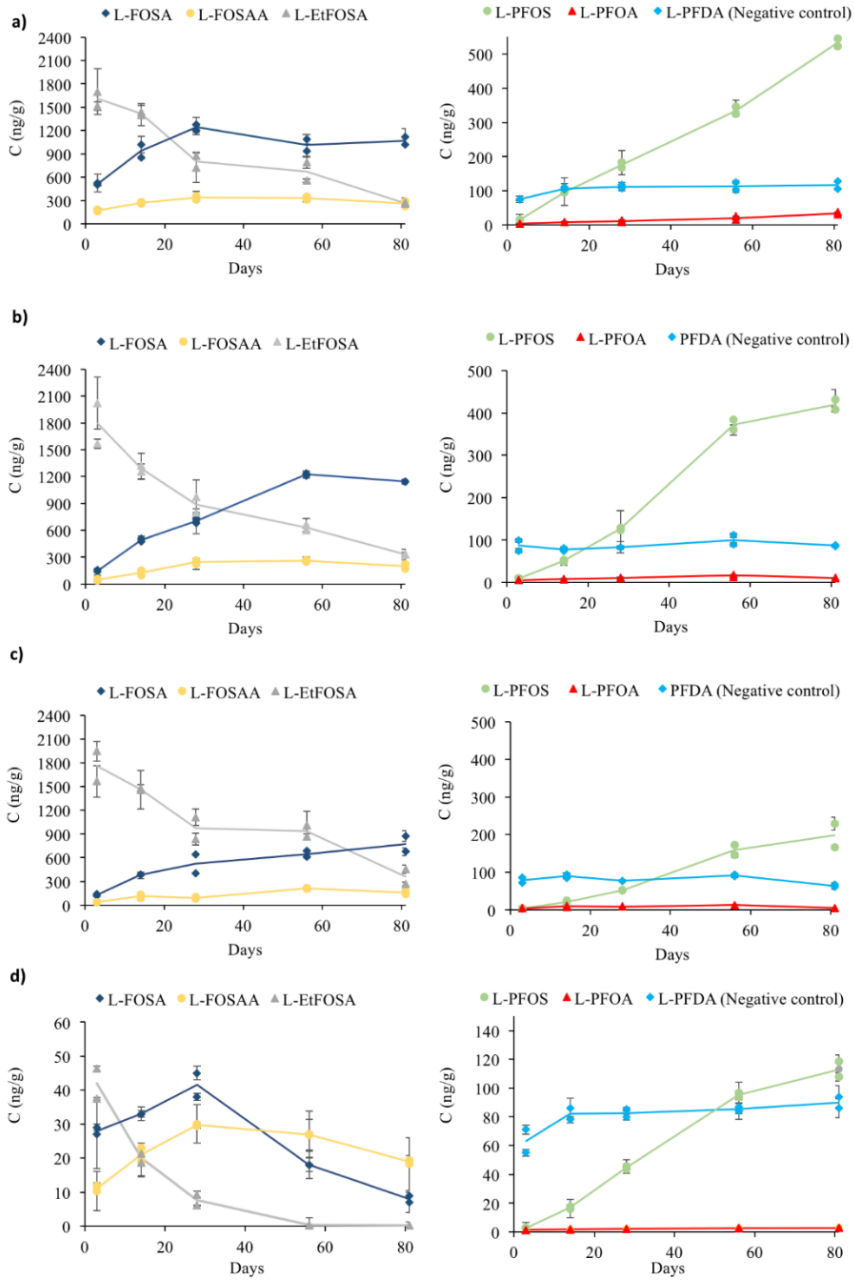


Figure 4.6: *Concentrations and standard deviations in ng/g of EtFOSA and its degradation products over time in soil from a) Experiment 1: Carrot/active soil mesocosm fortified with technical EtFOSA; b) Experiment 2: Active soil mesocosm fortified with technical EtFOSA; c) Experiment 3: Sterile soil mesocosm fortified with technical EtFOSA and d) Experiment 6: Active soil mesocosm with the addition of EtFOSA commercial baits.*

Table 4.6: Concentration (ng/g) of detected isomers ± standard deviation (n=3) in soil for Experiment 1 (technical EtFOSA incubated in active soil-carrot mesocosm).

Experiment 1										
	Day 0		Day 14		Day 28		Day 56		Day 81	
Analyte	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	16 ± 1	15 ± 1	95 ± 3	97 ± 6	167 ± 7	183 ± 16	326 ± 41	346 ± 35	523 ± 20	545 ± 14
6-PFOS	0.22 ± 0.01	0.19 ± 0.01	1.43 ± 0.04	1.30 ± 0.09	2.9 ± 0.2	4.0 ± 0.4	12 ± 1	17 ± 1	31 ± 2	33 ± 1
3,4,5-PFOS	0.28 ± 0.01	0.25 ± 0.03	1.33 ± 0.04	1.18 ± 0.06	2.5 ± 0.2	4.1 ± 0.4	11 ± 2	22 ± 2	36 ± 2	37 ± 1
1-PFOS	0.031* ±0.001	0.036* ± 0.005	0.063* ± 0.006	0.06* ± 0.01	0.073* ± 0.004	0.13 ± 0.01	0.15 ± 0.02	0.29 ± 0.01	0.46 ± 0.02	0.48 ± 0.02
L-PFOA	3.6 ± 0.2	3.6 ± 0.1	9 ± 1	8 ± 1	9 ± 1	13 ± 1	16 ± 2	25 ± 3	37 ± 1	31 ± 1
6-PFOA	0.136 ± 0.007	0.13 ± 0.02	0.45 ± 0.06	0.38 ± 0.05	0.48 ± 0.03	0.76 ± 0.09	1.0 ± 0.2	2.0 ± 0.2	3.2 ± 0.2	2.8 ± 0.2
5-PFOA	0.11 ± 0.01	0.12 ± 0.01	0.32 ± 0.03	0.28 ± 0.02	0.32 ± 0.03	0.55 ± 0.02	0.8 ± 0.1	1.7 ± 0.2	2.6 ± 0.2	2.4 ± 0.2
4-PFOA	0.096 ± 0.005	0.10 ± 0.02	0.29 ± 0.03	0.25 ± 0.05	0.29 ± 0.03	0.45 ± 0.02	0.8 ± 0.1	1.5 ± 0.1	2.4 ± 0.2	2.2 ± 0.2
L-FOSA	505 ± 17	522 ± 17	849 ± 56	1023 ± 82	1205 ± 106	1278 ± 114	940 ± 106	1086 ± 94	1120 ± 61	1076 ± 30
Br-FOSA	39 ± 3	40 ± 2	189 ± 15	209 ± 25	317 ± 24	359 ± 46	316 ± 41	369 ± 33	462 ± 28	461 ± 11
L-FOSAA	159 ± 27	182 ± 50	261 ± 51	286 ± 32	314 ± 17	359 ± 73	347 ± 91	312 ± 54	292 ± 43	229 ± 28
Br-FOSAA	1.51 ± 0.09	1.5 ± 0.3	3.5 ± 0.5	3.5 ± 0.4	5.3 ± 0.8	5 ± 1	8 ± 2	7 ± 1	9 ± 1	8 ± 1
L-EtFOSA	1699 ± 293	1514 ± 85	1439 ± 192	1403 ± 22	725 ± 60	875 ± 52	554 ± 141	789 ± 39	272 ± 75	258 ± 16
Br-EtFOSA	3123 ± 492	2923 ± 306	3092 ± 416	3284 ± 98	2352 ± 189	2739 ± 111	2305 ± 355	2804 ± 142	1937 ± 65	1678 ± 36

**Values below limits of quantification*

Table 4.7: Concentration (ng/g) of detected isomers ± standard deviation (n=3) in soil for Experiment 2 (technical EtFOSA incubated in active soil mesocosm).

Experiment 2										
	Day 0		Day 14		Day 28		Day 56		Day 81	
Analyte	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	9.6 ± 0.1	8.7 ± 0.5	53 ± 3	48 ± 2	128 ± 6	124 ± 7	385 ± 8	360 ± 45	408 ± 12	431 ± 24
6-PFOS	0.39 ± 0.01	0.32 ± 0.02	1.41 ± 0.09	1.13 ± 0.03	3.6 ± 0.2	3.7 ± 0.3	21 ± 1	22 ± 3	27 ± 1	21 ± 4
3,4,5-PFOS	0.69 ± 0.02	0.50 ± 0.03	2.40 ± 0.05	1.8 ± 0.1	5.9 ± 0.4	6.0 ± 0.3	15.7 ± 0.2	8 ± 1	9 ± 1	7 ± 1
1-PFOS	0.04* ± 0.01	0.032* ± 0.001	0.06* ± 0.01	0.042* ± 0.003	0.08* ± 0.01	0.08* ± 0.02	0.32 ± 0.01	0.35 ± 0.06	0.29 ± 0.03	0.24 ± 0.05
L-PFOA	6.2 ± 0.2	5.1 ± 0.2	9.1 ± 0.4	7.4 ± 0.3	10.8 ± 0.5	10.1 ± 0.6	13.1 ± 0.6	18 ± 3	9.5 ± 0.2	11.1 ± 0.2
6-PFOA	0.402 ± 0.003	0.32 ± 0.02	0.64 ± 0.07	0.43 ± 0.02	0.7 ± 0.1	0.68 ± 0.03	1.02 ± 0.03	1.4 ± 0.3	0.7 ± 0.1	0.73 ± 0.01
5-PFOA	0.31 ± 0.01	0.25 ± 0.01	0.47 ± 0.03	0.32 ± 0.01	0.52 ± 0.02	0.49 ± 0.02	0.7 ± 0.1	1.0 ± 0.2	0.51 ± 0.01	0.55 ± 0.02
4-PFOA	0.267 ± 0.009	0.211 ± 0.003	0.40 ± 0.02	0.2 ± 0.1	0.43 ± 0.04	0.42 ± 0.03	0.6 ± 0.1	0.8 ± 0.1	0.47 ± 0.04	0.52 ± 0.01
L-FOSA	160 ± 5	137 ± 5	478 ± 9	510 ± 22	730 ± 27	680 ± 41	1238 ± 28	1210 ± 119	1143 ± 26	1144 ± 15
Br-FOSA	34 ± 1	25 ± 1	154 ± 4	123 ± 25	231 ± 13	236 ± 31	606 ± 35	582 ± 47	626 ± 13	509 ± 73
L-FOSAA	40 ± 1	51 ± 14	154 ± 24	100 ± 12	262 ± 15	227 ± 20	251 ± 16	270 ± 63	227 ± 30	172 ± 25
Br-FOSAA	0.72 ± 0.01	0.8 ± 0.1	2.8 ± 0.4	3.0 ± 0.2	4.7 ± 0.3	4.8 ± 0.2	7.4 ± 0.3	8 ± 1	8 ± 1	8 ± 1
L-EtFOSA	2021 ± 137	1567 ± 116	1257 ± 150	1320 ± 122	973 ± 249	801 ± 72	602 ± 35	660 ± 150	327 ± 16	339 ± 50
Br-EtFOSA	3323 ± 219	2420 ± 127	2396 ± 325	2585 ± 191	2276 ± 339	2018 ± 159	2036 ± 133	2052 ± 215	1381 ± 36	1320 ± 29

**Values below limits of quantification*

Table 4.8: Concentration (ng/g) of detected isomers ± standard deviation (n=3) in soil for Experiment 3 (technical EtFOSA incubated in inactivated soil mesocosm).

	Experiment 3									
	Day 0		Day 14		Day 28		Day 56		Day 81	
Analyte	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	3.3 ± 0.2	3.0 ± 0.1	24.5 ± 0.3	16.1 ± 0.2	52 ± 5	51 ± 1	173 ± 9	146 ± 2	166 ± 6	229 ± 17
6-PFOS	0.21 ± 0.01	0.20 ± 0.01	0.72 ± 0.05	0.46 ± 0.02	1.0 ± 0.1	0.97 ± 0.02	5.0 ± 0.2	5.1 ± 0.1	7.2 ± 0.3	11 ± 1
3,4,5-PFOS	0.11 ± 0.01	0.12 ± 0.01	0.21 ± 0.02	0.10 ± 0.01	0.32 ± 0.03	0.33 ± 0.05	1.19 ± 0.07	1.25 ± 0.05	1.8 ± 0.1	2.6 ± 0.4
1-PFOS	0.031* ± 0.001	0.039* ± 0.001	0.057* ± 0.005	0.041* ± 0.004	0.042* ±0.005	0.042* ± 0.009	0.094 ± 0.004	0.086* ± 0.007	0.080* ± 0.009	0.114 ± 0.009
L-PFOA	4.0 ± 0.1	3.8 ± 0.1	10.1 ± 0.5	7.5 ± 0.3	8.2 ± 0.9	8.1 ± 0.2	12.1 ± 0.7	12.2 ± 0.4	4.5 ± 0.1	4.5 ± 0.5
6-PFOA	0.26 ± 0.01	0.23 ± 0.02	0.60 ± 0.03	0.38 ± 0.01	0.39 ± 0.04	0.42 ± 0.03	0.78 ± 0.03	0.72 ± 0.02	0.30 ± 0.02	0.30 ± 0.03
5-PFOA	0.20 ± 0.01	0.17 ± 0.01	0.44 ± 0.01	0.28 ± 0.01	0.29 ± 0.02	0.32 ± 0.01	0.57 ± 0.03	0.50 ± 0.02	0.19 ± 0.03	0.23 ± 0.02
4-PFOA	0.18 ± 0.01	0.14 ± 0.04	0.37 ± 0.03	0.24 ± 0.01	0.19 ± 0.06	0.27 ± 0.05	0.47 ± 0.02	0.42 ± 0.03	0.22 ± 0.03	0.16 ± 0.06
L-FOSA	140 ± 4	118 ± 4	385 ± 7	376 ± 10	641 ± 49	405 ± 9	610 ± 39	686 ± 5	676 ± 9	873 ± 68
Br-FOSA	24 ± 1	18 ± 1	70 ± 6	49 ± 3	94 ± 11	72 ± 2	163 ± 10	222 ± 2	242 ± 5	349 ± 29
L-FOSAA	34 ± 7	37 ± 1	134 ± 10	90 ± 17	103 ± 28	83 ± 26	216 ± 29	204 ± 24	174 ± 25	161 ± 22
Br-FOSAA	0.42 ± 0.09	0.33 ± 0.05	1.4 ± 0.2	0.7 ± 0.1	1.1 ± 0.1	1.2 ± 0.4	2.01 ± 0.06	2.08 ± 0.02	2.9 ± 0.3	3.4 ± 0.3
L-EtFOSA	1569 ± 198	1950 ± 243	1461 ± 76	1479 ± 184	834 ± 51	1114 ± 126	1006 ± 43	863 ± 103	457 ± 39	266 ± 22
Br-EtFOSA	2703 ± 348	3304 ± 359	2731 ± 65	2894 ± 168	2716 ± 205	2389 ± 73	2479 ± 133	2309 ± 128	1394 ± 42	1351 ± 131

**Values below limits of quantification*

Table 4.9: Concentration (ng/g) ± standard deviation (n=3) of detected isomers in soil for Experiment 4 (blank experiment in active soil mesocosm) and Experiment 5 (blank experiment in active soil-carrot mesocosm).

	Experiment 4					Experiment 5				
Analyte	Day 0 Pot 1	Day 14 Pot 1	Day 28 Pot 1	Day 56 Pot 1	Day 81 Pot 1	Day 0 Pot 1	Day 14 Pot 1	Day 28 Pot 1	Day 56 Pot 1	Day 81 Pot 1
L-PFOS	0.13 ± 0.03	0.12 ± 0.11	0.14 ± 0.02	0.22 ± 0.01	0.27 ± 0.03	0.17 ± 0.02	0.13 ± 0.01	0.15 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
6-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3,4,5-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	0.82 ± 0.11	0.71 ± 0.16	0.62 ± 0.06	0.87 ± 0.06	0.72 ± 0.03	1.45 ± 0.05	1.40 ± 0.04	1.27 ± 0.05	1.40 ± 0.09	1.10 ± 0.07
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Br-FOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSAA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Br-FOSAA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-EtFOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Br-EtFOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

< LOD: concentration lower than the detection limit
nd: not detected

Table 4.10: Concentration (ng/g) of detected isomers ± standard deviation (n=3) in soil for Experiment 6 (incubation of the commercial Sulfluramid formulation Grão Forte in active soil-carrot mesocosm).

Experiment 6										
	Day 0		Day 14		Day 28		Day 56		Day 81	
Analyte	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	3.1 ± 0.1	2.8 ± 0.1	17.9 ± 0.2	16 ± 1	44 ± 3	46 ± 4	94 ± 6	97 ± 5	108 ± 7	118 ± 5
6-PFOS	0.03* ± 0.01	0.04* ± 0.01	0.11 ± 0.01	0.07 ± 0.03	0.30 ± 0.02	0.34 ± 0.01	0.90 ± 0.05	0.92 ± 0.06	1.04 ± 0.11	1.36 ± 0.13
3,4,5-PFOS	0.06 ± 0.01	0.07± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.30 ± 0.02	0.29 ± 0.02	0.31 ± 0.03	0.46 ± 0.03
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	1.43 ± 0.05	1.3 ± 0.1	2.00 ± 0.04	1.8 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.2
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.05* ± 0.02	0.07 ± 0.01	0.060* ± 0.004	0.07 ± 0.01
5-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.03* ± 0.01	0.04* ± 0.01	0.042* ± 0.005	0.050* ± 0.004
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.0312* ± 0.0001	0.037* ± 0.009	0.034* ± 0.005	0.037* ± 0.004
L-FOSA	29 ± 1	27 ± 2	33 ± 1	33 ± 4	38 ± 3	45 ± 10	18 ± 1	18 ± 2	7 ± 2	9 ± 1
Br-FOSA	0.50 ± 0.04	0.42 ± 0.03	1.9 ± 0.1	1.9 ± 0.1	3.6 ± 0.2	4.3 ± 0.8	4.8 ± 0.3	4.9 ± 0.4	4.5 ± 0.3	4.2 ± 0.4
L-FOSAA	12 ± 1	10 ± 1	23 ± 1	22 ± 5	34 ± 1	30 ± 6	27 ± 4	27 ± 6	18 ± 5	19 ± 1
Br-FOSAA	0.15 ± 0.01	0.10 ± 0.01	0.36 ± 0.02	0.30 ± 0.08	0.6 ± 0.2	0.56 ± 0.02	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.84 ± 0.01
L-EtFOSA	37.5 ± 0.3	46 ± 4	19 ± 1	21 ± 2	9 ± 1	6 ± 1	0.297 ± 0.002	0.4 ± 0.2	0.297 ± 0.004	0.299 ± 0.004
Br-EtFOSA	24 ± 2	21 ± 2	18 ± 1	19 ± 1	13 ± 1	13 ± 3	5.0 ± 0.3	4.8 ± 0.2	2.2 ± 0.2	2.3 ± 0.2

< LOD: concentration lower than the detection limit

**Values below limits of quantification*

Table 4.11: Percentages ± standard deviation (n=3) of L-EtFOSA and its degradation products in soil obtained in experiments 1, 2, and 3 after 81 days.

Analyte	Experiment 1	Experiment 2	Experiment 3
L-EtFOSA	16.5 ± 0.6	18.6 ± 0.5	21 ± 8
L-FOSA	37 ± 4	58.6 ± 0.1	39 ± 8
L-FOSAA	5 ± 2	8 ± 2	7.1 ± 0.5
L-PFOS	34 ± 1	24 ± 1	12 ± 3

We also investigated whether the extent of L-EtFOSA depletion or product formation would be reduced using autoclaved soils (Experiment 3), in order to confirm unequivocally whether the transformation we observed was biological in nature. Considering that the mesocosms were open to the air and received water and unsterilised fertilizer over the course of the experiment, we expected some substrate loss and product formation (e.g. from volatilisation and/or re-activation of soil microbes), but to a lesser extent than Experiments 1 and 2. Indeed, the extent of product formation was considerably lower in Experiment 3 compared to Experiments 1 and 2 (***Figure 4.6(c)***). While these data do not rule out potential contributions from abiotic degradation processes, the observation of reduced substrate depletion and product formation with initially-sterilised soil indicates that biologically catalysed transformation played a significant role in the transformation of L-EtFOSA in Experiments 1 and 2.

Analysis of leachate (***Tables 4.12-4.16***) from Experiments 1 and 2 revealed low levels of L-EtFOSA (30 - 248 ng/L) over the course of the experiment (***Tables 4.12 and 4.13***), representing a small fraction (< 0.009 %) of the original dose. While filters were monitored for signs of sorption [32], the high quantities of soil which adhered to the filters prevented firm conclusions to be made regarding the impact of sorption on leachate concentrations. Chandramouli et al. [32] estimated that glass fiber filters might result in up to 10 % sorption of PFOS and FOSA; however, even using these estimates the mole balance remains unaffected, given the low concentrations in leachates relative to soil.

Table 4.12: *Concentration (ng/L) of leachate collected during Experiment 1 (technical EtFOSA incubated in active soil-carrot mesocosm).*

Experiment 1								
Analyte	0-14 days		14-28 days		28-56 days		56-81 days	
	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	225	206	453	504	487	491	704	613
6-PFOS	< LOD	< LOD	24*	23*	15*	27*	43*	37*
3,4,5-PFOS	20	16*	28	28	23	44	73	54
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	383	354	487	434	379	318	445	349
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	< LOD	< LOD	18*	21*	35*	26*
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSA	1652	1325	2843	2560	2001	2917	3461	2788
Br-FOSA	458	293	1056	803	403	1038	1012	960
L-FOSAA	84	95	803	873	295	1489	523	454
Br-FOSAA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
L-EtFOSA	51	30	114	54	51	248	228	210
Br-EtFOSA	64	41	190	100	98	416	329	288

< LOD: concentration lower than the detection limit

**Values below limits of quantification*

Table 4.13: *Concentration (ng/L) of leachate collected during Experiment 2 (technical EtFOSA incubated in active soil mesocosm).*

Experiment 2								
Analyte	0-14 days		14-28 days		28-56 days		56-81 days	
	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	168	192	703	921	6111	5975	3324	10487
6-PFOS	< LOD	< LOD	52	68	722	795	616	1731
3,4,5-PFOS	17*	21	82	115	1164	1262	1677	2701
1-PFOS	< LOD	< LOD	< LOD	< LOD	43*	58*	41*	106
L-PFOA	125	214	255	283	382	427	437	461
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	17*	20*	32*	37*	56	42*
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	31*	< LOD	39*
L-FOSA	1045	1388	2951	4325	13912	11740	15876	13728
Br-FOSA	341	472	1674	2334	8642	6200	10408	9267
L-FOSAA	247	357	749	596	1580	2714	1899	3110
Br-FOSAA	<LOD	<LOD	93	77	319	314	504	629
L-EtFOSA	33	95	90	110	104	112	41	68
Br-EtFOSA	36	108	161	178	281	258	157	263

< LOD: concentration lower than the detection limit

**Values below limits of quantification*

Table 4.14: *Concentration (ng/L) of leachate collected during Experiment 3 (technical EtFOSA incubated in inactivated soil mesocosm).*

Experiment 3								
Analyte	0-14 days		14-28 days		28-56 days		56-81 days	
	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	83	82	430	271	656	1075	581	879
6-PFOS	< LOD	< LOD	< LOD	< LOD	23*	39*	55	127
3,4,5-PFOS	10*	10*	18*	10*	24	38	53	126
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	321	335	425	199	392	437	422	512
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	< LOD	< LOD	17*	20*	21*	31*
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSA	1135	913	1609	820	1815	2781	2749	3985
Br-FOSA	< LOD	< LOD	406	208	400	896	978	1789
L-FOSAA	814	2048	1546	549	2378	4177	2784	2372
Br-FOSAA	<LOD	<LOD	< LOD	< LOD	111	273	188	290
L-EtFOSA	77	178	172	117	257	405	47	83
Br-EtFOSA	82	174	202	85	240	721	102	198

< LOD: concentration lower than the detection limits
**Values below limits of quantification*

Table 4.15: *Concentration (ng/L) of leachate collected during Experiment 4 (blank experiment in active soil mesocosm) and Experiment 5 (blank experiment in soil-carrot mesocosm).*

	Experiment 4				Experiment 5			
Analyte	0-14 days	14-28 days	28-56 days	56-81 days	0-14 days	14-28 days	28-56 days	56-81 days
	Pot 1	Pot 1	Pot 1	Pot 1	Pot 1	Pot 1	Pot 1	Pot 1
L-PFOS	< LOD	11*	22*	27*	25*	12*	23*	25*
6-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3,4,5-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	76	74	108	112	394	192	277	200
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSA	15	18	24	31	28	27	19	28
Br-FOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSAA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Br-FOSAA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-EtFOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Br-EtFOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

< LOD: concentration lower than the detection limit
**Values below limits of quantification*

Table 4.16: *Concentration (ng/L) of leachate collected during Experiment 6 (incubation of the commercial Sulfluramid formulation Grão Forte).*

Analyte	Experiment 6							
	0-14 days		14-28 days		28-56 days		56-81 days	
	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2	Pot 1	Pot 2
L-PFOS	35	26*	72	61	70	91	78	121
6-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3,4,5-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1-PFOS	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-PFOA	145	134	148	167	204	154	160	153
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
4-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSA	65	37	78	60	126	115	151	145
Br-FOSA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
L-FOSAA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Br-FOSAA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
L-EtFOSA	3*	2*	3*	2*	11	5	30	4*
Br-EtFOSA	< LOD	< LOD	< LOD	< LOD	5	4*	19	4*

< LOD: concentration lower than the detection limit
**Values below limits of quantification*

A high relative ratio of L-FOSA: L-PFOS (4-7 in Experiment 1 and 1-7 in Experiment 2) was observed in leachates, consistent with observations in Brazilian surface water [9]. Notably, PFASs recovered from leachate on day 81 were up to an order of magnitude higher in Experiment 2 (soil-only; ***Table 4.13***) compared to Experiment 1 (soil+carrot; ***Table 4.12***), highlighting the considerable lixiviation potential of carrot. This result has important implications for predicting the fate of EtFOSA transformation products following application of EtFOSA. While Gilljam et al. [9] predicted that 100 % of PFOS produced from Sulfluramid would be transported from soil to ground water, the present results indicate that uptake by plants may considerably reduce this fraction. Future work should investigate PFAS levels in plants grown around regions where Sulfluramid is applied and whether those intended for human consumption represent a significant source of human exposure to PFASs.

4.3.3 Observation of PFOA

To obtain a clear picture of the source(s) of PFOA observed in these experiments, the contribution of potential input and output sources to soil PFOA levels over the entire duration of the experiment were quantified and compared. Inputs included irrigation water and nutrient solutions, background contamination in soil, and the doses themselves. Outputs included leaching and plant uptake. Contamination introduced to the air was tracked through blank experiments (4 and 5). These data are collectively summarised in *Figure 4.7*.

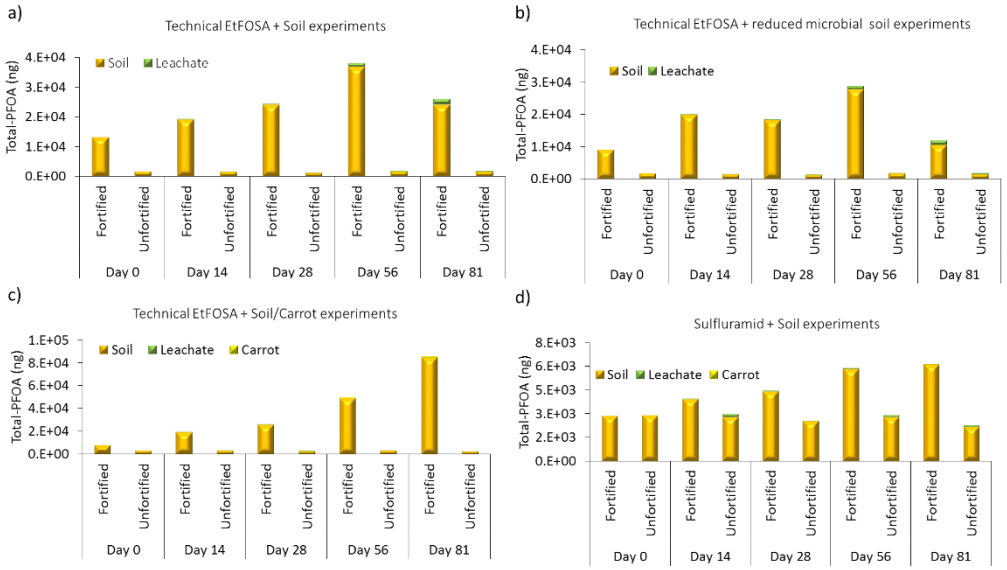


Figure 4.7: Formation of PFOA in a) Experiment 2: EtFOSA in active soil experiments, b) Experiment 3: EtFOSA in reduced microbial soil experiments, c) Experiment 1: EtFOSA + soil/carrot experiments, and d) Experiment 6: Sulfluramid + soil/carrot experiments.

PFOA was not present in the EtFOSA standard used for dosing, and in experiments involving commercial Sulfluramid (i.e. Experiment 6), only 4 % of PFOA in the entire system on day 0 was attributed to PFOA in the baits (see *Table 4.17*).

Table 4.17: Grão Forte baits characterisation using LC-MS/MS.

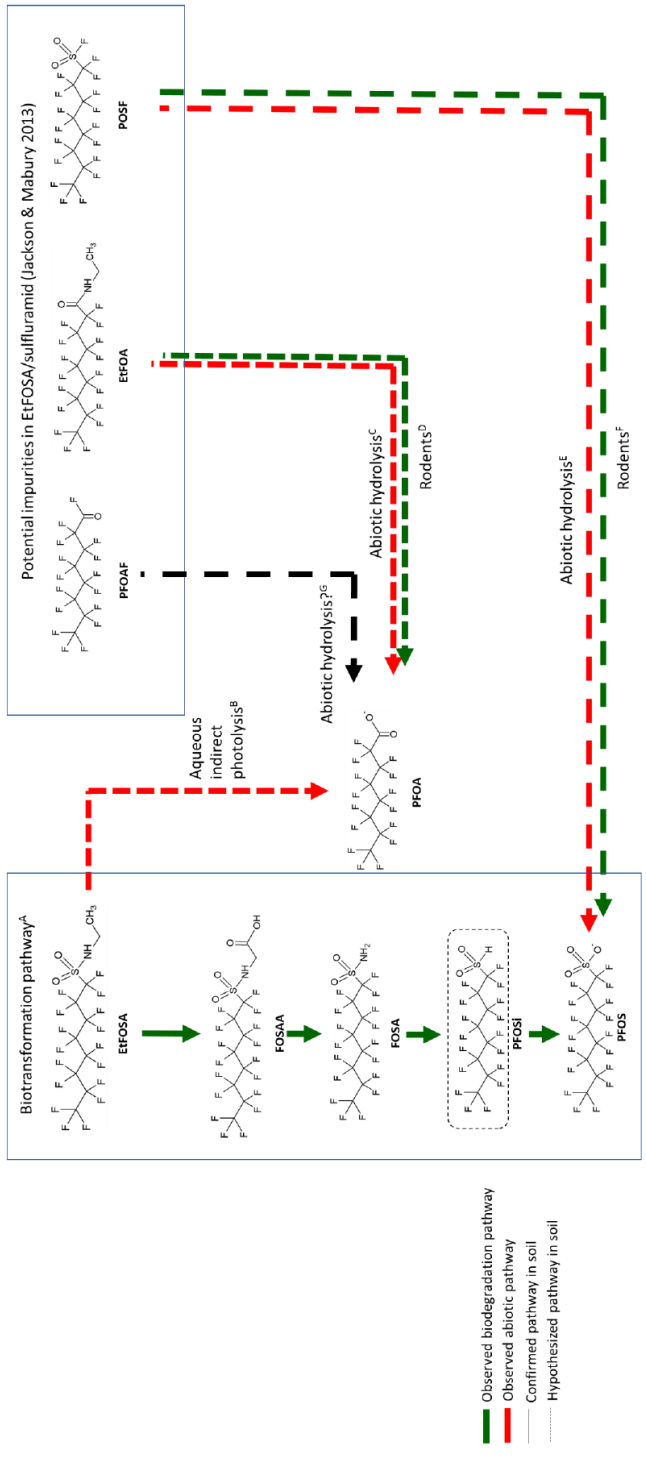
Analytes	Concentration ± s (ng/g)
L-PFOS	24 ± 2
6-PFOS	3.0 ± 0.8
3,4,5-PFOS	5.0 ± 0.9
1-PFOS	0.25 ± 0.09
dm-PFOS	0.30 ± 0.06
Br-PFOS (∑ individuals)	9 ± 2
L-PFOA	11 ± 2
6-PFOA	0.35 ± 0.03
5-PFOA	0.6 ± 0.1
4-PFOA	0.31 ± 0.01
dm-PFOA	nd
Br-PFOA (∑individuals)	1.3 ± 0.1
L-FOSA	445 ± 14
Br-FOSA	67 ± 6
L-FOSAA	nd
Br-FOSAA	nd
L-EtFOSA	$(17.1 \pm 1.2)\ 10^3$
Br-EtFOSA	$(7.2 \pm 0.5)\ 10^3$

nd: not detected

The quantity of PFOA introduced into the system from irrigation water and nutrient solutions over the course of the experiment was approximately 0.03 ng/day (day 81 cumulative total = 2.6 ng), representing a negligible contribution to levels in the soil. The elevated quantity of PFOA observed in experiments dosed with EtFOSA (i.e. experiments 1, 2, and 3) on day 0 relative to controls was attributed to the pre-incubation period in which the dosing solutions were mixed (together with solvent) with soil and left in a fume hood for 2 days to allow the solvent to evaporate. During this time, formation of PFOA occurred (see below for discussion on sources), resulting in a discrepancy between PFOA concentrations in fortified and unfortified experiments on day 0. This difference was not observed in experiments involving Sulfluramid since the baits were added directly to the surface of the soil, contained over an order of magnitude lower concentration of EtFOSA (and presumably residual impurities; see below), and contained PFOA concentrations which were very low (4 %) relative to the surrounding soil.

The maximum quantity of PFOA in experiment 1 (i.e. sum of PFOA in soil and carrot plus quantity of PFOA accumulated in leachate by day 81) was 37-fold higher than that observed in the corresponding unfortified experiment (Experiment 5; i.e. ~85301 vs 2275 ng, respectively). Subtracting the two values results in an overall yield of 83026 ng of PFOA by day 81. Lower yields of PFOA were observed in soil-only experiments (up to 25985 ng in active soils and up to 11946 ng in low-microbial soils), which is consistent with the relative order of decreasing yields of PFOS in Experiments 1, 2, and 3 (i.e. 34, 24, and 12 % yield respectively). PFOA yields in experiment 6 (Sulfluramid + soil/carrot) were lower than experiments 1-3 by approximately an order magnitude (6168 ng), which is not surprising considering the lower quantity of EtFOSA (and by extension, impurities; see below) introduced into the system in this experiment (i.e. 243 µg EtFOSA in Exp 6 versus 7600 µg EtFOSA in Exp 1).

Branched PFOA isomers (***Tables 4.6-4.10***) were only observed in dosed experiments, and tended to increase in concentration with time (see section on trends in isomer profiles). This observation is consistent with the transformation of an electrochemically-fluorinated precursor, not a telomer-manufactured substance. Few prior reports of such phenomena are available in the literature. In aerobic biodegradation experiments involving electrochemically-fluorinated *N*-ethyl perfluorooctane sulfonamidoethanol (EtFOSE), Lange [13,33] suggested that PFOA formed via abiotic hydrolysis of the intermediate perfluorooctane sulfinate (PFOSi). However, in subsequent biodegradation studies involving EtFOSE, PFOA was not produced [34,35]. PFOA may also be formed from indirect photolysis of a number of perfluorooctane sulfonamides [16,17] (see ***Figure 4.8***). Nguyen estimated a PFOA yield of 52 % for the 48 h aqueous indirect photolysis of EtFOSA at pH 6. Indirect photolysis in the top 2 mm of soil is known to occur [36]. We performed a back-of-the-envelope estimate of the total mass of PFOA produced from EtFOSA in the top 2 mm of soil assuming 100 % yield and obtained values of 92 µg for experiments 1-3 and 3 µg in experiment 6.



A. Aerobic soils. Present study and [11].
B. 52% yield of PFOA over 48hrs at pH 6. May be relevant for surface soils [16,17].
C. Quantitative conversion to PFOA at pH 14 (24hrs). No hydrolysis observed at pH 8.5 (8 days) [26].
D. Toxicokinetic study in rats involving structurally similar perfluorooctyl amide [37].
E. Slow hydrolysis in water; various chain lengths [38-40].
F. 0.1% inhaled POSF converted to PFOS in Sprague-Dawley rats [41].
G. Hypothesised by Jackson and Mabury. Has not been reported [26].

Figure 4.8: Overview of observed and hypothetised transformation pathways of EtFOSA and residuals in soils. Note that for simplicity, some pathways are not shown, for example EtFOSA can biodegrade directly to FOSA, and FOSAA has been shown to undergo aqueous indirect photolysis to form PFOA.

These values are similar to the maximum quantity of PFOA determined in soil (i.e. 28-84 µg for experiments 1-3 and 6 µg for experiment 6). However, indirect photolysis cannot explain the higher yield of PFOA produced in the presence of carrot, nor the lower yield of PFOA in inactive soil – both of which point to the role of biodegradation.

Perhaps the most likely explanation for the observed formation of PFOA is the presence of *N*-ethylperfluorooctanamide (EtFOA; ***Figure 4.8***), which is known to occur at a concentration of 150 ± 7 µg/g in EtFOSA produced by Lancaster synthesis [26]. Jackson and Mabury reported nearly complete conversion of EtFOA to PFOA in 24 h in a water/MeOH solution at pH 14 and postulated that EtFOA would readily undergo enzyme-catalysed hydrolysis to produce branched and linear isomers of PFOA, consistent with observations in the present work. They also pointed to a technical report by the 3M Co. in which a structurally similar substance (*N*- methylperfluorooctanamide; MeFOA) was metabolised to PFOA in Sprague-Dawley rats (***Figure 4.8***) [37]. Overall, we conclude that small quantities of PFOA may form from the use of Sulfluramid, either through indirect photolysis of EtFOSA in the surface of the soil or biodegradation of impurities such as EtFOA (see ***Figure 4.8***). Given the changes in PFOA yield with microbial activity and in the presence of carrot, the latter is a more likely explanation for the present work.

4.3.4 Translocation of EtFOSA from soil to carrot

Samples of carrot core, peel, and leaves were collected on days 56 and 81 in order to investigate the potential of EtFOSA and its transformation products to accumulate in agricultural crops. Concentrations in each of the carrot compartments are summarised in ***Tables 4.18-4.19*** and compartment-specific BCFs are provided in ***Tables 4.20-4.21***.

Table 4.18: Concentration (ng/g) ± standard deviation (n=3) of target analytes in different compartments of carrot (core, peel and leaves) for Experiment 1 (technical EtFOSA incubated in active soil-carrot mesocosm).

	Day 56						Day 81					
	Pot 1			Pot 2			Pot 1			Pot 2		
Analyte	Peel	Core	Leaves	Peel	Core	Leaves	Peel	Core	Leaves	Peel	Core	Leaves
L-PFOS	38 ± 10	63 ± 1	550 ± 83	43 ± 7	104 ± 15	630 ± 53	72 ± 13	73 ± 10	1049 ± 57	24 ± 3	33 ± 1	382 ± 52
6-PFOS	2.7 ± 0.5	1.9 ± 0.2	17 ± 3	3.0 ± 0.4	2.9 ± 0.3	17 ± 3	5.4 ± 0.9	1.9 ± 0.4	27 ± 5	3.1 ± 0.4	0.8 ± 0.3	9 ± 2
3,4,5-PFOS	3 ± 1	1.5 ± 0.1	10 ± 2	2.59 ± 0.05	3. 0 ± 0.2	15 ± 1	4.0 ± 0.6	2.0 ± 0.3	24 ± 4	2.2 ± 0.6	1.1 ± 0.1	8 ± 2
L-PFOA	2.1 ± 0.3	2.5 ± 0.3	16.1 ± 0.2	3.8 ± 0.6	4.7 ± 0.4	21 ± 2	3.3 ± 0.3	2.8 ± 0.2	35 ± 5	2.3 ± 0.3	2.3 ± 0.1	12 ± 3
6-PFOA	< LOD	< LOD	< LOD	< LOD	< LOD	2.9 ± 0.3	0.56 ± 0.05	< LOD	3.9 ± 0.5	0.494 ± 0.006	< LOD	1.8 ± 0.4
5-PFOA	< LOD	< LOD	1.61 ± 0.06	< LOD	< LOD	2.2 ± 0.3	< LOD	< LOD	2.9 ± 0.4	< LOD	< LOD	0.05 ±0.02
L-FOSA	131 ± 17	12 ± 1	25 ± 4	51 ± 3	30 ± 2	35 ± 7	69 ± 5	10 ± 1	14.4 ± 0.4	50 ± 3	4.4 ± 0.1	22 ± 3
Br-FOSA	19 ± 4	1.5 ± 0.2	4 ± 1	9 ± 1	3.3 ± 0.2	7 ± 1	11 ± 1	1.4 ± 0.2	4 ± 1	9 ± 1	0.9 ± 0.2	6 ± 1
L-FOSAA	1.5 ± 0.4	0.38 ± 0.08	0.030 ± 0.004	1.3 ± 0.2	0.7 ± 0.1	0.040 ± 0.007	1.5 ± 0.4	0.245 ± 0.001	0.070 ± 0.003	0.9 ± 0.2	0.14 ± 0.03	0.028 ± 0.009
Br-FOSAA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-EtFOSA	7 ± 2	< LOD	< LOD	9 ± 2	< LOD	< LOD	3 ± 1	< LOD	< LOD	2.78 ± 0.05	< LOD	< LOD
Br-EtFOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

< LOD: concentration lower than the detection limit
nd: not detected

Table 4.19: Concentration (ng/g) of target analytes ± standard deviation (n=3) in different carrot compartments (core, peel and leaves) for Experiment 6 (Incubation of the commercial Sulfluramid formulation Grão Forte).

	Day 56						Day 81					
	Pot 1			Pot 2			Pot 1			Pot 2		
Analyte	Peel	Core	Leaves	Peel	Core	Leaves	Peel	Core	Leaves	Peel	Core	Leaves
L-PFOS	9 ± 1	30 ± 6	105 ± 7	19 ± 5	36 ± 7	102 ± 28	7 ± 2	9 ± 2	202 ± 49	8.0 ± 0.8	7 ± 1	113 ± 29
6-PFOS	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3,4,5-PFOS	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-PFOA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6-PFOA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5-PFOA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-FOSA	2.49 ± 0.05	1.2 ± 0.2	1.3 ± 0.1	2.3 ± 0.5	0.75 ± 0.02	0.93 ± 0.08	1.4 ± 0.1	0.27 ± 0.07	0.7 ± 0.3	2.2 ± 0.2	0.38 ±0.08	0.87 ± 0.07
Br-FOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-FOSAA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Br-FOSAA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-EtFOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Br-EtFOSA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected

Table 4.20: Bioconcentration factors of target analytes ± standard deviation (n=3) in different carrot compartments (BCF_{Core}, BCF_{Peel} and BCF_{Leaves}) for Experiment 1 (technical EtFOSA incubated in active soil-carrot mesocosm).

	Day 56						Day 81					
	Pot 1			Pot 2			Pot 1			Pot 2		
Analyte	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}
L-PFOS	0.12 ± 0.03	0.19 ± 0.02	1.7 ± 0.3	0.12 ± 0.02	0.30 ± 0.05	1.8 ± 0.2	0.14 ± 0.02	0.14 ± 0.02	2.0 ± 0.1	0.044 ± 0.005	0.061 ± 0.003	0.7 ± 0.1
6-PFOS	0.23 ± 0.04	0.16 ± 0.02	1.4 ± 0.3	0.17 ± 0.03	0.17 ± 0.02	1.0 ± 0.2	0.18 ± 0.03	0.06 ± 0.01	0.9 ± 0.2	0.09 ± 0.01	0.026 ± 0.008	0.26 ± 0.05
3,4,5-PFOS	0.3 ± 0.1	0.13 ± 0.03	0.9 ± 0.2	0.12 ± 0.01	0.14 ± 0.01	0.7 ± 0.1	0.11 ± 0.02	0.055 ± 0.004	0.7 ± 0.1	0.06 ± 0.02	0.0310 ± 0.0009	0.21 ± 0.04
L-PFOA	0.13 ± 0.03	0.16 ± 0.02	1.0 ± 0.1	0.15 ± 0.03	0.19 ± 0.03	0.8 ± 0.1	0.09 ± 0.01	0.080 ± 0.005	1.0 ± 0.1	0.07 ± 0.01	0.074 ± 0.004	0.4 ± 0.1
6-PFOA	-	-	-	-	-	1.5 ± 0.2	0.17 ± 0.02	-	1.2 ± 0.2	0.17 ± 0.01	-	0.4 ± 0.1
5-PFOA	-	-	1.8 ± 0.2	-	-	1.3 ± 0.2	-	-	1.1 ± 0.2	-	-	0.021 ± 0.006
L-FOSA	0.4 ± 0.1	0.033 ± 0.004	0.07 ± 0.01	0.12 ± 0.01	0.07 ± 0.01	0.08 ± 0.02	0.16 ± 0.01	0.024 ± 0.002	0.033 ± 0.002	0.13 ± 0.01	0.0110 ± 0.0004	0.055 ± 0.007
Br-FOSA	0.06 ± 0.01	0.005 ± 0.001	0.013 ± 0.004	0.024 ± 0.003	0.009 ± 0.001	0.019 ± 0.003	0.024 ± 0.003	0.0030 ± 0.0005	0.009 ± 0.002	0.0195 ± 0.0007	0.0020 ± 0.0004	0.013 ± 0.002
L-FOSAA	0.004 ± 0.001	0.0011 ± 0.0004	0.00007 ± 0.00002	0.004 ± 0.001	0.0021 ± 0.0005	0.00084 ± 0.00003	0.005 ± 0.002	0.0008 ± 0.0001	0.00023 ± 0.00004	0.004 ± 0.001	0.0006 ± 0.0002	0.00010 ± 0.00004
L-EtFOSA	0.012 ± 0.004	-	-	0.010 ± 0.002	-	-	0.010 ± 0.005	-	-	0.0108 ± 0.0007	-	-
Br-EtFOSA	-	-	-	-	-	-	-	-	-	-	-	-

-: BCFs were not calculated because target compounds were not detected or values were < LOD

Table 4.21: Bioconcentration factors of target analytes ± standard deviation in different carrot compartments (BCF_{Core}, BCF_{Peel} and BCF_{Leaves}) for Experiment 6 (Incubation of the commercial Sulfluramid formulation Grão Forte).

	Day 56						Day 81					
	Pot 1			Pot 2			Pot 1			Pot 2		
Analyte	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}	BCF_{Peel}	BCF_{Core}	BCF_{Leaves}
L-PFOS	0.10 ±0.02	0.31 ± 0.06	1.1 ± 0.1	0.19 ± 0.05	0.37 ± 0.07	1.0 ± 0.3	0.07 ± 0.02	0.08 ± 0.02	1.9 ± 0.5	0.068 ± 0.007	0.06 ± 0.01	0.9 ± 0.2
6-PFOS	-	-	-	-	-	-	-	-	-	-	-	-
3,4,5-PFOS	-	-	-	-	-	-	-	-	-	-	-	-
L-PFOA	-	-	-	-	-	-	-	-	-	-	-	-
6-PFOA	-	-	-	-	-	-	-	-	-	-	-	-
5-PFOA	-	-	-	-	-	-	-	-	-	-	-	-
L-FOSA	0.09 ± 0.01	0.04 ± 0.01	0.050 ± 0.009	0.08 ± 0.02	0.027 ± 0.006	0.034 ± 0.008	0.034 ± 0.008	0.015 ± 0.006	0.04 ± 0.02	0.12 ± 0.01	0.020 ± 0.004	0.050 ±0.004
Br-FOSA	-	-	-	-	-	-	-	-	-	-	-	-
L-FOSAA	-	-	-	-	-	-	-	-	-	-	-	-
L-EtFOSA	-	-	-	-	-	-	-	-	-	-	-	-
Br-EtFOSA	-	-	-	-	-	-	-	-	-	-	-	-

-: BCFs were not calculated because target compounds were not detected or values were < LOD

While L-EtFOSA was only observed in peel (up to 9 ng/g on day 56 and up to 3 ng/g on day 81), L-FOSAA, L-FOSA, L-PFOA and L-PFOS were observed in core, peel and leaves. Hydrophobicity clearly played a significant role in partitioning of individual PFASs within the carrot. For example, PFOS (water solubility of 550 mg/L) [42] and PFOA (water solubility of 9500 mg/L) [42] were observed primarily in leaf (382 - 1049 ng/g for L-PFOS and 12-35 ng/g for L-PFOA), while compounds such as FOSA (water solubility of 4.4 mg/L) [43], FOSAA (water solubility unknown but presumed to be similar to FOSA) and EtFOSA (0.056 mg/L water solubility) [43] were observed primarily in carrot peel (50 - 131 ng/g for L-FOSA, 0.9 - 1.5 ng/g for L-FOSAA and 2.78 - 9 ng/g for L-EtFOSA) and core (4.4 - 30 ng/g for L-FOSA and 0.14 - 0.7 ng/g for L-FOSAA). Of the total burden of L-PFOS in carrot, 84 - 92 % was estimated to accumulate in carrot leaves, whereas 6-13 % and 2 - 3 % accumulated in carrot core and peel, respectively (*Figure 4.9*).

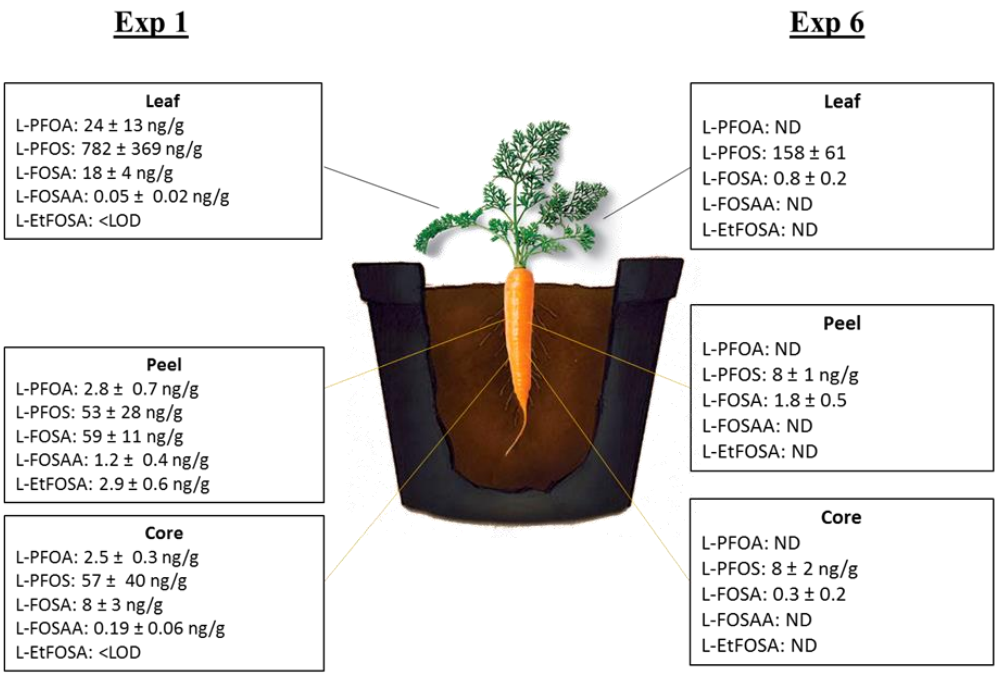


Figure 4.9. *Concentrations of EtFOSA and its transformation products in different carrot compartments (peel, core and leaves) in Experiment 1 (active soil mesocosm fortified with technical EtFOSA) and Experiment 6 (active soil mesocosm with the addition of EtFOSA commercial baits) after 81 days.*

The observations of increased translocation with water solubility are consistent with prior studies involving incubation with other PFASs such as PFAAs and polyfluoroalkyl phosphates (PAPs) in lettuce [12,25,44], legume [45] and carrot [12,44]. The highest carrot BCFs for L-PFOS and L-PFOA where determined in the leaves (0.7-2.0 and 0.4-1.0, respectively), while lower values where obtained for core (0.06-0.30 and 0.07-0.19, respectively) and peel (0.04-0.14 and 0.07-0.15, respectively). A similar tendency was observed in a previous study involving PFOA and PFOS uptake in a carrot/soil mesocosm [12]. In the case of L-FOSA and L-FOSAA, the highest BCFs were obtained in peel (0.1-0.4 and 0.004-0.005, respectively). Branched isomer BCFs could only be determined for FOSA, PFOA, and PFOS in Experiment 1 due to low concentrations FOSAA and EtFOSA in plant material. BCFs for Br-FOSA were an order of magnitude lower compared to L- FOSA, but similar BCFs were obtained for branched and linear isomers of PFOS and PFOA. These data are presented in ***Table 4.20*** but due to the few detects and high variability in these data, firm conclusions could not be drawn.

4.3.5 Trends in isomer profiles

Individual isomers of EtFOSA and its transformation products were tracked over the course of the experiment in soil (***Tables 4.6-4.10***), carrot (***Tables 4.18-4.19***), and leachate water (***Tables 4.12-4.16***). Given numerous prior studies which have clearly shown the tendency of branched isomers to degrade faster than the linear isomer [46-48], and the fact that reduced hydrophobicity [49] imparted by chain branching favours leaching of branched isomers, we expected to observe a more rapid depletion of branched-, as opposed to L-EtFOSA isomers. However, this was not the case in the present work. As shown in ***Figure 4.10***, L-EtFOSA depleted faster than Br-EtFOSA in soil from all experiments, resulting in an apparent enrichment of Br-EtFOSA by day 81 relative to day 0.

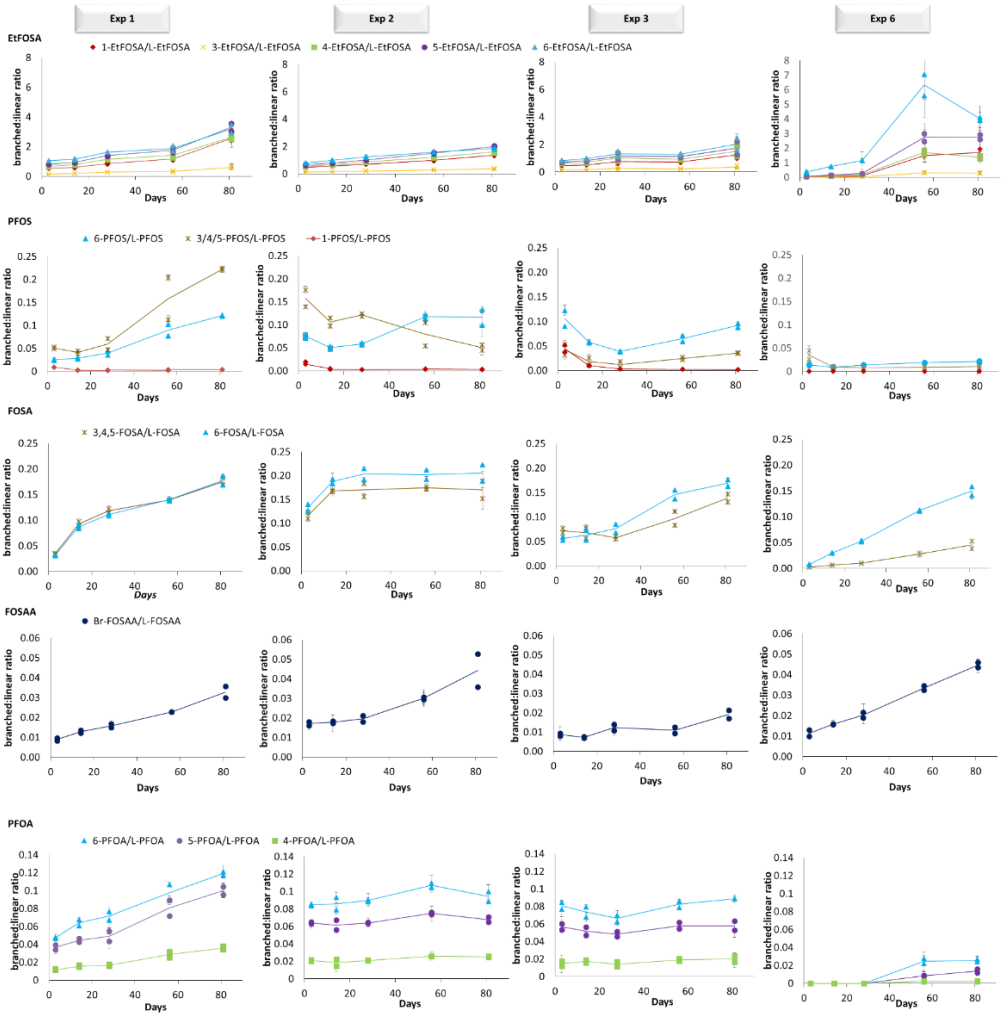


Figure 4.10: *Ratio between individual isomers and their corresponding linear target compound in soil.*

Half-lives for Br-EtFOSA were 127.6 ± 7.8 days, 88 ± 28 days, and 86.1 ± 15.8 days compared to 35.8 ± 3.7, 33.6 ± 9.0, 40.0 ± 7.8 days, for L-EtFOSA, in Experiments 1, 2, and 3, respectively. However, despite more rapid depletion of L-EtFOSA and enrichment of Br-FOSA and Br-FOSAA, an enrichment of the stable end products L-PFOS and L-PFOA was not observed in soil. In fact, in some cases, enrichment of branched isomers was observed (e.g. PFOS and PFOA in Experiment 1). This may be due to preferential biotransformation of one or more branched intermediates. Considering that formation

and biodegradation processes take place simultaneously for intermediates (e.g. FOSA, FOSAA), it is difficult to draw firm conclusions on the isomer-specific behaviour of substances other than EtFOSA.

Branched isomers were observed in leaching and were consistently enriched relative to soil for PFOS and in some cases FOSA and FOSAA, but not EtFOSA (see ***Figure 4.11***).

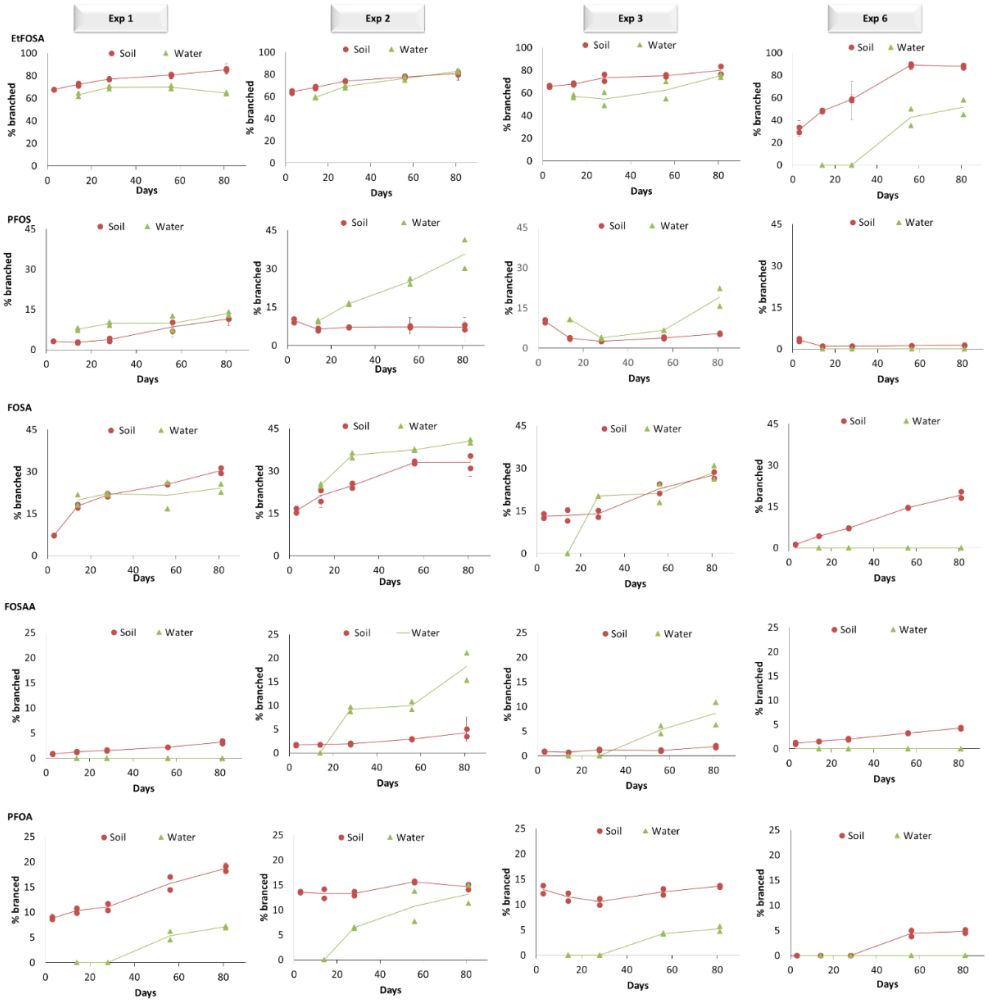


Figure 4.11: *Percentage (%) of branched isomers for soil and leaching water in the different experiments.*

For lower abundance targets (e.g. PFOA), a comparison of isomer profiles in leachate or plant material with that of soil must be interpreted cautiously since branched isomers below LODs can produce an apparent enrichment of the linear isomer, relative to soil. Overall, concentrations in leachate and carrot were very low relative to soil, and we conclude that isomer-specific leaching and plant translocation is unlikely to significantly affect the isomer profiles in soil. However, isomer profiles in soil appear to be affected by the presence of crop (see ***Figure 4.12***). For example, in Experiment 1 (soil+carrot), branched content of 15, 2.4, 0.2 and 0.32 % was observed for FOSA, PFOS, FOSAA and PFOA, respectively, in soil, while in Experiment 2 (soil only), branched content of up to 20, 1.1, 0.2 and 0.04 % were observed for FOSA, PFOS, FOSAA, and PFOA, respectively, in soil. The factor(s) contributing to these differences are unclear but may be related to enhancement of biodegradation in the presence of carrot.

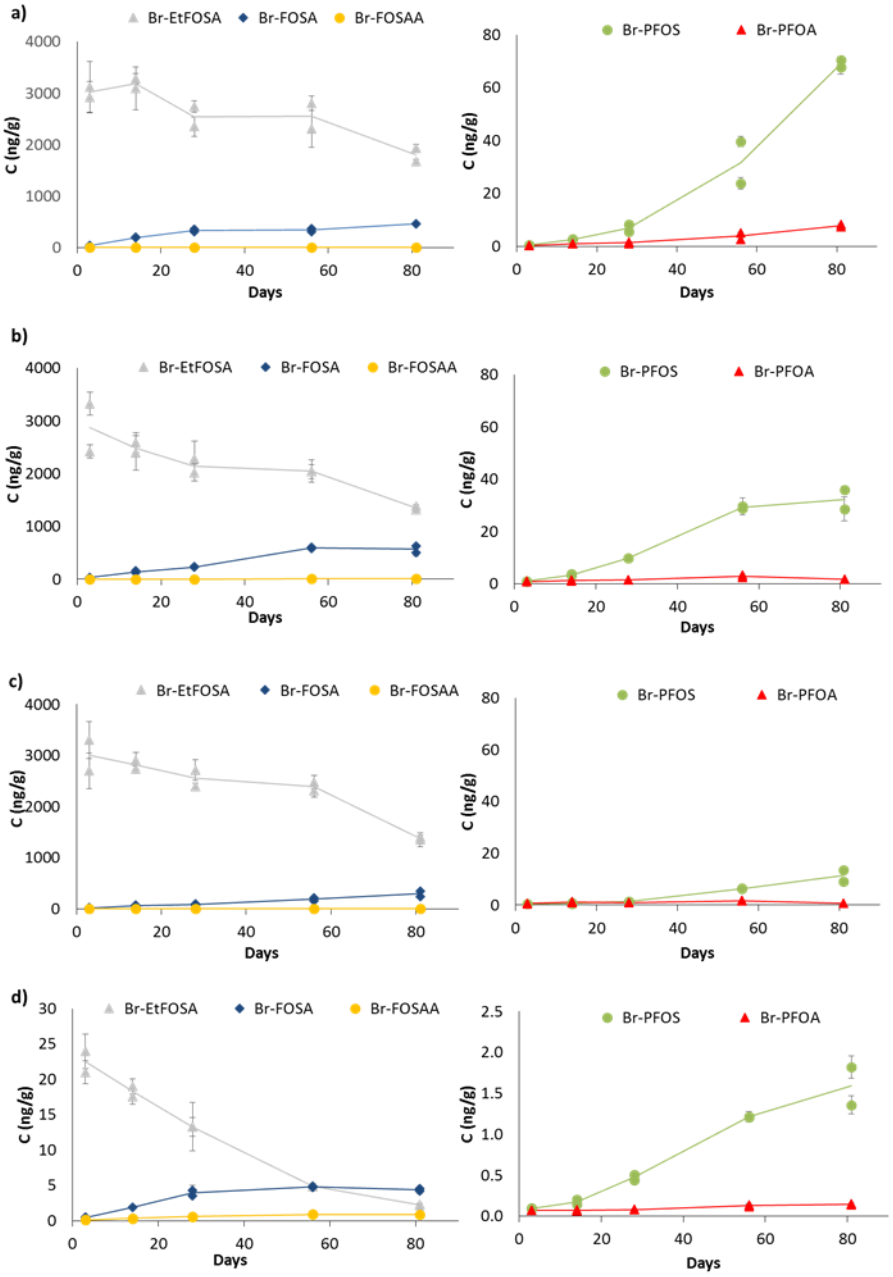


Figure 4.12. Degradation profiles of Br-EtFOSA and its metabolites in soil for the different experiments: a) Experiment 1, b) Experiment 2, c) Experiment 3 and d) Experiment 6.

4.3.6 Behaviour of commercial Sulfluramid in soil/carrot mesocosm

Incubation of Grão Forte in the soil-carrot mesocosm revealed a much shorter half-life for both L- and Br-EtFOSA (11.5 ± 2.1 days and 29.7 ± 3.1 days, respectively) compared to Experiment 1 (35.8 ± 3.7 days and 127.6 ± 7.8 days, respectively). These half-lives are similar to the estimates of Avendaño and Liu [11] in aerobic soils (13.9 ± 2.1 days). Transformation products observed at t=0 were attributed to their occurrence as residuals in the baits (see ***Table 4.17***). However, an increase in FOSAA and FOSA up to day 28, and an ongoing increase in PFOS throughout the experiment, indicated that these substances were also formed from transformation of EtFOSA over the course of the experiment (***Figure 4.7d***). In general, the relative levels and behaviour of transformation products (including branched and linear isomers) in Experiment 6 were similar to Experiment 1. For example, in both experiments 1 and 6, FOSA and FOSAA were the principal transformation products in soil, and in both experiments, the more hydrophilic transformation products (e.g. PFOS) tended to occur to a greater extent in leaves (***Figure 4.10***). Bioconcentration factors calculated for detectable targets in Experiment 6 (***Table 4.21***) were also fairly consistent with Experiment 1 (***Table 4.21***). However, unlike Experiment 1, the yield of PFOS was considerably higher (277 %) resulting in a mole balance of 176 ± 11 % by day 81, even after accounting for residual PFOS-precursors in the baits. Considering the high yield of PFOS, a significant fraction appears to be associated with one or more unidentified PFOS-precursors in the commercial bait. Ongoing research is focused on identifying this substance (or substances) along with a comprehensive characterisation of other Sulfluramid baits currently on the Brazilian market.

4.4 Conclusions

These data collectively show that the application of EtFOSA-containing Sulfluramid baits can lead to the occurrence of PFOS in crops and in the surrounding environment,

in considerably higher yields than previously thought. A longer exposure time is expected to produce even higher yields of PFOS. Furthermore, experiments involving commercial Sulfluramid indicate that an additional and as-of-yet characterised PFOS-precursor (or precursors) may be present in these baits. Identification of these substances is vital in order to accurately assess the risks related to the use of commercial Sulfluramid bait formulations. For now, our data support the hypothesis that the ratio of FOSA:PFOS is a suitable marker of Sulfluramid use. Future work will focus on commercial baits characterisation, occurrence of PFASs in agricultural regions, and investigating the potential uptake of PFASs into local fruits and vegetables as a potential pathway for human exposure.

4.5 References

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Chapter 5

Simultaneous determination of perfluorinated compounds and their potential precursors in mussel tissues, fish homogenate and liver samples by liquid chromatography–electrospray tandem mass spectrometry

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5.1 Introduction

Per- and polyfluoroalkyl substances (PFASs) have attracted increasing attention as emerging environmental contaminants in recent years due to their potential toxicity, persistence and bioaccumulation [1]. Their characteristic properties, such as water and grease repellency and high chemical and biological stability, are the responsible for their wide range of applications in consumer products. For instance, perfluorinated compounds (PFCs) are widely used in textile, carpet, paper and leather treatment and as performance chemicals in products such as fire-fighting foams, floor polishes, shampoos, paints and inks [2,3]. Furthermore, PFCs are also used in industrial applications as surfactants, emulsifiers, wetting agents, additives and coatings [4].

Commonly discussed fluorinated contaminants are the perfluorinated acids, including perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) [5]. Although PFCAs and PFSAs have been mostly studied in environmental samples, sulfonamides such as perfluorooctane sulfonamide (FOSA) and phosphonic acids such as perfluorooctane phosponic acid (PFOPA) are being included in monitoring programs [6,7]. PFCs are widely found in the environment, primarily resulting from anthropogenic sources. The numerous applications, followed by environmental persistence and bioaccumulation of these compounds, have resulted in their appearance in the aquatic systems, as well as biota samples, inhabiting not only locations in close proximity to pollution sources, but also in remote areas [8]. These compounds bioaccumulate in the aquatic organisms and high trophic level organisms accumulate greater concentrations of certain PFCs [9,10]. Since PFCs are generally hydrophobic but also lipophobic, they do not tend to accumulate in fatty tissues as in the case of persistent halogenated compounds and they are primarily retained in protein-rich compartments, such as blood, liver and kidneys of fish, birds and marine mammals [10].

Due to the growing concern about this class of chemicals, PFOS and its derivatives have been listed as priority hazardous substances in the field of water policy under the Directive 2013/39/EU [11]. Moreover, an environmental quality standard (EQS) value was established for PFOS in biota (9.1 μg/kg) [11].

Furthermore, an increasing attention is also being paid to PFC potential precursors, especially to polyfluoroalkyl phosphates (PAPs), a group of hydrophobic phosphates that are mainly mono-, di- and tri- substituted by partially fluorinated alkyl chains (mono-PAPs, di-PAPs and tri-PAPs, respectively) [12]. Recent studies reported the presence of PAPs in environmental samples, such as sewage sludge [12,13] and drinking water [14]. Moreover, biotransformation studies have been carried out in order to understand the relation between the presence of PFCs and their precursors [15].

Concerning to the extraction of PFCs from solid samples, alkaline digestion [16,17], ion-pair based extraction [18–21] or the extraction of the solid samples into an organic solvent assisted with an external energy source, such as pressurised liquid extraction (PLE) [16] or ultrasounds [22–25], have been mostly applied for the extraction of target compounds in biota samples. However, due to the lack of selectivity of the previously mentioned extraction techniques, a clean-up of the extracts is usually necessary. Different clean-up procedures have been applied in the literature. For instance, sulfuric acid washing and subsequent silica-column chromatography were applied after the ion-pairing extraction for lipid removal [26]. Moreover, a direct silica column clean-up was also developed [27]. In the case of extraction using an organic solvent, dispersive graphitised carbon (Envi-carb) and/or weak anion exchange (WAX) solid-phase extraction (SPE) are the clean-up approaches mostly used [22,24,28,29], although mixed mode (C8+ aminopropyl) SPE and a subsequent Envi-Carb clean-up was also applied for the determination of PFSAs and sulfonamide-based precursors in liver and muscle samples previously extracted in acetonitrile (ACN) under ultrasound energy [23].

There are currently only a few works for the determination of PAPs or other PFC precursors in environmental samples. For instance, Ding et al. [14] developed an analytical method for the determination of PAPs in drinking water by mix mode WAX SPE. Moreover, Liu et al. [12] employed extraction with (1:1) tetrahydrofuran: acetic acid mixture combined with ultra-sonication for sewage sludge samples with a posterior WAX or mixed-mode ion exchange (MAX) coupled to Envi-Carb clean-up approach for the determination of perfluoroalkyl phosphonic acids (PFPAs), monoPAPs and diPAPs, and for triPAPs, respectively. To the best of our knowledge, there is no method in the literature for the simultaneous determination of PAPs and other PFC precursors in biota samples.

Within this context, the aim of the present work was to overcome the challenge of developing an analytical method for the simultaneous determination of 14 PFCs and 10 potential precursors in fish liver, fish muscle and mussel samples. In order to achieve this objective, both, the optimisation of an instrumental method for PFC precursors and the comparison of different clean-up approaches for PFCs and their potential precursors by means of SPE using mix mode WAX, ENVI-Carb or a combination of them were carried out. In this sense, a previously optimised focused ultrasound solid-liquid extraction (FUSLE) method [25] was applied and the analyses were performed by liquid-chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS) in all the cases. Furthermore, grey mullet liver samples (*Chelon labrosus*) and mussels (*Mytilus galloprovincialis*) from different populations of the North Coast of Spain and Yellowfin tuna muscle samples (*Thunnus albacares*) from the Indian Ocean were analysed.

5.2 Experimental section

5.2.1 Reagents and materials

Potassium perfluoro-1-butane sulfonate (L-PFBS), sodium perfluoro-1-hexane sulfonate (L-PFHxS), potassium perfluoro-1-octane sulfonate (L-PFOS), perfluorooctane

phosphonic acid (PFOPA), perfluorohexane phosphonic acid (PFHxPA), perfluorodecane phosphonic acid (PFDPA), perfluoro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), sodium 1H, 1H, 2H, 2H-perfluorooctyl phosphate (6:2 monoPAP), sodium 1H, 1H, 2H, 2H-perfluorodecyl phosphate (8:2 monoPAP), sodium bis (1H, 1H, 2H, 2H-perfluorooctyl) phosphate (6:2 diPAP), sodium bis (1H, 1H, 2H, 2H-perfluorodecyl) phosphate (8:2 diPAP), 2-perfluorohexyl ethanoic acid (6:2 FTCA), 2-perfluorooctyl ethanoic acid (8:2 FTCA), 2H-perfluoro-2-octenoic acid (6:2 FTUCA), 2H-perfluoro-2-decenoic acid (8:2 FTUCA), 3-perfluoroheptyl propanoic acid (7:3 FTCA), 3-perfluoropentyl propanoic acid (5:3 FTCA), the surrogate mixture (sodium perfluoro-1-hexane [$^{18}O_2$] sulfonate (MPFHxS), sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octane sulfonate (MPFOS), perfluoro-n-[$^{13}C_4$] butanoic acid (MPFBA), perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid (MPFHxA), perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid (MPFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$] octanoic acid (MPFNA), perfluoro-n-[1,2-$^{13}C_2$] decanoic acid (MPFDA), perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid (MPFUnDA) and perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid (MPFDoDA)), 6-chloroperfluorohexyl phosphonic acid (Cl-PFHxPA), sodium bis (1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl) phosphate (M8:2diPAP), 2H-perfluoro-[1,2-$^{13}C_2$]-2-decenoic acid (M8:2 FTUCA), 2-perfluorohexyl-[1,2-$^{13}C_2$]-ethanoic acid (M6:2 FTCA), 2-perfluorooctyl-[1,2-$^{13}C_2$]-ethanoic acid (M8:2 FTCA) and 2-perfluorodecyl-[1,2-$^{13}C_2$]-ethanoic acid (M10:2 FTCA) were purchased from Wellington Laboratories (Ontario, Canada). FOSA was provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The purity of all the target analytes was > 98 % except for FOSA (97.5 %).

Methanol (MeOH, HPLC grade, 99.9 %) and methyl tert-butyl ether (MTBE, 99.8 %) were supplied by LabScan (Dublin, Ireland), acetonitrile (ACN, HPLC grade, 99.9 %) by Sigma Aldrich (Steinheim, Germany), acetic acid (HOAc, glacial, 100 %) by Merck (Darmstadt, Germany), formic acid (HCOOH, 98-100 %) by Scharlau (Barcelona, Spain)

and ammonium hydroxide (NH_4OH, 25 %) by Panreac (Barcelona, Spain). Ultra-pure water was obtained using a Milli-Q water purification system (<0.05 µS/cm, Milli-Q model 185, Millipore, Bedford, MA, USA).

For the clean-up step, Evolute-WAX (primary/secondary amine modified polystyrene-divinylbenzene incorporating non-ionisable hydroxyl groups, 200 mg) SPE cartridges and SPE column adapters were purchased from Biotage (Uppsala, Sweden) and bulk Superclean Envi-Carb sorbent (100 m^2/g, 120/400 mesh) and empty SPE tubes (6 mL) were purchased from Supelco (Bellefonte, PA).

For the mobile phase composition MeOH (Fisher Scientific, Loughborough, UK) was used. 1-methyl piperidine (1-MP, $\geq$ 98 %) was obtained from Merck and ammonium acetate (NH_4OAc ≥ 99 %) was purchased from Sigma Aldrich. High purity nitrogen gas (> 99.999 %) supplied by Messer (Tarragona, Spain) was used as collision gas and nitrogen gas (99.999 %) purchased from AIR Liquid (Madrid, Spain) was used as nebuliser and drying gas.

5.2.2 Sample collection and treatment

Fieldwork was conducted in June and July 2009 for mussel samples, in May and June 2010 in the case of grey mullet samples and during 2013 in the case of tuna muscle samples.

Mussels (*Mytilus galloprovincialis*, 5.7 ± 3.8 cm shell length) were obtained from harbor areas in Vigo (Galicia, Spain), Pasaia (Basque Country, Spain), Santurtzi (Basque Country, Spain) and Getxo (Basque Country, Spain) and from the estuary of Muskiz (Basque Country, Spain). All the sampling points are shown in ***Figure 5.1***. After collection, mussels were transported in an icebox to the laboratory, where the soft tissues were dissected.

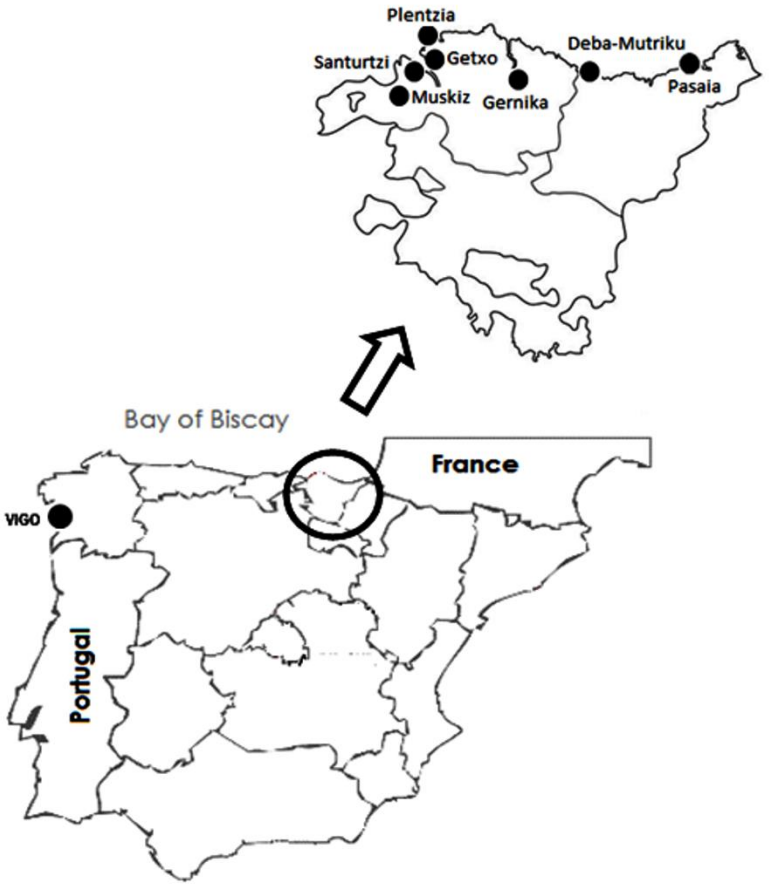

Figure 5.1. *Map of Iberian Peninsula showing sampling locations of mussel and liver samples.*

Adult thicklip grey mullets (*Chelon labrosus*) larger than 20-22 cm were captured by traditional rod (n=12-30) during May-June 2010 in the estuary of Deba-Mutriku, nearby the wastewater treatment plant (WWTP) of Gernika in the Biosphere Reserve of Urdaibai and in the harbors of Plentzia and Pasaia (see ***Figure 5.1***), all sites located in the Basque coast (South East Bay of Biscay, Spain). Once fished, thicklip grey mullets were immersed in a saturated solution of benzocaine and sacrificed by decapitation. Mullet processing was done according to the Bioethic Committee rules of the University of the Basque Country (UPV/EHU). Liver was dissected out, placed in sterile cryogenic

vials and kept in liquid N_2 until laboratory arrival, where it was stored at -80 °C until analysis.

Yellowfin tuna (*Thunnus albacares*) samples were captured by the Alakrana fishing boat during 2013 in the Indian Ocean. Traditional rods were used to capture the fishes. After collection, tuna samples were transported in an icebox to the laboratory. Tuna muscle tissue samples (one specimen) were homogenised using a food processor.

Before the analysis all the samples were freeze-dried and kept at 4 °C until analysis.

5.2.3 FUSLE

Under optimal conditions [25], 0.5 g of freeze-dried sample were placed together with 7 mL of an ACN: Milli-Q water (9:1) mixture in a 40 mL vessel and surrogate standards (MPFHxS, MPFOS, MPFBA, MPFHxA, MPFOA, MPFNA, MPFDA, MPFUdA, MPFDoA, Cl-PFHxPA, M8:2 diPAP, M8:2 FTUCA, M6:2 FTCA, M8:2 FTCA and M10:2 FTCA) were added (20 µL of a 0.5 ng/µL solution). The FUSLE step (Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogeniser equipped with a 3-mm titanium microtip, 20 kHz; Bandelin Electronic, Berlin, Germany) was performed for 2.5 min in duplicate, with a sonication time of 0.8 s and pulsed time off of 0.2 s and 10 % of amplitude. Extractions were carried out at 0 °C in an ice-water bath. After the extraction step, the supernatant was filtered through polyamide filters (0.45 µm, 25 mm, Macherey-Nagel, Germany) and the FUSLE extracts were evaporated to ~ 1 mL under a N_2 stream using a Turbo Vap LV Evaporator and submitted to the clean-up step.

5.2.4 Clean-up

For the optimisation of the clean-up approaches, FUSLE extracts of liver samples were spiked with PFOS, PFOA, FOSA and PFHxPA (chosen as target analytes) at a concentration level of 250 ng/mL before the clean-up step. The experiments were

performed in triplicate (n=3) and blanks were processed in parallel for signal subtraction.

5.2.4.1 Evolute-WAX

For Evolute-WAX clean-up four different protocols were tested:

(i) A first clean-up approach was performed according to the method published by Zabaleta et al. [25]. Briefly, the 200-mg Evolute-WAX cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water. After, the 1 mL extract diluted in 6 mL of Milli-Q water was loaded and, then, 1 mL of HCOOH (2 %) and 1 mL of Milli-Q water: MeOH (95: 5) mixture were added with cleaning purposes before the cartridge was dried for 1 h under vacuum. Then, the analytes were eluted using 4 mL of 2.5 % NH_4OH in acetone. After the elution, the extract was concentrated to dryness under a gentle stream of N_2 at 35 °C and reconstituted in 250 µL of LC-MS grade MeOH.

(ii) The same (i) clean-up procedure was performed using 4 mL of 2.5 % NH_4OH in MeOH as the elution solvent.

(iii) This clean-up approach was performed according to the method published by Ullah and co-authors for food samples [30]. Briefly, the 200-mg Evolute-WAX cartridges were conditioned with 3 mL of MeOH containing 0.1 % of 1-MP, 3 mL of pure MeOH and 1 mL of Milli-Q water. Once the 1 mL extract diluted in 6 mL of Milli-Q water was loaded, 2 mL of the (95:5) MeOH: MTBE mixture containing a 2 % of HCOOH followed by 1 mL pure MeOH were added with cleaning purposes and then the cartridges were dried for 1 h under vacuum. Then, the analytes were eluted using 8 mL of the (60: 40) MeOH: ACN mixture containing a 2 % of 1-MP.

(iv) Clean-up approach (i) was performed with the washing step of clean-up (iii). Briefly, the 200-mg Evolute-WAX cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water. Once the 1 mL extract diluted in 6 mL of Milli-Q water was loaded, 2 mL of the (95:5) MeOH: MTBE mixture containing a 2 % of HCOOH followed by 1 mL of pure MeOH were added with cleaning purposes and, then, the cartridges were dried for 1 h under vacuum. Finally, the analytes were eluted using 4 mL of 2.5 % NH_4OH in acetone.

In all the cases, the reconstituted extracts were filtered through a 0.2 µm polypropylene filter (13 mm, Pall, USA) before the LC-MS/MS analysis.

5.2.4.2 Envi-Carb graphitised carbon

This clean-up approach was a modification performed to the method published by Powley et al. [31]. Briefly, approximately 25 mg of Envi-Carb graphitised carbon sorbent was added to a 1.5 mL Eppendorf polypropylene tube and 50 µL of HOAc were added directly to the sorbent. The concentrated FUSLE extract was added to the Eppendorf polypropylene tube, the tube was capped, and the content was mixed using a vortex mixer. The sample was then centrifuged for 10 min at 2000 rpm in a microcentrifuge (Microlitre centrifuge, 230 V/50-60 Hz, Heraeus Instrument, Hanau, Germany). The supernatant was concentrated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 250 µL of LC-MS grade MeOH. Finally, the reconstituted extract was filtered through a 0.2 µm polypropylene filter before the LC-MS/MS analysis.

5.2.4.3 Evolute-WAX in-line coupled with Envi-Carb graphitised carbon

This clean-up approach was a modification performed to the method published by Liu et al. [12]. The extract evaporated to ~ 1 mL was diluted in 6 mL of Milli-Q water. The 200-mg Evolute-WAX cartridge was conditioned with 5 mL of MeOH and 5 mL of

Milli-Q water. After the sample was loaded, 1 mL of HCOOH (2 %) and 1 mL of the (95: 5) Milli-Q water: MeOH mixture were added with cleaning purposes and the cartridges were dried for 1 h under vacuum. Then, the WAX cartridges were coupled to the 5 mL MeOH preconditioned 250-mg Envi-Carb cartridges via adapter caps. Elution was performed using 4 mL of acetone with a 2.5 % NH_4OH and collected in a single vial. After elution, the extract was concentrated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 250 µL of LC-MS grade MeOH. Finally, the reconstituted extract was filtered through a 0.2 µm polypropylene filter before the LC-MS/MS analysis.

5.2.5 LC-MS/MS analysis

An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, autosampler and column oven coupled to an Agilent 6430 triple quadrupole (QqQ) mass spectrometer equipped with both electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) sources (Agilent Technologies, Palo Alto, CA, USA) was employed for the separation and quantification of PFCs and precursors. Two chromatographic columns were tested for analyte separation. An ultra-high performance liquid chromatographic (UHPLC) Agilent Zorbax Extend-C18 (2.1 mm, 50 mm, 1.8 µm) column (pH range 2.0-11.5) with an UHPLC Zorbax Eclipse XDB-C18 pre-colum (2.1 mm, 5 mm, 1.8 µm) and an ACE UltraCore 2.5 SuperC18 (2.1 mm x 50 mm, 2.5 µm) column (pH range 1.5-11) coupled to a pre-column filter (0.5 µm, Vici Jour). The column temperature was set at 35 °C.

Under optimised conditions [25], mobile phase A consisted of a (95:5) Milli-Q water: MeOH mixture and mobile phase B of a (95:5) MeOH: Milli-Q water mixture, both containing 2 mmol/L NH_4OAc and 5 mmol/L 1-MP. Precursors (PAPs, FTCAs, FTUCAs) and PFCs were analysed in two different runs. For PFCs, the gradient profile started with

90 % A (hold time 0.3 min) and continued with a linear change to 80 % A up to 1 min, to 50 % A up to 1.5 min and to 20 % A up to 5 min (hold time 5 min) followed with a linear change to 0 % A up to 13 min and a hold time until 16 min. Initial conditions were regained at 17 min followed by equilibration until 26 min. For precursors, the gradient profile started with 80 % A (hold time 0.3 min) and continued with a linear change to 20 % A up to 3 min and to 15 % A up to 5 min (hold time 3 min) followed with a linear change to 0 % A up to 13 min and a hold time until 17 min. Initial conditions were regained at 20 min followed by equilibration until 25 min. The flow rate was set at 0.3 mL/min and the injection volume was of 5 µL in both cases.

Quantification was performed in the selected reaction-monitoring (SRM) mode. N_2 was used as nebuliser, drying and collision gas. The instrument parameters used for PFCs in the present study are the parameters optimised elsewhere [25]. Briefly, ESI in the negative mode (NESI) was carried out using a capillary voltage of 3000 V, a drying gas flow rate of 10 L/min, a nebuliser pressure of 50 psi and a drying gas temperature of 350 °C. Moreover, NESI for precursors was carried out using a capillary voltage of 3500 V, a drying flow rate of 8 L/min, a nebuliser pressure of 50 psi and drying gas temperature of 300 °C. Detailed information of the optimised parameters (Fragmentor and Collision Energy) and monitored ion transitions for each analyte and surrogate standards are given in *Tables 5.1* and 5.*2*, respectively.

Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

Table 5.1: *Precursor and product ions (first ion was used as quantifier and the second as qualifier) at optimum fragmentor (V) and collision energy (eV), as well as the calibration ranges, the determination coefficients, the instrumental LODs and LOQs for each target analytes.*

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Calibration range (ng/mL)	Determination coefficient	LOD (ng/mL)	LOQ (ng/mL)
PFBA	213	169	60	5	0.46-1000	0.999	0.14	0.46
PFPeA	263	219	60	5	0.46-1000	0.999	0.14	0.46
PFHxA	313	269/119	60	5	0.16-1000	0.999	0.05	0.16
PFHpA	363	319/169	60	10	0.01-1000	0.999	0.004	0.01
PFOA	413	369/169	60	5	0.01-1000	0.999	0.004	0.01
PFNA	463	419/169	60	5	0.01-1000	0.999	0.004	0.01
PFDA	513	469/269	100	5	0.01-1000	0.999	0.004	0.01
PFBS	299	99/80	100	30	0.16-1000	0.999	0.05	0.16
PFHxS	399	99/80	150	20	0.16-1000	0.999	0.05	0.16
PFOS	499	99/80	150	45	0.01-1000	0.999	0.004	0.01
FOSA	498	78	220	5	0.12-1000	0.998	0.03	0.12
PFHxPA	399	79	100	10	0.40-1000	0.999	0.12	0.40
PFOPA	499	79	150	20	0.21-1000	0.998	0.06	0.21
PFDPA	599	79	100	5	1.41-1000	0.999	0.42	1.41
6:2 PAP	443	97/79	90	13	2.34-1000	0.999	0.70	2.34
8:2 PAP	543	97/79	90	21	2.13-1000	0.998	0.64	2.13
6:2 diPAP	789	97/443	120	41	0.005-1000	0.999	0.001	0.005
8:2 diPAP	989	97/543	135	41	0.02-1000	0.999	0.005	0.02
6:2 FTCA	377	293	75	9	1.93-1000	0.997	0.58	1.93
8:2 FTCA	477	393	75	9	0.90-1000	0.999	0.27	0.90
6:2 FTUCA	357	293/243	75	9	0.45-1000	0.999	0.13	0.45
8:2 FTUCA	457	393	75	9	0.22-1000	0.999	0.07	0.22
7:3 FTCA	441	337/317	75	9	0.41-1000	0.999	0.12	0.41
5:3 FTCA	341	237/217	75	9	0.41-1000	0.999	0.12	0.41

Table 5.2: Precursor and product ion at optimum fragmentor (V) and collision energy (eV) for surrogate standards, as well as which target analyte is corrected with each isotopic analogue.

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Corrected compounds
MPFBA	217	172	60	5	PFBA
MPFHxA	315	270	60	5	PFPeA, PFHxA, PFHpA
MPFOA	417	372	60	5	PFOA
MPFNA	468	423	60	5	PFNA, FOSA
MFPDA	515	470	100	5	PFDA
MPFUnDA	565	520	60	5	- [a]
MPFDoDA	615	570	100	5	- [a]
MPFHxS	403	103	150	30	PFBS, PFHxS
MPFOS	503	99	60	45	PFOS
Cl-PFHxPA	415	79	105	45	PFHxPA
M8:2 diPAP	993	97	150	41	6:2 monoPAP, 8:2 monoPAP, 6:2diPAP, 8:2 diPAP
M8:2 FTUCA	459	394	75	9	8:2 FTUCA
M6:2 FTCA	379	294	75	9	6:2 FTCA, 6:2 FTUCA, 5:3 FTCA
M8:2 FTCA	479	394	75	9	8:2 FTCA, 7:3 FTCA
M10:2 FTCA	579	494	75	9	- [a]

[a] *Surrogates not used for correction*

5.3 Results and discussion

5.3.1 Optimisation of LC-MS/MS

One of the major problems associated with trace-level analysis of PFCs is background contamination arising from the presence of a variety of fluoropolymer materials in the components of LC equipment or lab ware [17,32]. Therefore, blanks are duly needed to establish quantitation limits of perfluorinated compounds in environmental and biological matrices. In this case, avoiding the use of fluoropolymer materials in the lab during sample preparation, extraction and clean-up steps reduced procedural blank contamination. Moreover, there have been controversies about whether PFASs can adsorb in the glass surface. Although partial adsorption to glass containers of high concentrations standard solutions was reported [33], it is not expected to happen in samples with complex matrices [34]. In order to ensure that adsorption to glass surface

did not happen in the present work, the sensibility of a previously developed method [25] was checked using glass material in some of the stages of the method (extraction, sample collection vials, injection vials…) and avoiding it. No significant differences were observed and therefore glass material was used in some of the stages of the analysis protocol.

In the present study two different chromatographic columns were compared in order to ensure the best sensitivity and peak shape for PFCAs, PFSAs, FOSA, PFPAs, PAPs, FTCAs and FTUCAs. In this sense Zorbax Extend-C18 column and ACE UltraCore 2.5 SuperC18 column were tested (see some examples in ***Figure 5.2***).

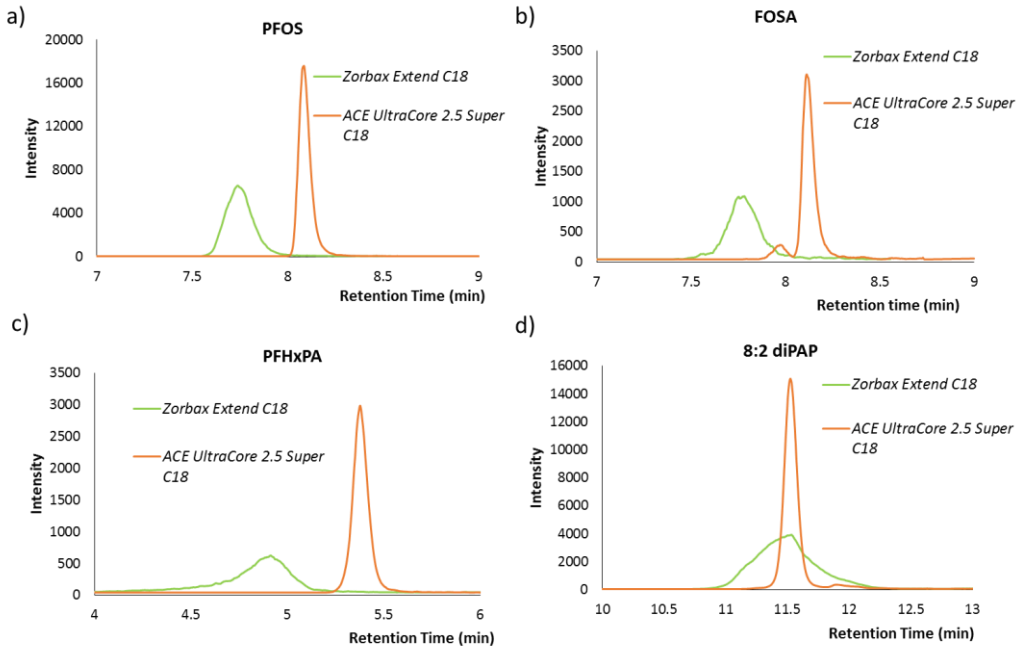

Figure 5.2. *Comparison of different chromatographic columns for (a) PFOS, (b) FOSA, (c) PFHxPA and (d) 8:2 diPAP.*

Significant improvement was observed in terms of sensitivity and peak shape for all the target analytes when ACE UltraCore 2.5 SuperC18 column was used. Therefore, the

ACE Ultracore column was further used for the analysis of the 24 PFASs analysed in the present work.

Moreover, according to the results obtained by Ullah et al. [35] and in our previous study for 14 PFC analytes [25], 1-MP can improve the chromatographic behaviour of PFCs since it generates ion-pairs that mask the negative charges of the phosponate group, leading to an increase in the retention on a C-18 stationary phase through hydrophobic interactions. In order to test the use of 1-MP in the mobile phase for the determination of PAPs, FTCAs and FTUCAs, different compositions of the mobile phase were tested. On the one hand, a mobile phase A consisting of a (95:5) water: MeOH mixture and mobile phase B consisting of a (95: 5) MeOH: water mixture with 2 mmol/L NH_4OAc and 5 mmol/L 1-MP in both A and B. On the other hand, a mobile phase A consisting of a (95:5) water: MeOH mixture and mobile phase B consisting of a (95: 5) MeOH: water mixture with 5 mmol/L NH_4OAc in both A and B. Mobile phase with 1-MP was selected since it significantly improved the chromatographic signal and peak shape for all the analytes except in the case of 6:2 FTCA (see ***Figure 5.3***). For instance, for 6:2 diPAP a ten-fold increase in the response signal was observed (see ***Figure 5.3***). Similarly, Gebbink et al. [36] added 1-MP to improve the chromatographic resolution of monoPAPs in food and packaging samples; however, this is the first time that 1-MP is used for the determination of FTCA and FTUCAs.

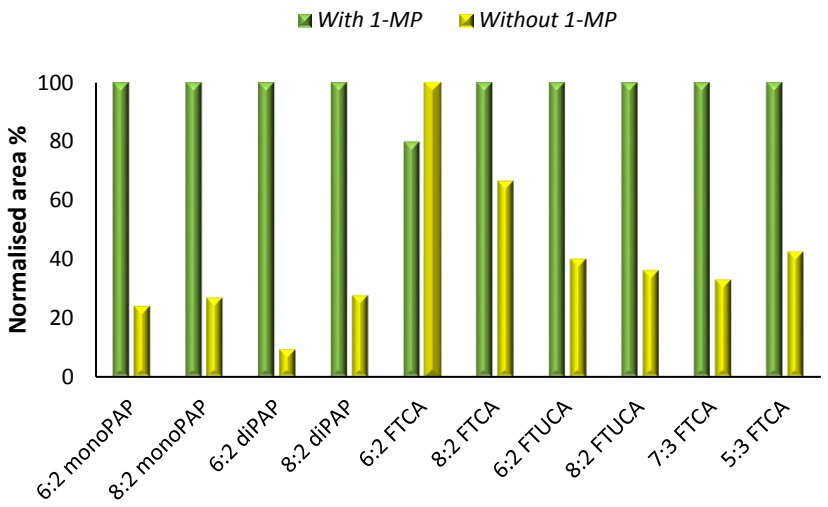

Figure 5.3. *Influence of 1-methyl piperidine (1-MP) on the chromatographic signal of PAPs, FTCAs and FTUCAs.*

In a first approach, different mobile phase gradients were tested in order to simultaneously determine the 24 target analytes but this goal could not be obtained since a poor chromatographic separation was achieved. Therefore, two different injections were performed similar to the literature [37].

Calibration curves were built with standard solutions in MeOH in the limit of quantification (LOQ)-1000 ng/mL range and at 10 concentration levels. As can be observed in ***Table 5.1***, determination coefficients in the range of 0.997-0.999 were obtained for all the target analytes without correction with the corresponding labeled standard. Instrumental limits of detection (LODs) were estimated as the lowest concentration for which the peak area was at least three times the signal to noise ratio (S/N=3). LOQs were established as the lowest concentration fulfilling all of the following criteria: (1) a linear calibration curve, (2) an acceptable peak shape, and (3) a signal-to-noise ratio of at least 10 (S/N=10). As can be observed in ***Table 5.1***, the LODs and LOQs obtained were below 0.7 and 2.3 ng/mL, respectively. LODs and LOQs were similar to

the values reported in the literature [14,38] but it should be highlighted that up to 24 target analytes were considered in the present work.

5.3.2 Sample clean-up optimisation and method validation

Different clean-up approaches using mix-mode Evolute-WAX cartridges, dispersive graphitised carbon (Envi-Carb) or an in-line combination of them were tested, while extraction conditions were maintained as described elsewhere [25].

In order to improve the efficiency of the clean-up step four different clean-up approaches were compared using a mix-mode WAX cartridge (see experimental section). The Evolute-WAX SPE cartridge was developed for sample preparation of strong acidic compounds. The mixed mode retention mechanism (both ion exchange and reverse phase) improves retention for strong acidic compounds (log pKa < 5) [12]. As shown in ***Figure 5.4***, the clean-up approach (iii) used by Ullah et al. [30], where neutral sulfonamides were not included, and the clean-up approach (iv) developed with the washing step of the same authors did not provide good recoveries for FOSA. Moreover, Ullah et al. [30] used a different mixed-mode co-polymeric sorbent (C8 + quaternary amine). On the other hand, clean-up methods (i) and (ii) provided similar recoveries. PFHxPA showed a high matrix effect in all the cases. Clean-up approach (i) was selected since it provided good recoveries for the simultaneous determination of the four families of analytes and the shortest evaporation time. It has to be mentioned that stock solutions of 2.5 % NH_4OH in acetone changed from colourless to red colour within a couple of weeks, probably due to the formation of acetone imine or other imine by-products due to the reaction between acetone and NH_4OH [39]. However, this colour change had no influence in the stability of the target analytes.

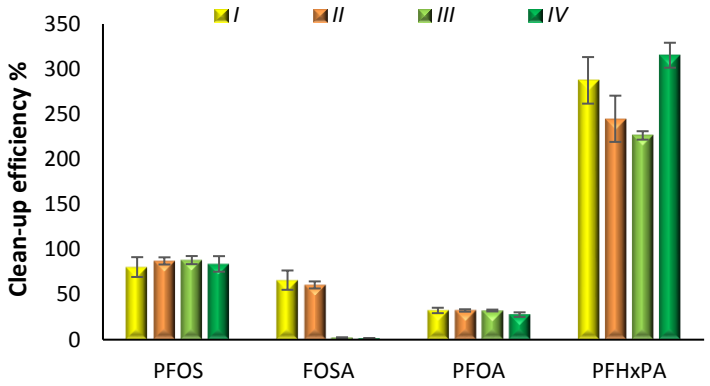

Figure 5.4. *Different clean-up approaches (see experimental section) using WAX cartridge in liver sample. FUSLE extracts of liver samples were spiked with PFOS, PFOA, FOSA and PFHxPA at a concentration level of 250 ng/mL. The experiments were performed in triplicate (n=3) and standard deviations are included.*

The retention mechanism of graphitised carbon is based on dispersive interactions with π electrons [12]. Since π electrons in PFASs are strongly associated with the highly electronegative fluorine atoms, most non-perfluorinated species with some degree of aromaticity strongly associate with the graphitised carbon, while PFASs remain unretained [32]. In order to improve the clean-up efficiency and to try a more exhaustive cleaning, different Envi-Carb sorbent amounts (25 mg and 50 mg) were tested. After FUSLE extraction, the liver extract evaporated to ~ 1 mL was added to a 1.5 mL Eppendorf polypropylene tube, which contained 25 mg or 50 mg of Envi-Carb, previously activated with 50 µL or 100 µL of acetic acid, respectively. Increasing the sorbent amount had no significant effect (F_{exp} = 1.4-11.3 < $F_{critical}$ = 18.5) according to the analysis of variance (ANOVA) in terms of extraction efficiency for all the target analytes. Moreover, neither cleaner chromatograms nor less colourful extracts were observed when increasing the amount of Envi-Carb. Therefore, 25 mg were fixed as sorbent amount for further experiments.

Once the optimal clean-up approaches using Evolute-WAX cartridges or Envi-Carb sorbent were established, they were evaluated for the 24 target analytes in terms of recovery and their cleaning ability.

Both WAX and Envi-Carb extracts showed a slight colour and no significant differences between the SCAN chromatograms were observed, but 1-2 fold higher sensitivity in terms of chromatographic response was obtained in the case of the WAX clean-up for most of the analytes.

Apparent recovery (n=3), defined as the recovery obtained after correction with the corresponding surrogate and using an external calibration approach for quantification, was calculated using liver samples spiked at 25 ng/g and 50 ng/g (see ***Figures 5.5 (a)*** and ***(b)***, respectively). However, the quantification of PFOPA and PFDPA was assessed relative to an external standard calibration due to the absence of a corresponding surrogate. Blanks were processed in parallel for signal subtraction. Acceptable apparent recoveries (78-110 % and 80-105 % for Evolute-WAX and Envi-Carb, respectively) were obtained for PFCAs, PFSAs and PFHxPA with the two clean-up approaches (see ***Figures 5.5 (a)*** and ***(b)***). However, apparent recoveries exceeded 120 % in the case of FOSA, PFOPA and PFDPA (see ***Figures 5.5 (a)*** and ***5.5 (b)*** for samples spiked at 25 ng/g and 50 ng/g, respectively) due to the matrix effect and the absence of a corresponding surrogate. Moreover, for monoPAPs and 6:2 diPAP low apparent recoveries ranging from 20-43 % (Envi-Carb) and 31-57 % (Evolute-WAX) were obtained, except for 6:2 monoPAP when Evolute-WAX was used (64-104 %). The reason for the low apparent recoveries could probably be the lack of a properly labeled standard for correction. However, 8:2 diPAP, FTCAs and FTUCAs provided good recoveries (50-114 %) using both Envi-Carb and Evolute-WAX.

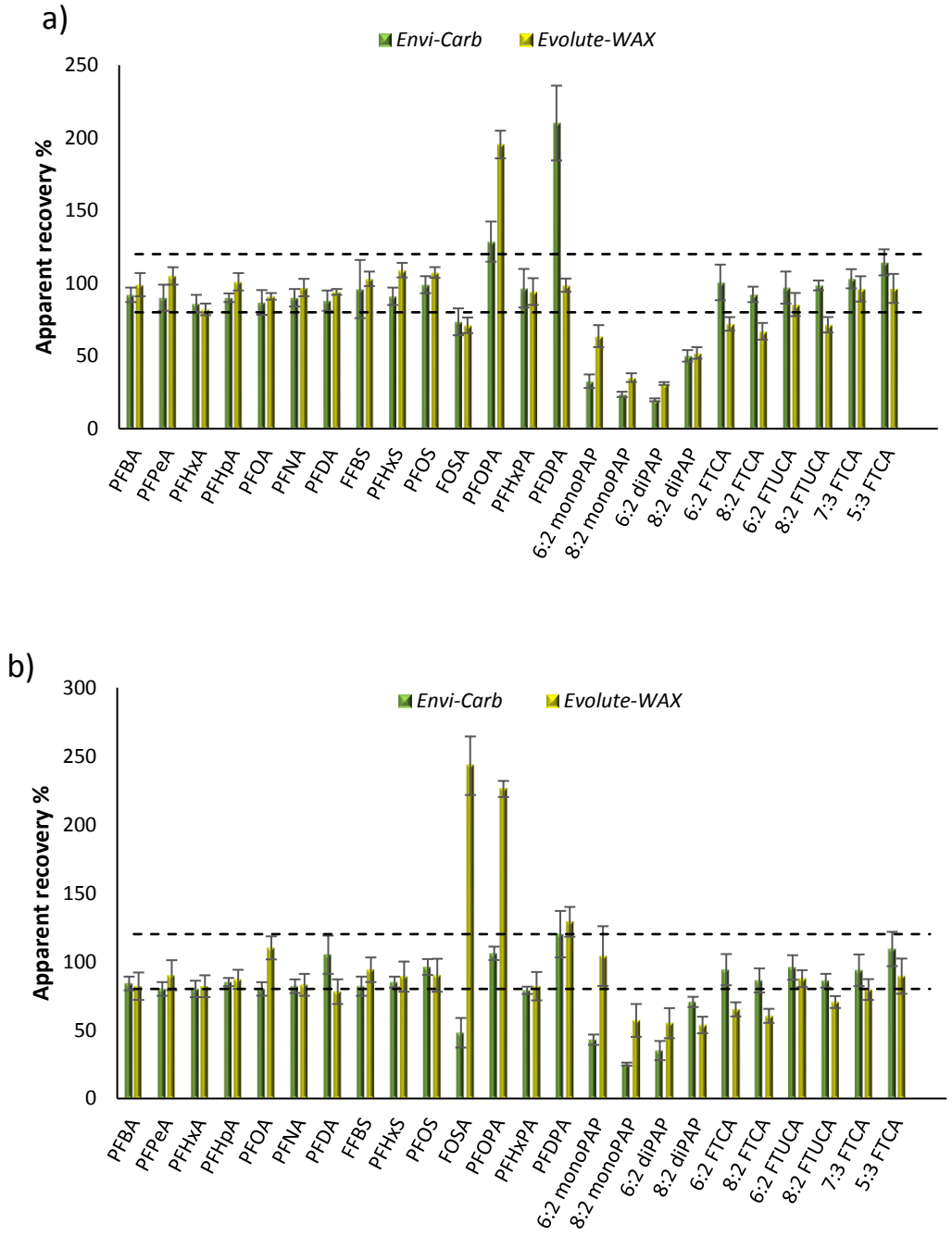

Figure 5.5: *Comparison of the apparent recoveries obtained for liver samples using Evolute-WAX and Envi-Carb clean-up approaches after correction with the corresponding surrogates and using an external calibration at different concentration levels: (a) 25 ng/g and (b) 50 ng/g.*

Although the figures of merit obtained for FUSLE coupled to both clean-up strategies were satisfactory for most analytes, a severe matrix effect was observed in the detection of FOSA, PFOPA, PFDPA and PAPs (***Figures 5.5 (a)*** and ***5.5 (b)***). Besides, the final extracts obtained were not colourless, the ESI interphase needed frequent cleaning when these two clean-up approaches were used and repeated injections often blocked the different connector tubes in the LC system. Therefore, Evolute-WAX in-line coupled with Envi Carb clean-up approach was studied. Extracts obtained when the combined clean-up was applied were colourless and in the case of FOSA matrix effect was corrected (see ***Table 5.3***). Therefore, this approach was finally selected for the clean-up of the FUSLE extracts.

Method validation was performed for liver, mussel and fish muscle tissue samples. In the case of liver samples apparent recovery was calculated at 25 ng/g (n=5) and 50 ng/g (n=4) and in the case of mussel and fish muscle tissue samples only at 25 ng/g level (n=4) (see ***Table 5.3***). Labeled standards were used for apparent recovery calculation, except for PFOPA and PFDPA whose concentration was again assessed relative to external standard calibration. Furthermore, matrix-matched calibration was also performed.

Table 5.3: *Apparent recoveries calculated with external calibration and recoveries calculated with matrix-matched calibration at, 25 ng/g and 50 ng/g, and MDL (as ng/g) for liver, mussels and muscle tissue.*

Analyte	Liver				
	Apparent recovery with external calibration 25 ng/g	Apparent recovery with external calibration 50 ng/g	Recovery with matrix-matched calibration 25 ng/g	Recovery with matrix-matched calibration 50 ng/g	MDL (ng/g)
PFBA	65	70	93	100	0.5
PFPeA	76	81	91	96	1.1
PFHxA	71	75	91	95	0.8
PFHpA	78	84	88	94	0.2
PFOA	73	92	77	95	0.1
PFNA	77	81	87	91	0.2
PFDA	72	80	89	92	0.2
PFBS	81	86	91	95	1.2
PFHxS	83	86	94	95	1.1
PFOS	99	100	90	96	0.3
FOSA	116	98	108	92	1.3
PFHxPA	81	105	72	103	0.9
PFOPA	140	216	77	111	2.2
PFDPA	129	199	68	110	1.9
6:2 monoPAP	57	51	66	75	2.7
8:2 monoPAP	37	35	69	86	1.0
6:2 diPAP	101	63	98	84	1.9
8:2 diPAP	63	60	78	85	4.1
6:2 FTCA	81	65	86	81	0.5
8:2 FTCA	50	62	86	83	1.2
6:2 FTUCA	98	84	98	92	0.9
8:2 FTUCA	57	68	100	101	1.6
7:3 FTCA	83	87	82	82	0.3
5:3 FTCA	104	106	76	89	0.9

	Mussels			Muscle tissue		
	Apparent recovery with external calibration 25 ng/g	Recovery with matrix-matched calibration 25 ng/g	MDL (ng/g)	Apparent recovery with external calibration 25 ng/g	Recovery with matrix-matched calibration 25 ng/g	MDL (ng/g)
PFBA	59	100	0.7	71	110	0.4
PFPeA	60	93	0.7	77	107	0.7
PFHxA	69	97	0.6	80	107	0.8
PFHpA	68	94	0.2	81	104	0.2
PFOA	69	95	0.1	80	100	0.2
PFNA	71	93	0.1	82	101	0.2
PFDA	65	93	0.1	76	105	0.3
PFBS	66	94	0.8	99	104	1.1
PFHxS	73	94	1.3	91	102	1.0
PFOS	98	98	0.2	102	98	0.4
FOSA	100	89	0.5	67	146	1.1
PFHxPA	72	111	0.6	134	126	0.2
PFOPA	392	80	2.4	37	116	0.5
PFDPA	456	118	3.8	45	105	0.7
6:2 monoPAP	23	112	1.7	46	106	1.2
8:2 monoPAP	12	100	0.6	50	99	1.5
6:2 diPAP	57	115	0.5	71	116	3.1
8:2 diPAP	99	111	1.3	55	108	1.4
6:2 FTCA	78	77	0.7	72	99	2.2
8:2 FTCA	110	97	0.3	65	99	2.6
6:2 FTUCA	128	108	0.4	54	83	1.6
8:2 FTUCA	113	119	0.4	63	99	2.6
7:3 FTCA	125	118	0.3	69	98	3.2
5:3 FTCA	215	115	0.7	78	107	2.3

In the case of PFCs, a correction of the matrix effect was observed for FOSA when the combined clean-up was used compared to the results obtained with the separate clean-up protocols. Recoveries obtained for PFCAs, PFSAs, PFHxPA and FOSA were in the range of 65-116 %, 59-100 % and 71- 134 % for liver, mussel and fish muscle tissue samples, respectively. Matrix-matched calibration remained necessary only for the quantification of PFOPA (37-392 %) and PFDPA (45-456 %). In the case of PAPs, FTCAs and FTUCAs, acceptable recoveries were obtained using surrogate correction in most of the matrices, except for 6:2 monoPAP and 8:2 monoPAP, which tended to show low recoveries (12-57 % and 37-57 %, respectively) probably due to the lack of the correct labeled standard for correction, as mentioned above. Satisfactory results were obtained, however, when matrix-matched calibration of PAPs, FTCAs and FTUCAs was performed. The precision of the method in terms of RSD, varied between 1-23 %, 3-17 % and 4-20 % for liver, mussel and fish muscle tissue samples, respectively.

Method detection limits (MDLs) were determined by fortification of five replicates of each blank matrix with each analyte at the lowest concentration (25 ng/g) used in the method validation, according to the USEPA (http://www.epa.gov/waterscience/methods/det/rad.pdf). The MDL was then calculated as MDL = $t_{(n-1,\ 1\ -\ \alpha\ =\ 0.95)}$ x s_d, where t = 2.13 corresponds to the Student's t-value for a 95 % confidence level and 4 degrees of freedom, whereas s_d is the standard deviation of the replicate analyses. The MDL values for liver, mussel and fish muscle tissue samples were in the range of 0.1 – 4.1 ng/g, 0.1 – 3.8 ng/g and 0.2 – 3.2 ng/g (see *Table 5.3*), respectively. PFOS MDL values were lower than the EQS value (9.1 µg/kg) established in biota under the Directive 2013/39/EU [11] for all the three matrices. Similar MDL values (0.2-1.4 ng/g) were reported by Bossi et al. when ion-pair extraction was performed for PFCAs, PFSAs and FOSA in biota samples [40]. Moreover, Liu et al. reported method quantification limit (MQL) values between 0.6-5.1 ng/g for PAPs and PFPAs when extraction with THF/HOAc and clean-up with Oasis WAX cartridge in-line

coupled with Envi-Carb was used for sewage sludge [12]. However, it has to be emphasised the number of analytes simultaneously determined in this work. To the best of our knowledge, this is the first time that up to 24 PFCs, PAPs and precursors are simultaneously determined in biota samples.

5.3.3 Application to environmental samples

Thicklip grey mullet (*Chelon labrosus*) liver samples from four sampling populations (Gernika, Pasaia, Plentzia and Deba-Mutriku) from the Basque Coast (North of Spain) were analysed (see ***Table 5.4***).

Of the 14 PFCs monitored PFOS, FOSA and PFDA were the only ones detected. PFOS was found in the 24-54 ng/g range, except in the case of Gernika sampling point, where the highest PFOS concentration was found (1062 ng/g). The high PFOS concentration obtained in Gernika could be due to the fact that fishes were collected nearby a WWTP. Similar results were obtained by Kannan et al. [41], who reported PFOS concentration ranging from 21 to 87 ng/g in livers of tuna (*Thunnus thynnus*) from the Italian Coast. Furthermore, Giesy and Kannan [19] reported that livers of Chinook salmon and lake whitefish from Michigan waters (USA) contained up to 170 and 81 ng/g of PFOS, respectively. Higher PFOS concentrations ranging from 3 to 7900 ng/g were also reported in the liver of fishes from Kin Bay (Okinawa, Japan) [21]. Moreover, Hoff et al. found 1822 and 9031 ng/g in carp (*Cyprinus carpio*) and eel (*Anguilla anguilla*) livers from Flanders (Belgium), respectively [42]. It has to be highlighted that concentrations of PFOS varied more than 100-fold, depending on the species and location.

Table 5.4: PFASs and precursors concentration ranges in fish liver, fish muscle tissue and mussel samples from various coastal locations of the world (including this study). Results are expressed in ng/g.

Location	Sampling period	Organism	Method	Concentration range (ng/g)							Reference
				PFOS	FOSA	PFDA	PFNA	8:2 monoPAP	6:2 diPAP	8:2 diPAP	
Basque Coast (Plentzia)	2010	Thicklip grey mullet liver (*Chelon labrosus*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	24-28	5.1-5.7	1.5-2.0	<MDL[a]	nd[c]	<MDL[a]	<MDL[a]	This study
Basque Coast (Gernika)	2010	Thicklip grey mullet liver (Chelon labrosus)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	918-1062	4-6	<MDL[a]	nd[c]	nd[c]	<MDL[a]	<MDL[a]	This study
Basque Coast (Pasaia)	2010	Thicklip grey mullet liver (*Chelon labrosus*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	33-37	10-15	1.5-1.7	nd[c]	nd[c]	<MDL[a]	<MDL[a]	This study
Basque Coast (Deba-Mutriku)	2010	Thicklip grey mullet liver (*Chelon labrosus*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	52-54	13-15	1.0-1.2	nd[c]	nd[c]	<MDL[a]	<MDL[a]	This study
Italian Coast (Reggio Calabria)	1999	Tuna liver (*Thunnus thynnus*)	IPE (MTBE)-LC/(-)ESI-MS/MS	21-87	<38	na[b]	na[b]	na[b]	na[b]	na[b]	[41]
USA (Michigan waters)	1999	Chinook salmon liver (*Oncorhynchus tshawytscha*)	IPE (MTBE)-LC/(-)ESI-MS/MS	33-170	-	na[b]	na[b]	na[b]	na[b]	na[b]	[19]
USA (Michigan waters)	1999	Lake whitefish liver (*Coregonus clupeaformis*)	IPE (MTBE)-LC/(-)ESI-MS/MS	33-81	-	na[b]	na[b]	na[b]	na[b]	na[b]	[19]
Swedish west coast (Fladen)	1991-2011	Herring liver (*Clupea harengus*)	Sonication (ACN)-SPE(mixed mode C8+aminopropyl)-Envi-Carb- LC/(-)ESI-MS/MS	4.3-9.3	6.5-18.2	na[b]	na[b]	na[b]	na[b]	na[b]	[23]
Japan (Okinawa)	2002	Ornate jobfish liver (*Tropidinius amoenus*)	IPE (MTBE)-LC/(-)ESI-MS/MS	593-7900	na[b]	na[b]	na[b]	na[b]	na[b]	na[b]	[21]
Belgium (Flanders)	2002	Carp liver (*Cyprinus carpio*)	IPE (MTBE)-LC/(-)ESI-MS/MS	11.3-1822	na[b]	na[b]	na[b]	na[b]	na[b]	na[b]	[42]
Belgium (Flanders)	2002	Eel liver (*Anguilla anguilla*)	IPE (MTBE)-LC/(-)ESI-MS/MS	17.3-9031	na[b]	na[b]	na[b]	na[b]	na[b]	na[b]	[42]
Spain (Vigo)	2009	Mussels (*Mytilus galloprovincialis*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	<MDL[a]	<MDL[a]	nd[c]	nd[c]	42-47	<MDL[a]	<MDL[a]	This study
Basque Coast (Pasaia)	2009	Mussels (*Mytilus galloprovincialis*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	1.9-2.4	4-5	nd[c]	nd[c]	65-86	<MDL[a]	<MDL[a]	This study

Table 5.4: Continuation.

Location	Sampling period	Organism	Method	Concentration range (ng/g)							Reference
				PFOS	FOSA	PFDA	PFNA	8:2 monoPAP	6:2 diPAP	8:2 diPAP	
Basque Coast (Muskiz)	2009	Mussels (*Mytilus galloprovincialis*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	nd[c]	3-4	nd[c]	nd[c]	31-34	<MDL[a]	<MDL[a]	This study
Basque Coast (Santurtzi)	2009	Mussels (*Mytilus galloprovincialis*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	1.5-2.0	7-8	nd[c]	nd[c]	48-53	<MDL[a]	<MDL[a]	This study
Basque Coast (Deportivo)	2009	Mussels (*Mytilus galloprovincialis*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	1.4-1.6	6-7	nd[c]	nd[c]	67.0-67.5	<MDL[a]	<MDL[a]	This study
Portugal (Vouga)	-	Mussels (*Mytilus galloprovincialis*)	IPE (MTBE)-LC/(-)ESI-MS/MS	36.8-125.9	na[b]	na[b]	na[b]	na[b]	na[b]	na[b]	[51]
Denmark	-	Mussels (*Mytilus Edulis*)	IPE (MTBE)-LC/(-)ESI-MS/MS	nd[c]	nd[c]	nd[c]	nd[c]	na[b]	na[b]	na[b]	[40]
Spain (Asturias, Cantabria and Basque Country)	2009	Mussels (*Mytilus galloprovincialis*) (caged)	Sonication (ACN)-Activated carbon UPLC/(-)ESI-MS/MS	nd[c] -0.06	na[b]	na[b]	nd[c]	na[b]	na[b]	na[b]	[48]
Mediterranean sea	-	Mussels (*Mytilus edulis*)	IPE (MTBE)-LC/(-)ESI-MS/MS	<2-3	na[b]	na[b]	na[b]	na[b]	na[b]	na[b]	[50]
Spain (Catalonia)	2006-2009	Mussels (*Dreissena polymorpha*)	Sonication (ACN)-activated carbon-LC/(-)ESI-MS/MS	nd[c]	na[b]	na[b]	nd[c]	na[b]	na[b]	na[b]	[49]
Indian Ocean	2013	Yellowfin tuna muscle (*Thunnus albacares*)	FUSLE-Evolute WAX coupled to Envi-Carb-LC/(-)ESI-MS/MS	nd[c]	nd[c]	<MDL[a]	nd[c]	nd[c]	<MDL[a]	11.4-11.5	This study
Lake Vätern (Sweden)	2001	Brown trout muscle *(Salmo trutta)*	Sonication (ACN)-graphitized carbon-LC/(-)ESI-MS/MS	1.0-6.9	0.4-0.7	0.2-0.4	0.08-0.3	na[b]	na[b]	na[b]	[52]
Guangzhou (China)	2004	Japanese mackerel muscle *(Pneumatophorus japonicas)*	IPE (MTBE)-LC/(-)ESI-MS/MS	2.18	na[b]	<LOQ[d]	<LOQ[d]	na[b]	na[b]	na[b]	[53]

[a] < MDL: concentrations below method detection limit value
[b] na: not analyzed
[c] nd: not detected
[d] < LOQ: concentrations below the limit of quantification value

In the case of FOSA concentrations ranging from 4 to 15 ng/g were obtained. Similar results were obtained by Ullah et al. [23], who reported FOSA concentrations ranging from 8.5 to 18.2 ng/g in herring liver samples (*Clupea harengus*) from the Swedish west coast between 1991 and 2011. FOSA is a possible precursor to PFOS in the environment and it seems to be transformed metabolically to PFOS. Therefore, it is not clear if these associations represent functions of metabolism or simple exposure [20].

Similar distribution pattern of PFCs in liver was obtained by Rubarth et al. [29] as average proportions of PFOS and FOSA accounted for ~ 90 % of the total PFC amount in the liver of red-throated divers. In addition, similar relative distributions were determined in previous studies for harbor seals (*Phoca* vitulina) [43], common guillemot (*Uria* aalge) [44] and glaucous gulls (*Larus* hyperboreus) [45].

Among PFCAs, PFBA, PFPeA, PFHxA, PFHpA and PFOA were not detected in any of the samples and PFNA was under MDL values in Plentzia. However, low concentrations of PFDA (1-2 ng/g) were detected.

On the other hand, studies on fluorotelomer-compound biotransformation in fish are limited; in fact, rainbow trout has been the only test species investigated so far [15]. It has been reported a rapid metabolisation of 8:2 FTCA with levels of 8:2 FTUCA, 7:3 FTCA, PFOA, PFNA and PFHpA. In this study only 6:2 diPAP and 8:2 diPAP were detected in liver samples although they were under MDL values. Moreover, none of the degradation products were detected. This may be a consequence of their short life times or their low concentrations [46,47].

In the case of mussel samples PFOS and FOSA were only detected (see ***Table 5.4***) at low concentrations (1.4-2.4 ng/g of PFOS and 3-8 ng/g of FOSA). Other studies carried out in Spain [48,49], the Mediterranean Sea [50] and Denmark [40] evidenced the low accumulation potential of such PFCs in mussels. However, Cunha et al. [51] detected

high concentrations of PFOS (125.9 ng/g) in mussels from north-central Portuguese estuaries crossing the most industrialised areas of the country. Moreover, a high concentration (31-86 ng/g) of 8:2 monoPAP was found in all the sampling points. In addition, 6:2 diPAP and 8:2 diPAP were under MDL values. To the best of our knowledge this is the first work that reports the presence of PAPs in mussels. Further research should be performed in order to understand the presence of PAPs in mussel samples, compared to the presence of PFOS in fish liver.

In the case of tuna samples (muscle tissue of the tuna was analysed) only 8:2 diPAP was detected (see *Table 5.4*). Similarly, low concentration of PFCs are reported in the literature [52,53]. Moreover, when tissue distribution of PFCs in fish has been determined, muscle is the tissue with the lowest PFC concentration [54,55]. On the other hand and according to our knowledge, this is the first time that precursors such as 8:2 diPAP are detected in fish muscle tissue samples.

5.4 Conclusions

A thorough optimisation and validation of different clean-up approaches was performed for the first time for the analysis of up to 24 PFASs, including PFCs and potential precursors in biota samples such as mussels, fish muscle tissue and fish liver. The combination of mixed mode WAX cartridges and Envi-Carb provided the cleanest extract, not only in terms of the absence of colour, but also in terms of good apparent recoveries and the prevention of frequent ESI interphase cleaning. To the best of our knowledge this is the first work where 7 families of PFCs are accomplished in three biota samples (mussels, fish muscle tissue and fish liver). When real fish liver samples of the North Coast of Spain were analysed, our study showed the evidence of widespread contamination by PFOS, FOSA and PFDA, in particular in Gernika, where samples were collected nearby a WWTP. PFC levels were in ranges similar to those reported in other European countries. Low concentrations of PFOS and FOSA were detected in the case

of mussel samples, showing a low accumulation in these species. However, high levels of 8:2 monoPAP and 8:2 diPAP were reported for the first time in mussel and fish muscle tissue samples, respectively. Further research should be performed in order to understand the presence of PAPs in mussel samples, compared to the presence of PFOS in fish liver.

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Chapter 6

Presence of fluorinated compounds in aquatic organisms of the Gulf of Biscay and the Portuguese coast

Ekaia 32 (2017) 51-65

6.1 Introduction

Last year, Greenpeace announced that considered toxic perfluorinated compounds had been detected in different well-known brand mountain clothing and materials [1]. In fact, per- and polyfluoroalkyl substances (PFASs, see some examples in ***Figure 6.1***) are widely used in our daily life due to their unique properties [2]. On the one hand, their fully (per-) or partially (poly-) fluorinated hydrophobic carbon chain confers them the ability to repeal water. On the other hand, the carbon chain is attached to one or more different hydrophilic functional groups that can be neutral, positively or negatively charged. These hydrophilic end groups provide PFASs the ability to repeal oil. Among their principal applications, they can be used as surface protectors in carpets, mountain clothing, food packaging materials or cookware. Moreover, they are also used as performance chemicals in products such as fire-fighting foams, shampoos, inks, and paints [3, 4].

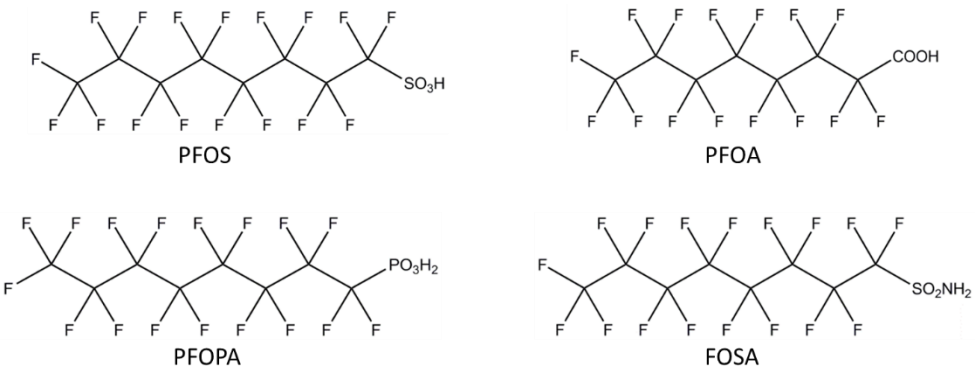

Figure 6.1: *PFASs with different hydrophilic end groups: PFOS (perfluorooctane sulfonic acid), PFOA (perfluorooctanoic acid), PFOPA (perfluorooctane phosphonic acid) and FOSA (perfluorooctane sulfonamide).*

Nowadays, an increasing concern has grown due to the toxicity of these compounds, which have been found to be endocrine disruptors and carcinogens [5–7], together with the high environmental persistence they present. In fact, the carbon-fluorine bond (one of the strongest bonds found in organic chemistry) confers them resistance towards

hydrolysis, photolysis, metabolism or biodegradation [8]. This stability and their low reactivity provide them with the ability to bioaccumulate.

In the year 2000, the largest producer of PFASs (3M Co.) announced the phase out of the production of long chain PFASs (C > 8). Since then, new shorter-chain PFASs (C_4-C_7) and their precursors have been introduced as replacements in the market since they are considered less persistent or toxic in humans [9]. In 2006, several North American PFAS manufacturers announced a voluntary stewardship program to reduce perfluorooctanoic acid (PFOA) and its precursors use by 2010 [10,11]. Moreover, in 2009, perfluorooctane sulfonic acid (PFOS) was listed as "restricted use" compound under the Stockholm Convention on persistent organic pollutants (POPs) [12]. Four years later, PFOS and its derivatives were listed as priority hazardous substances in the field of water policy under the Directive 2013/39/EU and an environmental quality standard (EQS) value (9.1 μg/kg) was established for PFOS in biota [13]. To date, PFOS and PFOA have been the mainly monitored PFASs in environmental compartments.

The above-mentioned restrictions have ended up with the use of new fluorinated compounds such as, polyfluoroalkyl phosphates (PAPs). However, since recent studies have demonstrated that PAPs could be PFOA and related PFASs potential precursors, their use has become a new source of PFASs to the environment and humans [15, 16], and therefore, these new fluorinated alternatives should also be included in monitoring studies [14].

PFASs are widely found in the environment due to anthropogenic sources. The presence of PFASs in remote locations such as the Arctic [17] or Antarctic [18] has raised the question on their transport. Two main pathways have been studied [19]. On the one hand, the first pathway involves the atmospheric transport of volatile precursors to remote areas. During atmospheric transport, the neutral precursors may be oxidised to

produce the ionic analogues. On the other hand, the second pathway involves long-range aqueous transport in their ionic form, directly by the oceanic currents, or associated to particle and/or sea-spray. In the last years, the detected PFAS levels in human serum [20–23] or human milk [24–26] has grown the social and scientific concern on them. Among the human exposure routes to PFASs, drinking water [27, 28], biota [29, 30], food packaging materials [31, 32] and air or dust [33, 34] should be highlighted.

Aquatic organisms are good bioindicators of the health of the aquatic environment where they live since they can bioaccumulate contaminants that are present in the water. Oysters, together with mussels, have been widely used in order to assess aquatic systems contamination [35]. Moreover, PFASs have been found to be biomagnified in higher trophic chain [36]. Due to PFASs properties (they are both hydrophobic and lipophobic) they do not tend to accumulate in fatty tissues, and they are mainly accumulated in protein rich tissues such as, liver, plasma or kidney. Within this context, grey mullet (*Chelon labrosus*) livers and oysters (*Ostrea edulis*) collected in the Spanish, Basque, French and Portuguese coasts were monitored in order to assess the aquatic health related to the PFAS presence.

6.2 Experimental section

In the present work the monitoring of 14 PFASs and 10 potential precursors (see *Table 6.1*) was carried out in grey mullet livers and oysters.

Table 6.1: *Acronyms, names, chemical formulas and method detection limits for the monitored PFASs.*

Acronyme	Analyte	Formula	Method detection limits (ng/g)*	
			Liver	Oyster
PFBA	Perfluorobutanoic acid	$CF_3(CF_2)_2COOH$	0.5	0.7
PFPeA	Perfluoropentanoic acid	$CF_3(CF_2)_3COOH$	1.1	0.7
PFHxA	Perfluorohexanoic acid	$CF_3(CF_2)_4COOH$	0.8	0.6
PFHpA	Perfluoroheptanoic acid	$CF_3(CF_2)_5COOH$	0.2	0.2
PFOA	Perfluorooctanoic acid	$CF_3(CF_2)_6COOH$	0.1	0.1
PFNA	Perfluorononanoic acid	$CF_3(CF_2)_7COOH$	0.2	0.1
PFDA	Perfluorodecanoic acid	$CF_3(CF_2)_8COOH$	0.2	0.1
PFBS	Perfluorobutane sulfonic acid	$CF_3(CF_2)_3SO_3H$	1.2	0.8
PFHxS	Perfluorohexane sulfonic acid	$CF_3(CF_2)_5SO_3H$	1.1	1.3
PFOS	Perfluorooctane sulfonic acid	$CF_3(CF_2)_7SO_3H$	0.3	0.2
PFHxPA	Perfluorohexane phosphonic acid	$CF_3(CF_2)_5PO_3H_2$	0.9	0.6
PFOPA	Perfluorooctane phosphonic acid	$CF_3(CF_2)_7PO_3H_2$	2.2	2.4
PFDPA	Perfluorodecane phosphonic acid	$CF_3(CF_2)_9PO_3H_2$	1.9	2.4
FOSA	Perfluorooctane sulfonamide	$CF_3(CF_2)_7SO_2NH_2$	1.3	3.8
6:2 monoPAP	1H, 1H, 2H, 2H-perfluorooctyl phosphate	$CF_3(CF_2)_5CH_2CH_2O\text{-}P(O)(OH)_2$	2.7	0.5
8:2 monoPAP	1H, 1H, 2H, 2H-perfluorodecyl phosphate	$CF_3(CF_2)_7CH_2CH_2O\text{-}P(O)(OH)_2$	1.0	1.7
6:2 diPAP	Bis (1H, 1H, 2H, 2H-perfluorooctyl) phosphate	$(CF_3(CF_2)_5CH_2CH_2O)_2\text{-}P(O)OH$	1.9	0.6
8:2 diPAP	Bis (1H, 1H, 2H, 2H-perfluorodecyl) phosphate	$(CF_3(CF_2)_7CH_2CH_2O)_2\text{-}P(O)OH$	4.1	0.5
6:2 FTCA	2-perfluorohexyl ethanoic acid	$CF_3(CF_2)_5CH_2COOH$	0.5	1.3
8:2 FTCA	2-perfluorooctyl ethanoic acid	$CF_3(CF_2)_7CH_2COOH$	1.2	0.7
6:2 FTUCA	2H-perfluoro-2-octenoic acid	$CF_3(CF_2)_4CF{=}CHCOOH$	0.9	0.3
8:2 FTUCA	2H-perfluoro-2-decenoic acid	$CF_3(CF_2)_6CF{=}CHCOOH$	1.6	0.4
5:3 FTCA	3-perfluoropentyl propanoic acid	$CF_3(CF_2)_4CH_2CH_2COOH$	0.3	0.4
7:3 FTCA	3-perfluoroheptyl propanoic acid	$CF_3(CF_2)_6CH_2CH_2COOH$	0.9	0.3

** Method detection limits were calculated in a previous work of the research group [37].*

6.2.1 Sample collection

Adult grey mullets (*Chelon labrosus*) larger than 20-22 cm were captured in different sampling points of the Basque Coast (see ***Figure 6.2***). The sampling campaigns were performed in Gernika, located at the Biosphere Reserve of Urdaibai (downstream the primary treatment wastewater treatment plant, WWTP), during spring 2007, 2009, 2010, 2012 and 2014, in the harbour of Plentzia during autumn 2009 and summer 2010,

in the harbour of Arriluze during spring and autumn 2007 and spring 2010 and in the harbour of Pasaia during autumn 2009, summer 2010 and spring 2012. Grey mullets were captured by traditional rod and processing was done according to the Bioethic Committee rules of the University of the Basque Country (UPV/EHU). Liver was dissected out, placed in sterile cryogenic vials and kept in liquid N_2 until laboratory arrival, where it was stored at -80 °C until analysis.

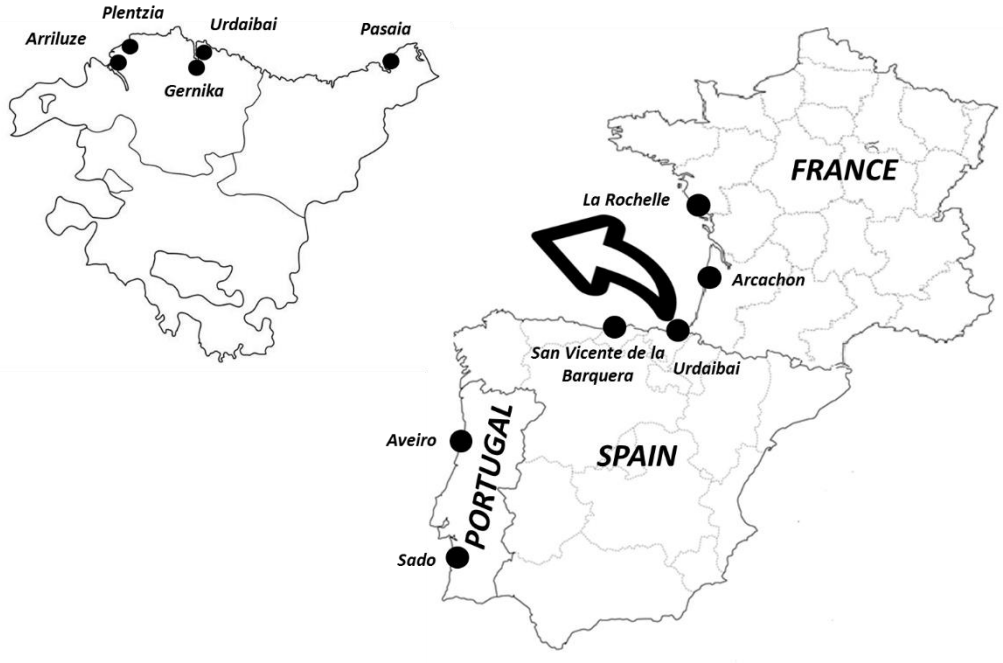

Figure 6.2: Sampling points of grey mullet liver and oysters.

Wild and cultured oysters were obtained from the French Coast (La Rochelle and Arcachon), the Basque Coast (Gernika estuary), the Spanish Coast (Ostranor, San Vicente de la Barquera) and the Portuguese Coast (Aveiro and Sado) during spring 2013.

6.2.2 Extraction and analysis

PFASs were extracted according to a previous work of the research group [37] based on focused ultrasound solid-liquid extraction (FUSLE) and a solid phase extraction (SPE) clean-up step (see ***Figure 6.3***). Briefly, 0.5 g of freeze-dried sample was placed together with 7 mL of an acetonitrile: Milli-Q water (9:1) mixture for FUSLE extraction. After the extraction step, the FUSLE extracts were evaporated to ~ 1 mL under a gentle stream of N_2 using a Turbo Vap LV Evaporator and submitted to the clean-up step. The 200 mg Evolute-WAX cartridges were conditioned with 5 mL of methanol and 5 mL of Milli-Q water. Afterwards, the 1 mL extract diluted in 6 mL of Milli-Q water was loaded and, then, 1 mL of formic acid (2 %) and 1 mL of Milli-Q water: methanol (95: 5) mixture were added with cleaning purposes before the cartridge was dried for 1 h under vacuum. Then, the analytes were eluted using 4 mL of 2.5 % ammonium hydroxide in acetone. After the elution, the extract was concentrated to dryness under a gentle stream of N_2, reconstituted in 250 µL of LC-MS grade methanol and analysed by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [37].

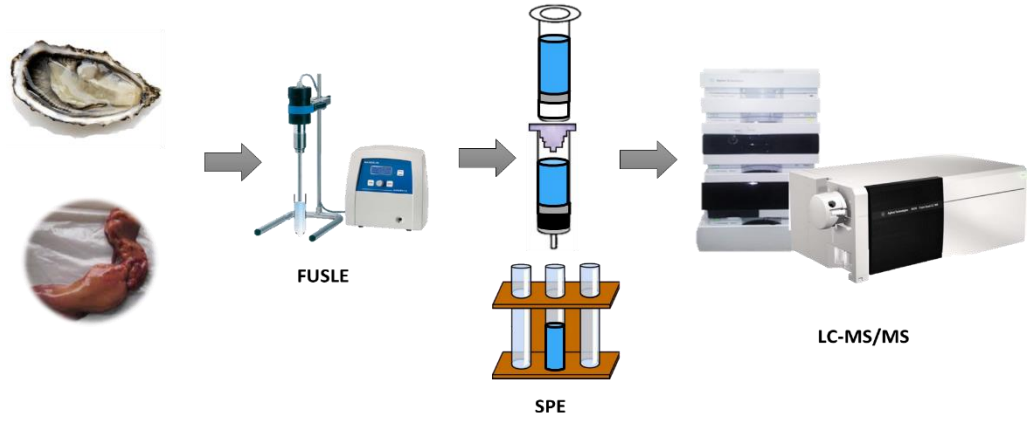

Figure 6.3: *Experimental procedure for the analysis of liver and oyster samples.*

6.3 Results and discussion

6.3.1 Grey mullet liver

In all the sampling points and among all the different sampling campaigns, PFOS was the PFAS detected at the highest concentration, followed by perfluorooctane sulfonamide (FOSA) and perfluorodecanoic acid (PFDA) (see ***Figure 6.4***). Liver collected during 2009 in the harbour of Plentzia was the only sampling point where FOSA concentrations were below the method detection limit (MDL) values (see ***Table 6.1***). Moreover, within the monitored precursors, 8:2 diPAP was detected in all the sampling points, while 6:2 diPAP was detected in Gernika and Pasaia. However, in both cases, concentrations were below the MDL values.

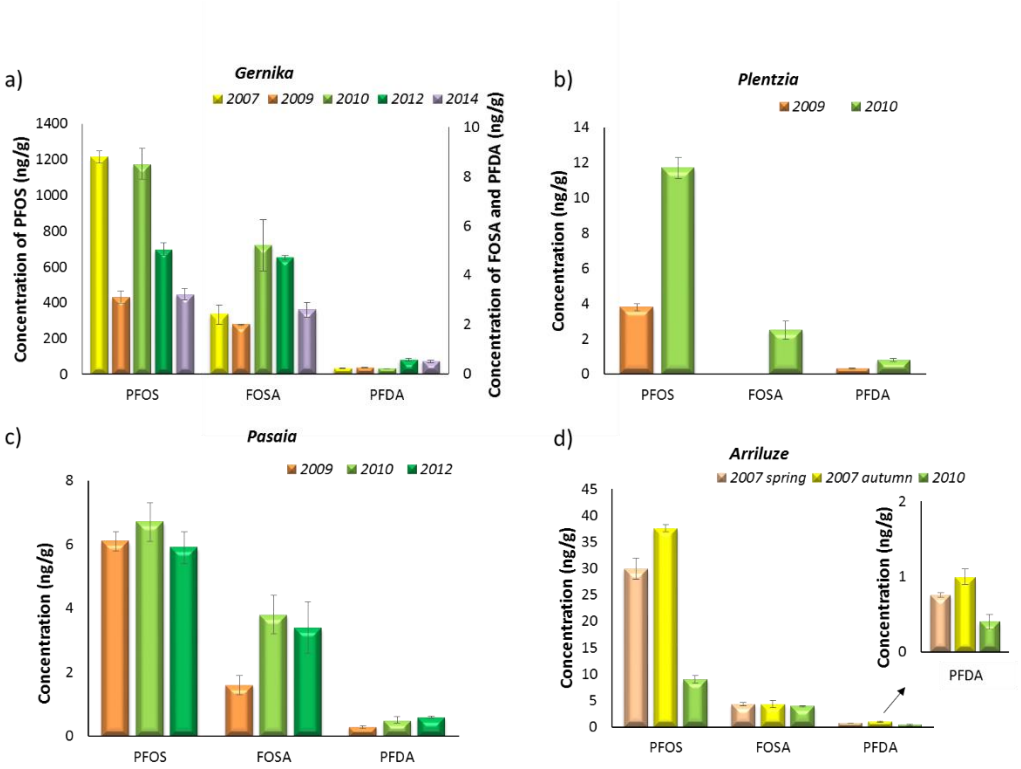

Figure 6.4*: PFAS concentrations (ng/g) quantified in grey mullet liver samples at the different sampling points: (a) Gernika, (b) Plentzia, (c) Pasaia and (d) Arriluze.*

Although FOSA (1.6-4.7 ng/g in Gernika, < MDL-2.5 ng/g in Plentzia, 1.6-3.8 ng/g in Pasaia and 3.9-4.3 ng/g in Arriluze) and PFDA (0.2-0.6 ng/g in Gernika, 0.3-0.8 ng/g in Plentzia, 0.3-0.6 ng/g in Pasaia and 0.4-1.0 ng/g in Arriluze) concentration levels were similar within the different sampling points, in the case of PFOS high differences were observed in terms of concentration. PFOS concentrations up to 1,214 ng/g were quantified in Gernika. However, this is not the first time that our research group detected high levels of contaminants at this sampling point. For instance, when grey mullet (*Chelon Labrosus*) livers of Gernika were collected for the determination of endocrine disrupting compounds, high concentrations of alkylphenols (629-679 ng/g), bisphenol-A (BPA, 97 ng/g) and phthalates (361 ng/g) were quantified [38]. Different sources can be identified as the contributors for such high PFASs levels in Gernika; on the one hand, a WWTP located nearby the sampling point discharges the treated (primary treatment) water to the estuary. On the other hand, different types of industry including, metallurgy, automotive industry and plastic industry, located upstream the sampling point, could contribute to the river contamination. Finally, it should be highlighted the presence of a fire station located nearby the sampling point, which could be an additional exposure source of PFOS to the water due to the use of this compound in fire-fighting foams. Similar or higher PFOS concentrations to the ones obtained in the present work have been reported in other countries; for instance, in Japanese fish (*Tropidinius amoenus)* livers PFOS concentrations up to 7,900 ng/g were determined [39]. The authors stated that an electric power plant and an army base were located nearby the sampling location, which could contribute to the high concentrations observed. Moreover, they reported that the use of PFOS in fire-fighting operations on army bases may provide a possible source of PFOS in fish liver [39]. Furthermore, in carp (*Cyprinus carpio*) and eel (*Anguilla anguilla*) liver collected near an industrial zone in Belgium, PFOS concentrations up to 1,822 and 9,031 ng/g were detected, respectively [40]. Finally, in Taiwan Tilapia fish PFOS concentrations up to

28,933 ng/g were reported in a river close to a WWTP which treats wastewater from an industrial area [41].

All these findings demonstrate that, although some regulations have been established with respect to the PFOS use, our aquatic organisms still present high levels of PFASs. In fact, in the case of PFOS, the EQS value established for biota (9.1 ng/g) was by far exceeded. It should be highlighted that although this value was exceeded 100 times in the case of Gernika sampling point, livers collected in Arriluze also exceeded it. The harbour of Arriluze is placed next to the marina of Getxo and in front of the commercial port of Santurtzi. Moreover, upstream of the estuary, apart from several fire stations, the WWTP of Galindo, the largest WWTP of the Basque Country and one of the largest WWTPs of Spain is located and connected with the estuary in a few meters. After a secondary treatment the WWTP discharges a flow of 289,000 m^3/day to the river Galindo. It is hard to work out the PFAS specific source since the WWTP, the fire stations and the port activity could be PFAS potential sources. Furthermore, due to the constant movement of grey mullets, the sources of PFASs can be different during their lifetime.

Plentzia and Pasaia are the sampling points where the lowest PFAS levels were detected. While in the harbour of Plentzia there is no industrial or commercial activity, in the harbour of Pasaia industrial activity (paper, metal and painting industry) are found. However, there is no evidence that these industries are major PFASs exposure sources.

Finally, a significant temporal trend was not observed during the different sampling years. In the case of Gernika, although PFOS concentration decreased from 2007 to 2009 ($p < 0.05$, according to the one-way analysis of variance, ANOVA), by the year 2010 concentrations similar to 2007 were once again regained ($p > 0.05$). Moreover, after 2010, a decrease in the concentrations of PFOS and FOSA was observed ($p < 0.05$).

However, in the rest of the sampling points, a similar trend was not observed; while in 2010 in the case of Plentzia, similar to Gernika, an increase in the target analyte concentration was detected ($p < 0.05$), in Arriluze the opposite trend was observed. Besides, in Pasaia there were no significant differences in the concentrations during the different years ($p > 0.05$) and only in the case of FOSA an increase was observed. Finally, fish collected in Arriluze during two different seasons (spring and autumn) of 2007 showed comparable levels for FOSA and PFDA ($p > 0.05$), but a small increase for PFOS was observed ($p < 0.05$). It could be concluded that, in order to study different temporal trends of PFASs, systematic monitoring campaigns are needed, which were not the aim of the present work.

6.3.2 Oysters

Wild and cultured oysters were collected from French, Spanish, Basque and Portuguese coasts. PFOS and FOSA were the only detected PFASs (see ***Table 6.2***) and they were only detected in oysters from the French Coast and the estuary of Gernika. For instance, Munschy and co-workers [42], who collected oysters (*C.gigas*) during 2010 along the three French coasts (English Channel, Mediterranean Coast and Atlantic Coast), including the sampling point of Arcachon, detected PFOS (0.03-0.1 ng/g), FOSA (0.57 ng/g) and PFDA (0.08 ng/g) along the Atlantic Coast. While in the present work higher PFOS concentrations (0.28-0.54 ng/g) were detected in the French Atlantic Coast, comparable FOSA concentrations (0.60-0.68 ng/g) were quantified. In this case, PFDA was not detected.

Table 6.2: Concentrations of detected PFASs and standard deviations in oysters (ng/g).

Sampling point	Sample	PFOS	FOSA
Arcachon	Wild oyster	0.54 ± 0.06	0.6 ± 0.1
	Cultured oyster	1.0 ± 0.2	< MDL
La Rochelle	Wild oyster	0.28 ± 0.06	0.68 ± 0.01
	Cultured oyster	1.72 ± 0.03	< MDL
Gernika estuary	Wild oyster	1.0 ± 0.2	< MDL
Ostranor	Cultured oyster	< MDL	< MDL
Aveiro	Cultured oyster	< MDL	< MDL
Sado	Wild oyster	< MDL	< MDL

< MDL: concentrations below method detection limit

When concentrations in cultured and wild oysters were compared, cultured oysters presented higher PFOS concentrations, which could be attributed to the seawater used to cultivate the oysters, since previously published works reported the presence of PFOS in seawater [43]. Moreover, FOSA was only detected in wild oysters. Due to the low concentrations detected, further hypothesis could not be withdrawn.

PFAS levels detected in oysters were lower compared with liver samples. On the one hand, as oysters and livers were not collected at the same sampling point, it is not possible to make a direct comparison between the obtained levels. However, taking into account the ability of PFASs to bind to proteins and their capacity of biomagnification in higher trophic levels [36], higher accumulation is expected for liver. From the results obtained, it could be concluded that fish liver is a good bioindicator in order to study the health of the aquatic environment in the case of PFASs pollution.

6.4 Conclusions

Aquatic organisms are excellent bioindicators of the health of the aquatic environment. In the present work, PFAS levels along different sampling points located in the French, Spanish, Basque and Portuguese coasts were reported. In the case of grey mullet livers collected in Pasaia, Gernika, Plentzia and Arriluze, PFOS, FOSA and PFDA were detected. PFOS was present at the highest concentration levels, especially in Gernika, where worrying levels were detected. The WWTP, the fire station or the

different types of industry placed upstream the sampling point could be the sources of the reported PFOS levels. In the case of the oysters, lower PFAS levels were observed, confirming the ability of these compounds to accumulate in protein rich tissues. In this case, PFOS and FOSA were the detected PFASs. It could be concluded that, in order to perform a monitoring study of this kind of compounds, fish liver seems to be an appropriate aquatic bioindicator.

6.5 References

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Chapter 7

Biotransformation of 8:2 polyfluoroalkyl phosphate diester in gilthead bream (Sparus aurata)

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7.1 Introduction

Per- and polyfluoroalkyl substances (PFASs) are a class of over 3000 chemicals with applications across a diverse range of commercial products and processes [1]. Among these substances, long chain perfluoroalkyl acids (PFAAs) have garnered the most international attention due to their ubiquitous occurrence in the global environment [2–4], including in humans [5,6] and wildlife [7,8]. Toxicological investigations involving laboratory animals (rats, mice, rabbits, fish, monkeys) have revealed significant hazards associated with long chain PFAAs [9,10], and a growing body of epidemiological data have demonstrated a link between PFAA exposure and adverse health effects in humans [11,12]. Based on the risks associated with these chemicals, the major fluorochemical manufacturer in North America (The 3M Co.) phased out perfluorooctane sulfonyl fluoride-based products in 2002 [13], and several major North American PFAS manufacturers entered in 2006 into a voluntary stewardship agreement to phase out the use and production of long-chain PFAAs by 2015 [14,15]. Three years later, perfluorooctane sulfonate (PFOS) was added to the United Nations Stockholm Convention on persistent organic pollutants (POPs) [16].

Polyfluoroalkyl phosphates (PAPs) are a sub-class of PFAS which are used as oil- and water-repellent coating agents for food-contact paper and board [17,18] as well surfactants in personal care and cosmetic products [19]. Phosphate-based fluorosurfactants were first introduced in 1974 with the perfluorooctane sulfonamido ethanol-based phosphate esters (SAmPAPs) [20]. SAmPAPs were phased out with other perfluorooctane sulfonyl fluoride-based substances by the 3M Co. in 2002, at which time telomer-manufactured PAPs became the predominant food contact fluorosurfactant. Recently, the European food packaging and paper industry has shifted to polymeric-based formulations [21], yet recent studies continue to detect PAPs and PFAAs not only in food packaging [22,23], but also food [24], house dust [25,26], and

human biofluids [27-28]. Moreover, recent studies have reported on the occurrence of PAPs in fish [29,30].

PAPs have been shown to transform to perfluoroalkyl carboxylic acids (PFCAs) in microbial systems [31–35] and in rats [36–38]. Surprisingly, no studies have investigated the fate and behaviour of diPAPs in fish, despite considerable work involving fluorotelomer alcohols [39,40] and fluorotelomer saturated and unsaturated acids (FTCAs and FTUCAs) [41], which are potential intermediates in the transformation of PAPs to PFCAs. Given the ongoing increase of long-chain (i.e. C_8-C_{13}) PFCAs in fish and other wildlife in some parts of the world [42,43], there is an urgent need to characterise potential routes of exposure. Moreover, some PFAA-precursors and reactive intermediates have been shown to be more toxic than PFAAs themselves [44,45], highlighting the importance of determining whether exposure is directly to PFAAs or via precursors [46].

Exposure of fish to PAPs via the diet could be an important route of exposure considering their historical usage, which in addition to food packaging, includes personal care and cosmetic products [47] and surface protection products (as surface tension lowering, wetting, and leveling surfactants) [48]. These materials have been identified among the numerous substances which contribute to microplastic contamination in the environment [49], and which have also been identified in the gut contents of fish [50,51]. Within this context, the aim of the present work was to perform the first dietary fish exposure involving 8:2 diPAP. Our objectives were to identify a) in which tissues 8:2 diPAP accumulates; and b) what transformation products are formed.

7.2 Experimental section

7.2.1 Standards and reagents

Solid 8:2 diPAP was obtained from Wellington Labs (Guelph, ON, Canada) and had a reported chemical purity of > 98 %. No residual traces of monoPAP, triPAP or PFAAs were present in the 8:2 diPAP standard. Native and isotopically-labeled PFASs quantified in the present study are provided in *Tables 7.1* and *7.2*, respectively.

A mixture containing 5 µg/mL of perfluoro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA) and individual standards containing 50 µg/mL of sodium 1H, 1H, 2H, 2H-perfluorodecyl phosphate (8:2 monoPAP), 2-perfluorooctyl ethanoic acid (8:2 FTCA), 2H-perfluoro-2-decenoic acid (8:2 FTUCA) and 3-perfluoroheptyl propanoic acid (7:3 FTCA) were purchased from Wellington Laboratories (Ontario, Canada). A surrogate mixture of perfluoro-n-[$^{13}C_4$] butanoic acid (MPFBA), perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid (MPFHxA), perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid (MPFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$] nonanoic acid (MPFNA) and perfluoro-n-[1,2-$^{13}C_2$] decanoic acid (MPFDA), and a surrogate mixture of 2-perfluorohexyl-[1,2-$^{13}C_2$]-ethanoic acid (M6:2 FTCA), 2-perfluorooctyl-[1,2-$^{13}C_2$]-ethanoic acid (M8:2 FTCA) and 2-perfluorodecyl-[1,2-$^{13}C_2$]-ethanoic acid (M10:2 FTCA) were obtained at 2 mg/L, while sodium bis (1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl) phosphate (M8:2diPAP), sodium 1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl phosphate (M8:2PAP) and 2H-perfluoro-[1,2-$^{13}C_2$]-2-decenoic acid (M8:2 FTUCA) were obtained individually at 50 mg/L. The purity of all the target analytes was > 98 %.

Table 7.1: *Target analyte structures and precursor and product ions (first ion was used as quantifier and the second as qualifier) at optimum fragmentor (V) and collision energy (eV) values.*

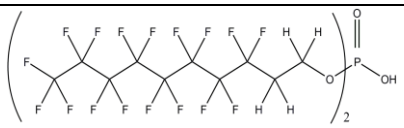
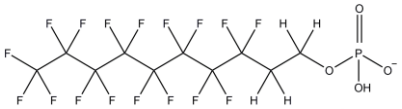
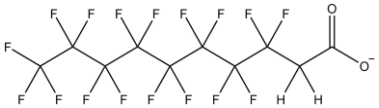
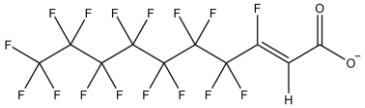
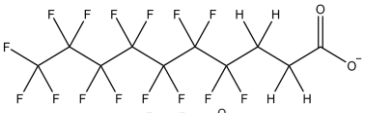
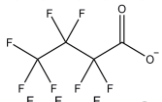
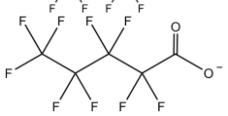
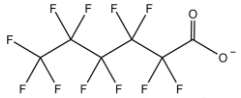
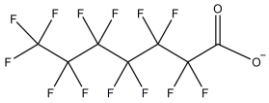
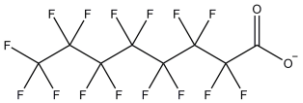
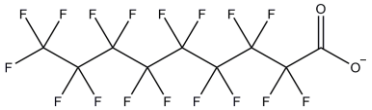
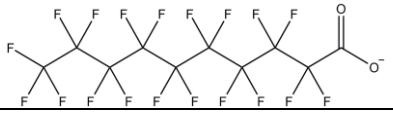
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
8:2 diPAP		989	97/543	16	24 (543), 42 (97)
8:2 monoPAP		543	523/97	16	12 (523), 20 (97)
8:2 FTCA		477	393	35	10
8:2 FTUCA		457	393	35	10
7:3 FTCA		441	337/317	20	10 (337), 11(317)
PFBA		213	169	20	10
PFPeA		263	219/169	20	10 (218), 15 (169)
PFHxA		313	269/119	20	10 (269), 15 (119)
PFHpA		363	319/169	20	11 (319), 17 (169)
PFOA		413	369/169	22	11 (369), 19 (169)
PFNA		463	419/169	24	11 (419), 15 (169)
PFDA		513	469/269	26	11 (469), 18 (269)

Table 7.2: *Precursor and product ions at optimum fragmentor (V) and collision energy (eV) for surrogate standards, as well as which target analyte is corrected with each isotopic analogue.*

Surrogate	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (eV)	Target analytes
MPFBA	217	172	20	10	PFBA
MPFHxA	315	270	20	10	PFPeA, PFHxA, PFHpA
MPFOA	417	372	22	11	PFOA
MPFNA	468	423	24	11	PFNA
MPFDA	513	470	26	11	PFDA
M8:2 diPAP	993	97	16	42	8:2 diPAP
M8:2PAP	545	97	16	20	8:2 monoPAP
M8:2 FTUCA	459	394	35	10	8:2 FTUCA
M8:2 FTCA	479	394	35	10	8:2 FTCA, 7:3 FTCA

In the case of the fish solid tissues, a Cryodos-50 laboratory freeze-dryer from Telstar Instrument (Sant Cugat del Valles, Barcelona, Spain) was used to freeze-dry the samples. For focused ultrasound solid-liquid extraction (FUSLE), a Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogeniser (20 kHz; Bandelin Electronic, Berlin, Germany) equipped with a 3-mm titanium microtip was used and in the case of liquid samples, an US bath (Axtor by Lovango) was used. Fractions were evaporated in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) using a gentle N_2 (> 99.999 % from Messer) blow-down. After the extraction step, the supernatant was filtered through polyamide filters (0.45 µm, 25 mm, Macherey-Nagel, Germany). For clean-up purposes, bulk Superclean Envi-Carb sorbent (100 m^2/g, 120/400 mesh) purchased from Supelco (Bellefonte, PA) and a microcentrifuge (Microlitre centrifuge, 230 V/50-60 Hz, Heraeus Instrument, Hanau, Germany) were used. Methanol (MeOH, HPLC grade, 99.9 %), ethyl acetate (EtOAc, HPLC grade, > 99.7 %), acetonitrile (ACN, HPLC grade, 99.9 %) and ethyl 3-aminobenzoate methanesulfonate (tricaine, < 98 %) were supplied by Sigma Aldrich (Steinheim, Germany), acetic acid (HOAc, glacial, 100 %) and sodium hydroxide pellets (NaOH, ≥ 99 %) by Merck (Darmstadt, Germany), ethanol (EtOH, 99 %) by Enma (Bilbao, Spain) and ethylenediaminetetraacetic acid (EDTA, ≥ 99 %) and sodium hydrogen carbonate ($NaHCO_3$, ≥ 99.9 %) by Panreac (Barcelona, Spain).

Ultra-pure water was obtained using a Milli-Q water purification system (< 0.05 µS/cm, Milli-Q model 185, Millipore, Bedford, MA, USA). Polypropylene microfilters (0.2 µm, 13 mm, Pall, USA) were used to filter extracts before LC-QqQ-MS/MS analysis. For water samples, Oasis-HLB (poly(divinylbenzene-co-*N*-vinylpirrilidone polymer, 200 mg) SPE cartridges were purchased from Waters (Milford, USA).

For the mobile phase composition, LiChrosolv® MeOH, ammonium acetate (NH_4OAc, 98 %) and 1-methyl piperidine (1-MP, purity > 98 %) were provided by Merck (Schuchardt OHG, Germany) and Chromasolv® ACN, was provided by Sigma-Aldrich (Steinheim, Germany).

7.2.2 Food fortification and measurement

Commercial fish feed was fortified with a solution of 8:2 diPAP in EtOH. The resulting slurry was stirred for 24 h, placed under a fume hood to allow the solvent to evaporate, and then aged for one week prior to use. The final concentration of 8:2 diPAP in the feed was 29 ± 5 µg/g, based on measurements in the feed before and after the exposure experiment using the method published by Zabaleta et al. [30]. The feed used in the control tank was prepared in the same manner, but without addition of the target compound.

7.2.3 Fish exposure and sampling

Juvenile gilt-head bream (approximately 30 g each) were purchased from Groupe Aqualande (Roquefort, France) and were allowed to acclimate for three weeks prior to exposure experiments. Fish were kept in the Aquatic Facility at the Plentzia Marine Station (PIE) in 250 L tanks under a flow-through system using seawater from Plentzia. A total of 35 fish were used in the exposed tank and another 35 in the control tank (70 in total). The water temperature was maintained at 13.5 ^{o}C and the photoperiod was set to a 14 h light /10 h dark cycle. All fish were fed once per day with a quantity of feed

equivalent to 1.5 % of the average body weight, which was maintained throughout the experiment. Feed pellets were slowly sprinkled into the tank and were consumed voraciously within a few seconds of offering. The total volume of water in the tank (~5,000 L) was renewed daily, and water samples were collected during days 2, 4 and 7. Ten fish were randomly collected before feeding from both the exposure- and control-groups after 2, 4 and 7 days. Animal handling was carried out according to the Bioethics Committee rules of the University of the Basque Country (UPV/EHU)**.** Fish were anesthetised in a tank containing 10 L of seawater with 200 mg/L tricaine and 200 mg/L $NaHCO_3$. The blood was immediately drawn using previously pre-treated syringes (0.5 M EDTA adjusted to pH=8 using NaOH), centrifuged and plasma was collected and stored at -80 ^{o}C until analysis. An incision was made along the ventral surface from the anus to the gills, and liver, bile, muscle, brain and gills were removed. Liver somatic index (LSI), which is a measure of the liver mass relative to the whole body (liver weight x 100/fish weight), as well as the condition factor (K= fish weight x 100/length) were determined, after which all tissues were stored at -80 ^{o}C prior to analysis.

7.2.4 Extraction procedure

7.2.4.1 Fish tissues

Extraction of PFASs from fish tissue was based on a method previously developed by our group [30], with minor modifications. Briefly, freeze-dried liver and muscle (~ 0.5 g each), brain and gills (~ 0.1 g each) were combined with 7 mL of a mixture of 9:1 ACN: Milli-Q water in a 40 mL vessel. FUSLE was performed for 2.5 min in duplicate, with a sonication on/off time of 0.8 / 0.2 s and 10 % of amplitude. Extractions were performed in an ice-water bath. After the extraction step, the supernatant was filtered and evaporated to ~1 mL under a N_2 stream using a Turbo Vap LV evaporator prior to clean-up. Approximately 25 mg of Envi-Carb graphitised carbon sorbent was added to a 1.5 mL

Eppendorf polypropylene tube and 50 µL of HOAc were added directly to the sorbent. The concentrated FUSLE extract was added to the Eppendorf polypropylene tube, the tube was capped, and the content was mixed using a vortex mixer. The sample extract was then centrifuged for 10 min at 2000 rpm in a microcentrifuge. The supernatant was concentrated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 250 µL of LC-MS grade MeOH. Finally, the reconstituted extract was filtered prior to LC-MS/MS analysis.

7.2.4.2 Biofluids

For fish biofluids, extraction was performed according to a previously described method [52] with minor modifications. Briefly, plasma (~ 500 µL) or bile (~ 100 µL) was combined with 7 mL of EtOAc in a 40 mL vessel and then sonicated for 15 min. After centrifugation for 10 min at 2000 rpm, the supernatant was transferred into another PP tube and the extract was concentrated and analysed following the same procedure used in the case of fish samples.

7.2.4.3 Seawater

Seawater extraction was carried out by solid phase extraction (SPE) following a slightly modified protocol reported previously [53]. Briefly, 200 mg Oasis-HLB cartridges were conditioned with 5 mL of MeOH and 5 mL of Milli-Q water, after which 100 mL of seawater spiked with 20 µL of IS (0.3 ng/µL) was added to the cartridge. The cartridge was subsequently rinsed with 5 mL of Milli-Q water (90:10) and then dried for 30 min under vacuum. Finally, target analytes were eluted using 10 mL of MeOH. After elution, the extract was concentrated, filtered and subjected to instrumental analysis.

7.2.5 LC-QqQ-MS/MS analysis

Instrumental analysis was carried out on an Acquity Ultra Performance Liquid Chromatograph (UPLC) coupled to a Xevo TQ-S triple quadrupole mass spectrometer

(Waters) operated in negative ion electrospray ionisation (ESI), selected reaction-monitoring (SRM) mode. The UPLC was equipped with a trapping column (Zorbax Extend C18 50 mm x 2.1 mm, 3.5 µm; Agilent Technologies, Santa Clara, CA, USA) installed between the eluent mixer and the injector to trap PFAS contamination from the pumps. Target analytes were chromatographed on a BEH C18 analytical column (2.1 mm × 50 mm, 1.7 µm; Waters) which was maintained at 40 °C. Mobile phases consisted of (A) 95 % water and 5 % MeOH and (B) 75 % MeOH, 20 % ACN, and 5 % water. Both mobile phases contained 2 mM NH_4OAc and 5 mM 1-MP. PFAAs-precursors and PFAAs were analysed in two different runs. For PFAAs, the gradient profile started at 90 % A (hold time 0.5 min) followed by a linear decrease to 20 % A by 5 min, to 0 % A by 5.1 min, and then held until 8 min. The gradient was returned to initial conditions by 10 min. For PFAAs-precursors, the gradient profile started at 80 % A, followed by a linear decrease to 0 % A by 4 min. The column was held for 2 min and then returned to initial conditions by 7.5 min, followed by 1.5 min of equilibration. The flow rate was set at 0.4 mL/min for PFAAs and 0.3 mL/min for PFAAs-precursors, with an injection volume of 5 µL in both cases.

The mass spectrometer was operated using a capillary voltage of 2.0 kV, source temperature of 100 °C and desolvation temperature of 400 °C. The desolvation and cone gas flow (nitrogen) were set at 600 and 150 L/h, respectively. Detailed information on the optimised parameters (fragmentor and collision energy) and monitored ion transitions for each analyte are given in ***Tables 7.1*** and ***7.2***. Instrumental operation, data acquisition and peak integration were performed with the MassLynx Software (Version V 4.1, Waters).

7.2.6 LC-q-Orbitrap analysis

Owing to a lack of authentic phase 2 metabolite standards, suspect screening was carried out using a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo

Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer equipped with a heated ESI source (Thermo, CA, USA). The column and mobile phase were identical to that reported above for LC-QqQ-MS/MS, while the LC gradient was identical to that described previously for PFAA-precursors. The flow rate was set to 0.3 mL/min at a column temperature of 40 °C. The injection volume was 5 μL and the autosampler was maintained at 5 °C. The Orbitrap was operated in full scan-data dependent MS2 (Full MS-ddMS2) acquisition mode. One full scan at a resolution of 120,000 full width at half maximum (FWHM) at m/z 200 over a scan range of m/z 100-1000 was followed by one ddMS 2 scans at a resolution of 30,000 FWHM at m/z 200, with an isolation window of 0.4 Da. The ddMS2 scans were acquired on an inclusion list of 20 ions in negative mode. If no ions from the inclusion list were detected in the previous full scan, the most intense ions from the full scan were chosen for fragmentation. The ddMS2 scans were run with an intensity threshold of $1.3x10^3$, a dynamic exclusion of 10 s and an apex trigger between 1 and 10 s. The HESI source parameters were set to 3.7 kV spray voltage, 350 °C capillary temperature, 45 arbitrary units (au) sheath gas (nitrogen), 5 au AUX gas and 350 °C AUX gas heater. External calibration of the instrument was conducted immediately prior to analysis using Pierce LTQ ESI Calibration Solutions (Thermo Scientific, Waltham, Massachusetts, United States). The instrument was controlled by Xcalibur 3.1 (Thermo) software.

7.2.7 Analyte quantification and quality control

Quantification of target analytes in fish biofluids and tissues was achieved using a matrix-matched calibration, while seawater samples were quantified using an isotope dilution/internal standard approach. Method accuracy and precision were evaluated through replicate (n = 5) spike/recovery experiments performed at 25 ng/g (tissues), 25 ng/mL (biofluids) and 125 ng/L (water). Blank contamination was monitored through the inclusion of blanks (n=3) in every batch. Instrumental limits of detection (LOD) and

quantification (LOQ) were estimated as the concentration producing a signal-to-noise ratio of 3 and 10, respectively (see ***Table 7.3***).

Table 7.3: *Apparent recoveries (%) and standard deviation (SD, n=5) for the target analytes in seawater, solid tissues and biofluids, as well as, instrumental LOD (ng/L) and LOQ (ng/L) values.*

Analyte	Seawater (%) ± SD	Solid tissues (%) ± SD	Fluids (%) ± SD	LOD (ng/L)	LOQ (ng/L)
PFBA	91 ± 1	116 ± 10	106 ± 8	50	166
PFPeA	86 ± 4	110 ± 9	101 ± 4	6	19
PFHxA	92 ± 4	104 ± 7	102 ± 7	18	60
PFHpA	90 ± 5	101 ± 10	99 ± 2	7	24
PFOA	95 ± 2	91 ± 7	97 ± 6	9	31
PFNA	90 ± 4	97 ± 7	101 ± 5	8	27
PFDA	90 ± 3	96 ± 8	98 ± 5	3	10
8:2 monoPAP	80 ± 16	108 ± 9	113 ± 11	27	89
8:2 diPAP	55 ± 4	79 ± 3	93 ± 6	7	24
8:2 FTCA	100 ± 13	97 ± 9	97 ± 9	12	41
8:2 FTUCA	75 ± 17	90 ± 8	91 ± 6	6	22
7:3 FTCA	100 ± 17	103 ± 11	90 ± 8	4	13

7.3 Results and discussion

7.3.1 Quality control

Replicate spike/recovery experiments revealed acceptable method accuracy and precision, with percent recoveries ranging from 79-116 % for solid tissues, 90-113 % for biofluids, 55-100 % for seawater (see ***Table 7.3***). Moreover, in the case of feed recoveries between 98 and 110 % were achieved. PFASs were not detectable in procedural blanks or feed (with the exception of 8:2 diPAP in the dosed feed only).

8:2 diPAP and its potential transformation products were not detected in control tank fish, with the exception of PFOA, which was present at concentrations up to 140 g/mL, 0.21 ng/g and 1.8 ng/g in bile, brain and liver, respectively (detected PFOA concentrations in plasma were below LOQ levels). In the case of bile and brain, these concentrations were negligible compared to PFOA measured in exposed animals; however, in the case of liver, concentrations were significant (up to 100 % of those observed in dosed animals in the last two sampling days). This was surprising

considering PFOA concentrations were below LOD in water. Fortunately, PFOA liver concentrations in control animals were consistent throughout the experiment and were subsequently subtracted from concentrations observed in exposed fish. All liver PFOA data reported herein were control-corrected. Moreover, statistical analysis was also performed in order to ensure significant differences between exposed and non-exposed fish PFOA levels. Sample chromatograms are provided in ***Figure 7.1***.

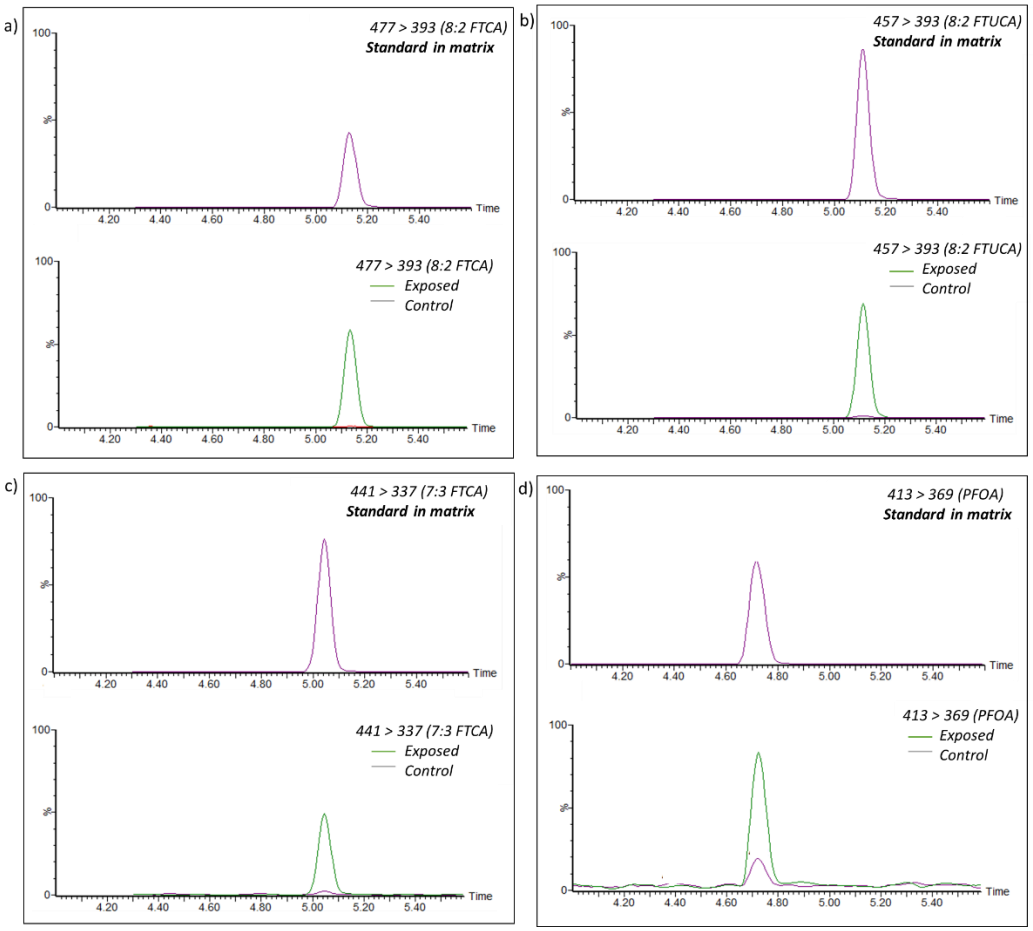


Figure 7.1: *Example chromatograms obtained in plasma on day 7 for (a) 8:2 FTCA, (b) 8:2 FTUCA, (c) 7:3 FTCA and (d) PFOA.*

7.3.2 Fish mortality and morphology

No mortality occurred in either exposed or control tanks. LSI, which is used as a marker of metabolic stress and was monitored throughout the experiment, was not significantly different between exposed and control fish (p = 0.12, one-way analysis of variance, ANOVA). The K factor was also not significantly different (p = 0.2) between exposed and control fish. Collectively, these data indicate that the health of the fish was maintained throughout the course of the experiment.

7.3.3 Water concentrations

8:2 diPAP ranged from 135 to 236 ng/L in water from the exposed tank, and was not detectable in water from the control tank over the course of the experiment. No degradation products were observed. The presence of 8:2 diPAP in the water from the exposed tank could be due to partitioning of the target compound from feed or feces to the water column. Depuration of 8:2 diPAP through the gills may also contribute to the occurrence of this analyte in water, as was previously observed following exposure of rainbow trout to perfluorophosphonates (PFPAs) and perfluorophosphinates (PFPiAs) [54]. To shed further light on the source of 8:2 diPAP in the water, 2 g of spiked feed were placed in 200 mL of Milli-Q water for one minute. Fish feed was unlikely to have exceeded this amount of time in the water during the dosing experiment due to rapid consumption by the fish. A negligible transfer of 8:2 diPAP (0.8 %) was observed from feed to water, which was not surprising considering the hydrophobicity of 8:2 diPAP (log K_{ow} = 10.93, [55]). Taking into account that the feed was consumed within a few seconds after offering, the high water turnover in the tanks (~5,000 L per day) and poor transfer of 8:2 diPAP from feed to water, the presence of 8:2 diPAP in the water is more likely to be from feces or gill depuration rather than the feed. However, as feces were not collected in the present work, this hypothesis could not be tested.

7.3.4 8:2 diPAP tissue distribution

The highest concentrations of 8:2 diPAP were observed in liver (up to 160 ng/g) followed by plasma (up to 94 ng/g) and gills (up to 119 ng/g) (see ***Table 7.4***).

Table 7.4*: Concentrations (ng/g) ± standard deviations of 8:2 diPAP in the different fish tissues and biofluids.*

Day	Plasma	Gills	Brain	Muscle	Bile	Liver
2	13 ± 1	43 ± 6	28 ± 6	19 ± 2	21.1 ± 0.3	54 ± 8
4	37 ± 3	62 ± 7	15 ± 3	4 ± 1	5.7 ± 0.5	57 ± 29
7	94 ± 3	119 ± 8	35 ± 7	19 ± 3	8 ± 2	160 ± 37

In previous experiments involving rainbow trout exposed to PFPiAs via the diet (PFPiAs are structurally similar to diPAPs in that they both have two perfluoroalkyl chains and a phosphorus containing acidic group), preferential partitioning was observed to blood and liver [54]. In that work, a liver-to-blood ratio (LBRs) of 3.24 ± 0.98 was reported for C8/C8 PFPiA by the end of the exposure period (day 31), suggesting the tendency of this substance to predominate in protein-rich compartments such as liver. Our results for 8:2 diPAP are in good accordance with this finding, with LBRs in the range of 1.5-4.2.

While 8:2 diPAP concentrations in gills and plasma increased gradually throughout the experiment, concentrations in liver were relatively steady (54-57 ng/g) during the first 4 days of exposure and then increased approximately 3-fold to 160 ng/g on day 7. Clearly, steady-state was unlikely to have been reached by day 7. Previous experiments involving the structurally-similar C8/C8 PFPiA in whole-body rainbow trout homogenate [54] did not attain steady state after 31 days. In that work, it was estimated that 115 days would be needed to achieve 90 % steady state for C8/C8 PFPiA. Thus, it is reasonable to assume that a longer exposure period is necessary to reach steady state for 8:2 diPAP.

Bile displayed low levels of 8:2 diPAP, so this route is unlikely to be the major route of excretion. Moreover, although increasing 8:2 diPAP levels were expected until reaching the steady state, bile concentration decreased over the course of the experiment. Finally, low accumulation was observed for muscle and brain during the exposure period. Several studies reported the ability of certain PFASs, including PFOA, to cross the blood-brain barrier (BBB). They suggested that PFASs are mainly bound to blood-proteins and the crossing of the BBB resembles the transport of free fatty acids [56,57]. While PFASs have recently been reported in fish brain [58,59], to the best of our knowledge, 8:2 diPAP has not been observed to date in this tissue.

This is the first work investigating 8:2 diPAP exposure in fish and is consistent with a previous 8:2 diPAP exposure study (oral gavage) in rodents that reported high concentrations of 8:2 diPAP in liver and blood [38].

7.3.5 Intermediate and terminal metabolites

The expected 8:2 FTOH precursors metabolic pathway in fish was proposed by Butt et al. [38,41] based on different biodegradation studies of different species (see *Figure 7.2*).

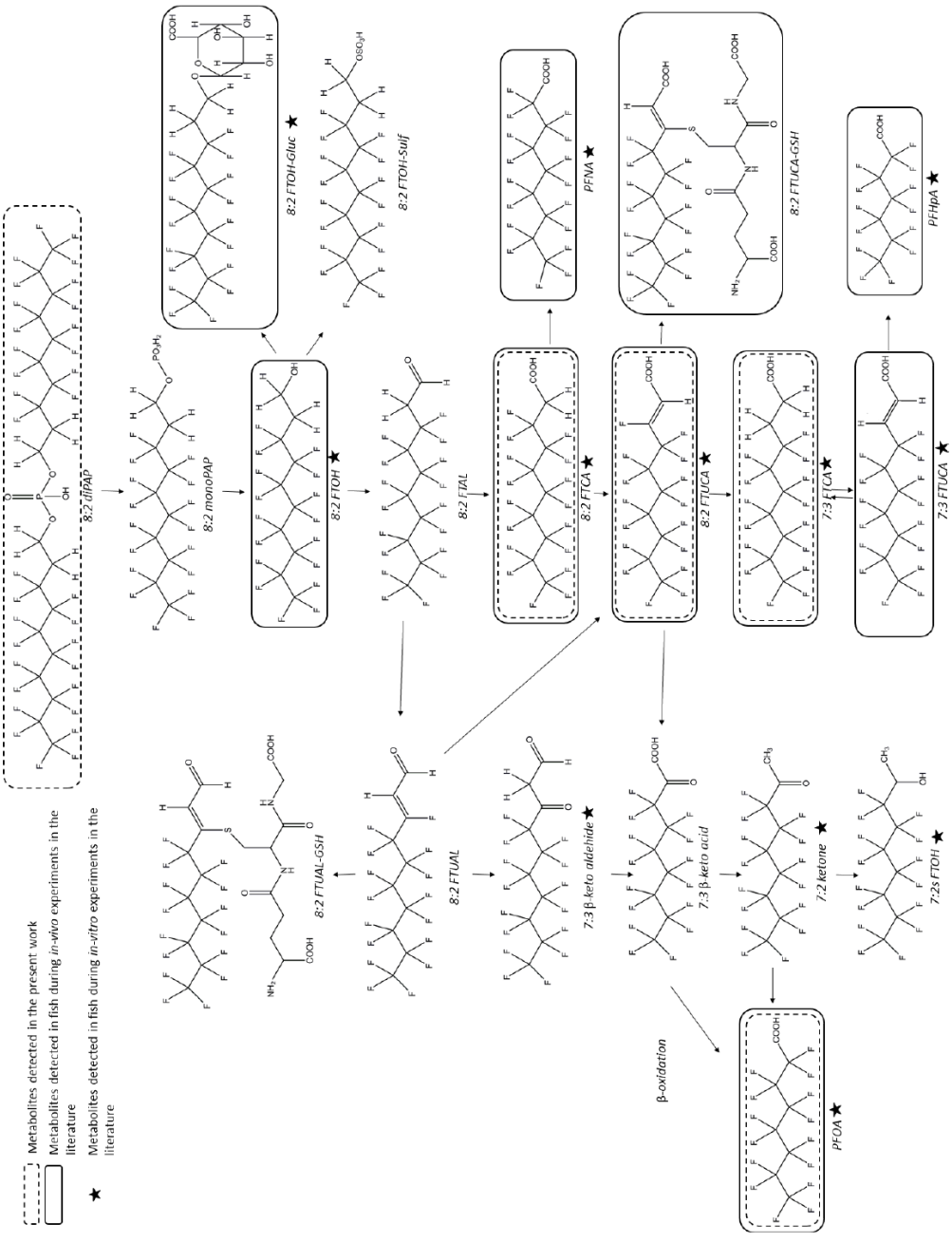


Figure 7.2: 8:2 diPAP metabolic pathway proposed for 8:2 FTOH precursors in fish (adapted from [41]).

However, some of the proposed metabolites have not been detected yet in fish after exposure experiments (the detected metabolites in fish in the literature are pointed out in ***Figure 7.2***). 8:2 FTCA, 8:2 FTUCA, 7:3 FTCA and PFOA were the intermediate and terminal metabolites detected in the present study.

Figure 7.3 includes the various intermediates and end-products of 8:2 diPAP transformation determined in the present study for all the evaluated fish tissues and fluids (raw data in ***Table 7.5***).

While 8:2 monoPAP was not observed in any of the samples of the exposed tank, 8:2 FTCA, 8:2 FTUCA, 7:3 FTCA and PFOA were all detected. PFNA and PFHpA were not observed in the present work, in contrast to previous experiments involving FTOHs and rainbow trout [41,52], presumably because of its relatively low formation yield. In plasma and gills, 8:2 FTCA was the major intermediate detected (2.2-3.5 ng/g for plasma and 1.4-2.8 ng/g for gills), followed by 8:2 FTUCA (0.20-0.37 ng/g for plasma and 0.09-0.20 ng/g in gills), 7:3 FTCA (0.07-0.22 ng/g for plasma and nd-0.21 for gills) and PFOA (0.05-0.12 ng/g for plasma and 0.57-0.60 ng/g for gills), which were at 10-fold lower levels. These results are consistent with the shorter biological half-life of FTUCAs in fish [41]. Increasing trends were observed for all target metabolites in plasma, while in gills, 8:2 FTCA appeared to reach steady state by day 4. Moreover, similar concentration ranges were achieved for both tissues, except for PFOA, which presented a higher constant concentration in gills (~ 0.6 ng/g).

In liver, 8:2 FTCA was also the major metabolite quantified (1.8-2.1 ng/g). PFOA was observed initially on day 2 (0.8 ± 0.1 ng/g) but was not detectable by day 4. Consistent with this result, bile PFOA concentrations increased over the course of the experiment (up to 1.3 ng/mL) indicating that biliary excretion was occurring. Interestingly, it appeared that 8:2 diPAP was metabolised in the liver during the first few days of the

exposure, while by the end of the experiment, 8:2 diPAP appeared to be accumulating (with limited biotransformation), since no PFOA was detected by day 7.

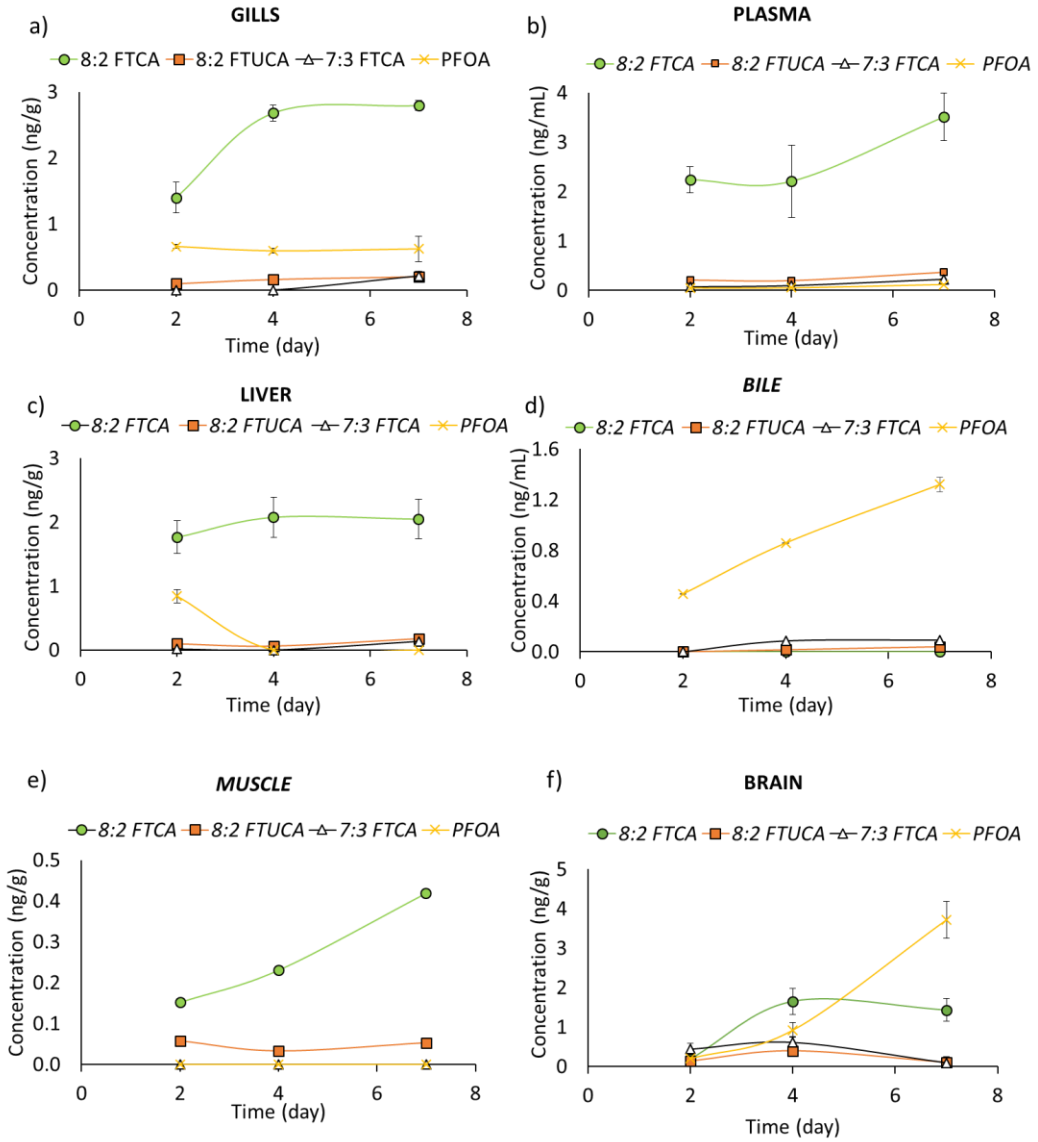


Figure 7.3: 8:2 diPAP metabolite distribution (in ng/g for solid tissues and ng/mL for biofluids including the standard deviation, n=3) in the different tissues: (a) gills, (b) plasma, (c) liver, (d) bile, (e) muscle and (f) brain.

Table 7.5: Concentrations (ng/mL) ± standard deviations of 8:2 diPAP metabolites in plasma.

Matrix	Day	8:2 monoPAP	8:2 FTCA	8:2 FTUCA	7:3 FTCA	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA
Plasma	2	nd	2.2 ± 0.3	0.21 ± 0.02	0.07 ± 0.01	nd	nd	nd	nd	0.048 ± 0.001	nd	nd
	4	nd	2.2 ± 0.7	0.20 ± 0.02	0.10 ± 0.03	nd	nd	nd	nd	0.05 ± 0.02	nd	nd
	7	nd	3.5 ± 0.5	0.37 ± 0.02	0.22 ± 0.01	nd	nd	nd	nd	0.12 ± 0.01	nd	nd
Gills	2	nd	1.4 ± 0.2	0.09 ± 0.02	nd	nd	nd	nd	nd	0.66 ± 0.03	nd	nd
	4	nd	2.7 ± 0.1	0.16 ± 0.03	nd	nd	nd	nd	nd	0.59 ± 0.04	nd	nd
	7	nd	2.8 ± 0.1	0.20 ± 0.03	0.21 ± 0.05	nd	nd	nd	nd	0.6 ± 0.2	nd	nd
Brain	2	nd	0.20 ± 0.04	0.14 ± 0.03	0.4 ± 0.1	nd	nd	nd	nd	0.20 ± 0.04	nd	nd
	4	nd	1.6 ± 0.3	0.4 ± 0.1	0.6 ± 0.2	nd	nd	nd	nd	0.9 ± 0.2	nd	nd
	7	nd	1.4 ± 0.3	0.11 ± 0.02	nd	nd	nd	nd	nd	3.7 ± 0.5	nd	nd
Muscle	2	nd	0.15 ± 0.05	0.06 ± 0.01	< LOQ	nd	nd	nd	nd	nd	nd	nd
	4	nd	0.23 ± 0.08	0.03 ± 0.01	< LOQ	nd	nd	nd	nd	nd	nd	nd
	7	nd	0.42 ± 0.03	0.05 ± 0.01	< LOQ	nd	nd	nd	nd	nd	nd	nd
Bile	2	nd	nd	nd	nd	nd	nd	nd	nd	0.457 ± 0.003	nd	nd
	4	nd	nd	< LOQ	0.09 ± 0.01	nd	nd	nd	nd	0.859 ± 0.002	nd	nd
	7	nd	nd	0.040 ± 0.006	0.09 ± 0.01	nd	nd	nd	nd	1.32 ± 0.06	nd	nd
Liver	2	nd	1.8 ± 0.2	0.10 ± 0.02	0.490 ± 0.007	nd	nd	nd	nd	0.8* ± 0.1	nd	nd
	4	nd	2.1 ± 0.3	0.08 ± 0.01	0.06 ± 0.02	nd	nd	nd	nd	nd*	nd	nd
	7	nd	2.0 ± 0.3	0.18 ± 0.03	0.17 ±0.04	nd	nd	nd	nd	nd*	nd	nd

nd: no detected
< LOQ: below limit of quantification
**subtracted control fish concentration*

In the present study, surprisingly high concentrations of PFOA (see ***Figure 7.3 (f)***) were quantified by day 7 in brain (3.7 ± 0.5 ng/g, the highest concentrations of PFOA were reported in this tissue). Moreover, 8:2 FTCA, 8:2 FTUCA and 7:3 FTCA were also detected in brain, with 8:2 FTCA being the major intermediate (up to 1.6 ng/g). Furthermore, these results showed a higher ability of PFOA in order to cross the BBB comparing to the rest of the target analytes, since higher relation in the concentration brain/plasma could be observed for PFOA in the different exposure days (4-31) followed by 7:3 FTCA (0.4-6.2), 8:2 FTUCA (0.3-2.0), 8:2 diPAP (0.3-1.3) and 8:2 FTCA (0.09-0.7).

Muscle (see ***Figure 7.3 (e)***) contained the lowest concentrations of all PFASs, with only 8:2 FTCA (0.15-0.42 ng/g) and 8:2 FTUCA (0.03-0.06 ng/g) being detectable. While concentrations up to 0.4 ng/g were achieved for 8:2 FTCA during the exposition period, 8:2 FTUCA was steady during the uptake phase. The relative profile for the target metabolites in each tissue over the course of the experiment is shown in ***Figure 7.4***.

Finally, conjugate metabolites (e.g. glucuronide, GSH and sulphate) were not detected in neither the tissues nor the biofluids.

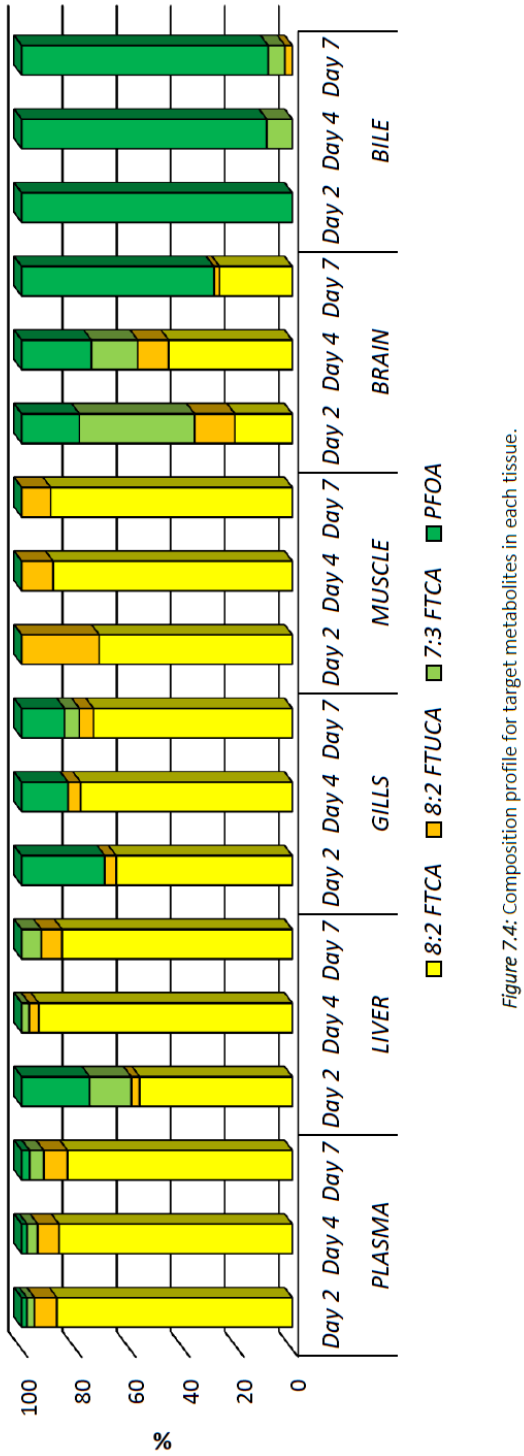


Figure 7.4: Composition profile for target metabolites in each tissue.

7.3.6 Mechanistic aspects of 8:2 diPAP transformation

Of the few studies investigating diPAP transformation, most have involved rodent models [36-38]. Among these studies, monoPAPs are rarely observed in the blood stream, consistent with the present work. In fact, the only study to observe monoPAPs in the bloodstream following diPAP exposure [38] suggested that this may actually be 8:2 FTOH-sulfate, which shares the same SRM transition as 8:2 monoPAP. D´eon and Mabury suggested that the absence of monoPAP in blood provides evidence that 8:2 diPAP is hydrolysed in the gut, after which FTOH is absorbed into the blood stream [37]. This hypothesis is supported by [36], who observed 8:2 monoPAP in feces but not serum of rodents, and by the present work, in which 8:2 monoPAP was absent from both tissues and biofluids of fish. Nevertheless, the detection of 8:2 diPAP in different tissues from the present work indicates that diPAPs may be absorbed into the blood; consequently we cannot rule out the possibility that some dephosphorylation occured in the liver.

An *in-vitro* study using bovine intestinal alkaline phosphatase (AP), the phosphatase enzymes catalyse monoester hydrolysis reactions, concluded that monoPAPs are efficiently hydrolysed by AP enzyme in the intestinal mucosa [60]. However, in the case of diPAPs, phosphodiesterase enzymes are responsible for catalyzing this reaction. These enzymes are ubiquitous within the body (e.g. brain, liver, gut, kidney, heart and muscle) and the lack of a nonspecific phosphodiesterase enzyme [61] may be the responsible for the slow hydrolysis of 8:2 diPAP in-*vivo*. In fact, D´eon and Mabury [38] reported that the 8:2 monoPAP-dosed rats had almost 1 order of magnitude more PFOA in their blood compared to rats dosed with 8:2 diPAP. This observation, together with the slower metabolic capacity of fish [40], provides a plausible explanation for the low concentrations of metabolites detected in the present work. Moreover, aside from PFOA, PFCAs were absent in the present work. D´eon and Mabury [38] also did not

detect PFCAs when rats were exposed to 8:2 diPAP. However, when they repeated the experiment with different diPAP congeners (including 8:2 diPAP), PFNA concentrations below LOQ values were detected [37]. Also consistent with our observations was the lack of phase II metabolites reported previously. To date the only phase II metabolites which have been reported are FTOH-sulfates [36–38] and 6:2 FTOH-glucuronide after 6:2 diPAP exposure [62]. The absence of phase II metabolites in the present work could be explained by interspecies differences or the short duration of exposure in the present work.

7.4 Conclusions

The current study presents for the first time the biotransformation of 8:2 diPAP in a model fish species (gilt-head bream) via dietary exposure. 8:2 diPAP displayed higher accumulation in liver, plasma and gills, compared with bile, muscle and brain. 8:2 FTCA was the major intermediate detected in most samples, followed by 8:2 FTUCA and 7:3 FTCA. PFOA, which was the only PFCA detected, occurred in plasma at low concentrations, and at higher concentrations in bile and brain. The highest concentration of PFOA was observed in brain on day 7. The absence of 8:2 monoPAP from tissues and biofluids supports the hypothesis that dephosphorisation of 8:2 diPAP occurs in the gut, similar to rodents. Further research is necessary to confirm this hypothesis. Finally, this work showed that fish can biotransform 8:2 diPAP to PFOA, indicating that this substance may be a source of PFCA exposure in fish.

7.5 References

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Chapter 8

Fast and simple determination of perfluorinated compounds and their potential precursors in different packaging materials

Talanta 152 (2016) 353-363

8.1 Introduction

Packaging has become essential in the food manufacturing process since it maintains food safe from external influences, offers preservation and ease transportation, and provides consumers with ingredient and nutritional information [1,2]. During recent years the production and use of packaging materials has increased enormously in order to meet the huge food industry demand. In fact, food packaging accounts for almost two-thirds of the total volume of packaging waste [1]. Although packaging manufacturing industry tries to produce food packaging materials that provide both a minimum environmental impact and food safety, recently, the packaging has been found to represent a source of contamination due to the migration of substances from the packaging into the food [3].

Among the different harmful chemicals reported in the recent literature, fluorochemical compounds have become a critical area of concern in terms of food safety due to their extended use as grease and water repellent coatings for food packaging [4]. The carbon-fluorine bond of these compounds makes them chemically and biologically stable [5]. This resistance confers them rigidity, low chemical reactivity and environmental persistence; therefore, fluorochemicals have the potential to bioaccumulate. Moreover, poly- and perfluoroalkyl substances (PFASs) have received an increasing attention during the recent years due to their toxicity. To date, toxicological information is available only for perfluorooctanosulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) congeners. These compounds have been reported to be peroxisome proliferators, disruptors of the reproductive development and endocrine system, and tumor promoters [6–8].

Although during the last years the focus has been set on the perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs) [9], it must be also considered that the majority of the commercial fluorochemical production involves the incorporation of

fluorinated precursors, such as polyfluoroalkyl phosphates (PAPs) or fluorotelomer alcohols (FTOHs) for use in different applications [10]. Furthermore, various studies have evidenced that PFCA precursors are more toxic than the PFCAs themselves [11,12]. Therefore, further attention should be paid in the monitoring of not only PFCAs and PFSAs but also their potential precursors. Besides, while the production of PFOS and PFOA was recently reduced or phased-out in Europe and North America [13–15], the production of fluorotelomer-based chemicals is still increasing. Thus, continued manufacturing of fluorinated precursors and subsequent biotransformation to PFCAs [16] is likely an ongoing pathway of human PFCA exposure [9,17].

Regarding food contact packaging materials, legislation is very limited. In Europe, the Framework Regulation (EC) No 1935/2004 is the basic legislation applied to all types of food contact materials. It requests that all parts of the food packaging chain must ensure that migration of chemicals from food contact materials to food should not occur in levels harmful to human health (EU Commission 2004) [18]. Moreover, despite the lack of specific EU limit values and rules for migration testing, the European Commission issued in March 2010 a recommendation (Commission Recommendation 2010/161/EU) [19], stating that fluorinated compounds should be monitored in food by all EU member states. In addition to the PFCA, PFSA and FTOH, it was recommended that polyfluoroalkyl phosphate diesters (diPAPs) and polyfluoroalkyl phosphate monoesters (monoPAPs) should also be included in the monitoring programs.

Concerning the extraction of PFASs from packaging materials, ion-pair based extraction [10], classical solid-liquid extraction (SLE) [20,21], ultrasound assisted extraction (USE) [22,23] or pressurised liquid extraction (PLE) [24–27] have been mostly applied for the extraction of target compounds in packaging materials. Moreover, a focused ultrasound solid-liquid extraction (FUSLE) was recently reported [28] for the determination of six PFCAs and PFOS in packaging. However, despite the extended use

of potential precursors such as PAPs in the manufacturing of packaging, to the best of our knowledge, there are currently only a few works for the determination of these kind of precursors in packaging materials [10,20,29].

Within this context, the aim of the present work was to overcome the challenge of developing a simple and fast analytical method for the determination of fourteen perfluorinated compounds (PFCs) and ten potential precursors in different packaging materials. In order to achieve this objective, the optimisation of FUSLE was carried out and the analyses were performed by liquid chromatography-triple quadrupole-tandem mass spectrometry (LC-QqQ-MS/MS) in all the cases. Furthermore, different plastic and cardboard materials from a local market were analysed, and the results obtained were compared with the ones reported in the literature.

8.2 Experimental section

8.2.1 Reagents and materials

A mixture of potassium perfluoro-1-butane sulfonate (L-PFBS), sodium perfluoro-1-hexane sulfonate (L-PFHxS), potassium perfluoro-1-octane sulfonate (L-PFOS), perfluorooctyl phosphonic acid (PFOPA), perfluorohexyl phosphonic acid (PFHxPA), perfluorodecyl phosphonic acid (PFDPA), perfluoro-n-butanoic acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA) and perfluoro-n-decanoic acid (PFDA) was obtained at 5 mg/L, sodium 1H, 1H, 2H, 2H-perfluorooctyl phosphate (6:2 monoPAP), sodium 1H, 1H, 2H, 2H-perfluorodecyl phosphate (8:2 monoPAP), sodium bis (1H, 1H, 2H, 2H-perfluorooctyl) phosphate (6:2 diPAP), sodium bis (1H, 1H, 2H, 2H-perfluorodecyl) phosphate (8:2 diPAP), 2-perfluorohexyl ethanoic acid (6:2 FTCA), 2-perfluorooctyl ethanoic acid (8:2 FTCA), 2H-perfluoro-2-octenoic acid (6:2 FTUCA), 2H-perfluoro-2-decenoic acid (8:2 FTUCA), 3-perfluoroheptyl propanoic acid (7:3 FTCA) and 3-perfluoropentyl propanoic acid

(5:3 FTCA) were obtained individualy at 50 mg/L and perfluorooctane sulfonamide (FOSA) was obtained as solid standard.

A surrogate mixture of sodium perfluoro-1-hexane [$^{18}O_2$] sulfonate (MPFHxS), sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octane sulfonate (MPFOS), perfluoro-n-[$^{13}C_4$] butanoic acid (MPFBA), perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid (MPFHxA), perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid (MPFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$] nonanoic acid (MPFNA), perfluoro-n-[1,2-$^{13}C_2$] decanoic acid (MPFDA), perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid (MPFUnDA) and perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid (MPFDoDA) and a surrogate mixture of 2-perfluorohexyl-[1,2-$^{13}C_2$]-ethanoic acid (M6:2 FTCA), 2-perfluorooctyl-[1,2-$^{13}C_2$]-ethanoic acid (M8:2 FTCA) and 2-perfluorodecyl-[1,2-$^{13}C_2$]-ethanoic acid (M10:2 FTCA) were obtained at 2 mg/L, while 6-chloroperfluorohexyl phosphonic acid (Cl-PFHxPA), sodium bis (1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl) phosphate (M8:2diPAP), sodium 1H, 1H, 2H, 2H-[1,2-$^{13}C_2$] perfluorodecyl phosphate (M8:2PAP) and 2H-perfluoro-[1,2-$^{13}C_2$]-2-decenoic acid (M8:2 FTUCA) were obtained individually at 50 mg/L. They were all purchased from Wellington Laboratories (Ontario, Canada), except for FOSA, which was provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The purity of all the target analytes was > 98 % except for FOSA (97.5 %).

Methanol (MeOH, HPLC grade, 99.9 %) and acetone (HPLC grade, 99.8 %) were supplied by LabScan (Dublin, Ireland), acetonitrile (ACN, HPLC grade, 99.9 %) by Sigma Aldrich (Steinheim, Germany), acetic acid (HOAc, glacial, 100 %) by Merck (Darmstadt, Germany) and ethanol (EtOH, super purity, > 99.8 %) by Romil (Cambridge, UK).

For the mobile phase composition MeOH (Fisher Scientific, Loughborough, UK) was used. 1-methyl piperidine (1-MP, $\geq$ 98 %) was obtained from Merck and ammonium acetate (NH_4OAc ≥ 99 %) was purchased from Sigma Aldrich. High purity nitrogen gas (> 99.999 %) supplied by Messer (Tarragona, Spain) was used as collision gas and

nitrogen gas (99.999 %) purchased from AIR Liquid (Madrid, Spain) was used as nebuliser and drying gas.

For extraction, a Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogeniser (20 kHz; Bandelin Electronic, Berlin, Germany) equipped with a 3-mm titanium microtip was used. Fractions were evaporated in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) using a gentle N_2 (> 99.999 % from Messer) blow-down. After the extraction step, the supernatant was filtered through a polyamide filter (0.45 µm, 25 mm, Macherey-Nagel, Germany) and polypropylene microfilters (0.2 µm, 13 mm, Pall, USA) were used to filter extracts.

8.2.2 Sample collection and treatment

Different packaging materials made of cardboard (microwave popcorn bag, greaseproof paper for French fries, cardboard box for pizza, cinema cardboard box for popcorn) and plastic (milk bottle, muffin cup, pre-cooked food wrapper, cup of coffee) were obtained randomly from local markets, restaurants and cinema. Before analysis, in the case the samples had a printed outside layer, this was removed when possible with the aid of a cutter. Subsequently, samples were cut into pieces of approximately 1 cm^2 with scissors.

Cardboard from popcorn bags was used for method optimisation and validation. For optimisation experiments, a known amount of matrix was weighed, covered with acetone, spiked with the target analytes and stirred during 24 hours. After that, acetone was evaporated and the sample was aged for one week.

8.2.3 FUSLE

Under optimised conditions, 0.5 g of sample was placed together with 7 mL of MeOH (1 % HOAc) in a 40 mL vessel and 20 µL of a 0.5 ng/µL of surrogate standard solution (MPFHxS, MPFOS, MPFBA, MPFHxA, MPFOA, MPFNA, MPFDA, MPFUdA, MPFDoA,

Cl- PFHxPA, M8:2 diPAP, M8:2 PAP, M8:2 FTUCA, M6:2 FTCA, M8:2 FTCA and M10:2 FTCA) were added. The FUSLE was performed for 2.5 min, with a sonication time of 0.8 s and a 30 % of amplitude. Extractions were carried out at 0 °C in an ice-water bath. After the extraction step, the supernatant was filtered through a polyamide filter and the FUSLE extracts were evaporated to dryness under a nitrogen stream using a Turbo Vap LV Evaporator and reconstituted in 250 µL of LC-MS grade MeOH. The reconstituted extracts were filtered through a 0.2 mm polypropylene filter before the LC-QqQ-MS/MS analysis.

8.2.4 LC-MS/MS analysis

An Agilent 1260 series HPLC chromatograph coupled to an Agilent 6430 triple quadrupole (QqQ) mass spectrometer equipped with both electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) sources (Agilent Technologies, Palo Alto, CA, USA) was employed for the separation and quantification of PFCs and precursors. An ACE UltraCore 2.5 SuperC18 (2.1 mm x 50 mm, 2.5 µm) column (pH range 1.5-11) coupled to a pre-column filter (0.5 µm, Vici Jour) was used for the separation of the target analytes at 35 °C.

Under previously optimised conditions [30,31], mobile phase A consisted of a Milli-Q water: MeOH (95:5) mixture and mobile phase B of MeOH: Milli-Q water (95:5), both containing 2 mmol/L NH_4OAc and 5 mmol/L 1-MP. Precursors (PAPs, FTCAs and FTUCAs) and PFCs were analysed in two different runs. For PFCs, the gradient profile started with 90 % A (hold time 0.3 min) and continued with a linear change to 80 % A up to 1 min, to 50 % A up to 1.5 min and to 20 % A up to 5 min (hold time 5 min), followed with a linear change to 0 % A up to 13 min and a hold time until 16 min. Initial conditions were regained at 17 min followed by equilibration until 26 min. For precursors, the gradient profile started with 80 % A (hold time 0.3 min) and continued with a linear change to 20 % A up to 3 min and to 15 % A up to 5 min (hold time 3 min), followed with a linear

change to 0 % A up to 13 min and a hold time until 17 min. Initial conditions were regained at 20 min followed by equilibration until 25 min. In both cases, the flow rate and the injection volume were set at 0.3 mL/min and 5 µL, respectively.

Quantification was performed in the selected reaction-monitoring (SRM) mode. Nitrogen was used as nebuliser, drying and collision gas. Instrument parameters used in the present work for PFCs and precursors were optimised elsewhere [30,31]. Briefly, for PFCs, ESI in the negative mode (NESI) was carried out using a capillary voltage of 3000 V, a drying gas flow rate of 10 L/min, a nebuliser pressure of 50 psi and a drying gas temperature of 350 °C. Moreover, NESI for precursors was carried out using a capillary voltage of 3500 V, a drying flow rate of 8 L/min, a nebuliser pressure of 50 psi and drying gas temperature of 300 °C. Detailed information of the optimised parameters (fragmentor and collision energy) and monitored ion transitions for each analyte and surrogate standards are given in ***Tables 8.1*** and ***8.2***, respectively.

Table 8.1: *Structures, precursor and product ions (first ion was used as quantifier and the second as qualifier) at optimum fragmentor (V) and collision energy (eV) for target analytes.*

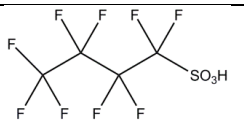
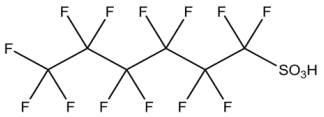
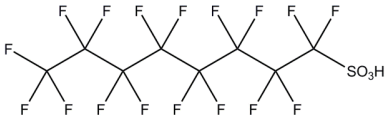
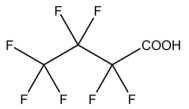
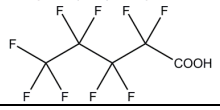
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
PFBS		299	99/80	100	30
PFHxS		399	99/80	150	20
PFOS		499	99/80	150	45
PFBA		213	169	60	5
PFPeA		263	219	60	5

Table 8.1: *Continuation.*

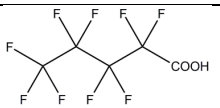
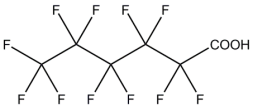
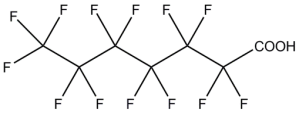
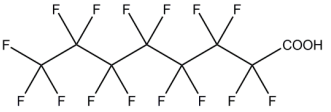
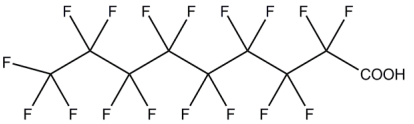
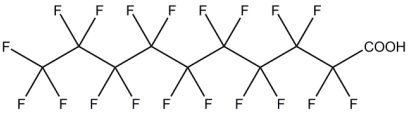
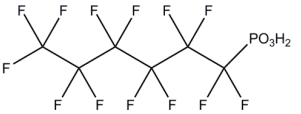
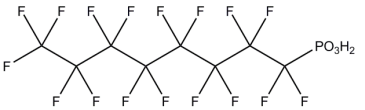
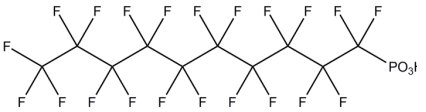
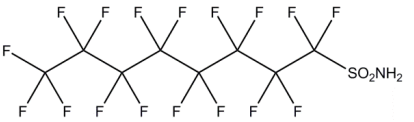
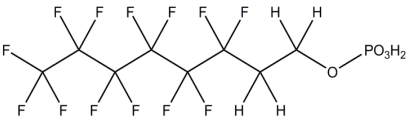
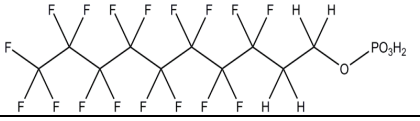
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
PFPeA		263	219	60	5
PFHxA		313	269/119	60	10
PFHpA		363	319/169	60	10
PFOA		413	369/169	60	5
PFNA		463	419/169	60	5
PFDA		513	469/269	100	5
PFHxPA		399	79	100	10
PFOPA		499	79	150	20
PFDPA		599	79	100	5
FOSA		498	78	220	5
6:2 monoPAP		443	97/79	90	13
8:2 monoPAP		543	97/79	90	21

Table 8.1: *Continuation.*

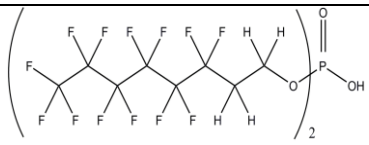
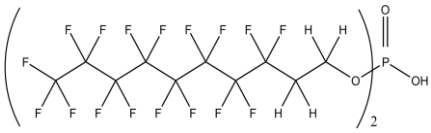
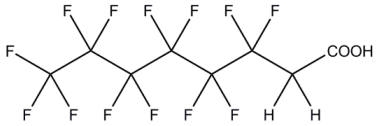
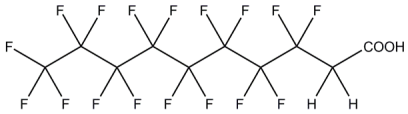
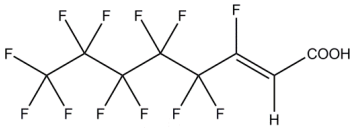
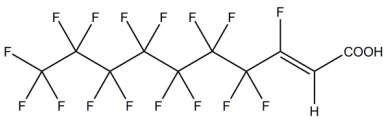
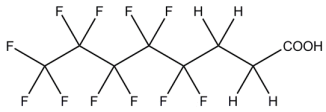
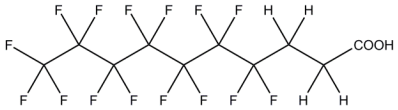
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
6:2 diPAP		789	97/443	120	41
8:2 diPAP		989	97/543	135	41
6:2 FTCA		377	293	75	9
8:2 FTCA		477	393	75	9
6:2 FTUCA		357	293/243	75	9
8:2 FTUCA		457	393	75	9
5:3 FTCA		341	237/217	75	5
7:3 FTCA		441	337/317	75	5

Table 8.2: *Precursor and product ion at optimum fragmentor (V) and collision energy (eV) for surrogate standards, as well as which target analyte is corrected with each isotopic analogue.*

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Corrected compounds
MPFBA	217	172	60	5	PFBA
MPFHxA	315	270	60	5	PFPeA, PFHxA, PFHpA
MPFOA	417	372	60	5	PFOA
MPFNA	468	423	60	5	PFNA, FOSA
MFPDA	515	470	100	5	PFDA
MPFUndA	565	520	60	5	- [a]
MPFDoDA	615	570	100	5	- [a]
MPFHxS	403	103	150	30	PFBS, PFHxS
MPFOS	503	99	60	45	PFOS
Cl-PFHxPA	415	79	105	45	PFHxPA
M8:2 diPAP	993	97	150	41	6:2diPAP, 8:2 diPAP
M8:2PAP	545	97	90	21	6:2 monoPAP, 8:2 monoPAP
M8:2 FTUCA	459	394	75	9	8:2 FTUCA
M6:2 FTCA	379	294	75	9	6:2 FTCA, 6:2 FTUCA, 5:3 FTCA
M8:2 FTCA	479	394	75	9	8:2 FTCA, 7:3 FTCA
M10:2 FTCA	579	494	75	9	- [a]

[a] *Surrogates not used for correction*

**PFOPA and PFDPA are not reported due to the lack of a corresponding labeled standard for correction*

Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

8.3 Results and discussion

For the method optimisation PFOS, PFOA, 8:2 diPAP, 8:2 FTCA, PFOPA and FOSA were selected as representative of each type of PFASs.

8.3.1 Sample fortification

Two different solvents were selected during sample fortification: ethyl acetate, based on several works in the literature [25,28] dealing with the determination of PFASs in packaging material, and acetone, according to the experience of our research group

[30–32] during the determination of the same target compounds in different environmental matrices. In this sense, samples were fortified by adding a standard solution of the target analytes in both acetone and ethyl acetate. The sample extraction was carried out according to the method published by Moreta and Tena [28]. Briefly, aliquots of 0.5 g of packaging were fortified at 100 ng/g and extracted with 8 mL of EtOH at 30 % of amplitude and 0.5 s of sonication time during 10 s. After the extraction step, the supernatant was filtered and evaporated to dryness before LC-QqQ-MS/MS analysis. Although comparable results were obtained according to the one-way analysis of variance (ANOVA) at 95 % of confidence interval (F_{exp} = 1.9-4.7 < $F_{critical}$ = 7.7) for all the evaluated analytes, the precision in terms of relative standard deviation (RSD %) of the replicates (n=3) of the samples fortified in ethyl acetate was higher than 20 % in the case of PFOS. Thus, acetone was selected for further experiments.

8.3.2 Optimisation of FUSLE

8.3.2.1 Extractant nature

Five extraction solvents were tested for the extraction of the target analytes: MeOH, acetone, EtOH, ACN and MeOH (1 % HOAc). Aliquots of 0.5 g (dry weight) of packaging material fortified at 100 ng/g were extracted with 7 mL of the different solvents mentioned above during 1 min (30 % of amplitude at 0.5 s/s of duty cycle). Three replicates per solvent were performed. ***Figure 8.1*** shows that the responses (normalised to the highest chromatographic signal) obtained were comparable when MeOH and MeOH (1% HOAc) were used for 8:2 diPAP, 8:2 FTCA, PFOA, PFOS and FOSA. However, in the case of PFOPA a significant signal improvement was observed using MeOH (1 % HOAc). This could be probably due to the partially neutralisation of the negative charges of this analyte (pKa_1: 2.4, pKa_2: 4.5) **[33]** in acidic media (~ pH=4), improving its extraction in the organic solvent. Therefore, MeOH (1 % HOAc) was chosen as extraction solvent for further experiments. Similarly, MeOH was the most common solvent to

extract PFAS from packaging material according to the literature [20,21,24–27]. Milli-Q water [34], EtOH [28,29,35] or a mixture of them (EtOH:Milli-Q water (50:50)) [22,23] were also used and reported in some works for the determination of PFASs.

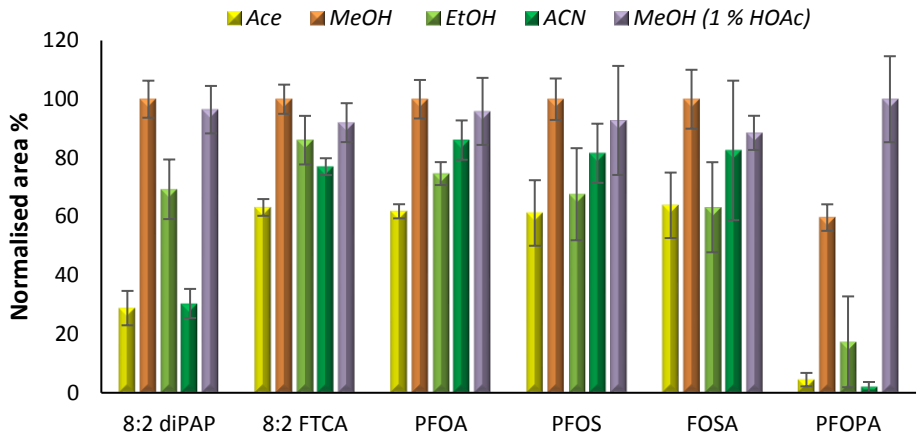


Figure 8.1. *Influence of the solvent type during FUSLE extraction. Average signals (n=3) were normalised to the highest chromatographic response. Standard deviations were also included.*

8.3.2.2 Optimisation of the amplitude, extraction time and sonication time

The influence of FUSLE main parameters (amplitude, extraction time and sonication time) were optimised by a central composite design (CCD) using the Statgraphics program (Statgraphics Centurion XV). In this sense, the extraction time was studied from 0.5 to 5 min, the amplitude from 10 to 56 % and the sonication time from 0.2 to 0.8 s. Extraction time is divided in different cycles. The sonication time is the fraction of the time unit during which ultrasound is applied. All the experiments (18 assays) were carried out using 0.5 g of spiked samples containing 100 ng/g of each target analyte. The ANOVA results indicated that the studied parameters had a positive effect at a 95 % of confidence level ($p > 0.05$) only for PFOPA (r^2= 73.6 %). For the rest of target analytes, no significant effects were observed for none of the parameters. According to the results obtained for PFOPA and based on our previously published works [30,31],

sonication time was fixed at the highest value (0.8 s). In the case of the amplitude, since it only affected the PFOPA response and in order to prolong the lifetime of the titanium tip, it was fixed at a 30 %. Finally, the influence of the extraction time was further studied and extraction times of 1, 2.5 and 4 minutes were tested at fixed sonication time (0.8 s) and amplitude (30 %). As shown in ***Figure 8.2***, no significant differences were observed between different extraction times for the majority of the analytes, except for PFOPA. In the case of the latter, although the average value was higher after 4 min extraction, comparable results were obtained according to ANOVA at a 95 % of confidence interval ($F_{exp} = 1.4 < F_{critical} = 7.7$) due to the high standard deviation obtained when the extraction time was 2.5 min. According to the results and as a consensus for all the target analytes extraction time was fixed at 2.5 min.

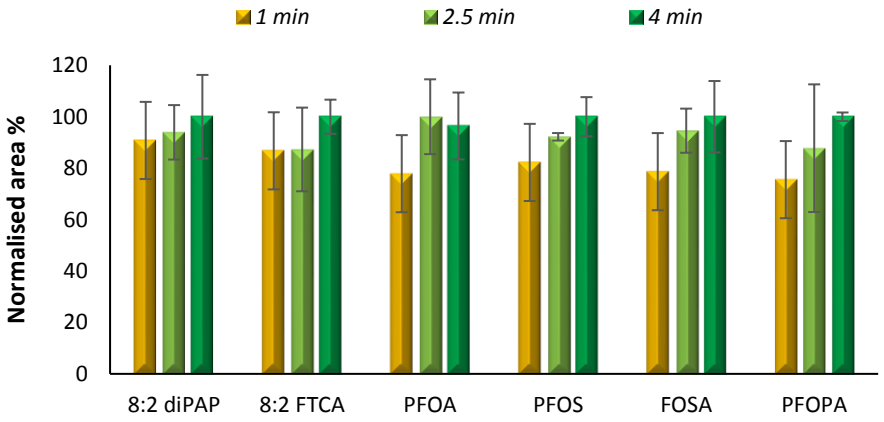


Figure 8.2. *Influence of extraction time during FUSLE extraction at fixed duty cycle (0.8 s/s) and amplitude (30 %). Signals were normalised to the highest chromatographic response. Average responses (n = 3) and standard deviations were used.*

In summary, optimum extraction conditions were fitted as follows: extraction time at 2.5 min, sonication time of 0.8 s and amplitude at 30 %.

Due to the lack of a certified reference material (CRM) and in order to determine whether exhaustive extraction was carried out under optimised conditions, up to three consecutive extractions (n=3) were performed on the same sample (cardboard popcorn bag). A single extraction was sufficient for quantitative extraction since recoveries up to 85-89 % were obtained for all the target analytes (see ***Figure 8.3***). Similar results were obtained by Moreta and Tena [28] for the determination of PFCAs and PFOS in packaging material where an unique FUSLE step was necessary. Thus, only a single extraction was selected and carried out in further experiments.

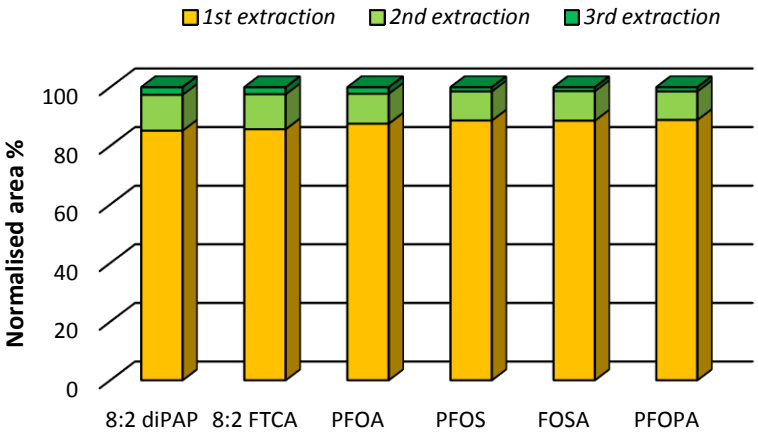


Figure 8.3. *Influence of the number of repeated extractions.*

8.3.3 Method validation

Method validation was performed in terms of linearity, limits of detection (LODs) and quantification (LOQs), method detection limits (MDLs), precision and recoveries, calculated with different calibration approaches, at two concentration levels (25 ng/g and 50 ng/g) using a cardboard popcorn bag matrix. Calibration curves were built with standard solutions in MeOH in the LOQ-1000 ng/mL range and at ten concentration levels. As can be observed in ***Table 8.3***, determination coefficients in the range of 0.997-

0.999 were obtained for all the target analytes without correction with the corresponding labeled standard. LODs were estimated as the lowest concentration for which the peak area was at least three times the background noise (S/N=3). LOQs were established as the lowest concentration fulfilling all of the following criteria: (1) linear calibration curve, (2) acceptable peak shapes, and (3) signal-to-noise ratio of at least 10 (S/N=10) [36,37]. As can be observed in ***Table 8.3***, the LODs and LOQs obtained were below 0.7 and 2.3 ng/mL, respectively. LODs and LOQs were similar to the values reported in the literature [10,38].

Table 8.3: *LODs (ng/mL), LOQs (ng/mL), determination coefficients (r^2), MDL values at 25 ng/g and RSD (%) at high (50 ng/mL) and low concentration (25 ng/mL) levels for PFSAs, PFCAs, PFPAs and potential precursors in fortified cardboard packaging samples*.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	r^2	MDL (ng/g)	RSD (%)	
					25 ng/g	50 ng/g
PFBA	0.1	0.5	0.999	1.6	10	17
PFPeA	0.1	0.5	0.999	2.1	12	15
PFHxA	0.05	0.2	0.999	1.8	10	16
PFHpA	0.004	0.01	0.999	1.1	9	15
PFOA	0.004	0.01	0.999	2.2	9	17
PFNA	0.004	0.01	0.999	1.4	10	16
PFDA	0.004	0.01	0.999	0.8	10	16
PFBS	0.05	0.2	0.999	1.0	11	24
PFHxS	0.05	0.2	0.999	0.6	9	20
PFOS	0.004	0.01	0.999	1.6	12	14
PFOSA	0.03	0.1	0.998	2.0	14	17
PFHxPA	0.1	0.4	0.999	1.9	20	12
PFOPA	0.06	0.2	0.998	0.9	6	7
PFDPA	0.4	1.4	0.999	2.0	14	5
6:2 monoPAP	0.7	2.3	0.999	1.3	7	8
8:2 monoPAP	0.6	2.1	0.998	2.0	9	9
6:2 diPAP	0.001	0.005	0.999	1.2	16	20
8:2 diPAP	0.005	0.02	0.999	0.8	14	9
6:2 FTCA	0.6	1.9	0.997	1.1	12	21
8:2 FTCA	0.3	0.9	0.999	1.1	12	17
6:2 FTUCA	0.1	0.4	0.999	1.3	14	22
8:2 FTUCA	0.07	0.2	0.999	1.0	11	21
7:3 FTCA	0.1	0.4	0.999	1.9	11	18
5:3 FTCA	0.1	0.4	0.999	1.7	10	23

MDLs were determined according to the USEPA using the samples fortified at the lowest concentration (25 ng/g) used in the validation. The MDL was then calculated as

MDL = $t_{(n-1,\ 1-\alpha=0.95)}$ x s_d, where t = 1.94 corresponds to the Student's t-value for a 95 % confidence level and 6 degrees of freedom, whereas s_d is the standard deviation of the seven replicate analyses. The MDL values for cardboard samples were in the range of 0.6-2.2 ng/g (see ***Table 8.3***) for all the analytes. Similar MDL values (0.5-2.2 ng/g) were reported by Moreta and Tena [28] when FUSLE extraction was performed for PFCAs and PFOS in packaging samples. Besides, these MDL values were lower than those reported by Martínez-Moral et al. (0.7-18 ng/g) [25] when PLE was used for the extraction of PFCAs and PFOS. Furthermore, it has to be emphasised the large number of analytes determined in this work. To the best of our knowledge, this is the first time that up to 24 PFCs, PAPs and precursors are determined in packaging samples.

Absolute recoveries at low (25 ng/g) and high (50 ng/g) concentration levels were in the range of 36-91 % and 36-97 %, respectively, when external calibration was used and no correction with the corresponding labeled standard was performed (see ***Table 8.4***). Moreover, labeled standards were used for the apparent recovery calculation, except for PFOPA and PFDPA due to the lack of the corresponding labeled standard for correction. Good apparent recoveries in the range of 69-103 % and 62-98 % were obtained for low and high concentration levels, respectively. Furthermore, matrix-matched calibration was also performed. Recoveries obtained for PFCs and potential precursors were in the range of 66-117 % for cardboard material at both concentration levels. Therefore, matrix-matched calibration quantification approach was only necessary in the absence of the corresponding labeled standards. In terms of precision, RSD values were in the 5-24 % range for all the analytes in the fortified samples at both concentration levels evaluated.

Table 8.4: *Recoveries (%) and apparent recoveries (%) at low (25 ng/g) and high (50 ng/g) concentration levels for PFCAs, PFSAs, PFPAs and potential precursors in fortified cardboard packaging samples.*

Analyte	Recovery with external calibration (%) ± s		Recovery with internal calibration (%) ± s		Recovery with matrix-matched calibration (%) ± s	
	25 ng/g	50 ng/g	25 ng/g	50 ng/g	25 ng/g	50 ng/g
PFBA	76 ± 12	60 ± 5	103 ± 10	95 ± 17	117 ± 21	99 ± 9
PFPeA	72 ± 8	62 ± 4	95 ± 11	85 ± 13	103 ± 15	96 ± 8
PFHxA	70 ± 8	61 ± 3	92 ± 10	84 ± 14	100 ± 15	94 ± 7
PFHpA	71 ± 9	63 ± 4	95 ± 9	87 ± 13	101 ± 14	96 ± 8
PFOA	73 ± 10	63 ± 4	94 ± 9	88 ± 15	105 ± 17	97 ± 8
PFNA	73 ± 10	63 ± 4	96 ± 10	90 ± 15	104 ± 18	96 ± 9
PFDA	68 ± 9	61 ± 5	90 ± 10	82 ± 13	100 ± 16	96 ± 10
PFBS	74 ± 8	65 ± 4	91 ± 11	82 ± 19	102 ± 15	96 ± 7
PFHxS	75 ± 8	65 ± 6	92 ± 9	82 ± 17	101 ± 17	96 ± 10
PFOS	75 ± 8	67 ± 4	95 ± 12	88 ± 12	100 ± 15	95 ± 7
PFOSA	57 ± 8	57 ± 4	76 ± 10	82 ± 14	99 ± 14	99 ± 9
PFHxPA	80 ± 15	62 ± 3	86 ± 18	62 ± 8	103 ± 21	80 ± 4
PFOPA	62 ± 4	55 ± 4	62 ± 4*	55 ± 4*	80 ± 6	74 ± 1
PFDPA	73 ± 10	72 ± 4	73 ± 10*	72 ± 4*	83 ± 11	84 ± 4
6:2 monoPAP	36 ± 2	36 ± 3	69 ± 5	80 ± 5	66 ± 5	68 ± 5
8:2 monoPAP	42 ± 4	38 ± 3	69 ± 8	73 ± 7	70 ± 8	66 ± 5
6:2 diPAP	80 ± 6	74 ± 11	87 ± 14	81 ± 16	100 ± 7	98 ± 5
8:2 diPAP	91 ± 7	97 ± 17	98 ± 14	84 ± 8	100 ± 7	108 ± 20
6:2 FTCA	62 ± 7	58 ± 4	100 ± 12	96 ± 20	114 ± 13	107 ± 8
8:2 FTCA	60 ± 5	60 ± 4	89 ± 11	97 ± 17	106 ± 9	103 ± 7
6:2 FTUCA	62 ± 5	56 ± 4	101 ± 14	93 ± 20	112 ± 10	108 ± 10
8:2 FTUCA	59 ± 5	56 ± 3	87 ± 10	84 ± 17	105 ± 9	104 ± 6
7:3 FTCA	58 ± 6	61 ± 4	86 ± 9	98 ± 18	108 ± 10	107 ± 8
5:3 FTCA	54 ± 6	50 ± 4	87 ± 9	83 ± 19	111 ± 13	109 ± 11

*Concentration without correction due to the lack of the correct labeled standard.

8.3.4 Application to real samples

Different plastic and cardboard packaging materials, including microwave popcorn bag, pizza box, greaseproof paper for French fries, cinema popcorn box, muffin cup, milk bottle, coffee cup and pre-cooked food wrapper, bought at local markets and cinemas, were analysed (n=3) and the results (average values in ng/g) obtained are included in ***Table 8.5***.

Table 8.5: PFASs and precursors concentration ranges in different packaging materials all around the world (including this study). Results are expressed in ng/g.

Country	Packaging type	Method	Concentration range (ng/g)											Reference
			PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFDA	8:2 diPAP	6:2 diPAP	6:2 FTCA	6:2 FTUCA	5:3 FTCA	Others	
Spain	Microwave popcorn bag	FUSLE-LC/(-)ESI-MS/MS	291.0	20.5	254.5	2.0	< MDL[a]	< MDL[a]	12.1[a]	< MDL[a]	161.6	114.4	24.6	-	This study
Spain	Pizza box	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	< MDL[a]	5.3	2.0	nd[b]	nd[b]	nd[b]	-	This study
Spain	French fries Wrapper	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	< MDL[a]	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
Spain	Cinema popcorn box	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	15.4	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
Spain	Muffin cup	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	16.9	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
Spain	Milk bottle	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	14.3	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
Spain	Coffee cup	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	13.3	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
Spain	Pre-cooked food wrapper	FUSLE-LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	< MDL[a]	nd[b]	15.0	< MDL[a]	nd[b]	nd[b]	nd[b]	-	This study
USA	Microwave popcorn bag	Sonication (Water: EtOH (50:50)) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	6-290	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[22]
USA	Hamburger wrapper	Sonication (Water: EtOH (50:50)) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[22]
USA	Sandwich wrapper	Sonication (Water: EtOH (50:50)) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[22]
USA	French fry box	Sonication (Water: EtOH (50:50)) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[22]
USA	Microwave popcorn bag	Shaking (MeOH)-LC/(-)ESI-MS/MS and shaking (EtOH)-GC/MS	nd[b]	130*	na[c]	320*	470*	140*	na[c]	na[c]	na[c]	na[c]	na[c]	PFNA: 210* PFUnDA: 70* PFDoDA: 40* 8:2 FTOH: 170*	[21]
China	PTFE packaging material	PLE-GC/MS	na[c]	na[c]	na[c]	na[c]	10.7-31.6	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 8.8-48.1	[37]
Australia	Microwave popcorn bag	Sonication (water)-SPE (C18)- LC/(-)ESI-MS/MS	na[c]	na[c]	nq[e]	nq[e]	9.2	nq[e]	na[c]	na[c]	na[c]	na[c]	na[c]	PFNA, PFOS and PFDoA detected	[34]

Table 8.5: Continuation.

Country	Packaging type	Method	Concentration range (ng/g)												Reference
			PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFDA	8:2 diPAP	6:2 diPAP	6:2 FTCA	6:2 FTUCA	5:3 FTCA	Others	
Australia	Baking paper	Sonication (water)-SPE (C18)- LC/(-)ESI-MS/MS	na[c]	na[c]	nd[b]	nd[b]	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[34]
Australia	French fry box	Sonication (water)-SPE (C18)- LC/(-)ESI-MS/MS	na[c]	na[c]	nd[b]	nd[b]	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[34]
Australia	Sandwich wrapper	Sonication (water)-SPE (C18)- LC/(-)ESI-MS/MS	na[c]	na[c]	nd[b]	nd[b]	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[34]
Denmark	Microwave popcorn bag	Sonication (95 % EtOH) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	nq[e]	na[c]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs and s-diPAPs detected	[29]
Denmark	Burger box	Sonication (95 % EtOH) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	nd[b]	na[c]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs detected	[29]
Denmark	Microwave popcorn bag	Sonication (95 % EtOH) -LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs, s-diPAPs and triPAPs detected	[35]
Spain	Microwave popcorn bag	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	37-99	53-198	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFNA: 30-61 PFOS: LOQ-23 PFUnDA: 3.7-13 PFDoDA: 33-90	[25]
Thailand	Instant food cup	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	2.4-9.6 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 7.2-9.8 *	[26]
Thailand	Microwave popcorn bag	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	0.1-1.7 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: nd-2.5 *	[26]
Thailand	Beverage cup	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	3.0-10.3 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 8.4-11.4 *	[26]
Thailand	Ice cream cup	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	3.4-16.9 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 6.4-7.4 *	[26]
Thailand	Fast food container	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	0.4-16.1*	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 0.9-92.5 *	[26]
Thailand	Dessert container	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	0.1-5.8 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: nd-10.0 *	[26]
Thailand	Baking paper	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	na[c]	1.3 *	na[c]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 1.2 *	[26]
Sweden	Microwave popcorn bag	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nq[e]	nq[e]	nq[e]	nq[e]	nq[e]	nq[e]	na[c]	na[c]	na[c]	PFNA, PFUnDA, PFDoDA, PFTrDA, PFTeDA, PFPeDA, monoPAPs, diPAPs and triPAPs detected	[20]

Table 8.5: Continuation.

Country	Packaging type	Method	Concentration range (ng/g)												Reference
			PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFDA	8:2 diPAP	6:2 diPAP	6:2 FTCA	6:2 FTUCA	5:3 FTCA	Others	
Sweden	Pizza grease proof paper	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	nq[e]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	6:2 monoPAP and diPAPs detected	[20]
Sweden	Thai food box	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nq[e]	nq[e]	nd[b]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs and 6:2 triPAPs detected	[20]
Sweden	Board cup	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nq[e]	nq[e]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	PFNA, 8:2 monoPAP and diPAPs detected	[20]
Sweden	Fries box	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nq[e]	nd[b]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs and triPAPs detected	[20]
Sweden	Burger box	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nq[e]	nq[e]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs detected	[20]
Sweden	Baking paper	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	nq[e]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	diPAPs detected	[20]
Sweden	Burger grease proof paper	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	nq[e]	nd[b]	nd[b]	nq[e]	na[c]	na[c]	na[c]	diPAPs detected	[20]
Sweden	Oven safe paper	Stirring (MeOH)- LC/(-)ESI-MS/MS	nd[b]	nd[b]	nd[b]	nd[b]	nq[e]	nd[b]	nq[e]	nq[e]	na[c]	na[c]	na[c]	monoPAPs and diPAPs detected	[20]
Spain	Microwave popcorn bag	FUSLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	nd[b]-23.1	nd[b]-14.0	nd[b]-4.6	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: nd[b]-5.9 PFDoDA: nd[b]-2.5	[28]
Spain	Microwave popcorn bag	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	nd[b]-21.5	nd[b]-15.2	nd[b]-4.3	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: nd[b]-7.7 PFDoDA: nd[b]-2.9	[28]
Spain	Carboard cup	FUSLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	9.8	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 5.7	[28]
Spain	Carboard cup	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	9.2	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 7.2	[28]
Spain	Ice cream tub	FUSLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	nd[b]	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 6.1	[28]
Spain	Ice cream tub	PLE-LC/(-)ESI-MS/MS	na[c]	na[c]	na[c]	nd[b]	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	PFOS: 6.9	[28]
Greece	Beverage cup	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]
Greece	Ice cream cup	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]	< LOD[d]	25.6	< LOD[d]	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]

Table 8.5: Continuation.

Country	Packaging type	Method	Concentration range (ng/g)												Reference
			PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFDA	8:2 diPAP	6:2 diPAP	6:2 FTCA	6:2 FTUCA	5:3 FTCA	Others	
Greece	Fast food paper box	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]
Greece	Fast food wrapper	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]-3.2	< LOD[d]	< LOD[d]-19.2	< LOD[d]-10.0	< LOD[d]	< LOD[d]-28.2	na[c]	na[c]	na[c]	na[c]	na[c]	PFNA: < LOD[d]-5.0 PFDoDA: < LOD[d]-19.1	[24]
Greece	Paper materials for baking	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]
Greece	Microwave bag	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]-275.9	< LOD[d]	< LOD[d]-341.2	< LOD[d]-5.2	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]
Greece	Aluminium foil bag/wrapper	PLE-SPE(fluorisil-alumina-Na_2SO_4)-LC/(-)ESI-MS/MS	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	< LOD[d]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[24]
Spain	Microwave popcorn bag	FUSLE-LC/(-)ESI-MS/MS	nd[b]-280	nd[b]-37.0	2.2-405.0	1.3-7.5	nd[b]	nd[b]	na[c]	na[c]	na[c]	na[c]	na[c]	-	[36]

[a] < MDL: concentrations below method detection limit value
[b] nd: not detected
[c] na: not analyzed
[d] < LOD: concentrations below the limit of quantification value
[e] nq: Detected compounds but not quantified
*values expressed in ng/dm²

Among the monitored materials, microwave popcorn bags contained the highest PFCs concentrations. PFBA, PFPeA, PFHxA, PFHpA, PFOA and PFDA were the ones detected. PFBA (291 ng/g) and PFHxA (254 ng/g) were found at a very high concentration level. Similar results were obtained by Moreta and Tena [38], who reported PFBA and PFHxA concentrations up to 280 ng/g and 405 ng/g, respectively. Furthermore, Zafeiraki et al. [24] reported that microwave popcorn bags from the Greek market contained up to 276 and 341 ng/g of PFBA and PFHxA, respectively. In the case of PFPeA and PFHpA concentrations, up to 20.5 ng/g and 2 ng/g were quantified. Similar results (PFPeA and PFHpA concentrations ranging from 27 to 37 ng/g and 1.3 to 7.5 ng/g, respectively) were reported by Moreta and Tena [38]. Furthermore, PFOA and PFDA were under MDL values and PFNA was the only PFCAs that was not detected. Moreover, neither PFSAs, PFPAs nor PFOSA were detected. Similar distribution pattern of PFCs in popcorn bags was reported in the literature, where the mainly quantified PFCs were PFCAs [20,21,24,38]. However, in some cases, PFOS has also been quantified [25,26,28,34,39].

In the case of the rest of packaging materials, PFCs were not detected or they were below MDL values. Similar results were obtained when 42 Greek market packaging samples were analysed [24]. Only PFHxA was found in ice cream cup and several PFCs were detected in fast food wrappers. However, for the rest of the packaging samples, PFCs were under LOD values [24]. Moreover, Dolman and Pelzing [34] did not detect any PFC in the packaging samples analysed, except for microwave popcorn bags.

On the other hand, studies focused on the determination of potential precursors in packaging materials are limited. However, in our study, the presence of these precursors was evidenced. 8:2 diPAP was quantified in all the packaging samples except for French fries wrapper. Nevertheless, 6:2 diPAP was found under MDL value in almost

all the samples. The presence, although not the quantification, of these compounds has also been reported in the literature [20,29,35].

Moreover, in the case of microwave popcorn bag high concentrations of 6:2 FTCA (162 ng/g), 6:2 FTUCA (114 ng/g) and 5:3 FTCA (24.6 ng/g) were detected. To the best of our knowledge, this is the first work that reports the presence of FTCAs and FTUCAs in microwave popcorn bags. Recent studies have evidenced the biotransformation pathways from fluorotelomer-based compounds to PFCAs in microbial incubations, mammals and fish [40]. Thus, our preliminary results could be an evidence of the potential degradation of 6:2 diPAP in microwave popcorn bags. Besides, other studies reported that PFHxA concentration in microwave popcorn bags increases after cooking [24,38] and this concentration increment could be explained by the degradation pathways of other PFASs such as 6:2 diPAP or 6:2 FTOH. In this sense, more effort should be made in order to monitor other precursors in packaging materials and in order to evaluate the PFCs migration ability into food.

8.4 Conclusions

A thorough optimisation and validation of a very fast (2.5 min) and simple FUSLE method was performed for the analysis of up to twenty four PFASs, including PFASs and potential precursors in packaging samples. Apparent recoveries corrected with a labeled standard or matrix-matched calibration rendered satisfactory results with a single 2.5 min extraction step using 7 mL of MeOH (1 % HOAc). To the best of our knowledge, this is the first work where seven families of PFASs are quantified in packaging materials. When different packaging samples were analysed, microwave popcorn bags showed the highest levels, in ranges similar to those reported in other European countries. However, high levels of 6:2 FTCA, 6:2 FTUCA and 7:3 FTCA in microwave popcorn bags were reported for the first time. This could evidence the potential degradation of 6:2 diPAP in these packaging materials. Nevertheless, further

research should be performed in order to study the source of these degradation products and their possible ability to migrate to food.

8.5 References

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Chapter 9

Screening and identification of per- and polyfluoroalkyl substances in microwave popcorn bags

Food Chemistry 230 (2017) 497-506

9.1 Introduction

Packaging is designed to give the food greater safety assurance from microorganisms and biological and chemical changes, to offer easy transportation and storage, and to provide information to the consumers about ingredients and nutritional data [1], and, as such, it has become an essential element in the food manufacturing process. Therefore, the demand for packaging materials has risen dramatically during the past decades. However, concerns about food safety have increased recently since packaging has been found to represent a source of contamination itself through the migration of substances from the packaging into food [2]. In this sense, characterisation of food packaging materials is important to support good manufacturing practices and compliance with food safety regulations.

Among the different components of food packaging materials, fluorochemicals have gained special attention during the recent years since they have been detected in human blood from all around the world [3–8]. Commercially available industrial blends (Zonyl FSE, Zonyl Ur, Zonyl NF, etc), which are commonly applied on paper and board materials to provide water and oil repellence, have been found to contain 20-100 % of fluorinated chemicals [9]. Industrial mixtures consist primary of disubstituted polyfluoroalkyl phosphate diesters (diPAPs), with polyfluoroalkyl phosphate monoesters (monoPAPs) and polyfluoroalkyl phosphate triesters (triPAPs) being present as by-products [10]. MonoPAPs, diPAPs, and triPAPs belong to the group of polyfluoroalkyl phosphates (PAPs), which can be present in different forms depending on the levels of phosphate ester substitutions. Moreover, according to some studies recently performed in microbial systems, in rat or mice and in fish [11], PAPs were identified as potential precursors of per- and polyfluoroalkyl substances (PFASs) such as perfluorooctanoic acid (PFOA) or perfluorononanoic acid (PFNA). This means that

continued manufacturing of PFAS precursors may result in further accumulation of PFAS residues in the environment, wildlife, and humans.

To date, most works concerning packaging material have focused on perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs) [12–16]. However, it must be considered that the majority of the commercial fluorochemical production involves the use of fluorinated precursors, such as PAPs or fluorotelomer alcohols (FTOHs) and only few works reported PFASs potential precursors in packaging material [9,17,18]. Furthermore, attention should also be paid on fluorotelomer saturated acids (FTCAs) and fluorotelomer unsaturated acids (FTUCAs), which are intermediate degradation products of PAPs that can subsequently break down to form PFCAs. To the best of our knowledge, only one study reported the presence of PAP degradation intermediates in popcorn bags [19].

Although PFASs are found to be peroxisome proliferators, disruptors of the reproductive development and endocrine system, and tumor promoters [20–23], only a few highly fluorinated substances are currently governed by regulations. In the year 2006, the major fluorochemical manufacturers of Canada and the United States entered into a voluntary stewardship agreement to phase out the use and production of long-chain PFCAs (8 consecutive perfluorinated carbons or longer), as well as precursors by 2015 [24,25]. Furthermore, in the case of Europe, the European Union (EU) issued a Directive that regulated from June 2008 the general use of perfluorooctane sulfonate (PFOS) and derivates [26]. Afterwards, in 2009, PFOS was listed as “restricted use” compound under the Stockholm Convention on persistent organic pollutants (POPs) [27]. Finally, PFASs have been announced as emerging contaminants in the food chain by the European Food Safety Authority (EFSA), which have recently established the tolerable daily intakes (TDI) of 150 ng/kg day^{-1} for PFOS and 1500 ng/kg day^{-1} for perfluorooctane carboxylic acid (PFOA) [28]. Furthermore, EFSA recommended that an

additional monitoring focused on PFASs is needed. On this account, Commission Recommendation 2010/161/EU [29] invited the Member States to monitor the presence of PFOS and PFOA, different chain length (C_4-C_{15}) PFASs similar to PFOS and PFOA, and their precursors, in order to estimate the relevance of their presence in food. However, to date, there is no strict regulation concerning PFASs and potential precursors in packaging material. In Europe, the EU regulation No 10/2011 (EU Commission 2011), concerning plastic material intended to come into contact with food, established the list of substances that can be used, including PFOA, with different restrictions and specifications; however, there is no regulation concerning paper or cardboard packaging material. Moreover, some countries have their own national legislation about coating materials that will be in direct contact with foodstuffs. For instance, among European countries, Germany has a national legislation for paper and board material [30], which also considers some fluorochemicals; however, commonly monitored PFASs are not included. Outside Europe, in the USA, the US Drug and Food Administration established regulations about paper and paperboard components (US FDA website).

Within this context, the aim of the present work was to identify not only different PAPs and their end products but also their degradation intermediate products in popcorn bags, in order to overcome the challenge of trying to establish a link between PAPs and their end products. In this sense, high accurate mass spectrometry (HAMS) was used for fluorochemical detection and identification. Moreover, quantification of different PFASs in microwave popcorn bags from twelve European countries (Spain, France, Austria, The Netherlands, Hungary, Germany, Italy, Ireland, Czech Republic, Sweden, England and Portugal), three American countries (Mexico, Brazil and United States) and two Asian countries (India and China) was performed by liquid chromatography-triple quadrupole-tandem mass spectrometry (LC-QqQ-MS/MS), in order to find any pattern in their composition.

9.2 Experimental section

9.2.1 Reagents and materials

The PFASs and isotopically mass-labeled compounds quantified in the present study are shown in ***Tables 9.1*** and ***9.2***, respectively.

Table 9.1: *Structures, precursor and product ions (first ion was used as quantifier and the second as qualifier) at optimum fragmentor (V) and collision energy (eV) for LC-QqQ analysis.*

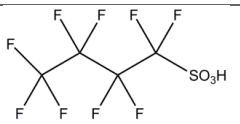
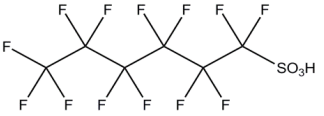
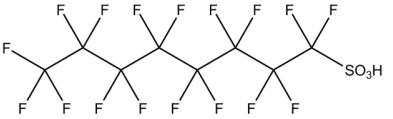
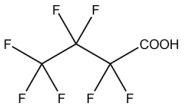
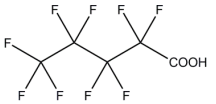
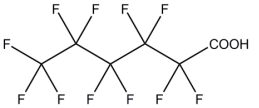
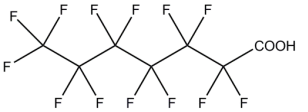
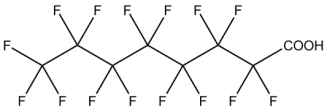
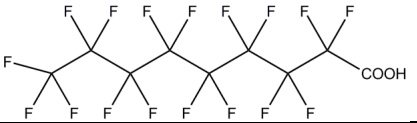
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
PFBS		299	99/80	100	30
PFHxS		399	99/80	150	20
PFOS		499	99/80	150	45
PFBA		213	169	60	5
PFPeA		263	219	60	5
PFHxA		313	269/119	60	10
PFHpA		363	319/169	60	10
PFOA		413	369/169	60	5
PFNA		463	419/169	60	5

Table 9.1: *Continuation.*

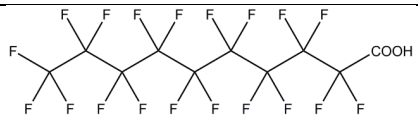
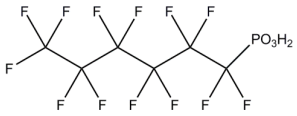
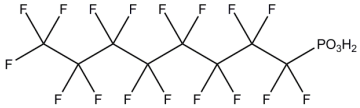
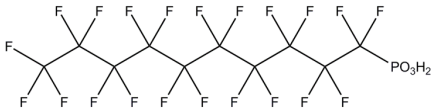
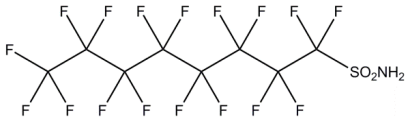
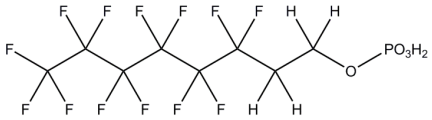
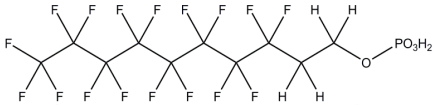
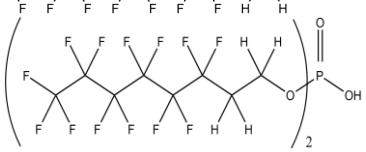
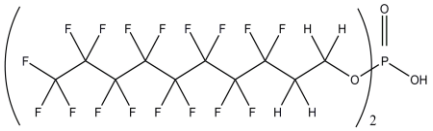
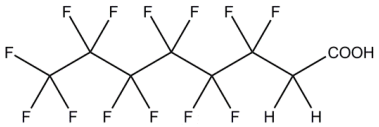
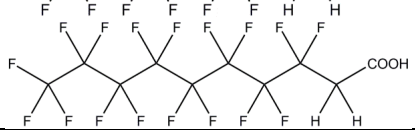
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
PFDA		513	469/269	100	5
PFHxPA		399	79	100	10
PFOPA		499	79	150	20
PFDPA		599	79	100	5
FOSA		498	78	220	5
6:2 monoPAP		443	97/79	90	13
8:2 monoPAP		543	97/79	90	21
6:2 diPAP		789	97/443	120	41
8:2 diPAP		989	97/543	135	41
6:2 FTCA		377	293	75	9
8:2 FTCA		477	393	75	9

Table 9.1: *Continuation.*

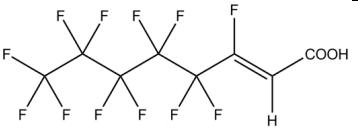
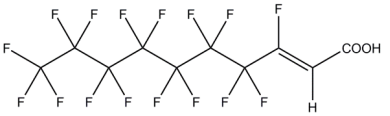
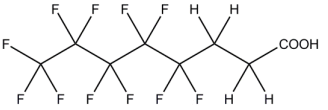
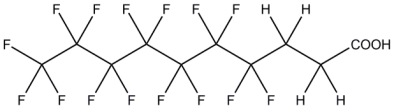
Analyte	Structure	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision Energy (eV)
6:2 FTUCA		357	293/243	75	9
8:2 FTUCA		457	393	75	9
5:3 FTCA		341	237/217	75	5
7:3 FTCA		441	337/317	75	5

Table 9.2: *Precursor and product ion at optimum fragmentor (V) and collision energy (eV) for surrogate standards, as well as which target analyte is corrected with each isotopic analogue.*

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Corrected compounds
MPFBA	217	172	60	5	PFBA
MPFHxA	315	270	60	5	PFPeA, PFHxA, PFHpA
MPFOA	417	372	60	5	PFOA
MPFNA	468	423	60	5	PFNA, FOSA
MFPDA	515	470	100	5	PFDA
MPFUnDA	565	520	60	5	- [a]
MPFDoDA	615	570	100	5	- [a]
MPFHxS	403	103	150	30	PFBS, PFHxS
MPFOS	503	99	60	45	PFOS
Cl-PFHxPA	415	79	105	45	PFHxPA
M8:2 diPAP	993	97	150	41	6:2diPAP, 8:2 diPAP
M8:2PAP	545	97	90	21	6:2 monoPAP, 8:2 monoPAP
M8:2 FTUCA	459	394	75	9	8:2 FTUCA
M6:2 FTCA	379	294	75	9	6:2 FTCA, 6:2 FTUCA, 5:3 FTCA
M8:2 FTCA	479	394	75	9	8:2 FTCA, 7:3 FTCA
M10:2 FTCA	579	494	75	9	- [a]

[a] *Surrogates not used for correction*

The PFASs and isotopically mass-labeled compounds quantified in the present study were all purchased from Wellington Laboratories (Ontario, Canada), except for perfluorooctane sulfonamide (FOSA), which was provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The purity of all the target analytes was > 98 %, except for FOSA (97.5 %). Methanol (MeOH, HPLC grade, 99.9 %) was supplied by LabScan (Dublin, Ireland) and acetic acid (HOAc, glacial, 100 %) from Merck (Darmstadt, Germany). For the clean-up step, a bulk Superclean Envi-Carb sorbent (100 m^2/g, 120/400 mesh) and empty SPE tubes (6 mL) were purchased from Supelco (Bellefonte, PA).

For the mobile phase composition, MeOH (UHPLC-MS, Scharlab S. L., Sentmenat, Barcelona) was used. 1-methyl piperidine (1-MP, $\geq$ 98 %) was obtained from Merck and ammonium acetate (NH_4OAc ≥ 99 %) was purchased from Sigma Aldrich (Madrid, Spain). High purity nitrogen gas (> 99.999 %) supplied by Messer (Tarragona, Spain) was used as collision gas and nitrogen gas (99.999 %), purchased from AIR Liquid (Madrid, Spain), was used as nebuliser and drying gas.

9.2.2 Sample collection and treatment

Microwave popcorn bags were purchased from Europe (Spain, France, Austria, The Netherlands, Hungary, Germany, Italy, Ireland, Czech Republic, Sweden, United Kingdom and Portugal), America (Mexico, Brazil and United States) and Asia (China and India) during 2015-2016 (all bags were ensured to be manufactured in the corresponding country). After removal of the food product, the paper was rinsed for salts with Milli-Q water and 1 dm^2 (~ 1 g) was collected and cut into small pieces.

Cardboard from popcorn bags was used for method validation. A known amount of matrix was weighed, covered with acetone, spiked with the target analytes and stirred during 24 hours. After that, acetone was evaporated and the sample was aged for one week.

9.2.3 Sample extraction and clean-up

The extraction procedure for the packaging material was a modification performed to the method published by Zabaleta et al. [19]. Briefly, 1 dm^2 of sample was placed together with 14 mL of MeOH (1 % HOAc) in a 40 mL vessel and surrogate standards (MPFHxS, MPFOS, MPFBA, MPFHxA, MPFOA, MPFNA, MPFDA, MPFUdA, MPFDoA, Cl- PFHxPA, M8:2 diPAP, M8:2 PAP, M8:2 FTUCA, M6:2 FTCA, M8:2 FTCA and M10:2 FTCA) were added (20 µL of a 0.3 ng/µL solution). The focused ultrasound solid liquid extraction (FUSLE, Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogeniser, 20 kHz; Bandelin Electronic, Berlin, Germany, equipped with a 3-mm titanium microtip) was performed for 2.5 min, with a sonication time of 0.8 s and a 30 % of amplitude. Extractions were carried out at 0 °C in an ice-water bath. After the extraction step, the supernatant was filtered through polyamide filters (0.45 µm, 25 mm, Macherey-Nagel, Germany) and the FUSLE extracts were evaporated to ~ 5 mL under a nitrogen stream using a Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA). In order to eliminate the ink of certain packaging materials a clean-up step was introduced using 500 mg Envi-Carb cartridges, previously conditioned with 5 mL of MeOH, where the extract was loaded and directly eluted. The eluate was concentrated to dryness under a gentle stream of N_2 at 35 °C and reconstituted in 350 µL of LC-MS grade MeOH. Polypropylene microfilters (0.22 µm, 13 mm, Phenomenex, California, USA) were used to filter extracts before LC-QToF or LC-QqQ analysis.

Blank sample extractions were simultaneously carried out in order to avoid any background contamination arising from any component of LC equipment or lab ware. Three blank samples were processed every 20 samples.

9.2.4 LC-QToF-MS analysis

Identification of fluorochemicals was performed using LC coupled to a QToF-MS with an electrospray ionisation (ESI) source. The apparatus consisted of a 1290 Infinity LC (Agilent Technologies, Wilmington, DE, USA) connected to a 6530 Accurate-Mass QToF- MS (Agilent Technologies) with a heated-ESI source (JetStream ESI). An ACE UltraCore 2.5 SuperC18 (2.1 mm x 100 mm, 2.5 µm) column (stable at pH range 1.5-11) coupled to a pre-column filter (0.5 µm, Vici Jour) was used and the column temperature was set to 35 °C.

Under optimised conditions, mobile phase A consisted of a Milli-Q water:MeOH (95:5) mixture and mobile phase B of MeOH:Milli-Q water (95:5), both containing 2 mmol/L NH_4OAc and 5 mmol/L 1-MP. The gradient profile started with 90 % A (hold time 0.3 min) and continued with a linear change to 80 % A up to 1 min, to 50 % A up to 1.5 min and to 20 % A up to 5 min (hold time 5 min), followed by a linear change to 0 % A up to 40 min and a hold time until 43 min. Initial conditions were regained at 44 min followed by equilibration until 55 min. The flow rate and the injection volume parameters were set at 0.3 mL/min and 10 µL, respectively.

The QToF-MS instrument was operated in the 2-GHz mode (extended dynamic range), which provides a full width at half-maximum (FWHM) resolution of approximately 4,700 at *m/z* 113 and 10,000 at *m/z* 1034. Negative polarity ESI mode was used under the following specific conditions: capillary voltage 3500 V, gas temperature 300 °C, gas flow 8 L/min, nebuliser pressure 50 psi, sheath gas temperature 300 °C and sheath gas flow 11 L/min. A reference calibration solution (provided by Agilent Technologies) was continuously sprayed into the ESI source of the QTOF-MS system. The ions selected for recalibrating the mass axis, ensuring the mass accuracy throughout the run were m/z 112.9856 and 980.0164 for the negative mode. The QToF-MS device acquired from m/z 50 to 1500 in data-dependent acquisition mode

(auto-MS/MS) using three different collision energies (5, 15, and 30 eV) for the fragmentation of the selected parent ions. For some fluorochemicals, additional injections in targeted MS/MS were necessary in order to obtain proper MS/MS fragmentation data.

Identification was based on mass accuracy and isotopic abundance obtained in MS mode, on the MS/MS fragmentation patterns and the accurate masses of the product ions. The raw data was examined by manual processing in MassHunter, using a mass window of 10 ppm around the precursor ion. When possible, the confirmation was also verified with the corresponding standard.

9.2.5 LC-QqQ analysis

Mobile phase, gradient profile, flow rate and instrument parameters and conditions were detailed in previously published works [31,32]. Fragmentor and collision energy parameters and monitored ion transitions for each analyte and surrogate standards are given in *Tables 9.1* and *9.2*, respectively.

9.3 Results and discussion

9.3.1 FUSLE-Envi Carb-LC-QqQ performance evaluation

Microwave popcorn bag samples were extracted using a modified version of the method developed in our previous work [19]. Due to the high pigment content of some of the packing material extracts, which blocked the LC injection due to overpressure, a clean-up step based on Envi-Carb was performed after the extraction. Method validation was performed and apparent recoveries were calculated at 10 ng/g (n=7). Labeled standards were used for apparent recovery calculation, except for perfluorooctane phosphonic acid (PFOPA) and perfluorodecane phosphonic acid (PFDPA) whose concentrations were assessed relative to external standard calibration. Good apparent recoveries were obtained for all target analytes (68-104 %), except for

PFOPA (39 %) and PFDPA (46 %) due to the lack of the corresponding labelled internal standards for correction. Precision in terms of RSD varied between 8-20 % for all target analytes. Moreover, method detection limit (MDL) values (calculated as MDL = $t_{(n-1,\ 1-\alpha\ =\ 0.95)}$ x s_d, where t = 1.94 corresponds to the Student's t-value for a 95 % confidence level and 6 degrees of freedom, whereas s_d is the standard deviation of the replicate analyses), in the range of 0.7-3.5 ng/g were obtained by means of LC-QqQ (see recoveries and MDL values in ***Table 9.3***). Similar MDL values (0.6-2.2 ng/g) were obtained when only a FUSLE methodology without a clean-up step was used for PFASs quantification in packaging materials [19].

Table 9.3. *Recoveries (%) and MDL values for PFCAs, PFSAs, PFPAs and potential precursors in fortified cardboard packaging samples at 10 ng/g concentration level. Results obtained using a LC-QqQ.*

Analyte	Recovery with external calibration (%) ± s	Apparent recovery with external calibration (%) ± s	MDL (ng/g)
PFBA	50 ± 5	93 ± 13	1.4
PFPeA	49 ± 5	98 ± 14	1.3
PFHxA	49 ± 6	96 ± 15	1.8
PFHpA	48 ± 5	94 ± 14	1.5
PFOA	50 ± 5	95 ± 14	1.9
PFNA	50 ± 6	96 ± 16	1.9
PFDA	48 ± 6	92 ± 14	2.3
PFBS	57 ± 7	103 ± 19	2.8
PFHxS	51 ± 7	94 ± 15	2.6
PFOS	55 ± 9	94 ± 17	3.5
PFOSA	46 ± 6	90 ± 12	2.8
PFHxPA	35 ± 4	77 ± 10	1.6
PFOPA	39 ± 3	39* ± 3	1.3
PFDPA	46 ± 9	46* ± 9	3.3
6:2 monoPAP	34 ± 5	81 ± 12	1.8
8:2 monoPAP	28 ± 2	68 ± 5	0.8
6:2 diPAP	43 ± 4	104 ± 12	2.0
8:2 diPAP	39 ± 6	94 ± 15	2.8
6:2 FTCA	31 ± 2	97 ± 15	0.9
8:2 FTCA	37 ± 3	88 ± 13	1.3
6:2 FTUCA	27 ± 1	87 ± 11	0.7
8:2 FTUCA	34 ± 2	84 ± 11	1.1
7:3 FTCA	30 ± 2	71 ± 10	1.1
5:3 FTCA	27 ± 2	86 ± 12	0.9

**Concentration without correction due to the lack of the correct labeled standard.*

9.3.2 Identification of fluorochemicals in popcorn bag

LC-QToF-MS analysis in auto-MS/MS acquisition mode was performed to identify different fluorochemicals. In this sense, different chain length of PFCAs, FTCAs, FTUCAs, monoPAPs, diPAPs, and triPAPs were found, which gave distinct peaks in the extracted ion current (EIC) chromatograms. The identified fluorochemicals, their theoretical masses, masses error (ppm), product ions and molecular formula are shown in ***Table 9.4***.

Table 9.4. *Identified fluorochemicals with the theoretical m/z, mass error (ppm), the product ions and the chemical formula.*

Analyte	Theoretical m/z $[M-H]^-$	Error (ppm)	Product ions	Chemical formula
Perfluorocarboxylate (PFCA)				
PFBA	212.9792	1.88	168.9878	$CF_3(CF_2)_2COOH$
PFPeA	262.9760	1.90	218.9854	$CF_3(CF_2)_3COOH$
PFHxA	312.9728	2.24	118.9928, 268.9846	$CF_3(CF_2)_4COOH$
PFHpA	362.9696	0.28	168.9884, 318.9819	$CF_3(CF_2)_5COOH$
PFOA	412.9664	-0.73	168.9878, 368.9781	$CF_3(CF_2)_6COOH$
PFNA	462.9632	3.02	168.9861, 418.9746	$CF_3(CF_2)_7COOH$
PFDA	512.9600	-3.12	268.9826, 468.9745	$CF_3(CF_2)_8COOH$
PFUnDA	562.9568	9.94	168.9889, 218.9849, 268.9805, 318.9772	$CF_3(CF_2)_9COOH$
PFDoDA	612.9537	-0.33	168.9905, 568.9646	$CF_3(CF_2)_{10}COOH$
PFTrDA	662.9505	0.90	-	$CF_3(CF_2)_{11}COOH$
PFTeDA	712.9423	-3.08	668.9565	$CF_3(CF_2)_{12}COOH$
PFPeDA	762.9441	-3.54	718.9516	$CF_3(CF_2)_{13}COOH$
PFHxDA	812.9409	3.69	268.9830, 768.9555	$CF_3(CF_2)_{14}COOH$
Fluorotelomer saturated and unsaturated carboxylate (FTCA and FTUCA)				
6:2 FTCA	376.9853	-0.27	292.9736	$CF_3(CF_2)_5CH_2COOH$
8:2 FTCA	476.9789	-4.19	392.9755	$CF_3(CF_2)_7CH_2COOH$
10:2 FTCA	576.9725	-3.12	-	$CF_3(CF_2)_9CH_2COOH$
6:2 FTUCA	356.9790	3.64	292.9836	$CF_3(CF_2)_4CF=CHCOOH$
8:2 FTUCA	456.9727	-1.31	392.9735	$CF_3(CF_2)_6CF=CHCOOH$
10:2 FTUCA	556.9663	2.15	-	$CF_3(CF_2)_8CF=CHCOOH$
5:3 FTCA	341.0041	-0.59	216.9887, 236.9950	$CF_3(CF_2)_4CH_2CH_2COOH$
7:3 FTCA	440.9977	0.22	336.9913	$CF_3(CF_2)_6CH_2CH_2COOH$
9:3 FTCA	540.9913	-1.48	-	$CF_3(CF_2)_8CH_2CH_2COOH$
5:3 FTUCA	338.9885	3.83	118.9935, 254.9861, 268.9853, 294.9937	$CF_3(CF_2)_4CH=CHCOOH$
7:3 FTUCA	438.9821	0.46	-	$CF_3(CF_2)_6CH=CHCOOH$
9:3 FTUCA	538.9757	1.48	-	$CF_3(CF_2)_8CH=CHCOOH$
Polyfluoroalkyl phosphate monoester (monoPAP)				
6:2 monoPAP	442.9723	-1.81	78.9588, 96.9703	$CF_3(CF_2)_5CH_2CH_2O\text{-}P(O)(OH)_2$
8:2 monoPAP	542.9659	-1.84	78.9586, 96.9692, 522.9563	$CF_3(CF_2)_7CH_2CH_2O\text{-}P(O)(OH)_2$
Polyfluoroalkyl phosphate diester (diPAP)				
6:2/6:2 diPAP	788.9751	-1.01	78.9585, 96.9690, 422.9632, 442.9691	$(CF_3(CF_2)_5CH_2CH_2O)_2\text{-}P(O)OH$

Table 9.4: *Continuation.*

Analyte	Theoretical m/z $[M-H]^-$	Error (ppm)	Product ions	Chemical formula
8:2/8:2 diPAP	988.9623	1.21	78.9594, 96.9705, 522.9565, 542,9625	$(CF_3(CF_2)_7CH_2CH_2O)_2$-P(O)OH
6:2/8:2 diPAP	888.9687	1.80	78.9587, 96.9687, 422.9643, 442.9715, 522.9579, 542.9654	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_7CH_2CH_2O)$
6:2/10:2 diPAP	988.9623	0.30	78.9584, 96.9694, 422.9684, 442.9752, 622.9569, 642.9557	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_9CH_2CH_2O)$
8:2/10:2 diPAP	1088.9559	-3.67	78.9591, 96.9696, 522.9581, 542.9640, 622.9503, 642.9573	$(CF_3(CF_2)_7CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_9CH_2CH_2O)$
6:2/12:2 diPAP	1088.9559	0.83	78.9584, 96.9694, 422.9630, 442.9695, 722.9443, 742.9472	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{11}CH_2CH_2O)$
10:2/10:2 diPAP	1188.9495	-0.67	78.9591, 96.9698, 622.9573, 642.9540	$(CF_3(CF_2)_9CH_2CH_2O)_2$-P(O)OH
8:2/12:2 diPAP	1188.9495	-0.42	78.9594, 96.9693, 522.9577, 542.9640, 722.9426, 742.9541	$(CF_3(CF_2)_7CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{11}CH_2CH_2O)$
6:2/14:2 diPAP	1188.9495	-0.59	78.9587, 96.9700, 422.9650, 442.9758, 822.9445, 842.9503	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{13}CH_2CH_2O)$
10:2/12:2 diPAP	1288.9431	-0.70	78.9594, 96.9696, 622.9533, 642.9581, 722.9443, 742.9529	$(CF_3(CF_2)_9CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{11}CH_2CH_2O)$
8:2/14:2 diPAP	1288.9431	1.47	78.9591, 96.9691, 522.9549, 542.9641, 822.9431, 842.9452	$(CF_3(CF_2)_7CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{13}CH_2CH_2O)$
6:2/16:2 diPAP	1288.9431	-1.47	78.9587, 96.9696, 422.9694, 442.9725, 922.9300, 942.9404	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)(OH)-$(CF_3(CF_2)_{15}CH_2CH_2O)$
Polyfluoroalkyl phosphate triester (triPAP)				
6:2/6:2/6:2 triPAP	788.9751	1.39	-	$(CF_3(CF_2)_5CH_2CH_2O)_3$-P(O)
6:2/6:2/8:2 triPAP	788.9751 888.9687	6.46 0.34	-	$(CF_3(CF_2)_5CH_2CH_2O)_2$-P(O)-$(CF_3(CF_2)_7CH_2CH_2O)$
6:2/8:2/8:2 triPAP	888.9687 988.9623	-0.79 0.00	-	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)-$(CF_3(CF_2)_7CH_2CH_2O)_2$
6:2/6:2/10:2 triPAP	788.9751 988.9623	-1.01 -2.12	-	$(CF_3(CF_2)_5CH_2CH_2O)_2$-P(O)-$(CF_3(CF_2)_9CH_2CH_2O)$
8:2/8:2/8:2 triPAP	988.9623	-1.11	-	$(CF_3(CF_2)_7CH_2CH_2O)_3$-P(O)
6:2/8:2/10:2 triPAP	888.9687 988.9623 1088.9559	1.46 -2.32 -0.46	-	$(CF_3(CF_2)_5CH_2CH_2O)$-P(O)-$(CF_3(CF_2)_7CH_2CH_2O)$ -$(CF_3(CF_2)_9CH_2CH_2O)$
6:2/6:2/12:2 triPAP	1088.9559	-4.41	-	$(CF_3(CF_2)_5CH_2CH_2O)_2$-P(O)-$(CF_3(CF_2)_{11}CH_2CH_2O)$

- *Accurate masses could not be measured.*

Industrial PAP mixtures consist primarily of diPAPs, with monoPAPs and triPAPs being present as by-products. In the present work, among diPAPs, precursors ions of

m/z 789, 889, 989, 1089, 1189, and 1289 were identified. Moreover, in the case of m/z 989, 1089, 1189 and 1289 ions, more than one structural isomer was found. For the identification of these structural isomers, different gradients were tested to achieve a proper peak separation. In this way, for the precursor ion at m/z 989, two structural isomers were observed (see **Figure 9.1**). From their product ion spectra, they could be described as the 8:2/8:2 diPAP (product ions at m/z 97, 523, 543) and 6:2/10:2 diPAP (product ions at m/z 97, 423, 443, 623, 643) isomers, respectively. Also for the precursor ion at m/z 1089 two structural isomers were separated (see **Figure 9.2**), which corresponded to the 8:2/10:2 diPAP (product ions at m/z 97, 523, 543, 623, 643) and 6:2/12:2 diPAP (product ions at m/z 97, 423, 443, 723, 743) isomers, respectively. In the case of the precursor ion at m/z 1189, three structural isomers were identified (see **Figure 9.3**) as the 10:2/10:2 diPAP (product ions at m/z 97, 623, 643), 8:2/12:2 diPAP (product ions at m/z 97, 523, 543, 723, 743) and 6:2/14:2 diPAP (product ions at m/z 97, 423, 443, 823, 843) isomers, respectively. Finally, with the precursor ion at m/z 1289, three structural isomers (see **Figure 9.4**) were described as the 10:2/12:2 diPAP (product ions at m/z 97, 623, 643, 723, 746), 8:2/14:2 diPAP (product ions at m/z 97, 523, 543, 823, 843) and 6:2/16:2 diPAP (product ions at m/z 97, 423, 443, 923, 943) isomers, respectively. In order to identify the different isomers, the product ions in the MS^2 spectra (see **Figures 9.1**, **9.2**, **9.3** and **9.4**) were studied. While the product ion at m/z 97 corresponded to the phosphonate group, ions at m/z 343, 443, 543, 643, 743, 843 and 943 corresponded to different length monoPAPs (4:2 monoPAP, 6:2 monoPAP, 8:2 monoPAP, 10:2 monoPAP, 12:2 monoPAP, 14:2 monoPAP, 16:2 monoPAP, respectively). Moreover, product ions at m/z 323, 423, 523, 623, 723, 823 and 923 corresponded to the monoPAP characteristic neutral loss of 20 u, representing HF, of 4:2 monoPAP, 6:2 monoPAP, 8:2 monoPAP, 10:2 monoPAP, 12:2 monoPAP, 14:2 monoPAP and 16:2 monoPAP, respectively. Several studies have also detected structural isomers of PAPs in popcorn bags [9,17,18].

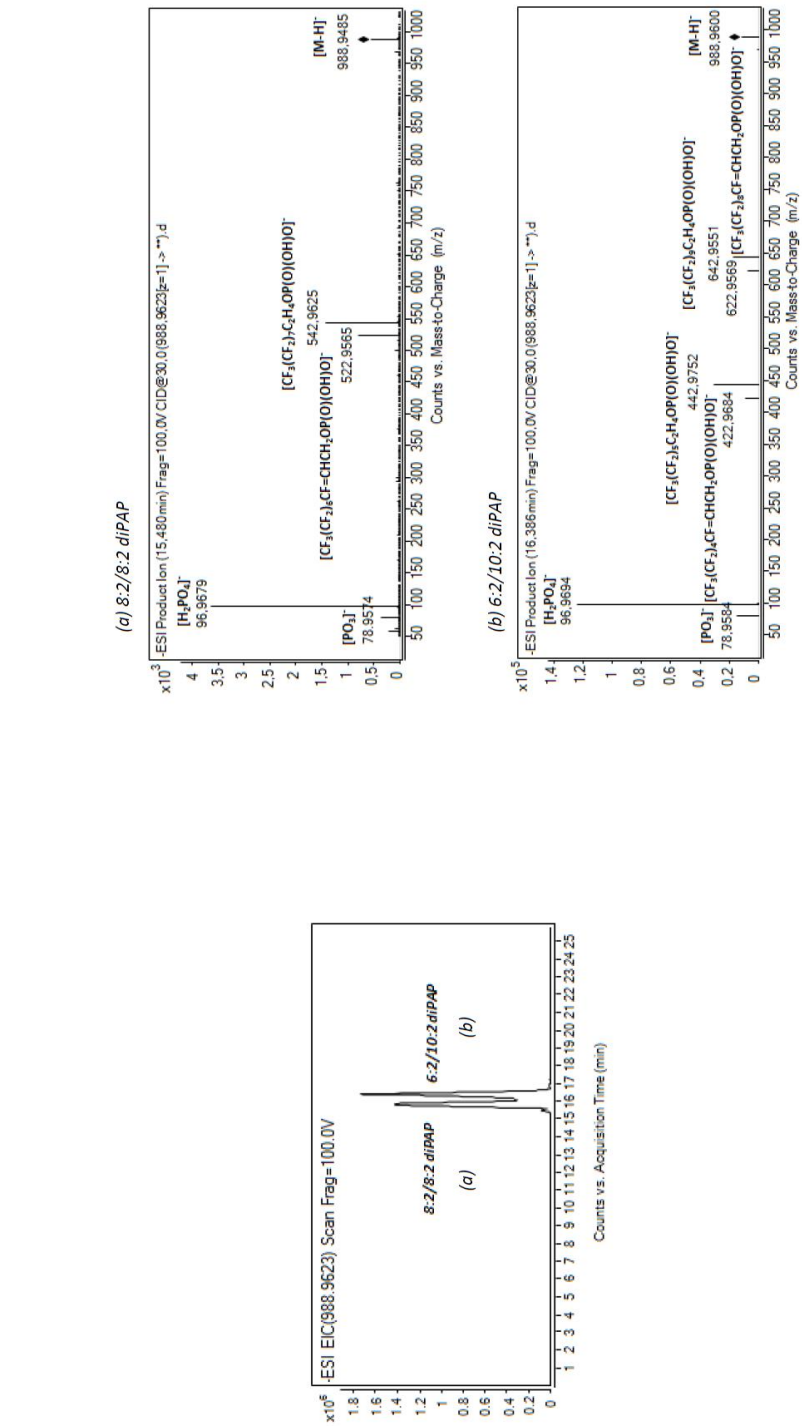


Figure 9.1: EIC chromatogram (left) of m/z 989, which belongs to the 8:2/8:2 diPAP and 6:2/10:2 diPAP, and MS² spectra (right) with molecular formula for both structural isomers found in an extract of microwave popcorn bag.

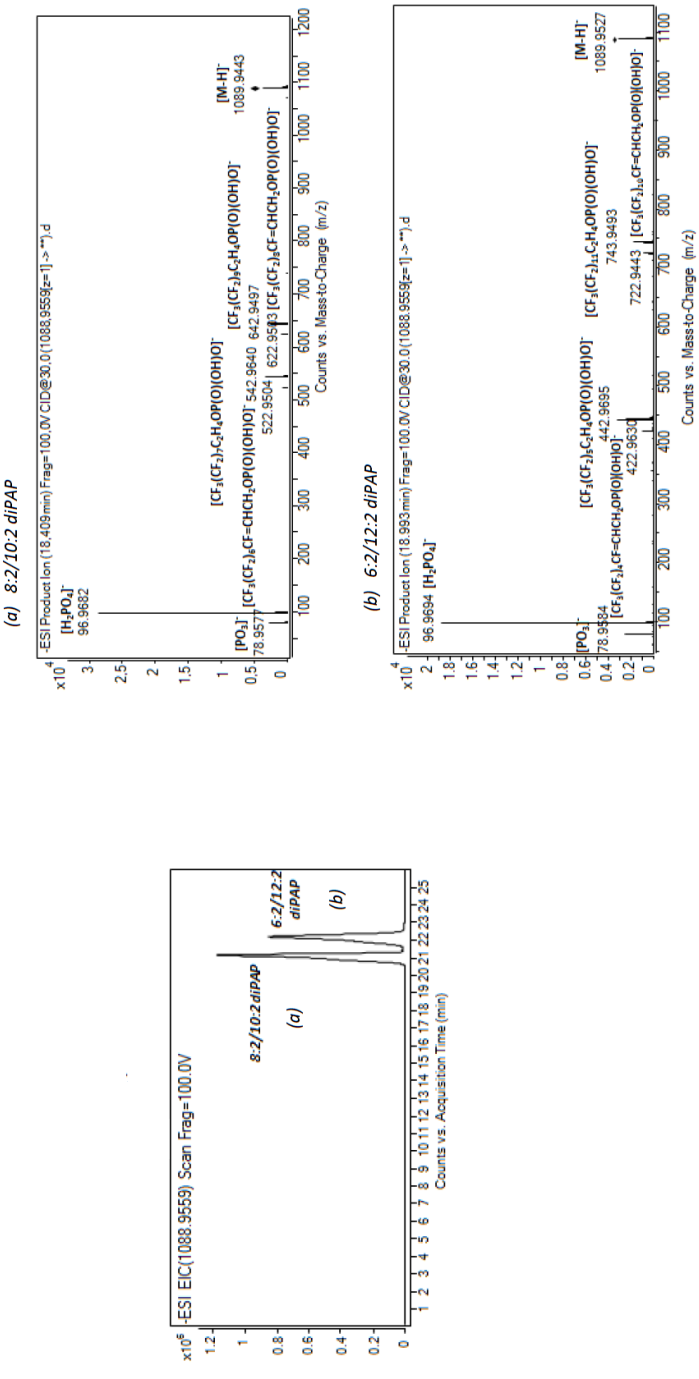


Figure 9.2: EIC chromatogram (left) of m/z 1089, which belongs to the 8:2/10:2 diPAP and 6:2/12:2 diPAP, and MS^2 spectra (right) with molecular formula for both structural isomers found in an extract of microwave popcorn bag.

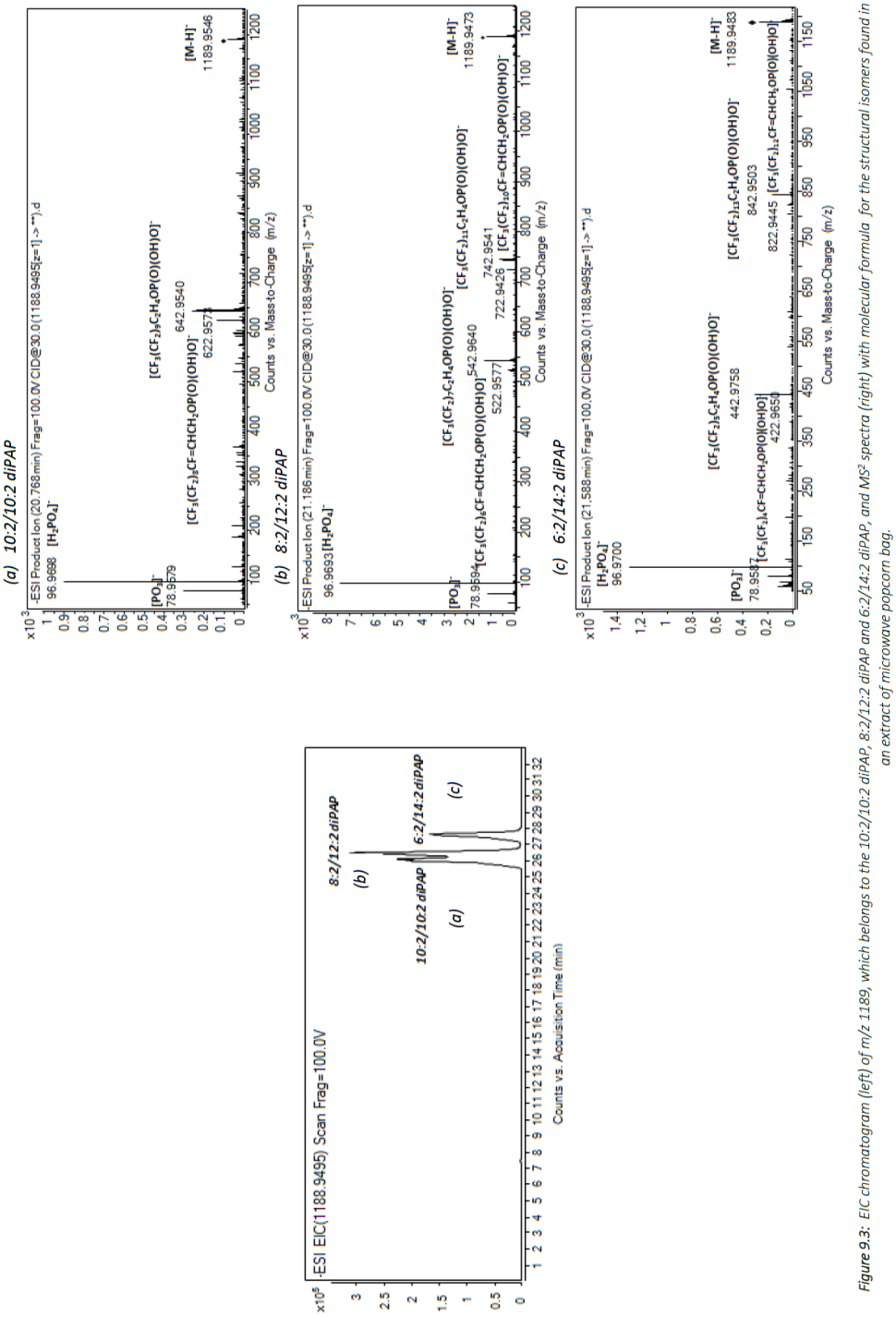


Figure 9.3: EIC chromatogram (left) of m/z 1189, which belongs to the 10:2/10:2 diPAP, 8:2/12:2 diPAP and 6:2/14:2 diPAP, and MS² spectra (right) with molecular formula for the structural isomers found in an extract of microwave popcorn bag.

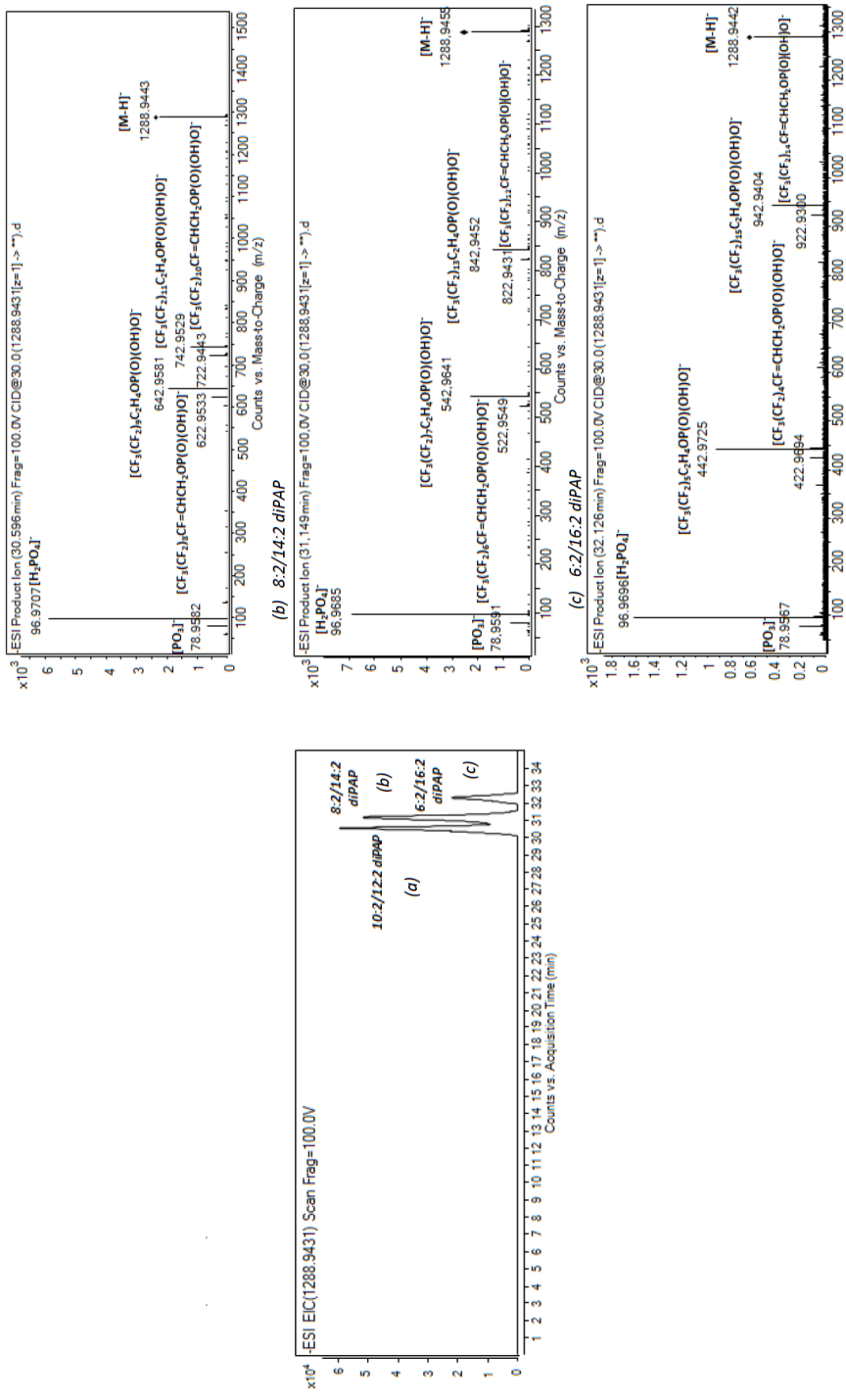


Figure 9.4: EIC chromatogram (left) of m/z 1289, which belongs to the 10:2/12:2 diPAP, 8:2/14:2 diPAP and 6:2/16:2 diPAP, and MS² spectra (right) with molecular formula for the structural isomers found in an extract of microwave popcorn bag.

Although different chain length monoPAPs were looked for, only 6:2 and 8:2 monoPAPs were identified. In the product ion spectrum, the representative ion at m/z 97 of the phosphonate group and the characteristic neutral loss of 20 u, representing HF, was observed (data not shown).

Low sensitive peaks corresponding to seven triPAP isomers were also observed (see Figure 9.5). To the best of our knowledge, this is the first method that achieved a proper chromatographic peak separation of seven triPAPs. Although Gebbink et al. [18] detected triPAPs in popcorn bags, five of the triPAP isomers coeluted in two peaks.

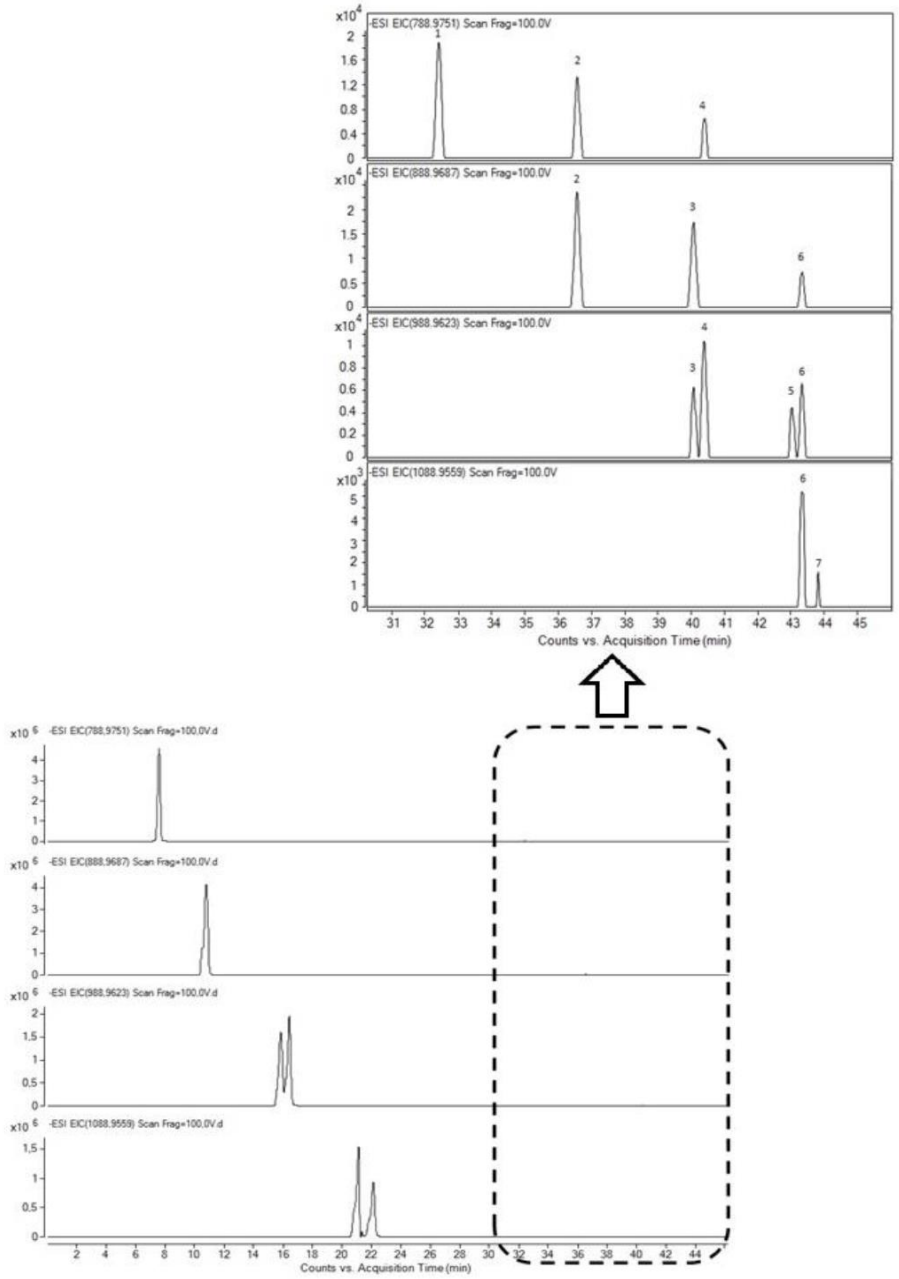


Figure 9.5: *EIC of triPAPs in an extract from a microwave popcorn bag. Seven triPAPs were tentatively identified: (1) 6:2/6:2/6:2 triPAP, (2) 6:2/6:2/8:2 triPAP, (3) 6:2/8:2/8:2 triPAP, (4) 6:2/6:2/10:2 triPAP, (5) 8:2/8:2/8:2 triPAP, (6) 6:2/8:2/10:2 triPAP and (7) 6:2/6:2/12:2 triPAP.*

It has been reported that triPAPs may form diPAP product ions due to in-source fragmentation [17]. In this sense, some triPAPs have more than one precursor ion depending on how they are ionised (Example *Figure 9.6*).

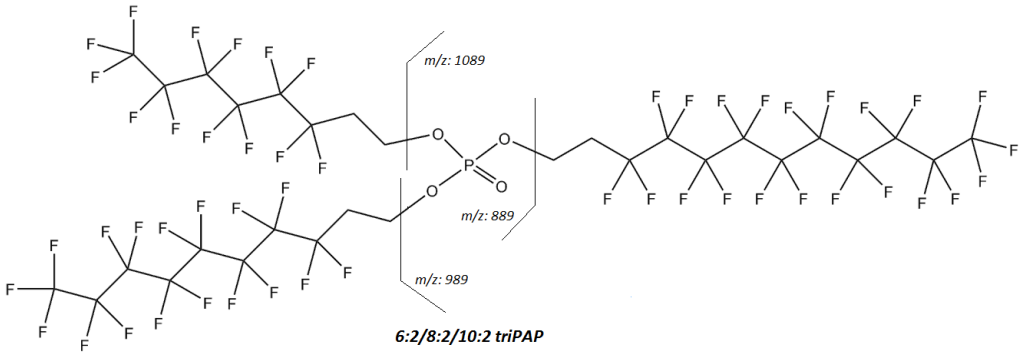


Figure 9.6: 6:2/8:2/10:2 triPAP structure showing the possible in-source fragmentations.

Thus, the precursor ion at m/z 789 and retention time (t_R) of 32.4 min represents 6:2/6:2/6:2 triPAP, precursor ions at m/z 789 and 889 and t_R of 36.5 min represent 6:2/6:2/8:2 triPAP, precursor ions at m/z 889 and 989 and t_R of 40.1 min represent 6:2/8:2/8:2 triPAP, precursor ions at m/z 789 and 989 and t_R of 40.4 min represent 6:2/6:2/10:2 triPAP, precursor ion at m/z 989 and t_R of 44 min represent 8:2/8:2/8:2 triPAP, precursor ions at m/z 889 and 989 and t_R of 43.3 min represent 6:2/8:2/10:2 triPAP and precursor ion at m/z 1089 and t_R of 43.8 min represent 6:2/6:2/12:2 triPAP. For the latter, a precursor ion at m/z 789 should also have appeared and the lack of this peak could be attributed to low sensitivity. These seven triPAPs have been only tentatively identified since, as they are found at very low sensitivity, accurate masses for the product ions could not be measured (Table 9.4).

FTCAs and FTUCAs represent the intermediate precursors of triPAPs, diPAPs or monoPAPs and can be transformed into PFCAs. In this case, 6:2 FTCA, 8:2 FTCA, 6:2 FTUCA, 8:2 FTUCA, 5:3 FTCA, 7:3 FTCA and 5:3 FTUCA were identified. Although

6:2 FTCA, 6:2 FTUCA and 5:3 FTCA had been already reported in popcorn bag in our previous work [19], to the best of our knowledge, this is the first time that the rest of the intermediates are identified in popcorn bags. All these intermediates were also confirmed with the corresponding standard, except for 5:3 FTUCA, whose standard is not available. However, the fragmentation pattern and the accurate masses of the precursor and product ions support its identity. The intermediate 5:3 FTUCA presented a neutral loss of 44 u (CO_2) and a subsequent neutral loss of 40 u (2 HF) (see ***Figure 9.7***).

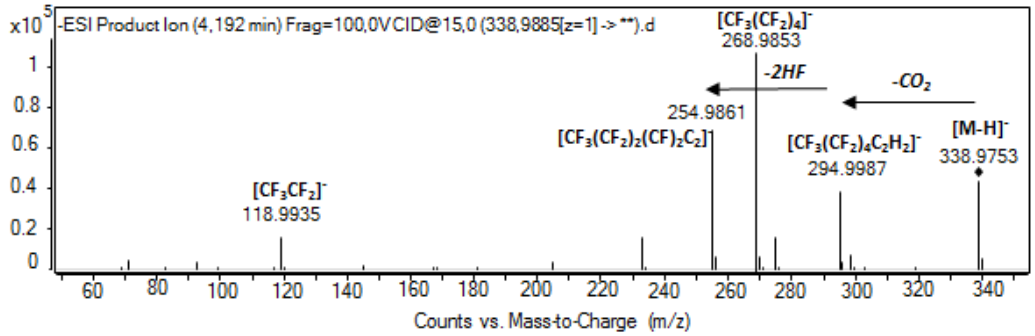


Figure 9.7: *MS^2 spectra with molecular formula for 5:3 FTUCA in an extract of microwave popcorn bag.*

These losses had been also reported for the intermediate 7:3 FTUCA detected in rat hepatocytes [33]. Moreover, product ion chromatogram for some other intermediates could not be obtained due to lack of sensitivity in the EIC chromatogram. Therefore, 7:3 FTUCA (mass error: 0.46 ppm), 10:2 FTCA (mass error: -3.12 ppm), 10:2 FTUCA (mass error: 2.15 ppm), 9:3 FTCA (mass error: -1.48 ppm), 9:3 FTUCA (mass error: 1.48 ppm) were only tentatively identified.

Finally, PFCAs represent the end products of fluorotelomer-based compound degradation. Different length (C_4-C_{16}) PFCAs were found in the EIC chromatograms. PFTrDA was the only analyte tentatively identified (error: 0.9 ppm).

To sum up, up to 46 fluorochemicals were identified in popcorn bags: 21 precursors (monoPAPs, diPAPs, and triPAPs), 12 intermediates (FTCAs and FTUCAs) and 13 PFCAs.

9.3.3 Quantification by LC-QqQ of fluorochemicals in popcorn bags around the world

Popcorn bags from twelve European countries, three American countries and two Asian countries were analysed in order to quantify fluorochemicals for which the corresponding standards were available (see *Table 9.5*). Blank samples were analysed in parallel and concentrations lower that the MDLs were obtained.

Among the European countries, PFASs with different lengths were quantified, the short chain PFCAs (C_4-C_8) being the predominant ones. Particularly in Spain three different brands of popcorn bags (Brands 1, 4 and 5) showed the highest concentrations of PFBA (250-820 ng/g) and PFHxA (174-811 ng/g). Similar results have been previously reported for these compounds in Spanish popcorn packaging at levels up to 280 ng/g and 405 ng/g of PFBA and PFHxA, respectively [34]. In addition, PFPeA, PFHpA and PFOA were quantified ranging from 15 to 73 ng/g, from MDL values to 15 ng/g and from 4 to 27 ng/g, respectively. Concentrations ranging from 37 to 99 ng/g and from 63 to 198 ng/g were also reported for PFHpA and PFOA in Spanish bags, respectively [15].The rest of the European countries (France, The Netherlands, Germany, Hungary, Portugal, Ireland, United Kingdom, Italy, Sweden, Czech Republic and Austria) showed low levels of PFCs, being PFHxA (< MDL-3.7 ng/g) and PFOA (<MDL-4.5 ng/g) the ones detected at the highest concentrations. It could be underlined that in popcorn bags from the Greek market up to 276, 341 and 5 ng/g levels of PFBA, PFHxA and PFHpA, respectively, were reported [12].

Table 9.5: *PFCA concentrations (ng/g) in popcorn bag samples from different countries around the world.*

Location	Samples	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA
European Countries								
Spain	Brand 1 (Salty)	250 ± 59	15 ± 3	174 ± 41	< MDL	27 ± 3	-	-
	Brand 1 (Light)	-	-	2.8 ± 0.8	-	< MDL	-	-
	Brand 2 (Salty)	-	-	2.5 ± 0.2	-	< MDL	-	-
	Brand 2 (Butter)	-	-	1.9 ± 0.1	-	< MDL	-	-
	Brand 3 (Salty)	-	-	0.6 ± 0.1	< MDL	4.6 ± 0.6	-	< MDL
	Brand 4 (Salty)	351 ± 32	36 ± 3	505 ± 52	14 ± 2	22 ± 2	< MDL	< MDL
	Brand 5 (Salty)	820 ± 124	73 ± 5	811 ± 232	15 ± 3	4.4 ± 0.6	< MDL	< MDL
France	Brand 6 (Salty)	< MDL	< MDL	3.7 ± 0.4	< MDL	3.9 ± 0.2	-	< MDL
	Brand 6 (Sweet)	< MDL	< MDL	2.8 ± 0.2	< MDL	4.2 ± 0.2	< MDL	< MDL
	Brand 6 (Sugar)	< MDL	< MDL	3.5 ± 0.7	< MDL	4.5 ± 0.6	< MDL	< MDL
	Brand 7 (Salty)	-	-	2.0 ± 0.1	< MDL	3.6 ± 0.8	< MDL	< MDL
	Brand 8 (Original)	-	-	< MDL	-	< MDL	< MDL	< MDL
	Brand 9 (Salty)	-	-	< MDL	-	< MDL	< MDL	< MDL
The Netherlands	Brand 10 (Salty)	< MDL	< MDL	2.5 ± 0.3	<MDL	4.1 ± 0.6	-	-
	Brand 11 (Salty)	-	< MDL	4 ± 1	< MDL	-	< MDL	-
Germany	Brand 12 (Salty)	< MDL	< MDL	3.1 ± 0.9	< MDL	4 ± 1	< MDL	< MDL
Hungary	Brand 13 (Salty)	-	-	< MDL	-	3.7 ± 0.3	< MDL	-
	Brand 14 (Salty)	-	-	2.1 ± 0.3	-	< MDL	-	< MDL
Portugal	Brand 15 (Salty)	-	-	< MDL	< MDL	3.0 ± 0.2	< MDL	< MDL
Ireland	Brand 16 (Salty)	-	-	-	-	-	< MDL	-
United Kingdom	Brand 17 (Salty)	< MDL	< MDL	3.5 ± 0.5	< MDL	< MDL	< MDL	< MDL
	Brand 17 (Butter)	-	< MDL	2.4 ± 0.1	< MDL	< MDL	< MDL	-
Italy	Brand 18 (Salty)	< MDL	< MDL	3.3 ± 0.4	< MDL	< MDL	< MDL	< MDL
Austria	Brand 19 (Salty)	< MDL	< MDL	1.9 ± 0.3	< MDL	< MDL	< MDL	< MDL
Czech Republic	Brand 20 (Salty)	< MDL	-	2.5 ± 0.9	-	< MDL	-	< MDL
Sweden	Brand 21 (Salty)	< MDL	< MDL	2.3 ± 0.6	< MDL	< MDL	< MDL	< MDL
	Brand 22(Original)	< MDL	-	< MDL	-	< MDL	< MDL	< MDL
	Brand 22 (Butter)	< MDL	-	< MDL	-	-	< MDL	< MDL
American Countries								
Mexico	Brand 23 (Butter)	-	-	< MDL	-	3.5 ± 0.5	-	-
	Brand 23 (Extra butter)	-	-	< MDL	-	3.8 ± 0.6	-	-
	Brand 24 (Natural)	-	-	< MDL	-	< MDL	-	< MDL
	Brand 25 (Natural)	-	-	-	-	< MDL	-	< MDL
	Brand 26 (Natural)	-	-	< MDL	-	< MDL	< MDL	< MDL
Brazil	Brand 27 (Natural)	6 ± 1	5.4 ± 0.8	27.5 ± 0.2	3.9 ± 0.4	< MDL	< MDL	-
	Brand 28 (Butter)	-	-	-	-	< MDL	-	-
	Brand 29 (Natural)	< MDL	< MDL	2.9 ± 0.3	4.2 ± 0.6	< MDL	-	2.8 ± 0.1
	Brand 30 (Butter)	3.8 ± 0.1	3.4 ± 0.1	15 ± 1	5.0 ± 0.3	< MDL	-	< MDL
USA	Brand 31 (Salty and butter)	-	-	< MDL	< MDL	< MDL	< MDL	-
	Brand 24 (Butter)	< MDL	-	< MDL	-	< MDL	-	< MDL
	Brand 32	-	-	-	-	-	-	-
Asian Countries								
India	Brand 24 (Cheddar Chease)	-	-	-	-	< MDL	-	14 ± 4
China	Brand 33 (Cream chocolate)	2.1 ± 0.1	3.2 ± 0.5	10.3 ± 0.6	6.0 ± 0.5	51 ± 3	8.7 ± 0.3	44 ± 4
	Brand 33 (Sweet strawberry)	1.8 ± 0.1	-	10.4 ± 0.4	6.6 ± 0.2	56 ± 4	7 ± 1	38 ± 3

< MDL: concentrations below method detection limit value.

- : not detected.

Among American countries, although Begley et al. [35] reported that PFOA content in fluorochemicals treated papers from the US market ranged from 300 to 1200 ng/g, in the present study, popcorn bags from the USA did not contain high levels of PFASs. It has to be mentioned that the USA bag (Brand 33) contained specific information in the box to ensure that it was free of PFOA, of chemicals and of plastic coating. In the case of the Brazilian popcorn bags, PFHxA (3-28 ng/g) and PFOA (4-5 ng/g) were the predominant PFASs detected. PFOA (3.5-4.0 ng/g) was the predominant in the case of Mexican ones. In Asian countries, a different pattern was observed since high levels of long chain PFASs (C_8-C_{16}) were detected, especially in Chinese bags. In these samples, PFOA (51-56 ng/g) and PFDA (38-44 ng/g) were the predominant PFASs. For India, PFDA (14 ng/g) was the only PFAS detected. PFASs containing C_{11}-C_{16} were also detected, but not quantified due to the lack of long chain PFAS standards. ***Figure 9.8*** shows a comparison of the use of long chain PFASs over all countries based on peak areas, being China the predominant user of these compounds.

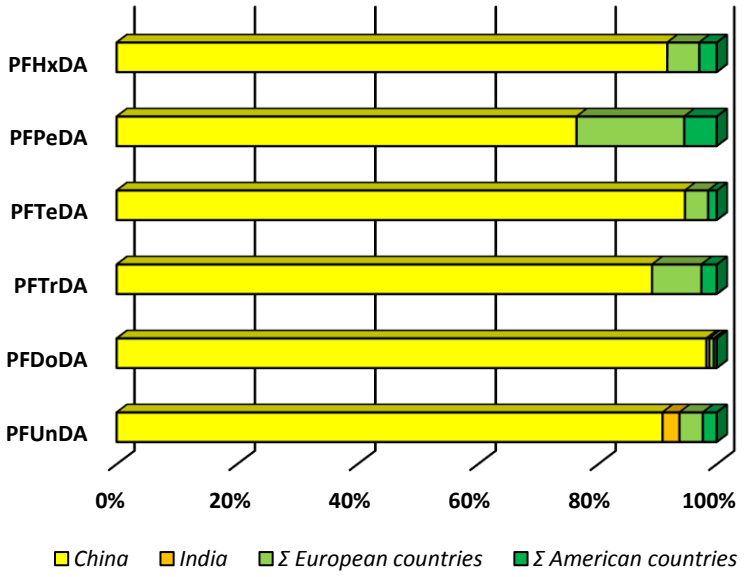


Figure 9.8: *Patterns of long chain PFCAs in microwave popcorn bags from different countries based on peak areas.*

It has to be mentioned that, although a voluntary stewardship agreement to phase out the use and production of long-chain PFASs has been established in some countries, their presence is still considerable, particularly in China. In the literature, PFOA concentrations ranging from 0.2 to 2 ng/g were reported in Thai bags [14]. However, longer chain PFASs were not analysed in their work. Although there are several works where PFOS was quantified in popcorn bags [13–15], in this work PFSAs, perfluorophosphonic acids (PFPAs) or FOSA were not detected in any popcorn bag. Finally, while in the present work European, Asian and American bags were reported, Dolman and Pelzing detected C_6-C_{11} length PFCAs in Australian bags [36]. However, as they only quantified PFOA (9.1 ng/g), any further comparison is not possible.

In addition to PFASs, their potential precursors were also quantified (see ***Table 9.6***). Among European and American countries, several Spanish and Brazilian bags presented high concentration of 6:2 diPAP (22-57 ng/g) and 6:2 monoPAP (3-27 ng/g). Moreover, intermediates such as 6:2 FTCA, 6:2 FTUCA and 5:3 FTCA were also detected at high levels, especially in Spanish brands. Furthermore, some Spanish brands also contained 8:2 diPAP and 8:2 monoPAP, despite intermediate compounds such as 8:2 FTCA, 8:2 FTUCA and 7:3 FTCA were not detected. In the case of Chinese bags, even if 8:2 diPAP and 8:2 monoPAP were not detected, 8:2 FTCA, 8:2 FTUCA and 7:3 FTCA were quantified. Moreover, 10:2 FTCA, 10:2 FTUCA, 9:3 FTCA, 9:3 FTUCA and 7:3 FTUCA were also qualitatively identified in Chinese bags (see ***Table 9.7***). Finally, triPAPs were only observed in the Spanish Brand 4 bag.

Table 9.6: PFCA potential precursor concentrations (ng/g) in popcorn bag samples from different countries around the world.

Location	Samples	8:2 diPAP	6:2 diPAP	8:2 monoPAP	6:2 monoPAP	8:2 FTCA	8:2 FTUCA	7:3 FTCA	6:2 FTCA	6:2 FTUCA	5:3 FTCA
European Countries											
Spain	Brand 1 (Salty)	< MDL	-	-	-	-	-	-	61 ± 16	39 ± 9	14 ± 3
	Brand 1 (Light)	< MDL	-	-	-	-	-	-	-	-	-
	Brand 2 (Salty)	< MDL	-	-	-	-	-	-	-	-	-
	Brand 2 (Butter)	< MDL	-	-	-	-	-	-	-	-	-
	Brand 3 (Salty)	9.8 ± 0.5	< MDL	-	-	-	-	-	-	-	-
	Brand 4 (Salty)	26 ± 9	57 ± 20	16 ± 5	13 ± 3	-	-	-	158 ± 45	383 ± 96	8 ± 2
	Brand 5 (Salty)	< MDL	< MDL	-	-	-	-	-	225 ± 65	700 ± 26	20 ± 9
France	Brand 6 (Salty)	< MDL	< MDL	-	-	-	-	-	1.3 ± 0.4	-	-
	Brand 6 (Sweet)	< MDL	< MDL	-	-	-	-	-	1.5 ± 0.3	-	-
	Brand 6 (Sugar)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 7 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 8 (Original)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 9 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
The Netherlands	Brand 10 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 11 (Salty)	< MDL	< MDL	-	-	-	-	-	-	2.3 ± 0.2	-
Germany	Brand 12 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
Hungary	Brand 13 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 14 (Salty)	< MDL	< MDL	-	-	-	-	-	-	1.4 ± 0.2	-
Portugal	Brand 15 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
Ireland	Brand 16 (Salty)	< MDL	< MDL	-	-	-	-	-	-	-	-
United Kingdom	Brand 17 (Salty)	< MDL	< MDL	-	-	-	-	-	-	2 ± 1	-
	Brand 17 (Butter)	< MDL	< MDL	-	-	-	-	-	-	-	-
Italy	Brand 18 (Salty)	< MDL	< MDL	-	-	-	-	-	-	2.5 ± 0.3	-
Austria	Brand 19 (Salty)	< MDL	< MDL	-	-	-	-	-	-	1.2 ± 0.3	-
Czech Republic	Brand 20 (Salty)	< MDL	< MDL	-	-	-	-	-	-	1 ± 2	-
Sweden	Brand 21 (Salty)	< MDL	< MDL	-	-	-	-	-	-	< MDL	-
	Brand 22 (Original)	< MDL	< MDL	-	-	-	-	-	-	-	-

Table 9.6: Continuation.

Location	Samples	8:2 diPAP	6:2 diPAP	8:2 monoPAP	6:2 monoPAP	8:2 FTCA	8:2 FTUCA	7:3 FTCA	6:2 FTCA	6:2 FTUCA	5:3 FTCA
	Brand 22 (Butter)	< MDL	< MDL	-	-	-	-	-	-	2 ± 1	-
American Countries											
Mexico	Brand 23 (Butter)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 23 (Extra butter)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 24 (Natural)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 25 (Natural)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 26 (Natural)	< MDL	< MDL	-	-	-	-	-	-	1.4 ± 0.6	-
Brazil	Brand 27 (Natural)	< MDL	29.8 ± 0.8	-	27 ± 4	-	-	-	2.1 ± 0.3	4.1 ± 0.6	-
	Brand 28 (Butter)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 29 (Natural)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 30 (Butter)	< MDL	22 ± 2	-	3 ± 1	-	-	-	-	2.3 ± 0.1	-
USA	Brand 31 (Salty and butter)	< MDL	< MDL	-	-	-	-	-	-	-	-
	Brand 24 (Butter)	< MDL	< MDL	-	-	-	-	-	-	1.2 ± 0.6	-
	Brand 32	-	-	-	-	-	-	-	-	-	-
Asian Countries											
India	Brand 24 (Cheddar Chease)	< MDL	< MDL	-	-	-	-	-	-	-	-
China	Brand 33 (Cream chocolate)	< MDL	< MDL	-	-	4 ± 1	10.2 ± 0.7	< MDL	-	-	-
	Brand 33 (Sweet strawberry)	< MDL	< MDL	-	-	6.3 ± 0.1	9 ± 2	< MDL	-	-	-

< MDL: concentrations below method detection limit value.

- : not detected.

Table 9.7: Detection of PFC precursors in microwave popcorn bags from different countries around the world.

Location	Samples	6:2/6:2 diPAP	6:2/8:2 diPAP	8:2/8:2 diPAP	6:2/10:2 diPAP	8:2/10:2 diPAP	6:2/12:2 diPAP	10:2/10:2 diPAP	8:2/12:2 diPAP	6:2/14:2 diPAP	10:2/12:2 diPAP	8:2/14:2 diPAP	6:2/16:2 diPAP	10:2 FTCA	10:2 FTUCA	9:3 FTCA	9:3 FTUCA	7:3 FTUCA
European Countries																		
Spain	Brand 1 (Salty)	X	-	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 1 (Light)	-	-	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 2 (Salty)	-	-	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 2 (Butter)	-	-	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 3 (Salty)	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 4 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 5 (Salty)	X	X	X	X	X	X	X	X	X	X	X	X	-	-	-	-	-
France	Brand 6 (Salty)	X	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 6 (Sweet)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 6 (Sugar)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 7 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 8 (Original)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 9 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
The	Brand 10 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Netherlands	Brand 11 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Germany	Brand 12 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Hungary	Brand 13 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 14 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Portugal	Brand 15 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Ireland	Brand 16 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
United Kingdom	Brand 17 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 17 (Butter)	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Italy	Brand 18 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Austria	Brand 19 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Czech Republic	Brand 20 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Sweden	Brand 21 (Salty)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 22(Original)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 22 (Butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
American Countries																		
Mexico	Brand 23 (Butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 23 (Extra butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 24 (Natural)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 25 (Natural)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 26 (Natural)	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brazil	Brand 27 (Natural)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 28 (Butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 29 (Natural)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 30 (Butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
USA	Brand 31 (Salty and butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 24 (Butter)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	Brand 32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asian Countries																		
India	Brand 24 (Cheddar Chease)	X	X	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
China	Brand 33 (Cream chocolate)	X	X	X	X	-	-	-	-	-	-	-	-	X	X	X	X	X
	Brand 33 (Sweet strawberry)	X	X	X	X	-	-	-	-	-	-	-	-	X	X	X	X	X

It has to be mentioned that different patterns were observed depending on the additives added to popcorn bags. In the case of the Spanish Brand 1, two different type of bag were analysed; a salty one and the other one free of any additive (light). In the case of the light one, only PFHxA was detected at low concentration (2.8 ng/g), while high concentrations of PFASs were detected in the salty one. However, when different flavour bags from the same brand (Spanish Brand 2, French Brand 6, English Brand 17, Swedish Brand 22, Mexican Brand 23 and Chinese Brand 33) were analysed, comparable concentrations were found. This could mean that the same treatment was used for popcorn bags even if they used different flavours; however, when no additives were added, popcorn bags seemed to be free of PFASs. Moreover, a same brand (Brand 24) manufactured in different countries (USA, Mexico and India) showed similar results for all PFAS concentrations (not detected or below MDL values), except for PFDA which was quantified (14 ng/g) in India, supporting that in Asian Countries (China and India) long PFASs are still being used for bag manufacturing purposes.

9.3.4 Relationship between PAPs and PFCAs

Several studies have demonstrated that PAPs are potential precursors of PFCAs [11]. However, there are not many studies focused on the detection of the intermediate compounds (FTCAs/FTUCAs). In this sense, this work presents for the first time a direct link between PAPs and PFCAs in popcorn bags. In our work, intermediates of 6:2 diPAP and 8:2 diPAP were identified. In this sense, 6:2 diPAP -> 6:2 monoPAP -> 6:2 FTCA -> 6:2 FTUCA -> 5:3 FTCA -> 5:3 FTUCA intermediates with their corresponding final degradation products (PFPeA, PFHxA and PFHpA) were observed. Moreover, 8:2 diPAP -> 8:2 monoPAP -> 8:2 FTCA -> 8:2 FTUCA -> 7:3 FTCA -> 7:3 FTUCA with their corresponding end degradation products (PFHpA, PFOA and PFNA) were also detected. Finally, in the case of Chinese bags, where PFDA was found at high concentration, intermediates as 10:2 FTCA-> 10:2 FTUCA-> 9:3 FTCA-> 9:3 FTUCA were detected (see *Table 9.7*).

Finally, it cannot be concluded that PAP degradation is due to microwave heating since all degradation compounds were already detected in microwave popcorn bags prior to use. Therefore, degradation probably occurred during the bag production.

9.4 Conclusions

A thorough identification of 46 fluorochemicals was performed in microwave popcorn bags from twelve European countries, three American countries and two Asian countries using a LC coupled to an accurate QTOF-MS. Moreover, a validation of an accurate analytical method was performed for the analysis of up to twenty-four PFASs, including PFCAs, PFSAs, PFPAs and potential precursors, in packaging samples. In this sense, different length PFCAs were quantified and different patterns were observed; while in European and American countries short chain PFCAs (C_4-C_8) were mostly detected, in Asian countries (specially in China) long chain PFCAs (C_8-C_{16}) were detected. Furthermore, potential precursors were also quantified; while in European and American countries 6:2 diPAP and its degradation intermediates were mostly found, in China degradation intermediates as 8:2 FTCA, 8:2 FTUCA and 7:3 FTCA were present, indicative of the use of longer chain PFCAs. It is worth to mention that a voluntary stewardship agreement in order to phase out the use and production of long-chain PFASs has been established in some countries but their presence is still considerable in Asian countries. To the best of our knowledge, this is the first work where intermediates, such as 8:2 FTCA, 10:2 FTCA, 8:2 FTUCA, 10:2 FTUCA, 7:3 FTCA, 9:3 FTCA, 5:3 FTUCA, 7:3 FTUCA and 9:3 FTUCA were detected in microwave popcorn bags. Nevertheless, further research should be performed in order to study the possible ability of PFASs to migrate to food.

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Chapter 10

Conclusions

The results obtained during this PhD Thesis have allowed the accomplishment of the objectives previously established. The following main conclusions could be highlighted from the present memory.

The use of focused ultrasound solid-liquid extraction (FUSLE) offered a simple and optimum extraction procedure of the target compounds in a variety of matrices, including biotic (vegetables, fish, mussels) and abiotic (soil) environmental samples, as well as, for packaging materials, requiring a low amount of sample (0.1–0.5 g), solvent (7 mL) and a short extraction time (2.5 min). However, since this extraction technique was not selective for the selected matrices, a clean-up step was necessary. In this sense, solid phase extraction (SPE) using a weak anion exchange sorbent (WAX) provided the best extraction efficiencies with the lowest matrix effect for vegetables, fish and soil. In the case of fish and shellfish, while WAX provided the best extraction efficiencies with the lowest matrix effect, the addition of an extra step using graphitized carbon was necessary in order to obtain colourless extracts and to prevent the frequent electrospray ionisation (ESI) interphase cleaning.

The method developed for fish and shellfish was applied to the analysis of grey mullet (*Chelon labrosus*) liver and mussel (*Mytilus galloprovincialis*) samples from the Basque Coast (North of Spain), oysters (*Ostrea edulis*) from the North Coast of Spain, French and Portuguese coasts and Yellowfin tuna muscle tissue (*Thunnus albacares*) samples from the Indian Ocean. In the case of grey mullet livers, perfluorooctane sulfonic acid (PFOS), perfluorooctane sulfonamide (FOSA) and perfluorodecanoic acid (PFDA) were detected in all the sampling points. Surprisingly high concentrations of PFOS (443-1,214 ng/g) were detected in the estuary of Gernika, which is located in the Natural Reserve of Urdaibai (Gulf of Biscay), highlighting the effect of the wastewater treatment plant (WWTP) and/or the industry close to the sampling point. Besides, the fire station located nearby the sampling point could be also an exposure source of

PFOS to the water due to the use of this compound in fire-fighting foams. Moreover, within the monitored precursors, 8:2 diPAP and 6:2 diPAP were detected, although in both cases concentrations were below the method detection limit (MDL) values. In the case of oysters and mussels, PFOS and FOSA were detected at levels lower than those found in liver, showing a low accumulation in these species. However, high levels of 8:2 monopolyfluoroalkyl phosphate (8:2 monoPAP) and 8:2 difluoroalkyl phosphate (8:2 diPAP) were reported for the first time in mussel and fish muscle tissue samples, respectively. Furthermore, due to the detected presence of 8:2 diPAP in fish, 8:2 diPAP tissue distribution was studied in gilthead bream (*Sparus aurata*), concluding that this analyte tended to accumulate in liver, plasma and gills, and to a lesser extent in muscle, bile and brain. Several transformation products were also detected in most tissues and biofluids, including fluorotelomer saturated and unsaturated acids (8:2 FTCA, 8:2 FTUCA, 7:3 FTCA), and PFOA. 8:2 FTCA was the major metabolite in all tissues/biofluids, except for bile, where PFOA occurred at the highest concentrations. Unexpectedly high PFOA levels (up to 3.7 ng/g) were also detected in brain. Phase II metabolites, were not observed in these experiments, probably due to their low abundance. Nevertheless, the detection of PFOA indicates that exposure to polyfluoroalkyl phosphates (PAPs) may be an indirect route of exposure to PFCAs in fish.

The experience acquired in the analysis of PFASs in solid samples using both low and high resolution mass spectrometry was also applied to study the fate and behaviour of the commercial ethyl-perfluorooctane sulfonamide (EtFOSA)-containing pesticide (Sulfluramid pesticide) in soil/carrot (*Daucus carota ssp sativus*) mesocosms. This study demonstrated that Sulfluramid use could lead to the occurrence of different fluorinated biodegradation products, including PFOS, in crops and in the surrounding environment. Moreover, the high yields of PFOS (277 %) obtained indicated that an additional PFOS-precursor (or precursors) may be present in the

pesticide baits and, therefore, additional research is needed for bait characterisation. Furthermore, the results obtained in the carrot crop uptake revealed that the more hydrophilic transformation products (e.g. PFOS) occurred primarily in the leaves, while the more hydrophobic products (e.g. FOSA, perfluorooctane sulfonamido acetate (FOSAA) and EtFOSA) occurred in peel and core. Overall, these results showed the risk that the use of the Sulfluramid pesticide could suppose for the environment and humans.

Finally, FUSLE also turned out to be a good approach for the determination of the target compounds in packaging materials. In this case, while the extraction was selective in the absence of pigments, the introduction of a clean-up step using graphitized carbon was necessary for high pigment containing materials. The developed method was applied to the analysis of plastic (milk bottle, muffin cup, pre-cooked food wrapper and cup of coffee) and cardboard materials (microwave popcorn bag, greaseproof paper for French fries, cardboard box for pizza and cinema cardboard box for popcorn). Microwave popcorn bags presented the highest PFASs concentrations. Additionally, several fluorotelomer saturated and unsaturated acids (6:2 FTCA, 6:2 FTUCA and 5:3 FTCA) were detected for the first time. In this context, the characterisation of microwave popcorn bags over twelve European countries, three American countries and two Asian countries was performed by means of liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QToF-MS). In this sense, up to 46 PFASs and precursors were identified. Moreover, different patterns in the microwave popcorn bag composition were observed within the countries; while in European and American countries short chain PFASs were detected, Asian countries seem to still use long chain PFASs. Nevertheless, further research should be performed in order to study the possible ability of PFASs to migrate to food.

Bearing in mind all the results obtained in the present PhD thesis, different future actions could be proposed. On the one hand, the use of fluorinated compounds based pesticides should be legislated taking as an example the particular risk that the use of the Sulfluramid pesticide could suppose to the environment or humans. On the other hand, a strict regulation for food packaging materials should be established since high PFASs levels have been quantified in microwave popcorn bags from some countries around the world. Finally, especial attention should be paid to fish and shellfish, since they are the principal PFASs contributors to our dietary, and they have shown the ability to accumulate PFASs and biodegraded PFCA potential precursors.