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DOCTORAL THESIS

Risk assessment of Perfluoroalkylated substances (PFASs) through the determination of their concentration in various food matrices.

EFFROSYNI ZAFEIRAKI

MSc CHEMIST

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Έκθεση αξιολόγησης κινδύνου Υπερφθοροαλκυλιωμένων ουσιών μέσω του προσδιορισμού της συγκέντρωσής τους σε δείγματα τροφίμων.

ΕΥΦΡΟΣΥΝΗ ΖΑΦΕΙΡΑΚΗ

MSc ΧΗΜΙΚΟΣ

Με τη χρηματοδότηση της Ελλάδας και της Ευρωπαϊκής ένωσης

ΑΘΗΝΑ 2016

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ABSTRACT

The presence of emerging environmental pollutants, like perfluoroalkylated substances (PFASs) in food products is one of the main issues for food safety. As the scientific interest on this topic has risen during the last decades, many studies have focused on the determination of PFASs in food. However, risk assessment of the dietary exposure to PFASs is hampered by the insufficient available information and thus further investigation is needed.

Thus, the main objective of this study is the risk assessment of PFASs through their detection in different food matrices, drinking water and food packaging materials. In the present study, both direct and indirect ways of food contamination were examined and evaluated. Human exposure to PFASs via the consumption of certain food items was also estimated. In order to detect very low levels of PFASs in all the aforementioned matrices, sensitive and selective analytical methods were developed.

The current thesis, apart from the development of novel analytical methods comprises of five distinct parts: (1) Determination of perfluorinated compounds (PFCs) in various foodstuff packaging materials used in the Greek market, (2) Levels of perfluorinated compounds in raw and cooked Mediterranean finfish and shellfish, (3) Perfluoroalkylated substances (PFASs) in home and commercially produced chicken eggs from the Netherlands and Greece, (4) Determination of perfluoroalkylated substances (PFASs) in drinking water from the Netherlands and Greece, (5) Perfluoralkylated substances in edible livers of farm animals, including accumulation kinetics in young sheep fed with contaminated feed. Hopefully this work will be an important contribution to the particular scientific field being explored, and also the trigger for further investigation of issues that have been addressed.

SUBJECT AREA: Environmental chemistry

KEYWORDS: Perfluoroalkylated substances, food, drinking water, food packaging materials, LC-MS/MS

ΠΕΡΙΛΗΨΗ

Η παρουσία περιβαλλοντικών ρύπων, όπως αυτή των υπερφθοροαλκυλιωμένων ενώσεων (PFASs) αποτελεί θέμα μείζονος σημασίας για την ασφάλεια τροφίμων. Τις τελευταίες δεκαετίες, το ενδιαφέρον της επιστημονικής κοινότητας σχετικά με τις PFASs αυξάνεται συνεχώς, ενώ παράλληλα αρκετές μελέτες προσανατολίζονται στον προσδιορισμό τους κυρίως σε δείγματα τροφίμων. Παρόλα αυτά, σαφή συμπεράσματα σχετικά με την αξιολόγηση του κινδύνου λόγω έκθεσης σε PFASs μέσω της διατροφής, δεν έχουν ακόμα εξαχθεί, καθώς οι διαθέσιμες πληροφορίες είναι ανεπαρκείς. Συνεπώς, περαιτέρω έρευνα στο συγκεκριμένο θέμα θεωρείται απαραίτητη.

Σκοπός της παρούσας εργασίας είναι η αξιολόγηση του κινδύνου των PFASs μέσω του προσδιορισμού των συγκεντρώσεών τους σε διάφορα είδη τροφίμων, πόσιμο νερό και υλικά συσκευασίας τροφίμων. Για την ανίχνευση των PFASs στα προαναφερθέντα δείγματα, αναπτύχθηκαν ευαίσθητες και επιλεκτικές αναλυτικές μέθοδοι.

Συγκεκριμένα, η παρούσα μελέτη, εκτός της ανάπτυξης αναλυτικών μεθόδων αποτελείται απο πέντε διαφορετικά τμήματα: (1) Προσδιορισμός των PFCs σε διάφορα υλικά συσκευασίας τροφίμων της ελληνικής αγοράς, (2) Επίπεδα PFCs σε ωμά και μαγειρεμένα ψάρια και οστρακοειδή από τη Μεσόγειο θάλασσα, (3) Επίπεδα PFASs σε αυγά κότας οικιακής ή εμπορικής παραγωγής από την Ελλάδα και την Ολλανδία, (4) Προσδιορισμός PFASs σε πόσιμο νερό από την Ολλανδία και την Ελλάδα, (5) Επίπεδα PFASs σε βρώσιμο ήπαρ από ζώα ελευθέρας βοσκής, συμπεριλαμβανομένης μελέτης της συσσώρευσης των ενώσεων σε πρόβατα που έχουν τραφεί με μολυσμένη τροφή.

Ευελπιστούμε η παρούσα μελέτη να αποτελέσει σημαντική συνεισφορά στο συγκεκριμένο επιστημονικό πεδίο, καθώς επίσης και έναυσμα για περαιτέρω διερεύνηση των ζητημάτων που πραγματεύθηκε.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Χημεία Περιβάλλοντος

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: υπερφθοροαλκυλιωμένες ενώσεις, τρόφιμα, πόσιμο νερό, πακέτα συσκευασίας τροφίμων, LC-MS/MS

To my family and friends

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PREFACE

This reseach has been conducted in the Mass Spectrometry and Dioxin Analysis Laboratory of NCSR Demokritos, Greece and at the Department of Contaminants and Toxins of RIKILT Institute of Food Safety – WUR, Netherlands, under a collaboration between the two countries.

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CHAPTER 1

Perfluoroalkylated substances (PFASs)

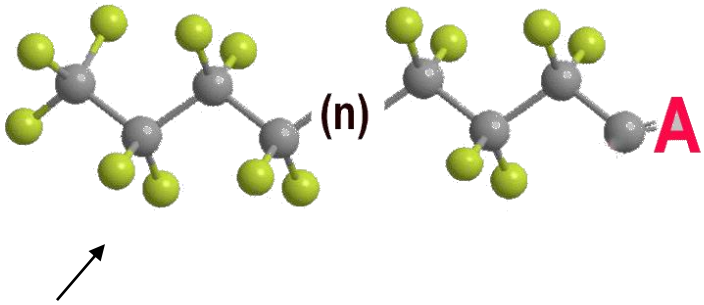
1.1 Terminology and classification of PFASs

Perfluoroalkylated substances (PFASs), also known as perfluorinated compounds (PFCs), are organic aliphatic compounds consisting of a carbon backbone in which all hydrogen atoms have been replaced by fluorine atoms, except for the ones consisting part of a functional group present. PFASs include perfluoroalkyl acids (PFAAs), perfluoroalkane sulfonyl fluorides (PASFs), perfluoroalkane sulfonamides (FASAs), perfluoroalkanoyl fluorides (PAFs), perfluoroalkyl iodides (PFAIs), perfluoroalkyl aldehydes (PFALs) and aldehyde hydrates (PFAL-H_2Os) (Table 1.1).

Table 1.1: **An overview of perfluoroalkyl substances[1].**

	Classification	**C_nF2_{n+1R}, R=**
Perfluoroalkyl acids (PFAAs)	Perfluoroalkyl carboxylic acids (PFCAs)	-COOH
	Perfluoroalkyl carboxyaltes (PFCAs)	$-COO^-$
	Perfluoroalkane sulfonic acids (PFSAs)	$-SO_3H$
	Perfluoroalkane sulfonates (PFSAs)	$-SO_3^-$
	Perfluoroalkane sulfinic acids (PFSIAs)	$-SO_2H$
	Perfluoroalkyl phosphonic acids (PFPAs)	$-P(=O)(OH)_2$
	Perfluoroalkyl phosphinic acids (PFPIAs)	$-P(=O)(OH)(C_mF_{2m+1})$
Perfluoroalkane sulfonyl fluorides (PASFs)		$-SO_2F$
Perfluoroalkane sulfonamides (FASAs)		$-SO_2NH_2$
Perfluoroalkanoyl fluorides (PAFs)		-COF
Perfluoroalkyl iodides (PFAIs)		-I
Perfluoroalkyl aldehydes (PFALs) and aldehyde hydrates (PFAL-H_2Os)		-CHO and $–CH(OH)_2$

PFASs have a hydrophilic group, such as carboxylate or sulfonate and a lipophilic perfluorinated carbon chain of varying length, usually between 4 and 14 carbon atoms fully fluorinated, that make them amphiphilic (Figure 1.1).



Fully fluorinated alkyl chain

Functional group A

A= -COOH

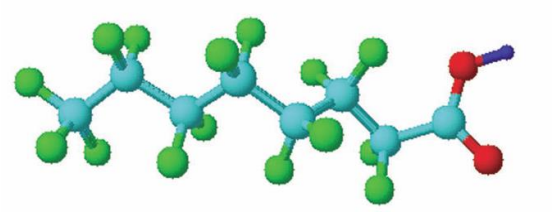
$-SO_3H$

$-CONH_2$

$-SO_2NH_2$ etc

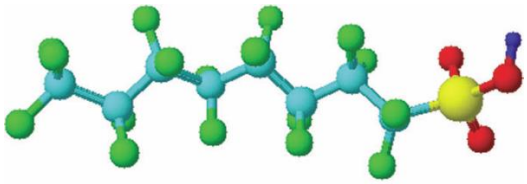
Figure 1.1: The chemical structure of PFASs.

The family of PFAAs includes perfluoroalkyl carboxylic, sulfonic, sulphinic, phosphonic and phosphinic acids (Table 1.1), with carboxylic and sulfonic compounds being the most frequently detected. Regarding perfluoroalkyl carboxylic acids (PFACs, $C_nF_{2n+1}COOH$) and perfluoroalkyl sulfonic acids (PFSAs, $C_nF2_{n+1}SO_3H$), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) respectively, are the two PFAAs that have drawn the greatest attention, because they have been produced in highest amounts in the past for several decades. PFOS and PFOA have a fully fluorinated alkyl chain of eight carbons and are extremely persistent in the environment, resistant to environmental degradation processes and with high ability of bioaccumulation (Figure 1.2).



PFOA

MW: 417.07



PFOS

MW: 500.13

Figure 1.2: The chemical structure of perfluorooctanoic acid (PFOA) and perfluorooctane sulphonate (PFOS).

1.2 PFASs physicochemical properties

PFASs have remarkable characteristics due to their structure. Fluorine atoms are characterized by high electronegativity. As a result the fluorine interacts with its surroundings via dipole and electrostatic interactions. This renders the bond between carbon and fluorine highly polarized. In addition, due to its electronic configuration ($1s^2 2s^2 2p^5$), the fluorine atom needs one electron to fill its outer shell. The 2s and 2p orbital of fluorine and carbon match very well, making the bond between the atoms very strong, and providing an effective shielding to the carbon atoms in a fully carbon chain [2].

Hence, PFASs possess thermal, chemical and biological resistance, as well as electric insulating properties of materials. In particular, due to the strong bond between carbon and fluorine atoms they are stable in the air at high temperatures, they are not readily degradable by strong acids, alkalising or oxidizing agents and they do not undergo photolysis [3].

PFASs vapor pressure also has an important impact on the environmental processes they undergo. As PFASs have relative low vapor pressure and they are water – soluble, they undergo two transport ways: a) direct transport via oceanic currents and/or sea spray, b) neutral, volatile precursors can undergo long range atmoshperic transport (LRAT) and be degraded to the persistant

compounds in remote regions. Vapor pressure depends on PFASs alkyl chain length. In particular, PFOS (potassium salt) and PFOA (free acid) vapor pressure is $2.48x10^{-6}$ Hg and 2.06 mm Hg, respectively [4].

The acid dissociation constant (pKa) of the different PFASs, especially of the acids, plays an important role in their environmental behavior. PFCAs are completely deprotonated at an environmental relevant pH (around 7), thus the ionic head greatly contributes to their solubility [5]. While they are lipid soluble, they are also moderately water-soluble. Especially, PFOS (potassium salt) water solubility is calculated at 550-570 mg L^{-1} in purified water, while in fresh and filtered sea water is 370 and 25 mg L^{-1} respectively. Accordingly, PFOA (free acid) water solubility has been measured in purified water and is equal to 9500 mg L^{-1} [4].

Apart from pKa, the octanol-water partitioning coefficient (Kow) is another important parameter in determining the behavior of PFASs environmental-wise. Considering PFASs hydrophobic and oleophobic character, the determination of Kow value remains a controversial topic. According to biomonitoring studies, there is a tendency of PFASs partition into organic fractions (biota), binding to proteins in blood serum rather than to fat. Kow values for PFOS and PFOA are not available yet.

Long chain PFASs are more likely to bioaccumulate and biomagnify than those with short alkyl chain http://www.epa.gov/risk_assessment/expobox/chemicalclass/other-pfc.htm. It has been found that PFOS half-life is 114 days in the atmosphere and more than 41 years in the water (at 25 ^{o}C), while PFOA half-life is calculated at 90 days in the atmosphere and higher than 92 years in the water (25 ^{o}C).

PFASs can also create surfaces of extremely low surface free energies, by modifying the surface characteristics of materials. In general, surfactants lower the surface tension of a liquid, or the interfacial tension between two liquids or a

liquid and a solid. PFASs surfactants are useful as leveling and wetting agents, emulsifiers and dispersants since they reduce the aqueous surface tension [6-8].

1.3 PFASs applications

PFASs are human made and they do not occur naturally in the environment. Their production has been taking place for more than 50 years by applying mainly two processes, Electro-Chemical Fluorination (ECF) and telomerisation (TM) [9]. Till now, PFASs have been used in a variety of consumer products, mainly due to their properties, like water and oil repellency, chemical inertia, non-wetting, nonstick properties as well as high fire-resistance.

In particular, PFASs find application as water and grease repellents for coating materials in textiles, food-contact paper and leather products. Taking advantage of their aqueous surface tension-lowering properties, they are also used as processing aids in the manufacturing of fluoropolymers such as polytetrafluoroethylene (PFTEE) and polyvinylidene fluoride (PVDF), in the photographic industry, in aqueous film-forming foams (AFFFs), in electronics, in the aviation industry (hydraulic fluids), etc [7,10,11]. PFASs are also used in fluoropolymer-coated cookware, Teflon products, sports clothing, extreme weather-resistant military uniforms, medical equipment, motor oil additives, paint and ink, self-shine floor polishes, cement, varnishes, gasoline, electrolytic plating plats, medical inhalers, fuel additives and air fresheners [12].

Especially, PFOS has been used in many industrial applications, including the photography and semiconductor industries, and in fire-fighting foams and hydraulic fluids in the aviation industry. On the other hand, PFOA has been widely used as a protective coating for carpets, textiles, leather etc. PFOA was also used in household and industrial products. However, its main application is in the production of fluoropolymers used in electronics, textiles and nonstick cookware.

1.4 PFASs sources and detection in the environment

PFASs can be released into the environment via direct or indirect sources. Basically, direct sources include the manufacturing (via air stack or Waste Water Treatment Plants, WWTP), or the leaching of PFASs, present as residuals or integral part of the formulation, from industrial and consumer products (like aqueous film-forming foam, (AFFF)), or from the use or disposal of consumer products that may contain them as impurity.

On the other hand, indirect sources comprise biotic or abiotic degradation of larger functional derivatives and polymers that contain a perfluoroalkyl moiety and degrade in the environment to form certain PFASs. These precursors are commonly used commercially and may be released in the environment from industrial raw materials and products and from consumer products and articles. In particular, N-ethyl perluorooctane sulfonamide ethanol (*N*-EtFOSE), *N*-methyl perfluorooctane sulfonamido ethanol (*N*-MeFOSE), perfluorosulfonamides and fluorotelomer (FTOHs) raw materials can degrade into PFOA [5]. The transformation pathways of PFOA include, apart from biodegradation [13,14], reaction with OHx, and ozonolysis [15,16]. As far as the indirect sources of PFOS are concerned, it can be formed by environmental microbial degradation [17] or by metabolism by higher organisms of PFOS-related substances. However, it is not clear yet how many substances are precursors to PFOS.

The release of PFASs in the environment, combined with their high applicability and chemical stability, has led to inevitable accumulation of PFASs in the environment and as a consequence to their entrance into the food chain and then to the human organism. Particularly, PFASs have been detected in environmental matrices, like air, dust, sewage, rivers, dust, etc [18-21] (Table 1.2). Thereafter, PFASs were also detected in drinking water [22-24] (Table 1.3) and in food products and food packages [25-29] (Table 1.4). Human samples, such as breast milk, blood/serum and urine were also found to be contaminated [30-33] (Table 1.5).

Table 1.2: Indicative examples of the detected concentrations of PFASs in environmental matrices.

Country	PFASs analysed	Matrix	Origin of samples	Method of analysis	Result of analysis	Reference
China	12	Surface water (n=30), surficial sediment (n=30), phytoplankton, zooplankton, two zoobenthos species (n=9) and Corbiculidae (bivalve) (n=8), white shrimp (n=18), fish samples of 9 different species (n=74), and two egret bird species	Collected from Taihu Lake	*Water:* Extraction: Oasis WAX cartridges *Sediment:* Extraction: MeOH Clean-up: Oasis WAX cartridges *Biota samples:* Alkaline digestion and Clean up with Oasis WAX cartridge HPLC-MS/MS: ZORBAX Eclipse C18 column.	*Water:* only PFCAs with 6-9 carbons were regularly detected. High concentrations of PFOA (28.1-16 ng L^{-1}), PFHxA (11.2 - 6.4 ng L^{-1}), PFNA (3.0-1.5 ng L^{-1}), and PFOS (3.5-2.6 ng L^{-1}). *Sediments:* PFOA, PFNA, PFDA, PFUnDA, PFDoA and PFOS were commonly detected, and PFOS was the dominant compound (0.92-1.4 ng g^{-1} dw). *Biota organism:* PFOS was the dominant compound (0.7 - 20.9 ng g^{-1} ww in the aquatic biota samples)	[21]

China	9	Water and sediment	Collected from Haihe river and Dagu Drainage Canal, Tianjin	*Water:* Extraction: Oasis WAX cartridges *Sediment:* Extraction: MeOH Clean-up: Envicarb HPLC-MS/MS: X-Terra MS C18 column (2.1 mm id., 150mm, 5 µm)	PFHxA, PFOA and PFOS were the predominant PFCs in the aqueous phase. PFOS was the major PFC in Haihe River sediments followed by PFOA, while PFHxA was the major PFC in Dagu Drainage Canal sediments.	[34]
Cantabrian sea (North Spain)	5	Water, sediment, mussels	Collected from estuarine areas of high urban and industrial impact from Northern Spain	*Water:* Extraction: Waters Oasis cartridges *Sediment:* Extraction: MeOH and 10 mL of a 1% glacial acetic acid solution Clean-up: activated carbon	PFCs ranged from 0.06 to 10.9 ng L^{-1} in water, with higher levels in wastewater treatment plants effluents and port waters than in submarine emissaries. Little accumulation was observed in sediments and mussels with PFCs ranging from 0.01–0.13 ng g^{-1} dw and 0.01–0.06 ng g^{-1} ww, respectively.	[35]

				Mussels: Extraction: ACN Clean-up: activated carbon UPLC–MS/MS Acquity UPLC BEH C18 column (1.7 μm particle size, 50mm*2.1 mm)		
Greece	18	Wastewater (dissolved and particulate phase) and sewage sludge samples	Collected from two WWTPs in Greece	*Wastewater sample*: filtration and SPE with Oasis HLB cartridges *Sewage sludge samples*: sonication, centrifugion and SPE with Oasis HLB cartridges LC-MS/MS	PFPeA, PFOA and PFOS were dominant in wastewater and sludge samples from both plants. In sewage sludge, the average concentrations ranged up to 6.7 ng g^{-1} dry weight for PFOS, while in wastewater the mean of PFOS was 13.4 ng L^{-1} for plant A (influence) and 3.5 ng L^{-1} for plant B (influence). Concentrations of most PFCs were higher in effluents than in influents.	[18]

Spain	27	House dust and indoor air	Collected from selected homes in Catalonia, Spain.	*Indoor and outdoor air samples and dust:* Extraction: MeOH Clean-up: Supelclean EnviCarb UPLC-MS/MS Acquity BEH C18 column (50 mm *2.1 mm, 1.7 µm)	Ten PFCs were detected in all house dust samples. The highest mean concentrations corresponded to PFDA and PFNA, are 10.7 ng g^{-1} (median: 1.5 ng g^{-1}) and 10.4 ng g^{-1} (median: 5.4 ng g^{-1}), respectively, while the 8:2 FTOH was the dominating neutral PFC at a concentration of 0.41 ng g^{-1} (median: 0.35 ng g^{-1}). The indoor air was dominated by the FTOHs, especially the 8:2 FTOH. A limited number of ionic PFCs were also detected in the indoor air samples.	[19]

U.K.	PFOS PFOA PFHxS FOSA MeFOSA EtFOSA MeFOSE EtFOSE	Indoor and outdoor air samples	Air samples were taken at a number of locations within the city of Birmingham, UK. These were: (a) homes (n=20), (b) offices (n= 12), and (c) outdoors at 10 different locations (n=10)	Extraction: shoxlet with hexane:acetone (60:40 v/v) Clean-up: Oasis WAX SPE LC–MS/MS C18 Metasil 3 Basic column (2.1 mm i.d.×150mm×3 µm)	EtFOSE and MeFOSE had the highest concentrations in both indoor and outdoor air. Concentrations of PFOS in offices exceed significantly those in homes.	[20]
U.S.A	16	39 house dust samples	Collected from houses in Wisconsin	Extraction: MeOH in the presence of graphitized carbon HPLC-MS/MS	PFOS, PFOA, PFHxS had the highest concentrations. Median (range) PFOS 47 (8.7–1100) ng g^{-1} PFOA 44 (6.5–420) ng g^{-1} PFHxS 16 (2.1–1000) ng g^{-1}	[36]
Canada	9 PFASs 6:2 FTOH 8:2 FTOH 10:2 FTOH MeFOSA EtFOSA	Indoor air, outdoor air, indoor dust, and clothes dryer lint	Collected from houses	Neutral PFCs Extraction: dichloromethane (DCM) Ionic PFCs Extraction: MeOH	Indoor air was dominated by 8:2 FTOH (mean: 2900 pg m^{-3}). Among the FOSAs and FOSEs, MeFOSE had the highest air concentration (mean of 380 pg m^{-3}). PFOA was the major ionic PFC and was	[37]

	MeFOSE EtFOSE			Clean-up: ENVI-Carb Neutral: GC-PCIMS Ionic: LC-MS/MS	detected in all indoor air samples (mean: 28 pg m^{-3}), whereas PFOS was <LOD. 8:2 FTOH was also dominated in house dust (mean of 88 ng g^{-1}). PFOS and PFOA were the most prominent compounds detected in dust samples.	
Norway	19 PFASs 6:2, 8:2, 10:2 FTUCA 6:2, 8:2 FTS 4:2, 6:2, 8:2, 10:2 FTOH PFOSA MeFOSA EtFOSA MeFOSE	House dust and indoor air samples	Samples of house dust and indoor air were collected from residences in Oslo. The dust was collected from elevated surfaces (bookselves and window sills) and not from the floor.	Extraction: methanol Clean-up: ENVI-Carb LC-TOF-MS ACE C18-column (150 x 2.1 mm, 3 µm, ACE)	The highest median concentrations in dust were observed for PFHxA (28 ng g^{-1}), PFNA (23 ng g^{-1}), PFDA (19 ng g^{-1}), and PFOA (18 ng g^{-1}). However, PFSAs were also frequently detected. FTOHs were the most prominent compounds found in indoor air, with median concentrations for 8:2 FTOH, 10:2 FTOH, and 6:2 FTOH of 5173, 2822, and 933 pg m^{-3} air, respectively.	[38]

Germany	FTOHs PFOS PFOA	31 house dust samples	Dust samples were collected from residences in Bavaria (Munich and nearby suburban and rural areas)	FTOHs:extraction acetone/MTBE (1/1). Clean-up: Envi-Carb cartridge PFOA and PFOS Extraction:MeOH FTOHs: GC-MS 60 m VMS column (0.25 mm inner diameter, 1.4 μm film thickness) PFOA and PFOS: LC–MS/MS (ReproSil-Pur ODS-3, 5 μm, 150 mm×2 mm)	PFOA was the dominant compound in 79% of the dust samples, followed by PFOS and 8:2 FTOH, while 4:2 FTOHwas not detected in any samples. The total concentration of per- and polyfluorinated compounds (PFCs) varied from 32.2 to 2456 ng g^{-1}.	[39]

Table 1.3: Indicative examples of the detected concentrations of PFASs in food and food packages.

Country	Analytes	Matrix	Origin of samples	Method of analysis	Results of analysis	Reference
Thailand	PFOS PFOA	34 samples of food packaging material made of paper	Domestic and international restaurants in Bangkok, Thailand	PLE: MeOH or saliva stimulant LC–MS/MS LC column: Agilent Eclipse XBD-C18 (4.6 mm * 50 mm ,1.8 μm)	PFOS and PFOA were detected in almost all paper packages. The highest concentration for PFOS (92.48 ng dm^{-2}) and PFOA (17.74 ng dm^{-2}) was found in a fried chicken box	[40]
Spain	7 PFASs	Microwave popcorn bags of three different brands	Supermarkets in Spain	PLE: MeOH LC–QTOF–MS/MS LC column: Waters Acquity C18 50 * 2.1 mm, 1.7 μm	Significant levels of PFOA (53–198 ng g^{-1}). Detectable levels of PFHpA, PFNA and PFDoA in some samples. All 7 PFCs were detected in two of the samples.	[41]

Greece	17 PFASs	42 samples including beverage and ice cream cups, fast food wrappers, paper boxes, baking paper, aluminum foil bags and wrappers, microwave bags	Retail sellers, fast food chain restaurants, coffee shops and multiplex cinemas in Athens, Greece	PLE: MeOH Clean-up: Florisil-Basic Alumina column LC–MS/MS LC column: Thermo Hypersil GOLD C8 (150 mm *2.1 mm i.d, 3 μm)	Neither PFOA nor PFOS was detected in any sample. PFTrDA, PFTeDA and PFHxDA were detected in fast food boxes. PFHxA was found in ice cream cup. Several PFCs were detected in fast food wrappers and microwave popcorn bag.	[28]
USA	PFOA	Popcorn bags, hamburger wrapper, French fry box, paper plates, perfluoro paper coatings, etc.	US retail market	Sonication with 50/50 ethanol/water LC–MS/MS LC column: Zobrax SB C8,100* 2.0 mm , 3.5 μm	PFOA was present in many samples, with highest amounts in popcorn bags (up to 290 μg kg^{-1}). The migration of PFCs from cookware and popcorn bags was studied.	[42,43]

					In all cases, PFOS was the most frequent compound (372–598 ng kg^{-1} in seafood and 104–478 ng kg^{-1} in fish)	
Belgium, Norway, Italy, Czech Republic	21 PFCs	Fish, meat, hen eggs, cheese and milk, and butter	Purchased from local supermarkets	Extraction: Quechers Clean-up: Envi-carb LC-MS: Acquity UPLC HSS T3 column (100 × 2.1 mm i.d., 1.8 µm)	Concentration ranges of individual compounds in the groups of PFASs were: 2.33–76.3 ng kg^{-1} for PFSAs (without PFOS), and 4.99–961 ng kg^{-1} for PFCAs. The contamination level in the analysed food commodities decreased in the following order: seafood > pig/bovine liver >> freshwater/marine fish > hen egg > meat >> butter.	[26]

Spain	18	40 items: meat and meat products, fish and shellfish, vegetables and tubers, fresh fruits, milk and dairy products, cereals, pulses, industrial bakery, eggs, oils and fats, and canned products	Purchased in 12 representative cities in Catalonia	Extraction: MeOH Clean-up: Oasis WAX cartridges UPLC-MS/MS: Acquity BEH C18 column (2.1*100 mm, 1.7 µm)	PFOS was the compound found in the highest number of samples (33 out of 80). Highest values of PFASs were found in fish and shellfish samples. (Highest level: 46,000 pg PFOA g^{-1} in a composite sample of mussels) The levels of detected PFASs in other groups of foodstuffs were considerably lower.	[25]
Greece	17	Anchovy, bogue, hake, picarel, sardine, sand smelt and striped mullet, Mediterranean mussel, shrimp and squid in raw and cooked form	Collected from various fishing sites in the Aegean Sea	PLE: MeOH Clean-up: Florisil-Basic Alumina column LC–MS/MS: Thermo Hypersil GOLD C8 (150 mm *2.1 mm i.d, 3 µm)	PFCs above the detection limit were found in all fish samples and in all shellfish except the mussel. PFOS was the most abundant PFC with values between <LOD and 44 ng g^{-1} ww	[27]

Italy	PFOS PFOA	81 pools of food products	Purchased in supermarkets in Sienna	Extraction: MTBE HPLC-ESI-MS: Betasil C18 column (50 *2.1 mm i.d., 5 µm)	Fish were the most contaminated samples (7.65 ± 34.2 ng g^{-1}); mean concentrations in meat and milk and dairy products were similar (1.43 ± 7.21 ng g^{-1} and 1.35 ± 3.45 ng g^{-1}, respectively). In all cereal-based food, eggs, vegetables, honey and beverages PFOS concentration was <LOD.	[44]
Netherlands Greece	11	171 house produced and commercially produced eggs	Collected from houses and supermarkets in the Netherlands and Greece	Extration: MeOH Clean-up: Oasis WAX cartridges LC-MS/MS: Fluorosep analytical column (50 mm * 2.1 mm, 5 µm)	In the house produced eggs PFOS was the predominant PFASs (highest concentration: 24.8 ng g^{-1}). The long-chain PFASs (C≥8) were the most frequently detected, while short-chain PFASs were rarely found.	[29]

					In the eggs collected for the supermarkets all PFASs levels were below the LOQ (0.5 ng g^{-1})	
Norway	12	21 samples of selected food and beverages such as meat, fish, bread, vegetables, milk, drinking water and tea	Purchased in Norwegian marked	Extration: MeOH Clean-up: Waters Oasis WAX LC-MS/MS: Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm i.d., 50 mm)	A wide range of PFCs were detected in the samples. PFOA was found in all the samples and PFOS concentrations were above LOD in all samples except in tea. The remaining PFCs were detected less frequent. The highest concentrations of PFOS were found in cod liver followed by cod, beef, salmon and canned mackerel	[45]

Netherlands	14	Pooled samples from 15 food categories: fatty fish, lean fish, crustaceans, butter, cheese, milk, eggs, pork, beef, chicken/poultry, bakery products, vegetables/fruit, flour, vegetable, industrial oil	Purchased in several Dutch retail stores with nation-wide coverage.	Extraction: THF:water Clean-up: Oasis WAX and Supelclean ENVI-carb LC-MS/MS: Fluorosep RP Octyl column (5 µm, i.d. 2.1mm, 15 cm)	Only PFHpA, PFOA, PFNA, PFDA, PFHxS, and PFOS were detected in the majority of the food categories. The food categories cheese, pork, chicken/poultry, bakery products, flour, vegetable oil, and industrial oil contained the lowest concentrations (<20 pg g^{-1} product for each compound). Highest concentrations of PFOS and PFOA were found in crustaceans and lean fish.	[46]
Netherlands	14	Fillets of raw fish and meat, whole-grain bread, vegetables, fruits, cheese and sunflower oil samples	Purchased in local supermarkets in Amsterdam	Extraction: THF:water Clean-up: Oasis WAX and Supelclean ENVI-carb LC-MS/MS: Fluorosep RP Octyl column (5µm, i.d. 2.1mm, 15 cm)	PFCs ranged between 4.5 to 75 pg g^{-1} in 25% of samples (fish and packaged spinach). C10–C14 PFCs were found in fish	[47]

Table 1.4: Indicative examples of the detected concentrations of PFASs in drinking water.

Country	PFASs analysed	Matrix	Origin of samples	Method of analysis	Result of analysis	Reference
Spain	15	30 samples of water and 21 composite samples of fish and shellfish	Water samples were collected at 3 points in each of 10 municipal water supply networks of Catalonia. One-third of those samples (drinking water for human consumption) were collected in public fountains from 10 different Catalan locations Fish and shellfish were collected from coastal areas of Catalonia.	Water: extraction with Oasis WAX cartridges Fish: extraction with ACN Clean-up: n-hexane and ENVI-Carb Quattro Premier XE MS/MS: Acquity BEH C18 analytical column	In water samples, the highest mean concentrations corresponded to PFOS and PFOA (1.81 and 2.40 ng L^{-1}, respectively) Among the analyzed PFCs in fish and shellfish, only seven compounds were detected in at least one composite sample. PFOS showed the highest mean concentration of 2.70 ng g^{-1} fw, being detected in all species with the exception of mussels.	[48]

France	10	331 samples of surface and groundwater used for public water systems and 110 of treated water	Collected from all the French departments	Extraction: Oasis WAX cartridges TSQ Quantum Ultra LC/MS-MS: BetaCil C18analytical column	In raw-water samples, the highest individual PFC concentration was 139 ng L^{-1} for PFHxA. PFOS, PFHxS, PFOA, and PFHxA predominated.	[22]
Spain	13	40 municipal drinking water samples	Collected from 40 different locations, from 5 different zones of Catalonia	Extraction: Oasis WAX cartridges Acquity UPLC – Quattro Premier XE tandem MS: Acquity BEH C18 analytical column	The most frequent compounds were PFOS and PFHxS, detected in 35 and 31 samples, with maximum concentrations of 58.1 and 5.30 ng L^{-1} respectively. PFBuS, PFHxA, and PFOA were also frequently detected with maximum levels of 69.4, 8.55, and 57.4 ng L^{-1}.	[49]

Netherlands	10	54 water samples	Collected from the source of drinking water for the city of Amsterdam (Netherlands), Lek canal , before and after treatment	Extraction: Oasis WAX cartridges HPLC-MS/MS: ACE 3 C18-300 Analytical column:	The finished water contained 26 and 19 ng L^{-1} of PFBA and PFBS. Other PFAAs were present in concentrations below 4.2 ng L^{-1}.	[23]
Greece Netherlands	11	80 tap water samples and 10 bottled water samples	Tap water was collected from different places around the Netherlands and Greece Bottled water samples were purchased from supermarkets in both countries	Extraction: Oasis WAX cartridges LC-MS/MS: Acquity UPLC BEH C18	Total PFAS concentrations for water samples from Greece ranged between <LOQ to 5.9 ng L^{-1}, while for the samples from the Netherlands ranged between <LOQ to 54 ng L^{-1}.	[24]

Faroe islands	15	Milk, yoghurt, crème fraiche, potatoes, fish, and fish feed, and water samples (surface water and purified drinking water)	Packaged dairy products were provided by the sole diary in the Faroe Islands. Potatoes were delivered from farms. Fish samples were collected from the Faroe Shelf area. Surface water was taken from four lakes. Raw cow's milk was taken from two major milk producers in the Faroe Islands	*Fish*: extraction with ACN Clean-up: n-hexane and ENVI-Carb *Water*: filtered extraction using Oasis WAX *Milk*: extraction with formic acid/water Clean up: Oasis WAX Acquity UPLC,coupled to a Quattro Premier XE MS: Acquity BEH C18 column (2.1×100 mm, 1.7 μm)	PFBA was a major contributor in water samples (mean concentration: 750 pg L^{-1}). PFUnDA was predominating in milk and wild fish with mean concentrations of 170 pg g^{-1}. PFOS was the most frequently detected compound in food items followed by PFUnDA, PFNA and PFOA.	[50]

Table 1.5: Indicative examples of the detected concentrations of PFASs in biological matrices.

Country	PFASs analysed	Matrix	Origin of samples	Method of analysis	Result of analysis	Reference
Canada	7	13 individual samples of human milk	Collected in the Kingston region of Ontario (Canada)	Extraction: MTBE LC-MS/MS: Discovery HS C18 (7.5 cm * 2.1 mm, 3 µm)	Only PFOA was detected in 85% individual human milk samples analyzed, with a concentration range of <0.072 to 0.52 ng ml^{-1}.	[30]
Sweden	PFOS PFHxS PFOA	20 pooled human milk	Collected from healthy native Swedish mothers by the Mothers' Milk Center (Stockholm, Sweden)	Primary extraction: ACN MTBE HPLC-MS/MS : ACE® C18 HPLC column (5 µm, 75 × 2.1 mm i.d.)	PFOS was the predominant analyte and the concentration ranged between 0.088–0.151 ng $mL^{-1.}$	[31]
Jordan	PFOS PFOA	Human breast milk and fresh cow milk	79 milk samples were collected from Breast milk breastfeeding mothers and 25 samples from local fresh cow milk in northern Jordan.	LLE: acetone Clean up: Oasis HLB cartridges LC-MS/MS: Agilent C18 columns (50 × 2.1 mm, 5 µm)	The measured concentrations ranged between n.d. and 178 ng L^{-1} for PFOS and between 24 and 1120 ng L^{-1} for PFOA in human milk and between nd-178 ng L^{-1} and LOQ-160 ng L^{-1} in fresh cow milk, respectively.	[51]

France	18	Breast milk, maternal and cord serum	Collected from 100 mother–newborn pairs recruited in France	*Serum and blood*: extraction with KOH 0.1 M in MeOH Clean up: Oasis® HLB cartridge *Breast milk*: extraction with acetone Clean-up with Oasis HLB cartridge LC-MS/MS: Gemini C18 (3 µm, 50 × 2.0 mm) analytical column	In serum, the cumulated concentrations of the 7 most frequently detected compounds were 5.70 ng mL^{-1} and 2.83 ng mL^{-1} (median values) in maternal and cord serum, respectively. PFOS, PFOA, PFHxS and PFNA contributed to around 90% of the total PFAAs contamination. Levels measured in breast milk were far lower (20 to 150 fold) than those determined in serum.	[32]
China	11	Human blood	Collected from non-occupationally exposed population.	Extraction with MTBE HPLC–MS/MS: Kinetex C18 column (100 mm × 4.6 mm internal diameter, 3.0 µm)	PFOA and PFOS were detected frequently in all of blood samples with median values of 1.28 and 4.66 ng mL^{-1}, respectively.	[52]

South Korea	16	Serum and urine	120 children aged 5-13 years from Daegu, Korea	Extraction: ACN and 1 mL of 2% formic acid Clean-up: Oasis WAX SPE LC-MS/MS	The total PFC concentrations in the serum were 4.26-29.70 ng mL^{-1}, and PFHxS, PFOA, PFOS, which was dominant overall, at 6.58 ng mL^{-1}, and PFUndA were detected in all serum samples.	[53]
Greece	PFOS PFOA	Serum	56 samples from Athens, 86 samples from Argolida and 40 samples from cancer patients from Greece.	Extraction: ACN Clean-up: C18 cartridge LC-MS/MS	PFOS and PFOA were detected in all samples examined. PFOS: 2.12-40.36 ng mL^{-1} PFOA: 0.48-10.21 ng mL^{-1}	[33]

1.5 Regulations on the elimination of PFASs production and emissions

Concerns about the potential environmental and toxicological impacts of the production and use of these compounds led to the implemention of preventing measures. 3M Company, the main manufacturer of PFOS based in America, phased out its production in 2002 and the compound is now used only in relatively small quantities for applications for which there is no acceptable substitute, such as in semiconductor manufacturing http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1867999/ [54-56]. However, PFOS and its derivatives are still manufactured in China [57]. Meanwhile, as part of the European Protection Agency (EPA) stewardship program, eight companies using PFOA (Arkema, Asahi, Ciba, Clariant, Daikin, DuPont, 3M/Dyneon, and Solvay Solexis) started reducing PFOA emissions, the use of precursor chemicals that break down into PFOA and other related higher homologue chemicals, and also PFOA product content by 95% by 2010, in order to eliminate their use by 2015 [58-59]. A similar agreement about the reduction of PFASs in products was also made between Canadian environmental and health authorities and five companies [60]. The European Union Marketing and Use Directive restricted the use of PFOS in the European Union [61] while other regulatory and voluntary initiatives intended to reduce environmental emissions of PFASs. Thus, other compounds of the same family started being used for the replacement of the long chain PFASs [62-65]. In particular, 3M Company started a new generation of PFAA products, by using shorter chain compounds (e.g. PFBA), as it has been reported that they have shorter half-lives in humans http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1867999/. However, these substitutes can also pose problems of their own, as for example some of them can be transformed into PFOA or PFOS as the result of metabolism or environmental biodegradation.

Moreover PFOS, owing to its characteristics, has been included in the Stockholm Convention on Persistent Organic Pollutant (POPs) as an Annex B substance [66] http://chm.pops.int/Programmes/NewPOPs/The9newPOPs/tabid/672/language/en-US/Default.aspx. The European Chemical Agency (ECHA) has included ammonium pentadecafluorooctanoate (APFO), PFOA, and C11 - C14 PFCAs in the candidate list of substances of very high concern [67], while recently, the Chinese Ministry of Environment Protection drafted a list of priority hazardous chemicals for environmental management

including PFOS and its salts [68]. However, no threshold limits for PFOS or other PFASs concentrations in environmental matrices have been specified yet.

1.6 Human health effects

PFASs frequent detection, their environmental persistency and their ability to bioaccummulate have raised warning signs for the human health. According to the literature, PFASs do not accumulate in adipose tissue like other POPs, but they are mainly distributed to the serum, kidney and liver, with the latter showing high levels of contamination. In particular, PFASs bind to β-lipoproteins, albumin and liver fatty acid-binding protein (L-FABP) [69].

One of the most remarkable features of PFASs pharmacokinetics, that can be also related to their partitioning in the liver and serum is the different elimination time among the species, with humans' half live reported as remarkably higher compared to the other species [70]. More specifically, half-lives of PFASs have been found to increase with increasing chain length. PFOS has a half-life of 100 days in rats, while the half life in humans is about 5 years. The elimination half-life of PFOA is 2-4 hours in adult female rats and about 3.5 years in human serum [3,71,72,73].

Since the late 1960s, when fluoride was detected in blood samples, the existence of PFASs in the human burden has been suspected. Human biomonitoring began focusing on occupational populations [74,75] and following on general population [33,72,76,77,78]. These studies were mainly based on PFASs detection in blood (whole blood, plasma and serum) samples, either individual or pooled, with PFOS and PFOA being the most frequently detected compounds. According to the results, PFASs levels were found to be higher in blood collected from workers occupationally exposed to PFASs than in blood from general population. Apart from the comparison between PFASs levels in serum from workers exposed to PFASs and from general population, the gender, the age, the diet of the blood donors, and the geographical place where they live, are also some of the factors that have been investigated [33,76,79,80,81]. However, information on the pharmacokinetic properties of PFASs, especially PFOS and PFOA, has been mainly provided by animal studies in rodents, mammals, monkeys and mice. According to these studies, the immunotoxic potential of PFOS and PFOA has been demonstrated in vitro and in a variety of species [82]. Reduction of body weight and cholesterol levels, elevation of liver weight, decreases in thymus and spleen

weight, and a steep dose-response curve for mortality have been reported as consequences of the exposure of rats to PFASs [83,84].

Adverse reproductive outcomes [85] like fetal weight reduction, cleft palate, cardiac abnormalities and delayed ossification of bones as well as postnatal growth are some of the symptoms related to PFASs exposure to rats and rabbits [86-89]. PFASs have also been associated with hormone disruption in rats. In particular, they cause significant reduction of the thyroid hormones T3 and T4 [90-91], and also cause changes in sex steroid hormone biosynthesis [92,93].

The available information on PFASs carcinogenicity does not prove PFASs carcinogenicity in humans, but the evidence is not conclusive. Although experiments in rats have shown that exposure to PFOS and PFOA causes tumor development, it is not proven yet that the same mechanism takes place in the human organism. Cancer evidence about pangreatic tumors and hepatocellular carcinomas caused by PFASs, stemming from proliferation, were first reported in animal studies at the late 1970s [94], while the first correlation between Leydig cell tumors and PFASs was also described in 1992 after measurements in rats [93]. A study conducted in workers exposed to PFASs showed that they have an increased risk of bladder cancer compared to the regular population. However, this outcome was not considered as reliable because of the worker's exposure also to other compounds and due to the limited cases of bladder cancer (three) observed [95]. According to a follow-up study, eleven cases of bladder cancer were identified in workers exposed to PFASs, but there were no statistically significant associations between PFOS exposure and an increased risk of bladder cancer [96]. In another study conducted in Greece, comparing the levels of PFOS in the blood of hospitalized cancer patients and healthy individuals no significant difference was observed between the groups [33].

While evidence on PFOS carcinogenicity is less extensive and conclusive, PFOA have been found to associate with kidney cancer [97,98] testicular, ovarian and prostate cancer and also with non-Hodgkin lymphoma [99].

Epidemiological studies on PFASs exposure and their health outcomes in humans are still inconclusive. However the absence of studies does not exclude the possibility of adverse effects. To this end, further investigation on PFASs exposure and human health risks is necessary.

1.7 Routes of human exposure to PFASs

Human exposure pathways to PFASs are dietary intake, consumption of drinking water, dermal exposure, inhalation of dust, indoor and outdoor air [19,25,38,100]. PFASs can also transfer from the mother to the fetus through the placenta and from the mother to the neonatal via breastfeeding [32,101,102].

Even if the relative contribution of each route of human exposure to PFASs is not yet well known, food ingestion has been reported as the main one [103,104]. To this end, several studies are focused on PFASs levels in food items and provide information on human dietary exposure to PFASs [25,26,45,46,104-109] (Table 1.3). Most of these studies are in accordance with the European Food Safety Authority (EFSA) report, presenting fish and other sea food as the most contaminated food item and PFOS as the dominant compound in most of the cases. Long-chain PFASs, including PFOA, PFNA, PFDA, PFUnA and PFDoA, are also detected frequently in the various food items. In all the studies, the calculated dietary intake for PFOS never exceeded the Tolerable Daily Intake (TDI) recommended by EFSA (150 ng kg^{-1} b.w. per day for PFOS and 1500 ng kg^{-1} b.w. per day for PFOA) [4].

1.8 Legislation on PFASs in food and drinking water

Concerns about the potential health effects of PFASs in humans, due to their exposure mainly via the consumption of food products and drinking water, has led non-governmental organizations, national and international authorities to address the threat of PFASs in food and drinking water via legislative actions.

EFSA published a health risk assessment for the two most important PFASs, PFOS and PFOA, and assigned a TDI (150 ng kg^{-1} b.w. per day for PFOS and 1500 ng kg^{-1} b.w. per day for PFOA) in 2008 [4].

In 2010 the EU recommended the monitoring of the presence of PFOS and PFOA and if possible, their precursors in food, during the years 2010 and 2011 (Recommendation 2010/161/EC:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:068:0022:0023:EN:PDF. To this end, a dietary exposure assessment, based on data submitted by EU member states between 2006 and 2012, was published by EFSA in 2012 [110]. This exposure assessment showed that the highest mean exposure to PFOS and PFOA was 5.2 and 4.3 ng kg^{-1} bw per day for adults, and 14 and 17 ng kg^{-1} bw per day for toddlers, the

highest exposed age group. The P95 levels were about two-times higher, demonstrating a reasonable margin of exposure even for highly exposed consumers. The main contributor to the dietary exposure was fish and fish products.

In the meantime, in 2009 the EPA's Office of Water established a provisional health advisory (PHA) of 0.2 μg L^{-1} for PFOS and 0.4 μg L^{-1} for PFOA to assess the potential risk from short-term exposure of the two compounds through the consumption of drinking water [111,112]. The Swedish National Food Agency has recently introduced a conservative "limit of action threshold" of 90 ng L^{-1} for the sum of PFBS, PFHxS, PFOS, PFPeA, PFHxA, PFHpA, and PFOA for drinking water [113]. Moreover, the EU Water Framework Directive has determined environmental quality standards (EQS) (0.65 ng L^{-1}) for PFOS [114] and proposed restrictions on manufacturing, use or market distribution of PFOA [115].

1.9 Sources of food contamination

Food can be contaminated with PFASs via direct and indirect sources. Direct contamination includes environmental exposure of animals and plants, and PFASs bioaccumulation through the food chain. On the other hand, indirect contamination includes cooking, food packaging and food processes.

As far as the direct contamination is concerned there are many different ways that PFASs can enter in drinking water and food. Considering their transport in the environment, PFASs can disseminate into plants and animals that will be further consumed by animals higher up in the food chain. As a consequence, the exposure of plants and animals to PFASs through i.e. contaminated water, feed or air comprises a very important route of PFASs input in the food chain. As contaminated water is one of the main sources of food contamination with PFASs, studies have focused on the investigation of water cycle contamination. According to the results, inefficient removal of ionic PFASs of wastewater, the use of sewage sludge as fertilizer and the run off can contribute to the contamination of drinking water and subsequently of food.

On the other hand, during the last years, the scientific interest has also turned to the investigation of the indirect sources of PFASs food contamination. The available information on this way of contamination is limited. Preliminary data show that domestic cookware does not influence PFASs levels in food during the preparation of food, while the procedure of

cooking may also reduce their levels [116]. PFASs can also migrate from the package to the food, as they are used in greaseproof materials in various food packaging materials [40,43].

1.10 Analytical methodologies

1.10.1 Sample preparation

The detectable PFASs levels in food matrices are high till now. For this reason, sensitive and reliable analytical methods for the determination of PFASs in food are needed in order to provide valid information and ensure consumer' safety. As food samples are quite complex matrices, they require some preliminary sample preparation before their analysis. Therefore, sample pretreatment can be realised in different steps like modification of the sample matrix, extraction of the analytes of interest and purification of the matrix by removing co-extracted components that can interfere in the analysis.

1.10.1.1 Matrix modification

Many different ways of pretreatment methods are applied in food containing PFASs, depending on each matrix. In particular, commonly the solid samples (fish, liver or meat) are weighed and chopped or homogenized in a blender. Also, other solid samples, like eggs, if not analysed raw, are subjected to freeze drying procedures, or are boiled before the analysis. On the other hand, liquid samples like water are usually extracted without any preparation. Food packaging materials are most of the times cut in small pieces and the outside layers or printings are removed before the extraction.

1.10.2 Sample extraction techniques

1.10.2.1 Liquid extraction

Liquid extraction (LE) is a technique used very often for the separation of compounds based on their different solubilities in two different immiscible liquids, usually water and an organic solvent. The selection of the extraction solvent has to be quite selective, considering the characteristics of the target compounds, in order to obtain the most optimal results and to avoid the extraction of matrix constituents that are going to prevent excessive matrix effects (ME). Apart from the target compounds, the selection of the extraction solvent depends also on the matrix.

1.10.2.2 Pressurized liquid extraction (PLE)

Automated methods are in general preferable as they are more reliable and at the same time faster in comparison to the manual ones. However, by applying automated methods the cost is usually higher. Pressurized liquid extraction (PLE) is the automated technique most frequently used for the extraction in food matrices.

The PLE technique that has been used for PFASs extraction is the accelerated solvent extraction (ASE). During this procedure temperature above the boiling point of the solvent and high pressure for the maintenance of the solvent in the liquid phase are applied. Thus, an efficient extraction of the target compounds from the matrix is achieved.

According to the majority of the studies that have focused on the detection of PFASs, organic solvents are normally used as extraction solvents. In particular, PFASs extraction from complex matrices like food is often based on ion-pair extraction. Tetra-n-butylammonium hydrogen sulfate solution and sodium carbonate buffer at pH 10 are used as the ion pairing agent and methyl tert-butyl ether (MTBE) as the extractor [117,118]. In other studies, the use of KOH digestion followed by solid phase extraction (SPE) has also been reported [119,120]. Other organic solvents like methanol (MeOH) and acetonitrile (ACN) have been also used as extractor solvents in various matrices, as they can precipitate the proteins contained in the matrix and at the same time extract the target compounds.

1.10.3 Sample clean-up/purification technique

1.10.3.1 Solid phase extraction (SPE)

Solid phase extraction is a technique designed for rapid, selective sample preparation and purification prior to chromatographic analysis. During the SPE process, the target compounds that are dissolved in a liquid mixture are separated from other compounds that exist in the mixture according to their physical and chemical properties. In the beginning, the desired analytes are retained on the stationary phase after elution of interfering compounds and then they are removed and collected by the use of an appropriate eluent. To this end, a wide variance of sorbents which rely on different mechanisms for the retention of analytes is available.

Due to the different polarities among PFASs, different solid phase extraction cartridges can be used for the separation of the analytes from undesired impurities. Oasis WAX cartridges, that

are based on weak anion exchange mechanism, are widely used and they yield good recoveries for short-chain PFASs. On the other hand, less polar phases, like C18 and Oasis HLB can be used for long-chain PFASs [121]. In general, sorbents based on weak anion exchange mechanisms, hydrophilic-lipophilic-balanced sorbents, or even just hydrophobic sorbents (florisil column) are used for PFASs purification depending on the matrix, and the polarity of the target compounds that are expected to be detected in each case.

1.10.4 Instrumental analysis

As far as the analytical detection method of PFASs is concerned, liquid chromatography combined with mass spectromentry (LC-MS) and with tandem mass spectrometry (LC-MS/MS) are the main choices for the detection of the anionic PFASs (including PFOS and PFOA). Gas chromatography combined with mass spectrometry (GC-MS) can be also used for the direct determination of both anionic and neutral PFASs, but is mainly used for neutral volatile PFASs, including several precursors of PFOS and PFOA e.g., PFOSA, fluorotelomer alcohols etc [122].

LC-MS/MS using a triple-quadrupole mass spectrometer (QqQ) is the most frequently applied technique concerning studies focused on anionic PFASs detection and also the best suited for the detection of PFASs in food matrices. Although LC with single quadrupole MS is also a sensitive technique, it requires a more thorough clean-up step of the sample in order to avoid interferences, because of its inherently lower selectivity [4].

Due to the acidic properties of PFCAs and PFSAs they can dissociate, and thus electrospray ionization in the negative mode (ESI^-) suits the detection of PFASs at low levels. Pseudomolecular ions, like [M-K]- for PFOS and [M-H]- for PFOA are formed, and they are usually precursor ions for MS/MS analysis with QqQ or ion trap (IT) instruments [121].

Apart from LC-MS/MS, other analysers have also been used by LC for the determination of PFASs. Quadrupole linear ion trap (QqLIT) usually allows the limit of qualification (LOQ) lower than QqQ, while by using atmospheric-pressure photoionization (APPI) the matrix effect is absent, but the limit of detection (LOD) is essentially higher compared to those of LC-MS/MS [123]. Quadrupole-time-of-flight (Q-TOF) MS analysers are less sensitive than QqQ MS/MS systems, but seem to be suitable for PFAS detection in the environment [124,125]. High resolution mass spectrometry (HRMS) has been used for quantification and screening.

Berger et al. compared three different analytical techniques, ion trap MS (IT-MS), QqQ-MS/MS and high resolution time of flight combined with LC for the detection of PFASs. According to the results of this study, IT-MS was suitable for the identification of branched isomers, QqQ-MS/MS was found to quantify telomer alcohols and PFASs at low levels (LOD: low pg and 10-100 pg respectively), while TOF-MS was the best choice for the quantification of PFASs, showing high selectivity and sensitivity [126].

A more recent study [126] was also conducted in order to compare QqQ, conventional 3D IT, and QqLIT. According to the results, the three aforementioned analytical methods were all accurate with high recoveries. QqLIT and QqQ were more precise and offered a more linear dynamic range than IT. In addition, QqLIT was found to be more sensitive than the two other systems.

1.10.5 Prevention of PFASs contamination

1.10.5.1 Sample conservation and pretreatment

A major analytical problem is the contamination of the samples during the sampling procedure and the analytical process. The use of the appropriate sample container, like glass containers [125], plays an important role concerning losses due to PFASs adsorption, possible biodegradation or biotransformation, and contamination due to the use of materials containing PFASs. Regarding sample conservation, it is usually achieved by freezing the samples till the day of the analysis, avoiding PFASs losses.

In addition, laboratory materials containing fluoropolymers, such as polytetrafluoroethylene Teflon or other fluoropolymers that can be used for vial caps, LC instrument tubing and internal instrument parts have to be avoided to prevent contamination. To this end, alternative materials such as polypropylene are used.

Investigation of blank samples has to be performed during the analysis of all the batches in order to monitor background contamination originating from various sources in the laboratory. As far as the contamination due to the fluoropolymer parts in the instrument is concerned, this can be overcome by the replacement of these parts, or the installation of an isolator column upstream of the LC-column to prevent PFASs contamination.

1.10.5.2 Matrix effect

The matrix effect when mass spectrometry techniques are applied, especially in complex matrices like food, is one of the main contamination problems encountered by LC-MS/MS. The matrix effect mainly appears as ion suppression. In particular, the evaporation efficiency of the ions of the analyte is decreased or increased due to competition between the co-extracted and co-eluted matrix components and the analytes. In the case of PFASs analysis, a common example of interference is the one between PFOS and taurodeoxycholic acid (TDCA) that is a bile salt. TDCA and PFOS have the same unit mass of 499 and they both contain a sulfonate group that delivers the same transition 299-80 when LC-MS/MS is used for the measurement of the samples. This interference can be overcome by using the transition 499-99 for PFOS quantification and also by introducing purification techniques that eliminate TDCA from the sample. Another option, is the use of fluorosep analytical column instead of C18, in the LC system, that according to previous studies [47] can separate the two different compounds by eluting them in a different retention time (RT). The use of accurate mass instrumentation can also provide a great peak separation due to the high resolution detection.

1.10.6 Validation

Method validation is the process of defining an analytical requirement, and confirming that the method under consideration has capabilities consistent with what the application requires. Specificity, selectivity, precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, ruggedness and recovery are some of the most common factors validated after the development of an analytical method http://www.eurachem.org/index.php/publications/guides/mv.

Specificity (Selectivity)

This parameter concerns the extent to which other substances interfere with the identification and, where appropriate, quantification, of the analytes of interest. It is a measure of the ability of the method to identify/quantify the analytes in the presence of other substances in a sample matrix under the stated conditions of the method.

The ion ratio of the relative response of the secondary mass transition to the primary mass transition and the retention time are recorded for each compound in order to identify (specify) the analytes, when LC-MS/MS is used.

Precision

Precision is a measure of how close repeat results are to one another and is usually expressed by statistical parameters which describe the spread of the results. Repeatability and reproducibility are the two common measures of precision which can be obtained. For the determination of the two aforementioned parameters, replicates of blank samples are fortified in different concentrations and are analysed in three different days in order to determine these two parameters. The precision of the method is then presented as the estimated relative standard deviation (RSD%) of the interday and intraday measurements.

Accuracy

Accuracy is used to describe the measure of exactness of an analytical method, or the closeness of agreement between the conventional true value or an accepted reference value and the value found. This is a measure of the difference between the expectation of the test result and the accepted reference value due to systematic method and laboratory errors and is usually expressed as the statistical error (E).

Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the matrix, compared to the detector response for the true concentration of the pure mass-labelled standard. In order to consider a method as valid, the recovery of the mass-labelled standards needs to vary between 60-120% for all the compounds.

Limit of detection

The limit of detection (LOD) is the lowest analyte concentration that can be detected and identified with a given degree of certainty. The LOD is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence. There are several methods of estimating the LOD, all of which depend on the analysis of blank spiked samples and the examination of the signal to noise ratio. A minimum requirement for signal-to-noise of three is widely accepted.

Limit of quantification

The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Like LOD, LOQ is expressed as a concentration at a certain specified signal-to-noise ratio, usually ten to one.

Linearity and working range

In general, methods are described as linear when there is a directly proportional relationship between the method response and concentration of the analyte in the matrix over the range of analyte concentrations of interest (working range). The working range is the interval over which the method provides results with an acceptable uncertainty and is predefined by the purpose of the method. High correlation coefficient (*r*) of 0.99 is used as criterion of linearity.

Ruggedness/Robustness

Ruggedness is a measure of the capacity of the analytical method to remain unaffected by small, but deliberate variations in method parameters. These parameters include different laboratories, analysts, instruments, reagents, days etc. Ruggedness provides an indication of the method's reliability and reproducibility of the results obtained.

CHAPTER 2

Scope and objectives

2.1 The problem

The concern over potential environmental and human health adverse effects increases the scientific interest and orients it towards the elucidation of PFASs environmental origin, fate and impact. Since diet is considered to be the main route of human exposure to PFASs, scientific research is lately focused on the analysis of food matrices and drinking water.

Till now, there is a limited number of available studies that are mainly focused on the detection of PFASs levels in various food items and on human dietary intake and potential exposure to PFASs due to the consumption of these food products. However, the sources of contamination (direct and indirect), that are of major importance and can provide information on PFASs behavior and transfer from the environment to food chain, including food origin, way of production and animals' exposure to PFASs which also lead to possible contaminated animal food products, are not well investigated and the available information is still missing from the literature. The extent to which each source contributes to the contamination of food items is also a crucial question that still remains unanswered.

In addition, the unique characteristics of the target compounds, the complexity of the matrix and the very low concentration levels at which PFASs should be analysed (ng g^{-1}), require the development of sensitive and selective analytical methodologies. In this context, sample pretreatment, extraction of the analytes and purification of the matrix are steps that have to be quite effective in order to detect PFASs in low levels. Contamination of the samples during the sampling procedure and the analytical process, including contamination due to the fluoropolymer parts of the instruments, and matrix effect, are the main difficulties during the analysis that has also to be taken under consideration and overcome, especially when complex matrices like food are analysed.

To this end, in the present study, risk assessment of PFASs through their detection mainly in food matrices was made in an effort to provide data that will fill this gap in the literature. In particular, PFASs ways of transport into the environment and then into the food chain, their presence in various food matrices and the estimation of the human daily dietary intake of

these compounds were examined and evaluated. For the analysis of the samples and the detection of PFASs in very low levels, selective and sensitive quantitative analytical methods were developed by evaluating different extraction and clean-up techniques and by using LC-MS/MS.

2.2 Research objective and scope

The work performed in the current study focus on:

- The development of selective analytical methods that can be applied in different matrices (food items, food packaging materials and drinking water).
- The collection of the samples considering their origin.
- The analysis of the samples and the processing of the results.
- The evaluation of PFASs detected levels.
- The assessment of PFASs pathways of transport into the environment.
- The estimation of the human dietary intake of PFASs, based on the detected PFASs concentrations and the frequency of consumption of each food product.

The selection of certain target compounds was made after an extensive literature review and the reason of this choice was mainly their high frequency of detection in various different matrices. PFASs analysed in the current study are presented in the following Table (Table 2.1).

Table 2.1: The selected perfluoroalkyl carboxylic acids and perfluoroalkyl sulfonic acids analysed in the present study.

Chemical name	Acronym	Molecular formula	Molecular weight
Perfluoroalkyl carboxylic acids (PFACs)			
Perfluorobutanoic acid	PFBA	$C_4F_7O_2H$	214.04
Perfluoropentanoic acid	PFPeA	$C_5F_9O_2H$	264.05
Perfluorohexanoic acid	PFHxA	$C_6F_{11}O_2H$	314.05
Perfluoroheptanoic acid	PFHpA	$C_7F_{13}O_2H$	364.06
Perfluorooctanoic acid	PFOA	$C_8F_{15}O_2H$	414.07
Perfluorononanoic acid	PFNA	$C_9F_{17}O_2H$	464.08
Perfluorodecanoic acid	PFDA	$C_{10}F_{19}O_2H$	514.08
Perfluoroundecanoic acid	PFUnDA	$C_{11}F_{21}O_2H$	564.09
Perfluorododecanoic acid	PFDoA	$C_{12}F_{23}O_2H$	614.10
Perfluorotridecanoic acid	PFTrDA	$C_{13}F_{25}O_2H$	664.11
Perfluorotetradecanoic acid	PFTeDA	$C_{14}F_{27}O_2H$	714.11
Perfluorohexadecanoic acid	PFHxDA	$C_{16}F_{31}O_2H$	814.13
Perfluorooctadecanoic acid	PFODA	$C_{18}F_{35}O_2H$	914.15
Perfluoroalkyl sulfonic acids (PFSAs)			
Perfluorobutane sulfonate	PFBuS	$C_4F_9SO_3H$	300.10
Perfluorohexane sulfonate	PFHxS	$C_6F_{13}SO_3H$	400.11
Perfluoroheptane sulfonate	PFHpS	$C_7F_{15}SO_3H$	450.12
Perfluorooctane sulfonate	PFOS	$C_8F_{17}SO_3H$	500.13
Perfluorodecane sulfonate	PFDS	$C_{10}F_{21}SO_3H$	600.14

Apart from the development of novel analytical methods, the current thesis comprises of 5 individual studies, whose short description is presented below.

1. In the first study, a new analytical method was developed for the detection of PFASs in various packaging materials. Materials made from paper, paperboard and aluminum foil used as wrapping materials of fast food items, chocolate, pharmaceutical products, yoghurt and marmalade lids were subsequently analysed. Beverage cups, ice cream cups, microwave bags for popcorn and rice, boxes of fast food and baking paper were also analyzed.
2. In the second study, a selective analytical method was developed and applied in different kind of fish samples that were cooked in two different ways. Evaluation of PFASs levels among the different fish species and a comparison between PFASs concentrations before and after the cooking procedure were made, while PFASs dietary intake based on fish consumption was also estimated.
3. In the third study, chicken eggs of different origin (home produced eggs and commercially produced eggs) were collected from houses and super markets and analysed. In this context, the difference of PFASs concentrations between the two categories and the PFASs contamination of eggs due to the chicken's different way of eating and living was investigated. In addition, PFASs dietary intake due to the consumption of chicken eggs was also estimated.
4. In the fourth study, drinking tap water samples were analysed for the detection of PFASs. The samples were divided basically into two categories (surface and underground water) and the found concentrations were evaluated mainly based on this separation. The different water treatment procedures, the profile of the area where the sample was collected (industrial or rural), and the contamination of the rivers in the case of surface water were also taken into account. A limited number of bottled water samples was also analysed in order to examine potential differences between tap and bottled water. PFASs intake due to the consumption of drinking water was also estimated.
5. In the fifth study, liver samples from sheep fed with contaminated grass pellets for a certain period of time were analysed in order to investigate PFASs transfer and accumulation from the animal feed to the liver of the animals. Both the contaminated

and the clean grass that were provided to the animals were analysed in the present study. In order to investigate potential PFASs contamination in the daily consumable liver, samples with different animal origin were also collected from the market and analysed. In particular, liver from free range animals and livestock were collected, in an effort to examine possible differences among PFASs levels due to animals' different living and eating habits.

The aforementioned topics were chosen after an overall literature review on the existing information on PFASs and in an effort to fill knowledge gaps of the ongoing research. However, risk assessment of PFASs, is still hampered by the insufficiency of available information and in any case further research is thought to be necessary.

CHAPTER 3

Determination of perfluorinated compounds (PFCs) in various foodstuff packaging materials used in the Greek market

3.1 Introduction

Despite the wide-spread use of PFCs in food packaging materials, a very limited number of studies have been published concerning PFCs concentrations in foodstuff packaging materials. More specifically, the samples that have been examined include polytetrafluoroethylene (PTFE) packaging materials and textiles [128] and/or the migration of PFCs from packaging materials and cookware to food [129,130]. Studies focusing on the detection of PFCs in paper packaging have demonstrated some amount of PFC contamination and PFC migration from the packaging materials to food [40-43,131]. In the present study we developed an analytical method suitable for the determination of trace level concentrations of PFCs in food packaging materials and we analyzed various packaging materials used in the Greek market. The method developed combines PLE, LC–MS/MS and isotope dilution method. In particular, the analytical protocol developed is suitable for quantitative determination of 12 perfluorinated compounds (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFBS, PFHxS and PFOS) and detection of 5 more (PFTrDA, PFTeDA, PFHxDA, PFODA and PFDS). The analyzed packaging materials from the Greek market were paper, paperboard and aluminum foil, and were used as wrapping materials of fast food items, chocolate, pharmaceutical products, and as yoghurt and marmalade lids. Beverage cups, ice cream cup, microwave bags for popcorn and rice, boxes of fast food and baking paper were also analyzed.

3.2 Materials and methods

3.2.1 Materials

The perfluorinated compounds analyzed in the present study are shown in Table 3.1. Standard solutions of $^{13}C_4$-labelled PFBA, PFOA and PFOS, $^{13}C_2$-labelled PFHxA, PFDA, PFUnDA and PFDoA, $^{13}C_5$-labelled PFNA and $^{18}O_2$-PFHxS were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Methanol, petroleum ether, sea sand, ammonium

acetate and sodium sulphate were purchased from Merck (Darmstadt, Germany). Florisil 60–100 mesh was purchased from Promochem (Germany) and Basic Alumina activity Super 1 from MP Biochemicals (Germany). Ultrapure water was provided by a Nanopure apparatus, (Barnstead/Thermolyne, USA). Basic alumina was activated in an oven at 200 °C overnight. Florisil sorbent was dried at 200 °C overnight and deactivated with 0.5% (w/w) ultrapure water prior to use.

3.2.2 Food packaging samples

42 samples of food packaging made of paper and/or aluminum were analyzed (beverage and ice cream cups, fast food wrappers for sandwiches, burgers etc., paper box for popcorn, french fries, pizza and sandwiches, non-stick baking paper, muffin cup, microwave bags for pop-corn and rice and aluminum foil bags and wrappers for chocolate, coffee, croissant, cereals, potato chips). All samples were obtained randomly from retail sellers, their exact composition was not stated and there were no information about perfluorochemicals used in their manufacturing process or not. More specifically, beverage and ice cream cups, wrappers and paper boxes were collected in Athens from October to December 2012 from the most popular in Greece fast food chain restaurants, coffee shops and multiplex cinemas with venues in many locations all over the country. Prevailing brands of muffin cups, baking papers and microwave pop-corn and rice bags were purchased from big super markets. All samples collected with the exception of microwave pop-corn and rice bags were manufactured in Greece. Most packaging materials were unused while some already contained food products.

Table 3.1: Mass transitions (parent ion/product ion) for target compounds.

Compound	RT	Primary ion transition (m/z)	Collision cell energy (eV)	Secondary ion transition (m/z)	Collision cell energy (eV)	Tube lens offset voltage (V)
PFBA	4.56	213 → 169	20		40	90
PFPeA[a]	6.11	263 → 219	20	263 → 69	40	90
PFHxA	7.18	312.9 → 268.9	20	312.9 → 118.9	40	90
PFHpA[b]	8.26	363.2 → 319	20	363.2 → 168.8	40	90
PFOA	9.27	412.9 → 368.8	20	412.9 → 218.9	40	90
PFNA	10.22	462.7 → 418.9	20	462.7 → 219	40	90
PFDA	11.10	512.9 → 468.9	20	512.9 → 268.9	40	90
PFUnDA	11.84	562.9 → 518.9	20	562.9 → 168.9	40	90
PFDoDA	12.52	612.9 → 568.8	20	612.9 → 168.9	40	90
PFTrDA[c]	13.13	662.8 → 619	20	662.8 → 268.9	40	90
PFTeDA[c]	13.67	712.9 → 668.9	20	712.9 → 168.9	40	90
PFHxDA[c]	14.47	813 → 769	20	813 → 269	40	90
PFODA[c]	15.21	913 → 869	20	913 → 169	40	90
PFBS[d]	6.31	298.9 → 99	50	298.9 → 80	50	146
PFHxS	8.33	398.9 → 99	50	398.9 → 79.9	50	146
PFOS	10.22	498.9 → 99.1	50	498.9 → 80	50	146
PFDS[e]	11.78	598.9 → 99	50	598.9 → 79.9	50	146
$^{13}C_4$-PFBA	4.56	217 → 172	20		40	90
$^{13}C_2$-PFHxA	7.25	315 → 270	20	315 → 118.9	40	90
$^{13}C_4$-PFOA	9.28	416.9 → 371.8	20	416.9 → 168.9	40	90
$^{13}C_5$-PFNA	10.22	467.9 → 422.9	20	467.9 → 168.9	40	90
$^{13}C_2$-PFDA	11.10	515 → 470	20	515 → 219	40	90
$^{13}C_2$-PFUnDA	11.84	565 → 520	20	565 → 168.9	40	90
$^{13}C_2$-PFDoDA	12.59	615 → 570	20	615 → 169.1	40	90
$^{18}O_2$-PFHxS	8.33	402.7 → 103	50	402.7 → 84.1	50	146
$^{13}C_4$-PFOS	10.23	502.9 → 99.1	50	502.9 → 80	50	146

a: ^{13}C4-PFBA is used as internal standard.

b: ^{13}C2-PFHxA is used as internal standard.

c: ^{13}C2-PFDoDA is used as internal standard.

d: ^{18}O2-PFHxS is used as internal standard.

e: ^{13}C4-PFOS is used as internal standard.

3.2.3 Sample preparation

3.2.3.1 Initial treatment

Before analysis, in the cases when the samples had a printed outside layer, this was removed when possible. Any food content was removed from the packaging, which was then rinsed with ultrapure water to remove salt and dried. Subsequently samples were cut into pieces of approximately 1 cm^2 with scissors.

3.2.3.2 Extraction

Food packaging samples were extracted by PLE, using an ASE Dionex 300 apparatus. Stainless steel ASE extraction cells (34 or 66 mL) were used. Two g of each sample were weighed and 200 µL of internal standard solution were added (200 ng mL^{-1} $^{13}C_4$-labelled PFBA, PFOA and PFOS, $^{13}C_2$-labelled PFHxA, PFDA, PFUnDA and PFDoA, $^{13}C_5$-labelled PFNA and $^{18}O_2$-PFHxS in methanol). Each sample was mixed with 35 g or 65 g of sea sand, depending on the extraction cell volume, and placed in the extraction cells with a cellulose fiber filter at the bottom. The cells were filled up with sea sand to reduce dead volume and minimize solvent quantity, capped and loaded on the ASE Dionex 300 apparatus. The extraction program included heating to 80 °C, 7 min static period, 3 cycles of extraction with MeOH, 100% flush volume, pressure at 1500 psi and purge to 1 min. The final extract was further cleaned up by SPE on florisil and basic alumina column as described below.

3.2.3.3 Clean-up

After completion of the ASE extraction, the methanol extract was centrifuged for 5 min at 5000 rpm (3857 × g), for precipitation and removal of insoluble particles. The extract was evaporated to dryness, redissolved in 3 mL of petroleum ether and brought onto the top of a glass column (30 cm length, 8 mm i.d.) plugged with precleaned glass wool and filled with 1.5 g florisil, 1 g basic alumina and 1 g of sodium sulphate. Prior to sample addition, the column was conditioned with 5 mL of methanol and 5 mL of petroleum ether. After sample addition the column was washed with 10 mL of petroleum ether and 8 mL of a MeOH/petroleum ether mixture (10:90 v/v). Target compounds were finally eluted with 8 mL of MeOH. The fraction collected was evaporated till dryness in a flash evaporator and the dry residue was dissolved in 200 µL of LC mobile phase (5 mM ammonium acetate – MeOH (80:20, v/v)). An aliquot of

100 µL of the redissolved residue was transferred to an auto-injector vial. A schematic presentation of the analytical protocol developed is shown in Figure 3.1.

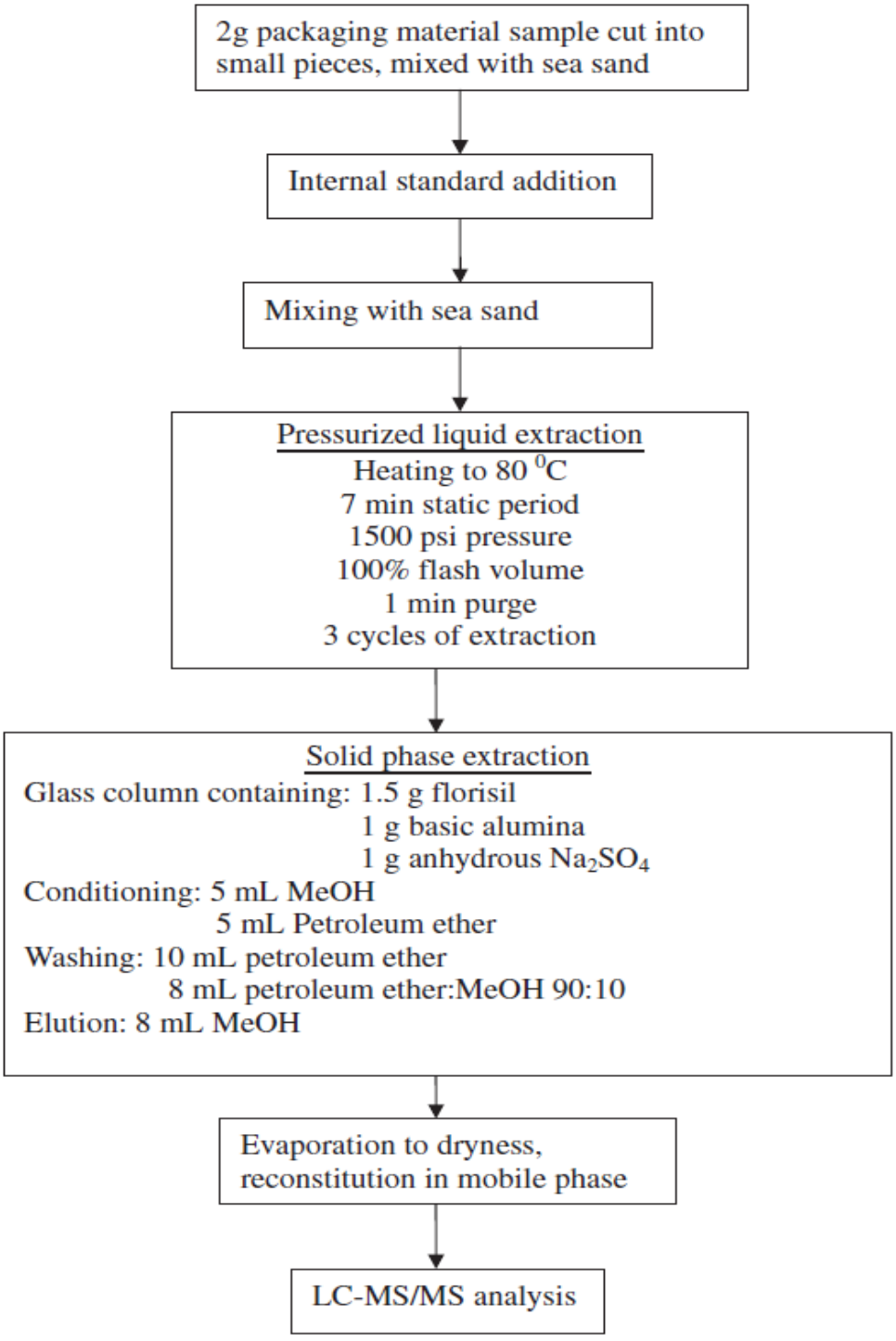


Figure 3.1: Schematic presentation of the analytical protocol for PFC analysis in food packaging materials.

3.2.4 Instrumental analysis

All sample extracts were analyzed by LC–MS/MS with ESI operating in negative mode. 35 µL were injected in a Hypersil GOLD C8 (150 mm × 2.1 mm i.d, 3 µm, Thermo) using a Surveyor MS Pump Plus (Thermo). The chromatographic gradient operated at a flow rate of 0.25 mL min^{-1} started with an initial condition of 80% 5 mM ammonium acetate – MeOH (80:20, v/v) (A) and 20% MeOH (B) and MeOH (B) increased to 50% (B) in 3 min. 100% (B) is reached in the next 12 min and held for 3 min. The oven temperature of the analytical column was set at 26 °C.

The HPLC was connected to a triple quadrupole mass spectrometer (TSQ QUANTUM ULTRA, Thermo) equipped with an Ion MAX-S thermoelectrospray source. The source temperature was maintained at 350 °C and the spray voltage at 3500 V. Analysis was performed by a multiple reaction monitoring (MRM) method that monitored two mass transitions (parent ion/product ion) for each analyte except for PFBA for which only one ion product was detected probably due to its small molecular weight. Ion transitions for target analytes and labeled standards are listed in Table 3.1. The values of the voltages applied to the tube lens offset and the collision cell were optimized for each ion transition. Confirmation of analyte identity was based on retention time, in addition to relative response of the secondary mass transition to the primary mass transition. Quantification of the target compounds was performed by the sum of areas of the two product ions using a response factor calibration curve vs the ^{13}C or ^{18}O-labelled standard.

3.2.5 Method validation

The method was validated for specificity, repeatability, reproducibility, recovery and sensitivity according to EURACHEM guide ‘‘The fitness for purpose of analytical methods – a laboratory guide to method validation and related topics’’. For analyte identity (specificity) confirmation, RT of the analyte should correspond to that of the labeled standard ±0.2 s. Repeatability and reproducibility of the method developed were tested by multiple analyses of spiked samples at concentrations of 5 ng g^{-1}, 10 ng g^{-1} and 30 ng g^{-1}. Recovery was estimated by the use of internal isotopically labeled standards and found to vary between 60% and 90%. Due to the very low noise in the LC–MS/MS system, the calculation of LOD and LOQ from a signal-to-noise ratio was not possible. Therefore, the LOD was calculated from the lowest

concentration with acceptable signal-to-noise ratio, and LOQ from the lowest concentration with ion abundance ratio within ±15% of the theoretical value and deviation of the relative response factor from the mean value ≤20%. The calculated LOD of the compounds analyzed ranged from 0.20 to 0.94 ng g^{-1}. Especially LOD for PFOS and PFOA were 0.49 and 0.60 ng g^{-1} respectively. Calculated LODs and LOQs are presented in Table 3.3. The laboratory participates successfully in international interlaboratory studies and is accredited for PFOS and PFOA analysis according to ISO/IEC 17025/2005.

3.3 Results and discussion

Up to now, a lot of studies have been carried out for the determination of PFCs in a wide range of matrices, including sewage treatment samples, air, sediment, soil, biological fluids, food and extending to consumer products (floor-polish waxes and impregnating agents, carpets and textiles). Initial studies focused on the determination of the two most abundant PFCs, PFOS and PFOA, however later studies gradually included several other volatile and non-volatile perfluorinated compounds of varying chain lengths. The diversity of analytes and matrices created the need to develop several methods of sample extraction and clean-up combined to instrumental techniques of quantification. The methods developed until 2007 have been reviewed extensively. Several limitations that render the analysis of PFCs especially challenging have been specified, including the impurity of the standards available, matrix effects and contamination through clean-up [132-134].

Several studies report the determination of PFCs in food packaging materials and other food-related items, such as cookware and vapors produced during cooking processes. Most of these studies are based on LC–MS/MS methodology. Their overview is presented in Table 3.2.

Table 3.2: Overview of the reported methods for analysis of PFCs in food packaging materials.

Country	Analytes	Matrix	Origin of samples	Method of analysis	Results of analysis	Reference
USA	PFOA	Popcorn bags, hamburger wrapper, French fry box, paper plates, perfluoro paper coatings, etc.	US retail market	Sonication with 50/50 ethanol/water LC–MS/MS LC column: Zobrax SB-C8, 100 × 2.0 mm × 3.5 µm	PFOA was present in many samples, with highest amounts in popcorn bags (up to 290 µg kg^{-1}). The migration of PFCs from cookware and popcorn bags was studied	[42,43]
USA	PFOA PFPeA PFHpA PFNA PFDA PFUnDA PFDoDA 6:2 FTOH 8:2 FTOH	3 samples of popcorn packaging materials	Not specified	Shaking with methanol and ethylacetate LC-MS/MS LC column: Keystone Betasil C18 50 x 2.0 mm x 5µm	PFOA and FTOHs were detected in vapors released by microwave popcorn. All analytes were found in one popcorn container at ng cm^{-2} concentrations. Only PFOA was detected in another.	[135]
China	PFOS PFOA	PTFE packaging material	Manufactured and purchased in China	PLE with acetonitrile GC–MS, derivatization by silylation	PFOA 17.5-45.9 ng g^{-1} PFOS 33.7-81.3 ng g^{-1}	[128]

Australia	PFHxA PFHpA PFOA PFNA PFDA PFUnDA PFOS	Microwave popcorn bags, popped popcorn after microwaving, non-stick backing paper, french fry box, sandwich wrapper, hamburger box	Retail stores and a major fast food company in Australia	Sonication with water LC–MS LC column: Luna Phenyl-Hexyl, 50 mm × 2 × 3 µm	PFOA was detected in one microwave popcorn bag (9 µg kg^{-1})	[136]
Denmark	Large number of PFCs	14 papers and board materials intended for contact with food at high temperatures	Retailers in Denmark	Sonication with ethanol LC–QTOF–MS LC column: Waters Acquity C18 150 × 2.1 mm × 1.7 µm	More than 115 polyfluorinated surfactants were detected	[131]
Thailand	PFOS PFOA	34 samples of food packaging material made of paper	Domestic and international restaurants in Bangkok, Thailand	PLE with methanol or saliva stimulant LC–MS/MS LC column: Agilent Eclipse XBD-C18 4.6 mm × 50 mm × 1.8 µm	PFOS and PFOA were detected in almost all paper packages. The highest concentration for PFOS (92.48 ng dm^{-2}) and PFOA (17.74 ng dm^{-2}) was found in a fried chicken box	[40]

Spain	PFHpA PFOA PFNA PFOS PFDA PFUnDA PFDoA	Microwave popcorn bags of three different brands	Supermarkets in Spain	PLE with methanol LC–QTOF–MS/MS LC column: Waters Acquity C18 50 × 2.1 mm × 1.7 μm	Significant levels of PFOA (53–198 ng g^{-1}). Detectable levels of PFHpA, PFNA and PFDoA in some samples. All 7 PFCs were detected in two of the samples	[41]
Greece	PFBA PFPeA PFHxA PFHpA PFOA PFNA PFDA PFUnDA PFDoA PFBS PHHxS PFOS PFTrDA PFTeDA PFHxDA PFODA PFDS	42 samples including beverage and ice cream cups, fast food wrappers, paper boxes, baking paper, aluminum foil bags and wrappers, microwave bags	Retail sellers, fast food chain restaurants, coffee shops and multiplex cinemas in Athens, Greece	PLE with methanol	Neither PFOA nor PFOS was detected in any sample. PFTrDA, PFTeDA and PFHxDA were detected in fast food boxes. PFHxA was found in ice cream cup. Several PFCs were detected in fast food wrappers and microwave popcorn bag	Present study, 2013

In this study, a method using PLE combined to LC–MS/MS for the determination of PFCs in foodstuff packaging materials is presented. Methanol as solvent has been shown efficient for the extraction of PFCs in several matrices, and an extensive study for the optimization of PFC extraction from polytetrafluoroethylene fluoropolymer has proven as optimal conditions the use of methanol in temperatures not exceeding 150 °C and at 12 min residence time [137]. In contrast to previous studies reporting methods of analysis of PFCs in packaging materials, we also deemed it necessary to include a clean-up step, especially since no precolumn clean-up

was included in our LC system, as is the case in some of the other previous methods [40,41,137]. The fact that this step adds to analysis time is counter-balanced by the short time needed for the PLE step. In-house florisil and alumina columns were used instead of prepacked C18 cartridges, reducing analysis cost. Although the use of florisil has not been reported in any of the other studies concerning the clean-up step in PFCs in food packaging materials, its use has been reported in clean-up method for the determination of PFCs in food samples [138] and in atmospheric air [139].

Instrumental analysis was carried out by LC–MS/MS using ESI ionization in the negative ion mode, a technique widely used for the analysis of anionic perfluorinated surfactants [134]. Crucial instrumental ionization parameters for detecting each one of the compounds of interest were optimized. These parameters included mainly voltages applied to the tube lens offset and the collision cell that are applied for the generation of the precursor and product ions of each ion transition. The developed method was applied for the quantification of 12 compounds: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFBS, PFHxS and PFOS, and detection of 5 compounds: PFTrDA, PFTeDA, PFHxDA, PFODA and PFDS. The transitions used for multiple reaction monitoring analysis of these analytes are presented in Table 3.1. The results for 42 samples of food packaging items are presented in Table 3.3. The two PFCs (PFOS and PFOA) most commonly found in many biological and environmental matrices analyzed (food samples, biological fluids, water and air samples) were not detected in any of our samples, unlike previous studies of food packaging materials where PFOA [40-42,131,135,136] and PFOS [40] i.e. the two most common PFCs, were detected in significant quantities. No PFCs were quantified in aluminum foil wrappers, baking paper materials or beverage cups. PFTrDA, PFTeDA and PFHxDA were detected in fast food boxes. Only PFHxA was found in the ice cream cup sample. On the other hand, several PFCs were quantified and detected in fast food wrappers while the highest levels of PFCs were found in the microwave popcorn bag sample (275.84 ng g^{-1} of PFBA, 341.21 ng g^{-1} of PFHxA and 5.19 ng g^{-1} of PFHpA).

Table 3.3: Concentrations (ng g^{-1}) of PFCs in packaging materials.

Compound	LOD	LOQ	Beverage cups (*n* = 8)	Ice cream cup (*n* = 1)	Fast food paper boxes[a] (*n* = 8)	Fast food wrappers (*n* = 6)	Paper materials for baking[b] (*n* = 2)	Microwave bags[c] (*n* = 3)	Aluminum foil bags/wrappers[d] (*n* = 14)
PFBA	0.51	1.54	<LOD	<LOD	<LOD	<LOD-3.19	<LOD	<LOD-275.84	<LOD
PFPeA	0.39	1.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxA	0.94	2.83	<LOD	25.56	<LOD	<LOD-19.17	<LOD	<LOD-341.21	<LOD
PFHpA	0.40	1.21	<LOD	<LOD	<LOD	<LOD-10.02	<LOD	<LOD-5.19	<LOD
PFOA	0.60	1.82	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFNA	0.42	1.25	<LOD	<LOD	<LOD	<LOD-4.97	<LOD	<LOD	<LOD
PFDA	0.69	2.08	<LOD	<LOD	<LOD	<LOD-28.25	<LOD	<LOD	<LOD
PFUnDA	0.70	2.11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFDoA	0.20	0.59	<LOD	<LOD	<LOD	<LOD-19.12	<LOD	<LOD	<LOD
PFTrDA	1.40		<LOD	<LOD	<LOD-detect.	<LOD-detect.	<LOD	<LOD	<LOD
PFTeDA	2.42		<LOD	<LOD	<LOD-detect.	<LOD	<LOD	<LOD	<LOD
PFHxDA	1.36		<LOD	<LOD	<LOD-detect.	<LOD-detect.	<LOD	<LOD	<LOD
PFODA	1.15		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBS	0.57	1.70	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxS	0.18	0.54	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	0.49	1.48	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFDS	2.65		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

a Pop-corn box, French fries box, pizza box, burger box.

b Baking paper, muffin cup.

c Pop-corn bag, rice bag.

d Chocolate wrapper, coffee bag, croissant wrapper, cereal bag, potato chips bag, aluminum foil.

The high concentration of PFCs in microwave popcorn bags is also reported in others studies. This food packaging item has been studied extensively, since it represents an extreme case of food in contact with its packaging during conditions of irradiation and high temperature in the presence of melted fats, and is therefore considered a model for the migration of PFCs from foodstuff packages to food. Indeed, all previous studies of microwave popcorn bags report the presence of PFCs. In 2005, Begley et al., determined PFOA and fluorotelomers in popcorn bags. PFOA concentration was between 6 and 290 μg kg^{-1} [42]. Migration studies showed that 1.4 mg kg^{-1} of fluorotelomers migrated to oil before microwaving, with an additional 2.1 mg kg^{-1} migrating after the microwaving procedure. Significant PFOA levels were also found in all three popcorn bags analyzed in the study of Martinez-Moral and Tena, 2012 (53 – 198 ng g^{-1}) and PFOS and PFOA were found in one of the two popcorn bags analyzed by Poothong et al. (2012) [40,41].

Dolman and Pelzing (2011) [136] also detected 9.1 μg kg^{-1} of PFOA in one of the two microwave popcorn bags analyzed, while no PFCs could be detected in the popped popcorn after microwaving, suggesting that either the PFCs did not migrate to the popcorn or that they could not be extracted from it. None of the above studies investigated further PFCs besides PFOS and PFOA.

We analyzed 17 PFCs in a microwave popcorn bag before and after the microwave cooking of the popcorn it contained. The results are presented in Table 3.4. PFOS and PFOA were not detected in the analyzed sample, but other PFCs were detected and showed different levels after cooking: PFBA (275.84 and 155.55 ng g^{-1}), PFPeA (<LOD and 60.76 ng g^{-1}), PFHxA (341.21 and 681.35 ng g^{-1}) and PFHpA (5.19 and 11.07 ng g^{-1}) before and after microwaving respectively. The concentrations of PFCs, except PFBA, on the surface of the bag are increased by microwave cooking conditions required for preparing popcorn. This could be explained by the release of these compounds from the matrix due to the temperature raise. The lowering of PFBA concentration after microwaving could be attributed to its higher volatility. In the study of Sinclair et al. (2007) [135], where several PFCs, including PFOA were detected in one of the 3 microwave popcorn bags studied, only fluorotelomer alcohols (FTOHs) were found at greater concentrations following cooking than before cooking.

Table 3.4: Concentrations (ng g^{-1}) of PFCs in microwave popcorn bag before and after cooking.

Compound	Popcorn bag before cooking	Popcorn bag after cooking
PFBA	275.84	155.55
PFPeA	<LOD	60.76
PFHxA	341.21	681.35
PFHpA	5.19	11.07
PFOA	<LOD	<LOD
PFNA	<LOD	<LOD
PFDA	<LOD	<LOD
PFUnDA	<LOD	<LOD
PFDoA	<LOD	<LOD
PFTrDA	<LOD	<LOD
PFTeDA	<LOD	<LOD
PFHxDA	<LOD	<LOD
PFODA	<LOD	<LOD
PFBS	<LOD	<LOD
PFHxS	<LOD	<LOD
PFOS	<LOD	<LOD
PFDS	<LOD	<LOD

3.4 Conclusions

A method based on PLE and LC–MS/MS was developed and applied in the determination of 17 PFCs in 42 samples of food packaging material from the Greek market. No PFCs were quantified in aluminum foil wrappers, baking paper materials or beverage cups. PFTrDA, PFTeDA and PFHxDA were detected in fast food boxes. In the ice cream cup sample only PFHxA was found. On the other hand, several PFCs were quantified and detected in fast food wrappers, while the highest levels of PFCs were found in the microwave popcorn bag. PFOA and PFOS were not detected in any of the samples. Compared to other studies from different countries, very low concentrations of PFCs were detected in the packaging materials analyzed. Most of the packaging materials studied were manufactured in Greece where perhaps PFC alternatives as fluorophosphates and fluorinated polyethers are used in the

manufacturing process. As the items analyzed were selected from the most popular chain restaurants, coffee shops and multiplex cinemas, we can assume that they are representative of the Greek market. Our results suggest that probably no serious danger for consumers' health can be associated with PFCs contamination of packaging material used in Greece.

CHAPTER 4

Levels of perfluorinated compounds in raw and cooked Mediterranean finfish and shellfish

4.1 Introduction

Although sources of human exposure to PFCs include household dust [38] and drinking water [48], it has been established that food is the most important source of PFC intake for non-occupationally exposed humans [103]. Studies in many countries including Poland, Germany, Norway, Sweden, United Kingdom, China, and Canada, have shown that the most important contributor to PFC exposure through food is fish, and investigated the potential correlation between fish consumption and PFC levels in human serum. These studies were reviewed extensively by Domingo in 2012. A more recent study in Sweden also confirms the existence of a strong correlation between PFC levels in blood serum and fish consumption [140]. The Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of EFSA has established TDI of 150 ng kg^{-1} b.w. day^{-1} for PFOS and 1500 ng kg^{-1} b.w. day^{-1} for PFOA. Despite the significant toxicity of PFCs, the number of studies focusing on their concentrations in items intended for human consumption still remains limited. Most of the studies concerning the levels of PFCs in edible fish and seafood have been conducted in raw muscle tissue. However it is possible that these levels may be altered in a non-predictable way by cooking processes, as shown in a limited number of recent studies [116,141].

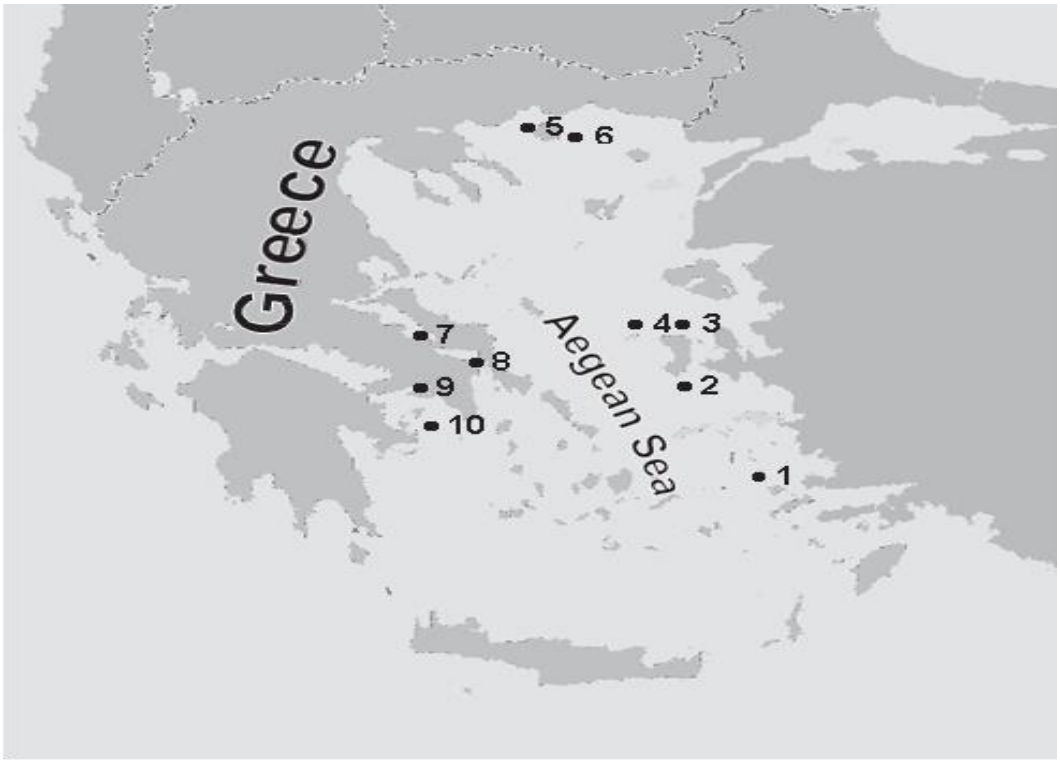
In the present study, the levels of PFCs were investigated in seven species of finfish and three species of shellfish, which are among the most commonly marketed species in the Aegean and Mediterranean Seas. To our knowledge, this is the first study reporting results from Greece, and the first one for most of these particular species of small Mediterranean fish and shellfish, which are quite often consumed in Mediterranean diet. The samples were analyzed raw as well as cooked following the most popular Greek culinary practices. Based on these results, the assessment of human exposure to PFCs through consumption of these fish species and the possible risk involved were attempted.

4.2 Materials and methods

4.2.1 Sample collection and preparation

The samples of the present study included finfish – anchovy, bogue, hake, picarel, sardine, sand smelt and striped mullet – and shellfish – Mediterranean mussel, shrimp and squid. The edible parts of these food items, which are all widely consumed in Greece, were analyzed raw, as well as cooked in the ways favored in Greek cuisine: pan-fried in olive oil (all samples), and grilled (anchovy, bogue, hake, sardine, striped mullet and squid).

All samples were obtained during the winter-early spring of 2011. Finfish, squids and shrimps were purchased from the local fish market in Kallithea, Athens, while mussels were obtained from a mariculture farm within the Saronikos Gulf, Attika. The fishing locations of the collected samples are shown in Figure 4.1 and provided in Table 4.1 along with additional information about biometric data and sample cooking and treatment before analysis. The quantity of each sample was 2–4 kg, comprising individuals of similar size. Following immediate transport to the laboratory and recording of biometric data, the samples were washed with cold water, scales were removed from the finfish and they were subsequently prepared according to the traditional Greek culinary practice. Mussels were first put for 2–3 min in boiling water in a casserole until they were opened and then their flesh was removed from the shells to be used for cooking and analysis.



1: Atherina boyeri, sand smelt
2: Boops boops, bogue
3: Loligo bulgaris, squid common
4: Merluccius merluccius, hake
5: Sardina pilchardus, sardine
6: Mullus barbatus, striped mullet
7: Engraulis encrasicholus, anchovy
8: Spicara smaris, picarel
9: Mytilus galloprovincialis, mussel (Mediterranean)
10: Parapenaeus longirostris, shrimp

Figure 4.1: The fishing locations of the collected samples.

Table 4.1: Scientific and common names, fishing and biometric data, water loss and frying oil uptake and pretreatment of fish and shellfish.

English common name	Scientific name	Fishing location	Length[a] (cm)	Weight[a] (g)	Water loss during grilling[b] (%)	Water loss during frying[b] (%)	Oil absorbed during frying[c] (%)	Pretreatment[d]
				Finfish				
Anchovy	*Engraulis encrasicolus*	Evoikos Gulf	10.4 ± 0.6	12.5 ± 1.8	15.2	76.4	34.4	1,3,4
Bogue	*Boops boops*	Chios island	17.7 ± 0.5	128.6 ± 8.0	18.5	40.1	4.0	1,2,3,5
Hake	*Merluccius merluccius*	Lesvos island	16.6 ± 1.5	43.6 ± 11.4	53.2	50.7	7.8	1,3,5
Picarel	*Spicara smaris*	Evoikos Gulf	9.5 ± 0.5	14.1 ± 2.3		50.9	24.8	1
Sand smelt	*Atherina boyeri*	Leros island	7.5 ± 0.9	4.2 ± 1.3		75.8	40.0	1
Sardine	*Sardina pilchardus*	Kavala	10.6 ± 0.8	18.3 ± 5.1	25.2	59.6	22.8	1,3,4
Striped mullet	*Mullus barbatus*	Kavala	9.2 ± 0.7	17.9 ± 5.1	29.3	56.8	10.8	1,2,3,5
				Shellfish				
Mediterranean mussel	*Mytilus galloprovincialis*	Saronikos Gulf	6.2 ± 0.5[e]	22.2 ± 6.2		56.7	11.6	1
Shrimp	*Parapenaeus longirostris*	Saronikos Gulf	12.1 ± 1.2	7.9 ± 2.4		59.2	7.0	1,4
Squid	*Loligo vulgaris*	Chios island	18.4 ± 1.9	68.1 ± 15.1	51.7	64.0	14.3	1,3,6

[a] Data obtained from 20 to 40 individuals.

[b] % w/w of raw food.

[c] % w/w of fried food.

[d] 1: Wash; 2: scales removal; 3: viscera removal; 4: head/cephalothorax removal; 5: gills removal; 6: internal pen (gladius) removal.

[e] Includes shell.

The washed fish and shellfish were pan-fried in Virgin Olive Oil (VOO), which was purchased in sealed plastic bottles from the local market. For this purpose, the samples were placed in a metal frying pan (30 cm diameter, 5 cm depth), which contained 300 mL VOO preheated at 170 °C and were fried until they were browned. To achieve uniform cooking the samples were turned and cooked in both sides by means of a wooden spatula, which was also used to remove the prepared food from the pan. The prepared fried seafood was placed in a clean plate covered with soft tissue paper to allow the excess of oil to drain. Both the frying oil and the food were weighed before and after frying to calculate water loss and oil uptake. After each frying operation, the used oil was discarded and the frying pan was thoroughly cleaned to be used for the next set of samples.

Five species of finfish as well as the squid were additionally grilled in a domestic electric oven at 180 °C. For this purpose, the food was placed on a grill and was heated from above by the oven's electric salamander. The metallic grill was covered with grease proof paper on which small holes had been opened, to allow juices from the cooked food to drain. The paper had previously been analyzed and found to be PFC free. Food was weighed before and after cooking in order to calculate water loss.

Quadruplicate composite samples, consisting of 4–6 items of raw or cooked fish or shellfish, were transferred to clean screw capped plastic containers and were freeze-dried for 48 h (Heto Lyolab 3000, Heto-Holten, Allerod, Denmark). Freeze-drying served also for moisture determination, as the water content of the freeze-dried samples was found to be less than 3%. The freeze dried samples were homogenized by means of a clean agate mortar and were subsequently analyzed.

4.2.2 Materials

The method of analysis used is suitable for quantitative determination of 12 perfluorinated compounds: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFBS, PFHxS and PFOS and the qualitative detection of 5 more: PFTrDA, PFTeDA, PFHxDA, PFODA and PFDS. Standard solutions of $^{13}C_4$-labelled PFBA, PFOA and PFOS, $^{13}C_2$-labelled PFHxA, PFDA, PFUnDA and PFDoA, $^{13}C_5$-labelled PFNA and $^{18}O_2$ PFHxS were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Methanol, petroleum ether, sea

sand, ammonium acetate and sodium sulphate were purchased from Merck (Darmstadt, Germany). Florisil 60–100 mesh was purchased from Promochem (Germany) and Basic Alumina activity Super 1 from MP Biochemicals (Germany). Ultrapure water was provided by a Nanopure apparatus, (Barnstead/Thermolyne, USA). Basic alumina was activated in an oven at 200 °C overnight. Florisil sorbent was dried at 200 °C overnight and deactivated with 0.5% (w/w) ultrapure water prior to use.

4.2.3 Sample preparation

4.2.3.1 Extraction

Lyophilized fish samples were extracted by PLE, using an ASE Dionex 300 apparatus. Stainless steel ASE extraction cells (34 mL) were used. Approximately 1 g of each sample was weighed and 200 µL of internal standard solution were added (200 ng mL^{-1} $^{13}C_4$-labelled PFBA, PFOA and PFOS, $^{13}C_2$-labelled PFHxA, PFDA, PFUnDA and PFDoA, $^{13}C_5$-labelled PFNA and $^{18}O_2$ PFHxS in methanol). Each sample was homogenized with 35 g of sea sand using a mortar and pestle, and placed in the extraction cells with a cellulose fiber filter at the bottom. The cells were filled up with sea sand to reduce dead volume and minimize solvent quantity, capped and loaded on the ASE Dionex 300 apparatus. The extraction program included heating to 80 °C, 7 min static period, 3 cycles of extraction with MeOH, 100% flush volume, pressure at 1500 psi and purge to 1 min. The final extract was further cleaned up by SPE on Florisil and basic alumina column as described below.

4.2.3.2 Clean-up

After completion of the ASE extraction, the methanol extract was centrifuged for 5 min at 5000 rpm (3857 × g), for precipitation and removal of insoluble particles. The extract was evaporated to dryness, redissolved in 3 mL of petroleum ether and brought to the top of a glass column (30 cm length, 8 mm i.d.) plugged with precleaned glass wool and filled with 1.5 g Florisil, 1 g basic alumina and 1 g of sodium sulphate. Prior to sample addition, the column was conditioned with 5 mL of methanol and 5 mL of petroleum ether. After sample addition the column was washed with 10 mL of petroleum ether and 8 mL of a MeOH/petroleum ether mixture (10:90 v/v). Target compounds were finally eluted with 8 mL of MeOH. The fraction collected was evaporated till dryness in a flash evaporator and the dry residue was dissolved

in 200 µL of LC mobile phase (5 mM ammonium acetate – MeOH (80:20, v/v)). An aliquot of 100 µL of the re-dissolved residue was transferred to an auto-injector vial.

4.2.4 Instrumental analysis

All sample extracts were analyzed by LC-MS/MS with ESI operating in negative mode. 35 µL were injected in a Hypersil GOLD C8 column (150 mm × 2.1 mm i.d, 3 µm, Thermo) using a Surveyor MS Pump Plus (Thermo). The chromatographic gradient operated at a flow rate of 0.25 mL min^{-1} starting with an initial condition of 80% 5 mM ammonium acetate – MeOH (80:20, v/v) (A) and 20% MeOH (B) and MeOH (B) increasing to 50% (B) in 3 min. 100% (B) is reached in the next 12 min and held for 3 min. The oven temperature of the analytical column was set at 26 °C.

The HPLC was connected to a triple quadrupole mass spectrometer (TSQ QUANTUM ULTRA, Thermo) equipped with an Ion MAX-S thermoelectrospray source. The source temperature was maintained at 350 °C and the spray voltage at 3500 V. Analysis was performed with a multiple reaction monitoring (MRM) method that monitored two mass transitions (parent ion/product ion) for every analyte except for PFBA. The information on ion transitions for both labelled and native PFASs, collision energies, tube lens voltages and the internal standards that were applied for each native analyte are illustrated in Table 4.2.

Confirmation of analyte identity was based on retention time, in addition to relative response of the secondary mass transition to the primary mass transition. Quantification of the target compounds was performed by the sum of areas of the two product ions using a response factor calibration curve vs the ^{13}C or ^{18}O-labelled standard.

Table 4.2: Mass transitions (parent ion/product ion) for target compounds.

Compound	RT	Primary ion transition (*m/z*)	Collision cell energy (eV)	Secondary ion transition (*m/z*)	Collision cell energy (eV)	Tube lens offset voltage (V)
PFBA	4.56	213 → 169	20		40	90
PFPeA[a]	6.11	263 → 219	20	263 → 69	40	90
PFHxA	7.18	312.9 → 268.9	20	312.9 → 118.9	40	90
PFHpA[b]	8.26	363.2 → 319	20	363.2 → 168.8	40	90
PFOA	9.27	412.9 → 368.8	20	412.9 → 218.9	40	90
PFNA	10.22	462.7 → 418.9	20	462.7 → 219	40	90
PFDA	11.10	512.9 → 468.9	20	512.9 → 268.9	40	90
PFUnDA	11.84	562.9 → 518.9	20	562.9 → 168.9	40	90
PFDoDA	12.52	612.9 → 568.8	20	612.9 → 168.9	40	90
PFTrDA[c]	13.13	662.8 → 619	20	662.8 → 268.9	40	90
PFTeDA[c]	13.67	712.9 → 668.9	20	712.9 → 168.9	40	90
PFHxDA[c]	14.47	813 → 769	20	813 → 269	40	90
PFODA[c]	15.21	913 → 869	20	913 → 169	40	90
PFBS[d]	6.31	298.9 → 99	50	298.9 → 80	50	146
PFHxS	8.33	398.9 → 99	50	398.9 → 79.9	50	146
PFOS	10.22	498.9 → 99.1	50	498.9 → 80	50	146
PFDS[e]	11.78	598.9 → 99	50	598.9 → 79.9	50	146
$^{13}C_4$-PFBA	4.56	217 → 172	20		40	90
$^{13}C_2$-PFHxA	7.25	315 → 270	20	315 → 118.9	40	90
$^{13}C_4$-PFOA	9.28	416.9 → 371.8	20	416.9 → 168.9	40	90
$^{13}C_5$-PFNA	10.22	467.9 → 422.9	20	467.9 → 168.9	40	90
$^{13}C_2$-PFDA	11.10	515 → 470	20	515 → 219	40	90
$^{13}C_2$-PFUnDA	11.84	565 → 520	20	565 → 168.9	40	90
$^{13}C_2$-PFDoDA	12.59	615 → 570	20	615 → 169.1	40	90
$^{18}O_2$-PFHxS	8.33	402.7 → 103	50	402.7 → 84.1	50	146
$^{13}C_4$-PFOS	10.23	502.9 → 99.1	50	502.9 → 80	50	146

[a] $^{13}C_4$-PFBA is used as internal standard.

[b] $^{13}C_2$-PFHxA is used as internal standard.

[c] $^{13}C_2$-PFDoDA is used as internal standard.

[d] $^{18}O_2$-PFHxS is used as internal standard.

[e] $^{13}C_4$-PFOS is used as internal standard.

4.2.5 Method validation

The method was validated for specificity, repeatability, reproducibility, recovery and sensitivity according to the EURACHEM: http://www.eurachem.org/index.php/publications/guides/mv. For analyte identity (specificity) confirmation, RT of the analyte should correspond to that of the labelled standard ± 0.2 s. Repeatability and reproducibility of the method developed were tested by multiple analyses of spiked samples at concentrations of 5 ng g^{-1}, 10 ng g^{-1} and 30 ng g^{-1}. Recovery was estimated by the use of internal isotopically labelled standards and found to vary between 60% and 90%. Due to the very low noise in the LC-MS/MS system, the calculation of LOD and LOQ from a signal-to-noise ratio was not possible. Therefore, the LOD was calculated from the lowest concentration with chromatographic peaks that clearly separate from the base- line and LOQ from the lowest concentration with ion abundance ratio within ±15% of the theoretical value and deviation of the relative response factor from the mean value ≤20%. The calculated LOD of the quantitatively analyzed compounds ranged from 0.20 to 0.94 ng g^{-1}. Especially LOD for PFOS and PFOA were 0.49 and 0.60 ng g^{-1} respectively. Calculated LODs and LOQs (for the compounds that were quantitated) are presented in Table 4.3. The laboratory participates successfully in international interlaboratory studies and is accredited for PFOS and PFOA analysis according to ISO/IEC 17025/2005.

Table 4.3: Moisture content (%) and PFCs concentrations (ng g^{-1} ww) in raw, fried and grilled fish and shellfish, on a fresh weight basis.

			Anchovy			Bogue			Hake		
	LOD	**LOQ**	**Raw**	**Fried**	**Grilled**	**Raw**	**Fried**	**Grilled**	**Raw**	**Fried**	**Grilled**
Moisture			76.3 ± 0.4	20.8 ± 9.2	51.0 ± 3.7	63.2 ± 2.7	51.6 ± 3.1	58.5 ± 1.3	82.5 ± 0.8	52.8 ± 9.7	66.9 ± 1.7
PFBA	0.51	1.54	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFPeA	0.39	1.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxA	0.94	2.83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHpA	0.40	1.21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOA	0.60	1.82	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFNA	0.42	1.25	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFDA	0.69	2.08	<LOD	<LOD	0.83 ± 0.01*	<LOD	<LOD	<LOD	<LOD	<LOD	0.82 ± 0.03*
PFUnDA	0.70	2.11	1.50 ± 0.04	1.75 ± 0.05*	2.73 ± 0.13*	0.24 ± 0.03	0.44 ± 0.02*	0.43 ± 0.03*	0.42 ± 0.05	LOD	1.11 ± 0.15*
PFDoA	0.20	0.59	1.86 ± 0.19	2.99 ± 0.22*	3.52 ± 0.10*	0.56 ± 0.08	1.12 ± 0.03*	0.63 ± 0.02	0.62 ± 0.08	<LOD	1.89 ± 0.05*
PFTrDA	1.40	[a]	Detected	Detected	Detected	<LOD	<LOD	<LOD	<LOD	<LOD	Detected
PFTeDA	2.42	[a]	Detected	Detected	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxDA	1.36	[a]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFODA	1.15	[a]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBS	0.57	1.70	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.45 ± 0.07	0.83 ± 0.03*	<LOD
PFHxS	0.18	0.54	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	0.49	1.48	3.06 ± 0.10	6.62 ± 0.14	6.29 ± 0.34*	0.82 ± 0.04	1.27 ± 0.06*	0.87 ± 0.07	0.84 ± 0.10	1.24 ± 0.06*	2.40 ± 0.13*
PFDS	2.65	[a]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

	Picarel		Sand smelt		Sardine			Striped mullet		
	Raw	**Fried**	**Raw**	**Fried**	**Raw**	**Fried**	**Grilled**	**Raw**	**Fried**	**Grilled**
Moisture	73.8 ± 2.1	41.3 ± 1.5	78.5 ± 0.3	16.4 ± 2.5	74.4 ± 1.5	31.8 ± 3.0	67.0 ± 0.7	62.3 ± 2.3	24.1 ± 1.0	51.3 ± 0.2
PFBA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFPeA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHpA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFNA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.60 ± 0.03	0.57 ± 0.11	0.50 ± 0.05
PFDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.87 ± 0.03*	0.65 ± 0.06	0.56 ± 0.07	<LOD
PFUnDA	0.70 ± 0.09	1.35 ± 0.08*	<LOD	0.74 ± 0.09*	<LOD	<LOD	1.70 ± 0.13*	1.05 ± 0.13	0.73 ± 0.20*	0.82 ± 0.02
PFDoA	<LOD	<LOD	1.08 ± 0.03	1.98 ± 0.04*	<LOD	0.93 ± 0.03	3.19 ± 0.09	<LOD	1.38 ± 0.07	<LOD
PFTrDA	<LOD	Detected	<LOD	Detected	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFTeDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFODA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	20.37 ± 2.47	44.69 ± 3.93*	1.16 ± 0.05	3.01 ± 0.13*	<LOD	<LOD	<LOD	5.66 ± 0.15	<LOD	10.23 ± 0.53*
PFDS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

	Mussel		Shrimp		Squid		
	Raw	**Fried**	**Raw**	**Fried**	**Raw**	**Fried**	**Grilled**
Moisture	78.4 ± 4.4	44.7 ± 3.5	76.5 ± 0.5	32.8 ± 6.4	84.4 ± 1.4	44.0 ± 6.5	63.9 ± 1.0
PFBA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFPeA	<LOD	<LOD	4.94 ± 0.26	14.88 ± 1.61*	<LOD	5.06 ± 0.19	<LOD
PFHxA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHpA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOA	<LOD	<LOD	<LOD	0.99 ± 0.21*	<LOD	<LOD	0.40 ± 0.01
PFNA	<LOD	<LOD	1.27 ± 0.07	1.52 ± 0.11*	<LOD	<LOD	<LOD
PFDA	<LOD	<LOD	1.73 ± 0.08	1.81 ± 0.19	<LOD	0.51 ± 0.04	<LOD
PFUnDA	<LOD	<LOD	2.76 ± 0.21	6.82 ± 0.22*	<LOD	1.04 ± 0.02*	<LOD
PFDoA	<LOD	<LOD	1.36 ± 0.09	2.31 ± 0.09*	<LOD	1.65 ± 0.07	1.09 ± 0.02
PFTrDA	<LOD	<LOD	<LOD	Detected	<LOD	Detected	Detected
PFTeDA	<LOD	<LOD	<LOD	Detected	<LOD	<LOD	<LOD
PFHxDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFODA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBS	<LOD	<LOD	1.37 ± 0.16	<LOD	<LOD	<LOD	<LOD
PFHxS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	<LOD	<LOD	5.15 ± 0.39	8.02 ± 0.42*	<LOD	1.56 ± 0.17	1.19 ± 0.17
PFDS	<LOD	<LOD	Detected	Detected	<LOD	<LOD	<LOD

Each value represents the mean ± standard deviation of four analyses.

The asterisk denotes statistically significant differences between raw and fried or raw and grilled samples ($p < 0.05$).

[a] Qualitative determination

4.2.6 Calculation of human intake of PFOS and PFOA

The daily food consumption of fish by adults was according to FAO: http://faostat.fao.org/. It was assumed to be 36 g per person (adult males and females) per day for fish, 9.80 g per person per day for cephalopod molluscs and 5.42 g per person per day for crustaceans. This data is in agreement with the daily consumption proposed by EFSA and was used as FIR (food intake rate) for the calculation of PFOS and PFOA intake. Average body weight (ABW) was equal to 70 kg, according to EFSA. The estimated daily intake (EDI) in ng kg^{-1} b.w. of PFOS and PFOA is calculated by the following equation:

$$EDI = (C \times FIR) / ABW$$

where C is the concentration of PFOS or PFOA (ng g^{-1} ww). Concentrations of zero were assigned when PFOS or PFOA was not detected above the LOD.

4.3 Results and discussion

The data available from the literature on PFC concentrations in fish is summarized in Table 4.4. Up to now, few studies report the levels of PFCs in edible fish, and to our knowledge none is available for Greece. Even fewer are the studies focusing on fish and shellfish from the Mediterranean Sea. More specifically, there are studies reporting PFC levels in shellfish from France [142], Spain [48] and Italy [44,143], farmed fish from Italy [144] and marine fish from Spain [48] and Italy [143]. The results reported from Italy by Nania et al., refer to different species of Mediterranean fish and shellfish, with the exception of Loligo vulgaris (common squid) and species of mussel and red mullet which are relatives of the species studied herein.

Table 4.4: Overview of recent studies of PFC levels in edible fish by chronological order.

Country	Sampling site	PFCs analyzed	Fish sample	Results of analysis	Reference
USA	Gulf of Mexico and Chesapeake Bay	PFOS	American oysters (*Crassostrea virginica)*	Concentrations of PFOS ranged from 42 to 1225 ng g^{-1} dw	[145]
Portugal	River estuaries in northern Portugal	PFOS	Mussels	PFOS concentrations between 36.8 and 125.9 ng g^{-1} ww	[146]
China	Purchased from local markets in Zhoushan and Guangzhou in 2004	9 PFCs	Twenty-seven samples including fish, mollusks, crabs, shrimp, oysters, mussels, and clams	PFOS was the predominant compound, with concentrations between 0.33 and 13.9 ng g^{-1} ww	[147]
Canada	Purchased from Canadian grocery stores and fast food restaurants	9 PFCs	Composite food containing freshwater, marine and canned fish and shrimp	PFOS was detected in all fish-containing samples, in levels between 1.3 and 2.6 ng kg^{-1}. PFOA was below LOD.	[130]
Spain	Purchased from retail stores from Tarragona County	11 PFCs	White fish (hake, whiting blue, sea bass, monkfish), seafood (mussel, shrimp), canned fish (tuna, sardine, mussel), blue fish (salmon, sardine, tuna)	PFOS, PFOA, and PFHpA were the only PFCs detected in at least one sample. Mean PFOS levels in fish and seafood ranged between 0.15 and 0.65 ng g^{-1} ww	[106]
Canada	Purchased from supermarkets and fish markets in Toronto, Mississauga and Ottawa	16 PFCs	Eighteen fish and shellfish species were analyzed raw and cooked	Several PFCs were detected. PFOS concentrations vary between 0.21 and 1.68 ng g^{-1} ww	[116]

Country	Sampling site	PFCs analyzed	Fish sample	Results of analysis	Reference
Italy	Coasts of Calabria and the Aeolian Islands in the Southern Tyrrhenian Sea	PFOS and PFOA	Muscle and liver of Mediterranean swordfish (*Xiphias gladius*)	PFOS and PFOA were below LOD	[148]
Sweden	Baltic Sea (BS) and Lake Vättern (LV)	11 PFCs	Perch (*P. fluviatilis*), burbot (*Lota lota*), whitefish (Coregonus lavaretus), salmon (*Salmo salar*) and brown trout (*Salmo trutta*)	PFOS was the predominant compound in all fish species with median levels between 1.0 ng g^{-1} ww and 12 ng g^{-1} ww for burbot. PFOS concentrations were higher in muscle tissue from LV fish than from BS fish	[149]
Italy	Mediterranean Sea	PFOS and PFOA	Twenty-six fish muscles, seventeen fish livers, five pooled samples of cephalopods and thirteen pooled samples of bivalves	62% and 67% of the samples had PFOA and PFOS levels below LOD, respectively. Concentrations were generally lower than those found in studies in different geographical areas	[143]
Canada	Purchased in local market, Nunavut, northern Canada	11 PFCs	Fish muscle and clams belonging to the traditional diet of Innuit	PFOS was found in concentrations up to 3.6 ng g^{-1}	[150]
Norway	Purchased in grocery stores in Oslo	16 PFCs	Fish sticks, canned mackerel, salmon, cod, and cod liver	PFOS and PFOA above LOQ were found in all samples. Other PFCs were also present. The highest PFOA value is 100 pg g^{-1} ww for cod and 310 pg g^{-1} ww for cod liver	[45]
China	High mountain lakes in the Qinghai-Tibetan Plateau	9 PFCs	Fish muscle	PFOS was detected in 96% of the samples (0.21 –5.20 ng g^{-1})	[151]
Spain	Cantabrian Sea in Northern Spain	5 PFCs	Mussels	Low levels of PFOS and PFOA in some of the samples	[35]

Country	Sampling site	PFCs analyzed	Fish sample	Results of analysis	Reference
Belgium	Belgian rivers and North Sea	PFOS, PFOA	Eels and cod	Fish was found to be a main contributor of PFOS and PFOA in human PFC intake	[152]
Spain	Purchased in 12 representative cities in Catalonia	17 PFCs	Fish and shellfish (sardine, tuna, anchovy, sword-fish, salmon, hake, red mullet, sole, cuttlefish, clam, mussel, and shrimp)	PFOS showed the highest mean concentration in fish and shellfish (2.70 ng g^{-1} ww)	[48]
China	Local markets in six Chinese coastal provinces (Liaoning, Shandong, Jiangsu, Zhejiang, Fujian and Guangdong)	13 PFCs	Fatty fish and shellfish	PFOS was the dominant PFC in fatty fish (maximum value 0.47 ng g^{-1}) and PFOA in shellfish (maximum value 1.45 ng g^{-1})	[153]
Belgium, Norway, Italy, Czech Republic	Purchased from local supermarkets	21 PFCs	Pooled farmed freshwater fish from Czech Republic, mixtures of farmed and wild marine fish from Belgium and Norway, pooled seafood from Norway, Italy and Belgium	In all cases, PFOS was the most frequent compound (372–598 ng kg^{-1} in seafood and 104–478 ng kg^{-1} in fish)	[154]
France	Selected locations in the English Channel, Atlantic and along Mediterranean coasts	7 PFCs	Oysters and mussels	PFOS was detected in all samples with values between 0.01 and 30.70 ng g^{-1} ww. PFDA was the second most frequently detected PFC (0.04 and 0.08 1.68 ng g^{-1} ww)	[142]
Italy	Purchased in supermarkets in Sienna	PFOS and PFOA	Fish and seafood	Only PFOS was detected (mean value = 7.65 ng g^{-1})	[44]

Country	Sampling site	PFCs analyzed	Fish sample	Results of analysis	Reference
Italy	Two fish farms in Liguria	PFOS and PFOA	Sea bass (*Dicentrarchus labrax* L.)	All samples were below or slightly above LOD	[144]
Canada	Sport fish from rivers in Ontario, Canada	12 PFCs	Chinook salmon (*Oncorhynchus tshawytscha*), common carp (*Cyprinus carpio*), lake trout (*Salvelinus namaycush*) and walleye (*Sander vitreus*) were analyzed raw and cooked	PFCs above the detection limit were found in all species. PFOS levels were 16–53 ng g^{-1} ww, about 1–2 orders of magnitudes higher than those of the other PFCs	[141]
Greece	Various fishing sites in the Aegean Sea	17 PFCs	Anchovy, bogue, hake, picarel, sardine, sand smelt and striped mullet, Mediterranean mussel, shrimp and squid in raw and cooked form	PFCs above the detection limit were found in all fish samples and in all shellfish except the mussel. PFOS was the most abundant PFC with values between <LOD and 44 ng g^{-1} ww	This study

4.3.1 PFC concentrations

PFCs above the detection limit were found in all raw samples except sardine, mussel and squid (Table 4.3). PFOS was the most abundant PFC, and the highest PFOS concentration was measured in picarel (20.4 ng g^{-1} fresh weight). This value exceeds the environmental quality standard (EQS) of 9.1 ng g^{-1} fresh weight that has been proposed by the European Commission for biota [155]. The PFOS values for the rest of the samples are between <LOD and 5.66 ng g^{-1} fresh weight, which are similar to results found in other studies of PFCs in raw fish muscle. In a study of PFC levels in various food products obtained in retail market in the Tarragona county of Spain, i.e. a location with dietary habits similar to those of Greece, a number of marine fish samples were analyzed, and PFOS levels up to 0.65 ng g^{-1} ww were reported [106]. Studies from China [146] reported slightly higher PFOS levels in marine fish and seafood (the maximum PFOS level reported is 13.9 ng g^{-1} in mantis shrimp). The same study detected several other PFCs in the analyzed samples, including PFUnDA and PFOA in levels similar to the ones found in the present study. Similar results were provided by a Canadian study of PFC levels in food [130], which detected up to 2.6 ng g^{-1} of PFOS in marine and freshwater fish, though in this case the samples were not raw fish, but food prepared for consumption. Similar levels were also reported in a study of fish from Qinghai-Tibetan Plateau in China [151]. However higher levels of PFOS have been reported in some cases, including a freshwater carp sample from Saginaw Bay in Michigan, USA (124 ng g^{-1} ww) [156], perch from Lake Mälaren in Sweden (44 ng g^{-1} ww) [157], and fish from the Western Scheldt in the Netherlands (>100 ng g^{-1}) [158]. In a comparative study analyzing fish muscle tissue from several species of marine fish from the Baltic Sea and freshwater fish from Lake Vättern in Sweden [149], PFOS concentrations were significantly higher in the former group (up to 12 ng g^{-1} ww) than in the latter (up to 2.1 ng g^{-1} ww). This difference was attributed to the fact that the specific lake is a nutrient poor ecosystem, with a long theoretical water residence time.

It is suggested that the high PFOS value in our picarel sample is due to the fact that it was caught in a fishing site near the most densely populated and industrially developed region of Greece, in water possibly burdened by anthropogenic discharges (Figure 4.1). Two other samples collected in other locations near the Attika area, anchovy and shrimp,

also had relatively high PFOS levels (3.06 and 5.15 ng g^{-1} ww respectively). The mussel sample, however, although cultured in the Saronikos Gulf, which is very near the urban zone of Athens, had no detected levels of PFCs. This may be due to the different feeding habits of this filter-feeder, compared to the above three fish species which feed on large zooplanctonic organisms. It has been established that PFOS and to a lesser degree PFOA have a bioaccumulation potential in aquatic organisms [156]. Interestingly, these same fish and shellfish samples, which were also used in a study of heavy metal concentrations [159] were found to have higher levels of Cr, Fe, Ni, Cd, Hg and Pb than other fish and shellfish samples, though in this case these high levels also included mussels, indicating the probably different pathways of contamination by metals and PFCs in aquatic species.

A previous study of PFCs in edible fish of the Mediterranean Sea, and particularly in Italy, reported a similar phenomenon: although most of the fish analyzed had PFOS and PFOA concentrations near or below the LOD, the muscle of horse mackerel and large scaled scorpion fish had extremely high PFOA concentrations (above 100 ng g^{-1}), and this fact was attributed to ‘‘dot-like’’ contamination affecting limited areas of the Mediterranean Sea [143].

4.3.2 Effect of cooking

The concentrations of the detected PFCs were in most cases higher after frying or grilling of the samples, and in most cases this increase was statistically significant (Table 4.3). The influence of cooking on the levels of PFCs has not been extensively investigated up to now. In 2008, Del Gobbo et al. studied PFC levels in raw, baked, boiled and fried samples of 18 fish species from the Canadian market. Of the 17 analytes, PFOS was the most frequently detected.All cooking methods appeared to reduce PFC concentrations. Baking seemed to cause the highest reduction [116].

A more recent study [141] investigates the effect of three cooking methods – baking, broiling, and frying – on the levels of PFCs in four species of sport fish from rivers in Ontario, Canada. These fish species were chosen because they have higher levels of PFCs than grocery store fish, such as those used in the study of Del Gobbo et al., and therefore it is less likely that data are influenced by analytical uncertainty. The study

showed that PFOS concentrations in all fish species increased significantly after cooking except for broiling and frying of common carp, which had no significant changes in PFOS concentrations. This fact was attributed to loss of moisture during cooking, PFCs being associated with proteins of biota and less likely to be removed via cooking. Our results, concerning a different group of fish, i.e., small Mediterranean finfish, are in agreement with this study.

Significant differences exist between seafood preparation in the present work and the report of Del Gobbo et al. In that study, prior to baking, the fish fillets were marinated in wine, resulting in significant exposure of fish muscle to marinate which could explain the reduction of PCFs via migration to the marinating solution. On the contrary, in our study small Mediterranean species are cooked as they are in their skins, a fact that minimizes components' loss other than the escaping of steam and small amounts of juices.

Moreover, studies investigating the possible transfer of PFCs from cookware and packaging materials to food, given the fact that some of these compounds are used as non-stick additives and water- and grease-repellents, do not provide a clear conclusion [103]. An overview of published results concerning packaging materials is presented by Zafeiraki et al., 2014 [28]. In a study of the levels of PFOA PFTE-coated cookware, although residual levels of PFOA are reported, these were not considered high enough to determine whether mass transfer of PFOA occurs from PTFE-coated cookware into water or oil at cooking temperatures even in worst case assumptions [42]. Another study investigated the presence of PFCs in the vapors released during cooking by non-stick cookware [135]. It was concluded that PFOA residues remain on the surface of PFC treated cookware and may be off-gassed when heated at normal cooking temperatures. In a study by Ericson-Jogsten et al. (2009), food was cooked in non-stick cookware and the PFC levels were compared to those of the non-cooked samples [160]. Although higher PFC levels were found in cooked food, in was not clear if non-stick cookware contribute to human exposure to PFCs. In the present study the greaseproof paper used during grilling was previously analyzed and found to be PFCs free. The influence of cooking on the levels of PFCs should depend not only on the cooking conditions and the composition of the cookware, but also on the particular food being cooked and the

culinary practice followed. Water loss, which is higher in frying than in grilling, is an important aspect of cooking. During frying there is the additional parameter of oil uptake. Both these factors (water loss and oil uptake) are inversely correlated to fish size [161]. Theoretically PFCs are not expected to be removed from the samples during cooking. Their ability to partition into the gas phase is minimal, as is proven by their low vapor pressure [162]. On the other hand, PFCs are known to bind to serum proteins and to have an affinity for lipoproteins [69], which enforces their ability to remain in samples after their processing. For the above reasons, the concentration of PFCs in cooked samples is expected to increase as a function of mass loss by water evaporation. In our study, this is indeed what was observed in most cases (Table 4.3). The same effect was observed in a study of heavy metal concentrations in the same fish samples, which showed that frying and grilling both increased metal concentrations compared to raw samples [160]. More specifically, we calculated the percentage true retentions of PFCs which were found above LOD in more than two fish samples (PFNA, PFDA, PFUdA, PFDoA, PFTrDA, PFOS) according to the formula proposed by Murphy et al. (1975) [163], for the calculation of nutrient retention and food yield (Figure 4.2). The retentions were between 44% and 92% after frying and between 102% and 135% after grilling. The percentage true retentions over 100 after grilling are consistent with the water evaporation and the slight decrease of retentions observed after frying can be explained by oil absorption.

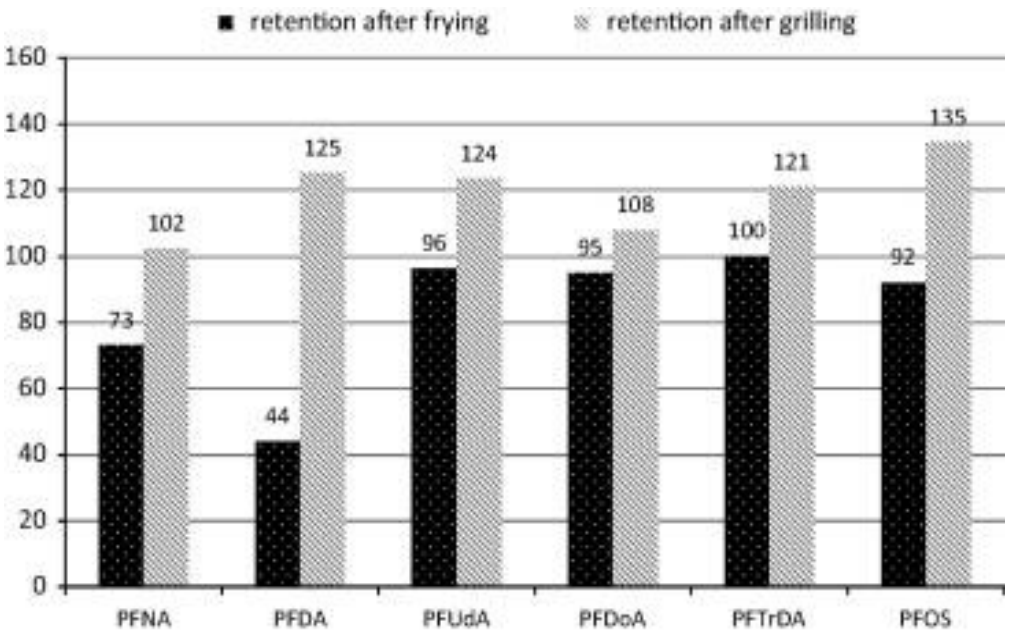


Figure 4.2: Percentage of true retentions of PFCs after frying and grilling.

4.3.3 Dietary intake of PFOS and PFOA

In the present study, the EDI of PFOS and PFOA by fish consumption was calculated according to Section 4.2.6 and the results are presented in Table 4.5. In order to calculate the EDI, the concentrations of PFOS and PFOA in raw fish and shellfish were used, as usually done in similar studies. All calculated values are well below the corresponding TDIs proposed by EFSA. However, in order to estimate the levels of human exposure to PFCs, it is necessary to take into account all items consisting daily human diet. The most complete studies of this kind performed until now include that of the U.K. Food Standards Agency: http://www.food.gov.uk/science/surveillance/fsisbranch2006/fsis1106, where 20 composites from the 2004 U.K. Total Diet Study (TDS) were analyzed, a study of several composite food samples prepared for consumption from Canada [130] and a study of 36 composite samples of the most frequently consumed foodstuffs by the population of Tarragona County, Spain [106]. The Canadian study concluded that the highest concentration of PFCs was found in fast food composites, while the studies in the UK and Spain concluded that fish is a major contributor to PFCs dietary intake. A recent study of pooled samples representing 15

different food commodities from four European countries concluded that seafood is the most important food source of PFC exposure, followed by pig and bovine liver and farmed fish [154], while a study of population exposure to PFCs in Belgium also showed fish as the most important edible PFC source [152]. Taking this into account, we estimate that even considering the contribution of other food items to overall PFC intake it is highly unlikely that consumers in Greece exceed the TDI for PFOS and PFOA.

Table 4.5: Estimated daily dietary intake of PFOS and PFOA for adult Greek population.

EDI (ng kg^{-1} bw)	PFOS	PFOA
Anchovy	1.57	0.05
Bogue	0.42	–
Hake	0.43	–
Picarel	10.48	–
Sandsmelt	0.60	–
Sardine	–	0.09
Stripped mullet	2.91	0.18
Mussel	–	–
Shrimp	2.65	0.20
Squid	–	–

4.4 Conclusions

The present study presents novel data about PFC concentrations in several species of edible fish that are very popular in the Mediterranean countries and in addition provides data on the impact of cooking on PFC levels. PFCs above the detection limit were found in all raw samples except sardine, mussel and squid. PFOS was the most abundant PFC in all samples. Frying and grilling resulted in elevation of PFC concentrations compared to raw samples. The EDI for PFOS and PFOA through consumption of Mediterranean fish and seafood was calculated to be well below the values proposed by EFSA.

CHAPTER 5

Perfluoroalkylated substances (PFASs) in home and commercially produced chicken eggs from the Netherlands and Greece

5.1 Introduction

Chicken eggs are important contributors to the human diet. Apart from commercially produced eggs from supermarkets, numerous people keep chickens for producing their own eggs. These chickens are most often free to be outside, picking their food or worms and small insects from the soil. The eggs are mostly consumed within the family. With chickens being exposed to the outdoor environment (e.g. soil), their products may become contaminated with pollutants. This has been clearly demonstrated for polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs), but much less information is available for PFASs [164-166].

The aim of the present study was to investigate the PFASs contamination in home produced chicken eggs from the Netherlands and Greece and to compare it to commercially produced eggs. To this end, eggs from people living in the Netherlands and Greece who rear chickens domestically, and eggs from supermarkets (organic, battery and free range eggs) were collected from both countries. For the analysis of these samples an LC-MS/MS method was developed for 11 PFASs. To our knowledge this is the first study presenting and comparing PFAS levels in such a large number (n=171) of commercially and especially home produced eggs. For Greece, this is the first study providing results on PFAS contamination in eggs.

5.2 Materials and methods

5.2.1 Sample collection

The egg samples of the present study were collected from different regions in the Netherlands (95 samples) and Greece (76 samples) from August 2013 until August of 2014. Home produced eggs in the Netherlands (n=73) and in Greece (n=45) were collected from volunteers who joined the study by providing the eggs. Commercial eggs were purchased from different supermarkets (n=22 from the Netherlands and n=31 from Greece). After the sampling, the eggs were brought to the laboratory. Every sample consisted in principle of 20

individual eggs, unless fewer eggs were provided. All the eggs were boiled and the yolk of each one was separated from the white part. The yolks of the same sample were pooled, homogenized and stored at 4°C until the analysis. The process of boiling the egg increased the sensitivity of the method and was also a convenient way of preserving and transportating of the Greek samples to the laboratory, as breaking of the samples was avoided and transportation under room temperature was also possible.

5.2.2 Chemicals

In the current study 11 PFASs: PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBuS, PFHxS, PFHpS and PFOS were analysed by applying LC-MS/MS and isotope dilution method. Native Perfluorosulfonic acids (PFSA) solution/mixture (PFS-MXA), native Perfluorinated carboxylic acids (PFCA) solution/mixture (PFC-MXA), mass-labelled internal PFCAs and PFSAs solution/mixture (MPFAC-MXA) and a $^{13}C_8$-PFOS solution were purchased from Wellington laboratories (Guelph, Ontario, Canada). ACN (Ultra LC-MS grade), MeOH (Ultra LC-MS grade) and HPLC water (Ultra LC-MS grade) were purchased from Actu-All chemicals (Oss, the Netherlands). Ammonium acetate (approx. 98%) (Sigma, St Louis, USA), sodium acetate trihydrate (Sigma, Germany), ammonium formate (≥99%) (Sigma, Switzerland) and sodium hydroxide (Sigma, Sweden) were all provided by Sigma. The ammonium solution and the hydrochloric acid (37%) were purchased from Merck (Darmstadt, Germany). SPE was carried out with Oasis WAX cartridges (3cc, 60mg, 60μm, Waters, USA).

5.2.3 Sample preparation

For each egg sample, 1 g of homogenized yolk was fortified with 25 μL of mass-labelled PFCAs and PFSAs solution/mixture (MPFAC-MXA) of 100 ng mL^{-1}. Subsequently, 2 mL of 200 mM sodium hydroxide were added to every sample for alkaline digestion. After adding 10 mL of MeOH as extraction solvent, the solution was vortexed for 1 min and shaken for 30 min at 250 rpm. To the methanol extract, 150 μL HCl 4 M were added in order to neutralize the solution, and then the extract was centrifuged for 10 min at 10,000 rpm for the precipitation and removal of insoluble particles. The supernatant was transferred to a new tube and 25 mL of milli-Q water were added. Then, clean-up was performed by SPE using weak anion

exchange Oasis WAX cartridges. SPE started with the conditioning of Oasis WAX cartridges with 4 mL of MeOH and 4 mL of HPLC water. Next, the extract was passed through the cartridge, which was then washed with 4 mL of 25 mM sodium acetate buffer (pH 4). PFASs were eluted from the cartridge with 2 mL of 2% NH_4OH in ACN. During all the SPE steps, the flow rate of the cartridge was constant at approximately 1–2 drops per second. The collected extract was evaporated till dryness under a gentle stream of N_2. The dry residue was dissolved in 775 µL of 2 mM ammonium formate in water and 200 µL of MeOH. Before the injection, 25 µL of $^{13}C_8$-PFOS solution 100 ng mL^{-1} were also added for monitoring the run to run MS response. The final solution was transferred into a vial for analysis by LC-MS/MS.

5.2.4 Instrumental analysis

For the analysis of the egg samples, LC-MS/MS was used, based on a Shimadzu LC system (Hertogenbosch, the Netherlands). A Fluorosep analytical column (50 mm * 2.1 mm, 5 µm, Waters, Etten-Leur, the Netherlands) was chosen in order to achieve better chromatographic separation between PFOS and cholic acids, present in the egg samples. In addition, a Symmetry C18 column (50 mm * 2.1 mm i.d., 5 µm, Waters) was used as guard column prior to the injector in order to isolate and delay potential PFASs traces from the LC system. The chromatographic gradient was operated at a flow rate of 0.300 mL min^{-1} starting from 80% 2 mM ammonium formate in water (A) to 95% MeOH (B) in 10 min. Each chromatographic separation lasted 15 min and the injection volume was 20 µL. Furthermore, the oven temperature of the analytical column was set at 35 °C.

The LC system was connected to a triple quadrupole MS (AB SCIEX QTRAP 5500 SYSTEM, Applied Biosystem - Analytical Technologies), equipped with a Turbo Spray source operating in negative mode. The source temperature was set at 350 °C and the ion spray voltage at -4500 V. The analyses were performed with an MRM method that monitored two mass transitions (parent ion/product ion) for every analyte. The information on ion transitions for both labelled and native PFASs, collision energies, retention times and which internal standards were applied for which native analyte are illustrated in Table 5.1.

Table 5.1: Instrumental mass spectrometry settings for the target compounds.

Compound	Molecular formula	Precursor ion (m/z)	Product ion 1 (m/z)	Collision energy (eV)	Product ion 2 (m/z)	Collision energy (eV)	Retention time	Internal standard
PFHxA	$C_6F_{11}O_2^-$	313	269	14	119	24	3.17	$^{13}C_2$-PFHxA
PFHpA	$C_7F_{13}O_2^-$	363	319	12	169	24	3.97	$^{13}C_2$-PFHxA
PFOA	$C_8F_{15}O_2^-$	413	369	14	169	24	4.42	$^{13}C_4$-PFOA
PFNA	$C_9F_{17}O_2^-$	463	419	16	169	26	4.99	$^{13}C_5$-PFNA
PFDA	$C_{10}F_{19}O_2^-$	513	469	16	219	26	5.77	$^{13}C_2$-PFDA
PFUnA	$C_{11}F_{21}O_2^-$	563	519	14	319	24	6.68	$^{13}C_2$-PFUnA
PFDoA	$C_{12}F_{23}SO_3^-$	613	569	20	169	40	7.56	$^{13}C_2$-PFDoA
PFBuS	$C_4F_9SO_3^-$	299	99	40	80	75	1.45	$^{18}O_2$-PFHxS
PFHxS	$C_6F_{13}SO_3^-$	399	99	42	80	104	3.94	$^{18}O_2$-PFHxS
PFHpS	$C_7F_{15}SO_3^-$	449	99	96	80	102	4.42	$^{18}O_2$-PFHxS
PFOS	$C_8F_{17}SO_3^-$	499	99	94	80	100	4.32	$^{13}C_4$-PFOS
					169	48		
$^{13}C_2$-PFHxA	$^{13}C_2{}^{12}C_4F_{11}O_2^-$	315	270	14			4.78	
$^{13}C_4$-PFOA	$^{13}C_4{}^{12}C_4F_{15}O_2^-$	417	372	24			3.17	
$^{13}C_5$-PFNA	$^{13}C_5{}^{12}C_4F_{17}O_2^-$	468	423	16			4.42	
$^{13}C_2$-PFDA	$^{12}C_2{}^{12}C_8F_{19}O_2^-$	515	470	16			4.99	
$^{13}C_2$-PFUnA	$^{13}C_2{}^{12}C_9F_{21}O_2^-$	565	520	14			5.77	
$^{13}C_2$-PFDoA	$^{13}C_2{}^{12}C_{10}F_{23}SO_3^-$	615	570	20			7.56	
$^{18}O_2$-PFHxS	$C_6F_{13}S[^{18}O_2]O^-$	403	103	42			6.67	
$^{13}C_4$-PFOS	$^{13}C_4{}^{12}C_4F_{17}SO_3^-$	503	80	100			4.78	
$^{13}C_8$-PFOS	$^{13}C_8F_{17}SO_3^-$	507	99	76			4.78	

5.2.5 Optimisation of the method

5.2.5.1 Sample preparation

During the development of the analytical method, different ways of sample preparation were applied. Initially, a blank yolk egg was spiked with native and labelled PFASs and was analysed with the same procedure in freeze-dried and raw form. The recoveries of PFASs were low in both cases of preparation (30-65% and 40-70% respectively). According to previous studies analysing raw and freeze-dried eggs, the range of the presented recoveries is quite wide, with percentages sometimes being even lower than the ones given in this study when the same ways of egg preparation were applied [45,104,106-109]. Therefore, in order to optimize the sensitivity of the method, the blank yolk egg was also boiled and analysed. In that case the recoveries of the compounds were higher, compared to the two previous ways of preparation (60-115%). By applying the process of boiling, the preservation and the transportation of the Greek eggs to the Netherlands was also facilitated. To our knowledge, this is the first study that this kind of egg preparation is applied for the determination of PFASs in egg samples.

5.2.5.2 Distribution pattern of PFASs in eggs

The distribution pattern of all the analysed PFASs compounds in egg yolk and egg white was also investigated by analysing separately the two parts of the same egg in almost all the analysed egg samples of this study. According to the results, 100% of the detectable PFASs were distributed in the egg yolk, while no PFASs were determined in the white part. This observation was in agreement with two previous studies conducted in eggs, where only PFOS concentration was examined in the two egg parts [167,168]. In both studies it was reported that 98%-100% of the PFOS was found in the yolk, whereas less than 1% was measured in the white part. To our knowledge this is the first study examining the distribution of various PFASs compounds (not only of PFOS) between the yolk and the white part of eggs. It is also worth mentioning that in the one aforementioned study the egg samples originated from other birds, in contrast

with the chicken eggs analysed in the present study. To this end, more studies on PFASs transfer between the two egg parts from different species are needed.

5.2.5.3 Selectivity

As far as the instrumental part of the method is concerned, different analytical columns were tested in order to achieve better chromatographic separation between cholic acids and PFOS. Taurodeoxycholic acid (TDCA) bile salts, present in eggs, have a molecular weight of 498.2968 g/mol, which resembles the molecular weight of PFOS (498.9297 g/mol). Unfortunately, TDCA may elute together with PFOS on a C18 analytical column and when using nominal mass MS it cannot be separated. Moreover, the TDCA molecule contains also a sulfonate group, leading to the same transition as for PFOS (m/z 499 – 80) [169]. In order to evaluate our chromatographic separation, an extract was analysed both on a C18 and on a Fluorosep analytical column, always combined with a guard column. On the C18 column TDCA co-eluted with PFOS (m/z 499 – 80). When Fluorosep column was used, PFOS chromatographic peak eluted approximately two minutes later than TDCA, thus resolving issue of interference. By this means, both PFOS transitions (m/z 499 – 80 and 499 – 99) could be used, allowing sensitive measurements combined with ion ratio qualification on the m/z 80 and 99 ions.

5.2.6 Quantification and quality assurance

The method was validated addressing repeatability, reproducibility, specificity, recovery and sensitivity. For the analysis of the samples, an isotope dilution method was applied. Eight mass-labelled compounds ($^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS) were used as internal standards in order to calculate the relative response factor of the corresponding native compound and to confirm the RT. For the native compounds with no corresponding mass-labelled compound, the one with best resembling structure was used (Table 5.1). Repeatability and reproducibility of the present method were tested by multiple analyses (five replicates for each concentration on three different days) of the same blank sample, spiked at four different concentrations (0.5, 1, 2 and 5 ng g^{-1}). The calculated interday RSD% for the concentration of 2 ng g^{-1} ranged between 1-6% and 2-7% for PFOS and PFOA respectively, while for the rest of the compounds ranged between 1-14% for the same spiked level (Tables 5.2 and 5.3). Calibration curves covering concentrations from 0.05 ng mL^{-1} to 10 ng mL^{-1} (9 points including 0 ng mL^{-1}) were used for the quantification of the PFASs concentration in the samples. The r^2 was greater than 0.99 for all the calibration curves. LOD was determined as at least 3 times the signal to noise ratio and it was set at 0.15 ng g^{-1} for all the compounds and LOQ was set at 0.5 ng g^{-1}. The recoveries ranged between 60-115% for all the mass-labelled compounds, except for $^{13}C_2$-PFDoA that was below 40%. Hence, this compound was just qualified in the present study. Quality-control (QC) standards (one blank yolk egg sample and one spiked at the concentration of 1 ng g^{-1}) were analysed in every batch of samples, controlling in this way the repeatability of the analytical method. The ion ratio of the secondary mass transition response relative to the primary mass transition response and the retention time were recorded for each compound and every sample, in order to identify the analytes. The response of the instrument was also monitored by adding $^{13}C_8$-PFOS into the vial just before the injection. The recovery of $^{13}C_8$-PFOS ranged from 90 to 120% in all the samples, verifying the sufficient ionisation of the compounds and the absence of matrix effects. Investigation of blank samples was also performed during the development of the method and then in every sequence of egg samples, in order to monitor background contamination originating from various sources in the the laboratory. In none of the blank samples PFASs were detected.

Table 5.2: Repeatability of the detected concentrations of PFASs in spiked egg yolk samples – Intraday measurements.
(1 blank egg yolk sample spiked with 4 different concentrations, 5 replicates for each concentration)

	Repeatability							
	0.5 ng g^{-1} ww		1 ng g^{-1} ww		2 ng g^{-1}ww		5 ng g^{-1}ww	
	Average	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**
PFBuS	0.45	8%	0.96	11%	1.95	14%	4.91	9%
PFHxA	0.49	3%	1.03	5%	2.05	4%	5.02	2%
PFHpA	0.52	3%	1.04	4%	2.09	1%	5.02	1%
PFHxS	0.53	4%	1.03	6%	2	2%	5.09	1%
PFOA	0.51	3%	1.03	4%	2.03	2%	5.01	0%
PFHpS	0.51	3%	1.02	3%	2.03	2%	5.00	1%
PFNA	0.51	8%	1.07	4%	2.02	3%	5.00	1%
PFOS	0.51	3%	1.03	3%	2.05	1%	5.01	2%
PFDA	0.51	2%	1.03	3%	2.03	4%	5.02	1%
PFUnA	0.53	2%	1.05	5%	2	4%	5.03	1%

Table 5.3: Reproducibility of the detected concentrations of PFASs in spiked egg yolk samples – Interday measurements.

(1 blank egg yolk sample spiked with 4 different concentrations (5 replicates each day) in three different days)

	Reproducibility							
	0.5 ng g^{-1} ww		1 ng g^{-1} ww		2 ng g^{-1} ww		5 ng g^{-1} ww	
	Average *	**RSD%****	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**
PFBuS	0.43	5-12%	0.86	7-11%	1.83	7-14%	4.75	9-15%
PFHxA	0.50	3-8%	0.98	5-14%	1.93	2-8%	4.83	2-7%
PFHpA	0.52	3-7%	1.08	4-6%	2.12	1-6%	5.20	1-4%
PFHxS	0.52	4-8%	1.02	5-9%	1.98	2-8%	4.95	1-8%
PFOA	0.53	3-5%	1.04	4-5%	1.99	2-7%	4.92	0-5%
PFHpS	0.51	3%	1.02	3-8%	1.99	2-5%	4.85	1-13%
PFNA	0.50	5-8%	1.00	4-9%	1.92	2-10%	4.84	1-13%
PFOS	0.52	3-5%	1.03	3-6%	2.01	1-6%	5.04	1-3%
PFDA	0.51	2-7%	1.02	3-6%	1.95	3-11%	4.80	1-7%
PFUnA	0.52	2-9%	0.99	5-10%	1.96	1-13%	4.85	1-10%

*Value calculated based on each day's average.

****** The range of RSD% among the 3 days of analysis.

5.3 Results and discussion

5.3.1 PFAS levels in egg samples

In the present study, 10 PFASs were quantified in 118 home produced egg samples from the Netherlands (n=73) and Greece (n=45). Compounds were analyzed in the yolks, as initial experiments demonstrated that the PFAS are primarily found in the egg yolks rather than the egg whites. This confirmed earlier studies [167,168] which demonstrated that PFOS is primarily found in the egg yolks. The concentrations of individual PFASs and the ∑PFASs (lower and upper bound principle) for each egg yolk sample are presented in Table A1 and A2. In 59 (out of 73) home produced eggs from the Netherlands and in 34 (out of 45) from Greece, one or more PFASs were detected above the LOQ (0.5 ng g^{-1}). PFAS levels were found to be higher in the eggs collected from homes in the Netherlands (∑PFAS: median 3.5, range <LOQ – 31.2 ng g^{-1}) compared to the Greek home-grown eggs (∑PFAS: median 1.1, range <LOQ – 15.0 ng g^{-1}). This difference was found to be statistically significant by application of one-way ANOVA ($p<0.005$) (MATLAB). Moreover, statistical analysis was performed to each analyte individually, and it was found that there was also a statistically significant difference in PFOS and PFOA concentrations between the Netherlands and Greece ($p<0.005$), while for the other PFASs the p value was higher than 0.005 and therefore considered as not relevant. However, it is unclear if such a difference between PFOS and PFOA concentrations points to a higher background contamination in general or whether it also depends on the areas where the samples were collected.

Besides the difference in the levels, PFAS patterns found in the home produced egg samples were the same in both countries. In particular, the long-chain PFASs (C≥8) were most frequently detected, while the short-chain ones were rarely found (Figures 5.1 and 5.2), being in line with previous studies [46,167]. PFOS was the predominant compound, detected in approximately 81% and 69% of the samples in the Netherlands and Greece respectively (Table 5.4), while for the other long-chain compounds (PFOA, PFNA, PFDA and PFUnDA) this ranged between 2% and 36% of the samples. Traces of PFDoA were found in some of the home produced eggs from both countries, but due to the low $^{13}C_2$-PFDoA recoveries, PFDoA could not be quantified. Overall, it appears that the pattern of contamination is very similar in the two countries.

Table 5.4: Ranges and frequency of detection of PFASs in domestic eggs from the Netherlands (n = 73) and Greece (n = 45).

	Range NL (ng g^{-1} ww)	Median value*	Frequency of detection (%)	Range Gr (ng g^{-1} ww)	Median value*	Frequency of detection (%)
PFHxA	<0.5	<0.5	0	<0.5	<0.5	0
PFHpA	<0.5	<0.5	0	<0.5	<0.5	0
PFOA	<0.5–2.7	1.1	27	<0.5–0.5	0.5	2
PFNA	<0.5–2.0	0.9	18	<0.5–1	0.8	20
PFDA	<0.5–3.0	0.9	32	<0.5–8.0	0.9	36
PFUnA	<0.5–2.3	0.9	21	<0.5–4.5	0.7	24
PFBuS	<0.5	<0.5	0	<0.5	<0.5	0
PFHxS	<0.5–5.2	1.1	7	<0.5	<0.5	0
PFHpS	<0.5	<0.5	0	<0.5	<0.5	0
PFOS	<0.5–24.8	3.5	81	<0.5–8.9	1.1	69
ΣPFASs (ng g^{-1} ww)	<0.5–31.2	4.4		<0.5–15.0	1.8	

*Median value is calculated based only on the concentrations above LOQ.

** PFDoA is not included in the table because it cannot be quantified in the present study.

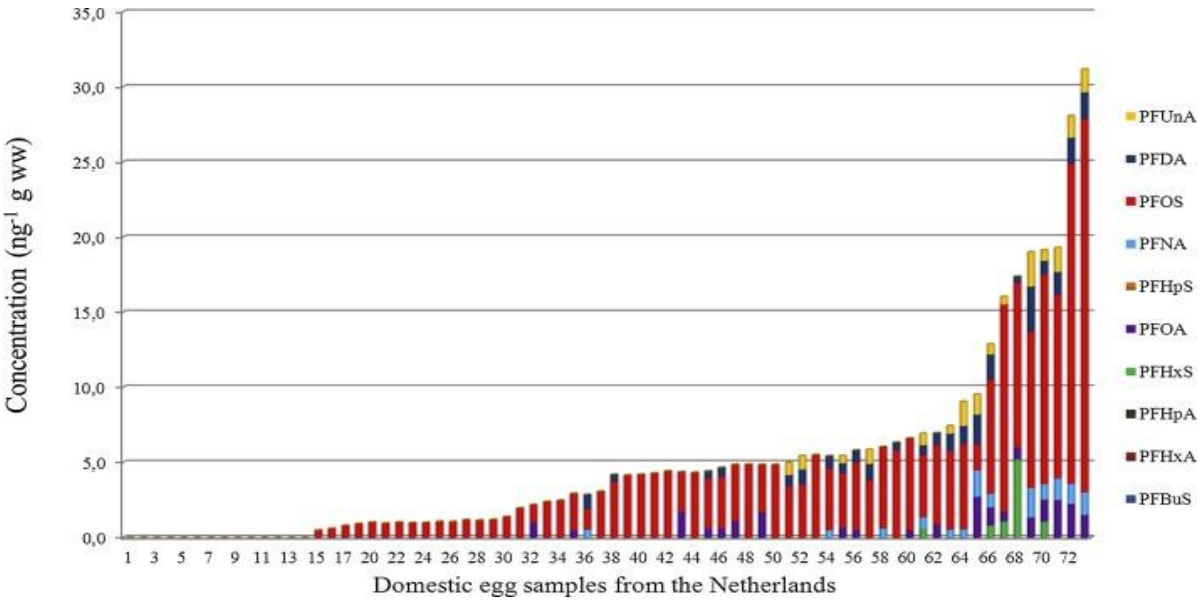


Figure 5.1: Concentrations of individual PFASs (ng g^{-1} ww) in yolk samples from home produced eggs from the Netherlands. In samples where no data are presented, all levels were below the LOQ. The samples have been presented in increasing PFASs level order.

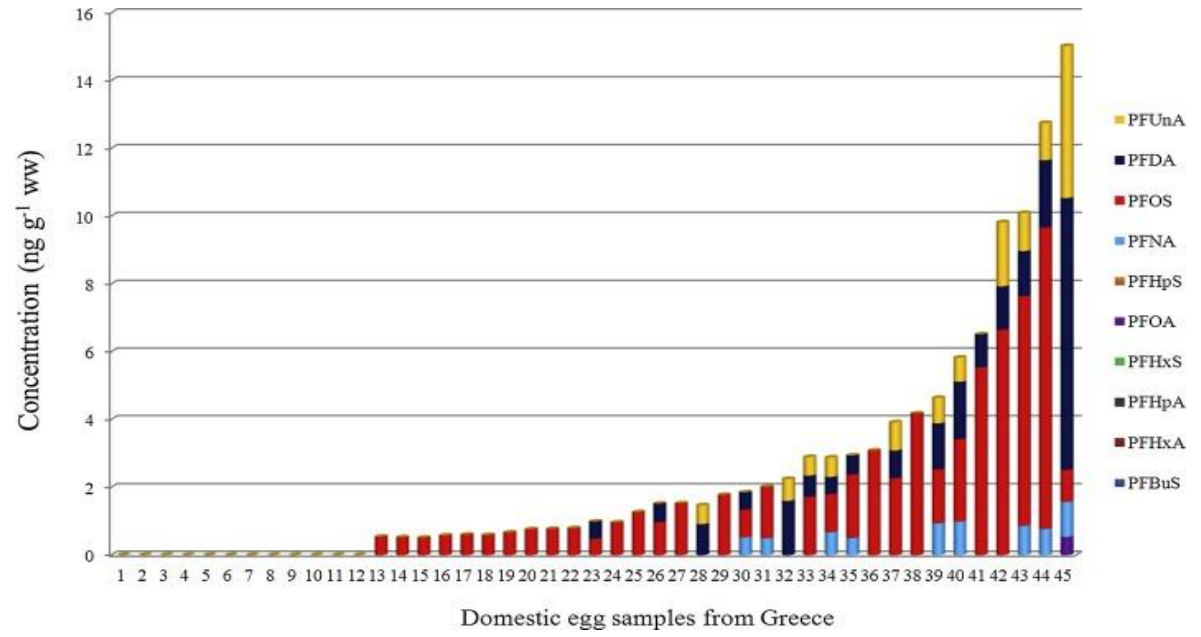


Figure 5.2: Concentrations of individual PFASs (ng g^{-1} ww) in yolk samples from home produced eggs from Greece. In samples where no data are presented, all levels were below the LOQ. The samples have been presented in increasing PFASs level order.

In order to reflect on the PFAS contamination in eggs from the Netherlands and Greece in a broader sense, also commercially produced eggs were investigated, including organic, battery and free-range poultry eggs from both countries. In contrast to the home produced eggs, in all the commercial egg samples, all PFASs were below the LOQ, except for one (out of 6) organic egg from the Netherlands and one (out of 11) free range egg from Greece where low levels of PFOS were detected (1.1 ng g^{-1} and 0.94 ng g^{-1} respectively) (Table A1 and A2).

5.3.2 Origin of the contamination

The differences between PFAS levels in the home produced and the commercially produced eggs could be explained by the living and eating habits of the hens in each case. It seems no surprise that eggs from free foraging hens are more contaminated, due to their intensive contact with the outside environment. Particularly soil intake combined with the ingestion of worms or insects, can be considered as the main contamination source. According to previous literature, examining the presence of environmental pollutants (PCDD/Fs, PCBs, heavy metals, PFASs, pesticides, etc) in home produced eggs, soil plays a very important role in the contamination of the eggs [164,165,170-176]. Given the widespread ubiquitous presence of PFASs in the environment, it was hypothesised that in a similar sense PFAS levels in home produced eggs may be higher than in commercial eggs. In fact, Hollander et al. demonstrated this in a study on home produced eggs in Belgium [165]. This seems contradicted by the non-detectable levels in commercial organic eggs, where chickens are obliged to forage outside. However, it should be mentioned that since a number of years, there is strict self-control on PCDD/Fs and PCBs in these eggs in the Netherlands, meaning that farms have to take measures to reduce the intake of contaminated soil, e.g. by replacing the soil in the courtyard when levels in eggs are too high. To reduce PFASs contamination of eggs, Brambilla et al. (2015) [170] also recommended the keeping of flocks in non PFASs contaminated areas, and the feeding with commercial feed. In principle, laying hens in commercial farms have a surplus of feed at their disposal, decreasing their need to collect food from the outside environment. Furthermore, hens living at private coops eat also food remains and bread. Some of the owners also give to the chicken mown grass and weeds in addition to commercial feed (mixture of grains) [164] . It would be interesting to further

investigate how the exposure of home kept chickens, is influenced by the consumption of kitchen waste, soil components and insects. As a start, the analysis of soil and waste from the individual coops would be needed.

5.3.3 Comparison of PFAS levels with studies from other countries

Compared with a previous study, conducted in home produced chicken eggs from Belgium [165], the concentrations of PFOS in the Dutch and Greek samples from this study showed similar levels, except for the eggs collected in the vicinity (<1 km) of a perfluorochemical plant in Zwijndrecht (Antwerp), where PFOS concentrations were extremely high, ranging from 53 to 3472 ng g^{-1}. No other studies were reported on home produced eggs. Most of the available data refer to chicken eggs purchased from supermarkets, as part of more general food studies. The results from other studies (Table 5.5) are generally in agreement with the current study for the commercially produced eggs, where all the PFAS levels were <LOQ in all samples, apart from two where only PFOS was detected. In particular, PFAS contamination in other countries like Norway [26,45,177,178], Spain [25,106], Italy [26,44,177,178], U.K. [105], U.S.A. [104], Belgium [26,152,177,178], France [109], the Netherlands [46], Sweden [107], China [179] and in EFSA reports on food [110,180] was also low and most of the compounds were <LOQ. However, in one study from China [167] PFOS was detected in high concentrations (87.6 - 107 ng g^{-1} ww in egg yolk samples and 34.7 – 86.9 ng g^{-1} ww in pooled egg samples). According to the authors [167] this variation among the countries might be due to different feed types and feeding habits of the chicken. However, a more local contamination cannot be excluded.

Table 5.5: Overview of the detected concentrations (ng g^{-1} ww) of PFASs in chicken egg samples from other countries.

Country	Origin of the eggs	Number of samples	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFBS	PFHxS	PFHpS	PFOS	References
China	Local market	2 (individual egg yolks)	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05	<0.01	<0.01		87.6-107	[167]
		8 (pooled samples. Whole egg)	<0.01	<0.01	<0.01-0.0914	<0.05-0.261	<0.01-0.312	<0.01-0.584	<0.05-0.164	<0.01	<0.01		34.7-86.9	
China	Local market or grocery stores	21 individual eggs		<0.13	0.26	<0.12	<0.02	<0.62	<0.52		<0.11		0.08	[179] (mean)
Norway	Grocery stores	1 (pooled sample)	0.013	<0.016	0.03	<0.0074	0.012	0.0099	<0.0081	0.002	0.0035		0.039	[45]
U.S.A.	Grocery stores	1 (pooled sample)	<0.04	<0.04	<0.04	<0.04	<0.04		<0.04	<0.04	<0.04		<0.04	[104]

Spain	Local market-large supermarkets-grocery stores	2 (pooled samples)	<0.005	<0.005	<0.055	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.082	[106] (mean)
U.K	Supermarkets and independent retailers	10 individual eggs	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1-1	[105] (range)
Italy	Supermarket	4 (pooled samples)			<0.5							<0.5	[44] (average)
Netherlands	Retail stores with nation-wide coverage	1 (pooled sample)	<0.054	<0.002	<0.032	0.006	0.011	<0.019	<0.013	<0.003	<0.006	0.029*	[46]
Spain	Local markets-supermarkets-small stores-grocery stores	2 (pooled samples)	<0.039	0.2	<0.39	<0.1	<0.01	<0.0038	<0.011	<0.0032	<0.002	<0.0053	[25] (mean)
Belgium	Chicken farms	8 (pooled samples)			0.86							6.86	[152] (average)

Netherlands	Domestic	73 individual yolks	<0.5	<0.5	<0.5-2.7	<0.5-2.0	<0.5-3.0	<0.5-2.3	<0.5	<0.5-5.2	<0.5	<0.5-24.8	Present study (range)
Greece	Domestic	45 individual yolks	<0.5	<0.5	<0.5-0.5	<0.5-1.0	<0.5-8.0	<0.5-4.5	<0.5	<0.5	<0.5	<0.5-8.9	Present study (range)
Netherlands	Supermarkets	22 individual yolks	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5-1.08	Present study (range)
Greece	Super market	31 individual yolks	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5-0.9	Present study (range)
Belgium	Domestic	29 individual eggs										0.4-3473	[165] (range)
Sweden	Spread out over Sweden (emphasis on the largest packaging plants)	36 yolks (pooled samples)	<0.008-0.013	<0.005-0.005	<0.014-0.225	<0.020-0.143	<0.006-0.067	<0.008-0.241	<0.006-0.051		<0.010-0.128		<0.026-6.48	[107] (range)

Europe	86 eggs and egg products			0.56-097						0.19-0.57	[180] mean (lower-upper bound)
Europe	Around 550 eggs (fresh)	0.0029-0.72	0.0034-0.7	0.088-0.76			0.0005-0.7		0.0001-0.69	0.037-0.7	[110] mean (lower – upper bound)
UK	10 eggs (caged, free range, organic)	<1	<1	<1	<1	<1	<1	<1	<1	<1	[181] (mean)

*Value between LOD and LOQ.

5.3.4 PFOS in comparison to PCDD/Fs and PCBs in home produced eggs

The home produced eggs from the Netherlands were collected to be analysed for PCDD/Fs and PCBs, in the framework of another study [182]. Since soil and soil organisms might be the source of both PFAS and PCDD/Fs and PCBs, it was of interest to compare the levels of these contaminants. Figure 5.3 and 5.4 show a comparison between levels of PFOS and PCDD/F-TEQ, resp. dl-PCB-TEQ in home produced eggs, expressed on a yolk basis. The relation between PFOS and PCDD/F-TEQ seems rather poor, as can be expected since the sources of contamination for these contaminants are likely to be different. Nevertheless, it is clear that samples with a low dioxin-TEQ contamination, in most cases also show low PFOS contamination levels. On the other hand, eggs with a higher PFOS contamination, generally are also more contaminated with PCDD/Fs, although there are clearly exceptions. As a result, consumption of home produced eggs may lead to elevated exposure to both PCDD/Fs and PFOS. The same is true for PFOS and PCB-TEQ (Figures 5.3 and 5.4), PFOS and total-TEQ and PFOS and the sum of ndl-PCBs (Figure 5.5. and 5.6). Possibly, other contaminants (e.g. brominated flame retardants, organophosphate flame retardants, etc) follow the same trend, as can be seen with e.g. wild eel [183]. More research is needed to confirm if the higher contaminated eggs from the present study are also contaminated with other contaminants.

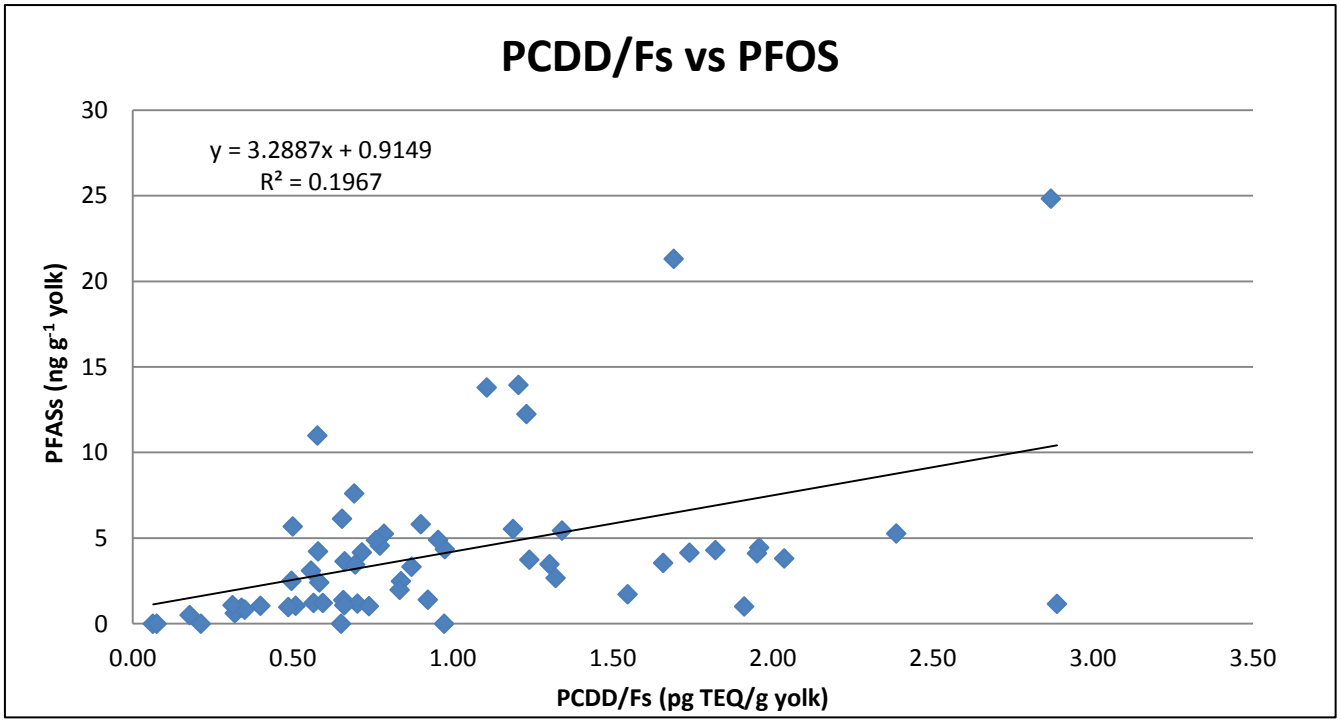


Figure 5.3: Contamination levels in home produced eggs from the Netherlands. PFOS levels (y-axis) are plotted versus (x-axis) dioxin-TEQ.

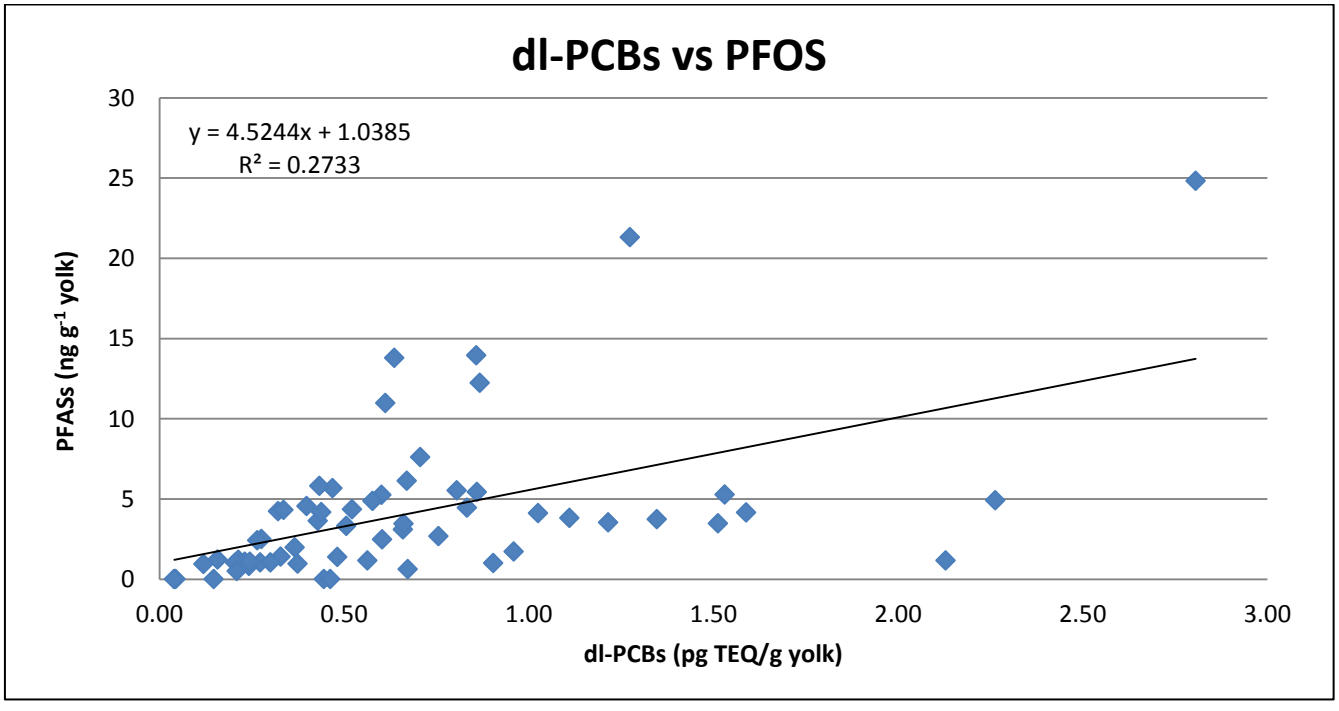


Figure 5.4: Contamination levels in home produced eggs from the Netherlands. PFOS levels (y-axis) are plotted versus (x-axis) dl-PCB-TEQ.

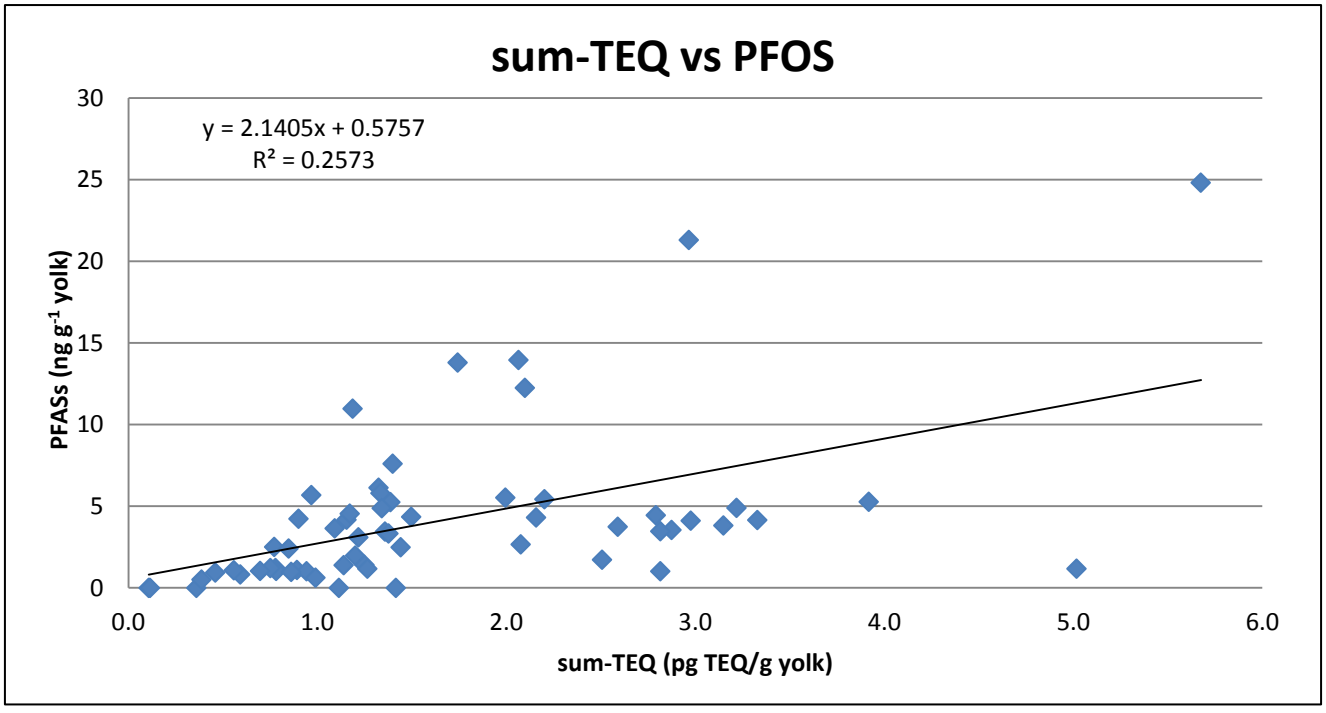


Figure 5.5: Contamination levels in home produced eggs from the Netherlands. PFOS levels (y-axis) are plotted versus (x-axis) sum-TEQ.

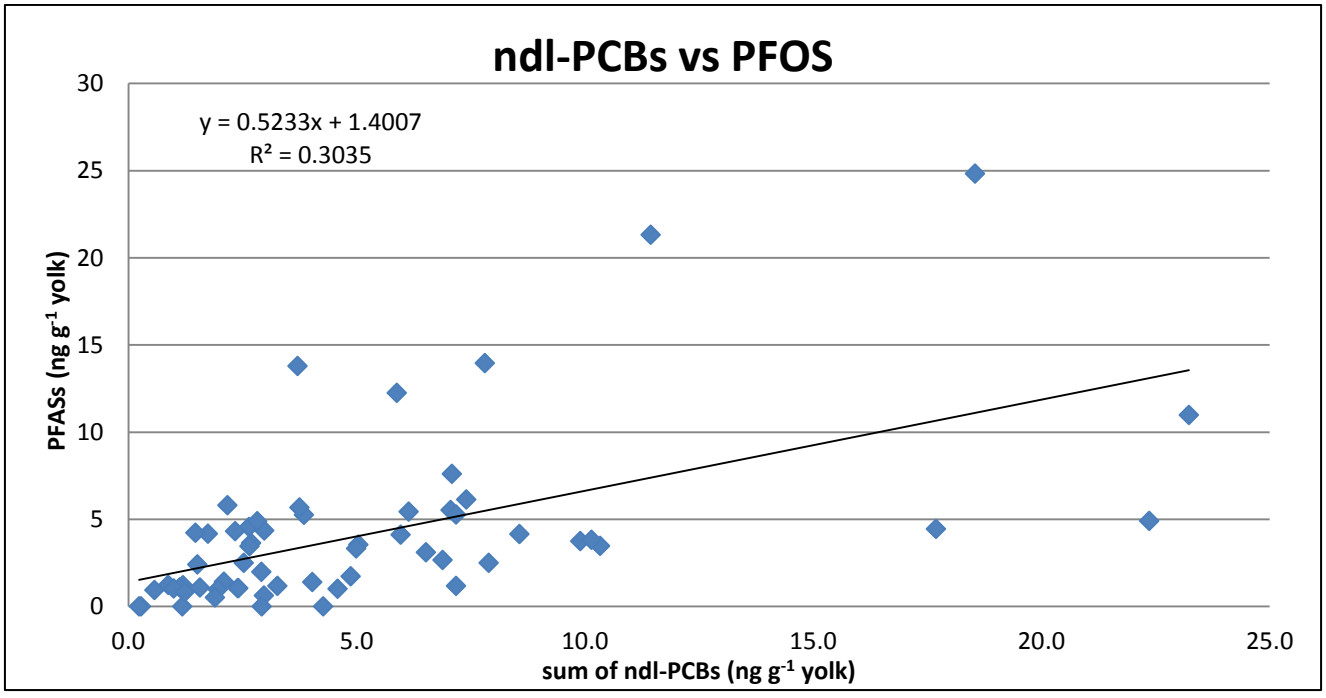


Figure 5.6: Contamination levels in home produced eggs from the Netherlands. PFOS levels (y-axis) are plotted versus (x-axis) ndl-PCBs-TEQ.

5.3.5 Potential exposure of consumers to PFASs from home produced eggs

EFSA set TDIs for PFOS and PFOA (150 ng kg^{-1} b.w. for PFOS, 1500 ng kg^{-1} b.w. for PFOA). PFOA levels in eggs were much lower than PFOS, and the TDI for PFOA is ten-fold higher. Therefore, the potential risk from PFOS is clearly of more concern. People who produce their own eggs are likely to have a higher consumption of eggs, since they have a surplus of these eggs. When asked most people indicated that they eat 3 to 4 of these eggs per week but higher consumption was not excluded. Since egg yolk represents 30% of an egg, daily consumption of one home produced egg would comprise a consumption of up to 20 gram egg yolk per day, resulting in an intake of 70 and 496 ng PFOS for respectively the median and highest observed level in the Netherlands. For a child and adult of respectively 20 and 65 kg b.w. the median intake would be 3.5 and 1.1 ng kg^{-1} b.w. per day, the maximum 24.8 and 7.6 ng kg^{-1} b.w. per day. These intakes are clearly lower than the TDI for PFOS. Levels of PFOS in eggs from Greece were lower and thus also the exposure. Even when combined with the exposure from other sources, as reported by EFSA [180], the intake of PFOS would be below the TDI. It can therefore be concluded that the PFOS and PFOA concentrations in home produced eggs from the two countries are believed not to be a risk for human health, when compared to the TDIs established by EFSA.

5.4 Conclusions

The present study is the first study reporting levels of PFASs in Greek egg samples and demonstrating a difference of PFAS levels between home and commercially produced eggs in both the Netherlands and Greece. Home produced eggs were contaminated with PFASs, and especially PFOS, while in the commercially produced eggs (organic, battery and free range eggs) all PFASs were below the LOQs in all the samples, except for two. The different levels of contamination between the two aforementioned categories can be mainly attributed to the intensive contact of the free-range home-kept laying hens with the outside environment, and basically to the consumption of contaminated soil and small organisms. The contamination of home produced eggs from Greece was lower (median 1.1, range <LOQ – 15.0 ng g^{-1}) than from the Netherlands (median 3.1, range <LOQ - 31.2 ng g^{-1}). The PFOS and PFOA concentrations in eggs from the two countries are believed not to be a risk for human health, based on the TDIs established by EFSA. A comparison of PFOS contamination in Dutch

home produced eggs versus PCDD/F-TEQ and PCB-TEQ showed that eggs with low contamination are also low in contamination with PCDD/Fs and PCBs and vice versa, so a co-exposure to both groups of contaminants is likely to occur for at least part of the home produced eggs.

CHAPTER 6

Determination of perfluoroalkylated substances (PFASs) in drinking water from the Netherlands and Greece

6.1 Introduction

Up to now, dietary intake is regarded as the main route for human exposure to PFOS and PFOA [39,184,185]. However, according to Pico et al. (2011) [186], one of the main inputs of PFASs in the food chain is the exposure of food producing animals or plants to these substances via environmental routes, with contaminated water being the most important one. Moreover, other studies point to the consumption of drinking water as one of the most important routes of exposure to PFASs, reporting a positive correlation between the consumption rate of PFASs-contaminated water and the PFASs concentration, especially of PFOA, in human serum [187].

The water supply system differs among the countries and also among different areas in the same country. Drinking water may be sourced from surface water (lakes or rivers), but also from groundwater. Surface water can be contaminated both via direct discharge of the contaminants (through industrial or municipal WWTPs) [18], or through industrially contaminated areas [188] and via indirect emissions (atmospheric degradation of precursor compounds) [189]. However, sources of PFASs contamination for the groundwater remain still uncertain. In a previous study, referring to groundwater from the Netherlands, landfill leachate and water draining from a military base were reported as PFASs contamination sources [190], while in another study conducted in drinking water sourced from groundwater in Uppsala, a military airport with fire-fighting training activities was reported as the most likely source of PFASs contamination [191]. Considering that PFASs are also quite soluble in water and that the purification treatment for drinking water cannot remove all of them [23], it is obvious that their presence in drinking water is a matter of great importance for human health.

To investigate the potential impact of some of these aspects, drinking water samples from the Netherlands and Greece were analysed for PFASs within a cooperative project between the two countries. In both countries, drinking water is produced from both groundwater and surface water sources. However, the two countries show different geomorphology, as the Netherlands is located on a river delta formed by the confluence of the Rhine, Meuse and Scheldt rivers, increasing in this way the possibility of PFASs presence in the surface water. Besides, the Netherlands is characterized by a slightly higher industrial activity compared to Greece [192] and certain industries can contribute to PFASs contamination of the water cycle. In addition, in a dietary exposure assessment of Dutch consumers [46], the contribution from drinking water was based on estimated PFASs levels due to the lack of measured levels. These estimated levels can now be evaluated against real measured values coming from this study. To our knowledge, this is the first study presenting PFASs levels in tap and bottled water samples from Greece.

6.2 Water supplying systems in the Netherlands and Greece

The water supply and sanitation in Greece is characterized by large diversity around the country. The metropolitan area of the capital Athens, where more than one third of the population of Greece lives, is supplied by five different water sources in order to have sufficient supply of water. The five water sources include the Lake of Marathon, the Lake Yliki, the Mornos reservoir and the Evinos reservoir. The fifth source consists of 105 boreholes in three wellfields that are used only in emergency situations, located in a range of 200 km away from Athens. Thessaloniki, the second biggest city in Greece, is mainly supplied by the Aliakmon river, that is, by surface water, similar to almost all Greek cities.

In contrast to big Greek cities, water resources are especially scarce on Greek islands. Most of the Aegean islands suffer from severe lack of good quality fresh water, mainly because of the low precipitation and their specific geomorphology [193]. Besides that, the problem becomes extremely imperative during the summer months, when tourism practically doubles the population of the islands

increasing the domestic water needs. At the same time, due to the climate conditions, irrigation needs to increase significantly. As a result, the temporary increase of population (in combination with the local activities), the low precipitation, the geomorphology and the over-exploitation of groundwater resources, all lead to extensive water shortage problems.

The medium-large sized islands, such as Syros, Andros, Mykonos and Kalymnos, with high development of residential and tourist infrastructure, have partially solved their water shortage problem with large scale projects, such as desalination plants, water dams and ground reservoirs. However, the smaller ones like Kythnos, are forced to adopt short-term solutions i.e. water transfer by ships and storage in water tanks. In addition, some of the Aegean islands also collect the rain water for domestic use and drinking after purification [194].

In the Netherlands, drinking water is supplied both from groundwater and surface water sources. In particular, 60% of the drinking water is provided from the ground, mainly in the eastern part of the Netherlands. Groundwater is generally supposed to be an attractive source for drinking water, because of its purification while passing through natural soil (removal of microorganism and chemical impurities)http://www.iwahq.org/uploads/iwa%20hq/website%20files/utilities/benchmarking_amsterdam_06/IWA%20conference%20on%20benchmarking%202006_04_Theo%20Schmitz.pdf. The remaining 40% of drinking water is obtained by surface water sources. The two main supplying points of surface water in the Netherlands are the Rhine, and its fed waters (Lek, Lek Canal, Amsterdam Rhine Canal, Haringvliet, IJssel and IJsselmeer), and the Meuse, including Harringvliet. [190,195].

6.3 Materials and methods

The sampling points for the tap water samples were chosen based on the origin of the water (ground- or surface water). For the analysis of the samples, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) and isotope dilution method was developed. In this study, 11 PFASs: PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFBS, PFHxS, PFHpS and PFOS were quantified.

6.3.1 Chemicals

In the current study eleven PFASs (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFBS, PFHxS, PFHpS, PFOS) were quantified by using standard solutions. Native PFSA solution/mixture (PFS-MXA), native PFCA solution/mixture (PFC-MXA), mass labelled internal PFCAs and PFSAs solution/mixture (MPFAC-MXA) and a $^{13}C_8$-PFOS solution were used, all purchased from Wellington laboratories (Guelp, Ontario, Canada). MeOH (Ultra LC-MS grade), ACN (Ultra LC-MS grade) and HPLC water (Ultra LC-MS grade) were purchased from Actu-All chemicals (Oss, Netherlands). The ammonium acetate (approx. 98%) was provided by Sigma (St. Louis, USA) and the ammonium solution 25% by Merck (Darmstadt, Germany). Solid-phase extraction (SPE) was carried out with Oasis WAX cartridges (3cc, 60 mg, 60 μm, Waters, U.S.A.).

6.3.2 Drinking water samples

Drinking water samples were collected from the Netherlands (37 tap water samples and 5 bottled water samples) and Greece (43 tap water samples and 5 bottled water samples) from August 2013 until January of 2014. For the collection of the tap water samples, different plastic bottles were tested. In particular, five plastic bottles were tested for PFASs contamination to the sample, PFASs adsorption and leaking. The bottles were filled with MeOH, weighted and shaking overnight. The next day the bottles were weighted again in order to check if there was any leak. The reason of this test was to avoid any PFASs loss during the transportation of the samples to the laboratory, especially of the Greek ones. In

addition, the bottles were tested for PFASs contamination to the sample, by evaporating the contained MeOH of each bottle till dryness, dissolving in the mobile phase and measuring in LC-MS/MS. The same procedure was repeated, by adding internal standards in each bottle before the evaporation of the MeOH. In this way, PFASs adsorption of the bottle was also tested. At this point the best bottle (polyethylene) in terms of leaking and contamination/adsorption was chosen for the sampling of all the water samples from both countries. The capacity of the bottle was at least the double of the needed water volume for the analysis (250 ml), so repetition of a sample was possible whenever necessary. The bottles were flushed three times with MeOH and three times with the sampled water before taking a sample. All the water samples were transferred to the laboratory and were directly stored at 4 °C until the analysis. The different brands of bottled water were collected from supermarkets in both countries, and were also stored in a refrigerator (4 °C) until the analysis. The sampling points of the tap water in Netherlands and Greece are illustrated in Figures 6.1a and 6.1b.

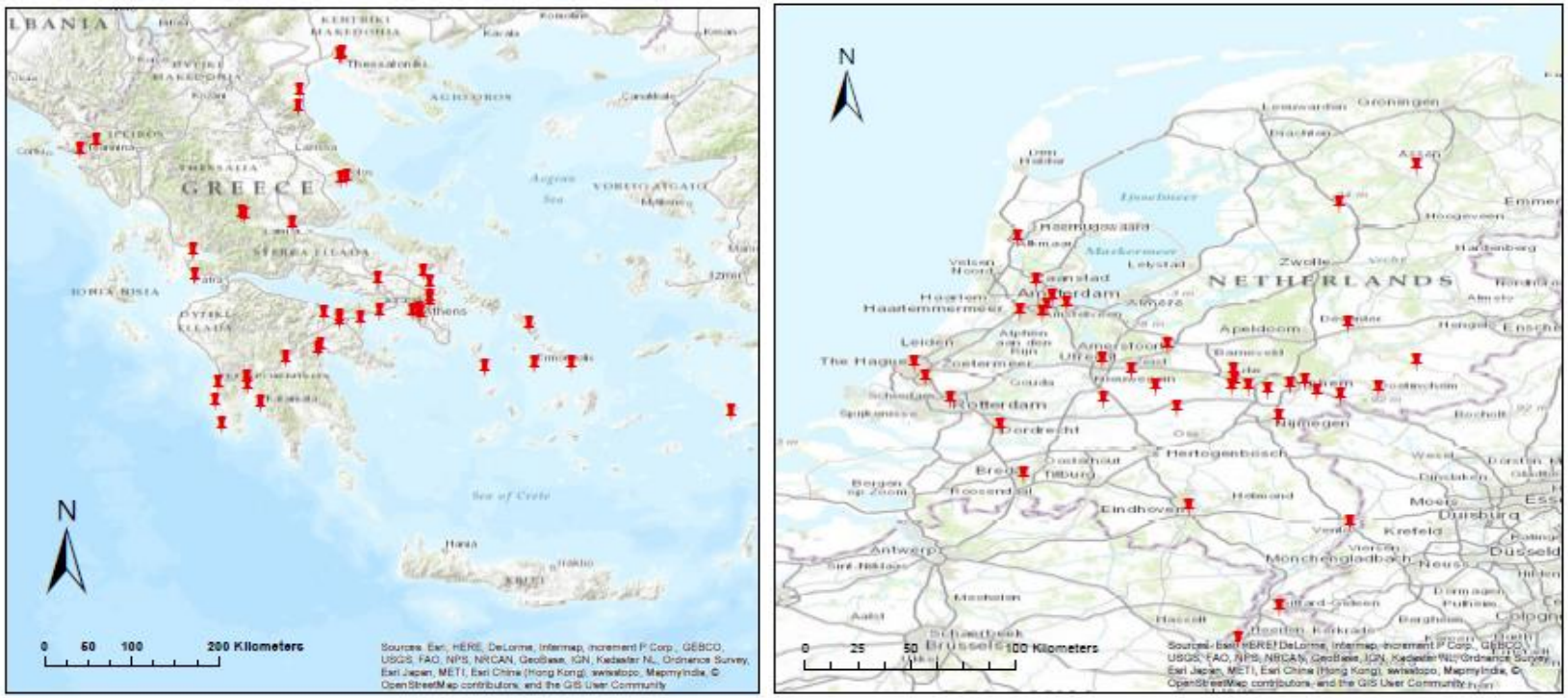


Figure 6.1: Drinking water sampling points in Greece and in the Netherlands. Maps were generated using Geographic Information System (GIS).

6.3.3 Sample preparation

For each water sample, 250 mL were fortified with 25 µL of mass-labelled PFCAs and PFSAs solution/mixture (MPFAC-MXA) of 100 ng mL^{-1} 1h before the analysis. Then SPE was performed by using weak anion exchange Oasis WAX cartridges. SPE started with the conditioning of Oasis WAX cartridges with 4 mL of MeOH and 4 mL of HPLC water. Consequently, the drinking water sample was passed through the cartridge and then washed with 4 mL HPLC water. The final SPE step was the elution from the cartridge with 3 mL NH_4OH in ACN. During all the SPE steps, the flow rate of the cartridge was constant at approximately 1–2 drops per second. The collected extract from SPE was centrifuged for 10 min at 10.000 rpm and 20 ^{o}C. After centrifugation, the supernatant was transferred to a new glass tube and was evaporated till dryness under N_2 and 60 ^{o}C. As the calculated recoveries for the labelled standards were high (85-115%), it can be assumed that no significant adsorption of PFASs to the glass tube took place. The dry residue was dissolved in 675 µL of 2 mM ammonium acetate in milli-Q water and 300 µL of ACN. Before the injection, 25 µL of $^{13}C_8$-PFOS solution 100 ng mL^{-1} were also added for monitoring the run to run MS response. The final solution was transferred into a vial for analysis by LC-MS/MS.

6.3.4 Instrumental analysis

For the analysis of all the water samples, LC-MS/MS was used, based on a Shimadzu LC system with an Acquity UPLC BEH C18 analytical column (50 mm * 2.1 mm i.d., 1.7 µm, Waters). In addition, a Symmetry C18 column (50 mm * 2.1 mm i.d., 5 µm, Waters) was used as guard column prior to the injector in order to isolate and delay interferences from the LC system. The chromatographic gradient was operated at a flow rate of 0.400 mL/min starting from 75% 2 mM ammonium acetate in water (A) to 100% ACN (B) in 6 min. Each chromatographic separation lasted 12 min and the injection volume was 20 µL. Furthermore, the oven temperature of the analytical column was set at 50 ^{o}C.

The LC system was connected to a triple quadrupole MS (AB SCIEX QTRAP 5500 SYSTEM, Applied Biosystem - Analytical Technologies), equipped with a Turbo Spray source operating in negative mode. The source temperature was set at 350 ^{o}C and the ion spray voltage at -4500 V. The analyses were performed with a multiple reaction monitoring (MRM) method that monitored two mass transitions (parent ion/product ion) for every analyte except for PFPeA. The information on ion transitions for both labelled and native PFASs, collision energies, retention times and which internal standards were applied for which native analyte are illustrated in Table 6.1.

6.3.5 Quantification and quality assurance

For the analysis of the samples, an isotope dilution method was applied. Eight mass-labelled compounds ($^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnDA, $^{13}C_4$-PFOS, $^{18}O_2$-PFHxS) were used in order to calculate the relative response factor of the corresponding native compound. For the native compounds with no corresponding mass-labelled compound, the one with the most similar structure was used (Table 6.1).

Table 6.1: Instrumental mass spectrometry settings for the target compounds.

Compound	Molecular formula	Precursor ion (m/z)	Product ion 1 (m/z)	Collision energy (eV)	Product ion 2 (m/z)	Collision energy (eV)	Retention time	Internal standard
PFPeA	$C_5F_9O_2^-$	263	219	15	-		2.62	$^{13}C_4$-PFBA
PFHxA	$C_6F_{11}O_2^-$	313	269	14	119	24	3.48	$^{13}C_2$-PFHxA
PFHpA	$C_7F_{13}O_2^-$	363	319	12	169	24	3.86	$^{13}C_2$-PFHxA
PFOA	$C_8F_{15}O_2^-$	413	369	14	169	24	4.13	$^{13}C_4$-PFOA
PFNA	$C_9F_{17}O_2^-$	463	419	16	169	26	4.37	$^{13}C_5$-PFNA
PFDA	$C_{10}F_{19}O_2^-$	513	469	16	219	26	4.59	$^{13}C_2$-PFDA
PFUnDA	$C_{11}F_{21}O_2^-$	563	519	14	319	24	4.81	$^{13}C_2$-PFUnA
PFBuS	$C_4F_9SO_3^-$	299	99	40	80	75	3.40	$^{18}O_2$-PFHxS
PFHxS	$C_6F_{13}SO_3^-$	399	99	42	80	104	4.17	$^{18}O_2$-PFHxS
PFHpS	$C_7F_{15}SO_3^-$	449	99	96	80	102	4.42	$^{18}O_2$-PFHxS
PFOS	$C_8F_{17}SO_3^-$	499	99	94	80 169	100 48	4.65	$^{13}C_4$-PFOS
$^{13}C_2$-PFHxA	$^{13}C_2{}^{12}C_4F_{11}O_2^-$	315	270	14			3.46	
$^{13}C_4$-PFOA	$^{13}C_4{}^{12}C_4F_{15}O_2^-$	417	372	24			4.13	
$^{13}C_5$-PFNA	$^{13}C_5{}^{12}C_4F_{17}O_2^-$	468	423	16			4.36	
$^{13}C_2$-PFDA	$^{12}C_2{}^{12}C_8F_{19}O_2^-$	515	470	16			4.58	
$^{13}C_2$-PFUnDA	$^{13}C_2{}^{12}C_9F_{21}O_2^-$	565	520	14			4.80	
$^{18}O_2$-PFHxS	$C_6F_{13}S[^{18}O_2]O^-$	403	103	42			4.16	
$^{13}C_4$-PFOS	$^{13}C_4{}^{12}C_4F_{17}SO_3^-$	503	80	100			4.56	
$^{13}C_8$-PFOS	$^{13}C_8F_{17}SO_3^-$	507	99	76			4.57	

Calibration curves covering concentrations from 0.05 ng mL^{-1} to 10 ng mL^{-1} (9 points including 0 ng mL^{-1}) were used for the quantification of the PFASs concentration in the samples. The regression coefficient (r^2) was greater than 0.99 for almost all calibration curves. For the validation of the method, a tap water sample fortified in five different concentrations (0.5 ng mL^{-1}, 1 ng mL^{-1}, 2 ng mL^{-1}, 5 ng mL^{-1}, 10 ng mL^{-1}) was analysed five times for each concentration and the analysis was repeated for three different days. The calculated interday RSD% for the concentration of 2 ng L^{-1} ranged between 3-14% and 5-10% for PFOS and PFOA respectively, while for the rest of the compounds it ranged between 2-16% for the same spiked level (Tables 6.2 and 6.3). QC standards (one blank water sample and two spiked water samples at the concentrations of 0.5 ng mL^{-1} and 1 ng mL^{-1}) were analysed in every batch of samples, monitoring in this way the repeatability of the analytical method. The ion ratio of the relative response of the secondary mass transition to the primary mass transition and the retention time were recorded for each compound after every batch, in order to identify the analytes. The response of the instrument was also monitored by adding $^{13}C_8$-PFOS into the vial just before the injection. Investigation of blank samples was also performed during the development of the method and then in every sequence of water samples, in order to monitor background contamination originating from various sources in the laboratory. In none of the blank samples PFASs were detected.

LOD was determined as at least 3 times the signal to noise ratio and was set at 0.2 ng L^{-1} for all the compounds. LOQ was accordingly determined as 10 times the signal to noise ratio and was set at 0.6 ng L^{-1}. The recoveries ranged between 85-115% for all the mass-labelled compounds except for the $^{13}C_2$-PFUnA (60-80%).

Table 6.2: Repeatability of the detected concentrations of PFASs in spiked tap water samples – Intraday measurements.
(1 blank sample spiked with 5 different concentrations, 5 times for each concentration)

Repeatability										
	0.5 ng L^{-1} (n=5)		1 ng L^{-1} (n=5)		2 ng L^{-1} (n=5)		5 ng L^{-1} (n=5)		10 ng L^{-1} (n=5)	
PFASs	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**
PFPeA	0.61	3%	1.16	16%	3.12	2%	5.35	18%	11.2	16%
PFHxA	0.43	8%	1.05	16%	2.28	4%	5.16	12%	10.0	9%
PFHpA	0.44	3%	1.04	15%	2.52	8%	5.04	7%	9.9	9%
PFOA	0.54	4%	1.00	8%	2.18	10%	4.62	10%	9.5	8%
PFNA	0.43	3%	0.98	9%	2.12	5%	5.10	6%	10.3	6%
PFDA	0.45	5%	1.08	15%	2.18	7%	5.55	10%	10.2	6%
PFUnDA	0.44	5%	1.13	26%	2.11	8%	5.79	7%	10.9	8%
PFBuS	0.51	13%	0.93	9%	1.88	7%	4.86	10%	10.0	12%
PFHxS	0.46	8%	1.04	4%	2.32	6%	4.81	8%	10.3	10%
PFHpS	0.34	17%	0.89	16%	1.98	8%	4.69	8%	9.8	6%
PFOS	0.46	14%	0.93	16%	2.16	3%	4.64	10%	10.2	5%

Table 6.3: Reproducibility of the detected concentrations of PFASs in spiked tap water samples – Interday measurements.
(1 blank sample spiked with 5 different concentrations (5 times each day) in three different days)

Reproducibility										
	0.5 ng L^{-1}		1 ng L^{-1}		2 ng L^{-1}		5 ng L^{-1}		10 ng L^{-1}	
PFASs	**Average***	**RSD%****	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**	**Average**	**RSD%**
PFPeA	0.74	3-9%	1.30	10-17%	2.96	2-16%	6.11	7-18%	10.1	2-16%
PFHxA	0.48	8-17%	1.08	8-16%	2.21	4-6%	5.28	5-17%	10.3	2-9%
PFHpA	0.44	3-22%	1.02	6-15%	2.32	6-8%	5.03	5-13%	9.6	2-9%
PFOA	0.47	4-15%	0.92	8-10%	1.97	5-10%	4.31	6-18%	9.7	3-8%
PFNA	0.42	3-15%	0.98	9-12%	2.04	5-12%	5.18	6-8%	10.1	3-7%
PFDA	0.42	5-17%	1.11	11-15%	2.16	3-7%	5.59	5-11%	9.8	5-6%
PFUnDA	0.42	5-28%	1.10	12-26%	2.09	6-9%	5.62	5-18%	10.4	3-9%
PFBuS	0.48	10-13%	0.95	6-9%	1.82	3-9%	4.77	2-16%	9.9	3-12%
PFHxS	0.48	3-8%	1.00	2-8%	2.09	2-8%	4.81	4-12%	10.0	5-10%
PFHpS	0.43	5-17%	0.95	9-16%	1.96	2-8%	4.93	7-15%	9.2	2-6%
PFOS	0.19	4-14%	0.70	8-56%	1.71	3-14%	4.38	8-16%	9.8	3-5%

*Value calculated based on each day's average.

** The range of RSD% among the 3 days of analysis.

6.4 Results and discussion

Eleven PFASs were determined in 80 samples of tap water and 10 samples of bottled water from the Netherlands and Greece. The concentrations of PFASs were summed (lower bound principle). The respective dataset is included in Tables B1 and B2, where the upper bound sums are also presented. The ∑PFASs levels in the drinking tap water of both countries are presented in Table 6.4. The results have been divided into different categories depending on the detected levels.

Table 6.4: Concentrations (ng L^{-1}) of PFASs in drinking (tap) water from the Netherlands and Greece.

∑PFASs (ng L^{-1})	No. samples (n)	Minimum (ng L^{-1})	Maximum (ng L^{-1})	Average (ng L^{-1})	Median (ng L^{-1})
Netherlands					
<LOQ - 15	26	<LOQ	7.5	0.6	1.4*
15-30	8	17	30	25	25
30-55	3	31	54	39	32
Greece					
<LOQ - 2	38	<LOQ	0.8	0.1	0.8*
2-6	5	2.4	5.9	4.5	5.3

*The median values are calculated taking into account only the concentrations >LOQ (lower bound principle).

In 48.6% of the samples from the Netherlands, PFASs were detected above the LOQ (0.6 ng L^{-1}). This was also the case for 20.9% of the samples in Greece. A statistically significant difference was found between the levels of the two countries after the application of one-way ANOVA ($p<0.001$) (MATLAB). Moreover, statistical analysis was performed to each analyte individually, and it was found that there was a statistically significant difference in the short chain PFASs ($p<0.001$ for PFBS, PFHxS, PFHxA, PFHpA and $p<0.005$ for PFPeA and PFOA) concentrations between the Netherlands and Greece. These results

render the identification of the different sources of pollution in the two countries as a matter of interest.

The ∑PFASs in the Greek samples ranged between <LOQ to 5.9 ng L^{-1}, with the highest concentration noted in the sample of an Aegean island (Mykonos), while in the Dutch samples concentrations ranged from <LOQ to 54 ng L^{-1}, with the highest concentrations being detected in water from the area around the Amsterdam Schiphol airport and the cities of Amsterdam and Rotterdam (Tables B1 and B2).

On the other hand, no contamination was observed in the water samples collected from highly populated cities in Greece (Athens and Thessaloniki). The maximum PFASs concentrations in the Greek samples were detected in the tap water from three Aegean islands, Mykonos (∑PFASs: 5.9 ng L^{-1}), Kalymnos (∑PFASs: 4.9 ng L^{-1}) and Syros (∑PFASs: 3.6 ng L^{-1}) and also from one town in the Peloponnese, Tripoli (∑PFASs: 5.7 ng L^{-1}) (Figure 6.2). Drinking water from Syros is originally sea water, treated by a local desalination plant, while water from Mykonos and Kalymnos was supplied from water dams (surface water). In the Aegean islands there is no industrial activity, so the contamination can probably be attributed to human activities in general. As far as the water sample from Tripoli is concerned, it was supplied from spring water (groundwater) and the PFASs contamination may be attributed to agricultural usage of contaminated fertilizers (soil improver or sewage sludge) in this area or to leachate from landfills [18,196]. However, further investigation is needed before solid conclusions can be drawn on the causes, as other sources cannot be excluded.

Concerning the Dutch water samples, they fall into two distinct categories: samples collected from the western part of the Netherlands, originating from raw surface water (lakes and rivers), and samples from the eastern area sourced from groundwater. The tap water samples from the western part showed higher levels (∑PFASs: 21.4 - 30.9 ng L^{-1}) than the ones collected from the eastern part (∑PFASs: <LOQ – 7.5 ng L^{-1}). The samples with levels <LOQ shown in Figure 6.3 were all derived from the eastern part of the Netherlands. Figure 6.4 also shows clearly that these non-detect samples originate from locations where

groundwater is used for preparation of drinking water. The samples where PFASs were detected (>LOQ) all originated from areas where purified surface water is used as drinking water. Concerning the Greek tap water samples, 12% and 7% of the tap water samples sourced from groundwater and surface water respectively, showed detectable levels of PFASs. According to previous studies, PFASs have been detected in rivers (surface water) from different countries and in drinking water prepared from contaminated river water [196,197]. In the present study, the detected PFASs concentrations in the drinking water samples of the western part of the Netherlands are in agreement with previously reported concentrations in rivers flowing through the Netherlands [23,198,199]. All the tap water samples in the western part of the Netherlands showed similar levels of PFASs without large variations in both concentration and composition. This indicates that the source of contamination in this area is the same (surface water), but further investigation is needed to confirm this hypothesis.

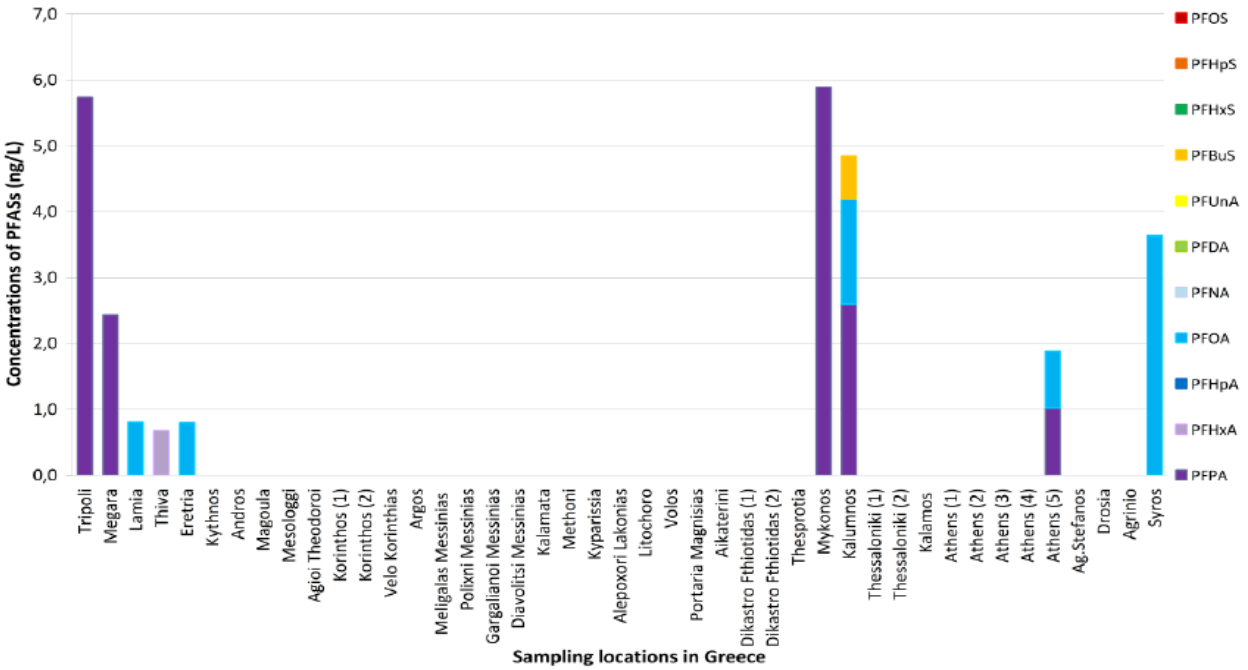


Figure 6.2: Concentrations of individual PFASs (ng L^{-1}) in the drinking (tap) water samples from Greece. In locations where no data are presented, all levels were below the LOQ.

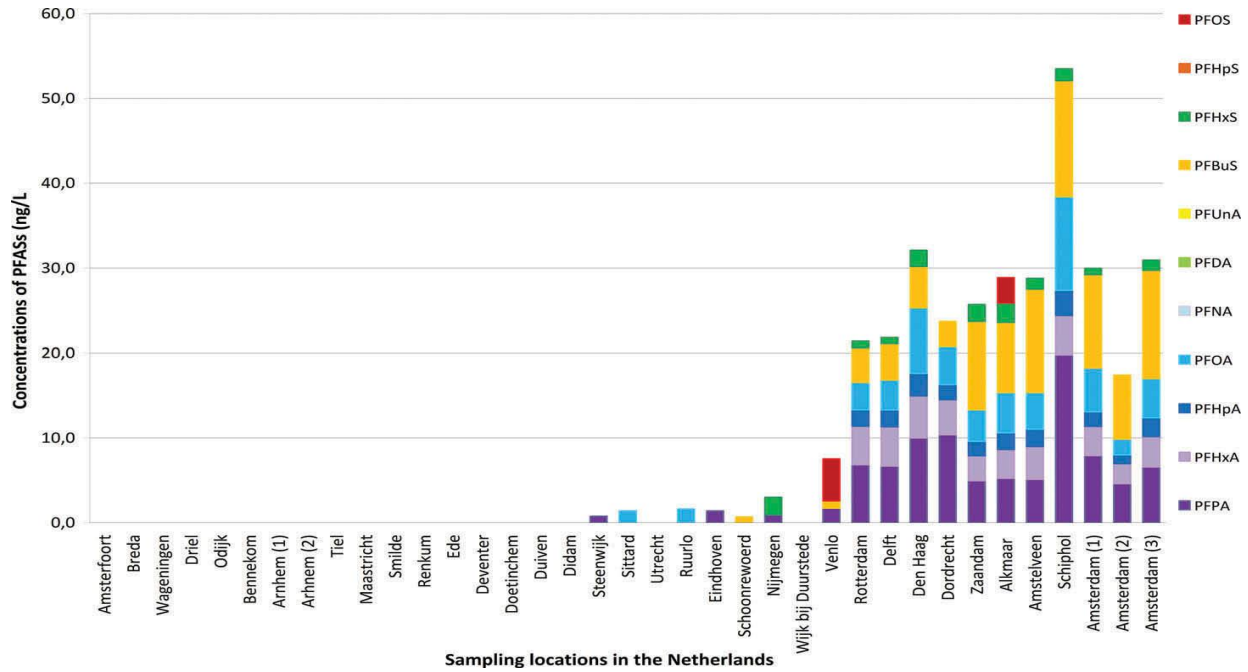


Figure 6.3: Concentrations of individual PFASs (ng L^{-1}) in the drinking (tap) water samples from the Netherlands. In locations where no data are presented, all levels were below the LOQ.

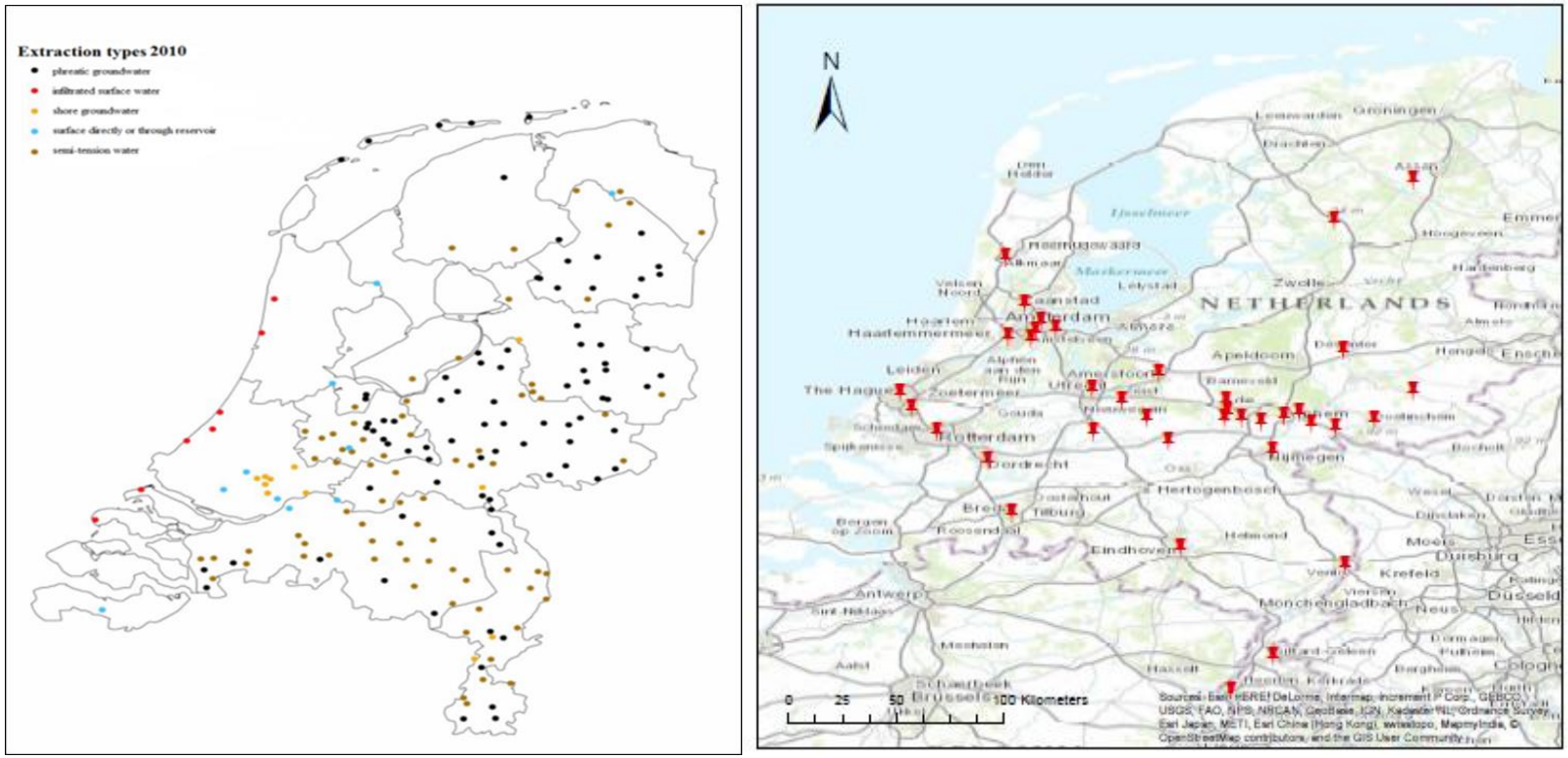


Figure 6.4: Groundwater and surface water supplying systems in the Netherlands (left panel). Contaminated and not contaminated drinking water sampling points in the Netherlands (right panel). Sampling points in red: detectable PFASs levels. Sampling points in green: PFASs concentration <LOQ.

(Rijksinstituut voor Volksgezondheid en Milieu, Geographic Information System (GIS)).

According to a comparison of the levels of PFASs in surface water samples, mainly in rivers of the European countries, it has been found that the contamination in Central Europe (Netherlands and Germany) is higher than in the rest of Europe [200]. Especially in the Netherlands there are some studies reporting high concentrations of PFASs (PFOA: 2-43 ng L^{-1} and PFOS: 4.7-33 ng L^{-1}) [199,201,202]. PFASs have been also detected, but in lower levels, in surface water from Spain (PFOA: 1.9-24.9 ng L^{-1} and PFOS: 1.59-5.88 ng L^{-1}) [203] and Poland (PFOA: 0.3-1.1 ng L^{-1} and PFOS: 0.24-19 ng L^{-1}) [204]. For surface water in Greece there are no monitoring data. However, there is a study conducted in two wastewater treatment plants in Greece (Mytilini and Athens), showing that PFASs, especially PFPeA, PFOA and PFOS, were detected in wastewater and sludge [18]. WWT plants have been already reported as a possible source of PFASs contamination in surface water [205,206].

In the present study, the short chain PFASs, particularly PFBS, PFHxS, PFPeA, PFHxA, PFHpA and PFOA, and in some cases PFOS, were detected more frequently, while the long-chain PFASs (C>8) were rarely detected (Tables B1 and B2, Figures 6.2 and 6.3). In comparison with previous studies on drinking water from European countries, the detected concentrations of PFASs are among the lowest levels observed (Figure 6.5). In particular, PFOS (average: 0.2 ng L^{-1}) and PFOA (average: 1.7 ng L^{-1}) in the drinking water from the Netherlands were detected at lower or almost equal concentrations compared to previous studies in the Netherlands (Amsterdam) [23,207], in Spain [49,103], Germany [196,208,209], Italy [197,207], Belgium [207], France [22], Sweden [207] and Norway [45,207]. In these previous studies the short-chain PFASs also dominated. Only the study from Germany [196] showed very high PFOA levels compared to the other studies, due to a local contamination from the use of contaminated soil improver on agricultural land.

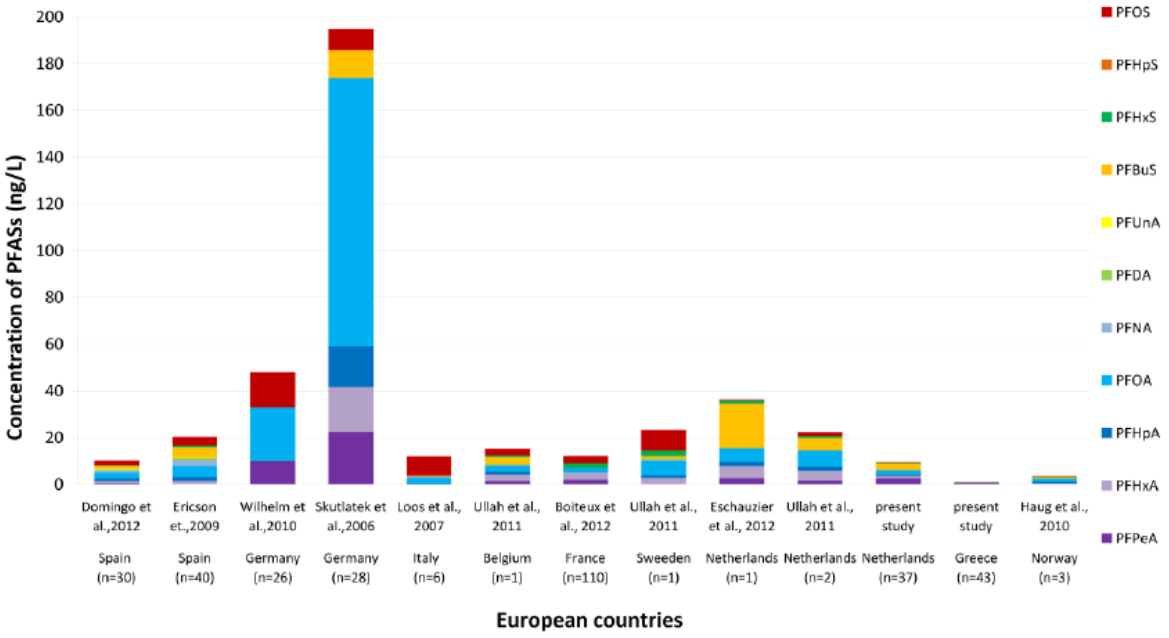


Figure 6.5: Overview of the detected concentrations of PFASs in drinking water in Europe.

The contamination levels of drinking water, apart from being influenced by the origin of the water (surface water or groundwater), may be affected by the applied water treatment procedure. However, till now, treatment methods, including ozonation, advanced oxidation and sand filtration, have been found inadequate in removing all PFASs [210]. On the other hand, reverse osmosis (RO) and nanofiltration (NF) have been reported as efficient techniques for the removal of PFASs with an alkyl chain longer than 5 carbons, but the application of membrane technology in water treatment is not so common because of the high costs and the problems of concentrate disposal [210].

Water filtration with granular activated carbon (GAC) filtration has been shown to be more effective for the removal of some PFASs, mainly the longer-chain ones (PFOS and PFOA average elimination efficiency is 64±11% and 45±19% respectively) [210,211] but not of the more hydrophilic short chain PFASs [23,209,212]. In particular, a previous study [23] on the impact of treatment processes during drinking water production from the Lek canal in the Netherlands, which is one of the sources of tap water in the western part of the

country, reported that sand filtration treatment didn't remove PFASs to an appreciable extent and ozonation didn't affect PFASs concentration. However, GAC filtration decreased the levels of the long-chain PFASs and thus the short-chain ones dominated, especially in the five finished water and in the one tap water (collected in Amsterdam) samples that were analysed. The observed PFASs concentrations and the pattern of contamination in the finished and tap water from this study are in agreement with the current results in tap water from the western part of the Netherlands, and also with PFASs concentrations in two tap water samples collected in Amsterdam [207]. To our knowledge, no data are available on drinking water treatment methods applied in Greece.

In the present study, a limited survey was performed on commercial bottled water. Five samples from each country were analysed in order to determine their PFASs levels. The ∑PFASs was below LOQ (0.6 ng L^{-1}) in all the samples. All the bottled water samples originated from ground wells (spring water), which may explain the absence of PFASs, as observed for water samples from the eastern part of the Netherlands (sourced from groundwater). The current results are in agreement with the study of Ericson et al., in 2008 [203], where 4 individual bottled mineral waters from Spain were tested and none of the 14 detected PFAS was found at levels >1 ng L^{-1}. Also, previous results of bottled mineral water from Japan and Poland, show that only ultratrace amounts of some PFASs were detected [204]. However, Gellrich et al. (2013) [208] tested 119 bottled mineral water samples, and in some of them PFASs were detected in relatively high levels (max PFBS: 13.3 ng L^{-1}). In another study, PFASs levels in bottled water from Thailand were found to be higher than those in the tap water [213]. The concentrations ranged from 0.22 to 10.55 ng L^{-1} with PFOA showing the highest concentration of all the PFASs. Therefore, further investigation is needed as the number of analysed samples is limited in the current study.

From a human exposure point of view, the currently found levels in drinking water from Greece and the Netherlands should be combined with levels of PFASs in food products in order to examine if they could cause a risk to human health, taking into account the TDIs established by EFSA (150 ng kg^{-1} b.w. for PFOS,

1500 ng kg^{-1} b.w. for PFOA) [4]. The current levels are even lower than the ones of a dietary exposure assessment, conducted in the Netherlands in 2011 [46] in which the exposure of Dutch consumers to PFOS and PFOA was examined, based on levels measured in various food products in the Netherlands, but levels for drinking water using data from EFSA. The applied values for the Dutch drinking water were 7 ng L^{-1} for PFOS and 9 ng L^{-1} for PFOA, which are higher than the current average concentrations of PFOS and PFOA of 4.0 ng L^{-1} and 4.4 ng L^{-1} respectively, based only on the contaminated samples. In the worst case scenario, where the concentrations of PFOS and PFOA were based only on the highest detected levels (western part of the Netherlands) (3.0 ng L^{-1} for PFOS and 4.9 ng L^{-1} for PFOA), the values were still lower than the ones used in Noorlander's study. In this study it was reported that the concentrations of 7 ng L^{-1} of PFOS and of 9 ng L^{-1} for PFOA contributed to 33% and 55% respectively of the combined PFOS and PFOA exposure from drinking water and food and that they did not cause a risk to human health. As a consequence the current detected PFOS and PFOA levels from the Netherlands contribute less to the total intake and do not imply a threat for human health. For Greece the exposure from drinking water seems even lower but this also depends on the consumption of water in comparison with the Netherlands. It should be noted that as EFSA has not derived a TDI for the short-chain PFASs, it is unknown what the consequences of the human exposure to these substances are.

6.5 Conclusions

The present study is the first study reporting levels of PFASs in Greek drinking water. It also provides missing data on PFASs levels in drinking water from the Netherlands, as a significant number of Dutch drinking water samples were analysed. According to the results, PFASs levels in the tap water from Greece were below the LOQs in most of the samples (79%). Considering the Dutch situation, tap water from the western region of the country has the highest levels of PFASs (∑PFASs: 21.4 - 30.9 ng L^{-1}), probably due to the origin of the water

(surface water) and to an inefficient removal from the raw drinking water. In the eastern part, drinking water is sourced from ground water, and almost no contamination was observed (∑PFASs: <LOQ – 7.5 ng L^{-1}). In the present study, five bottled water samples from each country were analysed, all of them sourced from ground wells. No PFASs were detected. The currently found PFOS and PFOA concentrations in drinking water from the two countries do not imply a risk for human health.

CHAPTER 7

Perfluoroalkylated substances in edible livers of farm animals, including depuration behaviour in young sheep fed with contaminated grass

7.1 Introduction

Dietary intake is considered as the main route of human exposure to PFASs. Thus, many studies focused on the detection of PFAS levels in food items during the last years. However, few studies provide data on PFAS levels in liver, either of wild animals or farm animals (Table 7.1). According to two EFSA reports, edible offal and especially liver are among the most contaminated food products, both in terms of frequency and mean levels [110,180]. According to these EFSA reports, PFAS levels were relatively high in edible offal (especially liver) from game animals, while the meat and offal from livestock animals was less contaminated. However, only a few studies provide data on PFAS levels in edible liver, either of wild or farm animals (Table 7.1). In addition, only a few animal studies have focused on the transfer of PFASs from contaminated feed in farm animals, in particular cows, sheep, broilers and pigs, and the consequences for food of animal origin, like milk and meat [214-218]. According to these studies, PFASs and especially PFOS accumulate in animal tissues and products. In particular, PFOS levels are higher in liver, followed by kidneys and muscles. In most of the cases its concentration decreased after exposure was stopped, which might be due to both excretion and further growth of the animals. In contrast to animal tissues, PFOS levels initially increase in blood/plasma during a depuration period. PFOS is also excreted via milk, but overall the elimination rate of PFOS has been reported as slow [214,216,217].

In terms of sources, the environment may be more important than compound feed, meaning that edible products from foraging animals may contain higher levels than those from animals raised inside. This was recently shown for eggs from private owners [29]. Among farm animals, sheep are the ones that spend in

general most time outside. However, the available information on kinetics of PFASs in sheep is very limited, as only one pilot study using three lactating sheep has been described [214].

In order to examine potential contamination of commercially available liver with PFASs, samples of different farm animal species were collected from local markets and slaughterhouses in the Netherlands. In addition samples were obtained from a study in which sheep were fed with grass harvested from a floodplain, initially aimed at obtaining more insight in the behaviour of polychlorinated dibenzo-*p*-dioxins and dibenzofurans and polychlorinated biphenyls (PCBs) [219]. Since the grass was also found to contain PFOS, the study enabled an investigation on the relationship between the intake of PFOS and its accumulation in the liver. To our knowledge this is the first study analysing such a large number (n=99) of liver samples from different farm animals, and also an extended study on PFOS kinetics in sheep.

7.2 Materials and methods

7.2.1 Sample collection

7.2.1.1 Liver samples from the market and slaughterhouses

In order to investigate potential PFASs contamination in commercially available liver, liver samples of different animal origin, including chicken, sheep, cow, pig and horse were purchased from local markets or obtained from slaughterhouses in the Netherlands in 2014. In order to avoid PFASs contamination of the sample, PFASs absorption and leaking of different plastic bottles were tested prior to the sampling process and in the end polypropylene bottles were chosen for the collection of all the samples. The plastic bottles were flushed three times with methanol and left to dry overnight before their use. After the collection, all the samples were transferred to the laboratory. Liver samples were homogenized and each sample was stored in a freezer (-20 °C) till the analysis.

7.2.1.2 Animal transfer study

Grass pellets, used in the sheep study, were previously shown to contain elevated levels of PCDD/Fs within the National Monitoring program on feed and feed ingredients in the Netherlands [219]. The grass was harvested on a floodplain of the river IJssel, where the soil was reported as contaminated with relatively high levels of PCDD/Fs. In the current study the samples were also analysed for PFASs. Part of the contaminated grass pellets, as well as clean grass pellets were purchased from the commercial grass dryer and shipped to the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, for the animal transfer study. Straw used in the study was bought by BfR from a local provider.

The details on the animal transfer study can be found elsewhere [219]. In short, young blackhead sheep were purchased by BfR and transferred to animal facilities. The animal experiment was authorized by the Landesamt für Gesundheit und Soziales in Berlin with approval G0030/12, complying with the German Animal Welfare Act (Tierschutzgesetz) and supervised by the BfR institutional animal welfare officer. During the first period all the sheep were fed with clean grass pellets, while later most of the sheep received the contaminated grass pellets, starting with about 0.6 kg and increasing to 1 kg per day at the end of the up to 113 days exposure period. However, most of the sheep, after 56 days of feeding with contaminated grass pellets, were switched to clean grass pellets. Apart from grass pellets, sheep also consumed part of the straw that was used as bedding in the cages. Also some control animals, receiving clean grass, were included. During the period of this study, sheep nearly doubled their body weight from about 24 kg to 40 kg, while liver weight was slightly increased. Animals from the control group were slaughtered at day 56 and 112. From the animals fed with contaminated feed, 4 animals were slaughtered after 8, 17, 29, 56 and 113 days, in order to collect their liver and other tissues. In addition, from the animals switched to clean grass at day 56, this was performed at day 8, 15, 36 and 57 thereafter. Unfortunately, not all liver samples were still available for PFASs analysis.

7.2.2 Chemicals

In the current study 11 PFASs: PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFBuS, PFHxS, PFHpS and PFOS were quantified by LC-MS/MS and isotope dilution method. Native PFSA solution/mixture (PFS-MXA), native PFCA solution/mixture (PFC-MXA), mass-labelled internal PFCAs and PFSAs solution/mixture (MPFAC-MXA) and a $^{13}C_8$-PFOS solution were purchased from Wellington laboratories (Guelp, Ontario, Canada).

Acetonitrile (Ultra LC-MS grade), methanol (Ultra LC-MS grade), petroleum ether 40-60 ^{o}C, and HPLC water (Ultra LC-MS grade) were purchased from Actu-All chemicals (Oss, Netherlands). Ammonium formate (≥99%) (Sigma, Switzerland), sodium hydroxide (Sigma, Sweden), and Florisil® (Sigma, St. Louis, USA) were all provided by Sigma. Aluminium oxide, sodium sulphate and ammonium solution 25% were purchased from Merck (Darmstadt, Germany).

7.2.3 Sample preparation

7.2.3.1 Liver samples

An amount of 1 g of each homogenized sample was fortified with 25 µL of mass-labelled PFACs and PFSAs solution/mixture (MPFAC-MXA) of 100 ng mL^{-1}. The extraction step was performed manually by adding 10 mL of acetonitrile (ACN) to each sample. Each tube was vigorously shaken for 30 min at 250 rpm and then centrifuged for 10 min at 3600 rpm. In order to remove the insoluble particles totally, centrifugation was performed followed by a filtration step. The extract was then evaporated till dryness and reconstituted in 3 mL of petroleum ether and brought to the top of a glass column filled with 1.5 g Florisil 0.5 w/w, 1 g basic alumina and 1 g of sodium sulphate. The column was first conditioned with 5 mL MeOH and 5 mL petroleum ether. After the conditioning the sample was added and the column was washed with 10 mL petroleum ether and 8 mL of MeOH/petroleum ether mixture (10:90 v/v). The final step was the elution of PFASs from the glass column with 8 mL of MeOH. The eluted fraction was evaporated till dryness in a flash evaporator. The dry residue was finally re-

dissolved in 775 µL of 2 mM ammonium formate in MeOH and 200 µL MeOH. Before the injection, 25 µL of a $^{13}C_8$-PFOS solution of 100 ng mL^{-1} were also added for monitoring the run to run MS response. The final solution was transferred into a vial for analysis by LC-MS/MS.

7.2.3.2 Grass samples

The grass pellet samples were analysed according to a previous published method [29]. Briefly, 1 g of each sample was fortified with 25 µL of mass-labelled PFCAs and PFSAs solution/mixture (MPFAC-MXA) of 100 ng mL^{-1} and 2 mL of 200 mM sodium hydroxide were added. 10 mL of MeOH were used as extraction solvent. The solution was vortexed for 1 min, shaken for 30 min at 250 rpm and then 150 µL HCl 4M were added. The extract was centrifuged for 10 min at 10,000 rpm and the supernatant was transferred to a new tube with 25 mL of milli-Q water. Clean-up was performed by SPE using weak anion exchange Oasis WAX cartridges and PFASs were eluted from the cartridge with 2 mL of 2% NH_4OH in ACN. The collected extract was evaporated till dryness under a gentle stream of N_2. The dry residue was dissolved in 775 µL of 2 mM ammonium formate in water and 200 µL of MeOH. Before the injection, 25 µL of $^{13}C_8$-PFOS solution 100 ng mL^{-1} were added and the final solution was transferred into a vial for analysis by LC-MS/MS.

7.2.4 Instrumental analysis

In the present study, quantification was performed by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). Every sample (20 µL) was injected in a Shimadzu LC system with a Fluorosep analytical column (50 mm * 2.1 mm, 5 µm, Waters). The chromatographic gradient was starting from 80% 2 mM ammonium formate in water (A) to 95% MeOH (B) in 10 min and each chromatographic separation lasted 15 min. The oven temperature of the analytical column was set at 35 °C and the flow rate at 0.3 mL min^{-1}.

The LC system was connected to a triple quadrupole MS (AB SCIEX QTRAP 5500 SYSTEM, Applied Biosystems - Analytical Technologies) equipped with a

Turbo Spray source operating in negative mode. Analysis was performed with a multiple reaction monitoring (MRM) method and two mass transitions (parent ion/product ion) were monitored for each analyte (Table 7.2). The source temperature was set at 350 °C and the ion spray voltage at -4500 V.

7.2.5 Quantification and quality assurance

The two analytical protocols were validated for repeatability, reproducibility, specificity, recovery and sensitivity. 11 PFASs were measured by applying isotope dilution method. Eight mass-labelled compounds ($^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS) were used as internal standards. For the native compounds with no corresponding mass-labelled compound, the one with closest resembling structure was used (Table 7.2). $^{13}C_8$-PFOS was also added in the vial just before the injection, in order to monitor the response of the instrument. The recoveries for all the mass-labelled compounds ranged between 60-115%, while the recovery of $^{13}C_8$-PFOS ranged from 90 to 120% in all the samples, verifying the sufficient ionisation of the compounds and the absence of matrix effects. For the quantification of PFAS in the liver and grass samples, calibration curves covering concentrations from 0.05 to 10 ng mL^{-1} (9 points including 0) were used and the r^2 was greater than 0.99 for all the calibration curves. The limit of detection (LOD) and the limit of quantification (LOQ) were determined as at least 3 and 10 times the signal to noise ratio respectively. The LOQ was set at 0.5 and 0.15 ng g^{-1} for the liver and feed samples respectively. Quality-control (QC) standards (one blank liver/grass sample and one spiked sample at concentration of 1 ng g^{-1}) were analysed in every batch of samples, controlling the repeatability of the analytical methods. For the identification of the analytes, both the ion ratio of the secondary mass transition response relative to the primary mass transition response and the retention time were recorded for each compound and every sample. During the development of the method, analysis of blank samples was also performed in order to monitor background contamination originating from

various sources in the laboratory. Blank samples were also included in every sequence of liver and grass samples. In none of the blank samples were PFASs detected.

7.3 Results and discussion

7.3.1 PFAS levels in liver samples from different farm animals

Concentrations of 11 PFASs were measured in 99 liver samples from different farm animal species, collected from various local markets and slaughterhouses in the Netherlands. Liver samples from chicken (n=20), sheep (n=18), pigs (n=20), cows (n=22) and horses (n=19) were collected and analysed.

A summary of the results is provided in Table 7.3 (see Table C1 for the individual sample results). PFOS was the only detected compound, while all other PFASs were <LOQ (0.5 ng g^{-1} ww). The PFOS concentrations ranged between <LOQ – 4.5 ng g^{-1} ww, with the highest concentrations and the highest frequency of detection in horse, sheep and cow liver samples. In fact, PFOS was not detected in chicken and pig liver in all but one sample of each animal origin (0.5 and 4.2 ng g^{-1} ww respectively). The results indicate that the liver samples can be divided in two distinct categories: livers from animals primarily raised indoors (chicken and pig) and from animals (sheep, cow, horse) spending most time outside. Apparently the exposure to PFOS is higher in the latter category, indicating that the environment is a more important source than compound feed.

Table 7.2: Instrumental mass spectrometry settings for the analytes.

Compound	Molecular formula	Retention time	Precursor ion (m/z)	Product ion 1 (m/z)	Collision energy (eV)	Product ion 2 (m/z)	Collision energy (eV)	Internal standard
PFHxA	$C_6F_{11}O_2^-$	3.17	313	269	14	119	24	$^{13}C_2$-PFHxA
PFHpA	$C_7F_{13}O_2^-$	3.97	363	319	12	169	24	$^{13}C_2$-PFHxA
PFOA	$C_8F_{15}O_2^-$	4.42	413	369	14	169	24	$^{13}C_4$-PFOA
PFNA	$C_9F_{17}O_2^-$	4.99	463	419	16	169	26	$^{13}C_5$-PFNA
PFDA	$C_{10}F_{19}O_2^-$	5.77	513	469	16	219	26	$^{13}C_2$-PFDA
PFUnA	$C_{11}F_{21}O_2^-$	6.68	563	519	14	319	24	$^{13}C_2$-PFUnA
PFDoA	$C_{12}F_{23}SO_3^-$	7.56	613	569	20	169	40	$^{13}C_2$-PFDoA
PFBuS	$C_4F_9SO_3^-$	1.45	299	99	40	80	75	$^{18}O_2$-PFHxS
PFHxS	$C_6F_{13}SO_3^-$	3.94	399	99	42	80	104	$^{18}O_2$-PFHxS
PFHpS	$C_7F_{15}SO_3^-$	4.42	449	99	96	80	102	$^{18}O_2$-PFHxS
PFOS	$C_8F_{17}SO_3^-$	4.32	499	99	94	80	100	$^{13}C_4$-PFOS
						169	48	
$^{13}C_2$-PFHxA	$^{13}C_2{}^{12}C_4F_{11}O_2^-$	4.78	315	270	14			
$^{13}C_4$-PFOA	$^{13}C_4{}^{12}C_4F_{15}O_2^-$	3.17	417	372	24			
$^{13}C_5$-PFNA	$^{13}C_5{}^{12}C_4F_{17}O_2^-$	4.42	468	423	16			
$^{13}C_2$-PFDA	$^{12}C_2{}^{12}C_8F_{19}O_2^-$	4.99	515	470	16			
$^{13}C_2$-PFUnA	$^{13}C_2{}^{12}C_9F_{21}O_2^-$	5.77	565	520	14			
$^{13}C_2$-PFDoA	$^{13}C_2{}^{12}C_{10}F_{23}SO_3^-$	7.56	615	570	20			
$^{18}O_2$-PFHxS	$C_6F_{13}S[^{18}O_2]O^-$	6.67	403	103	42			
$^{13}C_4$-PFOS	$^{13}C_4{}^{12}C_4F_{17}SO_3^-$	4.78	503	80	100			
$^{13}C_8$-PFOS	$^{13}C_8F_{17}SO_3^-$	4.78	507	99	76			

Table 7.3: Ranges and frequency of detection of PFOS in liver samples with different animal origin.

Animal origin	N	Frequency of PFOS detection (%)	Average (ng g^{-1} ww)	Median* (ng g^{-1} ww)	Range (ng g^{-1} ww)
Horse	19	89.5	1.5	1.4	<0.5 – 4.5
Sheep	18	77.8	1.5	1.4	<0.5 – 4.5
Bovine	22	41.0	0.4	0.0	<0.5 – 3.0
Pig	20	5.0	**	**	<0.5 – 4.2
Chicken	20	5.0	**	**	<0.5 – 0.5

*Median value is based on all the concentrations detected.

** In only 1 out of 20 samples PFOS was detected. The concentration in that sample is mentioned as the upper concentration of the range.

7.3.2 PFAS levels in sheep liver samples from a transfer study

Feed samples (n=23), including grass pellets prepared from grass collected on a floodplain of the river IJssel, batches of straw and non-contaminated (with PCDD/Fs) grass pellets were analysed for the presence of 11 PFASs. Among the three aforementioned feed categories, only the floodplain grass pellets were contaminated with PFASs; in fact, PFOS was the only detected compound. The concentration ranged between 0.4 to 0.7 ng g^{-1} in the various subsamples analysed (Table 7.4), showing that there was some fluctuation of PFASs contamination in the various subsamples. In the straw and clean grass samples, no PFASs were detected (LOQ: 0.15 ng g^{-1}).

The 36 remaining liver samples from the animal transfer study were analysed for the presence of 11 PFASs. PFOS was detected in all the liver samples, while the levels of the other PFASs were all below the LOQ (<0.5 ng g^{-1}). In particular, in the initial phase of the study (up to the start of the study at day 0), all sheep were only fed with clean grass. Then, they ate either only contaminated grass for 112 days (experimental group 1), or they started with contaminated feed for 56 days and then switched to clean grass for up to day 112 (experimental group 2). A third group was

fed only with clean grass (control group) during the whole period. There were no samples left from sheep slaughtered before the switch to contaminated grass (day 0). However, according to the levels in the other samples (Figure 7.1), average PFOS concentrations in these livers were probably around 2 ng g^{-1} ww. When sheep were fed with contaminated grass, PFOS average concentrations increased from about 2.4 at day 7 to 10.9 ng g^{-1} ww at day 112. The observed elevation in PFOS levels was positively correlated to the duration of the exposure to the contaminated grass but there was a clear leveling off after the first 4 weeks. When this exposure was stopped after 56 days (experimental group 2), a gradual decrease in PFOS concentrations from 9.2 to 4.7 ng g^{-1} ww after 63 and 112 days of feeding with non-contaminated grass respectively, was observed (Figure 7.1 and Table C2).

Table 7.4: Concentrations of PFOS (ng g^{-1}) in animal feed samples.

Animal feed	PFOS
Contaminated grass pellet	
1	0.7
2	0.6
3	0.6
4	0.5
5	0.4
6	0.5
7	0.6
8	0.6
9	0.4
10	0.7
11	0.6
12	0.5
13	0.5
14	0.6
15	0.6
16	0.5
Blank grass pellet	
1	<LOQ
2	<LOQ
Straw	
1	<LOQ
2	<LOQ
3	<LOQ
4	<LOQ

*Concentrations of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBuS, PFHxS, PFHpS were below LOQ (0.15 ng g^{-1}) in all the animal feed samples.

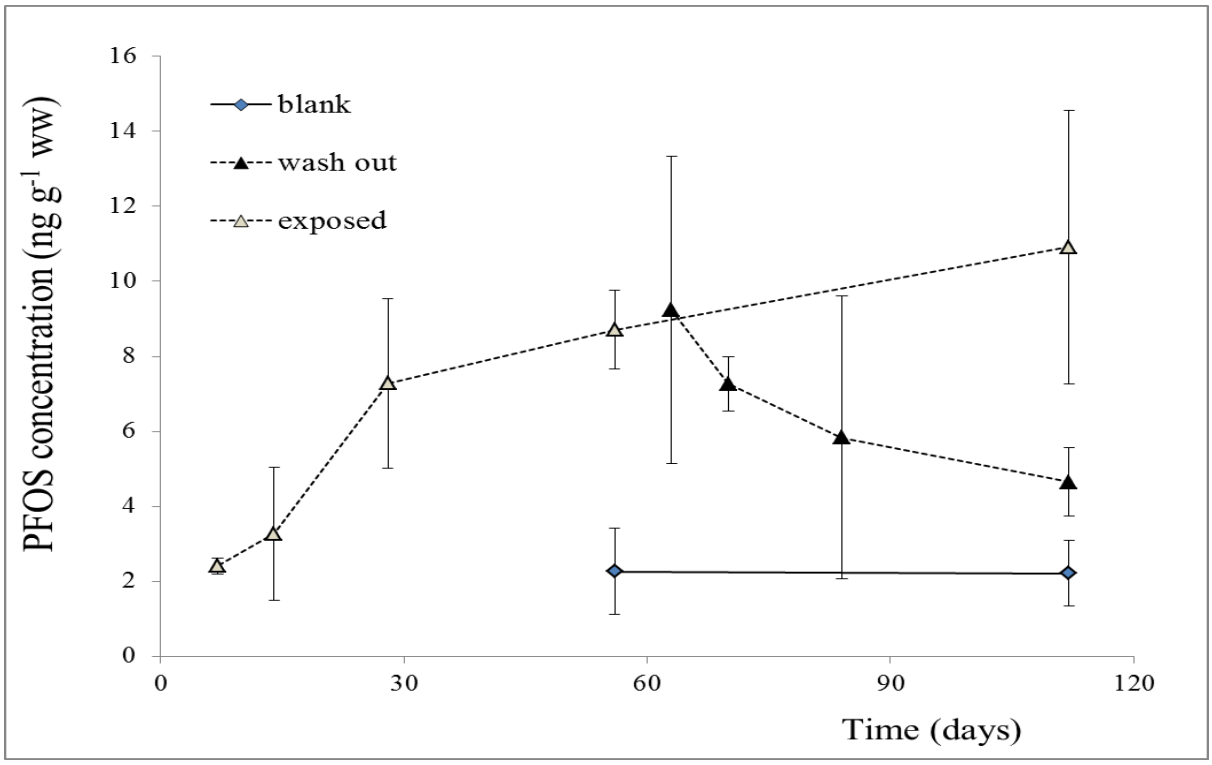


Figure 7.1: Levels of PFOS (average ± SD) in livers of sheep exposed for up to 112 days (white triangle), for 56 days followed by clean grass (black triangle), or only fed with non-contaminated grass (white diamonds).

7.3.3 Liver contamination in relation to foraging of animals

Considering the current data on PFASs in the grass pellet sample from the floodplain and increased levels in livers of sheep from the animal transfer study, it is clear that free ranging animals can be exposed to PFOS when foraging in certain areas. However, as also shown for PCDD/Fs, when they are fed with clean grass for several weeks prior to slaughter, contaminant levels can be reduced, approaching the levels of free ranging animals in a non-contaminated area. In fact, in the current study most PFOS concentrations in the sheep liver samples collected from markets in the Netherlands were even lower than the ones found in the control sheep livers from the animal transfer study. However, these findings imply that liver is susceptible to the animals' exposure to PFOS, which is ubiquitously present in the environment.

According to the present results for the liver samples collected from the Dutch market, it is also demonstrated that livers from free-ranging animals like sheep and horses are more contaminated compared to the ones normally fed with compound feed like chicken and pigs. This can probably be attributed to their intensive contact with the outside environment. These results are also in accordance with the previous study conducted in chicken eggs from private owners in the Netherlands [29], where soil intake was reported as the most likely source of PFASs contamination. Considering the absence of PFASs in livers from chicken in the present study and the absence of PFASs in commercially produced eggs reported in that study [29], it can be claimed that most farmed chickens are not exposed to PFASs and that compound feed seems not to be an actual source of these compounds. This may not apply to forage feed like grass and corn silage, harvested from contaminated soils.

Consequently, the living and eating habits of animals play an important role in their exposure to PFASs. Regarding the grazing behavior of sheep consuming potentially high amounts of soil [220], soil can also be considered as an important source of PFASs, as PFASs have been detected in soil samples in previous studies [221]. Although soil samples from the floodplain were not available for PFAS analysis in the present study, it was reported that the grass pellets contained a relatively high amount of soil. Therefore soil may be responsible for PFOS in the grass pellets used in this animal transfer study.

7.3.4 Comparison of PFAS levels in livers with previous animal studies

In the present animal transfer study, it is shown that PFASs and especially PFOS accumulate in the liver of animals when they are exposed to contaminated feed. This finding is in accordance with previous studies, conducted in lactating sheep, cows and chickens fed with contaminated feed, where PFASs were also detected in the liver [214,215,217]. In particular, in Kowalczyk's pilot study [214], PFOS showed marked accumulation in sheep tissue in an order of liver>kidney>muscle. The relative accumulation observed in the present study with young sheep is in agreement with that observed by Kowalczyk for the two lactating sheep.

Slightly different results were reported concerning PFOS levels in dairy cows fed with naturally contaminated corn silage, caused by the use of a highly contaminated soil improver. PFOS levels in liver from cows after 28 days of feeding were approximately nine times higher than in the feed. These levels are lower compared to those reported in the sheep animal studies. In particular, in the pilot study by Kowalczyk et al. (2012) and in the present study, PFOS concentrations in liver are approximately thirteen and fifteen times higher than the one detected in the feed respectively [214]. In addition, in the cow study, no decline in PFOS concentrations was observed in plasma and tissues of animals slaughtered after a period on clean feed. This seems in disagreement with the present study as a clear decrease of PFOS concentrations was observed in the wash-out phase of the study. The growth of the sheep during the study might explain this difference, but only to some extent since in the present study, liver weights increased only little, being about 0.44 ± 0.08 kg at day 0 (n=4), 0.47 ± 0.08 kg at day 56 (n=4) and 0.57 ± 0.04 kg at day 112 (n=12). The observed high constant PFOS concentrations in liver, kidney and muscles reported in Kowalcyk's study [215] were attributed to a possible PFOS release from non-examined tissues. Similarly, in a study with chickens [217], concentrations of PFOS in organs had not significantly changed, even after 4 weeks of depuration. Especially in liver the PFOS level was similar to the one in blood at the end of the depuration phase. It was hypothesized that either the mechanism of elimination of PFOS from liver is a slow process, or that blood-borne PFOS may be redistributed to the liver.

7.3.5 Comparison of PFAS levels in liver with previous food studies

The present results for livers collected from the Dutch market are in agreement with the EFSA 2011 report [180]. According to this report, PFASs concentrations are higher in edible offal from game animals compared to the ones from farmed animals. In particular, in edible offal from farmed animals, all PFAS concentrations were below 1 ng g^{-1}, except for PFOS and PFOA (range between 1 - 11 ng g^{-1} and 0.27 - 4.2 ng g^{-1} respectively), while PFOS concentrations in offal from game

animals ranged between 0.002 - 3480 ng g^{-1} while PFOA concentrations were between 0.5-161 ng g^{-1}.

PFOS concentrations in the present study showed similar or lower levels, compared to previous studies examining PFAS levels in liver samples from different animal origin (Table 7.1). In particular, Zhang et al. 2012 [178], reported PFOS concentrations ranging between 0.32 - 1.99 ng g^{-1}, with the maximum concentration being detected in liver from pork and the minimum in liver from chicken [179]. Ullah et al. (2012) detected PFOS in pig liver at a concentration of 0.182 ng g^{-1} [222]. Numata et al. showed in a transfer experiment that PFOS becomes more concentrated in the liver of pigs than all other tissues studied [218]. In a study conducted in Japan, higher levels of PFOS were detected in liver from chickens, pigs and cattle (67, 54 and 34 ng g^{-1} respectively) [118]. In previously published studies, mainly focused on PFAS levels in liver form wild animals, apart from PFOS also other PFASs compounds were detected (Table 7.1). However, in some of them the detected levels were lower than the LOQ of the present study.

Table 7.1: Overview of the detected concentrations (ng g^{-1}ww) of PFASs in liver samples from previous food studies.

Compounds	Country	Sample	Analytical method	LOQ	Results	Reference
10 PFASs	China	59 edible animal liver (pork, beef, chicken, duck, and goat liver)	extracted by ion-pair method Applied Biosystems API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS)	ng g^{-1}fresh weight: PFHxS: 0.21 ± 0.15 PFOS: 0.20 ± 0.14 PFDS: 0.19 ± 0.12 PFOSA:0.20 ± 0.15 PFHpA: 0.38 ± 0.23 PFOA: 0.25 ±0.13 PFNA: 0.26 ± 0.24 PFDA: 0.26 ± 0.21 PFUnDA:0.71 ±0.36 PFDoDA: 1.11±0.58	The highest mean concentration of PFOS was found in pork liver (1.99 ng g^{-1}). Total PFASs concentrations were >1 ng g^{-1} in all of the samples of liver (except for chicken liver).	[179]
10 PFASs	Japan	34 liver of chicken, cattle, pigs,goats and horses	Extraction with MTBE Agilent HP1100 liquid chromatograph interfaced with a Micromass_ (Beverly, MA, USA) Quattro Ultima Pt mass spectrometer. Betasil C18 analytical column (Thermo Hypersil-Keystone)	Ranging between 0.01 to 0.05 ng ml^{-1}	PFOS was the most prominant contaminant found in farm and pet animals. Chicken livers (67 ng g^{-1}) contained the highest mean PFOS concentration among the farm animals, followed by those of pigs (54 ng g^{-1}) and cattle (34 ng g^{-1}).	[118]

13 PFASs		Pig liver samples	Extraction with acetonitrile/ water and clean-up on a mixed-mode co-polymeric sorbent (C8+quaternary amine)	0.04 ng g^{-1}	PFOS was the predominant compound with detected concentration 0.182 ng g^{-1} PFHxA, PFOA, PFNA, PFDA, PFUnA and PFDoA were also detected but in concentrations lower than 0.04 ng g^{-1}	[222]
PFOS PFOA	Germany	592 liver samples of wild boar	Extraction with ACN and semineralised water. Add QuEChers mix. Clean up with: Oasis WAX Alliance 2695 separation module coupled to a Quattro-Micro tandem-mass spectrometer. Luna C18 HPLC analytical column (150 x 3 mm, 3 µm)	5 ng g^{-1}	PFOS: Range: <LOQ-1780 ng g^{-1} PFOA: Range : <LOQ-45 ng g^{-1}	[223]

11 PFASs	Germany	110 liver samples of roe deer	Extraction with ACN and dieionized water. Add QuEChers mix Clean up with: Oasis WAX Alliance 2695 separations module coupled to a Quattro-Micro tandem-mass spectrometer Luna C18 HPLC analytical column (150 x 3 mm, 3 µm)	0.5 ng g^{-1} for PFBA, PFHpA, PFDoDA, PFBS and PFHxS 0.2 ng g^{-1} for PFOA, PFOS, PFPeA, PFHxA, PFNA and PFDA	Mean (ng g^{-1}): PFBA: 0.7 PFPeA: <LOQ PFHxA: <LOQ PFHpA: <LOQ PFOA:0.7 PFNA: 1.3 PFDA: 0.4 PFDoDA: <LOQ PFBS: <LOQ PFHxS: <LOQ PFOS: 7	[224]

7.3.6 Potential exposure of consumers to PFASs from liver

PFOS was the only detected PFAS in all the liver samples analysed in this study. EFSA established a TDI for PFOS of 150 ng kg^{-1} b.w. per day. Assuming e.g. daily consumption of 100 gram liver by a person of 70 kg b.w., the highest observed level of 4.5 ng g^{-1} in commercial liver would result in an intake of 450 ng or 6.4 ng kg^{-1} b.w. per day, being 4% of the TDI. The exposure from the liver with the highest observed level in the sheep transfer study (15 ng g^{-1}) would be about 3-fold higher and still well below the TDI. Therefore, the potential risk from the PFOS levels observed in the present study seems clearly not a risk for human health even in the worst case scenario. It is unclear whether exposure of sheep foraging in floodplains can actually be higher than in the present animal transfer study, thus resulting in higher liver levels. However, based on the PFOS concentrations in the liver samples purchased from the market, it can be assumed that liver consumption will not cause adverse effects to human health because of PFASs, as the detected levels are very low and in most of the cases below the LOQ. In addition, for most consumers it seems unlikely that liver is consumed on a daily basis.

7.4 Conclusions

The present study shows that PFASs contamination of grass can cause elevated PFAS levels in livers of sheep. In the animal transfer study, PFOS was the only detected compound in both the grass and the sheep livers. PFOS concentrations in livers declined when the sheep were fed with grass free of PFOS, which only to some extent may be due to growth of the animal.

Commonly, in livers collected from the Dutch markets, only PFOS was detected. Livers from free ranging animals, like sheep, cows and horses were more contaminated than animals normally raised indoors like chicken and pigs. These findings demonstrate that the animals' contact with the environment, and the consumption of contaminated grass and soil, are the most likely sources of PFOS. However, overall PFOS levels in livers examined in the present study

seem rather low and even the levels in the sheep livers from the transfer study do not entail a risk for human health.

CHAPTER 8

Discussion

8.1 Summary of the thesis – general conclusions

PFASs are a class of emerging environmental pollutants that have gained scientific interest over the last decades, as their presence may pose a threat to the environment and human health. Since diet has been reported as the main route of human exposure to PFASs, research is directed towards the analysis of food matrices. Most of the recent available studies focus on PFASs detection in various food items and on human dietary intake and exposure to PFASs due to the consumption of these food products.

However, sound knowledge on PFAS behaviour and transport in the environment and therefore in the food chain is hampered by the insufficiency of available information. PFASs direct and indirect sources of contamination, food origin, way of food production, animals' exposure to PFASs that may also lead to contaminated food products are topics of major importance that are not yet well investigated.

In this context, in the present thesis, risk assessment of PFASs through their detection in various food matrices, food packaging materials and drinking water was made in an effort to provide new data and fill this gap in the literature.

To deal with the situation encountered, the current thesis focused on 5 individual studies that examine and evaluate PFASs presence in different food matrices, their ways of transfer into the environment and food chain, possible sources of contamination mainly of food products, and the human dietary intake due to the consumption of certain food items and drinking water.

In particular, novel data on PFASs concentrations in food products, including chicken eggs, edible fish and liver, in drinking water and in various food packaging materials were provided. PFASs were found in almost all the samples analysed, while the detected concentrations fluctuated in a wide range

depending on different factors each time. The detected compounds also depended mainly on the matrix analysed.

In particular, regarding the food packaging materials, several PFASs were detected in fast food wrappers, with the highest concentrations found in microwave popcorn bags. PFTrDA, PFTeDA and PFHxDA were the detected compounds in fast food boxes, while only PFHxA was found in ice cream cup samples. It is worth mentioning that the detected levels of PFASs in the food packages collected from Greece were very low compared to previous studies conducted in other countries, while PFOS and PFOA were not detected in any of the samples analysed. Concerning the fish samples analysed, PFASs were detected above the detection limit in all the raw samples, except for sardine, mussel and squid, and PFOS was the predominant compound in all the samples analysed. Similarly, in home produced chicken eggs, mostly the long chain PFASs were detected and PFOS was also the predominant compound detected, while in the commercially produced eggs no PFASs were detected in almost all the samples. In addition, PFOS was the only compound detected in liver samples of different animal origin.

Human exposure to PFASs due to the consumption of the aforementioned analysed food items was also investigated in the present study. According to the current results, it can be assumed that the consumption of the specific food products (including chicken eggs, fish and liver) and of the drinking water analysed cannot cause adverse effects to human health, as the calculated EDI for PFOS and PFOA was even in the worst case scenario less than the TDI values established by EFSA.

Both direct and indirect sources of food contamination with PFASs were other factors investigated in the present thesis. In an effort to examine and provide data on the indirect sources of PFASs contamination, food packaging materials were analysed, while different cooking processes were applied on edible fish in order to examine the impact of cooking on PFASs levels. According to the current results, low contamination of PFASs was detected in some of the packaging materials analysed, except for the microwave popcorn bag sample

where PFASs levels were high. On the other hand, the way of cooking was found to influence PFASs concentration in fish. Especially, grilling and frying were found to increase PFASs concentrations compared to raw samples.

Direct sources of PFASs contamination were also investigated in the current thesis mainly through the examination of the environmental exposure of animals to PFASs. In the same context, PFASs levels in home produced chicken eggs were compared to the detected concentrations in the commercially produced ones. In addition, liver from free range animals and livestock were collected and analysed in order to examine possible differences among PFASs levels due to animals' different way of living and eating. According to the findings, in both cases PFASs levels were found to be higher in the food products of the animals in intense contact with the open air, probably due to the intake of contaminated soil/grass and the ingestion of small organisms, like worms and insects. In particular, home produced eggs were contaminated with PFASs, and especially PFOS, while in the commercially produced ones no PFASs were detected in almost all the samples. Considering the free-range home-kept laying hens' living and eating habits, it can be assumed that this is the most likely source of eggs' contamination with PFASs. PFOS levels were higher in liver from free ranging animals, like sheep, cows and horses than in animals raised mainly indoors like chicken and pigs, indicating that the animals' intensive contact with the outside environment is the most likely source of PFOS.

In the present study, apart from the detection of PFASs levels in drinking water, it was also observed that there was a difference in PFASs contamination between two different regions of the Netherlands. This difference of PFASs levels could be probably attributed to the origin of the water, indicating that drinking water sourced from surface water is more contaminated than the one originated from ground water. This specific study not only provides data on possible sources of water contamination with PFASs, but also on PFASs way of transfer in the environment.

Furthermore, an animal study examining the kinetic behaviour of PFASs in foraging animals was conducted. Particularly, liver samples were obtained from a

study in which sheep were fed with either clean or contaminated grass. This study showed that even low-level contamination of grass and soil can lead to increased liver levels in foraging animals. It was also observed that PFOS concentrations in livers declined when the sheep were fed with grass free of PFOS.

Taking in consideration all the aforementioned results of the risk assessment made in the present thesis, it should be emphasized that the detected PFASs levels do not imply a risk for human health. However, the available information is still limited and further research in various directions on this field is needed, in order to draw more valid conclusions regarding PFASs presence and their effects in the environment and in human health.

8.2 Recommendations for future research

Future work in the field of PFASs calls for research in various directions, embracing environment, food safety and human health.

- Research on PFASs presence in food products is expected to continue and expand in the future, since scientific interest is oriented towards this direction. In this context, the analysis of a significant number of samples of each food category is necessary, since till now most of the food studies provide data deriving from the analysis of few individual samples.
- Regarding to indirect sources of food contamination with PFASs, further research on PFASs migration from food packaging materials to the food product is recommended. Besides the analysis of food before packaging, it would also be worthwhile analysing packaged food items. This could provide novel data in human exposure to PFASs through their diet.
- The investigation of a possible correlation between humans' exposure to PFASs and their way of living is also recommended. In this context, living areas' characteristics (industrial, urban and rural) and the working environment along with specific food products consumed, commercial

products used, altogether constitute factors that have to be taken under consideration in order to obtain a more solid conclusion.

- EFSA has already established TDIs for the two most frequently detected PFASs, PFOS and PFOA. However, TDIs regarding other PFASs that have been used during the last decades as alternatives of PFOS and PFOA by the manufactures have not yet been established, even though these compounds have been detected in various matrices, sometimes even in higher concentrations compared to PFOS and PFOA. Consequently, the establishment of additional TDIs seems necessary and is recommended.

ABBREVIATIONS

PFCs	Perfluorinated compounds
PFASs	Perfluoroalkylated substances
PFAAs	Perfluoroalkyl acids
PASFs	Perfluoroalkane sulfonyl fluorides
FASAs	Perfluoroalkane sulfonamides
PAFs	Perfluoroalkynoyl fluorides
PFAIs	Perfluoroalkyl iodides
PFALs	Perfluoroalkyl aldehydes
PFAL-H_2Os	Perfluoroalkyl aldehyde hydrates
PFACs	Perfluoroalkyl carboxylic acids
PFSAs	Perfluoroalkyl sulfonic acids
PFBA	Perfluorobutanoic acid
PFPeA	Perfluoropentanoic acid
PFHxA	Perfluorohexanoic acid
PFHpA	Perfluoroheptanoic acid
PFOA	Perfluorooctanoic acid
PFNA	Perfluorononanoic acid
PFDA	Perfluorodecanoic acid
PFUnDA	Perfluoroundecanoic acid
PFDoA	Perfluorododecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFTeDA	Perfluotetradecanoic acid
PFHxDA	Perfluorohexadecanoic acid
PFODA	Perfluorooctadecanoic acid
PFBuS	Perfluorobutane sulfonate
PFHxS	Perfluorohexane sulfonate
PFHpS	Perfluoroheptane sulfonate
PFOS	Perfluorooctane sulfonate
PFDS	Perfluorodecane sulfonate
LRAT	Long range atmospheric transport
ECF	Electro-Chemical Fluorination
TM	Telomerisation
PFTEE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride

AFFFs	Aqueous film-forming foams
WWTP	Waste water treatment plants
N-EtFOSE	N-Ethyl Perfluorooctane sulfonamido ethanol
N-MeFOSE	N-methyl perfluorooctane sulfonamido ethanol
POPs	Persistant organic pollutants
ECHA	European chemical agency
APFO	Ammonium pentadecafluorooctanoate
L-FABR	Liver fatty acid-binding protein
EFSA	European Food Safety Authority
TDI	Tolerable daily intake
EU	European Union
EPA	European Protection Agency
LE	Liquid extraction
ME	Matrix effect
PLE	Pressurized liquid extraction
ASE	Accelerator solvent extraction
MTBE	Methyl tert-butyl ether
SPE	Solid phase extraction
ACN	Acetonitrile
LC	Liquid chromatography
MS	Mass spectrometry
QqQ	Triple quadrupole
ESI	Electrospray ionization
IT	Ion trap
QqLIT	Quadrupole linear ion trap
APPI	Atmosphere pressure photoionization
Q-TOF	Quadrupole time of flight
HRMS	High resolution mass spectrometry
TDCA	Taurodeoxycholic acid
LOD	Limit of detection
LOQ	Limit of qualification
RT	Retention time
RSD	Relative standard deviation
E	Error
PTFE	Polytetrafluoroethylene

MRM	Multiplr reaction monitoring
VOO	Virgin olive oil
FIR	Food intake rate
ABW	Average body weight
EDI	Estimated daily intake
EQS	Environmental quality standard
TDS	Total diet study
WWTPs	Waste water treatment plants
QC	Quality control standard
RO	Reverse osmosis
NF	Nanofiltration
GAC	Granual activated carbon
PCDD/Fs	Polychlorinated dibenzo-p-dioxins and dibenzofurans
PCBs	polychlorinated biphenyls

APPENDIX A

Supplementary data to chapter 5:
Perfluoroalkylated substances (PFASs) in home and commercially produced chicken eggs from the Netherlands and Greece.

Table A1: Concentrations of individual PFASs (ng g^{-1} ww) in egg yolk samples from Greece.

Concentration (ng g^{-1} ww)	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFBuS	PFHxS	PFHpS	PFOS	∑PFAS (lower bound)	∑PFAS (upper bound)
Home producers												
1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
3	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
4	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
6	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
7	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
9	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
10	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
11	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
12	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
13	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	**0.5**	**5.0**
14	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	**0.5**	**5.0**
15	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	**0.5**	**5.0**
16	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6	**0.6**	**5.1**
17	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6	**0.6**	**5.1**
18	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6	**0.6**	**5.1**
19	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.7	**0.7**	**5.2**

20	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.8	**0.8**	**5.3**
21	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.8	**0.8**	**5.3**
22	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.8	**0.8**	**5.3**
23	<0.5	<0.5	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	0.5	**1.0**	**5.0**
24	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
25	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.3	**1.3**	**5.8**
26	<0.5	<0.5	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.5**	**5.5**
27	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.5	**1.5**	**6.0**
28	<0.5	<0.5	<0.5	<0.5	0.9	0.6	<0.5	<0.5	<0.5	<0.5	**1.5**	**5.5**
29	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.8	**1.8**	**6.3**
30	<0.5	<0.5	<0.5	0.5	0.5	<0.5	<0.5	<0.5	<0.5	0.8	**1.9**	**5.3**
31	<0.5	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.5	**2.0**	**6.0**
32	<0.5	<0.5	<0.5	<0.5	1.6	0.6	<0.5	<0.5	<0.5	<0.5	**2.2**	**6.2**
33	<0.5	<0.5	<0.5	<0.5	0.6	0.5	<0.5	<0.5	<0.5	1.7	**2.9**	**6.4**
34	<0.5	<0.5	<0.5	0.7	0.5	0.6	<0.5	<0.5	<0.5	1.1	**2.9**	**5.7**
35	<0.5	<0.5	<0.5	0.5	0.6	<0.5	<0.5	<0.5	<0.5	1.9	**2.9**	**6.4**
36	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	3.1	**3.1**	**7.6**
37	<0.5	<0.5	<0.5	<0.5	0.8	0.8	<0.5	<0.5	<0.5	2.3	**3.9**	**7.4**
38	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.2	**4.2**	**8.7**
39	<0.5	<0.5	<0.5	0.9	1.4	0.7	<0.5	<0.5	<0.5	1.6	**4.6**	**7.6**
40	<0.5	<0.5	<0.5	1.0	1.7	0.7	<0.5	<0.5	<0.5	2.4	**5.8**	**8.8**
41	<0.5	<0.5	<0.5	<0.5	1.0	<0.5	<0.5	<0.5	<0.5	5.5	**6.5**	**10.5**
42	<0.5	<0.5	<0.5	<0.5	1.3	1.9	<0.5	<0.5	<0.5	6.7	**9.8**	**13.3**
43	<0.5	<0.5	<0.5	0.9	1.3	1.1	<0.5	<0.5	<0.5	6.8	**10.1**	**13.1**
44	<0.5	<0.5	<0.5	0.8	2.0	1.1	<0.5	<0.5	<0.5	8.9	**12.8**	**15.8**

45	<0.5	<0.5	0.5	1.0	8.0	4.5	<0.5	<0.5	<0.5	0.9	**15.0**	**17.5**
Average (lower bound)	***<0.5***	***<0.5***	***<0.5***	***0.2***	***0.5***	***0.3***	***<0.5***	***<0.5***	***<0.5***	***1.4***		
Average (upper bound)	***0.5***	***0.5***	***0.5***	***0.6***	***0.8***	***0.7***	***0.5***	***0.5***	***0.5***	***1.6***		
Super market (commercial farms)												
1 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
2 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
3 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
4 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
5 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
6 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
7 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
8 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
9 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
10 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
11 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
12 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
13 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
14 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
15 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
16 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
17 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**

18 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
19 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
20 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
21 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
22 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
23 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
24 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
25 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
26 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
27 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
28 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
29 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
30 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.9	<0.5	**0.9**	**5.4**
31 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**

Table A2: Concentrations of individual PFASs (ng g^{-1} ww) in egg yolk samples from the Netherlands.

Concentration (ng g^{-1} ww)	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFBuS	PFHxS	PFHpS	PFOS	∑PFAS (lower bound)	∑PFAS (upper bound)
Home producers												
1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
3	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
4	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
6	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
7	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
9	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
10	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
11	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
12	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
13	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**
14	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**0.0**	**5.0**

15	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.5	**0.5**	**5.0**
16	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6	**0.6**	**5.1**
17	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.8	**0.8**	**5.3**
18	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.9	**0.9**	**5.4**
19	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.9	**0.9**	**5.4**
20	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
21	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
22	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
23	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
24	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.0	**1.0**	**5.5**
25	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.1	**1.1**	**5.6**
26	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.1	**1.1**	**5.6**
27	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.2	**1.2**	**5.7**
28	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.2	**1.2**	**5.7**
29	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.2	**1.2**	**5.7**
30	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.4	**1.4**	**5.9**
31	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.0	**2.0**	**6.5**
32	<0.5	<0.5	1.0	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.2	**2.2**	**6.2**
33	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.4	**2.4**	**6.9**

34	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.5	**2.5**	**7.0**
35	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.5	**2.9**	**6.9**
36	<0.5	<0.5	<0.5	0.6	0.9	<0.5	<0.5	<0.5	<0.5	1.4	**2.9**	**6.4**
37	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	3.1	**3.1**	**7.6**
38	<0.5	<0.5	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	3.7	**4.2**	**8.2**
39	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.2	**4.2**	**8.7**
40	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.2	**4.2**	**8.7**
41	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.3	**4.3**	**8.8**
42	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.4	**4.4**	**8.9**
43	<0.5	<0.5	1.7	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2.7	**4.4**	**8.4**
44	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.4	**4.4**	**8.9**
45	<0.5	<0.5	0.6	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	3.3	**4.4**	**7.9**
46	<0.5	<0.5	0.6	<0.5	0.6	<0.5	<0.5	<0.5	<0.5	3.5	**4.7**	**8.2**
47	<0.5	<0.5	1.1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	3.8	**4.9**	**8.9**
48	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.9	**4.9**	**9.4**
49	<0.5	<0.5	1.7	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	3.2	**4.9**	**8.9**
50	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4.9	**4.9**	**9.4**
51	<0.5	<0.5	<0.5	<0.5	0.7	0.8	<0.5	<0.5	<0.5	3.4	**5.0**	**8.5**
52	<0.5	<0.5	<0.5	<0.5	1.0	0.9	<0.5	<0.5	<0.5	3.5	**5.5**	**9.0**

53	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	5.5	**5.5**	**10.0**
54	<0.5	<0.5	<0.5	0.5	0.8	<0.5	<0.5	<0.5	<0.5	4.1	**5.5**	**9.0**
55	<0.5	<0.5	0.7	<0.5	0.7	0.5	<0.5	<0.5	<0.5	3.6	**5.5**	**8.5**
56	<0.5	<0.5	0.5	<0.5	0.8	<0.5	<0.5	<0.5	<0.5	4.5	**5.8**	**9.3**
57	<0.5	<0.5	<0.5	<0.5	1.1	0.9	<0.5	<0.5	<0.5	3.8	**5.9**	**9.4**
58	<0.5	<0.5	<0.5	0.6	<0.5	<0.5	<0.5	<0.5	<0.5	5.4	**6.1**	**10.1**
59	<0.5	<0.5	<0.5	<0.5	0.6	<0.5	<0.5	<0.5	<0.5	5.8	**6.4**	**10.4**
60	<0.5	<0.5	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	6.1	**6.6**	**10.6**
61	<0.5	<0.5	<0.5	0.8	0.6	0.8	<0.5	0.6	<0.5	4.2	**6.9**	**9.4**
62	<0.5	<0.5	0.9	<0.5	0.8	<0.5	<0.5	<0.5	<0.5	5.3	**7.0**	**10.5**
63	<0.5	<0.5	<0.5	0.5	1.1	0.5	<0.5	<0.5	<0.5	5.3	**7.4**	**10.4**
64	<0.5	<0.5	<0.5	0.6	1.2	1.6	<0.5	<0.5	<0.5	5.7	**9.1**	**12.1**
65	<0.5	<0.5	2.7	1.8	2.0	1.3	<0.5	<0.5	<0.5	1.7	**9.6**	**12.1**
66	<0.5	<0.5	1.2	0.9	1.7	0.7	<0.5	0.8	<0.5	7.6	**12.9**	**14.9**
67	<0.5	<0.5	0.7	<0.5	<0.5	0.5	<0.5	1.1	<0.5	13.8	**16.0**	**19.0**
68	<0.5	<0.5	0.8	<0.5	0.4	<0.5	<0.5	5.2	<0.5	11.0	**17.4**	**20.4**
69	<0.5	<0.5	1.4	2.0	3.0	2.3	<0.5	<0.5	<0.5	10.4	**19.0**	**21.5**
70	<0.5	<0.5	1.5	1.0	0.9	0.7	<0.5	1.1	<0.5	13.9	**19.2**	**21.2**
71	<0.5	<0.5	2.5	1.4	1.5	1.6	<0.5	<0.5	<0.5	12.2	**19.3**	**21.8**

72	<0.5	<0.5	2.3	1.3	1.7	1.5	<0.5	<0.5	<0.5	21.3	**28.1**	**30.6**
73	<0.5	<0.5	1.5	1.5	1.8	1.5	<0.5	<0.5	<0.5	24.8	**31.2**	**33.7**
Average (lower bound)	***<0.5***	***<0.5***	***0.3***	***0.2***	***0.3***	***0.2***	***<0.5***	***0.1***	***<0.5***	***3.6***		
Average (upper bound)	***0.5***	***0.5***	***0.7***	***0.6***	***0.7***	***0.6***	***0.5***	***0.6***	***0.5***	***3.7***		
Super market (commercial farms)												
1 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
2 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
3 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
4 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
5 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
6 organic	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
7 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
8 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
9 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
10 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
11 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
12 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**

13 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
14 battery	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
15 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
16 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
17 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
18 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
19 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
20 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.1	**1.1**	**5.6**
21 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**
22 free range	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	**<0.5**	**5.0**

APPENDIX B

Supplementary data to chapter 6:

Determination of Perfluoroalkylated substances (PFASs) in drinking water from the Netherlands and Greece.

Table B1: Concentrations of individual PFASs (ng L^{-1}) in drinking (tap) water samples from Greece.

Location	Origin of the water	PFPA	PFBuS	PFHxA	PFHpA	PFHxS	PFOA	PFHpS	PFNA	PFOS	PFDA	PFUnDA	Sum of PFASs (lower bound)	Sum of PFASs (upper bound)
Syros	Seawater desalination	<0.6	<0.6	<0.6	<0.6	<0.6	3.63	<0.6	<0.6	<0.6	<0.6	<0.6	**3.6**	**9.6**
Mykonos	Surface water (lake Marathos)	5.9	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**5.9**	**11.9**
Kalymnos	Surface water	2.6	0.7	<0.6	<0.6	<0.6	1.6	<0.6	<0.6	<0.6	<0.6	<0.6	**4.9**	**9.6**
Eretria	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	0.8	<0.6	<0.6	<0.6	<0.6	<0.6	**0.8**	**6.8**
Kythnos	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Andros	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Kalamos	Surface water (lake Marathonas)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Athens (1) (Gyzi)	Surface water (lake Marathonas)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Athina (2) (Zografou)	Surface water (lake Marthonas)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Athens (3)	Surface water	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**

(Ag.Paraskevi)	(Lake Marathonas)													
Athens (4) (Peyki)	Surface water (lake Marathonas)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Athens (5) (Egaleo)	Surface water (lake Marathonas)	1.0	<0.6	<0.6	<0.6	<0.6	0.9	<0.6	<0.6	<0.6	<0.6	<0.6	**1.9**	**7.3**
Ag.Stefanos	Surface water (lake Yliki)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Drosia	Surface water (lake Yliki)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Magoula	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Megara	Groundwater	2.4	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**2.4**	**8.4**
Agrinio	Surface water (river Acheloos)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Mesologgi	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Thiva	Groundwater	<0.6	<0.6	0.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.7**	**6.7**
Agioi Theodoroi	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Korinthos (1)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Korinthos (2)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Velo Korinthias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**

Argos	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Meligalas Messinias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Polixni Messinias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Gargalianoi Messinias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Diavolitsi Messinias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Kalamata	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Methoni	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Kyparissia	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Alepoxori Lakonias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Tripoli	Groundwater	5.7	<0.6	<0.6.	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**5.7**	**11.7**
Litochoro	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Volos	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Portaria Magnisias	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Aikaterini	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Lamia	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	0.8	<0.6	<0.6	<0.6	<0.6	<0.6	**0.8**	**6.8**
Dikastro Fthiotidas (1)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**

Dikastro Fthiotidas (2) (Veli)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Thesprotia	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Thessaloniki (1)	Surface water	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Thessaloniki (2) (Ampelokipoi)	Surface water	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Average (lower bound)		**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**		
Average (upper bound)		**0.9**	**06**	**0.6**	**0.6**	**0.6**	**0.7**	**0.6**	**0.6**	**0.6**	**0.6**	**0.6**		

Table B2: Concentrations of individual PFASs (ng L^{-1}) in drinking (tap) water samples from the Netherlands.

Location	Origin of the water*	PFPA	PFBuS	PFHxA	PFHpA	PFHxS	PFOA	PFHpS	PFNA	PFOS	PFDA	PFUnDA	Sum of PFASs (lower bound)	Sum of PFASs (upper bound)
Amsterfoort	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Breda	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Wageningen	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Driel	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Odijk	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Bennekom	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Arnhem (1)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Arnhem (2)	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Tiel	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Maastricht	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Smilde	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Renkum	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Ede	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Deventer	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Doetinchem	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Duiven	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Didam	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Steenwijk	Groundwater	0.8	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.8**	**6.8**
Sittard	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	1.4	<0.6	<0.6	<0.6	<0.6	<0.6	**1.4**	**7.4**
Utrecht	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**

Ruurlo	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	1.6	<0.6	<0.6	<0.6	<0.6	<0.6	**1.6**	**7.6**
Eindhoven	Groundwater	1.4	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**1.4**	**7.4**
Schoonrewoerd	Groundwater	<0.6	0.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.7**	**6.7**
Nijmegen	Groundwater	0.9	<0.6	<0.6	<0.6	2.1	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**3.0**	**8.4**
Wijk bij Duurstede	Groundwater	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	**0.0**	**6.6**
Venlo	Groundwater	1.7	0.9	<0,6	<0.6	<0.6	<0.6	<0.6	<0.6	5.0	<0.6	<0.6	**7.6**	**12.3**
Rotterdam	Surface water	6.8	4.0	4.5	2.0	0.8	3.2	<0.6	<0.6	<0.6	<0.6	<0.6	**21.3**	**24.4**
Delft	Surface water	6.7	4.2	4.6	2.0	0.7	3.5	<0.6	<0.6	<0.6	<0.6	<0.6	**21.7**	**24.8**
Den Haag	Surface water	10.0	4.9	4.9	2.7	1.9	7.7	<0.6	<0.6	<0.6	<0.6	<0.6	**32.1**	**35.1**
Dordrecht	Surface water	10.4	3.0	4.1	1.8	<0.6	4.5	<0.6	<0.6	<0.6	<0.6	<0.6	**23.8**	**27.3**
Zaandam	Surface water	4.9	10.4	2.9	1.7	2.0	3.7	<0.6	<0.6	<0.6	<0.6	<0.6	**25.6**	**28.7**
Alkmaar	Surface water	5.2	8.2	3.4	2.0	2.3	4.8	<0.6	<0.6	3.0	<0.6	<0.6	**28.9**	**31.2**
Amstelveen	Surface water	5.1	12.1	3.9	2.1	1.3	4.3	<0.6	<0.6	<0.6	<0.6	<0.6	**28.8**	**31.8**
Schiphol	Surface water	19.8	13.7	4.6	3.0	1.4	11.1	<0.6	<0.6	<0.6	<0.6	<0.6	**53.6**	**56.5**
Amsterdam	Surface water	7.9	11.0	3.4	1.7	0.7	5.2	<0.6	<0.6	<0.6	<0.6	<0.6	**29.9**	**32.9**

Amsterdam 2	Surface water	4.6	7.6	2.4	1.0	<0.6	1.9	<0.6	<0.6	<0.6	<0.6	<0.6	**17.5**	**21.0**
Amsterdam 3	Surface water	6.6	12.7	3.6	2.2	1.2	4.6	<0.6	<0.6	<0.6	<0.6	<0.6	**30.9**	**33.9**
Average (lower bound)		**2.5**	**2.5**	**1.1**	**0.6**	**0.4**	**1.7**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**		
Average (upper bound)		**2.9**	**2.9**	**1.6**	**1.0**	**0.8**	**1.9**	**0.6**	**0.6**	**0.8**	**0.6**	**0.6**		

*The origin of the water is based on the map provided by Rijksinstituut (see Figure 6.1). (Rijksinstituut voor Volksgezondheid en Milieu)

APPENDIX C

SUPPLEMENTARY DATA to chapter 7:

Perfluoralkylated substances in edible livers of farm animals, including depuration behaviour in young sheep fed with contaminated feed.

Table C1: Concentrations of PFOS (ng g^{-1}ww) in daily consumable liver with different animal origin.

Sheep (N=18)	PFOS	*Cow (N=22)*	PFOS	*Horse (N=18)*	PFOS	*Chicken (N=20)*	PFOS	*Pig (N=20)*	PFOS
1	1.5	1	<LOQ	1	1.4	1	<LOQ	1	<LOQ
2	2.0	2	<LOQ	2	2.7	2	<LOQ	2	<LOQ
3	1.3	3	0.5	3	1.4	3	<LOQ	3	<LOQ
4	2.7	4	0.6	4	1.7	4	<LOQ	4	<LOQ
5	4.3	5	<LOQ	5	1.6	5	<LOQ	5	<LOQ
6	0.7	6	<LOQ	6	<LOQ	6	<LOQ	6	<LOQ
7	<LOQ	7	<LOQ	7	0.8	7	<LOQ	7	<LOQ
8	<LOQ	8	0.9	8	2.1	8	<LOQ	8	<LOQ
9	2.0	9	1.1	9	0.9	9	<LOQ	9	<LOQ
10	<LOQ	10	0.7	10	1.4	10	<LOQ	10	4.2
11	1.4	11	3.0	11	2.9	11	<LOQ	11	<LOQ
12	<LOQ	12	0.8	12	0.7	12	<LOQ	12	<LOQ
13	1.3	13	0.5	13	1.6	13	<LOQ	13	<LOQ
14	1.4	14	1.7	14	0.8	14	<LOQ	14	<LOQ
15	4.5	15	<LOQ	15	0.7	15	<LOQ	15	<LOQ
16	2.4	16	<LOQ	16	<LOQ	16	<LOQ	16	<LOQ
17	1.6	17	<LOQ	17	4.5	17	<LOQ	17	<LOQ
18	0.9	18	<LOQ	18	2.5	18	<LOQ	18	<LOQ
		19	<LOQ	19	1.4	19	<LOQ	19	<LOQ
		20	<LOQ			20	0.5	20	<LOQ
		21	<LOQ						
		22	<LOQ						

*****Concentrations of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBuS, PFHxS, PFHpS were below LOQ (0.5 ng g^{-1} ww) in all the samples.

Table C2: Concentrations of PFOS (ng g^{-1}ww) in liver of sheep - Animal study.

Time (days)	*N*	*PFOS concentration (ng g^{-1})*	*PFOS average concentration (ng g^{-1})*	*Standard Deviation*
Contaminated feed				
112	1 2 3	8.3 9.4 15.1	10.9	3.6
56	1 2 3 4	8.7 9.1 9.8 7.3	8.7	1.0
28	1 2	5.7 8.9	7.3	2.3
14	1 2 3 4	2.8 5.9 2.3 2.2	3.3	1.8
7	1 2 3	2.2 2.7 2.8	2.4	0.2
Blank feed				
112	1 2 3	2.4 3.0 1.3	2.2	0.9
56	1 2 3	2.1 1.2 3.5	2.3	1.2

Blank feed after56 d of contaminated feed				
112	1	4.2	4.7	0.9
	2	6.0		
	3	4.0		
	4	4.5		
84	1	5.8	5.8	3.8
	2	11		
	3	4.5		
	4	2.1		
70	1	7.9	7.2	0.7
	2	6.5		
	3	7.4		
63	1	13.7	9.3	4.1
	2	5.6		
	3	8.4		

*Concentrations of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBuS, PFHxS, PFHpS were below LOQ (0.5 ng g^{-1} ww) in all the samples.

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