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PERFLUOROALKYLATED SUBSTANCES IN FOOD MATRICES: DEVELOPMENT OF MASS SPECTROMETRY BASED ANALYTICAL METHODS AND PRELIMINARY MONITORING

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Appendix

The following scientific publications derived from the work presented in this thesis:

- Veyrand B, Kadar H, Barbarossa A, Durand S, Marchand P, Antignac JP, Pagliuca G, Le Bizec B
  "*Compared analytical development and validation based on liquid chromatography coupled to tandem or high resolution mass spectrometry for measuring perfluorinated compounds in milk"*
  Organohalogen Compounds (2010), 72, 968-971
- Kadar H, Veyrand B, Barbarossa A, Pagliuca G, Legrand A, Bosher C, Boquien CY, Durand S, Monteau F, Antignac JP, Le Bizec B
  "*Development of analytical strategy on liquid chromatography-high resolution mass spectrometry for measuring perfluorinated compounds in human breast milk: Application to the generation of preliminary data regarding perinatal exposure in France"*
  Chemosphere (2011), 85 (3), 473-480
- Pollono C, Veyrand B, Barbarossa A, Durand S, Marchand P, Antignac JP, Pagliuca G, Le Bizec B
  "*Analysis of perfluoroalkyl precursors in fish samples: challenge and first application"*
  Organohalogen Compounds, in press
- Barbarossa A, Masetti R, Gazzotti T, Biagi C, Astolfi A, Veyrand B, Pagliuca G, Pession A
  "*Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in human breast milk: results of an Italian study"*
  Journal of Pediatrics, submitted manuscript

During the three years as PhD student, Dr. Barbarossa took part in several other research projects, which led to these scientific publications:

- Lugoboni B, Gazzotti T, Zironi E, Barbarossa A, Piva A, Pagliuca G
  "*Dati preliminari sulla presenza di Fumonisine in fegato suino"*
  Rivista dell'Associazione Italiana Veterinari Igienisti, vol 6.4, 33-36 (2009)
- Gazzotti T, Lugoboni B, Zironi E, Barbarossa A, Serraino A, Pagliuca G
  "*Determination of fumonisin B1 in bovine milk by LC-MS/MS"*
  Food Control (2009), 20 (12), 1171-1174
- Gazzotti T, Zironi E, Lugoboni B, Barbarossa A, Piva A, Pagliuca G
  "*Analysis of fumonisins B1, B2 and their hydrolysed metabolites in pig liver by LC–MS/MS"*
  Food Chemistry (2011), 125 (4), 1379-1384
- Zironi E, Gazzotti T, Lugoboni B, Barbarossa A, Pagliuca G
  "*Development of a rapid LC–MS/MS method for ribavirin determination in rat brain"*
  Journal of Pharmaceutical and Biomedical Analysis (2011), 54 (4), 889-892
- Lugoboni B, Gazzotti T, Zironi E, Barbarossa A, Pagliuca G
  "*Development and validation of a liquid chromatography/tandem mass spectrometry method for quantitative determination of amoxicillin in bovine muscle"*
  Journal of Chromatography B (2011), 879 (21), 1980-1986
- Lugoboni B, Barbarossa A, Gazzotti T, Zironi E, Pagliuca G
  "Development and validation of a liquid chromatography/tandem mass spectrometry method for quantitative determination of flunixin in bovine muscle"
  Manuscript

List of abbreviations

CABA-Lab	Laboratorio di Chimica Analitica Bio-Agroalimentare – Dipartimento di Scienze Mediche Veterinarie, Università di Bologna
CV%	coefficient of variation (%)
DI	daily intake
FASAs	perfluoroalkyl sulfonamides
FOSE	perfluorooctane sulfonamide ethanols
FTOH	fluorotelomer alcohols
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LABERCA	Laboratoire d'Etude des Résidus et Contaminants dans les Aliments – Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentacion Nantes-Atlantique
LC	liquid chromatography
LC_{50}	lethal concentration 50%
LOD	limit of detection
LOQ	limit of quantification
MRM	multiple reaction monitoring
MS/MS	tandem quadrupole mass spectrometry
NOAEL	no observed adverse effect level
ppb	parts per billion
ppm	parts per million
PFASs	perfluoroalkylated substances
PFSiAs	perfluoroalkyl sulfinates
PFCs	perfluorinated compounds
PFCAs	perfluoroalkyl carboxylic acids
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PFSAs	perfluoroalkyl sulfonates
RI	risk index
SPE	solid phase extraction
TDI	tolerable daily intake
UHPLC	ultra high performance liquid chromatography

1. Introduction

Perfluoroalkylated substances (PFASs) is the name of a vast and complex group of compounds consisting of a hydrophobic alkyl chain, whose length varies from C4 to C16, and a hydrophilic end group.

The hydrophobic part may be partially or fully fluorinated: if fully fluorinated, meaning that all hydrogen atoms have been replaced by fluorine atoms, molecules are called perfluorinated compounds (PFCs) (EFSA, 2011).

Depending on the charge of the hydrophilic group, which can be neutral, or negatively or positively charged, these amphiphilic compounds are non-ionic, anionic or cationic surface active agents.

PFASs manufacturing is mainly based on two processes, electrochemical fluorination and telomerisation, the latter becoming the most employed after the announcement by the major world producer of the termination of electrochemical fluorination production process by 2002 (EFSA, 2008).

The beginning of PFASs manufacturing dates back to the late 40s and from then on these molecules have been used in a wide range of industrial and commercial applications due to their chemical and physical properties. In fact, the chemical and thermal stability given to the perfluoroalkyl moiety by carbon-fluorine bounds, together with its hydrophobic and lipophobic nature, give these compounds useful and enduring features. Examples of their applicability include packaging, fire-fighting foams, insecticide formulations, impregnation agents for textiles, cleaning agents and floor polishes (EFSA, 2008; Buck et al., 2011).

The extensive use of PFASs led to their wide distribution into the environment, including animals and humans, as substances of anthropogenic origin. They hardly exist in nature: monofluorinated compounds can be produced by some moulds, as well as various perfluorinated compounds can be originated during some geochemical processes (such as volcanic activities), but in negligible amounts.

These contaminants have been detected in several environmental matrices, even in regions without anthropogenic activities as the arctic environment, due to the global ocean and atmospheric circulation (Butt et al., 2010; Schiavone et al., 2009).

Because of their amphiphilic properties, PFASs don't accumulate in fatty tissues, as other persistent halogenated compounds, but rather bind to proteins. They accumulate in the food chain and have been frequently found in human and plasma: diet is considered the main exposure route for the population, especially through seafood consumption, but also exposure via drinking water and inhalation must be taken into account.

The effects of these substances on human health haven't been fully ascertained yet, but the increasing interest of the scientific community for these emerging contaminants during the last ten years led to important discoveries on their toxic potential. Several animal studies proved that PFASs can be related to a wide range of pathologies in the exposed organisms, such as hepatotoxicity, developmental toxicity, neurobehavioral toxicity, immunotoxicity, reproductive toxicity, lung toxicity and hormonal effects (EFSA, 2011).

An important subset of PFASs is constituted by the perfluorinated organic surfactants, to which perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) belong. Due to their large employment, these two compounds are the most investigated PFASs, and their salts and precursors have been found in the environment, fish, birds and mammals. Furthermore, PFOS was recently added to the Stockholm Convention list of persistent organic pollutants.

The concern about potential PFASs impact on environmental and human health has led various authorities all over the world to launch research programs to better understand their fate in the environment and to evaluate their presence in food, collecting useful data for an accurate estimation of human exposure.

1.1 Perfluorinated compounds (PFCs)

1.1.1 Chemical identity

Perfluorinated compounds are PFASs presenting a fully fluorinated carbon chain; this can be linear or branched and its length generally varies between 4 and 16 carbon atoms.

The hydrophilic part can give the molecule negative, positive or neutral charge. Anionic end groups are, for example, the carboxylates ($-COO^-$, including PFOA), the sulfonates ($-SO_3^-$, to which PFOS belongs) and the phosphates ($-OPO_3^-$). Many among the neutral PFASs (thus considering not only perfluorinated but also partially fluorinated compounds) can be potential precursors of PFOA, for example 8:2 fluorotelomer alcohol, or PFOS, for example perfluorooctane sulfonamide and perfluorooctane sulfonamido ethanols.

Due to the high energy of the covalent carbon-fluorine bonds, PFCs are resistant to hydrolysis, photolysis and biological degradation; they are hardly metabolized, polymers can eventually degrade to lower molecular weight PFCs, such as PFOS (EFSA, 2008; Buck et al., 2011).

Since the names of perfluorinated compounds are quite long and complex, it is common to refer to them with abbreviations, deriving from the chemical structure of the molecule:

- type of molecule (“PF”, which stands for “PerFluoro”)
- number of fluorinated carbons (e.g. “O” when chain is made of 8 carbon atoms)
- hydrophilic end group (e.g. “A” for carboxylic acid, or “S” for sulfonate)

So, “PerFluoroOctanoic Acid” is also known as “PFOA” and “PerFluoroOctane Sulfonate” is rather called “PFOS”.

As previously described, PFOA and PFOS are the most important and widespread PFCs and the final degradation products of most of fluorinated compounds: for this reason this chapter will be focused mainly on these two molecules.

PFOA

Molecular formula: $C_8HF_{15}O_2$

It's a completely fluorinated organic acid, constituted by an eight carbons alkyl chain and a carboxylate group (see **Figure 1.1**).

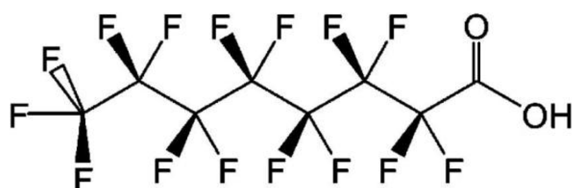

Figure 1.1 PFOA, perfluorooctanoic acid

In water, the free acid dissociates almost completely, while the perfluoroalkyl chain remains on the surface and the anionic carboxylate in the water; PFOA molecules are reported to associate on the water surface partitioning between the air/water interface (US EPA, 2005).

Some studies also reported water solubility for PFOA, but it's unclear whether it's due to a microdispersion of micelles rather than true solubility (3M, 2003a).

According to Prevedouros et al., the dissociated acid (PFO) has negligible vapor pressure, high solubility in water and moderate sorption to solids, thus being expected to accumulate in surface waters.

PFOA presence in the environment can derive directly from its production and use, or indirectly from degradation of related compounds: the transformation pathways include biodegradation, reaction with OH_x, ozonolysis (EFSA, 2008).

Property	Value
Appearance at normal P and T°	White powder / waxy white solid
Molecular weight	414.07 g/mol
Vapour pressure	0.1 kPa (20 °C)
Water solubility (at 20 °C)	3.4 g/L
Melting point	45-50 °C
Boiling point	189-192 °C
pK_a	2.5

Table 1.1 Physical and chemical properties of PFOA

PFOS

Molecular formula: $C_8F_{17}SO_3^-$

It's a fully fluorinated anionic compound, presenting an alkyl chain with eight carbon atoms and a sulfonate group (see **Figure 1.2**).

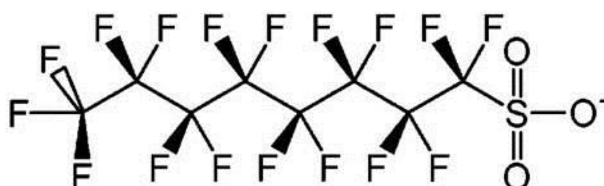

Figure 1.2 PFOS, perfluorooctane sulfonate

PFOS is generally used in salt form (potassium, sodium, ammonium), but can also be incorporated in bigger polymers. In water solution, at pH values form 3 to 8, it's completely dissociated.

PFOS is extremely stable: it resists to hydrolysis (estimated half-life >41 years), to photolysis (estimated half-life >3.7 years) and to biodegradation (several weeks). The only known degradation mechanism is incineration at high temperature (3M, 2003b).

Besides its industrial production, this compound can derive from degradation of various precursors, the so-called "PFOS-related substances", operated by environmental microorganisms or by the metabolism of higher organisms. The number of substances belonging to this family isn't clearly defined yet, but it's proven that there are lots of molecules having the potential to break down to PFOS (EFSA, 2008).

Among all PFCs, PFOS is by far the most frequently detected compound in food products, and at the highest concentrations.

Property	Value
Appearance at normal P and T°	White powder
Molecular weight	538.22 g/mol
Vapour pressure (at 20 °C)	3.31×10^{-4} Pa
Water solubility (at 20 °C)	519 mg/L
Melting point	>400 °C
Boiling point	Not measurable
pK_a	-3.3 (calculated value for acid)

Table 1.2 Physical and chemical properties of PFOS potassium salt (EFSA, 2008)

PFCs precursors

Since perfluorinated compounds presence in food has become of public concern, increasing attention is being given also to their precursors, as Recommendation 2010/161/EU demonstrates. With this document, issued on March 17 2010, the European Commission expresses the need to investigate also compounds like perfluorooctane sulfonamide (FOSA), N-ethyl perfluorooctane sulfonamido ethanol (N-EtFOSE) and 8:2 fluorotelomer alcohol (FOET).

- *FOSA (perfluorooctane sulfonamide)*

  Molecular formula: $C_8F_{17}S0_2NH_2$; Molecular weight: 499.15

  FOSA can enter the environment both being synthesized and as a metabolic by-product of perfluorooctane sulfonamido ethanols (Lehmler, 2005).

  Oxidation of FOSA can result in the formation of PFOS.

- *FOSEs (perfluorooctane sulfonamido ethanols)*

  The end group bound to the nitrogen atom can be a methyl or an ethyl, thus giving:

  - *N-MeFOSE (N-Methylperfluorooctane sulfonamido ethanol)*

    Molecular formula: $C_8F_{17}S0_2N(CH_3)CH_2CH_2OH$; Molecular weight: 557.23

  - *N-EtFOSE (N-Ethylperfluorooctane sulfonamido ethanol):*

    Molecular formula: $C_8F_{17}S0_2N(CH_2CH_3)CH_2CH_2OH$; Molecular weight: 571.25

  PFOS is the final product of FOSEs degradation.

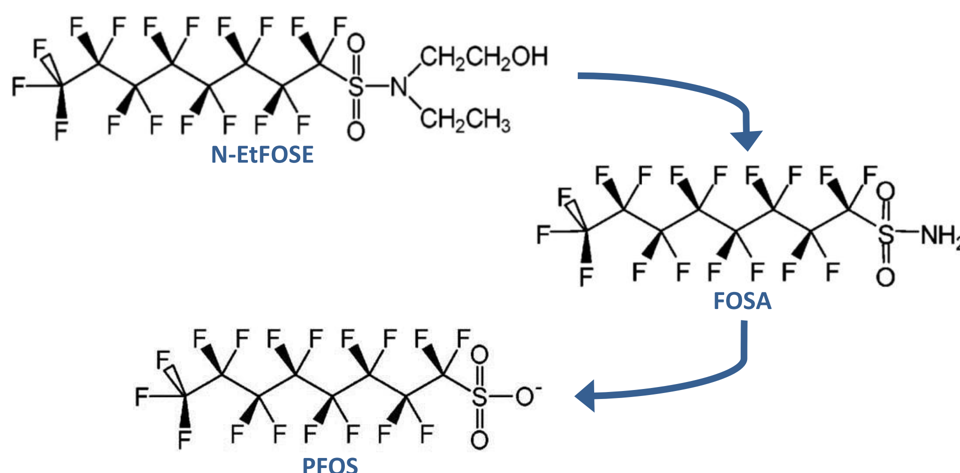

Figure 1.3 N-EtFOSE can degrade to FOSA, and then to PFOS

- *FTOHs (fluorotelomer alcohols)*

  They are fluorotelomers presenting an alcohol functional group.

  Depending on the length of the fluorinated carbon chain, the resulting compounds can be, for example:

  - *FHET (6:2 fluorotelomer alcohol, also called Perfuorohexyl ethanol)*

    Molecular formula: $C_6F_{13}CH_2CH_2OH$; Molecular weight 364.11
  - *FOET (8:2 fluorotelomer alcohol, also called Perfuorooctyl ethanol)*

    Molecular formula: $C_8F_{17}CH_2CH_2OH$; Molecular weight 464.12
  - *FDET (10:2 fluorotelomer alcohol, also called Perfuorodecyl ethanol)*

    Molecular formula: $C_{10}F_{21}CH_2CH_2OH$; Molecular weight 564.14

  FTOHs are volatile compounds, which being vulnerable to hydrolysis and microbial enzymes can break down to give perfluorinated carboxylic acids: FHET degrades to PFHxA and FOET to PFOA (as shown in **Figure 1.4**), FDET to PFDA.

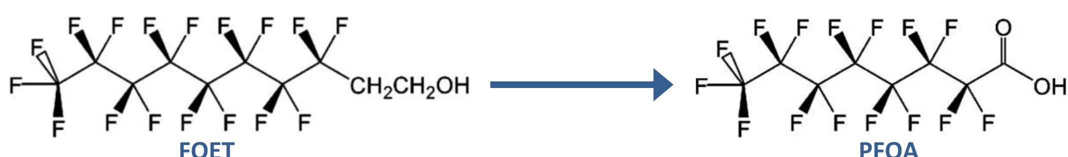

Figure 1.4 Degradation of 8:2 FTOH to PFOA

1.1.2 Production

PFCs are mainly produced by two processes: electrochemical fluorination (EF) and telomerisation (TM).

The EF process is based on the electrolysis of a hydrogen fluoride solution containing organic compounds, whose effect is that all the hydrogen atoms of the molecule are replaced by fluorine atoms. This reaction results in a 35-40% straight chain molecules and a mixture of various byproducts and waste. Moreover, due to variable conditions in the manufacturing process, the final product is a mix of isomers and homologues (of even or odd number of perfluorocarbons, depending on the variable length of the

initial compound's chain), consisting of approximately 70% linear and 30% branched molecules (3M, 1999; Brooke et al., 2004).

PFOS production is based on EF process (see **Figure 1.5**): octanesulfonyl fluoride reacts giving perfluorooctanesulfonyl fluoride (POSF), which is the intermediate product in the synthesis of PFOS and two PFOS-related substances, FOSA and N-EtFOSE (EFSA, 2008).

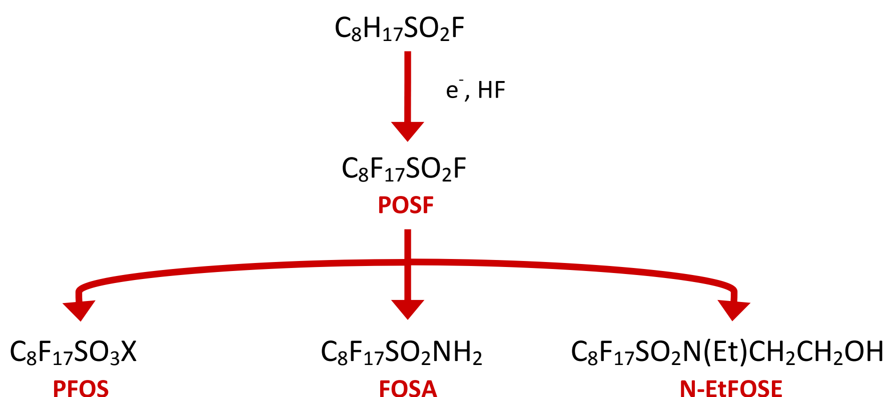

Figure 1.5 Electrochemical fluorination process

Similarly, electrochemical fluorination was employed also in PFOA manufacturing, mainly in the ammonium salt form. The process yields to a mixture of four to nine long carbon chains including linear and branched isomers (Prevedouros et al., 2006).

Introduced in the late 40's, EF has been widely employed during the following decades for the synthesis of perfluorinated compounds, also thanks to its low costs; nevertheless, the major world producers using this technique recently announced its termination, thus making telomerisation the main alternative.

Telomerisation is a process based on the reaction between tetrafluoroethylene (TFE) and iodine pentafluoroethyl iodide (PFEI), which produces perfluoroalkyl iodide (PFAI). This is often further reacted with the insertion of an ethylene, giving fluorotelomer iodide (FTI), which is characterized by a linear and even numbered chain. PFAI and FTI are intermediate products in the synthesis of fluorotelomer-based surfactants and polymers (Buck et al., 2011) (see **Figure 1.6**).

As far as PFOS and PFOA are concerned, this process can be only applied for the synthesis of the latter, but the advantage in comparison with EF is that the high purity of the starting material results in extremely pure (≥99%) linear PFOA (EFSA, 2008).

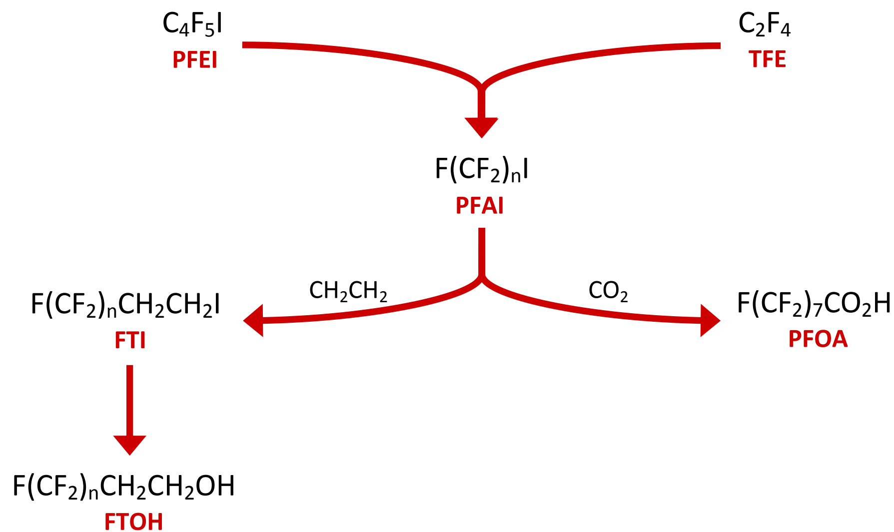

Figure 1.6 Telomerisation process: production of FTOH (left) and PFOA (right)

1.1.3 Applications

Perfluorinated compounds are extremely stable substances, presenting hydrophobic and oleophobic character as well as strong resistance to different types of degradation. Due to their properties, PFCs have been widely employed for more than fifty years in industrial applications and consumer products.

Until the year 2000, when the termination of its production was announced, PFOS was the most employed PFC. OECD (Organisation for Economic Co-operation and Development) grouped PFOS (and its related substances) applications in three categories: surface treatments, paper protection and performance chemicals.

Surface treatments, undertaken by textile mills, leather tanneries, finishers, fiber producers and carpet manufacturers, have the purpose to give water, oil and soil resistance to products like personal apparel and home furnishings. In addition, PFOS-related chemicals are also employed in aftermarket treatments by both general public and professional applicators.

Concerning paper protection treatments, these chemicals are included in sizing agent formulations providing grease, water and oil repellency to paper and paperboard, which are used in food contact products (such as plates, food containers, bags and wraps) as well as in other applications (like folding cartons, containers, carbonless forms and masking papers).

The third category includes several PFOS salts that are commercialized as finished products in a variety of applications, like fire fighting foams, mining and oil well surfactants, acid mist suppressants for metal plating and electronic etching baths, photolithography, electronic chemicals, hydraulic fluid additives, alkaline cleaners, floor polishes, photographic film, denture cleaners, shampoos, chemical intermediates, coating additives, carpet spot cleaners and insecticides (OECD, 2002).

PFOA has many applications too, mainly as a chemical intermediate. Its ammonium salt is mainly employed for the emulsion polymerization of fluoropolymers like polytetrafluoroethylene (PFTE) (whose most known brand name is Teflon) and ployvinilydene fluoride (PVDF), due to the high surface activity of fluorinated surfactants (Lehmler, 2005).

PFTE has an extremely low coefficient of friction and is very non-reactive: for these reasons this molecule is used for several applications, as non-stick coating, lubricant, water-resistant coatings for fabrics and many more.

Other PFOA applications include its use in the manufacturing of electronic components, as extraction agent in ion-pair reversed-phased liquid chromatography, as additive in oil and moisture resistant paper coatings used for food packaging, (EFSA, 2008; US EPA, 2002).

Concerning some PFCs precursors specific applications, fluorotelomer alcohols are involved in the production of acrylate polymers and fluorosurfactants, while FOSA was mainly used as a key ingredient in the original 3M's Scotchgard formulation. FOSA was also extensively employed for its water and oil repellent properties, as well as N-MeFOSE, mainly used on carpets and textiles, and N-EtFOSE, mainly used on paper (Boulanger et al., 2005; Martin et al., 2009; Rhoads et al., 2008).

1.1.4 Occurrence

During the second half of 20th century PFCs have been used in an ever larger number of applications, subsequently their production has continuously increased.

According to OECD, the manufacturing of PFOS and PFOS-related substances (almost entirely in Europe, United States and Japan) prior to the year 2000 amounted to around 4500 t per year, of which 50% for surface treatment applications and 30% for paper protection applications. Few information is available on PFOS production wastes; however, data collected by 3M on its biggest manufacturing plant (located in Alabama, USA) were used to make an estimation, expressed as PFOS equivalents (i.e. the amount of PFOS that could derive from the breakdown of fluorochemical products and residuals). Considering that about 90% of wastes were in solid form and that two thirds of this solid matter were disposed through incineration while the remaining fraction was sent to waste landfills, given an estimated total production of 96000 t between 1970 and 2002, global wastes were quantified in 26500 t, of which 24500 t solid, 435-575 t released to air and 230-1450 t to water (Paul et al., 2009).

With regard to PFOA, the estimated global production during the period 1951-2004 was around 3600-5700 t, with a value of 260 t in 1999. The majority of PFOA production (80-90% in 2000) derived from electronic fluorination process, while the remaining 10-20% was synthesized from about 1975 to present by telomerisation (Prevedouros et al., 2006).

At the beginning of the new millennium the major global producer of PFCs (3M Company) voluntarily decided to stop using the electrochemical fluorination process by 2002, thus phasing out the production of perfluoroalkyl sulfonate substances.

As a consequence of this decision and of the restrictions laid down by the European Union, the use of PFOS has significantly decreased and in some areas even ceased, being replaced by alternative substances providing the same functions or by other technologies (Brooke et al., 2004). **Figure 1.7** shows a comparison between PFOS equivalents production trends between 1970 and 2002 reported by four different studies.

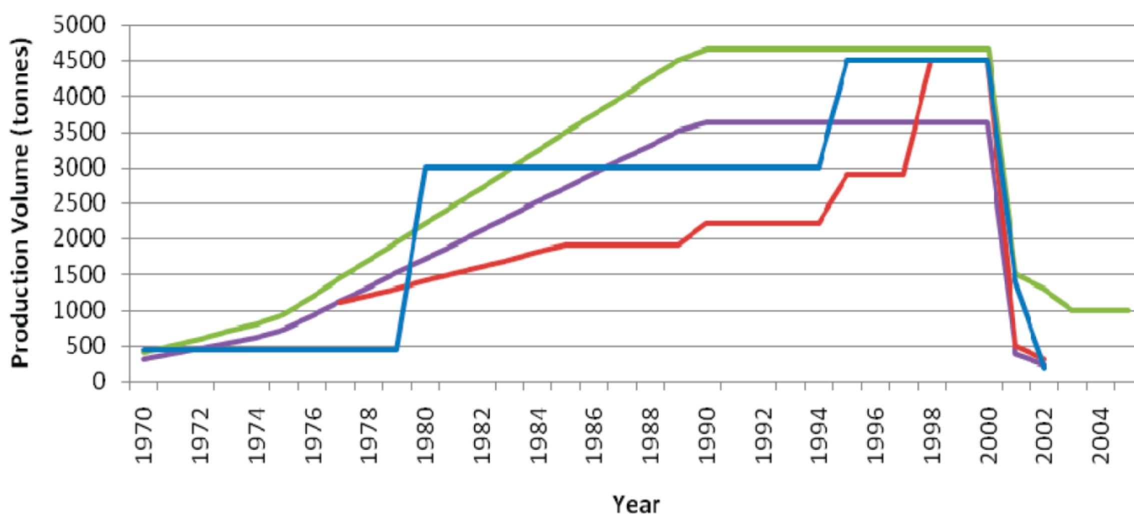

Figure 1.7 Estimated total global PFOS equivalents production volumes (Paul et al., 2009)

The termination of the electrochemical fluorination process led also to an increase in the production of PFOA by telomerisation, with the result that global direct emissions due to its manufacturing decreased from about 45 t in 1999 to about 15 t in 2004, with prospect of further reduction in the following years.

However, when talking about emissions to the environment, also indirect sources must be considered. In fact, in the case of perfluoroalkyl carboxylic acids (PFCAs, to which PFOA belongs), their presence in the environment is not only a consequence of their manufacture, use and disposal, but it's also partly due to the fact that PFCAs impurities are contained in perfluorooctyl sulfonyl-based products made by EF process, as well as they can be present at trace levels in fluorotelomer-based products as unintended reaction by-products. Degradation of these two groups of products and of relative raw materials is a further potential indirect source of PFCAs in the environment. More in details, estimated global PFCAs emissions in the year 2000 amounted to around 237 t, of which 200 consisting of PFOA (Prevedouros et al., 2006).

Degradation of perfluorooctyl sulfonyl-based products is an indirect source for PFOS too, as well as the synthesis of perfluorooctane sulfonyl fluoride itself. According to Paul et al., 85% of PFOS indirect emissions are associated to losses during use and disposal of consumer products, while the remaining 15% results from manufacturing

releases during secondary applications, such as start-up and shutdown operations or losses from production wastes.

The extensive use of perfluorinated compounds caused their global diffusion: PFCs are present in both urban areas with high population density and industrialization and regions far from anthropogenic activities, including the Arctic environment. Recent studies proved the presence of PFOS and PFOA (as well as their salts and precursors) in water, air and soil in many different geographical areas (Recommendation 161/2010/EU).

These substances can contaminate waters in many ways and virtually everywhere, thus their presence can involve also waters directed to human consumption: for this reason monitoring is important in order to prevent potential exposure due to contaminated drinking water.

Twelve PFCs have been investigated in surface waters collected by Skutlarek et al. from the rivers Moehne, Ruhr, Rhine and some of their tributaries, in Germany. PFOA was the compound detected at the highest concentrations, but, depending on the point where samples had been collected, other components were found as well and at different concentrations. The sum of the seven most detected PFCs didn't exceed 100 ng/L for the Rhine river and the lower reaches of the Ruhr river, but levels of contamination increased significantly in the upper part of the Ruhr and the Moehne river (which is an affluent of the Ruhr), reaching values of 446 and 4385 ng/L, respectively. Surveys on the causes of this situation proved that the widespread use of contaminated organic and inorganic material as fertilizer in various agricultural areas around the river Moehne was the source of its waters contamination. Also, further investigations reported high concentrations of PFCs in plasma samples collected from the local population (Skutlarek et al., 2006).

In light of the above, it's clear how PFCs presence in the soil can represent a source for contamination of surface waters. A later program of analysis carried out on 916 field samples collected in that area indicated levels of contamination between 100 and 500 µg/kg in just 8% of the processed samples, but in two farms (which have been subsequently sanitized) measured values exceeded 1500 µg/kg. The analysis of 199

samples collected in a non-contaminated area (Bavaria, Germany) showed PFOS concentrations always lower than 10 μg/kg.

In similar circumstances, increased PFOS soil concentrations were reported in Alabama in a 2007 study. Anyway, data on PFCs presence in soil are lacking to this day, therefore some Authors suggest the importance of monitoring soil and surface waters sampled in the same locations, in order to better understand the transfer of these contaminants (van Asselt et al., 2011).

In a recent study, Awad et al. focused the attention on the long-term persistence of PFOS following an accidental release of fire fighting foams which happened in 2000 in the area nearby Toronto airport. They collected samples of sediment, water and fish from 10 different locations between Etobicoke and Spring Creeks in 2003, 2006 and 2009. Even after ten years, PFOS levels were relevant in Spring Creek, but only in a confined area, due to Etobicoke's diluting action and to the pond's storm water management nature. In 2003 PFOS concentration in fish samples had decreased by 70%; in 2009 contamination levels had declined by 85% in fish and by >99.99% in waters collected downstream of Spring Creek. This drop was probably a consequence of the interruption of PFOS production and of the regulations on the use of this compound in fire fighting foams, resulting in a reduction of PFOS introduction in the environment. Anyway, the 2009 levels were 2-10 times higher than values measured in upstream locations, likely because of both the urbanization and the long-term consequences of the spill (Awad et al., 2011).

In order to verify the impact of fluorochemical producing facilities on measured environmental PFCs levels, Hansen et al. analyzed 40 samples collected in a stretch of the Tennessee river close to a manufacturing site. Mean PFOS concentrations were found significantly higher downstream the plant, increasing from around 30 to around 110 ng/L, thus confirming the effects of effluent from manufacturing on waters contamination (Hansen e al., 2002).

As reported by van Asselt et al., in samples collected in the Yodo River, next to Osaka airport (Japan), PFOS concentrations resulted relevant, reaching in 2004 the value of

526 ng/L. This was probably due to the closeness of a local source of contamination, presumably constituted by the use of aqueous film-forming foams (AFFF).

In a monitoring on the presence of PFOS and PFOA in the water environment of Singapore, the analysis of more than a hundred samples from coastal waters, rivers, reservoirs and wastewater treatment plants (WWTPs) reported significant variations between the different areas of the city. PFOS concentrations in the coastal area and in surface waters were in the range of 1.9-8.9 and 2.2-87.3 ng/L respectively, while for PFOA they were between 2.4-17.8 and 5.7-91.5 ng/L, respectively; wastewaters showed considerably higher values, ranging from 5.8 to 532 ng/L for PFOS and from 7.9 to 1060 ng/L for PFOA, with the highest levels given by effluents released by two WWTPs. This study remarked the role of WWTPs as key point in PFCs access to the oceans (Hu et al., 2011).

Data collected in the Netherlands, Germany, Sweden, Norway, Italy and other European locations showed levels of contaminations in surface water ranging from 0.02 to 56 ng/L for PFOS and from 0.65 to 57 ng/L for PFOA. The most relevant concentrations of PFOA were found in samples collected in Germany and the Netherlands, while Italian Alpine river and spring waters were the less contaminated; as for PFOS, Loos et al. reported close to non-detectable values (up to 0.1 ng/L) in Italian Alpine rivers, whereas in nearby Lake Maggiore its presence was in the range 7.2-8.6 ng/L (EFSA, 2008).

PFCs have been detected in different kinds of animals, including fish, mammals and birds. High concentrations of PFOS have been measured in apex predators of the food chain (including polar bears, seals and eagles) as well as in the lowest levels of the trophic chain, regardless of the distance from sources related to human activities.

A study published by Giesy and Kannan in 2001 investigated the presence of various PFCs (PFOS, FOSA, PFOA and PFHxS) in a wide number of wild animal species, including bald eagles, albatrosses, polar bears, seals and various species of fish. Samples were collected in multiple areas of the globe, from urbanized locations, such as the Great Lakes region and other coastal areas and rivers in North America and Europe, to less anthropized zones, like North Pacific Oceans and the Arctic. PFOS proved to be globally

present, while the other PFCs were detected less frequently and at minor concentrations. In more detail, it was observed that measured values of PFOS in serum depended on the area of origin, being significantly higher in animals living close to populated and industrialized locations than in those living in remote places far from human activities (10-230 against 5-50 ng/mL). Moreover, PFOS was present at different levels of concentration in fish from all the monitored areas, as well as in several bird species, but the highest values were detected in fish eating predators as minks (970-3680 ng/g wet weight of liver), bald eagles (1-2560 ng/mL of plasma), river otters (34-990 ng/g wet weight of liver) and polar bears (180-680 ng/g wet weight of liver). These results suggest that this contaminant is likely to accumulate in the highest food chain's levels (Giesy and Kannan, 2001).

This trend was confirmed also by a more recent study on PFCs presence in animals belonging to the food chain of the Great Lakes area. The monitoring indicated first of all a BCF (bioconcentration factor) for benthic invertebrates of around 1000, meaning that PFOS contamination in these animals was about 1000 times higher compared to that measured in the surrounding water. These invertebrates showed concentrations 2-4 times lower than those of fishes preying on them, which, in turn, were 10-20 lower than values measured in predator fishes, as lake whitefish and Chinook salmon. Also, PFOS was detected in minks and bald eagles at levels 5-10 times greater than their fish prey. These results confirmed the existence of PFOS biomagnification in liver and blood of higher trophic-level animals; as for PFOA, it was found in water as well, but showed a significantly lower biomagnification potential (Kannan et al., 2005).

Furthermore, in another study carried out in the New York State area, concentrations of various perfluorinated compounds, including PFOS and PFOA, were measured in a number of lake waters, in two species of sport fish and in ten species of waterfowl. While PFOA was detected in waters at higher levels of contamination (up to 173 ng/L) than PFHxS and PFOS (around 30 ng/L), this latter was more abundant in all fish and bird livers sampled. In addition, PFOS concentrations in birds reached 882 ng/g wet weight, up to 3-fold greater than fish, and piscivorous birds showed values around 2.5 times higher than those of the non-piscivorous species (Sinclair et al., 2006).

Perfluorinated compounds have been measured also in the air during several studies in different areas of the world. The range of monitored compounds often included, besides PFOS and PFOA, their volatile precursors fluorotelomer alcohols (FTOHs) and perfluorooctane sulfonamido ethanols (FOSEs). Because of the methyl groups present in their chain, these molecules are more vulnerable than perfluorinated compounds, thus representing a potential PFCs source when subjected to degradation in the atmosphere, as well as after inhalation or ingestion. According to the so called "precursors hypothesis", the extreme volatility of these precursors would allow indirect wide-range PFCs transport towards even the most remote areas of the planet, to be added to directly released perfluorinated compounds globally spread by oceanic currents (Jahnke et al., 2009; Barber et al., 2007; Ellis et al., 2004).

In a study conducted in 2007, PFCs presence was monitored in outdoor air samples collected in four different locations in the United Kingdom, Ireland and Norway. PFOA was generally the mainly detected compound in the particulate, with values ranging between 1-818 pg/m^3, while in the gas phase the highest levels were reported for fluorotelomer alcohols, in particular 8:2 FTOH (5-243 pg/m^3) and 6:2 FTOH (5-189 pg/m^3). Also, some perfluorooctane sulfonamido ethanols were found, mainly N-MeFOSE and N-EtFOSE, with concentrations ranging between 36-54 pg/m^3 and 16-33 pg/m^3, respectively. Even if the obtained data were significantly lower than those reported in literature for indoor air, the Authors underlined that, for some compounds, levels of contamination exceeded usual measured values of POPs (Persistent Organic Pollutants) (Barber et al., 2007).

This aspect was confirmed also by Jahnke et al., who measured neutral volatile perfluoroalkylated substances in environmental air samples collected in Hamburg and Waldhof, comparing contamination levels in respectively a metropolitan location and a rural site in northern Germany. Collected data were in-line, sometimes exceeding, with those found in the same area for POPs. More in details, 8:2 FTOH and 6:2 FTOH were reported to be the major pollutant ever measured in Waldhof, with the total measured values for fluorotelomer alcohols in that site varying between 64-311 pg/m^3 and between 150-456 pg/m^3 in Hamburg's urban area. Similarly, the highest FOSEs

concentrations were reported in the most anthropized environment, with values in the range of 29 to 151 pg/m^3 against 12 to 54 pg/m^3 of the rural area (Jahnke et al., 2007).
In another 14 months survey carried out in the Hamburg area, high concentrations were observed in air coming from highly populated and industrialized areas south of the town. 8:2 FTOH was the contaminant most found in the air, reaching concentrations of 600 pg/m^3, while PFOS was detected at a maximum of 13 pg/m^3 in the particle phase. In addition, higher concentrations in the gas phase were reported in summer compared to winter, probably due to temperature-dependent emissions of these volatile substances (Dreyer et al., 2009).
Stock et al. investigated volatile PFASs in North America, monitoring the environmental air of six big cities in the United States and Canada. Both fluorotelomer alcohols and perfluorinated sulfonamido ethanols were detected, with measured values ranging between 11-165 pg/m^3 for FTOHs and up to 359 and 199 pg/m^3 for N-MeFOSE and N-EtFOSE, respectively. Such high values were observed in only two towns, presumably because of the release of these substances by industrial plants in the nearby areas, suggesting the relevant role of point sources for their global diffusion (Stock et al., 2004).
The same Authors reported in a 2007 study the presence of fluorotelomer alcohols also in air samples from three arctic lakes in Canada, with mean concentrations between 2.8 and 29 pg/m^3 (Stock et al., 2007).
Another comparison between volatile PFASs presence in urban and rural environments was presented by Martin et al., who measured FTOHs and FOSEs in Toronto (highly populated area) and in Long Point (less anthropized zone). Reported environmental concentrations were 2 to 3 times higher in samples collected in the metropolis, with a maximum concentration of 87 pg/m^3 for 6:2 FTOH (Martin et al., 2002).
In Japan, an air sampling carried out in 33 different locations during 3 months for a monitoring of fluorotelomer alcohols proved that 8:2 FTOH was the dominant compound (up to 2466 pg/m^3, with a mean of 241 pg/m^3), followed by 6:2 FTOH (up to 768 pg/m^3, mean value 52 pg/m^3) and 10:2 FTOH (up to 113 pg/m^3, mean value 27 pg/m^3) (Oono et al., 2008).

Volatile PFCs concentrations have been measured also in a comparative study between samples coming from the Okinawa Island (Japan) and samples collected in Oregon (United States). Once more, fluorotelomer alcohols were the most detected contaminants, but with significantly greater concentrations in Oregon than in Okinawa. According to the Authors, taking into account the relative absence of high levels of FTOHs in trans-Pacific air masses compared to the air surrounding highly urbanized areas, this difference was due to a more relevant direct emission of pollutants from the western US region. FOSEs were found as well in both sites, but at lower concentrations and with minor frequency (Piekarz et al., 2007).

Harada et al. investigated PFOS and PFOA presence in air samples from Kyoto and Iwate, in Japan. These locations were chosen on the basis of the results of previous monitoring on serum, indicating the first as a typical extremely contaminated area and the second as a low polluted zone. The reported annual geometric means of the concentrations of PFOS and PFOA in the air were respectively 262.8 and 5.2 pg/m^3 in the urbanized area and respectively 2.0 and 0.7 pg/m^3 in the rural region (Harada et al. 2005).

Current information regarding the environmental sources of PFCs is incomplete, but the contamination of several different animal species and habitats suggests the existence of multiple sources.

Given its widespread presence, persistence and toxicity, in 2009 PFOS was included as POP (Persistent Organic Pollutant) in Annex B of the Stockholm Convention, which means that its employment is allowed exclusively for a limited list of applications. Although PFOS doesn’t tend to accumulate in lipids as other persistent halogenated compounds, in general its behavior is very similar to that of POPs: its concentration in blood is linked to its intake via food products and the measured values increase in relation to the age of the observed subject (Haug et al., 2010b).

The estimated half-life in the environment is 41 years for PFOS and 8 years for PFOA, therefore their presence and the subsequent contamination of multiple media will keep being of public interest in the decades to come (D’Hollander et al., 2010).

1.2 Exposure

Even if their production started around sixty years ago, it's only during the last decade that perfluorinated compounds have become of public concern, due to their high diffusion and persistence in the environment, resulting in multiple sources of human exposure, and to the first discoveries on their potential toxic effects.

As previously reported, PFCs are ubiquitous contaminants, being detected in the environment, in wildlife and in humans. However, exposure source for the population haven't been completely defined (EFSA, 2008).

Diet seems to be the major route of exposure, but the contribution of the different types of food still isn't clear (Haug et al., 2010b); according to EFSA opinion issued in 2008, data collected through the monitoring of food are insufficient and to this day it's not possible to characterize the levels of contaminations of the various foodstuffs.

During the recent past, different categories of food products have been investigated in various Countries to assess the eventual presence of perfluorinated compounds.

Fish products represent an important food source for humans and, being essential elements in various aquatic ecosystems such as rivers, lakes and seas, they are at the same time useful bio-indicators. Chronic exposure to high levels of contaminants by eating fish can be a risk for human health: according to Haug et al., fish consumption has proved to be one of the major causes of PFCs intake. However, also contaminated drinking waters can contribute to human exposure, especially in highly polluted areas (Fromme et al., 2009).

Besides food and drinking water, as reported by some Authors, a further potential route is represented by inhalation of outdoor air, indoor air and dust (EFSA, 2011).

PFOS is the most frequently found PFC in food, generally at higher concentrations than others. Non-food sources contribution to total PFOS exposure is estimated to be less than 2%, while for PFOA it could be as high as 50% compared to the predicted average dietary intake; for both compounds these values tend to decrease when moving from childhood into adulthood (EFSA, 2008).

1.2.1 Food sources

A number of studies have been carried out in the last decade on various categories of food from different parts of the world.

An evaluation of the exposure to PFCs through the diet was conducted monitoring 36 food samples collected in the Tarragona area (Spain). PFOS, PFOA and PFHpA were the only compounds detected; the most contaminated food resulted fish and, secondly, meat and dairy products, contributing to around 70% of the total intake. The Authors suggested a dietary intake of PFOS between 0.89 and 1.06 ng/kg b.w. per day (Ericson et al., 2008).

This value is significantly lower than that previously reported by a TDS (Total Diet Study) performed in the UK, in which PFOS and PFOA (and to a lesser extents other PFCs) had been found only in certain samples, mainly of foods containing potatoes, and the estimated intake of PFOS was around 100 ng/kg b.w. per day. In this survey, an average adult daily intake was calculated also for PFOA, corresponding to 70 ng/kg b.w. (UK Food Standard Agency, 2006).

Another survey was conducted more recently in the UK, monitoring 11 different perfluorinated compounds in 252 samples of multiple sorts of food. PFOS, FOSA and PFOA, were the most present contaminants, even if just in traces in almost 75% of the samples. They were found mainly in fish, crab, liver and kidney samples, while their presence in potato products was not reported. On the basis of the collected data, the combined estimated dietary intake via the diet of PFOS and PFOA adults was 20 ng/kg b.w. per day (Mortimer et al., 2009).

Tittlemier et al. analyzed 54 food samples part of a Canadian TDS, including fish, seafood, meat, fast-food and popcorn. The predominant presence of PFOS and PFOA was confirmed; a total dietary intake of all the detected PFCAs and PFOS equal to 250 ng/day was suggested (Tittlemier et al., 2007).

In a work by Zhang et al., the daily intake due to consumption of meat, meat products and eggs in China was estimated. Chicken meat showed the highest level of contamination (12.7 ng/g), followed by pork, pig liver and beef (6.38, 4.47 and 4.43

ng/g, respectively), while low concentrations were reported in eggs (0.38 to 1.21 ng/g). Estimated dietary intakes of 6-9.64 ng/day for PFOS and 254-576 ng/day for PFOA were suggested (Zhang et al., 2010).

A study evaluating the presence of perfluorinated compounds in food in the Netherlands reported the detection at quantifiable levels of 6 different substances (PFHpA, PFOA, PFNA, PFDA, PFHxS and PFOS). The highest total values were found in crustaceans and lean fish (825 and 481 ng/g, respectively), while in fatty fish, butter, eggs, flour and cheese the measured concentrations were smaller (20-100 pg/g), and even lower in milk, pork, chicken, bakery products and vegetable (<10 pg/g). PFOS and PFOA median dietary intake were estimated of 0.3 and 0.2 ng/kg b.w. per day (Noorlander et al., 2011).

According to Panel CONTAM, indicative daily dietary exposure to PFOS, calculated on the data on fish products available when the 2008 report was issued, would be around 60 ng/kg b.w. for the average population, increasing up to 200 ng/kg b.w. for high consumers. Based on these values, the Panel suggested that daily exposure is below the TDI (150 ng/kg b.w.), even if highly exposed subjects could reach and also exceed this value. As for PFOA, EFSA suggested a mean daily intake of 2 ng/kg b.w., with a maximum of 6, not statistically related to individual fish consumption (EFSA, 2008).

Observing the studies available in literature, it’s evident that there is a significant variability between the results obtained. This can be due to various factors, such as the area where the monitoring was conducted or the performances of the employed analytical methods. Also, it must be considered that the importance of certain categories of food in the diet is not constant, depending on the eating habits of the various Countries and regions, and consequently also their contribution to the intake of these contaminants can vary. For all these reasons it’s quite difficult to define representative values of exposure through the diet for the population.

However, fish and sea-food seem to be the most important contributors to the total dietary intake of these contaminants. In particular, PFOS is generally present at higher concentrations than PFOA and has been shown to accumulate in fish with a kinetic bioconcentration factor in the range 1000-4000, mainly in liver (EFSA, 2008).

In the recent past, an increasing number of works focused on the evaluation of PFCs contamination in this category of food: based on the data collected during the 2008 and 2009 monitoring in Europe, EFSA reported that PFOS and FOSA were the two most detected compounds in fish, being found at the highest concentrations in fish offal (47 and 15 µg/kg, respectively) but also, even if at lower levels, in fish meat (4.9 and 2.7 µg/kg, respectively). Concentrations similar to those measured in fish meat were observed also in crustaceans and mollusks, even if only a limited number of samples were analyzed (EFSA, 2011).

Domingo et al. reported that a survey on fish and seafood in Catalonia region (Spain) indicated the presence of PFOS (which resulted the predominant compound, representing 73% of the total), PFOA and PFHpA in this food item. Based on the results obtained, they estimated a mean dietary intake for adults living in that area of about 97 ng/day, largely deriving from sardine and red mullet (31.4 and 27.4 ng/day, respectively) (Domingo et al., 2011).

Within a recent ecological monitoring on river waters in a northern Germany region involved in a massive PFCs release a few years ago, 37 fish filet samples belonging to 6 different species were analyzed: PFOS and PFDA were detected in all samples, at concentrations up to 63.8 ng/g the former and 19.1 ng/g the latter. Other PFSAs and PFCAs, including PFOA, were found only in few samples and at relatively low levels, often close to the limits of quantification (Ehlers et al., 2011).

PFOS was the most detected compound (up to 121 ng/g) also in trout samples from the Great Lakes, in the United States, showing correlations between measured level and body weight. Also PFDS was present in most of cases, at a maximum of 9.8 ng/g, while various PFCAs were found in all samples, with a highest total concentration of 19 ng/g. Based on data concerning lake waters levels of contamination, the Authors calculated BAFs (Bio-Accumulation Factors) of 4.1 for PFOS, 3.9 for PFDA, 3.8 for FOSA and 3.2 for PFOA (Furdui et al., 2007).

Various studies evaluated the presence of PFCs also in fish and seafood from Asia. For example, the analysis of samples of seven types of seafood from China allowed the identification of several PFCs, among which PFOS was the most detected in terms of

both frequency (it was found in all 27 samples) and concentration (with the highest level of 13.9 ng/g measured in mantis shrimps) (Gulkowska et al., 2006).

A study focused on the occurrence of perfluorinated compounds in marine coastal ecosystem was conducted in an estuarine area in the south of Japan. PFOS and PFOA showed the highest frequency, but they showed different exposure and bioaccumulation trends: while the former was the most abundant contaminant in animals living in shallow waters, the latter was mainly detected in tidal flat species. PFNA, FOSA and PFHpS as well were found in lots of the analyzed samples (Nakata et al., 2006).

Even if at significantly lower concentrations, PFCs levels in seafood have been measured also in remote and allegedly less contaminated areas such as Sri Lanka, where the maximum concentrations measured for PFOS, PFHxS and FOSA were 0.012, 0.310 and 0.231 ng/g, respectively (Manage et al., 2005), and even the eastern Arctic, where the reported values for PFOS didn't exceed 1.4 ng/g and were consistently lower for PFOA (Tomy et al., 2004).

Based on the results of a multi-site monitoring of various farmed species in Europe, South America and Southeast Asia, van Leeuwen et al. observed interesting aspects concerning PFCs contamination. Concentrations were sensibly higher in fish than in shrimps, and in carnivorous species (salmon and trout) compared to omnivorous species. In addition, concentrations found in farmed salmon and trout were greater than those measured in lean wild marine fish, while levels detected in farmed shrimp, tilapia and pangasius were generally lower. Finally, within the group of considered species, salmon was believed to be responsible for 97% of human exposure to a range of pollutants, because of the much higher contamination levels and average consumption compared to the other species (van Leeuwen et al., 2009).

Within a study conducted in Norway on 21 samples of local foods, the highest levels of PFCs were detected in cod, cod liver, meat, canned salmon and mackerel. This underlines once more the importance of fish as source of exposure for the population, which is even more significant considering the wide fish consumption in this Country. On the basis of the collected data, a rough total PFCs average dietary intake of 100

ng/day was suggested, higher in male subjects compared to females (Haug et al., 2010a). A further survey by the same Authors investigated the relations between the consumption of certain categories of food (mainly seafood) and serum concentrations of 19 PFCs in 175 subjects, showing that measured levels were associated with the estimated dietary intake of these contaminants. After a 12 months monitoring, it was proved that fish and shellfish were the main responsible for PFCs seric concentrations, contributing to 38% and 93% of total intake, respectively for PFOA and PFOS. Measured levels depended also on other factors, including age and place of origin of the subject: concentrations were higher increasing the age of the observed subjects and in those living in areas near the coast, probably due to direct fishing in more contaminated waters. This fish, in fact, presented greater levels of contamination compared to that caught in open sea for commercial purposes. The estimated dietary intakes of PFOA and PFOS were 0.6 and 1.5 ng/kg b.w. per day, respectively, and were significantly related to the corresponding serum concentrations (Haug et al., 2010b).

Various other studies had already suggested the role of diet as major route of exposure, but in the two investigating on a potential correlation between estimated PFCs intake through diet and seric concentrations no tendency was observed: this was probably due to the limited period of observation (7 days in the work by Fromme et al., 2007a; 1 day in the work by Kärmann et al., 2009), not sufficient to highlight trends in concentrations, which are the consequence of several years of exposure.

The increasing concern on diet-related exposure to these contaminants resulted in the production of specific investigations also for other, apparently less relevant, sources of PFCs, which proved their wide diffusion in several food products.

A recent monitoring explored the presence of perfluorinated compounds in chicken eggs produced in Belgium, highlighting that home-produced eggs contained higher levels of contaminants than commercially produced eggs. Considering that the average egg consumption of people who own chickens is about twice the mean value reported for the Belgian population (20.3 and 10.0 g/day, respectively), the Authors estimated a median intake for home-produced eggs consumers of 142 ng/kg b.w. per day, but this

value was higher for those subjects living near a perfluorochemical production site (D'Hollander et al., 2011).

PFCs have been detected also in tomatoes and grown lettuce, with higher concentrations in the roots compared to leaves and fruits, and it was observed that short-chain compounds were better transferred from the roots to the leaves compared to longer molecules (Felizeter et al., 2011).

Even game animals have been recently indicated by EFSA as significantly contaminated by perfluorinated compounds. High concentrations of PFOS (up to 216 ng/g), PFNA (up to 10.3 ng/g), PFOA (up to 7.1 ng/g), PFDA (up to 6.0 ng/g) and PFDoA (up to 3.7 ng/g) were measured in edible offal of these species, while lower levels of PFOS and PFOA were observed in their meat. These matrices resulted significantly more contaminated in game animals, both birds and mammals, compared to farmed ones. However, from the dietary exposure point of view, it must be considered that they represent a not very significant component of the diet for the general population. Moreover, analysis were conducted only on a small number of samples, so it's difficult to make firm deductions on the real contamination levels in this food (EFSA, 2011).

Since diet is likely to be the most important factor when estimating human exposure to PFCs, their concentrations should be monitored not only in the final food products, but also through all the food chain. **Figure 1.8** summarizes how PFOS can enter the food chain and being then transferred from one step to the other, influencing the total consumer intake.

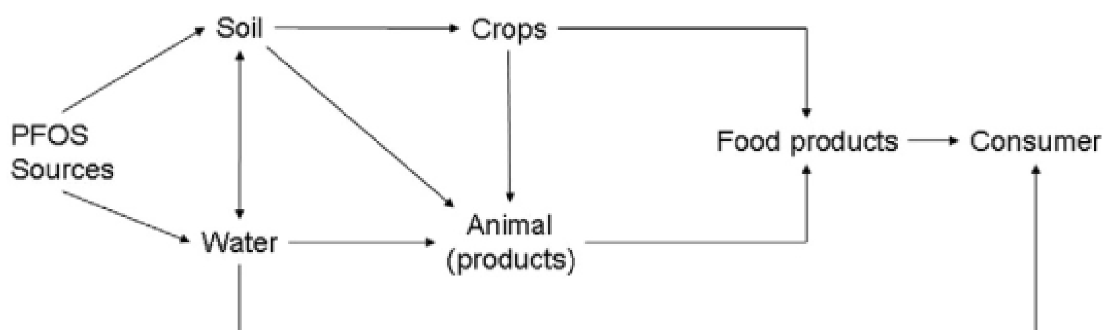

Figure 1.8 PFOS transfer through the food productive chain (van Asselt et al., 2011)

Moreover, it must be considered that food products, even at the end of the food chain, can be contaminated by the same packaging in which they are contained, as well as by the cookware in which they are prepared. In fact, grease and water repellent coatings applied on these items are often manufactured using PFCs precursors, which can transfer to food and then, through degradation, contribute to increase human body burdens of substances like PFOS (Fromme et al., 2009).

In a total diet study carried out in Canada during the 1992-2004 period, individual perfluorooctane sulfonamides were detected in food contained in treated paper packaging, such as pizza and French fries. However, concentrations of these molecules in food are reported to have decreased during the sampling period, being replaced in these applications by fluorotelomer alcohols after the termination of perfluorooctyl sulfonyl compounds production (Tittlemier et al., 2006).

A recent survey on fluorinated and perfluorinated compounds in food contact materials from the Munich area, in Germany, proved the high FTOHs content in these items. After analyzing 47 paper-based packaging samples previously identified as containing fluorine, the Authors observed fluorotelomer alcohols (6:2, 8:2, 10:2 FTOH) concentrations ranging from 9 to 29500 ng/g, as well as lower levels of contamination for PFCAs, mainly PFNA (up to 1500 ng/g), PFOA (up to 619 ng/g) and PFDA (up to 390 ng/g) (Wolz et al., 2010).

A study conducted in 2009 by Jogsten et al. tried to assess the effects of some food processing and packaging on the exposure to perfluorinated compounds through the diet: PFOS and PFHxA were detected in various food items, but the obtained data were not sufficient to understand if these factors can actually influence human exposure.

An investigation on potential migration from PTFE containing items reported a PFOA concentration in cookware in the range 4-75 ng per g of coating (Begley et al., 2005).

Perfluoroalkyl surfactants and fluorotelomer alcohols contained in nonstick cookware and food packaging can also be released to the air while cooking. Testing 4 different nonstick pans, it has been measured a pan-to-gas-phase transfer for PFOA of up to 337 ng, detecting also 6:2 FTOH and 8:2 FTOH; a decrease of PFOA and FTOH release was observed after the fist use. Moreover, high amounts of 6:2 FTOH (up to 223 ng per

bag) and 8:2 FTOH (up to 258 ng), as well as lower quantities of PFOA (5-34 ng) were found in the vapors produced by microwave popcorn bags and on the packaging internal surface. According to the Authors, these results indicate that residues of the cited contaminants remained on the surface of treated coatings may migrate to the gas phase at normal cooking temperatures (Sinclair et al., 2007).

The French Food Safety Agency (AFSSA) investigated the potential risks for the population associated to the residual PFOA presence in cookware provided with non-stick coatings, concluding that this represents a minor route of exposure for the consumers (EFSA, 2011).

Milk

Milk has been treated in this specific sub-section, being the matrix investigated in the present work and representing a particular issue when it comes to perfluorinated compounds exposure through the diet. In fact, milk and dairy products (mostly from cow, buffalo, sheep and goat but, in certain areas, also from other animal species such as camel) are important components of the diet for adults and particularly for children, while human breast milk represents virtually the only source of nutrition for newborns. Based on the above aspects, the importance of evaluating the role of PFCs as potential contaminants also in this matrix is evident.

PFOS has been reported to have strong affinity, as well as for albumin, also for β-lactoglobulin, therefore contaminated milk and dairy products constitute a possible source of exposure (Wang et al., 2010).

A first multi-city monitoring performed by 3M in 2001 on a wide range of food products (with a LOQ of 0.5 ng/g) reported PFOS presence only in 4 milk and 1 ground beef samples, with concentrations reaching 0.85 ng/g (EFSA, 2008).

During the following years a number of works have been published, using more sensitive methods and providing data on a larger number of samples.

According to EFSA's report on the 2008-2009 monitoring of PFCs in food, including 121 milk samples, 87 fermented milk samples and 43 cheese samples, PFOS contaminations was observed only in 2 (out of 18) sheep milk samples, with

concentrations of 140 and 260 pg/mL (LOQ 20 pg/mL). However, it was highlighted that the animals from which milk was collected were usually grazing near an industrial settlement (EFSA, 2011).

Within the previously mentioned survey on food products available on the Catalan market conducted by Ericson et al., also milk and dairy products were monitored. More specifically, whole milk, semi-skimmed milk, 3 kinds of cheese, yogurt, creamy yogurt, cream caramel and custard samples were analyzed, reporting PFOS presence mainly in dairy products, at mean concentration of 121 pg/g. PFOA and PFHpA were detected in two samples of whole milk, at mean levels of 56 and 15 pg/g, respectively, and at even lower levels in the other products. Based on the collected data, the Authors estimated the daily intake of PFOS for the local population: it was suggested a value ranging between 1.3-3.7 ng/day for milk and from 8.4 to 16.5 ng/day for dairy products, depending on the age and sex of the subjects (Ericson et al., 2008a).

A monitoring performed in the United Kingdom on a wide range of food products included also 11 milk samples, showing PFOS and PFOA concentrations below the limit of detection, which corresponded to 1 ng/g (Mortimer et al., 2009).

Wang et al. recently evaluated the presence of 9 perfluorinated compounds in milk, milk powder and yogurt from China. PFHpA and PFNA were found in 68% of the 84 milk samples at mean concentrations of 54 and 67 pg/g respectively, while PFOS and PFOA were present at lower frequencies (<50%) and concentrations (24 and 26 pg/g). Concerning milk powder, PFOA was found in 12 of the 36 samples with a mean level of 46 pg/g, while PFOS and PFNA were less frequently detected (mean concentrations 22 and 30 pg/g respectively). As for yogurt, only PFOA was significantly present, with a mean concentration of 32 pg/g. Considering a mean milk and dairy products consumption for a Chinese adult of 59.2 g/day, the Authors calculated a daily intake for PFOS and total PFCs of 23 and 167 pg/kg b.w., assuming that milk was the only contributor (this value would decrease if also dairy products were considered as contributors to the daily consumption) (Wang et al., 2010).

Eleven different brands of milk (12 total samples) purchased in retail stores from the United States were analyzed, showing concentrations below the limits of detection for

all the PFCs investigated a part for 8 sample presenting extremely low levels of PFHXs (up to 4 pg/mL); similarly, among 21 samples of 5 different infant formula brands collected in the same Country, in only few cases low concentrations of PFOS (up to 11 pg/mL) and PFHxS (up to 4 pg/mL) were found (Tao et al., 2008a).

Among the samples included in their mentioned monitoring conducted in Norway, Haug et al. analyzed also 1 sample of milk and 1 sample of cheese, detecting only few of the 12 target compounds. The most abundant in milk was PFOS (7 pg/g), followed by PFOA (5 pg/g) and PFDA (4 pg/g); in cheese, PFNA showed the highest concentration (16 pg/g) and slightly lower levels were measured for PFOS (13 pg/g) and PFOA (0.012 ng/g). A total intake through milk and dairy products was estimated of 4.7 ng/day for PFOS and of 4.4 ng/day for PFOA and PFNA (Haug et al., 2010a).

The data available so far on milk are not many; however they seem to prove that this food doesn't represent a significant source of PFCs for the population, even if it must be considered that, in general, milk consumption is sensibly higher in children. In this context, the risk of exposure is even more relevant for breastfed infants, whose major source of food is human breast milk.

Several works have demonstrated the presence of various PFCs in blood and milk of breastfeeding women and, even if the transfer mechanism from the former to the latter isn't clear, it has been reported that PFOS levels in milk are about 100 times lower compared to blood (Kärrman et al., 2007). This is probably due to the fact that these compounds have great affinity for the protein fraction of blood, which is higher compared to the lactalbumin and casein content of breast milk, resulting in limited migration and accumulation into milk (Völkel et al., 2008; Fromme et al., 2009).

The risk that persistent contaminants can reach a newborn through breastfeeding, after the in-utero exposure due to transplacental passage, has raised the concern on the topic, leading to a number of studies on PFCs presence in human breast milk.

Kärrman et al. analyzed 12 breast milk samples collected from as many Swedish mothers during the third week after delivery. PFOS was present in all the samples with concentrations between 60 and 470 pg/mL (median 166 pg/mL), showing a positive association with values measured in the corresponding serum. Also PFHxS was

detected (range 31-172 pg/mL, median 70 pg/mL), while FOSA, PFNA and PFOA were present in 8, 2 and 1 samples, respectively. A total PFCs intake by lactation of around 200 ng/day was estimated (Kärrman et al., 2007).

Völkel et al. analyzed 57 human breast milk samples from Germany and 13 from Hungary, detecting PFOS in all 70 samples but at significantly different levels between the two Countries. In fact, while samples from Germany showed concentrations between 28-309 pg/mL (median 119 pg/mL), those from Hungary presented levels of contaminations ranging from 96 to 639 pg/mL (median 330 pg/mL). As for PFOA, it was detected less frequently, with only 11 German samples showing concentrations between 201 and 460 pg/mL. The Authors calculated a PFOS mean daily intake through breast milk of 100 ng for 5 kg infants, based on the data from Germany (Völkel et al., 2008).

Another study conducted in Germany monitored 203 human breast milk samples from women living in the North Rhine-Westphalia region, where a soil improver containing highly contaminated PFCs industrial waste had been previously used by local farmers on their fields. PFOS was found in 99 samples, with median and maximum concentrations of 82 and 284 pg/mL, respectively; PFOA was detected in 120 samples, with median and maximum levels of 137 and 610 pg/mL. PFHxS was present only in two cases, at low concentrations (Bernsmann and Fürst, 2008).

Fromme et al. collected breast milk samples from German women during the first 5 months of newborn's life (201 samples in total) to investigate PFCs presence. PFOS showed the highest frequency (72%), with concentrations in the range <30-110 pg/mL (median 40 pg/mL); PFOA and PFHxS were found in 2 and 3% of samples, respectively, with maximum concentrations of 25 pg/mL for the former and 30 pg/mL for the latter.

In addition, 4 infant formulas were analyzed as well, but none of the compounds was measured above its correspondent LOQ (Fromme et al., 2010).

A monitoring conducted in Barcelona (Spain) included 20 breast milk samples and 3 powder milk-based infant formulas. PFOS was found in 95% of breast milk samples, with concentrations generally between 28 and 865 pg/mL ; PFOA was detected in only 40% of samples, but often presented high concentrations (up to 907 pg/mL).

Concerning powder milk, PFDA showed the highest concentrations, ranging from 693 to 1289 pg/kg, followed by PFOS, PFOA and PFNA (Llorca et al., 2010).

Tao et al. measured the concentrations of 9 PFCs in 45 breast milk samples from Massachusetts. The mean levels of PFOS and PFOA (the two predominant substances) were 131 and 438 pg/mL, respectively, and a total average daily PFCs intake of 23.5 ng/kg b.w. was estimated (highest intake = 87.1 ng/kg b.w. per day). Comparing the ratio PFOS/PFOA resulting from the analysis of these samples with that reported for human serum in the US female population, the Authors suggested a preferential transfer of PFOA to milk. Also, it was observed that PFOA concentrations were greater in samples originating from primiparous mothers (Tao et al., 2008b).

Various surveys have been carried out in Asian Countries as well. The results of a monitoring performed in China on 19 primiparous mothers revealed that PFOS and PFOA were the most present PFCs, with concentration ranging from 45 to 360 pg/mL for PFOS and from 47 to 210 pg/mL for PFOA. Other PFCs were detected at lower levels, with maximum measured levels of 100 ng/mL for PFHxS, 62 pg/mL for PFNA, 56 pg/mL for PFUnA and 15 pg/mL for PFDA (So et al., 2006).

A Japanese study reported PFOS presence in all the human breast milk samples analyzed (51), with concentrations ranging between 8 and 401 pg/g, evidencing how these outcomes suggest an important exposure for infants. Even if only in certain samples, also PFOA, PFNA and PFHxS were detected, at maximum concentrations of 339, 150 and 25 pg/mL, respectively (Nakata et al., 2007).

The analysis of a large number of breast milk samples from 7 different Asian Countries was carried out by Tao et al. in 2008. PFOS was present in 178 of the 184 samples (only in 6 out of 39 samples from India it was not detected), showing significant variability in the mean concentrations between the different Countries: the lower value was reported for India (461 pg/mL) while the highest for Japan (232 pg/mL). PFOA was detected in almost all samples from Japan (mean 777 pg/mL), but rarely in breast milk coming from the other 6 Countries. Great variability was reported for PFHxS frequency, whose levels didn’t exceed 158 pg/mL (Tao et al., 2008a).

Another monitoring was recently performed in China on 24 pools originating from 1237 individual human breast milk samples. A mean concentration of 46 pg/mL was reported for both PFOS and PFOA, but a significant variability depending on the area of sampling was observed: Samples from subjects living in the Shanghai region resulted the most contaminated, with PFOA concentrations up to 814 pg/mL. The Authors estimated mean and highest dietary intakes of total PFCs of 178 and 129 pg/kg per day, respectively; moreover, they highlighted that their estimated PFOA intake of 88.4 ng/kg b.w. per day resulted close to some proposed TDIs (Liu et al., 2010).

Human milk contamination by PFCs was investigated in pooled milk samples from 19 developing Countries as part of a WHO project. PFOS was detected in almost all samples, with the highest concentrations measured in Moldova (65 pg/mL). PFOA was found less frequently, with the highest concentration equal to 192 pg/mL and obtained from Antigua, but it must be observed that this compound had a sensibly higher LOD (80 pg/mL) (Kärrman et al., 2011).

Some Authors investigated the trends of PFCs content in human breast milk and serum during lactation. Thomsen et al. observed that PFOA and PFOS concentrations in milk decreased by 94 and 37%, respectively, during 12 months of breastfeeding (Thomsen et al., 2010). Similarly, Haug et al. indicated that breastfeeding for a period longer than 4 months considerably reduced levels of various PFCs in serum (Haug et al., 2010b). In the light of the above, lactation history can represent a useful instrument in studies on both mother and child exposure.

An interesting study recently published by Haug et al. presented further interesting aspects. After monitoring PFCs presence in milk and serum of 19 Norwegian women, they investigated the partitioning between the two media, observing positive correlations: PFOS and PFOA mean concentrations were 1.4% and 3.8%, respectively, of the corresponding values measured in serum. This suggested that PFOA migration from blood to milk is about two times greater than that of PFOS, which is in agreement with what previously reported by Thomsen et al. Moreover, considering also the potential exposure of infants through house dust inhalation, the Authors calculated that maximum estimated intakes were quite close to proposed TDIs (even if it must be

observed that these TDIs are set for lifelong exposure) and demonstrated that breast milk consumption contributes to more than 94 and 83% of total exposure to PFOS and PFOA, respectively (Haug et al., 2011a).

Based on the above information, it's clear that postnatal exposure to these pollutants can have a relevant impact on health, therefore further surveys employing more sensitive and precise analytical techniques are needed to assess the risks for infants deriving from lactation.

1.2.2 Non-food sources

In a wide perspective, to produce accurate estimations of global exposure to these contaminants it's important to evaluate, other than diet, also significant sources related to the environment where humans live. These include drinking water, indoor air and house dust (Haug et al., 2010b).

Although drinking water is subjected to many controls on various contaminants, there are no regulations concerning perfluorinated compounds, thus it may represent a relevant route of exposure for the population.

Drinking water is estimated to contribute to less than 16% of total PFOA intake and to a more modest extent (<0.5%) of PFOS exposure (EFSA, 2008).

Even if activated carbon adsorption is a promising technique for the removal of PFOS from dilute aqueous streams, this treatment is not so common (van Asselt et al., 2011); the slight differences found by Loos et al. between PFCs levels in drinking water generated from Lake Maggiore (Italy) and those measured directly in the lake indicated the inefficiency of chlorination and sand filtration processes operated by the local waterworks. Therefore, surveys are required to detect possible drinking waters contaminations.

A number of studies on drinking water contamination by perfluorinated compounds in Europe reported values in the range 0.4-9.7 ng/L for PFOS and between 1-4 ng/L for PFOA (EFSA, 2008).

A survey conducted in Spain to assess the role of drinking water as contributor to dietary intake of perfluorinated compounds for the population of Tarragona revealed the presence of various compounds belonging to this family. In tap water samples PFOA was the most detected analyte, with levels in the range 0.32-6.28 ng/L; PFOS was also detected (between 0.39 and 0.87 ng/L), as well as PFHxA, PFHpA and PFNA. Concentrations were sensibly lower in bottle water, with reported values for PFOA <1 ng/L and PFOS not detected at all. Assuming a water consumption of 2 L per day, the Authors calculated a potential intake of around 12.6 ng/day for PFOA and of 0.78-1.74 ng/day for PFOS (Ericson et al., 2008b).

Similarly, after analyzing several drinking waters from public fountains in Catalonia region (Spain) Domingo et al. identified water consumption as one of the predominant PFCs source for people living in that area. Various PFCs were identified: PFOA and PFOS showed the greatest mean concentrations (2.42 and 1.95 ng/L, respectively), with samples from the highly populated and industrialized Barcelona resulting 3-4 times more contaminated than those from the other observed areas, reaching peak concentrations of 9.60 ng/L for PFOA and 6.20 ng/L for PFOS (Domingo et al., 2011).

In the previously mentioned monitoring in the Ruhr river area, Skutlarek et al. reported the presence of various PFCs also in local drinking water. PFOA and PFOS were detected at maximum concentrations of 519 and 22 ng/L respectively, but also PFPA (up to 77 ng/L), PFHxA (up to 56 ng/L) and PFBS (up to 56 ng/L) were found. The relevance of these results is evident when compared to data collected from the analysis of drinking waters outside the Ruhr area, in which maximum PFOS and PFOA concentrations were in the order of 5 ng/L (Skutlarek et al., 2006).

3M Company investigated PFOS and PFOA levels in drinking water in four American cities where these compounds were produced or industrially employed (Columbus, Decatur, Mobile and Pensacola) and in two other cities, used as control (Cleveland and Port St. Lucie). Data indicated PFOS and PFOA contamination only in Columbus (up to 59 and 27 ng/L, respectively) and in Pensacola (only PFOS detected, with concentrations ranging between 0-45 ng/L) (EFSA, 2008).

A monitoring on drinking water treatment facility samples from different locations in the United States was performed by Quiñones and Snyder. Depending on the sampling site, total detected PFCs concentrations and profile were variable. The highest global concentration reported was around 80 ng/L, with measured levels of PFOS and PFOA of 29 and 25 ng/L respectively; in some cases significant amounts were observed for some other PFCs as well, including PFHxA (up to 29 ng/L) and PFHxS (up to 12 ng/L) (Quiñones and Snyder, 2009).

Perfluorinated compounds have been measured also in drinking waters from the Rio de Janeiro area, in southeast Brazil, showing profiles different from those commonly reported. PFOS levels were in fact comparable, or even higher, to those of PFOA and a relevant contribution to total PFCs contamination was given by PFHxS: these three compounds were detected in all the analyzed samples, at concentrations ranging between 0.58-6.70, 0.35-2.82 and 0.15-1.00 ng/L, respectively for PFOS, PFOA and PFHxS (Quinete et al., 2009).

Mak et al. performed between 2006 and 2008 a large-scale investigation on drinking water sampled in the United States, Canada, China, Japan and India in order to evaluate levels of contamination by 20 PFCs. Samples from China showed the highest concentrations of PFOA (mean value in Shanghai water was 78.4 ng/L) and PFOS (mean value in Shenzhen was 10.6 ng/L, while in Chinese tap water in general it was 3.9 ng/L), as well as the presence of various short-chain perfluoroalkyl carboxylic acids, which might have been employed as replacements for PFOS and PFOA. In all the other Countries measured concentrations were lower than those from China and certain compounds were not detected at all. However, also in the United States and Canada the composition profiles of PFCs was dominated by PFOS and PFOA (Mak et al., 2009).

A monitoring on tap water and bottle water samples collected in Örebro (Sweden), Vancouver and Calgary (Canada), and various Asian locations was conducted by Tanaka et al. in 2006. The Authors reported tap water PFOS and PFOA average concentrations in Sweden equal to 1 and 0.4 ng/L, respectively, while in samples from Canada PFOS was not detected and PFOA mean concentration was around 0.2 ng/L. Measured values in Malaysia, Thailand and Singapore were in the range between 0 and 2.5 ng/L

for both contaminants, while in Vietnam they weren't detected at all. PFOA was detected at a mean of 7 ng/L in two Japanese towns, while the highest values were observed in China, with average concentrations of 6.3 ng/L for PFOS and 3 ng/L for PFOA (Tanaka et al., 2006).

In a work published in 2003, Harada et al. measured PFOS concentrations in drinking waters in four cities treating fresh water from the Tama river (Japan), which had proved to be contaminated by sewage plant discharges, into drinking water: in most of cases PFOS levels were lower than 4 ng/L, but in one case it was measured at 50.9 ng/L (Harada et al., 2003).

A research conducted in the Osaka area (Japan) to evaluate PFCs contaminations in drinking water reported the presence of PFOA at concentrations in the range 5.4-40.0 ng/L and of PFOS with values up to 12 ng/L (Saito et al., 2004).

In another study in the same town, a comparison between raw and treated tap waters collected from 14 different drinking water treatment plants was performed. PFOA concentrations were in the range 5.2-92.0 ng/L in raw waters, decreasing to values between 2.3-84.0 ng/L after being treated; measured levels of PFOS were in the range 0.26-22.0 ng/L before treatment and between 0.16-22.0 after. On the basis of the observed correlations between PFCs levels in raw and tap waters, the Authors highlighted the inefficiency of the removal treatment applied, even if no risks were expected in relation to such a limited PFOA contamination (Takagi et al., 2008).

Qiu et al. monitored 8 different PFCs in tap water from 12 locations near Lake Taihu, in the eastern part of China. Again, PFOA and PFOS were the dominant PFCs in all samples, but a significant difference was reported in their concentrations depending on the sampling location: in fact, measured levels in Shanghai area (22-260 ng/L for the former and 0.62-14.0 ng/L for the latter) were on average around 10 times higher than in Nanjing (2.1-2.4 and 0.33-0.38 ng/L, respectively) (Qiu et al., 2010).

A survey was recently carried out in order to estimate for the first time human exposure to PFCs through drinking water in Australia, by the analysis of 62 samples collected from 34 different locations across the Country. PFOS and PFOA were detected in about half of the samples and also PFHxS was often detected (27% of the

samples, generally at higher concentration than PFOA but lower than PFOS). Total PFCs concentrations were in the range 1-5 ng/L for the majority of sampling sites, but in the Sidney area values up to 36 ng/L were reported (Thompson et al., 2011).

On the basis of the available data, drinking water doesn't seem to be a significant source for human exposure. However, water contribution can become relevant in those areas close to sources of contamination and in case of local pollution events (van Asselt et al., 2011). For this reason, advisory guidelines on PFOS and PFOA presence in drinking water have been set by certain authorities in the United Kingdom, Minnesota and Germany.

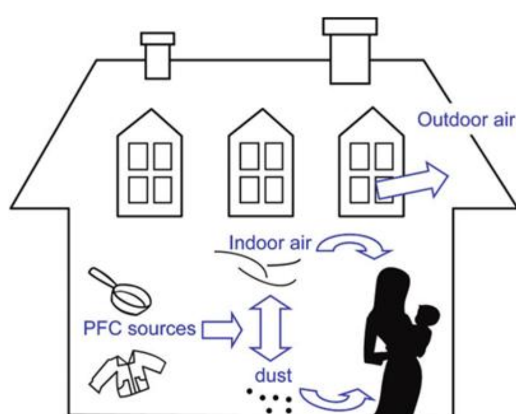

Figure 1.9 Domestic environment-related PFCs pathways to humans (Shoeib et al., 2011)

As previously reported, besides food and drinking water, a potential route of exposure to perfluorinated compounds for the population is represented by inhalation, especially of indoor air and house dust (see **Figure 1.9**).

During the last decade Shoeib et al. conducted different surveys on the presence of perfluoroalkylated substances (mainly volatile precursors of PFOS and PFOA) in these matrices, highlighting significant differences compared to outdoor air.

In a 2004 study the Authors reported mean indoor air concentrations of 2590 pg/m^3 for N-MeFOSE and of 770 pg/m^3 for N-EtFOSE, calculating a ratio compared to environmental air of 110 for the former and 85 for the latter (Shoeib et al., 2004).

These data were confirmed by a monitoring performed the following year on a larger number of air and dust samples, collected in 59 houses in Ottawa (Canada) and in 7

outdoor locations in the same town. Measured levels of contaminations for FOSEs in indoor air were around 10-20 times higher than outdoor concentrations, with mean values of 1490 and 740 pg/m^3 for N-MeFOSE and N-EtFOSE respectively. Concerning indoor dust, reported geometric mean concentrations were of 110 ng/g for N-MeFOSE and 120 ng/g for N-EtFOSE (Shoeib et al., 2005).

In a recent study the same group performed an even more accurate investigation, simultaneously monitoring PFOS, PFOA and their volatile neutral precursors, including FOTHs and FOSEs, in 152 houses in Vancouver (Canada). Between neutral compounds, 8:2 FTOH was widely the most present in air samples, with a mean concentration of 2900 pg/m^3, while N-MeFOSE was found at a mean concentration of 380 pg/m^3. PFOA was measured in all indoor air samples (mean value 28 pg/m^3), while PFOS was not detected. On the contrary, high concentrations were reported for ionic PFCs in house dust, reaching 4700 ng/g for PFOS and 1400 ng/g for PFOA. Concerning their precursors, 8:2 FTOH was the most abundant in house dust as well, with a geometric mean of 88 ng/g. According to the Authors, while inhalation of contaminated air represents a more relevant route of exposure in adults, dust ingestion (e.g. by contact with hands) can be a consistent source of PFCs for children (Shoeib et al., 2011).

A Norwegian study estimated the contribution of various routes of exposure in a group of 41 women, proving the role of the indoor environment as a relevant source to be added to diet. In fact, if on the one hand diet was responsible for 88-99% and 67-84% of the total intake of PFOS and PFOA, on the other hand for certain subjects the contribution of indoor environment reached about 50% of the total. The highest values in house dust were reported for PFHxA (28 ng/g), PFNA (27 ng/g), PFDoA (19 ng/g) and PFOS (18 ng/g), while fluorotelomer alcohols were the most detected in the air, with concentrations of 5173, 2822 and 933 pg/m^3, respectively for 8:2, 10:2 and 6:2 FTOH. Also, the Authors observed correlations between levels of contamination of house dust and corresponding concentrations in blood, as well as between the age of the house and measured values in both indoor air and dust (Haug et al., 2011a and b).

An interesting research compared the presence of several perfluoroalkylated substances in air samples collected in residential and non-residential indoor

environments. Data obtained reported great variability between the different samples, with total concentrations ranging between 8.2-458 ng/m^3, but a deeper analysis highlighted that the highest concentrations (both individual and total) were found in shops selling outdoor equipment, furniture and carpets (Langer et al., 2010).

A recent study investigated the content of PFOA and FTOHs in various consumer products which can be employed in households, suggesting that impregnating sprays for shoes and textiles, generating inhalable aerosols, can contribute to the environmental distribution of these contaminants, thus resulting an additional source for people using them (Schramm et al. 2010).

Fromme et al. produced an exposure assessment collecting data on all the significant media for human exposure: food (which confirmed to be the most relevant source), drinking water, indoor air and house dust. Average and upper daily intake values of 1.6 and 8.8 ng/kg b.w. for PFOS and of 2.9 and 12.6 ng/kg b.w. for PFOA were reported. In addition, they estimated mean global dietary and non-dietary intakes of volatile precursors equal to 0.14 ng/kg b.w. per day for FTOHs and 1.6 ng/kg b.w. per day for FOSEs (Fromme et al., 2009).

Also Haug et al. estimated total intakes for PFCs within the previously cited study on 41 Norwegian women, including all the potential sources of exposure, i.e. food, water, dust and air; dermal uptake was discarded, having been reported to be a negligible route. Three different scenarios were used to estimate house dust intake, since there's still little information on the real contribution of this route and on volatile precursors biotransformation. PFOS median total intakes varied, depending on the scenario, between 0.64 and 0.77 ng/kg b.w. per day; similarly, those of PFOA ranged from 0.27 to 0.36 ng/kg b.w. per day (Haug et al., 2011a).

In order to obtain more representative and consistent data, there's need for further monitoring on perfluorinated compounds, extending the variety of products and matrices examined and investigating a wider range of analytes, including as well their precursors.

1.2.3 Occurrence in humans

Following the increasing interest towards the global spread of perfluorinated compounds and the related risks for health, several studies have been conducted in order to evaluate human exposure to these contaminants measuring their levels in blood, plasma or serum.

If on the one hand it has been demonstrated that PFCs concentrations in plasma and serum are comparable, on the other hand reported ratios between those two media and whole blood levels are not consistent. According to some Authors, the median plasma to whole blood ratios for PFOS and PFOA are 2.3 and 2.0, respectively, while lower values (1.2 and 1.4, respectively) have been suggested by others (Fromme et al., 2009).

Various studies suggested sex-related differences in blood levels of PFOA and PFOS: higher concentrations were observed in male compared to women, even if measured levels in women seemed to increase with age. However, other experiments didn't confirm this aspect. Some works reported also differences in PFOA and PFOS serum levels related with the place of origin of the donor, but it's difficult to ascertain if this variability was due to ethnic differences or rather to a combination of factors, including also lifestyle and diet (Fromme et al., 2009; EFSA, 2008).

Kannan et al. conducted an extended investigation, monitoring the presence of PFOS, PFOA, FOSA and PFHxS in 473 human blood, serum and plasma samples from Italy, Belgium, Poland, United States, Brazil, Colombia, India, Malaysia and Korea. PFOS resulted the predominant substance, with the highest concentrations found in samples from the United States and Poland (>30 ng/mL) and the lowest in those from India (<3 ng/mL, in only 51% of which at levels >1 ng/mL). The other analytes showed smaller frequencies and concentrations. PFOA, the second most abundant compound, was generally found at levels 2-7 times lower than PFOS; however, in lots of serum samples from Korea it resulted the most detected analyte, suggesting the existence of specific sources of exposure in that area. The highest concentrations of PFHxS were found in samples from the United States, Japan and Korea, ranging between 1.5 and 3

ng/mL in these Countries; a significant variability of the ratios between PFHxS serum levels and those measured for PFOS was observed among the various Countries (Kannan et al., 2004).

60 human blood samples collected in the Gulf of Gdañsk area (Poland) were analyzed by Falandysz et al. within a monitoring on exposure for people living on the Baltic Coast. The results evidenced that PFOS and PFOA had the highest concentrations, in the range 5.2-84.0 and 1.2-8.7 ng/g, respectively, but also other PFCs were present, even if at lower concentrations (for PFHxS between 0.2 and 3.7 ng/mL; for PFNA between 0.16 and 3.8 ng/mL) (Falandysz et al., 2006).

A survey carried out in Germany evaluated concentrations of PFOS and PFOA in 105 plasma samples, reporting median concentrations of 22.3 ng/mL for the former and 6.8 ng/mL for the latter. Higher levels were observed in men compared to women (Midasch et al., 2006).

Slightly lower values were obtained analyzing 356 samples of human plasma collected in southern Bavaria (Germany), whose PFOS concentrations were between 2.5 and 30.7 ng/mL (median 10.9 ng/mL) and those of PFOA between 1.5 and 16.2 ng/mL (median 4.8 ng/mL). Again, the Authors reported higher concentrations in male subjects (Fromme et al., 2007b).

Hölzer et al. evaluated the levels of PFCs in 170 children, 317 women and 204 men who had been exposed to PFCs through contaminated drinking water in the Arnsberg area (Germany). The Authors reporting PFOA levels in plasma 4.5-8.3 times greater than concentrations measured in the reference population. One year after this monitoring, a follow-up survey was conducted on a large portion of the subjects, showing a slow decline of PFOA levels: mean concentrations decreased by around 21.3% in children, 19.7% in women and 7.5% in men (Hölzer et al., 2008 and 2009).

During a monitoring conducted by Kärrman et al. in Sweden, 12 different PFCs were investigated in 66 whole blood samples collected from the local population. PFOS, PFOA, FOSA, PFHxS and PFNA were present in 92-100% of cases and showed higher concentrations in men compared to women; PFOS showed the highest concentrations, ranging between 1.7-37.0 ng/mL (median 17.1 ng/mL). PFDA and PFUnA were found in

65% of the samples, while the other analytes were detected sporadically. Assuming a plasma to whole blood ratio of 2, the Authors suggested that calculated plasma concentrations of PFOS and PFOA based on their data were similar to those reported in other studies (Kärrman et al., 2004).

Ericson et al. tried to investigate potential correlations between PFCs levels in blood of 48 people living in Catalonia (Spain) and their age and gender. PFOS showed the highest mean concentration (7.6 ng/mL), but significant levels were observed also for PFHxS and PFOA (mean concentrations 3.6 and 2.8 ng/mL, respectively). Once again, samples collected from male subjects resulted more contaminated; PFHxS levels presented age-related differences, being higher in the 25±5 years group (Ericson et al., 2007).

Both PFOS and PFOA were found in 56 serum samples collected in Athens (Greece), at median concentrations of 13.7 ng/mL in males and 7.0 ng/mL in females the former and of 3.1 and 1.7 ng/mL the latter. PFOS values were significantly higher in samples belonging to the "over 40" group, while no age-related trend was observed for PFOA (Vassiliadou et al., 2009).

An increase of PFOS seric levels with age was indicated as well by Ingelido et al., who highlighted the same situation also for PFOA after the analysis of 230 serum samples from two Italian cities, Brescia and Rome. More precisely, measured concentrations of both PFOS (range 0.06-29.6 ng/g, median 6.3 ng/g) and PFOA (range 0.2-51.9 ng/g, median 3.6 ng/g) were higher in the 36-50 and 51-65 years ranges, with a significant increase in females belonging to the 51-65 years group. Moreover, PFOS and PFOA levels showed a strong correlation, with the former always significantly more present than the latter (Ingelido et al., 2010).

The previously mentioned survey by Haug et al. on 175 serum samples from Norway reported the presence of PFOA, PFNA, PFDA, PFUnA, PFHxS and PFOS in all samples; PFHxS and FOSA were almost always detected as well. PFOS was the most found (mean 32 ng/mL, significantly higher than any other analyte; maximum 133 ng/mL), followed by PFOA, PFHxS and PFNA (means 4.1, 2.2 and 1.1 ng/mL, respectively) (Haug et al., 2010b).

In 2001, Hansen et al. performed a monitoring on 65 serum samples collected in the United States, detecting PFOS in all the samples, while PFOA and PFHxS in 52% and 47% of cases, respectively. Mean and maximum measured concentrations were respectively 28.4 and 81.5 ng/mL for PFOS, 4.8 and 35.2 ng/mL for PFOA, 5.1 and 21.4 ng/mL for PFHxS (Hansen et al., 2001).

The analysis of 20 serum samples collected in Atlanta (United States) reported comparable values: PFOS ranged between 3.6 and 164.0 ng/mL, PFOA between 0.2 and 10.4 ng/mL, PFHxS between 0.4 and 11.2 ng/mL (Kuklenyik et al., 2004).

A number of surveys have been presented by Olsen et al. on PFCs presence in serum of the Unites States population. A monitoring on 645 Red Cross blood donors from six different centers showed PFOS mean concentration of 34.9 ng/mL (maximum value 1626 ng/mL), while other detected PFCs were present at 10-fold lower levels (Olsen et al., 2003a). Similar values were reported in a survey on 238 aged subjects (65-96 years old) from Seattle: PFOS mean concentration was 31 ng/mL, with a maximum level of 175 ng/mL. No sex-related differences were observed (Olsen et al., 2004). In a more recent work, the Authors analyzed 100 serum samples collected in Minneapolis in 2000 and 40 plasma samples collected in the same town in 2005, in order to evaluate if PFCs levels had decreased after the termination of the electrochemical fluorination process by 3M Company. The mean concentration decreased from 33.1 to 15.1 ng/mL for PFOS and from 4.5 to 2.2 ng/mL for PFOA (Olsen et al., 2007a).

The same Authors have also conducted investigations on the levels of PFCs in occupationally exposed workers from various production sites, highlighting extremely higher concentrations in these subjects compared to the general population. A monitoring on 263 3M Company employees from the Decatur (United States) plant and 255 from the Antwerp (Belgium) plant showed 2 times higher concentrations in subjects working in the American site, with PFOS and PFOA concentrations ranging between 60-10060 and 40-12700 ng/mL, respectively, and mean values of 1320 ng/mL for PFOS and 1780 ng/mL for PFOA (Olsen et al., 2003b). Another survey on 126 workers reported mean concentrations of 941 ng/mL (range 787-1126 ng/mL) for PFOS and 899 ng/mL (722-1220 ng/mL) for PFOA (Olsen et al., 2003c). The analysis of other

506 serum samples from exposed subjects showed PFOA levels between 7 and 92030 ng/mL, with a mean concentration of 2210 ng/mL (Olsen and Zobel, 2007).

Two survey projects have been carried out by Calafat et al. on serum samples collected from United States residents belonging to three major ethnic groups (non-Hispanic whites, non-Hispanic blacks and Mexican Americans), in order to evaluate potential ethnicity-depending differences in PFCs levels. The analysis of 54 pooled samples (from 1832 donors) collected between 2001-2002 reported significantly higher mean concentrations of PFOS in non-Hispanic white subjects (40.2 ng/mL in males and 24.0 ng/mL in females) compared to non-Hispanic black subjects (18.3 and 18.0 ng/mL, respectively); as for Mexican Americans, mean levels were even lower, being 13.7 ng/mL in men and 10.4 ng/mL in women. Similar trends were observed for PFHxS as well (Calafat et al., 2006a). In a similar investigation, 1562 samples collected between 1999-2000 showed the constant presence of PFOS, PFOA, PFHxS and FOSA, with significantly lower levels in Mexican American donors. Sex-related trends were observed, while no age-related variations resulted (Calafat et al., 2007). In another research by the same Authors, serum samples collected in the United States were compared to serum samples from Peru, which showed extremely lower PFCs frequencies and concentrations. In particular, PFOS and PFOA were detected in all the US samples while in only 20 and 25%, respectively, of the Peruvian samples; also, median concentrations of PFOS and PFOA were 31.1 ng/mL and 11.6 ng/mL for the US residents, while 0.7 mg/mL and 0.1 ng/mL for the Peruvians (Calafat et al., 2006b).

A preliminary monitoring in Canada on 56 serum samples showed results similar to those of the majority of studies, with PFOS detected in all samples with concentrations between 3.7 and 65.1 ng/mL (mean 28.8 ng/mL). PFOA was present at significantly lower concentrations and only in 29% of cases (Kubwabo et al., 2004).

An extended survey, involving 3802 serum samples collected in Australia between 2002-2003, reported the highest mean concentrations for PFOS, PFOA, PFHxS, PFNA and FOSA (20.8, 7.6, 6.2, 1.1 and 0.7 ng/mL, respectively). Increase of PFOS levels with age was highlighted in both genders; curiously, PFNA showed higher values in female pools (Kärrman et al., 2006).

Another huge monitoring was conducted in Australia between 2006-2007 collecting 2420 serum samples, subsequently pooled based on donor's age. The most detected PFCs were, in descending order, PFOS (mean concentration 15.2 ng/mL), PFOA (6.4 ng/mL), PFHxS (3.1 ng/mL) and PFNA (0.8 ng/mL). Gender differences were observed (men concentrations were higher compared to those of women) except for the <12 years pool; an interesting difference was observed between PFOS, which showed the highest concentrations in adults >60 years, and PFOA, PFNA, PFDA and PFHxS, which on the contrary were higher in children <15 years (Toms et al., 2009).
More recently, Toms et al. integrated the results of these two studies with further samples collected in Australia between 2008-2009, in order to evaluate PFCs trends in the population during an 8 years period. Based on the data obtained, concentrations had significantly decreased during that span of time in both adults and children. Moreover, some interesting trend differences were observed: no age-related trend was found in the earlier data for PFOS, while the most recent samples indicated that its level increases with age; at the same time, the early samples suggested a decrease of PFOA from youngest age groups towards oldest age groups, which was absent in the 2008-2009 samples. According to the Authors, these differences may be due to a faster response to changing exposure profiles in younger subjects (Toms et al., 2010).
Concerning Asian Countries, a monitoring carried out in the Tokyo area on 10 whole blood samples indicated a mean concentration of PFOS of 8.3 ng/mL, while PFHxS and PFBS were not detected (Taniyasu et al., 2003).
Again in Japan, Harada et al. observed significant differences between serum levels measured in Miyagi (lowest), Akita and Kyoto (highest): mean levels of PFOS were in the range 5.7-28.1 ng/mL in males and 3.5-13.8 ng/mL in females; as for PFOA, ranges were 3.3-12.4 and 2.5-7.1 ng/mL, respectively (Harada et al., 2004).
Within a wide monitoring in China, 1437 serum samples were collected from different locations. PFOS and PFOA showed correlated mean concentrations, equal to 4.3 and 3.6 ng/mL, respectively; however, a significant zone-related variability was observed: PFOS mean levels varied from 0.3 ng/mL in a rural area to 18.8 ng/mL in a big town; similarly, PFOA ranged between 0.5 and 25.4 ng/mL (Jin et al., 2011).

1.3 Toxicity

Several investigations have been conducted during the last decade in order to deepen the knowledge on the toxic effects caused by perfluorinated compounds.

Most of the available information concerns PFOS and PFOA, which have been reported to have a rather long half-life and to accumulate in liver and blood. Studies on these substances proved their immunotoxicity and hepatotoxicity, negative effects on reproductive, respiratory and nervous systems, as well as potential to cause development and hormonal alterations (OECD, 2002; EFSA, 2011).

Adverse effects have been studied mainly on rats, but some data are available also on rabbits and non-human primates. Moreover, in a recent study Sonne observed a negative impact on health related to exposure to this these contaminants also in animals from the Arctic Circle, such as polar bears, sled dogs and arctic foxes (Sonne, 2010).

Some epidemiological studies have been conducted on exposed populations, such as workers from plants producing fluorinated substances, but collected data are still fragmented and incomplete.

OECD published in 2002 a hazard assessment on PFOS and its salts, concluding that it's a persistent and bioaccumulative pollutant, with toxic potential towards mammals. Consequently, a NOAEL (No Observed Adverse Effect Level) was set at 0.1 mg/kg/day, based on the results of a 2-generation reproductive toxicity study performed on rats (OECD, 2002).

In 2003, the United States Environmental Protection Agency (US EPA) issued a preliminary evaluation on the toxic effects on development associated with exposure to PFOA and its salts, declaring a NOAEL for females and males of 10 and 3 mg/kg/day, respectively.

EFSA's Panel on Contaminants in the Food Chain (CONTAM) in the 2008 report indicated the lowest NOAEL value for PFOS at 0.03 mg/kg b.w. per day and for PFOA at 0.06 mg/kg b.w. per day. Concerning PFOA, the Panel observed also that in various

studies in rats the 95% lower confidence limit of the values for the benchmark dose for a 10% increase in effects on the liver (BMDL10) was in the range 0.3-0.7 mg/kg b.w. per day. Based on these values, Tolerable Daily Intakes (TDIs) for these substances were estimated, resulting of 150 and 1500 ng/kg b.w., respectively for PFOS and PFOA (EFSA, 2008).

Based on the available data, CONTAM panel concluded that there's little likelihood that adverse effects due to these compounds are occurring in the population, but more data are needed to ascertain this statement (EFSA, 2008).

This opinion was shared also by other authorities. The Bundensinstitut für Risikobewertung (BfR, the German Federal Institute for Risk Assessment) expert panel estimated a provisional TDI of 100 ng/kg b.w. for both compounds and confirmed that PFCs exposure through diet is very low, adding that suggested intake levels could be indicated for those groups of people living in highly contaminated areas (BfR, 2010). The UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, based on the effects on liver, kidney, hematological and immune systems, recommended a TDI of 3000 ng/kg b.w. for PFOA and 300 ng/kg b.w. for PFOS (Committee on Toxicity, 2009 and 2010).

According to data reported by Haug et al., dietary exposure to PFOS and PFOA is respectively 100 and 2500 times lower than their relative TDIs indicated by EFSA, but this margin could be smaller for people consuming high amounts of fish products; it must also be highlighted that these values didn't take into account non-food sources (Haug et al., 2010b).

Fromme et al. (2007a), who referred to both food and non-food sources, assessed that global daily intakes are significantly lower than even the lowest recommended TDI values, at least for adults, since children exposure profile isn't well defined yet.

However, it must be noticed that most of the epidemiological studies on humans available in literature have been conducted by the major manufacturer of fluorinated chemicals and may thus report tendentious results. Further studies will clarify the potential correlations between PFCs exposure and risks for human health.

1.3.1 Toxicokinetics

1.3.1.1 PFOS

In animals

PFOS kinetics has been evaluated through oral administration of PFOS-^{14}C in rats. After 48 h, around 5% of the radioactivity was found in the feces and the intestine, therefore it was assumed that 95% was absorbed. Measured PFOS concentrations after 89 days reported significant levels only in liver (20.6 µg/g, corresponding to 25.21% of the dose) and plasma (2.2 µg/g, corresponding to 2.81% of the dose); less significant amounts were found in kidney (1.1 ng), lung (1.1 ng) and other tissues, and they were probably due to residual blood in these organs when homogenized (OECD, 2002; EFSA, 2008).

Seacat et al. demonstrated, through daily repeated PFOS administration to rats, that it tends to accumulate, showing 31-42% higher serum levels in female rats and no relevant differences as regards values measured in the liver (Seacat et al., 2003).

Several studies in mice and rats reported PFOS transfer from dam to fetus during pregnancy and indicated that fetal liver content of PFOS was about 50% compared to maternal liver, while serum levels were similar (EFSA, 2008).

PFOS is not metabolized, after absorption it binds to serum proteins (mainly albumin) and distributes in serum and liver; on the contrary, precursors N-MeFOSE and N-EtFOSE are reported to be metabolized to PFOS (3M, 1999).

PFOS is mainly eliminated through the kidney and partially in the feces; its half-life was estimated of >90 days in male rats after a single oral dose and of around 200 days in monkeys after a 183 days administration (EFSA, 2008; OECD, 2002).

In humans

A 2004 study on Japanese pregnant women proved that PFOS can partially transfer from maternal to fetal circulation; more recently, it was demonstrated that it can reach the fetus also slowly crossing the placenta (EFSA, 2008).

Renal elimination has been demonstrated to be negligible in humans; several studies evaluated the elimination half-life of PFOS, with quite variable results. According to 3M Company survey on 3 former workers, it would be almost 4 years, while an investigation on other 9 former employees reported a value of 8.67 years, with a relevant variability between the subjects. According to EFSA Panel, the most reliable estimation is that proposed by Olsen et al., suggesting a value of 5.44 years (EFSA, 2008, Olsen et al., 2007b).

1.3.1.2 PFOA

In animals

Also to determinate PFOA kinetics a single oral dose of radioactive-labeled molecule on rats was used, which resulted in absorption of 93% after 24 h. Similarly to PFOS, it mainly distributed in serum, due to its interaction with albumin, and in liver (in female rats also in kidneys), due to its affinity for liver fatty acid-binding protein (L-FABP). PFOA is not metabolized, but precursors 8:2 FTOH can metabolize to PFOA, which has the potential to accumulate (EFSA, 2008; Luebker et al., 2002).

It was reported that PFOA can be transferred to the fetus via the placenta, with measured concentrations in fetal plasma around 50% of those in maternal plasma; also dam-to-pup transfer by lactation was proved, reporting PFOA levels in milk corresponding to about 10% of the plasmatic concentrations (Hinderliter et al., 2005).

A significant difference concerning the urinary elimination of this compound was observed in rats depending on the gender: during the first 24 h after administration, in fact, female rats eliminated 91% of the dose, while male rats only 6%. These resulted in sex-related elimination half-life values, calculated in less than 1 day for female rats and 15 days for male rats (Vanden Heuvel et al., 1991).

This difference was due to a hormone-depending secretory mechanism involving organic anion transporters, which testosterone was supposed to competitively inhibit. A demonstration was given by the fact that in castrated male rats and female rats renal

elimination was comparable and, if they were all treated with testosterone, it was reduced in both genders (Kudo et al., 2002).
A Butenhoff et al. study on monkeys feed a 26 weeks diet containing PFOA reported high variability in measured levels in liver; however, estimated half-life was around 30 days for female monkeys and 21 for male monkeys (EFSA, 2008).

In humans

As for animals, also in humans PFOA rapidly distributes in serum (according to Han et al., 2003, more than 90% is transported by albumin), but in this case its renal excretion is irrelevant compared to rats and monkeys. Also, it was observed that PFOA plasmatic concentrations in 20-50 years old people were more significant in males, while in >50 years old subjects there was no difference between genders, but it must noticed that the reported Japanese study was conducted on a small number of people and therefore its statistical values is not certain. Although only little information is available, PFOA can cross the placenta and bioaccumulate in the fetus (EFSA, 2008).
A study concerning serum half-life of PFOA reported highly discordant values (between 1.5 and 13.5 years), with a mean of 4.37 years (EFSA, 2008). With less variability on the 26 subjects in his survey, Olsen et al. calculated a half-life for elimination from serum equal to 3.8 years (Olsen et al., 2007b). According to a monitoring on 138 subjects who had been exposed to PFOA contaminated drinking water few years ago in Arnsberg (Germany), mean half-life of this contaminant in plasma would be 3.26 years. Also, a recent study investigated PFOA trends in the serum of 200 subjects from two highly contaminated locations in Ohio and West Virginia, after the beginning of activated carbon water filtration: the obtained data allowed the Authors to estimate a PFOA half-life of 2.3 years (Bartell et al., 2010).

1.3.2 Mechanisms of action

Even if the mechanisms by which PFCs generate toxic effects are not well known, these compounds have been proved to be responsible for adverse effects such as peroxisome proliferation and changes in enzymatic activity.

Various Authors indicated that PFOS and PFOA are capable of activating peroxisome proliferator-activated receptors α (PPARα), which are ligand dependent transcription factors acting on genes implicated in lipid metabolism, lipid and glucose homeostasis, inflammation, cell proliferation and differentiation. In animals exposed to these contaminants, also production of cytokines, reduction of lymphoid organs weight, altered inflammatory response and antibody synthesis were observed. Some of these effects have been demonstrated to be PPARα independent (Shipley et al., 2004; De Witt et al., 2009).

PFCs can affect the metabolism of fat acids interfering with their β-oxidation, probably due to their chemical structure being very similar to that of endogenous fat acids. It has been shown that PFCs cause alterations in some hepatic enzymes activities, including acyl-CoA oxidases and dehydrogenases, resulting also in decreased amounts of triglycerides and cholesterol in the blood circle and in oxidative DNA damage. (Hu et al., 2005; EFSA, 2008).

PFOS and PFOA are suspected endocrine disruptors, whose interference on sexual hormones causes increased levels of oestradiol and decreased levels of testosterone; PFCs have shown oestrogenic effects on cell cultures (Jensen and Leffers, 2008).

It has been also observed in some tests, performed in vitro on rat liver and dolphin kidney epithelial cell lines and in vivo on rats treated with PFCs, that they can be incorporated into the cellular membrane and reversibly inhibit gap junction intercellular communication (Hu et al., 2002).

Since no genotoxicity for these compounds has been reported by neither in vivo nor in vitro studies, it’s probable that their carcinogenicity is related to an indirect mechanism (EFSA, 2008).

1.3.3 Toxic effects of PFOS

1.3.3.1 Effects on animals

Acute toxicity

Acute studies have been observed by various studies in rats and rabbits.

In a 1979 work, a lethal concentration (LC_{50}, i.e. the dose causing the death of half the treated animals) of 5.2 mg/L was estimated administering PFOS through inhalation to several groups of rats, generating also signs of toxicity, as emaciation, breathing problems and nasal secretion; moreover, post-mortem examination showed a variable liver discoloration. In another study rats were treated with a single dose through feeding tube, showing alterations in the nervous system and a LD_{50} of 271 and 251 ng/kg, respectively for male and female rats. Skin and eye irritation possibly caused by PFOS was investigated on rabbits, but without significant results (OECD, 2002).

Subacute toxicity

During administration of PFOS through a 14 weeks diet, significantly increased glycemia and liver weight (mainly in male rats) were observed; histological analysis highlighted hypertrophy and vacuolization of the hepatocytes (Seacat et al., 2003).

Other studies on rats showed also a reduction in body weight, as well as, in some cases, decreased levels of cholesterol and tryglicerides in blood.

Monkeys appeared to be more sensitive to PFOS than rats: they seemed to present a steep dose-response curve, which in some cases led them to death if treated with doses of few mg/kg/day. Alterations in thyroid hormones were also observed in these animals (EFSA, 2008).

Chronic toxicity

A study on male and female rats being given PFOS in the diet for 104 weeks reported hepatotoxicity in both genders within the highest doses groups (but in male rats also at lower administered concentrations) and carcinogenicity, being responsible for raised incidence of hepatocellular and thyroid follicular cells adenomas. Also for mammary

adenomas and fibroadenomas increased incidences were observed, but the obtained data were not sufficient to ascertain if this was due to PFOS exposure (EFSA, 2008).
Based on the results concerning liver toxicity, a NOAEL for PFOS was estimated of 0.5 and 2 ppm for male and female rats, respectively (OECD, 2002).

Developmental and reproductive toxicity

Experiments conducted on rodents evaluated the effects of exposure to PFOS on dam, fetus and newborn. Female rats and mice received PFOS at different doses via feeding tube during the entire gestation, resulting in a dose-dependent maternal weight gain decline and in a decrease of T_3 and T_4 in plasma after the first week. PFOS levels in maternal circulation increased with dosage and were around 25% of the hepatic concentrations, which in turn were twice higher than those measured in fetal liver. At the highest doses (10 mg/kg b.w. per day in rat, 20 mg/kg b.w. per day in mouse), the Authors observed reduction of fetal body weight and higher incidence of anasarca, heart defects and cleft palate, with pups becoming pale and inactive after birth and dying in 4-6 h. Subjects whose dams had been treated with lower concentrations survived for a longer time (8-12 h). PFOS plasmatic concentrations in newborns were comparable to those in dams at day 21, decreasing in the following days. In surviving pups, persistent growth and eye-opening delays were observed, as well as hypothyroxinemia (Thibodeaux et al., 2003; Lau et al., 2003).
In another study, reduced duration of the gestation and pup viability were observed in female rats treated with PFOS from 6 weeks before mating till the fourth day of lactation (Luebker et al., 2005).
Delays in physical development were observed also in rabbits, as well as in both generations during a two-generation reproductive study in rats (EFSA, 2008).

Neurotoxicity

One dose of PFOS (of 0.75 or 11.3 mg/kg) was given by gavage to a group of 10 days old male mice, in order to evaluate its neurotoxic potential. Alterations of the behavior, mainly resulting in hyperactivity, were observed during experiments carried

out when they were 2 and 4 months old: further tests proved that these alterations were due to the involvement of the cholinergic system (Johansson et al., 2008).

1.3.3.2 Effects on humans

Developmental toxicity

Due to PFOS wide diffusion, capacity to cross the placenta and long half-life in humans, and to the outcomes concerning its adverse effects on the development in animals, a number of studies have been conducted in order to verify the occurrence of similar alterations in the population.

In a 2009 review by Olsen et al., the results of different epidemiological studies on general population and on occupationally exposed population were summarized. Potential correlations were studied in the general population between PFOS presence in maternal blood or umbilical cord and anthropometric parameters of the newborns, such as birth weight, birth length, head circumference and ponderal index. The investigations conducted on occupationally exposed population focused on associations between PFOS exposure of female workers from a perfluorochemical production facility and the birth weight of their sons. According to the Authors, the results of the mentioned works were inconsistent and, due also to the weakness of the epidemiological analysis (relevant factors as sex of the newborn or mother's age often weren't taken in account), couldn't prove the real existence of any of these associations (Olsen et al., 2009a).

Data collected by the Danish National Birth Cohort (DNBC) during a 1996-2002 monitoring were used to verify whether PFOS exposure could influence fecundity in humans. Blood concentrations of this contaminant were measured in 1240 women in early pregnancy and compared to their TTP (time to pregnancy, a commonly used surrogate of follow-up studies to estimate fecundity), defining infertility as a reported TTP of at least 12 months. Based on the results obtained, PFOS exposure at levels commonly found in the developed Countries seemed to reduce fertility (Fei et al., 2009).

A recent work on 123 paired samples of maternal and cord blood from Norwegian women showed the presence of various PFCs. Concentrations in cord blood corresponded to 30-79% of those in maternal blood, proving placental passage; however, a more efficient transfer to the fetus was observed for short-chain compounds and non sulfonated molecules, but also for branched PFOS isomers (Gützkow et al., 2011).

Other effects on health

A monitoring on 2083 3M Company workers in Alabama suggested that those whose job implied a relevant exposure to PFOS based substances had higher risk of death from bladder cancer, but this assertion was grounded on only 3 cases. A further follow-up study on those subjects identified 11 cases of bladder cancer, but without any significant correlations with PFOS exposure. Occupationally exposed employees were also subjected to cross-sectional analysis, which showed a positive correlation between exposure to these substance and increased serum T3 and triglycerides concentrations. Moreover, episodes of medical care in that plant had been more frequent in the most exposed workers. However, it's quite difficult to make firm deductions, due to various shortcomings of these investigations, related for example to the low number of participants and the lack of information on potential concurrent exposure to other compounds (EFSA, 2008).

The consequences of fetal exposure to PFCs on atopic dermatitis (AD) and levels of immunoglobulin E (IgE) were recently evaluated in Taiwan. Data were collected from several 2 years old children, correlating their serum IgE and PFCs levels and the potential development of AD with previously measured cord blood IgE and PFCs concentrations. A relation between pre-natal PFOS exposure and cord blood levels of IgE was observed, while it seemed not to have significant association with AD (Wang et al., 2011).

1.3.4 Toxic effects of PFOA

1.3.4.1 Effects on animals

Acute toxicity

Studies conducted in rats suggested a lethal concentration (LC_{50}) by inhalation of 980 mg/m^3, as well as an oral LD >500 mg/kg for male rats and >250 mg/kg for female rats, causing moderate acute toxicity. Symptoms observed after 4 h of exposure to PFOA via inhalation included increased liver size and opacity of the cornea; upon prolonged treatment (10 days), increased liver weight and reduction of body weight gain were observed. A feeble skin irritation was reported in rabbits, which resulted more sensitive than rats (EFSA, 2008).

Subacute toxicity

PFOA concentrations of 30 mg/kg through diet and of 50 mg/kg through drinking water during 28 days resulted in increased liver weight and limited body weight gain (and sometimes death) in rats and mice. In more extended studies, increased activity of hepatic palmitoyl CoA oxidase (marker for peroxisome proliferation) was reported after a 90 days oral administration of PFOA. At the histological level, hepatocellular hypertrophy and necrosis of liver cells were observed (EFSA, 2008).

An investigation was conducted on the different responses of mice and rats exposed to linear (now in use), 80% linear/20% branched (used in the past) and branched (synthesized for this study) PFOA via feeding tube during 14 days. The three forms showed similar toxicity, even if completely branched PFOA resulted less potent compared to the others (Loveless et al., 2006).

In monkeys, oral PFOA administration of up to 30 mg/kg b.w. per day for 26 weeks (during which weight loss and decreased food consumption were observed) caused dose-dependent increases in liver weight, resulting from mitochondrial proliferation, and the death of two subjects; no further macroscopic nor microscopic alterations were observed (Butenhoff et al, 2002).

Chronic toxicity

Two different 2 years-long studies were carried out on rats to evaluate chronic toxicity of PFOA. A first test on 50 male rats and 50 female rats, treated with doses of up to 14.2 and 16.1 mg/kg b.w. per day, respectively, showed dose-related decrease in body weight gain in both sexes (but more relevant in male rats) and increase in ataxia in female rats. Blood analysis showed decreased hematocrit, red blood cells count and hemoglobin values in the high-dose subjects, as well as increased levels of some enzymes in male rats. From the histological point of view, lesions and nodules were mainly observed in the liver (including also hepatomegalocytosis, hepatocellular necrosis, portal mononuclear cell infiltration and hepatic cystoids degeneration), testis (with a remarkably increased incidence of Leydig cell adenomas), mammary tissue and ovary. Based on the collected data, NOAELs of 1.3 and 1.6 mg/kg b.w. per day were calculated for male and female rats, respectively (US EPA, 2005).

A second experiment, involving 153 male rats subjected to a dietary exposure to PFOA of 14 mg/kg b.w. per day, confirmed the higher incidence of Leydig cell adenomas but highlighted also a significant rise in the incidence of liver adenomas and pancreatic acinar cell tumors. Concerning this last aspect, further investigations on pancreatic acinar cells revealed that PFOA was responsible for increasing the incidence of hyperplasia but not of tumor onset (EFSA, 2008).

Developmental and reproductive toxicity

Pregnant mice treated by oral gavage with PFOA doses of up to 40 mg/kg b.w. per day during the entire gestation showed increased liver weight; dams exposed to the highest concentration reabsorbed their litters, while those receiving medium or low concentrations had decreased percentage of live fetuses. These latter, in turn, showed reduced postnatal survival and body weight, as well as dose-dependent growth deficits and delayed eyes opening (Lau et al., 2006).

Later works by other Authors reported the same results and indicated that, in addition to intrauterine exposure, also lactation can contribute to the occurrence of the symptoms described in newborns. NOAELs were calculated of 30 mg/kg b.w. for

reproductive function, 10 mg/kg b.w. for pup mortality, birth weight and sexual maturation, <1 mg/kg b.w. for male body weight and organ weight changes (EFSA, 2008).

Neurotoxicity

In the previously mentioned study by Johansson et al. on PFOS neurotoxicity, also a single dose of PFOA (of 0.58 or 8.7 mg/kg) was administered to 10 days old mice, monitoring the effects when they were 2 and 4 months old. Observed symptoms included hyperactivity and lack of habituation; tests on the response to nicotine showed that these alterations were mediated by the cholinergic system (Johansson et al., 2008).

1.3.4.2 Effects on humans

PFOA effects on the population were investigated at the same time as PFOS in all the previously described works concerning the toxicity of this last. Lots of these studies were conducted on 3M Company workers occupationally exposed to PFOA, reporting, as for PFOS, potential correlations of this contaminant with the occurrence of various symptoms, sometimes inconsistent with each other and often quite poor in terms of statistical relevance.

However, similarly to PFOS, PFOA may be responsible for reduced fecundity in subjects exposed to average environmental levels and to increased levels of IgE in cord blood (Fei et al., 2009; Wang et al., 2011).

Also, PFOA showed a more efficient transfer to cord blood than PFOS during the study conducted by Gützkow et al. in Norway, which results in a more relevant fetal exposure to this compound.

Examining potential correlations of PFOA and PFOS concentrations in cord blood with gestional age and anthropometric parameters of the newborn in 293 subjects, Apelberg et al. suggested an association between these substances and birth weight and birth size (Apelberg et al., 2007).

According to EFSA, further investigations are needed to ascertain the actual responsibilities of PFOA and other PFCs for the described adverse effects on human health, since other factors may contribute significantly (EFSA, 2008).

1.3.5 Other PFCs and precursors toxicity

Besides PFOS and PFOA, other PFSAs (perfluoroalkyl sulfonates) and PFCAs (carboxylic acids) were included by some Authors in the range of analytes investigated but, due to their less relevant presence compared to the two most found PFCs, the scientific community has given minor attention to the potential adverse effects of these compounds. However, in many cases they have been associated to the same effects observed for PFOS and PFOA.

Half-life time is a specific parameter for each individual compound and, according to various Authors, in all the species it resulted sensibly shorter in shorter-chain molecules: therefore, PFBS and PFBA (whose chain is made of 4 carbon atoms) showed much shorter half-lives than those of the correspondent perfluorooctane-compounds, as well as values reported for PFNA and PFDA (9 and 10 carbon atoms, respectively) were higher than those observed for PFOA (Ohmori et al., 2003; Olsen et al., 2009b; Wilhelm et al., 2010).

This aspect was reflected also on the expression of the toxic potential: the longer the chain of the molecule, the greater were the effects observed. Experiments on rats indicated that PFBS and PFHxS, similarly to PFOS, increase the acyl CoA oxidase activity, even if a 50 times higher concentration of PFBS compared to the other two compounds was needed to generate similar effects; it was proved that PFHxA (only on male rats), PFNA and PFDA induce peroxisomal β-oxidation and hepatomegaly, depending on their concentrations in the liver (Lau et al., 2007).

Probably due to the fact that precursors FTOHs and FOSEs can be metabolized, representing a further source of PFCAs and PFSAs, respectively, only few studies investigated the direct toxicity of these compounds.

Some studies proved that 8:2 FTOH is a peroxisome proliferator and produces the same alterations as PFOA on the hepatic metabolism and enzymatic activity; FTOHs seem to interact with estrogen receptors. An in vitro research on hepatocytes showed that FTOHs are extensively metabolized in rats and mice, while clearance rate is significantly lower in humans (EFSA 2008; Harrad, 2009). In addition, assays performed on daphnia suggested that intermediate metabolites of their degradation are up to 10000 times more toxic than the correspondent PFCAs.
FOSA resulted more toxic than other PFCs, significantly suppressing DNA production and causing cellular oxidative stress. Its higher toxicity may be due to its potential to easily cross cell membranes, being more hydrophobic than other compounds. Similarly to certain PFCs, FOSA was found to inhibit gap junctional intercellular communication (Fields, 2007; Hu et al., 2002).
Toxic effects on development and enzymatic activity in animals were reported also for N-EtFOSE but, unlike PFOS, it doesn't apparently induce peroxisome proliferation. Moreover, according to in vivo and in vitro studies, also the carcinogenic activity of FOSEs seems related to an indirect (non-genotoxic) mechanism (Xie et al., 2009; EFSA 2008).

1.4 Legislation

An assessment made by OECD (Organization for Economic Cooperation and Development) in 2002 according to information available at that time concluded that PFOS is a persistent contaminant, presenting bioaccumulative potential and toxic effects on mammals.
On the basis of these statements, confirmed also by SCHER (Scientific Committee on Health and Environmental Risks), in order to safeguard human health and the environment the European Union decided to establish restrictions in the use and marketing of PFOS, issuing on December 12, 2006, Directive 2006/122/EC. The

measures contained in this document, which is a modification of Council Directive 76/769/EEC concerning restrictions that must be applied to various dangerous substances and preparations, should have been applied by Member States starting from June 27, 2008. These restrictions concern all those non-food products to which PFOS is added on purpose (also considering that it could have been used only in singular parts of a finished item) and refer exclusively to new products. More in details, it's not allowed to sell or use this compound in concentrations greater than 0.005% by mass; also, it's not allowed to place on the market semi-finished items, or parts, containing concentrations of PFOS higher than 0.1% by mass. However, some minor uses of PFOS are not subjected to these limitations, because they don't seem to be a risk and since no alternative substances are available: these applications include coatings for photolithography processes, photographic coatings, plating processes (which should anyway be minimized) and hydraulic fluids for aviation. In addition, the use of existing stocks of fire-fighting foams containing PFOS was allowed until June 27, 2011. Lastly, this Directive pointed out the need to focus the attention also on PFOA and its salts, which are believed to have a risk potential similar to PFOS.

What reported above was subsequently included in Commission Regulation (EC) No 552/2009 of June 22, 2009, on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).

There are currently no restrictions set by the European Community on PFCs presence in food and their use in plastic and paper used for food packaging is allowed in Germany and the Netherlands.

After issuing a first opinion on PFOA ammonium salt food contamination deriving from non-stick cookware, in 2008 EFSA (European Food Safety Authority) published a scientific opinion on PFOS and PFOA. This document, besides being a complete and updated source of information on perfluorinated compounds, reported the conclusions drawn by the CONTAM Panel: the risk for humans related to exposure to these contaminants was considered moderate, but potential effects on the development aren't clear yet. Moreover, it was recommended to collect further data on PFCs concentrations in food and in the population.

In 2009 PFOS and its salts were included in Annex B of the Stockholm Convention, which lists persistent organic pollutants (POPs) subjected to restrictions in use and production.

All these factors induced the European Commission to call Member States (through Recommendation 2010/161/EU, released on March 17, 2010) to monitor during 2010 and 2011 the presence of perfluoroalkylated substances in a wide variety of foodstuffs of both animal and plant origin, in order to allow a reliable estimation of human exposure. The monitoring should be directed towards PFOS and PFOA and, when possible, their precursors (including FOSA, N-EtFOSE and 8:2 FTOH) and similar compounds, such as homologues with different chain length and PAPS (polyfluoroalkyl phosphate surfactants). Member States are also recommended to periodically provide the collected data to EFSA, including available monitoring from previous years, in order to expand the European database.

In January 2011, EFSA published an intermediate report on the monitoring, summarizing the collected data and making recommendations to adjust current investigations. The final report will be available in 2012.

Concerning the United States, in 2002, after the termination of PFOS manufacturing by 3M Company, the Environmental Protection Agency (US EPA) issued two SNURs (Significant New Use Rules) in order to limit production or importation of 88 PFOS-related chemicals. 183 additional compounds were added to the list in 2007. Similarly to European Directive 2006/122/EC, these regulations permitted the continuation of a limited number of extremely technical applications of these substances, resulting in very low volumes and negligible releases, since no alternatives exist. In the event of any other use, manufacturers and importers are required to notify the US EPA a minimum of 90 days before it occurs (US EPA, 2010).

In Canada, after the insertion of PFOS and its related substances in the list of toxic compounds of the 1999 Canadian Environmental Protection Act (whose purpose was to prevent pollution and protect the environment), a regulation of 2009 added these molecules to the Virtual Elimination List. This means that Canada is the first Country proposing a complete ban of PFOS (SOR/2009-15).

“Kashinhou” is the name of the in use regulation law for chemicals and dangerous substances in Japan. Being hazardous compounds for human health, based on this law PFOS and PFOA were designated as Type II Monitoring Chemicals, therefore manufacturers and importers need to annually report their production or import volume. However, after the insertion of PFOS in Annex B of the Stockholm Convention, it has been classified as Class I Specified Chemical: as a consequence, similarly to what happens in Europe and in the United States, its manufacturing and use is prohibited except for specific essential uses (Yamazaki, 2009).

1.5 Methods of analysis

Perfluoroalkylated substances may significantly differ from each other concerning their chemical and physical features, therefore multiple analytical methods exist for their determination.

When performing analysis on these substances, the adoption of few simple but effective measures it’s suggested in order to reduce the risk of contaminations or losses and to guarantee the reliability of the results. During sample processing, laboratory equipment made of glass or Teflon should not be used, since the former can absorb PFASs while the latter represents a source of contamination (Hansen et al., 2001); polypropylene is thus preferable, being a non-interacting material. Similarly, contamination can occur during instrumental analysis, for this reason it’s advisable to use also polypropylene vials and, if PTFE (polytetrafluoroethylene) tubing are mounted on the LC system, to replace them with PEEK (polyether ether ketone) or stainless steel tubing (Tittlemier and Braekevelt, 2011).

Samples are usually stored in refrigerators and, when performing analysis of volatile compounds such as some PFCs precursors, it’s suggested to place them in completely filled tubes and then in freezers, as to prevent evaporation. Fresh or thawed samples

are generally analyzed, but in some studies food have been freeze-dried prior to extraction, without causing analyte losses.

To reduce the matrix-effect, complex samples are often pretreated: protein precipitation, for example, can be achieved through the addition of acetonitrile, formic acid or trifluoroacetic acid, followed by centrifugation.

Sample extraction can be performed in many ways, including solid phase extraction (SPE), liquid-liquid extraction (LLE), pressurized liquid extraction (PLE), ion pairing extraction (IPE) and solid phase microextraction (SPME, only for gas chromatography analysis). It may be followed by a further purification of the extract, generally involving SPE cartridges or SPE dispersive phase, aimed at the elimination of residual interferents.

Neutral volatile perfluoroalkylated substances, presenting high vapor pressures, can be measured with gas chromatography (GC) using medium or high polarity columns, while anionic PFCs need to be derivatized prior to analysis with this technique. Some of the anionic compounds have low vapor pressure and their derivatization proved to be poorly reproducible, therefore liquid chromatography (LC) resulted a better choice for these substances (EFSA, 2008). LC separation is performed in most cases on reversed phase C18 columns, using a mixture of an organic solvent (such as methanol or acetonitrile) and an aqueous solvent (typically ammonium acetate, at concentrations between 1-20 mM) as mobile phase (de Voogt and Sáez, 2006).

Mass spectrometry (MS) has allowed relevant improvement in the analysis of perfluoroalkylated substances, in particular since the introduction of triple quadrupole mass spectrometers (MS/MS), which are considered the most suitable detectors for this purpose. For PFASs detection in LC-MS(MS) systems, the most commonly used interface is electrospray ionization (ESI), working in negative mode to generate the pseudomolecular ions $[M\text{-}H]^-$. In GC-MS instruments, depending on the molecules, electronic impact (EI) as well as chemical ionization (CI) sources can be employed, in both positive and negative mode, with methane or ammonia as reagent gas; however, negative chemical ionization is the most widespread configuration, due to its sensitivity.

Other detectors have been tested with both LC (time of flight, ion trap, fluorescence and conductometric detectors) and GC (flame ionization and electron capture detectors), but triple quadrupole mass spectrometer was preferred in most of cases.

Besides mass spectrometry, also other analytical techniques have been used for PFASs analysis, including combustion methods, neutron activation and x-ray fluorescence (all three proving to be non-specific), and later nuclear magnetic resonance (quite unreliable in quantifications), ion exclusion chromatography (only on PFCAs) and Fourier transform infrared spectroscopy (EFSA, 2008; de Voogt and Sáez, 2006).

To this day, not many methods have been specifically developed for the determination of these substances in food; however, most of the techniques employed for the analysis of biological samples can generally be used also with food items (Tittlemier et al., 2007). One of the most frequently applied is the ion pairing extraction into methyl tert-butyl ether (MTBE) proposed by Hansen et al. (2001), which showed great flexibility in terms of matrices analyzed (liver, muscle, egg and others), but results laborious and requires considerable time. Also SPE cartridges (often weak anion exchange type) are employed in many studies; Powley et al. (2005) proposed a time saving alternative for the detection of PFCAs based on dispersive solid phase extraction with graphitized carbon, significantly reducing matrix-effect and maintaining good recovery values. Taniyasu et al. (2005a) introduced the extraction by alkaline digestion with potassium hydroxide (KOH), later modified by other Authors employing, for example, sodium hydroxide (NaOH) (Haug et al., 2010b). A simple and rapid pretreatment technique using pressurized liquid extraction was recently introduced by Llorca et al. (2009): the Authors suggested that this method improves LOQs and at the same time allows to easily process a large number of samples. Lastly, Luque et al. (2010) developed a new approach based on solvent microextraction, which allows simple and fast PFCs extraction with reduced solvent consumption.

LC-MS/MS resulted the analytical technique of choice for the detection of anionic PFCs in food matrices, working in MRM (multiple reaction monitoring) mode and using negative electrospray ionization, due to the strong electronegative character of the fluorinated chain (Tittlemier et al, 2007).

1.5.1 PFCs determination in milk

Most of the works available in literature investigating the presence of perfluorinated compounds in milk referred to human breast milk rather than milk from cow, goat or other animals. This is easily understandable considering what described in the previous sections: given their long half-life, PFCs enter the body through multiple routes accumulating for years and, during lactation, migrate in relevant amounts to breast milk, which therefore represents an alarming source of exposure for infants.

All the proposed methods were able to detect PFOS and PFOA, being the two most found and studied molecules of the group, but in many cases other perfluoroalkyl sulfonates (mainly PFHxS and PFDS) and carboxylic acids (in particular PFHxA, PFNA and PFDA) were monitored as well. Moreover, also FOSA (PFOS precursor) is often included among the target analytes, due to its recurrent non-negligible presence in food matrices.

Various techniques have been used for the extraction of these analytes from milk. Several methods employed weak anion exchange SPE cartridges, preceded by a protein precipitation step through formic acid (So et al., 2006; Nakata et al., 2006; Kärrman et al., 2007 and 2011; Tao et al., 2008a and b; Liu et al., 2010; Wang et al., 2010), acetonitrile (Völkel et al., 2008; Thomsen et al., 2010) or methanol (Bernsmann and Fürst, 2008; Ericson et al., 2008 Haug et al., 2010a). Some Authors performed also a final clean up by dispersive solid phase extraction with EnviCarb (Ericson et al., 2008; Haug et al., 2010a). Moreover, others among the cited methods included initial enzymatic digestion through lipase and protease addition and overnight incubation at 37°C (Bernsmann and Fürst, 2008; Mosch et al., 2010) or alkaline digestion (Haug et al., 2010a; Llorca et al., 2010).

The ion pairing extraction technique, introduced by Hansen et al., has been used for the detection of PFOS and PFOA in human breast milk samples by Guerranti et al. (2011).

A recently proposed approach employs an extraction with formic acid, acetonitrile, magnesium sulfate and sodium chloride, followed by the already mentioned clean up with dispersive EnviCarb (Lacina et al., 2011).

All the existing methods employ LC systems: more in particular, HPLC (high performance liquid chromatography) results the standard equipment for this kind of analysis being used in most of cases, even if few of the most recent works the method was optimized for UHPLC (ultra high performance liquid chromatography) instruments, significantly reducing the time of analysis (Haug et al., 2010a; Liu et al., 2010; Kärrman et al., 2011; Lacina et al., 2011).

Separation was generally achieved through C18 columns, due to their versatility and efficiency; the only exception is the method proposed by Lacina et al., which employed a T3 column, specifically conceived for the retention of polar organic compounds.

Also the choice of the mobile phase is a common point between the various techniques: a mixture of methanol and ammonium acetate aqueous buffer (at different concentrations, generally in the range 1-20 mM) was used by all the Authors except for Nakata et al., who chose acetonitrile as organic phase.

As for the detector, triple quadrupole mass spectrometer (equipped with ESI source in negative mode) working in MRM mode was the shared choice in almost all cases. Some Authors employed quadrupole-linear ion trap (Q-LIT) mass spectrometers as well, but always working in MRM mode; however Llorca et al. (2010) tested also the enhanced production ion (EPI) and MS^3 modes that this kind of instrument offers, reporting its limitation given by the low stability of fragment ions in the LIT.

Concerning the performances of the different approaches, various Authors reported limits of detection in the range of 0,01-0,1 ng/mL, depending on the compound; few works declared even better performances, with limits of detection <0,01 ng/mL (Thomsen et al., 2010; Liu et al., 2010; Lacina et al., 2011).

1.5.2 Analysis of PFCs volatile precursors

FOSES and FTOHs are more difficult to ionize than PFCs and tend to form adducts easily, therefore their analysis in LC-MS/MS it's quite difficult and requires all buffers to be removed from the system (Taniyasu et al., 2005b; Szostek et al., 2006).

Perfluorooctane sulfonamido ethanols (N-MeFOSE and N-EtFOSE) have been investigated mainly in air (Martin et al., 2002; Barber et al., 2007; Jahnke et al., 2007; Loewen et al., 2008) and house dust samples (Shoeib et al., 2005; Haug et al., 2011b), while no studies on food matrices have been performed. Analytes extraction was obtained using glass/quartz-fiber filters and XAD-2 resin inserted between polyurethane foam plugs or through Isolute ENV+ solid phase extraction cartridges. GC-MS/MS systems, equipped with chemical ionization sources, were employed for the analysis.

Fluorotelomer alcohols have often been investigated in air and house dust samples together with FOSEs by the previously cited Authors. However, FTOHs have been analyzed also in other matrices, such as water (Taniyasu et al., 2005b; Szostek et al., 2006; Motegi et al., 2010), soil (Ellington et al., 2009), plants (Yoo et al., 2011), food contact materials (Fengler et al., 2011) and biological matrices including plasma, liver, muscle and egg (Szostek and Prickett, 2004; Taniyasu et al., 2005b; Chu and Letcher, 2008). Different types of SPE cartridges (often Oasis® WAX and Oasis® HLB) were used for the extraction, while Szostek and Prickett, as well as Ellington et al., employed methyl tert-butil ether (MTBE). Gas chromatography was generally chosen for the analysis, but in some cases they were performed in LC-MS/MS systems; in particular, Chu and Letcher reported very good results employing a LC-APPI-MS/MS system (atmospheric pressure photoionization), which showed increased ionization capability and decreased matrix-effect, resulting in higher sensitivity and linearity compared to ESI (Chu and Letcher, 2008).

2. Objectives of the experiment

Perfluorinated compounds are a group of chemicals that have been largely employed during the last 60 years in several applications, widely spreading and accumulating in the environment due to their extreme resistance to degradation. As a consequence, they have been found also in various types of food as well as in drinking water, proving that they can easily reach humans through the diet. The available information concerning their adverse effects on health has recently increased the interest towards these contaminants and highlighted the importance of investigating all the potential sources of human exposure, among which diet was proved to be the most relevant. This need has been underlined by the European Union through Recommendation 2010/161/EU: in this document, Member States were called to monitor the presence of perfluoroalkylated substances in food, in order to produce accurate estimations of exposure.

In consideration of the above, the purpose of the research presented in this thesis was to develop efficient tools for the analysis of these pollutants in food, to be used for generating useful data on potentially contaminated matrices.

This work is the result of a partnership between two laboratories: CABA-Lab – Laboratorio di Chimica Analitica e Bio-Agroalimentare (Department of Veterinary Medical Sciences, University of Bologna, Italy) and LABERCA – Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (Oniris Nantes Atlantique, France).

The first phase of the project consisted of a 3 months period at LABERCA, during which an analytical method for the quantification of several perfluorinated compounds in cow milk, human breast milk and powder milk by means of liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) and high resolution mass spectrometry (HRMS) was optimized and validated in compliance with European Regulations. Moreover, within this experiment, data on PFCs presence in some French

milk samples were collected in order to be compared to those from other countries and also to estimate the risk of exposure for breastfed infants.

The second work package was carried out in the following months at CABA-Lab and its purpose was to transfer the previously developed method to the instrumentation available in the Italian laboratory, an ultra high performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS) system. Then, the same technique was used for the quantification of the two most important and frequently found PFCs (PFOS and PFOA) in a number of cow and human breast milk samples from Italy, in order to obtain data on these matrices also for this country.

The third part of the activity was conducted during another 3 months at LABERCA and was focused not on the already investigated substances, but rather on their precursors. In fact, as reported by the previously mentioned European Commission Recommendation 2010/161/EU, in order to make complete and reliable evaluations of the presence of PFCs in food matrices also other molecules, which can break down to give PFCs, should be considered. Therefore, preliminary tests were conducted for the detection of these precursors in fish (which is indicated as the most contaminated food by perfluoroalkylated substances), leading to the development of one of the first methods ever for their analysis in food matrices.

3. Material and methods

3.1 PFCs analysis at LABERCA

The purpose of this first work package was to optimize and validate a method for the detection of a wide range of PFCs in milk, comparing two different analytical approaches: liquid chromatography coupled to tandem mass spectrometry on a triple quadrupole instrument and liquid chromatography coupled to high resolution mass spectrometry on a LTQ-Orbitrap™ system. On the one hand, MS/MS is considered the current standard for this kind of analysis (EFSA, 2008), while on the other hand, the presence of some matrix effect using this kind of detectors and the interest in monitoring an increasing number of contaminants, make it interesting to evaluate the potential of HRMS instruments in this field.

Target compounds belonged to different families, as shown in **Table 3.1**:

Family	Chemical structure	Monitored compounds
Perfluoroalkyl carboxylic acids (PFCAs)	$F(CF_2)_x-C(=O)OH$	9
Perfluoroalkyl sulfonates (PFSAs)	$F(CF_2)_x-S(=O)_2-O^-$	5
Perfluoroalkyl sulfonamides (FASAs)	$F(CF_2)_x-S(=O)_2-NH_2$	1
Perfluoroalkyl sulfinates (PFSiAs)	$F(CF_2)_x-S(=O)-O^-$	1

Table 3.1 Groups of investigated compounds and relative chemical structure

The analysis of various cow milk, human breast milk and powder milk samples from France, carried out during method development and validation, provided also preliminary data on PFCs contamination of these three matrices in this country.

3.1.1 Material

Standards

All standards employed were from Wellington Laboratories (Wellington, Canada) with a purity grade >98% in methanol. Standards of the following PFCs were used:

- PFBA Perfluoro-n-butanoic acid [a]
- PFPA Perfluoro-n-pentanoic acid [a]
- PFHxA Perfluoro-n-hexanoic acid [a]
- PFHpA Perfluoro-n-heptanoic acid [a]
- PFOA Perfluoro-n-octanoic acid [a]
- PFNA Perfluoro-n-nonanoic acid [a]
- PFDA Perfluoro-n-decanoic acid [a]
- PFUnA Perfluoro-n-undecanoic acid [a]
- PFDoA Perfluoro-n-dodecanoic acid [a]
- PFBS Potassium perfluoro-1-butanesulfonate [b]
- PFHxS Sodium perfluoro-1-hexanesulfonate [b]
- PFHpS Sodium perfluoro-1-heptanesulfonate [b]
- PFOS Sodium perfluoro-1-octanesulfonate [b]
- PFDS Sodium perfluoro-1-decanesulfonate [b]
- FOSA Perfluoro-1-octane sulfonamide [c]
- PFOSi Sodium perfluoro-1-octanesulfinate acid [c]

[a] *in “PFC-MXA” mixture solution in methanol at a concentration of 2 µg/mL (1.2 mL)*
[b] *in “PFS-MXA” mixture solution in methanol at a concentration of 2 µg/mL (1.2 mL)*
[c] *in individual solution in methanol at a concentration of 50 µg/mL (1.2 mL)*

As internal standards, these ^{13}C-labeled compounds in individual methanol solutions (50 µg/mL, 1.2 mL) were purchased:

- M-PFBA Perfluoro-n-[1,2,3,4-$^{13}C_4$]butanoic acid
- M-PFOA Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid
- M-PFNA Perfluoro-n-[$^{13}C_9$]nonanoic acid
- M-PFDA Perfluoro-n-[1,2-$^{13}C_2$]decanoic acid
- M-PFUnA Perfluoro-n-[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid
- M-PFDoA Perfluoro-n-[1,2-$^{13}C_2$]dodecanoic acid
- M-PFHxS Sodium perfluoro-1-[$^{18}O_2$]hexanesulfonate
- M-PFOS Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate
- M-FOSA Perfluoro-1-[$^{13}C_8$]octane sulfonamide
- M-PFOSi Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfinate

After preparing 10 µg/mL stock solutions for all the compounds, the following working solutions were obtained by dilution:

- MIX PFC 10 ng/mL and MIX PFC 1 ng/mL, containing all the 16 PFCs listed above
- MIX M-PFC 10 ng/mL, containing the 10 M-PFCs listed above

A fluorometholone solution in methanol, at 10 µg/mL, was used as external standard.
All solutions were stored in the refrigerator at 4 °C and away from light.

Reagents and chemicals

Solvents employed for sample preparation and LC analysis:

- Methanol Picograde® (Promochem)
- Acetone Picograde® (Promochem)
- Ammonium acetate, Reag. Ph Eur (Merck)
- Ammonia solution 32%, extrapure (Merck)
- Glacial acetic acid (SDS)
- Formic acid, reagent grade (Sigma Aldrich)
- Ultrapure water (produced directly in the laboratory)

To extract the analytes from milk, two different solid phase extraction (SPE) cartridges were used:

- Oasis® HLB cartridges: 6 cc, 500 mg, 60 µm (Waters, Milford MA, USA)
- Supelclean™ ENVI-Carb™ cartridges: 6 cc, 500 mg, 120-400 mesh (Supelco, Bellefonte PA, USA)

Prepared solutions

- Ammonium acetate solution 20 mM: 1.54 g of ammonium acetate was dissolved in 1 L of ultrapure water and shaken vigorously
- Formic acid 0.1 M: 3.8 mL of formic acid and 996.2 mL of pure water were mixed in a 1 L bottle and shaken vigorously

Samples

Milk samples employed for method development and validation included:

- *Human breast milk*: 11 samples, obtained from a hospital in Nantes
- *Cow milk*: 9 commercial samples, purchased from various large retailers in the area of Nantes
- *Powder milk*: 4 samples, purchased from large retailers in the Nantes area (diluted 1:3 in ultrapure water)

Collected samples were transferred into polypropylene bottles, in order to prevent the risk of PFCs contamination caused by the original packaging material, and then stored in a freezer at -18 °C until analysis.

3.1.2 Equipment

HPLC-MS/MS system

An Agilent HP 1200 series HPLC pump (Agilent Technologies, Santa Clara CA, USA), provided with binary LC-pump (G1312B), vacuum degasser (G1379B), temperature controlled autosampler (G1367D) and thermostated column compartment (G1316B), was interfaced with an Agilent 6410 triple quadrupole mass spectrometer, equipped with ESI interface (Agilent Technologies, Santa Clara CA, USA).
A Phenomenex Gemini® reversed-phase C18 column (50 x 2.0 mm, 3.0 µm), fitted with a Phenomenex guard column with the same packing (10 x 2.0 mm, 3.0 µm) (Phenomenex, Torrance CA, USA), was employed for chromatographic separation.
Agilent Mass Hunter Workstation software was used for data acquisition and processing (Agilent Technologies, Santa Clara CA, USA).
A Schmidlin N2-Mistral-4 generator (Schmidlin Labor & Service, Neuheim, Switzerland) supplied the nitrogen needed for the ion source and used as collision gas.

HPLC-HRMS system

The HPLC system was an Agilent HP 1200 binary pump (Agilent Technologies, Santa Clara CA, USA), equipped with binary LC-pump (G1312B), vacuum degasser (G1379B), temperature controlled autosampler (G1367D) and thermostated column compartment (G1316B). This separation module was interfaced with a Thermo LTQ-Orbitrap™ Discovery system, consisting of a linear ion trap coupled with an orbital trap, equipped with an ESI ion source (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was achieved using a Phenomenex Gemini® reversed-phase C18 column (50 x 2.0 mm, 3.0 µm), fitted with a Phenomenex guard column with the same packing (10 x 2.0 mm, 3.0 µm) (Phenomenex, Torrance CA, USA).
Data were acquired and processed using Thermo Xcalibur™ 2.0 software (Thermo Fisher Scientific, Bremen, Germany).

Nitrogen required for instrument operation was produced by a Schmidlin N2-Mistral-4 generator (Schmidlin Labor & Service, Neuheim, Switzerland).

Other equipment

The following equipment was used during the development of the method and for samples preparation:

- Solid phase extraction manifold (Supelco, Bellefonte PA, USA)
- Centrifuge (Jouan, Winchester VA, USA)
- Microcentrifuge (Thermo Fisher Scientific, Bremen, Germany)
- Water purification system (Barnstead, Germany)
- Nitrogen sample concentrator
- Thermostated dry bath (Fisher Scientific, Illkirch, France)
- Automatic pipettes (Eppendorf, Hamburg, Germany)
- Vortex mixer
- Ultrasonic bath

3.1.3 Instrumental conditions

LC conditions

The mobile phase consisted of:

- Phase A: ammonium acetate solution 20 mM
- Phase B: methanol

Analysis were carried out under programmed conditions, at flow rate of 0.6 mL/min. After 2 min at 70% A and 30% B, the gradient switched linearly to 0% A and 100% B over 5 min, remained in this conditions for 4 min and then returned to 30% A and 70% B in 4 min. A further 5 min at the starting conditions were needed to equilibrate the column before the following injection.

The following table resumes the chromatographic gradient.

Time (min)	Phase A (%)	Phase B (%)
0	70	30
2	70	30
7	0	100
11	0	100
15	70	30
20	70	30

Table 3.2 Mobile phase gradient program

Column was thermostated at 40 °C in order to keep not too high back pressure values, while samples were kept at 6 °C in the autosampler during the day of analysis.
Injection volume varied between the two systems, being 5 µL on the triple quadrupole and 20 µL on the LTQ-Orbitrap.

MS/MS conditions

The device operated in negative electrospray ionization (ESI-) mode.
Acquisitions were performed in MRM (multiple reaction monitoring) mode, following, when possible, two transitions for each molecule.
The table on the next page shows the precursor-to-product transitions, with relative cone voltage and collision energy values, expressed in eV.
Mass spectrometer settings:

- Capillary voltage: 3.00 kV
- Source temperature: 250 °C
- Desolvation temperature: 300 °C
- Desolvation gas flow: 10 L/min
- Nebulization gas flow: 45 psi

Analyte	Transition 1 (*m/z*)	Cone voltage	Collision energy	Transition 2 (*m/z*)	Cone voltage	Collision energy
PFBA	212.9 > 168.9	60	5			
M-PFBA	216.9 > 171.9	60	5			
PFPA	262.9 > 218.9	60	5			
PFHxA	312.9 > 268.9	60	5	268.9 > 118.9	100	15
PFHpA	362.9 > 318.9	70	5	318.9 > 168.9	100	10
PFOA	412.9 > 368.9	80	5	412.9 > 169.1	80	15
M-PFOA	416.9 > 371.9	80	5			
PFNA	462.9 > 418.9	90	5	418.9 > 168.9	120	15
M-PFNA	471.9 > 426.9	90	5			
PFDA	512.9 > 468.9	90	5	468.9 > 218.9	130	15
M-PFDA	514.9 > 469.9	90	5			
PFUnA	562.9 > 518.9	80	5	562.9 > 268.9	120	15
M-PFUnA	569.9 > 524.9	80	5			
PFDoA	612.9 > 568.9	100	5	612.9 > 168.9	120	25
M-PFDoA	614.9 > 569.9	100	5			
PFBS	298.9 > 99.0	120	40	298.9 > 80.0	120	40
PFHxS	398.9 > 99.0	80	50	398.9 > 80.0	80	50
M-PFHxS	402.9 > 84.0	80	50			
PFHpS	448.9 > 99.0	60	45	448.9 > 80.0	60	45
PFOS	498.9 > 80.0	60	45	498.9 > 99.0	60	45
M-PFOS	502.9 > 502.9	60	15			
PFDS	598.9 > 80.0	80	50	598.9 > 99.00	80	50
FOSA	497.9 > 78.1	150	35	497.9 > 219.0	150	25
M-FOSA	506.0 > 78.1	150	35			
PFOSi	483.0 > 419.0	80	15	483.0 > 219.0	80	5
M-PFOSi	487.0 > 423.0	80	15			

Table 3.3 Monitored transitions and their individual parameters

HRMS conditions

The instrument operated in negative electrospray ionization mode (ESI-), recording mass spectra from 200 to 900 *m/z* in full scan mode, with a resolution of 15000 FWHM at 400 *m/z* in centroid mode.

For each analyte and the corresponding internal standard, the pseudomolecular ion $[M-H]^-$ at its exact mass was extracted from full scan recording, using the *m/z* values reported in **Table 3.4**.

Mass spectrometer parameters:

- Capillary voltage: 4.00 kV
- Cone voltage: -14 V
- Nebulization gas temperature: 280 °C
- Sheath gas flow (nitrogen): 6 (arbitrary unit)
- Tube lens voltage: -85 V

Analyte	**$[M-H]^-$ exact mass (*m/z*)**	**Analyte**	**$[M-H]^-$ exact mass (*m/z*)**
PFBA	212.9792	PFDoA	612.9536
M-PFBA	216.9926	M-PFDoA	614.9603
PFPA	262.9760	PFBS	298.9429
PFHxA	312.9728	PFHxS	398.9366
PFHpA	362.9696	M-PFHxS	402.9450
PFOA	412.9664	PFHpS	448.9334
M-PFOA	416.9798	PFOS	498.9302
PFNA	462.9632	M-PFOS	502.9302
M-PFNA	471.9934	PFDS	598.9238
PFDA	512.9600	FOSA	497.9462
M-PFDA	514.9667	M-FOSA	505.9730
PFUnA	562.9568	PFOSi	482.9353
M-PFUnA	569.9803	M-PFOSi	486.9487

Table 3.4 Pseudomolecular ions HRMS diagnostic signals

3.1.4 Extraction procedure

First of all 3 mL of milk were transferred into a polypropylene tube and spiked with 50 µL of MIX M-PFC (0.01 µg/mL) solution, then 9 mL of acetone were added to perform a protein precipitation.

After vortex shaking for 30 sec, the sample was placed during 5 min in an ultrasonic bath to facilitate the extraction and centrifuged at 2000 g for 10 min at room temperature.

The supernatant was transferred to a new polypropylene tube and evaporated to around 3 mL under gentle nitrogen stream at 45 °C, then 8 mL of formic acid 0.1 M solution were added in order to adjust the pH for the first purification step on the Oasis HLB® cartridge.

After conditioning the cartridge with 10 mL of methanol and 10 mL of formic acid 0.1 M, always avoiding the solid phase to go dry, the sample was loaded.

Once all the solution had passed through the column, two washings were performed with 5 mL of formic acid 0.1 M and with 5 mL of formic acid 0.1 M/methanol 50/50 solution, then vacuum was applied for 5 min to remove eventual residual drops.

The analytes were eluted with 6 mL of a mixture of methanol/ammonia solution 32% 99/1 and subsequently concentrated to around 2 mL under nitrogen.

A second purification was achieved using a Supelclean™ ENVI-Carb™ cartridge, previously activated with 10 mL of methanol.

After placing a new tube under the column, the sample was loaded and then an elution with 6 mL of a methanol/glacial acetic acid 80/1 solution was performed.

The eluate was evaporated to dryness under nitrogen and reconstituted in 100 µL of fluorometholone methanolic solution. After vortex shaking for 30 sec, 100 µL of ammonium acetate solution 20 mM were added and the solution was agitated for another 15 sec.

The content of the tube was transferred into a microtube and centrifuged at 12000 rpm during 45 min, then 150 µL were collected and added to 50 µL of ammonium acetate solution 20 mM in a polypropylene vial.

The final solution, thus consisting of methanol/ammonium acetate 20 mM 37.5%/62.5%, was injected in the two instruments.

The procedure is schematically resumed in **Figure 3.1**.

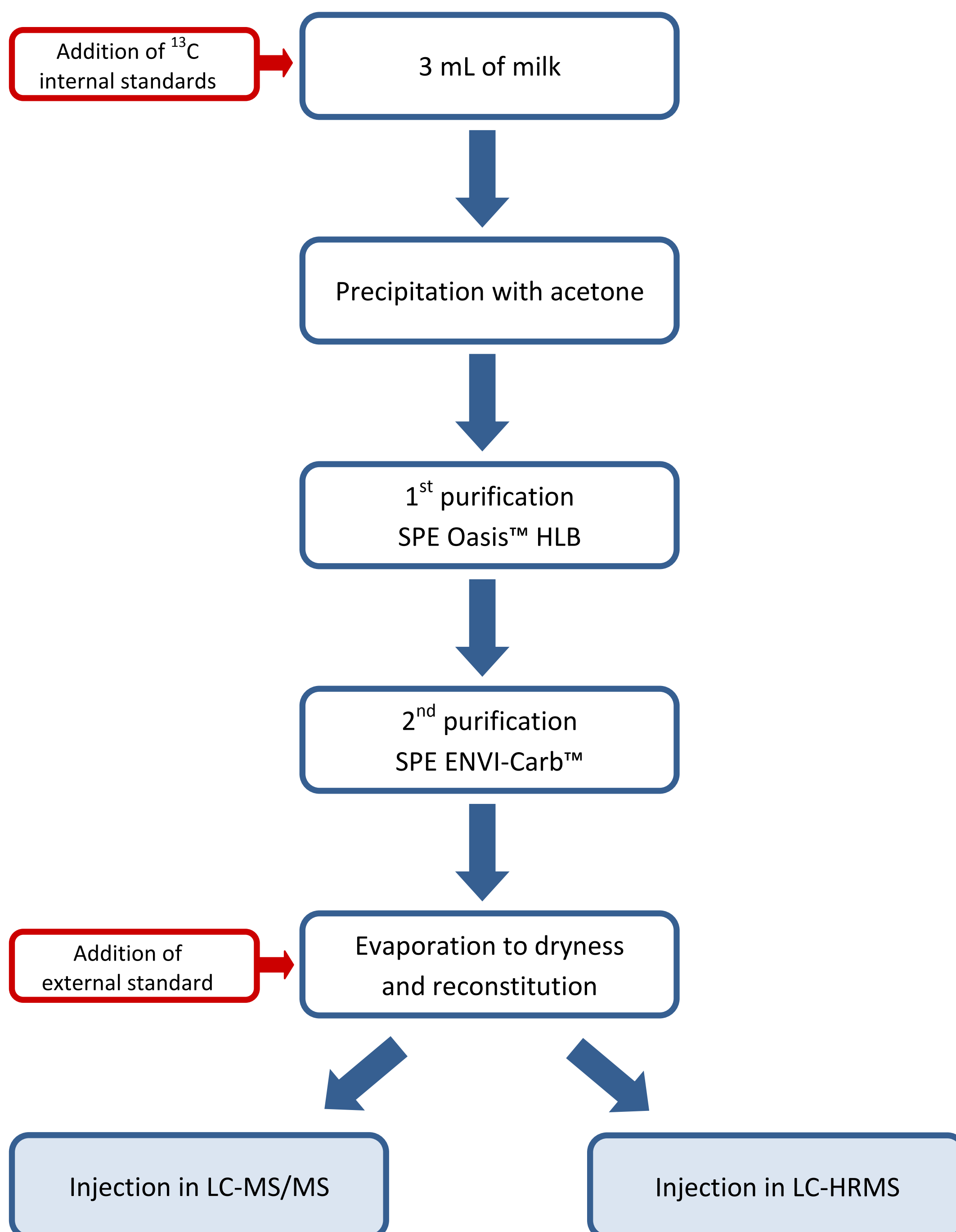

Figure 3.1 Extraction procedure

3.1.5 Method validation

The described method was validated on both LC-MS/MS and LC-HRMS systems according to current European regulations (Commission Decision 2002/657/EC).

10 different ^{13}C-labeled internal standards were employed, so that each compound was quantified calculating the ratio between its area and the area of the relative internal standard.

Specificity

In order to assess the specificity of the method, the analysis of milk samples presenting low levels of contamination (8 of human breast milk, 4 of cow milk and 4 of powder milk) was carried out. The screening was performed twice, changing the starting amount of milk: 1 mL and 3 mL.

Identification criteria

In accordance with 2002/657/EC guidelines, requirements for mass spectrometric detection were verified. These included:

- Retention time: for a sample analyzed in LC, the relative retention time of the analyte shall correspond to that of the standard solution at a tolerance of ±2.5%
- Relative ion intensity: tolerances, depending on the ratio between the intensity of the two monitored transitions, are reported in the following table

Ratio	Tolerance (%)
R ≤10%	50
10%< R <20%	30
20%< R <50%	25
R ≥50%	20

Table 3.5 Maximum permitted tolerances for relative ion intensities

Linearity

Linearity was evaluated on each matrix through the analysis of calibration curves, prepared during three different days: 1 mL milk samples were spiked at 7 different levels of concentration (plus blank), following the scheme reported in **Table 3.6**.

A linear regression model was applied, associating the relative response of target compound to the concentration.

Name	Concentration (ng/mL = ppb)	µL MIX M-PFC (10 ng/mL)	µL MIX PFC (1 ng/mL)	µL MIX PFC (10 ng/mL)
G 0	0	50	-	-
G 0.025	0.025	50	25	-
G 0.05	0.05	50	50	-
G 0.1	0.1	50	100	-
G 0.2	0.2	50	200	-
G 0.5	0.5	50	-	50
G 1	1	50	-	100
G 2	2	50	-	200

Table 3.6 Calibration curve preparation

Limits of detection

Limits of detection were determined for both systems taking into account the chromatograms resulting from the analysis of cow milk's calibration curve.

For LC-MS/MS system, limits were calculated as the concentration giving a signal-to-noise ratio of 3; for LC-HRMS system, being important that no noise is observed in the chromatograms, limits were evaluated on the basis of the differences between peaks areas.

Recovery

To verify the recovery of the analytical procedure, for each matrix two samples spiked at 0.5 ng/mL, one before extraction and the other immediately after, were compared.

Precision

Taking into account the contamination levels likely to be found in the considered matrices, precision was evaluated with two different approaches depending on the type of milk.

For cow and powder milk, the 4 less contaminated samples were employed, performing two tests at different spiking levels (0.1 ng/mL and 0.5 ng/mL, n = 2*4); for human breast milk, 8 samples taken from a pool prepared using the 8 less contaminated samples available were spiked at 0.5 ng/mL (n = 8).

Relative standard deviation to the mean (CV%) should be lower than the value calculated by the Horwitz equation:

$$CV\% = 2^{(1-0.5\log C)}$$

being C the mass fraction expressed as a power of 10. For mass fractions lower than 100 µg/kg this formula generates excessively high values, therefore in those cases CV% shall be as low as possible.

Trueness

Since to this day no milk reference material with certified PFCs concentrations is available, it was decided to verify trueness using 2 human breast milk samples from an international ring test, one from Sweden and one from Germany.

In addition, being available in the laboratory an optimized protocol for the analysis of perfluorinated compounds in serum implying a similar extraction and purification procedure to that developed for milk, a certified serum sample (NIST SRM 1957) was analyzed twice to obtain further data on trueness.

Trueness was expressed as bias, that is the difference between the mean measured value and the reference value.

Uncertainty

Measurement uncertainty for PFOA and PFOS was evaluated on the LC-HRMS system, considering precision, trueness and standards purity.

- Uncertainty in precision ($U_{precision}$) was calculated on the basis of the repeatability data obtained from the analyzed samples and from three further series of eight human breast milk samples (spiked at 0.1, 0.25 and 0.75 ng/mL), added to increase the statistical significance of the value. To calculate CV% the following formula was used:

$$U_{precision} = CV\% = \sqrt{\frac{(n_1 - 1) * {CV_1}^2 + (n_2 - 1) * {CV_2}^2 + \ \dots}{(n_1 - 1) + (n_2 - 1) + \ \dots}}$$

- For trueness, uncertainty ($U_{trueness}$) was evaluated as a ratio between the bias and the certified value (in this case, the mean of the values obtained for the 2 ring test's human breast milk samples was used):

$$U_{trueness} = \frac{(certified\ value - measured\ value) \times 100}{certified\ value}$$

- Uncertainty related to standard solutions purity (U_{purity}) was calculated through this formula:

$$U_{purity} = \frac{1 - (purity/100)}{\sqrt{3}}$$

Combining the obtained factors, relative combined uncertainty (U_{RC}) resulted from this equation:

$$U_{RC} = \sqrt{(U_{precision})^2 + (U_{trueness})^2 + (U_{purity})^2}$$

Then, final uncertainty (U_F), with a 95% confidence interval, was calculated as twice the relative combined uncertainty:

$$U_F = 2 * U_{RC}$$

3.1.6 Samples quantification

For each day of analysis, a calibration curve in ultrapure water (to avoid potential interferences due to milk contamination) was prepared following the same procedure described for calibration curves in milk, employed to verify method's linearity (see **Section 3.1.5**). Moreover, in compliance with 2002/657/EC guidelines, the previously mentioned identification criteria were verified.

In order to perform a correct quantification of perfluoroalkyl sulfonates and perfluoroalkyl sulfinates, a specific correction factor was applied to each molecule (see **Table 3.7**), since they are sold in salt form and thus their concentrations are always referred to those of the anionic form. This specific factor was calculated as the ratio between the molar mass of the anionic form of the compound and that of its salt.

Compound	Correction factor
$PFBS^-$	0,929
$PFHxS^-$	0,945
$PFHpS^-$	0,951
$PFOS^-$	0,956
$PFDS^-$	0,963
$PFOSi^-$	0,952

Table 3.7 Corrections factors for sulfonic acids

3.2 PFCs analysis at CABA-Lab

During this second work package the method developed at LABERCA was transferred to CABA-Lab to verify its performances in the analysis of PFOA and PFOS in milk, being the two most important molecules of this group, using the UHPLC-MS/MS system available in the laboratory.

Once the efficiency of the method had been ascertained, it was employed for the analysis of several cow milk and human breast milk samples from Italy, in order to obtain some preliminary data on PFCs contamination also for this country. Moreover, taking into account the available information on such samples, the monitoring tried to reveal potential correlations between the characteristics of each kind of milk and its level of contamination.

3.2.1 Material

Standards

To verify the method performance and for samples quantification, PFOA, PFOS and relative $^{13}C_4$-labeled M-PFOA e M-PFOS standards (employed as internal standards) were used.

All standards solutions were purchased from Wellington Laboratories (Guelph, Canada) with a purity grade >98% in methanol:

- PFOA Perfluoro-n-octanoic acid: 50 µg/mL, 1.2 mL
- PFOS Sodium perfluoro-1-octanesulfonate: 50 µg/mL, 1.2 mL
- M-PFOA Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid: 50 µg/mL, 1.2 mL
- M-PFOS Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate: 50 µg/mL, 1.2 mL

Starting from these products, the following standard solutions were prepared:

- Stock solutions: for each compound a 5 µg/mL solution was prepared diluting 1:10 in methanol the relative 50 µg/mL standard
- PFC stock solution (0.5 µg/mL): 20 µL of PFOS (50 µg/mL) and 20 µL of PFOA (50 µg/mL) were dissolved in 2 mL of methanol.

  Thereafter, diluting this PFC stock solution in methanol, the following working solutions were obtained:
  - PFC working solution (50 ng/mL)
  - PFC working solution (5 ng/mL)
- M-PFC working solution (50 ng/mL): 100 µL of M-PFOA tune solution (5 µg/mL) and 100 µL of M-PFOS tune solution (5 µg/mL) were dissolved in 10 mL of methanol

All solutions were stored in refrigerator at 4 °C and away from light.

Reagents and chemicals

All solvents used for mass spectrometry analysis were LC-MS grade:

- Methanol (Sigma Aldrich)
- Ammonium acetate (Fluka)
- Ultrapure water (produced directly in the laboratory)

Solvents employed during sample treatment were all analytical grade:

- Acetone (VWR)
- Ammonia solution 33% (Sigma Aldrich)
- Methanol (Sigma Aldrich)
- Glacial acetic acid (Sigma Aldrich)
- Formic acid (Sigma Aldrich)
- Pure water (produced directly in the laboratory)

Two different solid phase extraction (SPE) cartridges were used to extract the analytes from milk:

- Oasis® HLB cartridges: 6 cc, 500 mg, 60 µm (Waters, Milford MA, USA)
- Supelclean™ ENVI-Carb™ cartridges: 6 cc, 500 mg, 120-400 mesh (Supelco, Bellefonte PA, USA)

Prepared solutions

- Ammonium acetate solution 20 mM: 1.54 g of ammonium acetate was dissolved in 1 L of ultrapure water and shaken vigorously
- Formic acid 0.1 M: 3.8 mL of formic acid and 996.2 mL of pure water were mixed in a 1 L bottle and shaken vigorously

Samples

To test and optimize at CABA-Lab the previously developed method, milk purchased in a store located in the province of Bologna was used, being stored in the refrigerator for a limited number of hours prior to analysis.

The preliminary monitoring included cow and human breast milk:

- *Human breast milk*: 13 samples, provided by the Department of Gynecology, Obstetrics and Pediatrics of Bologna University. Sampling was authorized by the Independent Ethics Committee of Bologna University Hospital Authority Sant'Orsola-Malpighi Polyclinic (clinical trial # 49/2011/U/Tess).
- *Cow milk*: 22 samples, including 16 different varieties of milk purchased from large retailers in the province of Bologna, 1 raw milk purchased at a vending machine in the province of Bologna and 5 milk samples provided by the experimental farm of Ozzano dell'Emilia (Bologna, Italy), owned by the Faculty of Veterinary Medicine of Bologna University.

Collected samples were transferred into polypropylene bottles, in order to prevent the risk of PFCs contamination caused by the original packaging material, and then stored in freezer at -18 °C until analysis.

Sample	Primipara / Multipara	Sample	Primipara / Multipara
A	Primipara	I	Multipara
B	Primipara	J	Multipara
C	Primipara	K	Multipara
D	Primipara	L	Multipara
E	Primipara	M	Multipara
F	Primipara		
G	Primipara		
H	Primipara		

Table 3.8 Human milk samples and relative mother's primipara/multipara status

Sample	Type	Sample	Type
A	UHT whole	L	High Quality* whole
B	Pasteurized whole	M	Microfiltered partially skimmed
C	Microfiltered whole	N	High digestibility
D	Organic whole	O	UHT partially skimmed
E	Organic whole	P	Pasteurized partially skimmed
F	Organic whole	Q	Raw
G	Pasteurized whole	R	Sampled during milking
H	UHT skimmed	S	Sampled during milking
I	Pasteurized partially skimmed	T	Sampled during milking
J	Whole	U	Sampled during milking
K	Organic whole	V	Sampled during milking

** According to Italian D.M. 185/91, a milk can be given the "High Quality" appellation if the following requirements are satisfied:*
- fat content not lower than 3.50%
- protein content not lower than 32.0 g/L
- bacteria count at 30 °C lower than 100000/mL (mean value monitored during two months at least twice per month)
- somatic cell count lower than 300000/mL (mean value monitored during two months at least twice per month)
- lactic acid content lower than 30 ppm

Table 3.9 Cow milk samples and type specifications

3.2.2 Equipment

UHPLC-MS/MS system

The system employed for PFCs analysis consisted of a Waters Acquity UPLC® binary pump (provided with degasser, thermostated autosampler and column compartment), coupled with a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA).

Chromatographic separation was achieved using a Waters Acquity UPLC® BEH C18 reversed-phase column (50 x 2.1 mm, 1.7 µm), fitted with a Waters VanGuard™ guard column with the same packing (5 x 2.1 mm, 1.7 µm) (Waters Corporation, Milford MA, USA).

Data were acquired and processed using Waters MassLynx™ 4.1 software (Waters Corporation, Milford MA, USA).

The nitrogen needed for the operation of mass spectrometer's interface was produced by a DBS N2-Mistral-4 generator (DBS Strumenti Scientifici, Padova, Italy).

Other equipment

During method transfer and milk samples preparation, the following equipment was employed:

- Solid phase extraction manifold (Waters Corporation, Milford MA, USA)
- Centrifuge (Hettich, Kirchlengern, Germany)
- Water purification system (Human Corporation, Seoul, Korea)
- Nitrogen sample concentrator
- Waterbath (Grant Instruments, Cambridge, GB)
- Automatic pipettes (Gilson, Middleton WI, USA)
- Vortex mixer (Velp Scientifica, Monza e Brianza, Italy)
- Ultrasonic bath (AGE Elettronica, Milano, Italy)

3.2.3 Instrumental conditions

LC conditions

The mobile phase consisted of:

- Phase A: ammonium acetate solution 20 mM
- Phase B: methanol

Analysis were carried out under programmed conditions, at flow rate of 0.5 mL/min.
The gradient started with 30 sec at 70% A and 30% B, switched to 0% A and 100% B over 1 min and hold for 1.5 min. Then conditions went back to 30% A and 70% B in 0.5 min and hold for further 1.5 min, in order to equilibrate the column before the following injection.
The chromatographic gradient is resumed in the following table:

Time (min)	Phase A (%)	Phase B (%)
0	70	30
0.5	70	30
1.5	0	100
3	0	100
3.5	70	30
5	70	30

Table 3.10 Mobile phase gradient program

Injection volume was 10 µL, in “full loop” mode; samples were kept at 6°C in the autosampler during the day of analysis, while the column was kept at 45 °C to avoid excessive backpressures.

MS/MS conditions

Mass spectrometer operated in negative electrospray ionization (ESI-) mode.
Analysis were performed in MRM (multiple reaction monitoring) mode, following two transitions for PFOS and PFOA and one for each internal standard. Argon was used as collision gas.

In **Table 3.11** the precursor-to-product transitions are reported, with the correspondent cone voltage and collision energy, expressed in eV.

Analyte	Transition 1 (*m/z*)	Cone voltage	Collision energy	Transition 2 (*m/z*)	Cone voltage	Collision energy
PFOA	412.82 > 368.97	12	10	412.82 > 168.98	12	17
PFOS	498.64 > 498.64	52	10	498.64 > 79.98	52	43
M-PFOA	416.93 > 372.00	11	10			
M-PFOS	502.86 > 502.86	50	11			

Table 3.11 Monitored transitions and their relative specific parameters

Mass spectrometer settings:

- Capillary voltage: 2.00 kV
- Extractor voltage: 3.00 V
- Source temperature: 150 °C
- Desolvation temperature: 220 °C
- Cone gas flow: 50 L/h
- Desolvation gas flow: 700 L/h

3.2.4 Extraction procedure

The previously described extraction procedure (refer to **Section 3.1.4**) was transferred to CABA-Lab without significant changes, except for the different amount of internal standard added to the samples, being in this case 30 µL of M-PFC working solution at 50 ng/mL.

Moreover, it was chosen not to use an external standard, therefore after evaporation to dryness the sample was reconstituted in methanol/ammonium acetate.

3.2.5 Performances of the transferred method

An apparently not contaminated cow milk sample was employed for the preparation of an 8 levels matrix-matched calibration curve, in order to assess linearity and limits of detection of the transferred method.

To this purpose, starting with 3 mL fractions of milk, 30 µL of M-PFC working solution were added as internal standard, followed by increasing amounts of the two PFC working solutions according to the scheme reported in **Table 3.12**.

The 8 solutions obtained, with concentrations from 0 to 2 ng/mL, were then injected in the UHPLC-MS/MS system.

Name	Concentration (ng/mL = ppb)	µL M-PFC (50 ng/mL)	µL PFC (5 ng/mL)	µL PFC (50 ng/mL)
C 0	0	30	-	-
C 0.025	0.025	30	15	-
C 0.05	0.05	30	30	-
C 0.1	0.1	30	60	-
C 0.2	0.2	30	120	-
C 0.5	0.5	30	-	30
C 1	1	30	-	60
C 2	2	30	-	120

Table 3.12 Calibration curve preparation

3.2.6 Samples quantification

As well as during the analysis carried out at LABERCA, quantification at CABA-Lab was performed in accordance with 2002/657/EC criteria (see **Section 3.1.6**).

An 8 levels calibration curve in ultrapure water was prepared each day of analysis to quantify samples, following the above scheme used for the linearity test.

To perform a correct quantification, a correction factor was applied to PFOS.

3.3 PFCs precursors analysis at LABERCA

With Recommendation 2010/161/EU, the European Commission called Member States to monitor the presence of perfluoroalkylated substances in food, in order to detect the presence of PFOA and PFOS, but also, when possible, their precursors, such as perfluorooctane sulfonamide (FOSA), perfluorooctane sulfonamido ethanols (FOSEs) and fluorotelomer alcohols (FTOHs).

Having already included FOSA in the list of molecules monitored with the previously developed method for PFCs analysis (see **Section 3.1**), during this third work package preliminary tests were performed in order to investigate other precursor compounds (see **Table 3.13**) in fish, trying if possible to include them in the range of perfluoroalkylated substances monitored with the already existing LABERCA procedures.

Family	Chemical structure	Monitored compounds
Perfluorooctane sulfonamido ethanols (FOSEs)	$F(CF_2)_8-S(=O)_2-N(R)-CH_2CH_2-OH$	2
Fluorotelomer alcohols (FTOHs)	$F(CF_2)_x-CH_2CH_2-OH$	3

Table 3.13 Groups of precursors considered and relative chemical structure

On the basis of the few works available in literature and of some experiments conducted in the laboratory, gas chromatography was considerate the most suitable technique for the analysis of these molecules, which are extremely volatile.

3.3.1 Material

Standards

The employed standards solutions of FOSEs and FTOHs were from Wellington Laboratories (Wellington, Canada), with a concentration of 50 µg/mL in ethanol and a purity grade >98%:

- N-MeFOSE 2-(N-methylperfluoro-1-octanesulfonamido)-ethanol
- N-EtFOSE 2-(N-ethylperfluoro-1-octanesulfonamido)-ethanol
- FHET 2-Perfuorohexyl ethanol (6:2 FTOH)
- FOET 2-Perfuorooctyl ethanol (8:2 FTOH)
- FDET 2-Perfuorodecyl ethanol (10:2 FTOH)

As internal standards, one labeled standard for FOSEs and one for FTOHs (50 µg/mL in methanol, purity grade >98%) were purchased from Wellington Laboratories (Wellington, Canada):

- d7-N-MeFOSE 2-(N-deuteriomethylperfluoro-1-octanesulfonamido)-1,1,2,2-tetradeuterioethanol
- M-FOET 2-Perfuorooctyl-[1,1,-$^{2}H_{2}$]-[1,1,-$^{13}C_{2}$]-ethanol (M-8:2 FTOH)

Stock solutions at 10 µg/mL of all the compounds were prepared in methanol; the following working solutions were obtained by dilution:

- FOSE+FTOH 1 µg/mL, FOSE+FTOH 0.1 µg/mL and FOSE+FTOH 0.01 µg/mL, containing the all the 5 analytes investigated
- IS FOSE+FTOH 0.1 µg/mL, containing both internal standards employed

All solutions were stored in the refrigerator at 4 °C and away from light.

Reagents and chemicals

In addition to solvents already described in **Section 3.1.1**, Dichloromethane Picograde® (Promochem) was employed.

Other chemicals needed for sample extraction:

- Supelclean™ ENVI-Carb™ SPE bulk packing 120-400 mesh (Supelco)
- Silica gel 70-230 mesh (Fluka)

Preparation of silica column

The required hydrated silica (1.5%) was obtained shaking several times during one day, each time for 5 min, a flask containing 98.5 g of silica gel and 1.5 mL of water.
After placing a thick layer of glass wool on the bottom of the column, previously positioned on the manifold, two washings with 5 mL of acetone and 5 mL of dichloromethane were performed, then 4 g of hydrated silica were added.

3.3.2 Equipment

GC-MS/MS system

FOSE and FTOH analysis were performed using a system composed of an Agilent 7890 gas chromatographer coupled to an Agilent 7000 triple quadrupole mass spectrometer (equipped with chemical ionization interface) and provided with an Agilent 7693 autosampler (Agilent Technologies, Santa Clara CA, USA).
A Varian CP-WAX 57CB capillary column (25 m, 0.25 mm I.D., 0.20 µm film thickness) (Varian, Palo Alto CA, USA) was used for chromatographic separation.
Data were acquired and processed with Agilent Mass Hunter Workstation software (Agilent Technologies, Santa Clara CA, USA).

Other equipment

The same equipment described in **Section 3.1.2** was employed, with the addition of:

- Grinder
- Horizontal mechanical shaker

3.3.3 Instrumental conditions

GC-MS/MS conditions

A volume of 2 µL was injected in pulsed splitless mode at a temperature of 225 °C, with helium as carrier gas (1 mL/min flow).

The oven temperature gradient started with 4 min at 60 °C, increased to 70 °C in 2 min and then to 200 °C at 15 °C/min, held for 6 min, as resumed in the following table:

Time (min)	Temperature (°C)	Ramp (°C/min)
0	60	-
4	60	0
6	70	5
14.7	200	15
20.7	200	0

Table 3.14 Oven temperature gradient program

The detector operated in positive chemical ionization (CI+), with source temperature set at 200 °C and methane employed as reagent gas.

Acquisition was performed in MRM (multiple reaction monitoring) mode; collision gas consisted of a mixture of nitrogen and helium, at a flow of 1.5 and 2.25 mL/min respectively. In **Table 3.15** the monitored transitions and the relative collision energy values, expressed in eV, are reported.

Analyte	Transition 1 (*m/z*)	Collision energy	Transition 2 (*m/z*)	Collision energy
N-EtFOSE	571.7 > 553.7	10	571.7 > 571.7	1
N-MeFOSE	557.7 > 539.7	10	557.7 > 557.7	1
d7-N-MeFOSE	564.7 > 546.7	10	564.7 > 564.7	1
6:2 FTOH	364.6 > 326.7	10	364.6 > 364.6	1
8:2 FTOH	464.6 > 426.7	10	464.6 > 464.6	1
10:2 FTOH	564.6 > 526.7	10	564.6 > 564.6	1
M-8:2 FTOH	468.6 > 430.7	10	468.6 > 468.6	1

Table 3.15 Monitored transitions and corresponding collision energy

3.3.4 Extraction procedure

1 g of freeze-dried fish was transferred to a polypropylene tube, added of 100 µL of IS FOSE+FTOH solution and 15 mL of methanol, then mechanically shaken for 15 min and centrifuged at 4000 g during 15 min.

In a new tube, 800 mg of ENVI-Carb™ phase were activated with 1 mL of glacial acetic acid, then the supernatant was transferred and the tube agitated for 1 min and centrifuged at 4000 g for 5 min.

After transferring the extract into another tube, it was reduced to around 500 µL being heated at 35 °C under gentle nitrogen stream and added of 3 mL of dichloromethane.

The solution was then loaded onto a self-prepared silica column (see **Section 3.3.1**), previously activated with 10 mL of dichloromethane.

Then, a washing with 15 mL of dichloromethane was performed and the analytes were eluted with 30 mL of acetone.

The eluate was placed into a dry bath at 35 °C and carefully evaporated to 200 µL by a delicate nitrogen stream, then transferred to a microtube and centrifuged at 12000 rpm during 45 min.

Finally, 150 µL were transferred into a polypropylene GC vial and injected.

A scheme resuming the whole procedure is presented on next page.

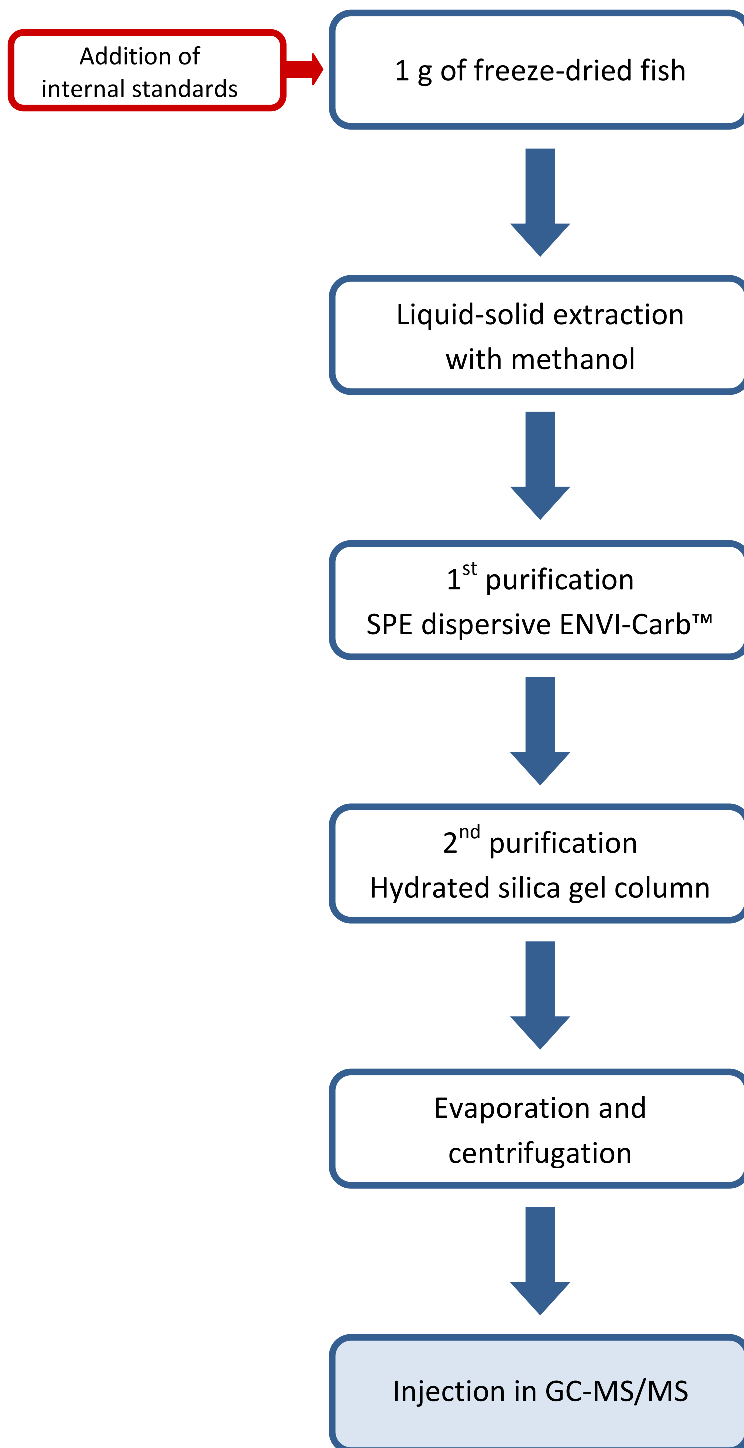

Figure 3.2 Extraction procedure

3.3.5 Performances of the method

After optimizing the detection of all the target compounds, some preliminary tests were performed to assess the performances of the method.

Concerning linearity and limits of detection, a 6 points matrix-matched calibration curve ranging from 0 to 50 µg/kg of dry matter was prepared, as reported in the following table:

Name	Concentration (µg/kg d.m.)	µL IS FOSE+FTOH (0.1 µg/mL)	µL FOSE+FTOH (0.01 µg/mL)	µL FOSE+FTOH (0.1 µg/mL)	µL FOSE+FTOH (1 µg/mL)
F 0	0	100	-	-	-
F 0.5	0.5	100	50	-	-
F 1	1	100	100	-	-
F 5	5	100	-	50	-
F 10	10	100	-	100	-
F 50	50	100	-	-	50

Table 3.16 Calibration curve preparation

In order to evaluate absolute extraction recovery for FOSEs, two samples were prepared with the addition of 50 µL of FOSE+FTOH 1 µg/mL solution, the first being spiked at the beginning of the extraction procedure and the other being spiked at the end, during the final evaporation prior to injection.

4. Results and discussion

4.1 PFCs analysis at LABERCA

A method based on liquid chromatography coupled to two different mass spectrometry detectors for the quantification of perfluorinated compounds in milk was optimized and fully validated in accordance with European regulations (2002/657/EC). This technique allows to simultaneously measure 16 different analytes at ultra-trace levels and is currently under evaluation for ISO 17025 accreditation.

LC-MS/MS is nowadays the technique of election for the analysis of these substances, but the LC-HRMS system resulted even more performing in terms of sensitivity and specificity for some compounds. This instrument thus represents a useful alternative for both quantitative and confirmatory purposes, especially considering the low concentrations at which these analytes are often measured.

4.1.1 Instrumental analysis

Chromatographic separation

The chromatographic part was in common between the two systems and the choice of the column fell on a reversed phase C18 Gemini column, due to its high stability and efficiency at variable gradients and pH. The interactions between the silica phase and target analytes allowed to successfully separate the latter, whose retention time increased with increasing polarity.

The mobile phase, as well, has a major role when trying to obtain a good chromatographic separation and the choice of its components must take into account both the characteristics of the compounds investigated and the detector’s interface.

Similarly to what indicated in previous works on PFCs analysis, in order to improve the elution of the molecules and their ionization, ammonium acetate was added to the methanol/water mobile phase: the concentration of 20 mM in aqueous solution was in line with various studies, however also lower concentrations (between 2 and 20 mM) have been reported as well. This salt interacts with target compounds to form ion-pairs, helping their passage through the column; at the same time it facilitates their deprotonation, and consequently the formation of pseudo-molecular ions.

From the initial methanol/ammonium acetate 30%/70% mixture, mobile phase gradually switched to 100% methanol and held like that for 4 min, in order to let all the compounds elute from the column.

These conditions allowed to satisfactorily separate 26 different molecules (16 perfluorinated compounds and 10 relative internal standards) with a 20 min chromatographic run, including column re-equilibration.

Ionization

Once they have been eluted from the column and before they enter the detector, target analytes undergo ionization, which can be operated by different types of sources. Concerning perfluorinated compounds, the most employed technique is electrospray ionization, which is based on the effects of a strong electric field (at kV levels) applied to the chromatography eluate exiting from a capillary at controlled flow and atmospheric pressure. By means of a nebulization gas (nitrogen), the mixture of mobile phase and produced ions is transformed into spray droplets, which move towards the entrance cone of the detector being simultaneously hit by the desolvation gas (again, nitrogen). This causes a progressive evaporation of the solvent, until the droplets explode releasing the highly charged molecules, which finally enter the detector. Depending on the polarity of the applied potential difference, positive or negative ions can be generated: in the case of perfluorinated compounds, negative ionization is generally used, since they tend to give up protons forming pseudo-molecular ions $[M\text{-}H]^-$. Thus, ESI- was employed on both systems.

MS/MS detection

The principle of operation of tandem mass spectrometry, operating in MRM mode, is illustrated if **Figure 4.1** and is based on a linear series of 3 quadrupoles. The first one (Q1) filters ions coming from the source according to their mass-to-charge ratio (*m/z*), then a collision gas (an inert gas, such as argon or nitrogen) collides with selected ions in Q2 generating fragments, which are then filtered in Q3. Fragmentation pathways are characteristic for each compound, thus this technique allows a selective identification of target analytes since only specific fragments reach the detector.

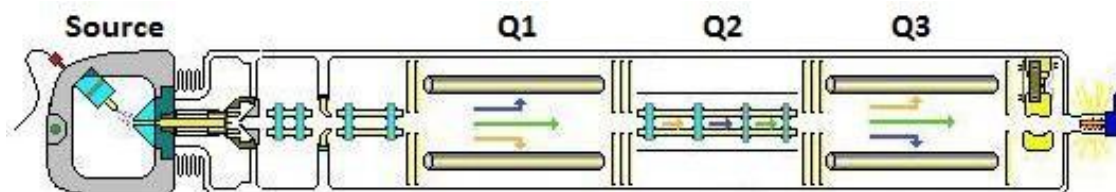

Figure 4.1 Scheme of a triple quadrupole mass spectrometer

In the proposed method, when possible, for an unambiguous determination of the investigated substances, 2 transitions (parent ion giving fragment ion) were monitored for each compound, the most abundant being used for quantification and the other for confirmatory purpose. This was achieved optimizing for each transition the energy applied to the analytes before the entrance of the detector and to the selected parent ion in the collision cell, on which the generation of product ions depends.

However, the high stability of perfluorinated compounds causes in some cases a relatively poor and non-specific fragmentation pathway, resulting in parent-to-parent or parent-to-small fragment transitions to be used for the identification of certain analytes. The consequence of this is a lack of specificity of MS/MS detectors, which is the main limit of this kind of instrument in PFCs analysis, potentially causing in some matrices overestimations due to interfering co-eluting compounds.

Moreover, performing MRM analysis on such a large number of molecules and their relative internal standards (40 transitions in total) implies significantly decreased dwell time values, resulting in a loss of sensitivity.

In more detail, 4 different families of perfluorinated compounds were considered in this study, each of them showing a characteristic fragmentation pathway depending on the chemical structure. The analysis of their product ions mass spectrum provided useful information about the fragments generated after precursor ion's break up.

Perfluoroalkyl carboxylic acids fragmentation is easily obtained even at relatively low collision energy values, producing mainly the loss of a CO_2 group and secondly parts of the chain constituted by 3 or 4 fluorinated carbons (see **Figure 4.2**).

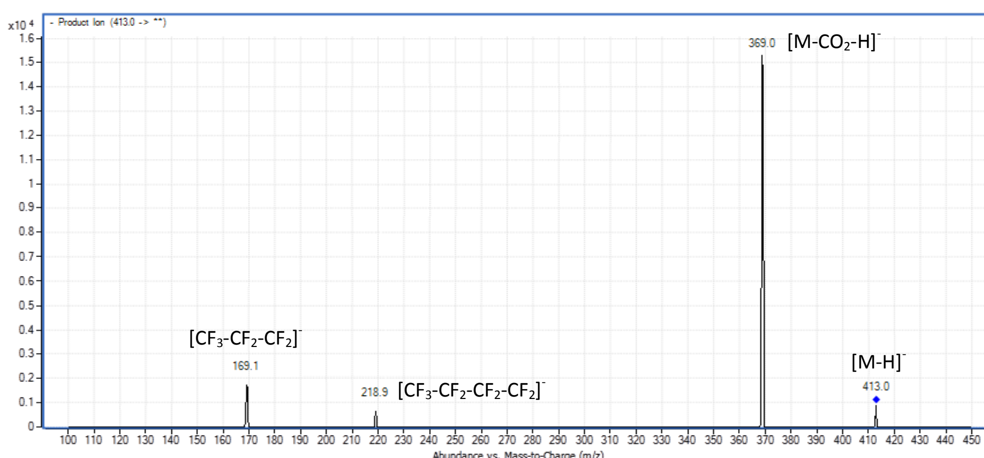

Figure 4.2 Product ions scan for PFOA, applying a collision energy of 15 eV

Perfluoroalkyl sulfonates showed higher stability than PFCAs, having the sulfonate group a stronger interaction with the alkyl chain, due to its greater electronegativity. Applying high collision energy, $[SO_3]^-$ and $[FSO_3]^-$ are the two most detected product ions, proving that PFSAs fragmentations lacks in specificity (see **Figure 4.3**).

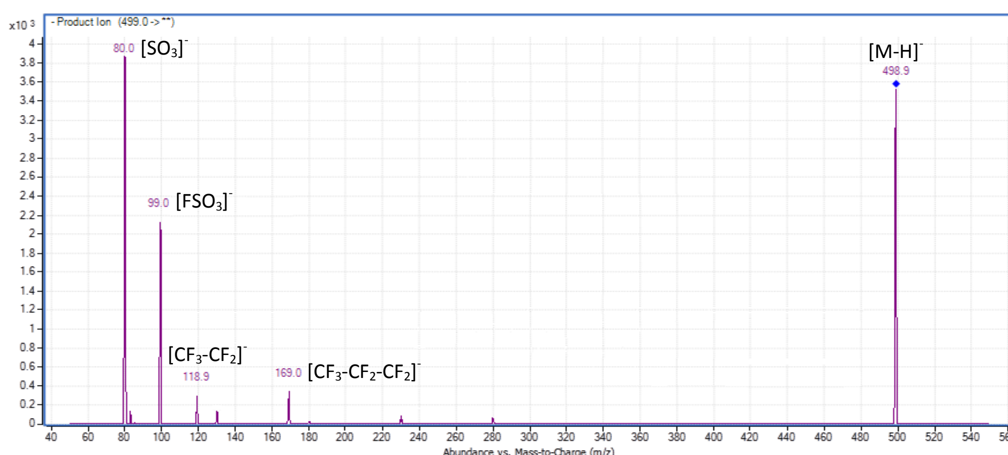

Figure 4.3 Product ions scan for PFOS, applying a collision energy of 45 eV

Also perfluoroalkyl sulfonamide's predominant product ion is a small fragment, given by a $[SO_2N]^-$ group; however, also fragments of the alkyl chain are observed, mainly constituted by 3 fluorinated carbons (see **Figure 4.4**).

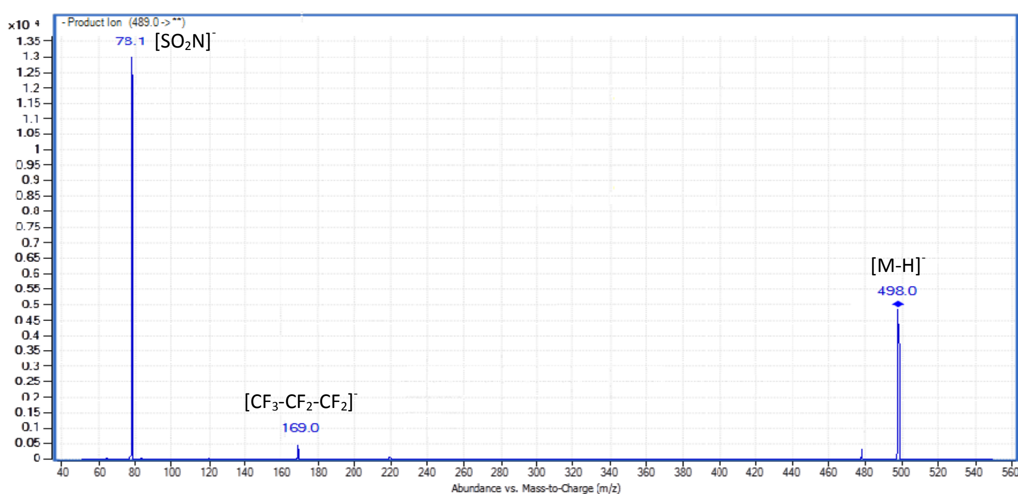

Figure 4.4 Product ions scan for FOSA, applying a collision energy of 35 eV

Concerning perfluoroalkyl sulfinates, they show the loss of a $[SO_2]^-$ group and chain fragments of different lengths, as shown in **Figure 4.5** (in this work the transition $[M\text{-}H]^-$ > $[CF_3\text{-}(CF_2)_3]^-$ was chosen for confirmatory purpose); their fragmentation requires a lower energy compared to PFSAs, indicating the lower stability of these family of compounds.

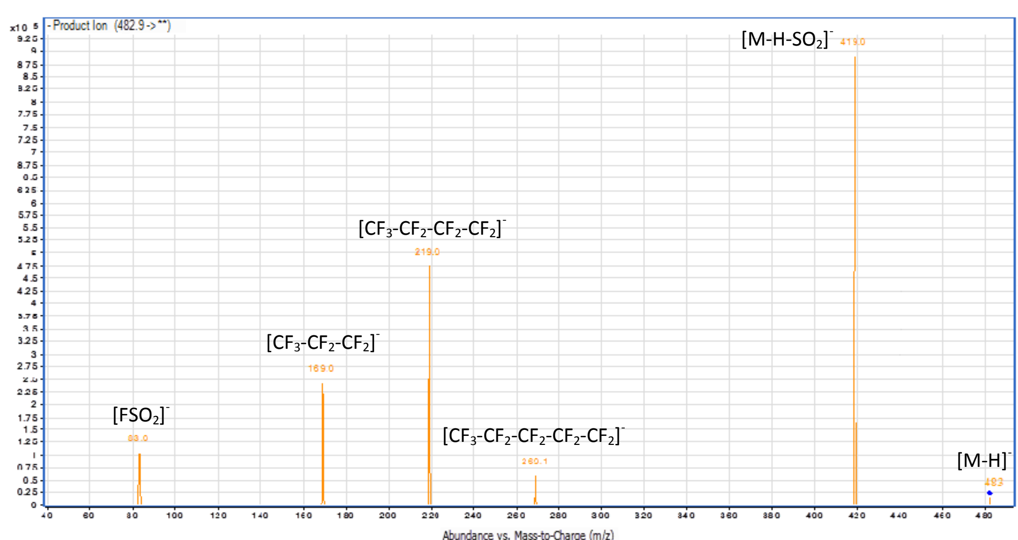

Figure 4.5 Product ions scan for PFOSi, applying a collision energy of 15 eV

HRMS detection

The term “resolution” in mass spectrometry indicates the mass filter capacity to distinguish two ions having very close mass-to charge (*m/*z) ratios. Triple quadrupole mass spectrometers are often employed to resolve ions differing by a single atomic mass unit, being thus considered low resolution instruments. High resolution mass spectrometers, such as the LTQ-Orbitrap™ employed in this study, allow much higher resolution, achieving resolving power >100000 FWHM (meaning the ratio between the measured value and the width of the peak measured at half of its height) and mass accuracy <5 ppm.

In **Figure 4.6** the principle of operation of the LTQ-Orbitrap™ is resumed. This system combines a linear ion trap, based on the quadrupole principle but capable to perform MS^n analysis, with an Orbitrap™. After exiting the linear trap, ions are orientated by a curved trap (c-trap) and reach the Orbitrap™, within which are trapped beginning to move in circular trajectories around the central electrode and back and forth along its axis, due to the form of this device and the inside electric field. Their oscillation frequency is inversely proportional to the square root of their *m/z* and is converted into a signal by means of the Fourier transform.

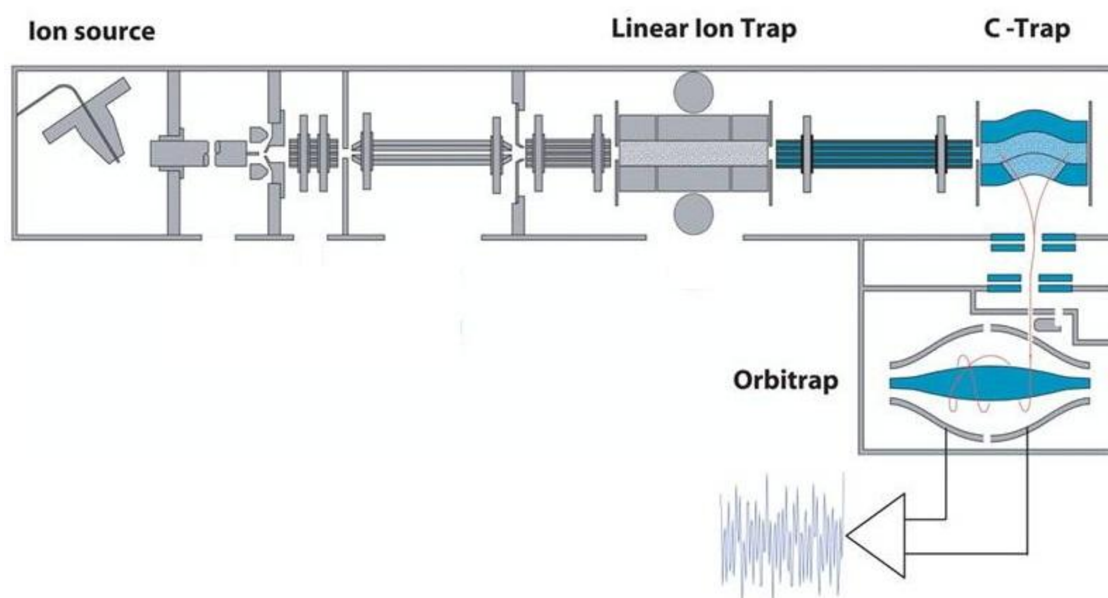

Figure 4.6 Scheme of the LTQ-Orbitrap™ system

After optimizing ionization parameters for the detection of the various target compounds, analysis were performed in full scan mode. From the acquired mass spectrum, the exact masses of the pseudo-molecular ions $[M-H]^-$ were extracted with a resolution of 15000 FWHM at 400 *m/z*. The high mass accuracy of this instrument provided a great specificity, allowing extremely selective identification even in this kind of complex multi-residue analysis.

4.1.2 Extraction procedure

The extraction procedure is essentially based on a LLE (liquid-liquid extraction) followed by two purifications on SPE (solid phase extraction).

The LLE step is very important when analyzing milk samples, since perfluorinated compounds tend to bind to proteins, whose content in this matrix is relevant. In order to separate them, an extraction with acetone was performed, denaturing proteins and causing their precipitation. This could have been achieved in many ways, but acetone was chosen due to its low boiling point, which is an advantage considering that samples should be concentrated before being loaded on the SPE cartridge.

Waters Oasis® HLB was the first SPE cartridge employed for sample purification because of the macroporous copolymer constituting its stationary phase, which combines hydrophilic and lipophilic properties, providing strong interactions with PFCs. The mechanisms underlying their retention are both dipole-dipole and cation exchange interactions, the latter being facilitated by sample acidification obtained through addition of formic acid solution before loading.

The second SPE step is a modification of the technique proposed by Powley et al. (2005), but instead of using dispersive graphitized carbon phase, in this case purification was carried out on columns containing the same sorbent.

For both types of cartridges, elution profiles were studied to optimize sample preparation, decreasing as much as possible the amount of eluting solutions employed. In more detail, 2mL fractions of formic acid/methanol mixture (for Waters

Oasis® HLB) and methanol/glacial acetic acid mixture (for Supelclean™ ENVI-Carb™) were loaded one after the other on the respective cartridge and collected separately, in order to quantify the amount of analytes eluted by each fraction. This experiment proved that 6 mL volume of the above mentioned solutions were sufficient to completely elute the analytes, which translates into, besides reduced solvents consumption, shorter time required for the following evaporation steps.

Some tests were conducted also to decide the initial amount of sample to process, extracting 1, 2 and 5 mL milk samples (added of proportional volumes of acetone, to ensure protein precipitation). 3 mL was considered the optimum compromise to obtain very good limits of detection without generating relevant matrix effect.

Prior to injection, a centrifugation was performed in order to further reduce the risk of potential residual components of the matrix being present in the injected solution.

During all the experiments that have been conducted, glass tubes and vials were replaced by polypropylene ones, as well as Teflon lined caps and septa (potentially releasing small amounts of residual perfluorinated compounds), to prevent from alterations and interferences affecting the analysis. Moreover, standards solutions and samples were stored away from light and at refrigerator or freezer temperature.

4.1.3 Method validation

Validation of the described method for the identification and quantification of 16 PFCs was performed on both systems as described in **Section 3.1.5**, in compliance with Commission Decision 2002/657/EC, concerning the performances of analytical methods and establishing the required parameters.

Specificity

No significant differences were observed between samples processed starting from 1 mL of milk and those starting from 3 mL. Both instruments allowed to efficiently

separate target analytes but, in general, the LC-HRMS system generated better signals, with low to no background noise, demonstrating a higher specificity.

PFOS and PFOA were detected without relevant problems, while PFUnA analysis was affected by the presence of a coeluting compound, interfering with its quantification at low concentrations. However, according to data available in literature, this contaminant seems not to be present in milk. The short retention time of PFBA and PFPA, together with the availability of only one MS/MS transition, negatively influenced the specificity of their analysis.

In the following figure it's possible to appreciate the higher S/N ratio obtained with LC-HRMS compared to LC-MS/MS for the detection of PFOA in a milk sample spiked at a concentration of 0.020 ng/mL.

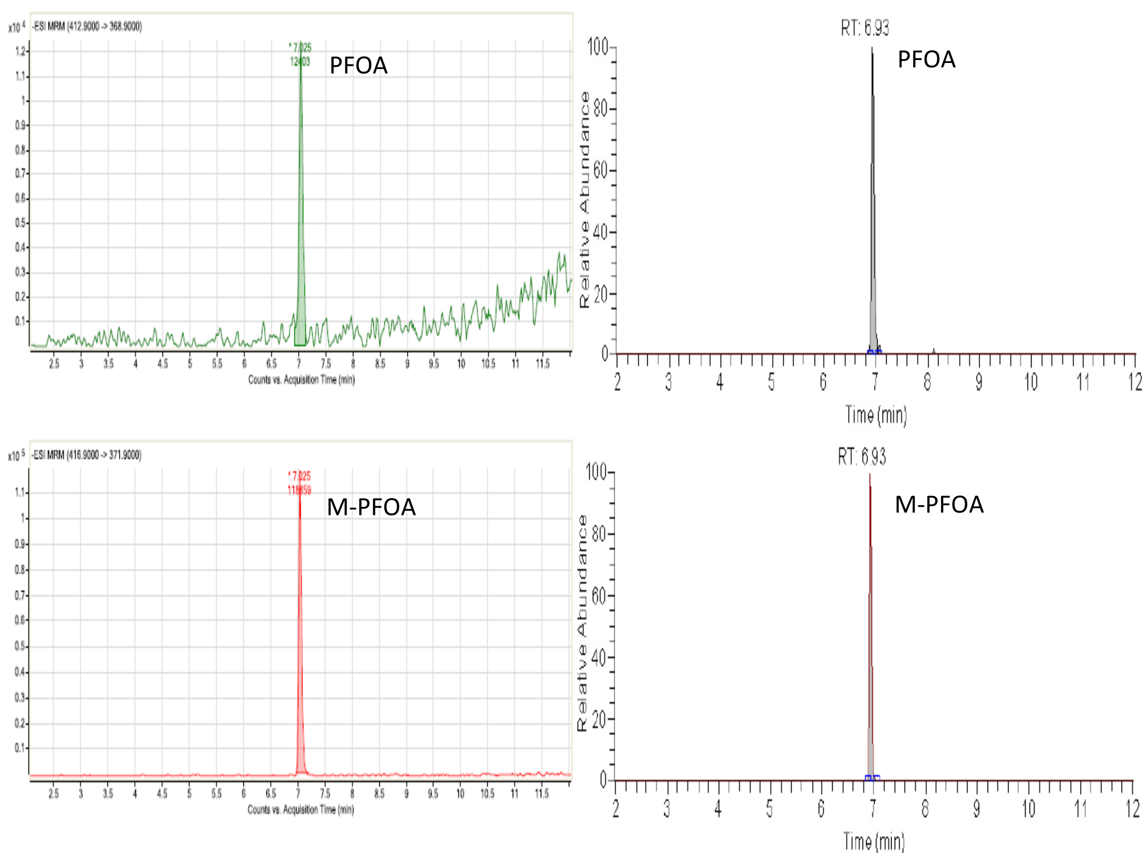

Figure 4.7 Chromatograms of the most representative transitions of PFOA (412.9 > 368.9) and M-PFOA (416.9 > 371.9), resulting from the analysis of a milk sample spiked at 0,020 ng/mL in LC-MS/MS (left) and LC-HRMS (right)

Identification criteria

The results obtained analyzing 16 milk samples spiked with PFCs standards and corresponding internal standards at 0.5 ng/mL are reported in the following table.

Analyte	Relative retention time		Transitions ratio	
	Mean (min)	CV (%)	Mean (min)	CV (%)
PFBA	1.00	0.49	n/a	n/a
PFPA	0.55	1.22	n/a	n/a
PFBS	0.64	4.09	26.15	11.10
PFHxA	0.85	0.15	3.90	12.74
PFHpA	0.94	0.63	5.09	10.49
PFHxS	1.00	0.04	32.32	10.45
PFHpS	0.96	0.19	44.56	9.02
PFOA	1.00	0.01	28.16	5.15
PFNA	1.00	0.01	9.77	24.14
PFOS	1.00	0.04	52.76	8.86
PFDA	1.00	0.01	7.54	20.21
PFDS	1.07	0.31	2.63	6.09
PFUnA	1.00	0.03	n/a	n/a
PFDoA	1.00	0.02	8.39	21.29
PFOSi	1.00	0.03	6.22	11.10
FOSA	1.00	0.16	n/a	n/a

Table 4.1 Results for the required identification criteria in LC-MS/MS analysis

Concerning relative retention time, only for PFBS it didn't fell within the established range (±2.5%). This was due to the non negligible differences between this analyte, whose alkyl chain is constituted by 4 carbon atoms, and the internal standard to which it was associated ($^{13}C_4$-PFOS, whose chain presents 8 carbon atoms), resulting in PFBS having an extremely shorter and highly variable retention time. This could probably be solved with the adoption of labeled PFBS as internal standard.

No problem was observed for the relative ion intensity, with CV% values resulting lower than the maximum allowed tolerance for all compounds.

Linearity

Coefficient of determination (R^2) and slope (a) values obtained from the injection of calibration curves prepared for each type of milk are shown in **Tables 4.2, 4.3 and 4.4**. Results were very satisfying, with R^2 values >0.99 in almost all cases; in particular, for PFOS and PFOA they were generally around 0.999 even in LC-MS/MS.

As for FOSA, significantly lower values were observed in LC-MS/MS compared to LC-HRMS. Also in this case, the reason was that the labeled internal standard $^{13}C_4$-FOSA wasn't available in the laboratory at the time when this experiment was conducted.

Analyte	**LC-MS/MS signal 1**		**LC-MS/MS signal 2**		**LC-HRMS**	
	R²	**a**	**R²**	**a**	**R²**	**a**
PFBA	0.9989	2.00	n/a	n/a	0.9988	1.81
PFPA	0.9996	0.48	n/a	n/a	0.9988	3.20
PFBS	0.9791	0.01	0.9995	0.06	0.9914	1.55
PFHxA	0.9998	0.81	0.9961	0.03	0.9962	1.75
PFHpA	0.9982	1.68	0.9952	0.08	0.9975	1.78
PFHxS	0.9971	1.00	0.9920	3.69	0.9999	2.33
PFHpS	0.9996	0.04	0.9968	0.09	0.9988	1.73
PFOA	0.9979	2.98	0.9963	0.80	0.9997	2.01
PFNA	0.9996	1.60	0.9982	0.12	0.9999	1.60
PFOS	0.9963	0.09	0.9996	0.05	0.9998	1.98
PFDA	0.9993	1.65	0.9933	0.06	0.9998	1.44
PFDS	0.9950	0.06	0.9950	0.03	0.9323	0.73
PFUnA	0.9968	0.16	n/a	n/a	0.9979	1.59
PFDoA	0.9978	1.56	0.9987	0.13	1.000	1.52
PFOSi	0.9996	7.08	0.9950	0.40	0.9999	2.29
FOSA	0.9758	0.02	n/a	n/a	0.9998	4.22

Table 4.2 Regression parameters of calibration curves in cow milk

Analyte	LC-MS/MS signal 1		LC-MS/MS signal 2		LC-HRMS	
	R^2	a	R^2	a	R^2	a
PFBA	0.9985	1.89	n/a	n/a	0.9997	1.69
PFPA	0.9998	0.50	n/a	n/a	0.9995	2.74
PFBS	0.9968	0.02	0.9961	0.07	0.9930	1.33
PFHxA	0.9975	0.89	0.9915	0.04	0.9894	1.44
PFHpA	0.9997	1.57	0.9995	0.07	0.9990	1.64
PFHxS	0.9988	1.16	0.9955	3.76	0.9997	2.34
PFHpS	0.9936	0.04	0.9963	0.10	0.9992	1.73
PFOA	0.9984	3.05	0.9990	0.82	0.9995	2.07
PFNA	0.9998	1.67	0.9990	0.12	0.9997	1.59
PFOS	0.9916	0.09	0.9924	0.05	0.9998	2.01
PFDA	0.9997	1.67	0.9950	0.07	0.9994	1.46
PFDS	0.9929	0.07	0.9809	0.05	0.9915	1.49
PFUnA	0.9983	0.16	n/a	n/a	0.9997	1.63
PFDoA	0.9997	1.61	0.9878	0.12	0.9994	1.50
PFOSi	0.9998	7.12	0.9972	0.42	0.9995	2.29
FOSA	0.9832	0.04	n/a	n/a	0.9992	4.24

Table 4.3 Regression parameters of calibration curves in human breast milk

Analyte	LC-MS/MS signal 1		LC-MS/MS signal 2		LC-HRMS	
	R^2	a	R^2	a	R^2	a
PFBA	0.9932	2.06	n/a	n/a	0.9980	1.75
PFPA	0.9995	0.53	n/a	n/a	0.9991	2.97
PFBS	0.9992	0.02	0.9986	0.07	0.9928	1.99
PFHxA	0.9970	0.94	0.9902	0.04	0.9945	1.82
PFHpA	0.9953	1.88	0.9870	0.09	0.9953	1.82
PFHxS	0.9835	1.18	0.9956	3.23	0.9998	2.33
PFHpS	0.9993	0.04	0.9948	0.09	0.9996	2.08
PFOA	0.9977	3.30	0.9961	0.91	0.9999	2.05
PFNA	0.9997	1.68	0.9924	0.13	0.9999	1.65
PFOS	0.9994	0.09	0.9991	0.04	0.9998	2.00
PFDA	0.9988	1.70	0.9941	0.07	0.9999	1.49
PFDS	0.9953	0.05	0.9943	0.02	0.9882	0.40
PFUnA	0.9982	0.13	n/a	n/a	0.9993	1.73
PFDoA	0.9989	1.68	0.9855	0.11	0.9997	1.52
PFOSi	0.9992	7.57	0.9980	0.48	0.9998	2.28
FOSA	0.9222	0.01	n/a	n/a	0.9921	4.81

Table 4.4 Regression parameters of calibration curves in powder milk

Limits of detection

Predictably, due to its higher sensitivity, the LC-HRMS system showed lower limits of detection, varying between 0.002 and 0.025 ng/mL (see **Table 4.5**).

Concerning PFOS and PFOA, LOD resulted identical with the two instruments for the former (0.005 ng/mL), while for the latter it was slightly lower with the LTQ-Orbitrap™ system (0.002 ng/mL against 0.010 ng/mL). These values are extremely significant in consideration of the levels of contamination likely to be found in milk.

For some compounds the obtained values were not so good, as for PFUnA, whose limit of detection is affected by the presence of an interferent with the same retention time, as previously mentioned.

Analyte	**LC-MS/MS**	**LC-HRMS**	**Analyte**	**LC-MS/MS**	**LC-HRMS**
	ng/mL	**ng/mL**		**ng/mL**	**ng/mL**
PFBA	0.040	0.015	PFNA	0.010	0.005
PFPA	0.100	0.025	PFOS	0.005	0.005
PFBS	0.050	0.002	PFDA	0.010	0.010
PFHxA	0.025	0.015	PFDS	0.025	0.010
PFHpA	0.025	0.010	PFUnA	0.040	0.025
PFHxS	0.020	0.003	PFDoA	0.050	0.025
PFHpS	0.010	0.002	PFOSi	0.010	0.002
PFOA	0.010	0.002	FOSA	0.050	0.025

Table 4.5 Limits of detection of the two instruments

Recovery

Although the use of internal standards significantly increases the reliability of the results, extraction recoveries were evaluated to assess the efficiency of the extraction procedure, giving satisfying results.

Measured values were between 30 and 111%, with mean recoveries varying from 69 to 87%. Concerning PFOS and PFOA, the obtained values were in the ranges 56-95% and 69-89%, respectively.

The results for each of the 3 matrices are reported in the table presented on the next page.

Analyte	Cow milk (%)		Human breast milk (%)		Powder milk (%)	
	LC-MS/MS	LC-HRMS	LC-MS/MS	LC-HRMS	LC-MS/MS	LC-HRMS
PFBA	75	73	92	97	68	66
PFPA	85	82	85	97	83	87
PFBS	92	90	99	106	87	92
PFHxA	91	88	85	96	81	88
PFHpA	89	81	74	84	81	89
PFHxS	82	89	83	87	101	96
PFHpS	92	92	69	76	86	90
PFOA	84	86	69	77	88	89
PFNA	84	86	58	61	82	85
PFOS	95	89	56	57	91	89
PFDA	83	86	61	55	92	87
PFDS	89	78	38	30	88	86
PFUnA	85	86	48	51	93	85
PFDoA	87	82	59	51	83	83
PFOSi	86	81	58	56	74	90
FOSA	57	54	69	35	111	48

Table 4.6 Extraction recoveries in milk, expressed as percentages

Precision

Coefficients of variation were globally very good on both systems (see **Table 4.7**, for the LC-MS/MS instrument the most abundant transition was considered).

In particular, for PFOS and PFOA they ranged between 1.1-20.8% and 1.0-5.5%, respectively. Moreover, except for PFDS and FOSA, all compounds showed values lower than 20.4% in samples fortified at 0.5 ng/mL and up to 30.3% in milk spiked at 0.1 ng/mL.

As it can be observed, compounds which were associated to the correspondent labeled internal standard generally presented very low CV%, proving that the use of molecules with highly similar characteristics and behaviors allows to significantly reduce response variability.

This aspect explains the poor results shown by the previously mentioned PFDS and FOSA, whose internal standards were not available at the time of validation; however, the LC-HRMS system demonstrated good repeatability even for FOSA.

As for PFBA, the decreased precision reported in cow and powder milk samples spiked at 0.1 ng/mL can depend on the already described difficulties in the separation and detection of this compound, being extremely rapidly eluted.

In general, the higher precision observed in human breast milk was probably due to the fact that analysis in this matrix were conducted on a pool and not on individual samples.

Analyte	Cow + powder milk CV% (0.1 ng/mL, n=4+4)		Cow + powder milk CV% (0.5 ng/mL, n=4+4)		Human breast milk CV% (0.5 ng/mL, n=8)	
	LC-MS/MS	LC-HRMS	LC-MS/MS	LC-HRMS	LC-MS/MS	LC-HRMS
PFBA	22.8	25.8	8.3	3.0	8.6	3.4
PFPA	5.74	4.2	7.1	5.7	4.2	3.0
PFBS	16.2	15.7	8.1	8.8	2.0	7.3
PFHxA	9.9	13.9	6.6	6.4	5.0	18.0
PFHpA	5.4	8.0	4.3	1.7	4.7	9.5
PFHxS	7.8	1.6	8.0	1.7	8.3	6.0
PFHpS	7.4	8.3	8.9	6.5	4.7	7.0
PFOA	5.5	2.9	2.9	1.0	3.1	1.4
PFNA	7.5	1.1	3.8	1.2	4.4	1.8
PFOS	20.8	3.7	7.7	1.3	4.6	1.1
PFDA	4.8	3.6	3.3	1.1	4.1	1.2
PFDS	50.9	50.1	34.0	41.3	11.6	17.7
PFUnA	28.8	4.9	20.4	1.7	6.3	1.9
PFDoA	15.9	30.3	9.2	6.7	4.5	2.8
PFOSi	10.9	1.6	4.4	1.0	4.8	1.6
FOSA	23.1	8.3	51.3	4.5	53.7	4.2

Table 4.7 Precision (CV%) in milk, calculated in cow and powder milk spiked at 0,1 and 0.5 ng/mL and in human breast milk spiked at 0.5 ng/mL

Trueness

The 2 milk samples analyzed showed satisfying results: deviations of 13.8% and 11.8% were measured for PFOS, while for PFOA they were of 21.9% and 25.8%.

In **Tables 4.8 and 4.9**, trueness data for the monitored compounds, obtained comparing concentrations measured in LC-HRMS to those reported in the international ring test, are shown.

Analyte	Reported values		Measured values	
	Median (ng/mL)	**SD (ng/mL)**	**Conc. (ng/mL)**	***Bias (%)***
PFHpA	0.007	0.006	0.005	*28.6*
PFOA	0.146	0.137	0.114	*21.9*
PFHxS	0.086	0.042	0.116	*34.9*
PFOS	0.094	0.102	0.081	*13.8*

Table 4.8 Comparison between measured values and other labs median values in the human breast milk sample from Sweden

Analyte	Median values		Measured values	
	Median (ng/mL)	**SD (ng/mL)**	**Conc. (ng/mL)**	***Bias (%)***
PFOA	0.066	0.080	0.049	*25.8*
PFOS	0.051	0.245	0.045	*11.8*

Table 4.9 Comparison between measured values and other labs median values in the human breast milk sample from Germany

As for the analysis of the certified serum, the deviations of the mean measured concentrations fell in the range indicated by InterCal. In particular, PFOS and PFOA showed a bias of 0.7% and 15.7%, respectively.

Analyte	Reported values		Measured values	
	Mean (ng/mL)	**SD (ng/mL)**	**Mean (ng/mL)**	***Bias (%)***
PFHpA	0.318	0.405	0.544	*71.1*
PFOA	2.976	0.634	0.114	*15.7*
PFNA	0.815	0.233	0.116	*10.9*
PFDA	0.225	0.078	0.116	*9.0*
PFHxS	3.281	0.804	0.116	*13.9*
PFOS	9.952	3.294	0.081	*0.7*

Table 4.10 Comparison between measured values and reported concentrations of the certified serum sample (NIST SRM 1957)

Uncertainty

Measure uncertainty was evaluated for PFOS and PFOA correlating various factors resulting from the validation process, as described in **Section 3.1.5**; it was estimated only on the LC-HRMS system, since it showed better performances.

Concerning uncertainty in trueness, it was evaluated on the basis of the data obtained for milk, being the range of concentrations of certified serum significantly different.

Sample	n	PFOS CV (%)	PFOA CV (%)
Cow + powder milk (0.1 ng/mL)	8	3.7	2.9
Cow + powder milk (0.5 ng/mL)	8	1.3	1.0
Human breast milk (0.5 ng/mL)	8	1.1	1.4
Human breast milk (0.1 ng/mL)	5	7.3	11.0
Human breast milk (0.25 ng/mL)	5	0.6	1.2
Human breast milk (0.75 ng/mL)	5	1.4	2.0
$U_{precision}$		***3.2***	***4.2***

Table 4.11 Uncertainty in precision

Sample	PFOS bias (%)	PFOA bias (%)
Ring test sample (Sweden)	13.8	21.9
Ring test sample (Germany)	11.8	25.8
$U_{trueness}$	***12.8***	***23.8***

Table 4.12 Uncertainty in trueness

Standard solution purity (according to Wellington Labs)	**PFOS (%)**	**PFOA (%)**
	>98	>98
U_{purity}	***1.2***	***1.2***

Table 4.13 Uncertainty in trueness

Combining the uncertainty factors expressed in the above tables, relative combined uncertainty resulted of 13.2% for PFOS and 24.2% for PFOA.

Based on these values, final uncertainty (95% confidence interval) in LC-HRMS was calculated equal to 26.5 and 48.5%, respectively for PFOS and PFOA. Considering the

extremely low levels at which these compounds are found in milk (<1 ng/mL), results were judged satisfying.
In conclusion, the described method for the analysis of 16 different PFCs was successfully validated in compliance with current European guidelines, demonstrating very good performances with both LC-MS/MS and LC-HRMS systems. However, the latter showed higher sensitivity and specificity, and resulted more suitable for those compounds presenting a difficult and unspecific fragmentation, since the extracted mass signal corresponds unambiguously to that of the target analyte while MS/MS transitions can be common to those of interfering substances.
PFOS and PFOA, the two most important analytes of the group, showed extremely good results in all the considered parameters, but also for the majority of the other target molecules the obtained values were satisfying.
In particular, the very low limits of detection measured for most of the compounds are suitable with the concentrations that are generally measured in milk and comparable to those reported by the most performing methods described in literature.
Finally, the adoption of a wider range of specific internal standards (when available) could lead to a further improvement of the performances of the method.

4.1.4 Samples analysis

All the available samples at the time of validation were processed applying the developed method, in order to assess perfluorinated compounds contamination in the three types of milk.
The choice of preparing for each day of analysis a calibration curve in ultrapure water was due to the difficulty in finding milk containing negligible PFCs levels: instead of risking to affect quantifications with an unreliable matrix-matched calibration curve, it was preferred to prepare one processing a non-milk solution, spiked at the same previously described levels, after verification of its non-contamination.

4.1.4.1 Human breast milk from France

The analysis of the 11 samples available at the time of validation highlighted the predominance of PFOS and PFOA in human breast milk. They were found in all the samples, with mean concentrations of 139 pg/mL (median 127 pg/mL) for PFOS and of 121 pg/mL (median 116 pg/mL) for PFOA. Measured levels ranged from 32 to 433 pg/mL for the former, and from 43 to 297 pg/mL for the latter.

Even if at lower concentrations, PFHxS and PFNA were detected at quantifiable levels with a frequency of 91% and 82%, respectively. PFHxS highest concentration was 77 pg/mL, while for PFNA it was 69 pg/mL.

PFBA, PFPA and PFDA were found in some samples at trace levels: in one case PFBA was quantified at 33 pg/mL and PFDA at 20 pg/mL.

These data were obtained with the LC-HRMS system, but comparing PFOS and PFOA concentrations with those resulting from the analysis in LC-MS/MS a strong correlation was observed (0.91 and 0.87, respectively).

4.1.4.2 Cow milk from France

PFCs levels observed in the analyzed cow milk samples resulted significantly lower compared to those found in human breast milk.

PFOS was quantified in 4 out of 9 samples, with the highest measured concentration equal to 40 pg/mL, and detected at trace levels in 2 cases; slightly lower values were reported for PFOA, which was quantifiable in only 3 samples, at a maximum concentration of 23 pg/mL, and detected with just a weak signal in 1 further sample. The higher sensitivity of LC-HRMS allowed to detect PFOA at levels that couldn't otherwise have been observed with the LC-MS/MS instrument.

In addition, PFHxS and PFNA were detected in few cases, at levels up to 11 and 24 pg/mL, respectively. All the other target analytes weren't found, except for one sample presenting trace levels of PFDA.

4.1.4.3 Powder milk from France

A part for one sample, for which a PFOS concentration of 17 pg/mL was reported, in the few powder milks analyzed PFCs were absent or detected at trace levels (PFOS, PFOA and PFNA).

Measured levels of PFCs which showed relevant presence in the processed milk samples are summarized in the following table.

Sample		PFOS	PFOA	PFHxS	PFNA
Human breast milk	A	32	43	Traces	Traces
	B	152	142	26	26
	C	140	115	21	19
	D	127	152	77	69
	E	105	94	20	23
	F	71	66	18	22
	G	48	53	16	Traces
	H	209	155	24	38
	I	108	101	12	20
	J	433	297	50	30
	K	92	116	28	25
Cow milk	L	37	23	<LOD	<LOD
	M	Traces	<LOD	<LOD	<LOD
	N	25	<LOD	11	24
	O	<LOD	<LOD	Traces	<LOD
	P	19	16	<LOD	Traces
	Q	<LOD	<LOD	<LOD	<LOD
	R	40	Traces	<LOD	Traces
	S	21	17	<LOD	16
	T	Traces	<LOD	<LOD	<LOD
Powder	U	Traces	<LOD	<LOD	<LOD
	V	<LOD	Traces	<LOD	<LOD
	W	17	Traces	<LOD	Traces
	X	<LOD	<LOD	<LOD	<LOD

Table 4.14 Concentrations (pg/mL for human breast and cow milk; pg/g for powder milk) of the detected PFCs analyzing milk samples with the LC-HRMS system. “Traces” means that value resulted >LOD and <LOQ (calculated as 3 times the LOD)

4.2 PFCs analysis at CABA-Lab

The developed method for PFCs detection in milk was transferred to the CABA-Lab laboratory, adapting it to the UHPLC-MS/MS system of which that structure is provided. During this first experiment, it was chosen to reduce the range of target compounds to PFOS and PFOA, being the two most important PFCs.

The good performances obtained after the optimization of the analytical procedure allowed to conduct a preliminary monitoring on the levels of contamination by these pollutants in several cow milk and human breast milk samples collected in Italy.

4.2.1 Method transfer

4.2.1.1 Instrumental analysis

The most critical aspect of the transfer concerned the liquid chromatography system. The method was in fact initially developed on a HPLC (high performance liquid chromatography) instrument, while analysis at CABA-Lab were conducted on a UHPLC (ultra high performance liquid chromatography) system. The latter is the result of the recent technological implementations in liquid chromatography, which led to produce stationary phases containing much smaller particles and mechanical components able to operate at higher pressure, allowing a more efficient chromatographic separation and significantly reduced injected volume and analysis time.

In order to reproduce as much as possible the chromatographic conditions, a Waters Acquity BEH C18 column was chosen, having very similar properties to the column used for HPLC separation at LABERCA; moreover, the same column was employed also in most of the few available studies on PFCs detection in milk through UHPLC instruments (Haug et al., 2010a; Liu et al., 2010; Kannan et al., 2011).

Ammonium acetate 20 mM (A) and methanol (B) were kept as mobile phases, with a gradient that similarly started from 70% A and 30% B, switching to 100% B and then

going back to the initial conditions. Thanks to the performances of the UHPLC system, the analysis time was significantly reduced from 20 to 5 min: in this way it's thus possible to process a larger number of samples, which can be useful in case of large scale monitoring.

Concerning the detector part, negative electrospray ionization was employed and all the parameters of the mass spectrometer were optimized using the standard solutions of the two analytes and of their corresponding labeled compounds.

For PFOS, the parent-to-parent transition (498.64 > 498.64 *m/z*) was chosen for quantification, since on this instrument it showed a slightly better signal compared to the 498.64 > 80.00 *m/z* transition previously used.

4.2.1.2 Extraction procedure

Concerning samples extraction, the developed procedure was reproduced following all the steps and critical points previously underlined.

To reduce potential interferences, polypropylene tubes and vials were preferred to glass-made ones, and Teflon septa and caps were avoided.

In order to prevent alterations, all collected samples were stored in the freezer at -18 °C before processing; similarly, prior to injection vials were stored away from light in the refrigerator at 4 °C and kept at 6 °C during analysis.

A slightly higher amount of internal standards was added when preparing both standard solutions and samples, without affecting the results in any way.

Unlike previously described for the analysis conducted at LABERCA, it wasn't employed the external standard (fluorometholone, added just before sample injection) since it was not considered necessary.

4.2.1.3 Performances of the transferred method

Some tests were performed to verify the performances of the transferred procedure before employing it for the analysis of milk samples.

The analysis of fresh cow milk purchased from a local store was conducted in order to verify if it was contaminated with PFOS and PFOA: it didn't contain any of the analytes, demonstrating the specificity of the method (chromatograms of the detected signals for PFOS and PFOA in that sample are reported in **Figures 4.8** and **4.9**).

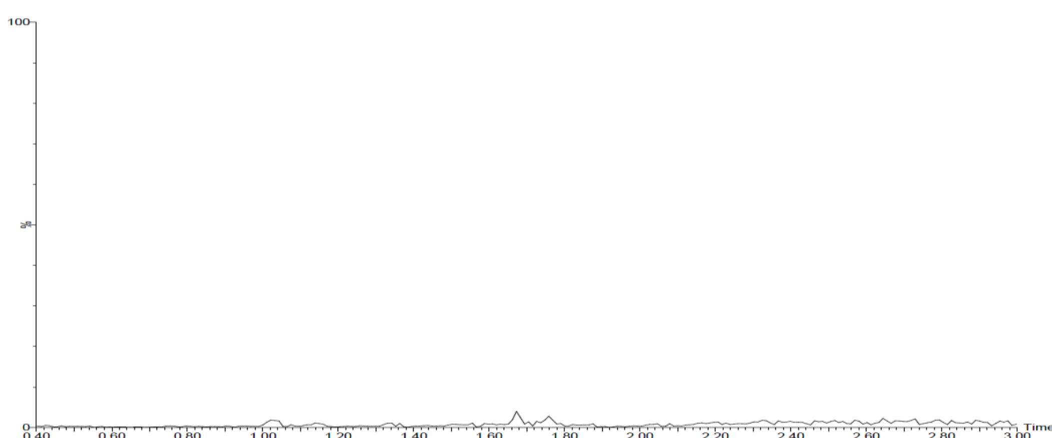

Figure 4.8 Chromatogram of the diagnostic ion of PFOS in a blank cow milk sample

Figure 4.9 Chromatogram of the diagnostic ion of PFOA in a blank cow milk sample

Fractions of this milk were subsequently fortified at different concentrations of PFOS and PFOA in order to obtain a matrix-matched calibration curve. The injection of the curve proved the good linearity of the method, with R^2 values greater than 0.99 for both PFOS and PFOA, as shown in **Figures 4.10** and **4.11**.

Moreover, limits of detection, calculated as the concentrations showing a signal-to-noise ratio of 3, were extremely good. For PFOS, LOD was equal to that obtained

during method validation at LABERCA (0.005 ng/mL); as for PFOA, it was of 0.008 ng/mL, resulting comprised between those of the two previously used instruments. These values were therefore comparable to those of the most sensitive methods in literature and compatible with concentrations potentially measured in milk.

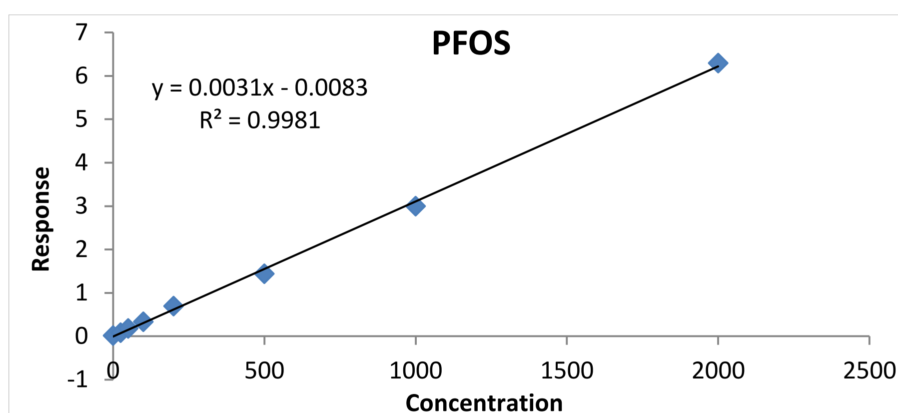

Figure 4.10 Matrix matched calibration curve for PFOS

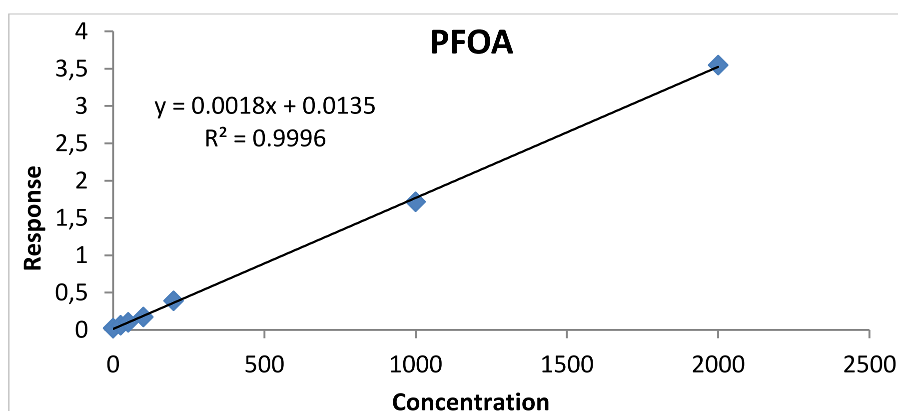

Figure 4.11 Matrix matched calibration curve for PFOA

4.2.2 Samples analysis

The preliminary monitoring consisted in the analysis of 22 cow milk samples and 13 human breast milk samples, previously listed in **Section 3.2.1**. As described in **Section 3.2.6**, freshly prepared calibration curves in water (which confirmed the excellent linearity of the method) allowed to perform accurate quantifications.

4.2.2.1 Human breast milk from Italy

Table 4.15 shows the concentrations of PFOS and PFOA found in human breast milk. Both compounds were detected at quantifiable levels (≥15 pg/mL for PFOS, ≥24 pg/mL for PFOA) in all the analyzed samples.

Sample	PFOS (pg/mL)	PFOA (pg/mL)
A	44	25
B	98	103
C	102	65
D	86	94
E	68	84
F	76	50
G	63	33
H	143	181
I	40	63
J	92	57
K	106	77
L	60	62
M	42	24

Table 4.15 PFOS and PFOA concentrations in human breast milk samples from Italy

PFOS mean concentration was 78 pg/mL (median 76 pg/mL), with values comprised between 40 and 143 pg/mL.

In **Figure 4.12** the chromatograms of the most representative transition of PFOS (498.64 > 498.64 *m/z*) and of the internal standard M-PFOS (502.86 > 502.86 *m/z*) in a human breast milk sample are presented.

As for PFOA, measured levels were in the range 24-281 pg/mL, with a mean concentration of 71 pg/mL (median 63 pg/mL).

Figure 4.13 shows the signals obtained for the diagnostic ions of PFOA (412.82 > 368.97 *m/z*) and of its correspondent internal standard M-PFOA (416.93 > 372.00 *m/z*) analyzing a human breast milk sample.

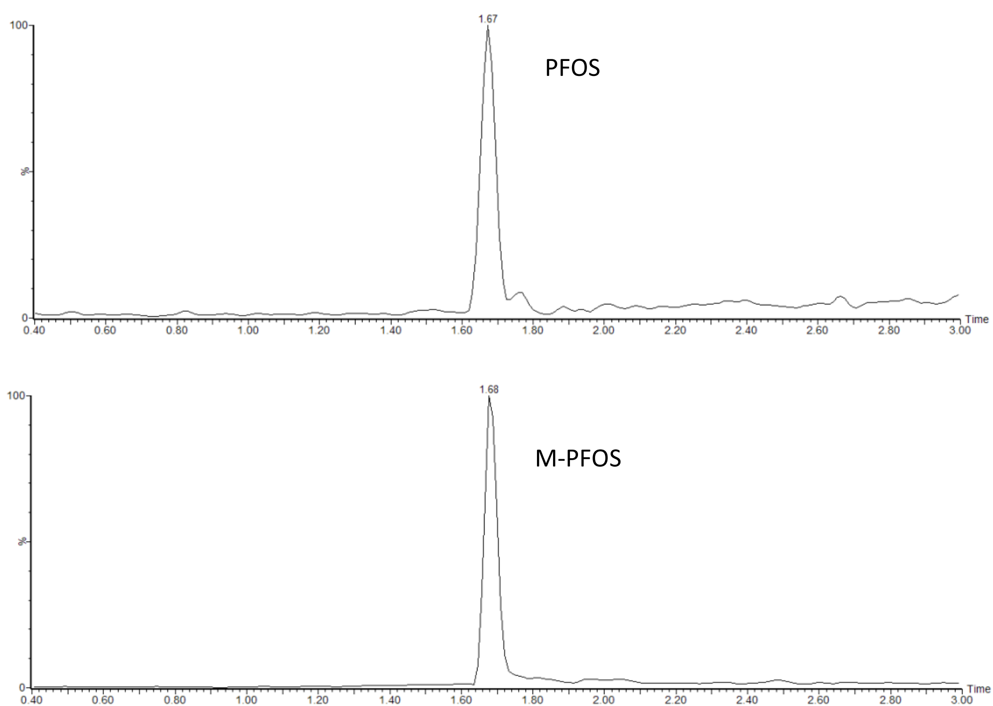

Figure 4.12 Chromatograms of the human breast milk sample “C” (PFOS concentration = 102 pg/mL): diagnostic ions for PFOS and M-PFOS

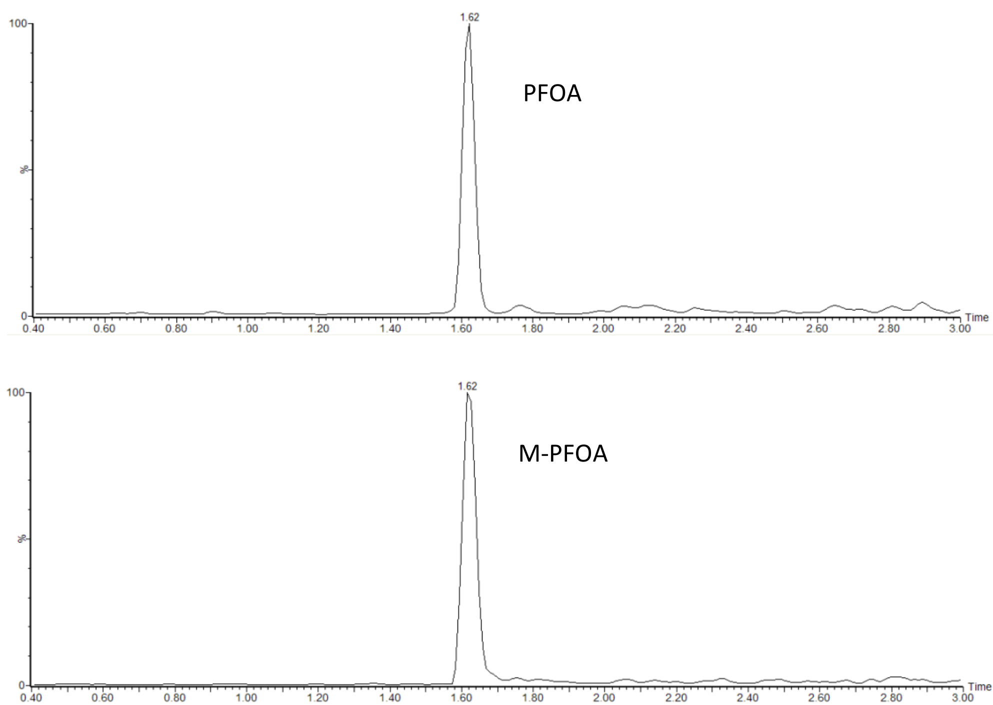

Figure 4.13 Chromatograms of the human breast milk sample “L” (PFOA concentration = 181 pg/mL): diagnostic ions for PFOA and M-PFOA

4.2.2.2 Cow milk from Italy

PFOS and PFOA levels measured in cow milk samples are reported in **Table 4.16**. Concentrations higher than the LOD but lower than the LOQ (calculated as 3 times the LOD) were reported as traces.

Sample	PFOS (pg/mL)	PFOA (pg/mL)
A	Traces	<LOD
B	26	25
C	29	24
D	Traces	Traces
E	Traces	Traces
F	22	32
G	21	Traces
H	Traces	Traces
I	15	Traces
J	31	27
K	32	29
L	17	<LOD
M	18	Traces
N	16	<LOD
O	15	<LOD
P	26	Traces
Q	67	Traces
R	Traces	Traces
S	Traces	Traces
T	16	Traces
U	22	Traces
V	25	<LOD

Table 4.16 PFOS and PFOA concentrations in cow milk samples from Italy

As it can be seen, PFOS was the predominant substance, having been always detected at least at trace levels. In 73% of samples it was found with concentrations in the range 15-67 pg/mL, with an arithmetic mean of 25 pg/mL (median 22 pg/mL). The most contaminated sample resulted a raw milk purchased at a local vending machine.

PFOA was present in almost all samples, but could only be quantified in 6 out of 22 (27%). The mean concentration was 27 pg/mL, with a maximum level of 32 pg/mL, measured in a pasteurized whole milk sample.

4.3 Data interpretation

4.3.1 Human breast milk

A comparison between PFCs levels in the human breast milk samples processed during the present study (collected in 2010) and those reported in other works available in literature so far is presented in the table on the next page.

Even if the various analytes gave a variable contribution to total PFCs content, PFOS was the most detected compound, being found in almost all samples, with median concentrations around 100 pg/mL but reaching also values in the order of ng/mL in some cases. PFOA was measured at levels generally slightly lower compared to PFOS and showed great variability in terms of frequency among the different studies, probably partially due to the fact that also limits of detection for this analyte varied significantly. In particular, in the case of the present work, the high sensitivity of the employed instruments allowed to confirm its presence in all the samples, even at very low concentrations. PFHxS was often detected as well, in some cases even in a greater percentage of samples and at higher concentrations than PFOA. No correlation was observed between the concentrations of the various compounds.

Although during this research project only a limited number of samples were analyzed, these data are among the first information available on the presence of these contaminants in human breast milk from France and Italy.

A research group at LABERCA laboratory recently analyzed 30 samples of French human breast milk employing the analytical method here described and performing analysis in LC-HRMS, reporting the presence of PFOS and PFOA in 100% of the samples and at levels in line with previous data (Kadar et al., 2011).

In Italy, Guerranti et al. reported in 2011 the results of an investigation on PFOS and PFOA presence in human breast milk form the Siena area (Italy), which gave out of average results: PFOS concentrations ranged between the LOD of 0.5 ng/mL and 4.28 ng/mL, while PFOA was found in 1 case at 7.78 ng/mL.

Country	#	PFOS (pg/mL)	PFOA (pg/mL)	PFHxS (pg/mL)	PFNA (pg/mL)	*Reference*
France	11	32-433(127) [100%]	43-297(116) [100%]	<12-77(22.5) [91%]	<19-69(25) [82%]	*Present study*
Italy	13	40-143(76) [100%]	24-281(63) [100%]	-	-	
France	30	24-171(74) [100%]	18-102(57) [100%]	0 [0%]	-	*Kadar et al. (2011)*
Spain	20	<28-865(84) [95%]	<21-907(nd) [40%]	-	0 [0%]	*Llorca et al. (2010)*
Germany	203	<50-284(82) [55%]	<80-610(137) [56%]	<160-180(nd) [1%]	0 [0%]	*Bernsmann and Fürst (2008)*
Germany	57	28-309(119) [100%]	<201-460(nd) [19%]	-	-	*Völkel et al. (2008)*
Germany	201	<30-110(40) [72%]	<150-250(nd) [2%]	<20-30(nd) [3%]	-	*Fromme et al. (2010)*
Hungary	13	96-639(330) [100%]	0 [0%]	-	-	*Völkel et al. (2008)*
Sweden	12	60-470(166) [100%]	<209-492(nd) [6%]	31-172(70) [100%]	<5-20(nd) [17%]	*Kärrman et al. (2007)*
USA	45	<32-617(106) [96%]	<30-161(36) [89%]	<12-64(12) [51%]	<5-18(7) [64%]	*Tao et al. (2008b)*
China	19	45-360(110) [100%]	47-210(110) [100%]	4-100(11) [100%]	6-62(18) [100%]	*So et al. (2006)*
China	24*	6-137(49) [100%]	<LOQ-814(35) [88%]	<LOQ-15(6) [83%]	6-76(13) [100%]	*Liu et al. (2010)*
Japan	51	8-401(nd) [100%]	<LOQ-339(nd) [44%]	<LOQ-25(nd) [64%]	<LOQ-150(nd) [86%]	*Nakata et al. (2007)*
Japan	24	140-523(196) [100%]	<43-170(67) [92%]	<2-18(6) [92%]	<9-24(nd) [13%]	*Tao et al. (2008a)*
Malaysia	13	49-350(111) [100%]	<43-90(nd) [23%]	<2-13(7) [85%]	<9-15(nd) [8%]	*Tao et al. (2008a)*
Philippines	24	27-208(104) [100%]	<43-183(nd) [29%]	<2-59(13) [92%]	<9-25(nd) [17%]	*Tao et al. (2008a)*
Indonesia	20	25-256(67) [100%]	0 [0%]	<2-6(nd) [45%]	<9-135(nd) [5%]	*Tao et al. (2008a)*
Vietnam	40	17-393(59) [100%]	<43-89(nd) [3%]	<2-27(4) [73%]	<9-11(nd) [5%]	*Tao et al. (2008a)*
Cambodia	24	17-327(40) [100%]	<43-132(nd) [4%]	<2-19(nd) [13%]	<9-12(nd) [13%]	*Tao et al. (2008a)*
India	39	<11-120(39) [85%]	<43-335(nd) [8%]	<2-13(nd) [36%]	0 [0%]	*Tao et al. (2008a)*
Developing countries	19	<9-65(nd) [84%]	<80-192(nd) [31%]	<9-14(nd) [11%]	<25-55(nd) [21%]	*Kärrman et al. (2011)*

Table 4.17 Comparison between measured levels of the most found PFCs in human breast milk samples and other published data: range, (median) and [frequency]

** 24 pooled samples, consisting of 1237 individual samples*

Taking into account the primipara/multipara status of the Italian donors, it was observed that mean concentration for PFOS in milk from primiparous mothers was 85 pg/mL (median 81 pg/mL), while in that from multiparas it decreased to 68 pg/mL (median 60 pg/mL). Similarly, PFOA mean level in primiparas was 79 pg/mL (median 75 pg/mL), decreasing to 57 pg/mL (median 62 pg/mL) in women nursing for at least the second time. **Figure 4.14** resumes graphically the described results.

Even if the number of analyzed samples is rather low to draw statistically significant conclusions, it can be observed a trend for both compounds to decrease in breast milk after the first lactation, as reported also by Tao et al. (2008b). This suggests the role of breast feeding as a route of gradual elimination of perfluorinated compounds, which entails a potentially higher exposure for first-born infants.

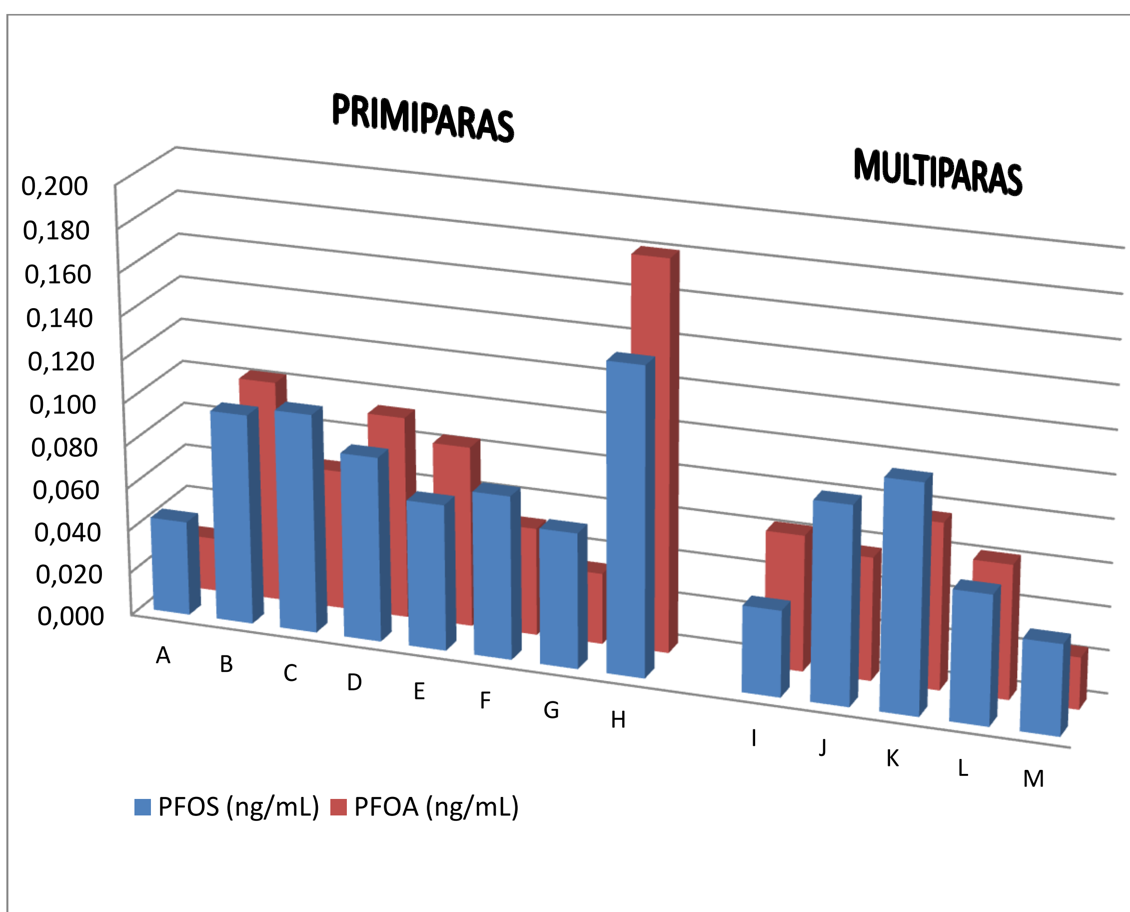

Figure 4.14 Graphic representation of measured levels of PFOS and PFOA in human breast milk samples from Italy

Based on the data obtained for France and Italy, PFOS and PFOA daily intakes (DIs) for newborns, whose diet consists almost exclusively of breast milk, were calculated using this formula (expressed as ng/kg b.w./day):

$$Daily\ intake = \frac{mean\ consumption\ (mL/day) * mean\ concentration\ (ng/mL)}{body\ weight\ (kg)}$$

The resulting values, considering an average milk consumption of 780 mL/day during the first 6 months of infant's life, with a mean body weight of 5 kg (SCF, 2003), are reported in **Table 4.18**. For each compound, DIs were calculated on the basis of the lowest, highest and mean concentrations measured in the two Countries.

Country	PFOS			PFOA		
	Min DI	Max DI	*Mean DI*	Lower DI	Upper DI	*Mean DI*
France	4.99	67.5	*21.53*	6.71	46.33	*18.88*
Italy	6.24	22.31	*12.17*	3.74	43.84	*11.08*

Table 4.18 PFOS and PFOA daily intake (ng/kg b.w./day)

Daily intakes were then related to TDIs suggested by EFSA (150 ng/kg b.w. for PFOS, 1500 ng/kg b.w. for PFOA) in order to estimate the risk index (RI) for newborns:

$$Risk\ index = \frac{DI}{TDI}$$

Country	PFOS			PFOA		
	Min RI	Max RI	*Mean RI*	Lower RI	Upper RI	*Mean RI*
France	0.03	0.45	*0.14*	<0.01	0.03	*0.01*
Italy	0.04	0.15	*0.08*	<0.01	0.03	*0.01*

Table 4.19 Risk index for PFOS and PFOA

Higher risk index values were observed for PFOS, but even the maximum RIs were significantly lower than 1, meaning that no toxicological risk should be expected to derive from PFOS and PFOA intake through breastfeeding. At the same time, it must be also observed that RIs are based on TDIs, which for these contaminants have not yet been defined, existing rather different values suggested by various authorities and referred to adult population. Therefore, what the preliminary results of this study bring out is that the daily intake of these substances for infants via breast milk seems to be several times higher than those reported for adults through diet.

These first outcomes suggest the interest in further investigations on the existence of potential correlations between PFCs levels in breast milk and mother's history, not only concerning lactation, but taking into account also other possible influencing factors, such as age at delivery, length of the interval between two deliveries, diet habits and place of origin, in order to better define the real exposure for newborns.

4.3.2 Cow milk

In general, according to data available in literature, PFCs concentrations measured in cow milk are significantly lower than those found in human breast milk. This was confirmed also by the results of both the investigations performed in the present work. One possible explanation for this difference is that these contaminants have a reduced possibility to accumulate, in terms of years of life before the first lactation, in bovines compared to humans. Moreover, the continuous lactation, interrupted only by the dry period, leads to an almost constant elimination over time in these animals.
In **Table 4.20**, the results of the current experiment are compared to data reported in the previously mentioned works on cow milk.

Country	#	PFOS (pg/mL)	PFOA (pg/mL)	PFHxS (pg/mL)	PFNA (pg/mL)	PFHpA (pg/mL)	*Reference*
France	9	<19-40 [56%]	<16-23 [33%]	<LOQ-11 [11%]	<16-24 [22%]	0 [0%]	*Present Study*
Italy	22	<15-67 [73%]	<24-32 [27%]	-	-	-	
Norway	1	7 [100%]	5 [100%]	<LOQ [0%]	<LOQ [0%]	<LOQ [0%]	*Haug et al. (2010)*
Spain	4	<19 [0%]	<55-58 [50%]	-	-	<14-16 [50%]	*Ericson et al. (2008a)*
UK	11	<1000	<1000	-	-	-	*Mortimer et al. (2009)*
USA	12	<11 [0%]	<48 [0%]	<1-4 [67%]	<2 [0%]	<1 [0%]	*Tao et al. (2008a)*
China	84	<5-695 [36%]	<18-178 [46%]	-	<27-476 [68%]	<13-312 [68%]	*Wang et al. (2010)*

Table 4.20 Comparison between measured levels of the most found PFCs in cow milk samples and other published data: range and [frequency]

Measured levels show a certain variability, depending on the Country but often also on the sensitivity of the employed methods.

Although also other PFCs were detected in some cases (PFHxA and PFDA, Haug et al.; PFDA and PFUnA, Wang et al.), the global cow milk contamination by these pollutants appears negligible. This was recently confirmed also by EFSA, on the basis of the results of the European monitoring on perfluoroalkylated substances in food, reporting PFOS presence in only 2 sheep milk samples out of 121 analyzed samples (including cow, sheep and goat milk). However, in the cited work Mortimer et al. estimated a total PFASs upper bound concentration of 11 ng/g. Therefore, further investigations are needed in order to ascertain the potential risks deriving from the consumption of different types of milk in the various Countries.

Moreover, surveys should include also cheese and other dairy products, since they represent an important component of the diet for most of the population and are likely to concentrate PFCs, which seem to have high affinity for proteins.

Within the monitoring of Italian cow milk samples, no significant correlations were observed between the type of milk and its level of contamination by perfluorinated compounds.

4.3.3 Powder milk

The importance of evaluating potential PFCs contaminations in powder milk is evident considering the fact that, sometimes by choice but most of the times by necessity (in case of mothers unable to produce milk), it can have a major role in newborns diet.

Among all the surveys that have been conducted on the presence of these contaminants in food, only three included powder milk in the monitored matrices, reporting extremely different results.

After analyzing 21 samples (diluted 1:1 in water) from 5 different brands available in the United States, representing 99% of the US market, Tao et al. (2008a) reported PFOS presence above the LOQ (10 pg/mL) with a frequency of 24% and a maximum

concentration of 11 pg/mL; PFHxS was found in 48% of samples, with measured levels lower than 4 pg/mL.

Higher levels were observed by Wang et al. (2010) within a monitoring on 36 Chinese powder milk samples, reporting the presence of PFOS, PFOA and PFNA with frequencies of 33, 25 and 22%, respectively. Mean concentration in quantifiable samples was 46 pg/g (range <36-482 pg/g) for PFOS, 22 pg/g (<10-175 pg/g) for PFOA and 30 pg/g (<54-192 pg/g) for PFNA.

In the 4 powder milk samples processed in the presented work, only PFOS was quantified in one sample (at 17 pg/g), while it was present in some cases at trace levels, as well as PFOA and PFNA.

Comparing the above data, even if a certain variability in the presence of the various analytes can be observed, PFCs concentrations result globally about an order of magnitude lower than those likely to be found in human breast milk. Therefore, purely in terms of exposure for newborns, these results suggest that powder milk may be potentially less dangerous for newborns.

Nevertheless, in the study conducted by Llorca et al. (2010) 3 infant formulas were analyzed, showing surprisingly high PFCs concentrations. PFDA was the predominant substance, with concentrations between 693 and 1289 pg/g; also PFOS and PFOA (ranges 229-1098 and 374-723 pg/g, respectively) were found at relevant levels.

On the contrary, Fromme et al. could not quantify any of the PFCs monitored in the 4 different infant formula samples included in their study (Fromme et al., 2010).

It's difficult to explain the reasons for such big differences, however when comparing this kind of food products, it must be considered that the various items can be extremely different from each other in terms of composition (e.g. milk- or soy-based), state of conservation (e.g. powder or concentrated liquid) and packaging material (e.g. glass, plastic, cardboard). All these factors can therefore translate into different amounts of contaminants deriving from various sources.

However, further data, on a wider range of samples, are needed to draw reliable conclusions on perfluorinated compounds presence in this matrix.

4.4 PFCs precursors analysis at LABERCA

As previously reported, fluorotelomer alcohols and perfluorooctane sulfonamido ethanols have been mainly analyzed in air and house dust, few Authors investigated their presence in water as well, but little information is available to this day concerning their potential contamination of food items. For this reason, in compliance with what expressed by Recommendation 2010/161/EU, a method for the detection of some of these PFCs precursors in fish (which is considered the most affected food item by perfluoroalkylated substances) was developed.

Within this work package, various tests were performed also on another group of perfluorinated compounds precursors, the so-called PAPS (polyfluoroalkyl phosphate surfactants), which were mentioned in the above EU Recommendation as well. These compounds were detected in LC-MS/MS, but various problems have been encountered trying to optimize their extraction, which needs further experiments, and for this reason they will not be discussed in this thesis.

4.4.1 Instrumental analysis

Some initial experiments were conducted to detect FOSEs and FTOHs on the LC-MS/MS system in negative electrospray mode. Even after the removal of all the buffer solutions, acetate adducts were the only detected signals for N-MeFOSE and N-EtFOSE, and neither specificity nor sensitivity were satisfying; as for fluorotelomer alcohols, they are known to be difficult to ionize in ESI and weren't detected at all. Optimization on GC-MS/MS was therefore preferred.

The initial tests were performed using a medium-high polarity column (50% phenyl / 50% dimethylpolysiloxane), but it showed a lack of retention for FTOHs, whose peaks overlapped with the solvent front. For this reason it was replaced by a Varian CP-WAX 57CB column: the extreme inertness of this highly polar column, made of 100%

chemically-bonded polyethylene glycol, allowed an accurate separation and very good peak shapes for all compounds.

Pulsed splitless mode was chosen as injection technique, since it combines the advantages of splitless, which allows a higher amount of sample to deposit on the column resulting in a greater response, and split, which provides better chromatographic resolution thanks to a greater number of theoretical plates.

Also for the ionization technique various options have been weighed up. Electron impact (EI), based on a beam of electrons which collide with the vaporized sample transforming its molecules in ions, appeared a too strong method especially for FTOHs: they were in fact subjected to on-source fragmentations generating small and non-specific ions. Chemical ionization (CI) was thus tested, in both negative and positive mode. In this technique the electron impact is applied to a reagent gas (in this case methane) which then interacts with the target molecules, causing a softer ionization that results in the formation of pseudo-molecular ions. These species are ions of the type $[M+H]^+$ or $[M-H]^-$, depending if a positive or a negative ionization has been employed, and tend to be relatively more stable than their corresponding molecular ions. As a consequence, their fragmentation is sparse and it's easier to detect them. In this case positive CI was preferred, since it allowed to detect all compounds in full scan mode and to produce two specific fragments for each analyte after fragmentation in the collision cell. **Figure 4.15** and **Figure 4.16** show the full scan mass spectrum of N-EtFOSE and 8:2 FTOH, respectively, obtained after positive chemical ionization.

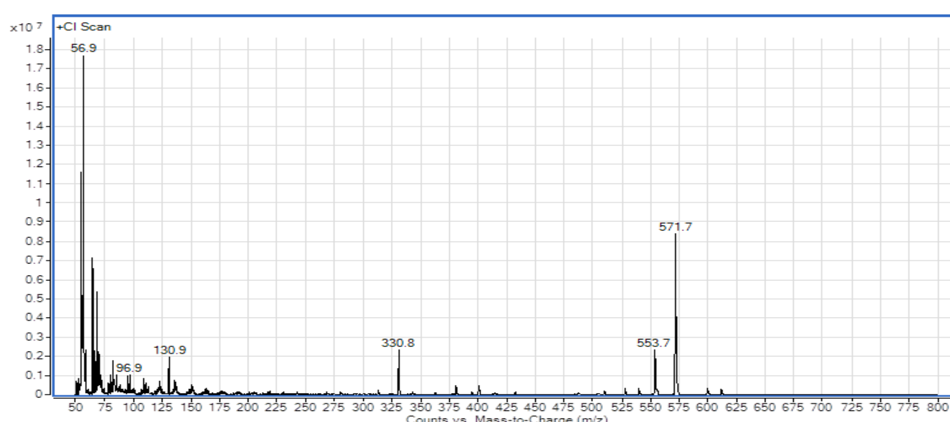

Figure 4.15 Mass spectrum of N-EtFOSE in full scan GC-(CI+)-MS

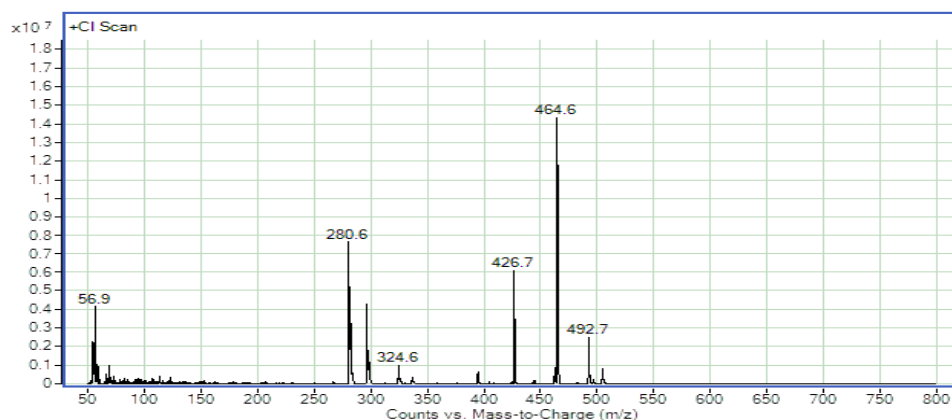

Figure 4.16 Mass spectrum of 8:2 FTOH in full scan GC-(CI+)-MS

For the unambiguous identification of the compounds, two transitions were monitored in MRM mode. The most intense signal was given for all the analytes by a big fragment obtained applying a relatively low collision energy (10 V): for example, for N-EtFOSE and 8:2 FTOH the observed transitions were 571.7 > 554.0 *m/z* and 464.6 > 426.7 *m/z*, respectively. Parent-to-parent transitions (obtained with the lowest collision energy, equal to 1 V) were used for confirmatory purposes, being preferred to the significantly less intense secondary fragments produced with a high collision energy (30 V).

The following picture shows the peak of the most representative transition and its retention time for each compound, analyzed in gas chromatography (using the previously mentioned column) coupled to tandem mass spectrometry, operating positive chemical ionization and acquiring in multiple reaction monitoring mode.

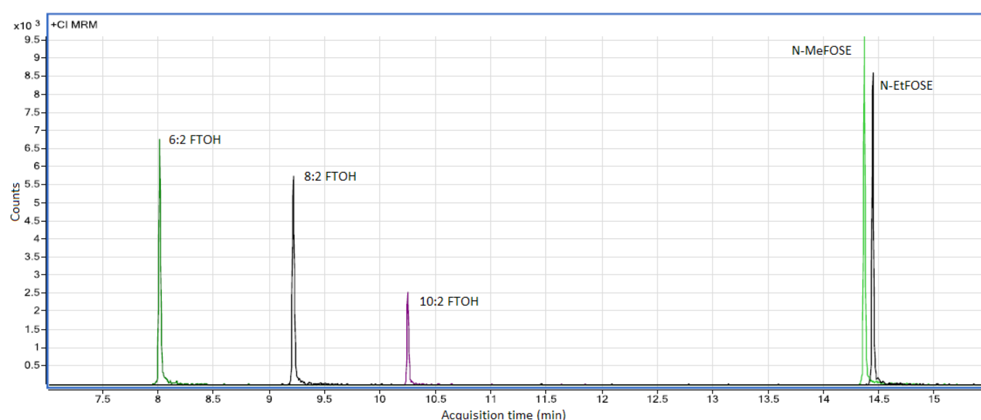

Figure 4.17 Separation of the diagnostic ions of the 3 FTOHs and the 2 FOSEs monitored. 0.02 ng injected in GC-(CI+)-MS/MS

4.4.2 Optimization of the extraction procedure

The performed preliminary tests aimed at the inclusion of these substances in the range of analytes extracted through an already existing procedure developed at LABERCA for PFCs in fish. This method is basically an evolution of that proposed by Powley et al. (2005), which was based on an extraction with methanol followed by graphitized carbon clean up. Here, a further purification through hydrated silica gel was added, followed by centrifugation.

In order to balance the effects of the natural variability deriving from the different phases of the extraction procedure, an internal standard for each family was added at the beginning of the procedure. In particular, being extremely similar molecules, both FOSEs were associated to deuterated N-MeFOSE, while labeled 8:2 FTOH was used for the 3 FTOHs.

Due to the extreme volatility of both FTOHs and FOSEs, it resulted impossible to extract them from the samples with this technique without causing their almost total evaporation, therefore some changes have been made to try to reduce losses during the various concentration steps. Instead of evaporating to dryness, the volume of the extracts were just reduced to around 500 µL after the ENVI-Carb™ purification and to 200 µL before the injection. In addition, decreasing the temperature of the dry bath from 45 to 35 °C and applying a more gentle nitrogen flow significantly increased the recovery for N-MeFOSE and N-EtFOSE; as for FTOHs, even after these preventive measures the efficiency of the extraction remained unsatisfactory.

To reduce fluorotelomer alcohols losses, some tests using a keeper solvent were performed as well, but without improvements.

The extraction procedure previously described for PFCs in milk was tested on these molecules as well but, even if elution profile assays seemed encouraging, the analytical results were poor and thus the method was discarded.

4.4.3 Performances of the method

The performances of the developed method for N-EtFOSE and N-MeFOSE were evaluated through the injection of a 6 levels matrix-matched calibration curve (0, 0.5, 1, 5, 10 and 50 µg/kg of dry matter).

Linearity was very good for both compounds, with R^2 (coefficient of determination) values higher than 0.99, as highlighted in **Figure 4.18**.

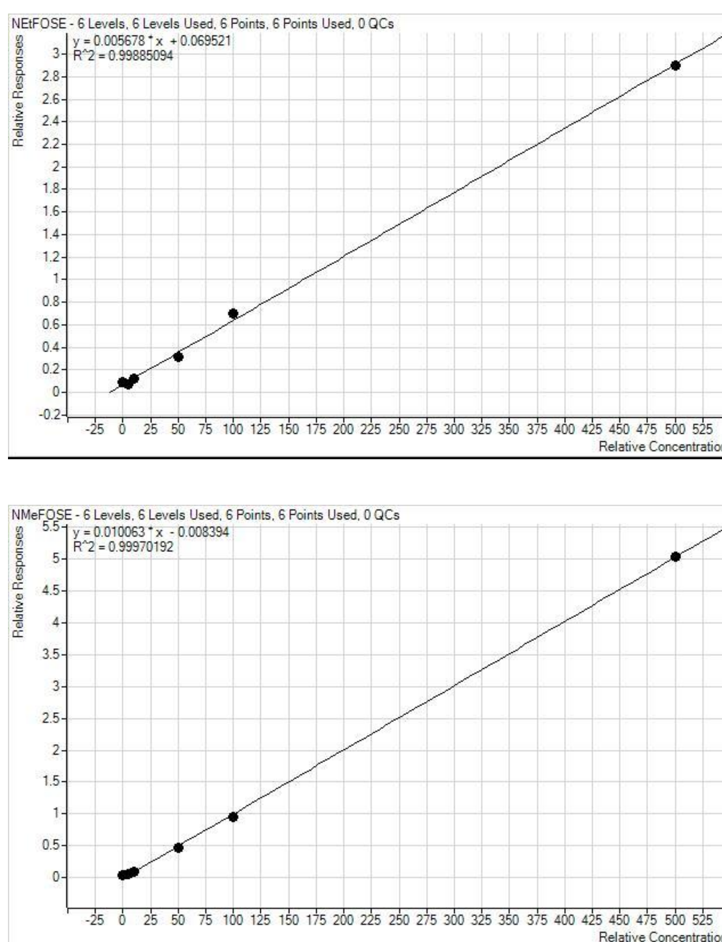

Figure 4.18 Calibration curves obtained for N-EtFOSE and N-MeFOSE in a fish sample

As for the limits of detection, they were evaluated as the concentrations showing a signal-to-noise ratio of 3, resulting equal to 0.03 ng/g of wet matter (corresponding to 0.118 ng/g of dry matter) for N-EtFOSE and 0.04 ng/g of wet matter (0.156 ng/g of dry matter) for N-MeFOSE.

The chromatograms in **Figure 4.19** show the signals obtained monitoring the most representative transitions of N-EtFOSE, N-MeFOSE and of their internal standard (d7-N-MeFOSE) in a spiked fish sample, demonstrating, besides the sensitivity at this level of contamination (0.25 ng/g of wet matter), also the specificity of the signal.

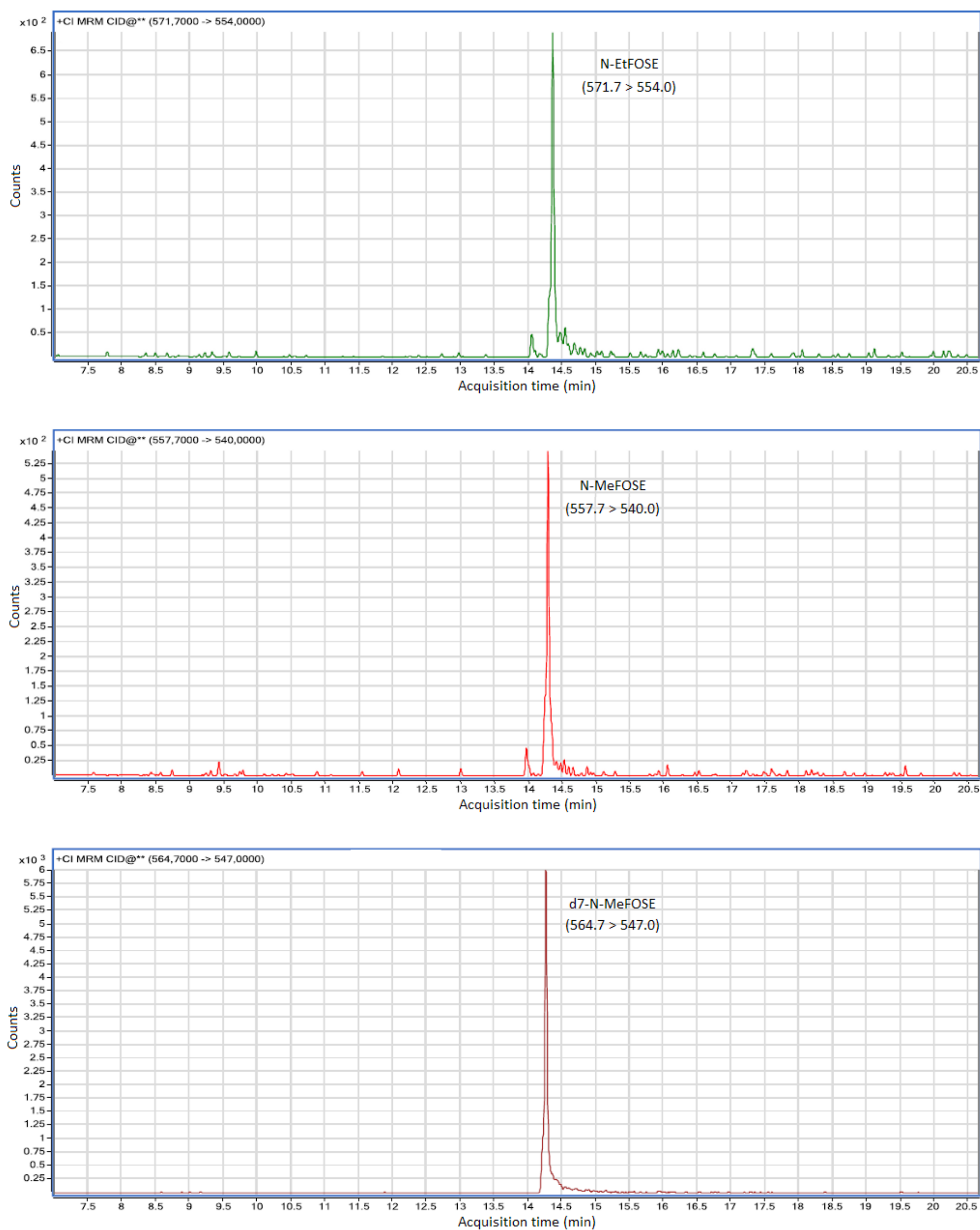

Figure 4.19 Diagnostic ion chromatograms for N-EtFOSE, N-MeFOSE and d7-N-MeFOSE in a fish sample spiked at 0.25 ng/g (2.5 ng/g for d7-N-MeFOSE) of wet matter

Absolute extraction recoveries were calculated comparing two samples processed in parallel, of which one had been spiked at the beginning and the other at the end of the extraction procedure, and resulted between 40 and 50% for both compounds.

Further tests are needed to confirm the reliability of the method and to evaluate all the parameters required for its complete validation. However, a preliminary monitoring on 15 fish samples using the described technique has been recently performed by Pollono et al. (2011), who reported the presence of N-EtFOSE in certain samples at concentrations up to 5 ng/g of wet matter.

In conclusion, a promising method for perfluorooctane sulfonamido ethanols detection in fish was developed, allowing to monitor the presence of these PFOS precursors even at trace levels in what is considered the most relevant contributor to perfluoroalkyl substances dietary intake. This is a further achievement meeting the requests expressed by Commission Recommendation 2010/161/EU on the monitoring of these substances in food.

5. Conclusions

An efficient method based on liquid chromatography-mass spectrometry for the detection of 16 different perfluorinated compounds in milk has been validated in accordance with current European regulation guidelines (2002/657/EC) and is currently under evaluation for ISO 17025 accreditation.

The analytical part was optimized at the French laboratory LABERCA and was developed in parallel on a HPLC-MS/MS system, which is considered the standard solution for PFCs measurement, and on a HPLC-HRMS instrument (the Thermo Scientific LTQ-Orbitrap™), which proved to be a good alternative, providing in some cases even better performances in terms of specificity and sensitivity. In fact, the accurate extracted mass signal obtained with this high resolution detector allowed ultra trace level quantification of such a large number of analytes despite their difficult and unspecific fragmentation.

The described method represents a useful instrument for large-scale surveys on the contamination of this matrix and was applied to cow, powder and human breast milk samples from France to produce a limited and preliminary monitoring. Subsequently, the procedure was successfully transferred to the Italian laboratory CABA-Lab and employed for a similar pilot survey on PFOS and PFOA levels in cow and human breast milk samples collected in Italy.

The obtained data, among the first produced in these two Countries, resulted in line with those of most of the studies available in literature, proving the presence of PFOS and PFOA in all the human breast milk samples analyzed, at concentrations (range 30-400 pg/mL) several times higher than those measured in cow milk. These early results thus confirmed the importance of breastfeeding as a major route of exposure for infants and were used to perform an evaluation of the risk index, calculated as the ratio between the daily intake and the TDI (tolerable daily intake) suggested by EFSA, for 0-6 months old subjects, whose diet is constituted almost exclusively by breast

milk. Calculated risk indices suggested that there's apparently no risk related to PFCs intake via breastfeeding for newborns, but it must be noticed that a number of different TDIs have been proposed for these contaminants and they all referred to the adult population.

Moreover, the analysis of the Italian milk samples, for which the primipara/multipara status of the donor was known, suggested that milk produced by mothers breastfeeding for the first time was more contaminated, highlighting the role of lactation as elimination route.

In consideration of the above, further investigations on larger numbers of samples are needed to completely understand health risks for infants deriving from breast milk consumption and to define potential correlations between measured PFCs levels and individual parameters related to the mother.

In accordance with the recent European Commission Recommendation 2010/161/EU on the monitoring of perfluoroalkylated substances in food, in which Member States are required to focus not only on PFOS and PFOA but as well on their precursors, this project led also to the development of a promising technique for the quantification on N-MeFOSE and N-EtFOSE (perfluorooctane sulfonamido ethanols, precursors of PFOS) in fish. This method showed extremely satisfying performances in terms of linearity and limits of detection, allowing to measure these two contaminants at ultra trace levels in this matrix, and will be a useful tool for future surveys.

The increasing interest on these emerging contaminants and on their adverse effects on human health has led to the need for extensive monitoring of their presence in food, in order to enable an accurate hazard evaluation deriving from dietary exposure. The research project presented in this thesis is in line with this aim, providing some preliminary data and reliable methods to be employed for further investigations.

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