UiT The Arctic University of Norway

Faculty of Health Sciences
Department of Community Medicine

Human Exposure to PFAS and Other Anthropogenic Organofluorine Chemicals in Tromsø between 1986 and 2015

Addressing exposure changes during three decades with a combination of analytical methods

Lara Cioni

A dissertation for the degree of Philosophiae Doctor

September 2023

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Summary

In the last century, there has been a dramatic increase in chemical production and number and diversity of chemicals produced. Especially, organofluorine chemistry has increased its importance due to applications in pharmaceuticals, agrochemicals, refrigerant gases and in consumer products and industry with fluoropolymers and per- and polyfluoroalkyl substances (PFAS).

PFAS are receiving international attention due to ubiquitous detection in the environment and in humans, their persistence and potential health and environmental impacts. Due to these concerns, production of some PFAS has been reduced internationally and their human blood concentrations are declining globally. However, PFAS production shifted towards new chemistries. Since >4700 PFAS exist and there is growing evidence about the presence of unknown organofluorine in human blood, there are concerns about PFAS human exposure underestimation.

The overall thesis aim was to improve the description of PFAS and organofluorine exposure over three decades (1986-2015), covering a relevant timeframe for PFAS legislation and production changes.

To achieve our goal, a new method to measure unknown PFAS in human blood had to be developed and we developed the total oxidizable precursors (TOP) assay for human serum. Pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015 were analysed using a fluorine mass-balance approach that included total fluorine (TF), extractable organic fluorine (EOF), target PFAS, suspect screening and, for the first time, TOP and fluorinated pharmaceuticals.

Our study shows that TF concentrations did not change significantly between years, while EOF decreased between 1986-2007 and did not change between 2007-2015. However, the composition of EOF has been changing through years. While PFAS concentrations were highest in 2007, TOP concentrations were low and did not change between years and fluorinated pharmaceuticals and metabolites concentrations increased between 1986-2015. Further, suspect screening revealed only one additional PFAS with low concentrations.

Sammendrag

I det siste århundre har det vært en dramatisk økning i produksjon av kjemikalier, både i antall og mangfold. Spesielt har organofluorkjemi fått økt betydning på grunn av gruppen med fluorforbindelser kjent som per- og polyfluoralkylforbindelser (PFAS). PFAS brukes i produksjon av farmasøytiske produkter og agrokjemikalier, gasser til kjøleanlegg, i en rekke forbrukerprodukter og polymerindustri .

Den internasjonale oppmerksomheten rundt PFAS skyldes at de er funnet overalt i miljøet og i mennesker, demmes stabilitet i miljøet og potensielt helseskadelige. På grunn av disse bekymringene har produksjonen av noen PFAS blitt faset ut eller redusert. Som en følge av dette har konsentrasjonen av noen av disse PFAS målt i blodet hos mennesker avtatt på en globalt skala. Etter initiativene til utfasing av noen PFAS skiftet produksjonen av PFAS-til nye varianter. I dag eksisterer det over 4700 PFAS, og flere studier indikerer en tilstedeværelse av ukjente organiske fluorforbindelser i humant blod.

Det overordnede målet med oppgaven var å forbedre forståelsen av PFAS- og organofluoreksponeringen over tre tiår (1986-2015). Denne tidsperioden dekker også den relevante tidsrammen for PFAS-lovgivning og produksjonsendringer.

For å oppnå vårt mål, en måtte vi utvikle ny metode for å måle ukjente PFAS i human plasma. Vi utviklet metoden total oxidizable precursors (TOP) for serum for dette formålet. Sammenslåtte serumprøver fra Tromsø-undersøkelsen samlet inn i 1986, 2007 og 2015 ble analysert ved hjelp av en fluormassebalansemetode som inkluderte totalfluor (TF), ekstraherbar organisk fluor (EOF), kjente PFASer, mulige PFASer og, for første gang, totale oksiderbare forløpere (TOP) og fluorerte legemidler.

Vår studie viser at TF-konsentrasjonene endret seg ikke vesentlig mellom årene, men mengden EOF avtok mellom 1986-2007 og var uendret mellom 2007-2015. Sammensetningen av EOF har imidlertid vært i endring gjennom årene. Mens PFAS-konsentrasjonene var høyest i 2007, var TOP-konsentrasjonene lave og endret seg ikke mellom år, og bidraget til fluorerte legemidler og metabolitter økte mellom 1986-2015. Videre viste analysen av mulige PFASer bare én ekstra PFAS med lave konsentrasjonene.

Sommario

Nell'ultimo secolo, la produzione chimica e il numero e la diversità di sostanze prodotte sono drasticamente aumentate. In particolare, la chimica degli organofluoruri ha guadagnato importanza grazie alle sue applicazioni nei prodotti farmaceutici, agrochimici, nei gas refrigeranti e nei prodotti di consumo e nell'industria grazie a fluoropolimeri e sostanze per- e polifluoroalchiliche (PFAS).

Le PFAS sono oggetto di attenzione a livello internazionale a causa della presenza ubiquitaria nell'ambiente e negli esseri umani e dei potenziali impatti sulla salute e sull'ambiente. A causa di queste preoccupazioni, la produzione di alcune PFAS è stata ridotta internazionalmente e le loro concentrazioni nel sangue umano stanno diminuendo in tutto il mondo. Tuttavia la produzione di PFAS si è spostata verso nuove sostanze. Poiché esistono > 4700 PFAS e vi sono sempre più prove a sostegno della presenza di organofluorori sconosciuti nel sangue umano, ci sono preoccupazioni sulla sottostima dell'esposizione umana alle PFAS.

L'obiettivo generale della tesi è quello di migliorare la descrizione dell'esposizione umana alle PFAS e agli organofluorori durante tre decenni (1986-2015), in cui la legislazione e la produzione delle PFAS sono cambiate.

Per raggiungere questo obiettivo, è stato necessario sviluppare un nuovo metodo per misurare i PFAS sconosciuti nel sangue umano e in questo studio abbiamo sviluppato il test dei precursori ossidabili totali (TOP) per il siero umano. Pool di siero umano provenienti dal Tromsø Study raccolti nel 1986, 2007 e 2015 sono stati analizzati utilizzando un approccio basato sul bilancio di massa del fluoro, includendo fluoro totale (TF), fluoro organico estraibile (EOF), PFAS conosciuti, suspect screening e, per la prima volta, TOP e farmaci fluorurati.

Le concentrazioni di TF non sono cambiate in modo significativo tra gli anni analizzati, mentre l'EOF è diminuito tra1986-2007 e non è cambiato tra 2007-2015. Tuttavia, la composizione dell'EOF è cambiata nel corso degli anni. Mentre le concentrazioni di PFAS erano più alte nel 2007, le concentrazioni di TOP erano basse e non sono cambiate nel corso degli anni e le concentrazioni dei farmaci fluorurati e dei loro metaboliti sono aumentate tra 1986-2015. Inoltre, il suspect screening ha rivelato solo una PFAS aggiuntiva in basse concentrazioni.

Resumen

En el último siglo, se ha verificado un incremento significativo de la producción química y del número y diversidad de sustancias químicas producidas. Especialmente, la química organofluorada ha aumentado su importancia debido a sus aplicaciones en productos farmacéuticos, agroquímicos, gases refrigerantes y en productos de consumo e industria con fluoropolímeros y sustancias perfluoroalquiladas (PFAS).

Las PFAS son objeto de atención internacional debido a su detección ubicua en el medio ambiente y en los seres humanos y por su posible impacto ambiental y en la salud. Debido a estas preocupaciones, la producción de algunas PFAS se ha reducido a nivel internacional y las concentraciones en sangre humana están disminuyendo a nivel mundial. Sin embargo, la producción de PFAS se ha desplazado hacia nuevas sustancias. Dado que existen >4700 PFAS y hay evidencia sobre la presencia de organofluorados desconocidos en la sangre humana, existe preocupación sobre la subestimación de la exposición humana a las PFAS.

El objetivo general de la tesis es mejorar la descripción de la exposición humana a las PFAS y a los organofluorados durante tres décadas (1986-2015), en las que la legislación y la producción de PFAS han cambiado.

Para lograr nuestro objetivo, fue necesario desarrollar un nuevo método para medir PFAS desconocidos en sangre humana y desarrollamos el ensayo de precursores oxidables totales (TOP) para suero humano. Se analizaron pools de suero sanguíneo del Tromsø Study recolectados en 1986, 2007 y 2015 utilizando un balance de masa de flúor que incluyó flúor total (TF), flúor orgánico extraíble (EOF), PFAS conocidos, suspect screening y, por primera vez, TOP y productos farmacéuticos fluorados.

Las concentraciones de TF no cambiaron significativamente en los años investigados, mientras que el EOF disminuyó entre 1986-2007 y no cambió entre 2007-2015. Sin embargo, la composición del EOF ha ido cambiando a lo largo de los años. Si bien las concentraciones de PFAS eran más altas en 2007, las concentraciones de TOP eran bajas y no cambiaron en los años investigados y la concentracion de productos farmacéuticos y metabolitos fluorados aumentó entre 1986-2015. Además, el suspect screening detectó solo una PFAS adicional con concentraciones bajas.

List of papers

This thesis is based on the following papers:

1. Cioni L., Nikiforov V., Coêlho A.C.M.F., Sandanger T.M., Herzke D. **Total oxidizable precursors assay for PFAS in human serum.** *Environ Int.* 2022; 170.

2. Cioni L., Plassmann M.M., Benskin J.P., Coêlho A.C.M.F., Nøst T.H., Rylander C., Nikiforov V., Sandanger T.M., Herzke D. **Fluorine mass balance, including total fluorine, extractable organic fluorine, oxidizable precursors and target PFAS, in pooled human serum from the Tromsø population in 1986, 2007 and 2015**. Manuscript accepted at Environmental Science and Technology.

3. Cioni L., Nikiforov V., Benskin J.P., Coêlho A.C.M.F., Dudášova S., Lauria M.Z., Lechtenfeld O.J., Nøst T.H., Plassmann M.M., Reemtsma T., Sandanger T.M., Herzke D. **Combining advanced analytical methodologies to uncover the contribution of suspect PFAS and fluorinated pharmaceuticals to extractable organic fluorine in pooled human serum from the Tromsø Study between 1986 and 2015.** Manuscript.

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Abbreviations

ACN	Acetonitrile
AMAP	Arctic Monitoring and Assessment Programme
CIC	Combustion ion chromatography
ddMS2	Data dependent acquisition
EOF	Extractable organic fluorine
Et-FOSAA	2-N-ethyl-perfluorooctane sulfonamido acetic acid
FASA	Perfluoroalkane sulfonamides
FASAA	Perfluoroalkane sulfonamido acetic acids
FOSA	Perfluorooctane sulfonamide
FOSAA	Perfluorooctane sulfonamido acetic acid
FTCA	Fluorotelomer carboxylic acids
FT-ICR-MS	Fourier-transform ion cyclotron resonance
FTS	Fluorotelomer sulfonates
FTUCA	Fluorotelomer unsaturated carboxylic acids
HRMS	High-resolution mass spectrometry
LOD	Limit of detection
Me-FOSAA	2-N-methyl-perfluorooctane sulfonamido acetic acid
MTBE	Methyl tert-butyl ether
NorPD	Norwegian prescription database
PFAS	Per- and polyfluoroalkyl substances
PFBS	Perfluorobutane sulfonic acid
PFCA	Perfluoro carboxylic acids
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFECA	Perfluoroalkyl ether carboxylic acids
PFECHS	Perfluoroethylcyclohexane sulphonate
PFHpA	Perfluoroheptanoic acid
PFHpS	Perfluoroheptane sulfonic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFPeA	Perfluoropentanoic acid
PFSA	Perfluoroalkyl sulfonic acids
PFUnDA	Perfluoroundecanoic acid
PFAA	Perfluoroalkyl acids
POPs	Persistent organic pollutants
TF	Total fluorine
TFA	Trifluoroacetic acid
TOP	Total oxidizable precursors
UEOF	Unidentified EOF

1 Introduction

1.1 Human exposure to anthropogenic organic chemicals

Every day, we are exposed to many anthropogenic organic chemicals through the air we breathe, the food we eat, the water we drink, and the products we use. In the last century, we have been exposed to some of these chemicals, like polychlorinated biphenyls (PCBs), organochlorines (OCs) and DDTs [1, 2]. Over the years, there has been a dramatic increase in chemical production, with a growth of a factor of 50 between 1950 and 2010 and further increases in more recent years [3, 4]. In fact, in 2017 the chemical industry was the second-largest manufacturing sector worldwide, with the European Union being the second largest chemical producer by sales value in the world after China [3]. Projections indicate that chemical sales will almost double between 2017 and 2030 [5]. Consequently, the number and diversity of chemicals available on the market has significantly increased in recent decades [6], with estimates from 2020 suggesting that there are over 350 thousands chemicals registered globally [7]. Many of these chemicals play an important role in our society, contributing to our health, food security and industrial production. However, the growing number of chemicals also brings the risk of chemical pollution and potential harm to human health and ecosystems [8]. Chemical pollution is one of the drivers for biodiversity loss [9] and was estimated to cause 9 million human deaths in 2019 [10]. Since many anthropogenic chemicals are showing global impacts on ecosystems and on human health, chemical pollution has also been identified as a planetary boundary, i.e., one of the processes that could generate unacceptable environmental change [11].

One example of chemical pollutants recognized as a global concern are persistent organic pollutants (POPs) [12]. POPs have gained attention due to their high persistence, that comes from their high resistance to degradation in the environment. Persistence gives them the capability to accumulate in various environmental compartments and be transported over long distances, reaching also remote regions, far from the places where they are produced and used. POPs are not only persistent, but also bioaccumulative, toxic and mobile across air, water and soil. As a result, POPs are distributed worldwide and have been detected in humans, wildlife and in all environmental compartments. In response to this global challenge, chemicals recognized as POPs have been regulated internationally under the Stockholm Convention since 2004 [12]. Following this regulation, concentrations in humans and in the environment of the

POPs initially included in the convention, such as PCBs, DDTs and PCDD/PCDF, have been declining globally [13]. However, more chemicals are showing similar properties and have been included or are proposed for listing under the convention.

1.1.1 Organofluorine chemicals

In the last century, organofluorine chemistry has significantly increased its importance in the chemical industry, due to many developments in the synthesis of fluorine containing compounds, to the important role of fluorinated substances in many fields and to its economic value [14-16]. In the production of pharmaceuticals and agrochemicals, the C-F bond is used to fine-tune active ingredient properties, such as lipophilicity, metabolic stability, bioavailability and binding affinity, for improved effectiveness [15, 17]. This fine-tuning is possible thanks to the high electronegativity and compact size of fluorine and its ability to form stable bonds with carbon, that allow for a greater range of electronic properties modifications than any other element [17]. In these two fields the growing importance of organofluorine chemistry is evident. For agrochemicals, organofluorine compounds account for 53 % of all active ingredients commercially available between 1998 and 2020 [18]. For pharmaceuticals, the percentage of fluorine containing active substances increased from 2 to 25 % between 1970 and 2021 and the percentage is expected to increase further since around 30 % of the newly approved drugs contains at least one fluorine atom [14, 19]. Notably, among the most prescribed drugs the proportion of fluorinated substances is even higher [14]. Healthcare takes advantage of organofluorine chemistry also from the routine use of fluorinated anesthetics in medical operations [15].

Other important organofluorine chemicals are hydrofluorocarbons and hydrochlorofluorocarbons, that are widely used in air conditioning and refrigeration systems as substitutes for chlorofluorocarbons that have ozone layer depleting properties [14]. Organofluorine chemistry also finds many applications in industrial processes and consumer products with fluorinated polymers and per- and polyfluoroalkyl substances. Fluorinated polymers are polymers in which one or more of the monomer units contains fluorine and include fluoropolymers, side-chain-fluorinated polymers and perfluoropolyethers. Fluorinated polymers are used for a wide range of applications including the production of plastic and rubber, manufacturing of metal products, coatings, paints, lubricants and automotive industry [20].

1.1.2 Per- and polyfluoroalkyl substances (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are a group of diverse synthetic chemicals produced since the late 1940s including solids, liquids and gases, polymers and non-polymers, volatile and non-volatile substances [21, 22]. PFAS have hundreds of (still increasing) uses both in industrial applications and consumer products, such as firefighting-foams, food-contact materials, textiles, metal plating, cosmetics, medical equipment and electronics [20, 23]. PFAS are widely used because of their special properties, like high chemical and thermal stability, surfactant properties and water and oil repelling capabilities [24]. However, these properties also make PFAS problematic from an environmental and human health perspective.

PFAS started to attract attention from this point of view in the early 2000s, when the two most well-known PFAS, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), started being reported in environmental and human blood samples from all over the world. PFAS global occurrence was first demonstrated in 2001 by Giesy and Kannan [25] that detected PFOS in a wide variety of wildlife tissues collected from both industrialized and remote regions. Also in 2001, Hansen et al. [26] detected PFOS and PFOA in human blood. Due to their ubiquitous presence and concerns about their potential toxic effects, production and use reduction initiatives for these PFAS have been introduced since the early 2000s. Between 2000 and 2002, PFOS and PFOA have been voluntarily phased-out by their major manufacturer [21]. PFOS use has been restricted in the United States and in the European Union from 2001 and 2006, respectively [27, 28]. PFOA and related compounds have also been banned in the European Union in 2020 and in the United States some of the major producers took part to the "PFOA Stewardship Program" and committed to achieve a 95% reduction of emissions of PFOA and its precursors by 2010 [29, 30]. Additionally, PFOS and PFOA have been listed under the Stockholm Convention on Persistent Organic Pollutants (POPs) in 2009 and 2019, respectively [31, 32]. Due to similar concerns as with PFOA, perfluorohexane sulfonic acid (PFHxS) has also been listed under the Stockholm Convention in 2022 and long-chain perfluoro carboxylic acids (PFCA) containing 9 to 14 carbons atoms have been classified as very persistent and bioaccumulative chemicals and are now listed as substances of very high concern in the European REACH [33]. Long-chain PFCA with carbon chain lengths from 9 to 21 have also been proposed for listing under the Stockholm Convention [34].

Following these initiatives, PFAS production has been shifting away from PFOS, PFOA and their precursors and moving towards shorter perfluoroalkyl chain lengths and new chemistries

[35]. Short-chain PFAS (<C_6) have lower bioaccumulation potential [36, 37] but have higher affinity for the water phase and have the potential to accumulate in the water cycle [38]. Short-chain PFAS have been detected in seas [39-41], rivers [42-45], rain [46, 47], snow [48], groundwater [49] and drinking water [50, 51]. Other new PFAS, like for example the fluoroalkyl ethers, GenX, ADONA and F-53B, have also been widely detected in water samples [52-57]. Some of these new PFAS have also been detected in biota and human serum [58-63], showing that alternative PFAS could also be bioaccumulative.

Over 750 different PFAS have been identified in consumer products, environmental and biological samples [64, 65]. However, the number of existing PFAS is even higher. In 2018, the OECD reported over 4700 PFAS available in the global market and stated there could also be more commercially available PFAS for which structural information is claimed as confidential [66]. In addition, the total number of existing PFAS can vary between several thousands and millions of compounds depending on which definition is used. Indeed, the PFAS definition has been evolving through the last decades (Figure 1) and at the moment there is no agreement on a common one.

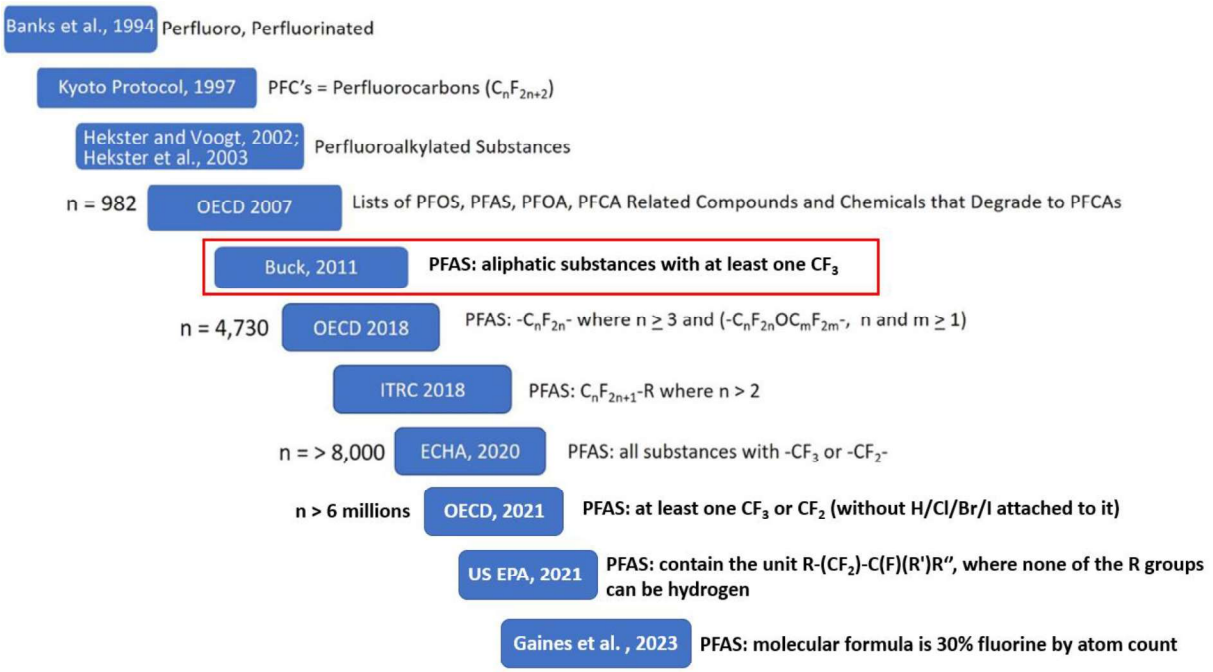

Figure 1 - PFAS definition evolution (figure adapted from Buck et al. [20]). The red rectangle highlights the definition used in this thesis.

The most accepted definition was introduced by Buck et al. [21] in 2011 and included "aliphatic substances containing one or more C atoms on which all the H substituents (present in the

nonfluorinated analogues from which they are notionally derived) have been replaced by F atoms, in such a manner that PFAS contain the perfluoroalkyl moiety C_nF_{2n+1}". In 2018 the OECD reported the existence of PFAS that were not following the Buck et al. definition and for this reason the PFAS definition has been expanded by the European Chemical Agency in 2020 [22] to include "substances containing at least one aliphatic CF_2 or CF_3 element" and by the OECD in 2021 [67] to include "substances that contain at least one fully fluorinated methyl ($-CF_3$) or methylene ($-CF_2-$) carbon atom (without any H/Cl/Br/I atom attached to it)". The latter OECD definition includes over 6.5 million structures on PubChem (as of July 2023) [68]. However, different groups are arguing that this definition is too wide [22, 69, 70]. Buck et al. [22] argue that substances containing only an isolated CF_2 in the carbon chain should not be called PFAS since these would not degrade to persistent end-products. Wallington et al. [69] suggest that the PFAS definition should avoid not only including compounds with single CF_2 groups, but also structures with isolated CF_3 or CF groups. Going in the direction of a less wide definition, in 2021, the US EPA defined PFAS as "substances that structurally contain the unit R-(CF_2)-C(F)(R')R". Both the CF_2 and CF moieties are saturated carbons and none of the R groups (R, R' or R") can be hydrogen". Recently, Gaines et al. [70] suggested another alternative approach to narrow down the PFAS definition including substances with a molecular formula containing 30% fluorine by atom count.

Throughout this thesis we will use the PFAS definition and classification introduced by Buck et al. [21] (Figure 1). The major PFAS groups discussed in this thesis are defined as follows. Perfluoroalkyl carboxylic acids (PFCA), perfluoroalkyl sulfonic acids (PFSA) and perfluoroalkyl ether carboxylic acids (PFECA) are perfluoroalkyl acids (PFAA). PFAA are also divided into short-chain PFAA, with less than 6 perfluorinated carbon atoms, and long-chain PFAA, with a number of perfluorinated carbon atoms equal to or higher than 6. PFAA precursors are a group of PFAS that can be transformed to PFAA biotically and/or abiotically. Precursors discussed in this thesis include fluorotelomer carboxylic acids (FTCA), fluorotelomer unsaturated carboxylic acids (FTUCA), fluorotelomer sulfonates (FTS), perfluoroalkane sulfonamides (FASA) and perfluoroalkane sulfonamido acetic acids (FASAA). For the scope of this thesis, pharmaceuticals containing at least one CF bond will be generally referred to as fluorinated pharmaceuticals, even if some of them (8 out of 360 fluorinated pharmaceuticals evaluated by Hammel et al.[71]) would be classified as PFAS according to the Buck et al. definition. Organic compounds containing fluorine, but not following the PFAS definition from Buck et al. will be generally referred to as organofluorine compounds.

1.1.3 Organofluorine chemicals production volumes

All fluorine used to produce organofluorine chemicals comes from anhydrous hydrogen fluorine (HF), that is produced from mined fluorite (CaF_2). Almost 70% of the produced HF is used for synthesis of organofluorine chemicals: up to 1 million tonnes/year for fluorinated gases, 100 thousand tonnes/year for fluoropolymers, 10 thousand tonnes/year for fluorotelomer based derivatives, up to 1000 tonnes/year for PFAA and estimates lacking for perfluoropolyethers, fluorinated aromatics (that include many fluorinated pharmaceuticals and agrochemicals) and other organofluorine chemicals [72]. A synthesis of production volumes of PFAA and their precursors, fluorinated gases and fluorinated polymers in the EU is provided in the EU PFAS restriction proposal [73]. Estimated manufacturing volumes ranged from 53902 to 118051 tonnes/year for PFAA and their precursors, from 15000 to 176548 tonnes/year for fluorinated gases and from 49000 to 101763 tonnes/year for fluorinated polymers. There are no estimates available for the production volumes of fluorinated pharmaceuticals and pesticides.

1.2 Human exposure to PFAS

1.2.1 Exposure assessment

Human exposure can be assessed by measuring external or internal exposure [74, 75]. External exposure is evaluated by measuring the chemicals of interest in different exposure media (for example air, water, food or dust) in combination with exposure factors (for example the rate of inhalation) and estimates of exposure frequency and duration. Internal exposure is evaluated by measuring the concentrations of the chemicals of interest (or their metabolites) in a biological tissue sample (for example blood, urine or hair) in combination with questionnaire data to evaluate associations between measured concentrations and sources of exposure (for example consumption of specific food items or use of personal care products). External exposure gives information about the total intake from multiple exposure pathways, while internal exposure gives an estimate of the total body burden [74, 75]. Both approaches have strengths and limitations. When studying external exposure, it is possible to evaluate the relative significance of different exposure pathways and the direct link with the sources can be used to design interventions to reduce exposure. However, external exposure is expensive and time-consuming to measure when several exposure sources are considered and can have uncertainties related to challenges in achieving statistically representative sampling for many exposure routes

and uncertainties related to exposure factors, duration, and frequency. On the other hand, internal exposure has the advantage of accounting for all sources of exposure and processes affecting the transfer of chemicals from the environment to the human body using only a single measurement. Another advantage of internal exposure is that this can be used to assess health effects. However, the internal exposure approach has more ethical requirements and has limited capability in identifying the sources of exposure, especially because of confounding effects between different exposure routes and uncertainties related to questionnaire data [74-76]. Since these two approaches are complementary, a combination results in a more detailed description of human exposure, allowing the identification of the dominant exposure routes and the application of suitable mitigation measures.

1.2.2 PFAS exposure pathways

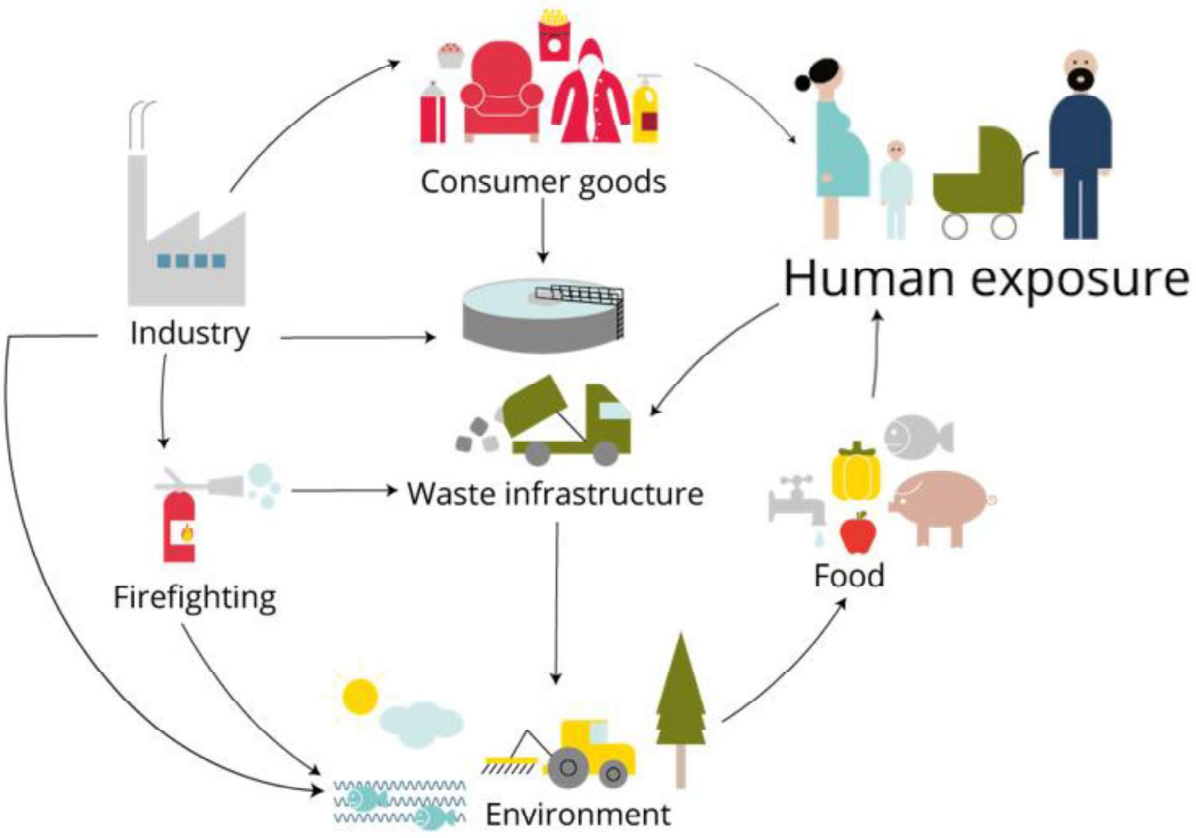

Figure 2 - PFAS human exposure pathways scheme (figure adapted from European Environment Agency [77]).

Figure 2 shows a simplified scheme of typical PFAS exposure pathways. PFAS can be released during their whole lifecycle, going from their production and use in industrial processes and consumer products [78-81] to the waste disposal of PFAS containing waste [82-85]. One particular case, that has resulted in PFAS contamination of soil, groundwater, surface waters and biota worldwide, is the production, use and waste handling of aqueous film forming foams (AFFF) for firefighting and training [86-91]. Once released into the environment, different PFAS accumulate in different environmental compartments depending on their physical-chemical properties. Short-chain PFAS, that are highly soluble and mobile in water, can easily reach our water supply, while long-chain PFAS, that are less polar and more particle-bound and bioaccumulative, can be found in our water but also in our food supply and in the dust in our homes [92-96]. Neutral PFAS, that are volatile and highly mobile in the gas phase, can evaporate from products and are mainly detected in the air [97-100].

Human exposure to PFAS can be distinguished into direct and indirect exposure. Direct exposure happens through dietary and non-dietary ingestion, inhalation and dermal absorption of PFAS [101]. Indirect human exposure to PFAS happens through metabolic formation from their precursors. Many precursors have been detected in consumer products [102-105], water [50, 106, 107] and air [97, 108] and exposure to some of these precursors has been confirmed by their detection in human blood [109-111]. However, the contribution of precursors metabolism to the overall PFAS exposure is still unclear [112, 113].

Looking at the relative importance of different exposure pathways, several studies have found diet and drinking water to be the main routes of exposure to PFAS [101, 114-118] with seafood consumption being recognized as one of the major contributors to PFAS exposure through the diet [119-125]. Ingestion of house dust and inhalation of indoor air are also known to contribute to PFAS exposure [101, 114-118]. Their contribution is lower compared to diet but differs across studies, with large variability especially because of dust ingestion estimates [75, 117]. In the few studies investigating the contribution of dermal absorption to the overall exposure to PFAS, this pathway is found to be a minor contributor [101, 126]. However, in a recent review Ragnarsdóttir et al. [127] highlighted the lack of PFAS dermal exposure data and an increasing number of scenarios under which dermal exposure to PFAS could happen (e.g.; contact with PFAS containing clothing and cosmetics), suggesting that the importance of this pathway might be underestimated.

Additionally, the uptake of PFAS in the human body from different exposure pathways can also be influenced by their bioaccessibility (i.e., the fraction of a chemical that is available for absorption from a matrix) and their bioavailability (i.e., the fraction of a chemical that reaches the systemic circulations). Bioaccessibility and bioavailability of PFAS have been shown to change depending on PFAS structural properties, such as chain length and functional group connected to the perfluoroalkyl chain, and on the matrix of exposure (e.g., the bioaccessibility of PFAS in food was associated with nutrient composition of the food) [128-131].

1.2.3 PFAS in human blood

Once taken up in the human body, blood circulation can transfer PFAS to organs, tissues and biofluids [132]. Due to their high stability most PFAS are not metabolized in the human body and are only being slowly excreted through urine, feces, menstruation and breastfeeding [133-138]. Indeed, half-lives of PFAS in the human body vary between few days for short-chain PFAS and several years for long-chain PFAS [139-142]. PFAS with long half-lives have been shown to accumulate in the human body, specifically in blood and protein-rich organs, such as liver and kidney. While PFAS that are eliminated faster have been rarely detected in blood and more often detected in urine [143].

Due to the high affinity of many PFAS for serum proteins, blood is the preferred matrix for PFAS biomonitoring. PFAS have been detected in human blood since 2001 when PFOA, PFOS, PFHxS and perfluorooctane sulfonamide (FOSA) were first detected in human serum [26]. Since then, PFAS have been detected in blood from humans worldwide [144]. PFOS and PFOA are usually the dominating PFAS in human blood [143] and their concentrations in human blood have been shown to follow the history of production and use of these chemicals with a time lag [27]. For example, in Tromsø (northern Norway) human blood concentrations of PFOS and PFOA increased significantly between 1979 and 2001, followed by a decrease between 2001 and 2007 [145]. Similar decreasing trends have been observed worldwide [27, 144].

Other PFAS are also found in human blood. For example, long-chain PFCA are also widely detected, and several studies are showing increasing concentrations following PFOS and PFOA regulation in early 2000s [27, 146]. However, only 1% or less of the known PFAS is currently analyzed in human biomonitoring studies, including mainly the perfluoroalkyl acids (PFAA) and few other PFAS, like perfluorooctane sulfonamides (FOSA), fluorotelomer sulfonates (FTS) and fluorotelomer alcohols (FTOH) [93]. In addition, several studies measuring

extractable organic fluorine (EOF) in human blood have shown that measured target PFAS account only for a small percentage of the EOF concentrations [147-152]. One of these studies, on German plasma samples, has also observed an increasing proportion of unidentified EOF following year 2000 [148]. In the light of this mounting evidence, there are increasing concerns about the likely underestimation of PFAS human exposure.

1.2.4 PFAS health concerns

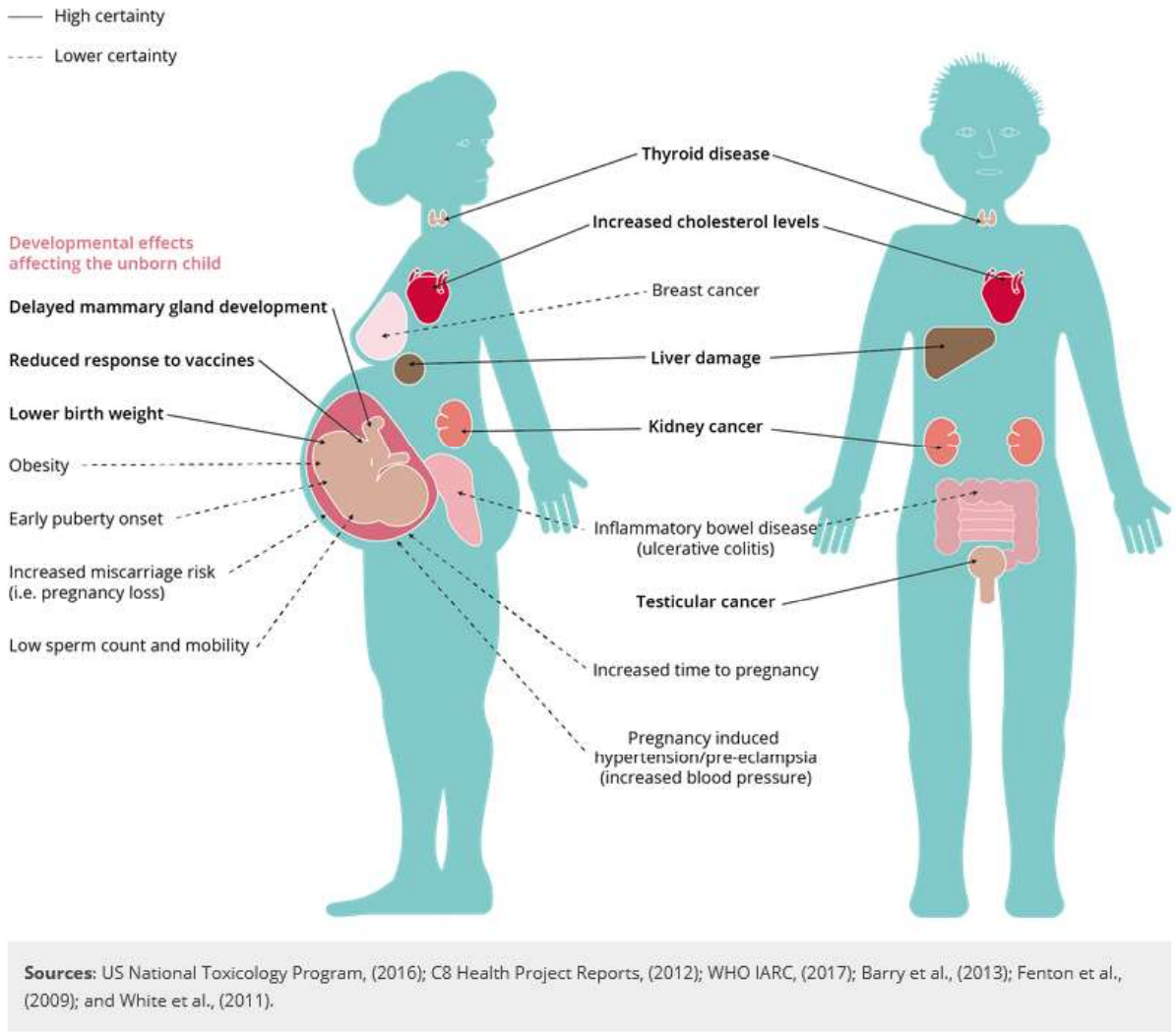

Figure 3 - Effects of PFAS on human health (figure from European Environment Agency) [77]

PFAS have been associated with several toxic health effects (Figure 3), including immune system dysfunction, liver damage, thyroid disease, increased cholesterol levels, kidney and testicular cancer and developmental and reproductive effects [142, 144]. For these effects,

associations with PFAS exposure are reported both in epidemiological and animal studies, but causality of these relationships has not been established and there are still gaps of knowledge about the mode of action of these compounds [142, 153]. Additionally, it is still unclear whether these health effects are a result of lifelong cumulative exposure or exposure during sensitive time-periods that could include in-utero, childhood, puberty or adulthood [154]. Some studies also report associations between PFAS exposure and breast cancer, ulcerative colitis, pregnancy induced hypertension, type-2 diabetes, cardiometabolic disease and effects critical for reproduction like early puberty onset, increased miscarriage risk and low sperm count. However, for these end-points contradictory results are reported [142, 155]. Additionally, in a systematic review, Rappazzo et al. [156] highlighted consistent evidence for associations between children PFAS exposure and dyslipidemia, reduced response to vaccines, asthma, impaired renal function and delayed menarche.

Most of the health effect data currently available for PFAS is for the two most-well known compounds, PFOS and PFOA, and few other substances (mostly PFAA) [157]. However, the few studies available for alternative PFAS are showing that these new compounds could also be toxic. For example, for GenX, liver and kidney toxicity, immune and developmental effects have been observed in mice and rats after oral exposure [158]. Additionally, most studies are investigating individual PFAS and the effects of PFAS mixtures on human health are still poorly understood [159].

1.3 Analytical methods for biomonitoring of fluorine compounds

The increasing number of PFAS in production and use has made PFAS monitoring in environmental and biological samples more complex. Looking for thousands of substances with targeted methods is not feasible and complementary analytical tools are needed to screen for a large number of known and unknown PFAS. To go in this direction, both sum parameters methods, like total fluorine (TF), extractable organic fluorine (EOF) and total oxidizable precursors (TOP) assay, as well as suspect and non-target screening strategies are being developed. These methods have different degrees of specificity towards PFAS and are often combined in what is called a fluorine mass-balance approach (Figure 4).

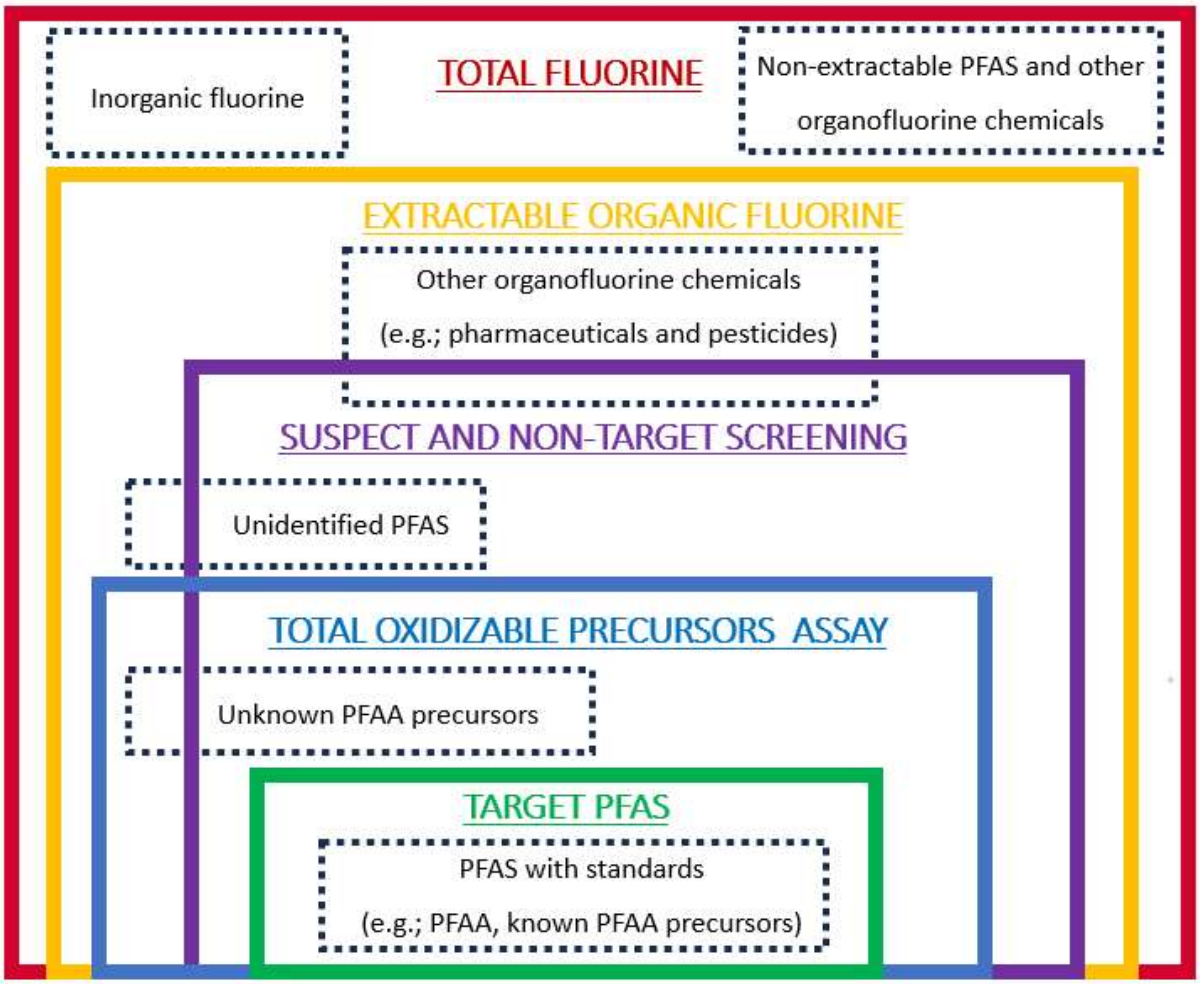

Figure 4 - Analytical methods scheme showing the inclusivity and specificity of different methods.

1.3.1 Target PFAS

The development of traditional target methods is often impaired by the lack of both native and labelled standards for recently identified PFAS and for PFAS for which the structure remains unknown [15]. Additional challenges in traditional target PFAS analyses also arise from the wide variety of PFAS chemical structures, that make challenging to develop one-fits-all methods. For example, short-chain PFAS are highly polar and have low retention in reverse phase chromatography commonly employed for long-chain PFAS determination. Therefore, short-chain PFAS analysis requires the use of different chromatographic methods, such as the use of mixed-mode columns, supercritical fluid chromatography or the use of derivatization and GC separation [160]. The variety of PFAS chemical physical properties also requires additional developments for extraction methods since the methods traditionally used for PFAS

can show low recoveries for short-chain PFAS and zwitterionic and neutral compounds [160, 161].

1.3.2 Total oxidizable precursors assay

The total oxidizable precursors (TOP) assay is a method that determines the concentrations of PFAA precursors by measuring the PFAA resulting from the oxidation of precursors under controlled conditions (Figure 5) [162].

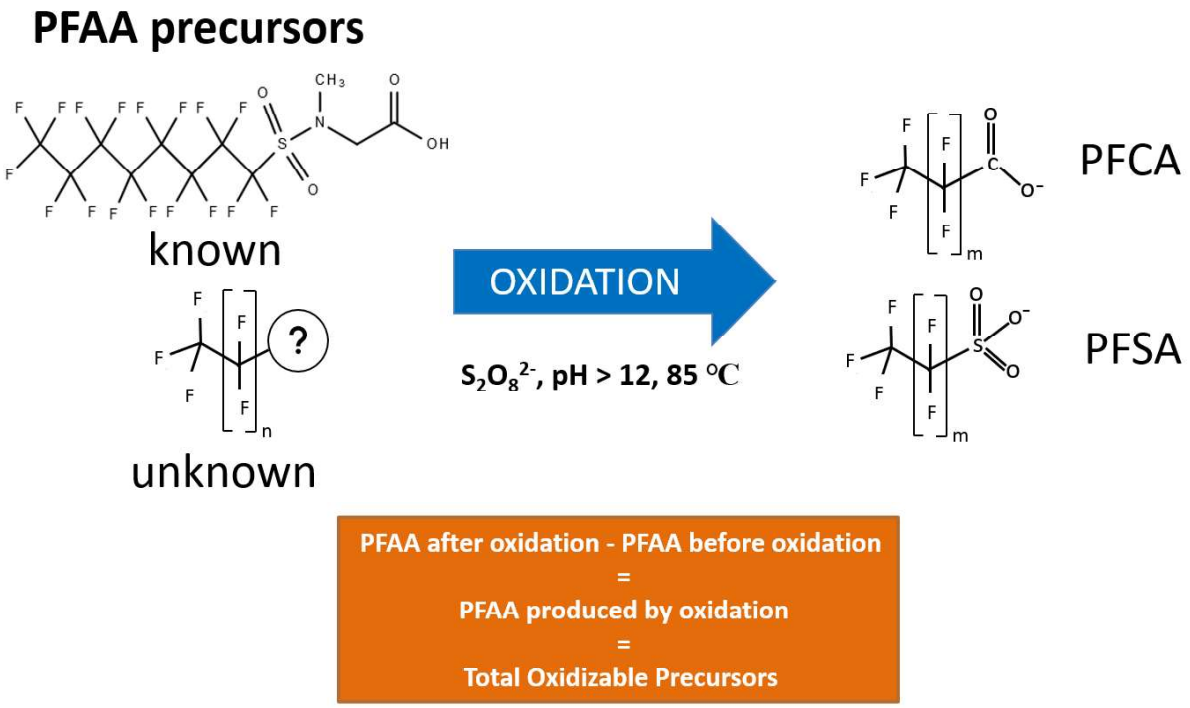

Figure 5 – TOP assay concept scheme.

The difference in PFAA concentrations before and after oxidative treatment is considered to be an indicator of the total concentration of oxidizable PFAA precursors, because PFCA and PFSA that are present in the original sample remain intact under the conditions of the assay. The advantage of this method is that it allows the identification of known precursors for which we have standards, but also precursors for which we do not have standards or for which the structure is unknown. However, the TOP assay is less specific than target PFAS analysis because the exact precursor's structure is lost upon oxidation. The TOP assay has been successfully applied to detect PFAA precursors in water [162-166], soil [163, 167], biota [168-171] and consumer products [172-175]. However, existing methods use sample amounts and oxidation conditions that are unsuitable for the small volume of serum commonly available for biomonitoring studies.

1.3.3 Extractable organic fluorine

EOF determination consists in measuring the amount of organofluorine compounds left after removal of inorganic fluorine upon extraction of the sample with an organic solvent [176]. Therefore, EOF concentrations are often used as a proxy for total PFAS concentrations and in combination with target PFAS measurements to evaluate the presence of yet unknown PFAS [151]. However, organofluorine compounds other than PFAS, such as fluorinated pharmaceuticals and pesticides, can also contribute to the EOF concentrations. In a recent study, Spaan et al. [177] observed that around 22% of the EOF in wastewater treatment plant sludge was attributable to these substances. EOF is commonly measured in human blood using combustion ion chromatography (CIC). In this method, the sample is combusted at 900-1000 °C to convert all fluorinated chemicals to hydrogen fluoride, that is adsorbed in a solution where the concentration of fluoride ions is measured by ion chromatography [147].

1.3.4 Total fluorine

TF is the most inclusive and least specific method as this measurement includes all chemicals containing fluorine, both organic and inorganic. The most common method for measuring TF in human blood is CIC [150, 176]. TF is measured by CIC also in environmental samples and consumer products [176, 178-180]. Other methods to measure TF include particle-induced γ-ray emission (PIGE) spectroscopy, instrumental neutron activation analysis, inductively coupled plasma (ICP) mass spectrometry, molecular absorption spectroscopy, and X-ray photoelectron spectroscopy, but these are mainly applied to environmental samples and consumer products [181].

1.3.5 Suspect and non-target screening

Suspect and non-target screening approaches using high-resolution mass spectrometry (HRMS) have been used to identify novel PFAS in human blood, environmental samples and consumer products [64, 65]. Modern HRMS instruments have high resolving power and mass accuracy and allow us to screen for unknown chemicals without standards or even without knowing their structure. Strategies for identification of PFAS from HRMS data include both suspect and non-target screening approaches. In suspect screening full scan and MS/MS data are screened against a list of PFAS candidate formulas based on accurate mass, isotope patterns and MS/MS data from the literature, public databases or in-silico predictions [64]. This approach is limited to the list of compounds included in the suspect-list. A more inclusive strategy consists of the

use of non-target analysis. In this case, potential PFAS features are preselected based for example on mass defect, homologue series searching, study design or presence of diagnostic fragments or neutral losses [64]. After these filtration steps, possible molecular formulas for the selected features can be proposed based on the measured m/z using MS/MS data or authentic standards [64]. PFAS identified through suspect and non-target screening methods can be confirmed with different levels of confidence based on the Schymanski scale, in which the highest level of confidence (level 1) is assigned to suspects confirmed with authentic standards, while the lowest level of confidence (level 5) is assigned to suspects with an accurate mass match but no other evidence supporting the assignment [182, 183].

1.4 The PERFORCE3 project

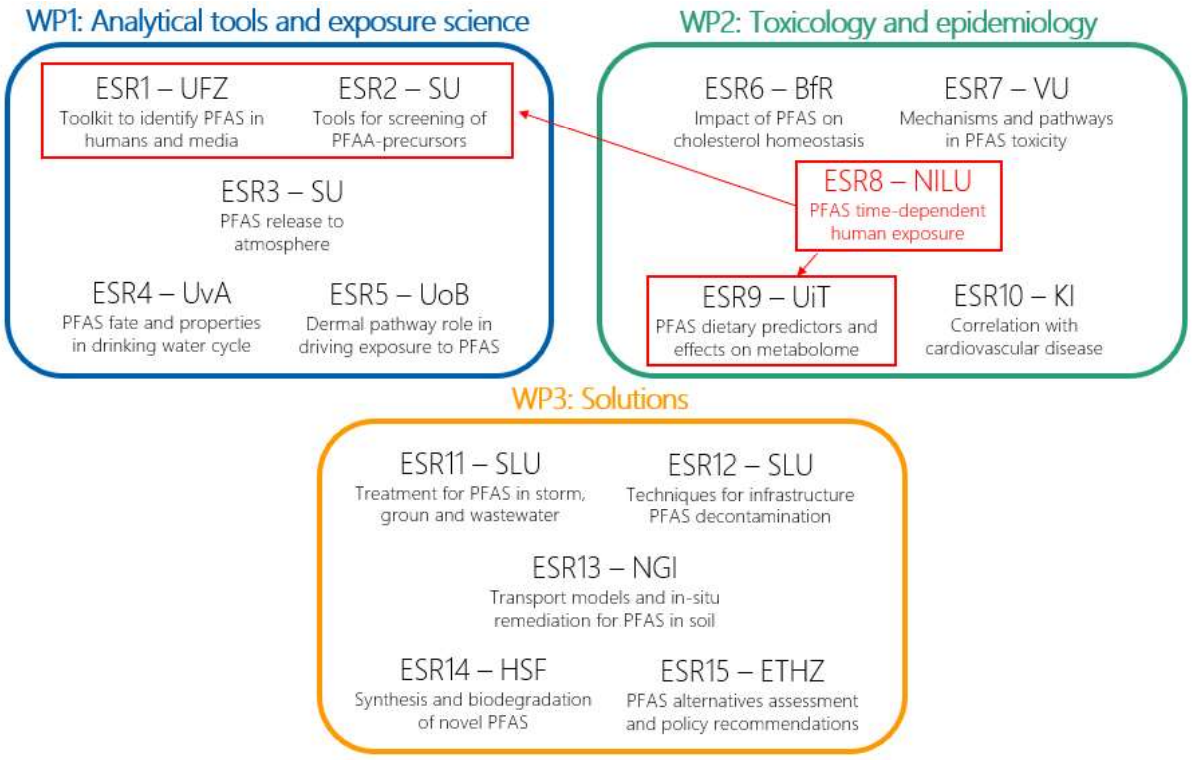

Figure 6 – Scheme of the PERFORCE3 work packages. The red text highlights the project discussed in this thesis and the red arrows and boxes represent the collaborations that are part of the work presented in this thesis.

To address some of the knowledge gaps on the risks that PFAS can pose to the environment and human health and investigate novel solutions to PFAS contamination, the EU funded Marie Skłodowska Curie ITN project, PERFORCE3 (PER and polyfluorinated alkyl substances (PFASs) towards the Future Of Research and its Communication in Europe 3), was launched

in 2020. The PERFORCE3 project brings together 15 early-stage researchers (ESR) across Europe, their supervisors, industry and policy partners to investigate different aspects of PFAS contamination. The project is organized in 3 work packages: analytical tools and exposure science (WP1), toxicology and epidemiology (WP2) and solutions (WP3). The work presented in this thesis was conducted as part of WP2 with collaborations within WP2 and with WP1 (Figure 6).

2 Aim of the thesis

The overall aim of this thesis was to contribute to the description of the full extent of PFAS and organofluorine human exposure and provide novel insights into the complexity of this exposure. With the analysis of serum samples collected between 1986 and 2015 within the frame of the Tromsø Study we aimed to describe the changes in exposure over three decades, including relevant periods of changing legislation and PFAS content in products and industrial production.

Specific goals were to:

1. Develop a method to evaluate the presence of oxidizable precursors in human serum by adapting the TOP assay protocol for PFAS in human serum (Paper 1)
2. Evaluate the human exposure to total fluorine and known and unknown extractable organofluorine compounds between 1986 and 2015 in Tromsø with respect to sex and age (Paper 2)
3. Identify emerging PFAS and fluorinated pharmaceuticals contributing to the unknown organofluorine measured in pooled serum from the Tromsø population between 1986 and 2015 (Paper 3)

3 Material and methods

3.1 Tromsø study serum samples

This thesis is based on serum samples and data from the Tromsø Study. The Tromsø Study is an on-going cohort study started in 1974 to examine the causes for the high incidences of cardiovascular diseases in the population of Tromsø, the largest city in Northern Norway. Since then, 7 surveys have been completed every 7th year and the research hypotheses included have been expanded to encompass several aspects of human health. During each survey, the participants have answered a questionnaire, donated a blood sample and gone through a physical examination [184].

The work, presented in Paper 2 and Paper 3, was based on a selection of 529 individual Tromsø Study serum samples collected in 1986 (n=201), 2007 (n=198) and 2015 (n=130) (Figure 7). The samples were selected based on a case-control study design on type-2 diabetes. The cases were diagnosed between 2001 and 2007, while the controls had no diagnosis recorded in the local registry. The sample selection included 104 women and 97 men in 1986, 113 women and 86 men in 2007 and 72 women and 58 men in 2015. The age of the selected individuals ranged from 17 to 61 years old in 1986 (mean: 46), from 38 to 81 in 2007 (mean: 67) and from 46 to 89 in 2015 (mean: 72).

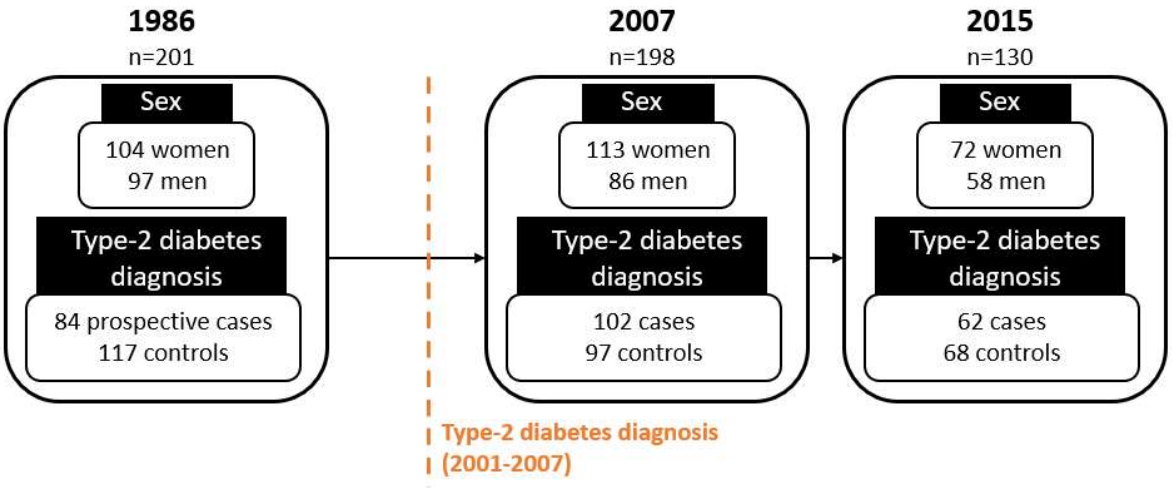

Figure 7 – Tromsø Study serum samples selection (figure from Paper 2).

3.1.1 Pooling strategy

To allow the analysis of the serum from the Tromsø Study using a combination of multiple analytical techniques a volume of at least 750 µL was required. This volume was not available for individual samples and a pooling approach had to be used. The selected samples included 104 women and 97 men in 1986, 113 women and 86 men in 2007 and 72 women and 58 men in 2015. The age of the individuals ranged from 17 to 61 years old in 1986 (mean: 46), from 38 to 81 in 2007 (mean: 67) and from 46 to 89 in 2015 (mean: 72). From the above-described selection, 472 individual samples (1986 [n=167], 2007 [n=175], 2015 [n= 130]) were pooled based on sampling year, sex, age and type-2 diabetes diagnosis. As variables for pooling, sampling year, sex, and age were chosen because these variables have been shown to affect PFAS concentrations in human blood [146]. The case/control status for type-2 diabetes diagnosis was used as a variable for pooling because some studies have reported associations between this endpoint and PFAS concentrations, although the evidence for these associations is contradictory [185].

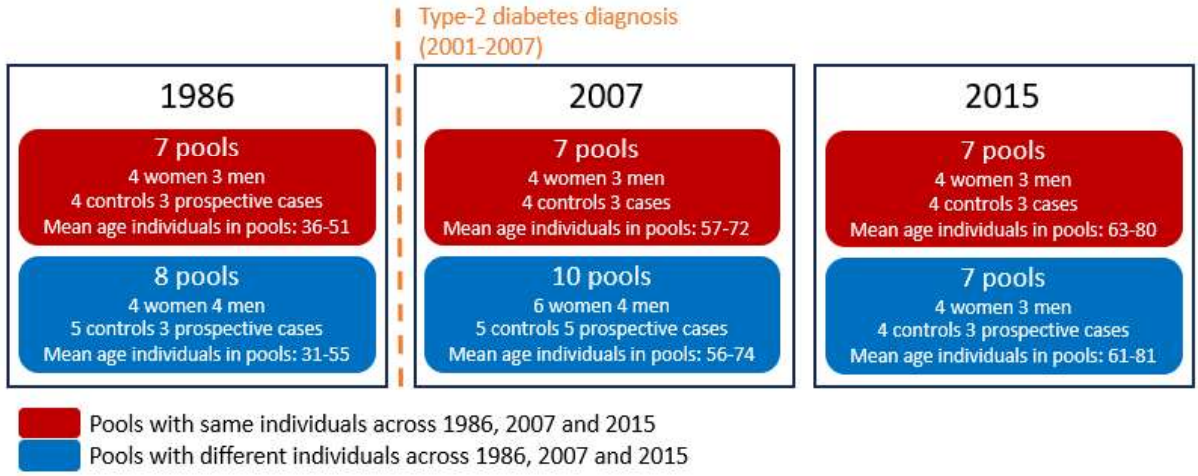

Figure 8 – Pooling strategy summary and sex, age and type-2 diabetes diagnosis distribution among pools (Figure from Paper 2).

Pools 1 to 7 at each sampling year included the same individuals in 1986, 2007 and 2015 (Figure 8). To have the largest possible number of pools including the same individuals, these pools were obtained mixing variable volumes (50, 100 or 150 µL) of individual serum samples, but keeping the volume per individual constant throughout the sampling years. For the remaining pools, it was not possible to follow the same individuals through time and 15 participants (with matching sampling year, sex, age and type-2 diabetes diagnosis) were included in each pool mixing 50 µL of serum per individual (Figure 8). Detailed information about the serum pools

characteristics (number of individuals, age range and mean, and type-2 diabetes status) are reported in Paper 2.

3.2 Analytical methods

3.2.1 Fluorine mass-balance approach

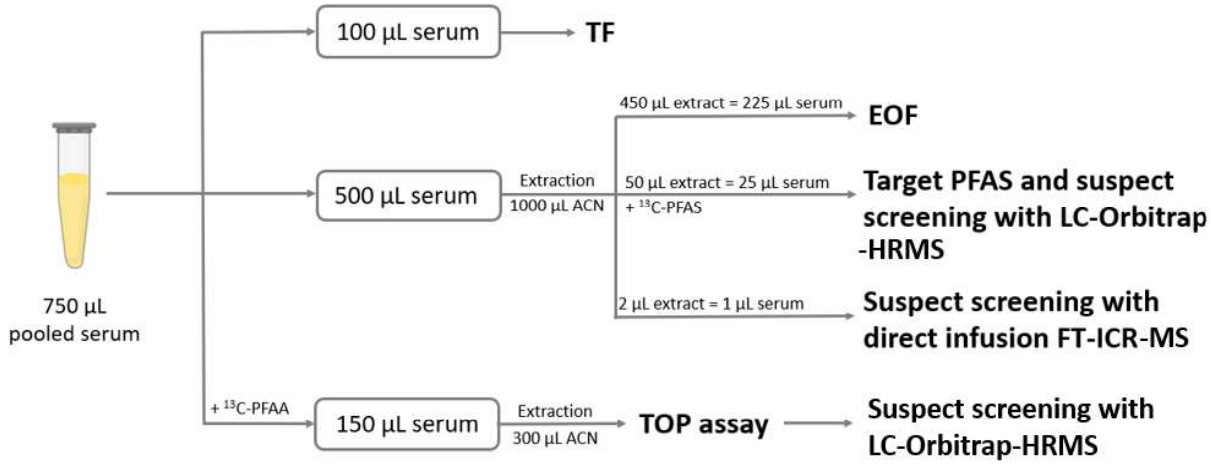

Figure 9 – Fluorine mass-balance scheme (figure adapted from Paper 2 and Paper 3).

The serum pools were analyzed with a combination of analytical techniques to evaluate different fluorine fractions (Figure 9). The pools were split into 3 portions: (1) 100 µL for TF, (2) 500 µL for EOF extraction, (3) 150 µL for the TOP assay. Target PFAS analysis was performed on the TOP assay extracts (before and after oxidation), and on the EOF extracts after addition of internal standard. The EOF and TOP assay extracts were also used for suspect screening using direct infusion FT-ICR-MS and LC-Orbitrap-HRMS.

3.2.2 Total fluorine

TF was measured on 100 µL of serum using a combustion ion chromatograph with the method described by Miaz et al. [150]. A 9-point calibration curve ranging from 2.5 to 2500 ng of NaF in water (R^2>0.999) was included at the beginning and end of each run. Quality control measures for each run included: (1) three sample boat blanks for limit of detection (LOD) determination, (2) two sample boats spiked with 100 ng of PFOS standard, and (3) three measurements of a certified reference material (fluorine in clay, CRM 461). Blanks ranged between 18 and 21 ng F/mL (n=9) and LOD (average boat blanks + 3 times the standard deviation of the blanks) ranged between 23 and 25 ng F/mL. The recovery of the PFOS standard

(120 ± 6 %, n=6) confirmed complete combustion and measurements of the certified reference material showed good accuracy and precision (recovery: 123 ± 9 %, n=9).

3.2.3 Extractable organic fluorine

For EOF measurements, 500 µL of pooled serum were extracted with 1 mL of acetonitrile (ACN). For the extraction, the samples were vortexed and sonicated (10 minutes) for 3 times and the supernatants were transferred to 2 mL glass vials. EOF analyses were performed on 450 µL of the extracts with the same CIC used for TF analyses and the method described by Miaz et al. [150]. For each extraction batch (14 serum samples), the quality control measures included: (1) three extraction blanks, (2) three reference serum samples not spiked, (3) one reference serum sample spiked with 239 ng of PFOS, (4) one reference serum sample spiked with 500 ng of NaF. The reference serum was from the Arctic Monitoring and Assessment Programme (AMAP) Ring Test for Persistent Organic Pollutants [186]. Each batch was run separately and included a calibration curve at the beginning and end of the run (2.5-1000 ng of NaF in water, R^2>0.999) and two sample boats spiked with 100 ng of PFOS standard. The extraction blanks ranged from 5 to 7 ng F/mL (n=12) and the EOF LOD (average extraction blanks + 3 times the standard deviation of the blanks) ranged from 6 to 9 ng F/mL. The analysis of the reference serum spiked with PFOS confirmed good recovery and reproducibility (recovery: 77 ± 14 %, n=8). The analysis of the reference samples spiked with NaF confirmed the removal of fluoride upon extraction (NaF recoveries ranging from 0 to 2 %, n=4).

3.2.4 Total oxidizable precursors assay

TOP assay protocol for human serum

The TOP assay was originally developed for large volumes of water [162] and had to be adapted to be applied to small volumes of human serum. The detailed description of the adaptation of the TOP assay protocol for human serum can be found in Paper 1. Briefly, our experiments were designed as follows: 250 µL of serum were spiked with 20 µL of 0.5 ng/µL ^{13}C-PFAA mixture as internal standard and vortexed. Samples were extracted with methanol, and, after centrifugation, the supernatants were transferred to 2 mL glass vials. The extracts were split into two portions: 50 µL were used for PFAS analysis before oxidation and 450 µL were treated for the TOP assay. The TOP assay aliquots were evaporated to dryness and oxidation reagents were added to the dry residues. Potassium persulfate was added as solid by weight, while sodium persulfate was added as a 0.8 M solution. For sodium hydroxide, a 10 M solution was

used. The vials were tightly capped, vortexed, and subsequently heated in an oven at 85 ℃ for a certain time (as defined in Table 1). After oxidation, the samples were acidified with 50 µL of concentrated HCl and extracted with methyl tert-butyl ether (MTBE). Subsequently, 200 µL of the organic phase were transferred to glass vials with insert and 30 µL of 2 % ammonia in methanol were added, followed by 20 µL of 0.1 ng/µL recovery standard in methanol. The MTBE was evaporated until the residual volume was 50 µL.

Table 1 - Oxidation conditions tested on human serum samples (Table from Paper 1).

Parameters	Method			
	A	**B**	**C**	**D**
Heating time (hours)	8	24	8	24
10 M NaOH (µL)	20	20	40	120
MilliQ H_2O (µL)	100	100	200	-
$K_2S_2O_8$ (mg)	20	20	40	-
0.8 M $Na_2S_2O_8$(µL)	-	-	-	500
Model precursors (ng)	20	20	20	200*

* Tested also for serum spiked with 4 ng of 7:3 FTCA and 6:2 FTS + 10:2 FTS mix

Oxidation conditions optimization

Four different adaptations of the method were tested on reference serum samples from the AMAP Ring Test for Persistent Organic Pollutants spiked with model PFAS substances known to act as precursors to stable PFAA. We included a selection of fluorotelomer compounds and two perfluoroalkyl ether carboxylic acids (PFECA) as described in Figure 10 and Paper 1. The oxidation conditions tested are summarized in Table 1 and included changes in length of heating (method B), degree of dilution and in oxidant amount and type (method C and D). More detailed information about the model precursors and conditions tested can be found in Paper 1.

For the final method conditions from method D were selected (see chapter 5.2.6 for more details) and applied to 150 µL of pooled serum samples from the Tromsø Study as described in Paper 2.

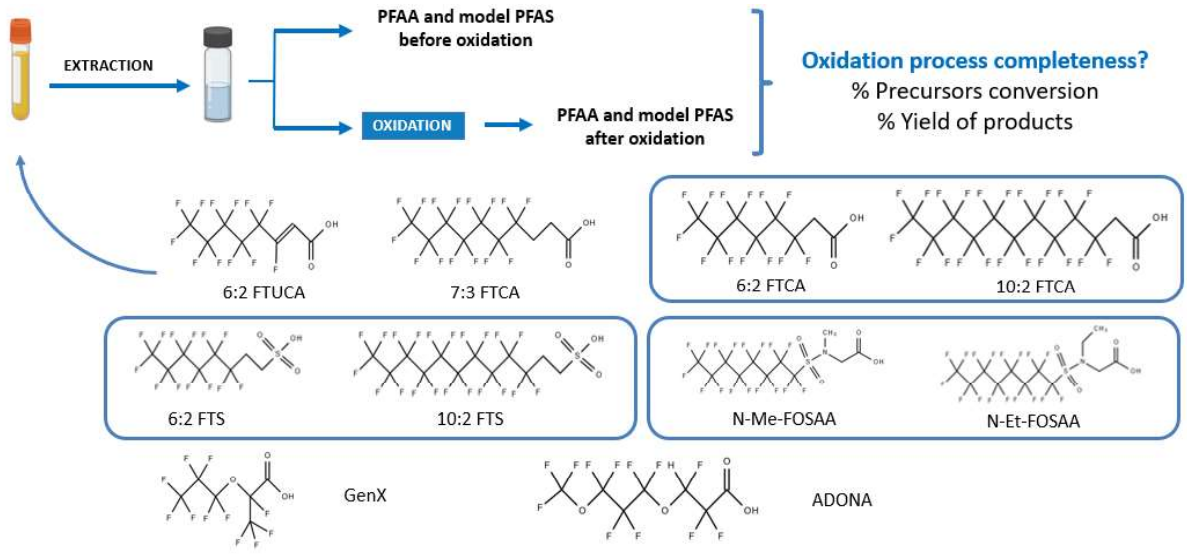

Figure 10 – TOP assay method testing with reference serum spiked with model PFAS.

TOP assay for fluorinated pharmaceuticals and pesticides

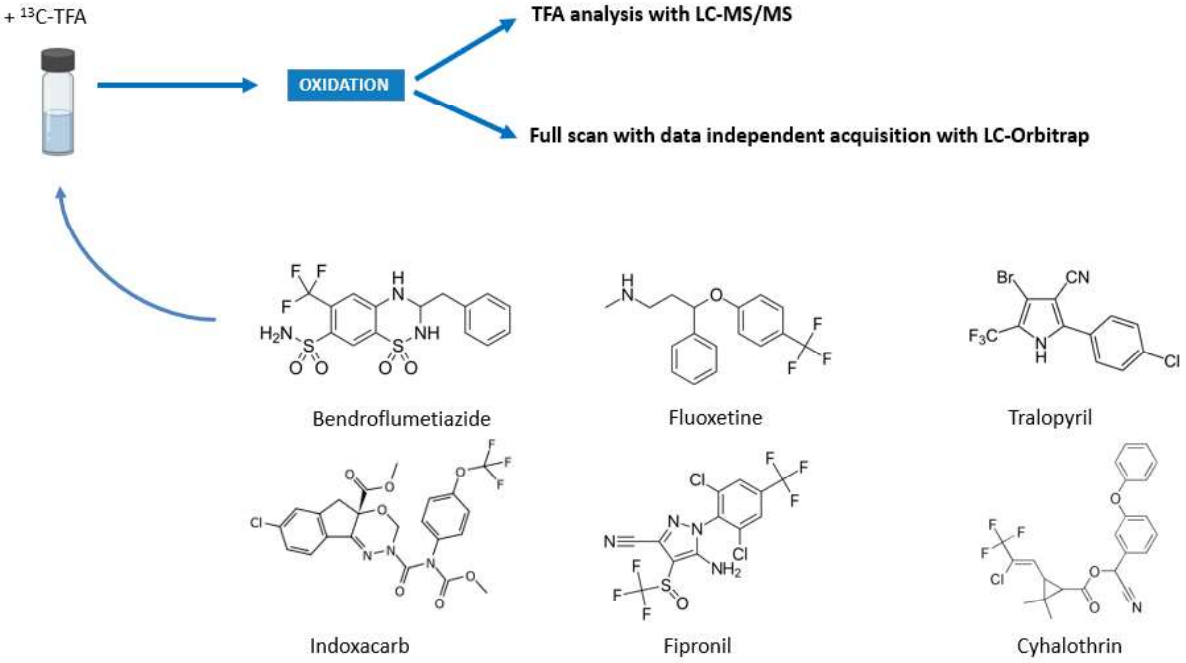

Figure 11 – Fluorinated pharmaceuticals and pesticides oxidation testing scheme.

Since fluorinated pharmaceuticals and pesticides containing CF_3 groups are potentially trifluoroacetic acid (TFA) precursors, some of these substances were also tested for oxidation to understand their behavior in the TOP assay, as described in Figure 11 and Paper 3. Briefly, standard substances were transferred to 2 mL glass vials and spiked with ^{13}C-TFA. After

evaporation to dryness the samples were mixed with the TOP assay reagents and heated at 85 ℃ for 24 hours (method D). After oxidation samples were extracted with MTBE and residues of salts and water were settled by adding anhydrous sodium sulphate. The samples were centrifuged at 10000 rpm for 10 minutes and the organic phase was transferred to glass vials with insert. The samples were spiked with 50 µL of 2 % ammonia in methanol and the MTBE was evaporated until the residual volume was 50 µL. The samples were analyzed for TFA using LC-MS/MS and on LC-Orbitrap-HRMS in full scan with data independent acquisition (DIA) to monitor the formation of other transformation products.

3.2.5 Target PFAS

Target analyses on the EOF extracts included 54 PFAS (list of analytes available in Paper 2) and were performed using a Dionex UltiMate 3000 Ultrahigh performance liquid chromatograph coupled to a Q Exactive HF hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described by Miaz et al. [15]. For these analyses, 50 µl of EOF extracts were mixed with 10 µl of internal standard and 50 µl of 4 mM NH_4OAc in MilliQ water. Since the internal standard was added after extraction, these concentrations were not recovery corrected and were only used for fluorine mass balance calculations.

Target analyses on the TOP assay extracts from method testing and the Tromsø Study pools included 34 PFAS (including PFAA and a selection of precursors and new PFAS reported in Paper 1) and were performed using a quaternary Accela 1250 pump with a PAL Sample Manager coupled to a Vantage TSQ MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) as described elsewhere [32]. After oxidation the Tromsø Study extracts were also analysed for C_2 and C_3-PFAA using a Raptor Polar X column. TFA was analyzed in a 5 minute isocratic run with 80 % 2mM ammonium acetate in methanol and 20 % 2mM ammonium acetate in 90:10 water:methanol. Perfluoropropionic acid (PFPrA), trifluoromethane sulfonic acid (TFMS), difluoro (perfluoromethoxy) acetic acid (1,2-PFECA), difluoroacetic acid (DiFA) and chlorodifluoro acetic acid (Cl-DiFA) were analyzed in a 10 minute isocratic run using 80% 60:40 methanol:water with 0.05% formic acid and 20% 10 mM ammonium formate in water with 0.05% formic acid, based on an application note from Restek [187].

3.2.6 Suspect-screening

Pooled serum samples from the Tromsø Study were analyzed for suspect screening using direct infusion Fourier-transform ion cyclotron resonance (FT-ICR-MS) and LC-Orbitrap-HRMS.

For FT-ICR-MS analysis, 20 serum pools with the highest unidentified EOF (as absolute value and/or percentage) were selected. For the analyses, 2 μL of EOF extracts were diluted with 198 μL of 50:50 MeOH:MilliQ H_2O and analyzed on a 12 tesla FT-ICR-MS using direct infusion. All samples were injected twice: once for full scan acquisition and once for ocular method acquisition. In the ocular method the mass range is divided into segments to maintain near constant resolving power and increase sensitivity. The mass range width of the segments was 30 Da from 150 to 300 m/z, 50 Da from 300 to 600 m/z and 150 Da from 600 to 900 m/z.

The full scan data (m/z=150-1800) acquired on LC-Orbitrap-HRMS for target PFAS analyses of the EOF extracts was screened for the suspects identified by FT-ICR-MS and a list of 326 PFAS compiled from literature on human serum and biota using patRoon and a mass tolerance of 2 ppm (Paper 3). This data was also screened for a list of 340 fluorinated pharmaceuticals from the NORMAN Suspect List exchange [188].

For the FT-ICR-MS and LC-Orbitrap-HRMS suspect matches, MS2 spectra were obtained by running the samples before and after TOP assay in a different LC-Orbitrap-HRMS instrument using the same LC method described for PFAS analyses. Data were acquired in full scan with data dependent acquisition (ddMS2) for the suspect matches. The MS2 spectra were inspected and annotated with patRoon and Freestyle. Detection before and after TOP assay was used to confirm or discard suspect assignments as described in Paper 3.

For suspect fluorinated pharmaceuticals with confirmatory MS2 data, metabolites were predicted using Biotransformer in patRoon. Suspect screening was also performed for this list of metabolites, that included also known human metabolites found in the literature. For suspects with confirmatory MS2 data, confirmation on level 1 and quantification were also performed using analytical standards as described in Paper 3.

3.3 Data treatment

3.3.1 Evaluation of the TOP assay oxidation performance

For each method modification, the completeness of oxidation was evaluated using the percentage of conversion of spiked precursors (i.e., the disappearance of the precursor) and the yield of products (i.e., the production of PFAA) with the equations described in Paper 1.

3.3.2 Fluorine mass-balance calculations

EOF values were subtracted from TF concentrations to estimate the amount of inorganic and non-extractable organic fluorine. For this comparison, samples with TF below LOD were excluded. To estimate the unidentified EOF (UEOF), the ∑PFAS concentrations obtained from the EOF extracts, converted to fluorine equivalents, were subtracted from the EOF concentrations. PFAS concentrations below LOD were set to LOD/√2.

The total amount of oxidizable precursors (ΔPFAA) was estimated as described by Coêlho et al. [189]. To estimate the contribution of total oxidizable precursors and fluorinated pharmaceuticals to EOF, their concentrations were also converted to fluorine equivalents.

3.3.3 Statistical analysis

Statistical analyses were performed using R 4.1.2 (R Core Team). Prior to statistics calculations, concentrations below the LOD were substituted with LOD/√2. Differences in TF, EOF, TOP, ∑PFAS, ∑F-pharmaceuticals and UEOF between sampling years and sex and age (as weighted mean of the age of the individuals in the pools expressed in years) groups were assessed by multiple linear regression. When sex was a significant predictor, differences in concentrations between men and women at each sampling year were assessed adding an interaction term. The inclusion of the type-2 diabetes diagnosis (case/control status) to the multiple linear regression model was tested using Akaike information criterion (AIC) model selection. Since the model with lowest AIC score never included the type-2 diabetes diagnosis variable, this was not included. TF, EOF, $\sum_{12}$PFAS and $\sum_{13}$PFAS concentrations were log-transformed before performing regression analyses. Statistical significance was set at $p < 0.05$ and post-hoc power calculations were performed using the pwr package. The multiple linear regression equations are described in Paper 2.

3.4 Ethics

The Tromsø Study obtained informed consent from all participants for the scientific use of data and link to the health registries. The study described in this thesis was approved by the Regional Committee for Medical Research Ethics (REK, case number: 2020/13188).

4 Results

4.1 Paper 1

This study investigated the applicability of the TOP assay to small volumes of human serum, the reaction conditions needed to ensure complete oxidation of model precursors and the qualitative and quantitative information obtainable from the TOP assay application.

Oxidative conditions similar to previous TOP assay methods (method A) were not sufficient for complete oxidation of model precursors. While complete conversion was observed for the FTCA with 2 non-fluorinated carbons (6:2 FTCA, 10:2 FTCA and 6:2 FTUCA), incomplete conversion was observed for all the remaining model precursors. In addition, independently from the percentage of conversion, a 100% yield of PFAA was never observed under these conditions. Prolonging the heating time (method B) and increasing the oxidant amount (method C) increased the conversion of precursors and/or the PFAA yields. The improvement observed upon increasing the amount of oxidant was larger than the one observed by prolonging the heating time and a further increase of oxidant amount (method D) was tested with a heating time of 24 hours. As an additional new aspect in method D, we used $Na_2S_2O_8$ as a 0.8 M solution in water instead of neat $K_2S_2O_8$ to ensure good intermixing with the sample. Using method D full conversion was observed for all precursors, except 10:2 FTS for which a satisfactory conversion of 91% was reached. With this final method the yield of PFAA ranged between 35 and 100%. These yields were judged satisfactory, and a further increase of oxidant amount was not tested since this would require a scale-up of the experiment or a downsize of the sample. After optimization for human serum, the TOP assay was still not fully quantitative since some precursors are not fully converted to PFAA. Therefore, the TOP assay can only provide semi-quantitative estimates of oxidizable precursors in human serum. However, the TOP assay can also provide qualitative information about the structure of the precursors present in human serum. Indeed, even if the precursors' structure is lost upon oxidation the patterns of PFAA formed differ for different precursors, providing indications about the length of the perfluoroalkyl chain length and the presence of functional groups. Our experiments also highlighted the possible formation of additional stable end-products and the importance of including, among the TOP assay target analytes, PFSA and PFECA, that are not routinely analyzed as TOP assay products.

4.2 Paper 2

This study investigated exposure to total fluorine and known and unknown organic fluorinated compounds over time with respect to sex and age using a fluorine mass-balance approach. The mass-balance included TF, EOF, TOP and selected target PFAS in pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015.

TF concentrations were comparable in 1986 (<25.0 to 1330 ng F/mL, mean: 112 ng F/mL), 2007 (<25 to 1212 ng F/mL, mean: 74.8 ng F/mL) and 2015 (<25.0 to 265 ng F/mL, mean: 68.3 ng F/mL), even if more variability was observed in TF concentrations in 1986 compared to 2007 and 2015. EOF concentrations in 1986 (13.3 to 45.3 ng F/mL, mean: 23.3 ng F/mL) were significantly higher than in 2007 (16.2 to 30.3 ng F/mL, mean: 20.5 ng F/mL) and 2015 (12.6 to 22.6 ng F/mL, mean: 18.4 ng F/mL) and between 2007 and 2015 EOF concentrations were comparable. Out of 54 PFAS included in the target analyses only 12 PFAS were detected, including 6 PFCA (PFHpA, PFOA, PFNA, PFDA, PFUnDA and PFDoDA), 3 PFSA (PFHxS, PFHpS and PFOS) and 3 precursors (FOSAA, Me-FOSAA and Et-FOSAA). For all PFAA, except PFHpA, concentrations in 2007 were higher than in 1986. Lower concentrations in 2015 compared to 2007 were observed for PFOA and the PFSA, while for the longer chain PFCA the same decreasing trend was not observed. The sulfonamido acetic acids concentrations rapidly decreased between 1986 and 2007 and in 2015 none of these compounds were detectable. Total target PFAS ($\sum_{12}$PFAS) concentrations were higher in 2007 (38.7 to 75.7 ng/mL, mean: 48.2 ng/mL) than in 1986 (23.7 to 40.3 ng/mL, mean: 30.2 ng/mL) and 2015 (22.9 to 52.4 ng/mL, mean: 36.3 ng/mL). This was the first fluorine mass-balance study in human serum to include TOP and the results suggest that precursors with more than 4 perfluorinated carbon atoms contribute only to 0-4 % of the EOF. The contribution of TOP to the EOF did not change between sampling years. Taken together, $\sum_{12}$PFAS and PFAA precursors did not fully describe the concentrations of EOF found in human serum from the Tromsø Study at any of the sampling years. The unidentified EOF (UEOF) concentrations in 1986 (2.93 to 34.8 ng F/mL, mean: 10.9 ng F/mL), were significantly higher than in 2007 (0.00 to 10.9 ng F/mL, mean: 3.17 ng F/mL) and in 2007 UEOF concentrations were significantly lower than in 2015 (0.00 to 9.74 ng F/mL, mean: 5.32 ng F/mL). While TF, EOF and TOP concentrations were not influenced by sex, $\sum_{12}$PFAS and UEOF concentrations differed between men and women. Women had lower concentrations of $\sum_{12}$PFAS but higher concentrations of UEOF than men.

4.3 Paper 3

This study investigated the presence of emerging PFAS and fluorinated pharmaceuticals using suspect screening strategies relying on direct infusion FT-ICR-MS and LC-Orbitrap-HRMS measurements on pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015.

From the FT-ICR-MS data 365 suspect PFAS masses out of 5000 suspects included in the suspect screening were observed in pooled serum from the Tromsø Study with a ppm error < 0.5 ppm. However, only 4 of these masses could also be observed in the LC-Orbitrap-HRMS data with a mass error < 2 ppm. The LC-Orbitrap-HRMS data was also screened for a list of 332 PFAS masses compiled from the literature about PFAS in biota and human serum and 3 of these suspects were detected. Two suspects, $C_8HF_{15}O_3S$ (m/z=460.9334) and $C_8HF_{15}O_4S$ (m/z=476.9283), were part of both PFAS suspect lists and were detected both by FT-ICR-MS and LC-Orbitrap-HRMS. The first suspect, corresponding to the cyclic PFAS known as PFECHS, was confirmed on level 1 and quantified using an authentic standard. PFECHS concentrations ranged from 0.34 to 0.64 ng/mL and, similarly to PFOA and the PFSA, were higher in 2007 compared to 1986 and 2015.The second PFAS suspect could only be confirmed on level 5 due to lack of MS2 diagnostic fragments and standards but could be detected before and after TOP assay. No other emerging PFAS were found, but the fluorinated pharmaceuticals suspect screening allowed the identification of 3 additional fluorinated compounds contributing to EOF in pooled serum samples from 2007 and 2015. Two of these pharmaceuticals (teriflunomide and lansoprazole) contained a CF_3 group while the third one (pantoprazole) contained a CF_2 group. All these fluorinated pharmaceuticals were confirmed on level 1 using standards and the pooled serum samples were also screened for the presence of potential metabolites of these pharmaceuticals. Five metabolites of these pharmaceuticals retaining the CF_3 and CF_2 groups were also detected and confirmed on level 3 based on diagnostic MS2 fragments. Overall, fluorinated pharmaceuticals and their metabolites largely contributed to the EOF (0-56%) and their contribution to the EOF increased significantly between 1986 (none of the pharmaceuticals was detected), 2007 (0-50%; mean 5.3%) and 2015 (0-56%; mean: 31%). Additionally, the oxidation of 6 model pharmaceuticals and pesticides containing CF_3 groups showed that these substances were oxidizable in the TOP assay, but not converted to TFA and no TFA was observed in pooled serum samples from the Tromsø Study after TOP assay.

5 Discussion

5.1 Temporal changes in human exposure

The combined application of targeted and group-wise analyses and suspect screening approaches on pooled serum samples from the Tromsø Study (Paper 2 and Paper 3) enabled the evaluation of the contribution of known and so far unidentified organofluorine compounds in human serum through time. Exposure through time to TOP and fluorinated pharmaceuticals and their contribution to organofluorine exposure were for the first time analyzed in human serum.

The temporal changes in concentrations of TF, EOF, TOP, target PFAS ($\sum_{13}$PFAS) and fluorinated pharmaceuticals concentrations in pooled serum samples from the Tromsø Study between 1986, 2007 and 2015 are summarized in Figure 12.

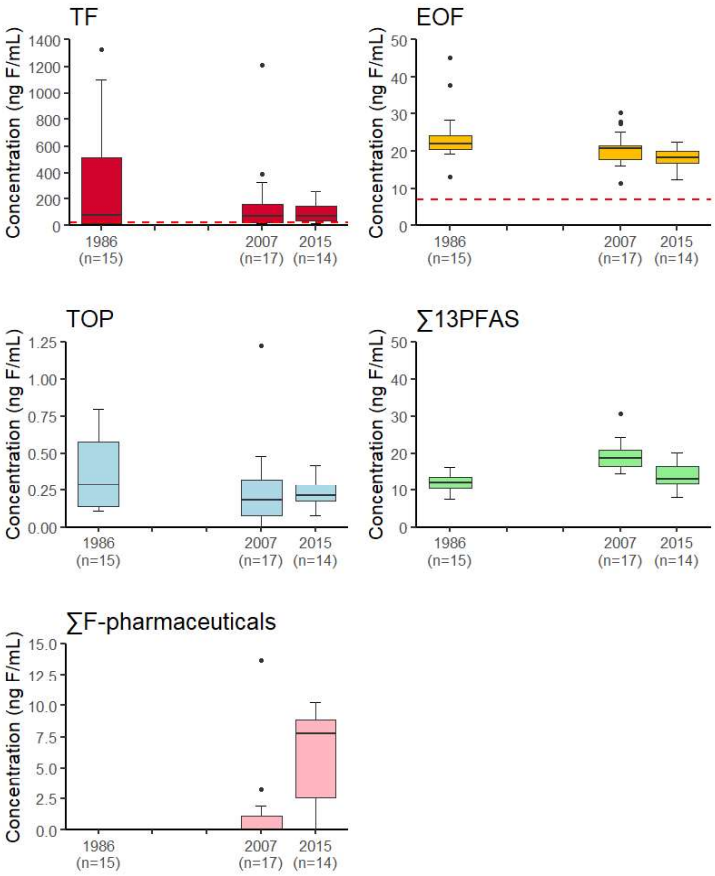

Figure 12 - TF, EOF, TOP, $\sum_{13}$PFAS and ∑F-pharmaceuticals concentrations in pooled serum samples from the Tromsø Study from 1986, 2007 and 2015 (Figure adapted from Paper 2 and Paper 3).

5.1.1 Total fluorine

TF in pooled serum from the Tromsø Study, that includes both inorganic and extractable and non-extractable organofluorine chemicals, ranged from <25 to 1330 ng F/mL, with a narrower range observed in 2015 compared to 1986 and 2007 (Paper 2). The percentage of pools with TF below LOD (25 ng F/mL) was 33 % in 1986, 24 % in 2007 and 7% in 2015. TF concentrations stayed stable between 1986, 2007 and 2015 based on multiple linear regression analysis (Figure 12, Paper 2). This observation differed from the only other study reporting temporal changes for TF in human serum, in which declining TF concentrations were observed between 1996 and 2017 [150]. In that study samples were collected from Swedish women that were consuming PFAS-contaminated drinking water up until mid-2012 and that might have influenced the TF trend. However, the different temporal changes might also be explained by the range of observed for TF concentrations in the Tromsø Study pools in 1986 and in 2007 being wider than in 2015 and the presence in 1986 of a higher number of pools than in 2007 and 2015 with both elevated concentrations and concentrations below LOD.

5.1.2 Extractable organic fluorine

EOF concentrations in pooled serum from the Tromsø Study in 1986 were significantly higher than in 2007 and 2015, while between 2007 and 2015 concentrations were not significantly different (Figure 12). Other two studies on EOF temporal changes in pooled serum samples from Swedish women collected between 1996 and 2017 and in individual plasma samples from two German cities collected between 1982 and 2009 showed no significant differences in concentrations between sampling years [148, 150]. The different temporal changes observed between our study and these studies might be due to differences in the sampling years, differences in overall exposure to organofluorine chemicals but also to different extraction methods being used to measure EOF, since different extraction methods may perform differently for individual fluorinated substances [190].

When comparing EOF with TF concentrations, a large difference was observed at all time points. The EOF accounted for 20 to 99% of the TF and the TF not explained by EOF ranged from 5 to 1194 ng F/mL. The TF not explained by EOF can include inorganic fluoride and organic fluorinated compounds not extracted or partially extracted with ACN. Water in Norway is not fluorinated and fasting plasma fluoride concentrations in areas with non-fluorinated water (water fluoride concentrations <0.3 mg/L) range from 9.3 to 24 ng F/mL [14, 197]. In humans the fluoride metabolism is not homeostatically regulated and plasma concentrations vary

depending on levels of intake, deposition in hard tissues and excretion [197, 198]. After ingestion, plasma concentrations take 3 to 6 hours to return to baseline values [198]. This could contribute to explaining the variability observed in the TF, because the serum collected in the Tromsø Study is from non-fasting individuals.

5.1.3 Total oxidizable precursors

All pooled serum samples (except one from 2007) had detectable concentrations of oxidizable precursors, even if the concentrations were low (ranging between 0.02 and 1.85 ng/mL). Based on multiple linear regression, TOP concentrations in 1986, 2007 and 2015 were comparable (Figure 12, Paper 2).

Looking at the increase of individual PFAA concentrations after TOP assay, increases were observed for 8 PFCA (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA and PFDoDA) and 3 PFSA (PFBS, PFHxS and PFHpS), but only PFDoDA, PFBS and PFHpS increased in at least 50% of the pools of one time-point. Increases in PFDoDA and PFHpS were observed at all time-points, while increases in PFBS were only observed in pools from 2015. Increases in concentrations of multiple PFAA following oxidation were more common than increases in only one PFAA but 8 pools showed an increase only in PFHpS (5 samples), PFDoDA (2 samples) and PFBS (1 sample) (Figure 13). No increases in concentrations of PFAA with less than 4 fluorinated carbons were observed after TOP assay. In particular, no TFA was found in the pooled samples after TOP assay, indicating also its absence in serum before oxidation. However, serum might not be the optimal matrix to measure TFA, since short-chain PFAS have faster elimination half-lives compared to long-chain PFAS.

The patterns of PFAA increases after oxidation differed from those observed for model substances in Paper 1 and could not be used to tentatively identify the precursors in pooled serum from the Tromsø Study. However, the profile of the oxidation products offered indications about the chain length of the precursor(s) and the presence of sulfonic groups. For example, increases in PFDoDA after TOP assay point to the presence of precursors with 11 or more perfluorinated carbons, while increases in PFBS and PFHpS suggest the presence of precursors containing sulfonic groups attached to 4 or 7 perfluorinated carbons.

The patterns of PFAA in pooled serum from the Tromsø Study were also different from those reported for plasma collected from women from all over Norway between 2003 and 2006, even if also in this case the concentrations of TOP were low (0.41-2.72 ng/mL) [189]. In contrast to

our study, no increases in PFDoDA and PFBS were observed. Also, in the Tromsø Study pools, the concentrations of branched PFOA and PFOS did not increase after the TOP assay and the detection of increases of PFHpA, PFNA, PFDA and PFUnDA was limited, while in the plasma collected from Norwegian women 7 PFAA increased after oxidation (PFHpA, branched-PFOA, PFNA, PFDA, PFUnDA, PFHpS, branched PFOS) with the greatest concentration differences observed for PFHpA, branched PFOA and PFDA. The differences in patterns of PFAA observed after TOP assay could be due to exposure to differences among the two studied groups, since here we measured serum samples collected from both men and women living in Tromsø, while Coêlho et al. [189] measured samples only from women and from all over Norway. The differences could also be due to the use of serum instead of plasma and to the use of different extraction methods.

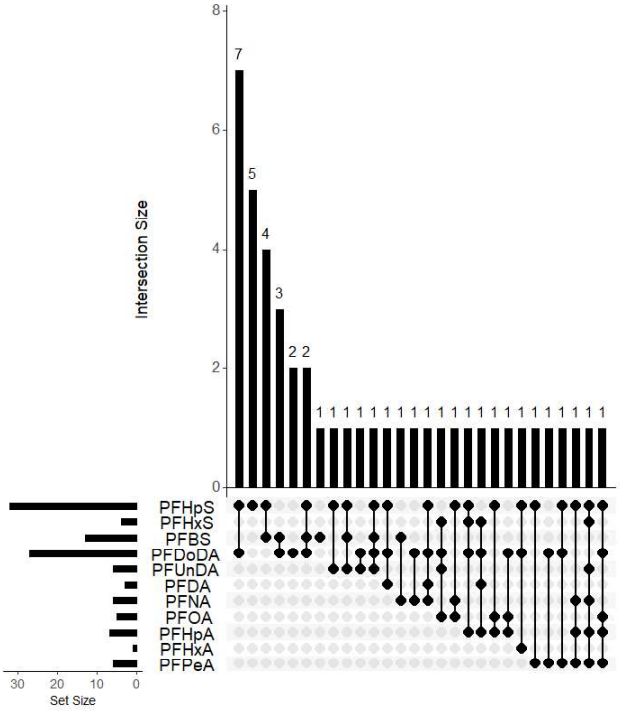

Figure 13 - UpSet plot showing the intersection of PFAA with increased concentrations after oxidation. The bar chart shows the number of pools with increases in concentrations of a combination of PFAA. The graphical table underneath indicates the PFAA combinations (black dots and lines). The frequency count of each PFAA across all subsets is shown as a smaller bar chart on the left side of the graphical table (Figure from Paper 2).

5.1.4 Known PFAS

In total, 13 known PFAS were identified in pooled serum samples from the Tromsø Study. The identified PFAS included six PFCA (PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA),

three PFSA (PFHxS, PFHpS and PFOS), three sulfonamidoacetic acids (FOSAA, Me-FOSAA, Et-FOSAA) and one cyclic compound (PFECHS). PFECHS was identified through the suspect screening workflow described in Paper 3, while the other PFAS were found through conventional target PFAS analyses described in Paper 2. Through suspect screening, an additional suspect PFAS mass was found, corresponding to the formula $C_8HF_{15}O_4S$, but this assignment could not be confirmed or discarded due to the lack of MS2 fragmentation and due to the unavailability of analytical standards.

The total known PFAS concentrations ($\sum_{13}$PFAS) in pooled samples from 2007 were significantly higher than in samples from 1986 and 2015 (Figure 12). Focusing on individual PFAS changes over time (Paper 2), concentrations of all PFAA in 2007 were higher than in 1986, except for PFHpA. Between 2007 and 2015, PFSA (PFHxS, PFHpS and PFOS), PFOA and PFECHS concentrations decreased, as opposed to the longer chained PFCA (PFNA, PFDA, PFUnDA and PFDoDA), for which concentrations increased. Concentrations of sulfonamidoacetic acids increased from 1986 to 2007 but none was detected in 2015. PFHpA concentrations were comparable in 1986, 2007 and 2015. The increase in $\sum_{13}$PFAS and individual PFAA concentrations between 1986 and 2007 points to increased PFAS exposure between these years. However, we know from previous PFAS analyses in serum from the Tromsø Study, including individual samples from 1994 and 2001, that target PFAS concentrations peaked in 2001 with an increase between 1979 and 2001, followed by a decrease between 2001 and 2007 [145, 146].

5.1.5 Fluorinated pharmaceuticals and their metabolites

Three organofluorine pharmaceuticals (teriflunomide, lansoprazole and pantoprazole) were detected in some of the pooled samples from the Tromsø Study through suspect-screening and confirmed with native standards. None of these pharmaceuticals was detected in pooled samples from 1986, while in 2007 two of them (lansoprazole and pantoprazole) were found and in 2015 all of them were detected (Paper 3).

The detection frequencies of these pharmaceuticals in pooled serum samples from 2007 and 2015 were lower than those of PFAS and agreed with the prescription data from the NorPD database (Paper 3). Teriflunomide, that had a higher number of users in the Troms and Finnmark region in 2015 compared to earlier years and had a higher number of users among women, was detected only in 2 pools from 2015 including women. Lansoprazole, for which the number of users in 2015 was lower than in 2007 and only slightly higher in women than in men,

was detected in 4 pools from 2007 (2 including men and 2 including women) and in 2 pools from 2015 including women. Lastly, pantoprazole, for which the number of users in 2015 was higher than in 2007 and higher in women than in men, was detected in 1 pool from 2007 and in 10 pools from 2015.

Additionally, for all fluorinated pharmaceuticals detected in pooled serum, some metabolites containing the CF_2 or CF_3 groups were also detected. A total of 5 fluorinated pharmaceuticals metabolites (4-hydroxy-teriflunomide, lansoprazole sulfide, 5-hydroxy-lansoprazole/lansoprazole sulfone, pantoprazole sulfone and 4-demethyl pantoprazole-4-hydrogen sulfate) were confirmed based on diagnostic MS2 fragments and quantified using their parent compound.

The concentrations of fluorinated pharmaceuticals and their metabolites in pooled serum from the Tromsø Study varied. For teriflunomide the concentrations in pooled serum ranged from <LOD to 39.6 ng/mL. The concentration of the metabolite 4-hydroxy-teriflunomide was almost 2 orders of magnitude lower (range: <LOD-0.56 ng/mL). For lansoprazole, concentrations ranged from <LOD to 1.68 ng/mL. Higher concentrations were observed for the lansoprazole metabolites, lansoprazole sulfide (range: <LOD-12.2 ng/mL) and lansoprazole sulfone (range: <LOD-77.4 ng/mL). For pantoprazole, concentrations ranged from <LOD to 16.7 ng/mL. Concentrations of the metabolite pantoprazole sulfone (range: <LOD-105 ng/mL) were higher than those of pantoprazole while concentrations of the metabolite 4-demethyl pantoprazole-4-hydrogen sulfate (range: <LOD-14.8 ng/mL) were comparable. In total pantoprazole and its metabolites accounted for <LOD and 10.2 ng F/mL.

Overall, the ∑F-pharmaceuticals (including both parent compounds and metabolites) concentrations in pooled serum from the Tromsø Study did not increase significantly between 1986 and 2007 but increased significantly between 2007 and 2015 (Figure 12, Paper 3). The increase in concentrations and detection frequencies of fluorinated pharmaceuticals over these three decades might be explained by the increasing number of fluorinated pharmaceuticals available in the market, since the percentage of fluorinated pharmaceuticals increased from around 2% in 1970 to 25% in 2021 [14, 19]. In addition, the increase in concentrations of ∑F-pharmaceuticals observed in our study between 1986 and 2015 might also reflect an increased use of these pharmaceuticals among older individuals, since the pooled serum samples from 2015 (mean age individuals in the pools: 61-81 years) in our study included individuals that were older compared to the individuals included in the pools from 1986 (mean age individuals

in the pools: 31-55 years) and 2007 (mean age individuals in the pools: 56-74 years). For example, for pantoprazole in 2015, the percentage of users in the Troms and Finnmark region was higher among the older age groups (Figure 14).

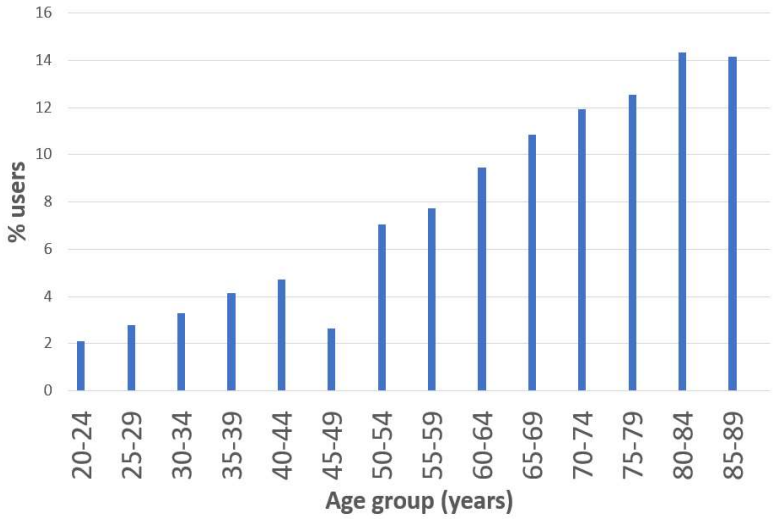

Figure 14 – Percentage of users (number of users/population base from NorPD database) of pantoprazole in different age groups in the Troms and Finnmark region in 2015 [191].

5.1.6 Known PFAS, TOP and fluorinated pharmaceuticals contribution to EOF

Known PFAS accounted for a large part of the EOF ranging from 24 to 100 %. The $\sum_{13}$PFAS accounted for 24-82% (mean: 53 %) of the EOF in 1986, 62-100 % (mean: 88%) of the EOF in 2007 and 46-100% (mean: 75 %) in 2015. The contribution of oxidizable PFAA precursors to the EOF in human serum was minimal, ranging from 0 to 4%. Overall, fluorinated pharmaceuticals and their metabolites accounted for a significant portion of the EOF ranging from 0 to 56 %. Even if the fluorinated pharmaceuticals and metabolites detected in pooled serum from the Tromsø Study only contained 2 or 3 fluorine atoms, their concentrations were higher than those of target PFAS and showed a large contribution to the EOF in 2007 (0-50 %, mean: 5.3 %) and 2015 (0-55 %, mean: 31 %).

5.1.7 Unidentified EOF

After inclusion of fluorinated pharmaceuticals and their metabolites to the fluorine mass-balance, the portion of EOF left unexplained was notably reduced compared to the values reported in Paper 2 (Figure 15).

UEOF concentrations were highest in 1986 (mean: 10.3 ng F/mL = 46 %, range: 2.47–34.3 ng F/mL = 18-76 %), when the target PFAS concentrations were lowest, and no fluorinated pharmaceuticals were detected. In 2007 (mean: 2.04 ng F/mL = 9.2 %, range: 0.00–6.74 ng F/mL = 0-31 %) and in 2015 (mean: 0.54 ng F/mL = 3 %, range: 0.00–5.96 ng F/mL = 0-30 %) the UEOF portion was significantly lower than in 1986 (Figure 15, Paper 3). In 7 pools from 2007 and 10 pools from 2015 the fluorine mass-balance was closed (UEOF=0%).

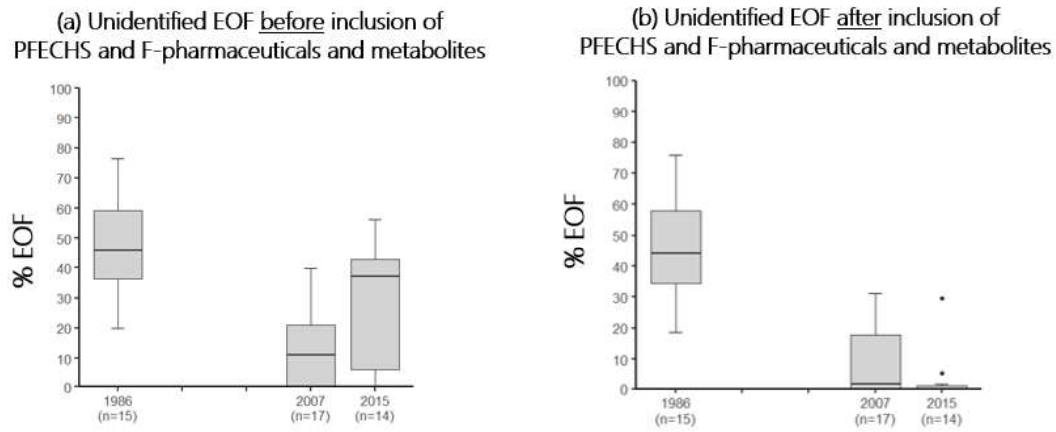

Figure 15 - UEOF before (a) and after (b) inclusion of PFECHS and F-pharmaceuticals in the mass balance (Figure adapted from Paper 2 and Paper 3).

The high fraction of UEOF observed in the 1986 Tromsø Study samples, followed by a decrease in 2007 could be explained by the presence of PFOS-related substances in the serum which use was also reduced following the restriction of PFOS and PFOA in early 2000s. According to the PubChem PFAS Tree [68], there are 1297 chemicals registered in PubChem that would be restricted under Annex B of the Stockholm Convention. However, among these chemicals, C8-precursors can be excluded since no increases in PFOS and limited increases in PFOA were observed after the TOP assay in 1986. No additional C8-PFAS were found through suspect screening. To fully investigate this hypothesis, a possibility would be to screen the HRMS data using a non-target approach, which is not limited by a list of possible structures. Non-target screening could widen the investigation to potential PFAS features based for example on mass defect, homologue series searching or presence of diagnostic fragments or neutral losses. Another possible explanation for the high UEOF observed in 1986 could be the presence in human serum of fluorinated pesticides not assessed in our study.

5.1.8 Relationship with age

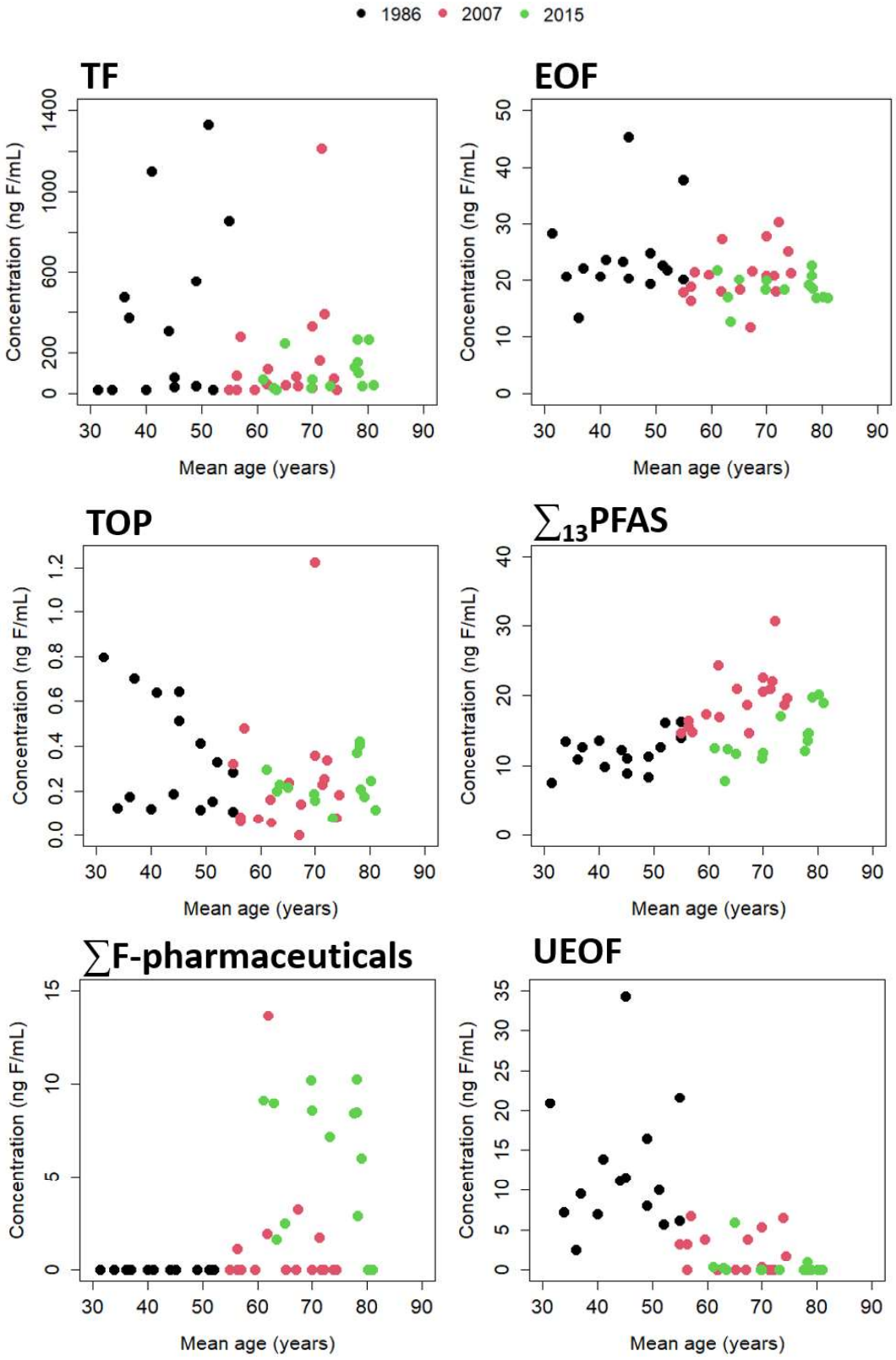

Figure 16 – Concentrations (ng F/mL) of TF, EOF, TOP, $\sum_{13}$PFAS, ∑F-pharmaceuticals and UEOF in pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015 in relationship with mean age in years of the individuals in the pools.

Based on multiple linear regression analysis, mean age of the individuals in the pools was a predictor of the $\sum_{13}$PFAS with the highest concentrations at each sampling year observed in the pools with highest mean age (Figure 16, Spearman's rank correlation coefficient=0.48 in 1986, 0.60 in 2007 and 0.79 in 2015). This has been explained by higher exposure in the older birth cohorts compared to the younger ones due to the history of changing PFAS production [145].

For TF, EOF, TOP, ∑F-pharmaceuticals and UEOF concentrations no relationship with mean age was found (Figure 16). For TF and EOF concentrations, this might be explained by these two measurements being sum-parameters that can include chemicals with different properties. This explanation can also be extended to the UEOF concentrations, that can potentially include a mixture of chemicals as well. For TOP and ∑F-pharmaceuticals concentrations, the lack of relationship with age might be explained by these chemicals having shorter half-lives than known PFAS (e.g., leflunomide half-live: 2 weeks; lansoprazole half-live: 2 hours, pantoprazole half-live: 1 hour [192, 193]) and showing no bioaccumulation.

However, it must be noted that with the study design used in Paper 2 and Paper 3, the relationship with age on the different fluorine fraction concentrations could not be properly assessed due to the use of pooled samples. The pools analyzed included individuals covering a wide range of ages and this limited the investigation of the influence of age and birth cohorts on the different fluorine fractions measured, because age–period–cohort effects could not be assessed.

5.1.9 Sex differences

No significant differences in TF and EOF concentrations based on sex were found in the Tromsø Study pools using multiple linear regression analyses (Paper 2, Figure 17). EOF measurements in Chinese whole blood samples also showed no significant differences based on sex and age [147], but in serum samples collected in Sweden in 2021 EOF concentrations were higher in women than in men [152]. TOP and ∑F-pharmaceuticals also did not differ significantly between men and women (Paper 2, Paper 3, Figure 17). Men had significantly higher $\sum_{13}$PFAS concentrations than women. However, when looking at the difference in $\sum_{13}$PFAS concentrations at each time-point, men had significantly higher concentrations only in 2007 ($\Delta\sum_{13}PFAS_{men\text{-}women}$=3.78 ng F/mL). Higher concentrations in men compared to women were observed for most PFAS (PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFHxS, PFHpS and PFOS), but for PFHpA and the three sulfonamido acetic acids the concentrations were comparable between men and women (Paper 2). Berg et al. [146] also reported lower

PFAS concentrations in women compared to men in the Tromsø Study. This difference between men and women can be explained by the contribution of placental transfer, breast feeding and menstruation to PFAS elimination [194-197].

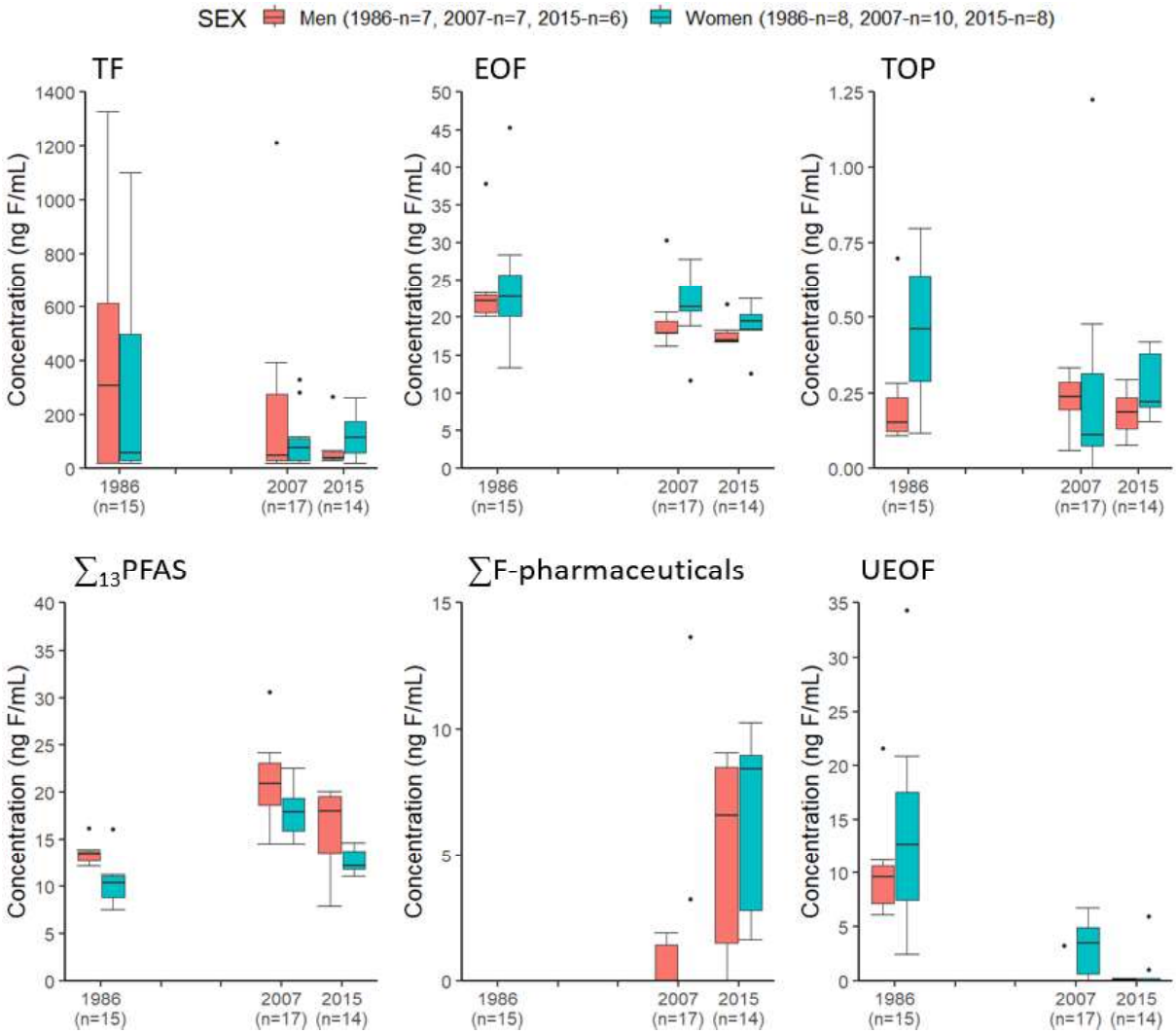

Figure 17 - TF, EOF, TOP, ∑13PFAS, ∑F-pharmaceuticals and UEOF concentrations (ng F/mL) in pooled serum from men and women from the Tromsø Study in 1986, 2007 and 2015 (n=number of pools) (Figure adapted from Paper 2 and Paper 3).

For UEOF women had higher concentrations than men, even after inclusion in the mass-balance of PFECHS and fluorinated pharmaceuticals (Figure 17). As for target PFAS, the evaluation of differences in UEOF concentrations between men and women at each time-point was limited by statistical power and significant differences were observed only in 2007 ($\Delta UEOF_{women-men}$=2.68 ng F/mL=11%). The sex difference observed for UEOF is the opposite of what we observed for PFAA. Higher UEOF in women compared to men have also been reported in whole blood collected in Sweden, where the highest UEOF was reported in women aged 18-44

[16]. Two hypotheses were proposed by Aro et al. [16] to explain the different UEOF concentrations between men and women. The first hypothesis is that a more frequent use of cosmetics and personal care products containing precursors (like PAPs) and other unknown PFAS [43, 68] could lead to higher blood concentrations of unknown PFAS. This hypothesis is also supported by studies reporting associations between PFAS concentrations in the blood and the use of cosmetics and personal care products [69, 70]. However, in our study, the TOP assay showed only a minor contribution of precursors to the EOF in human serum with no differences between men and women and therefore this first hypothesis regarding precursors exposure can be discarded. Still, the more frequent use of cosmetics might be a possible explanation for the higher UEOF in women compared to men since cosmetics could also lead to exposure to yet unknown PFAS that are not oxidizable and therefore non-detectable in the TOP assay. A second explanation could lie in a difference in use of fluorinated pharmaceuticals between men and women resulting in fluorinated metabolites that were not assessed in this study, since sex differences in prescription are reported for several groups of pharmaceuticals [71-76]. For example, sex differences in prescription of leflunomide and pantoprazole can be observed in the Troms and Finnmark region (Paper 3). Additionally, differences in elimination kinetics between men and women for these yet unidentified fluorinated chemicals could also play a role.

5.1.10 Current understanding of total fluorine, organofluorine and PFAS internal exposure measured in human serum

Overall, these findings contribute to a better understanding of internal exposure measured in human serum. TF concentrations did not increase between 1986, 2007 and 2015 (Figure 18), indicating no significant increases in internal exposure to new fluorinated compounds in the Tromsø population. The fact that there was no observed increase in EOF concentrations between 1986, 2007 and 2015 (Figure 18) also indicates that the overall internal exposure to organofluorine compounds has not been increasing over the studied period. Also, for PFAS exposure, no increases in exposure in recent years were observed and we can observe that PFAS peak internal exposure in the Tromsø population was in 2001. Nøst et al. [145] and Berg et al. [146] analyzed PFAS in the Tromsø Study including also serum samples from 1994 and 2001 and found that target PFAS concentrations peaked in 2001 with an increase between 1979 and 2001, followed by a decrease between 2001 and 2007. In this thesis, when adding up together the target PFAS concentrations, including the newly identified PFECHS, and unknown TOP, the total PFAS concentrations in human serum are increasing between 1986 and 2007 and declining between 2007 and 2015 (Figure 18). This observation about PFAS peak exposure is

also confirmed by no emerging PFAS being detected in pooled serum from 2007 and 2015 and by the increasing contribution to the overall organofluorine exposure of fluorinated pharmaceutical and their metabolites (Figure 18), that were found in the serum probably due to an increased use in recent years and an increased use in aging individuals. When looking at individual PFAS concentrations it is important to highlight that, while PFOA and PFSA concentrations have been declining between 2007 and 2015 (driving the total PFAS exposure decline), the concentrations of longer chain PFCA have been increasing between 2007 and 2015 (Paper 2) showing the importance of continuing PFAS biomonitoring.

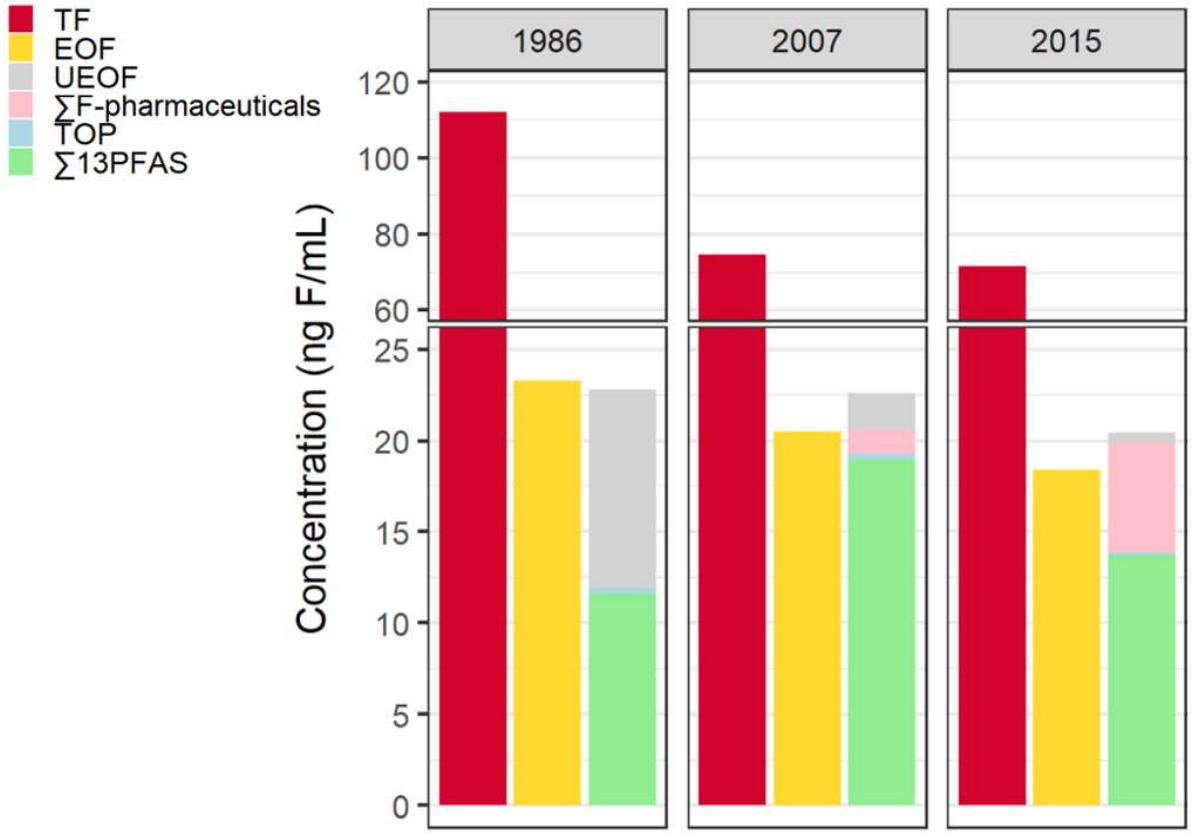

Figure 18 - Summary of mean concentrations (ng F/mL) in pooled serum samples from the Tromsø Study in 1986, 2007 and 2015 (Figure adapted from Paper 2 and Paper 3).

5.2 Methodological considerations

5.2.1 Sample selection bias

A selection bias is introduced when there is a systematic difference between the people that participate in a study and those who do not [198]. Participation in the Tromsø Study is voluntary and differences between responders and non-responders are an inevitable source of selection bias. In general, individuals that voluntarily participate in epidemiological studies are often

healthier, younger and have a higher socioeconomic status than non-participants [198]. The first survey of the Tromsø Study from 1974 only included men and focused on the study of the high mortality from cardiovascular diseases in Norway [184]. In the following surveys, with the intent of mitigating the selection bias, both genders and other health conditions have been included. From the six surveys between 1974 to 2007 it was observed that among the Tromsø Study participants the proportion of men and of younger birth-cohorts was lower than among non-participants [184]. Additionally, a selection bias is introduced in the Tromsø Study longitudinal design by a higher rate of response among returning participants compared to those who never participated in the previous surveys [184].

The sub-selection of individual Tromsø Study samples used in this thesis was based on a case-control study design on type-2 diabetes. The cases were selected among individuals that had type-2 diabetes diagnosed between 2001 and 2007 and since the controls were selected randomly among individuals that had no type-2 diabetes diagnosis recorded in the local registry, no significant biases are expected from this selection. Based on the sub-selection of samples used to obtain the pools for Paper 2 and Paper 3, there could be a selection bias coming from having 7 of the pools for each sampling year following the same individuals across the 3 surveys included in our study (1986, 2007 and 2015) and the remaining pools including many individuals that participated in at least two of the surveys.

5.2.2 Generalizability

Absolute PFAS and TOP concentrations can be generalized to the Tromsø population but might lack external validity for other populations or Norway as a whole country since for example, individuals living in the coastal areas of Norway tend to have higher PFAS concentrations than those living in the inland areas [199]. However, the temporal changes in PFAS and TOP concentrations are expected to be generalizable because for PFAA the trends that we observed are in agreement with trends in production and use of these compounds. For the detection of emerging PFAS the results might not be generalized to other populations but are expected to be generalizable to other populations with background exposures, similar to Tromsø.

For TF and EOF, that are sum parameters, the results might lack external validity due to the contribution of different chemicals to these parameters. Populations with fluorinated water might have different TF values than those observed in this study and populations exposed to PFAS might have higher EOF values. Also, for EOF the results might vary depending on the contribution of fluorinated pharmaceuticals.

The contribution of fluorinated pharmaceuticals to the EOF in the Tromsø Study pools from 2007 and 2015 might be generalized to populations with similar age distribution. However, for populations including younger individuals the contribution of these compounds to the organofluorine mass-balance might be lower. This fraction might also vary depending on the incidence of diseases that are treated with fluorinated pharmaceuticals.

5.2.3 Use of pooled samples

Using pooled serum instead of individual samples comes with both some advantages and limitations. The main advantage that drove the choice towards the use of pooled samples, was the use for the pools of small volumes of individual samples (in our case 50, 100 or 150 μL). The combined application of multiple analytical techniques described in this thesis required the availability of 750 μL of serum and this amount from individual samples was not available for our study and is often not available for biobanked blood/serum samples. Indeed, the use of pools allowed for the screening of the Tromsø Study a combination of multiple state-of-the-art analytical methods in a time- and cost-efficient manner.

However, the use of pooled samples was also a limitation, since it was not possible to perform complex statistical analysis due to the limited number of pools. The small number of pools also limited the statistical power of multiple linear regression analyses. To obtain a power of 80% (i.e., 80% probability of rejecting a false null hypothesis when the alternative hypothesis is true) in multiple linear regression with large effect size (Cohen's f^2=0.35) and alpha=0.05 (i.e., 5% probability of rejecting a true null hypothesis), at least 39 samples are necessary. The number of pools (46 in total) was sufficient to assess differences between the three time-points examined and the general effect of sex and age on the concentrations measured. However, the number of pools at each time-point (n=15 in 1986, n=17 in 2007 and n=14 in 2015) was lower than the number of pools needed to achieve a power of 80% and was not sufficient to detect concentrations differences between men and women at each sampling year. Additionally, using pools, the effect of many variables known to influence PFAS exposure (e.g., dietary habits, parity) could not be assessed and, since the individuals in each pool covered a wide range of ages, the influence of age and birth cohorts (age–period–cohort effects) could also not be properly studied.

5.2.4 Use of pooled samples containing the same individuals across time

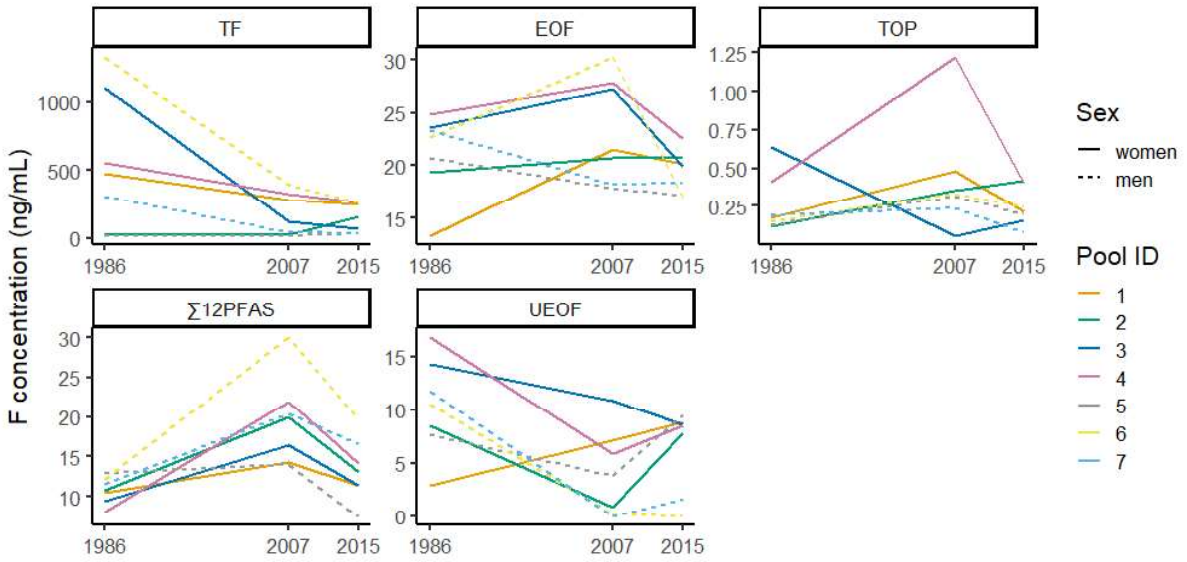

Figure 19 – TF, EOF, TOP, $\sum_{12}$PFAS and UEOF concentrations (ng F/mL) in serum pools from the Tromsø Study containing the same individuals at each sampling year.

The concentrations of the different fluorine fractions in the pools containing the same individuals in 1986, 2007 and 2015 are reported in Figure 19.

For target PFAS, the changes in concentrations observed were consistent in all pools with increasing concentrations between 1986 and 2007 followed by a decline in 2015 as described with multiple linear regression when including all pools. In pool 5, the tendency was not as pronounced since in this pool almost comparable $\sum_{12}$PFAS concentrations in 1986 and 2007 were observed. This could be due to this pool containing a lower number of individuals (10) compared to the other ones (11-14). With a lower number of individuals in a pool, even just one outlier could have a higher impact on the measured target PFAS concentrations.

For TOP, the time differences observed in pools from same individuals were not consistent across pools and this could be to a higher variability in precursors exposure, but also to the low concentrations of precursors present. Additionally, for this method a higher variability compared to target PFAS measurements is expected since the TOP concentrations are estimated by comparing two PFAA concentrations measurements, before and after oxidation.

Also, for EOF and UEOF, the time differences observed in the pools from the same individuals were not consistent. This is probably due to EOF being a sum parameter that includes not only PFAS that are detectable in most individuals. For example, the detection of fluorinated pharmaceuticals is expected to result from the inclusion in the pools of one or few individuals using the detected pharmaceuticals, and this could influence the variability observed for these fractions.

For TF, there were also inconsistencies in the time differences observed for pools with the same individuals and, as for EOF, this could also be explained by TF being a sum parameter. In pools 2 and 5, differences in TF concentrations over time differed from the rest of the pools because these were below or close to LOD at all sampling years.

5.2.5 Use of serum for PFAS and organofluorine compounds biomonitoring

The use of serum compared to whole blood and plasma has some important implications in this study. The most-well studied PFAS are known to accumulate in the blood and blood-rich organs. In the blood these PFAS are mainly distributed to the serum and plasma and only to a limited extent to the blood cells due to their high affinity for serum proteins. However, Poothong et al. [200] showed that the distribution between the different blood fractions is not the same for all PFAS. For example, while the highest PFOA and PFOS concentrations were detected in serum, PFHxA could only be detected in whole blood. Additionally, for FOSA the highest concentrations were observed in whole blood and for 6:2 PAP and 6:2 diPAP frequency of detection was highest in plasma with concentrations following the order plasma > serum > whole blood [200]. These observations show that serum might not be the most appropriate matrix to study precursors exposure. Therefore, the analysis of plasma/whole blood instead of serum could lead to different conclusions than those reported in this thesis for EOF, TOP and emerging PFAS exposure.

The analysis of different blood fractions would probably also account for the presence of different fluorinated pharmaceuticals. The three pharmaceuticals detected in pooled serum from the Tromsø Study (teriflunomide, lansoprazole and pantoprazole) have a high plasma protein binding (respectively, >99.3%, 97% and 98%). However, other fluorinated pharmaceuticals can have lower protein binding and could go undetected in serum but not in whole blood.

5.2.6 TOP assay oxidation optimization for human serum

The TOP assay method development for human serum showed that matrix specific protocols need to be developed to apply this method to samples with high content of organic matter. Human serum, from a chemical point of view, consists of mainly water (>90 %) with proteins (>5 %), as well as electrolytes, hormones and exogenous substances [201]. To remove interfering matrix, proteins were denatured and removed during extraction with methanol, while residual serum components that could further consume the oxidant needed to be oxidized using a suitable excess of oxidant.

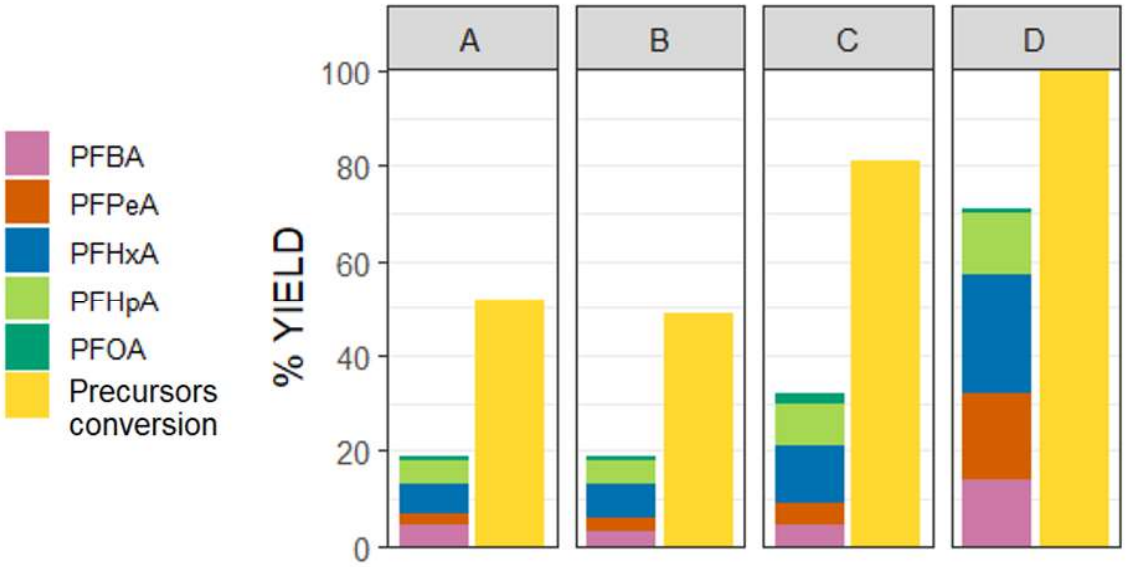

Figure 20 – Yield of PFAA (first bar) and conversion of precursors (second bar) for 7:3 FTCA in human serum with method A, B, C and D (Figure adapted from Paper 1).

As an example, the results of the different oxidation methods tested for 7:3 FTCA are shown in Figure 20. The test with oxidation conditions similar to the ones reported in the literature (method A) showed incomplete oxidation of the model precursors in human serum. Incomplete oxidation under similar conditions has been also reported for precursors in other matrices with high content of organic components, such as laying hens' eggs and biosolids [168, 202, 203]. To ensure complete oxidation the heating time was increased to 24 hours (method B) and the amount of oxidant was doubled (method C). Both modifications increased the conversion and/or the yield of PFAA for the model precursors, but the improvement observed by increasing the amount of oxidant was larger than the one achieved by increasing the heating time. Therefore, in method D a further increase of oxidant amount was tested using a heating time of 24 hours. The yields of PFAA and the conversion of model precursors using method D are

reported in Figure 21. Using this method, full conversion was observed for all precursors, except 10:2 FTS for which a 91% conversion was measured. The yield of PFAA was 100% only for Me-FOSAA, Et-FOSAA and ADONA. However, the yield of PFAA for the other model precursors was above 50% and this was considered satisfactory for the application of the method for high-throughput screening of PFAA precursors in human serum.

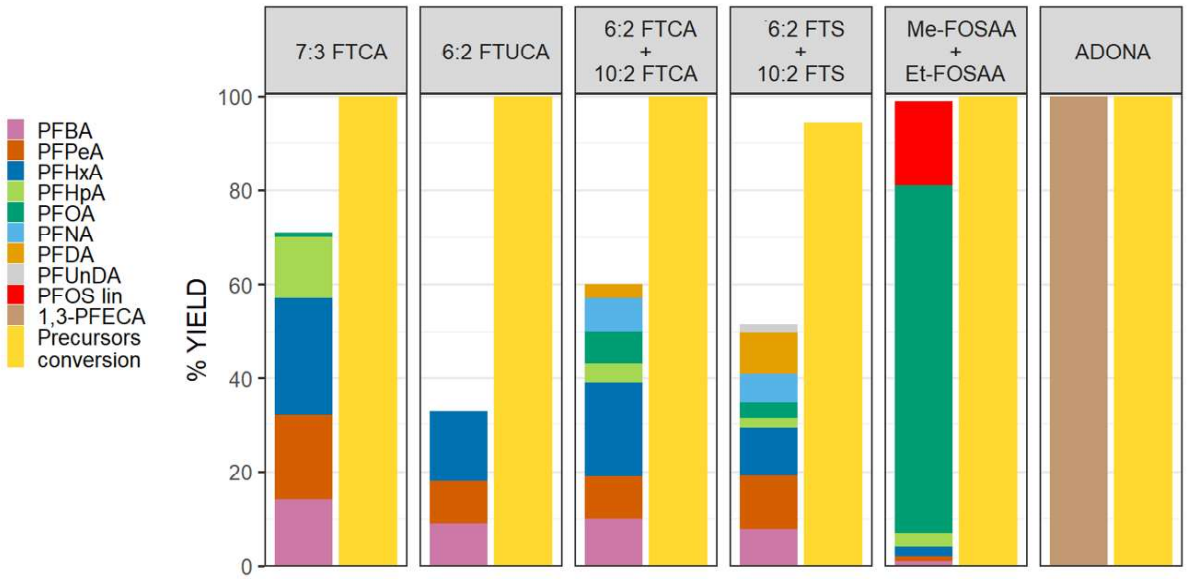

Figure 21 – Yield of PFAA (first bar) and conversion of precursors (second bar) for the model precursors in the optimized TOP assay for human serum (method D) (Figure from Paper 1).

5.2.7 PFAA precursors and organofluorine pharmaceuticals in the TOP assay

Model precursors with different chemical structures resulted in different PFAA patterns after oxidation in the final TOP assay method (Figure 21). For the fluorotelomer carboxylic acids, mixtures of PFCA were observed. For the fluorotelomer carboxylic acids with even number of fluorinated carbon atoms and 2 non-fluorinated carbon atoms (6:2 FTUCA, 6:2 FTCA and 10:2 FTCA), PFCA with n-1 or less (n=number of fluorinated carbons in the precursor) fluorinated carbon atoms were detected with n-1 PFCA as main oxidation product. For 7:3 FTCA, a small percentage of PFOA was also formed and the main product was the n-2 PFCA. For the fluorotelomer sulfonates (6:2 FTS and 10:2 FTS) a mixture of PFCA was also observed. The longest PFCA observed was PFUnDA, that has the same number of fluorinated carbon atoms of 10:2 FTS, and the dominant products were the n-1 and n-2 PFCA. For all these fluorotelomer compounds a yield of 100% could not be reached with the expanded list of targets PFAA. The first step to reach full conversion and make the assay fully quantitative would be the inclusion

of TFA and PFPrA to the set of target PFAS analyzed in the TOP assay, since it has been shown by others that short-PFAA can also be relevant oxidation products [164, 167]. However, the formation of intermediates and/or additional stable oxidation products is also a possibility. For Me-FOSAA and Et-FOSAA, full conversion to PFAA was achieved with the final method, but with method A, B and C, some oxidation intermediates (FOSA, Me-FOSA and Et-FOSA) were also detected (Paper 1). This observation highlights the possible formation of unknown intermediates in the TOP assay.

As an additional new aspect, the oxidation of Me-FOSAA and Et-FOSAA showed that PFCA are not the only end-products of the TOP assay with PFOS accounting for 18% of the yield (Figure 21, Paper 1). The formation of PFOS or any other PFSA in the TOP assay has previously been disregarded, reporting only PFCA as oxidation products [204-206]. The formation of PFSA could be due to base-catalyzed hydrolysis of sulfonamides [207] and has been observed also upon application of the TOP assay to suspended particulate matter, food packaging, surface and waste water [208-211]. Taken together, these findings show that PFSA should also be included in the target PFAS analysis for the TOP assay. Further, to detect oxidizable precursors containing ether groups, the inclusion in the target analysis for the TOP assay of stable PFECA is also recommended, since ADONA is oxidized and fully converted to 1,3-PFECA (Paper 1).

The application of the TOP assay to model pharmaceuticals and pesticides containing CF_3 groups showed that these substances are oxidizable with the TOP assay. However, after oxidation, no TFA was observed, showing that the TOP assay cannot be used to detect these compounds. Compounds with CF_3 could be fully oxidized/degraded to form fluoride under the TOP assay conditions [212] or could be converted to other stable end-products, that were not identified withing the scope of this thesis.

5.2.8 TOP assay strengths and limitations

The modified version of the TOP assay for human serum allowed to screen for conventional PFAS and known and unknown PFAA precursors in small volumes of serum without needing additional instrumentation or standards.

The TOP assay application can provide both qualitative and semi-quantitative information about known and unknown oxidizable PFAA precursors in human serum. The original precursor's structure is lost during oxidation and additional methods will be needed to elucidate

the identity of the precursors present. However, the products observed after oxidation can give indications about some of the precursor's structural features. For example, the chain lengths of the PFAA increased after TOP assay can give indications about the lengths of the precursors perfluoroalkyl chain. The TOP assay products can also be used to give an estimate of the TOP present in human serum, but it must be highlighted that this is a low-bound estimate since the PFAA yield of some precursors might not be 100% and oxidation intermediates might be formed.

5.2.9 Analytical methods uncertainty

Target PFAS were measured in the EOF extracts without internal standard addition before extraction and in the TOP assay extracts before oxidation with internal standard added before extraction. The target concentrations and detection frequencies from the EOF extracts are lower and less accurate than those from the TOP assay extracts, because of the lack of recovery correction for procedural losses. However, the use of PFAS concentrations not corrected for recovery for fluorine mass balance calculations provides a more representative and accurate result in terms of mass balance. This is because the EOF concentrations cannot be recovery corrected since the addition of internal standard before extraction would increase the LOD and it is not possible to correct for the recovery of unknown fluorinated chemicals present. Measured PFAA concentrations before oxidation in the reference AMAP serum samples were within -/+ 20% of the reference values. For fluorinated pharmaceuticals and PFECHS the concentrations were measured only without recovery correction. As for the target PFAS, the lack of recovery correction probably resulted in less accurate absolute concentrations values but more representative and accurate results in terms of mass balance.

The results of the TOP assay method evaluation reported in Paper 1 showed good repeatability and accuracy as well as good recoveries and low LODs for all target PFAA both before and after oxidation. However, the TOP assay measurements have more uncertainties compared to target PFAS analyses. The method is based on the calculation of TOP concentrations based on PFAA concentrations before and after oxidation and this comparison will introduce some uncertainties. To reduce these uncertainties TOP concentrations were calculated by comparing PFAA concentrations before and after oxidation that were recovery corrected and to reduce the influence of analytical uncertainties on apparent PFAA concentrations increases a cut-off of 20% change in PFAA concentrations was applied as described by Coêlho et al.[189]. The

comparison of TOP concentrations with EOF concentrations has some uncertainty because the TOP assay data are corrected for procedural losses, but the EOF data are not.

For EOF and TF, the quality control measures described in chapter 3.2 showed good accuracy and repeatability. However, some uncertainties in the analysis of EOF and TF using CIC have to be taken into account. In these measurements it is assumed that all PFAS are fully incinerated, but Aro et al. [213] have shown that the combustion efficiencies of PFAS can vary between 66 and 110 %. Additionally, in our study the calibration curve for EOF and TF was based on NaF in water and Aro et al. [213] also showed that different results can be obtained with CIC using organofluorine calibration instead of inorganic fluorine calibration.

5.2.10 Analytical toolbox considerations

All methods described and applied in this thesis come with advantages and disadvantages and here we want to provide some guidance on the choice between these methods when studying internal exposure with blood/serum samples.

For evaluation of the total exposure to organofluorine compounds, EOF can only account for the portion of these compounds that is extractable with a specified extraction protocol. TF has the advantage of accounting not-only the organofluorine compounds that are included in the EOF, but also non-extractable or partially extractable organofluorine compounds. However, TF also includes inorganic fluoride and to be able to use TF for monitoring of the total exposure to organofluorine compounds, the contribution of inorganic fluoride needs to be measured, since the fluoride metabolism in humans is not homeostatically regulated [214, 215].

When inorganic fluoride cannot be measured, EOF can be a good marker for the overall organofluorine exposure. As discussed by Aro et al. [151], EOF can be good a method to screen for elevated PFAS exposures and identify contamination hot-spots, since in their study the EOF concentrations in a PFAS exposed group were 9 times higher than those of a control group with background PFAS exposure. However, the results presented in this thesis highlight that care must be taken in interpreting EOF concentrations in human blood as a measurement of “total PFAS exposure”, since the contribution of organofluorine pharmaceuticals to EOF can be comparable to the PFAS contribution. This observation is expected to be particularly important for blood samples collected in more recent years, since the contribution of organofluorine pharmaceuticals could be even higher due to the rapidly increasing number of approved pharmaceuticals containing fluorine [14, 19].

On the contrary, the results from this thesis show that the TOP assay is a PFAS-specific method, since fluorinated pharmaceuticals containing CF_3 groups, that are considered potential TFA precursors, were not converted to TFA after oxidation. However, even if the TOP assay provides insights about the presence of known and unknown PFAA precursors, it might underestimate PFAS exposure in populations exposed to PFAS that are not oxidizable (e.g., GenX) or to precursors that are not fully converted to PFAA.

In cohorts with background PFAS contamination, such as the Tromsø population, the measurement of target PFAA might be sufficient to describe human exposure to PFAS. The concentrations of the sulfonamido acetic acids detected in the Tromsø Study pools from 1986 have been rapidly declining and are not detected anymore in samples from 2015. Also, the concentrations of PFECHS, not included in our initial target analysis, have been declining between 2007 and 2015.

Suspect screening approaches allow to screen for a large number of new PFAS and other organofluorine compounds, such as fluorinated pharmaceuticals. However, these methods are hard to use for routine biomonitoring, since these are expensive, time-consuming and require HRMS instrumentation as well as expert knowledge to interpret the data. Therefore, these methods are recommended as a complementary approach when the EOF is not fully explained by known PFAS and there is evidence that additional unidentified compounds might be present.

6 Conclusions

Overall, the results described in this thesis contribute to a more detailed understanding of the full extent of exposure to PFAS and other organofluorine chemicals in background exposed human populations, such as the one of Tromsø, and how this exposure has been changing during three decades covering a timeframe in which PFAS production and use has been evolving. The application of a fluorine mass balance approach, including for the first time the TOP assay and the analysis of fluorinated pharmaceuticals, to human serum collected in 1986, 2007 and 2015 from the Tromsø population provided novel insights about the contribution of PFAS to the overall fluorine exposure.

First of all, we observed that TF exposure did not decrease or increase significantly between 1986 and 2015 and that even if the EOF in pooled serum has decreased between 1986 and 2007, the overall exposure to organofluorine compounds has remained stable between 2007 and 2015. Secondly, even when EOF concentrations remained comparable, our analysis clearly shows that its composition has been changing substantially between sampling years. While PFAS concentrations were highest in 2007, the TOP assay showed that precursors are found in low concentrations in human serum with no temporal changes and the identification of three fluorinated pharmaceuticals and their metabolites has shown that their concentrations were significantly higher in 2015 compared to 2007 and 1986. Further, suspect screening for additional 5000 PFAS, revealed only one additional PFAS, showing no detection of emerging PFAS compounds, other than oxidizable precursors. This finding was also supported by the observation of only a small portion of the EOF left unidentified in pooled serum from 2007 and 2015.

The TOP assay adaptation to human serum allowed for the screening of the presence of known and unknown precursors. By using a small volume of serum and without requiring additional sample amounts, instrumentations, or standards, traditional target PFAS analyses can be complemented with novel data on the contribution of PFAA precursors to PFAS exposure. However, our results show that care must be taken in using the TOP assay as an accurate measurement of the total amount of precursors present, as there can be PFAS that are not at all or not completely converted to PFAA. The PFAA produced in the TOP assay can therefore only provide an estimate of precursors concentrations and indications about their structure and type (such as length of the perfluoroalkyl chain and presence of functional groups, like sulfonic and ether functionalities).

7 Future perspectives

Certain knowledge gaps remain in our understanding of total fluorine and organofluorine exposure in the Tromsø Study with special attention to known and unknown PFAS.

First of all, the differences between TF and EOF concentration are large and highly variable and future fluorine mass-balance studies in human blood that want to use both these measurements should include fluoride measurements so that is possible to assess the non-extractable organofluorine fraction. In pooled serum samples from 1986 a large portion of the EOF remained unidentified and lower UEOF concentrations were also found in 2007 and 2015. This fraction might be explained by unknown PFAS not included in our suspect lists but also by fluorinated pesticides which presence in human serum might also lead to human health concerns. To address this gap a possible strategy is to analyze the HRMS data using non-target screening to identify potential PFAS features, like mass defect filtering, homologue series identification and presence of diagnostic fragments and neutral losses.

Secondly, there is a discrepancy between the low concentrations of PFAA precursors in human serum and their wide detection in the environment and consumer products. Future studies should address this discrepancy and investigate the uptake potential and extent of metabolism and elimination of precursors in the human body.

Furthermore, no TFA was found in the human serum pools after TOP assay suggesting also its absence in serum before oxidation and that the human metabolism of fluorinated pharmaceuticals might not result in the formation of TFA. However, serum might not be the preferred compartment for TFA, precursors and emerging PFAS circulation and future studies should try to understand if serum is the most suitable matrix for monitoring of PFAS other than PFAA or other matrices should be preferred to assess exposure to these chemicals.

Lastly, while the TOP assay protocol for human serum provides a step forward for the application of this method for biomonitoring and to other matrices with high organic content, the TOP assay is still far from standardization and efforts should be made to better understand the effect of matrix on the oxidation process and evaluate the possible formation of other stable end-products and intermediates to make the assay fully quantitative. Additional investigations are also needed to understand the fate of CF_3-containing chemicals, like pharmaceuticals and agrochemicals, in the TOP assay.

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Total oxidizable precursors assay for PFAS in human serum

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a class of chemicals including over 4700 substances. As a limited number of PFAS is routinely analyzed in human serum, complementary analytical methods are required to characterize the overlooked fraction. A promising tool is the total oxidizable precursors (TOP) assay to look for precursors by oxidation to perfluoroalkyl acids (PFAA). The TOP assay was originally developed for large volumes of water and had to be adapted for 250 μL of human serum. Optimization of the method was performed on serum samples spiked with model precursors. Oxidative conditions similar to previous TOP assay methods were not sufficient for complete oxidation of model precursors. Prolonged heating time (24 h) and higher oxidant amount (95 mg of $Na_2S_2O_8$ per 225 μL of serum) were needed for complete conversion of the model precursors and accomplishing PFAA yields of 35–100 %. As some precursors are not fully converted to PFAA, the TOP assay can only provide semi-quantitative estimates of oxidizable precursors in human serum. However, the TOP assay can be used to give indications about the identity of unknown precursors by evaluating the oxidation products, including perfluoroalkyl sulfonic acids (PFSA) and perfluoroalkyl ether carboxylic acids (PFECA). The optimized TOP assay for human serum opens the possibility for high-throughput screening of human serum for undetected PFAA precursors.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals with hundreds of applications in industry and consumer products (Gluge et al., 2020; Kissa, 2001). PFAS have been extensively used because of the special properties, like high chemical and thermal stability, surfactant and water and oil repelling properties (Buck et al., 2012). Due to their widespread use and stability, PFAS are ubiquitous in the environment. Humans are easily exposed to these substances through food and drinking water consumption, dust ingestion, air inhalation and dermal contact (Poothong et al., 2020). Exposure to PFAS can result in adverse health effects, that have been observed both in toxicological and epidemiological studies (Fenton et al., 2021). For example, exposure to perfluorooctanoic acid (PFOA), one of the most studied PFAS, has been linked to kidney and testicular cancer (Barry et al., 2013; Shearer et al., 2021), pregnancy-induced hypertension (Darrow et al., 2013), ulcerative colitis (Steenland et al., 2013) and hypothyroidism (Lopez-Espinosa et al., 2012).

PFAS have been detected in humans since 2001 when PFOA, PFHxS, PFOS and FOSA were reported for the first time in human serum (Hansen et al., 2001). PFOS and PFOA have been listed under the Stockholm Convention on Persistent Organic Pollutants in 2009 and 2019, respectively (UNEP, 2009; UNEP, 2019). As a result of these restrictions and of the voluntary phase-out of PFOS and its precursors by their main manufacturer (3M) between 2000 and 2002, the production of PFAS shifted towards new structures and now over 4700 PFAS have been listed (Land et al., 2018; OECD, 2007). Despite the numerosity of PFAS, in most epidemiological studies only a limited number of these chemicals is analyzed, including the perfluoroalkyl acids (PFAA) and few other PFAS, like perfluorooctane sulfonamides (FOSA), fluorotelomer

Abbreviations: AMAP, Arctic Monitoring and Assessment Programme; FOSA, Perfluorooctane sulfonamide; FTCA, Fluorotelomer carboxylic acid; FTOH, Fluorotelomer alcohol; FTS, Fluorotelomer sulfonate; FTUCA, Fluorotelomer unsaturated carboxylic acid; HCl, Hydrochloric acid; ISTD, Internal standard; $K_2S_2O_8$, Potassium persulfate; LOD, Limit of detection; LOQ, Limit of quantification; MeOH, Methanol; MTBE, Tert-butyl methyl ether; $Na_2S_2O_8$, Sodium persulfate; NaOH, Sodium hydroxide; NH_4OAc, Ammonium acetate; PFAS, Per- and polyfluoroalkyl substances; PFCA, Perfluoroalkyl carboxylic acids; PFECA, Perfluoroalkyl ether carboxylic acids; PFSA, Perfluoroalkyl sulfonic acids; PFAA, Perfluoroalkyl acids; RSTD, Recovery standard; TOP, Total Oxidizable Precursors.

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sulfonates (FTS) and fluorotelomer alcohols (FTOH) (Sunderland et al., 2019; EFSA, 2020). Measuring only these compounds is not sufficient to describe the full extent of internal exposure to PFAS. In serum of Swedish women only 11–75 % of extractable organic fluorine could be explained by 17 target PFAS (Miaz et al., 2020). Complementary analytical tools are required to characterize the unaccounted fraction.

One promising tool is the total oxidizable precursors assay (TOP assay), that was developed to analyze oxidizable PFAA precursors in water (Houtz and Sedlak, 2012). Precursors are a group of chemicals that can be transformed to PFAA biotically and/or abiotically (Butt et al., 2014; Houtz and Sedlak, 2012; Nilsson et al., 2013; Zhang et al., 2021). The TOP assay allows to determine the presence of both known and unknown PFAA precursors by oxidizing them under controlled conditions to their end-products PFAA (Houtz and Sedlak, 2012). The PFAA are well known and easy to measure with routine methods, using instrumentation available to most analytical laboratories. By comparing PFAA concentrations before and after oxidation, the TOP assay allows to calculate the additional amount of PFAA formed by oxidation and to indicate the content of precursors with different chain length (Houtz and Sedlak, 2012). This approach has been successfully applied to detect PFAA precursors in wastewater (Houtz et al., 2016), groundwater (Houtz et al., 2013; Martin et al., 2019), surface water (Meng et al., 2021); stormwater (Chen et al., 2019), landfill leachate (Wang et al., 2020), soil (Janda et al., 2019), textiles (Zhu and Kannan, 2020), fire-fighting foams (Houtz et al., 2013), impregnation sprays (Sorli et al., 2022), insecticide formulations (Lasee et al., 2022), and biota (Gockener et al., 2020) but to our knowledge has not been applied to human serum before.

In this paper we describe the development of a modified version of the TOP assay for human serum. The aim of our study was to evaluate the applicability of the TOP assay to small volumes of human serum, the reaction conditions needed to ensure complete oxidation and the qualitative and quantitative information obtainable.

2. Materials and methods

2.1. Chemicals and consumables

Methanol (MeOH, LiChrosolv), *tert*-butyl methyl ether (MTBE, Suprasolv), fuming hydrochloric acid (HCl, p.a. 37 %) and sodium hydroxide (NaOH, EMSURE, ≥ 99.0 %) were obtained from Merck (Darmstadt, Germany). Potassium persulfate ($K_2S_2O_8$, trace metals basis, 99.99 %, lot #MKCH6998), sodium persulfate ($Na_2S_2O_8$, reagent grade, ≥ 98 %, lot #BCCC8760) and ammonium acetate (NH_4OAc, LiChropur) were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia (NH_3, solution 25 %, AnalaR NORMAPUR) was purchased from VWR (Fontenay-sous-Bois, France). All native and isotopically labelled PFAS standards were obtained from Wellington Laboratories Inc. (Guelph, Ontario, Canada).

2.2. Adaptation of the TOP assay protocol for human serum

The TOP assay protocol as published in the literature (Houtz and Sedlak, 2012) was optimized using human serum samples from the Arctic Monitoring And Assessment Programme (AMAP) Ring Test for Persistent Organic Pollutants (AMAP, 2021). As general steps in all experiments (Fig. 1), aliquots of 250 μL of serum were spiked with 20 μL of 0.5 ng/μL ^{13}C-PFAA mixture (containing C_4 to C_{14} ^{13}C-PFCA and C_6, C_8 ^{13}C-PFSA) as internal standard and vortexed. For the extraction, 500 μL of methanol were added and samples were sonicated 3 times for 10 min. Before each repetition samples were vortexed. Samples were centrifuged for 10 min at 10000 rpm and the supernatants were transferred to 2 mL glass vials. The extracts were split into two portions: the first aliquot (50 μL) was used for PFAS analysis before oxidation without any additional clean-up step and the second aliquot (450 μL) was treated for the TOP assay. Prior to oxidation, the TOP assay aliquots were evaporated to dryness to remove the methanol that would otherwise be the primary target for the oxidant instead of the precursors. Reagents were added to the dry residues. Potassium persulfate was added as solid by weight, while sodium persulfate was added in form of a 0.8 M solution (made of 7.6 g of $Na_2S_2O_8$ and MilliQ water so that the final volume was 40 mL). For sodium hydroxide, a 10 M solution in MilliQ water was used. The vials were tightly capped, vortexed, and subsequently heated in an oven at 85 °C for a certain time (as defined in Table 1). In a separate experiment shaking during the oxidation was shown to have no effect on conversion or yield (Figure S1). After oxidation, the samples were acidified with 50 μL of concentrated HCl (pH = 1–2 in each batch) and extracted with a liquid–liquid extraction with methyl *tert*-butyl ether (MTBE): 500 μL of MTBE were added to the samples, that were vortexed

Table 1
Oxidation conditions tested on human serum samples.

Parameters	Method			
	A	B	C	D
Heating time (hours)	8	24	8	24
10 M NaOH (μL)	20	20	40	120
MilliQ H_2O (μL)	100	100	200	–
$K_2S_2O_8$ (mg)	20	20	40	–
0.8 M $Na_2S_2O_8$(μL)	–	–	–	500
Model precursors (ng)	20	20	20	200*

* Tested also for serum spiked with 4 ng of 7:3 FTCA and 6:2 FTS + 10:2 FTS mix.

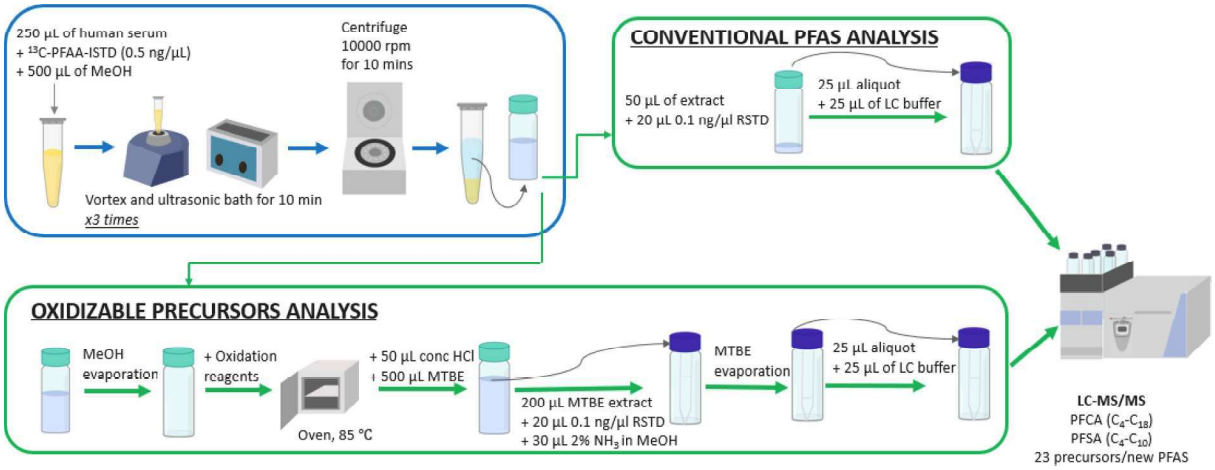

Fig. 1. Scheme of the TOP assay protocol for human serum.

and sonicated for 10 min. Subsequently, 200 μL of the organic phase were transferred to glass vials with insert and 30 μL of 2 % ammonia in methanol were added, followed by 20 μL of 0.1 ng/μL recovery standard in methanol. The vials were left uncapped for approximately 2 h to let the MTBE evaporate, until the residual volume was 50 μL.

2.3. Optimization of oxidation conditions

The oxidation conditions tested are summarized in Table 1.

Method A was the closest to those reported in the literature (Houtz and Sedlak, 2012; Janda et al., 2019; Martin et al., 2019; Zhang et al., 2019). In method B the reaction time was increased from 8 to 24 h and in method C the amount of $K_2S_2O_8$ was doubled to 40 mg. In method D the amount of oxidant was further increased to 100 mg. As an additional new aspect in method D, we also switched from using neat $K_2S_2O_8$ to adding 500 μL of 0.8 M $Na_2S_2O_8$ solution in MilliQ water. $Na_2S_2O_8$ has higher water-solubility than $K_2S_2O_8$ and allows for the preparation of higher concentrated solutions that can be easily added to the reaction vial and ensure good intermixing with the sample. The same molar concentration of $K_2S_2O_8$ and $Na_2S_2O_8$ in the reaction solution gave the same oxidation results (Figure S2). For methods A, B and C, serum samples were spiked with 20 ng of precursors. In method D serum samples were spiked with 10 times higher concentrations (200 ng of precursors). However, to also cover lower concentration, closer to real life PFAS serum concentrations, method D was also tested on serum samples spiked with 4 ng of 7:3 FTCA and 6:2 FTS + 10:2 FTS.

2.4. Model precursors

The method was tested on a selection of fluorotelomer compounds and two perfluoroalkyl ether carboxylic acids (PFECA). Some chemicals were spiked as single compound solutions, while others were spiked as a mixture of two compounds to represent both short and long fluorinated carbon chains. In Table S2 the list of model precursors is provided.

2.5. Instrumental analysis

Extracts before and after the oxidation were analyzed using ultrahigh pressure liquid chromatography triple-quadrupole mass-spectrometry (UHPLC-MS/MS) using the method described by Hanssen et al. (Hanssen et al., 2013). The instrument was a quaternary Accela 1250 pump (Thermo Fisher Scientific, Waltham, MA, USA) with a PAL Sample Manager (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Vantage TSQ MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). The MS method was modified to include the model substances used for the method testing and perfluoro alkyl ether carboxylic acids. The list of compounds measured, including the internal standards used for the quantification and the monitored mass transitions can be found in Table S3 of the Supporting Material. For the analysis before and after oxidation, 25 μL of the extracts were mixed with 25 μL of 2 mM NH_4OAc in MeOH. For each sample (before and after oxidation) 10 μL were injected two times, once for PFCA and PFSA determination and once for model precursors and PFECA analysis. The analytes were quantified using the software LC Quan (v.2.6, Thermo Fisher Scientific, Waltham, MA, USA).

2.6. QA/Qc

For each oxidation test, triplicate method blanks were collected and analyzed before and after oxidation to evaluate possible contamination issues. LODs were calculated as the average concentration in the blanks plus 3 times the standard deviation of the blanks, and LOQs as the average concentration in the blanks plus 10 times the standard deviation of the blanks. In case of no detection in the blanks, LODs and LOQs were calculated by multiplying the noise of the blanks by 3 and 10, respectively. Each test was performed in triplicate for all the model precursors to assess the reproducibility of the method. The accuracy of target PFAS analyses was evaluated by comparing the measured concentrations before oxidation to the concentrations declared in the AMAP Ring Test report for PFHxA, PFOA, PFNA, PFUnDA, PFHxS, PFOS (sum of branched and linear isomers). Recoveries of target PFAA were evaluated using labelled standards and the recovery of model precursors was evaluated by comparing the measured concentrations before oxidation to the theoretical spiked amount. To confirm the stability of PFAA under the final oxidation conditions, 10 human serum samples were oxidized in duplicate: one replicate was spiked with the PFAA internal standard mixture before the oxidation, while the second one was spiked after oxidation and prior to the liquid–liquid extraction with MTBE. Both aliquots were spiked after MeOH extraction to eliminate the influence of this step on the recoveries.

3. Results and discussion

The original TOP assay was developed for large volumes of water and had to be adapted to be applied to small aliquots of human serum. We tested oxidative conditions similar to the ones previously reported in the literature as well as increasing amounts of oxidant and heating time to achieve higher reaction yields. The method was tested on fluorotelomer compounds of different chain length and with different functional groups as well as on GenX and ADONA.

3.1. Optimization of oxidation method

Chemically, human serum is mainly water (>90 %) with proteins (>5 %), as well as electrolytes, hormones, etc.; and exogenous substances with a normal pH of 7.4 (Barrett et al., 2010) To assure for complete oxidation of precursors to target PFAA we had to use excess of oxidant for all oxidizable matter in the sample. An elementary calculation shows that stochiometric oxidation of 1 mol of carbon would require 540 g of potassium persulfate (2 mol), or 45 mg per 1 mg of carbon. A hydrocarbon with brutto-formula CH_2 would require 810 g of potassium persulfate per 14 g of substance, or 58 mg per mg of substrate. Typical lipids, cholesterol and tristearin would require 53 mg and 43 mg per mg, respectively. Oxidation of 1 mg of serum albumin would require from 25 mg (if all nitrogen gets converted to ammonia) to 45 mg (if all nitrogen gets converted to nitrate) of the oxidant. Carbohydrates (CH_2O) would require just 18 mg of the oxidant per mg of substrate. One should evaluate amount and composition of their samples and calculate the required amount of an oxidant to determine the starting point in the TOP assay development for samples of specific kind.

In our case, the residue from evaporation of methanol from serum extracts was merely visible, we judged it was less than 1 mg and we began testing from 20 mg of the oxidant per sample. In each run, the completeness of oxidation was evaluated using the percentage of conversion of spiked precursors (i.e., the disappearance of the precursor) and the yield of products (i.e., the production of PFAA) as described in the Supplementary Material (Equation S1 and S2). The results are presented in Table 2 except for GenX (stable to oxidation).

Oxidation test A showed that conditions similar to the ones commonly used in previous TOP assay studies (Houtz and Sedlak, 2012; Janda et al., 2019; Martin et al., 2019; Zhang et al., 2019) were not sufficient for complete oxidation of any of the precursors tested. Complete conversion was observed only for the fluorotelomer carboxylic acids with 2 non-fluorinated carbons (6:2 FTCA, 10:2 FTCA and 6:2 FTUCA), independently of the saturation status of the carbon chain. All the remaining model precursors showed incomplete conversion. For 7:3 FTCA, that has one additional non-fluorinated carbon compared to the other fluorotelomer carboxylic acids tested, conversion reached only 52 %. The fluorotelomer sulfonates (6:2 FTS and 10:2 FTS) were also only partially converted and were less reactive compared to the fluorotelomer carboxylic acids with same number of fluorinated carbons. Correlation between the reactivity and calculated bond dissociation

Table 2
Conversion of model precursors and yield of products in human serum with TOP assay method A, B, C and D (all values are reported in percentages).

Test ID	Conversion	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFOS linear	1,3 -PFECA	Total yield
7:3 FTCA (n = 7)		**n-4**	**n-3**	**n-2**	**n-1**	**n**	–	–	–	–	–	
A	52 ± 3	4.1 ± 0.5	2.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.2	1.0 ± 0.2	0	0	0	0	0	19 ± 1
B	49 ± 5	3.3 ± 0.7	3.2 ± 0.8	6 ± 1	5.2 ± 0.8	1.0 ± 0.4	0	0	0	0	0	19 ± 4
C	81 ± 9	3.9 ± 0.9	5 ± 2	12 ± 3	9 ± 2	2.3 ± 0.4	0	0	0	0	0	33 ± 8
D	100 ± 0	13.5 ± 0.2	17.5 ± 0.3	25.4 ± 0.5	13.0 ± 0.4	1.2 ± 0.1	0	0	0	0	0	71 ± 1
6:2 FTUCA (n = 6)		**n-3**	**n-2**	**n-1**	**n**	–	–	–	–	–	–	
A	100 ± 0	4.8 ± 0.8	4.6 ± 0.8	11 ± 1	0	0	0	0	0	0	0	20 ± 2
B	100 ± 0	5 ± 1	4 ± 2	10 ± 1	0	0	0	0	0	0	0	19 ± 4
C	100 ± 0	4.7 ± 0.5	5 ± 1	11 ± 2	0	0	0	0	0	0	0	20 ± 3
D	100 ± 0	8.9 ± 0.1	7.6 ± 0.1	18.5 ± 0.6	0	0	0	0	0	0	0	35 ± 1
6:2 FTCA (1) and 10:2 FTCA (2) mix (n_1 = 6, n_2 = 10)		**n_1-3 n_2-7**	**n_1-2 n_2-6**	**n_1-1 n_2-5**	**n_1 n_2-4**	**- n_2-3**	**- n_2-2**	**- n_2-1**	**- n_2**	– -	– -	
A	1. 100 ± 0 2. 100 ± 0	5.4 ± 0.4	5.3 ± 0.3	12 ± 1	1.6 ± 0.1	3.1 ± 0.3	4.1 ± 0.3	1.7 ± 0.1	0	0	0	33 ± 2
B	1. 100 ± 0 2. 100 ± 0	8 ± 1	8.0 ± 0.8	18 ± 3	1.8 ± 0.2	3.8 ± 0.9	3.9 ± 0.3	2.7 ± 0.3	0	0	0	46 ± 6
C	1. 100 ± 0 2. 100 ± 0	7.5 ± 0.6	8 ± 1	17 ± 3	3 ± 1	5 ± 1	5 ± 1	3 ± 1	0	0	0	49 ± 7
D	1. 100 ± 0 2. 100 ± 0	9.5 ± 0.1	8.8 ± 0.2	20.4 ± 0.4	3.9 ± 0.1	7.5 ± 0.1	7.2 ± 0.1	3.4 ± 0.1	0	0	0	61 ± 1
6:2 FTS (1) and 10:2 FTS (2) mix (n_1 = 6, n_2 = 10)		**n_1-3 n_2-7**	**n_1-2 n_2-6**	**n_1-1 n_2-5**	**n_1 n_2-4**	**- n_2-3**	**- n_2-2**	**- n_2-1**	**- n_2**	**- n_2-2**	– -	
A	1. 62 ± 4 2. 45 ± 4	1.6 ± 0.3	1.5 ± 0.4	3.1 ± 0.4	0.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0	0	0	7 ± 1
B	1. 85 ± 3 2. 73 ± 1	1.7 ± 0.3	2 ± 1	3 ± 2	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0	0	0	8 ± 3
C	1. 95 ± 4 2. 79 ± 6	1.4 ± 0.9	3 ± 1	6 ± 1	1 ± 1	0.5 ± 0.1	0.7 ± 0.4	1.7 ± 0.6	2 ± 1	0	0	16 ± 5
D	1. 100 ± 0 2. 91 ± 1	7.2 ± 0.1	11.3 ± 0.6	10.4 ± 0.6	1.3 ± 0.1	3.3 ± 0.8	6.3 ± 0.4	8.6 ± 0.2	1.1 ± 0.3	0	0	50 ± 2
Me-FOSAA (1) and Et-FOSAA (2) ($n_1 = n_2 = n = 8$)		**n-5**	**n-4**	**n-3**	**n-2**	**n-1**	**n**	–	–	**n**	–	
A	1. 23 ± 3 2. 28 ± 3	1.5 ± 0.4	0.4 ± 0.3	1.1 ± 0.7	0.6 ± 0.2	4 ± 1	0	0	0	4.8 ± 0.5	0	13 ± 3
B	1. 46 ± 5 2. 43 ± 4	1.0 ± 0.4	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	6 ± 2	0	0	0	4.5 ± 0.2	0	13 ± 2
C	1. 79 ± 9 2. 75 ± 8	0.5 ± 0.1	0.6 ± 0.2	2 ± 1	3 ± 2	33 ± 2	0	0	0	8.9 ± 0.5	0	48 ± 4
D	1. 100 ± 0 2. 100 ± 0	1.0 ± 0.1	1.1 ± 0.1	2.2 ± 0.1	3.3 ± 0.1	74 ± 2	0	0	0	17.8 ± 0.8	0	99 ± 3
ADONA		–	–	–	–	–	–	–	–	–	–	
A	66 ± 3	0	0	0	0	0	0	0	0	0	61 ± 9	61 ± 9
B	76 ± 6	0	0	0	0	0	0	0	0	0	80 ± 18	80 ± 18
C	81 ± 5	0	0	0	0	0	0	0	0	0	83 ± 12	83 ± 12
D	100	0	0	0	0	0	0	0	0	0	130 ± 15*	130 ± 15*

n, n1, n2 = number of perfluorinated carbons in the precursor's structure.
All reported values are based on triplicate experiments.
* Higher than 100 % apparent yield for 1,3-PFECA likely results from analytical uncertainty. There was no good internal standard for this compound. Accordingly, apparent yields of 13-PFECA by methods A, B, C can somewhat lower as well.

energies for fluorotelomer carboxylic acids and sulfonates has been observed by Liu et al. (Liu et al., 2021). Further, the 10:2 FTS was more recalcitrant to oxidation compared to 6:2 FTS and this is also consistent with previous fluorotelomer oxidation experiments that showed lower reactivity for longer fluorotelomers (Liu et al., 2021). The two sulfonamidoacetic acids tested showed low conversion but similar reactivity, independently from the methyl or ethyl substitution (conversion of 23 % for Me-FOSAA and 28 % for Et-FOSAA). GenX was stable during the reaction, while ADONA concentrations decreased by 66 % after oxidation (Table 2).

However, independently from the completeness of the precursor's conversion, a 100 % yield of PFAA was never observed in method A (Table 2, Fig. 2). No increase in PFAA concentrations was observed for GenX and ADONA. However, while GenX was not affected at all by the oxidation process, ADONA showed formation of perfluoro-3-methoxypropanoic acid (1, 3-PFECA) as end product (Figure S4).

Incomplete oxidation under similar conditions has also been observed for precursors in laying hens' eggs and biosolids and could be due to the presence of other organic molecules consuming the oxidant and interfering with the oxidation process (Casson and Chiang, 2018; Gockener et al., 2020; Hutchinson et al., 2020). To prevent the scavenging of oxidant within the sample, two different approaches are described in literature. A direct TOP assay is suggested as an option, by oxidizing small amounts of sample without any extraction using a large excess of oxidant to also break down all the matrix components (Gockener et al., 2020, 2021). A second approach consists of the use of a hydrogen peroxide pretreatment prior to extraction and oxidation, not suitable for small volumes of serum, since it would involve an additional dilution step (Hutchinson et al., 2020).

In our case, oxidant scavenging components of human serum samples can, beside other matrix compounds, consist of either proteins or the methanol used for extraction of the samples. Proteins are removed by denaturation during the methanol extraction, while the methanol is removed prior to the TOP assay by evaporation. Methanol was chosen as extraction solvent instead of acetonitrile both to make this evaporation step faster and to be able to measure GenX, that is not stable in acetonitrile (Liberatore et al., 2020; Zhang et al., 2022). Any residual serum related compounds able to scavenge the persulfate have to be oxidized by the use of excess amounts of a suitable oxidant and harsh conditions.

To ensure that complete oxidation was accomplished, we increased the heating time and the amount of oxidant added to human serum extracts in method B and C, respectively. By extending the time at 85 °C in method B from 8 to 24 h, it was possible to increase conversion and/or yield of products for 6:2 FTCA, 10:2 FTCA, 6:2 FTS, 10:2 FTS, Me-FOSAA and Et-FOSAA. No improvement was observed for 7:3 FTCA and 6:2 FTUCA. Doubling the amount of $K_2S_2O_8$ in method C showed an improvement for all tested precursors, except 6:2 FTUCA, that showed constant low yields of products. Even under these harsher conditions, GenX concentrations were unchanged after oxidization and this compound was not further tested, as its stability in the TOP assay has been reported independently (Zhang et al., 2019). In general, the effect of increasing the amount of the oxidant was larger than the improvement observed by increasing the heating time.

To follow up on this, a further increase of oxidant amount was tested under heating time of 24 h (method D).

With method D, all but one precursor, the 10:2 FTS, were fully converted. Conversion of 10:2 FTS was 91 %. The yield of the oxidation end products, the PFAA, reached 100 % only for the sulfonamidoacetic acids, resulting in the TOP assay being fully quantitative for these precursors in human serum. For all the other precursors the transformation to PFAA was not complete, but product yields above 50 % were achieved. The only precursor showing a lower PFAA yield of 35 % was 6:2 FTUCA (Fig. 2, Table 2).

To test the final conditions of method D on lower precursors concentrations, the procedure was repeated on samples spiked with 4 ng of 7:3 FTCA and 6:2 FTS and 10:2 FTS mix. These experiments showed that the oxidation process was independent of the starting precursors concentration and yields of PFAA stayed the same (Figure S3).

Further increase of amount of oxidant would lead to scale-up of the experiment (larger glassware etc.), or sample downsize. Average yield of the products was circa 2/3, and we judged it satisfactory for high-throughput screening of human serum.

3.2. Oxidation products patterns

After optimization of the oxidation process, the TOP assay for human serum performed with routine PFAA analyses was still not fully quantitative for most of the model precursors. Despite this limitation, the evaluation of the oxidation products for the selected model substances can give interesting insights for the interpretation of TOP assay experiments in human serum and the identity of the respective precursors present.

For the fluorotelomer carboxylic acids in human serum with method D, mixtures of PFCA were observed (Fig. 3, Table 2). For 6:2 FTCA, 10:2

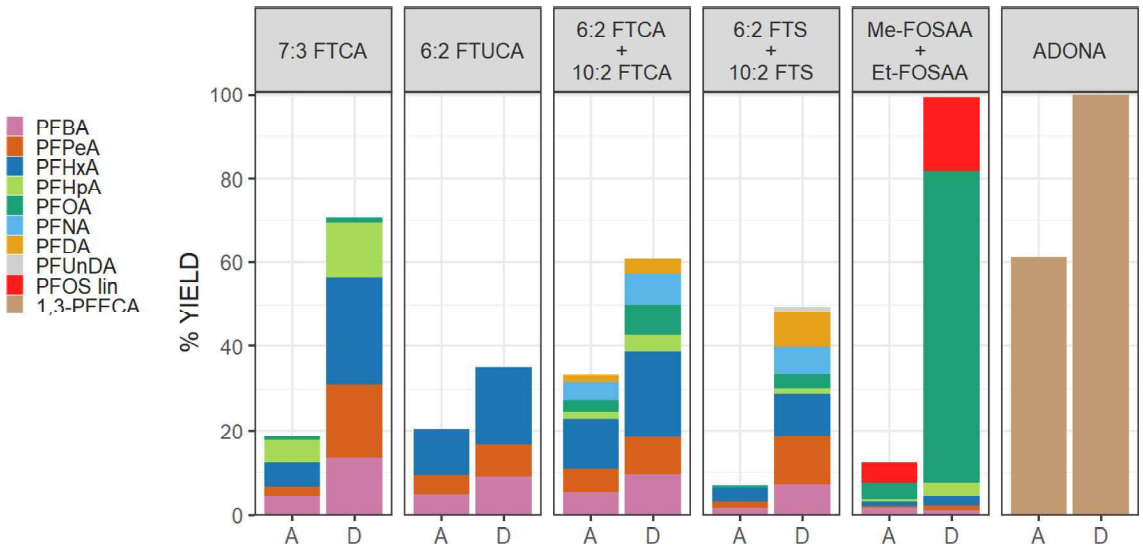

Fig. 2. Yield of oxidation products from the model precursors in the initial test method (method A) and in the optimized method for TOP in human serum (method D).

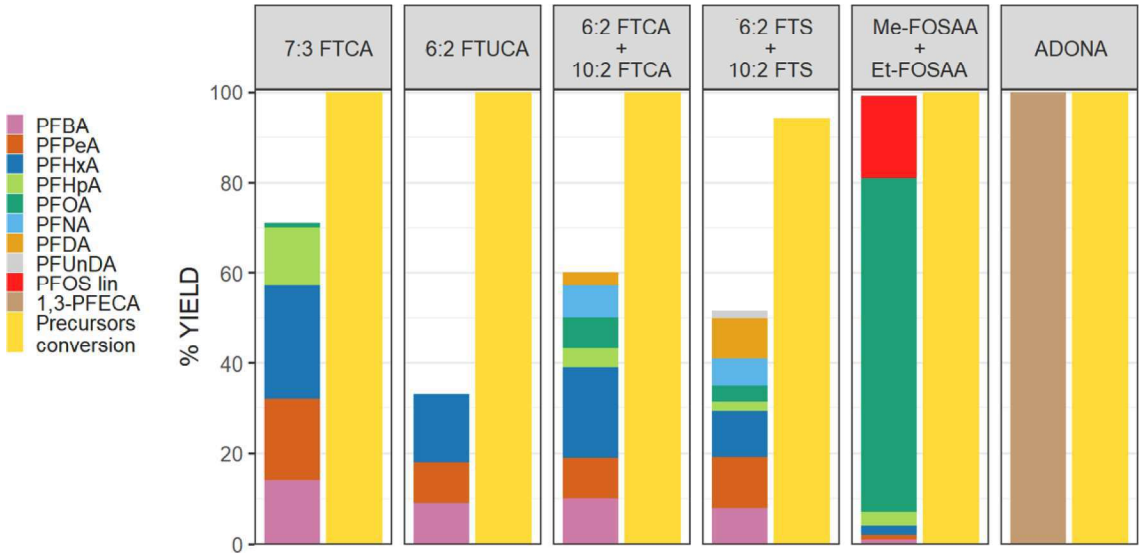

Fig. 3. Yield of products (first bar) and conversion of precursors (second bar) for the model precursors in the optimized method for TOP in human serum (method D).

FTCA and 6:2 FTUCA, PFCA with n-1 fluorinated carbons (where n is referring to the number of fluorinated carbons in the precursor as in Table 2) and shorter carbon chains were detected after oxidation, while for 7:3 FTCA the formation of a small percentage of PFOA (n = 7) was also observed. The dominant product of 7:3 FTCA was the n-2 PFCA, while for fluorotelomer carboxylic acids with 2 non-fluorinated carbon atoms the dominant product was the n-1 PFCA (Fig. 3). Similar PFCA patterns for 7:3 FTCA and 6:2 FTUCA have been observed in ultrapure water by Martin et al. (Table S4), but in this case also PFPrA was included, showing that the ultra-short PFAA can also be relevant oxidation products (Martin et al., 2019). For example, the PFPrA accounted for 21 % of the oxidation yield for 6:2 FTUCA and for 12 % of the yield for 7:3 FTCA in ultrapure water (Martin et al., 2019).

In the case of the fluorotelomer sulfonates 6:2 FTS and 10:2 FTS, a mixture of PFCA was also observed after oxidation. The longest PFCA formed was PFUnDA, that has the same number of fluorinated carbons of 10:2 FTS, and the dominant products were the n-1 and n-2 PFCA (Fig. 3, Table 2). Higher yields were reported in the literature for all products (Table S4), even if also in these studies the total PFAA yields did not reach 100 % for 6:2 FTS (73 % Houtz et al. (Houtz and Sedlak, 2012) and 87 % Martin et al. (Martin et al., 2019)). Similar to the fluorotelomer carboxylic acids, the lower yields could be due to the formation of TFA and PFPrA, not assessed in this study. The contribution of PFPrA and TFA can be small for long chain fluorotelomer sulfonates but can be relevant for short chained precursors. In ultrapure water Martin et al. reported PFPrA yields of 23 % and 35 % for 6:2 FTS and 4:2 FTS, respectively (Martin et al., 2019).

The inclusion of TFA and PFPrA to the target PFAS analyses list for the TOP assay has been proven to be beneficial also for other precursors (Janda et al., 2019; Martin et al., 2019) and it is an essential step to make the assay fully quantitative in any matrix, especially when short PFAA precursors are present (Meng et al., 2021; Wang et al., 2020). However, the formation of intermediate and additional stable oxidation products should also be considered. As it can be observed for Me-FOSAA and Et-FOSAA, full oxidation was observed under the final TOP assay conditions, but in method A, B and C, FOSA, Me-FOSA and Et-FOSA were identified as intermediates of the oxidative treatment (Fig. 4). These intermediates have been observed in hydroxyl radical oxidation

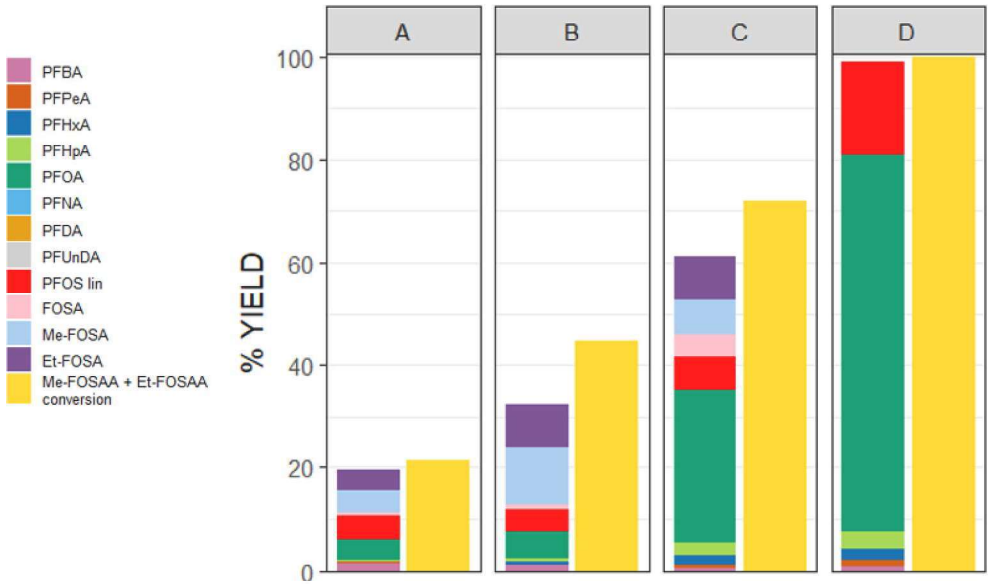

Fig. 4. Yield of products (first bar) and conversion of precursors (second bar) for Me-FOSAA and Et-FOSAA in human serum with method A, B, C and D.

experiments before (Plumlee et al., 2009) and their detection in our tests highlights the possible formation of unknown intermediates in the TOP assay.

Our testing on Me-FOSAA and Et-FOSAA also showed the importance of considering the possible formation of stable end products, other than PFCA. In the original TOP assay, Me-FOSAA and Et-FOSAA were quantitatively converted to PFOA (Houtz and Sedlak, 2012). This was not the case in our experiments, where PFOA was still the dominant product, but shorter chain PFCA accounted for 8 % of the yield and, interestingly, PFOS was the second dominant product accounting for 18 % of the yield (Fig. 4). The formation of PFCA shorter than PFOA has also been observed by Göckener et al. (Gockener et al., 2020) for Et-FOSAA and by Martin et al. (Martin et al., 2019) that observed the formation of PFHpA from Me-FOSAA and Et-FOSAA (Table S4). While Martin et al. used the original TOP assay method in ultrapure water, Göckener et al. used a modified direct TOP assay with increased amount of oxidant on eggs. A possible explanation for these differences in products could be the use of different oxidation conditions as well as the application of the method to different matrices. The formation of PFOS or any other perfluorinated sulfonate by the TOP assay has earlier been disregarded, reporting only PFCA as oxidation products (Gockener et al., 2022; Lazcano et al., 2020; Simonnet-Laprade et al., 2019; Sivaram et al., 2022). In one application of the TOP assay to suspended particulate matter, the PFOS increase after oxidation was attributed to the release of non-extractable PFOS during the oxidation, because precursor conversion to PFSA in the TOP assay had not been described before (Gockener et al., 2022). A report from an interlaboratory study of the TOP assay suggests that the formation of PFSA could be due to base-catalyzed hydrolysis of sulfonamides (Ventia Utility Services Ptw Ltd, 2019). These findings together with the results from our experiments show that PFSA can also be relevant end products in the TOP assay. Therefore, we recommend the inclusion of PFSA to the target PFAS portfolio after oxidation, as these could also be end products of additional known or unknown precursors that have not been tested before.

In addition to the PFSA inclusion to the target PFAS analyses after oxidation, other stable end products besides PFCA should be considered. For example, ADONA is not stable in the oxidation and is fully converted to 1,3-PFECA (Table 2 and Figure S4), showing that the TOP assay can also be used to detect oxidizable precursors with ether groups by including stable PFECA among the PFAS analyses portfolio (Zhang et al., 2019).

3.3. Method evaluation

Low levels of PFAA were detected in the blanks before and after oxidation (Table S5). LODs and LOQs before and after oxidation were comparable for most compounds. Variation in LODs ranging from 0.02 to 0.07 ng/mL and in LOQs ranging from 0.02 to 0.18 ng/mL were observed for PFNA, PFDA, PFUnDA and PFDoDA, because these compounds were not detected in the blanks before oxidation but were present in low levels (0.02–0.03 ng/mL) after oxidation.

The method showed good repeatability and accuracy. Relative standard deviations both before and after oxidation were always below 20 % for all detected PFAS (Table S6 and S7). Measured concentrations before oxidation were in good agreement with the ones reported by AMAP (deviations ranging from 2 to 24 %), even with no clean-up step was included after the MeOH extraction (Table S8).

Recoveries were satisfactory for all the available internal standards, with an average of 73 % (ranging from 52 to 92 %) before the oxidation and an average of 60 % (ranging from 41 to 75 %) after the oxidation (Table S9). Recoveries after the oxidation were lower than those before the oxidation due to the additional MTBE extraction step needed after the TOP assay. This was confirmed by a PFAA stability test performed, using parallel human serum samples spiked with the internal standard either before or after the oxidation step. No significant drop in concentrations of labelled PFAA were observed, evidencing that the oxidation step does not affect the present PFAA (Figure S5).

Recoveries for model precursors were comparable to the recoveries for PFAA, ranging from 64 to 107 % (Table S10). However, we only tested a limited set of precursors, and our extraction protocol was not tested, for example, for zwitterionic and neutral precursors (Nickerson et al., 2020).

3.4. TOP assay for human serum strengths and limitations

The here presented TOP assay method allows for the processing of small volumes of a large series of samples in a short time, opening for the possibility of high-throughput screening of human serum for otherwise undetected PFAA precursors. The method can potentially be applied (using the aforementioned guidelines regarding oxidant/substrate ratio) to other valuable biological samples, like extracts from tissues and serum from other species or whole human blood, even if in this case the oxidation of precursors might be more difficult due to the presence of red cells in the sample.

By using only one extract of a small volume of human serum, conventional PFAS and oxidizable precursors can be measured without the need of additional instrumentation, analytical methodology or standards in a time efficient manner. The TOP assay application on human serum can provide both qualitative and semi-quantitative information about the presence of unknown oxidizable PFAA precursors.

In most of the cases, the structural identification of precursors from the PFAS formed during the TOP assay will not be possible. As it was shown in our study, many precursors produce mixtures of PFAA, and mixtures of precursors would produce even more complex mixtures of PFAA. However, even if the exact precursors' identity is lost by oxidation, the reaction product pattern can still give indications about some of the precursors' structural features, like the length of the fluorinated chain length (e.g., for precursors with 7 perfluorinated carbons, like 7:3 FTCA, PFAA longer that PFOA will never be observed) or the presence of specific functional groups (e.g., PFOS is observed for Me-FOSAA and Et-FOSAA but PFSA are not observed for precursors that do not contain sulfonic groups). The inclusion of PFSA and PFECA as target analytes in the TOP assay can potentially provide more information to provisionally identify a precursor but additional techniques, as for example the use of hydrolysis of precursors (Nikiforov, 2021) or suspect and non-target screening tools, will be needed to identify the precursors.

The determined change in PFAA concentrations can be used to give an estimate of the total oxidizable precursors present in human serum. As some precursors are not fully converted to PFAA, the TOP assay can only provide a lower bound estimate of oxidizable precursors. In addition, the total amount of precursors present might also be underestimated due to potential losses of precursors that are either non-extractable with methanol or volatile and semi-volatile evaporating during the methanol evaporation step needed prior to oxidation. The addition of PFSA and stable PFECA to the analytes after oxidation can help provide better estimates of the total amount of oxidizable precursors in human serum and they are recommended to always be included in the post-TOP assay PFAS analyses. However, it is of utmost importance to fully comprehend, that the TOP assay can yield only semi-quantitative estimates since the nature of precursors in the sample is *a priori* unknown.

To conclude, the TOP assay can be used to reveal human exposure to unknown oxidizable PFAA precursors. To fully describe human exposure to potentially harmful PFAA, it is important to understand the contribution of their precursors as indirect exposure source. The TOP assay does not necessarily reproduce the metabolism of precursors in human blood but can point out the presence of additional fluorinated organic substances with the potential to form PFAA. The application of the TOP assay to human serum can shed further light into yet unknown oxidizable PFAA precursors in humans, adding insights into the holistic assessment of human exposure to PFAS.

4. Notes

A preprint version of this article is available on ChemRxiv (Cioni et al., 2022).

CRediT authorship contribution statement

Lara Cioni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Vladimir Nikiforov:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Ana Carolina M.F. Coêlho:** Investigation, Writing – review & editing. **Torkjel M. Sandanger:** Writing – review & editing, Supervision, Funding acquisition. **Dorte Herzke:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107656.

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Supplementary Material

Total Oxidizable Precursors Assay for PFAS in Human Serum

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1. Material and methods

Each oxidation test was evaluated for conversion of precursor (Equation S1) and the yield of products (Equation S2). [p] and [P] are the molar concentrations of precursors and products respectively, while the subscripts *bo* and *ao* stand for before and after oxidation.

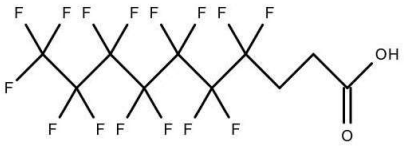
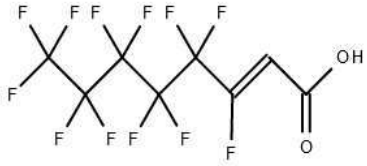
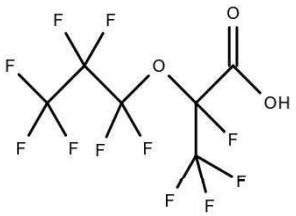
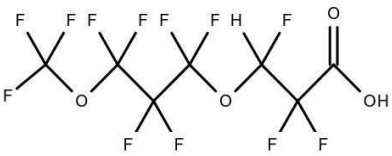
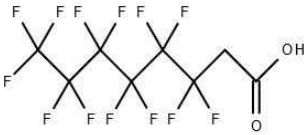
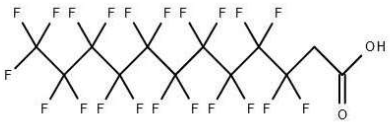
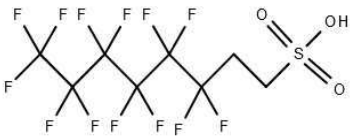
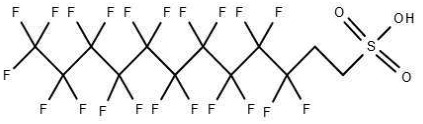
$$Precursor\ conversion\ (\%) = \frac{[p]_{bo} - [p]_{ao}}{[p]_{bo}} \cdot 100 \quad \text{(S1)}$$

$$Product\ yield\ (\%) = \sum_{i=1}^{n} \left(\frac{[P_i]_{ao} - [P_i]_{bo}}{[p]_{bo}} \right) \cdot 100 \quad \text{(S2)}$$

Table S1 – Table of abbreviations.

Abbreviation	Definition
1,3-PFECA	Perfluoro-3-methoxypropanoic acid
AMAP	Arctic Monitoring and Assessment Programme
FOSA	Perfluorooctane sulfonamide
FTCA	Fluorotelomer carboxylic acid
FTOH	Fluorotelomer alcohols
FTS	Fluorotelomer sulfonate
FTUCA	Fluorotelomer unsaturated carboxylic acid
HCl	Hydrochloric acid
ISTD	Internal standard
$K_2S_2O_8$	Potassium persulfate
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MTBE	Tert-butyl methyl ether
$Na_2S_2O_8$	Sodium persulfate
NaOH	Sodium hydroxide
NH_4OAc	Ammonium acetate
PFAS	Per- and polyfluoroalkyl substances
PFDoDA	Perflurododecanoic acid
PFECA	Perfluoroalkyl ether carboxylic acids
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFSA	Perfluoroalkyl sulfonic acids
PFUnDA	Perfluoroundecanoic acid
PFAA	Perfluoroalkyl acids
RSTD	Recovery standard
TOP	Total Oxidizable Precursors

Table S2 - Model precursors used for the TOPA experiments.

Name	Abbreviation	CAS number	Chemical structure
Single compounds			
2H,2H,3H,3H-perfluoro-decanoic acid	7:3 FTCA	812-70-4	
2H-perfluoro-2-octenoic acid	6:2 FTUCA	70887-88-6	
Perfluoro-2-methyl-3-oxahexanoic acid	GenX	13252-13-6	
3H-perfluoro-4,8-dioxanonanoic acid	ADONA	958445-44-8	
Mixtures			
2H,2H-perfluoro octanoic acid	6:2 FTCA	53826-12-3	
2H,2H -perfluoro decanoic acid	10:2 FTCA	53826-13-4	
1H,1H,2H,2H-perfluoro octanesulfonic acid	6:2 FTS	27619-97-2	
1H,1H,2H,2H-perfluoro dodecane sulfonate	10:2 FTS	120226-60-0	

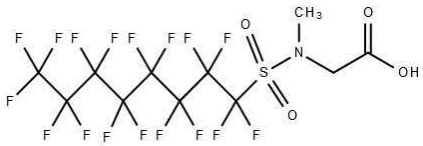
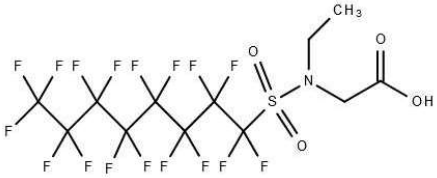
N-methyl-perfluoro-1-octanesulfonamidoacetic acid	Me-FOSAA	2355-31-9	
N-ethyl-perfluoro-1-octanesulfonamidoacetic acid	Et-FOSAA	2991-50-6	

Table S3- Target analytes, respective internal standards (used for the quantification) and MS-MS transitions.

Analyte	ISTD	Mass transitions
PFBA	^{13}C-PFBA	213 → 169
PFPeA	^{13}C-PFPeA	263 → 219
PFHxA	^{13}C-PFHxA	313 → 269 313 → 119
PFHpA	^{13}C-PFHpA	363 → 319 363 → 169
PFOA	^{13}C-PFOA	413 → 369 413 →169
PFNA	^{13}C-PFNA	463 → 419 463 → 219
PFDA	^{13}C-PFDA	513 → 469 513 → 269
PFUnDA	^{13}C-PFUnDA	563 → 519 563 → 269
PFDoDA	^{13}C-PFDoDA	613 → 569 613 → 169
PFTrDA	^{13}C-PFDoDA	663 → 619 663 → 169
PFTeDA	^{13}C-PFTrDA	713 → 669 713 → 169
PFBS	^{13}C-PFHxS	299 → 80
PFPS	^{13}C-PFHxS	349 → 80
PFHxS	^{13}C-PFHxS	399 → 80 399 → 99
PFHpS	^{13}C-PFHxS	449 → 99 449 → 80
PFOS	^{13}C-PFOS	499 → 80 499 → 99
PFNS	^{13}C-PFOS	549 → 80 549 → 99
PFDS	^{13}C-PFOS	599 → 80 599 → 99

FOSA	^{13}C-PFOS	498 → 78 498 → 498
Me-FOSA	^{13}C-PFOS	512 → 219 512 → 169
Et-FOSA	^{13}C-PFOS	526 → 219 526 → 219
FOSAA	^{13}C-PFOS	556 → 498 556 → 419
Me-FOSAA	^{13}C-PFOS	570 → 419 570 → 483
Et-FOSAA	^{13}C-PFOS	584 → 419 584 → 526
6:2 FTS	^{13}C-PFOS	427 → 80 427 → 407
10:2 FTS	^{13}C-PFOS	627 → 80 627 → 607
6:2 FTCA	^{13}C-PFOA	377 → 293 377 → 313
10:2 FTCA	^{13}C-PFOA	577 → 493 577 → 514
7:3 FTCA	^{13}C-PFOA	441 → 317 441 → 337
6:2 FTUCA	^{13}C-PFOA	357 → 243 357 → 357
GenX	^{13}C-PFOA	285 → 169 285 → 185
ADONA	^{13}C-PFOA	251 → 85 251→ 251
1,3-PFECA	^{13}C-PFOA	229 → 85 229 → 229

2. Results and discussion

Table S4 – Yield of products from this study (method D) and from the literature.

	Study	PFPrA	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFOS
7:3 FTCA	Martin et al., 2019	12 ± 1	15 ± 1	18 ± 2	36 ± 3	8 ± 1	3 ± 1	0	0	0	0
	This study	-	13.5 ± 0.2	17.5 ± 0.3	25.4 ± 0.5	13.0 ± 0.4	1.2 ± 0.1	0	0	0	0
6:2 FTUCA	Martin et al., 2019	21 ± 2	21 ± 1	17 ± 1	31 ± 1	0	0	0	0	0	0
	This study	-	8.9 ± 0.1	7.6 ± 0.1	18.5 ± 0.6	0	0	0	0	0	0
6.2 FTS	Houtz et al., 2012	-	22 ± 5	27 ± 2	22 ± 2	2 ± 1	0	0	0	0	0
	Martin et al., 2019	23 ± 2	21 ± 1	24 ± 1	17 ± 1	2 ± 0.1	0	0	0	0	0
10:2 FTS	Martin et al., 2019	1 ± 1	2 ± 1	3 ± 1	6 ± 2	14 ± 3	16 ± 1	29 ± 6	28 ± 8	3 ± 2	0
6:2 FTS + 10:2 FTS	This study	-	7.2 ± 0.1	11.3 ± 0.6	10.4 ± 0.6	1.3 ± 0.1	3.3 ± 0.8	6.3 ± 0.4	8.6 ± 0.2	1.1 ± 0.3	0
Me-FOSAA	Houtz et al., 2012	-	0	0	0	0	92 ± 4	0	0	0	0
	Martin et al., 2019	-	0	0	0	1.7 ± 0.2	94 ± 10	0	0	0	0
Et-FOSAA	Houtz et al., 2012	-	0	0	0	0	110 ± 8	0	0	0	0
	Martin et al., 2019	-	0	0	0	1.2 ± 0.1	95 ± 6	0	0	0	0
	Gockener et al., 2020	-	3.1 ± 3.1	5.5 ± 1.5	3.4 ± 0.7	7.1 ± 0.1	62.8 ±1.8	0	0	0	0
Me-FOSAA +Et-FOSAA	This study	-	1.0 ± 0.1	1.1 ± 0.1	2.2 ± 0.2	3.3 ± 0.1	74 ± 2	0	0	0	17.8 ± 0.8

Houtz et al., 2012 [1]
Martin et al., 2019 [2]
Gockener et al., 2020 [3]

Table S5 - Average blank concentrations, LODs and LOQs in ng/ml of serum (n=3).

	Before oxidation			After oxidation		
	Blank concentration	LOD	LOQ	Blank concentration	LOD	LOQ
PFBA	0.23	0.47	0.51	0.26	0.30	0.55
PFPeA	0.29	0.32	0.39	0.13	0.18	0.30
PFHxA	0.03	0.10	0.13	0.06	0.09	0.16
PFHpA	0.01	0.02	0.05	0.06	0.10	0.19
PFOA	0.05	0.10	0.26	0.04	0.11	0.27
PFNA	0.00	0.02	0.06	0.03	0.09	0.23
PFDA	0.00	0.02	0.06	0.02	0.08	0.24
PFUnDA	0.00	0.02	0.06	0.02	0.04	0.08
PFDoDA	0.00	0.02	0.06	0.02	0.04	0.08
PFTrDA	0.00	0.07	0.23	0.00	0.09	0.30
PFTeDA	0.00	0.13	0.42	0.00	0.13	0.42
PFBS	0.00	0.04	0.20	0.00	0.12	0.40
PFPeS	0.00	0.07	0.23	0.00	0.07	0.23
PFHxS	0.10	0.13	0.21	0.06	0.15	0.35
PFHpS	0.00	0.03	0.10	0.00	0.03	0.10
PFOS	0.19	0.26	0.38	0.06	0.14	0.33
PFNS	0.00	0.04	0.20	0.00	0.04	0.20
PFDS	0.00	0.05	0.17	0.00	0.05	0.17

Table S6 – Reproducibility before oxidation (n=24). Relative standard deviation values are reported only for detected compounds.

Compound	Relative standard deviation (%)
PFBA	-
PFPeA	7
PFHxA	9
PFHpA	10
PFOA	6
PFNA	9
PFDA	13
PFUnDA	18
PFDoDA	-
PFTrDA	-
PFTeDA	-
PFBS	-
PFPeS	-
PFHxS	6
PFHpS	10
PFOS branched	7
PFOS linear	6
PFNS	-
PFDS	-

Table S7 – Reproducibility after oxidation (n=3 for each precursor). Relative standard deviation values are reported only for detected compounds.

Compound	7:3 FTCA	6:2 FTUCA	6:2 FTCA 10:2 FTCA	6:2 FTS 10:2 FTS	Me-FOSAA Et-FOSAA	ADONA
PFBA	-	2	2	1	4	-
PFPeA	2	2	2	1	2	3
PFHxA	2	3	1	2	3	3
PFHpA	2	6	1	6	2	3
PFOA	3	1	2	3	3	8
PFNA	1	2	2	2	4	6
PFDA	4	8	8	8	11	11
PFUnDA	5	11	16	5	6	9
PFDoDA	-	-	-	-	-	-
PFTrDA	-	-	-	-	-	-
PFTeDA	-	-	-	-	-	-
PFBS	-	-	-	-	-	-
PFPeS	-	-	-	-	-	-
PFHxS	2	3	2	2	5	3
PFHpS	4	2	6	7	10	7
PFOS branched	2	5	2	5	4	7
PFOS linear	2	2	2	3	4	3
PFNS	-	-	-	-	-	-
PFDS	-	-	-	-	-	-

Table S8 – Method accuracy before oxidation. Concentrations declared by AMAP, measured concentrations and respective deviation from the declared value.

AMAP ID		PFHxA	PFOA	PFNA	PFUnDA	PFHxS	Tot PFOS
	Declared value (ng/ml)	8,67	7,59	2,87	4,71	14,30	83,60
1401	Measured value (ng/ml)	7,47	6,47	2,21	3,62	10,90	71,26
	Deviation (%)	-14	-15	-23	-23	-24	-15
	Declared value (ng/ml)	2,25	28,50	4,75	0,98	16,40	137,00
1404	Measured value (ng/ml)	2,33	26,35	4,13	0,85	14,97	132,33
	Deviation (%)	3	-8	-13	-13	-9	-3
	Declared value (ng/ml)	6,75	4,48	3,22	4,20	18,40	59,60
1406	Measured value (ng/ml)	7,24	4,49	3,14	3,80	15,78	57,68
	Deviation (%)	7	0,3	-2	-10	-14	-3
	Declared value (ng/ml)	2,32	22,00	1,35	0,99	6,37	59,70
1703	Measured value (ng/ml)	2,20	19,51	1,05	0,82	4,86	47,71
	Deviation (%)	-5	-11	-22	-17	-24	-20
	Declared value (ng/ml)	1,62	3,24	2,59	2,31	11,80	34,30
1705	Measured value (ng/ml)	1,82	3,49	2,92	2,21	11,40	33,65
	Deviation (%)	12	8	13	-5	-3	-2
	Declared value (ng/ml)	1,99	11,60	1,40	3,22	5,56	25,00
1707	Measured value (ng/ml)	2,46	12,26	1,31	2,75	5,02	27,27
	Deviation (%)	23	6	-7	-15	-10	9

Table S9 - Recoveries before and after oxidation for the final TOPA method for human serum (n=24).

Compound	Recovery before oxidation (%)	Recovery after oxidation (%)
^{13}C-PFBA	92 ± 10	75 ± 7
^{13}C-PFPeA	87 ± 2	71 ± 6
^{13}C-PFHxA	73 ± 2	62 ± 2
^{13}C-PFOA	69 ± 4	64 ± 3
^{13}C-PFNA	68 ± 5	63 ± 5
^{13}C-PFDA	66 ± 3	57 ± 8
^{13}C-PFUnDA	73 ± 6	52 ± 3
^{13}C-PFDoDA	68 ± 9	52 ± 6
^{13}C-PFTeDA	52 ± 8	41 ± 9
^{13}C-PFHxS	76 ± 5	61 ± 1
^{13}C-PFOS	74 ± 3	60 ± 6

Table S10 – Recoveries of model precursors before oxidation in human serum (n=3 for each precursor)

Compound	Recovery (%)
7:3 FTCA	73 ± 4
6:2 FTUCA	85 ± 3
6:2 FTCA	83 ± 2
10:2 FTCA	64 ± 1
6:2 FTS	79 ± 3
10:2 FTS	92 ± 3
Me-FOSAA	70 ± 2
Et-FOSAA	69 ± 1
ADONA	107 ± 8

Figure S1 – Yield of products (first bar) and conversion of precursors (second bar) for 6:2 FTS + 10:2 FTS and 7:3 FTCA in human serum with and without shaking using method C (n=3).

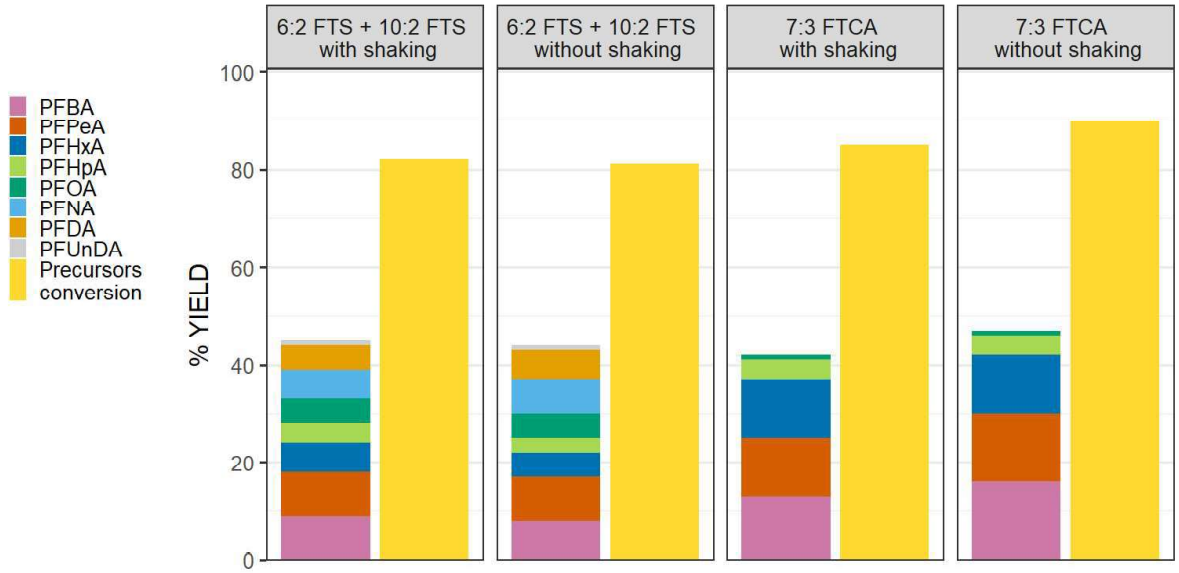


Figure S2 – Yield of products (first bar) and conversion of precursors (second bar) for 6:2 FTS + 10:2 FTS and 7:3 FTCA in human serum with $K_2S_2O_8$ or $Na_2S_2O_8$ as oxidant using method C (n=3).

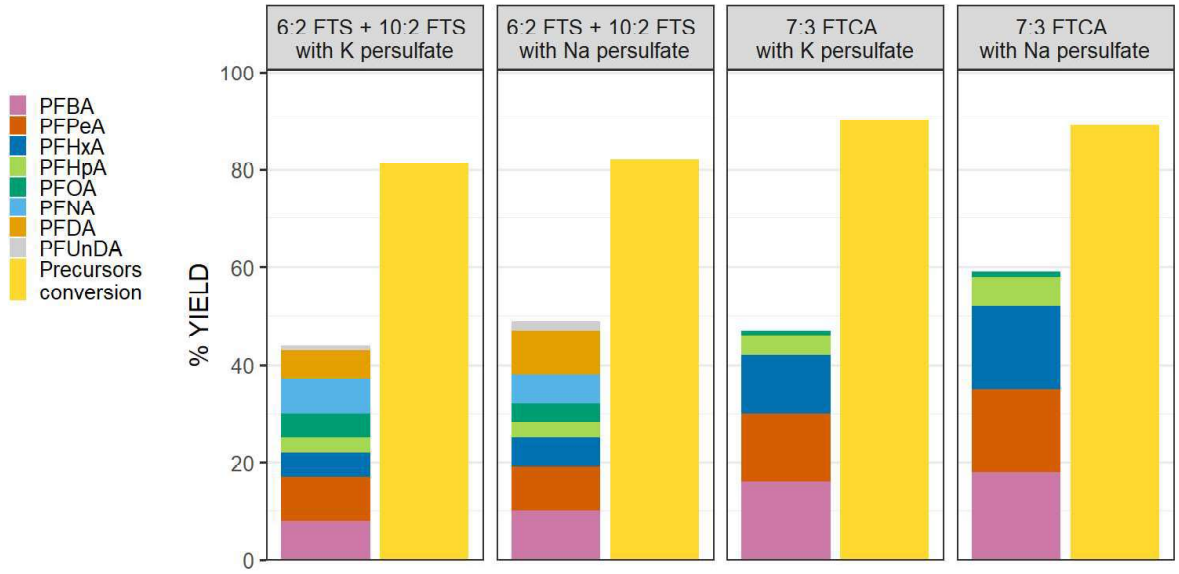


Figure S3 – Yield of products (first bar) and conversion of precursors (second bar) for human serum spiked with 200 ng of 6:2 FTS + 200 ng of 10:2 FTS, 4 ng of 6:2 FTS + 4 ng of 10:2 FTS, 200 ng of 7:3 FTCA and 4 ng of 7:3 FTCA using method D (n=3).

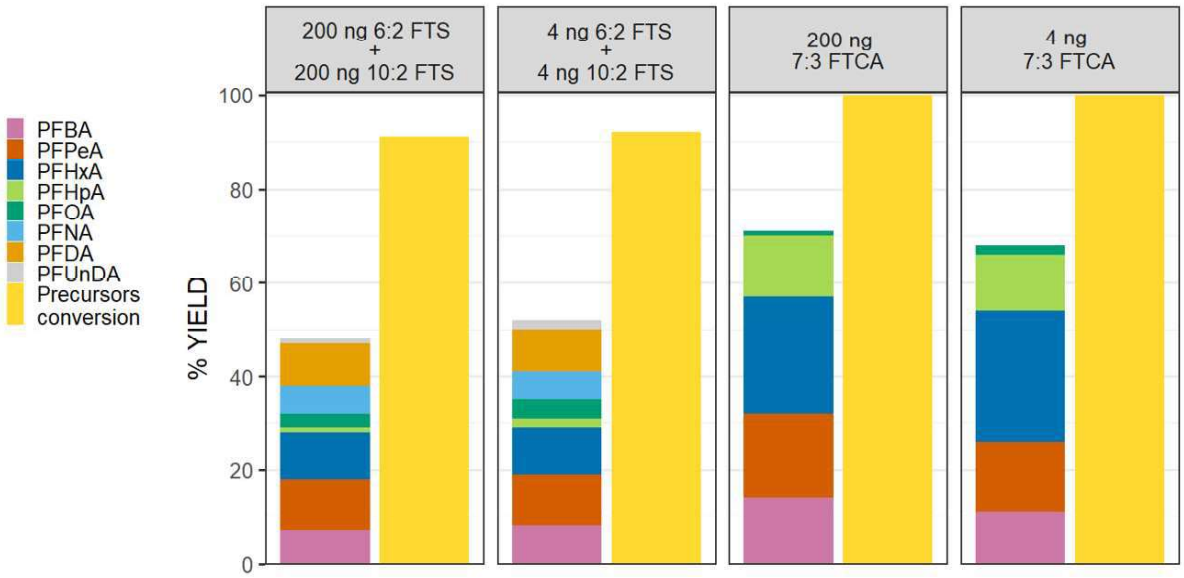


Figure S4 – Chromatograms for ADONA and 1,3-PFECA standards and for human serum spiked with ADONA before and after TOPA oxidation.

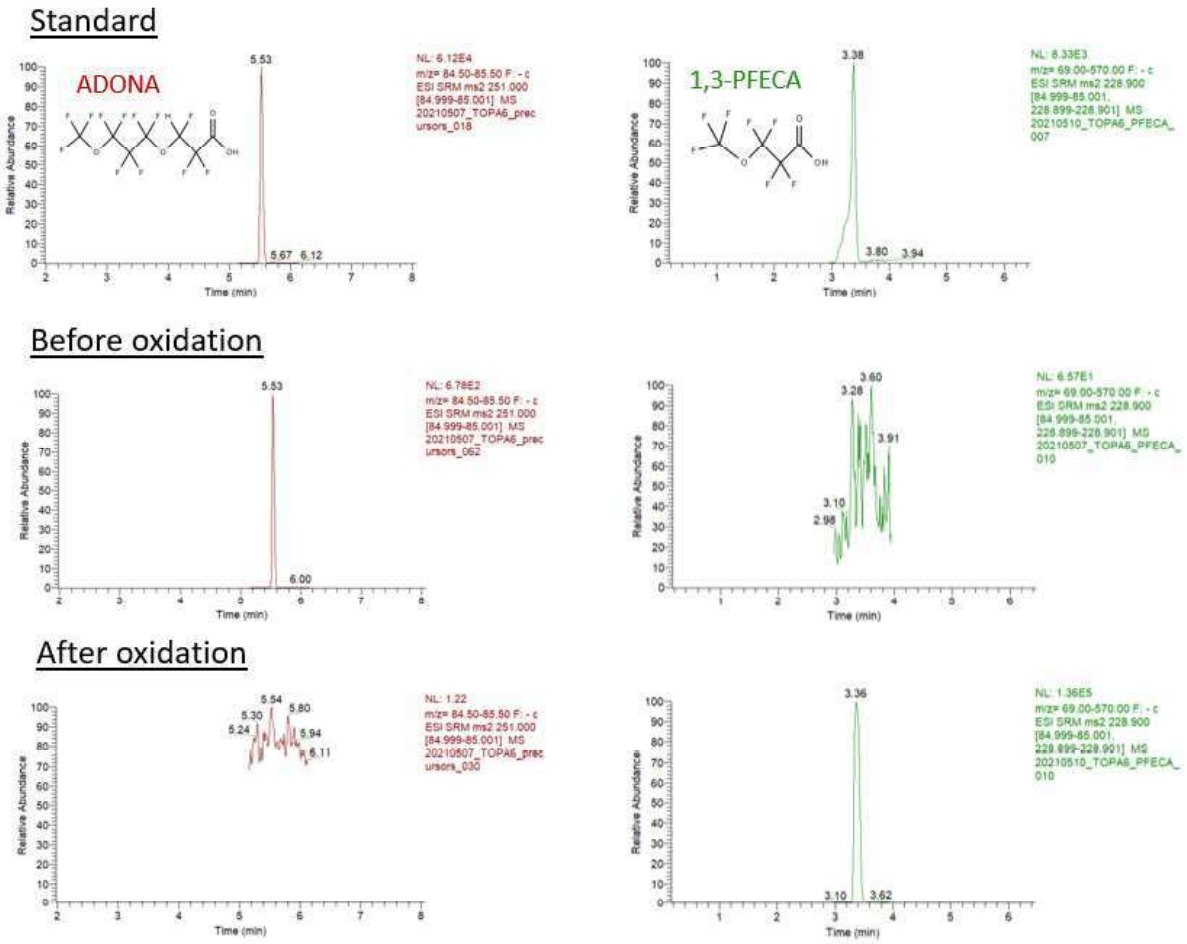
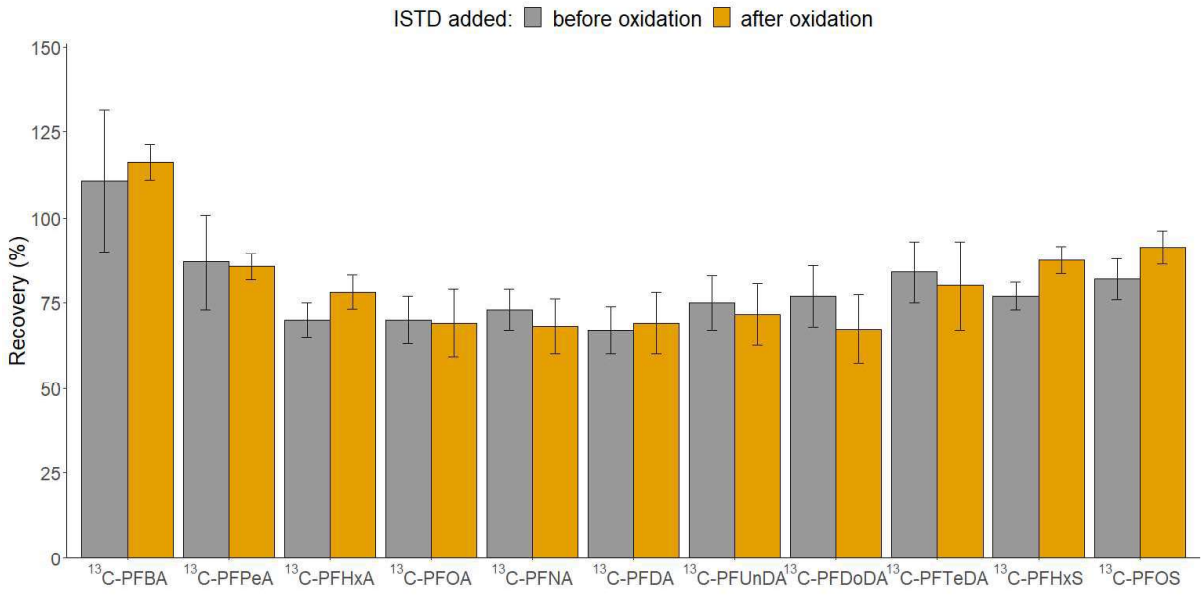


Figure S5 – Recoveries for ^{13}C-labelled PFAA added to the TOPA extracts before or after oxidation.



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Paper 2

Fluorine mass balance, including total fluorine, extractable organic fluorine, oxidizable precursors and target PFAS, in pooled human serum from the Tromsø population in 1986, 2007 and 2015

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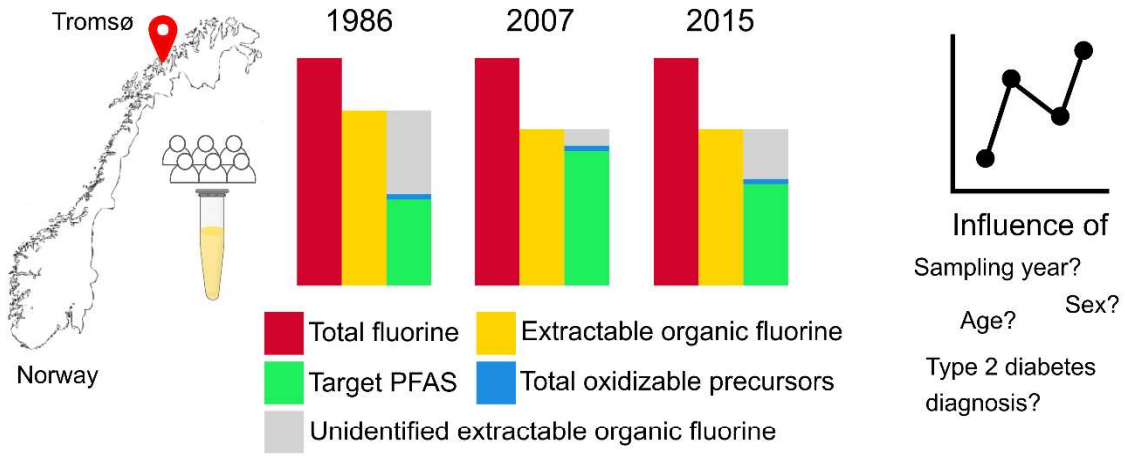

Abstract

Of the thousands of per- and polyfluoroalkyl substances (PFAS) known to exist, only a small fraction (≤1%) are commonly monitored in humans. This discrepancy has led to concerns that human exposure may be underestimated. Here we address this problem by applying a comprehensive fluorine mass balance (FMB) approach – including total fluorine (TF), extractable organic fluorine (EOF), total oxidizable precursors (TOP) and selected target PFAS – to human serum samples collected over a period of 28 years (1986, 2007 and 2015) in Tromsø, Norway. While concentrations of TF did not change between sampling years, EOF was significantly higher in 1986 compared to 2007 and 2015. Sum target PFAS were highest in 2007 compared to 1986 and 2015 and unidentified EOF (UEOF) decreased from 1986 (46 %) to 2007 (10 %) and then increased in 2015 (37%). While TF and EOF were not influenced by sex, women had higher UEOF compared to men, opposite to target PFAS. This is the first FMB in human serum to include TOP and suggests that precursors with > 4 perfluorinated carbon atoms make a minor contribution to EOF (0-4%). Additional tools are therefore needed to identify substances contributing to UEOF in human serum.

Keywords

Human exposure, PFAS, PFAA precursors, TF, EOF, TOP assay, time trend

Synopsis

The combined application of targeted and group-wise analyses on pooled serum samples enables the evaluation of the contribution of known and so far unidentified fluorinated compounds in human serum through time.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals with over 200 applications in industrial processes and consumer products [1]. Due to their widespread use and high persistence, PFAS have been observed throughout the environment, including wildlife and human blood globally [2]. PFAS ubiquity has led to concerns surrounding their ongoing production and use, in particular because some of them have been linked to adverse health effects, both in epidemiological and animal studies [3]. These effects include impaired immune system, thyroid dysfunction, liver disease, lipids dysregulation, kidney disease and adverse reproductive and developmental outcomes [3].

PFAS production and use restrictions were introduced in the United States and European Union in early 2000s, following the phase-out of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) by 3M [4, 5]. PFOS was subsequently added to the Stockholm Convention on Persistent Organic Pollutants (POPs) in 2009, followed by PFOA in 2019 [6, 7]. While PFOA and PFOS concentrations in human blood have declined globally in response to these initiatives [2, 5], longer perfluoroalkyl carboxylic acids (PFCA) are not following the same trend [5, 8]. Moreover, as fluorochemical manufacturers shift towards production of unregulated PFAS, novel PFAS may become increasingly relevant for human exposure [9].

Of the ~4600 PFAS registered on the global market in 2018 [10], ≤1% are routinely analysed in human biomonitoring studies [2, 11]. This discrepancy has led to doubts about whether targeted methodologies are sufficient to describe the full extent of PFAS exposure. Indeed, a growing number of fluorine mass balance (FMB) studies in human blood have quantified large fractions of extractable organic fluorine (EOF) that cannot be explained by targeted PFAS analyses [12-18]. One possible explanation for this gap are perfluoroalkyl acids (PFAA) precursors, such as perfluorooctane sulfonamides, fluorotelomer alcohols and polyfluoroalkyl phosphate esters. Many of these substances have been detected in human blood using targeted

methodologies [19], but as-of-yet unidentified precursors may also be important. The total oxidizable precursors (TOP) assay, in which PFAA concentrations are measured before and after controlled oxidation [20], offers a promising mean for quantifying the total contribution from both known and unknown precursors. While the TOP assay has been used successfully to determine PFAA precursors in environmental samples [21-26] and consumer products [27-31], there are few examples of its application to human serum [32, 33], and in particular no examples when used in conjunction with a FMB.

Here we build upon previous analyses of PFAS time-trends in serum from the Tromsø Study, which showed that PFAS concentrations in the Tromsø population changed according to the history of production and use of these chemicals and that time-trends differed depending on birth cohort, age group and study design [8, 34]. In the present study, serum samples from the Tromsø Study collected in 1986, 2007 and 2015 were pooled and for the first time analysed for total fluorine (TF), EOF, TOP and selected target PFAS. Through the combined application of a set of targeted and group-wise analyses, we aimed to evaluate exposure to total fluorine and known and unknown organic fluorinated compounds over time with respect to sex and age.

2. Materials and methods

Information on chemicals and consumables is provided in the SI.

2.1. Serum samples and pooling strategy

The Tromsø Study is a cohort study in the population of Tromsø, the largest city in Northern Norway. Details on the Tromsø Study are provided by Jacobsen et al. [35]. The study obtained informed consent from all participants and was approved by the Regional Committee for Medical Research Ethics (REK, case number: 2020/13188).

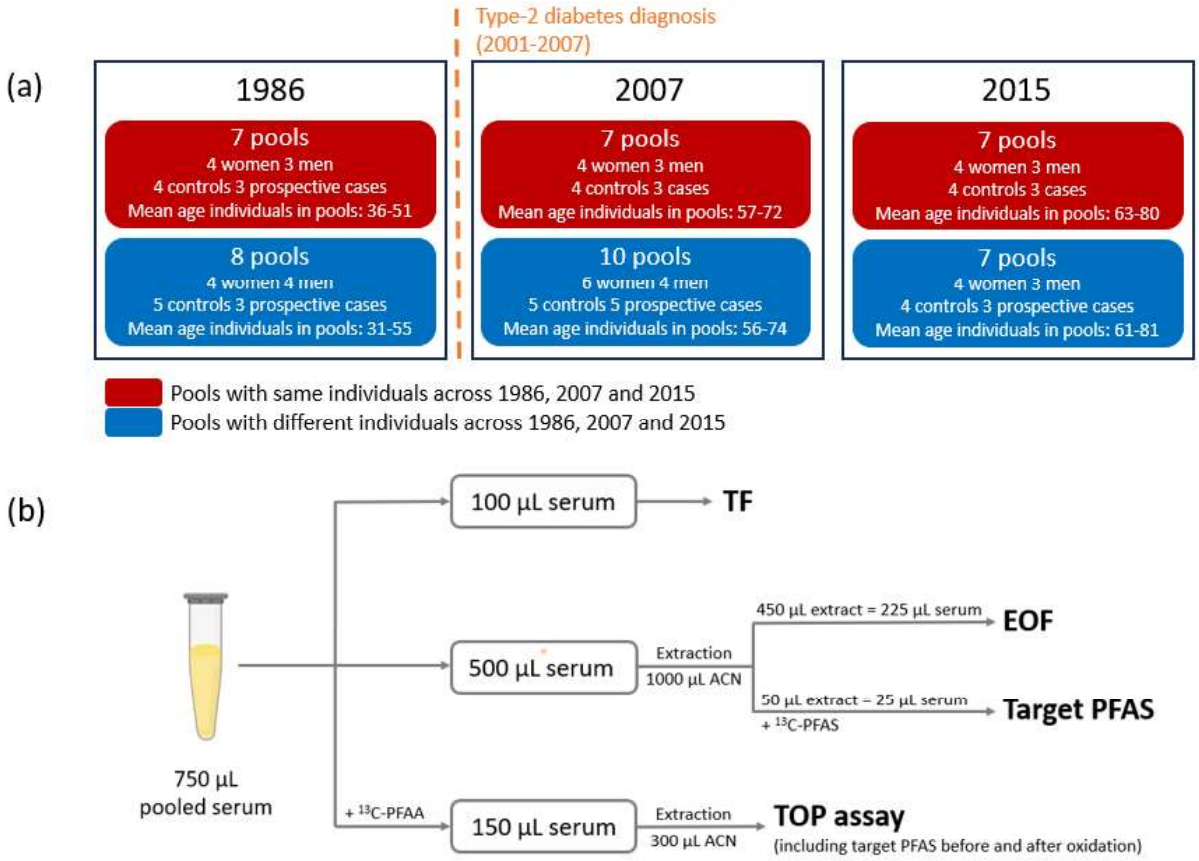

Figure 1 – (a) Pooling strategy summary, (b) fluorine mass balance approach.

The present work utilized 529 individual Tromsø Study serum samples collected in 1986 (n=201), 2007 (n=198) and 2015 (n=130) (Figure S1). The samples were selected based on a case-control study design on type-2 diabetes: the cases (1986 [n=84], 2007 [n=102], 2015 [n=62]) were diagnosed between 2001 and 2007, while the controls (1986 [n=117], 2007 [n=97], 2015 [n=68]) had no diagnosis recorded in the local diabetes registry. The selected samples included 104 women and 97 men in 1986, 113 women and 86 men in 2007 and 72 women and 58 men in 2015. The age of the individuals ranged from 17 to 61 years old in 1986 (mean: 46), from 38 to 81 in 2007 (mean: 67) and from 46 to 89 in 2015 (mean: 72).

From this selection, 472 individual samples (1986 [n=167], 2007 [n=175], 2015 [n= 130]) were pooled based on sampling year, sex, age and type 2 diabetes diagnosis (Figure 1, Table S1). Sampling year, sex and age were chosen as variables for pooling because these are known to

influence PFAS concentrations in human blood. Type-2 diabetes diagnosis was used as a variable for pooling because some studies have reported associations between this endpoint and PFAS concentrations, but it is important to note that evidence for these associations is contradictory [36]. Pools 1 to 7 at each sampling year included the same individuals in 1986, 2007 and 2015. To have the largest possible number of pools including the same individuals, these pools were obtained mixing variable volumes (50, 100 or 150 μl) of individual serum samples, but keeping the volume per individual constant throughout the sampling years. For the remaining pools, it was not possible to follow the same individuals through time and 15 participants (with matching sampling year, sex, age and type 2 diabetes diagnosis) were included in each pool mixing 50 μl of serum per individual.

2.2. Fluorine mass balance

Each pool was analysed using a combination of analytical techniques to evaluate different fluorine fractions (Figure 1). The pools were split into 3 portions: (1) 100 μl for TF, (2) 500 μl for EOF, (3) 150 μl for the TOP assay. Target PFAS analysis was performed on the TOP assay extracts (before and after oxidation), and on the EOF extracts after addition of internal standard.

2.2.1. Total fluorine

For TF measurements, 100 μL of serum were transferred to a sampling boat for analysis using a Thermo-Mitsubishi combustion ion chromatograph (CIC) with the method described by Miaz et al. [15], which was previously demonstrated to produce fluorine-specific responses [37]. Details about quality control measures (including calibration, blank values, LODs, accuracy and precision evaluation) are reported in the SI.

2.2.2. Extractable organic fluorine

For EOF determination, 500 µL of serum were transferred to Eppendorf tubes and extracted once with 1 mL of ACN. Samples were vortexed and sonicated (10 minutes) for 3 times and, after centrifugation at 10000 rpm for 10 minutes, supernatants were transferred to 2 mL glass vials. EOF analyses were performed on 450 µL of the extracts with the same CIC used for TF analyses and the method described by Miaz et al. [15]. Details about quality control measures (including calibration, blank values, LODs, evaluation of PFOS recovery, reproducibility and removal of fluoride upon extraction) are reported in the SI.

2.2.3. Total oxidizable precursors assay

For the TOP assay, 150 µL of serum were processed using a previously published protocol [32]. Briefly, samples were spiked with ^{13}C-PFAA and extracted with ACN. After vortexing, sonication and centrifugation, the supernatant was collected and split into 2 portions: one for target analyses before oxidation and one was oxidized for TOP determination. Prior to oxidation, ACN was removed by evaporation and the dry extracts were reconstituted with 0.8 M $Na_2S_2O_8$ and 10 M NaOH. Post oxidation, the samples were acidified and extracted with MTBE. Aliquots of the organic phase were transferred to vials with insert and spiked with recovery standard and 2% ammonia in methanol. The MTBE was evaporated prior analyses. Details about quality control measures (including blanks, LODs and recoveries before and after TOP assay, and summary of method validation with model precursors) are reported in the SI.

2.2.4. Target PFAS

Target analyses on the EOF extracts included 54 PFAS (Table S5) and were performed using a Dionex UltiMate 3000 Ultrahigh performance liquid chromatograph coupled to a Q Exactive HF hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described elsewhere [15]. For these analyses, 50 µl of EOF extracts were mixed with

10 µl of internal standard and 50 µl of 4 mM NH_4OAc in MilliQ water. Since the internal standard was added after extraction, these concentrations were not recovery corrected and were only used for FMB calculations. LODs and accuracy of these analysis are reported in the SI.

Target analyses on the TOP assay extracts included 34 PFAS and were performed using a quaternary Accela 1250 pump with a PAL Sample Manager coupled to a Vantage TSQ MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) as described elsewhere [32].

After oxidation the extracts were also analysed for C_2 and C_3-PFAA using a Raptor Polar X column. Details about these analyses and the quality control measures (including blank concentrations and LODs) are provided in the SI.

2.3. Data treatment

2.3.1. Fluorine mass balance calculations

EOF values were subtracted from TF concentrations to estimate the amount of inorganic and non-extractable organic fluorine. For this comparison, samples with TF below LOD were excluded. To estimate the unidentified portion of EOF (UEOF), the $\sum_{12}$PFAS concentrations obtained from the EOF extracts (Table S6) were subtracted from the EOF concentrations. For this comparison PFAS concentrations were converted to fluorine equivalents using equation S1. PFAS concentrations below LOD were set to LOD/$\sqrt{2}$. The $\sum_{12}$PFAS concentrations and detection frequencies from the EOF extracts are lower and less accurate than those from the TOP assay extracts (Table 2 and S6), because of the lack of recovery correction for procedural losses. However, the use of $\sum_{12}$PFAS concentrations not corrected for recovery for fluorine mass balance calculations provides in a more representative and accurate result in terms of mass balance. This is because the EOF concentrations cannot be recovery corrected since the addition of internal standard before extraction would increase the LOD and it is not possible to correct for the recovery of unknown fluorinated chemicals present.

The total amount of oxidizable precursors (ΔPFAA) was estimated as described by Coêlho et al. [33]. To establish if there was an increase in PFAA concentrations after oxidation, the ratio between the concentration after oxidation and the concentration before oxidation ($PFAA_{after\ TOPA}/PFAA_{before\ TOPA}$) was calculated. To avoid the possibility that apparent changes were influenced by analytical uncertainties, a cut-off of 20% change in PFAA concentrations was applied. Specifically, if the ratio was ≥1.2, the difference (ΔPFAA) was calculated as the PFAA concentration after oxidation minus the PFAA concentration before oxidation. If the ratio was <1.2, ΔPFAA was set to zero.

To estimate the contribution of total oxidizable precursors to EOF, ΔPFAA concentrations were converted to F equivalents with the same equation used for target PFAS (Equation S1). This comparison has some uncertainty because the TOP assay data are corrected for procedural losses, but the EOF data are not.

2.3.2. Statistical analyses

Statistical analyses were performed using R 4.1.2 (R Core Team). Prior to statistics calculations, concentrations below the LOD were substituted with LOD/√2. Differences in TF, EOF, sum PFAS, unidentified EOF and TOP between sampling years, sex and age (as weighted mean of the age of the individuals in the pools expressed in years) groups were assessed by multiple linear regression using equation S2. When sex was a significant predictor, differences in concentrations between men and women at each sampling year were assessed adding an interaction term (equation S3). The inclusion of the type-2 diabetes diagnosis (case/control status) to the multiple linear regression model was tested using Akaike information criterion (AIC) model selection. Since the model with lowest AIC score never included the diabetes diagnosis variable, this was not included. TF, EOF and $\sum_{12}$PFAS concentrations were log-

transformed before performing regression analyses. Statistical significance was set at $p < 0.05$. Post-hoc power calculations were performed using the pwr package.

3. Results and discussion

3.1. Total fluorine

TF in pooled serum from the Tromsø Study ranged from <25 to 1330 ng F/mL, with a narrower range observed in 2015 compared to 1986 and 2007 (Figure 2–a, Table S7). The percentage of pools with TF below LOD (25 ng F/mL) was 33 % in 1986, 24 % in 2007 and 7% in 2015. Based on multiple linear regression, there were no significant differences in TF concentrations between 1986, 2007 and 2015 and no significant effect of sex and age (Table S8). For TF the time differences observed in the pools with same individuals were not consistent and this could be explained by TF being a sum parameter, including both inorganic and organic compounds containing fluorine for which the contribution might be vary between individuals. In two of these pools the concentrations temporal changes clearly differed from the rest of the pools with same individuals because these were below or close to LOD at all sampling years (Figure S2). In contrast with the results of our study, Miaz et al. [22] observed declining TF concentrations in pooled serum samples from Swedish women collected between 1996 and 2017 (3.2 % decline per year), although in that study the cohort was consuming PFAS-contaminated drinking water up until mid-2012.

The range of observed TF concentrations in 1986 and 2007 was wider than those reported in the literature, but in 2015 it was comparable (Table S7). However, the mean concentrations of TF in 1986, 2007 and 2015 were comparable to those reported for blood samples from China in 2008 and lower than those reported for serum from Japan in 2003-2004 and plasma from the USA in 2001 (Table S7).

3.2. Extractable organic fluorine

EOF in serum from the Tromsø Study ranged from 12.6 to 45.3 ng F/mL across all time-points (Figure 2–b, Table S7). Unlike TF, EOF was detected in all pools (LOD = 7 ng F/mL). EOF concentrations in 1986 were significantly higher than in 2007 and 2015, while no significant differences were found between 2007 and 2015 (Table S7, Table S8).

For EOF the time differences observed in the pools from the same individuals were not consistent (Figure S2) and, as for TF, this could be explained by EOF being a sum parameter, including potentially different PFAS and organofluorine chemicals.

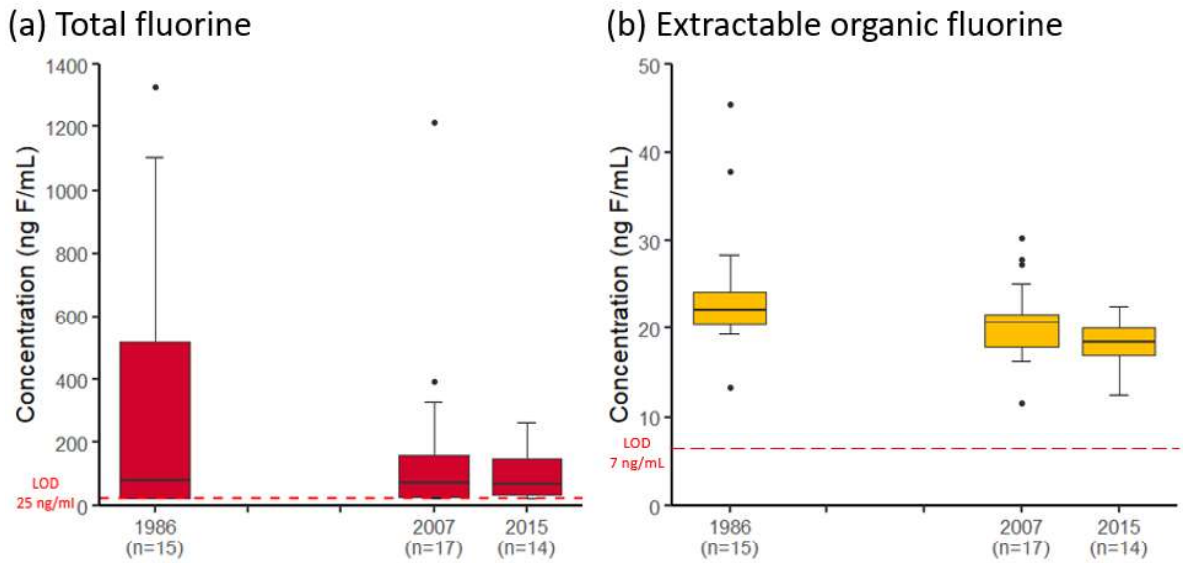

Figure 2 – TF (a) and EOF (b) concentrations (ng F/ml) in pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015 (n = number of pools). The boxes represent the interquartile range between the 25th and 75th percentiles, containing the middle 50% data. The line in the boxes represents the median. The whiskers extend from the smallest observation greater than/equal to the 25th percentile minus 1.5 times the interquartile range to the largest observation lower than/equal to the 75th percentile plus 1.5 times the interquartile range. The points outside the whiskers represent outliers with values outside these limits.

The EOF concentrations observed in our study were in the same range of those observed in plasma from Germany collected between 1982-2009 and in pooled serum samples from Swedish women collected between 1996-2017. However, no significant time trends were observed for EOF in the German (1982-2009) and Swedish (1996-2017) samples [14, 15]. EOF concentrations in 2007 and 2015 were also comparable to those in whole blood collected in China in 2004 and in Sweden in 2015 and between 2018-2019. The EOF at all sampling years was higher than in whole blood from Japan (2003) and pooled serum from Austria (2021), but lower than the EOF in plasma from the USA (2001) and in whole blood from people living in Ronneby, where the drinking water has been contaminated from firefighting foams (Table S7). However, apparent differences in EOF measurements between studies must be interpreted cautiously since different extraction methods may perform differently for individual fluorinated substances [38]. In addition, different EOF values can be measured from different blood fractions, since some PFAS, like for example perfluorohexanoic acid (PFHxA) and perfluorooctane sulfonamide (FOSA), bind minimally to serum proteins and are usually detected in whole blood rather than serum or plasma [39].

Based on multiple linear regression, sex and age were not associated with EOF. This observation agrees with EOF measurements in samples from China that also showed no sex- and age-related differences [13]. On the contrary, EOF concentrations in samples from Sweden in 2021 were higher in women compared to men [16].

3.3. Total oxidizable precursors

The pooled samples from the Tromsø Study were also analysed with the TOP assay to evaluate the contribution of oxidizable precursors. Even if the increases in PFAA concentrations (ΔPFAA) were low (0.02-1.85 ng/mL), all pools (except one from 2007) contained detectable

oxidizable precursors (Table 1). No significant differences in TOP concentrations were found between 1986, 2007 and 2015 and sex and age did not influence the TOP measured (Table S8). For TOP, the time differences observed in pools from same individuals were not consistent and this could be to a higher variability in precursors exposure or to the low concentrations of precursors present. Additionally, for this method a higher variability compared to target PFAS measurements is expected since the TOP concentrations are estimated by comparing PFAA concentrations before and after oxidation (Figure S2).

Increases in concentrations after oxidation were observed for 8 PFCA and 3 PFSA (Table 1). Perfluorododecanoic acid (PFDoDA), perfluorobutanoic sulfonic acid (PFBS) and perfluoroheptanoic sulfonic acid (PFHpS) were the only compounds to display increased concentrations after oxidation in more than 50% of the pools in at least one time-point. While ΔPFDoDA and ΔPFHpS were observed at all the examined time-points, ΔPFBS was only detected in serum pools from 2015. Increases in concentrations after oxidation were also detected for perfluoropentanoic acid (PFPeA), PFHxA, perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA) and perfluorohexanoic sulfonic acid (PFHxS), but in a limited number of pools. Increases in concentrations of multiple PFAA following oxidation were more common than increases in only one PFAA, even if 8 pools showed an increase only in PFHpS (5 samples), PFDoDA (2 samples) and PFBS (1 sample) (Figure S3). The pattern of oxidation products differed from those observed for model precursors spiked into human serum [32] and could not be used to tentatively identify precursors in serum from the Tromsø Study. However, even if the structure of the precursor(s) is lost upon oxidation, the profile of the oxidation products offered clues about the chain length of the precursor and the presence of sulfonic groups. For example, ΔPFDoDA points to the presence of precursors with 11 or more

perfluorinated carbons, while ΔPFBS and ΔPFHpS suggest the presence of precursors containing sulfonic groups attached to 4 or 7 perfluorinated carbons [32].

Table 1 – Differences in PFAA concentrations before and after TOP assay oxidation ($\Delta PFAA = PFAA_{afterTOP} - PFAA_{beforeTOP}$) in pooled serum samples from the Tromsø Study (n=number of pools).

	1986 (n=15)				2007 (n=17)				2015 (n=14)			
	DF (%)	Median	Mean	Range	DF	Median	Mean	Range	DF	Median	Mean	Range
ΔPFPeA	5/15 (33%)	0.00	0.03	0.00-0.09	1/17 (6%)	0.00	0.01	0.00-0.11	0/14 (0%)	-	-	-
ΔPFHxA	0/15 (0%)	-	-	-	1/17 (6%)	0.00	0.08	0.00-1.32	0/14 (0%)	-	-	-
ΔPFHpA	7/15 (47%)	0.00	0.04	0.00-0.12	0/17 (0%)	-	-	-	0/14 (0%)	-	-	-
ΔPFOA	5/15 (33%)	0.00	0.23	0.00-1.00	0/17 (0%)	-	-	-	0/14 (0%)	-	-	-
ΔPFNA	4/15 (27%)	0.00	0.04	0.00-0.18	1/17 (6%)	0.00	0.02	0.00-0.40	1/14 (7%)	0.00	0.02	0.00-0.36
ΔPFDA	1/15 (7%)	0.00	0.00	0.00-0.05	2/17 (12%)	0.00	0.01	0.00-0.15	0/14 (0%)	-	-	-
ΔPFUnDA	3/15 (20%)	0.00	0.03	0.00-0.15	0/17 (0%)	-	-	-	2/14 (14%)	0.00	0.02	0.00-0.18
ΔPFDoDA	9/15 (60%)	0.04	0.03	0.00-0.09	12/17 (71%)	0.06	0.07	0.00-0.15	6/14 (43%)	0.00	0.03	0.00-0.14
ΔPFBS	0/15 (0%)	-	-	-	0/17 (0%)	-	-	-	13/14 (93%)	0.21	0.19	0.00-0.35
ΔPFHxS	4/15 (27%)	0.00	0.04	0.00-0.26	0/17 (0%)	-	-	-	0/14 (0%)	-	-	-
ΔPFHpS	9/15 (60%)	0.05	0.08	0.00-0.21	14/17 (82%)	0.18	0.19	0.00-0.43	8/14 (57%)	0.11	0.11	0.00-0.32
ΔPFAA	15/15	0.43	0.52	0.17-1.16	16/17	0.26	0.38	0.00-1.85	14/14	0.36	0.38	0.13-0.66

DF = detection frequency: number and % of pools with increase in concentration after oxidation ($PFAS_{after\text{-}TOP}/PFAS_{before\text{-}TOP} \geq 1.2$)

The TOP assay has previously been applied to plasma samples collected between 2003 and 2006 from Norwegian women [33]. The patterns of PFAA that increased after oxidation were different from those observed in this study. In contrast to our study, no increases in PFDoDA and PFBS were observed. Also, in the Tromsø Study pools the concentrations of branched isomers of PFOA and PFOS did not increase after the TOP assay and the detection of ΔPFHpA, ΔPFNA, ΔPFDA and ΔPFUnDA was limited, while in the plasma collected from Norwegian women 7 PFAA increased after oxidation (PFHpA, branched-PFOA, PFNA, PFDA, PFUnDA, PFHpS, branched PFOS) with the greatest concentration differences observed for PFHpA, branched PFOA and PFDA. There are several possible explanations for these differences. First, there could be differences in exposure among the studied groups. The samples in this study were collected from both men and women living in Tromsø, while in the Coêlho et al. study

samples were collected only from women but from all over Norway. Additionally, the sampling years were different in the two studies. Secondly, the use of serum in the present study and plasma in the other could lead to the detection of different precursors. Another possible explanation could be the different extraction methods used in the two studies resulting in different extraction effectiveness of the precursors present.

3.4. Target PFAS

In the pooled samples, 12 out of 54 target PFAS were detected: six PFCA (PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA), three PFSA (PFHxS, PFHpS, PFOS) and three sulfonamidoacetic acids (FOSAA, Me-FOSAA and Et-FOSAA). Branched isomers were above LOD only for PFOS. It is interesting to note that, in agreement with the TOP assay results showing low concentrations of precursors, no precursors included in the target analyses other than the sulfonamidoacetic acids (including fluorotelomer sulfonates, fluorotelomer carboxylic acids and fluorotelomer phosphate esters) were detected in pooled serum. Other biomonitoring studies investigating the presence of these precursors in human blood, have also reported no detection or detection at trace levels (pg/mL) [40-42]. However, some of these precursors have been widely detected in consumer products, such as cosmetics and food packaging [43, 44]. This discrepancy between wide detection in consumer products and low detection in human blood might be due to a low uptake potential, rapid metabolism or elimination of precursors in humans, but the contribution of precursor metabolism to indirect PFAA exposure remains unknown [19, 45, 46].

Based on the analysis of the Tromsø Study pools, the $\sum_{12}$PFAS concentrations in 2007 were significantly higher than in 1986 and 2015 (Table 2, Table S8). Focusing on individual PFAS changes over time, concentrations of all PFAA in 2007 were higher than in 1986, except for PFHpA. Between 2007 and 2015, PFSA (PFHxS, PFHpS and PFOS) and PFOA concentrations

decreased, as opposed to the longer chained PFCA (PFNA, PFDA, PFUnDA and PFDoDA), for which concentrations increased. Concentrations of the sulfonamidoacetic acids increased from 1986 to 2007 but none was detected in 2015. PFHpA concentrations were comparable in 1986, 2007 and 2015 (Table 2, Figure S4).

Table 2 – PFAS concentrations (ng/mL) in pooled serum samples from the Tromsø Study before TOP assay oxidation (n=number of pools).

	1986 (n=15)				2007 (n=17)				2015 (n=14)			
	DF	Median	Mean	Range	DF	Median	Mean	Range	DF	Median	Mean	Range
PFHpA	13/15	0.06	0.06	<0.02-0.25	17/17	0.06	0.05	0.03-0.08	13/14	0.05	0.04	<0.02-0.09
PFOA	15/15	2.44	2.35	1.53-3.30	17/17	3.59	3.66	3.26-4.55	14/14	2.34	2.46	1.86-3.34
PFNA	15/15	0.56	0.59	0.39-1.08	17/17	1.71	1.65	1.27-2.31	14/14	1.99	2.03	1.43-1.89
PFDA	15/15	0.20	0.19	0.11-0.37	17/17	0.65	0.64	0.33-1.09	14/14	0.75	0.76	0.41-1.32
PFUnDA	15/15	0.61	0.63	0.48-1.05	17/17	1.04	1.02	0.55-2.16	14/14	1.08	1.06	0.43-1.96
PFDoDA	5/15	<0.02	<0.02	<0.02-0.08	9/17	0.06	0.03	<0.02-0.14	11/14	0.06	0.05	<0.02-0.13
PFHxS	15/15	0.74	0.69	0.38-1.17	17/17	2.31	2.37	1.61-6.36	14/14	1.99	2.13	1.18-4.74
PFHpS	10/15	0.10	0.07	<0.03-0.32	17/17	0.29	0.29	0.10-0.61	14/14	0.23	0.24	0.10-0.58
br-PFOS	15/15	9.53	9.16	6.63-12.3	17/17	14.9	14.5	10.6-20.5	14/14	8.92	9.73	7.54-14.3
lin-PFOS	15/15	15.9	15.5	12.0-21.5	17/17	22.6	23.5	15.8-42.6	14/14	15.5	17.3	9.34-29.0
FOSAA	14/15	0.12	0.12	<0.04-0.32	0/17	-	-	-	0/14	-	-	-
Me-FOSAA	15/15	0.20	0.18	0.07-0.35	17/17	0.11	0.11	0.05-0.21	0/14	-	-	-
Et-FOSAA	15/15	0.43	0.41	0.25-0.58	0/17	-	-	-	0/14	-	-	-
∑12 PFAS	15/15	30.9	30.2	23.7-40.3	17/17	47.0	48.2	38.7-75.7	14/14	34.0	36.3	22.9-52.4

DF = detection frequency: number of pools with PFAS concentration > LOD.

The increase in $\sum_{12}$PFAS and individual PFAA concentrations between 1986 and 2007 points to increased exposure between these years. However, we know from previous PFAS analyses in serum from the Tromsø Study, including individual samples from 1994 and 2001, that target PFAS concentrations peaked in 2001 with an increase between 1979 and 2001, followed by a decrease between 2001 and 2007 [8, 34]. Divergent trends between PFOA and longer chained PFCA were also reported in the aforementioned studies: while PFOA concentrations peaked in 2001, long chained PFCA were increasing between 2001 and 2007. These trends in the Tromsø Study samples have been shown to follow trends of PFAS production and use [26].

The $\sum_{12}$PFAS concentrations in the pools with the same individuals followed the temporal changes described by multiple linear regression, except in one pool, that showed comparable $\sum_{12}$PFAS concentrations in 1986 and 2007. This deviation could be due to this pool containing a lower number of individuals (10) compared to the other ones (11-14). With a lower number of individuals in a pool, even just one outlier could have a bigger impact on the measured target PFAS concentrations (Figure S2).

Mean age of the individuals in the pools was a predictor of the $\sum_{12}$PFAS concentrations between 1986 and 2015 (Table S8). The highest $\sum_{12}$PFAS concentrations were found in the pools with highest mean age (Figure S5). This has been explained by higher exposure in the older birth cohorts compared to the younger ones due to the history of changing PFAS production [8].

Men had significantly higher $\sum_{12}$PFAS concentrations than women (Table S8). When looking at the difference in $\sum_{12}$PFAS concentrations at each time-point, men had significantly higher concentrations only in 2007 (Table S9). However, the difference might be not observed at all time-points due to limited statistical power. To obtain a power of 80% with large effect size (0.35), at least 39 samples are necessary and the number of pools at each time-point is lower than this value. Higher concentrations in men compared to women were observed for most of the individual PFAS (PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFHxS, PFHpS and PFOS), but comparable concentrations were observed for PFHpA and the three sulfonamido acetic acids (FOSAA, Me-FOSAA and Et-FOSAA) (Figure S4). Higher PFAS concentrations in men compared to women were already reported in the Tromsø Study by Berg et al. [34], which also noted higher PFAS concentrations in women that had not given birth compared to multiparous women. Placental transfer [47-52], breast feeding [53-56], and menstruation [57-60] are known PFAS elimination pathways in women and could all contribute to explain sex differences in PFAS concentrations.

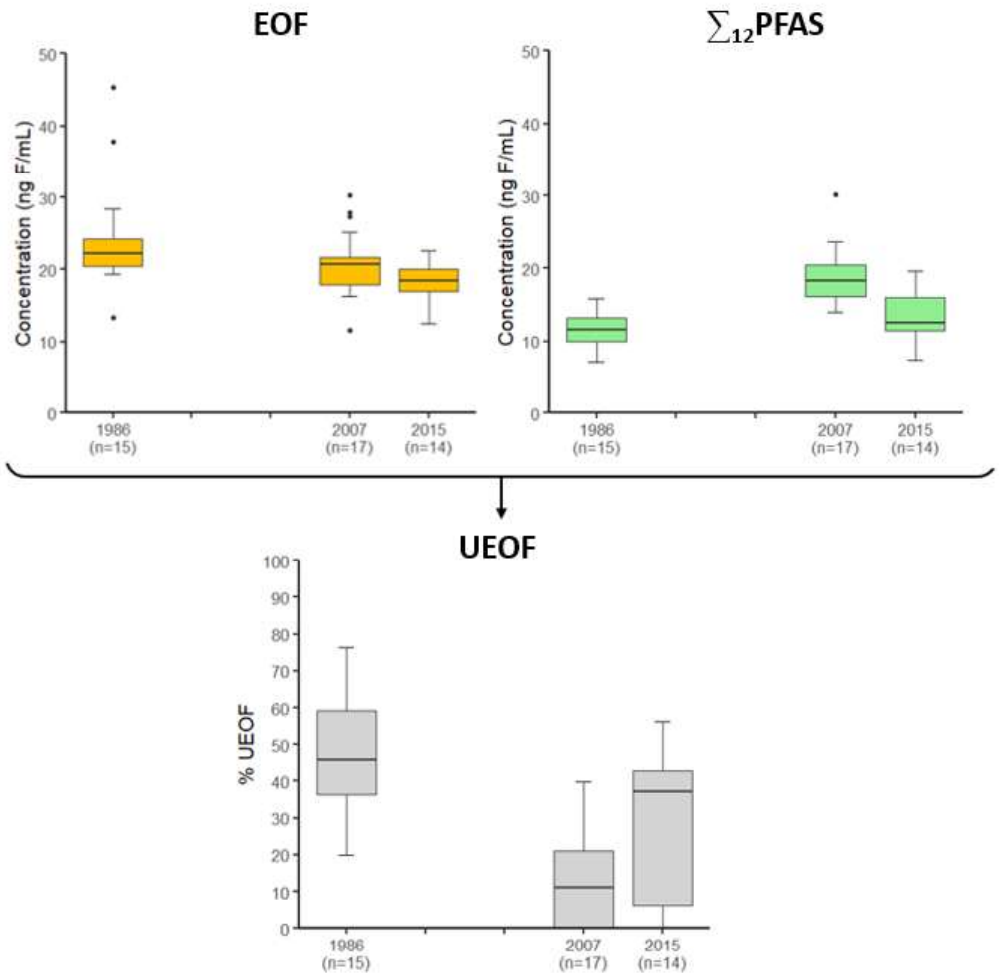

Figure 3 – Comparison between extractable organic fluorine (EOF) and $\sum_{12}$PFAS concentrations in ng F/mL and unidentified EOF (UEOF) percentage in pooled serum samples from the Tromsø Study in 1986, 2007 and 2015 (n=number of pools).

3.5. Fluorine mass-balance

The comparison of EOF and target PFAS concentrations revealed the presence of unidentified organofluorine at all time-points. This unidentified EOF (UEOF) ranged from 0.00 to 34.8 ng F/mL, accounting for 0 to 77 % of the EOF (Table S10, Figure 3).

UEOF concentrations were highest in 1986, when the target PFAS concentrations were lowest. In 2007 the UEOF portion was significantly lower than in 1986, while between 2007 and 2015 a significant increase in UEOF was observed (Figure 3, Table S8, Table S10).

For comparison, the UEOF fractions from other studies available in the literature are summarized in table S10. While the UEOF in the Tromsø Study pools was higher in 1986 than in 2007, no time-trends were observed for the UEOF in German plasma between 1982 and 2000. The high fraction of UEOF observed in the 1986 Tromsø Study samples, followed by lower concentrations in 2007, could be explained by the presence in the serum of PFOS-related substances that have been restricted with PFOS in early 2000s. According to the PubChem PFAS Tree [61], there are 1297 chemicals registered in PubChem that would be restricted under Annex B of the Stockholm Convention. However, among these chemicals, C8-precursors can be excluded since no increases in PFOS and limited increases in PFOA were observed after the TOP assay in 1986 (Table 1). An increasing trend for UEOF following PFOS and PFOA production and use reduction, has been observed between 2000 and 2009 plasma samples coming from Germany [14] and in pooled serum samples from Swedish women, for which a 3.9% increase in UEOF per year between 1986 and 2017 has been modelled [15]. The increase in UEOF that we observed between 2007 and 2015 (both in percentage and absolute concentration) is in agreement with these findings and could be explained by increasing exposure to novel PFAS which have not yet been identified. However, fluorinated chemicals other than PFAS could also contribute to explain the elevated UEOF. Fluorine substitution is often used in the agrochemical and pharmaceutical industry. Among the halogenated agrochemicals available in the market between 1940 and 2003, around 28% contained fluorine [62]. While, for pharmaceuticals, the percentage of globally used active substances containing fluorine increased from around 2% in 1970 to 25% in 2021 [62, 63]. This percentage is expected to increase further, since 25-30 % of the newly approved drugs contain one or more fluorine atoms. In addition, among the most prescribed drugs, the proportion of fluorinated pharmaceuticals is even higher [62]. While we are not aware of studies investigating the contribution of pharmaceuticals and pesticides towards the EOF mass

balance in human blood, a recent study determined that ~22% of the EOF in wastewater treatment plant sludge (which mirrors societal use of chemicals) was attributable to these substances, many of which contain no fluoroalkyl functionalities [64].

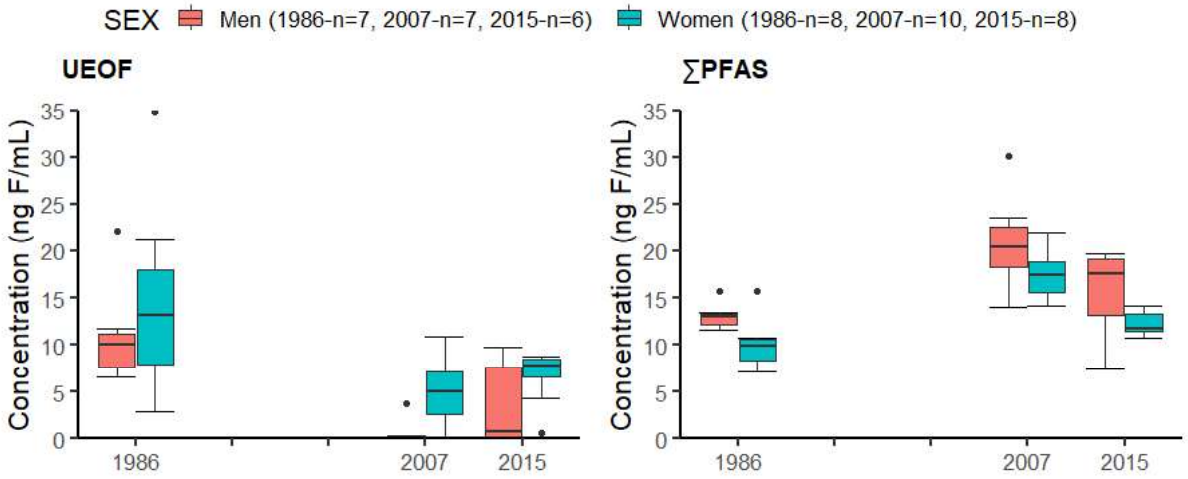

Figure 4 – UEOF and $\sum_{12}$PFAS in ng F/mL in men and women from the Tromsø Study in 1986, 2007 and 2015 (n=number of pools).

Mean age was not a significant predictor of UEOF, but women had higher UEOF than men (Table S8, Figure 4). As for target PFAS, the evaluation of differences in concentrations between men and women at each time-point was limited by statistical power and significant differences were observed only in 2007 (Table S9). The sex difference observed for UEOF is the opposite of what we observed and what is reported in the literature for PFAA, for which concentrations are higher in men than in women [65-67]. Higher UEOF in women compared to men have also been reported in whole blood collected in Sweden, where the highest UEOF was reported in women aged 18-44 [16]. Two hypotheses were proposed by Aro et al. [16] to explain the different UEOF concentrations between men and women. The first hypothesis is that a more frequent use of cosmetics and personal care products containing precursors (like

PAPs) and other unknown PFAS [43, 68] could lead to higher blood concentrations of unknown PFAS or precursors metabolism intermediates that are not investigated in the target PFAS analyses. This hypothesis is also supported by studies reporting associations between PFAS concentrations in the blood and the use of cosmetics and personal care products [69, 70]. In our study, the TOP assay showed only a minor contribution of precursors to the EOF in human serum with no differences between men and women and therefore this first hypothesis regarding precursors exposure can be discarded. However, the more frequent use of cosmetics might still be a possible explanation for the higher UEOF in women compared to men since cosmetics could also lead to exposure to yet unknown PFAS that are not oxidizable and therefore non-detectable in the TOP assay. A second explanation could lie in a difference in use of fluorinated pharmaceuticals between men and women, since sex differences in prescription are reported for several pharmaceuticals groups [71-76]. Additionally, differences in elimination kinetics between men and women for these yet unidentified fluorinated chemicals could also play a role.

The TOP assay showed a limited contribution of oxidizable precursors to the EOF. The TOP ranged from 0.00 to 1.85 ng F/ml and accounted for a portion of the EOF ranging from 0 to 4 % and for 0 to 100% of the UEOF. While the percentage contribution of TOP to the EOF stayed the same in 1986 (median: 1%, range: 1-3%), 2007 (median: 1%, range: 0-4%) and 2015 (median: 1%, range: 0-2%), the contribution to the UEOF changed between time points, ranging from 1 to 7% in 1986 (median: 2%), from 0 to 100% in 2007 (median: 18%) and from 0 to 37 % in 2015 (median: 3%).

The TOP assay results suggest the absence of pharmaceuticals containing -CF_3 groups, since these should be oxidizable to TFA, which was not detected after oxidation. However, Hammel et al. [77] found that, among 360 organofluorine pharmaceuticals approved and used globally between 1954 and 2021, 50% of these chemicals contained a single fluorine, 35% contained a

single aromatic fluorine and 10% contained more than three fluorine atoms. As most of these fluorinated pharmaceuticals contains only one fluorine, this large number of substances would go undetected in the TOP assay and fluorinated pharmaceuticals could still contribute to the observed UEOF.

The EOF accounted for 20 to 99% of the TF and the unidentified TF (UTF) ranged from 5 to 1194 ng F/mL. This fraction did not change between time-points and was not influenced by sex and mean age (Table S8). The UTF can include both inorganic fluoride and organic fluorinated compounds not extracted or partially extracted with acetonitrile. Fasting plasma fluoride concentrations reported in the literature range from 9.3 to 24 ng F/mL in areas with non-fluorinated water (water fluoride concentrations <0.3 mg/L) [62, 78]. Water in Norway is not fluorinated and a study from 2017 found that only 4 of 201 registered waterworks had fluoride exceeding the regulatory limit of 1.5 mg/L [79]. In humans the fluoride metabolism is not homeostatically regulated and plasma concentrations vary depending on levels of intake, deposition in hard tissues and excretion [80]. After ingestion, plasma concentrations take 3 to 6 hours to return to baseline values [78] . This could contribute to explain the variability observed in the UTF, since the serum collected in the Tromsø Study is from non-fasting individuals. Overall, these observations indicate the need of measuring fluoride when conducting FMB studies using TF.

4. Implications and limitations

The combined application of a set of targeted and group-wise analyses enabled the assessment of known and thus far unidentified organic fluorinated substances in human serum over three decades. No significant changes in TF were observed between 1986, 2007 and 2015. TF has the advantage of including both extractable and non-extractable fluorinated compounds. However, this advantage is lost if the fluoride contribution is not measured in human serum.

Therefore, in this case, EOF provides a better estimate of the overall exposure to organic fluorinated chemicals. The EOF concentrations were significantly higher in 1986 than in 2007 and 2015. At the same time, the relative contribution of target PFAS and UEOF varied across the time-points examined. While target PFAS concentrations were highest in 2007, the highest UEOF concentrations were observed in 1986.

Interestingly, the UEOF concentrations were higher in women than in men, opposite to what is commonly observed for target PFAS. UEOF concentrations might reflect exposure to unknown PFAS, to fluorinated pharmaceuticals, and elimination kinetics for these yet unidentified chemicals. The difference in sex for UEOF deserves attention also because Kaiser et al. [18] found UEOF in placental tissue and cord serum.

The addition of the TOP assay to the FMB added valuable information about the contribution of PFAA precursors to human exposure. Precursors accounted only for 0–4 % of the EOF, explaining a minor portion of the UEOF. However, it is important to highlight that the TOP assay provides only a lower bound estimate of precursors concentrations since conversion of precursors to PFAA can be incomplete [32, 81]. The TOP assay also provided key information on the structure of precursors, namely, minimal length of perfluorinated carbon chain and presence of sulfur.

The UEOF found in pooled serum clearly indicates the need for additional tools to assess previously unidentified fluorinated compounds. The use of suspect and non-target screening can be helpful in elucidating previously unidentified compounds. To close the FMB these screening strategies should not only focus on PFAS, but also on fluorinated pharmaceuticals and pesticides. In the present study, the lack of TFA increases after TOP assay points to the absence of CF_3-containing pharmaceuticals and pesticides. However, the yields of TFA from these chemicals in the TOP assay are not known yet and many pharmaceuticals and pesticides

containing a single fluorine cannot be detected with the TOP assay. Further studies are needed to understand the contribution of these chemicals to the EOF measured in human blood.

The use of pools instead of individual samples allowed for the screening of the Tromsø Study using the amounts of serum available from the biobank with a combination of multiple state-of-the-art analytical methods in a time- and cost-efficient manner. However, this was also a limitation, since the effect of many variables known to influence PFAS exposure (e.g., dietary habits, parity) could not be assessed using pools. In addition, the individuals in each pool covered a wide range of ages and this limited the investigation of the influence of age and birth cohorts on the different fluorine fractions measured.

Supporting information

Chemicals and consumables; characteristics of Tromsø Study samples and pools; quality control measures for TF, EOF, TOP assay and target PFAS; data evaluation equations; PFAS concentrations used for FMB calculations; TF and EOF concentrations in human blood from this study and from the literature; multiple linear regression coefficients estimates and 95% confidence intervals for ln(TF), ln(EOF), ln(∑12 PFAS), % UEOF and TOP; multiple linear regression (including sex and sampling year interaction terms) coefficients estimates and 95% confidence intervals for ln(∑12 PFAS) and % UEOF; UEOF concentrations in human blood from this study and from the literature;TF, EOF, TOP, ∑12PFAS and UEOF concentrations in serum pools containing the same individuals in 1986, 2007 and 2015; UpSet plot showing the intersection of PFAA with increased concentrations after oxidation; individual target PFAS in pooled serum from 1986, 2007 and 2015; $\sum_{12}$PFAS concentrations in relationship with mean age of the individuals in the pools; individual target PFAS concentrations in men and women.

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Supporting Information

Fluorine mass balance, including total fluorine, extractable organic fluorine, oxidizable precursors and target PFAS, in pooled human serum from the Tromsø population in 1986, 2007 and 2015

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Summary: 21 pages, 6 figures 10 tables

The following information is included:

1. Materials and methods

1.1. Chemicals and consumables

Acetonitrile (ACN, LiChrosolv®), tert-butyl methyl ether (MTBE, Suprasolv®), fuming hydrochloric acid (HCl, p.a. 37%) and sodium hydroxide (NaOH, EMSURE®, ≥ 99.0%) were obtained from Merck (Darmstadt, Germany). Sodium persulfate ($Na_2S_2O_8$, reagent grade, ≥ 98%, lot #BCCC8760) and ammonium acetate (NH_4OAc, LiChropur™) were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia (NH_3, solution 25%, AnalaR NORMAPUR) was purchased from VWR (Fontenay-sous-Bois, France). All native and isotopically labelled PFAS standards were obtained from Wellington Laboratories Inc. (Guelph, Ontario, Canada).

1.2. Serum samples and pooling strategy

Figure S1– Tromsø Study serum samples selection.

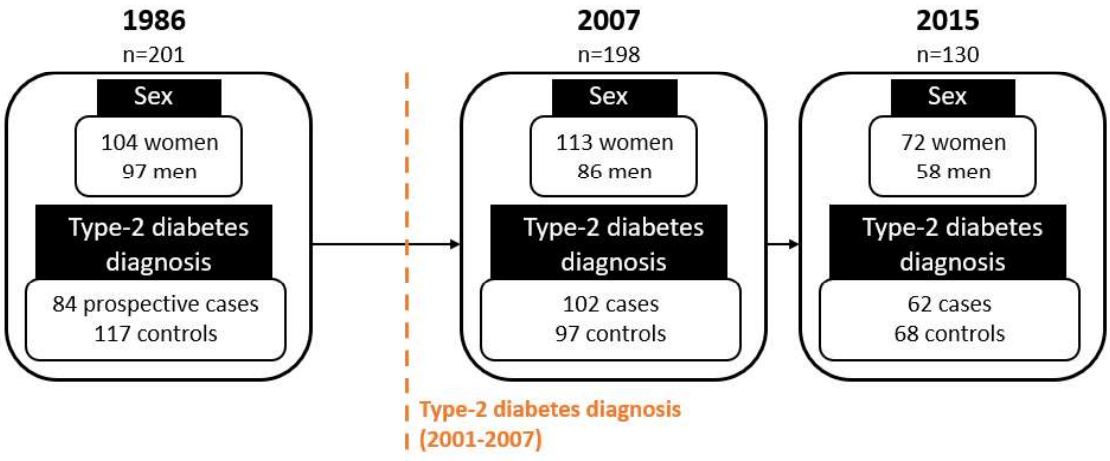

Table S1 – Characteristics of the Tromsø Study serum pools.

1986 (n=167)					2007 (n=175)					2015 (n=130)				
Pool ID	***n***	***Sex***	***Age mean (range)***	***Diabetes***	***Pool ID***	***n***	***Sex***	***Age mean (range)***	***Diabetes***	***Pool ID***	***n***	***Sex***	***Age mean (range)***	***Diabetes***
1	14	Women	36 (25-45)	Controls	**1**	14	Women	57 (46-66)	Controls	**1**	14	Women	65 (54-74)	Controls
2	12	Women	49 (46-57)	Controls	**2**	12	Women	70 (67-78)	Controls	**2**	12	Women	78 (75-86)	Controls
3	11	Women	41 (30-45)	Prospective cases	**3**	11	Women	62 (51-66)	Cases	**3**	11	Women	70 (59-74)	Cases
4	8	Women	49 (46-53)	Prospective cases	**4**	8	Women	70 (67-74)	Cases	**4**	8	Women	78 (75-82)	Cases
5	10	Men	34 (17-47)	Controls	**5**	10	Men	55 (38-68)	Controls	**5**	10	Men	63 (46-76)	Controls
6	10	Men	51 (48-55)	Controls	**6**	10	Men	72 (69-76)	Controls	**6**	10	Men	80 (77-84)	Controls
7	13	Men	44 (33-58)	Prospective cases	**7**	13	Men	65 (54-79)	Cases	**7**	13	Men	73 (62-87)	Cases
8	15	Women	31 (25-43)	Controls	**8**	15	Women	56 (46-64)	Controls	**8**	15	Women	63 (54-72)	Controls
9	15	Women	45 (43-47)	Controls	**9**	15	Women	67 (65-69)	Controls	**9**	15	Women	78 (74-82)	Controls
10	15	Women	52 (48-60)	Controls	**10**	15	Women	74 (70-81)	Controls	**10**	15	Women	70 (59-74)	Cases
11	15	Women	45 (43-48)	Prospective cases	**11**	15	Women	60 (51-64)	Cases	**11**	15	Women	78 (75-83)	Cases
12	15	Men	37 (17-49)	Controls	**12**	15	Women	67 (65-69)	Cases	**12**	15	Men	61 (46-73)	Controls
13	15	Men	55 (50-61)	Controls	**13**	15	Women	74 (70-81)	Cases	**13**	15	Men	79 (74-84)	Controls
14	15	Men	40 (25-48)	Prospective cases	**14**	15	Men	56 (38-66)	Controls	**14**	15	Men	81 (75-89)	Cases
15	15	Men	55 (49-60)	Prospective cases	**15**	15	Men	72 (68-76)	Controls					
					16	15	Men	62 (54-66)	Cases					
					17	15	Men	71 (67-76)	Cases					

Pool ID: cells highlighted in green indicate pools with same individuals across 1986, 2007 and 2015
n = number of individuals

1.3. TF quality control

A 9-point calibration curve ranging from 2.5 to 2500 ng of NaF in water (R^2>0.999) was included at the beginning and end of each run. Quality control measures for each run included: (1) three sample boat blanks for limit of detection (LOD) calculation, (2) two sample boats spiked with 100 ng of PFOS standard, and (3) three measurements of a certified reference material (fluorine in clay, CRM 461). Blanks ranged between 18 and 21 ng F/mL (n=9) and LOD (average boat blanks + 3 times the standard deviation of the blanks) ranged between 23 and 25 ng F/mL. The recovery of the PFOS standard (120 ± 6 %, n=6) confirmed complete combustion and measurements of the certified reference material showed good accuracy and precision (recovery: 123 ± 9 %, n=9).

1.4. EOF quality control

For each extraction batch (14 serum samples), the quality control measures included: (1) three extraction blanks, (2) three reference serum samples not spiked, (3) one reference serum sample spiked with 239 ng of PFOS, (4) one reference serum sample spiked with 500 ng of NaF. The reference serum was obtained from the Arctic Monitoring and Assessment Programme (AMAP) Ring Test for Persistent Organic Pollutants [1]. Each extraction batch was run separately and included a calibration curve at the beginning and end of the run (2.5-1000 ng of NaF in water, R^2>0.999) and two sample boats spiked with 100 ng of PFOS standard. The extraction blanks ranged from 5 to 7 ng F/mL (n=12) and the EOF LOD (average extraction blanks + 3 times the standard deviation of the blanks) ranged from 6 to 9 ng F/mL. The analysis of the reference serum samples spiked with PFOS confirmed good recovery and reproducibility of the EOF analysis in human serum (recovery: 77 ± 14 %, n=8). The analysis of the controls spiked with NaF confirmed the removal of fluoride upon extraction (NaF recoveries ranging from 0 to 2 %, n=4).

1.5.TOP assay quality control

For each TOP assay batch (18 samples), a blank and an AMAP reference serum sample were included and processed as the samples. Blanks before and after oxidation showed low levels of PFAA (Table S2). LODs were calculated as the average concentration in the blanks plus 3 times the standard deviation of the blanks and in case of no detection in the blanks, LODs were calculated by multiplying the noise of the blanks by 3. LODs before and after oxidation were comparable for most compounds (Table S2). Measured PFAA concentrations before oxidation in the AMAP serum samples were within -/+ 20% of the reference values. Mean recoveries before TOP assay ranged from 61 to 78 % and mean recoveries after TOP assay ranged from 55 to 65 %. Model precursors spiking oxidation experiments were performed as part of the

validation described in our method paper and showed complete conversion for all spiked precursors and yields of PFAA ranging from 35-100% [2].

Table S2 - Average blank concentrations and LODs before and after TOP assay in ng/mL of serum (n=3).

Compound	Before TOP assay		After TOP assay	
	Blank concentration	LOD	Blank concentration	LOD
PFBA	0.15	0.47	0.12	0.49
PFPeA	0.20	0.32	0.26	0.47
PFHxA	0.03	0.10	0.13	0.39
PFHpA	0.01	0.02	0.02	0.13
PFOA	0.05	0.10	0.08	0.18
PFNA	0.00	0.02	0.01	0.03
PFDA	0.00	0.02	0.03	0.10
PFUnDA	0.00	0.02	0.00	0.04
PFDoDA	0.00	0.02	0.00	0.04
PFTrDA	0.00	0.07	0.00	0.09
PFTeDA	0.00	0.13	0.00	0.13
PFBS	0.00	0.04	0.02	0.08
PFPeS	0.00	0.07	0.00	0.07
PFHxS	0.10	0.13	0.01	0.04
PFHpS	0.00	0.03	0.00	0.03
PFOS	0.09	0.16	0.06	0.14
PFNS	0.00	0.04	0.00	0.04
PFDS	0.00	0.05	0.00	0.05

Table S3 – Recoveries in pooled serum samples before and after TOP assay (n=46).

Compound	Before TOP assay	After TOP assay
^{13}C-PFBA	74 ± 7	58 ± 10
^{13}C-PFPeA	78 ± 5	62 ± 5
^{13}C-PFHxA	75 ± 7	63 ± 4
^{13}C-PFHpA	70 ± 5	65 ± 4
^{13}C-PFOA	73 ± 6	62 ± 6
^{13}C-PFNA	71 ± 5	58 ± 5
^{13}C-PFDA	78 ± 5	55 ± 3
^{13}C-PFUnDA	61 ± 8	57 ± 5
^{13}C-PFDoDA	72 ± 6	61 ± 7
^{13}C-PFTeDA	75 ± 6	58 ± 7
^{13}C-PFHxS	74 ± 5	62 ± 6
^{13}C-PFOS	75 ± 3	61 ± 8

1.6.Target PFAS quality control

Target PFAS analyses on the EOF extracts included also the EOF extraction blanks (n=9). No PFAA were detected in the blanks and the LODs were calculated using the standard error of the regression divided the slope of the calibration curve multiplied by 3. LODs ranged from 0.03 to 0.13 ng/mL (Table S4). Measured PFAA concentrations in the AMAP serum samples use as quality control were within -/+ 20% of the reference values.

After the TOP assay the extracts were also analysed for C_2 and C_3-PFAA using a Raptor Polar X column. Trifluoroacetic acid (TFA) was analysed in a 5 minute isocratic run with 80 % 2mM ammonium acetate in methanol and 20 % 2mM ammonium acetate in 90:10 water:methanol. Perfluoropropionic acid (PFPrA), trifluoromethane sulfonic acid (TFMS), difluoro (perfluoromethoxy) acetic acid (1,2-PFECA), difluoroacetic acid (DiFA) and chlorodifluoro acetic acid (Cl-DiFA) were analysed in a 10 minute isocratic run using 80% 60:40

methanol:water with 0.05% formic acid and 20% 10 mM ammonium formate in water with 0.05% formic acid, based on an application note from Restek [3]. For these analyses, serum extracts were spiked with ^{13}C-TFA before oxidation and recoveries ranged from 56 to 65 % (n=46). Concentrations in the blanks ranged from 0.00 to 0.25 ng/mL. LODs were calculated as the average concentration in the blanks plus 3 times the standard deviation of the blanks and in case of no detection in the blanks, LODs were calculated by multiplying the noise of the blanks by 3 (Table S4).

Table S4 – Target PFAS analysed on EOF extracts by UHPLC-Orbitrap.

Abbreviation	Name	LOD (ng/mL)
PFCA (Perfluoroalkyl carboxylic acids)		
PFBA	Perfluorobutanoic acid	0.07
PFPeA	Perfluoropentanoic acid	0.06
PFHxA	Perfluorohexanoic acid	0.07
PFHpA	Perfluoroheptanoic acid	0.07
PFOA	Perfluorooctanoic acid	0.06
PFNA	Perfluorononanoic acid	0.07
PFDA	Perfluorodecanoic acid	0.09
PFUnDA	Perfluoroundecanoic acid	0.10
PFDoDA	Perfluorododecanoic acid	0.10
PFTrDA	Perfluorotridecanoic acid	0.10
PFTeDA	Perfluorotetradecanoic acid	0.13
PFPeDA	Perfluoropentadecanoic acid	0.13
PFHxDA	Perfluorohexadecanoic acid	0.14
PFOcDA	Perfluorooctadecanoic acid	0.13
PFSA (Perfluoroalkyl sulfonic acids)		
PFBS	Perfluorobutane sulfonic acid	0.06
PFPeS	Perfluoropentane sulfonic acid	0.06
PFHxS	Perfluorohexane sulfonic acid	0.06
PFHpS	Perfluoroheptane sulfonic acid	0.06
PFOS	Perfluorooctane sulfonic acid	0.03
PFNS	Perfluorononane sulfonic acid	0.04
PFDS	Perfluorodecane sulfonic acid	0.05
PFUnDS	Perfluoroundecane sulfonic acid	0.06
PFECA (Perfluoroalkyl ether sulfonic acids)		
GenX	Ammonium perfluoro-4,8-dioxa-3H-nonanoic acid	0.08
ADONA	Perfluoro-4,8-dioxa-3H-nonanoic acid	0.08
FTCA (Fluorotelomer carboxylic acids)		
3:3 FTCA	3:3 Fluorotelomer carboxylic acid	0.06
5:3 FTCA	5:3 Fluorotelomer carboxylic acid	0.08
7:3 FTCA	7:3 Fluorotelomer carboxylic acid	0.08
FTS (Fluorotelomer sulfonates)		
4:2 FTS	4:2 Fluorotelomer sulfonic acid	0.06
6:2 FTS	6:2 Fluorotelomer sulfonic acid	0.08
8:2 FTS	8:2 Fluorotelomer sulfonic acid	0.08
Perfluorooctane sulfonamido substances		
FOSA	Perfluorooctane sulfonamide	0.07
Me-FOSA	N-Methyl perfluorooctane sulfonamide	0.07
Et-FOSA	N-Ethyl perfluorooctane sulfonamide	0.07
FOSAA	Perfluorooctane sulfonamidoacetic acid	0.06
Me-FOSAA	N-Methyl perfluorooctane sulfonamidoacetic acid	0.06
Et-FOSAA	N-Ethyl perfluorooctane sulfonamidoacetic acid	0.06
Me-FOSE	N-Methyl perfluorooctane sulfonamido ethanol	0.08
Et-FOSE	N-Ethyl perfluorooctane sulfonamido ethanol	0.08
Cl-PFAES		
9Cl-PF3ONS	9Cl-Perfluoro-3-oxononane sulfonic acid	0.10
11Cl-PF3OUdS	11Cl-Perfluoro-3-oxoundecane sulfonic acid	0.10
PAPs		
4:2 monoPAP	4:2 Fluorotelomer phosphate monoester	0.10
4:2 diPAP	4:2 Fluorotelomer phosphate diester	0.10
6:2 monoPAP	6:2 Fluorotelomer phosphate monoester	0.10
6:2 diPAP	6:2 Fluorotelomer phosphate diester	0.10
6:2/8:2 diPAP	6:2/8:2 Fluorotelomer phosphate diester	0.10
6:2/10:2 diPAP	6:2/10:2 Fluorotelomer phosphate diester	0.12
6:2/12:2 diPAP	6:2/12:2 Fluorotelomer phosphate diester	0.12

6:2/14:2 diPAP	6:2/14:2 Fluorotelomer phosphate diester	0.12
8:2 diPAP	8:2 Fluorotelomer phosphate diester	0.13
8:2/10:2 diPAP	8:2/10:2 Fluorotelomer phosphate diester	0.13
10:2 monoPAP	10:2 Fluorotelomer phosphate monoester	0.13
10:2 diPAP	10:2 Fluorotelomer phosphate diester	0.13

Table S5 - Average blank concentrations and LODs before and after TOP assay in ng/mL of serum (n=3).

Compound	Blank concentration	LOD
TFA	0.28	0.32
PFPrA	0.10	0.13
TFMS	0.00	0.07
1,2-PFECA	0.00	0.07
DiFA	0.00	0.07
Cl-DiFA	0.00	0.07

1.7. Data evaluation

For comparison with EOF values, target PFAS concentrations measured in the EOF extracts and ΔPFAA concentrations from the TOP assay were converted to F equivalents using the following equation:

$$Concentration\left(\frac{ng\ F}{mL}\right) = \frac{concentration\left(\frac{ng}{mL}\right) \cdot \mathrm{nF} \cdot AW_F}{MW_{PFAS}} \quad \text{(S1)}$$

where nF is the number of fluorine atoms in the PFAS structure, A_F is the atomic weight of fluorine and MW_{PFAS} is the molecular weight of the PFAS which concentration is being converted.

Differences in TF, EOF, $\sum_{12}$ PFAS, unidentified EOF and TOP between sampling years were assessed by multiple linear regression to account for the influence of sex and age (as weighted mean of the age of the individuals in the pools expressed in years) using the following equation:

$$y = \beta_0 + \beta_1 dummy\ 1 + \beta_2 dummy\ 2 + \beta_3\ sex + \beta_4\ age \quad \text{(S2)}$$

where y is the log transformed concentration for TF, EOF and $\sum_{12}$ PFAS, the ΔPFAA concentration in ng/mL for TOP and the percentage contribution to EOF for UEOF; β0 is the intercept of the multiple linear regression; β_1, β_2, β_3 and β_4 are the regression coefficients for the predictor variables; dummy 1 is a dummy variable equal to 1 if sampling year is 1986, equal to 0 if sampling year is 2007 or 2015; dummy 2 is a dummy variable equal to 1 if sampling year is 2015, equal to 0 if sampling year is 1986 and 2007; sex is categorical variable equal to 0 for women and equal to 1 for men; age is the weighted mean age of the individuals making up each pool expressed in years.

When sex was a significant predictor, differences in concentrations between men and women at each sampling year were assessed by adding an interaction term between sex and each sampling year dummy variable as described by equation S3.

$$y = \beta_0 + \beta_1 dummy\ 1 + \beta_2 dummy\ 2 + \beta_3\ sex + \beta_4\ age + \beta_5\ dummy1\ sex + \beta_6\ dummy2\ sex \quad \text{(S3)}$$

2. Results

Table S6 – PFAS concentrations (ng/mL) used for fluorine mass-balance calculations (concentrations are not recovery corrected).

	1986 (n=15)				2007 (n=17)				2015 (n=14)			
	DF	Median	Mean	Range	DF	Median	Mean	Range	DF	Median	Mean	Range
PFHpA	0/15	-	-	-	0/17	-	-	-	0/14	-	-	-
PFOA	15/15	1.60	1.50	0.88-2.04	17/17	2.32	2.40	2.00-2.96	14/14	1.52	1.56	1.12-2.24
PFNA	15/15	0.24	0.25	0.08-0.64	17/17	1.04	0.99	0.72-1.52	14/14	1.14	1.18	0.68-1.60
PFDA	1/15	<0.09	<0.09	<0.09-0.24	17/17	0.40	0.39	0.20-0.84	14/14	0.50	0.50	0.20-0.84
PFUnDA	15/15	0.32	0.32	0.12-0.56	17/17	0.60	0.60	0.24-1.56	14/14	0.62	0.58	0.24-1.20
PFDoDA	0/15	-	-	-	0/17	-	-	-	0/14	-	-	-
PFHxS	15/15	0.40	0.38	0.16-0.72	17/17	1.44	1.59	1.04-4.68	14/14	1.24	1.36	0.72-3.12
PFHpS	4/15	<0.03	<0.03	<0.03-0.08	17/17	0.20	0.17	0.04-0.36	14/14	0.08	0.09	0.04-0.20
br-PFOS	15/15	3.68	3.58	2.48-5.16	17/17	5.44	5.26	3.68-7.16	14/14	3.14	3.40	2.56-4.88
lin-PFOS	15/15	10.9	10.4	6.72-15.4	17/17	16.2	16.5	11.1-30.2	14/14	10.4	11.3	5.52-18.8
FOSAA	9/15	0.08	0.08	<0.06-0.20	0/17	-	-	-	0/14	-	-	-
Me-FOSAA	14/15	0.16	0.13	<0.06-0.28	10/17	0.08	0.08	<0.06-0.20	0/14	-	-	-
Et-FOSAA	15/15	0.28	0.27	0.12-0.52	0/17	-	-	-	0/14	-	-	-
∑ 12 PFAS	15/15	17.8	17.2	11.0-24.1	17/17	27.9	28.3	21.5-46.1	14/14	19.2	20.3	11.4-30.0

DF = detection frequency: number of pools with PFAS concentration > LOD.

Table S7 – Descriptive statistics for TF and EOF concentrations (ng F/mL) in the Tromsø Study pooled serum samples from 1986, 2007 and 2015 and in samples from previous studies available in the literature (n=number of pools/number of individual samples).

Study	Country	Sampling year	Matrix	n	TF (ng F/mL)			EOF (ng F/mL)		
					Median	Mean	Range	Median	Mean	Range
This study	Norway	1986	Serum (pooled)	15	79.1	112	<25.0-1330	22.2	23.3	13.3-45.3
This study	Norway	2007	Serum (pooled)	17	74.2	74.8	<25.0-1212	20.8	20.5	16.2-30.3
This study	Norway	2015	Serum (pooled)	14	68.3	71.6	<25.0-265	18.5	18.4	12.6-22.6
Miyake et al. (2007)	Japan	2003-2004	Whole blood	3	208	214	181-262	<6	<6	<6-8.89
Miyake et al. (2007)	USA	2001	Plasma	4	149	163	140-189	45.2	38.3	17.8-59.0
Yeung et al. (2008)	China	2004	Whole blood	30	-	-	60.6-166	-	-	<6-43.4
Yeung and Mabury (2016)	China	2004	Whole blood	34	-	-	-	17	18.4	8.22-94.4
Yeung and Mabury (2016)	Germany (Halle)	1995-2009	Plasma	42	-	-	-	-	15.9	5.29-43.9
Yeung et al. (2016)	Germany (Munster)	1982-2009	Plasma	80	-	-	-	-	23.7	9.20-115
Miaz et al. (2020)	Sweden	1996-2017	Serum (pooled)	57	-	-	-	-	-	8.10-32.0
Aro et al. (2021)	Sweden	2015	Whole blood	9	-	-	-	-	24.8	17.6-37.8
Aro et al. (2021)	Sweden (Ronneby)	2014-2016	Whole blood	20	-	-	-	-	234	<107-592
Aro et al. (2021)	Sweden	2018-2019	Whole blood	130	-	-	-	-	-	0.51-48.7
Kaiser et al. (2021)	Austria	2021	Serum (pooled)	6	-	-	-	-	3.83	2.85-7.17

Table S8 – Multiple linear regression coefficients estimates and 95% confidence intervals for ln(TF), ln(EOF), ln(∑12 PFAS), % UEOF and TOP in pooled serum samples from the Tromsø Study.

	ln(TF)	ln(EOF)	ln((∑12 PFAS)	% UEOF	TOP
	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)
β_0 (intercept)	1.17 (2.68 to 5.03)	2.55 (1.91 to 3.20)	2.76 (2.33 to 3.08)	61.4 (18.8 to 104)	0.49 (-0.48 to 1.45)
β_1 (1986-2007)	1.41 (-0.15 to 2.97)	0.29* (0.03 to 0.55)	-0.48*** (-0.61 to -0.35)	22.8* (5.60 to 40.0)	0.14 (-0.25 to 0.53)
β_2 (2015-2007)	-0.39 (-1.47 to 0.68)	-0.16 (-0.34 to 0.02)	-0.41*** (-0.51 to -0.30)	18.2** (6.35 to 30.0)	0.02 (-0.24 to 0.29)
β_3 (sex)	0.04 (-0.77 to 0.85)	-0.05 (-0.18 to 0.09)	0.18*** (0.10 to 0.26)	-14.3** (-23.3 to -5.32)	-0.16 (-0.36 to 0.05)
β_4 (age mean)	0.05 (-0.01 to 0.11)	0.01 (-0.002 to 0.017)	0.02*** (0.01 to 0.02)	-0.64 (-1.27 to 0.01)	-0.001 (-0.015 to 0.013)
R^2	0.084	0.209	0.796	0.594	0.091
F-test p-value	0.445	0.042	0.000	0.000	0.409

*p < 0.05
**p < 0.01
*** p < 0.001

Table S9 – Multiple linear regression (including sex and sampling year interaction terms) coefficients estimates and 95% confidence intervals for ln(∑12 PFAS) and % UEOF in pooled serum samples from the Tromsø Study.

	ln((∑12 PFAS)	**% UEOF**
	Estimate (95% CI)	Estimate (95% CI)
β_0 (intercept)	2.72 (2.34 to 3.11)	64.4 (20.3 to 109)
β_1 (1986-2007)	-0.17 (-0.35 to 0.01)	18.3 (-2.27 to 38.8)
β_2 (2015-2007)	-0.40*** (-0.53 to -0.27)	17.1* (1.91 to 32.3)
β_3 (2007 sex)	0.16* (0.02 to 0.28)	-18.3* (-33.4 to -3.14)
β_4 (age mean)	0.02*** (0.01 to 0.02)	-0.66* (-1.31 to -0.01)
β_5 $(1986\ sex)$	0.08 (-0.11 to 0.27)	9.24 (-12.7 to 31.2)
β_6 $(2015\ sex)$	-0.01 (-0.02 to 0.18)	3.04 (-19.4 to 25.5)
R^2	0.802	0.602
F-test p-value	0.000	0.000
*p < 0.05 **p < 0.01 *** p < 0.001		

Table S10 – Descriptive statistics for UEOF concentrations (ng F/mL and/or %) in the Tromsø Study pooled serum samples from 1986, 2007 and 2015 and in samples from previous studies available in the literature (n=number of pools/number of individual samples).

Study	**Country**	**Sampling year**	**Matrix**	**n**	**UEOF**		
					Median	Mean	Range
This study	Norway	1986	Serum (pooled)	15	10.5 ng F/mL 46%	10.9 ng F/mL 46%	2.93-34.8 ng F/mL 21-77%
This study	Norway	2007	Serum (pooled)	17	2.26 ng F/mL 10%	3.17 ng F/mL 14%	0.00-10.9 ng F/mL 0-40%
This study	Norway	2015	Serum (pooled)	14	7.54 ng F/mL 37%	5.32 ng F/mL 27%	0.00-9.74 ng F/mL 0-56%
Miyake et al. (2007)	Japan	2003-2004	Whole blood	3	-	-	0.00-1.38 ng F/mL 0-15%
Miyake et al. (2007)	USA	2001	Plasma	4	-	-	0.00-4.40 ng F/mL 0-15%
Yeung et al. (2008)	China	2004	Whole blood	30	-	-	15-43%
Yeung and Mabury (2016)	China	2004	Whole blood	34	-	-	14-69%
Yeung and Mabury (2016)	Germany (Halle)	1995-2009	Plasma	42	-	-	0.0-9.5 ng F/mL
Yeung et al. (2016)	Germany (Munster)	1982-2009	Plasma	80	-	-	0.0-9.9 ng F/mL
Miaz et al. (2020)	Sweden	1996-2017	Serum (pooled)	57	-	-	11-75%
Aro et al. (2021)	Sweden	2015	Whole blood	9	-	84%	71-97%
Aro et al. (2021)	Sweden (Ronneby)	2014-2016	Whole blood	20	-	37%	0-76%
Aro et al. (2021)	Sweden	2018-2019	Whole blood	130	-	0-99%	-
Kaiser et al. (2021)	Austria	2021	Serum (pooled)	6	-	1.17 ng F/mL 24%	-

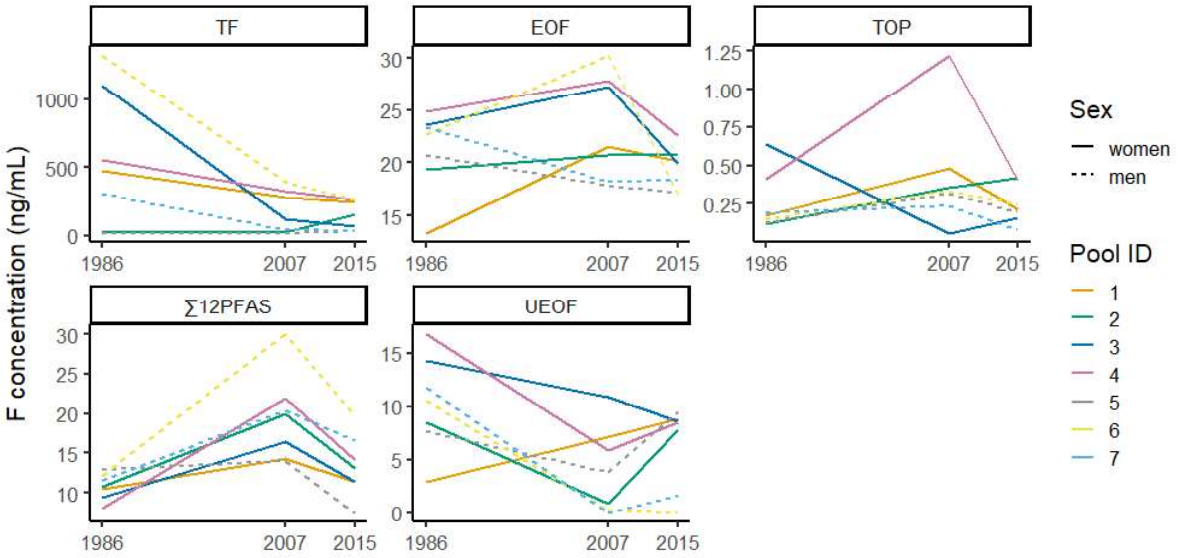

Figure S2 – TF, EOF, TOP, $\sum_{12}$PFAS and UEOF concentrations (ng F/mL) in serum pools from the Tromsø Study containing the same individuals in 1986, 2007 and 2015.

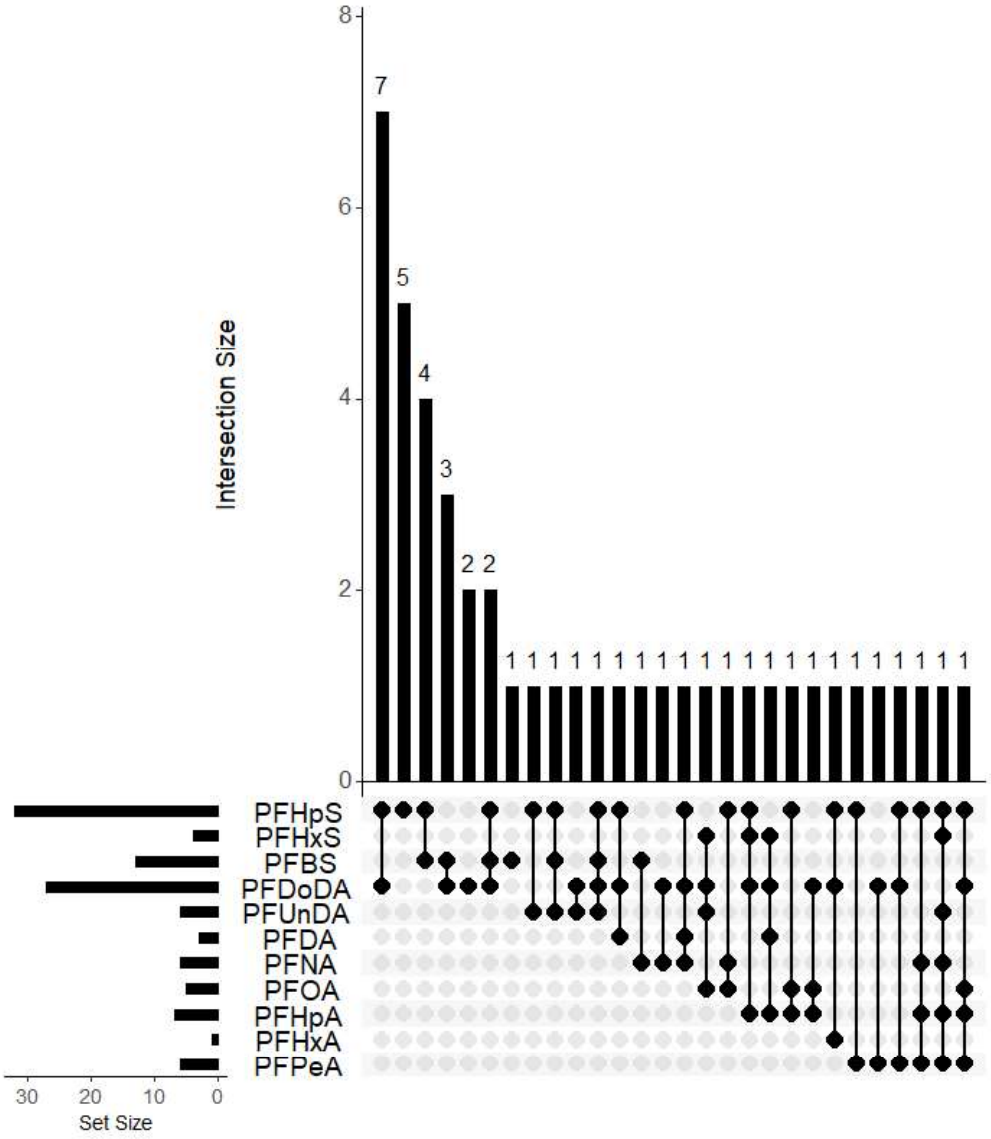

Figure S3 - UpSet plot showing the intersection of PFAA with increased concentrations after oxidation. The bar chart shows the number of pools with increases in concentrations of a combination of PFAA. The graphical table underneath indicates the PFAA combinations (black dots and lines). The frequency count of each PFAA across all subsets is shown as a smaller bar chart on the left side of the graphical table.

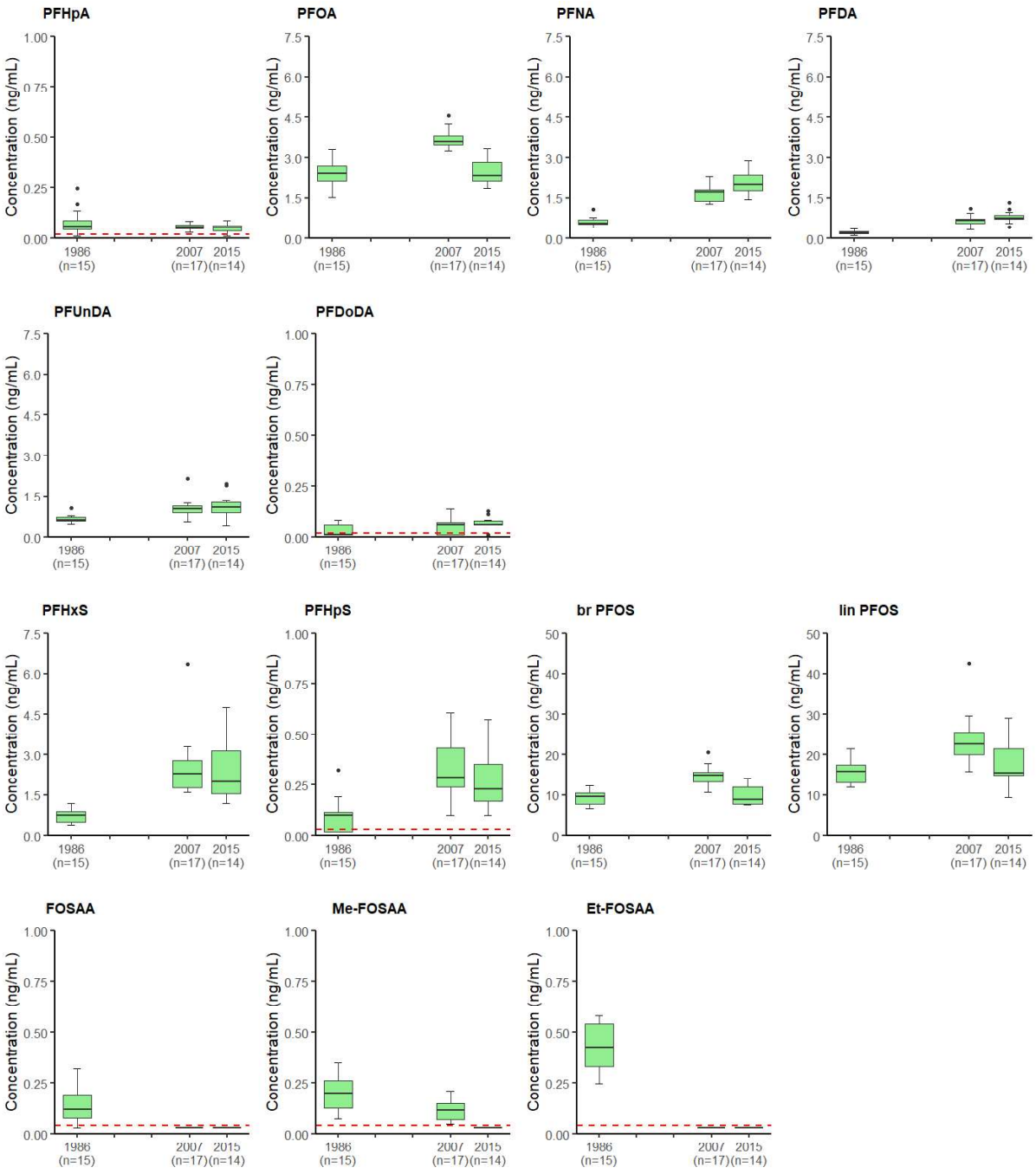

Figure S4 – Target PFAS (ng/ml) in pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015.

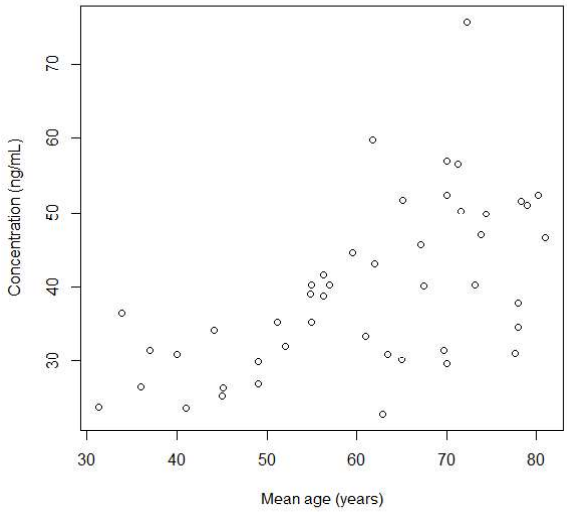

Figure S5 – Concentrations of ∑12 PFAS in pooled serum samples from the Tromsø Study collected in 1986, 2007 and 2015 in relationship with mean age of the individuals in the pools in years.

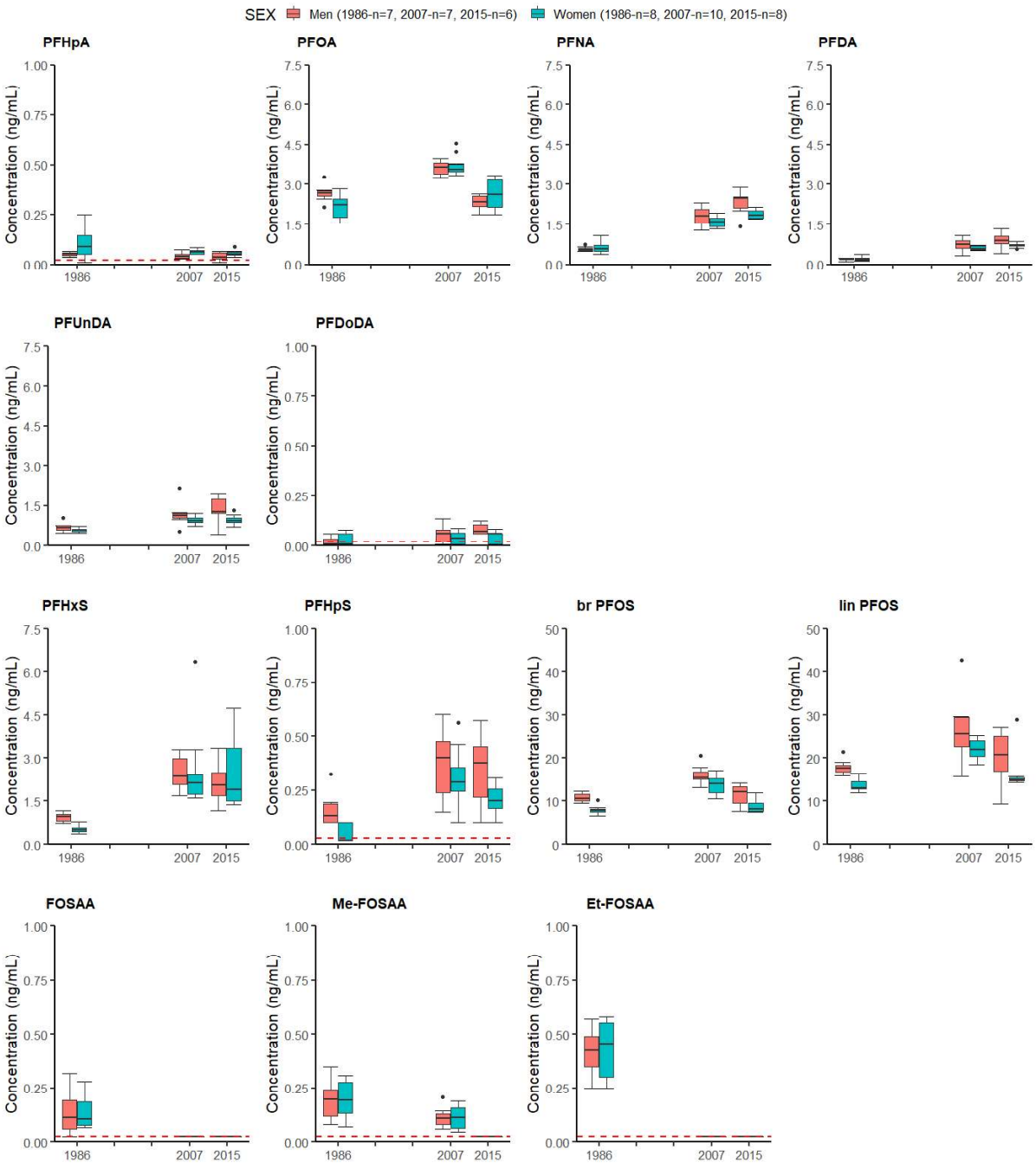

Figure S6 – Target PFAS concentrations (ng/mL) in men and women from the Tromsø Study in 1986, 2007 and 2015.

References

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Paper 3

Combining advanced analytical methodologies to uncover the contribution of suspect PFAS and fluorinated pharmaceuticals to extractable organic fluorine in pooled human serum from the Tromsø Study between 1986 and 2015

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1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of industrial chemicals that have received growing international attention due to their potential health and environmental impacts. PFAS are used throughout society, including both industrial processes and consumer products [1]. The most well studied PFAS, the perfluoroalkyl acids (PFAA), are highly persistent and have been detected globally in humans and wildlife, including remote environments [2, 3]. Exposure to PFAA has been linked to a variety of adverse health effects, such as immune system dysfunction, liver damage, thyroid disease, increased cholesterol levels, renal and testicular cancer, reproductive and developmental effects [4, 5].

Based on the concern surrounding PFAS exposure, a number of PFAA have been regulated nationally and/or internationally. Use and production of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been reduced in the European Union and the United States in the early 2000s [6]. Additionally, PFOS, PFOA and perfluorohexane sulfonic acid (PFHxS) were listed in the Stockholm Convention on persistent organic pollutants (POPs) in 2009, 2019 and 2022 respectively [7, 8]. Long chain PFCA with carbon chain lengths from 9 to 21 have also been proposed for listing under the Stockholm Convention [9]. Most recently, a class-wide restriction proposal for PFAS was submitted for consideration by the European Chemicals Agency (ECHA), which is expected to go into force as early as 2025 [10].

While temporal trend studies have shown that PFOS and PFOA concentrations in human blood have been declining globally in response to changes in production and regulatory initiatives [6, 11-13], a growing number of studies has also reported significant quantities of unidentified extractable organic fluorine (UEOF) in human blood [11, 14-19], which appears to be increasing in recent years [11, 16, 20]. This discrepancy suggests the occurrence of PFAS which are not captured by targeted analyses. Over 4700 PFAS were available in the global market as of 2018 [21] and over 750 different PFAS have been identified in consumer products,

environmental and biological samples [22, 23] but are not routinely analysed in biomonitoring studies [24].

However, the UEOF in human serum could also be explained by the presence of fluorinated pharmaceuticals. The C-F bond is increasingly used in pharmaceuticals to improve their effectiveness and 25% of the pharmaceuticals globally available in 2021 contained at least one fluorine atom [25-27]. Recently, Spaan et al. [28] found that fluorinated pharmaceuticals and pesticides contributed up to 22% of the EOF in wastewater treatment plant sludge. Additionally, Pennoyer et al. [29] modelled that the contribution of the nine most prescribed fluorinated pharmaceuticals in the United States to EOF in human serum could range from 0.1 to 55.6 ng F/mL but observed discrepancies between these estimates and the measured EOF (2.02-11.22 ng F/mL). Since there is no agreement on a common PFAS definition and different fluorinated pharmaceuticals cannot uniformly be classified as PFAS [30], for the scope of this paper, all fluorinated pharmaceuticals will not be included among PFAS independently from their structure.

The present study builds upon a previous fluorine mass-balance study we performed on pooled serum samples from the Tromsø Study collected between 1986 and 2015, which showed that EOF concentrations were only partially explained by 12 known target PFAS (23-100 %) and unknown total oxidizable precursors (0-4 %) [20]. In the present study, the same extracts were analysed using direct infusion Fourier-transform ion cyclotron resonance (FT-ICR-MS) and liquid chromatography–high resolution mass spectrometry (LC-Orbitrap-HRMS). These measurements were used to perform suspect screening of more than 5000 PFAS and 342 fluorinated pharmaceuticals and their known metabolites. The goal was to identify novel PFAS and fluorinated pharmaceuticals in human serum and estimate their contribution to the EOF. Additionally, a selection of model CF_3-containing pharmaceuticals and pesticides was oxidized

with the total oxidizable precursors (TOP) assay to understand if this method is applicable for the detection of their presence in human blood.

2. Materials and methods

2.1. Pooled serum samples

In the present work, pooled serum samples from a previous fluorine mass-balance study were used [20]. These pools were obtained from a selection of individual serum samples from the Tromsø Study collected in 1986, 2007 and 2015 based on a case-control study design on type 2 diabetes. The cases were diagnosed with diabetes between 2001 and 2007, while the controls had no diagnosis reported in the local diabetes registry. The selection of samples included 104 women and 97 men in 1986, 113 women and 86 men in 2007, and 72 women and 58 men in 2015. The age of the individuals ranged from 17 to 61 years old in 1986 (mean: 46), from 38 to 81 in 2007 (mean: 67) and from 46 to 89 in 2015 (mean: 72). From this selection, 472 individual samples (1986 [n=167], 2007 [n=175], 2015 [n= 130]) were pooled based on sampling year, sex, age and type 2 diabetes diagnosis. Detailed information about the pools can be found in our previous study [20]. The present study obtained informed consent from all participants and was approved by the Regional Committee for Medical Research Ethics (REK, case number: 2020/13188).

2.2. Suspect screening and fluorine mass-balance

Pooled serum samples were analyzed using a three-step suspect screening approach to evaluate the presence of novel PFAS and fluorinated pharmaceuticals (Figure S1). The first step consisted of a broad suspect screening using FT-ICR-MS measurements and a suspect list of 5000 PFAS. From this first step, a reduced list of suspect PFAS was prioritized for the second step, that consisted of a more focused screening using LC-Orbitrap-HRMS. This second

suspect screening step also included a second list of PFAS compiled from the literature and a list of fluorinated pharmaceuticals. The third step was to confirm the suspects with standards or other diagnostic evidence (such as MS2 spectra, retention time, presence/absence after TOP assay) and assign suspect identification confidence levels (CLs) according to the Schymanski scale [31]. The suspects confirmed with a CL≥3 were quantified and the concentrations were compared to previous fluorine mass-balance measurements, including total fluorine (TF), EOF, TOP assay and target PFAS measured in the same pools (Figure 1) [20].

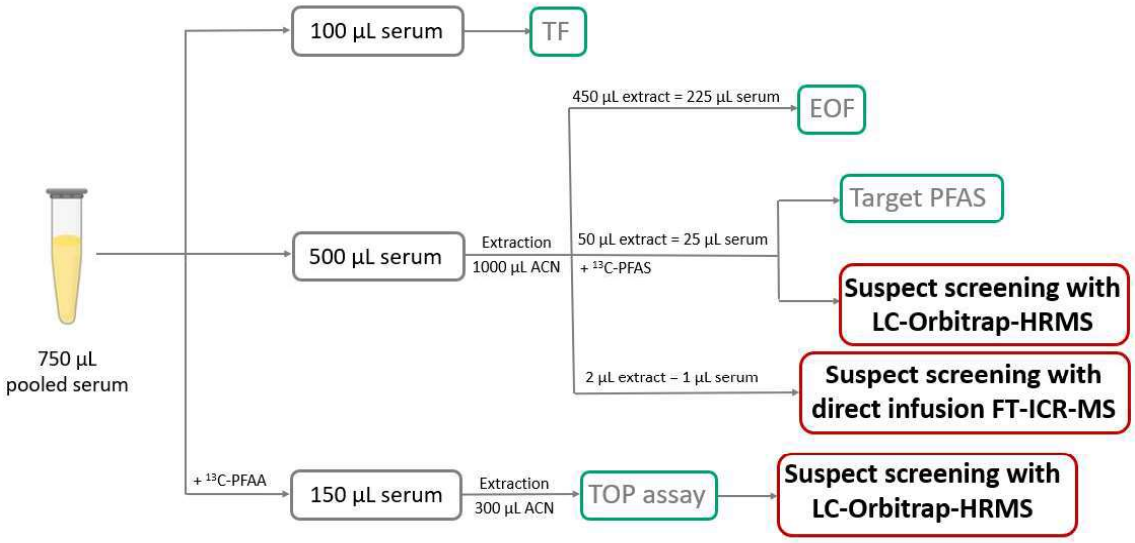

Figure 1 – Fluorine mass-balance study design. The measurements highlighted in red are discussed in the present study while the measurements highlighted in green are discussed in a previous fluorine mass-balance paper [20].

2.3.1. FT-ICR measurements

For FT-ICR measurements, 20 pools with the highest UEOF in absolute value and/or percentage were selected. An aliquot of 2 µL of EOF extract was diluted with 198 µL of 50:50 methanol:milliQ water prior to injection into an FT-ICR mass spectrometer using a nano-LC system. The mass spectrometer was equipped with a dynamically harmonized analyzer cell

(solariX XR, Bruker Daltonik GmbH, Bremen, Germany) and 12 Tesla superconducting magnet (Bruker Biospin, Wissembourg, France). The capillary voltage was 4.2 kV, the nebulizer gas pressure 1.0 bar, the drying gas temperature 250 °C and the dry gas flow rate 8.0 L/min. Data acquisition was performed with the ocular method developed by Palacio Lozano et al. [32] In the ocular method the mass range is divided in segments to maintain near constant resolving power and increase sensitivity. In this case the mass range from 150 to 900 m/z was divided into 16 mass segments. The mass range width of the segments was 30 Da from 150 to 300 m/z, 50 Da from 300 to 600 m/z and 150 Da from 600 to 900 m/z.

2.3.2. LC-Orbitrap measurements

All 46 serum pools were first analyzed using a Dionex UltiMate 3000 Ultrahigh performance liquid chromatograph coupled to a Q Exactive HF hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described by Miaz et al. [11] in full scan with data dependent MS2 (ddMS2) acquisition. Thereafter, serum pools were re-analyzed on a different LC-Orbitrap system for ddMS2 of suspect masses identified from the above described HRMS measurements for which MS2 was not collected in the first LC-Orbitrap-HRMS run (see suspect screening data processing section). This run also included the extracts of the pools after processing with the TOP assay. The instrument was a Vanquish UHPLC coupled with an Orbitrap Exploris 120 (Thermo Fisher Scientific, Waltham, MA, USA). The LC was operated with an Acquity UPLC HSS T3 column (2.1×100 mm, 1,8 μm) equipped with a Waters Van guard HSS T3 guard column (2.1×5 mm, 1.8 μm). The LC gradient described by Hanssen et al. [33] using 2mM NH_4OAc in 90:10 water:acetonitrile and 2mM NH_4OAc in 99:1 acetonitrile:water as mobile phases. The MS acquisition parameters are reported in table S1.

2.3.3. Suspect screening data processing

The FT-ICR-MS data were screened for 5000 suspect PFAS masses using a workflow developed by Dudášová et al. Data were calibrated using a list of 226 fatty acids and sulfonates. After calibration the data were screened for suspect masses using a mass error < 0.5 ppm. This threshold was chosen based on the mass accuracy observed for PFAS previously identified through target analysis (Table S2).

The suspect masses identified by FT-ICR-MS were used as a suspect list for the LC-Orbitrap-HRMS data. The LC-Orbitrap-HRMS data were also screened using a suspect list compiled from PFAS literature on human serum and biota samples [34-39] containing 332 unique masses. This screening was performed using patRoon (an R-based open-source software platform) [40]. Feature detection and retention time alignment were performed using the OPENMS algorithm. Features were filtered based on intensity (intensity > 10000), blank threshold (intensity in the samples > 3 times the intensity in the blanks) and detection frequency (detection in at least 30% of the pools of a sampling year). The filtered features were screened for the masses included in the suspect list using a mass error < 2 ppm. This threshold was chosen based on the mass accuracy observed for target PFAS (Table S3). Suspect screening for a list of 342 fluorinated pharmaceuticals, including a list of 340 fluorinated pharmaceuticals part of the WHO ATC (Anatomical Therapeutic Chemical) [41] and 2 additional pharmaceuticals used to treat diabetes (ATC=A10B), was also performed using patRoon. The workflow was the same as that described for suspect PFAS but without detection frequency feature filtering prior to suspect screening to allow for the potential detection of fluorinated pharmaceuticals with low number of users.

For all suspects with an accurate mass match (ppm error <2), ddMS2 data were acquired as described above. The MS2 spectra were annotated using the PubChem and CompTox libraries available in patRoon. For suspects with diagnostic MS2 fragments, authentic standards were

purchased for confirmation. Additionally for fluorinated pharmaceuticals confirmed with standards, the presence of metabolites predicted using BioTransformer in patRoon and described in the literature, was evaluated using the same suspect screening workflow used for fluorinated pharmaceuticals.

2.3.4. Suspects quantification and fluorine mass-balance calculations

Suspect PFAS and pharmaceuticals confirmed with authentic standards were quantified using standard calibration curves without internal standard recovery correction. For the fluorinated pharmaceuticals metabolites, the concentrations were estimated using the calibration curve of the parent pharmaceutical. Peaks were integrated using TraceFinder 5.1 (Thermo Fisher Scientific). No confirmed suspects were detected in the blanks and the limits of detection (LODs) were calculated using the standard error of the regression divided the slope of the calibration curve multiplied by 3. Finally, to allow a comparison between concentrations of EOF and suspect PFAS, fluorinated pharmaceuticals and metabolites, molecular concentrations (i.e. ng substance per mL of serum) were converted to fluorine equivalents (i.e. ng fluorine per mL of serum) using equation S1.

2.4. Fluorinated pharmaceuticals prescription data

For fluorinated pharmaceuticals confirmed with standards, prescription data was obtained from the Norwegian Prescription Database (NorPD) at the Norwegian Institute of Public Health [42]. The database includes data about drugs dispensed with a prescription in Norway starting from 2004. Drugs that are purchased without prescription or supplied to hospitals and nursing homes are not included. The number of users in the Troms and Finnmark region between 2004 and 2015 split by sex was extracted from the database. A user is defined as a person who has had at least one prescription dispensed in a pharmacy during the year.

2.5. TOP assay on model CF_3-pharmaceuticals and agrochemicals

To understand if the TOP assay for human serum could be used to detect the presence of CF_3-containing pharmaceuticals, a selection of six model pharmaceuticals and agrochemicals containing at least one CF_3 group were oxidized using a previously published TOP assay protocol for human serum [43]. The model substances were bendroflumethiazide, fluoxetine, tralopyril, indoxacarb, fipronil and cyhalothrin. For each substance, 100 ng of standard (10 µL of 10 ng/µL solutions) were transferred to 2 mL glass vials and spiked with 10 ng of ^{13}C-TFA (20 µL of 0.5 ng/µL solution). After evaporation to dryness the samples were mixed with the TOP assay reagents and heated at 85 °C for 24 hours. After oxidation samples were extracted with MTBE and residues of salts and water were settled by adding anhydrous sodium sulphate. The samples were centrifuged at 10000 rpm for 10 minutes and the organic phase was transferred to glass vials with insert. The samples were spiked with 50 µL of 2 % ammonia in methanol and the MTBE was evaporated until the residual volume was 50 µL. Each model substance was oxidized in triplicate. The samples after oxidation were analyzed for trifluoroacetic acid (TFA) using a quaternary Accela 1250 pump with a PAL Sample Manager coupled to a Vantage TSQ MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). TFA was analysed with a Raptor Polar X column with a 5 minute isocratic run with 80 % 2mM ammonium acetate in methanol and 20 % 2mM ammonium acetate in 90:10 water:methanol. The samples after oxidation were also run on LC-Orbitrap Exploris in full scan with data independent acquisition (DIA) to screen the samples for the presence of the model substances and transformation products other than TFA.

2.6. Statistical analysis

Statistical analyses were performed using R 4.1.2 (R Core Team). Prior to statistics calculations, concentrations below the LOD were substituted with LOD/√2. Differences in concentrations of perfluoroethylcyclohexane sulfonate (PFECHS), $\sum_{13}$PFAS, ∑F-pharmaceuticals and UEOF between sampling years, sex and age (as weighted mean of the age of the individuals in the pools expressed in years) groups were assessed by multiple linear regression as described in the SI. Statistical significance was set at $p < 0.05$.

3. Results and discussion

A total of 46 pooled serum was analysed with a suspect screening approach using direct infusion FT-ICR-MS and LC-Orbitrap-HRMS. The samples were screened for the presence of over 5000 PFAS and 342 fluorinated pharmaceuticals and the contribution of newly identified compounds to EOF in human serum was quantified.

3.1. Suspect PFAS

Out of 5000 suspect PFAS, a total of 365 unique masses were observed in the 20 pooled serum samples analyzed by FT-ICR-MS with a mass error of < 0.5 ppm. However, only 4 of these masses could also be observed by LC-Orbitrap with a mass error < 2 ppm (Table S4). It is important to note that the LC-Orbitrap-HRMS data processing included some filtering steps (i.e., intensity filter and detection frequency filter) that were not part of the FT-ICR-MS suspect prioritization, and some suspects might be lost during this filtering. However, the discrepancy could also be due to differences in ionization source conditions, formation of in-source fragments and interferences coming from other serum components that are not separated due to the absence of liquid chromatography prior to FT-ICR-MS. In direct infusion FT-ICR-MS only the exact mass can be used as diagnostic evidence for suspect identification, therefore suspects not observed by LC-Orbitrap-HRMS could not inspected further. From the PFAS

suspect list compiled from the literature 3 out of 332 unique masses were observed by LC-Orbitrap-HRMS (Table S4).

The suspects with formula $C_8H_6ClF_4$ and $C_{25}F_8O_2Cl_1H_{18}N_3$ were excluded from further analyses due to the absence of the M+2 peak from ^{37}Cl. After this filtering steps, ddMS2 spectra were acquired for the remaining 3 suspects in samples before and after the TOP assay. The presence/absence of the suspect after TOP assay and spectra annotation using the PubChem and CompTox libraries available on patRoon were used to confirm/exclude suspects.

For the formula $C_9H_{13}F_7O$ (m/z=269.0782) there are 18 entries in PubChem all containing non-fluorinated alkyl parts. This suspect could indicate the presence of a PFAS precursor. However, since all the possible structures should be oxidizable, due to the presence of non-fluorinated alkyl parts, and the suspect was still detected after TOP assay, this assignment was discarded (Figure S2).

Two suspects, $C_8HF_{15}O_3S$ (m/z=460.9334) and $C_8HF_{15}O_4S$ (m/z=476.9283), were part of both suspect lists used and were detected both by FT-ICR-MS and LC-Orbitrap-HRMS. The first suspect, PFECHS ($C_8HF_{15}O_3S$, m/z=460.9334) was confirmed on level 1 by re-running the serum samples with a PFECHS standard (Figure S3). The retention time and MS2 spectra for PFECHS in serum matched those in the standard except for one fragment (m/z=79.9573, SO_3^-) only observed in the serum samples. As an additional confirmation, PFECHS, that is expected to be resistant to oxidation, was also detected after TOP assay and the MS2 spectra also matched the spectra from the standard except for the SO_3^- fragment.

The second PFAS suspect ($C_8HF_{15}O_4S$, m/z=476.9283) was detected both before and after TOP assay but could not be confirmed with a level of confidence higher than 5, since the MS2 spectra did not show any diagnostic fragments that could be used to confirm or discard the assignment (Figure S4). This suspect has been previously reported in human serum and wildlife [37, 44-46]. In wildlife this suspect has been observed as part of a homologue series with

formula $C_nF_{2n-1}O_4^-$ (n=7-9), with homologues from 7 to 9 in polar bear serum, 8-10 in polar bear liver and 7-11 in liver of cetaceans from Sweden. In these studies reported in the literature, MS/MS analysis revealed typical PFSA fragments but was not sufficient to fully elucidate the structure that remained ambiguous since the formula $C_8HF_{15}O_4S$ could match an unsaturated ether, a cyclic ether or a carbonyl PFSA [37, 45].

Table 1 – Suspect fluorinated compounds detected in pooled serum samples from the Tromsø Study in 1986, 2007 and 2015.

ID	Molecular formula	m/z	Mass error (ppm)	RT (min)	CL[1]	DF[2] 1986	DF[2] 2007	DF[2] 2015
PFAS								
PFECHS	$C_8HF_{15}O_3S$	460.9334	1.01	6.81	1	15/15	17/17	14/14
Carbonyl/ether/cyclic-ether-PFSA	$C_8HF_{15}O_4S$	476.9283	0.52	7.02	5	1/15	15/17	2/14
Fluorinated pharmaceuticals								
Teriflunomide	**$C_{12}H_9F_3N_2O_2$**	**269.0543**	**0.49**	**5.42**	**1**	**0/15**	**0/17**	**2/14**
4-Hydroxy-Teriflunomide	$C_{12}H_9F_3N_2O_3$	285.0493	0.40	7.01	3	0/15	0/17	2/17
Lansoprazole	**$C_{16}H_{15}F_3N_3O_2S$**	**368.0686**	**0.14**	**6.51**	**1**	**0/15**	**4/17**	**2/14**
Lansoprazole sulfide	$C_{16}H_{14}F_3N_3OS$	352.0737	1.30	7.38	3	0/15	4/17	2/14
5-Hydroxy-lansoprazole /lansoprazole sulfone	$C_{16}H_{15}F_3N_3O_3S$	384.0635	0.65	6.39	3	0/15	4/17	2/14
Pantoprazole	**$C_{16}H_{15}F_2N_3O_4S$**	**382.0679**	**0.57**	**5.95**	**1**	**0/15**	**1/17**	**10/14**
Pantoprazole sulfone	$C_{16}H_{15}F_2N_3O_5S$	398.0628	0.33	5.34	3	0/15	1/17	10/14
4-Demethyl pantoprazole-4-(hydrogen sulfate)	$C_{15}H_{13}F_2N_3O_7S_2$	448.0090	1.12	4.52	3	0/15	1/17	10/14

[1]CL = confidence level

[2]DF = detection frequency (number of pools)

3.2. Suspect pharmaceuticals

From the list of 342 fluorinated pharmaceuticals included in the WHO ATC classification (Anatomical Therapeutic Chemical), nine were found in full scan with a mass error < 2 ppm.

None of the fluorinated pharmaceuticals available to treat diabetes (ATC=A10B) was detected in the pools including individuals diagnosed with type-2 diabetes. Using analytical standards, three of the suspect pharmaceuticals (teriflunomide, lansoprazole and pantoprazole) could be confirmed with CL1 based on retention time and MS2 spectra matches (Table 1 and Figures S5, S7 and S10).

Teriflunomide, which is the active metabolite of leflunomide (an immunosuppressive drug used to cure rheumatoid arthritis) [47], was detected in 2 of the pools from 2015 including women (Table 1). This observation agrees with prescription data for the Troms and Finnmark region from the NorPD database, which shows a higher number of leflunomide users in 2015 compared to earlier years and a higher number of users among women than in men (Figure 2). Additionally, in the 2 pools where teriflunomide was detected, 4-hydroxy-teriflunomide, an additional metabolite of this pharmaceutical, was found. The detection of 4-hydroxy-teriflunomide was confirmed with CL3 based on the observed MS2 fragmentation (Figure S6). The second pharmaceutical confirmed with CL1 was lansoprazole, which is a proton pump inhibitor used worldwide for ulcer treatment and gastroprotection. Lansoprazole was detected in 4 serum pools from 2007 and in 2 serum pools from 2015 (Table 1). This observation was also in agreement with data from the NorPD database, that showed lower number of users in 2015 compared to 2007 (Figure 2). The number of pools containing lansoprazole in 2007 was the same for men and women and in this year the number of users of lansoprazole among women was only slightly higher then among men (1652 men, 1829 women). In 2015 the 2 pools where lansoprazole was detected were made up from women and in this year the number of users among women was still slightly higher than among men (1096 men, 1185 women). Lansoprazole is mainly metabolized in the liver to 5-hydroxy lansoprazole and lansoprazole sulfone [48], both of which have the formula $C_{16}H_{14}F_3N_3O_3S$. The $[M-H]^-$ peak corresponding to this formula was observed in all the pools where lansoprazole was detected and the MS2

spectra showed fragments that support this assignment (CL3), but it was not possible to distinguish between these two metabolites (Figure S8). Lansoprazole sulfide (another metabolite of lansoprazole) was also detected in all pools containing the parent compound and could be confirmed with CL3 based on the observed MS2 fragmentation (Figure S9).

Lastly, pantoprazole, another proton pump inhibitor widely used for ulcer treatment and gastroprotection, was detected and confirmed with a standard (CL1) in one pool from 2007 and 10 pools from 2015 (Table 1). This observation was also in agreement with the NorPD data since the number of users for this drug in the Troms and Finnmark region has been increasing from 3414 users in 2007 to 12744 users in 2015 (Figure 2). For pantoprazole the detection frequency was higher in the pools containing men than in the pools containing women both in 2007 and 2015, even if in both years the number of users among women was higher than among men (2007: 509 men, 543 women; 2015: 5829 men, 6915 women). Some of pantoprazole metabolites were also observed. The main metabolic pathway for pantoprazole is demethylation followed by sulfation and 4-demethyl-pantoprazole-4-(hydrogen-sulfate) was detected in the pooled samples containing pantoprazole (Figure S11 and S12) with CL3 based on the observed MS2 fragmentation. Another metabolic pathway is oxidation to pantoprazole sulfone, that was also detected in the pools containing pantoprazole with CL3 based on MS2 fragmentation (Figure S11).

The higher detection frequency in pooled serum from 2007 and 2015 of pantoprazole compared to lansoprazole and leflunomide probably reflected the higher number of users of pantoprazole compared to the other two drugs. Pantoprazole was the 14th most used drug in Norway in 2015 [49]. None of the fluorinated pharmaceuticals found in the pools from 2007 and 2015 were detected in pooled samples from 1986 and this was not surprising since leflunomide, lansoprazole and pantoprazole were approved to the market in Norway in 1999, 2003 and 2001 respectively [50-52].

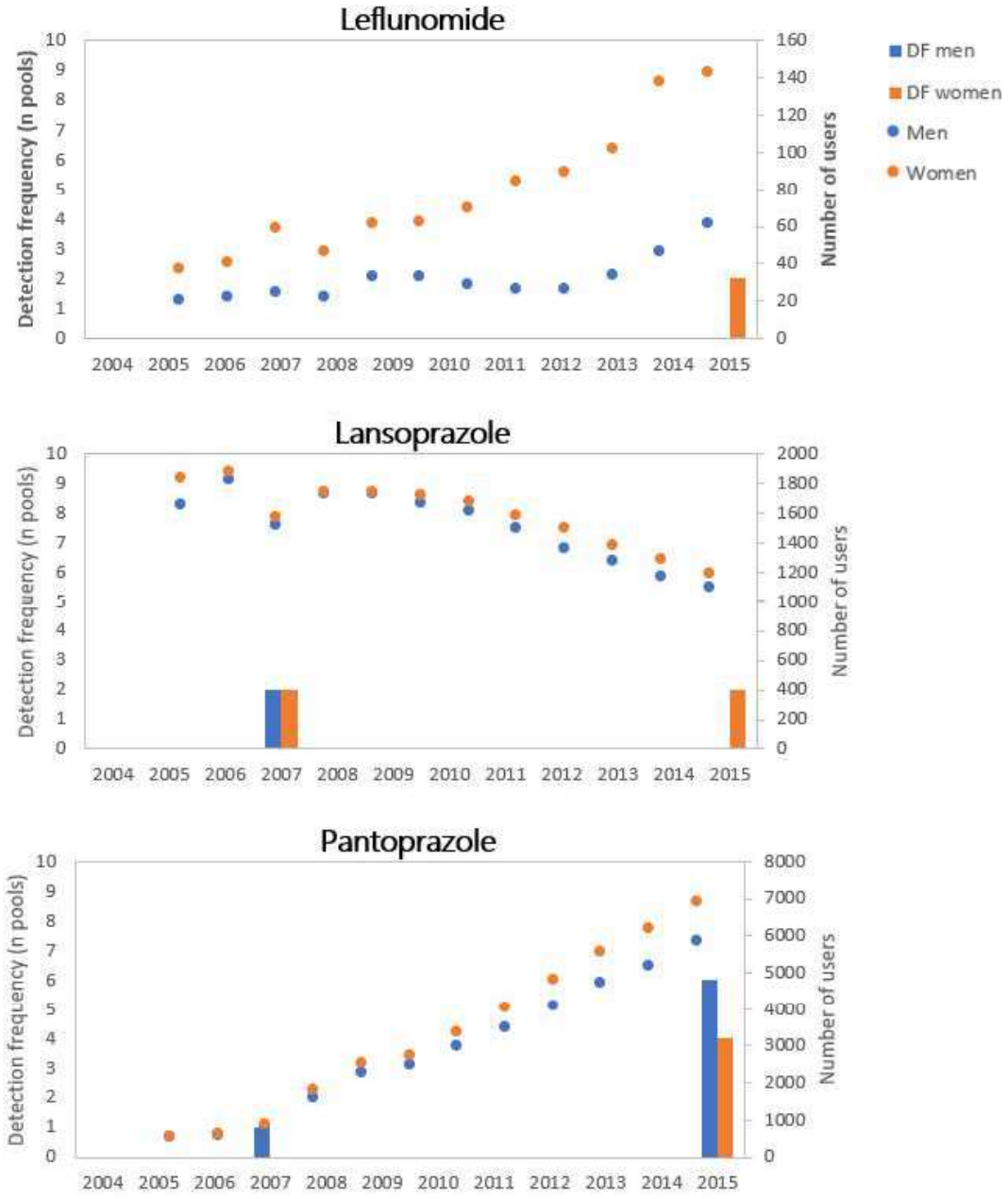

Figure 2 – Detection frequency (DF), as number of pools, in pooled serum samples from the Tromsø Study collected in 2007 and 2015 and number of users per year for teriflunomide, lansoprazole and pantoprazole.

3.3. Contributions of suspect PFAS and fluorinated pharmaceuticals to EOF

PFECHS concentrations ranged from 0.52 to 1.03 ng/mL and changed over time, with the highest concentrations observed in 2007, consistent with observations for PFOA, PFHxS, PFHpS and PFOS reported previously [20]. Similar to PFAA, men had higher PFECHS concentrations than women. PFECHS concentrations in pooled serum from the Tromsø Study were higher than those reported by Miaz et al. [11] for pooled serum samples from Swedish women (0.06-0.28 ng/mL) between 1986 and 2015.

Table 2 – Concentrations (ng F/mL) of PFECHS, $\sum_{12}$PFAS (from Cioni et al.[20]), $\sum_{13}$PFAS, teriflunomide, lansoprazole, pantoprazole and their metabolites in pooled serum samples from 1986, 2007 and 2015 (n=number of pools).

	1986 (n=15)				2007 (n=17)				2015 (n=14)			
ID	DF	Mean	Median	Range	DF	Mean	Median	Range	DF	Mean	Median	Range
PFECHS	15/15	0.48	0.48	0.41-0.59	17/17	0.51	0.53	0.32-0.64	14/14	0.42	0.42	0.38-0.45
$\sum_{12}$PFAS	15/15	11.2	11.6	7.28 – 15.7	17/17	18.5	18.2	14.1 – 30.1	14/14	13.3	12.6	7.51 – 19.6
$\sum_{13}$PFAS	**15/15**	**11.6**	**12.2**	**7.52-16.3**	**17/17**	**19.0**	**18.7**	**14.6-30.7**	**14/14**	**13.7**	**13.0**	**7.87-20.1**
Teriflunomide	0/15	-	-	-	0/17	-	-	-	2/14	1.19	<LOD	< LOD-8.35
4-Hydroxy-Teriflunomide	0/15	-	-	-	0/17	-	-	-	2/14	<LOD	<LOD	<LOD-0.11
Lansoprazole	0/15	-	-	-	4/17	<LOD	<LOD	<LOD-0.26	2/14	<LOD	<LOD	<LOD-0.12
Lansoprazole sulfide	0/15	-	-	-	4/17	0.17	<LOD	<LOD-1.96	2/14	0.12	<LOD	<LOD-1.13
5-Hydroxy-lansoprazole /lansoprazole sulfone	0/15	-	-	-	4/17	0.96	<LOD	<LOD-11.45	2/14	0.43	<LOD	<LOD-3.78
Pantoprazole	0/15	-	-	-	1/17	<LOD	<LOD	<LOD-0.51	10/14	0.56	0.60	<LOD-1.65
Pantoprazole sulfone	0/15	-	-	-	1/17	<LOD	<LOD	<LOD-1.17	10/14	3.93	3.13	<LOD-9.99
4-Demethyl pantoprazole-4-(hydrogen sulfate)	0/15	-	-	-	1/17	<LOD	<LOD	<LOD-0.23	10/14	0.59	0.38	<LOD-2.50
∑ F-pharmaceuticals	**0/15**	**-**	**-**	**-**	**5/17**	**1.33**	**<LOD**	**<LOD-13.7**	**13/14**	**6.78**	**7.62**	**<LOD -11.9**

PFECHS contributed to 2 to 4 % of the EOF. PFECHS concentrations were added to the $\sum_{12}$ PFAS concentrations to evaluate the known PFAS ($\sum_{13}$PFAS) contribution to the EOF (Table

2). The $\sum_{13}$PFAS accounted for 24-82% (mean: 53 %) of the EOF in 1986, 62-100 % (mean: 88%) of the EOF in 2007 and 46-100% (mean: 75 %) in 2015.

The concentrations of fluorinated pharmaceuticals varied. In the two pools where teriflunomide was detected the concentrations were 39.6 and 39.2 ng/mL. The concentration of the metabolite 4-hydroxy-teriflunomide was almost 2 orders of magnitude lower (0.54 and 0.56 ng/mL). In total teriflunomide and its metabolite accounted for 8.39 and 8.46 ng F/mL in the pools where they were detected.

For lansoprazole, concentrations ranged from <LOD to 1.68 ng/mL. Higher concentrations were observed for the lansoprazole metabolites, lansoprazole sulfide (range: <LOD-12.2 ng/mL) and lansoprazole sulfone (range: <LOD-77.4 ng/mL). In total lansoprazole and its metabolites accounted for <LOD to 13.7 ng F/mL.

For pantoprazole, concentrations ranged from <LOD to 16.7 ng/mL. Concentrations of pantoprazole sulfone (<LOD-105 ng/mL) were higher than those of pantoprazole while concentrations of 4-demethyl pantoprazole-4-hydrogen sulfate (<LOD-14.8 ng/mL) were comparable. In total pantoprazole and its metabolites accounted for <LOD and 10.2 ng F/mL.

Overall, fluorinated pharmaceuticals accounted for 0 to 56 % of the EOF. The portion of EOF explained by fluorinated pharmaceuticals increased significantly from 1986 (0 %), over 2007 (0-50%; mean: 5.3 %) to 2015 (0-56 %; mean 31 %) (Figure 3 and Table S6). These changes reflect the increase in production and use of organofluorine pharmaceuticals in more recent years. Between 1979 and 2021 the percentage of pharmaceuticals containing at least one fluorine atom increased from 2 to 25% and the percentage is expected to increase further since around 30% of newly approved drugs contain fluorine [25, 53].

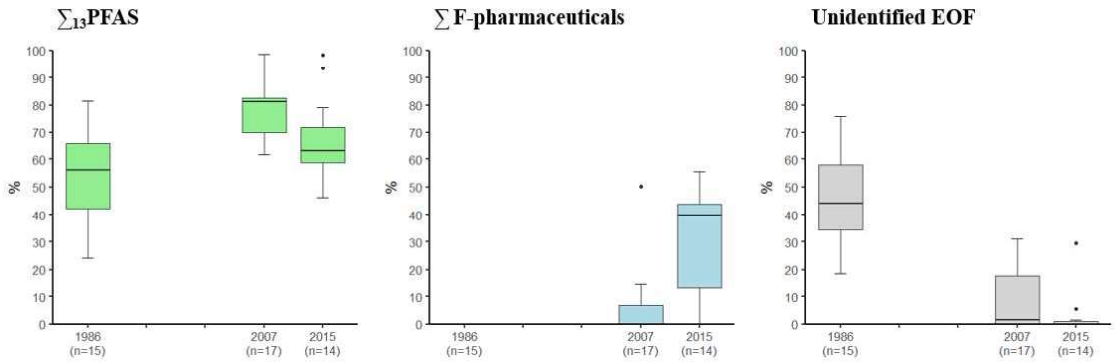

Figure 3 – Percentage contribution to EOF from $\sum_{13}$PFAS, $\sum$ fluorinated pharmaceuticals and unidentified EOF (after inclusion of $\sum_{13}$PFAS, TOP and $\sum$F-pharmaceuticals) in pooled serum samples from the Tromsø Study from 1986, 2007 and 2015.

In pooled serum samples from 1986 between 18 and 76 % of EOF remained unidentified. This fraction might be explained by unknown PFAS not included in our suspect lists. To address this gap a possible strategy is to analyze the HRMS data using non-target screening strategies to identify potential PFAS features, like mass defect filtering, homologue series identification and presence of diagnostic fragments and neutral losses [22].

3.4.Pharmaceuticals in the TOP assay

The oxidation of model pharmaceuticals and pesticides containing CF_3 groups showed that these substances are oxidizable with the TOP assay. With the exception of fipronil, all parent compounds were not detected after oxidation. However, following oxidation, the expected oxidation product TFA was also not observed, leaving these organofluorine compounds undetected in the TOP assay. Additionally, from the evaluation of the high-resolution mass spectrometry data, no potential intermediates could be identified, leaving these not identified within the scope of this project. One possible explanation is that these compounds are fully mineralized to fluoride under the TOP assay conditions. Bhat et al. [54] studied the photolysis

of fluoxetine and observed fluoride as major product under a wide variety of conditions. In their photolysis experiments TFA formation from fluoxetine was observed at pH 7 (with and without H_2O_2), but no TFA was formed under basic conditions at pH 10 (with and without addition of SO_3^{2-}). Furthermore, no TFA was found in the human serum pools post TOP assay, suggesting the absence of TFA also before TOP assay and indicating that metabolic processes of fluorinated pharmaceuticals in humans are also not causing the formation of TFA or that serum is not the preferred compartment for TFA circulation.

4. Implications

Suspect screening using FT-ICR-MS and LC-Orbitrap-HRMS in combination with the TOP assay allowed to screen for the presence of over 5000 PFAS prioritizing only a limited number of suspect features for which the MS2 spectra had to be evaluated. The TOP assay not only provided valuable information about the presence/absence of oxidizable precursors, but also helped to confirm/exclude suspects based on their chemical structure and presence/absence after TOP assay oxidation.

In pooled serum samples from the Tromsø study collected in 1986, 2007 and 2015, PFAS (including also the newly quantified PFECHS) only explained a portion of the EOF measured. In 2007 and 2015 the EOF portion not explained by PFAS was largely explained by 3 fluorinated pharmaceuticals. This observation and the non-detection of newly emerging PFAS (e.g., short-chain PFAA, ether PFAS and other PFAS included in the suspect screening lists) in the pools of 2007 and 2015 is suggesting that target PFAA analysis might be sufficient to describe human exposure to PFAS in the Tromsø population between 2007 and 2015.

The detection and quantification of fluorinated pharmaceuticals and their metabolites in human serum also showed that even if these compounds often contain only 1 to 3 fluorine atoms, they can still contribute significantly to the EOF due to their higher concentrations in human serum

compared to PFAS. The contribution of fluorinated pharmaceuticals highlights that care must be taken in interpreting EOF concentrations in human blood as a measurement of "total PFAS exposure", since these might include fluorinated chemicals that are not classified as PFAS. The TOP assay can be used to detected PFAA precursors in human serum, but fluorinated pharmaceuticals containing CF_3 groups remained undetected in this method since these were not converted to TFA after oxidation. This observation does not rule out completely possible formation of TFA from precursors with isolated CF_3-group (such as pharmaceuticals and agrochemicals) but indicates the need for careful investigation of environmental transformations for risk assessment of precursors in particular of those carrying a CF_3 moiety.

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Supporting Information

Combining advanced analytical methodologies to uncover the contribution of suspect PFAS and fluorinated pharmaceuticals to extractable organic fluorine in pooled human serum from the Tromsø Study between 1986 and 2015

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1. Materials and methods

Table S1 – Orbitrap Exploris 120 ion source and full scan and ddMS2 acquisition parameters**.**

Ion source parameters	
Ion source type	H-ESI
Spray voltage	Static
Negative ion voltage (V)	2500
Gas mode	Static
Sheath gas (arb)	40
Aux gas (arb)	5
Sweep gas (arb)	0
Ion transfer tube temperature (°C)	200
Vaporizer temperature (°C)	300
Full scan parameters	
Orbitrap resolution	120000
Scan range (m/z)	150-700
RF lens (%)	65
Normalized AGC target (%)	100
Maximum injection time (ms)	100
Microscans	1
Data type	Profile
Polarity	Negative
ddMS2 parameters	
Isolation window (m/z)	0.8
Isolation offset	Off
Collision energy mode	Stepped
Collision energy type	Absolute
HCD collision energies (V)	15,35,60,75
Orbitrap resolution	15000
Scan range mode	Auto
Normalized AGC target (%)	100
Maximum injection time (ms)	100
Microscans	1
Intensity threshold	1.0e4
Apex detection desired window (%)	30

Table S2 – Target PFAS ppm error in FT-ICR.

Compound	Molecular formula	Theoretical m/z	ppm error
PFHpA	$C_7HF_{13}O_2$	362.9696	0.16
PFOA	$C_8HF_{15}O_2$	412.9664	0.13
PFNA	$C_9HF_{17}O_2$	462.9632	0.44
PFDA	$C_{10}HF_{19}O_2$	512.9600	0.18
PFUnDA	$C_{11}HF_{21}O_2$	562.9568	0.15
PFDoDA	$C_{12}HF_{23}O_2$	612.9537	Not detected
PFHxS	$C_6HF_{13}O_3S$	398.9366	0.13
PFHpS	$C_7HF_{15}O_3S$	448.9334	0.20
PFOS	$C_8HF_{17}O_3S$	498.9302	0.15
FOSAA	$C_{10}H_4F_{17}NO_4S$	555.9517	Not detected
Me-FOSAA	$C_{11}H_6F_{17}NO_4S$	569.9673	Not detected
Et-FOSAA	$C_{12}H_8F_{17}NO_4S$	583.9830	0.22

Table S3– Target PFAS ppm error in LC-Orbitrap.

Compound	Molecular formula	Theoretical m/z	ppm error
PFHpA	$C_7HF_{13}O_2$	362.9696	0.79
PFOA	$C_8HF_{15}O_2$	412.9664	0.74
PFNA	$C_9HF_{17}O_2$	462.9632	1.08
PFDA	$C_{10}HF_{19}O_2$	512.9600	0.20
PFUnDA	$C_{11}HF_{21}O_2$	562.9568	0.44
PFDoDA	$C_{12}HF_{23}O_2$	612.9537	0.65
PFHxS	$C_6HF_{13}O_3S$	398.9366	0.52
PFHpS	$C_7HF_{15}O_3S$	448.9334	0.93
PFOS	$C_8HF_{17}O_3S$	498.9302	0.14
FOSAA	$C_{10}H_4F_{17}NO_4S$	555.9517	0.96
Me-FOSAA	$C_{11}H_6F_{17}NO_4S$	569.9673	0.18
Et-FOSAA	$C_{12}H_8F_{17}NO_4S$	583.9830	0.55

For comparison with EOF values, suspect concentrations measured in the EOF extracts were converted to F equivalents using the following equation:

$$Concentration\left(\frac{ng\ F}{mL}\right) = \frac{concentration\left(\frac{ng}{mL}\right) \cdot \mathrm{nF} \cdot AW_F}{MW_{suspect}} \quad \text{(S1)}$$

where nF is the number of fluorine atoms in the suspect structure, A_F is the atomic weight of fluorine and $MW_{suspect}$ is the molecular weight of the suspect which concentration is being converted.

Differences in $\sum_{13}$ PFAS, ∑F-pharmaceuticals and UEOF and TOP between sampling years were assessed by multiple linear regression to account for the influence of sex and age (as weighted mean of the age of the individuals in the pools expressed in years) using the following equation:

$$y = \beta_0 + \beta_1 dummy\ 1 + \beta_2 dummy\ 2 + \beta_3\ sex + \beta_4\ age \quad \text{(S2)}$$

where y is the log transformed concentration for $\sum_{13}$ PFAS, ∑F-pharmaceuticals and the percentage contribution to EOF for UEOF; β0 is the intercept of the multiple linear regression; β_1, β_2, β_3 and β_4 are the regression coefficients for the predictor variables; dummy 1 is a dummy variable equal to 1 if sampling year is 1986, equal to 0 if sampling year is 2007 or 2015; dummy 2 is a dummy variable equal to 1 if sampling year is 2015, equal to 0 if sampling year is 1986 and 2007; sex is categorical variable equal to 0 for women and equal to 1 for men; age is the weighted mean age of the individuals making up each pool expressed in years.

When sex was a significant predictor, differences in concentrations between men and women at each sampling year were assessed by adding an interaction term between sex and each sampling year dummy variable as described by equation S3.

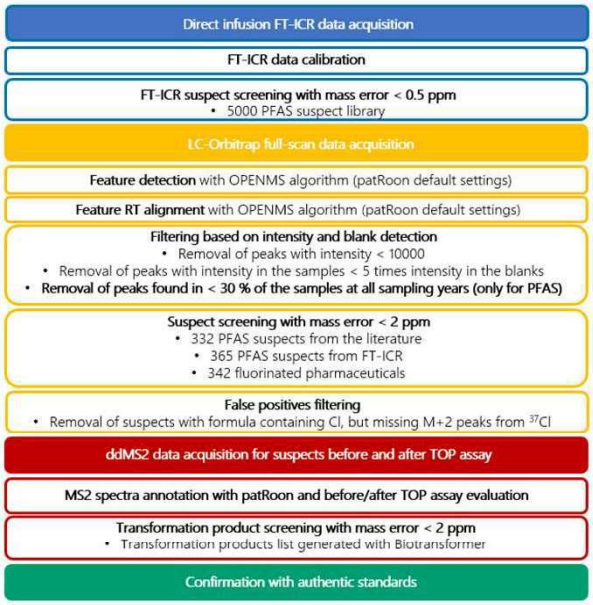
$$y = \beta_0 + \beta_1 dummy\ 1 + \beta_2 dummy\ 2 + \beta_3\ sex + \beta_4\ age + \beta_5\ dummy1\ sex + \beta_6\ dummy2\ sex \quad \text{(S3)}$$

2. Results and discussion

Table S4 - Suspect PFAS with mass error < 2 ppm.

Molecular formula	Theoretical m/z	Mass error (ppm)	Retention time (min)	Suspect list	Confidence level
$C_8H_6ClF_4$	212.0021	1.30	2.17	FT-ICR-MS	-
$C_9H_{13}F_7O$	269.0782	0.52	5.23	PFAS literature	-
$C_8HF_{15}O_3S$	**460.9334**	**1.01**	**6.81**	**FT-ICR-MS and PFAS literature**	**Level 1**
$C_8HF_{15}O_4S$	**476.9283**	**0.52**	**7.02**	**FT-ICR-MS and PFAS literature**	**Level 5**
$C_{25}F_8O_2Cl_1H_{18}N_3$	578.0887	0.77	6.47	FT-ICR-MS	-

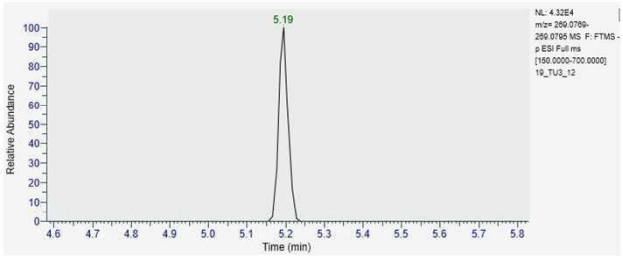
Figure S1 – Suspect screening workflow.



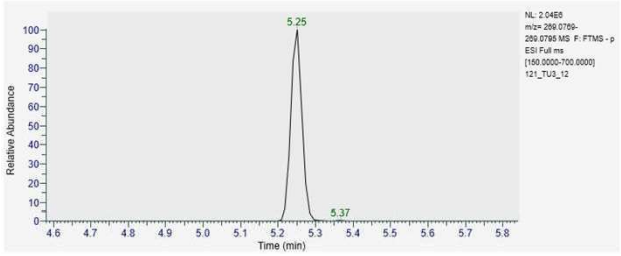
1

Figure S2 – Chromatogram of suspect $C_9H_{13}F_7O$ in a pooled serum sample before TOP assay (a) and in a pooled sample after TOP assay (b).

(a)

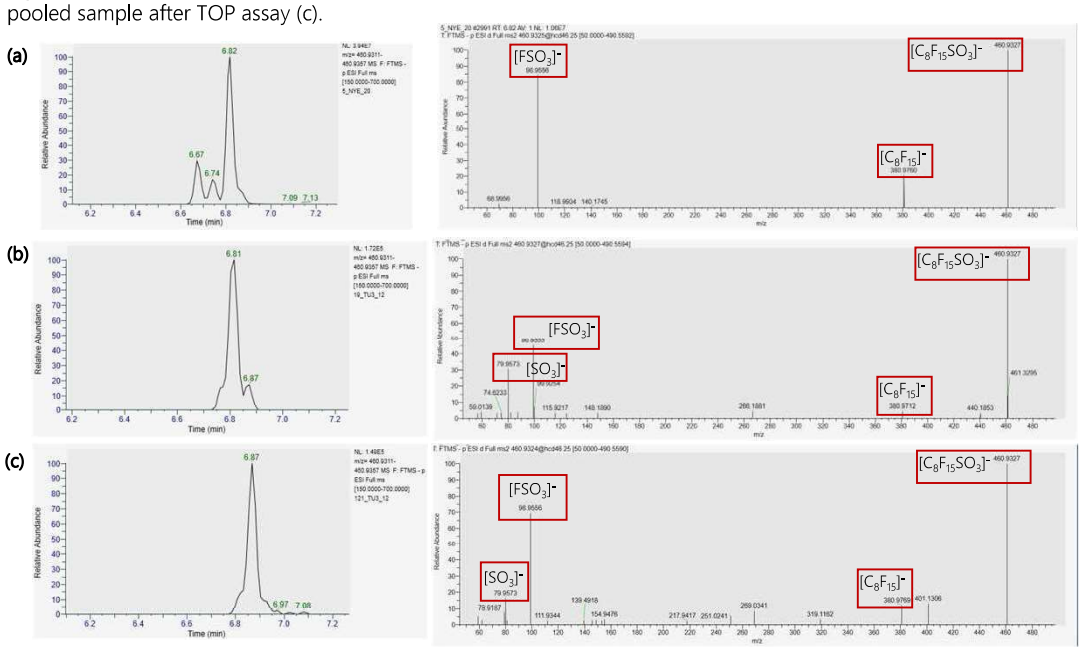


(b)



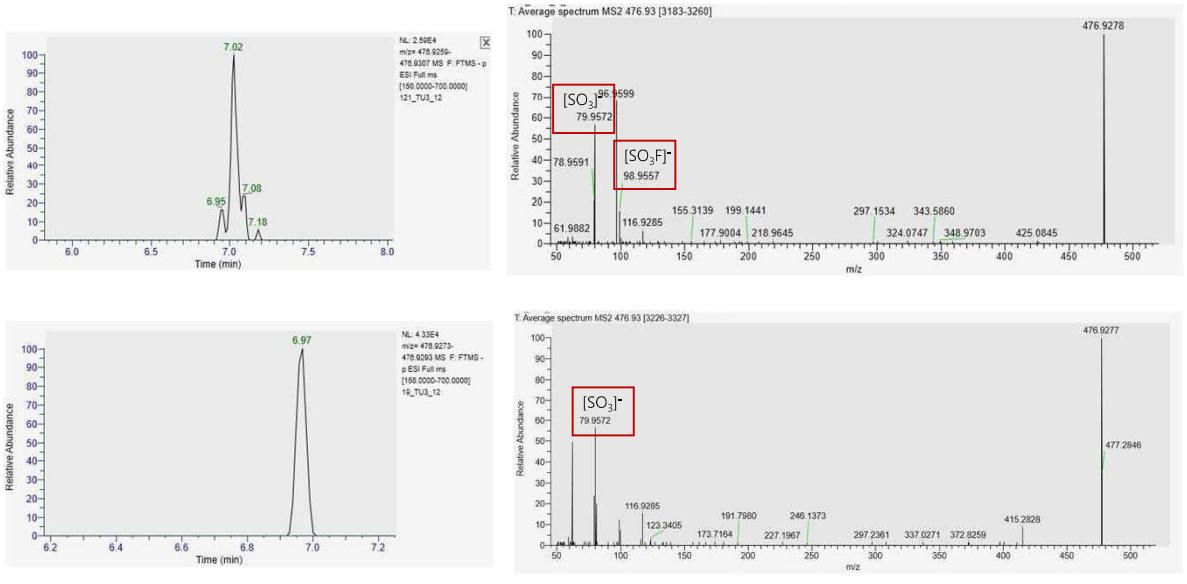
2

Figure S3– Chromatogram and mass spectra of PFECHS in a standard (a) in a pooled serum sample before TOP assay (b) and in a pooled sample after TOP assay (c).



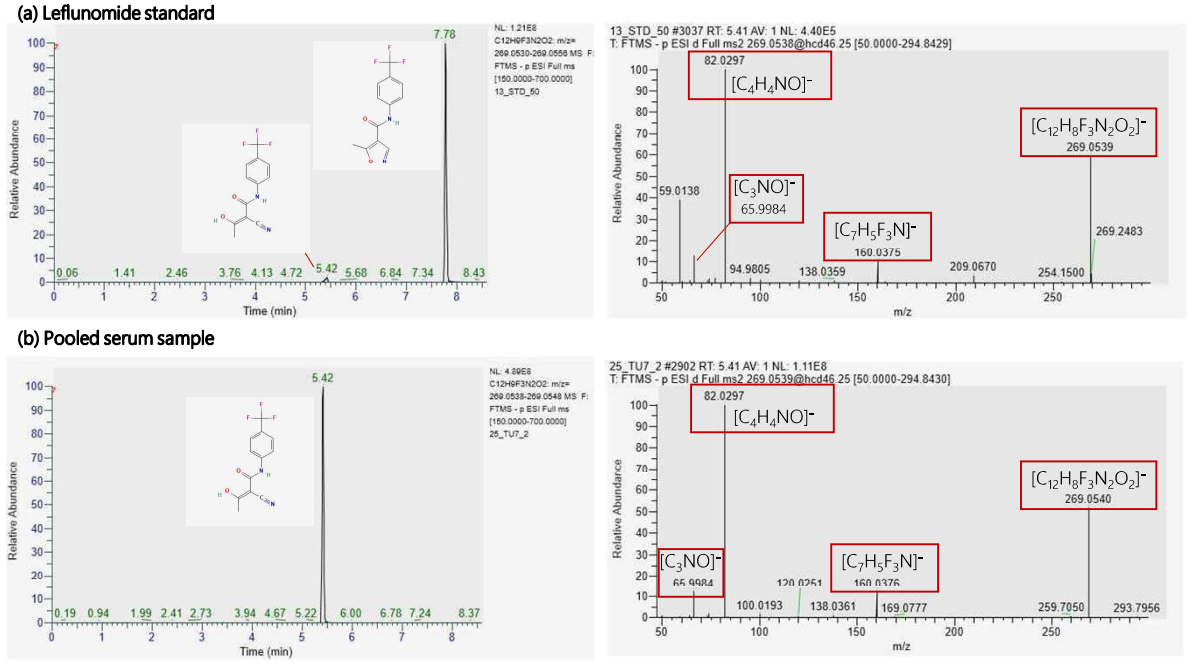
3

Figure S4 – Chromatogram and mass spectra of $C_8HF_{15}O_4S$ in a standard (a) in a pooled serum sample before TOP assay (b) and in a pooled sample after TOP assay.



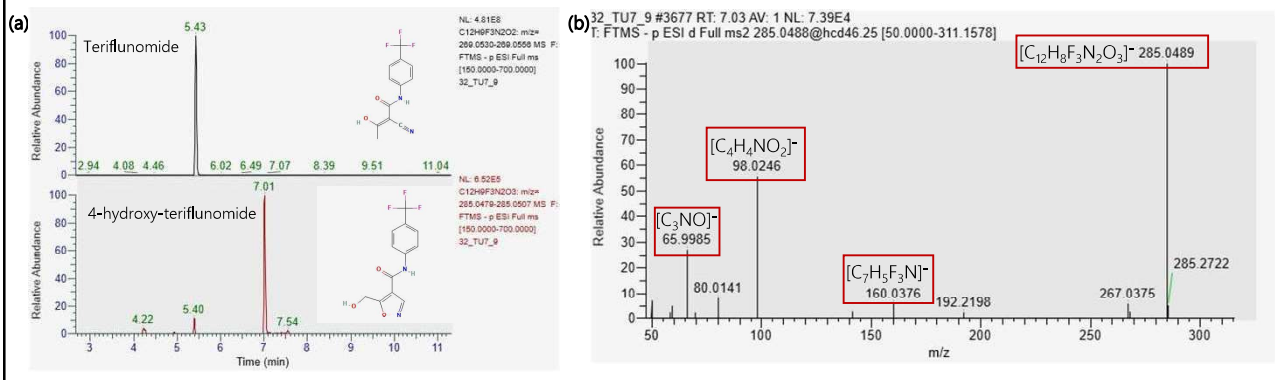
4

Figure S5 – Chromatogram and mass spectra of leflunomide and teriflunomide in a standard (a) and a pooled sample (b).



5

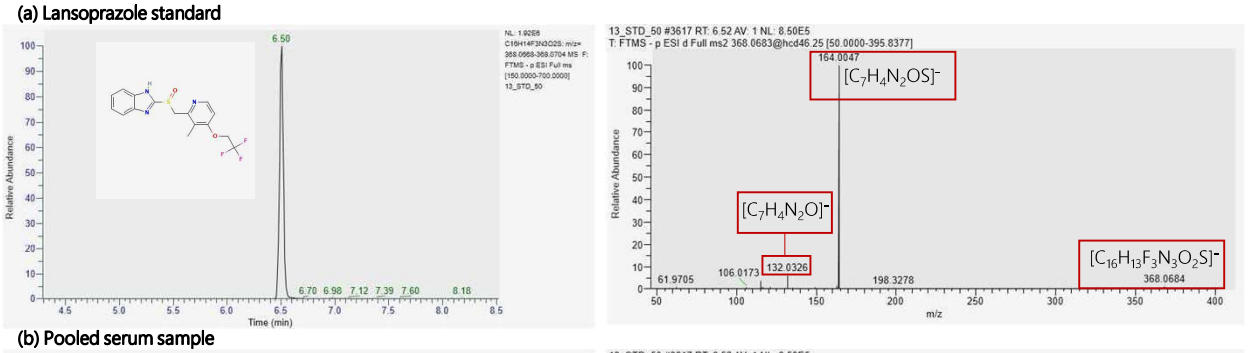
Figure S6 – Chromatogram of teriflunomide and 4-hydroxy-teriflunomide detected in a pooled sample (a) and MS2 spectra of 4-hydroxy-teriflunomide detected in a pooled sample (b).



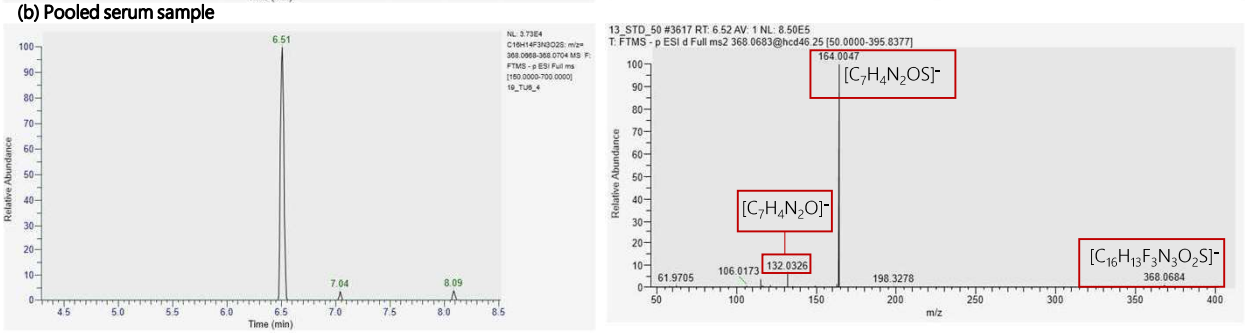
6

Figure S7 – Chromatogram and mass spectra of lansoprazole in a standard (a) and a pooled sample (b).

(a) Lansoprazole standard

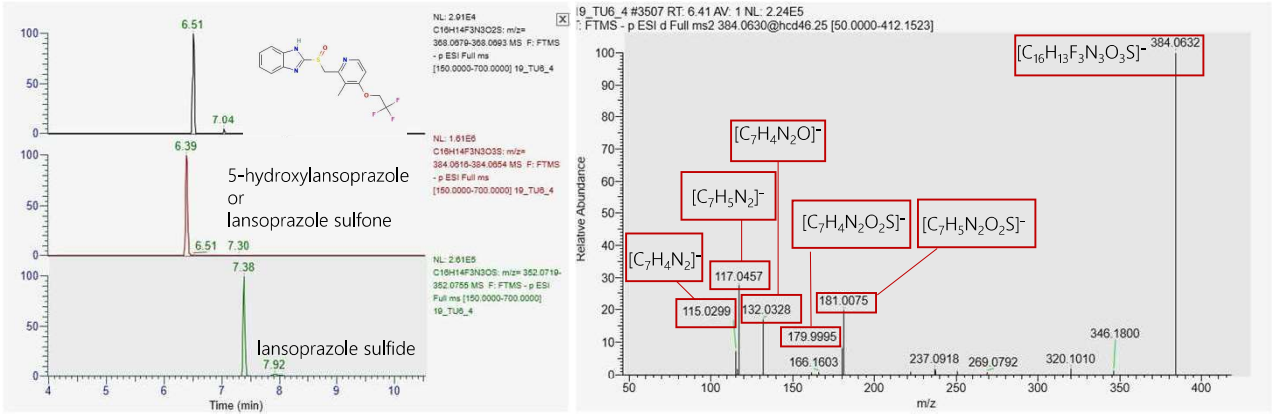


(b) Pooled serum sample



7

Figure S8 – Chromatogram of lansoprazole, 5-hydroxylansoprazole/lansoprazole sulfone and lansoprazole sulfide detected in a pooled sample (a) and MS2 spectra of 5-hydroxylansoprazole/lansoprazole sulfone detected in a pooled sample (b).

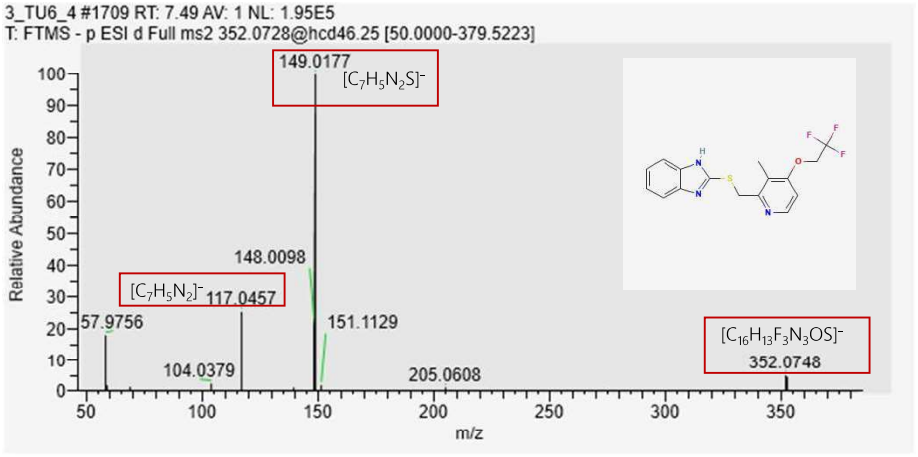


5-hydroxylansoprazole

Lansoprazole sulfone

8

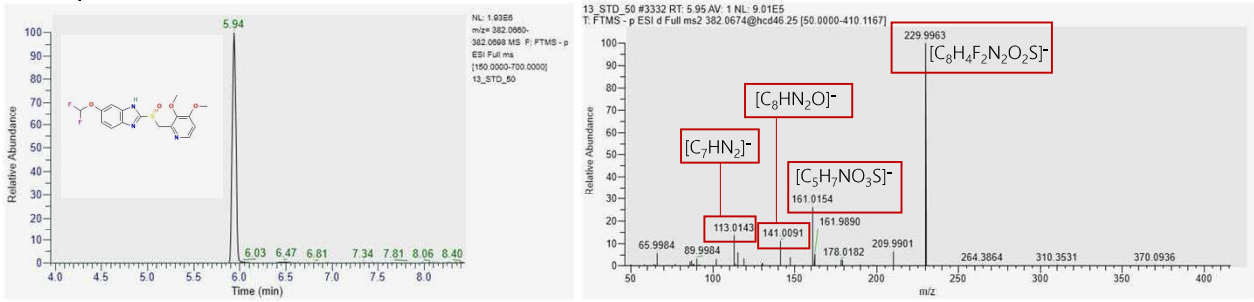
Figure S9 – MS2 spectra of lansoprazole sulfide detected in a pooled sample.



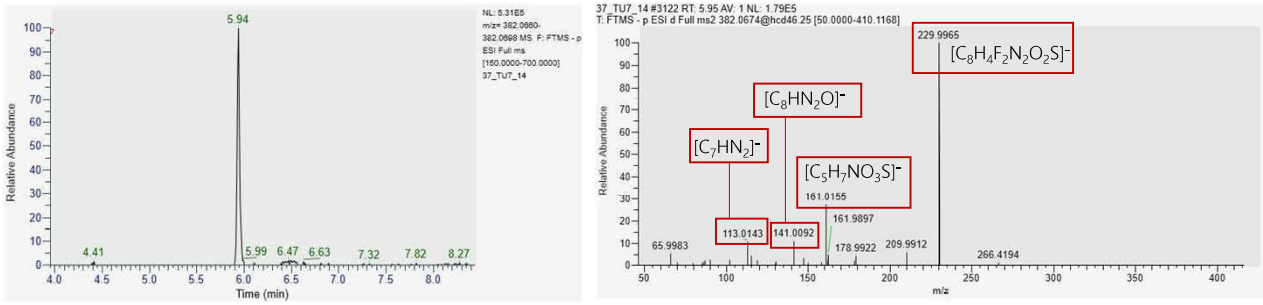
9

Figure S10 – Chromatogram and mass spectra of pantoprazole in a standard (a) and a pooled sample (b).

(a) Pantoprazole standard

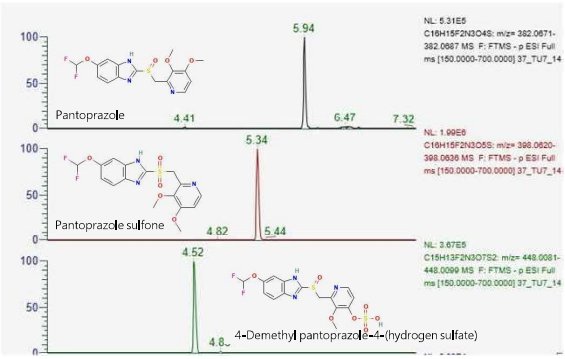


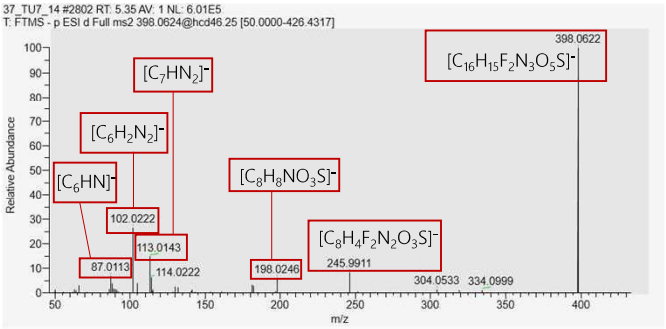
(b) Pooled serum sample



10

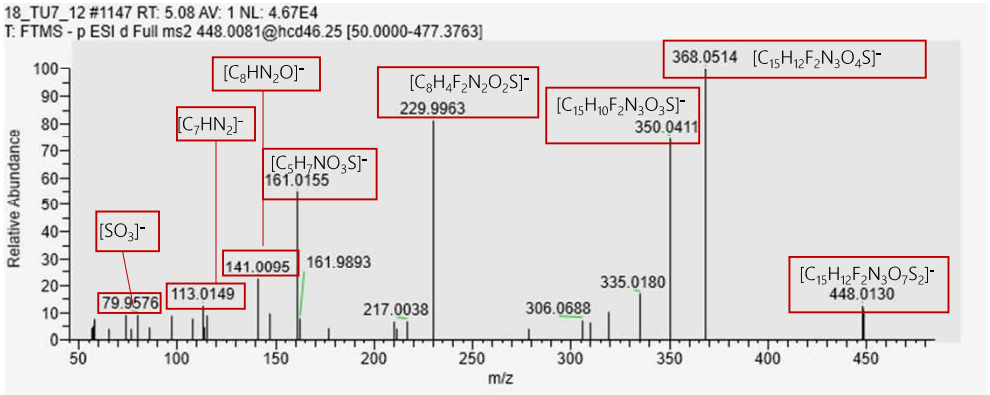
Figure S11 – Chromatogram of pantoprazole and its transformation products detected in a pooled sample (a) and MS2 spectra of pantoprazole sulfone in a pooled sample (b).

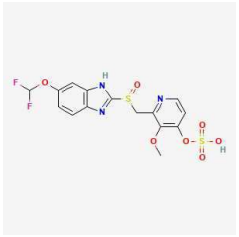




11

Figure S12 – MS2 spectra of 4-Demethyl pantoprazole-4-(hydrogen sulfate) detected in a pooled sample.





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