Total Oxidizable Precursors Assay for PFAS in Human Serum

Lara Cioni[1,2*], Vladimir Nikiforov[1], Ana Carolina M. F. Coêlho[2], Torkjel M. Sandanger[1,2], Dorte Herzke[1]

1. Norwegian Institute for Air Research (NILU), Fram Centre, Tromsø, Norway

2. UiT – The Arctic University of Norway, Department of Community Medicine, Tromsø, Norway

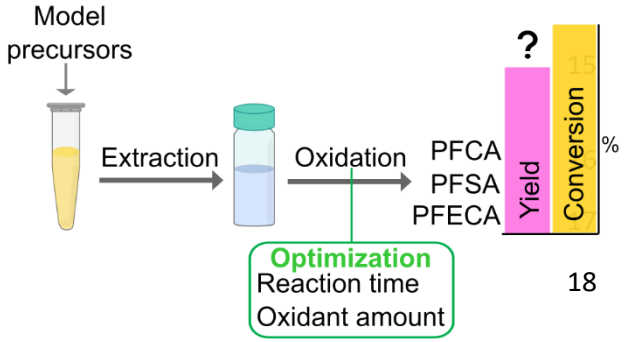
***Corresponding author**

Lara Cioni

Norwegian Institute for Air Research (NILU), Fram Centre, Tromsø, Norway

[*]lci@nilu.no

Table of Contents (TOC)/Abstract art



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 assay (TOPA) to look for precursors by oxidation to perfluoroalkyl acids (PFAA). The TOPA 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 TOPA methods were not sufficient for complete oxidation of model precursors. Prolonged heating time (24 hours) 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 TOPA can only provide semi-quantitative estimates of oxidizable precursors in human serum. However, the TOPA can provide indications about the identity of unknown precursors by evaluating the oxidation products, including PFSA and PFECA. The optimized TOPA for human serum opens for high-throughput screening of human serum for undetected PFAA precursors.

Synopsis

The total oxidizable precursors assay was optimized to evaluate the presence of unknown oxidisable PFAA precursors in small volumes of serum samples.

Keywords

TOPA, PFAS, PFAA, precursors, human exposure, method, oxidation, blood

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals with hundreds of applications in industry and consumer products [1, 2]. PFAS have been extensively used because of the special properties, like high chemical and thermal stability, surfactant and water and oil repelling properties [3]. 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 [4]. Exposure to PFAS can result in adverse health effects, that have been observed both in toxicological [5-10] and epidemiological studies [11-17]. For example, exposure to perfluorooctanoic acid (PFOA), one of the most studied PFAS, has been linked to kidney and testicular cancer [18, 19], pregnancy-induced hypertension [20], ulcerative colitis [21] and hypothyroidism [22].

PFAS have been detected in humans since 2001 when PFOA, PFHxS, PFOS and FOSA were reported for the first time in human serum [23]. PFOS and PFOA have been listed under the Stockholm Convention on Persistent Organic Pollutants in 2009 and 2019, respectively [24, 25]. 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 [26, 27]. 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 sulfonates (FTS) and fluorotelomer alcohols (FTOH) [28, 29]. 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 [30]. Complementary analytical tools are required to characterize the unaccounted fraction.

One promising tool is the Total Oxidizable Precursors Assay (TOPA), that was developed to analyze oxidizable PFAA precursors in water [31]. Precursors are a group of chemicals that can be transformed to PFAA biotically and/or abiotically [31-34]. The TOPA allows to determine the presence of both known and unknown PFAA precursors by oxidizing them under controlled conditions to their end-products PFAA [31]. 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 TOPA allows to calculate the additional amount of PFAA formed by oxidation and to indicate the content of precursors with different chain length [31]. This approach has been successfully applied to detect PFAA precursors in wastewater [35], soil [36], textiles [37], firefighting foams [38], impregnation sprays [39] and biota [40] 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 TOPA 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 TOPA protocol for human serum

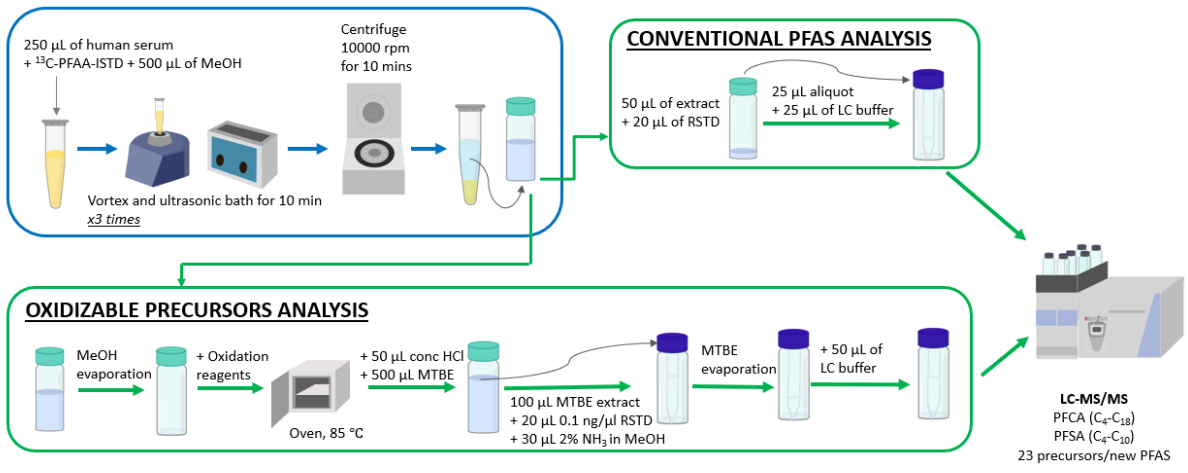


Figure 1 – Scheme of the TOPA protocol for human serum.

The TOPA protocol as published in the literature [31] was optimized using human serum samples from the AMAP Ring Test for Persistent Organic Pollutants [41]. As general steps in all experiments (Figure 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 minutes. Before each repetition samples were vortexed. Samples were centrifuged for 10 minutes 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 TOPA 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 16 % solution (made of 7.6 g of $Na_2S_2O_8$ and 40 ml of MilliQ water). For sodium hydroxide, a 29 % solution was used (made of 20 g of NaOH and 50 ml of MilliQ water). The vials were tightly capped, vortexed, and subsequently heated in an oven at 85 °C for a certain time. 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 and extracted with 500 µl of methyl tert-butyl ether (MTBE). Subsequently, 200 µl of the organic phase was 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. The vials were left uncapped for approximately 2 hours to let the MTBE evaporate and the residue was reconstituted in methanol.

2.3. Optimization of oxidation conditions

The oxidation conditions tested are summarized in Table 1.

Table 1 - Oxidation conditions tested on human serum reference samples.

Parameters	Method			
	A	**B**	**C**	**D**
Heating time (hours)	8	24	8	24
29 % NaOH (µl)	20	20	40	120
MilliQ H_2O (µl)	100	100	200	-
$K_2S_2O_8$ (mg)	20	20	40	-
16 % $Na_2S_2O_8$ (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

Method A was the closest to those reported in the literature [31, 36, 42, 43]. In method B the reaction time was increased from 8 to 24 hours 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 16 % $Na_2S_2O_8$ 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 perfluoro alkyl 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 S1 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 instrumental set-up and the method described by Hanssen et al. [44]. The MS method was

153 modified to include the model substances used for the method testing and perfluoro alkyl ether
154 carboxylic acids (PFECA). The list of compounds measured, including the internal standards
155 used for the quantification and the monitored mass transitions can be found in Table S2 of the
156 Supporting Information. For the analysis 25 µl of the extracts were mixed with 25 µl of 2 mM
157 NH_4OAc in MeOH. For each sample 10 µl were injected two times, once for PFAA
158 determination and once for selected precursors and PFECA analysis. The analytes were
159 quantified using the software LC Quan (v.2.6, Thermo Fisher Scientific Inc., Waltham,
160 Massachusetts, USA).

161 **2.6. QA/QC**

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

176 **3. Results and discussion**

The original TOPA 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. Evaluation of the completeness of oxidation

After each method alteration, the completeness of the oxidative treatment was evaluated using the percentage of conversion of spiked precursors and the yield of products as described in the Supporting Information. The results are presented in Table 2 except for GenX (stable to oxidation).

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

Test ID	Conversion	Total yield	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFOS linear	1,3 - PFECA
7:3 FTCA (n=7)			n-4	n-3	n-2	n-1	n	-	-	-	-	-
A	52 ± 3	19 ± 1	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
B	49 ± 5	19 ± 4	3.3 ± 0.7	3.2 ± 0.8	6 ± 1	5.2 ± 0.8	1.0 ± 0.4	0	0	0	0	0
C	81 ± 9	33 ± 8	3.9 ± 0.9	5 ± 2	12 ± 3	9 ± 2	2.3 ± 0.4	0	0	0	0	0
D	100 ± 0	71 ± 1	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
6:2 FTUCA (n=6)			n-3	n-2	n-1	n	-	-	-	-	-	-
A	100 ± 0	20 ± 2	4.8 ± 0.8	4.6 ± 0.8	11 ± 1	0	0	0	0	0	0	0
B	100 ± 0	19 ± 4	5 ± 1	4 ± 2	10 ± 1	0	0	0	0	0	0	0
C	100 ± 0	20 ± 3	4.7 ± 0.5	5 ± 1	11 ± 2	0	0	0	0	0	0	0

D	100 ± 0	35 ± 1	8.9 ± 0.1	7.6 ± 0.1	18.5 ± 0.6	0	0	0	0	0	0	0
6:2 FTCA and 10:2 FTCA 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	100 6:2 FTCA 100 10:2 FTCA	33 ± 2	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
B	100 6:2 FTCA 100 10:2 FTCA	46 ± 6	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
C	100 6:2 FTCA 100 10:2 FTCA	49 ± 7	7.5 ± 0.6	8 ± 1	17 ± 3	3 ± 1	5 ± 1	5 ± 1	3 ± 1	0	0	0
D	100 6:2 FTCA 100 10:2 FTCA	61 ± 1	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
6:2 FTS and 10:2 FTS 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	62 ± 4 6:2 FTS 45 ± 4 10:2 FTS	7 ± 1	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
B	85 ± 3 6:2 FTS 73 ± 1 10:2 FTS	8 ± 3	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
C	95 ± 4 6:2 FTS 79 ± 6 10:2 FTS	16 ± 5	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
D	100 ± 0 6:2 FTS	50 ± 2	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

	91 ± 1 10:2 FTS											
Me-FOSAA and Et-FOSAA (n_1= n_2= n =8)			n-5	n-4	n-3	n-2	n-1	n	-	-	n	-
A	23 ± 3 Me-FOSAA 28 ± 3 Et-FOSAA	13 ± 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
B	46 ± 5 Me-FOSAA 43 ± 4 Et-FOSAA	13 ± 2	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
C	79 ± 9 Me-FOSAA 75 ± 8 Et-FOSAA	48 ± 4	0.5 ± 0.1	0.6 ± 0.2	2 ± 1	3 ± 2	33 ± 2	0	0	0	8.9 ± 0.5	0
D	100 ± 0 Me-FOSAA 100 ± 0 Et-FOSAA	99 ± 3	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
	ADONA		-	-	-	-	-	-	-	-	-	-
A	66 ± 3	61 ± 50	0	0	0	0	0	0	0	0	0	61 ± 50
B	76 ± 6	80 ± 50	0	0	0	0	0	0	0	0	0	80 ± 50
C	81 ± 5	83 ± 50	0	0	0	0	0	0	0	0	0	83 ± 50
D	100	130 ± 50	0	0	0	0	0	0	0	0	0	130 ± 50

n, n_1, n_2 = number of perfluorinated carbons in the precursor's structure

All reported values are based on triplicate experiments.

Oxidation test A showed that conditions similar to the ones commonly used in previous TOPA studies [31, 36, 42, 43] were not sufficient for complete oxidation in human serum for 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 energies for fluorotelomer carboxylic acids and sulfonates has been observed by Liu et al. [45]. 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 higher reactivity for fluorotelomers with shorter fluorinated chains [45]. 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 of 66% after oxidation (Table 2, Figure 2).

However, independently from the completeness of the precursor's conversion, a 100 % yield of PFAA was never observed in method A (Table 2, Figure 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 interfere with the oxidation process [40, 46, 47]. To prevent the scavenging of oxidant within the sample, two different approaches are described in literature. A direct TOPA 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 [40, 48]. A second approach consists of the use of a hydrogen peroxide pretreatment prior to extraction and oxidation, not suitable for our serum samples [47].

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 TOPA 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 [49, 50]. 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 hours, 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 TOPA has been reported independently [43]. 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 hours (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 TOPA 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 (Figure 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).

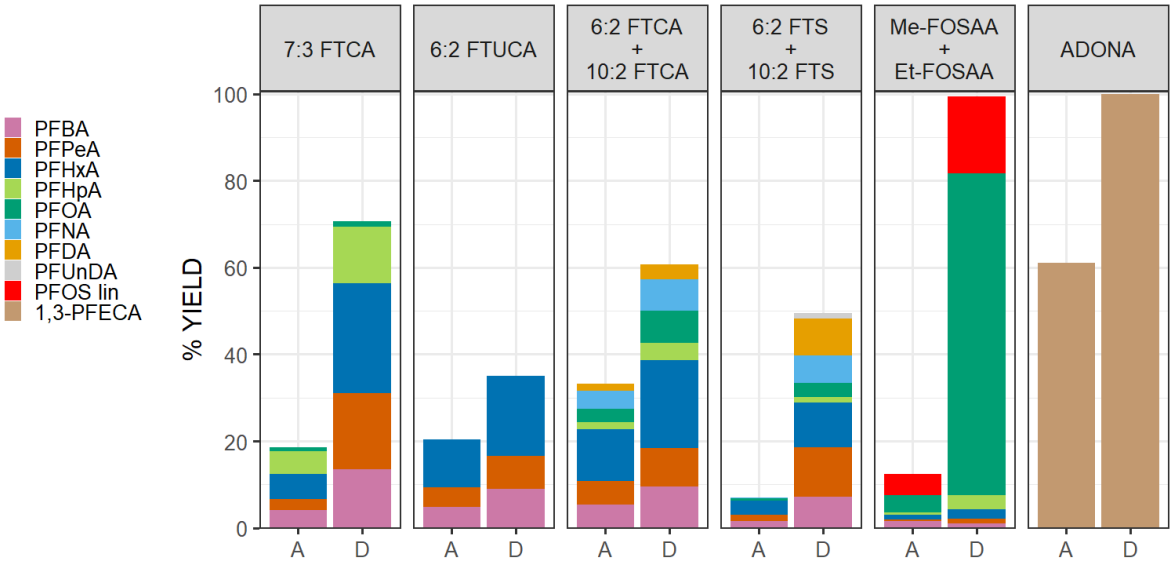


Figure 2 – Yield of oxidation products from the model precursors in the initial test method (method A) and in the optimized method for TOP in human serum (method D).

3.2. Oxidation products patterns

After optimization of the oxidation process, the TOPA 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 TOPA experiments in human serum and the identity of the respective precursors present.

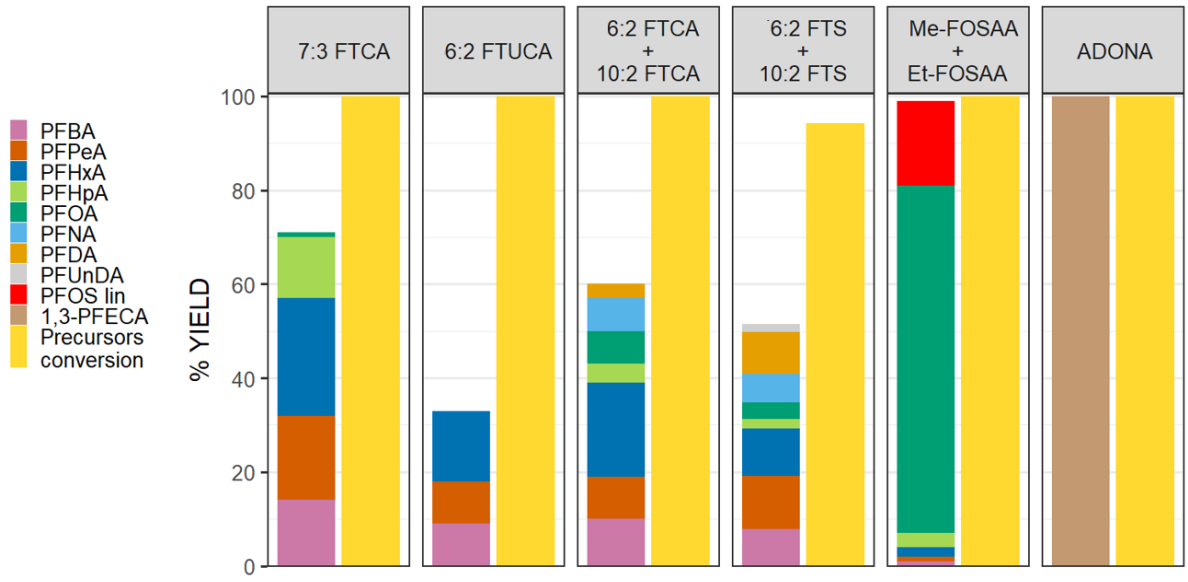
For the fluorotelomer carboxylic acids in human serum with method D, mixtures of PFCA were observed (Figure 3, Table 2). For 6:2 FTCA, 10:2 FTCA and 6:2 FTUCA, PFCA with n-1 fluorinated carbons (where n is referring to the number of fluorinated carbons in the precursor as in Table 2) and shorter carbon 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 (Figure 3). Similar PFCA patterns for these model substances have been observed in ultrapure water, but in this case also TFA and PFPrA were included, showing that the ultra-short PFAA can also be relevant oxidation products [42]. 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 [42].

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 were the ones with the number of fluorinated carbons preserved, and the dominant products were the n-2 PFCA (Figure 3, Table 2). Higher yields were reported in the literature, even if also in these studies the PFAA yields did not reach 100 % [31, 42]. 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 [42].

283 The inclusion of TFA and PFPrA to the target PFAS analyses list for the TOPA has been proven
284 to be beneficial also for other precursors [36, 42] and it is an essential step to make the assay
285 fully quantitative in any matrix, especially when short PFAA precursors are present [51, 52].
286 However, the formation of intermediate and additional stable oxidation products should also
287 be considered. As it can be observed for Me-FOSAA and Et-FOSAA, full oxidation was
288 observed under the final TOPA conditions, but in method A, B and C, FOSA, Me-FOSA and
289 Et-FOSA were identified as intermediates of the oxidative treatment (Figure 4). These
290 intermediates have been observed in hydroxyl radical oxidation experiments before [53] and
291 their detection in our tests highlights the possible formation of unknown intermediates in the
292 TOPA.

293 Our testing on Me-FOSAA and Et-FOSAA also showed the importance of considering the
294 possible formation of stable end products, other than PFCA. In the original TOPA, Me-FOSAA
295 and Et-FOSAA were quantitatively converted to PFOA [31]. This was not the case in our
296 experiments, where PFOA was still the dominant product, but shorter chain PFCA accounted
297 for 8% of the yield and, interestingly, PFOS was the second dominant product accounting for
298 18% of the yield (Figure 4). The formation of PFOS or any other perfluorinated sulfonate by
299 the TOPA has earlier been disregarded, reporting only PFCA as oxidation products [54-57]. In
300 one application of the TOPA to suspended particulate matter, the PFOS increase after oxidation
301 was attributed to the release of non-extractable PFOS during the oxidation, because precursor
302 conversion to PFSA in the TOPA had not been described before [54]. Our experiments show
303 that PFSA can also be relevant end products in the TOPA. Therefore, we recommend the
304 inclusion of PFSA to the target PFAS portfolio after oxidation, as these could also be end
305 products of additional known or unknown precursors that have not been tested before.

306



307

308 **Figure 3 –** Yield of products (first bar) and conversion of precursors (second bar) for the model

309 precursors in the optimized method for TOP in human serum (method D).

310

311 In addition to the PFSA inclusion to the target PFAS analyses after oxidation, other stable end

312 products besides PFCA should be considered. For example, the formation of PFECA would

313 enable the use of the TOPA to evaluate the presence of oxidizable precursors containing ether

314 groups [43]. ADONA is not stable in the oxidation, but it is not transformed to PFAA. ADONA

315 was fully converted to 1,3-PFECA (Table 2 and Figure S4), showing that the TOPA can also

316 be used to detect oxidizable precursors with ether groups by including stable PFECA among

317 the PFAS analyses portfolio.

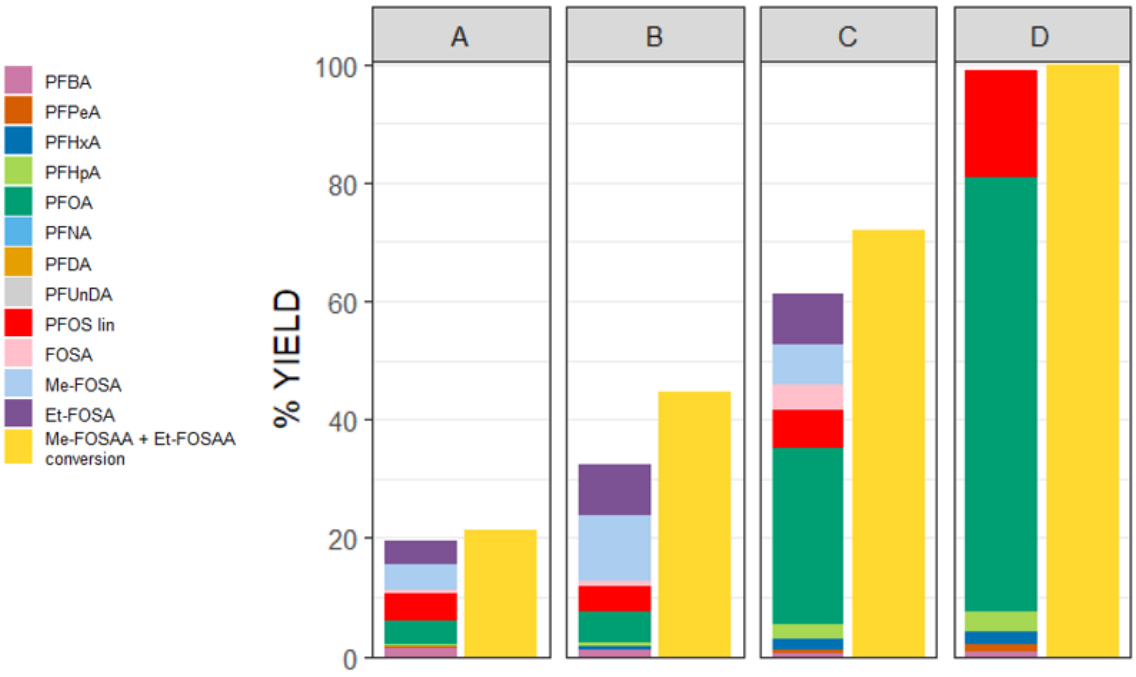


Figure 4 – Yield of products (first bar) and conversion of precursors (second bar) for Me-FOSAA and Et-FOSAA in human serum with method A, B, C and D.

3.3. Method evaluation

Low levels of PFAA were detected in the blanks before and after oxidation (Table S3). 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. RSDs both before and after oxidation were always below 20% for all detected PFAS (Table S4 and S5). 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 S7).

Recoveries were satisfactory for all the available internal standard, 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 S6). Recoveries after the oxidation were lower than before the oxidation due to the additional MTBE extraction step needed after the TOPA. 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 labelled PFAA concentrations were observed, evidencing that the oxidation step does not affect the present PFAA (Figure S5).

3.4. TOPA for human serum strengths and limitations

The here presented TOPA method allows for the processing of a large series of samples in a short time, opening for high-throughput screening of human serum and other valuable biological samples for otherwise undetected PFAA precursors. By using only one extract from a small volume of human serum, conventional PFAS and oxidizable precursors can be measured at the same time without the need of additional instrumentation, analytical methodology or standards in a time efficient manner. The TOPA application on human serum can provide both qualitative and semi-quantitative information about the presence of unknown oxidizable PFAA precursors.

Even if the complete precursors' identity is lost by oxidation, the reaction products patterns can give indications about some of the precursors' structural features, like the length of the fluorinated chain or the presence of specific functional groups. The inclusion of PFSA and PFECA as target analytes in the TOPA will increase the probability to provisionally identify a precursor. Even provisional identification might not be possible in every case: as it was shown here, many precursors produce mixtures of PFAA, and mixtures of precursors would produce

even more complex mixtures of PFAA. Other techniques, as for example the use of hydrolysis as a pre-treatment, could be considered as additional tools for identification of precursors [58].

Further, the determined change in PFAA and PFECA concentrations can be used to give an estimate of the total oxidizable precursors present in human serum. It is of utmost importance to fully comprehend, that the TOPA can yield only semi-quantitative estimates since the nature of precursors in the sample is *a priori* unknown.

To conclude, the TOPA can be used to reveal human exposure to unknown oxidizable PFAA precursors. To fully describe human exposure to potentially harmful PFAA and PFECA, it is important to understand the contribution of their precursors as indirect exposure source. The TOPA 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 TOPA 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.

Supporting Information

Equations for conversion of precursors and product yield; list of model PFAS; list of target PFAS; blanks, LODs and LOQs before and after oxidation; reproducibility before and after oxidation; recoveries before and after oxidation; method accuracy before oxidation; yield of products and conversion of precursors with and without shaking; yield of products and conversion of precursors with $K_2S_2O_8$ or $Na_2S_2O_8$ as oxidant; yield of products and conversion of precursors with 200 ng and 4 ng of selected precursors; chromatograms for ADONA and 1,3-PFECA.

Acknowledgements

This work received funding from the PERFORCE3 Innovative Training Network, funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement 860665, and from the PERFORCE-North project, funded by the program "Hazardous substances – effects on ecosystem and health" from the Fram Centre.

We thank Wendy van Dreunen from UiT and Unni Mette Nordang from NILU for their contribution to the laboratory work. We also want to thank Ian Cousins (SU) for leading the PERFORCE3 project.

Notes

The authors declare no competing financial interest.

References

1. Gluge, J., et al., *An overview of the uses of per- and polyfluoroalkyl substances (PFAS).* Environ Sci Process Impacts, 2020.
2. Kissa, E., *Fluorinated Surfactants and Repellents: Second Edition, Revised and Expanded Surfactant Science Series. .* Journal of the American Chemical Society, 2001. 123(36): p. 8882-8882.
3. Buck, R.C., P.M. Murphy, and M. Pabon, *Chemistry, Properties, and Uses of Commercial Fluorinated Surfactants*, in *Polyfluorinated Chemicals and Transformation Products*. 2012. p. 1-24.
4. Poothong, S., et al., *Multiple pathways of human exposure to poly- and perfluoroalkyl substances (PFASs): From external exposure to human blood.* Environ Int, 2020. 134: p. 105244.
5. Blake, B.E., et al., *Evaluation of Maternal, Embryo, and Placental Effects in CD-1 Mice following Gestational Exposure to Perfluorooctanoic Acid (PFOA) or Hexafluoropropylene Oxide Dimer Acid (HFPO-DA or GenX).* Environ Health Perspect, 2020. 128(2): p. 27006.
6. Guillette, T.C., et al., *Elevated levels of per- and polyfluoroalkyl substances in Cape Fear River Striped Bass (Morone saxatilis) are associated with biomarkers of altered immune and liver function.* Environ Int, 2020. 136: p. 105358.
7. Zhang, Y., et al., *Exposure of female mice to perfluorooctanoic acid suppresses hypothalamic kisspeptin-reproductive endocrine system through enhanced hepatic fibroblast growth factor 21 synthesis, leading to ovulation failure and prolonged dioestrus.* J Neuroendocrinol, 2020. 32(5): p. e12848.
8. van Esterik, J.C., et al., *Programming of metabolic effects in C57BL/6JxFVB mice by in utero and lactational exposure to perfluorooctanoic acid.* Arch Toxicol, 2016. 90(3): p. 701-15.
9. Caverly Rae, J.M., et al., *Evaluation of chronic toxicity and carcinogenicity of ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate in Sprague-Dawley rats.* Toxicol Rep, 2015. 2: p. 939-949.
10. Filgo, A.J., et al., *Perfluorooctanoic Acid (PFOA)-induced Liver Lesions in Two Strains of Mice Following Developmental Exposures: PPARalpha Is Not Required.* Toxicol Pathol, 2015. 43(4): p. 558-68.
11. Fenton, S.E., et al., *Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research.* Environ Toxicol Chem, 2021. 40(3): p. 606-630.
12. Lee, J.E. and K. Choi, *Perfluoroalkyl substances exposure and thyroid hormones in humans: epidemiological observations and implications.* Ann Pediatr Endocrinol Metab, 2017. 22(1): p. 6-14.
13. Darrow, L.A., et al., *Modeled Perfluorooctanoic Acid (PFOA) Exposure and Liver Function in a Mid-Ohio Valley Community.* Environ Health Perspect, 2016. 124(8): p. 1227-33.
14. Abraham, K., et al., *Perfluorobutanoic acid (PFBA): No high-level accumulation in human lung and kidney tissue.* Int J Hyg Environ Health, 2021. 237: p. 113830.
15. Grandjean, P., et al., *Serum Vaccine Antibody Concentrations in Adolescents Exposed to Perfluorinated Compounds.* Environ Health Perspect, 2017. 125(7): p. 077018.
16. Kvalem, H.E., et al., *Perfluoroalkyl substances, airways infections, allergy and asthma related health outcomes - implications of gender, exposure period and study design.* Environ Int, 2020. 134: p. 105259.
17. Song, X., et al., *Biomonitoring PFAAs in blood and semen samples: Investigation of a potential link between PFAAs exposure and semen mobility in China.* Environ Int, 2018. 113: p. 50-54.
18. Barry, V., A. Winquist, and K. Steenland, *Perfluorooctanoic acid (PFOA) exposures and incident cancers among adults living near a chemical plant.* Environ Health Perspect, 2013. 121(11-12): p. 1313-8.

19. Shearer, J.J., et al., *Serum concentrations of per- and polyfluoroalkyl substances and risk of renal cell carcinoma.* J Natl Cancer Inst, 2020.
20. Darrow, L.A., C.R. Stein, and K. Steenland, *Serum perfluorooctanoic acid and perfluorooctane sulfonate concentrations in relation to birth outcomes in the Mid-Ohio Valley, 2005-2010.* Environ Health Perspect, 2013. 121(10): p. 1207-13.
21. Steenland, K., et al., *Ulcerative colitis and perfluorooctanoic acid (PFOA) in a highly exposed population of community residents and workers in the mid-Ohio valley.* Environ Health Perspect, 2013. 121(8): p. 900-5.
22. Lopez-Espinosa, M.J., et al., *Thyroid function and perfluoroalkyl acids in children living near a chemical plant.* Environ Health Perspect, 2012. 120(7): p. 1036-41.
23. Kristen J. Hansen, L.A.C., Mark E. Ellefson, and Harold O. Johnson, *Compound-Specific, Quantitative Characterization of Organic Fluorochemicals in Biological Matrices.* Environmental Science & Technology, 2001. 35 (4): p. 766-770.
24. UNEP, *SC-4/17: Listing of perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride.* 2009.
25. UNEP, *SC-9/12: Listing of perfluorooctanoic acid (PFOA), its salts and PFOA-related compounds.* 2019.
26. Land, M., et al., *What is the effect of phasing out long-chain per- and polyfluoroalkyl substances on the concentrations of perfluoroalkyl acids and their precursors in the environment? A systematic review.* Environmental Evidence, 2018. 7(1).
27. OECD, *Toward a new comprehensive global database of per- and polyfluoroalkyl substances (PFASs): Summary report on updating the OECD 2007 list of per- and polyfluoroalkyl substances (PFASs)*, in *Series on Risk Management No. 39*. 2018.
28. Sunderland, E.M., et al., *A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects.* J Expo Sci Environ Epidemiol, 2019. 29(2): p. 131-147.
29. EFSA, *Risk to human health related to the presence of perfluoroalkyl substances in food*, in *EFSA J*. 2020. p. e06223.
30. Miaz, L.T., et al., *Temporal trends of suspect- and target-per/polyfluoroalkyl substances (PFAS), extractable organic fluorine (EOF) and total fluorine (TF) in pooled serum from first-time mothers in Uppsala, Sweden, 1996-2017.* Environ Sci Process Impacts, 2020. 22(4): p. 1071-1083.
31. Houtz, E.F. and D.L. Sedlak, *Oxidative conversion as a means of detecting precursors to perfluoroalkyl acids in urban runoff.* Environ Sci Technol, 2012. 46(17): p. 9342-9.
32. Butt, C.M., D.C. Muir, and S.A. Mabury, *Biotransformation pathways of fluorotelomer-based polyfluoroalkyl substances: a review.* Environ Toxicol Chem, 2014. 33(2): p. 243-67.
33. Zhang, W., et al., *Biotransformation of perfluoroalkyl acid precursors from various environmental systems: advances and perspectives.* Environ Pollut, 2021. 272: p. 115908.
34. Nilsson, H., et al., *Biotransformation of fluorotelomer compound to perfluorocarboxylates in humans.* Environ Int, 2013. 51: p. 8-12.
35. Houtz, E.F., et al., *Poly- and perfluoroalkyl substances in wastewater: Significance of unknown precursors, manufacturing shifts, and likely AFFF impacts.* Water Res, 2016. 95: p. 142-9.
36. Janda, J., et al., *Closing the gap - inclusion of ultrashort-chain perfluoroalkyl carboxylic acids in the total oxidizable precursor (TOP) assay protocol.* Environ Sci Process Impacts, 2019. 21(11): p. 1926-1935.
37. Zhu, H. and K. Kannan, *Total oxidizable precursor assay in the determination of perfluoroalkyl acids in textiles collected from the United States.* Environ Pollut, 2020. 265(Pt B): p. 114940.
38. Houtz, E.F., et al., *Persistence of perfluoroalkyl acid precursors in AFFF-impacted groundwater and soil.* Environ Sci Technol, 2013. 47(15): p. 8187-95.

39. Sorli, J.B., et al., *Risk assessment of consumer spray products using in vitro lung surfactant function inhibition, exposure modelling and chemical analysis.* Food Chem Toxicol, 2022. 164: p. 112999.
40. Gockener, B., et al., *Transfer of Per- and Polyfluoroalkyl Substances (PFAS) from Feed into the Eggs of Laying Hens. Part 1: Analytical Results Including a Modified Total Oxidizable Precursor Assay.* J Agric Food Chem, 2020. 68(45): p. 12527-12538.
41. AMAP, *AMAP Assessment 2021: Human Health in the Arctic.* 2022, Arctic Monitoring and Assessment Programme (AMAP): Tromsø, Norway. p. 240.
42. Martin, D., et al., *Zwitterionic, cationic, and anionic perfluoroalkyl and polyfluoroalkyl substances integrated into total oxidizable precursor assay of contaminated groundwater.* Talanta, 2019. 195: p. 533-542.
43. Zhang, C., et al., *Fate of Per- and Polyfluoroalkyl Ether Acids in the Total Oxidizable Precursor Assay and Implications for the Analysis of Impacted Water.* Environ Sci Technol Lett, 2019. 6(11): p. 662-668.
44. Hanssen, L., et al., *Partition of perfluoroalkyl substances (PFASs) in whole blood and plasma, assessed in maternal and umbilical cord samples from inhabitants of arctic Russia and Uzbekistan.* Sci Total Environ, 2013. 447: p. 430-7.
45. Liu, Z., et al., *Near-Quantitative Defluorination of Perfluorinated and Fluorotelomer Carboxylates and Sulfonates with Integrated Oxidation and Reduction.* Environ Sci Technol, 2021. 55(10): p. 7052-7062.
46. Casson, R. and S.-Y.D. Chiang, *Integrating total oxidizable precursor assay data to evaluate fate and transport of PFASs.* Remediation Journal, 2018. 28(2): p. 71-87.
47. Hutchinson, S., T. Rieck, and X. Wu, *Advanced PFAS precursor digestion methods for biosolids.* Environmental Chemistry, 2020.
48. Gockener, B., et al., *Exploring unknown per- and polyfluoroalkyl substances in the German environment - The total oxidizable precursor assay as helpful tool in research and regulation.* Sci Total Environ, 2021. 782: p. 146825.
49. Liberatore, H.K., et al., *Solvent Suitability for HFPO-DA ("GenX" Parent Acid) in Toxicological Studies.* Environ Sci Technol Lett, 2020. 7(7): p. 477-481.
50. Zhang, C., et al., *Stability of Per- and Polyfluoroalkyl Substances in Solvents Relevant to Environmental and Toxicological Analysis.* Environ Sci Technol, 2022. 56(10): p. 6103-6112.
51. Meng, Y., et al., *Legacy and emerging per- and polyfluoroalkyl substances (PFASs) in Dagang Oilfield: Multimedia distribution and contributions of unknown precursors.* Journal of Hazardous Materials, 2021. 412.
52. Wang, B., et al., *Per- and polyfluoroalkyl substances and the contribution of unknown precursors and short-chain (C2-C3) perfluoroalkyl carboxylic acids at solid waste disposal facilities.* Sci Total Environ, 2020. 705: p. 135832.
53. Plumlee, M.H., K. McNeill, and M. Reinhard, *Indirect Photolysis of Perfluorochemicals: Hydroxyl Radical-Initiated Oxidation of N-Ethyl Perfluorooctane Sulfonamido Acetate (N-EtFOSAA) and Other Perfluoroalkanesulfonamides.* Environmental Science & Technology, 2009. 43(10): p. 3662-3668.
54. Gockener, B., et al., *Long-Term Trends of Per- and Polyfluoroalkyl Substances (PFAS) in Suspended Particular Matter from German Rivers Using the Direct Total Oxidizable Precursor (dTOP) Assay.* Environ Sci Technol, 2022. 56(1): p. 208-217.
55. Kim Lazcano, R., et al., *Characterizing and Comparing Per- and Polyfluoroalkyl Substances in Commercially Available Biosolid and Organic Non-Biosolid-Based Products.* Environ Sci Technol, 2020. 54(14): p. 8640-8648.
56. Simonnet-Laprade, C., et al., *Biomagnification of perfluoroalkyl acids (PFAAs) in the food web of an urban river: assessment of the trophic transfer of targeted and unknown precursors and implications.* Environ Sci Process Impacts, 2019. 21(11): p. 1864-1874.

57. Sivaram, A.K., et al., *Per- and polyfluoroalkyl substances (PFAS) in commercial composts, garden soils, and potting mixes of Australia.* Environmental Advances, 2022. 7.