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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.

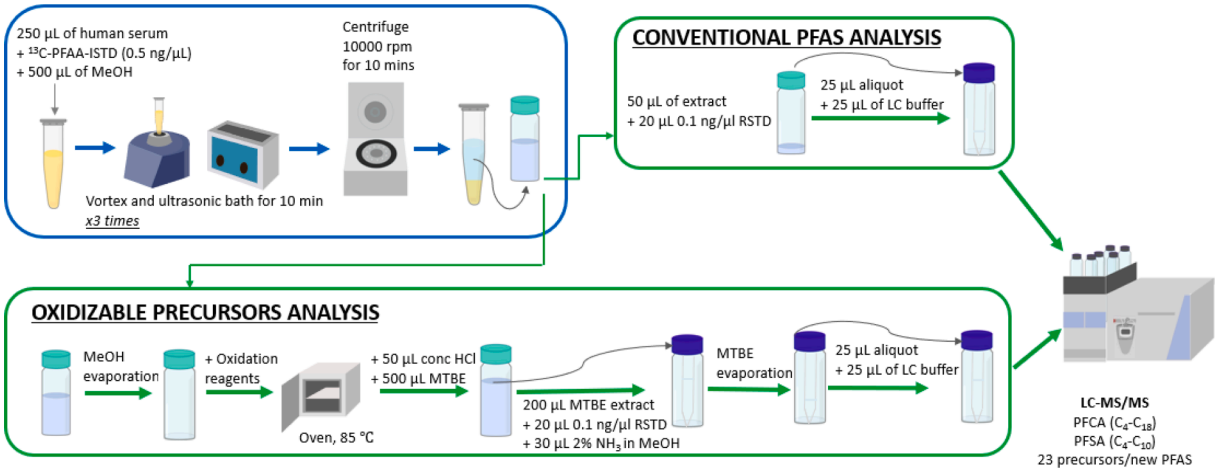

Fig. 1. Scheme of the TOP assay protocol for human serum.

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 ℃ 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

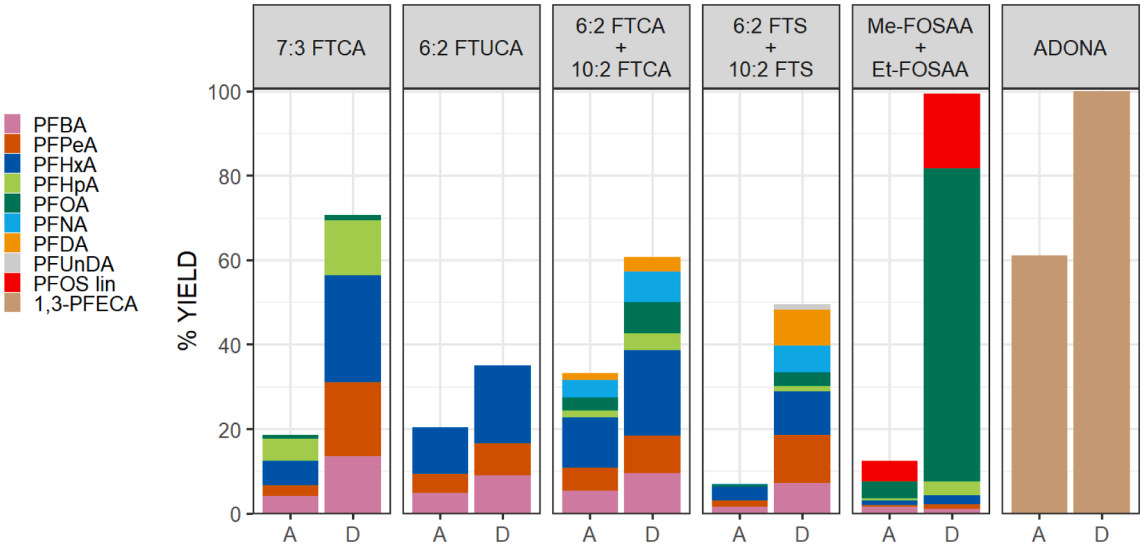

Fig. 2. Yield of oxidation products from the model precursors in the initial test method (method A) and in the optimized method for TOP in human serum (method D).

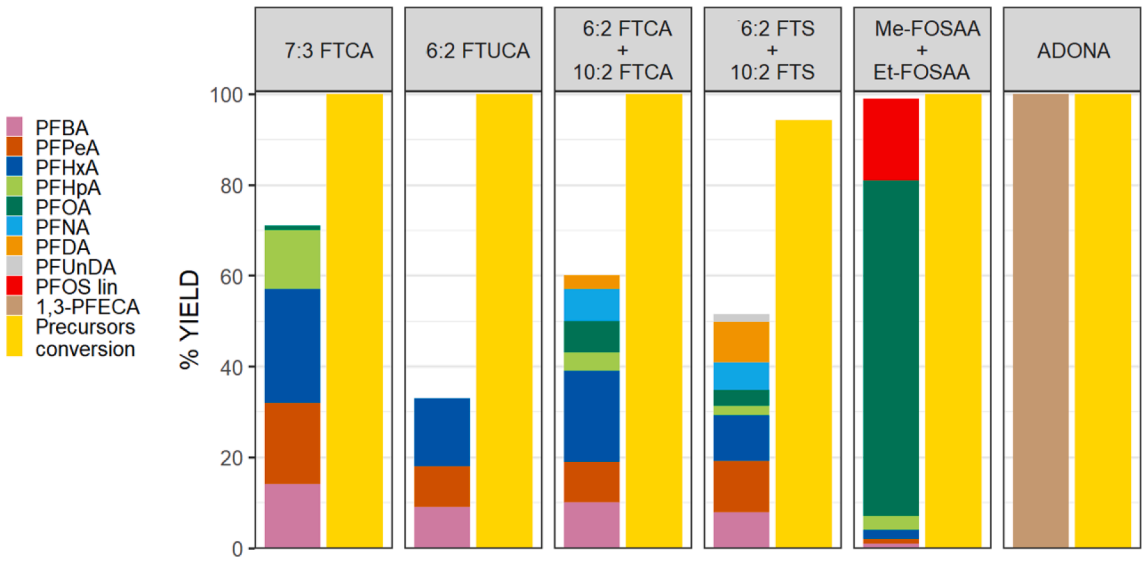

Fig. 3. Yield of products (first bar) and conversion of precursors (second bar) for the model precursors in the optimized method for TOP in human serum (method D).

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

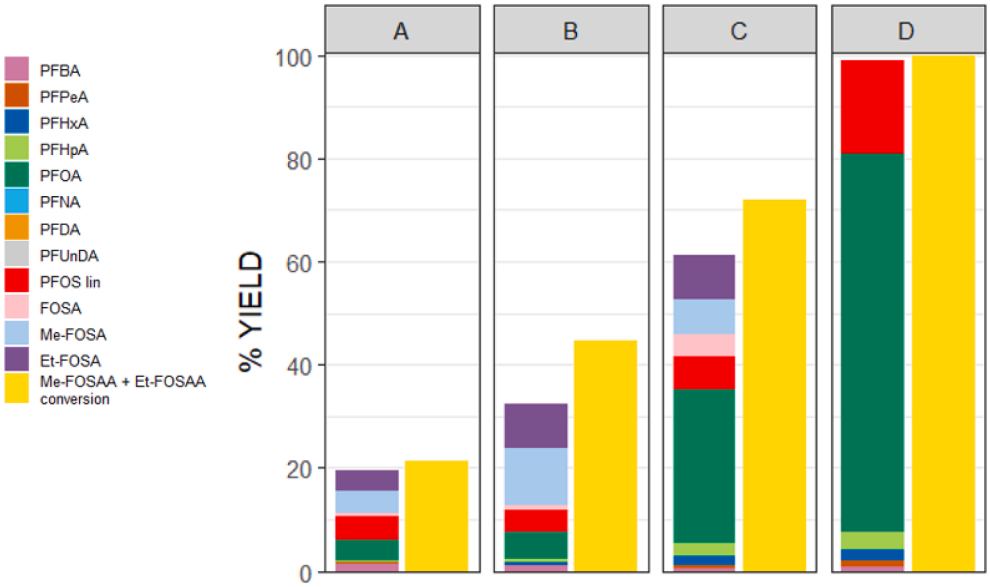

Fig. 4. Yield of products (first bar) and conversion of precursors (second bar) for Me-FOSAA and Et-FOSAA in human serum with method A, B, C and D.

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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