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Technical annex on the determination of Per- and polyfluoroalkyl substances (PFASs) in biota

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1. Background

Perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA) and other perfluoroalkyl acids (PFAAs) are global environmental contaminants. PFOS and PFOA are chemically and biologically inert and very stable (Poulsen et al. 2005). PFOS meets the P (Persistent) and vP (very Persistent) criteria due to slow degradation. PFOS is also bioaccumulative (B) and toxic (T) (OSPAR 2005). PFOA is considered very persistent (vP) and toxic (T) (Van der Putte et al. 2010). PFOA also has the capacity to undergo long-range transportation.

Per- and polyfluoroalkylsubstances (PFAS) can bind to serum albumin and accumulate in blood and organs (Jones et al., 2003). Accumulation in the marine food web starts from the bottom of the food chain, with invertebrates such as zooplankton and molluscs, followed by crustaceans, and then fish (Van de Vijver et al., 2003). The highest PFAS concentrations have been found in blood and internal organs (e.g. liver, kidney) of top predators, such as marine mammals (Martin et al., 2004; Ahrens et al., 2009) and fish-eating birds (Kannan et al., 2001). Fish species widely used for monitoring of

organic contaminants are also suitable for PFAS analysis. Based on the literature, liver is the first choice of tissue for monitoring purposes but PFAS has also been detected in other organs (e.g. blood and muscle at lower concentrations) (Ahrens et al., 2010).

The following guideline focuses on the sampling and extraction of PFASs from biota and address the special aspects of the sampling matrix and is based on a previous ICES guideline for PFAS analysis in biota (Ahrens et al., 2010). It also provides advice for the analysis of PFASs in biota which includes the following steps:

- sampling and tissue selection;
- pretreatment;
- analytical determination.

1.1 Introduction

PFOS and PFOA are members of the larger family of PFASs (in the past referred to as "PFCs"). PFASs are diverse in terms of structure and other categorization elements (Figure 1). Categorization, as well as the terminology related to PFASs, is challenging and furthermore many of the compounds are not commercially available. Compounds manufactured from the starting material perfluorooctyl sulfonyl fluoride (POSF) can degrade to PFOS, and are therefore referred to as PFOS-related compounds. The Swedish Chemicals Agency (KEMI) estimate that there are 3000 available PFASs on the global market (KEMI 2015). OECD has identified and manually categorized 4730 PFAS-related CAS numbers, including several new groups of PFASs that fulfil the common definition of PFASs (i.e. they contain at least one perfluoroalkyl moiety) but have not yet been commonly regarded as PFASs. In particular, it focuses on those PFASs, including perfluorocarbons, that contain a perfluoroalkyl moiety with three or more carbons (i.e. $–C_nF_{2n}–$, $n \geq 3$) or a perfluoroalkylether moiety with two or more carbons (i.e. $–C_nF_{2n}OC_mF_{2m}–$, n and $m \geq 1$) (OECD 2018).

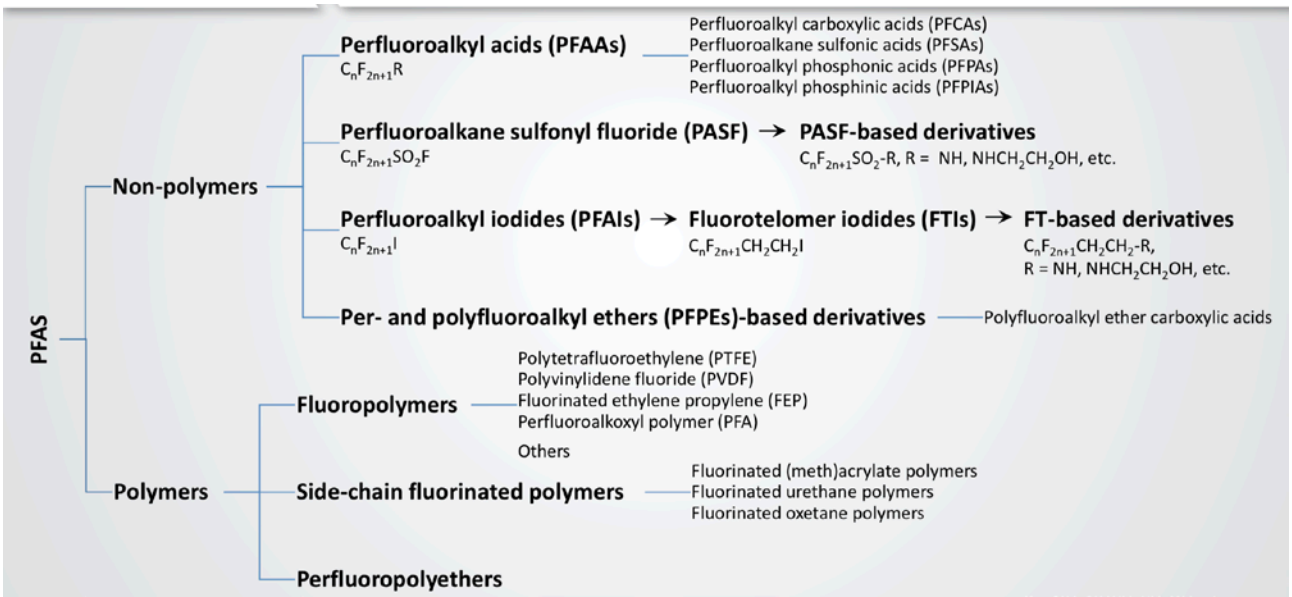

Figure 1 Schematic division of PFASs (EPA 2018[1]).

PFASs are widely detected in the Baltic Sea Region (Sahline 2017). Table 1 provides an overview of PFAS that are environmentally relevant in water and biota and provides information on chemical names, acronyms, formula, and Chemical Abstracts Service (CAS) numbers. The list has been compiled from previous studies worldwide.

Table 1. List of possible PFASs to be monitored based on previous findings.

ANALYTE	***ACRONYM***	***FORMULA***	***CAS-NUMBER***
Perfluorobutanoate	*PFBA*	$C_3F_7COO^-$	*375-22-4*
Perfluorohexanoate	*PFHxA*	$C_5F_{11}COO^-$	*307-24-4*
Perfluorooctanoate	*PFOA*	$C_7F_{15}COO^-$	*335-67-1*
Perfluorononanoate	*PFNA*	$C_8F_{17}COO^-$	*375-95-1*
Perfluorodecanoate	*PFDA*	$C_9F_{19}COO^-$	*335-76-2*
Perfluoroundecanoate	*PFUnDA*	$C_{10}F_{21}COO^-$	*2058-94-8*
Perfluorododecanoate	*PFDoDA*	$C_{11}F_{23}COO^-$	*307-55-1*
Perfluorotridecanoate	*PFTrDA*	$C_{12}F_{25}COO^-$	*72629-94-8*
Perfluorotetradecanoate	*PFTeDA*	$C_{13}F_{27}COO^-$	*376-06-7*
Perfluorobutane sulfonate	*PFBS*	$C_4F_9SO_2O^-$	*29420-49-3 (potassium salt)*
Perfluorohexane sulfonate	*PFHxS*	$C_6F_{13}SO_2O^-$	*3871-99-6 (potassium salt)*

[1] Marc A. Mills, Ph. D. EPA Office of Research and Development PFAS 101: An Introduction to PFAS and EPA research on PFAS Presentation to "Per- and Polyfluoroalkyl Substances (PFAS) Heartland Community Engagement Meeting" https://www.epa.gov/sites/production/files/2018-09/documents/final_epa_pfas_leavenworth_kansas_presentations_september_5_2018.pdf

Perfluorooctane sulfonate	*PFOS*	*$C_8F_{17}SO_2O^-$*	*2795-39-3 (potassium salt)*
Perfluorooctane sulfonamide	*FOSA (same as PFOSA)*	*$C_8F_{17}SO_2NH_2$*	*754-91-6*

In addition to PFOS analysis that fulfills the HELCOM indicators needs, a broad target list of substances should be preferred for monitoring of PFASs in biota. Wider range of substances addresses the large number and diversity of PFASs. Long-chain PFASs (≥ C_6) should be included because of their potential to bioaccumulate. Only through using longer lists of PFASs in biota monitoring, a data can be collected that can help to fully understand and characterize PFAS exposure in environment.

1.2 Purpose and aims

The aim of the monitoring is to identify spatial variations and temporal trends of PFASs in biota and to provide information on the core indicator "perfluorooctane sulfonate (PFOS)". Monitoring of PFASs in biota samples provides information on the contaminant load of the Baltic Sea and the possible need for development of a PFAS indicator (expand existing PFOS indicator to the PFAS indicator).

Based on the current core indicator, good status is achieved when the concentration of PFOS in fish muscle is below 9.1 µg/kg fish wet weight. The threshold value is an environmental quality standard (EQS), derived at EU level as a substance included on the list of priority substances under the Water Framework Directive (2000/60/EC and 2013/39/EC). Good environmental status within the Marine Strategy Framework Directive (MSFD) is defined as 'concentrations of contaminants at levels not giving rise to pollution effects'. EQSs are derived from ecotoxicological studies to protect freshwater and marine ecosystems from potential adverse effects of chemicals, as well as adverse effects on human health via drinking water and food from aquatic environments (2018 Helcom core indicator report[2]).

2 Monitoring methods

2.1 Monitoring features

The main focus of monitoring coastal and open sea biota for PFAS contamination.

2.2 Time and area

Monitoring covers the entire Baltic area.

2.3 Monitoring procedure

2.3.1 Sampling, selection of species and tissue for PFAS analysis

In general sampling of biota should be in accordance with Manual for Marine Monitoring in the COMBINE Programme of HELCOM "Monitoring guideline on biological material sampling and

[2] http://www.helcom.fi/Core%20Indicators/Perfluorooctane%20sulphonate%20PFOS%20HELCOM%20core%20indicator%202018.pdf

sample handling for the analysis of persistent organic pollutants (PAHs, PCBs and OCPs) and metallic trace elements"[3].

Each biota sample should be characterized and described in a suitable sample protocol. For fish species, length, weight and, if possible, sex should be reported as a minimum. After the preparation of each sample, including the preparation of different organs from the same individual, the tools should be changed or cleaned.

Fish species widely used for monitoring of organic contaminants in the HELCOM area are suitable for PFAS analysis. Fish species recommended for monitoring in the Baltic Sea are dab, flounder, cod, herring, perch and eelpout. The fish species should be chosen dependent on the sampling method and availability in the area (coastal/off-shore). The number of fish individuals needed depends on species, but should be targeted to suitable size and age class and preferably two composite samples analyzed for contaminants (preferably 15 - 20 individual (but a minimum of 12 individuals)). The amount of material is also dependent on what other analyses should be performed from the same sample preparations. PFAS analyses can be performed with ca. 5 g, but PCDD/F analyses may require up to 100 g. Sex, size and age should be defined to reduce biological variability for long term trend analysis.

The biological variability (sex, size and age) should be defined in each country's monitoring program. Biological variability should be as low as possible and suitable for long term trend analysis. PFASis accumulate differently in species. Even though no significant difference in PFOS liver:muscle ratio has been found between species, it might not be appropriate to use a mean ratio for all the species, since only some of the species did show a linear relationship between the liver and muscle. For herring and perch (both marine and limnic), there were strong linear relationships between liver and muscle, therefore these can be grouped (Faxneld et al. 2014).

Muscle is the recommended tissue for PFAS monitoring to fulfill the HELCOM core indicator needs. Muscle is chosen based on harmonization purposes of MSFD criteria's monitoring of D8 and D9. Another reason is the WFD EQS being "Human Health" - related and harmonization for coastal waters monitored for WFD and MSFD purposes needs to be taken in to account. Muscle samples should be used for monitoring in the HELCOM area despite the suggestion from the literature that liver should be the first choice of tissue for PFAS analyzis in biota since PFAS concentrations may be an order of magnitude lower than liver in most fish species. For example PFOS in herring and perch has to be multiplied with 17 to convert the wet weight in edible parts of fish to liver concentrations. PFAS often is measured in liver, while the EQS is set to protect human health via consumption of fishery products and thus is more relevant for the evaluation of measured concentrations in edible parts (e.g., muscle or muscle+skin), it is possible to use calculated conversion factors between liver and muscle(Faxneld et al. 2014).

Sampling should be carried out by trained personnel who are aware of the risk of sample contamination posed by incorrect handling. When analyzing PFASs from biologically active samples, it is highly recommended to prepare the samples for analysis as soon as possible after sampling without further manipulation or store them in closed containers below -20 °C until sample preparation. Moreover, samples should not be transferred into new containers before extraction.

3 https://www.helcom.fi/wp-content/uploads/2019/08/Manual-for-Marine-Monitoring-in-the-COMBINE-Programme-of-HELCOM_PartB_AnnexB12_Appendix1.pdf

Handling time at room temperature should be minimal in order to prevent any possible degradation of PFAA-precursors (Tomy et al., 2004, Ahrens et al., 2010).

Materials and clothes that contain, or may adsorb, fluorinated compounds, such as polytetrafluoroethylene (PTFE), must be avoided during sampling and sample processing. In particular, the containers that come into direct contact with the sample must not contain any fluorinated polymers (e.g. PTFE). Containers and equipment made of polypropylene (according to ISO 21675:2019), polyethylene, glass, or stainless steel should be used. However, especially glass and polypropylene sampling and storage containers should be carefully checked for PFASs, as longer chained PFASs tend to adsorb to glass walls, and cleaned before use (e.g. the sample container or filtration equipment must be rinsed with a polar solvent, such as methanol and dried before use) in order to minimize contamination. All material that may come into contact with the sample must be free of fluorinated compounds. Dissection of samples should be carried out with stainless-steel tools.

2.3.2 Sample handling and analysis

All the steps are susceptible for cross contamination. All the equipment used for sampling should be tested for suitability in PFAS analysis with un using blanks, analysis of reference materials and/or spiked samples.

Homogenization of the samples is necessary and a homogenizator with non-fluorinated plastic dispersing components (e.g. polycarbonate and polysulfone) should be used in order to obtain samples free of contamination. Samples can be homogenized and then kept below -20 °C until extraction.

Spiking with internal standards
Samples should be spiked with internal standards (see Table 2) before extraction, at concentrations close to the environmental level, in order to correct for losses during extraction, extract clean-up and concentration, and for matrix effects during analysis. It is advisable that there is a corresponding internal standard (ideally, structurally matched) for each of the PFASs as the recoveries and matrix effects differentiate between analytes. After spiking and before extraction, biota samples should be left to equilibrate for approximately 30 minutes to 1 hour at 4 °C (Kaupmees, 2017). Isotopically labelled internal standards help to take into account recovery as well as matrix effects.

Table 2. Possible internal standards for PFAS analysis.

INTERNAL STANDARDS		
Perfluoro-n-(1,2,3,4-$^{13}C_4$)butanate	*[$^{13}C_4$]-PFBA*	*(2,3,4-$^{13}C_3$)$F_7{}^{13}COO^-$*
Perfluoro-n-(1,2-$^{13}C_2$)hexanate	*[$^{13}C_2$]-PFHxA*	*C_4F_9(2-^{13}C)$F_2{}^{13}COO^-$*
Perfluoro-n-(1,2,3,4-$^{13}C_4$)octanate	*[$^{13}C_4$]-PFOA*	*C_4F_9(2,3,4-$^{13}C_3$)$F_6{}^{13}COO^-$*
Perfluoro-n-(1,2,3,4,5,6,7,8-$^{13}C_8$)octanate	*[$^{13}C_8$]-PFOA*	*(2,3,4,5,6,7,8-$^{13}C_7$)$F_{15}{}^{13}COO^-$*
Perfluoro-n-(1,2,3,4,5-$^{13}C_5$)nonanate	*[$^{13}C_5$]-PFNA*	*C_4F_9(2,3,4,5-$^{13}C_4$)$F_8{}^{13}COO^-$*
Perfluoro-n-(1,2-$^{13}C_2$)decanate	*[$^{13}C_2$]-PFDA*	*$C_8F_{17}{}^{13}CF_2{}^{13}COO^-$*
Perfluoro-n-(1,2-$^{13}C_2$)undecanate	*[$^{13}C_2$]-PFUnDA*	*$C_9F_{19}{}^{13}CF_2{}^{13}COO^-$*
Perfluoro-n-(1,2-$^{13}C_2$)dodecanate	*[$^{13}C_2$]-PFDoDA*	*$C_{10}F_{21}{}^{13}CF_2{}^{13}COO^-$*
Perfluoro-n-[1,2-$^{13}C_2$]tetradecanoic acid	*[$^{13}C_2$]-PFTeDA*	*$C_{12}F_{25}{}^{13}CF_2{}^{13}COOH$*
Perfluoro-1-hexane($^{18}O_2$)sulfonate	*[$^{18}O_2$]-PFHxS*	*$C_6F_{13}S(^{18}O_2)O^-$*
Perfluoro-1-(1,2,3,4-$^{13}C_4$)octanesulfonate	*[$^{13}C_4$]-PFOS*	*C_4F_9(1,2,3,4-$^{13}C_4$)$F_8SO_2O^-$*
Perfluoro-1-(1,2,3,4,5,6,7,8-$^{13}C_8$)octanesulfonate	*[$^{13}C_8$]-PFOS*	*(1,2,3,4,5,6,7,8-$^{13}C_8$)$F_{17}SO_2O$*
Perfluoro-1-(1,2,3,4,5,6,7,8-$^{13}C_8$)octanesulfonamide	*[$^{13}C_8$]-FOSA*	*(1,2,3,4,5,6,7,8-$^{13}C_8$)$F_{17}SO_2NH_2$*
N-deuterioethylperfluoro-1-octanesulfonamidoacetic acid	*d_5-N-EtFOSAA*	*$C_8F_{17}SO_2N(C_2D_5)CH_2CO_2H$*

Blanks and contamination

Due to the significant handling during sample preparation, every sample batch should include a procedural blank that has been prepared in the same way as the samples. If measurable blanks occur, the analytical instrumentation and every sample preparation step must be checked for contamination and eliminated. If elimination is not possible and the sources of contamination are known and constant for all the samples, the blank concentrations can be subtracted from the samples in cases where they have been tested during the validation procedure.

In order to minimize the risk of sample contamination, especially when blanks are a problem, sample treatment and processing should be carried out on a clean bench or in a clean room containing no fluorinated compounds (e.g. PTFE) and it is strongly recommended to pretreat all used glassware and adsorption material (for example by rinsing with HPLC grade water and methanol (Kaupmees, 2017)). This also includes washing syringes and syringe filters used for filtration of extracts prior chromatographic analysis. Solvents, including ultrapure water and the internal standard spiking solution should be tested for PFASs and additionally, a dispenser (check for PFAS) can be used for solvents so that constant opening and closing of the containers will not contaminate.

Extraction and clean-up

It is advisable to keep sample preparation as simple as possible in order to avoid any contamination. Methods commonly used for the extraction of PFASs from biota samples.

1) ion pair extraction with tetrabutylammonium (TBA) and the extraction solvent methyl *tert*-butyl ether (MTBE).(Hansen *et al*., 2001) This method avoids contamination by skipping the clean-up step. The matrix-effects of a dirtier extract are taken into account by using IS-s (Kaupmees, 2017).

2) ultrasonic extraction (UE) with acetonitrile or methanol with subsequent clean-up. Non-polar lipids can be removed from methanol or acetonitrile extracts by precipitation at − 20 °C (Powley *et al*., 2008).

3) additional extract cleaning with different solid phase extraction cartridges containing HLB, WAX or ENVI-Carb resin[4] and such as by So et al (So *et al*., 2006).

There is no universal, standardised method for PFAS analysis in biota. There is therefore a constant development of new analysis methods for PFASs in complex matrices. It is recommended to look for new methods in peer-reviewed articles.

Sample extracts should be concentrated in order to meet the required quantification limits. Concentration techniques at low temperature (< 40 °C) and controlled pressure conditions are preferred in order to avoid losses of volatile PFASs. Evaporation to dryness should be avoided if volatile PFAS are present. Before analysis, if necessary (eg contain solid particle), filter extracts (So 2006; Kaupmees 2017) or use centrifugation.

Instrumental analysis

For detection of PFASs in biota, the standard for water quality (ISO 21675:2019) is also preferred as the proposed liquid chromatography coupled with a tandem mass spectrometer is also suitable for extracts obtained from biota. Tandem MS and QTOF-MS have the advantage of providing low signal-to-noise ratio and high selectivity at low concentrations.

However, it must be checked that the solvent composition of the final extract corresponds to the mobile phase of the LC method in order to obtain a satisfactory peak shape of the compounds, in particular of short-chain PFASs eluting early from the column. If necessary, sonication of the sample extracts can be used for better dissolving and extracts should be filtered before analysis. Unless the samples are analysed immediately, the vials should be kept at a temperature below 4 °C. If glass vials are used, PFCs can be adsorbed onto the glass surface with solvents containing a greater amount of water. It is therefore advisable to use polypropylene vials (as some long-chained PFASs may be absorbed, check how long the solutions can be stored in the vials).

For chromatographic conditions, the standard for water analysis (ISO 21675:2019) is also recommended for biota. However, for biota samples, more interfering compounds might be present and to overcome separation problems (e.g. co-eluting matrix compounds), it may be helpful to use columns with polar groups instead of C 8 or C 18 columns. For eluents, use of PFAs trapping column is highly recommended as it delays analyte peaks originating from eluents (Stone et al. 2010).

For MS conditions, refer to ISO 21675:2019 but be aware that for biota samples MS analysis might suffer from matrix effects (signal suppression by co-eluting compounds). To overcome this problem, quantitation can be done using isotope dilution or matrix-matched calibration curve. The most promising approach for minimizing matrix effects is reducing the volume of the initial sample and the amount of extract injected.

Co-elution of matrix constituents (e.g. taurocholate bile salts) with the same transition ions as perfluorohexane sulfonate (PFHxS) and PFOS may lead to a significant bias in the quantification of these compounds in biota samples (Benskin et al., 2007). However, bias in PFHxS and PFOS levels can be avoided by separating the interferences from the target analytes and/or by using the

[4] https://doi.org/10.1016/j.trac.2019.02.011

interference-free transition to a mass-to-charge ratio (m/z) of 119 in order to verify results obtained with the product ions at m/z 80 and 99 (Chan et al., 2009). The PFCAs and PFSAs are almost completely dissociated in environmental matrices. If salts are used for the preparation of calibration standards, quantification results should be calculated for the corresponding acids. (Ahrens et al., 2010)

3 Data reporting and storage

Data is reported annually to the HELCOM COMBINE database, hosted by ICES. Data must be flagged as for HELCOM Combine.

4 Quality control

4.1 Quality control of methods

Quality assurance (QA) is a relevant part of all procedures from sampling to the final chemical analytical measurement. All procedures must be evaluated and controlled on a regular basis. For this purpose a quality assurance procedure scheme must be established and documented in each laboratory.

4.1.1 Quality assurance during sampling

Training of personnel is part of the quality assurance and of special importance with regards to sampling. Only experienced personnel aware of possible contamination sources and trained in biological sampling should carry out sampling and storage. Suitable sampling equipment not contaminated with PFASs should be used. Transport and storage times should be in accordance with requirements of the PFAS analysis. If necessary consultation with testing laboratory before sampling should be made.
The sampling plan should include the following parts according to the country's monitoring plan: Selection of species; minimizing natural variability, age and size, migration behavior, gender (WFD guidance 32).

Detailed sampling schemes (Species, sex, numbers, sizes etc.) and sampling/storage protocols should be available as documents on the ship and clearly communicated to the person in charge before the sampling starts.

4.1.2 Quality assurance during PFAS measurement

A number of measures should be taken to ensure sufficient quality of the analysis. Six main areas can be identified:

a) Extraction efficiency and clean-up

The use of isotopically labeled internal standards is recommended in order to take any losses or matrix effects into account. For the determination of the recovery rates of the clean-up and concentration steps, it is recommended to pass a standard solution through the entire procedure. If major losses have occurred, the results should not be reported. In addition, blanks should be run.

b) Calibrant and calibration

For preparation of standard solutions, refer to ISO 21675:2019. However, some PFSAs and sulfonamides demonstrate more than one peak in the chromatogram, owing to the presence of branched isomers. The ratio of linear and branched isomers can differ between the calibration standard and environmental samples. Branched isomers should be quantified separately if calibration standards are available (Riddell et al., 2009). If there are no proper calibration standards

and the peak area of the branched isomer exceeds 10 % of that of the linear isomer, it is advisable to estimate its concentration based on the response factor of the linear standard. However, the response factors of the linear and branched isomers may be different. It must be indicated with the results whether the reported concentration refers to the sum of the linear and branched isomers, or to the linear or branched isomer only (ICAS, 2010). When using a PFOS standard solution of mixed isomers, it should be determined whether the nominal concentration corresponds to the linear PFOS or to the total amount of isomers (Kaupmees 2017).

c) Long-term stability

One laboratory reference sample should be included in each series of samples. A quality control chart should be recorded for selected PFASs. If warning limits are exceeded, the method should be checked for possible errors and the obtained sample results should not be reported.

d) Internal standards

Internal standards should be added to all standards and samples either in a fixed volume or by weight and should not interfere with the target analytes.

A number of isotopically labeled PFASs were proven to be suitable for LC-MS analysis (Table 2).

e) Duplicates, triplicates

There are also applications where works use triplicates and duplicates.

f) Interlaboratory proficiency testing

Frequent participation in interlaboratory proficiency testing schemes and analysis of certified reference materials (CRM) is necessary. There are multiple of proficiency testing schemes for PFASs in biota (e.g. QUASIMEME BT-10 for biota and MS-8 for sediment) and there are reference materials are available for PFASs in water and fish.

4.2 Quality control of data and reporting

Measurement uncertainty should be estimated using ISO 11352:2012 or according to the Nordtest report TR537. Estimation should be based on within-laboratory reproducibility, data from proficiency tests, IRM, and, when available, CRM.

Data must be flagged if normal QA routines or recommended storage conditions cannot be followed.

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5.3 Additional literature

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