Guidance Document on Analytical Paramters for the Determination of Per- and Polyfluoroalkyl Substances (PFAS) in Food and Feed

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ANNEX

Version 1.0

11 May 2022

Example of Methodology for the Determination of PFAS in Food and Feed

Document history

Action	Date	Version
Development of annex by EURL/NRL network	2021-2022	
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Disclaimer

This annex refers to a number of products (analytical standards and equipment) as examples of items that may be used in the described procedures. Alternative sources of these or similar products may be available, and the annex does not endorse or recommend any particular product for use in the described procedures.

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Abbreviations

Abbreviation	Definition
dMRM	Dynamic multiple reaction monitoring
dSPE	Dispersive solid-phase extraction
ESI	Electrospray ionisation
GCB	Graphitized carbon black
HILIC	Hydrophilic interaction chromatography
HRMS	High resolution mass spectrometry
ILIS	Isotope-labelled internal standard
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
PEEK	Polyether ether ketone
PES	Polyethersulfone
PFAS	Per- and polyfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylic acids
PFSA	Perfluoroalkyl sulfonic acids
PP	Polypropylene
PRM	Parallel reaction monitoring
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
QQQ	Triple quadrupole
rpm	Rotation per minute
RRF	Relative response factor
RS	Recovery standard
RT	Retention time
sMRM	Scheduled multiple reaction monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TIC	Total ion current
TOF	Time-of-flight
(U)HPLC	(Ultra) High performance liquid chromatography
w.w.	Wet weight

1. DESCRIPTION AND SCOPE

The methodologies/modules described in this annex are examples of the procedures that may be used for the determination of per- and polyfluoroalkyl substances (PFAS) in food and animal feed samples. It is based on the use of isotope labelled standards that are commercially available, analyte separation using (ultra) high performance liquid chromatography ((U)HPLC) and detection using tandem mass spectrometry (MS/MS) or high resolution mass spectrometry (HRMS). The target analytes are perfluoroalkyl carboxylic acids (PFCA; C4 - C14), perfluoroalkyl sulfonic acids (PFSA; C4 – C12), FOSA and PFAS substitutes which are reported as individual and optionally as summed concentrations (∑PFOA, PFNA, PFOS, PFHxS) as described in the main guidance document.

In order to allow flexibility of use and incorporation of individual laboratory practices, the methodology for sample extraction, purification and measurement by different LC-MS techniques is presented in modular form. Laboratories may choose modules based on available equipment. The description of analytical standards, quantitation and reporting format, described in other sections of this annex is however, common, notwithstanding which modules are chosen.

Module X: **Sample pre-treatment** (chapter 6.2)

Module 1: **Description of extraction procedures** (chapter 6.3)

Module 1A: Solid-liquid extraction

Module 1B: Solid-liquid extraction based on ion-pair extraction

Module 2: **Description of purification procedures** (chapter 6.4)

Module 2A: Solid-phase extraction (SPE), manual

Module 2B: Dispersive solid-phase extraction (dSPE)

Module 2C: Solid-phase extraction (SPE), automated

Module 3: **Description of measurement procedures** (chapter 6.5)

Module 3A: Measurement by LC-MS/MS

Module 3B: Measurement by LC-HRMS

Assuming competence in trace analysis and LC-MS, the first steps for a laboratory wishing to set up PFAS determination would be procurement of the required standards and the use of these to establish the (U)HPLC-MS conditions required to measure PFAS. Modules may then be chosen to complement the equipment available in individual laboratories. The modules are given as examples of working methodology that will allow users to meet the performance parameters given in the main guidance document but as these are performance based, the procedures described here may also be adapted to suit the prevailing circumstances/equipment in individual laboratories.

NOTE: The methods described in this annex have been developed and successfully applied by different laboratories of the EURL/NRL network. Methods have been developed prior to the design of the main guidance document and thus, have not been validated according to the criteria given in the main document. They might not have been optimised for all analytes of interest listed in **Table 1**. Laboratories must carefully evaluate if a method works and are responsible by themselves to show their performance by validation.

2. ANALYTICAL STANDARDS

The analytical standards that would be required for determination listed in the guidance document are commercially available from current suppliers of persistent organic pollutant standards. An example of a full set of standards that could be used for routine determination is listed in **Table 1**. Some of the analytes are available as both standard mixtures and individual standards. All ^{13}C-labelled standards listed in **Table 1** are given as examples and could be substituted with ^{18}O-, ^{15}N- and D-mass labelled standards.

Mixed standard solutions may be prepared in methanol and can be stored at refrigerator temperatures in polypropylene (PP) vessels for about one year. NOTE: Long chain PFCA (>C_{10}) are known to adsorb to container/vial walls unless ≥ 50% organic solvent (e.g. methanol) is present [1–4].

Table 1: Example of a set of analytical standards (commercially available native and isotope labelled standards) for the determination of PFAS

Standard type	PFAS standard description[a)]	CAS-number[b)]
Native standard	**Perfluoroalkyl carboxylic acids (PFCA)**	
	Perfluoro-n-butanoic acid (PFBA)	375-22-4
	Perfluoro-n-pentanoic acid (PFPeA)	2706-90-3
	Perfluoro-n-hexanoic acid (PFHxA)	307-24-4
	Perfluoro-n-heptanoic acid (PFHpA)	375-85-9
	Perfluoro-n-octanoic acid (PFOA)	335-67-1
	Perfluoro-n-nonanoic acid (PFNA)	375-95-1
	Perfluoro-n-decanoic acid (PFDA)	335-76-2
	Perfluoro-n-undecanoic acid (PFUnDA)	2058-94-8
	Perfluoro-n-dodecanoic acid (PFDoDA)	307-55-1
	Perfluoro-n-tridecanoic acid (PFTrDA)	72629-94-8
	Perfluoro-n-tetradecanoic acid (PFTeDA)	376-06-7
	Perfluoroalkyl sulfonic acids (PFSA)	
	Perfluoro-1-butanesulfonate (PFBS)	375-73-5
	Perfluoro-1-pentanesulfonate (PFPeS)	2706-91-4
	Perfluoro-1-hexanesulfonate (PFHxS)	355-46-4
	Perfluoro-1-heptanesulfonate (PFHpS)	375-92-8
	Perfluoro-1-octanesulfonate (PFOS)	1763-23-1
	Perfluorooctanesulfonate (technical grade, T-PFOS)[c)]	-
	Perfluoro-1-nonanesulfonate (PFNS)	474577-07-4
	Perfluoro-1-decanesulfonate (PFDS)	335-77-3
	Perfluoro-1-undecanesulfonate (PFUnDS)	749786-16-1
	Perfluoro-1-dodecanesulfonate (PFDoDS)	79780-39-5
	Perfluoro-1-tridecanesulfonate (PFTrDS)	-
	Perfluoroalkane sulfonamides	
	Perfluoro-1-octanesulfonamide (FOSA)	754-91-6

Table 1 (continued)

Standard type	PFAS standard description[a)]	CAS-number[b)]
	PFAS substitutes	
	2,2,3-Trifluoro-3-[1,1,2,2,3,3-hexafluor-3-(trifluoromethoxy)propoxy]-propionic acid (DONA)	919005-14-4
	2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoic acid (HFPO-DA)	13252-13-6
	Potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (F-53B)	73606-19-6
	Potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate (minor component of F-53B)	83329-89-9
	1-Propanaminium, N,N-dimethyl-N-oxide-3-[[(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)sulfonyl]amino]-, hydroxide (Capstone A)	80475-32-7
	1-Propanaminium, N-(carboxymethyl)-N,N-dimethyl-3-[[(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)sulfonyl]amino]-, hydroxide (Capstone B)	34455-29-3
^{13}C-labelled internal standard	Perfluoro-n-[$^{13}C_4$]butanoic acid (M4PFBA)	1017281-29-6
	Perfluoro-n-[$^{13}C_5$]pentanoic acid (M5PFPeA)	2283397-79-3
	Perfluoro-n-[1,2,3,4,6-$^{13}C_5$]hexanoic acid (M5PFHxA)	2328024-54-8
	Perfluoro-n-[1,2,3,4-$^{13}C_4$]heptanoic acid (M4PFHpA)	2328024-55-9
	Perfluoro-n-[$^{13}C_8$]octanoic acid (M8PFOA)	1350614-84-4
	Perfluoro-n-[$^{13}C_9$]nonanoic acid (M9PFNA)	2283397-80-6
	Perfluoro-n-[$^{13}C_9$]decanoic acid (M9PFDA)	-
	Perfluoro-n-[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid (M7PFUnDA)	-
	Perfluoro-n-[1,2 -$^{13}C_2$]dodecanoic acid (M2PFDoDA)	960315-52-0
	Perfluoro-n-[1,2 -$^{13}C_2$]tetradecanoic acid (M2PFTeDA)	-
	Perfluoro-1-[2,3,4-$^{13}C_3$]butanesulfonate (M3PFBS)	2708218-84-0
	Perfluoro-1-[1,2,3-$^{13}C_3$]hexanesulfonate (M3PFHxS)	2708218-86-2
	Perfluoro-1-[$^{13}C_8$]octanesulfonate (M8PFOS)	2522762-16-7
	Perfluoro-1-[$^{13}C_8$]octanesulfonamide (M8FOSA)	-
	2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-$^{13}C_3$-propanoic acid (M3HFPO-DA)	-
^{13}C-labelled recovery standard	Perfluoro-n-[2,3,4-$^{13}C_3$]butanoic acid (M3PFBA)	2483735-33-5
	Perfluoro-n-[1,2-$^{13}C_2$]octanoic acid (M2PFOA)	864071-08-9
	Perfluoro-n-[1,2-$^{13}C_2$]decanoic acid (M2PFDA)	960315-50-8
	Perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate (M4PFOS)	960315-53-1

a) if not mentioned the standards refer to the linear isomers only

b) Some PFAS are commercially available as protonated, ammonium, sodium or potassium salts. An overview of compounds and CAS-numbers can be found elsewhere [5]. The CAS-numbers mentioned here refer to the protonated native PFCA/PFSA. Note, however, that the anions should be reported (see chapter 9).

c) should not be included in mixed standard solution and only be used for confirmation of the retention time of br-PFOS (see section 2.3 of main guidance document)

3. GENERAL ADVICE ON STANDARDS AND REAGENTS

Recommendations for reliable analysis with respect to analytical standards, reagents and other conditions used during sample extraction, purification and analysis are given below.

- Reagents of recognized analytical grade and purity (both in terms of PFAS and other contamination) should be used.
- Check of purity of the reagents and reference materials (e.g. standard solutions) by performing a procedural blank test under the same conditions as used in the method. The resulting chromatograms should be clear of interferences at the retention time of compounds of interest, otherwise the source of contamination needs to be identified and further steps to contain the blank problem should be taken.
- Regular check of concentration of standard solutions.
- Addition of isotope-labelled internal standards (ILIS): at the very beginning following weighing to compensate for analyte losses during the whole sample preparation procedure and matrix effects/absorption effects/variability of injection volume during measurement.
- Optionally: Addition of recovery standards (RS) after sample processing and prior to instrument analysis.

4. REDUCING THE IMPACT OF BACKGROUND CONTAMINATION

When conducting the analysis of PFAS in food and feed, background contamination ('blanks') at all stages of the analysis can compromise the analytical result. Particularly when aiming for low and sub-µg/kg levels (even down to ng/kg levels), the analyst should be aware of possible causes of PFAS contamination, throughout the analytical procedure. Below, guidance is provided on possible causes, and best practices to control, or monitor background contamination. Although all PFAS may cause the blanks to rise, mostly encountered blank issues include PFBA, PFPeA and PFOA.

NOTE: The following list of causes is not exhaustive. Other sources may also be possible.

4.1 Workspace and laboratory environment

- Dust particles contain PFAS. Therefore, the impact of dust particles that enter tubes, SPE columns etc. should be minimized by covering the sample, covering solvent bottles and flasks, covering tubes etc. It could be consider to reserve lab space and fume-hoods solely for (low level) PFAS analysis.
- Freeze-drying of sample material may be critical as regards contamination. Particularly when the vacuum of the freeze dryer is vented, air is sucked in. Such air may contaminate the sample. Moving the equipment to a low-contamination room may overcome this issue.

4.2 Equipment

- LC systems and parts thereof (e.g. degasser, tubing, valves) may contain fluoropolymer parts, e.g. made of polytetrafluoroethylene (PTFE; a.k.a. Teflon). PTFE should be replaced by steel, polyether ether ketone (PEEK) or other non-fluoropolymer tubing to minimize the blanks. Moreover, the installation of a trap column preceding the injector valve may prevent LC-system related PFAS from raising the blank level.
- Equipment or equipment's parts coming into contact with a sample should be carefully cleaned prior to usage. For example glassware and needles from SPE manifolds can be rinsed with methanol to reduce PFAS blank levels. Commercial automatic SPE systems may contain fluoropolymer parts that can contaminate sample extracts and alter blank levels.
- Bottle-top dispensers of solvents can contain PFAS and may alter PFAS blanks.

4.3 Materials

- Solvents (e.g. methanol or acetonitrile) may contain traces of PFAS from the manufacturing process. Changing to another supplier can resolve the problem. Moreover, solvent bottle caps may contain PTFE inlays or rings that will cause the blanks to rise.
- Water purifying systems (e.g. MilliQ), and parts thereof (e.g. cartridges) may contain PFAS. Tapping water from these systems for sample extraction may result in elevated blanks. This can be overcome by post-treatment of this water (e.g. cleaning over a weak anion exchange column) or by switching to another source of ultra-pure water (e.g. bottled HPLC water).

- ILIS are known to contain very small amounts of impurities, including the native analogue. In order to reduce the level of these impurities in the sample, it should be considered to add lower amounts of the ILIS to the sample.
- Some PFAS are reported to be sensitive to degradation. For example, fluorotelomer diphosphat esters (di-PAPs) may degrade to perfluorocarboxylates such as PFBA and PFPeA, and may as such be a source of contamination.
- The use of non-fluoropolymer disposables may be preferred for sample handling (extraction, clean-up, solvent evaporation) over multiple use laboratory glassware. Polypropylene sample tubes are commonly used to that end.
- It is advised to keep administrative records of batches of materials, solvents and chemicals. Whenever a new batch of material is used, it should be ensured that this is recorded. This will help to trace back possible sources of PFAS in the event that unexpected blanks are encountered. Moreover, when low-contaminated materials are used in your laboratory, it should be ensured that such material is only used for PFAS analysis. For example, when blank analysis of methanol showed that a specific batch of methanol in the lab is suitable for PFAS analysis, it should be ensured that multiple bottles are kept apart, and reserved solely for PFAS analysis.

4.4 How to investigate the origin of a blank contribution?

- Check batch numbers of materials. Possibly a new batch of material is contaminated whereas previous batches were not.
- Investigate every single step of the sample storage, sample preparation and analysis, to see where blank contamination rises. Try to pinpoint the exact cause by investigating every solvent, chemical, material or lab equipment part that comes into contact with the sample.
- It is helpful to monitor blank levels of procedural blanks in control charts, to keep track of progressing blank levels in time.
- Finally, blanks can originate from all stages of sample handling and analysis. It takes awareness and attention of the analyst to reduce and prevent as much as possible blank contributions to the sample result. Nevertheless, even with a very cautious attitude towards blanks, interfering background may appear unexpectedly, and may disappear unexpectedly without pinpointing the cause. This is a fact of life of ultra-trace analysis of pollutants in food and feed, and is sometimes beyond control of the analyst.

5. PREPARATION OF SAMPLE MATERIAL

Advice on representative sampling is not provided in this annex – guidelines such as those described in

- COMMISSION IMPLEMENTING REGULATION laying down methods of sampling and analysis for the control of perfluoroalkyl substances in certain foodstuffs [6],
- COMMISSION DIRECTIVE 2002/63/EC for pesticides residues in food in and on products of plant and animal origin [7],
- COMMISSION REGULATION (EC) No 333/2007 for lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs [8],
- COMMISSION REGULATION (EC) No 152/2009 and (EU) 2017/644 for PCDD/Fs and PCBs in feed/food [9,10], or
- EN ISO 6498 animal feeding stuffs — guidelines for sample preparation [11]

may be helpful.

NOTE: Wherever possible, apparatus and equipment coming into contact with the sample shall not contain any fluoropolymer materials (e.g. PTFE), polyvinylidene fluoride (PVDF) and others) to minimize the risk of contamination.

5.1 Food material

Representative samples of high moisture products such as, meat, fish, vegetables, fruits, eggs should be thoroughly mixed in a knife mill (e.g. Grindomix, Retsch) so that the material is homogenous. Alternatively, the sample can be initially blended, homogenised and then freeze-dried (air-drying may be adequate for feed). After drying, the lyophilised or air dried material may be re-homogenised to yield a dry and representative sample.

Dry or low moisture products such as, bread, nuts, cereals and cereal products should be ground carefully so that the material can pass through a 1 mm mesh sieve. Post-grinding, the material should be homogenised to yield a representative ground sample. Some laboratories may prefer to use a drying agent such as anhydrous Na_2SO_4 or polyacrylate when using small aliquots for analysis.

High lipid content foods such as butter, fish and vegetable oils, animal fats etc. may be homogenised by blending after melted at 37 – 45 °C in an incubator to ensure a representative sample and used without further treatment.

5.2 Feed material

Representative samples of dry or low moisture products such as, mixed feeds, and hay should be ground carefully so that the material can pass through a 1 mm mesh sieve. Post-grinding, the material should be homogenised to yield a representative ground sample.

High water content feed materials such as grasses and silages and liquid feed may be initially blended, homogenised and then freeze-dried (air-drying may be adequate for feed). After drying, the lyophilised or air dried material may be re-homogenised to yield a dry and representative sample. Some laboratories may prefer to use a drying agent such as anhydrous Na_2SO_4 or polyacrylate when using small aliquots for analysis.

High lipid content feed such as fish products, vegetable oils, animal fats etc. may be homogenised by blending after melted at 37 – 45 °C in an incubator to ensure a representative sample and used without further treatment.

For feed samples the determination of the moisture content (optional) may be useful if the calculation of analyte results based on 12% moisture content is required.

6. INDEPENDENT MODULES ON SAMPLE PRE-TREATMENT, EXTRACTION, PURIFICATION AND MEASUREMENT BY LC-MS TECHNIQUES

6.1 General aspects

The equipment used for sample preparation and analysis should not allow removal of the extracted PFAS (e.g. adsorption on container walls) or contamination of the extract (e.g. through PTFE capillaries).

Depending on the equipment available, either manual or automated procedures may be used. Each module (1, 2, and 3) describes a part of the whole PFAS determination method. For sample preparation each extraction method described in **Module 1** can be combined with each purification method described in **Module 2**. Each combination out of **Module 1 and 2** can be combined with each determination method described in **Module 3**.

All modular procedures described here are given as examples and could be adapted or substituted with alternative methods to match the equipment and expertise available in the laboratory.

6.2 Module X: Pre-treatment

6.2.1 Freeze-drying/lyophilization

Before sample extraction, the samples can be freeze-dried to remove all traces of water. The samples are then finely ground to obtain a fine powder. The percentage of dry matter is determined for each sample in order to determine the analyte content in µg/kg wet weight.

Lyophilization of test samples is usually not required for the determination of PFAS but may be done for the purpose of e.g. determination of water content/moisture, homogenization, sample storage or other determinations to be carried out on the sample. Lyophilized test samples should be weighed in, taking into account the removed water in the sample requiring lower sample weight compared to a wet/fresh sample. The weighed test portion should be wetted with an appropriate volume of water to reconstitute the approximate original water content in the sample, followed by shaking/whirl mixing, before spiking.

6.3 Module 1: Extraction

The procedures described in this module are extraction methods for the isolation of PFAS from the sample of food or feed. The extraction efficiency is affected by the properties of the chosen extraction solvent, the solvent-to-solid ratio and the extraction duration/repetitions. The extraction procedures described are divided in solid-liquid and ion-pair extraction and can each be applied to properly extract PFAS from any kind of food or feed sample matrix. Before extraction the preparation recommendations for different types of samples should be followed as described in chapter 5.

6.3.1 Module 1A: Solid-liquid extraction

Similarly other techniques e.g. liquid-liquid extraction for milk may be used if the complete extraction of PFAS is validated.

Principle

Solid-liquid extraction is an easy and relatively inexpensive procedure to isolate the analytes of interest from the raw material. The optimum solvent for this purpose penetrates into the solid or wet matrix, the analytes dissolve in the solvent and diffuse out of the matrix. The supernatant, containing the analytes of interest, can be collected. Multiple different mechanical techniques e.g. shaking, stirring or ultrasonication are known and may be used if they allow the complete extraction of PFAS.

6.3.1.1 Extraction procedure: Example 1

Accurately weigh a test portion of the homogenized test sample material into a 50-mL PP centrifuge tube. The weight will depend on the sensitivity of the measurement process, but for most samples, 0.5 - 5 g of material should be used to ensure a representative sample. Fortify the weighed sample with e.g. 100 µL ILIS solution by spiking small droplets of the solution over the cross-section of the sample surface. The concentration of the ILIS solution depends on the level of expected contamination.

Add 15 mL of a mixture of methanol/KOH (0.01 M) and shake it vigorously by vortexing for 1 min. The alkaline digestion is performed by leaving the tubes 15 h at room temperature.

Centrifuge the sample 10 min. Transfer 3 mL of the supernatant in a new polypropylene tube of 15 mL. Evaporate the extract to 1 mL under a gentle stream of nitrogen and add 4 mL of water. The extract can be used for purification e.g. by manual SPE (see **Module 2**, section 6.4.1).

6.3.1.2 Extraction procedure: Example 2

Accurately weigh a test portion of the homogenized test sample material into a 15-mL PP centrifuge tube. The weight will depend on the sensitivity of the measurement process, but for most samples, 0.5 - 5 g of material should be used to ensure a representative sample.

Fortify the weighed sample with e.g. 100 µL ILIS solution by spiking small droplets of the solution over the cross-section of the sample surface. The concentration of the ILIS solution depends on the level of expected contamination. Mix by vortexing and allow the fortified sample to soak for 5 min before extraction.

Add 5 mL acetonitrile or methanol to the fortified sample, shake it briefly by hand and place it in an ultrasonic bath at room temperature for 15 min. Centrifuge the sample for 5 to 10 min. Transfer the supernatant in a new polypropylene centrifuge tube and repeat the extraction once again by adding 5 mL acetonitrile or methanol to the sample. Combine the supernatants. The method was developed in combination with a purification step by dSPE (see **Module 2**, section 6.4.2).

6.3.1.3 Extraction procedure: Example 3

Accurately weigh a test portion of the homogenized test sample material into a 50-mL PP centrifuge tube. The weight will depend on the sensitivity of the measurement process and on the level of expected contamination, but for most samples, 0.5 - 5 g of material should be used to ensure a representative sample.

Fortify the weighed sample with e.g. 100 µL ILIS solution by spiking small droplets of the solution over the cross-section of the sample surface. The concentration of the ILIS solution depends on the level of expected contamination. Mix by vortexing and allow the fortified sample to soak for 5 min before extraction. Add 2 mL of water to samples with a low water content.

Add 20 mL 0.1 % NH_3 in acetonitrile to the fortified sample, shake it briefly by hand and shake it with a laboratory shaker at room temperature for 2 hours. Centrifuge the sample for 5 to 10 min. Transfer the supernatant in a 15-mL PP centrifuge tube and evaporate the supernatant under a gently stream of nitrogen at a temperature of 40 °C to a final volume of 5 mL. The method was developed in combination with a purification step by automated SPE (see **Module 2**, section 6.4.3.1).

6.3.2 Module 1B: Solid-liquid extraction based on ion-pair extraction

Principle

The procedure described in this section is a method for the isolation and purification of PFAS from other components and co-extractives present in the sample of food or feed. It is based on the distribution of ionic compounds to an organic phase with the aid of counterions of opposite charge. This procedure combines extraction and preliminary purification in a single stage.

Extraction procedure

Accurately weigh a test portion of the homogenized test sample material into a 50-mL PP centrifuge tube. The weight will depend on the sensitivity of the measurement process, but for most samples, 0.5 - 5 g of material should be used to ensure a representative sample.

Fortify the weighed sample with e.g. 100 µL ILIS solution by spiking small droplets of the solution over the cross-section of the sample surface. The concentration of the ILIS solution depends on the level of expected contamination. Mix by vortexing and allow the fortified sample to soak for 5 min before extraction.

Add 10 mL water to the fortified sample and shake briefly by hand. Afterwards, add 2 mL of 0.5 M tetrabutyl ammonium hydrogen sulphate solution, adjusted to pH 10 using sodium hydroxide, 4 mL of 0.25 M sodium carbonate solution and 20 mL methyl tert-butyl ether to the tube. Place the tube in an orbital shaker and mix for 25 min at room temperature. Centrifuge the sample for 10 min.

NOTE: The supernatant can now be analysed by (U)HPLC-MS without further purification or be evaporated and further purified using one of the extract purification methods described in **Module 2**.

Without further purification: Aliquot 10 mL of the supernatant in a 15-mL PP centrifuge tube and evaporate the extract to dryness under a gently stream of nitrogen at 40 °C. Reconstitute the residue with 500 µL HPLC mobile phase (1:1, v/v) and centrifuge the final extract for 5 min. Prior to instrumental analysis, transfer the supernatant in PP vials. The method has been validated for food of plant origin.

6.4 Module 2: Extract purification

6.4.1 Module 2A - Solid-phase extraction (SPE), manual

Principle

The procedure in this section describes the purification of the extracts obtained in **Module 1**. In addition to the purification from other components and co-extractives present in the sample, the method is also used to concentrate the present PFAS in the sample.

The solid-phase extraction is based on the interaction of PFAS with a mixed-mode, weak anion-exchange sorbent (e.g. a primary and secondary diamines bound to a hydrophobic polystyrene divinylbenzene backbone). The retention mechanism is based on ionic and hydrophobic interactions. Negatively charged PFAS like PFCAs and PFSAs interact with the positive charged amines which results in a retention. By changing the condition to an alkaline environment PFAS will be eluted from the sorbent.

The combination of SPE with graphitized carbon black (GCB; ENVI-Carb) material proved to add further purification to the extracts or eluates [12]. This method is widely used for food matrices of animal origin, but may also be applied for matrices of plant origin [13,14].

6.4.1.1 Purification procedure: Example 1

Adjust the pH of the sample extract obtained by **Module 1** to a range of 4 - 5 using e.g. 1 % formic acid. NOTE: Different matrices or blank samples will have different starting pH values. Alternatively, instead of water, an ammonium acetate buffer in the pH range from 4 - 6 can be added. NOTE: Due to the acidic conditions, proteins can precipitate. If needed (e.g. protein precipitation), centrifuge the sample for 10 min at room temperature.

Condition the SPE sorbent using e.g. 4 mL methanol followed by 4 mL water. Load the supernatant of the freshly centrifuged sample onto the SPE cartridge. If necessary, apply vacuum to the extraction manifold chamber. Wash the cartridges with e.g. 4 mL 25 mM ammonium acetate (pH 4) followed by 4 mL water. If further purification is desired connect the ENVI-Carb cartridge with the weak-anion exchange cartridge. NOTE: The ENVI-Carb purification step can also be done prior to the weak-anion exchange. Slowly elute neutral PFAS with e.g. 4 x 1 mL methanol and charged PFAS with 4 x 1 mL 3 % ammonium hydroxide in methanol.

Evaporate the eluates to dryness under a gentle stream of nitrogen at 40 - 50 °C. If using recovery standards, reconstitute the residue with 450 µL HPLC mobile phase (1:1, v/v). Add 50 µL of recovery standard and mix on a Vortex mixer. If recovery standards are not used, reconstitute in 500 µL final volume and transfer the final extract into a PP vial. (**Figure 1**)

NOTE: If the final extract is not clear, filtrate the sample prior to instrumental analysis by using e.g. a polyethersulfone (PES) syringe filter (0.2 µm). In case of high fat content, freeze the final extract for at least 3 h at -20 °C prior to the filtration step.

6.4.1.2 Purification procedure: Example 2 and 3

Further purification procedures based on manual solid-phase extraction are published e.g. by Berendsen et al. [15] and Sadia et al. [12].

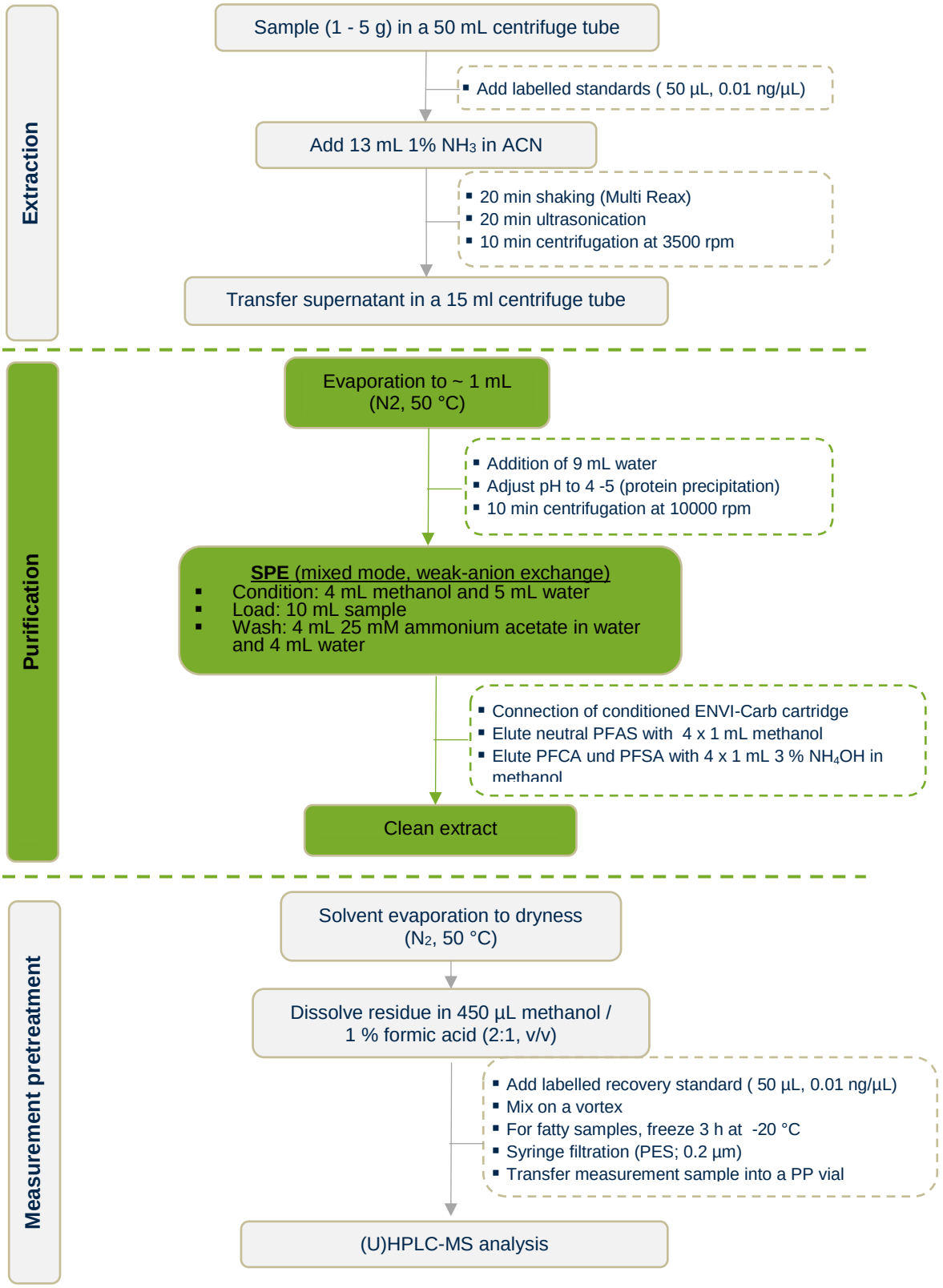

Figure 1: Flowchart of a sample preparation method for the determination of PFAS. Extraction is based on the method described in **Module 1**, section 6.3.1.3 and purification on the SPE method described in **Module 2**, section 6.4.1.1.

6.4.2 Module 2B – Dispersive solid-phase extraction (dSPE)

Principle

The procedure described in this module is a manual method for the purification of PFAS from other components and co-extractives present in the sample of food or feed. The procedure is comparable to the widely known QuEChERS method for pesticides [16].

After extraction (**Module 1**), removal of residual water and clean-up are performed in one step by means of a dispersive solid-phase extraction (dSPE). For the dSPE a mixture of different salts (e.g. anhydrous $MgSO_4$, NaCl) and sorbents (e.g. primary secondary amine, ENVI-Carb) can be used. An example of a dSPE method is described below.

Purification procedure

The following purification method can be applied to extracts from plant and animal food matrices from the extraction **Module 1** and provides purified extracts for PFAS. The method is validated for food matrices of animal origin such as fish (muscle), meat, egg, milk, offal, and milk based infant formula.

Weigh 2.0 g anhydrous $MgSO_4$, 0.5 g NaCl, 0.1 g C18 adsorbent and 0.1 g ENVI-Carb graphitized carbon adsorbent in e.g. a 15 mL centrifuge tube and add the salts to the sample extract. NOTE: Addition of the sample extract to the salts is also possible. Cap the centrifuge tube tightly and shake immediately and vigorously by hand. NOTE: Shaking immediately is important to prevent formation of $MgSO_4$ conglomerates. Mix on a Vortex mixer for 1 min. Centrifuge the extract for 15 min to separate solids from solution and evaporate the extract to dryness under a gently stream of nitrogen at 40 °C. Reconstitute the residue with 500 µL HPLC mobile phase (1:1, v/v) in an ultrasonic bath for 3 min and centrifuge the final extract for 5 min. Prior to instrumental analysis, transfer the supernatant in PP vials. (**Figure 2**)

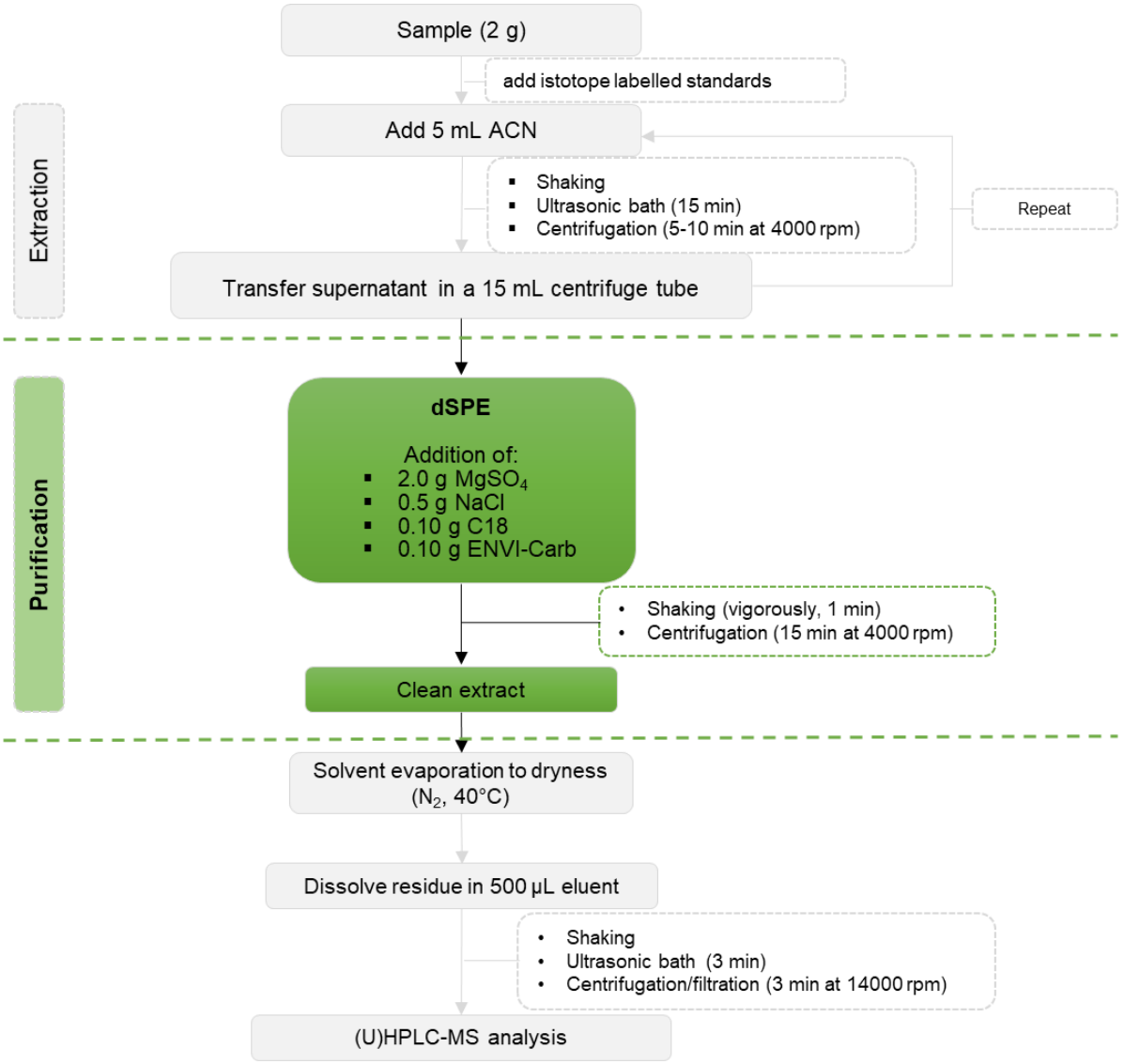

Figure 2: Flowchart of a sample preparation method for the determination of PFAS. Extraction is based on the method described in **Module 1**, section 6.3.1.2 and purification on the dSPE method described in **Module 2**, section 6.4.2.

6.4.3 Module 2C – Solid-phase extraction (SPE), automated

Principle

This module describes procedures for the automated clean-up of PFAS from other components and co-extractives present in a sample of food or feed. This procedure is carried out using equipment that is commercially available. Examples of automated procedures are described using LC-Tech™ or Gilson equipment, but other automated procedures may also be used.

Although differing in the detail and order of procedures used, the principle is essentially the same as that described in **Module 2A** (section 6.4.1).

6.4.3.1 Example 1: LCTech™ Freestyle

Use Freestyle robotic system for PFAS analysis from LCTech™ or other automated systems suitable for PFAS analysis. For SPE cleanup, use two different SPE cartridges (1. ENVI-Carb and 2. STRATA-X AW). Use the ENVI-Carb as a not retaining SPE. Transfer the whole sample extract (**Module 1**) to the ENVI-Carb and collect the eluate directly.

1. ENVI-Carb (not retaining SPE)

Settings of Freestyle robotic system for PFAS analysis from LCTech™ for the automated steps:

1. Conditioning
    a. 0.1 % NH_3 in methanol (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
    b. Methanol (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
    c. Water (0.1 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 0.1 mL/min
2. Load

    Transfer Sample-Aliquot (5 mL) over sample loop
        - Suction speed: 5 mL/min
        - Dispensing speed: 5 mL/min
        - Waiting time after step: 150 sec
        - Dispense: into vial
3. Washing

    2 x 0.1 % NH_3 in methanol (3 mL)
        - Suction speed: 5 mL/min
        - Dispensing speed: 1 mL/min
        - Waiting time after dosage: 10 sec

- Dispense: stay on actual position
- Waiting time after step: 5 sec

4. Drying - drying by defined air volume (4.5 mL)
    - Suction speed: 100 mL/min
    - Dispensing speed: 15 mL/min
    - Dispense: stay on actual position

Evaporate the sample extract to 1 mL under a gently stream of nitrogen at a temperature of 40 °C. Adjust the final volume of 10 mL by addition of 9 mL water[1].

2. Strata-X AW (retaining SPE)

Settings of Freestyle robotic system for PFAS analysis from LCTech™:

1. Conditioning
    a. 0.1 % NH_3 in methanol (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
    b. Methanol (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
    c. Water (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
    d. Sodium acetate buffer (25 mM; pH 4) (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 2 mL/min
2. Load
    Transfer sample-aliquot (10 mL) over sample loop
        - Suction speed: 20 mL/min
        - Dispensing speed: 5 mL/min
        - Waiting time after step: 150 sec
        - Dispense: into waste
3. Drying
    Drying by defined air volume (6 mL)
        - Suction speed: 100 mL/min
        - Dispensing speed: 2.5 mL/min
        - Dispense: stay on actual position

[1] Matrices like fruits/vegetables: adjust pH 5 – 5.5 (with formic acid and NaOH)

4. Washing
    a. Sodium acetate buffer (25 mM; pH 4) (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 1 mL/min
        - Dispense: into waste
    b. Methanol (4 mL)[2]
        - Suction speed: 15 mL/min
        - Dispensing speed: 1 mL/min
        - Dispense: into waste
5. Drying

    Drying by defined air volume (4 mL)
        - Suction speed: 100 mL/min
        - Dispensing speed: 5 mL/min
        - Dispense: stay on actual position
6. Eluting

    0.1 % NH_3 in methanol (4 mL)
        - Suction speed: 15 mL/min
        - Dispensing speed: 1 mL/min
        - Waiting time after step: 60 sec
        - Dispense: into vials
7. Drying

    Drying by defined air volume (2 mL)
        - Suction speed: 100 mL/min
        - Dispensing speed: 2.5 mL/min
        - Dispense: stay on actual position
8. Drying

    Drying by defined air volume (10 mL)
        - Suction speed: 100 mL/min
        - Dispensing speed: 50 mL/min
        - Dispense: into waste

Add 100 µL of 10 % ethylene glycol in acetonitrile to the purified sample extract and mix by vortexing. Evaporate the extract to near dryness under a gently stream of nitrogen at a temperature of 40 °C. Add 40 µL water and 50 µL recovery standard and transfer it in a PP microvial insert (final volume 100 µL: 10 µL ethylene glycol; 40 µL water; 50 µL recovery standard).

[2] Elution of FOSA → if FOSA is to be analyzed, the wash solution must be collected

6.4.3.2 Example 2: Gilson ASPEC®

Filter extracts from **Module 1** through a 0.45 µm nylon syringe filter by decanting the extracts into a syringe with appropriate volume fitted with the filter and dispense the syringe contents into a 50 mL polypropylen-tube. Add water to the filtrated solution to make up the total volume to 36 mL before mixing the contents of the tube. Divide the solution equally into two 20 mL plastic ASPEC tubes.

Table 2: Example of general parameters in automated SPE using Gilson ASPEC®

Method Type	Fixed tray
Configuration	ASPEC GX-274
Tray	345 (source) 373 (collect) 373 mobile rack
Solvent A	1% (v/v) ammonium hydroxide in methanol
Solvent B	Methanol
Solvent C	Water
Solvent D	2% (v/v) formic acid in water
Reservoir	30 % acetone in water

Table 3: Example of task parameters in ASPEC® method

Parameter		Rinse Needle	Condition	Condition	Condition	Load	Load	Wash	Elute
Dec name		-	373	373	373	373	373	373	373
Collect name		-	-	-	-	-	-	-	-
Rinse:	Inside Volume	1000 µL	-	-	-	-	-	-	-
	Outside Volume	1000 µL	-	-	-	-	-	-	-
Solvent/ Source:	Select	From Reservoir	From Tray	From Tray	From Tray	From Tray	From Tray	From Tray	From Tray
	Name	-	SolventA	SolventB	SolventC	Sample1	Sample2	SolventD	SolventA
	Volume (mL)	-	5	5	5	18	18	5	1
	Disp Flow Rate (mL/min)	-	6	6	6	3	3	6	3
	Asp Flow Rate (mL/min)	-	1	1	1	1	1	1	1

Table 3 (continued)

Parameter	Rinse Needle	Condition	Condition	Condition	Load	Load	Wash	Elute	Parameter
Air Push:	Method	-	Using Syringe	Using Syringe	Using Syringe	Using Syringe	Using Syringe	Using Syringe	Using Syringe
	Air Push Volume* (mL)	-	0	0	0	0	20*	10	10
	Air Push Disp Flow Rate (mL/min)	-	6	1	6	6	6	6	6

6.5 Module 3 – Measurement of extracted PFAS

The procedures described in this module assume competence in the practice of (ultra) high performance liquid chromatography ((U)HPLC) and tandem mass-spectrometry (MS/MS) or high resolution mass spectrometry (HRMS). The equipment required for this module is a combined (U)HPLC-MS/MS or (U)HPLC-HRMS system consisting of:

- A liquid chromatograph equipped with a pump, autosampler, and column oven.
- A triple quadrupole (QQQ) or ion trap mass analyser equipped with an electrospray ionisation (ESI) source and operated in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode which support fast MS/MS transition scan speed (e.g. > 300 MS/MS transitions/s). Or in case of HRMS a high resolution mass spectrometer e.g. time-of-flight (TOF) or orbitrap mass analyser, high gain detection and acquisition capacity and integrated data handling capability.

6.5.1 Module 3A – Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Before measurement, the (U)HPLC-MS/MS system should be adjusted and calibrated with the appropriate calibrant to the guidelines provided by manufacturer and the adequate ion transmission through the ion optics should be verified within the mass range of selected MS/MS transitions.

The MS method should comprise at least two specific MS/MS transitions for each native analyte (exceptions e.g. PFBA and PFPeA, see chapter 2.4.3 of main guidance document). Compound parameters including precursor ion, fragment ion, and collision energies should be optimised for each analyte. An appropriate retention time window for each analyte should be established (use of dynamic MRM mode or equivalent). This should be based on measurements of actual retention time variation for each method analyte in calibration standards analysed on the LC over the course of time. A value of plus or minus three times the standard deviation of the retention time obtained for each method analyte while establishing the initial calibration can be used to calculate a suggested window size. However,

the experience of the analyst should weigh heavily on the determination of the appropriate retention window size [1].

When the performance of the (U)HPLC-MS/MS system has been verified, the extracts can be analysed. Typically the sequence of injections should commence and end with the set of PFAS calibration standards. The batch of samples including a procedural blank and reference material may be run in between these standards separated by solvent blanks (after every five samples) to ensure that there is no carryover.

Examples of suitable (U)HPLC-MS/MS conditions are given below. Example 1 and 2 describe a chromatographic separation of target analytes using a C18 reversed-phase column. Retention of target analytes described in example 3 is based on hydrophilic interaction chromatography (HILIC).

6.5.1.1 Example 1: HPLC-ESI-QQQ (C18 column and autosampler injector program)

Table 4: Example 1 of HPLC gradient program for PFAS measurement by LC-MS/MS (1290 Infinity II-system; Agilent)

Type	HPLC capable of at least 600 bar
Pump	Binary pump
Autosampler (temperature)	15 °C
Column oven (temperature)	50 °C
Analytical column	Acquity UPLC BEH C18, 150 x 3.0 mm, 2.7 µm
Trap column or PFAS kit[3]	InfinityLab PFC Delay Column 30 x 4.6 mm
Injection volume	10 µL

Table 5: Example 1 of HPLC gradient program for PFAS measurement by LC-MS/MS (1290 Infinity II-system; Agilent)

Time (min)	Mobile phase A (2 mM ammonium acetate in water, 5% ACN)	Mobile phase B (acetonitrile/methanol 60:40, v/v)	Flow rate (mL/min)
0.00	90	10	0.25
0.01	60	40	0.25
4.00	40	60	0.25
6.00	25	75	0.25
15.00	5	95	0.25
16.00	2	98	0.25
17.00	2	98	0.25
18.00	90	10	0.25

[3] PFAS kit: all parts of the LC system that contain PFAS (e.g. solvent lines) are, if possible, replaced by stainless steel, PEEK or PP parts to reduce contamination of samples

Table 6: Example of autosampler injector program for PFAS measurement (1290 Infinity II-system; Agilent)

	Function	Parameter
1	Wash	Wash needle in flushport with 0.1 % formic acid in acetonitrile for 3 s
2	Draw	Draw 10.00 µL methanol with default speed using default offset
3	Wash	Wash needle flush with methanol for 3 s
4	Eject	Eject 10.00 µL to waste with default speed
5	Wash	Wash needle in flushport with 0.1 % formic acid in acetonitrile for 3 s
6	Draw	Draw 10.00 µL methanol with default speed using default offset
7	Wash	Wash needle flush with methanol for 3 s
8	Eject	Eject 10.00 µL to waste with default speed
9	Wash	Wash needle in flushport with 0.1 % formic acid in acetonitrile for 3 s
10	Draw	Draw 15.00 µL water with default speed using default offset
11	Eject	Eject 15.00 µL to waste with default speed
12	Wash	Wash needle flush with methanol for 3 s
13	Draw	Draw 2.00 µL from air with default speed
14	Draw	Draw default volume from sample with default speed using default offset
15	Draw	Draw 2.00 µL from air with default speed
16	Wash	Wash needle in flushport with 0.1 % formic acid in acetonitrile for 3 s

Table 7: Example 1 of analytes and retention times (RT) for PFAS measurement by LC-MS/MS (1290 Infinity II-system; Agilent)

Analyte	Peak # (Figure 3)	RT (min)
PFBA	1	3.6
PFPeA	2	4.1
PFBS	3	4.7
4:2 FTS	4	4.7
PFHxA	5	4.9
HFPO-DA	6	5.2
PFHpA	7	5.8
PFHxS	8	6.5
6:2 FTS	9	6.5
PFOA	10	6.8
PFHpS	11	7.4
PFNA	12	7.7
L-PFOS	13	8.2
8:2 FTS	14	8.3
PFDA	15	8.5
PFUnDA	16	9.1
PFDS	17	9.4
FOSA	18	9.4
PFDoDA	19	9.8
PFTrDA	20	10.5
L-PFDoDS	21	10.8
PFTeDA	22	11.2

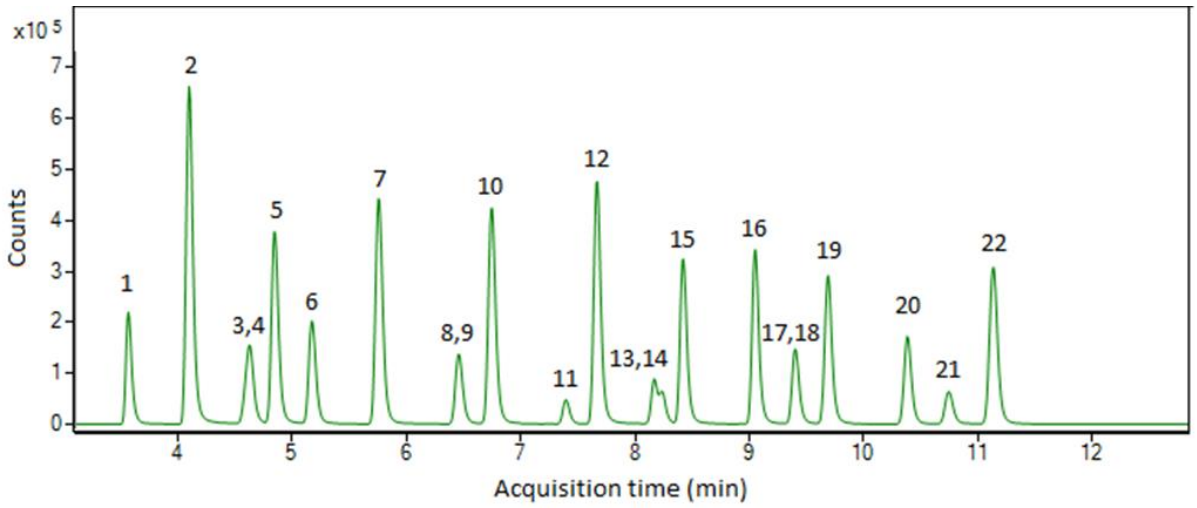

Figure 3: Total ion current (TIC)-chromatogram of 22 PFAS fortified at 5 µg/kg in solvent (methanol/water (1:1, v/v). Numbered peaks are identified in **Table 7**.

Table 8: Example 1 of mass spectrometer parameters used for PFAS measurement by LC-MS/MS (QQQ 6470; Agilent)

Parameter	
Type	Triple quadrupole
Ionization Mode	ESI negative
Nebulizer	20 psi
Capillary Needle Voltage	3000 V (tune)
Gas Temperature	400 °C (tune)
Gas Flow	12 L/min (tune)
MS1/MS2 Resolution	Unit/Unit
Gas Temperature	240 °C
Gas Flow	4 L/min (tune)
Nozzle Voltage	0 V (tune)
Scan Type	Dynamic multiple reaction monitoring (dMRM)

Table 9: Example 1 of MS/MS transitions and optimised mass spectrometer parameters used for PFAS measurement by LC-MS/MS (QQQ 6470; Agilent)

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Fragmentor (V)	Collision energy (eV)
PFBA	213	169	75	5
PFPeA	263	219	80	5
PFBS	299	80	145	34
	299	99	145	28
PFHxA	313	269	80	5
	313	119	80	21
PFPeS	349	80	160	41
	349	99	160	35
HFPO-DA	285	185	50	20
	285	169	65	5
PFHpA	363	319	80	7
	363	169	80	15
DONA	377	251	70	9
	377	85	70	33
PFHxS	399	80	175	39
	399	99	175	45
PFHpS	449	80	190	48
	449	99	190	44
PFOA	413	369	85	7
	413	169	85	17
PFOS (linear and branched)	499	80	190	47
	499	99	190	51
	499	169	190	44
Capstone B	569	549	60	11
	569	223	60	13
PFNA	463	419	95	7
	463	219	95	15
Capstone A	527	507	85	7
	527	181	85	11
9Cl-PF3ONS (F-53B major)	531	351	150	29
	531	83	150	31
PFNS	549	80	200	55
	549	99	200	53
PFDA	513	469	90	7
	513	269	90	17
PFDS	599	99	215	54
	599	80	215	56
PFUnDA	563	519	100	9
	563	269	100	17
	563	169	100	17
FOSA	498	78	165	39
	498	478	120	21
11Cl-PF3OUdS (F-53B minor)	631	451	180	31
	631	83	180	37
PFUnDS	649	99	100	55
	649	80	100	43
PFDoDA	613	569	100	9
	613	319	100	21
PFDoDS	699	99	215	61
	699	80	215	61

Table 9 (continued)

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Fragmentor (V)	Collision energy (eV)
PFTrDA	663	619	105	19
	663	169	105	19
PFTrDS	749	99	180	59
	749	80	180	35
PFTeDA	713	669	115	11
	713	169	115	29
4:2 FTS	327	307	150	20
	327	81	150	36
6:2 FTS	427	407	150	30
	427	81	150	32
8:2 FTS	527	507	200	30
	527	81	200	46
$^{13}C_4$-PFBA	217	172	75	5
$^{13}C_5$-PFPeA	268	223	80	5
$^{13}C_3$-PFBS	302	80	145	38
$^{13}C_6$-PFHxA	319	274	80	5
$^{13}C_4$-PFHpA	367	322	80	7
$^{13}C_3$-PFHxS	402	99	175	39
$^{13}C_8$-PFOA	421	376	85	7
$^{13}C_8$-PFOS	507	99	190	47
$^{13}C_9$-PFNA	472	427	95	7
$^{13}C_9$-PFDA	522	477	90	7
$^{13}C_9$-PFUnDA	572	528	100	9
$^{13}C_2$-PFDoDA	615	570	100	9
$^{13}C_2$-PFTeDA	715	670	115	11
$^{13}C_8$-FOSA	506	78	165	39
$^{13}C_3$- HFPO-DA	287	185	64	20
$^{13}C_2$-4:2 FTS	329	309	150	24
$^{13}C_2$-6:2 FTS	429	409	150	28
$^{13}C_2$-8:2 FTS	529	509	200	28

6.5.1.2 Example 2: HPLC-ESI-QQQ (C18 column)

Table 10: Example 2 of HPLC system and conditions for PFAS measurement by LC-MS/MS (ACQUITY UPLC I-Class system; Waters™)

Type	UPLC capable of at least 1200 bar
Pump	Binary pump
Autosampler (temperature)	10 °C
Column oven (temperature)	50 °C
Analytical column	C18, 100 x 2.1 mm, 1.7 µm
Trap column or PFAS kit[4]	C18, 50 x 2.1 mm, 1.7 µm
Injection volume	2 µL

[4] PFAS kit: all parts of the LC system that contain PFAS (e.g. solvent lines) are, if possible, replaced by stainless steel, PEEK or PP parts to reduce contamination of samples

Table 11: Example 2 of HPLC gradient program for PFAS measurement by LC-MS/MS (ACQUITY UPLC I-Class system; Waters™)

Time (min)	Mobile phase A (water/methanol 98/2 v/v 2 mM ammonium acetate)	Mobile phase B (methanol)	Flow rate (mL/min)
0.00	90	10	0.4
1.00	90	10	0.4
13.00	5	95	0.4
14.00	5	95	0.4
14.10	90	10	0.4
16.00	90	10	0.4

Table 12: Example 2 of analytes and retention times (RT) for PFAS measurement by LC-MS/MS (ACQUITY UPLC I-Class system; Waters™)

Analyte	RT (min)	Analyte	RT (min)
PFBA	3.10	**PFOA**	9.70
PFPeA	6.00	**PFHpS**	9.75
PFBS	6.60	**PFNA**	10.35
4:2-FTS	7.60	**L-PFOS**	10.40
PFHxA	7.70	**9Cl-PF3ONS (F-53B)**	10.70
PFPeS	8.00	**PFNS**	10.90
HFPO-DA	8.10	**PFDA**	10.90
PFHpA	8.85	**8:2-FTS**	10.90
DONA	8.95	**PFDS**	11.40
PFHxS	9.00	**PFUnDA**	11.40
6:2-FTS	9.65	**PFDoDA**	11.85

Table 13: Example 2 of mass spectrometer parameters used for PFAS measurement by LC-MS/MS (Xevo TQ-S; Waters™)

Parameter	
Type	Triple quadrupole
Ionization Mode	ESI negative
Capillary Needle Voltage	0.5 kV (tune)
Source Temperature	150°C
Desolvation Temperature	400 °C (tune)
Cone Gas Flow	150 L/hr (tune)
Desolvation Gas Flow	800 L/hr
MS1/MS2 Resolution	Unit/Unit
Scan Type	Scheduled multiple reaction monitoring (sMRM)

Table 14: Example 2 of MS/MS transitions and optimised MS parameters used for PFAS measurement by LC-MS/MS (Xevo TQ-S; Waters™)

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Cone Voltage (V)	Collision energy (eV)
PFBA	213	169	20	11
PFPeA	263	219	20	8
PFBS	299	80	20	26
	299	99	20	26
4:2-FTS	327	307	20	20
	327	81	20	28
PFHxA	313	269	20	9
	313	119	20	26
PFPeS	349	80	20	30
	349	99	20	26
HFPO-DA	285	169	60	7
	329	285	60	5
PFHpA	363	319	20	16
	363	169	20	10
DONA	377	251	15	13
	377	85	15	29
PFHxS	399	80	20	34
	399	99	20	30
6:2-FTS	427	407	20	20
	427	81	20	28
PFHpS	449	80	20	35
	449	99	20	30
PFOA	413	369	20	10
	413	169	20	18
PFOS	499	80	20	44
	498	99	20	38
PFNA	463	419	20	12
	463	219	20	18
9Cl-PF3ONS (F-53B	531	351	58	24
major)	531	83	58	24
PFNS	549	80	20	44
	549	99	20	38
PFDA	513	469	20	11
	513	269	20	18
8:2-FTS	527	507	20	20
	527	81	20	28
PFDS	599	80	20	50
	599	99	20	42
PFUnDA	563	519	20	12
	563	269	20	18
PFDoDA	613	569	20	14
	613	269	20	14

Table 14 (continued)

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Cone Voltage (V)	Collision energy (eV)
$^{13}C_4$-PFBA	217	172	20	11
$^{13}C_5$-PFPeA	268	223	20	8
$^{13}C_2$-PFHxA	315	270	20	9
$^{18}O_2$-PFHxS	403	84	20	30
$^{13}C_4$-PFOA	417	372	20	10
$^{13}C_4$-PFOS	503	99	20	38
$^{13}C_5$-PFNA	468	423	20	12
$^{13}C_2$-PFDA	515	470	20	11
$^{13}C_2$-PFUnDA	565	520	20	12
$^{13}C_2$-PFDoDA	615	570	20	14
$^{13}C_2$-6:2-FTS	429	409	20	20
$^{13}C_3$-HFPO-DA	287	169	60	7

6.5.1.3 Example 3: HPLC-ESI-QQQ (HILIC column)

An example method for analysis of PFAS using HILIC separation is described elsewhere [17].

Mass spectrometer parameters and MS/MS transitions are similar to those described in sections 6.5.1.1 and 6.5.1.2.

6.5.2 Module 3B – Liquid chromatography-high resolution mass spectrometry (LC-HRMS)

Alternatively to low resolution mass spectrometry, HRMS could also be effectively used for routine analysis of PFAS. The same requirements to (U)HPLC equipment as for (U)HPLC-MS/MS are applicable in case of using (U)HPLC-HRMS.

Procedure

Introduce an appropriate calibrant (according to the guidelines provided by manufacturer) into the stabilised source and tune the MS to a minimum required resolving power of 10 000 (10 % valley) at a mass that is within the mass range of the PFAS ions, e.g. *m/z* 554.9659. Verify the resolution at different masses within the range (*m/z* 121.051 – *m/z* 922.092). Calibrate and record the resolution and mass deviation for the full set of ion masses corresponding to the required PFAS from C4 to C14 (**Table 1**). For a satisfactory calibration, the resolution should be greater than or equal to 10 000, and the deviation between the exact *m/z* and the theoretical *m/z* for each exact *m/z* monitored should be equal or less than 5 ppm. Set up an MS monitoring programme to include a minimum of two masses per analyte (two for native and ^{13}C labelled each) and using the appropriate PFAS standards, adjust the time windows for each analyte. Examples of (U)HPLC-HRMS conditions are given below.

Inject a medium concentration range PFAS calibration standard into the (U)HPLC-HRMS system and ensure that all the ions corresponding to the required analytes from C4 to C14 are recorded with the required sensitivity. Sensitivity is dependent on the cleanliness of the ESI source. If required take appropriate action, e.g. cleaning the ion source.

When the performance of the (U)HPLC-HRMS system has been verified, the extracts can be analysed. Typically the sequence of injections should commence and end with the set of PFAS calibration standards. The batch of samples including a procedural blank and reference material or a QC sample may be run in between these standards separated by solvent blanks (after every five samples) to ensure that there is no carryover.

Example: (U)HPLC-ESI-Orbitrap

Table 15: Example of HPLC system and conditions used for PFAS measurement by LC-HRMS

Type	HPLC capable of at least 1000 bar
Pump	Binary pump
Autosampler (temperature)	4 °C
Column oven (temperature)	45 °C
Analytical column	C18, 150 x 2.1 mm, 1.8 µm
Injection volume	5 µL

Table 16: Example of HPLC gradient program used for PFAS measurement by LC-HRMS

Time (min)	Mobile phase A (2 mM ammonium acetate in water)	Mobile phase B (2 mM ammonium acetate in methanol)	Flow rate (mL/min)
0.00	85	15	0.2
0.10	85	15	0.2
0.11	25	75	0.2
11.00	10	90	0.2
11.10	0	100	0.2
17.00	0	100	0.2
17.10	85	15	0.2
30.00	85	15	0.2

Table 17: Example of mass spectrometer parameters used for PFAS measurement by LC-HRMS (Orbitrap Exploris 120; Thermo Scientific)

Parameter	
Scan Type	Parallel reaction monitoring (PRM): MS1 Full Scan – Targeted MS2
ION SOURCE PROPERTIES	
Ionization Mode	H-ESI negative
Spray voltage	3000 V
Sheat gas (Arb)	40
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion Transfer Tube Temperature	320 °C
Vaporizer Temperature	300 °C
FULL SCAN PROPERTIES	
Orbitrap Resolution	60 000
Scan Range (*m/z*)	100-750
RF Lens (%)	70
TARGETED MS2 SCAN PROPERTIES	
HCD Collision Energies (V)	20, 60, 100
Orbitrap Resolution	30 000

Table 18: Example of mass list and retention times of unlabelled and labelled PFAS (Orbitrap Exploris 120; Thermo Scientific)

Analyte	Full scan MS1 ion (*m/z*)	Fragment ion (*m/z*)	Retention time (min)
PFBA	212.9792	171.9994	3.96
PFBS	298.9430	79.9574	4.02
PFHxA	312.9728	118.9927	4.15
PFHxS	398.9366	79.9574	4.25
PFHpA	362.9696	168.9896	4.28
PFOA	412.9664	168.9894	4.48
PFOS	498.9302	79.9574	4.69
PFNA	462.9632	168.9894	4.75
PFDA	512.9600	168.9894	5.13
PFDS	598.9238	79.9574	5.53
PFUnDA	564.9636	168.9893	5.63
PFDoDA	612.9537	168.9894	6.30
PFTrDA	662.9505	168.9894	7.11
FOSA	497.9462	77.9655	7.49
PFTeDA	712.9473	168.9893	8.04
N-MeFOSA	511.9619	168.9888	9.70
N-MeFOSE	616.0092	59.0138	9.80
N-EtFOSA	525.9774	218.9855	10.70
N-EtFOSE	630.0249	59.0138	10.70

Table 18 (continued)

Analyte	Full scan MS1 ion (*m/z*)	Fragment ion (*m/z*)	Retention time (min)
$^{13}C_4$-PFBA	216.9926		3.96
$^{13}C_6$-PFHxA	317.9896		4.15
$^{13}C_3$-PFHxS	401.9467		4.25
$^{13}C_4$-PFHpA	366.9830		4.28
$^{13}C_8$-PFOA	420.9933		4.48
$^{13}C_8$-PFOS	506.9571		4.69
$^{13}C_9$-PFNA	471.9934		4.75
$^{13}C_9$-PFDA	518.9802		5.13
$^{13}C_9$-PFUnDA	569.9803		5.63
$^{13}C_2$-PFDoDA	614.9604		6.30
$^{13}C_8$-FOSA	505.9730		7.49
$^{13}C_2$-PFTeDA	714.9540		8.04
D3-N-MeFOSA	514.9807		9.70
D7-N-MeFOSE	623.0531		9.75
D5-N-EtFOSA	531.0089		10.65
D9-N-EtFOSE	639.0813		10.63

7. CONFIRMATION OF PFBA AND PFPEA

As described in section 2.4.3 of the main guidance document, PFAS with only one specific MS/MS transition (e.g. PFBA and PFPeA) should be verified using a second chromatographic separation method or another MS method. If analysis of PFAS is performed using a C18 column (sections 6.5.1.1 and 6.5.1.2) confirmation of PFBA and PFPeA may be done using e.g. HILIC separation (section 6.5.1.3) or high resolution mass spectrometry (section 6.5.2).

8. QUANTITATION AND PRESENTATION OF RESULTS

Quantitation is based on the internal standard method, which provides a measure of the analyte content that is automatically corrected for matrix-effects and any losses during extraction, purification or measurement. The use of ILIS also allows for calculation of the recovery of the analytical process when measured against the analytical recovery standard(s) (**Table 1**). For analytes without ILIS (e.g. PFNS), another ILIS may be used.

PFAS are identified from the individual ion chromatograms based on retention time of the native and corresponding labelled standard, exact mass or at least two transitions and ion ratio (identification requirements are given in section 2.4.3 of the main guidance document). These parameters should be established before quantitation. The retention times of all PFAS of interest should be checked and, if necessary, modified in the processing method that is usually provided with the instrument software. Use the modified method to integrate all ion chromatograms that were produced during the analytical sequence. It is strongly recommended that the output is manually checked for correct integration of each individual compound.

Prepare a calibration curve encompassing the concentration range to be determined and verify the linearity of the PFAS standard calibration for each compound. If the relative response for any PFAS is constant (less than 20 % coefficient of variation) over the calibration range, an averaged relative response factor (RRF) may be used for that analyte. The RRF for a native analyte (iN) in the analytical standard may be determined using equation 1 below.

$$RRF_{iN} = \left(\frac{S_{iN}}{conc_{iN}}\right) \div \left(\frac{S_{ILIS}}{conc_{ILIS}}\right) = \frac{S_{iN} \times conc_{ILIS}}{S_{ILIS} \times conc_{iN}} \quad (1)$$

S_{iN}: response of the native analyte (iN)
S_{ILIS}: response of the corresponding isotope labelled internal standard (ILIS)
$conc_{iN}$: concentration of the native analyte (iN)
$conc_{ILIS}$: concentration of the corresponding isotope labelled internal standard (ILIS)

The amount (m) in [µg] of the native analyte (iN) in the sample may be determined from equation 2.

$$m_{iN} = \frac{m_{ILIS}}{S_{ILIS}} \times \frac{1}{RRF_{iN}} \times S_{iN} \quad (2)$$

m_{ILIS}: amount (in µg) of the corresponding isotope labelled internal standard added to the test sample aliquot
S_{iN}: response of the native analyte (iN)
S_{ILIS}: response of the corresponding isotope labelled internal standard (iIS)
RRF_{iN}: relative response factor of the native analyte (iN)

The content (c) in [µg/kg] of the native analyte (iN) in the sample may then be determined using equation 3.

$$c_{iN} = \frac{m_{iN}}{w} \quad (3)$$

m_{iN}: amount (in µg) of the native analyte in the sample
w: weight (in kg) of the test portion taken for analysis

The quantitation software on most LC-MS systems allows the above calculation process to be automated, so that a collated list of the PFAS concentration values for the measured sample extract is obtained.

For quality control purposes, the analytical recovery (R) in [%] of an isotope labelled internal standard (ILIS) may be calculated using equation 4 below:

$$R_{IILS} = \left(\frac{S_{ILIS}}{m_{ILIS}}\right) \div \left(\frac{S_{RS}}{m_{RS}}\right) \times \frac{1}{RRF_{ILIS}} \times 100 \quad (4)$$

m_{ILIS}: amount (in µg) of the internal standard (ILIS) added to the test portion
m_{RS}: amount (in µg) of the recovery standard (RS) in the final extract
S_{ILIS}: response of the internal standard (ILIS)
S_{RS}: response of the recovery standard (RS)
RRF_{ILIS}: relative response factor of the internal standard (ILIS)

9. REPORTING OF RESULTS - FORMAT

An example of a reporting format for PFAS concentrations in food and feed samples is given below (**Table 19**).

The conventional units for reporting PFAS concentrations are µg/kg wet weight (w.w.). The definition of lower bound sums is described in section 1.7 of the main guidance document which also provides more information on reporting in section 2.5.

Table 19: Example of a reporting format for PFAS samples

Sample ID	
Origin of sample	
Sample type	
Year of sampling	
Routine or incident related	

Analyte	**Content[d)] [µg/kg w.w.]**	Measurement uncertainty [%]	Apparent recovery [%]	**LOQ [µg/kg w.w.]**
PFBA				
PFPeA				
PFHxA				
PFHpA				
PFOA				
PFNA				
PFDA				
PFUnDA				
PFDoDA				
PFTrDA				
PFTeDA				
PFBS				
PFPeS				
PFHxS				
PFHpS				
L-PFOS				
br-PFOS				
PFNS				
PFDS				
PFUnDS				
PFDoDS				
PFTrDS				
Total PFOS (sum of L-PFOS and br-PFOS)				
Sum of total PFOS, PFOA, PFHxS, PFNA (lower bound)				

Table 19 (continued)

Analyte	**Content[d] [µg/kg w.w.]**	Measurement uncertainty [%]	Apparent recovery [%]	**LOQ [µg/kg w.w.]**
Other measured analytes				
FOSA				
DONA				
GenX				
F-53B				
Capstone A				
Capstone B				
Etc.				
% Moisture content (feed)				
Extraction method used				
Purification method used				
Quantitation method used				
Other relevant information				
Level of identification confirmation for e.g. PFBA and PFPeA				
NOTE: Fields in grey font are optional				

[d] Results shall be reported as anions (e.g. PFCA, PFSA) or neutral compounds (e.g. FOSA) and to two significant figures (see chapter 2.5. of the main guidance document).

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