Application Note

Environmental

Determination of 40 PFAS in Tilapia Tissue Following EPA 1633 Method Guidance

Using Agilent Captiva EMR PFAS Food II passthrough cleanup and LC/MS/MS detection

Authors

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Abstract

This application note presents the development and validation of a multiresidue method for the analysis of 40 per- and polyfluoroalkyl substances (PFAS) residues in tilapia tissue. The method uses QuEChERS extraction followed by enhanced matrix removal (EMR) mixed-mode passthrough cleanup using Agilent Captiva EMR PFAS Food II cartridges followed by LC/MS/MS detection. The method features simplified and efficient sample preparation, sensitive LC/MS/MS detection, and reliable quantitation using neat standard calibration curves. The method was validated under U.S. Environmental Protection Agency (EPA) method 1633 guidance, meeting all the method quality control requirements. The method was compared with the traditional weak anion exchange (WAX) solid phase extraction (SPE) method described in EPA 1633 and the modified carbon/WAX SPE method, showing improved method quantitation accuracy and precision and over 80% savings in the time and cost of sample preparation.

Introduction

Determination of PFAS residues in tissue, especially in fish, has been an important avenue for monitoring and regulating PFAS residues in the environment. In 2021, the EPA published method 1633 for the quantitative analysis of PFAS in aqueous, solid, biosolid, and tissue samples using LC/MS/MS[1] for 40 PFAS targets. The acidic groups contained in PFAS compounds enable them to be ionized easily and efficiently under negative mode, providing advantages in method sensitivity and selectivity. Method quantitation based on the use of stable labeled internal standards (ISTD) allows easy and reliable quantitation using neat calibration standard curves. Two sets of internal standards were used: extracted internal standards (EIS) and nonextracted internal standards (NIS). The EIS compounds included 24 isotopically labeled PFAS compounds and were used for PFAS target quantitation. EIS spiking solution was spiked directly into the sample matrix before extraction so EIS standards could track PFAS targets through the sample preparation procedure. NIS compounds included seven different isotopic PFAS compounds and were used for EIS recovery calculation. NIS solution was spiked into the final sample extract, thus only tracking the matrix effect.

WAX polymer sorbent based SPE methods have been used widely in many EPA methods for PFAS analysis of water, soil, and other environmental media; these methods include the EPA 533 method, published in 2009 and 2019, for determination of PFAS targets in drinking water[2,3]; the EPA's Multi-Industry Per- and Polyfluoroalkyl Substances (PFAS) Study – 2021 Preliminary Report[4]; and EPA 1633 method[1], released in 2021, for PFAS analysis of aqueous, solid, biosolid, and tissue samples. The WAX SPE method provides a robust and reliable solution for aqueous sample preparation for PFAS analysis. However, the SPE methodology is challenging for complex solid tissue sample preparation. Tissue samples first need to be extracted using an organic solvent such as methanol (MeOH) or acetonitrile (ACN). The crude extract subsequently requires a solvent switch before SPE loading from a high percentage organic medium to a high aqueous medium, which is accomplished through either a dry-and-reconstitute step or high dilution with water. The typical SPE conditioning-equilibrium-loading-washing-eluting procedure is followed. Both the solvent switch and SPE procedure make the entire method a time-consuming and labor-intensive procedure.

QuEChERS extraction has been reported for PFAS analysis in food sample preparation.[5,6] Compared to the alkaline MeOH digestion and extraction procedure used in EPA method 1633, QuEChERS extraction reduces the extraction time from about 20 hours to approximately 1 hour, uses approximately 80% less organic solvent, and delivers high extraction efficiency. Captiva EMR PFAS Food II cartridges were developed and optimized specifically for PFAS analysis in animal-origin food matrices. The crude extract after QuEChERS extraction is mixed with 10% water, followed by passthrough cleanup on the Captiva EMR PFAS Food II cartridge. The PFAS targets flow through the cartridge, while the unwanted matrix co-extractives are retained on the cartridge based on the mixed-mode interactions with the sorbents. The method demonstrated a validated analysis of PFAS in animal-origin food matrices.[7,8]

The objectives of this study were to apply this method to the analysis of 40 PFAS in fish tissue and validate it following EPA 1633 method guidance to meet the acceptance criteria. Considering the different requirements on method limits of quantitation (LOQs) for environmental tissue analysis, the previous sample preparation method used for food analysis[7] was modified accordingly, saving time by eliminating the drying step. An Agilent 6495D Triple Quadrupole LC/MS was used for LC/MS/MS detection and quantitation, and instrumental conditions were adjusted to accommodate differences in the samples.

Experimental

Chemicals and reagents

EPA 1633 native PFAS, EIS, and NIS stock solutions were purchased from Wellington Laboratories (Guelph, Ontario, Canada). The concentrations of compounds in the stock solution vary and are shown in Appendix Table 1. MeOH, ACN, and isopropyl alcohol (IPA) were purchased from VWR (Radnor, PA, USA). Acetic acid, ammonium acetate, ammonium hydroxide, and potassium hydroxide were procured from MilliporeSigma (Burlington, MA, USA).

Solutions and standards

The native PFAS, EIS, and NIS spiking solutions were prepared by diluting the corresponding stock solutions with MeOH. The concentrations of native PFAS targets in the spiking solution were 25 ng/mL and above in MeOH. The concentrations of EIS compounds in the spiking solution I and II were 5 ng/mL and above in MeOH and 25 ng/mL and above in MeOH. The concentrations of NIS compounds in the spiking solution were 5 ng/mL and above in MeOH. The concentrations in the spiking solutions correspond to the compounds with the lowest concentration in the stock solutions. For other compounds in the mix, concentrations were proportionally higher based on the corresponding concentrations in the stock solutions, which are listed in Appendix Table 1.

The native PFAS spiking, EIS spiking I, and NIS spiking solutions were used for preparation of calibration curve neat standards at 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/mL for native PFAS targets, 0.1 ng/mL for EIS, and 0.1 ng/mL for NIS in ACN with 1% acetic acid extraction solvent. Similarly, the concentrations listed above correspond to the lowest compounds in the stock solutions. The other compounds in the mix were in proportionally higher concentrations based on their concentrations in the stock solution mix. After all calibration standards were prepared, the calibration standards were diluted with 10% water, mixed well, then used for injection in LC/MS/MS detection. This step was to correspond to the additional 10% dilution of the sample during sample cleanup on EMR cartridges.

All standards were stored at 4 °C and used for no more than two weeks. The solutions need to be warmed up thoroughly to room temperature before use. Appropriate sonication can be applied to expedite the warming of the standard solutions. For running routine calibration curve standards, an aliquot of calibration solutions was transferred to another set of vials with polypropylene (PP) inserts, then used for instrument injections.

The ACN with 1% acetic acid extraction solvent was prepared by adding 10 mL of glacial acetic acid into 990 mL of ACN and stored at room temperature. LC mobile phase A was 5 mM NH_4OAc in water, and mobile phase B was ACN. Needle wash solvents included IPA, water, and ACN.

Equipment and material

The study was performed using an Agilent 1290 Infinity II LC system consisting of an Agilent 1290 Infinity II binary pump (G4220A), an Agilent 1290 Infinity II high-performance autosampler (G4226A), and an Agilent 1290 Infinity II thermostatted column compartment (G1316C). The LC system was coupled to an Agilent triple quadrupole LC/MS system (G6495D) equipped with an Agilent Jet Stream iFunnel electrospray ion source (ESI). Agilent MassHunter workstation software was used for data acquisition and analysis.

Other equipment used for sample preparation included:

- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Geno/Grinder (Metuchen, NJ, USA)
- Multi Reax test tube shaker (Heidolph, Schwabach, Germany)
- Pipettes and repeater (Eppendorf, NY, USA)
- Agilent positive pressure manifold 48 processor (PPM-48; part number 5191-4101)
- Ultrasonic cleaning bath (VWR, PA, USA)

The 1290 Infinity II LC system was modified using an Agilent InfinityLab PFC-free HPLC conversion kit (part number 5004-0006), including an InfinityLab PFC delay column, 4.6 × 30 mm (part number 5062-8100). Chromatographic separation was performed using an Agilent ZORBAX RRHD Eclipse Plus C18 column, 95Å, 2.1 × 100 mm, 1.8 µm (part number 959758-902) and an Agilent UHPLC guard column (part number 821725-901).

The sample preparation and other consumables used included:

- Agilent Bond Elut QuEChERS EN extraction kit, EN 15662 method, buffered salts, ceramic homogenizers (part number 5982-5650CH)
- Agilent Captiva EMR PFAS Food II cartridges, 6 mL cartridges, 750 mg (part number 5610-2232)
- PP snap caps and vials, 1 mL (part numbers 5182-0567 and 5182-0542)
- PP screw cap style vials and caps, 2 mL (part numbers 5191-8121 and 5191-8151)
- Tubes and caps, 50 mL, 50/pk (part number 5610-2049)
- Tubes and caps, 15 mL, 100/pk (part number 5610-2039)

All the consumables used in the study were tested and verified with acceptable PFAS cleanliness.

LC/MS/MS instrument conditions

Table 1. LC pump conditions for LC/MS/MS.

Parameter	Setting			
Mobile Phase A	5 mM NH_4OAc in water			
Mobile Phase B	ACN			
Gradient	Time (min)	A%	B%	Flow (mL/min)
	0.00	90	10	0.400
	2.00	70	30	0.400
	8.50	55	45	0.400
	11.50	25	75	0.400
	13.25	0	100	0.460
Stop Time	15.50 min			
Post Time	2.0			

Table 2. LC multisampler program for LC/MS/MS.

Parameter	Setting				
Injection Program	– Draw 15.00 µL of water – Draw 5.00 µL of sample – Wash needle as defined in method – Draw 10.00 µL of water – Mix 10.00 µL from air five times – Inject				
Multiwash	Step	Solvent	Time (s)	Seat Backflush	Needle Wash
	1	IPA	10	Enabled	Enabled
	2	ACN	10	Enabled	Enabled
	3	Water	10	Enabled	Enabled

LC column compartment: isothermal temperature 55 ± 0.8 °C.

Mass spectrometer (MS) acquisition parameters: negative ion mode with constant fragmentor setting at 166 V and iFunnel standard mode.

ESI source settings:

- **Drying gas:** 200 °C, 18 L/min
- **Sheath gas:** 300 °C, 11 L/min
- **Nebulizer gas:** 15 psi
- **Capillary voltage:** 2,500 V (NEG) and 3,000 V (POS)
- **Nozzle voltage:** 0 V (NEG) and 1,500 V (POS).

The MS acquisition conditions for PFAS targets, EISs, NISs, and cholic acids were drawn from the Agilent PFAS MRM Database for LC/TQ (part number G1736AA).

Sample preparation procedure

Fresh tilapia fillet was purchased from local grocery stores. Samples were frozen at –20 °C for 1 to 2 hours, then cut into small pieces. The frozen chops were then homogenized using a mechanical blender. The homogenized fish paste was kept at –20 °C until use.

Five grams of homogenized fish sample was weighed into a 50 mL PP tube. The PFAS standard and EIS were spiked to all prespiked quality control samples (QCs) appropriately, and EIS to matrix zero blanks (MBs). For procedure blanks (PBs), only 5 mL of water and spike EIS were added.

Table 3 shows the prespiked sample spiking details. Since the sample preparation procedure introduced 2x dilution, the concentrations in the final extract were 50% of the concentrations in the original sample. To achieve the correct quantitation results using the previously prepared calibration curve, it was important to keep the theoretical EIS and NIS concentrations equivalent to those in the calibration standards, which were 0.1 ng/mL of EIS and NIS in the final ACN extract. The 10% water mixing for the sample ACN extract prior to EMR cleanup was matched by calibration curve standard dilution with 10% water (see previous section, "Solutions and standards"), so its impact on the sample concentration was not required. The concentration listed in Table 3 was for the targets and EIS compounds that have the lowest concentration in the stock solutions. Other targets or EIS compounds had proportional higher concentrations based on their original concentrations in the stock solutions.

After sample spiking, all samples were vortexed for 2 to 3 minutes for equilibrium. Samples were then ready for extraction using the developed procedure, which is shown in Figure 1.

After the sample extraction and cleanup procedure, a 1.111 mL aliquot of the sample was transferred to a 2 mL PP vial, which corresponded to 1 mL of sample extract in ACN. Then, after spiking with 20 µL of NIS spiking solution (5 ng/mL), the vial was capped and the mixture was vortexed for 10 to 20 seconds. This created an NIS concentration in samples equivalent to that in calibration curve standards. All samples were then ready for injection for LC/TQ analysis.

Table 3. Prespiked sample spiking with native PFAS targets and EIS compounds.

Samples	Native PFAS Target Spiking				EIS Compound Spiking				Replicates (N)
	Spiking Solution and Concentration (ng/mL)	Spiking Volume (µL)	Concentration in Sample (µg/kg)	Concentration in Sample Extract (ng/mL)	Spiking Solution and Concentration (ng/mL)	Spiking Volume (µL)	Concentration in Sample (µg/kg)	Concentration in Sample Extract (ng/mL)	
PB	NA	NA	NA	NA	EIS spiking solution II (25 ng/mL)	40	0.2	0.1	3
MB	NA	NA	NA	NA		40	0.2	0.1	7
QC-LOQ	PFAS spiking solution (25 ng/mL)	10	0.05	0.025		40	0.2	0.1	6
QC-Low (4x LOQ)		40	0.2	0.1		40	0.2	0.1	6
QC-Mid (40x LOQ)	PFAS stock solution (250 ng/mL)	40	2	1		40	0.2	0.1	6

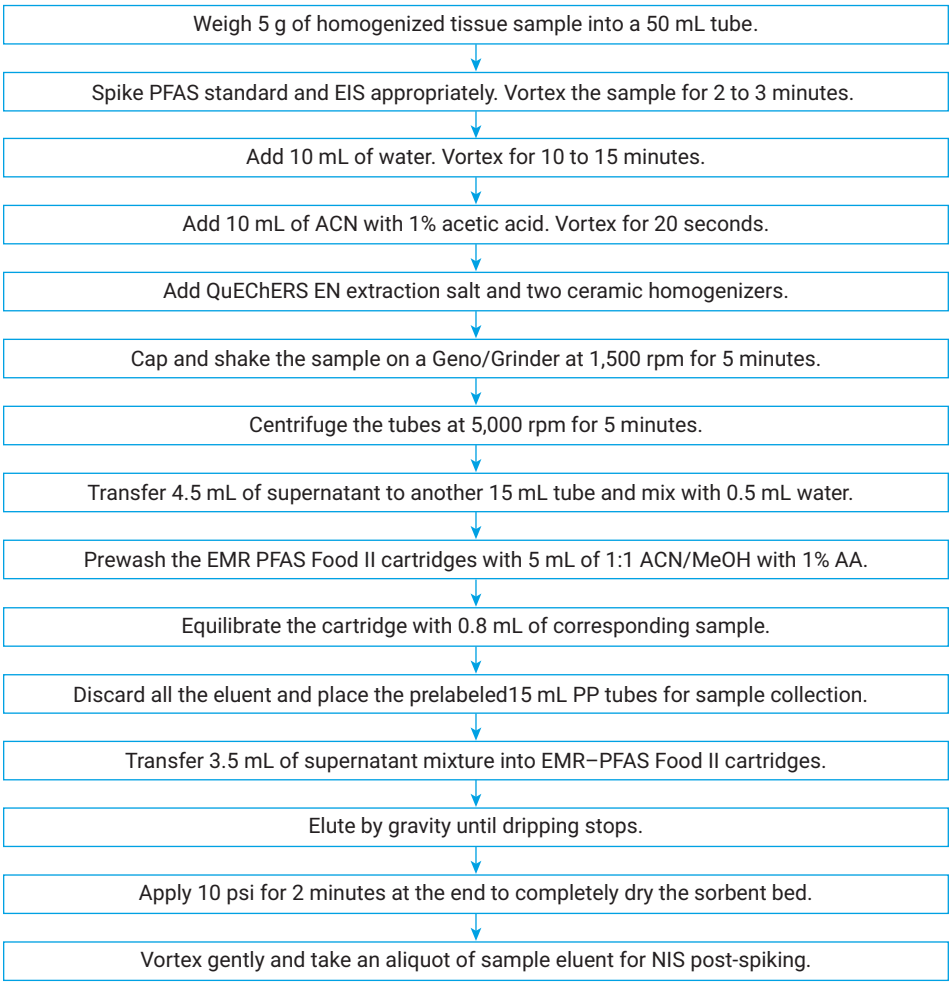

Figure 1. Sample preparation procedure using QuEChERS extraction followed by EMR mixed-mode passthrough cleanup on an Agilent Captiva EMR PFAS Food II cartridge.

For a comparison study using the traditional EPA 1633 sample preparation method, both the original method using alkaline MeOH extraction followed by carbon dispersive SPE (dSPE) plus WAX SPE cartridges and a modified method using the same sample extraction followed by dual-phase carbon/WAX SPE cartridges were tested. The preparation procedure for the original method was followed exactly according to the procedure listed in the EPA 1633 method.[1] For the modified SPE method, the preparation procedure was similar to the original method but with two modifications. First, the carbon material dispersive cleanup step was skipped. Second, the carbon/WAX dual-phase SPE cartridge was used to replace the original WAX SPE cartridge in the SPE steps. For a comparison study, only MB and QC-LOQ samples were prepared in replicates of four. Since the dilution factor introduced from the SPE methods was 2.5 times and the final samples after SPE preparation were in MeOH, a separate set of calibration curve standards were prepared in MeOH and used for comparison sample quantitation. All the calibration curve standard concentrations were kept the same except for the first standard, which was changed to 0.02 ng/mL to match the QC-LOQ samples after SPE method preparation with 2.5-fold dilution.

Results and discussion

LC/TQ instrument method

The MS detection method used here was adopted directly from previous studies[7,8] but with additional targets and ISTD compounds from the Agilent PFAS MRM database. More modifications were applied on the LC method side. The LC method still used the same LC column as the previous studies, but with different mobile phase B, gradient, and injection programs. The modified LC method provided better chromatographic distributions on native targets, EIS, and NIS compounds within the acquisition window. It also improved the chromatographic separation for some targets with their isomers, and provided baseline separation for PFOS and cholic acid interferences. Figure 2 shows the chromatogram of all the targets, EIS, and NIS peaks with partial identification (A), and PFOS isomer and cholic acid interferences (B), demonstrating improved peak distribution over the retention time window and baseline separation for critical targets and possible matrix interferences.

Sample preparation procedure

The method using QuEChERS extraction followed with EMR mixed-mode passthrough cleanup on Captiva EMR PFAS Food II cartridges greatly simplified the entire sample preparation procedure. The ACN solvent extraction followed with salt partition improved extraction efficiency and thus reduced the extraction time. Comparing the long extraction process with alkaline MeOH (~ 18 hours) used in the traditional EPA 1633 method, the QuEChERS method significantly reduced the extraction process time down to approximately 1 hour for the same number of samples, without compromise in extraction efficiency. The salt partition step played a critical role for more polar PFAS target extraction, and also helped with cleaning the matrix polar co-extractives, as the polar matrix co-extractives were retained in the aqueous phase after the salt partition.

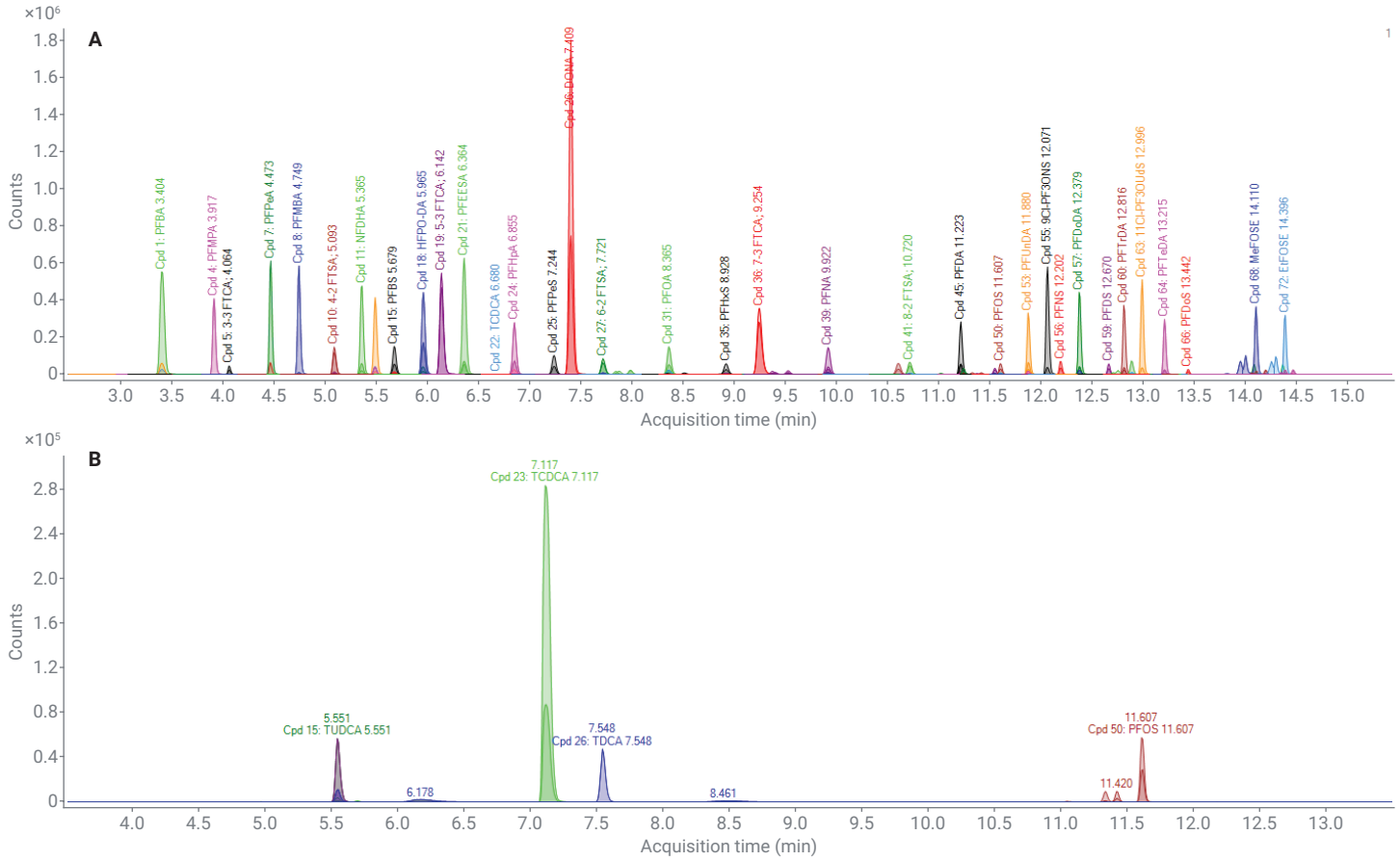

Figure 2. MRM chromatogram for all PFAS targets, EIS, and NIS compounds (A), and PFOS (RT = 11.607 minutes) and cholic acids (B), i.e. TUDCA (RT = 5.551 minutes), TCDCA (RT = 7.117 minutes) and TDCA (RT = 7.548 minutes).

After QuEChERS extraction, the crude ACN extract was mixed with 10% water, then cleaned by passthrough cleanup on Captiva EMR PFAS Food II cartridges. The eluent was subject for direct injection for LC/TQ analysis. No drying step was used in the sample cleanup procedure. The entire sample cleaning procedure takes approximately 1 to 2 hours, depending on the number of samples being processed.

After sample extraction, the traditional EPA 1633 method uses a WAX SPE procedure to further clean the sample extract. For the original method using the WAX SPE cartridge, the crude extract was first further cleaned using a small amount of carbon sorbent, then dried down for solvent medium switch to prepare for sample loading. For the modified method, the dispersive carbon material cleanup step was skipped, but the sample crude extract still needed to go through the drying process prior to sample loading. The drying process usually takes 1 to 3 hours due to a 25 mL total volume for each sample, depending on the number of samples being processed. After getting samples ready for the SPE process, the same SPE procedure was used for either WAX or carbon/WAX dual-phase SPE cartridges, which included cartridge preconditioning, equilibrating, sample loading, washing, and final elution. Depending on the number of samples being processed, the entire SPE procedure usually takes 2 to 4 hours. The SPE procedure also used much more organic solvent.

Table 4 shows the sample preparation method comparison in time consumption, organic solvent use, and other consumables needed to prepare one sample. For a simplified description of each method, abbreviation terms based on each method's critical feature are used. The newly developed method is represented as $QuEChERS_{ext}$-EMR, the original EPA 1633 preparation method is represented as $Solvent_{ext}$-Carbon dSPE-WAX SPE, and the modified method is represented as $Solvent_{ext}$-Carbon/WAX SPE.

As shown in the table, the traditional SPE methods required a significantly longer preparation procedure, while the $QuEChERS_{ext}$-EMR method demonstrated a simplified procedure, saving > 80% of the time needed. For solvent and chemicals use per sample, the new method saved ~ 80% on solvent and chemical use. The new method also offers a > 50% saving on consumables use. For multiple sample preparation, the use of organic solvent and consumables is directly multiplied by the number of samples. The time needed for preparing multiple samples depends on the preparation procedure complexity, and the more complex procedure the more time needed. For example, in the comparison study, it took the analyst ~ 2 to 2.5 hours to prepare a batch of 10 to 15 samples when using the $QuEChERS_{ext}$-EMR sample preparation method. But to prepare even fewer samples (8 to 10) using traditional SPE-based methods, completing the preparation work took the same analyst 2 to 3 days.

Table 4. Comparing time, organic solvent, and consumables needed to prepare one sample across three quantitation methods.

	$QuEChERS_{ext}$-EMR Method				$Solvent_{ext}$-Carbon/WAX SPE Method				$Solvent_{ext}$-Carbon dSPE-WAX SPE Method			
Preparation Main Phases	Main Steps	Time (hr)	Solvent Used (mL)	Consumables	Main Steps	Time (hr)	Solvent Used (mL)	Consumables	Main Steps	Time (hr)	Solvent Used (mL)	Consumables
Prework	Make two reagents	0.25			Make six reagents, pack glass wool in SPE cartridge	1		Glass wool	Make six reagents, pack glass wool in SPE cartridge	1		Glass wool
Sample Extraction	One-step QuEChERS extraction	0.75	10	50 mL Tube (1), Extraction salt (1), CHs (2)	Three-step solvent extraction and digestion	17.5	25	15 mL Tube (1), 50 mL Tube (1)	Three-step solvent extraction and digestion, carbon dSPE cleanup	18	25	15 mL Tube (1), 50 mL Tube (2), Carbon material (10 mg)
Transition Step	Dilution with 10% water	0.25		5 mL Tube (1)	Drying and redissolving, pH check and adjustment	1.25		pH paper (1-2)	Drying and redissolving, pH check and adjustment	1.25		pH paper (1–2)
Sample Further Extraction or Cleanup	EMR passthrough cleanup	0.25		EMR cartridge (1), 15 mL Tube (1)	Carbon/WAX SPE extraction and cleanup	2	25	Carbon/WAX SPE cartridge (1), Loading 50 mL tube (1), Connection fitting (1), 15 mL tube (1)	WAX SPE extraction and cleanup	1.25	25	WAX SPE cartridge (1), Loading 50 mL tube (1), Connection fitting (1), 15 mL tube (1)
Sample Post-Treatment	NIS post-spike	0.25		2 mL PP vial (1)	Neutralization sample, NIS post-spike, sample filtering	0.5		2 mL PP vial (1), 5 mL Syringe (1), Syringe filter (1)	Neutralization sample, NIS post spike, sample filtering	0.5		2 mL PP vial (1), 5 mL Syringe (1), Syringe filter (1)
Total (Per Sample)		1.75	10			22.25	50			22	50	

Quantitation method

The EPA 1633 quantitation method is based on the use of both isotopic EIS and NIS compounds, where the EIS compounds are used for target quantitation and NIS compounds are used for EIS recovery calculations. The method has recommendations on the assignment of quantification reference compounds for each target and EIS compound. The exact method recommendation was used to create the quantitation method and then applied to matrix sample quantitation. The majority of recommended reference compounds worked well, but several exceptions caused a quantitation failure. First, the $^{13}C_3$-HFPO-DA (EIS compound) was not a well-behaved IS compound, with more significant variations in its response in the sample matrix. The method assigned this EIS for quantitation of HFPO-DA, ADONA, 9Cl-PF3ONS, and 11Cl-PF3OUdS. It delivered acceptable quantitation results for its corresponding target, HFPO-DA. However, the other three targets failed due to the variations in the IS compound. Therefore, other EIS compounds with closer retention time and more consistent responses were used for the other three targets. This correction resolved the failure of those three targets and delivered successful quantitation results. All the PFAS targets and EIS compound quantification reference compounds are listed in Table 5.

Table 5. Comparison of QuEChERS$_{ext}$-EMR method quantification results with published EPA 1633 results.[1]

Target	RT (min)	Quantification Reference IS	MDL-EMR (µg/kg)	MDL-EPA (µg/kg)	LOQ-EMR (µg/kg)	LOQ-EPA (µg/kg)
PFHxA	5.56	$^{13}C_5$-PFHxA	0.002	0.111	0.05	0.4 to 0.5
PFHpA	6.94	$^{13}C_4$-PFHpA	0.006	0.099	0.05	0.4 to 0.5
PFOA isomers	8.5	$^{13}C_8$-PFOA	0.005	0.105	0.05	0.4 to 0.5
PFNA isomers	10.1	$^{13}C_9$-PFNA	0.009	0.119	0.05	0.4 to 0.5
PFDA	11.34	$^{13}C_6$-PFDA	0.006	0.149	0.05	0.4 to 0.5
PFUnA	11.99	$^{13}C_7$-PFUdA	0.007	0.125	0.05	0.4 to 1.0
PFDoA	12.51	$^{13}C_2$-PFDoA	0.007	0.101	0.05	0.4 to 0.5
PFTrDA	12.96	$^{13}C_2$-PFDoA	0.009	0.142	0.05	0.4 to 0.5
PFTeDA	13.36	$^{13}C_2$-PFTeDA	0.009	0.159	0.05	0.4 to 1.0
PFOSA isomers	12.88	$^{13}C_8$-PFOSA	0.005	0.069	0.05	0.4 to 0.5
N-MeFOSA isomers	14.19	D_3-N-MeFOSA	0.011	0.162	0.05	0.4 to 0.5
N-EtFOSA isomers	14.45	D_5-N-EtFOSA	0.008	0.163	0.05	0.4 to 1.0
N-MeFOSAA isomers	11.32	D_3-N-MeFOSAA	0.016	0.145	0.05	0.4 to 0.5
N-EtFOSAA isomers	11.63	D_5-N-EtFOSAA	0.01	0.148	0.05	0.4 to 0.5
PFBS	5.76	$^{13}C_3$-PFBS	0.007	0.097	0.05	0.4 to 0.5
PFPeS	7.36	$^{13}C_4$-PFHpA	0.004	0.076	0.05	0.4 to 0.5
PFHxS isomers	9.09	$^{13}C_3$-PFHxS	0.013	0.081	0.05	0.4 to 0.5
PFHpS	10.82	$^{13}C_9$-PFNA	0.004	0.119	0.05	0.4 to 0.5
PFOS isomers	11.74	$^{13}C_8$-PFOS	0.005	0.145	0.05	0.4 to 2.0
PFNS	12.32	$^{13}C_7$-PFUdA	0.009	0.108	0.05	0.4 to 0.5
PFDS	12.81	$^{13}C_2$-PFDoA	0.018	0.114	0.05	0.4 to 0.5
PFDoS	13.61	$^{13}C_8$-PFOS	0.021	0.153	0.05	0.4 to 0.5
PFPeA	4.5	$^{13}C_5$-PFPeA	0.005	0.155	0.1	0.8 to 1.0
PFEESA	6.47	$^{13}C_4$-PFHpA	0.007	0.123	0.1	0.8 to 1.0
PFMPA	3.95	$^{13}C_4$-PFBA	0.007	0.273	0.1	0.8 to 2.0
PFMBA	4.79	$^{13}C_5$-PFPeA	0.005	0.168	0.1	0.8 to 1.0
NFDHA	5.43	$^{13}C_5$-PFHxA	0.01	0.216	0.1	0.8 to 1.0
PFBA	3.47	$^{13}C_4$-PFBA	0.019	0.208	0.2	1.6 to 4.0
HFPO-DA	6.05	$^{13}C_2$-HFPO-DA	0.014	0.339	0.2	1.6 to 2.1
4:2 FTS	5.14	$^{13}C_2$-4:2 FTS	0.024	0.369	0.2	1.6 to 2.0
6:2 FTS	7.82	$^{13}C_2$-6:2 FTS	0.019	0.537	0.2	1.6 to 2.0
8:2 FTS	10.87	$^{13}C_2$-8:2 FTS	0.039	0.378	0.2	1.6 to 2.0
ADONA	7.52	$^{13}C_8$-PFOA	0.013	0.274	0.2	1.6 to 2.0
9Cl-PF3ONS	12.2	$^{13}C_7$-PFUdA	0.019	0.362	0.2	1.6 to 2.0
11Cl-PF3OUdS	13.15	$^{13}C_8$-PFOS	0.021	0.352	0.2	1.6 to 2.0
N-MeFOSE isomers	14.09	D_7-N-MeFOSE	0.046	0.832	0.5	4.0 to 5.0
N-EtFOSE isomers	14.37	D_9-N-EtFOSE	0.028	1.77	0.5	4.0 to 5.0
3:3 FTCA	4.07	$^{13}C_5$-PFPeA	0.034	0.716	0.25	2.0 to 4.0
5:3 FTCA	6.17	$^{13}C_4$-PFHpA	0.12	2.38	1.25	10 to 20
7:3 FTCA	9.26	$^{13}C_3$-PFHxS	0.199	2.02	1.25	10 to 12.5

For PFAS targets with isomers, the method requires summation of all isomers' peaks for integration and quantitation. This requirement can be satisfied by using spectrum summation to integrate all the peaks in the defined integration window. This strategy was used for all targets with linear and branched isomers, including PFOA, PFNA, PFOSA, N-MeFOSA, N-EtFOSA, N-MeFOSAA, N-EtFOSAA, N-MeFOSE, N-EtFOSE, PHFxS, and PFOS. Figure 3 shows representative examples of summative integrations of critical targets and their isomers for quantitation.

Method validation

The developed method was validated for the quantitative determination of 40 PFAS targets in tissue using tilapia as a representative matrix. The selection of tilapia for method validation was made after screening multiple kinds of fish for PFAS background. Tilapia was found to be a representative fish tissue with the lowest positive PFAS background. A method calibration curve was established using the neat calibration curve standards prepared in the extraction solvent, i.e., ACN with 1% AA. Considering the 2-fold dilution factor introduced during sample preparation, the method calibration dynamic range in the real sample matrix should be 2 times the range in the neat calibration curve standards. Given the various concentrations of targets in the stock mix solution, the calibration dynamic ranges also varied. For the first group of 22 targets with 250 ng/mL concentration in the stock solution, from PFHxA to PFDoS in the Native PFAS column of Appendix Table 1, their calibration dynamic range was 0.05 to 20 µg/kg. For the next group of five targets with 500 ng/mL concentration in the stock solution, including PFPeA, PFEESA, PFMPA, PFMBA, and NFDHA, the calibration dynamic range was 0.1 to 40 µg/kg. For the third group of eight targets with 1,000 ng/mL concentration in the stock solution, including PFBA, HFPO-DA, 4:2 FTS, 6:2 FTS, 8:2 FTS, ADONA, 9Cl-PF3ONS, and 11Cl-PF3OUdS, the calibration dynamic range was 0.2 to 80 µg/kg. The calibration dynamic range for two FOSE targets was 0.5 to 200 µg/kg. For 3:3 FTCA, it was 0.25 to 100 µg/kg, and for 5:3 FTCA and 7:3 FTCA it was 1.25 to 500 µg/kg. All calibration curves used linear regression with $1/x^2$ weight, giving $R^2 > 0.99$ or relative standard error (RSE) < 20.

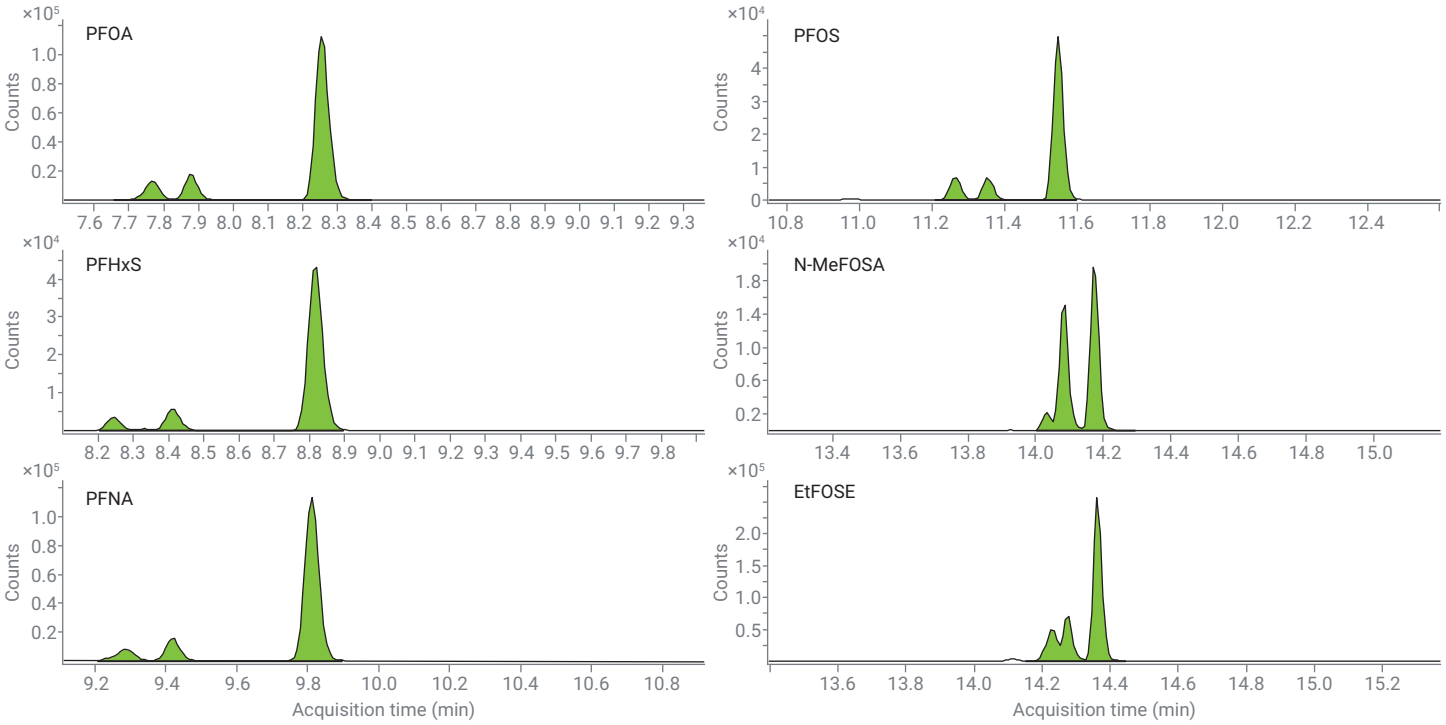

Figure 3. Summative integrations of critical PFAS targets to include all isomers for quantitation.

Method quantitation accuracy and precision was validated through the three levels of prespiked QC samples, which were LOQ, 4x LOQ, and 40x LOQ. For each level, six replicates were prepared for method precision validation. Method validated LOQ was determined based the lowest prespiking concentration with acceptable accuracy and precision. Method detection limits (MDL) were then calculated based on published U.S. Environmental Protection Agency procedure[10], specifically the equation below for six replicates with 99% confidence.

$$MDL = SD_{LOQspiking} \times 3.365 \quad \textbf{(Eq. 1)}$$

Where $SD_{LOQspiking}$ is the standard deviation of the validated method LOQ based on prespiking.

Table 5 lists the method MDL and LOQ results using the QuEChERS$_{ext}$-EMR method and their comparison with published results using the EPA 1633 traditional method.[1] Results show that the method delivered lower method MDL and LOQ than the published pooled study results from the EPA 1633 traditional method.

The EIS and NIS compound recoveries were assessed using all the matrix samples in the validation batch, including seven matrix blanks, six prespiked QC samples at each level. In total, 25 samples were used to calculate average results. Figure 4 shows the average EIS and NIS recovery in the tilapia matrix using the QuEChERS$_{ext}$-EMR method. The red dotted lines show the acceptance criteria upper and lower limits specified in EPA 1633 for PFAS in tissue analysis. The blue line shows the EIS (24 compounds on the left of the X-axis) and NIS (seven compounds on the right of the X-axis) compounds from the validation batch, which are within the acceptable range.

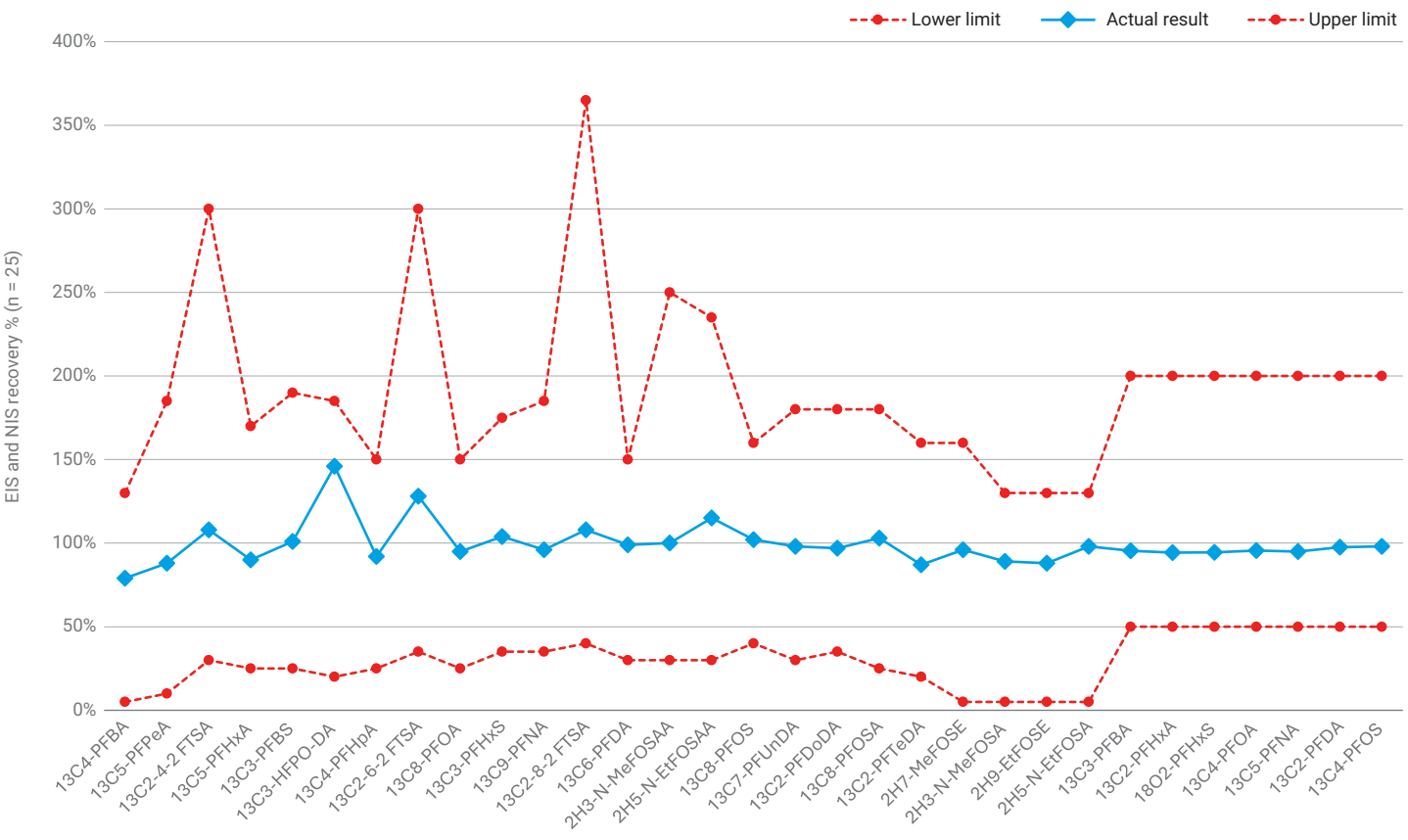

Figure 4. EIS and NIS compound average recoveries in tilapia using the QuEChERS$_{ext}$-EMR method.

Figures 5 and 6 show the quantitation accuracy and precision results for 40 PFAS targets in tilapia using the developed QuEChERS$_{ext}$-EMR sample preparation method. All targets delivered excellent results on both quantitation accuracy and precision for three prespiking levels of QC samples. The quantitation accuracy results for 3:3 FTCA and 5:3 FTCA were relatively high but still within the acceptance range. For 3:3 FTCA at the LOQ prespiking level, it was slightly above the upper acceptance limit. This is because the isotopic EIS analog compound used for quantitation does not correct the matrix effect on the actual targets. It is believed that the corresponding isotopic ISTD can resolve the issue and provide more accurate quantitation results.

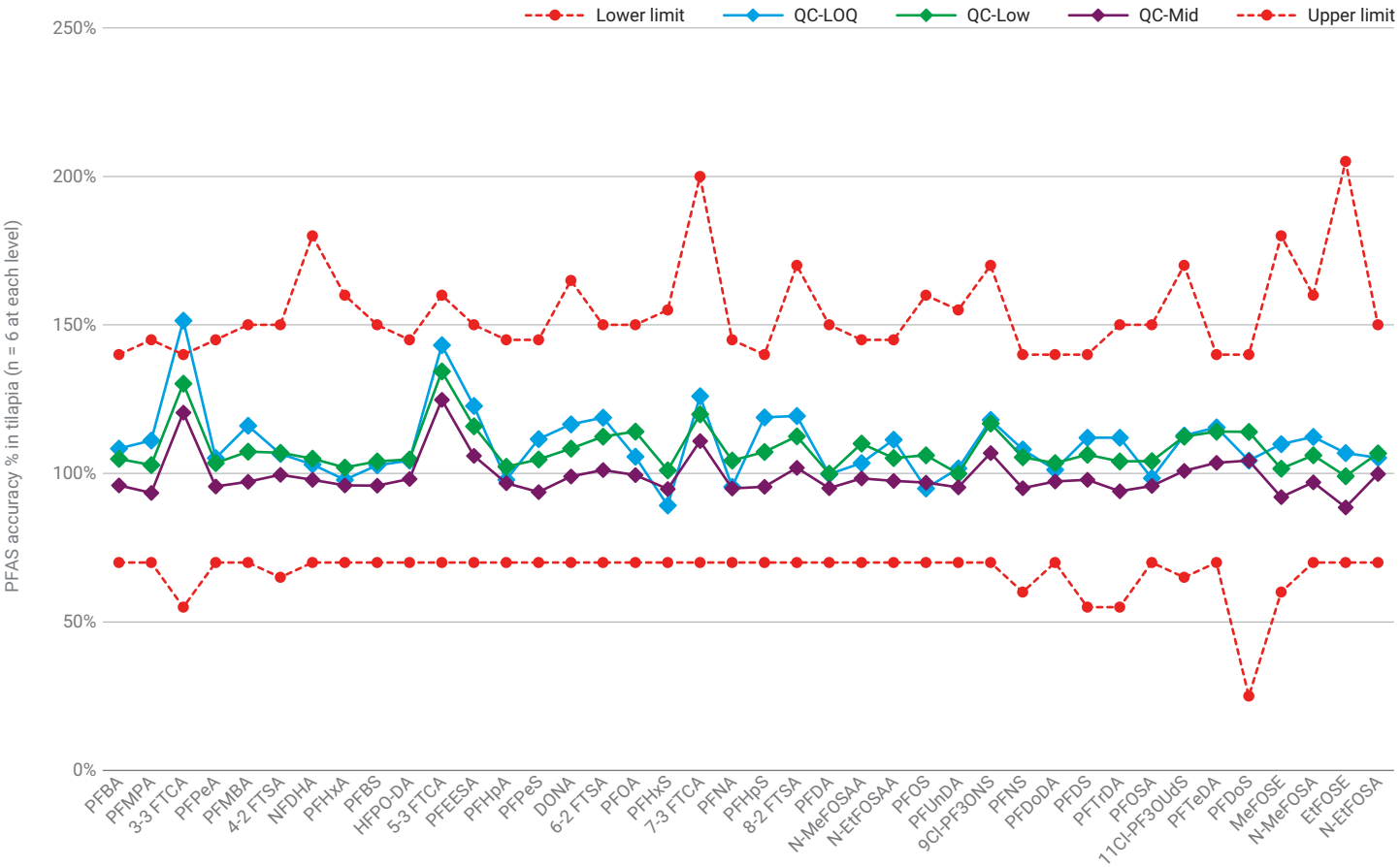

Figure 5. Quantitation accuracy (recovery %) results for analysis of 40 PFAS targets in tilapia at three spiking levels using the QuEChERS$_{ext}$-EMR method.

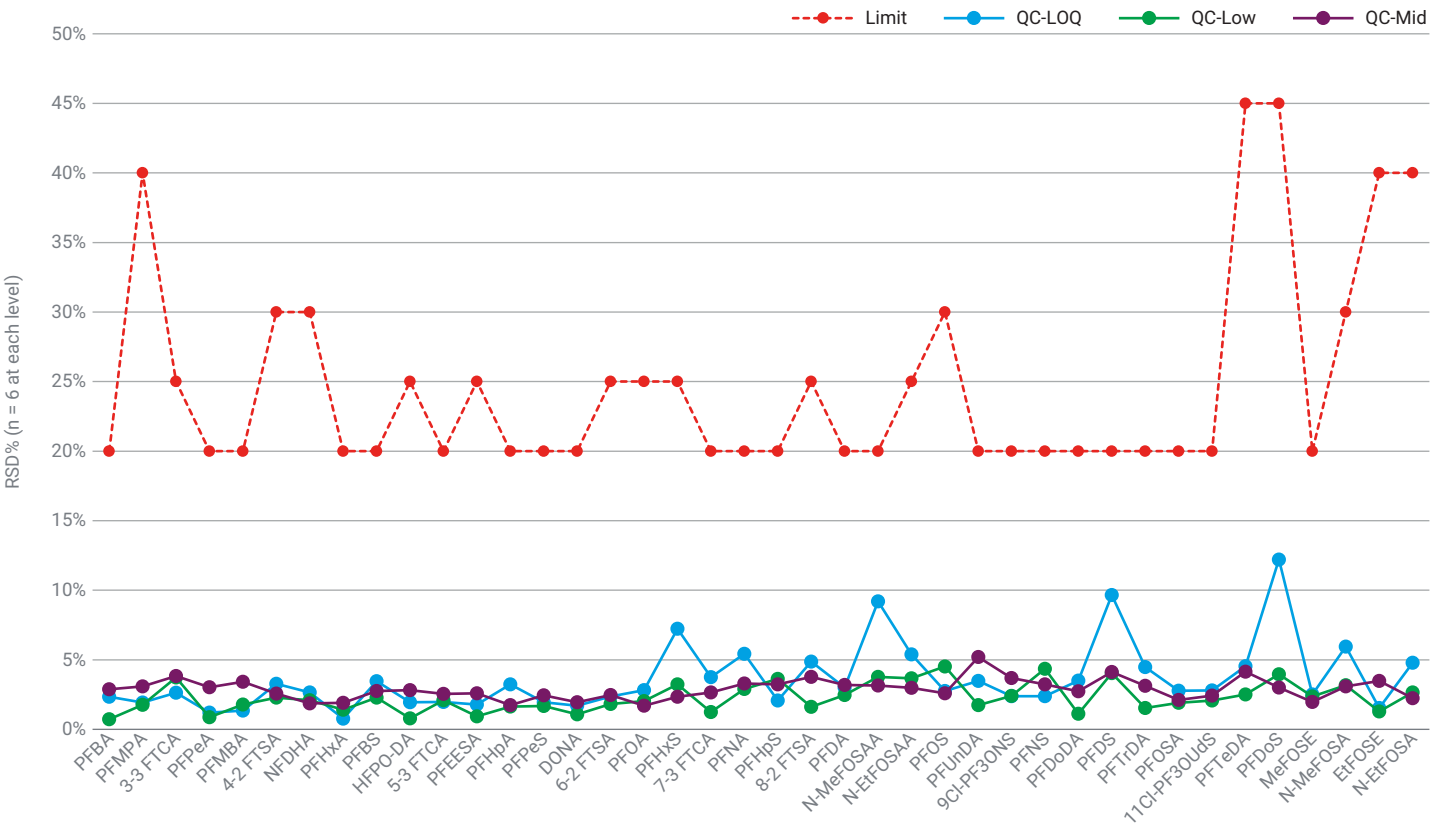

Figure 6. Quantitation precision (RSD%) results for analysis of 40 PFAS targets in tilapia at three spiking levels using the QuEChERS$_{ext}$-EMR method.

Method performance comparison

In addition to comparing the sample preparation procedure in the QuEChERS$_{ext}$-EMR method with the traditional SPE-based methods, which is discussed in previous sections, method performance was also compared. Given the complexity of native PFAS target comparison, the performance comparison is focused on the EIS results. There were seven NIS compounds, and they all delivered excellent recoveries between 92 and 101% with all three sample preparation methods. NIS results were thus not used for method comparison.

Figure 7 shows the comparison of EIS compound recovery in tilapia using different sample preparation methods. The EIS compound recovery is a critical indicator of sample preparation method performance. First, because they are isotopically labeled, their recovery is not affected by any PFAS present in the sample matrix. Second, the 24 EIS compounds are labeled analogs for seven of the nine PFAS target classes included in the EPA 1633 method, making them representative of the target analytes. Third, the seven NIS compounds only represent two PFAS classes, which limits their effectiveness in correcting for matrix effects. Therefore, EIS recoveries are indicative of the overall method performance.

The results shown in the figure were based on the four or six replicates of prespiked LOQ QC samples. Blue bars show the results from the QuEChERS$_{ext}$-EMR method, while orange bars show the results from the traditional Solvent$_{ext}$-Carbon/WAX SPE method, and green bars show the results from traditional Solvent$_{ext}$-Carbon dSPE-WAX SPE method. Overall, the developed method delivered 79 to 128% recoveries, while the traditional Solvent$_{ext}$-Carbon/WAX SPE method delivered 18 to 169% recoveries and the Solvent$_{ext}$-Carbon dSPE-WAX SPE method delivered 5 to 90% recoveries. This method clearly outperformed two traditional methods.

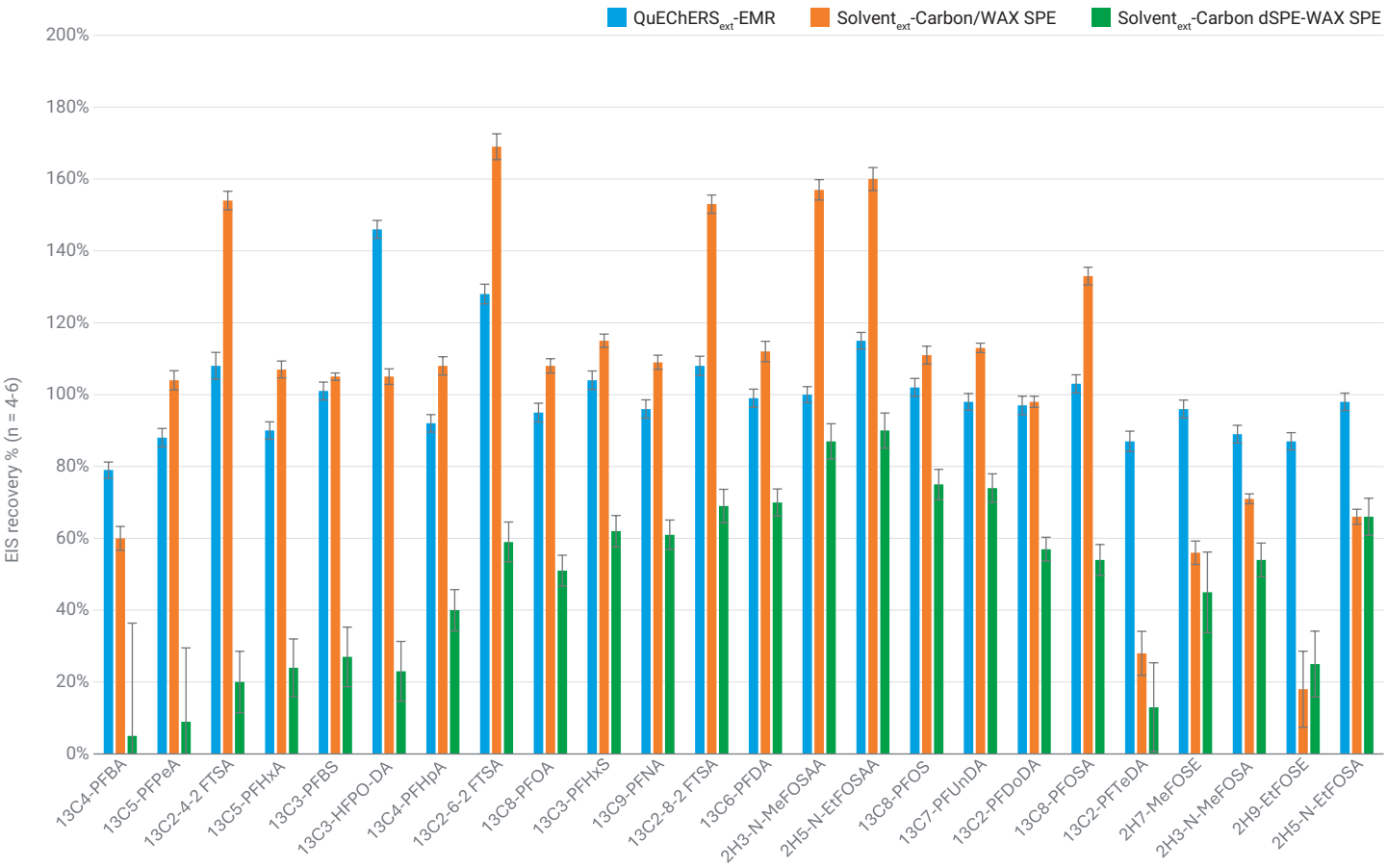

Figure 7. Sample preparation method comparison for EIS compound recovery in tilapia.

Conclusion

A simplified, rapid, and reliable method using QuEChERS extraction followed by Agilent Captiva EMR PFAS Food II passthrough cleanup was developed and validated for 40 PFAS targets in fish tissue (tilapia) by LC/MS/MS under EPA 1633 guidelines. When comparing to traditional EPA 1633 SPE-based sample preparation methods, the newly developed method saved > 80% time, reduced solvent and chemical consumption by ~ 80%, and uses fewer consumables. The method was validated following the EPA 1633 guideline, providing acceptable EIS and NIS recoveries and native PFAS acceptable quantitation accuracy and precision. The MDL and LOQ levels for all targets using this method are lower than those reported by the EPA 1633 method. The method described here demonstrates an alternative solution with improved performance, efficiency, and cost compared to traditional EPA 1633 method sample preparation for PFAS analysis in tissue samples.

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Appendix Table 1. Concentrations of native PFAS, EIS, and NIS compounds in the stock mix solutions.

Native PFAS Compound	Concentration (ng/mL)	EIS Compound	Concentration (ng/mL)
PFHxA	250	$^{13}C_4$-PFBA	2,000
PFHpA	250	$^{13}C_5$-PFPeA	1,000
PFOA isomers	250	$^{13}C_5$-PFHxA	500
PFNA isomers	250	$^{13}C_4$-PFHpA	500
PFDA	250	$^{13}C_8$-PFOA	500
PFUnA	250	$^{13}C_9$-PFNA	250
PFDoA	250	$^{13}C_6$-PFDA	250
PFTrDA	250	$^{13}C_7$-PFUdA	250
PFTeDA	250	$^{13}C_2$-PFDoA	250
PFOSA isomers	250	$^{13}C_2$-PFTeDA	250
N-MeFOSA isomers	250	$^{13}C_8$-PFOSA	500
N-EtFOSA isomers	250	D_3-N-MeFOSA	500
N-MeFOSAA isomers	250	D_5-N-EtFOSA	500
N-EtFOSAA isomers	250	D_3-N-MeFOSAA	1,000
PFBS	250	D_5-N-EtFOSAA	1,000
PFPeS	250	D_7-N-MeFOSE	5,000
PFHxS isomers	250	D_9-N-EtFOSE	5,000
PFHpS	250	$^{13}C_2$-HFPO-DA	2,000
PFOS isomers	250	$^{13}C_3$-PFBS	500
PFNS	250	$^{13}C_3$-PFHxS	500
PFDS	250	$^{13}C_8$-PFOS	500
PFDoS	250	$^{13}C_2$-4:2 FTS	1,000
PFPeA	500	$^{13}C_2$-6:2 FTS	1,000
PFEESA	500	$^{13}C_2$-8:2 FTS	1,000
PFMPA	500	**NIS Compound**	**Concentration (ng/mL)**
PFMBA	500	$^{13}C_3$-PFBA	1,000
NFDHA	500	$^{13}C_2$-PFHxA	500
PFBA	1,000	$^{13}C_4$-PFOA	500
HFPO-DA	1,000	$^{13}C_5$-PFNA	250
4:2 FTS	1,000	$^{13}C_2$-PFDA	250
6:2 FTS	1,000	$^{18}O_2$-PFHxS	500
8:2 FTS	1,000	$^{13}C_4$-PFOS	500
ADONA	1,000		
9Cl-PF3ONS	1,000		
11Cl-PF3OUdS	1,000		
N-MeFOSE isomers	2,500		
N-EtFOSE isomers	2,500		
3:3 FTCA	1,250		
5:3 FTCA	6,250		
7:3 FTCA	6,250		

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