Application Note

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A Fully Automated Workflow for PFAS Analysis in Seafood for Regulatory Screening

PFAS quantitation using CTC PAL3 with 6495D LC/TQ

Suitable for Agilent
1290 Infinity III LC

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Abstract

This application note details a fully automated workflow for the quantitative analysis of per- and polyfluoroalkyl substances (PFAS) in seafood using a CTC PAL3 Series 2 RTC autosampler and an Agilent 6495D triple quadrupole LC/MS system. The workflow demonstrates high precision and accuracy in complex analytical tasks, including calibration preparation, QuEChERS salting out-assisted solvent extraction, and micro-SPE cleanup. Linearity, method sensitivity, matrix-spiked quality control (QC) recovery, and method reproducibility were evaluated for 73 PFAS analytes. The analytical performance for critical PFAS and other regulated compounds met the validated performance from the United States Food and Drug Administration (U.S. FDA), and the requirements from European Union (EU), European Union Reference Laboratory for Halogenated Persistent Organic Pollutants in Feed and Food (EURL POPs), and the Association of Official Analytical Chemists (AOAC). A method detection limit (MDL) of ≤ 10 ng/kg (ppt) was achieved for all 28 mandatory targets from the U.S. FDA. Validated limits of quantitation (LOQ_{vali}) of ≤ 0.3 µg/kg (ppb) with $\%RSD_R$ ≤ 12 were obtained for 30 regulated PFAS, except for 6:2 FTSA, which had an LOQ_{vali} of 1.0 µg/kg. The LOQs for all mandatory analytes meet regulatory requirements/recommendations. These results underscore the robustness and efficiency of the PAL3 Series 2 RTC autosampler and the 6495D triple quadrupole LC/MS in providing reliable data for PFAS monitoring in seafood, and safeguarding public health.

Introduction

PFAS are synthetic chemicals that are widely used in various industrial and consumer products due to their resistance to heat, water, and oil. These properties contribute to their persistence in the environment, where they can accumulate in aquatic ecosystems and contaminate marine life. Seafood, such as fish and shellfish, can therefore absorb PFAS, leading to potential human exposure through consumption. Studies have detected PFAS in various seafood items, including clams, cod, crab, pollock, salmon, shrimp, tilapia, and tuna.[1] Surveys by the FDA have found detectable levels of PFAS in a significant percentage of seafood samples.

The U.S. FDA, the European Food Safety Authority (EFSA), EURL POPs, and AOAC are actively involved in discovering the extent of PFAS contamination in seafood to establish guidelines to protect public health.[2-6] For instance, EU 2022/1431, EURL POPs, and AOAC have set the required or recommended limits of quantitation (LOQs) at 0.3 µg/kg for four individual PFAS (PFOS, PFOA, PFNA, and PFHxS) in seafood matrices.[4-6] The U.S. FDA has also published validation data for MDLs at the parts per trillion (ppt) level for all 28 regulated PFAS in seafood.[2]

Detecting trace levels of PFAS in food, particularly seafood, poses significant challenges due to the complexity of the matrices. PFAS analysis typically involves QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction followed by solid-phase extraction (SPE) cleanup. SPE is a widely used protocol due to its efficiency in extracting a broad range of analytes from food matrices.[2,7] However, these manual steps can be labor-intensive and prone to errors, impacting the accuracy and reliability of results. Skilled analysts are required to perform these tedious extractions and operate the instruments. Variation in skill level can also lead to inconsistent results, reducing the reliability and repeatability of PFAS analysis, especially when high precision at trace levels is needed.

This study discusses a fully automated workflow for the quantitative analysis of PFAS in seafood using a PAL3 Series 2 RTC autosampler and 6495D LC/TQ. Solvent extraction followed by QuEChERS salting-out and micro-SPE cartridge cleanup was automatically performed by the PAL3 platform, while data analysis was conducted on the LC/TQ in parallel mode. The method performance was thoroughly evaluated based on EU 2023/915, EU 2022/1431, EURL POPs, the U.S. FDA, and AOAC SMPR 2023.003.

Experimental

Chemicals and reagents

The methanol (MeOH) and ammonium acetate used for this study were LC/MS grade and purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Ultrapure LC/MS acetonitrile (ACN) and water (H_2O) were used from Agilent (part numbers 5191-4496 and 5191-4498).

Native and isotopically labeled PFAS standards were sourced from Wellington Laboratories Inc. (Guelph, ON, Canada) and Toronto Research Chemicals (Toronto, ON, Canada) as stock solutions, solution mixes, or powdered standards.

Consumables

Consumables are crucial for trace-level analysis of PFAS, as their composition can greatly impact background levels and contribute to false positive high results for the targets. All consumables used in this work were therefore tested and verified for their suitability in PFAS analysis to deliver ultralow background.[8] The following consumables were used:

- Agilent QuEChERS extraction salt packets, EN 15662 method (part number 5982-6650)
- Agilent micro-SPE cartridges (part number G6074-67013)
- Vial, screw top, 20 mL (part number 5188-2753)
- Vial, screw top, 10 mL (part number 5188-5392)
- Screw cap for 20/10 mL vial, magnetic (part number 5188-2759)
- Agilent polyfluorinated compound (PFC)-free polypropylene vials, 2 mL (part number 5191-8150)
- Agilent cap, screw style, bonded, magnetic (part number 5191-8160)
- Agilent polypropylene vials and caps, 250 µL (part numbers 5190-2242 and 5191-8151)
- Preslit cap (part number 5183-2076)
- Agilent ZORBAX Rapid Resolution High Definition Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm, part number 959758-902)

Instrumentation

An integrated PAL3 Series 2 RTC autosampler coupled with a 6495D LC/TQ (Figure 1) was used for the fully automated workflow of PFAS quantitation from seafood matrix in this study.

A 160 cm PAL3 Series 2 RTC autosampler was used as an automated liquid handling platform for preparing calibration standards, sample extraction, and for performing injections onto the LC/TQ system. The PAL3 platform was equipped with various tools and modules, providing the necessary capabilities to achieve its designated functions. The following tools and modules were used in this study:

- Two PAL Park Stations with three Liquid Syringe Tools, Dilutor Tool, micro-SPE Tool, and LC/MS Tool
- Vortex Mixer
- Centrifuge
- Dilutor Multi
- Tray Cooler (for 2/10/20 mL vials)
- Tray Holders with Rack R60 (for 10/20 mL vials)
- Micro-SPE Tray (for 2 mL vials and micro-SPE cartridges)
- Solvent Module and Fast Wash Module
- LC Injection Valve

The LC Injection Valve was configured on the PAL3 platform, and all liquid syringes were cleaned using a Fast Wash Module. All solvent tubing used in the PAL3 platform was PFAS-free. Extra modules and tools can be added to meet specific sample preparation needs.

Chromatographic separation was achieved using a ZORBAX RRHD Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm) installed on an Agilent 1290 Infinity II UHPLC system. This system consisted of the following two modules (Figure 1):

- Agilent 1290 Infinity II high-speed pump (part number G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (part number G7116B)

An Agilent 1290 Infinity II Multisampler (part number G7167B) was not required for this work, as the sample handling and injection were carried out by the PAL3 platform. To minimize background PFAS contamination from the LC flow path and mobile phases, an Agilent InfinityLab PFC-free HPLC conversion kit (part number 5004-0006) was installed on the UHPLC system.[9] This kit includes PFC-free bottle head assemblies, a pump head adapter, an inline filter, multiwash tubing, and a delay column. A 12-minute gradient elution, as outlined in the Agilent PFAS MRM Database for LC/TQ (part number G1736AA), was used with 5 mM ammonium acetate in water as mobile phase A and 100% methanol as mobile phase B at a flow rate of 0.4 mL/min.

Figure 1. CTC PAL3 Series 2 RTC autosampler with an Agilent 6495D triple quadrupole LC/MS.

A 6495D LC/TQ equipped with an Agilent Jet Stream Technology ion source (AJS) was used for target acquisition in negative ionization mode. Autotuning was performed in Standard Quadrupole mode to optimize instrument parameters. The operating conditions and parameters are listed in Table 1. The integrated PAL3 Series 2 RTC autosampler and 6495D LC/TQ was operated using Agilent MassHunter acquisition software for LC/MS systems, version 12.1 update 3. Data analysis was conducted using Quantitative Analysis software, version 12.1.

Table 1. Instrument operating conditions and MS source parameters.

CTC PAL3 Series 2 RTC Autosampler and Agilent 1290 Infinity II LC Conditions	
Analytical Column	Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm, 1,200 bar pressure limit (p/n 959758-902)
UHPLC Guard	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm, 1.8 µm, 1,200 bar pressure limit, UHPLC guard, (p/n 821725-901)
Column Temperature	55 °C
Injection Volume	10 µL
PAL Tray Cooler Temperature	5 °C
Mobile Phase A	5 mM Ammonium acetate in water
Mobile Phase B	100% Methanol
Mobile Phase Flow Rate	0.4 mL/min
Timetable	Time (min) %A %B 0.00 85 15 1.00 85 15 1.50 45 55 5.50 30 70 7.00 20 80 12.00 0 100 14.40 0 100 14.50 85 15
Stop Time	14.5 min
Post Time	2.5 min
PAL Injection Needle Wash	Multiwash
Wash Solvent 1 (S1)	15:85 Methanol:water
Wash Solvent 2 (S2)	1:1 Acetonitrile:2-propanol
Agilent 6495D Triple Quadrupole LC/MS System Parameters	
Ion Source	AJS ESI
iFunnel Mode	Standard
Polarity	Negative
Q1 and Q3 Resolution	Unit
Cycle Time	720 ms
Gas Temperature	250 °C
Gas Flow	11 L/min
Nebulizer	25 psi
Sheath Gas Temperature	375 °C
Sheath Gas Flow	11 L/min
Capillary (Negative)	3,000 V
Nozzle Voltage	0 V

End-to-end automation procedure using PAL3 Series 2 RTC autosampler and 6495D LC/TQ

The fully automated workflow was developed for PFAS quantitation in shrimp matrix, encompassing calibration preparation and analysis, followed by sample preparation and analysis.

Automated calibration preparation: A total of 12 calibration levels were automatically prepared by the PAL3 platform for this study. Three individual intermediate stock solutions were manually prepared: a mix of 73 analytes, a mix of 34 surrogates, and a mix of three internal standards (ISTDs), all in a solvent mixture of ACN:MeOH:H_2O (60:15:25, v:v:v). This solvent mixture was also used as the diluent for the experimental work. Submixes A, B, C, and D were prepared from the intermediate stock solution of analytes by the PAL3 platform in serial dilution. The 12 calibration standards were then prepared from different submixes, with constant amounts of surrogates and ISTDs spiked into each level. A calibration blank was prepared by adding surrogates and ISTDs to the solvent mixture only. All stock solutions, submixes, and calibration standards were stored in the PAL Tray Cooler at 5 °C to maintain the stability of the PFAS compounds and prevent evaporation. Once the preparation of the calibration standards was completed, the worklist was automatically activated to run the full range of calibrators.

Automated sample preparation methodology: Seafood is one of the regulated matrices by the U.S. FDA, EU, EURL POPs, and AOAC. Shrimp, a typical crustacean seafood, was used as a sample to test the performance of automated extraction by the PAL3 platform (Figure 2). Solvent extraction followed by QuEChERS salting-out is a widely recognized methodology for PFAS analysis in food matrices.[2,7,10]

Fresh shrimp was purchased from a local grocery store, cut into small pieces, and frozen at –20 °C. The samples were then blended to obtain a fine powder before sample extraction. Approximately 4 ± 0.1 g of the fine shrimp sample was manually weighed into a 20 mL sample vial and placed into the PAL Tray Cooler, set at 5 °C. This maintained the shrimp samples in optimum conditions for further extraction.

As illustrated in Figure 2, the remaining steps were carried out by the PAL3 platform. A surrogates mix (used as extracted internal standards, EIS) was spiked into the 20 mL sample vial, followed by the spiking of PFAS targets if matrix-spiked QCs were needed.[2,11] In this study, QC samples with low, middle, and high analyte concentrations of 0.1 µg/kg (LSQ), 0.3 µg/kg (MSQ), and 1.0 µg/kg (HSQ) were prepared in duplicate technical preparations by the PAL3 platform following the entire automation workflow. A matrix blank (MB) was prepared without spiking target analytes. Using the PAL dilutor module, 8 mL of extraction solvent ACN:H_2O (50:50, v:v) was added into the sample vial and vortexed vigorously at 2,000 rpm in pulse mode. Next, the sample vial was centrifuged at 4,500 rpm for 3 minutes. One milliliter of supernatant was transferred to a 2 mL polypropylene vial with preweighed QuEChERS salt, followed by immediate vortexing. The 2 mL vial was then centrifuged. The micro-SPE cartridge was conditioned with 100 µL of ACN and drained by air blowing. Next, 150 µL of extract from the 2 mL polypropylene vial was taken and the micro-SPE cartridge was fixed on top of a 250 µL polypropylene injection vial (with a preslit cap). The 150 µL extract was carefully loaded through the micro-SPE cartridge at 5 µL/s and everything was eluted into an injection vial by air blowing. An appropriate amount of ISTDs (used as nonextracted internal standards, NIS) were spiked into the injection vial, which was then filled to a final volume of 250 µL with diluent. The mixture was vortexed and 10 µL was directly injected into the 6495D LC/TQ.

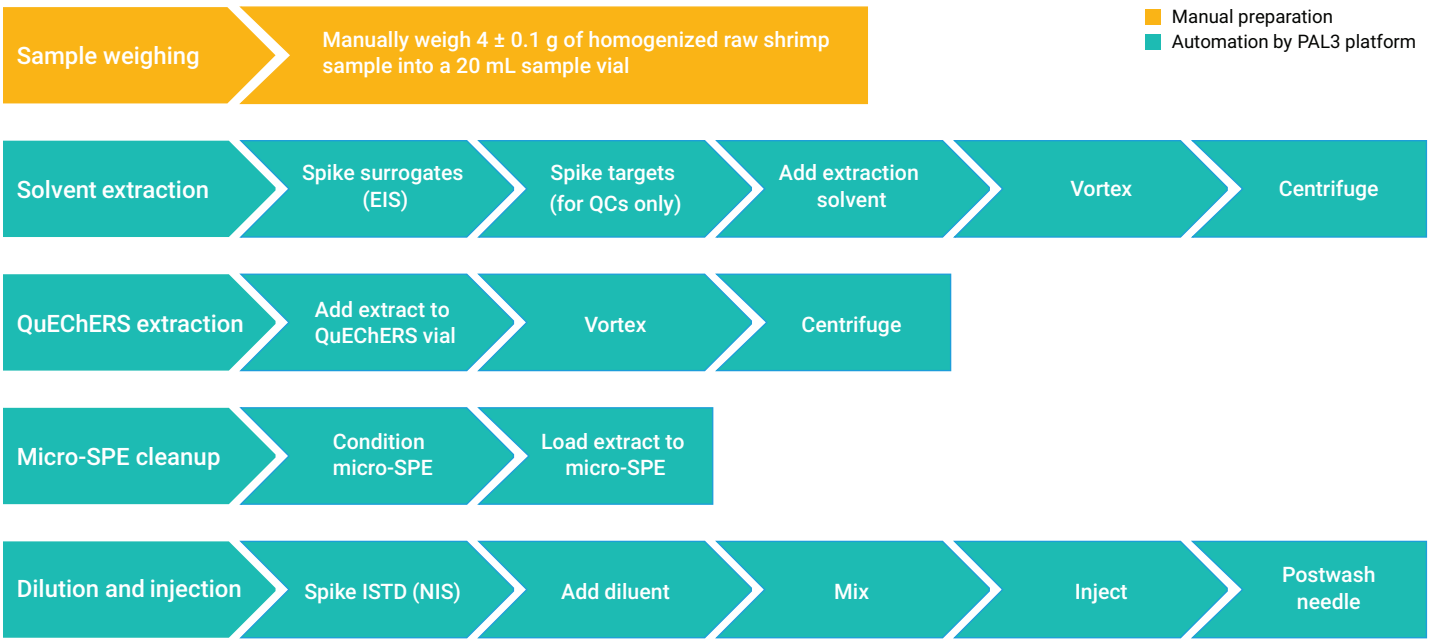

Figure 2. Automated sample preparation for shrimp matrix by the CTC PAL3 Series 2 RTC autosampler.

No further drying or reconstitution was required in this workflow, which significantly shortened the sample preparation time and eliminated target loss due to heating/evaporation. The LC/MS Tool on the PAL3 platform was automatically moved to the Fast Wash Module for postwash using S1 and S2 (as shown in Table 1). The PAL3 platform was then ready for the next cycle of sample preparation while the LC/TQ was acquiring data for the sample.

Online analysis sequence: The entire automated workflow was managed using MassHunter software, which facilitated the creation of an online analysis worklist batch, as shown in Figure 3. The routine worklist typically includes calibration standards, a reagent blank (RB), a matrix blank (MB), optional matrix-spiked QCs, and unknown samples 1 to n. The RB, also referred to as procedural blank, was prepared without a matrix in the sample vial to monitor the contamination throughout the extraction process.

As shown in Figure 3, the PAL3 platform began with the preparation of calibrators. Before calibration analysis, a solvent blank was injected and analyzed to monitor the quality of the solvent used and the background contamination. When the entire range of calibration analyses was completed, the PAL3 autosampler initiated RB preparation followed by TQ analysis. At the same time, the PAL3 platform continued with the sample preparation without affecting live TQ data acquisition. As a result, the integrated PAL3 platform and 6495D LC/TQ enabled parallel sample preparation and analysis, increasing overall lab productivity through automation and eliminating waiting time between runs.

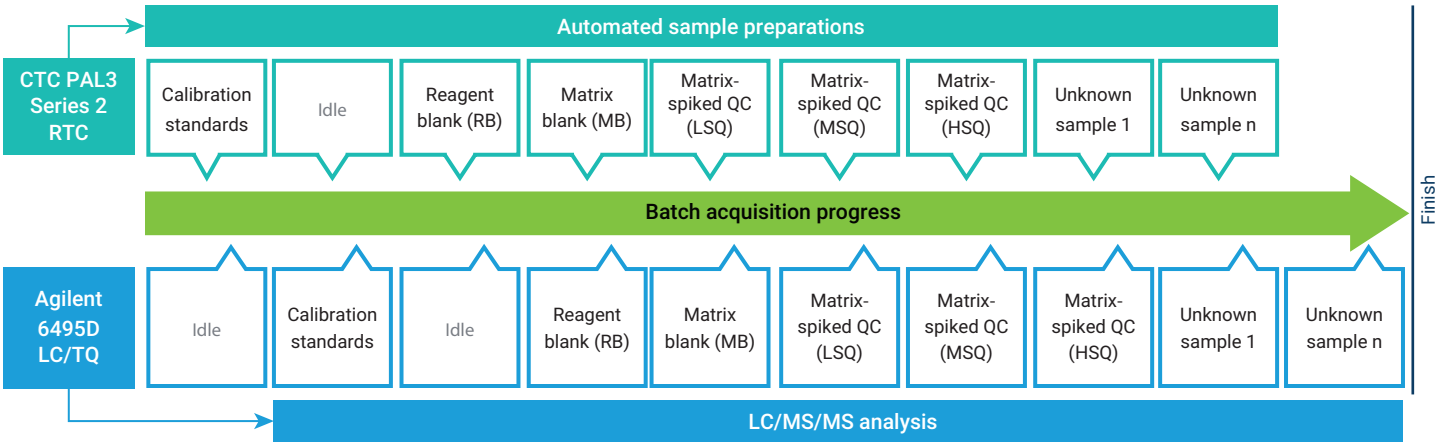

Figure 3. Online analysis sequence on the integrated CTC PAL3 Series 2 RTC autosampler and Agilent 6495D triple quadrupole LC/MS.

Results and discussion

Performance of automated calibrations

The performance of automated calibrations, ranging from 1 to 50,000 ng/L (ppt), was evaluated in terms of linearity, accuracy, and precision. The linearity for all 73 analytes met the stringent criterion of $R^2 \geq 0.99$ with a minimum of five calibration points (four points for 10:2 FTCA and 8:2 FTCA) (see Table 2). The surrogate recoveries across the linearity range were well within 70 to 130%, and the ISTD response RSD was ≤ 20%, demonstrating excellent accuracy and precision of calibrations prepared by the PAL3 platform.

Figure 4A shows the linearity of PFNA covering the full calibration range from levels 1 to 12. Figure 4B displays the MRM overlay of $^{13}C_9$-PFNA (surrogate for PFNA) from levels 1 to 12. Figure 4C displays the MRM overlay of $^{13}C_2$-PFOA (ISTD for $^{13}C_9$-PFNA surrogate) from levels 1 to 12. These figures indicate that the automated calibration preparation by the PAL3 platform was precise and accurate, ensuring the reliability and robustness of the calibration process. Moreover, the successful automation of this process eliminated tedious labor and reduced potential human error when handling complex analytical tasks; this improved throughput, and ensured consistent, accurate calibration.

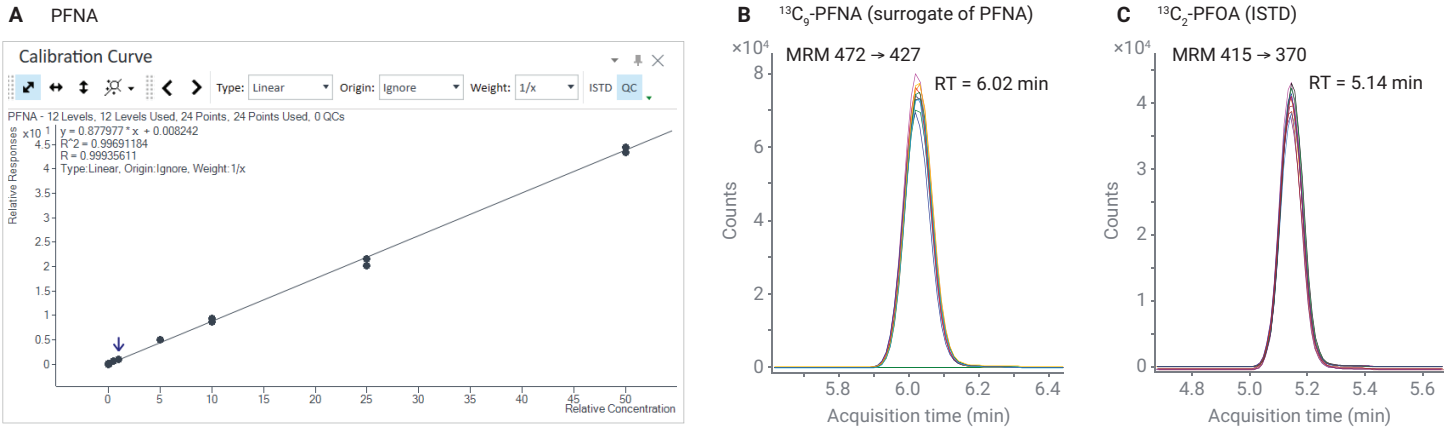

Figure 4. (A) The linearity of PFNA covering the full calibration range from levels 1 to 12; (B) the MRM overlay of $^{13}C_9$-PFNA (surrogate) from levels 1 to 12; (C) the MRM overlay of $^{13}C_2$-PFOA (ISTD) from levels 1 to 12.

Method sensitivity

The sensitivity of the automation workflow was evaluated in terms of MDL and LOQ. The analytical performance criteria for PFAS vary depending on different regulatory guidelines. According to the U.S. FDA method C-010.03, MDLs and LOQs were calculated by multiplying the standard deviation of replicated low-level QCs by factors 3.14 and 10, respectively.

In this study, calculated MDLs (MDL_{cal}) and calculated LOQs (LOQ_{cal}) were obtained from nine replicates of LSQs (except for 6:2 FTSA, which used HSQ) from three batches. Table 2 lists the values of MDL_{cal} and LOQ_{cal} for all analytes, while the highlighted compounds in blue were included in different regulations/guidelines. Overall, 86% and 95% of targets met $MDL_{cal} \leq 10$ ng/kg and $LOQ_{cal} \leq 50$ ng/kg, respectively.

Table 2. Summary of method linearity, MDL, LOQ, regulatory requirements/recommendations. The blue highlighted targets are currently listed in the U.S. FDA/EU/EURL POPs/AOAC.

No.	Compound Name	CAS Number	Surrogate	CF R^2	Method MDL_{cal} (ng/kg)	MDL U.S. FDA (ng/kg)	Method LOQ_{vali} (µg/kg)	LOQ EU 2023/915 (µg/kg)	Recommended LOQ EU 2022/1431 (µg/kg)	LOQ EURL POPs (µg/kg)	LOQ AOAC SMPR 2023.003 (µg/kg)
1	PFBPA	52299-24-8	Cl-PFOPA	0.993	ND	NA	ND	NA	NA	NA	NA
2	PFBA	375-22-4	$^{13}C_4$-PFBA	0.997	6	NA	0.1	NA	NA	NA	3.0
3	PFMPA	377-73-1	$^{13}C_4$-PFBA	0.995	7	NA	0.1	NA	NA	NA	NA
4	PFPeA	2706-90-3	$^{13}C_5$-PFPeA	0.994	10	NA	0.1	NA	NA	NA	3.0
5	3:3 FTCA	356-02-5	$^{13}C_5$-PFPeA	0.992	10	NA	0.1	NA	NA	NA	NA
6	PFBS	375-73-5	$^{13}C_3$-PFBS	0.996	6	6	0.1	NA	NA	NA	3.0
7	PFHxPA	40143-76-8	Cl-PFOPA	0.992	6	NA	0.3	NA	NA	NA	NA
8	PFMBA	863090-89-5	$^{13}C_5$-PFPeA	0.997	4	NA	0.1	NA	NA	NA	NA
9	Cl-PFHxPA	NA	Cl-PFOPA	0.995	7	NA	0.1	NA	NA	NA	NA
10	PFEESA	113507-82-7	$^{13}C_3$-PFBS	0.997	4	NA	0.3	NA	NA	NA	NA
11	NFDHA	151772-58-6	$^{13}C_5$-PFHxA	0.994	3	NA	0.1	NA	NA	NA	NA
12	4:2 FTSA	757124-72-4	$^{13}C_2$-4:2 FTSA	0.995	4	8	0.1	NA	NA	NA	3.0
13	PFHxA	307-24-4	$^{13}C_5$-PFHxA	0.997	5	32	0.1	NA	NA	NA	3.0
14	PFPeS	2706-91-4	$^{13}C_3$-PFHxS	0.997	8	17	0.1	NA	NA	NA	3.0
15	HFPO-DA	13252-13-6	$^{13}C_3$-HFPO-DA	0.991	8	10	0.1	NA	NA	NA	3.0
16	FBSA	30334-69-1	$^{13}C_3$-PFHxS	0.997	6	NA	0.3	NA	NA	NA	NA
17	P5MeODIOXOAc	1190931-41-9	$^{13}C_3$-HFPO-DA	0.991	13	NA	0.1	NA	NA	NA	NA
18	PFHpA	375-85-9	$^{13}C_4$-PFHpA	0.996	6	8	0.1	NA	NA	NA	3.0
19	PFHxS	355-46-4	$^{13}C_3$-PFHxS	0.996	7	9	0.3	1.5	0.3	0.3	0.3
20	DONA	919005-14-4	$^{13}C_4$-PFHpA	0.999	3	5	0.1	NA	NA	NA	3.0
21	PFOPA	40143-78-0	Cl-PFOPA	0.993	7	NA	0.3	NA	NA	NA	NA
22	5:3 FTCA	914637-49-3	$^{13}C_2$-6:2 FTUCA	0.991	4	NA	0.1	NA	NA	NA	NA
23	6:2 FTUCA	70887-88-6	$^{13}C_2$-6:2 FTUCA	0.991	5	NA	0.1	NA	NA	NA	NA
24	6:2 FTCA	53826-12-3	$^{13}C_2$-6:2 FTCA	0.993	ND	NA	ND	NA	NA	NA	NA
25	4-PFecHS	646-83-3	$^{13}C_8$-PFOS	0.995	6	NA	0.3	NA	NA	NA	NA
26	6:2 FTSA	27619-97-2	$^{13}C_2$-6:2 FTSA	0.995	4	25	1.0	NA	NA	NA	3.0
27	PFOA	335-67-1	$^{13}C_8$-PFOA	0.995	6	51	0.1	0.7	0.3	0.3	0.3
28	PFHpS	375-92-8	$^{13}C_8$-PFOS	0.995	4	19	0.1	NA	NA	NA	3.0
29	MeFBSA	68298-12-4	$^{13}C_8$-PFOSA	0.999	12	NA	0.1	NA	NA	NA	NA
30	FHxSA	41997-13-1	$^{13}C_8$-PFOS	0.996	4	NA	1.0	NA	NA	NA	NA
31	PFNA	375-95-1	$^{13}C_9$-PFNA	0.995	7	25	0.1	1.0	0.3	0.3	0.3
32	PFOS	1763-23-1	$^{13}C_8$-PFOS	0.995	5	14	0.1	3.0	0.3	0.3	0.3
33	8:2 FTUCA	70887-84-2	$^{13}C_2$-8:2 FTUCA	0.996	6	NA	0.1	NA	NA	NA	NA
34	PFDPA	52299-26-0	Cl-PFOPA	0.992	6	NA	0.3	NA	NA	NA	NA
35	7:3 FTCA	812-70-4	$^{13}C_2$-8:2 FTUCA	0.995	7	NA	0.1	NA	NA	NA	NA
36	HFPO-TA	13252-14-7	$^{13}C_9$-PFNA	0.994	8	NA	0.1	NA	NA	NA	NA

No.	Compound Name	CAS Number	Surrogate	CF R^2	Method MDL_{cal} (ng/kg)	MDL U.S. FDA (ng/kg)	Method LOQ_{vali} (µg/kg)	LOQ EU 2023/915 (µg/kg)	Recommended LOQ EU 2022/1431 (µg/kg)	LOQ EURL POPs (µg/kg)	LOQ AOAC SMPR 2023.003 (µg/kg)
37	8:2 FTCA	27854-31-5	$^{13}C_2$-8:2 FTCA	0.996	ND	NA	ND	NA	NA	NA	NA
38	9Cl-PF3ONS	756426-58-1	$^{13}C_8$-PFOS	0.994	6	7	0.1	NA	NA	NA	3.0
39	FOSAA	2806-24-8	$^{2}H_3$-N-MeFOSAA	0.991	6	NA	0.1	NA	NA	NA	NA
40	8:2 FTSA	39108-34-4	$^{13}C_2$-8:2 FTSA	0.995	5	14	0.1	NA	NA	NA	3.0
41	PFNS	68259-12-1	$^{13}C_8$-PFOS	0.991	5	5	0.1	NA	NA	NA	3.0
42	PFDA	335-76-2	$^{13}C_6$-PFDA	0.996	9	25	0.1	NA	NA	NA	3.0
43	8:3 FTCA	34598-33-9	$^{13}C_6$-PFDA	0.995	8	NA	0.1	NA	NA	NA	NA
44	N-MeFOSAA	2355-31-9	$^{2}H_3$-N-MeFOSAA	0.990	9	NA	0.1	NA	NA	NA	NA
45	MeFHxSA	68259-15-4	$^{13}C_8$-PFOSA	0.993	8	NA	0.1	NA	NA	NA	NA
46	PFDS	335-77-3	$^{13}C_8$-PFOS	0.990	7	9	0.1	NA	NA	NA	3.0
47	PFUnDA	2058-94-8	$^{13}C_7$-PFUnDA	0.991	9	26	0.1	NA	NA	NA	3.0
48	N-EtFOSAA	2991-50-6	$^{2}H_5$-N-EtFOSAA	0.996	4	NA	0.1	NA	NA	NA	NA
49	PFOSA	754-91-6	$^{13}C_8$-PFOSA	0.995	8	8	0.1	NA	NA	NA	3.0
50	10:2 FTUCA	70887-94-4	$^{13}C_2$-10:2 FTUCA	0.998	7	NA	0.1	NA	NA	NA	NA
51	11Cl-PF3OUdS	763051-92-9	$^{13}C_8$-PFOS	0.994	6	11	0.1	NA	NA	NA	3.0
52	PFUnDS	749786-16-1	$^{13}C_7$-PFUnDA	0.996	7	9	0.1	NA	NA	NA	3.0
53	PFDoDA	307-55-1	$^{13}C_2$-PFDoDA	0.992	10	17	0.1	NA	NA	NA	3.0
54	10:2 FTSA	120226-60-0	$^{13}C_2$-8:2 FTSA	0.993	10	12	0.1	NA	NA	NA	3.0
55	10:2 FTCA	53826-13-4	$^{13}C_2$-10:2 FTCA	0.991	ND	NA	ND	NA	NA	NA	NA
56	6:6 PFPi	40143-77-9	$^{13}C_2$-PFDoDA	0.995	9	NA	0.1	NA	NA	NA	NA
57	PFDoS	79780-39-5	$^{13}C_8$-PFOS	0.996	9	10	0.1	NA	NA	NA	3.0
58	PFTrDA	72629-94-8	$^{13}C_2$-PFDoDA	0.995	10	77	0.1	NA	NA	NA	3.0
59	N-MeFOSA	31506-32-8	$^{2}H_3$-N-MeFOSA	0.991	10	NA	0.1	NA	NA	NA	NA
60	FDSA	NA	$^{13}C_8$-PFOSA	0.992	4	NA	0.1	NA	NA	NA	NA
61	MeFOSE	24448-09-7	$^{2}H_7$-MeFOSE	0.996	11	NA	0.1	NA	NA	NA	NA
62	PFTrDS	791563-89-8	$^{13}C_2$-PFTDA	0.992	9	10	0.1	NA	NA	NA	3.0
63	6:2 diPAP	57677-95-9	$(^{13}C_2)_2$-6:2 diPAP	0.995	12	NA	0.1	NA	NA	NA	NA
64	PFTDA	376-06-7	$^{13}C_2$-PFTDA	0.993	9	17	0.1	NA	NA	NA	3.0
65	6:8 PFPi	610800-34-5	$(^{13}C_2)_2$-6:2 diPAP	0.995	12	NA	0.1	NA	NA	NA	NA
66	N-EtFOSA	4151-50-2	$^{2}H_5$-N-EtFOSA	0.994	10	NA	0.1	NA	NA	NA	NA
67	EtFOSE	1691-99-2	$^{2}H_9$-EtFOSE	0.991	16	NA	0.1	NA	NA	NA	NA
68	6:2/8:2 diPAP	943913-15-3	$(^{13}C_2)_2$-6:2 diPAP	0.995	6	NA	ND	NA	NA	NA	NA
69	8:8 PFPi	40143-79-1	$(^{13}C_2)_2$-6:2 diPAP	0.995	5	NA	0.1	NA	NA	NA	NA
70	PFHxDA	67905-19-5	$^{13}C_2$-PFHxDA	0.994	9	NA	0.1	NA	NA	NA	NA
71	8:2 diPAP	678-41-1	$(^{13}C_2)_2$-8:2 diPAP	0.996	5	NA	0.1	NA	NA	NA	NA
72	PFODA	16517-11-6	$^{13}C_2$-PFHxDA	0.993	5	NA	0.1	NA	NA	NA	NA
73	diSAmPAP	2965-52-8	$(^{13}C_2)_2$-8:2 diPAP	0.997	10	NA	ND	NA	NA	NA	NA

NA: Not available from the regulations/guidelines
ND: Not determined from the method

For the 28 regulated PFAS analytes from U.S. FDA, MDL_{cal} ≤ 10 ng/kg (Figure 5A) and LOQ_{cal} ≤ 35 ng/kg (Figure 5B) were achieved. These numbers were lower than or equivalent to the validation data for the crustacean matrix category.[2] The results confirmed that the automated workflow developed for the quantitative analysis of PFAS in shrimp matrix was highly sensitive and capable of achieving U.S. FDA-validated analytical performance.

According to the EU, EURL POPs, and AOAC, the required or recommended LOQ was established based on method validated LOQ (LOQ_{vali}) adhering to certain identification criteria.[5,6] In this study, the LOQ_{vali} was claimed based on the simultaneous fulfillment of the following identification criteria:

- Target recovery of 80 to 120% for PFOS, PFNA, PFOA, and PFHxS; 65 to 135% for other regulated PFAS
- Recovery %RSD of ≤ 20
- Intrabatch retention time (RT) tolerance of 1%
- Signal-to-noise ratio (S/N) of ≥ 3:1
- Ion ratio of quantifier and qualifier within ± 30%

The required or recommended LOQs across different regulations and guidelines may vary depending on the specific PFAS compound and the category of the sample matrix. An LOQ of ≤ 0.3 µg/kg was defined or recommended for four critical PFAS compounds (PFNA, PFOA, PFOS, and PFHxS) by EU, EURL POPs, and AOAC guidelines under the seafood category, including crustaceans and mollusks. Meanwhile, an LOQ of ≤ 3 µg/kg was required for the other 26 PFAS compounds, according to AOAC guidelines under the same matrix group. Method LOQ_{vali} for all 73 PFAS analytes are listed in Table 2, along with the LOQs from EU, EURL POPs, and AOAC for mandatory compounds.

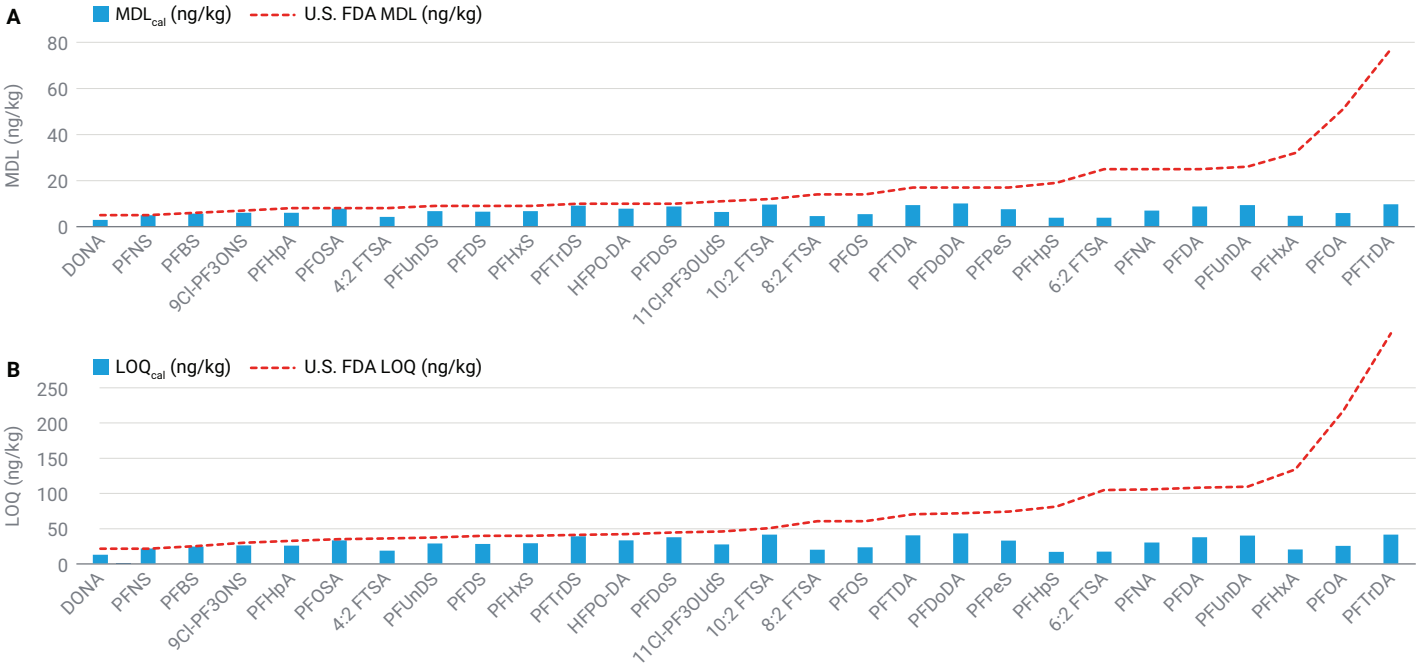

Figure 5. Sensitivity comparison for 28 mandatory PFAS targets from the U.S. FDA in terms of MDL (A) and LOQ (B).

As shown in Figures 6 and 7, the LOQ_{vali} met the requirements and recommendations from EU, EURL POPs, and AOAC for all regulated compounds. Among these targets, an LOQ_{vali} of 0.1 µg/kg was achieved for PFOS, PFNA, and PFHxS, which is below the regulated level. Due to the high positive residue of PFOA determined from the shrimp matrix blank, an LOQ_{vali} of 0.3 µg/kg was obtained for PFOA, meeting the required/recommended specifications exactly. For the remaining 26 mandatory targets from AOAC, the LOQ_{vali} was lower than the required LOQs (Figure 7).

Besides this, the method LOQ_{vali} was obtained from spiked QCs, based on micro-SPE cleanup followed by a dilute-and-shoot approach on the 6495D LC/TQ. This eliminated the drying and reconstitution steps and offered a fast and easy analytical protocol. All results confirmed that the integrated PAL3 Series 2 RTC autosampler and 6495D LC/TQ are highly capable of performing a sensitive, automated workflow for PFAS analysis in crustacean matrices, including sample preparation and target quantitation.

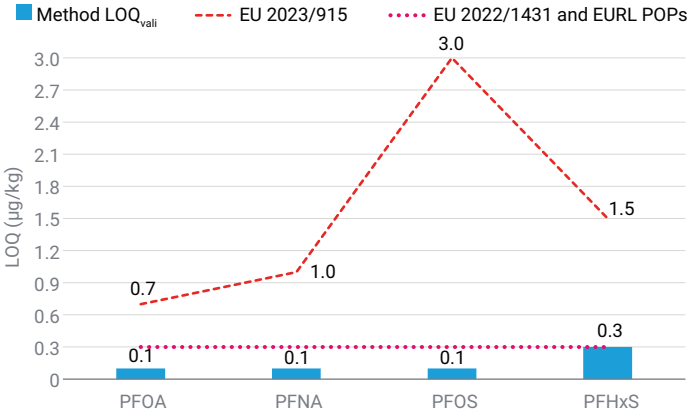

Figure 6. Method LOQ_{vali} versus LOQ requirements/recommendations for PFOA, PFNA, PFOS, and PFHxS from EU and EURL POPs.

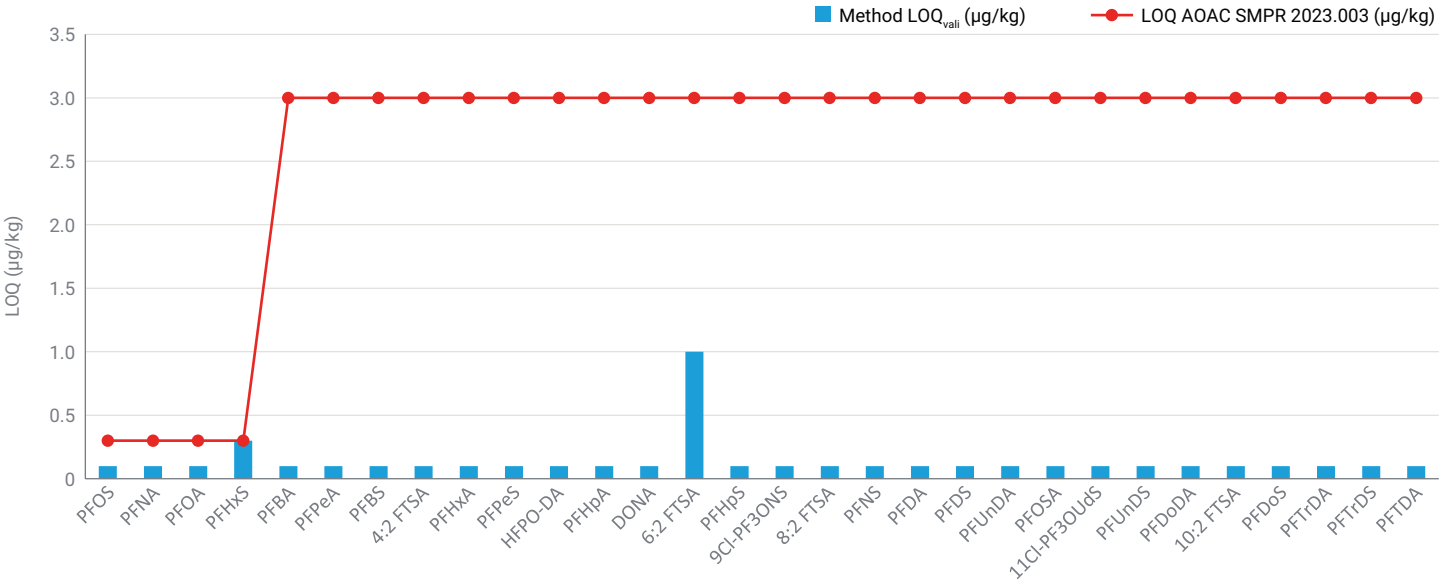

Figure 7. Method LOQ_{vali} versus LOQ requirements for 30 mandatory PFAS analytes from AOAC.

Matrix-spiked recovery

Matrix-spiked QC recovery was used to evaluate the automated sample extraction efficiency and accuracy for the target analytes in the shrimp sample matrix. Matrix-spiked QC samples were prepared by spiking a 73-PFAS analyte mix and 34-surrogate mix (EIS) using the PAL3 platform. The EPA 533 isotope performance standard mix, including three labeled PFAS ($^{13}C_3$-PFBA, $^{13}C_2$-PFOA, and $^{13}C_4$-PFOS) used as NIS, were postspiked into the final sample extract, as indicated in Figure 2. QC samples, including LSQ (0.1 µg/kg), MSQ (0.3 µg/kg), and HSQ (1.0 µg/kg), were prepared in duplicate technical preparations by the PAL3 platform following the entire automation workflow. The measured concentration of each analyte in QC samples was corrected by subtracting its presence (if above the MDL) in the unspiked shrimp blank sample. The method recovery for each QC was calculated based on the mean percent recovery (n = 6, three injections per technical preparation).

Recoveries of 65 to 135% were achieved for 79% of analytes at LSQ, 89% at MSQ, and 92% at HSQ. This demonstrates the high extraction efficiency and accuracy of the automation workflow for PFAS in shrimp samples. Figure 8 illustrates the recovery distribution of mandatory PFAS at MSQ, which was equivalent to or lower than the LOQ requirements and recommendations from the EU, EURL POPs, and AOAC. For the critical compounds PFOS, PFNA, PFOA, and PFHxS, MSQ recoveries were all well within the acceptable range of 80 to 120%. For the remaining 26 compounds from AOAC, recoveries ranged from 71 to 99%, meeting the guidelines/requirements of 65 to 135%. HSQ was used for 6:2 FTSA due to significant matrix interference from shrimp samples, which impacted the recoveries of LSQ and MSQ. However, 1.0 µg/kg of HSQ was still three times lower than the 3 µg/kg LOQ requirement for 6:2 FTSA from AOAC guidelines.

These recovery results confirm the outstanding performance of the automation workflow developed on the PAL3 Series 2 RTC autosampler and 6495D LC/TQ. This workflow offers superior sample preparation efficiency through solvent extraction, followed by the QuEChERS principle and micro-SPE cleanup, for PFAS analysis in complex seafood matrices. Significant matrix interference affected target integration, resulting in poor recovery (42 to 55%) for diSAmPAP in interbatch analysis, and recovery was not determined for PFAS compounds PFBPA, 10:2 FTCA, 8:2 FTCA, and 6:2 FTCA. Nevertheless, these compounds are currently not listed in any of the regulatory guidelines/methods discussed here.

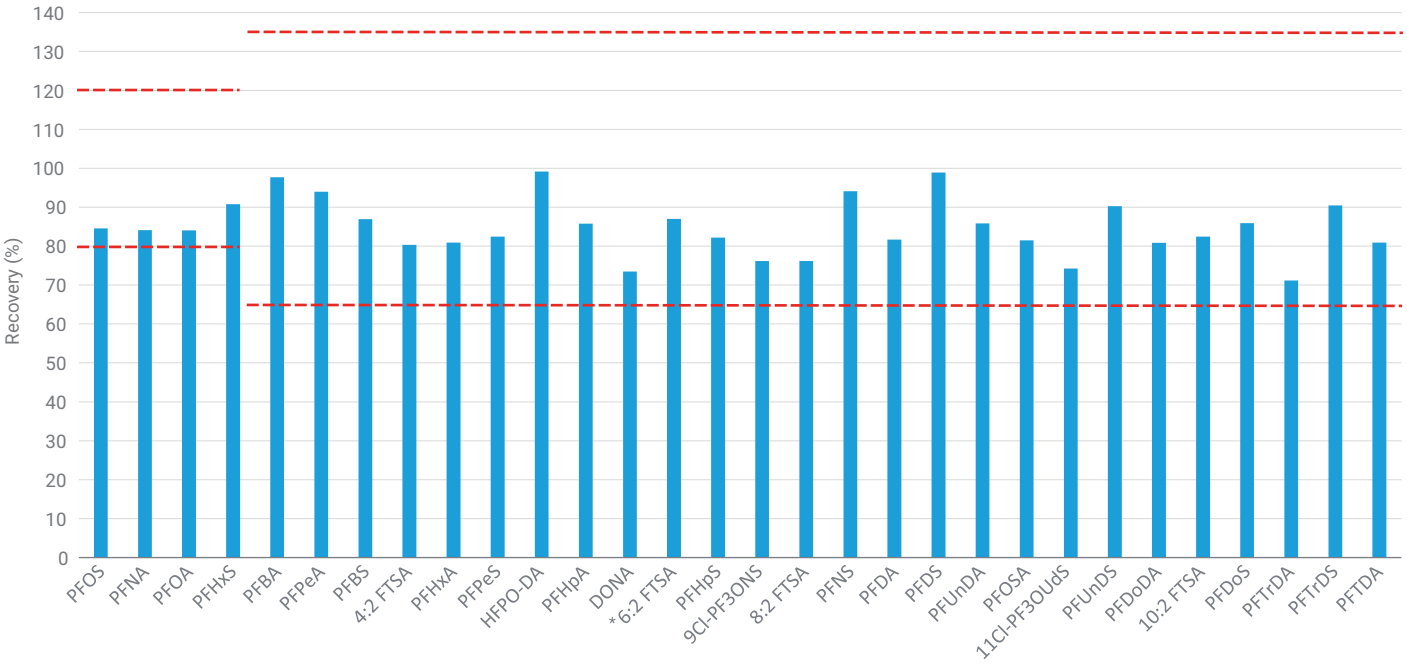

Figure 8. MSQ recovery distribution of mandatory PFAS from EU, EURL POPs, and AOAC (*recovery of HSQ for 6:2 FTSA was used). The recovery limit is marked using a red dotted line.

Method repeatability and reproducibility

The entire workflow repeatability (RSD_r) and reproducibility (RSD_R) were evaluated based on the relative standard deviation (RSD) of spiked QC recoveries from intra and interbatch analyses, respectively. RSD_r was calculated from triplicate injections of duplicate technical preparations (n = 6) within a batch for LSQ, MSQ, and HSQ. Overall, more than 93% of the targets achieved an RSD_r ≤ 19% across three QC levels, meeting the acceptance criteria of ≤ 20% from EURL POPs, U.S. FDA, and AOAC. An RSD_r of ≤ 12% was also achieved for the 30 regulated PFAS analytes from EU, EURL POPs, and AOAC. Figure 9 illustrates the MRM overlay of six injections from two technical preparations of (A) PFNA at LSQ, (B) PFOS at LSQ, and (C) PFOA at MSQ, which were equivalent to their LOQ_{vali} (shown in Table 2). The RSD_r of recoveries for PFNA, PFOS, and PFOA was even ≤ 3%, demonstrating excellent intrabatch repeatability over within-vial injection and between-vial preparations performed by the PAL3 Series 2 RTC autosampler and 6495D LC/TQ. Recovery repeatability was not available for PFBPA, 10:2 FTCA, 8:2 FTCA, and 6:2 FTCA due to undetermined recoveries from the shrimp matrix caused by significant matrix interference.

Interbatch recovery RSD_R was used to assess the day-to-day robustness of the developed automation workflow. RSD_R was obtained based on MSQ (except 6:2 FTSA, which used HSQ) and calculated from mean recoveries of three consecutive batches (n = 3) over different days. An RSD_R of ≤ 20% was achieved for 68 out of 73 targets (93%), demonstrating highly reliable analytical results for most PFAS analytes using this fully automated system. Meanwhile, the 30 regulated PFAS compounds achieved an RSD_R of ≤ 12% (Figure 10), underscoring the requirement of an RSD_R of ≤ 20% for four critical PFAS, according to EURL POPs, and ≤ 40% for all regulated PFAS per AOAC guidelines. These findings confirm that the automation workflow developed on the PAL3 Series 2 RTC autosampler and 6495D LC/TQ is reproducible, reliable, and robust when handling complex analytical tasks for PFAS analysis in challenging seafood matrices.

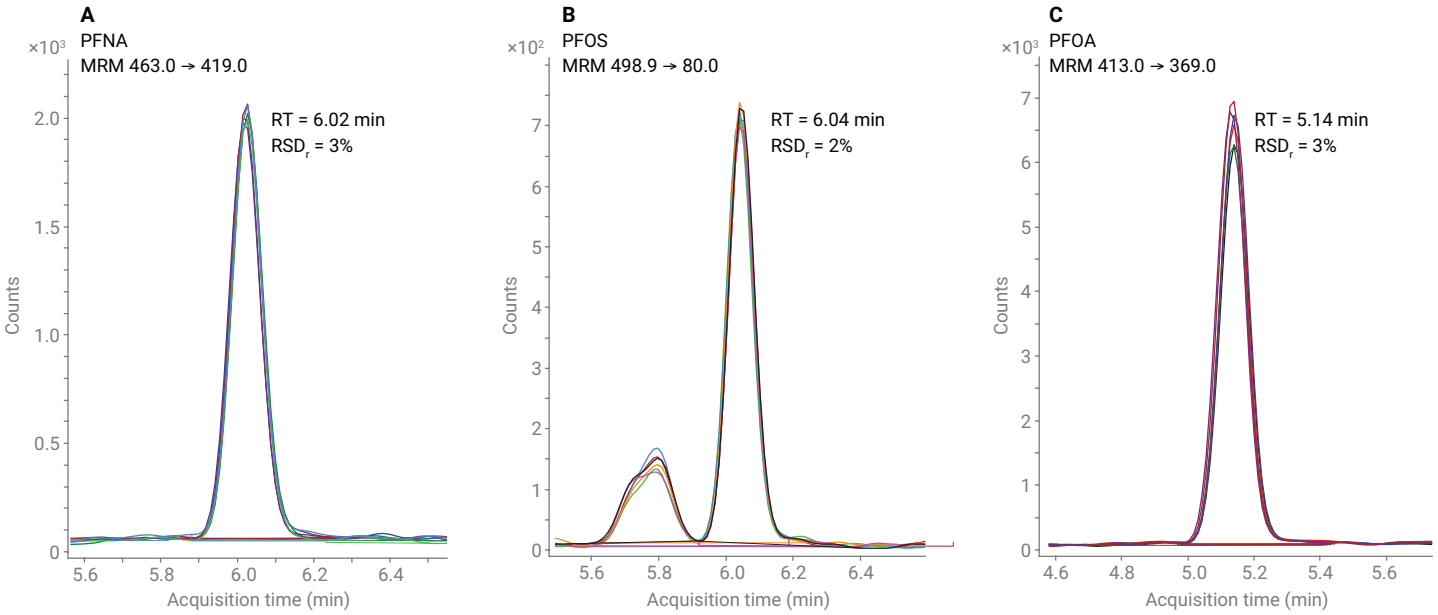

Figure 9. Six overlaid injections of MRM traces of (A) PFNA at LSQ, (B) PFOS at LSQ, and (C) PFOA at MSQ from two technical preparations within an analytical batch.

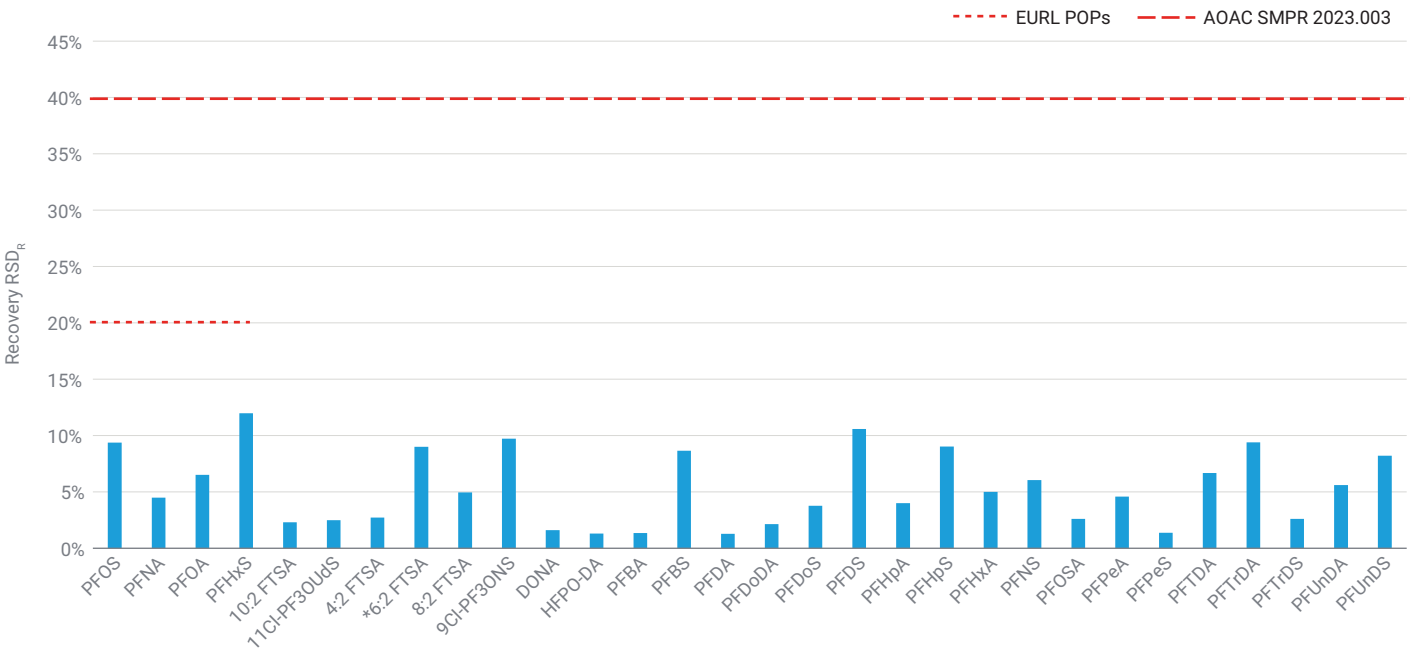

Figure 10. Interbatch recovery reproducibility (RSD_R) at MSQ (0.3 µg/kg) for the 30 regulated PFAS (*HSQ at 1.0 µg/kg was used to calculate RSD_R for 6:2 FTSA). The RSD_R limit is marked with a dotted red line.

Conclusion

In this study, the newly developed analytical method protocol provided a fully automated PFAS analysis using the integrated CTC PAL3 Series 2 RTC autosampler and 6495D LC/TQ. This approach transferred labor-intensive tasks to the robotic system, covering calibration preparation, sample extraction, and target analysis. The Agilent QuEChERS salting-out-assisted solvent extraction and micro-SPE cleanup were successfully executed by the PAL3 platform, eliminating tedious manual tasks in the sample preparation process. The method exhibited excellent linearity, sensitivity, accuracy, repeatability, and reproducibility, consistently meeting the stringent regulatory requirements and recommendations for PFAS in seafood matrices set out by the U.S. FDA, EU, EURL POPs, and AOAC. The exceptional performance across key analytical metrics even met the specific requirements for PFAS in other matrix categories such as eggs, coffee, fish oil, and feed. These results confirmed the robustness and reliability of the automated system in delivering high-quality analytical data.

The automated workflow significantly reduces manual intervention, which minimizes human error and enhances the precision of the analysis. The integrated system allows sample preparation and data analysis to run in parallel, offering a streamlined workflow and improving productivity for routine laboratory operations. Also, the integration of advanced automated sample preparation techniques with the highly sensitive 6495D LC/TQ ensures consistent and reproducible results, which are critical for meeting the regulatory requirements.

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