Application Note

Automated SPE Extraction and UPLC-MS/MS Analysis PFAS in Milk

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Abstract

The following work describes an automation workflow for the solid phase extraction of per- and polyfluoroalkyl substances (PFAS) in milk samples using the Andrew+™ Pipetting Robot and the Extraction+ connected device which saves analyst time, reduces experimental errors, and improves reproducibility across users. The total PFAS workflow solution including the enhanced sensitivity of the ACQUITY™ Premier UPLC™ System and Xevo™ TQ Absolute MS System, along with automated workflow using the Andrew+ Extraction+ robot and Oasis™ GCB/WAX SPE gave excellent recoveries of 98–110% for PFOA, PFOS, PFNA and PFHxS, and the LOQ was validated at 0.005 µg/kg in milk which meets EU Commission Recommendation 2022/1431.

Benefits

- Simplified creation of automation protocols through OneLab for making of calibration standard solutions and PFAS extractions through SPE
- Unique features in Andrew+ Extraction+ robot to ensure that the automation workflow is suitable for PFAS extractions in food

- Automation of PFAS workflow saves analyst time, improves reproducibility and reduces errors across multiple operators
- Low limits of quantification (down to 0.005 µg/kg) in milk, adhering to EURL POP guidelines, and meeting EU 2022/1431

Introduction

PFAS are a group of man-made chemicals that have multiple fluorine atoms attached to an alkyl chain. Due to its unique properties such as high heat and chemical stability, ability to reduce surface tension of aqueous solutions, and surfactant and oleophobic nature, PFAS are used in many areas for various applications such as packaging, surfactants, and firefighting fluids. However, studies have shown potential links between PFAS exposure and detrimental health effects on humans and animals.[1] As such, there is a pressing need to reduce the usage and prevalence of PFAS in all materials, and one important area is to reduce PFAS in food. As PFAS compounds are resistant to degradation and bioaccumulate in the tissues of mammals[2], they can be transferred from food products to humans through consumption. The European Food Safety Authority (EFSA) predicts food to be a predominant channel for PFAS exposure, and regulatory bodies around the world have been placing increasingly stringent regulations on the testing and monitoring of PFAS in food. Milk, being the staple food product for toddlers and children, would affect them disproportionately compared to adults, and as such it has been argued that there is a need to monitor PFAS levels in milk matrices.[3]

The European Recommendation 2022/2388 has set maximum levels of PFAS of PFOS, PFOA, PFNA, and PFHxS in eggs, fish meat, crustaceans, bivalve molluscs, meat and offal.[4] As PFAS analysis in fruits, vegetables, milk and baby food require sensitive methods and is not achievable in many laboratories, the European commission has adopted European Recommendation 2022/1431 to monitor PFAS at indicative levels in fruits, vegetables, milk and baby food. In milk, the indicative levels are 0.020 µg/kg for PFOS, 0.010 µg/kg for PFOA, 0.050 µg/kg for PFNA and 0.060 µg/kg for PFHxS.[5] Preparing milk samples for PFAS analysis using LC-MS/MS requires many pipetting tasks and is time-consuming. Therefore, it is desirable to automate as many of the parts of the workflow to improve throughput and reduce human experimental errors. Recent methods have established optimized analytical methods for the quantification of PFAS in fish, vegetable, fruit and baby food as per European Commission recommendations.[6,7] Solid-phase extraction (SPE) with dual-phase graphitized carbon black (GCB) and weak anion exchange (WAX) was used to extract and concentrate PFAS in food extracts and these sample clean-up steps are amenable to automation. The Andrew+ pipetting Robot, along with the Extraction+ connected device has also been used to automate bioanalytical solid phase extraction sample preparation.[8] The focus of this study is to build on previous work for quantifying PFAS in food matrices by incorporating automation with the Andrew+ pipetting Robot coupled with an Extraction+ vacuum manifold and vacuum pump, with a focus on milk samples.

Experimental

Standards and Solutions

All standards were purchased from Wellington Laboratories. Standards in Table 1 were used to prepare stock solutions.

Name of commercial standard	Purpose
PFAC30PAR	Native standard mix
L-PFUdS	Native standard
L-PFDoS	Native standard
L-PFTrDS	Native standard
M3-HFPODA	Extraction internal standard mix
MPFAC-24ES	Extraction internal standard mix
MPFAC-C-IS	Injection internal standard mix

Table 1. PFAS standards.

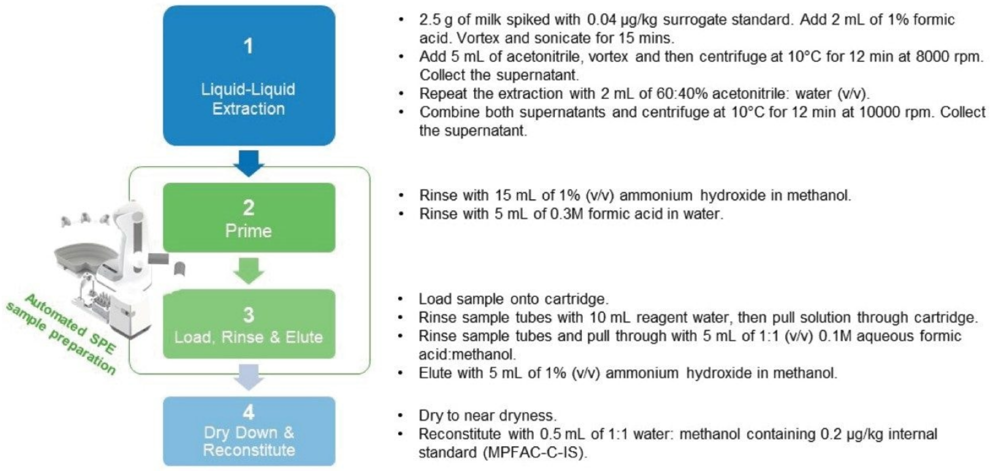
A solvent calibration curve in the range of 0.00125–5 ng/mL (equivalent to 0.00025–1 µg/kg in actual sample) was prepared and used for sample analysis.

Sample Preparation

Sample preparation was carried out as shown in Figure 2. Liquid-liquid extraction of milk samples were carried out as per *Rawn et. al.* with slight modifications.[9] 2.5 g of milk samples were placed in a 15 mL centrifuge tube and spiked with EIS at a concentration of 0.04 µg/kg. 2 mL of 1% formic acid (v/v) was added, and the mixture vortexed for a few seconds, followed by sonication for 15 mins. 5 mL of acetonitrile was added, and the mixture vortexed for a few seconds followed by centrifugation at 10 °C for 12 min at 8000 rpm, and the supernatant collected. Repeat the extraction with 2 mL of 60:40% acetonitrile: water (v/v). Combine both supernatants and centrifuge at 10°C for 12 min at 10000 rpm. Collect the supernatant.

Oasis GCB/WAX cartridges were used for sample clean-up. Cartridges were primed with 15 mL of 1% methanolic ammonium hydroxide followed by 5 mL of 0.3 M formic acid in water. Milk extract was then loaded onto the cartridge. The cartridge was washed with 10 mL water and 5 mL of 1:1 0.1 M formic acid:methanol. Finally, analytes were eluted with 5 mL of 1% methanolic ammonium hydroxide, collecting the eluate in a clean 5 mL collection tube.

The eluent was evaporated to near dryness, and reconstituted with 0.5 mL of 1:1 water:methanol. Extracted internal standards were spiked into each vial at a concentration of 0.04 µg/kg. The analytical sequence of each batch was composed of two different sets for solvent calibrants (≥6 points per each curve, excluding blank) bracketing incurred, and fortified samples.



- 2.5 g of milk spiked with 0.04 µg/kg surrogate standard. Add 2 mL of 1% formic acid. Vortex and sonicate for 15 mins.
- Add 5 mL of acetonitrile, vortex and then centrifuge at 10°C for 12 min at 8000 rpm. Collect the supernatant.
- Repeat the extraction with 2 mL of 60:40% acetonitrile: water (v/v).
- Combine both supernatants and centrifuge at 10°C for 12 min at 10000 rpm. Collect the supernatant.

- Rinse with 15 mL of 1% (v/v) ammonium hydroxide in methanol.
- Rinse with 5 mL of 0.3M formic acid in water.

- Load sample onto cartridge.
- Rinse sample tubes with 10 mL reagent water, then pull solution through cartridge.
- Rinse sample tubes and pull through with 5 mL of 1:1 (v/v) 0.1M aqueous formic acid:methanol.
- Elute with 5 mL of 1% (v/v) ammonium hydroxide in methanol.

- Dry to near dryness.
- Reconstitute with 0.5 mL of 1:1 water: methanol containing 0.2 µg/kg internal standard (MPFAC-C-IS).

Figure 2. Workflow for the extraction of PFAS in milk.

LC Conditions

LC system:	ACQUITY™ Premier System with PFAS Analysis Kit

Vials:	Polypropylene autosampler vial (p/n: 186005230)
Analytical column:	ACQUITY Premier UPLC BEH™ C_{18}, 2.1 x 50 mm, 1.7 µm (p/n: 186009452)
Isolator column:	Atlantis™ Premier BEH C_{18} AX 2.1 x 50 mm, 5.0 µm (p/n: 186009407)
Column temperature:	35 °C
Sample temperature:	10 °C
Injection volume:	5 µL
Flow rate:	0.3 mL/min
Mobile phase A:	2 mM ammonium acetate in water
Mobile phase B:	2 mM ammonium acetate in methanol/acetonitrile 1/1 (v/v)

Time (min)	%A	%B	Curve
0.0	95	5	0
0.5	75	25	6
3.0	50	50	6
6.5	15	85	6
7.0	5	95	6
8.5	5	95	6
9.0	95	5	6
11.0	95	5	6

Table 2. LC gradient.

MS Conditions

MS system:	Xevo TQ Absolute
Ionization mode:	Electrospray -
Source temperature:	100 °C
Capillary voltage:	0.5 kV
Desolvation temperature:	350 °C
Desolvation flow rate:	900 L/hr
Cone flow rate:	150 L/hr

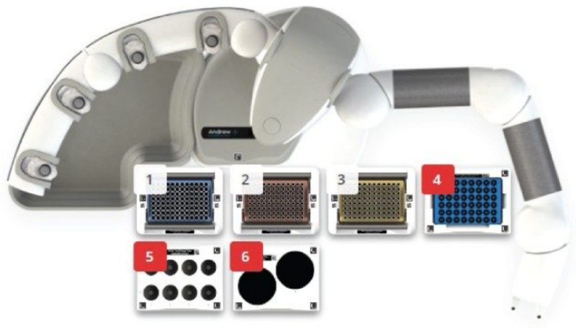
MRM transitions were as previously described by Dreolin *et. al.*[7]

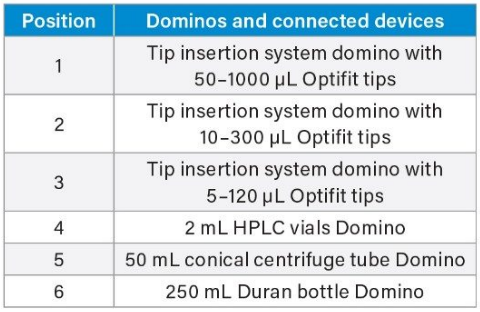
Data Management

waters_connect™ was used to acquire LC-MS/MS data and for processing.

Results and Discussion

Preparation of Solvent Standards for Calibration Curve with Andrew+ Pipetting Robot





Position	Dominos and connected devices
1	Tip insertion system domino with 50–1000 µL Optifit tips
2	Tip insertion system domino with 10–300 µL Optifit tips
3	Tip insertion system domino with 5–120 µL Optifit tips
4	2 mL HPLC vials Domino
5	50 mL conical centrifuge tube Domino
6	250 mL Duran bottle Domino

Figure 3. Andrew+ OneLab deck set up for preparation of solvent standards for calibration curve.

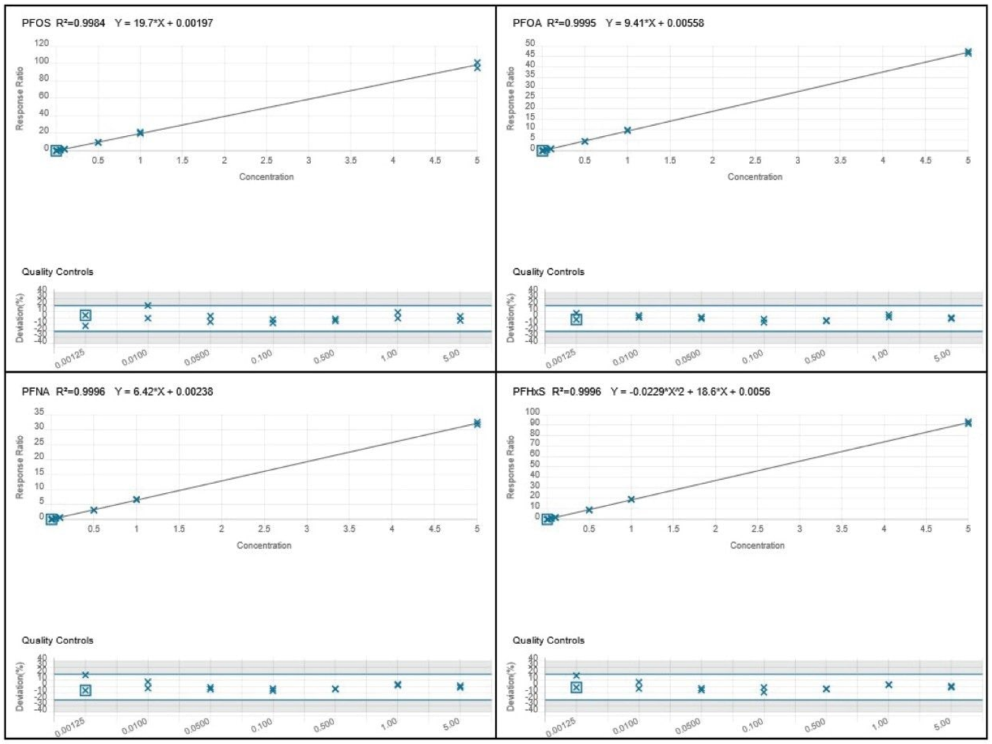


Figure 4. Calibration curves and residual plots for EU mandatory PFAS (PFOS, PFOA, PFNA, and PFHxS).

A OneLab protocol was developed to dilute stock solutions of native PFAS, and a mixture of surrogate and internal standards to create calibration standards, blanks, and QC samples.[10] Altogether 101 pipetting steps were automated saving approximately 1 hr 28 mins of laboratory time. Coefficients of determination (R^2) for all the calibration curves were >0.99 and residuals within ±20% for EU mandatory PFAS (PFOS, PFOA, PFNA, and PFHxS), and within ±35% for all other PFAS to be monitored, adhering to EURL POPs PFAS guidelines.[11]

Automated PFAS Extraction with Andrew+ Extraction+ Robot and Oasis GCB/WAX

Position	Dominos and connected devices
1,2	5000 µL Tip rack holder Domino
3	Collection Labware rack Domino with VWR® 5 µL snap-cap tube, racked
4	15 mL Conical centrifuge tube Domino
5	Extraction+ device with Oasis GCB/WAX 6 cc cartridges
6–8	50 mL conical centrifuge tube Domino
9	250 mL Duran bottle Domino

Figure 5. Andrew+ OneLab deck set up for automated PFAS extraction with Oasis WAX/GCB.

A OneLab protocol <https://onelab.andrewalliance.com/app/lab/YkaglE83/library/automation-of-pfas-samples-in-milk-matrices-using-spe-VbwYYOwZ> was developed to automate priming, loading, rinsing, and eluting of the sample from Oasis GCB/WAX cartridges.[10] 236 steps were automated, saving approximately 2 hours of laboratory time when 12 milk samples are prepared by SPE.

PFAS Contamination Minimization with Automated PFAS Extraction Workflow

Environmental PFAS contamination is minimized through the design of the extraction manifold, and automated steps programmed into the workflow. Eluent from Oasis GCB/WAX cartridges drip straight into collection tubes with no contact with surfaces, therefore minimizing surface transfer of PFAS contamination. In addition, methanol rinse steps for all pipette tips are programmed into the automated workflow which ensures that tips are clean of PFAS before pipetting solutions. A comparison between automated and manual extractions was done on process blanks and spiked water to evaluate the suitability of the Andrew+ Extraction+ robot for PFAS extractions (Figure 6). No notable differences were seen in process blanks and water spiked with 0.005 µg/kg native PFAS. As previously reported, PFBA is present in process blank samples at high levels and is attributed to solvent contamination. PFBS, PFHxA, PFPeA, PFOA, and ADONA contamination were observed in process blanks for both Andrew+ Extraction+ and manual extractions but was less than 30% of 0.005 µg/kg native spiked PFAS and is attributed to solvent purity adhering to EURL POPs PFAS guidelines.[11]

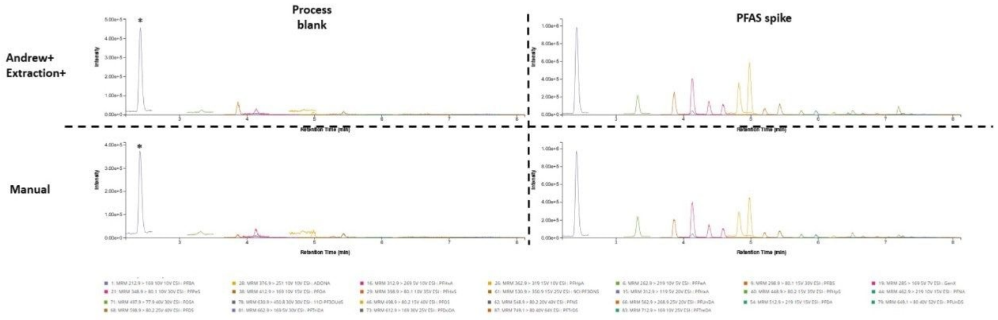


Figure 6. PFAS contamination in process blanks compared to 0.005 µg/kg PFAS spiked water through automated of manual extractions.

** denotes PFBA contamination in process blank*

Maximizing PFAS Recovery with Automated PFAS Extraction Workflow

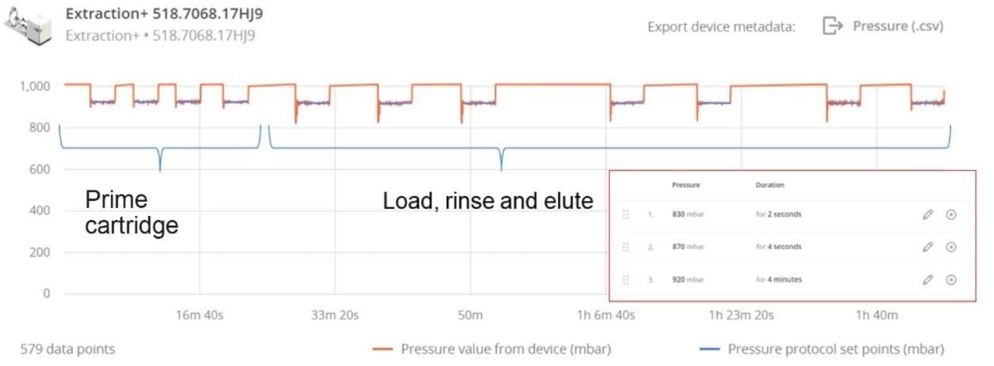
dispense on the rim:
-7mm
0mm
2mm
1250uL
0mm
-6.5mm
2mm
1250uL
6.5mm

Figure 7. OneLab guidelines to dispense solvent along 4 positions of solvent dispensing (right).

To maximize the recovery of PFAS that might adhere to the walls of the sample tube, the protocol makes use of a script within ‘Guidelines’ to wash the inside of the sample tubes (Figure 7). By specifying 4 points along the inside of the sample tubes, solvent is dispensed at these points allowing wash solvents to run down the sides of the tubes to collect as much PFAS as possible. Thereafter, a mixing step is introduced to allow wash solvents to be thoroughly agitated in the sample tube, and the wash solvents are then applied to Oasis GCB/WAX cartridge to be pulled through.

Optimizing Extraction+ Negative Pressure Profile to Maintain Consistent Flow

Each solvent pull-through step is programmed to have a stepwise pressure differential with a higher pressure difference at the beginning which is then reduced after a few seconds (Figure 8), which kickstarts the solvent pull-through and allows solvents to be pulled through in a drip-wise manner. During sample loading and the subsequent solvent addition steps, matrix build up hinders flow through the Oasis GCB/WAX cartridge and can become stuck. The OneLab software allows different pressure differentials to be specified at each stage of the extraction, therefore after samples is loaded onto the cartridges, a higher-pressure differential is applied at the beginning of the pull-through step and the total amount of time for the pull-through is increased to maintain flow through the cartridges (Figure 8). This combination of pressures was found to attain the optimal flow through the cartridges.



	Pressure	Duration
1.	830 mbar	for 2 seconds
2.	870 mbar	for 4 seconds
3.	920 mbar	for 4 minutes

Figure 8. Programmable stepwise pressure gradient to ensure optimal flow through cartridges.

Method Validation

In EU 2022/1431, indicative levels for PFOS, PFOA, PFNA, and PFHxS in milk are 0.020 µg/kg, 0.010 µg/kg, 0.050 µg/kg and 0.060 µg/kg respectively. A recent application note has shown that the method LOQs in vegetable, fruit and baby food range from 0.0005 to 0.005 µg/kg. Building on that work, milk matrices were validated at an

LOQ of 0.005 µg/kg if it meets the EURL POP Guidance document on PFAS in Food and Feed for the following parameters for identification, trueness and precision: 2 product ion with ion ratio within ± 30% of calibration standards, signal-to-noise ratio ≥ 3, trueness ± 20% for compliance testing and ± 35% for monitoring purposes, precision ≤ 20% for compliance testing and ≤ 25% for monitoring purposes. Fresh cow's milk was fortified with PFAS at 1x target LOQ (0.005 µg/kg), 10x target LOQ (0.05 µg/kg) and 100x target LOQ (0.5 µg/kg). All PFAS species apart from PFTrDS fulfilled the validation requirements at 0.005 µg/kg (Table 3). When considering the entire panel of native PFAS together, mean percentage recovery across all fortification levels was 102 ± 18% for milk (min = 40.6%, max = 123.6%). For the four mandatory compounds for monitoring, PFOS, PFOA, PFNA, and PFHxS, apparent recoveries were between 98 and 118%. (Figure 9). The poor apparent recovery of PFTrDS for all conditions is due to M2 PFTreDa being used as an extraction internal standard, and both analytes respond differently in milk matrix during extraction, which indicates the need for corresponding isolabelled isotopes for all PFAS species.

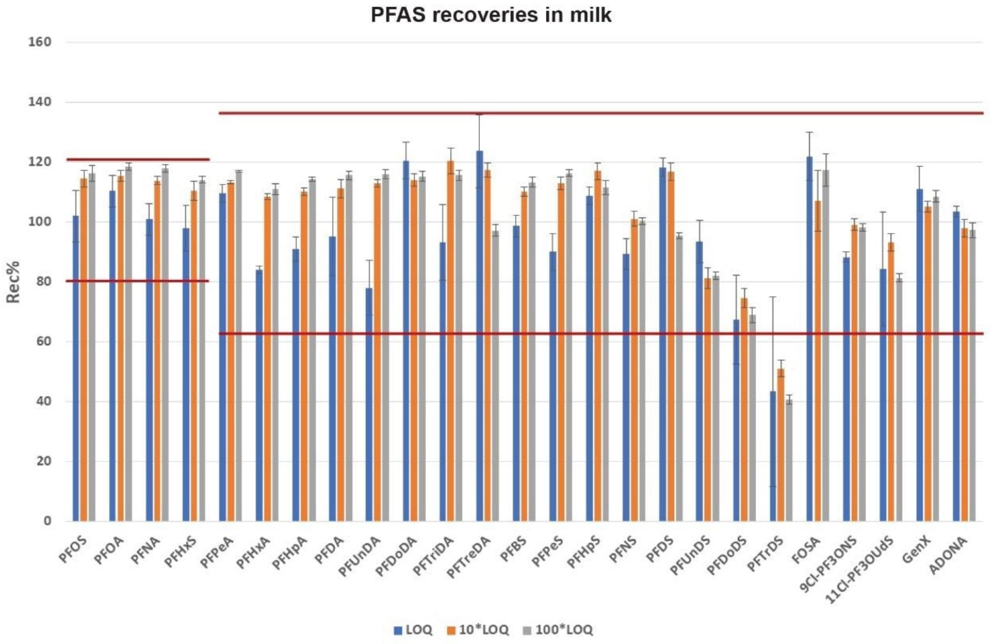


Figure 9. Bar-plots representing the recovery of PFAS in milk at three fortification levels. Red lines represent the thresholds set by the EURL POPs guidelines.

The use of automation improves efficiency and reduces errors especially in laboratories with multiple operators. A within-laboratory matrix group validation was carried out with 2 operators, over 3 non-consecutive days, and with 5 different milk matrices (Table 4). Each milk matrix was fortified at 1x target LOQ (0.005 µg/kg), 10x target LOQ (0.05 µg/kg) and 100x target LOQ (0.5 µg/kg) in duplicates. Where PFAS compounds were found in matrix blanks, blank subtraction was used to calculate recoveries. For PFOS, PFOA, PFNA, and PFHxS, most of the apparent recovery values were within ± 20% of the expected values. However, PFOS at 10x target LOQ in full fat UHT milk (71.1%), and 1x target LOQ (142.2%), 10x target LOQ (135.7%) and 100x target LOQ (144.8%) in vanilla ice-cream flavor milk were not within ± 20% of the expected values (Figure 10). Vanilla ice-cream flavor milk is heavily processed, and the results suggests that heavily processed milk will need extra sample pre-preparation steps to reduce any potential interferences from PFAS.

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Milk sample	Full fat UHT	Fresh milk	Low Fat UHT	Goat milk	Vanilla Ice cream flavor milk
Day	1	1	2	2	3
Operator	A	B	A	B	B

Table 4. Milk matrix group validation.

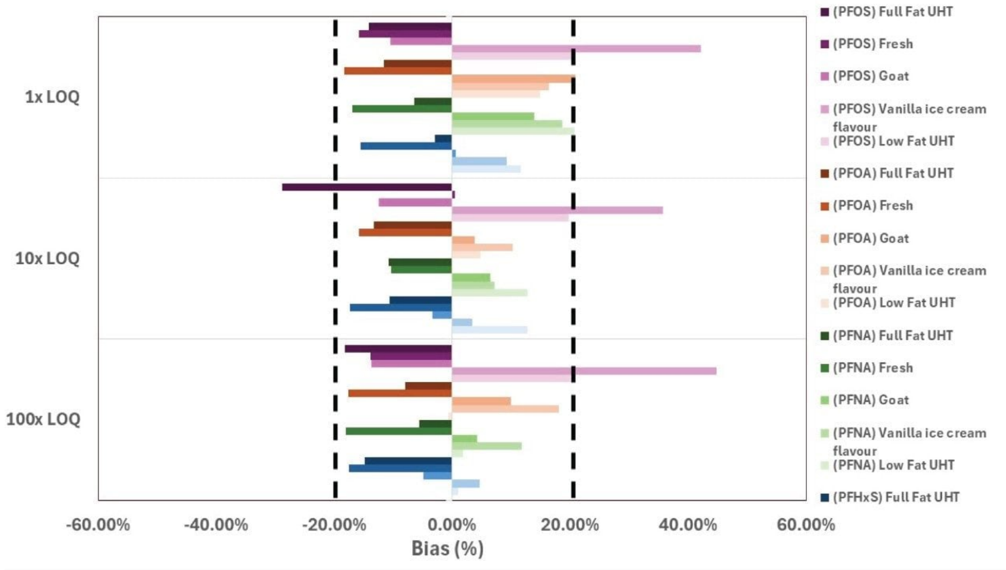


Figure 10. Milk matrix group validation at 1x, 10x, and 100x LOQ.

Conclusion

In this study, an automated workflow using the Andrew+ Pipetting Robot coupled with the Extraction+ vacuum manifold and vacuum pump was developed to extract PFAS in milk samples using Oasis GCB/WAX bilayer cartridges. The automated workflow includes creating calibration standard solutions, blanks and QC samples, and PFAS extraction through Oasis GCB/WAX SPE cartridges. Automated steps are programmed to minimize environmental PFAS contamination and maximize recovery of PFAS from samples. For 12 milk samples, approximately 337 pipetting steps are automated, saving approximately 3.5 hours of analyst time. In addition, the use of automation improves efficiency and reduces errors especially in laboratories with multiple operators.

In this study, the total workflow solution of Andrew+ Extraction+ automation robot, Oasis GCB/WAX bilayer cartridges, Acquity Premier UPLC coupled with Xevo TQ-Absolute was used to validate PFAS in milk at an LOQ of 0.005 µg/kg, adhering to the compounds listed in EU 2022/1431 and EURL POP guidelines. A within-laboratory milk matrix group validation was carried out, and the majority of milk samples were validated with this method at 1x LOQ, 10x LOQ, and 100x LOQ of PFAS fortified milk samples. Unfortunately, apparent recoveries for PFOS in vanilla ice cream flavor milk were consistently above 130%, suggesting matrix effect on milk samples with flavouring. Additional sample pre-processing steps will need to be investigated in future for heavily processed milk samples.

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