AUTOMATED SOLID PHASE EXTRACTION AND UHPLC-MS/MS ANALYSIS OF PER- AND POLY- FLUOROALKYL SUBSTANCES IN MILK

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INTRODUCTION

The European Food Safety Authority (EFSA) predicts that food is a predominant source of per- and poly- fluoroalkyl substances' (PFAS) exposure. As a result, regulatory agencies worldwide have implemented increasingly stringent monitoring requirements. The European Regulation 2022/2388 has set maximum levels of PFAS for PFOS, PFOA, PFNA, and PFHxS in eggs, fish meat, crustaceans, bivalve molluscs, meat and offal. [1] As PFAS analysis in fruits, vegetables, milk, and baby food require sensitive methods for complex samples, the European Commission has adopted European Recommendation 2022/1431 to monitor PFAS at indicative levels in fruits, vegetables, milk, and baby food. [2] 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. Milk, a dietary staple for toddlers and children, may disproportionately contribute to exposure in vulnerable populations, highlighting the need for robust PFAS analysis in milk matrices.

The purpose of this study was to develop and validate an automated workflow for determining 25 PFAS in milk using solid-phase extraction (SPE) clean-up followed by LC-MS/MS. Sample cleanup was performed using Waters Oasis™ PFAS GCB/WAX SPE Cartridges automated on the Andrew+™ Pipetting Robot and Extraction+™ Vacuum Manifold.

This study aimed to evaluate the benefits of automating liquid handling to improve reproducibility, reduce manual labor, and support labs operating under strict regulatory turnaround pressures.

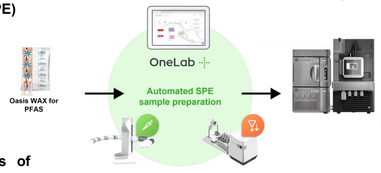

Figure 1 *A pictorial display of the analytical workflow applied in this study for the determination of PFAS in milk based foods.*

DISCUSSION

Given the chemical nature of PFAS compounds, the analysis is renowned as challenging, so best practices are advised. Some considerations are highlighted here, with additional information curated by Dreolin, *et al.* [6] In this study, PFBA, PFBS, PFHxA, PFPeA, PFOA, and ADONA were detected at analytically significant levels, due to solvent purity and not contributed by the Andrew+ system as seen in the similarity of the PFAS intensities in the automated and manual test, as shown in **Figure 3**. These analytes were observed in process blanks for both Andrew+ Pipetting Robot with Extraction+ Vacuum Manifold and manual extractions (**Figure 3a**) but were less than 30% of 0.005 µg/kg native spiked PFAS (**Figure 3b**), adhering to EURL POPs PFAS guidelines. [4]

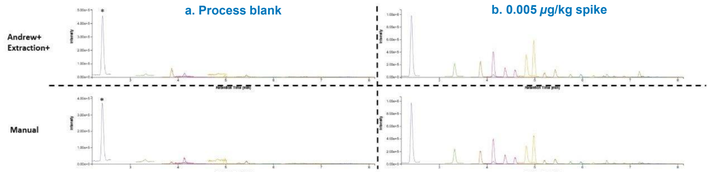

Figure 3 *PFAS contamination in process blanks compared to 0.005 µg/kg PFAS spiked water through automated of manual extractions. * denotes PFBA contamination in process blank*

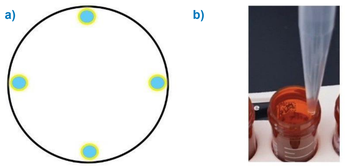

Figure 4 *a) OneLab software guidance points for Andrew+ Pipetting Robot and b) the automated dispensing in action.*

To maximize the recovery of PFAS and reproducibility of the automated method, guidelines and protocols in OneLab software were evaluated. One such optimization includes rinsing the inside of the collection tubes, where analytes may adhere to the walls. This is shown in **Figure 4**, where 4 points along the inside of the sample tubes were specified, allowing solvent to be dispensed at these points for wash solvents to run down the sides of the tubes to collect as much PFAS as possible.

Additional optimiziation of the automated SPE protocol was completed and is discussed further in the relevant content. [3,5] These developed protocols are available for download for streamlined implementation in routine laboratory operation. [5]

RESULTS

The method performance, achieved by both the manual and automated liquid handling approaches showed comparable results. This included the preparation of stock, working and calibration curve series using Andrew+ Pipetting Robot, where the 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.

Based on indicative levels for the priority 4 PFAS (namely PFOS, PFOA, PFNA, and PFHxS) in EU Recommendation 2022/1431 milk matrices were validated in this study at an LOQ of 0.005 µg/kg using the EURL POP Guidance document on PFAS in Food and Feed for parameters around identification, trueness and precision.

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. 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%).

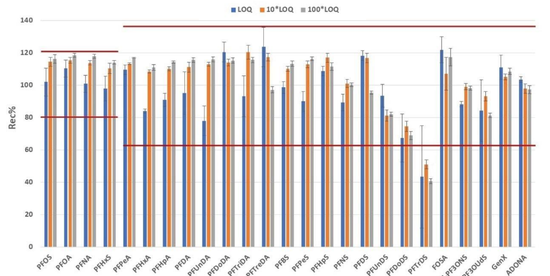

Figure 5 *Bar-plots representing the recovery of PFAS in milk at three fortification levels. Red lines represent the thresholds set by the EURL POPs guidelines.*

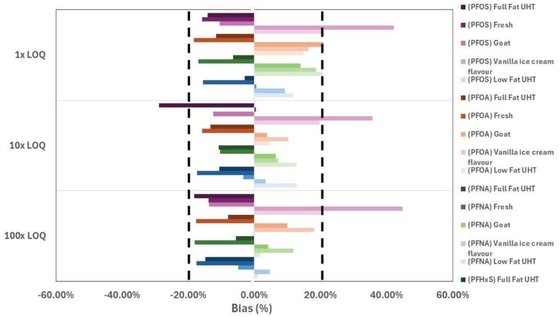

Figure 6 *Milk matrix group for intra-lab validation at 1x, 10x, and 100x LOQ for 5 sample types, prepared by 2 analysts across 3 days, all prepared using the automated workflow.*

For the four mandatory compounds, apparent recoveries were between 98 and 118%. The poor apparent recovery of PFTrDS for all conditions was due to M2 PFTreDa being used as an extraction internal standard, with both analytes responding differently in milk matrix during extraction, indicating the need for corresponding stable labelled isotopes for all PFAS species.

A within-laboratory matrix group validation was carried out with 2 operators, over 3 non-consecutive days, and with 5 different milk matrices. Each milk matrix was fortified at the same three levels (1x target LOQ, 10x target LOQ, and 100x target LOQ), in duplicate.

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. Vanilla ice cream flavor milk is heavily processed, and the results suggests the heavily processed milk would require extra sample pre-preparation steps to reduce any potential interferences with PFAS.

EXPERIMENTAL

One of the main factors that may affect LC-ESI-MS/MS analysis is matrix effects due to presence of different interferences in the foodstuffs. To reduce matrix effects, sample clean-up was carried out using Waters Oasis GCB/WAX SPE Cartridges – both manually and using automated techniques.

Five samples of dairy based foodstuffs were purchased from a local retail outlet, including fresh cow's milk, goat's milk, full fat UHT, low fat UHT and vanilla ice-cream flavored milk. Details of the sample preparation are summarized in **Figure 2**, with more detailed information available in the associated application note. [3] Method validation was conducted in accordance with the EURL for POPs guidance on PFAS analysis. [4]

PFAS native standards and isotope-labelled standards were obtained from Wellington Laboratories. A standard calibration series, containing internal standards, was prepared manually and using the Andrew+ Pipetting Robot over a concentration range of 0.00125 to 5 ng/mL (equivalent to 0.00025 to 1 µg/kg in actual food samples).

LC conditions:

System: ACQUITY™ Premier System with PFAS analysis kit installed
Analytical: ACQUITY Premier BEH™ C_{18} 2.1 x 50 mm, 1.7 µm column
Isolator: Atlantis™ Premier BEH C_{18} AX 2.1 x 50 mm, 5 µm column
Injection volume: 5 µL
Flow rate: 300 µL/ min
Mobile phase A: 2 mM ammonium acetate in water
Mobile phase B: 2 mM ammonium acetate in methanol/acetonitrile
Runtime: 11 minutes

MS parameters:

System: Xevo™ TQ Absolute MS
Ionization mode: ESI negative
Acquisition mode: Time windowed MRM

Analytes: 25 PFAS, please see app note [3] for relevant reference

Software control: Data acquisition, processing and review were performed using waters_connect ™ for Quantitation Software.

The SPE workflow, highlighted in green in **Figure 2**, was fully automated using the Andrew+ Pipetting Robot with Extraction+ Vacuum Manifold. The design and execution of the protocol was completed through OneLab™ Software (an intuitive software which allows user the full control of vacuum pressure setting, thus eliminating the need for user intervention in the procedure) and is available for download by scanning the QR code. [5]

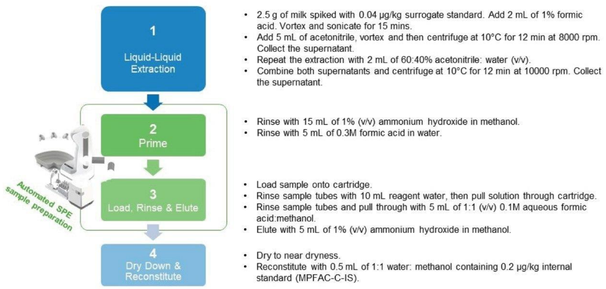

Figure 2 *Workflow summarized for the preparation of milk-based samples for the determination of PFAS.*

EURL for POPs guidance on method performance criteria applied:

Identification using 2 product ions with ion ratio within ± 30% of calibration standards and signal-to-noise ratio ≥ 3; trueness ± 20% for compliance testing and ± 35% for monitoring purposes ; and precision ≤ 20% for compliance testing and ≤ 25% for monitoring purposes. Method validation was completed at fortification levels of 1x target LOQ, 10x target LOQ and 100x target LOQ.

CONCLUSION

This poster describes a validated method using an automated workflow for the SPE clean-up of PFAS in milk samples using the Andrew+ Pipetting Robot and the Extraction+ Vacuum Manifold, providing benefits in terms of:

- **Reducing analyst time, minimizing experimental errors and enhancing reproducibility across users, where:**
  - **more than 101 steps were automated and approximately 88 minutes of hands-on time was saved in standard preparation.**
  - **236 steps were automated, saving approximately 2 hours when preparing a batch of 12 samples.**
- **Meeting the limit of quantitation for milk requirements of EU Recommendation (2022/1431) and method performance proposed in the EURL for POPs guidance.**
- **Simplified automation protocols are available for download through OneLab software for consistent calibration standard preparation and PFAS extractions through SPE, saving additional time and reducing risk of error in high throughput working environments.**

Scan the QR code to access the OneLab Protocol [5]

References
[1] Commission Regulation (EU) 2022/2388, maximum levels of perfluoroalkyl substances in certain foodstuffs, L 316/38, 8.12.2022.65. [2] Commission Recommendation (EU) 2022/1431, on the monitoring of perfluoroalkyl substances in food. L 221/105, 26.8.2022. [3] Ng D., Devakishen M., Kuah K. X., Adams S., Hird S., Ho J. Automated SPE Analysis of PFAS in milk. App note 720008740. [4] EURL for halogenated POPs in feed and food: Guidance Document on Analytical Parameters for the Determination of Per- and Polyfluoroalkyl Substances (PFAS) in Food and Feed, May 2022. [5] OneLab Software Protocol available for download at https://onelab.andrewalliance.com/library/automation-of-pfas-samples-in-milk-matrices-using-spe-VbwYYOwZ [6] Dreolin N., Foddy H., Organtini K., Adams S., Rosnack K., Hancock P., Best practices for monitoring PFAS contamination in a routine shared-space. White paper 720007905.

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