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VALIDATION OF A UHPLC-HRMS METHOD FOR THE ASSESSMENT OF PER AND POLY-FLUOROALKYL SUBSTANCES (PFAS) IN BIOTA

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Abstract

PFAS are present in freshwater fish in the Danube River, posing concerns about aquatic ecosystem contamination and adverse effects on human health. These substances also referred to as "forever chemicals" have the potential to accumulate in the food chain and provoke several health issues, including hormonal imbalance and cancer. In this work, the QuEChERS extraction and clean-up method combined with UHPLC-HRMS (Ultra-High-Performance Liquid Chromatography – High-Resolution Mass Spectrometry) was applied for the determination of 18 PFAS in muscle tissue of freshwater fish (European wels catfish – Silurus glanis). The method was validated in terms of Specificity, Linearity, Precision (% RSD), Recovery, and Accuracy (mean spike recovery, %) at two levels of concentration: 0.1 and 5 ng. Additionally, the study assessed the impact of matrix effects on PFAS detection in fish tissue.

Key words*: Danube River, freshwater fish, mass spectrometry, PFAS, QuEChERS.*

INTRODUCTION

Per and poly-fluoroalkyl substances (PFAS) are a class of thousands of environmental contaminants that contain carbon-fluorine bonds, known in organic chemistry as one of the strongest chemical bonds (Figure 1) (*Per- and Polyfluoroalkyl Substances (PFAS) - ECHA*, n.d.). They have been detected in freshwater fish from European rivers, the contamination with these substances is mainly the result of bioaccumulation in aquatic food chains (*PFAS Pollution in European Waters*, 2024). Given the increased anthropogenic pressures on aquatic ecosystems, fish meat and fish products have been observed to accumulate various contaminants, which can pose toxicity risks if transferred to humans via consumption (Panda et al., 2023). PFAS can accumulate in fish via two pathways: direct uptake from contaminated water and indirect uptake through the consumption of contaminated sediments and feed. In time, this can lead to bioaccumulation especially in vulnerable fish species tissue, causing disruption of the aquatic ecosystem.

The Danube River, one of the most important rivers in Europe, has been exposed to environmental pollutants like PFAS mainly through agriculture, industrial processes and wastewater discharges. In the Danube River Basin, a comprehensive study has focused on the occurrence and distribution of 4,777 PFAS in river water, wastewater, groundwater, and biota samples. Results revealed the presence of 82 PFAS, with perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) being widespread in surface water. Furthermore, the risk assessment led to identifying 18 PFAS of environmental concern, highlighting the

ubiquity of these substances throughout the river basin (Ng et al., 2022). Another study conducted in the Lower Danube River region focused on the occurrence of PFAS in surface water and ground water from two sites. PFAS concentrations were mostly low with the exception of PFOA concentrations that exceeded 10 ng/L at one site (Obeid et al., 2023).

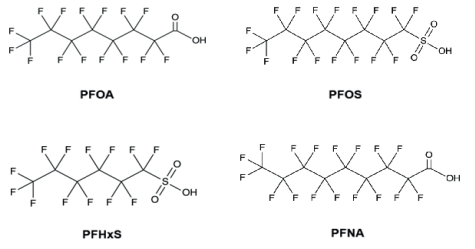

Figure 1. Chemical structure of the most studied PFAS

In the literature (Chaudhary et al., 2024), it is found that the absorption of PFAS at the level of biota depends on several factors that characterize water quality, e.g. heavy metals. It is necessary to associate studies on heavy metals from sensitive areas (Burada et al., 2015) with studies including statistical processing of the results of physicochemical determinations from surface ecosystems (Popa et al., 2018) and determinations of certain pollutants in biota.

As a consequence, regulations regarding PFAS are increasingly strict in Europe due to growing concern about their environmental and health impacts. The European Union has proposed restrictions on the use for specific PFAS under the REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) regulation (*REACH Annex XVII: REACH Restricted Substance List 2023*, n.d.). In addition, the European Chemicals Agency (ECHA) is actively identifying and imposing limitations for the use of PFAS substances of high concern (*Per and Polyfluoroalkyl Substances (PFAS) - ECHA*, n.d.). These restrictions will have as an impact an increased demand for targeted assessment of PFAS in different environmental matrices, like water, sediment and biota. Moreover, researchers and environmental agencies will update their monitoring strategies to comply with these regulations with focus on the most harmful and widespread PFAS.

The aim of the present work was to develop and validate a method for the determination of 18 PFAS in fish muscle using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and clean-up followed by UHPLC-HRMS (Ultra-High-Performance Liquid Chromatography – High-Resolution Mass Spectrometry) analysis.

MATERIALS AND METHODS

The fish tissue used in this validation study was muscle from three different wels catfish (*Silurus glanis*). The fish were purchased from a local fisherman in the Lower Danube River Region, Galați. 5 grams of each homogenized tissue (wet weight) were submitted to the QuEChERS extraction and dSPE (dispersive solid-phase extraction) clean-up. PFAS compounds were extracted from the fish tissue using acetonitrile and extraction salts (6.0 g $MgSO_4$ and 1.5 g Na-acetate). The resulted extract was submitted to clean-up (150 mg $MgSO_4$, 50 mg Primary and Secondary Amines) and concentrated under a gentle nitrogen flow. All resulted extracts were stored in polypropylene vials to prevent sample contamination. Figure 2 depicts the general workflow for the PFAS analysis in fish samples. For the method validation, spiked samples of 0.1 and 5 ng PFAS were also prepared in triplicate using the same protocol.

The final extracts were separated on Accucore aQ C18 (100 mm x 2.1 mm, 2.6 μm) analytical column in a gradient mode, and the mobile phase consisted of methanol and ultrapure water, both acidified with 0.1% formic acid. The flow rate was 0.5 mL/min. The temperature of the autosampler was 6°C and the analytical column temperature was set to 45°C. Furthermore, the extracts were analysed using a Thermo Fisher Scientific Chromatographic System consisting of Vanquish Flex Liquid Cromatograph coupled to Orbitrap Exploris 120 Mass Spectrometer with heated electrospray ionization (H-ESI II), operated in Full Scan at 120,000 (FWHM) at m/z 200, negative ion mode. The mass measurement accuracy was 5ppm. The chromatographic separation and detection were

performed within 21 min of analysis time. All chromatograms were processed using Chromeleon 7.3 software.

Table 1 contains a list of the target analytes; their acronyms and chemical formula and they are presented in their elution order. Also, the table presents the negative monitored ion (MS Quantitation peak) for each of the 18 PFAS.

The following validation parameters were tested according to Commission Decision 657/2002: specificity, linearity, precision, recovery, accuracy and matrix effects (*Decision - 2002/657 - EN - EUR-Lex*, n.d.). The obtained data represent a helpful tool in evaluating the reliability of the analytical method, in particular for low concentrations quantification of compounds in environmental samples.

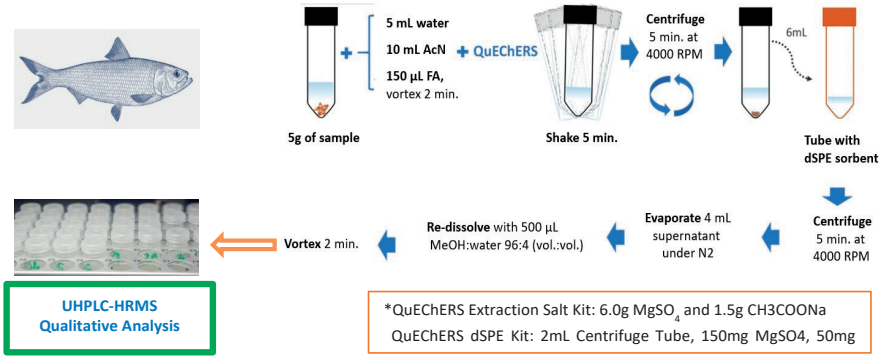

Figure 2. General targeted PFAS analysis workflow in biota samples

RESULTS AND DISCUSSIONS

The specificity of the method was investigated by injecting standard solution and solvent (a mixture of methanol and ultrapure water). Also, a chromatogram of the mobile phase was analysed. The obtained chromatograms demonstrated the absence of interference in the analyte elution. Figure 3 and 4 show a Total Ion Chromatogram (TIC), respectively an Extracted Ion Chromatogram of the PFAS standard mixture at a concentration of 100 ng/mL.

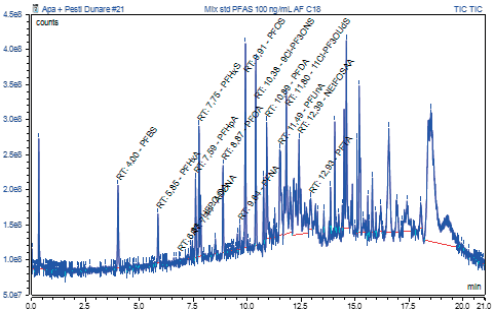

Figure 3. Total Ion Chromatogram of the PFAS standard mixture at 100 ng/mL

They both show a good separation of the compounds which is an indicator for an appropriate choice of the mobile phase, gradient and chromatographic column.

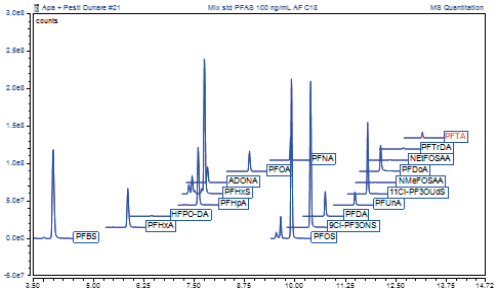

Figure 4. Extracted Ion Chromatogram of the PFAS standard mixture at 100 ng/mL

Linearity of the method was evaluated by testing calibration standards of PFAS on 8 concentration levels. These were prepared by dilutions of the stock standard solution prepared in 96%: 4% methanol: ultrapure water (vol./vol.) and comprise a mixture of target analytes in a calibration range of 0.5-100 ng/mL for most analytes, except for HFPO-DA (5-100 ng/mL).

The coefficient of determination was R^2>0.995, which complies with the acceptance criteria (Table 2). This indicates a strong linear relationship between the measured concentration and expected concentration in the method used for analysis.

Precision was measured as %Relative Standard Deviation. Higher RSD values indicate lower precision, while lower values suggest less variability in measurements. 2 levels of spike were tested: 0.1 and 5 ng. The analysis was performed in triplicate for each spike level. Results for most compounds were reasonably low, especially for the 0.1ng spike. Precision values range from about 2.74% to 18.62% for different compounds and are below 20%. (Tabel 2). For example, PFBS at the 0.1 ng spike has an RSD of 8.14% and at the 5 ng spike of 8.13%, which indicates that the measurements are consistent. Some compounds like PFOS have a relatively low % RSD of 3.11 at 5 ng, indicating good precision, while others like HFPO-DA have higher % RSD values of 18.06 at 0.1 ng, which suggests poorer precision in the measurements at lower concentrations.

Recovery represents a measure of the analytical method efficiency in detecting the spiked compound and it is the measured concentration compared to the spiked concentration, expressed as a percentage. The recovery for some compounds at the 0.1 ng spike is lower than ideal (100%). For example, PFH x A has a recovery of 66.53% at the 0.1 ng spike, suggesting that the method may not be as efficient at detecting lower concentrations. Many compounds (e.g., PFHpA, PFDA, NMeFOSAA) have recoveries slightly lower than 100% at the 5 ng spike, which indicates that the method has good efficiency at higher concentrations.

Accuracy, measured as mean spike recovery %, shows how close a result is to the true value. The obtained results are presented in Table 2. % RSD of the % recovery was within a range of 1.97 to 16.15%.

Table 1. Targeted analytes list

Compound name	Acronym	Chemical formula	Retention Time, min	MS quantitation peal
Perfluoro-1-butanesulfonic Acid	PFBS	C4HF9O3S	3.72	298.94300
Perfluorohexanoic Acid	PFHxA	C6HF11O2	5.59	268.98370
Undecafluoro-2-methyl-3-oxahexanoic Acid	HFPO-DA	C5HF11O	6.17	284.97790
Perfluoroheptanoic Acid	PFHpA	C7HF13O2	7.34	318.97849
Perfluorohexanesulfonic Acid	PFHxS	C6HF13O3S	7.47	398.93660
2,2,3-trifluoro-3-[1,1,2,2,3,3-hexafluoro-3 (trifluoromethoxy) propoxy] propanoic Acid	ADONA	C7H2F12O4	7.59	376.96890
Pentadecafluorooctanoic Acid Hydrate, Linear	PFOA	C8HF15O2	8.59	412.96640
9-chlorohexadecafluoro-3-oxanone-1-sulfonic Acid, K Salt Unlabeled As Free Acid	PFNA	C9HF17O2	9.63	462.96320
Perfluorooctanesulfonic Acid	PFOS	C8HF17O3S	9.63	498.93020
Perfluorononanoic Acid	9Cl-PF3ONS	C8HClF16O4S	10.11	530.89510
Perfluorodecanoic Acid	PFDA	C10HF19O2	10.50	512.96000
Potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate As Free Acid	PFUnA	C11HF21O2	11.24	562.95680
N-methyl Perfluorooctanesulfonamidoacetic Acid	11Cl-PF3OUdS	C10HClF20O4S	11.55	630.88920
Perfluoroundecanoic Acid	NMeFOSAA	C11H6F17NO4S	11.85	569.96730
N-ethyl Perfluorooctanesulfonamidoacetic Acid	PFDoA	C12HF23O2	11.90	612.95370
Perfluorododecanoic Acid	NEtFOSAA	C12H8F17NO4S	12.21	583.98300
Perfluorotridecanoic Acid	PFTrDA	C13HF25O2	12.47	662.95050
Perfluorotetradecanoic Acid	PFTA	C14HF27O2	12.97	712.94730

Table 2. Method validation results for the target PFAS evaluated in wels catfish muscle

Compound name	Coeff. of determination	Precision (%RSD)		Recovery (%)			
		Spike 0.1 ng	Spike 5 ng	Spike 0.1 ng	RSD, %	Spike 5 ng	RSD, %
PFBS	0.9993	8.14	4.16	71.75	8.13	97.48	4.16
PFHxA	0.9989	10.45	7.81	66.53	10.43	92.53	7.80
HFPO-DA	0.9951	18.06	12.19	77.89	12.06	87.13	9.19
PFHpA	0.9991	9.93	15.49	82.26	9.92	102.19	15.48
PFHxS	0.9991	5.63	16.16	72.27	5.62	78.82	16.15
ADONA	0.9989	11.66	15.71	89.84	11.66	98.58	15.71
PFOA	0.9986	6.74	9.26	118.55	5.92	135.21	8.71
PFNA	0.9989	6.66	5.18	113.47	5.84	127.43	4.86
PFOS	0.9991	18.62	14.12	89.23	3.11	87.78	7.44
9Cl-PF3ONS	0.9975	6.55	2.74	68.13	6.55	78.76	2.74
PFDA	0.9993	16.10	7.73	92.82	7.48	119.43	5.01
PFUnA	0.9990	7.99	6.57	115.82	4.41	131.70	4.91
11Cl-PF3OUdS	0.9979	7.68	2.86	67.96	7.68	81.36	2.86
NMeFOSAA	0.9984	15.06	6.71	124.60	14.67	110.64	6.65
PFDoA	0.9992	16.34	12.50	102.55	3.11	128.71	1.97
NEtFOSAA	0.9982	10.07	5.88	98.83	10.07	131.55	5.88
PFTrDA	0.9992	16.07	7.14	127.79	8.21	121.02	4.89
PFTA	0.9992	4.79	7.67	103.74	3.54	128.05	6.62

Matrix effects were significantly reduced by the Orbitrap 120 Mass Spectrometer due to its high resolution, sensitivity, and accurate mass measurements, along with its ability to optimize ionization conditions, whereas in traditional quadrupole MS, matrix effects can lead to ion suppression or enhancement, causing inaccurate quantification and reduced sensitivity due to interference from co-eluting compounds in the sample. Therefore, the proposed method is well-suited for environmental testing, such as analysing fish extracts for contaminants.

The carryover effect was evaluated by injecting two solvent blanks right after the analysis of the highest calibration standard concentration. There was no carryover effect observed.

CONCLUSIONS

An optimized analytical method based on QuEChERS extraction and UHPLC-HRMS analysis of PFAS was successfully validated. However, there are some limitations to this method. While it demonstrates high sensitivity and specificity, it may not detect all PFAS, particularly those with low ionization. Future research could focus on expanding its scope by refining extraction protocols or incorporating complementary extraction techniques. Additionally, further validation on a broader range of environmental matrices would also be valuable for improving the method's practical applications in monitoring PFAS contamination.

The proposed method is highly suitable for the accurate quantification of PFAS in biota samples, offering accurate measurement of these contaminants in fish. This makes it an effective tool for environmental monitoring, risk assessment, and studies on the bioaccumulation and potential health impacts of PFAS exposure in ecosystems and human populations. PFAS determinations in biota are part of the quality indicators for surface aquatic ecosystems and must be included in integrative systems such as the Water Quality Index (Iticescu et al., 2016) due to their persistence, bioaccumulation potential, and widespread distribution in aquatic ecosystems. This will give a correct global picture of the interactions between the different pollutants in complex systems such as the Danube.

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REFERENCES

Burada, A., Topa, M.C., Georgescu, L., Teodorof, L., Despina (Nastase), C., Seceleanu-Odor, D., & Iticescu, C. (2015). Heavy Metals Environment Accumulation in Somova-Parches Aquatic Complex from the Danube Delta Area. *Revista de Chimie - Bucharest - Original Edition*, *66*, 48–54.

Chaudhary, A., Usman, M., Cheng, W., Haderlein, S., Boily, J.-F., & Hanna, K. (2024). Heavy-Metal Ions Control on PFAS Adsorption on Goethite in Aquatic Systems. *Environmental Science & Technology*, *58*(45), 20235–20244. https://doi.org/10.1021/acs.est.4c10068

Decision - 2002/657 - EN - EUR-Lex. (n.d.). Retrieved February 24, 2025, from https://eur-lex.europa.eu/eli/dec/2002/657/oj/eng

Iticescu, C., Murariu, G., Georgescu, L., Burada, A., & Topa, M.C. (2016). Seasonal Variation of the Physico-chemical Parameters and Water Quality Index (WQI) of Danube Water in the Transborder Lower Danube Area. *Revista de Chimie -Bucharest-Original Edition*, *67*(9), https://revistadechimie.ro/Articles.asp?ID=5199

Ng, K., Alygizakis, N., Androulakakis, A., Galani, A., Aalizadeh, R., Thomaidis, N., & Slobodnik, J. (2022). Target and suspect screening of 4,777 per and polyfluoroalkyl substances (PFAS) in river water, wastewater, groundwater and biota samples in the Danube River Basin. *Journal of Hazardous Materials*, *436*, 129276. https://doi.org/10.1016/j.jhazmat.2022.129276

Obeid, A. A., Oudega, T. J., Zoboli, O., Gundacker, C., Blaschke, A. P., Zessner, M., Saracevic, E., Devau, N., Stevenson, M. E., Krlovic, N., Liu, M., Nagy-Kovács, Z., László, B., Lindner, G., & Derx, J. (2023). *Occurrence and Distribution of PFAS in the River and Groundwater at Two Danube Sites*. EGU-11264. EGU General Assembly Conference Abstracts. https://doi.org/10.5194/egusphere-egu23-11264.

Panda, B. P., Mohanta, Y. K., Parida, S. P., Pradhan, A., Mohanta, T. K., Patowary, K., Wan Mahari, W. A., Lam, S. S., Ghfar, A. A., Guerriero, G., Verma, M., & Sarma, H. (2023). Metal pollution in freshwater fish: A key indicator of contamination and carcinogenic risk to public health. *Environmental Pollution*, *330*, 121796. https://doi.org/10.1016/j.envpol.2023.121796

Per and polyfluoroalkyl substances (PFAS)-ECHA. (n.d.). Retrieved February 17, 2025, from https://echa.europa.eu/hot-topics/perfluoroalkyl-chemicals-pfas

PFAS pollution in European waters. (2024, December 9). https://www.eea.europa.eu/en/analysis/publications/ pfas-pollution-in-european-waters

Popa, P., Murariu, G., Mihaela, T., Georgescu, L., & Iticescu, C. (2018). Multivariate statistical analyses of water quality of Danube river at Galati, Romania. *Environmental Engineering and Management Journal*, *17*(5), https://doi.org/10.30638/eemj.2018.124

REACH Annex XVII: REACH Restricted Substance List 2023. (n.d.). Retrieved July 18, 2024, from https://www.chemsafetypro.com/Topics/EU/REACH_annex_xvii_REACH_restricted_substance_list.html