Preliminary results from monitoring perfluoroalkyl substances contamination in the livers of broiler chickens raised in Italy

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Abstract

This study aimed to demonstrate the efficacy of the analytical method proposed for the assessment of the amount of European Union (EU)-regulated perfluoroalkyl substances (PFAS) in broiler chicken livers and to perform preliminary monitoring of hepatic contamination in chickens raised in Italy under different rearing systems. A total of 21 liver samples were analyzed, revealing widespread PFAS contamination, with perfluorooctanoic acid detected in all samples at 0.48-0.66 µg/kg. Perfluorononanoic acid and perfluorohexane sulfonic acid were also found, while perfluorooctanesulfonic acid contamination was observed only in specific samples. The total PFAS amount varied across groups, even though none of the samples exceeded the EU regulatory limits. The PFAS content in livers from rural free-range chickens tended to be higher than that of their indoor counterparts (upper bound: 0.83 *vs*. 0.67 µg/kg; lower bound: 0.87 *vs*. 0.71 µg/kg, respectively; p=0.08 and p=0.06), suggesting that access to outdoor environments is a key factor involved in PFAS contamination. Further monitoring across more diverse samples is needed to confirm these preliminary findings and define mitigation strategies to reduce the risk of PFAS contamination.

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Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large group of synthetic chemicals extensively used in various industrial processes and products due to their unique properties, such as resistance to heat, water, and oil (Ghelli *et al*., 2019; Death *et al*., 2021; Peritore *et al*., 2023). However, these same characteristics also contribute to their persistence in the environment, earning them the designation of "forever chemicals" (Ghelli *et al*., 2019; Death *et al*., 2021). In recent years, PFAS have become a global concern due to their potential toxicity to humans and wildlife (Ghelli *et al*., 2019; Death *et al*., 2021; Peritore *et al*., 2023).

Human exposure to PFAS occurs primarily through the ingestion of contaminated food and water (Ghelli *et al*., 2019; Death *et al*., 2021; Peritore *et al*., 2023). These persistent chemicals readily infiltrate the food chain and accumulate in organisms, posing significant health risks (Ghelli *et al*., 2019; Death *et al*., 2021; Peritore *et al*., 2023). Research has shown that PFAS can accumulate in poultry liver, which can be consumed by humans (Qi *et al*., 2019; Death *et al*., 2021). The liver is a key site for PFAS accumulation due to its role in the synthesis of albumin, a protein to which PFAS rapidly bind (Death *et al*., 2021). This is concerning as some PFAS, such as perfluorooctanoic acid (PFOA), have been classified as carcinogenic and are linked to several adverse health effects, including liver damage, thyroid disease, obesity, fertility problems, and cancer (Death *et al*., 2021; Peritore *et al*., 2023).

Recognizing these risks, regulators are working to understand and mitigate PFAS contamination in foods, with a particular focus on products of animal origin (Death *et al*., 2021). Regulation (EU) 2023/915 sets maximum levels for perfluorooctanesulfonic acid (PFOS), PFOA, perfluorononanoic acid (PFNA), and perfluorohexane sulfonic acid (PFHxS) (6.0, 0.70, 0.40, and 0.50 µg/kg, respectively) in the offal of bovine animals, sheep, pigs, and poultry, with a combined limit for the sum of these PFAS compounds set at 8.0 µg/kg (European Commission, 2023). For this latter limit, the maximum levels refer to lower-bound concentrations, assuming that all values below the limit of quantification (LOQ) are zero.

The Commission Recommendation (EU) 2022/1431 highlights

the importance and the urgency of identifying reliable and standardized analytical methods to monitor PFAS levels in food supply chains with the aim of safeguarding food safety and public health (European Commission, 2022a). Monitoring PFAS in chicken liver provides valuable insights into the contamination of this human-edible tissue in Italy and helps investigate factors influencing PFAS hepatic accumulation (Death *et al*., 2021; Gazzotti *et al*., 2021; Xing *et al*., 2023).

The study aimed to demonstrate the efficacy of the analytical method employed for assessing PFAS contamination in broiler chicken livers and to conduct preliminary monitoring of regulated PFAS (as per Regulation EU 2023/915) in livers from chickens raised in Italy under various rearing systems.

Materials and Methods

Chemicals and reagents

High-purity PFAS standards, specifically L-PFHxS, PFOA, PFNA, L-PFOS, and deuterated standards perfluoro-n-(1,2-$^{13}C_2$)octanoic acid (M2-PFOA), and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$)octanesulfonate (M-PFOS), were acquired from Wellington Laboratories (Guelph, Ontario, Canada). Acetonitrile, methanol, formic acid, ammonium hydroxide, and sodium hydroxide, used for sample preparation and cleanup, were sourced from Merck (Milan, Italy). Oasis WAX SPE cartridges (6 cc/150 mg) were procured from Waters (Milford, MA, USA). Nylon syringe filters (0.22 µm) were obtained from FAVS (Bologna, Italy). For ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis, ammonium acetate, methanol, and ultra-pure water were purchased from Sigma Aldrich (Milan, Italy).

Working solution

The PFAS stock solutions (2 mg/L in methanol) of each analyte were serially diluted in methanol to prepare the working solutions containing all four analytes necessary for calibrators and quality control (QC) samples. Specifically, the working solutions had concentrations of 2.0 and 20.0 µg/L. The internal standard (IS) working solution was prepared by diluting the IS stock solutions 10-fold in methanol. All standard solutions were stored at -20°C.

Sampling

A total of 21 livers were obtained from broiler chickens raised in Italy under different rearing systems. The main distinction between rearing systems was the possibility for the animals to be in contact with the farm's external environments, which has been identified as a relevant factor for PFAS contamination (Ghelli *et al*., 2019; Death *et al*., 2021; Gazzotti *et al*., 2021). In the "indoor" systems (either intensive or semi-intensive), chickens were raised within a commercial barn with no access to the outdoor areas. On the contrary, the "free-range rural" category identifies chickens that were raised outside and thus in contact with the external environment and the soil. A total of 7 groups were defined based on rearing system, genetic type, age, commercial category, and geographical area, as shown in Table 1. These samples were stored at -20°C and thawed in a refrigerator (4°C) overnight prior to extraction.

Sample preparation

Sample preparation followed the protocol outlined by Organtini *et al*. (2022) with minor modifications. Specifically, a 2-gram liver sample, Previously homogenized using an Ultra Turrax, was weighed into a 50 mL centrifuge tube and spiked with 1 ng/g of internal standard (M2-PFOA and M-PFOS). 10 mL of 0.02 M sodium hydroxide in methanol was added, and the sample was magnetically stirred for 1 hour. Subsequently, the sample was centrifuged at 4000 RPM for 10 minutes at room temperature. After extraction, 1 mL of the supernatant was diluted with 14 mL of water and prepared for solid-phase extraction using Oasis WAX for PFAS, 6 cc, 150 mg cartridges. Finally, the eluate was dried and redissolved in methanol:ultra-pure water (50:50) and analyzed by UPLC-MS/MS. To avoid potential interactions between PFAS and glass, all procedural steps were conducted using polypropylene materials

Liquid chromatography-mass spectrometry conditions

The chromatographic analysis was performed using a Waters Acquity UPLC® binary pump system (Waters Corporation, Milford, MA, USA). The system was equipped with a perfluorinated compounds (PFC) isolator kit to prevent background contamination. Chromatographic separation was achieved using a Waters Acquity UPLC® BEH Shield C18 analytical column (50×2.1 mm, 1.7 µm particle size), fitted with a Waters VanGuard guard pre-column packed with the same stationary phase material. Both the analytical column and guard column were maintained at a temperature of 35°C during the analysis.

The mobile phase consisted of 5 mM ammonium acetate in water (solvent A) and methanol (solvent B). The separation was achieved using a linear gradient elution at a constant flow rate of 0.3 mL/min. The gradient started at 50% A and 50% B and changed linearly over 8 minutes to 10% A and 90% B. This final composition was held for 2 minutes. The method then included a 0.5-minute gradient return to the initial 50% A and 50% B conditions, which were held for an additional 1.5 minutes. Finally, the

Table 1. Detailed description of the samples.

Group	Rearing system	n. samples	Genetic type	Age (days)	Commercial category	Province
A	Indoor (intensive)	5	Ross 308	35	Rotisserie	Forlì-Cesena
B	Indoor (intensive)	5	Ross 308	45	Medium	Forlì-Cesena
C	Indoor (semi-intensive)	1	Golden	190	Light chicken	Forlì-Cesena
D	Indoor (semi-intensive)	1	Lohmann	180	Capon	Forlì-Cesena
E	Outdoor (free-range rural)	2	Ross 308	55	Heavy	Forlì-Cesena
F	Outdoor (free-range rural)	3	Rustic	90	Chicken	Caserta
G	Outdoor (free-range rural)	4	Campese	70	Slow-growing	Perugia

column was equilibrated at the starting conditions for 1.5 minutes before the next injection.

The UPLC system was coupled to a Xevo® TQ-S micro triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The mass spectrometer was operated in negative electrospray ionization mode with a capillary voltage of 0.50 kV.

The analyses were performed in multiple reaction monitoring (MRM) mode. Two MRM transitions were monitored for each target analyte, along with one transition for the IS. The specific MRM transitions, cone voltages, and collision energies are provided in *Supplementary Table 1*. Data acquisition and processing were conducted using MassLynx 4.2 software (Waters Corporation, Milford, MA, USA).

Method performance assessment

The analytical method was verified to comply with the EURL POPs guidance for PFAS determination in food and feed (EURL POPs, 2024), as outlined in Commission Implementing Regulation (EU) 2022/1428, which establishes general criteria to ensure reliable and consistent official controls of perfluoroalkyl substances in certain foodstuffs (European Commission, 2022b).

Matrix-matched calibration curves, including blank and 5 concentration levels (0.04, 0.10, 0.50, 1.00, and 5.00 µg/kg), were prepared daily by adding 20 µL of IS working solution. Peak area ratios of PFAS to IS were plotted against their concentrations. To account for the analyte levels naturally present in the sample matrix, blank concentrations were subtracted from the measured values.

A maximum 20% deviation between back-calculated and true concentrations was allowed for linearity verification at each of the five calibration points.

Method intra- and inter-day trueness and precision were evaluated through repeated analysis of QC samples at three concentration levels (0.04, 0.50, and 5.00 µg/kg) over multiple days. QC samples were prepared and analyzed in triplicate during each analytical session alongside calibration standards and authentic samples.

According to EURL POPs guidelines, trueness must fall within the range of -20% to +20% for compliance testing of maximum levels, while for monitoring purposes, a range of -35% to +35% is acceptable. For compliance testing of maximum levels, within-laboratory reproducibility should not exceed 20%. A tolerance of 25% is allowed for monitoring purposes.

For LC-MS/MS analysis using MRM, peak identification is assessed by ensuring the analyte's retention time ratio to the IS matches the calibration standard within 1%. Additionally, the ion ratios of sample extracts must be within ±30% of the average ion ratio in calibration standards from the same analytical sequence.

LOQ was estimated as the lowest validated level meeting all the aforementioned criteria of trueness, precision, and identification.

The European Commission recommends a LOQ≤0.50 µg/kg wet weight for PFOS, PFOA, PFNA, and PFHxS in the edible offal of terrestrial animals (European Commission, 2022).

The limit of detection (LOD) was determined by the concentration at which the signal-to-noise ratio reached a value of 3.

Data management and statistical analysis

The method outlined in the scientific report by the European Food Safety Authority titled "Management of left-censored data in dietary exposure assessment of chemical substances" (EFSA, 2010) was applied for handling left-censored data. This guidance recommends using the lower bound (LB) and upper bound (UB) approach for chemicals expected to be present in food. The LB is calculated by assigning a value of zero to all samples reported below the LOD or LOQ. The UB is determined by assigning the LOD to values reported as <LOD and the LOQ to values reported as <LOQ (the maximum possible value), based on the laboratory's reported LOD or LOQ. The Student *t*-test was applied to LB and UB mean values to evaluate the effect of the rearing system (indoor *vs*. free-range). To this purpose, data from groups A, B, C, and D were considered as a single group (indoor) and compared with those from groups E, F, and G (free-range). The liver sample was the experimental unit, and differences were considered statistically significant when the p-value was lower than 0.05.

Results and Discussion

Method performance

The method has fully satisfied all the established acceptance criteria, thus demonstrating its applicability to the analyzed matrix, ensuring the reliability of the data obtained.

The method exhibited excellent linearity within the validated concentration range, with back-calculated concentrations consistently falling within the range -8% and +4% of the expected values. The performance assessment carried out on QC levels under both intra-day and inter-day conditions consistently demonstrated excellent results. Deviation of the back-calculated concentration was always ≤±10%, demonstrating the method's linearity across the validated concentration range.

Furthermore, the method demonstrated highly satisfactory performance in terms of trueness and precision. For all QC levels, under both intra-day and inter-day conditions, trueness and precision were consistently within the acceptable limits, with bias values falling within a narrow range of -10% to +4% and coefficient of variation values ranging from 0% to 5%. (*Supplementary Table 2*). The LOQ for the four PFAS was 0.04 µg/kg, while the LOD was 0.01 µg/kg.

Perfluoroalkyl substance levels in broiler chicken liver

Farmland contamination is a significant risk factor for PFAS accumulation in poultry and livestock via the environment, drinking water, and feeding (Death *et al*., 2021; Xing *et al*., 2023). Studies show that PFAS levels are consistently higher in liver tissues than in muscle, as the liver plays a key role in plasma albumin synthesis, to which PFAS bind efficiently (Lau, 2015; Xing *et al*., 2023). Since PFAS accumulate in edible tissues like the liver, which may be considered the main target organ, further data and risk assessments are needed (Xing *et al*., 2023).

Table 2 provides, for each group, the percentage of left-censored results (where PFAS levels were below the LOQ) and the concentration range of the four regulated PFAS compounds within the quantifiable samples.

The results of the sample analysis reveal a widespread presence of PFAS in poultry liver. PFOA, a particularly concerning PFAS compound, was consistently detected in all samples, with concentrations ranging from 0.48 to 0.66 µg/kg. While PFNA and PFHxS were also found, their levels were generally lower, suggesting a less significant accumulation in poultry liver. PFOS, on the other hand, was detected only in specific groups, indicating a more localized contamination or exposure pattern. Notably, the total PFAS contamination varied widely among the groups, highlighting the potential influence of different factors and conditions on PFAS

uptake in poultry. Limited data is available on PFAS contamination in farmed broiler liver. Zafeiraki *et al.* (2016) analyzed 99 liver samples from various farm animal species in the Netherlands, including 20 from indoor-raised chickens. They detected PFOS at 4.2 µg/kg in only one chicken liver sample. However, based on data collected from a range of farm animals, their findings demonstrated a distinct difference in contamination levels between animals raised indoors and those raised outdoors. Mikołajczyk *et al.* (2024) analyzed PFAS concentrations in chicken liver samples collected from various regions of Poland (with no information on the rearing system). Their findings identified PFOA as the predominant PFAS (0.17 µg/kg), followed by PFNA (0.10 µg/kg), PFOS (0.090 µg/kg), and PFHxS (0.047 µg/kg). These results are consistent with the findings of the present study.

Table 3 presents the data on the LB and UB mean concentrations of PFOS, PFOA, PFNA, PFHxS, and ∑(PFAS) in the analyzed groups. The mean value and standard deviation are reported only in cases where multiple samples of the same type were available. In the last row, the maximum level set by Commission Regulation (EU) 2023/915 for the offal of bovine animals, sheep, pigs, and poultry is reported.

The data comparison demonstrates that, although PFAS contamination was extensive, none of the poultry offal samples exceeded regulatory limits.

Preliminary evidence suggests that the rearing system is a crucial factor influencing PFAS concentrations in poultry liver. Free-

Table 2. Concentration of perfluorooctanesulfonic acid, perfluorooctanoic acid, perfluorononanoic acid, and perfluorohexane sulfonic acid in poultry liver samples.

	PFOS		PFOA		PFNA		PFHxS	
Group	LC (%)	Quantifiable samples range (µg/kg)	LC (%)	Quantifiable samples range (µg/kg)	LC(%)	Quantifiable samples range (µg/kg)	LC (%)	Quantifiable samples range (µg/kg)
A	100	/	0	0.48-0.59	0	0.040-0.12	0	0.040-0.070
B	100	/	0	0.49-0.56	0	0.060-0.090	0	0.030-0.10
C*	na	/	0	0.53	0	0.070	na	0.32
D*	na	/	0	0.53	0	0.060	na	/
E	0	0.50-0.58	0	0.60-0.66	0	0.10-0.10	100	/
F	0	0.17-0.19	0	0.50-0.53	0	0.070-0.080	66	0.060
G	100	/	0	0.50-0.53	0	0.050-0.060	75	0.21

LC, left censored (%); PFOS, perfluorooctanesulfonic; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; na, not applicable. *Group with one sample.

Table 3. Lower bound and upper bound mean concentration of perfluorooctanesulfonic acid, perfluorooctanoic acid, perfluorononanoic acid, perfluorohexane sulfonic acid, and ∑(perfluoroalkyl substances) in chicken liver, and maximum level set by Commission Regulation (EU) 2023/915.

	PFOS		PFOA		PFNA		PFHxS		Σ(PFAS)	
Group	LB (µg/kg)	UB (µg/kg)	LB (µg/kg)	UB (µg/kg)	LB (µg/kg)	UB (µg/kg)	LB (µg/kg)	UB (µg/kg)	LB (µg/kg)	UB (µg/kg)
A	0.00	0.04±0.00	0.53±0.05	0.53±0.05	0.07±0.03	0.07±0.03	0.06±0.01	0.06±0.01	0.66±0.08	0.70±0.08
B	0.00	0.04±0.00	0.52±0.03	0.52±0.03	0.07±0.01	0.07±0.01	0.06±0.03	0.06±0.03	0.65±0.06	0.69±0.06
C*	0.00	0.04	0.53	0.53	0.07	0.07	0.32	0.32	0.93	0.97
D*	0.00	0.04	0.53	0.53	0.06	0.06	0.00	0.04	0.59	0.67
E	0.54±0.06	0.54±0.06	0.63±0.04	0.63±0.04	0.10±0.02	0.10±0.02	0.00	0.04	1.28±0.02	1.32±0.02
F	0.18±0.09	0.18±0.09	0.52±0.02	0.52±0.02	0.07±0.01	0.07±0.01	0.02±0.03	0.05±0.01	0.80±0.06	0.82±0.04
G	0.00	0.04±0.00	0.51±0.01	0.51±0.01	0.05±0.01	0.05±0.01	0.05±0.10	0.08±0.09	0.62±0.09	0.69±0.07
ML	6.00	0.70	0.40	0.50	8.00					

LB, lower bound; UB, upper bound; PFOS, perfluorooctanesulfonic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid ; ∑(PFAS), perfluoroalkyl substances sum; ML, maximum level set by Commission Regulation (EU) 2023/915 for offal of bovine animals, sheep, pig, and poultry; LC, left censored (%); na, not applicable. *Group with one sample.

Table 4. Lower bound and upper bound mean concentration of perfluorooctanesulfonic acid, perfluorooctanoic acid, perfluorononanoic acid, perfluorohexane sulfonic acid, and ∑(perfluoroalkyl substances) in chicken liver.

		PFOS		PFOA		PFNA		PFHxS		Σ(PFAS)	
Rearing system	n. samples	LB±SE (µg/kg)	UB±SE (µg/kg)	LB±SE (µg/kg)	UB±SE (µg/kg)	LB±SE (µg/kg)	UB±SE (µg/kg)	LB±SE (µg/kg)	UB±SE (µg/kg)	LB±SE (µg/kg)	UB±SE (µg/kg)
Indoor	12	0.00±0.00	0.04±0.00	0.53±0.01	0.53±0.01	0.07±0.01	0.07±0.01	0.00±0.00	0.08±0.02	0.67±0.03	0.71±0.03
Outdoor	9	0.18±0.07	0.20±0.07	0.54±0.02	0.54±0.02	0.07±0.01	0.07±0.01	0.00±0.00	0.06±0.02	0.83±0.09	0.87±0.09
p-value		<0.01	<0.01	0.41	0.41	0.76	0.76	.	0.59	0.08	0.06

LB, lower bound; UB, upper bound; SE: standard error; PFOS, perfluorooctanesulfonic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; ∑(PFAS), perfluoroalkyl substances sum.

range rural poultry shows significantly higher detectable PFOS (LB: 0.18 *vs*. 0.00 µg/kg; UB: 0.20 *vs*. 0.04 µg/kg, respectively; p<0.01; Table 4), while no significant difference was observed for PFOA, PFNA, and PFHxS. Overall, total PFAS content tended to be higher in the livers of outdoor birds than in those of the indoor counterparts (UB: 0.83 *vs*. 0.67 µg/kg; LB: 0.87 *vs*. 0.71 µg/kg, respectively; p=0.08 and p=0.06). These findings further strengthen the link between PFAS contamination and the potential for chickens to access and explore outdoor environments and be in contact with the soil (Death *et al*., 2021; Gazzotti *et al.*, 2021). In contrast, poultry raised in indoor intensive and semi-intensive systems display similar PFAS levels, with minimal variation in PFOA concentrations, suggesting that controlled environments and feed can contribute to more uniform PFAS exposure in these systems. Although older poultry, particularly those raised free-range, tend to present higher PFAS concentrations, the limited data available call for further research to confirm this age-related association.

Conclusions

The method applied in this study successfully met all acceptance criteria, confirming its effectiveness in analyzing the target matrix and providing reliable data.

Preliminary results, albeit based on a limited sample size, suggest that farming systems can influence PFAS accumulation in liver tissue, with potential implications for human consumption, even though none of the analyzed samples exceeded the EU regulatory limits. In particular, chickens raised in rural free-range systems presented greater hepatic PFAS content, further reinforcing the importance of access to outdoor environments and soil contact on PFAS contamination. Therefore, it is necessary to continue monitoring activities to confirm these findings, expanding the investigation to a larger sample of subjects and diverse geographic areas to assess the impact of various environmental factors (*e.g.*, feed, soil, and water quality) on PFAS contamination in chicken offal, with the aim of identifying appropriate mitigation actions to reduce food safety risks.

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Online supplementary material:
Supplementary Table 1. MS/MS detection parameters
Supplementary Table 2. Method trueness (bias%) and precision (CV%) under intra-day and inter-day conditions.