Assessment of Per- and Polyfluoroalkyl Substances (PFAS) in Traditional, Organic and Protein Flours

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1. Introduction

Human exposure to per- and polyfluoroalkyl substances (PFAS) is strictly connected with food consumption. To gather occurrence data in foods is of the utmost importance and on this base, much research has been conducted (Mohamad et al.,2023; Surma et al., 2023; Piva et al., 2022) in this fields. Among PFAS, the group composed by potassium perfluoro-1-octanesulfonate (PFOS), perfluoro-n-octanoic acid (PFOA), perfluoro-n-nonanoic acid (PFNA) and potassium perfluorohexanesulfonate (PFHxS) is considered at higher risk to cause developmental effects and to have adverse effects on serum cholesterol, the liver and the immune system and birth weight in humans (Sunderland et al., 2019; Rickard et a., 2022). For this reason, the European Food Safety Authority (EFSA) established a tolerable weekly intake (TWI) of 4.4 ng/kg body weight per week for the sum of 4 frequently detected PFAS (PFOA, PFNA, PFHxS, and PFOS) in serum. As PFAS are known to be persistent in the body, the EFSA derived a TWI rather than a Tolerable Daily Intake (TDI). The EFSA guidelines derived TWI amounts considered protective against the effects of those substances. European Regulation (Commission Recommendation EU, 2022) set limits for PFOS, PFOA, PFNA and PFHxS concentration and the corresponding sum for eggs, fish meat, crustaceans, bivalve molluscs, meat and offal of farmed and wild animals. Moreover, EU recommend Member States to monitor the presence of PFAS in food during the years 2022, 2023, 2024 and 2025 (2022) also including fluorotelomers and other emerging PFAS. Specimen should reflect all consumption habits and thus cover all types of food, such as fruits, vegetables, starchy roots and tubers, seaweed, cereals, nuts, oilseeds, food for infants and young children, food of animal origin, non-alcoholic drinks, wine and beer. Particular attention should also be paid to feed provided to animals devoted to human consumption in consideration of the biomagnification process (Goeritz et al., 2013). The term

biomagnification is used to describe trophic enrichment of contaminants within food webs and refers to the progressive increase in chemical concentrations through the animal trophic chain. For example, biomagnification has been reported in the eggs of hens raised and fed on a contaminated site. The highest concentration of contaminants was observed in the egg yolk (Göckener et al., 2020). In fact, although the EU requested method performance limits are very low for fruits, vegetables and baby-food, protein-based food is allowed to contain higher values in view of the biomagnification process. EFSA concluded that PFAS transfer from feed to animal derived food, with differences between species and the type of PFAS, but it can occur also from soil ingested by foraging farm animals and from drinking water for animals. As a conclusion, required limits of quantification (LOQ) in µg/kg (w/w) for the four individual PFOS, PFOA, PFNA, PFHxS in vegetables, fruits and baby food are 0.010 µg/kg for PFOS, 0.010 µg/kg for PFOA, 0.005 µg/kg for PFNA and 0.015 µg/kg for PFHxS (EURL, 2022). Values greater are possible for fish, eggs, edible offal of terrestrial animals.

Notwithstanding flours are the base of many local diets, including the Mediterranean diet, the available data on the incidence of PFAS in this type of food are scarce, and as a consequence, flours themself are not provided by specific limits. To the best of the authors' knowledge, most studies have so far focused on animal-derived food, which generally represents the highest source of ingested PFAS. Only recently research has shifted to the monitoring of these contaminants also in vegetables and baked processed foods. Flours have not been extensively investigated and data on their presence is difficult to find in literature.

This study aims to collect informative data on the presence of these contaminants in flours, reflecting their widespread consumption in many areas of the world and to address the need to infer information regarding the total exposure deriving from this staple food.

In addition to traditional flours made from cereals such as wheat, oat, and corn, protein-based flours have also been included in this study due to the rising popularity of alternative foods across Europe. For instance, cricket flour has been examined, taking into account the various types of feed provided to the insects. This inclusion aims to provide a comprehensive understanding of the presence of contaminants in both conventional and emerging food products, reflecting the evolving dietary trends and consumption patterns. Moreover, an experimental setup has been established to investigate the effects of different feed types on animal-derived flours. Crickets were harvested and fed a variety of diets, including shrimp, cookies, dog kibble, and oats, to assess how these feed variations influence the characteristics of the resulting flours. Organic cereals-based flours have also been included in the study.

The analytical method leverages a previously developed LC-MS method, with few modifications, already published, for determining PFAS and fluorotelomers in vegetal food materials (Piva et al., 2023). Results are presented as type and amount of the detected PFAS in the different classes of flours.

2. Materials and methods

2.1 Materials

Analytes and isotopically labelled standards (> 98% purity) were purchased from Wellington Laboratories (Guelph, Canada). Native analytes included perfluoro-n-butanoic acid (PFBA); perfluoro-n-pentanoic acid (PFPeA); perfluoro-n-hexanoic acid (PFHxA); perfluoro-n-heptanoic (PFHpA); PFOA; PFNA; perfluoro-n-decanoic acid (PFDA); perfluoro-n-undecanoic acid (PFUnA); perfluoro-n-dodecanoic acid (PFDoA); potassium perfluoro-1-butanesulfonate (PFBS); sodium perfluoro-1-pentanesulfonate (PFPeS); PFHxS; sodium perfluoro-1-heptanesulfonate (PFHpS); PFOS; 1H,1H, 2H,2H-perfluoro-1-hexanesufonate (4:2 FTS); 1H,1H, 2H,2H-perfluoro-1-octanesufonate (6:2 FTS); 1H,1H,

2H,2H-perfluoro-1-decanesufonate (8:2 FTS); potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS); potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate (11Cl-PF3OUdS); potassium perfluoro (2-ethoxyethane) sulfonate (PFEESA). Isotopically labelled compounds, added to the sample before extraction in known amount (surrogate) were used. Since not all target PFAS have an isotopically labelled analogue, an alternate labelled compound is used as recommended in EPA-533 method (for details see Supplementary material).

2.2 Sample Collection

A total of 17 samples were collected as: organic flours (n. 4: wheat flour, whole wheat flour, chickpeas flour, oat flour); traditional flour (n.6: durum wheat flour, wheat flour, durum wheat semolina, glute-free flour, corn flour, chickpeas flour); flour from animal origin used for animal feed (n.3: trout flour, poultry flour, cricket flour); n.4 in-house harvested cricket flour (for their husbandry see section 2.3). All commercial flour were acquired in local stores. All samples were stored at dark and cool conditions until the analysis.

2.3 Cricket husbandry

In order to investigate the potential correlation between diet and the presence of PFAS in the house cricket, *Acheta domesticus* (Linneaus, 1758), one of the insects currently included in the list of European insects suitable for human consumption, a laboratory breeding experiment was conducted. One hundred specimens of *Acheta domesticus* were purchased from a local fisherman's warehouse and were provided with four different diets: 1) dog dry food (kibble) (Companino, EXPERT Mini); 2) fish meal; 3) dry biscuits (Dolce mattino, Carrefour); and 4) oat flakes (Carrefour). Additionally, catnip (*Nepeta cataria*) was provided to ensure an adequate water intake for the specimens. The breeding was conducted in plastic boxes furnished with egg cartons to offer a sheltered

environment for the crickets. At the end of the husbadry, crickets were collected and placed in the freezer inside polypropylene Falcon tubes to limit PFAS contaminations. Subsequently, the specimens were placed in a controlled and constant temperature dryer at 50°C for one week to completely dehydrate and facilitate their transformation into flour. The dried material was then grounded inside polypropylene Falcon tubes.

2.4 Sample preparation

One ± 0.10 gram of flour was sampled after homogenisation from each product and added of 3 µL of isotopically mass-labelled standards into a 50 mL polypropylene tube. Samples were extracted by using 5 mL of acetonitrile acidified with 0.1% of formic acid and placed in an ultrasound bath for 30 minutes. Subsequently samples were centrifuged at 8000 g for 15 min. Supernatants were purified by weak anion exchange solid-phase extraction (SPE) according to the validated procedure (Piva *et al.*, 2023). Briefly, column conditioning was carried out by using 5 mL of methanol and 3 mL of formic acid 2% in water. After sample loading, the cartridge was washed with 3 mL of formic acid 2% in water and 3 mL of methanol. Cartridge was dried for 15 min and then a 2-step elution was performed with 2 mL of methanol / NH4OH (90:10, v/v), twice. Extracts were taken to dryness under a stream of nitrogen at 45°C. Dried extracts were reconstituted in 300 µL methanol / water (10:90, v/v).

2.5 Instrumentation and analysis

Analyses were carried out in a LC-MS/MS system composed of 1290 Infinity II coupled to an 6495C triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). Separations were achieved in a ZORBAX Eclipse Plus C18 2.1 x 50 mm, 1.8 µm (Agilent Technologies). The mobile phase A was composed of 20 mM ammonium acetate in water

and mobile phase B consisted in methanol. The column temperature was set at 50°C and flow rate was 0.250 mL/min. The gradient was as follow: 10%B at time 0 min; 30%B at 2 min, 95%B at 14 min, 100%B at 14.50 min, 10%B at 15.50, isocratic at 90% for 1 min, post run up to 17 minutes. The injection volume was 5 µL. The delay column was introduced after the pump and was a ZORBAX Eclipse Plus C18 3.0 x 50 mm, 1.8 µm (Agilent Technologies) to trap any system related interferences. Mass spectrometer operated in negative acquisition mode and source parameters were as follow: capillary Voltage 2500V, gas temperature 250°C, gas flow 11 L/min, Nebulizer 25 psi, sheath gas temperature 375°C, sheath gas flow 11 L/min, capillary voltage 3000 V. All source parameters were optimized under LC conditions. Acquisition was performed in multiple reaction monitoring (MRM) by acquiring at least two transitions for each compound (except PFBA and PFPeA, due to their chemical structure). Data analysis was carried out by MassHunter software (version 10.0, Agilent Technologies). Details on retention times and multiple reaction monitoring data are reported in the Supplemental material.

2.6 Sample extraction optimization

Different solvents were tested to optimize extraction from flours. Methanol, acetonitrile, methanol/acetonitrile 50/50 (v/v) were selected on the basis of the literature as extraction solvents for PFAS. The addition of formic acid at 0.1% in each solvent and dehydration of flour prior extraction were also evaluated.

2.5 Method Validation

The present research represents a new application of the previously validated method (Piva et al., 2023) and for this reason, the tested parameters were reduced to selectivity, sensitivity, linearity, accuracy, precision, matrix effect and stability. The results

were compared to acceptance criteria reported in the “Guidance Document on Analytical Parameters for the Determination of Per- and Polyfluoroalkyl Substances (PFAS) in Food and Feed” (European Union Reference Laboratory, 2022) for monitoring purposes. The selectivity of the method was evaluated in one sample of flour for each category taken into consideration (traditional, organic, protein and cricket) to check for possible interfering compounds. The sensitivity of the method, expressed as limit of detection (LOD) and limit of quantification (LOQ), was calculated for each compound by signal to noise ratio (S/N) of the qualifier transition (when present). LOD and LOQ were defined as signal-to-noise ratio (S/N) equal to 3 and 10, respectively and verified experimentally by spiking mass-labelled surrogates at the calculated concentrations. Linear calibration curves were prepared using standard solution consisting of a concentration series of 0.05, 0.125, 0.25, 0.50, 1.25, 2.5, 5 ng/ml for each analyte. Origin was not included and a weight factor of 1/x was applied. Matrix-matched calibration was not required due to the isotope-labelled surrogate used as internal standard for each target analyte. Accuracy, precision, matrix effect and stability were assessed on in-house quality controls (QC) prepared by spiking mass-labelled surrogates in chickpeas flour at the concentration of 1 and 3 ng/ml to avoid any contribution of the native PFAS (n =6). Matrix-effect was evaluated through isotope-labelled surrogate performance. Stability of the processed samples was studied by analysing samples stored at -80°C for 4 days. Prepared procedural blanks were processed in parallel with the samples to evaluate contamination throughout the analytical procedure.

2.6 Statistical analysis & data interpretation

Descriptive statistics was provided for all compounds. Samples were then classified as organic, normal or protein flours and for each PFAS, median and interquartile ranges (IQ) in the three types of flours were calculated. In order to include in the statistical evaluation results < LOQ, these were calculated as LOD/√2. Statistical differences among organic,

normal and protein flours considering single compounds and PFAS sum were calculated by Kruskal-Wallis test, followed by post-hoc analysis. Only compounds detected in at least 50% of the samples were considered for this analysis. Finally, the Kruskal-Wallis comparison was repeated by considering organic, normal and protein flours only for human consumption, with the exclusion of trout flour and poultry flour.

3. Results and discussion

3.1 Method optimization and validation

Best results in terms of absolute recovery were obtained in acidified acetonitrile without the need for dehydration before extraction (see Figure 1 in Supplementary material). Recovery fitted the established limits of 65-135% for all compounds, except for mass-labelled surrogates of the fluorotelomers 4:2FTS, 6:2FTS, 8:2FTS, which showed absolute areas above 150% and for this reason the present method should be considered only qualitative for these analytes.

Method validation data obtained under the described analytical parameters proved suitable for most of the analytes. Limit of detection was in the range 0.003 – 0.01 ng/g, while limit of quantification was fixed at the lowest calibration point of the curve corresponding to a concentration of 0.015 ng/g, except for PFDoA, which was calculated at 0.038 ng/g. LOQ was routinely re-examined throughout the method development and analysis to ensure that the reported limits would be representative of the system under operational conditions. Accuracy results were in the range 1-25% for all PFAS at both QC levels. Precision was comprised in the range 3-25%; accuracy and precision data proved suitable for the monitoring purposes. Matrix-effect was in the range 80-120%, except for PFHxA and PFOS. Stability of processed samples was in the range of -35% and +12%. Procedural blanks

225 reported PBFA contamination above LOQ and for this reason data on PFBA are not reported
226 further. All data from validation procedure are reported in Supplementary Material.
227

228 3.2 Samples Analysis

229 Among the tested PFAS, 15 were found in the analysed material, although some of
230 them were scarcely represented due to concentration below LOQ or found in only one
231 sample (PFPeS, 9Cl-PF3ONS) (details in table 5 of Supplemental material). The most
232 detected PFAS at quantifiable concentrations were PFOA and PFDA in the range 0.015-
233 0.134 ng/g, and 0.015-0.219 ng/g, respectively. A graphical representation of the detection
234 frequency and concentration for each compound is reported in figure 1 and 2.
235 The sum of PFAS in all samples ranged from 0.015 ng/g in organic whole wheat flour,
236 organic oat flour and durum wheat semolina up to 2.014 ng/g in flour obtained from flour
237 produced in-house from cricket harvested with shrimps. The sum for the PFAS considered
238 in the EFSA document for the tolerable week intake, PFOS, PFOA, PFHxS and PFNA,
239 concentration varied from 0.029 ng/g in wheat flour up to 1.469 ng/g in cricket flour. When
240 considering only commercial flour the concentration ranged 0.015- 0.958 ng/g for all PFAS
241 and 0.029 ng/g-0.663 ng/g for the sum of the 4 PFAS. The highest concentrations were
242 found in flours derived from fish 0.958 ng/g and 2.014 ng/g for trout flour and cricket
243 harvested with shrimps, respectively. Samples were arbitrarily grouped in the following
244 categories: organic, traditional and protein flours for the statistical analysis. The median
245 and interquartile ranges for single compounds and for the sum of compounds are shown in
246 Table 6 of Supplemental material. Organic flours were characterized by a median of 0.026
247 ng/g (interquartile range 0.019-0.037), while traditional and protein flour showed median
248 values of 0.094 ng/g (interquartile range 0.031-0.183) and 0.522 ng/g (interquartile range
249 0.077-0.881). By comparing organic, normal and protein flours, only PFOS and the sum of

PFAS showed a statistically significant difference, with p = 0.0146 and p = 0.0062 respectively. In both cases, the post-hoc analysis showed that protein flours had significantly higher amount of the compound compared to the organic ones (p = 0.0462 and p = 0.0108 respectively). All the performed comparisons are shown in Figure 3. PFUnA and PFDoA were excluded due to their detection in less than 50% of the samples. Comparisons were repeated by considering, among protein flours, only those for human consumption (i.e. excluding trout and poultry flours), confirming that PFOS and the sum were significantly higher in protein flour compared to organic one (Figure 4). Additionally, PFBS and PFOA appeared significantly higher in protein flour for human consumption (p = 0.0154 and p = 0.0224), as shown by the Kruskal-Wallis test.

4. Discussion

Consumers are increasingly concerned about food safety, particularly regarding the presence of contaminants recognized as toxic for humans. PFAS are recognised as endocrine-disrupting chemicals (EDC) and are also associated to chronic pathologies such as dyslipidaemia and recently to birth defects. They have been found accumulating in almost all human tissues, including the most peripherical such as hair, placenta, semen and breast milk (Di Giorgi et al., 2023; Di Giorgi et al., 2024; LaKind et al., 2022; Pascali et al., 2023). Due to their widespread distribution and persistence in the environment, they are constantly detected in food even after their phase out in Europe and Unites States of America for some of them. As a result, food regulations primarily concentrate on setting safety limits for these contaminants, with a foundation in the precautionary principle. The paradigm of tolerable daily intake for compounds associated to health effects effectively addresses the issue of food-related exposure, which is one of the most significant routes of exposure for PFAS. Both EFSA and European Commission (EC) are steadily working on regulation to set PFAS maximum levels in foodstuffs and on this ground large-scale

food monitoring is encouraged by the European Commission through the years 2022-2025. The monitored foodstuffs should respond to consumption habits, including fruits, vegetables, starchy roots and tubers, mushrooms, seaweed, cereals, nuts, oilseeds, food for infants and young children, food of animal origin, non-alcoholic drinks, wine and beer. Flour is a versatile ingredient that can be derived from various sources, including cereals and proteins. Its diverse composition allows for a wide range of culinary applications, making it a staple in many diets worldwide.

Additionally, consumers are increasingly interested in protein-based flours and gluten-free products due to specific health concerns. These health-driven preferences often arise from the need to avoid allergens or manage conditions like celiac disease. However, it's important to note that these specialized products can sometimes concentrate contaminants present in the original raw materials, raising the need for stringent quality control and thorough testing to ensure their safety and purity.

Flours are also commonly used in animal feed as a protein-rich food source, such as trout or poultry flours. These animal-based flours play a crucial role in the nutrition of livestock and poultry, enhancing their growth and overall health. As a result, they ultimately enter the human food chain, highlighting the importance of monitoring and regulating the quality of these flours to ensure the safety and nutritional integrity of the food consumed by humans.

The flour daily intake can be very high in many diets across the world in form of processed food or pasta. Is has been estimated that the average daily consumption of flour per person can reach approximately 200-250 grams per day, translating to around 6-7.5 kilograms per month, depending on dietary habits and cultural practices across regions. Based on this, the amounts of contaminants ingested through the consumption of flour, whether in at-home products or processed into various foods, could potentially reach considerable levels.

In the frame of offering monitoring data as encouraged by the EC, a study has been conducted on the presence of PFAS in flours of different origin commonly found and consumed by the population. As preliminary research 17 samples among traditional, organic and protein flours have been monitored for 16 PFAS. PFAS were encountered in all samples from < LOQ up to 1.963 ng/g, the occurrence of PFAS was 6:2FTS>PFOA=PFDA>PFHxA>PFOS=PFNA=PFuNA>PFBS=PFDoA. Our data revealed that some compounds were preferentially present only in protein-based flours such as crickets and trout flours and almost absent in other types of flours (i.e. PFOS). Differently, PFOA was present almost in all samples, although mostly at <LOQ. Organic flours contained lower amounts of contaminants, and this was confirmed also by the statistics. When organic and traditional food had been compared, lower amounts of PFAS were present in organic preparation, suggesting a contribution due to pesticides. The discrepancies were even more pronounced when considering protein-based flours, which contained significantly higher concentrations of PFOA, PFOS, and PFBS compared to organic flours.

According to the European Food Safety Authority (EFSA), a tolerable weekly intake (TWI) of 4.4 ng/kg body weight per week has been established for the sum of PFOS, PFOA, PFNA, and PFHxS. For a 70 kg individual, this translates to an estimated total intake of 308 ng per week. Our data indicated that the sum of these compounds, for a 200 g/day diet, can reach 40 ng/week in wheat flour, 102 ng/week in gluten-free flour, and 2,058 ng/week in crickets harvested with shrimps. The amounts detected in flours can significantly impact a diet, particularly given the maximum protective limit of 308 ng/week. Therefore, it is crucial to monitor raw flours, including new varieties, to ensure they remain within safe consumption limits. To the best of the authors' knowledge, data on flours are very scarce since many studies take into consideration the processed food such as bakery and pizza, etc. A recent paper (Surma et al., 2022) considered wheat and rye flours,

beside other processed foodstuffs, finding very high concentrations of PFBA, up to 119.68 ng/g and PFOA as the most frequent compound with the highest concentration of 0.430 ng/g. PFOS was found only in processed foodstuff, while PFPeA was frequently found at concentration below LOQ. Aside this study it is very difficult to infer other information on PFAS presence in flours, however assessing PFAS content in these foodstuff merits more attention since flours are at the base of the food pyramid and in consideration of the total intake, amounts may not be negligible. In recent years, an increasing number of consumers have started embracing edible insects, not just as a snack but also as a side dish or an ingredient for other foods (Acosta-Estrada et al., 2021; Castro Delgado et al., 2020). Crickets' based flours have been included because are considered a good candidate protein source (Kouatcho et al., 2022) at low environmental impact. Unfortunately, insects can be a vector for PFAS accumulation depending on the adopted feed. It has been recognized in literature that invertebrates represent the base of the environmental food web in the way that can be used also to infer PFAS contamination up to higher trophic levels (McDermett et al., 2022). In our study both commercial crickets' flour and in-house harvested crickets' flour have been considered being the PFAS amounts not very different (range 0.06-0.166 ng/g) except for crickets fed with dog kibbles and shrimps (0.318-1.963 ng/g), confirming a higher amount of PFAS for feed from animal origin. Of course, more studies are needed to confirm our results both in terms of occurrence and concentration of PFAS in this specimen and it not possible to infer data or suggest any decisional threshold on the base of the results. In fact, this application was intended as a proof-of-concept study to determine whether it is worthwhile to investigate on flours. Notwithstanding the intrinsic limits of this study (i.e. limited number of samples, samples collected in only one country) it is in the authors' opinion to consider flours and how there are produced when evaluating the PFAS total intake in consideration of the amounts consumed worldwide.

5. Conclusions

This research examined the PFAS content in organic, traditional and protein flours. 6:2FTS, PFOA, PFDA and PFHxA were frequently detected in all the analysed samples. Organic flours contained the lowest amounts of PFAS. Protein flours showed the highest values for PFAS, especially PFOS. The impact of flours content on PFAS total intake should be considered for human consumption, but also as feed for animals since it represents the entrance into the food chain.

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Statements and Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design [Jennifer Pascali, Arianna Giorgetti, Susan Mohamed, Michele Pozzebon, Stefano Vanin, Filippo Pirani, Guido Pelletti, Paolo Fais]. Material preparation, data collection and analysis were performed by [Jennifer Pascali, Stefano Vanin, Arianna Giorgetti, Susan Mohamed]. The first draft of the manuscript was written by [Michele Pozzebon and Jennifer Pascali] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

Data are available upon request.