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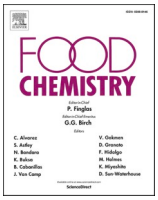

A new method for determining PFASs by UHPLC-HRMS (Q-Orbitrap): Application to PFAS analysis of organic and conventional eggs sold in Italy

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ABSTRACT

Per- and polyfluoroalkyl substances (PFASs) are ubiquitous environmental pollutants with the ability to uptake to food and feed. Among food, fish, fruits and eggs are considered as major contributors to human dietary exposure. A new method was developed and validated for the simultaneous determination of 18 PFASs in eggs using isotope dilution followed by ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry. The analysis of 132 samples (organic, barn and caged eggs) was performed. Levels were always close to the detection limits and no significant difference emerged among the 3 groups. The highest PFAS concentration in eggs was used to estimate the dietary exposure of different Italian population groups. As expected, children were more highly exposed than adults due to lower body weight. This data suggests that the recent tolerable weekly intake of 4.4 ng kg^{-1}b.w. could be exceeded when the cumulative intake arising from other food products is considered.

1. Introduction

Per- and poly-fluoroalkyl substances are compounds with fluorinated alkyl chain (length usually C4-C16) ending with a polar group (typically carboxylic or sulfonic). The presence of perfluoro-alkyl chain together with hydrophilic end group gives to these compounds surface-active properties, while the strength of carbon–fluorine bond justifies thermal and chemical stability (Buck et al., 2011; Cousins et al., 2020). Due to their characteristics, PFASs are widely used in many industrial applications and in many consumer products (Buck et al., 2011; Cousins et al., 2020; DeLuca, Angrish, Wilkins, Thayer, & Cohen Hubal, 2021; Glüge et al., 2020) such as surfactants and repellents of water, dirt and oil (Death et al., 2021). Since the early 2000's PFASs have drawn public attention, and global manufacturers, already, tend to replace long-chain PFASs, in particular Perfluorooctane sulfonic acid (PFOS) and Perfluorooctanoic acid (PFOA), with alternative chemicals. These analytes, in fact, are considered persistent organic pollutants (POPs), listed in Stockholm Convention on POPs (United Nations Environment Programme (UNEP), 2019) and their manufacturing, commercialization and use are regulated in Europe (Parliament, 2019). They are considered "emerging contaminants" with potential long-term adverse effects on humans including increased cholesterol levels, liver disease, reduced fertility, thyroid disorders, changes in hormone functioning, changes in the immune system, and adverse developmental effects (DeLuca et al., 2021; EFSA, 2018; EFSA, 2020; Kwiatkowski et al., 2020). In addition, there are other compounds known as precursors that have been used in

Abbreviations: b.w., body weight; dd-MS2, data-dependent scan mode; HESI-II, electrospray interface; ISi, standards for injection; ISp, standards for processing; LB, Lower bound; LOD, limit of detection; LOQlimit of quantification, MRL, minimal risk level; MS/MS, tandem mass spectrometry; MU, measurement uncertainty; PFAS, Per- and polyfluoroalkyl substances; PFBA, Perfluorobutanoic acid; PFDA, Perfluorodecanoic acid; PFDoDA, Perfluorododecanoic acid; PFDoDS, Perfluorododecane sulfonic acid; PFDS, Perfluorodecane sulfonic acid; PFHpA, Perfluoroheptanoic acid; PFHpS, Perfluoroheptane sulfonic acid; PFHxA, Perfluorohexanoic acid; PFHxS, Perfluorohexane sulfonic acid; PFNA, Perfluorononanoic acid; PFNS, Perfluorononane sulfonic acid; PFOA, Perfluorooctanoic acid; PFOS, Perfluorooctane sulfonic acid; PFPeA, Perfluoropentanoic acid; PFTrDA, Perfluorotridecanoic acid; PFUnDA, Perfluorobutane sulfonic acid, POP, persistent organic pollutant; QqQ, triple quadrupole; RSD, relative standard deviation; TWI, tolerable weekly intake; UB, Upper bound; UHPLC-HRMS, ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry.

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industrial processes and commercial product manufacturing. Perfluorooctane sulfonamidoacetic acids (FOSAAs) and perfluorooctane sulfonamides (FOSAs), for example, can be biotransformated in perfluorooctane sulfonic acid (PFOS), as well as polyfluoroalkyl phosphate esters (PAPs) can be biotransformated into perfluoroalkyl carboxylic acids like PFHxA, PFOA, PFDA. These chemicals are less persistent in the environment, but should be taken into account due to their transformation into PFASs of interest which can result in indirect contributions to human dietary exposure (EFSA, 2018, EFSA, 2020). It has been suggested that food and drinking water are the main contributors to human exposure, although it has been recently observed that volatile PFASs present indoors could also have an important role on a total risk exposure to humans via inhalation of air and dust particles ingestion (De Silva et al., 2021; DeLuca et al., 2021; Jian et al., 2017; Morales-McDevitt et al., 2021; Schwanz, Llorca, Farré, & Barceló, 2016; Zafeiraki et al., 2015). During the past few years, directive (EU) 2020/2184 fixed minimum requirements for parametric values related to "total PFASs" and "sum of PFASs" to assess the quality of water intended for human consumption (European Union, 2020). Despite this, there are still no enforced legal limits regulating the PFAS maximum concentration in food and foodstuffs, but a major attention is required to monitor their levels.

In 2020, the Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency (EPA) revised the estimate of daily human exposure (MRL – minimal risk level) for PFOA and PFOS to 3.0 and 2.0 ng/kg bodyweight (b.w.) and set new MRLs for Perfluorohexane sulfonic acid (PFHxS) and Perfluorononanoic acid (PFNA) to 3.0 and 20 ng/kg b.w., respectively (ATSDR, 2021).

In the same year, the European Food Safety Authority (EFSA) used a combined exposure method to set a new tolerable weekly intake (TWI) limit of 4.4 ng/kg b.w. for a mixture of four PFASs (PFOS, PFOA, PFNA, and PFHxS) (EFSA, 2020). The new TWI was lower than the values set in the previous opinion (EFSA, 2018), where it was expressed separately for PFOS and PFOA at 13 and 6 ng/kg b.w., respectively.

Among food categories, EFSA identified 'Fish meat', 'Fruit and fruit products' and 'Eggs and egg products' as major contributors to human PFAS dietary exposure (EFSA, 2020). Hen eggs are considered as food of high nutritional value and are widely consumed worldwide (Réhault-Godbert, Guyot, & Nys, 2019). The global marketplace of organics has grown rapidly over the last few decades and consumer demand for organic products is increasing globally (Vigar et al., 2020). Despite the fact that consumers consider organic food, including eggs, healthier than conventional ones, this food is equally exposed to environmental contaminants which depend on their closeness to anthropogenic sources of contamination. Some studies reported higher levels of environmental pollutants in organic foods than in conventional ones, and this means that it is necessary to constantly monitor contaminant levels also in organic food (D'Hollander, De Voogt, & Bervoets, 2011; González, Marquès, Nadal, & Domingo, 2019). Previous studies focused on investigation of home produced eggs as an indicator of PFAS environmental contamination, and consequently as an estimation of human intake associated with their consumption (D'Hollander et al., 2011; Gazzotti et al., 2021; Zafeiraki et al., 2016). To date, few researchers have investigated contamination levels of commercial eggs that are the main source of supply for Italian general population (ISMEA, 2021).

The difficulties associated with the analysis of PFASs at ultra-trace levels in food samples have hampered the understanding of human exposure. Among the analytical challenges related to PFASs in food, special attention should be paid to high sensitivity and selectivity of analytical methods. In food such as milk, liver, fish and egg, the classic methodology for determining PFASs involves extraction and clean-up procedures followed by liquid chromatography coupled to different mass analyzers (Chiesa et al., 2018; Zacs & Bartkevics, 2016). Among them, tandem mass spectrometry (MS/MS) with triple quadrupole (QqQ) is the most frequently used detection technique. However, special care should be taken to avoid the interference of endogenous compounds present in biological matrices that co-elute with the analytes and share the same MS/MS transitions. In particular, in some food matrices such as eggs and liver, taurodeoxycholic acid as bile salt has been observed to coelute with PFOS. Due to its similar mass, it is difficult to discriminate these compounds in QqQ tandem mass spectrometry with a consequent overestimation of PFOS. In this study, the high resolution mass spectrometry (HRMS) technique allows discriminating between two exact masses (Ballesteros-Gómez, Rubio, & van Leeuwen, 2010).

Recent advances in analytical chemistry and increased availability of high quality labelled standards have significantly improved the performance on PFAS analysis. In general, the use of HRMS techniques have been shown to afford trace analysis of different compounds in complex matrices, ensuring high sensitivity and selectivity. To date, just a few studies used Orbitrap-MS technique to implement analysis of targeted PFASs in food matrices (Barola et al., 2020; Chiesa et al., 2018; Zacs & Bartkevics, 2016).

Since EFSA suggests determining PFASs with the lowest possible quantification limits in food, a more sensitive analytical method is needed to measure PFASs in these matrices. Furthermore, more data are needed concerning the PFAS content in eggs, one of the major contributors to dietary exposure.

This study developed a sensitive method for the simultaneous determination of 18 PFASs in eggs using isotope dilution followed by ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). Furthermore, this study evaluated contamination levels of PFASs in egg samples taken from the Italian market, and potential differences between organic and conventional (barn and caged) eggs were also investigated. Finally, the occurrence data generated from this study were used to estimate the contribution of eggs to the dietary intakes of PFASs for children, adolescents and adults in Italy.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (LC–MS grade, ≥ 99.9 %), methanol (LC-MS grade, ≥ 99.95 %), 30 % ammonia solution, ammonium acetate (≥99 %), acetic acid (≥99 %), and sodium acetate (≥99 %) were purchased from Sigma (Darmstadt, Germany), while water (HPLC grade) was purchased from Biosolve chimie (Dieuze, France). QuEChERS Extraction Packets, EN Method (4 g of magnesium sulphate, 1 g of sodium citrate, 1 g of sodium chloride, 0.5 g sodium hydrogen citrate sesquihydrate) were obtained from Agilent Technologies (Santa Clara, USA). SPE Strata X-AW (up)/XL (bottom) (150 mg/150 mg, 6 mL) cartridges were purchased from Phenomenex (Torrance, USA). Native PFASs for this study were 10 perfluoroalkyl carboxylic acids (chain length, C5-C14) and 8 perfluoroalkane sulfonic acids (chain length, C4-C12). The native PFASs and corresponding internal standards for processing (ISp) and injection (ISi) are shown in Table 1. Native PFASs (chemical purities > 98 %), ISp and ISi (chemical purities > 98 %, isotopic purities > 99 %,) were obtained as three different mixtures with a concentration of 2 μg mL^{-1} in methanol from Wellington Laboratories (Guelph, Canada).

2.2. Egg sampling

PFAS contamination was evaluated in 132 egg samples collected in the period between October 2018 and February 2020. Samples were purchased in supermarkets because this is the main sales channel for eggs consumed in Italy (ISMEA, 2021). Samples came from various locations in Italy, even if the majority were produced in Northern Italy (e.g. Lombardy, Veneto and Emilia-Romagna). Eggs were divided into 3 groups, namely organic eggs (n = 57), barn eggs (n = 48), and caged hen eggs (n = 27), as indicated by the code stamped on the shells. After collection, the samples were taken to the laboratory where they were

Table 1
The 18 perfluorinated alkylated substances tested in this study with the corresponding isotopically labelled internal standards (ISs) for processing (ISp) and injection (ISi).

Chemical class	Chemical name	Molecular formula	ISp	ISi
Perfluoroalkyl carboxylic acids				
PFPeA	Perfluoropentanoic acid	$C_5HF_9O_2$	[$^{13}C_5$] PFPeA	[$^{13}C_2$] PFOA
PFHxA	Perfluorohexanoic acid	$C_6HF_{11}O_2$	[$^{13}C_5$] PFHxA	[$^{13}C_2$] PFOA
PFHpA	Perfluoroheptanoic acid	$C_7HF_{13}O_2$	[$^{13}C_4$] PFHpA	[$^{13}C_2$] PFOA
PFOA	Perfluorooctanoic acid	$C_8HF_{15}O_2$	[$^{13}C_8$] PFOA	[$^{13}C_2$] PFOA
PFNA	Perfluorononanoic acid	$C_9HF_{17}O_2$	[$^{13}C_9$] PFNA	[$^{13}C_2$] PFOA
PFDA	Perfluorodecanoic acid	$C_{10}HF_{19}O_2$	[$^{13}C_6$] PFDA	[$^{13}C_2$] PFDA
PFUnDA	Perfluoroundecanoic acid	$C_{11}HF_{21}O_2$	[$^{13}C_7$] FUnDA	[$^{13}C_2$] PFDA
PFDoDA	Perfluorododecanoic acid	$C_{12}HF_{23}O_2$	[$^{13}C_2$] PFDoDA	[$^{13}C_2$] PFDA
PFTrDA	Perfluorotridecanoic acid	$C_{13}HF_{25}O_2$	[$^{13}C_2$] PFDoDA	[$^{13}C_2$] PFDA
PFTeDA	Perfluorotetradecanoic acid	$C_{14}HF_{27}O_2$	[$^{13}C_2$] PFTeDA	[$^{13}C_2$] PFDA
Perfluoroalkane sulfonic acids				
PFBS	Perfluorobutane sulfonic acid	$C_4HF_9SO_3$	[$^{13}C_3$] PFBS	[$^{13}C_4$] PFOS
PFPeS	Perfluoropentane sulfonic acid	$C_5HF_{11}SO_3$	[$^{13}C_3$] PFHxS	[$^{13}C_4$] PFOS
PFHxS	Perfluorohexane sulfonic acid	$C_6HF_{13}SO_3$	[$^{13}C_3$] PFHxS	[$^{13}C_4$] PFOS
PFHpS	Perfluoroheptane sulfonic acid	$C_7HF_{15}SO_3$	[$^{13}C_3$] PFHxS	[$^{13}C_4$] PFOS
PFOS	Perfluorooctane sulfonic acid	$C_8HF_{17}SO_3$	[$^{13}C_8$] PFOS	[$^{13}C_4$] PFOS
PFNS	Perfluorononane sulfonic acid	$C_9HF_{19}SO_3$	[$^{13}C_8$] PFOS	[$^{13}C_4$] PFOS
PFDS	Perfluorodecane sulfonic acid	$C_{10}HF_{21}SO_3$	[$^{13}C_8$] PFOS	[$^{13}C_4$] PFOS
PFDoDS	Perfluorododecane sulfonic acid	$C_{12}HF_{25}SO_3$	[$^{13}C_8$] PFOS	[$^{13}C_4$] PFOS

homogenized and then stored at −18 °C until analysis.

2.3. Sample preparation

Samples were treated according to the modified QuEChERS method for samples of animal origin developed in 2017 by the European Union Reference Laboratory for food of animal origin (EURL, 2017), with some modifications to the purification step. Briefly, 5 g homogenate of whole egg was spiked with 50 μL of ISp in methanol (100 ng mL^{-1}) and extracted with 10 mL water and 10 mL acetonitrile. The sample was shaken using an automatic axial extractor (Agytax Cirta, Madrid, Spain) for 15 min. Then, one QuEChERS extraction packet, EN Method was added to each extract and shaken again with the automatic axial extractor (10 min). After centrifugation at 4500 rpm for 10 min at 4 °C, 5 mL of supernatant was evaporated to dryness under a nitrogen stream (40 °C) and 0.25 mL of 1 % acetic acid in methanol was added. The solid phase extraction (SPE) procedure involved different steps: (1) removing contaminants from the cartridge with 40 mL of 1 % NH_4OH in methanol, (2) conditioning with 6 mL of methanol and 6 mL of water, (3) loading the sample, (4) washing with 2 mL of 10 mM ammonium acetate in water, and (5) elution with 10 mL 1 % NH_4OH in methanol. Finally, the eluted solution was evaporated to dryness using a nitrogen stream and dissolved in 100 μL of ISi in acetonitrile (10 ng mL^{-1}), 60 μL of ammonium acetate 10 mM and 40 μL of acetonitrile.

2.4. Instrumental analyses

The analyses were carried out using an ultrahigh performance liquid chromatography (UHPLC, Ultimate 3000 Thermo Fisher Scientific) system coupled to a high-resolution mass spectrometer (Q-Orbitrap, Thermo Fisher Scientific). First, 5 μL of each extract was injected, and analytes were separated on a Luna Omega PS C_{18} column (2.1 × 100 mm, 1.6 μm; Phenomenex). In addition, Gemini C_{18} guard column (4.6 × 100 mm, 5 μm; Phenomenex) was inserted between the pump and injector in order to delay potential PFAS traces due to LC instrument and mobile phase. Mobile phase A was 10 mM ammonium acetate and mobile phase B was acetonitrile-methanol (1:1, vol./vol.). The flow rate was 0.3 mL min^{-1} for a total run time of 30 min, and the column temperature was set at 40 °C. Analytes were separated using a gradient elution scheme: for 3 min, phase B was maintained at 10 % and then it was increased to 50 % over 2.5 min; then, it was increased to 75 % over 1 min and kept constant for 10.5 min. Phase B was increased up to 85 % for 0.5 min and kept constant for 7.5 min. The latter switched back to the initial 10 % in 0.5 min and was kept constant for 4 min.

The UHPLC system was connected to Q-Orbitrap mass analyser (Q Exactive, Thermo Fisher Scientific) through a heated electrospray interface (HESI-II) operating in negative ionization mode. The parameters of HESI-II were the following: source and capillary temperature, 300 °C; electrospray voltage, 3.8 kV; S-lens, 50 arbitrary units (AU); sheath gas, 30 AU; auxiliary gas, 4 AU. The MS acquisition was performed in full scan MS/data-dependent scan mode (dd-MS2). The parameters of full scan were the following: mass range, 150–950 *m/z*; resolving power, 70,000 FWHM; automatic gain control (AGC), 1.0 × 10^6 (maximum number of ions filling C-Trap) with a maximum injection time of 100 ms using a mass accuracy ≤ 5 ppm. The dd-MS2 parameters were the following: resolving power, 17,500 FWHM; AGC 2.0 × 10^5 with a maximum injection time of 50 ms. The collision energies were set at 10 eV and 50 eV for perfluoroalkyl carboxylic acids and perfluoroalkane sulfonic acids, respectively. The monitored ion species are listed in Table 2. Identification criteria of targeted compounds included retention time, mass accuracy (≤5 ppm for both precursor and product ions), and fragment ions (at least detection of one qualifier ion reported in Table 2). Further details are reported in Supplementary Material (Table S5).

2.5. Method validation

According to SANTE/12682/2019 Guidelines (European Commission, 2020), the parameters to be checked for method were linearity, limit of detection (LOD), limit of quantification (LOQ), precision and trueness, and estimation of measurement uncertainty (MU). Linearity was tested by injecting four calibration points in triplicate into a range from 0.2 to 20 ng mL^{-1} for native compounds, and a fixed concentration of 5 ng mL^{-1} for ISp and ISi. LOD was determined using two approaches chosen on the basis of the presence or absence of the analytes of interest in the procedural blank (EPA, 2016). For the first approach including PFPeA, PFHxA, PFHpA and PFOA, LODs were estimated as three times the standard deviation of the background concentrations of procedural blank (n = 10); instead, for the other analytes, LODs were determined using the lowest spiked samples at 0.01 ng g^{-1} (n = 6). LOQ was estimated as the lowest concentration of the sample fortified with acceptable precision and trueness, by applying the complete analytical method and identification criteria. Precision and trueness were obtained by spiking each sample with all PFASs at three concentration levels (0.05 ng g^{-1}, 0.10 ng g^{-1}, 0.50 ng g^{-1}). Each level was analysed three times on two different days for a total of 18 tests. The inter-day precision was evaluated as the relative standard deviation (RSD, expressed as a percentage) for each level, and the trueness was obtained from the average recovery for each level. The expanded measurement uncertainties were obtained using a top-down approach as reported in the Guidance Document on Measurement Uncertainty (confidence level of 95 %)

Table 2
The ion species of native perfluorinated alkylated substances tested in this study, the labelled internal standards (ISs) for processing (ISp) and injection (ISi).

Chemical class	Quantifier ion $[M-H]^-$ (*m/z*)	F1 (*m/z*)	F2 (*m/z*)
Native compounds			
PFPeA	262.9760	218.9862	–
PFHxA	312.9728	268.9830	118.9926
PFHpA	362.9696	318.9799	168.9894
PFOA	412.9664	368.9766	168.9892
PFNA	462.9632	418.9734	218.9862
PFDA	512.9600	468.9702	218.9862
PFUnDA	562.9568	518.9670	268.9835
PFDoDA	612.9537	568.9638	268.9837
PFTrDA	662.9505	618.9606	318.9803
PFTeDA	712.9473	668.9574	368.9769
PFBS	298.9430	98.9558	79.9574
PFPeS	348.9398	98.9558	79.9574
PFHxS	398.9366	98.9558	79.9574
PFHpS	448.9334	98.9558	79.9574
PFOS	498.9302	98.9558	79.9574
PFNS	548.9270	98.9558	79.9574
PFDS	598.9238	98.9558	79.9574
PFDoDS	698.9174	98.9558	79.9574
Internal standards (ISp)			
[$^{13}C_5$] PFPeA	267.9928	–	–
[$^{13}C_5$] PFHxA	317.9896	–	–
[$^{13}C_4$] PFHpA	366.983	–	–
[$^{13}C_8$] PFOA	420.9937	–	–
[$^{13}C_9$] PFNA	471.9934	–	–
[$^{13}C_6$] PFDA	518.9802	–	–
[$^{13}C_7$] FUnDA	569.9803	–	–
[$^{13}C_2$] PFDoDA	614.9604	–	–
[$^{13}C_2$] PFTeDA	714.954	–	–
[$^{13}C_3$] PFBS	301.9531	–	–
[$^{13}C_3$] PFHxS	401.9467	–	–
[$^{13}C_8$] PFOS	506.9571	–	–
Injection standards (ISi)			
[$^{13}C_2$] PFOA	414.9933	–	–
[$^{13}C_2$] PFDA	514.9668	–	–
[$^{13}C_4$] PFOS	502.9571	–	–

F1 = Qualifier ion 1; F2 = Qualifier ion 2.

(Eppe, Schaechtele, Haedrich, & Fernandes, 2017).

2.6. Sample analyses

A total of 132 egg samples were analyzed using developed and validated method. A procedural blank and a quality control at LOQ concentration (0.05 ng g^{-1}) were added to each batch, consisting of 10 samples. Precautions have been taken to avoid cross-contamination during analysis in the laboratory. Taking into account the possibility of contamination background during sample preparation, efforts were made to use materials known as free of fluoropolymer materials (e.g. PTFE, PVDF and others) or glass that could adsorb PFAS analytes. More information on quality control in routine analysis is provided in the Supplementary Material.

2.7. Dietary intake estimation

The contribution of eggs to dietary intakes of PFASs was estimated for three different age groups (children, adolescents and adults) of the Italian population. Food consumption data for eggs were taken from the Italian national food consumption survey INRAN-SCAI 2005–06 (Leclercq, Arcella, Piccinelli, Sette, & Le Donne, 2009). The details on age groups are as follows: a) children 3–10 years (average body weight 26.1 ± 8.3 kg); b) adolescents 10–18 years (average body weight 52.6 ± 12.5 kg); c) adults 18–65 years (average body weight 69.7 ± 13.5 kg). For each age group, the mean consumption of eggs is 139.3 g per week for children, 145.6 g per week for teenagers and 149.1 g per week for adults (Leclercq et al., 2009). The weekly intake (WI) for the sum of PFOS, PFOA, PFNA, and PFHxS, expressed as ng kg^{-1} b.w. per week, was estimated according to the following formula: $WI = C^*Ac^*Bw^{-1}$ where C is the highest value of the contamination for PFOS, PFOA, PFNA, and PFHxS in eggs; Ac is the average consumption of eggs per age group, and Bw stands for the mean weight of the different age groups (children, adolescents, and adults). In all cases an upper bound scenario was applied, using the LOD value for the contribution of non-detected analytes.

3. Results

We developed an analytical method for PFAS determination in eggs based on isotope dilution and processing by UHPLC with high-resolution mass spectrometry (Q-Orbitrap). The method was validated for 18 analytes, including 10 perfluoroalkyl carboxylic acids and 8 perfluoroalkane sulfonic acids (Table 3). Validation addressed linearity, LOD, LOQ, precision, trueness, and uncertainty. For native PFASs, the detector response was linear in the range 0.2–20 ng mL^{-1} with a coefficient of determination (R^2) in the range 0.980–0.999. LODs ranged from 0.005 to 0.036 ng g^{-1}. At LOD level, identification criteria of compounds were met, and the instrumental response was in the order of magnitude of 1×10^4 for the qualifier ion (Figure S1 of Supplementary Material). LOQs were assessed at the lowest spiked level for all analytes set at 0.05 ng g^{-1}. At three spiked concentrations (0.05 ng g^{-1}, 0.10 ng g^{-1}, 0.50 ng g^{-1}), recoveries of native analytes were within the range 81–128 %. Precision, expressed as RSD, was in the range 4–21 %. Expanded uncertainties (coverage factor $k = 2$, level of confidence $p \sim 95$ %) were found to be in the range 26–48 %. Furthermore, the method was tested in several proficiency tests on food organized by the European Union Reference Laboratory for Halogenated Persistent Organic Pollutants in Feed and Food between 2019 and 2021, obtaining satisfactory z-scores. Details of validation method are reported on Supplementary Material (Table S1 – S4).

The method was used to determine PFAS content in eggs from chickens raised in different settings (Table 4): organic eggs (n = 57), caged hen eggs (n = 27) and barn eggs (n = 48). PFASs were found in all types of eggs, although the frequency and magnitude of detection varied depending on the analyte. Among the 18 PFASs investigated, no analyte was quantified above LOQ while only 7 were above LOD. Overall, 6 analytes were found in organic eggs, 4 were detected in caged hen eggs and 5 were detected in barn eggs. The remaining 11 analytes were under LOD for all 3 groups. The analytes found in samples were all long-chain PFASs (PFHpA, PFNA, PFDA, PFDoDA, PFHxS, PFOS, PFDoDS), while the short-chain ones were never found. Among perfluoroalkyl carboxylic acids, PFNA and PFDA were detected in all 3 groups. Instead, for perfluoroalkane sulfonic acids, none was found in all 3 groups, but PFOS was the most frequently detected. The highest concentration was 0.042 ng g^{-1} for PFOS and was found in barn eggs.

The present study allowed to calculate the weekly dietary intake for the sum of PFOS, PFOA, PFNA, and PFHxS in eggs, notwithstanding the high frequencies of values below LOD. Exposure levels were calculated considering the "worst case" scenario for the four PFASs by applying an upper bound approach. Results show that exposure is higher for children with a value equal to 0.76 ng kg^{-1} b.w., followed by adolescents and adults with 0.40 and 0.31 ng kg^{-1} b.w., respectively.

4. Discussion

A sensitive and accurate UHPLC-HRMS method was developed for the determination of 18 PFASs at the trace level range (fraction of ng g^{-1}) in eggs. The method was applied to 132 samples of commercial eggs divided into 3 groups (organic, barn and caged hen eggs) to evaluate the contamination levels of these compounds and to assess the risk for the Italian population consuming this food. Only a few of 18 analytes were

Table 3
Analytical performance data for PFAS achieved during validation study. LOQs for all analytes were equal to 0.05 ng g^{-1}.

Analyte	LOD (ng g^{-1})	Linearity (R^2)	Trueness (inter-day precision) [a]			Uncertainty (%)
			Spiking Level 1 [b]	Spiking Level 2 [b]	Spiking Level 3 [b]	
Perfluoroalkyl carboxylic acids						
PFPeA [c]	0.030	0.999	105 (11)	110 (10)	104 (6)	30
PFHxA [c]	0.036	0.999	112 (7)	112 (6)	111 (7)	31
PFHpA [c]	0.012	0.999	128 (5)	119 (8)	115 (9)	48
PFOA [c]	0.030	0.999	101 (8)	103 (12)	107 (6)	27
PFNA [d]	0.006	0.980	128 (21)	116 (12)	108 (10)	42
PFDA [d]	0.013	0.999	111 (8)	107 (9)	103 (8)	29
PFUnDA [d]	0.012	0.999	109 (7)	110 (6)	109 (6)	27
PFDoDA [d]	0.006	0.999	114 (10)	113 (9)	113 (9)	39
PFTrDA [d]	0.012	0.998	102 (12)	105 (7)	109 (4)	27
PFTeDA [d]	0.032	0.998	110 (9)	108 (12)	109 (6)	32
Perfluoroalkane sulfonic acids						
PFBS [d]	0.007	0.999	110 (9)	107 (7)	111 (5)	29
PFPeS [d]	0.008	0.990	107 (11)	97 (12)	106 (8)	32
PFHxS [d]	0.005	0.999	111 (9)	112 (9)	111 (7)	33
PFHpS [d]	0.019	0.998	111 (10)	107 (10)	107 (10)	33
PFOS [d]	0.006	0.999	108 (6)	114 (5)	108 (6)	27
PFNS [d]	0.008	0.998	112 (7)	101 (10)	101 (6)	26
PFDS [d]	0.015	0.993	105 (11)	100 (10)	103 (11)	31
PFDoDS [d]	0.010	0.991	81 (16)	87 (14)	100 (10)	45

[a] The trueness was obtained from the percentage average recovery for each level, and the inter-day precision was evaluated as the relative standard deviation (RSD %) for each level.
[b] Spiking levels were equal to 0.05, 0.10, and 0.50 ng g^{-1} respectively.
[c] LODs were estimated as three times the standard deviation of the background concentrations of procedural blank.
[d] LODs were determined using the lowest spiked samples.

Table 4
PFAS contamination in organic and conventional eggs.

Analyte [a]	Organic eggs (n = 57)		Caged hen eggs (n = 27)		Barn eggs (n = 48)	
	Content [b] ng/g	Samples, n > LOD	Content [b] ng/g	Samples, n > LOD	Content [b] ng/g	Samples, n > LOD
Perfluoroalkyl carboxylic acids						
PFHpA	0.019 (0.014–0.028)	3	< 0.012	0	0.017 (0.013–0.020)	5
PFNA	0.018 (0.007–0.037)	3	0.008 (0.007–0.010)	4	0.015	1
PFDA	0.021 (0.014–0.030)	3	0.026 (0.018–0.032)	4	0.014	1
PFDoDA	0.017 (0.013–0.022)	5	0.016 (0.009–0.022)	4	< 0.006	0
Perfluoroalkane sulfonic acids						
PFHxS	< 0.005	0	0.006	1	0.020 (0.006–0.034)	2
PFOS	0.016 (0.007–0.025)	4	< 0.006	0	0.015 (0.007–0.042)	5
PFDoDS	0.011	1	< 0.010	0	< 0.010	0

[a] Contamination levels of analytes over LOD are reported while for PFPeA, PFHxA, PFOA, PFUnDA, PFTrDA, PFTeDA, PFBS, PFPeS, PFHpS, PFNS and PFDS analytical values were below LOD in all samples.
[b] Values are mean (range).

detected in eggs regardless of the type of farming and, moreover, the concentrations were always close to the detection limits.

The validation parameters, such as precision and trueness, are in agreement with recent studies (Bao et al., 2019; Berendsen, Lakraoui, Leenders, & van Leeuwen, 2020; Chen, Bai, Chang, Chen, & Chen, 2018; Sadia, Yeung, & Fiedler, 2020), where the isotope dilution technique shows the ability to control and measure method performance. Furthermore, the LOD and LOQ values found in this work are lower than those of other studies (Bao et al., 2019; Chen et al., 2018; Gazzotti et al., 2021; Zafeiraki et al., 2016). Our method allows the sensitive detection of all PFASs, especially PFOA, PFNA, PFHxS and PFOS, and satisfies the testing requirements for the tolerable intake set by EFSA, ATSDR and U. S. EPA (EFSA, 2020; ATSDR, 2021).

For some analytes such as perfluorobutanoic acid (PFBA), it was not possible to reach the required validation levels because of blank contamination during extraction and clean-up procedures. In fact, the procedural blank was heavily contaminated by PFBA found at a concentration range from 0.53 to 1.33 ng g^{-1} considering sample intake, which was higher than all levels investigated.

The developed procedure could also be implemented in other testing laboratories with experience in the detection of traces of persistent organic pollutants in food and environmental matrices. However, it should be noted that this is a relatively expensive method where the use of advanced equipment (e.g. HRMS) and labelled standards, might represent a weakness in the applicability of the method by a small sized environmental laboratory.

First investigations regarding PFAS levels in eggs collected in different European countries were carried out early 2000s (Table 5). These studies were extremely heterogeneous in terms of number of samples, origin of eggs, methodological approach, number of analyzed PFASs, and often eggs were only a part of larger studies. In general, there was a low incidence of detection in eggs and only a few samples showed detection with values close or below the respective LODs /LOQs. In some cases, high levels have been found in home produced eggs or from farms

Table 5
PFAS concentrations (ng/g) in eggs reported in the literature. All results are showed as mean and/or range when it is available.

Country	SampleType	Year	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	PFBS	PFHxS	PFHpS	PFOS	PFDS	Reference
Belgium	Home produced eggs	–	–	–	–	–	–	–	–	–	–	–	–	–	0.4–3472.8	–	D'Hollander et al. (2011)
Sweden	Food basket sample	2010	0.0036	<LOD	0.039	<LOD	0.0033	<LOD	<LOD	<LOD	<LOD	–	0.0025	–	0.039	–	Vestergen et al. (2012)
		2005	0.0051	<LOD	0.0072	0.0056	0.0049	0.0033	<LOD	<LOD	<LOD	–	<LOD	–	0.0072	–	
		1999	0.0051	<LOD	0.031	0.022	0.015	0.038	0.010	0.014	<LOD	–	0.039	–	1.28	–	
Belgium	Eggs from chickens farms	2008	–	–	0.86 (<0.055–5.0)	–	–	–	–	–	–	–	–	–	6.86 (<0.12–22)	–	Cornelis et al. (2012)
Italy	Eggs from supermarket	–	–	–	<0.5	–	–	–	–	–	–	–	–	–	<0.5	–	Guerranti et al. (2013)
Sweden	Eggs from packaging plants	1999–2010	<0.008–0.013	<0.005–0.005	<0.014–0.225	<0.020–0.143	<0.006–0.067	<0.008–0.241	<0.006–0.051	<0.004–0.102	<0.005–0.010	<0.010–0.128	–	–	43–6480	–	(Johansson et al., 2014)
Netherlands	Home produced eggs (yolk)	2013–2014	<0.5	<0.5	1.1 (<0.5–2.7)	0.9 (<0.5–2.0)	0.9 (<0.5–3.0)	0.9 (<0.5–2.3)	–	–	–	<0.5	1.1 (<0.5–5.2)	<0.5	3.5 (<0.5–24.8)	–	Zafeiraki et al. (2016)
Greece	Home produced eggs (yolk)	2013–2014	<0.5	<0.5	0.5 (<0.5–0.5)	0.8 (<0.5–1)	0.9 (<0.5–8.0)	0.7 (<0.5–4.5)	–	–	–	<0.5	<0.5	<0.5	1.1 (<0.5–8.9)	–	Zafeiraki et al. (2016)
Netherlands	Commercially eggs (yolk)	2013–2014	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	–	–	–	<0.5	<0.5	<0.5	<0.5–1.8	–	Zafeiraki et al. (2016)
Greece	Commercially eggs (yolk)	2013–2014	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	–	–	–	<0.5	<0.5	<0.5	<0.5–0.9	–	Zafeiraki et al. (2016)
Spain (Catalonia)	Local markets-supermarkets-small stores-grocery stores	2011	<0.039	0.2	<0.39	<0.1	<0.01	<0.0.038	<0.011	<0.017	<0.017	–	<0.002	–	<0.0053	<0.0006	Jian et al. (2017)
		2006	–	<0.005	<0.055	–	–	–	–	–	–	–	–	–	0.082	–	Jian et al. (2017)
Norway	Grocery stores	2008–2009	0.013	<0.016	0.03	<0.0074	0.012	0.0099	<0.0081	–	–	0.002	0.0035	–	0.039	–	Jian et al. (2017)
Netherlands	Retail stores with nation-wide coverage	2009	<0.054	<0.002	<0.032	0.006	0.011	–	<0.013	<0.107	<0.005	–	<0.006	–	0.029	–	Jian et al. (2017)
Italy	Eggs from commercial lying hens (yolk)	2017	–	–	UB[a] 0.11 LB[a] 0.00	UB 0.11 LB 0.00	–	–	–	–	–	–	UB 0.12 LB 0.00	–	UB 0.11 LB 0.01	–	Gazzotti et al. (2021)
Italy (north)	Eggs from backyard chickens (yolk)	2018–2019	–	–	UB 0.15 LB 0.01	UB 0.19 LB 0.03	–	–	–	–	–	–	UB 0.16 LB 0.05	–	UB 0.86 LB 0.79	–	Gazzotti et al. (2021)
Italy (centre-south)	Eggs from backyard chickens (yolk)	2018–2019	–	–	UB 0.11 LB 0.00	UB 0.16 LB 0.04	–	–	–	–	–	–	UB 0.13 LB 0.00	–	UB 0.57 LB 0.49	–	Gazzotti et al. (2021)
European countries	Eggs from European countries	2000–2016	–	–	UB 0.21 LB 0.106	UB 0.10 LB 0.00	–	–	–	–	–	–	UB 0.06 LB 0.00	–	UB 0.35 LB 0.27	–	EFSA opinion (2020)

Dashes identify values that are not available.
[a] UB, upper bound; LB, lower bound.

nearby fluorochemical plants (D'Hollander et al., 2011; Gazzotti et al., 2021).

In Italy PFOS and PFOA levels of commercial eggs were previously investigated in four pooled samples (Guerranti, Perra, Corsolini, & Focardi, 2013). Zafeiraki et al. analyzed the egg yolk on a larger number of samples collected in Greece and Netherlands, measuring the concentration of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBS, PFHxS, PFHpS, PFOS (Zafeiraki et al., 2016). In both studies the PFAS levels were below the limit of quantitation, apart from two samples in Zafeiraki's study, where only PFOS was detected. Zafeiraki et al. also investigated yolk of home produced eggs and results showed a contamination with PFASs, especially PFOS. Recently, in Italy yolk of eggs from backyard hens were analyzed for PFOA, PFNA, PFHxS and PFOS, indicating higher PFAS contamination in this egg category respect to commercial eggs (Gazzotti et al., 2021). Some studies, in fact, showed that PFASs transfer through the food chain from feed and water to eggs (Death et al., 2021; Kowalczyk et al., 2020; Wilson et al., 2021) and PFAS contamination in home-produced chicken eggs was higher than in commercial eggs (Gazzotti et al., 2021; Zafeiraki et al., 2016). These findings may be because these hens are free to move outdoors and eat worms, small insects and soil that represent $>$ 80 % of the exposure in outdoor poultry and eggs (Brambilla, D'Hollander, Oliaei, Stahl, & Weber, 2015; D'Hollander et al., 2011). We compared 3 different groups of commercial eggs to assess consumer exposure, since EFSA identified "eggs and egg products" as a main food category contributing to PFAS intake. Our findings, carried out on a large number of samples, corroborate previous studies that found PFAS contamination levels in commercial eggs generally low and close to the LODs for all analytes detected (Guerranti et al., 2013; Jian et al., 2017; Zafeiraki et al., 2016). Furthermore, these values were lower than those reported by recent EFSA opinion (EFSA, 2020).

No difference emerged from the comparison of the 3 groups of commercial eggs available in the Italian market. It could be supposed that organic and home-produced eggs have a similar PFAS content owing to the fact that in both cases hens can move freely outdoors and have free access to soil. However, this does not occur, probably because hens from organic farming have an abundance of commercial feed that is free of PFAS contamination, so they do not need to search other sources of food (Zafeiraki et al., 2016). As discussed above, the main exposure pathways for hens are soil, water and feed. It is reasonable that found values are close to detection limit for many samples, suggesting that Italian commercial eggs are at background levels on the presence of PFASs. Several studies focused on the detection of PFAS in egg yolk, because of the large abundance of lipoproteins and phosphoproteins that have high affinity to linear isomers compared to albumen (Wang et al., 2019). One third of an egg consists of egg yolk and if the PFASs tend to accumulate in the egg yolk, it would increase the detection frequency in the sample. On the contrary, in this study whole eggs were analyzed to have an overview of dietary exposure of Italian consumers to eggs.

We found mostly long-chain PFASs, which could be due to two possible reasons. Firstly, the potential bioaccumulation in eggs for long-chain analytes is greater than for the short ones (Kowalczyk et al., 2020; Kwiatkowski et al., 2020). Secondly, as described by Kowalczyk et al. (2020), some short-chains such as PFBS have a shorter half-life and lower transfer rate in eggs than long-chains such as PFHxS, PFHpS and PFOS; these long chains have a higher chance to be detected. Zafeiraki et al. also found long-chain PFASs in home produced eggs and suggested that the source of contamination is the ingestion of soil through pecking (Zafeiraki et al., 2016).

Diet has been considered one of the major non-occupational source of PFAS intake for the general population (Vestergren, Cousins, Trudel, Wormuth, & Scheringer, 2008). A number of recent studies on human dietary exposure have been carried out (Chen et al., 2018; Gazzotti et al., 2021; Jian et al., 2017; Pasecnaja, Bartkevics, & Zacs, 2022; Zafeiraki et al., 2016). However, comparison among studies is challenging due to differences in food types, sampling design and data treatment. Moreover, different sets of analytes, high variation of LOD/LOQ values as well as high proportion of non-detects hamper an accurate determination of average levels of PFASs in food leading to a significant uncertainty in the exposure assessment.

In the present study an upper bound exposure was proposed considering the highest PFAS concentration for the three age groups (children, adolescents and adults). The exposure levels were calculated taking into account the cumulative intake of four PFASs targeted by EFSA and ATSDR. In this scenario, children have around twofold higher exposure levels than the older age groups. As expected, children may be more exposed to environmental toxicants because they consume more food per unit of body weight than adults. Considering the lowest TWI limit of 4.4 ng kg^{-1}b.w., the contribution to exposure from eggs stands at 17.3 % in children, 9.0 % in adolescents and 7.0 % in adults. In this "worst case" scenario, where the highest PFAS levels in eggs have been taken into account, eggs contribution to total dietary intake should not be an issue in adolescents, and adults, while could be of greater concern in children.

5. Conclusion

In conclusion, our UHPLC-HRMS method allows the detection of PFASs at low concentrations (fraction of ng g^{-1}) that satisfy EFSA requests and provides accurate results on PFAS contamination of eggs. These results indicate that Italian commercial eggs have a generally low contamination levels regardless of the production systems. The resulting dietary exposure to the Italian age groups shows that children are the most exposed to PFASs. Furthermore, the developed method can be used to analyze other food categories and other emerging PFASs for an overall assessment of PFAS dietary exposure.

CRediT authorship contribution statement

Francesco Chiumiento: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Mirella Bellocci:** Methodology, Formal analysis, Data curation. **Roberta Ceci:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Silvia D'Antonio:** Methodology, Formal analysis, Data curation. **Alfonso De Benedictis:** Methodology, Formal analysis, Data curation. **Manuela Leva:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Luigi Pirito:** Methodology, Formal analysis, Data curation. **Roberta Rosato:** Methodology, Formal analysis, Data curation. **Rossana Scarpone:** Methodology, Formal analysis, Data curation. **Giampiero Scortichini:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Giulio Tammaro:** Methodology, Formal analysis, Data curation. **Gianfranco Diletti:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134135.

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