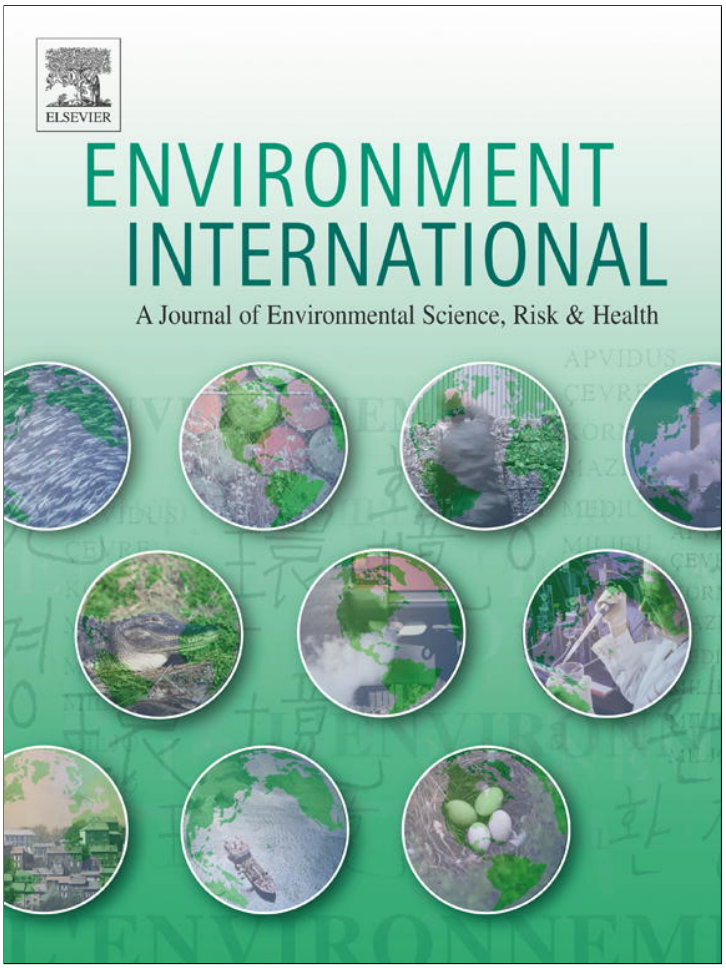

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Perfluoroalkyl substances in human milk: A first survey in Italy

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ABSTRACT

Due to their widespread diffusion, perfluoroalkyl substances (PFASs) have been frequently found in the environment and in several animal species. It has been demonstrated that they can easily reach also humans, mainly through diet. Being lactation a major route of elimination of these contaminants, their occurrence in human milk is of particular interest, especially considering that it generally represents the unique food source for newborns. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), the two most important compounds of this family, have been frequently found in human milk at variable concentrations, but still limited data are available. The present study, the first conducted in Italy capable to detect these pollutants at ultra-trace levels by UPLC-MS/MS, confirmed the role of lactation as a relevant source of exposure for breastfed children. The measured concentrations ranged between 15 and 288 ng/L for PFOS and between 24 and 241 ng/L for PFOA. Moreover, mean concentrations and frequencies of both analytes resulted higher in milk samples provided by primiparous women, suggesting that the risk of intake might be higher for first-borns. Finally, comparing these results with previous data, PFOS gradual decrease over time since year 2000 was confirmed.

1. Introduction

Perfluoroalkyl substances (PFASs) is the name of a wide group of synthetic substances that, due to their physicochemical properties, have been largely employed during the last 60 years in multiple common use products. The extensive use of these compounds led to their global distribution as persistent contaminant into the environment, including animals and humans, and even in regions far from anthropogenic activities (EFSA, 2008; Prevedouros et al., 2005). Several studies highlighted the bioaccumulation potential of certain PFASs, as perfluorinated sulfonates and long-chain (>7 fluorinated carbons) perfluorinated carboxylates, even if further investigations are needed to fully understand the behavior of the different types of compounds (Conder et al., 2008). Because of their amphiphilic properties, unlike other persistent halogenated compounds which accumulate in fatty tissues, PFASs bind to proteins and have been frequently found in human plasma: diet is considered the main exposure route for the population, especially through seafood consumption, but other high protein content food categories, including milk, eggs and meat, might also represent a risk (EFSA, 2011). The effects of these substances on human health haven't been fully ascertained yet, but they seem to be related to a wide range of pathologies in the exposed organisms, such as hepatotoxicity, neurobehavioral toxicity, immunotoxicity, lung toxicity, reproductive toxicity and hormonal effects (OECD, 2002).

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the two most studied and frequently found molecules belonging to this family. Their half-life in humans has been estimated to be of more than 5 years for PFOS and around 4 years for PFOA (Olsen et al., 2007).

Due to their high persistency in the environment, tendency to bioaccumulate in organisms and potentially dangerous effects on human health, PFOS and its salts have been recently included as persistent organic pollutant (POP) in Annex B of the 2009 Stockholm Convention and their manufacturing and use have been restricted (Wang et al., 2009).

The ability of PFOS and PFOA to cross the placental barrier and to be eliminated through lactation has been observed in multiple animal species and recently confirmed in humans as well (Midasch et al., 2007; Völkel et al., 2008). Studies on rodents showed that prenatal exposure to these chemicals delays development and reduces survival and weight gain (Lau et al., 2007). The potential toxicity of PFASs in human fetuses and in newborns raised the interest of the scientific community on their occurrence in breast milk, since this latter represents in most cases the sole food source during the first year of life.

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Several studies evaluated the occurrence of these pollutants in human milk in different countries, detecting PFOS and PFOA in almost every sample (Fromme et al., 2009). In addition, up to now no data from Italy are available.

In this context, the aims of the present work were: 1) to perform a survey on the presence of perfluoroalkyl chemicals in human milk from Italy, in order to overcome the lack of information concerning this country and contributing at the same time to the collection of useful data for risk assessment on breastfed children's exposure to these emerging contaminants; 2) to assess potential correlations between the primipara/multipara status of the donor and measured levels of PFOS and PFOA.

2. Material and methods

2.1. Reagents and chemicals

Perfluoro-n-octanoic acid (PFOA), sodium perfluoro-1-octanesulfonate (PFOS) and their relative ^{13}C-labeled internal standards (purity greater than 98%) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Methanol and ammonium acetate were of LC–MS grade and were both acquired from Fluka (Sigma Aldrich, St. Louis, MO, USA); acetone was from VWR (Radnor, PA, USA), while glacial acetic acid, formic acid and ammonia solution (33%) were from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water was produced in-house with a Human Power I system (Seoul, Korea). Oasis® HLB 500 mg SPE cartridges were purchased from Waters (Milford, MA, USA), while Envicarb® 500 mg SPE cartridges were from Supelco (Sigma Aldrich, St. Louis, MO, USA).

2.2. Sample collection

All the breast milk samples were provided by the Department of Paediatrics of the Sant'Orsola-Malpighi Hospital of Bologna, with the authorization of the Independent Ethics Committee of the hospital (clinical trial # 49/2011/U/Tess). Among the 37 samples, collected during the second semester 2010, 21 were from primipara donors and 16 from women nursing for at least the second time. Collected samples were transferred to polypropylene tubes and stored in the dark at −20 °C.

2.3. Sample preparation

Samples were prepared following the extraction procedure described by Kadar et al. (2011). First of all 3 mL of milk was transferred into a polypropylene tube and spiked with the two internal standards, then 9 mL of acetone was added to perform protein precipitation. After being shaken for 30 s, the sample was placed for 10 min in an ultrasonic bath to facilitate the extraction and centrifuged at 2000×*g* for 10 min at room temperature. The supernatant was transferred to a new polypropylene tube and evaporated to around 3 mL under gentle nitrogen stream at 45 °C, then 8 mL of 0.1 M formic acid solution was added in order to adjust the pH for the first purification step on the Oasis® HLB cartridge. After conditioning the cartridge with 10 mL of methanol and 10 mL of 0.1 M formic acid, always avoiding the solid phase to go dry, the sample was loaded. Once all the solution had passed through the column, two washings were performed with 5 mL of 0.1 M formic acid and with 5 mL of 0.1 M formic acid/methanol (50/50, *v*/*v*) solution, then vacuum was applied for 5 min to remove eventual residual drops. The analytes were eluted with 6 mL of a mixture of methanol/ammonia solution 33% (99/1, *v*/*v*) and subsequently concentrated to around 2 mL under nitrogen. A second purification was achieved using a Supelclean™ ENVI-Carb™ cartridge, previously activated with 10 mL of methanol. After placing a new tube under the column, the sample was loaded and then an elution with 6 mL of a methanol/glacial acetic acid (80/1, *v*/*v*) solution was performed. The eluate was evaporated to dryness under nitrogen and reconstituted in 200 μL of methanol/water (50/50, *v*/*v*) solution. The content of the tube was transferred into a microtube and centrifuged at 12,000 rpm for 45 min, then 150 μL was collected and added to 50 μL of water in a polypropylene vial prior to analysis in UPLC–MS/MS.

2.4. UPLC–MS/MS analysis

Analysis was performed on an UPLC–MS/MS system, including a Waters Acquity UPLC® binary pump, equipped with built-in vacuum degasser, thermostated autosampler and column heater. Chromatographic separation was achieved using a Waters Acquity UPLC® BEH C18 reversed-phase column (50×2.1 mm, 1.7 μm), fitted with a Waters VanGuard guard column with the same packaging (Waters Corporation, Milford, MA, USA). The mobile phase consisted of ammonium acetate 20 mM aqueous solution (solvent A) and methanol (solvent B). The gradient (constant flow rate at 0.5 mL/min) started with 30 s at 70% A and 30% B, then switched to 100% B over 1 min and held for 1.5 min, then went back to the initial conditions in 0.5 min and held for a further 1.5 min, in order to equilibrate the column before the following injection. Samples were kept at 6 °C in the autosampler and 10 μL was injected in "full loop" mode; the column was kept at 45 °C to avoid excessive backpressures.

The chromatographer was interfaced with a Waters Quattro Premier XE tandem mass spectrometer, equipped with an ESCi multi-mode ionization source (Waters Corporation, Milford, MA, USA) and operating in negative electrospray ionization (ESI −) mode. Analysis was performed in MRM (multiple reaction monitoring) mode, following two transitions for PFOS and PFOA and one for each internal standard: the selected values of cone voltage, collision energy and transitions are reported in Table 1. The following parameters were applied: capillary voltage was set at 2.00 kV and cone voltage at 3.00 V, while source and desolvation temperatures were 150 and 220 °C, respectively. Nitrogen flow was set at 50 L/h on the cone and 700 L/h for desolvation; argon was used as collision gas at 0.35 mL/min. Data acquisition and processing were performed using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

The presence of potential background contaminations was prevented by instrumental analysis of the employed solvents, performed at the beginning of the experiment in order to assess the absence of signals corresponding to our target analytes, and constantly monitored through the injection of a mobile phase sample every five milk samples during each analysis session.

2.5. Method validation

The method was validated in accordance with current European guidelines set by 2002/657/EC, employing selected cow milk samples

Table 1
Monitored transitions and their relative specific parameters.

Analyte	Transition 1 (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Transition 2 (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
PFOA	412.8 > 369.0	12	10	412.8 > 169.0	12	17
PFOS	498.6 > 498.6	52	10	498.6 > 80.0	52	43
^{13}C PFOA	416.9 > 372.0	11	10			
^{13}C PFOS	502.86 > 80.0	50	11			

Table 2
Summary of the results of human milk sample analysis.

		No of samples	Samples<LOQ	Lowest conc. (ng/L)	Highest conc. (ng/L)	Mean conc. ± SEM[a] (ng/L)
PFOA	Primiparas	21	4	24	241	76 ± 14
	Multiparas	16	5	24	100	43 ± 6
PFOS	Primiparas	21	2	15	288	57 ± 13
	Multiparas	16	6	15	116	36 ± 7

[a] SEM: standard error of mean.

which had been proved to be non-contaminated by target analytes, since no blank breast milk was available. A six point (range 0–2000 ng/L) matrix-matched calibration curve was prepared each day of analysis, proving the method's linearity: the coefficient of determination R^2 was always greater than 0.99 and relative standard deviations were lower than 17%. Specificity was assessed excluding the presence of potential interferences around PFOS and PFOA retention times in the chromatograms of non-contaminated samples. The injection of four replicates of blank milk spiked at four different levels (50, 200, 400 and 800 ng/L) demonstrated satisfying precision, with maximum relative standard deviations to the mean (CV%) of 8%, as well as good trueness values, expressed as relative difference between the mean value measured and the spiked concentration, resulting at always below 10%. Limits of quantification (LOQs) of the method, defined as the concentrations providing a chromatographic signal with a signal-to-noise (S/N) ratio equal to 10, were 15 ng/L for PFOS and 24 ng/L for PFOA.

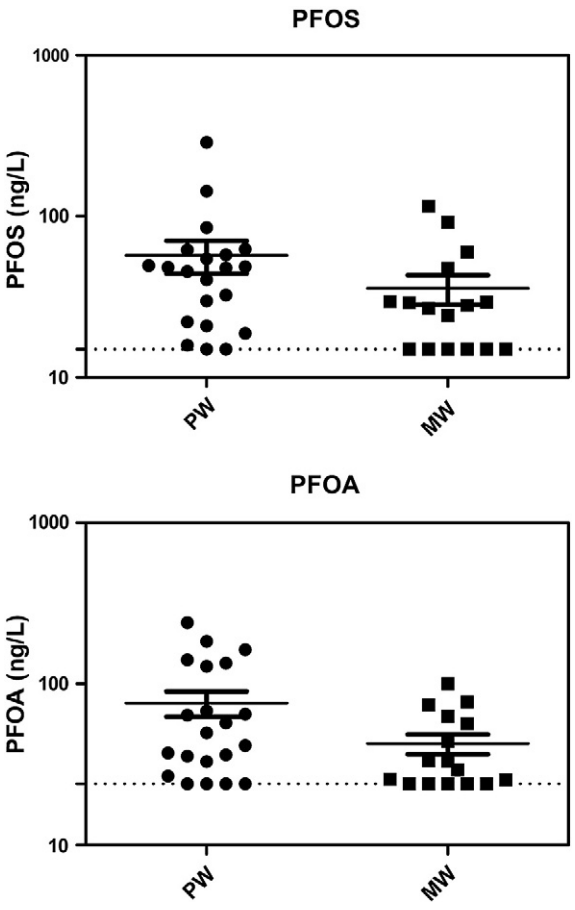

Fig. 1. PFOS and PFOA in breast milk samples of primiparous women (PW) and multiparous women (MW). Dashed line indicates the limit of quantification that was 15 ng/L for PFOS and 24 ng/L for PFOA.

3. Results and discussion

All the samples resulted contaminated by the investigated compounds. PFOS and PFOA were present at trace levels (lower than the LOQ) in 22 and 24% of samples, respectively. In more detail, PFOS was quantified in 90% of the samples provided by primiparous mothers and in 62% of samples given by multiparas. Concentrations ranged from 15 to 288 ng/L, with mean values of 57 ng/L, in the case of mothers lactating for the first time and from 15 to 116 ng/L (mean 36 ng/L) in the case of women who had already breastfed previously. Concerning PFOA, quantifiable levels were observed in 81% of cases in primiparas and 46% of cases in multiparas, with concentrations between 24 and 241 ng/L (mean 76 ng/L) and 24 and 100 ng/L (43 ng/L), respectively (see Table 2 and Fig. 1).

The highest values were obtained for both PFOS (288 ng/L) and PFOA (241 ng/L) in samples which had been provided by primiparous women.

This survey was the first conducted in Italy on the presence of perfluoroalkyl substances in breast milk capable of detecting ultra-trace concentrations. The obtained data are in line with those reported in similar studies performed in other developed countries, such as France (Kadar et al., 2011), Germany (Bernsmann and Fürst, 2008; Fromme et al., 2010; Völkel et al., 2008), Sweden (Kärrman et al., 2007), Spain (Llorca et al., 2010), Hungary (Völkel et al., 2008), USA (Tao et al., 2008a), Japan (Nakata et al., 2007; Tao et al., 2008b) and China (Liu et al., 2010; So et al., 2006). In some of these works other PFCs were also investigated, but PFOS always resulted as the most found contaminant, being present in almost all the analyzed samples. Moreover, considering PFOS median concentration for each monitoring and taking into account the relative approximate time period of collection, a slight decrease of the measured values over time can be noticed. In more detail, in the most recent surveys (from year 2010 onwards) the median was lower than 100 ng/L (76 ng/L in the present one), while the older works (up to year 2008) reported values generally greater than 100 ng/L and up to over 300 ng/L. The presented data are therefore consistent with the latest results from other developed countries, confirming the gradual decrease of this contaminant over time since year 2000 in consequence of the almost complete end of its manufacturing, as highlighted in a temporal trend study recently conducted in Sweden (Sündstrom et al., 2011).

PFOA has been measured at levels generally slightly lower than PFOS, but with a relevant variability in terms of frequency among the various studies, which is likely to be partially related to the high variability of the limits of quantification. In particular, the good sensitivity of the equipment used in the present research allowed us to determine this substance in all the analyzed samples, even at extremely low concentrations.

PFASs levels in blood are significantly higher than in breast milk, however their transfer from the former to the latter during breastfeeding has been confirmed and the existence of correlations between measured values in the two media has been proved (Fromme et al., 2010).

Taking into account the primipara/multipara status of this study's donors, it was observed that the mean concentration and frequency were higher for both the monitored analytes in milk provided by women nursing for the first time. In particular, the mean measured

level of PFOS was 57 ng/L in samples from primiparas and 36 ng/L in those from multiparous women, and for PFOA, this value decreased from 76 to 43 ng/L. Therefore, although the number of processed samples is rather low to draw statistically significant conclusions, a trend for both compounds to decrease in breast milk after the first lactation can be noticed, as previously reported by Tao et al. (2008a) in a similar cohort of patients. On the one hand this confirms the role of breastfeeding as a route of gradual elimination of perfluoroalkyl substances, while on the other hand it suggests a potentially higher exposure to these contaminants for first-born infants. However, as recently reported by Whitworth et al. (2012), it seems likely that the amount of PFASs body burden among parous women is significantly influenced by the length of the interpregnancy interval, meaning that, in case of extended elapsed time since the previous birth, breast milk contamination may get close to that of the first lactation.

Given all these outcomes, further investigations would be useful to better understand the importance of infant exposure to PFASs and identify potential influencing factors, especially considering the fact that breast milk represents almost the entire diet for children during the first months of life.

4. Conclusions

Data on the worldwide burden of the presence of these emerging contaminants in human breast milk are limited: this study provides a first picture of the situation in Italy, which resulted in contributing to PFASs exposure risk assessment for infants. In particular, the role of lactation as a source of both PFOS and PFOA for newborns was confirmed, suggesting a higher risk of exposure for first-born children as a consequence of the accumulation of these chemicals in maternal body during the lifetime until the first nursing, although it has been hypothesized that a long interval between pregnancies may allow the mother's body burden to significantly increase again.

The results of this preliminary monitoring, as well as the data available from recent studies performed in other developed countries, suggest that the toxicological risk related to the intake of these emerging pollutants through breastfeeding is moderate and is apparently decreasing with time. However, it must be considered that breast milk represents almost the entire diet for one of the most sensitive group of population, small children; moreover, a certain variability in the measured levels can be observed from country to country and still no data are available from several regions all around the globe. All these aspects highlight the need for further investigations on the existence of potential correlations between PFASs levels in breast milk and mother's history, taking into account, besides age at delivery and interpregnancy interval, also other possible influencing factors, such as length of breastfeeding, place of origin and diet habits, in order to better define the real exposure for newborns.

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