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Assessment of perfluoroalkyl substances in placenta by coupling salt assisted liquid-liquid extraction with dispersive liquid-liquid microextraction prior to liquid chromatography-tandem mass spectrometry

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ABSTRACT

The widespread use of perfluoroalkyl substances (PFAS) is resulting in a broad human exposure to these endocrine disrupting chemicals (EDCs), prompting biomonitoring research to evaluate its magnitude and impact, especially during critical windows of exposure such as fetal and perinatal periods. This study was focused on developing a method to determine 10 PFAS in placental tissue by combining salt-assisted liquid-liquid extraction with dispersive liquid-liquid microextraction and using liquid chromatography-tandem mass spectrometry. Chemometric strategies were applied to optimize the experimental parameters. The limit of quantification was 0.02 ng g^{-1} for all analytes, and the inter-day variability (as relative standard deviation) ranged from 7.9% to 13.8%. Recoveries ranged from 88.2% to 113.9%. The suitableness of the procedure was demonstrated by assessing the targeted compounds in 20 placenta samples. The highest concentrations were recorded for perfluorooctanoic acid and perfluorooctane sulfonate, with maximum concentrations of 0.62 and 1.02 ng g^{-1} and median concentrations of 0.13 and 0.53 ng g^{-1}, respectively. Median concentrations of the other PFAS ranged from detected values to 0.08 ng g^{-1}. This analytical procedure yields useful data on fetal exposure to PFAS.

1. Introduction

Perfluoroalkyl substances (PFAS) are widely utilized in the manufacture of numerous types of domestic and industrial materials. Their high thermal and chemical stability has led to their incorporation in firefighting foams, impregnating agents, and surface coatings for furniture, textiles, paper products, and kitchenware, among others [1]. The resulting continuous human exposure to PFAS is well documented in biomonitoring studies [2–4] and there is increasing evidence of their negative impact on health. For instance, PFAS exposure has been implicated in various endocrine disorders, including subfertility in women [5], reduced testosterone levels in men [6], and insulin resistance or elevated serum lipids [7,8]. Indeed, two of the most common PFAS, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), have been designated as endocrine disrupting chemicals (EDCs) [9–11].

The persistence, bioaccumulation, and possible harmful effects of PFAS have prompted to officially avoid or minimize exposure to PFOA, PFOS, perfluorohexanoic acid (PFHxA), and perfluorohexane sulfonate (PFHxS) [12–14]. In this line, the European Food Safety Authority (EFSA) [14] recently established the tolerable weekly intake (TWI) of PFOA and PFOS to 6 ng [kg bw]$^{-1}$ and 13 ng [kg bw]$^{-1}$, respectively. In addition, the European Human Biomonitoring Initiative HBM4EU (https://www.hbm4eu.eu) has been launched to generate high-quality evidence on the exposure of Europeans to priority chemicals, including PFAS, and on the health repercussions.

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The health impact of PFAS on the fetus, new-born, and child is of special concern. PFAS exposure has been associated with abnormal fetal growth and behavioral disorders in children [15–17], and prenatal exposure to PFOA and PFOS has been linked to low birth weight, although the results were not conclusive [18,19]. In addition, maternal serum concentrations of the long-chain PFAS perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA) have been related to a higher risk of miscarriage [20]. Some gender differences in their effects have been observed. Thus, cord blood concentrations of PFOS and PFOA were associated with an increased risk of congenital cerebral palsy in Danish boys but not girls [21], and it was suggested that cord plasma PFOA may increase thyroxine hormone levels in new-born girls but not boys [22]. Rodent studies have associated exposure to PFOA and PFOS during gestation with reduced postnatal survival, lower birth weight, decreased pup growth, altered lactation, and disrupted thyroid function [23–25].

Research on the effects of exposure during pregnancy has revealed the presence of PFAS in placental tissue, a complex matrix in which xenobiotics are usually present at low concentrations, requiring an adequate technique for their detection and measurement [26–28]. Large-scale biomonitoring studies need methods that are not only accurate and sensitive but also rapid, inexpensive, and easy to apply. To date, biomonitoring studies have used classical extraction with solvents [26,28], sometimes followed by solid phase extraction (SPE) to concentrate and/or clean-up samples [27]. These procedures offer low limits of detection (LODs) and reliable results, but they require long extraction times and repeated extraction cycles, reducing their usefulness for extensive biomonitoring. In relation to other typical biomonitoring matrices as serum or breast milk, most of proposed procedures are based on several manual and automated SPE modalities [29–31]. Nevertheless, some microextraction methodologies, with a remarked PFAS extraction specificity, have been applied in environmental matrices [32,33].

The combination of salt-assisted liquid-liquid extraction (SALLE) and dispersive liquid-liquid microextraction (DLLME) has achieved remarked extraction efficiencies for numerous sorts of compounds in various complex matrices [34–37] and may offer an alternative. This approach has demonstrated high extraction efficacy for most non-hydrophilic compounds, combining the capacity of SALLE to extract chemicals with the ability of DLLME to provide elevated enrichment factors. This combination has been used to measure pesticide and herbicide concentrations in fish [38], maize [39], vegetables [40] and milk [41], among other complex matrices, and to analyze the presence of bisphenol A in canned foods [42,43]. However, this binary extraction system has been little used to measure xenobiotic concentrations in human matrices and, to our best knowledge, it has not previously been used to study PFAS concentrations in human placenta samples.

The purpose of this study was to develop and validate a method to assess 10 PFAS in placental tissue using SALLE combined with DLLME and LC-MS/MS. The proposed procedure was applied to 20 placenta samples from anonymous donors.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA). Perfluorohexanoic (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrA), perfluorohexane sulfonate (PFHxS), and perfluorooctane sulfonate (PFOS) were purchased from Sigma-Aldrich (Madrid, Spain). Mass-labelled internal standards ($^{13}C_5$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_2$-PFDA, $^{13}C_2$-PFDoA, $^{13}C_4$-PFOS) were supplied by Wellington Laboratories (Ontario, Canada). Individual solutions of target analytes and internal standards (200 mg L^{-1}) were prepared in acetonitrile and stored at 4 °C. Solutions were stable for at least six months. Standard mixtures were prepared with acetonitrile. A set of solutions from 2.0 to 200 μg L^{-1} were used for optimization, calibration, and validation purposes.

HPLC-grade hexane and trichloromethane (TCM) were purchased from Merck (Darmstadt, Germany), and phosphate buffer saline (PBS), sodium chloride, calcium chloride, magnesium sulfate, ammonium acetate, LC–MS grade acetonitrile, and acetic acid from Sigma-Aldrich (Madrid, Spain). Collagenase type-I from *Clostridium histolyticum* was supplied by Sigma-Aldrich. The enzymatic solution was prepared immediately before use by dissolving 1 mg of enzyme powder in 10 mL of PBS medium (0.01 M, pH = 7.4) with the presence of 5 mM Ca^{2+}.

2.2. Analytical equipment and software

LC–MS/MS analyses were conducted with a NexeraXR LC-20A liquid chromatography instrument (Shimadztu, Japan) and a QTRAP MS/MS4500 mass spectrometer (AB Sciex, Framinham, MA). Statgraphics Centurion XVI 16.0.07 (Manugistics Inc., Rockville, MD) was used for statistical analyses. Placenta samples were homogenized with a Mixer B-400 (Buchi Labortechnik AG, Switzerland), and the collagenase enzymatic treatment was performed using a Hei-MIX incubator 1000 (Heidolph Instruments GmbH & Co, Germany).

2.3. Sample collection and storage

Placental samples were collected from 20 volunteers after the delivery (San Cecilio University Hospital of Granada, Spain). Informed consent documents, approved by the Institutional Ethical Committee of San Cecilio University Hospital, were signed by all donors. After being examined and accurately weighed, half of the placenta (maternal and fetal sides as well as central and peripheral parts were included) was homogenate in a glass container of the Mixer B-400. Aliquots of 35 g were coded and stored at −86 °C until chemical analysis.

2.4. Preparation of positive control (spiked) samples

The method was optimized and validated using a pool of three homogenized placenta samples. Pooled samples were spiked at 0.5 ng g^{-1} for optimization purposes and at concentrations ranging from 0.02 to 2.0 ng g^{-1} for validation purposes (calibration curves and recovery assay). Spiking was carried out by adding 10 μL of the corresponding solution (see section "chemical and reagents") to 1-g aliquots of the pooled sample. In the case of mass-labelled internal standards, samples were spiked at 0.25 ng g^{-1} with 25 μL of a solution containing $^{13}C_5$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_2$-PFDA, $^{13}C_2$-PFDoA, and $^{13}C_4$-PFOS at 10 μg L^{-1}.

2.5. Sample liquefaction by enzymatic treatment

For this treatment, 1 g of homogenate was placed in a polypropylene centrifuge tube, followed by the addition of 2 mL of collagenase solution and incubation at 37 °C for 4 h.

2.6. Sample treatment

First, 6.0 mL of acetonitrile (saturated with hexane) was poured over the liquefied sample, and the resulting mixture was agitated by vortex for 30 s. Then, 600 mg of NaCl were added and 60 s of manual shaking was performed, with the subsequent centrifugation at 4000 rpm for 10 min (2600×*g*). The supernatant was deposited in a 7 mL glass vial, concentrated to 1 mL under a nitrogen stream, and poured into a 15 mL screw-cap glass test tube, with the addition of 10.0 mL of 6% NaCl aqueous solution (w/v) at pH of 2, being ready for the DLLME process. Next, 800 μL of TCM were rapidly injected using a syringe, and the

mixture was gently shaken for 60 s and centrifuged for 5 min at 4000 rpm (2600×*g*). All the sedimented phase was transferred to a glass vial and dried under a nitrogen stream. The residue was dissolved with 100 μL of a 30:70 (v/v) mixture of 5 mM ammonium acetate (pH 4.5) and acetonitrile and then vortexed for 30 s, being ready for injection into the LC-MS/MS system.

2.7. Chromatography and mass spectrometry conditions

A Gemini C18 column (100 mm × 2 mm i.d., 3 μm particle) from Phenomenex (Torrance, CA) was used for chromatographic purposes. Injection volume and temperature maintained in the column were 10 μL and 25 °C, respectively. PFAS were separated using a gradient mobile phase consisting of 5 mM ammonium acetate (pH = 4.5) aqueous solution (solvent A) and acetonitrile (solvent B). Gradient conditions were as follows: 0.0–1.0 min, 30% B; 1.0–5.0 min, 30–60% B; 6.0–8.0 min, 70% B; 8.0–8.50, 70–90% B; 8.50–9.50, 90% B and back to 30% in 0.1 min. Flow rate was 0.35 mL min^{-1}. Total run time was 12.0 min.

Compounds were determined in negative ion mode, using the selected reaction monitoring (SRM) mode and unit mass resolution for Q1 and Q3. Perfusion of standard solutions (50 μg L^{-1}) were conducted in order to optimize mass spectrometry conditions. Ion source temperature and capillary voltage were set at 400 °C at −4.5 kV respectively. Nitrogen was used as curtain gas at 35 psi and as ion source gas 1 and 2 at (40 psi). All electric potentials related to the spectrometric process were conveniently adjusted for each compound. Regarding dwell time, a value of 50 ms was adopted. Table 1 compiles the optimal values for each compound and their corresponding diagnostic signals (SRM MS/MS transitions).

2.8. Quality control

Background contamination was controlled by analyzing procedural blanks. No quantifiable concentrations of target PFAS were recorded. In addition, a pool of placental tissue spiked at 0.5 ng g^{-1} and 2.0 ng g^{-1} was injected in triplicate every 15 injections.

Table 1
Selected transitions and optimized potentials.

Compound	Transitions	DP (V)	EP (V)	CE (V)	CXP (V)
PFHxA	313.0 → 269.0[a]	−43	−8	−12	−10
	313.0 → 119.0[b]	−43	−8	−27	−9
$^{13}C_5$-PFHxA	318.0 → 272.9[a]	−32	−10	−12	−11
	318.0 → 121.0[b]	−32	−10	−30	−10
PFHpA	363.0 → 319.1[a]	−40	−8	−12	−9
	363.0 → 169.0[b]	−40	−8	−25	−11
PFOA	413.0 → 369.0[a]	−40	−9	−13	−10
	413.0 → 219.2[b]	−40	−9	−24	−10
$^{13}C_4$-PFOA	417.1 → 371.8[a]	−44	−10	−12	−9
	417.1 → 222.1[b]	−44	−10	−27	−10
PFNA	463.0 → 419.0[a]	−40	−10	−13	−10
	463.0 → 219.2[b]	−40	−10	−35	−9
PFDA	513.1 → 469.0[a]	−51	−9	−13	−10
	513.1 → 269.2[b]	−51	−9	−32	−9
$^{13}C_2$-PFDA	515.0 → 470.1[a]	−46	−9	−15	−9
	515.0 → 220.2[b]	−46	−9	−33	−8
PFUnA	563.0 → 519.0[a]	−60	−9	−15	−10
	563.0 → 269.2[b]	−60	−9	−31	−10
PFDoA	613.0 → 569.0[a]	−62	−10	−15	−8
	613.0 → 319.1[b]	−62	−10	−36	−9
PFTrA	662.9 → 619.0[a]	−52	−11	−17	−9
	662.9 → 369.1[b]	−52	−11	−40	−8
$^{13}C_2$-PFDoA	615.0 → 570.0[a]	−54	−9	−14	−11
	615.0 → 320.2[b]	−54	−9	−32	−10
PFHxS	399.1 → 79.9[a]	−52	−11	−68	−13
	399.1 → 98.8[b]	−52	−11	−54	−10
PFOS	499.0 → 80.1[a]	−80	−10	−97	−9
	499.0 → 98.9[b]	−80	−10	−80	−11
$^{13}C_4$-PFOS	502.9 → 80.1[a]	−81	−10	−97	−11
	502.9 → 99.1[b]	−81	−10	−75	−10

[a] SRM transition used for quantification.
[b] SRM transition used for confirmation; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

3. Results and discussion

3.1. Optimization of SALLE conditions

The acetonitrile volume and manual shaking extraction time were optimized using univariate experimental designs. Figs. S1 and S2 depict the upward trend of extraction efficacies at larger volumes and longer times, reaching a plateau from 6.0 mL of acetonitrile and 60 s of extraction time.

The influence of NaCl and $MgSO_4$ masses was studied using a composite experimental design with six replicates at the central point (two-level factorial 2^2 experimental design with star points). The experimental domain of this design is summarized in Table S1. Extraction efficacies for all target PFAS were found strongly dependent on NaCl mass, whereas the effect of $MgSO_4$ mass was not significant. As an example, Fig. 1 shows that the highest extraction efficacies were obtained at the maximum NaCl mass and that selection of a null mass of $MgSO_4$ did not reduce the extraction yield. Therefore, 600 mg of NaCl was adopted as optimal value, with no addition of $MgSO_4$.

3.2. Optimization of DLLME conditions

After establishing SALLE conditions, multivariate experiments were performed to optimize the response (absolute chromatographic peak area) for each PFA in relation to the experimental DLLME parameters, *i.e.*, TCM volume, pH of aqueous solution, percentage NaCl in aqueous solution, and extraction time. The effect of these variables on signal response intensity (peak area) were examined using a two-level 2^4 factorial experimental design, with six replicates of the central point (the design was executed twice). Range and domain of this assay are shown in Table S2. Fig. 2 depicts the standardized effects of the evaluated parameters for four PFAS, taken as examples. A clear positive influence of NaCl mass and TCM volume was observed for all target compounds, while the standardized effects of pH and extraction time were not statistically significant for any compound except for PFOS, which showed a small effect of extraction time. Therefore, a pH value of 2 and extraction time of 60 s were selected to evaluate the optimal values of TCM volume and percentage NaCl by the use of a quadratic response surface design.

A Doehlert design was executed (six central point replicates), because of practical advantages of this type of chemometric procedure compared to other response surface designs [44]. The experimental domain is described in Table S3. Maximum peak area values were obtained between central and high levels of the studied variables, as illustrated in Fig. S3. Consequently, the desirability function was used to obtain the optimal values. This chemometric procedure makes it possible to determine the best compromise values of experimental factors for multiple simultaneous responses. Thus, the ideal desirability value would be the unit (i.e. all individual responses would be optimized). In the present case, a maximum desirability value of 0.76 was obtained with 800 μL TCM and 6% NaCl. Fig. 3 depicts the response surface associated with the desirability function obtained.

3.3. Analytical performance and method validation

According to US Food and Drugs Administration guidelines [45] and the criteria specified in EU Commission Decision 2002/675/EC [46] a proper evaluation of analytical merits was performed in terms of linearity, sensitivity, accuracy (trueness and precision) and selectivity.

A calibration function was established for each PFA using ten concentration levels (four replicates) from 0.02 to 2.0 ng g^{-1}, plotting the analyte/mass-labelled surrogate peak area ratio against the analyte concentration. The matrix effect (ME) was evaluated by comparing the

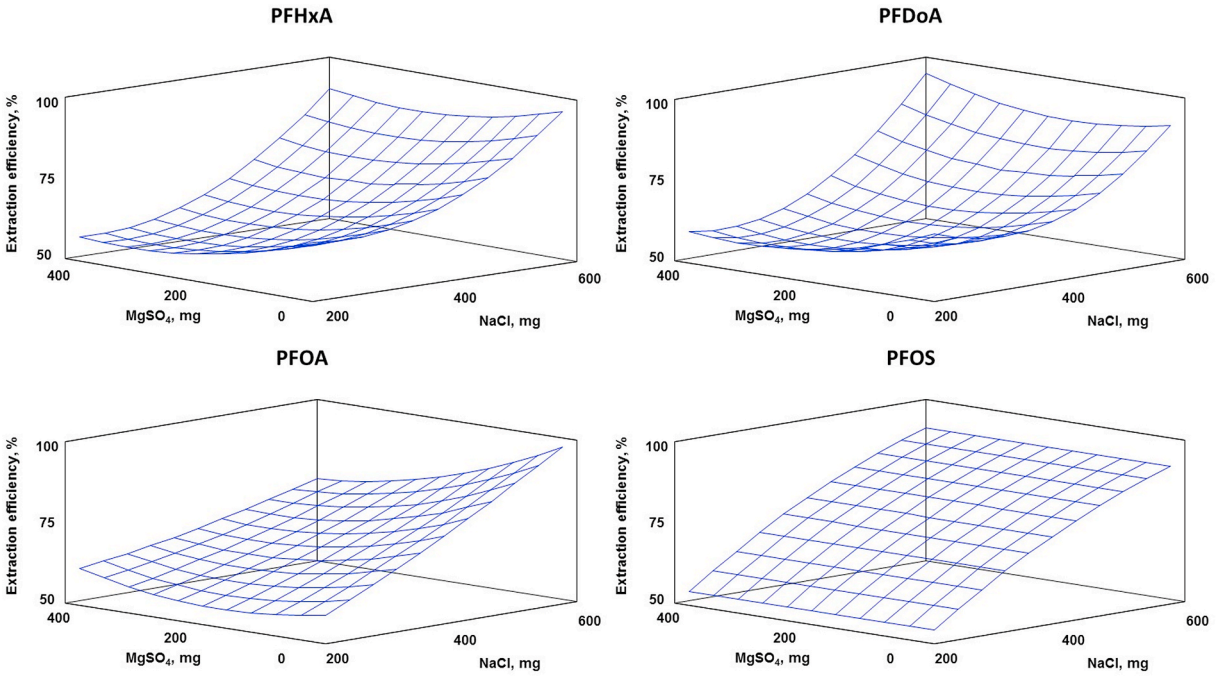

Fig. 1. Examples of response surfaces obtained for extraction efficacy in the NaCl mass optimization experiment.

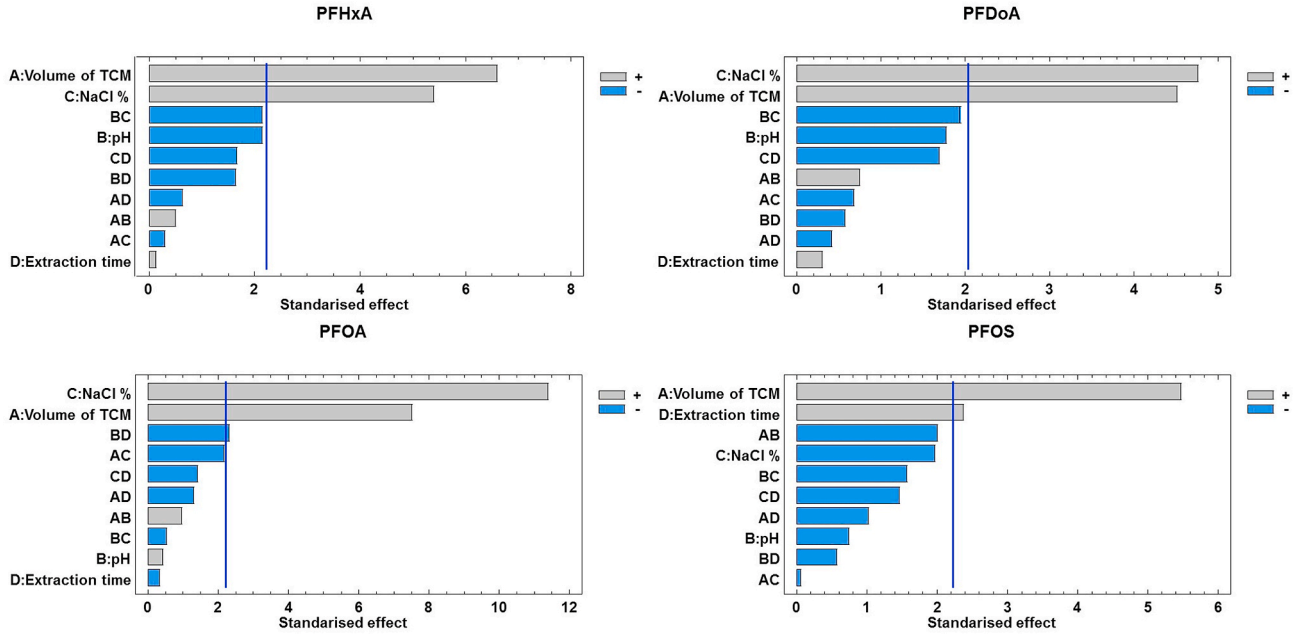

Fig. 2. Standardized effects of DLLME conditions on peak areas.

slopes of two calibration curves for each compound, one in milliQ water (W) and the other in placenta (P). The percentage ME was assessed as follows:

ME (%) = [1 - (Slope of calibration in P/Slope of calibration in W)] x 100.

Negligible ME values were obtained for all PFAS, ranging from −7.6 to 5.2%. This fact is especially important, given that it is very difficult to find a placenta with no detectable concentrations of PFAS. Hence, milliQ water was used as a matrix for calibration purposes. Fig. 4 depicts the chromatograms obtained from water and placenta spiked at 0.25 ng g^{-1} with all the target compounds.

Accuracy (precision and trueness).

A recovery study with spiked pooled placenta samples (0.02, 0.25, 1.0 and 2.0 ng g^{-1}) was performed on three consecutive days. As

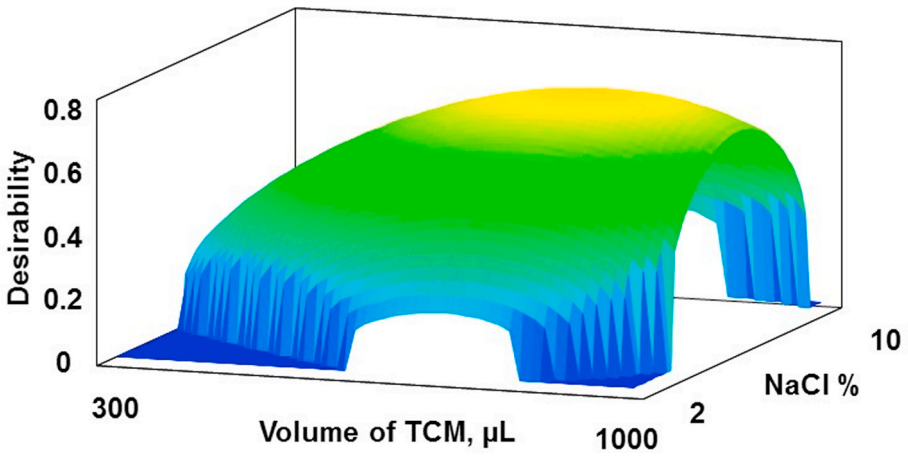

Fig. 3. Estimated response surface for desirability function.

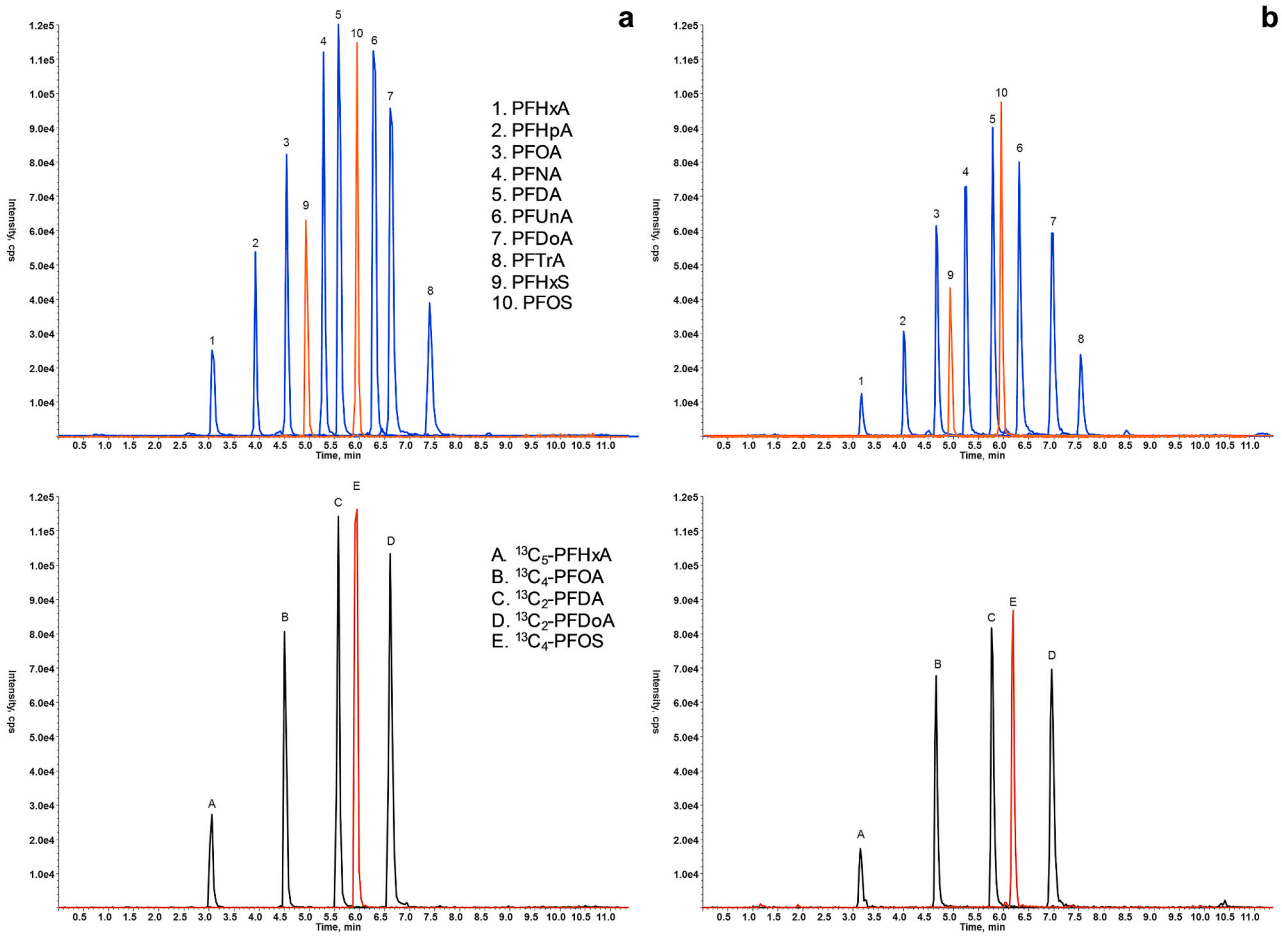

Fig. 4. Chromatograms obtained from a) milliQ water and b) placenta, spiked at 0.25 ng g^{-1}.

Table 2
Recovery assay, precision and trueness of the method.

	Spiked (ng g^{-1})	Found[a] (ng g^{-1})	Recovery (%)	RSD (%)		Spiked (ng g^{-1})	Found[a] (ng g^{-1})	Recovery (%)	RSD (%)
PFHxA	0.02	0.019	94.5	11.0	PFUnA	0.02	0.018	88.9	10.4
	0.25	0.265	106.1	11.7		0.25	0.262	104.9	9.2
	1.0	1.025	102.5	10.7		1.0	1.012	101.2	10.1
	2.0	2.162	108.1	13.8		2.0	2.168	108.4	9.1
PFHpA	0.02	0.019	95.5	9.4	PFDoA	0.02	0.019	95.5	11.9
	0.25	0.243	97.2	8.9		0.25	0.273	109.3	12.4
	1.0	0.971	97.1	12.4		1.0	1.047	104.7	8.5
	2.0	2.046	102.3	13.1		2.0	2.036	101.8	9.7
PFOA	0.02	0.018	88.5	10.4	PFTrA	0.02	0.019	94.2	10.4
	0.25	0.224	89.6	10.1		0.25	0.259	103.7	9.6
	1.0	0.924	92.4	9.0		1.0	1.004	100.4	12.7
	2.0	2.064	103.2	9.8		2.0	2.091	104.5	10.9
PFNA	0.02	0.020	100.9	12.4	PFHxS	0.02	0.022	109.7	9.4
	0.25	0.261	104.4	11.2		0.25	0.264	105.6	11.9
	1.0	1.079	107.9	12.0		1.0	1.103	110.3	8.9
	2.0	2.256	112.8	13.2		2.0	2.184	109.2	10.4
PFDA	0.02	0.021	102.9	13.4	PFOS	0.02	0.018	88.2	11.7
	0.25	0.279	111.5	8.7		0.25	0.227	90.7	10.7
	1.0	1.139	113.9	9.1		1.0	1.017	101.7	11.2
	2.0	2.203	110.2	11.6		2.0	1.998	99.9	7.9

[a] Mean of 18 determinations.

Table 3
Analytical and statistical parameters.

	b (g ng^{-1})	s_b (g ng^{-1})	R^2 (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})	LDR (ng g^{-1})
PFHxA	3.54	0.04	99.6	0.006	0.02	0.02–2.0
PFHpA	3.62	0.07	99.1	0.006	0.02	0.02–2.0
PFOA	5.18	0.06	99.5	0.006	0.02	0.02–2.0
PFNA	1.77	0.03	99.4	0.006	0.02	0.02–2.0
PFDA	3.18	0.04	99.3	0.006	0.02	0.02–2.0
PFUnA	9.17	0.10	99.7	0.006	0.02	0.02–2.0
PFDoA	4.42	0.05	99.5	0.006	0.02	0.02–2.0
PDTrA	2.86	0.03	99.1	0.006	0.02	0.02–2.0
PFHxS	4.74	0.07	99.2	0.006	0.02	0.02–2.0
PFOS	3.85	0.06	99.2	0.006	0.02	0.02–2.0

b, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range.

reported in Table 2, the precision of the method can be stated on account of relative standard deviation (RSD) values were lower than 15%. In an analogue way, the method trueness was supported by recovery values ranging from 85% to 115%.

3.4. Limits of detection and quantification

The limit of quantification (LOQ) was determined as the lowest concentration at which trueness and precision were within ±20%. The limit of detection (LOD) was defined as the lowest concentration at which signals were three times greater than background noise. For the present method, LOQ and LOD values were, respectively, 0.02 and 0.006 ng g^{-1} for all analytes, as shown in Table 3.

3.5. Linearity

Since p-values of the lack-of-fit test (%P_{lof}) were >5% and determination coefficient (R^2) ranged from 99.1% to 99.7%, a good linearity could be declared. Thus, linear dynamic range (LDR) of concentrations from the LOQ to 2.0 ng g^{-1} was established (Table 3).

3.6. Selectivity

The selectivity of the method was evaluated by analyzing the chromatograms of the procedure blank. No interferences were observed at the analyte retention times, as it is shown in Fig. S4. In addition, there was not any carry-over phenomenon (see Fig. S5).

3.7. Method application

The proposed method was used to determine the target PFAS in 20 placenta samples. Table 4 shows that all samples contained detectable concentrations of several analytes under study.

PFHxA and PFHpA were detected in more than 70% of samples, while the remaining PFAS were detected in almost 100% of samples. PFOA and PFOS showed the highest concentrations, with median concentrations of 0.14 and 0.60 ng g^{-1} and maximum concentrations of 0.62 and 1.02 ng g^{-1} (samples M16 and M10), respectively. Median concentrations of the other PFAS ranged from detected values to 0.08 ng g^{-1}. This profile is in agreement with previous studies reporting significantly higher concentrations of PFOS and PFOA than of other PFAS and the detection of PFNA, PFDA, PFUnA, and PFDoA in almost all placenta samples [26–28]. However, variations in exposure levels among study populations hamper the comparison of results. For instance, Mamsen et al. [26] reported median concentrations of 0.30 ng g^{-1} PFOA and 1.24 ng g^{-1} PFOS in 71 placenta samples from a Danish population, and substantially higher median concentrations of 1.22 ng g^{-1} PFOA and 3.64 ng g^{-1} PFOS [27] and 1.41 ng g^{-1} PFOA and 7.32 ng g^{-1} PFOS [28] have been recorded in Chinese populations.

3.8. Comparison with previous methods

The LOQs obtained with the present method appear similar to those achieved with others, as shown in Table 5. However, it does not require a specific extraction device and it offers significantly shorter pre-

Table 4
Application of the proposed method to placenta samples.

Sample	Found concentration, ng g^{-1} (RSD %)[a]									
	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTrA	PFHxS	PFOS
M01	D	D	0.22 (11.2)	0.10 (12.8)	0.06 (13.2)	0.22 (9.6)	0.08 (10.0)	0.55 (7.5)	0.05 (13.4)	0.91 (8.0)
M02	D	D	0.05 (7.9)	0.02 (13.5)	D	0.02 (12.7)	D	0.02 (10.3)	0.09 (9.0)	0.24 (10.4)
M03	D	D	0.11 (13.5)	0.04 (7.4)	0.02 (12.7)	0.02 (13.5)	D	0.05 (7.8)	0.04 (8.9)	0.24 (10.2)
M04	0.03 (11.4)	D	0.06 (12.2)	0.03 (11.3)	D	0.05 (10.0)	D	0.07 (12.5)	0.03 (13.3)	0.27 (7.2)
M05	D	D	0.22 (7.8)	0.06 (6.9)	0.03 (9.1)	0.05 (7.5)	0.02 (12.8)	0.08 (11.8)	0.10 (10.0)	0.93 (8.0)
M06	ND	0.39 (7.1)	0.09 (9.4)	0.04 (9.4)	0.02 (14.0)	0.03 (7.8)	0.02 (14.0)	0.07 (7.2)	D	0.16 (12.4)
M07	D	ND	0.17 (10.7)	0.07 (12.6)	0.04 (11.9)	0.10 (6.8)	0.03 (7.8)	0.13 (9.2)	0.08 (8.3)	0.86 (7.2)
M08	ND	ND	0.13 (12.9)	0.05 (8.1)	0.03 (10.1)	0.12 (11.6)	0.03 (8.6)	0.13 (9.9)	0.06 (13.5)	0.84 (11.0)
M09	ND	D	0.32 (8.7)	0.09 (12.7)	0.05 (7.0)	0.06 (9.2)	0.02 (11.9)	0.10 (10.4)	0.05 (10.3)	0.50 (9.4)
M10	D	D	0.10 (11.0)	0.06 (7.1)	0.03 (12.6)	0.09 (9.9)	D	0.03 (11.4)	0.17 (6.9)	1.02 (7.1)
M11	D	0.39 (9.4)	0.15 (12.5)	0.07 (7.3)	0.04 (8.7)	0.03 (13.6)	D	0.02 (12.6)	0.05 (12.1)	0.61 (11.5)
M12	0.07 (8.1)	0.02 (12.4)	0.10 (11.6)	0.04 (10.7)	0.02 (9.4)	0.04 (11.9)	0.05 (7.9)	0.08 (11.5)	0.05 (9.0)	0.26 (11.1)
M13	ND	ND	0.14 (9.4)	0.09 (8.7)	0.05 (10.1)	0.11 (8.2)	0.02 (13.8)	0.10 (6.4)	0.05 (11.5)	0.91 (7.5)
M14	0.33 (10.7)	0.07 (8.3)	0.21 (9.9)	0.08 (12.8)	0.04 (12.2)	0.10 (10.7)	0.14 (8.3)	0.17 (7.9)	0.08 (8.1)	0.63 (6.9)
M15	0.03 (7.8)	D	0.13 (13.4)	D	D	D	D	0.02 (12.1)	0.04 (12.9)	0.24 (6.2)
M16	D	0.03 (9.9)	0.62 (7.2)	0.19 (8.5)	0.11 (13.1)	0.08 (11.2)	0.04 (12.2)	0.08 (12.7)	0.11 (7.4)	0.81 (8.8)
M17	ND	D	0.14 (12.0)	0.05 (12.9)	0.03 (9.3)	0.06 (7.3)	0.02 (12.0)	0.09 (7.4)	0.04 (13.5)	0.59 (7.9)
M18	ND	D	0.14 (9.1)	0.07 (9.6)	0.04 (13.2)	0.05 (12.5)	0.02 (9.9)	0.07 (8.6)	0.07 (9.5)	0.47 (9.8)
M19	D	D	0.13 (7.6)	0.07 (13.1)	0.04 (10.4)	0.09 (10.3)	0.03 (7.6)	0.15 (8.3)	0.06 (9.9)	0.61 (12.6)
M20	0.03 (12.2)	ND	0.05 (12.6)	0.04 (7.9)	0.02 (11.7)	0.10 (7.1)	0.03 (11.2)	0.19 (10.1)	0.03 (12.2)	0.48 (10.0)
Det. (n, (%))[b]	14 (70)	16 (80)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)
Median	D	D	0.14	0.06	0.03	0.06	0.02	0.08	0.05	0.60
C.range[c]	ND-0.33	ND-0.39	0.05–0.62	D-0.19	D-0.11	D-0.22	D-0.14	0.02–0.55	D-0.17	0.16–1.02

[a] Mean of 3 determinations; RSD: relative standard deviation; ND, not detected (<LOD); D, detected (>LOD and <LOQ).
[b] Detected.
[c] Concentration range.

Table 5
Comparison of this SALLE-DLLME procedure with previous methods for determining PFAS in placenta samples.

PFAS	Sample pre-treatment	Treatment techniques[a]	Sample amount	Extraction-cleanup global time/solvent consumption	Instrumental technique[b]	LOQ[c] (ng g^{-1})	Ref.
PFOA, PFNA, PFDA, PFUnA, PFHxS, PFOS	Homogenisation by ultrasonication	LLE	0.1 g	30 min/—	LC-MS/MS	0.09–0.60	26
PFOA, PFHxS, PFOS	-Mechanical homogenisation. -Lyophilisation, 72 h. -Treatment with 1 N NaOH in methanol, 24 h. -Drying and reconstitution.	SPE (HLB)	10 g	—/7 mL	LC-MS/MS	0.01–0.05	27
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS	-Lyophilisation, 24 h	Ion pairing-LLE	10 g	60 min/11 mL	LC-MS/MS	0.01–0.03	28
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFHxS, PFOS	-Enzymatic treatment with collagenase, 4 h.	SALLE-DLLME	1.0 g	2 min/6.8 mL	LC-MS/MS	0.02	This study

[a] LLE: liquid liquid extraction; SPE: solid phase extraction.
[b] LC: liquid chromatography; MS: mass spectrometry.
[c] LOQ: limit of quantification.

treatment and extraction times. For instance, sample pre-treatment took four days in the study by Chen et al. [27] and one day in the study by Zhang et al. needed one [28]. This time-saving is especially advantageous when a high number of samples must be treated every day, as in large-scale biomonitoring studies.

4. Conclusions

Concentrations of 10 PFAS were successfully measured in placenta samples from 20 randomly selected women using SALLE coupled to DLLME and LC–MS/MS. The experimental parameters were optimized by chemometric procedures, and the procedure was validated. The operational advantages of this approach offer major benefits for biomonitoring research on exposure to these prevalent EDCs, especially in early life. This is the first application of SALLE-DLLME combination to determine PFAS in placental tissue, which is able to provide significant operational improvements compared to previous methods. The proposed procedure can be used to explore PFAS exposure of embryos and fetuses through placenta and relate it to health effects, a line of research that needs further study.

Authorship contributions

F. Vela-Soria: conceptualization, methodology, validation, formal analysis, investigation, writing-original draft, writing-review and editing. J. García-Villanova: conceptualization, formal analysis, investigation, resources, writing-original draft. V. Mustieles: validation, formal analysis, writing-original draft, writing-review and editing. T. de Haro: resources, writing-review and editing, supervision. J.P. Antignac: conceptualization, resources, writing-original draft, writing-review and editing, supervision, project administration. M.F. Fernandez: conceptualization, resources, writing-original draft, writing-review and

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.

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Appendix A. Supplementary data

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