# Validated method for the determination of perfluorinated compounds in placental tissue samples based on a simple extraction procedure prior to ultra–high performance liquid chromatography–tandem mass spectrometry analysis

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ABSTRACT Exposure to xenobiotics during pregnancy is inevitable. Determination of perfluorinated compounds (PFCs), renowned environmental contaminants by Public Health Authorities due to its persistence, bioaccumulative effect and toxicity, is a challenge. In the present work, a method based on a simplified sample treatment involving steps of freeze-drying, solvent extraction and dispersive clean-up of the extracts with C18 prior to ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis for the determination of 5 perfluorinated carboxylic acids (from C4 to C8) and perfluorooctane sulfonate (PFOS) in placental tissue samples is developed and validated. The most influential parameters affecting the extraction method and the clean-up step were optimized using design of experiments. The method was validated using matrix-matched calibration. Found detection limits (LOD) ranged from 0.03 to 2 ng $g^{-1}$ and quantification limits (LOQ) from 0.08 to 6 ng $g^{-1}$, while inter- and intra-day variability was under 14% in all cases. Recovery rates for spiked samples ranged from 94 to 113%. The method was satisfactorily applied for the determination of compounds in human placental tissue samples collected at the moment of delivery from 25 randomly selected women.

*Keywords:* Perfluorinated compounds; Placental tissue; Dispersive solid phase extraction; UHPLC–MS/MS

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# 1. Introduction

Exposure to xenobiotics during pregnancy is inevitable. They are likely to act not only directly on the fetus but also on the placenta itself which may affect its ability to support pregnancy [1]. Endocrine disruptors (EDs) are ubiquitous in the environment around us and as a consequence in human bodies. There is a growing concern about the potential health effects of exposure to various environmental chemicals during pregnancy and infancy. Mono-2-ethylhexyl phthalate (MEHP), octylphenol (OP), 4-nonylphenol (4-NP) or polybrominated diphenyl ethers (PBDEs) have been detected in physiologically relevant compartments within pregnant women and the developing fetuses, such as maternal urine, cord blood, breast milk, meconium, placenta and amniotic fluid in several studies [2-8], showing that pregnant women and their fetuses are exposed to those chemicals.

Perfluorinated compounds (PFCs) have been recognized as an important class of potential contaminants by Public Health Authorities due to its persistence, bioaccumulative effect and toxicity [9]. Since their first production in 1947, PFCs have been used in a wide range of commercial and industrial applications such as polymers, metal plating and cleaning, surfactants, lubricants, pesticides, coating formulations, inks, varnishes, firefighting foam, and stain/water repellents for leather, paper and textiles. Worldwide human exposure to PFCs has been confirmed [10-13]. For the general population the major source of contamination arises from food and sometimes drinking water [12, 14-16]. Human exposure also arises from indoor and ambient air and house dust. Perfluorooctane sulfonate (PFOS) is generally the most abundant PFCs found in humans, the second usually being perfluorooctanoic acid (PFOA) [9]. An advanced estimation (2012) of dietary exposure was ~5–10 ng/kg bw/day for PFOS, 4–7 ng/kg bw/day for PFOA, the most important contributors being fish and seafood, fruit and fruit products [17]. Children, especially toddlers, are more exposed than adults (2-3 times higher) on a body weight basis, due to higher relative food consumption and to hand-to-mouth transfer from impregnated carpets and ingestion of dust, resulting in higher PFCs serum levels than for adults [14, 18, 19]. *In utero* and postnatal exposures are of particular concern. PFCs cross the placental barrier, exposing neonates via their mother's blood [20-22].

In addition, first scientific evidences suggesting that PFCs would be responsible for reproductive disorders in humans have been recently published [23-29]. Butenhoff et al. [26] published a work on the role of PFCs in the onset of health troubles affecting the reproductive function when exposure occurs during critical stages of the development, *i.e.* the perinatal

period. Fei et al. [25] observed an association between high serum levels of PFOS and PFOA and a longer time to pregnancy. Effects of *in utero* exposure to PFCS on the female reproductive functions have also been reported. In a recent study, carried out by Kristensen and co-workers [29], daughters who were exposed *in utero* to levels of PFOA that were higher than the reference group showed a later age of menarche. In particular, a number of animal toxicology studies have shown that exposure to PFOS and PFOA can alter ovarian function [27] and affect the development of mammary gland tissue [28].

In this context, the characterization of the PFCs transfer, if any, from the mother to the fetus (through placenta) and/or to the newborn (through breastfeeding) is acutely expected. Biomonitoring data reflecting the internal PFCs exposure levels in the general population have already been provided in breast milk, maternal and cord serum from different states/countries [30]. However, there is limited data on the *in utero* exposure levels of newborns to these PFCs so far. Most of the researches on placental transfer of PFCs were conducted by animal or *in vitro* experiments instead of human studies. Therefore, the validation of analytical methods to determine PFCs in human placental tissue is of special scientific interest.

The preferred technique for the analysis of these compounds has been high-performance liquid chromatography-tandem mass spectrometry in negative electrospray ionization mode (LC/ESI–MS/MS) [31-32]. The isolation of analytes from this complex biological matrix is a critical aspect even when a chromatographic technique is employed due to selectivity and sensitivity issues. Some techniques such as microwave-assisted extraction (MAE) or pressurized liquid extraction (PLE) could be good alternatives to maximize the extraction efficiency, but the application of these techniques involves drastic conditions, specific technical resources and not very clean final extracts including ion suppression, which is critical when mass spectrometry is employed as detection technique [33]. Solvent extraction with vortex homogenization followed by a clean-up of the extract based on dispersive solid-phase extraction (d-SPE) was chosen in our study as extraction technique because its simplicity, low cost, short extraction time, low volume of solvents required and its widespread use in most of the laboratories focused on routine analyses.

To our knowledge, there is a lack of published literature on PFCs determination in placental tissue. The aim of the present work was to validate a fast, simple, accurate and sensitive UHPLC–MS/MS method for the determination of PFCs in freeze-dried human placental tissue samples. After validation, the method was satisfactorily applied to determine the free content of target compounds in samples collected from 25 unknown volunteers.

## 2. Experimental

### *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, USA). Perfluorobutanoic acid (PFBuA) (98%), perfluoropentanoic acid (PFPeA) (97%), perfluorohexanoic acid (PFHxA) (≥ 97%), perflurorohеptanoic acid (PFHpA) (99%), perflurorooctanoic acid (PFOA) (96%), and perfluorooctanesulfonic acid (PFOS) (≥ 98%) and perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid (MPFOA) were purchased from Sigma-Aldrich (Madrid, Spain). Stock solutions of each compound, at a concentration of 1000 mg $L^{-1}$, were prepared in methanol and stored at -20 ºC. Working solutions were prepared by diluting the stock standard solutions in methanol or in the initial mobile phase immediately before use. These solutions were stored at 4 ºC and prepared fresh weekly. All solutions were stored in dark glass bottles to prevent photodegradation. Anhydrous magnesium sulfate was provided by Panreac (Barcelona, Spain). PSA sorbent (primary secondary amine, 40–60 μm) was purchased from Scharlab (Barcelona, Spain) and BAKERBONDs octadecyl C18 sorbent (40 μm particle size) was provided by J. T. Baker (Deventer, The Netherlands). LC–MS grade methanol, water and acetonitrile, acetic acid and ammonia (25%, w/v) were purchased from Sigma-Aldrich.

### *2.2. Instrumentation and software*

UHPLC–MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class (Waters, Manchester, UK), consisting of Acquity UPLC™ binary solvent manager and Acquity UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for PFCs detection. An Acquity UPLC® BEH $C_{18}$ (50 mm × 2.1 mm i.d., 1.7 μm particle size) and a CORTECS UPLC™ C18 (Waters) column (50 mm × 2.1 mm; 1.6 μm particle size) from Waters (UK) were assayed as chromatographic columns. Placental tissue samples were freeze-dried using a SCANVAC CoolSafe™ freezedryer (Lynge, Denmark). A Branson digital sonifier (Danbury, CT, USA), a vortex-mixer (IKA, Staufen, Germany), an ultrasound-HD bath (Selecta, Barcelona, Spain), a Spectrafuge™ 24D centrifuge from Labnet

International, Inc. (New Jersey, USA) and a sample concentrator (Stuart, Staffordshire, UK) were also used. Statgraphics Plus software version 5.1 (Statpoint Technologies Inc., Virginia, USA) was used for statistical treatment of data.

### *2.3. Sample collection and storage*

Placenta tissue samples were obtained from health women living in Granada, Spain. Samples were anonymized, frozen at -20 ºC and stored until analysis in our laboratory. The study was performed in compliance with the Ethical Principles for Medical Research Involving Human Subjects issued by the World Medical Association, and all volunteers signed the informed consent form. ~~Human placental tissue samples were collected from volunteers at the moment of delivery.~~ In order to ensure the homogeneity and representativeness of the whole placenta tissue, each one was accurately examined, weighed and fragmented. Then, half of the placenta (including maternal and fetal sides and central and peripheral parts) was placed in the glass container of a mixer for its homogenization. Once homogenized, aliquots of 25 g were stored frozen at –86 ºC within 90 min of its collection. Samples were anonymized to preserve the confidentiality of patients. All volunteers signed their informed consent to participate in the study.

Before processing, placental tissue aliquots were additionally homogenized using an ultrasonic spindle. The container was placed in a glass full of ice in order to avoid sample heating and the spindle was in direct contact with the placental tissue. Ultrasound setting consisted in ~~pulses~~ duty cycles of 30 s followed by 30 s without sonication, until complete 5 min of effective radiation. Once the samples were homogenized, they were frozen at −86 ºC and stored confidentially and anonymously until analysis.

### *2.4. Basic procedure*

#### *2.4.1. Preparation of spiked samples*

Due to the absence of certified materials for these compounds in this matrix, 6 g of spiked sample were prepared containing the analytes at seven different concentration levels, from 0.025 to 62.5 ng $g^{-1}$, for calibration standards and 1.25, 6.25, 12.5 and 25 ng $g^{-1}$, for quality control and validation of the method. Spiked samples were accurately stirred and slightly heated (35 ºC) to homogenize. Then, aliquots of 1 g were weighted in 8 mL glass

vials and fortified with 200 µL of a methanolic solution (62.5 ng $mL^{-1}$) of the surrogate MPFOA (final concentration 12.5 ng $g^{-1}$ in placental tissue). After shaking for 10 min, the spiked samples were ready for the experiments.

*2.4.2. Sample treatment*

An aliquot of placental tissue sample (1 g) was weighed in an 8 mL glass vial, fortified with 200 µL of methanol containing 62.5 ng $mL^{-1}$ of MPFOA, shaken for 5 min and freeze-dried. The sample was homogenized with 5 mL of acetonitrile in vortex for 1 min. Then, the mixture was centrifuged for 10 min at 4050 × *g*.

In order to decrease the matrix co-extractives in the extract that could cause the matrix effect, a clean-up of the extract based on d-SPE was carried out. The extract was transferred to an 8 mL polypropylene conical tube containing 108 mg of C18 sorbent. The mixture was hand-shaken for 2 min and centrifuged for 5 min at 4050 ~~4050 ×~~ *g*. The organic phase (acetonitrile) containing the analytes was separated into a glass vial and then evaporated under a nitrogen stream at room temperature and the final residue was dissolved with 250 µL of the initial mobile phase. After stirring for 30 s in vortex and centrifugation for 5 min at 16,300 × *g*, the sample was ready to be injected into the LC system.

*2.4.3. Ultra-high performance liquid chromatography–tandem mass spectrometry conditions*

Chromatographic separation of compounds was performed using a CORTECS UPLC™ C18 (Waters) column (50 mm × 2.1 mm; 1.6 µm particle size). The compounds were separated using a gradient mobile phase consisting of a buffer solution acetic acid/ammonium acetate (pH 4.4) (solvent A) and methanol (solvent B). Gradient conditions were: 0.0-5.0 min, 20-100% B and back to 20% in 0.1 min and kept for 1.9 min to equilibrate the column. Flow rate was 0.3 mL $min^{-1}$. The injection volume was 10 µL. The column temperature was maintained at 40 ºC. Total run time was 7 min.

The tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. ESI was performed in negative ion mode. The ion source temperature was maintained at 150 ºC. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 ºC; desolvation temperature, 500 ºC; cone gas flow, 150 L $h^{-1}$; desolvation gas flow, 500 L $h^{-1}$; collision gas flow, 0.15 mL $min^{-1}$, and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was

used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell time was 25 ms. Table 1 shows the parameters and the mass transitions for each compound.

**Table 1**

*2.4.4. Method validation*

Validation in terms of linearity, selectivity, accuracy (trueness and precision) and sensitivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [34].

Linearity of the calibration graphs was tested using the determination coefficients (%, $R^2$) and the P-values of the *lack-of-fit* test (%, $P_{lof}$) [35]. The specificity of the method was determined by comparing the chromatograms of blank with the corresponding spiked placental tissue. Due to the absence of certified materials, in order to evaluate the trueness and the reproducibility of the method, a study with spiked placenta samples, at three concentrations levels (6.25, 12.5 and 25 ng $g^{-1}$ for PFBuA, PFPeA and PFHxA and 1.25, 6.25 and 12.5 ng $g^{-1}$ for PFHpA, PFOA and PFOS) was performed. Precision, expressed as relative standard deviation (%, RSD) was determined from triplicate spiked samples during the same day and in six different days, and the trueness was evaluated by a recovery assay. The recovery of the tested compounds in placenta samples was evaluated by comparing the known concentration in spiked samples with the concentration of each compound determined using the method proposed. Finally, LODs and LOQs were calculated by taking into consideration the standard deviation of residuals, $s_{y/x}$, the slope, b, of the calibration graphs and an estimate $s_o$ obtained by extrapolation of the standard deviation of the blank (Analytical Methods Committee). The LOD was $3 \cdot s_o$ and the LOQ was $10 \cdot s_0$.

*2.4.54. Quality assurance and quality control*

Validity of the analytical results was verified by some simple quality assurance and quality control (QA/QC) measures. Procedural blanks were injected to monitor for background contamination. Blanks were processed in the same way as the samples and injected into the UHPLC–MS/MS system. No quantifiable amounts of target compounds were detected. On the other hand, in order to evaluate possible contaminations and the variability of the instrumental analysis, standards (spiked blank samples at 0 and 25 ng $g^{-1}$) and a standard in the initial mobile phase (100 ng $mL^{-1}$) were injected by triplicate every 20 samples.

## 3. Results and discussion

### *3.1. Liquid chromatographic-mass spectrometric analysis*

A BEH $C_{18}$ UPLC™ column (50 mm × 2.1 mm i.d., 1.7 µm particle size) and a CORTECS UPLC™ C18 (Waters) column (50 mm × 2.1 mm; 1.6 µm particle size) were tested. Although both columns offered similar resolution for all the analytes investigated, at the retention time of the analytes eluted CORTECS UPLC™ column provided better peak shape in the shortest time with, even this last generated pressures 1000 psi lower than BEH $C_{18}$ UPLC™. Consequently, this column was the one we selected for further experiments.

The effect of the mobile phase on chromatographic separation was also studied. Our aim was to obtain high sensitivity and selectivity in a short time. First, the pH of the mobile phase was studied and deionized water with different additives was studied as solvent A. Acetic acid (from 0% to 0.2%, v/v), ammonia (from 0% to 0.050%, w/v) and mixtures of them were assayed. Higher responses and better peak shapes were obtained using a mixture of 1:1 (v/v) of acetic acid (0.1%, v/v) and ammonia (0.025% w/v). MeOH, ACN and mixtures of these solvents were evaluated as organic mobile phases (solvent B). MeOH was selected because of the sensitivity, peak shapes and separation achieved. A linear gradient, as described in the previous section, was used. The injection volume was studied in order to enhance the analytical signal and consequently the LOD of the method. A range from 2.5 to 10 µL was analyzed and 10 µL was chosen as injection volume since a marked increase in sensitivity without loss of resolution was obtained. Finally, the increase of temperature from 30 to 50 ºC did not improve significantly the characteristics of chromatographic method, therefore 340 ºC was chosen as optimum.

The MS/MS detection method was set up by continuous infusion of standard solutions of each individual compound (1 mg $L^{-1}$) to optimize the response of the precursor ion. The mass spectrometric conditions were optimized for each compound. ESI and ESCI interfaces in positive and negative modes were evaluated. ESI interface in negative mode was selected because it showed higher sensitivity for all compounds of interest. For each compound two product ions (two reactions) were monitored: one for quantification and the other for confirmation. The most abundant transition ion was selected to obtain maximum sensitivity

for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1.

### *3.2. Optimization of the extraction procedure*

Key variables, such as the extraction step (solvent and technique) and the clean-up step (type and amount of sorbent) were optimized using aliquots of 1 g of human placental tissue spiked with the selected compounds at a concentration level of 250 ng $g^{-1}$, in triplicate.

#### *3.2.1. Selection of the extraction solvent*

Acetone, acetonitrile, ethyl acetate and methanol, widely used for the extraction of different families of ECDs from biological and environmental samples [33, 34], were evaluated. Spiked human placental tissue was mixed with 5 mL of each studied solvent. Any clean-up step was used. Results are shown in Figure 1.

**Figure 1**

The best extraction efficiencies were obtained using acetonitrile, followed by acetone and ethyl acetate, respectively. Methanol extracts was characterized by the highest extraction of matrix components, making difficult the handling of the extracts and were not analyzed further. Therefore, acetonitrile was selected for further optimization.

#### *3.2.2. Selection of the extraction procedure*

An additional step based on ultrasonic bath for 10 min, after the addition of 5 mL of acetonitrile and shaken in a vortex-mixer for 1 min, was tested to improve the extraction of the studied compounds.

**Figure 2**

Slightly higher extraction recoveries were achieved with the additional ultrasonic step for most of the compounds (from 100 to 114%, versus 90% to 106% when using only vortex mixer), but also more interfering compounds were extracted as well, affecting the chromatography. Therefore, only vortex extraction was selected due to its simplicity, low cost and general use in most of the laboratories focused on routine analyses. Extraction times of 1, 2, 5 and 10 min were assayed. No significant difference was observed between the shortest and the longest extraction time, hence, for practical reasons, 1 min was enough to obtain the homogenization of the freeze-dried placental tissue.

*3.2.3. Optimization of the clean-up sorbent: experimental design*

A clean-up of the extract based on d-SPE was carried out for decrease/remove the matrix co-extractives in the extract. A 15-run Box–Behnken experimental design including three replicates at the central point was used for fitting a second-order response surface (Table S1). Three factors and three levels for each one were selected: amount of $MgSO_4$ (0, 250 and 500 mg), amount of PSA (0, 150 and 300 mg) and amount of C18 (0, 150 and 300 mg).

The data were analyzed using ANOVA, which provided determination coefficients ($R^2$) greater than 0.90 in all cases. P values for the *lack-of-fit* test also were >0.05, what makes the model satisfactory with the 95% of confidence level. Pareto charts were also obtained and statistically significant effects of the variables were screened using a Student's t-test. Variables having a confidence greater than 95% were considered to have a significant effect on the extraction efficiency. Figure 3 shows the statistically significant effect of each variable.

**Figure 3**

The PSA amount and some of their interactions, especially the negative one, resulted to be the most significant variable for analytes. In this case, the addition of PSA sorbent caused a decrease of the extraction efficiencies values for PFCs. This could be due to the ion-pair formation between the primary secondary amine with the carboxylic groups of PFCs. There were not differences in optimal values for two of the three factors, amount of PSA and $MgSO_4$, the optimal values was situated at minimal levels of PSA and $MgSO_4$ (0 mg) between selected analytes. Different behaviors were obtained between the amount of C18 sorbent and analytes. Perfluorinated carboxylic acids (from C5 to C8) providing slightly higher extraction

efficiencies without C18, while PFOS showed higher values when the amount of C18 is raised to 300 mg. See supplementary material, Figure S1.

The combination of the optimized experimental values obtained for each compound was obtained using the desirability function. Responses for each compound in the experiments of the Box–Behnken design were first normalized between 0 and 1, and the global desirability function was defined as their geometric mean. The plot of this function: (A) amount of PSA *vs.* amount of C18; (B) amount of $MgSO_4$ *vs.* amount of C18 and (C) amount of $MgSO_4$ *vs.* amount of C18 are shown in Figure S2. The optimal values was situated at minimal levels of PSA and $MgSO_4$ (0 mg) and medium level of C18 mass (108 mg).

**Figure S2**

*3.2.4. Optimization of the reconstitution step*

Finally, the effect of different reconstitution solvents (methanol, water and the initial mobile phase) and volumes (50, 100, 250, 500 and 1000 µL) were evaluated. The optimal results, providing better peak shape, were achieved with the mixture of buffer solution acetic acid/ammonium acetate (pH 4.4) and methanol (80:20, v/v).

On the other hand, selectivity aspects, including ion suppression, are critical when mass spectrometry is employed as detection technique. It was noted that the use of volume higher than 250 µL provided a cleaner extraction solution with lower background; conversely the use of 50 and 100 µL made the extraction solution turbid and the noise in the analysis was extremely high and made quantification difficult. Since a constant response was observed from 250 µL for all analytes, this was the selected as reconstitution volume.

*3.3. Analytical performance*

For calibration purposes, seven concentration levels (from 0.025 to 62.5 ng $g^{-1}$) were prepared and calibration curve was built. The calibration standards were prepared adding 500 µL of methanol containing the analytes to 1 g of the placental tissue prior freeze-drying. Each level of concentration was made in triplicate. The samples were vigorously stirred and slightly heated in order to remove the methanol until they recovered their original weight. Then, the extraction procedure previously explained was applied.

Calibration curves were constructed using analyte/surrogate peak area ratio versus concentration of analyte. MPFOA (12.5 ng $g^{-1}$) was used as surrogate. Calibration graphs were made using SRM mode. In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound: one in solvent (initial mobile phase) and the other one in the matrix. A Student's t-test was applied in order to compare the calibration curves. First, we had to compare the variances estimated as $s^2_{y/x}$ by means of a Snedecor's *F*-test. Then, the Student's *t*-test was applied to compare the slopes of calibration curves. The *t* calculated was compared with the two-tailed tabulated value, $t_{tab}$ for the appropriate number of degrees of freedom at P (%) confidence. Typical values are k = 2 for 95% confidence [~~33~~37], so: If $t < k$, the ratio of the slopes is not significantly different from 1; and, if $t > k$, the ratio of the slopes is significantly different from 1 and the matrix-matched calibration must be used. The Student's *t*-test showed statistical differences among slope values for the calibration curves in cases of PFBuA and PFOS and, consequently, the use of matrix-matched calibration was necessary. Table 2 shows the statistical and analytical parameters obtained for each analyzed compound.

**Table 2**

*3.4. Method validation*

*3.4.1. Linearity*

A concentration range from the minimal quantified amount to 62.5 ng $g^{-1}$ was selected. The values obtained for $R^2$ ranged from 99.3 for PFPeA to 99.9% for PFBuA and PFHxA, and $P_{lof}$ values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges.

*3.4.2. Selectivity*

No interferences from endogenous substances were observed at the retention time of the analytes eluted. These finds suggest that the spectrometric conditions ensured high selectivity of the LC–MS/MS method. Figure ~~54~~A shows the SRM chromatograms obtained for a spiked blank.

**Figure ~~5~~4**

*3.4.3. Accuracy: precision and trueness*

The precision and the trueness of the proposed analytical method are shown in Table 3.

**Table 3**

The recoveries were close to 100% (94-113%) in all cases. Inter-day precision was lower than 14%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data demonstrated that the proposed method is reproducible. Precision and trueness data indicate that the methodology to determine the target compounds in placenta samples is accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

*3.4.4. Sensitivity*

Two fundamental aspects, which need to be examined in the validation of any analytical method, are the LOD and LOQ in order to determine if an analyte is present in the sample. Found LODs ranged from 0.03 ng $g^{-1}$ for PFHpA, PFOA and PFOS to 2 ng $g^{-1}$ for PFPeA. Table 2 shows the values obtained.

*3.5. Method application*

After validation, the method was applied to the determination of PFCs in 25 samples obtained from unknown women at the moment of delivery. The concentrations found expressed as mean of six determinations are summarized in Table 4. Figure ~~5~~4B shows a SRM chromatogram obtained using the quantification transition for a real human placental tissue sample (mother 24).

**Table 4**

As it is shown in Table 4, PFOS was the compound more frequently found, it was detected in 56% (n = 14/25) of the analyzed samples, however it was quantified (amount > LOQ) in only 6 of them in concentrations ranging from 0.21 to 1.2 ng $g^{-1}$; PFHxA was detected in 9 samples (36%) and quantified in 2 of them at a concentration level close to 5 ng $g^{-1}$; PFOA and PFBuA were detected in 6 samples (24%) and quantified in 2 of them. It is a remarkable fact that in the case of PFBuA, the two samples were contaminated with abnormally high amounts of the compound (≈ 30 ng $g^{-1}$). PFHpA was detected in 4 samples but not quantified and finally, regarding to PFPeA , this compound was not detected in any of the analyzed samples. Pérez et al. [38] studied the presence of PFCs in other biologic matrices such as human hair and urine, and they found the same pattern of PFCs than in our study, but at much higher concentrations in urine. PFOS and PFOA were the compounds more frequently found. PFOS was present at concentrations in the range 3.7–7.0 ng $g^{-1}$ among the positive samples, whereas PFOA was between 0.1 and 6.0 ng $g^{-1}$. It should be also pointed out that some of the less frequently found compounds were present at high concentrations, as it happens in this work with PFBuA reaching concentration levels up to 30 ng $g^{-1}$.

## 4. Conclusions

A method for the determination of 5 perfluorinated carboxylic acids (from C4 to C8) and perfluorooctane sulfonate (PFOS) in placental tissue samples has been successfully performed. One of the main advantages of the present study is the possibility of performing a complex analytical determination using a reduced number of steps and giving the sensitivity and selectivity necessary to the detection of these compounds at biological relevant concentrations in the few nanogram per gram level in placental tissue.

The proposed method has been applied to samples collected from 25 randomly selected women, being PFOS the most frequently detected and quantified compound. Studies on human exposure to PFCs are needed to address the question of whether maternal exposure to these compounds can lead to adverse health effects in the offspring.

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| Name | Abbreviation | Retention time (min) | Precursor Ion (m/z) | SRM 1[a] (m/z) | SRM 2[b] (m/z) | CV (V) | CE (eV) |
|---|---|---|---|---|---|---|---|
| Perfluorobutanoic acid | PFPuA | 1.60 | 213 | 169 | 51.6 | 36 | 20 |
| Perfluoropentanoic acid | PFPeA | 3.18 | 263 | 219 | 89.7 | 36 | 20 |
| Perfluorohexanoic acid | PFHxA | 3.98 | 313 | 269 | 119 | 36 | 20 |
| Perfluoroheptanoic acid | PFHpA | 4.49 | 363 | 319 | 333 | 36 | 20 |
| Perfluorooctanoic acid | PFOA | 4.87 | 413 | 369 | 194 | 36 | 20 |
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**Table 2**
Analytical and statistical parameters.

| | PFBuA | PFPeA | PFHxA | PFHpA | PFOA | PFOS |
|---|---|---|---|---|---|---|
| $b_{MP}$ (g $ng^{-1}$) | 0.066 | 0.12 | 0.24 | 0.19 | 0.19 | 0.59 |
| $s_{bMP}$ (g $ng^{-1}$) | $8.5 \cdot 10^{-4}$ | $9.4 \cdot 10^{-4}$ | $3.1 \cdot 10^{-3}$ | $2.2 \cdot 10^{-3}$ | $2.2 \cdot 10^{-3}$ | $6.9 \cdot 10^{-3}$ |
| $b_{PT}$ (g $ng^{-1}$) | 0.036 | 0.11 | 0.24 | 0.20 | 0.20 | 0.30 |
| $s_{bPT}$ (g $ng^{-1}$) | $1.1 \cdot 10^{-3}$ | $6.4 \cdot 10^{-3}$ | $4.4 \cdot 10^{-3}$ | $7.0 \cdot 10^{-3}$ | $7.0 \cdot 10^{-3}$ | $1.1 \cdot 10^{-2}$ |
| $t_{student}$ | 13 | 1 | 2 | 1 | 1 | 13 |
| % $R^2$ | 99.9 | 99.3 | 99.9 | 99.8 | 99.8 | 99.6 |
| LOD (ng $g^{-1}$) | 1.5 | 2 | 0.5 | 0.03 | 0.03 | 0.03 |
| LOQ (ng $g^{-1}$) | 4 | 6 | 2 | 0.08 | 0.08 | 0.08 |
| LDR (ng $g^{-1}$) | 4.0-62.5 | 6.0-62.5 | 1.8-62.5 | 0.08-62.5 | 0.08-62.5 | 0.08-62.5 |

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| | 25 | 28 (2) | 6 | 113 |
| PFPeA | 6.25 | 6.61 (1) | 9 | 106 |
| | 12.5 | 12.3 (2) | 10 | 98 |
| | 25 | 24 (3) | 9 | 94 |
| PFHxA | 6.25 | 6.44 (1) | 10 | 103 |
| | 12.5 | 12.4 (2) | 12 | 99 |
| | 25 | 23 (3) | 12 | 94 |
| PFHpA | 1.25 | 1.32 (0.1) | 1 | 105 |
| | 6.25 | 6.24 (1) | 4 | 100 |
| | 12.5 | 11.8 (1) | 12 | 95 |
| PFOA | 1.25 | 1.30 (0.1) | 1 | 104 |
| | 6.25 | 6.24 (1) | 4 | 100 |
| | 12.5 | 11.8 (1) | 12 | 95 |
| PFOS | 1.25 | 1.30 (0.1) | 1 | 104 |
| | 6.25 | 6.11 (1) | 13 | 98 |
| | 12.5 | 12.6 (2) | 14 | 101 |

SD: Standard deviation; RSD: Relative standard deviation

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Concentrations of PFCs determined in the placenta samples.

| Sample | *Found amount (ng $g^{-1}$) (SD) | | | | | |
|---|---|---|---|---|---|---|
| | PFBuA | PFPeA | PFHxA | PFHpA | PFOA | PFOS |
| Mother 01 | D | ND | D | ND | D | D |
| Mother 02 | ND | ND | ND | ND | ND | ND |
| Mother 03 | ND | ND | ND | ND | ND | ND |
| Mother 04 | ND | ND | D | ND | ND | 1.1 (0.01) |
| Mother 05 | ND | ND | ND | ND | ND | D |
| Mother 06 | ND | ND | ND | ND | ND | D |
| Mother 07 | ND | ND | ND | ND | ND | 1.0 (0.01) |
| Mother 08 | 30 (2) | ND | 4.9 (0.5) | ND | D | ND |
| Mother 09 | ND | ND | ND | ND | ND | 1.2 (0.01) |
| Mother 10 | D | ND | D | ND | ND | ND |
| Mother 11 | ND | ND | ND | ND | ND | ND |
| Mother 12 | ND | ND | ND | ND | ND | ND |
| Mother 13 | ND | ND | ND | ND | ND | D |
| Mother 14 | ND | ND | D | D | D | D |
| Mother 15 | 28 (1.5) | ND | 5.1 (0.4) | ND | ND | D |
| Mother 16 | ND | ND | D | ND | ND | D |
| Mother 17 | ND | ND | ND | ND | ND | ND |
| Mother 18 | ND | ND | D | ND | D | 1.1 (0.01) |
| Mother 19 | ND | ND | ND | ND | ND | ND |
| Mother 20 | D | ND | D | ND | ND | D |
| Mother 21 | D | ND | ND | D | 0.37 (0.03) | 1.2 (0.01) |
| Mother 22 | ND | ND | ND | D | ND | ND |
| Mother 23 | ND | ND | ND | ND | ND | ND |
| Mother 24 | ND | ND | ND | D | 0.37 (0.02) | 0.21 (0.01) |
| Mother 25 | ND | ND | ND | ND | ND | ND |

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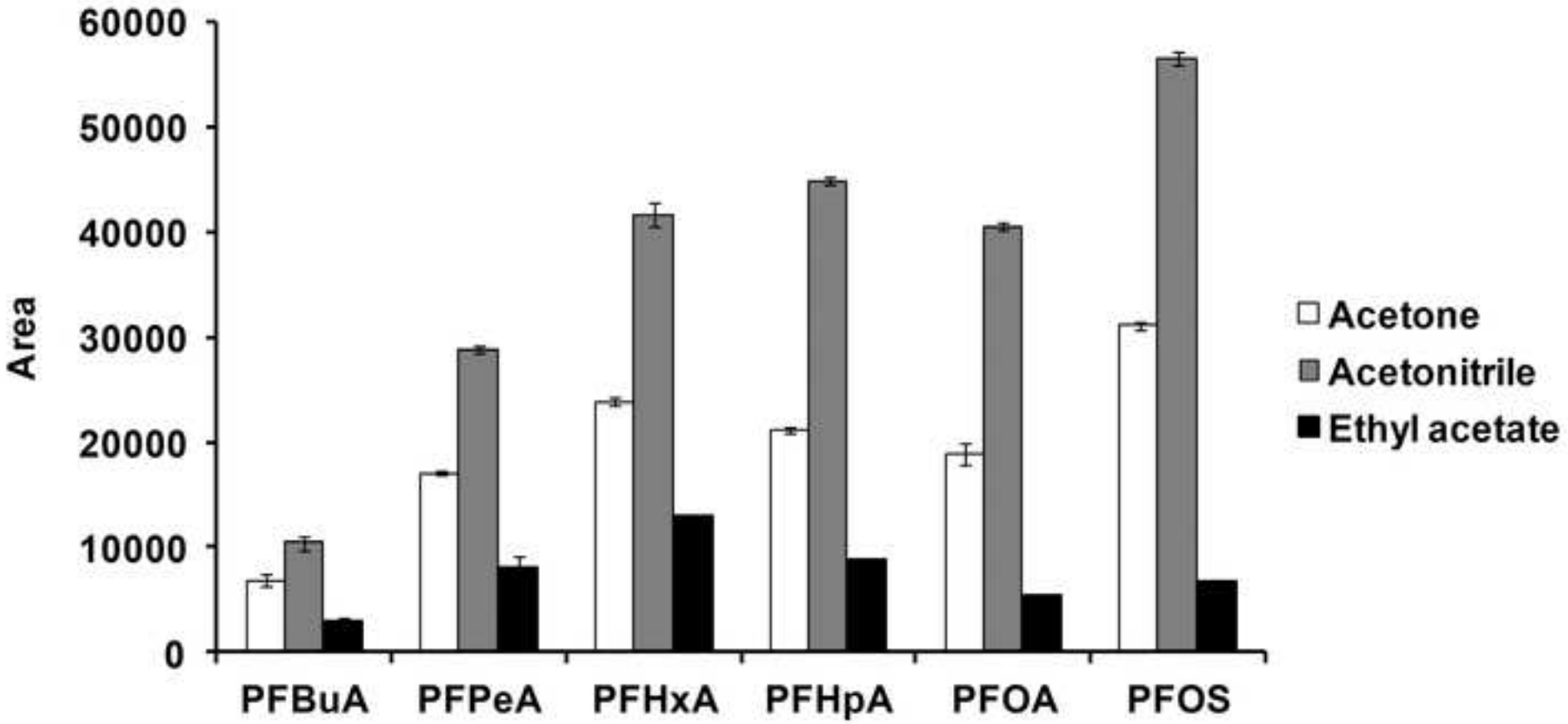

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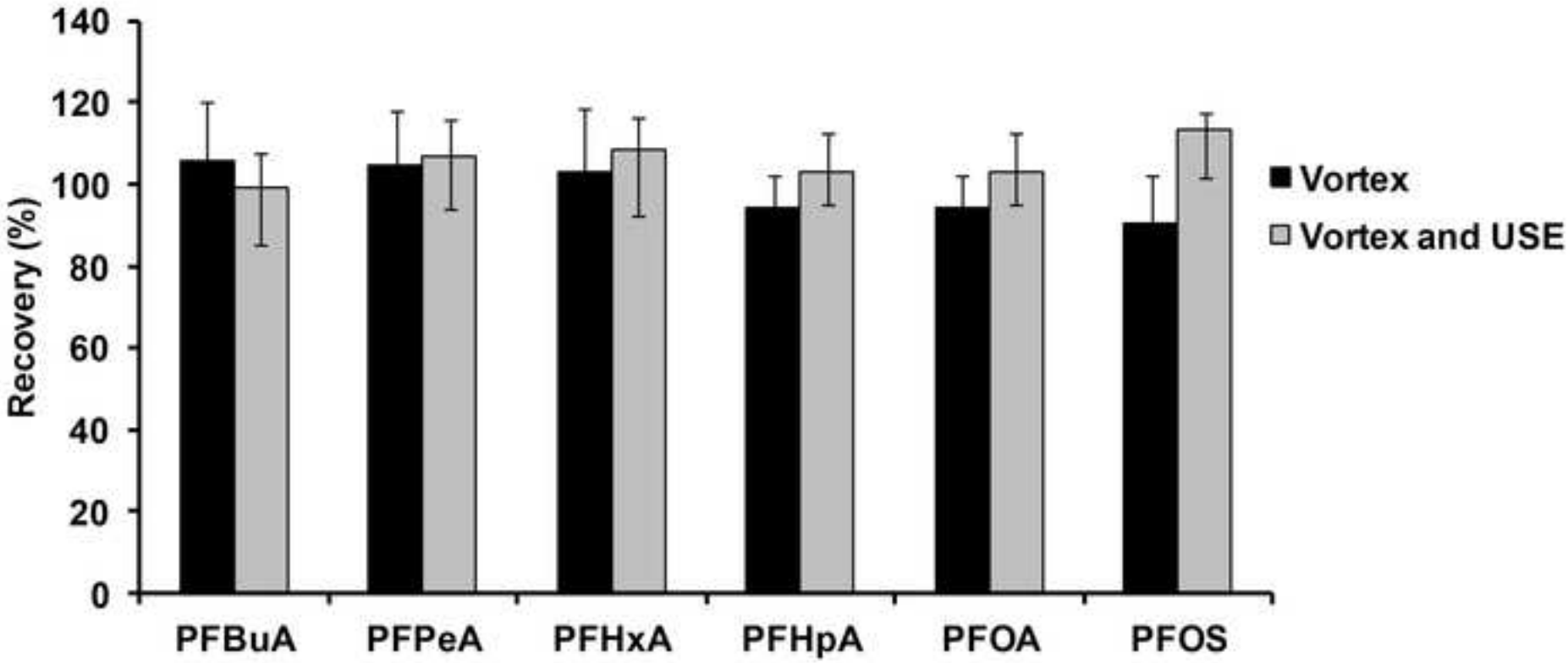

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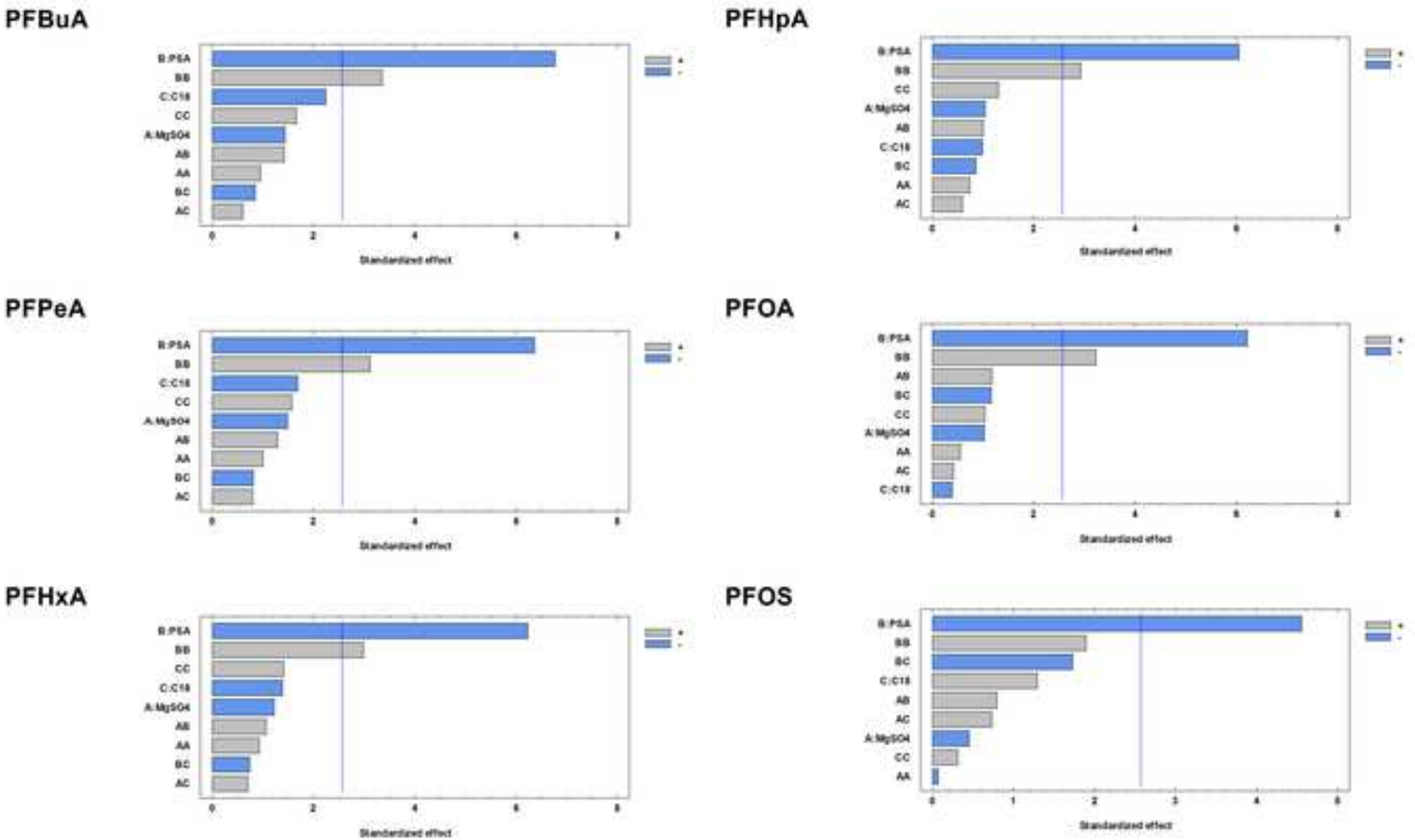

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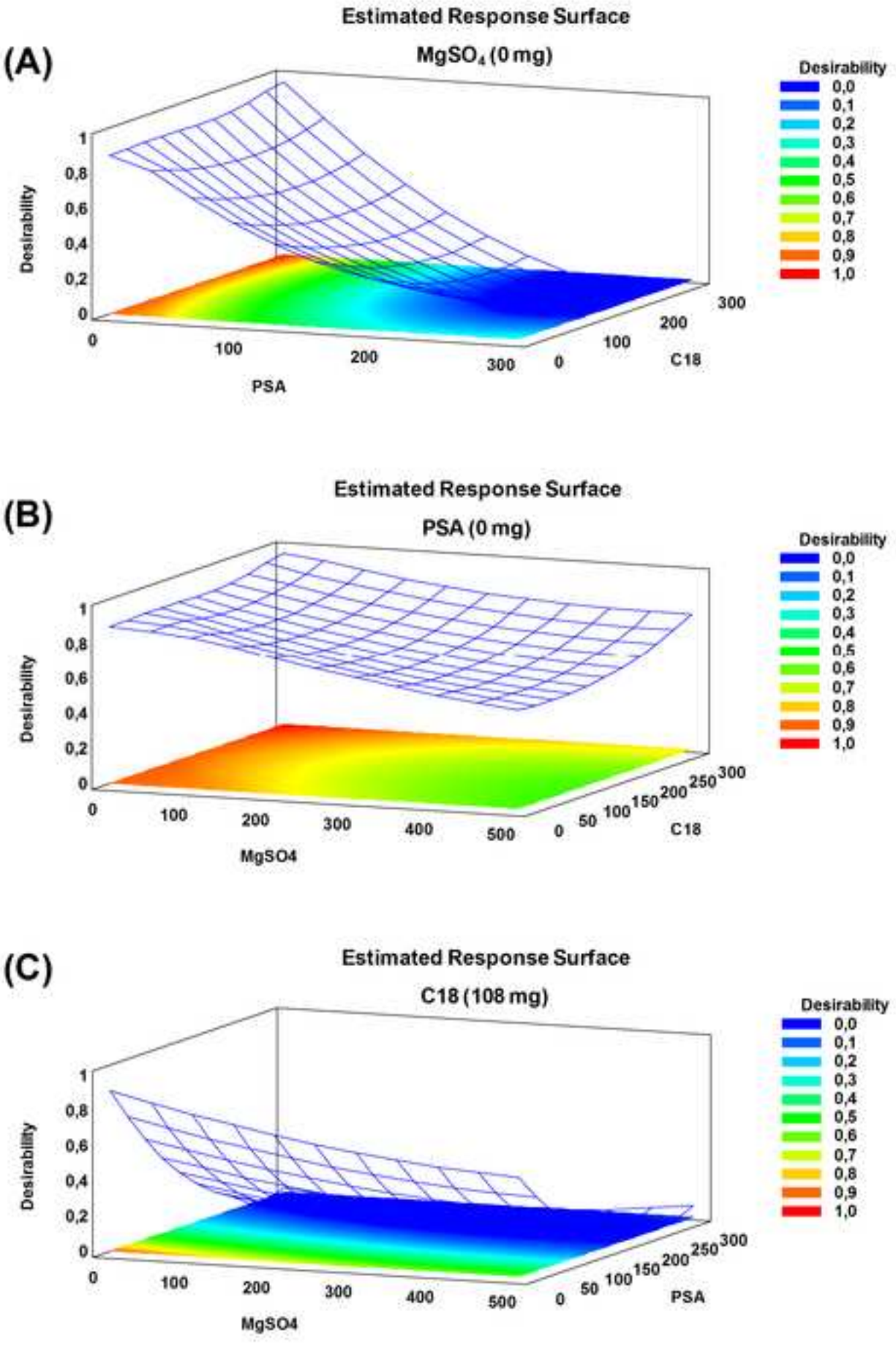

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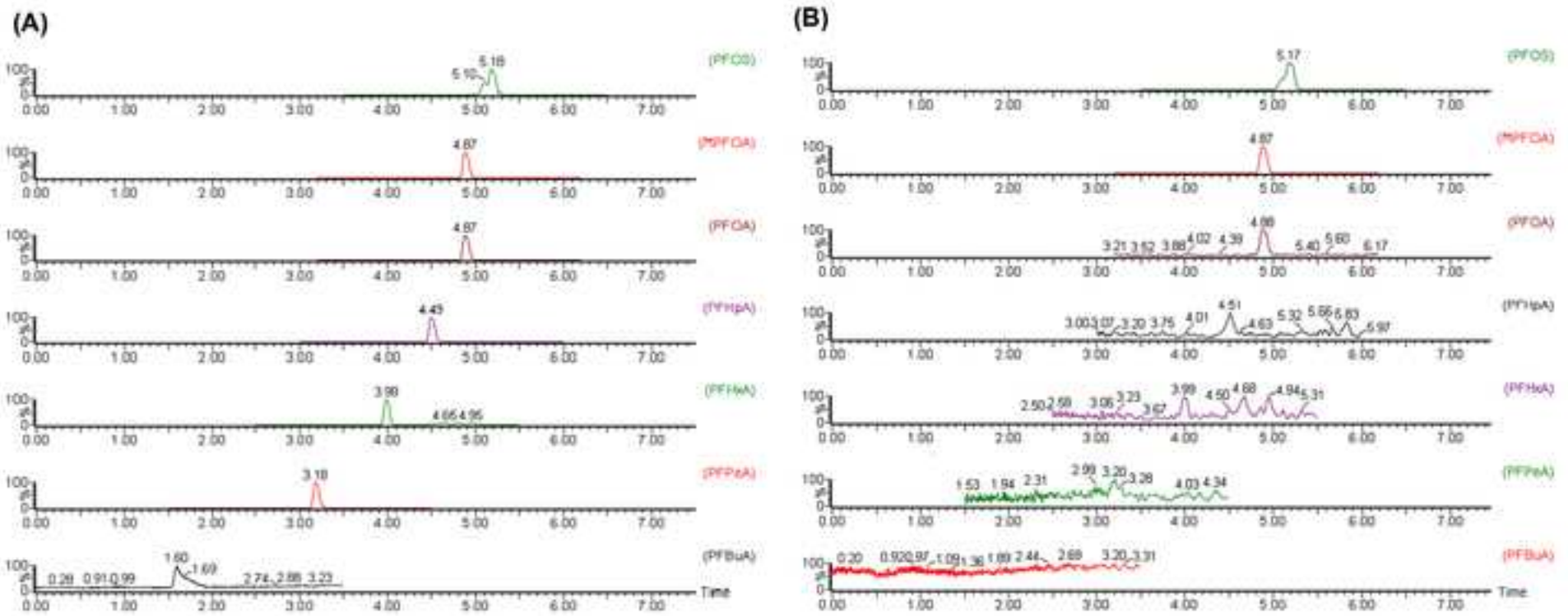

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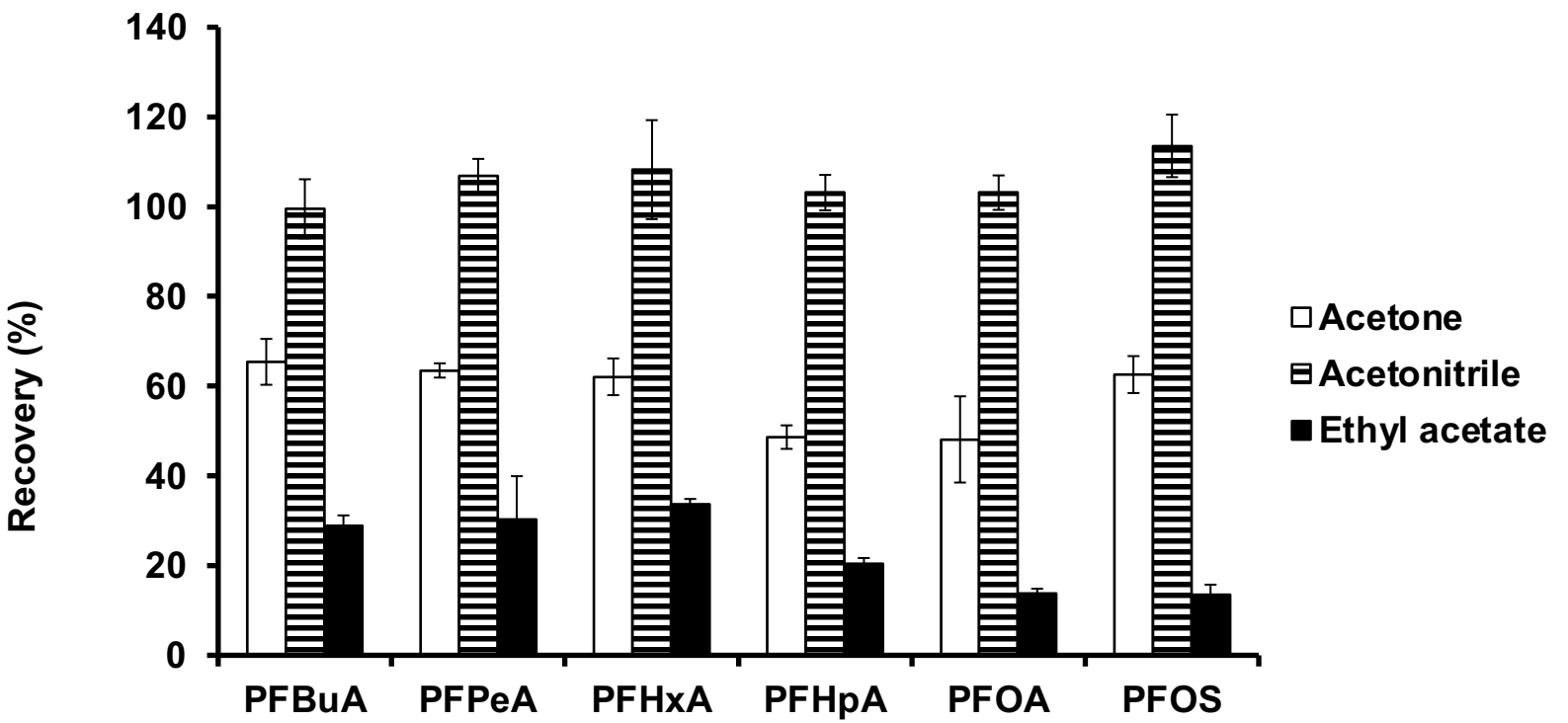
140
120
100
80
60
40
20
0
Recovery (%)
PFBuA
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PFOA
PFOS
Acetone
Acetonitrile
Ethyl acetate

Figure
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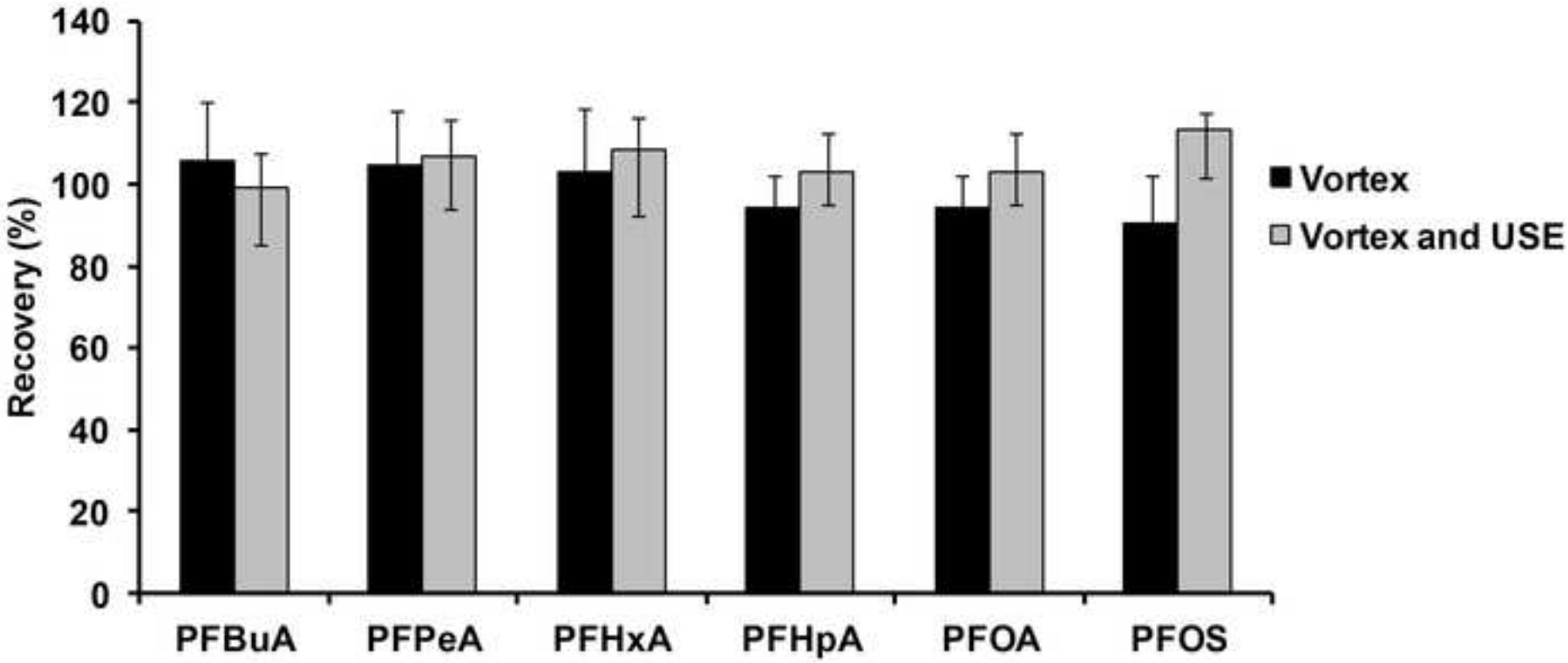

Figure

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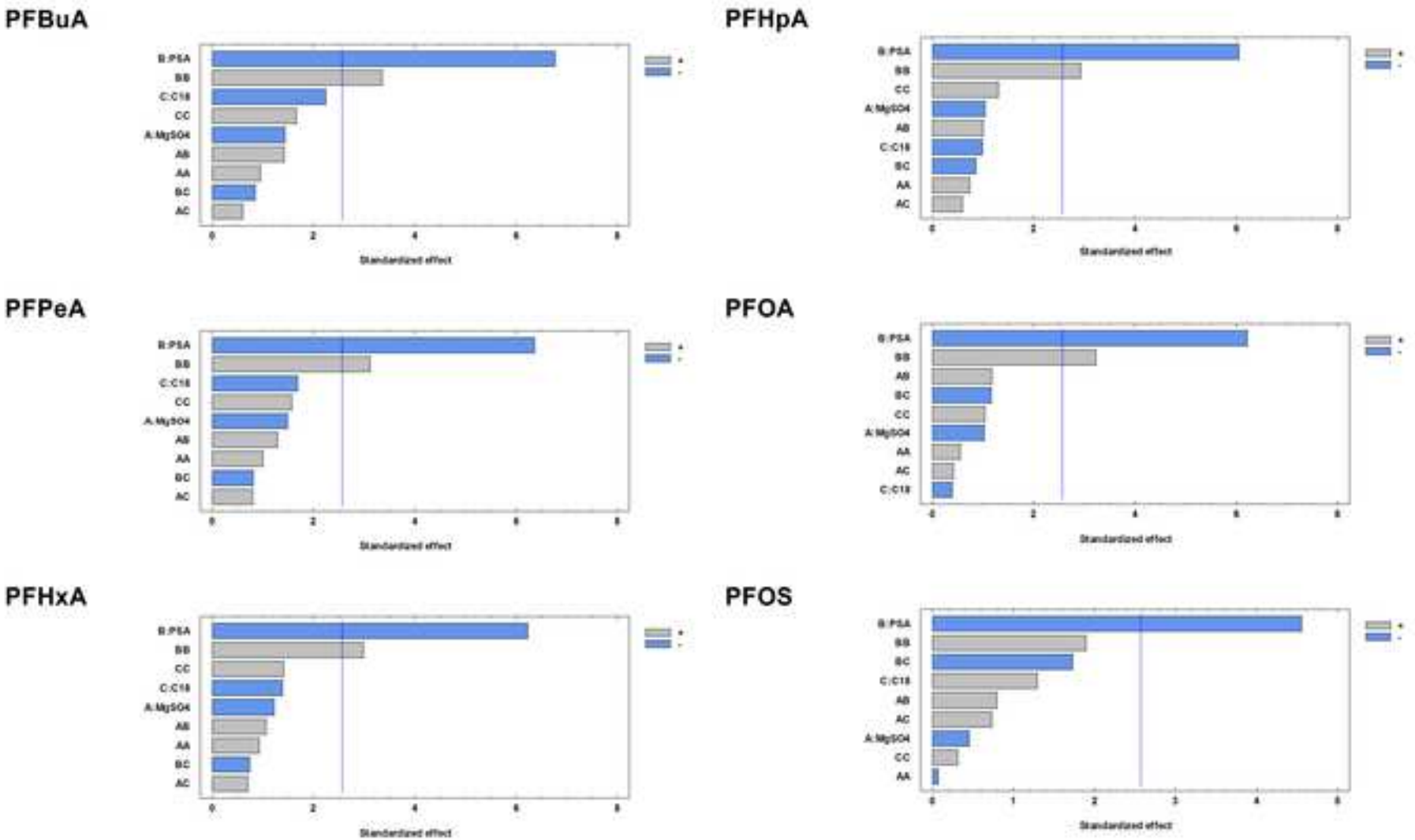
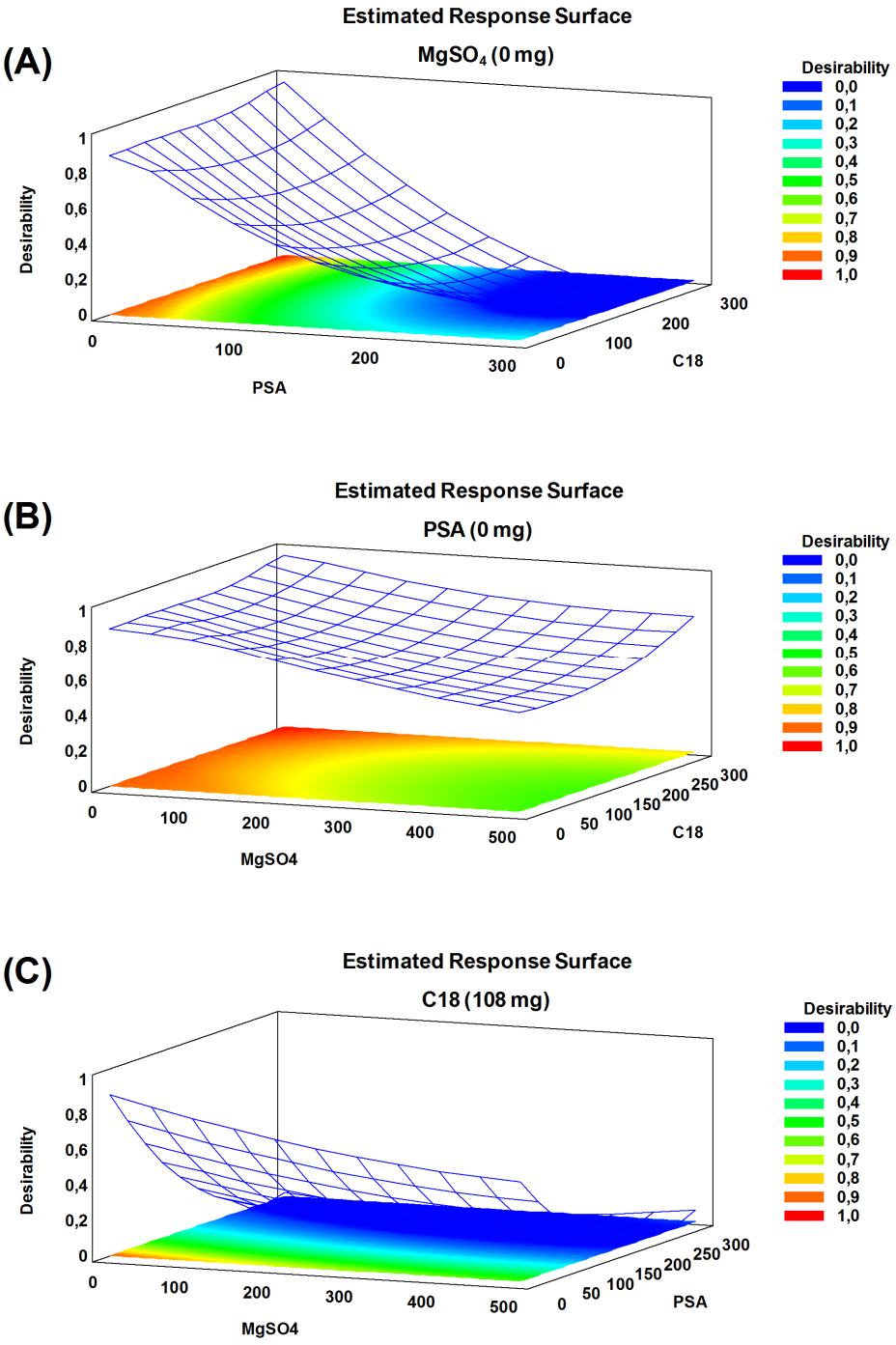



(A)
Estimated Response Surface
MgSO4 (0 mg)
Desirability
1
0,8
0,6
0,4
0,2
0
0
100
200
300
PSA
0
100
200
300
C18
Desirability
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
(B)
Estimated Response Surface
PSA (0 mg)
Desirability
1
0,8
0,6
0,4
0,2
0
0
100
200
300
400
500
MgSO4
0
50
100
150
200
250
300
C18
Desirability
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
(C)
Estimated Response Surface
C18 (108 mg)
Desirability
1
0,8
0,6
0,4
0,2
0
0
100
200
300
400
500
MgSO4
0
50
100
150
200
250
300
PSA
Desirability
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0

Figure

Click here to download high resolution image

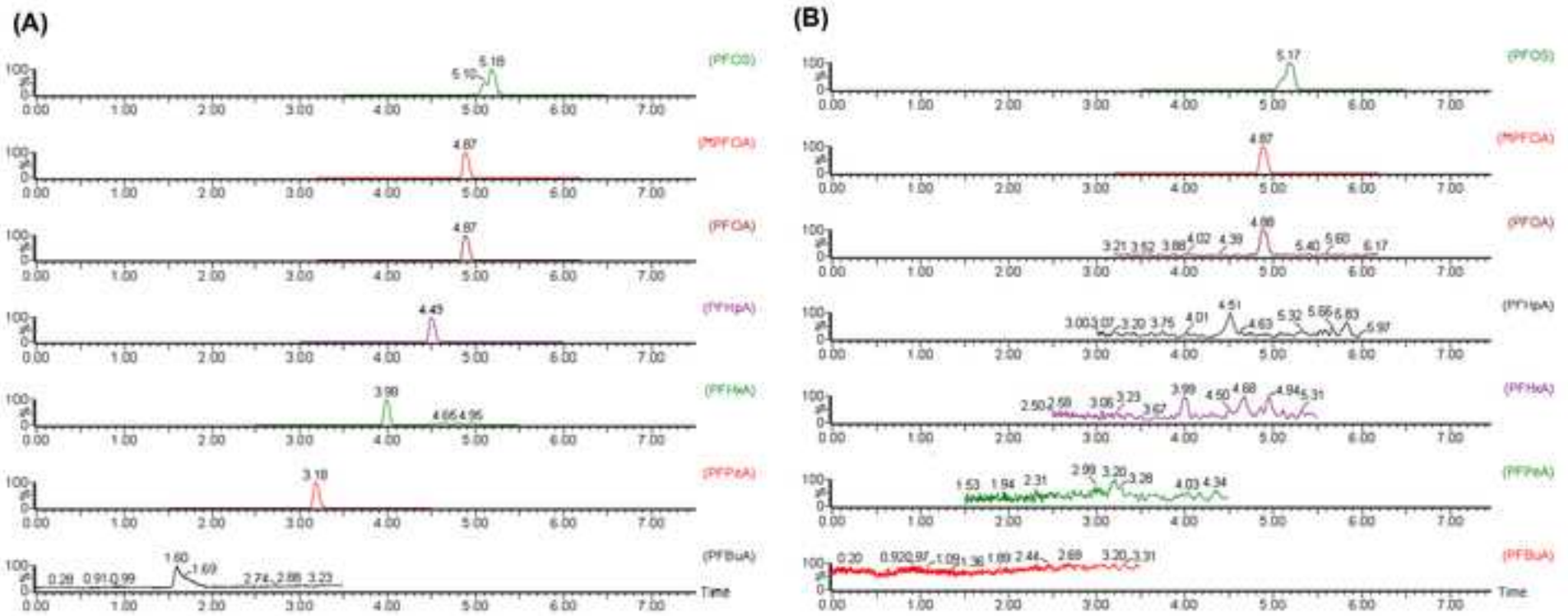


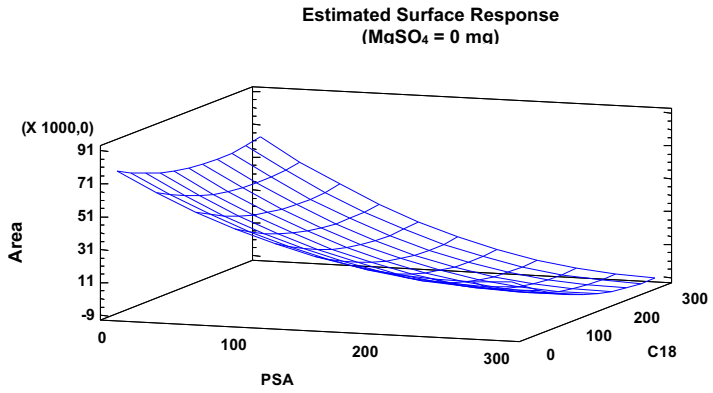
**Table S1**

Box–Behnken design matrix for optimization of clean-up step with d-SPE sorbents.

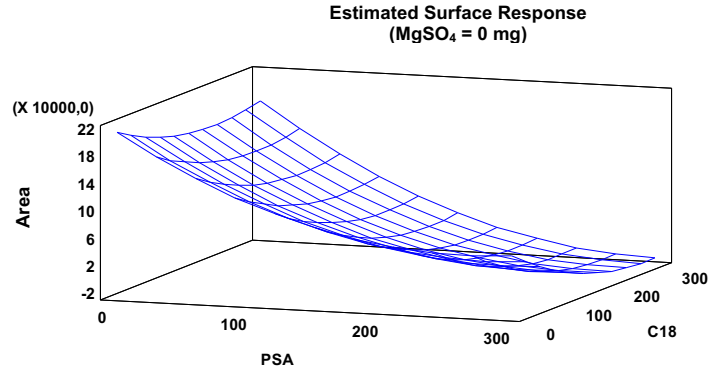
| Experiment | $MgSO_4$ (mg) | PSA (mg) | C18 (mg) |
|---|---|---|---|
| 1 | 500 | 0 | 150 |
| 2 | 500 | 300 | 150 |
| 3 | 500 | 150 | 300 |
| 4 | 250 | 0 | 300 |
| 5 | 0 | 150 | 300 |
| 6 | 250 | 300 | 0 |
| 7 | 500 | 150 | 0 |
| 8 | 0 | 0 | 150 |
| 9 | 250 | 300 | 300 |
| 10 | 250 | 150 | 150 |
| 11 | 250 | 150 | 150 |
| 12 | 0 | 150 | 0 |
| 13 | 0 | 300 | 150 |
| 14 | 250 | 150 | 150 |
| 15 | 250 | 0 | 0 |

**Figure S1**. Optimization of clean-up step with d-SPE sorbents (C18, PSA and $MgSO_4$). Response surfaces for PFCs according to Box–Behnken experimental design. Placental tissue samples were spiked with 250 ng $g^{-1}$ of each PFC.

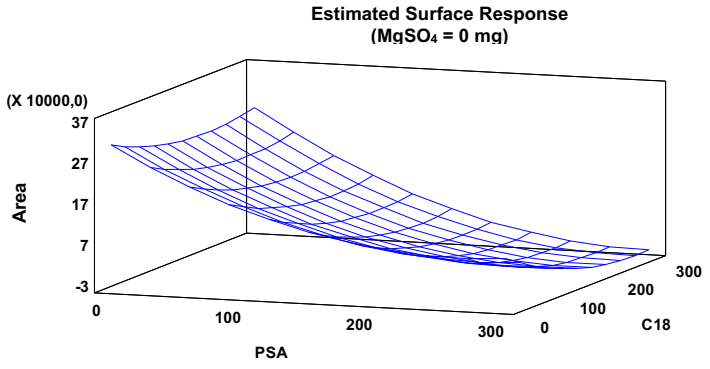
**PFBuA**



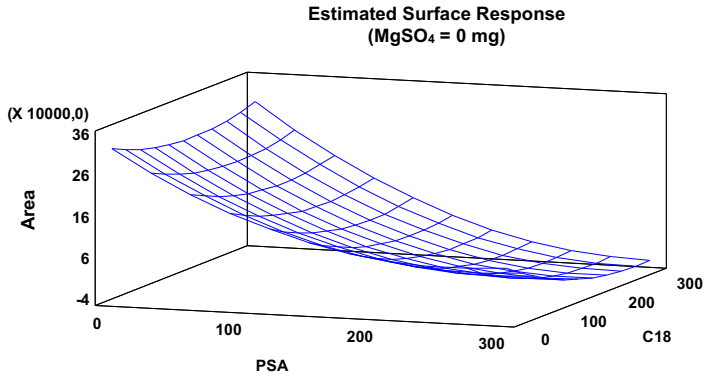

**PFPeA**



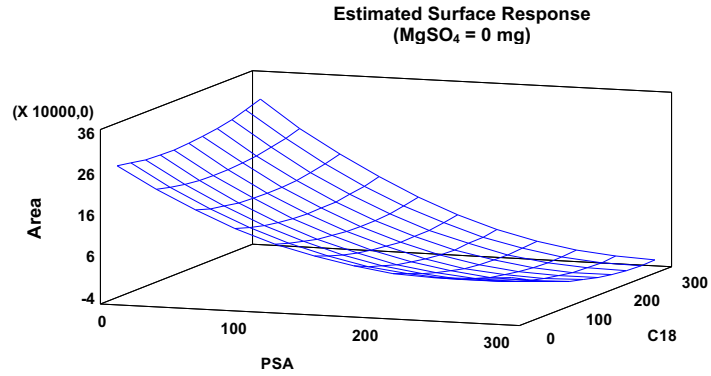

**PFHxA**



## PFHpA




## PFOA




## PFOS

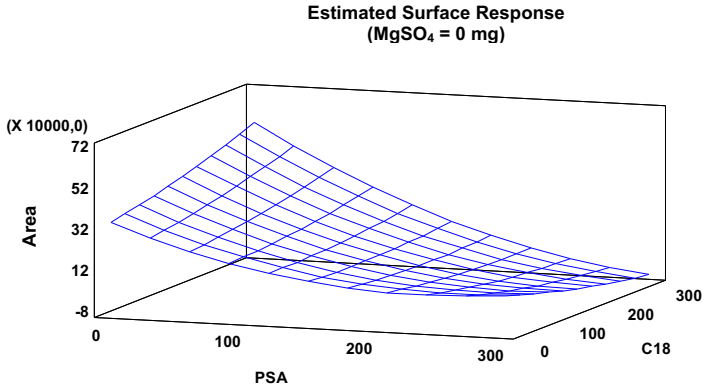


**Figure S2.** Response surface plots corresponding to the desirability function when optimizing the following pair of factors from the clean-up step with d-SPE sorbents: (A) amount of PSA vs. amount of C18; (B) amount of MgSO4 vs. amount of C18 and (C) amount of MgSO4 vs. amount of C18. Results were evaluated using a 95% confidence interval.