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Analytical strategies to profile the internal chemical exposome and the metabolome of human placenta

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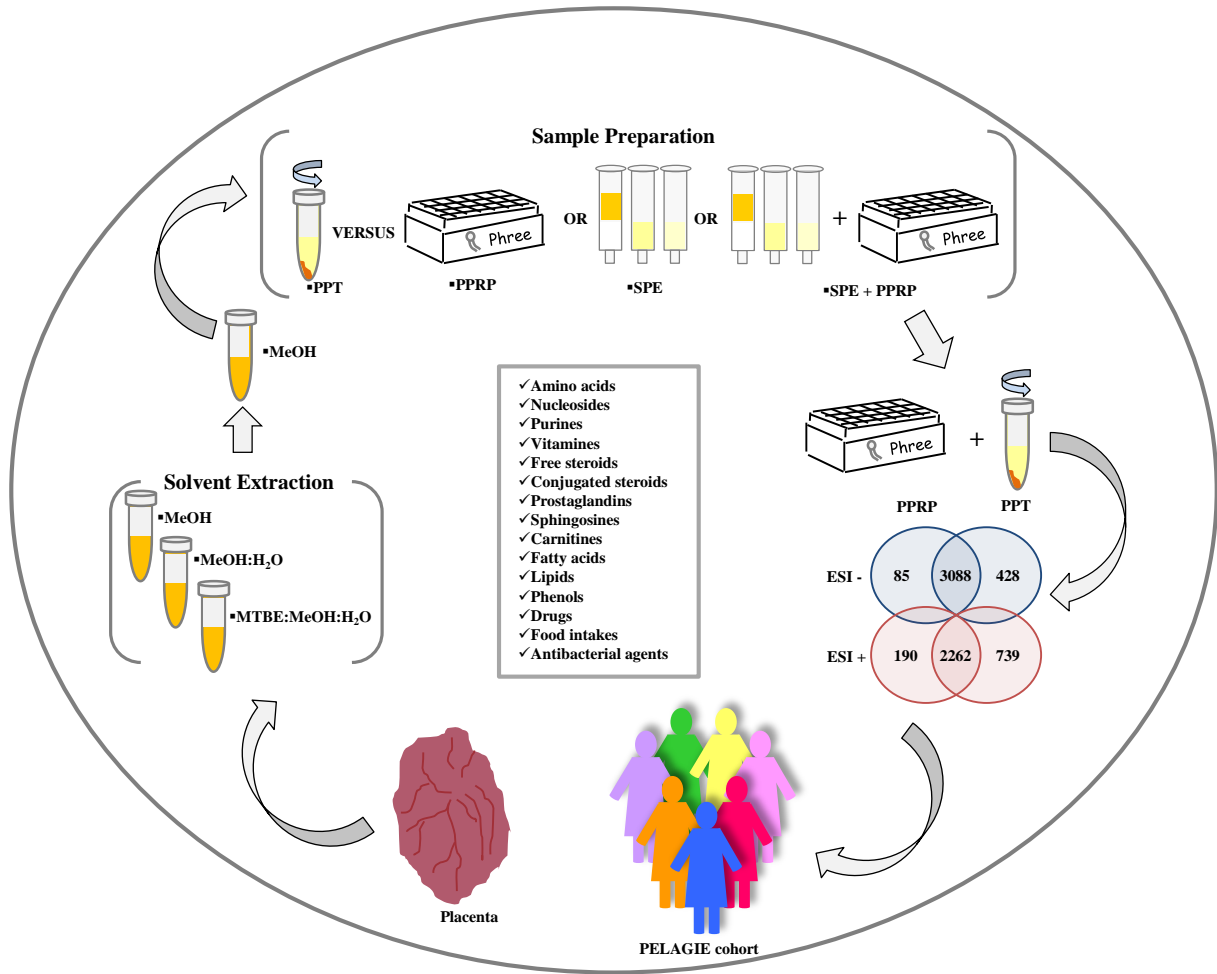
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Credit Author Statement

- Raghad Al-Salhi: Experimental design, data collection, data interpretation, manuscript drafting - editing & revision.
- Christine Monfort: Experimental design, data collection and management, editing.
- Nathalie Bonvalot: Data interpretation, manuscript drafting - editing & revision.
- Arthur David: Conceptualization, experimental design, data analysis, writing – editing & revision, supervision, funding acquisition & resources.



Highlights

- A robust analytical method based on high-resolution mass spectrometry was developed for profiling placenta.
- Suspected and non-target screening were combined to annotate endogenous metabolites and new exogenous chemicals.
- Sample extract split into protein precipitation and phospholipid removal fractions enable complementary profiling.
- The methodology enabled to annotate 90, confirmed 12, and probable 78 chemicals in placenta samples.

Abstract

As a non-invasive biological matrix, the placenta offers great and novel opportunities to monitor fetal exposure to exogenous chemicals and their biotransformation products (the internal chemical exposome), as well as the biological responses associated, in large-scale epidemiological studies. However, it is first crucial to ensure that analytical methods based on high-resolution mass spectrometry (HRMS) can detect the low abundant components of the internal chemical exposome present in these complex biological matrices. In this study, we aimed to develop a robust analytical method (extraction and sample preparation) sensitive enough to profile the internal chemical exposome and the metabolome of placenta using high-resolution mass spectrometry (HRMS) for future application in mother child cohorts. Several extraction solvents (methanol, methanol/H₂O (50/50 v/v), methyl tert-butyl ether/methanol/H₂O) were tested and their ability to extract components of the internal exposome and metabolome were compared. Then, sample preparation methods commonly used for metabolomics application (methanolic protein precipitation) were compared with solid phase extraction (SPE), protein and phospholipid removal plates (PPRP) and combination of SPE and PPRP. The methods were compared and validated using qualitative (i.e., numbers of features and chemical classes ID), quantitative parameters adopted from targeted multi-residue analysis (recovery experiments, repeatability and matrix effect) as well as the ability of these methods to be implemented for high-throughput applications. The analytical repeatability of the two most effective methods (methanolic extraction followed by either protein precipitation or PPRP) were tested at the batch level to determine the best concentration factors to be used for improving detection of components of the internal chemical exposome and metabolome without impacting on the analytical response. Finally, these two methods based on protein precipitation and PPRP were tested on 40 placenta samples from the French PELAGIE birth cohort, and annotation was performed on the related datasets to compare the respective impacts of PPT and PPRP. A wide range of exogenous (e.g., biocides, pharmaceuticals, personal care products) and endogenous chemicals (steroids, prostanoids, lipids, carnitins) could be detected and annotated (some of them for the first time in placenta). We show that both methods are complementary but that PPRP allows the injection of more concentrated extracts without impacting the LC repeatability and therefore improve the detection (presence and signal area fold change) of many exogenous and endogenous chemicals.

Key words: High-resolution mass spectrometry, non-targeted analyses, suspect screening, placenta, sample preparation

1. Introduction

Environmental exposures and their interactions with genetic factors can play an important role in many common chronic diseases [1, 2]. However, compared to genetic factors, the accurate assessment of many environmental exposures (including chemical mixtures from various origins and sources) in epidemiology is currently underdeveloped [3]. To address this, new methodologies such as non-targeted analyses and suspect screening based on high-resolution mass spectrometry (HRMS) are emerging [4-7]. HRMS-based chemical profiling methods enable the broad measurement without *a priori* of small molecules (generally 50-1500 Da) present in human biospecimen (e.g., blood and urine), encompassing endogenous metabolites (the metabolome) as well as exogenous chemicals and their transformation products (the internal chemical exposome) [8, 9]. Hence, the use of HRMS-based methods on biological samples from epidemiological studies sounds very promising in order to identify complex exposure signatures associated with disease susceptibility, and at the same time study of the underlying biological mechanisms involved in the development of chronic diseases [1, 8, 10]. In particular, the chemical profiling of maternal and/or fetal biological samples from birth cohorts using HRMS-based method would be a step forward to improve the monitoring of fetal exposure to chemical mixtures during the most sensitive period of development [11].

Monitoring fetal exposure is often performed using maternal biological samples such as blood, urine or hair as a substitute. Nevertheless, profiling placenta using HRMS-based methods could offer many advantages; this unique maternal-fetal unit is the primary demarcation between the maternal system and fetal system where many exogenous chemicals are known to cross, enter the fetal bloodstream and, for some of them, accumulate throughout the pregnancy [11]. Furthermore, the placenta plays a vital role for the immature fetus (i.e., supply of oxygen, nutrients and hormones), and HRMS-based methods could also identify changes in the profiles of metabolites involved in important signaling pathways (e.g. steroids, thyroid hormones or eicosanoids). So far, it has been demonstrated that many exogenous chemicals such as persistent organic pollutants (POPs) (e.g., PCBs, PBDEs, PCNs, and PBDD/Fs) can cross the placenta and partition between the maternal blood, the fetal blood and the placenta tissues [12, 13]. Non-persistent organic pollutants (e.g., bisphenol A, parabens and UV filters) have also been measured in placenta tissues and cord blood but the potential of the placenta to store these chemicals during the pregnancy is understudied [11-14].

Despite the many promises that offer HRMS-based methods for improving exposure assessment, some limitations remain and have to be overcome so that these methods can provide unbiased detection of chemical exposures. One of the current limitations involves the lack of versatility of analytical techniques used for HRMS-based methods and the lack of analytical sensitivity which hinders the detection of low abundant components of the internal chemical exposome (e.g., environmental contaminants) and the metabolome (e.g. steroids, eicosanoids) that are often present at trace levels (low

or sub ng/g in tissues). To date, the analytical part of the complex HRMS workflow, which includes the sample preparation, has often been overlooked to the benefits of the annotation/identification step, meaning that many endogenous and exogenous chemicals remain undetected with current analytical methods [15-18]. Investing time in the development of new analytical methods sensitive and robust enough to profile the internal chemical exposome and the metabolome of placenta at the population level is a prerequisite for future applications in epidemiological studies.

So far, the monitoring of fetal exposure to organic contaminants using placenta have been done almost exclusively using targeted approach based on GC or LC-MSMS methods. These studies aimed to quantify persistent organic pollutants such as organochlorine pesticides (OCPs), PAHs, PCBs, brominated flame retardants (PBDEs), perfluoroalkyl substances [12, 19-21], and non-persistent chemicals such as UV filters, bisphenols, parabens, pyrethroids and organophosphorus flame retardants [14, 20, 22-24]. The number of studies using HRMS-based methods to assess chemical exposure in placenta is currently very scarce. Furthermore, these studies did not use full scan methods for the detection of the exogenous chemicals, limiting the potential of discovery of new biomarkers of exposure and effect. These include a study in which a method based on SPE and UHPLC-ESI-QTOF was optimized to detect tobacco markers in placenta in MSMS mode (MSe) [25], and another study in which POPs were quantified in placenta using high resolution gas chromatography–high resolution mass spectrometry (HRGC/HRMS) in selected ion monitoring mode after accelerated solvent extraction and SPE purification [26]. Other non-targeted profiling applications of placenta tissues have been performed using LC-ESI-HRMS, but for metabolomics applications. These include studies aiming at detecting changes in endogenous metabolites related to preeclampsia and small for gestational age conditions [27-30] or spontaneous preterm birth [31]. These studies, focusing on endogenous metabolites, used minimal sample preparation based on solvent extraction (biphasic solution) as it is often recommended for metabolomics. Hence, the combination of non-targeted and suspect screening HRMS-based methods to profile the internal chemical exposome of placenta is yet to be implemented.

To improve the detection of low abundant components of the internal chemical exposome and the metabolome, sample preparation with minimal selectivity such as solid phase extraction (SPE) or lipid depletion plates can be used alone or in combination with liquid-liquid extraction as an alternative to conventional methods used in metabolomics (e.g., protein precipitation) [7]. In particular, these methods have shown their potential to increase the chemical coverage as well as the repeatability of HRMS-based analytical methods compared to protein precipitation [16, 32-37]. To date, applications of these sample preparation for HRMS-based methods have been tested on blood plasma and serum [16, 35] and tissues such as testes, liver or kidney [32] but not on placenta.

The aim of this study was to develop a robust analytical method (extraction and sample preparation) sensitive enough to profile the internal chemical exposome and the metabolome of placenta for application in mother child cohorts. Several extraction methods (sonication and bead beating) and

solvents (acetonitrile, methanol, methanol/H₂O (50/50 v/v), methyl tert-butyl ether (MTBE)/methanol/H₂O) were tested and their ability to extract components of the internal exposome and metabolome were compared. Then, sample preparation methods commonly used for metabolomics application (methanolic protein precipitation) were compared with more selective sample preparation (SPE, protein and phospholipid removal plates (PPRP) and combination of SPE and PPRP). Qualitative and quantitative parameters based on targeted MS/MS strategies (e.g., recoveries, matrix effect) were applied to compare the methods. We also compared the methods based on their ability for high-throughput implementation (automation options using 96-well plates and total time for preparation). The LC repeatability of the two most effective methods (methanolic extraction followed by either protein precipitation or PPRP) were tested at the batch level using composite samples (QCs) and analytical replicates to determine the best concentration factors to be used for improving detection of components of the internal chemical exposome and metabolome without impact on the analytical response. Finally, these two methods based on protein precipitation and PPRP were tested on 40 placenta samples from the French PELAGIE cohort, and annotation was performed on the related datasets to compare the respective impacts of both methods.

2. Material and methods

2.1. Chemicals

Details of chemicals and solvents used in this study are provided in table S1.

2.2. Human placenta samples

Human placenta used for method development and the final application study (n=40) were obtained from pregnant women of the PELAGIE birth cohort including 3,421 women from Brittany (France) from the general population enrolled by gynecologists before the 19th week of gestation between 2002 and 2006 [38]. The collections of placenta were performed just after the delivery. Sections of placenta were sampled (about 10 cm³ starting from the umbilical cord) by hospital staff from the maternity unit following a standardized procedure. Placenta samples were kept at -20°C in the maternity wards and then placed at -80°C in the PELAGIE team laboratory for long term storage.

2.3. Placental tissue homogenization and extraction methods

- *Bead beating and solvents of extraction*

For the method development work, several portions of a frozen placenta were cut (10 g in total), placed in a mortar filled with liquid nitrogen and then grounded to a fine powder. Once homogenized, several aliquots (100 mg) were prepared from this homogenate and stored at -80°C for all the method development work. For the final application, study on 40 placenta samples from PELAGIE, five sections

from different positions from each sample were cut, homogenized as described above to obtain a more representative sample of placental tissues.

Placental tissues were disrupted and extracted using a bead beater (MM400, RETSCH Technologies) that allows the extraction of 12 samples at a time. Placenta samples (~100 mg) were loaded after weighing into safe-lock Eppendorf tubes pre-loaded with three prewashed with methanol (MeOH) tungsten carbide beads (3 mm, QIAGEN, Germany). In first work, the efficiency of solvent to extract components of the internal chemical exposome and metabolome was assessed by comparison of pre-chilled 1) 100% MeOH, 2) 1:1 MeOH:H₂O (v/v) and 3) 2:1:1 MTBE:MeOH:H₂O (v/v/v) in quadruplicate for each solvent. Biphasic (MTBE:MeOH:H₂O) extracts were in the end analysed as one extract combining polar and non-polar phases in our experiments, to observe the full benefits of extracting compounds from the aqueous and the organic phase in a relevant amounts, and to minimise the technical variance that could generate from separating the high volatile phase (i.e., MTBE). Also, chloroform was replaced by MTBE solvent due to its ability of solubilising water (i.e., 1.4%) [39], low cost and less toxic compared to chloroform.

The volume of the solvent was adjusted to the accurate mass of the sample to reach a final ratio equivalent to 400 μ L of solvent/100 mg of tissue. Labeled versions of standards used for the spiking experiments (n= 24 internal standards (ISTD), see Table S1) was added to the solvent before extraction (equivalent to 20 ng/g tissue) to monitor analytical repeatability during the LC-HRMS injections. Spiking experiments at environmentally relevant concentrations (equivalent to 20 ng/g tissue) were performed for each solvent condition (n=4) using a mixture of 26 exogenous chemicals including pharmaceuticals, fungicides, herbicides, insecticides, biocides, personal care products, plasticizers covering a wide range of polarity (logP -0.43 to 5.17) (see table S1 for the full list). The vibration of the bead beater was set at 25 Hz for 2 min and 2 cycles were applied with chilling on ice between cycles. After bead beating, Eppendorf tubes were vortexed for 1 min to complete solvent extraction, centrifuged (accuSpin Micro 17R, Fisher Scientific, Germany) at 13,300 g for 20 min at 4°C and then the supernatants were transferred to another Eppendorf tube. Further ~400 μ L of chilled solvent was added to the pellet to re-extract any remaining exogenous chemicals/endogenous metabolites. The combined supernatants were mixed thoroughly and evaporated by vacuum centrifugation to dryness (SAVANT SPD121P, SpeedVac concentrator puls RVT5105 Refrigerated vapour Trap, Thermo Scientific), then reconstituted with 20 μ L of the initial mobile phase conditions prior to UHPLC-Q-TOFMS analysis.

- Assessment of extraction solvents

The efficiency of the different extraction methods was assessed based on absolute recoveries of spiked chemicals, repeatability of extraction, matrix effects, detection frequencies of extracted features, and detection responses. Absolute recoveries of test chemicals (n=26) from spiked placenta were determined

using external calibration. Injections of matrix matched calibration points (n=5, from 25 to 150 ng/mL) were performed using standards spiked into the same amount of placenta matrix which had previously been extracted using the 3 different solvent methods. The concentrations of any analytes detected in unspiked placenta samples was also determined and subtracted from spiked concentrations to estimate the true absolute recovery of the test chemical. Integration of peak areas, linear regression, coefficients of correlation (R^2) and quantification of analytes were generated using Sciex OS software. Repeatability was assessed for each analyte to evaluate the possible differences between the studied methods using the coefficient of variation (CV) of IS-corrected peak area on four preparation replicates. Matrix effect (ME) was calculated using Equation 1 for each compound, where A represents the peak area of compound X at a given concentration C.

$$ME[X, C] (\%) = \frac{A[X, C]_{solvent} - A[X, C]_{matrix}}{A[X, C]_{solvent}} * 100$$

Using Want et al. [40] criteria, repeatability of the abundant features (>80%) among sample preparation replicates and QC samples was calculated considering CV values of <30%, to exclude chromatographic peaks that are not detected in all preparation and injection replicates. For each individual sample (n=4 replicates), all peak areas were summed together to give a total peak area to determine the global detection responses. The mean log₂-fold change of selected annotated features was calculated to visualize the differences in exogenous chemical/endogenous metabolite detection responses among the studied methods.

2.4. Sample preparation of placenta extracts for method development

A methanolic protein precipitation (PPT) commonly used for metabolomics applications was tested as a reference and then compared with alternative sample preparations (SPE, PPRP and a combination of SPE with PPRP) that present different levels of selectivity designed to remove specifically analytical interferences (e.g. lyso- and phospholipids, salts and proteins).

- Protein precipitation (PPT)

Extracts were kept at -80 °C for 1 h to help precipitate protein followed by centrifugation at 13,300 x g for 20 min at 4°C. The process was repeated to ensure the removal of the majority of proteins. Extracts were dried down using a vacuum concentrator at 35°C, then reconstituted in 20:80 ACN:H₂O (v:v) prior to UHPLC-Q-TOFMS analysis. The final resuspension volume of the tissue samples was adjusted to the initial mass of tissue used to ensure that the same ratio of placenta to solvent was used to compare the methods.

- Protein/p-lipid removal (PPRP)

The Phree 96-Well Plate (Phenomenex, USA) were selected based on the results of previous work that showed better recoveries and more repeatable results for these plates for a large range of chemicals with

various physico-chemical properties [41]. First, ultrapure water was added to organic MeOH extracts (80/20 MeOH/H₂O final ratio) after the extraction as the manufacturer recommends it [41]. Extracts were then acidified with formic acid (1%), mixed thoroughly then dispensed into the Phree 96-Well Plate. Phree plate was placed onto a 96-well sample manifold and a 96-well collection plate was positioned inside the manifold. Vacuum was applied until filtrate is collected. Hundred μ l of MeOH/H₂O (80/20) containing 1% FA was then added to the wells to elute the remaining analytes potentially present in the dead volume [16]. All filtrates were collected in glass tubes, dry down using the vacuum concentrator, and then reconstituted in the mobile phase composition 20:80 ACN:H₂O (v:v).

- Solid phase extraction (SPE)

Polymeric Strata-X (Phenomenex, USA) SPEs were selected based on the results of previous work that demonstrated that these sorbents extract chemicals with a large range of physico-chemical properties and provide very repeatable results for HRMS-based applications [16, 41]. MeOH extracts were first dry down using the vacuum concentrator and resuspended in an aqueous solution (4 mL ultrapure water with 5% MeOH) and then purified using Strata-X cartridges that were conditioned with 4 mL MeOH and equilibrated with 4 mL ultrapure water. After vortexing and sonication, extracts were loaded on the cartridges and sample tubes were rinsed with 6 mL ultrapure water with 5% MeOH which was added to the SPE cartridges (washing step), then cartridge sorbents were dried under vacuum for 1 h. The elution was made using 4 mL MeOH, then the eluates were dry down, reconstituted in 20:80 ACN:H₂O (v:v) and frozen at -80°C prior to analysis.

Finally, a method combining a two-step solid-phase extraction and protein/p-lipid removal (SPE-PPRP) was also tested using the protocols previously described to study the potential of these methods to remove more efficiently the analytical interferences.

- Assessment of sample preparation methods

The four preparation protocols were evaluated using four replicates of placenta homogenate spiked with the mix of 26 exogenous chemicals at 20 ng/g. Likewise, the mix of ISTD was used to monitor the analytical repeatability during the injections. The same criteria than the ones used for the solvent extraction were applied to compare the different methods. Absolute recovery of spiked chemicals from the complete procedure (i.e., extraction followed by one the sample preparation method) in placenta was determined. Background contamination was determined using blanks (i.e., solvent and matrix blanks). The CV distribution of the spiked chemicals intensities was compared among the replicates. Equation 1 was used to identify the chemicals that are influenced by coeluting compounds. Detection of frequencies of the features was computed using Want et al. protocol. Speed and ease of implementation was also reported for each preparation protocols.

2.5. Final study comparing PPT and PPRP on placenta from PELAGIE birth cohorts

- Adjustment of the volume of reconstitution using within-batch repeatability

In this experiment, dried extracts were reconstituted in three different volumes: 20, 50 and 100 μL of ACN:H₂O (20:80) to monitor any analytical drifts (retention time or signal intensity) at the batch level related to the dilution/concentration of final extracts. Batches included quality control (QC) samples, solvent and extracted ultrapure water blanks, and placental extracts (n=8 for each group) injected in triplicate. Analytical repeatability was assessed at the targeted scale (i.e., IS peak areas in QC and sample replicates), and at the non-targeted scale using Want et al. criteria to which repeatability of the features (>80%) with CV values of 20, 30, and 40% were computed. In addition to these criteria, the distances between sample preparation replicates and QC samples in PCA score space were measured using Euclidean distances formula to assess the analytical repeatability.

- Application on placenta samples from PELAGIE

In the final study, the two methods that provide the best results in terms of efficiency but also ease of implementation, i.e. the PPT and the PPRP were tested on a larger subset of placenta samples to study their potential to profile the placenta internal exposome and metabolome. A total of 40 placenta samples from the PELAGIE cohort were selected randomly and extracted using PPT (reference method) and the PPRP. The volume of the solvent of reconstitution used was adjusted based on the previous repeatability experiment and resulted on injection of PPRP extracts five time more concentrated than PPT extracts. T-test was used to determine the significant differences between the two sample preparation methods. The resultant data subsets were used for annotating suspect and non-targeted screening to characterize the method that best reflects placenta's internal exposome and metabolome. Annotated features' signal-to-noise (S/N) and fold changes (FC) between methods were also calculated.

2.6. Data acquisition and quality assurance procedures

- UHPLC-QTOFMS analyses

An Exon UHPLC system (AB Sciex, USA) coupled to an AB Sciex X500R Q-TOFMS system (Sciex technologies, Canada), equipped with a DuoSpray ion source was employed for the analysis of placental extracts. Extracts of 2 μL were loaded and separated on an acquity UPLC HSS-T3 column, 1.0 x 150 mm x 1.8 μm (Water technologies, Saint Quentin, France). The column oven and the auto-sampler were maintained at 40°C and 4°C, respectively. A flow rate of 100 $\mu\text{L}/\text{min}$ was used with mobile phase consisting of 0.01% aqueous formic acid (as solvent A) and 100% ACN containing 0.01% formic acid (as solvent B). The gradient was as follows: 2.5-20 min, 20-30% B; 20-38 min, 30%-45% B; 38-45 min, 45%-100% B; 45-55 min, 100% B; and equilibration to initial conditions in 5 min. Recalibration of the Q-TOF was done automatically after each measurement using the manufacturer's solution (i.e., ESI positive/negative calibration solution) via an automated calibrant delivery system (CDS). Samples were

analysed twice in order to be ionized in positive and negative modes in full scan experiment with a mass range of 50-1100 Da. The Q-TOF parameters were set as follows: the collision energy, 35 V; collision energy spread, 15 V; curtain gas, 35 V; ion source gas 1, 50 psi; ion source gas 2, 70 psi; temperature, 550°C; declustering potential, 80 V; mass resolution of 50 000 FWHM for both positive and negative modes.

- SWATH MS data acquisition

A selected extracts were re-analysed for chemical elucidation by performing data independent acquisition via sequential window acquisition of theoretical mass spectrum (SWATH) as well as in-source fragmentation applying different voltages (i.e., 10, 20 and 40eV) for further validation based on structural elucidation. A variable window acquisition method was computed via SCIEX SWATH variable window calculator using a previous placental extract MS dataset, to optimize the Q1 isolation window for the samples of interest. SWATH parameters were set as follows: MS1 accumulation time, 80 ms; MS2 accumulation time, 30 ms; collision energy, 35 V; collision energy spread, 15 V; cycle time, 469 ms; mass range, m/z 50–900 in both positive and negative ionization modes.

- Quality controls

Carryover of the UHPLC system was monitored by injecting blank samples (i.e., solvent blank prepared from initial mobile phase condition 80:20 H₂O:ACN (v/v) and matrix blank prepared replacing placenta sample with ultrapure water) with each sample batch. Pooled QC samples were run, to condition the analytical platform, at the beginning of the sample batch and between every 5 samples. Samples were randomly injected. Internal standards were considered for peak area drift assessment.

- Data processing

Raw data obtained from UHPLC-QTOFMS was preprocessed using vendor software (i.e., MarkerView software packages (V.1.3.1, AB, SCIEX), to extract, deconvolute and align all the spectral peak (the extraction parameters can be found in Table S2). In addition, an open-source tool (i.e., MS-DIAL) was employed for expanding MS/MS searching in which 13,303 and 12,879 compounds were searched in positive and negative respectively. To do so, raw data were first converted to mzML files employing ProteoWizard [42] then converted to the analysis base files (ABF format files) using Reifycs Abf converter which then loaded into MS-DIAL 4.48 in which MS/MS spectra were deconvoluted using MS2Dec. Principal component analysis (PCA) was performed to provide an overview of the data and identify any analytical drifts and potential outliers. Data were log transformed and pareto scaled prior to statistical analysis. The significance of the discriminatory features detected in placental tissue using both sample preparation methods was determined by conducting the student's t-test. Features were ranked according to the lowest p-values and highest S/N and FC.

To reducing search space and increasing efficiency in metabolite annotation, clustering features with similar elution profiles together at the same retention time, as their presences are likely to be generated from the same metabolite (i.e., adducts, isotopes and ion-source fragments) was implemented. Potential features were verified by manual investigation of MS/MS spectra. The resulted putative metabolites (the precursor ions with low EV=10) were searched against the fragment ions obtained by means of the SWATH fragmentations (high EV=35) on the basis of retention time. This process facilitated to an extent the decomposition of the acquired SWATH fragmentations spectra into individual MSMS spectrum. Confidence feature levels were assigned based on the annotation scale proposed by Schymanski et al. [43]. The acquired spectra were searched in online available experimental MS/MS spectral libraries for potential matches such as: MS-DIAL [44], Metlin [45], HMDB [46] or MassBank [47]. Suspect features were confirmed by matching retention time, MS and MS/MS spectral with available authentic standards (level 1) or using *in silico* MSMS prediction software such as MetFrag (level 2) [48].

The Analytics tool (Sciex OS v.1.6) was used to achieve the integration of spiked compounds and internal standards (see table S2 for integration parameters). Quantification was based on the ratio between the responses of the compound to the internal standard for four biological replicates of each spiked compound.

3. Results and discussion

3.1. Evaluation of solvent extractions for profiling placenta tissues

It is worth mentioning that preliminary investigations demonstrated the better potential of bead-beating over ultrasound probe in terms of maximizing the sample high-throughput, eliminating cross-contamination, and avoiding the large amount of heat that can be generated by ultrasonic probe (data not shown). These preliminary investigations also demonstrated the poor suitability of acetonitrile to extract placenta samples using bead-beating (data not shown). Hence, three extraction solvents for bead-beating were then examined to assess the efficiency of extraction methods across placental tissue at both targeted and non-targeted scales via compound/metabolite recovery (i.e., count and response of detected compound), repeatability (i.e., CV) and matrix effect.

In terms of detection yield, MeOH had higher recovery rates (i.e. numbers of spiked compounds with recoveries included in the range 65-120%) compared to MeOH:H₂O or MTBE:MeOH:H₂O solvents (Fig. 1 and table S3). The lowest recovery rates were observed for MeOH:H₂O that did not recover well some chemicals with log Kow >1.5 such as azoxystrobin, tebuconazole, acetochlor, and propiconazole. The matrix effect was also evaluated for all spiked compounds (i.e., comparison of signal areas in

solvent and matrix-matched calibration points), and MeOH showed the highest proportion of compounds with a lower matrix effect (i.e. below 60%) compared with the other tested solvents (Fig. 1, Table S3). In particular, higher matrix effect was observed across non-polar compounds with the MTBE:MeOH:H₂O solvent, which could be explained by higher analytical interferences caused by co-elution with many endogenous lipids extracted at high levels by this solvent.

At the non-targeted scale, MeOH and MTBE:MeOH:H₂O methods extracted similar numbers of features that were both higher than the one observed for MeOH:H₂O (Fig. S1). Likewise, MeOH and MTBE:MeOH:H₂O had similar total feature peak area that were both higher than MeOH:H₂O method (Fig. S2). An annotation experiment was then performed to follow the trends for specific classes of metabolites and exogenous chemicals. Classes of chemicals annotated included amino acids, nucleosides, purines, steroids, vitamins, carnitines, and sphingosines in positive mode, while prostaglandins, lipids, conjugated steroids, xenobiotics (e.g., food intakes, phenols, and drugs) were mainly detected in negative mode. The Log₂ plots were constructed to visualize the distribution of metabolite intensity for each solvent method in both ionization modes. MeOH and MTBE:MeOH:H₂O methods had higher analytical responses for all metabolites identified at level 1 as well as MS/MS-annotated (level 2) compared to MeOH:H₂O (which is also in agreement with the results of the recovery experiment). Overall, MeOH and MTBE:MeOH:H₂O extracted these metabolites and exogenous chemicals to the same extent (i.e. in terms of detection and responses, Fig. 2 a&b), even though it was observed that MeOH performed better (i.e. higher signal areas) in extracting classes such as; amino acids, carnitines, conjugated steroids, and phenols whereas MTBE:MeOH:H₂O performed better for steroids (logP > 2.5), lipids (logP > 6), and prostaglandins (logP > 3). As for MeOH:H₂O, extraction performances was constantly lower than these of MeOH and MTBE:MeOH:H₂O, as illustrated for carnitines CR14:0, CR16:1, CR18:0, CR20:0 and sphingosine that showed 4-fold decrease in comparison to the other two solvents.

Regarding the repeatability observed for the extractions using the solvent-based methods, MeOH performed better than the other solvents since the proportion of spiked compounds having CVs within the 30% cut-off for the recovery experiments was higher in comparison with the other solvents (Fig. 1, Table S3). Likewise, at the non-targeted scale, the percentage of repeatable features with CV<40% using the Want method were calculated in both ESI⁺ and ESI⁻, and the MeOH method appeared to be the most reproducible method as compared to the other investigated solvents. The change of polarity for MeOH:H₂O and the potential problem of repartition in the biphasic extract obtained with MTBE:MeOH:H₂O could explain the lower repeatability observed for these compounds. The clustering of replicate samples extracted using the different methods in the PCA score plots also confirmed the higher repeatability observed with MeOH extractions (Fig. S3 a&b).

Hence, our results show that the MeOH method seems as effective as the MTBE:MeOH:H₂O method in terms of feature counts and total peak area detection responses but MeOH slightly outperformed it when considering more targeted criteria such as recovery and matrix effects experiments performed for specific compounds. As for the MeOH:H₂O, this solvent did not perform well for many non-polar chemicals and does not seem recommended for placenta since important chemicals could be missed. Both methods using MeOH and MTBE:MeOH:H₂O have their respective merits since we observed with the annotation experiments that some chemicals will be favoured (mainly in terms of signal area as opposed to detection frequencies) with one or the other methods. However, the MeOH method was selected as the extraction solvent for the subsequent studies as it provides more reproducible results than the biphasic extraction, which is a key criteria for non-targeted analysed.

The MeOH extraction method was then further optimised to ensure the best recovery of components of the internal chemical exposome and the metabolome. Two consecutive extractions of placental tissue with MeOH solvent and effect of vortexing time (i.e., 1, 5, or 10 min) were applied to test extraction efficiency. An enhancement of spiked chemical and metabolite detection response was observed after the two consecutive extractions for spiked compounds such as venlafaxine (1.6-fold), cyprodinil (1.6-fold) and diazinon (1.9-fold), as well as for compounds originating from the matrix, e.g., cresol sulfate (1.6-fold), sphingosine (2.2-fold) and arachidonic acid (1.6-fold). Therefore this additional step was then included in the method. However, the vortexing time did not affect the chemical recoveries (Fig. S4 & S5).

3.2. Evaluation of sample preparation methods on extraction of metabolites from placental tissues

Four preparation methods, namely: protein precipitation (PPT), protein/p-lipids removal (PPRP), solid phase extraction (SPE), and combined SPE with PPRP (SPE-PPRP) were evaluated for better performance and comprehensive metabolite profiling of placental tissue.

In terms of detection yield, the PPT method, which is considered as the reference method in metabolomics, showed higher recovery rates (i.e., within the range 65-120%) compared to the other three preparation methods: PPRP, SPE, and SPE-PPRP (Fig. 3 and table S4), which could be expected as this is the less selective method. More specifically, among the 24 spiked compounds, 16, 12, 14, 6 compounds were recovered in the range 65-120% by PPT, PPRP, SPE, and SPE-PPRP, respectively. A detection frequency experiment using spiked and non-spiked placenta extracts was also performed to study the impact of the sample preparation to detect chemicals in real-life situation. These frequencies of detection of spiked compounds were higher for PPT and PPRP (22 and 21 out of 24, respectively) than for the SPE (16 out of 24) and SPE-PPRP (6 out of 24) (Fig. 3, Table S4). Triclosan, cyprodinil, malathion, and diazinon had low detection response for all tested methods which is probably due either

to poor ESI ionization of the compound (e.g., triclosan) or ion suppression caused by co-eluting endogenous lipids which is reflected in their high ME% (Fig. 3, Table S4). Nicotine and chlorpyrifos methyl could not be detected by SPE and SPE-PPRP methods at all. This could be explained by the protonation of nicotine during the acidification step which may have prevented its retention on the SPE sorbent, and the retention of chlorpyrifos methyl on the PPRP plate, which has already been demonstrated in previous studies [16], [41]. This PPRP step has also been shown to disadvantage diazinon which could also explained its low detection response. Hence, even though we show that matrix effect was improved for two preparation methods using SPE (probably as it allows the removal of interfering matrix components and enrichment of metabolites), these results also show that SPE can be detrimental for the recovery of specific compounds due to retention issues on the sorbent.

At the non-targeted scale, a higher number of features were retrieved with SPE and SPE-PPRP compared to PPT and PPRP methods (Fig. 3, Table S4). However, this could be explained by the higher number of feature introduced from the SPE experimental blanks (probably caused by higher plastic leaking from the 96-well plates and collectors). Further analysis of total peak area of the four tested methods also showed higher detection response of SPE method and its respective experimental blanks compared to the other three methods (Fig. S6). An annotation experiment was then performed to observe the differences in responses and detection of more specific classes of metabolites. Considering metabolite ion detection responses, PPT and PPRP methods had similar responses for most of the detected endogenous metabolites, except for long-chain carnitines, sphingosines and lipids that presented better responses with PPT (Fig. 4 a&b). However, lower abundant exogenous chemicals such as amoxicillin and its metabolites (log fold change between 2.5-4.2), acetaminophen and its metabolites (log fold change between 1.6-2.2), lidocaine (log fold change 2.6), bupivacaine (log fold change 4.0), triclosan glucuronide (log fold change 2.1) and triclosan sulphate (log fold change 5.6) presented high responses with PPRP overall. Similar results were obtained when comparing PPRP and SPE methods, i.e., PPRP plates favoured overall the extraction of exogenous chemicals compared to SPE sample preparation as opposed to some endogenous compounds such as long-chain carnitines. We suspect that the lack of recovery of long-chain carnitines by PPRP could be explained by a mechanism of retention similar to those of PL and LPL with these plates (the exact mechanism of retention with the Phree plates is not known) since acylcarnitines are composed of a polar part (i.e., a quaternary ammonium) linked to a non-polar part (i.e., fatty acyl chain). To illustrate this, the short-chain carnitines with a more hydrophilic character (< C12-chain) were recovered by PPRP plates while long-chain carnitines were retained potentially by its hydrophobic character.

In terms of repeatability for the different sample preparation methods, PPT showed the best repeatability with a higher proportion of spiked chemical having a CV within the 30% cut-off (92%), followed by PPRP (88%) and then SPE and SPE-PPRP (67%, and 79%, respectively) (Fig. 3, Table S4). This loss

of repeatability for the SPE methods could be explained by the loading/elution steps that may introduce variabilities. The percentage of repeatable features with $CV < 40\%$ according to the Want method for both ESI+ and ESI- modes observed at the non-targeted scale confirmed that PPT and PPRP methods provide more robust results than the SPE methods (Fig. 3, Table S4). It is however worth mentioning that an overview of the placental replicates extracted by the tested methods (represented in Fig. S7 a&b) on the PCA shows that acceptable repeatability was achieved for all evaluated methods. The percentage of repeatable features with $CVs < 40\%$ in positive and negative mode were ranged between 66% and 84% for PPT and PPRP method, while 54% and 62% were the percentages for SPE and SPE-PPRP methods, respectively.

Overall, PPT and to some extent PPRP perform better in terms of recoveries compared to both SPE methods. The loading/elution steps of SPE induced some selectivity issues for specific chemicals even though it is worth mentioning that overall polymeric phases such as Strata-X recovered a large range of the tested chemicals nevertheless. This has to be balanced with the better removal of matrix effect observed for SPE methods that can be useful for the detection of some chemical classes in the case of targeted metabolomics (e.g. steroidomics). SPE methods introduced more variability due to the multiple step involved even though it was still within an acceptable range. The time required for each method was also estimated as it is a key factor for large-scale application studies. The extraction time for the four methods spanned between 51 min and 7h30 (Fig. 3, Table S4). PPT was the most time-saving extraction method, followed by PPRP as compared to the other two methods. It is also worth mentioning that the PPRP methods can be automatized using robots as it is using 96-well plates.

Our results tend to show that PPRP and PPT methods proved to be the best methods overall for application in large-scale epidemiological studies, with short preparation time ($< 2\text{hr}$) compared to the SPE and SPE-PPRP methods, and better detection yield and repeatability. Both methods were therefore selected for further optimisation.

3.3. Final study on placenta sample comparing PPT and PLR

- Optimizing volume of reconstitution using within-batch repeatability

Since PPT and PPRP showed specificities that could both help to extend the coverage of the internal chemical exposome and the metabolome, we then focused on optimizing both PPT and PPRP methods in terms of maintaining robustness and metabolite detection by selecting the most appropriate volume of reconstitution for the injection. For a given injection volume, the volume of reconstitution will impact upon the detection limits, chromatographic dilution and therefore analytical interferences (ion suppression), the saturation of UHPLC columns or the carryover between injections for highly abundant

or non-polar compounds (which can increase analytical drifts at the batch level). We therefore tested different volume of reconstitution (20, 50 and 100 μL of ACN:H₂O (20:80)) to find the best compromise between concentrating the extracts for the best analytical sensitivity and within-batch analytical repeatability. QCs (composite sample) and 8 placental extracts were then injected in triplicate for both PPRP and PPT, and different methods were used to assess the analytical repeatability.

The results showed that the most appropriate volume reconstitution was different for the two sample preparation methods. PPRP method allowed the injection of 5-times more concentrated extracts than the PPT method without affecting the analytical repeatability. The most appropriate volume for PPT was 100 μL (PPT₁₀₀) and 20 μL for PPRP. PPT₁₀₀ was the only volume for which acceptable analytical repeatability could be observed considering the Euclidean distances calculated for analytical replicates on the PCA, CVs of the area of spiked IS or the CVs of the area for the total number of features detected in all QC samples (Fig. 5). To illustrate this, at the non-targeted scale in QCs, the percentage of repeatable features (+60% of features in both modes with CVs < 20%) for PPT₁₀₀ method was similar to the percentage observed with the PPRP₂₀ (Table S5). Euclidean distances were also calculated to assess the variance across the sample preparation replicates. Similar results revealed from PPRP₂₀ and PPT₁₀₀ (<12%) that were less than PPT₂₀ and PPT₅₀ (<40%) protocols (Table S5) (Fig. S8-12), indicating comparable reproducibility the used PPRP₂₀ and PPT₁₀₀ protocols. These differences in volume of reconstitution for both methods could be explained by the presence of remaining proteins in the case of PPT where no filtration is applied.

The base peak total ion chromatograms generated from extracts of the two optimised methods are presented in Fig. S13. PPRP extracts showed higher abundant ion peaks (i.e., polar and semi-polar metabolites) within t_{R} 2-35 min, while PPT method had a superior effect on extracting metabolites eluted over non-polar region (i.e., t_{R} >35 min). This could show the better performance of using both PPT and PPRP that could complement each other to cover a broader scope of placental metabolites (i.e., metabolome and exposome). This will be discussed in the next section as for the final study, a simple analytical workflow in series was elaborated to provide two extracts from the PPT and the PPRP that were both analysed separately for comparison.

- Comparison of PPT and PPRP on PELAGIE samples

The optimized methods were applied for the analysis of placental tissue extracts and an annotation experiment (based on confidence levels from Schymanski et al. [43]) was performed to study on hand the relevance of both analytical methods to detect the components of the internal chemical exposome and the metabolome in these cohort placenta.

In total, more than 100 endogenous and exogenous metabolites were annotated from a range of chemical classes, namely: amino acids, nucleosides, purines, vitamins, free steroids, conjugated steroids, prostaglandins, sphingosines, carnitines, fatty acids, lipids and, for exogenous chemicals, e.g., phenols, drugs, food intakes, and antibacterial agents. It is worth mentioning that some of these annotations are new for the placenta. Most of the annotated metabolites were detected in both ionisation modes (MS-scan and MS/MS), which provides another layer of confidence for the structural elucidation. On the other hand, some chemical classes were ionized only in positive MS mode (e.g., acylcarnitines and some steroids), whereas classes such as conjugated metabolites and free fatty acids were detected in only negative MS mode. Screening the neutral loss of conjugated ions (mainly glucuronides and sulphates) also facilitated the detection of conjugated endogenous and exogenous metabolites. This suspect screening strategy let to detect more markers of exposure compared to its deconjugated forms due to their high polarity and high abundance MS response (e.g., triclosan sulphate and glucuronide). Many features remained unknown due either to their poor MS/MS spectra or absence from online databases. A summary table of the metabolites identified or annotated can be found in Table S6.

Twelve metabolites were confidently identified at level 1 by comparing their chemical predictors (including MS/MS patterns and retention times) with those of authentic standards. 128 metabolites were MS/MS-annotated at level 2 ($a = 78$ and $b = 50$) using match with experimental MS/MS from online databases, 26 unknown metabolites were putatively-annotated at level 3 (26), and at level 5 (31) by matching accurate m/z to online MS databases and isotopic patterns. Some chemicals were detected in positive mode including, phenols ($n=10$), drugs ($n=3$), while others in negative mode mainly including conjugated metabolites (e.g., acetaminophen).

Comparison of identified and MS/MS-annotated metabolites/exogenous chemicals's areas in placental tissues extracted by PPRP and PPT were done by log fold change calculation. Results are presented in Fig. 6 a&b. Chemical classes having a better analytical responses in PPRP in positive mode (P) and negative mode (N) were: amino acids (log fold change (P) ; 0.3-3.4), nucleosides (P ; 2.3-2.7), purines (P ; 1.4-2.1), vitamins (P; 1.9-2.5), steroids (P ; 0.4-2.7), conjugated steroids (N; 1.4-4.2), prostaglandins (N; 1.4-2.1), phenols (N; 1.0-2.8), antibacterial agents (N ; 2.1-5.6), food intakes (N; 0.8-3.3), drugs (P ; 2.5-4.2 and N ; 1.7-2.2), as well as some unknown chemicals (P ; 0.9-2.5 and N ; 1.3-3.5). On the other hand, sphingosines (P ; 1.3-10.8), fatty acids (N ; 0.3-0.7), lipids (N ; 1.0-5.0) were higher in PPT. In the carnitines class, short-chain carnitines (i.e., CR :0-12) had higher log fold change in PPRP (P ; 0.6-2.5), whereas long-chain carnitines (i.e., CR :14-20) were higher in PPT (P ; 1.8-8.6) which can be explained by the retention of the latter on PPRP plates as demonstrated earlier. Hence, both PPRP and PPT approaches (in both ionization modes) would provide complementary information on the placental endogenous and exogenous chemicals. However, if the study is focused on profiling the internal chemical exposome, the PPRP should be favoured as it offered a better detection of exogenous chemicals which can be explained by the injections of more concentrated extracts.

In comparison with previous studies, among the identified (level 1; n=12) and MS/MS-annotated (level 2a; n=78) metabolites, twelve exogenous chemicals and their biotransforms products had not been previously detected in placenta tissues, including four pharmaceuticals and their metabolites (i.e., amoxicillin, acetaminophen, lidocaine, and bupivacaine), two food intakes and their metabolites (i.e., caffeine, piperine, and paraxanthine), two vitamins (i.e., lumichrome and pantothenic acid), one antibacterial agents (i.e., triclosan sulphate and triclosan glucuronide), and three phenols (i.e., indoxyl sulfate, cresol sulfate, and phenol sulfate). Some of the pharmaceuticals such as the anesthetics and the analgesics could be directly linked to the delivery.

4. Conclusions

To our knowledge, this is the first study to apply suspect and non-targeted screening based on high-resolution mass spectrometry (HRMS) to profile endogenous and exogenous chemicals in placenta tissues. This study comprehensively assessed and examined three solvent extraction methods and four sample preparation methods for the extraction of endogenous and exogenous chemicals from placental extracts. Using targeted and non-targeted criteria, we demonstrate that methanolic extractions provides the best coverage of the internal chemical exposome/metabolome as well as the best repeatability compared to a biphasic solvent and a mix of MeOH/H₂O. For the sample preparation, methods based on protein precipitation and phospholipid/protein removal plates offer better performances compared to SPE in terms of chemical recoveries even though each method provides its own specificities. Both PPT and PPRP are high-throughput and robust but, importantly, phospholipid/protein removal plates allow the injection of more concentrated extracts without affecting the analytical repeatability, increasing the sensitivity and the coverage of low abundant chemicals. In the end, both methods could be implemented in a simple workflow and injected separately to deliver complementary information and achieve wider metabolome/exposome coverage of placenta tissues.

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CRedit authorship contribution statement

Raghad Al-Salhi: Experimental design, data collection, data interpretation, manuscript drafting - editing & revision.

Christine Monfort: Experimental design, data collection and management, editing.

Nathalie Bonvallot: Data interpretation, manuscript drafting - editing & revision.

Arthur David: Conceptualization, experimental design, data analysis, writing – editing & revision, supervision, funding acquisition & resources.

Declaration of competing interest

The author(s) declare no competing financial interests.

Appendix A. Supplementary data

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Fig. 1. Recovery and matrix effect of spiked analytical standards in placenta and repeatability assessment for different extraction solvents. Radar chart illustrating the recovery ranges for 26 compounds spiked at 20 ng/g (% of mean recovery (n=4) between 65-120%), recovery repeatability for four replicates (% of spiked compounds with coefficient of variation CV < 30%), matrix effect (% of spiked compounds with ME < 60%, n=4), repeatability (% of MS signals with coefficient of variation < 40%), and features extracted in both ESI modes for the differences between the extracting solvents: 100% methanol (100%MeOH; blue), 1:1 methanol:water (1:1 MeOH:H₂O; red), 2:1:1 methyltert-butylether:methanol:water (2:1:1 MTBE:MeOH:H₂O; green).

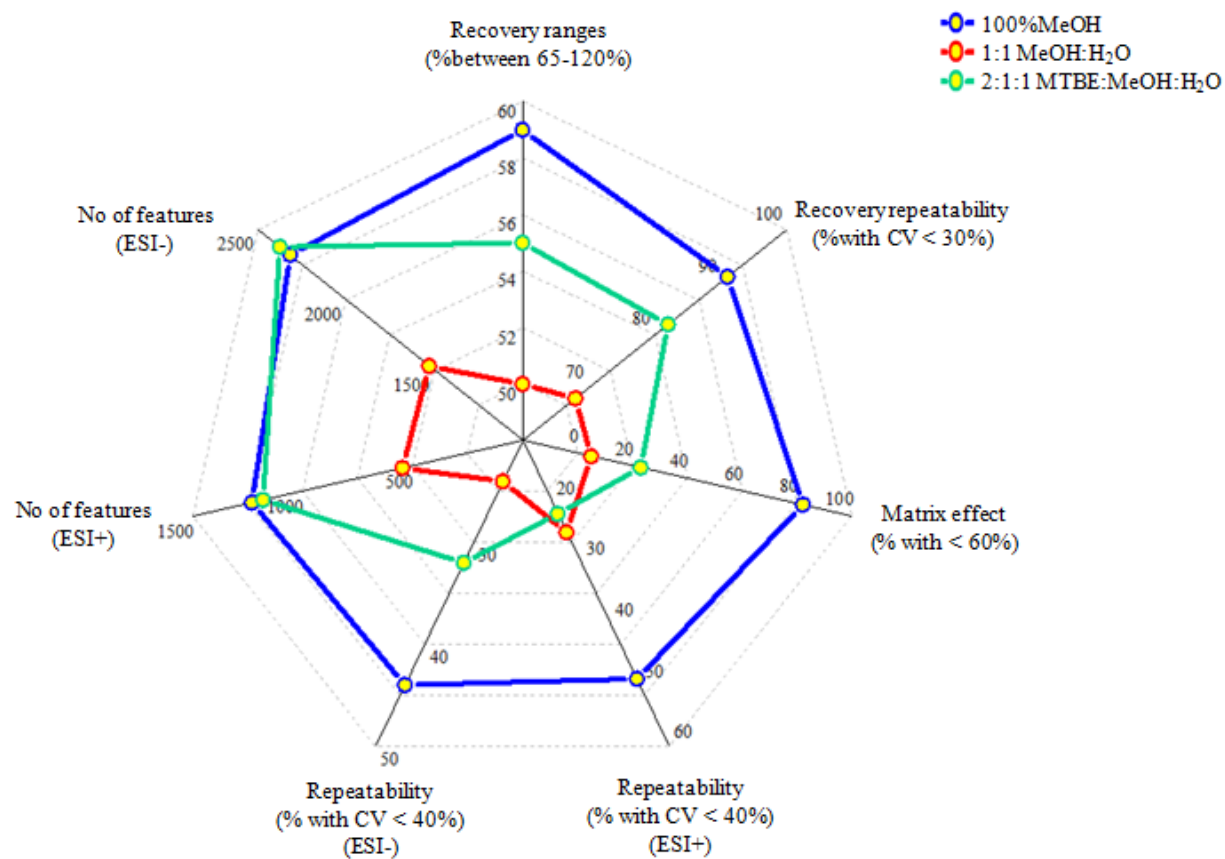
Fig. 2. Placental endogenous chemicals (A) and exogenous chemicals (B) area fold changes using three different solvents. Bar plots illustrating the metabolite levels as log₂ fold change between two comparison groups: 100% methanol versus 1:1 methanol:water (MeOH vs MeOH:H₂O), 100%MeOH versus 2:1:1 methyltert-butylether:methanol:water (MeOH vs MTBE:MeOH:H₂O) and 2:1:1 methyltert-butylether:methanol:water versus 1:1 methanol:water (MTBE:MeOH:H₂O vs MeOH:H₂O). Four replicates were analysed in positive ESI mode. For each pairwise comparisons, log₂ fold change with positive values indicate higher abundances whereas negative values indicate lower abundances.

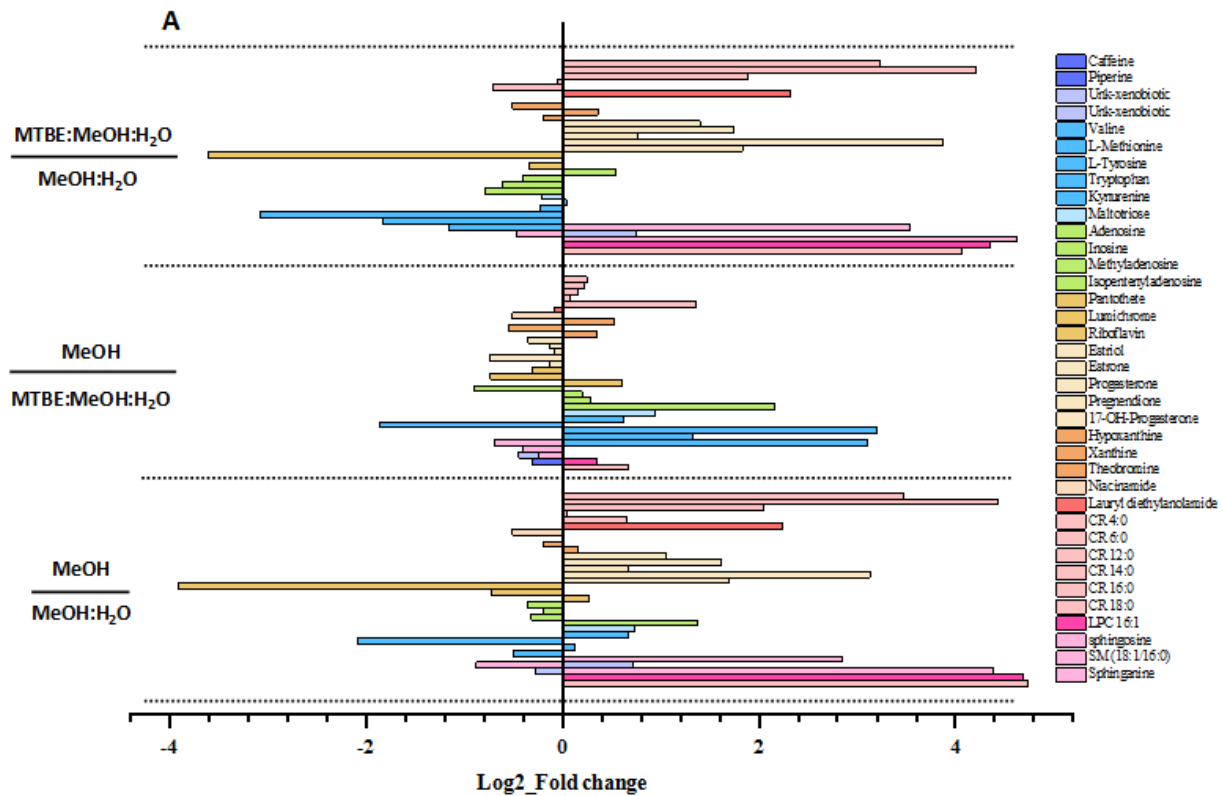
Fig. 3. Recovery and matrix effect of spiked analytical standards in placenta and repeatability assessment for different samples preparation methods. Radar chart illustrating the recovery ranges for 26 compounds spiked at 20 ng/g (% of mean recovery (n=4) between 65-120%), recovery repeatability for four replicates (% of spiked compounds with CV < 30%), matrix effect (% of spiked compounds with ME < 40%, n=4), repeatability (% of MS signals with CV < 40%), features extracted, and estimated time (semi-optimised) for the four extraction methods: protein precipitation (PPT; black), protein/p-lipids removal (PPRP; red), solid phase extraction (SPE; green), and combined SPE with PPRP (SPE-PPRP; blue)

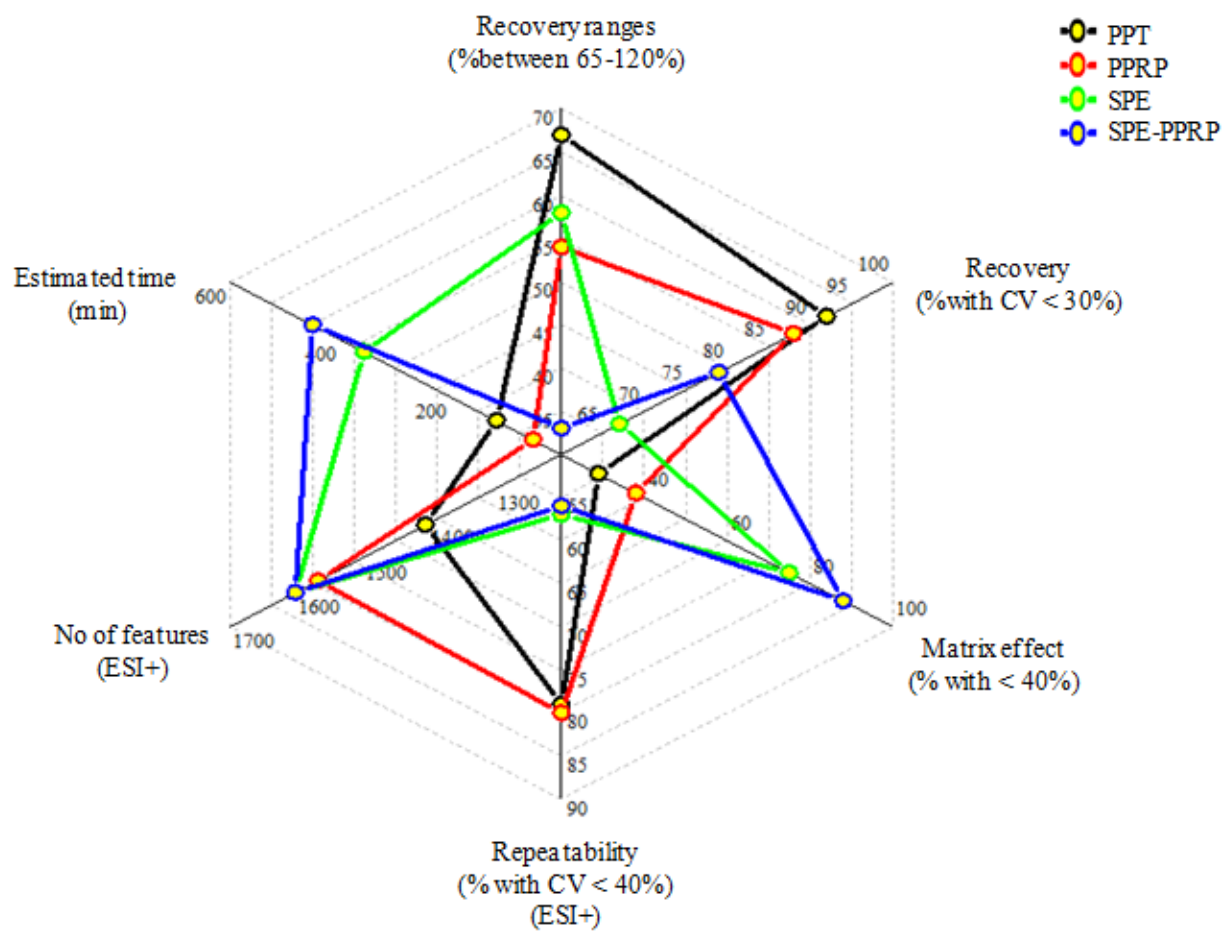
Fig. 4. Placental endogenous chemicals (A) and exogenous chemicals (B) area fold changes using four extraction methods. Bar plots illustrating the metabolite levels as log₂ fold change between two comparison groups: (1) PPRP versus PPT, (2) PPRP versus SPE, (3) PPRP versus SPE-PPRP, (4) SPE versus PPT, (5) SPE-PPRP versus PPT, and (6) SPE-PPRP versus SPE. Four replicates were analysed in negative ESI mode. For each pairwise comparisons, log₂ fold change with positive values indicate higher abundances whereas negative values indicate lower abundances.

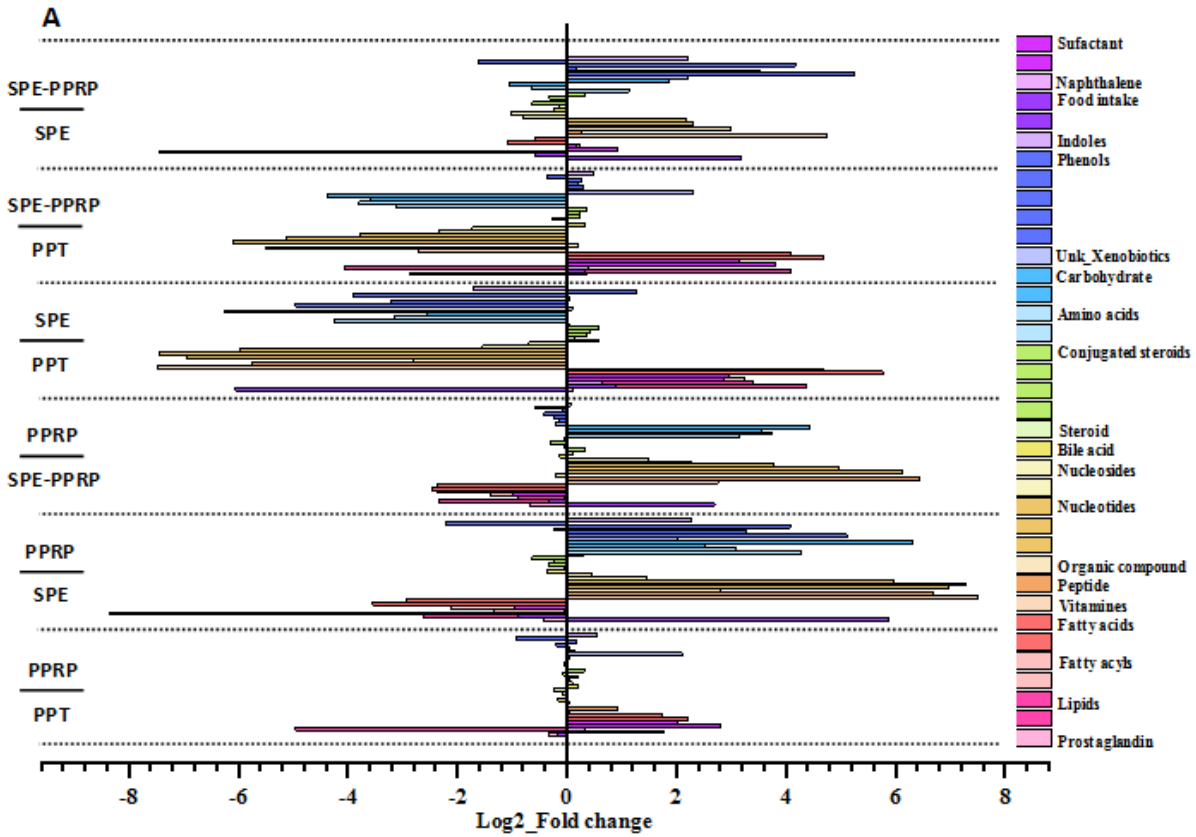
Fig. 5. Sample preparation method repeatability for placental tissue profiled in (A) positive and (B) negative ESI modes. Radar chart illustrating the repeatability (% of MS signals with CV < 20, 30 and 40%), Euclidean distances (PCA) (mean ± SD), variability area internal standards (with CV% <20) for the four dilution protocols: protein/p-lipids (PPRP20; black), protein precipitation (PPT20; red, 50; green and 100; blue), where 20, 50 and 100 values represent the volume of the final reconstituted extracts prior to injection.

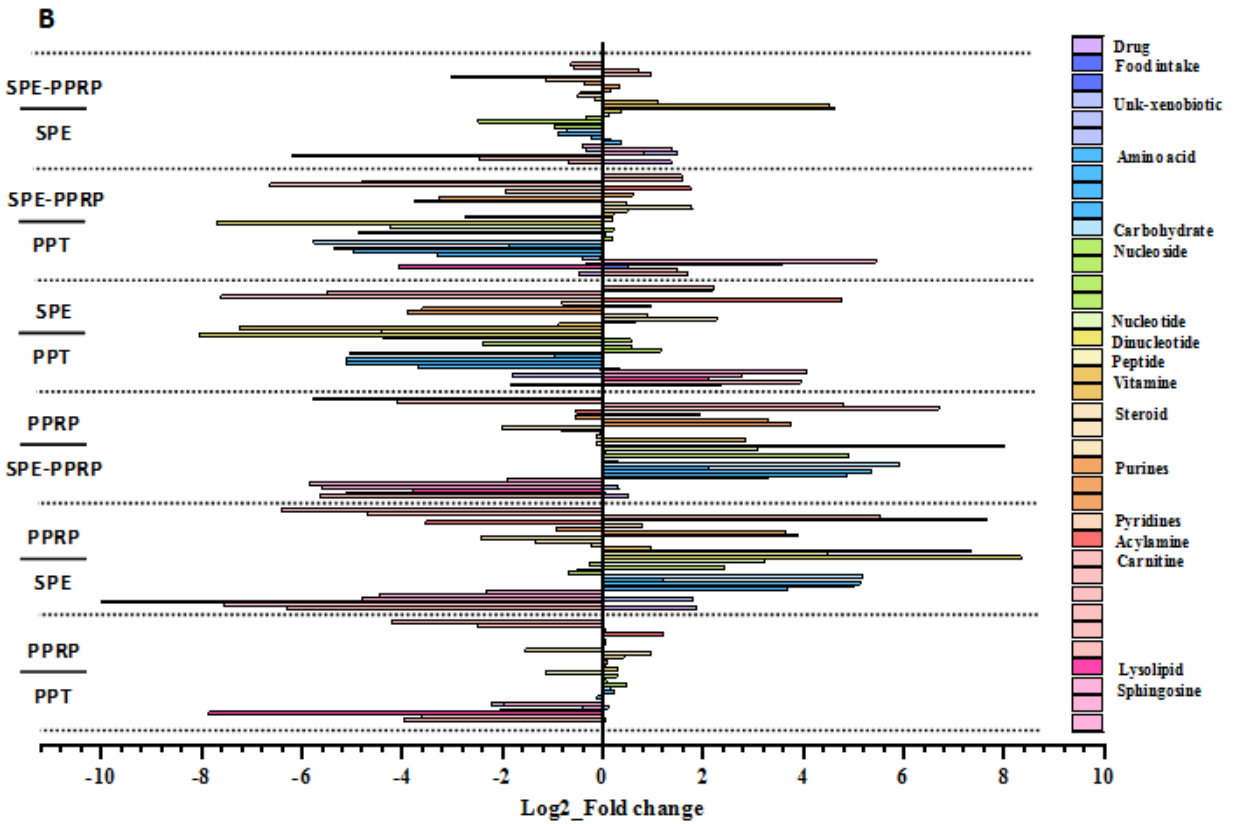
Fig. 6. Comparison of detected placental chemicals extracted by the optimised methods. Each bar represents mean of 40 samples extracted using PPRP in comparison to PPT method and analysed in a) negative and b) positive ESI mode.

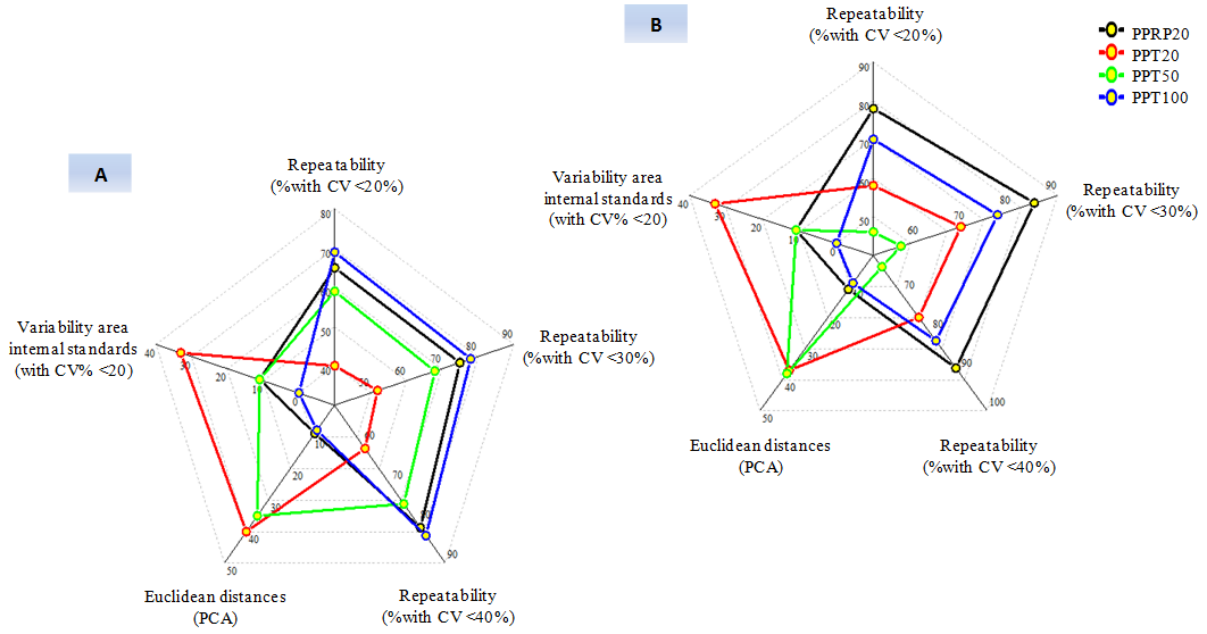


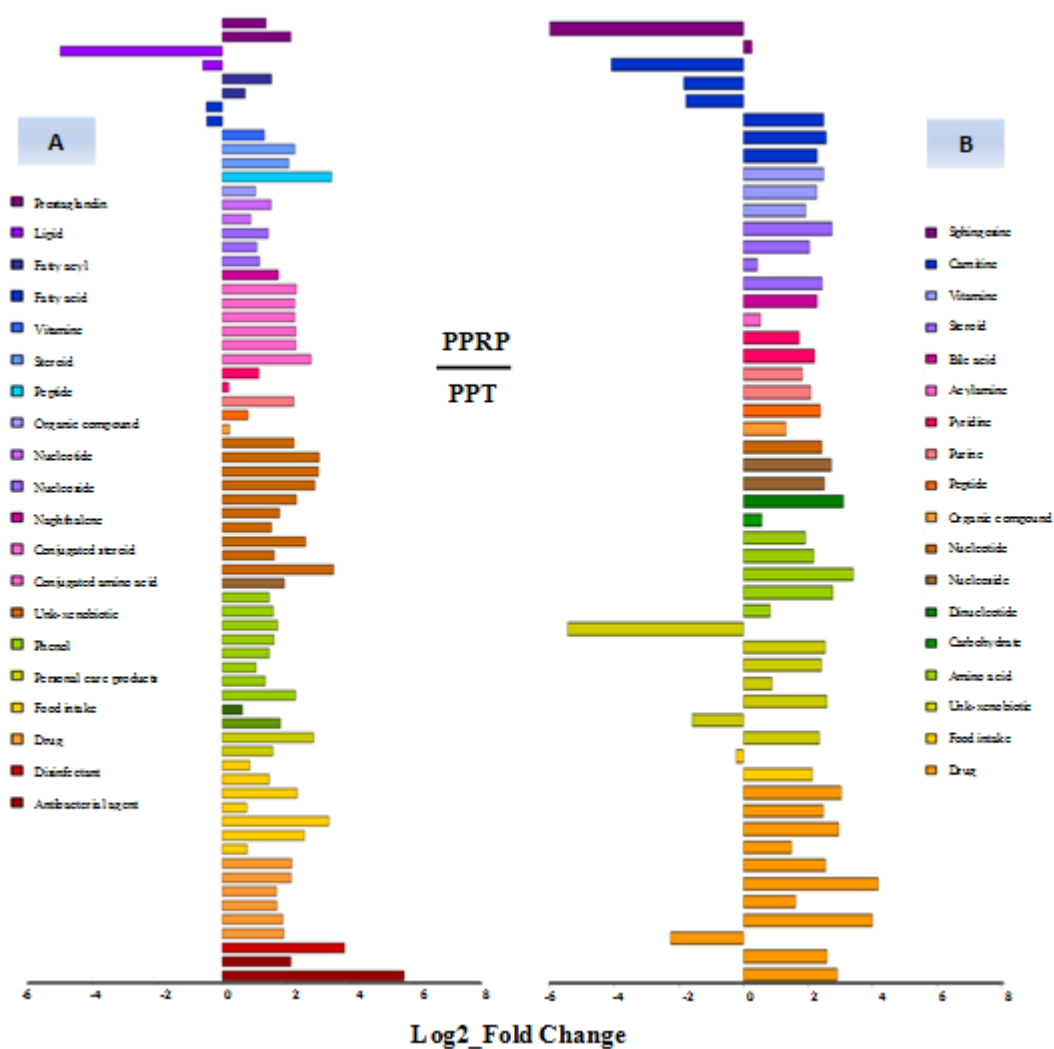












Highlights

- A robust analytical method based on high-resolution mass spectrometry was developed for profiling placenta.
- Suspected and non-target screening were combined to annotate endogenous metabolites and new exogenous chemicals.
- Sample extract split into protein precipitation and phospholipid removal fractions enable complementary profiling.
- The methodology enabled to annotate 90, confirmed 12, and probable 78 chemicals in placenta samples.

Declaration of competing interest

The author(s) declare no competing financial interests.