One-step dispersive solid phase extraction (dSPE) and protein precipitation to streamline high-throughput reversed-phase metabolic phenotyping of blood samples

Mark David[1,2], Elena Chekmeneva[1,2], María Gómez-Romero[1,2], Stephane Camuzeaux[1,2], Benjamin Cooper[1,2], Ada H.Y. Yuen[1,2], Ravi Mehta[3], Shiranee Sriskandan[3], Miguel Reis Ferreira[4], Olivier Cloarec[5], Caroline Sands[1,2], Gonçalo D.S. Correia[1,2,6,7], Zoltan Takats[1,2], Matthew R. Lewis[1,2]

[1] *National Phenome Centre, Imperial College London, London W12 0NN, UK*
[2] *Division of Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College London, London W12 0NN, UK*
[3] *Department of Infectious Disease, Imperial College London, London W12 0NN, UK*
[4] *Guys and St Thomas NHS Foundation Trust, King's College London, London, SE1 9RT, UK*
[5] *Sartorius Corporate Research, Sartorius Stedim Cellca GmbH, 89081 Ulm, Germany*
[6] *Institute of Reproductive and Developmental Biology, Department of Metabolism, Digestion and Reproduction, Imperial College London, London W12 0NN, UK*
[7] *March of Dimes Prematurity Research Centre at Imperial College London, London, UK*

Abstract

The chemical analysis of blood products (plasma and serum) is commonplace in metabolic phenotyping studies. The diversity of analytes in blood products characterized by varying physico-chemical properties, stability, and solubility presents an analytical challenge when attempting to achieve comprehensive analyte coverage by liquid chromatography mass spectrometry (LC-MS). While reversed-phase chromatography (RPC) of lipid analytes does not suffer from the presence of small molecules eluting mostly early in the chromatogram, the RPC-based analysis of low-molecular-weight metabolites (LMWMs) in minimally processed blood products is hindered by the presence of proteins and lipid species which fail to cleanly elute and negatively impact the assay. Here, we propose a novel application of dispersive solid phase extraction (dSPE) for the one-step single-phase depletion of proteins and lipids from plasma and serum samples without detrimental effect to the composition of LMWMs, overcoming challenges of conventional SPE. Using this approach, we demonstrate in two clinical studies the paired use of C18 RPC LC-MS for LMWM profiling enabled by dSPE and C8 RPC LC-MS for lipid profiling, providing 650+ annotated LMWMs and lipid species in plasma and serum samples.

Introduction

Human blood products (plasma and serum) provide a gateway to the study of endogenous and exogenous substrate metabolism, including microbial co-metabolism, as influenced through the expression of human and microbial genetics, changes in nutrition, exposure to environmental factors, infection and disease, and other stimuli[1]. Both plasma and serum are commonly studied[2] for the discovery of diagnostic and prognostic metabolic biomarkers which can be translated into clinical practice or used in the development of therapeutics. The chemical compositions of plasma and serum are complex, ranging from volatile transient signaling molecules (e.g. nitric oxide), small polar metabolites (e.g. organic acids), larger and more hydrophobic molecules (e.g. sterols, steroids, bile acids, drug metabolites), complex and neutral lipids, peptides and larger macromolecules (e.g. protein and lipoprotein species). This broad chemical expanse, together with the great physiological range of blood metabolites[3], poses an analytical challenge for researchers aiming to construct a complete image of the metabolome, as no single technology or profiling method is truly comprehensive[4].

Where LC-MS is employed, RPC has long been the staple of metabolomics methodology providing an exceptionally broad coverage of the metabolome[3] through a combination of more hydrophobic interactions of analytes with alkyl chains of RP column and more hydrophilic interactions with residual silanols. RPC is especially well suited for the analysis of moderately hydrophobic endogenous molecules[5-7] and much of the exposome: dietary metabolites[8,9], xenobiotics[10], synthetic chemicals[11], and microbiome products[12]. However, direct application of RPC in the analysis of human blood products is challenging in practice due to the abundance of protein and complex lipids such as glycerophospholipids and triacylglycerols. Proteins are removed by organic solvent precipitation[13,14] allowing their separation by centrifugation or filtering from the small molecule-containing supernatant. Lipids are strongly retained under RPC conditions and highly ionizable in electrospray ionization (ESI). Consequently, complex and neutral lipid species can accumulate in the LC system during analyses of low-molecular-weight metabolites (LMWMs) in blood products, eluting unpredictably from the column and exhibiting suppressive effects on the ionization of other metabolites[15-19]. To better accommodate lipids in profiling analyses, the established standard is to design methods which measure LMWMs and cleanly elute lipids[17] either simply preventing their problematic accumulation or allowing their additional measurement. The latter may be impractical due to the differences in solubilization of LMWMs and hydrophobic lipids in the sample diluent.

Independent lipidomics and metabolomics analyses for one sample[20-22] require separation of lipids and LMWMs using various sample preparation strategies. The gold standard here is liquid-liquid extraction (LLE) using different organic solvent mixtures allowing to achieve biphasic separation with

lipids partitioned into the organic phase and LMWMs into the aqueous phase[23-25]. To avoid the use of harsh organic solvents (e.g. chloroform in Folch and Bligh-Dyer methods), methyl tert-butyl ether (MTBE) was used by Matyash in 2008[26] allowing a phase distribution with the upper layer being the organic solvent (lower density MTBE) rich in lipid species, the lower aqueous layer rich in LMWMs, and the protein pellet collected at the bottom of the vial. However, sample extraction using two immiscible solvents has the risk of metabolites being incompletely partitioned between the two phases. It has been shown that lysophospholipids (LPC and LPE) as well as some fatty acids were highly recoverable in Matyash and Bligh-Dyer aqueous phase, and that some LMWMs, such as acylcarnitines, were recovered in both organic and aqueous phases of Matyash method[24,27,28]. It has also been suggested that LMWMs were lost in biphasic extraction by all Folch, Bligh-Dyer, and Matyash methods[25], and some lipid classes (especially more polar phospholipids) were not extracted quantitatively[28]. This emphasizes the need for more efficient, safe, easy, and reproducible serum and plasma content separation to achieve a dramatic reduction in lipids abundance required for sustainable RPC metabolic profiling of LMWMs.

One practical solution for mitigating lipid-based matrix is to deplete the samples of those lipids. Recently, some porous materials such as covalent organic frameworks[29] or polyanion-metal ion systems[30] were proposed for lipid removal. However, conventional SPE materials remain as the most common method for the depletion of lipids and enrichment of certain compounds in metabolomics studies, with varying degrees of extraction efficiency[24,31-36]. While SPE selectivity is very advantageous for targeted experiments, it presents a challenge for untargeted global profiling as can lead to the depletion of analytes of interest and introduce contaminants[33,37]. SPE is typically performed by loading samples onto a packed bed of sorbent material and eluting unbound metabolites for downstream analysis. So-called “dispersive SPE” (dSPE), based on the addition of a sorbent directly into the analytical sample extraction solution[38], allows for easier handling and rapid sample processing. Equilibrium is reached quickly, reducing time significantly when compared to conventional SPE and LLE protocols[39] and allowing high-throughput applications. The dSPE components, the sorbent and the extraction solvent, can be tuned to target different classes of analytes.

In this work, we developed and optimized a C18 dSPE-based method that simultaneously depletes lipids and proteins from blood products with a minimal perturbance to the LMWM composition. The proposed approach is rapid, inexpensive, and fit for large-scale deployment. Using this method, we were able to apply an established C18 RPC method developed for large-scale human urine metabolic profiling[40,41] to the measurement of LMWMs in blood products, leveraging a singular in-house metabolite annotation resource. Together with an established C8 lipidomic profiling [14,41,42], the two RPC methods cover a broad range of the blood metabolome with robust analytical performance.

Experimental section

1. Design

The development, validation, and application of the dSPE protocol are summarized in **Figure 1**.

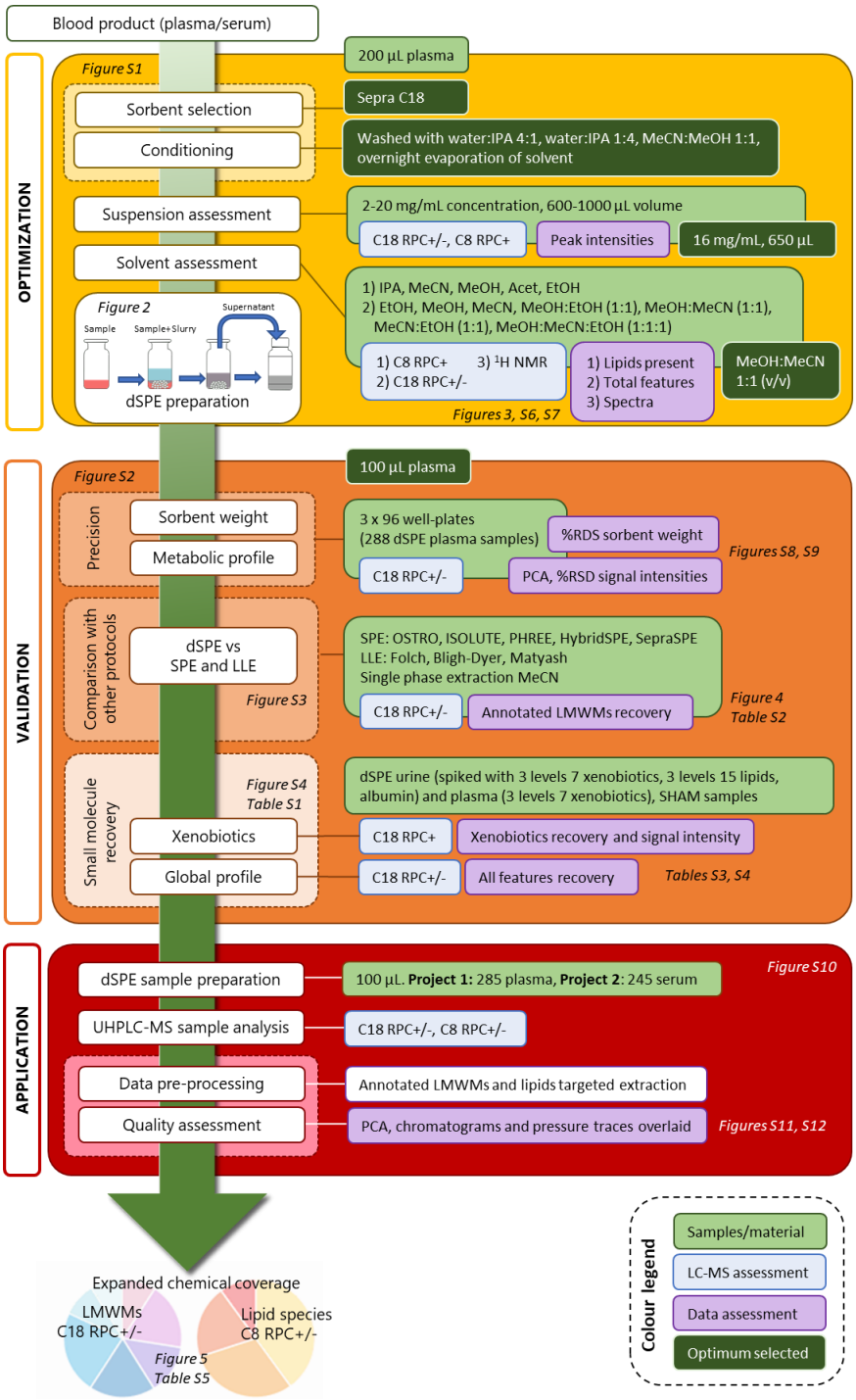

Figure 1. Workflow of the methodology used to optimize and validate the lipid removal dSPE protocol and its application to serum and plasma sample sets. The samples or material used in each experiment (light green boxes), the UHPLC-MS method they were subjected to (light blue boxes), the parameters being assessed (violet boxes) and the optimal conditions selected (dark green boxes) are shown in each of the steps.

2. Data acquisition by UHPLC-MS

All dSPE optimization, validation and application experiments were performed using ACQUITY ultra-performance liquid chromatography (UPLC) instruments coupled to Xevo G2-S orthogonal acceleration time-of-flight (oa-TOF) mass spectrometers via a Z-spray electrospray ionization (ESI) source (Waters Corp., Manchester, UK). LMWM analysis was achieved using a Waters ACQUITY UPLC HSST3 (1.8 µm, 2.1 x 150 mm) column and data across the range of 50 to 1200 *m/z* was collected in both ESI positive and negative ion modes (C18 RPC+/-). Lipid analysis was performed using a Waters ACQUITY UPLC BEH C8 (1.7 µm, 2.1 x 100 mm) column across the 50 to 2000 *m/z* range (C8 RPC+/-). Detailed instrumental conditions of both methods have been described previously[41]. Details of the reagents used can be found in **Experimental S1**.

All datasets generated were filtered[43] using two criteria: precision of individual features, with relative standard deviation (%RSD) in pooled samples < 30%, and Pearson correlation to dilution factor > 0.7 (see **Experimental S2** for more details).

3. dSPE protocol development

3.1 Representative biological samples

All optimization and validation experiments were performed using pooled EDTA anticoagulated human plasma purchased from Sera laboratories international (West Sussex, U.K). Plasma from six individual donors was pooled and subsequently subaliquoted in 1 mL tubes before storing at -80°C.

3.2 dSPE conditioning and suspension preparation

Sepra™ C18-E (50 µm, 65Å, bulk packing) (Phenomenex, Torrance, USA) was washed twice with water and isopropanol (IPA), firstly at 4:1 (v/v), then at 1:4 (v/v) in Falcon tubes. The sorbent was further washed and conditioned three times with the extraction solvent, and the supernatant (after centrifugation) was decanted and discarded after each step. The solvent selected after the development of the protocol was a mixture 1:1 (v/v) of acetonitrile (MeCN) and methanol (MeOH). After the final wash, the sorbent was left overnight for complete evaporation of solvent and stored in an airtight container ready for use.

The creation of the suspension and how it is dispensed into a 96 well-plate is shown in **Figure 2**. Pipette tips required an initial pre-wetting with the suspension material (three iterations of aspirating and

dispensing the suspension back into the stock) for maximal precision. Replicates ranged from three to six for each experimental condition.

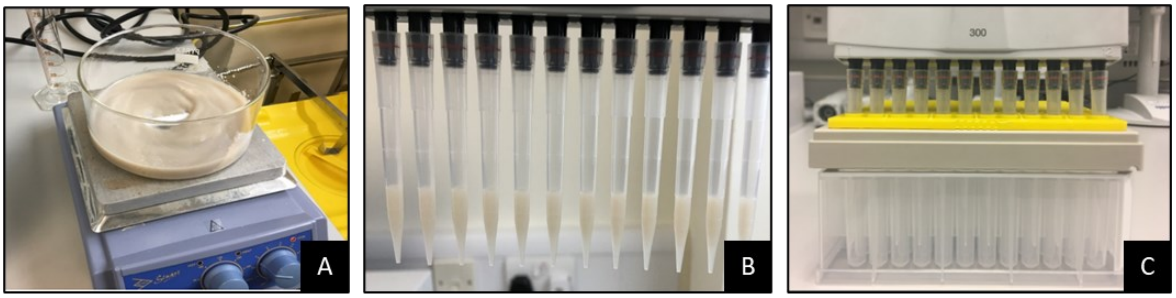

Figure 2. Suspension creation and dispensing in 96 well-plates. A) Sorbent and solvent are mixed using a magnetic stirrer and plate in a 300 mL Pyrex borosilicate glass crystallizing basin with flat bottom and without spout. B) Suspension aspiration using a 12-channel multichannel pipette 30-300 µL. C) Suspension dispensing in a 2 mL 96-well plate with a top rack used as a "jig".

3.3 dSPE protocol optimization experiments

A comprehensive overview of the workflow used during the dSPE optimization stage is presented in **Figure S1.**

3.3.1 Suspension composition optimization

Before starting the extraction, the required amount of dry sorbent was weighed based on the number of samples and then washed. The tested suspension concentration ranged from 2 to 20 mg/mL and the suspension volume ranged from 600 to 1000 µL for a 200 µL sample volume. Suspension was vortexed to ensure homogeneity at every instance prior to sample addition. Plasma samples were prepared for each condition, with the inclusion of sham (prepared using sorbent-free solvent) samples and analyzed by C18 RPC+/- and C8 RPC+.

3.3.2 Solvent composition optimization

Acetone (Acet), acetonitrile (MeCN), 2-propanol (IPA), methanol (MeOH), and ethanol (EtOH) were used (see details of the reagents in **Experimental S1**). Solvents used for various extraction conditions were kept at -20°C. Six replicates were included for each solvent condition.

The sample-suspension mixture was vortexed and incubated for two hours at 4°C, then centrifuged for 10 minutes at 3214 x g, and 50% of the total supernatant was collected and dried under a gentle flow of nitrogen. After drying, the sample was resuspended in water in half of the original sample

volume to maintain the sample dilution for the C18 RPC+/- analysis. The remaining supernatant was subjected to C8 RPC+ to assess the lipid species present. Sham control samples were also prepared.

Further assessment was undertaken with the best performing solvents: MeCN, MeOH, and EtOH, individually and in combination, resulting in seven different extraction conditions: EtOH, MeOH:EtOH (1:1), MeOH:MeCN (1:1), MeCN:EtOH (1:1), MeOH, MeCN, and MeOH:MeCN:EtOH (1:1:1).

3.3.3 Lipid removal assessment by nuclear magnetic resonance (NMR) spectroscopy

Sample preparation details for 1D ^{1}H NMR spectroscopic assessment of lipid removal from the dSPE-treated plasma and comparison to untreated plasma are presented in **Experimental S3**.

3.3.4 Optimized dSPE sample preparation protocol

The following procedure describes the final optimized experimental conditions for dSPE. Plasma or serum samples (100 µL in 96-well preparation plates) were removed from the -80°C freezer and allowed to thaw at 4°C for approximately two hours prior to extraction. A two-point internal standard solution in water was spiked into the MeOH:MeCN 1:1 (v/v) extraction solvent prior to the addition of sorbent to obtain the final concentrations of 0.05 µM L-phenylalanine-$^{13}C_9$,^{15}N and 0.04 µM N-benzoyl-d_5-glycine. The sorbent was washed and equilibrated as previously described. MeOH:MeCN 1:1 (v/v) was added to the sorbent to create a 16 mg/mL suspension. Two 96-well plates (192 samples) require 100 mL of suspension solution (1.6 g of sorbent), prepared in cold solvent (-20°C). The suspension (325 µL) was added to each sample well, and the sample-suspension mixture were vortexed thoroughly and left to incubate for 2 hours at 4°C. After centrifugation for 10 minutes at 3214 x g and 4°C, 212.5 µL of the supernatant (1/2 of the suspension-sample volume) were collected and dried under a gentle flow of nitrogen at room temperature. Resuspension was undertaken in 100 µL of water, containing a mixture of eight method reference standards including L-glutamic acid-$^{13}C_5$; L-isoleucine-$^{13}C_6$, ^{15}N; L-leucine-$^{13}C_6$; L-tryptophan-$^{13}C_{11}$, $^{15}N_2$; L-glutamine-$^{13}C_5$; creatinine-(methyl-d_3); cytidine-5,6-d_2; and benzoic acid-phenyl-$^{13}C_6$ (at concentrations specified previously[40,41]).

3.4 dSPE protocol validation experiments

A comprehensive overview of the workflow used during the dSPE validation stage is presented in **Figure S2**.

3.4.1 Feasibility and reproducibility

The precision of the sorbent weight distribution and of the plasma sample profile were assessed (**Experimental S4**). For sorbent weight precision, the suspension was dispensed into three 96-well PCR

tube racks. Each tube was weighed before and after the addition of the suspension, the difference calculated, and the %RSD reported. The precision of the LMWM profile of 288 replicates of the EDTA pooled plasma sample prepared using the optimized dSPE protocol and analyzed by C18 RPC+/- was assessed using principal component analysis (PCA) and %RSD calculated for the measured signal intensities of individual molecular species.

3.4.2 Comparison with commercially available SPE plates and LLE

Phospholipid removal plates tested included OSTRO 25 mg (Waters, Milford, MA, USA), ISOLUTE C18 50 mg (Biotage, Uppsala, Sweden), PHREE 30 mg (Phenomenex, Macclesfield, U.K.) and HybridSPE-Phospholipid 15 mg (Courtesy of Sigma-Aldrich; Hybrid SPE-ppt, USA). Phenomenex also supplied 5 mg of Sepra C18 material packed in a 96-well SPE plate format (Sepra-SPE). The supernatants from dSPE treated samples were also compared to the hydrophilic fractions from biphasic LLE protocols, which included Folch, Bligh-Dyer (BD) and Matyash extractions[28] and to a monophasic MeCN with 0.1% formic acid (FA) extraction as a control (**Figure S3**). Extraction conditions are summarized in **Experimental S5**.

All dSPE, LLE and SPE preparation protocols were performed using seven replicates of the pooled EDTA plasma. Aqueous extracts from the proposed dSPE method, SPE plates, and LLE aqueous extracts were analyzed by C18 RPC+/-. Evaluation of the LLE and SPE protocols against the dSPE technique were based on recovery measurements for annotated LMWMs using the R package peakPantheR[41,44]. Recovery of each annotated metabolite was calculated as a ratio of the average intensity from each extraction protocol to the average intensity from the reference monophasic 0.1% FA in MeCN extraction. Violin plots were used to illustrate the distribution of average recoveries for each preparation protocol.

3.4.3 Assessment of the LMWMs recovery in a urine-based artificial plasma matrix

LMWMs recovery was assessed using a dSPE-treated spiked urine and sham samples. The aim here was to create an artificial mixture resembling blood by spiking lipids and albumin into a matrix rich in LMWMs. A pooled urine sample, collected from six individual donors of mixed genders at multiple time points and free from investigated xenobiotics, was spiked with four concentrations of seven xenobiotics, three concentrations of 15 lipids and albumin. Details can be found in **Experimental S6, Figure S4** and **Table S1.** Spiked xenobiotic recoveries, and global small molecule recoveries were calculated by taking the quotient of the average intensity from six replicates measured in the dSPE-treated sample, against the average intensity measured in the corresponding sham treated sample.

4. Application

We applied the developed dSPE method for metabolic profiling of the samples from two different cohorts of blood products.

Plasma samples were collected from male participants enrolled in a cross-sectional prostate cancer and pelvic radiotherapy study (Microbiota- and radiotherapy-induced gastrointestinal side-effects, MARS, study; London-Bromley Research Ethics Committee (REC) no. 13/LO/1527)[45]. A subset of this pilot study, consisting of 285 plasma samples, were provided for metabolic profiling. After collection, plasma samples were stored at -80°C prior to their analysis.

Serum samples (245) from two parallel studies assessing biomarkers of host response in infection at Imperial College Healthcare NHS Trust (ICHT), the Bioresource for Adult Infectious Diseases (BioAID, South Central – Oxford C REC no. 14/SC/0008 and 19/SC/0116), and the Microbial Products in Infection study (West London REC no. 06/Q0406/20), were collected from 161 patients at the point of admission and from 13 healthy controls (ICHT Tissue Bank, approved by Wales REC3, 17/WA/0161, to release human material for research from subcollection MED_SS_12_023)[46].

Sample handling and pooled quality control (QC) sample preparation were done in accordance with guidelines previously reported[40,41]. Samples were analyzed by the two complementary C18+/- and C8 RPC+/- methods. The pooled QC sample was used for data quality monitoring and feature filtering. To assess the metabolome coverage, targeted extraction, and integration of annotated metabolites in the generated datasets was done using the R package peakPantheR[41,44]. Further details of data pre-processing and analysis are described in **Experimental S2.**

Results and Discussion

This study aimed to develop a protocol for blood product sample preparation enabling sustainable large-scale metabolic profiling using conventional RPC on a C18 column. This approach offers significant expansion of metabolic coverage in blood analysis, particularly for moderately hydrophobic metabolites, that are often poorly retained and detected using established RPC methods for lipid profiling or HILIC for polar LMWMs. However, C18 RPC analysis of plasma and serum samples suffer from the presence of proteins and lipids that, if not removed, can accumulate on the column in a RP system, negatively impacting the analysis of LMWMs through increased LC backpressure and ionization suppression. **Figure S5** exemplifies this challenge by showing C18 RPC+ chromatograms, pressure traces, and spectra of an untreated plasma sample compared to blank samples analyzed before and after.

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1. Establishing dSPE conditions for blood plasma preparation

1.1 Suspension optimization

Sepra C18 sorbent was selected from all commercially available C18 sorbent materials owing to its high carbon load, its full endcapping (minimizing the residual silanol group activity and ensuring the separation selectivity based on analyte hydrophobicity), and its availability as a bulk material. The concentration and total volume of the suspension were optimized to yield conditions favorable for lipid removal whilst maintaining maximum recovery of the other LMWMs. A value of 16 mg/mL was determined to be optimal for liquid handling purposes, with more concentrated suspensions becoming too dense and non-fluid. A 1:3 sample-to-solvent ratio (accounting for the sorbent volume) was used to facilitate solvent-based protein removal[13,14] at the same time as lipid depletion. The optimal conditions were 650 µL of suspension volume for a 200 µL sample (or 325 µL suspension volume / 100 µL sample).

1.2 Effect of organic solvent choice on dSPE-treated samples

Determination of the solvent(s) most suitable for C18 dSPE-based lipid depletion and LMWM profile preservation required empirical assessment. Acet, MeCN, IPA, MeOH, and EtOH were tested owing to their routine use in LC-MS workflows. For each set of solvent conditions, the residual lipid profiles were analyzed by C8 RPC+ and visually inspected (**Figure 3**). IPA and Acet failed to adequately deplete

the lipids present, owing to their efficient disruption of lipid-to-sorbent binding. The remaining solvents tested (MeCN, MeOH, EtOH) resulted in greater lipid depletion, especially for more hydrophobic lipid species (e.g. two and three acyl chain lipids predominant in chromatographic regions 2 and 3). In all conditions tested, the less hydrophobic lipid species (e.g. lysophospholipids predominant in chromatographic region 1) were the least affected by depletion. This is desirable, as this region may contain important LMWMs including lipophilic xenobiotics and endogenous metabolites (e.g. bile acids and acylcarnitines).

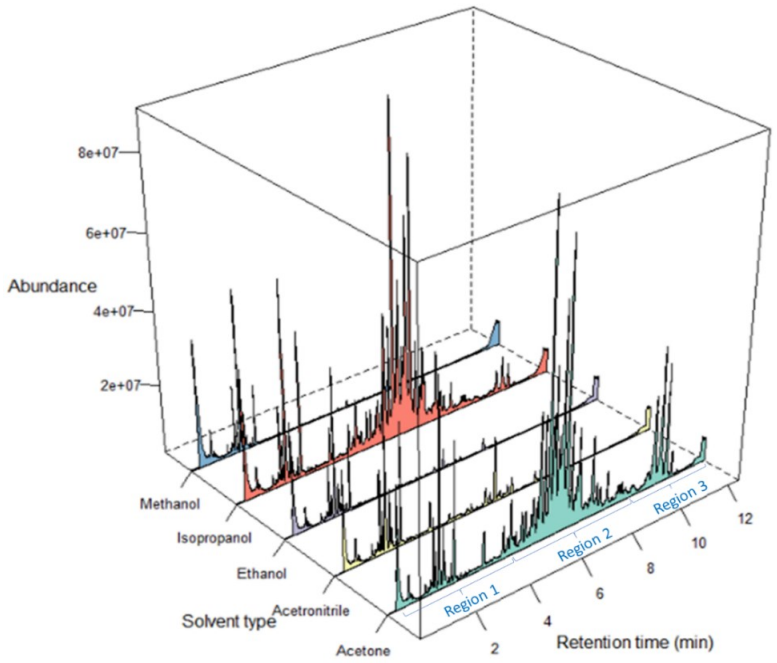

Figure 3. C8 RPC+ total ion chromatograms (TICs) comparing the effect of MeOH, IPA, EtOH, MeCN and Acet as extraction solvents used in dSPE-treated plasma. Three retention time regions relevant to C8 RPC analysis were examined: 0-4 minutes (**Region 1**), lysophosphatidylcholines (LPC), monoglycerides (MG), lysophosphatidylethanolamines (LPE), lipophilic endogenous metabolites and xenobiotics; 4-9 minutes (**Region 2**), phospholipids [phosphoglycerols (PG), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS)], sphingomyelins (SM), ceramides (Cer) and diglycerides (DG)]; and 9 minutes and onwards (**Region 3**), triglycerides (TG) and cholesteryl esters (ChE).

The effects of MeCN, MeOH, and EtOH solvents (individually, in pairwise volumetric 1:1 combination and altogether) on the resulting LMWM profiles were assessed using C18 RPC+/- analyses. Solvent compositions containing EtOH produced the highest number of features, but also produced a higher baseline and large asymmetrical macromolecule-derived peaks which eluted from seven minutes onward (**Figure S6.A**), ultimately resulting in carryover and increased system pressure during sequential injections. The same effects were observed to a lesser extent with the other EtOH and MeOH solvent compositions. It was hypothesized that the combination of water and these alcohols

was not sufficient in reducing the dielectric constant, allowing proteins to remain in solution. Extraction with MeCN yielded a greatly improved baseline with no evidence of residual protein, consistent with the reporting of MeCN as superior to other solvents for protein precipitation[13]. Although MeCN was the most efficient of the tested solvents for lipid and protein removal, its addition to samples containing high salt concentrations is known to result in inconsistent biphasic partitioning of metabolites, leading to poor reproducibility[47]. A 1:1 combination of MeOH:MeCN provided a good balance in terms of adequate protein removal and high recovery of LMWMs (**Figure S6.B**), with the methanolic component also serving to eliminate biphasic partitioning. This result validates the observations from Southam and coworkers[48], who compared several monophasic and biphasic extraction methods for the analysis of LMWMs from plasma and urine samples by hydrophilic interaction chromatography (HILIC) and concluded that monophasic MeOH:MeCN 1:1 (v/v) was the optimal solvent composition in terms of reproducibility and number of detected putative metabolites.

To provide additional evidence of the efficiency of lipid removal from blood products with the developed protocol, ^{1}H 1D NMR spectroscopy was used to compare spectra obtained for the dSPE-treated and untreated plasma (**Figure S7**). It can be clearly observed from the 1D spectra that the broad lipid signals, that can be assigned in the untreated plasma samples to the terminal CH_3 and various types of CH_2 and CH groups from long-chain saturated and unsaturated fatty acyls of lipoproteins[49,50], are to a large extent removed from the dSPE-treated plasma sample. The singlet at 2.04 ppm, corresponding to acetyl groups of glycoproteins, is not present on the spectrum of dSPE-treated plasma due to the precipitation of proteins occurring simultaneously with the lipid removal. The LMWM profile of the dSPE-treated plasma shows enhanced resolution in the absence of the broad signals of the lipid species and protein-affected baseline. The results of ^{1}H 1D NMR analyses corroborate the protein and lipid removal observed in the LC-MS experiments described above.

2. Validation

2.1 Feasibility and reproducibility

The practicality of conducting dSPE is of paramount importance for its implementation in the laboratory as a one-step high-throughput solution for lipid depletion. To ensure applicability in large-scale studies, the preparation performance of the dSPE material was evaluated in a 96-well plate-based format. The reproducible addition of loose C18 particle material from a single large-scale suspension was demonstrated by aliquoting the suspension using a standard 8-channel electronic pipette, resulting in an 7.7% RSD of sorbent weight measured across the 96 wells in plate.

To test the reproducibility of the method in terms of lipid depletion and preservation of LMWM features, 288 replicate aliquots of the pooled human EDTA plasma sample, from three 96-well plates, were prepared using the dSPE protocol and analyzed with the C18 RPC+/- assay. This LC-MS data was processed and %RSD calculated for the detected features. The median %RSD values for C18 RPC+/- were 4.5% and 4.2%, respectively (**Figure S8**), showing no significant variation across all detected features passing the dilution series filter. Additionally, PCA was used to examine whether the main sources of variability could be associated with sample preparation parameters. PCA scores plots (**Figure S9**) demonstrated no trends in variation between the samples when colored by addition of suspension (added column-wise using an 8-channel electronic pipette) or addition of the resuspension water (added row-wise using a 12-channel electronic pipette).

Chromatographic performance across these tests indicated that the product of the dSPE treatment was well suited for large-scale UHPLC-MS analysis by the C18 RPC method, with no observable pressure spikes throughout the analytical run, low background, a stable baseline and negligible drift in retention time.

2.2. Comparison with commercially available SPE plates and LLE

Numerous commercially available SPE materials exist for the depletion of lipids from blood product samples prior to UHPLC-MS analysis. These materials are most often applied in targeted quantitative analyses where any unintended depletion effects on the analytes of interest can be directly tested during method development and validation, which is impossible in downstream assessment of the global LMWM profile[37,33]. In addition, SPE-based sample preparation in untargeted metabolomics can have the potential risk of selectively remove LMWMs, introduce contaminants, be time- and resource-consuming, and suffer from lower reproducibility in large-scale analyses[33, 37]. Still, these solutions, when used in conjunction with the manufacturer's recommended protocols, represent a valuable reference point against which our proposed dSPE methodology was evaluated.

To deconvolve the effects of packed vs. free C18 coated particle material, a 96-well SPE plate was packed by Phenomenex (Sepra-SPE) with the amount equivalent to the optimized dry weight (5 mg/well). As illustrated by the violin plots in **Figure 4**, both Sepra-dSPE and Sepra-SPE yielded a greater number of LMWMs with mean recoveries at 100% or higher. Higher recoveries can potentially be explained by the Sepra methods having a greater capacity to extract LMWMs whilst simultaneously removing both protein and lipids. However, packing the Sepra particles in SPE format increases sample preparation costs. Of the four SPE plates tested (Hybrid-SPE, PHREE, ISOLUTE and OSTRO), Hybrid-SPE

showed the poorest performance. The violin plots for the remaining three SPE methods demonstrated distributions of recoveries in the 50-100% range.

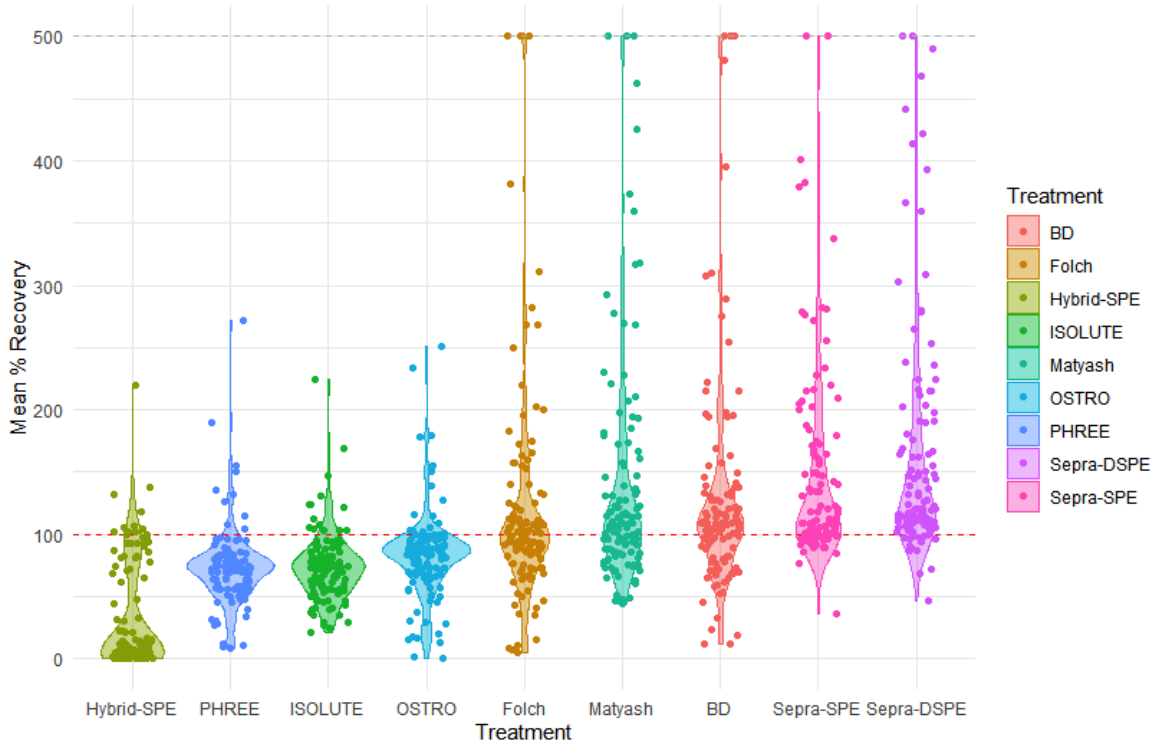

Figure 4. Violin plots representing the distribution of mean recoveries calculated for annotated LMWMs in the dSPE, SPE and LLE (aqueous phase) extracts. Each scatter point represents the recovery for an individual LMWM compared to the monophasic control extraction with MeCN + 0.1%FA, measured using C18 RPC+/-. The threshold indicated by the horizontal red dashed line represents a 100% recovery (i.e. no change between the extraction treatment and the control).

Biphasic extractions are popular techniques used in the analysis of lipids on blood products. Folch, Bligh and Dyer (BD), and Matyash are the most commonly used extraction protocols[28,51]. The aqueous phase produced in LLE also allows the measurement of polar metabolites from the hydrophilic fraction.

In this work, plasma samples prepared by Folch and BD LLE methods exhibited the presence of multiply charged signals from residual protein, which led to increased system pressure and disruption to the analysis due to exceeded pressure limits. We assumed that protein precipitate formed on the walls of the glass tubes, which was difficult to avoid during aspiration of the upper hydrophilic aqueous phase. In the Matyash extraction, the precipitate was neatly deposited at the bottom of the tube after centrifugation and therefore was easily avoided when aspirating the aqueous material. The organic LLE extracts were analyzed by C8 RPC+, requiring the resuspension of the dried organic phase in the method appropriate solution of water:IPA 1:4 (v/v). The analyzed organic fractions showed no

evidence of protein in the spectra (in accordance with the literature, where lipid analysis from LLE has never indicated the presence of protein). In the less frequent cases where the aqueous phase is analyzed, HILIC methodologies are used, requiring further addition of MeCN to the aqueous extract[52,55]. In both cases, the use of organic solvents plays a protective role by ensuring protein precipitation. This is not necessarily the case in the RPC analysis of the hydrophilic fraction. To mitigate these procedural issues and allow completion of the comparison experiments, all hydrophilic LLE extracts required an additional protein precipitation step using cold MeCN in the 1:3 (v/v) sample:solvent proportion. The summary of our observations during sample preparation can be found in **Table S2**.

Comparison of dSPE and LLE performed using Folch, BD, and Matyash methods demonstrated similarity in the recovery distributions as shown in **Figure 4**. However, a greater number of LMWMs detected in the LLE methods had lower recoveries (<100%) compared to the dSPE method. Notably, the Matyash protocol demonstrated the most comparable performance to dSPE amongst the LLE methods tested. However, LLE methods typically require a larger investment of time and resources for sample preparation, and the observed lower recoveries of LMWMs render them less favorable for lipid removal, especially when downstream analysis of the hydrophilic fraction by C18 RPC is desired.

2.3 Validation of performance in a complex matrix

To validate the effect of the dSPE method on the LMWM profile in a biochemically complex matrix, human urine was supplemented with controlled amounts of albumin, a lipid mixture and a set of lipophilic xenobiotics (**Table S1**). The results were compared to a sham control (i.e. sorbent free solvent added to the sample).

The possible depletion effects in the entire LMWM profile (all feature peak integrals passing filtering/QC) of the dSPE procedure were assessed by calculating the median recovery for both C18 RPC+/- analyses. The calculated values were between 102-104% (**Table S3**), indicating no broad unintended depletion of LMWMs content by dSPE.

The impact of the dSPE lipid depletion on selected xenobiotics, especially more lipophilic lansoprazole, amitriptyline, terbinafine and diclofenac, eluting across the whole retention range and added to the sample before processing was assessed by calculating their recovery at four different concentrations (**Table S4**). dSPE-treated and sham control samples demonstrated no significant difference in mean signal intensities for the exemplary set of xenobiotics with recoveries ranging 80-110%, except for ibuprofen. Its mean intensity between sham and dSPE-treated samples varied significantly at different

concentrations. Ibuprofen elutes after 10 min, co-eluting with the lipophilic fraction, which may produce an unstable signal due to mutual ion suppression and high background signal (in agreement with the observations made during the extraction solvent optimization).

Overall, regardless of the concentration of the lipid mixture, sham and dSPE-treated samples demonstrated little significant difference in mean signal intensities for this exemplar set of xenobiotics.

3. Application: analysis of serum and plasma samples from two clinical studies

The optimized dSPE method allowed to expand the coverage of chemical space representing blood products metabolome. The newly developed dSPE method was applied to enable the C18 RPC metabolic profiling of two exemplary cohorts of human plasma (MARS study, investigating acute and late radiation enteropathy[45]) and serum (BioAID and Microbial Products in Infection studies, to support the assessment of the host response in infection[46]) samples. The samples were additionally prepared using IPA protein precipitation[14] and analyzed by C8 RPC tailored for lipid profiling. The metabolome coverage (**Figure S10**) and analysis stability (**Figure S11**) using the two complementary C18 and C8 RPC+/- methods as well as the system performance, robustness and high degree of analytical precision when analyzing dSPE prepared samples by C18 RPC+/- (**Figure S12**) are exemplified using the serum pooled QC sample of the BioAID and Microbial Products in Infection studies. The tight clustering of the QC samples relative to the dispersion of the study samples on the PCA score plots demonstrates the instrument's stability and high quality of the acquired data.

To assess the metabolome coverage, targeted extraction, and integration of annotated endogenous LMWMs, xenobiotics and lipids was performed (**Table S5**). Note that our aim was to demonstrate the applicability of the dSPE-C18 RPC method for high-throughput analysis of both blood products, and not to compare the composition of two blood sample types.

In terms of the number of annotated endogenous and exogenous LMWMs, the results for the two blood products are very similar (**Figure 5A**). Using the new dSPE method, 190 and 212 endogenous LMWMs and xenobiotics were annotated using C18 RPC in plasma (MARS) and serum (BioAID and Microbial Products in Infection) samples, respectively. In addition, 482 and 460 lipid species from different lipid classes were measured with a C8 RPC lipidomic assay in plasma (MARS) and serum (BioAID and Microbial Products in Infection) samples, respectively (**Figure 5B**). Except for minimal overlap of medium- and long-chain acylcarnitine species detected in both C18 and C8 RPC+, jointly the two assays yielded 661 unique annotations for both plasma and serum.

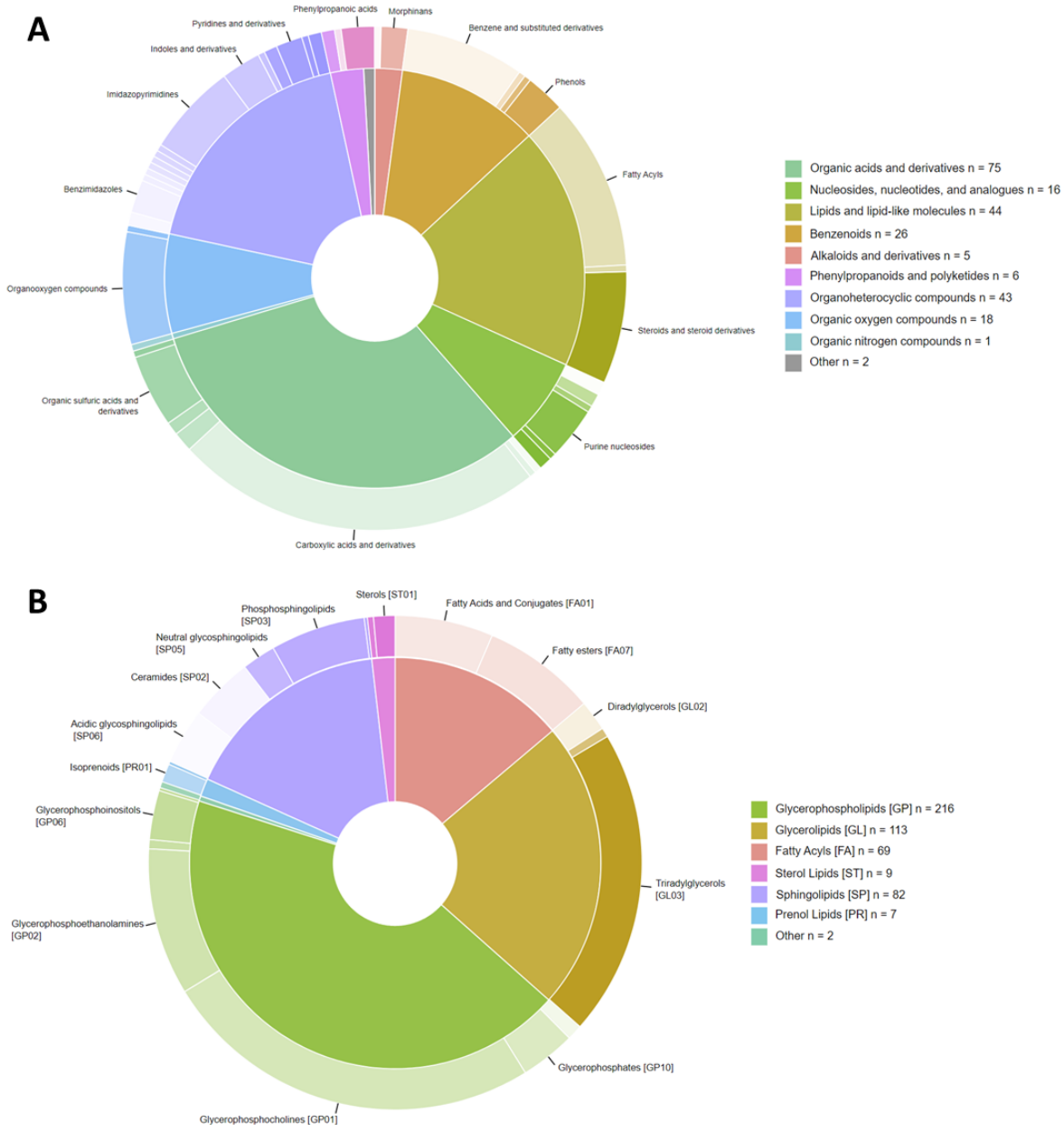

Figure 5. Summary of the annotated chemical coverage for the C18 RPC LMWM (A) and C8 RPC lipid profiling (B) assays. LMWM annotations are grouped by their ClassyFire[56] superclass and class, and lipids by LIPID MAPS[57] main class and sub class (inner and outer pie chart, respectively).

By developing the reported herein dSPE-C18 RPC method, we were able to efficiently close the gap in coverage of moderately hydrophobic metabolites and enable the detection of a wide variety of chemical structures representing the blood metabolome.

Conclusion

The untargeted nature of metabolomics allows measurement of biofluid chemistry related to both endogenous metabolism and host-environment exposures. Comprehensive coverage of chemically diverse constituents of human blood products benefits from the use of multiple methods, each oriented toward metabolite subsets generally segregated by polarity and hydrophobicity. Whilst recent developments in UHPLC-MS profiling methodologies have delivered numerous solutions for the analysis of polar molecules (e.g., via HILIC-MS) and complex lipids, the analysis of moderately hydrophobic and amphipathic molecules in plasma and serum by RPC methodology is complicated by the suppressive effects of lipids on the ionization of LMWMs. SPE techniques offer a solution to remove lipophilic species, but can often be expensive, affect recoveries of the other small molecules and introduce contamination. This study offers a solution for one of the major remaining gaps in end-to-end comprehensive metabolome coverage. The proposed high-throughput and reproducible dSPE sample preparation technique provides a way to efficiently remove highly lipophilic species from the sample, but with minimal effect on moderately hydrophobic, amphipathic and polar LMWMs. Currently, the average cost of the sorbent material used for removing lipids and proteins from plasma or serum is $2.55 per sample when using a 96-well SPE commercial plate, while using dSPE reduces the cost down to $0.16/sample. Moving forward, packing the Sepra particle in a SPE format may provide a more concentrated sample and increased sensitivity, however this would increase sample preparation costs. For large-scale application, using a 96 multichannel pipetting robot for aspirating the supernatant may be convenient and beneficial to the observed precision of LMWM profiles, as it minimizes the risk of disturbing the sorbent pellet after centrifugation by ensuring a steady draw rate from a constant depth across the plate.

The dSPE approach enables the use of RPC methodology tailored for small molecule metabolites measurement and has both the advantages of being cheaper and more robust than conventional SPE and LLE methodologies, making it a highly suitable way to cover a wider space of the blood metabolome.

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