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The dispersive micro-solid phase extraction method for MS-based lipidomics of human breast milk

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ABSTRACT

A simple and rapid microextraction method ensuring high lipidome coverage was developed for liquid chromatography mass spectrometry (LC-MS)-based lipidomics of human breast milk. The dispersive microsolid phase extraction (D-μ-SPE) technique, coupled with the design of experiment (DoE) method, enabled the study of the influence of several conditions (desorption solvent, stationary phase ratio, and sorption and desorption time) on the lipid extraction process of various lipid classes. The D-μ-SPE-based method, which used a mixture of C18 and zirconia-coated silica gel as the sorbent, allowed for the extraction of a wide range lipid classes characterized by different concentration levels. The developed method simplified the extraction procedure for lipidomics without loss of good reproducibility (70% of the MFs had peak volume%RSD $<20\%$ for all the tested stationary phases). The highest lipidome coverage was achieved when 100 μL of the human breast milk (HBM) sample was extracted using 27 mg of C18 mixed with 3 mg of zirconia-coated silica gel as the sorbent and methanol:2-propanol: ammonium hydroxide (*14:81:5 v/v/v*) mixture as the desorption solvent. The sorption and desorption time did not influence the number of extracted molecular features. The advantages of the present method over the traditional SPE and liquid-liquid extraction (LLE) commonly used in lipidomics are the possibility of mixing sorbents with various sorption mechanisms, which ensures high lipidome coverage, and the use of a small number of materials, including the sorbent and organic solvent.

1. Introduction

Lipidomics is the branch of "omics" science that is the study of the biochemical and molecular characterization of lipids present in a given biological system and the lipid changes that are induced by various factors [1]. Lipids consist of several structurally and functionally diverse molecular species that cover a broad range of polarity, from nonpolar (e.g., glycerolipids) to polar (e.g., glycerophospholipids), and are present in biological samples in significantly varying levels, from the femtomole level to the micromole level. The differences in the lipid structures and concentration levels pose considerable challenges to complete and efficient lipidome extraction. Careful sample preparation in lipidomics helps to isolate the analytes of interest to simultaneously achieve high lipidome coverage and avoid signal suppression. In addition, the sample preparation method for lipidomics should be reproducible, robust, and rapid and should enable the extraction of a wide range of analytes of markedly varying polarity, molecular weight, and concentration levels. Additionally, the possibility of the automation of the sample preparation step is beneficial, due to the high-throughput character of lipidomic studies.

The most commonly used techniques for biofluid sample processing for MS-based global lipidomics analysis include single organic solvent extraction (SOSE) [2], two-phase liquid-liquid extraction (LLE) [3,4], SPE [5] and solid-phase microextraction (SPME) [6]. Other techniques, such as ultrasonic-assisted extraction (UAE) [7], microwave-assisted extraction (MAE) [8], and supercritical fluid extraction (SFC) [9], are used much less frequently. Lipids can be efficiently extracted with a superabsorbent polymer (SAP)-integrated microfluidic lipid extraction platform [10]. However, the complex nature of biological samples sometimes requires a combination of two or more different extraction techniques [11,12]. Although LLE and SPE have been broadly applied to extract lipids from biological samples, they suffer certain drawbacks, including the need for time-consuming procedures and the use of considerable amounts of potentially toxic organic solvents.

The dispersive microsolid phase extraction (D-μ-SPE) technique, a modified version of dispersive solid-phase extraction (DSPE) has been

In Memoriam: We would like to honor the memory of Prof. Jacek Namieśnik, who has sadly passed away. We salute you for your tenacity of purpose and outstanding leadership qualities.

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recently developed and applied to extract and enrich compounds such as quinolones [13], tetracyclines [14], and polycyclic aromatic hydrocarbons (PAHs) [15]. The extraction process includes i) trapping the analytes in the sorbent dispersed directly in the sample, ii) isolation of the sorbent by centrifugation or filtration and iii) elution/desorption of the analytes by an appropriate desorption solvent. The major advantages of D-μ-SPE in comparison to conventional SPE are less solvent consumption, simple and short procedures, effective cleanup, and the possibility of mixing the sorbents. Thus, the D-μ-SPE technique can be considered as an alternative sample preparation method for MS-based lipidomic analysis. In this work, we present for the first time a new extraction method for lipidomics based on this technique. We implemented the design of experiment (DoE) approach to study the influence of the conditions of the extraction process in terms of the lipidome coverage and repeatability. The studied factors that influenced the extraction were the desorption solvent, stationary phase ratio, and the sorption and desorption time. The main intent of using DoE in this work was to simultaneously examine the various factors affecting the lipid extraction efficiency. Human breast milk (HBM) was used as a model biological matrix containing a wide range of biomolecules, including lipids with considerable differences in abundance and chemical structure as well as interfering substances such as proteins and carbohydrates.

2. Material and methods

2.1. Reagents and chemicals

LC-MS grade methanol and HPLC grade hexane were purchased from Merck (Darmstadt, Germany), and LC-MS grade 2–propanol, ammonium formate (99.9% purity), ammonium hydroxide solution (28.0–30.0%) and formic acid (≥98% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was purified by an HLP5 system (Hydrolab, Wiślina, Poland). The lipid standards 1,2,3-tripentadecanoyl-glycerol, 1,2-dipalmitoyl-glycerol, and 1,2-distearoyl-glycero-3-phosphocholine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample and sample treatment

The pooled HBM sample, which was prepared by mixing 150 μL of previously collected ($n = 71$) human breast milk samples [11], was used for the lipid extraction method optimization using DoE. The HBM samples were obtained from healthy volunteer mothers ($n = 6$) of healthy term infants. The HBM samples were collected for 5–7 days (twice a day, at the same time in the evening and morning) after full expression from one breast using a milk pump while the baby was fed on the other breast. Approximately 10 mL aliquots were transferred to polypropylene tubes and stored at −20 °C before transport to the laboratory for storage at −80 °C. The sample preparation involved extraction of the lipids contained in the HBM using the D-μ-SPE technique. For the protein precipitation, 100 μL of the HBM sample was transferred to a polypropylene tube (15 mL, VWR International, Gdansk, Poland) and mixed with 900 μL of 1% formic acid in methanol for the 30 s using a vortex mixer. Then, the sample was centrifuged for 5 min at 10,000 × g, and 900 μL of the supernatant was transferred to a 1.7 mL centrifuge microtube (VWR International, Gdansk, Poland) containing the stationary phase (30 mg). For further extraction, the mixture was stirred using a vortex mixer for a given time (*sorption time)*. After the stationary phase sedimented at the bottom of the tube, the supernatant was carefully discarded using a glass Pasteur pipette (150 mm, VWR International, Gdansk, Poland). Then, 1000 μL of desorption solvent was added to the stationary phase, and the system was mixed using a vortex mixer for a given time (*desorption time*). After desorption, the extract was carefully collected using a syringe with a needle (1 mL, Terumo, Laguna Technopark, Binan, Laguna, Philippines), filtrated with a 0.2 μm syringe filter (Puradisk, GE Healthcare UK limited, Amersham Place, UK) and transferred to a chromatographic vial (2.0 mL, 9 mm short-cap, screw-thread vials with PTFE/silicone screw-vial closures, Restek Corporation, U.S., 110 Benner Circle, Bellefonte, PA 16,823). One extraction blank (no matrix) and two test samples were prepared for each studied condition.

2.3. Instrumentation

The HPLC system used was an Agilent 1290 LC system equipped with a binary pump, an online degasser, an autosampler and thermostated column compartment coupled to a 6540 Q-TOF-MS with a dual electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out by using an Agilent Poroshell 120 EC−C8, (150 mm × 2.1 mm I.D., 1.9 μm particle size) column equipped with 0.2 μm in-line filter. The elution program was generated with a mixture of 5 mM ammonium formate in water and methanol (1/4, v/v) (component A) and a mixture of 5 mM ammonium formate in water, n-hexane and 2-propanol (1/20/79, v/v/v) (component B) as follows: 0–15 min, B (%) 10–50 (linear increase); 15–20 min, B (%) 50–100 (linear increase). Subsequently, the column was washed for 0.5 min at 100% B and the gradient returned to starting conditions and the system was re-equilibrated for 10 min. The flow rate was 0.5 mL min^{-1} and the injection volume was 0.5 μL. The column was kept at a constant temperature of 45 °C. Data were acquired in ESI + (SCAN) mode in the range from 200–1700 m/z in the high-resolution mode (4 GHz). The ESI source condition applied was optimized earlier and described in detail elsewhere [14].

2.4. Data treatment

The data preprocessing was done using MassHunter Workstation Software Qualitative Analysis, version B 14.9.1 (Agilent technologies, Santa Clara, CA, USA). The parameters for the molecular feature extraction (MFE) were as follows: extraction algorithm, small molecule; input data range, restricted retention time 0.90 − 15.00 min, restricted m/z 200 − 1700 m/z; peak filters; peak with height ≥ 1000, ion species, + H, -H; peak spacing tolerance 0.0025 m/z plus 7.0 ppm; isotope model, common organic molecules charge state, 2. The background noise limit was set to 1000 counts. The molecular feature (MF) alignments were carried using Mass Profiler Professional (Agilent technologies, Santa Clara, CA, USA), and the MFs were filteredusing Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) based on frequency; only the MFs present in both test samples were included for further analysis. The MFs in the extraction blank with the peak volume values greater than 5% of the mean value in the samples were discarded. The multivariate data analysis was done using the Sirius software package from Pattern Recognition Systems (PRS AS, Bergen, Norway).

2.5. Lipid identification

The compounds were tentatively identified by comparing the accurate mass of the obtained MF against the database of HBM lipids. The mass error was set to 7 ppm. The database was created as previously described, based on the theoretical fatty acyl substituents potentially present in HBM [16]. The database contained lipid groups with unique chemical formulas and unique exact masses that were not distinguished by stereochemistry, unsaturated bond position, or the position and length of the fatty acyl chains. Lipid groups with theoretically possible