Microchemical Journal 152 (2020) 104269

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

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

Inal Bakhytkyzy, Weronika Hewelt-Belka*, Agata Kot-Wasik

Department of Analytical Chemistry, Chemical Faculty, Gdansk University of Technology, ul. Gabriela Narutowicza 11/12, Gdansk, 80-233, Poland

ARTICLE INFO

Keywords:
Dispersive microsolid phase extraction
Experimental design
Lipid extraction optimization
Lipidomics

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.

* Corresponding author.
E-mail address: weronika.belka@pg.edu.pl (W. Hewelt-Belka).

https://doi.org/10.1016/j.microc.2019.104269
Received 25 July 2019; Received in revised form 13 September 2019; Accepted 16 September 2019
Available online 14 October 2019

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

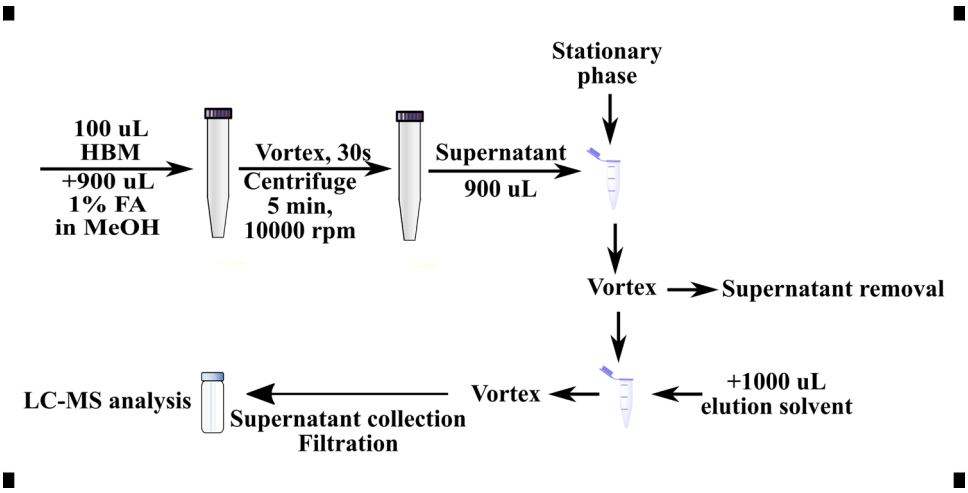

Fig. 1. The procedure of lipid extraction from HBM based on the D-μ-SPE method.

Table 1
Extraction conditions for preliminary studies and comparison of the number of total MF and compounds with saturated MS signals using different stationary phases.

Stationary phase	Desorption solvent	Sorption vortex time	Desorption vortex time	Total number of MFs	% of saturated MF
C18	2-propanol, *1000μL*	1 min	1 min	710	10%
HybridSPE-Phospholipid	methanol:NH_3, 95:5 *(v/v)*			291	4%
Mix, C18: HybridSPE, 1:9 (*w/w*)	methanol:NH_3, 95:5 *(v/v)*			435	9%

fatty acyl combinations and a number of unsaturated bonds were added to the database, based on the available literature [17–23]. The molecular formulas and the exact masses of the compounds were calculated using Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). The generated database contained 6 lipid classes of glycerophospholipids, 3 lipid classes of glycerolipids, and 9 lipid classes of sphingolipids, giving 3542 lipid groups (database hits). The database did not contain the fatty acyls category, as we did not focus on this category of lipids.

3. Results and discussion

Lipid extraction in lipidomics is a highly laborious, time-consuming process, which utilizes highly hazardous organic solvents. However, due to the diverse physical and chemical properties of different lipid classes found in biological systems, it is still a challenge to extract the complete lipidome in one extraction step. Human breast milk is an example of a complex biological matrix that contains lipids with substantially diverse physicochemical properties and concentrations, as it consists of approximately 98–99% triacylglycerols and 0.2–1% phospholipids, including glycerophospholipids (GPs) and sphingolipids (SPs) [24]. Therefore, it is essential to develop a lipid extraction method that can extract the maximum possible number of both nonpolar and polar lipid classes in one step, with appropriate concentrations of both, so that one class does not saturate the MS signal, allowing the second class to be detected as well. Various sample preparation techniques were applied for the extraction of lipids for the MS-based lipidomic study of human milk samples, mainly liquid-liquid extraction [12], single phase liquid extraction [2], solid phase extraction [22] and solid phase microextraction [25]. Liquid-liquid extraction is the gold standard for sample preparation for lipidomic studies. Although this method enables highly repeatable extraction of a wide range lipid classes, it does not provide extraction of low abundant phospholipids from human milk samples. Liquid-liquid extraction is effective only for the extraction of highly abundant glycerolipids. This limitation is an issue especially for samples collected in the later period of lactation, as phospholipid content decreases with the stage of lactation. This limitation also applies to single phase extraction and SPME with the use of C18 as a stationary phase. Solid phase extraction with a selective sorbent that enables enrichment of the phospholipids, e.g., a silica-based stationary phase modified with zirconia atoms, allows the extraction of low abundant phospholipids; however, it results in the removal of unretained lipids without a phosphate moiety, such as glycerolipids. This situation limits the employment of commonly used sample preparation techniques in lipidomics for the lipid extraction from human milk samples to be used for nontargeted MS-based lipidomic analysis. Recently, we developed an analytical approach that overcomes these issues [11]. Although this procedure enables obtaining extracts covering a wide range of lipids, including low and high abundant species, and it is suitable for detection of all HBM lipids in one analytical run, it also has some limitations. Generally, it is highly laborious and time-consuming. Thus, the applicability of D-μ-SPE for the MS-based lipidomic study of HBM samples has been studied, as it enables the mixing of the stationary phases with different sorption mechanisms to expand the lipidome coverage and simplify the extraction process. The developed method consists of 3 main steps, including protein precipitation, sorption, and desorption of the analytes (Fig. 1).

Preliminary studies were performed to evaluate the performance of this method. Two stationary phases were chosen, C18 reversed-phase stationary phase and zirconia-coated silica (HybridSPE-Phospholipid) stationary phase, which selectively retains phospholipids [26]. The applied conditions are described in Table 1.

First, the lipidome coverages obtained with the use of both stationary phases separately and their mixture in a ratio of 1:9 C18 to zirconia stationary phase were compared. The lipidome coverage of

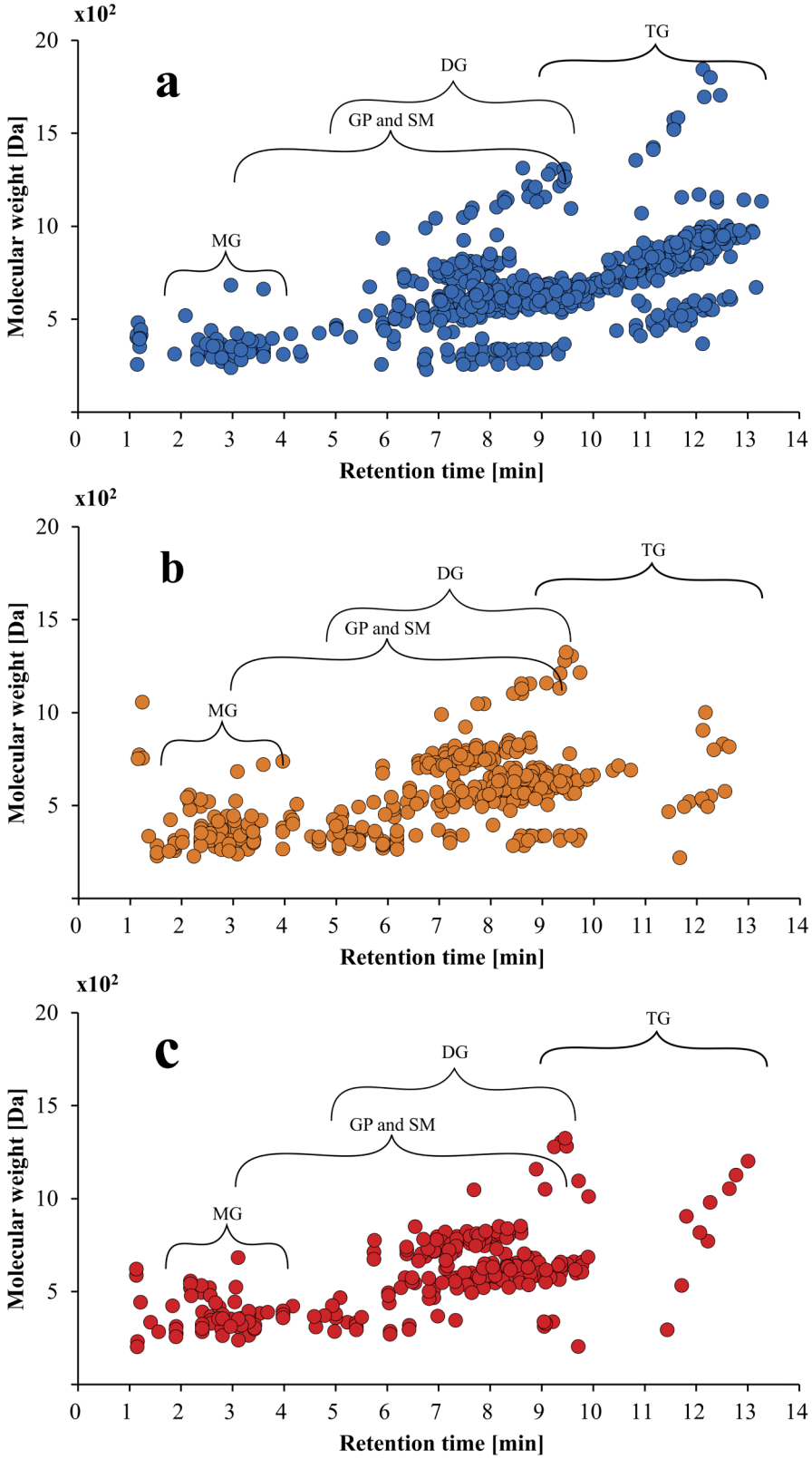

Fig. 2. Comparison of the lipidome coverage obtained by the D-μ-SPE method using different stationary phases: **(a)** C18, **(b)** mixed stationary phase with a ratio of 1:9 C18 to zirconia, and **(c)** zirconia.

each extract is presented in Fig. 2. The lipidome coverage graph presents the distributions of different MFs on the chromatogram. Each circle represents a different MF.

As expected, the results show that the type of sorbent used influences the obtained lipidome coverage, the total number of detected MFs and their MS signal intensity.

The use of C18 stationary phase resulted in an increased number of peaks and increases in their intensities (exceeding the limit of MS

Table 2
Experimental variables and levels in the 2^4 full factorial screening design for lipid extraction from HBM.

Variable	Coded	Level Low (-)	High (+)
Desorption solvent, methanol:propanol-2:NH_3	X_1	81:14:5 *(v/v/v)*	14:81:5 *(v/v/v)*
Stationary phase ratio, C18:HybridSPE	X_2	1:9 (w/w)	9:1 (w/w)
Sorption time	X_3	1 min	5 min
Desorption time	X_4	1 min	5 min

saturation level) mainly in the region of TG and DG elution, while the use of zirconia-coated silica resulted in a considerable decrease in the number of detected peaks and decreases in their intensities in this region. The total number of detected MFs was the highest for the C18 stationary phase (710) and the lowest for the zirconia-coated silica (291). This phenomenon is easily explained, as C18 is a good sorbent for hydrophobic substances such as glycerolipids and improves their extraction. In addition, 2-propanol facilitates the desorption of glycerolipids from C18 sorbent more than from methanol because of lower polarity. On the other hand, zirconia-coated silica particles only interact with lipids containing a phosphate moiety; thus, a higher number of peaks near the phospholipid (GP and SP) elution was observed. Only a small portion of the glycerolipids were detected, probably as a result of incomplete removal of the supernatant after the sorption step. The combination of two stationary phases led to a decrease of up to 435 in the number of total MFs, but, as the tentative identification showed, it still provided high lipidome coverage for both the phospholipids (GP and SM) and glycerolipids. However, 9% of the MFs were still above the MS signal saturation level, which resulted in the loss of the signal-to-concentration relationship for those MFs.

3.1. Experimental design and multivariate data analysis

Four variables affecting the extraction efficiency were selected to define the experimental domain. These variables were as follows:

1 desorption solvent,
2 stationary phase ratio (HybridSPE-Phospholipid and C18 (both from Sigma-Aldrich, St. Louis, MO, USA)),
3 sorption time, and
4 desorption time.

Four variables at 2 levels gave a 2^4 full factorial design. The total number of experiments, including replicates and one extraction blank sample for each set, was 48. The variables considered, the code used, and the low and high levels studied are shown in Table 2.

3.2. Extraction yield

First, the results obtained for different extraction conditions were compared based upon several responses: the number of total and identified MFs, total MS signal intensities of the identified and total MFs, MS signal saturation levels of the total and identified MFs (Fig. 3), number of tentatively identified GLs, GPs and SPs and peak volumes of the tentatively identified individual lipids from each class (Fig. 4). The extracted compound chromatograms of the HBM lipidome for each experimental run are presented in Figure S1 in the Supplementary Materials.

The bar graph of the total and tentatively identified number of MFs in each run (Fig. 3a) shows that the number of total and identified MFs followed a similar trend, showing that a large number of MFs were extracted in experimental runs 4, 8, 12 and 16. As shown in Table 3, all these experimental runs shared high levels in the desorption solvent (methanol:propanol-2:NH3, 14:81:5 (v/v/v)) and stationary phase (Zr:C18, 1/9, (w/w)), differing only in the sorption and desorption times. The mixing times using the vortex mixer did not influence the number of extracted MFs. The combination of a higher ratio of C18 stationary phase with a lower polarity desorption solvent positively influenced the number of extracted MFs. This trend followed that observed in the preliminary study, and the high number of MFs could be attributed to the glycerolipids. However, to confirm the correctness of the above assumption, multivariate data analysis was performed. The significance of the variables and their interactions was evaluated utilizing Lenth's method using the Sirius software package from Pattern Recognition Systems (PRS AS, Bergen, Norway). The bar graphs of the regression coefficient of each variable and their interactions are shown in Fig. 3(b). The horizontal lines in the plot represent the margin of error (ME), and they define the limit of significance. Any effects below these lines are not significant. In addition to the information about the significance, the sign of the regression coefficient of a factor gives additional useful information: a positive sign indicates that a high value for this factor is favorable for the design, and a negative sign indicates the opposite.

The regression coefficients of the total and identified MFs followed a similar trend and mainly had positive signs. According to Lenth's method, the most significant factors were the desorption solvent (X1) and stationary phase ratio (X2). The positive effects favoring high levels (more 2-propanol and more C18) were favorable for extracting more MFs, which confirmed the previous assumption. The third largest effect was the interaction of the desorption solvent and stationary phase ratio (1×2). When an interaction effect is positive and the two variables in question are at the same level, an overall positive effect on the response is observed. Therefore, when more C18 stationary phase is used, more 2-propanol should be used. C18 is the hydrophobic stationary phase and retains hydrophobic glycerolipids strongly, and 2-propanol is a more suitable solvent in comparison to methanol in this case.

Fig. 3(c and d) shows the bar graph of the MS total signal of the identified MFs and all the MFs. The trend observed in this graph is similar to the one presented in Fig. 3(a). Although a high total MS signal is preferred, it does not always represent a large number of MFs. When the number of MFs is low and the total MS signal is high, the saturation of the MS signal is most likely to occur. Thus, care should be taken to consider all types of responses and to compromise between efficiency and the quality of the result when developing a new extraction method.

The percent of saturation of the total MFs and triacylglycerols (TG) in each experiment is shown in Fig. 3(e and f). For both the total MFs and triacylglycerols (TG), a higher MS signal saturation was observed in the experimental setups N 2, 4, 6, 8, 10, 12, 14 and 16, in which a high level of desorption solvent (methanol:propanol-2:NH_3, 14:81:5 *(v/v/v)*) was used. This phenomenon can be ascribed to a high abundance of glycerolipids in the HBM and their affinity to a solvent with low polarity. However, false results can be caused by saturation of the MS signal, and it should be avoided if possible.

Although a higher MS signal saturation was observed in similar experiments for total MFs and TGs, the regression coefficient suggests that a higher MS signal saturation of total MFs was achieved when high levels of desorption solvent (X1), stationary phase ratio (X2) and their interaction (X1*X2) were used. Whereas the MS signal saturation of the TGs differed according to the ratio of the stationary phase, the MS signals of the TGs were more saturated when more zirconia stationary phase was used. This phenomenon may have been a result of the limited data available on lipids in databases, as according to the bar graph, experimental setups with more C18 stationary phase had more MS signal saturation.

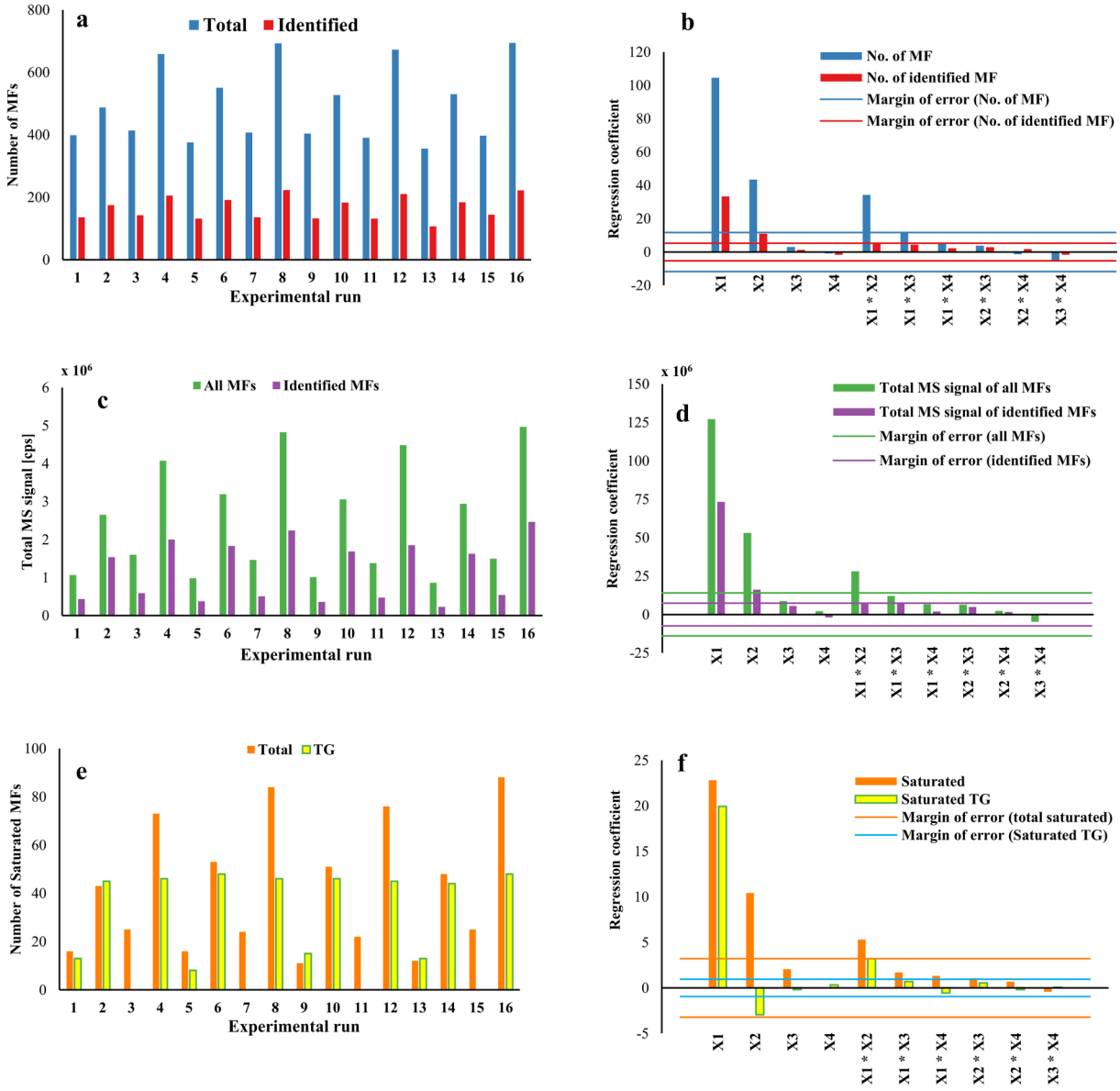

Fig. 3. Comparison of the number of extracted total (identified and nonidentified) and identified MFs: (a) bar graph of the extracted molecular features in each run, (b) regression coefficients of the variables and comparison of the MS total signal of all the MFs and the identified MFs; (c) bar graph of the total MS signal in each run; (d) regression coefficients of the variables and comparison of the MS saturation levels of the total MFs and triacylglycerols; (e) bar graph of the MS signal saturation level in each run; and (f) regression coefficients of the variables.

Selecting an appropriate response is crucial when designing an experiment, because, depending on the scope of the study, various responses can be used in the design. The number of MFs is a commonly used response for the optimization of extraction in lipidomics. However, since the newly developed lipid extraction method was assumed to concurrently extract both polar and nonpolar lipids from HBM, the number of extracted glycerolipids and phospholipids (GP and SP) and the extractability of different lipid classes were used as a response for the experimental design.

The number of identified GLs varied between 100 and 175 units (Fig. 4(a)), following the trend observed in Fig. 3(a). This result was confirmed by the regression coefficients (Fig. 4(b)), showing that more GLs were extracted when high levels of elution solvent and stationary phase ratios were used. Regarding the GPs and SPs, the identified numbers of MFs varied between 25 and 50, which was expected, due to the matrix used for the method development. The regression coefficients of the model suggested that the stationary phase ratio had a positive effect on the number of extracted phospholipids, meaning that more GPs and SPs were extracted when more C18 stationary phase was used. Although phospholipids are considered comparatively polar compounds among other lipids, they still consist of long hydrophobic fatty acid chains, which makes them hydrophobic. This property could justify their affinity to the C18 stationary phase.

The behavior of individual lipid classes during different extraction conditions was assessed. The peak volumes of three tentatively identified representatives from the TG, DG, MG, PC, PE, LysoPC and LysoPE lipid classes were compared. Different lipids from the same class showed similar trends in the extraction (Figure S2). The comparisons of

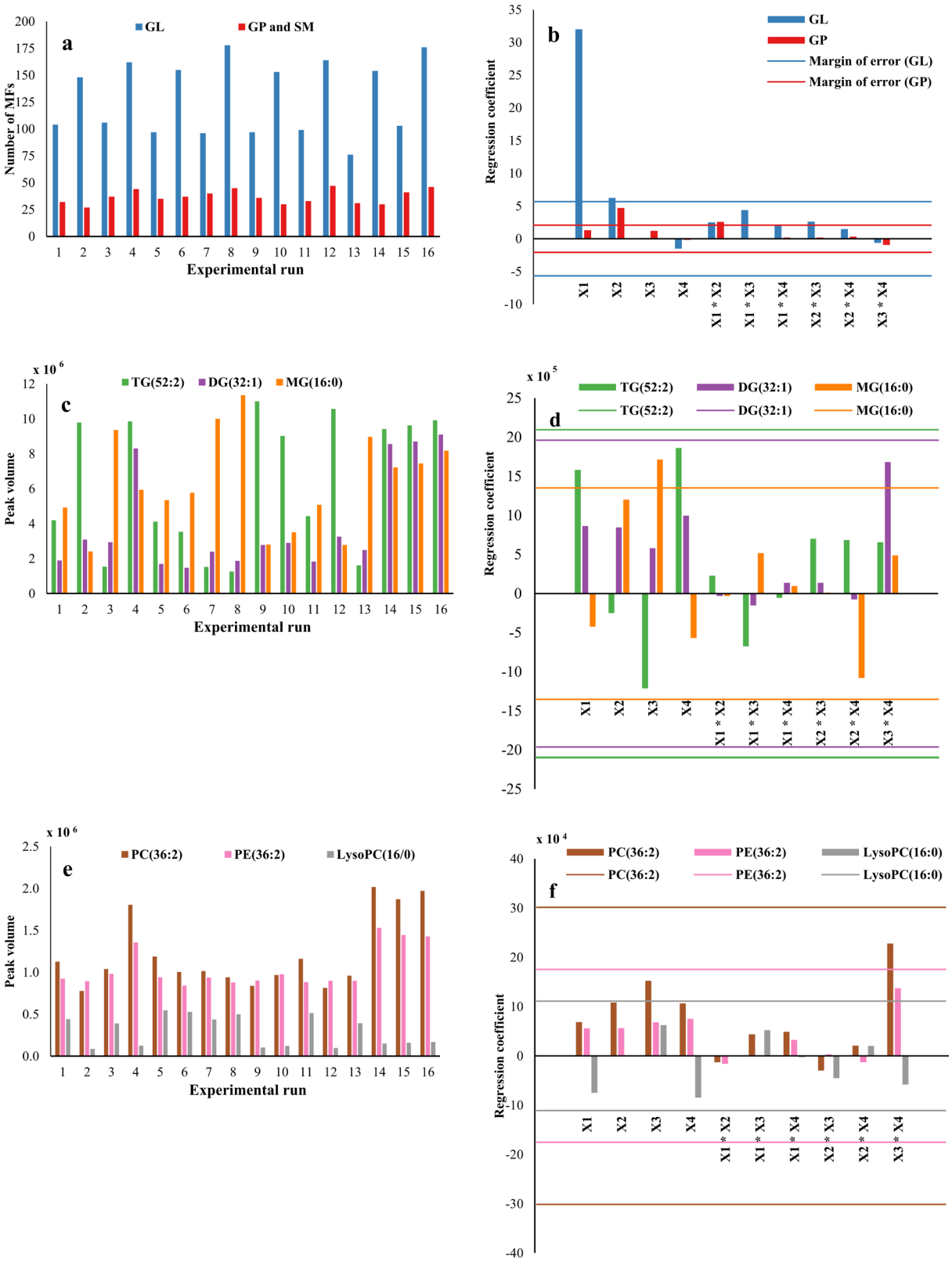

Fig. 4. Comparison of the number of extracted glycerolipids and phospholipids (GP and SP): (a) bar graph of the number of tentatively identified GLs and phospholipids in each experimental run; (b) regression coefficients of the variables and comparison of the extractability of individual GLs; (c) bar graph of the peak volume of tentatively identified TG(52:2), DG(32:1) and MG(16:0) in each experimental run; (d) regression coefficients of the variables and comparison of the extractability of individual GPs; (e) bar graph of the peak areas of tentatively identified PC(36:2), PE(36:2) and LysoPC(16:0) in each experimental run; and (f) regression coefficients of the variables.

Table 3
Design matrix in the 2^4 full factorial screening design for lipid extraction from HBM.

Variables	Experimental run															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
X1	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
X2	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+
X3	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+
X4	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+

Table 4
The%RSD for LC-MS and extraction triplicates of evaluated MFs peak volume extracted with the developed extraction procedure.

LC-MS %RSD	number MFs	%MFS	Extraction %RSD	number MFs	%MFS
<5%	123	34%	<5%	47	12%
5–10	99	27%	5–10	120	30%
10–20	73	20%	10–20	135	34%
20–30	16	4%	20–30	41	10%
> 30	55	15%	> 30	55	14%
total	366	100%	total	398	100%

the GLs and phospholipids (GPs and SPs) are presented in Figs. 4(c and d) and 4(e and f), respectively. The comparisons of the peak volumes of the individual lipids show that the lipids were extracted differently and that there was no clear trend, unlike that shown in Fig. 3(a). The comparisons of the different experimental conditions and their interpretation were clear and straightforward when the number of MFs was used as a response. However, in the case of the peak volumes of individual lipids, the conclusions were not so obvious. The regression coefficients of the variables showed that the MG, unlike the DG and TG, was better extracted when a higher polarity desorption solvent and shorter desorption time were applied. In addition, the TG was better extracted when the sorption time was shorter. The regression coefficients of the main variables for the PC and PE were positive, meaning that they were better extracted when more 2-propanol, more C18 stationary phase, and longer sorption and desorption times were applied. However, the LysoPC, like the MG, preferred a higher polarity desorption solvent and shorter desorption time. Both the MG and LysoPC had 16 carbon atoms in their hydrophobic tails, which could be the reason for their similar extraction behavior.

3.3. Analytical performance of the extraction method

The repeatability of the developed extraction method was assessed using the calculation of the MF volume percent relative standard deviation (%RSD) for triplicate extractions (Table S1). The number of MFs characterized with MF volume%RSD < 30 was higher than 86% of the total MFs; thus, the method met the criteria of acceptance for semi-quantitative lipidomic studies [27,28]. Detailed information about the evaluated MF volume%RSDs for the LC-MS and triplicate extractions are included in Table 4.

We carried out the analysis of a series of diluted, pooled HBM samples to explore the analytical performance of the proposed extraction protocol in terms of linearity. The lipid constituents in diluted samples (2, 5, 10 and 20 × fold) were extracted, and the obtained total lipid extracts were analyzed by LC-MS-Q-TOF. The results (Supplementary Materials, Figure S3) showed that the method was linear for the detected lipids in the tested range (R^2 higher than 0.99). Additionally, three commercially available lipid standards, 1,2,3-tripentadecanoyl-glycerol (TG (45:0)), 1,2-dipalmitoyl-glycerol (DG(32:0)) and 1,2-distearoyl-glycero-3-phosphocholine (PC (36:0)), were used to assess the basic validation parameters. The linearity, accuracy, and precision were assessed during the validation. The linearity of the method was measured by the analysis of standard solutions with concentrations in the range 0.5–10.0 μg/mL. The calibration curves for each analyte were constructed. Within a day, the precision and accuracy were also calculated for a selected concentration. A summary of the validation parameters is presented in the Supplementary Material in Table 5. All the studied analytes exhibited a linear relationship between concentration and peak area in the tested concentration range. The precision and accuracy values were lower than 5% for all the compounds, which is consistent with typical requirements for bioanalytical assays.

4. Conclusion

We present an alternative lipid extraction method for LC-MS-based lipidomics of human breast milk samples. A simple and rapid microextraction method employed dispersive microsolid phase extraction (D-μ-SPE). Due to the possibility of mixing the stationary phases of different retention mechanisms, this method ensures high lipidome coverage for complex samples with a wide concentration range of various classes of lipids. The repeatability of the method allows for semi-quantitative studies. The need for only a small quantity of materials, including the sorbent and organic solvent used for the extraction, is an additional advantage of the D-μ-SPE method. The use of DoE enabled testing of the lipid behavior under different extraction conditions, including the desorption solvent and stationary phase ratio, which had a large influence on the lipid extraction yield. The applicability of the D-μ-SPE method for lipidomic studies was demonstrated on a human milk sample, which was characterized by great compositional diversity, including the chemical structure and concentration range of lipids. However, this method can be easily adapted for other biofluids, as it enables simple optimization of the extraction conditions, such as the sorbent type and ratio and the desorption solvent, to obtain higher lipidome coverage.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research received funding from the National Science Centre, Poland (2018/29/B/NZ7/02865).

Table 5
A summary of the validation parameters obtained for the lipid standards.

	1,2,3-tripentadecanoylglycerol, TG(45:0) R_t 29.04, $[M+NH_4]^+$ m/z = 782.7232	1,2-dipalmitoylglycerol, DG(32:0) R_t 12.75, $[M+NH_4]^+$ m/z = 586.5405	1,2-distearoyl-glycero-3-phosphocholine, PC(36:0) R_t 11.13, $[M+H]^+$ m/z = 790.6320
Linearity (R^2)	0.9901	0.9992	0.9914
Range	0.5–10 μg/mL	0.5–10 μg/mL	0.5–10 μg/mL
Precision, CV%	2.14	2.37	1.54
Accuracy, CV%	3.83	3.83	3.8

Author Contributions

W.H-B and I.B contributed equally to this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.microc.2019.104269.

References

[1] M.R. Wenk, Lipidomics: new tools and applications, Cell 143 (2010) 888–895, https://doi.org/10.1016/j.cell.2010.11.033.

[2] A. Villaseñor, I. Garcia-Perez, A. Garcia, J.M. Posma, M. Fernández-López, A.J. Nicholas, N. Modi, E. Holmes, C. Barbas, Breast milk metabolome characterization in a single-phase extraction, multiplatform analytical approach, Anal. Chem. 86 (2014) 8245–8252, https://doi.org/10.1021/ac501853d.

[3] J. Godzien, M. Ciborowski, E.G. Armitage, I. Jorge, E. Camafeita, E. Burillo, J.L. Martín-Ventura, F.J. Rupérez, J. Vázquez, C. Barbas, A single in-vial dual extraction strategy for the simultaneous lipidomics and proteomics analysis of HDL and LDL fractions, J. Proteome. Res. 15 (2016) 1762–1775, https://doi.org/10.1021/acs.jproteome.5b00898.

[4] K. Sandra, A. dos, S. Pereira, G. Vanhoenacker, F. David, P. Sandra, Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry, J. Chromatogr. A. 1217 (2010) 4087–4099, https://doi.org/10.1016/j.chroma.2010.02.039.

[5] M.A. López-Bascón, M. Calderón-Santiago, J. Sánchez-Ceinos, A. Fernández-Vega, R. Guzmán-Ruiz, J. López-Miranda, M.M. Malagon, F. Priego-Capote, Influence of sample preparation on lipidomics analysis of polar lipids in adipose tissue, Talanta 177 (2018) 86–93, https://doi.org/10.1016/j.talanta.2017.09.017.

[6] N. Reyes-Garcés, E. Gionfriddo, Recent developments and applications of solid phase microextraction as a sample preparation approach for mass-spectrometry-based metabolomics and lipidomics, TrAC Trends Anal. Chem. 113 (2019) 172–181, https://doi.org/10.1016/J.TRAC.2019.01.009.

[7] C. Pizarro, I. Arenzana-Rámila, N. Pérez-del-Notario, P. Pérez-Matute, J.-.M. González-Sáiz, Plasma lipidomic profiling method based on ultrasound extraction and liquid chromatography mass spectrometry, Anal. Chem. 85 (2013) 12085–12092, https://doi.org/10.1021/ac403181c.

[8] F. González-Illán, G. Ojeda-Torres, L.M. Díaz-Vázquez, O. Rosario, Detection of fatty acid ethyl esters in skin surface lipids as biomarkers of ethanol consumption in alcoholics, social drinkers, light drinkers, and teetotalers using a methodology based on microwave-assisted extraction followed by solid-phase microextraction and gas chromatography-mass spectrometry. J. Anal. Toxicol. 35 (2011) 232–237 http://www.ncbi.nlm.nih.gov/pubmed/21513617 accessed August 31, 2018.

[9] T. Uchikata, A. Matsubara, E. Fukusaki, T. Bamba, High-throughput phospholipid profiling system based on supercritical fluid extraction–supercritical fluid chromatography/mass spectrometry for dried plasma spot analysis, J. Chromatogr. A. 1250 (2012) 69–75, https://doi.org/10.1016/J.CHROMA.2012.06.031.

[10] G. Bang, Y.H. Kim, J. Yoon, Y.J. Yu, S. Chung, J.A. Kim, On-chip lipid extraction using superabsorbent polymers for mass spectrometry, Anal. Chem. 89 (2017) 13365–13373, https://doi.org/10.1021/acs.analchem.7b03547.

[11] W. Hewelt-Belka, D. Garwolińska, M. Belka, T. Bączek, J. Namieśnik, A. Kot-Wasik, A new dilution-enrichment sample preparation strategy for expanded metabolome monitoring of human breast milk that overcomes the simultaneous presence of low- and high-abundance lipid species, Food Chem. (2019), https://doi.org/10.1016/j.foodchem.2019.03.001.

[12] N.J. Andreas, M.J. Hyde, M. Gomez-Romero, M.A. Lopez-Gonzalvez, A. Villaseñor, A. Wijeyesekera, C. Barbas, N. Modi, E. Holmes, I. Garcia-Perez, Multiplatform characterization of dynamic changes in breast milk during lactation, Electrophoresis 36 (2015) 2269–2285, https://doi.org/10.1002/elps.201500011.

[13] W.-H. Tsai, H.-Y. Chuang, H.-H. Chen, J.-J. Huang, H.-C. Chen, S.-H. Cheng, T.-C. Huang, Application of dispersive liquid–liquid microextraction and dispersive micro-solid-phase extraction for the determination of quinolones in swine muscle by high-performance liquid chromatography with diode-array detection, Anal. Chim. Acta. 656 (2009) 56–62, https://doi.org/10.1016/j.aca.2009.10.008.

[14] W.-H. Tsai, T.-C. Huang, J.-J. Huang, Y.-H. Hsue, H.-Y. Chuang, Dispersive solid-phase microextraction method for sample extraction in the analysis of four tetracyclines in water and milk samples by high-performance liquid chromatography with diode-array detection, J. Chromatogr. A. 1216 (2009) 2263–2269, https://doi.org/10.1016/j.chroma.2009.01.034.

[15] E.M. Reyes-Gallardo, R. Lucena, S. Cárdenas, M. Valcárcel, Magnetic nanoparticles-nylon 6 composite for the dispersive micro solid phase extraction of selected polycyclic aromatic hydrocarbons from water samples, J. Chromatogr. A. 1345 (2014) 43–49, https://doi.org/10.1016/J.CHROMA.2014.04.033.

[16] W. Hewelt-Belka, J. Nakonieczna, M. Belka, T. Baczek, J. Namieśnik, A. Kot-Wasik, Comprehensive methodology for Staphylococcus aureus lipidomics by liquid chromatography and quadrupole time-of-flight mass spectrometry, J. Chromatogr. A. (2014) 1362, https://doi.org/10.1016/j.chroma.2014.08.020.

[17] A. López-López, M. López-Sabater, C. Campoy-Folgoso, M. Rivero-Urgell, A. Castellote-Bargalló, Fatty acid and sn-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas, Eur. J. Clin. Nutr. 56 (2002) 1242–1254, https://doi.org/10.1038/sj.ejcn.1601470.

[18] N. Blaas, C. Schüürmann, N. Bartke, B. Stahl, H.-.U. Humpf, Structural profiling and quantification of sphingomyelin in human breast milk by HPLC-MS/MS, J. Agric. Food Chem. 59 (2011) 6018–6024, https://doi.org/10.1021/jf200943n.

[19] M.A. Masood, C. Yuan, J.K. Acharya, T.D. Veenstra, J. Blonder, Quantitation of ceramide phosphorylethanolamines containing saturated and unsaturated sphingoid base cores, Anal. Biochem. 400 (2010) 259–269, https://doi.org/10.1016/j.ab.2010.01.033.

[20] X.L. Pan, T. Izumi, Variation of the ganglioside compositions of human milk, cow's milk and infant formulas, Early Hum. Dev. 57 (2000) 25–31, https://doi.org/10.1016/S0378-3782(99)00051-1.

[21] L. Ma, A.K.H. MacGibbon, H.J.B. Jan Mohamed, S. Loy, A. Rowan, P. McJarrow, B.Y. Fong, Determination of ganglioside concentrations in breast milk and serum from Malaysian mothers using a high performance liquid chromatography-mass spectrometry-multiple reaction monitoring method, Int. Dairy J. 49 (2017) 62–71, https://doi.org/10.1016/j.idairyj.2015.05.006.

[22] C. Jiang, B. Ma, S. Song, O.-M. Lai, L.-Z. Cheong, Fingerprinting of phospholipid molecular species from human milk and infant formula using HILIC-ESI-IT-TOF-MS and discriminatory analysis by principal component analysis, J. Agric. Food Chem. 66 (2018) 7131–7138, https://doi.org/10.1021/acs.jafc.8b01393.

[23] J. Jiang, K. Wu, Z. Yu, Y. Ren, Y. Zhao, Y. Jiang, X. Xu, W. Li, Y. Jin, J. Yuan, D. Li, Changes in fatty acid composition of human milk over lactation stages and relationship with dietary intake in Chinese women, Food Funct. 7 (2016) 3154–3162, https://doi.org/10.1039/c6fo00304d.

[24] B. Koletzko, M. Rodriguez-Palmero, H. Demmelmair, N. Fidler, R. Jensen, T. Sauerwald, Physiological aspects of human milk lipids, Early Hum. Dev. 65 Suppl (2001) S3–S18, https://doi.org/10.1016/S0378-3782(01)00204-3.

[25] D. Garwolińska, W. Hewelt-Belka, J. Namieśnik, A. Kot-Wasik, Rapid characterization of the human breast milk lipidome using a solid-phase microextraction and liquid chromatography-mass spectrometry-based approach, J. Proteome. Res. 16 (2017) 3200–3208, https://doi.org/10.1021/acs.jproteome.7b00195.

[26] F. Wei, X. Wang, H. Ma, X. Lv, X. Dong, H. Chen, Rapid profiling and quantification of phospholipid molecular species in human plasma based on chemical derivatization coupled with electrospray ionization tandem mass spectrometry, Anal. Chim. Acta. 1024 (2018) 101–111, https://doi.org/10.1016/j.aca.2018.04.012.

[27] W.B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-Mcintyre, N. Anderson, M. Brown, J.D. Knowles, A. Halsall, J.N. Haselden, A.W. Nicholls, I.D. Wilson, D.B. Kell, R. Goodacre, Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry, Nat. Protoc. 6 (2011) 1060–1083, https://doi.org/10.1038/nprot.2011.335.

[28] D. Broadhurst, R. Goodacre, S.N. Reinke, J. Kuligowski, I.D. Wilson, M.R. Lewis, W.B. Dunn, Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies, Metabolomics 14 (2018) 72, https://doi.org/10.1007/s11306-018-1367-3.