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Rapid Characterization of the Human Breast Milk Lipidome Using a Solid-Phase Microextraction and Liquid Chromatography−Mass Spectrometry-Based Approach

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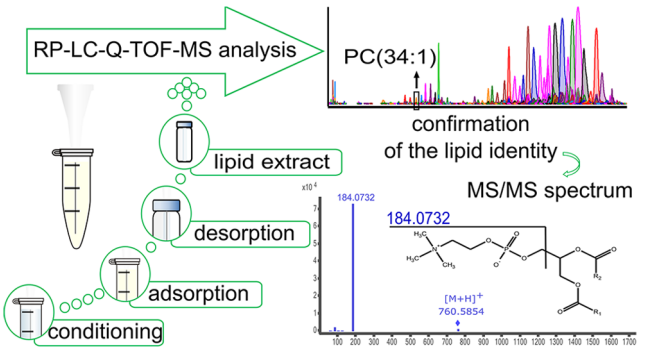
ABSTRACT: Human breast milk (HBM) is a biofluid consisting of various biomolecules such as proteins, lipids, carbohydrates, minerals and bioactive substances. Due to its unique and complex composition, HBM provides not only nutritional components required for the growth of the infant, but also additional protection against infections. Global insight into the composition of HBM is crucial to understanding the health benefits infants receive from breastfeeding and could be used to improve the composition of milk formula for babies that cannot be breastfed. To improve global profiling of the HBM lipidome, a new analytical approach based on solid-phase microextraction (SPME) and liquid chromatography−mass spectrometry (LC−MS) was developed. The new extraction method allows for the rapid and simple extraction of a broad range of lipids directly from HBM samples. Moreover, the optimized two-step lipid extraction protocol ensures high lipidome coverage without using toxic solvents such as chloroform. The use of liquid chromatography−quadrupole time-of-flight mass spectrometry (LC−Q-TOF-MS) and an automated search of a lipid database allows comprehensive identification of the lipids contained in HBM. The demonstrated analytical approach based on SPME sample preparation and LC−Q-TOF-MS is rapid, free of toxic solvents, and suited for the qualitative analysis of the HBM lipid composition.



KEYWORDS: *human breast milk, lipidomics, SPME, lipids*

1. INTRODUCTION

Increased interest in lipid analysis has driven the development of a new field of science—lipidomics.[1] Modern analytical tools enable the detection and determination of hundreds of lipid compounds in one analytical cycle. The extraction of lipids from biological material is the crucial step in lipidomics analysis. Sample preparation in lipidomics research is focused on the isolation and enrichment of lipids from biological matrices and the removal of interfering substances such as proteins, sugars and low molecular weight compounds. The most commonly used lipid extraction techniques in lipidomics are liquid−liquid extraction (LLE) and solid-phase extraction (SPE). Other techniques such as solid-phase microextraction (SPME),[2] ultrasonic-assisted extraction (UAE),[3] and dispersive liquid−liquid microextraction (DLLME)[4] are sporadically used.

The advantages of SPME have attracted much interest from chemists since the development of this extraction technique in the 1990s.[5−7] The use of SPME reduces the sample volume, total analysis time (by shortening the sample preparation step) and solvent usage (also eliminating toxic solvent use), thereby decreasing the costs of solvent purchasing and disposal. SPME can also improve detection limits. The devices used in SPME are convenient and simple to use and can be combined with other analytical techniques (e.g., gas chromatography (GC) and high-performance liquid chromatography (HPLC)). For example, SPME has been employed for the analysis of volatile compounds,[8,9] peptides,[10] and metabolites,[11] among others.[12]

Human breast milk (HBM) is a complex biofluid, of which the composition depends on many factors. HBM not only contains nutrients (lipids, proteins, carbohydrates and minerals) that are necessary for proper infant growth but also bioactive substances (hormones, antibodies and antimicrobial substances) that protect the infant against infection and inflammation and contribute to maturation of the immune system.[13,14] Due to the contents of these bioactive and nutritional components, HBM is considered the only food that can satisfy all nutritional requirements of an infant. Moreover, the composition of HBM varies from woman to woman and within an individual woman constantly during the lactation period.[15] Understanding the health benefits of breastfeeding to neonates is impossible without global insight

into HBM composition, and extensive knowledge of HBM contents is crucial to improving the composition of infant formula.

To date, several studies have investigated HBM, including determining substances that are essential for proper infant growth (such as antibodies, hormones and nutrients) as well as undesirable chemicals (such as parabens, phenols, flame retardants and chlorinated organic chemicals). For these purposes, GC, LC and mass spectrometry (MS) have been employed.[16−21] Research aimed at assessing the long-term changes in the biochemical composition of HBM has mainly involved determining only selected biomolecules (essentially proteins[22−24]) or the nutritional value (protein, fat or sugar concentrations).[25,26] While much research on the HBM composition has been performed, new components of mother's milk are constantly being identified. Despite the important role of HBM lipids in child development, the molecular composition of these compounds is not well-known.

According to the literature, few studies involving lipidomics analysis of HBM have been performed to date, and most have been targeted analyses, including determination of the contents of fatty acids or other specific lipid classes (such as phospholipids) in HBM using thin-layer chromatography (TLC), LC or GC.[27−32] To the best of our knowledge, the study by Villasenor et al.[33] is the only untargeted lipidomics analysis that has been carried out to date.

Herein, we present an analytical approach for the rapid, qualitative lipidomics analysis of HBM samples. The use of SPME and LC−MS allows for a fast and comprehensive characterization of the HBM lipidome. The demonstrated analytical approach is simple and almost free of organic solvents and utilizes convenient SPME sample preparation. A liquid chromatography-quadrupole time-of-flight mass spectrometry (LC−Q-TOF-MS)-based untargeted method was developed to separate and detect HBM lipids.

To the best of our knowledge, this is the first lipidomics analytical approach based on SPME sample preparation that enables global analysis of HBM lipids, including fatty acyls (FAs), glycerolipids (GLs), glycerophospholipids (GPs), sphingolipids (SPs), prenol lipids (PRs) and sterol lipids (STs). Using the developed analytical procedure, we characterized the HBM lipidome at the molecular level.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents

LC−MS-grade methanol and HPLC-grade chloroform and hexane were purchased from Merck (Darmstadt, Germany), and HPLC-grade 2-propanol and ammonium formate (99.9% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified on an HLP5 system (Hydrolab, Wiślina, Poland).

2.2. Sample Preparation

The sample preparation protocol was developed using HBM that was produced after one year postpartum by one woman. Samples were stored at −80 °C before analysis. Several lipid extraction conditions were examined to optimize the extraction protocol to ensure high lipidome coverage.

First, the SPME tip that consisted of a fiber coated with a silica-based sorbent modified with C18 groups (Supelco, Sigma-Aldrich, St. Louis, MO, USA) was preconditioned in a mixture of MeOH/H_2O (1/1, *v*/*v*) for a minimum of 20 min to solvate the stationary phase prior to use. Then, the SPME tip was immersed in 1 mL of HBM for lipid adsorption. Different adsorption times (5, 15, 30, 40, and 60 min) with and without shaking (vortex agitation at 267 rpm) were tested (Heidolph Promax 2020, Heidolph, Schwabach, Germany). After the adsorption step, the SPME tip was transferred to a LC vial containing a glass insert filled with 100 μL of 2-propanol for lipid desorption. Different desorption times (5, 15, 30, and 60 min) with 267 rpm vortex agitation were tested. After the desorption step, the SPME tip was removed. The obtained lipid extract was subsequently analyzed using LC−Q-TOF-MS.

2.3. Instrumental Conditions

The reversed-phase (RP)-LC−Q-TOF-MS analysis was performed on an Agilent 1290 LC system equipped with a binary pump, an online degasser, an autosampler and a thermostated column compartment coupled with a 6540 Q-TOF-MS with a dual electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, USA). Lipid compounds were chromatographically separated on an Agilent ZORBAX SB-C18 column (50 mm × 2.1 mm, 1.8 μm particle size) with a 0.2 μm in-line filter. The column and autosampler temperature throughout the analysis were maintained at 90 and 4 °C, respectively. The injection volume was 0.5 μL. The mobile phase consisted of 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, hexane and 2-propanol (1/20/79, *v*/*v*/*v*) (component B) and was pumped at 0.6 mL/min within a total run time of 81 min. The gradient elution program was initiated with 0% eluent B during the first 10 min and was then ramped from 0% to 30% B from 10 to 30 min, 30% to 40% B from 30 to 60 min and 40% to 100% B from 60 to 70 min. Then, after 1 min, the gradient was switched to 0% eluent B for 10 min of equilibration prior to the next injection.

The MS analyses were carried out with a capillary voltage of 3500 V and fragmentor voltage of 120 V for positive ionization mode, using the SCAN acquisition mode. The remaining MS parameters were set as follows: nebulizer gas pressure of 35 psi, drying gas flow rate of 10 L/min and temperature of 300 °C. Mass spectra were acquired from 200 to 1700 *m*/*z* in centroid and profile modes using the high-resolution mode (4 GHz). The TOF detector was calibrated daily prior to sample analysis to ensure constant mass correction of the data and accurate mass in each injection. During the experiment, the reference masses at *m*/*z* 121.0509, 149.0233, 922.0098, and 1221.9906 were continuously detected.

Control samples were used (deionized water instead of HBM) to monitor and exclude the introduction of artifacts into the analytical process from the chemicals used for extraction or in LC−MS analysis (compounds that did not originate from HBM). Preparation of the control samples was the same as for the studied samples

2.4. Data Analysis

Initial data processing was performed using MassHunter Workstation Software, Qualitative Analysis, version B.03.01 (Agilent Technologies, Santa Clara, CA, USA). Cleaning of background noise and obtaining the molecular entities from the resulting LC−MS data were performed using the molecular feature extraction (MFE) algorithm. The MFE parameters were as follows: extraction algorithm, small molecule; input data range, restricted retention time of 0.2−71 min and restricted *m*/*z* 200−1700; peak filters; peak high ≥2000; ion species, + H; peak spacing tolerance, 0.025 *m*/*z* plus 5.0 ppm; isotope

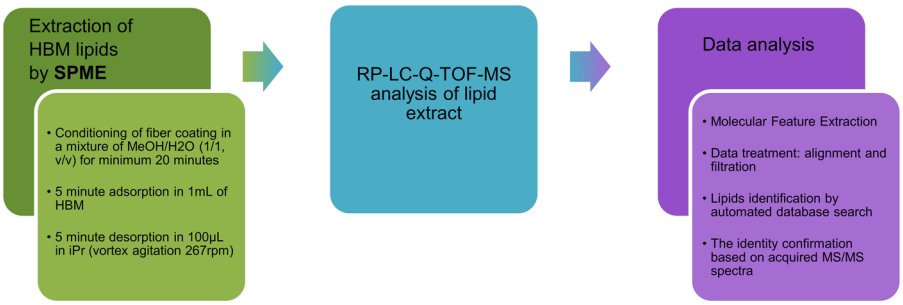


Figure 1. Simplified workflow of the developed analytical approach for the rapid characterization of the HBM lipidome.

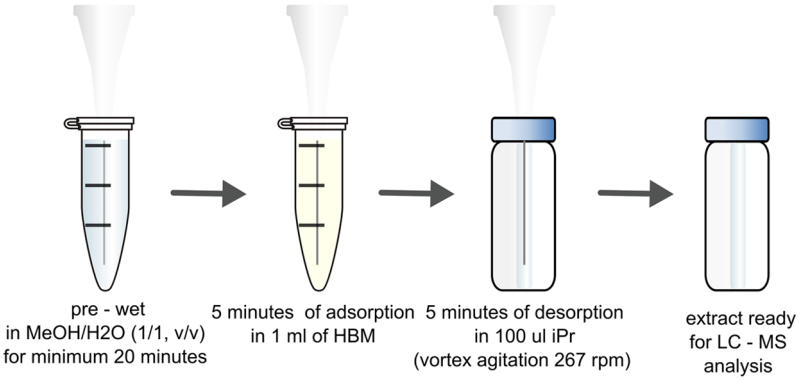


Figure 2. Optimized protocol for HBM lipid extraction.

model, common organic molecules; and charge state, 1−2. The MFE results consisted of a list including all molecular entities with the full mass spectral data (retention time, mass and volume).

Alignment and filtering of the primary data were performed with MassHunter Mass Profiler, version B.02.00 (Agilent Technologies, Santa Clara, CA, USA) and Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). The preanalysis parameters were as follows: number of ions, ≤ 2; charge state, any; and alignment, mass tolerance with an intercept of 2 mDa and a slope of 5 ppm and retention time tolerance with an intercept of 0.3 min and a slope of 0.5%. The resulting list of aligned molecular features (MFs) was exported to a.csv file, which was subjected to MF filtering in Microsoft Excel. Data were filtered by selecting only the MFs that were present in at least 5 from 6 samples. Further comparison between three groups of samples (HBM, cow milk and formula milk) were performed using Venn diagram (a web-tool for the generation for the Venn diagram, bioinformatics.psb.ugent.be).

2.5. Compound Identification

Tentative lipid identification was performed using MassHunter Qualitative Software by comparing the mass accuracy of potential lipids (obtained MFs) against the online database LIPID MAPS (www.lipidmaps.org). An automatic searching of the online database was performed with the following parameters: values to match, mass only; match tolerance, 5 ppm; charge carriers, H^+, Na^+, NH_4^+ and K^+; and charge state, 1−2. Potential hits were confirmed by LC−MS/MS analysis. Experiments were performed with the same chromatographic conditions as in the primary analysis. The MS analyses were carried using the auto MS/MS acquisition mode in the 30−1700 m/z mass range. The parameters of the ion source were identical to those used in the MS analysis. The collision energy was set at 35 V. The spectra of the two most abundant ions selected for fragmentation were excluded from MS/MS for 0.3 min. The obtained MS/MS spectra were compared with the fragmentation patterns of lipid compounds.

3. RESULTS AND DISCUSSION

During the development of the LC−MS method for the rapid characterization of the HBM lipidome, special attention was paid to the sample preparation step (SPME). The chromatographic separation and MS detection conditions were optimized simultaneously to achieve both high resolution and high MS signal intensity of HBM lipids. Important issues regarding the identification of lipid compounds are described below. The proposed workflow for the rapid lipidomics investigation of HBM, including all analytical steps, is shown in Figure 1, so that the proposed methodology may be easily followed.

3.1. Lipid Extraction Based on SPME

In traditional lipidomics analysis, sample preparation is based on LLE, which requires substantially large volumes of toxic organic solvents. In laboratory practice, the classic lipid extraction procedure is based on the use of a mixture of methanol and chloroform (2:1, v/v). This method was introduced by Folch et al.[34] and modified by Bligh and Dyer (addition of water and acetic acid to the extraction mixture).[35] This method enables the fast and effective isolation of polar and nonpolar lipids from biological matrices. Other solvents such as *tert*-butyl methyl ether (MTBE),[36] a mixture of butanol and methanol (BUME)[37] and a mixture of hexane and isopropanol (3:2, v/v)[38] have been used less commonly. However, these solvents are less toxic than chloroform, and their use in extraction procedures provides better recoveries compared with the Folch or Bligh and Dyer methods.

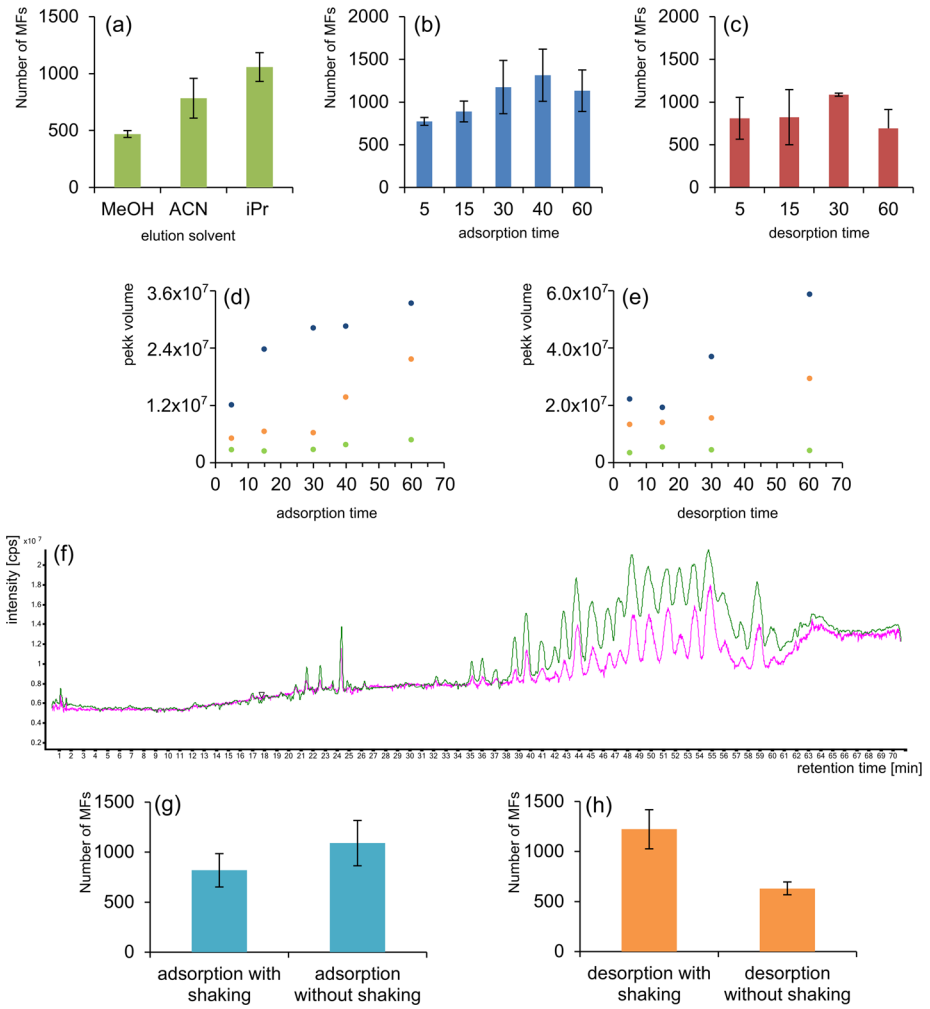


Figure 3. Comparison of desorption solvents (a), different adsorption times at a constant desorption time (30 min) (b), and different desorption times at a constant adsorption time (60 min) (c) considering the number of extracted MFs. Comparison of the peak volumes of three lipids differing in lipophilicity (glycerophosphatidylcholine (PC) (34:1) (green spots), diacylglycerol (DG) (34:4) (orange spots) and triacylglycerol (TG) (51:1) (blue spots)) at different adsorption times and a 30 min desorption time (d). Comparison of the peak volumes of PC (34:1) (green spots), DG (34:4) (orange spots) and TG (51:1) (blue spots) at a 60 min adsorption time and different desorption times (e). TIC obtained after 5 (pink line) and 60 (green line) minutes of adsorption with a 30 min desorption time in both cases (f). Comparison of the obtained number of extracted MFs with and without shaking during 5 min of adsorption (g) and 5 min of desorption (h).

Importantly, LLE enables the simultaneous extraction of many classes of lipids, so this type of sample preparation is particularly useful for nontargeted analysis. Differently, SPE, which has been used less frequently, enables the extraction of selected lipid classes and is thus primarily used in targeted analysis. Selecting the optimal sample preparation technique essentially depends on the chosen analytical strategy and research goal.

Increasing awareness of the risks associated with use of toxic solvents has led scientists to investigate new, practical, solventless sample preparation methods such as SPME, which was invented by Pawliszyn and Arthur[5] in 1989.

During method development, special attention was paid to the sample preparation step. We used SPME to extract HBM lipids instead of the commonly used LLE technique. The SPME technique was chosen because of its advantages over LLE. The SPME technique is described in the literature as a solvent-free sample preparation technique. Therefore, the utilization of costly high-purity solvents is minimized, and the method is simple to use and more efficient in terms of time and cost. Moreover, very small sample volumes are required for SPME analysis, which is important considering the often limited volumes of biological samples.[6,39] Here, we present the optimized method for lipid extraction from HBM samples using SPME. The proposed extraction parameters were selected to minimize the use of organic and toxic solvents, reduce sample manipulation and attain high lipidome coverage. A simplified diagram of the extraction protocol for the optimized method is shown in Figure 2.

Different lipid extraction conditions were tested including the desorption solvent, adsorption and desorption times and mixing conditions. Each experiment was performed in triplicate. An SPME coating with a silica-based sorbent modified with C18 groups was selected for lipid extraction. The obtained results were compared in terms of the number and intensity of extracted MFs and the degree of obtained lipidome coverage. LC−MS analysis of the obtained lipid extracts showed that isopropanol as a desorption solvent extracts a greater number of lipid species than methanol or acetonitrile (Figure 3a). Comparison of the number of extracted MFs at different adsorption (Figure 3b) and desorption (Figure 3c) times with shaking revealed that a 5 min adsorption time and 5 min

desorption time with shaking are sufficient to obtain a broad profile of HBM lipids. Although the number of MFs increased with longer adsorption times, 5 min of adsorption provided sufficient lipidome coverage. Moreover, comparison of the peak volume of three different lipids at different adsorption (Figure 3d) and desorption (Figure 3e) times and visual analysis of the TIC (Figure 3f) showed that the adsorption and desorption times impact the MS signal intensity of extracted lipids. To summarize, high lipidome coverage can be obtained after a few minutes but can be extended by increasing the adsorption time. However, the MS signal intensity also increased as the adsorption time increased. In the last step, the adsorption and desorption efficiencies, with or without shaking (Figure 3g and 3h), were compared. The number of extracted MFs at the same adsorption time with or without shaking and at the same desorption time was higher without shaking. Therefore, shaking is not required during adsorption. Regarding desorption, comparison of the MFs obtained from desorption with or without shaking at the same adsorption time revealed that vortex agitation during desorption is required to obtain a higher number of MFs.

Comparison of the specific lipid classes extracted using different extraction procedures (modified Bligh and Dyer method, single-phase liquid extraction with MTBE and our SPME procedure) revealed that all extraction procedures provide similar lipidome coverage (protocols provided in the Supporting Information (SI)).

The total time of the presented two-step (adsorption/desorption) procedure is 10 min (5 min of adsorption and 5 min of desorption). The choice of on-tip SPME mode is convenient and minimizes sample manipulation. This indicates that the developed extraction procedure is suitable for the high-throughput lipid analysis of HBM samples. The use of organic solvents is reduced to 100 μL of isopropanol, instead of highly toxic chloroform. Most importantly, adsorption can be performed directly in the HBM sample without prior protein precipitation. Additionally, the obtained lipid extract does not need to be concentrated and can be injected directly on the LC column after the desorption step.

The relative standard deviation (% RSD) of the MF volumes, which were detected in all three extraction replicates of the same biological sample, demonstrates that the main disadvantage of the proposed extraction procedure is its insufficient repeatability, which prohibits the use of this sample preparation technique for quantitation (69% of 459 MFs had % RSD > 20%, which does not meet the criteria for semiquantitative analysis). Furthermore, the same matrix had limitations. The wide concentration range of different lipid classes in the HBM samples prohibited the simultaneous quantification (as well as relative quantification) of all lipid classes in one analytical cycle, e.g., the MS signal obtained for some lipid classes was saturated (such as TGs and DGs), necessitating dilution for quantification, but the MS signals for other lipid classes (such as GPs) were very low, prohibiting dilution.

Despite the quantification limitations, the presented lipid extraction method can be useful for the rapid, global characterization of the HBM lipid composition.

3.2. Development of the LC–Q-TOF-MS Method for HBM Lipid Fingerprinting

RP chromatography combined with Q-TOF-MS was chosen for the analysis of HBM lipids. Chromatographic separation was performed to minimize ion suppression and to resolve isobaric and isomeric species. The Q-TOF mass analyzer yielded the molecular formulas of unknown peaks based on measured m/z values (with high accuracy) and isotope peak patterns, thereby enabling a qualitative analysis of all HBM lipids without any predetermination of whether the lipids had been sufficiently ionized by the ion source of the instrument.

The chromatographic conditions had to be optimized to obtain sufficient resolving power. To achieve high resolving power in the chromatographic system, an efficient C_{18} column with a 1.8 μm particle diameter was chosen. Several solvent compositions of the mobile phase were investigated to provide sufficient elution strength and to ensure lipid elution from the chromatographic column without any carry-over effects. The initial chromatographic conditions were adapted from Hewelt-Belka et al.[40] The best chromatographic resolution and sufficient elution strength of the mobile phase were provided by a mixture of 5 mM ammonium formate in water/methanol (1/4, v/v) as component A and a mixture of 5 mM ammonium formate in water/hexane/2-propanol (1/20/79, $v/v/v$) as component B. The addition of 5 mM ammonium formate to the mobile phase was fundamental to ensuring the efficient ionization of lipid compounds such as DGs and TGs, for which the $[M + NH_4]^+$ ions had higher MS signal intensity than the $[M + H]^+$ ions ($[M + H]^+$ were not observed for TGs). The appropriate amount of water in a mixture of 5 mM ammonium formate in water/methanol (component A of the mobile phase) was maintained to separate the polar lipid compounds. A high content of organic solvent in component A of the mobile phase and the addition of hexane to component B of the mobile phase ensured the complete elution of lipids from the chromatographic column. To ensure the elution of the most hydrophobic compounds (TGs), the temperature was increased to 90 °C. At lower temperatures, a carry-over effect of this lipid class was observed. The MS working parameters were optimized. simultaneously with the chromatographic conditions to achieve high-intensity MS signals and to detect the greatest number of lipid compounds.

The selected chromatographic column, column temperature, mobile phase composition and gradient elution program provided sufficient separation of all lipid compounds in a reasonable analysis time (71 min). To examine the repeatability of the developed LC–MS method, the results obtained from triplicate injections of one sample (HBM lipid extract) were compared in terms of the calculated MF volume RSD% and the total number of detected MFs (Table 1). The results presented in Table 1 show that 85% of the detected MFs had acceptable RSD% values (below 20%).

Table 1. Comparison of the Calculated MF Volume RSD% and the Total Number of Detected MFs Obtained from Triplicate Injections of One HBM Lipid Extract

	number	percentage [%]
total number of detected MFs present in all samples	622	100
% RSD < 5%	179	26
5% < % RSD < 10%	216	32
10% < % RSD < 15%	108	16
15% < % RSD < 20%	73	11
% RSD > 20%	99	15

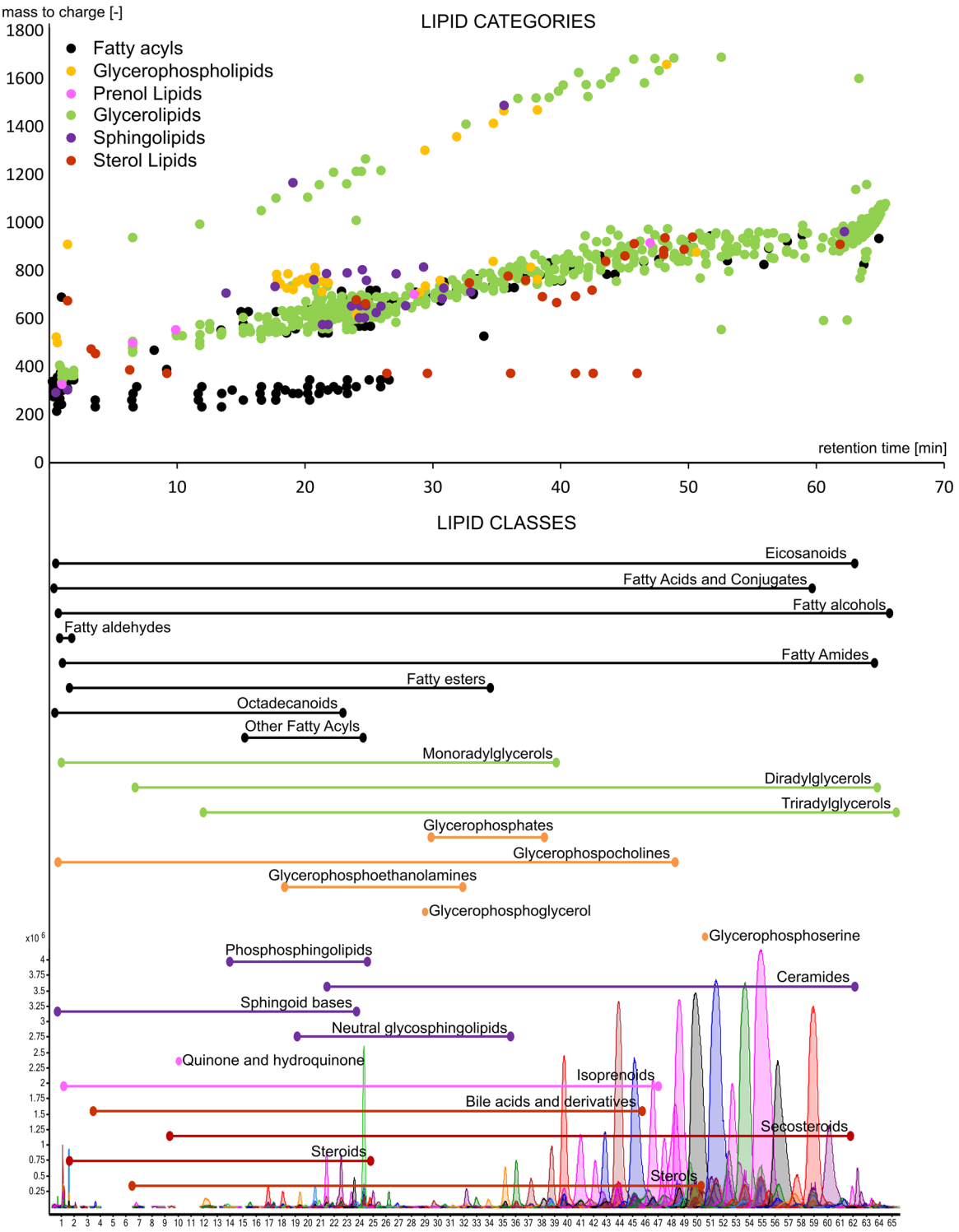


Figure 4. Retention time range of specific lipid categories and classes extracted using the developed analytical approach based on SPME and LC−Q-TOF-MS and identified by an automated LIPID MAPS database search.

3.3. HBM Lipidome Coverage

The aim of our study was to develop an analytical tool for the rapid characterization of the composition of HBM lipids. The presented analytical procedure, which includes an automated search of a lipid database, can be used to assess the lipid classes and groups (sum of carbon atoms and number of double bonds in the FA chains of lipids[41]) contained in HBM lipid extract. The application of MS/MS confirms the identity of the detected lipid compounds through comparison of the obtained MS/MS spectra with typical fragment ions, which have been extensively described in the literature.[42−47] The automated search of the database imported from LIPID MAPS enables quick identification of lipids based on accurate mass measurements (assignment of the headgroup, total FA carbon number and total degree of FA unsaturation of the lipid group). This automatic database search revealed 25 classes of lipids, from 6

lipid categories, FAs, GLs, GPs, SPs, PRs and STs, detected in the HBM lipid extracts. Hits from the database with the corresponding retention time ranges are presented in Figure 4. Additionally, the identified lipids are summarized in Table S1 in the SI. The lipidome coverage obtained using the optimized analytical approach with SPME is comparable to that described in previous reports.[33]

To confirm the identity of specific lipid compounds and to determine the structure of the acyl substituents, additional MS/MS experiments were performed. Manual investigation of the acquired MS/MS spectra enabled verification of the lipid identity based on a comparison of the fragment ions in the MS/MS spectra with the structures of the identified lipid compounds. The fragmentation patterns of lipid classes belonging to specific categories are shown in the SI. Notably, manual inspection of the obtained MS/MS spectra revealed that some DGs and TGs fragment the ion source and form smaller fragments such as fatty acids with the same retention times. Therefore, some FA species are products of DG and TG fragmentation in the ion source.

Employing MS/MS confirmed the identity of GPs, including the differentiation of isobaric species such as phosphoethanolamines (PEs) and PCs. The distinction between these two lipid classes is possible due to their characteristic fragmentation patterns. For the determination of diacylglycerophosphoethanolamines (subclass of PEs), a neutral loss of 141 Da, which is the result of the loss of the PE headgroup, has been used as a diagnostic tool. MS and MS/MS spectra of diacylglycerophosphoethanolamines are presented in Figure S1. The MS and MS/MS spectra of PC are presented in Figure S2. The unambiguous identification of PC species is possible because of the formation of the characteristic ion (184.0733 m/z) resulting from the loss of the polar headgroup (PC-specific headgroup) during fragmentation. Similarly, the presence of ion 184.0733 m/z in the MS/MS spectra of sphingomyelins (SMs), a class of SPs, enabled the differentiation of this lipid species from its isobaric species, ceramide phosphoethanolamines (PE-Cer). MS and MS/MS spectra of SMs are presented in Figure S3.

Comparing the accurate measured m/z of MFs against the online database enabled the identification of GLs, such as DGs and TGs, at the category, class and lipid group levels, MS/MS analysis enabled structural determination of the acyl substituents. MS and MS/MS spectra of DGs and TGs are also presented in the SI, Figures S4 and S5.

The identity of cholesterol esters, a class of STs, was confirmed after fragmentation, which resulted in mass spectra containing a characteristic fragment (369.3516 m/z) formed by the loss of the fatty ester constituent. The MS and MS/MS spectra of a cholesteryl ester (18:2)[48] are presented in Figure S6

MS/MS experiments did not provide unambiguous identification of FAs and PRs because the signal intensities of these compounds were too low to obtain a clear fragmentation spectrum.

For the first time, we present a complete characterization of HBM lipids. Identification was performed in terms of the polar headgroup, number of double bonds and number of carbon atoms in the lipid FA chains. The developed method was used to determine the following lipid classes: 91 fatty acids and conjugates, 6 fatty alcohols, 4 fatty aldehydes, 17 fatty amides, 6 fatty esters, 10 octadecanoids, 7 eicosanoids, 5 other FAs, 14 monoradylglycerols, 204 diradylglycerols, 304 triradylglycerols, 4 glycerophosphates, 18 glycerophosphocholines, 10 glycerophosphoethanolamines, 1 glycerophosphoglycerol, 1 glycerophosphoserine, 6 phosphosphingolipids, 13 ceramides, 3 sphingoid bases, 5 neutral glycosphingolipids, 1 quinone, 1 hydroquinone, 4 isoprenoids, 7 bile acids and derivatives, 8 secosteroids, 3 steroids and 11 sterols. The identified lipid classes are presented in Table S1.

3.4. Comparative Lipidomics of HBM, Milk Formula and Cow Milk

The optimized solid phase microextraction method with LC−MS detection was applied to qualitative comparison of lipid composition in 3 months postpartum HBM obtained from one healthy volunteer, formula milk (FM) for babies under 6 months and cow milk (CM). For the comparative lipidomics of three types of milk, a data set containing 6 extraction replicates of HBM, FM and CM were analyzed. The data were processed with the MFE algorithm, then imported to the MassProfiler software, and subsequently aligned. MFs detected in the control sample were not included in further steps of data analysis. A table containing feature identification numbers, retention times, masses and MF volumes was imported to MS excel. MFs present in less than 5 or 6 replicates within one group (HBM, MF or CM) were discarded. The obtained list of MFs after data treatment contained 301 lipids characteristic for each group. Clear qualitative differences between lipidomic composition of HBM, FM and CM were visualized using Venn diagram created using the web tool (bioinformatics.psb.ugent.be).

The Venn diagram corresponding to the data obtained from SPME-LC−MS of HBM, FM and CM (Figure 5) demonstrated different lipid composition for each group and presence of unique lipids in HBM (72) and additional lipids in FM and CM that not occur in HBM (48 and 58 respectively). To further confirm uniqueness of 72 lipids presented in HBM, the list of 72 neutral mass of indicated lipids was once again extracted by MFE algorithm with decreased peak filter: peak high ≥100 counts. The results obtained after additional data filtration revealed that 5 MFs are present also in the other types of milk. The 67 MFs unique for HBM were not detected in FM and CM (these MFs were below applied criteria of the MFE algorithm). The list of identified unique HBM lipids is presented in SI, Table S2.

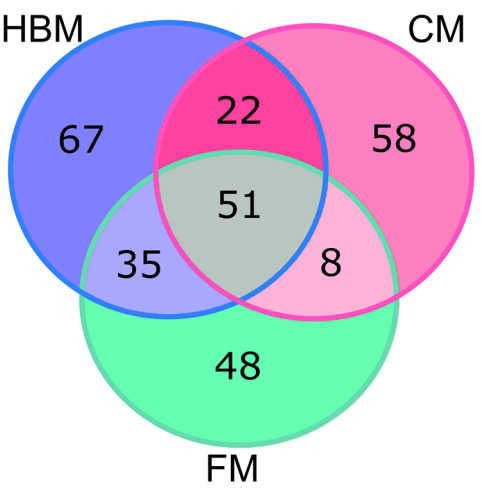


Figure 5. Qualitative differences between lipidomic composition of HBM, FM and CM visualized by Venn Diagram.

Further investigation of additional lipids is necessary, to examine influence of additional lipids in FM on child

development and to improve the composition of milk formula for babies that cannot be breastfed.

4. CONCLUSION

In this paper, we demonstrated a rapid and simple method for the comprehensive characterization of lipids in HBM samples. We also presented for the first time the use of SPME for qualitative lipid analysis of HBM. Employing LC−Q-TOF-MS enabled the separation, detection and identification of the major HBM lipids including FAs, GLs, GPs, SPs, PRs and STs. The two-step procedure (adsorption/desorption) for lipid extraction from HBM samples that does not require protein precipitation minimizes the time required for sample preparation and greatly reduces the use of organic solvents, offering significant improvements over other published methods for HBM lipid extraction.

We have applied the developed method to indicate qualitative differences between HBM, FM and CM lipid composition. The obtained results revealed that 68 lipids are unique for HBM including TGs, DGs, PCs and SMs. The impact of any changes in natural lipid composition of milk that constitute food for infant on child development should be further investigated.

This manuscript can serve as a ready-to-use tool for lipidomics investigations of HBM because we describe herein all analytical steps as well as postanalysis data treatment used for identification.

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