Ultrasound-assisted three-phase hollow fiber microextraction-based method for untargeted metabolomics

M.C. Villegas-Álvarez[a], A. Arias-Borrego[a,b*], I. Velasco[c], T. García-Barrera[a*].

[a]Research Center on Health and The Environment (RENSMA), Department of Chemistry "Prof. J.C. Vílchez Martín", University of Huelva, Fuerzas Armadas Ave., 21120 Huelva, Spain.

[b]Department of Analytical Chemistry, Faculty of Chemistry, University of Sevilla, Profesor García González Ave., 41012 Seville, Spain

[c]Department of Gynecology & Obstetrics, Hospital Universitari Germans Trias i Pujol, Badalona, Spain.

Corresponding Author

* Prof. Tamara García Barrera
Research Center on Health and The Environment (RENSMA), Department of Chemistry "Prof. J.C. Vílchez Martín", University of Huelva, Fuerzas Armadas Ave., 21120 Huelva, Spain.
tamara@dqcm.uhu.es. ORCID: 0000-0002-8859-9550. Tel. +34 959219962
*Dr. Ana Arias Borrego
Department of Analytical Chemistry, Faculty of Chemistry, University of Seville, Profesor García González Ave., 41012 Seville, Spain. aarias1@us.es ORCID: 0000-0002-0054-5639. Tel. +34 954556442

ABSTRACT

Sample treatment for untargeted metabolomics is still the bottleneck of the analytical procedure, especially for complex samples like food or biofluids. The main pitfalls are the suppression of minor compounds, low metabolite coverage and the impossibility to detect certain families, combined with the requirement of non-selective approaches for untargeted analysis. Herein, we developed a new analytical extraction method for untargeted metabolomics using a non-selective procedure based on three phase hollow fiber liquid phase microextraction aided by ultrasound (three-phase-UA-HF-LPME) followed by gas chromatography-mass spectrometry. As a proof of concept, the method has been validated for human milk (HM). The analytical method allows extracting organic nitrogenous compounds from HM, which was not possible using the conventional liquid-liquid extraction (LLE) and provided cleaner chromatographic profiles and mass spectra with lower background noise. Moreover, three-phase-UA-HF-LPME enhanced the extraction of a high number of metabolites from 1.65 to 3.27-fold compared to LLE, namely: fatty acids and derivatives (decanoic acid, lauric acid, palmitic acid, tetradecanoic acid, palmitoleic acid, stearic acid, myristic acid, linoleic acid, azelaic acid), lipids and lipid-like molecules (oleamide, monopalmitin), organic acids and derivatives (lactic acid, citric acid), amino acids and peptides (glycine, proline, glutamine). Otherwise, the extraction of carbohydrates and derivatives (urea, phosphoric acid, galactose) decreased the fold changes from 0.15 to 0.25. Results show that our proposed method is attractive owing to its limits of detection from 0.08 to 0.16 mg L^{-1}, recoveries of 99-86 %, enrichment factors up to 123 and fold changes very satisfactory when compared with LLE. The validation by targeted metabolomics as well as the untargeted procedure showed that three-phase-UA-HF-LPME presents better sensitivity, enrichment factors, sample throughput and metabolite coverage than the conventional LLE method

considered the gold-standard for untargeted metabolomics. Our results show insights into the possible application of HF for untargeted metabolomics with important analytical advantages in comparison with the LLE.

Keywords: Untargeted metabolomics, Hollow-fiber liquid phase microextraction, ultrasound

1. Introduction

Untargeted metabolomics measures global sets of low-molecular-weight metabolites that result from cellular activity and, therefore, they serve as a snapshot of relevant biological processes [1]. These molecules usually belong to the families of amino acids, lipids, organic acids, steroids, fatty acids, vitamins, and small peptides among others. In contrast to proteins or genes, which require the activation of post-translational modifications or epigenetics, respectively, the metabolites can serve as biomarkers of cell activity, which reflect changes in the phenotype and therefore the function [2]. Metabolite profiles play a critical role in diverse biological functions and they have been shown to be good biomarkers for the early diagnosis and prognosis of several diseases [2–5] as well as for environmental pollution monitoring using free-living organisms [6–7], to evaluate the biological response against xenobiotics [8] or the food quality [9–11]. Biological fluids like urine, serum or plasma are often used in metabolomics studies due to their ease sampling and because they are a direct reflection of the metabolism of an individual or organism [12].

Nevertheless, there are other less common biofluids, such as human milk (HM), which can provide a valuable and complementary information, due to the potential consequences for maternal–infant health [13–16]. However, the analysis of HM, is complex and challenging from the analytical viewpoint, especially the sample preparation since there are fats and other major components that could hide important biomarkers as well as differences in concentrations and great structural diversity among metabolites. Thus, cleaning the sample to remove protein interferences, as well as a subsequent separation into multiple fractions to reduce sample complexity is usually required before the untargeted metabolomic analysis by mass spectrometry (MS) or nuclear magnetic resonance (NMR), but not other enrichment or clean-up procedures are applied to obtain a wide metabolite coverage by untargeted and non-selective procedures [13,17]. Nevertheless, this fact can lead to signal suppression by major components, other matrix effects, and interferences that difficult the detection of minor metabolites. Otherwise, it has been shown that protein precipitation alone, even when solvents of different polarities are used, cannot solve this problem completely [18,19]. A previous study has reported the metabolic profile of HM using liquid-liquid extraction (LLE) with a chloroform-methanol mixture obtaining two different phases (lower phase containing nonpolar metabolites and upper phase containing polar metabolites) by NMR as the analytical platform [20,22]. Subsequently, an extraction of metabolites has been proposed using methyl tert-butyl ether (MTBE) and methanol that were later centrifuged and then analyzed by mass spectrometry after both, gas and liquid chromatographic separations (GC-MS, LC-MS)

providing a very good metabolite coverage from polar metabolites to fatty acids and lipids [23]. This sample preparation analytical method has been successfully employed to determine the changes in the HM metabolome during lactation [14,23,24], as well as the potential impact of maternal iodine deficiency [13] and COVID-19 in the HM metabolome [15,25–27].

The hollow fiber liquid phase microextraction (HF-LPME) is a promising membrane-based analytical technique that has been successfully applied to solve many analytical problems [28]. It is remarkable the reliability of the diverse HF-LPME modes and the simplicity of the assembly that enables low sample consumption. This aspect is particularly significant when dealing with biological samples. Furthermore, these modes exhibit high enrichment factors for analytes of diverse nature, while simultaneously reducing matrix effects, cross contamination, and memory effects. This is primarily attributed to the disposable nature of the fiber, which eliminates the need for subsequent cleaning steps and minimizes solvent usage. All these features of HF led to an enhancement of the parameters of quality of the analytical method against others such as LLE or solid-phase microextraction (SPME) [29,32]. Furthermore, the application of ultrasound energy is a powerful aid in the acceleration of various steps in the analytical process [33,34].

Accordingly, ultrasound-assisted (UA) extraction has been extensively used to improve mass transfer between immiscible phases [35] and few research so far have used ultrasound-assisted extraction combined with HF-LPME and they are related to the targeted analysis trace elements in urine samples [36] and estrogens from aqueous matrices [37].

Herein, a new analytical sample preparation method has been developed for HM untargeted metabolomics. The extraction and preconcentration of the HM metabolites along with fat and proteins removal were carried out in a single step by HF-LPME using the three-phase mode three-phase-HF-LPME. Moreover, the simultaneous extraction and preconcentration of HM metabolites were aided by ultrasound (three-phase-UA-HF-LPME). HM metabolites were annotated by GC-MS and the figures of merit of the new analytical approach were compared with the existing methods for HM metabolomics regarding metabolite coverage, extraction efficiency, repeatability, sensitivity, and feature detection. This sample treatment provides a handy analytical tool for the extraction of features in HM metabolomics and opens further research for the pretreatment of complex biological samples.

2. Material and methods

2.1. Chemicals and reagent solutions

Ultrapure water, employed for the preparation of all aqueous solutions, was procured from a Milli-Q system (Millipore Milli-Q, Watford, UK). Pure standards were individually dissolved in methanol (MeOH, Fisher Scientific, Nepean, Canada) to attain a final concentration of 1000 mg L^{-1} and subsequently stored at -20ºC. Intermediate working solutions were prepared by diluting the aforementioned solutions with methanol and kept in darkness at 4ºC until analysis. Sigma-Aldrich (Steinheim, Germany) was the source of all standards utilized for method validation, including lactic acid, urea, glycerol, proline, glutamic acid, glucose, citric acid, and serine. Additionally, O-methylhydroxylamine hydrochloride, N,O-bis(trimethylsilyl)trifluoroacetamide

(BSTFA) + 1% trimethylchlorosilane (TCMS), as well as solvents such as pyridine anhydride, n-decane, and methyl tert-butyl ether (MTBE), were also procured from Sigma-Aldrich. The hollow fiber employed for the immobilization of the supported liquid membrane and housing the acceptor solution was an S6/2 Accurel® polypropylene hollow fiber (1800µm i.d., 450µm wall thickness, and 0.2µm pore size) purchased from Membrana (Wuppertal, Germany). Acetonitrile (MeCN), utilized for the extraction, was acquired from Fisher Chemical (Madrid, Spain).

2.2. Sample collection

Breast milk (HM) samples were collected during the first four months of infancy and subsequently stored at −80 °C until analysis. The collection of HM samples took place between 2019 and 2020 at the University Hospital Germans Trias I Pujol, Badalona, Spain. This study received approval from the Local Ethics Committee, and written informed consent was obtained from all participating women (Ethics Committee Reference: PI-18-266). Comprehensive oral and written information regarding the study was provided to all participants. Women with multiple gestations, diseases, or perinatal complications were excluded from the study. The exclusion criteria encompassed women who were unable to breastfeed due to severe symptomatology requiring intensive care unit support, women who required the use of medications with potential adverse effects on the infant, and those for whom obtaining HM was not feasible.

2.3. Human milk liquid-liquid extraction

The extraction protocol used was previously developed by Villaseñor et al. [23]. A total of 50 µL of HM was vortex-mixed with 175 µL of methanol (MeOH) and 175 µL of MTBE (~8-fold dilution). The mixture was vortex and mixed for 1 min. Then the extract was centrifuged at 4000 g for 15 min at 15 ºC and then analyzed by GC-MS after derivatization. The derivatization process was carried out with methoxyamine in pyridine and the silylation was performed with MSTFA following a previously developed method [38].

2.4. Ultrasound-assisted three-phase hollow fiber microextraction of human milk metabolites

The extraction procedure was optimized prior to the application. The solvent bar configuration of the fiber and the three-phase mode was used for three-phase-UA-HF-LPME (Figure 1). The fibers were cut into 3 cm pieces and later cleaned into an ultrasound bath with acetone for 5 min. The clean air-dried fiber was then sealed at one end with the help of a thermal welder. Once sealed, it was immersed in n-dodecane solvent for 20 seconds to facilitate the formation of the supported liquid membrane (SLM). Then, the acceptor phase (50 µL of MeOH) was injected into the fiber lumen and thus, the analytes were extracted from HM to n-dodecane and then to MeOH. After that, the other end was sealed and introduced into the donor phase (175 µL of HM sample up to 1.4 mL with Milli-Q water, ~8-fold dilution). Then, it was placed into an ultrasound bath for the optimal extraction time at 5 min. Once the extraction time was elapsed, the collected acceptor solutions was discharged into an Eppendorf tube and dried under a nitrogen stream. The derivatization procedure was performed in the same way as in the previous section. Methoxyamine in pyridine was used for the initial reaction, followed by silylation with MSTFA.

2.5. *Mass spectrometric metabolomic analysis*

Extracts were analyzed by gas chromatograph (Trace GC ULTRA) equipped with an ion trap analyzer model ITQ 900 (Thermo Fisher Scientific). The capillary column used was a VF-5MS of dimensions: 30 m × 0.25 mm I.D. and 0.25 μm of film thickness (Agilent Technologies, Tokyo, Japan). Splitless mode was selected for the injections in the injector oven at 250ºC and helium was used as carrier gas at 1.2 mL min^{-1}. The oven temperature program started at 60 °C during 1 min, then ramped at 10 °C min^{-1} to 325°C, and cooled down during 10 min before the next injection. The electron ionization (EI) source was operated at 70 eV mode. The MS detection was performed in full scan mode in the m/z range 50−650 at a rate of 1 spectra/s. The total chromatographic time was 30 minutes. The temperature of the MS detector was kept at 280ºC, using nitrogen as auxiliary gas at 60 mL min L^{-1}. The acquisition, evaluation and calculation of chromatographic peak areas were performed with the ChemStation software (version A0903).

2.6. *Data Treatment and Metabolite Identification*

GC–MS raw data was processed as reported previously [11]. In brief, CDF format was selected to convert the files using the Thermo File Converter tool and later, GC–MS data matrix with normalized peak areas was imported into the SIMCA-P (version 11.5, Umetrics). Principal component analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were used to obtain predictive models to visualize the metabolic information as well as to explore the differences in the number features extracted using different extraction times (5, 10 and 15 min) by three-phase-UA-HF-LPME versus LLE. Besides, the fold change values were calculated for each metabolite, in order to estimate the variation in their abundance within the comparison. The quality of the model was assured by the class separation (R^2) and predictive (Q^2) parameters. The differences between the types of extraction (three-phase-UA-HF-LPME vs LLE) were evaluated for each individual metabolite by One-way ANOVA and Tukey's test by using Statistica 8 (Statsoft, Tulsa, OK, USA) to find statistically significant comparisons and to investigate the trend of metabolite concentrations. Then, the Benjamini-Hochberg (FDR correction) method was also carried out to adjust the *p*-values. NIST Mass Spectral Library (version 08) was used for metabolites annotation. Those metabolites with spectrum scores > 80% and concordant retention index (using the n-alkane scale) (Table S1), were used for the annotation of metabolites. Target ions with higher intensities and masses were chosen from each metabolite mass spectrum since they are affected by matrix into a lesser extent. Moreover, we checked the area of the qualifier/target ion ratio for each metabolite and those with a variation < 20% were selected. Before any statistical analysis, the internal standard at 10 mg L^{-1} (IS, Ribitol) abundance was used to normalize sample concentrations to correct any instrumental variability. In addition, coefficient of signal variation (CV) in quality controls (QCs) was used to filter data, considering values < 30% as acceptable. Metabolites that led to the discrimination between the extraction type, THREE-PHASE-UA-HF-LPME and LLE, were considered as statistically significant using a variable importance in the projection (VIP values, a weighted sum of squares of the PLS weight) ≥1 and p-values ≤ 0.05.

2.7. *Validation of the methodology by targeted metabolomics*

To evaluate the practical reliability of the extraction method, the figures of merit were determined, namely: linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability (intra-day), reproducibility (inter-day), enrichment factor (Ee) and recovery. For method validation, the sample phase was composed by 175 µL of HM spiked with the commercial standards at different concentrations (10 mg L^{-1} of lactic and glutamic acid; 25 mg L^{-1} of glucose; 50 mg L^{-1} of urea; 100 mg L^{-1} of glycerol; 5 mg L^{-1} of citric acid and serine; 2.5 mg L^{-1} proline) in quintuplicate (n=5) to a final volume of 1.4 mL with ultrapure water (18.2 MΩ cm). The linearity was obtained by plotting calibration curves of the relative area (analyte peak area/internal standard peak area) versus the concentration of each analyte. The analytical curves were constructed at six levels in triplicate, at the following concentration ranges: 2-25 µg mL^{-1} for lactic and citric acids; 30-260 µg mL^{-1} for urea; 60-500 µg mL^{-1} for glycerol; 0.5-7 µg mL^{-1} for proline; 6-60 µg mL^{-1} for glutamic acid; 15-150 µg mL-1 for glucose and 1.5-15 µg mL^{-1} for serine. LODs and LOQs were calculated as a + 3 $S_{y/x}$ and a + 10 $S_{y/x}$, respectively, where "a" is the origin ordinate and "$S_{y/x}$" the random errors in the values for the slope and intercept [39]. Element concentrations below LOQ were excluded from statistical analysis. Repeatability and reproducibility were evaluated with the analysis of samples (n = 5) on the same and on different days. Also, the enrichment factor (E_e) of the extraction was measured with the ratio ($E_e = C_{a,f}/C_{d,}$) where , $C_{a,f}$ is the final concentration of each standard in the acceptor phase and $C_{d,i}$ is the initial concentration of each standard in the sample solution. The instrumental stability was determined by multiple injections in quintuplicate of a QC sample. In addition, These QC samples were injected at the beginning, in the middle, and at the end of each run.

3. Results and discussion

3.1. Optimization of the ultrasound-assisted three-phase hollow fiber microextraction of human milk metabolites

3.1.1. Optimization of the three-phase-UA-HF-LPME general configuration. Since HF-LPME is the miniaturization of LLE, initially we plan to reproduce the extraction using MeOH mixed with MTBE as the acceptor phase. The reason is that the previously reported successful LLE method is based in a mixture of MeOH, for polar metabolites, MTBE, for lipophilic metabolites and then, the diluted HM sample is mixed for a mono-phasic extraction followed by the later precipitation of proteins by centrifugation [23]. However, the extraction of HM metabolites by HF-LPME using MeOH/MTBE in the acceptor phase is not possible because the two phases (acceptor and donor) are mixed, preventing the proper functioning of SLM. Therefore, the HF-LPME for HM metabolites was configured in the three-phase mode using n-dodecane immobilized in the HF pores, thus providing a SLM. The acceptor phase (MeOH) was filled in its lumen and the diluted HM sample as the donor phase. The centrifugation step used after the traditional LLE was not required in the three-phase-UA-HF-LPME, since unlike small molecules such as metabolites, the proteins and other interfering molecules (e.g. fat components) cannot cross the membrane pores. The optimization of the variables affecting HM metabolites by three-phase-UA-HF-LPME was carried out taking into account the number of metabolites later identified by GC-MS in the extracts as the main objective. Later, the number of families of compounds to which these metabolites belong and the peak areas of the metabolites was compared with the LLE method. Thus, several variables were

considered namely: acceptor phase, SLM, HM dilution factor, extraction time, fiber length, ionic strength and sample pH. These variables were optimized by changing one parameter at a time while utilizing standard conditions for the other ones: MeOH (acceptor phase), n-dodecane (SLM), 8-fold HM dilution with Milli-Q water, 5 minutes of extraction time, 3 cm of fiber length and the absence of ionic strength and pH adjustment.

3.1.2. Acceptor phase selection. One of the most important parameters affecting HF-LPME is the type of extracting solvent. The acceptor phase must be selective regarding the nature of the analytes, which must have a high tendency to dissolve them and leave the sample matrix. In addition, previous studies reported the use of different acceptor phases such as MeOH or acetonitrile (MeCN) to deprotenize and to extract metabolites [37,38]. Taking these considerations into account, MeOH was first selected as it is used in the traditional LLE method, but MeCN was also assayed. Since MeOH provided much better results in terms of the number of extracted features conserving the compound families, it was selected as the acceptor phase (Figure 2a).

3.1.3. Supported liquid membrane selection. The SLM must be compatible with the fiber to form a very thin organic membrane film by filling completely the pores in the fiber wall. In addition, the SLM must be immiscible with the aqueous donor phase and with the acceptor organic phase. Moreover, the use of a mixture of MeOH/n-dodecane solvents has been previously reported as it favors the extraction of fatty acids [40,41]. Based on the above considerations and in agreement with previous works [42], n-dodecane was selected as the SLM.

3.1.4. Driving force. To speed up the extraction, stirring is usually selected as the driving force as in most of the HF-LPME methods [18,43]. However, in order to use a minimal sample volume, the magnetic stirring was ruled out for incompatibility with the miniaturized model because HF was not immersed properly into the donor solution and the stirrer did not rotate properly in such a low donor phase volume affecting the repeatability of the three-phase-HF-LPME. Alternatively, an ultrasound bath was used to speed up the HF-LPME process. The use of sonication usually led to short extraction times, probably due to a combined process of cavitation and agitation attained inside the ultrasound bath [44]. Moreover, it also made possible to work with low sample volumes and higher sample throughput than using stirring. Using the new three-phase-UA-HF-LPME arrangement, the other variables were optimized as follows.

3.1.5. Extraction time. The extraction efficiency was studied at 1, 5, 10, and 15 min under the same conditions. As can be seen in Figure2a, the times 5 and 10 min were found to be the most optimal. The extraction efficiencies were equivalent at 5 and 10 min using the total number of features detected, (2411 and 2405, respectively). For this reason, 5 minutes was chosen to shorten the analysis time.

3.1.6. Fiber length. The extraction was carried out with different fiber lengths from 1 to 4 cm, and the optimum was 3 cm the since the metabolite coverage and the total number of features slightly better (Figure2b.), and no discernible influence was changed when the fiber length increased from 3.5 to 4 cm. Accordingly, 3 cm was adopted as optimized fiber length in the subsequent experiments.

3.1.7. HM dilution factor. The composition in HBM differs in terms of chemical structure and metabolites concentration, which require often dilution of the sample and enrichment before analysis. Therefore, the dilution factor is an important parameter in the extraction

process. Initially, we used the same dilution factor (~8-fold) as previously reported with the LLE method [23]. The results obtained demonstrated good enrichment factors and wide metabolite coverage detected with low sample consumption, which are explained in the following sections. Therefore, it was decided to carry out the experiments under these conditions.

3.1.8. Ionic strength adjustment and pH adjustment. Non-selective protocols were applied to three-phase-UA-HF-LPME with the purpose to maximize the ability to detect the higher number of metabolites and compare them with the reported extraction techniques used until now in metabolomics, in which there is no prior information about the target metabolites that can be extracted with a focus of untargeted analysis. Therefore, it was decided not to adjust the pH and ionic strength of the sample in the future experiments, like in the traditional LLE applied to untargeted metabolomics, unless a certain low metabolite coverage was observed in the final application of the procedure. However, this fact did not happen, as can be seen in the following section and supplementary information (Figure S1).

3.2. Comparison of LLE and three-phase-UA-HF-LPME for HM untargeted metabolomics regarding metabolite coverage

Figure 3 shows the main families of compounds to which the annotated metabolites belong and the percentage of each class against the total number of metabolites using both LLE and three-phase-UA-HF-LPME. As we can see, the three-phase-UA-HF-LPME provides greater metabolite coverage and allows extracting a greater number of metabolites (three-phase-UA-HF-LPME *vs* LLE, Table S1). Interestingly, three-phase-HF-LPME allows the extraction of organic nitrogenous compounds from HM (3% of the total number of metabolites annotated by three-phase-UA-HF-LPME), which was not possible using LLE. These metabolites detected only with three-phase-UA-HF-LPME, such as *butylamine*, may be of special interest because there is scientific evidence of their possible relationship with neonatal hypergastrinemia [45]. On the other hand, three-phase-UA-HF-LPME was more efficient for the extraction of fatty acids and derivatives (23% vs 7% using LLE) as well as for lipids (17% vs 14% LLE). As one family of compounds (organic nitrogen compounds) cannot be extracted by LLE and the total number of metabolites extracted by this analytical technique was also lower, the percentages of each family of compounds referred to the latter were high as in the case of non-metallic compounds (15%), carbohydrates and derivatives (29%) and amino acids and peptides (14%) compared to three-phase-UA-HF-LPME (7%, 20% and 7%, respectively).

3.3. Comparison of the chromatographic profiling and annotated metabolites by GC-MS after LLE and three-phase-UA-HF-LPME

HM chromatographic profiles were obtained using both extraction methods, LLE and three-phase-UA-HF-LPME by GC-MS analysis (Figure S1). As can be seen in SI, the chromatographic profile obtained by three-phase-UA-HF-LPME remove background noise compared to the LLE method. In order to obtain good reliability and stability of the metabolomic results, the analysis was evaluated using QCs, which consisted of a pool of all the samples. PCA plots displayed a good QCs clustering (Figure S2a). PLS-DA showed good separation between the different extraction methods included in this study and analyzed by GC-MS (Figure S2b). In addition, three pairwise comparisons between

LLE versus three-phase-UA-HF-LPME at three different times (5, 10, and 15 min) were performed to identify the metabolites responsible for the discrimination between both analytical techniques. The values of R^2 and Q^2 confirmed the discrimination power of the models (Table S2). The efficiency of the extraction method was evaluated by the total number of extracted metabolites and the total intensity of the extracted features. Figure 3c and 3d shown the total number of features extracted and the total number of annotated metabolites with the different extraction techniques compared in this study. As can be seen, LLE and three-phase-UA-HF-LPME allow extracting 2345 and 2411 features respectively, and finally, 25 and 30 metabolites were annotated by LLE and three-phase-UA-HF-LPME. Using the new three-phase-UA-HF-LPME method, the metabolites were annotated using Kovats retention indices, tandem mass spectrometric experiments, and NIST database MS searching (Table S1). Additionally, we used the fold change to explore the differences in the total intensity of the m/z signal of metabolites between both analytical extraction techniques (Table S3). Fold changes were computed simply as the ratio between m/z peak intensity using the three-phase-UA-HF-LPME against the peak intensity using LLE for metabolite. Thus, the three-phase-UA-HF-LPME technique enhanced the extraction of fatty acids and derivatives such as decanoic acid (1.65-fold), lauric acid (2.87-fold), tetradecanoic acid (3.05-fold), palmitic acid (3.02-fold), palmitoleic acid (2.73-fold), stearic acid (2.65-fold), myristic acid (2.43-fold), linoleic acid (1.54-fold), and azelaic acid (3.27). Likewise, we also found an increase of lipids and lipid-like molecules such as oleamide (2.06-fold) and monopalmitin (2.75-fold), organic acids and derivatives such as lactic acid (3.25-fold) and citric acid (2.67-fold), amino acids and peptides such as glycine (2.01-fold), proline (2.11-fold) and glutamine (3.02-fold). The extraction capability of these metabolites were significantly enhanced with the three-phase-UA-HF-LPME method in comparison with LLE. These compounds are bioactive molecules necessary for key metabolic and physiological functions related with the proper development, growth and health of the newborn [46–48]. Otherwise, LLE seems to be greater for the extraction of carbohydrates and derivatives such as urea (0.15-fold), phosphoric acid (0.02), and galactose (0.25-fold).

3.4. Validation of the three-phase-UA-HF-LPME /GC-MS by targeted metabolomics

To evaluate the applicability of the proposed three-phase-UA-HF-LPME, the following figures of merit were determined and compared with LLE: repeatability, reproducibility, recoveries, enrichment factor, linearity and detection limits (LODs) and limits of quantification (LOQs), by means of targeted metabolomics using standard solutions of lactic acid, urea, glycerol, serine, proline, glutamic acid, citric acid, and glucose. Ribitol was included as an internal standard to correct possible errors in the signal. Linear ranges were evaluated by analyzing five different levels of concentrations of standard solutions in triplicate, where the linearity coefficients were all greater than 99% (R^2> 0.99). The recoveries were estimated by comparing the values of the samples enriched in triplicate (n=3), obtaining recoveries within the range 86-117% [37]. The LODs and LOQs were in the ranges 0.08-0.16 and 0.26-0.54 mg L^{-1}, respectively (Table 1). The Ee results are very high, thus enhancing the extraction efficiency of LLE. The results have confirmed that the features of the method are very good and the methodology that can be applied successfully for the analysis of complex samples.

4. Conclusions

The new method optimized for HM untargeted metabolomics, based on three-phase-UA-HF-LPME by GC-MS provides a wide metabolite coverage with higher features and family of compounds annotated than LLE, probably because the former analytical technique achieves lower background noise and higher peak signal to noise ratio in chromatograms and mass spectra, thus preventing the common signal suppression. The analytical validation of the analytical method using commercially available standards and targeted metabolomics showed lower detection limits, higher enrichment factors and high fold changes in comparison with LLE. Moreover, the new optimized approach allows higher sample throughput with shorter extraction times aided by ultrasound and because with HF the common LLE step for proteins precipitation by centrifugation is not required. Thus, HF is a potential analytical technique that can be applied to untargeted metabolomics with comparable results to LLE combining both, a non-selective procedure and high enrichment factors, that are ideal for untargeted analysis and metabolite coverage, respectively. Our results provide insights into the possible application of HF to HM untargeted metabolomics and open further research related to other samples, especially complex biofluids in which the sample consumption and sample throughput is critical. Further research will be focused on the analysis of the obtained extract with LC-MS since it is also compatible with the polarity and volume of the obtained extract using three-phase-UA-HF-LPME .

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest

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Appendix A. Supplementary Information

Supplementary data to includes detail about the material and method, results and discussion sections. Two supplementary Figures and three supplementary Tables are including in this file. The Supplementary data is available free of charge at https://doi.org/

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Figures

Figure 1. Schematic drawing of the three-phase-UA-HF-LPME process used for the extraction

Figure 2. Total number of molecular features extracted by three-phase-UA-HF-LPME and later annotated by GC-MS depending; a) effect of solvents as acceptor phase (MeOH or MeCN) and extraction times, b) effect of fiber length on the extraction efficiency at different extraction times (gray line=5min, red line=10min and blue line=15 min).

Figure 3. Metabolite coverage according to the families of compounds using both a) LLE and b) three-phase-UA-HF-LPME for human milk metabolomics. Venn diagram comparing c) total number extracted features and d) total number annotated metabolites using three-phase-UA-HF-LPME and LLE by GC-MS.

666 **Table 1.** Validation parameters of the proposed method in comparison with LLE.

Validation parameters	**Lactic acid**	**Glutamic acid**	**Urea**	**Glycerol**	**Glucose**	**Citric acid**	**Serine**	**Proline**
LLE (*)								
LOD mg L^{-1} *	0.53	1.51	3.22	4.63	0.53	0.20	0.70	0.13
LOQ mg L^{-1} *	1.87	5.03	10.72	15.44	1.60	0.61	2.32	0.64
Intra-day (%RSD) n=5 *	3.5	3	4.1	4	4.7	5.8	3.9	6.8
Inter-day (%RSD) n=5 *	5.0	6.6	4.7	5.2	6.7	7.8	4.7	10.8
Recovery (%) n=3	97.5	95.6	100.5	98.4	90.1	80.3	103.2	82.4
Averange concentration (mg L^{-1}± S.E.M)	12.4± 0.5	98.3±3.2	160.4±8.9	420.3±26.4	99.3±7.5	70.9±5.7	6.8±4.5	2.2±0.7
three-phase-UA-HF-LPME								
LOD (mg L^{-1})	0.16	0.08	0.08	0.16	0.14	0.09	0.14	0.09
LOQ (mg L^{-1})	0.54	0.26	0.27	0.53	0.48	0.53	0.46	0.48
Intra-day (%RSD) n=5	0.24	10.46	6.74	3.64	6.93	4.37	2.42	7.96
Inter-day (%RSD) n=5	6.31	8.09	3.71	5.20	4.93	8.94	5.99	6.11
Recovery (%) n=3	86.4	99.2	98.1	90.6	98.2	98.5	97.8	98.4
Ee (RSD%) n=3	15 (12)	123 (5)	8 (7)	107 (10)	75 (4)	84 (6)	103 (11)	78.9 (16)
Linearity								
Regression equation	Y=0.79X+1.75	Y=0.06X+2.48	Y=0.08X+1.48	Y=0.12X-4.74	Y=0.03X+2.27	Y=0.03X+0.29	Y=0.34X+3.21	Y=0.25X+0.98
Determination coefficient (R^2)	0.998	0.996	0.997	0.991	0.993	0.991	0.994	0.997
Range (mg L^{-1})	0.54-23	0.26-60	0.27-260	0.53-500	0.48-125	0.53-25	0.46-15	0.48-7
Averange concentration	15.3± 0.9	120.6±1.3	149.4±6.2	450.4±29.6	104.3±4.7	85.2±1.2	5.6±0.6	3.1±0.4

mg L^{-1}± S.E.M)								

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669 Enrichment factors, Ee ; S.E.M, standard error of the mean; (*)Validation parameters in LLE according to literature [21].

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