

# Untargeted Human Milk Metabolomics

Subjects: [Biochemical Research Methods](#)

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Human milk (HM) is considered the gold standard for infant nutrition. HM contains macro- and micronutrients, as well as a range of bioactive compounds (hormones, growth factors, cell debris, etc.). The analysis of the complex and dynamic composition of HM has been a permanent challenge for researchers. The use of novel, cutting-edge techniques involving different metabolomics platforms has permitted to expand knowledge on the variable composition of HM. Here, the state-of-the-art in untargeted metabolomic studies of HM, with emphasis on sampling, extraction and analysis steps is presented.

human milk

metabolome

sampling

extraction

liquid chromatography–mass spectrometry

## 1. Introduction

Human milk (HM) has been markedly established as the optimal way of providing infants with the necessary nutrients and bioactive factors for their early development. Many health associations and organisms, including World Health Organization, recommend exclusive breastfeeding for the first six months of life <sup>[1]</sup>. Health benefits of HM for infants include reduced mortality and morbidity, including sepsis, respiratory diseases, otitis media, gastroenteritis, and urinary tract infections, among others <sup>[2]</sup>. In addition, studies reporting on long-term benefits of HM consumption such as lower risk of suffering from type 1 diabetes and inflammatory bowel disease or overweight in adulthood emerged <sup>[3]</sup>. HM may also be associated with a slightly improved neurological outcome as cohort studies report <sup>[4]</sup>, especially in preterm infants <sup>[5]</sup>, although potential confounders must be accounted for <sup>[6]</sup>.

HM composition is dynamic and influenced by several factors including genetics, gestational and infant's age, circadian rhythm, maternal nutrition, or ethnicity. It provides a series of nutrients such as lipids, proteins, carbohydrates, and vitamins, jointly with a number of bioactive factors that contribute to several physiological activities in the newborn infant as well as to short- and long-term outcomes <sup>[7][8]</sup>. Living cells including stem cells, hormones, growth factors, enzymes, microbiota, and even genetic material are part of this vast array of HM components with impact in early development, particularly the immune system <sup>[9]</sup>. In addition, HM appears to be one of the richest sources of microRNAs <sup>[10]</sup>. On the other hand, because of the maternal environmental exposure and lifestyle, the presence of some contaminants such as persistent organic pollutants or pharmacologically active substances in HM has been described <sup>[11][12]</sup>.

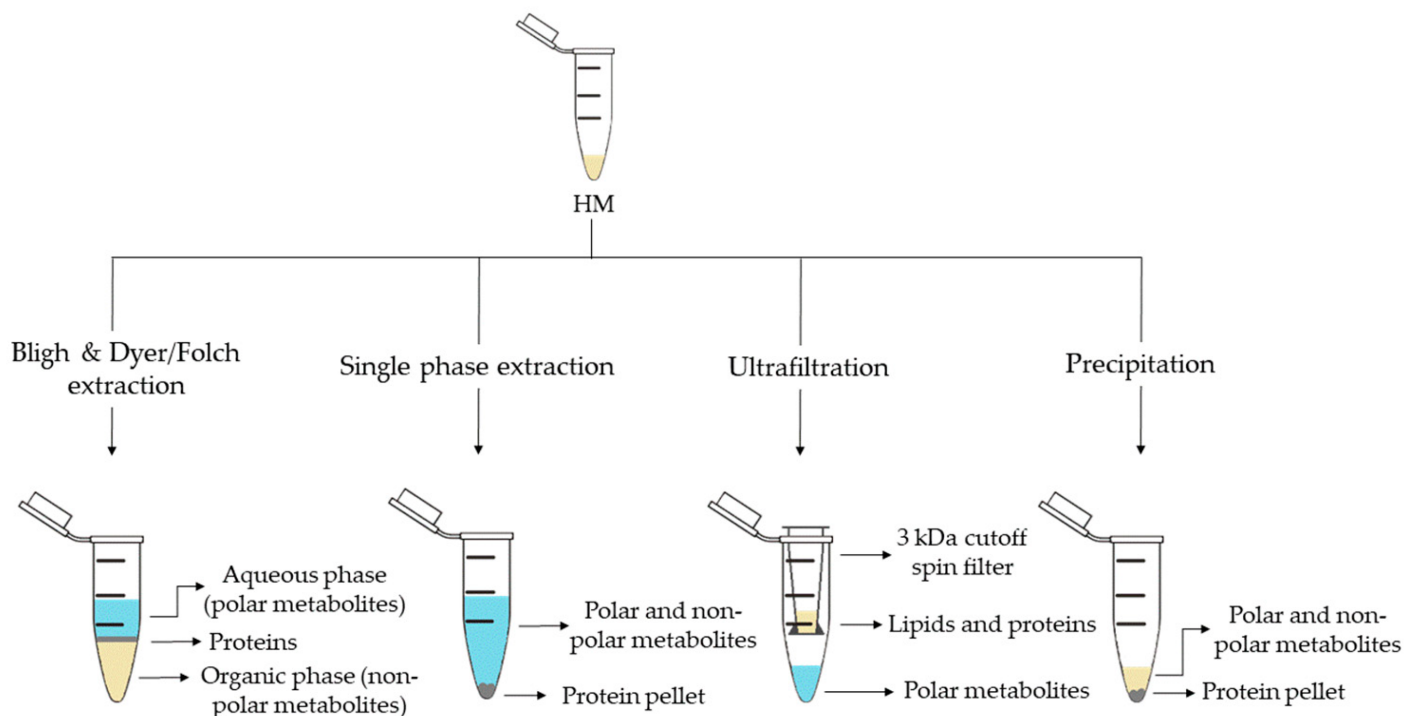
Due to its complex composition, the analysis of HM is not straightforward. While the advent of “omics” approaches has offered valuable insights into the composition of this unique biofluid, untargeted metabolomic and lipidomic studies have only recently been applied to HM [13]. The comprehensive study of the HM metabolome, which includes the intermediate and end products of metabolism, can shed light on maternal status or phenotype [14][15]. The generation, analysis, and integration of large and complex data sets obtained in metabolomic studies go hand in hand with the following challenges: (i) the intrinsic complexity of the sample: a rich variety of jointly present, structurally heterogeneous compounds at concentrations that strongly vary covering several orders of magnitude; (ii) pre-analytical steps related to sampling, storage, and pre-processing (e.g., extraction, clean-up); and (iii) the diversity of platforms currently available including nuclear magnetic resonance (NMR), as well as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) coupled to mass spectrometry (MS). The analysis of the HM metabolome has been approached employing a variety of extraction and analytical techniques to respond to a spectrum of clinically relevant questions. Several studies have compared HM metabolome with formula milk [13][16][17][18][19][20] or with milk from other mammalian species including monkey [21], donkey [17], and cow [18], whereas others have made efforts in defining the metabolome of preterm milk [13][16][22][23][24][25][26] and the evaluation of the HM metabolome during the course of lactation [15][23][27][28][29][30]. Furthermore, the influence of maternal diet [14][15][31], phenotype [14][32], obesity [30], or atopy status [33], as well as geographical location [33][34], time of the day [29][35], chemotherapy [36], or preeclampsia during pregnancy [31] on the HM metabolome have been reported.

## 2. Metabolite Extraction from HM

HM is a biofluid characterized by a dynamically varying composition according to several factors including lactation time, time of the day, throughout each feed, maternal status, and the environmental exposure. Although compositional variations have been mainly studied regarding the protein content of HM [37], changes of other compound classes such as fat or vitamins have been also reported [38][39]. Considering the intrinsic variability of HM, the complexity of obtaining representative HM samples is not negligible. Sources of variation related to sample manipulation and compositional variation can be minimized using standard operational procedures (SOPs). SOPs are fundamental to maintain quality assurance (QA) and quality control (QC) process and facilitate repeatable and reproducible research within and across laboratories. However, biologically meaningful results across studies will only be obtained if several key factors during the sample collection process are successfully controlled. This is of special importance in untargeted approaches, where the interpretation of results is especially challenging, and confounding factors introduced by a non-exhaustive sampling protocol can be wrongly attributed to differences between subjects of a studied population. Conversely, biologically meaningful information can be missed or remain unnoticed due to unwanted bias introduced during sample collection.

For metabolite extraction from HM, an array of methods has been reported. An overview of the employed approaches is shown in **Figure 1**. The selection of the extraction method is conditioned by the study objective and the subsequent analysis method. As in other untargeted metabolomics workflows, for HM metabolomics, the selected sample preparation approach should enable a high degree of metabolome coverage while making the

sample matrix compatible with the analytical platform. Other considerations might include the available amount of sample volume and the use of one sample extraction procedure for subsequent analysis by multiple, complementary analytical platforms [13][27][28].



**Figure 1.** Sample preparation approaches employed in human milk (HM) metabolomics.

Liquid-liquid extraction (LLE) is the classical extraction method employed in metabolomics and lipidomics. This method, developed by Folch et al. [40] in 1957, uses a chloroform-methanol mixture (2:1, v/v), which results in two differentiate phases: an upper phase containing polar metabolites and a lower phase containing nonpolar metabolites. Subsequently, in 1959 Bligh and Dyer [41] developed a modified method using a miscible chloroform-methanol-water mixture and later separated into two phases by adding chloroform or water. Both approaches enable the separation of polar and nonpolar metabolites, thus, allowing the analysis of a wide range of metabolites and making them compatible with several analytical platforms. While the use of Bligh and Dyer LLE is widely extended for HM metabolomics studies (see **Table 1**) [13][16][17][18][19][24][25][29][32], only Andreas et al. [28] used a modified Folch extraction protocol for processing HM samples.

**Table 1.** Sample preparation steps and platforms employed in untargeted analysis of HM metabolome.

Sample Preparation (1st. Preparation step)	Sample Preparation (2nd. Preparation step)	Compound Class	Platform	Column/Capillary	References
Bligh & Dyer extraction	Deuterated solvent addition to aqueous phase	Polar metabolites	$^1\text{H-NMR}$	-	[13][16][29][32]

Sample Preparation (1st. step)	Sample Preparation (2nd. step)	Compound Class	Platform	Column/Capillary	References
	Derivatization of aqueous phase: methoximation and silylation	Polar metabolites and FAs	GC-MS	DB-5ms	[17][18][19]
	Derivatization of organic phase: methylation	FAs	GC-MS	DB-5ms	[13]
	Direct injection of aqueous phase	Polar metabolites	LC-QTOF-MS (+)	HILIC	[35]
	Redissolution of aqueous phase in H <sub>2</sub> O:ACN (95:5)	Polar metabolites	LC-Orbitrap-MS (+, -)	C18	[24]
	Redissolution of organic phase in (ACN:IPA:H <sub>2</sub> O (65:30:5)	Lipidic metabolites	LC-Orbitrap-MS (+,-)	C18	[25]
Folch extraction	Deuterated solvent addition to aqueous and organic phases	Hydrophobic and polar metabolites	<sup>1</sup> H-NMR	-	
	Redissolution of aqueous phase in formic acid and centrifugation	Polar metabolites (amino acids)	CE-TOF-MS (+)	60 m × 50 μm I.D.	[28]
	Redissolution of organic phase in IPA:H <sub>2</sub> O:ACN (2:1:1) and centrifugation	Lipidic metabolites	UPLC-QTOF-MS (+,-)	C18	
Single phase extraction	Derivatization: methoximation and silylation	Polar metabolites and FAs	GC-MS	DB-5ms	[27][28]
	Direct injection	Lipidic (and polar) metabolites	LC-QTOF-MS (+,-)	C8	[27][28]
			UPLC-QTOF-MS (+)	C18	[15]

Sample Preparation (1st. step)	Sample Preparation (2nd. step)	Compound Class	Platform	Column/Capillary	References
Fat extraction with n-hexane/IPA	Deuterated solvent addition	TGs	<sup>13</sup> C-NMR; <sup>1</sup> H-NMR	-	[20]
Filtration 3 kDa cutoff spin filter	Deuterated solvent addition	Polar metabolites	<sup>1</sup> H-NMR	-	[14][21][22][29][33]
Protein precipitation	Derivatization: methoximation and silylation	Polar metabolites	GC-MS	DB-5ms	[36]
	Hybrid SPE-Phospholipid extraction and redissolution in diluted organic phase of Bligh & Dyer extraction	Lipidic metabolites	LC-QTOF-MS (+)	C8	[35]
	Fat removal with CH <sub>2</sub> Cl <sub>2</sub> and dansylation of aqueous phase	Polar metabolites (amine/phenol submetabolome)	Chemical isotope labelling LC-QTOF-MS (+)	C18	[42][43]
	Direct injection	Polar metabolites and FAs	UPLC-QTOF-MS (+,-)	C18	[18]
Fat removal by centrifugation	Two additional centrifugations and deuterated solvent addition	Polar metabolites	<sup>1</sup> H-NMR	-	[34]
[27]	Filtration 10 kDa cutoff spin filter and deuterated solvent addition	Polar metabolites	<sup>1</sup> H-NMR	-	[23][26]
Homogenization	Deuterated solvent addition	Polar metabolites	<sup>1</sup> H-NMR	-	[31]
H <sub>2</sub> O-dilution	NaBH <sub>4</sub> -reduction and PGC cartridge	Oligosaccharides [15][27][28]	UPLC-TQD-MS (+)	Hypercarb®	[24][28]

complementary analytical platforms.

Ultrafiltration makes use of centrifugal molecular weight cutoff filters. Different molecular weight cut-off filters are commercially available for this purpose and repeated centrifugation steps might be employed to remove proteins and lipids (see **Table 1**). Unlike single-phase extraction, ultrafiltration allows to separate polar metabolites from the chromatography; IPA, 2-propanol; I.D., inner diameter; LC, liquid chromatography; MS, mass spectrometry; <sup>13</sup>C-

NMR without a built-in reference compound. The major challenge in the analysis of HM metabolites is the low abundance of metabolites, which requires the use of sensitive detection methods. At present, LC-MS is the only method used for the analysis of HM metabolites, with LC-MS/MS being the most common method. LC-MS/MS is a combination of LC and MS, where the metabolites are first separated by LC and then analyzed by MS. The most common LC method used for HM metabolite analysis is reversed-phase LC (RPLC), which separates metabolites based on their hydrophobicity. The most common MS method used for HM metabolite analysis is electrospray ionization (ESI), which ionizes metabolites by spraying them into a fine mist. The combination of RPLC and ESI is known as LC-ESI-MS/MS. Other MS methods used for HM metabolite analysis include atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption/ionization (MALDI).

Precipitation with organic solvents separates the polar and nonpolar metabolites of the proteins that settle at the bottom of the tube which can then be easily removed by centrifugation. This simple method has been employed for the analysis of polar metabolites by GC-MS after derivatization [36] as well as for the analysis of polar and nonpolar metabolites by LC-MS without further pre-processing [18]. Furthermore, this approach has been implemented in more sophisticated workflows as recently shown by Hewelt-Belka et al. [35]. Here, the authors combined LLE and a protein precipitation and solid-phase extraction (SPE) procedure to prepare HM samples, thereby, enabling the detection of high- and low-abundant lipid species (e.g., glycerolipids and phospholipids) in one LC-MS run.

### 3. The HM Metabolome: Compound Annotation and Coverage

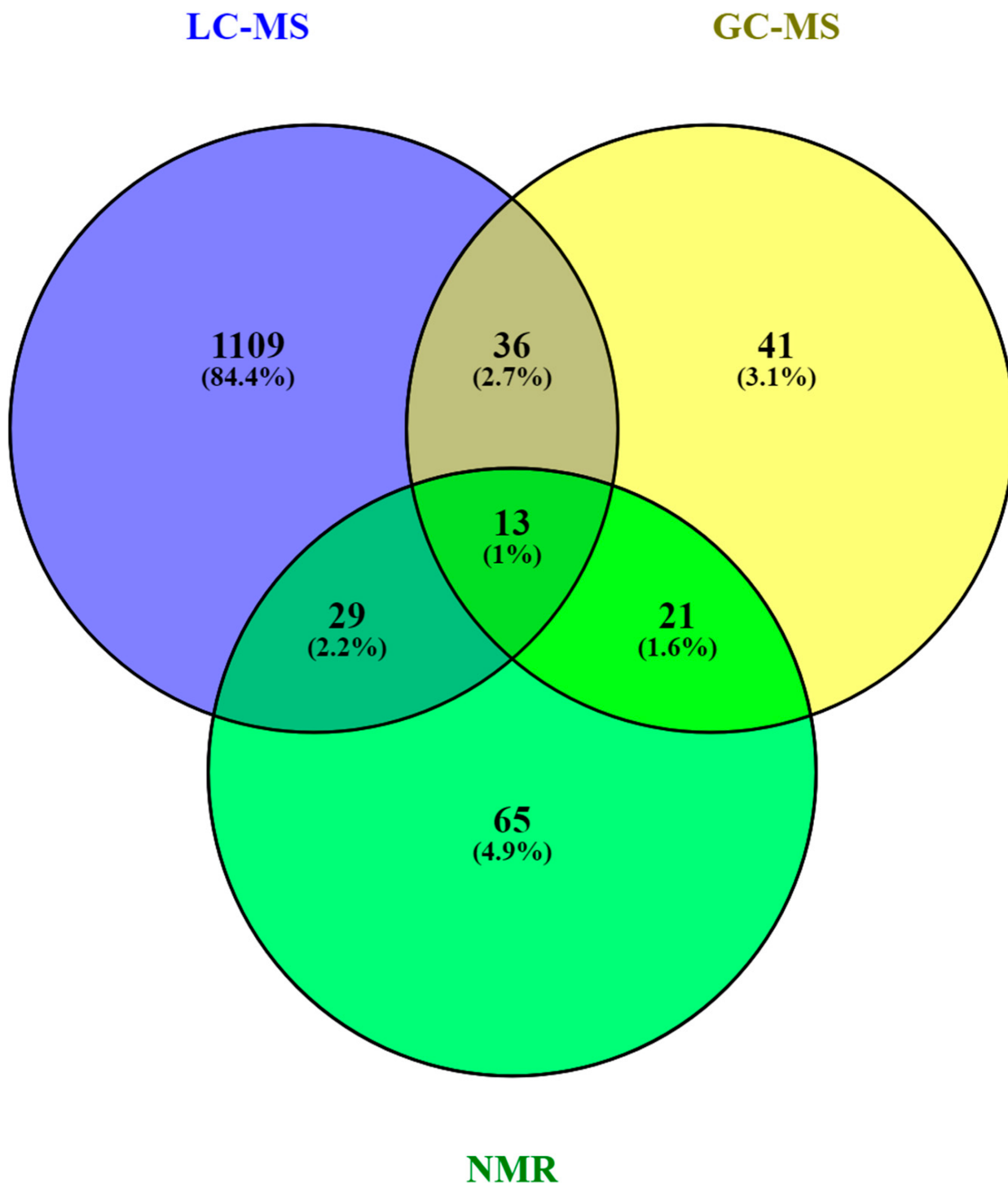
As in other areas of metabolomic research, compound identification is still a major bottleneck in data analysis and interpretation. The Metabolomics Standards Initiative's (MSI) defines four levels of metabolite identification, which include: identified metabolites (level 1); putatively annotated compounds (level 2); putatively annotated compound classes (level 3); and unknown compounds (level 4) [44]. Due to the limited availability of pure analytical standards required to reach level 1, biological databanks and spectral databases are the most important resources for metabolite annotation (levels 2 and 3). A large number of databases are available today, providing different levels of information and complementary data on chemical structures, physicochemical properties, biological functions, and pathway mapping of metabolites [45]. The metabolomics community classifies these resources in several categories: (i) chemical databases; (ii) spectral libraries; (iii) pathway databases; (iv) knowledge databases; and (v) references repositories [46].

Regarding HM metabolomics, the most frequently used databases and libraries are: Human Metabolome Database (HMDB) [47], Metabolite and Chemical Entity Database (METLIN) [48], National Institute of Science and Technology (NIST) library, Fiehn RTL Library [49], LipidMAPS Structure Database (LMSD) [50], Milk Metabolome Database (MCDB) [51][52], Kyoto Encyclopedia of Genes and Genomes (KEGG) [53], MycompoundID with the evidence-based metabolome library (EML) [54], Chemomx NMR Suite Profiles and other online university databases, such as CEU-mass mediator [55][56].

Metabolite assignment in NMR spectra has been performed based on literature data and commercial resonance databases, such as Chemomx NMR Suite Profiles. Metabolite annotation was contrasted with in-house libraries containing pure compound spectra. Some of the proposed assignments were confirmed by two-dimensional NMR spectra, such as Correlation Spectroscopy (COSY) [13][29][31][32], Homonuclear Correlation Spectroscopy (TOCSY) [13][31][32][34], Diffusion-Ordered Spectroscopy (DOSY) [32], Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) [32][34], and Heteronuclear Multiple Bond Correlation (HMBC) [32].

In LC-MS and CE-MS-based studies of the HM metabolome, tentative metabolite annotation has been carried out by matching of accurate masses, isotopic profiles, and/or fragmentation patterns to candidate metabolites in online databases such as KEGG, METLIN, LipidMAPS, and HMDB [18][24][25][27][28][35]. In-house built databases generated by the analysis of commercial standards are also commonly employed [24][25]. In GC-MS, retention index (RI) corrections are made by analyzing a fatty acid methyl ester (FAME) mixture standard solution and assigning a match score between the experimental FAME mixture and theoretical RI values based on the values contained in the Fiehn RTL library. Furthermore, metabolites were complementarily annotated by comparing their mass fragmentation patterns with those available in Fiehn RTL and NIST libraries [13][17][18][19][27][28][36].

A comprehensive list of annotated and/or identified metabolites in HM from untargeted metabolomics studies [14][15][17][18][19][21][22][23][24][25][26][27][28][29][31][32][33][34][35][36] is reported. This table contains information about the metabolites reported in each reference, such as their molecular formula, IDs (LipidMAPS and/or HMDB IDs), the extraction procedure performed, the analytical platform used, and the detected metabolite class. Readers can select metabolites dynamically by filtering data according to the latter information. A total of 1187, 111, and 128 metabolites were reported using LC-MS, GC-MS, and NMR, respectively (see **Figure 2**). As shown in the Venn diagram, LC-MS and GC-MS allowed the detection of 36 common metabolites (mainly carbohydrates and FAs); a total of 29 metabolites overlapped between LC-MS and NMR (principally oligosaccharides); and 21 metabolites (predominantly amino acids and organic acids) were commonly reported in GC-MS and NMR based studies. Only 13 metabolites were reported by all three platforms, i.e., creatine, tyrosine, arabinose, galactose, glucose, lactose, maltose, capric acid/caprate, caprylic acid/ caprylate, citric acid/citrate, pyruvic acid/pyruvate, hippuric acid/hippurate, and myo-inositol. These metabolites were assigned to different classes including amino acids, carbohydrates, FAs, and organic acids.

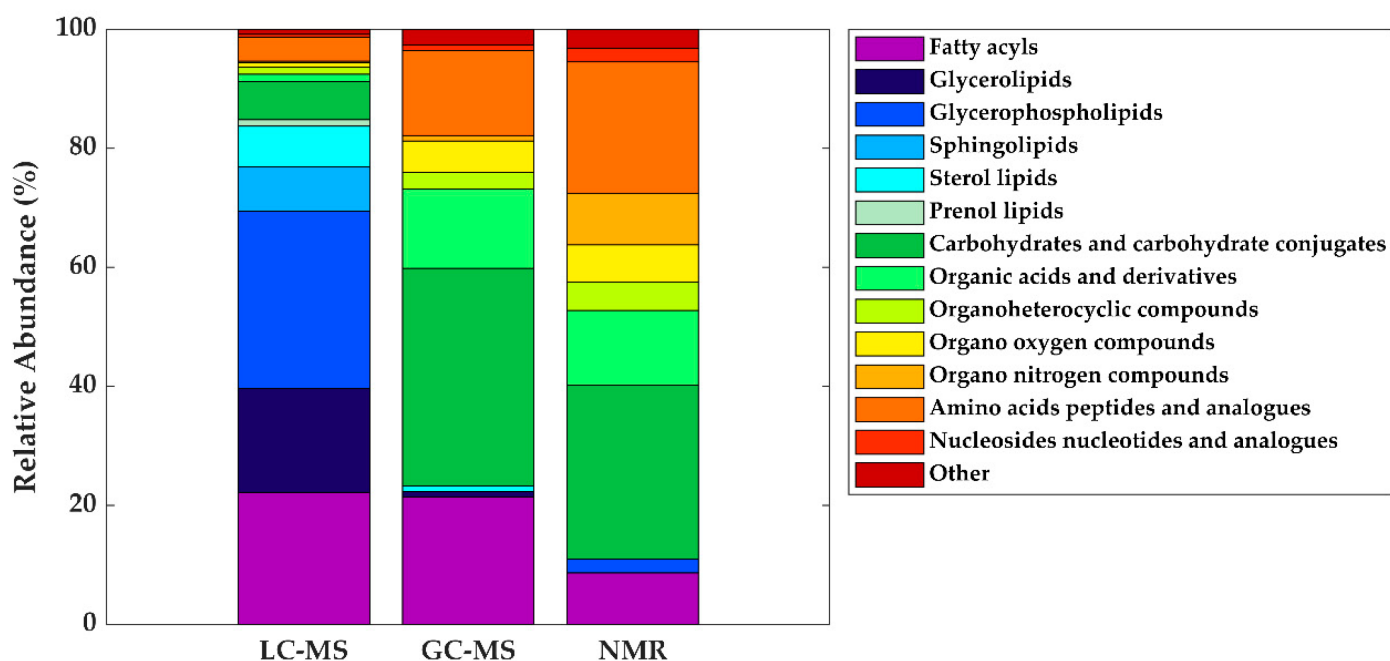


**Figure 2.** Venn diagram of metabolites reported in human milk (HM) according to the technique in [57]. Note: GC-MS, gas chromatography—mass spectrometry; LC-MS, liquid chromatography—mass spectrometry; NMR, nuclear magnetic resonance.

Based on the available data from the literature, the distribution of metabolite classes present in HM according to each technique was assessed. As can be seen in **Figure 3**, the difference in detected metabolite classes as observed by LC-MS in comparison to GC-MS and NMR is evident. Using GC-MS and NMR, carbohydrates are the most reported metabolites in HM, followed by amino acids, organic acids, organooxygen compounds, and



organoheterocyclic compounds, with all these metabolite classes being certainly less abundant in LC-MS studies. In the case of NMR, organonitrogen compounds have also been reported, as well as nucleosides and nucleotides on a smaller scale. In the case of lipid classes, fatty acyls have been identified by LC-MS and GC-MS with similar incidence and in lesser extent by NMR. It is indubitable that lipid classes are more comprehensively studied by LC-MS assays, where glycerophospholipids, glycerolipids, and fatty acyls are detected at relatively high abundances, followed by sphingolipids, sterol lipids, and, to a lesser extent, prenol lipids.



**Figure 3.** Distribution of metabolite classes annotated and/or identified in HM according to technique. Note: GC-MS, gas chromatography—mass spectrometry; LC-MS, liquid chromatography—mass spectrometry; NMR, nuclear magnetic resonance.

**Table 2** shows a list of metabolites reported in > 80% of studies employing either LC-MS, GC-MS, or NMR-based assays. This table is intended to aid method development of future untargeted metabolomics workflows tailored to the study of the HM metabolome, as it shows a shortlist of metabolites that should be detected by each platform regardless of the instrumental settings employed. It should be noted that due to the high versatility of LC-MS, there is a greater variation in metabolites recorded and in return, the list of consistently reported metabolites in HM across studies is shorter than for NMR and GC-MS, where differences in experimental conditions and variations between the employed detection parameters and instruments are smaller. Again, this table represents the high orthogonality between the detected metabolites using NMR and LC-MS. While the use of LC-MS is clearly of advantage for the measurement of different lipids, NMR provides information on amino acids and small organic acids. Metabolome coverage provided by GC-MS falls in-between the other two platforms, consistently providing information on lipids, sugars, amino acids, and organic acids.

**Table 2.** Most frequently reported metabolites (>80% of studies) according to technique.

Metabolite class	LC-MS	GC-MS	NMR
Fatty acyls	Linoleic acid (C18:2)	Oleic acid (C18:1)	
	Oleic acid (C18:1)	Palmitic acid (C16:0)	-
	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	
Glycerolipids	DG (36:1)	-	-
Glycerophospholipids	LysoPC (16:0)	-	-
Carbohydrates and carbohydrate conjugates	-	Fructose	
		Fucose	Lactose
		Ribose	
Organic acids and derivatives	-	Malic acid	Acetate
		Urea	Citrate
			Lactate
Organo nitrogen compounds	-	-	Choline
Amino acids, peptides, and analogues	-	Alanine	Alanine
		Glutamate	Creatine
		Glycine	Glutamate
		Pyroglutamic acid	Glutamine
		Serine	Isoleucine
		Valine	Leucine
		Tyrosine	
		Valine	

GC-MS, gas chromatography—mass spectrometry; LC-MS, liquid chromatography—mass spectrometry; NMR, nuclear magnetic resonance; DG, diacylglycerol; PC, phosphatidylcholine.

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## 4. Conclusions and Future Perspectives

In less than a decade, 26 research papers have been published trying to shed light on the complex and dynamic composition of HM and the feasibility of different options for sample extraction and metabolite detection has been demonstrated. Due to the many factors that influence HM composition, a thorough study design including SOPs for milk extraction, collection, and storage is indispensable for obtaining biologically meaningful results. Multi-platform approaches are encouraged for providing adequate metabolome coverage, as the diversity of compounds and Der, G., Batty, G.D., Draper, J. Effect of breast feeding on intelligence in children: Prospective study, sibling pairs analysis, and meta-analysis. *BMJ* 2006, 333, 945–948. The implementation of QA/QC procedures. Automated metabolite annotation and identification with pure chemical standards is warranted

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