Advanced Analytical Techniques Applied in Cow Milk Metabolomics

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Abstract
Milk metabolomics provides a comprehensive analysis of the biochemical content of cow's milk, revealing both the animal's physiological condition and the quality of the end product. Through the identification and quantification of low-molecular-weight metabolites, such as amino acids, fatty acids, sugars, and organic acids, metabolomics enables the detection of biomarkers associated with mastitis, nutritional imbalances, and milk authenticity. The establishment of reliable metabolic profiles relies on advanced analytical techniques, including nuclear magnetic resonance spectroscopy and liquid or gas chromatography coupled with mass spectrometry. This study synthesizes and critically reviews recent research on bovine milk metabolomics employing these state-of-the-art methodologies, aiming to elucidate the biochemical diversity and metabolic complexity underlying milk composition.

Keywords: biomarkers, cow, chromatography, milk, metabolomics, spectrometry.

1. Introduction

Milk is a complex biological fluid whose composition reflects both the dairy cow's physiological state and the quality of the end product. Aside from standard measurements like fat, protein, and lactose content, milk contains a wide variety of low-molecular-weight metabolites that provide biological information. Milk metabolomics, or the detailed investigation of these metabolites, has emerged as a valuable tool for assessing animal health, diagnosing disease, monitoring nutritional status, and confirming milk authenticity.

Metabolomics is the study of substances such as amino acids, organic acids, sugars, fatty acids, nucleotides, and other small molecules produced by gene expression and cellular activity. Because the metabolome responds swiftly to physiological and environmental changes, milk metabolomics is well-suited to discovering biomarkers of mastitis, metabolic diseases, and dietary imbalances, as well as improving dairy quality management [1, 2].

Modern techniques, such as nuclear magnetic resonance (NMR) and liquid or gas chromatography with mass spectrometry (LC-MS, GC-MS), can detect and measure amino acids, fatty acids, sugars, organic acids, and other bioactive substances.

Thus, metabolomics is growing as an interdisciplinary field that integrates biology, analytical chemistry, and bioinformatics to provide a comprehensive assessment of milk quality and cattle health. Metabolomics is the systematic study of small molecules in a biological system. Metabolomics in bovine milk captures the downstream effects of gene expression, protein activity, nutrition, microbiota, and environmental factors [3]. Unlike specialized

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chemical investigations, metabolomics provides a full metabolic fingerprint of milk.

Milk metabolites are produced by mammary gland metabolism, blood transport, microbial activity, and biochemical reactions that occur after secretion. As a result, changes in metabolite concentrations can reflect physiological characteristics such as lactation stage, energy balance, inflammatory reactions, and feed composition. As a result, milk metabolomics has become an important tool for quality control, animal health monitoring, and precision dairy production [4].

This paper synthesizes and critically examines recent research on bovine milk metabolomics using cutting-edge approaches, with the goal of elucidating the biochemical diversity and metabolic complexity that underpin milk composition.

2. Results and discussion

Metabolomic analysis of cow's milk provides a complex insight into the biochemical dynamics that reflect the health of the animal and the quality of the final product.

Metabolomics is emerging as an integrative field, capable of connecting chemical and biological data into a coherent system for assessing animal health and the nutritional value of milk. Figure 1 summarizes the workflow of metabolomics studies in the literature, from biological sample collection and processing to instrumental analysis and statistical interpretation of data.

2.1. Analytical techniques used in milk metabolomics

2.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a popular approach in milk metabolomics due to its excellent reproducibility, minimal sample preparation, and non-destructive nature. NMR enables the simultaneous identification and quantification of key milk metabolites, including lactose, citrate, lactate, acetate, choline, and free amino acids.

While NMR has lower sensitivity for metabolite detection compared to mass spectrometry-based approaches, its quantitative robustness makes NMR well-suited for untargeted metabolomic profiling and longitudinal research, where accurate quantification is essential. As a result, NMR-based metabolomics has been successfully used to differentiate milk samples based on health state, lactation stage, and feeding regimen [5].

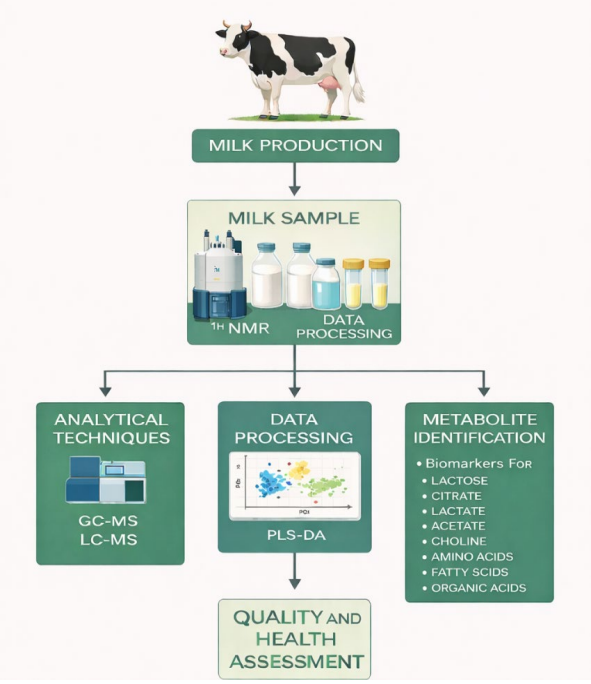

Figura 1. Schematic representation of the metabolomic workflow applied to bovine milk

Minimal sample preparation represents another key advantage of NMR-based milk metabolomics. Typically, milk samples require only centrifugation and dilution before analysis, reducing analytical variability and preserving the native metabolic profile. Moreover, the non-destructive nature of NMR allows samples to be reanalyzed or combined with complementary techniques such as mass spectrometry.

Although NMR exhibits lower sensitivity compared to LC-MS or GC-MS, the study emphasizes that its detection limits are sufficient for the majority of abundant milk metabolites. This makes NMR particularly well suited for untargeted metabolomic fingerprinting, where the objective is to capture global metabolic variation rather than low-abundance compounds.

Importantly, NMR-based metabolomic profiling was shown to effectively discriminate milk samples according to lactation stage, metabolic status, and feeding regimen.

Metabolic shifts observed between early and late lactation reflected changes in energy balance and mammary metabolism, highlighting the potential of milk metabolomics as a non-invasive indicator of cow health and physiological adaptation. Researchers also recommend a complementary multi-platform method that combines NMR and mass spectrometry to gain greater metabolite coverage [5, 6].

Metabolic analysis using ^{1}H-RMN demonstrated that milk metabolites are strongly influenced by biological factors such as genetics, lactation stage, and udder health. Metabolites solubilized in milk represent both the metabolic activity of the mammary glands and the permeability of epithelial barriers, which is altered in inflammatory conditions such as mastitis. Studies demonstrated that RMN allows for the differentiation of milk based on the animal's physiological state, providing comprehensive information on the energetic, proteic, and glucidic metabolism [7].

The intricacy of milk's matrix might cause analytical interferences and matrix effects, particularly when determining the presence of chemicals at low concentrations [8, 9].

To circumvent these limits, modern analytical techniques are combined with pretreatment and purification procedures such as solid-phase extraction. The integration of omics technologies (proteomics, lipidomics, and metabolomics) with modern analytical techniques enables a comprehensive investigation of milk constituents and their interactions [10-13].

Lipids are one of the most common types of milk constituents, with a wide range of saturated, unsaturated, and branched fatty acids and a predominant composition of triacylglycerols [14]. Lactose, the principal carbohydrate in milk, is an important source of energy and contributes significantly to the technical qualities of dairy products [15-18]. Proteins, especially caseins and whey proteins, significantly increase the nutritional and functional value of milk [19–22]. Both conventional and modern extraction techniques have been developed to separate these components, ranging from organic solvent extraction to more ecologically friendly strategies like supercritical CO_2 extraction or microwave-assisted procedures [13, 23].

Over the past years, research has moved away from straightforward descriptive metabolic profiling and toward more focused applications concerning interspecies variations, udder health, lactation physiology, and the assessment of commercial dairy products. For instance, Du et al. (2024) found 54 metabolites in a comparative 1H-NMR metabolomic investigation of human milk, cow milk, goat milk, and baby formula [24]. Their findings demonstrated that whereas cow and goat milk have species-specific metabolic profiles, human milk has a unique metabolic signature marked by increased quantities of fucosylated oligosaccharides and certain amino acids. One of the most active areas of research in bovine milk is the identification of intramammary infections and mastitis. According to a thorough analysis by Ametaj et al. (2010), milk metabolomic research has previously found a core set of metabolites that are consistently changed during mastitis. Promising biomarker candidates include acetate, arginine, β-hydroxybutyrate, carnitine, citrate, lactate, leucine, isoleucine, phenylalanine, proline, riboflavin, and valine [25].

This research direction has been further strengthened by recent studies specifically using NMR-based metabolomics. For instance, Bonfatti et al. (2025) investigated subclinical mastitis using 1H-NMR analysis of milk samples collected at the mammary quarter level and demonstrated that the metabolic signature of milk, together with the immune cell profile, can reveal not only subclinical inflammation but also a potential functional interdependence between mammary quarters [26].

In a more recent study, Lisuzzo et al., (2026) looked at metabolomic alterations in cow's milk as it changed from a healthy state to clinical mastitis. Their results demonstrated the metabolic connections between intramammary infection, subclinical mastitis, and clinical mastitis, indicating that NMR is helpful for both tracking the course of disease and differentiating between healthy and sick animals. Another important research direction concerns lactation physiology and the transition period [27].

Boccia et al. (2026) showed that the physiological state of dairy cows throughout the dry-off and peripartum period has a bigger influence on the milk metabolomic profile than the treatment options investigated using ^{1}H-NMR spectroscopy and chemometric analysis. Before dry-off, there were higher quantities of choline, butyrate, branched-chain amino acids, and N-acetylated

substances; following calving, there were higher amounts of sugars, citrate, phosphorylcholine, and galactose-1-phosphate [28]. The development of better annotation and quantification tools for milk NMR spectra is one of the most significant recent methodological advancements.

HRMAS-NMR has been effectively used to distinguish yogurt made from cow and goat milk, finding 21 metabolites and clearly distinguishing the two groups using OPLS-DA analysis, according to Kandasamy et al. (2024) [29].

In 2025, the same methodological approach was applied to monitor the ripening process of sheep milk cheeses. Although these studies focus on processed dairy products, they confirm a clear trend: NMR applications in dairy research are expanding from raw milk toward the entire dairy production chain.

Recent studies by Homobono Brito de Moura et al. [30] and Sen et al. [31] emphasize the increasing significance of integrative frameworks connecting feed, biological fluids, and food items, as well as data fusion techniques combining NMR with mass spectrometry. While mass spectrometry enhances the analytical workflow by offering greater sensitivity and the ability to identify metabolites at low concentrations, NMR remains crucial in practice for robustness, quantitative repeatability, and batch comparability.

In general, optimizing milk's nutritional and technological quality as well as encouraging sustainable dairy business practices requires a deep grasp of milk composition and the analytical techniques used to classify it.

2.1.2. Mass Spectrometry-Based Techniques (LC–MS and GC–MS)

Mass spectrometry-based metabolomics, notably liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), has emerged as a key analytical method for comprehensively characterizing the composition of cow milk.

These techniques provide high sensitivity and broad metabolite coverage, enabling the detection of hundreds of chemicals across a wide concentration range, including low-abundance metabolites that are not readily accessible by NMR-based methods [32].

LC-MS is particularly effective at profiling polar and semi-polar metabolites, lipids, and bioactive compounds, making it a valuable tool for studying lipid metabolism, inflammatory mediators, and metabolic adaptations associated with lactation, nutrition, and health status in dairy cows [32, 33].

Because of its high chromatographic resolution and well-established mass spectral libraries, GC-MS is ideal for analyzing volatile and derivatized metabolites such as organic acids, amino acids, sugars, and fatty acids [3].

Applications of LC–MS- and GC–MS-based metabolomics in bovine milk can offer valuable insights into energy balance, mammary gland metabolism, immune response, and metabolic disorders, including mastitis and negative energy balance. These approaches have also been successfully applied to assess the impact of feeding strategies, lactation stage, and management practices on milk metabolic profiles [32, 34]. Compared to NMR, mass spectrometry-based methods require more intricate sample preparation, stringent quality control, and sophisticated data processing, despite their greater analytical capacity. For accurate metabolite identification and biological interpretation, current research highlights the significance of standardized procedures and the integration of chemometric and bioinformatic techniques [35].

In order to achieve thorough metabolome coverage and reliable biomarker identification, the reviewed literature emphasizes LC-MS and GC-MS as essential methods in milk metabolomics, especially when paired with NMR in a multi-platform strategy [32].

Ma et al. (2025) did a study on the non-target metabolic analysis of milk metabolites by LC–MS/MS analysis [36]. Collectively, the study expands metabolite coverage for under-characterized dual-purpose cattle populations and supports the view that milk metabolomics can capture physiologically meaningful variation, especially across lactation stages, while also revealing candidate metabolite signatures associated with milk composition traits and SCC. These findings are directly relevant to breed characterization, milk traceability, nutritional profiling, and biomarker-based health monitoring in dairy systems.

Mass spectrometry-based techniques, particularly liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS), represent some of the most powerful analytical platforms used in milk metabolomics. These techniques are widely applied due to their

high sensitivity, broad metabolite coverage, and ability to detect compounds present at very low concentrations. Compared with NMR spectroscopy, LC–MS and GC–MS provide significantly greater analytical sensitivity and enable the identification of a much wider range of metabolites, including lipids, organic acids, amino acids, and volatile compounds [37]. Liquid chromatography-mass spectrometry (LC-MS) is especially useful for identifying polar and semi-polar metabolites, peptides, and lipids. According to Becchi et al., [37], LC-MS is being used in many metabolomic investigations in dairy science because to its high sensitivity and comprehensive metabolite coverage, which allows researchers to investigate complicated biochemical networks related to milk quality and animal physiology. Modern LC-MS systems, such as UHPLC-QTOF-MS and LC-MS/MS platforms, can detect hundreds of metabolites in a single analysis, yielding full metabolic fingerprints of milk samples.

A recent study has shown that LC-MS-based metabolomics may reveal precise metabolic profiles of milk from various species, breeds, and physiological circumstances. For example, Ma et al. [36] used LC-MS/MS metabolomic profiling to characterize milk metabolites in several cattle breeds and identified over 4000 metabolites, indicating significant metabolic heterogeneity between breeds and emphasizing the genetic influence on milk composition. Similarly, comparative LC-MS studies have been performed to uncover metabolic differences between camel and cow milk, suggesting that LC-MS metabolomics may define species-specific milk composition. [38].

Gas chromatography-mass spectrometry (GC-MS) is another important platform used in milk metabolomics, particularly for the detection of volatile chemicals, fatty acids, and other minor metabolites following derivatization. GC-MS has great chromatographic resolution and repeatability, making it ideal for detecting compounds found in energy metabolism and fermentation processes. According to Mangroliya et al. (2025), GC-MS-based metabolomics has been successfully used to assess the effects of feeding techniques and environmental factors on the metabolite composition of milk [39].

Both LC-MS and GC-MS have been used to study physiological and metabolic changes related to lactation and animal health. Studies have shown that metabolomic profiles produced using mass spectrometry techniques can identify changes in milk composition associated with metabolic disorders, nutritional status, and illness states in dairy cows. For example, metabolite levels such as lactate, citrate, and short-chain fatty acids have been shown to vary in cows with subclinical mastitis, indicating that milk metabolomics can provide significant indicators for early illness diagnosis [40].

Mass spectrometry-based metabolomics has also been used to investigate metabolic alterations during lactation. Previous research using GC-MS and NMR has shown that milk composition varies dramatically between early and late lactation, with many chemicals involved in energy metabolism and mammary gland activity exhibiting differential expression. Such research emphasizes the importance of metabolomic profiling in understanding the metabolic pathways that underpin milk production and physiological response in dairy cows [41].

Beyond metabolite profiling, LC-MS techniques are rapidly being applied in milk proteomics and lipidomics. Nano-flow LC combined with tandem mass spectrometry (nLC-MS/MS) is commonly used to characterize milk proteins and post-translational changes, such as glycosylation and phosphorylation. These analyses shed light on the biological activities of milk proteins and how they differ among populations and lactation stages [42].

Despite their analytical advantages, mass spectrometry-based approaches have some drawbacks. Sample preparation processes are typically more sophisticated than those necessary for NMR, and may include extraction, derivatization, or chromatographic separation steps.

Additionally, data processing and metabolite identification can be difficult due to the vast number of detected spectrum characteristics and the scarcity of reference spectral libraries. Nonetheless, developments in bioinformatics tools, spectrum databases, and machine learning methodologies are gradually enhancing metabolite annotation and data interpretation in LC-MS and GC-MS metabolomics research. In recent years, there has been an increasing interest in combining mass spectrometry platforms with other analytical methods, particularly NMR and multi-omics tactics. According to Zhang et al. [43], integrating

LC-MS, GC-MS, and NMR in metabolomics workflows enables a more comprehensive characterisation of the milk metabolome. It increases our understanding of the biological mechanisms that underpin milk production, quality, and safety.

GC-MS and LC-MS are practical methods in modern milk metabolomics. Their great sensitivity, broad metabolite coverage, and interoperability with multi-omics techniques make them ideal for studies on milk composition, animal health, dairy product quality, and metabolic reactivity to environmental or nutritional factors.

3. Conclusions

Studies in the specialized literature confirm metabolomics' role as an interdisciplinary field that correlates chemical and biological data for a holistic evaluation of cow's milk.

The diversity of macro- and micro-constituent compounds in milk requires the use of a wide range of complementary analytical techniques. The choice of analytical platform depends mainly on the physicochemical nature and concentration of the target analyte. Major compounds, such as proteins, lipids, and carbohydrates, are present in high concentrations and can be analyzed by simple extraction methods and spectrophotometric techniques.

More precise extraction methods and highly sensitive analytical methods, such as LC-MS or GC-MS, are needed for small molecules, including vitamins and other bioactive metabolites. LC-MS is a crucial instrument for advanced research on the composition and functionality of milk, as well as for quality control, due to its exceptional sensitivity and selectivity.

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