Proteome Science

REVIEW

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Mass-spectrometry based metabolomics: an overview of workflows, strategies, data analysis and applications

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Abstract

Background Metabolomics, a burgeoning field within systems biology, focuses on the comprehensive study of small molecules present in biological systems. Mass spectrometry (MS) has emerged as a powerful tool for metabolomic analysis due to its high sensitivity, resolution, and ability to characterize a wide range of metabolites thus offering deep insights into the metabolic profiles of living systems.

Aim of review This review provides an overview of the methodologies, workflows, strategies, data analysis techniques, and applications associated with mass spectrometry-based metabolomics.

Key scientific concepts of review We discuss workflows, key strategies, experimental procedures, data analysis techniques, and diverse applications of metabolomics in various research domains. Nuances of sample preparation, metabolite extraction, separation using chromatographic techniques, mass spectrometry analysis, and data processing are elaborated. Moreover, standards, quality controls, metabolite annotation, software for statistical and pathway analysis are also covered. In conclusion, this review aims to facilitate the understanding and adoption of mass spectrometry-based metabolomics by newcomers and researchers alike by providing a foundational understanding and insights into the current state and future directions of this dynamic field.

Keywords Metabolomics, Mass spectrometry, LC–MS, Metabolites, Metabolic fingerprinting, Analytes

Introduction

The central dogma refers to the flow of information from DNA through mRNA transcripts, which are then translated to proteins. The enzymes amongst these proteins catalyze metabolic reactions and thus influence the concentrations of *metabolites* in metabolic pathways. The turnover rate of metabolites through a metabolic pathway, that is "flux", generates a phenotype. Just as "omics" of DNA, mRNA and protein are referred to as genomics, transcriptomics and proteomics, respectively, *metabolomics* is the study of metabolome of a cell, tissue, organ, biofluid, media or organism (Fig. 1). *Metabolome* refers to the total complement of metabolites present in a sample under a particular set of conditions. *Metabolites* are small organic molecules with low molecular weight (50–1500 Da) that are involved in biochemical reactions as a substrate, intermediate and product, e.g. sugars, lipids, fatty acids, amino acids, nucleotides, etc. Related to metabolomics is *metabonomics* which measures and compares the overall metabolic profile, not the individual metabolites, of a sample or organism in response to drugs, nutrition, and disease. The term "metabolome" was first used in 1998 [1] whereas the terms "metabonomics" and "metabolomics" were coined in 1999 and 2000

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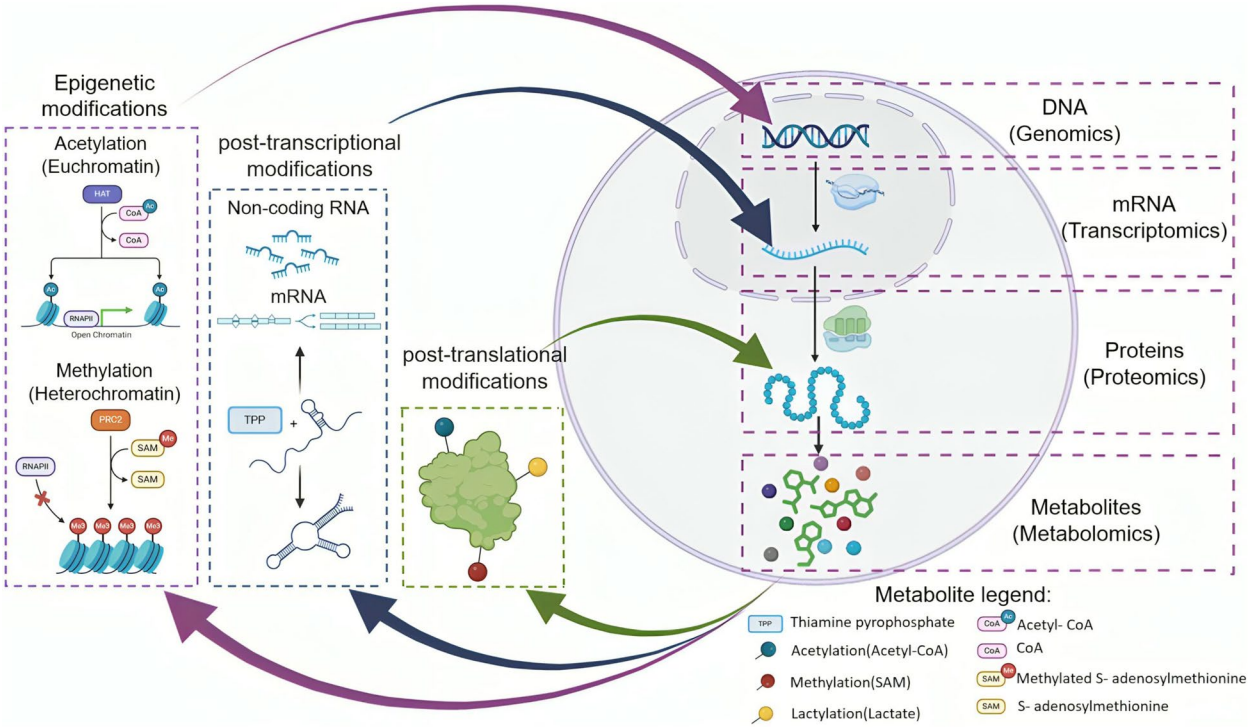

Fig. 1 Four major omics fields, from genomics, transcriptomics, proteomics and metabolomics. Metabolites like S-adenosyl methionine (SAM) and acetyl-CoA play important regulatory roles in epigenetic modifications (e.g. methylation, acetylation, respectively). TPP (Thiamine Pyrophosphate) is involved in riboswitch activation. Metabolites like SAM, lactate and acetyl-CoA are involved in post-translational modifications of proteins (e.g. methylation, lactylation and acetylation)

respectively [2, 3]. Metabolomics is an advance omics technology that aims at studying the metabolites, their identification and concentration, thus, representing the biochemical scenario in a biological sample. It is an analytic method that employs an interdisciplinary approach involving basic sciences, bioinformatics, epidemiology and clinical research.

Global metabolomic alterations reflect upon the cellular or organismal response to changes due to disease, nutrition, environment, genetic variation, enzyme kinetics, metabolic pathways and, changes in gut microflora [4]. Accordingly, metabolomic alterations represent changes in the phenotype and molecular physiology [5]. Owing to the efficient sample preparations and rapid analysis of the samples, metabolomics is a powerful omics technology that has great potential to impact clinical health practices [6]. The correlation of metabolomics data with that of genomics and proteomics data may provide useful insights into the biology of the disease. Metabolic profiling has been referred to as either metabolomics or metabonomics where metabolomics refers to the total measurable metabolite pool that exists in a sample [7] and metabonomics is the quantitative measurement of the dynamic metabolic response of living systems to pathophysiological stimuli [2]. Metabolomics is highly informative as the metabolite pool detected in a biological sample could reflect not only the genetics but also the effect of factors like diet, drugs, exercise, disease status, gut microbiota, hormonal homeostasis, drug toxicity, and age [8]. Based on the detected metabolites and their concentration, activity of a particular metabolic pathway could be assessed in a particular disease [9].

Metabolomics workflow and procedures

A systematic workflow is essential for conducting metabolomic studies effectively, ensuring the accurate identification and quantification of metabolites. This section discusses the key steps in a typical metabolomics workflow, from sample processing, analytics to data analysis and interpretation.

Sample collection, processing and metabolite extraction

Sample collection and preparation are critical steps in the metabolomics workflow (Fig. 2), as they directly impact the quality and reliability of the metabolomic data. The choice of sample (cell, tissue, blood, urine etc.) depends on the research question and metabolites of interest. For example, if one is interested in intracellular metabolic

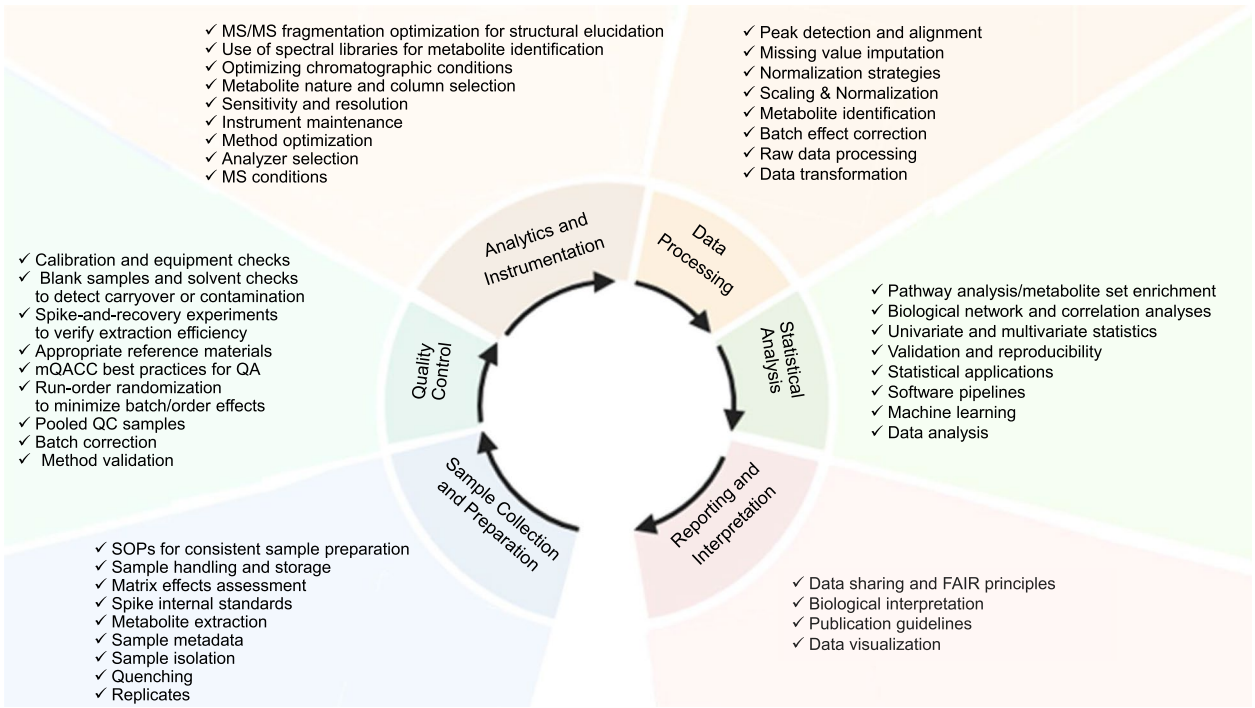

Fig. 2 Steps involved in a typical metabolomics workflow

pathway, cells or tissue would be appropriate choice whereas urine may be relevant for biomarkers of bladder cancer and/or kidney cancer [10, 11]. It is crucial to avoid contamination of samples and therefore sterile techniques, and appropriate collection containers should be used. To minimize variability and for the purpose of standardization, it is preferred to collect samples at the same time of day, under similar conditions, and in a consistent manner. Samples should be processed as soon as possible to minimize changes in metabolite levels.

The first and a vital step in sample processing is the rapid quenching (enzymatic inhibition) of total metabolism followed by the extraction of metabolites in such a way that extract obtained should quantitatively reflect the endogenous metabolite levels originally present in the sample [12, 13]. Because living cells and tissue are metabolically active systems, the quenching step becomes particularly important but not so much with biological fluids like blood, plasma, urine etc. There are several ways of quenching e.g. flash freezing in liquid N_2, pouring liquid N2 directly onto sample (if cells), using chilled methanol (−20˚C or −80 ˚C) and ice-cold PBS [14–17]. Quick quenching should be done as soon as possible after sample collection, delay may result in deviation of metabolic scenario from the one desired to be investigated. The efficiency of quenching can be estimated by determining the abundance of (stable isotope-labeled) standards spiked into the quenching solvent [18]. Samples can be stored followed by quenching at −80 ˚C till the application of extraction solvent.

Followed by quenching is organic solvent-based precipitation of proteins and extraction of metabolites. Efficient sample processing is crucial to prevent degradation of labile metabolites and to achieve high quality data. Reproducible quantification of metabolites depends on the quality of sample processing, therefore, optimization of extraction method in accordance with the sample type and metabolomics strategy (targeted or untargeted) is important [19, 20]. For instance, non-targeted metabolomics needs extraction methods that should capture broad range of metabolites, however, physico-chemical diversity of metabolites makes extraction methods challenging [21]. During extraction compounds of interest (metabolites) are separated from other, often undesired, compounds like proteins. A commonly used extraction method is liquid–liquid extraction, which relies on differential immiscibility of solvents. Compounds can be separated based on their differential solubilities in immiscible solvents, thus, leading to partitioning. Polar, aqueous solutions are often paired with non-polar organic solvents such as chloroform to form a two-phase system for liquid–liquid extraction [22]. This allows the separation of polar and non-polar metabolites for subsequent analytical analysis. Traditionally, "Folch" method and its variant, "Bligh & Dyer "method, has been used for extraction of lipids from tissues [23, 24]. However, other solvents

Table 1 Types of extraction solvents with examples, characteristics and target metabolites

Solvent type	Characteristics	Target metabolites
Polar solvents • Water • Methanol • Ethanol • Acetonitrile • Isopropanol • Acetone	• High polarity • Miscible with water • Effective for polar metabolites • Biocompatible • Versatile mixtures possible	• Amino acids • Sugars • Sugar phosphates • Nucleotides • Polyamines etc.
Non-polar solvents • Chloroform • Methyl tert-butyl ether (MTBE) • Hexane • Dichloromethane	• Low polarity • Hydrophobic	• Lipids • Fatty acids • Ceramides • Hormones • Cholesterol etc.
Biphasic or mixed solvents • Ethanol–water • Methanol-chloroform • Acetone–water • Methanol/isopropanol/water	• Combination of polar and non-polar properties	

like MTBE (methyl tert-butyl ether) are also used for lipid extraction. MTBE is a non-polar solvent that has a high affinity for lipids and is commonly used for extracting lipophilic metabolites from biological samples [25].

Usually, biphasic liquid–liquid extraction is used to extract metabolites [26]. However, the nature of the organic and aqueous solvents, their volumes, solvent ratios, and aqueous solvent pH, must be considered carefully because these parameters can significantly affect the total number of metabolites extracted and data reproducibility. Various solvents used for liquid–liquid extraction methods are: methanol (MeOH), methanol/chloroform ($MeOH/CHCl_3$), methanol/chloroform/water ($MeOH/CHCl_3/H_2O$), acetone (CH_3COCH_3), acetone/water (CH_3COCH_3/H_2O), Methanol/isopropanol/water (MeOH/IPA/H2O), Acid–base methanol (acid–base MeOH), acetonitrile (ACN), ethanol (CH_3CH_2OH), perchloric acid ($HClO_4$) [16, 20, 21]. Methanol/chloroform is the classical and most widely used for biphasic extraction of metabolites. Polar metabolites get extracted in methanol whereas non-polar metabolites (lipids) are extracted in chloroform [20]. The methanol-to-chloroform ratio can be adjusted to optimize the extraction of polar and non-polar metabolites. For lipid extraction, chloroform is preferable, with typical ratios such as 1:1 or 2:1 or 3:1 MeOH: $CHCl_3$ [27, 28]. Since lipids exhibit both polar and non-polar characteristics, methanol is essential alongside chloroform for effective extraction. Conversely, 100% MeOH or 9:1 MeOH:$CHCl_3$ would be preferred for extracting highly polar metabolites [16]. Typically, liquid–liquid extraction of metabolites is performed at neutral pH. However, by taking advantage of the differences in metabolite acid–base chemistry and varying the pH of the aqueous extraction solvent, extraction of metabolite classes from either biofluids or cells can be significantly improved. Table 1 compares different extraction solvents, their characteristics and target metabolites.

To manage the variations in extraction and other experimental variations, internal standards (usually labeled isotopes of metabolites or a structurally similar metabolite (not present in biological sample naturally) should be added at known concentrations to extraction buffer prior to sample processing. Internal standards enable the accurate quantification of metabolites by providing a reference for comparison [29, 30]. By compensating for variability, internal standards enhance the accuracy of metabolite quantification. Consequently, internal standards increase the robustness of metabolomic analyses, making it easier to compare results across different samples or studies. The internal standard selection is based on the type of metabolites studied, at a minimum, one representative internal standard could be taken for a particular class of metabolites. The internal standard should have similar chemical properties to the target metabolites and should be stable throughout the sample preparation and analysis to ensure comparable behavior during extraction and analysis.

Quality assurance and quality control

Quality Assurance (QA) and Quality Control (QC) are critical components in metabolomics, ensuring the reliability, reproducibility, and integrity of data generated in metabolomic studies. Given the complexity of biological samples and the myriad of metabolites present, implementing robust QA and QC protocols is essential for drawing meaningful conclusions. Accordingly, the Metabolomics Quality Assurance and Quality Control Consortium (mQACC) is a collaborative effort dedicated

to defining and advancing best practices in quality assurance (QA) and quality control (QC) within the field of metabolomics [31, 32]. Recognizing the complexity and variability inherent in metabolomic studies, the mQACC seeks to enhance data reliability, reproducibility, and overall scientific rigor. Similarly, the lipidomics standards initiative consortium is developing common standards for minimum acceptable data quality and reporting for lipidomics [33].

QA in metabolomics involves a series of systematic processes implemented well before sample collection. It encompasses all activities that contribute to the overall quality of the data and the experimental processes. Establishing robust QA practices at this initial stage is crucial for ensuring that the resulting data are reliable and reproducible. QA activities include: formal design of experiment; selection of appropriate biological sample; analyses to determine the appropriate sample size needed for statistical significance; standard operating procedures for biobanking, sample handling, and instrument operation; instrument system suitability tests (SSTs) and calibration; preventative instrument maintenance; and standardized computational workflows [34].

QC involves the operational techniques and activities used to fulfill quality requirements during the metabolomics study. It focuses on detecting and correcting defects in the data. Examples of QC measurements include, but are not limited to, analysis of QC samples such as reference standards, replicate extracted samples, pooled samples and blanks. Pooling samples is a vital step in QC, and it is achieved by combining aliquots from multiple individual samples. They serve as a reference for monitoring instrument performance and data integrity [35]. Analysis of pooled QC samples allows researchers to correct for analytical variance introduced during sample preparation and data acquisition stages [35].

Another important aspect of a good QA/QC system is the use of good reference materials (RMs) which are essential for ensuring the quality and reliability of analytical results [34, 36]. RMs are substances with well-defined characteristics that serve as benchmarks for method validation, calibration, and quality control [37]. Their incorporation into metabolomics studies helps researchers achieve consistent and reproducible results. RMs are used to calibrate analytical instruments, ensuring accurate quantification of metabolites. This involves preparing calibration curves using known concentrations of reference materials. RMs help validate analytical methods by confirming their accuracy, precision, and sensitivity. By analyzing RMs alongside experimental samples, researchers can monitor instrument performance and detect any deviations or systematic errors that may arise during analysis. RMs allow for regular performance assessments of analytical methods, ensuring they remain reliable over time and across different batches of samples [34]. Using RMs enables researchers to compare results across different studies and laboratories, promoting standardization and reproducibility in metabolomics.

Different types of RMs can be used in metabolomics-i) *certified reference materials (CRMs)* have certified values for specific metabolites and are produced under strict quality control conditions. They are traceable to international standards, making them ideal for method validation. ii) standards *internal standards*: these are compounds added to samples in known quantities. They help compensate for variability in sample preparation and instrument response, improving quantification accuracy. iii) *QC samples*, which may consist of pooled biological samples, are analyzed regularly to monitor instrument performance and ensure consistent results. iv) *matrix reference materials* mimic the biological matrix (e.g., blood, urine) and help assess the effect of the matrix on metabolite recovery and quantification (different RMs, and methodologies used within untargeted metabolomics reviewed in ref. [37].)

In a large-scale metabolomics study, the processes of sample collection, preparation, and analysis can extend over several days or even months. Such a study may lead to apparent batch effects in the acquired metabolomics data. Variability between batches can arise from differences in instrument performance, sample handling, and environmental factors, potentially confounding the true biological relationships among metabolites and thus obscuring obscure real metabolic changes. To mitigate batch effect, randomization, pooled QC sampling and normalization approaches can be used [38]. One of the most effective ways to minimize batch effects is through careful experimental design. Randomizing the assignment of samples to batches can help ensure that any variability is evenly distributed across experimental conditions [39]. Computational tools like QComics [40], MetaboDrift [41], MetaboAnalyst 5.0 [42] have also been developed to facilitate quality control and batch effect correction in metabolomics data analysis. By avoiding systematic grouping of similar samples within specific batches, researchers can reduce the likelihood of batch-specific trends influencing the results.

Analytical technique and instrumentation

Once the metabolites are extracted and dissolved in appropriate *analytical grade* (high purity and contaminant free) solvent, multiple replicates of sample are injected into a liquid chromatography coupled mass spectrometer (LC–MS) or gas-chromatography-mass spectrometry (GC–MS) instrument, commonly used for data acquisition in metabolomics. LC–MS and GC–MS

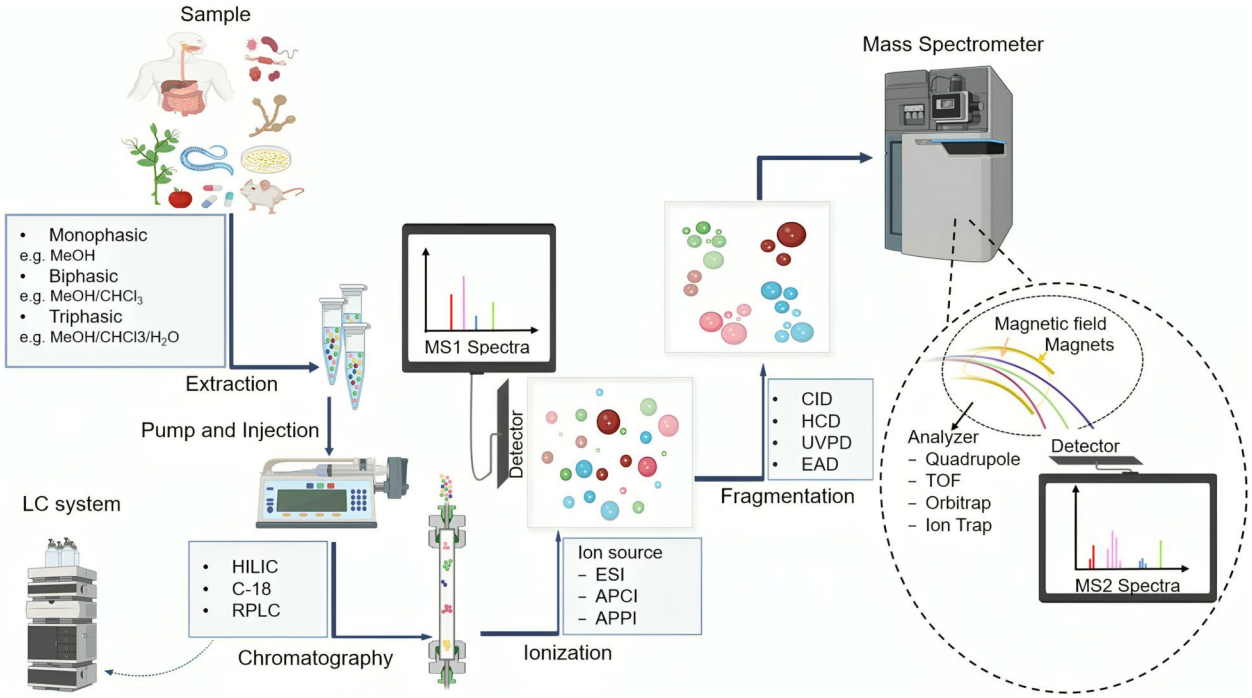

Fig. 3 Setup and principle of a typical LC–MS instrument

are hyphenated techniques combining the separation by high performance liquid chromatography (HPLC) and detection by mass spectrometry. The key difference between LC–MS and GC–MS lies in the *mobile phase*: LC–MS utilizes a liquid mobile phase, while GC–MS employs a gaseous mobile phase. Despite this distinction, both techniques rely on a column to serve as the *stationary phase*, facilitating separation and analysis of compounds. The purpose of chromatography is to separate *analytes* (metabolites being measured) present in sample before they enter the mass-spectrometer for ionization and detection. The chromatography part is important and therefore use of appropriate column for separation of analytes is crucial [43]. In LC–MS, hydrophilic interaction chromatography (HILIC) column is used for chromatographic separation of polar and ionic metabolites, whereas octadecyl carbon chain (C-18) column chemistry, a reverse phase chromatography column, is preferred for non-polar metabolites like lipids and plant secondary metabolites [30, 44]. In GC–MS, selecting a column is not easy and is rather complex, variety of GC columns are used depending on metabolite chemistry and type of chromatography [45]. For gas-chromatography-mass spectrometry (GC–MS), additional step of *derivatization* of metabolites (in order to make them volatile) is required for chromatography before injecting the sample into a GC–MS system [46]. Generally, GC–MS is compatible with less polar metabolites whereas, LC–MS is more compatible with polar metabolites [47, 48]. Table 2 lists the pros and cons of different metabolomics technologies.

The mass-spectrometer instrument is designed to separate gas phase ions, produced from sample molecules through ionization process by *ion source* part of MS, these ions which are essentially in gaseous phase are then separated by *analyzer*, according to their m/z (mass to charge ratio) value. The first ionization generates *MS1* signal; further fragmentation of ions generates *MS2* signals (Fig. 3). Finally, the *detector* detects the ions and produces a signal which is represented as peaks on the attached computer screen (Fig. 3). The MS conditions are important in determining the coverage, quality and accuracy of data obtained. Two important parameters- ionization mode and mass range are often optimized to get the coverage of ions needed. The ionization mode of MS could be set to positive or negative and data could be acquired in either or both modes. Negative mode is preferred for anionic metabolites (e.g. phosphate sugars) while positive mode is a good choice for cationic metabolites like amino acids and polyamines [49, 50]. Data acquisition in both modes is needed for non-polar metabolites (lipids) [51]. The mass range (m/z) depends on the metabolomics approach i.e. targeted or untargeted; a wider mass range will give more coverage, therefore, more data to handle and process. Often, most anionic

and cationic metabolites fall within 50–1200 m/z [52], whereas 200–2000 m/z is used for non-polar lipids [53, 54]. Sensitivity and resolution are two important parameters of MS, *sensitivity* refers to detection limit of MS; *resolution* refers to the ability of MS to separate two ions (i.e. spectral peaks). Mass accuracy is tightly linked to resolution; higher resolution is required to achieve high mass accuracy which is the difference between the measured mass and theoretical mass and is often expressed as parts per million (ppm). Identification of an analyte is dependent on how accurately a MS could measure its mass; therefore, higher resolution is desired where identification of analyte is required. Depending on the nature of metabolomics experiment, higher sensitivity may be preferred over greater resolution and vice-versa.

Various types of ionization methods and mass analyzers are used in MS, with different principles, advantages, and limitations (Table 3). Vendors like Agilent, Waters, ThermoScientific, AB Sciex, Bruker, Shimadzu manufacture LC–MS and GC–MS platforms with different features and technologies. Because metabolomics relies heavily on mass spectrometry (MS) for analyzing the complex mixture of metabolites, the choice of mass analyzer can significantly influence the sensitivity, resolution, and overall performance of the metabolomic analysis.

Quadrupole mass analyzers utilize oscillating electric fields to filter ions based on their mass-to-charge ratio (m/z). Ions are accelerated into the quadrupole, and only those with specific m/z ratios can pass through the electric fields to reach the detector. Quadrupoles are robust, relatively inexpensive, and can be used in multiple stages (e.g., tandem MS) for structural elucidation [55]. They are well-suited for targeted metabolomics due to their high sensitivity and fast scanning capabilities [56]. Quadrupole analyzers are of different types, for example, single quadrupole and triple quadrupole. Single quadrupole is the simplest form, used for single-stage mass spectrometry, where ions are filtered by their m/z and then detected. Triple quadrupole is a tandem mass spectrometer that consists of three quadrupoles. This setup is ideal for targeted analysis and sensitive quantification, commonly used in quantitative LC–MS/MS.

Time of flight (TOF) mass analyzers measure the time it takes for ions to travel a fixed distance. Ions are accelerated into a drift region, and their time of flight is inversely proportional to their m/z ratio.

TOF analyzers offer high resolution and a wide dynamic range, making them ideal for non-targeted metabolomics [57]. They can provide accurate mass measurements, which are critical for the identification of metabolites. While TOF analyzers excel in resolution, they can be less sensitive than quadrupoles, especially for low-abundance metabolites [58].

Orbitrap mass analyzers trap ions in an electrostatic field and measure their oscillation frequencies to determine their m/z ratios. This unique trapping mechanism allows for high-resolution mass measurements. Orbitraps offer exceptional mass accuracy and resolution, making them ideal for the detailed characterization of complex metabolomic profiles [59]. They can analyze a broad range of metabolites simultaneously [60].

Ion trap mass analyzers capture ions in a three-dimensional (3D) or linear trap and can manipulate them using electric fields to isolate and analyze specific ions. These analyzers allow for extensive fragmentation studies, making them suitable for structural elucidation of metabolites. They are also capable of performing multiple rounds of MS analysis, providing detailed information about complex mixtures [61]. While ion traps can achieve high sensitivity, they typically have lower resolution and mass range compared to TOF and orbitrap analyzers [62]. Their data acquisition speed may also be slower, impacting high-throughput analyses.

The choice of mass analyzer in metabolomics is critical, as each type has its own set of advantages and limitations. Quadrupole, TOF, Orbitrap and ion trap analyzers serve distinct purposes within the field, catering to various analytical needs, from targeted quantification to detailed structural analysis. Understanding these differences allows researchers to select the most appropriate technology for their specific metabolomic studies, ultimately enhancing the understanding of metabolic pathways and their implications in health and disease. As technology advances, the integration of different mass analyzers may further improve the capabilities and applications of metabolomics in biological research.

Data processing and statistical analysis

To ensure high-quality data, multiple biological, technical, and analytical replicates of a sample should be analyzed to eliminate contaminant peaks. All samples should be processed sequentially, using the same instrument, under identical LC-MS parameters and conditions, ideally within the same day [63]. Biological replicates are independent samples taken from different biological entities (e.g., different individuals, tissues, experiments under identical conditions) to account for natural variability. Technical replicates are repeated measurements of the same biological sample under identical conditions to assess precision and minimize measurement error. That is, replicates of independent performance of the complete experimental process whereas repeat injections of the same sample are analytical replicates. While analytical replicates are useful in evaluating machine performance,

Table 2 Comparison of different metabolomics technologies *[modified from Wishart, DS, Nat Rev Drug Discov 15, 473–484 (2016)]*

Technology	Pros	Cons
LC–MS	• Excellent sensitivity • Flexible • Can detect some inorganic molecules • Small sample volumes (10–100 µl) • Can be coupled to metabolite imaging • Direct sample injection without separation possible • Can detect largest portion of metabolome • Mostly automated • Compatible with solids and liquids	• Sample not recoverable • Start-up cost high • Slow sample run time (15–40 min) • Usually requires separation (LC) • Not compatible with gases • Less robust compared to GC–MS or NMR • Most spectral features not identifiable • Identification is difficult for volatile compounds
GC–MS	• Robust technology • Modest start-up cost • Quantitative • Sample volume modest (0.1–0.2 ml) • Good sensitivity • Software and databases for identification available • Detection of inorganic moieties limited to compounds that are part of organic structures (e.g., halogenated compounds) • Excellent separation reproducibility • Many spectral features identifiable • Automation compatible • Compatible with gases and liquids	• Sample not recoverable • Sample needs to be derivatized • Requires separation (GC) • Slow (20–40 min per sample) • Cannot be used in imaging, unlike LC–MS • Not compatible with solids • Novel metabolite identification limited to volatile compounds, not suitable for polar or thermally labile compounds
NMR	• Quantitative • Less run time per sample (2–3 min per sample) • No derivatization required • No separation required • Detects most organic compound classes • Identification of novel compounds easier • Most spectral features identifiable • Robust technology • Magnetic resonance imaging compatible (e.g. Functional Magnetic Resonance Imaging (fMRI)) • Full automation possible • Compatible with solids and liquids • Long instrument life (> 20 years)	• Lower sensitivity • High start-up cost • Large space required for instrument set-up • Cannot detect or identify salts and inorganic ions • Requires larger sample volumes (0.1–0.5 ml)

technical replicates allow a far more comprehensive assessment of any experimental variance in data generation [64]. It is highly recommended to run a pilot experiment to evaluate the variation (biological and/or technical) and determine an appropriate number of samples and/or replicates required for robust statistical analysis [65]. In absence of pilot data, number of replicates or sample size can also be determined using algorithms/softwares available for this purpose [66, 67]. In a typical metabolomics experiment, a large amount of raw data is generated, especially in an untargeted approach. To handle such data and to extract useful information out of it, data processing and analysis becomes crucial. Initial steps in processing include conversion of raw data files (from data acquisition softwares) into formats like mzXML or NetCDF or mzData files, by softwares from MS manufacturers, readable by freely available XCMS online software [68]. Followed by conversion, XCMS has options for statistical analysis, pathway analysis and metabolite identification through METLIN database. Other good softwares with a streamlined workflow for metabolomics data analysis are MetaboAnalyst [42, 69], MAVEN [70], GNPS [71], SIRIUS [72], BioPAN [73], MASST [74], MS-DIAL [75, 76] and MzMINE [77]. The purpose of these softwares is to process, analyze and normalize data followed by multivariate statistical analysis for meaningful interpretation. For metabolite identification various database libraries are available like, METLIN [78], HMDB (Human metabolome database) [79], MassBank [80], GMD (Golm metabolome database) [81], LIPID Maps (for lipidomics) [82] and CHEBI (Chemical entities of biological interest) [83]. Once the metabolites are identified, biological interpretation of the data can be done using various online resources- IMPaLA [84], MSEA (metabolite gene set enrichment) [85], KEGG database [86], Recon1 [87] and Biocyc [88].

Types of metabolomics

Metabonomics focuses on the systematic study of metabolites in biological systems and their role in physiological processes. It is a subset of metabolomics, emphasizing the dynamic responses of metabolites to external stimuli, environmental changes, and biological interventions. By providing a comprehensive view of metabolic

Table 3 Different types of ion source, analyzers and detectors used in LC–MS

Ionization	Analyzers	Detection
Electrosparay ionization (ESI) • Use of electric field (electrospray) to convert ions into gas phase. • Most common ionization method. • Good for charged or polar compounds.	**Quadrupole** • Sample ions filtered by mass filter based on their m/z ratio. • Mass filter is made up of four parallel metal rods (quadrupole). • Used in MS/MS.	**Electron multiplier** • A conversion dynode is used to convert either -ve or + ve ions into electrons to produce a current which is measured. • Used in quadrupole and ion trap instruments.
Atmospheric pressure chemical ionization (APCI) • A gas phase chemical ionization process where sample solution is sprayed into a heater (400 °C), for vaporization, using a gas such as N2. • Good for less polar compounds like lipids	**Triple quadrupole** • Made up of two quadrupoles separated by a collision cell. • Better quantitation, accuracy and reproducibility compared to single quadrupole. • Used in MS/MS.	**Photomultiplier** • Ions strike a dynode which results in electron release. These electrons strike a phosphor to emit photons are detected by photomultiplier. • Better lifetime.
Atmospheric pressure photo ionization (APPI) • For compounds that do not ionize well by ESI and APCI. • Sample ionised using UV light. • Good for non-polar compounds.	**Ion trap** • Sample ions trapped in trapping electric fields and then released progressively from the trap as per their m/z ratio. • Better sensitivity compared to quadrupole. • Used in MS/MS.	**Microchannel plate** • Electrons pass through very small capillaries, fused to form a disc, releasing secondary electrons which are measured. • Used in modern mass spectrometers.
Particle beam electron ionization (PB-EI) • Particle beam separates the sample from solvent and allows the sample entry as dry particle for ionization. • Used for organic and inorganic compounds.	**Time of flight (TOF)** • Ions are accelerated to a high velocity by an electric field through a tube. The time taken by an ion to reach detector is proportional to its m/z ratio. Thus, each m/z value has its characteristic time–of–flight from the source to the detector. • Used in single MS. • Can be coupled to quadrupole (QTOF) or ion trap (QIT/TOF) for MS/MS.	
	Fourier transform ion cyclotron resonance (FTICR) • Measures the cyclotron frequency of ions in a fixed magnetic field, different m/z ratio of ions produce different frequency. • Highest mass accuracy and resolution.	

profiles, metabonomics offers valuable insights into health, disease, and the effects of therapeutic interventions. Metabonomics involves the quantitative analysis of metabolites—small molecules produced during metabolic processes. This field is particularly interested in the relationships between metabolic changes and biological states, making it an essential tool for understanding complex biological systems. For instance, metabonomics is employed in studying impact of pathologies on the diversity of gut microbiota [89], in studying neurobiology of stroke [90], in studying the process of ageing [91], in the field of toxicological studies [92], establishing food safety standards [93], to name a few.

Lipidomics, a specialized branch of metabolomics, focuses on analyzing lipids and their metabolic derivatives [27, 94]. As key biomolecules, lipids play vital roles in cellular structure, energy storage, and signaling pathways. With growing insights into lipid biology, lipidomics has become an indispensable tool across disciplines such as medicine, nutrition, and environmental science. It aids in identifying disease-associated lipid profiles, serving as biomarkers for diagnosis and prognosis, particularly in conditions like cancer [95–97]. Additionally, lipidomics has contributed to assessing cardiovascular disease risks [98] and has enabled tissue imaging [99] as well as single-cell analysis, offering deeper insights into cellular heterogeneity and single cell analysis for improved understanding of cellular heterogeneity [100]. Advances in analytical and fragmentation techniques now allow for detailed characterization of complex isomeric lipids [101–104]. Given these developments, lipidomics is poised to play an increasingly significant role in biomedical and clinical research.

Fluxomics is an innovative approach that measures the rates of all intracellular fluxes in the central metabolism of biological systems. It focuses on the quantitative measurement of metabolic fluxes and provides vital information regarding the rates of metabolic reactions, nutrient distribution, metabolic pathways crosstalk, and the overall functioning of metabolic networks [105–107]. A key technical element in fluxomics is the use of stable isotopes (e.g., ^{13}C-glucose) to label substrates. By tracking

the distribution of these labeled substrates through metabolic pathways, researchers can deduce the flux rates of various reactions and the product-precursor relationship in metabolic pathways [108]. For example, ^{13}C glucose can enter glycolysis to form pyruvate or pentose phosphate pathway to form ribose sugars, ^{13}C labeled carbons of pyruvate and ribose can reveal the fractions of glucose going into glycolysis and pentose phosphate pathway. Further, fluxomics can also help in metabolic networks modelling through computational models, such as flux balance analysis (FBA), which is used to predict metabolic fluxes based on known stoichiometry and thermodynamics of metabolic reactions [109, 110]. A recent study used single-cell RNA-seq data to develop a neural network model to infer cell-wise fluxome, enabling metabolic classification of cell groups [111].

Metabolomics strategies: targeted and untargeted

Metabolomics strategies fall into two categories *targeted* and *untargeted*. Targeted approach refers to analysis of all the metabolites (in a sample) that are chemically characterized and biochemically annotated i.e. known metabolites, which could be identified [112]. Besides, studying the metabolites of a particular metabolic pathway(s) e.g. glucose metabolism, is also considered as targeted metabolomics. By contrast, untargeted approach investigates all the measurable metabolites in a sample, including the unknowns; therefore, untargeted metabolomics is also called "discovery metabolomics" [63, 113]. However, the nature of analytical technique, its sensitivity, methods of sample preparation and abundance of metabolites, limits the detection of novel metabolites in untargeted metabolomics. Further, due to the all-inclusive nature, untargeted metabolomics generates extensive raw datasets which are not easily manageable, therefore, advanced chemometric techniques, such as multivariate analysis, are needed to analyze and interpret the data. Another bottleneck in untargeted metabolomics is identification of unknown metabolites and their characterization [114].

Compared to untargeted metabolomics, targeted metabolomics offers a less complex approach to metabolite identification, quantification, and data analysis. This is because the metabolites or metabolic pathways under investigation are already known and well-characterized, allowing for more precise and streamlined analysis [114]. Further, sample preparation in targeted metabolomics can be optimized as per the chemical nature of metabolites (polar or non-polar), which is difficult to do in untargeted metabolomics due to its extensive coverage of metabolites. As predefined set of metabolites are studied in targeted metabolomics, novel relationships between metabolites could be established in the context of physiological state of sample e.g. stress, nutrition, drugs, exercise, pathological conditions [115]. Moreover, relative changes in metabolite concentrations can also be studied in healthy and diseased states [116]. Based on the quantitative changes in metabolites, changes in flux of a metabolic pathway could also be examined in targeted metabolomics [117].

Applications of metabolomics

Metabolome is the final product of genomic and proteomic interactions and therefore is a measure of an organism's phenotype [118]. Due to the dynamic and highly sensitive nature of metabolome, it becomes useful in studying the response of an organism or a cell to changes in the environment, e.g. in response to a pathological or physiological condition. Metabolomics has emerged as a very powerful omics technique having applications in the fields of biomedical research, medicine, healthcare, pharmacology agriculture, toxicology, forensics, plant biology and food industry.

Serum and plasma metabolomics of patients has revealed metabolic fingerprints of various cancers [119–121]. Metabolomics of urine identified prognostic and diagnostic markers for lung cancer [122]. The mechanistic basis of cancer progression has been delineated by metabolomics and the role of specific metabolites -called *oncometabolites*- is identified [123, 124]. The metabolic changes associated with heart diseases have been studied using metabolomics for staging of heart failures and predicting the risk [125]. Ischemic heart diseases, which account for nearly half of cardiovascular disease burden, have been studied using metabolomics, for their understanding, detection, and treatment [126–128]. Lipidomics has been used to identify diagnostic lipidomic signature of pediatric inflammatory bowel disease [129]. In addition, lipidomics has been exploited in biomarkers identification, prediction and treatment response in a variety of cancers [95, 130, 131] cardiovascular diseases [132, 133], Alzheimer's [134], traumatic brain injury [135], liver diseases [136], neurological disorders [137] and for a variety of preclinical, clinical and translational studies [138, 139]. Metabolic signatures for diseases like depression and schizophrenia have also been reported [140]. Subclasses of disease have been characterized using metabolomics [141]. The role of atherotoxin called trimethylamineN-oxide (TMAO) in development of atherosclerosis has been highlighted through untargeted metabolomics [142, 143]. Using both targeted and untargeted metabolomics approach, several research groups have identified association of amino acids with the risk of developing type 2 diabetes [144, 145]. Metabolomics has a great potential in the field of drug research and

development, metabolomics highlighted the dysregulated metabolism in several diseases and identified culprit metabolites e.g. oncometabolites in cancer, TMAO in atherosclerosis, and thus, paved path for drug discovery and development. Metabolomics is used in assessing drug toxicity, drug purity, monitor patient compliance (detecting drugs in blood) [146, 147]. In precision or personalized medicine, metabolomics has been successfully used not only in screening of newborns for disease but to design optimal therapy (enzyme replacement or dietary restrictions) accordingly [148–150]. Pharmacometabolomics is another emerging application where metabolomics can be used to complement pharmacogenomics [151]. In nutrigenomics, interaction of dietary components with the genetic background and resulting effect on metabolism has been studied by metabolomics [152]. Food safety and standards are being monitored globally to detect presence of chemical contaminants and other harmful or forbidden substances by employing metabolomics [153, 154]. Several dietary biomarkers have been identified by metabolomics, e.g. TMAO in urine samples following fish consumption, creatinine, carnitine, TMAO in meat intake, proline betaine as a marker of citrus fruit consumption, biomarkers for tea consumption [155]. Growing antibiotic resistance in bacteria and emergence of superbugs is a threat to human health, specific metabolic profiles have been associated with antibiotic resistance, exogenous supply of metabolites has been shown to restore the antibiotic susceptibility in bacteria [156]. In plants, the metabolic fingerprints and the metabolic response of plants to stress conditions has revealed insights into plant physiology [157, 158]. In essence, metabolomics is gaining recognition as the most powerful among the omics sciences, thanks to its wide-ranging applications and unparalleled ability to capture phenotypic measurements, thus, changing the research in biomedical sciences. However, there is a need to make metabolomics portable and cost-effective for further pushing the limits of its applications.

Conclusion and future directions

In conclusion, mass spectrometry-based metabolomics has rapidly evolved into a powerful and versatile tool for analyzing the complex metabolic landscape of biological systems. This review highlights the diverse workflows, strategies, and data analysis techniques that could enable researchers to execute a metabolomic workflow. The integration of high-resolution mass spectrometry with advanced computational methods has greatly enhanced the sensitivity, accuracy, and throughput of metabolomic studies. However, challenges remain, including the need for standardized workflows, improved annotation of metabolites, and the integration of multi-omics data to provide a more comprehensive understanding of metabolic networks.

Future directions in mass spectrometry-based metabolomics are likely to focus on improving technological advancements, such as the development of more sensitive, high-throughput instruments and the application of AI-driven data analysis approaches for better interpretation of complex datasets. Furthermore, the integration of metabolomics with other"omics"technologies, such as genomics and proteomics, will continue to provide more holistic insights into the underlying biology. As the field matures, there is also a growing emphasis on personalized metabolomics for clinical applications, where metabolite profiling could serve as a tool for disease diagnosis, prognosis, and treatment monitoring. With ongoing advancements in both instrumentation and computational techniques, mass spectrometry-based metabolomics is poised to have a transformative impact on both basic and applied biological research.

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MAI and KH conceived, designed and wrote the manuscript. MAI and KH involved in manuscript revision. MAI critically reviewed the manuscript for the intellectual content and approved the final submission.

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Declarations

Ethics approval ad consent to participate
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