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Advances in mass spectrometry-based metabolomics for investigation of metabolites

Jun-Ling Ren,^a Ai-Hua Zhang,^a Ling Kong^a and Xi-Jun Wang *^{abc}

Metabolomics is the systematic study of all the metabolites present within a biological system, which consists of a mass of molecules, having a variety of physical and chemical properties and existing over an extensive dynamic range in biological samples. Diverse analytical techniques are needed to achieve higher coverage of metabolites. The application of mass spectrometry (MS) in metabolomics has increased exponentially since the discovery and development of electrospray ionization and matrix-assisted laser desorption ionization techniques. Significant advances have also occurred in separation-based MS techniques (gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, capillary electrophoresis-mass spectrometry, and ion mobility-mass spectrometry), as well as separation-free MS techniques (direct infusion-mass spectrometry, matrix-assisted laser desorption ionization-mass spectrometry, mass spectrometry imaging, and direct analysis in real time mass spectrometry) in the past decades. This review presents a brief overview of the recent advanced MS techniques and their latest applications in metabolomics. The software/websites for MS result analyses are also reviewed.

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1 Introduction

Metabolomics is a booming omics field arising after genomics, transcriptomics, and proteomics, and it is a vital part of systems biology. Biological fluids (*e.g.*, serum, plasma, urine, saliva, cerebrospinal fluid, bile, amniotic fluid, tears, pancreatic juice, intestinal fluid, and breast milk), human tissues, and cells are the common analysis objects in metabolomics. Metabolites are the end products of complex cellular regulation networks,¹ and they can also influence or even alter regulation *via* feedback loops.² Through the overall analysis of metabolites, the insightful knowledge of physiological, pathological, and biochemical status can be directly understood, which can then be combined with chemical and informatics methods. Further, endogenous small-molecule metabolic changes can also be determined, and the corresponding biomarkers can be obtained, which can be used to characterize or reveal the functional states for a particular time or environment.³ Thus, metabolomics may provide the “fingerprint” of metabolic

systems that can reveal the clinical phenotype in the best possible manner.⁴ The studies of metabolomics can help us to enhance the understanding of disease mechanisms and drug effects, as well as improve the ability to predict personal disease progression or variation in drug response phenotypes.^{5–8}

Metabolomics refers to a large number of compounds that belong to multiple categories, such as amino acids, lipids, nucleotides, carbohydrates, and organic acids. The physical and chemical properties of metabolites are diverse and are often distributed over a wide range of concentrations.⁹ Consequently, metabolomics research is a challenge in analytical chemistry. The most common analytical techniques used in metabolomics are nuclear magnetic resonance spectroscopy and mass spectrometry (MS).¹⁰ Due to the evident superiorities of speed, sensitivity, and wide dynamic range over other techniques, MS has provided new dimensionality to medical research. The applications of MS are rapidly broadening, encompassing molecular and cellular biology. MS-based metabolomics technologies (Fig. 1) aid the analysis of *in vivo* metabolite changes, understanding of the possible pathogenesis and mechanism of diseases, and identification of potential biomarkers that may improve the diagnosis and treatment of diseases.¹¹

The large-scale commercialization of two soft ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), has made MS the dominant analytical technique in metabolomics research, and has proven to be particularly helpful in large-scale omics studies.^{12,13} The development of an ESI source extends the applications of MS, as it offers a conventional method by generating gas-phase

^aSino-America Chinmedomics Technology Collaboration Center, National TCM Key Laboratory of Serum Pharmacochimistry, Chinmedomics Research Center of State Administration of TCM, Laboratory of Metabolomics, Department of Pharmaceutical Analysis, Heilongjiang University of Chinese Medicine, Heping Road 24, Harbin, China. E-mail: xijunwangls@126.com

^bState Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau

^cNational Engineering Laboratory for the Development of Southwestern Endangered Medicinal Materials, Guangxi Botanical Garden of Medicinal Plant, Nanning, Guangxi, China



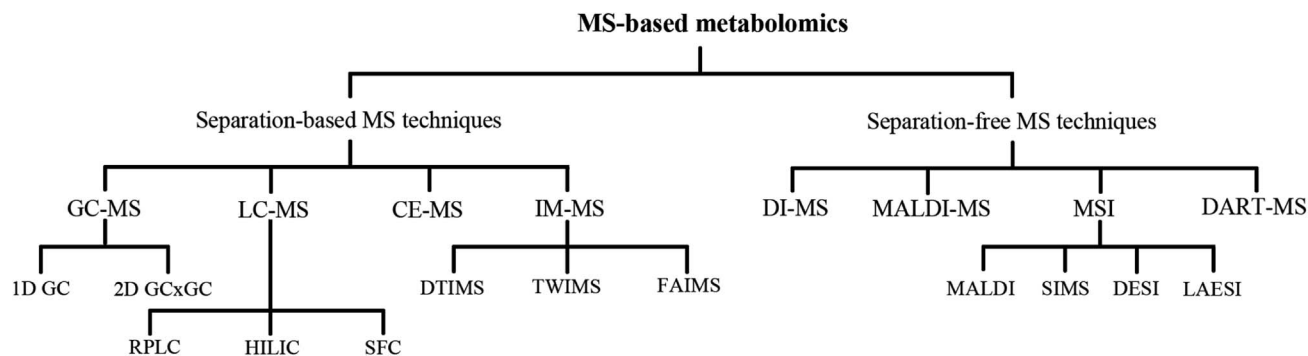


Fig. 1 MS-based technologies currently used for metabolomics. CE: capillary electrophoresis; DART: direct analysis in real time; DESI: desorption electrospray ionization; DI: direct infusion; DTIMS: drift-time ion mobility-mass spectrometry; FAIMS: field asymmetric ion mobility-mass spectrometry; GC: gas chromatography; HILIC: hydrophilic interaction chromatography; IM-MS: ion mobility-mass spectrometry; LAESI: laser ablation electrospray ionization; LC: liquid chromatography; MALDI: matrix-assisted laser desorption ionization; MSI: mass spectrometry imaging; RPLC: reversed-phase liquid chromatography; SFC: supercritical fluid chromatography; SIMS: secondary ion mass spectrometry; TWIMS: traveling-wave ion mobility-mass spectrometry.

solute ions from the solution droplets by solution evaporation.^{14,15} MALDI is widely used primarily because of the rise of proteomics; in the last decade, MALDI has been extensively used in the detection and quantification of small molecular metabolites.^{16–18} Mass spectrometry imaging (MSI) opened a new chapter in the intuitive presentation of the metabolites distribution in tissues or cells. Herein, we categorically review the MS-based technologies employed in metabolomics and the corresponding analysis using software/website. Table 1 summarizes the advantages and disadvantages of these MS techniques in order to facilitate research applications.

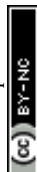
2 Separation-based MS techniques

2.1 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the preferred instrument for volatile metabolites analysis, which yields high sensitivity and resolution, prominent reproducibility, and highly repeatable fragmentation. The availability of a spectral library (such as NIST and AMDIS) makes the identification of biomarkers more convenient and helpful for the elucidation of subsequent mechanisms of biology or pathology alterations.^{19,20} Furthermore, although low cost and ease of

Table 1 Advantages and disadvantages of MS-based metabolomics

	Advantages	Disadvantages
GC-MS	Suitable for the detection of volatile metabolites Highly repeated retention times Universal database facilitates the identification of the structure	Unsuitable for non-volatile and thermally unstable metabolites Sample pre-processing process is tedious and often requires derivatization
LC-MS	Simple sample preparation Can be matched with multiple MS detectors Wide coverage of detectable metabolites	Ion suppression Metabolites can not be detected without form adduct ions
CE-MS	Low sample volume required for the detection, especially suitable for precious samples	System stability is less stable than LC-MS, GC-MS Affected by salt in the sample
IM-MS	Isomers can be distinguished Fast detection speed The three-dimensional structure of metabolites can be determined in dynamic motion	Determination structural information indirectly from CCS values
DI-MS	High-throughput detection of samples The data processing process is relatively simple	Ion suppression Isomers cannot be distinguished
MALDI-MS	Short sample analysis time Low sample consumption High salt tolerance	Reproducibility is greatly affected by the matrix and sample processing Quantitative analysis is difficult to achieve
MSI	Enables <i>in situ</i> detection and presented <i>in situ</i> metabolites information	Imaging effect is affected by resolution It takes longer time to complete the detection under the high resolution
DART-MS	Samples can be analyzed directly without the extraction process Low sample consumption Sample analysis cycle was sharply shortened	Polar compounds are difficult to ionize Ion suppression



operation are its merits, GC-MS usually requires tedious sample processing and derivatization when the analysis targets are nonvolatile constituents. To increase the volatility and thermal stability of the analytes, various derivatizations, such as alkylation, acylation, and silylation, can be employed to “protect” functional groups.^{1,9} Among these derivatization methods, methoximation and trimethylsilylation are commonly used in large-scale metabolomics studies with GC-MS.²¹ On account of the difference in the derivatization efficiencies of metabolites, the reproducibility of the overall analysis may be affected.^{9,22} It should also be noted that the formation of byproducts and structural transformation in the derivatization process may lead to the misinterpretation of the generated data. The simple scheme of the GC-MS technique is illustrated in Fig. 2.

Zhao *et al.* developed an automated high-throughput sample derivatization and quantitative method by using the GC-MS platform, and simultaneously measured over one hundred microbial metabolites in human serum, urine, feces, and *Escherichia coli* cell samples within 15 min for each sample.²³ This established method is suitable for future microbiome metabolomics research that require good linearity, reproducibility, and stability. Hadi *et al.* profiled serum metabolites for the prediction of breast cancer diagnosis, grading, staging, and neoadjuvant status by GC-MS.²⁴ The robust analytical approach highlights the potential of these metabolic features as prognostic factors, thus helping to improve the treatment of breast cancer. Yan *et al.* performed GC-MS to investigate urinary metabolic alterations related to systemic lupus erythematosus: 70 endogenous metabolites were identified that involved nucleotide metabolism, energy metabolism, oxidative stress, and gut-microbiome-derived metabolism.²⁵ The present study manifested that GC-MS is a promising technique for metabolites analysis and understanding the pathology of diseases.

Since 1991, the use of comprehensive GC (GC × GC) has increased both peak capacity and sensitivity, which aid the identification of metabolites.^{26–28} In GC × GC, the compounds eluted from the primary column are imported into the secondary column to perform a second independent separation.²⁹ Most of the reported applications of GC × GC are executed using the nonpolar stationary phase in the first

dimension of separation and polar stationary phase in the second dimension. This choice is usually made as the elution behavior of the compound class is predictable, and these compounds are subjected to the first-dimension separation according to the different boiling points. Due to the similar boiling points, first-dimension separation fails to isolate the compounds. Therefore, these compounds were separated according to polarity in the second-dimension separation; consequently, the separation power was significantly enhanced.³⁰ Miyazaki *et al.* examined the temporal changes of serum metabolites in neonatal calves after the first ingestion of colostrum by GC × GC-MS.³¹ Approximately 1400 calf serum metabolites were detected, the number of which was significantly higher than that of conventional GC-MS. About 180 metabolites were detected in the same serum sample, thus enhancing our understanding of serum metabolites.

2.2 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) has become the dominating analytical technique in the field of global metabolite profiling. As compared to GC-MS, the mobile phase changes from gas to liquid, eliminating the need for metabolites to be volatile. Thus, no sample derivatization is required, which indicates that a broader range of analytes can be detected. In 2004, the introduction of commercialized ultra-high-performance LC (UPLC) with sub-2 μm-particles drastically increased the throughput of regular LC methods.³² UPLC usually employs sub-2 μm or 2.6–2.8 μm (fused-core) stationary phase particles at the chromatography column and can withstand high solvent flow rates and high pressures in the range of 6000–19 000 psi, which can reduce the peak width, yield shorter analytical run times, increase the peak capacity, provide better ionization, and reduce mass spectral overlap, leading to improved structural determination and confirmation.^{33,34} It is reported that the separation of human serum metabolites with UPLC-MS brings about the detection of 20% more metabolites when compared with those detected by HPLC-MS.³⁵ Currently, UPLC-MS has become the mainstay in metabolites separation and identification. The simple scheme of the LC-MS technique is illustrated in Fig. 2.

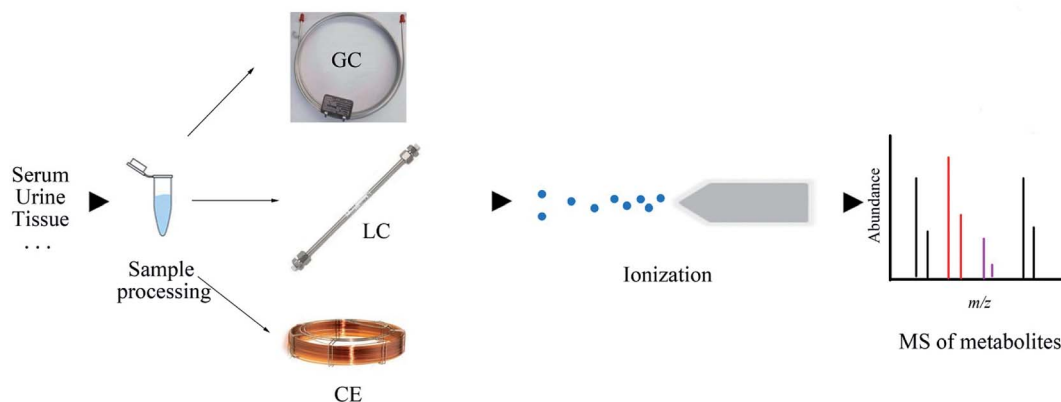


Fig. 2 Flowchart depicting the basic workflow of GC-MS, LC-MS, and CE-MS techniques.



2.2.1 Reversed-phase liquid chromatography-mass spectrometry. At present, most of the LC-MS-based metabolomics studies have been performed using RPLC column with C18 or C8 materials.³⁶ Reversed-phase liquid chromatography (RPLC) provides an extensive range of selectivity values for the analysis of diverse metabolites, which are highly compatible for the analysis of organisms.³⁷ For RPLC separation, gradient elution is often employed, which starts with highly aqueous and organic contents.³⁸ For the sake of improving the separation efficiency, various buffer modifiers (*e.g.*, formic acid, acetic acid, and ammonium acetate) can be added into the mobile phase to adjust the separation selectivity or detection sensitivity.³⁹

Wang *et al.* investigated a comprehensive metabolome of jaundice syndrome by RP-UPLC-MS, which established a specific metabolite phenotype and identified 44 potential biomarkers. The disturbance of alanine, aspartate, and glutamate metabolism, as well as the synthesis and degradation of ketone bodies were found in jaundice syndrome patients, which can yield a better understanding of the pathophysiology of the jaundice syndrome, and laid the foundation for the scientific interpretation of the pathology of traditional Chinese medicine syndrome.⁴⁰ Based on the chinmedomics analysis method, Li *et al.* analyzed the global constituents of *Phellodendri amurenensis* cortex and serum metabolites by RP-UPLC-MS.⁴¹ Herein, 34 different metabolites were found to be related with prostate cancer, and *Phellodendri amurenensis* cortex can observably reverse the abovementioned biomarkers to the normal level, indicating its therapeutic effect. Zhang *et al.* profiled urine metabolites by RP-UPLC-MS to probe the phenotypic characteristics of nanshi oral liquid, altering the metabolic features of the Kidney-Yang deficiency syndrome.⁴² The established metabolomics platform characterized the metabolic pathways of the Kidney-Yang deficiency syndrome and evaluated the intervention effect of the nanshi oral liquid against this syndrome, which is conducive to further understanding the pathogenesis of traditional Chinese medicine syndrome, and supplied novel information for studying the pharmacology of traditional Chinese medicine.

However, highly polar and ionic compounds are difficult to retain in RPLC; the analysis of polar compounds is practicable using RPLC by adding ion-pairing agents into the mobile phase by means of ion-pairing liquid chromatography (IPLC).⁴³ The ion-pair reagent is a compound that has an opposite charge when compared with the target metabolites in an aqueous solution.⁴⁴ As for cation analysis, the ion-pair reagent usually includes hydrochloric acid, perchloric acid, perfluorocarboxylic acids, pentane sulfonic acids, hexane sulfonic acids, heptane sulfonic acids, and octane sulfonic acids, while for anion analysis, it usually includes tetramethylammonium, tetraethylammonium, tetrapropylammonium, tetrabutylammonium, tributylamine, and hexylamine.⁴⁵ Guo *et al.* exploited the IP-RP-UHPLC-MS method to separate and analyze biochemical-related metabolites that contain phosphate and carboxylic acid in cellular metabolism.⁴⁶ Diisopropylethylamine and hexafluoroisopropanol were used as the IP reagents, which enhanced the sensitivity and chromatographic separation, thus

assisting in the precise analysis of metabolites in cell center energy metabolism and proving the in-depth knowledge of mechanisms related to metabolic adaptations. Though the usage of an ion-pair reagent is helpful, the remaining unpaired reagent may contaminate the ion source that may influence the sensitivity or reproducibility of LC-MS.^{44,47}

2.2.2 Hydrophilic interaction chromatography-mass spectrometry. Hydrophilic interaction chromatography (HILIC) utilizes a polar stationary phase and a high proportion of organic mobile phase, which usually requires at least 3% water, followed by increasing the percentage of water, as the eluent.^{9,48} Contrary to RPLC, in HILIC, the more lipophilic compounds are barely attracted to the stationary phase and get rapidly eluted, whereas the more polar compounds are retained and only eluted when the aqueous phase content increases. HILIC-MS is complementary to RPLC-MS since early eluting analytes in RPLC-MS are usually well retained in HILIC-MS. However, HILIC usually generates a wider peak than RPLC, leading to lower peak capacity and higher dependence on the peak resolution of the mass spectrometer.⁴⁴ Overall, HILIC-MS analysis provides a broader view for metabolites analysis.

It is well known that the simultaneous quantitative measurements of amino acids, neurotransmitters, purines, and pyrimidines exhibit immense difficulties. Chen *et al.* established a novel method based on HILIC-MS to target the quantification of the abovementioned metabolites in rat brain and serum, which displayed high selectivity, high throughput, and better chromatographic behavior than existing approaches.⁴⁹ The results demonstrated that HILIC-MS can monitor a wide range of metabolites. Boelaert *et al.* developed a HILIC-MS-based metabolomics study in searching for novel potential biomarkers of chronic kidney disease.⁵⁰ Herein, glyco-cursodeoxycholic acid, 2-hydroxyethane sulfonate, and pregnenolone sulfate were the newly identified metabolites in uremic retention solutes, which extends the range of metabolites of chronic kidney disease, opening a new perspective for future study. Cífková *et al.* systematically optimized the separation of acidic lipid classes and other lipid classes by HILIC-MS.⁵¹ It is a fairly tough task to separate acidic lipid classes due to the broadening or tailing of the chromatographic peaks by them. The established method obtained a higher number of lipids, providing a basis for the future detailed research on lipids.

2.2.3 Supercritical fluid chromatography-mass spectrometry. A substance having its temperature and pressure above a critical point is defined as a supercritical fluid.⁵² Supercritical fluid chromatography (SFC) uses supercritical fluids, usually CO₂, as the primary mobile phase, which possesses a modest supercritical point (31.1 °C and 7.38 MPa). Moreover, the polarity of supercritical CO₂ is similar to that of hexane, which can be adjusted by polar solvents (such as methanol). Its non-toxicity and ease of handling makes CO₂ to be often used as the mobile phase.^{53,54} The properties of low viscosity and high diffusivity of supercritical fluids permits higher flow rates and lower pressure drops than that those encountered in traditional HPLC.⁵⁵ The use of CO₂ makes the SFC environmentally friendly, which requires only a small quantity of organic solvent



as the auxiliary solvent. However, this was once considered to be the main reason that restricts SFC in the separation of nonpolar and relatively low polar compounds. At present, SFC is recognized as a suitable means for nonpolar as well as polar compound separation.^{55,56}

Lisa *et al.* developed a novel analytical strategy for high throughput and comprehensive analysis of lipids by SFC-MS, which enables a quick separation of 30 classes of nonpolar and polar lipids within 6 min.⁵⁷ Taguchi *et al.* established a rapid method to target profile bile acids in rat serum with SFC-MS.⁵⁸ Twenty four bile acids were simultaneously quantified without any solid-phase extraction and complex sample preparation within 13 min. These applications confirm that SFC-MS is an alternative to existing analytical methods for metabolomics research.

2.3 Capillary electrophoresis-mass spectrometry

Electrophoresis is defined as the migration of ions under the influence of a spatially uniform electric field.⁵⁹ Capillary electrophoresis (CE) requires the application of high voltage to produce an electrophoretic flow of different ionic species in a narrow-bore capillary (20–200 μm i.d.).⁶⁰ CE-MS is a robust technique for polar and charged metabolite analyses, which is a complementary tool to the chromatographic separation technique.⁶¹ Capillary zone electrophoresis, the most common pattern of CE, when combined with MS, initially separates metabolites on the basis of their charge-to-size ratios and then on the basis of the mass-to-charge ratios, thus providing an alternative to LC.^{62,63} CE is considered to be a new technique among metabolomics and till date, it has not been routinely applied in metabolites profiling due to certain drawbacks, such as poor system stability and easy blockage of narrow capillaries by salt. While CE is a valuable analytical tool for those who have mastered the required skills and knowledge,⁶⁴ it has some distinct advantages over other instrumental techniques employed for metabolomics: low separation volume, which is particularly suitable for the study of biological fluids in small experimental animals, homogeneous separation environment, easy sample preparation process, and capability of concentrating analytes.⁶² CE-MS exhibits semblable or even better separation efficiency than GC-MS and LC-MS due to the analysis time. The simple scheme of the CE-MS technique is illustrated in Fig. 2.

Significant sensitivity enhancement of modern MS and optimized methods have transformed CE-MS into a potential tool for metabolomics analysis, and the application of CE-MS in metabolomics has drastically increased in the past decade.⁶⁵ Onjiko *et al.* developed “microprobe single-cell CE-ESI-MS” for the *in situ* analysis of metabolites in single cells in a freely developing vertebrate embryo.⁶⁶ Only <0.02% of the single cell content can allow the detection of ~230 different molecules in the positive ion mode, which extends the biological toolbox of the system cell biology and opens a new direction toward understanding normal and impaired developments of cell biology.⁶⁶ Zeng *et al.* employed CE-MS to profile metabolites in hepatocellular carcinoma patients, which is helpful to cover

polar metabolites. Few studies have used CE-MS to evaluate serum polar metabolites in hepatocellular carcinoma, and this study may provide novel insights into the complicated biological processes in hepatocellular carcinoma.⁶⁷ Fujii *et al.* applied CE-MS to detect metabolic changes in the postmortem brain of schizophrenia patients, and the results pointed out that glucose metabolism and proteolysis were altered, revealing that several pathways are changed in the brains of schizophrenia patients.⁶⁸

2.4 Ion mobility-mass spectrometry

Ion mobility-mass spectrometry (IM-MS), a gas-phase electrophoretic technology, enables the differentiation of ions in the gas phase and electric field on the basis of their size, shape, charge, and mass, and can provide important supplementary information regarding molecular chromatographic separation and ion mass spectrometry separation.^{69–71} The advantages of IM-MS include distinguishing isobars and isomers, increased signal-to-noise ratio, and determining the conformational state caused by the charge location.⁷² The separation of IM-MS usually occurs in milliseconds, making it suitable for collaborating with MS, which adds a new dimension in the analysis. The scheme of IM-MS technique is displayed in Fig. 3.

There are three major ion mobility spectrometry (IMS) techniques used in IM-MS: drift-time IMS (DTIMS), traveling-wave IMS (TWIMS), and field asymmetric IMS (FAIMS). DTIMS is the most straightforward method for measuring ion mobility. In the presence of an axial linear electric-field gradient, ions migrate through the buffer gas, thus demonstrating high resolution.⁷³ However, DTIMS suffers from two deficiencies that limit its broad applications: low sensitivity and difficulty in coupling with commercial instruments.⁷⁴ TWIMS is similar to DTIMS; however, in TWIMS, a sequence of applied voltages engenders a traveling wave that drives the ions through the buffer gas.¹³ This characteristic facilitates its coupling with the MS platform, greatly enhancing the sensitivity results.⁷⁵ Since it is difficult to quantify traveling wave voltages, the measurement accuracy of the ion collision cross-section value (CCS: distinct physicochemical property of a compound, with which the compound's molecular structure can be predicted) is the primary disadvantage of TWIMS.⁷⁶ It is possible for TWIMS to determine CCS accurately, which can be calibrated with the highly accurate CCS obtained from DTIMS.^{77–79} As the name suggests, FAIMS employs an asymmetric voltage waveform composed of a low- and high-field component to induce ions that can migrate between two fields when they are swept through the flow of gas. Only the stable ions in the applied waveform can pass through FAIMS, thus becoming capable of producing an effective narrow band-pass ion mobility filtering device. When compared with DTIMS and TWIMS, FAIMS operates with high sensitivity when the ions pass through it in a space-dispersive mode by varying the compensation voltage, making it suitable as a commercial technique for several MS instrument platforms.^{74,80}

At present, numerous metabolomics studies using IM-MS have been published. Zhang *et al.* used ESI IM-MS to study the striatal metabolomics in Parkinson's-like disease for a rat



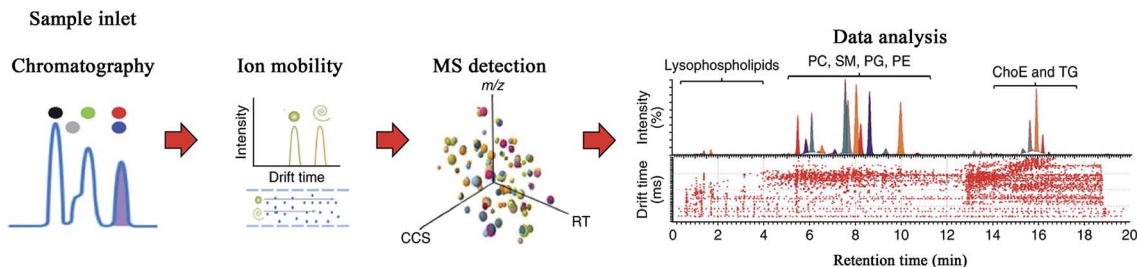


Fig. 3 Scheme of IM-MS technique. Reprinted with permission from ref. 72. Copyright (2017) Nature.

model, and 9 metabolites were selected as potential biomarkers.⁸¹ A major discovery of this research was the finding of a dopamine isomer, which has not been reported previously, which was separated using IM-MS. This finding implied that the rangeability of dopamine in Parkinson's disease may be masked by the presence of its isomer. With the maturity of metabolomics, it is found that the function of isomers is not exactly the same, making isomer recognition particularly important. Bowman *et al.* explored FAIMS-MS to separate lipid isomers (the main differences including *sn*, chain length, double bond position, and *cis/trans*), and a success rate of approximately 75% was found in the four main types of glycerolipids and phospholipids.⁸² This study demonstrates FAIMS-MS as a powerful tool for lipid isomer analysis. Dwivedi *et al.* profiled human blood by IM-MS with about 1100 metabolites ions detected and 300 isomeric metabolites separated.⁸³ Here, the peaks of low-abundance metabolites that may vanish in MS noise can be distinguished in the mobility space. Furthermore, the peak capacity of IM-MS is enhanced six-fold when compared with individual MS analysis. Thus, IM-MS seems to be a promising analytical technique for assessing metabolomics.

3 Separation-free MS techniques

3.1 Direct infusion-mass spectrometry

Direct infusion-mass spectrometry (DI-MS) is an analysis platform based on the direct injection or infusion of samples into MS, particularly ultra-high-resolution and accurate MS (such as Fourier transform ion cyclotron resonance-MS and Orbitrap-MS), without prior chromatographic or electrophoretic separation, which is clearly advantageous for high-throughput screening.^{3,84} This feature considerably shortens the total analysis time, thus minimizing the drift of sequence analysis, and improves the repeatability and accuracy among the inter-samples.^{9,85} In addition, subsequent data processing is also simplified because it needs no alignment of the retention time.

Anand *et al.* detected and confirmed serum lipid biomarkers for preeclampsia using DI-MS, which provides a high throughput method for the analysis of lipid species without a chromatographic separation step.⁸⁶ It represents a versatile method that requires relatively simple sample preparation and sufficient reproducibility. González-Domínguez *et al.* analyzed regional metabolic abnormalities in the brains of transgenic APP/PS1 mice with DI-MS; when compared with wild-type

control mice, the levels of phospholipids, fatty acids, acylcarnitines, purine and pyrimidine metabolites, sterols, and amino acids in transgenic APP/PS1 mice significantly changed, which is helpful to explore the possible pathogenesis of Alzheimer's disease.⁸⁷

Although DI-MS can achieve rapid sample analysis, the ion source contamination of sample residue is tedious, and requires a long time to remove. The emergence of chip-based nano-ESI has excellently resolved this problem.⁸⁸ The nozzle of chip-based nano-ESI is engraved on a silicon board, and the analyzed liquid is passed through the nozzle and sprayed under a relatively gentle pressure.⁸⁹ Since the requirement of the sample volume is very small and each nozzle can be used only once, the sample residue effect is eliminated. It has been reported that this technique can reduce or even eliminate the differences in the MS response among analytes and adduct ions.⁹⁰

Zhang *et al.* employed chip-based direct-infusion nano-electrospray Fourier transform ion cyclotron resonance-mass spectrometry to simultaneously quantify 6 free fatty acids in healthy controls, 5 types of cancer (colorectal, gastric, pancreatic, breast, and lung cancer) patients, and corresponding benign diseases. The results suggest that this platform could provide high sensitivity and high throughput analysis of free fatty acids, and serum unsaturated free fatty acids might have important clinical significance for the early detection of the abovementioned cancers.^{91–95}

Although DI-MS is easy to perform, since it lacks chromatographic or electrophoretic separation capabilities, ion suppression becomes the main obstacle and isomeric compounds cannot be separated.

3.2 Matrix-assisted laser desorption ionization-mass spectrometry

MALDI, another powerful soft ionization source, has become an indispensable analytical tool for complex sample analyses.^{96–99} This technique provides distinguishing features such as rapid analysis ability, low sample consumption, and relatively high tolerance towards salts.¹⁰⁰ The sample analysis time of MALDI-MS is mainly dependent on the amount of laser shots required for the experiment to be suitable for a high-throughput analysis (Fig. 4). In addition, 0.1–1 μL sample or the sample and matrix mixture is deposited on the target plate for MALDI-MS analysis, and only a small fraction of the spotted sample was consumed



during the analysis. For LC-MS, usually, 2–20 μL sample volume is infused into the LC, which separates and concentrates the compounds prior to ionization.

Due to the poor reproducibility of the signal intensities of the analytes, the quantitative analysis of MALDI-MS is not very outstanding; also, the reproducibility of the signal intensities is largely determined by the type of matrices used and sample preparation.^{102,103} The most commonly used MALDI matrices are α -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). The selection of the matrix is also an important aspect that affects the experimental results. MALDI is an essential tool for analyzing macromolecule metabolites, such as proteins, peptides, and nucleic acids. With the discovery of various novel matrices, MALDI can also be used for the analysis of small molecule metabolites in the low mass region. Nanomaterials, such as carbon nanotubes,¹⁰⁴ carbon dots,¹⁰⁵ graphene,¹⁰⁶ and graphene oxide,¹⁰⁷ have been extensively developed as effective matrices for the MALDI profiling of smaller molecules.

He *et al.* adopted MALDI-MS for the *in situ* determination of the membrane constituents of human mammary epithelial cells and 6 breast cancer cell lines without any extraction and separation steps, thus providing a new rapid method for analyzing membrane lipids.¹⁰⁸ Zhang *et al.* employed an ammonia-treated *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDC) as a new matrix for the rapid quantitative and qualitative determination of serum free fatty acids by MALDI-MS. Here, 9 free fatty acids were quantified in 339 serum samples, including healthy controls and non-hyperglycemia and hyperglycemia patients.¹⁰⁹ This study indicated that MALDI-MS is not only suitable for metabolomics analysis, but can also quantitatively analyze the contents of metabolites. Ren *et al.* used graphene oxide as the matrix to rapidly and simultaneously quantify serum non-esterified and esterified fatty acids in 1440 subjects by MALDI-MS.¹⁸ Further, 12 non-esterified and esterified fatty acids were quantified within an analysis time of less than 1 min, and the potential biomarker panels presented good diagnostic ability to distinguish benign lung diseases from lung cancer with respect to the clinical uses of carcinoembryonic antigen or cytokeratin 19 fragment antigen 21-1.

3.3 Mass spectrometry imaging

MSI is a powerful tool for determining metabolites *in situ*, which can simultaneously map multiple molecules, from low-

molecular-weight metabolites and drugs to large proteins while simultaneously maintaining the morphological integrity of the analyzed tissue slides.^{12,17,110} Typically, the spatial information of the biological processes in the tissue was obtained from histology staining, or immunohistochemistry. However, histology staining is non-molecular-specific, and immunohistochemistry requires the knowledge of the target analyte prior to investigation and limited to the simultaneous investigation of only a small number of analytes at any given time.¹¹¹ Currently, MSI is an emerging technology that can enhance the understanding of the *in situ* distribution of metabolites or biomarkers directly in tissue sections or cells.

The general workflow of MSI can be briefly summarized as follows: the tissue is cut into thin slices, usually 10–20 μm , and placed on an indium tin oxide-coated slide; then, according to the analysis' requirements, it is selected to either wash the tissue or not and spray an appropriate matrix or not on the basis of the selected ion source; and then, the analysis is performed.^{112–114} Tissue section is detected according to a predefined raster, which generates mass spectrum at each measurement point; then, the measured information is used to perform image reconstruction, yielding the distribution of molecules in the tissue.¹¹⁵

A variety of ionization methods have been employed for MSI, such as MALDI,¹⁷ secondary ion mass spectrometry (SIMS),^{116,117} desorption electrospray ionization (DESI),^{118–120} and laser ablation electrospray ionization (LAESI).^{121,122}

3.3.1 MALDI mass spectrometry imaging. Many techniques are capable of carrying out MSI, among which MALDI is the most commonly used (Fig. 5).^{112,123,124} MALDI is a so-called “soft” ionization technology, which allows the analysis of metabolites across a wide mass range.¹²⁶ In MALDI MSI, the laser beam is rastered across the matrix-coated tissue surface to achieve tissue scanning.¹²⁷ Moreover, the lateral resolution is limited by the diameter of the laser beam, the raster step size, and the diffusion of the biomolecules due to matrix addition.¹²⁸ One of the most important factors affecting MALDI MSI's performance is the manner in which an appropriate substrate can be added to the tissue sample.¹²⁹ The selected matrix should form good co-crystals with the tissue biomolecules while maintaining the analytes localization within the tissue, which is critical for good imaging resolution.¹³⁰

Guo *et al.* investigated 134 tissue samples from 6 types of cancer patients by MALDI MSI.¹⁷ The analysis of such a large

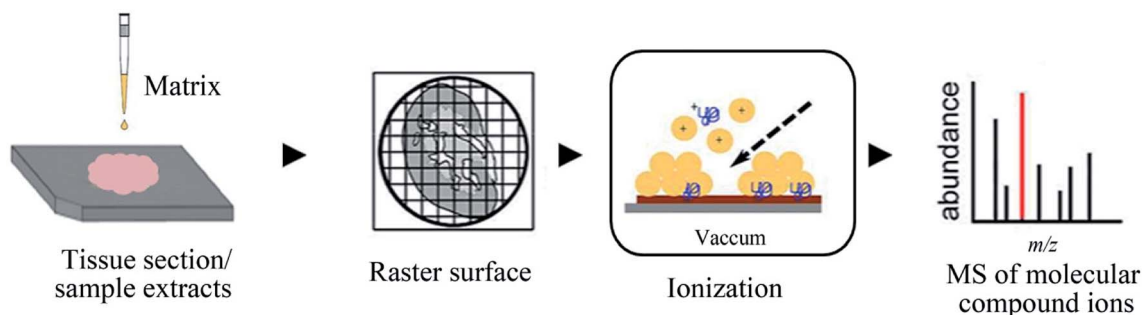


Fig. 4 Flowchart depicting the basic workflow of MALDI-MS. Reprinted with permission from ref. 101. Copyright (2018) ACS Publications.



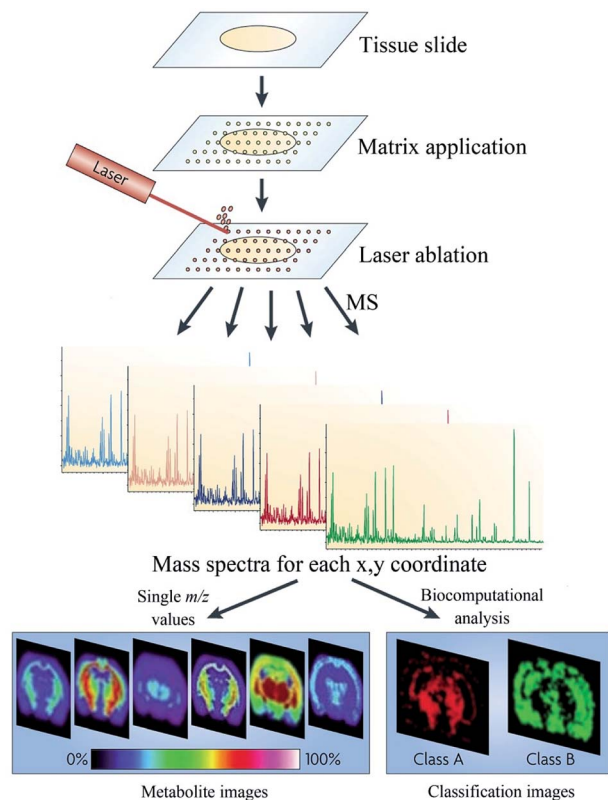


Fig. 5 Flowchart depicting the basic workflow of MALDI MSI. Reprinted with permission from ref. 125. Copyright (2010) Nature.

sample size revealed that monosaturated lipids were significantly increased relative to polyunsaturated lipids and the associated enzymes were overexpressed in the tumor microenvironment, which also indicated that the MALDI MSI platform is a powerful tool to study metabolomics and cancer mechanisms. Zhou *et al.* employed graphene oxide as a matrix to image small molecules in the tissue; here, in the negative ion mode, 212 small molecules were detected and spatially visualized in mouse brain tissues, which reiterated the robust characteristics of MALDI MSI.¹³¹

3.3.2 Secondary ion mass spectrometry imaging. Secondary ion mass spectrometry (SIMS), as the “oldest” MSI technique, works by exposing the tissue sample surface to a high-energy primary ion beam (such as Ar^+ , Ga^+ , and In^+) and then assembling and analyzing the generated secondary ions. This technique currently provides the highest lateral spatial resolution for cellular or subcellular exploration of metabolomics (Fig. 6).^{12,132} Without applying any matrix, SIMS is capable of acquiring higher spatial resolution than that by MALDI, thus reducing the possibilities of diffusion. However, this usually leads to lower chemical specificity at the expense of molecular fragments.¹³³ Traditionally, SIMS has been the primary method for inorganic surface analysis. However, SIMS has recently undergone a revival in pursuing new chemical information on tissue, single cell, and microbial systems. Phan *et al.* performed the SIMS of lipids across drosophila brain and obtained a detailed understanding of the biomolecular

distribution as well as their chemical structures with high accuracy based on the ion images of its fragments.¹³⁴ The present study is also useful in studying the relationship between biomolecules and their synthetic precursors or metabolite products.

3.3.3 Desorption electrospray ionization mass spectrometry imaging. Desorption electrospray ionization (DESI), an atmospheric pressure ionization technique developed in 2004, is derived from electrospray ionization and desorption ionization. It is performed by conducting electrosprayed charged droplets and solvent ions onto the surface to be analyzed, *i.e.*, DESI relies upon the regional desorption of analyte molecules into the liquid bridge formed between two fused silica capillaries, and the extracted analytes were transferred to spray ionization and mass spectrometer inlet (Fig. 7).^{121,135} In contrast to MALDI, multicharged ions of molecules (*i.e.*, proteins) may be observed on the spectra of DESI. As a matrix-free technique, DESI is not hampered by matrix-analyte co-crystallization issues and can serve as an alternative platform when compounds cannot be ionized in MALDI.¹³⁷ With further research, the spatial resolution of DESI has been improved to 10 μm .¹³⁸ The development of ambient pressure ionization of DESI further expands the applications of MSI.

Jarmusch *et al.* examined human brain tumors by DESI MSI. A cluster of lower abundance signals were observed, which aided in differentiating brain parenchyma and gliomas.¹³⁹ This operation can also be performed using tissue smears during intraoperative analyses, which may provide surgeons with near-real-time pathological information and guide the intraoperative resection of tumors whose perimeters are difficult to detect. Lanekoff *et al.* performed *in situ* imaging by nano-DESI MSI, obtained efficient separation, and identified difficult-to-isolate isomeric phospholipids in the full scan mode.¹⁴⁰ This novel feature opens up exciting opportunities for metabolomics.

3.3.4 Laser ablation electrospray ionization mass spectrometry imaging. Laser ablation electrospray ionization (LAESI) is a mixed ambient ionization source grounded on mid-infrared laser ablation with charged droplets produced by ESI.¹⁴¹ When rapid micro-ablation is driven by phase explosions, the mixture of molecules and particulate matter is emitted from the samples.¹⁴² The molecules are then bonded with the charged droplets generated by the electrospray, and a part of these molecules is transformed into gas-phase ions.¹⁴³ LAESI is particularly suitable for biological samples with high water content, and it usually employs a focused mid-infrared laser beam of 2940 nm wavelength to excite OH vibrations of water molecules in the sample. Here, the water acts as an “intrinsic matrix” (Fig. 8).¹⁴⁴ Li *et al.* utilized 3D LAESI MSI to characterize the distributions of a wide array of metabolites and lipids in *E. coli* and *B. subtilis* model microorganisms interacting with antibiotics in Kirby–Bauer-type experiments. This provided a more in-depth molecular understanding of microbe-antibiotic interactions and improved the quantitative properties of antibiotic susceptibility testing while conspicuously reducing the required incubation time.¹⁴⁵

Nowadays, MSI has evolved from a simple detection technique for different molecules present on various tissue surfaces



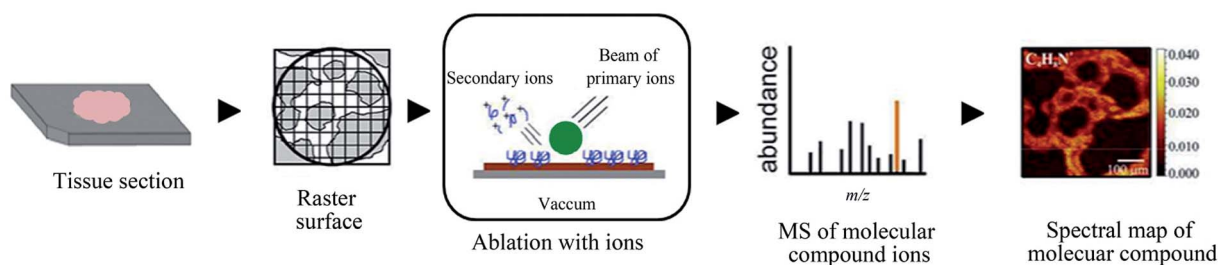


Fig. 6 Flowchart depicting the basic workflow of SIMS MSI. Reprinted with permission from ref. 101. Copyright (2018) ACS Publications.

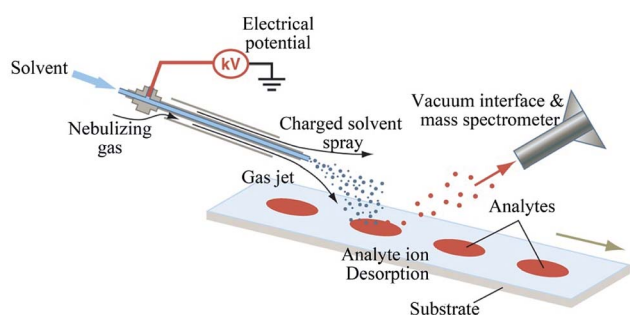


Fig. 7 Scheme of DESI MSI technique. Reprinted with permission from ref. 136. Copyright (2006) Science.

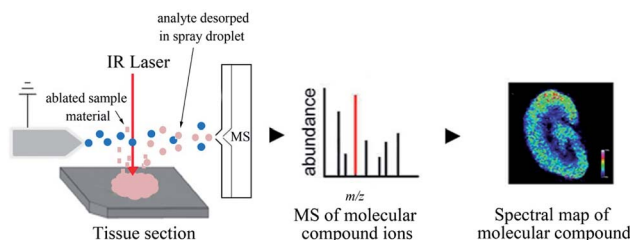


Fig. 8 Flowchart depicting the basic workflow of LAESI MSI. Reprinted with permission from ref. 101. Copyright (2018) ACS Publications.

or cells to a complex, in-depth imaging technique, revealing a mature analytical technology.

3.4 Direct analysis in real time-mass spectrometry

Direct analysis in real time (DART) is another versatile, new ambient ionization technology demonstrated by Cody *et al.* in 2005, which enables the rapid analyzing of solids, liquids, and gases at atmospheric pressure without sample preparation.¹⁴⁶ Helium is conducted *via* an axial tube and supports a corona discharge that engenders ions, electrons, and excited atoms. Then, helium passes through the other two chambers, where the electrons, cations, and anions are removed, passing into the atmospheric reaction zone that include only electronically excited neutral substances (Fig. 9).^{147,148} These released atoms in the atmosphere will cause the environmental gas (*e.g.*, atmospheric water or solvent) to undergo gas-phase reaction ionization cascade. Further, these ions serve as chemical ionizing reagents near the surface of the analyzed sample, resulting in

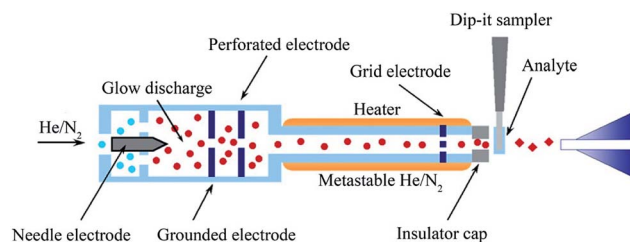


Fig. 9 Scheme of DART MSI technique. Reprinted with permission from ref. 149. Copyright (2011) Elsevier.

analyte ions finally getting transferred to the mass analyzer.^{147,149} Penning ionization is the most important step in DART ionization, and the flow rate of carrier gas and temperature are the two major factors that affect the DART ionization performance.¹⁰ The prominent features of DART include high throughput, minor cross-contamination, and simplicity. In fact, DART is typically used to analyze small molecular compounds with m/z of 50–1200.¹⁵⁰

From the very beginning, DART was utilized in plant component analysis, vegetable pesticide residue monitoring, forensics, and now gradually used in metabolomics research. Cajka *et al.* employed DART-MS, where the flow rate of carrier gas and temperature were optimized, as a rapid tool to evaluate the response of dietary supplementation in fish metabolomics.¹⁵¹ Distinguishing carp muscles according to dietary supplementation was practicable when using DART-MS, which represents a powerful analytical technique in metabolomics-based aquaculture research. Zhou *et al.* reported metabolomics fingerprinting research of human serum by DART-MS.¹⁵² In this approach, volatile metabolites were derivatized from protein precipitate, followed by DART-MS analysis. The detection of each sample required only 1.2 min, during which more than 1500 different spectral characteristics were observed. This shows that DART-MS is an invaluable tool for metabolomic fingerprinting. Exploring the changes in human metabolism with DART-MS may provide a better outlook on the amount of biological information.

4 Tools for MS result analysis

There are various open access and commercial software/websites available for MS data processing and analysis (Table 2). These software/websites involve peak alignment, peak



Table 2 MS-based metabolomics data processing and analysis software/websites

Software/website	Accepted data forms	Statistics	Pathway analysis	Data visualization	Source
MarkerLynx	.raw	✓		✓	Waters
MarkerView	.d	✓		✓	AB Sciex
MassHunter	all	✓		✓	Aglient
Mass Profiler	all	✓	✓	✓	Aglient
MetQuest	.raw	✓		✓	Thermo
SIEVE	.raw	✓		✓	Thermo
IDEOM	all	✓		✓	https://mzmatch.sourceforge.net/ideom.html
MathDAMP	all	✓		✓	https://mathdamp.iab.keio.ac.jp/
MAVEN	all	✓	✓	✓	https://maven.princeton.edu
MetAlign	all	✓		✓	https://www.metalign.nl
MetboAnalyst	all	✓	✓	✓	https://www.metaboanalyst.ca/
MET-COFEA	all	✓		✓	https://bioinfo.noble.org/manuscript-support/metcofea/
MET-XAlign	all	✓		✓	https://bioinfo.noble.org/manuscript-support/metxalign
MS-DIAL	all	✓		✓	https://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL
MsXelerator	all	✓		✓	https://www.msmetrix.com/
MZmine	all	✓		✓	https://mzmine.sourceforge.net/
msCompare	all	✓			https://trac.nbic.nl/mscompare
OpenMS	all	✓		✓	https://www.openms.de/
SMART	all	✓		✓	https://www.stat.sinica.edu.tw/hsinchou/metabolomics/SMART.htm
XCMS	all	✓	✓		https://xcmsonline.scripps.edu/

extraction, metabolites identification, and metabolic pathway analysis by searching metabolomics databases. The commonly used metabolomics databases include KEGG, HMDB, Metlin, and Massbank. However, there is no currently available standardized software/website for MS data processing and analysis. Using different software, the results will be very different,^{153–155} which shows that data processing and analysis software/websites have a great influence on data processing. Data processing is a vital part of metabolomics research and each software/website has its own characteristics. Researchers should choose the software/website to process and analyze data according to their own needs. It is believed that with the development of metabolomics methodologies and databases, data processing and analysis software/websites can be continuously enriched and perfected.

5 Limitations and challenges

MS-based metabolomics technology plays an important role in metabolites profiling due to its high sensitivity, high speed, and high throughput. However, some problems are constantly present in the continuous exploration of this field. First, since the concentration of metabolites in organisms varies greatly and some metabolites with lower concentrations may have important regulatory effects, the detection of trace-level metabolites presents a challenge for MS-based metabolomics. Therefore, the combined use of multiple separation, extraction methods, and MS detectors is imperative. Second, with the continuous improvement of sample processing methods, chromatographic separation capabilities, and MS sensitivity, massive amounts of data can be obtained. Thus, data processing will become another challenge for metabolomics. Third, on account of the different forms of adduct ions, the large amount

of data obtained after peak alignment, and the imperfection in the databases and standards, the identification of metabolites is considerably challenging. Fourth, accurate concentration data are roughly relevant to the experimental conditions and data collection methods, which are considered to be comparable among different groups. At present, most MS-based metabolomic studies are limited to the relative quantification of metabolites, which is also regarded as the Achilles heel of metabolomics.¹⁵⁶ The constant enrichment of isotopic affinity tags and standards will be conducive to the quantification of metabolites. Last, with the upgrading of the MS instrument, the construction and popularization of the Metabolomics Standards Initiative should be accelerated to achieve data sharing, effective use, and verification of experimental results.^{157–160}

6 Conclusion and future perspective

As a part of systems biology, metabolomics has received extensive attention in the field of life sciences, which represents the final omics level, supplying “functional” information among the omics studies.^{161–169} The latest advances in novel MS techniques have greatly improved the progression of metabolomics over the past decade (Fig. 10). The high selectivity, flexibility, and lower detection limits of MS along with its compatibility with various separation techniques and the capacity to provide qualitative and quantitative data makes MS an ideal instrument for metabolomics applications. At present, MS not only allows chemical information analysis of the extract content by LC-MS or DI-MS methods, but also can use imaging methods to analyze intact tissue or cells to provide spatial information for the location of given metabolites. In order to achieve better matching with clinical results, the sample processing, data analysis, method validation, and other details



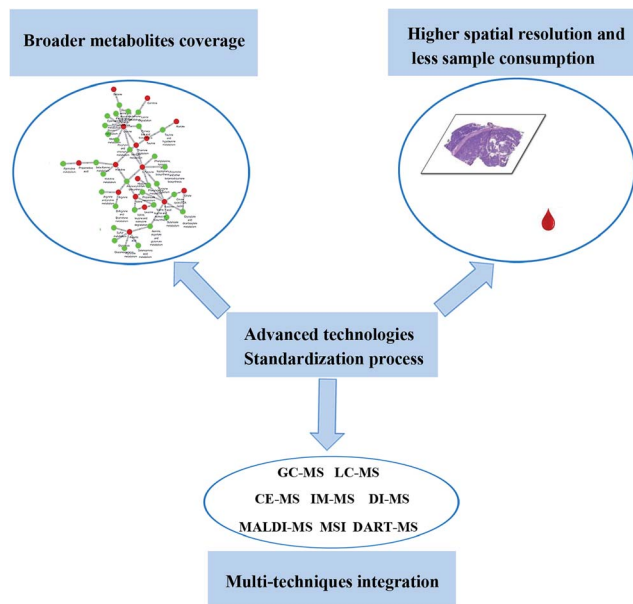


Fig. 10 Trends in MS-based metabolomics.

need to be standardized. In the future, it is necessary to continually develop new technologies to expand the coverage of metabolites and improve data quality. The measurement sensitivity, spatial resolution, and sample consumption should be improved constantly. Up to now, numerous published studies have demonstrated the promising potential of MS in metabolomics research. As another rapidly growing field of omics, it is expected that there will be more exciting new MS technologies applied in metabolomics in the near future.

Conflicts of interest

The authors declare no competing financial interests.

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