REVIEW

Mass Spectrometry for Metabolome Analysis

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Abstract : Metabolomics has become an important research field with many areas of applications ranging from disease biomarker discovery to global biology systems study. A key step in metabolomics is to perform metabolome analysis to obtain quantitative information on metabolic changes among comparative samples. Mass spectrometry (MS) is widely used for highly sensitive detection of many different types of metabolites. In this review, we highlight some of the more commonly used MS techniques for metabolome analysis.

Keywords : Metabolite, metabolome, metabolomics, mass spectrometry, targeted analysis, untargeted analysis.

From Genomics to Metabolomics

A living system is quite complicated with multiplex characteristics of life and scales of biological organizations. Biological functions are able to be explored through the molecules involved in physical and biochemical reactions in the body. The comprehensive assessment of a set of molecules can be achieved with various "omics" technologies, which include genomics, transcriptomics, proteomics and metabolomics.[1] The schematic representation of the omics cascade is shown in Figure 1.

Genomics was the first omics to appear and the most mature omics field. It studies the whole genomes and genetic variants caused by disease or medical treatment, through DNA sequencing and bioinformatics.[2] Nonetheless, many parts of genes have not been experimentally characterized and some of the relations between gene assignments and biochemical functions are still not clear, which may also be affected by other factors, such as environment.[3]

Transcriptomics is the connection between genomics and proteomics. It focuses on identifying RNA transcripts and measuring and quantifying the expression of genes in different organisms and under different conditions.[4] The limitation of transcriptomics is that some knowledge of transcriptome is still based on gene predictions, and relatively small changes in RNA level may lead to significant protein changes in the organisms.[5] This indirect correlation needs to be further studied.
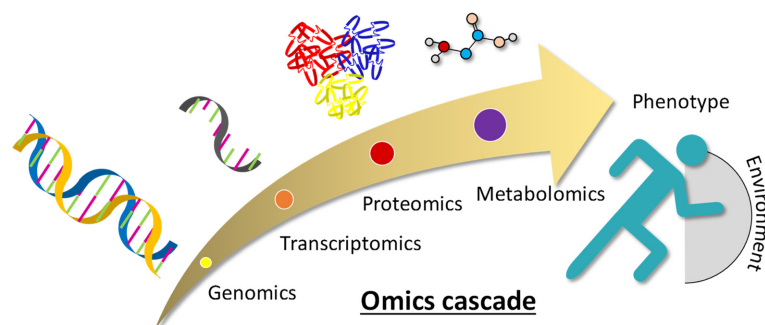
Proteomics explores the entire proteome and analyzes protein's abundance and interaction, and reflects the underlying genomics and transcriptomics.[6] Post-translational modifications can greatly increase the complexity of proteome studies.

Metabolomics is the end point of the omics cascade.[7] It quantitatively studies the entire set of metabolites, which are endogenous and exogenous compounds with mass <1500 Da, including amino acids, short peptides, carbohydrates, nucleic acids, organic acids and other products of metabolic functions. The significance of metabolomics lies in the fact that it is an ultimate reflection of organisms influenced by both genetic and environmental factors. In comparison with genomics or proteomics, which predicts a process may happen, metabolomics studies what is really occurring.[8] It is sensitive to the small changes in organisms' processes (e.g., DNA duplication, RNA expression) or diet or environment, which may cause huge metabolite concentration variances.[9] Therefore, it is the most appropriate and closest description of phenotype. Metabolomics is a powerful tool for global study of composition, dynamics and responses of metabolites in cells, biofluids, tissues and organs.[10] It can be used for studying the effects of system perturbations on metabolic functions by environmental influences or toxin effects, as well as for diagnosing diseases or monitoring treatment.[11] Thus, metabolomics studies have evolved exponentially since the concept was introduced.
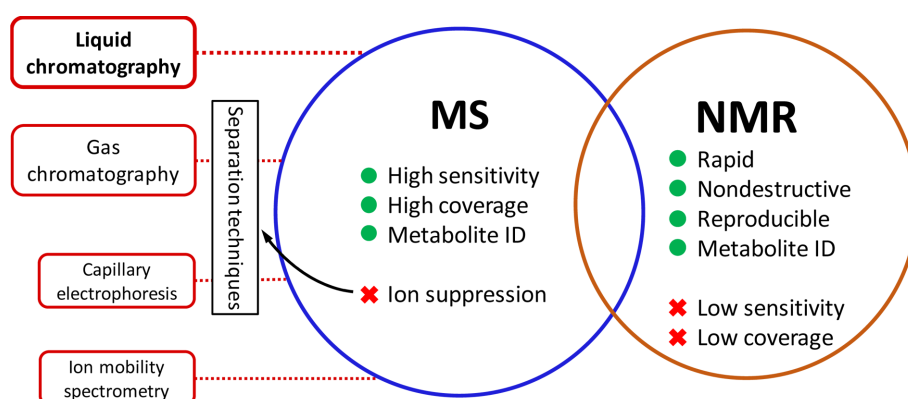
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Figure 1. Schematic representation of the omics cascade.

Figure 2. Analytical platforms for metabolomics.

Analytical platforms for metabolomics

Normally, the analytical techniques for metabolomics include detection and separation processes, as shown in Figure 2. For detection, there are mainly two platforms, nuclear magnetic resonance (NMR) and mass spectrometry (MS). For separation, there are mainly three kinds of chromatography methods, high-performance (HP) or ultra-high-performance (UP) liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), and a spectrometry-based method, ion mobility spectrometry (IMS). These high-throughput separation techniques are usually coupled to MS for metabolic profiling. No single technique can provide an ideal analysis for all metabolites. Each technique has its advantages and disadvantages. Next, we briefly introduce these platforms.

NMR is a spectroscopic technique. NMR is based on energy absorption and re-emission of the atom nuclei affected by the changing of an external magnetic field.[12] With rich natural abundance of hydrogen in biological samples, high resolution ^{1}H NMR is the most widely used NMR technique. It can rapidly quantify and identify a wide range, from low-molecule weight to high-molecule weight, of metabolites in a single run with little requirement for sample preparation, while simultaneously detect lots of

other compounds with high reproducibility. NMR is an information-rich method especially for providing structural information, which can be used for characterizing and exploring biological processes.[13] Besides, it is a non-destructive technique. As a result, samples can be recovered for further analysis if necessary. NMR has been widely used for biomarker discovery and disease studies.[11,14] However, the major limitation of NMR is the relatively low sensitivity. The detection can only be achieved above the micro-molar range, while in real world analysis, many metabolites will be below its detection limit.[15]

MS detects metabolites in the form of spectral peaks with mass-to-charge ratios (*m/z*). MS is the most widely used platform in metabolomics. It can provide high sensitivity and selectivity, as well as reproducible quantitative analysis and the possibility for metabolite identification. There are several different mass analyzers. Quadrupole (Q), ion trap (IT), time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), Orbitrap and linear ion trap (LIT) are the common ones used for metabolite analysis. Quadrupole and ion trap MS are relatively easy to maintain and offer good sensitivity but with limited resolution. High-resolution MS, such as TOF, FTICR and Orbitrap, can provide accurate mass measurement, allowing for precise identification.[16] FTICR-

MS offers extremely high resolution (200,000–5,000,000), but the slow data acquisition rate leads to low sensitivity. Besides, the high instrumental cost limits its application. In comparison, TOF-MS can provide high resolution as well as high sensitivity with a relatively low cost. Thus TOF-MS is more popular for metabolomics. Orbitrap MS is also widely used. Hybrid instruments, such as quadrupole-TOF-MS, are widely used for structural analysis and rapid screening analysis.[17] Besides generating structure information, triple quadrupole (QqQ) and quadrupole linear trap (QTrap) tandem MS are excellent instruments for quantification of metabolites.

Different kinds of MS-based methods have been developed. Direct injection MS is a rapid technique that can analyze a large number of metabolites.[18] The obvious drawbacks of this method involve co-suppression and low ionization efficiencies.[19] Thus, MS is usually combined with separation techniques such as LC,[20] GC,[21] CE[22] and IMS[23] to reduce sample complexity.

LC-MS is the most widely used technology with the ability to detect a wide range of compounds. The recent UPLC system can further improve chromatographic resolution. Compared with GC-MS, LC-MS doesn't require sample volatility and has lower analysis temperature. Sample derivatization is usually not required, but when necessary, it can be useful to provide better separation and sensitivity.[24] Metabolites can be detected in both positive and negative ion modes, which can achieve high metabolome coverage. It usually uses electrospray ionization (ESI) as the ionization source, which is a soft ionization technique and leads to little fragmentation; but ESI is easily affected by ion suppression.[25] Various column chemistries have been developed. For example, hydrophilic interaction liquid chromatography (HILIC) can be used to separate polar metabolites, while reversed phase liquid chromatography (RPLC) can separate non-polar metabolites. However, one kind of column can be beneficial to analyze only one kind of polarity of metabolites, which makes detection more complicated and time-consuming.

GC-MS is a high-throughput technique and generally detects low-molecular-weight, volatile and thermally stable compounds, such as for the analysis of breath.[26] The high-molecular-weight, non-volatile metabolites cannot be analyzed directly, and they need multiple chemical derivatization procedures to increase volatility and chemical stability.[27] Because GC analysis is usually done under high temperatures, the sample stability is a major concern.

CE-MS is an emerging tool for metabolomics studies and has significant potential.[28] CE-MS can provide extremely high-resolution and analyze for almost all charged compounds.[28] However, the repeatability needs to be further improved.[29]

Over the past 2-3 decades, IM-MS has been obtaining great interest in applying for metabolomic analysis. IMS is a gas-phase electrophoretic technique.[23] Based on the different shape, size, and charge state of different ions, it can achieve rapid separation. Since the separation mechanisms of IMS is orthogonal to that of MS, IM-MS can enormously enhance signal-to-noise ratio, separation and identification, especially when further combined with LC. Nevertheless, high instrument-to-instrument variation, particularly for metabolite quantification, is the current limitation.[30] And more software tools need to be developed to process the complicated data.[31]

Metabolites can have very different chemical and physical properties. In general, one MS system cannot analyze all types of metabolites. For analyzing relatively polar or ionic molecules such as sugars, nucleotides and amino acids, hyphened techniques such as LC-MS and CE-MS are often used. The type of mass analyzer used depends on the requirement of the analysis. For example, for accurate mass measurement, high resolution and high accuracy mass analyzers such as FTICR, Orbitrap and TOF can be used. To generate chemical structure information, tandem MS (or MS/MS) instruments may be used. For analyzing relatively nonpolar metabolites such as many classes of lipids (e.g., fatty acids), GC-MS and LC-MS are commonly used. For GC-MS, high resolution TOF or Orbitrap MS is available for accurate mass measurement. GC-MS/MS can be helpful for structural analysis and metabolite identification.

MS-based targeted and untargeted metabolomics

Based on experimental methods, metabolomics can be divided into two major categories: targeted and untargeted. Figure 3 shows targeted and untargeted metabolome analysis workflow. Targeted metabolomics only focuses on a specific class of compounds or particular biological pathways.[32] It is a hypothesis-driven approach and explores abundance changes of selected and pre-known metabolites. Generally, triple quadrupole (QqQ) MS with selected reaction monitoring (SRM) or even multiple reaction monitoring (MRM) is applied for routine targeted analysis.[33] In this case, only certain *m/z* values in mass spectra or certain regions of chromatograms are analyzed.[34] Targeted metabolomics is not a global approach, but can provide high sensitivity, high throughput and capability for absolute quantification.

In comparison, untargeted metabolomics is global in scope and cares about the entire chromatogram and all *m/z* values.[35] It is usually hypothesis-generating instead of hypothesis-driven, aiming at detecting as many metabolites as possible.[33] Untargeted metabolomics determines the relative amount of all measurable known and unknown metabolites and carries out the identification. This technique is significantly attractive and suitable for diagnostic biomarker discovery as well as nonbiased
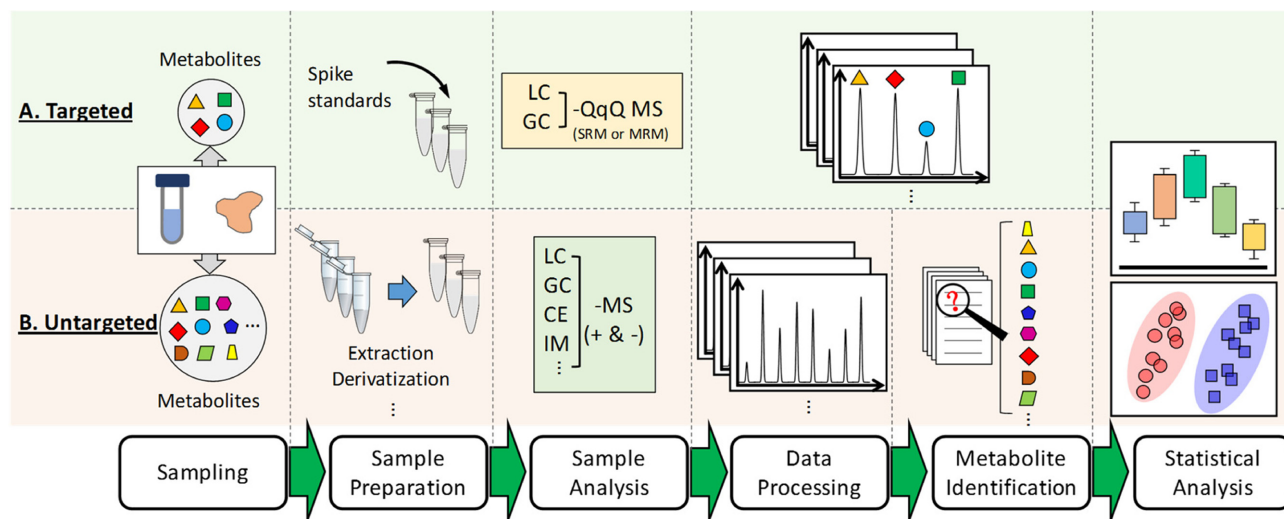
Figure 3. (A) Targeted and (B) untargeted metabolome analysis workflow.

metabolite fingerprinting in response to disease or genetic alterations.[29] Different kinds of biological samples have been studied, such as tissue,[36] serum,[37] urine,[38] cell,[39] etc. with different advantages. Biofluids are usually easy to collect and widely used for different analysis, which are considered as a pool of metabolites of the organisms and can reflect systemic metabolic changes. In comparison, tissue samples can reveal organ-specific metabolic fingerprints,[40] and tissue metabolomics plays an important role in investigating specific diseases[41] and sites of toxicity.[42] Although untargeted metabolomics is promising and significantly developed in the last couple of decades, no one single method can achieve the non-targeted analysis of all the metabolites, due to their diverse polarities, molecular weights and concentrations.

Workflow for MS-based untargeted metabolomics

As Figure 3B shows, MS-based untargeted workflow involves several steps. Generally, the experimental steps include sampling, sample preparation, sample analysis, data processing, metabolite identification and statistical analysis.[16] Next, each step is briefly discussed.

The first step is sampling, which primarily depends on the experiment type and experimental design. Sampling is the basis of the project and plays important roles. Many aspects should be considered, such as, how much sample would be sufficient, what kind of samples could be analyzed (e.g., serum/plasma, urine, tissue, cell, saliva, etc.), what would be the effects of sex, age and diet, and what would need to be done to inhibit enzymatic activity and stop metabolism after sample collection (e.g., freezing or acidic treatments). Otherwise, biological variability and metabolite degradation would be major issues.

The second step is sample preparation, which mainly extracts metabolites from complex matrix and removes interfering components. The conventionally analyzed sample types include cells, tissues and biofluids (e.g., urine, saliva, blood). Different kinds of samples need to be processed differently. For example, cells need to be efficiently lysed and tissues need to be completely homogenized in order to detect all the metabolites. For blood samples, such as serum or plasma, protein precipitation with organic solvent is critical to remove the interference of high-abundance proteins. Urine and saliva are usually analyzed directly, but need to be diluted if concentration is too high. When necessary, further extraction methods, such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE), and derivatization can be used to pre-concentrate certain metabolites and improve the detection limit.

After sample preparation, the third step is sample analysis and data acquisition, in which samples are analyzed by different kinds of platforms, such as, direct injection MS, IM-MS, CE-MS, GC-MS or LC-MS, with LC-MS as the most popular one. In this process, metabolites are detected as peaks with *m/z* values in mass spectra and the intensities of chromatographic peaks are related to their concentrations. Then, metabolic data in mass spectra are exported to a standard and uniform format for further analysis.

The fourth step involves peak extraction and data processing. The process includes peak picking, noise and background exclusion, alignment of chromatograms and mass spectra. One widely used software is XCMS, a web-based software.[43] Here, each peak is referred to as a metabolite feature and extracted information is displayed in a peak table with information of each feature's retention time, *m/z*, intensity, as well as *p*-values and fold changes representing relative concentration differences.

The next step is the identification of metabolites, which still remains a big challenge. In this step, accurate mass of metabolites is firstly searched in metabolite database, such as METLIN[44] and Human Metabolome Database (HMDB)[45] to get putative identification information. Here, depending on the different mass accuracy and resolution power of different kinds of MS instruments, various mass-tolerance criteria can be applied. For example, for low-resolution MS, such as triple quadrupole, the mass window is usually around 0.5 Da, while for high-resolution MS, such as TOF, it can be 0.005 Da or 10 ppm, and for even higher resolution MS, such as FTICR and Orbitrap, it can be as low as 2 ppm. A putative ID can be achieved if the detected m/z is within the tolerance range to the m/z value of a metabolite in the database. Then, further experimental data are used, including retention time and tandem mass spectra (MS/MS to get fragmentation patterns), before the data are compared with standards to definitely identify those metabolites of interest. Currently, large numbers of metabolites still cannot be matched and comprehensive metabolite identification is a challenge.

Finally, multivariate and univariate statistical tools are applied for sample classification and significant markers determination, such as principal component analysis (PCA), partial least square discriminant analysis (PLS-DA), analysis of variance (ANOVA) and volcano plot. The commonly used software includes SIMCA-P (Umetrics AB, Umeå, Sweden) and Metaboanalyst[46] (https://www.metaboanalyst.ca).

Increasing metabolome coverage

Because of great diversity of chemical and physical properties as well as metabolite concentrations in complex biological samples, high-coverage metabolome analysis is a major analytical challenge. Due to the reproducible and robust separation characteristics, RPLC-MS is the most widely used untargeted metabolomics technique. It has been applied for the studies of various kinds of samples, such as serum,[47] urine,[48] tissue[49] and cells[50] etc., with the ability to detect thousands of features. However, conventional RPLC-MS can only handle medium polar and non-polar compounds. It is not appropriate for extremely polar metabolites. In this case, HILIC-MS develops rapidly as a complementing approach to separate and detect polar compounds.[51] And from the aspect of MS, to enable comprehensive analysis, metabolite ionization can be performed in both positive and negative modes.

In order to further increase metabolome coverage of LC-MS, the combination of multi-platform analytical techniques was thought to be a good way. It can be two-dimensional liquid chromatography (2DLC) techniques, combining orthogonal separation techniques, to analyze polar and non-polar metabolites.[52] It can also be LC with other separation techniques, for example, LC-MS and GC-

MS were integrated to analyze zebrafish embryos,[53] because GC-MS is advanced in analyzing small volatile molecules and has larger metabolome databases for identification.[54] And, LC-MS can be incorporated with CE-MS[55] to take advantage of CE-MS's power in detecting polar and charged metabolites. Also, LC-IM-MS has been developed with the potential for additional selectivity and extending measurable identifiers to include analyte ion-specific collision cross section (CCS) values from IMS.[56] Many other efforts have been taken to increase metabolome coverage, mainly focusing on optimizing metabolite extraction methods and chromatography conditions.[57-60] However, all of these setups make the experiments complicated and low-throughput. And, although with these techniques, tremendous number of features (a unique m/z with retention time) are detected, many of them are actually from a single compound detected in different forms, such as in-source fragments, adducts, isobaric compounds (e.g., dimers, trimers). It makes the results difficult to interpret and explain. Also, it is still a challenge to differentiate the weak signals of low-abundant metabolites from background noise.[61] Previously, our lab developed a high-performance differential chemical isotope labeling (CIL) method coupled with LC-MS, which can significantly increase the metabolome coverage. This is discussed in the next section.

Chemical isotope labeling metabolomics

CIL LC-MS is a "divide and conquer" technology, in which the whole metabolome is divided into different submetabolomes based on different chemical functional groups. It can be used to analyze each submetabolome with high coverage and the combined results are used for the complete metabolome analysis. Figure 4 shows the typical workflow of the CIL LC-MS. Dansyl chloride (DnsCl) for amine/phenol submetabolome[24] is used here for the discussion of the CIL LC-MS process and its benefits.

In this approach, individual experimental samples are labeled with ^{12}C-DnsCl, while a pooled sample, combining aliquots from individual samples, is labeled with ^{13}C-DnsCl, working as the reference and internal standard. Then, LC-UV is used for the metabolite quantification and pre-acquisition sample normalization. After mixing ^{12}C-labeled individual samples and ^{13}C-labeled pooled sample together in equal mole amount, samples are analyzed by LC-MS. Metabolites are detected as peak pairs, which contain light peaks (^{12}C-labeled, from individual samples) and heavy peaks (^{13}C-labeled, from pooled sample) with an m/z difference of 2.0067 Da. The intensity ratio of light and heavy peaks is used for relative quantification of metabolites. This method has many advantages for the detection of amine/phenol containing metabolites. Firstly, the aromatic group makes the metabolite more hydrophobic which increases retention in RPLC. Secondly, the tertiary
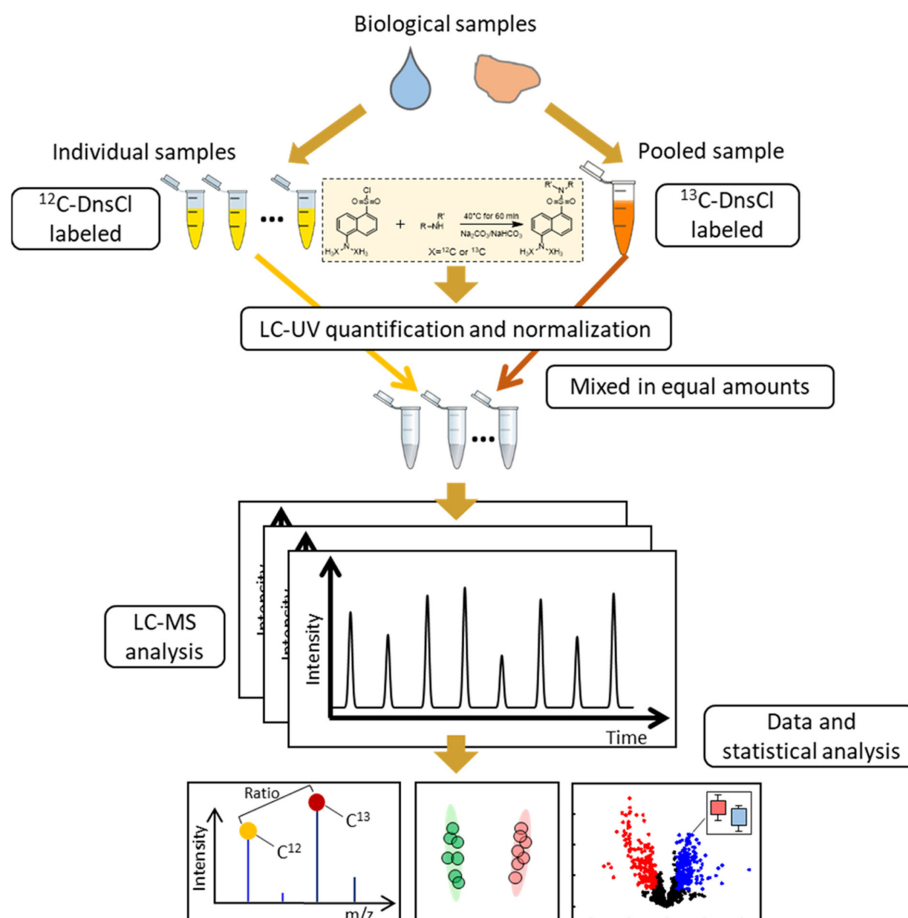
Figure 4. Workflow of chemical isotope labeling LC-MS.

amine enhances ESI chargeability, thus enhancing MS signals. Thirdly, the two carbons on the tag are either ^{12}C or ^{13}C, which are used for relative quantification. This method simultaneously improves separation, detection and relative quantification, in which case, only positive ion mode RPLC is required, increasing throughput. In the last decade, we have also developed ^{12}C-/^{13}C DnsCl with base-activation for analyzing the hydroxyl submetabolome,[62] ^{12}C-/^{13}C-dimethylaminophenacyl (DmPA) bromide labeling for the carboxyl submetabolome[63] and ^{12}C-/^{13}C dansylhydrazine labeling for the carbonyl submetabolome.[64] These four submetabolomes cover more than 95% of the whole metabolome. Thus, this comprehensive technique is applicable and promising for untargeted metabolomics applications. Development of new and improved CIL reagents has been an active research area for both targeted and untargeted metabolome analysis.[65-74]

Concluding remarks

Compared with other omics fields, metabolomics is still an emerging and fast developing research area. Profiling the metabolome with MS as completely as possible remains a major task. At the same time, other higher requirements are needed. The goal now is not only to detect significantly changed metabolites and determine biomarkers, but it is more important to study the underneath biological stories, such as which metabolic pathways and networks are those metabolites related, how those metabolites regulate and affect organism functions and phenotypic outcomes. Besides, on the sampling and instrumentation part, new and advanced analytical methods and technologies continue evolve to handle samples and detect metabolites with high throughput and time efficiency. Moreover, metabolite identification is still a challenge and more synthetic standards and computational tools are required to confirm the identities of unknown metabolites. Although with these issues and challenges, as it moves forward, MS-based metabolomic study is promising and will be more and more practical for real-world applications.

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