CHAPTER 2

Chemical Derivatization for Polar Metabolome Analysis

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2.1 Introduction

There are diverse polar compounds present in biological systems. Their high solubility in water helps them to participate in many metabolic reactions commonly taking place in the aqueous environment of an organism. Serving as multifunctional elements (*e.g.*, substrate, product, intermediate, cofactor, *etc.*) in metabolic pathways, relatively polar metabolites play fundamental roles in physiological and pathological processes. Therefore, analytical methods to measure these metabolites with high performance (*i.e.*, highly sensitive detection, accurate and precise quantification) are extremely important in biological studies.[1]

Liquid chromatography–mass spectrometry (LC–MS) has become one of the most commonly used platforms for small molecule analysis.[2] It offers several attractive features, including the ability to detect a wide range of different types of molecules, compatibility with various sample types, consumption of small amounts of samples, and operational robustness with possible automation. However, even with the most cutting-edge LC–MS instrumentation, it is still challenging to analyze many polar

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metabolites with high sensitivity and specificity. Because of their hydrophilic properties, polar metabolites cannot maintain strong hydrophobic interactions with the stationary phase of reversed-phase liquid chromatography (RPLC) (*e.g.*, C18 column), resulting in very weak retention on RPLC. To address this issue, two approaches are traditionally used. The first approach is to use hydrophilic interaction liquid chromatography (HILIC) to target the analysis of polar compounds.[3] Although many methods have been reported, HILIC analysis still suffers from relatively low performance of separation, compared to RPLC. The other approach is to use ion-pairing RPLC.[4] By adding ion pairing reagents as counterions, which are usually large ionic molecules with opposite charge to the analytes of interest and the hydrophobic part, the retention on RPLC can be enhanced. However, the ion pairing reagents often introduce severe ion suppression on the electrospray ionization (ESI) process, which decreases detection sensitivity. In addition to the challenge of separating polar metabolites with high efficiency in LC, many polar metabolites (*e.g.*, sugar molecules) are not readily ionized by ESI. They suffer strong matrix interferences in complex biological samples. Some polar metabolites are readily fragmented in the interface region of a mass spectrometer, which may result in the reduction of molecular ion signals, as well as cause confusion in determining the molecular ions.

Alternatively, instead of optimizing the instrumental setup and running conditions, chemical derivatization can be combined with LC–MS analysis to measure polar metabolites in complex biological samples.[1,5] In this approach, metabolites are derivatized with one or several chemical reagents prior to the LC–MS analysis (see Figure 2.1). As the physicochemical properties of the metabolites are altered, improved analysis performance can be achieved, including enhanced retention and separation on RPLC, higher

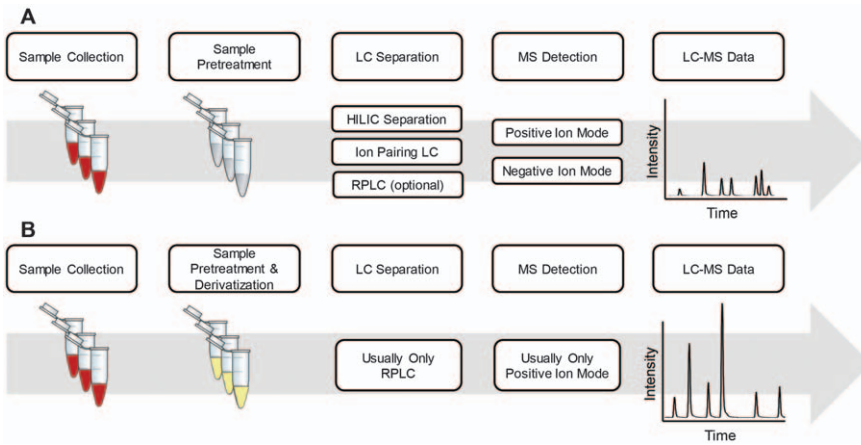

Figure 2.1 Comparison of (A) conventional LC–MS method and (B) chemical derivatization LC–MS method for polar metabolome analysis.

detection sensitivity, and better quantification ability. In this chapter, we present some chemical derivatization methods targeting several important classes of polar metabolites. We then discuss a high-performance chemical isotope labeling (CIL) LC–MS approach[6] as a robust method for high-coverage quantitative analysis of both polar and less-polar metabolites in one instrumental setup.

2.2 Chemical Derivatization LC–MS Analysis of Targeted Polar Metabolites

2.2.1 Amino Acid and Derivative Analysis

In addition to being the basic constituent units of proteins, free amino acids and their analogs and derivatives serve in many biological functions, such as central carbon metabolism, neurotransmitters, *etc.* As a zwitterionic metabolite, the amine group and carboxyl group in this type of molecule greatly increase its hydrophilicity. Derivatization is commonly used in amino acid analysis. There are mainly three types of derivatization reagents: 1) sulfonyl chlorides or acyl chlorides, 2) NHS ester-based reagents, and 3) isothiocyanates. In all three types of reactions, the amine group in amino acids serves as a nucleophile to attack the electrophilic region of a derivatization reagent, forming a stable derivative.

For example, a broadly applicable method of using benzoyl chloride (BzCl) labeling for analyzing neurologically relevant compounds was developed.[7] Many polar metabolites were well separated by a C18 column and detected with greater sensitivity, including amino acids, catecholamines, polyamines, and their metabolites. This approach was successfully applied in studying dopamine regulation in learning and motivation.[8] Multiplex isobaric tags reagents [*e.g.*, isobaric tags for relative and absolute quantification (iTRAQ),[9,10] tandem mass tags (TMT),[11] *N*,*N*-dimethyl leucine (DiLeu)[12,13]] combined with LC–MS/MS have been used in several studies for amino acid analysis. By using multiplex reagents containing different cleavable reporter ions for samples and standards, a wide range of amino acids and amines can be quantified with MS/MS fragmentation in relatively higher sample throughput.

Another highly important area of metabolite analysis is related to the determination of chiral metabolites, such as the analysis of D/L amino acids. Instead of using special chiral chromatography, chiral derivatization reagents that contain another chiral center can be used to label the metabolites, followed by conventional LC separation and MS detection. By forming diastereomers during the derivatization, original enantiomers (*i.e.*, D/L amino acids) can be well separated through achiral chromatography. Toyo'oka and co-workers have developed a number of chiral derivatization reagents for various metabolites.[14,15] For example, by using DMT-(*S* or *R*)-Pro-OSu for chiral amines and DMT-(*S* or *R*)-Apy for chiral carboxylic acids, chiral biomarker candidates were found for Alzheimer's disease.[16]

2.2.2 Nucleotide Analysis

Nucleotides are small molecules consisting of three moieties: a nucleobase, a five-carbon sugar, and a phosphate(s) group. Serving as the basic building blocks of macromolecules such as DNA, nucleotides play essential roles in many biological processes, including DNA/RNA/lipids synthesis, cell signaling, *etc.* However, robust analysis of these molecules by LC–MS is challenging, due to their high polarity. Several derivatization methods combined with RPLC–MS have been reported to improve the detection of nucleotides. Most of the methods use a strategy of blocking the polar and ionic groups in the nucleotides.

A method of using propionyl or benzoyl acid anhydride to esterificate the free hydroxyl groups in AMP, ADP, and ATP was reported.[17] The method could also be used for other types of polar compounds, such as cytokinins, showing the improvement of both ESI response and chromatographic properties on RPLC. Similarly, by using acetone as the derivatization reagent, a simple and low-cost derivatization method was reported for ribonucleoside determination.[18] Improvement of separation and enhancement of sensitivity were achieved. A methylation reagent, (trimethylsilyl)-diazomethane (TMSD), was developed to attach methyl groups to the phosphate groups, primary amines, and secondary amines on nucleotides, leading to much increased hydrophobicity of the metabolites.[19] The derivatized nucleotides could be separated on a C18 column with a common acetonitrile–water elution system. This method also offered a possibility of exhibiting unique ion transition for each metabolite to avoid mutual interferences from each other in MS/MS acquisition, thereby increasing the detection specificity.

2.2.3 Sugar Analysis

Low molecular weight sugars (monosaccharides) and sugar phosphates are key components of many critical energy metabolism pathways, including glycolysis, the pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, *etc.* However, reliable measurement of these compounds by conventional LC–MS is difficult, due to their extremely polar nature. In addition, some sugar phosphates are chemically unstable and easily degraded during sample preparation. Sugars are not ionized by ESI efficiently. To overcome these challenges, derivatization methods of sugars and related compounds followed by LC–MS analysis have been reported.

A two-step derivatization method has been developed, using methoxylamine and propionic acid anhydride to react with the free carbonyl group and hydroxyl groups in the sugar compounds, respectively.[20] After derivatization, the compounds showed good retention behavior on RPLC without the need to use ion pairing reagents, which eliminates the ion suppression effect caused by ion pairing reagents. The detection sensitivity was also increased. The authors successfully measured sugar phosphates in *Populus*

leaf extracts. Both reducing and non-reducing sugar phosphates could be analyzed. Similarly, a method using 3-amino-9-ethylcarbazole (3-AEC) to derivatize the sugars and sugar phosphates was reported.[21] The labeled sugars were analyzed on a pentafluorophenyl core–shell LC column. Selective determination of free and phosphorylated reducing sugars could be achieved by this method.

Researchers also used chemical derivatization to improve HILIC separation of sugar compounds. For example, Bawazeer *et al.* found that, if using d_5-aniline to tag the sugars, better separation and peak shape of monosaccharides with a ZIC-HILIC column were observed.[22] With this derivatization reagent, they could perform separation of fructose, glucose, galactose, and mannose with baseline resolution and good peak shape. The approach was validated by applying the method to analyze sugars in urine and brain samples.

2.2.4 Small Organic Acid Analysis

Small carboxylic acids are another important category of molecules that are involved in many essential pathways, including short-chain fatty acids, keto acids, hydroxyl acids, *etc.* Because of the presence of relatively large polar or ionizable groups in small molecules, small organic acids also have weak chromatographic retention behavior on RPLC. Detection of underivatized carboxylic acids is usually conducted in negative ion mode, which suffers from less sensitivity and more interference than positive ion mode detection. Therefore, better methods than the conventional LC–MS approach are needed for small organic acid analysis.

Chemical derivatization can improve the analysis. Many reactions in derivatizing carboxylic acids are based on the condensation reaction with amines and esterification reaction with hydroxyls. The method of using ^{12}C-/^{13}C-3-nitrophenylhydrazine (3-NPH) to analyze carboxylic acids was reported.[23] The labeled metabolites gained significant enhancement of detection in negative ion mode. It was applied in the detection and quantitation of many important categories of acids, including many carboxylic acids in central carbon metabolism, short-chain fatty acids, and bile acids.[24]

Zhao *et al.* developed dansylhydrazine (DnsHz) labeling for untargeted profiling of the carboxyl submetabolome.[25] A labeled-carboxyl-standard library consisting of 193 endogenous human metabolites was constructed for metabolite identification, including various amino acids, short-chain fatty acids, keto acids, polycarboxylic acids, *etc.* With this method, high-performance relative quantification of two comparable groups of urine samples was achieved. Using the similar labeling reaction, Chen *et al.* developed a method with DnsHz labeling to carry out absolute quantification of 25 free fatty acids and 13 acylcarnitines in human plasma samples within 12 min of an LC–MS run.[26]

Among many different types of organic acids, short-chain fatty acids (SCFAs) and their derivatives, as crucial intermediates and elements for

metabolism, have attracted great interest. Many reagents have been developed to profile and quantify SCFAs. For example, Girard's reagent T (GT) derivatization can provide a permanent cationic charge to an acid, resulting in a significant increase in detection sensitivity.[27] A fast derivatization reaction was developed with d_0/d_6-DHPP for SCFAs.[28] The authors reported the analysis of 40 SCFA and OH-SCFA species in liver, feces, and serum samples.

2.3 Untargeted Analysis of Polar and Less-polar Metabolites

The examples described in the previous section show that chemical derivatization can significantly improve the analysis of targeted polar metabolites. For untargeted metabolome analysis, the goal is to detect and quantify as many metabolites as possible, including both polar and less-polar metabolites with diverse chemical structures. An ideal method should be able to analyze many compounds with different functional groups (*i.e.*, high metabolic coverage), enable satisfactory retention and separation by LC for a complex matrix (*i.e.*, reducing interference), detect metabolites with wide concentration ranges in samples (*i.e.*, high detection sensitivity), and render reliable quantification (*i.e.*, high accuracy and precision). In the meantime, a compatible data processing method to facilitate data interpretation (*e.g.*, data processing software, databases, *etc.*) is required.

Our laboratory has been working on the development of a high-performance (HP) chemical isotope labeling (CIL) LC–MS platform for high-coverage quantitative metabolome analysis. Chemical derivatization is the central theme of the platform. However, differing from the conventional strategy of chemical derivatization to analyze selected metabolites, HP-CIL is a holistic approach to use chemical derivatization to simultaneously improve the performance of multiple steps in the whole metabolome analysis workflow. For example, a derivatization method used to attach a permanent charge to a metabolite may result in an increase in ionization efficiency. However, the derivatized metabolite is ionic and may not be retained on RPLC, adversely affecting its separation. A preferred method is to rationally design the chemical structure of a derivatization reagent that would allow concomitant enhancement in both ionization and separation efficiency after a metabolite is derivatized. Similarly, a derivatization method can increase the hydrophobicity of polar metabolites; however, we also need to balance the extent of such an increase so that we will not make the less-polar metabolites too hydrophobic to be effectively separated by RPLC. Another consideration is related to the incorporation of isotopic atoms to the reagents. A simple route of synthesizing a reagent would reduce the reagent cost. Robustness of reaction conditions used for chemical derivatization also needs to be carefully weighed. Thus, only a chemical derivatization method that enhances the performance of all different aspects of the whole metabolome analysis workflow can be viewed to have high performance.

Development of the reagents and reaction conditions to perform high-performance metabolome analysis is an active research area. We have developed the 4-channel chemical isotope labeling (CIL) LC–MS technique for metabolomics.[6] We use the term, chemical isotope labeling, to be in line with the metabolic isotope labeling (MIL) approach. MIL uses metabolic reactions in a cell culture containing heavy isotope materials (*e.g.*, $^{15}NH_3$) to introduce heavy isotope atoms into molecules of interest such as metabolites, proteins, *etc.*, while CIL uses chemical reactions to introduce an isotope tag to metabolites. By using four derivatization reactions targeting several functional groups (*i.e.*, amine, phenol, hydroxyl, carboxyl, and carbonyl group), the whole metabolome can be profiled with very high coverage. The rationally designed derivatization reagents ensure the overall improvement in analyte separation and detection. By using a pair of isotope reagents (*i.e.*, $^{12}C_2$-reagent and $^{13}C_2$-reagent) for each derivatization reaction, internal standards can be created.

The workflow of using the CIL LC–MS method to measure both polar and less-polar metabolites in samples is shown in Figure 2.2. Briefly, after general sample pretreatment procedures for biological samples (*e.g.*, protein precipitation for serum/plasma samples, metabolite extraction for tissue/cell samples), derivatization reagents are mixed with samples for chemical labeling. All individual samples are derivatized by $^{12}C_2$-reagents (light labeling), and a reference sample is derivatized by $^{13}C_2$-reagents (heavy labeling). Then the $^{13}C_2$-labeled reference sample is mixed with each $^{12}C_2$-labeled individual sample, followed by LC–MS analysis of the mixture. In the LC–MS data, each metabolite is detected as a peak pair: the light peak originates from a $^{12}C_2$-labeled individual sample and the heavy peak from the $^{13}C_2$-labeled reference sample. This differential isotope labeling approach allows accurate relative quantification. If the reference sample is a standard or a mixture of standards, with known concentration(s), absolute quantification of metabolites in individual samples can also be carried out. With dedicated software, such as IsoMS Pro, peak pair information is extracted, and the chromatographic intensity ratio of a peak pair is calculated to represent the metabolite amount for quantification. Metabolite identification is finally done using a database search approach.

2.3.1 Main Features of CIL LC–MS

Chemical derivatization of the high-performance CIL LC–MS method is based on using different reactions to target four submetabolomes: dansylation reaction for amine/phenol submetabolome,[29] DmPA labeling for carboxyl submetabolome,[30] base-activated dansylation reaction for hydroxyl submetabolome,[31] and dansylhydrazine labeling for carbonyl submetabolome[32] (see Figure 2.3). The derivatization reagents used in these reactions, dansyl chloride, dimethylaminophenacyl (DmPA) bromide, and dansylhydrazine, share several major features for analysis, especially for polar compound analysis.

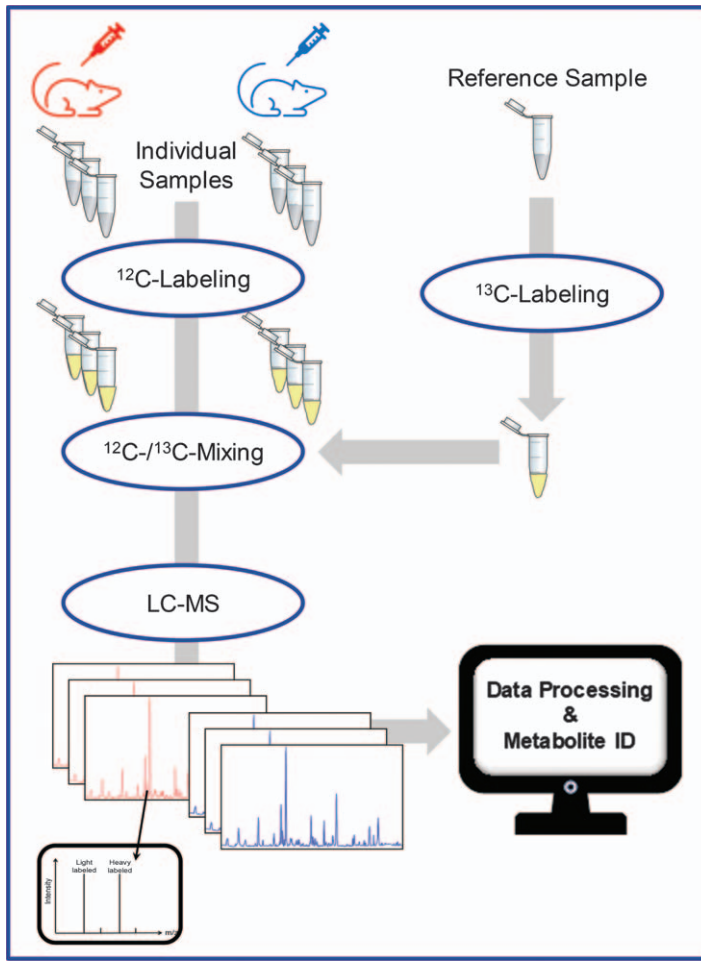

Figure 2.2 General workflow of CIL LC–MS method for metabolome analysis.

First of all, all the reagents contain an aromatic moiety (benzene ring in DmPA and naphthalene ring in the other two reagents), which can significantly increase the hydrophobicity of derivatized metabolites. Thus, only the C18 LC column is required for the 4-channel CIL LC–MS method to separate all labeled compounds, even for the very polar ones, such as amino acids, sugars, *etc.* Compared to the underivatized approach, there is no need for using ion pairing reagents or HILIC column to improve the performance for these compounds. This strategy of using a proper reagent to label polar and less-polar metabolites to alter their chemical and physical properties to an extent that they all can be retained and separated by RPLC decreases the instrumental and operational cost. During the entire metabolome analysis process, there is no need to switch columns and solvents, simplifying the instrument setup and operation.

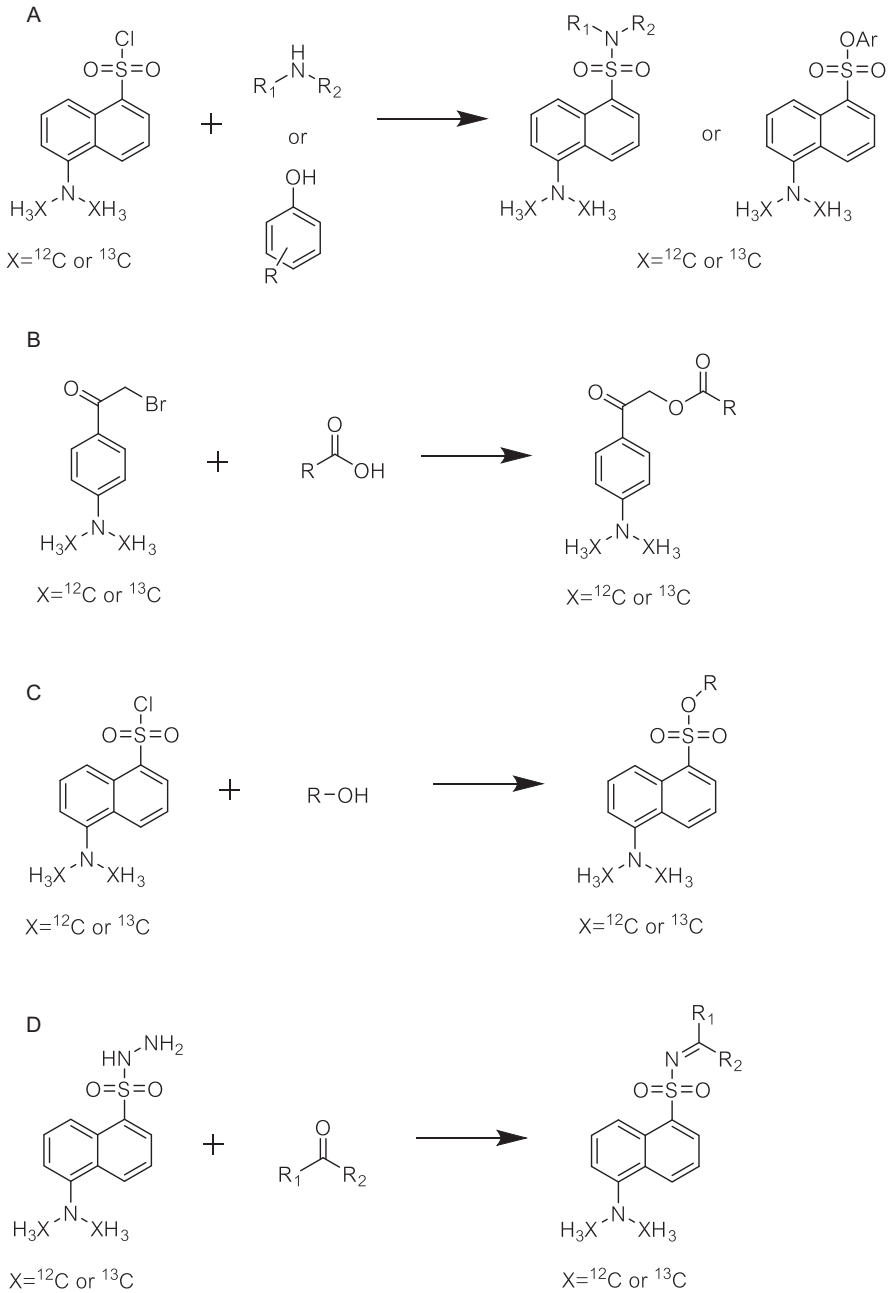

Figure 2.3 Reaction schemes for high-performance CIL LC–MS methods. (A) Dansylation reaction for amine/phenol submetabolome. (B) DmPA labeling for carboxyl submetabolome. (C) Base-activated dansylation reaction for hydroxyl submetabolome. (D) Dansylhydrazine labeling for carbonyl submetabolome.

Secondly, there is a dimethylamino structure located in the labeling reagents. Once introduced into the derivatized metabolite, as an easily ionizable group, it can dramatically increase the detection signal by improving the electrospray ionization process in positive ion mode. In this case, all derivatized metabolite can be analyzed in positive ion mode with much higher detection sensitivity, including those small carboxylic acids that are normally detected in the negative ion mode. Furthermore, for the metabolites with low concentration or low ESI response, they show enhanced signals with better peak shapes, which benefit the metabolite recognition and quantification.

Thirdly, two ^{13}C atoms can be incorporated into the methyl groups in the reagents to generate $^{13}C_2$-reagents for labeling. When the $^{13}C_2$-labeled reference sample is spiked into each individual $^{12}C_2$-labeled sample, internal standards for all labeled metabolites are created. Thus, much improved quantification accuracy and precision can be achieved.

2.3.2 Data Processing in CIL LC–MS

When performing untargeted metabolome analysis, one of the challenging tasks is to extract qualitative and quantitative information of many metabolites from a large batch of LC–MS data. Thanks to generating the heavy-labeled internal standard for every light-labeled metabolite, the metabolite signal picking and quantitative information extraction become more reliable. Because each metabolite is shown as a peak pair (*i.e.*, light peak from $^{12}C_2$-labeling and heavy peak from $^{13}C_2$-labeling) in the MS spectra, an algorithm can be developed to easily differentiate true metabolite signals from the noise and background signals which are shown as singlet peaks. By measuring the peak intensity ratio of light peak and heavy peak, accurate metabolite amount information can be generated. Software, such as IsoMS,[33,34] has been developed to achieve these functions in a batch mode, without needing manual intervention in data processing.

Metabolite identification is essential to obtain qualitative information to understand the biological meaning of the dataset. A metabolome database containing 3-tiered libraries has been constructed for the 4-channel CIL LC–MS method. This database includes information from more than 1500 authentic metabolite standards (1st-tier),[31,35,36] *in-silico*-predicted information for more than 9000 metabolites, covering most of the metabolism pathways (2rd-tier) and a comprehensive evidence-based metabolome library (3rd-tier).[37] By matching the information such as mass and retention time of unknown metabolites from experimental samples with the libraries, rapid and high-confidence metabolite identification can be achieved.

2.3.3 Applications of CIL LC–MS

The CIL LC–MS methods have been applied for metabolomic profiling of various samples, such as urine,[38] blood,[39] sweat,[40] cerebrospinal fluid,[41]

feces,[42] tissue,[43] and cells.[44] By using the 4-channel CIL LC–MS method, Zhao *et al.* profiled human plasma and yeast metabolome.[6] A total of 7431 and 5629 metabolite candidates were detected. Among them, 670 and 431 metabolite candidates can be identified by matching the library with high confidence. The metabolites that can be analyzed by this method include many polar metabolites such as amino acids and their derivatives, dipeptides, various carboxylic acids, sugars, *etc.*

In another study, Han *et al.* used a dansylation reaction to measure the amine/phenol-containing metabolites to determine potential metabolite biomarkers associated with Parkinson's disease (PD) and PD with incipient dementia.[45] By analyzing 43 serum samples from PD patients and 42 serum samples from healthy controls, the authors successfully found significantly changed metabolites associated with catecholamine metabolism, tryptophan metabolism, caffeine metabolism, and oxidative stress. By using five small molecules (vanillic acid, 3-hydroxykynurenine, isoleucyl-alanine, 5-acetylamino-6-amino-3-methyluracil, and theophylline) as a biomarker candidate panel, the PD patients and controls can be successfully differentiated. The receiver operating characteristic curve produced an area-under-the-curve value of 0.955 with 87.5% sensitivity and 93.0% specificity. This clearly shows that though only one or two of the four-channel CIL methods were used in some studies, satisfied metabolite coverage can still be achieved with very high detection and quantification performance.

The 4-channel CIL LC–MS method has also been applied to absolute quantification of many targeted compounds. For example, El-Aneed and co-workers performed absolute quantification of potential biomarkers for asthma and chronic obstructive pulmonary disease using dansyl chloride and DmPA bromide.[46,47]

2.4 Concluding Remarks

Chemical derivatization LC–MS can be used to improve the analysis of polar metabolites. Moreover, by rationally designing the chemical labeling reagents, both polar and less-polar metabolites can be derivatized together and detected simultaneously in one LC–MS run. Differential isotope labeling of individual samples and a reference sample (*e.g.*, a pooled sample or standards) can be performed for accurate relative (and absolute) quantification. Using high-performance chemical isotope labeling to target different chemical groups of metabolites or submetabolomes, high-coverage quantitative metabolome analysis can be done.

The limitations of the chemical derivatization approach include 1) the derivatization reaction requires extra time and cost, 2) a small portion of metabolites are not easily derivatized, and 3) labeling efficiencies may not be high for some metabolites. However, much improved analysis performance offered by chemical derivatization often outweighs these limitations. We envisage that this approach will be further advanced in the near future,

and many applications in biological studies and biomarker discovery research will emerge.

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