Chemical Isotope Labeling LC-MS for Metabolomics

Shuang Zhao and Liang Li

1 LC-MS for Metabolomics: Conventional Approach

The main goal of metabolomics is to characterize the metabolome by qualitative and quantitative analysis of as many metabolites as possible [1, 2]. Achieving this goal requires highly sensitive metabolite detection, accurate relative or absolute quantification, and confident metabolite identification. In conventional LC-MS-based metabolomics approach, increasing the number of metabolites detected and quantified (i.e., metabolomic coverage) is achieved by the use of different instrumental platforms and methods to analyze the same samples [3, 4]. The combined results from individual analyses represent the overall metabolome covered. These individual analyses are usually carried out according to the metabolites' physical and chemical properties. In a typical LC-MS workflow, after sample collection and pretreatment (e.g., protein removal, metabolite extraction, etc.), reversed phase (RP) LC is used for separating hydrophobic metabolites, while hydrophilic interaction LC (HILIC) is used for separating more polar compounds [5]. In each case, the eluted analytes are ionized in positive ion mode and negative ion mode. In this way, four LC-MS runs are required to analyze one sample. The main advantage of this approach is that it is easy to implement by using readily available LC and MS instruments to collect data and commercial or freely available software to process the resultant data. The major limitation of this approach is that the metabolome coverage is still low even after combining all the multiple analysis data. Although tens of thousands of features can be detected in LC-MS analysis of a metabolome sample, only a small fraction of them (hundreds) are originated from unique metabolites. Many redundant peaks, such as adduct ions and fragment ions, are detected from one metabolite. This is not surprising considering that electrospray ionization (ESI), the most widely used method for LC-MS, does not offer the same ionization efficiency for all metabolites. In fact, a large number of metabolites cannot be ionized with high efficiency. The combined low ionization efficiency and low metabolite concentration result in low or no MS signals.

Another challenge in conventional LC-MS for metabolome analysis is on quantification which relies on the use of internal standards to correct for sample loss, instrument drift, matrix effect, and ion suppression in the whole workflow. Compared with chemical structural analogue, stable isotope labeled (SIL) internal standard is a better choice due to its nearly identical physical and chemical properties to the analyte of interest. However, SIL internal standards are generally

S. Zhao · L. Li (✉)
Department of Chemistry, University of Alberta, Edmonton, AB, Canada
e-mail: liang.li@ualberta.ca

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expensive and their availability is limited. Particularly in metabolomics area, it is impossible to purchase or synthesize isotopic internal standards for all the metabolites. Thus, conventional LC-MS is widely used for quantification of a small number of metabolites with SIL (i.e., targeted analysis). Untargeted analysis of metabolites without using SIL offers limited quantification precision and accuracy.

2 Stable Isotope Labeling LC-MS for Metabolomics

In order to perform quantitative metabolomics using LC-MS, methods that can simultaneously introduce stable isotopes into many metabolites have been developed. In one approach, cells are cultured with a stable isotope-labeled substrate (e.g., D, ^{13}C, ^{15}N, etc.) in growth medium. During cultivation, the substrate-related metabolites and metabolic networks are "in vivo" labeled with enriched heavy isotopes. As a result, each heavy-isotope-labeled metabolite can potentially serve as the internal standard. This cell-culture-based metabolic isotope labeling (MIL) is not only useful for quantitative metabolite measurement but also an important tool for metabolic flux analysis [6]. For example, growing cells using ^{13}C-glucose or ^{13}C- or ^{15}N-glutamine as main energy sources can be used for monitoring and analyzing many cellular pathways, such as glycolysis and pentose phosphate pathway [6, 7]. This MIL approach is particularly convenient for metabolomic analysis of cells or organisms that can be cultured in isotope-enriched media, including microbial, yeast, some mammalian cell cultures [7] or plants [8]. However, this approach is not easily applicable for samples that cannot be readily cultured, such as human biofluids including urine, blood, etc. [9] In addition, this approach does not alter the chemical and physical property of a metabolite to improve its detectability in LC-MS. Metabolites that are not ionized efficiently will still not be detected. In other words, MIL can improve quantification in analyzing special types of samples, but cannot overcome the limitation of low coverage.

An alternative approach to create isotope internal standards for LC-MS-based quantitative metabolomics is to use chemical-reaction-based isotope labeling or chemical isotope labeling (CIL), which is, in principle, suitable for all categories of samples. In contrast to MIL, biological samples are "in vitro" labeled in CIL using a reagent which targets a specific chemical functional group. Ideally, all the metabolites containing the same functional group react with the reagent to form the corresponding derivatized metabolites. Many reagents can incorporate one or more isotopic atoms (e.g., H/D, ^{12}C/^{13}C, ^{14}N/^{15}N, etc.) in the molecular structure, thereby introducing the isotopic moiety into the labeled metabolites after chemical derivatization. The metabolites derivatized by a heavy isotope reagent can serve as the internal standards for light isotope reagent labeled metabolites. This CIL strategy has been widely used in relative quantification for untargeted metabolomics as well as absolute quantification for targeted metabolomics.

3 Chemical Isotope Labeling LC-MS Metabolomics Workflow

The general workflow using CIL LC-MS for metabolomics is shown in Fig. 1. In this workflow, for metabolome profiling or relative quantification in two comparative groups of samples, a control sample (e.g., a pooled sample) is labeled by a heavy isotope reagent, while individual samples are derivatized by a light isotope reagent. Then the two derivatized samples are mixed and injected into LC-MS for analysis. Since the light-labeled derivative and heavy-labeled derivative of a metabolite have nearly identical properties, they elute out at the same time from LC. In the mass spectra, the two derivatives of the same metabolite are shown as a peak pair. The relative amount of the metabolite can be determined from the comparison of peak areas of two derivatives. Combined with database identification and statistical analysis, this approach has been successfully used for biological studies and biomarker discovery [10].

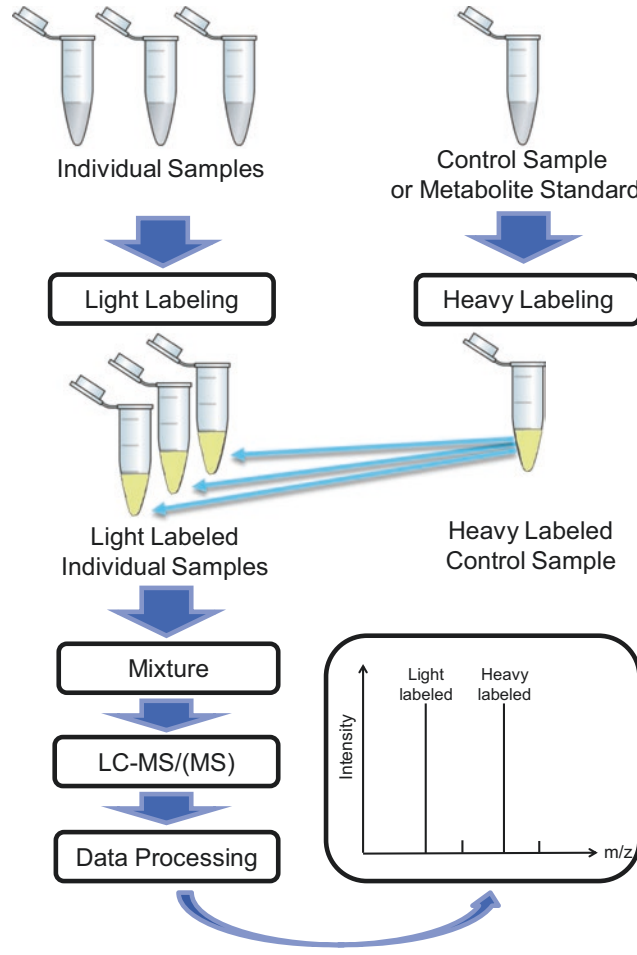

Fig. 1 General workflow of CIL LC-MS-based metabolomic analysis

For absolute quantification of metabolites in samples, similar to the above-described method, samples are derivatized with a light isotope labeling reagent and the analyte standards of known concentrations are labeled with a heavy isotope reagent. The resulting heavy derivatives are used as CIL internal standards for performing quantification with MS. For absolute quantification of a small number of metabolites of interest, tandem MS can also be used to increase sensitivity and quantification dynamic range by using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM).

4 Derivatization Reagents for CIL LC-MS Metabolomics

Many derivatization reagents are developed for different functional groups and have been widely used for biological studies. Table 1 lists a number of reagents that are used for CIL LC-MS-based metabolomics. We highlight some examples of reagents below. Detailed information for each reagent can be found in the references listed in the table.

For amine-containing metabolites, sulfonyl chloride, acyl chloride, NHS esters, and isothio-

Table 1 Reagents that are used for CIL LC-MS based metabolomics

Reagent	Isotope reagent	Targets	Applications	References
DnsCl	$^{13}C_2$-DnsCl	Amines/phenols	- Constructed library containing 273 dansylated metabolites - Profiled and positively identified metabolites in urine, blood, serum, fecal, CSF, cells - Targeted absolute quantification of 19 metabolites as asthma/CPOD potential biomarkers	Guo and Li [27], Chen et al. [44], Han et al. [10], Xu et al. [47], Guo et al. [46] and Khamis et al. [42]
	$^{13}C_2$-DnsCl	Hydroxyls	- Constructed library containing 85 hydroxyl standards - Untargeted profiled urinary hydroxyl submetabolome and positively identified 20 metabolites	Zhao et al. [22]
DensCl	$^{13}C_2$/$^{13}C_4$-DensCl	Amines/phenols		Zhou et al. [52]
MASC	d_3-MASC	Amino acids	- Absolute quantification of amino acids and monoamine neurotransmitters using MRM mode	Song et al. [53] and Zheng et al. [54]
Benzoyl chloride	$^{13}C_6$-BzCl	Neurochemicals	- Absolute quantification of 70 neurochemicals	Wong et al. [11]
MBAA-NHS	$^{13}C_2$-MBAA-NHS	Amines	- Untargeted profiling of urinary amine submetabolome - Absolute quantification of amino acids in human urine	Zhou et al. [55]
DBAA-NHS	$^{13}C_2$-DBAA-NHS	Amines	- Untargeted profiling of urinary amine submetabolome - Absolute quantification of amino acids in human urine	Zhou et al. [55]
BZ-NHS	$^{13}C_6$-BZ-NHS	Amines/thiols/phenols	- Untargeted metabolome profiling of cell extracts - Positively identified 10 metabolites	Wagner et al. [56]
DIPP-L-Ala-NHS	$^{18}O_2$-DIPP-L-Ala-NHS	Amines	- Determination of 20 L-amino acids and 10 D-amino acids	Zhang et al. [57]
iTRAQ	iTRAQ	Amines	- Analysis of 44 amino acids in plasma, urine and tissue	Takach et al. [35]
DiLeu	4-plex DiLeu	Amines	- Profiling and relative quantification of amine submetabolome of mouse urine	Hao et al. [58]
DMAP	d_4-DMAP	Amines	- Constructed library containing 118 amine compounds - Positively identified 46 amine metabolites in fecal sample	Yuan et al. [34]
Cyanuric chloride/methylamine	Methyl-d_3-amine	Amines	- Determined concentrations of 27 metabolites in HepG2 cells	Lee and Chang [59]
Acetone	d_6-acetone	Phosphatidyl-ethanolamine	- Identified and quantified 45 PE species in rat livers using double neutral loss scan.	Wang et al. [60]
MPBS	d_3-MPBS	Amino acids	- Analysis of amino acids in newborn bloodspot	Johnson [28]

(continued)

Table 1 (continued)

Reagent	Isotope reagent	Targets	Applications	References
DMABS	d_3/d_6-DMABS	Amino acids	- Analysis of amino acids in newborn bloodspot	Johnson [28]
C_n-NA-NHS	C_4d_9-NA-NHS	Amines		Yang et al. [61]
Methyl acetimidate	$^{13}C_2$-methyl acetimidate	Amines	- Relative quantification of primary and secondary amines in Arabidopsis seed extracts	Shortreed et al. [62]
Formaldehyde	^{13}C-formaldehyde	Amines	- Analysis of 20 amino acids and 15 amines - Profiling human urine amine-containing metabolites	Guo et al. [63]
Acetyaldehyde	d_4-acetaldehyde	Monoamine neurotransmitters	- Determination of neurotransmitters in brain microdialysate	Ji et al. [64]
PEG-OPFP	^{13}C-PEG-OPFP	Primary amines	- Quantification of intracellular amino acids	Abello et al. [65]
TAHS	d_3-TAHS	Amino acids	- Determination of amino acids in rat plasma	Shimbo et al. [66]
L-PGA-OSu	L-PGA(d_5)-OSu	Chiral amines	- Differential analysis of DL-amino acids in serum and yogurt	Mochizuki et al. [67]
DMED	d_4-DMED	Carboxylic acids	- Constructed library containing 184 carboxyl metabolites - Positively identification of 83 carboxyl metabolites	Yuan et al. [34]
Cholamine	d_9-cholamine	Carboxylic acids	- Relative quantification of fatty acids from hydrolyzed egg lipid using nanoLC	Lamos et al. [68]
	^{15}N-cholamine	Carboxylic acids	- Analysis of 48 carboxylic acids	Tayyari et al. [69]
3-NPH	$^{13}C_6$-3-NPH	Short-chain fatty acids	- Quantification of short-chain fatty acids in human feces	Han et al. [14]
Butanolic HCl	d_9-butanol	Carboxylic acids	- Profiling and relative quantification of human plasma metabolites	O'Maille et al. [16]
Aniline	$^{13}C_6$-aniline	Carbonyl, phosphoryl, and carboxyl	- Quantification 33 intermediate metabolites in central carbon and energy metabolism	Yang et al. [21]
BAMP/HAMP	d_9-BMAP	Carboxylic acids	- Metabolome profiling of rat urine sample and positively identified 32 metabolites	Yang et al. [70]
BMP/CMP	d_3-CMP	Fatty acids	- Metabolome profiling, relative quantification and absolute quantification of human serum	Yang et al. [71]
DmPA bromide	$^{13}C_2$-DMPA	Carboxylic acids	- Constructed library containing 113 carboxylic acid - Positively identified 51 metabolites in urine	Guo and Li [17]
HMEP	d_5-HMEP	Fatty acids	- Monitored changes of metabolite levels in plasma of individuals	Koulman et al. [72]
DBD-PZ-NH2	d_6-DBD-PZ-NH2	Carboxylic acid	- Determination and relative quantification of fatty acids in plasma	Tsukamoto et al. [73]

(continued)

Table 1 (continued)

Reagent	Isotope reagent	Targets	Applications	References
DMPP	d_6-DMPP	Carboxylic acid	- Determined trace free fatty acids in human urine and thyroid tissues	Leng et al. [74] and Leng et al. [75]
T3	d_{20}-T3	Fatty acids	- Relative quantification of FAs with general MRM conditions. - Discovered FA species related to the ageing process	Tie et al. [76]
HIQB	d_7-HIQB	Carbonyls	- Constructed library containing 147 carbonyl compounds - Untargeted profiling of carbonyl submetabolome of human serum and fecal sample using double precursor ion scan. 12 and 50 metabolites were positively identified, respectively	Guo et al. [77] and Yuan et al. [34].
4-APC	d_4-4-APC	Aldehydes	- Profiled aldehyde submetabolome using double neutral loss scan of urine, beer, and wine samples	Zheng et al. [78], and Yu et al. [79]
DnsHz	$^{13}C_2$-DnsHz	Carbonyls	- Constructed library containing 78 carbonyl compounds - Profiling of urinary carbonyl submetabolome and positive identification of 33 metabolites	Zhao et al. [37]
Aniline	$^{13}C_6$-aniline	Carbonyls, phosphoryls and carboxyls	- Quantification of 33 intermediate metabolites in central carbon and energy metabolism	Yang et al. [21]
Girard P	d_5-GP	Steroid hormones	- Quantified steroid hormones in human follicular fluid	Guo et al. [29]
	d_5-GP/isobaric mass	Sterols/oxysterols	- Profiled plasma sterols/oxysterols to identify inborn errors	Crick et al. [80]
HMP	d_3-HMP	Neurosteroids	- Relative and absolute quantification of allopregnanolene and pregnenolone levels in brain	Higashi et al. [81]
T3	D3 (d_{20}-T3)	Fatty aldehydes	- Globally profiling of fatty aldehyde in plasma and brain tissue	Tie et al. [82]
QAO	d_3-QAO	Ketosterols	- Absolute quantification of ketosterol in very small volumes of plasma	DeBarber et al. [83]
DMBA	d_4-DMBA	Hydroxyl-containing steroid hormones	- Measurement of 17 derivatized free steroid hormones in urine	Dai et al. [23]
MDMAES	$^{13}C_4$-MDMAES	Steroids	- Quantitative and comparative analysis of SIRS and sepsis clinical samples	O'Maille et al. [16]
Acetone	d_6-acetone	Ribonucleosides	- Profiled urinary metabolome and positively identified 56 ribonucleosides - Metal oxide-based dispersive SPE applied for enrichment of ribonucleosides	Li et al. [33], and Chu et al. [84]

(continued)

Table 1 (continued)

Reagent	Isotope reagent	Targets	Applications	References
Pyridine and Tf2O	d_5-pyridine	Steroids, fatty alcohols and carbohydrates	- Ten pairs of d_0/d_5 ion peaks were identified as cholesterol and fatty alcohols	Wang et al. [85], and Wang et al. [86],
BQB	d_7-BQB	Thiols and oxidized thiols	- Constructed library containing 27 thiol metabolites - Profiled thiol submetabolome in urine, beer and fecal samples - Positively identified 14 and 8 thiol metabolites in fecal and urine, respectively	Yuan et al. [34],, Huang et al. [87], and Liu et al. [26]

cyanates are used for CIL. For example, Kennedy's group developed a method targeting 70 neurochemicals using LC-MS/MS and benzoyl chloride (BzCl) derivatization [11]. The commercially available ^{13}C-BzCl was used for labeling internal standards. The reagent offers very fast reaction (seconds at room temperature). This approach has been proved to be effective in various matrix, including tissue, serum, CSF, and microdialysate [12].

Many reactions for labeling carboxylic acids are based on condensation reaction with amines. In this type of reaction, carboxyl groups in metabolites are activated by a condensation reagent (e.g., carbodiimide), followed by reacting with amine groups in the labeling reagents, generating a stable amide bond [13]. The method of using ^{12}C/^{13}C-3-nitrophenylhydrazine (3-NPH) to analyzed carboxylic acids was reported. The labeled metabolites gain significant enhancement of detection in negative ion mode. It has been successfully applied in the detection and quantitation of many important categories of acids, including carboxylic acids in central carbon metabolism [13], short-chain fatty acids [14] and bile acids [15]. In addition, esterification reaction was also used to derivatize carboxyl group [16, 17].

Various hydrazine or hydrazide reagents have been proven to be effective on labeling ketones or aldehydes, such as Girard reagents. Girard's reagent P was used in quantitative glycomics with its pentadeuterated (d_5-) counterpart [18]. In this method, reducing glycans were labeled with either nondeuterated (d_0-) or deuterated (d_5-) Girard's reagent P, followed by online HILIC-MS analysis to achieve rapid and sensitive relative quantitation of reducing glycans between two comparative groups. It was also used for analyzing oxysterol [19, 20]. The signal enhancement factor after Girard derivatization was more than 30-folds. Several amine-containing reagents are also developed for carbonyl group derivatization, such as $^{12}C_6/^{13}C_6$-aniline [21].

For hydroxyl group, which is a weaker nucleophile compared with amine groups, electrophilic reagents were also used for chemical isotope labeling, such as sulfonyl chloride (e.g., ^{12}C-/^{13}C-dansyl chloride [22]) or carboxylic acids (e.g., d_0/d_4-(dimethylamino)-benzoic acid [23]).

At last, thiol groups are easily oxidized by autoxidation and disulfide formation during the sample preparation process [24]. Several reagents were reported to be useful in both isotopic labeling and stabilizing for thiol-containing metabolites [25, 26].

5 Key Features of High-Performance CIL LC-MS

In addition to the introduction of isotopic moiety into metabolites to create isotope internal standards, many CIL LC-MS methods also provide enhancement in other aspects of the LC-MS analysis, including detection and separation. Therefore, using a rationally designed labeling reagent with proper structure can improve the overall performance of LC-MS analysis for a complex metabolomic system.

Improving metabolite detectability is a common advantage of many CIL LC-MS methods. This is achieved by introducing permanent charge or easily ionizable moiety to metabolites in order to increase their ionization efficiency. For example, ^{13}C-/^{12}C-dansyl chloride (DnsCl) was developed as a high-performance CIL reagent for derivatizing amine-, phenol- [27] and hydroxyl-containing metabolites [22]. The dimethylamino structure is an easily ionizable group and can increase the detection ability by 10–1000-folds compared with unlabeled metabolites. Figure 2 shows the ion chromatograms comparison between dansyl-labeled and unlabeled human urine. Enhancement of detection can be clearly observed. The presence of permanently charged moieties can also greatly improve ionization efficiency of labeled molecules, such as quaternary ammonium [28], pyridinium [29] and phosphonium [30] salts for positive ion mode detection. After labeling, the labeled metabolites can be charged readily in ESI, requiring only positive ion mode detection.

Improvement of separation is another important feature of high-performance CIL LC-MS. In conventional LC-MS metabolomics, RPLC and HILIC are usually used to separate complex metabolome. However, this approach suffers from poor retention of polar compounds on RPLC, less-than-ideal reproducibility of HILIC separation [31] and requirement of multiple instruments or changing of columns. Some high-performance CIL methods have been proved to be effective to overcome these problems by improving the separation ability of a complex biological sample. If the introduced moiety dur-

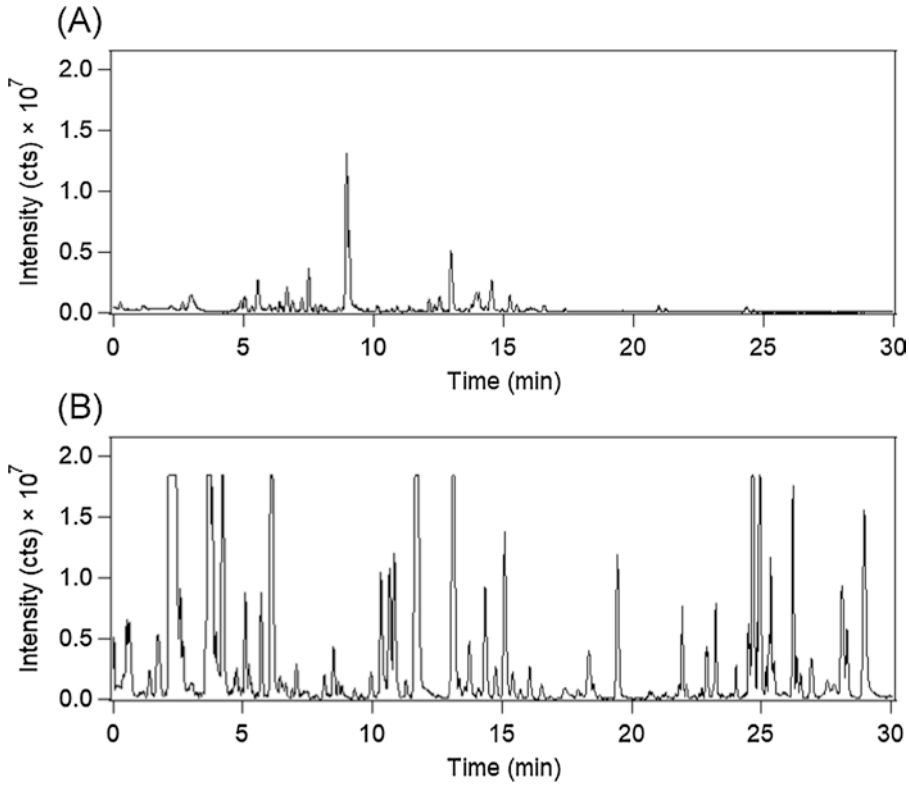

Fig. 2 Comparison of ion chromatograms obtained from (**a**) unlabeled human urine with the injection of 5 μL of sample containing 5 μL of the original urine and (**b**) dansyl-labeled human urine with the injection of 2 μL of sample containing 0.48 μL of the original urine. Despite the injection of 10.4-fold less sample in (**b**), the labeled urine sample gave significantly higher intensities with peaks distributed along the entire gradient elution window

ing derivatization contains an aromatic ring (e.g., dansyl chloride for amine/phenol/hydroxyl group derivatization [22, 27]), an alkyl chain (e.g., N,N′-dimethylethylenediamine for carboxyl group derivatization [32]), or parts that can block polar groups (e.g., acetone for ribonucleosides derivatization [33]), the hydrophobicity of the labeled metabolites would increase, resulting in better retention and peak shapes in RPLC. As a result, polar metabolites in a biological sample can be retained and separated effectively only using RPLC. We note that the consideration of retention properties of metabolites on RPLC favors the use of a reagent without a permanently charged moiety to labeled metabolites. A reagent with permanent charges will increase the hydrophilicity of labeled metabolites and thus poor retention on RPLC. The use of HILIC or other mode LC is required for separation. For the convenience of a user to perform LC-MS, sticking with one mode of separation (i.e., RPLC) for all sample analyses offers a significant advantage.

Introduction of hydrophobic moiety into metabolites also increases the detection ability. This is because the labeled metabolites will elute out at higher composition of organic phase on RP column, leading to more efficient desolvation process and less surface tension of droplets in ESI. In the meantime, increased hydrophobicity prompts metabolite molecules to reside on the surface of the droplets. Both of them benefit the ionization process.

Another significant improvement in metabolite detection offered by CIL is that adding one or more labeling moieties or tags increases the mass of the analyte, thereby avoiding severe interferences in the low m/z region from the low mass impurities and backgrounds in solvents and reagents.

Finally, CIL allows data processing to be done relatively easier with much higher confidence in determining true metabolite peaks, compared to analyzing LC-MS data of unlabeled samples. If differential isotope labeling (e.g., mixture of heavy-labeled pooled sample and light-labeled individual sample) is used for CIL LC-MS metabolome profiling, all metabolite peaks are detected in pairs of light-labeled and heavy-labeled metabolite ions. Impurity and background peaks are detected in singlet peaks. Thus, special software program can be developed to filter out the singlet peaks to improve the data quality. For example, IsoMS was developed to pick the peak pairs from all the peaks detected in mass spectra, followed by removing redundant peaks such as adduct ions, dimers, multimers, etc. These redundant peaks are detected as peak pairs with different m/z values in the same mass spectrum as the peak pair of $[M + H]^+$. A simple algorithm can be used to filter these additional peak pairs to retain only one peak pair for one labeled metabolite.

Instead of directly extracting peak pairs from MS spectra, using common MS/MS fragmentation pattern is another approach to recognize metabolite peaks. For example, Feng and coworkers developed stable isotope labeling combined with double precursor ion scan/double neutral loss scan in MS to selectively analyze a particular group of metabolites [34].

The isobaric tag for relative and absolute quantification (iTRAQ) reagents has been widely used in proteomics. These reagents have been applied for analyzing amino acids [35] and other amine-containing metabolites. Unlike the reagents used in MS-based peak pair detection, iTRAQ reagent consists of a reporter group, a mass balance group, and an amine-reactive group which could be NHS ester. Two differential iTRAQ reagents use different isotope coding patterns in both reporter group and balance group; however, the overall mass of the reagents keeps constant. In derivatization for absolute quantification, metabolites of a sample will be labeled by the reagent containing a light reporter group and a heavy mass balance group. The metabolite standards are labeled by the reagent containing a heavy reporter group and a light mass balance group, which are spiked into the labeled sample as internal standards for LC-MS analysis. The labeled standards and corresponding metabolites in the sample elute out at the same time and have the same m/z in mass spectra. Upon MS/MS fragmentation, the reporter group will be cleaved from the labeled compounds and produce unique product ions, which can be quantified in the SRM mode to reflect the metabolite quantity in a sample.

6 Multichannel CIL LC-MS Metabolome Analysis

In CIL LC-MS-based metabolomics approach, several different submetabolomes targeting different groups of metabolites can be analyzed separately, and the combined datasets can be used to represent the entire metabolome. Zhao et al. have recently reported a study of analyzing the chemical structures of compound entries in several well-used databases to determine the distributions of chemical groups in each database [36]. Because of the interest in studying endogenous metabolites of a metabolome, they removed the lipids, inorganic species, and other molecules that are unique to drug, food, plant, and environmental origins from database compound entries. They found that five groups, namely, amine, phenol, hydroxyl, carboxyl, and carbonyl, are the dominant classes in the remaining endogenous metabolites. In the databases of MCID (2683 filtered metabolites), HMDB (5506), KEGG (11,598), YMDB (1107), and ECMDB (1462), 94.7%, 85.7%, 86.4%, 85.7%, and 95.8% of the filtered metabolites were found to be belonging to one or more of the five groups, respectively. Figure 3 shows an example of the chemical group distribution analysis for the 2683 filtered metabolites in MCID. Thus, in-depth analysis of these five groups of metabolites can result in a very high coverage of a metabolome.

Zhao et al. described a 4-channel high-performance CIL LC-MS approach (Fig. 4) for targeting amine/phenol- [27], carboxyl- [17], carbonyl- [, 37] and hydroxyl-containing metabolites [22] using dansyl and DmPA labeling reagents (Fig. 5) [36]. They showed the detection of a total of 7431 peak pairs with 6109 unique-mass pairs in labeled human plasma using 4-channel CIL LC-MS. Among them, 670 peak pairs (9.0%) could be identified with high confidence and 6256 (84.3%) could be mass-matched to metabolome database entries. In the case of yeast samples, a total of 5629 pairs with 4955 unique-mass pairs were detected. There were 431 peak pairs (7.6%) identified with high confidence and 4836 peak pairs (85.7%) mass-matched to database entries. These results illustrated that the combined datasets from the analyses of four submetabolomes can detect many metabolites. While four different labeling methods were used for profiling four submetabolomes, the LC-MS setup

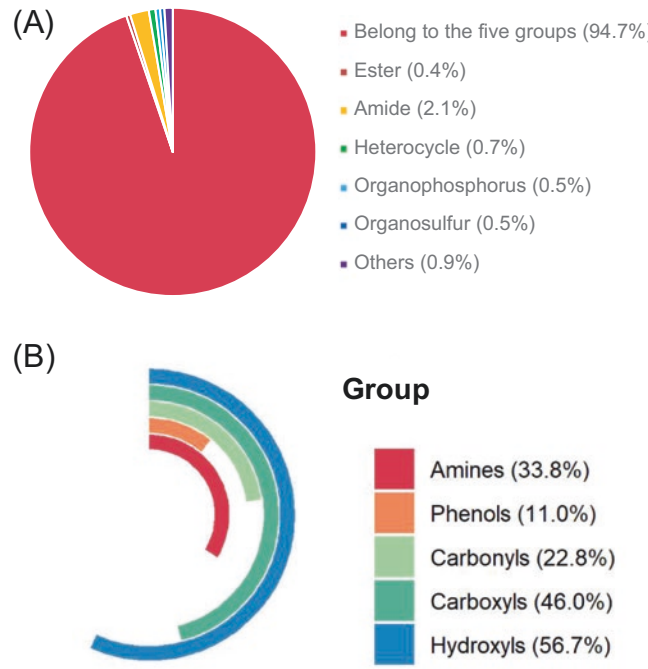

Fig. 3 (**a**) Classification of chemical groups of 2683 known human endogenous metabolites from the MyCompoundID library. (**b**) Percent distributions of metabolites belonging to the five groups

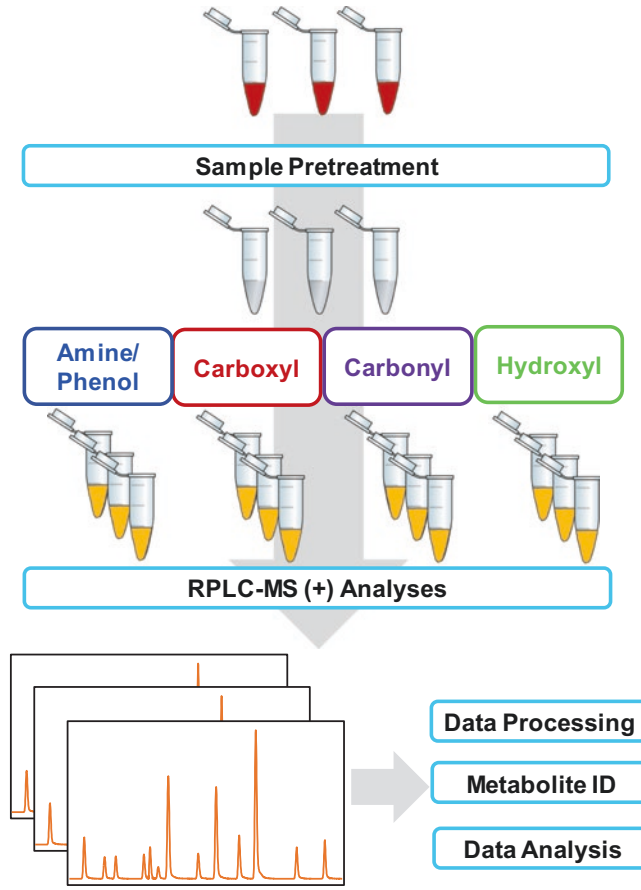

Fig. 4 Workflow of 4-channel high-performance CIL LC-MS-based metabolomics

remained to be the same; all labeled samples were analyzed using RPLC-MS with positive ion detection.

In a similar way, Yu-Qi Feng's group reported profiling of mice fecal samples using integrated derivatization combined LC-MS strategy [34]. In their study, different reagents targeting amine-, carboxyl-, carbonyl-, and thiol-submetabolome were applied. 2302 metabolite candidates were detected and 308 metabolites were further confirmed.

7 Limitations and Future Direction of CIL LC-MS Metabolomics

One presumed weakness of CIL LC-MS is the requirement of performing chemical derivatization. However, due to the need of carrying out multiple steps in a typical sample workup procedure leading to LC-MS analysis, the addition of a chemical labeling step does not necessarily increase the workload significantly. If a very robust and convenient labeling method is used, the chemical derivatization step can be viewed like those of other sample handling steps, such as methanol precipitation of proteins used for analyzing serum samples, creatinine measurement sample normalization in urine sample analysis, cell lysis and metabolite extraction in cellular metabolomics, etc. Thus, developing a reproducible labeling procedure that can be easily implemented in a laboratory and carried out by personnel with little chemistry expertise is critical for wide usage of CIL LC-MS in metabolomics.

Although the reactions that are chosen for derivatization have relatively rapid reaction speed, the required time for many reactions is in still in hours to ensure that the complex subme-

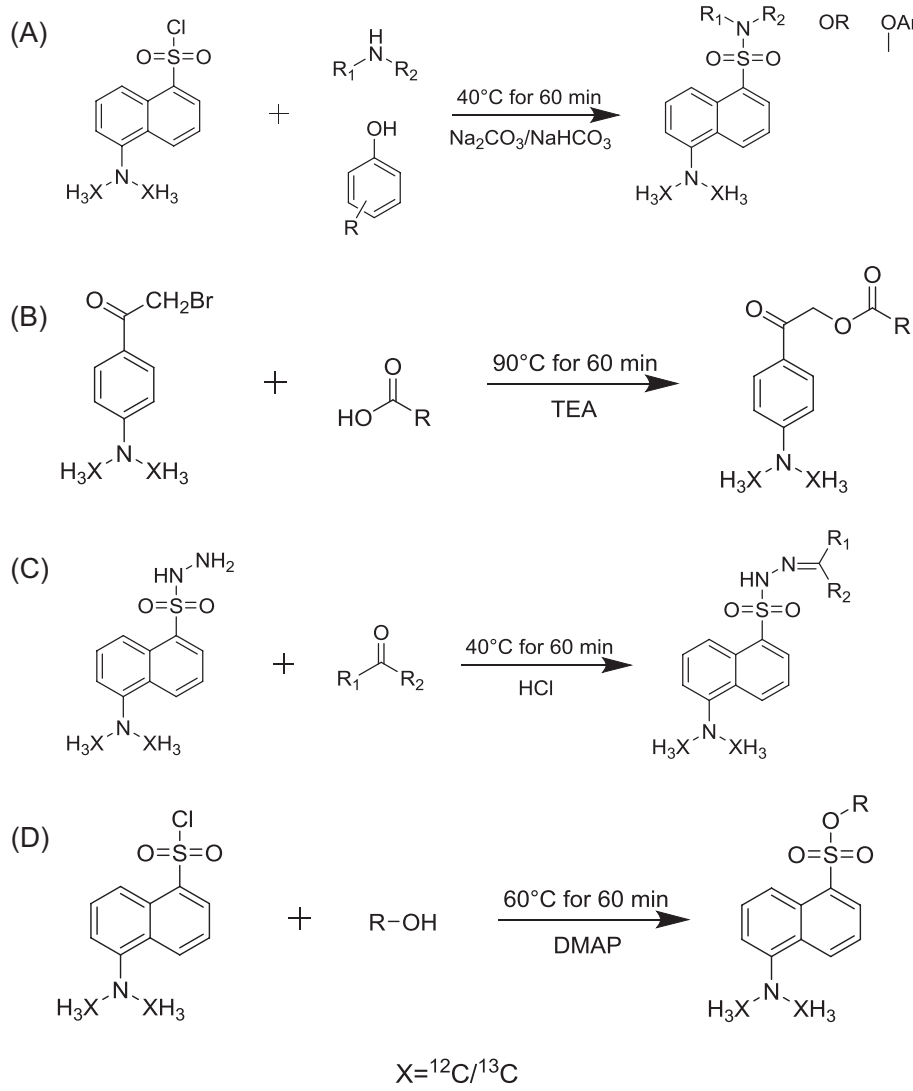

Fig. 5 Reaction schemes of (**a**) dansylation labeling for amine/phenol-containing metabolites; (**b**) DmPA bromide labeling for carboxyl-containing metabolites; (**c**) dansylhydrazine labeling for carbonyl-containing metabolites; (**d**) base-activated dansylation labeling for hydroxyl-containing metabolites

tabolome can be fully labeled and analyzed. One way to address this is to develop and employ reaction conditions that can achieve very fast derivatization. For example, Bian et al. used cholamine derivatization coupled with LC-MS to determine long-chain free fatty acids in complex biological samples [38]. The derivatization step can be finished within 1 min at room temperature. Increase in 2000-fold of sensitivity was obtained and the limits of detection of femtogram level were achieved. The feasibility of one-minute derivatization has been validated using both targeted quantification and untargeted profiling approach with serum samples. Further the authors discovered several metabolites that have significantly differences between healthy and asthma groups. We note that while fast reaction is beneficiary for some applications, parallel labeling of multiple samples can be done, and thus the labeling step is usually not the rate-limiting step in determining the overall sample analysis throughput in metabolomics.

Using robotic liquid handling systems to automatically perform derivatization can be very helpful to achieve high throughput. For example, an autosampler-in-needle-derivatization technique was reported by Siegel et al. [39]. The authors used p-toluenesulfonylhydrazine to derivatize aldehydes and ketones in a UHPLC autosampler. The labeling reagents and samples were consecutively drawn into the autosampler needle and mixed. The complete derivatization can be finished within 10 min and the solution is ready to be injected for analysis. This automatic derivatization approach has been proved to be useful in simultaneously quantifying and identifying molecules containing carbonyl groups.

Relative quantification by CIL LC-MS can be very accurate as the same metabolite in the pooled sample and an individual sample would encounter the same matrix (e.g., urine matrix for urine metabolome analysis). Moreover, the concentration of the metabolite in a pool should be similar to that in an individual sample (usually less than ten-fold changes among different samples). If we perform the labeling of the pool and an individual sample in parallel, the minor differences in matrix effect and labeling efficiency can be accounted for using the intensity ratio of a peak pair of the same metabolite in LC-MS. However, for absolute quantification using CIL LC-MS, we need to be aware of potential pitfalls. In conventional LC-MS-based quantification approach, an internal standard, either SIL or chemical structural analogue, is added to the sample at the very beginning of the sample workup process. Therefore, the internal standard and analyte share the identical preparation steps and, theoretically, the same recovery rate. However, an internal standard created by CIL is usually added to the sample just before the instrumental analysis. Thus, the analyte recovery rate from a sample is assumed to be 100%. Moreover, sample matrix effect, if any, on the labeling efficiency is not accounted for; labeling the internal standard dissolved in a clean solvent may be more or less efficient than labeling the analyte with the presence of many other components in a sample. To address these issues, sample pretreatment procedures need be optimized to obtain high recovery rates of analytes. Matrix effect on labeling efficiency should be measured. Another approach is to use the standard addition method for quantification. However, this method has a low throughput for quantifying many metabolites in a metabolomic sample.

Another potential pitfall of using CIL internal standards is the isotope effect in chromatography when using deuterium [40, 41]. The retention of deuterium derivatized species is generally less than its hydrogen coded counterpart on RPLC column due to weaker hydrophobic interactions with the stationary phase. The lack of co-elution leads to different matrix for ionization, which may be detrimental for quantification. The isotope effect of ^{13}C-, ^{18}O-, or ^{15}N- is usually negligible. Thus, in high-performance CIL methods, these isotope atoms are preferred; however, the synthesis of the reagents containing these isotopes may be more expensive than making the deuterium-coded reagents.

Finally, current CIL methods do not cover all chemical groups. There are several chemical groups such as amides and esters that require the development of efficient and robust labeling methods. Prior to the available of these methods, targeted analysis using LC-MRM-MS may be used to analyze some important metabolites that are not covered with current labeling methods.

8 Applications of CIL LC-MS for Cancer Metabolomics

Because of the possibility of performing highly accurate relative quantification of many different metabolites in metabolome samples, CIL LC-MS is a powerful technique in many metabolomics applications, including cancer metabolomics. For example, dansylation isotope labeling LC-MS has been widely used in various metabolomics studies. Dansyl chloride is one of the most commonly used reagents to derivatize primary, secondary amines, phenol [27] and alcoholic hydroxyl [22]. It provides all the features mentioned above that improve the analytical power for metabolome analysis. In addition, the concentration of dansyl-labeled metabolites can be

determined using UV absorbance, which can be very useful for sample normalization. The workflow of using DnsCl for metabolites profiling and biomarker discovery in various samples have been developed, including urine [42, 43], blood [44], serum [10], sweat [45], cerebrospinal fluid [46], faces [43, 47] and cell extracts [48, 49]. This method has been used for cancer biomarker discovery research.

One example is the work reported by Huan et al. describing a method of metabolomic profiling of prostate tissue samples using DnsCl labeling and conducted a proof-of-principle study for metabolic classification of prostate cancer [50]. In this study, the authors presented a typical workflow of high-performance CIL LC-MS method for metabolomics biomarker discovery of disease. In this workflow, metabolites were first extracted from prostate needle biopsies using molecular preservation by extraction and fixation techniques. Then samples from patients were individually labeled with ^{12}C-dansyl chloride and the total concentration of the labeled metabolites was determined using LC-UV. Based on the total concentration information, the same amount of ^{13}C-labeled universal metabolome standard generated from a pooled tissue extract was spiked into each labeled individual sample, served as internal standard for metabolome comparison. The generated ^{12}C-/^{13}C- mixtures were analyzed by RPLC-QTOF-MS. The data were then processed using a set of in-house programs in batch mode, including peak pair picking and chromatographic peak ratio measurement. Metabolite identification was performed either using labeled standard library [51] for definitive identification or other metabolite database (e.g., HMDB, MyCompoundID) for putative matches. At last, various statistical tools were used to visualize the separation between groups, find significant metabolites, and understand biological meanings.

Using this workflow, three batches of samples were analyzed. In the first batch of experiment, 2900 metabolites were consistently detected in more than 50% of the samples and 88 metabolites were positively identified. Then the panel of significant metabolites was refined using the second batch of samples. Receiver operating characteristic (ROC) analysis showed area under the curve (AUC) of 0.896 with sensitivity of 84.6% and specificity of 83.3% using 7 metabolites. At last, a blind study of validation samples was conducted, providing specificity of 90.9% and sensitivity of 84.6%. Although the sample numbers in this proof-of-concept studies is limited (in total 85 samples), it was still a good example showing the analysis power of high-performance chemical isotope labeling LC-MS method in disease biomarker discovery.

9 Conclusions

CIL LC-MS is a very powerful method for improving metabolomic coverage and quantification accuracy and precision. The presumed disadvantage of adding an extra step of performing chemical labeling of samples in the metabolome analysis workflow is often outweighed by the benefits offered by CIL. We envisage a wide use of this method for comprehensive and quantitative metabolomics in many areas of applications including discovery studies of biomarkers and therapeutic targets. For technical development, work on expanding the labeled standard library to identify more metabolites is needed. In addition, developing new labeling methods targeting chemical groups that are not covered by the current methods will further increase the overall metabolome coverage.

References

1. Peng, B., Li, H., & Peng, X. X. (2015). Functional metabolomics: From biomarker discovery to metabolome reprogramming. *Protein & Cell, 6*(9), 628–637.
2. Caldwell, G. W., & Leo, G. C. (2017). Can untargeted metabolomics be utilized in drug discovery/development? *Current Topics in Medicinal Chemistry, 17*(24), 2716–2739.
3. Aretz, I., & Meierhofer, D. (2016). Advantages and pitfalls of mass spectrometry based metabolome profiling in systems biology. *International Journal of Molecular Sciences, 17*(5), 14.
4. Ortmayr, K., Causon, T. J., Hann, S., & Koellensperger, G. (2016). Increasing selectivity and coverage in

LC-MS based metabolome analysis. *Trac-Trends Analitical Chemistry, 82*, 358–366.
5. Tang, D. Q., Zou, L., Yin, X. X., & Ong, C. N. (2016). HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS. *Mass Spectrometry Reviews, 35*(5), 574–600.
6. Antoniewicz, M. R. (2018). A guide to C-13 metabolic flux analysis for the cancer biologist. *Experimental & Molecular Medicine, 50*, 13.
7. Nilsson, R., & Jain, M. (2016). Simultaneous tracing of carbon and nitrogen isotopes in human cells. *Molecular BioSystems, 12*(6), 1929–1937.
8. Freund, D. M., & Hegeman, A. D. (2017). Recent advances in stable isotope-enabled mass spectrometry-based plant metabolomics. *Current Opinion in Biotechnology, 43*, 41–48.
9. Sun, R. C., Fan, T. W. M., Deng, P., Higashi, R. M., Lane, A. N., Le, A. T., Scott, T. L., Sun, Q. S., Warmoes, M. O., & Yang, Y. (2017). Noninvasive liquid diet delivery of stable isotopes into mouse models for deep metabolic network tracing. *Nature Communications, 8*, 10.
10. Han, W., Sapkota, S., Camicioli, R., Dixon, R. A., & Li, L. (2017). Profiling novel metabolic biomarkers for Parkinson's disease using in-depth Metabolomic analysis. *Movement Disorders, 32*(12), 1720–1728.
11. Wong, J. M. T., Malec, P. A., Mabrouk, O. S., Ro, J., Dus, M., & Kennedy, R. T. (2016). Benzoyl chloride derivatization with liquid chromatography-mass spectrometry for targeted metabolomics of neurochemicals in biological samples. *Journal of Chromatography. A, 1446*, 78–90.
12. Mohebi, A., Pettibone, J. R., Hamid, A. A., Wong, J.-M. T., Vinson, L. T., Patriarchi, T., Tian, L., Kennedy, R. T., & Berke, J. D. (2019). Dissociable dopamine dynamics for learning and motivation. *Nature, 570*(7759), 65–70.
13. Han, J., Gagnon, S., Eckle, T., & Borchers, C. H. (2013). Metabolomic analysis of key central carbon metabolism carboxylic acids as their 3-nitrophenylhydrazones by UPLC/ESI-MS. *Electrophoresis, 34*(19), 2891–2900.
14. Han, J., Lin, K., Sequeira, C., & Borchers, C. H. (2015). An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta, 854*, 86–94.
15. Han, J., Liu, Y., Wang, R. X., Yang, J. C., Ling, V., & Borchers, C. H. (2015). Metabolic profiling of bile acids in human and mouse blood by LC-MS/MS in combination with phospholipid-depletion solid-phase extraction. *Analytical Chemistry, 87*(2), 1127–1136.
16. O"Maille, G., Go, E. P., Hoang, L., Want, E. J., Smith, C., O'Maille, P., Nordstrom, A., Morita, H., Qin, C., Uritboonthai, W., Apon, J., Moore, R., Garrett, J., & Siuzdak, G. (2008). Metabolomics relative quantitation with mass spectrometry using chemical derivatization and isotope labeling. *Spectroscopy an International Journal, 22*(5), 327–343.
17. Guo, K., & Li, L. (2010). High-performance isotope labeling for profiling carboxylic acid-containing metabolites in biofluids by mass spectrometry. *Analytical Chemistry, 82*(21), 8789–8793.
18. Wang, C. J., Wu, Z. Y., Yuan, J. B., Wang, B., Zhang, P., Zhang, Y., Wang, Z. F., & Huang, L. J. (2014). Simplified quantitative Glycomics using the stable isotope label Girard's reagent P by electrospray ionization mass spectrometry. *Journal of Proteome Research, 13*(2), 372–384.
19. Griffiths, W. J., Hornshaw, M., Woffendin, G., Baker, S. F., Lockhart, A., Heidelberger, S., Gustafsson, M., Sjoall, J., & Wang, Y. Q. (2008). Discovering oxysterols in plasma: A window on the metabolome. *Journal of Proteome Research, 7*(8), 3602–3612.
20. DeBarber, A. E., Sandlers, Y., Pappu, A. S., Merkens, L. S., Duell, P. B., Lear, S. R., Erickson, S. K., & Steiner, R. D. (2011). Profiling sterols in cerebrotendinous xanthomatosis: Utility of Girard derivatization and high resolution exact mass LC-ESI-MSn analysis. *Journal of Chromatography B, 879*(17–18), 1384–1392.
21. Yang, W. C., Sedlak, M., Regnier, F. E., Mosier, N., Ho, N., & Adamec, J. (2008). Simultaneous quantification of metabolites involved in central carbon and energy metabolism using reversed-phase liquid chromatography-mass spectrometry and in vitro C-13 labeling. *Analytical Chemistry, 80*(24), 9508–9516.
22. Zhao, S., Luo, X., & Li, L. (2016). Chemical isotope labeling LC-MS for high coverage and quantitative profiling of the hydroxyl submetabolome in metabolomics. *Analytical Chemistry, 88*(21), 10617–10623.
23. Dai, W. D., Huang, Q., Yin, P. Y., Li, J., Zhou, J., Kong, H. W., Zhao, C. X., Lu, X., & Xu, G. W. (2012). Comprehensive and highly sensitive urinary steroid hormone profiling method based on stable isotope-labeling liquid chromatography mass spectrometry. *Analytical Chemistry, 84*(23), 10245–10251.
24. Comini, M. A. (2016). Measurement and meaning of cellular thiol:Disufhide redox status. *Free Radical Research, 50*(2), 246–271.
25. Ortmayr, K., Schwaiger, M., Hann, S., & Koellensperger, G. (2015). An integrated metabolomics workflow for the quantification of sulfur pathway intermediates employing thiol protection with N-ethyl maleimide and hydrophilic interaction liquid chromatography tandem mass spectrometry. *Analyst, 140*(22), 7687–7695.
26. Liu, P., Huang, Y. Q., Cai, W. J., Yuan, B. F., & Feng, Y. Q. (2014). Profiling of thiol-containing compounds by stable isotope labeling double precursor ion scan mass spectrometry. *Analytical Chemistry, 86*(19), 9765–9773.
27. Guo, K., & Li, L. (2009). Differential C-12/C-13-isotope Dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Analytical Chemistry, 81*(10), 3919–3932.

28. Johnson, D. W. (2011). Free amino acid quantification by LC-MS/MS using derivatization generated isotope-labelled standards. *Journal of Chromatography B, 879*(17–18), 1345–1352.
29. Guo, N., Liu, P., Ding, J., Zheng, S. J., Yuan, B. F., & Feng, Y. Q. (2016). Stable isotope labeling - liquid chromatography/mass spectrometry for quantitative analysis of androgenic and progestagenic steroids. *Analytica Chimica Acta, 905*, 106–114.
30. Woo, H. K., Go, E. P., Hoang, L., Trauger, S. A., Bowen, B., Siuzdak, G., & Northen, T. R. (2009). Phosphonium labeling for increasing metabolomic coverage of neutral lipids using electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 23*(12), 1849–1855.
31. Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. *Analytical and Bioanalytical Chemistry, 402*(1), 231–247.
32. Zhu, Q. F., Hao, Y. H., Liu, M. Z., Yue, J., Ni, J., Yuan, B. F., & Feng, Y. Q. (2015). Analysis of cytochrome P450 metabolites of arachidonic acid by stable isotope probe labeling coupled with ultra high-performance liquid chromatography/mass spectrometry. *Journal of Chromatography. A, 1410*, 154–163.
33. Li, S. F., Jin, Y. B., Tang, Z., Lin, S. H., Liu, H. X., Jiang, Y. Y., & Cai, Z. W. (2015). A novel method of liquid chromatography-tandem mass spectrometry combined with chemical derivatization for the determination of ribonucleosides in urine. *Analytica Chimica Acta, 864*, 30–38.
34. Yuan, B. F., Zhu, Q. F., Guo, N., Zheng, S. J., Wang, Y. L., Wang, J., Xu, J., Liu, S. J., He, K., Hu, T., Zheng, Y. W., Xu, F. Q., & Feng, Y. Q. (2018). Comprehensive profiling of fecal metabolome of mice by integrated chemical isotope labeling-mass spectrometry analysis. *Analytical Chemistry, 90*(5), 3512–3520.
35. Takach, E., O'Shea, T., & Liu, H. L. (2014). High-throughput quantitation of amino acids in rat and mouse biological matrices using stable isotope labeling and UPLC-MS/MS analysis. *Journal of Chromatography B, 964*, 180–190.
36. Zhao, S., Li, H., Han, W., Chan, W., & Li, L. (2019). Metabolomic coverage of chemical-group-submetabolome analysis: Group classification and 4-channel chemical isotope labeling LC-MS. *Analytical Chemistry*.
37. Zhao, S., Dawe, M., Guo, K., & Li, L. (2017). Development of high-performance chemical isotope labeling LC-MS for profiling the carbonyl submetabolome. *Analytical Chemistry, 89*(12), 6758–6765.
38. Bian, X. Q., Sun, B. Q., Zheng, P. Y., Li, N., & Wu, J. L. (2017). Derivatization enhanced separation and sensitivity of long chain-free fatty acids: Application to asthma using targeted and non-targeted liquid chromatography-mass spectrometry approach. *Analytica Chimica Acta, 989*, 59–70.
39. Siegel, D., Meinema, A. C., Permentier, H., Hopfgartner, G., & Bischoff, R. (2014). Integrated quantification and identification of aldehydes and ketones in biological samples. *Analytical Chemistry, 86*(10), 5089–5100.
40. Turowski, M., Yamakawa, N., Meller, J., Kimata, K., Ikegami, T., Hosoya, K., Tanaka, N., & Thornton, E. R. (2003). Deuterium isotope effects on hydrophobic interactions: The importance of dispersion interactions in the hydrophobic phase. *Journal of the American Chemical Society, 125*(45), 13836–13849.
41. Szarka, S., Prokai-Tatrai, K., & Prokai, L. (2014). Application of screening experimental designs to assess chromatographic isotope effect upon isotope-coded derivatization for quantitative liquid chromatography-mass spectrometry. *Analytical Chemistry, 86*(14), 7033–7040.
42. Khamis, M. M., Adamko, D. J., & El-Aneed, A. (2017). Development of a validated LC-MS/MS method for the quantification of 19 endogenous asthma/COPD potential urinary biomarkers. *Analytica Chimica Acta, 989*, 45–58.
43. Su, X. L., Wang, N., Chen, D. Y., Li, Y. N., Lu, Y. F., Huan, T., Xu, W., Li, L., & Li, L. J. (2016). Dansylation isotope labeling liquid chromatography mass spectrometry for parallel profiling of human urinary and fecal submetabolomes. *Analytica Chimica Acta, 903*, 100–109.
44. Chen, D. Y., Han, W., Su, X. L., Li, L., & Li, L. J. (2017). Overcoming sample matrix effect in quantitative blood metabolomics using chemical isotope labeling liquid chromatography mass spectrometry. *Analytical Chemistry, 89*(17), 9424–9431.
45. Hooton, K., Han, W., & Li, L. (2016). Comprehensive and quantitative profiling of the human sweat submetabolome using high-performance chemical isotope labeling LC-MS. *Analytical Chemistry, 88*(14), 7378–7386.
46. Guo, K., Bamforth, F., & Li, L. A. (2011). Qualitative metabolome analysis of human cerebrospinal fluid by C-13-/C-12-isotope Dansylation labeling combined with liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry. *Journal of the American Society for Mass Spectrometry, 22*(2), 339–347.
47. Xu, W., Chen, D. Y., Wang, N., Zhang, T., Zhou, R. K., Huan, T., Lu, Y. F., Su, X. L., Xie, Q., Li, L., & Li, L. J. (2015). Development of high-performance chemical isotope labeling LC-MS for profiling the human fecal metabolome. *Analytical Chemistry, 87*(2), 829–836.
48. Luo, X., & Li, L. (2017). Metabolomics of small numbers of cells: Metabolomic profiling of 100, 1000, and 10000 human breast Cancer cells. *Analytical Chemistry, 89*(21), 11664–11671.
49. Luo, X., Zhao, S., Huan, T., Sun, D. F., Friis, R. M. N., Schultz, M. C., & Li, L. (2016). High-performance chemical isotope labeling liquid chromatography mass spectrometry for profiling the Metabolomic reprogramming elicited by ammonium limitation in yeast. *Journal of Proteome Research, 15*(5), 1602–1612.

50. Huan, T., Troyer, D. A., & Li, L. (2016). Metabolite analysis and histology on the exact same tissue: Comprehensive Metabolomic profiling and metabolic classification of prostate Cancer. *Scientific Reports, 6*, 13.
51. Huan, T., Wu, Y. M., Tang, C. Q., Lin, G. H., & Li, L. (2015). DnsID in MyCompoundID for rapid identification of Dansylated amine- and phenol-containing metabolites in LC-MS-based metabolomics. *Analytical Chemistry, 87*(19), 9838–9845.
52. Zhou, R. K., Guo, K., & Li, L. (2013). 5-Diethylaminonaphthalene-1-sulfonyl chloride (DensCl): A novel triplex isotope labeling reagent for quantitative metabolome analysis by liquid chromatography mass spectrometry. *Analytical Chemistry, 85*(23), 11532–11539.
53. Song, C. H., Zhang, S. J., Ji, Z. Y., Li, Y. P., & You, J. M. (2015). Accurate determination of amino acids in serum samples by liquid chromatography-tandem mass spectrometry using a stable isotope labeling strategy. *Journal of Chromatographic Science, 53*(9), 1536–1541.
54. Zheng, L. F., Zhao, X. E., Zhu, S. Y., Tao, Y. D., Ji, W. H., Geng, Y. L., Wang, X. A., Chen, G. A., & You, J. M. (2017). A new combined method of stable isotope-labeling derivatization-ultrasound-assisted dispersive liquid-liquid microextraction for the determination of neurotransmitters in rat brain microdialysates by ultra high performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B, 1054*, 64–72.
55. Zhou, R., Huan, T., & Li, L. (2015). Development of versatile isotopic labeling reagents for profiling the amine submetabolome by liquid chromatography-mass spectrometry. *Analytica Chimica Acta, 881*, 107–116.
56. Wagner, M., Ohlund, L. B., Shiao, T. C., Vezina, A., Annabi, B., Roy, R., & Sleno, L. (2015). Isotope-labeled differential profiling of metabolites using N-benzoyloxysuccinimide derivatization coupled to liquid chromatography/high-resolution tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 29*(18), 1632–1640.
57. Zhang, S. S., Shi, J. W., Shan, C. K., Huang, C. T., Wu, Y. L., Ding, R., Xue, Y. H., Liu, W., Zhou, Q., Zhao, Y. F., Xu, P. X., & Gao, X. (2017). Stable isotope N-phosphoryl amino acids labeling for quantitative profiling of amine-containing metabolites using liquid chromatography mass spectrometry. *Analytica Chimica Acta, 978*, 24–34.
58. Hao, L.; Zhong, X. F.; Greer, T.; Ye, H.; Li, L. J., Relative quantification of amine-containing metabolites using isobaric N,N-dimethyl leucine (DiLeu) reagents via LC-ESI-MS/MS and CE-ESI-MS/MS. Analyst 2015**,** 140 (2), 467–475.
59. Lee, D. Y., & Chang, G. D. (2015). Quantitative liquid chromatography-electrospray ionization-mass spectrometry analysis of amine-containing metabolites derivatized with cyanuric chloride and methylamine isotopologues. *Journal of Chromatography. A, 1388*, 60–68.
60. Wang, X., Wei, F., Xu, J. Q., Lv, X., Dong, X. Y., Han, X. L., Quek, S. Y., Huang, F. H., & Chen, H. (2016). Profiling and relative quantification of phosphatidylethanolamine based on acetone stable isotope derivatization. *Analytica Chimica Acta, 902*, 142–153.
61. Yang, W. C., Mirzaei, H., Liu, X. P., & Regnier, F. E. (2006). Enhancement of amino acid detection and quantification by electrospray ionization mass spectrometry. *Analytical Chemistry, 78*(13), 4702–4708.
62. Shortreed, M. R., Lamos, S. M., Frey, B. L., Phillips, M. F., Patel, M., Belshaw, P. J., & Smith, L. M. (2006). Ionizable isotopic labeling reagent for relative quantification of amine metabolites by mass spectrometry. *Analytical Chemistry, 78*(18), 6398–6403.
63. Guo, K., Ji, C. J., & Li, L. (2007). Stable-isotope dimethylation labeling combined with LC-ESI MS for quantification of amine-containing metabolites in biological samples. *Analytical Chemistry, 79*(22), 8631–8638.
64. Ji, C. J., Li, W. L., Ren, X. D., El-Kattan, A. F., Kozak, R., Fountain, S., & Lepsy, C. (2008). Diethylation labeling combined with UPLC/MS/MS for simultaneous determination of a panel of monoamine neurotransmitters in rat prefrontal cortex microdialysates. *Analytical Chemistry, 80*(23), 9195–9203.
65. Abello, N., Geurink, P. P., van der Toorn, M., van Oosterhout, A. J. M., Lugtenburg, J., van der Marel, G. A., Kerstjens, H. A. M., Postma, D. S., Overkleeft, H. S., & Bischoff, R. (2008). Poly(ethylene glycol)-based stable isotope labeling reagents for the quantitative analysis of low molecular weight metabolites by LC-MS. *Analytical Chemistry, 80*(23), 9171–9180.
66. Shimbo, K., Yahashi, A., Hirayama, K., Nakazawa, M., & Miyano, H. (2009). Multifunctional and highly sensitive Precolumn reagents for amino acids in liquid chromatography/tandem mass spectrometry. *Analytical Chemistry, 81*(13), 5172–5179.
67. Mochizuki, T., Todoroki, K., Inoue, K., Min, J. Z., & Toyo'oka, T. (2014). Isotopic variants of light and heavy L-pyroglutamic acid succinimidyl esters as the derivatization reagents for DL-amino acid chiral metabolomics identification by liquid chromatography and electrospray ionization mass spectrometry. *Analytica Chimica Acta, 811*, 51–59.
68. Lamos, S. M., Shortreed, M. R., Frey, B. L., Belshaw, P. J., & Smith, L. M. (2007). Relative quantification of carboxylic acid metabolites by liquid chromatography - mass spectrometry using isotopic variants of cholamine. *Analytical Chemistry, 79*(14), 5143–5149.
69. Tayyari, F., Gowda, G. A. N., Gu, H. W., & Raftery, D. (2013). N-15-Cholamine-a smart isotope tag for combining NMR- and MS-based metabolite profiling. *Analytical Chemistry, 85*(18), 8715–8721.
70. Yang, W. C., Regnier, F. E., & Adamec, J. (2008). Comparative metabolite profiling of carboxylic acids in rat urine by CE-ESI MS/MS through positively pre-charged and H-2-coded derivatization. *Electrophoresis, 29*(22), 4549–4560.
71. Yang, W. C., Adamec, J., & Regnier, F. E. (2007). Enhancement of the LC/MS analysis of fatty acids

through derivatization and stable isotope coding. *Analytical Chemistry, 79*(14), 5150–5157.
72. Koulman, A., Petras, D., Narayana, V. K., Wang, L., & Volmer, D. A. (2009). Comparative high-speed profiling of carboxylic acid metabolite levels by differential isotope-coded MALDI mass spectrometry. *Analytical Chemistry, 81*(18), 7544–7551.
73. Tsukamoto, Y.; Santa, T.; Yoshida, H.; Miyano, H.; Fukushima, T.; Hirayama, K.; Imai, K.; Funatsu, T., Synthesis of the isotope-labeled derivatization reagent for carboxylic acidsr 7-(N,N-dimethylaminosulfonyl)-4-(aminoethyl)piperazino-2,1,3-benzoxadiaz ole (d(6)) DBD-PZ-NH2 (D) , and its application to the quantification and the determination of relative amount of fatty acids in rat plasma samples by high-performance liquid chromatography/mass spectrometry. *Biomed. Chromatogr.* **2006,** *20* (4), 358–364.
74. Leng, J. P., Wang, H. Y., Zhang, L., Zhang, J., Wang, H., & Guo, Y. L. (2013). A highly sensitive isotope-coded derivatization method and its application for the mass spectrometric analysis of analytes containing the carboxyl group. *Analytica Chimica Acta, 758*, 114–121.
75. Leng, J. P., Guan, Q., Sun, T. Q., Wang, H. Y., Cui, J. L., Liu, Q. H., Zhang, Z. X., Zhang, M. Y., & Guo, Y. L. (2015). Direct infusion electrospray ionization-ion mobility-mass spectrometry for comparative profiling of fatty acids based on stable isotope labeling. *Analytica Chimica Acta, 887*, 148–154.
76. Tie, C., Hu, T., Zhang, X. X., Zhou, J., & Zhang, J. L. (2014). HPLC-MRM relative quantification analysis of fatty acids based on a novel derivatization strategy. *Analyst, 139*(23), 6154–6159.
77. Guo, N., Peng, C. Y., Zhu, Q. F., Yuan, B. F., & Feng, Y. Q. (2017). Profiling of carbonyl compounds in serum by stable isotope labeling double precursor ion scan - mass spectrometry analysis. *Analytica Chimica Acta, 967*, 42–51.
78. Zheng, S. J., Wang, Y. L., Liu, P., Zhang, Z., Yu, L., Yuan, B. F., & Feng, Y. Q. (2017). Stable isotope labeling-solid phase extraction-mass spectrometry analysis for profiling of thiols and aldehydes in beer. *Food Chemistry, 237*, 399–407.
79. Yu, L., Liu, P., Wang, Y. L., Yu, Q. W., Yuan, B. F., & Feng, Y. Q. (2015). Profiling of aldehyde-containing compounds by stable isotope labelling-assisted mass spectrometry analysis. *Analyst, 140*(15), 5276–5286.
80. Crick, P. J., Bentley, T. W., Abdel-Khalik, J., Matthews, I., Clayton, P. T., Morris, A. A., Bigger, B. W., Zerbinati, C., Tritapepe, L., Iuliano, L., Wang, Y. Q., & Griffiths, W. J. (2015). Quantitative charge-tags for sterol and oxysterol analysis. *Clinical Chemistry, 61*(2), 400–411.
81. Higashi, T., Aiba, N., Tanaka, T., Yoshizawa, K., & Ogawa, S. (2016). Methods for differential and quantitative analyses of brain neurosteroid levels by LC/MS/MS with ESI-enhancing and isotope-coded derivatization. *Journal of Pharmaceutical and Biomedical Analysis, 117*, 155–162.
82. Tie, C., Hu, T., Jia, Z. X., & Zhang, J. L. (2016). Derivatization strategy for the comprehensive characterization of endogenous fatty aldehydes using HPLC-multiple reaction monitoring. *Analytical Chemistry, 88*(15), 7762–7768.
83. DeBarber, A. E., Luo, J., Star-Weinstock, M., Purkayastha, S., Geraghty, M. T., Chiang, J., Merkens, L. S., Pappu, A. S., & Steiner, R. D. (2014). A blood test for cerebrotendinous xanthomatosis with potential for disease detection in newborns. *Journal of Lipid Research, 55*(1), 146–154.
84. Chu, J. M., Qi, C. B., Huang, Y. Q., Jiang, H. P., Hao, Y. H., Yuan, B. F., & Feng, Y. Q. (2015). Metal oxide-based selective enrichment combined with stable isotope labeling-mass spectrometry analysis for profiling of ribose conjugates. *Analytical Chemistry, 87*(14), 7364–7372.
85. Wang, H., Wang, H. Y., Zhang, L., Zhang, J., & Guo, Y. L. (2011). N-Alkylpyridinium isotope quaternization for matrix-assisted laser desorption/ionization Fourier transform mass spectrometric analysis of cholesterol and fatty alcohols in human hair. *Analytica Chimica Acta, 690*(1), 1–9.
86. Wang, H., Wang, H. Y., Zhang, L., Zhang, J., Leng, J. P., Cai, T. T., & Guo, Y. L. (2011). Improvement and extension of the application scope for matrix-assisted laser desorption/ionization mass spectrometric analysis-oriented N-alkylpyridinium isotope quaternization. *Analytica Chimica Acta, 707*(1), 100–106.
87. Huang, Y. Q., Ruan, G. D., Liu, J. Q., Gao, Q., & Feng, Y. Q. (2011). Use of isotope differential derivatization for simultaneous determination of thiols and oxidized thiols by liquid chromatography tandem mass spectrometry. *Analytical Biochemistry, 416*(2), 159–166.