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# Metabolite Annotation through Stable Isotope Labeling

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ABSTRACT

Stable isotope labeling (SIL) is a powerful tool for probing cellular metabolism and has been increasingly employed for metabolite annotation in mass spectrometry-based metabolomics. Herein, we outline the principles and methods of designing and performing SIL-based experiments in metabolomics studies. Further, we summarize different SIL approaches and highlight recent examples of their use for metabolite annotation, including biological feature recognition, degenerate feature annotation, elemental composition determination and metabolite structure elucidation, across diverse biological systems. Throughout, the opportunities, open challenges, and potential pitfalls of each SIL workflow are discussed. Finally, we summarize and compare the recently developed software tools for the analysis of SIL-based metabolomics data.

## 1. Introduction

As the end products of cellular regulatory processes, metabolites represent the ultimate response of biological systems associated with genetic and environmental changes. Thus, through metabolomics, the comprehensive analysis of metabolites, insightful information of physiological, pathological and biochemical status can be obtained [1,2].The depth of metabolome coverage, throughput and sensitivity of mass spectrometry (MS)-based metabolomics has steadily improved over the past decade; yet metabolite annotation remains the major bottleneck [3]. This holds true for 'simple' organisms such as bacteria with relatively small-sized metabolomes (in the hundreds) and even more for plants that contain 100,000 to 1 million structurally-diverse metabolites [4–6].

In metabolomics the term annotation generally refers to the assignment of each mass feature with an elemental formula and putative chemical structure [7,8]. Advances in ultra-high resolution accurate mass instruments enable direct determination of the elemental formula based on accurate mass and natural isotope abundance [9,10], while it is generally limited for metabolites up to about 500 Da [11,12]. In many cases, even with correctly assigned elemental formula it is challenging to identify the chemical structure due to the presence of multiple isomers. For a given elemental formula, depending on the number and type of elements in the chemical composition, several to billions of structures can be theoretically constructed. For instance, a simple formula such as $C_{100}H_{202}$ could have more than $5.9 \times 10^{39}$ possible structures [13].

In this review, we will summarize the latest advances in using stable isotope labeling for metabolite annotation in MS-based metabolomics, focusing on three key aspects: biological mass feature recognition, degenerate feature annotation, elemental composition determination, and metabolite structural elucidation.

## 2. Stable isotope labeling in MS

Stable isotopes are non-radioactive form of elements with the same number of protons as common elements; but they differ in masses due to the difference in the number of neutrons [14–16]. Most elements have naturally occurring isotopes. For example, the most abundant stable isotope of carbon is $^{12}C$, which constitutes 98.89 % of all carbon found on the planet. The nucleus of this carbon has six protons and six neutrons. A less abundant stable carbon species (1.11 %) exists as $^{13}C$ with six protons but seven neutrons. Additionally, a trace amount of $^{14}C$, an unstable radioactive isotope of carbon with six protons and eight neutrons, also exist in nature (Fig. 1A). Due to biological perturbations, safety concerns and incompatibility with MS, the radioisotope is limited in its applications in MS nowadays [17]. Instead, stable isotopes have

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been widely used in MS-based metabolomics. Heavy isotopes such as hydrogen ($^{2}$H), carbon ($^{13}$C), nitrogen ($^{15}$N), oxygen ($^{18}$O) and sulfur ($^{34}$S), which have one or two more neutrons than their most abundant naturally occurring counterparts (i.e., $^{1}$H, $^{12}$C, $^{14}$N, $^{16}$O, and $^{32}$S), are the most commonly used stable isotopes in biology [17,18] (Fig. 1A).

Due to the wide presence of natural stable isotopes, the mass spectrum for an unlabeled metabolite typically contains several mass peaks, including the most abundant monoisotopic peak and several low abundance isotopic peaks representing various combinations of all the naturally occurring isotopes [14]. For instance, phenylalanine ($C_9H_{11}NO_2$) naturally possesses a $^{13}$C peak (*m/z* 167.0896) which is 1.00335 Da higher in mass, and at roughly 10 % abundance (9 × 1.11 %), compared with the monoisotopic peak (*m/z* 166.0863) (Fig. 1B). The exact abundance of each isotopic peak can be calculated based on binomial distribution, i.e., the natural abundance of single $^{13}$C phenylalanine peak is $9 \times (1.11\ \%)^{1} \times (100\ \%–1.11\ \%)^{9\text{-}1} = 9.06\ \%$, and the monoisotopic peak is $(100\ \%–1.11\ \%)^{9} = 90.52\ \%$. Additional peaks representing the natural hydrogen ($^{2}$H), nitrogen ($^{15}$N) or oxygen ($^{18}$O)

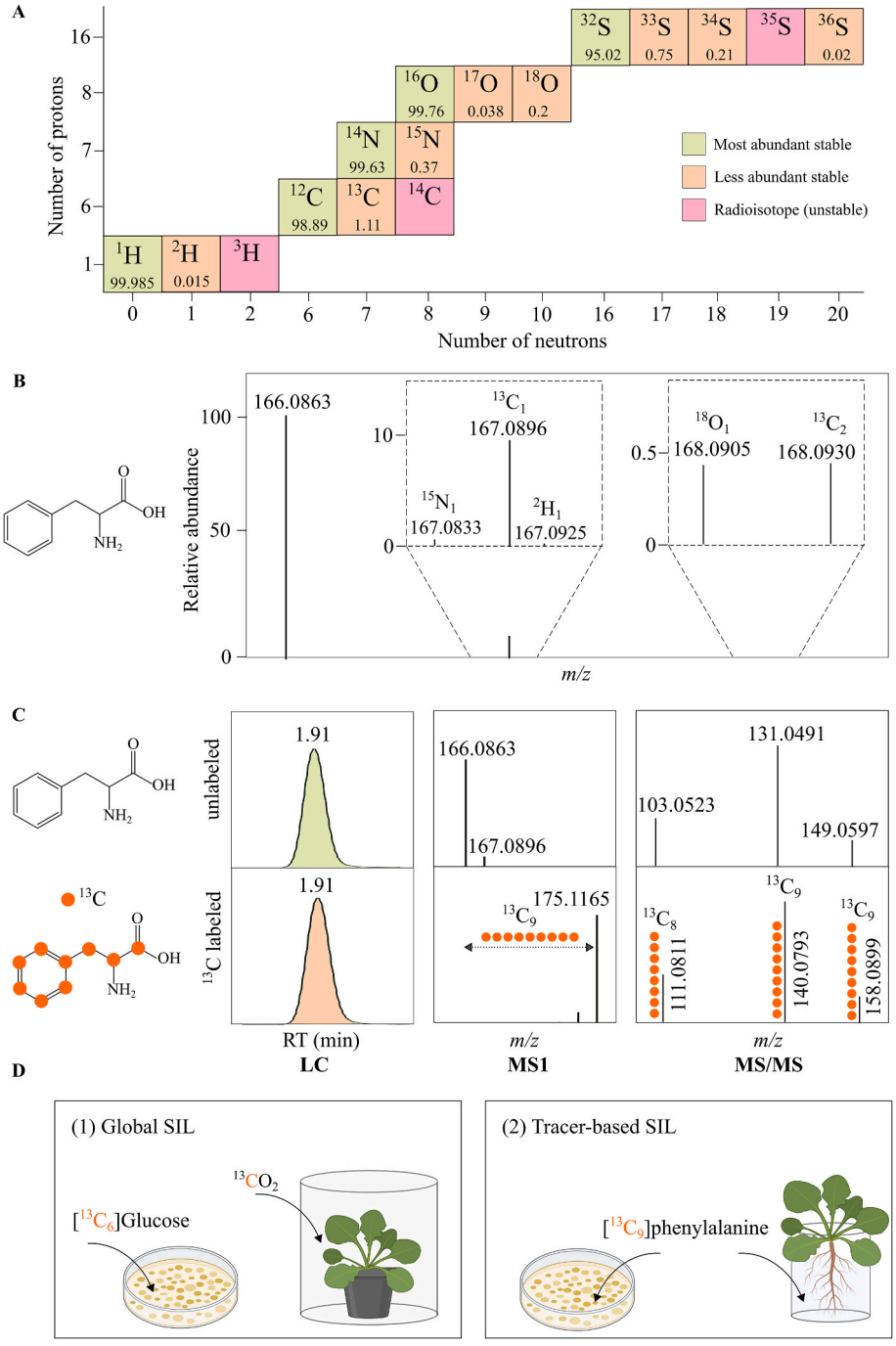



**Fig. 1.** An overview of stable isotope labeling (SIL). (**A**) Common elements and their naturally occurring isotopes. (**B**) For a given metabolite, the mass spectrum of the metabolite (e.g., phenylalanine) usually comprises multiple mass peaks, encompassing the prevalent monoisotopic peak and several less abundant isotopic peaks representing various combinations of naturally occurring isotopes. (**C**) The unlabeled metabolites and their labeled forms (e.g., $^{12}C_9$-phenylalanine and $^{13}C_9$-phenylalanine) exhibit identical retention time in liquid chromatography but show distinct masses in mass spectra. The labeling reveals not only the mass shift of the precursor ion but also that of the product ions in mass spectra, as demonstrated with $^{12}C_9$-phenylalanine and $^{13}C_9$-phenylalanine. (**D**) Global SIL and tracer-based SIL are the most commonly used *in vivo* SIL approaches in biology.

isotopes, and a peak containing two $^{13}C$ atoms, are also present at low abundance (Fig. 1B).

Stable isotope labeling (SIL) is a technique that introduces heavy isotopes into biological systems. It takes advantage of the fact that isotopes are different in mass, but they generally behave chemically identically. Therefore, isotopologues and their unlabeled counterparts share the same elemental formula and structure, and generally co-elute during chromatographic separation (except for $^{2}H$ labeled metabolites that can differ from their unlabeled form due to the deuterium isotope effects [19]). However, they can be readily distinguished by the mass shift associated with the labeled atoms in MS. For example, unlabeled and uniformly $^{13}C$-labeled phenylalanine standards are eluted at identical retention time (RT) in liquid chromatography (LC). While the fully $^{13}C$-labeled phenylalanine would have an mass-to-charge ratio ($m/z$) of 175.1165 ($[M+H]^+$) in positive ion mode Electrospray Ionization (ESI)-MS, which is 9.0302 Da ($9 \times 1.00335$, $^{13}C_9H_{11}NO_2$) higher than the unlabeled phenylalanine detected at $m/z$ 166.0863 ($[M+H]^+$) (Fig. 1C). In addition, mass shifts are also observed for product ions that carry $^{13}C$ isotopes in tandem MS (MS/MS) (Fig. 1C).

Depending on the labeling strategy, organisms can be labeled uniformly or non-uniformly with different degrees of enrichment [20–22]. Through global SIL, all endogenously produced metabolites are uniformly labeled (i.e., metabolites with all atoms of a given element are replaced by heavy isotopes) by introducing stable isotopes to the biological systems via primary nutrient sources (e.g., uniformly $^{13}C$-labeled glucose) [20]. Fully labeled $^{13}C$ plant samples can also be achieved via a continuous supply of $^{13}CO_2$ in an airtight cultivation chamber (Fig. 1D). In addition to $^{13}C$ as the most comprehensive stable isotope label, global SIL with $^{15}N$ or $^{34}S$ enriched nutrient solutions can also be used to study the nitrogen or sulfur containing metabolites of cell cultures or whole plants [23,24]. With tracer-based SIL, isotopically labeled tracer (or precursor), is administered to the biological system via infusions/injections, gavage or solid/liquid feeding [25,26] (Fig. 1D). The labeled and the native form of the substance are then both metabolized. Consequently, downstream metabolites derived from the labeled metabolite will contain labeled atoms of some enrichment depending on metabolic flux and labeling time [17,20].

## 3. Discrimination of biological from non-biological mass features

A typical LC-MS based metabolomics study can detect more than 10,000 mass features [27–29]. However, the maximal number of detected features does not necessarily correspond to the greatest metabolome coverage due to the presence of a significant amount of non-biological features. Non-biological features refer to mass peaks that arise from contaminants, chemical noise and bioinformatic artifacts. Biological features are peaks that are derived from endogenous metabolites in the sample [30]. It has been estimated that over 80 % of the peaks detected in *Saccharomyces cerevisiae* and *E.coli* are non-biological features [27,31]. Therefore, the first step towards metabolite annotation is to detect biological features. Despite numerous attempts to filter out non-biological features, their elimination proves challenging. This difficulty is mainly due to the similar analytical characteristics shared with biological features, especially those associated with chemical contaminants [31].

Global SIL is an effective approach to detect biological features through a credentialing-type strategy. This workflow compares the

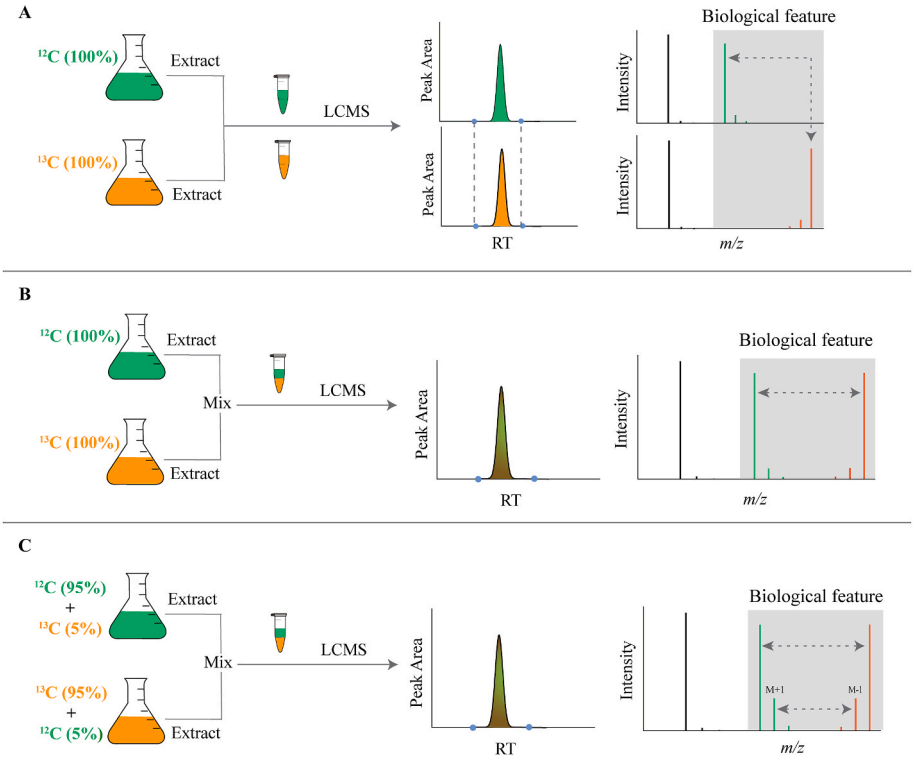



**Fig. 2.** Representative workflows of stable isotope labeling (SIL)-based credentialing strategies. (**A**) Demonstration of a separate sample approach for credentialing. Two groups of *E. coli* underwent SIL, one group grown on natural abundance $^{12}C_6$-glucose and the second grown on $^{13}C_6$-glucose as the sole carbon source. The two groups were subjected to LC-MS analysis. Biological features can be recognized based on whether the peaks exhibit mass shifts. (**B**) Demonstration of a mixed sample approach for credentialing. Two groups of *E. coli* underwent SIL, one group grown on natural abundance $^{12}C_6$-glucose and the second grown on $^{13}C_6$-glucose as the sole carbon source. The two cultures were combined equally for LC-MS analysis. Peaks showing mirrored isotope patterns are identified as biological features. (**C**) Workflow of isotopic ratio outlier analysis (IROA). Two groups of *E. coli* underwent SIL, one group grown on medium containing 95 % $^{12}C_6$-glucose and 5 % $^{13}C_6$-glucose, and the second grown on medium containing 5 % $^{12}C_6$-glucose and 95 % $^{13}C_6$-glucose. The two cultures were combined equally for LC-MS analysis. Peaks showing mirrored isotope patterns with enhanced M+1 and M − 1 peaks are identified as biological features.

labeled and unlabeled samples, and biological peaks can be readily distinguished from non-biological peaks based on whether the peaks exhibit mass shifts [8,32] (Fig. 2A). To minimize sample-to-sample variations and prevent potential errors in matching labeled and unlabeled peaks, labeled and unlabeled samples are generally pooled prior to sample extraction and analysis. In such cases, peaks showing mirrored isotope patterns are identified as biological features (Fig. 2B). This approach has been applied to detect biological features in *Triticum durum* wheat [20,33], *E.coli* [10], and *Pichia pastoris* [34]. An innovative variation of the mixing sample approach includes cultivating organisms in two sets of media containing uniformly labeled carbon sources of defined $^{12}C$:$^{13}C$ ratios (e.g., 95:5 vs. 5:95). This method, termed isotopic ratio outlier analysis (IROA), leads to more predictable and diagnostic isotopic patterns for all biological features [35]. For instance, metabolites labeled at 5 % $^{13}C$ medium show a strongly enhanced M+1 peak, while those labeled at 95 % $^{13}C$ exhibit a markedly increased M − 1 peak. In contrast, artifacts adhere to the isotopic pattern of natural abundance, thus providing a more stringent criterion for biological feature recognition. The promise of IROA for biological feature detection has been demonstrated in both LC-MS [10,36] and gas chromatography (GC)-MS based metabolomics studies [37,38]. Another strategy is to mix labeled and unlabeled samples at various ratios (e.g., 1:1 and 1:2) for an additional peak intensity check. This method requires that the ratios of unlabeled and labeled isotopologues align proportionally with the mixed ratios of each sample, thus further improving the confidence in credentialing. Using this approach, the authors found that the number of biological features in an *E. coli* extract was less than 4 % of all features detected [30,39]. In addition to mixing extracts from labeled and unlabeled samples, they can be also analyzed separately. For instance, the peak annotation and verification engine (PAVE) was specifically developed for credentialing in separate cell extracts cultivated in unlabeled, $^{13}C$, $^{15}N$, and $^{13}C$+$^{15}N$ media [27]. In the proof-of-concept study, the authors showed that over 80 % features in *S. cerevisiae* and *E. coli* extracts were non-biological features [27]. Favilli et al. (2023) integrated a high-throughput, multi-well cultivation method with PAVE, enabling conducting large-scale credentialing experiments. Their finding demonstrated that, out of the approximately 37,000 total detected features in both positive and negative ion modes from *S. cerevisiae*, only 3–7% were credentialed [31].

## 4. Degenerate feature annotation

Typically, a single metabolite could generate multiple mass peaks in MS due to the presence of adducts, in-source fragments, and the natural abundance of heavy isotopes [40–42]. For instance, glutamate was found to produce over 100 peaks and exhibited complex adduct formation in the negative ion mode [40]. Mass features derived from the same metabolite are referred to as degenerate features. They constitute a significant source of complexity that challenges MS data interpretation [8,28,39,40]. As such, a crucial need is to properly detect and identify these peaks, which can then be either used collectively for metabolite structure elucidation or eliminated to focus on only the protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) ions [27].

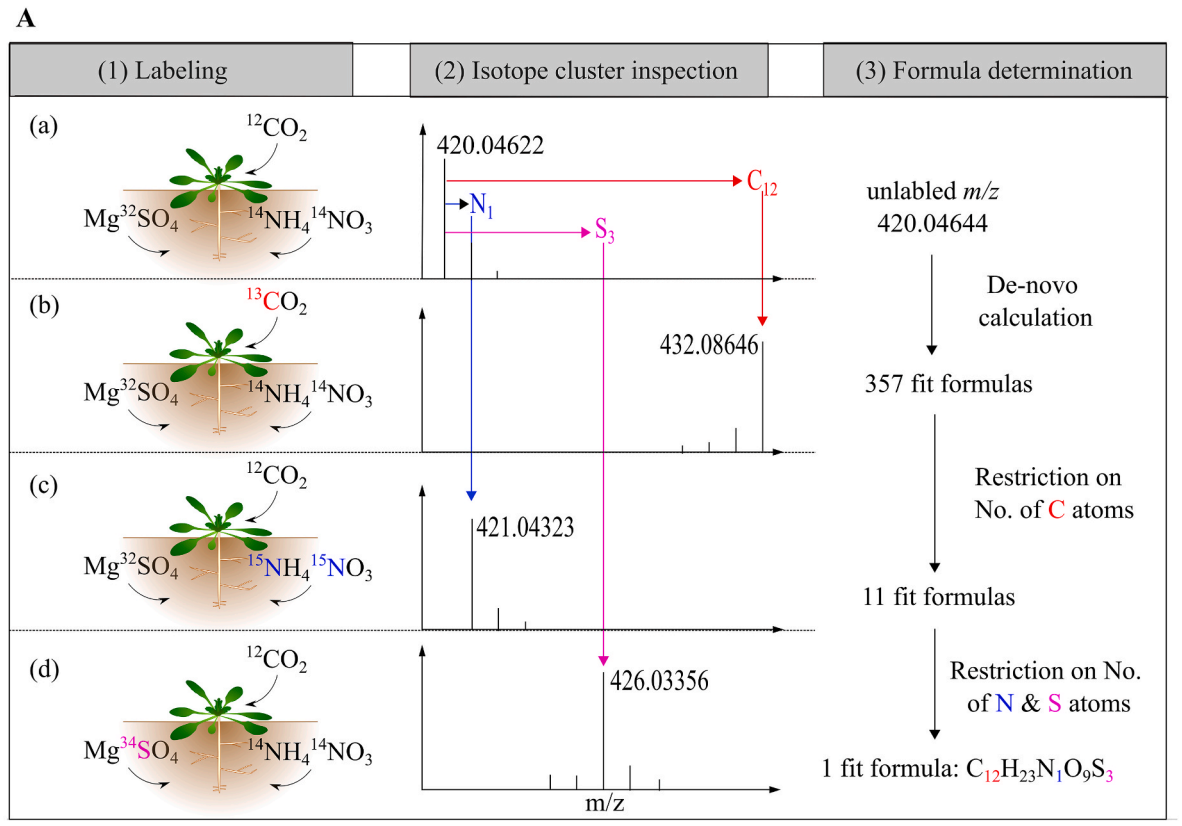
While global SIL-based credentialing approaches are powerful in detecting biological features, they often fail to effectively annotate degenerate mass features. Nevertheless, the labeling information can be used for degenerate feature identification. The rationale is that degenerate peaks co-elute, where adduct peaks exhibit higher masses but share the same labeling patterns (i.e., the same number of labeled atoms) as their metabolite peak ($[M+H]^+$ or $[M-H]^-$), whereas in-source fragments have smaller masses and may display different labeling patterns. The PAVE approach takes into account the information from calculated carbon and nitrogen atom counts, commonly observed adduct relationships, and weak fragmentation for degenerate feature annotation. When applied to *S. cerevisiae* and *E. coli*, PAVE successfully credentialed between 2 and 5 % unique biological features, respectively. The majority of the detected signals were recognized as non-biological features, with 80 % attributed to background signals, along with 4 % each for adducts and isotopes [27].

In addition to *in vivo* SIL approaches, an LC-MS-based buffer modification workflow (BMW) was developed to identify buffer-derived adduct ions [28]. In a BMW workflow, the same sample is analyzed by LC-MS in both liquid chromatography solvent with $^{14}NH_3$-acetate buffer, and in solvent with the buffer modified with $^{15}NH_3$-formate. The modification of the LC buffer leads to robust mass and intensity shifts for adduct peaks, while it has minimal impact on the intensities of metabolite peaks ($[M+H]^+$ or $[M-H]^-$), thereby facilitating adduct peak annotation. Application to mouse liver data annotated both known metabolite and known adduct peaks with 95 % accuracy [28]. Another advantage of BMW is that it can be applied to any samples including those are not amenable to global SIL, e.g., human tissues.

## 5. Elemental composition determination

SIL-based credentialing generally reduces the tens of thousands features detected in an untargeted LC-MS experiment to a few hundred biological features, which can then be prioritized for metabolite annotation [31]. The next step of metabolite annotation is elemental composition determination. Even without SIL, the stable isotope pattern from naturally distributed isotopes has long been used for constraining accurate mass-based elemental composition assignment [17,43,44]. For instance, the SIRIUS software utilizes isotope pattern along with the fragmentation pattern to determine elemental compositions of measured masses [45]. This approach is particularly appealing for metabolites containing elements of distinct isotopic distribution patterns in MS, such as chlorine (76 % $^{35}Cl$ *vs* 24 % $^{37}Cl$) and bromine (50.6 % $^{79}Br$ *vs* 49.4 % $^{81}Br$). For example, a mass peak with an isotopic mass distance of 2 Da and equal isotopic abundance most likely corresponds to a metabolite containing one bromine atom. Unfortunately, the natural abundance of hydrogen, carbon, nitrogen and oxygen exhibit a low abundance of heavy isotopes (Fig. 1A), therefore do not contribute significantly to the predictive power in elemental composition assignment [17].

With global SIL, it becomes feasible to accurately pinpoint the number of labeled atoms in each molecule by comparing the labeled and unlabeled peak pairs. This approach greatly improves the confidence in elemental composition assignment [17]. For instance, the number of carbon atoms contained in a metabolite can be counted by subtracting the *m/z* value of $^{12}C$ ion signal from its corresponding $^{13}C$ fully labeled signal. In this regard, a multi-isotope labeling approach, e.g., combing $^{13}C$ and $^{15}N$ labeling, can further decrease the ambiguity in elemental composition assignment by restricting the number of both C and N atoms in the formula [46]. As illustrated in Fig. 3A, parallel analysis of three groups of Arabidopsis plants labeled respectively with $^{13}C$, $^{15}N$ and $^{34}S$, along with one unlabeled group, enables direct readout of the underlying elemental composition of detected metabolites [47]. For example, an isotopologues cluster was detected at RT of approximately 4.16 min, with *m/z* values of 420.0622 in unlabeled group, 432.0846 in $^{13}C$-labeled group, 421.04323 in $^{15}N$-labeled group and 426.03356 in $^{34}S$-labeled group, respectively (Fig. 3A). A closer examination of the mass peaks revealed that the *m/z* values were shifted according to the number of carbon (+12 = $C_{12}$), nitrogen (+1 = $N_1$) and sulfur (+6 = $S_3$) atoms. After having calculated the number of the labeled elements, the number of the remaining elements, such as hydrogen, phosphate and oxygen atoms, can easily be deduced from the unlabeled *m/z* 420.0622 using a *de novo* elemental composition calculation. Consequently, the elemental composition of this metabolite was unambiguously identified as $C_{12}H_{23}N_1O_9S_3$. To further demonstrate the power of multi-isotope labeling in assisting elemental composition assignments, *de novo* elemental composition calculation was performed from the unlabeled *m/z* 420.0622 without constraining the number of labeled elements. This resulted in the identification of 357 possible elemental



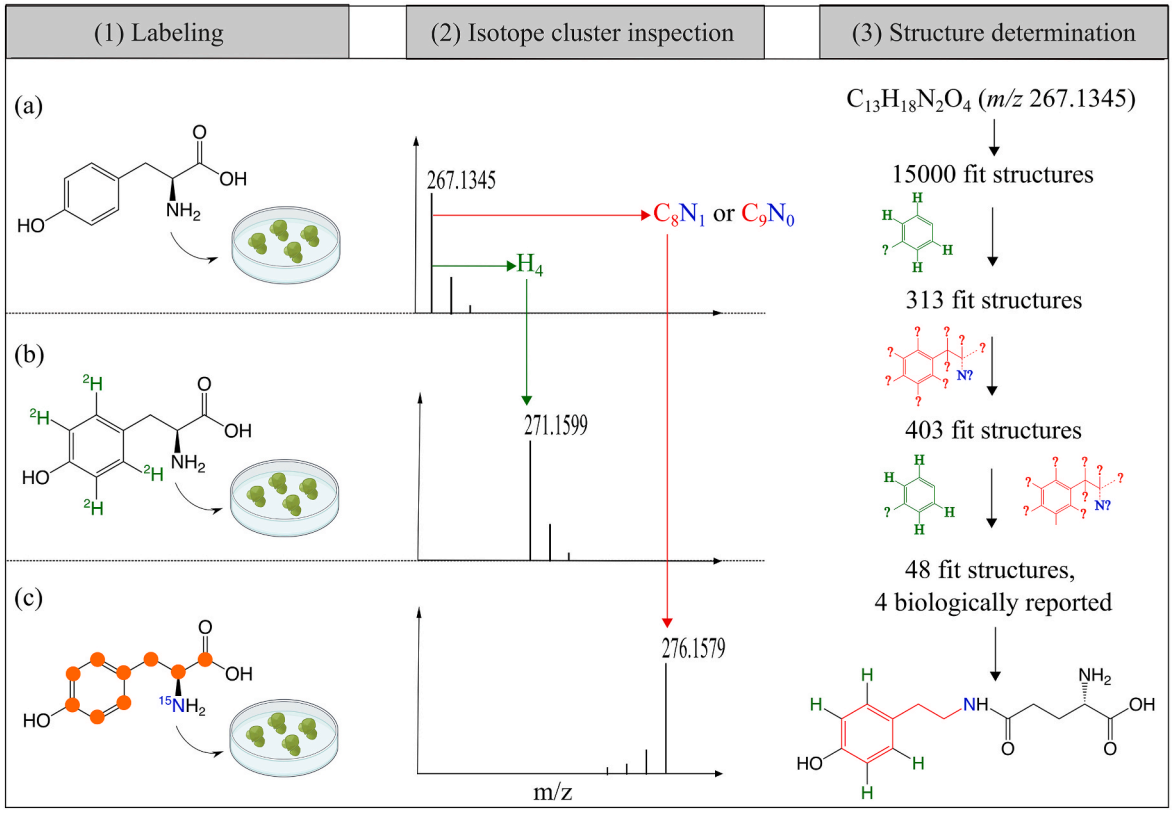




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**Fig. 3.** Representative workflows of the use of stable isotope labeling (SIL) for metabolite annotation. (**A**) An example of a global SIL workflow for elemental composition determination. (1) Four groups of *Arabidopsis* plants underwent SIL: unlabeled group A was grown under $^{12}CO_2$ (a), $^{13}C$-labeled group B was grown under $^{13}CO_2$ (b), $^{15}N$-labeled group C was administered with $^{15}NH_4^{15}NO^3$ (c), and $^{34}S$-labeled group D were administered with $Mg^{34}SO$ (d). (2) An isotopologues group with an unlabeled *m/z* value of 420.04622 was used to demonstrate the efficacy of SIL in elemental composition determination. (3) The flow chart illustrated the stringency in elemental composition determination. The number of labeled atoms, discerned from the labeling information, was utilized to narrow down the possible elemental compositions. Adapted with permission from Ref. [47]. (**B**) An example of a positionally-labeled tracer-based SIL workflow for metabolite structure elucidation. (1) Three groups of Lemna plants underwent SIL: group A was fed with unlabeled tyrosine (a), group B with tyrosine where all hydrogen atoms on the aromatic ring were labeled (b), and group C with tyrosine where all carbon and nitrogen atoms were labeled. (2) An isotopologues group with an unlabeled *m/z* value of 267.1345 was used to demonstrate the efficacy of SIL in metabolite structure elucidation. (3) The flow chart illustrated the stringency in metabolite structure annotation. The structural information from the labeling groups was utilized to narrow down the candidates found in a database (i.e., SciFinder). Adapted with permission from Ref. [49].

compositions with a mass tolerance of 10 ppm. In contrast, limiting the *de novo* calculation to 12 carbon atoms narrowed the list down to 11 possible elemental compositions. Further *de novo* calculation with fixed isotope-labeled elements ($C_{12}$, $N_1$, and $S_3$) ultimately led to the identification of a single unique elemental composition [47] (Fig. 3A). Hegeman et al. (2007) modeled the numbers of assignable formulas as a continuous function of accurate mass (with 3 ppm mass tolerance window), both with and without labeled atom constraints. These models were compared with calculations of formula counts for thousands of formulas from a verified metabolite database. Out of approximately 5000 formulas, double SIL ($^{15}N$ and $^{13}C$) with atom constraints resulted in unique assignments in 87 % of instances, compared to 20 % with the typical unconstrained approach [48]. It is crucial to emphasize that uniform labeling is necessary to accurately infer the correct number of elements as partial labeled mass peaks would lead to wrong element counting. In addition, it is important to note that high-resolution accurate mass measurement is crucial for determining elemental composition. This ensures that masses are measured for a single species, rather than for unresolved or partially resolved peaks, allowing for the assignment of a unique formula, or limiting the number of theoretically assignable formulas.

## 6. Structure elucidation

Often, an elemental composition alone is not sufficient for metabolite structural elucidation due to the presence of multiple structural isomers [14,20,49]. Tracer-based SIL provides unique advantages for metabolite identification from a pathway-centric perspective [25]. It allows to reduce metabolomics datasets to focus on metabolites derived from the tracer (or precursor) and specific metabolic pathways [50,51]. Uniformly-labeled tracers (i.e.,tracers with all atoms of a given element are replaced by stable isotopes) are widely used due to their potential to probe all the tracer-derived metabolites at once. For example, to explore the biotransformation products of deoxynivalenol (DON) in human body, both uniformly $^{13}C$-labeled DON and unlabeled DON were administered to cell cultures of a liver carcinoma cell line (HepG2) and a human colon adenocarcinoma cell line (HT29). All DON-derived metabolites formed in the cell cultures would exist in both native and $^{13}C$-labeled forms, while non-DON-derived metabolites were exclusively in their native form. Thus, this characteristic allowed for the recognition of all DON biotransformation products. Using this approach, the authors identified nine DON-derived metabolites in both cell lines [52]. Similar studies involve the use of $^{13}C_6$-labeled ortho-quinone to detect all quinone reaction products in wines [53], and the use of $^{13}C_6$,$^{15}N_4$-labeled L-arginine or $^{13}C_6$,$^{15}N_2$-labeled L-lysine to identify ribosomally synthesized and posttranslationally modified peptides (RiPPs) with L-arginine or L-lysine as the starter unit for the fatty acyl groups [54]. In another study, a comparison between $^{15}N$ labeled and unlabeled cell extracts of Nostocsp. strain UIC 10630 enabled the detection of all nitrogen containing metabolites in the strain. Among them, four metabolites with the same number of nitrogen atoms as predicted in the biosynthetic gene clusters (BGCs) were identified, of which two were novel [23].

Despite the widespread use of uniformly labeled tracers, positionally-labeled tracers (i.e., tracers with only specific atoms replaced by stable isotopes) are more powerful in metabolite identification [25,50]. The rationale is that the prior knowledge of the precursor structure could be used to reduce the search space when identifying the precursor-derived metabolites [50]. The substructure of a tracer can be tagged with stable isotopes, and in reactions where the tracer is modified, these labels are only transferred to the specific reaction products which (partially) contain the labeled substructure. This, in return, facilitates tracking and identification of these products [55]. Recent examples include the use of ring-[$^{13}C_6$]-labeled phenylalanine to identify phenylpropanoids in *Arabidopsis* [50], and the use of ring-[$^{13}C_6$]-labeled phenylalanine and ring-[$^{13}C_6$]-labeled tyrosine to identify phenylalanine- and tyrosine-derived metabolites in sorghum tissues, respectively [56,57]. Tagging different substructures of the tracer with unique and distinguishable labeling schemes can further enhance its potential to identify the tracer-derived metabolites. Following this principle, Feldberg et al. (2009) developed an approach named DLEMMA (Dual Labeling of Metabolites for Metabolome Analysis), utilizing a dual-labeled tracer for high-confidence metabolite identification [49,55]. For instance, three groups of giant duckweed (*Spirodela polyrhiza*) were fed with tyrosine: group A was fed with unlabeled tyrosine, group B with $^2H_4$-tyrosine (all unsubstituted hydrogen atoms on the aromatic ring were labeled), and group C with $^{13}C_9$,$^{15}N_1$-tyrosine (all carbon and nitrogen atoms were labeled). The extracts were then analyzed separately with LC-MS for metabolite identification (Fig. 3B). A detected isotopologues cluster was used to demonstrate the effectiveness of DLEMMA for metabolite annotation. In the example, the results showed that one metabolite of *m/z* 267.1345 exhibited the labeling patterns of +4 (*m/z* 271.1599, Group B) and +9 (*m/z* 276.1579, Group C) (Fig. 3B). The elemental composition of this metabolite was assigned based on its unlabeled accurate *m/z* value and natural isotope pattern. However, database search (i.e., using SciFinder) unveiled over 15,000 candidates for the elemental composition $C_{13}H_{18}N_2O_4$. Given that this metabolite was labeled with 4 deuterium in Group B, suggesting the presence of an aromatic ring like tyrosine, database search with this substructural information thus reduced the number of the candidates from 15,000 to 313. Likewise, the number of candidates was narrowed down to 403 considering the substructural information obtained from group C. By using the substructural information from both group B and C, the number of candidates was substantially reduced to 48, with 4 being reported in biological samples. The final identification of this metabolite was confirmed based on MS/MS information (Fig. 3B). With this approach, 60 tyrosine-derived metabolites were successfully identified [49]. The same approach has been applied to identify tyrosine-derived metabolites in tomato [49], phenylalanine-derived metabolites in *Arabidopsis* [58] and cannabigerolic acids in *H. umbraculigerum* [59]. While the examples provided above are about metabolite identification, the use of SIL for lipid identification is more challenging. This is because the mass shift associated with the labeled atoms is often insufficient to distinguish the labeled lipid from other lipids within the lipidome because most lipids occur within a narrow mass range. To address this challenge, Reimers et al. (2023) developed a dual-isotope labeling approach that used an equimolar mix of two different deuterated arachidonic acids (e.g., $^2H_5$- and $^2H_{11}$-arachidonic

acid). These arachidonic acids were equally incorporated into the HT-1080 human cells, producing mass spectra featuring distinctive doublet or triplet peaks. This unique labeling signature enables the robust detection and identification of arachidonic acid-derived lipids with high confidence [60]. The efficacy of metabolite identification through positionally-labeled tracer depends on both the selection and labeling of the tracer. Tracer selection should be guided by the identification of the metabolic pathways critically for addressing the biological question at hand [25]. In terms of labeling, it is crucial to cover as many substructures of the tracer as possible while ensuring that different substructures can be clearly distinguished by distinct labeling schemes. One limitation of tracer-based approach is its targeted nature, restricting the identification to specific classes of metabolites.

In addition to tracer-based SIL, global SIL can also be used for metabolite structural elucidation. The mass shifts associated with labeling can assist the interpretation of MS fragmentation spectra by revealing the number of labeled atoms in each fragment. This information enhances the interpretation of mass spectral fragmentation and the elucidation of molecular sum formulas, both of which are crucial for narrowing down possible chemical structures of yet unidentified compounds. Alongside *in vivo* SIL, in vitro chemical isotope labeling (CIL) is another popular approach for metabolite identification [22,61]. Biological samples are "in vitro" labeled in CIL using a reagent designed to target a specific chemical functional group. Ideally, all the metabolites containing the targeted functional group react with the reagent to form the labeled derivatized metabolites, allowing for their recognition and identification [62–64].

## 7. SIL-assisted metabolite identification in NMR experiments

While MS coupled to chromatography is the dominant technique in metabolomics studies, nuclear magnetic resonance (NMR) spectrometry remain a gold standard for metabolite structure elucidation [65]. SIL has also been applied in NMR-based metabolomics studies to aid in metabolite identification. Isotopically labeled metabolites generate unique NMR signals compared to their unlabeled counterparts due to changes in nuclear spin, enabling the detection of isotopically enriched metabolites by comparing the NMR spectra of labeled and unlabeled samples [66]. Furthermore, unlike MS, NMR allows researchers to pinpoint the precise positions of labeled atoms within molecules by analyzing the spectral patterns and chemical shifts of NMR peaks. This capability provides critical insights into specific metabolic pathways, especially in cases where the position of the labeled atom reveals important information about its metabolic origin [67–69]. Rationally, 1D $^{1}$H (proton) NMR is the most common NMR experiments due to its high sensitivity. However, the molecular complexity often leads to a large degree of spectral overlap observed in 1D $^{1}$H NMR, making metabolite identification challenging. While heteronuclear NMR experiments, which utilize the greater spectral dispersion of $^{13}$C and $^{15}$N, can overcome this issue, they typically have lower sensitivity due to the low natural abundance of these NMR-active nuclei [70–73]. The use of SIL could largely improve the sensitivity and reduce the spectral overlap, allowing for more efficient statistical analysis and improved database matching for metabolite identification [71]. Although the principles of using SIL in MS- and NMR-based studies for metabolite identification differ, the two techniques are complementary and can be cross validated. For instance, combining NMR and IROA LC-MS experiments enables more confident identification of truly unknown metabolites [71]. It is important to note that, as the primary focus of this review is on the application of SIL in MS-based metabolite identification, the applications of SIL in NMR-based metabolite identification are only briefly mentioned here. Readers interested in a deeper exploration of SIL in NMR applications are directed to other sources [71,73,74].

## 8. Software tools

A myriad of free open-source software tools has been developed for SIL-assisted metabolite annotation, and an overview of these tools is provided in Table 1. These tools employ different algorithms and possess specific nuances for metabolite annotation (Table 1). Nevertheless, they collectively apply the same principle that the native and labeled mass features exhibit unique mass increments and highly characteristic artificial labeling patterns to automatically detect all possible isotopologue clusters. The performance of some of the software have been compared elsewhere [32,75,76]. For users, selection of software tools depends on the overall objective and experimental design of their studies, as well as the user-friendliness of the software.

## 9. Conclusion

SIL has found extensive applications in MS-based metabolomics, significantly enhancing the precision and reliability of various analytical processes. It is widely used for metabolite quantification, providing accurate and absolute measurements by correcting for matrix effects and analytical variabilities, thus enabling more robust comparisons across different biological samples and conditions [77,78]. Additionally, SIL is integral to metabolic flux analysis, where it allows researchers to trace the flow of metabolites through biochemical pathways in real-time, offering insights into the dynamic regulation of metabolic networks under different physiological or pathological states [79,80]. Furthermore, SIL plays a crucial role in determining structure of the metabolic pathway and networks. The ability to identify and characterize novel metabolic pathways by tracking the distribution of heavy atoms from isotopologues through the metabolic network reveals previously unrecognized connections between metabolites, thereby contributing to a deeper understanding of complex biological systems [55,81,82].

In this review, we highlight the potential of SIL for metabolite annotation in MS-based metabolomics studies by providing an up-to-

**Table 1**
Overview of software tools developed for SIL-assisted metabolite annotation.

| Name | Function[a] | Pooled or Separate Samples | Global or Tracer-based SIL | Language | Reference |
|---|---|---|---|---|---|
| X$^{13}$CMS | 1, 4 | Separate | Both | R | [84] |
| geoRge | 1, 4 | Separate | Both | R | [85] |
| El-MAVEN | 1, 4 | Separate | Both | C++ | [86] |
| MetTracer | 1, 4 | Separate | Both | R | [32] |
| SIAMA | 1, 4 | Separate | Both | Fortran | [87] |
| HiTIME | 4 | Pooled | Tracer-based SIL | C++ | [88,89] |
| MetExtract II | 1, 4 | Pooled | Both | Python | [90] |
| 2H-SIAM | 4 | Pooled | Tracer-based SIL | Visual Basic | [91] |
| MS-DIAL | 1, 2 | Separate | Global SIL | C[b] | [92] |
| Pave | 1, 2, 3 | Separate | Global SIL | MATLAB | [27] |
| CPExtract | 4 | Pooled | Tracer-based SIL | Python | [93] |
| MISO | 1, 4 | Separate | Both | R | [94] |
| HiResTEC | 1, 4 | Separate | Both | R | [95] |
| PODIUM | 4 | Separate | Tracer-based SIL | R | [50] |
| D-Tracer | 1 | Pooled | Tracer-based SIL | Python | [60] |
| IsoMS | 4 | Pooled | Tracer-based SIL[b] | R | [96] |
| MS-IDF | 4 | Pooled | Tracer-based SIL[b] | MATLAB | [97] |

[a] Functions: (1) Credentialing; (2) Count labeled atoms or assign elemental composition; (3); Annotate degenerate features; (4) Detect tracer-derived features.

[b] Because the principle of chemical isotope labeling (CIL) is the same as that of tracer-based stable isotope labeling, CIL is viewed as tracer-based SIL here.

date summary of different innovations and applications in this field. The examples discussed demonstrate the effectiveness of SIL for metabolite annotation across different aspects, including biological feature recognition, degenerate feature annotation, elemental composition determination and metabolite structure elucidation. Leveraging the particularly developed software tools, SIL maximizes the true biological information extracted from complex metabolomics datasets and enhances the interpretation of the information, leading to the discovery of novel metabolites and pathways. This review primarily focusses on the use of SIL for metabolite annotation in LC (GC)-MS-based metabolomics, while SIL finds utility in metabolite identification within mass spectrometry imaging (MSI) studies [16,83]. However, due to the absence of retention time information, accurately and efficiently pairing an unlabeled peak with its labeled counterpart in MSI is generally challenging. Despite the existing challenges and limitations associated with different SIL approaches, with the increasing availability of stable isotope-labeled compounds and samples, SIL is anticipated to become a routine technique in future metabolomics, continuing to play a significant role, particularly for metabolite annotation.

## CRediT authorship contribution statement

**Yonghui Dong:** Writing – review & editing, Writing – original draft, Conceptualization. **Liron Feldberg:** Writing – review & editing. **Asaph Aharoni:** Writing – review & editing. **Uwe Heinig:** Writing – review & editing, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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