Title:

Recent Advances in Stable Isotope-Enabled Mass Spectrometry-Based Plant Metabolomics

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Abstract:

Methods employing isotope labeled compounds have been an important part of the bioanalytical canon for many decades. The past fifteen years have seen the development of many new approaches using stable (non-radioactive) isotopes as labels for high-throughput bioanalytical, ‘omics-scale’ measurements of metabolites (metabolomics) and proteins (proteomics). This review examines stable isotopic labeling approaches that have been developed for labeling whole intact plants, plant tissues, or crude extracts of plant materials with stable isotopes (mainly using ^2H , ^{13}C , ^{15}N , ^{18}O or ^{34}S). The application of metabolome-scale labeling for improving metabolite annotation, metabolic pathway elucidation, and relative quantification in mass spectrometry-based metabolomics of plants is also reviewed.

Introduction:

Researchers engaged in the application of metabolomics in a wide range of disciplines have identified both analytical and informatics limitations that, when addressed, will yield a dramatic improvement in information quality and quantity [1]. Grand challenge areas have emerged from the metabolomics community for methodological improvement including: 1) metabolite annotation; 2) metabolite quantification; 3) metabolic pathway elucidation; 4) high resolution spatial localization; 5) temporal analysis including metabolite turnover and flux [2]. In this review we will examine how stable (non-radioactive) isotopes have been used as mass labels for mass spectrometry (MS)-based metabolomics in order to make analytical improvements in these challenge areas. Most often, metabolomics research uses MS, nuclear magnetic resonance (NMR) spectroscopy, or some combination of the two methodologies, although other spectroscopies and approaches are occasionally employed. Stable isotopic labeling techniques are also employed for NMR [3]; it is worth following both MS and NMR disciplines as procedures used for labeling can be found in either. General MS-based metabolomics approaches and concepts have been reviewed elsewhere [4].

The metabolomic analysis of plants and plant-derived materials particularly benefits from isotope-enabled methodology for several reasons. First, plant metabolomes are large and chemically diverse and they are under-represented in public database resources, which are often more heavily populated with clinical, pharmaceutical, or petrochemical related compounds. Plant metabolite annotation can, therefore, be difficult and error prone and thus benefits from the additional information provided by using isotopic labeling strategies. Furthermore, quantification can be much more error prone due to interference from complex

plant matrices much of which can be controlled using stable isotopes. Second, it is possible to provide a plant with relatively inexpensive isotopically labeled nutrients and completely substitute a specific element with nearly 100% enrichment of a stable isotope. A number of plant metabolomics specific reviews have been published fairly recently [1,5].

Metabolic turnover and flux analysis are important techniques that rely heavily on labeling information. Metabolic flux analysis can be performed in many different ways depending on the types of analytical techniques used and the constraints and assumptions employed. We will not cover this in depth here, as there are excellent recent reviews on this topic [6,7].

In the **Box** are definitions for commonly used terms in the field of metabolomics and mass spectrometric analysis of stable isotope labeled metabolites. Here we will examine recent work using various different stable isotopic labeling strategies to improve the depth and precision of plant metabolomics studies with a particular focus on challenges associated with metabolite annotation and quantification and metabolic pathway elucidation.

Using Isotopes as Labels

Stable isotopic labeling is sometimes confused with radioactive (unstable) isotopic labeling (using ^3H , ^{14}C , ^{35}S , ^{32}P or ^{33}P), which has historically been used to great effect for metabolic pathway elucidation and turnover or flux analysis. Use of radioisotopes has limitations in its application due to biological perturbations [8], safety concerns and incompatibility with MS and NMR. Stable isotopes such as ^2H , ^{13}C , ^{15}N , ^{18}O , and ^{34}S , which have one or two more neutrons than the most abundant naturally occurring isotopes (^1H , ^{12}C , ^{14}N , ^{16}O , and ^{32}S) have been widely used as labels for MS analysis as they can easily be

distinguished by mass. While radioisotopes are detectable as signal from their radiation without any contribution of noise from unlabeled compounds, stable isotopically labeled compounds are detected with the same sensitivity as their unlabeled counterparts, which results in relative advantages and disadvantages between the two labeling types. The comparison between radioisotope labels and stable isotope labels and the complementarity of their utility has been reviewed recently [9].

Labeling Metabolites with Stable Isotopes at a Metabolomics Scale

Simultaneous introduction of stable isotopes into large numbers of metabolites can be accomplished using either *in vivo* or *in vitro* approaches. **Figure 1** summarizes common labeling approaches in these two categories. *In vitro* labeling strategies make use of metabolic labeling [10] where endogenous metabolism is used to propagate label from some entry point into and throughout the metabolic network. This is accomplished easily in plant cell culture by reformulation of growth media to include labeled nutrients [11].

In several *in vivo* labeling approaches (see **Figure 1, A**) an intact plant is provided with labeled nutrients (such as $^{13}\text{CO}_2$ or K^{15}NO_3) normally obtained from the environment or growth medium in unlabeled form. With this method labeling can be performed in bursts, for a short time, or for longer given adequate growth chamber equipment. While different isotopes of the same element at the same position within a molecular structure may be regarded as chemically indistinguishable (having identical electronic configurations), differences in mass due to different numbers of neutrons between isotopes alter the frequencies of molecular vibrations resulting in different rates for some chemical transitions (bond breaking/forming and rehybridization), or what is more generally referred to as

‘isotope effects’ [12]. The magnitude of isotope effects for ^{13}C , ^{15}N , ^{18}O , ^{34}S are small enough that they generally do not either perturb the biological system (when compared with unlabeled) or have significant analytical consequences within typical error of metabolomics measurements. Deuterium (^2H) labeling on the other hand can cause significant isotope effects and can be toxic to the organism depending on the chemical form incorporated and the isotopic enrichment [13] and thus requires proper considerations of these limitations.

Labeling with ^{13}C is most generally useful due to the omnipresence of carbon in metabolites, although ^{15}N has been used for metabolic studies of nitrogen metabolism [14]. Comprehensive metabolic labeling of plants with ^{15}N is facile and has been used widely for quantitative proteomics [15] and protein turnover [16,17]. Labeling with $^{13}\text{CO}_2$ typically requires an enclosure to maintain an atmosphere of $^{13}\text{CO}_2$ around the plant. Labeling can be accomplished on a fairly short term (2 hours) using a simple enclosure [18]. Labeling with $^{13}\text{CO}_2$ can also be accomplished under field conditions for pulse-labeling experiments for organisms as large as trees [19]. Growth enclosures have been constructed for longer term labeling experiments that require additional functionality to regulate temperature, humidity, light and remove ethylene and other volatile organic compounds from the chamber that can buildup over time and adversely affect plant growth [20–26]. Our laboratory has a website (<http://13carbon.com>) to share information regarding chambers that we have developed for this purpose.

Another mode of *in vitro* labeling (see **Figure 1, B**) involves cutting tissue and applying a solution containing a labeled compound to the cut site where it is taken up through the vasculature or absorbed directly and metabolized. This typically does not result in high specific activity labeling, but does provide the opportunity to introduce metabolic

intermediates that can be critical for pathway elucidation rather than relying on normal modes of environmental nutrient uptake that are limited to mineral nutrients and CO₂. This approach has been used with soybean nodules [27], *A. thaliana* leaf disks [28,29] and detached leaves [30,31], and *Vitis vinifera* berries [32].

In vitro labeling is performed after metabolites have been extracted from plant tissue and is accomplished by chemical derivatization with an isotopically labeled reagent, or by isotope exchange with labeled solvent. Chemical derivatization can be accomplished in many ways with different benefits and detractors. Two examples are shown in **Figure 1 C** and **D**. In **C** amines are derivatized with labeled methyl [¹³C₂]acetimidate resulting in cationic derivatives, which may help increase sensitivity in positive ionization mode electrospray ionization [33]. In **D** a range of functional groups are modified by N-methyl-N-*tert*-butyl [²H₆]dimethyl-silyl trifluoroacetamide (MTBSTFA-*d*₆) to yield volatile *tert*-butyl [²H₆]dimethyl-silyl (TBDMS-*d*₆) derivatives for GC-MS analysis [34]. Deuterium isotope effects can cause, in addition to biological toxicity, significant shifts in chromatographic retention; deuterated and undeuterated TBDMS derivatives, for example, are nearly baseline-resolved. Chemical derivatization strategies are advantageous because they can be used to target specific chemical classes such as polyphenols [35], carboxylic acids, and amines [33,36].

Isotope exchange is another *in vitro* labeling approach that targets specific functionalities and can help with chemical classification. Isotope exchange reactions occur between metabolites and isotopically labeled solvent such as D₂O (²H₂O) and usually involve hydrogen/deuterium (H/D) exchange. It is possible to exchange ¹⁸O as well, and while H₂¹⁸O is costly, ¹⁶O/¹⁸O exchange is used in proteome quantitation [37]. H/D exchange is

accomplished by incubating metabolites at a pH or temperature that facilitates exchange with labeled solvent but that can be readjusted to stop the back-exchange reaction from occurring when deuterated metabolites are dissolved in proteated water (H₂O). Deuterated standard compounds have been made using this approach for many decades [38], and have been employed on a metabolomics scale for labeling polyphenolic compounds via an electrophilic aromatic substitution reaction [39].

Stable Isotopes and Metabolite Annotation

Stable isotopic labeling has a number of different benefits for metabolite annotation and identification [40]. Natural abundance stable isotope abundance has been used to constrain elemental composition assignment to augment assignment based on accurate mass measurement without adding exogenous isotopes. The shape of the isotopic envelope can be calculated for multiple elemental compositions matching the accurate mass within error to find the best match to the measured spectral envelope [41]. This is particularly helpful for compounds that have elements with diagnostic isotopic patterns (S, Cl, Br, some transition metals [42]); unfortunately natural abundance hydrogen, carbon, nitrogen and oxygen only have a low abundance of heavy isotopes and do not contribute very much predictive power using this approach. With stable isotope enrichment it becomes possible to compare labeled and unlabeled metabolites and effectively count the number of labeled atoms in each molecule to decrease the ambiguity in elemental composition assignment in MS [43,44] and MS/MS [45]. A modification of this approach uses the unique isotope pattern of 5% ¹³C vs. 95% ¹³C to generate a characteristic Isotope Ratio Outlier Analysis (IROA) ‘smile’ pattern that is easily identified for annotation and relative quantification [46]. An added benefit of

annotation strategies using metabolic labeling is that peaks from labeled compounds can be unambiguously identified as metabolites vs. chemical background or contaminants [47].

Labeling strategies have also been employed for production of labeled standard compounds for analysis of natural products that are not commercially available such as amanita toxins [48] or polyunsaturated fatty acids [49]. Lastly, H/D exchange in polyphenolics has been shown to occur at different rates depending on the regiochemistry and the presence of glycosylation and acylation; this information can be used to differentiate between structural isomers in the absence of standard compounds or suitable material for NMR [50].

Metabolic Network Elucidation and Tracers

While radioisotopes were critically important for the initial elucidation of core metabolic pathways, stable isotopes are now being used routinely to extend that knowledge [51]. Dual Labeling of Metabolites for Metabolome Analysis (DLEMMA) is an effective strategy that uses two differently labeled forms of a metabolic tracer (eg. [$^2\text{H}_5$, $^{13}\text{C}_6$]phenylalanine and [$^{13}\text{C}_6$]phenylalanine) and its unlabeled form to examine differences in metabolism between two different genotypes [52]; a similar approach was used to examine sources of indole-3-acetic acid production [53]. Untargeted tracer experiments have been used extensively in various species and tissues types [29,54–57].

Metabolome-Scale Quantification

Absolute quantities (moles/L) require the use of standard compounds to generate response curves both to define a range for signal to concentration linearity and to provide a response factor to convert raw signal into a concentration within the linear range. With

complex mixtures internal standards are often needed to control for matrix effects. Isotope dilution, where a sample is spiked with a known concentration of a stable isotope labeled internal standard has been used for many decades in mass spectrometric quantification because it provides an ideal internal control with a standard for absolute quantification. This approach depends on labeled standard availability. For many applications absolute quantification is too laborious, limited by the lack of standard compounds, or not relevant due to the desire to observe and quantify new and unknown chemicals. Relative quantities (eg. sample A has 3 times more of compound X than sample B) are often sought in these cases but can also be erroneous in the absence of internal controls due to complex sample matrix effects.

Stable isotopic metabolic labeling provides an ideal control for relative quantification on a metabolomics scale because relative quantities are calculated from the labeled (heavy) and unlabeled (light) peak intensities for every metabolite [58]. **Figure 2** illustrates several workflows for relative quantification using *in vivo* and *in vitro* labeling approaches. In each case relative quantities are derived from the relative intensities of heavy and light peaks with excellent internal control for matrix effects. Studies have demonstrated approaches for applying *in vivo* labeling for relative quantification in untargeted metabolomics workflows that also improve annotation [44,54]. MetExtract [47] and X¹³CMS [59] are publically available software packages for relative quantification analysis of this sort. **Figure 2** shows a variant of metabolic labeling where the labeled standard from an exogenous source is spiked into many experimental samples. Experimental samples and the spiked labeled standard, in this case, do not have to be obtained from the same biological source. For example, IROA is often employed using a labeled yeast extract spiked into samples from other species [60].

While this strategy has some advantages, one may miss large segments of metabolism that are not present in the labeled standard as the experimental and standard materials constitutionally diverge.

Conclusions

Stable isotopic labeling strategies have been used in many creative ways to meet challenges in metabolomics. Here we have reviewed the major recent advances in plant stable isotopic labeling strategies as well as approaches for using labeling to improve annotation, metabolic pathway elucidation, and relative quantification for plant metabolomics. Obstacles for broader community implementation are the lack of accessible tools for some labeling experiments and for data analysis and interpretation. Further development and distribution of chemical standards and reagents, growth apparatus and easily employed bioinformatics tools will help make stable isotope enabled metabolomics approaches more accessible to biologists and the metabolomics community.

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Figure Legends:

Figure 1. Stable Isotopic Labeling Strategies.

In vivo labeling strategies are shown in panels **A** and **B**. Panel **A** describes metabolic labeling of intact plants by incorporation of a labeled element from a labeled form of naturally utilized nutrient source such as $^{13}\text{CO}_2$ or K^{15}NO_3 . Metabolic labeling can also be accomplished using parts of plants or dissected tissues soaked in a labeling medium containing a labeled metabolic intermediate as shown in panel **B**. *In vitro* labeling strategies are shown in panels **C**, **D** and **E**. Panel **C** shows the reaction of a primary amine with labeled or unlabeled methyl acetimidate to provide an amine-specific isotopic tagging strategy. Panel **D** shows a more general tagging strategy using (N-methyl-N-tert-butyldimethylsilyl)-trifluoroacetamide (MTB-STFA) to generate volatile tert-butyldimethylsilyl (TBDMS) derivatives that can be analyzed by GC-MS. Panel **E**. shows hydrogen/deuterium exchange of the aromatic ring protons of phenolic compounds with solvent via an electrophilic aromatic substitution reaction.

Figure 2. Stable Isotope-Enabled Relative Quantification Workflows.

Three workflows are presented for ‘*in vivo* labeling’, ‘*in vitro* labeling’ and introduction of a ‘spiked labeled standard.’ The *in vivo* labeling workflow shows how a plant (experimental or control) can be grown with natural abundance nutrients **A** and the another (control or experimental) with nutrients formulated with a heavy isotope **B** and then mixed, extracted and analyzed by MS. It is often desirable to perform a reciprocal labeling experiment where the experimental and control samples are reversed with regard to which label is used so that any isotope effects, or more likely differences in nutrient formulation or growth environment,

will become apparent. The ***in vitro* labeling** workflow shows a similar workflow to accommodate relative quantification using *in vitro* labeling. Note that fewer steps are internally controlled in the *in vitro* workflow because the samples are combined later in the process. The extraction process is, for example internally controlled in the *in vivo* but not *in vitro* workflow, so more care needs to be taken to make the uncontrolled steps as consistent as possible. The **spiked labeled standard** workflow shows another approach where the labeled internal standard from some biological source, **S**, is introduced into many test samples (**A**, **B**, ...) and the relative abundance information is derived from the ratio of ratios.

Box:**Definitions:**

Isotopes are atoms of the same element with different numbers of neutrons so that different isotopes of the same element have different masses.

Isotopic abundance indicates the relative amount of an isotope present in a chemical; this often refers to a specific atom or atoms in a molecular structure.

Isotopic envelope refers to the complete set of mass spectral peaks observed for all of the isotopomers of a chemical.

Isotopomers refer to chemically identical molecules differing only in their isotope composition. Each molecule with n carbon atoms has a total of 2^n possible stable carbon isotopomers.

One **mass isotopomer** is the set of isotopomers having the same molecular mass. Each molecule with n carbon atoms has $n + 1$ carbon mass isotopomers.

Metabolic flux refers to the net flow of substance (moles/time) through the reactions and compartments or metabolic processes.

Metabolic labeling refers to methods where the endogenous biosynthetic apparatus of living cells is used to incorporate chemical or isotopic tags into biomolecules.

Metabolome is a term conceptualized by extrapolation from ‘genome’, and refers to the complete set of metabolites in a biological system often as they correspond to a complete set of genes.

Metabolomics is the characterization of the metabolome.

A **monoisotopic peak** is a peak in an isotopic envelope that measures a single isotopomeric form of a chemical; this usually refers to the lowest m/z peak of the envelope, which (if populated) measures only isotopomers completely composed of the lightest stable isotopes of each element at every atom (ie. ^1H , ^{12}C , ^{14}N , ^{16}O , ^{31}P and ^{32}S).

Natural isotopic abundance refers to the variable composition of isotopes in naturally occurring substances. It is typically dominated by the lightest stable isotopes of each element and has slightly different amounts of the heavier stable isotopes depending on cosmic origin and depletion and enrichment from isotope effects that depend on a given substance’s chemical history.

Stable isotopes are atom types of the same element with nuclear configurations that do not break down spontaneously through the process of radioactive decay.

Tracers are labeled chemicals that are used to help understand the reactions that occur in metabolism.

Graphical abstract:

FIGURE 1.

In Vivo Labeling

A.

^{13}C -metabolic labeling
from $^{13}\text{C}\text{O}_2$ using enclosed
plant growth chamber

^{15}N -metabolic labeling
from $^{15}\text{NO}_3^-$ and/or
 $^{15}\text{NH}_4^+$ using hydroponics

Every atom of the labeled
element is substituted with
the stable isotope in all
metabolites to different
degrees of enrichment
depending on metabolic flux,
growth rate, and labeling time

B.

^{13}C or ^{15}N -metabolic labeling
by treatment with exogenous
metabolic intermediate

incubate leaf or other tissue
in medium w/ labeled
compound

Every metabolite derived
from the labeled
compound will contain labeled
atoms of some enrichment
depending on metabolic
pathway utilization and flux,
and labeling time

In Vitro Labeling

C.

Fixed positive
charge increases
sensitivity in
LC-MS

D.

Labeled derivatives
are volatile and can be
analyzed by GC-MS

E.

Hydrogen/deuterium exchange

Extent and rate
of labeling can also
provide structural
information

FIGURE 2.