Chapter 7

Isotopomer Measurement Techniques in Metabolic Flux Analysis II: Mass Spectrometry

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Abstract

Mass spectrometry (MS) offers a sensitive, reliable, and highly accurate method for measurement of isotopic labeling, which is required for generating comprehensive flux maps using metabolic flux analysis (MFA). We present protocols for assessing isotope labeling in a wide range of biochemical species, including proteinogenic amino acids, free organic and amino acids, sugar phosphates, lipids, starch-glucose, and RNA-ribose. We describe the steps of sample preparation, MS analysis, and data handling required to obtain high-quality isotope labeling measurements that are applicable to MFA. By selecting target analytes that maximize identifiability of the key fluxes of interest, MS measurements of isotope labeling can provide a powerful platform for assessing metabolic fluxes in complex biochemical networks.

Key words Mass spectrometry, Metabolic flux analysis, Isotopomer, Proteinogenic amino acids, Sugar phosphates, Lipids, Starch, RNA, Ribose, GC-MS, LC-MS/MS

1 Introduction

The ability to quantitatively map intracellular fluxes using metabolic flux analysis (MFA) is critical for identifying pathway bottlenecks and elucidating network regulation in biological systems, especially those that have been engineered to alter their native metabolic capacities [1]. MFA experiments involve feeding isotopically labeled substrates to cells, tissues, or whole organisms and subsequently measuring the patterns of isotope incorporation that occur in intracellular metabolites or secreted products. Both mass spectrometry (MS) and nuclear magnetic resonance (NMR) can be used to quantify the relative abundance of different "isotopomers" (i.e., isotope isomers) associated with each measured biomolecule. MS provides a highly sensitive and accurate method for quantifying isotope incorporation, and has been increasingly used for MFA studies over the past decade. MS instruments are widely available and can be maintained and operated by individual labs, whereas NMR instruments are typically only available through

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shared user facilities due to their high cost and operational complexity. This consideration along with the reduced sensitivity and longer analysis times of NMR has likely contributed to the increasing popularity of MS [2]. It should be noted, however, that datasets produced by MS and NMR often provide complementary information, and MFA strategies that combine both measurement technologies have been applied to maximize flux identifiability in complex networks [3, 4].

A mass spectrometer is an instrument that measures the mass-to-charge ratio (m/z) of gas-phase ions and quantifies the abundance of each ionic species. For most low molecular weight analytes, the ionic charge is inevitably +1 or −1, so the instrument is essentially a mass analyzer capable of resolving ions that differ by only 1 Da or less. Because the presence of heavy isotopes results in a mass shift toward increasing molecular weight, MS can differentiate between "mass isotopomers" of the same ionic species, which have the same chemical composition but differ in the number of heavy atoms they contain (Fig. 1). The relative abundance of these mass isotopomers is represented by a "mass isotopomer distribution" (MID), which can be directly quantified using MS. If different metabolic pathways give rise to distinct patterns of isotope incorporation, MID measurements can be used to infer the relative fluxes through these pathways. As a result, mass isotopomer data obtained from MS in combination with direct measurements of extracellular uptake or secretion fluxes can be computationally analyzed to reconstruct comprehensive flux maps describing intracellular metabolism, which is the essence of tracer-based MFA.

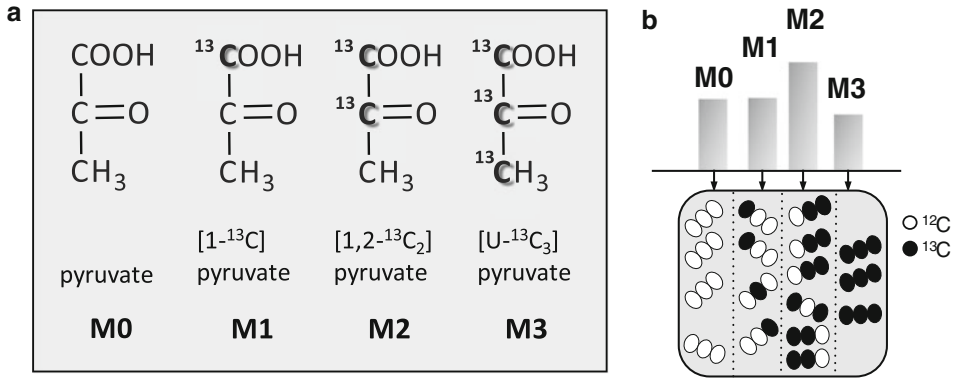

Fig. 1 Mass isotopomers and MID measurement. (**a**) Different mass isotopomers of pyruvate result from incorporation of ^{13}C and are denoted M0, M1, M2, etc. in order of increasing mass. (**b**) Mass isotopomer distributions (MIDs) represent the relative abundance of different mass isotopomers. MIDs are quantified using mass spectrometric methods such as GC-MS or LC-MS. These methods can determine the total number of heavy atoms present but not necessarily their position within the molecule

To reduce potential overlaps between the MIDs of isobaric species, stable isotope enrichment is best analyzed when only one chemical compound enters the MS at a time. However, biological samples typically contain mixtures of many compounds that have been extracted from a tissue or cell population. Although off-line processes for sample purification are available, the power and throughput of MS analysis is maximized when it is interfaced with an on-line method for separating samples into their pure components. Chromatographic methods such as gas chromatography (GC) and liquid chromatography (LC) can be directly interfaced with MS instruments, which enable high-resolution multicomponent separations to be achieved just prior to MS analysis (Fig. 2). These methods rely upon the partitioning of analytes between a mobile phase (gas or liquid) and a stationary phase (typically a solid or high-molecular-weight liquid coated on a solid support). The mobile phase sweeps the sample through a column that contains the stationary phase, and the chemical components elute from the column at different retention times (RT) depending upon their relative affinities for the two phases. Several excellent references are available that describe in further detail the operation of GC- and LC-MS instruments, as well as their application to mass isotopomer analysis and metabolomics [5–8]. In particular, one should note that the scan rate, sensitivity, mass resolution, and linear dynamic range of the instrument can vary greatly depending on the MS design (e.g., quadrupole, time-of-flight, ion trap, Orbitrap, etc.).

To date, most MFA studies have relied upon GC-MS measurements of proteinogenic amino acids to assess labeling of intracellular metabolite pools [2, 9]. This approach involves the administration of an isotope tracer for a sufficiently long period of time so that steady-state labeling of cellular protein is achieved. The amino acid building blocks can then be obtained by extracting and hydrolyzing the protein fraction from a biomass sample. The labeling patterns of these building blocks serve as proxies of the precursor metabolites from which they were biosynthetically derived (*see* Table 2). Because proteinogenic amino acids provide a stable and abundant pool that can be reliably sampled and analyzed, this has become the most popular method for isotopomer analysis in plant and microbial systems. However, there are limitations to this approach. First, because of the slow turnover of cellular protein, the labeling of proteinogenic amino acids occurs over a long time scale and can take several days to achieve steady state. Aside from the time and cost of these extended tracer experiments, this precludes the use of proteinogenic amino acids for assessing dynamic changes in metabolite labeling, such as is required for isotopically nonstationary MFA [10]. Second, the

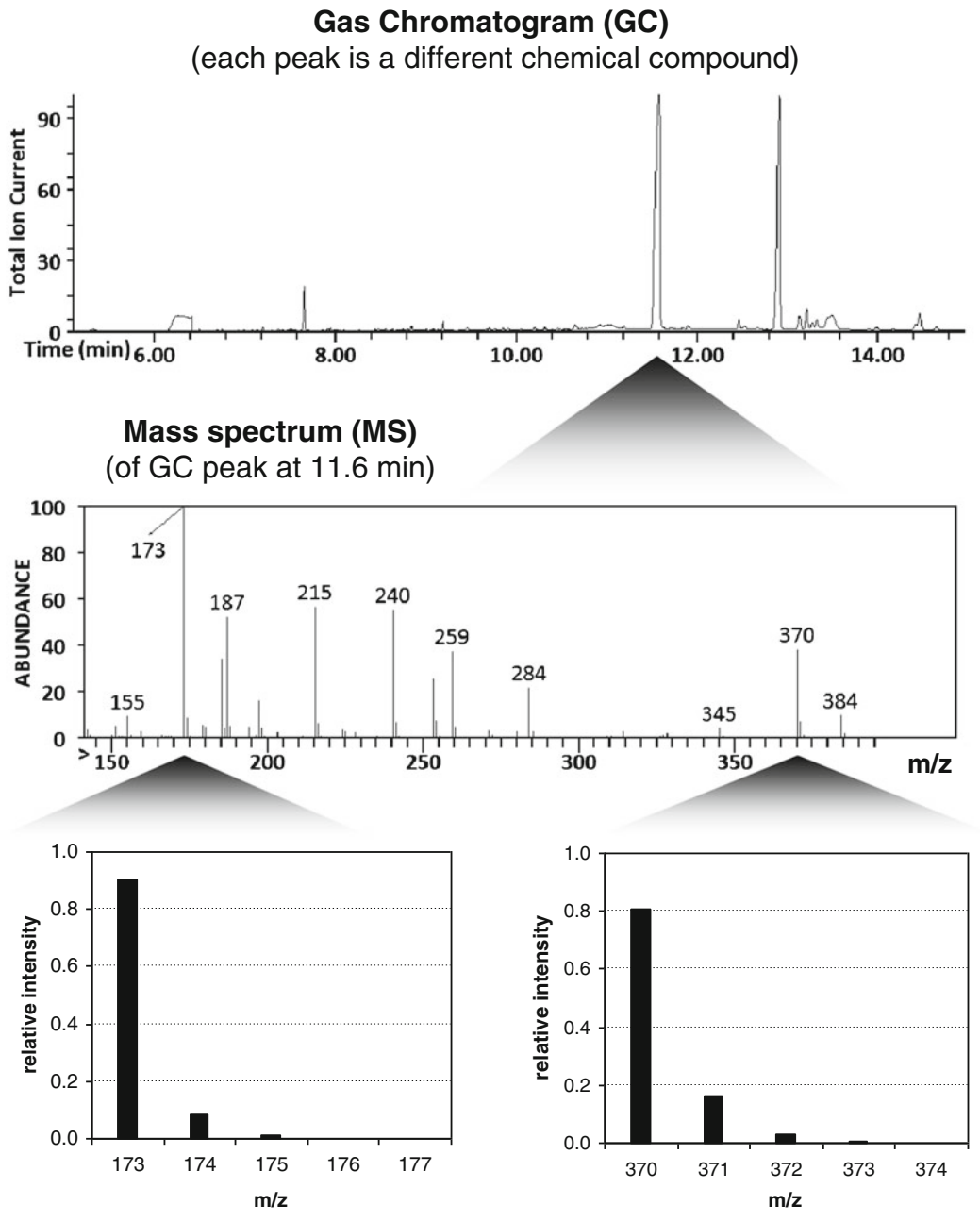

Fig. 2 Coupling GC with MS enables efficient purification and quantification of mass isotopomers in complex biological samples. Chemical compounds are separated according to their retention times using gas chromatography. The mass spectrum of each compound is detected on-line as it elutes from the GC column. The MS data can be used to identify each compound based on the fragment ions it produces. MIDs of specific fragment ions can be extracted from the full MS scans and used to quantify isotope labeling

highly compartmented nature of plant cells places a higher burden on the experimentalist to obtain labeling information that can be used to resolve parallel fluxes occurring simultaneously in separate organelles. This typically requires labeling data on carbohydrates, lipids, and other compounds that provide compartment-specific information complementary to that obtained from proteinogenic amino acids [11].

In light of the diverse array of MFA approaches and applications that are now being considered, we have assembled a collection of MS protocols applicable to several important classes of intracellular metabolites and macromolecules: proteinogenic amino acids, sugars from cellular RNA or starch, fatty acids from cellular lipid, and free intracellular metabolites (sugar phosphates, organic acids, amino acids, sugars). We do not attempt to provide protocols for extracting these compounds from plant tissues, as the appropriate methods can vary greatly depending on the tissue type. Typically, extraction of 20–100 mg of plant tissue is required to obtain sufficient material for MS analysis, depending upon the target compounds to be analyzed. For example, labeling in abundant pools such as protein and lipid can be readily determined by extracting 20 mg of plant tissue, whereas free metabolites may require 100 mg or more to achieve adequate signal-to-noise ratios. Furthermore, if free intracellular metabolites (e.g., sugar phosphates or organic acids) are to be extracted from cells, care must be taken to rapidly quench all enzymatic activities immediately upon sampling so that the measured MIDs will provide an accurate reflection of the in vivo labeling state [8].

Because GC-MS is a mature technology that has been used for qualitative and quantitative chemical analysis for over five decades, we restrict our attention mainly to "tried-and-true" GC-MS methods. However, GC-MS is only applicable to analytes that are volatile or can be made so through chemical derivatization, and which furthermore do not thermally degrade at typical GC temperatures. For this reason, compounds such as sugar phosphates and CoA-containing molecules are often analyzed using LC-MS, which has the further advantage of not requiring any chemical derivatization steps. For example, the protocol presented in Subheading 3.8 is based on an LC-MS method we have used for analysis of sugar phosphates in cyanobacterial extracts. It should also be noted that much recent progress has been made toward developing LC-MS methods for simultaneous analysis of metabolites from multiple chemical classes, and these "untargeted" metabolomics approaches are becoming increasingly important in MFA studies [12, 13]. However, the protocols provided in this chapter will mainly describe "targeted" methods that have been used to analyze groups of similar compounds within a single chemical class.

2 Materials

Aside from the MS instrument itself, very little in the way of specialized equipment or reagents is necessary to perform mass isotopomer analysis. Most of the required materials are readily available in typical biological or chemical laboratories (e.g., dry block heater, microcentrifuge and tubes, liquid sonicator, Pasteur pipettes, analytical evaporator, autoclave, and a vacuum source). Supplier and catalog information are provided below for specialty items (e.g., reagents for silylation or methoximation). Other items can be obtained from common laboratory supply companies. The chromatographic parameters provided below for GC-MS or LC-MS analysis should be treated only as initial suggestions. Typically, these will need to be further optimized depending on the target analytes of interest and the complexity of the sample matrix.

2.1 Hydrolysis of Cellular Protein to Amino Acids

1. 1 mL vacuum hydrolysis tube (Pierce #29550).
2. 6 N hydrochloric acid, HCl.
3. 13 mm syringe filter with PVDF membrane, 0.2 μm pore size.
4. 1 mL syringe.

2.2 Hydrolysis of Cellular RNA to Ribose

1. 1 mL vacuum hydrolysis tube (Pierce #29550).
2. 6 N hydrochloric acid, HCl.

2.3 Hydrolysis of Starch to Glucose

1. 0.1 M acetate buffer adjusted to pH 4.8.
2. Solution of amyloglucosidase and amylase (1 mg/mL each) in acetate buffer.
3. Ethanol.

2.4 GC-MS Analysis of Amino Acids

1. MTBSTFA +1 % TBDMCS, 1 mL ampules (Pierce #48927).
2. 2 mL amber glass injection vial.
3. 150 μL insert for injection vial.
4. Single quadrupole GC-MS with the following settings:
   (a) Injection volume: 1 μL.
   (b) Split injection mode, 10:1 ratio (*see* **Note 1**).
   (c) Column: HP-5MS (or similar), 30 m × 0.25 mm ID, 0.25 μm film.
   (d) Column flow: He at 0.75 mL/min (*see* **Note 2**).
   (e) Inlet temp: 270 °C.
   (f) Interface temp: 300 °C.
   (g) Temp profile: 150 °C for 2 min, ramp at 5 °C/min to 280 °C, hold at 280 °C for 2 min (30 min run time).
   (h) MS settings: electron impact (EI) ionization, full-scan detection (100–500 *m/z*), 5 min solvent delay.

2.5 GC-MS Analysis of Organic Acids

1. MOX reagent (Pierce #45950) or anhydrous pyridine (*see* **Note 3**).
2. MTBSTFA +1 % TBDMCS, 1 mL ampules (Pierce #48927).
3. 2 mL amber glass injection vial.
4. 150 μL insert for injection vial.
5. Single quadrupole GC-MS with the following settings:
   (a) Injection volume: 1 μL.
   (b) Purged splitless mode, set to activate at 1 min (*see* **Note 1**).
   (c) Column: HP-5MS (or similar), 30 m × 0.25 mm ID, 0.25 μm film.
   (d) Column flow: He at 1 mL/min (*see* **Note 2**).
   (e) Inlet temp: 270 °C.
   (f) Interface temp: 300 °C.
   (g) Temp profile: 80 °C for 2 min, ramp at 20 °C/min to 140 °C, ramp at 4 °C/min to 280 °C, hold at 280 °C for 5 min (45 min run time).
   (h) MS settings: Scan mode (100–550), 5 min solvent delay.

2.6 GC-MS Analysis of Sugars

1. 2 wt % hydroxylamine hydrochloride in pyridine solution (may be refrigerated up to 1 year).
2. Propionic anhydride.
3. Ethyl acetate.
4. 2 mL amber glass injection vial.
5. 150 μL insert for injection vial.
6. Single quadrupole GC-MS with the following settings:
   (a) Injection volume: 1 μL.
   (b) Purged splitless mode, set to activate at 1 min (*see* **Note 1**).
   (c) Column: HP-5MS (or similar), 30 m × 0.25 mm ID, 0.25 μm film.
   (d) Column flow: He at 0.88 mL/min (*see* **Note 2**).
   (e) Inlet temp: 250 °C.
   (f) Interface temp: 300 °C.
   (g) Temp profile: 80 °C for 1 min, ramp at 20 °C/min to 280 °C, hold at 280 °C for 4 min (15 min run time).
   (h) MS settings: Scan mode (100–500), 5 min solvent delay.

2.7 GC-MS Analysis of Fatty Acids

1. 5 % (v/v) sulfuric acid dissolved in anhydrous methanol (fresh).
2. 0.2 % solution of butylated hydroxytoluene (BHT) in methanol.
3. Toluene.
4. Hexane.

5. Screw-cap glass vial, with Teflon-lined cap.
6. 2 mL amber glass injection vial.
7. Single quadrupole GC-MS with the following settings:
   (a) Injection volume: 1 μL.
   (b) Purged splitless mode, set to activate at 1 min (*see* **Note 1**).
   (c) Column: DB-23 (or similar), 30 m × 0.25 mm ID, 0.25 μm film (*see* **Note 4**).
   (d) Column flow: He at 1 mL/min (*see* **Note 2**).
   (e) Inlet temp: 250 °C.
   (f) Interface temp: 300 °C.
   (g) Temp profile: 80 °C for 2 min, ramp at 20 °C/min to 140 °C, ramp at 10 °C/min to 240 °C, hold at 240 °C for 5 min (20 min run time).
   (h) MS settings: Scan mode (50–500), 5 min solvent delay.

2.8 LC-MS/MS Analysis of Sugar Phosphates

1. 13 mm syringe filter with PVDF membrane, 0.2 μm pore size.
2. 1 mL syringe.
3. 2 mL amber glass injection vial.
4. Eluent A: solution of 10 mM tributylamine +15 mM acetic acid (*see* **Note 5**).
5. Eluent B: HPLC-grade methanol.
6. Linear ion-trap triple quadrupole LC-MS/MS with the following settings:
   (a) Injection volume: 10 μL.
   (b) Column: Phenomenex Synergi Hydro-RP (or similar), 150 mm × 2.1 mm ID, 4 μm particle size.
   (c) Column flow: 0.3 mL/min.
   (d) Column temp: 25 °C.
   (e) Gradient profile: 0 % B (0 min), 8 % B (8 min), 22 % B (18 min), 40 % B (28 min), 60 % B (32 min), 90 % B (34 min), 90 % B (37 min), 0 % B (39 min), 0 % B (49 min).
   (f) MS settings: Negative-mode electrospray ionization (ESI), MRM mode (*see* Table 1 and **Note 6**).

2.9 GC-MS Data Analysis

1. Computer equipped with AMDIS (freeware available at http://chemdata.nist.gov/mass-spc/amdis), Wsearch32 (freeware available at http://www.wsearch.com.au/wsearch32/wsearch32.htm), or commercial software for searching and integrating GC-MS data.
2. Mass spectral library such as the NIST/EPA/NIH Mass Spectral Database [14], Golm Metabolome Database [15], or FiehnLib [16] for compound identification (optional).

Table 1
LC-MS/MS ion transitions and method parameters

Analyte	RT	MS1	MS2	DP	EP	CP	CXP
G6P	11.3	259–265	97	−50	−10	−22	−5
GAP	11.7	169–172	97	−35	−10	−14	−7
R5P	12.0	229–234	97	−50	−10	−22	−5
S7P	12.1	289–296	97	−50	−10	−22	−5
E4P	12.1	199–203	97	−50	−10	−22	−5
F6P	12.3	259–265	97	−50	−10	−22	−5
X5P	13.1	229–234	97	−50	−10	−22	−5
Ru5P	13.8	229–234	97	−50	−10	−22	−5
DHAP	15.7	169–172	97	−35	−10	−14	−7
2/3PGA	25.9	185–188	79	−35	−35	−10	−10
2PG	26.2	155–157	79	−35	−35	−10	−10
FBP	26.3	339–345	97	−60	−60	−10	−10
RuBP	26.8	309–314	97	−60	−60	−10	−10
PEP	27.1	167–170	79	−35	−35	−10	−10

Labeling of sugar phosphates is measured by monitoring transitions from selected precursor ions to PO3- (mass 79) or H2PO4- (mass 97) product ions in multiple reaction monitoring (MRM) mode. Compound-specific MS parameters are abbreviated as follows: retention time (RT), declustering potential (DP), collision energy (CE), entrance potential (EP), and cell exit potential (CXP). Retention time is expressed in minutes and all potentials are in volts

2.10 LC-MS/MS Data Analysis

1. Computer equipped with either (1) freeware LC-MS/MS analysis software and an appropriate raw data file converter (*see* **Note 7**) or (2) commercial software for searching and integrating LC-MS/MS data.
2. Access to MS/MS mass spectral library such as Metlin [17], HMDB [18], or MassBank [19] for compound verification (optional).

3 Methods

3.1 Hydrolysis of Cellular Protein to Amino Acids

GC-MS analysis of proteinogenic amino acids requires that the protein fraction is first isolated from cells and hydrolyzed by incubating with hydrochloric acid. It is recommended to perform the reaction in a vacuum hydrolysis tube to prevent oxidative degradation of the sample. Alternatively, the acidified sample can be purged with nitrogen for 5–10 min to exclude oxygen and quickly capped prior to the incubation step.

1. Aspirate liquid from protein pellet.
2. Transfer protein to vacuum hydrolysis tube using Pasteur pipette.
3. Add 700 μL of 6 N HCl to hydrolysis tube.
4. Apply vacuum to hydrolysis tube.
5. Place sample on dry block heater for 20 h at 110 °C.
6. Let the hydrolysis tube cool down for several minutes.
7. Transfer sample to microcentrifuge tube using a Pasteur pipette.
8. Centrifuge for 10 min at 18,000 × *g* to remove cell debris.
9. Transfer supernatant into a clean microcentrifuge tube using a Pasteur pipette.
10. Evaporate sample to dryness under air flow at 60 °C.
11. Dissolve in 150 μL ultrapure water (sonicate if necessary to fully dissolve).
12. Transfer sample to a syringe equipped with a 0.2 μm filter using a Pasteur pipette.
13. Filter sample into a clean microcentrifuge tube.
14. Evaporate sample to dryness under air flow at 60 °C.

3.2 Hydrolysis of Cellular RNA to Ribose

In principle, the amino acid histidine provides direct information on the labeling of pentose-5-phosphate (P5P) intermediates of the Calvin cycle. However, histidine is often present at low abundance in protein hydrolysates, which results in insufficient ion counts for accurate mass isotopomer quantification. GC-MS analysis of the ribose moiety of RNA provides an alternative P5P proxy that can be reliably measured. Prior to analysis, cellular RNA is isolated (e.g., using TRIzol® reagent) and hydrolyzed in much the same way as in the preparation of proteinogenic amino acids, although the required reaction conditions are less severe.

1. Decant solvent from RNA pellet.
2. Dry under air flow at room temperature.
3. Add 400 μL ultrapure water.
4. Sonicate briefly to dissolve RNA.
5. Use Pasteur pipette to transfer solution to vacuum hydrolysis tube.
6. Add 200 μL of 6N HCl to hydrolysis tube.
7. Apply vacuum to the tube.
8. Heat at 100 °C for 2 h in dry block heater.
9. Transfer to microcentrifuge tube.
10. Evaporate to dryness under air flow at 60 °C (approx. 120 min).

3.3 Hydrolysis of Starch to Glucose

Starch synthesis occurs exclusively in the plastid [11]. Therefore, isotopomer analysis of glucose monomers derived from starch hydrolysis provides compartment-specific information on plastidic carbohydrate metabolism. Starch hydrolysis can be achieved by steam pretreatment followed by enzymatic degradation, as described below.

1. Add 200 µL acetate buffer to starch sample and cap loosely (*see* **Note 8**).
2. Autoclave at 121 °C for 1 h and cool to room temperature.
3. Add 400 µL amyloglucosidase/amylase solution and vortex.
4. Incubate for 1 h at 55 °C in dry block heater.
5. Add 400 µL ethanol and vortex.
6. Incubate for 15 min at 15 °C to denature proteins.
7. Centrifuge at 5,000 × *g* for 15 min.
8. Remove supernatant to a clean microcentrifuge tube using Pasteur pipette.
9. Evaporate to dryness under air flow at 60 °C.

3.4 GC-MS Analysis of Amino Acids

GC-MS analysis of amino acids, whether derived from protein hydrolysis or free intracellular extracts, is most readily achieved following conversion to *tert*-butyldimethylsilyl (TBDMS) derivatives [20]. TBDMS derivatives are approximately 1,000 times more stable than trimethylsilyl (TMS) derivatives and produce characteristic $[M\text{-}57]^+$, $[M\text{-}85]^+$, and $[M\text{-}157]^+$ fragment ions that facilitate identification (*see* Tables 2, 3) [2, 9]. Figure 3 shows an example chromatogram obtained using this method.

1. Dissolve dried sample in 50 µL anhydrous pyridine in a fume hood.
2. Add 70 µL MTBSTFA +1 % TBDMCS.
3. Sonicate for 5 min.
4. Incubate for 30 min at 60 °C on dry block heater.
5. Centrifuge for 5 min at 18,000 × *g* to remove solid debris.
6. Transfer supernatant to injection vial containing a 150 µL microvolume insert.
7. Run sample on GC-MS.

3.5 GC-MS Analysis of Organic Acids

Organic acids can be converted to their TBDMS derivatives and analyzed in much the same was as amino acids. However, it is recommended to first convert all ketone and aldehyde functional groups to their associated methyloxime derivatives. This prevents keto-enol tautomerization, which would otherwise result in multiple TBDMS derivatives.

Table 2
GC-MS ions of TBDMS-derivatized amino acids that have been previously validated for accuracy by Antoniewicz et al. [20] and in our own labs (*see* Note 15)

Metabolite	Mass	Carbon atoms	Composition	Precursor(s)
Ala	232	2-3	$C_{10}H_{26}ONSi_2$	Pyr
Ala	260	1-2-3	$C_{11}H_{26}O_2NSi_2$	Pyr
Asn	417	1-2-3-4	$C_{18}H_{41}N_2O_3Si_3$	Oac
Asp	302	1-2	$C_{14}H_{32}O_2NSi_2$	Oac
Asp	376	1-2	$C_{16}H_{38}O_3NSi_3$	Oac
Asp	390	2-3-4	$C_{17}H_{40}O_3NSi_3$	Oac
Asp	418	1-2-3-4	$C_{18}H_{40}O_4NSi_3$	Oac
Glu	330	2-3-4-5	$C_{16}H_{36}O_2NSi_2$	Akg
Glu	432	1-2-3-4-5	$C_{19}H_{42}O_4NSi_3$	Akg
Gln	431	1-2-3-4-5	$C_{19}H_{43}N_2O_3Si_3$	Akg
Gly	218	2	$C_9H_{24}ONSi_2$	3PGA
Gly	246	1-2	$C_{10}H_{24}O_2NSi_2$	3PGA
Ile	200	2-3-4-5-6	$C_{11}H_{26}NSi$	Pyr + Oac
Ile	274	2-3-4-5-6	$C_{13}H_{32}ONSi_2$	Pyr + Oac
Leu	274	2-3-4-5-6	$C_{13}H_{32}ONSi_2$	Pyr
Met	218	2-3-4-5	$C_{10}H_{24}NSiS$	Oac
Met	292	2-3-4-5	$C_{12}H_{30}NOSi_2S$	Oac
Met	320	1-2-3-4-5	$C_{13}H_{30}NO_2Si_2S$	Oac
Phe	234	2-3-4-5-6-7-8-9	$C_{14}H_{24}NSi$	PEP + E4P
Phe	302	1-2	$C_{14}H_{32}O_2NSi_2$	PEP + E4P
Phe	308	2-3-4-5-6-7-8-9	$C_{16}H_{30}ONSi_2$	PEP + E4P
Phe	336	1-2-3-4-5-6-7-8-9	$C_{17}H_{30}O_2NSi_2$	PEP + E4P
Ser	288	2-3	$C_{14}H_{34}ONSi_2$	3PGA
Ser	302	1-2	$C_{14}H_{32}O_2NSi_2$	3PGA
Ser	362	2-3	$C_{16}H_{40}O_2NSi_3$	3PGA
Ser	390	1-2-3	$C_{17}H_{40}O_3NSi_3$	3PGA
Thr	376	2-3-4	$C_{17}H_{42}O_2NSi_3$	Oac
Thr	404	1-2-3-4	$C_{18}H_{42}O_3NSi_3$	Oac
Tyr	302	1-2	$C_{14}H_{32}O_2NSi_2$	PEP + E4P
Val	260	2-3-4-5	$C_{12}H_{30}ONSi_2$	Pyr
Val	288	1-2-3-4-5	$C_{13}H_{30}O_2NSi_2$	Pyr

MS ions recommended for isotopomer analysis. Nominal masses, carbon backbone atoms, and molecular composition of each ion are listed. *See* **Note 14** for metabolite abbreviations. The precursor metabolites from which the amino acids are biosynthetically derived are also shown

Table 3
GC-MS ions of MOX/TBDMS-derivatized organic acids commonly observed in cell extracts

Metabolite	Mass	Carbons	Composition
Akg	346	1-2-3-4-5	$C_{14}H_{28}O_5NSi_2$
Cit	459	1-2-3-4-5-6	$C_{20}H_{39}O_6Si_3$
Fum	287	1-2-3-4	$C_{12}H_{23}O_4Si_2$
Lac	233	2-3	$C_{10}H_{25}O_2Si_2$
Lac	261	1-2-3	$C_{11}H_{25}O_3Si_2$
Mal	419	1-2-3-4	$C_{18}H_{39}O_5Si_3$
Pyr	174	1-2-3	$C_6H_{12}O_3NSi$
Suc	289	1-2-3-4	$C_{12}H_{25}O_4Si_2$

MS ions recommended for isotopomer analysis. Nominal masses, carbon backbone atoms, and molecular composition of each ion are listed. *See* **Note 14** for metabolite abbreviations.

Table 4
GC-MS ions of aldonitrile pentapropionate derivatives of glucose and ribose

Metabolite	Mass	Carbons	Composition
Glucose	173	5-6	$C_8H_{13}O_4$
Glucose	259	4-5-6	$C_{12}H_{19}O_6$
Glucose	284	1-2-3-4	$C_{13}H_{18}O_6N$
Glucose	370	1-2-3-4-5	$C_{17}H_{24}O_8N$
Ribose	173	4-5	$C_8H_{13}O_4$
Ribose	259	3-4-5	$C_{12}H_{19}O_6$
Ribose	284	1-2-3-4	$C_{13}H_{18}O_6N$

MS ions recommended for isotopomer analysis. Nominal masses, carbon backbone atoms, and molecular composition of each ion are listed. *See* **Note 14** for metabolite abbreviations.

1. Dissolve dried sample in 50 µL MOX reagent in a fume hood (*see* **Note 3**).
2. Sonicate for 5 min.
3. Incubate for 90 min at 40 °C on dry block heater.
4. Add 70 µL MTBSTFA +1 % TBDMCS.
5. Incubate for 30 min at 70 °C on dry block heater.

Table 5
GC-MS ions of common fatty acid methyl esters (*see* Note 16)

Metabolite	Mass	Carbons	Composition
α-Linolenic	292	1–18	$C_{19}H_{32}O_2$
Linoleic	294	1–18	$C_{19}H_{34}O_2$
McLafferty	74	1–2	$C_3H_6O_2$
Oleate	264	1–18	$C_{18}H_{32}O$
Palmitate	270	1–16	$C_{17}H_{34}O_2$
Stearate	298	1–18	$C_{19}H_{38}O_2$

MS ions recommended for isotopomer analysis. Nominal masses, carbon backbone atoms, and molecular composition of each ion are listed. *See* **Note 14** for metabolite abbreviations.

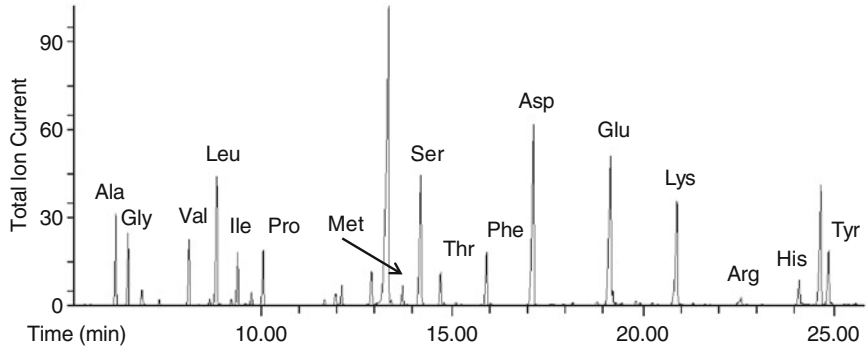

Fig. 3 Representative GC-MS chromatogram (total ion count) of a protein hydrolysate sample. Sixteen amino acids are identified in samples prepared and analyzed using Subheadings 3.1 and 3.4. Glutamine, asparagine, cysteine, and tryptophan are degraded during the acid hydrolysis procedure. As a result, glutamine is converted to glutamate and asparagine to aspartate

6. Remove from heating block and incubate overnight at room temperature (*see* **Note 9**).
7. Centrifuge for 5 min at 18,000 × *g* to remove solid debris.
8. Transfer liquid to injection vial containing a 150 μL microvolume insert.
9. Run sample on GC-MS.

3.6 GC-MS Analysis of Sugars

There is a wealth of information on carbohydrate metabolism that can be obtained from isotopomer analysis of sugars derived from RNA (*see* Subheading 3.2), starch (*see* Subheading 3.3), or free intracellular extracts. Furthermore, Allen et al. [11] have shown

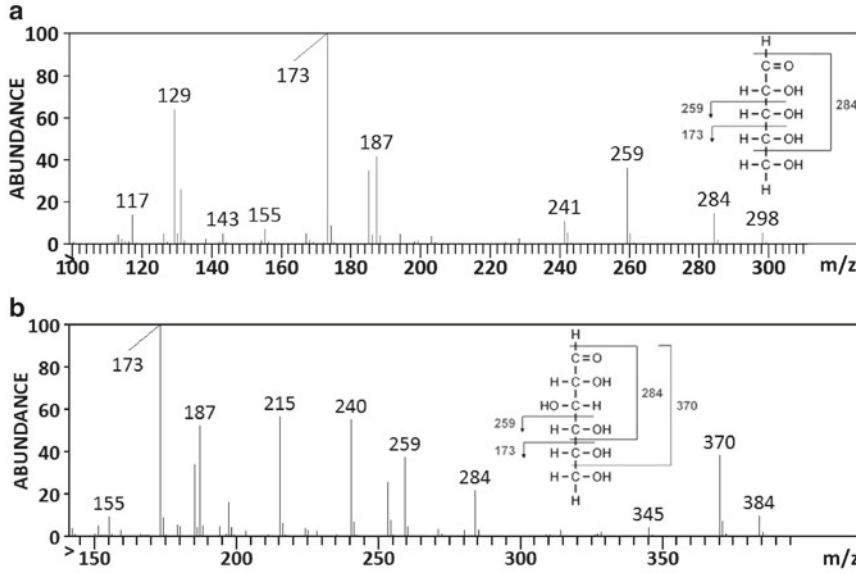

Fig. 4 Mass spectra of aldonitrile pentapropionate sugar derivatives. *Insets* show the backbone atoms included in the main fragment ions of (**a**) glucose and (**b**) ribose

that sugar monomers derived from cell wall and protein glycans can provide compartment-specific readouts on cytosolic fluxes. Price [21] has evaluated several sugar derivatives including alditol acetates, aldonitrile acetates, and peracetates. Here we provide a slight variation on the aldonitrile acetate approach, which replaces the acetate functionality with a propionate group. Antoniewicz has shown that this substitution leads to improved accuracy in mass isotopomer quantification [22]. Example mass spectra of both ribose and glucose derivatives obtained using this method are shown in Fig. 4.

1. Add 50 μL hydroxylamine/pyridine solution to dried sample in a fume hood.
2. Sonicate for 5 min.
3. Incubate for 60 min at 90 °C on dry block heater (*see* **Note 10**).
4. Centrifuge for 30 s at 18,000 × *g* to pull liquid to bottom of tube.
5. Add 100 μL propionic anhydride.
6. Sonicate for 5 min.
7. Incubate for 30 min at 60 °C on dry block heater.
8. Centrifuge for 30 s at 18,000 × *g* to pull liquid to bottom of tube.
9. Transfer supernatant to new microcentrifuge tube.
10. Evaporate to dryness under air flow at 60 °C (approx. 30 min).

11. Add 100 μL of ethyl acetate.
12. Sonicate for 5 min (*see* **Note 11**).
13. Centrifuge for 10 min at 18,000 × *g* to remove solids.
14. Transfer supernatant to GC injection vial containing a 150 μL insert.
15. Run sample on GC-MS.

3.7 GC-MS Analysis of Total Fatty Acids

Isotopomer analysis of fatty acids derived from cellular lipids provides compartment-specific information on the labeling of acetyl-CoA pools [11]. Typically, lipids are extracted and transesterified to generate fatty acid methyl esters (FAMEs), which are subsequently analyzed by GC-MS [6, 23]. Other derivatization protocols can be found in the literature including the formation of butylamides [11]. Here, we present a commonly used acid-catalyzed transmethylation approach that is straightforward to perform.

1. Add 2 mL 5 % sulfuric acid in methanol (v/v) to dried sample in glass vial.
2. Add 0.5 mL toluene.
3. Add 25 μL 0.2 % BHT in methanol.
4. Vortex and heat at 95 °C for 2 h.
5. Add 3 mL ultrapure water and shake to quench reaction.
6. Extract twice with 3 mL hexane, using a Pasteur pipette to remove extracts.
7. Pool hexane extracts and dry under air flow at room temperature.
8. Dissolve in 1 mL hexane and transfer to GC injection vial.
9. Run sample on GC-MS.

3.8 LC-MS/MS Analysis of Sugar Phosphates

Although analysis of labeling in macromolecule components such as protein, starch, and lipid has dominated the MFA literature due to their high abundance in tissues, recent advances in MS technology have facilitated direct analysis of free intracellular metabolites. This approach enables quantification of MIDs in sugar phosphate intermediates that participate directly in glycolysis and Calvin cycle reactions, providing comprehensive and dynamic information on flux through these important pathways. (However, for intermediates found in multiple subcellular compartments this will only provide the *average* labeling among the various intracellular pools.) The vast majority of sugar phosphate analysis has been performed using LC-MS/MS, which avoids degradation of these heat-labile analytes [24]. The LC-MS/MS conditions provided in

Subheading 2.8 are based on the method of Luo et al. [13], which has been subsequently modified and applied to analyze labeling in cyanobacteria extracts by Shastri [25].

1. Evaporate solvent from deproteinized cell extract.
2. Dissolve in 1 mL ultrapure water and filter to remove particulates.
3. Transfer to LC injection vial.
4. Run sample on LC-MS/MS.

3.9 GC-MS Data Analysis

Analysis of GC-MS data requires (1) identification of chromatographic peaks and fragment ions associated with target analytes of interest, (2) integration of ion chromatograms over time to quantify relative abundance of specific isotope peaks, and (3) assessment of measurement standard errors. In many cases, it is also desirable to "correct" the raw MIDs to account for the presence of naturally occurring stable isotopes. Corrected mass isotopomer data provides a more intuitive picture of the labeling that is attributable to the introduction of a tracer compound and is generally the preferred method for presenting data from an isotope labeling experiment. However, note that some MFA software will perform these corrections internally, and therefore it is only necessary to input the raw, uncorrected MIDs in this case.

1. *Identify the chromatographic peaks associated with analytes of interest.* Identification of chromatographic peaks is based on both the retention time (RT) and the MS fingerprint of the peak. If targeted analysis of a relatively small number of compounds (for which pure standards are available) is to be performed, then it is not necessary to use a comprehensive library search. The retention time and main fragment ions can be determined by running pure standards of each compound under the relevant GC-MS conditions, and then the sample chromatograms can be manually searched using available software tools to locate the corresponding peaks. However, untargeted analysis is facilitated by using a mass spectral library to automatically search the GC-MS dataset for uniquely identified "hits". Retention time locked libraries are now available that contain both RT and MS information, which can further improve search reliability [16].
2. *Identify ions to be used for mass isotopomer analysis.* Once the chromatographic peaks of interest have been identified, it is necessary to select the ions that will be used for mass isotopomer analysis and to determine their molecular composition. The best candidates are highly abundant ions with masses greater than 150 Da, since these are less likely to be contaminated by interfering fragment ions of similar mass. Determining the

elemental composition of these ionic species is facilitated by references that list common fragmentation patterns and molecular rearrangements obtained for particular classes of compounds and derivatization groups (e.g., *see* Kitson et al. [6]). Tables 2–5 lists several important fragment ions that are obtained using the methods described in this chapter, many of which have been utilized in previous MFA studies.

3. *Integrate mass isotopomer peaks.* In order to maximize the accuracy of mass isotopomer data, it is necessary to integrate each ion chromatogram over its full peak width. This involves integrating all single ion traces over all scans of the peak, including masses up to 3 Da heavier than the fully labeled fragment ion (*see* **Note 12**). For example, to quantify the MID of a fragment with monoisotopic mass 200 *m/z* and up to three labeled carbons, extract and independently integrate the ion traces of 200, 201, 202,…, 206. Normalize the integrated areas such that the sum of all mass isotopomers for a given fragment ion is 1 (i.e., 100 mol%).
4. *Correct MIDs for natural isotope abundance (optional).* There has been much confusion in the literature over how to properly correct mass isotopomer data for the presence of naturally occurring isotopes. Most errors have resulted from improper handling of the "skew correction factor" [7]. We recommend the approach of Fernandez et al. [26], which can be readily implemented in Matlab or any other appropriate programming language. Coplen et al. [27] provide data on the natural isotope abundance of all elements commonly found in biological samples (*see* **Note 13**).
5. *Assess the precision and accuracy of MIDs.* In order to perform statistical analysis of best-fit flux solutions, MFA software requires the user to input standard errors of each mass isotopomer measurement. Typically, the precision (i.e., repeatability) of these measurements is superior to their absolute accuracy [20]. Inaccurate MIDs can occur due to interference from overlapping fragment ions or gas-phase proton exchanges that contaminate the mass spectrum of the target ions [11]. Therefore, it is important to assess both precision and accuracy using standards of known isotope labeling. At minimum, it is necessary to run samples from naturally labeled cell extracts and compare the experimentally determined MIDs to theoretically predicted values. The approach of Fernandez et al. [26] can be used to predict MIDs of unlabeled samples based on reported values of elemental isotope abundance [27]. A more thorough error assessment would also involve analyzing mixtures of labeled standards to quantify the uncertainty in measuring MIDs that differ from natural labeling (e.g., see Antoniewicz et al. [20]). In general, fragment ions used for

MFA should be accurate to within 1.5 mol% (and preferably 0.8 mol%) of the predicted value [2].

3.10 LC-MS/MS Data Analysis

1. *Identify the chromatographic peaks associated with analytes of interest.* Similar to GC-MS data, chromatographic peaks from LC-MS/MS data can be identified based on RT and MS/MS fragment spectra. One key difference, however, is that MS/MS data is only obtained for selected parent ions and therefore cannot be used for untargeted profiling. As a result, RT information and parent ion mass are initially used to locate candidate peaks of interest, which can be further subjected to MS/MS analysis to confirm the identity of these peaks using a mass spectral library that contains ESI-MS/MS data.
2. *Identify ions to be used for mass isotopomer analysis.* The precursor ions formed in negative-ESI mode typically result from simple proton extraction. Current applications of LC-MS/MS to mass isotopomer analysis only make use of product ions that are formed without breaking the carbon backbone of their precursor ions. Therefore, the product ion spectra reflect the MIDs of the intact precursor ions when ^{13}C is used as tracer [28]. A list of LC-MS/MS ions used for analysis of sugar phosphate labeling can be found in Table 6, with the composition of each precursor and product ion shown.
3. *Correct MIDs for natural isotope abundance* (*optional*). Correction for naturally occurring stable isotopes can be performed by the same approach described for GC-MS under Subheading 3.9, **step 4**. In general, the correction is less dramatic for LC-MS/MS data due to the lack of chemical derivatization.
4. *Assess the precision and accuracy of MIDs.* This can be accomplished by analyzing naturally labeled extracts or isotopically labeled standards, as described for GC-MS data under Subheading 3.9, **step 5**.

4 Notes

1. The split ratio (in split mode) or purge activation time (in splitless mode) can be varied to achieve total ion counts in the desired range. Split mode is appropriate for concentrated samples while splitless mode is most appropriate for dilute samples. Typical split ratios vary from 1:5 to 1:100, with 1:10 representing a good initial value. Typical purge times range from 0.5 to 2 min, with 1 min representing a good initial value. Note that GC inlet liners are specific for either split or splitless operation and are not interchangeable, in principle. However, we have used splitless liners for both operation modes without

Table 6
LC-MS/MS ions of sugar phosphates observed in cyanobacterial cell extracts

Metabolite	$[M-H]^-$	Carbons	Composition	Production
2PG	155	1-2	$C_2H_4O_6P$	PO_3^-
2/3PGA	185	1-2-3	$C_3H_6O_7P$	PO_3^-
DHAP	169	1-2-3	$C_3H_6O_6P$	$H_2PO_4^-$
E4P	199	1-2-3-4	$C_4H_8O_7P$	$H_2PO_4^-$
F6P	259	1-2-3-4-5-6	$C_6H_{12}O_9P$	$H_2PO_4^-$
FBP	339	1-2-3-4-5-6	$C_6H_{13}O_{12}P_2$	$H_2PO_4^-$
G6P	259	1-2-3-4-5-6	$C_6H_{12}O_9P$	$H_2PO_4^-$
GAP	169	1-2-3	$C_3H_6O_6P$	$H_2PO_4^-$
PEP	167	1-2-3	$C_3H_4O_6P$	PO_3^-
R5P	229	1-2-3-4-5	$C_5H_{10}O_8P$	$H_2PO_4^-$
RuBP	309	1-2-3-4-5	$C_5H_{11}O_{11}P_2$	$H_2PO_4^-$
S7P	289	1-2-3-4-5-6-7	$C_7H_{14}O_{10}P$	$H_2PO_4^-$
Ru5P	229	1-2-3-4-5	$C_5H_{10}O_8P$	$H_2PO_4^-$
X5P	229	1-2-3-4-5	$C_5H_{10}O_8P$	$H_2PO_4^-$

MS ions recommended for isotopomer analysis. Nominal masses, carbon backbone atoms, and molecular composition of each ion are listed. *See* **Note 14** for metabolite abbreviations. The compositions of precursor and product ions are shown

noticeable deterioration in performance. Also note that when using splitless mode, the initial column temperature should be near or below the boiling point of the solvent in which the sample is dissolved [6].

2. The optimal linear velocity is in the range 20–40 cm/s for helium. Agilent's FlowCalc tool or the instrument control software can help determine what the linear velocity will be for a particular combination of flowrate, column diameter, temperature, and pressure.
3. The methoxyamine (MOX) reaction protects ketone and aldehyde functional groups, and thereby prevents the formation of multiple TBDMS derivatives. This step is unnecessary if no ketone or aldehyde functional groups are present in the analytes of interest. In this case, dissolve the dried sample in 50 μL anhydrous pyridine and skip Subheading 3.5, **step 3**.

4. Although polar phases are preferred for lipid analysis due to their enhanced resolving power, standard nonpolar phases have also been used successfully for less stringent separations.
5. In order to dissolve tributylamine completely in water, the tributylamine and acetic acid are first mixed together in a dry flask before the requisite amount of ultrapure water is added. The solution is then filtered through a 0.45 μm membrane prior to use. The final pH should be 4.5–5.
6. To increase sensitivity, the method should be divided into multiple time segments with different MRM transitions scanned in each interval. Dwell time of each transition should be optimized such that the total cycle time in each time segment does not exceed 2 s. This will provide at least 10–15 scans of each chromatographic peak as it elutes from the column.
7. Two popular freeware programs for LC-MS/MS data analysis are MZmine and XCMS, the latter of which runs in the R statistical programming environment. Both programs require the user to convert raw data files into a nonproprietary format such as mzXML, NetCDF, or mzData. Conversion to mzXML format can be accomplished using one of several instrument-specific software tools developed and maintained by the Seattle Proteome Center (http://tools.proteomecenter.org/software.php).
8. Typically, this pellet is what remains after performing previous extractions to remove oil, protein, and free metabolites. Refer to Allen et al. [11] for details.
9. This step may be necessary for dilute samples in order to ensure complete conversion. However, it may be skipped for concentrated samples.
10. Ensure tube lid remains secure. Periodically check to ensure that solution is in contact with pellet and hasn't condensed on the lid of the tube. Shake liquid down if it has.
11. In some instances, a gel-like precipitate forms that requires additional time to dissolve. Leave overnight to fully dissolve, if necessary.
12. Many software packages will integrate ion chromatograms, but they often differ in the way they select the time-window of integration and perform baseline correction for each mass peak. If these parameters are not determined consistently for all mass isotopomers of a given fragment ion, substantial errors in the MID can occur (e.g., see Antoniewicz et al. [20]). This will give unacceptable results when performing the error analysis of Subheading 3.9, **step 5**. Therefore, choose an integration algorithm that is known to produce consistent datasets and has been validated using samples of known isotope labeling.

13. There are slight variations in natural ^{13}C-enrichment depending upon the process of carbon fixation utilized [29]. Plant RuBisCO exhibits a kinetic preference for ^{12}C over ^{13}C, with the effect being more pronounced in C3 plants relative to C4 plants [27]. However, the differences are in the range of 0.01–0.03 mol%, which are not measurable by typical quadrupole MS instruments and therefore are insignificant in most MFA studies. These variations are only important when using GC-isotope ratio mass spectrometry (GC-IRMS) or similar approaches that provide highly accurate mass isotopomer measurements.
14. Abbreviations: *2PG*, 2-phosphoglycolate; *2PGA*, 2-phosphoglycerate; *3PGA*, 3-phosphoglycerate; *Akg*, alpha-ketoglutarate; *Cit*, citrate; *DHAP*, dihydroxyacetone phosphate; *E4P*, erythrose 4-phosphate; *F6P*, fructose-6-phosphate; *FBP*, fructose 1,6-bisphosphate; *Fum*, fumarate; *G6P*, glucose 6-phosphate; *GAP*, glyceraldehyde 3-phosphate; *Lac*, lactate; *Mal*, malate; *Oac*, oxaloacetate; *PEP*, phosphoenolpyruvate; *Pyr*, pyruvate; *R5P*, ribose 5-phosphate; *Ru5P*, ribulose 5-phosphate; *RuBP*, ribulose 1,5-bisphosphate; *S7P*, sedoheptulose 7-phosphate; *Suc*, succinate; *X5P*, xylulose 5-phosphate.
15. Gln and Asn are converted to Glu and Asp during acid hydrolysis and cannot be measured individually in protein hydrolysates. However, they can be measured as free amino acids in cell extracts.
16. Although the McLafferty rearrangement ion at *m/z* 74 has been used extensively as a proxy of plastidic acetyl-CoA labeling, it should be treated with caution due to interferences that can lead to significant errors in the MID measurement. For example, Lonien and Schwender [30] have addressed this problem by adjusting the EI voltage to 15 eV from the standard 70 eV to reduce interference and by applying statistical corrections to improve the accuracy of MID measurements.

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