Published in final edited form as:
Methods Mol Biol. 2014 ; 1198: 147–167. doi:10.1007/978-1-4939-1258-2_11.

Stable Isotope Labeled Tracers for Metabolic Pathway Elucidation by GC-MS and FT-MS

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Abstract

Advances in analytical methodologies, principally nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), over the last decade have made large-scale analysis of the human metabolome a reality. This is leading to the reawakening of the importance of metabolism in human diseases, particularly widespread metabolic diseases such as cancer, diabetes, and obesity. Emerging NMR and MS atom-tracking technologies and informatics is poised to revolutionize metabolomics-based research because they deliver the high information throughput (HIT) that is needed for deciphering systems biochemistry. In particular, Stable Isotope Resolved Metabolomics (SIRM) enables unambiguous tracking of individual atoms through compartmentalized metabolic networks, in a wide range of experimental systems, including human subjects. MS offers a wide range of initial capital outlay and operating costs, ranging from gas-chromatography (GC) MS affordable by many individual laboratories, to the HIT-supporting Fourier-transform (FT) class of MS that rivals NMR in cost and infrastructure support. This chapter will focus on sample preparation, instrument, and data processing procedures for these two extremes of MS instrumentation used in SIRM.

Keywords

Metabolomics; stable isotope; mass spectrometry; FT-MS; GC-MS

1. Introduction

Advances in analytical methodologies, principally nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), over the last decade have made large-scale analysis of the human metabolome a reality. This is leading to the reawakening of the importance of metabolism in human diseases, particularly widespread metabolic diseases such as cancer, diabetes, and obesity. The metabolome represents a functional readout of the genome and proteome; by interactions with the proteome, the metabolome is an integral component of homeostatic regulation, at the molecular level. The analysis of the metabolome, “known as “metabolomics”, is a comparatively recent addition to systems biology, now being applied to numerous diseases, mostly by non-biased or targeted metabolite profiling for biomarker discovery (1-7), but also in pharmacology and therapeutics (1, 8-15).

Most importantly, the advances in instrumentation coupled with the emerging atom-tracking technologies and informatics is poised to revolutionize metabolomics-based research because they deliver the high information throughput (HIT) that is needed for deciphering systems biochemistry. In particular, Stable Isotope Resolved Metabolomics (SIRM) enables unambiguous tracking of individual atoms through compartmentalized metabolic networks, in a wide range of experimental systems, including human subjects (10, 15-25) (**Figure 1**). This and other stable isotope tracer-based approaches (26-28) can resolve the complex human disease metabolome in unprecedented detail (29).

Biologically-mediated labeling is needed for many studies because each metabolite typically participates in multiple pathways, e.g. glutamate is found in some 51 mammalian pathways (30), which cannot be resolved based on total metabolite concentrations alone. Just as critical are the dynamics and compartmentation of metabolic events, which requires recording the "history" of these events by isotopic labeling. For example, as shown in **Figure 2**, the authors tracked the behavior of seven isotopic forms (isotopologues) of citrate, which enabled us to resolve the contribution of anaplerotic pyruvate carboxylation (PC) to the Krebs cycling but also uncover a novel Krebs cycle-independent pathway that is important to the function of the *MYC* oncogene (31). This would not have been possible without the SIRM approach. Similarly, the authors also discovered the up regulation of the PC pathway in lung cancer by using *in situ* stable-isotope tracing in human patients (32), which also demonstrated that SIRM is practical in clinical translational studies.

Among the analytical techniques amenable to conducting SIRM, MS techniques stand out in terms of low metabolite detection limits, small sample size requirements, and throughput. In comparison with the other viable technique, NMR, mass spectrometry has disadvantages of difficulty in assigning the actual position(s) of stable isotope enrichment(s) and sample destruction. For the difficult task of metabolite identification, MS and NMR are highly complementary such that any advantages of one over the other depend on the sample and metabolite in question. Practical differences are also of great – and sometimes overarching – importance to conducting SIRM; MS offers a much wider range of initial capital outlay and operating costs, ranging from gas-chromatography (GC) MS affordable by many individual laboratories, to the HIT-supporting Fourier-transform (FT) class of MS that rivals NMR in cost and infrastructure support. This chapter will focus on sample preparation, instrument, and data processing procedures for these two extremes of MS instrumentation used in SIRM.

1.1. Sample Processing Strategic Considerations

Sample processing has been the silent partner alongside the rapid metabolomics developments, but its goals remain unchanged: 1) to maintain biochemical integrity during sampling; 2) to efficiently and reproducibly recover metabolites in large-scale from biospecimens with high throughput; 3) to increase the coverage of the metabolome on limited quantities of biospecimens; 4) to determine trace level and/or labile metabolites in the presence of stable and abundant species, with high throughput; 5) to enable large-scale metabolite identification and automation of metabolite assignment; 6) to identify de novo or obtain crucial structural information of unknowns, with or without the use of metabolite

databases; 7) to facilitate large-scale metabolite quantification without the need for authentic standards.

Goals 1 and 2 involve minimizing any systematic variation and error introduced by sampling processing itself (33). Goals 3 to 7 deal with maximizing the accessible biological information content from analytical and data analysis. Together, the first six goals are common to all metabolomics methods, but the seventh is particularly acute for SIRM. Consider the biological ^{13}C labeling of 3-carbon metabolite lactate: the SIRM target analytes would be the unlabeled form, a single ^{13}C label in each of the 3 carbon positions, two ^{13}C labels in the 1,2 or 2,3 or 1,3 positions, and three ^{13}C labels in all 3 positions. Thus, if authentic standards were to be required, even this rudimentary 3-carbon metabolite would require eight standards. For a single C_{48} glycerophospholipid structure, this could require 49 authentic standards to represent the isotopologues, or about 2.8×10^{14} authentic standards to cover every possibility! Of course, not all permutations of labeling actually occur in a given experiment – for the study depicted in **Figure 2**, we observed seven ^{13}C labeled citrate species out of scores of possible labeling patterns – but how many, and which ones, will not be known *apriori*. Indeed, how many and which ones are labeled is the unknown that is desired from SIRM analysis. Thus, the synthesis and maintenance of many millions of ^{13}C labeled authentic standards for even just a handful of metabolites is simply not feasible, and the overall analytical method must address this issue.

The sample processing procedures described herein reasonably meets all seven needs, however it should be noted that specific experimental designs and biospecimens may require empirical adjustments to the general procedure. Critically, the procedures described here are also suitable for NMR analysis, which is an important strategic decision, even if the original aim was only for MS analysis.

1.2. GC-MS Strategic Considerations

GC-MS has been used extensively for metabolomics (34-37), including SIRM (31, 32, 38-47), and features low initial cost and relatively simple maintenance. Most metabolites are too polar or otherwise not sufficiently volatile to transit a gas chromatograph column, so in general some form of chemical derivatization is required; if metabolites of interest are >800 Da, it is highly unlikely to be amenable to analysis by GC-MS, derivatized or not. Additionally a basic requirement of SIRM is to measure the isotopologue distribution in an intact metabolite, which in turn means that fragmentation must be minimized in the GC-MS unless the purpose is to identify the isotopomer position (48). In order to achieve minimal fragmentation in the most common electron-ionization MS instruments, many investigators – including the authors – have long (34) turned to N-methyl N-(*tert*-butylsilyl)trifluoroacetamide (MTBSTFA) derivatization (49, 50). This reagent reacts with all non-sterically hindered active hydrogen functional groups (e.g. –COOH, –OH, $–NH_2$, –NH, –SH), thus rendering the target analyte volatile via the -(*tert*butylsilyl) derivatization, and providing a *tert*-butyl group that is readily cleaved upon electron impact, yielding a fragment ion that harbors the entire original metabolite. This “pseudo-molecular ion” cluster represents the distribution of isotopic labels in GC-MS data, as illustrated in **Figure 3**. An obvious disadvantage of such large derivatization agents is that it adds to the isotopologue

complexity of the derivative by adding Si as well as more carbons and protons, which must be accurately accounted for in the data analysis by natural abundance stripping.

1.3. FT-MS Strategic Considerations

FT-MS encompasses, at present, two types of instruments at the retail level: the ion cyclotron resonance and Markarov trap ("Orbitrap") mass spectrometers. The FT-MS instruments, as a class, presently achieve the highest m/z resolution and most accurate m/z measurements among all types of MS. For the most common analytes in "metabolomics", which are <1000 Da, a resolving power ($R = m/\Delta m$) of >200,000 preferably >400,000 is required to unambiguously analyze stable isotope enrichments such as ^{13}C or ^{15}N; currently this is possible only on FT-MS instruments. The unambiguous assignment is, in turn, required for SIRM bioinformatics (51, 52).

Because FT-MS is not limited to the very small metabolites of GC-MS, the main analytical thrust of FT-MS has been larger metabolites such as intact lipids, for which a majority is amenable to ionization by electrospray. Thus derivatization is not required for FT-MS analysis. However, as stated earlier, most medium-sized metabolites such as nucleotides and lipids actually encompass large number of isotopologues in SIRM studies, which cannot be adequately analyzed in the short time frames of chromatography. Thus the best approach for FT-MS in SIRM studies is direct infusion by e.g. nanoelectrospray. The generalized procedure for accomplishing this is therefore described. Furthermore, FT-ICR-MS does not require derivatization, making isotopologue analysis more straightforward.

2. Materials

Materials are specific to each of the methods and are stated for each subsection below.

3. Methods

3.1. Initial Sample Processing

Maintaining sample integrity prior to analysis while avoiding addition of excessive non-volatile salts is prerequisite to analysis by MS. Since the 1980's (17, 20, 34, 53-60), we have been tackling this problem for large-scale metabolite profiling of a wide range of biospecimens. For example, freshly resected or biopsy tissues from human subjects should be immediately (<5 min) freeze-clamped in liquid N_2 to minimize alteration of metabolite profiles (17). We have experimentally optimized many such protocols by comparing metabolite profiles of biospecimens handled with different methods.

Most importantly, we have learned from our past 25 years of experience – ranging from microorganisms, plants, invertebrates, and animals to humans, biofluids, and cell cultures – that sample processing requirements for metabolite analyses should be optimized based on the research objectives, and not blindly following a protocol intended for different research goals. Clearly, initial sampling and sample processing varies widely depending on the specific study (human surgery or biopsy tissue specimens, cell culture, bacterial chemostat, plant tissues, marine alga, soil bacteria and fungi, airborne microorganisms, etc), and no generalized procedure will suffice, other than that metabolism in the samples must be halted

such as by freeze-clamping as soon as possible. Otherwise, the aspect of initial sampling and sample processing is well beyond the scope of this chapter, and has been treated elsewhere (32, 49, 61, 62)

After the initial sample processing, efficient and reproducible recovery of metabolites from biospecimens on large-scale remains a key issue. The extremely large diversity of metabolites differing in functional groups, polarity, size, stability, and abundance precludes the use of a single extraction method. However, a common strategy is to use cold solvents to quench metabolism rapidly, followed by different solvent extractions (57, 60, 63-71). We found that cold acetonitrile followed by aqueous $CHCl_3$ partitioning is a versatile method for simultaneous quenching and extraction of polar and non-polar metabolites, including the protein fraction (20, 72, 73). This method is compatible with various cells, tissues, and biofluids that we have tested, and provides efficient, reproducible recovery of a wide range and large number of metabolites including labile compounds (e.g. NADPH and oxylipins). The resulting extracts are suited for NMR as well as MS analyses. A similar $H_2O/CHCl_3$/methanol partitioning method, better suited for MS analysis, gives polar extracts heavily contaminated with lipids, which interferes with ^1H NMR analysis of polar extracts (57, 60).

3.2. Cell Culture and Biofluids Sample Processing

Our current standardized processing protocols for adherent and suspension cell cultures are described respectively in (21, 73) and (74). For biofluids such as blood plasma, saliva, urine, bronchoalveolar lavage fluid (BALF), nipple aspirate fluid, the same protocols as described also apply. Briefly, growth medium components are removed from cells by washing cells quickly in cold PBS, followed by quenching of cell metabolism in cold (–20°C) CH_3CN, and extraction for polar and non-polar metabolites with solvent partitioning in H_2O:CH_3CN:$CHCl_3$ (1.5:2:1). To obtain just the polar fraction quickly, an alternative 60% aqueous CH_3CN or cold 10% trichloracetic acid (TCA) extraction (21, 55, 60) can be performed but the latter leads to loss of acid-labile metabolites. Denatured proteins will be obtained as precipitates and quantified for normalization of metabolite concentrations; we have successfully used it for Western blotting or proteomic analysis.

3.2.1. "Small" Metabolite Processing—Keeping in mind the above considerations, a useful sample processing for cell cultures and biofluids is given below.

Equipment: Retsch (Haan, Germany) MM200 tissue pulverizer

Refrigerated centrifuge with swinging bucket rotor for 15mL conical centrifuge tubes

Vacuum centrifuge for evaporation, e.g. Eppendorf (Hamburg, Germany) Vacufuge

0.01mg resolution balance, e.g. Mettler (Toledo, OH, USA) Toledo AX 105

200 µL pipettor

Materials: Solid glass beads e.g. Kimble (Vineland, NJ, USA) Glass inc. Art. n° 13500-3 size/cap 3mm

acetonitrile (CH_3CN), mass spectrometry grade

chloroform ($CHCl_3$) HPLC grade

18 MΩ water

butylated hydroxyl toluene (BHT), ACS reagent grade or better

Liquid nitrogen (LN_2)

fine tip transfer pipette (e.g. Samco 204 or 235) (ThermoFisher, Waltham, MA, USA)

1.5 mL microcentrifuge tubes

2.0 mL screwcap microcentrifuge tubes

1.5ml polypropylene centrifuge tube (e.g. Sarstedt, Nümbrecht, Germany)

11mm crimp top 2mL chromatography glass vials (recommended: National Scientific, Rockwood, TN, USA, Target ID vials)

11mm crimp tops with Teflon lined rubber septa

11m crimper and decapper

Procedure

1. For each sample, tare one labeled microcentrifuge tube to 0.01 mg.
2. Harvest cells by a method appropriate to the culture system and research goals, obtain wet cell mass by transferring cells to the tared tube for weight determination using the balance;
3. Immediately freeze cells by immersing the capped microcentrifuge tube in LN_2.
4. The cell pellet is quenched in metabolism by adding cold CH_3CN (kept @ -15°C). The volume of CH_3CN is 0.5-1ml for cell wt of 20 to 60 mg.
5. Add 2 or 3 glass beads to each tube
6. Use the ball mill to pulverize the cell pellet in CH_3CN for 1 min at 30 Hz; the glass beads assist the process; repeated twice or three times until the pellet is broken into fine particles
7. Transfer the cell suspension into a 15 ml polypropylene centrifuge tube; wash with additional CH_3CN (0.5 or 1 ml at a time) for more complete transfer. For 60 mg cell wt, the total volume of CH_3CN should not exceed 5 ml; for 20 mg cell wt, the volume of CH_3CN is 2 ml.
8. Continue washing the tube with 18 MΩ water (1 ml at a time; up to 3.25 ml for 60 mg cell wt and 1.25 ml for 20 mg cell wt)
9. Add 0.2 mM Tris-HCl (pH 8) (0.5 ml for 60 mg cell wt & 0.25 ml for 20 mg cell wt).

10. Shake the aqueous CH_3CN mixture rigorously before adding $CHCl_3$ (2.5 ml for 60 mg cell wt & 1 ml for 20 mg cell wt). The final ratio of CH_3CN:H_2O:$CHCl_3$ should be 2:1.5:1 (v/v)
11. Shake the mixture rigorously again (at least 60 sec) to extract lipids from the pellet; the cell-containing upper aqueous layer should have a milky consistency.
12. The mixture is centrifuged in a swinging bucket rotor in a refrigerated centrifuge (set at 4°C) at 3000 g for 20 minutes
13. Carefully transfer the majority of the top layer (CH_3CN -Water, polar fraction) into two 2 ml screw-cap centrifuge tubes using a fine tip transfer pipette. Use larger polypropylene vials for storing the polar fraction, if necessary. Save the transfer pipette for step g below. Reduce the volume by vacuum centrifugation.
14. Distribute the polar fraction in aliquots appropriate for analysis (determined empirically). For aliquots bound for GC-MS analysis, be sure to use 2mL glass chromatography crimp top vials.
15. Freeze the aliquots in LN_2 and lyophilize until dry.
16. Cap tightly and store all samples at –80°C until use.
17. Transfer the majority of the lower layer (chloroform, lipids fraction) into a 2 ml glass screw-thread vial using a 200 µl pipettor with a gel loading tip. Use larger glass vials with aluminum or Teflon-lined caps for storing the $CHCl_3$ fraction, if necessary. Avoid pipetting the aqueous layer. Add appropriate volume of 100 mM BHT to the collected chloroform fraction to make 1 mM BHT final as an antioxidant.
18. Transfer the remaining protein precipitate along with a small amount of both layers into a 1.5 ml microfuge tube (tare weight recorded to 0.01mg).
19. Wash the 15 ml tube with 200-500 µl (depending on how much precipitate is left) of chloroform:methanol:BHT (2:1:1mM)
20. Pool wash with the middle fraction and shake rigorously again to extract remaining lipids from the precipitate
21. Cap tightly and store all samples at –80°C until use.

3.2.2. RNA Processing—RNA is extracted and purified from the cells using the TRIzol reagent and RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Typically 100 µg RNA (length > 200 nt) is obtained from one 10 cm cell culture plate. Purified RNA is assayed by absorption at 260 and the absorption ratio 260/280 nm, which should be ≥ 2 for a pure preparation.

1. Lyophilize the extracted RNA
2. Redissolve in 0.1 M sodium acetate buffer containing 1 mM $ZnCl_2$, pH 5.3.

3. The conditions for digestion are determined using unlabeled Sigma RNA. Two units of P1 micrococcal nuclease (US Biological) are added to 1 ml RNA solution in the assay buffer (A_{260} = 0.5 to 1 unit) in a capped quartz cuvette
4. Incubate at 50 °C.
5. Periodically measure the absorbance at 260 nm (or closest available wavelength) as a function of time for the progress of hydrolysis in a spectrophotometer.
6. After the reaction stops (absorbance levels off), add another aliquot of the enzyme to ensure complete digestion.
7. Divide the solution is into two parts, and lyophilize each.
8. One freeze dried powder sample is redissolved in 350 μL D_2O containing 30 nmol DSS-d_6 for analysis by NMR (75). The NMR analysis provides quality control, and a check on the atom labeling in the ribose subunit of the free nucleotides.
9. The other lyophilized aliquot is analyzed for free nucleotides by direct-infusion electrospray MS analysis of polar samples, as in section **3.5.2.**

3.3. Generalized Tissue Sample Processing

Biological tissues can vary extremely widely in structure and composition, for example with vascular plants the need to disrupt polysaccharidic cell walls being a great distinction from e.g. mammalian samples; in many cases sample processing methods must be empirically adapted. Our procedures for collecting and processing mammalian **tissue** samples are described in (17, 20). Briefly, tissues are flash-frozen in liquid N_2 within 5-10 min of collection, pulverized to 10 μm particles in liquid N_2, and stored in liquid N_2 until extraction using the same protocol as described above for cells. Our extraction protocol is more efficient in separating lipids from polar metabolites and yields much cleaner ^{1}H NMR spectra than the alternative H_2O/methanol/$CHCl_3$ methods, particularly for fatty tissues such as liver (60).

Again, keeping in mind the above considerations, the most versatile sample processing for mammalian tissues is given below.

Equipment and Materials—Spex (Metuchen, NJ) 6750 Freezer/Mill

All other equipment and materials are the same as in section 3.1.1

Procedure

1. Grind frozen tissues in LN2 to >10 μm particles in a Freezer/Mill and extracted simultaneously for soluble and lipidic metabolites as follows.
2. Transfer up to 20 mg of frozen tissue powder into 15 ml centrifuge tube containing three glass beads and vigorously shake in 2 ml of cold acetonitrile to denature proteins
3. Add 1.5 ml 18 MΩ water, and 1 ml $CHCl_3$ and shake vigorously until achieving a milky consistency (ca. 5 min),

4. Centrifuge at 3,000g for 20 min at 4°C to separate the polar (top), lipidic (bottom), and tissue debris layers (interface).
5. Continue as in section 3.2 from step 13.

3.4. GC-MS Analysis

3.4.1. Derivatization—The procedure below was revised in 2005 (76) and currently used by the authors.

Equipment: Lyophilizer

Materials: Sigma (St. Louis, MO, USA) A6407 (1mL): 2.5 mM acidic and neutral amino acids

Sigma A6282 (5mL): 2.5 mM basic amino acids

Norleucine, highest grade possible

Glutamine, highest grade possible

Organic acids of e.g. TCA cycle (such as pyruvic, citric, fumaric, malic, alpha-ketoglutaric, oxaloacetic, succinic acids)

MTBSTFA (N-methyl-N-[tert-butyl-dimethylsilyl]trifluoroacetamide) (Regis Chemical, Morton Grove, IL)

acetonitrile (CH_3CN), mass spectrometry grade

11mm crimp top 2mL chromatography glass vials (recommended: National Scientific Target ID vials)

11mm crimp tops with Teflon lined rubber septa

Glass conical inserts for 2mL glass vials to reduce volume

11m crimper and decapper

Disposable glass pipettes with bulb, prerinsed with CH_3CN

18 MΩ water

Procedure for Amino Acid Standards

1. Prepare 15 mM glutamine (146.15 g/mol) standard (2.192 mg in 1 mL) in water.
2. Prepare 5 mM norleucine (131.17 g/mol) standard (0.6559 mg in 1 mL) in water.
3. Mix together 60 μL of Sigma A6407 + 60 μL of A6282 + 60 μL glutamine standard + 60 μL norleucine standard.
4. Add H_2O to get a final volume of 300 μL

5. Distribute in 10 μL aliquots in crimp top glass vials. Crimp vials must be used as screwcap vials can work loose in the derivatization step.
6. Freeze in LN2 and lyophilize overnight
7. Cap all aliquots with crimp caps and store at –80°C until use.

Procedure for Organic acid standard

1. Prepare 0.5 mM each of desired organic acids in water.
2. Prepare 5 mM norleucine (131.17 g/mol) standard (1.312 mg in 1 mL) in water.
3. Mix together 240 μL organic acid standard + 60μL norleucine
4. Distribute in 10 μL aliquots in crimp top glass vials. Crimp vials must be used as screwcap vials can work loose in the derivatization step.
5. Freeze in LN2 and lyophilize overnight

Cap all aliquots with crimp caps and store at –80°C until use.

Procedure to derivatize standards and samples.

1. Thaw the stored samples to room temperature while capped to avoid condensation of water from the air.
2. Uncrimp the 2mL glass vials of amino acid and organic acid standards as appropriate, and samples one at a time.
3. Add MTBSTFA reagent (25 μL CH_3CN + 25 μL MTBSTFA) to one vial at a time and crimp cap immediately. Crimp vials must be used as screwcap vials can work loose in the next step.
4. Derivatize by sonication in 100W sonic bath for 3 h and let stand overnight
5. Uncap one vial at a time to prevent volatilization and reaction with water in the air.
6. Transfer using a disposable glass pipette to another chromatography vial with glass insert.
7. Centrifuge 1500 g for 10 min to settle any precipitates (inorganic salts).
8. Examine each sample for excessive precipitate that might interfere with the GC-MS injection syringe. If the precipitate height exceeds the autoinjector sampling depth, carefully transfer supernatant to another glass insert and repeat step 6.

3.4.2. GC-MS Instrument Method—The procedure below was revised in 2005 (76) and currently used by the authors.

Equipment: PolarisQ (Thermo Scientifc, Austin, TX) GC-ion trap MSn

SGE 0.15 mm i.d. x 50 m fused silica open-tubular column, coated with 0.25 μm BPX-5 (5% phenylmethyl- phenylsiloxane)

Materials: acetonitrile (CH_3CN), mass spectrometry grade

Procedure

1. Inject 1μL each standard or sample
2. GC-MS conditions: injector at 280 °C, column at 60 °C for first 2 min, followed by a 20 °C/min ramp to 150 °C, then 6 °C/min to 300 °C, splitless vent held for 1.5 min, He carrier gas velocity 30 cm/s at 60 °C, transfer line=280 °C, electron energy=70 eV, source heated to 220 °C, automatic gain control target value=50, maximum inject time=50 ms, He damping gas=1.5 mL/ min, full scan acquisition from 140 to 650 m/z at a rate of 5 spectra/s which were averaged into one. Mass calibration preformed by perfluorotributylamine and a mass defect of 1.0 mmu/amu was applied to all spectra.

3.4.3. GC-MS Data Processing—The procedure below was revised in 2005 (76) and currently used by the authors.

Data system requirements: Windows PC capable of running the GC-MS software – in this case Thermo Scientific Xcalibur 2.0

Procedure

1. Metabolites are identified and quantified automatically using Xcalibur Quan Browser software (ThermoFinnigan), based on their GC retention times and mass fragmentation patterns matched against an in-house database and external standards. In order to accomplish this, the user must set up a Processing method for all the target analytes, following instructions for Xcalibur
2. Identities should be extensively verified by manual inspection of analytes of interest, to ensure that the processing method developed in step 1 is working properly.
3. GC-MS quantification of total abundance of metabolites was accomplished by comparing ion response for each metabolite and each isotopologue in the samples with that for the corresponding standard of known concentration. It is assumed that isotopologues of each metabolite derivative have the same molar response as the monoisotopic pseudo-molecular ion, as authentic standards of each isotopologue are not available.

3.5. FT-MS Analysis

3.5.1. Hydrophobic Samples—The procedure below (77) is currently used by the authors.

Instruments: Thermo Scientific (Bremen, Germany) LTQ-FT (FT-MS) with Advion (Ithaca, NY, USA) Triversa Nanomate nanoelectrospray

Materials: Mass spectrometry grade methanol (MeOH_

Butylated hydroxytoluene (BHT) – serves as preservative

Reserpine – internal standard

Procedure

1. Thaw the stored hydrophobic samples to room temperature while capped to avoid condensation of water from the air.
2. Dilute samples with a solution of 1mM BHT in methanol with 1ng/μL of reserpine added as a standard. Dilution depends on the concentration of the extract. Typically 10x to 20x dilution is sufficient for cell extract samples and 10x to 100x dilution for tissue extracts.
3. Centrifuge at 1500 g for 2 minutes.
4. Directly infuse the samples using the Nanomate.
5. Spectra are acquired in the both – positive and negative ion modes for 15 minutes each. The Advion Nanomate parameters are: (+) mode voltage +1.5kV, head pressure 0.5; (-) mode voltage -1.6kV, head pressure 0.7psi. The mass range is typically set to 150-1500 Da and AGC is set to 1000ms, and usually 30 sec ion trap scans precede and follow a 14 minute FT acquisition. The resolving power is set to 400,000 at m/z = 400 (10% valley). 5 microscans are recorded per saved spectrum.
6. Acquired spectra are summed as appropriate and exported as an accurate mass text file for further data processing.

3.5.2. Polar Samples—The procedure below (78) is currently used by the authors.

Instrument: Thermo Scientific LTQ-FT (FT-MS) with Advion Triversa Nanomate nanoelectrospray

Materials: 18 MΩ water

Buffer [I] (5mM hexylamine in H_2O pH adjusted to 6.3 with AcOH).

Methanol (Fisher HPLC Optima Grade or Burdick & Jackson – suitable for FTMS)

Buffer [II] consisting of 70% Buffer [I] and 30% Buffer composed of MeOH +10% 10mM NH_4Ac, pH adjusted to 8.5 with NH_4OH.

Buffer [III] - mixture of 1 part buffer [II] and 2 parts MeOH

C_{18} bed pipet tips (Pierce) – 100 μL capacity.

Procedure: Sample processing should be done on ice.

1. The previously freeze dried extracts should be dissolved up in 50μL of buffer [I].
2. Set pipet volume to 40-45 μL (to avoid bubbling and foaming).
3. Condition/equilibrate tips by aspirating 5 times in MeOH and buffer [I]

4. Without allowing the tip to dry slowly aspirate the sample solution in buffer [I]. Once aspirated, wait ~5 seconds and release the solution. Repeat four more times.
5. Rinse the tip twice by quickly aspirating and releasing 50 μL of buffer [I]
6. Elute from the tip by washing it with two 50 μL portions of buffer [II] (10 quick aspirations)
7. Combine the eluate and freeze dry.
8. Reconstitue in 75μL of buffer [III]
9. Centrifuge sample at 1500 g for 2 minutes.
10. Processed samples can be stored in the freezer (–80°C long term storage, –20°C up to 4 hours)
11. Directly infuse the samples using the Nanomate.
12. Spectra are acquired in the negative ion mode for 15 minutes. The Advion Nanomate parameters are: voltage -1.6kV, head pressure 0.7psi. The mass range is 150-850 Da. 30sec ion trap scans precede and follow a 14 minute FT acquisition. The resolving power is set to 400,000 at m/z = 400 (10% valley). 5 microscans are recorded per cycle. AGC set to 1000ms.

11 Acquired spectra are summed as appropriate and exported as an accurate mass text file for further data processing.

3.5.3. FT-MS Data Processing

Data system requirements: x86 (or x86_64) based CPU architecture running a current major Linux distribution (RedHat, CentOS, Fedora, Suse, Ubuntu, etc) with: i) Perl 5.12 or later installed and ii) LibreOffice installed.

Procedure

1. For a given metabolite, organize exported FT-MS peak intensities in a spreadsheet using software that is capable of exporting spreadsheets in a space-delimited text format (i.e. Excel, LibreOffice, etc.). Each column will indicate a specific isotopologue of the metabolite, discretely increasing in labeling isotope content from left to right. Each row will include isotopologues of a metabolite derived from a single MS histogram. Typically, a set of histograms derived from a time series of collected samples will be ordered with increasing time of collection from top to bottom rows. Put a “#” at the beginning of each header and comment row.
2. Fill in missing isotopologue intensities with a zero value.
3. Normalize (i.e. divide) each isotopologue peak intensity to the sum of the isotopologue peak intensities derived from the same histogram (i.e. in the same row) using formulas in additional rows in the spreadsheet. For example, the following formula in an Excel spreadsheet cell would normalize the m+0 isotopologue of metabolite with 14 possible label atoms in the second row of the spreadsheet: “=A1/@SUM(A1:N1)”.

4. Where multiple analytical or sampling replicates were collected, compare peak intensities between replicates. If a time series of replicates were run, compare each time point. Look for replicates with a significant number of zero or low intensity isotopologues as compared to other replicates. If a simple and smooth buildup and/or decay curve is expected in a time series, look for significantly nonsmooth behavior across time points for specific isotopologues. If significantly deviated replicates are detected, decide whether to exclude these replicates or repeat the experiments.

5. Save each metabolite spreadsheet as a separate tab delimited text file.

6. Copy the text files to a computer running the Linux operating system and the natural abundance stripping algorithm (52). If the text files originated from a computer running the Windows operating system, use the command dos2unix to convert each DOS text file to a UNIX text file.

7. Run the natural abundance stripping algorithm on each metabolite text file to remove isotopologue intensity arising from the natural isotope abundances.

4. Notes

This section is intended to provide notes for successful execution of the procedures; we have embedded some practical guidelines with the procedures. However, even the perfect execution of an analytical procedure is “unsuccessful” if it is inappropriate or inadequate for the intended experimental design. With the network of intersecting and parallel pathways in metabolism often utilizing identical metabolites, we have found that SIRM requires a full interaction of the analytical design with the experimental design. Therefore, in this section we will outline overarching considerations for successful SIRM analyses, the topic of this chapter.

The first consideration is to recognize that analysis of the “metabolome” is one of the most chemically diverse – and therefore one of the most demanding – analytical tasks. Consider the following:

a) The metabolome consists not just of metabolites that are matched to the subject organism proteome, but also include xenobiotic compounds such as nutrients, toxins, non-nutrient metabolites from the environment and food, metabolites from co-habitating organisms (e.g. gut fauna, disease), non-enzymatic degradates of metabolites (oxidized compounds), as well as synthetic compounds such as drugs and pesticides.

b) Inorganic carbon (e.g. CO_2) as well as non-carbon compounds (e.g. NO) can be key metabolites to analyze for a given experimental design (79).

c) Not all metabolites are “small” molecules. For example, key metabolites to analyze might be macromolecular pools impacting major metabolic pathways such as amylose or glycogen (44) or metabolic pathways of the essential amino acid selenocysteine that must account for its pool in proteins (80, 81). Note that these cases mentioned call for analysis of macromolecules with a highly diffuse

structure, sequence, branching, and molecular weight, and thus not readily amenable to the typical macromolecular (e.g. proteomics, biophysics) approaches.

d) Metabolites range 12 orders of magnitude in abundance from signaling to structural compounds, many of them transient, posing an unprecedented analytical challenge.

e) Determining compartmentalization and localization, in combination with all of the above issues, may often hold the key to a metabolomic study (76, 82).

The second consideration follows from the first, namely that a handful of defined analyses cannot provide the necessary metabolome coverage for many experimental designs. Even for a very limited SIRM study keying on external ^{13}C glucose and primary metabolism, a particular experimental design may require accounting for the pools of e.g. ^{13}C alanine (via glycolysis and pyruvate) in proteins, transient storage of the ^{13}C glucose in glycogen, and generation of $^{13}CO_2$. Thus a standard list of analytical procedures may not address such information needs.

Acknowledgements

This work was supported in part by National Science Foundation EPSCoR grants# EPS-0447479 (TWMF) and EPS-0132295 (R.J. Wittebort), NIH NCRR 5P20RR018733 (D.M. Miller, ANL), 1R01CA118434-01A2, 1RO1CA101199-01, R01ES022191-01 and 3R01CA118434-02S1 (TWMF), R21CA133668-02, and P01CA163223-01A1 (ANL) from the National Cancer Institute, the University of Louisville CTSPGP/ARRA grants 20044, the Kentucky Lung Cancer Research Program (OGMB090354B1 and OGMB101380) (TWMF and ANL), the Robert W. Rounsavall Jr. Family Foundation, the Kentucky Challenge for Excellence. We thank Jin Lian Tan, Alex Belshoff, Katherine Sellers, and Radhika Burra for technical assistance and/or comments on the manuscript.

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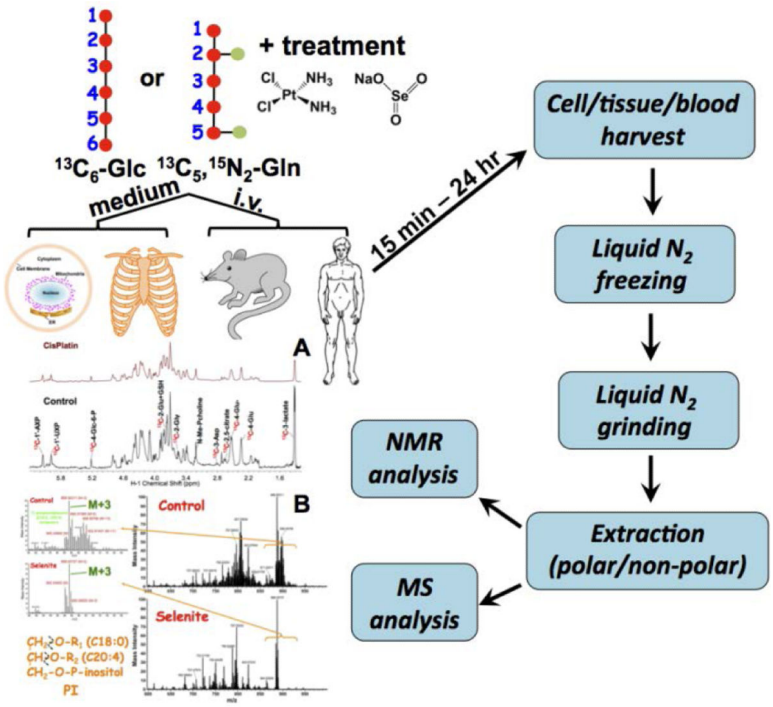

Figure 1.

Stable isotope-resolved metabolomics (SIRM) pipelines. Redrawn from ref (15). SIRM approach is implemented by administering stable isotope tracers such as uniformly ^{13}C-labeled glucose ($^{13}C_6$-Glc) or uniformly ^{13}C,^{15}N-labeled glutamine ($^{13}C_5$,$^{15}N_2$-Gln) to cell cultures, excised tissues, animals, and even human subjects. Therapeutic agents can be included in the treatment to observe their impact on the metabolic networks. The tracers are allowed to be metabolized in situ for a period appropriate to the experimental design, followed by cell harvest or tissue resection via surgery. Polar and lipophilic metabolites are extracted and analyzed by NMR and MS for labeling patterns of various metabolites. Illustrated are the 1-D 1H-11-47 HSQC NMR spectra of polar extracts acquired from control and cisplatin-treated human lung adenocarcinoma A549 cells (A) and the high-resolution exact mass FT-ICR-MS spectra of lipid extracts obtained from control and selenite-treated A549 cells grown in $^{13}C_6$-Glc tracer (B). M+3: represents excess of three ^{13}C neutron masses, which corresponds to $^{13}C_3$-glycerol backbone; PI: phosphatidylinositol; curved dashed arrows: cleavage of fatty acyl chains by tandem MS to confirm C18:0 (stearate) and C20:4 (arachidonate) acyl chain composition. Peaks with exact mass higher than M+3 in the insets are derived from ^{13}C-labeled fatty acyl chains. They are greatly reduced under selenite treatment.

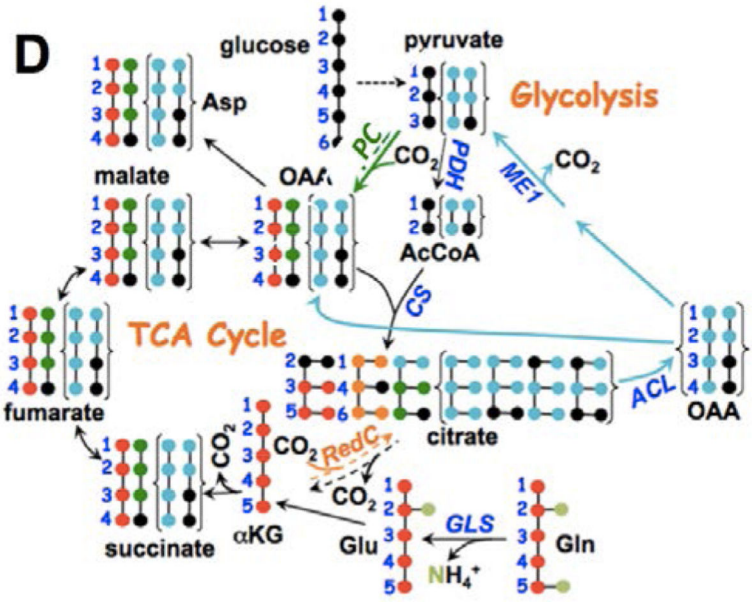

Figure 2.

Fate of ^{13}C and ^{15}N from fully labeled glutamine through the TCA cycle and ACL-ME pathway. Redrawn from ref (25). Red: ^{13}C fate from glutamine in the first forward TCA turn; Light green: ^{15}N; light blue: ^{13}C derived from the glutamine tracer via the ATP-citrate lyase (ACL)-malic enzyme (ME) pathway in the cytoplasm; orange: ^{13}C labeling of citrate +5 from reductive carboxylation (RedC); Green: ^{13}C labeling of TCA cycle intermediates catalyzed by pyruvate carboxylase (PC); double headed arrows: reversible reactions; dashed arrows: multiple step reactions; not all possible ^{13}C isotopologues of the cycle intermediates are shown; the ^{13}C isotopologues of malate, fumarate, and succinate depicted include those from the reverse direction of OAA to succinate.

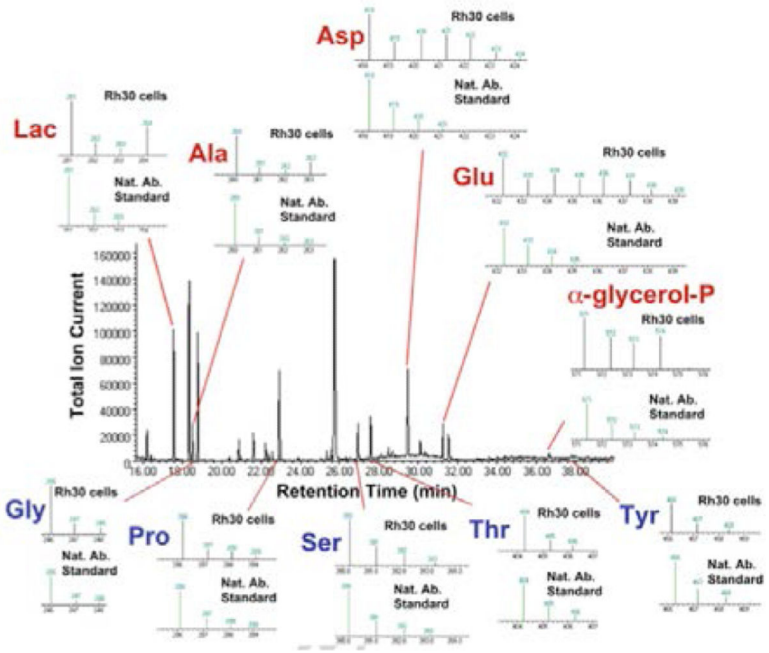

Figure 3.

GC-MS analysis of silyl-derivatized organic and amino acids, revealing isotopologue patterns from crude extracts of rhabdomyosarcoma cells grown in the presence of [U-^{13}C]-glucose. For each metabolite, the corresponding molecular ion region of the tert-butyldimethylsilyl derivative is shown for analyses of both natural isotopic abundance chemical standard and cellular extract ("Rh 30 cells"). The total ion chromatogram is shown for the Rh 30 sample while the chromatogram for the standards is not shown. By comparison of the spectra, it is immediately apparent that some metabolites are heavily and multiply labeled (red lettering) while others are not (blue lettering), rapidly providing a glimpse into the glucose-utilization networks by these cancer cell lines. This would not have been apparent using unlabeled cell cultures, which would instead require extensive experimentation, tedious quantification of concentrations, and modeling of pathways to interpret the concentrations, just to obtain a similar glimpse. Redrawn from ref (49).