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Applications of Chromatography-Ultra High-Resolution MS for Stable Isotope-Resolved Metabolomics (SIRM) Reconstruction of Metabolic Networks

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Abstract

Metabolism is a complex network of compartmentalized and coupled chemical reactions, which often involve transfers of substructures of biomolecules, thus requiring metabolite substructures to be tracked. Stable isotope resolved metabolomics (SIRM) enables pathways reconstruction, even among chemically identical metabolites, by tracking the provenance of stable isotope-labeled substructures using NMR and ultrahigh resolution (UHR) MS. The latter can resolve and count isotopic labels in metabolites and can identify isotopic enrichment in substructures when operated in tandem MS mode. However, MS² is difficult to implement with chromatography-based UHR-MS due to lengthy MS¹ acquisition time that is required to obtain the molecular isotopologue count, which is further exacerbated by the numerous isotopologue source ions to fragment. We review here recent developments in tandem MS applications of SIRM to obtain more detailed information about isotopologue distributions in metabolites and their substructures.

Keywords

Ion chromatography; ultra high-resolution FT-MS; data independent MS²; ¹³C/¹⁵N positional isotopologues; multiplexed stable isotope resolved metabolomics (mSIRM); pathway reconstruction; nucleotides

1. Introduction

1.1 Metabolic network analysis via Stable Isotope Resolved Metabolomics (SIRM)

Metabolomics is the study of intracellular and extracellular metabolite profiles in biological systems, from which a systematic understanding of molecular physiology in cell, tissue, or organisms can putatively be achieved [1]. As such, metabolomics plays important roles in

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biomarker discovery for disease diagnosis/drug responses [2–4] and metabolic pathway analysis in response to perturbations [5, 6].

However, the reconstruction of metabolic pathways based on total metabolite profiles is challenging as given metabolite levels are governed by many factors including rates of synthesis and degradation, multiple input and output pathways, and compartmentalized reactions including exchanges across compartments [7]. That is, metabolism is a complex network of coupled chemical reactions, which transfer or alter substructures of molecules, requiring that metabolite substructures to be tracked. To greatly reduce the ambiguities in metabolic network analysis, the use of stable isotope tracers coupled with metabolomics analysis such as stable isotope-resolved metabolomics (SIRM) has been developed to track individual atoms in precursors through metabolic transformations (cf. Figure 1) most effectively using a combination of MS and NMR methods. This approach has been successfully applied to rigorously define and uncover reprogrammed metabolic activities in response to perturbations in a wide range of biological systems ranging from microbial systems [8–11] to the full gamut of mammalian models including 2D/3D cell cultures [12–21], human tissues *ex vivo* [22–24], patient-derived xenograft (PDX) mice and other models *in vivo* [25–27]) to human subjects *in vivo* [23, 28–35]. Such studies of course must be prospective in nature, and isotope labeling may not always be feasible in large cohort studies and non-experimental settings such as natural populations of organisms.

In and of itself, stable isotope tracing does not imply flux analysis, though it greatly facilitates the mapping of metabolic pathways which is an essential prerequisite- in its simplest form the SIRM approach permits the tracing of atoms from source through metabolic pathways, but does not directly provide flux information. In contrast, metabolic flux analysis, is explicitly directed to measuring (usually relative) fluxes through metabolic pathways, for which there are numerous mathematical approaches, all of which require an explicit metabolic model [36] which may require metabolic, and/or isotopic steady state [37], or with greater flexibility dynamic non-steady state systems at a much higher experimental and computational cost [38–42]. The use of the loose term “fluxomics” often conflates flux (rate) with tracers, and implies the study of flux. However flux analysis is a complex field in its own right, here we restrict ourselves to the use of tracers to define metabolic pathways in an experimental setting.

SIRM studies best utilize a combination of NMR and MS methods to define the number (isotopologues) and position(s) (isotopomers) of label atoms in as many metabolites as practical. Positional information can be very useful for distinguishing routes of carbon and nitrogen in from multiple sources in both catabolic and anabolic metabolism, which can be greatly enhanced by using multiple isotopes in the same experiment [43–45]. NMR has the advantages in isotope-selective detection, positional isotopomer determination, robust standard-free quantification, and is non-destructive [46]. However, it may not have sufficient sensitivity and resolution for detecting certain labeled metabolites [47]. In comparison, MS has much lower detection limits with small sample size requirements and high metabolite resolution, but is destructive and does not readily yield label position information [1, 48].

A typical SIRM workflow is shown in Scheme 1. The basic workflow for SIRM experiments is the same as for non-tracer metabolic experiments, and begins with experimental design. For SIRM this requires consideration of which tracers to use to address optimally the most important hypotheses proposed. Starting with an assumed metabolic network model, particular metabolites and expected isotopes can be preselected in data analyses for testing the main or secondary hypotheses, which avoids unrestricted discovery mode by reducing the multiple testing statistical problem of untargeted metabolomics. Unlike the data analysis, the data acquisition itself can be as broad or narrow as desired as determined by the instrument capabilities and subject to quality assurance (QA) and control (QC) considerations. That is, far more metabolites and their isotopologue distributions can be detected and quantified than is needed for testing the main hypotheses. The remaining data can either be used for cross validation, or for further discovery and hypothesis generation (which would likely require new sets of experiments, possibly with different isotopically enriched sources). The choice of enriched precursors for evaluating different metabolic pathways has been discussed elsewhere [44, 49, 50]. Polar (water soluble) and non-polar (predominantly lipids) are readily partitioned using the Folch method. Generally, both should be analyzed as complex lipids are made from Acetyl CoA and several other common polar metabolites deriving from e.g. glucose or amino acids, including glycerol (phosphate) serine, inositol among other intermediates of central metabolism. Analysis of the media or blood sampled at multiple time points post administration affords a means of assessing nutrient uptake and compound excretion, including lactate and alanine, but also other amino acids that are commonly exchanged across the plasma membrane [51–53]. With blood from animal experiments it is possible also to assess metabolic scrambling though inter-organ transfers. Compound identification is based on accurate mass, fragmentation patterns and retention times in mass spectrometry [54], and preferably with comparison to standards [55]. Whenever feasible internal standards are used for quantification, such as isotope labeled compounds that do not interfere with those labeled in the experiment. Good practice for QC and QA must be followed including blanks and controls interspersed between samples, which should be randomized [56].

Similarly, NMR based identification is based on chemical shifts, coupling patterns and with 2D NMR molecular connectivity. With $^{13}\text{C}/^{15}\text{N}$ enrichments, a wide range of spin-topology-based experiments is possible with NMR to identify the molecules and its isotopomer patterns [50, 57, 58]. Standards for all possible isotopologues are generally not available, but identification of isotopologues from the accurate mass increments is relatively straightforward [1, 59].

This review will focus on the current state of SIRM employing tandem MS for identifying isotope labeling patterns in metabolism experiments.

1.2 LC-MS/MS

Separation technology such as – but not limited to – liquid chromatography (LC) [60–64], gas chromatography (GC) [65–68], or capillary electrophoresis (CE) [69, 70] are deployed to effectively introduce a nominally interference-free sample to the MS. However, they each have limitations (cf Table 1). For example, high-resolution LC-MS methods can analyze a

wide range of metabolites; but since it still has difficulty in separating polar and charged structural isomers with common LC method [71], samples may need to be analyzed twice with Reverse-Phase Liquid Chromatography (RPLC) and Hydrophilic Interaction Liquid Chromatography (HILIC) in order to achieve the reasonable metabolome coverage [72]; GC-MS is well-suited for analyzing volatiles or semi-volatile derivatives of amino acids/organic acids/sugars with high chromatographic resolution but the latter requires derivatization and even so, polar metabolites such as hexose phosphates, nucleotides, and permanently charged cholines remain low in volatility and cannot be analyzed [73]; CE-MS is a useful technique for profiling highly polar and charged metabolites but its application is currently limited by sensitivity and/or reproducibility for metabolomics applications [74] [75, 76]. More recently ion mobility devices interfaced to MS have been incorporated into metabolomics workflows [77, 78], which can have high sample throughput, and high chromatographic resolution, albeit currently with relatively low mass resolution ($<200,000$) that is unsuitable for mSIRM.

LC MS/MS using different high resolution separation columns such reverse phase C_{18} , C_8 , or hydrophilic columns such as HILIC [54] are generally the workhorse of metabolic profiling, though the push for ever higher resolution chromatography increasingly constrains the mass resolution attainable by the MS. In addition, global untargeted MS/MS is impractical for SIRM studies because there are far more features than the unlabeled cases due to the presence of labeled isotopologues and their adducts in addition to chemical noise [79, 80]. For example, from our typical ^{13}C , ^{15}N , 2H mSIRM experiment, there are $>14,500$ targeted analytes in MS^1 across 88 metabolites, an average of about 165 co-eluting analytes per chromatographic peak, such that sub-ppm resolving power in MS^1 is required to quantify these isotopologues before any MS/MS is engaged. These isotopologues are not independent metabolites (so they are not independent variables), instead they represent time-integrated interweaved biosynthesis/utilization and are vital to pathway reconstruction.

Tandem MS is often used to increase the reliability of compound identification [54]. Where isotopologue distributions in metabolic subunit(s) are required, tandem MS is recommended [81], and is greatly enhanced by ultrahigh-resolution (UHR) MS ($>200,000$). UHR-MS is compatible with ion chromatography (IC) MS in anion mode, which was introduced for metabolomics studies with excellent separation properties for charged molecules such as those found in glycolysis, the pentose phosphate pathway, the Krebs cycle and hexosamine pathway [82]. This is enhanced by the fact that anion IC is adduct-free, mobile phase gradient-free, and has extremely low electrospray ion suppression presentation of chromatographic peaks to the MS. The combination of IC with tandem UHR-MS in the context of SIRM studies [83, 84] is the next logical and exciting new development that will be described below with specific applications.

1.3 Ion Chromatography (IC) coupled with ultrahigh-resolution Fourier transform mass spectrometry (UHR-MS) enhances stable isotope tracing for metabolic networks

For elucidating metabolic networks, it is highly advantageous to multiplex biologically compatible tracer atoms such as ^{13}C , ^{15}N and 2H [85] in the same (e.g. $[^{13}C_5, ^{15}N_2]$ -Gln) or different substrates (e.g. $[^{13}C_6]$ -glucose + $[^{15}N_2]$ -Gln) to expand the metabolic pathway

coverage [45]. Moreover, the expanded coverage for each sample circumvents sample batch effects, helps to avoid confounding data due to biological variability, and the labeling provides a hedge against (but does not eliminate) contamination from unlabeled sources; non-SIRM experiments have little recourse to address the latter. However, the neutron mass difference between these tracer atoms (e.g. mass = 0.006995 Da for ^{13}C and ^{15}N) requires UHR-MS to reliably resolve them [1, 13]. One approach to implement UHR-MS is direct infusion UHR-FTMS with a resolving power setting of >200,000 at 400 m/z in SIRM or multiplexed SIRM (mSIRM) studies [86, 87]. However, direct-infusion UHR-MS cannot readily resolve isomers because they have identical mass to charge ratio (m/z) such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) (m/z 259.02244) or UDP-GlcNAc and UDP-GalNAc (m/z 606.07429). The distinction between such metabolites could be crucial in metabolic studies. In addition, the presence of labeled isotopologue species can multiply the number of molecular ions by one to two orders of magnitude, with often one to two orders of magnitude difference in abundance, which creates serious data analysis problems due to overcrowding and ion suppression [71], not to say of taxing the limits of data processing numerous co-eluting ions. For example, a typical ^{13}C , ^{15}N , ^2H mSIRM experiment in our laboratory has >450 isotopologues for just ATP alone, and requires resolution of >14,500 isotopologue target analytes per sample.

The required analyte resolution is achieved by coupling IC to UHR-MS, which affords sufficient resolution and sensitivity under electrospray MS for analyzing polar and charged organic and inorganic compounds. It is particularly compatible with electrospray MS detection due to the decades of steady improvement of the eluent suppressor, which removes the mobile phase salt (e.g. KOH for anion exchange) from column eluent, thereby greatly reducing electrospray ion suppression while presenting analytes under relatively gradient-free, adduct-free conditions to the MS, and thus improving the MS quantification limits [71, 88, 89].

For SIRM studies, medium resolution IC is best suited because of the need for long-scan-time UHR-MS¹ to first analyze any and all isotopologues of metabolites bearing single or multiple tracer atoms. A much-overlooked need for metabolomics in general has been the analysis of inorganic anions such as nitrate, nitrite, sulfate, and phosphate, and the current IC implementation also accomplishes this task simultaneously with separation of the polar organic metabolites. The IC-UHR-MS method has been successfully applied to analysis of a large number of charged metabolites and their isotopologues, including amino/organic acids, glutathione, sugar phosphates, nucleotides, inorganic anions, and sugar nucleotides [18, 22, 71, 89–94], as stated earlier currently totaling >14,500 targeted analytes per injection.

2. MS² analysis for determining positional labeling

Many single isotope-enriched metabolites can be readily determined by various chromatography-MS platforms including IC-UHR-MS operated in MS¹ full scan mode [22, 26]. However, this provides no information on the label position in metabolites [1], which limits the ability to reconstruct intersecting or cyclical, coupled chemical reactions that comprise the metabolic pathways.

For example, UDP-GlcNAc comprises four organic metabolic subunits that are derived from several intersecting metabolic pathways (Figure 1) [95, 96]. The uracil ring (subunit U in Fig 1) is synthesized from glucose and glutamine via aspartate through the Krebs cycle and pyrimidine synthesis; the ribose ring (subunit R in Fig 1) can be generated from glucose via the pentose phosphate pathway (PPP); the glucosamine unit (subunit G in Fig 1) is derived from glucose and glutamine through the hexosamine biosynthesis pathway (HBP); and the acetyl group (Subunit A in Fig 1) can be derived from multiple sources, including glucose, glutamine, and fatty acids [96]. Without the use of tracers such as [$^{13}\text{C}_6$]-glucose or [$^{13}\text{C}_5$, $^{15}\text{N}_2$]-Gln and label number information, it is not tenable to reconstruct the activity of the multiple biochemical pathways leading to UDP-GlcNAc synthesis.

Rigorous interpretation on the intersecting pathways can be accomplished by obtaining information on the isotopically enriched positions via fragmenting metabolites in the MS² mode and analyzing the isotopologue distributions of relevant fragmented metabolic subunits [81, 96–98].

2.1 Need for Data Independent Mode to perform MS² analysis for profiling isotopologues of fragments

Data-dependent analysis (DDA) and data-independent analysis (DIA) are two main strategies for targeted and untargeted identification and quantification of metabolites in metabolomic studies [99–102]. Although the DDA method can provide accurate and sensitive mass information on both precursor and product ions in a single LC-MS run [103], it suffers from poor reproducibility and insufficient MS/MS coverage [104–106] since only those precursor ions that meet defined criteria after MS¹ scans can be selected for fragmentation, which is stochastic under the DDA method. Unlike in direct infusion, the signal intensity of every compound in each LC-MS¹ scan varies across the chromatography peak, which may give rise to different precursor ions selection and thus poor reproducibility between runs [106]. In addition, the most abundant ions in the full MS¹ scans are typically chosen for fragmentation, while many relevant low abundance species often elude fragmentation [105]. The latter is not a serious issue since for non-SIRM studies, because the natural-abundance isotopologues can be ignored. In contrast, for SIRM studies the co-eluting dozens of often low-abundance isotopologues are the very ions sought after for MS/MS.

Alternatively, DIA in principle can activate fragmentation of all ion species regardless of their abundance and m/z . This is because for each cycle, all detectable precursors within a pre-defined m/z window are selected, fragmented, and analyzed. However, fragmenting all precursor ions in a wide window loses the direct link between precursor and their product ions, which makes the MS² spectra far more difficult to analyze. Nevertheless DIA has the important advantages of reproducibility and accuracy for fragment identification and quantification while requiring much less acquisition time than DDA [107].

When applied to SIRM studies, DIA offers the capability of fragmenting any and all isotopologue ions of given metabolites regardless of their abundance, which is impractical to perform with DDA but crucial to the determination of isotopic label position(s) in metabolites. Because the UHR-MS¹ must continue to be acquired to quantify the total

isotopologues, it is highly desirable to acquire UHR-MS¹ and MS² simultaneously, especially when considering sample limitations in mSIRM studies and lengthy IC injection cycles (60 min per sample). Unfortunately, the UHR-MS¹ data require relatively long acquisition times, so that very limited time is available for MS² data acquisition in each cycle, which greatly restricts the number of ions that can be fragmented via DDA. These considerations compelled us to choose DIA-MS² for developing the method for the IC-UHR-MS-based positional isotopomer profiling. As there is presently no literature on this next-level approach for SIRM, we illustrate below the principles of the method and its biochemical interpretive values.

2.2 Ultra High Resolution Mass spectrometry with Data-independent measurements in SIRM studies

For targeted fragment quantification of major metabolites in polar extracts, DIA can be performed in the very restricted time between full MS¹ scans. DIA is best suited for limited biological samples and much greater isotopologue coverage for pathway interpretation. Thus, we developed a full scan-DIA data acquisition method balancing full high resolution MS¹ and MS² scans to generate sufficient spectral information on both precursor and fragments. The following requirements needed to be met: (1) the cycle time of at most 2–3 seconds in order to acquire 10–15 points across chromatographic peaks for reliable quantification of precursors and their isotopologues; (2) sufficient resolving power in full scan (500,000) and MS² (60,000) modes to separate isotopologues of precursors and fragments; (3) full isotopologue coverage for each metabolite in selecting the precursor mass range for MS² scan. To meet those requirements, the Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific) was employed. For full MS scan, the settings were 500,000 resolution, automatic gain control (AGC) target at 2.0e5, *m/z* range of 80 to 700 and the maximal injection time of 100 ms. The heated electrospray source parameters were as follows: the sheath gas flow rate was 35 Arbitrary units (Arb), auxiliary gas flow rate was 4 Arb, negative ion mode voltage at –2.8 kV, vaporizer temperature at 400 °C, and the ion transfer tube temperature at 300 °C. For DIA, precursor mass range is set to 280–440 *m/z* with the quadrupole isolation window of 200 *m/z*, HCD stepped collision energies of 25/30/35, detection with Orbitrap with its resolution set to 60,000, scan range of 50–650 *m/z*, S-lens at 60%, AGC target at 5.0 e4; maximal injection time of 100 ms, microscan of 1, and EASY-IC for internal mass correction.

2.3 Data analysis and quantification

The precursor-product ion spectra acquired for individual metabolite standards were analyzed to establish an in-house compound database. Metabolomics databases, including Human Metabolome DataBase (HMDB) [108], Kyoto Encyclopedia of Genes and Genomes (KEGG) [109] and METLIN [110] as well as Mass Frontier were used to help determine potential fragments for aiding spectral interpretation. Of course, none of these databases contained the MS patterns for the numerous biochemically produced isotopologues, nor any hint of their positional isotopomer ions. For example, just for reduced glutathione there are 351 isotopologues of a ²H+¹³C+¹⁵N mSIRM experiment that must be detected under MS¹, translating to thousands of MS² patterns. Our in-house empirical database was incorporated into TraceFinder v3.3 (Thermo Scientific) to assign and obtain the peak areas of precursor

ions in MS¹ spectra and fragment ions in MS² spectra of targeted metabolites for further quantification. Mass accuracy was set to 5 ppm for both precursors and fragments. Other than TraceFinder, some open source software packages and platforms, such as Skyline [111], EI-Maven [112], *etc.*, could be adapted for data analysis after necessary format conversion. A critical step is visual curation of integrated peaks, because the isotopologue molecular ion cluster for a single metabolite can stretch out over hundreds of m/z, making interference with some of them likely. Very critically, peak areas of isotopologues were corrected for natural abundance as previously described, which yields the experimental isotope-enriched intensities [96, 113]. Other natural abundance correction algorithms can be used [114, 115]. However, for multiplexed SIRM experiments where ultrahigh resolution is required, an exact analytical solution is not possible [116], requiring iterative algorithms that has been validated for at least three isotopes simultaneously [43, 117].

2.4. Isotope enrichment distributions of metabolites from MS¹ and MS² for metabolic pathway reconstruction

Isotope enrichment distributions of major metabolites from glycolysis, the Krebs cycle, PPP, and nucleotides metabolism can be obtained from the UHR-MS¹ and MS² spectra of [¹³C₆]-glucose and [¹³C₅,¹⁵N₂]-Gln traced samples. Here we show an example of glycolytic and gluconeogenic products to illustrate the principles.

Glycolysis converts glucose to pyruvate via a series of phosphorylated intermediate metabolites, whereas gluconeogenesis converts 3-carbon metabolites via PEP to glucose, ordinarily in the liver and kidney (Fig. 2). These organs produce glucose for maintaining tissue homeostasis. However, many other tissues are also gluconeogenic, except that they do not express the phosphatase, thus retaining the gluconeogenic products in the cell for anabolic purposes such as glycogen synthesis from G6P, PRPP synthesis (from G6P or F6P) and NADPH generation (from G6P) via the pentose phosphate pathway, as well as glucosamine synthesis (from F6P) via the hexosamine pathway.

The IC column cannot resolve glucose from other neutral hexoses, which elute as a single peak right after the void volume. However, the negatively charged phosphorylated intermediates were resolved by IC except for the isobaric 2-phosphoglycerate (2PG) and 3-phosphoglycerate (3PG) pair, which had similar retention times. We were able to quantify the isotopologue distribution of all other glycolytic products using the MS¹ data i.e. glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6P), 1,3-bisphosphoglucerate (1,3-BPG), phosphoenolpyruvate (PEP), pyruvate, and lactate.

In [¹³C₆]-Glc traced A549 spheroids (Fig. 3A), these products were dominated by their uniformly ¹³C labeled isotopologues, indicating active glycolysis. Selenite treatment altered these isotopologue distributions for G6P, F6P, 1,3-BPG, pyruvate, and lactate, which suggested altered activity of enzymes involved in their metabolism, including hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructose kinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase, and/or lactate dehydrogenase. In addition, low levels of ¹³C scrambled isotopologues of all glycolytic products were evident including ¹³C₂₋₅-F6P (c) -F1,6BP (d), and -G6P (a) (Fig. 3A). The ¹³C labeling patterns of the MS² fragments for these metabolites are consistent with their derivation from PPP via

the transketolase (TK) and/or transaldolase (TA) activity, as pentose phosphate pathway intermediates including S7P, E4P are resolved by IC. The $^{13}\text{C}_3\text{-C}_{1,4-6}$ fragment of G6P (**b**) and $^{13}\text{C}_2\text{-C}_{1-3}$ fragment of F1,6BP (**e**) suggest the presence of the parent $^{13}\text{C}_4\text{-C}_{3,4,5,6}$ and/or $^{13}\text{C}_2\text{-C}_{1,2}$ species (Fig. 3A), which are respective products of the TA and TK reactions. Selenite blocked the production of these ^{13}C -scrambled products of sugar phosphates, which reflected its inhibitory effect on the PPP activity in A549 spheroids.

Turning now to a multiplexed SIRM experiment on [$^{13}\text{C}_5, ^{15}\text{N}_2$]-Gln traced BEAS-2B cells, very low level of ^{13}C enrichment was evident in the glycolytic intermediates (Fig. 3B), which suggested very low levels of gluconeogenesis in these cells. Arsenite significantly depleted the unlabeled intermediates after the GAPDH step, which could result from a block of this and subsequent enzymes in the pathway. It is likely that malic enzyme (ME) was also inhibited as evidenced from the depletion of $^{13}\text{C}_1$ -lactate. However, arsenite did not appear to affect gluconeogenesis based on the ^{13}C labeling patterns of PEP and F1,6BP.

3. Conclusions

For both pathway and flux analysis, tracer methods are indispensable. SIRM and mSIRM analysis based on MS¹ and tandem MS together are particularly valuable for adequate metabolic coverage and localization of heavy atoms in molecules at the metabolic subunit level. To date there are very few workflows described that combine high resolution tandem MS with chromatography, in part due to hardware and software limitations [81, 84], and none have been reported for mSIRM.

We have demonstrated a quantitative IC-UHR-MS¹/DI-MS² method for mSIRM studies in mammalian cells to analyze for metabolite substructures harboring biosynthetic provenance. This method apportioned the required lengthy scan time for UHR-MS¹ with sufficient DI-MS² operation to obtain positional label information. The combined IC-UHR-MS¹/DI-MS² method is both time- and sample-frugal by simultaneously providing the required overall label distribution of metabolites via MS¹ and positional labeling in metabolite subunits via DI-MS² with a single injection, which we illustrates with analysis of glycolysis and gluconeogenesis in human lung cells.

We expect that the general approach of LC-MS/MS combined with NMR and appropriate informatics will be very powerful for analyzing altered metabolic pathways associated with disease states, which will be very valuable for drug discovery and robust disease diagnostics.

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Abbreviations

DDA	Data dependent analysis
DIA	Data independent analysis

CDB	compound database
SIRM	Stable Isotope Resolved Metabolomics
IC	ion chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance
HCD	higher-energy collisional dissociation
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
<i>m/z</i>	mass to charge ratio
UHR	ultra high-resolution

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Figure 1. Metabolic Subunits: UDP-N Acetyl Glucosamine

UDP-N Acetyl Glucosamine is synthesized from a series of sequential and parallel metabolic pathways producing the major substructures glucosamine (G), acetyl (A), ribose (R), and the uracil pyrimidine ring (U). For a summary of the biochemical pathways with provenance of the atoms, please see [95, 96]. The reference illustrates that aspartate, acetyl CoA is derived from pyruvate or fatty acid oxidation, and UTP, which is itself synthesized from pentose phosphate pathways (ribose, R) and the pyrimidine pathways (U) from Asp, Gln and bicarbonate. Carbon sources for Asp may include both glucose and glutamine. PRPP is 5-phosphoribose-1-pyrophosphate.

Figure 2. ^{13}C isotopologues in glycolysis and gluconeogenesis

^{13}C atoms are traced from $[^{13}\text{C}_6]\text{-Glc}$ or $[^{13}\text{C}_5, ^{15}\text{N}_2]\text{-Gln}$ into glycolytic metabolites. G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F1,6P: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde-3-phosphate; 1,3-BPG: 1,3-bisphosphoglycerate; 2 or 3PG: 2 or 3-phosphoglycerate; PEP: phosphoenolpyruvate. The gluconeogenesis-specific enzymes are PEP carboxykinase (PEPCK), fructose bisphosphatase (FBPase) and in liver and kidney, Glucose 6-phosphatase.

Figure 3. ^{13}C isotopologue analysis via FTMS¹ and MS² shows the response of glycolytic and gluconeogenic pathways to selenite in A549 spheroids or to arsenite transformation in BEAS-2B cells.

A. A549 Spheroids. B BEAS-2B cells

^{13}C atoms are traced from [$^{13}\text{C}_6$]-Glc (**A**) or [$^{13}\text{C}_5, ^{15}\text{N}_2$]-Gln (**B**) into glycolytic metabolites. The ^{13}C (●) products in brackets in **A** are derived from PPP and those in **B** come from gluconeogenesis (●) or the ME pathway (●). The polar extracts were analyzed by IC-FTMS¹ and DI-MS². G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F1,6BP: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde-3-phosphate; 1,3-BPG: 1,3-bisphosphoglycerate; 2 or 3PG: 2 or 3-phosphoglycerate; PEP: phosphoenolpyruvate.

Scheme 1. SIRM workflow

The workflow shows the progression from experimental design through sample processing, analytical data collection and analysis and bioinformatics.

Table 1.

Comparison of separation techniques coupled to MS systems used in metabolomics

Separation Technique	Typical Application in metabolomics	Limitations	Separation + MS Compatible with mSIRM	Compatible with UHR MS/MS for SIRM
GC	Volatile compounds, derivatized polar compounds such as amino acids, organic acids, fatty acids	Samples need derivatization	Yes	Severe limitations
LC	Wide coverage of metabolites	Time-consuming to analyze samples twice with RPLC and HILIC	Yes	Yes
CE	Highly polar and charged metabolites	Low sensitivity and/or reproducibility	Yes	Severe limitations
IC	Polar and charged small molecules, such as organic acid, sugar phosphates, nucleotides, etc.	Need separate IC systems to analyze samples in positive and negative ESI modes	Yes	Yes
IMS	Wide coverage of metabolites and lipid classes, especially isomers and isobars	Unproven utility in mSIRM; lack of software tools to interpret multidimensional data	Not Demonstrated	Not Demonstrated
Direct Infusion	“Shotgun” lipidomics, polar metabolites	Isomers difficult to distinguish	Yes	Yes