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Exploring the use of gas chromatography coupled to chemical ionization mass spectrometry (GC-CI-MS) for stable isotope labeling in metabolomics

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Abstract

Isotopic labeling experiments have been utterly valuable to monitor the flux of metabolic reactions in biological systems, which is crucial to understand homeostatic alterations with disease. Experimental determination of metabolic fluxes can be inferred from a characteristic rearrangement of stable isotope tracers (e.g., 13C or 15N) that can be detected by mass spectrometry (MS). Metabolites measured are generally members of well-known metabolic pathways, and most of them can be detected using both gas chromatography (GC)-MS and liquid chromatography (LC)-MS. In here, we show that GC methods coupled to chemical ionization (CI) MS have a clear advantage over alternative methodologies due to GC's superior chromatography separation efficiency and the fact that CI is a soft ionization technique that yields identifiable protonated molecular ion peaks. We tested diverse GC-CI-MS setups, including methane and isobutane reagent gases, triple quadrupole (QqQ) MS in SIM mode or selected ion clusters using optimized narrow-windows (~10 Da) in scan mode, and standard full scan methods using high resolution GC-(q)TOF and GC-Orbitrap systems. Isobutane as reagent gas in combination with both low-resolution (LR) and high-resolution (HR) MS showed the best performance, enabling precise detection of isotopologues in most metabolic intermediates of central carbon metabolism. Finally, with the aim of overcoming manual operations, we developed an R-based tool called isoSCAN that automatically quantifies all isotopologues of intermediate metabolites of glycolysis, TCA cycle, amino acids, pentose phosphate pathway and urea cycle from LRMS and HRMS data.

Introduction

Metabolomics is nowadays a well-established branch of the omics field[1]. The ability to detect and quantify hundreds of different small organic compounds in complex biological mixtures makes metabolomics a powerful source of biological information. Due to the chemical diversity of naturally occurring metabolites, metabolomics is rich in analytical instrumentation and configurations, with GC-MS and LC-MS/MS being the most common techniques[2].

GC-MS has been the analytical platform of choice for measuring volatile compounds for decades[3]. However, with the introduction of chemical derivatization, and the inherent high-resolution chromatographic separations and low MS background noise, GC-MS has evolved into a powerful analytical platform to produce compound abundance data in multiple types of targeted and untargeted metabolomic experiments[4–8]. GC systems are typically coupled to single (GC-sQ) or triple quadrupole mass spectrometers (GC-QqQ) for targeted metabolomics, while untargeted analyses are generally performed using time-of-flight (GC-TOF) or Orbitrap (GC-Orbitrap) mass spectrometers. The most widely used ionization method in GC-MS is electron impact ionization (EI), a hard ionization strategy that generates highly reproducible and characteristic fragmentation spectra[3]. However, softer ionization techniques based on chemical ionization (CI) are alternative methods, where molecular ions of analyzed compounds are kept (mostly) intact[9].

The determination of relative or absolute metabolite concentrations by GC-MS can also be used to investigate the rate at which these compounds are being transformed into other intermediates by metabolic (i.e., enzymatic) activity, a field known as stable-isotope labeling and fluxomics[10]. Stable isotopes, such as ^{13}C and ^{15}N, are used to track the fate of a labeled nutrient, e.g. ^{13}C-glucose or $^{13}C/^{15}N$-glutamine, being a much safer and informative alternative to the formerly used radioactive isotopes[11–13]. Metabolites enriched with stable isotopes maintain the original chemical structure and biochemical properties, but the different isotopic composition (i.e., isotopologues) causes a shift in their mass, which is detectable by MS. In this regard, GC-EI MS has become a platform of choice for stable isotope tracing studies and fluxomics approaches[14–20]. The coverage of GC-EI MS is comprehensive enough to cover most central carbon metabolism (i.e., glycolysis, TCA, amino acids, pentose phosphate pathway) thanks to the use of chemical derivatization. Several software tools for automated analysis of GC-EI MS data are available[21–25], including tools for stable-isotope labeling data analysis[26–32],

which must address the inherent difficulty of deconvoluting the intensity of overlapping isotopologues from EI mass spectral fragmentation. Interestingly, the use of GC-CI MS for stable-isotope tracing is largely unexplored[33–37]. In here, we have studied the suitability of GC-CI-MS for stable-isotope tracing using multiple analytical configurations based on low-resolution (LRMS) and high-resolution (HRMS) mass spectrometry. We prove that isobutane is an ideal ionization gas by its ability to yield intact protonated molecular ions, and coupled to cutting-edge high-resolution GC-MS instruments, currently provide the best configuration for isotopologue quantification. In addition, we have developed an R-package called isoSCAN capable of processing GC-CI MS data from LRMS and HRMS stable-isotope labeling studies.

Methods

Cell cultures

For isotope-labeling experiments, glucose or arginine was replaced with U-13C-glucose (Cambridge Isotope Labs) or U-13C-Arginine (Sigma Aldrich). Refer to previously published work for further details on cell culturing[38,39].

Metabolite extraction

Cells were scrapped, collected and frozen. Cell pellets were resuspended with 300 µl of cold methanol/water (8:1, v/v) containing 13C-glycerol (5 µl/ml) as internal standard. Metabolites were extracted with three rounds of liquid N2 immersion and bath sonication, followed by 1 h in ice before centrifugation at 22,800 × *g* (10 min at 4 °C). The supernatant was set aside for derivatization in a new tube.

Derivatization

Samples were dried under a stream of N2 gas and lyophilized before chemical derivatization with 40 µl of methoxyamine in pyridine (30 µg/ml) for 45 min at 60 °C. Samples were also silylated using 25 µl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Thermo Fisher Scientific) for 30 min at 60 °C to increase volatility of metabolites.

LRMS acquisition

A 7890A GC system coupled to a 7000 QqQ mass spectrometer (Agilent Technologies) was used for isotopologue determination for the low-resolution datasets using SIM and NS acquisition modes. Derivatized samples were injected (1 µl) in the gas chromatograph system with a split inlet equipped with a J&W Scientific HP-5ms stationary phase column (30 m × 0.25 mm i.d., 0.1 µm film, Agilent Technologies). The

injector split ratio was adjusted to 1:5. Helium was used as a carrier gas at a flow rate of 1 mL/min in constant flow mode. In the method to quantify TCA metabolites, the oven temperature was programmed to sit at 70 °C for 1 min and afterwards increase at 10 °C/min to 325 °C. On the other hand, in the polyamines method (Supplementary table 1) the temperature gradient was the following: from 70 to 150 °C at a heating rate of 5 °C/min, from 150 to 250 °C at 10 °C/min and from 250 to 325 °C at 50 °C/min. Metabolites were ionized using positive chemical ionization (CI) with isobutane or methane as reagent gas.

HRMS acquisition

A 7890B GC system coupled to a 7250 QTOF mass spectrometer (Agilent Technologies) was used. Derivatized samples were injected (1 µl) in the gas chromatograph system equipped with an Agilent 19091S-433UI HP5-ms Ultra Inert stationary phase column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies). Helium was used as a carrier gas at a flow rate of 1.5 mL/min in constant flow mode. The temperature gradient used was: from 70 to 190 °C at a heating rate of 11 °C/min, and from 190 to 325 °C at 21°C/min, finally holding for 4 min. Metabolites were ionized using positive chemical ionization (CI) with isobutane as reagent gas. Mass spectral data were acquired in full scan mode (110 – 800 m/z) at and an acquisition rate of 3 Hz.

A TRACE 1300 Series GC coupled to an Orbitrap Q Exactive mass spectrometer (Thermo Scientific) was used for isotopologue determination. Derivatized samples were injected (1 µl) in the gas chromatograph system with a split inlet 1:5 equipped with a TraceGOLD-5MS stationary phase column (30 m x 0.25 mm x 0.25µm film, Thermo Scientific). Helium was used as a carrier gas at a flow rate of 1.5 mL/min.

The temperature gradient used was: from 70 to 190 °C at a heating rate of 11 °C/min, and from 190 to 325 °C at 21°C/min, finally holding for 2.7 min. Mass spectral data were acquired in full scan mode (150 – 750 m/z) and the AGC target was set to 1 million ($1x10^6$). The analyzer resolution settings were 60,000 at 200 m/z. Metabolites were ionized using positive chemical ionization (CI) with isobutane as reagent gas, also low-energy (15eV) and high-energy (70eV) electron impact (EI) ionization was used (Supplementary Figure 1).

Results

Comparing Electron Ionization (EI) with Chemical Ionization (CI) for stable isotope labeling

In order to optimize the detection of isotopologues using GC-MS, we first compared the performance of the most commonly used ionization sources in GC-MS: EI and CI. Furthermore, we tested two different reagent gases for CI, namely methane and isobutane. Taking lactate as a reference metabolite in a complex biological mixture such as 13C-U-glucose labeled and non-labeled cell cultures, we found that CI with isobutane yielded most suitable spectra to detect isotopic enrichments in comparison with EI (Figure 1A). Notably, the use of isobutane as reagent gas produced mass spectra in which the abundance of a metabolite is typically concentrated in its $[M+H]^+$ isotopic envelop, facilitating the detection of isotopic enrichment, unlike methane gas that induced unwanted fragmentation of the $[M+H]^+$ ion (Figure 1B). This observation also applied to all intermediates of the glycolytic pathway and TCA cycle, where the $[M+H]^+$ ion was, on average, >5-fold more intense using isobutane than methane (Figure 1C). In contrast, EI at 70 eV is a hard ionization technique causing extensive fragmentation of compound structures in both non-labeled and labeled samples. This complicates the process of determining the amount of isotope labeling because the isotopic enrichment is scattered throughout multiple fragments. Similarly, low-energy EI at 15 eV[31] also produced greater fragmentation of the molecular ion than CI-isobutane (Suppl. Figure S1).

Finally, we show that CI-isobutane can ionize efficiently most relevant metabolites found in central carbon metabolism pathways, including intermediates of glycolysis, TCA cycle, pentose phosphate pathway, amino acids, urea cycle and polyamines (Supplementary Table1). In summary, CI-isobutane resulted in a broad coverage of metabolites in addition to greater signal intensity of the $[M+H]^+$ molecular ion for all metabolites tested.

Optimizing data acquisition modes in GC-CI MS for stable isotope labeling

The collection of mass analyzers used in hyphenated mass-spectrometry is quite broad nowadays. To test the capabilities of GC-CI MS for stable isotope labeling, we compared the sensitivity, selectivity and isotope ratio fidelity of different mass analyzers and data acquisition modes. In particular, we used a low-mass resolution configuration based on GC-CI triple quadrupole MS, and two high-mass resolution

configurations based on GC-CI qTOF MS and GC-CI qOrbitrap MS to analyze the same labeled and non-labeled complex biological mixtures. In the case of the quadrupole configuration, we explored two acquisition modes to find the most sensitive and selective for isotopologue analysis: selected ion monitoring (SIM) and narrow scan (NS) acquisition.

SIM acquisition method was set to monitor single ions, that is, every isotopologue based on the number of carbons in each metabolite. In contrast, NS acquisition method was set to register a cluster of ions that covers the full isotopic envelope (typically 8-10 Da) of the derivatized metabolite, e.g. in the case of lactate-2TMS (m/z 235) the quadrupole was set to scan a mass range from m/z 232 to 240. Despite the greater signal intensity of NS for all metabolites tested, SIM yielded better signal-to-noise ratios (SNR), likely due to its greater ion selectivity (Figure 2A). The isotope ratio fidelity of NS and SIM, calculated as the relative deviation to the theoretical isotopic ratio, was similar in both acquisition methods, showing a median value of 5% from a total of 14 metabolites detected in 9 cell culture replicates (Figure 2B).

Next, we compared the sensitivity, selectivity and isotope ratio fidelity of two high-mass resolution instruments: a GC-qOrbitrap mass analyzer acquiring in full scan mode at 60.000 resolution (at m/z 200) and a GC-qTOF mass analyzer acquiring in full scan mode at ~16.000 resolution. The median deviation of isotopologue abundances in relation to theoretical natural abundances was 19% for GC-CI qOrbitrap MS and 26% for GC-CI qTOF MS, yet only the Orbitrap mass analyzer was capable of clearly resolving natural isotopes of silicon (29Si and 30Si from the trimethylsilyl derivatization) from carbon, while in the case of the TOF mass analyzer silicon and carbon isotopes were practically indistinguishable and thus were summed up (Figure 2C). As expected, the Orbitrap showed a better mass accuracy (<1.5 ppm) compared to the TOF mass analyzer (<8 ppm).

Interestingly, when the quadrupole of the GC qOrbitrap was set to filter the same cluster of ions that covers the full isotopic envelope of the derivatized metabolites as in NS, with the aim of reducing the ion population in the Orbitrap mass analyzer, we did not observe an increase in sensitivity in comparison with full scan acquisition (data not shown). As expected, both high-mass resolution instruments have the additional advantage that are more selective than low-mass resolution methods SIM and NS, because the former can distinguish artifacts (such as background noise) or compounds that could interfere with the detection of the isotopologues of interest. This is

particularly important when detecting labeled isotopologues of low abundant compounds in biological samples (see aconitate 3TMS and dihydroxy-acetone phosphate 3TMS+MA in Figure 2D).

In summary, our results demonstrate that GC CI-isobutane is an optimal configuration for the detection of isotopologues, and the latest generation of high-mass resolution GC-MS instruments in full scan mode have similar sensitivity than quadrupole-based mass analyzers. In counterpoint, the isotope ratio fidelity is superior in LRMS than HRMS.

isoSCAN: an R package to process GC-CI-MS data from low-mass and high-mass resolution acquisition methods

To extend high throughput capabilities of GC-CI-MS for stable isotope labeling studies, we have developed *isoSCAN*, an open-source R-based computational tool (https://github.com/jcapelladesto/isoSCAN) that encloses a complete workflow to perform high-throughput compound-driven isotopologue quantification in GC-MS data, either from high- or low-mass resolution instrumentation. The performance of isoSCAN has been assayed in several stable isotope labeling studies so far[38–43].

isoSCAN computational workflow is summarized in Figure 3A. isoSCAN requires GC–MS files in open standard formats (mz(X)ML) as input data, and a list of compounds to target with their associated molecular formulas, monoisotopic mass values, and estimated retention times (Supplementary Table1). The software processes each file independently, targeting each singular isotopologue peak of every targeted compound within a specified retention time range through local maxima detection. This process differs slightly from low to high mass resolution data. In the case of low-resolution instruments, *autoQ* computes the isotopologue mass by adding the corresponding enrichment isotope mass difference (i.e., 1.003355 for 13C) as many times as the maximum number of atoms present in the non-derivatized molecular formula (i.e., up to six times in the case of glucose (C6H12O6)). To perform on low-resolution data, such data must be in profile mode, as this facilitates the elucidation of spectral peaks. For high-resolution data, isotopologue quantification supports centroided data. We observed that isotopologue mass calculation need to be refined as mass spectral data may include other isotopic peaks from Si, N or O that are relatively less intense but still must be considered for accurate quantification. Therefore, *autoQ* makes use of enviPat[44] functionalities to calculate and merge the theoretical isotopic distribution given an estimated mass resolution. Each different isotopologue m/z is then searched separately

by minimizing the cumulative relative mass error (ppm) in order to avoid misassignations. Afterwards, isotopologue abundances are added by considering the number of labeled atoms to which each isotopologue corresponds (see Figure 3B for an example in the case of isotopologues M+0 to M+3 of lactate 2TMS).

Discussion and conclusions

MS is currently the preferred analytical technique used for measuring stable-isotope labeling of metabolites. In this regard, GC-MS is characterized by low cost and relatively simple maintenance, which makes it more affordable than LC-MS equipment by many individual laboratories. A desirable scenario of stable-isotope labeling is to measure isotopologue distributions in intact metabolites, however, the inherent fragmentation of EI (the most widespread used ionization source in GC-MS) cause scattered isotope patterns. To minimize fragmentation, different analytical strategies have been implemented, including chemical derivatization with N-methyl N-(*tert*-butylsilyl)trifluoroacetamide (MTBSTFA)[15], EI ionization at 15 eV [31] or the use of chemical ionization[37]. The latter has been traditionally implemented using methane as reagent gas[31], however, isobutane produces superior intact analyte signal abundance to methane[45], resulting also in a predominant protonated adduct ion and less fragmentation. Here we have used this previous knowledge to demonstrate that CI-isobutane outperforms CI-methane, EI at 70eV and EI at 15eV for isotopologue analysis by GC-MS. We have also shown that the metabolic coverage of CI-isobutane allows analysis of most relevant metabolites in central carbon metabolism with good ionization efficiency, including intermediates of glycolysis, TCA cycle, pentose phosphate pathway, amino acids, urea cycle and polyamines. In addition, we have benefited from this better suited ionization source to explore different acquisition modes from the most common mass analyzers in metabolomics: time-of-flight (TOF), orbitrap, and quadrupole.

Quadrupoles act as low-resolution mass filters and can be placed in series (e.g., triple quadrupoles) to measure only a predefined subset of ions, offering in principle the best sensitivity for measuring a single metabolite. Our results showed that monitoring every isotopologue by selected ion monitoring (SIM) or opening the quadrupole 8-10 Da to scan the whole cluster of isotopologues of a metabolite, yielded similar results with regard to sensitivity; that is to say, despite the better signal-to-noise ratios of SIM or the higher absolute intensity counts of the narrow scan (NS) acquisition, the same isotopologues were detected for low abundant metabolites. Remarkably, our results also

indicate that, despite LRMS shows higher isotopic fidelity than HRMS, cutting-edge high-resolution GC-MS instruments improve detection of certain isotopologues in low abundant compounds. Barely distinguishable isotopologues in SIM and/or NS showed a considerably higher and selective response in full scan acquisition mode with HRMS instruments such as the GC Orbitrap, probably as a result of increased capacity to distinguish m/z interferences.

Finally, we have developed a versatile open-source software that can automatically process low- and high-resolution isotopic labeling data produced with GC CI-isobutane coupled to quadrupole, TOF and orbitrap instruments.

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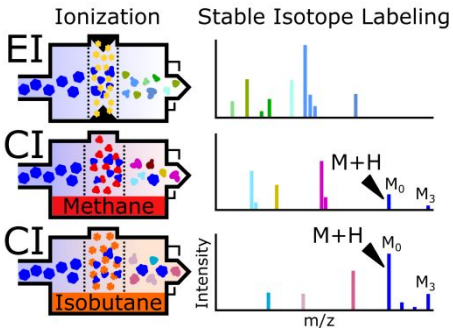


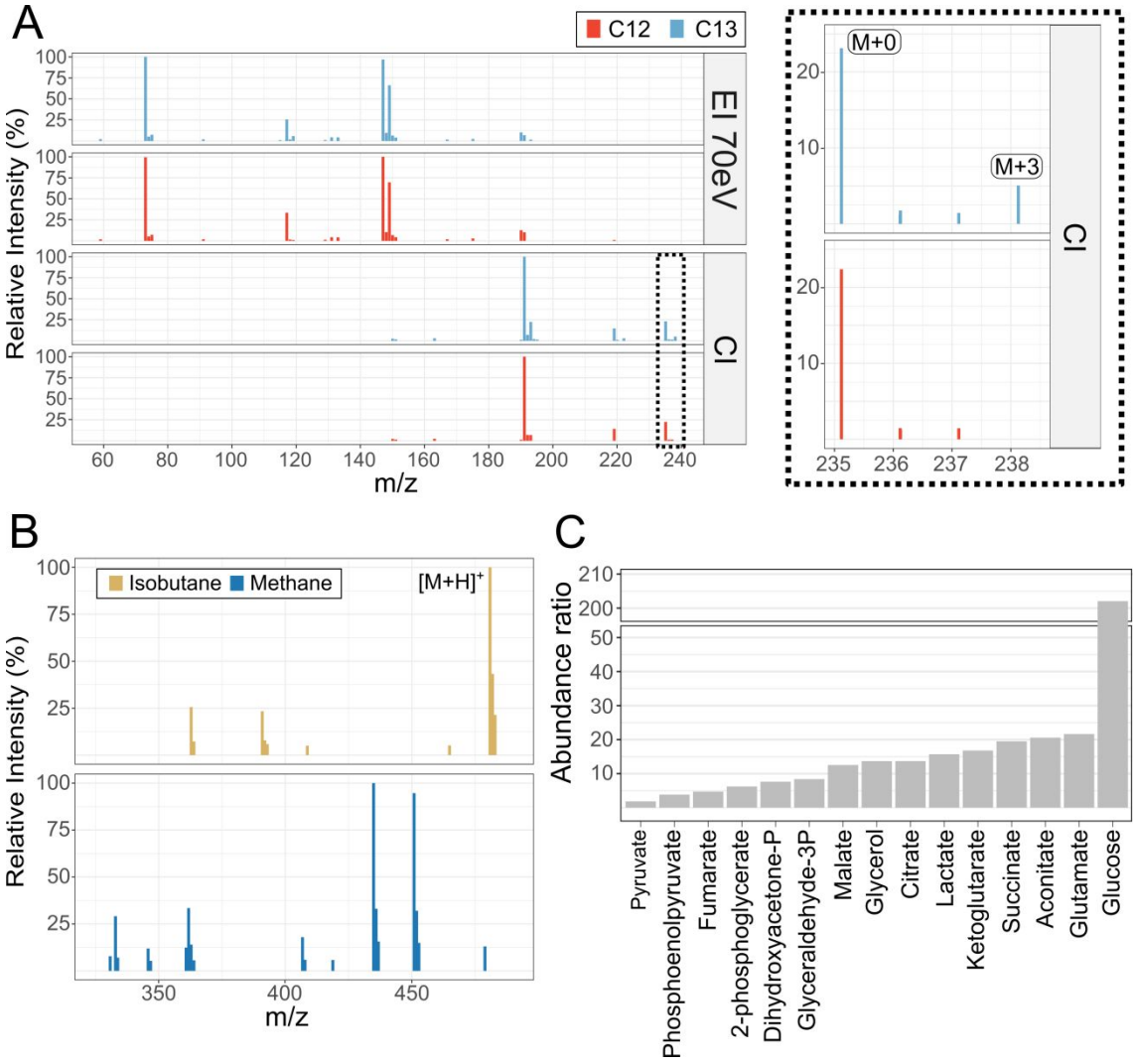


Figure 1. Comparison of EI and CI. (A) Lactate 2TMS spectra obtained from non-labeled (red) and $^{13}C_6$-Glucose labeled (blue) cell cultures. Top spectra show electron impact ionization mass spectral fragments of lactate. Bottom spectra show ions produced by chemical ionization, where the isotopic envelope of intact $[M+H]^+$ ion is squared and zoomed in. (B) Chemical ionization spectra of Citrate 4TMS using isobutane and methane as reagent gases. (C) Protonated $[M+H]^+$ ion abundance ratios (isobutane/methane) of glycolysis and TCA cycle compounds.

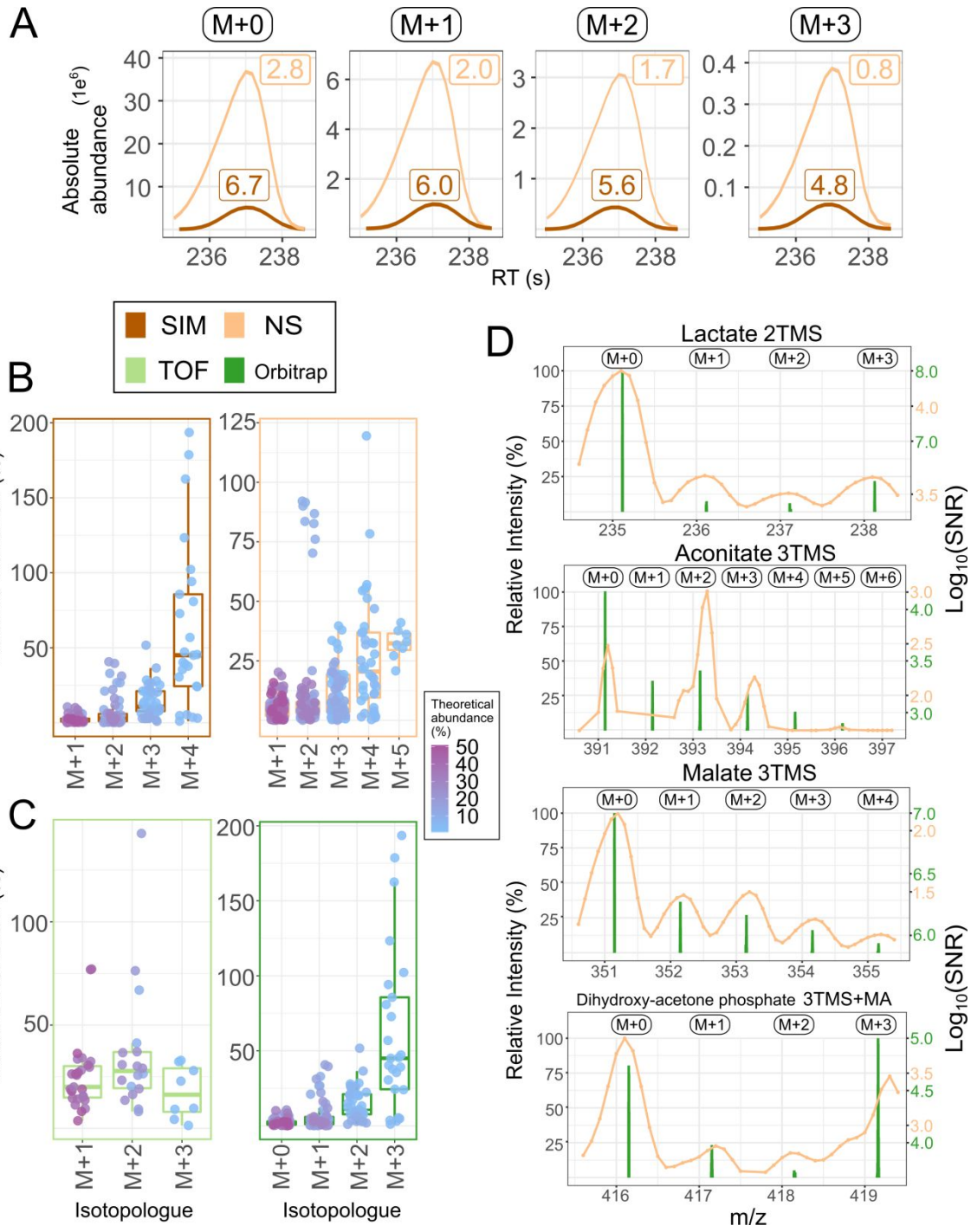
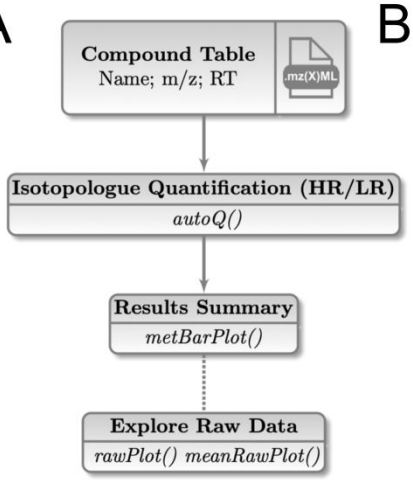


Figure 2. Low-mass and high-mass resolution methods for isotopologue analysis using GC CI-isobutane. (A) Extracted Ion Chromatograms (EICs) of lactate 2TMS isotopologues from NS and SIM acquisition using a QqQ mass analyzer. Squared values are rounded log10 Signal-to-Noise (SNR) values calculated per each isotopologue. (B) Relative deviation of isotopologue abundances in relation to theoretical natural abundances, experimentally calculated from 14 metabolites using

a QqQ mass analyzer (n=9). (C) Relative deviation of isotopologue abundances in relation to theoretical natural abundances, experimentally calculated from 8 and 23 metabolites, using TOF and Orbitrap mass analyzers, respectively (n=5-10). Blue shade indicates the range of relative theoretical abundance per ion. (D) Relative abundances of isotopologue ions of lactate 2TMS, malate 3TMS, aconitate 3TMS and dihydroxy-acetone phosphate 3TMS+methoxyamine (MA) from NS acquisition using a QqQ mass analyzer, and full scan using an Orbitrap mass analyzer. In these, the spectra belong to a 13C-labeled sample.

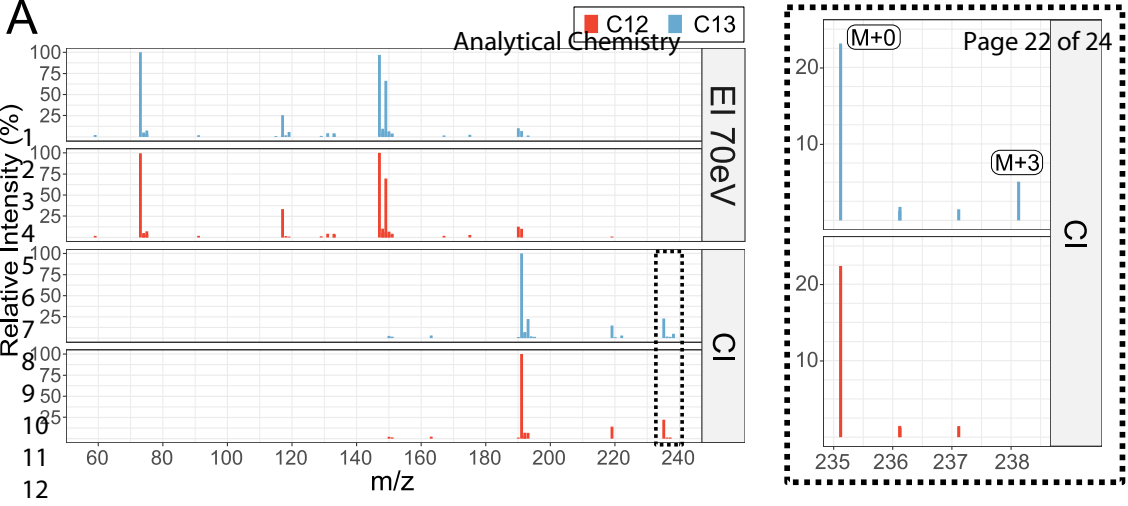
A

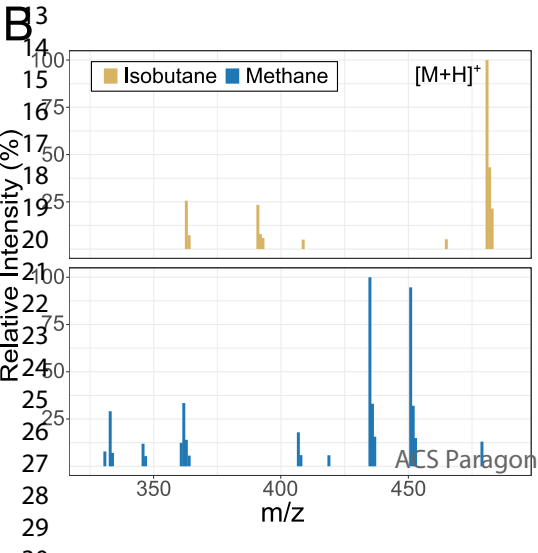


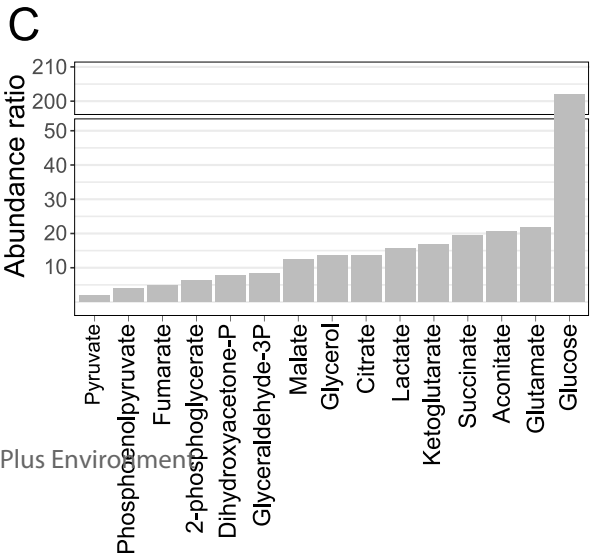
B

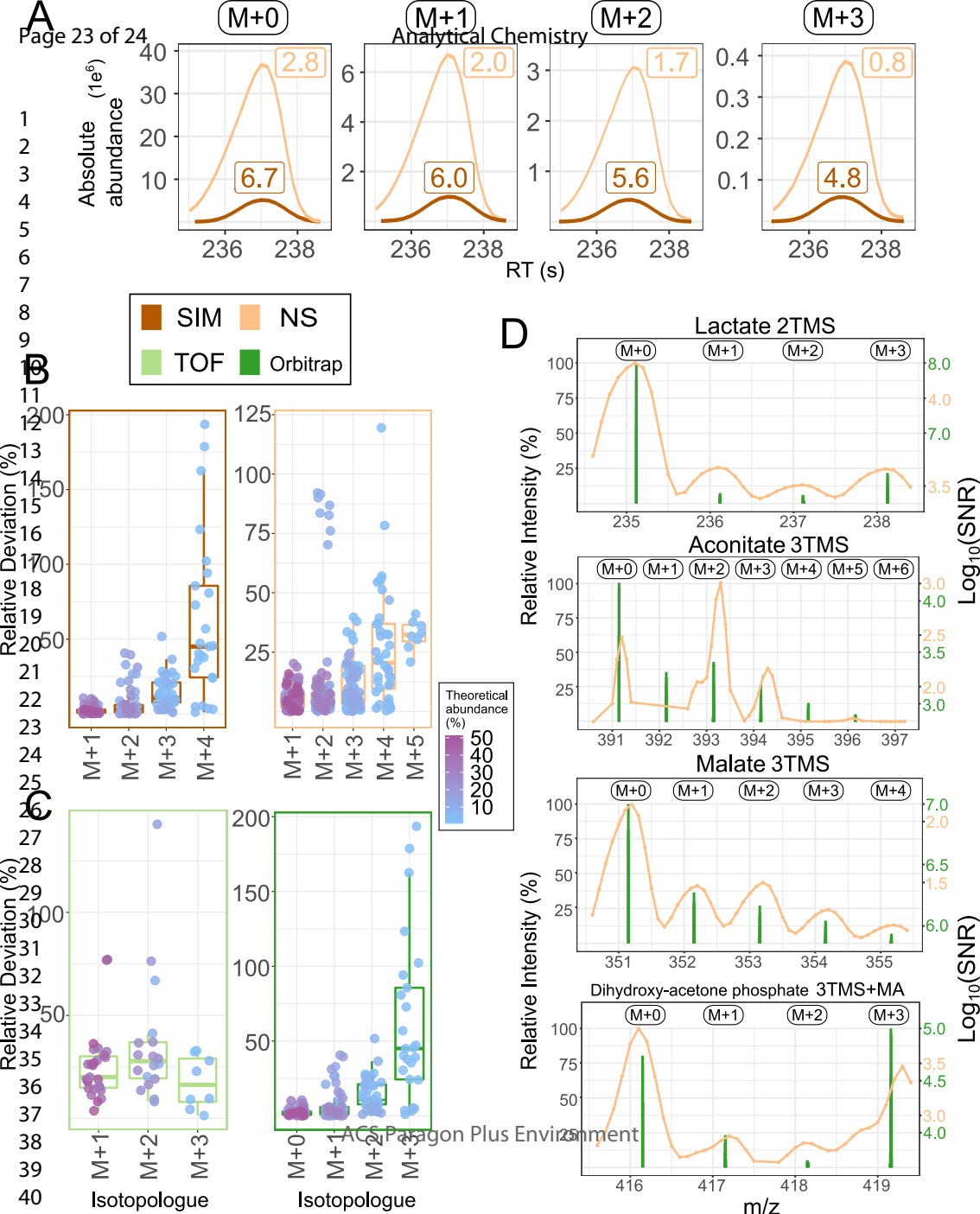
m/z	$^{12}C_n$	$^{13}C_n$	$^{28}Si_n$	$^{29}Si_n$	$^{30}Si_n$
236.1258	9	0	2	0	0
237.1254	9	0	1	1	0
237.1292	8	1	2	0	0
238.1226	9	0	1	0	1
238.1287	8	1	1	1	0
238.1325	7	2	2	0	0
239.1261	8	1	1	0	1
239.1321	7	2	1	1	0
239.1359	6	3	2	0	0
240.1294	7	2	1	0	1
240.1354	6	3	1	1	0
241.1327	6	3	1	0	1

Figure 3. Data input and function hierarchy of isoSCAN. A) isoSCAN R-package main workflow: *autoQ* function performs quantification for both low (LR) and high-resolution (HR) data. The input of *autoQ* is a list of targeted compounds and mz(X)ML files, and its results can be plotted using *metBarPlot*. In addition, raw data can be explored by separate functions. B) Isotopologues to consider in HRMS for Lactate 2TMS (C9H24O3Si2). Each color determines ions that should be summed for correct isotopologue estimation if 13C enrichment is to be quantified.

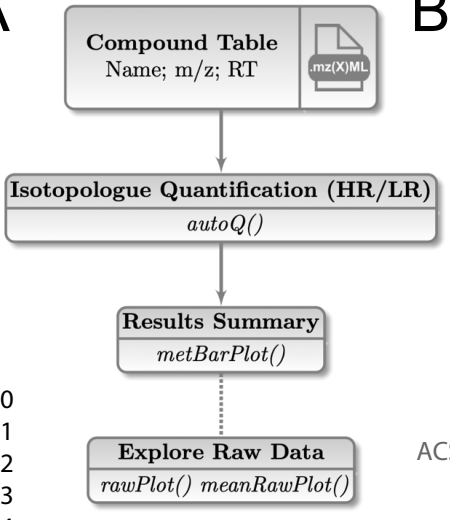







A
M+0
M+1
M+2
M+3
Absolute abundance (1e6)
2.8
6.7
2.0
6.0
1.7
5.6
0.8
4.8
RT (s)
SIM
NS
TOF
Orbitrap
B
Relative Deviation (%)
Theoretical abundance (%)
C
Isotopologue
D
Lactate 2TMS
Aconitate 3TMS
Malate 3TMS
Dihydroxy-acetone phosphate 3TMS+MA
Relative Intensity (%)
Log10(SNR)
m/z

A



B

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