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Mass spectrometry

A guide to implementing targeted and standardized clinical metabolomics using stable isotope-labeled standards and triple quadrupole mass spectrometry

Authors

Scott M. Peterman[1], Andrew J. Percy[2], Susan S. Bird[1], Jifang Zhao[3], Masoumeh Dorrani[3], Nihel Bekhti[3], Jurre J. Kamphorst[3]

[1]Thermo Fisher Scientific, San Jose, CA, USA; [2]Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA; [3]Olaris, Inc., Framingham, MA, USA

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Executive summary

Metabolomics is a nascent field of “omics” research with the potential to drive new developments in clinical biomarkers, diagnostics, and precision medicine. A variety of powerful techniques have been developed to characterize the metabolome, leading to many new insights and observations. However, “translation” of these findings to a clinical setting has proven difficult, in part due to a lack of standardized metabolomics approaches that can be applied to large patient cohorts. Could the combined use of internal standards and targeted analysis bridge this translational gap?

Continued developments in triple quadrupole mass spectrometry (QqQ MS) now enable comprehensive metabolome analysis with unparalleled sensitivity, accuracy, and linear range. This is maximized by using stable isotope-labeled internal standards (IS) to help compensate for matrix effects, allowing for more accurate comparisons of bio-samples, both within and between study cohorts. This white paper describes the basis of targeted metabolomics, from the selection of the metabolite panel and its internal standards to their analysis with appropriate QqQ MS settings and quality control (QC) measures.

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Introduction

Metabolomics can be defined as the large-scale study of small molecules (metabolites) present in biological systems. Metabolites are dynamically formed, broken down, and regulated by a variety of processes. This includes endogenous metabolism and signaling, metabolism of the microbiome, diet, and exposure to the environment and drugs. In essence, the metabolome provides a real-time readout of the integrated effects of a subject's genetics, microbiome, lifestyle, and environmental exposure. Because of this, metabolomics uniquely complements other branches of "omics," including genomics and proteomics. Since virtually every disease and its therapy impinges on metabolism, there are high expectations for metabolomics to help uncover new biomarkers for drug development, diagnostics, and precision medicine. Indeed, some of the widely used assays in clinical chemistry today have metabolites as their readouts. This includes glucose for diabetes, creatinine for kidney disease, and cholesterol for cardiovascular disease.

Due to the wide diversity of chemistry and concentrations within the metabolome, a variety of analytical techniques have been applied in metabolomics, including NMR and MS, to profile metabolites in clinical samples. High-resolution, accurate-mass (HRAM) MS has been a powerful tool for characterizing the metabolome, leading to countless new insights and candidate biomarkers across many disease areas. Non-targeted data acquisition using HRAM-tandem MS improves metabolite detection and annotation with high-quality precursor and product ion measurements. State-of-the-art HRAM instruments have very high data acquisition speeds, increasing the sample coverage. However, non-targeted data acquisition can be limited when high numbers of metabolites are profiled across wide dynamic ranges, especially at low levels.

To be impactful in the clinic, observations in discovery cohorts need to be validated across large patient sample sets. This requires an analytical platform that provides high sensitivity, accuracy, and linear response across a wide dynamic range and that produces data that can be analyzed relatively easily and is amenable to automation. Triple quadrupole (QqQ) MS, in conjunction with stable isotope-labeled standards, are well suited to fill the "gap" between discovery and clinical implementation. Moreover, with the improvements in cycle times, newer generations of QqQ MS instruments can be used independently to comprehensively quantify hundreds of known metabolites in clinical samples.

This white paper introduces the concepts and considerations of a standardized clinical metabolomics approach based on QqQ MS. It will address the merits of this approach and how it can work in concert with other MS-based metabolomics methods. We will describe the approach in a stepwise manner, beginning with metabolite panel and IS selection, followed by strategies for analytical platform method selection and optimization (LC-MS/MS on QqQ) then concluding with measures for assuring data quality.

QqQ MS in metabolomics—context-of-use guides method of choice

In the context of clinical metabolomics applications, QqQ MS offers multiple advantages over other types of mass spectrometers. Foremost amongst its merits is its unrivaled sensitivity, enabling lower limits of detection and quantification. Together with its wide linear dynamic range, QqQ MS is an excellent tool to use in conjunction with stable isotope standards. This enhances the quantitative accuracy of human sample analysis where disease-specific metabolite signatures can be subtle against a background of large patient-to-patient differences in metabolite levels. Having been in use for many years, QqQ MS technology is extremely robust. In fact, QqQ mass spectrometers are often regarded as the "workhorses" of mass spectrometry, making them well suited for the analysis of large batches (100s–1000s) of samples. Finally, QqQ MS instruments provide a simple-to-interpret data analysis workflow and are therefore relatively easy to implement in non-expert metabolomics labs.

As data acquisition on a QqQ MS is fundamentally targeted, one first needs to first consider what metabolites to measure. The list of targeted metabolites is highly context-dependent but can broadly be divided into either information-driven or from non-targeted, comprehensive profiling metabolite experiments. (Figure 1) Information-driven applications are based on discovery studies performed on other types of mass spectrometers (e.g., HRAM as introduced above) or on published literature. An example of the former case is the work by Schwaiger-Haber et al[1], who demonstrated how profiling data obtained on the high-resolution Thermo Scientific™ Orbitrap™ ID-X Tribrid™ mass spectrometer (MS) could be used to establish selected reaction monitoring (SRM) methods on QqQ mass spectrometers (i.e., Thermo Scientific™ TSQ Altis™ mass spectrometer and an Agilent™ 6460 MS. From the Orbitrap MS data, the authors were able to determine both optimal SRM transitions and collision energies for the metabolites of interest. The approach was found to be suitable to not only known metabolites, for which standards are available, but also for unknowns annotated during the discovery experiments. The use of such an approach maximizes the strengths of both instruments, with in-depth discovery work being performed on an HRAM instrument and highly precise follow-up studies of large sample sets conducted on a QqQ instrument.

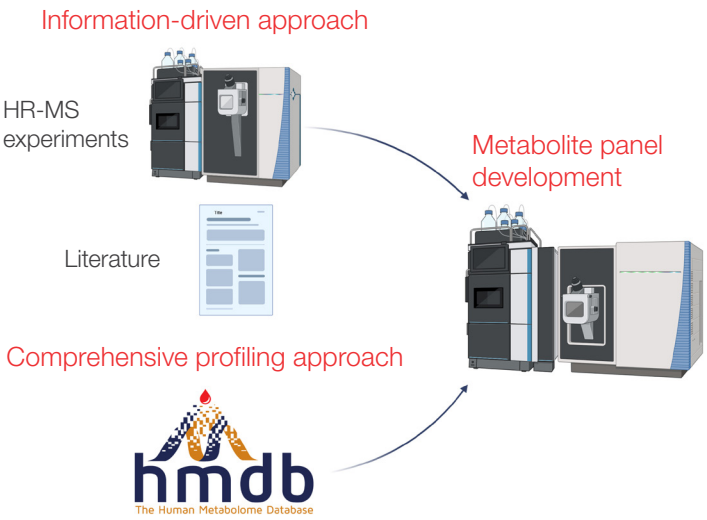


Figure 1. Approaches for developing a targeted metabolite panel can be broadly categorized as (I) information-driven where the goal is to validate findings from discovery experiments or literature in larger sample sets, or (II) comprehensive profiling where the intent is to measure as manyof the known metabolites to uncover new metabolite signatures and for precision medicine.

A separate information-driven approach bypasses the need for untargeted discovery experiments, truncating method development time. All experiments are performed on the QqQ MS method targeting metabolite biomarker candidates reported in published literature in a disease-area of interest and the SRM transition information is imported. For instance, a laboratory interested in biomarkers for kidney disease could set up a method targeting uremic toxins.[2] Existing collision energy (CE) conversion equations can be used to translate reported data on different vendors' instruments. A few replicate injections of the sample are used to map the targeted metabolite retention times to finish the scheduled/timed SRM parameters.

While information-driven approaches seek to verify previously established candidate biomarkers, QqQ MS can also be extremely powerful in comprehensive profiling applications where the goal is to identify novel relationships between clinical conditions/subsets and metabolite levels (i.e., precision medicine). As QqQ MS technology continues to evolve, effective dwell times needed to acquire high-quality SRM data continue to decrease (e.g., 100 ms down to 5 ms), making it feasible to confidently quantify hundreds of metabolites in a single LC-MS run. Importantly, recent work by multiple teams has established that the size of the known metabolome is within scope of targeted profiling methods. The tens of thousands of metabolite features reported for non-targeted metabolomics studies are a gross inflation of the actual number of metabolites observed. This is perhaps best demonstrated by a study by Mahieu et al.[3] In this example, their rigorous examination of a metabolomics data set with 25,000 features established that most features were isotopes, adducts, artifacts, or contaminants, and that the actual number of unique metabolites was less than 1,000. Similarly, our own analysis of the Human Metabolome Database (HMDB)[4], focusing on endogenous metabolites that have been detected and quantified in human plasma and/or urine, revealed a total of 1,200 metabolites. Of these, 36% are lipids, leaving approximately 750 polar metabolites. A deeper evaluation of these 750 metabolites teaches us that a non-negligible proportion ionizes poorly with ESI and some subsets require dedicated methods to be optimally resolved (e.g., sugars). Thus, comprehensive metabolome profiling is feasible using QqQ MS in combination with a few modes of separation (e.g., HILIC and RPLC).

Constructing a metabolite SRM panel

As QqQ mass spectrometers provide fundamentally targeted workflows, specific information about metabolites needs to be considered in the instrument method: individual SRMs, CE values, and retention times (RTs) if RT scheduling is used. A SRM transition contains the *m/z* of both the precursor and product ion, and the CE is the voltage offset with which the precursor is fragmented. Typically, a second and sometimes a third SRM for the same metabolite is used to improve the sensitivity and specificity of the measurement. The ratios of the metabolite SRM signal intensities are metabolite-specific and can be used to confirm the annotation of a peak (Figure 2). For this reason, we monitor two SRMs for nearly all the metabolites in our panel, with one further serving as the quantifier and the other the qualifier in quantitative metabolomic applications.

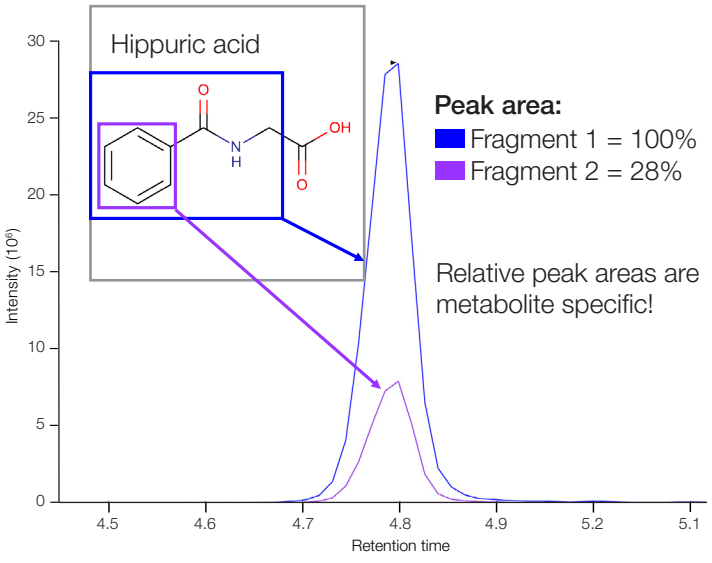


Figure 2. Monitoring multiple fragments for each metabolite (as is shown here for hippuric acid) increases confidence in annotation. Additionally, the relative peak areas of the SRMs are metabolite-specific and large deviations may indicate the measurement is unreliable. For guidance on SRM ratio acceptance criteria, please see WADA Technical Document – TD2021IDCR.

There are multiple ways to establish SRMs, CEs, and RTs. One way is to use authentic standards of each analyte of interest. The breadth of metabolite standards available is continuously expanding, both through the larger consumable providers, such as Cambridge Isotope Laboratories, Inc. (CIL), and smaller specialty companies, such as MetaSci (MetaSci.ca). Entire metabolite standard libraries, such as IROA's Mass Spectrometry Metabolite Library of Standards (MSMLS), are also available commercially. Using one of these MSMLS kits (e.g., for microbiome), the unlabeled standards can be infused individually or in a self-formulated mix to determine the optimal SRMs and CEs, and then injected onto a LC-MS platform for RT determinations. While the use of authentic standards is the most direct way to obtain these necessary method parameters, doing so can be time consuming and labor-intensive. Furthermore, standards for certain metabolites of interest, particularly in their stable isotope-labeled form, might not be available commercially or may be costly.

An alternative approach to establish targeted method parameters involves transferring information obtained on an untargeted HRAM mass spectrometer to a QqQ, as exemplified by the work of Schwaiger-Haber et al.[1] In brief, the authors used the Orbitrap ID-X MS to identify metabolites of interest then used this information for method creation on a multiple vendor QqQ MS, including the TSQ Altis MS, for follow-up confirmation. The metabolite fragmentation data from the Orbitrap ID-X MS was used to determine the SRM transitions, while the optimal CEs were established using a conversion equation based on a set of known metabolites analyzed on both types of instruments. Since the LC methods were identical between the instruments, the metabolite RTs could be directly transferred. The utility of this was demonstrated for 100s of known and unknown metabolites in a human plasma profiling study. It's important to bear in mind that refinement of the targeted LC-MS method may be necessary to remove metabolite interference[5], so careful inspection of the SRM data is critical to ultimately render accurate quantitation in a clinically driven MS metabolomics application.

Finally, many metabolite SRMs can be obtained from literature, vendors, and publicly available resources. For example, numerous publications report metabolite SRM parameters in their supplemental information.[1,6-10] CIL provides SRM-associated parameters in user manuals for their omics kits, as is the case of their U-^{13}C and unlabeled metabolite and lipid yeast extract products (metabolites: catalog no. ISO1 and ISO1-UNL; lipids: catalog no. L-ISO1 and L-ISO1-UNL). The Thermo Scientific™ mzCloud™ advanced mass spectral databases (mzcloud.org) is also extremely useful. The mzCloud database is a manually curated database, containing MS fragmentation trees for over 30,000 compounds. The database contains over two million fragmentation spectra for these compounds, and this information can be used to shortlist the most promising SRMs for confirmation on QqQ mass spectrometers. Since many of the fragment ions in this database are annotated with chemical structures, the *m/z* of ^{13}C-labeled product ions can be readily determined. The main limitation of this approach is that metabolite RTs are typically unknown. Additionally, optimal CEs are instrument-specific and cannot be directly transferred to a QqQ from an alternate vendor. To address this, RTs can be established through scouting experiments on samples that contain the metabolites of interest, and only when individual compounds yield non-unique SRMs showing multiple peaks, should standards then be obtained for conclusive annotation. A similar scouting approach can be used to identify the instrument-specific optimal CEs. To aid this, the developers of targeted MS processing software, such as Skyline, have included functionality for CE optimization.[11,12]

Importance, design, and implementation of stable isotope-labeled standards

To facilitate accurate MS-based measurements in metabolomics, as well as standardization across batches and laboratories, stable isotope-labeled standards must be incorporated. The ideal approach is to add the labeled standard(s) in precise amounts to the experimental and QC (e.g., pooled matrix, see below) samples to function as IS. This helps the researcher assess matrix effects and extraction efficiency. Only by adding an IS can recovery differences within and between samples, batches, and instrument platforms be effectively evaluated and normalized. With IS use, the type and its point of insertion are two critical factors that a researcher faces in designing an analytically robust metabolomics method. This is critical for evaluating the assay's effectiveness and to help guide corrective actions, when and if necessary.

The nature of IS can take many forms, but is conventionally a compound, or mixture of compounds, that has been labeled with one or more stable isotopes (commonly ^{13}C, ^{15}N, and/or D). The type of labeling must be carefully selected in the study design as it can be impacted by the pre-analytical and analytical methods used. If, for instance, D-labeling is selected (as is typical in newborn screening tests), the labels must be inserted at non-exchangeable positions to mitigate the effects of hydrogen-deuterium exchange. Labeling with ^{13}C (and/or ^{15}N) is generally preferred over deuterium due to its chemical stability, which helps ensure that the isotope remains intact throughout an experimental method. In other words, the ^{13}C (and ^{15}N) isotope remains positioned at its point of synthesis throughout all phases of an analytical workflow (from matrix spike through analysis). This provides flexibility to the researcher as there is no limitation on the choice of preparation strategy nor mode of MS/MS analysis. Since ^{13}C (and/or ^{15}N) standards have exceptional isotopic stability, these can be inserted at an early stage of sample preparation. Of additional benefit is that this type of labeled

compound co-elutes with its corresponding unlabeled metabolite (in a particular matrix) during chromatographic separation. This co-eluting behavior is optimal in correcting for both ion suppression and matrix effects. Furthermore, ^{13}C (and/or ^{15}N) standards are not affected by isotope scrambling or loss during ionization and collisional activation in the mass spectrometer. Owing to these collective merits, ^{13}C (and/or ^{15}N) standards have proven to be of great value in MS applications and should be considered the preferred standard in a researcher's study design process.

Regardless of the type of isotope selected, the labeled standards in metabolomics should ideally have a minimum mass shift of 3 Da from its unlabeled counterpart. Uniform labeling is favored over positional labeling because it affords greater opportunity for differentiation of precursor and/or product ions in mass spectrometric analysis of labeled to their unlabeled. This is particularly beneficial in SRM transition selections, as is the case when utilizing QqQ mass spectrometers. Prior to use, the isotopically labeled standards must be well characterized (in terms of chemical and/or chiral purity and isotopic enrichment) to enable the accurate accounting of losses or errors in method application.[13] This relates to human (e.g., pipetting), chemical (e.g., analyte extraction, hydrolysis), and/or instrument (e.g., ion suppression, matrix effects) errors. For optimum results in application, the standards should be added as early in the analytical workflow as possible, such that they can effectively normalize the variations that may arise throughout the experimental stages (Figure 3).

In terms of the number of labeled standards to be implemented, it is recommended that the number of IS equates to the number of analytes in a user's target panel. While this is generally practical for small panels, it becomes extremely labor intensive and cost-prohibitive when working with large panels, as is the case with the metabolomics approach described here. In those situations, a suitable approach is to use a smaller set of stable isotope-labeled IS that is still representative of the chemical diversity of the measured panel of metabolites. These labeled standards serve as direct IS to their unlabeled counterparts. Additionally, they can act as "surrogate" standards for those metabolites that lack a direct labeled analogue. This practice is considered acceptable in quantification exercises provided that the surrogates exhibit similar elution times, and thus similar physicochemical properties to their native targets in an endogenous sample. The commercially available U-^{13}C metabolite yeast extract (CIL catalog no. ISO1) provides a stable source of uniformly labeled ^{13}C metabolites of high isotopic enrichment (≥98% as defined by longitudinal HRMS analysis). The 100s of biologically relevant metabolites present span broad metabolic classes (e.g., amino and organic acids, sugar phosphates, coenzymes), biochemical pathways (e.g., citrate and glyoxylate

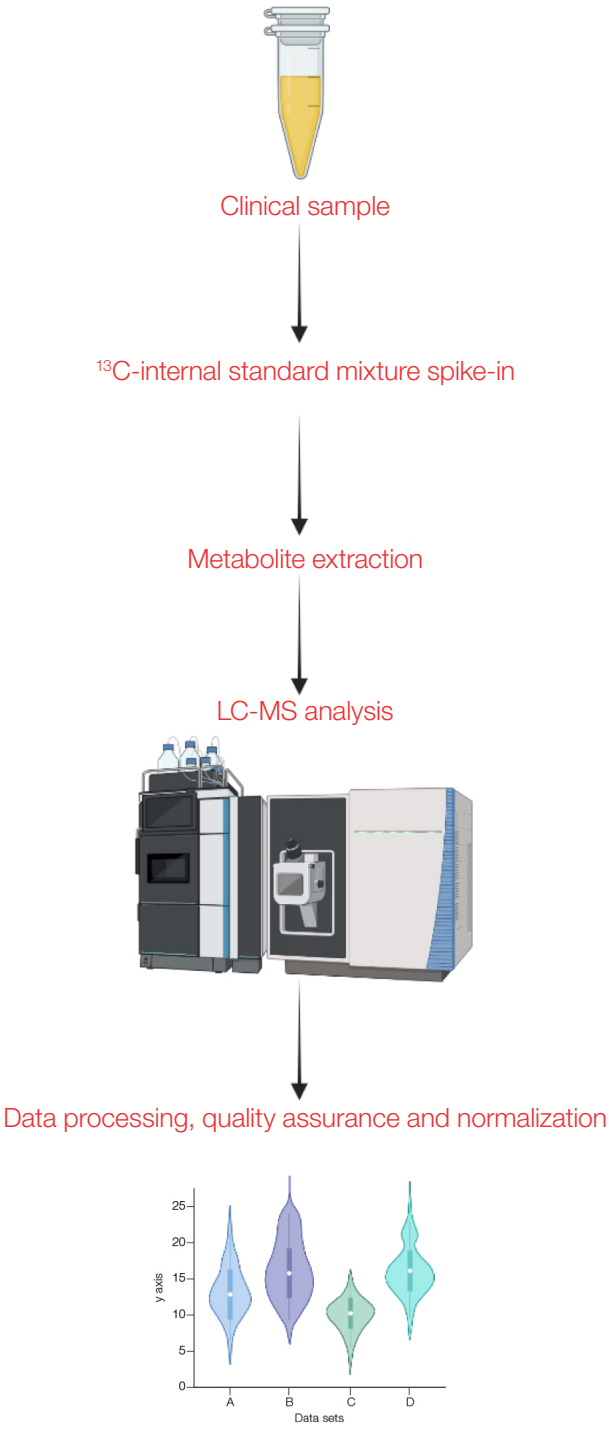


Figure 3. Sample preparation and LC-MS procedure. The U-^{13}C-internal standard mixture (e.g., CIL's metabolite yeast extract—cat no. ISO1) is spiked into each study sample as early as possible (i.e., before sample extraction and LC-MS analysis), to correct for error introduced during the sample analysis process.

cycle, amino acid and nucleotide metabolism, pentose phosphate), and cellular/molecular processes (e.g., immune system, blood coagulation, DNA metabolism). As yeast is eukaryotic, its metabolite composition is largely conserved with the human metabolome making this material a well suited and acceptable source of labeled IS for human-directed research studies. Further to its benefits, the highly enriched extracts have been characterized and validated by various analytical methodologies, which enhances its implementation and standardization in MS metabolomics (from basic research to clinical translation). In experimental applications, an aliquot of a solubilized U-^{13}C metabolite yeast extract can be added early in the analytical workflow serving as effective IS to their endogenous analogues. These IS also serve as potential surrogates to endogenous metabolites lacking a direct IS in a given sample, such as human urine. This provides a useful means to maximize the quantity of analytes to be quantified in a single run using a singular, consistently prepared, product source.

QqQ MS optimization for large-scale metabolite panels

To maximize the analytical performance of the LC-MS method, the liquid chromatographic performance must be carefully optimized. Maximizing the peak capacity along the gradient profile provides significant benefits, including greater sensitivity, reduced ion suppression, and the potential for increased specificity resulting from separating isomeric compounds, such as leucine and isoleucine. In addition, narrow chromatographic peak shapes with high reproducibility enable tighter scheduled windows that can reduce the number of concurrent SRM transitions monitored in a given time window. Historically, reversed-phase liquid chromatography (RPLC) has been the method of choice for metabolomics research. This mode of separation works well for the mid- to non-polar segment of the metabolome but does not retain or separate polar metabolites very well. This can be addressed by using metabolite ion-pairing or derivatizing reagents.[14-17] More recently, maturation of hydrophilic interaction liquid chromatography (HILIC) separation technology has made it a viable alternative to RPLC, with the benefit that polar metabolites can be retained and separated without the need for ion-pairing or derivatization.[18-22] Since the primary focus of our research was on the more polar metabolites, we developed a rapid and robust HILIC-based MS method for a panel of polar metabolites that can baseline resolve isomeric metabolites (Figure 4). Improved confidence in annotation and quantification was achieved using metabolites from the U-^{13}C metabolite yeast extract as IS in this experimental workflow.

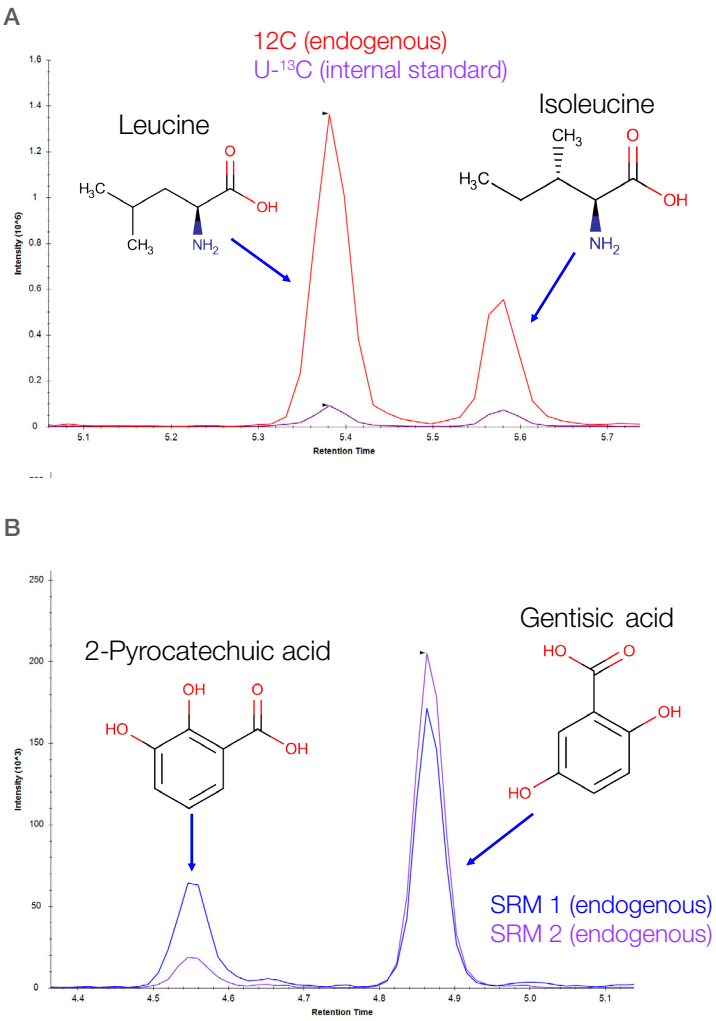


Figure 4. Two examples of isomeric pairs that can be separated using our chromatographic method. Column: Atlantis Premier BEH Z-HILIC (2.1 × 150 mm, 1.7 µm particles), H_2O-ACN gradient with ammonium acetate (pH 7). (A) SRMs: 132.2 → 86.2 (^{12}C), 138 → 90.9 (^{13}C), (B) SRMs: 153.2 → 109 (^{12}C, SRM1), 153.2 → 107.9 (^{12}C, SRM2). Notice that SRM1 has higher intensity than SRM2 for 2-pyrocatechuic acid and vice versa for gentisic acid.

Coupling premier QqQ mass spectrometers to liquid chromatographic systems delivering UHPLC separations becomes imperative to handle the increased peak capacity. Targeting 100's of metabolites and their corresponding ^{13}C-labeled IS can still result in large numbers of concurrent SRM transitions despite using narrow RT window scheduling. The goal of a quantitative method is to ensure a minimum of seven to ten data points per LC peak width for peak profiling. Thus, the cycle time must be determined by specifying the number of data points and the average LC peak width. From these two user-defined parameters, an equivalent dwell time setting is determined for all SRM transitions in a given time-scheduled window, minimizing the preliminary work needed in development.

The TSQ Altis mass spectrometer has an additional software tool to streamline method refinement for dwell time settings. The Dwell Time Prioritization (DTP) setting introduces a prioritization option for the user to enable automated changes to dwell times per SRM transition by manually changing the prioritization. Upon the initial method creation using the process outlined above, each SRM transition has a DTP setting of 3 (normal) to acquire an initial test data set. Following processing, those SRM transitions with lower intensity/area values and/or coefficient of variation (%CV) can be changed from a DTP setting of 3 to 2 or 1, increasing the priority. Likewise, for SRMs with extremely abundant signals, DTP can be lowered from 3 to 4 or even 5. The onboard processor donates dwell time from all other SRM transitions in the scheduled time window with lower DTP settings to those SRM transitions with high DTP settings. Conversely, it minimizes time spent on abundant signals, making more time available for those transitions with higher priority DTP. The overall response is to improve low-level SRM measurements while the higher abundant SRM transitions measured using shorter dwell times on the Thermo Scientific™ TSQ Altis™ mass spectrometer, Thermo Scientific™ TSQ Altis™ MD Series mass spectrometer, and Thermo Scientific™ TSQ Altis™ Plus mass spectrometer can maintain acceptable quantitative reproducibility (Figure 5).

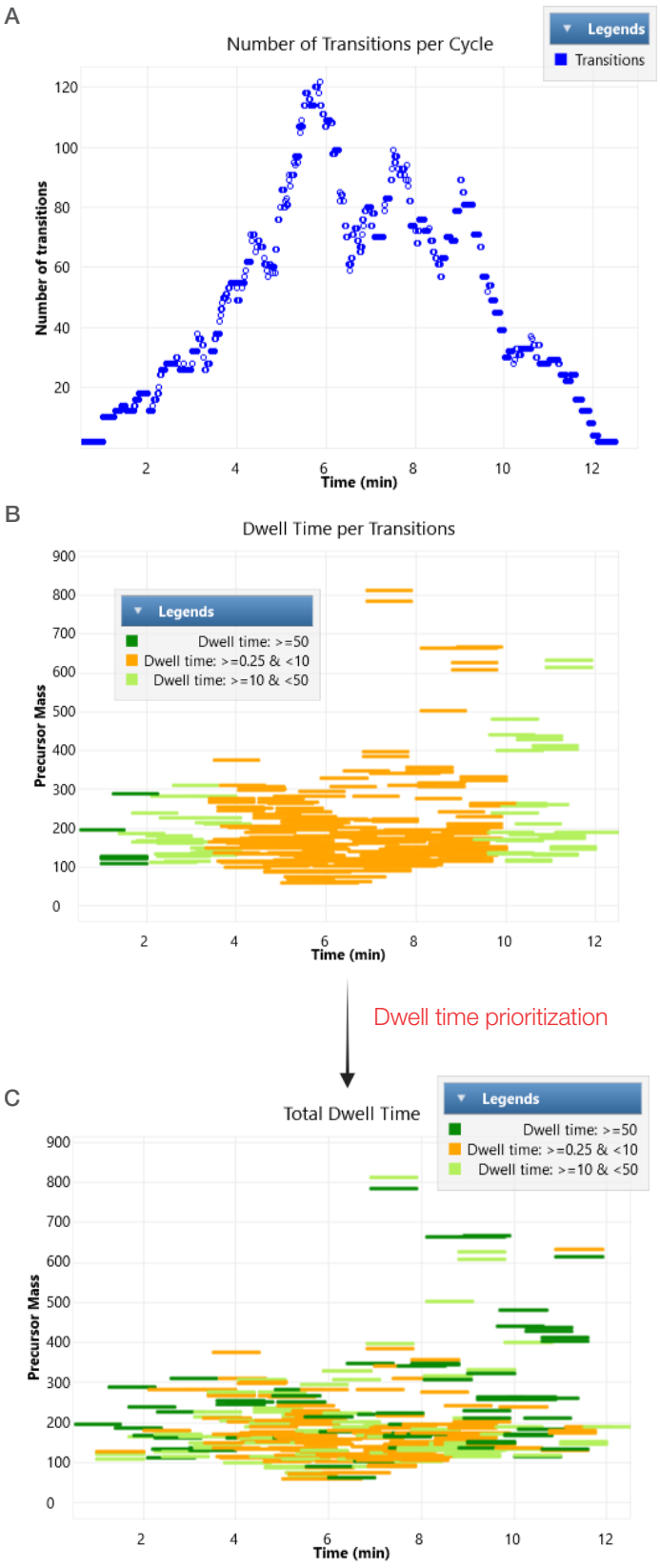


Figure 5. Retention time scheduling and dwell time prioritization (DTP). (A) The number of SRMs (transitions) during each measurement cycle during the LC-MS run (600 total SRMs and 1 min scheduling windows). (B) The impact of the transition 'load' (number of concurrent SRMs during a cycle) on dwell time. The more SRMs per cycle, the lower the dwell time (based on 12s peak width at base and 10 datapoints per peak). (C) Dwell times post DTP. This improves measurement quality of low abundance SRMs.

Data quality control and normalization

Ensuring high data quality is of utmost importance for accurate interpretation and decision making.[23-25] In particular, three elements need to be considered:

1. Confirming that the LC-MS performance is within specifications,
2. Monitoring data quality over the course of the run to detect analytical issues and to see if signal correction needs to be performed so that samples within a run can be compared to each other, and
3. Being able to compare data from different LC-MS batches, which is important for analysis of large clinical datasets.

Each of these elements can be evaluated with specific samples that should be included in every single LC-MS batch:

1. **System suitability sample** – This is typically a well-defined sample with a small set of stable metabolites, such as those present in CIL's QReSS quality control mixes (catalog no. MSK-QReSS-KIT). Use of system suitability samples helps facilitate direct comparison of a run to historical data from the same sample, to determine LC (e.g., retention time, peak shape and width, back pressure) and MS (e.g., signal intensity, S/N, mass accuracy) performance. Applications like Skyline's AutoQC provide ready-to-use solutions to compare the most recent system suitability run against prior analyses, enabling the immediate evaluation of trends in the system's performance and the identification of technical issues.[26,27]
2. **QC sample** – This is typically a pooled sample of the study samples from that batch, ideally with the incorporation of an isotopically labeled metabolite mix (e.g., CIL's QReSS mixes[25]), that will be run after every 8–10 study samples. The main advantage of using a pooled QC sample is that it enables the assessment of retention time and signal stability for each metabolite that is studied (Figure 6). With large batches, it is not uncommon to observe some signal loss over the course of the run, and the QC sample data can be used to effectively apply signal correction algorithms. It is also recommended to run a QC sample dilution series at the start of a run, for example undiluted, 2× diluted, 4× diluted, and 8× diluted. This helps to confirm linear response for the metabolites being investigated.
3. **Reference sample** – A sample that is run at least once during each batch. Data from this sample can act as a "reference" to assess significant differences that exist in the data from the different batches and hence if batch correction is needed. This sample ideally has a very similar composition to the study samples and could be a biological sample. It is best to prepare many aliquots of this sample and to store these as cold as possible (≤-80°C), to minimize freeze-thaw cycles and metabolite degradation. Of note, the use of stable isotope-labeled IS will alleviate the need for batch correction.

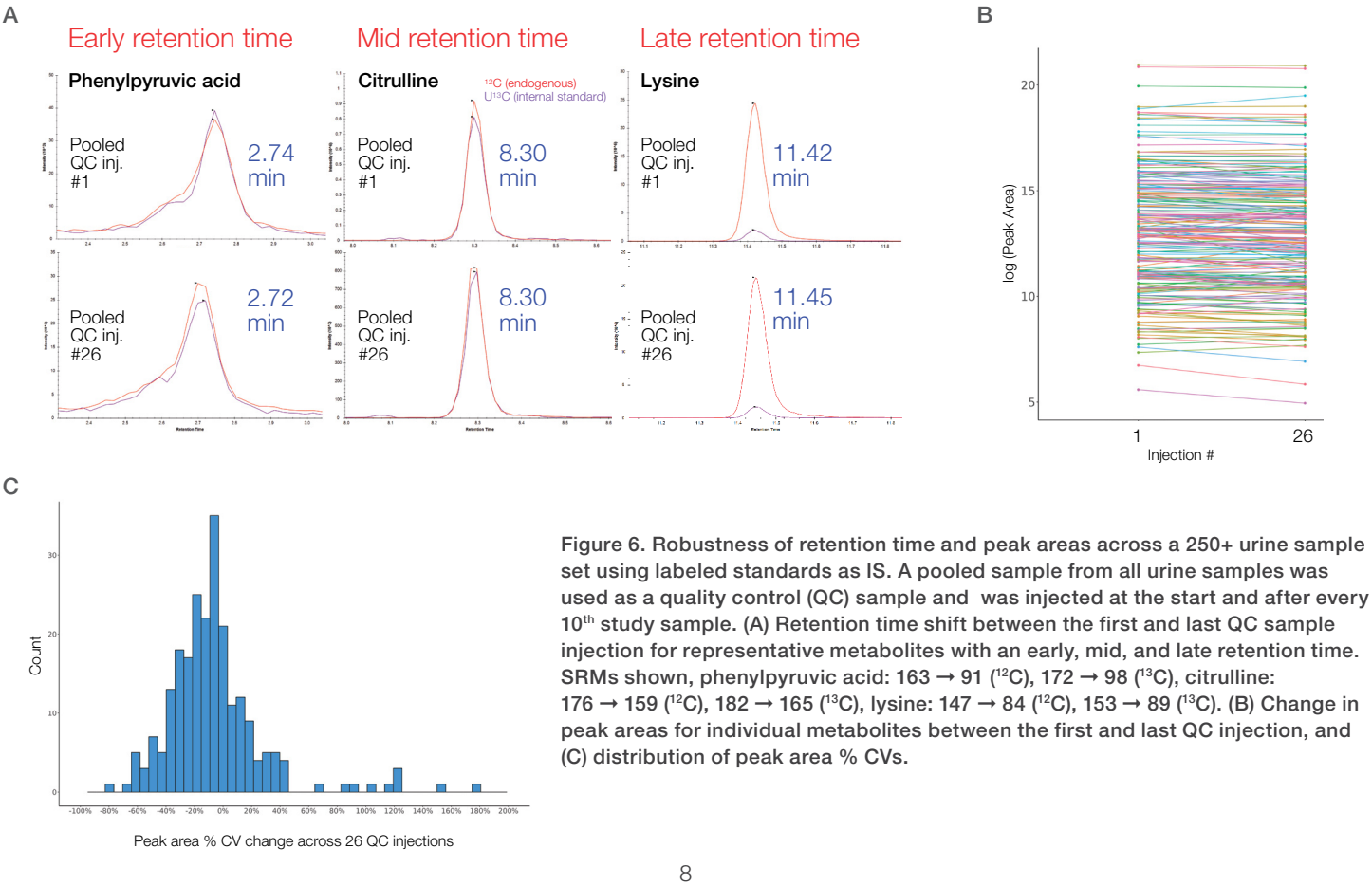
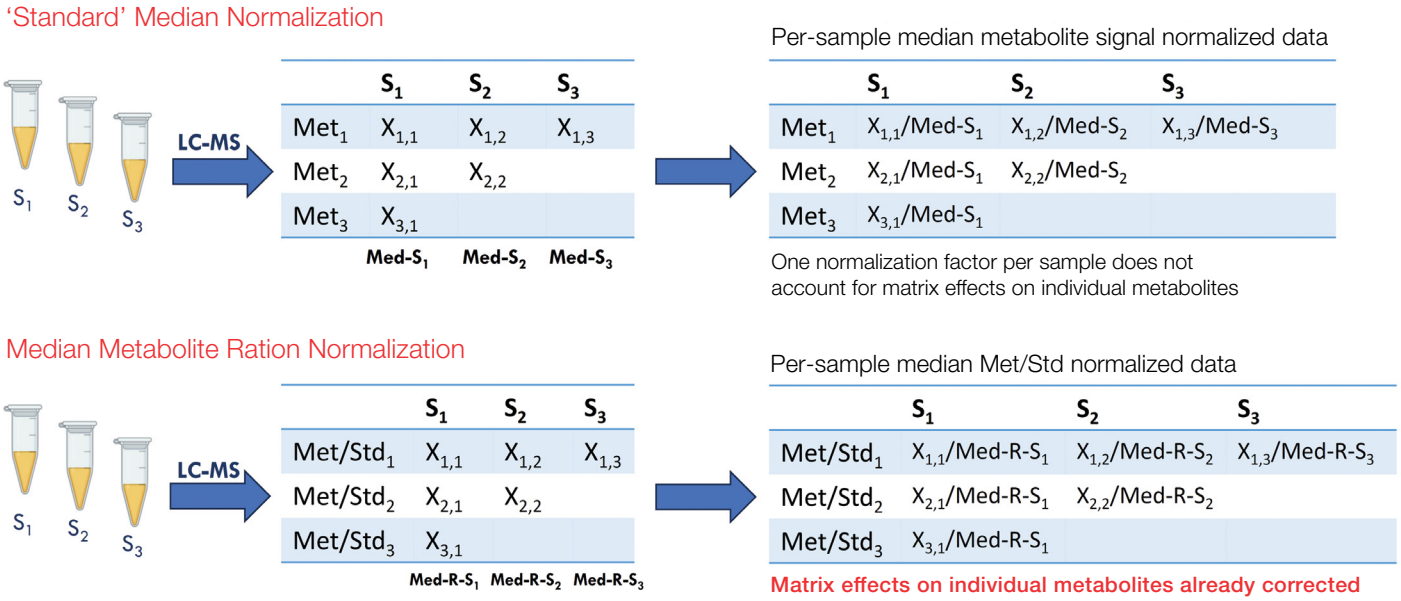


Figure 6. Robustness of retention time and peak areas across a 250+ urine sample set using labeled standards as IS. A pooled sample from all urine samples was used as a quality control (QC) sample and was injected at the start and after every 10th study sample. (A) Retention time shift between the first and last QC sample injection for representative metabolites with an early, mid, and late retention time. SRMs shown, phenylpyruvic acid: 163 → 91 (^{12}C), 172 → 98 (^{13}C), citrulline: 176 → 159 (^{12}C), 182 → 165 (^{13}C), lysine: 147 → 84 (^{12}C), 153 → 89 (^{13}C). (B) Change in peak areas for individual metabolites between the first and last QC injection, and (C) distribution of peak area % CVs.

Post-acquisition data normalization is often performed in metabolomics studies and serves to eliminate sample-to-sample variability that is introduced during sample preparation and LC-MS analysis. In addition, normalization can also correct for differences in gross total metabolite abundances between clinical samples. For instance, total metabolite levels vary significantly in urine depending on the donor's hydration status, and this needs to be corrected either pre (sample concentration adjustment) or post (normalization) analysis, before meaningful comparison between samples can be performed. A variety of normalization procedures exist, and this has been effectively reviewed elsewhere.[28] One popular procedure is median normalization, where for each LC-MS run the median metabolite peak area is identified and all the metabolite peak areas in that run are expressed as a ratio (normalized) to the median peak area. The main downside of this approach is that all metabolites in a sample are subjected to the same normalization factor while these metabolites may experience vastly different matrix effects, introducing error and making normalization less effective. We therefore piloted the use of median normalization using metabolite ratios to stable isotope-labeled IS, rather than endogenous metabolite peak areas only (Figure 7A). As the stable isotope-labeled standards experience the same matrix effects as their endogenous metabolites, their ratio is not affected by ion suppression effects, and hence normalization using ratios should lead to introduction of fewer errors. Indeed, we find that normalization of metabolite to IS ratios outperforms standard normalization approaches (Figure 7B).

A



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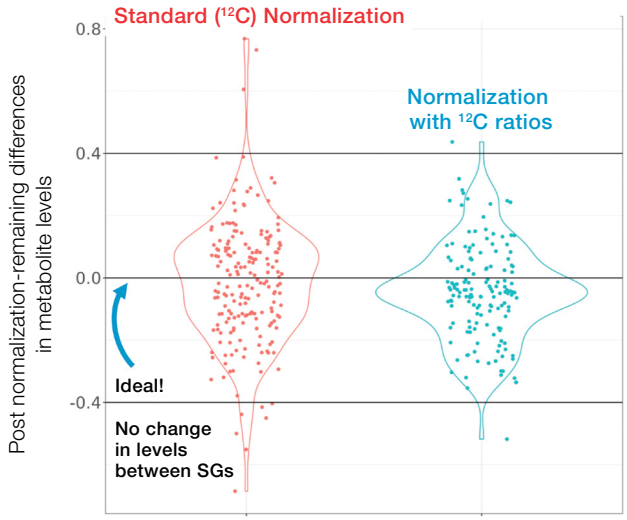

Figure 7. Use of ^{13}C-metabolite standard ratios improves normalization. (A) Standard median normalization procedure and median normalization using metabolite/^{13}C-internal standard ratios. Median normalization on ratios eliminates error due to matrix effects. (B) Quality of median normalization without (red) and with (blue) internal standard ratios. A dilution series (1×, 2×, 4× diluted) of commercial pooled urine with equal amounts of U^{13}C-yeast metabolite extract spike-in into each sample was analyzed by LC-MS. The data was normalized using the procedure of panel A. The plot shows the residual fold differences for individual metabolites between the sample dilutions. The residual differences are considerably smaller using ^{13}C-standard ratios.

Summary and conclusion

The rise of metabolomics provides a promising avenue to study a group of molecules that render a real-time readout of the integrated effects of a subject's genetics, microbiome, lifestyle, and environmental exposure. As such, it is expected to deliver new biomarkers for drug development and diagnostics, and thus serve as an essential component of multi-omics endeavors that empower precision medicine. A variety of analytical methodologies are necessary to truly realize the potential of metabolomics. In addition to HRAM MS-based methods aimed at identifying new metabolite biomarkers and characterizing unknowns, there is a growing need for targeted approaches that enable standardized/quantitative analysis of metabolite signatures across large sample sets, batches, and laboratories.[29] As described in this white paper, QqQ MS methods with stable isotope-labeled standards are well positioned to address this ongoing need.

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