A Workflow to Perform Targeted Metabolomics at the Untargeted Scale on a Triple Quadrupole Mass Spectrometer

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ABSTRACT: The thousands of features commonly observed when performing untargeted metabolomics with quadrupole time-of-flight (QTOF) and Orbitrap mass spectrometers often correspond to only a few hundred unique metabolites of biological origin, which is in the range of what can be assayed in a single targeted metabolomics experiment by using a triple quadrupole (QqQ) mass spectrometer. A major benefit of performing targeted metabolomics with QqQ mass spectrometry is the affordability of the instruments relative to high-resolution QTOF and Orbitrap platforms. Optimizing targeted methods to profile hundreds of metabolites on a QqQ mass spectrometer, however, has historically been limited by the availability of authentic standards, particularly for “unknowns” that have yet to be structurally identified. Here, we report a strategy to develop multiple reaction monitoring (MRM) methods for QqQ instruments on the basis of high-resolution spectra, thereby enabling us to use data from untargeted metabolomics to design targeted experiments without the need for authentic standards. We demonstrate that using high-resolution fragmentation data alone to design MRM methods results in the same quantitative performance as when methods are optimized by measuring authentic standards on QqQ instruments, as is conventionally done. The approach was validated by showing that Orbitrap ID-X data can be used to establish MRM methods on a Thermo TSQ Altis and two Agilent QqQs for hundreds of metabolites, including unknowns, without a dependence on standards. Finally, we highlight an application where metabolite profiling was performed on an ID-X and a QqQ by using the strategy introduced here, with both data sets yielding the same result. The described approach therefore allows us to use QqQ instruments, which are often associated with targeted metabolomics, to profile knowns and unknowns at a comprehensive scale that is typical of untargeted metabolomics.

KEYWORDS: Untargeted metabolomics, targeted metabolomics, triple quadrupole mass spectrometry, high-resolution mass spectrometry, collision energy conversion

INTRODUCTION

Global profiling of the metabolome, referred to as “untargeted metabolomics”, is most commonly performed by coupling liquid chromatography with quadrupole time-of-flight (QTOF) and Orbitrap mass spectrometers. QTOF and Orbitrap instruments are well suited for untargeted metabolomics because they have the capability to acquire MS1 data over a wide m/z range in a single scan, thereby providing a broad survey of chemicals present in a sample. Additionally, QTOFs and Orbitraps provide accurate mass, high resolving power, and the potential to acquire MS2 spectra, all of which are required for confident identification of an unknown compound.1,2 Depending on the model, some QTOFs and Orbitraps may also be equipped with other technologies to facilitate the identification of unknown compounds such as ion mobility3–5 and MSn capabilities.5,7 In contrast to QTOF and Orbitrap platforms, triple quadrupole (QqQ) mass spectrometers are not designed to acquire MS1 data over a wide m/z range. Moreover, QqQ instruments have low resolution and provide only nominal mass measurements. As a consequence, it is challenging to discover unexpected chemicals in a sample or to determine the identity of an unknown compound when using QqQ mass spectrometry. Notwithstanding, QqQ instruments are widely used in the field of metabolomics to profile known molecules by using multiple reaction monitoring (MRM), also known as selected reaction monitoring on some instrument platforms.8–10 Indeed, for applications such as newborn screening where the accumulation of specific metabolites is diagnostic,
QqQ assays have been used since the 1990s and are still considered to be the gold standard.\textsuperscript{1,12} In most cases, only a relatively small number of known metabolites are profiled by QqQ mass spectrometry and the experiments are therefore referred to as a type of “targeted metabolomics”.\textsuperscript{9}

Given their ability to characterize unknown molecules, the latest QTOF and Orbitrap platforms are the most attractive for performing untargeted metabolomics. A practical limitation, however, is the cost of an instrument. The list price of a QTOF or an Orbitrap may be more than twice as high as that of a QqQ. Here, we wished to explore a potentially more cost-effective workflow for performing untargeted metabolomics where some of the experimental burden is transferred from a QTOF or an Orbitrap to a QqQ. Our rationale is that the unique technical capabilities of QTOF and Orbitrap instruments are only required for two purposes in untargeted metabolomics: (i) to establish which compounds can be measured in the samples and (ii) to determine the chemical identities of those molecules. We reasoned that these functions can be accomplished by the analysis of pooled-reference samples. On the basis of those results, the individual research samples can then be profiled by using a QqQ instrument. For untargeted metabolomics studies in which hundreds to thousands of research samples are being analyzed, this will reduce the load on QTOF or Orbitrap instruments considerably and also reduce the time required for processing the data after acquisition.

The challenge of the proposed workflow is that establishing MRM methods to profile metabolites on a QqQ mass spectrometer has historically required authentic standards, which are used to experimentally determine what fragments should be monitored at which voltage settings. Beyond the abundance of resources required to purchase and subsequently analyze hundreds of authentic standards, a fundamental issue of feasibility remains. Even with the most advanced QTOF and Orbitrap instrumentation, many of the compounds measured by untargeted metabolomics cannot be identified.\textsuperscript{13−15} Thus, it is not possible to obtain a complete set of authentic standards to design MRM methods. A key advantage of untargeted metabolomics is that these so-called “unknowns” can be profiled, potentially revealing interesting biological differences between samples. Here, building upon earlier work,\textsuperscript{16−20} we sought to perform metabolite profiling on QqQ mass spectrometers without sacrificing coverage of unknowns. To this end, we developed an approach to design MRM transitions without using authentic standards. We demonstrate that fragmentation data from research samples analyzed on an Orbitrap ID-X are sufficient to build MRM methods that have equivalent quantitative performance to those built with conventional methods relying on authentic standards. The approach allows us to use QqQ instruments, which are commonly associated with targeted metabolomics, to profile knowns and unknowns at a comprehensive scale that is typical of untargeted metabolomics.

\section*{EXPERIMENTAL SECTION}

\subsection*{Standards and Chemicals}

Liquid chromatography/mass spectrometry (LC/MS) grade acetone, methanol, and water were purchased from Millipore Sigma or Fisher Scientific. Ammonium bicarbonate, ammonium hydroxide, and methylenediphosphonic (medronic) acid were purchased from Millipore Sigma and used as eluent additives for LC/MS. Metabolite standards were purchased from Sigma-Aldrich. Authentic standards were weighed and dissolved in an appropriate solvent. A 10 μM standard mix was prepared in 50:50 acetonitrile/water (v/v).

\subsection*{Human Plasma Collection and Extraction}

Blood was collected from 11 volunteers into dipotassium ethylenediaminetetraacetic acid (K2-EDTA) plasma collection tubes. No Institutional Review Board approval was required from the University of Minnesota because samples were collected as part of quality assurance testing. To mimic conditions under which samples are often shipped via courier service, the tubes were placed on a frozen gel pack after collection for 0, 24, 48, or 72 h prior to the isolation of plasma fractions via centrifugation. The frozen gel pack was not cooled or refreshed and thus gradually came to room temperature over time, as would occur during shipment. Plasma was then stored at −80 °C. After thawing on ice, a 50 μL aliquot of each condition (n = 44) was transferred to a solid-phase-extraction (SPE)-system CAPTIVA EMR-Lipid plate (Agilent Technologies). Next, 200 μL of 1:1 acetonitrile/methanol containing internal standards at a concentration of 10 μM was added. The internal standards included 16 amino acids that were uniformly \textsuperscript{13}C and \textsuperscript{15}N labeled, purchased from Cambridge Isotope Laboratories, Inc. The samples were mixed for 1 min at 360 rpm on an orbital shaker at room temperature prior to a 10 min incubation period at 4 °C. Afterward, 150 μL of 2:2:1 acetonitrile/methanol/water (v/v/v) was added to the samples, and they were mixed on an orbital shaker (360 rpm) for an additional 10 min at room temperature. The samples were then eluted into a 96 deep-well plate by using a positive-pressure manifold (Biotage). A second elution step into the same well plate was performed with 100 μL of 2:2:1 acetonitrile/methanol/water (v/v/v).

A pooled-reference sample was prepared by mixing 20 μL aliquots of all 44 samples prior to extraction. A 50 μL aliquot of pooled-reference sample was then added to the SPE-system CAPTIVA EMR-Lipid plate and extracted by using the same methods detailed above for research samples. Blank samples were prepared by adding a 50 μL aliquot of water (without biospecimen) to the SPE-system CAPTIVA EMR-Lipid plate. The blank samples were also subjected to the same extraction steps detailed above for research samples. The polar eluates of all samples were analyzed in a randomized order by LC/MS. The pooled-reference sample was injected after every tenth sample.

\subsection*{Liquid Chromatography}

In all experiments, metabolites were separated via hydrophilic interaction liquid chromatography (HILIC) by using a SeQuant ZIC-phILIC column (100 mm × 2.1 mm, 5 μm, polymer, Merck-Millipore) with a ZIC-phILIC guard column (20 mm × 2.1 mm, 5 μm, polymer, Merck-Millipore). The column compartment temperature was 40 °C, and the flow rate was set to 250 μL·min\textsuperscript{−1}. The mobile phases consisted of A (95% water, 5% acetonitrile, 20 mM ammonium bicarbonate, 0.1% ammonium hydroxide solution 2.5 μM medronic acid) and B (95% acetonitrile, 5% water, 2.5 μM medronic acid). The ammonium hydroxide solution was purchased as 25% ammonia in water. The following linear gradient was applied: 0−1 min, 90% B; 12 min, 35% B; 12.5−14.5 min, 25% B; 15 min, 90% B followed by a re-equilibration phase of 4 min at 400 μL·min\textsuperscript{−1} and 2 min at 250 μL·min\textsuperscript{−1}. The samples were kept at 6 °C in the autosampler. The injection volume was 2 μL for standard solutions and 4 μL for plasma samples.

\subsection*{High-Resolution Mass Spectrometry}

A Vanquish UHPLC system was coupled to an Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific) via electrospray ionization with the following source conditions: sheath gas flow 50 arbitrary units (Arb), auxiliary gas flow 10 Arb, sweep gas flow 1 Arb, ion transfer tube temperature 300 °C, and vaporizer temperature 200 °C. The RF lens value was 60%. Data were acquired in positive and negative polarity with spray voltages of 3.5 and 2.8 kV, respectively. To generate a list of unique metabolites for the plasma samples, the AcquireX workflow was used with a pooled-reference sample and a blank sample as described previously.\textsuperscript{21} MS1 data were acquired at a resolution of 120 K with an automatic gain control (AGC) target of 2e5 and a maximum injection time of 200 ms. MS1 scans for data-
temperature $300^\circ$C. Following source parameters were used: gas temperature $250^\circ$C. The system was coupled to a 6460A Triple Quadrupole MS system. The described above was carried out. For the Agilent platform, a 1260 LC coupled to a Vanquish UHPLC system and the HILIC method respectively. A cycle time of 1 s was used. The Q1 and Q2 resolutions voltage was 3.5 and 2.8 kV for positive and negative polarity, fl-flushing gas flow 11 L/min. In total, the MS2 data were acquired with 15 K resolution, an AGC target of 2.5e4, a maximum injection time of 40 ms, and a dynamic exclusion of 8 s. A 5 ppm mass tolerance was used for the detection of inclusion list entries, and the intensity threshold was set to 2.5e4. For each cycle, 40 MS2 spectra were acquired.

**Extracting Product Ions and Collision Energies from High-Resolution Data**

The automated generation of MRM transitions from high-resolution mass spectrometry (HRMS) data was accomplished with the HRMS2QQQ software, which is freely available on the Patti Lab Web site (http://pattilab.wustl.edu/software/HRMS2QQQ/). HRMS2QQQ was built on the framework of DecoID.22 A detailed description can be found in the Supporting Information.

**QQQ Mass Spectrometry**

We performed experiments by using two different QQQ mass spectrometers: a TSQ Altis from Thermo Scientific and an Agilent 6460. Separate standard solutions were prepared for each of 81 authentic standards and introduced by flow injection. To find the optimal fragments and collision energies (CE) to be used for MRM transitions, the vendor optimization software was applied. For the TSQ Altis, the following source settings were used: sheath gas flow 50 Ar, auxiliary gas flow 10 Ar, sweep gas flow 1 Arb, ion transfer tube temperature 300 °C, and vaporizer temperature 200 °C. The spray voltage was 3.5 and 2.8 kV for positive and negative polarity, respectively. A cycle time of 1 s was used. The Q1 and Q2 resolutions were 0.7 and 1.2 Da, respectively. For the CID gas pressure, 1.5 mTorr was used. To accomplish LC separation, the TSQ Altis was coupled to a Vanquish UHPLC system and the HILIC method described above was carried out. For the Agilent platform, a 1260 LC system was coupled to a 6460A Triple Quadrupole MS system. The following source parameters were used: gas temperature 250 °C, drying gas flow 11 L·min⁻¹, nebulizer pressure 45 psi, sheath gas temperature 300 °C, sheath gas flow 12 L·min⁻¹, capillary 3500 V, and VCharging 500. HILIC separation was accomplished according to the method described above.

**Data-Processing Software**

For both high-resolution and QQQ data, peaks were extracted by using Skyline 20.2.23 Compound Discoverer 3.2 was used to determine the number of features. Graphpad Prism 8.3 and Agilent MassHunter Profiler Professional 15.0 were used for statistical analysis.

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**RESULTS**

**Overall Workflow**

Our proposed workflow for performing untargeted metabolomics can be considered in two phases: a chemical-characterization phase and a chemical-profiling phase (Figure 1). The chemical-characterization phase uses a reference material that is ideally created by mixing a small aliquot of the research samples together and is hence referred to as a “pooled-reference sample”. One or more pooled-reference samples are analyzed on a QTOF or an Orbitrap in MS1 mode, and the data are processed to generate a comprehensive list of features that is representative of the molecular diversity of the research samples. Depending upon the sample matrix, instrument settings, software parameters, etc., it is common to detect 10,000 or more features. Most of the features, however, do not correspond to unique metabolites of biological interest. There are numerous software programs available (such as XCMS, MS-DIAL, mzMine, MetaboAnalyst, CAMERA, BFF, and many others) to process untargeted metabolomics data, to filter contaminants, and to collapse features redundant for the same metabolite (e.g., adducts, fragments, naturally occurring isotopes, etc.). MS2 data are then collected on features corresponding to unique biological metabolites by analyzing pooled-reference samples on a QTOF or an Orbitrap instrument in DDA mode at several different collision energies. The QTOF or Orbitrap fragmentation data are subsequently used to compute MRM transitions as described in detail below. MRM transitions are calculated irrespective of whether the feature has been structurally identified. Finally, in the chemical-profiling phase, each research sample is analyzed by using the calculated MRM transitions on a QQQ instrument. The objective is to profile all of the unique biological metabolites that appear in the QTOF or the Orbitrap data set in a single analytical QQQ run.

In this work, we used an Orbitrap ID-X to acquire MS1 and MS2 data on human plasma samples. To filter contaminants and features redundant for the same metabolite, we applied the AcquireX software as previously described. In brief, a blank sample and a pooled-reference sample were analyzed in MS1 mode. After peak detection, isotopes and adducts were grouped. Features that were not at least three times higher in the pooled-reference sample than in the blank sample were discarded. We then collected fragmentation spectra on the remaining features by using DDA and, on the basis of these data, designed MRM methods for both a Thermo Scientific QQQ and an Agilent QQQ.
Determining Optimal QqQ Transitions and Collision Energies from Orbitrap Data

To establish a ground truth for optimal MRM methods, we first analyzed 81 authentic standards on a TSQ Altis QqQ mass spectrometer. The authentic standards used represented a physicochemically diverse set of compounds that spanned a wide m/z range (Table S1). To find optimal fragment ions and collision energies, we applied the typical strategy for developing an MRM method. The process involves finding voltages in which precursor and product ion intensities are maximal, independent of in-source fragmentation. For each authentic standard, a solution of 10 μM was created. Using the built-in optimization software, fragment ions and collision energies were identified by analyzing 25 μL of each standard solution three times. The process resulted in a list of precursor and product ions with corresponding CE values and S-lens RF voltages (Table S2).

We next aimed to determine whether the same optimal MRM methods for a TSQ Altis QqQ could be determined from Orbitrap ID-X MS2 data. Prior to Orbitrap ID-X analysis, we subjected a single 10 μM mix of our authentic 81 standards to HILIC separation. We then collected MS2 data at 10 different collision energies by using DDA (Figure S1A). Inclusion lists were constructed on the basis of m/z values and retention times, and separate runs were performed for positive and negative polarities. Next, we computationally identified the five most abundant fragments for each precursor and the NCE values that led to the highest intensity for each of those product ions (Figure 2A). For more details, see Figure S1B and Supporting Information. The top five most abundant fragments from each precursor on the Orbitrap ID-X were generally consistent with the MRM transitions identified by the built-in optimization software on the TSQ Altis (Figure 2B). We note that we did not consider the few transitions having fragments less than 50 m/z because they cannot be measured on an Orbitrap ID-X. Without any conversion, the optimal NCE values determined from the Orbitrap ID-X data did not match the optimal CE values returned from the optimization software on the TSQ Altis (Figure 2C). Thus, using multivariate linear regression, we derived an equation that converted optimal NCE values from an Orbitrap ID-X into optimal CE values from a TSQ Altis QqQ. We found that the equation was suitable for use in both positive and negative modes. Using this formula, we accurately predicted the CE values when compared to the CE values experimentally determined by the built-in optimization software on a TSQ Altis (Figure 2D).

\[
CE_{\text{Altis}} = 0.0267 \cdot \text{m/z} + 0.408 \cdot \text{NCE} - 0.17
\]

Extending the Approach to Other Instrument Platforms

Equation 1 enables the determination of optimal TSQ Altis CE values from Orbitrap ID-X data, but we recognize that researchers may want to use MS2 data from other Orbitrap or QTOF instruments to derive optimal CE values for alternative QqQ systems. While the approach described
First, we utilized the built-in optimization software on an software were highly consistent with the optimal product ions selected by the MRM optimization analysis of standard solutions for our 81 authentic metabolites. The optimal RF voltages for a QqQ could theoretically be set for each precursor to improve sensitivity. Although a QqQ instrument, on the contrary, an optimal RF voltage can be set for each precursor to improve sensitivity. Although larger compounds, such as acetyl coenzyme A (Figure S5). When performing untargeted metabolomics on a QTOF or an Orbitrap, one RF voltage has to be selected as a compromise for all m/z values because all ions are measured simultaneously. Given that each metabolite is analyzed sequentially on a QqQ instrument, on the contrary, an optimal RF voltage can be set for each precursor to improve sensitivity. Although optimal RF voltages for a QqQ could theoretically be determined on the basis of Orbitrap ID-X data, we found

above could be applied to any pair of instruments, the analysis of 81 authentic standards is a time-consuming process. Accordingly, we sought to determine whether the same strategy could be successfully applied when using a smaller number of authentic standards. To determine the smallest number of authentic standards needed to accurately predict optimal CE values, we again derived an equation to convert Orbitrap ID-X data to optimal TSQ Altis CE values. This time, however, equations were determined by using different numbers of authentic standards. The equations were then applied to the authentic standards that were not used in the derivation (Figure S2A). Highly accurate predictions with $R^2 > 0.70$ were achieved between the predicted and experimental CE values when just 10 randomly selected compounds were used. We found that better results were obtained when the precursors spanned a larger $m/z$ range (Figure S2B). We further predicted that chemically diverse standards would improve the results. We therefore manually chose 10 authentic standards with the following properties: (i) metabolites that represented different compound classes, (ii) $m/z$ values that spanned 90–809 Da, and (iii) standards that are typically available in metabolomics laboratories or can be readily purchased from companies. The 10 standards selected were acetyl coenzyme A, adenine, adenosine, adenosine diphosphate, adenosine monophosphate, citrate, cyclic adenosine monophosphate, glucose 6-phosphate, lactate, and phenylalanine. When constructing an equation for the prediction of optimal TSQ Altis CE values on the basis of Orbitrap MS2 data from only these 10 metabolites, we again found that predicted CE values were consistent with the experimental CE values, having an $R^2$ of 0.86 (Figure S2C). We then applied the equation to the remaining 71 compounds and found that it accurately predicted CE values with an $R^2$ value equal to that of the training data (Figure S2D). The data demonstrate that predicting CE values from just these 10 standards provides comparable results as when CE values are predicted from 81 standards.

To demonstrate that our approach is effective for other mass spectrometers, we applied it to two different QqQ instruments. First, we utilized the built-in optimization software on an Agilent 6460 to find optimal MRM methods on the basis of the analysis of standard solutions for our 81 authentic metabolites. The optimal product ions selected by the MRM optimization software were highly consistent with the five most abundant fragments in the Orbitrap ID-X MS2 data (Figure S3A). Similar to the results from the Altis QqQ, the NCE values determined from the Orbitrap ID-X data showed only a weak correlation with the optimal CE values found by the built-in optimization software on the Agilent QqQ (Figure S3B). We then used the same 10 authentic standards listed above to derive an equation to convert Orbitrap ID-X MS2 data to optimal Agilent 6460 CE values. When applying the equation to all 81 authentic standards, an $R^2$ value of 0.87 was achieved (Figure S3C). Equation 2 shows the conversion formula, which had equal performance whether it was trained on our subset of 10 authentic standards or the full set of 81.

\[
\text{CE}_{6460} = 0.0227 \cdot m/z + 0.473 \cdot \text{NCE} – 3.03
\]

As a second independent validation of our approach, we used optimized MRM data from an Agilent 6495 QqQ that was included in a study by Medina et al.\textsuperscript{11} We determined that the majority of optimal product ions found by the Agilent 6495 QqQ were also among the five most abundant fragments in the Orbitrap ID-X MS2 data (Figure S4A). As expected, we were able to improve our ability to accurately predict optimal Agilent 6495 CE values from Orbitrap ID-X data by using the conversion formula derived from the analysis of the 10 authentic metabolites listed above (Figure S4B,C). Equation 3 shows the conversion formula, which had comparable performance whether it was trained on our subset of 10 authentic standards or the full set of 81.

\[
\text{CE}_{6495} = 0.0207 \cdot m/z + 0.454 \cdot \text{NCE} + 1.26
\]

### Optimization of RF Voltages is Not Required

In addition to selecting product ions and their optimal CE values, the built-in QqQ software can use data from authentic standards to set optimal RF voltages for each precursor. This RF-voltage setting, which has different names depending on the system (e.g., S-lens RF voltage, fragmentor voltage, etc.), is responsible for the successful transfer of ions through the filtering/focusing lenses, and its value is dependent upon the $m/z$ of the ion entering the instrument. Smaller molecules, such as pyruvate, require lower voltages than larger compounds, such as acetyl coenzyme A (Figure S5). When performing untargeted metabolomics on a QTOF or an Orbitrap, one RF voltage has to be selected as a compromise for all $m/z$ values because all ions are measured simultaneously. Given that each metabolite is analyzed sequentially on a QqQ instrument, on the contrary, an optimal RF voltage can be set for each precursor to improve sensitivity. Although optimal RF voltages for a QqQ could theoretically be determined on the basis of Orbitrap ID-X data, we found...
that creating an equation for such a conversion is not necessary. Instead, we can rely on the predetermined RF voltages that are set by the QqQ on the basis of the precursor m/z value. We compared the optimized RF voltages experimentally determined by the built-in QqQ software to the predetermined RF voltages selected on the basis of m/z value alone and found that the resulting peak areas were nearly identical with an $R^2$ of 0.986 (Figure 3A). The results demonstrate that, in contrast to CE values, it is not necessary to predict RF voltages from experimental data.

**Predicted MRM Methods Provide Comparable Sensitivity to Conventional MRM Methods**

After establishing a strategy to design MRM methods for a QqQ instrument from Orbitrap ID-X data, we sought to quantitate the success of our approach. We analyzed a mixture of our 81 authentic standards on the TSQ Altis QqQ by using two different sets of MRM transitions. The first set of MRM transitions was provided by the built-in optimization software, derived from analyses of each authentic standard separately. The second set of MRM transitions was calculated from Orbitrap ID-X data obtained through analysis of a mixed solution of the standards. Highly similar peak areas were found, irrespective of which set of MRM transitions was applied ($R^2 = 0.98$, Figure 3B). These data show that we can successfully predict MRM methods from Orbitrap ID-X data alone without losing sensitivity.

**Extension to Unknown Compounds**

A major benefit of the developed approach is that it can be applied to generate MRM methods for compounds in high-resolution data prior to identifying them (so-called “unknowns”). As an example, we applied the experimental workflow to human plasma. When analyzing the extracted metabolites of a pooled-reference sample on an Orbitrap ID-X in positive and negative polarity, we detected 17,752 features. After background subtraction and filtering of features that are redundant for the same metabolite (e.g., adducts, naturally occurring isotopes, etc.), we reduced the list to 987 features representing potentially unique biological metabolites. Comparable reductions in data have been described in prior studies, which reported more than an order of magnitude fewer metabolites than features.21,32 In this work, we filtered nonbiological and redundant features by using the AcquireX workflow, but any data-reduction strategy can be applied. Given that low-abundant ions typically do not yield high-quality MS2 spectra, we also applied an intensity threshold of 25,000 in this work. While low-abundant signals could potentially be of biological relevance, they provide limited insight without MS2 data because orthogonal data (such as MS2 spectra) are required for confident identification
The maximum of three transitions were proposed-reference sample. All peaks were manually inspected. A coefficient of variation (CV) greater than 20% after three replicate analyses of the pooled-reference sample. The preferred polarity for each precursor, the manual by generating updated inclusion lists after each run. Orbitrap ID-X and used to create an MRM method. We converted into CE values for the TSQ Altis QqQ. Transitions contained 1243 transitions for 554 unique metabolites as identified at level 1 with authentic standards and 116 were identified at level 2 with online libraries.2

We used a scheduled MRM method in which precursor-to-product transitions were monitored over defined retention-time windows where they were expected to elute. By using such scheduled MRM methods on modern QqQ mass spectrometers, it is possible to acquire several hundred transitions in one run without sacrificing sensitivity or reproducibility. 3,4 As such, we were able to monitor at least two product ions per compound, which is generally recommended for specificity. 3,5,6 Thus, applying our workflow to profile samples on a QqQ instrument has equivalent throughput to profiling samples on an Orbitrap ID-X. Moreover, the workflow we established enables samples to be profiled on a QqQ instrument by using MRM methods without losing global coverage of the unique biological metabolites that were profiled on a high-resolution QTOF or Orbitrap.

Evaluation of Sample-Handling Methods for Human Plasma

As a representative application, we aimed to characterize metabolic alterations in human plasma that occur due to differences in preanalytical sample handling. Although the stability of plasma during sample handling and storage has been previously investigated, 37–40 we were specifically interested in plasma metabolites that change as a function of the time between blood collection and centrifugation. This analysis was motivated by studies in which participant blood must be collected outside of a clinic where there is no immediate access to centrifuges. Examples of settings where blood is collected without access to centrifuges may include personal residences, blood drives, mobile medical units, and healthcare facilities in developing countries. In some instances, samples may need to be shipped to a research laboratory via a courier service and the time between blood collection and centrifugation could be several days. Hence, in this study, we tested whether delaying centrifugation by up to 72 h after blood collection affects a sample’s metabolic profile. After collecting specimens from 11 participants in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, an aliquot of blood was immediately centrifuged, and plasma was isolated prior to storage in a −80 °C freezer. An aliquot of blood from the same participants was also kept unprocessed on a frozen gel pack for 24, 48, or 72 h prior to centrifugation, and their polar metabolites were extracted in parallel as detailed in the Experimental Section. To validate the workflow we developed above, polar metabolites extracted from a pooled-reference sample were analyzed with an Orbitrap ID-X, and these high-resolution data were used to design an MRM method for a TSQ Altis QqQ. Metabolites extracted from each of the 44 samples were subsequently profiled by using a TSQ Altis instrument and the previously developed MRM method. Alternatively, metabolites extracted from each of the 44 samples were independently profiled by using an Orbitrap ID-X.

Whether metabolites were profiled by using our computed MRM method on a QqQ or by using full-scan mode on an
Orbitrap ID-X, similar results were obtained. The CVs of a repeatedly injected pooled-reference sample \((n = 6)\) were lower for QqQ data compared to ID-X data, with more than half of the compounds having a CV < 5% and more than 85% of the compounds having a CV < 10% (Figure 4B). A principal component analysis revealed consistent global patterns between both instrument data sets, with samples clustering according to the time between blood collection and centrifugation (Figure 4C and Figure S6). When comparing blood that was immediately centrifuged to the other delayed time points, 209 and 195 compounds were found to be significantly altered in the Orbitrap ID-X and TSQ Altis data, respectively. The overlap between instruments was 155 compounds (Figure 4D). Additionally, metabolites that were determined to be altered with statistical significance between groups exhibited comparable fold changes independent of the analytical workflow in which they were profiled (Figure 4E).

Considering individual compounds, we identified metabolites such as glutamate that increased (Figure 5A), metabolites such as arginine that decreased (Figure 5B), and metabolites such as citrate that did not change in abundance (Figure 5C) as precentrifugation processing time was delayed. A benefit of our approach for designing MRM methods is that we can use a QqQ to profile unidentified compounds, which also showed similar trends between processing times compared to data from an Orbitrap ID-X (Figure S7). A comprehensive list of identified and putatively annotated metabolites together with fold changes over time is provided as Table S4. The results reveal that extended periods of time between blood collection and centrifugation lead to considerable metabolic changes. We conclude that it is preferable to centrifuge specimens as rapidly after blood collection as possible. When the rapid processing of the specimens is not feasible, data from the metabolites found to be altered in this quality-assurance study should be discarded. It may also be possible to normalize metabolite levels in samples where centrifugation is delayed on the basis of their degradation and accumulation kinetics, but more work is required to validate whether such an approach is effective as not all compounds show a linear trend over time (Figure S7). Notwithstanding, this representative application shows that profiling metabolites by using computed MRM methods derived from reference Orbitrap data provides the same level of insight as profiling metabolites in MS1 mode on a high-resolution instrument.

A transition list for human plasma with optimized CE values for three different QqQ instruments (TSQ Altis, Agilent 6460, and Agilent 6495) is given in Table S3. Should one wish to develop a comprehensive set of MRM transitions to profile another sample type, our HRMSS2QQQ python package can be readily applied. In our hands, we were able to carry out the entire workflow in 3 days. This included the acquisition of high-resolution data from a pooled-reference sample and the subsequent calculation of a comprehensive set of optimal MRM transitions for a QqQ instrument. Our data demonstrate the importance of converting the optimal CE values that are experimentally determined from high-resolution data to optimal CE values for a QqQ instrument. Using our equation to transform optimal NCE values from an Orbitrap ID-X to optimal CE values for a TSQ Altis increased the peak areas of 554 compounds by an average value of more than 6-fold compared to when the optimal CE values from an Orbitrap ID-X were used for a TSQ Altis directly (Figure 6). We also note that, because we are profiling two to three transitions for most compounds, we can monitor the ratio between fragment ions to assess the possibility of interference (Figure S8).

**DISCUSSION**

The latest QTOF and Orbitrap instruments have impressive analytical capabilities that range from ion mobility to MS^n. These platforms require a significant investment of capital, however, and are generally beyond what is needed for the simple quantitative application of profiling metabolite levels. In untargeted metabolomics studies with hundreds to thousands of biospecimens, profiling metabolite levels may take weeks to months of instrument time. It is therefore not cost effective to conduct large-scale studies by using only QTOF or Orbitrap mass spectrometers for quantitative analysis. A more economical approach is to profile metabolites from individual samples with less expensive instrumentation and prioritize the unique capabilities of QTOF or Orbitrap platforms for metabolite characterization (i.e., qualitative analysis).

An attractive alternative to QTOF and Orbitrap instruments is QqQ mass spectrometers, which require less of a financial investment, generally have a smaller physical footprint, are already widely available in most clinical laboratories, and enable the acquisition of highly specific transitions. Historically, QqQ systems have had the gold standard for the quantitative analysis of small molecules in fields such as newborn screening, but a limitation of QqQ instruments is that they have required establishing MRM methods with authentic standards. While this is practical to do for targeted metabolomics studies focused on dozens of compounds, obtaining hundreds of authentic standards to match the scope of untargeted metabolomics creates practical challenges. Moreover, many of the compounds measured in a typical untargeted metabolomics experiment performed by QTOF or Orbitrap mass spectrometry cannot be identified, which means it is not even possible to obtain authentic standards for the complete set of chemicals being profiled.

Here, we established a workflow to overcome this barrier by enabling the design of MRM methods on the basis of QTOF or Orbitrap fragmentation data derived from the analysis of biological samples rather than authentic standards. We demonstrate that our MRM methods built without authentic standards have comparable quantitative performance to those.
that were designed with the conventional approach using the built-in QqQ optimization software. A major benefit of the experimental workflow is that it allows metabolites to be profiled by QqQ mass spectrometry irrespective of whether they have been structurally identified. Further, known compounds of low abundance can be added to the transition list. In this work, an equation is provided to convert MS2 data from an Orbitrap ID-X to optimal MRM transitions for a TSQ Altis QqQ. We recognize that the applicability of this equation is limited because other laboratories will have different instrumentation. We therefore developed a simpler strategy to recalibrate our equation to different instrument platforms on the basis of the analysis of just 10 authentic metabolite standards. Data are provided to show that the recalibration procedure works effectively for two different Agilent mass spectrometers. Additionally, even though we used an Orbitrap ID-X here, the approach can be readily adapted to any high-resolution instrument such as a QTOF or Q Exactive Orbitrap, given that each step of the workflow is independent of vendor software. In cases where lower scan rates are required to collect high-quality MS2 spectra, more iterative runs can be performed to achieve comprehensive coverage.

Having the ability to profile knowns and unknowns with MRM methods allows QqQ instruments to achieve the same metabolite coverage as QTOF and Orbitrap systems. When analyzing human plasma by untargeted metabolomics on an MRM platform, millions of MS2 spectra have already been collected beyond predicting MRM methods from pooled-reference standards. We anticipate that this could prove to be especially useful for applications where MRM methods have not yet been developed for many compounds of interest (e.g., toxicants in exposure analysis).

Another important characteristic of our workflow is that we analyzed a pooled-reference sample on a high-resolution instrument to survey the chemical space of the sample matrix. Although thousands of features are often detected, we generally find that there are only a few hundred unique metabolites in an untargeted metabolomics data set after nonbiological compounds and redundant signals are filtered. Targeting only unique biological metabolites with MRM methods allows us to achieve comprehensive metabolite coverage in a single QqQ run while still monitoring the ratios of two or more fragments for each precursor, which is essential for specificity (Figure S8).

A limitation of our approach is that, even though individual research samples are profiled on a QqQ instrument, high-resolution data are still needed. The amount of time required on a high-resolution instrument, however, is limited to the analysis of only a pooled-reference sample. We expect that the high-resolution measurements can be made in less than a day or two, thereby making it possible to perform the experiments in another laboratory or in a core facility. It is important to recognize that the high-resolution instrument used will determine the sensitivity of the QqQ profiling experiments because it will not be possible to predict MRM methods for compounds whose abundance is below the intensity threshold required to collect reliable high-resolution MS2 data. Metabolites known to be present at levels below the MS2 threshold on the high-resolution instrument may be spiked into the sample matrix to overcome this limitation, or already established MRM transitions for such compounds may be manually added to the final method.

Finally, we point out that our workflow has applicability beyond predicting MRM methods from pooled-reference samples. Millions of MS2 spectra have already been collected from authentic standards on QTOF and Orbitrap instruments. These data are publicly available through resources such as mzCloud, which includes breakdown curves that enable the identification of the most intense fragments at different NCE values. By using our approach, these existing MS2 data can be applied to create MRM methods for compounds of interest without the need for purchasing a large number of authentic standards. We anticipate that this could prove to be especially useful for applications where MRM methods have not yet been developed for many compounds of interest (e.g., toxicants in exposure analysis).
Table of MRM transitions for metabolite standards for TQ SIs (XLSX)
Table of MRM transitions for plasma with CEs for three different QqQs (XLSX)
Table of compounds with annotations and fold changes found in the plasma stability study (XLSX)

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