A simultaneous exploratory and quantitative amino acid and biogenic amine metabolic profiling platform for rapid disease phenotyping via UPLC-QToF-MS

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ABSTRACT

Metabolic phenotyping using mass spectrometry (MS) is being applied to ever increasing sample numbers in clinical and epidemiology studies. High-throughput and robust methods are being developed for the accurate measurement of metabolites associated with disease. Traditionally, quantitative assays have utilized triple quadrupole (QQQ) MS based methods; however, the use of such focused methods removes the ability to perform discovery-based metabolic phenotyping. An integrated workflow for the hybrid simultaneous quantification of 34 biogenic amines in combination with full scan high-resolution accurate mass (HRAM) exploratory metabolic phenotyping is presented. Primary and secondary amines are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate prior to reversed-phase liquid chromatographic separation and mass spectrometric detection. Using the HRAM-MS data, retrospective phenotypic data mining could be performed, demonstrating the versatility of HRAM-MS instrumentation in a clinical and molecular epidemiological environment. Quantitative performance was assessed using two MS detector platforms: Waters TQ-XS (QQQ; \(n = 3\)) and Bruker Impact II QToF (HRAMS-MS; \(n = 2\)) and three human biofluids (plasma, serum and urine). Finally, each platform was assessed using a certified external reference sample (NIST SRM 1950 plasma). Intra- and inter-day accuracy and precision were comparable between the QQQ and QToF instruments (\(<15\%)\), with excellent linearity (\(R^2 > 0.99\)) over the quantification range of 1–400 \(\mu\)mol L\(^{-1}\). Quantitative values were comparable across all instruments for human plasma, serum and urine samples, and calculated concentrations were verified against certified reference values for NIST SRM 1950 plasma as an external reference. As a real-life biological exemplar, the method was applied to plasma samples obtained from SARS-CoV-2 positive patients versus healthy controls. Both the QQQ and QToF approaches were equivalent in being able to correctly classify SARS-CoV-2 positivity. Critically, the use of HRAM full scan data was also assessed for retrospective exploratory mining of data to extract additional biogenic amines of biomarker interest beyond the 34 quantified targets.

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1. Introduction

The metabolic phenotype (or metabotype [1]) of an individual is determined by their complex history of gene-environment interactions. Such metabotypes are linked to disease risks at both the individual and population levels and are strongly influenced by disease processes themselves [1,2]. Metabolic phenotyping has been widely applied to expand the biochemical knowledge of a range of diseases including cancers [3,4], cardiovascular disease [5], type 2 diabetes mellitus [6], obesity [7] and liver disease [8]. Mass spectrometry is one of the most commonly adopted techniques for the detection and investigation of metabolic changes, often combined with directly-coupled chromatographic separation [9,10]. In discovery studies the majority of mass spectrometric metabolite phenotyping studies take advantage of high-resolution accurate mass (HRAM) instrumentation (mass resolution > 10,000), commonly quadrupole-time-of-flight (QToF) and Orbitrap technologies, for the detection and characterization of unknown analytes. HRAM instruments afford a high number of scan/s and are selective and sensitive in full scan acquisition mode, allowing multiple accumulations to be recorded in parallel over a large mass range (e.g. m/z from 100 to 1000) and consequently enable a global snapshot of metabolic status. A key advantage of full scan HRAM data is the ability to revisit the dataset to perform retrospective mining and analysis should future unanswered questions arise.

Conversely, low or unit mass resolution triple quadrupole (QQQ) mass analyzers are employed for the quantification of known metabolites of interest. This approach requires high sensitivity and specificity and is regarded as the ‘gold standard’ for quantitative measurements in clinical research [11] and routine analysis [12–14]. Operation via selected or multiple reaction monitoring (SRM/MRM) enables sensitive and selective detection, however, unlike HRAM, SRM/MRM approaches require a priori knowledge of the precursor and product ions and optimized collision energies. Since SRM/MRM-based methods can only acquire specific data channels for known metabolite transitions, no retrospective data mining is possible.

While HRAM platforms have been primarily dedicated to untargeted analysis in metabolic phenotyping, their application for targeted measurements in routine and research analysis have been documented [15–17]. Advancements in QToF technology have resulted in enhanced quantitative performance, with improved sensitivity and dynamic range, and while the use of QToF MS has been shown to be successful for quantitative analyses, reports of their use for simultaneous small molecule quantification with additional full scan profiling in metabolite phenotyping studies remain rare [18]. A wide variety of applications can benefit from simultaneous screening and quantification, including toxicology [19], forensic [20], anti-doping [21] and pesticide screening [22] research. The ability of HRAM platforms to record full scan data together with accurate mass MS/MS fragmentation information using data dependent acquisition (DDA) or data independent acquisition (DIA), such as sequential window acquisition of all the theoretical mass spectra (SWATH [23]) and broadband collision induced dissociation techniques for all ion fragmentation (e.g. bbCID [24], MS² [25]), enables additional retrospective data mining and the potential for detection and identification of novel biomarkers in parallel with generating quantitative data for known targets. The high versatility and performance of most HRAM instruments has placed them as attractive solutions to answer new analytical challenges in systems biology and personalized medicine requiring more holistic, untargeted approaches while simultaneously allowing for quantification of key metabolites or molecular classes of interest and a priori hypotheses.

Metabolic profiling is typically not subject to rigid quality standards as the analytical platforms and methods used are often optimized in-house for each laboratory or research group. Thus, although recommendations and frameworks have been proposed [26], widely accepted standardization does not yet exist. As such, the degree of validation performed varies greatly amongst laboratories, making a global standardization difficult, with few analytical laboratories validating assays to stringent standards laid out by governing bodies such as the US-Food and Drug Administration and European Medicines Agency. In discovery metabolite phenotyping, a pragmatic tiered approach to method validation has been suggested, with appropriate parameters verified using a fit-for-purpose approach ensuring robust analysis suitable for early discovery research [27]. Performing appropriate method validation is becoming critical as metabolic phenotyping moves into epidemiology settings, whereby methodologies are applied to large population cohorts with potential to combine metabolic measurements acquired across multiple sites. To be successful, this requires a new level of analytical rigor and standardization. Confidence that multiple platforms will generate consistent results is critical to the biological interpretation of metabotypes and comparison of data across different instruments and laboratories is also critical for translation into the clinical environment.

Increasingly, clinical laboratories and phenome centres [28] are challenged by large sample numbers and the need for high-throughput, robust analysis with ever-shorter turn-around times. Comprehensive phenotyping demands multiple analytical strategies and tools, such as specific sample preparation and optimization of chromatographic and mass spectrometry parameters tailored to the analytes of interest. Targeted analyses in particular offer the ability to optimize method parameters for particular metabolites or pathways of interest to obtain detailed insights into metabolism, providing unequivocal identification and absolute quantification. Combining hybrid quantitative/exploratory assays in parallel using HRAM platforms, however, is increasingly desirable to answer specific clinical questions as well as allowing for additional discovery phenotyping.

Here, a simultaneous exploratory and quantitative method for the phenotyping of amino acids and biogenic amines, designed for rapid disease profiling via UHPLC-HRAM (QToF-MS) is described. The resultant hybrid quantitative/exploratory assay was adapted from a targeted assay that uses 6-aminoquinolinyl-N-hydroxysuccinimidy carbamate for the derivatization of primary and secondary biogenic amines prior to chromatographic separation [29,30]. The described assay underwent comprehensive validation across five MS platforms; three QQQ (Waters TQ-XS) and two HRAM-QToF (Bruker Impact II) mass analyzers. The performance across the multiple platforms was evaluated, with consideration given to the quantitative accuracy and precision of HRAM instrumentation compared with unit mass QQQ instruments in multiple human biofluids (plasma, serum and urine). Importantly, quantification performance of all platforms was also compared to an externally certified reference material (NIST SRM 1950). Emphasis was placed on analytical data quality which is appropriate for reliable interpretation in metabolite phenotyping studies, informing clinical decisions with translational potential, and the ability to simultaneously capture untargeted data for retrospective and exploratory data mining with HRAM MS analyzers.

Amino acids and biogenic amines are metabolites of great physiological significance, with alterations being reported in many disease states including metabolic disease [31], liver failure [30], neurodegenerative disease [32], sepsis [33] and pathogenic infections [34]. Furthermore, there is currently much interest in the detailed analysis of biogenic amine metabolism in relation to the global SARS-CoV-2 pandemic, with emerging studies reporting significant changes in plasma and serum biogenic amines in patients with COVID-19 [35–37]. Given the urgent need to improve understanding of SARS-CoV-2 infection, we have recently applied targeted QQQ analysis to plasma samples from individuals who tested positive for Covid-19 compared with healthy controls to reveal perturbed amino acid metabolism associated with the disease [38]. Therefore, as an exemplar biological application for the hybrid simultaneous quantitative/exploratory method described, we applied the assay to the same sample set that underwent analysis via QQQ-MS. Our data showed that both platforms provided equivalent results and were able to separate the
participant classes, however in addition the HRAM-MS hybrid approach allowed for retrospective data mining and facilitated the acquisition of additional metabolic information from the sample set.

2. Materials and methods

2.1. Reagents

Unlabelled amino acid standards (physiological amino acids; acids, basics and neutrals), ammonium acetate, sodium hydroxide and formic acid were purchased from Sigma-Aldrich (North Ryde, NSW, Australia). Stable isotope labelled internal standard non-canonical and canonical amino acid mixes were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Water, acetonitrile, methanol and isopropanol (all Optima grade) were purchased from Thermo Fisher Scientific (Melbourne Western Australia). AccQTag Ultra 3X derivatization kit was purchased from Waters Corporation (Milford, MA, USA).

2.2. Calibrators and quality controls

Calibrators and quality controls were prepared from a stock solution of physiological amino acids (acids, basics and neutrals) at 500 μmol L⁻¹ (Sigma-Aldrich, North Ryde, NSW, Australia). On each day, asparagine and glutamine were prepared at 5 mmol L⁻¹ due to instability. A working stock solution containing all amino acids was prepared at 400 μmol L⁻¹. The stable isotope labelled internal standard solution (ISTD, 12.5 μmol L⁻¹ in water) was prepared from stocks of canonical and non-canonical amino acids at 2.5 mmol L⁻¹ in water and stored at -20 °C until use.

2.3. Sample preparation

Calibrators, quality control (QC) and biofluid samples (plasma, serum and urine) were treated using a Biomek i5 sample automation system. Calibrators and QCs were prepared from a working stock solution at the upper limit of quantification, 400 μmol L⁻¹ in water, and diluted to 200, 100, 40, 20, 10, 4, 2 and 1 μmol L⁻¹ for calibrators and 300, 75, 15, and 3 μmol L⁻¹ for quality controls. Biofluids were vortex mixed and centrifuged at 13,000 g for 10 min and 10 μL was transferred to a PCR 96-well plate (Axygen, Fisher Biotech, Wembley, WA, Australia) together with 10 μL of water to result in a 1:1 (v/v) dilution which brings the physiological concentrations within the analytical calibration range. The dilution factor is corrected for following data processing by multiplying the calculated concentrations by two. The dilution factor is corrected for following data processing by multiplying the calculated concentrations by two.

2.4. Liquid chromatography-mass spectrometry

Waters Acquity I-class UHPLC systems (comprising a Binary Solvent Manager, thermostatic Column Manager and FL Sample Manager) coupled to Waters TQ-XS triple quadrupole (Waters Corporation, Wilsnlow, UK), or Bruker Impact II quadrupole time-of-flight (Q-ToF) (Bruker Daltonics, Bellerica, MA) mass analyzers were employed.

2.4.1. Liquid chromatography

The chromatographic separation of analytes was performed on an Acquity UPLC HSS T3 1.8 μm 2.1 × 150 mm column (Waters Corporation, Milford, MA, USA). Eluent A consisted of 2 mM ammonium acetate in water and eluent B consisted of 2 mM ammonium acetate/acetonitrile/water 95/5 (v/v). The flow rate was 0.6 mL/min and column temperature was maintained at 45 °C. The autosampler compartment was cooled to 4 °C and 2 μL injection volume was performed using full-loop injection mode. Gradient elution was performed starting with 5% B for 0.2 min, increasing to 30% B at 5 min, 100% B at 5.1 min for 1 min before returning to 5% B until 7.5 min. The weak and strong washes were water/acetonitrile 95/5 (v/v) and isopropanol respectively.

2.4.2. Triple quadrupole mass spectrometry

Triple quadrupole (QQQ) mass spectrometers (Waters TQ-XS (Waters Corporation, Wilsnlow, UK), were operated in positive electrospray ionization (ESI) and in multiple reaction monitoring (MRM) mode. MS/MS settings were optimized by infusion of each analyte and ISTD with 0.2 mL/min of eluent A/B 50/50 (v/v). MS/MS settings including precursor and product ions and the collision energies optimized for the Waters TQ-XS are detailed in Supporting Information Table S1. The final ion source settings were: capillary voltage = 1.0 kV; cone voltage = 30 V; desolvation gas flow = 1000 L h⁻¹; cone gas flow = 150 L h⁻¹; nebulizer gas = 7.0 bar; desolvation temperature = 650 °C; source temperature = 150 °C.

2.4.3. Quadrupole time-of-flight mass spectrometry

Positive electrospray ionization (ESI) was performed on quadrupole time-of-flight (QToF) mass spectrometers (Bruker Impact II (Bruker Daltonics, Bellerica, MA)). QToF instruments were operated to collect full scan MS and MS/MS fragmentation spectra in the same analytical run using a data independent modality offered by the Bruker broadband collision-induced dissociation (bbCID) function. The collision energy for the MS scan was set to 6.0 eV and alternating low and high energy for MS/MS were set at 20 and 50 eV. The ion source settings were: capillary voltage = 4.5 kV; end plate offset = 500 V; drying gas flow = 12.0 L min⁻¹; nebulizer gas = 5.0 bar; drying temperature = 250 °C. The data acquisition rate was set to 8 Hz over the mass range of m/z 30–1000. An internal calibration was performed by injection of 5 mM sodium formate solution in water/isopropanol (50:50 v/v) at the beginning of every run.

2.5. Method validation

Validation of the method was performed to verify various parameters and the reliability of the developed assay for analysis of samples with either QQQ or QToF detection. The method was validated for selectivity, linearity, accuracy, precision and matrix effects based on a previously reported protocol adapted from FDA and EMA bioanalytical guidelines [30,39]. Stability of processed samples at various storage conditions has previously been investigated and reported elsewhere [30]. Since analyte-free matrix is not available, aqueous external calibration curves and quality control (QC) samples were used to calculate the concentration values during the method validation as described in Section 2.3. The intra-day precision and accuracy were determined within one day by analysing replicates at each QC level of concentrations 3, 15, 75 and 300 μmol L⁻¹. The inter-day accuracy and precision were determined on three separate days by analysing three replicates of each QC at each concentration level. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the QCs values respective to nominal values expressed as a percentage (% bias) and the assay precision was calculated by the percent coefficient of variation (CV %). We performed validation for human plasma, serum and urine, using 12 individual sources of each to assess matrix effects. Matrix effects were determined by comparing the response of the stable isotope labelled internal standards spiked into biological samples compared
with the response in aqueous standards at the equivalent concentrations.

2.5.1. Cross-platform comparison

To evaluate cross-platform comparability, we performed two comparisons. The first compared the extracts of 12 individual human plasma, serum and urine samples for measurement of 34 target metabolites using two different analytical platforms (Waters TQ-XS QQQ and Bruker Impact II QToF). The calculated concentrations were then compared to assess the level of agreement in biological samples across the different instrument platforms. The second comparison applied both mass spectrometry platforms to a biological exemplar consisting of a cohort of individuals infected with SARS-CoV-2 and healthy controls. The samples used in this cross-platform comparison are fully described in Supporting information with clinical characteristics detailed in Table S2.

2.5.2. Comparison with reference material

To further investigate the robustness and performance of our method, we performed replicate determinations of a commercially available reference plasma (NIST SRM 1950) as an external QC. Mean calculated concentrations obtained from the different MS instrument types were compared with the certified reference values for 12 available amino acids.

2.6. Data and statistical analyses

QQQ mass spectrometric data were collected with MassLynx 4.2 (Waters package, Milford, MA, USA) and processed using the TargetLynx package to generate calculated concentrations. Calibration curves were linearly fitted with a weighting factor of 1/x^2. QToF mass spectrometric data were collected with Compass HyStar 5.1 and O-TOF Control version 5.2. Data were processed using TASQ 2.2 (Bruker Daltonics, Bremen, Germany). The molecular formula or exact mass of the derivatized amino acid was used to extract the precursor ions with a mass error of <3 mDa. Calibration curves were linearly fitted with a weighting factor of 1/x. For each biological fluid (i.e., serum, plasma and urine) Bland-Altman analysis [40] was performed to determine mean bias as well as the upper and lower limits of agreement with 95% confidence limits of agreement between the QTOF and QQQ measurements for each of the 34 biogenic amines. Further data analyses and the generation of figures was performed in R Studio (version 1.2.5) running R (version 3.6.1) using in-house pipelines.

3. Results and discussion

High-resolution accurate mass (HRAM) analyzers are widely used mass spectrometry platforms for qualitative global metabolic phenotyping. Here we evaluated QToF technology using a simultaneous quantitative/exploratory data acquisition workflow for the metabolic profiling of amino acids and biogenic amines, with a particular focus on their quantitative performance compared with that of “gold-standard” QQQ mass analyzers. The assay underwent extensive bioanalytical validation for multiple human biofluids (plasma, serum and urine) across multiple MS platforms, considering selectivity, linearity, accuracy, precision and matrix effects. The analytical method was designed to adopt a 7.5 min analysis time (Figure S1), streamlining study planning and sample throughput to complement a practical 24-h cycle in a 96-well plate format (2 x 96-well plate throughput in 24-h), well suited to high-throughput analysis for large-scale population cohorts. The targeted workflow integrates automated sample preparation, including the preparation of calibration standards, quality controls, addition of stable isotope labelled internal standards, protein precipitation and derivatization, which not only affords increased throughput but also consistency in the processes, omitting any inter-individual analytical traits. In addition to quantitative performance, the HRAM platforms were assessed for their ability to simultaneously collect quantitative and qualitative data, allowing for retrospective data mining.

As a biological application exemplar, we compared the performance of QQQ targeted approaches and the hybrid quantitative/exploratory HRAM-MS assay for the investigation a cohort of individuals infected with SARS-CoV-2 and healthy controls, with recent reports of significant differences in circulating amino acids and biogenic amines following SARS-CoV-2 infection [35–37]. Given the global urgency to enhance mechanistic understanding and accurate testing of SARS-CoV-2 infection, this HRAM-MS workflow, where full scan and quantitative targeted data are collected in the same analytical run, will offer optimal productivity and data capture where sample volume is understandably precious and limited.

3.1. Multi-instrument method assessment of quantitative performance

3.1.1. Linearity

The QQQ and QToF platforms demonstrated good linearity over the concentration range 1–400 μmol L⁻¹, with correlation coefficients (r²) higher than 0.99 and residuals lower than 15% (or 20% at the limits of quantification) in all cases, illustrating the quantitative performance of the QToF instruments over the desired concentration range.

3.1.2. Sensitivity and specificity

Despite the difference in absolute sensitivity between the QQQ and QToF platforms, the same lower limit of quantification (LLLOQ) was achieved across all instruments. Following derivatization, standards and samples were either diluted 1:4 (v/v) for injection onto the QToF instruments or 1:49 (v/v) for the QQQ, resulting in absolute lower limit of quantification (LLOQ) concentrations of 400 nmol on column for the QToF and 40 nmol on column for the QQQ. The chromatography was unaffected by the increase in on-column concentration used for QTOF detection.

While specificity and selectivity with QQQ are offered by selection of precursor and multiple fragment ions, ideally one quantifier and one qualifier transition, HRAM provides specificity and robust quantification based on the accurate mass of precursors with narrow mass extraction windows. With QQQ multiple reaction monitoring (MRM) there are instances where interferences are detected within the same transition as the analyte of interest, and unless a unique fragment ion can be found, the components must be completely chromatographically resolved or removed using more extensive sample clean-up procedures for accurate quantification. An example of an unknown co-eluting interference was detected in the development of the QQQ assay for arginine in human urine and is illustrated in Fig. 1. Two out of the three monitored transitions for arginine suffered from a co-eluting matrix interference, while one was found to be selective for arginine to provide accurate quantification. With the use of the accurate mass of the precursor ion from the QToF data, there was no interference detected highlighting the specificity afforded by HRAM with a narrow mass window for analyte extraction (±0.005 Da) (Fig. 1).

3.1.3. Accuracy and precision

The intra- and inter-run accuracy (expressed as mean percentage bias (%)) and precision (expressed as coefficient of variance (CV %)) data are summarized in Supporting Information Table S3 – S7 for the three triple quadrupole (QQQ) and two quadrupole-time-of-flight (QToF) mass platforms considered in this multi-instrument comparison. The pre-defined acceptance criteria for our assay validation was ±20% for mean bias and CV for three replicates of the four QC levels, as proposed by the European Bioanalysis Forum for the analysis of metabolites in plasma [27]. Representative radar plots are shown in Fig. 2 for a sub-set of analytes for which NIST SRM 1950 plasma reference values are available (additional analytes are displayed in Supporting Information Figure S2), illustrating the accuracy (expressed as mean % bias) of inter-day QCs across all five instruments.
3.1.4. Matrix effects

Matrix effects have been previously evaluated on the QQQ platform, reporting no interference from human plasma [30]. Here, this has been expanded to include human serum and urine matrices, as well as comparing between both analytical platforms given their different ion source designs. Matrix effects were evaluated through comparison of the 24 stable isotope labelled (SIL) internal standards (IS) peak areas spiked into individual sources of matrix (plasma, serum or urine) or reference solution at the same concentration (12.5 μmol L$^{-1}$), expressed as a percentage matrix effect (ME %). The ME (%) are displayed in Supporting Information Figure S3 to illustrate the degree of ion suppression/enhancement of each SIL on each of the mass analyzers evaluated. Of the 24 SIL, only cystine was found to suffer from ion suppression in urine on the QToF instruments due to a co-eluting compound. Investigation using the HRAM full scan data revealed a predicted chemical formula of C$_{10}$H$_{9}$N$_{3}$O ($m/z$ 188.0824 [M + H$^+$]), which was identified as the 6-aminoquinoline derivative of ammonia. While SIL IS have been included to correct for matrix effects for those analytes considered for absolute quantification, it is interesting to note this observation on the QToF instruments with the ability to reveal and identify co-eluting species with the full scan accurate mass data capture.

3.2. Comparison of quantitative performance of QQQ and QToF instruments

To evaluate the level of agreement between the calculated concentrations from real matrix samples on each of the five instruments considered in this study, amino acid determinations from 12 plasma, serum and urine samples were compared. A correlation matrix is shown in Fig. 3 which illustrates the high level of agreement between the calculated concentrations of amino acids in real plasma samples across the instrument platforms, demonstrating a correlation coefficient of $>0.849$ (data for serum and urine are displayed in Figure S4). Furthermore, a Bland-Altman analysis [40] was completed presenting results from a QQQ and a QToF for agreement of measurement for each analyte in 12 different plasma, serum and urine samples (Figure S5), which demonstrated that all measurements were within the limits of agreement.

3.3. Verification of amino acid quantification using QQQ and QToF platforms

NIST SRM 1950 plasma was used as a biological reference material to confirm the validity of the assay. Six replicates of NIST SRM 1950 plasma were prepared and analyzed on each system and the results compared to certified reference values available for 12 amino acids. The level of agreement is given as the average concentration from each
3.4. Comparison of multivariate data analysis

In metabolite phenotyping, principal component analysis (PCA) is often employed as an unsupervised statistical tool to interpret large datasets. PCA was applied to amino acid data collected as part of a SARS-CoV-2 infection research project, discussed elsewhere [38], for 34 biogenic amine targets generated from the analysis of plasma samples collected from individuals who were tested and confirmed to be positive or negative for SARS-CoV-2 (study details described in Supporting Information Section 1). The PCA scores plot in Fig. 5 describes the variance between the two biological states and highlights the clustering of samples that were analyzed on a QQQ or a QToF. Together with the patient samples, a long-term reference (LTR) [41] plasma was analyzed.

![Radar plots illustrating accuracy (expressed as mean % bias) of inter-day QCs across all five instruments evaluated. Displayed here are the amino acids for which NIST SRM 1950 certified reference values are available, additional analytes are shown in Supporting Information Figure S2.](image-url)
Fig. 3. Correlation plots demonstrating the level of agreement in calculated amino acid concentrations generated from 12 plasma samples across the three QQQ and two QToF platforms. Additional correlation plots are shown for measurements made from serum and urine samples in Supporting Information Fig. 4 S.
at various intervals throughout the analysis as a quality control to evaluate analytical stability and can be seen to tightly cluster regardless of the platforms used for analysis. The visualization of the data in this way indicates that, by PCA, there is no significant variation attributed to the analytical platform, and that regardless of whether data were acquired on a QQQ or QToF the resulting pattern was recreated due to the

Fig. 4. Mean and 95% CI comparing NIST SRM 1950 plasma amino acid concentrations measured on each type of instrument with certified reference values. The solid horizontal line represents the NIST SRM 1950 reference concentrations and dashed horizontal lines represent confidence boundaries.

Fig. 5. Principal component analysis (PCA) scores plot illustrating the comparison of analytical platform (QQQ or QToF) on the variance in the projection. Individual plasma samples analyzed on either a QQQ (circles) or a QToF (triangles) group together, while the biological variance is not affected by the analytical platform employed as illustrated by positive (green) or negative (purple) SARS-CoV-2 individuals. Long-term reference (LTR) plasma samples used to evaluate analytical stability are shown in red from the QQQ (circles) and QToF (triangles). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
biological variance between infected and non-infected individuals.

This data highlights the great promise of the assay when applied to the metabolite phenotyping of disease. The capability to differentiate between healthy controls and patients who tested positive for Covid-19 demonstrates the value of this methodology for further Covid-19 studies. In the case of Covid-19, much is unknown about the molecular mechanisms relating to clinical outcomes, so combined targeted and exploratory analysis is attractive especially where limited sample volumes are available.

3.5. QToF technology for simultaneous qualitative and quantitative data acquisition

The major advantage of performing targeted analyses on a HRAM instrument is the ability to simultaneously acquire untargeted data that can be mined retrospectively for additional analytes of interest not originally considered in targeted assay development. This can be achieved, for example, using untargeted metabolite phenotyping workflows within MetaboScape 5.0 software (Bruker Daltonics, Bremen, Germany) to reveal unknown features associated with particular sample groups. Using this approach, full scan accurate mass data from the QToF indicated an additional 2700 features detected in the plasma samples. The additional scan functionalities allow for the capture of fragmentation spectra in the same analytical run to enhance the annotation of unknown species. The value of simultaneous untargeted data capture here is illustrated in Fig. 6. The full scan data facilitated the retrospective extraction of an additional related metabolite, kynurenine, from human plasma. Kynurenine was not included in the targeted assay development, and hence data pertaining to this analyte are not acquired using SRM performed on the QQQ instruments. Extraction of the derivatized molecular formula of kynurenine (C_{20}H_{18}N_{4}O_{4}, theoretical mass m/z 379.1401 ([M + H^+]^+)) revealed detection in human plasma, with identification through its acquired fragmentation spectrum and chemical formula prediction, with further subsequent confirmation using an authentic reference standard. Accurate mass measurements in both full scan mode and MS/MS modes confer enhanced confidence in analyte identification. With the derivatization of all primary and secondary amines using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, accurate mass in MS/MS mode generates a common fragment ion (m/z 171.0558 ([M + H^+]^+)) which is valuable for the reliable structural elucidation of unknown metabolites.

This example is particularly pertinent given the current interest in amino acid and biogenic amine signatures associated with SARS-CoV-2 infection. Significant changes in circulating tryptophan have recently been reported [35–37], and hence the ability to retrospectively mine and perform exploratory biomarker discovery for related pathway metabolites such as kynurenine, in combination with targeted quantification, offers great potential for providing mechanistic insights and potential markers of infection.

4. Conclusions

The HRAM paradigm has become crucial for understanding the complexity of biological systems and for delivering better personalized diagnostics and medicine, with combined capabilities of recording a global and quantitative view of the metabolome simultaneously. HRAM instrumentation today shows suitable sensitivity and reliable quantification, with the advantage of capturing a global profile in the same analytical run.

Primarily we assessed the quantitative performance of HRAM-MS when compared to the gold standard QQQ workflow. A multi-platform validation was performed, demonstrating accurate and reliable measurements across different QQQ and QToF instruments. The present workflow describes optimized sample preparation allowing automation for maximum throughput and reproducibility, applied to the quantification of biogenic amines in biological matrices. The validation data and application to real samples show excellent agreement between QQQ and QToF platforms. This indicates that QToF technology can be successfully
utilized for robust and accurate quantification, whilst critically allowing for additional simultaneous untargeted profiling for retrospective data mining and exploratory analysis of unknown metabolites. Sample preparation includes derivatization of primary and secondary amines, enabling sensitive detection for reliable quantification on QToF instrumentation and assisting with identification through the generation of a common fragment ion. However, it is noteworthy to mention that the simultaneous exploratory and quantitative approach will not be universal for all applications given the inherent lower sensitivity compared with QQQ platforms, thereby potentially restricting limits of quantification. While the simultaneous collection of exploratory and quantitative data is highlighted here for amine profiling, the utility of which is demonstrated through the discovery of additional related amines not originally targeted for quantification, future work will focus on the effect of the derivatization reaction on non-derivatized metabolites. In order to fully realize the potential of the exploratory workflow, it will be important to determine the influence of the derivatization reaction on metabolites other than primary and secondary amines for untargeted data processing. Although there remains a lack of global standardization and harmonization amongst metabolic phenotyping laboratories, the quantification performed here for amino acids in human plasma, serum and urine shows excellent agreement across multiple platforms and different technologies, providing confidence that well characterized and validated assays will generate consistent results for reliable biological interpretation, critical for understanding human metabolism in health and disease.

CRediT author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data can be found at https://doi.org/10.1016/j.talanta.2020.121872.

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