

● Rapid metabolic phenotyping in SARS-CoV-2 patients using the impact II QTOF MS system

High mass-accurate MS and MS/MS data collection for targeted analyses and flexibility for discovery data mining to confidently drive critical metabolic research workflows

Abstract

The impact II QTOF system meets the critical elements of sensitivity, selectivity, accuracy, and precision for targeted metabolite analysis. The con-

tinuous collection of both MS and MS/MS data provides an exponentially deeper and broader pool of data valuable for subsequent retrospective discovery or targeted analyses. This analytical flexibility is particularly

valuable in metabolic phenotyping of biological samples when molecular mechanisms are poorly understood and knowledge is emerging at a rapid pace, as with the SARS-CoV-2 global pandemic.

Keywords:
impact II QTOF MS, metabolic phenotyping, quantitative metabolomics, retrospective discovery, SARS-CoV-2, MetaboScape®2021, TASQ®, cross-platform validation

Introduction

The impact II QTOF MS system has been established to have high utility in many critical analyses, including toxicology, forensic medicine, antidoping, and pesticide screening [1-3]. This application flexibility is driven in part by the system's robustness and ability to simultaneously record full, high accurate mass, MS scan data together with accurate mass MS/MS fragmentation information for confident identification of both known and novel targets. Further, robust hardware and software components support high sample throughput and subsequent rapid data processing requirements.

Within the "omics" scope, interest in metabolic phenotyping has dramatically increased in recent years. Individual metabolic phenotypes, determined by a complex history of gene-environment interactions, may be linked to disease risks [4], and population-based profiling has been widely applied to expand the biochemical knowledge of a range of diseases as well as in effort to understand patterns of disease severity. However, a targeted approach of quantitating particular metabolite panels linked to a given disease state or a metabolic pathway of interest can provide detailed insights to support biomedical research or to specifically characterize an individual's current state of health. This workflow often employs LC-TQ MS systems due to their low detection limits and wide dynamic range in targeted analyses.

In this study, the quantitative performance of the impact II UHR-QTOF (Ultra-High Resolution Quadrupole-Time-Of-Flight) for metabolite analyses was compared to that of a

Table 1. Instrument parameters

Liquid chromatography			
Instrument	UPLC I-Class		
Column	UPLC HSS T3 1.8 μ m 2.1 x 150 mm		
Column oven temp.	45°C		
Mobile phases	A: 2 mM ammonium acetate in water B: 2 mM ammonium acetate in acetonitrile/water (95/5)		
Flow rate	0.6 mL/min		
Gradient	0 min	5% B	
	0.2 min	5% B	
	5.0 min	30% B	
	5.1 min	100% B	
	6.1 min	100% B	
	7.5 min	5% B	
Injection volume	2 μ L		
Mass spectrometry			
MS	impact II UHR QTOF	Triple quadrupole (TQ)	
Ionization (V)	ESI (+) 4500	ESI (+) 1000	
Dry gas (L/hr)	720	1000	
Nebulizer gas (Bar)	5	7	
Desolvation temperature (°C)	250	650	
Cone voltage (V)	-	30	
Cone gas (L/hr)	-	150	
Source temperature (°C)	-	150	
MS mode	Quantitation Full MS scan + bbCID*	Discovery Auto MS/MS	Multiple Reaction Monitoring (MRM) of 34 biogenic amines
Acquisition rate	8 Hz	12 Hz (MS) 16-30 Hz (MS/MS)	
Collision energy	6 eV (MS) 20 and 50eV (MS/MS)	6eV (MS) 20-50eV (MS/MS)	See Reference 6
Mass range	30 – 1000 m/z		

* bbCID - Broadband Collision Induced Dissociation, where both TOF MS full scan data and MS/MS fragments are continuously generated in two independent, rapidly alternating data channels, without either precursor ion or threshold selection. See Discussion.

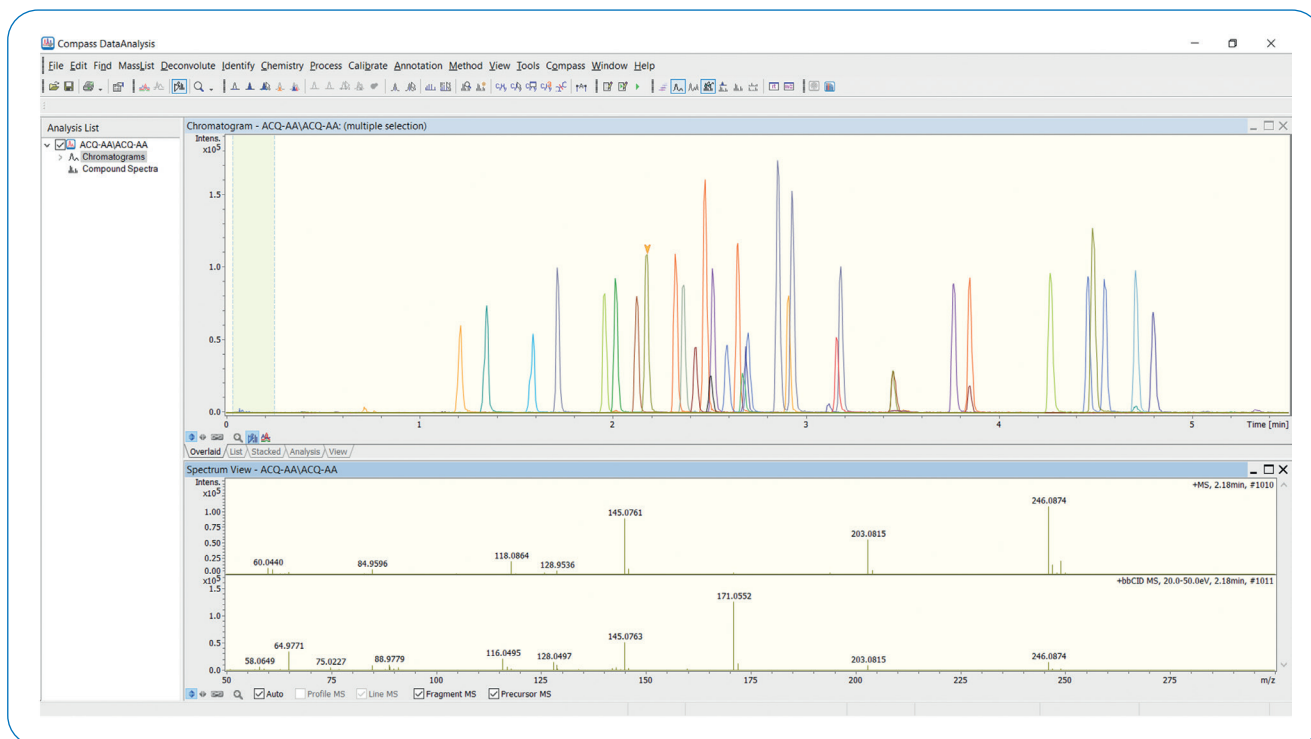


Figure 1. Extracted ion chromatogram (EIC) of 34 biogenic amines shown in the Chromatogram view (upper panel). The Spectrum view (lower panel) shows the MS and MS/MS spectra of glycine at 2.2 min.

current TQ MS system in the quantitative detection of 34 biogenic amines reported to be altered in COVID-19 positive individuals [5]. Three human biofluids (plasma, serum, and urine) were profiled on multiple instruments (two impact II QTOF systems, three TQ systems) within a certified laboratory (The Australian National Phenome Centre, ANPC). A certified external reference sample (NIST SRM 1950 plasma) was also profiled on all systems.

The elements of analytical method sensitivity, detection accuracy, and reproducibility, as examined, are critical for confidence in any screening workflow, including metabolic phenotyping. The extension of analytical scope within the data collected is also of high value in order to fully explore any unique biochemical signatures, particularly in the case of new or emerging clinical challenges. In this study, the accurate mass MS and MS/MS data sets provided by the impact II systems could be

retrospectively examined to identify an additional bioamine of clinical significance.

Experimental

Sample preparation

Briefly, 10 μ L of biofluids (plasma, serum, and urine) were centrifuged and transferred onto a 96-well plate. For the analysis of the cohorts of SARS-CoV-2 (positive and negative) plasma samples, a pooled sample was also prepared. Each plate included calibrators (1 to 400 μ mol/L) and quality controls (QC; 300, 75, 15, and 3 μ mol/L) prepared in DI water. An internal standard mixture was added to each well, followed by protein precipitation with methanol. An aliquot of the supernatant was transferred into a new 96-well plate and derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to transform primary and secondary amines into highly stable derivatives. The subsequent derivatized samples

were diluted 1:4 and 1:49 with water for impact II QTOF and TQ analysis, respectively. Refer to reference [6] for detailed methodology.

Data acquisition

Instrument parameters are listed in Table 1.

Data processing

QTOF MS and MS/MS data from the impact II data were processed using TASQ[®] 2.2. 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 $1/x$. A representative QTOF Extracted Ion Chromatogram is shown in Figure 1. MRM data collected on the TQ systems was processed using the manufacturer's software, and calibration curves were linearly fitted with a weighting factor of $1/x^2$.

Method validation

A multidimensional evaluation of the analytical performance of the impact II QTOF systems was conducted using the QC samples and compared directly with the performance of the TQ systems. Metabolite target selectivity, calibration linearity, intra- and inter-day accuracy, precision, and matrix effects were each examined, based on a previously reported protocol adapted from FDA and EMA bioanalytical guidelines [7,8]. The quantitation accuracy of the method was investigated with the analysis of reference plasma (NIST SRM 1950) for comparison to the certified reference values for 12 available amino acids.

Authentic biofluid analysis

For a more comprehensive comparison between the two analytical platforms, 34 target biogenic amines (Table 2) were sought in 12 individual healthy human plasma, serum, and urine samples, with data collection as previously defined for each platform. Further statistical analyses were applied identically to all data sets using ANPC's in-house pipelines.

For a broader, more systemic comparative view, targeted quantitative metabolite analyses were made on both the QTOF and TQ platforms on plasma samples from a cohort of individuals infected with SARS-CoV-2 (n=7) and a cohort of healthy controls (n=8). Unlike previous comparisons, the levels of the 34 target biogenic amines as detected on both platforms were compared together within a multivariate PCA. Reference plasma (NIST SRM 1950) was also analyzed as a control.

Table 2. Targeted biogenic amine and amino acids

Analyte	Formula	Accurate mass [M+H] ⁺ (Da)	RT (min)	IS
1-methylhistidine	C ₁₇ H ₁₇ N ₅ O ₃	340.1404	2.73	Arginine- ¹³ C ₆ , ¹⁵ N ₄
2-(α)aminobutyric acid	C ₁₄ H ₁₅ N ₃ O ₃	274.1186	3.23	Proline- ¹³ C ₅ , ¹⁵ N
3-(β)aminoisobutyric acid	C ₁₄ H ₁₅ N ₃ O ₃	274.1186	2.96	Proline- ¹³ C ₅ , ¹⁵ N
3-methylhistidine	C ₁₇ H ₁₇ N ₅ O ₃	340.1404	2.62	Histidine- ¹³ C ₆ , ¹⁵ N ₃
4-(γ)aminobutyric acid	C ₁₄ H ₁₅ N ₃ O ₃	274.1186	2.87	Proline- ¹³ C ₅ , ¹⁵ N
4-hydroxyproline	C ₁₅ H ₁₅ N ₃ O ₄	302.1135	1.78	Asparagine- ¹³ C ₄ , ¹⁵ N ₂
Alanine	C ₁₃ H ₁₃ N ₃ O ₃	260.103	2.7	Alanine- ¹³ C ₃ , ¹⁵ N
Amino adipic acid	C ₁₆ H ₁₇ N ₃ O ₅	332.1241	1.66	Serine- ¹³ C ₃ , ¹⁵ N
Arginine	C ₁₆ H ₂₀ N ₆ O ₃	345.167	2.69	Arginine- ¹³ C ₆ , ¹⁵ N ₄
Asparagine	C ₁₄ H ₁₄ N ₄ O ₄	303.1088	2.02	Asparagine- ¹³ C ₄ , ¹⁵ N ₂
Aspartic acid	C ₁₄ H ₁₃ N ₃ O ₅	304.0928	1.26	Aspartic acid- ¹³ C ₄ , ¹⁵ N
β-Alanine	C ₁₃ H ₁₃ N ₃ O ₃	260.103	2.38	β-Alanine- ¹³ C ₃ , ¹⁵ N
Carnosine	C ₁₉ H ₂₀ N ₆ O ₄	397.1619	2.71	Sarcosine- ¹³ C ₃ , ¹⁵ N
Citrulline	C ₁₆ H ₁₉ N ₅ O ₄	346.151	2.41	Citrulline- ¹³ C ₅
Cystine	C ₂₆ H ₂₄ N ₆ O ₆ S ₂	291.0672	3.22	Cystine- ¹³ C ₆ , ¹⁵ N ₂
Ethanolamine	C ₁₂ H ₁₃ N ₃ O ₂	232.1081	3.49	Proline- ¹³ C ₅ , ¹⁵ N
Glutamic acid	C ₁₅ H ₁₅ N ₃ O ₅	318.1084	1.4	Glutamic acid- ¹³ C ₅ , ¹⁵ N
Glutamine	C ₁₅ H ₁₆ N ₄ O ₄	317.1244	2.18	Glutamine- ¹³ C ₅ , ¹⁵ N
Glycine	C ₁₂ H ₁₁ N ₃ O ₃	246.0873	2.24	Glycine- ¹³ C ₂ , ¹⁵ N
Histidine	C ₁₆ H ₁₅ N ₅ O ₃	326.1248	2.44	Histidine- ¹³ C ₆ , ¹⁵ N ₃
Isoleucine	C ₁₆ H ₁₉ N ₃ O ₃	302.1499	4.51	Isoleucine- ¹³ C ₆ , ¹⁵ N
Leucine	C ₁₆ H ₁₉ N ₃ O ₃	302.1499	4.6	Leucine- ¹³ C ₆ , ¹⁵ N
Lysine	C ₂₆ H ₂₆ N ₆ O ₄	244.1081	4.53	Lysine- ¹³ C ₆ , ¹⁵ N ₂
Methionine	C ₁₅ H ₁₇ N ₃ O ₃ S	320.1063	3.91	Methionine- ¹³ C ₅ , ¹⁵ N
Ornithine	C ₂₅ H ₂₄ N ₆ O ₄	237.1008	4.32	Ornithine- ¹³ C ₅
Phenylalanine	C ₁₉ H ₁₇ N ₃ O ₃	336.1343	4.77	Phenylalanine- ¹³ C ₉ , ¹⁵ N
Proline	C ₁₅ H ₁₅ N ₃ O ₃	286.1186	2.95	Proline- ¹³ C ₅ , ¹⁵ N
Sarcosine	C ₁₃ H ₁₃ N ₃ O ₃	260.103	2.52	Sarcosine- ¹³ C ₃ , ¹⁵ N
Serine	C ₁₃ H ₁₃ N ₃ O ₄	276.0979	2.08	Serine- ¹³ C ₃ , ¹⁵ N
Taurine	C ₁₂ H ₁₃ N ₃ O ₄ S	296.07	2.58	Histidine- ¹³ C ₆ , ¹⁵ N ₃
Threonine	C ₁₄ H ₁₅ N ₃ O ₄	290.1135	2.57	Threonine- ¹³ C ₄ , ¹⁵ N
Tryptophan	C ₂₁ H ₁₈ N ₄ O ₃	375.1452	4.86	Tryptophan- ¹³ C ₁₁ , ¹⁵ N ₂
Tyrosine	C ₁₉ H ₁₇ N ₃ O ₄	352.1292	3.5	Tyrosine- ¹³ C ₉ , ¹⁵ N
Valine	C ₁₅ H ₁₇ N ₃ O ₃	288.1343	3.81	Valine- ¹³ C ₆ , ¹⁵ N

Retrospective data mining

Retrospective analysis of the QTOF MS data for additional analytes of interest (not originally considered in the targeted comparative profiling) was initiated by the analysis of the pooled plasma sample from the cohort study using the auto MS/MS method (Table 1) and processed on MetaboScape®2021. A table of mass features, or 'buckets,' was generated, grouping together all related adducts (e.g., [M+H]⁺, [M+Na]⁺, etc.) for all detected precursor compounds. To allow easier viewing of derivatized compounds, the dataset was filtered within MetaboScape to show/display only buckets containing MS/MS fragments of the AQC adducts (C₈H₁₀N₂O₂; 171.055 *m/z*), which is a common fragment signature from the cleavage at the ureide bond formed upon derivatization. Subsequently, all buckets from the previously targeted biogenic amines were excluded. Compounds that were not annotated were further investigated using accurate mass MS and MS/MS data for tentative identification using SmartFormula. The identity of one compound (tentatively identified) to be of clinical interest was confirmed by comparison with a commercial standard.

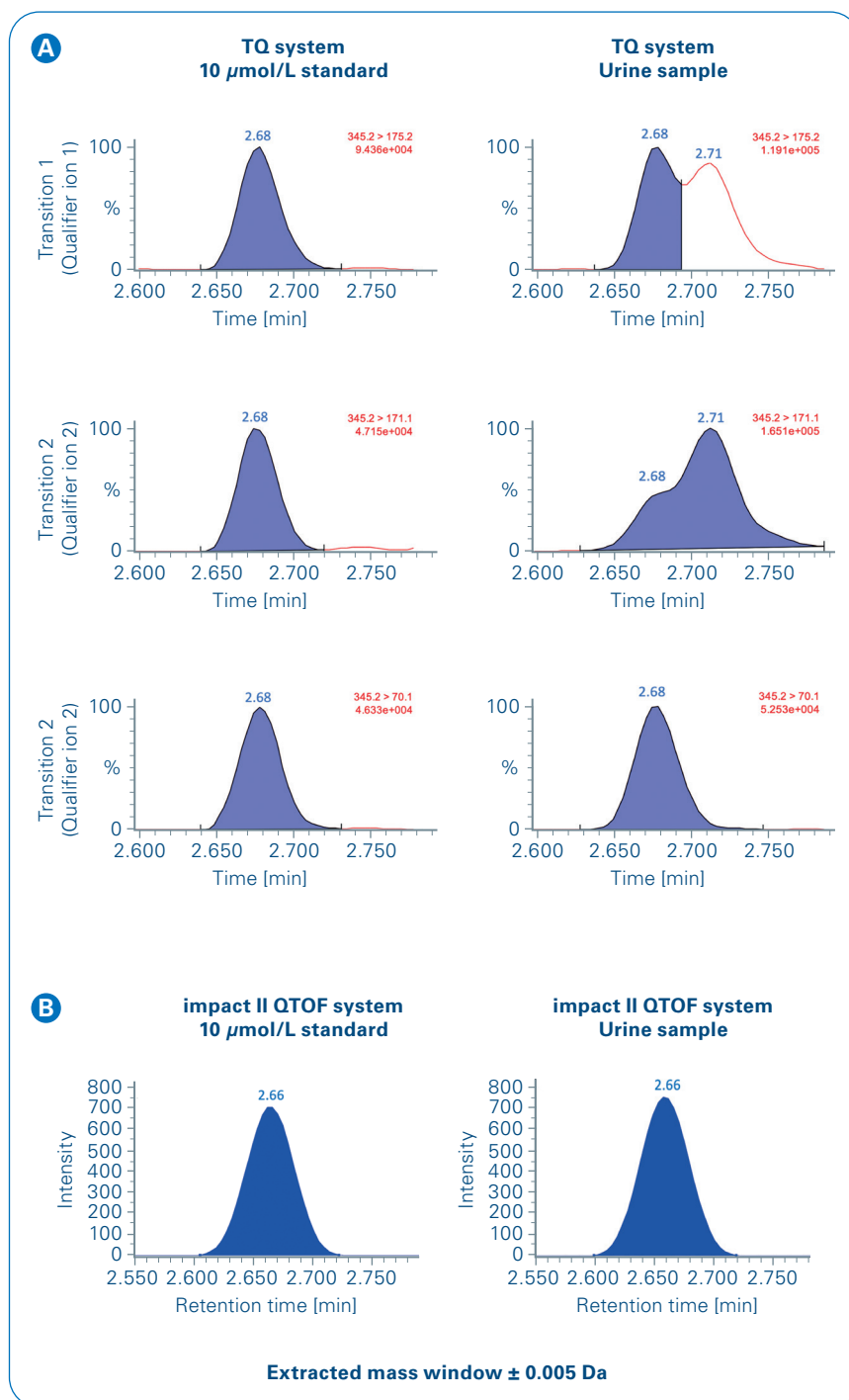


Figure 2. **A** Example of TQ and QTOF specificity for arginine in human urine. Top: Three TQ MRM transitions monitored for arginine in 10 µmol/L standard solution (left panel), and in urine samples (right panel). Peak interference is present in two of the three TQ monitored transitions. The interference is not present in the standard solution (left panel). **B** Using mass accurate data extraction on the QTOF MS, no interference in the urine sample (right panel) is detected. (Adapted from Reference 6. Reprinted with permission).

Results and Discussion

Comparative analytical performance for method validation

Target selectivity

TQ MS systems are often the instrument of choice for routine targeted and quantitative workflows as they are generally considered to be more sensitive and robust than QTOF MS

platforms. In this study, the absolute lower limits of quantitation (LLOQ) for all targeted amino acids and bioamines by the QTOFs (400 nmol on-column) were higher than in the TQ (40 nmol on-column). However, the multiple reaction monitoring (MRM) of the TQ systems was challenged by an unknown co-eluting matrix interference in two of three monitored transitions for the arginine target in many of the urine samples

(Figure 2A). This interference was not detected in the QTOF system. The high mass accuracy of the impact II permits the application of a narrow mass extraction window (± 0.005 Da), as shown in the extracted ion chromatogram (EIC) (Figure 2B). All target compounds could be quickly and cleanly isolated from the complete data pool, regardless of sample type.

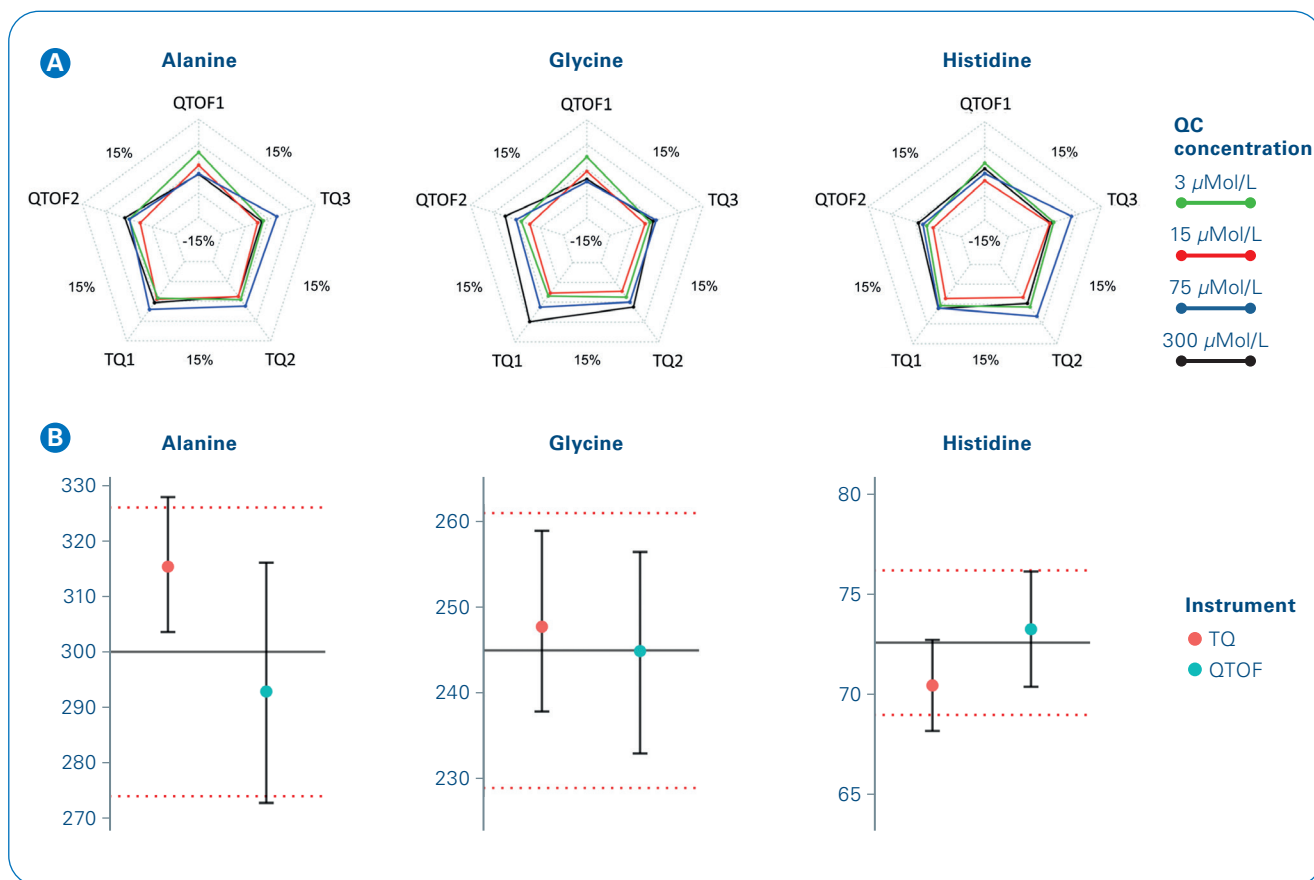


Figure 3. (A) Representative radar plots illustrating accuracy (expressed as mean % bias) of inter-day QCs of three amino acid targets across all five instruments evaluated. (B) Mean and 95% CI comparing plasma amino acid concentrations of reference serum as measured on each type of instrument with certified reference values. The solid horizontal line represents the NIST SRM 1950 reference concentrations and dashed horizontal lines represent confidence boundaries. (Adapted from Reference 6. Reprinted with permission).

Linearity

The impact II QTOF MS demonstrated excellent quantitative performance with linearity over the concentration range (1 – 400 $\mu\text{mol/L}$) with correlation coefficients (r^2) higher than 0.99 and residuals lower than 15% for all metabolite targets, similar to those observed in TQ.

Accuracy and Precision

The intra- and inter-run accuracy, expressed as mean percentage bias (%), and precision, expressed as coefficient of variance (% CV), were evaluated based on the recommendations of the European Bioanalysis Forum [9] for metabolites in plasma samples. The mean bias of the 34 amino acids was within $\pm 20\%$ in analyses on both system types,

as were the % CV values for three replicates of the four QC levels, as demonstrated by the radar plots in Figure 3A.

Through the parallel analyses of the NIST SRM 1950 standard on both platforms, the quantitation accuracy of 12 amino acids was found to be in good agreement with the certified reference values. The accuracy of the concentrations observed by the instruments was within 12.9% bias for all 12 amino acids. Values for alanine, glycine, and histidine are shown in Figure 3B.

Matrix effects

Sample matrices are known to cause ion suppression in many MS analyses. To evaluate this effect, 24 stable isotope labeled (SIL) internal

standards were spiked into individual matrices (serum, plasma, and urine) or reference solution. The matrix effect (% ME), expressed as a percentage, illustrates the degree of ion suppression/enhancement as shown in Figure 4. Of the 24 SIL investigated, only cystine in urine was found to be adversely affected by ion suppression ($>25\%$). Further investigation using the high-resolution accurate mass full scan data predicted a molecule with a chemical formula of $\text{C}_{10}\text{H}_9\text{N}_3\text{O}$ (m/z 188.0824 $[\text{M}+\text{H}^+]$), which was identified as the 6-aminoquinoline derivative of ammonia. This finding illustrates a key advantage of full scan accurate mass data acquisition of QTOF instruments – the ability to detect and identify co-eluting species.

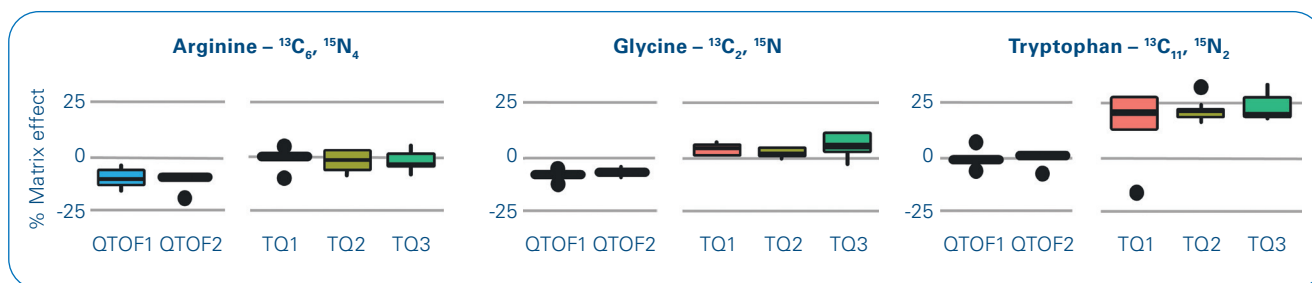


Figure 4. Box plots illustrate the degree of matrix effect (%) in plasma samples across the instruments. Matrix effects shown are evaluated by calculating the percentage difference between the peak area of stable isotope labeled internal standards spiked into plasma or standard solution. (Adapted from Reference 6. Reprinted with permission).

Quantitative robustness and parity for authentic biofluids

The robustness of the methodologies was further investigated with three common types of biofluids: serum, plasma, and urine (n=12 each). A high level of agreement was observed between the calculated amino acid concentrations in serum, plasma, and urine as measured across the various platforms, with a correlation coefficient of >0.85 (Figure 5). Furthermore, Bland-Altman analysis demonstrated that all measurements were also within the limits of agreement for all platforms.

As mentioned, the 34 metabolic targets evaluated within the course of this work had been previously shown to be modified in SARS-CoV-2 positive individuals [5]. In

some cases, monitoring individual metabolites may be of particular interest, potentially serving as analytical guideposts for specific metabolic pathways or organ function. Concentration differences in several metabolites demonstrated to be altered by SARS-CoV-2 [5,9,10,11], such as tryptophan and taurine, were also observed in plasma from the cohort of individuals who had tested positive for SARS-CoV-2 and the cohort of healthy controls (Figure 6). In this study, the small sample cohort size precludes rigorous evaluation of statistical significance; however, the trends of these differentiating metabolites are evident and independent of the analytical platform.

Multivariate analysis of the 34 targeted bioamines and amino acids in the SARS-CoV-2 positive and

negative cohorts (Figure 7) further illustrates the robustness of the QTOF MS system. Individual patient samples cluster similarly on both platforms; thus, the separation of diseased and healthy cohorts was equitable between the QTOF (data shown as triangles) and TQ (shown as circles) MS systems. This analysis also included replicates of the certified reference plasma, which clustered very tightly together (Figure 7, red triangles and circles). This discriminatory parity between the two platforms is an important factor to consider, as metabolites may be able to serve as diagnostic biomarkers or help to guide care based on identified systemic effects.

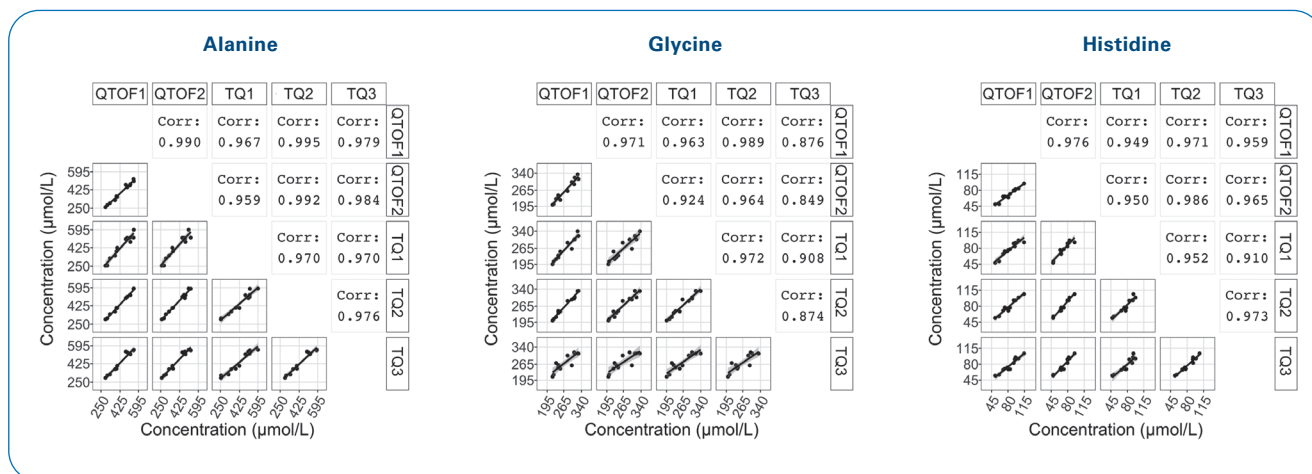


Figure 5. Correlation plots demonstrating the level of agreement in calculated amino acid concentrations in plasma samples generated across the three TQ and two QTOF platforms. (Adapted from Reference 6. Reprinted with permission).

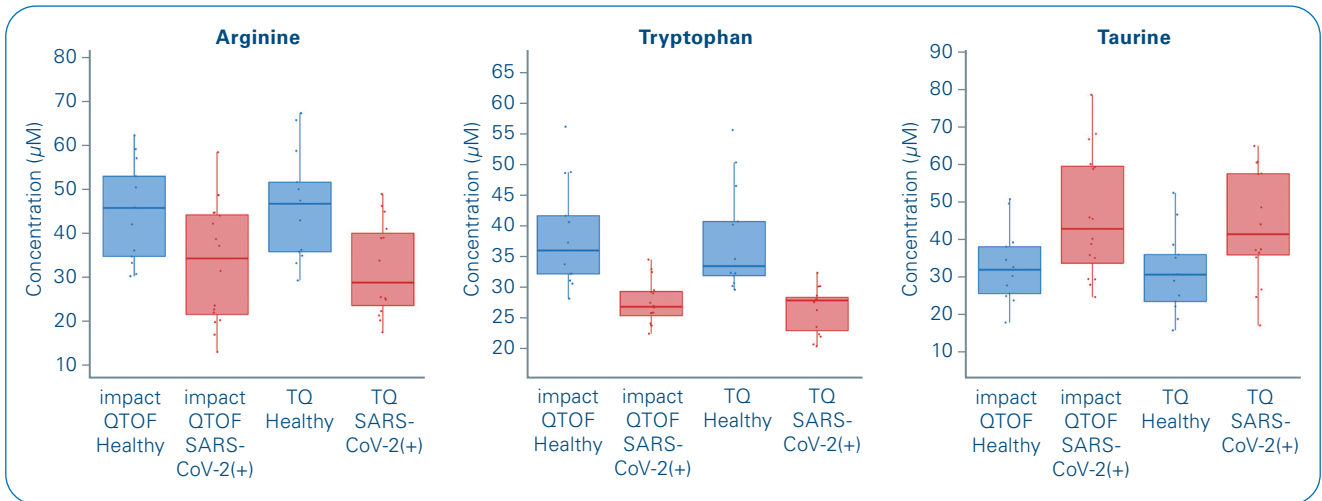


Figure 6. Example plasma bioamine concentration differences between cohorts of SARS-CoV-2 positive and healthy individuals as observed on the QTOF and TQ platforms.

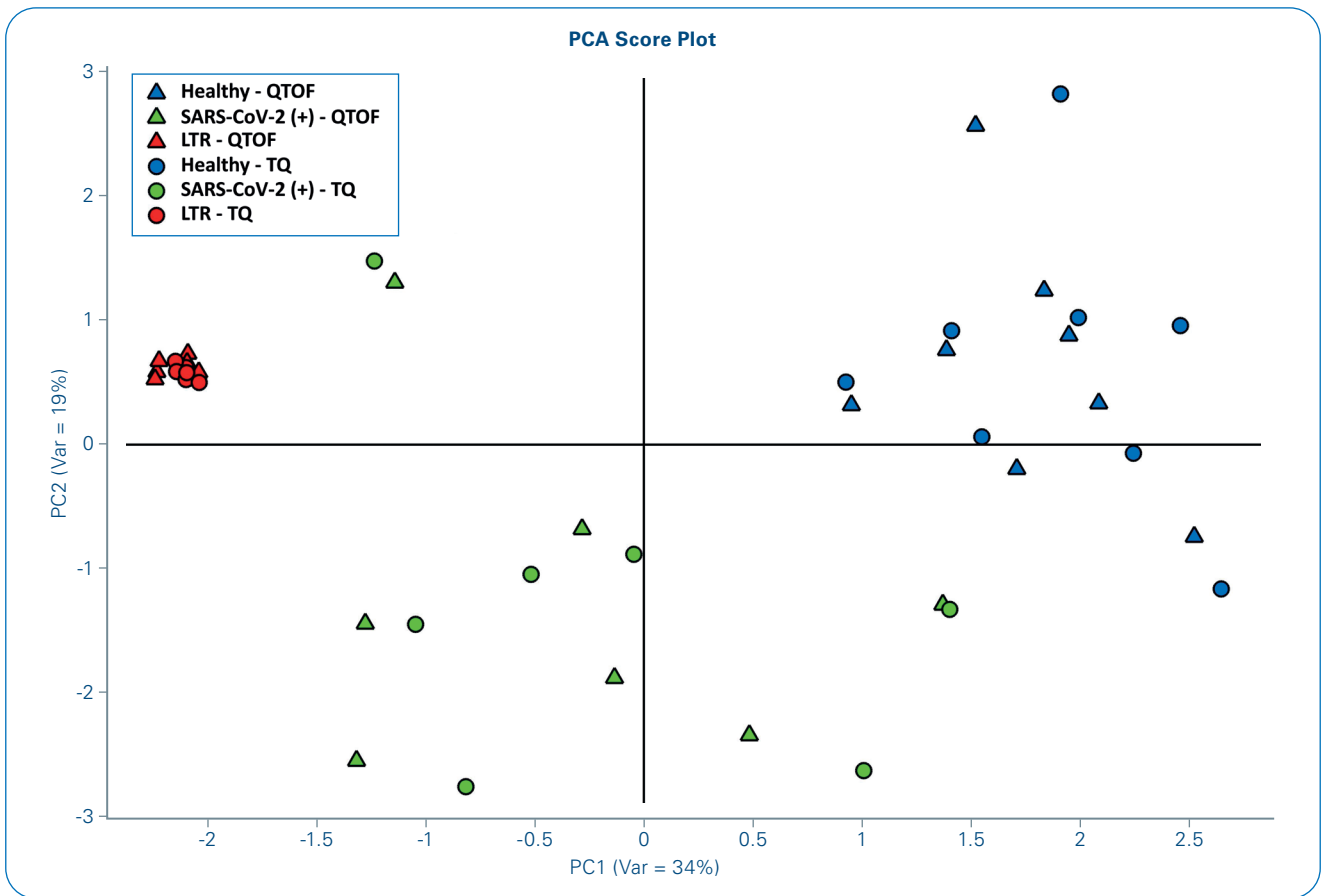
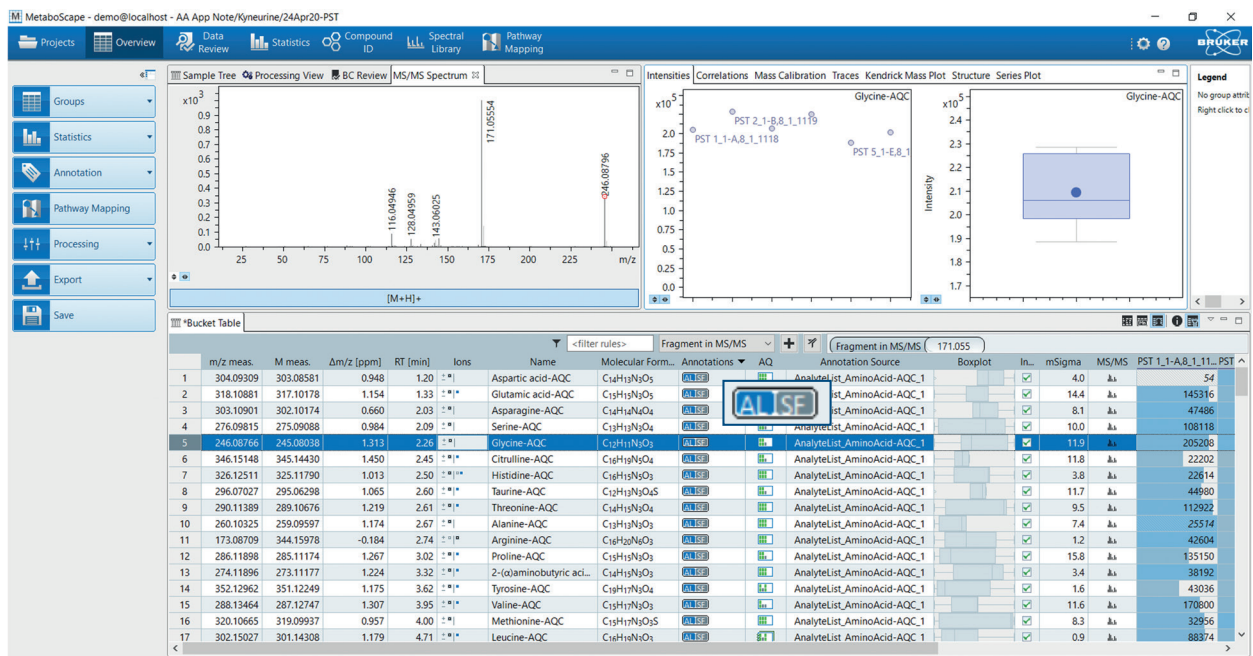


Figure 7. Principal component analysis (PCA) scores plot illustrating the comparison of analytical platform (TQ or QTOF) on the quantitative assessment of biological variance between the positive (green) or negative (blue) SARS-CoV-2 individuals. As shown, all negative or positive individuals cluster within their respective diagnostic group. Additionally, plasma samples from each individual group together whether analyzed on a TQ (circles) or a QTOF (triangles). The certified external reference plasma (red) clusters tightly together, regardless of instrument. (Adapted from Reference 6. Reprinted with permission).

A**B**

m/z meas.	M meas.	$\Delta m/z$ [ppm]	RT [min]	Ions	Name	Molecular Formula	Annotations	AQ	Annotation Source	Boxplot	In...	mSigma	MS/MS
102	379.14047	378.13319	1.017	4.78		C ₂₀ H ₁₈ N ₄ O ₄	SB	✓		✓	21.3	Δ	

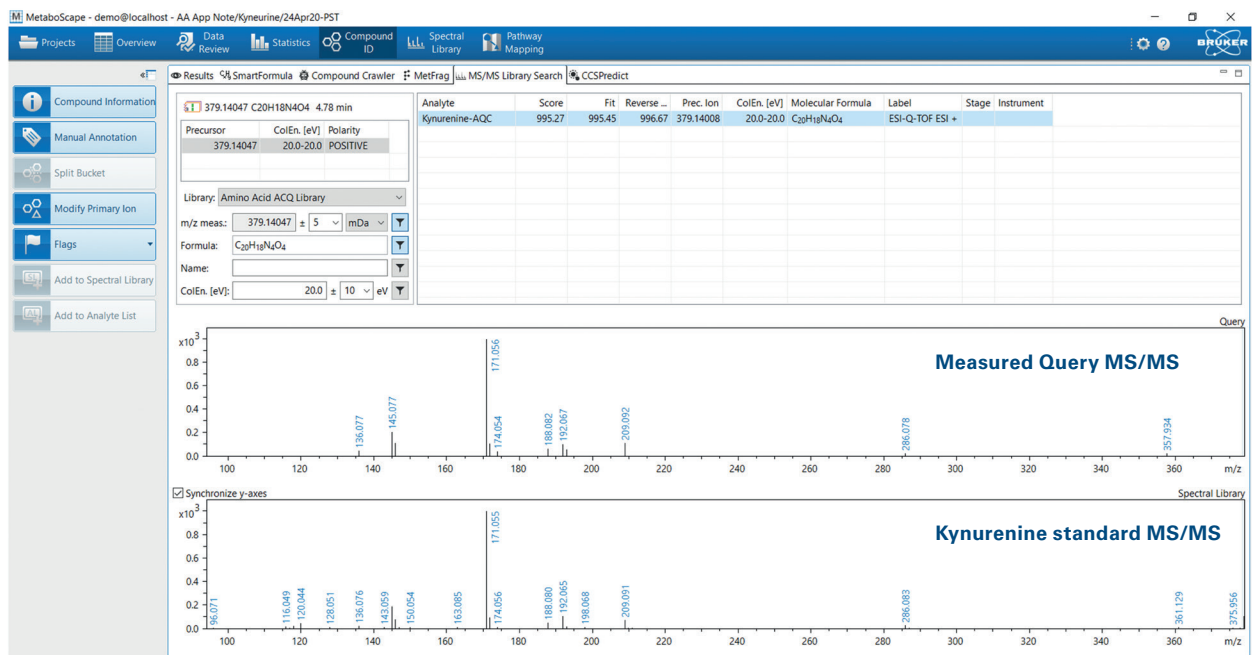
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Figure 8. **A** Dashboard view of MetaboScape 2021 showing MS/MS spectrum of glycine and a condensed Bucket Table based on the presence of MS/MS fragment of the AQC adducts (171.055 m/z). Annotation of derivatized metabolites were performed using a customized derivatized amino acid Analyte List and was in agreement with SmartFormula (in box). **B** Molecular formula prediction using SmartFormula of a tentative kynurenine metabolite. **C** Measured MS/MS spectrum of kynurenine (top) and commercial standard (bottom).

Discovery metabolomics (Retrospective data mining)

As the data collected on the impact II QTOF system 1) was untargeted, and 2) included both high mass accurate MS and MS/MS data, analytes that were not originally considered in targeted assay development could be retrospectively sought. In a subsequent analysis of the pooled plasma samples from the



SARS-CoV-2 cohorts, an additional 2700 features were detected using MetaboScape 2021. The feature list was further reduced using the unique MS/MS fragment of the AQC adducts and annotated with derivatized amino acid Analyte List (Figure 8A). With the simultaneous acquisition of high accuracy mass measurement of the MS and MS/MS fragmentation data, among these “un-annotated” features was kynurenine (C₂₀H₁₈N₄O₄,

theoretical mass m/z 379.1401 ([M+H⁺]), which has been shown to be altered in SARS-CoV-2 individuals [11]. Its chemical formula was accurately predicted using Smart Formula and confirmed with a commercial kynurenine standard (Figure 8B and 8C).

Conclusion

- The precision and accuracy of the impact II QTOF system in the analysis of reference samples were sufficient to meet current metabolite profiling recommendations and were comparable to a contemporary TQ platform. Performance was robust and reliable, suitable to support the sample throughput (around 190 samples/day) necessary for the study of larger sample cohorts.
- High accurate mass MS detection on the QTOF system enabled narrow mass window filtering to eliminate co-eluting matrix interference, which prohibited clear detection of arginine in urine samples within the TQ workflow. Further, the full scan data collection of the QTOF permitted identification of a co-eluting ammonia derivative which had suppressed the detection of cystine in urine in comparative matrix effect evaluations.
- The simultaneous collection of full scan (complete m/z range) accurate mass MS and MS/MS data utilized within the QTOF workflow offers unique flexibility and analytical power. MS and MS/MS data for kynurenine, previously reported to be altered by SARS-CoV-2, were extracted from the complete data pool.
- Multivariate profiling of target metabolites previously found to be linked to SARS-CoV-2 was comparable in the analysis of true human samples (SARS-CoV-2 positive versus healthy controls), reinforcing the utility of the QTOF platform within the metabolic phenotyping community.

Further reading



• **Proven robustness for large-scale metabolomics studies using the 'Phenomics Workhorse'**

HRAM LC-MS profiling of > 1000 urine samples demonstrates the outstanding analytical skills of the **Impact II**

Introduction

The metabolome is the final manifestation of biochemical pathways. The temporal and spatial changes of metabolites reflect the outcome (phenotype) of interactions at the genomic, transcriptomic, and proteomic levels. The immense diversity of the metabolome can be further influenced in virtually countless ways. In order to understand the answers or changes related to e.g. a disease or therapeutic intervention at the level of the metabolome, large profiling studies with generally hundreds of samples are required.

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