



Standardizing Quantitative Metabolomics Analyses Through the QReSS™ Kit

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Introduction

One of the incessant challenges in mass spectrometry (MS)-based “omics” is the qualification of methods and instrument platforms. Required are materials that can be used to routinely test platform performance over time and to test assay effectiveness throughout all phases of a work list.^{1,2} Such evaluations are necessary as it provides an indicator to the effectiveness of an analytical method and platform. Errors or deficiencies observed in the performance would then cue corrective action. This could help save precious samples and experimental resources, while also extend column lifetimes.

In metabolomics, the test materials would be metabolites, with the end product being a mixture that can be readily (and routinely) applied for quality control (QC) and system suitability testing (SST). The metabolites should be carefully selected and the mix well characterized to enable reliable and continuous application. Ideally, the metabolites should be diverse and embody broad coverage, while not being overly complex in number as that could complicate the multiplexed analysis of experimental samples. A commercially available kit that embodies these requisites, while additionally offering the benefit of identification and quantification in MS/MS applications, is QReSS™ (Quantification, Retention, and System Suitability).

The QReSS kit (CIL catalog no. **MSK-QReSS-KIT**) comprises two dried-down mixtures of stable isotope-labeled metabolites and a document package. Included with the package is a user manual, which outlines example procedures and illustrates sample results for user reference. These mixes are well suited for qualification applications due to the carefully selected compounds, their inherent characteristics (e.g., molecular weight), and their experimental tendencies (e.g., broad elution behavior, absent from solubility issues or stability concerns). Discussed here is the

development of the QReSS mixes, experimental methods, and example reversed phase (RP) LC-MS/MS results to illustrate the products utility in untargeted/targeted MS metabolomics.

Key Features/Benefits of QReSS Kits

- stable and reproducibly prepared mixes
- predominantly ¹³C and/or ¹⁵N metabolites
- concentration-balanced metabolites
- user manual supplied for method guidance
- broad coverage in metabolic compound class, molecular weight, and retention times
- enables reliable performance tracking
- allows qualification and quantification

Experimental

Materials

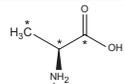
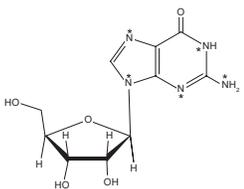
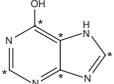
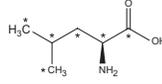
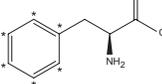
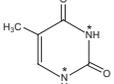
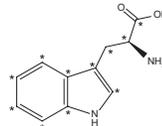
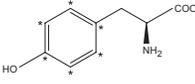
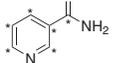
All chemicals were of the highest reagent grade available and were stored in accordance to the suppliers' recommendations. The solvents used in the sample/eluent preparations were LC-MS grade. Isotope-labeled QReSS mixes (see vial snapshots below and **Table 1** for composition details) were used as the metabolite standards in the MS analyses. These were stored at ambient temperature (protected from light and moisture) until use. Human plasma (NIST SRM 1950) and human urine (BioIVT) served as the test matrices.

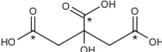
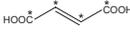
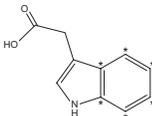
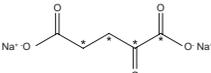
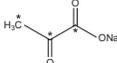
MSK-QReSS-Kit



Continued ►

Table 1. Composition details for the stable isotope-labeled mixes in the QReSS kit. Isotopic enrichments are all $\geq 98\%$ and chemical purities $>98\%$, unless otherwise noted (see *). **Note:** The concentrations reflect 1 mL solvent reconstitutions.

Vial 1				
Abbrev. Description	Abbrev./Alt. Name	Chemical Structure	Metabolic Class	Conc. ($\mu\text{g/mL}$)
L-Alanine ($^{13}\text{C}_3/^{15}\text{N}$)	Ala		Amino acid	100
1,4-Butanediamine-HCl ($^{13}\text{C}_4$)	Putrescine		Other (polyamine)	10
Creatinine (D_3)	Crn		Amino acid	100
Ethanolamine-HCl (D_4)	ETA		Other (1,2-aminoalcohol)	10
Guanosine ($^{15}\text{N}_5$)*	Guo		Nucleoside	2
Hypoxanthine ($^{13}\text{C}_5$)	HPX		Nucleobase (purine)	10
L-Leucine ($^{13}\text{C}_6$)	Leu		Amino acid	5
L-Phenylalanine ($^{13}\text{C}_6$)	Phe		Amino acid	100
Thymine ($^{15}\text{N}_2$)	T		Nucleobase (pyrimidine)	20
L-Tryptophan ($^{13}\text{C}_{11}$)	Trp		Amino acid	100
L-Tyrosine ($^{13}\text{C}_6$)	Tyr		Amino acid	100
Vitamin B ₃ ($^{13}\text{C}_6$)	Nicotinamide		Vitamin	5

Vial 2				
Abbrev. Description	Abbrev./Alt. Name	Chemical Structure	Metabolic Class	Conc. (µg/mL)
Citric acid ($^{13}\text{C}_3$)	CA		Organic acid	10
Fumaric acid ($^{13}\text{C}_4$)	FA		Organic acid	100
Indole-3-acetic acid ($^{13}\text{C}_6$)	IAA		Hormone	5
α -Ketoglutaric acid, disodium salt ($^{13}\text{C}_4$)*	α -KG		Organic acid	100
Sodium palmitate ($^{13}\text{C}_{16}$)	PA (or 16:0)	$^*\text{CH}_2(^*\text{CH}_2)_{14}^*\text{COO}^-\text{Na}^+$	Fatty acid	10
Sodium pyruvate ($^{13}\text{C}_3$)	Pyr		Organic acid	100

Sample Preparation

Each dried-down QReSS mix was reconstituted with 1 mL of 50% methanol. Complete solubilization was achieved by injecting the solvent directly through the vial septum, followed by brief vortexing and centrifugation. Aliquots of the concentrated stocks were then diluted 100-fold in buffer (individually or combined) prior to LC-MS/MS analysis. The concentrated and working stock mixtures were stored at 4°C. In the matrix applications, the QReSS mixes (individually or combined) were diluted 100-fold into plasma and 100-fold into 10× diluted urine. All dilutions were achieved with methanol. The metabolites in plasma were additionally extracted (in methanol) and the supernatant dried prior to reconstitution (in 100 µL water) and LC-MS/MS analysis.

Chromatography

Metabolite separations (at 1 µL injections) were performed by RPLC on a Kinetex F5 column (150 × 2.1 mm i.d., 2.6 µm particles; Phenomenex®). Please see www.phenomenex.com/info/page/f5 for more information. Briefly here, the column and autosampler were maintained at 30 and 4°C, respectively. The separations occurred at standard flow rates (0.2 mL/min) using a linear acetonitrile (ACN) gradient. The mobile phase compositions were 0.1% formic acid (FA) in water for eluent A and 0.1% FA in ACN for eluent B. Each run was followed by a 3.9 min column re-equilibration.

Mass Spectrometry

The Kinetex F5 column (contained within a Shimadzu LC-30AD system) was hyphenated to a low (i.e., QTRAP® 6500+, 7500 triple quadrupole LC-MS system) or high (i.e., TripleTOF® 6600+, X500B QTOF) resolution (R) SCIEX mass spectrometer via an ESI source. In an example low R operation, the MS/MS measurements on the QTRAP were performed in the scheduled MRM mode with unit mass resolution (0.7 Da) used for both Q1 and Q3. All transitions and corresponding collision energies (CEs) were empirically determined from tuning experiments with authentic standards. The general and specific MS/MS parameters utilized in the optimized QTRAP 6500+ assays are shown in **Tables 2** and **3**, respectively. In an example high R operation, SWATH® experiments on the TripleTOF 6600+ and X500B QTOF were performed using fixed or variable precursor ion isolation windows, fixed CEs (35±15 V in positive ESI), 1 *m/z* window overlaps (on the window edges), and cycle times approximating 667 ms.

Table 2. Source and gas parameters for the QTRAP 6500+ operated under positive and negative ESI conditions. Ultra-high-purity nitrogen serves as the carrier gas in all settings. **Note:** With the exception of the IonSpray voltage (5.5 kV, +ESI; -4.5 kV, -ESI), identical parameters were used for +ESI and -ESI.

Parameter	Value
Temperature	500°C
Curtain gas	30 psi
Collision gas (CAD)	12 psi
Ion source gas 1 (GS1)	80 psi
Ion source gas 2 (GS2)	80 psi

Table 3. Specific acquisition parameters for the QReSS metabolites monitored by LC-MRM/MS on the QTRAP 6500+. Tabulated are the isotope-labeled $[M+H]^+$ and $[M+H]^-$ ions. **Note:** Equivalent transitions and CEs were used on the 7500 triple quadrupole LC-MS/MS system. Further, alternate transitions may be possible, with those ideally being empirically optimized using the labeled metabolite mixes as MS infusion solutions. *NM = not monitored*

Analyte	Target +ESI Parameters			Target -ESI Parameters		
	Q1 → Q3	DP (V)	CE (V)	Q1 → Q3	DP (V)	CE (V)
$^{13}C_3/^{15}N$ Ala	94 → 47	30	17	92 → 92	-30	-10
	94 → 29	30	50			
$^{13}C_4$ Putrescine	93 → 76	50	15	NM	NM	NM
	93 → 31	50	30			
D_3 Crn	117 → 89	50	20	115 → 68	-50	-20
	117 → 47	50	30	115 → 41	-50	-30
D_4 ETA	66 → 48	25	15	NM	NM	NM
	66 → 66	25	5			
$^{15}N_5$ Guo	289 → 157	50	18	287 → 155	-50	-20
	289 → 139	50	35	287 → 137	-50	-35
$^{13}C_5$ HPX	142 → 124	60	30	140 → 96	-60	-23
	142 → 114	60	28	140 → 68	-60	-35
$^{13}C_6$ Leu	138 → 91	30	15	NM	NM	NM
	138 → 46	30	30			
$^{13}C_6$ Phe	172 → 126	30	20	170 → 109	-40	-20
	172 → 109	30	35	170 → 72	-40	-20
$^{15}N_2$ T	129 → 85	60	30	NM	NM	NM
	129 → 55	60	35			
$^{13}C_{11}$ Trp	216 → 155	40	25	214 → 124	-40	-22
	216 → 126	40	35	214 → 76	-40	-22
$^{13}C_6$ Tyr	188 → 142	30	19	NM	NM	NM
	188 → 97	30	35			
$^{13}C_6$ Vitamin B ₃	129 → 85	60	25	NM	NM	NM
	129 → 83	60	30			
$^{13}C_3$ CA	NM	NM	NM	194 → 113	-40	-17
				194 → 88	-40	-22
$^{13}C_4$ FA	NM	NM	NM	119 → 74	-60	-10
				119 → 29	-60	-20
$^{13}C_6$ IAA	182 → 136	40	20	180 → 136	-30	-16
	182 → 109	40	40	180 → 134	-30	-20
$^{13}C_4$ α-KG	NM	NM	NM	149 → 105	-40	-10
				149 → 60	-40	-15
$^{13}C_{16}$ PA	273 → 61	50	40	271 → 271	-50	-5
	273 → 46	50	40	271 → 253	-50	-30
$^{13}C_3$ Pyr	NM	NM	NM	90 → 45	-30	-15
				90 → 32	-30	-15

Data Processing and Analysis

Data acquisitions were collected in Analyst (version 1.7), while the analysis was performed with SCIEX OS software (version 1.6.1). The individual and combined mixes were processed in replicate (triplicate minimum) for development testing and in application, with sequence order randomized to reduce systematic bias. To reduce carryover, a blank injection of 0.1% FA was inserted between each type of sample. All peak selections and integrations in data analysis were validated manually before extraction and interpretation. Compound identifications in the untargeted experiments was performed with the all-in-one HR-MS/MS library (version 2.0, SCIEX) and the NIST 2017 MS/MS library.

Results and Discussion

The QReSS product is designed for an array of research applications, spanning from QC to quantification. To ensure suitability of the metabolites and their mixes, a number of upfront tests were first conducted in the development phase before extending to the sample applications. Presented here is an overview of those tests and application explorations.

Metabolite Selection and Mix Testing

The metabolite selection criteria for the QReSS mixes involved evaluating the isotope labeling, their solubility and stability, molecular weight and hydrophathy, and metabolic class spread. In terms of the labeling, ^{13}C and/or ^{15}N was desired with a total mass shift of ≥ 3 Da from its unlabeled counterpart. This preference aids isotope stability and analytical reliability. In cases where D-labels were necessary, H/D exchange and stability were experimentally assessed for optimal performance. The chosen metabolites were tested individually (in the absence and presence of matrix) and their MS/MS parameters empirically optimized before the QReSS mix preparations (see compositions in **Table 1**).

The QReSS mixes were tested first for detectability and reproducibility. This was conducted initially on the QTRAP and TripleTOF instruments operated in the MS1/MS2 mode using positive/negative ESI. **Figure 1** illustrates an example of the combined mix analyses.

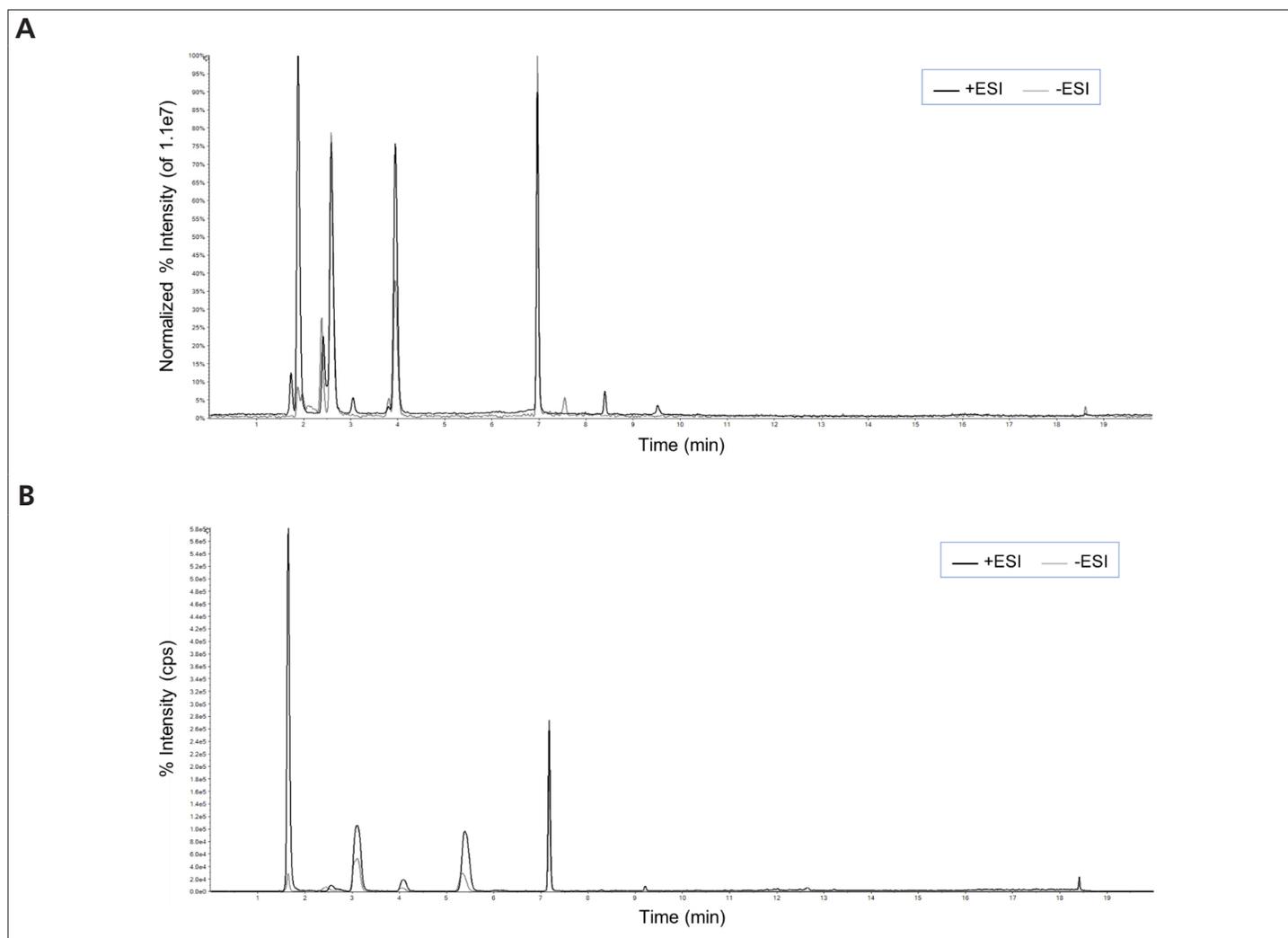


Figure 1. Total ion chromatograms (TICs) of the matrix-free, combined QReSS mix analyses measured by RPLC-MS/MS. An example of the QTRAP 6500+ analysis is in **A** and the TripleTOF 6600+ in **B**, with the positive ESI operations shown in the black trace and negative ESI in gray.

On the QTRAP 6500+, for instance, 14 metabolites were observed in the positive mode and 12 in the negative ion mode. Although not all metabolites were targeted in both ionization modes (see **Table 3**), all 18 metabolites were measured overall and were observed to be distinct from the void volume. The RP separations spanned from ethanolamine (at ~1.6 min) to palmitate (at ~18.6 min). The basic gradient employed returned a decent spread of metabolite elutions on the LC-MS platforms. Further separation could be achieved by RPLC (using a step-wise gradient or adjusted eluent conditions) or HILIC.

In terms of the reproducibility, the metabolite detections were observed to be highly reliable across replicate platform and kit analysis (see **Figure 2** examples).

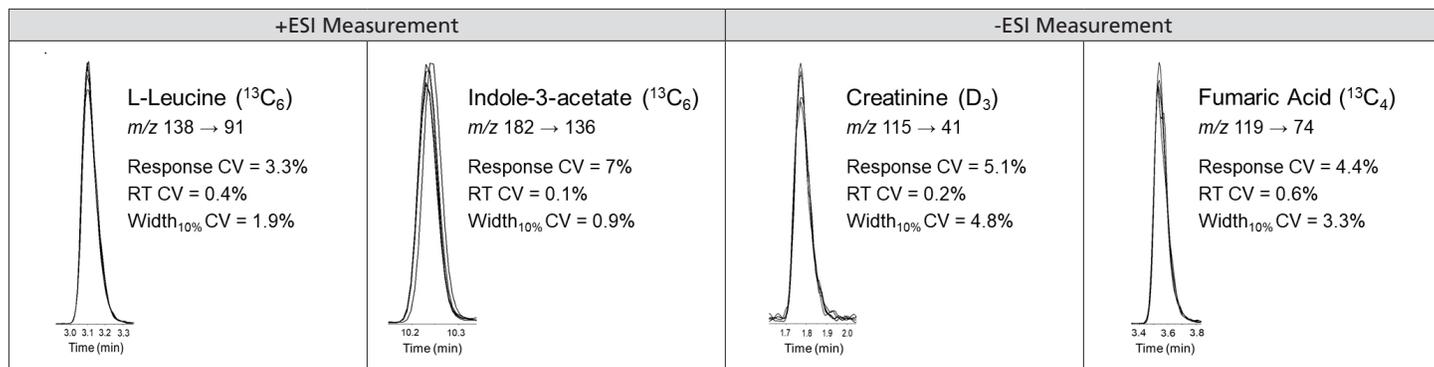


Figure 2. Example XICs from the inter-vial testing of combined QReSS mixes measured in replicate by RPLC-MRM/MS (QTRAP 6500+). Experimentally, five labeled QReSS kits (selected at random) were measured in triplicate, with vial/replicate numbers interspersed. Total CVs were calculated from the sum of the squares of the intra- and inter-vial variabilities.

The total variabilities of this intra-/inter-vial testing were found to be an average of <9%, for response and width_{10%}, and <0.7%, for RT, across the target panel. This high level of precision highlights the reproducibility of the formulation prep and the consistency of the LC-MS/MS analysis.

An additional aspect of the testing phase was the concentration evaluations in a reference material of extracted control human plasma. As shown in **Table 1**, these concentrations are variable because the responses of the quantifier ions were assigned relative to their endogenous analogues in normal human plasma. Using the described analytical workflow, the labeled metabolites in the combined QReSS mixes were largely found to be within an order of magnitude of their corresponding natives in the human plasma test sample (see **Figure 3** examples).

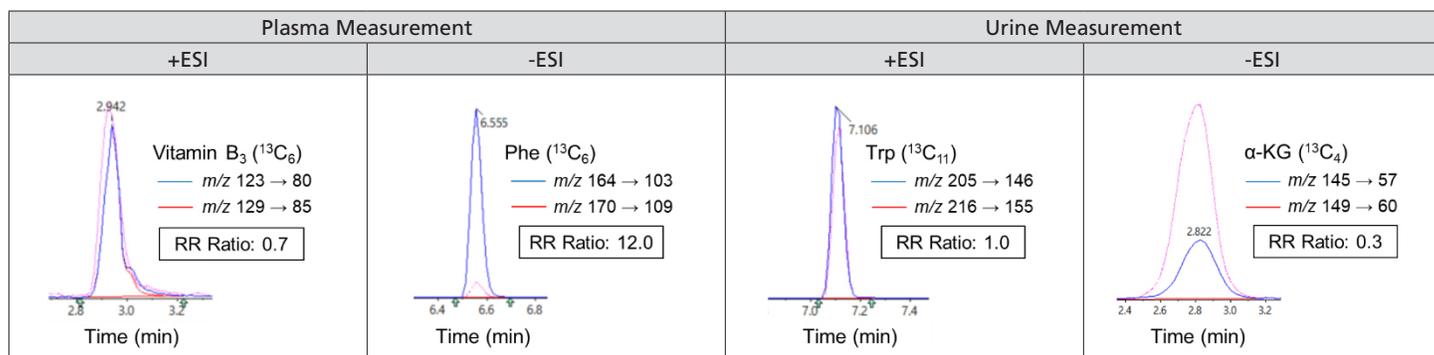


Figure 3. XIC overlays of example QReSS metabolites measured in matrix by RPLC-MRM/MS. The labeled and unlabeled signals are the red and blue traces, respectively. The ratio of the relative responses (RR; light/heavy) are displayed for reference.

It's noteworthy to indicate that the measured response ratios of the secondary transition, where relevant, were also largely found to be within an order of magnitude. As evidenced by our ratio testing in human urine, these mixes were additionally found to be applicable (see **Figure 3** examples), which helps extend potential QReSS applications.

Application Overview

In practice, an aliquot of the combined QReSS mix can be spiked at different method points, with its insertion dictating the nature of the application (e.g., metabolite extraction efficiency in matrix vs. LC-MS performance evaluation). General product implementations extend from assay fitness assessments (at assay or systems level) to identification/quantification applications. **Figure 4** illustrates a simplistic metabolomic workflow highlighting a few of the different entry points for an individual or combined QReSS mix.

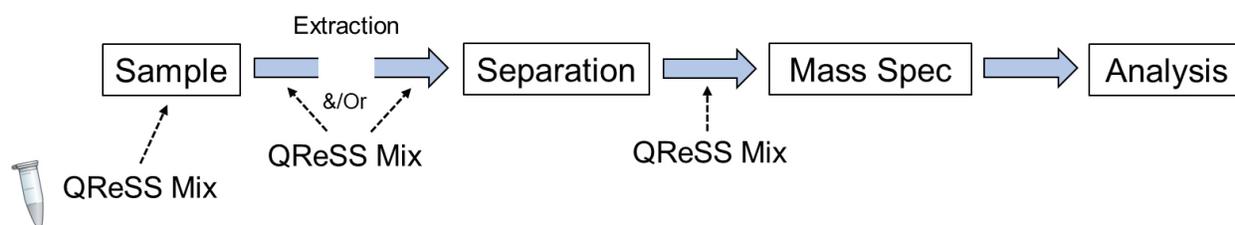


Figure 4. Schematic of a simplistic metabolomics workflow highlighting example insertion points for the QReSS mix. Depending on the nature of application, the mass spectrometer can be operated in an untargeted or targeted manner using positive and/or negative ESI.

In theory, the QReSS mix(es) could be implemented in multiple ways within the same batch sequence. This could involve matrix-free and matrix-containing QReSS samples that would be processed for quality control insights and identification/quantification exercises. Discussed in the following sections is the application of QReSS to system suitability testing and quantification studies. The purpose of these applications differs (i.e., performance check vs. concentration determination), which is why the insertion points of the QReSS mix(es), as shown in **Figure 4**, are also different. The entry points and application fundamentals of the QReSS mix(es) are discussed below.

System Suitability

The efficient and successful operation of an analytical platform is a critical component of the experimental pipeline. This is imperative toward obtaining reproducible data of high quality. Platform evaluations could be achieved by analyzing the combined QReSS mix (e.g., in absence of matrix) by LC-MS, with the mass spectrometer operated in the MS1 or MS2 mode under an untargeted or targeted regime (see **Figure 4** for mix insertion possibilities). The system suitability tests should be run independent of the study calibrators, QCs, and samples, with the data ultimately being retained with the study records.³ Before implementation, it is recommended that baseline values be first determined for each metabolite by measuring the QC mix by technical replicate (e.g., $n \geq 5$). Once acceptance criteria are established (e.g., $\pm 7\%$ and $\pm 14\%$ CV for warning and action limits on peak response), system suitability tests can be routinely run. These should be performed throughout an acquisition batch (i.e., before, during, and after sample measurement). In practice, it is recommended to run a set of QCs (e.g., 5-10) first for system conditioning and then intermittently throughout a sequence batch (e.g., every 4-10 injections),² with performance metrics tracked over time. For thoroughness, the metrics should encompass chromatographic (e.g., response, retention time, peak width and symmetry, capacity factor) and mass spectrometric (e.g., signal intensity, mass accuracy, fragmentation patterns, transition ratios) parameters. The nature of the MS metric tracking is predicated on the technique (e.g., targeted vs. untargeted), with the results ideally being displayed pictorially using Pareto plots or Shewhart control charts,⁴ for instance. Through the longitudinal monitoring of performance metrics, deviations in data quality relating to deficits (e.g., signal drift or offset, peak tailing or splitting) or issues (e.g., column integrity, mass calibration, fragmentation efficiency) in the LC and/or MS system can be revealed. This would cue corrective maintenance, with follow-up tests run thereafter both before and throughout experimental measurements. It must be noted that metric analysis should occur in a timely manner (not long after the batch sequence is acquired), such that corrective action on any out-of-limit occurrences can immediately be conducted and sample volumes potentially saved. To aid this monitoring, QC measurements on the 7500 triple quadrupole and X500B QTOF, for example, can be tracked in real-time with autoprocessing by the SCIEX OS. In this software, once the rules are entered by the user (e.g., response, retention time, mass accuracy), signs of erroneous data in the LC-MS measurements would be illuminated and action(s) automatically triggered (e.g., stop batch run, blank injection, sample reinjection). The use of the QReSS mixes (in combination with software) help serve as an effective means to monitor/track system suitability toward enabling fit-for-purpose applications.

Identification and Quantification

The QReSS mixes can help facilitate metabolite identification and quantification in MS metabolomic experiments. Procedurally, an aliquot of the working QReSS mix can be spiked to the extraction solvent or directly to an experimental sample before proceeding with the analytical workflow (see **Figure 4**). In this framework (i.e., as internal standards), the stable isotope metabolites in QReSS possess equivalent physicochemical behavior as their unlabeled counterpart in matrix (e.g., $^{15}\text{N}_5$ guanosine to native guanosine). This helps compensate for ion suppression effects and eliminate experiment/instrument bias. Due to the careful selection of the standards, the labeled and unlabeled metabolites co-elute and are of sufficient mass difference to avoid isotopic interferences. These merits help facilitate the identification and relative quantification of endogenous metabolites in matrix samples, as illustrated in **Figure 3**.

The quantification potential of the QReSS mixes can extend beyond the 18 total compounds. This would involve using the labeled standards as surrogates for metabolites bearing similar physicochemical properties. These properties manifest at the structural, chromatographic (e.g., retention), and mass spectrometric (e.g., ionization) levels. In this context, $^{13}\text{C}_6$ leucine (from QReSS) could serve as a surrogate for isoleucine in a matrix analysis, for example. Given the separation spread of the QReSS components (result of basic RPLC gradient in **Figure 1**), a target panel of broadly dispersed endogenous metabolites in sample matrices could be analyzed (via MRM, for example) in quantitative applications using the labeled metabolites as standards. The mix can also be implemented in untargeted quantitative applications using LC-MS or -MS/MS performed in the data-independent acquisition (DIA) mode. To demonstrate this, RPLC-MS metabolomic experiments were performed on the TripleTOF 6600+ and X500B QTOF using plasma and urine as test matrices (see **Figure 5** for full scan MS1 data).

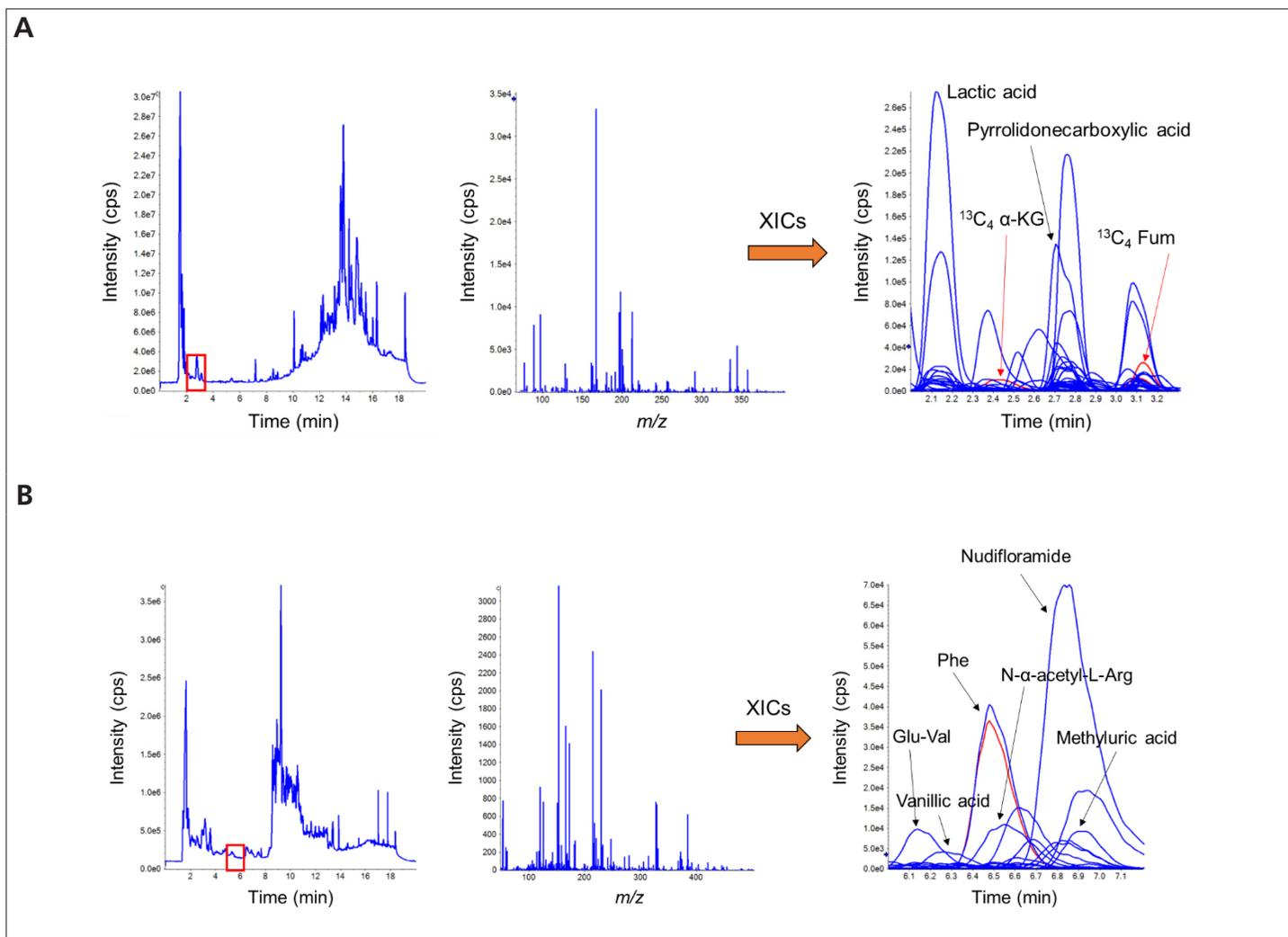


Figure 5. Overview of matrix MS1 scans on high R hybrid mass spectrometers operated under negative ESI. The QReSS spiked plasma analysis is shown in **A** (collected on a TripleTOF 6600+) and urine in **B** (collected on a X500B QTOF), with the endogenous signals in blue and labeled in red. Example compound annotations are shown for reference.

At this MS1 level, the labeled standards could serve as surrogates for their neighboring unlabeled compounds in the matrices. As evidenced by the example chromatograms, the quantity of precursors at a given time can be quite complex, which could pose quantitation issues. By collecting MS2 spectra in the SWATH (sequential window acquisition of all theoretical spectra) acquisition mode, fragmentation spectra of all precursor ions within defined m/z isolation windows throughout a chromatographic run can be obtained.⁵ This helps reduce potential interference issues and partial overlap concerns on target precursor ions, while allowing you to collect both MS1 and MS2 data in a single injection; thus, aiding not only identification but quantification. Further, if interferences are observed, alternate fragment ions can be selected retrospectively since a complete dataset has already been acquired. **Figure 6** shows a collection of XICs obtained from a plasma and urine SWATH-MS analysis of example m/z ranges.

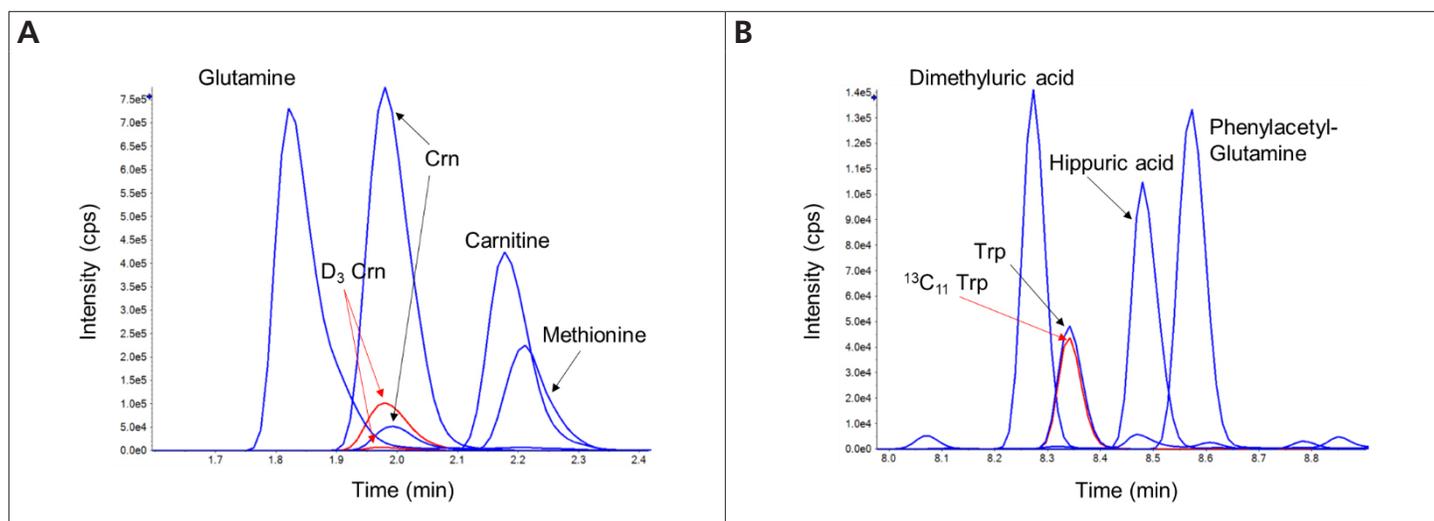


Figure 6. QReSS-spiked matrix analysis on high resolution instruments by SWATH-MS. The plasma analysis is shown in **A** (collected on a TripleTOF 6600+) and urine in **B** (collected on a X500B QTOF), with the endogenous signals in blue and labeled in red. Example compound annotations are shown for reference.

As illustrated, D_3 Cre could serve as a quantitative surrogate for its neighboring compounds in the plasma analysis, while $^{13}C_{11}$ Trp could similarly act as a surrogate in the urine analysis. One caveat to this extension is that there cannot be a coeluting compound with a similar product ion m/z in a given SWATH window of reference. While the accuracy and reliability of this approach on a larger scale would need to be vetted, the extension of the combined QReSS mix in this manner could serve as a means to reduce costs and expand the quantitative potential of a given application.

Conclusions

The two mixes in the QReSS kits contain a total of 18 metabolite standards. These have been carefully selected and characterized for adaption to various MS-based workflows and applications. The value of these mixes resides in its production reproducibility, the compound characteristics, and the breadth of application potential. As discussed herein, the applications could encompass QC evaluations (of method and/or platform) and identification/quantification exercises. Although alternate methods, instrument platforms, and applications are possible,⁴ this note should provide a glimpse into the utility of the QReSS mixes in MS metabolomics and analytical/bioanalytical chemistry.

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Related Products

Catalog No.	Description	Unit Size
MSK-QReSS-KIT	Metabolomics QReSS Kit	1 kit
MSK-QReSS1	Metabolomics QReSS Standard 1	1 vial
MSK-QReSS2	Metabolomics QReSS Standard 2	1 vial
MSK-QReSS-US-KIT	Metabolomics QReSS Kit (unlabeled)	1 kit
MSK-QReSS1-US	Metabolomics QReSS Standard 1 (unlabeled)	1 vial
MSK-QReSS2-US	Metabolomics QReSS Standard 2 (unlabeled)	1 vial

Please refer to isotope.com/products for pricing and isotope.com/applications (Metabolic Research → Metabolomics Mixtures and Kits → QReSS Kits) for FAQs and additional information.

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