Simultaneous Quantitation and Discovery (SQUAD) Orbitrap MX mass detector workflow for comprehensive, single-injection metabolomics insight

Authors

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Keywords

Liquid chromatography - mass spectrometry (LC-MS), liquid chromatography - high-resolution accurate-mass mass spectrometry (LC-HRAM-MS), Orbitrap Exploris MX mass detector, targeted metabolomics, untargeted metabolomics, Simultaneous Quantitation and Discovery (SQUAD), small molecule quantitation

Application benefits

Enhances metabolomics productivity by enabling a single sample injection method for the accurate quantitation of known compounds and annotation and/or identification of potentially biologically significant unknown species

Goal

Demonstrate use and productivity of the Simultaneous Quantitation and Discovery (SQUAD) workflow that combines identification, accurate quantitation of preselected metabolites, and retrospective data analysis using the HRAM capability of Thermo Scientific™ Orbitrap™-based mass spectrometers

Introduction

Liquid chromatography (LC) - mass spectrometry (MS)-based metabolomics approaches fall into two general categories: untargeted discovery and targeted quantitative analyses. Untargeted methods are used for hypothesis generation and involve the identification of a large number of unique compounds of interest. Targeted methods are used for hypothesis-driven studies where a particular subset of metabolites is prioritized based on the specific needs of the study. While untargeted methods

allow researchers to retrospectively analyze data when new compounds of interest arise, targeted methods have the benefit of providing the most accurate quantification when the necessary standards are available.

Traditionally, two separate LC-MS runs are used to carry out untargeted profiling and targeted quantification because it can be difficult to acquire a sufficient number of data points over an eluting LC peak to carry out both with only a single injection. To enhance study productivity and conserve samples in situations in which they are limited, it is desirable to be able to simultaneously perform untargeted and targeted analyses on a single sample injection. The Simultaneous Quantitation and Discovery (SQUAD) workflow,[1] which relies on the HRAM capabilities of Orbitrap mass spectrometers, offers a solution with MS^1-level identification, accurate quantitation of preselected metabolites, and the ability to retrospectively mine the acquired data. The workflow uniquely streamlines metabolomics studies and accelerates time-to-insight by extracting more complete information from one sample injection.

This application note applies the SQUAD workflow to LC-MS profiling and quantitation of isotopically labeled internal standards (i.e., amino acids and organic acids) spiked in NIST SRM 1950 plasma reference standard. LC-MS analysis was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system and a Thermo Scientific™ Hypersil GOLD™ VANQUISH™ HPLC column for separation. Data acquisition was performed on a Thermo Scientific™ Orbitrap Exploris™ MX mass detector. MS data were acquired in full-scan MS^1 using rapid alternating positive and negative polarity electrospray ionization (ESI(+) and ESI(-)) modes over the entire runtime to ensure broad analyte coverage.

Experimental

Reagents and consumables

- Metabolite Reference Standard NIST™ SRM™ 1950 plasma sample, Sigma (NIST1950)
- Isotope-labeled amino acids and organic acids, CIL, (MSK-CAA-1 and MSK-OA-1, respectively)
- Water (H_2O), UHPLC-MS grade, Thermo Scientific (P/N W81)
- Methanol (MeOH), UHPLC-MS grade, Thermo Scientific (P/N A4581)
- Formic acid (FA), Pierce™ LC-MS grade, Thermo Scientific (P/N TS-28905)

Sample preparation

As illustrated in Figure 1, Metabolite Reference Standard NIST SRM 1950 plasma was spiked with a dilution series (1 nM–2.5 mM) of isotope-labeled amino acids and organic acids (CIL MSK-CAA-1 and MSK-OA-1, respectively), covering the expected endogenous biological range. This range was used to determine the lower limit of detection (LLOD), the lower limit of quantitation (LLOQ), and the linear dynamic range of the Orbitrap mass detector-based SQUAD workflow. The authentic isotope-labeled amino acids and isotope-labeled organic acids standards were used for confident identification and absolute quantitation. To obtain high-quality data, QC samples were incorporated into the experimental design. Extraction was performed in an excess of methanol, where 200 µL of each spiked in plasma were added to 800 µL of methanol and mixed for three minutes at room temperature using a bench vortex. The mixture was then centrifuged for 15 min (21 k × g) at 4 °C to separate the precipitated protein layer. Finally, an aliquot of 100 µL was transferred to an LC-MS vial with an insert before LC-MS analysis.

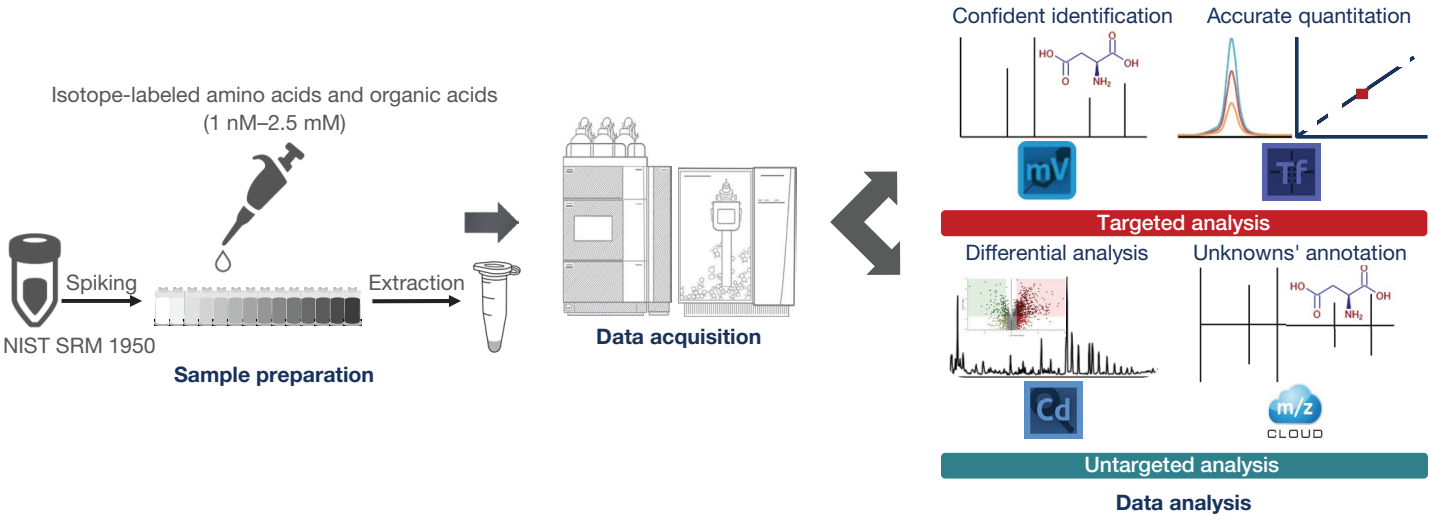

Figure 1. SQUAD analysis workflow on an Orbitrap MX mass detector for comprehensive, single-injection metabolomics insight

Chromatography

Reversed-phase liquid chromatography (Table 1) was carried out on a Vanquish Horizon UHPLC system and Hypersil GOLD VANQUISH C18 column. Samples were injected in triplicate.

Table 1. LC and autosampler conditions

Parameter	Value
UHPLC column	Hypersil GOLD VANQUISH C18 UHPLC column, 150 × 2.1 mm, 1.9 µm (P/N 25002-152130-V)
Column temperature	45 °C
Flow rate	0.3 mL/min
Solvent A	0.1% (v) FA in LC-MS grade water
Solvent B	0.1% (v) FA in LC-MS grade methanol
Gradient	Time (min) Solvent B 0.00 0 8.00 50 9.00 98 13.00 98 13.10 0 15.00 0
Run time (data acquisition time)	15 min
Injection volume	2 µL
Divert valve	To waste: 0–0.2 min; to MS: 0.2–15.0 min
Autosampler temperature	5 °C

Mass spectrometry

Detection and data acquisition were performed on an Orbitrap Exploris MX mass detector equipped with a heated ESI probe and the Thermo Scientific™ Easy-IC™ ion source, which delivered an internal reference mass. Scan-to-scan internal lock mass correction on the Easy IC ion source was used to ensure accurate mass assignments. Acquisition used rapid alternating positive and negative ESI mode scanning over the 15-minute runtime. During method development, the RF (%) on the S-lens in the ion source was optimized for best transmission of a broad range of compounds and *m/z* in a single scan. The MS method parameters used are listed in Table 2.

Table 2. Orbitrap Exploris MX mass detector method parameters (Note: Unless otherwise indicated, default parameters were used.)

Parameter	Value
Ion source parameters	
Mode	Polarity switching
Spray voltage	Positive: 3.5 kV; negative: 3.0 kV
Vaporizer temperature	320 °C
Transfer tube temperature	275 °C
RF lens	70%
Sheath gas	40
Aux gas	8
Sweep gas	1
Autosampler temperature	5 °C
Scan parameters	
Scan range	70–800 *m/z*
Resolution	120,000
AGC target	Standard (1e6)
Maximum injection time	Auto
Microscans	1

Data processing and analysis

Untargeted data analysis and interpretation used Thermo Scientific™ Compound Discoverer™ software version 3.3 with the Thermo Scientific™ mzCloud™ advanced mass spectral database for compound extraction, differential analysis, and confident metabolite annotation. Targeted quantification used Thermo Scientific™ TraceFinder™ software version 5.1. The data processing and analysis scheme is shown in Figure 1. Though not used in the experiments presented here, Thermo Scientific™ mzVault application can be used for offline searching mzCloud's MS^2-level spectral data in either Compound Discoverer software or TraceFinder software if desired. The annotation in this Orbitrap Exploris MX SQUAD analysis is done using predicted composition, ChemSpider, and mass list search using HRAM MS[1].

Results and discussion

Quantification performance

Peak responses of the spiked-in isotope-labeled compounds and the corresponding plasma endogenous compounds were used to build calibration curves to determine the sensitivity (i.e., LLOD and LLOQ) and the linear dynamic range of the Orbitrap Exploris MX mass detector.

In both positive and negative ESI modes, the quantitative results for the target compounds provided excellent sensitivity and linearity (R^2 > 0.99 for all compounds) over a wide dynamic range.

As presented in Table 3, the linearity for the target compounds in the plasma matrix ranged from four to five orders of magnitude, with low LLOQ and LLOD and sufficient data points for accurate, precise, and sensitive quantitation since advancements in scanning speed have been achieved in hybrid-based Orbitrap instruments, enabling polarity switching (Figure 2) even in conjunction with high-throughput LC methods. This breakthrough innovation allows the hybrid mass spectrometers to achieve wider metabolome coverage through the utilization of fast polarity switching.

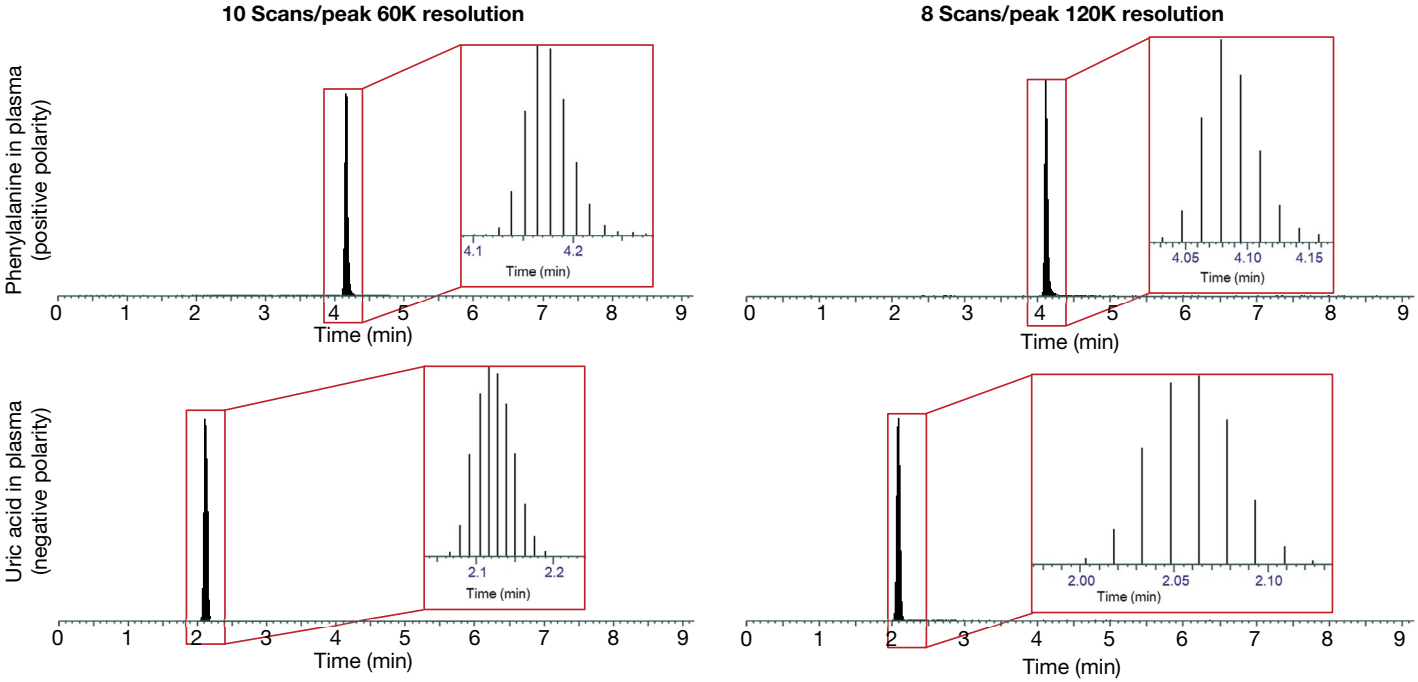

Figure 2. Fast polarity switching on the Orbitrap Exploris MX platform enables accurate and sensitive quantitation with wide coverage within a single injection analysis.

Table 3. Linear dynamic range in plasma, lower limits of quantitation (LLOQ), and lower limits of detection (LLOD) using the SQUAD workflow on the Orbitrap Exploris MX mass detector

Analyte	Polarity	Calibration linear dynamic range	LLOD (femtomole on column)	LLOQ (femtomole on column)
Phenylalanine	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	2.5	50
Tryptophan	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	5	50
Isoleucine	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	10	50
Leucine	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	10	50
Tyrosine	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	10	50
Valine	Positive	2.5 nM – 2.5 mM (5 orders of magnitude)	25	50
Creatine	Negative	25 nM – 2.5 mM (5 orders of magnitude)	25	50
Hippuric acid	Negative	12.5 nM – 0.5 mM (4 orders of magnitude)	25	50
Uric acid	Negative	25 nM – 2.5 mM (5 orders of magnitude)	25	50
α-Keto-isocaproic acid	Negative	50 nM – 2.5 mM (4 orders of magnitude)	50	100
α-Keto-isovaleric acid	Negative	50 nM – 1.25 mM (4 orders of magnitude)	50	100
Succinic acid	Negative	50 nM – 0.5 mM (4 orders of magnitude)	100	100
3-Hydroxybutyric acid	Negative	250 nM – 2.5 mM (4 orders of magnitude)	250	500

Figure 3 shows the calibration curves for phenylalanine (positive ESI) and α-keto-isovaleric acid (negative ESI) over the 1 nM to 2.5 mM concentration range.

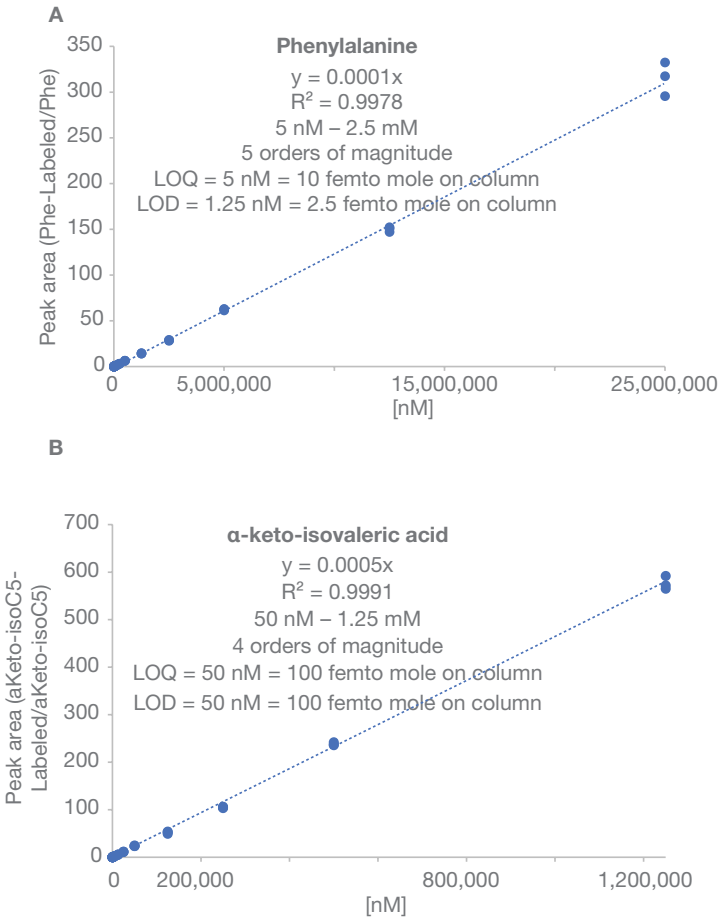

Figure 3. Absolute quantitation results for (A) phenylalanine and (B) alpha-keto-isovaleric acid spiked in NIST SRM 1950 plasma reference standard

Compound detection and identification

Data processing using Compound Discoverer software with mzCloud spectral database resulted in a high number of detected unique compounds, 20% of which were putatively identified using HRAM full-scan MS1-level data (Figure 4). Putative identifications are generated based on the high MS^1 isotope fidelity and high mass accuracy of the instrument (Figure 5).

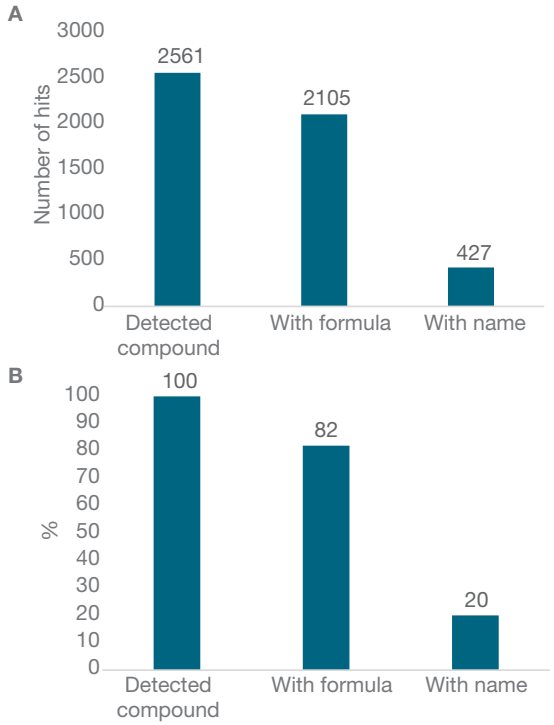

Figure 4. Unique compound detection and identification were obtained using HRAM full-scan MS^1-level data from the SQUAD workflow with data processing using Compound Discoverer software and the mzCloud spectral database. (A) The absolute number of hits, and (B) the percentage of overall detected unique compounds. Only unique compounds are shown; background ions, charge states, and adducts were excluded. Compound is defined by detected feature with at least an *m/z* and retention time values.

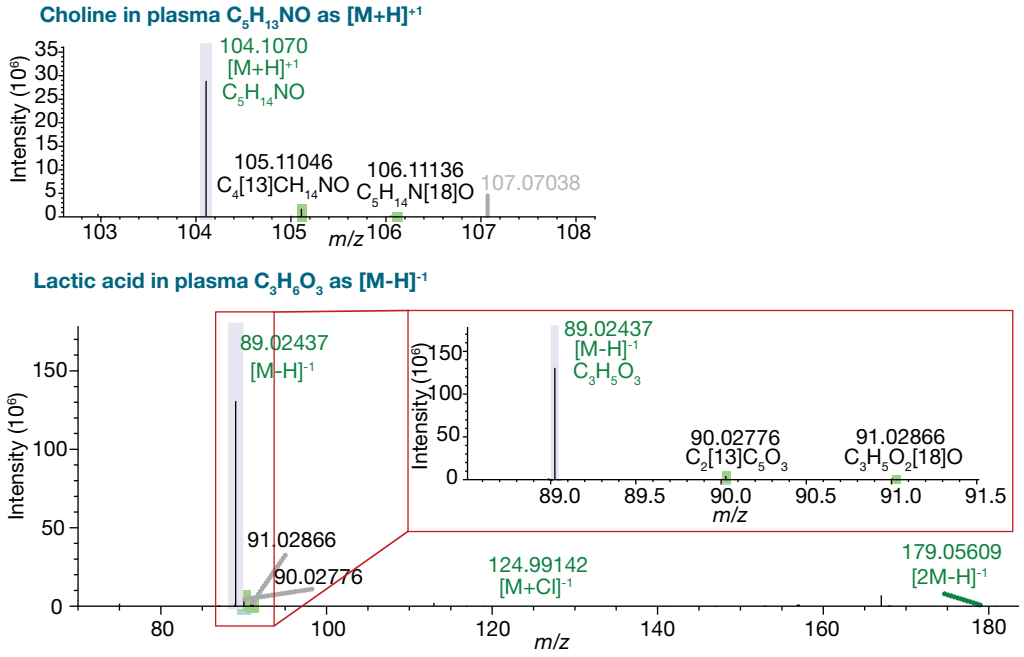

Figure 5. Two annotated compounds in plasma using the SQUAD analysis workflow on an Orbitrap Exploris MX mass detector, which provided high isotopic fidelity for compound identification. Lavender bars indicate the spectral peak in the isotope pattern with the highest intensity. Usually the most intense peak in an isotope pattern is the A0 peak for the monoisotopic ion. Green rectangles indicate matching centroids for isotopic ions.

Conclusion

The SQUAD workflow for the Orbitrap Exploris MX mass detector supports the paradigm shift in metabolomics toward increased productivity and accelerated time-to-insight by performing untargeted discovery profiling and targeted quantitation on one MS platform and in one analytical run. The HRAM capability of the Orbitrap Exploris MX mass detector provides high specificity, isotopic fidelity, and sensitivity, which are essential to reliable compound detection for untargeted profiling, while outstanding HRAM MS^1-level spectral quality enables robust targeted analysis. Fast polarity switching of alternating positive and negative ESI scans over the entire run ensured broad analyte coverage while acquiring sufficient data points for accurate, precise, and sensitive quantitation.

Compound Discoverer software, mzCloud advanced mass spectral database, and TraceFinder software worked together to streamline processing and analysis of HRAM data acquired in single experimental runs, providing more comprehensive sample insights.

Reference

1. Amer, B.; Deshpande, R.; Bird, S. Review: Simultaneous Quantitation and Discovery (SQUAD) Analysis: Combining the Best of Targeted and Untargeted Mass Spectrometry-Based Metabolomics, *Metabolites* **2023,** *13,* 648. https://doi.org/10.3390/metabo13050648

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