Metabolomics and Research

Simultaneous Quantitation and Discovery (SQUAD) Metabolomics: Orbitrap-based methods to empower your research

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Abstract

A single injection, high-throughput, and accurate metabolomics approach that enhances productivity by utilizing the Thermo Scientific™ Orbitrap™ instruments for parallel quantitation of predefined compounds as well as unknown identifications of potential biological significant features was developed.

LC-MS quantitation of isotopically labeled amino acids spiked in NIST SRM 1950 plasma reference standard and untargeted characterization of the NIST plasma metabolites was performed on multi Thermo Scientific™ Orbitrap™ platforms including Hybrid, Tribrid, and the novel Orbitrap™ Astral™ mass spectrometers.

Within a single injection approach, the SQUAD analysis demonstrated a wider coverage of the metabolome by utilizing the fast polarity switching of the Orbitrap Hybrid platforms, and an increased percentage of fragmented compounds using the advanced deep scan Thermo Scientific™ AcquireX™ workflow on the Orbitrap Hybrid and the Tribrid resulting in improved annotation capability. Finally, the novel Astral mass analyzer increased the percentage (i.e., 90%) of fragmented compounds leading to improved annotation capability for deeper discovery analysis. The linear Ion trap of the Tribrid systems showed excellent sensitivity (e.g., LOQ down to 5 femtomoles) and a great linear dynamic range (6 orders of magnitude) for phenylalanine in plasma. On the other hand, faster and high-quality Orbitrap MS^1 scans on the Orbitrap Astral mass spectrometer enabled a more sensitive quantitative analysis (i.e., LOQ down to 10 femtomoles with 5 orders of magnitude linearity range) compared to the Orbitrap of the Hybrid systems (i.e., LOQ down to 50 femtomoles with 5 orders of magnitude linearity range) of phenylalanine in plasma.

Introduction

The field of Metabolomics has been advancing at an impressive rate, inspiring key analytical innovations designed to keep pace with the biological needs of a study. Despite these advances, challenges remain around compound annotation and identification as well as streamlined quantification in a single analysis. Without internal standards and libraries, untargeted metabolomics lacks accurate quantitation and identification of metabolites that are needed to study biological systems. Thus, many researchers target a few analytes and risk missing significant unknown compounds with potential biological significance.

Here we introduce a single injection **simultaneous quantitation and discovery (SQUAD)** metabolomics method that combines targeted and untargeted workflows on the Orbitrap-based mass spectrometers, Figure 1. SQUAD is used for the confident identification and accurate quantitation of targeted metabolites. It also allows the untargeted discovery analysis to look for global metabolic changes that were not part of the original focus. This offers a way to strike the balance between targeted and untargeted approaches in one single experiment[1].

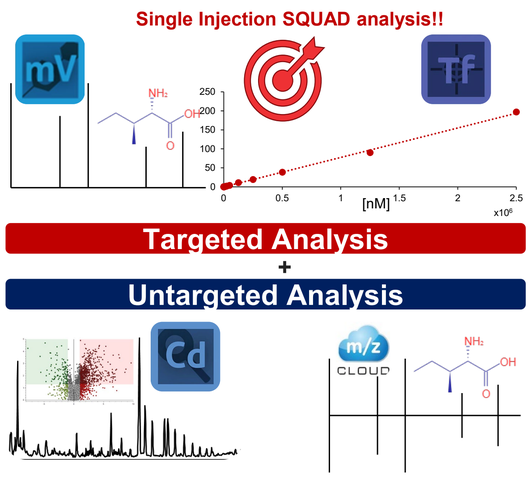

Figure 1. SQUAD workflow provides both targeted quantitation and untargeted discovery data analysis.

When available, analyzing authentic standards of target compounds, provides absolute quantitation, standardizing response across instrument platforms and laboratories providing additional quality control and assurance to the study. The simultaneous acquisition of HRAM full scan data, future proofs the discovery of metabolites through data retro mining as new targets of interest are discovered.

Materials and methods

Metabolite Reference Standard NIST SRM 1950 plasma sample and isotope-labeled amino acids were purchased from Sigma and CIL, respectively. The plasma was spiked with a dilution series (1 nM – 2.5 mM) of isotope-labeled amino acids before extraction with 80% methanol, Figure 2.

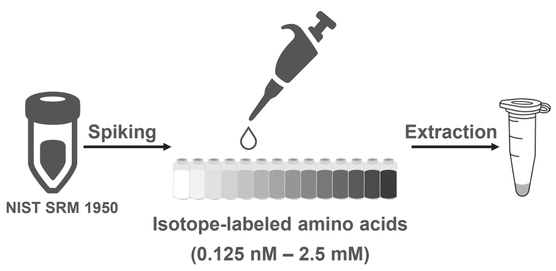

Figure 2. Sample preparation for SQUAD analysis on multi Orbitrap platforms.

Reversed-phase chromatography was applied as the technique of choice for the pre-separation of the metabolites using Thermo Scientific™ Hypersil GOLD™ column attached to Thermo Scientific™ Vanquish™ Horizon UHPLC as a pre-separation technique. Data were acquired in full-scan Orbitrap MS^1 on Thermo Scientific™ Exploris MX and Thermo Scientific™ Exploris 240 while switching the polarity of ion source ionization. Similar data acquisition was conducted but with single polarity on the Thermo Scientific™ IQ-X and Ascend in parallel to tMS^2 targeted quantitation using the linear Ion trap of the Tribrid systems. The data analysis on Exploris 240 and the Tribrid systems was accompanied by intelligent data acquisition via the advanced deep scan AcquireX workflow, Figure 3. Finally, full-scan Orbitrap MS^1 data acquisition in parallel to MS^2 (top 30) was conducted on a Thermo Scientific Orbitrap Astral mass spectrometer. Thermo Scientific™ TraceFinder™ 5.1 and Compound Discoverer™ 3.3 software were used for data processing, analytes quantitation, and unknown annotation, Figure 1.

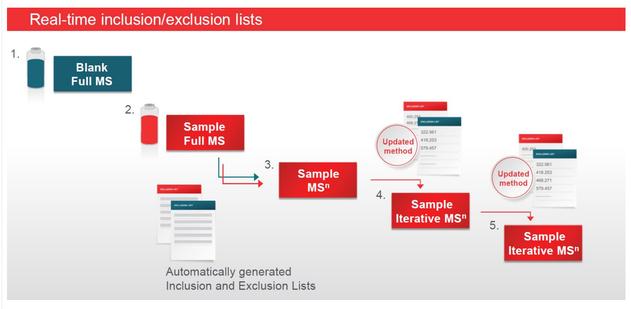

Figure 3. Thermo Scientific™ AcquireX Deep Scan mode for intelligent data acquisition to maximize the number of relevant compounds interrogated by MS/MS, resulting in higher coverage and confidence annotation.

Results

SQUAD on Orbitrap Hybrid mass spectrometers

Absolute quantitation results of the SQUAD analysis on the Orbitrap Exploris MX were exemplarily shown for spiked isotope-labeled phenylalanine (detected in positive ionization mode) and spiked isotope-labeled uric acid (detected in negative ionization mode), Table 1. While applying fast polarity switching for Orbitrap detection, both modes could be applied in the same run while acquiring sufficient data points for accurate and precise quantitation exhibiting excellent sensitivity in both ionization modes, Figure 4.

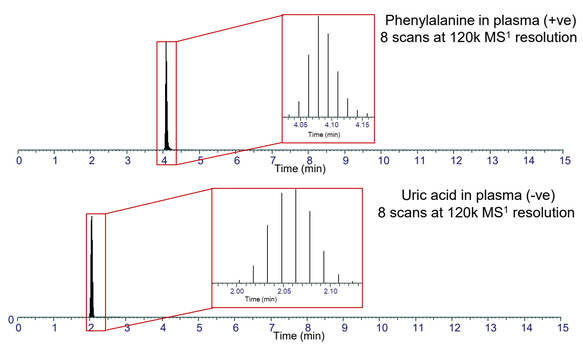

Figure 4. Fast polarity switching on the Orbitrap Hybrid instruments enables wider coverage of the metabolome by securing sufficient scans per peak at high MS^1 resolution (i.e., 120k).

Data processing of the HRAM MX Orbitrap MS^1 utilizing the mzCloud™ spectral library resulted in a high rate of detected unique compounds with a putative identification based on the accurate mass capabilities and the excellent isotope fidelity of the assay, Figure 5.

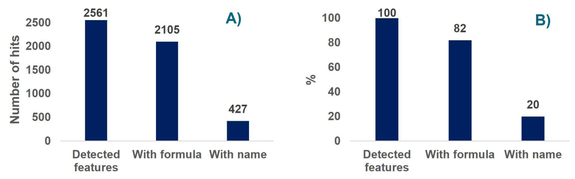

Figure 5. Identification capabilities of the full MS^1 SQUAD approach.

SQUAD analysis on Orbitrap Exploris 240, Figure 6, has the advantage of performing an MS^2 fragmentation experiment to increase unknowns' annotation accuracy especially when using the AcquireX workflow, which increased the number of putatively annotated compounds.

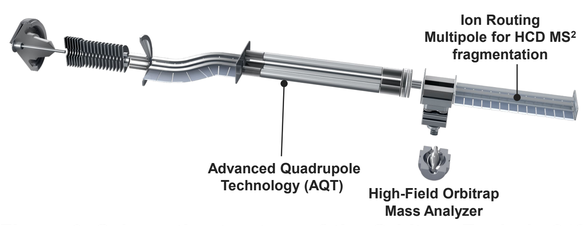

Figure 6. Schematic structure of the Orbitrap Exploris 240 mass spectrometer.

SQUAD on Orbitrap Tribrid mass spectrometers

In parallel, the Thermo Scientific Ascend (Figure 7) and IQ-X Tribrids can run a quantitative assay using the sensitive linear Ion trap without sacrificing the untargeted assay performed on the high-resolution accurate mass Orbitrap analyzer. This fast alternating eliminates the variability of using multiple instruments and the need to re-inject limited biological samples.

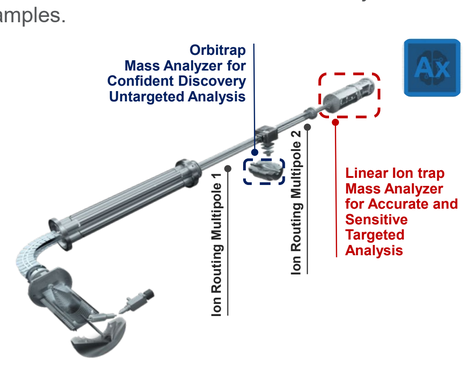

Figure 7. Schematic structure of the Orbitrap Ascend mass spectrometer.

The linear Ion trap of the Tribrid system showed a higher sensitivity compared to the Orbitrap analyzer, Table 1. This was demonstrated by its higher ability to detect and quantify selected metabolites at low levels compared to the Orbitrap, Figure 8.

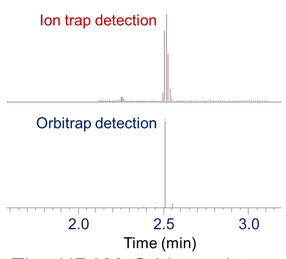

Figure 8. Linear Ion trap mass analyzer for accurate and sensitive targeted analysis. At low concentrations, the Ion trap records reproducible multi-scans across the peak for quantitation while the Orbitrap records one scan only. SM (d18:1/16:0).

The HRAM Orbitrap data and the increased percentage of fragmented compounds using the advanced deep scan AcquireX workflow resulted in improved annotation capability compared to traditional DDA on a wider dynamic range of plasma compounds, Figure 9.

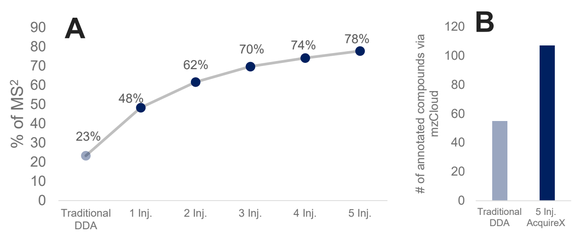

Figure 9. (A) Percentage of compounds with MS/MS spectra, and (B) number of annotated compounds utilizing the AcquireX deep scan intelligent data acquisition workflow compared to traditional DDA.

SQUAD on the novel Orbitrap Astral mass spectrometer

The parallel data acquisition utilizing the two mass analyzers (i.e., Orbitrap and Astral, Figure 10) results in faster Orbitrap HRAM scans (sub-ppm mass accuracy), this empowers the Orbitrap to measure sufficient and high-quality scans per peak at low concentrations that are required for accurate and sensitive MS^1-based quantitation. As a result, the Orbitrap Astral mass spectrometer demonstrated high sensitivity and extended linear dynamic range MS^1-based quantitative analysis, Table 1.

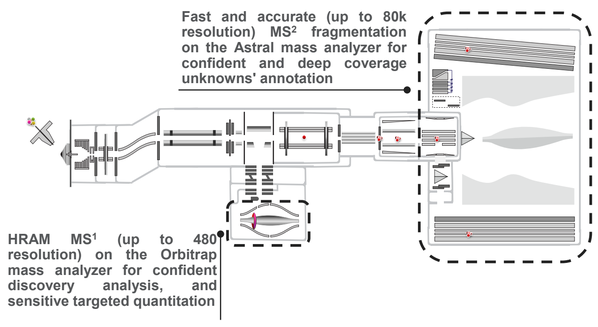

Figure 10. Schematic structure of the Orbitrap Astral mass spectrometer.

In addition, the Orbitrap Astral platform allows for reducing the LC gradient (i.e., 3 times shorter) without compromising the number of MS^1 scans and signal-to-noise (Figure 11), nor the spectra MS^2 fragmentation ratios (Figure 12). Thus, providing a golden opportunity for the development of high-throughput SQUAD analysis on the Orbitrap Astral mass spectrometer.

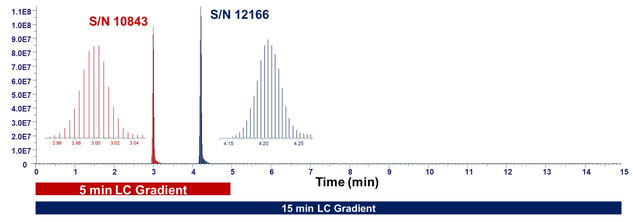

Figure 11. Signal-to-noise and the number of scans per peak for phenylalanine in NIST plasma using two different LC gradients and Orbitrap-Astral SQUAD analysis.

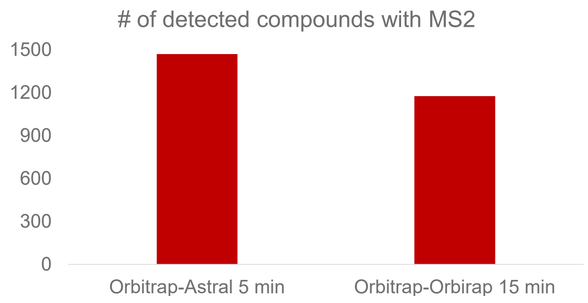

Figure 12. The total number of detected compounds in NIST plasma with MS2 fragmentation using parallel Orbitrap MS^1 and Astral DDA top 30 MS^2 with 5-min LC gradient, and Orbitrap MS^1 and Orbitrap MS^2 with 15-min LC gradient. Data collected from Compound Discoverer analysis using ≥ 5 spectra/peak threshold.

Table 1. Absolute quantitation results (i.e., linear dynamic range, LOQ, and LOD) for isotope-labeled phenylalanine spiked in NIST SRM 1950 plasma reference standard using different Orbitrap-based instruments.

Mass spectrometer	Calibration linear dynamic range	LOQ (fmole on column)	LOD (fmole on column)
Orbitrap Exploris MX and 240*	25 nM – 2.5 mM (5 orders of magnitude)	50	25
Orbitrap IQ-X and Ascend**	2.5 nM – 2.5 mM (6 orders of magnitude)	5	0.5
Orbitrap Astral	5 nM – 2.5 mM (5 orders of magnitude)	10	5

*Data is valid for uric acid in negative polarity. Quantitation is performed via HRAM Orbitrap MS^1.
**Quantitation is performed via tMS^2 linear Ion trap.
***Quantitation is performed via HRAM Orbitrap MS^1.

Conclusions

The recently developed SQUAD metabolomics technique is a promising alternative, offering researchers a new way to merge untargeted and targeted approaches in one single experiment. It also offers laboratories a new way to approach metabolomics. This workflow enables the annotation and quantification of a pre-selected group of metabolites in a sample. In addition, the data can then be reanalyzed (or retro-mined) to look for global metabolic changes that were not part of the original focus.

SQUAD workflows were developed on multi-Thermo Scientific Orbitrap mass spectrometers which enabled a wider coverage by using the fast polarity switching on the Exploris series, extended sensitivity and linear dynamic ranges by using the Tribrid platforms with Ion trap quantitation, and high throughput analysis achieved on the novel Orbitrap Astral mass spectrometer.

References

1. Amer, B., Deshpande, R., and Bird, S., Simultaneous Quantitation and Discovery (SQUAD) Analysis: Combining the Best of Targeted and Untargeted Mass. *Metabolites*, **2023,** *13*, 648.

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