Simultaneous Quantitation and Discovery (SQUAD) analysis: Combining targeted and untargeted metabolomics on Orbitrap-based mass spectrometers

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Keywords

Orbitrap Astral mass spectrometer, Orbitrap IQ-X mass spectrometer, Orbitrap Exploris 240 mass spectrometer, untargeted metabolomics, targeted metabolomics, metabolites

Table of contents

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Executive summary

Metabolomics research involves two primary approaches: untargeted analysis, which provides a broad overview of molecular changes but lacks precision, and targeted analysis, which focuses on specific metabolite groups with high accuracy. A novel method called Simultaneous Quantitation and Discovery (SQUAD) analysis has emerged to strike a balance between these approaches.

SQUAD analysis combines the strengths of both methods. It quantifies a predefined set of metabolites like targeted analysis by using calibration curves with isotopically labeled standards and chemical standards, but also confidently annotates unknown metabolites as in untargeted analysis. Thus, it enhances our understanding of molecular relationships within biological systems.

A SQUAD workflow was developed on multiple Thermo Scientific™ Orbitrap™ mass spectrometers. These instruments offer several advantages that enhance the capabilities of the SQUAD analysis. The fast polarity switching feature available on the Thermo Scientific™ Orbitrap Exploris™ series instruments enables broader coverage of metabolites, while the Thermo Scientific™ Orbitrap Tribrid™ platforms, with two detectors that can be utilized in parallel, provide a middle ground that blends the superior features of targeted and untargeted metabolomics. The SQUAD approach was also developed on the advanced Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer, offering streamlined processes and precise results. This system combines comprehensive full-scan Orbitrap HRAM MS^1 and rapid, accurate DDA MS^2 analysis, enabling confident SQUAD analysis in a single injection, which allows for shorter LC gradients without compromising data quality.

In conclusion, SQUAD analysis offers a versatile solution by integrating targeted and untargeted approaches, enabling researchers to simultaneously quantify specific metabolites and discover new ones while maintaining data accuracy and reliability. Optimization of analytical parameters enhances its effectiveness in metabolomics research.

1. Introduction

Metabolomics is defined as the measurement of small molecule substrates, intermediates, and/or end products of cellular metabolism (i.e., metabolites) and provides an immediate and dynamic response to genetic and/or environmental perturbations in biological systems including biofluids, cells, tissues, and/or organisms.[1] Traditionally, mass spectrometry (MS)-based metabolomics studies are performed using either an untargeted discovery or a targeted hypothesis-driven approach. While both methods have their strengths and weaknesses, the greatest challenge for the untargeted analysis is maximizing the detection and accurate identification of thousands of metabolites, while for the targeted analysis, it is maximizing the linear dynamic range and quantification sensitivity.[2]

This review discusses the opportunities and challenges of the two metabolomics workflows; in addition, it presents a composite approach (i.e., SQUAD analysis: Simultaneous Quantitation and Discovery) that combines the advantages of both. The SQUAD analysis involves the simultaneous quantification of a pre-defined set of metabolites but also allows for the identification of new metabolites that were not included in the defined list.

1.1. Untargeted metabolomics

Untargeted metabolomics aims to determine all measurable metabolites in a biological sample. The untargeted analyses are often used for biomarker discovery and are hypothesis-generating approaches that focus on acquiring data for as many compounds as possible and reviewing both known and unknown metabolic changes in the studied system. Data from the untargeted approach are normally used for relative quantification across sample groups which could be further validated with targeted analysis.[3]

The extraction, separation, and detection of metabolites in untargeted metabolomics face challenges due to the diverse chemical properties and range of concentrations present in the biological samples. Often researchers must compromise on the analytical parameters, such as the stationary phases and ionization modes. This can enhance the detection of certain substances while hindering the detection of others. Another approach involves combining different extraction and separation methods (e.g., reversed-phase (RP) and hydrophilic interaction liquid chromatography (HILIC)-based MS), but this extends analysis time and requires integrating information from various analytical techniques. Accurate identification of numerous metabolites poses an additional challenge, alongside signal bias and mass drift caused by analyzing complex sample matrices, resulting in reduced sensitivity. Therefore, it is crucial to employ high resolution accurate mass (HRAM) detection, such as that offered by Orbitrap mass analyzers, whenever possible, to precisely identify and determine the elemental and isotopic composition of samples. Advanced pathway analysis tools are still needed to effectively interpret metabolomics data and address complex biological questions.[4] Lastly, the complex nature of untargeted metabolomics data demands sophisticated statistical and bioinformatics tools for thorough analysis and interpretation.

1.2. Targeted metabolomics

Targeted metabolomics aims to measure defined groups of chemically characterized and biochemically annotated metabolites.[5] This analysis requires a hypothesis-driven experiment, where there is a recognized rationale for selecting the metabolite group under study.[6] The preferred analytical technologies for an ideal targeted metabolomic analysis should provide a combination of high sensitivity, specificity, linear dynamic range, and throughput.

Targeted metabolomics serves as a valuable tool for validating biomarkers, specifically focusing on confirming the distinct presence of metabolites identified through untargeted metabolomics. This approach commonly employs MS-based techniques in conjunction with separation methods like liquid chromatography (LC) and gas chromatography (GC). For instance, in a study aiming to develop bacterial consortia for treating chronic immune-mediated colitis and restoring intestinal balance, a targeted LC-MS approach was utilized to determine the concentration levels of bile acids, short-chain fatty acids (SCFAs), and tryptophan/indole metabolites in mouse feces and/or cecal contents.[7] Conversely, a GC-MS-based targeted metabolomics strategy was employed to identify highly sensitive biomarkers in the early detection of pancreatic cancer. Additionally, there are targeted metabolomics kits available that can quantify as many as 500 compounds.[8,9]

Despite the benefits of targeted metabolomics, its scope is constrained, resulting in the exclusion of biologically significant metabolites that fall outside the predetermined subset. However, acquiring all the necessary pure chemical standards and internal standards for the metabolites of interest can be challenging, leading to a restricted range of detected metabolites in targeted metabolomics.[2]

1.3. SQUAD analysis

In the preceding sections, we discussed the merits and drawbacks of untargeted and targeted methods in the context of metabolomics research. Researchers often face a trade-off between obtaining a general understanding of overall molecular changes with limited accuracy (i.e., untargeted analysis) or gaining a focused and detailed view of specific metabolite groups (i.e., targeted analysis). However, a promising and novel approach called SQUAD analysis has recently emerged, aiming to strike a balance by integrating both untargeted and targeted workflows.[10] This innovative method combines the strengths of the traditional approaches while addressing their limitations, offering a more comprehensive and versatile solution (Figure 1). This technique involves the quantification of a pre-defined set of metabolites, as in targeted metabolomics, but also allows for the identification of new metabolites that were not included in the targeted list.

The main objective of this approach centers around two key aspects: the reliable identification of metabolites using authentic chemical standards and the precise measurement of their quantities via calibration curves utilizing both chemical standards and isotopically labeled standards. Simultaneously, a secondary aim is to uncover novel molecular relationships within the biological system by conducting untargeted analysis, leveraging the existing recorded data from a single injection. As discussed in our previous review,[10] similar approaches have been used within the metabolomics community but without a predefined structure and were reported under various names that might cause some uncertainty. To this end, we found it urgent to present an expressive term for this workflow that can be used by the community without any confusion. Based on that, we proposed the name: Simultaneous Quantitation and Discovery (SQUAD) analysis.

The quantitation aspect of this approach offers flexibility, where the user has the freedom to determine the desired level of accuracy and the number of targets to be quantified. Absolute quantitation of metabolites can be achieved when both isotopically labeled (internal standards (IS)) and unlabeled standards are used to create calibration curves. The IS are added to the samples at known concentrations to account for variations in ionization efficiency and other losses/variations during sample preparation. However, due to factors like high costs or limited availability, it may not always be practical to have a dedicated IS for each metabolite of interest. In such cases, calibration curves are generated using standards alone to understand how the signal intensity changes with analyte concentration within the linear range.[11] Users can also investigate using one IS per metabolite or lipid class; however, this might compromise accuracies in comparison to truly quantitative methods. Additionally, a relative quantitation (relative quantification with one-point calibration) can be performed by comparing the results to a reference sample. (The actual concentration of analyte in the sample is estimated by extrapolating on the closeness to the standard, which is quick and easy to apply but provides less accurate data.) It is important to note that absolute quantitation, when possible, holds greater value as it allows for the measurement of metabolic reactions' thermodynamics[12] and the molecular dynamics involved in the flow of atoms through a metabolic network[13]. Moreover, absolute quantitation facilitates a more robust comparison of results across instruments and laboratories by adjusting for ionization and instrumentation variabilities using stable labeled coeluting standards.

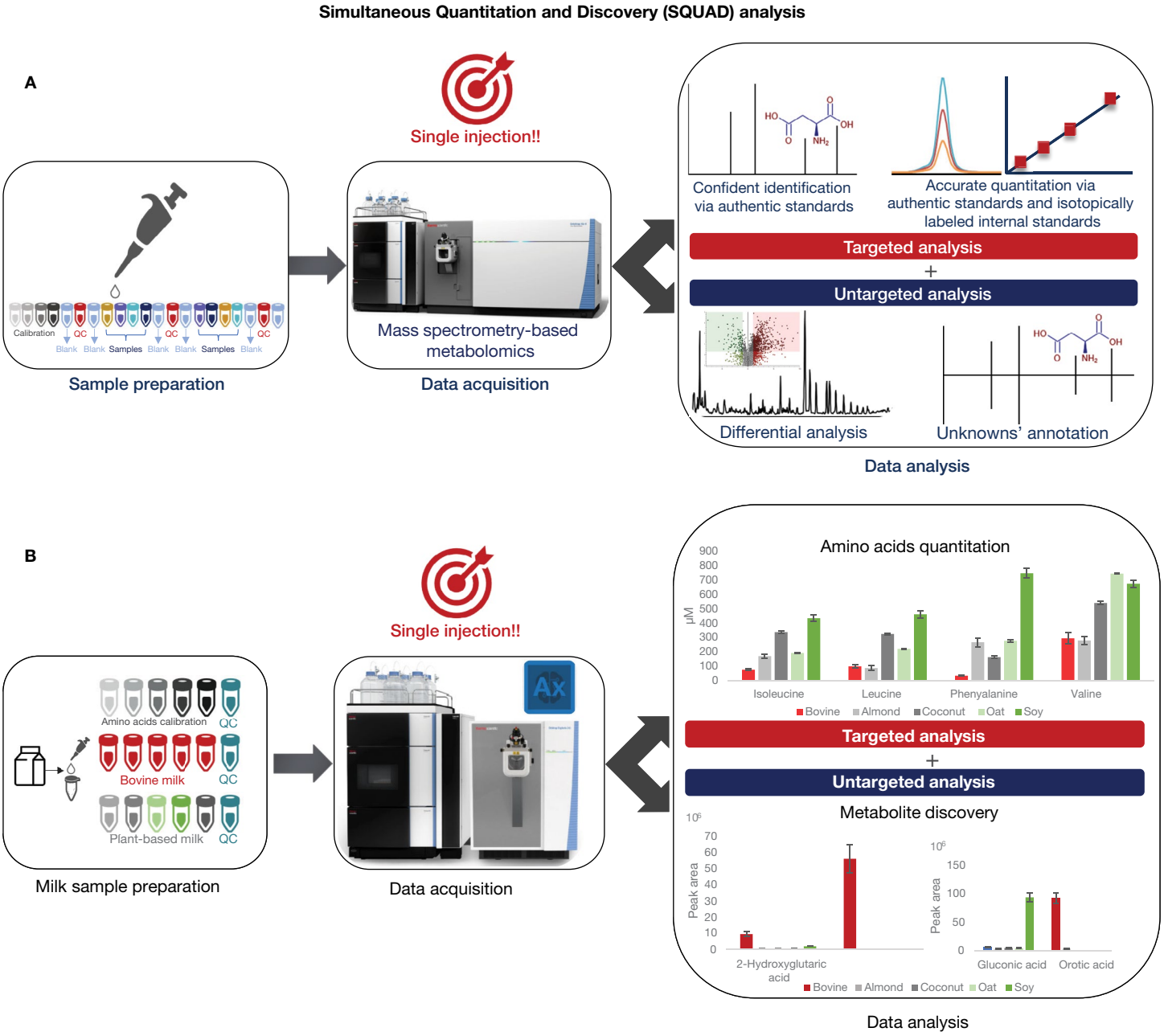

Figure 1. (A) The novel single-injection SQUAD metabolomics combines targeted and untargeted workflows; (B) a SQUAD case study presents the absolute quantitation of amino acids in milk samples, meantime, identifying markers via a discovery approach of the acquired data.

In SQUAD analysis, quality control (QC) samples, such as pooled mixes of biological samples, are employed to assess the stability and reliability of the analytical system. These QC samples serve multiple purposes, including correction and normalization of data, as well as the collection of compound fragmentation information that aids in the identification of unknown metabolites. Like untargeted metabolomics, acquiring HRAM data is of utmost importance in SQUAD analysis. This ensures the ability to confidently annotate and identify unknown metabolites during the analysis process.

To expand the scope of quantified metabolites in a SQUAD analysis, it is essential for users to optimize the separation method (such as LC or GC) and the mass detection methods accordingly. The optimization of experimental methods aims to ensure the recording of an adequate number of scans per peak (≥8 scans) for precise peak integration, achieving sufficient sensitivity for measuring trace amounts and establishing a linear dynamic range that encompasses the levels of the targeted analytes. For instance, adjusting the Orbitrap resolution from 240,000 to 120,000 in an Orbitrap-based mass spectrometer can notably increase the number of scans per peak while maintaining the quality of MS^1 accuracy during the discovery phase. In addition, utilizing a fast-scanning linear ion trap for target quantitation in parallel to Orbitrap untargeted discovery in an Orbitrap Tribrid mass spectrometer can improve the sensitivity of SQUAD analysis but also increase the number of targeted metabolites without compromising the Orbitrap discovery analysis. On the other hand, an optimized separation method can facilitate the quantification of isomers through MS^1 scanning, eliminating the need for an MS^2 technique like parallel reaction monitoring (PRM). We will expand on these considerations further in the discussion.

2. SQUAD analysis opportunities

One of the major advantages of SQUAD analysis is its capability to perform both targeted and untargeted analysis in a single sample injection. This feature proves particularly beneficial for laboratories that face limitations in sample availability, time, and resources. It provides an efficient and powerful approach to extracting more valuable information from biological samples.

SQUAD analysis on HRAM platforms also facilitates discovery in clinical studies by capturing high-quality MS^1 full scans, thereby enhancing the sensitive and reliable quantification of a wide range of analytes. The development of intelligence-driven data acquisition strategies allows scientists to delve deeper into samples while obtaining an overview of known metabolites. To this end, we have developed an advanced and user-friendly method called Thermo Scientific™ AcquireX™ intelligent data acquisition workflow (Figure 2), which integrates independent experiments into an automated workflow. The AcquireX workflow enhances real-time, selective LC-MS^2 data acquisition, facilitating comprehensive sample and study characterization. With five different routines, the AcquireX workflow significantly increases productivity for various small-molecule applications, ranging from comprehensive structural annotation to screening. These approaches optimize MS^2 interrogation of relevant compounds and offer multiple benefits to users, such as seamless integration of independent experiments into automated workflows. Ultimately, these advancements improve the efficiency and ease of LC-MS-based SQUAD analysis.

The availability of comprehensive metabolite databases, including the Thermo Scientific™ mzCloud™ mass spectral fragmentation library, NIST™, ChemSpider™, KEGG™, and others, coupled with advancements in multivariate data analysis methods and software, such as Thermo Scientific™ Compound Discoverer™ software, has revolutionized the analysis of vast amounts of metabolic profiling data. These tools now allow for the identification of clusters based on specific features.

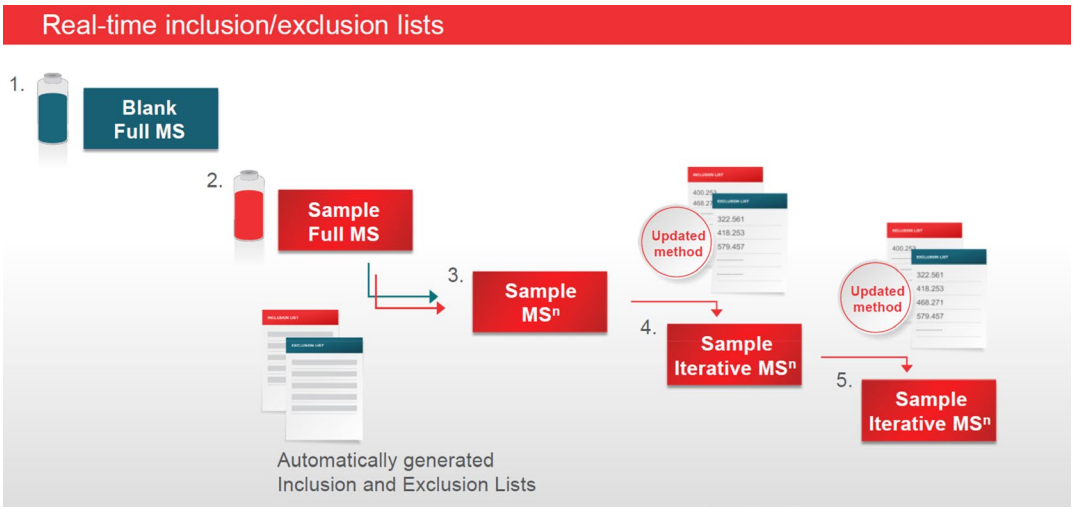

Figure 2. The AcquireX workflow for intelligent data acquisition maximizes the number of relevant compounds interrogated by MS^2, resulting in higher coverage and confidence annotation.

Besides its clinical applications, the SQUAD analysis has potential in the analysis of bacterial metabolism. The human gut microbiota plays a crucial role in various physiological processes, including nutrient digestion and immune system regulation.[2] By measuring traditional microbiota metabolites like short-chain fatty acids and bile acids in the gut, along with conducting a discovery-based analysis, we can gain deeper insights into the activity of gut microbes. This information can enhance our understanding of their impact on human health. Moreover, SQUAD analysis can be applied to investigate microbial metabolism and pharmacomicrobiomics, which involves studying the interaction between foreign compounds (xenobiotics) and the gut microbiome. Such research would offer valuable insights into how an individual's gut microbiome influences drug efficacy within specific patient populations.

SQUAD analysis holds great potential for the field of metabolic engineering. This innovative workflow can effectively pinpoint metabolic pathway bottlenecks and facilitate the identification of diagnostic biomarkers. These biomarkers can be leveraged to optimize various aspects of microbial performance, including growth rate, strain tolerance, stress regulation and adaptation, substrate utilization, and product yield, rate, and titer improvement. Simultaneously, SQUAD provides quantitative information about the desired production, akin to traditional targeted approaches.

Furthermore, SQUAD analyses are anticipated to play a crucial role in the realm of natural products, encompassing compounds like flavonoids and steroids. Particularly on platforms equipped with multiple fragmentation methods and intelligent MS fragmentations for unknown identification, such as the Orbitrap Tribrid mass spectrometers. These instruments offer various fragmentation techniques like higher-energy collisional dissociation (HCD), collision-induced dissociation (CID), and ultraviolet photodissociation (UVPD). The latter, UVPD, produces fragment ions that provide valuable insights into double bond locations and other unique structurally diagnostic information. This is especially beneficial for analyzing compound classes like lipids and glucuronides. Moreover, these instruments incorporate real-time library search capabilities, which involve matching MS^2 spectra against spectral libraries for confident decision-based MS^n triggering. This leads to enhanced metabolite annotation and improved characterization of unknown compounds.

3. Barriers to adopting SQUAD analysis

Although SQUAD analysis offers notable advantages for metabolomics, its widespread adoption may face certain barriers that necessitate attention from vendors and researchers to enable its broader application in laboratories. One key barrier is the availability of pure and diverse chemical standards, including labeled standards, which are essential for confident identification and, if required, accurate absolute quantitation. These authentic standards play a crucial role in confirming the identity of metabolites by matching various properties such as separation retention time, precise molecular mass, and MS fragmentation patterns. While numerous authentic metabolite standards are commercially accessible, there remains a significant number that are not readily available. This poses a major hurdle as custom chemical synthesis becomes necessary when encountering true unknown compounds, thereby significantly increasing costs.

Nevertheless, the untargeted aspect of the SQUAD analysis workflow can contribute to the scientific community by providing valuable insights into important and relevant metabolites that warrant synthesis. This information can then inform and guide the targeted portion of future SQUAD analysis studies. By bridging the gap between untargeted and targeted approaches, SQUAD analysis can facilitate a more efficient allocation of resources for chemical synthesis, focusing on metabolites of greater significance and potential impact.

The achievement of high-quality data is paramount to ensure a reliable interpretation of results in SQUAD analysis. Therefore, selecting a mass spectrometer that provides HRAM and robustness in both biological and statistical aspects is crucial for confident annotation of unknown metabolites and accurate differential analysis. Mass spectrometers such as the HRAM Orbitrap-based systems are ideal choices, while nominal mass instruments like the QQQ and quadrupole traps may not offer the desired level of performance.

Moreover, robust data processing capabilities are essential for effective SQUAD analysis, particularly in retro-mining data. Powerful data analysis solutions are necessary to enable fast processing and accurate quantification of metabolites. These tools should facilitate differential analysis and ensure confident metabolite annotation by leveraging high-quality spectral libraries and databases for precise discovery analysis. Furthermore, these solutions should offer metabolic pathway analysis to enable accurate biological interpretation of the acquired data, enhancing the understanding of metabolic processes and their implications.

4. SQUAD analysis structure

This section covers the configuration of the SQUAD analysis workflow across various Orbitrap-based mass spectrometers, including hybrid systems that combine a quadruple and Orbitrap analyzer such as the Thermo Scientific™ Orbitrap Exploris™ MX mass spectrometer, Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, and Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer, as well as the Orbitrap Tribrid mass spectrometers that combine a quadruple, linear ion trap, and Orbitrap analyzer, such as the Thermo Scientific™ Orbitrap™ IQ-X™ Tribrid™ mass spectrometer and Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer. Additionally, it discusses the SQUAD analysis framework on the recently developed Orbitrap Astral mass spectrometer.

Each Orbitrap mass spectrometer offers distinct features that enhance SQUAD analysis. The hybrid mass spectrometers facilitate comprehensive coverage of the metabolome within a single injection through rapid polarity switching analysis. The Orbitrap Tribrid mass spectrometers, on the other hand, enable concurrent SQUAD analysis with sensitive quantitation and discovery, utilizing the linear ion trap and the HRAM Orbitrap, respectively. Lastly, the novel Orbitrap Astral mass analyzer enables faster scanning at the MS^2 level, resulting in a higher rate of annotation, while ensuring sufficient data points across the peak at the Orbitrap MS^1 level for precise and extended analyte quantitation.

4.1. SQUAD analysis on Orbitrap Exploris mass spectrometers

When the SQUAD analysis is conducted on hybrid mass spectrometers, the instruments combine the quadrupole and Orbitrap technologies (Figure 3), employing a comprehensive HRAM MS^1 analysis for each sample. This analysis serves both quantitation and untargeted discovery purposes. Incorporating authentic chemical standards and IS into the Orbitrap SQUAD analysis facilitates highly sensitive absolute quantitation of metabolites in plasma. The quantitation range is broad and exhibits a good linear dynamic range. In addition, HRAM mass spectrometers that can achieve greater than 60K resolution facilitate the resolution of low abundant metabolites in a complex matrix and are, therefore, a good choice in this approach.

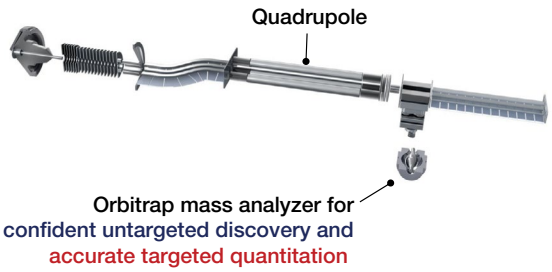

Figure 3. SQUAD analysis utlizes a high field Orbitrap mass analyzer for high-resolution confident untargeted discovery and accurate targeted quantitation within single-injection analysis on Orbitrap Exploris (i.e., Orbitrap Exploris MX, Orbitrap Exploris 120, and Orbitrap Exploris 240) mass spectrometers.

Furthermore, advancements in scanning speed have been achieved in hybrid-based Orbitrap instruments, enabling polarity switching (Figure 4) even in conjunction with high-throughput LC and GC methods. This breakthrough innovation allows the hybrid mass spectrometers to achieve wider metabolome coverage through the utilization of fast polarity switching.

The advanced deep scan AcquireX workflow enables a higher percentage of compounds to undergo MS^2 fragmentation, thereby enhancing annotation capability and structural elucidation. By performing deep fragmentation analysis using QC pools individually, samples can be profiled in both polarities within a single injection. This approach enhances throughput while maintaining the integrity of both targeted and nontargeted analyses.

Utilizing Orbitrap MS^1-based quantitation on the hybrid platforms has proven to exhibit excellent sensitivity, showcasing the ability to detect low limits of quantification (LLOQ) as low as 50 femtomoles while achieving a low limit of detection (LLOD) of 25 femtomoles on the column for phenylalanine in plasma. This remarkable sensitivity provides a dynamic range spanning 6 orders of magnitude. When greater sensitivity is required for targets of interest, the Thermo Scientific™ Orbitrap Exploris™ family of mass spectrometers can provide enhanced sensitivity through either single ion monitoring (SIM) or PRM experiments. This compromise in target sensitivity versus untargeted compound discovery maintains less restriction than a fully targeted assay without any full scan hypothesis generating acquisition. The power of SQUAD analysis can be seen in this example where the design of an experiment showcases what is most needed scientifically while still employing a more flexible acquisition technique.

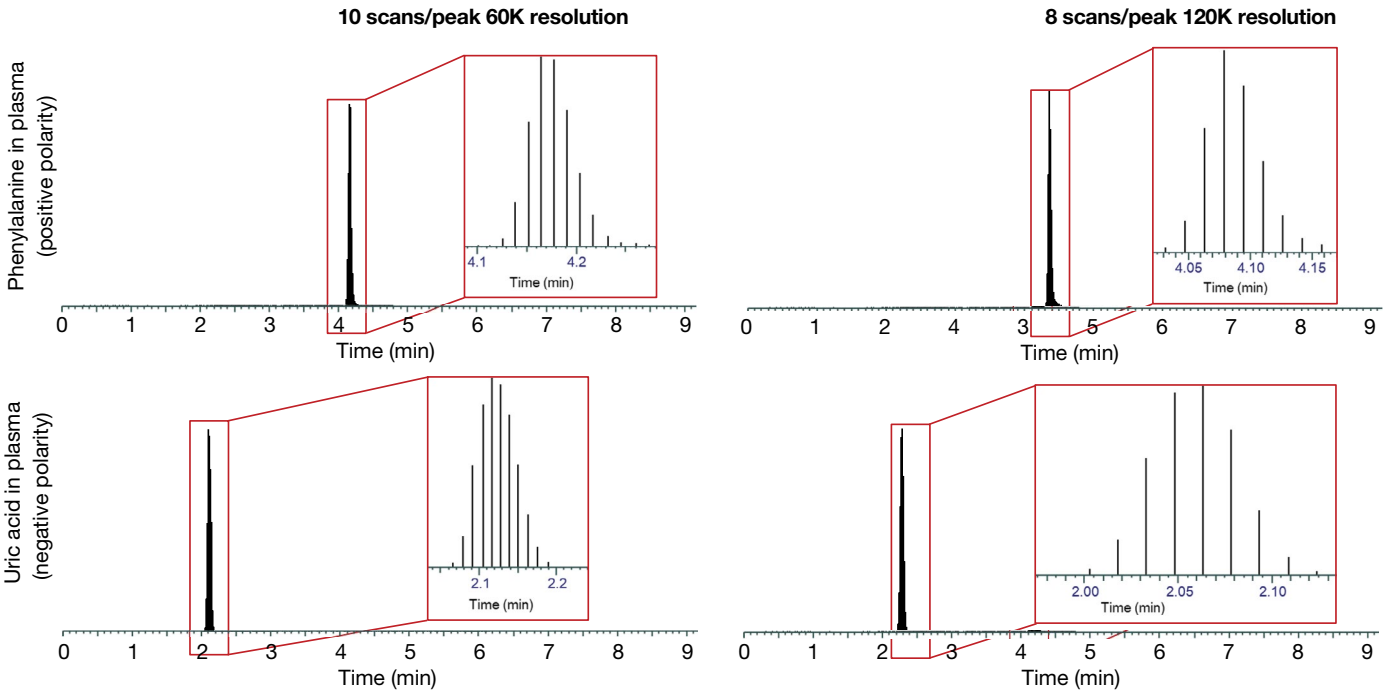

Figure 4. Fast polarity switching on the Orbitrap Exploris series MS platforms enables accurate and sensitive quantitation with wide coverage within a single-injection analysis.

4.2. SQUAD analysis on Orbitrap Tribrid mass spectrometers

The recent advancements in Orbitrap Tribrid mass spectrometers present an excellent opportunity to employ SQUAD analysis, leveraging the sensitive linear ion trap for metabolite quantitation while simultaneously conducting the discovery phase of the untargeted assay on the HRAM Orbitrap analyzer. As illustrated in Figure 5, this approach eliminates the need for multiple instruments and avoids the requirement of re-injecting limited biological samples, thus reducing variability.

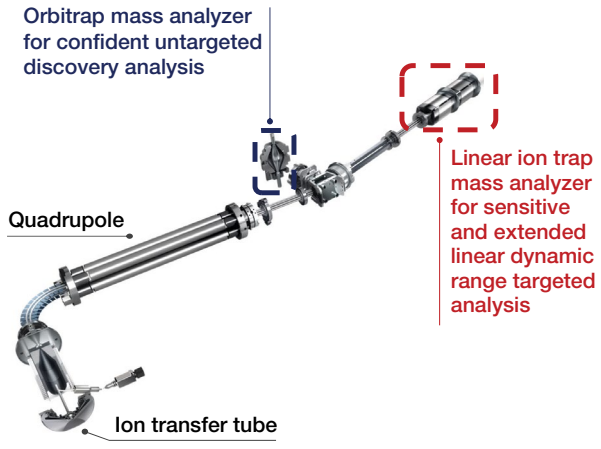

Figure 5. The SQUAD analysis is demonstrated on an Orbitrap IQ-X Tribrid mass spectrometer, where the sensitive linear ion trap is employed for metabolite quantitation while maintaining the discovery phase of the untargeted assay on the high-resolution accurate mass orbitrap analyzer.

The Orbitrap Tribrid mass spectrometers offer multiple methods for MS^2 fragmentation, including HCD, CID, and UVPD. Additionally, the instruments enable MS^n fragmentation, leading to enhanced confidence in the identification of unknown compounds. Particularly, the combination of UVPD with HCD and/or CID enables the generation of fragment ions that provide valuable information for the determination of double bond positions and other structurally diagnostic features across different compound classes such as lipids and glucuronides.

Furthermore, Thermo Scientific mass spectrometers provide real-time library search functionality, which utilizes spectral matching against spectral libraries for decision-based MS^n acquisition. This feature significantly enhances confidence in metabolite annotation and improves the characterization of unknown compounds.

The linear ion trap of the Orbitrap Tribrid mass spectrometer exhibits remarkable sensitivity, demonstrated by its ability to detect LLOQ down to 5 femtomoles and an LLOD of 0.5 femtomoles on the column for phenylalanine in plasma via a PRM (or targeted MS^2) quantitation approach. Additionally, it showcases an impressive linear dynamic range spanning 6 orders of magnitude. Like the analysis performed on the hybrid mass spectrometers, QC pools are utilized for deep fragmentation analysis through the intelligent data acquisition tool, i.e., AcquireX intelligent data acquisition. This approach enhances throughput while preserving the integrity of both targeted and nontargeted analyses.

4.3. SQUAD analysis on the novel Orbitrap Astral mass spectrometer

An advanced SQAUD metabolomics approach that offers several benefits, including a streamlined process, improved efficiency, and precise results, was also developed on the state-of-the-art Orbitrap Astral mass spectrometer (Figure 6). The Orbitrap Astral MS can perform comprehensive full-scan Orbitrap HRAM MS^1 analysis as well as rapid, accurate, HRAM and sensitive DDA MS^2 in the Astral analyzer. By combining these capabilities, our approach enables confident and deep coverage of untargeted and accurate targeted analysis using a single injection. This eliminates any potential variability associated with using iterative fragmentation of QC pooled samples for unknown annotation due to sample dilution in large studies.

Additionally, the Astral analyzer achieves a high percentage of compound fragmentation (approximately 90%) compared to the traditional DDA method utilizing an Orbitrap mass analyzer for both MS^1 and MS^2 detection (Figure 7), ensuring the fragmentation of lower-abundance compounds even in complex matrices such as plasma (Figure 8) results in higher annotation rate of metabolites (Figure 9).

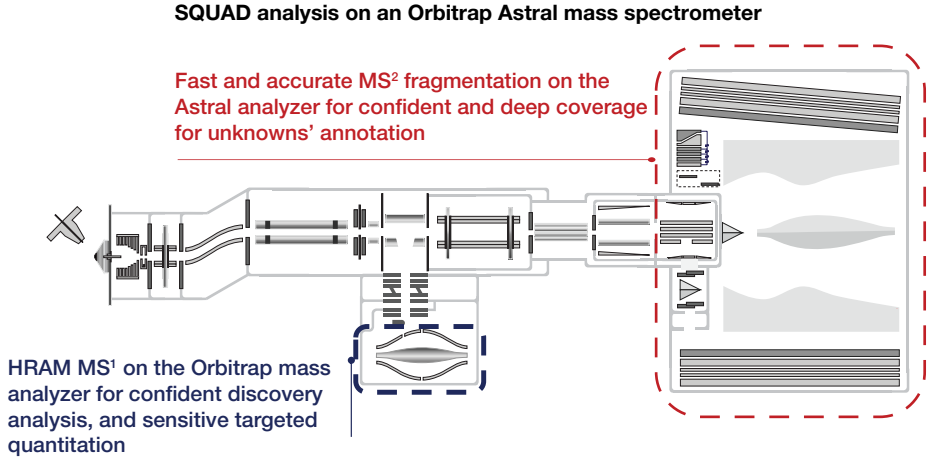

Figure 6. SQUAD analysis on an Orbitrap Astral mass spectrometer. Fast and accurate MS^2 fragmentation on the Astral analyzer was utilized for confident and deep coverage for unknowns' annotation while securing sensitive MS^1 Orbitrap targeted quantitation.

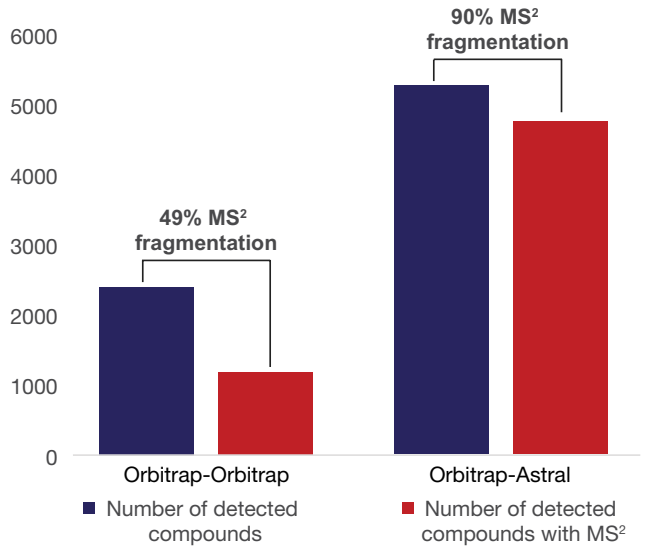

Figure 7. The percentage of compound fragmentation in data-dependent MS^2 analysis (DDA) on an Orbitrap Astral (Orbitrap-Astral) mass spectrometer and traditional DDA on an Orbitrap mass analyzer only (Orbitrap-Orbitrap). The presented data represent plasma metabolites analyzed using a reversed-phase 15-minute LC method in postive ionization mode with the Orbitrap collecting at 120K resolution for MS^1 and 30K resolution for MS^2. Compound is defined by detected feature with at least an *m/z* and retention time values.

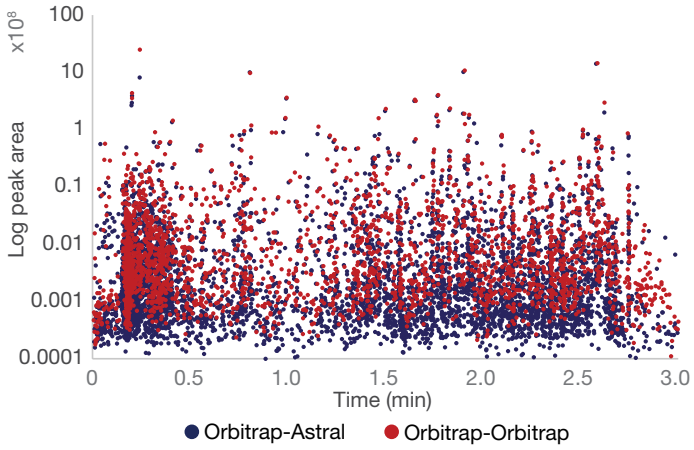

Figure 8. The data-dependent MS^2 analysis (DDA) on an Orbitrap Astral (Orbitrap-Astral) mass spectrometer ensures the fragmentation of lower-abundance compounds compared to traditional DDA on an Orbitrap mass analyzer only (Orbitrap-Orbitrap). The presented data is for plasma metabolites analyzed using a HILIC 4-minute LC method in postive ionization mode with the Orbitrap collecting at 120K resolution for MS^1 and 30K resolution for MS^2. Compound is defined by detected feature with at least an *m/z* and retention time values.

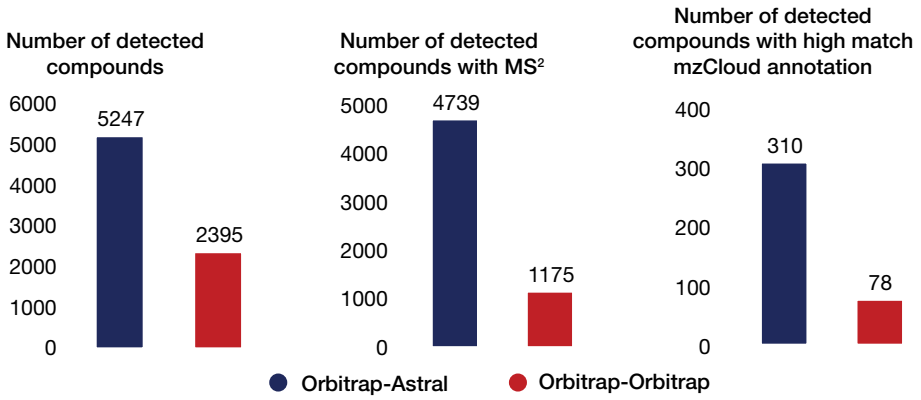

Figure 9. The data-dependent MS² analysis (DDA) on an Orbitrap Astral (Orbitrap-Astral) mass spectrometer resulted in a higher annotation rate of plasma metabolites compared to traditional DDA on an Orbitrap mass analyzer only (Orbitrap-Orbitrap). The presented data represent plasma metabolites analyzed using a reversed-phase 15-minute LC method in postive ionization mode with the Orbitrap collecting at 120K resolution for MS¹ and 30K resolution for MS². Compound is defined by detected feature with at least an *m/z* and retention time values.

Moreover, the Orbitrap MS¹ level of a DDA method provides sufficient data points across the peak (Figure 10A), ensuring precise and extended quantitation of the analytes. This offers an impressive dynamic range of 5 orders of magnitude, with an LLOQ of 10 femtomoles and an LLOD of 5 femtomoles of phenylalanine in plasma on the column (Figure 10B), compared to an LLOQ of 50 femtomoles using the Orbitrap Exploris series (i.e., Orbitrap Exploris MX, Orbitrap Exploris 120 MS, and Orbitrap Exploris 240 mass spectrometers). This could also be attributed to the wider entrance of the ion transfer tube (letterbox shape) of the Orbitrap Astral mass spectrometer compared to the round shape of the three Orbitrap Exploris instruments mentioned before.

Furthermore, with the introduction of the Orbitrap Astral mass spectrometer, it becomes possible to significantly reduce the LC gradient duration, by a factor of three for example, while still maintaining enough MS¹ scans and signal-to-noise ratio for sensitive and accurate quantitation (Figure 11A). Additionally, the fragmentation ratios of MS² spectra remain unaffected (Figure 11B).

This presents an excellent opportunity to leverage the capabilities of the Orbitrap Astral mass spectrometer for high-throughput SQUAD analysis. Such advancement is crucial for achieving extensive coverage and confident untargeted discovery analysis. Moreover, when compared to the Orbitrap-Orbitrap 15-minute LC gradient experiment, the Orbitrap-Astral 5-minute LC gradient experiment exhibited a 25% increase in the number of detected compounds with MS² (Figure 12A). This, in turn, leads to a higher number of annotated unknowns when utilizing the high-throughput SQUAD analysis on Orbitrap-Astral mode (Figure 12B), owing to its enhanced scan speed and improved peak quality (Figure 12C).

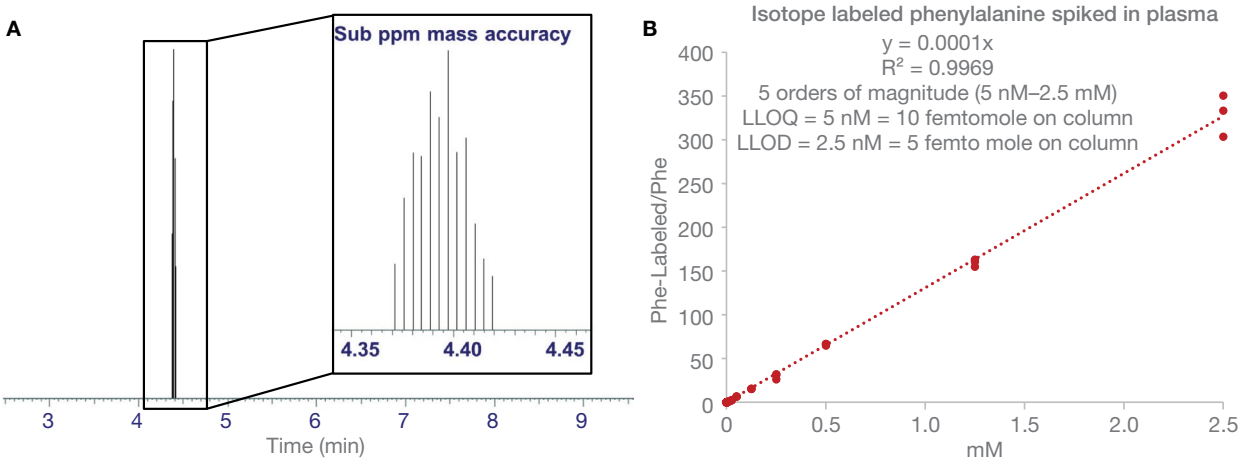

Figure 10. (A) Number of Orbitrap MS¹ scans across the peak and (B) linear calibration curve of isotope-labeled phenylalanine spiked in plasma analyzed using the Orbitrap Astral mass spectrometer data-dependent MS² analysis (DDA). The presented data represent plasma metabolites analyzed using a reversed-phase 15-minute LC method in postive ionization mode with the Orbitrap collecting at 120K resolution for MS¹ and 30K resolution for MS².

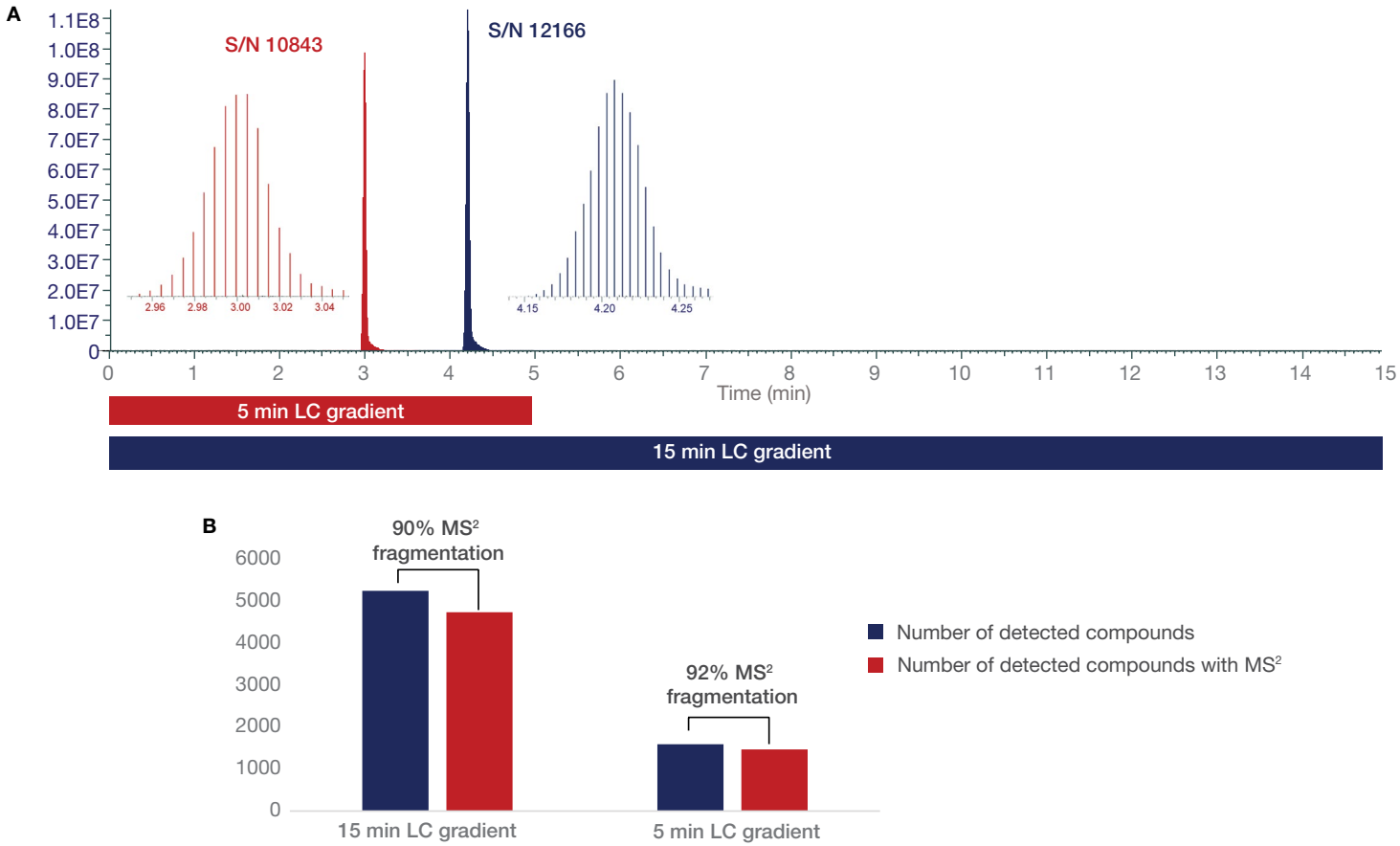

Figure 11. (A) Number of Orbitrap MS¹ scans across the plasma phenylalanine peak of 15- and 5-minute LC gradients and (B) the percentage of compound fragmentation in data-dependent MS² analysis (DDA) on an Orbitrap Astral mass spectrometer using 15- and 5-minute reversed-phase LC gradients. Compound is defined by detected feature with at least an *m/z* and retention time values.

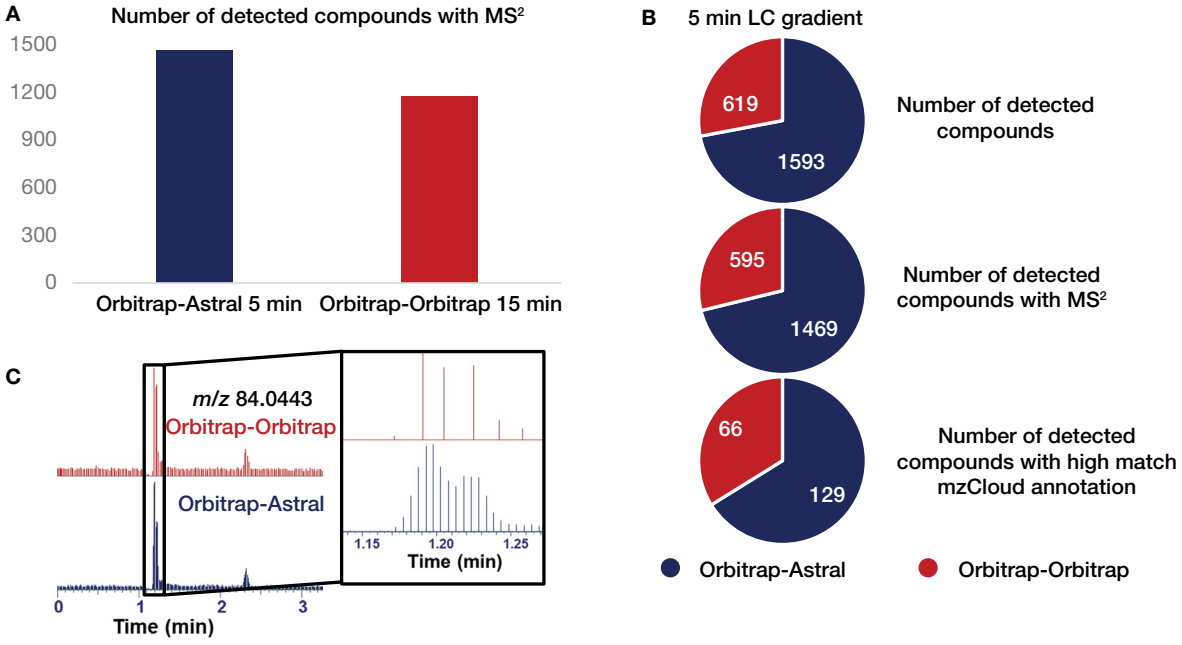

Figure 12. (A) Number of detected and annotated plasma compounds with MS² using a 5-minute LC gradient with Orbitrap-Astral data-dependent MS² analysis (DDA) vs. a 15-minute LC gradient with an Orbitrap-Orbitrap DDA experiment, (B) annotation rate of plasma metabolites, and (C) number of scans per peak via DDA on an Orbitrap Astral mass spectrometer vs. traditional DDA on an Orbitrap mass analyzer only (Orbitrap-Orbitrap) using a reversed-phase 15-minute LC method. Compound is defined by detected feature with at least an *m/z* and retention time values.

5. Conclusion

The innovative SQUAD analysis metabolomics technique has emerged as a promising solution, offering researchers the ability to integrate untargeted and targeted approaches within a single experiment. This approach not only revolutionizes the way laboratories approach metabolomics but also enables the annotation and quantification of a pre-selected group of metabolites in a sample. Moreover, the data obtained can be reanalyzed or retro-mined to unveil global metabolic changes that were not initially the main focus. By simultaneously acquiring hypothesis-led and discovery-led datasets, scientists can gain a deeper understanding of biological systems through a single experiment. Historically, the dilemma of choosing between untargeted and targeted metabolomics has limited the vast potential of metabolomics in life sciences research. However, the SQUAD analysis now offers a remarkable solution, combining the best aspects of both approaches to unlock the full potential of metabolomics for scientists worldwide.

SQUAD analysis workflows were designed specifically for utilization on a range of Orbitrap mass spectrometers. These instruments offer several advantages that enhance the capabilities of the SQUAD analysis. The fast polarity switching feature available on the Orbitrap Exploris series instruments enables broader coverage of metabolites. The Orbitrap Tribrid platform instruments, with two detectors that can be utilized in parallel, provide an optimum merging between the targeted and untargeted portion of the study while also highlighting extended sensitivity and linear dynamic ranges through their ion trap quantitation capabilities (Table 1). Additionally, the novel Orbitrap Astral mass spectrometer, with its fast second HRAM detector, allows for high-throughput analysis, further optimizing the efficiency of SQUAD workflows.

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

Mass spectrometer	Calibration linear dynamic range	LLOQ (fmole on column)	LLOD (fmole on column)	Quantitation approach	Untargeted discovery	Max Orbitrap resolution	MS^n	AcquireX workflow
Orbitrap Exploris MX	25 nM – 2.5 mM (5 orders of magnitude)*	50*	25*	HRAM Orbitrap full scan MS^1	HRAM Orbitrap full scan MS^1	180,000	No	No
Orbitrap Exploris 120	25 nM – 2.5 mM (5 orders of magnitude)*	50*	25*	HRAM Orbitrap full scan MS^1	HRAM Orbitrap full scan MS^1 + DDA top 4	120,000	No (MS^2 only)	Optional
Orbitrap Exploris 240	25 nM – 2.5 mM (5 orders of magnitude)*	50*	25*	HRAM Orbitrap full scan MS^1	HRAM Orbitrap full scan MS^1 + DDA top N	240,000	No (MS^2 only)	Yes
Orbitrap IQ-X	2.5 nM – 2.5 mM (6 orders of magnitude)	5	0.5	Linear ion trap tMS^2	HRAM Orbitrap full scan MS^1 + DDA top N	500,000 (optional 1,000,000)	Yes	Yes
Orbitrap Ascend	2.5 nM – 2.5 mM (6 orders of magnitude)	5	0.5	Linear ion trap tMS^2	HRAM Orbitrap full scan MS^1 + DDA top N	500,000 (optional 1,000,000)	Yes	Yes
Orbitrap Astral	5 nM – 2.5 mM (5 orders of magnitude)	10	5	HRAM Orbitrap full scan MS^1	HRAM Orbitrap full scan MS^1 + DDA top N	480,000	No (MS^2 only)	No

*Data is valid for uric acid in negative polarity.

6. References

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