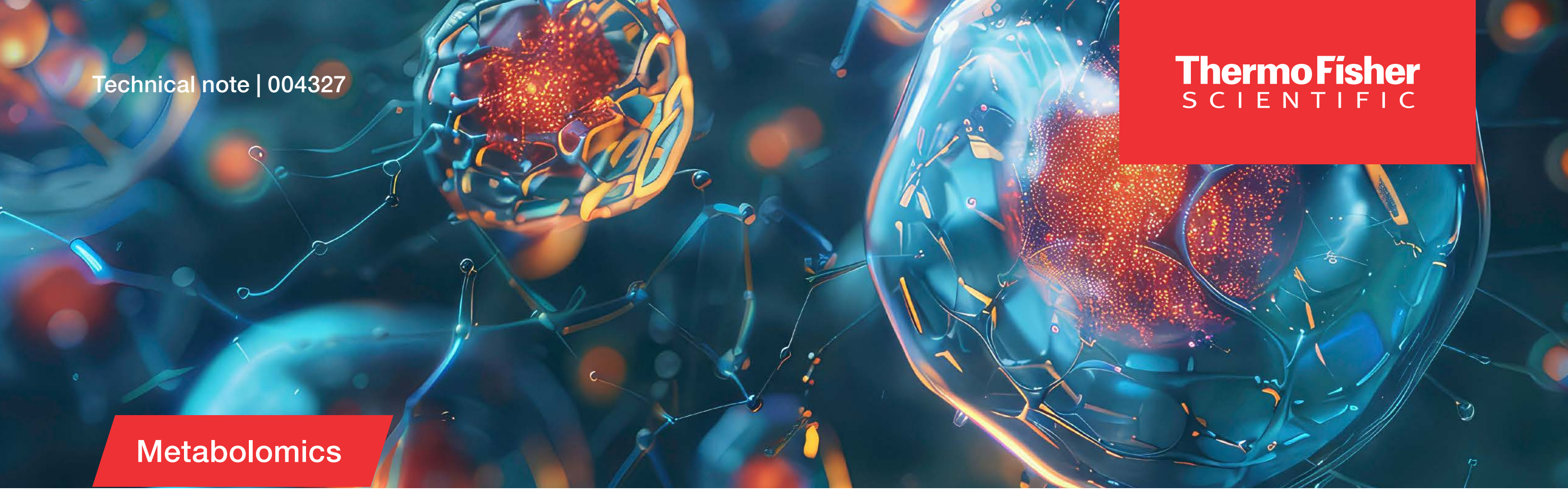
Technical note | 004327



Metabolomics

Simultaneous Quantitation and Discovery analysis: Combining targeted and untargeted metabolomics on Orbitrap mass spectrometers

Authors

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1. Introduction

Metabolomics, the study of metabolites within biological systems, involves analyzing small molecules that reflect cellular metabolic activity. This field encompasses untargeted and targeted approaches, each with strengths and challenges.

Untargeted metabolomics seeks to identify and quantify all measurable metabolites in a sample, providing a broad overview of metabolic changes. This approach is useful for discovering new biomarkers and understanding complex biological processes but struggles with detecting a vast array of metabolites and accurately identifying them due to the diverse nature of biological samples and the limitations of analytical techniques.

Targeted metabolomics, on the other hand, focuses on measuring specific metabolites with high sensitivity and accuracy. This method is driven by hypotheses and is well-suited for validating known biomarkers or studying defined metabolic pathways. It uses technologies like mass spectrometry (MS) and chromatography to achieve high sensitivity and specificity. However, it can miss biologically relevant metabolites that fall outside the predefined set, and acquiring necessary chemical standards can be challenging.

To address the limitations of these traditional methods, we developed a hybrid approach called Simultaneous Quantitation and Discovery (SQUAD) analysis (Figure 1). SQUAD integrates the strengths of both untargeted and targeted metabolomics. It allows for the quantification of a predefined set of metabolites while also identifying novel metabolites not included in the predefined list. This dual functionality is achieved through the simultaneous use of targeted quantification and untargeted discovery techniques, leveraging high-resolution mass spectrometry (HRMS) and advanced data analysis tools.

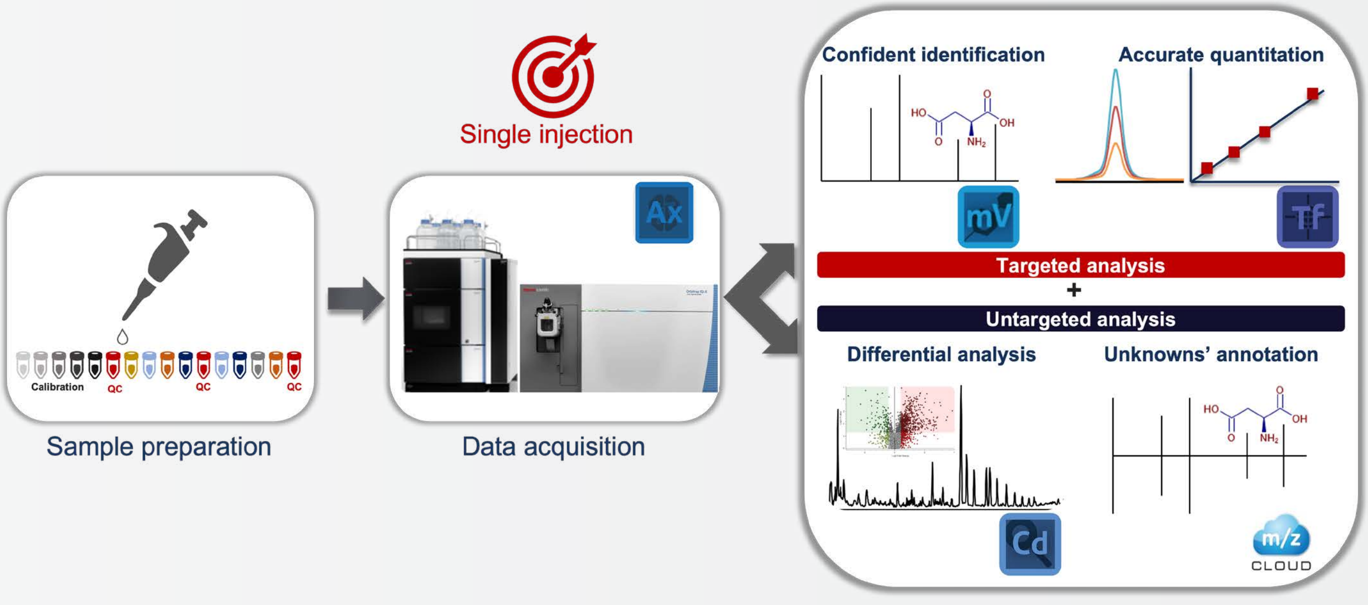


Figure 1. The SQUAD metabolomics approach combines targeted and untargeted workflows.

SQUAD analysis is particularly beneficial for many fields, including clinical research, the nutritional field, and microbial studies. In clinical settings, for example, it can enhance biomarker discovery and monitoring by providing detailed metabolic profiles. In microbiome research, it can offer insights into microbial metabolism and interactions, which are crucial for understanding gut health and drug efficacy, while targeting certain compounds such as bile acids. Additionally, SQUAD analysis has applications in metabolic engineering, where it helps identify pathway bottlenecks and optimize microbial performance by measuring substrate and products, while simultaneously performing untargeted discovery.

This technical note provides guidelines on performing a SQUAD analysis on various Thermo Scientific™ Orbitrap™ mass spectrometers. It covers the design of the experiment, sample preparation, method setup for data acquisition, and data analysis. Additionally, a case study using NIST SRM 1950 spiked with different concentrations of isotope-labeled amino acids will be demonstrated.

2. Design of SQUAD experiment

Proper design is critical for MS-based targeted metabolomics, as it greatly influences the number of metabolites measured and the reproducibility of results. Key aspects include optimizing sample preparation, which involves determining the appropriate concentration of internal standards (IS) to be added for absolute quantitation and selecting suitable extraction and cleanup procedures.

2.1 Design of SQUAD experiment

In SQUAD analysis, a predefined set of metabolites is quantified using calibration curves with both authentic and isotopically labeled standards. This enables precise measurement of metabolite quantities and supports accurate cross-laboratory comparisons. Quantifying metabolites can vary in accuracy depending on the method used.

The most precise approach involves absolute quantitation using authentic standards (STD), which are analyzed in a dilution series, along with IS, such as isotopically labeled compounds. Those labeled IS are typically added to the sample matrix, with calibration dilutions of the STD prepared at concentrations like those expected in the biological sample. The labeled IS facilitates ionization normalization by coeluting with the target compounds, which enhances measurement accuracy.

In contrast, using only calibration curves of STD provides a less accurate quantification. The least accurate method is a semi-quantitative approach employing a one-point calibration strategy, which is quick and straightforward but yields less reliable data. Many metabolites lack commercially available IS, whether isotopically labeled or unlabeled. As a result, researchers sometimes use a single IS for a metabolite or lipid class, which can compromise accuracy compared to truly quantitative methods. Nonetheless, these quantifications are generally more reliable and comparable between different laboratories and studies than untargeted metabolomics.

Accurate quantitative capability is a key strength of targeted metabolomics. Additionally, authentic chemical standards are essential for precise identification (level 1 identification) in targeted metabolomics, involving retention times, MS fragmentation patterns, peak shapes, and high-resolution accurate mass measurements of molecular ions.

Multiple reaction monitoring (MRM) and selected reaction monitoring (SRM) are commonly employed for quantitation in targeted metabolomics, typically using triple-quadrupole mass spectrometers. SRM focuses on a single fixed mass window, while MRM rapidly scans multiple narrow mass windows to acquire data on several fragment ion masses simultaneously. Parallel reaction monitoring (PRM) is another approach, analyzing all product ions from a precursor ion with high resolution and mass accuracy. Both MRM/SRM and PRM support relative and absolute quantification of metabolites. High-Resolution Accurate Mass (HRAM) spectrometry, although more common in untargeted metabolomics, is also increasingly used in targeted metabolomics due to advancements in Orbitrap technology, offering greater selectivity, improved scan speed, and enhanced sensitivity. HRAM allows for PRM with benefits such as better data confirmation through MS/MS, retroactive data analysis, and reduced method development.

Ultra-high resolution mass spectrometry (UHMS) is crucial for targeted metabolomics due to its precision and reliability. It offers exceptional resolution, enabling accurate separation and quantitation of metabolites with similar mass-to-charge (*m/z*) ratios (e.g., isobars), and high sensitivity for detecting low-abundance metabolites. UHMS ensures accurate mass measurements, minimizes spectral interferences, and enhances data reproducibility, leading to clearer data and consistent results. It supports comprehensive metabolite profiling and advanced detection techniques, providing detailed insights into metabolic pathways and aiding accurate metabolite identification. Overall, UHMS is essential for precise metabolic analysis and advancing biomarker discovery.

2.2 Untargeted discovery

Effective metabolite extraction should be tailored to the specific class of metabolites under investigation, as only a subset of all metabolites will be analyzed. The extraction method must be aligned with the physicochemical properties and relative abundances of the target metabolites, while avoiding the inclusion of macromolecules (e.g., proteins) and irrelevant metabolites. Parameters to consider include the choice between monophasic or biphasic liquid-liquid extraction, the volume and ratio of organic to aqueous solvents, and the pH and temperature conditions during extraction. Separation of analytes before MS detection is optimized based on their physicochemical properties, using techniques such as hydrophilic interaction liquid chromatography (HILIC), reversed-phase liquid chromatography (RPLC), ion chromatography (IC), or gas chromatography (GC) to minimize ion suppression effects. Within the mass spectrometer, different ion treatment methods are used. For targeted analysis, analyte fragmentation is often required to enhance specificity. Fragmentation can occur during ionization (in-source fragmentation) or by selecting ions for collision-induced fragmentation (tandem MS).

UHMS is pivotal for untargeted metabolomics due to its exceptional resolution and accuracy, which allow for the precise differentiation and identification of metabolites with similar *m/z* ratios (Figure 2A). This high resolution helps in distinguishing between closely related compounds and improves the accuracy of mass measurements. Additionally, UHMS enhances sensitivity, making it possible to detect low-abundance metabolites and identify them at lower concentrations, which is crucial for capturing a comprehensive metabolic profile. It also reduces ion suppression effects and spectral interferences, leading to more reliable and reproducible data (Figure 2B).

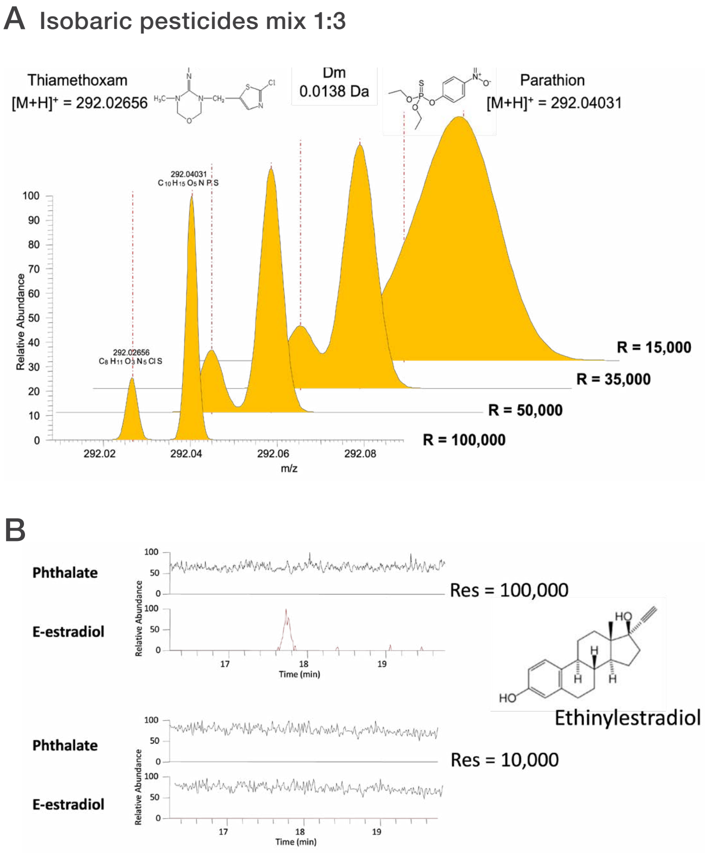
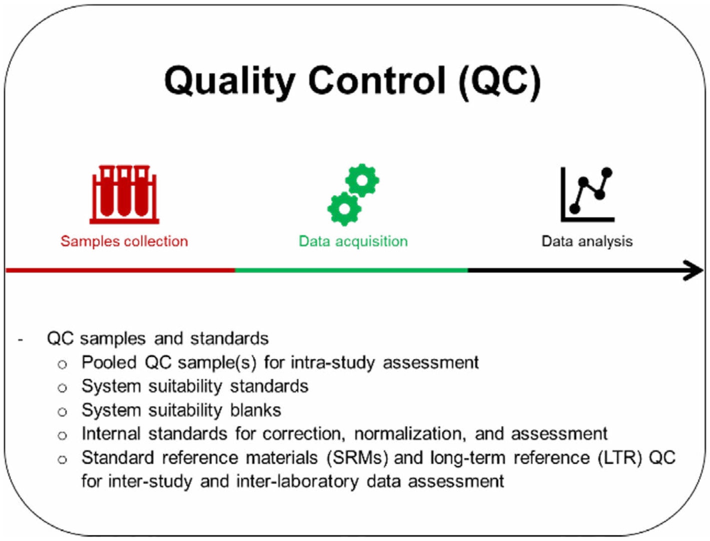


Figure 2. The role of UHMS in untargeted metabolomics. (A) High resolution and accuracy enable precise differentiation and identification of metabolites with similar *m/z* ratios. (B) Enhanced sensitivity improves the detection of low-abundance metabolites while reducing ion suppression effects and spectral interferences, ensuring more reliable and reproducible data.

With its ability to provide precise mass measurements and molecular formulas, UHMS facilitates advanced metabolite identification and annotation. This comprehensive profiling capability supports the discovery of novel biomarkers and aids in understanding metabolic changes, ultimately advancing diagnostic and therapeutic research.

2.3 Quality control

To ensure data reliability and confidence in metabolomics analysis, quality control (QC) measures must be integrated throughout the study (Figure 3). To learn more about QC samples in metabolomics, read Amer et al., technical note 001771 Quality assurance and quality control in metabolomics: achieving high-quality data for high-quality results. QC samples, based on reference standards, assess system accuracy and stability, and are typically included at regular intervals throughout the batch sequence to evaluate measurement robustness and identify potential drift or accuracy issues. Processing blanks are essential for identifying contamination, monitoring baseline noise, and checking the purity of reagents. QC samples, containing known concentrations of analytes, assess instrument performance, method reproducibility, and calibration accuracy. Together, these controls ensure the analysis is free from external contamination, the instrument performs consistently, and the quantification of target compounds is reliable.



- QC samples and standards
  - Pooled QC sample(s) for intra-study assessment
  - System suitability standards
  - System suitability blanks
  - Internal standards for correction, normalization, and assessment
  - Standard reference materials (SRMs) and long-term reference (LTR) QC for inter-study and inter-laboratory data assessment

Figure 3. Integration of QC measures in metabolomics to ensure data reliability and confidence.

In metabolomics, QC measures also include the use of pooled biological samples, which are aliquots from all samples in the study combined. These pooled samples help monitor the consistency of the analytical process and can be used for unknown annotation, such as collecting MS^2 data. Additionally, system suitability tests (SSTs) are performed before and during the analysis to ensure the instrument is functioning correctly. These tests include checking the mass accuracy, resolution, and sensitivity of the mass spectrometer (Figure 4).

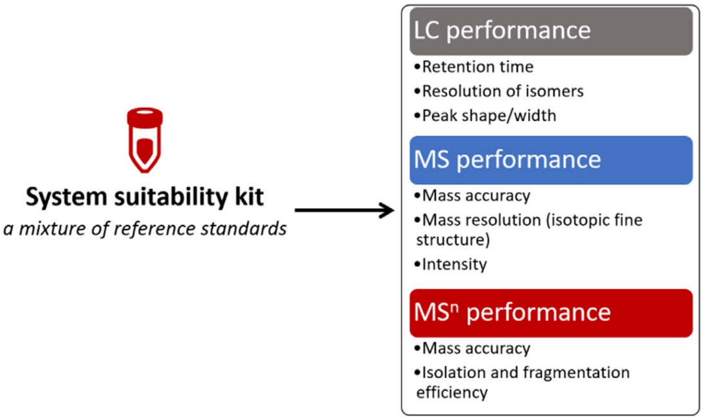


Figure 4. Role of pooled samples and SSTs in metabolomics. Pooled samples help monitor analytical consistency and aid in unknown annotation by collecting MS^2 data. SSTs, conducted before and during analysis, ensure proper instrument performance by assessing mass accuracy, resolution, and sensitivity.

Furthermore, IS, which are compounds that are not present in the biological sample, are spiked into each sample to monitor extraction efficiency, instrument performance, and data normalization. The use of isotopically labeled standards can further enhance the accuracy of quantification by compensating for matrix effects and variations in sample preparation and analysis.

Regular calibration of the mass spectrometer and chromatographic system is also crucial to maintain data quality. Calibration ensures that the instrument's measurements are accurate and reproducible. Additionally, the implementation of data quality checks, such as evaluating peak shapes, retention times, and signal-to-noise ratios, helps identify any anomalies or issues that may affect the results.

Overall, integrating comprehensive QC measures throughout the metabolomics workflow is essential for generating reliable, reproducible, and accurate data, ultimately leading to more robust and meaningful biological interpretations.

3. Sample preparation

Sample preparation in metabolomics workflows directly influences the accuracy, precision, and reliability of downstream analyses. It encompasses multiple stages designed to ensure optimal quantification of target metabolites and the generation of high-quality data. Key processes include the construction of calibration curves, the extraction of metabolites from biological samples, and the preparation of processing blanks and QC samples. Calibration curves, created using pure authentic chemical standards and isotope-labeled internal standards, are essential for achieving precise quantification of metabolites. Metabolite extraction leverages organic solvents to separate small molecules while precipitating proteins, with methods tailored to the sample matrix and target compound classes. Processing blanks and QC samples are indispensable for identifying contamination, assessing instrument performance, and monitoring analytical reproducibility. Together, these carefully orchestrated steps form the foundation of robust and reproducible metabolomics analyses.

3.1 Calibration curve

Pure authentic chemical standards and isotope-labeled IS are fundamental for constructing dilution series used in calibration curves for targeted metabolomics in general and for SQUAD analysis. These calibration curves are essential for quantifying metabolites with high accuracy and precision.

To begin, prepare a stock solution of the target compounds by dissolving the pure STD in an appropriate solvent. The choice of solvent depends on the polarity of the target compounds, ensuring their complete dissolution. Similarly, prepare a separate stock solution for the available IS compounds, using a suitable solvent that matches their polarity characteristics.

Once the stock solutions are ready, create the STD dilution series by mixing specific aliquots of the STD stock solution with a desired volume of solvent to achieve the required concentration range (Figure 5). This step ensures the calibration curve spans the expected concentration levels of the target analytes in your samples. For each dilution of the STD, add a fixed concentration of IS to normalize variability in sample preparation and instrument response.

Figure 5. Preparation of the STD dilution series to achieve the desired concentration range. Specific concentrations of the IS are mixed with each STD solution.

Mix each dilution thoroughly for one minute using a bench vortex to ensure homogeneity. Consistency in mixing is crucial for reliable data. To account for variability, prepare multiple replicates of the calibration dilutions; typically, orders to five replicates for each concentration. This repetition enhances the robustness of the calibration curve and improves statistical reliability.

By carefully following these steps, you can generate precise and reproducible calibration curves for quantifying metabolites in complex biological samples.

3.2 Analytical samples

Metabolite extraction from biological samples involves using organic solvents that simultaneously extract small molecules and precipitate proteins. The choice of extraction technique depends on the target compound classes and the complexity of the sample matrix. For plasma metabolites, 80% methanol is commonly employed due to its efficiency in extracting a broad range of metabolites while precipitating proteins.

If the biological samples are frozen, begin by thawing them at a low temperature, such as 4 °C, to preserve metabolite integrity. Once thawed, mix an aliquot of the sample with the IS solution, ensuring the IS concentration matches that used in the calibration standards. Add pure LC-MS-grade methanol to this mixture at a 2:8 ratio (sample to methanol). The use of high-purity solvents minimizes contamination and enhances reproducibility in downstream analyses.

Thoroughly mix the sample-methanol solution to ensure complete metabolite extraction. Centrifuge the mixture at a low temperature (e.g., 4 °C) to separate the supernatant, which contains the extracted metabolites, from the precipitated protein pellet. Proper centrifugation is critical to achieving a clean separation and avoiding carryover of proteins into the metabolite-rich phase.

To prepare the extracted metabolites for analysis, dry an aliquot of the supernatant under a gentle stream of nitrogen. This step removes the solvent without degrading the metabolites. Reconstitute the dried extract in a solvent compatible with the chromatography method used for analysis, ensuring the solvent choice aligns with the polarity and stability of the target metabolites. This careful preparation ensures optimal performance in LC-MS analysis, yielding reliable and reproducible data for metabolomics studies.

3.3 Processing blanks and QC samples

In metabolomics analysis, processing blanks and QC samples plays a critical role in ensuring the reliability, reproducibility, and accuracy of the generated data. These controls are indispensable for identifying potential sources of error, evaluating instrument performance, and maintaining the integrity of the analytical workflow.

Processing blanks are prepared by following the same extraction and sample preparation procedures used for biological samples but without adding the biological matrix. They serve multiple purposes, including identifying contamination from reagents, solvents, or laboratory equipment, monitoring baseline noise, and confirming the purity of reagents. Any unexpected peaks in the blanks can indicate contamination or carryover, which must be addressed to avoid false-positive results in sample analyses.

QC samples, on the other hand, are designed to assess the performance of the analytical method and instrument. These samples typically contain known concentrations of selected analytes, enabling the evaluation of method reproducibility, calibration accuracy, and instrument sensitivity. QC samples are essential for detecting deviations in performance over the course of a batch analysis and for ensuring data consistency across different runs or instruments.

Pooled samples, created by combining aliquots of multiple biological samples, are often used as QC samples. They represent an average metabolic profile and can help monitor batch-to-batch variability. Additionally, pooled samples are invaluable for unknown annotation, such as acquiring MS^2 data to support structural elucidation of unidentified metabolites.

To maximize the effectiveness of blanks and QC samples, include them strategically in the analytical sequence. For example, processing blanks should be analyzed at the beginning and end of each run to detect contamination or carryover, while QC samples should be interspersed at regular intervals throughout the run to monitor instrument drift and variability. Together, these controls ensure that the metabolomics data generated is accurate, reproducible, and free from external interference, ultimately enhancing confidence in downstream biological interpretations.

The Thermo Scientific™ Pierce™ Small Molecule System Suitability Standard (Part No. A51740) is a powerful QC tool designed to assess system performance in metabolomics workflows. This kit contains nine small-molecule standards spanning a mass range from *m/z* 76 to 1,279, making it suitable for evaluating both positive and negative ionization modes. When paired with chromatographic separation using the Thermo Scientific™ Hypersil GOLD™ C18 Selectivity HPLC Column (Part No. 25002-052130), the standard enables rapid analysis with a runtime of under 5.5 minutes. By providing a comprehensive assessment of system performance, the SST helps identify suboptimal conditions, enhances troubleshooting efficiency, and ensures standardized operation across different analytical setups. Regular use of this kit supports the generation of higher-quality, reproducible metabolomics data by confirming that the instrument is operating within optimal parameters, ultimately reducing variability and improving confidence in downstream analyses.

4. Data acquisition

The untargeted component of SQUAD analysis involves discovering new metabolites and understanding their roles within the biological system, all from a single sample injection. This method enhances the efficiency and depth of analysis by combining targeted and discovery approaches, providing a more comprehensive view of the metabolic landscape.

The SQUAD approach offers several advantages. It maximizes the use of limited sample volumes and resources, making it ideal for scenarios with sample constraints. It also improves the sensitivity and reliability of metabolic profiling by capturing both known and unknown metabolites. Innovations like the Thermo Scientific™ AcquireX™ intelligent data acquisition workflow further enhance the SQUAD analysis by automating data acquisition and integrating multiple experimental routines, thus increasing productivity and data quality.

4.1 Orbitrap Exploris mass spectrometers

SQUAD analysis on hybrid mass spectrometers leverages the combined capabilities of quadrupole and Thermo Scientific™ Orbitrap™ technologies for high-resolution MS1 analysis. This powerful approach supports both quantitation and untargeted metabolite discovery, enabling comprehensive profiling of the metabolome. The high-resolution Thermo Scientific™ Orbitrap™ analyzer ensures accurate mass measurements, while the quadrupole facilitates selective ion filtering for precise targeting of metabolites.

The faster scanning speeds of Thermo Scientific™ Orbitrap™ hybrid quadrupole mass spectrometers allow rapid polarity switching, even in high-throughput methods. This capability significantly expands metabolome coverage by efficiently alternating between positive and negative ionization modes, enabling the detection of a broader range of metabolites in a single analysis. The AcquireX workflow (Figure 6) further enhances this capability by increasing MS^2 fragmentation, which improves compound annotation and structural elucidation.

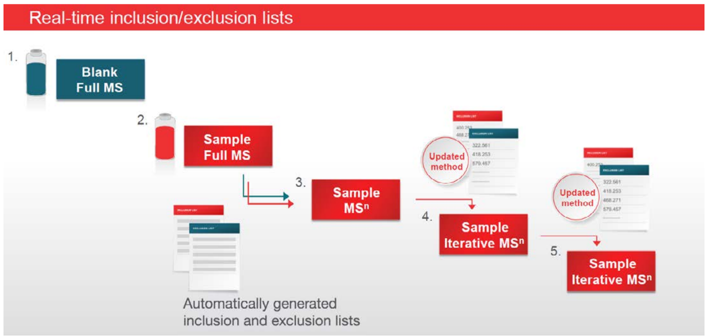


Figure 6. Enhanced metabolome coverage with the AcquireX workflow, which increases MS^2 fragmentation for improved compound annotation and structural elucidation.

Through the analysis of QC pools, the AcquireX workflow extends intelligence-driven mass spectrometry by integrating independent experiments into an automated workflow. This approach facilitates real-time, selective LC-MS^2 data acquisition, enabling efficient and comprehensive characterization of samples and studies. By connecting experimental data, the AcquireX workflow enhances the depth and specificity of metabolomics analysis while maintaining high efficiency.

An MS^1-based SQUAD method employing polarity switching is recommended on the Thermo Scientific™ Orbitrap™ Exploris™ mass spectrometer series. Additionally, AcquireX methods are applied to enhance MS^2 fragmentation, providing deeper coverage for untargeted analysis and enabling confident annotation of unknown compounds within SQUAD workflows. This integration of advanced mass spectrometry technologies ensures robust and detailed metabolomics investigations. SQUAD sensitivity and selectivity can be improved by adding a tMS^2 experiment for the targeted compounds. Similarly, a tSIM experiment can be added to the SQUAD method for improved sensitivity of the targeted compounds. To access example SQUAD method files, please create an account on the Thermo Fisher Scientific **AppsLab™ Library** and download them directly from there.

4.2 Orbitrap Tribrid mass spectrometers

SQUAD analysis on Orbitrap Exploris mass spectrometers and Thermo Scientific™ Orbitrap™ Tribrid™ mass spectrometers integrates quadrupole, Orbitrap, and linear ion trap technologies to deliver unparalleled analytical performance. This advanced setup enables high-resolution Orbitrap MS^1 analysis for discovery and sensitivity, rapid ion trap tMS^2 analysis for quantitation (Figure 7). The combination of these technologies supports both untargeted metabolite discovery and accurate quantitation, making Orbitrap Tribrid mass spectrometers ideal for comprehensive metabolomics studies. The integration of the AcquireX workflow further enhances the analysis by increasing MS^n fragmentation, leading to improved compound annotation and structural elucidation.

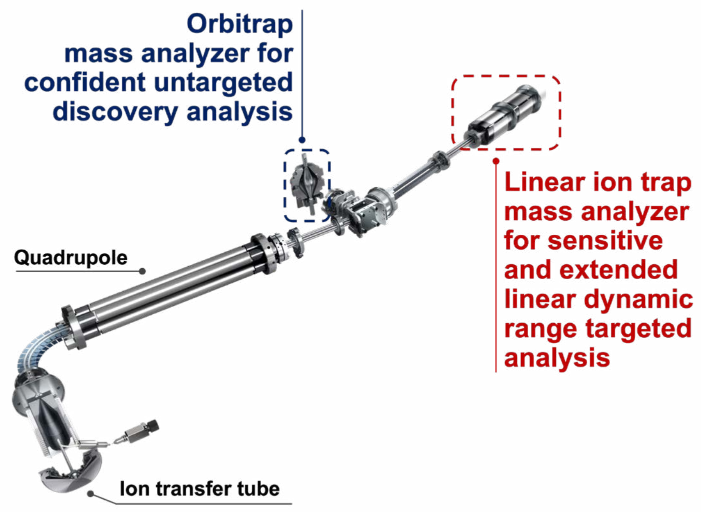


Figure 7. SQUAD analysis on Tribrid mass spectrometers. The integration of quadrupole, Orbitrap, and linear ion trap technologies enables high-resolution Orbitrap MS^1 analysis for discovery and sensitive, rapid ion trap tMS^2 analysis for quantitation.

The AcquireX workflow on Orbitrap Tribrid mass spectrometers utilizes multi-fragmentation methodologies, including higher-energy collisional dissociation (HCD), collision-induced dissociation (CID), and ultraviolet photodissociation (UVPD), to improve the confidence of unknown metabolite annotations. This capability allows for a more detailed and accurate characterization of complex metabolomic profiles. Additionally, Real-Time Library Search (RTLS) technology can be integrated into any method to guide the instrument toward collecting more relevant and informative data (Figure 8). RTLS facilitates on-the-fly spectral searching against either an offline Thermo Scientific™ mzCloud™ mass spectral library database or a local, customizable Thermo Scientific™ mzVault™ library, enabling decision-based triggering of MS^n scans. This filter provides targeted guidance during untargeted analyses, helping to maximize the utility of the acquired data for annotating known compounds and characterizing unknowns.

To use RTLS effectively, users must first create an MS^2 library. This can be achieved by analyzing data from pure analytical standards or utilizing the offline mzCloud mass spectral library. The MS^2 library is generated on an Orbitrap Tribrid mass spectrometer by employing a library creation template that incorporates multiple fragmentation techniques, such as HCD and CID, at various collision energies. Once the data is acquired, the Thermo Scientific™ Compound Discoverer™ Software deconvolutes the raw data and exports it as an MS^2 library in mzVault library software format. The mzVault software can then be used to curate and refine the library. Finally, this curated library is imported into the AcquireX method template on the Orbitrap Tribrid mass spectrometer, enabling advanced, guided analyses for both known and unknown metabolites.

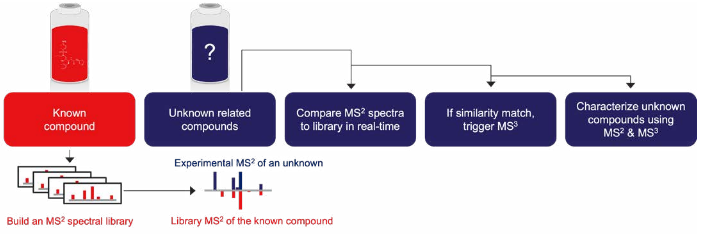


Figure 8. RTLS technology. RTLS enhances data acquisition by enabling on-the-fly spectral searching against an offline mzCloud database or a local mzVault library, allowing decision-based triggering of MS^n scans for more relevant and informative data collection.

Similar to SQUAD analysis on the Orbitrap Exploris mass spectrometer series, the AcquireX workflow can be employed on Orbitrap Tribrid mass spectrometers for deeper coverage and confident unknown annotation. This workflow requires separate MS^1 and ddMS^2 methods, each operating in a single polarity. The RTLS filter can be incorporated to limit additional data collection (such as ddMS^3) to scenarios where it is most beneficial, improving efficiency without sacrificing analytical depth. To access example SQUAD method files, please create an account on the AppsLab Library and download them directly from there.

4.3 Orbitrap Astral mass spectrometers

The Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer combines comprehensive HRAM Orbitrap MS1 analysis with rapid and sensitive HRAM data-dependent acquisition (DDA) MS^2 in the Thermo Scientific™ Astral™ analyzer (Figure 9). This innovative approach enables both untargeted and targeted metabolomics analysis in a single injection, eliminating the variability associated with iterative fragmentation of QC pooled samples for unknown annotation. By avoiding repeated sample preparation or injection, this method mitigates issues such as sample dilution, particularly in large-scale studies.

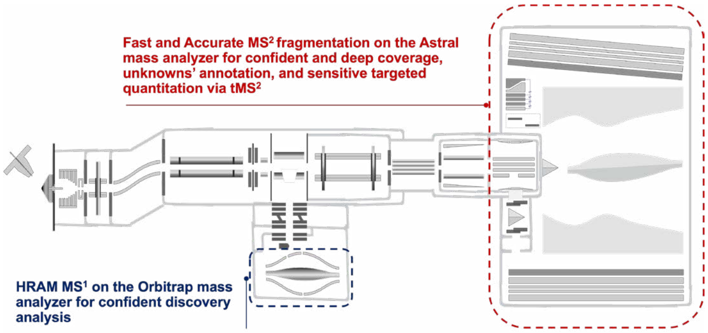


Figure 9. The Orbitrap Astral MS. This system integrates HRAM Orbitrap MS^1 analysis with rapid and sensitive HRAM DDA MS^2 in the Astral analyzer.

The Astral analyzer achieves a fragmentation efficiency of ≥90%, ensuring that even low-abundance compounds in complex biological matrices, such as plasma, are fragmented. This capability significantly enhances metabolite annotation rates, making it especially valuable for comprehensive metabolomics studies where detecting a broad range of metabolites is crucial.

To access example SQUAD method files, please create an account on the AppsLab Library and download them directly from there.

5. Data analysis

The goal of the targeted analysis component of SQUAD is to accurately quantify known compounds and precisely measure the differences in their levels between groups under study. This ensures a detailed understanding of specific metabolites' roles and variations, making it an essential aspect of focused metabolomics research.

In contrast, the untargeted analysis component of SQUAD aims to accurately identify and determine the elemental and isotopic composition of unknown metabolites in biological samples. This approach also seeks to map their metabolic pathways, which is crucial for interpreting metabolomics data and addressing complex biological questions. By uncovering these unknowns, untargeted analysis significantly broadens the scope of discovery in metabolomics studies. Consequently, SQUAD data analysis requires advanced statistical and bioinformatics tools for comprehensive evaluation and interpretation, such as differential analysis and reliable metabolite annotation.

To meet these challenges, we have developed specialized software platforms, Thermo Scientific™ TraceFinder™ Software and Compound Discoverer software. These tools are specifically designed to facilitate the interpretation of complex metabolomics datasets. They integrate data analytics with retro-mining processes and leverage spectral libraries and databases for compound identification, annotation, statistical analysis, and biological interpretation.

5.1 Targeted quantitation with TraceFinder software

TraceFinder software is tailored for targeted quantitation of metabolites, offering an efficient and streamlined workflow. This software enables quick access to results, boosting productivity across the laboratory. Key features like smart sample flagging, flexible data review, and custom report generation eliminate bottlenecks in data review and simplify sample analysis at every step. By standardizing operating procedures and reducing training needs, TraceFinder software makes lab operations easier and more efficient.

One of the standout features of TraceFinder software is its automated peak integration, which minimizes human input and reduces errors while ensuring consistent results. The software adapts to varying sample conditions, accurately identifying and quantifying peaks even in noisy or complex data. Robust calibration methods further enhance the accuracy of quantitation, ensuring reliable results across a wide concentration range.

TraceFinder software also supports comprehensive reporting by generating detailed summaries of peak detection and quantitation results. This facilitates easier data interpretation and compliance with regulatory standards. Visual tools within the software help confirm peak identity and integrity, enabling analysts to make informed decisions about data quality.

With its workflow-focused approach, TraceFinder software automates and accelerates method creation, sample loading, data generation, manual result review and editing, and the final data review and reporting process. This high-throughput quantitation system is ideal for targeted metabolomics workflows, helping researchers achieve accurate, reproducible results efficiently and effectively.

The TraceFinder software data analysis workflow consists of three major steps:

1. **Building a quantitative processing method**
   This step involves creating a Compound Database containing the metabolites of interest for quantitation. From this database, a **Master Method** is constructed, which includes detailed workflow parameters such as the quantitation type (e.g., MS^1- or tMS^2-based), calibration curve levels, calibration methods, peak detection and integration algorithms, peak quality settings, and optional matching against an MS^2 library for enhanced confidence. These settings provide a robust foundation for accurate and consistent data analysis. A compound database could be built by using the TraceFinder software wizard by clicking the **Method Development** tab, then **Compound Database**, or by importing a compound list available in .csv format (Figure 10).

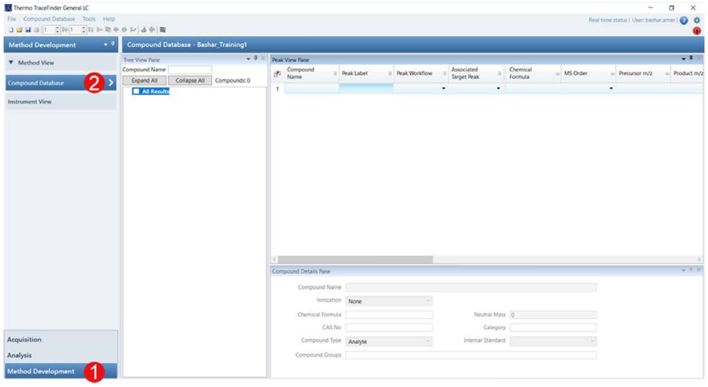
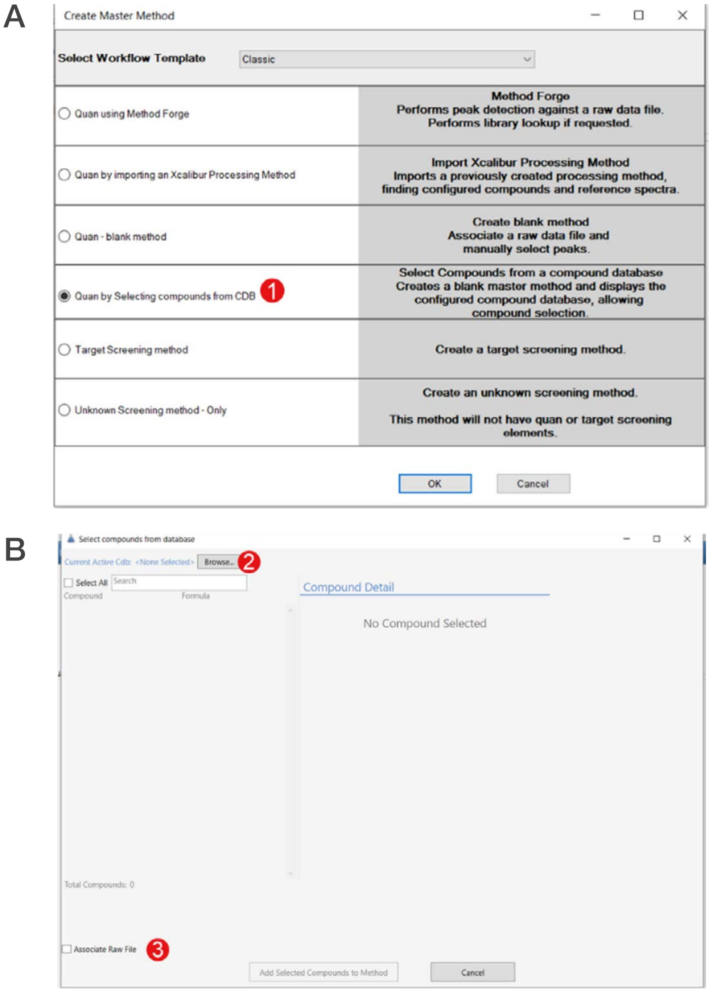


Figure 10. Building a compound database using the TraceFinder software wizard.

Figure 11A shows the option of using a **Compound Database** to build a **Master Method** in the TraceFinder software. A raw data file, typically an STD sample, can also be associated with the **Master Method** (Figure 11B) for cross-validation of the parameters while setting and updating them before the analysis of the whole analytical sample.



Select Workflow Template: Classic

○ Quan using Method Forge	Method Forge Performs peak detection against a raw data file. Performs library lookup if requested.
○ Quan by importing an Xcalibur Processing Method	Import Xcalibur Processing Method Imports a previously created processing method, finding configured compounds and reference spectra.
○ Quan - blank method	Create blank method Associate a raw data file and manually select peaks.
● Quan by Selecting compounds from CDB ①	Select Compounds from a compound database Creates a blank master method and displays the configured compound database, allowing compound selection.
○ Target Screening method	Create a target screening method.
○ Unknown Screening method - Only	Create an unknown screening method. This method will not have quan or target screening elements.

OK Cancel

Current Active Cdb: <None Selected>

Figure 11. Master Method options. (A) Using a **Compound Database** to build a **Master Method** in TraceFinder software. (B) Associating a raw data file, typically from an STD sample, with the **Master Method** for cross-validation of parameters before analyzing the full set of analytical samples.

2. **Building and processing an analytical batch:**
The analytical batch is constructed using the previously developed **Master Method**. This is done by clicking on the **Analysis** tab, selecting **File** and **New Batch** in the TraceFinder software wizard (Figure 12).

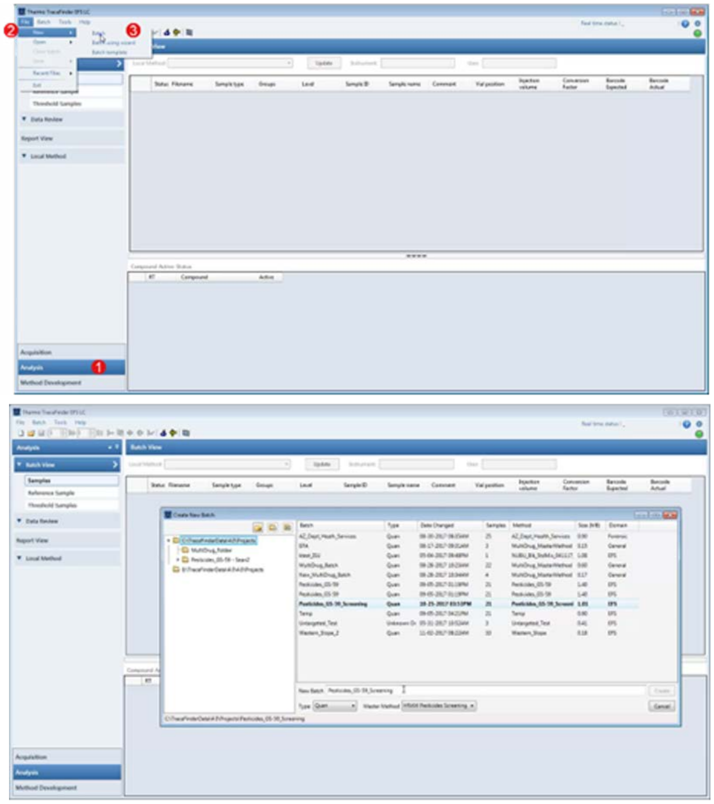


Figure 12. Constructing an analytical batch using the Master Method in TraceFinder software. This is done by selecting the **Analysis** tab, then choosing **File** and **New Batch** in the TraceFinder software wizard.

During this step, raw data files are imported, and sample types are assigned (e.g., matrix blank, solvent, calibration standards, unknown samples, quality control samples). The software processes the batch based on the predefined method, ensuring a streamlined analysis workflow.

3. **Reviewing and reporting results**
In the final step, users review the results to fine-tune settings such as peak integration if necessary. TraceFinder software offers flexible reporting options, allowing users to customize the output format to meet specific requirements. This step ensures that results are accurate, comprehensive, and presented in a format suitable for further interpretation or regulatory compliance.

The TraceFinder software user guide provides a step-by-step manual to create a study and process your SQUAD data for an intelligent targeted analysis using TraceFinder software

5.2 Untargeted discovery with Compound Discoverer software

Compound Discoverer software streamlines compound identification and comparative analyses, providing extensive filtering and data visualization capabilities in easy-to-use, powerful workflows. These workflows are designed to drive rapid insights from valuable SQUAD data. The software enhances certainty with multi-factorial peak quality scoring for confident detection, identification, and quantification. It identifies elemental composition through isotopic fine structure analysis of full-scan HRAM data and boosts identification confidence with automated MS^n tree searches in the mzCloud mass spectral library.

The software automatically identifies compounds by searching spectral libraries using integrated identity and similarity search modes. It annotates spectra with predicted fragmentation and queries chemical databases for potential candidates, ranking them by spectral properties to speed up compound identification.

In addition, Compound Discoverer software facilitates differential analysis using univariate t-tests, ANOVA, multiple hypothesis testing with adjusted p-values, and fold-change analyses. Users can delve deeper with multivariate analyses, including HCA, PCA, PLS-DA, and visual plots. Statistical results can be visualized with volcano plots, box-and-whisker plots, and heatmaps. Trend charts allow visualization of peak areas, average peak areas per group, or fractional label incorporation over time.

Compound Discoverer software enhances data processing through customizable workflows. Workflow nodes (Figure 13) can be easily dragged and dropped from a wide range of processing tools into workflow trees to create the desired data flows. Workflows can be saved as templates, and numerous pre-made templates exist for easier setup. The **Study and Analysis Wizard** allows for quick initiation; users simply add their data, define their study and sample groups, select a workflow template, and start processing. Additionally, users can save time by re-running only part of a workflow if necessary.

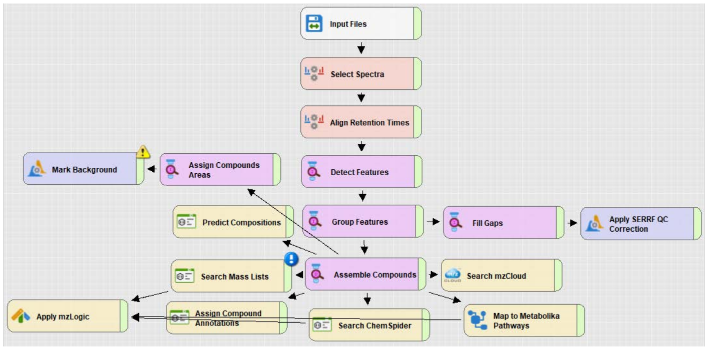
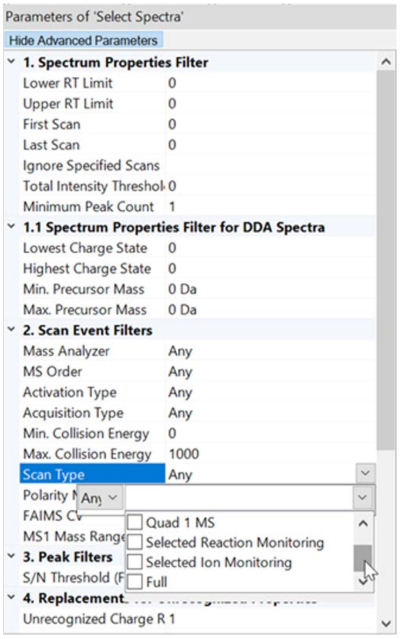


Figure 13. Compound Discoverer software workflow nodes. These customizable nodes enhance data processing by allowing tailored workflows for metabolomics analysis.

Finally, mzCloud mass spectral library is a high-resolution and accurate-mass MS^n fragmentation library used to identify unknown compounds when reference standards are unavailable. It provides confident identification of those compounds, even in challenging scenarios.

A Compound Discoverer software **tutorial** provides a step-by-step guide to create a study and process SQUAD data for an intelligent untargeted analysis using Compound Discoverer software. The tutorial provides default values for each node parameter as a starting point. This includes value descriptions and some hints; however, the users can adjust those values based on their needs and the type of data. One important point to mention is to select **Full** as the **Scan Type** under the **Select Spectra** node (Figure 14) in case you run a SQUAD method with multiple experiments to secure using the MS^1 data and not using the SIM or tMS^2 data.



1. Spectrum Properties Filter
Lower RT Limit 0
Upper RT Limit 0
First Scan 0
Last Scan 0
Ignore Specified Scans
Total Intensity Threshol 0
Minimum Peak Count 1

1.1 Spectrum Properties Filter for DDA Spectra
Lowest Charge State 0
Highest Charge State 0
Min. Precursor Mass 0 Da
Max. Precursor Mass 0 Da

2. Scan Event Filters
Mass Analyzer Any
MS Order Any
Activation Type Any
Acquisition Type Any
Min. Collision Energy 0
Max. Collision Energy 1000
Scan Type Any
Polarity M Any
FAIMS CV
MS1 Mass Range
☐ Quad 1 MS
☐ Selected Reaction Monitoring
☐ Selected Ion Monitoring
☐ Full

3. Peak Filters
S/N Threshold (F

4. Replacement
Unrecognized Charge R 1

Figure 14. Selecting Full **as the** Scan Type **under the** Select Spectra **node**. This ensures the use of MS^1 data and prevents the use of SIM or tMS^2 data when running a SQUAD method with multiple experiments.

6. Case study

This section details the SQUAD analysis of NIST SRM 1950 plasma, which was spiked with varying concentrations of isotope-labeled amino acids and organic acids. The primary objective of this study was twofold: first, to achieve targeted quantification of the spiked isotope-labeled compounds, and second, to conduct untargeted metabolic profiling of the intrinsic composition of the NIST plasma. Both objectives were pursued using a single-injection SQUAD analysis on several Orbitrap mass spectrometers.

The NIST SRM 1950 plasma samples were spiked with labeled compounds across a concentration range spanning from 1 femtomole to 2.5 millimoles. These spiked plasma samples were subsequently analyzed using the SQUAD methodology on a Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer, a Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ Mass Spectrometer, and an Orbitrap Astral mass spectrometer. The experimental procedures employed for data acquisition and analysis adhered to the methods outlined in the respective sections.

6.1. Materials and methods

Sample preparation

The NIST SRM 1950 plasma sample and isotope-labeled amino acids and organic acids were obtained from Sigma-Aldrich and Cambridge Isotope Laboratories (CIL), respectively. A dilution series of isotope-labeled compounds, ranging from 1 femtomole to 2.5 millimoles, was prepared and spiked into the plasma to facilitate quantitation against the corresponding endogenous metabolites. Plasma samples were extracted using methanol (80% v/v), ensuring efficient protein precipitation and metabolite recovery.

Chromatographic separation

The chromatographic analysis was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC System equipped with an autosampler set to 5 °C. The separation was achieved on a Hypersil GOLD C18 selectivity HPLC column (2.1 x 150 mm, 1.9 µm) maintained at a column temperature of 45 °C.

The mobile phase consisted of (A) 0.1% (v/v) formic acid (FA) in LC-MS grade water and (B) 0.1% (v/v) FA in LC-MS grade methanol. A total injection volume of 2 µL was used for all samples. The flow rate was set at 0.30 mL/min, and the following gradient elution program was applied:

Time (min)	%A	%B
0.00	100	0
8.00	50	50
9.00	2	98
13.00	2	98
13.10	100	0
15.00	100	0

Mass spectrometry

The MS methods described in sections 4.1, 4.2, and 4.3 were applied on an Orbitrap Exploris 240 mass spectrometer, an Orbitrap IQ-X Tribrid mass spectrometer, and an Orbitrap Astral mass spectrometer, respectively.

6.2 Data analysis

TraceFinder software version 5.1 and Compound Discoverer software version 3.3, utilizing the mzCloud mass spectral library database, were used for data processing, analyte quantitation, and unknown annotation as described in sections 5.1 and 5.2.

6.3 Results

SQUAD analysis on Orbitrap Exploris 240 mass spectrometer

The SQUAD analysis conducted on hybrid mass spectrometers, combining quadrupole and Orbitrap technologies, integrates high-resolution MS^1 analysis for both quantitation and untargeted discovery. Incorporating authentic standards and internal standards enables highly sensitive absolute quantitation of plasma metabolites with a broad linear dynamic range. High-resolution Orbitrap mass spectrometers (>60,000 resolution) resolve low-abundance metabolites in complex matrices, while advancements in scanning speed allow fast polarity switching (Figure 1), enabling wider metabolome coverage and compatibility with high-throughput LC and GC methods.

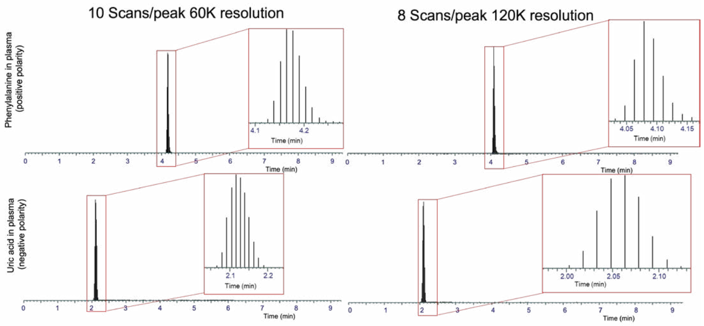


Figure 15. High-resolution Orbitrap mass spectrometers (>60K resolution) enable the detection of low-abundance metabolites in complex matrices. Enhanced scanning speed and fast polarity switching expand metabolome coverage and improve compatibility with high-throughput methods.

Orbitrap MS1-based quantitation demonstrates exceptional sensitivity, detecting low limits of quantification (LLOQ) down to 50 femtomoles and low limits of detection (LLOD) at 25 femtomoles, achieving a dynamic range of five orders of magnitude. For enhanced sensitivity, the Orbitrap Exploris mass spectrometer series offers tSIM or tMS^2 experiments, balancing targeted sensitivity and untargeted discovery flexibility. The SQUAD approach exemplifies adaptable experimental designs to meet diverse scientific needs effectively. Finally, the deep scan AcquireX workflow enhances structural annotation through extensive MS^2 fragmentation, increasing metabolome coverage in single injections for both polarities. This method supports high-throughput profiling while preserving targeted and untargeted analysis integrity.

SQUAD analysis on an Orbitrap IQ-X mass spectrometer

Advancements in Orbitrap Tribrid mass spectrometers enable SQUAD analysis by combining the sensitive linear ion trap for metabolite quantitation with untargeted discovery on the HRAM Orbitrap analyzer. This approach minimizes variability by eliminating the need for multiple instruments or re-injecting limited biological samples.

The linear ion trap demonstrates exceptional sensitivity, detecting LLOQ as low as 5 femtomoles and LLOD at 0.5 femtomoles, with a dynamic range spanning six orders of magnitude. Similar to hybrid mass spectrometers, the AcquireX workflow is used for deep fragmentation analysis with QC pools, ~80% coverage (Figure 16), ensuring high throughput while maintaining the integrity of targeted and untargeted analyses.

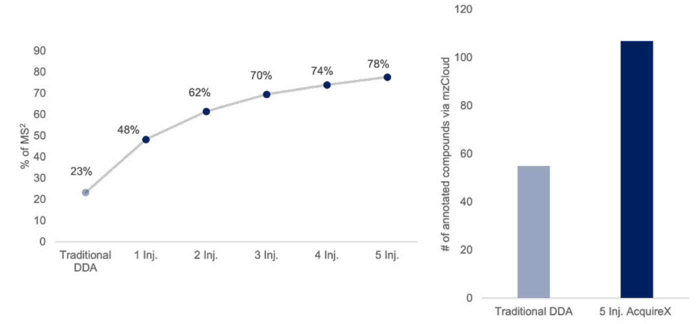


Figure 16. The AcquireX workflow for deep fragmentation analysis with QC pools, achieving ~80% coverage.

SQUAD analysis on an Orbitrap Astral mass spectrometer

The advanced SQUAD metabolomics approach on the Orbitrap Astral mass spectrometer offers a streamlined workflow, enhanced efficiency, and precise results. The Astral analyzer enables comprehensive HRAM, rapid and sensitive DDA MS^2, supporting confident untargeted and targeted analyses in a single injection. This eliminates variability associated with iterative QC sample fragmentation and achieves a higher compound fragmentation rate (~90%) compared to traditional Orbitrap-based DDA methods. This improvement facilitates the fragmentation and annotation of low-abundance metabolites in complex matrices like plasma, in addition to sensitive tMS^2-based quantitation.

The Astral analyzer provides exceptional sensitivity, achieving an LLOQ of 1 femtomoles and an LLOD of 5 attomoles of phenylalanine in plasma while maintaining an impressive dynamic range of six orders of magnitude, and ensures sufficient data points across peaks for precise quantitation.

The Orbitrap Astral MS also supports significantly reduced LC gradient durations, up to threefold shorter, without compromising MS^1 scan coverage, signal-to-noise ratio, or MS^2 fragmentation quality. This capability enhances throughput and coverage, detecting 25% more compounds with MS^2 in a 5-minute gradient compared to a 15-minute Orbitrap-Orbitrap gradient (Figure 17). These advancements result in improved peak quality, increased detection of annotated unknowns, and extensive metabolome coverage, making the Orbitrap Astral mass spectrometer an excellent choice for high-throughput SQUAD analysis.

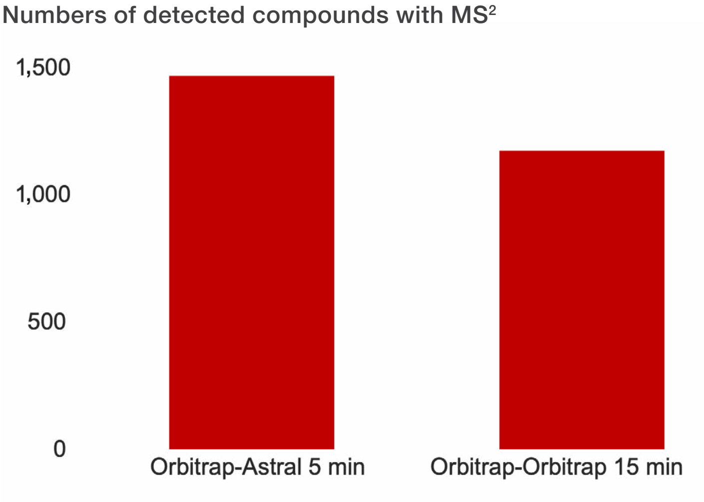


Figure 17. The Orbitrap Astral MS reduces LC gradient durations by up to threefold, enhancing throughput and detecting 25% more compounds with MS^2 in a 5-minute gradient compared to a 15-minute Orbitrap-Orbitrap gradient.

6.4 Conclusion

The innovative SQUAD analysis metabolomics technique represents a groundbreaking approach, enabling the seamless integration of untargeted and targeted methodologies within a single experiment. This dual-purpose strategy redefines how laboratories conduct metabolomics research, facilitating the simultaneous annotation and quantification of pre-selected metabolites while allowing retrospective data analysis to uncover broader metabolic changes. By generating both hypothesis-driven and discovery-focused datasets in a single experiment, researchers can gain comprehensive insights into biological systems.

Traditionally, the need to choose between untargeted and targeted metabolomics has constrained the potential of metabolomics in life sciences research. SQUAD analysis resolves this limitation by merging the strengths of both approaches, unlocking new opportunities for scientific discovery. Designed for Orbitrap mass spectrometers, SQUAD workflows leverage the unique capabilities of these instruments to enhance performance. The fast polarity switching feature of the Orbitrap Exploris mass spectrometer series broadens metabolite coverage, while Orbitrap Tribrid mass spectrometers, equipped with dual detectors operating in parallel, balance targeted and untargeted analyses with extended sensitivity and linear dynamic ranges using ion trap quantitation.

The Orbitrap Astral mass spectrometer further elevates SQUAD workflows by incorporating a high-speed second HRAM detector, enabling high-throughput analysis and optimizing workflow efficiency. Together, these advancements ensure that SQUAD analysis maximizes the potential of metabolomics for scientists worldwide, offering unparalleled precision, flexibility, and depth of analysis.

Learn more at **thermofisher.com/squad**