Metabolomics via SQUAD

Simultaneous quantitation and discovery (SQUAD) metabolomics workflow on the Orbitrap IQ-X for the analysis of fecal bile acids

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Abstract

Purpose: Develop and implement a comprehensive metabolomics workflow for the simultaneous quantitation and discovery (SQUAD) of fecal bile acids (BAs) and BA conjugates to quantify a selected list of primary and secondary BAs and their conjugates in fecal samples from mice subjected to different diets.

Methods: Thermo Scientific™ Vanquish™ Horizon LC and Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer were used to develop a SQUAD workflow. Additionally, the workflow incorporates AcquireX™ and Real-Time Library Search (RTLS) to enhance confidence in annotating relevant unknowns, thereby enabling the detection and annotation of BA-related metabolites that may be implicated in pathophysiological conditions due to microbiome perturbations.

Results: Calibration curves were generated using both unlabeled and labeled BA standards to achieve absolute quantitation. This approach enables accurate quantification using an ion trap across a broad dynamic range, spanning five orders of magnitude, for the targeted BAs in feces. Most of the targets exhibited a lower limit of quantification (LLOQ) of 5 pg on-column. In the mice dietary intervention study, the method successfully provided absolute quantification of the targeted BAs and their conjugates in fecal samples. Additionally, untargeted analysis was employed to detect and annotate relevant compounds in the different experimental groups.

Introduction

BAs are synthesized from cholesterol in the liver and are integral to lipid digestion and absorption. In the gastrointestinal tract, fecal bile acids serve as critical biomarkers and signaling molecules, interacting intricately with the gut microbiota. Perturbations in the gut microbiome can modify the composition and volume of the bile acid pool, resulting in the generation of various conjugated bile acids and structurally analogous metabolites, which may be implicated in pathophysiological conditions.

This study presents a metabolomics workflow for the SQUAD (Figure 1A), of BA standards and their conjugates (Cambridge Isotope Laboratories, Inc.), utilizing the Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer. The workflow incorporates RTLS to assess spectral similarity, thereby enhancing the confidence in relevant unknowns' annotation during method execution.

The developed SQUAD method was employed to quantify a selected list of primary and secondary BAs and BA conjugates in fecal samples collected from 8-week-old mice subjected to different diets with varying fat sources for 29 days (Figure 1B). Additionally, the method utilized an untargeted discovery approach to detect and annotate BA-related unknowns in the fecal samples.

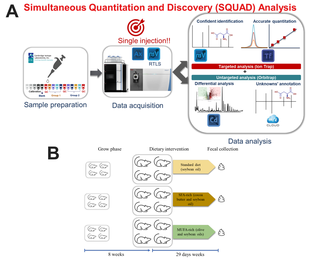

Figure 1. The developed SQUAD of fecal BAs and BA conjugates to quantify a selected list of primary and secondary BAs and their conjugates in fecal samples from mice subjected to different diets.

Materials and methods

Sample preparation

Unlabeled and stable-isotope-labeled BA standards were acquired from CIL. These standards were then utilized to prepare dilution series solutions in a 50% methanol, which were used to create calibration curves for the quantitation of BAs in fecal samples collected from 8-week-old mice. These mice were subjected to different diets with varying fat sources for a duration of 29 days; standard diet (soybean oil), saturated fatty acids (SFA)-rich diet (cocoa butter and soybean oil), and monounsaturated fatty acids (MUFA)-rich diet (olive and soybean oils). Before analysis, metabolites were extracted using 80% methanol. Extracts were dried down and reconstituted in 50% methanol solution containing BA-labeled internal standards (IS).

The BA standards also played a crucial role in constructing an MS^2 spectral library. This library facilitated intuitive MS^3 fragmentation via the RTLS workflow. Additionally, aside from their use in generating calibration curves, the labeled BAs served as quality control measures. Their retention time and peak area robustness and reproducibility were measured in the various samples, ensuring the reliability and consistency of the analysis.

Test method(s)

The development of a SQUAD workflow involved the utilization of the Thermo Scientific Vanquish Horizon LC and Orbitrap IQ-X Tribrid mass spectrometer. A Thermo Scientific™ Hypersil GOLD C18 reversed-phase column was used for the separation of BAs and other small molecules extracted from the fecal samples (Figure 2). To further enhance confidence in annotating relevant unknowns, the workflow incorporates AcquireX and RTLS features.

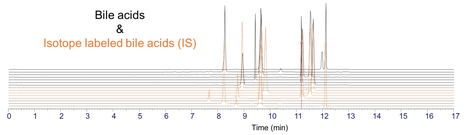

Figure 2. Chromatographic separation of BAs and isotope-labeled BAs from fecal samples using a Thermo Scientific Hypersil GOLD C18 reversed-phase column.

Data analysis

Thermo Scientific™ TraceFinder™ 5.1 and Thermo Scientific™ Compound Discoverer™ 3.4 software were used for data processing, analyte quantitation, and unknown annotation.

Results

SQUAD method

By assessing the performance of isotopically labeled bile acids over time using metric tracking (such as retention time and signal response), the developed SQUAD method showcased excellent data quality, reliability, and robustness of measurement. The IS exhibited minimal chromatographic shift and consistent signal responses, as indicated by % CVs below 10% across analyzed samples (Figure 3). These stable metrics provide strong assurances regarding the quality of data obtained from the targeted and untargeted MS-based workflow.

To achieve absolute quantitation, calibration curves were created utilizing both unlabeled and labeled BA standards. This methodology allows for precise quantification using an ion trap instrument over a wide dynamic range, encompassing five orders of magnitude, specifically targeting BAs in fecal samples. The majority of the targets demonstrated an LLOQ of 5 pg on-column (Figure 4).

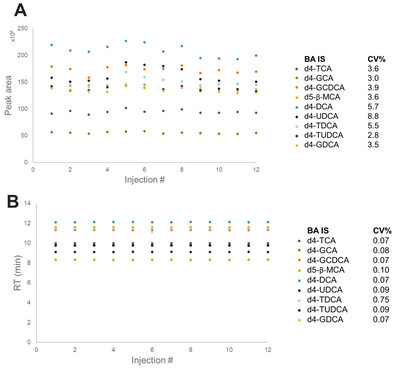

Figure 3. QC evaluation of stable isotope-labeled BAs over time using peak area (A) and retention time (B), demonstrating the excellent data quality, reliability, and robustness of measurement achieved by the developed SQUAD method.

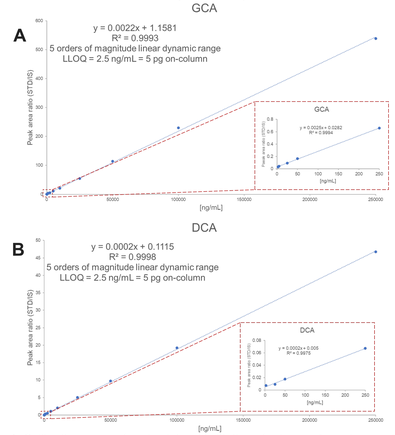

Figure 4. Calibration curves for absolute quantitation of primary (A) and secondary (B) BAs using both unlabeled and labeled BA standards. This approach enables precise quantification across a wide dynamic range, spanning five orders of magnitude, utilizing an ion trap instrument. The majority of the targets exhibit an LLOQ of 5 pg on-column.

Bile Acids Quantitation

The developed SQUAD method was employed to quantify a selection of primary and secondary bile acids in fecal samples obtained from a dietary-based intervention study conducted on mice. The results reveal distinct variations in the levels of these bile acids across the different experimental groups, as depicted in Figure 5.

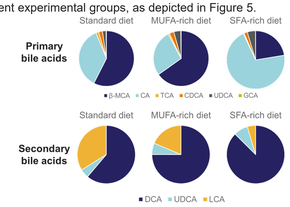

Figure 5. Quantification of primary and secondary bile acids in fecal samples from a dietary-based intervention study in mice using the developed SQUAD method. The figure illustrates notable variations in the levels of these bile acids among the different experimental groups.

Untargeted Discovery

Figure 6 displays the results of the untargeted discovery analysis within the SQUAD workflow, showcasing variations in the fecal metabolome among the samples from different dietary groups. This is evidenced by the PCA scores (Figure 6A) and loadings heat map plots (Figure 6B). Further data analysis highlights distinct patterns and differences among the groups as shown in Figure 6C, which presents a clear variation in the level of annotated fatty acids among the different groups.

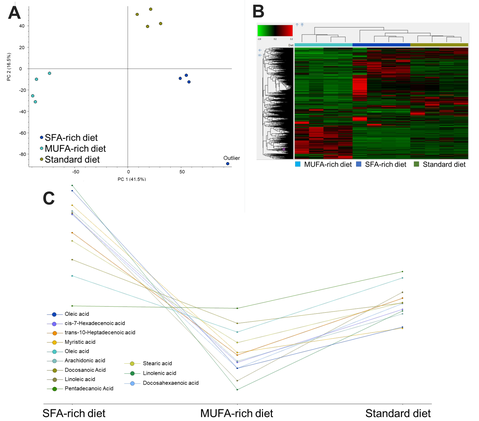

Figure 6. PCA scores (A) and loadings heat map (B) plots highlight distinct patterns and differences among the groups, which are further revealed in (C), providing valuable insights into the metabolic profiles.

Moreover, the RTLS workflow empowered the SQUAD method to selectively initiate MS^3 fragmentation of unknown compounds that exhibit MS^2 fragmentation similarities to BAs. Figure 7 demonstrates the selective triggering of a DCA-like compound for MS^3 fragmentation, enabling subsequent structural elucidation.

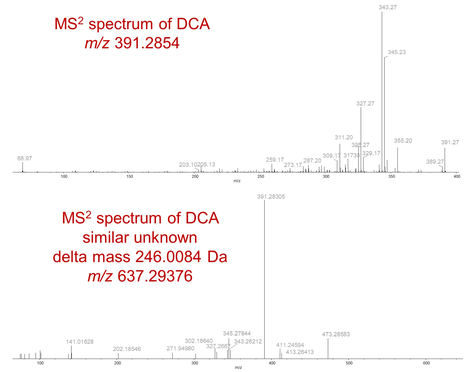

Figure 7. An example of a selected compound for MS^3 fragmentation using the RTLS workflow within the SQUAD method.

Conclusions

We have successfully developed the SQUAD workflow for simultaneous quantitation and discovery of fecal BAs and BA conjugates. This comprehensive metabolomics approach utilizes advanced instrumentation, including the Thermo Scientific Vanquish Horizon LC and Orbitrap IQ-X Tribrid mass spectrometer, and isotopically labeled standards, to achieve accurate quantification and confident annotation of unknowns. Incorporating AcquireX and RTLS enhances the detection and annotation of BA-related metabolites, potentially relevant to pathophysiological conditions resulting from microbiome perturbations.

Acknowledgments

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Trademarks/licensing

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