Metabolomics and Research

Simultaneous quantitation and discovery (SQUAD): an intelligent combination of targeted and untargeted metabolomics workflow for the analysis of fecal metabolites using Orbitrap Astral MS

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Abstract

Purpose: Develop and implement a comprehensive metabolomics workflow for the simultaneous quantitation and discovery (SQUAD) to quantify a selected list of metabolites in fecal samples from mice subjected to different diets in parallel to an untargeted evaluation of the metabolic profiles of those samples.

Methods: Thermo Scientific™ Vanquish™ Horizon LC and Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer were used to develop a SQUAD workflow for the analysis of fecal metabolites (Figure 1). The workflow parallelizes the acquisition of high-resolution accurate mass full scans with high dynamic range using the Orbitrap analyzer and fast and sensitive high-resolution accurate mass MS^2 scans with the Astral analyzer.

Results: Calibration curves were established using pure authentic standards of fecal metabolites to achieve absolute quantitation. This approach facilitates precise quantification through Astral-sensitive tMS2 data acquisition. In a mice dietary intervention study, the method effectively provided absolute quantification of the targeted metabolites in fecal samples. Additionally, untargeted analysis was utilized to detect and annotate relevant compounds across the different experimental groups.

Introduction

Recent research highlights the crucial role of the gut microbiome in gastrointestinal health and disease, driving interest in fecal metabolomics for its potential in clinical diagnostics. This link emphasizes the diagnostic value of metabolomic insights.

Discovery fecal metabolomics using mass spectrometry aims to explore the metabolic diversity of samples, identifying a wide range of molecules and potentially uncovering new compounds. While this approach can enhance clinical diagnostics by linking the microbiome and health, challenges like the speed, sensitivity, and accuracy of mass spectrometers can hinder the quality of MS^2 spectra and reduce annotation confidence.

Targeted metabolomics, on the other hand, identify and quantify various fecal metabolites, including amino acids, fatty acids, lipids, and bile acids. However, traditional MS approaches often face limitations in speed and selectivity, which can restrict the number of metabolites analyzed.

To address these issues, a SQUAD fecal metabolomics approach was established using the Orbitrap Astral mass spectrometer (Figure 1A), which combines faster and more sensitive Astral MS^2 scanning with high-resolution accurate mass full scan Orbitrap detection for parallel sensitive quantitation and deep coverage discovery analysis.

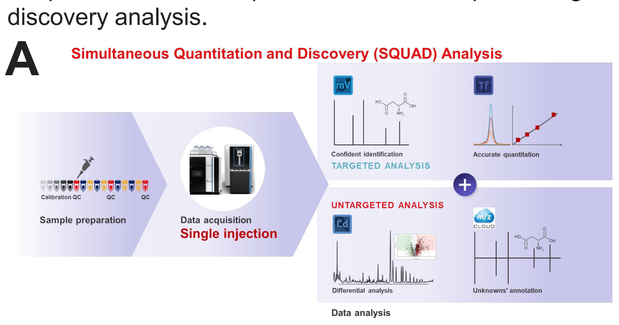


Figure 1. (A) SQUAD workflow was developed to quantify a selected list of fecal metabolites in parallel to untargeted analysis of the extracted metabolites in (B) fecal samples from mice subjected to different diets.

Materials and methods

Sample Preparation

Fecal metabolite standards were acquired from MetaSci. These standards were then utilized to prepare dilution series solutions in a 50% acetonitrile, which were used to create calibration curves for quantitation 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% acetonitrile solution containing.

Test Method(s)

The development of a SQUAD workflow involved the utilization of the Thermo Scientific Vanquish Horizon LC and Orbitrap Astral mass spectrometer. A Thermo Scientific™ Accucore™-150-Amide-HILIC column was used for the separation of the analytes and other small molecules extracted from the fecal samples. Data acquisition was performed via both positive and negative ionization; +ESI and –ESI.

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

To achieve absolute quantitation, calibration curves were created utilizing chemical standards. This methodology allows for precise quantification using the Astral mass analyzer over a wide linear dynamic range (4 orders of magnitude). The majority of the targets demonstrated a high sensitivity down to LLOQ of less than 3 femtomole on column (Figure 2).

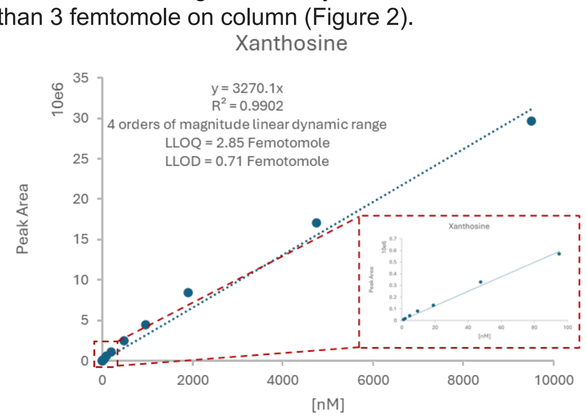


Figure 2. Calibration curves for absolute quantitation of Xanthosine as an example of the targeted fecal analytes. This approach enables precise quantification across a wide dynamic range, spanning four orders of magnitude, utilizing the Astral analyzer for tMS2 quantitation. The developed SQUAD method showed an LLOQ and an LLOD of 2.85 and 0.71 femtomoles on the column, respectively.

Fecal Metabolites Quantitation

The developed SQUAD method was employed to quantify a selection of metabolites 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 3.

Compound [nM]	SFA-rich diet	MUFA-rich diet	Standard diet
Phenylalanine	2297	3631	2339
Proline	13425	13533	5651
Tyrosine	18907	19809	12875
Carnitine	31	59	57
Kynurenic acid	108	90	166
Creatinine	4190	3602	3799
Choline	2398	1521	1962
Urocanic acid	1101	441	842
Taurine	93137	45160	31245
Nicotinic acid	6376	6109	3347
N-Acetylserotonin	285	381	92
N-a-Acetyl-L-arginine	183	58	47
Alpha-Aminobutyric acid	2388	1499	1947
Inosine	3009	2115	1685
Cytidine	2220	2301	1214
Citrulline	1809	1180	400
Atenolol	100	0	0
Isoleucine	31064	12871	9621
Adenine	2270	2193	1297
Inosine	6557	3547	2982
Xanthosine	89	190	107
Cyclic AMP	132	0	0
Guanosine	232	278	161
Glycyl-glycine/L-Asparagine	1248	906	318
Uridine	2708	1023	1832
Deoxycytidine	4411	3920	1082
Cytidine	360	465	312

Figure 3. Quantifying selected analytes 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 compounds among the different experimental groups.

Untargeted Discovery

The parallel fast analysis utilizing both the Orbitrap and the Astral analyzers secures a higher number of high-quality (80k resolution) Astral MS^2 scans. This is crucial for deep coverage and confident untargeted discovery analysis (Figure 4).

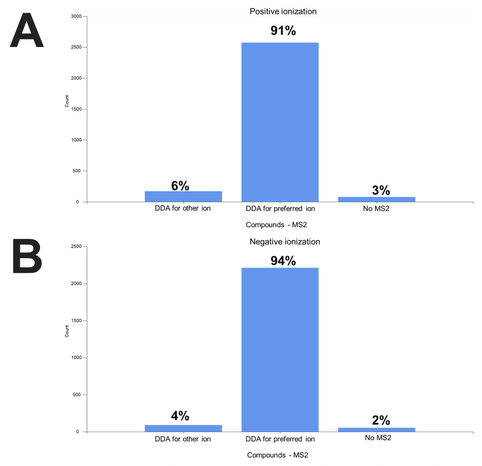


Figure 4. Number of detected fecal metabolites with orbitrap full scan only (No MS2) and fecal metabolites with both Orbitrap full scan and Astral MS2 spectra (either for the preferred ion or other adducts) in positive (A) and negative (B) polarity SQUAD data. The data was observed from Compound Discoverer 3.4.

The untargeted discovery analysis within the SQUAD workflow showcases variations in the fecal metabolome among the samples from different dietary groups. This is evidenced by the PCA scores (positive and negative polarity; Figure 5 and Figure 6, respectively) and corresponding loadings heat map plots.

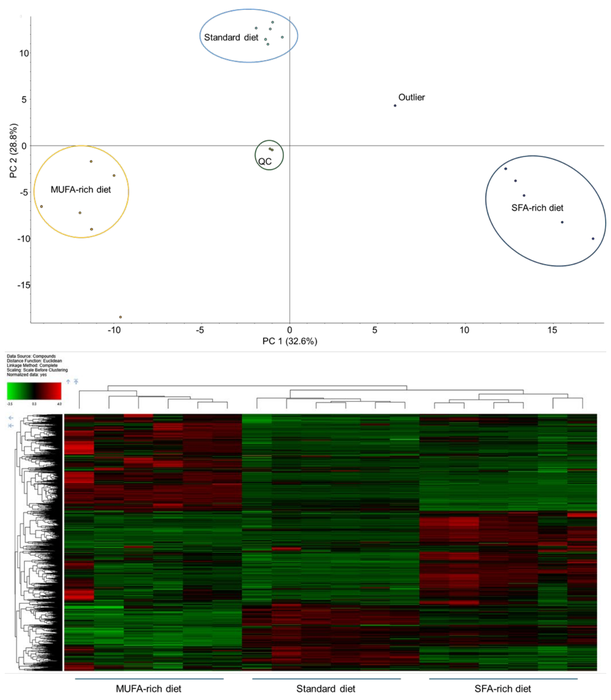


Figure 5. PCA scores (A) and loadings heat map (B) plots highlight distinct patterns and differences among the groups as shown in the positive polarity data.

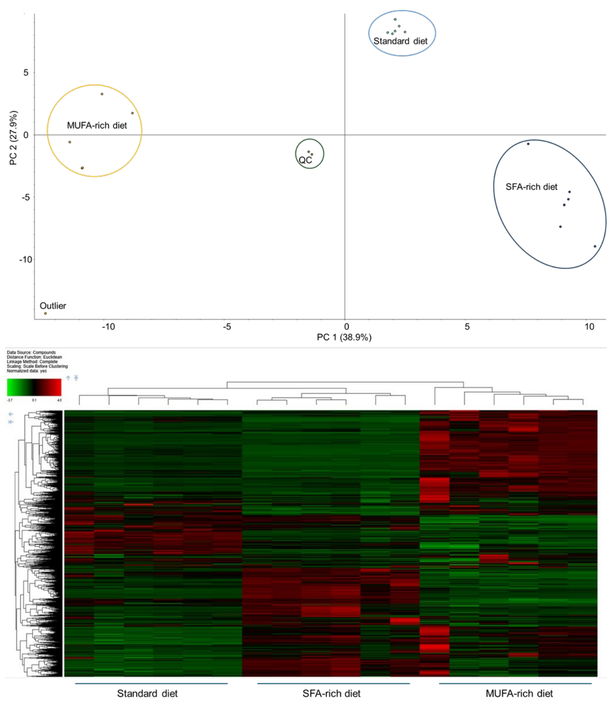
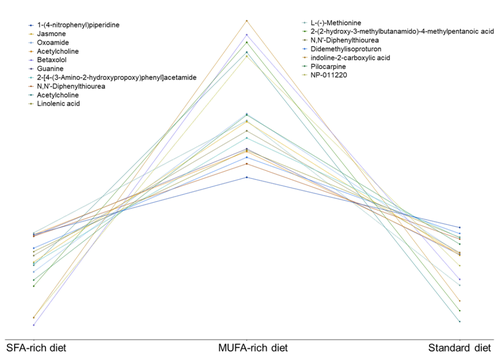


Figure 6. PCA scores (A) and loadings heat map (B) plots highlight distinct patterns and differences among the groups as shown in the negative polarity data.

Further data analysis highlights distinct patterns and differences among the groups as shown in Figure 7 and Figure 8, which presents a clear variation in the level of annotated fatty acids among the different groups.



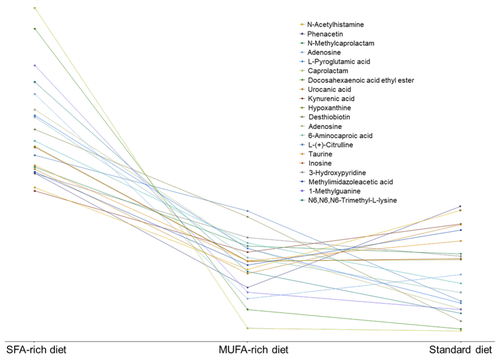


Figure 6. Selected annotated unknown fecal metabolites (+ve polarity) that provide valuable insights into the variation of metabolic profiles in the fecal samples collected from the mice dietary experiment. The data was observed from Compound Discoverer 3.4 utilizing mzCloud library.

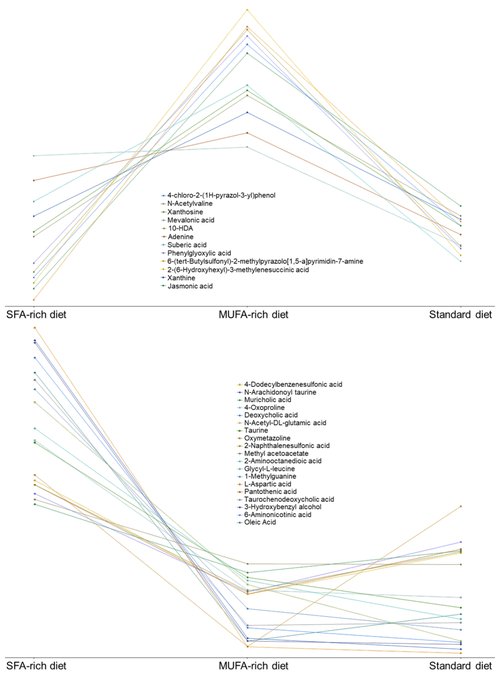


Figure 6. Selected annotated unknown fecal metabolites (-ve polarity) that provide valuable insights into the variation of metabolic profiles in the fecal samples collected from the mice dietary experiment. The data was observed from Compound Discoverer 3.4 utilizing mzCloud library.

Conclusion

We have successfully developed the SQUAD workflow for simultaneous quantitation and discovery of fecal metabolites. This comprehensive metabolomics approach utilizes the advanced Orbitrap Astral mass spectrometer, to achieve sensitive and accurate quantification, and deep coverage with confident annotation of unknowns in the fecal extract.

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