Simultaneous quantitation and discovery (SQUAD) of fecal bile acids and their conjugates in children with autism spectrum disorder (ASD)

Bashar Amer[1], Alexey Melnik[2], James B Adams[3], Rosa Krajmalnik-Brown[3], Khemlal Nirmalkar[3], Brandon Bills[1], Rafael Melani[1], and Susan S. Bird[1]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Bileomix Inc., Farmington, CT, United States; [3]Biodesign Center for Health Through Microbiomes, Arizona State University, Tempe, Arizona, United States

Abstract

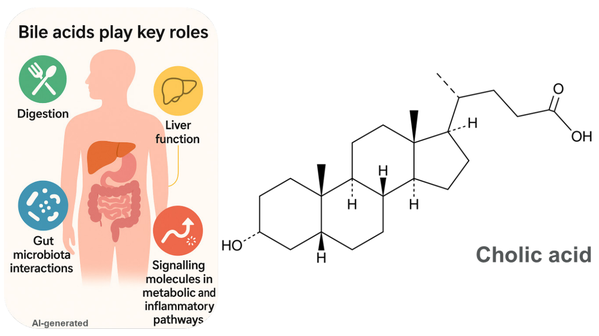
Purpose: We assessed a single-injection SQUAD workflow for simultaneous quantitative and discovery metabolomics. This workflow was applied to analyze bile acids and their conjugates in fecal extracts from children with Autism Spectrum Disorder who underwent microbiome transfer therapy.

Methods: Fecal samples from adults with and without Autism Spectrum Disorder (ASD) were analyzed. Bile acid (BA) and microbially conjugated bile acid (MCBA) standards from Bileomix Inc. were separated using a reversed-phase column integrated into a liquid chromatography (LC) system. Data acquisition was performed using parallel reaction monitoring (PRM) in the linear ion trap for quantitation, alongside high-resolution MS1 scanning in the Orbitrap for improved metabolite annotation.

Results: The SQUAD approach improves productivity by enabling high-throughput, accurate analysis for both targeted quantitation of predefined compounds and untargeted discovery of unknown metabolic features.

Introduction

Bile acids (BAs), synthesized from cholesterol, are essential for lipid metabolism and play a vital role in host–microbiota interactions. The gut microbiota modifies BAs into secondary and microbially conjugated bile acids, which can influence immune function, metabolic regulation, and gut-brain signaling.



Alterations in the microbiota, common in individuals with Autism Spectrum Disorder (ASD), have been linked to disruptions in BA metabolism, potentially contributing to the neurodevelopmental and gastrointestinal symptoms observed in ASD (Figure 1).

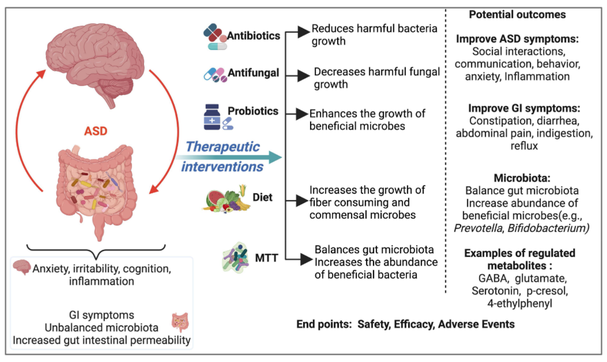


Figure 1. Gut microbiota and metabolic imbalances have been linked to both GI and neurodevelopmental issues in ASD; Takyi et al., 2025.

To explore these connections, we developed and implemented a simultaneous quantitation and discovery (SQUAD) metabolomics workflow using a Tribrid mass spectrometer (Figure 2). This workflow combines targeted and untargeted analysis and incorporates Real-Time Library Search (RTLS) to improve spectral matching and confidence in molecular identification during acquisition.

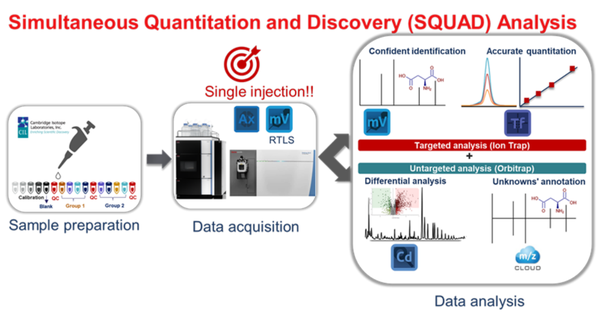


Figure 2. The developed SQUAD of fecal BAs and BA conjugates to quantify a selected list of primary and secondary BAs and their conjugates.

We applied the SQUAD workflow to the analysis of bile acids and their conjugates in fecal samples from children with ASD who underwent microbiota transfer therapy (MTT). This approach enabled high-throughput, high-resolution profiling of known and novel BA-related metabolites, providing insight into the biochemical impact of microbial modulation on host metabolism and neurodevelopmental health.

Materials and methods

Sample preparation

Bile acids, their conjugates, and other metabolites were extracted from fecal samples collected from the ASD cohort (Figure 3) using a methanol–water mixture with methanol in excess. Isotopically labeled bile acid internal standards were spiked into the samples to support accurate quantitation.

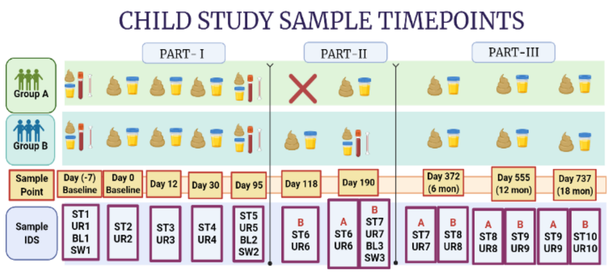


Figure 3. Overview of the microbiota transfer therapy (MTT) cohort of children with Autism Spectrum Disorder (ASD).

Data acquisition

The extracted samples were analyzed using the developed SQUAD workflow on the Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer (Figure 4) coupled to a Thermo Scientific™ Vanquish™ Horizon UHPLC system.

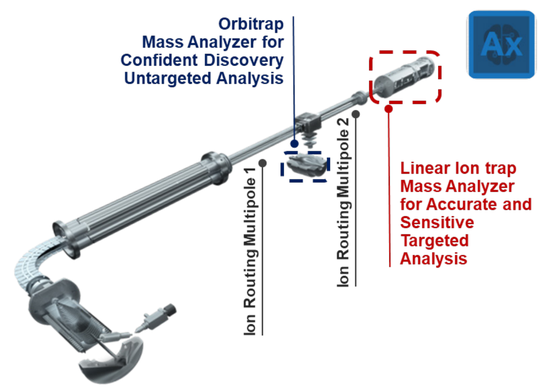


Figure 4. Structural schematic of the Orbitrap Ascend Tribrid mass spectrometer. The system integrates three mass analyzers: quadrupole, linear ion trap, and Orbitrap, to enable advanced fragmentation strategies and high-resolution, accurate-mass (HRAM) analysis.

Data analysis

Data processing, including quantitation of analytes and annotation of unknowns, was performed using Thermo Scientific™ TraceFinder™ 5.2 software and Thermo Scientific™ Compound Discoverer™ 3.4 software.

Results

To achieve absolute quantitation, calibration curves were created utilizing bile acid standards. This methodology allows for precise quantification using the linear trap mass analyzer over a wide linear dynamic range (6 orders of magnitude). The majority of the targets demonstrated a high sensitivity down to LLOQ of less than 1.8 femtomole on column (Figure 5).

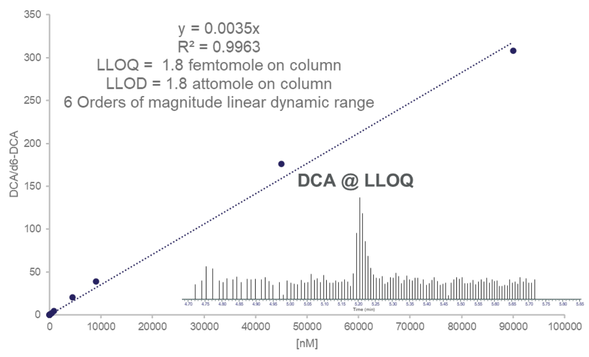


Figure 5. Sensitivity and dynamic range performance of the SQUAD workflow using the Orbitrap Ascend Tribrid mass spectrometer. The method demonstrates exceptional quantitative sensitivity with a lower limit of quantitation (LLOQ) of 1.8 femtomoles and a lower limit of detection (LLOD) of 1.8 attomoles on-column. A linear dynamic range spanning six orders of magnitude was achieved, supporting robust quantitation across a broad concentration range.

Quantitative analysis of bile acids in fecal samples revealed notable variations in their levels. Interestingly, MTT resulted in distinct changes in bile acid profiles, suggesting a potential link between these metabolic shifts and the observed improvement in ASD symptoms following treatment (Figure 6). These findings highlight the importance of carefully interpreting bile acid alterations to better understand their role in therapeutic outcomes.

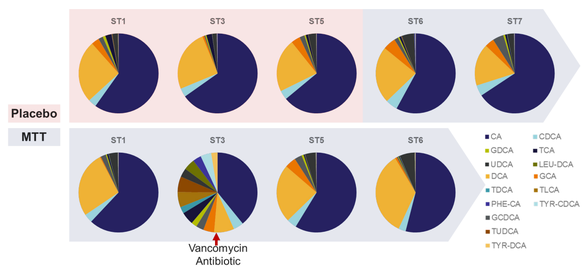


Figure 6. Changes in fecal bile acid profiles following microbiota transfer therapy (MTT) in children with Autism Spectrum Disorder (ASD).

Quantitation using the linear ion trap offers enhanced selectivity through its MSn capability, which is particularly advantageous for distinguishing and quantifying co-eluting isomers that share identical MS² fragmentation patterns. This is especially relevant in cases such as leucine-LCA and isoleucine-LCA conjugates, where higher-order fragmentation is required to resolve structural isomers accurately (Figure 7).

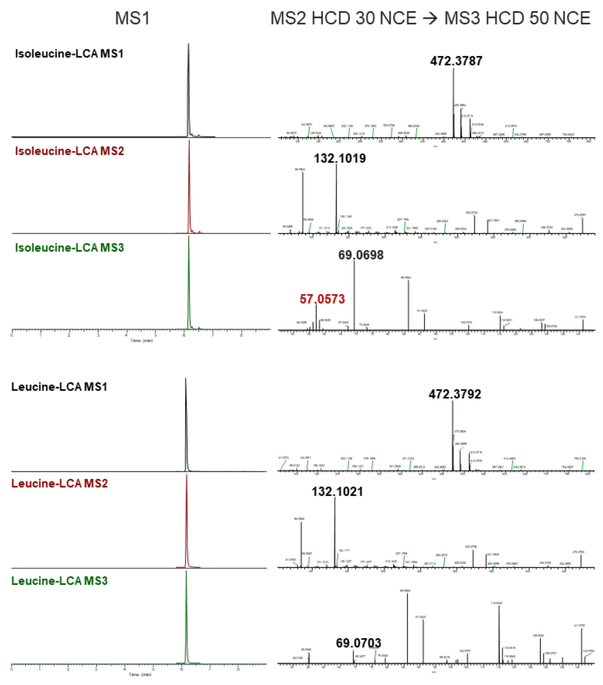


Figure 7. Enhanced selectivity of linear ion trap quantitation using MSⁿ for isomer resolution.

To validate the use of MS3 fragmentation for the specific quantitation of the isoleucine-LCA isomer, mixtures of the two isomers were prepared. The concentration of leucine-LCA was held constant at a high level, while the concentration of isoleucine-LCA was varied. Using the MS3-based quantitation approach, we observed a strong linear response across the tested concentration range, demonstrating no interference from the leucine isomer (Figure 8). These results confirm the specificity and reliability of MS3 for diagnostic quantitation of structurally similar, co-eluting isomers

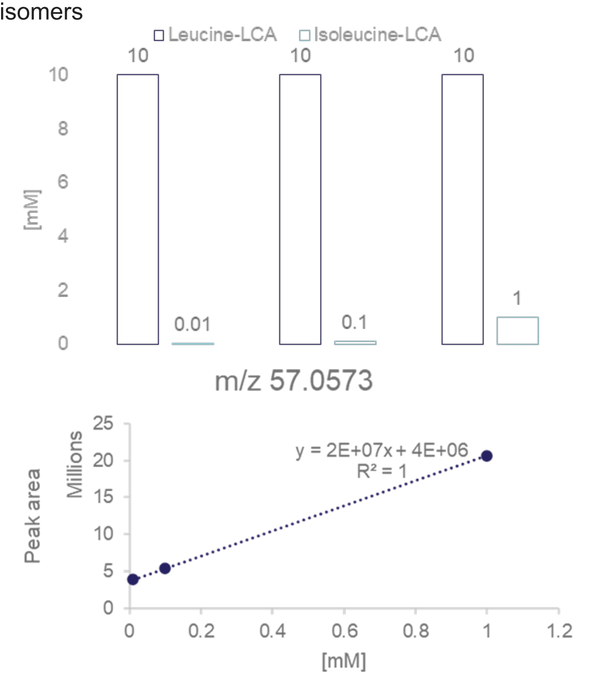


Figure 8. Validation of MS3 quantitation for isoleucine-LCA in the presence of leucine-LCA.

Discovery analysis of the data obtained through the SQUAD workflow revealed distinct shifts in the metabolic profiles of fecal samples following MTT. Principal component analysis (PCA) demonstrated clear separation between pre- and post-treatment samples, as shown in Figure 9. Notably, no such separation was observed in the placebo group, indicating that the metabolic changes were specific to the MTT intervention.

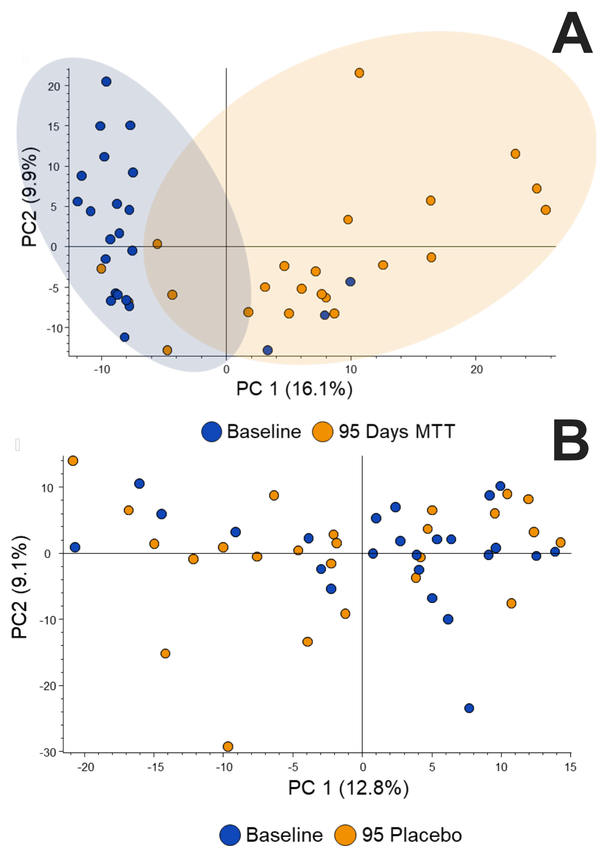


Figure 9. Principal component analysis (PCA) of fecal metabolomic profiles before and after treatment. (A) PCA of the MTT-treated group shows clear separation between pre- and post-treatment samples, indicating distinct metabolic shifts following microbiota transfer therapy. (B) PCA of the placebo group shows no significant separation, suggesting minimal metabolic changes in the absence of treatment. These results highlight the specific impact of MTT on the fecal metabolome in children with ASD.

A subset of annotated metabolites showing significant contribution to the observed separation was selected from the multivariate data analysis. Notably, fatty acids and related lipid metabolites were among the key drivers of this metabolic shift (Figure 10). Further interpretation and pathway-level analysis are ongoing to deepen our understanding of the biological relevance of these changes following MTT.

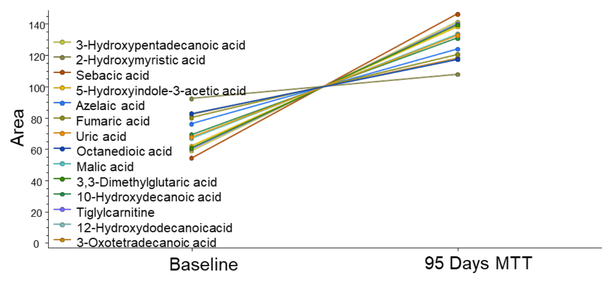


Figure 10. Key metabolites contributing to metabolic shifts following microbiota transfer therapy (MTT). Multivariate data analysis identified a subset of annotated metabolites significantly associated with the separation observed in PCA. Fatty acids and lipid-related metabolites were among the top contributors to these changes.

Conclusion

We have successfully developed the SQUAD workflow for the simultaneous quantitation and discovery of fecal bile acids and other metabolites. This comprehensive metabolomics approach leverages the capabilities of advanced Orbitrap Ascend Tribrid mass spectrometers to deliver highly sensitive and accurate quantification, along with deep metabolome coverage and confident annotation of unknown compounds in fecal extracts.

Acknowledgments

We gratefully thank all individuals with ASD and their families for participating in the study and Joel Meraz for laboratory assistance at ASU.

General Laboratory Equipment – Not For Diagnostic Procedures.

Trademarks/licensing

Do you want to learn more about SQUAD metabolomics? Scan here

Learn more at thermofisher.com/squad