Advancing bile acid quantitation: High-specificity MS2 and MS3 analysis using the Stellar Mass Spectrometer

Lauren Bishop[1], Brittany Lee[1], Jingshu Guo[1], Bashar Amer[1], Alexey V. Melnik[2], James B. Adams[3], Rosa Krajmalnik-Brown[3], Khemlal Nirmalkar[3], Charles E. Maxey[1], Cristina C. Jacob[1], Scott M. Peterman[1], Kerry Hassell[1], Susan S. Bird[1]

[1]Thermo Fisher Scientific, San Jose, CA 95134; [2]BileOmix Inc., Farmington, CT 06032; [3]Biodesign Center for Health Through Microbiomes, Arizona State University, Tempe, AZ 85287

Abstract

Purpose: Development of a highly selective MS-based approach for the quantitation of bile acids and their conjugates in biological specimens.

Methods: A single method combining both targeted-MS2 and targeted-MS3 scan modes and using both HCD (collision cell-based) CID (resonance) as activation type was developed on Stellar mass spectrometer.

Results: Calibration curves were generated using both unlabeled and labeled bile acids standards to achieve absolute quantitation. This approach enabled accurate quantitation using Stellar MS across a broad dynamic range, spanning five orders of magnitude. Further, this demonstrates enhanced sensitivity and selectivity for the accurate quantification of bile acids in human serum.

Introduction

Bile acids (BA) are cholesterol-derived molecules synthesized in the liver that play a crucial role in lipid digestion, absorption, and signaling. Beyond their traditional function, they serve as key regulators of gut microbiome interactions while also modified by gut microbiota into secondary and conjugated forms, providing insights into various states of health and disease. Alterations in bile acid profiles may contribute to digestion, immune function, and neurological development differences observed in autism spectrum disorder (ASD). Given the growing recognition of bile acid-microbiome interactions, there is an increasing need for analytical tools capable of differentiating structurally similar bile acid isomers with high specificity. The Thermo Scientific™ Stellar™ mass spectrometer (**Figure 1**), incorporating both MS2 and MS3 capabilities with complementary activation types, provides a powerful solution for enhanced bile acid quantitation and disease characterization.

Materials and methods

Sample Preparation

Unlabeled and stable-isotope-labeled bile acids and conjugated standards were purchased from Cambridge Isotopes Laboratories. Amino acids conjugates were also included in this method. These standards were then used to prepare dilution series solutions in a 50% methanol and generate calibration curves. NIST 1950 plasma and patient samples were extracted with 0.1% formic acid in isopropanol, evaporated to dryness and reconstituted in 50% methanol prior LC-MS analysis.

Data Acquisition

Samples were chromatographically separated using a reverse-phase column (**Figure 2**) and a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to Stellar mass spectrometer. Data was acquired utilizing both tMS2 and tMS3 scan functions and HCD and CID fragmentation. Our LC-MS/MS method included 15 primary bile acids and conjugates,11 amino acid-conjugates and 12 isotope-labeled bile acids. **Figure 2** shows the overlaid extracted ion chromatograms of all bile acids included in the method.

Figure 1. Thermo Scientific Stellar MS diagram.

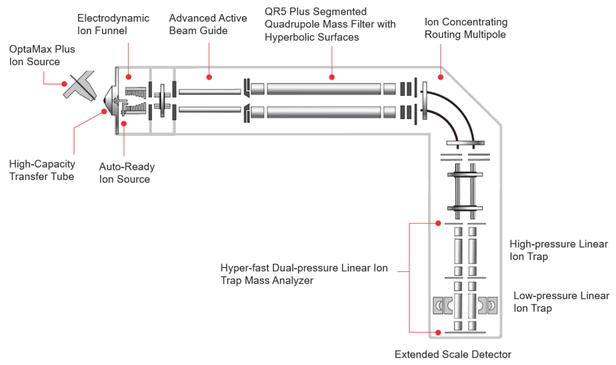
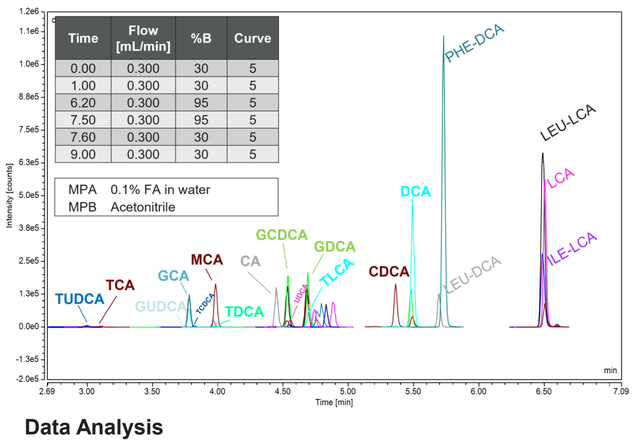


Figure 2. Overlaid extracted ion chromatograms of BAs and isotope-labeled BAs using a reversed-phase column. LC separation was over 9 minutes run time (inserted table).



Time	Flow [mL/min]	%B	Curve
0.00	0.300	30	5
1.00	0.300	30	5
6.20	0.300	95	5
7.50	0.300	95	5
7.60	0.300	30	5
9.00	0.300	30	5

MPA 0.1% FA in water
MPB Acetonitrile

Data Analysis

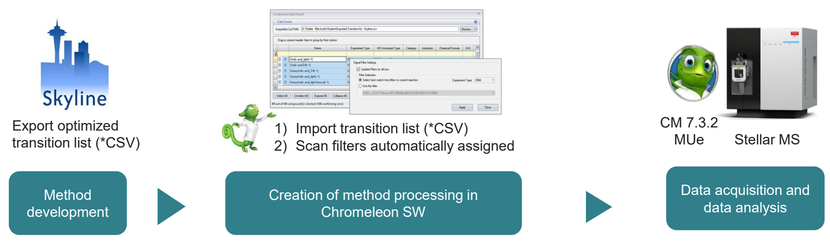
Skyline software was used during method development. A refined transition list was then transferred to Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3.2 Software, a CRF21 compliant software ready, to create the data processing method. Chromeleon Software was then used for data acquisition and data analysis. Data was also processed using Thermo Scientific™ TraceFinder™ software version 5.2.

Results

LC-MS/MS Method Development

MS parameters were optimized to achieve ultimate sensitivity and selectivity. The list of transitions for each compound were generated using the Thermo Scientific™ mzVault™ application and then imported in Skyline Software for visualization and evaluation of the progress of method optimization. Selected MS^2 or MS^3 fragments designated for quantitation were then imported into Chromeleon CDS software for final assay evaluation.

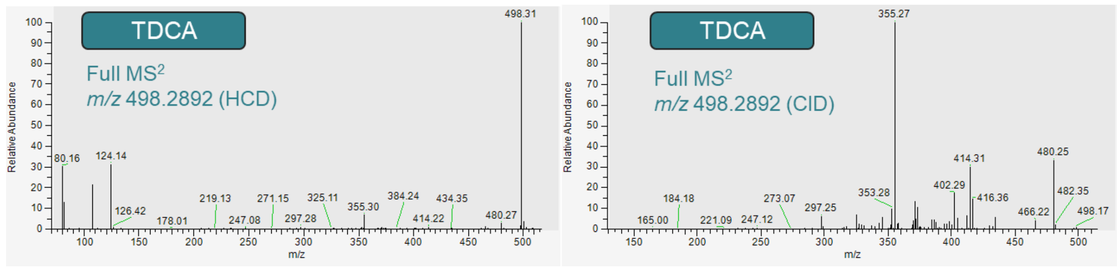
Figure 3. Skyline to Chromeleon SW workflow.



Combined tMS2 and tMS3 approach

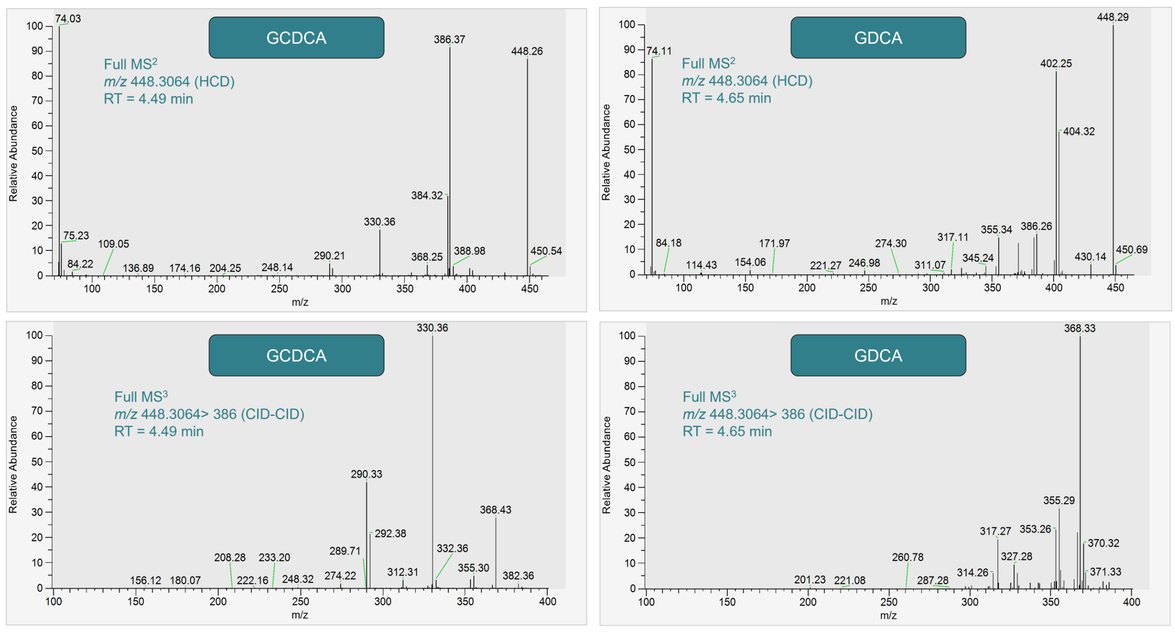
The versatility of the instrument control software of Stellar MS allows the user to define a combination of different activation types for both tMS2 and tMS3 experiments for optimized results.

Figure 4. Example of MS^2 spectra with CID and HCD activation types of TDCA.



MS^3 fragmentation enhances the specificity of certain BA isomers. For instance, unique MS^3 fragments were observed for GDCA and GCDCA. **Figure 5** illustrates the MS^2 and MS^3 spectra of GDCA and GCDCA.

Figure 5. MS^2 and MS^3 spectra of GCDCA and GDCA.



Quantitative Performance

Calibration standards were prepared in 50% methanol. The plot of response ratios for labeled versus unlabeled bile acids was linear over the concentration range of 0.5 – 1000 nM. Correlation coefficients were greater than 0.99 for most bile acids and the respective residuals were within 20% of the nominal values.

Figure 6. Selected calibration curves based on MS^2 fragments for primary bile acids over the range 0.2 to 1000 nM.

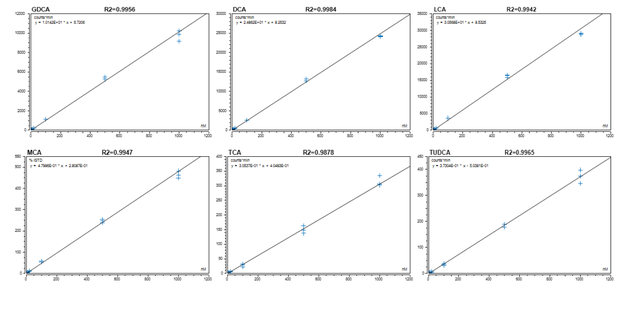


Figure 7. Selected calibration curves based on MS^3 fragments for Amino Acids-conjugates over the range 0.2 to 1000 nM.

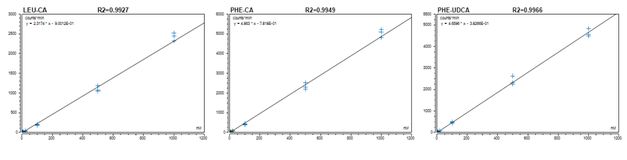


Table 1. Limit of quantitation achieved for selected BA and conjugates.

Compound	LOQ (nM) (CV%<20%)	pg on column	Internal Standard	RSD% (N=78)
CA	2	1.63	NA	NA
DCA	0.5	0.39	d6-DCA	7%
GCA	1	0.93	d4-GCA	6%
GCDCA	1	0.90	d4-GCDCA	7%
GDCA	1	0.90	d6-GDCA	6%
LCA	0.5	0.38	13C4-LCA	14%
TCA	2	2.06	d4-TCA	16%
TCDCA	0.5	0.50	d4-TCDCA	12%
TDCA	1	1.00	d6-TDCA	21%
TLCA	1	0.97	N/A	N/A
TUDCA	2	2.00	d4-TUDCA	9%
UDCA	10	7.85	d4-UDCA	10%
MCA	5	3.92	d5-MCA	9%
LEU-CA	1	0.82	N/A	N/A
LEU-DCA	0.5	0.52	N/A	N/A
LEU-LCA	0.2	0.20	N/A	N/A
LEU-UDCA	0.5	0.49	N/A	N/A
PHE-CA	0.5	0.51	N/A	N/A
PHE-DCA	0.5	0.56	N/A	N/A

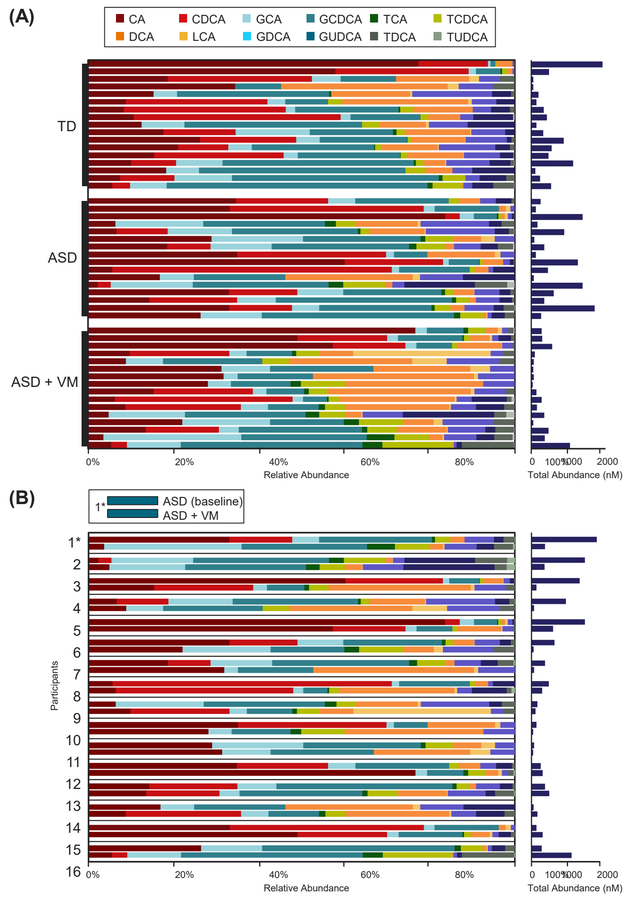
Table 2. BA concentrations measured in NIST human plasma reference material (2-fold overall dilution was taken into account).

Compound	Measured concentrations (nM)	RSD% (N=6)
CA	129.2	8%
CDCA	60.13	1%
DCA	54.42	7%
GCA	72.53	6%
GCDCA	273.55	7%
GDCA	105.32	4%
GUDCA	105.94	4%
LCA	0.62	21%
TDCA	12.1	5%
TCDCA	0.92	15%
LEU-DCA	0.24	14%

Quantification of Bile Acids in Human Serum

The optimization of Stellar MS tMS^2/tMS^3 capabilities enables accurate and robust quantification of bile acids in human serum, even at low levels. **Figure 8** shows the relative abundance of 12 bile acids across 49 serum samples from typically developing (TD) children and children with autism spectrum disorder (ASD) before and after vitamin-mineral supplement treatment (ASD + VM). Total bile acid abundance ranged from 5 nM to 2100 nM. Comparison revealed that 11 of 16 participants had a >30% decrease in total bile acids post-treatment, matching trends observed in the absolute abundance of primary and primary conjugated bile acids – CDCA, GCA, GCDCA, TCDCA.

Figure 8. Bile Acid profiles in children of typical development (TD) and with autism spectrum disorder (ASD). (A) Relative abundance of 12 quantified bile acids for each sample (TD = 17, ASD = 16, ASD+VM = 16). The right panel shows the total bile acid abundance (in nM) per sample. (B) Profiles of participants with ASD before (baseline) and after vitamin-mineral supplementation (+ VM).



Conclusions

A combined tMS2 and tMS3 method was successfully developed using Stellar MS to measure bile acids and conjugates with linearity, accuracy and reproducibility. The targeted MS^2/MS^3 acquisition enabled sensitive and selective quantification of bile acids and their conjugates in human serum of individuals with ASD, providing bile acid profiles.

References

1. Remes, P. M., et. al. Hybrid Quadrupole Mass Filter–Radial Ejection Linear Ion Trap and Intelligent Data Acquisition Enable Highly Multiplex Targeted Proteomics, *J. Proteome Res.* **2024**, 5476.

Acknowledgements

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

Trademarks/licensing