# High-throughput LC-HRMS method for the quantitation of 20 Bile Acids in Human Serum and Urine

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Danielle Ronnow[1], Chris Hardcastle[1], Alexander Schwartz[1], Brandon Wilcock[1], Troy Voelker[1], and Scott Reuschel[1]

[1]Aliri Bioanalysis, Salt Lake City, UT, USA

The authors declare no competing financial interests.

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## Introduction

Recent research has revealed the involvement of bile acids in several pathophysiological pathways, including liver disease, diabetes, tumors, and Alzheimer's disease, leading many to consider the potential of bile acids as biomarkers for a range of diseases. The composition of bile acid varies significantly depending on the biological matrix and much remains to be discovered regarding their compositional differences across individuals. Bile acids exhibit remarkable structural diversity, characterized by variations in hydroxylation patterns and glycine or taurine conjugation states. Consequently, the study of bile acids requires methods that cover their structural diversity and liquid chromatography-mass spectrometry (LC-MS) has emerged as the method of choice for distinguishing structural analogues and isobars owing to its high sensitivity and specificity.

## Challenges

Bile acids are commonly extracted from complex biological samples with methanol-based extraction procedures. However, with these simple extraction procedures, numerous reports have found that accumulating lipids on the analytical column interferes with LC/MS analyses of bile acids. In our experience, these adverse effects included increasing column backpressure, retention time drift and peak shape broadening over multiple injections. In lieu of employing an alternative offline solid-phase extraction (SPE) cleanup, we investigated several column chemistries and mobile phase combinations in conjunction with the simple protein precipitation extraction.

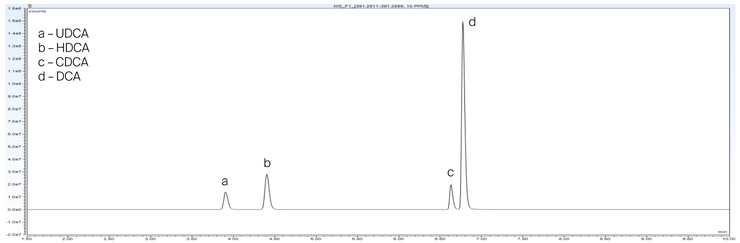
## Method Overview

A qualified liquid chromatography-high resolution mass spectrometry (LC-HRMS) method was developed for the quantitation of 20 bile acids in human serum and urine. A surrogate matrix was utilized for the calibration standards and quality control samples to allow for the analysis of multiple biological matrices in a single run. The method utilizes a "solvent first" protein precipitation plate to reduce matrix effects in both the native quality control samples and unknown samples. Extracts are analyzed by reversed-phase LC coupled to electrospray ionization in negative mode full scan MS. Ten stable-isotopically labeled bile acids were used as internal standards. The analytical run time is 13 minutes.

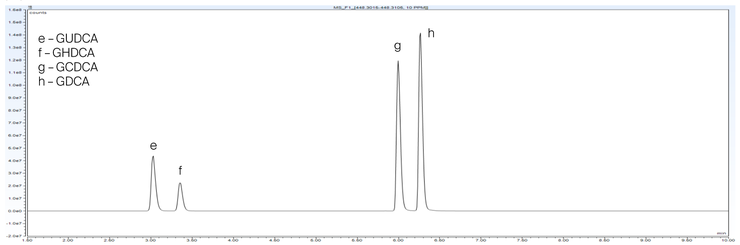
| LC Parameters | | |
|---|---|---|
| Analytical Column | Phenomenex Kinetex Phenyl-Hexyl 100 Å, 2.1 x 100 mm, 1.7 µm | |
| Mobile Phase A | 5 mM Ammonium Acetate in water | |
| Mobile Phase B | Methanol/Acetonitrile 50/50 | |
| Initial A/B Total Flow Rate | 0.50 mL/min | |
| LC Gradient | | |
| Time | A/B Total Flow Rate | Percent B Concentration |
| 0.00 | 0.50 | 33 |
| 0.75 | 0.50 | 33 |
| 4.75 | 0.60 | 40 |
| 6.25 | 0.60 | 55 |
| 9.00 | 0.60 | 65 |
| 9.50 | 0.60 | 95 |
| 11.00 | 0.60 | 95 |
| 11.50 | 0.60 | 33 |
| 13.00 | 0.50 | 33 |

## Results

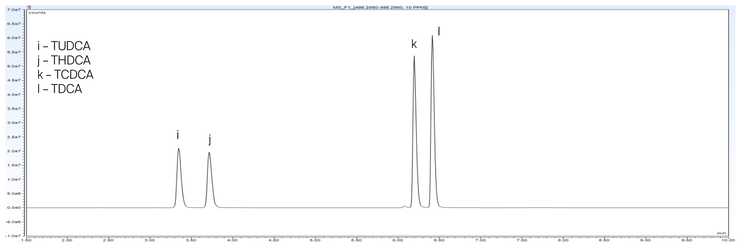
(A)




(B)




(C)




(D)

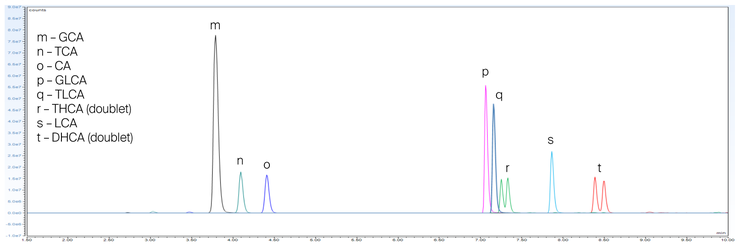



Figure 1. Representative Chromatograms of Bile Acids ULOQ. (A) Isobaric Bile Acids with m/z of 391.28; (B) Isobaric Bile Acids with m/z of 448.30; (C) Isobaric Bile Acids with m/z of 498.28; (D) Overlay of non-isobaric Bile Acids quantitated by the method.

## Results (continued)

| | 26-Nov-25 LQC | 27-Nov-25 LQC | 30-Jan-26 LQC | Inter-Assay LQC | 26-Nov-25 HQC | 27-Nov-25 HQC | 30-Jan-26 HQC | Inter-Assay HQC |
|---|---|---|---|---|---|---|---|---|
| CA | | | | | | | | |
| Mean | 1.48 | 1.38 | 1.48 | 1.45 | 407 | 403 | 417.0 | 409 |
| Bias | -1.3 | -8.0 | -1.3 | -3.5 | 1.8 | 0.8 | 4.3 | 2.2 |
| %CV | 5.3 | 2.9 | 1.9 | 4.1 | 2.0 | 1.3 | 1.3 | 2.1 |
| CDCA | | | | | | | | |
| Mean | 1.45 | 1.73 | 1.47 | 1.45 | 407 | 407 | 420 | 410 |
| Bias | -3.3 | -4.7 | -2.0 | -3.4 | 1.8 | 1.8 | 5 | 2.6 |
| %CV | 3.1 | 4.0 | 1.8 | 3.1 | 2.4 | 2.2 | 1.8 | 2.4 |
| DCA | | | | | | | | |
| Mean | 7.29 | 7.26 | 7.39 | 7.31 | 2080 | 2010 | 2030 | 2045 |
| Bias | -2.8 | -3.2 | -1.5 | -2.5 | 4 | 0.5 | 1.5 | 2.3 |
| %CV | 2.4 | 1.3 | 1.4 | 1.8 | 3.3 | 3.9 | 4.3 | 3.8 |
| DHCA | | | | | | | | |
| Mean | 1.44 | 1.40 | 1.49 | 1.44 | 402 | 394 | 437 | 409 |
| Bias | -4 | -6.9 | -0.7 | -4.2 | 0.5 | -1.5 | 9.3 | 2.3 |
| %CV | 2.4 | 4.7 | 3 | 4.3 | 2 | 2.4 | 1.5 | 4.9 |
| GCA | | | | | | | | |
| Mean | 7.24 | 7.10 | 7.37 | 7.23 | 2010 | 2000 | 2110 | 2035 |
| Bias | -3.5 | -5.3 | -1.7 | -3.6 | 0.5 | 0.0 | 5.5 | 1.8 |
| %CV | 2.5 | 1.9 | 1.9 | 2.5 | 3.5 | 2.0 | 1.8 | 3.5 |
| GCDCA | | | | | | | | |
| Mean | 7.3 | 7.11 | 7.57 | 7.31 | 2010 | 2020 | 2080 | 2029 |
| Bias | -2.7 | -5.2 | 0.9 | -2.5 | 0.5 | 1 | 4 | 1.5 |
| %CV | 1.5 | 1.2 | 1.6 | 2.9 | 6.7 | 2.2 | 3.4 | 4.4 |
| GDCA | | | | | | | | |
| Mean | 7.39 | 7.24 | 7.47 | 7.36 | 2070 | 2050 | 2110 | 2072 |
| Bias | -1.5 | -3.5 | -0.5 | -1.9 | 3.5 | 2.5 | 5.5 | 3.6 |
| %CV | 1.7 | 2.3 | 1.0 | 2.1 | 3 | 2.6 | 39.4 | 2.9 |
| GLCA | | | | | | | | |
| Mean | 1.47 | 1.46 | 1.49 | 1.47 | 418 | 422 | 426.2 | 422 |
| Bias | -2.0 | -2.7 | -0.9 | -1.9 | 4.5 | 5.5 | 6.5 | 5.5 |
| %CV | 2.2 | 1.6 | 2.6 | 2.2 | 2.7 | 2.1 | 2.3 | 2.4 |
| TCDCA | | | | | | | | |
| Mean | 2.99 | 2.92 | 3.0 | 2.98 | 805 | 804 | 833.7 | 811 |
| Bias | -0.3 | -2.7 | 0.8 | -0.8 | 0.6 | 0.5 | 4.2 | 41.4 |
| %CV | 2 | 2 | 1.9 | 2.4 | 5.8 | 1.7 | 2.9 | 4.0 |
| TDCA | | | | | | | | |
| Mean | 2.99 | 2.88 | 3.0 | 2.95 | 824 | 815 | 845 | 827 |
| Bias | -0.3 | -4.0 | -0.9 | -1.8 | 3 | 1.9 | 5.6 | 3.4 |
| %CV | 3.6 | 2.5 | 2.6 | 3.3 | 2.5 | 2 | 3.1 | 2.9 |

Table 2. Accuracy and Precision Quality Control Data for a Subset of Bile Acids in Surrogate Matrix. Analytical statistics for n=6 at both LQC and HQC for each assay.

## Conclusion

This qualified LC-HRMS method demonstrates that high-throughput, multi-analyte quantitation of structurally diverse bile acids across multiple biological matrices is both feasible and analytically robust. By integrating a solvent-first protein precipitation workflow with a carefully optimized reversed-phase LC separation, the method effectively mitigates lipid-related matrix effects while providing baseline resolution of isobaric bile acids. The consistent quantification across multiple runs over a period of several months demonstrates this method is well suited for clinical applications.