Having your cake and eating it, too: A novel quantitative LC-HRAM bile acids workflow incorporating robust quantitative and discovery capabilities

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STABLE ISOTOPE LABELED STANDARDS
32 bile acids
16 conjugated and non conjugated bile acids, and their sulfated analogs

TOTAL BA TARGETS
155 compounds
Including 32 quantitative and 123 semi-quantitative bile and fatty acids

TOTAL LC-MS TIME
10 minutes per sample
Inject-to-inject time is 10 minutes per sample

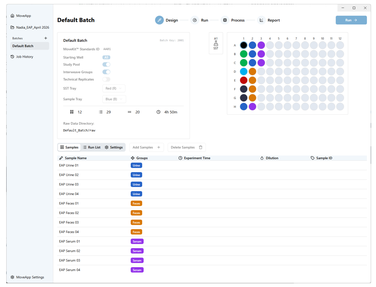
DATA ANALYSIS TIME
< 30 minutes per plate
Automated peak assignment takes only seconds per sample

01 INTRODUCTION
What is new about this bile acids approach?

Recent publications have highlighted the biological importance of bile acids (BAs), as well as a dizzying array of possible (and potentially important) BA conjugates, in a variety of diseases. However, significant challenges remain in reliably identifying and quantifying bile acids using nontargeted metabolomics, due to their generally poor fragmentation and lack of method standardization. Here, we combine the best practices of a gold-standard quantitative bile acids liquid chromatography-mass spectrometry (LC-MS) assay with nontargeted high-resolution accurate mass (HRAM) data collection. Using this approach, the goal was to create a highly simplified kit and software workflow that can be easily implemented for absolute bile acids quantification across laboratories, while remaining flexible enough for unique discoveries to be obtained from the LC-MS/MS data.

METHOD AND WORKFLOW

Calibration and QC materials (MoveKit™ BA, Move Analytical LLC) were formulated from pooled biological matrices (Goldenwest Biologics) spiked with unlabeled BA mixes (MSK-BA1-US to -BA4-US, CIL). Additional standards for building the bile acids library included in MoveKit™ BA were purchased from IROA Technologies, Cayman Chemical, and Bileomix. The kit extraction incorporated methanol spiked with stable isotope-labeled (SIL) bile acids (MSK-BA1 to -BA4, CIL), ammonium acetate, and a glass fiber filter plate (Move Analytical LLC). The extraction was optimized for 96-well plates. The approach was validated using multi-day extraction of a wide variety of sample matrices, including human and mouse serum (BioIVT), human fecal (NIST), and SRM1950 (NIST). Chromatographic separation used a 9.5-minute gradient on a BEH C18 column (Waters) at 0.3 mL/min. Method cross-validation was performed in six laboratories utilizing a variety of Thermo Scientific™ Orbitrap™ platforms and Stellar™ Mass Spectrometers (MS).



MoveKit™ BA consumables (above) include all reagents and standards needed for the fully quantitative workflow. The MoveApp™ (right) provides a seamless, integrated experience from design of your experiment through quantification and

SAMPLES DISTRIBUTED
- Human Serum
- Human Urine
- Human Fecal Extract

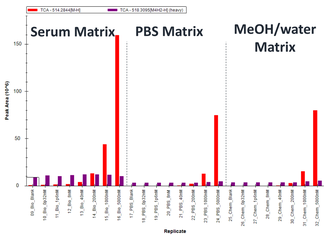
OTHER SAMPLE TYPES
- Mouse serum
- Human, mouse plasma
- Cecum
- Brain, Liver, other tissue

SAMPLE PREPARATION
1. 140 µL Extraction Reagent (MeOH+IS) to plate
2. 20 µL sample specimen (shake 15 min)
3. 40 µL focusing reagent (aq ammonium acetate)
4. Chill for 10 mins
5. Filter to collect

SOFTWARE WORKFLOW
- **Design**
  - List to MS Seq in 30 sec
- **Run** (automated)
  - SST
  - Data Check for samples
- **Process** (~15 min)
  - Quant and QC
- **Report**
  - Statistical Analysis

02 QUANTITATIVE RIGOR WITHOUT THE HASSLE
Single-Point External Calibration (SPEC)

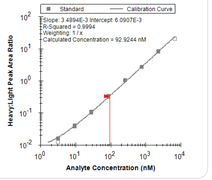
Calibration Matrix Selection



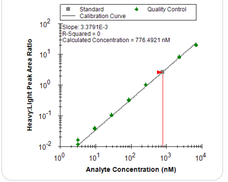
Matrix-based calibrators showed improved recovery and accuracy compared to aqueous, salt-based, or MeOH/water based calibration systems (above). At right, we show the process for validating the SPEC workflow. Linear calibration was compared to single-point with respect to bias, with all points calculated within 20% (01, 02). We then utilize the single-point external calibration with multi-point QC in the kit, and surface the accuracy calculations with high visibility in the app (03, 04).

SPEC Implementation

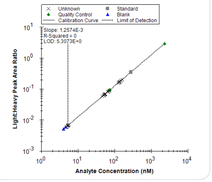
01 Traditional Multi-Point Calibration



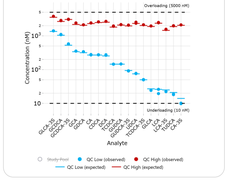
02 Validation of Single-Point External Calibration



03 Application of SPEC with multi-point QC in MoveKit

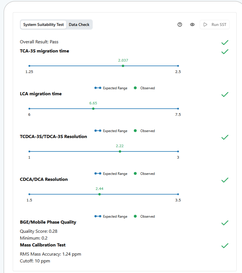


04 Visualization of QC Performance in MoveApp



03 PERFORMANCE IMPROVEMENTS
How MoveApp™ translates to faster decisions

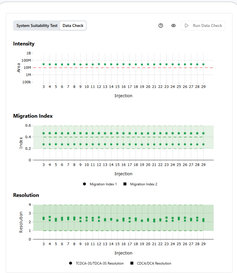
SST Dashboard



Remove the guesswork when evaluating System Suitability Test (SST) results.

We boiled down the parameters critical for success of the assay to the fewest actionable metrics. MoveApp checks retention time, critical pair resolution, gradient shape, and mass calibration, and prominently displays results on the SST dashboard.

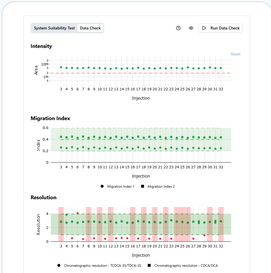
Data Check Dashboard



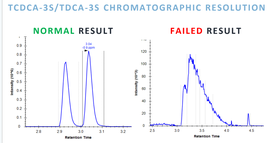
Traditional metabolomics quality assessment is forensic. We believe QC should become operational.

With a deep bench of data and dedicated processing logic, a real-time data quality assessment can be made quickly and with minimal user input. This allows early intervention, before a failure gets expensive.

A Failed Resolution Check

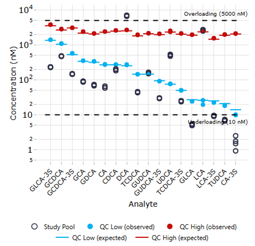


TCDCA-3S/TDCA-3S CHROMATOGRAPHIC RESOLUTION

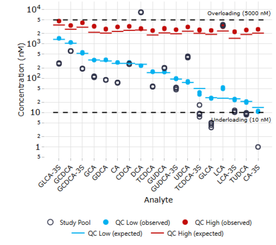


04 QC MONITORING PLOT
A New Visualization to Assess Experimental Quality

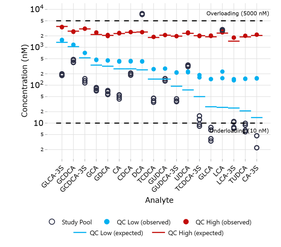
Example 1 – "Perfect"



Example 2 – Very Good



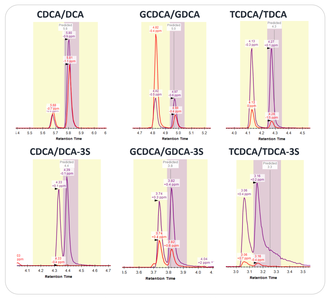
Example 3 – QC Low Failure

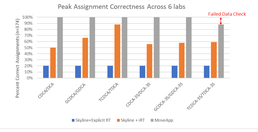


The **QC Monitoring Plot** displays automatically after data processing, and allows a fast, visual assessment of: 1) QC Low/High accuracy versus expected, and 2) Study Pool QC (study average) versus linear dynamic range of the assay. Example 1 shows good accuracy for all QCs and study pools with good precision mostly in the linear range. Example two shows a small positive bias in QC high (likely pipetting) and high LCA in the QC low (likely carryover). Example 3 shows significant positive bias in all QC low samples (likely sample prep issue).

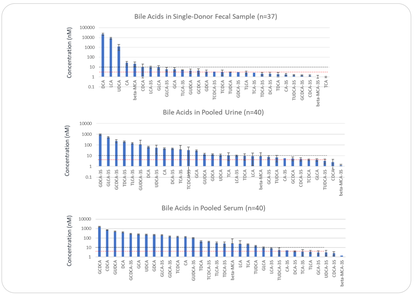
05 EARLY ADOPTER PROGRAM
Results of MoveKit™ BA in 6 laboratories

Peak Assignment Accuracy





Coverage and Precision Across Matrices



Across all experiments, MoveApp was able to correctly assign the 6 critical bile acid pairs (top left) **in every case that passed the data check**, compared to roughly coin-flip performance of using iRT in Skyline (bottom left). Explicit retention times do not perform well across laboratories. Top right shows average and standard deviation for bile acid concentrations determined across the 6 laboratories in 3 different matrices.

Acknowledgements

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