Automated and Robust CE-MS Metabolomics with Stable Isotope-Labeled Standards: From QC to Quantification

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Introduction

Stable-isotope labeled (SIL) standards have long been the utilized for ionization response correction in small molecule quantification. Independently, retention time standards are a robust tool for correcting elution time variance in gas and liquid chromatographic systems.[1,2] Capillary electrophoresis has been less commonly used in metabolomics, despite potential advantages in separation efficiency and sensitivity. The standardization of CE-MS workflows with robust microchip separations along with utilization of SIL standards can potentially help to overcome adoption hurdles. Here we demonstrate the importance of SIL standards in migration time normalization and response correction in CE-MS, and investigate the additional use of SIL standards for automated sample QC to assess missed injection, sample overloading, and other technical issues.

CE-HRMS Metabolomics

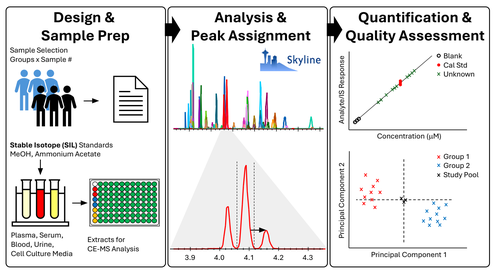
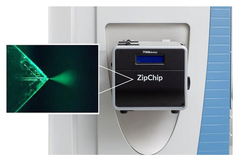
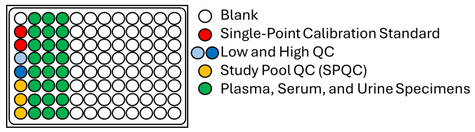


Diagram for the microchip CE-MS metabolomics workflow (above). The ZipChip CE source and detailed plate layout for the study is below.

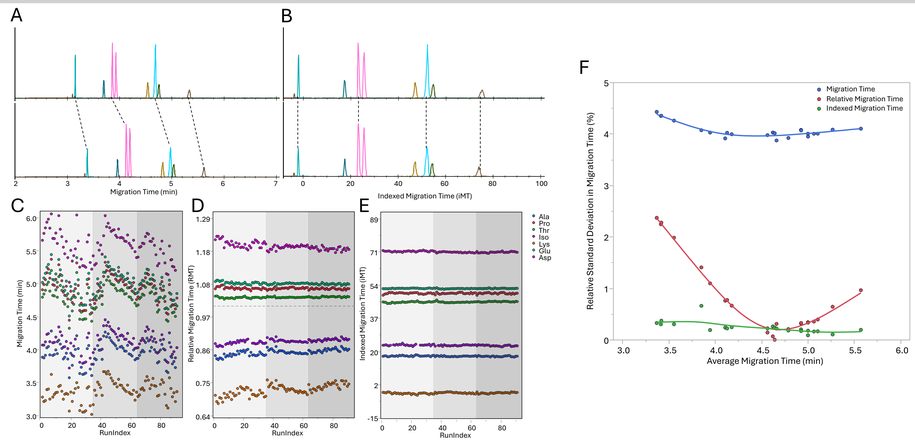




Materials and Methods

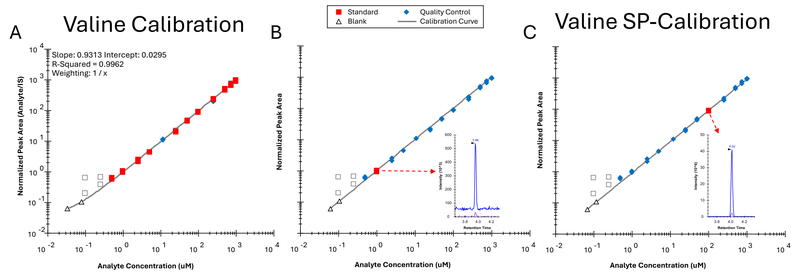
Biological matrices (20 µL per plasma, serum, and urine; BioIVT), SRM 1950 (NIST), calibration standards, and quality control materials were prepared with methanol precipitation in ammonium acetate using a custom stable isotope-labeled standard mixture (Cambridge Isotope Laboratories, Inc.). The 36-plex SIL mix comprised canonical and non-canonical amino acids as well as carnitine/acylcarnitine standards. Calibrants were value-assigned using a third-party laboratory. A ZipChip CE system (Repligen Corporation) coupled to an Orbitrap Exploris 240 mass spectrometer (Thermo Scientific) was used for sample processing. We performed a minimum of 3 preparations of each matrix across 3 batches. Skyline and ZipChip ACE software (908 Devices) were among the platforms used for data analysis.

Migration Time Indexing

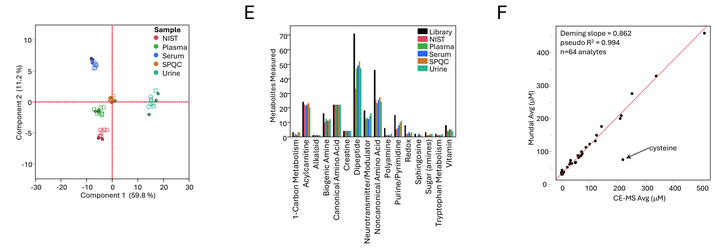


Using iRT procedure of Escher et al[2] to correct for migration time drift. (A) Raw migration drift for 8 example SIL amino acids from QC sample between batch 1 (top) and batch 3 (bottom) versus (B) the same samples plotted with indexed migration time. C) The raw migration time variability for 7 amino acids (see legend) D). The same measurements as C but using relative migration time (RMT)[3] to correct for migration time variability, compared to E) indexed migration time (iMT) to correct for variability. Panel F shows migration time variability (%RSD) for each method across 22 amino acids for each approach. Normalization using effective electrophoretic mobility (ε_0), as previously described[4], is not possible with this CE-MS method because the separation is performed under conditions of near-zero electroosmotic flow.

Single-Point External Calibration

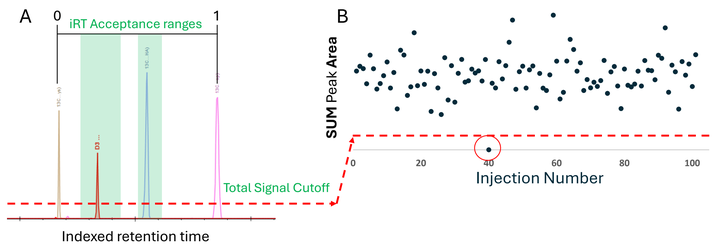


A separate validation experiment was performed as a proof-of-concept for single-point external calibration, using a standardized 13-point calibration curve for amino acids. Results for the typical calibration approach using linear regression and 1/x weighting (A) were compared against single-point calibration fit through zero using the 1 µM point as the calibrator (B) and the 100 µM point as the calibrator (C). All analyses performed in Skyline.[5] At ≥1 µM, 95% of all results measured were within 20% bias (514/541 measurements).



Using single-point external calibration, quantitative data for 126 metabolites showed good inter-day precision using PCA (D). Breakdown of these metabolites across classes for each matrix is shown in panel E. A quantitative comparison of the NIST SRM-1950 results showed excellent comparability to Mundal et al. [6]

Single-Sample Data Quality Check



A preliminary 'data check' can be run on each sample using a small set of SIL standards (four shown in Panel A). The green regions represent the range where the two 'indicator' SIL standards are expected to elute. In (B), a minimum total peak area for the four internal standards, can be used to estimate sample prep and proper injection volume. Either of these metrics, or in combination, can be used to flag a run for reanalysis and if necessary, repeat prep.

Conclusions

- **Indexed migration time** (iRT approach) using SIL standards for the regression, shows superior performance to RMT for correcting migration variability
- **Single-point external calibration**, using stable-isotope labeled internal standards in each sample to correct for response, has the following **advantages**:
  - Simpler to deploy and automate, with no calibration curve curation
  - Takes less space on the plate
  - Can utilize matrix-based calibration standards
- With the following **requirements and limitations**:
  - There must be enough of the analyte in the calibrator to estimate response (hard for low-abundant analytes)
  - Less clear cutoffs for upper- and lower-limits of quantification
- SIL standards represent a powerful tool for individual-sample data quality checks using iRT and intensity cutoffs

References

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6. Mundal R, et al. *Anal. Chem*. **2025**; 97(1): 667-675.

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