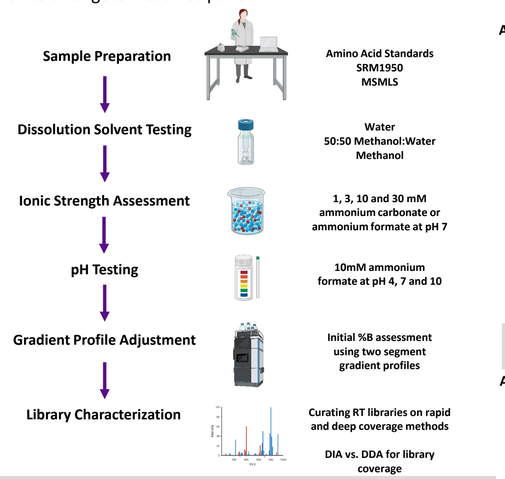
Evaluation of a UPLC zwitterionic HILIC stationary phase for deep coverage and high throughput metabolomics

Rebecca E. Jones[1], Lia Ficaro[1], Tori Rodrick[1], Manor Askenazi[2], Yik Siu[1], Thomas H. Walter[3], Jonathan Turner[3], and Drew R. Jones[1]

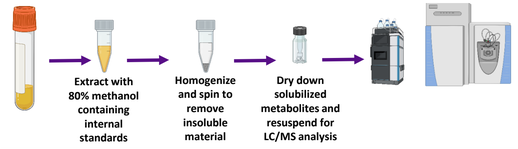
[1]NYU Langone Health, New York; [2]Biomedical Hosting LLC, Arlington, MA; [3]Waters Corporation, Milford, Milford, MA

Overview

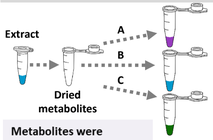
Great advances in hydrophilic interaction liquid chromatography (HILIC) have improved the separation of previously challenging polar analytes. While chromatographic performance has been lacking with only HPLC or near-UPLC particles being available, we have been able to validate deep coverage and high throughput "rapid" methods for the detection of metabolites using Atlantis™ Premier BEH Z-HILIC Columns (1.7 µm 2.1 x 150 mm) produced by Waters™. Rapid coverage offers MSMLS retention library and exploration of DIA pseudo-spectra. Deep coverage method offers panels of named metabolites searched against NIST and METLIN databases as well as untargeted features panels.

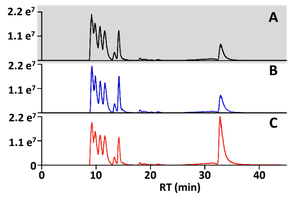


Sample Preparation



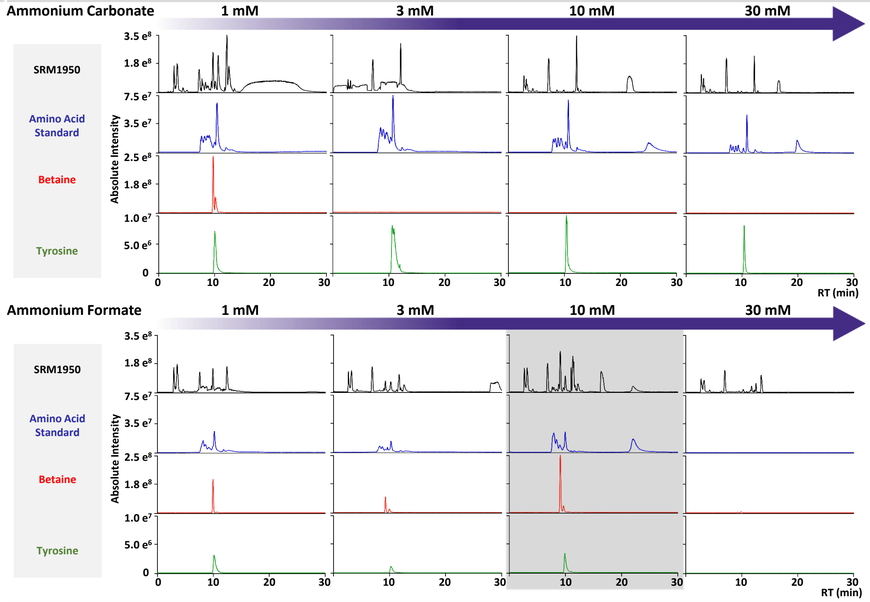
Dissolution Solvent Testing





Metabolites were reconstituted in A) water, B) 50:50 methanol: water, or C) methanol. Nearly identical profiles showed dissolution solvent had minimal effect on performance.

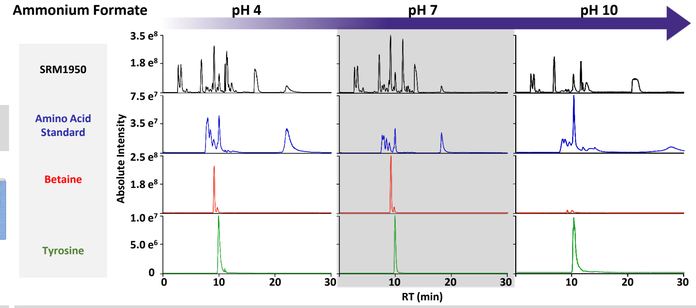
Ionic Strength Assessment

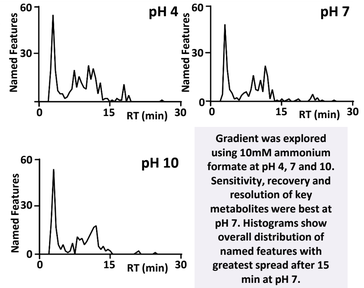


Using a gradient from our previously established HILIC method, we studied the effects of increasing ammonium carbonate ionic strength from 1-30mM. Data shows better resolution of complex matrices as ionic strength increased, however we began to lose signal/sensitivity for key metabolites of interest such as betaine.

The same study was repeated using 1-30mM ammonium acetate. Overall sensitivity of the base peak was comparable to the ammonium carbonate. Peak shape and sensitivity was best at 10mM. At 30mM, betaine and tyrosine dropped drastically in sensitivity. Thus, 10mM ammonium formate was chosen for future analyses.

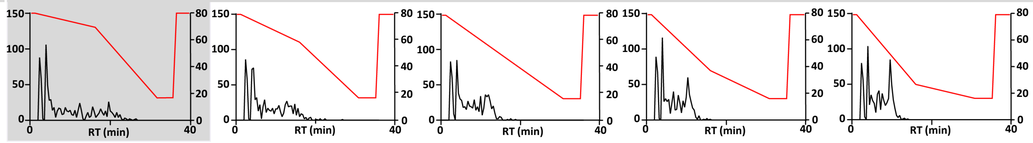
pH Testing





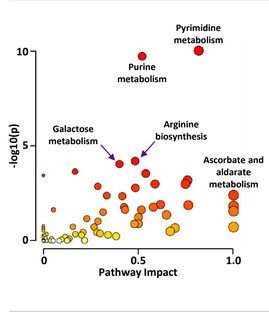
Gradient was explored using 10mM ammonium formate at pH 4, 7 and 10. Sensitivity, recovery and resolution of key metabolites were best at pH 7. Histograms show overall distribution of named features with greatest spread after 15 min at pH 7.

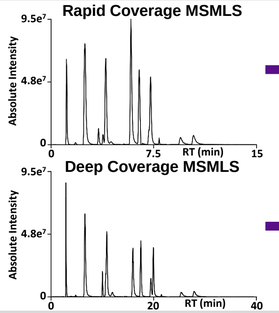
Gradient Profile Adjustment

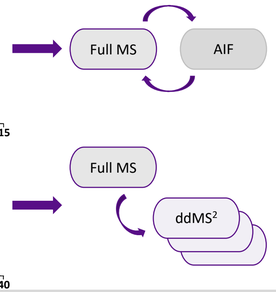


Initial %B studies (not shown) yielded elution of unwanted lipids in the first gradient segment when B was >80%. Therefore, 80% B was chosen to force elution of lipids within the first few minutes. Two segment gradients were explored using varying slopes (shown above). Less steep starting slopes show larger spread of metabolites across the first 15 min of run time. More aggressive slopes in the second segment showed greatest spread of less polar compounds. Therefore, we chose to use the first gradient for greatest distribution of metabolites and enhanced use of MS duty cycle for fragmentation.

Library Characterization



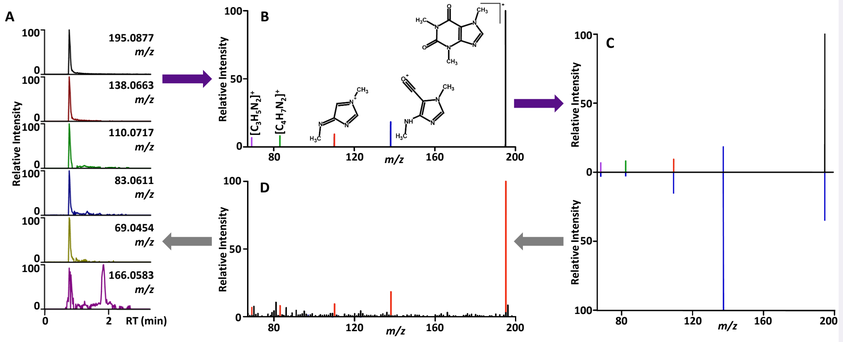




- Full scan pos/neg MS
- DIA in positive and negative modes
- Validated library search with known RTs of over 450 metabolites.
- Explore pseudo-spectral searches against known databases.

- Full scan pos/neg MS
- Top 5 DDA in positive and negative modes
- Named features annotation using MS^2 searches against NIST and METLIN databases with custom scoring.
- Untargeted features annotation using in-house software

Data Independent Acquisition



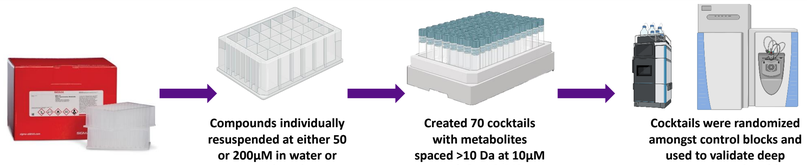
DIA data was investigated for correlation using XICs of fragments acquired at the same RT (A). Reconstruction of peaks showing similar shape enabled creation of pseudo MS^2 spectrum (B) with plausible structure predictions. Pseudo-spectrum was searched against NIST database to confirm putative metabolite (C).

Process can be reversed by taking known database spectra with known peak correlations (C lower plot), and looking for these patterns within AIF data (D). This can allow for reconstruction of correlated XICs and putative identification of large amounts of known metabolites. In-house software is currently under development to handle both directions.

Library Generation

MSMLS Library Contents:
634 compounds in seven 96-well plates at 5µg each:

- Carboxylic acids
- Amino acids
- Biogenic amines
- Polyamines
- Nucleotides, coenzymes
- and vitamins
- Mono- and disaccharides
- Fatty acids, lipids,
- steroids, and hormones



Compounds individually resuspended at either 50 or 200µM in water or methanol

Created 70 cocktails with metabolites spaced >10 Da at 10µM each for LC/MS validation

Cocktails were randomized amongst control blocks and used to validate deep coverage and rapid metabolomics assays

Conclusions

- Sample solvent does not effect retention/sensitivity
- Increasing ionic strength showed better peak shape in formate and carbonate studies.
- Some key metabolites became undetected at higher ionic strengths of carbonate, so we chose 10mM ammonium acetate.
- Low/neutral pH studies with formate gave best peak shape and greater metabolite IDs. Neutral pH was chosen due to large variety of pK_as in complex matrices of interest.
- Gradient investigation showed most exclusion of undesirable lipid peaks with >80% initial B.
- Library cocktails showed greatest metabolite distribution with more shallow slope at beginning of gradient elution.
- Rapid method allows for discrimination of metabolites in MSMLS library and investigation of pseudo-spectra from DIA.
- Deep coverage allows for increased duty cycle and broader coverage of named and untargeted features by MS^2.
- In-house software will create and search pseudo-spectra against known databases. We are updating script to include reverse searching to allow blast searches of database correlations against all AIF data.