Selection of Mobile Phase Modifiers for High Efficiency HILIC Separations

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Summary

In high performance liquid chromatography (HPLC) mobile phase modifiers (buffers, and other additives) are common practice to achieve better performance in both Reverse Phase (RP) and Hydrophilic Interaction Chromatography (HILIC). Trifluoroacetic acid (TFA) has proven to be a better modifier for improved peak symmetry, peak widths, and column overloading. It is a widely used modifier, but for separations using liquid chromatography-mass spectrometry (LC-MS) this modifier is often problematic. Formic acid (FA) has become popular, as it is volatile and broadly compatible with electrospray ionization (ESI) MS. Recent research has shown that the addition of the volatile neutral buffer ammonium formate (AmmForm), can provide enhanced separation performance. Mixtures of FA and AmmForm are also compatible with ESI MS. Another acidic modifier, difluoroacetic acid (DFA), is an alternative to TFA, exhibiting weak ion pairing properties. The use of FA , AmmForm, and DFA modifiers were examined for HILIC separations of small basic molecules (nucleobases, nucleosides, and derivatives) and peptides (mixture of four Angiotensins). HPLC separations used highly efficient superficially porous particles (HALO™ Fused-Core) bonded with the Penta-HILIC stationary phase. The use of this high performance material, along with ***appropriate*** mobile phase additives produced separations that exhibited excellent peak symmetry, decreased peak widths, and high column efficiency. Examples are shown of these improvements can be applied to LC-MS analyses. Future research will focus on expanding and evaluating new mobile phase modifiers (and their combinations) for use in LC-MS applications.

Objectives

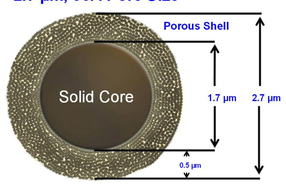
Apply different mobile modifiers and combinations for:

- ❖High performance HILIC separations (e.g. symmetrical peaks and high column efficiency)
- ❖Resolving mixtures of structurally similar small molecules and peptides
- ❖Compatibility with LC-MS applications

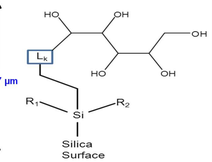
HALO® Penta-HILIC

Penta-HILIC is a novel stationary phase that uses 5 hydroxyl groups to create a polar surface for use in HILIC separations. The ligand is attached to the surface with a proprietary linker, L_k, and silane side chains, R_1 and R_2. HALO® Fused-Core particles bonded with the Penta-HILIC Stationary phase allow for high performance HILIC separations. This material is available in the new 2 µm particle size, as well as the 2.7 and 5 µm particle sizes. The dimensions of the 2.7 µm particle are shown below.

Fused-Core Particles
2.7 µm, 90Å Pore Size



Bonded Phase Ligand



Now available in 2µm particle size

Experimental

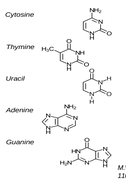
Instrumentation: HPLC experiments (unless otherwise stated) were performed on a Shimadzu Nexera UFLC equipped with a SPD-M30A Diode Array Detector. Integration and data analysis were done through the Shimadzu LabSolutions software. LC-MS experiments were performed on a Shimadzu LCMS-2020 single quadrupole mass spectrometer, with Electrospray Ionization (ESI).

HPLC Columns: All 4.6x100 and 2.1x100mm columns were prepared in-house (Advanced Materials Technology).These columns were loaded with 2.7µm, 90Å Penta-HILIC Fused-Core particles.

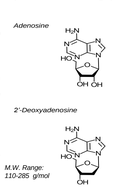
Analytes: Standard nucleobases, nucleosides, and derivatives were obtained from Sigma and prepared in either 0.1% formic acid, or in 50/50 ACN/H$_2$O mixtures. Angiotensin peptides were obtained from Sigma and prepared in aqueous solutions. All samples were made up in the weak mobile phase used for experiment.

Mobile Phases: Mobile phases are expressed as concentration of buffer in final solution, with pH in the aqueous component. 0.1% acidic solutions were measured by volume.

Nucleobase:



Nucleoside:
(Nucleobase + ribose sugar)



M.W. Range: 110-285 g/mol

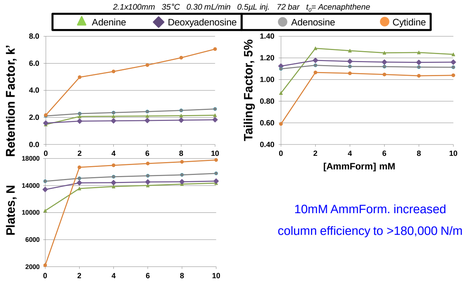
Angiotensin Peptide Forms

- **Angiotensin I, A-I** *(1296.48 g/mol)*
  Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
- **Angiotensin Fragment 1-7, A (F 1-7)** *(899.00 g/mol)*
  Asp-Arg-Val-Tyr-Ile-His-Pro
- **Angiotensin II, A-II** *(1046.18 g/mol)*
  Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
- **Angiotensin III, A-III** *(931.09 g/mol)*
  Arg-Val-Tyr-Ile-His-Pro-Phe

High Efficiency Separations with Ammonium Formate

Effect of [AmmForm]

Mobile Phase: 90/10 ACN/H$_2$O + 0.1% FA (with specified concentration of AmmForm)
2.1x100mm 35°C 0.30 mL/min 0.5µL inj. 72 bar t_o= Acenaphthene

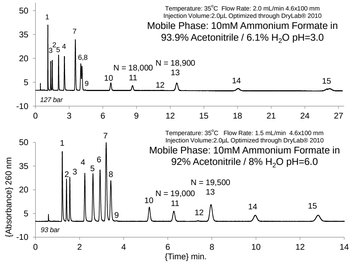


10mM AmmForm. increased column efficiency to >180,000 N/m

Appropriate AmmForm concentration resulted in highly efficient and symmetrical peaks, along with adjustable selectivity

Isocratic Separations at 2 pH's

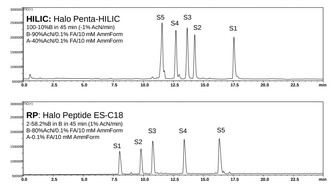
Analytes
1 Thymine
2 Uracil
3 Thymidine
4 2'-Deoxyadenosine
5 Adenine
6 Uridine
7 Adenosine
8 Hypoxanthine
9 Xanthine
10 Cytosine
11 2'-Deoxycytidine
12 Guanine
13 2'-Deoxyguanosine
14 Cytidine
15 Guanosine



Fully resolved isocratic separation of all 15 components was achieved on a high efficiency Penta-HILIC column, using an ***appropriate*** low ionic strength mobile phase modifier

HILIC and RP Peptide Comparison

RP and HILIC Peptide Separations
2.1 mm ID x 100 mm, 0.35 mL/min, 40°C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s



- 2.7 µm Penta-HILIC columns demonstrate high performance separations of peptides using LC-MS compatible mobile phases
- The above separation of 5 synthetic peptides demonstrates the comparable peak width and symmetry between the two modes of separation
- Penta-HILIC columns provide a high performance option for varying the selectivity in peptide separations

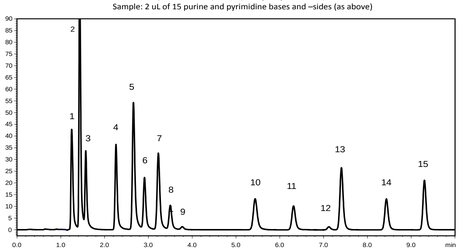
Mobile Phase Considerations for LC-MS

- **A concern in LC-MS separations of peptides and other biomolecules is the correct selection of mobile phase and modifiers.**
  The mobile phase should provide conditions for enhanced separations and detection.
- **TFA is a commonly used acidic modifier in the separation of biomolecules, that improves peak symmetry and width through ion pairing.**
  This modifier can be a poor choice for ESI sources in LC-MS.
- **DFA is a novel alternative to TFA that exhibits similar improvements in chromatography.**
  Previous in-house experiments with this modifier have shown good compatibility with ESI MS for RP and HILIC separations of peptides and proteins.
- **A comparison peptide separation between mobile phases modified with DFA and FA is shown below.**
  As was seen with the previous comparison of DFA and FA, the addition of AmmForm was useful for symmetrical peaks, retention, and efficiency.

HILIC Separation of Nucleobases, Nucleosides, and Derivatives

Gradient Separation of 15 Component Mixture

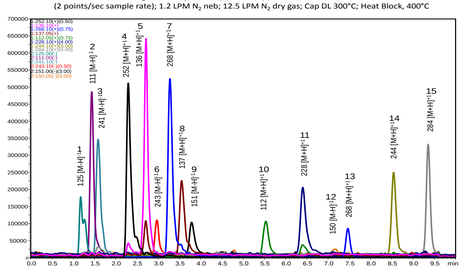
Shimadzu Nexera LC: 2.1 mm ID x 100 mm Halo Penta HILIC column; 35°C ; Abs. 260 nm
Gradient: 6% B for 3.5 min, 65-100% B 3.5 to 9.5 min., hold 100% B for 0.5 min;
A – 10mM Ammonium Formate in 93% AcN/7% H$_2$O, pH 6.0; B – A at 85% AcN
Flow 0.31 mL/min 0-10 min, 1.0 mL/min (6%B) 10-16 min, return to initial
Sample: 2 uL of 15 purine and pyrimidine bases and –sides (as above)



LC-MS Detection of Molecular Ions

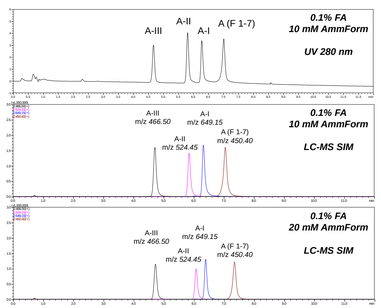
Resolution and detection of all molecular ions can enable further development of methods for identification of these compounds in biological samples

Shimadzu LCMS-2020 Single Quadrupole: ESI (+) 4.5 kV, (-) 3.5 kV; SIM 1 amu; 0.25 sec each SIM Event
(2 points/sec sample rate); 1.2 LPM N_2 neb; 12.5 LPM N_2 dry gas; Cap DL 300°C; Heat Block, 400°C



Angiotensin LC-MS

Separation Performance

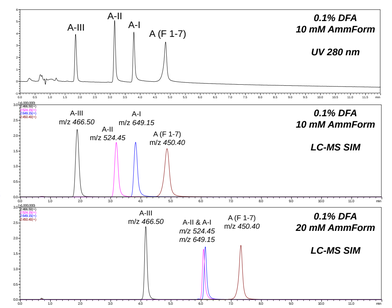


2.1x100 mm, 35ºC, 0.30 mL/min, 2.0 µL inj., 85 bar

B= 90/10 ACN/H$_2$O + (Specified Modifiers)
A= 50/50 ACN/H$_2$O + (Specified Modifiers)
20-60% A over 12 min

Addition of AmmForm

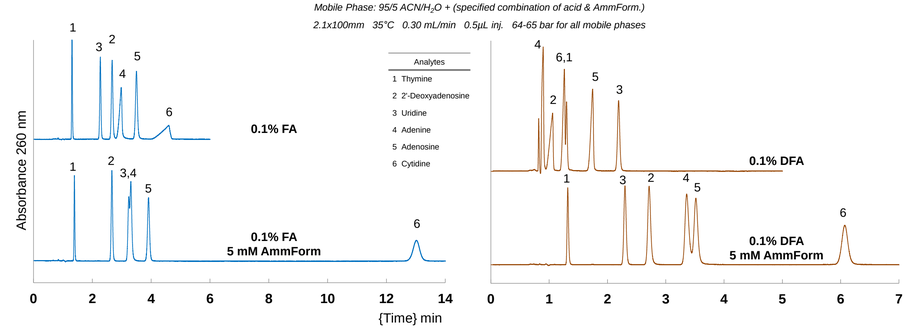
- Increased R_T
- Improved peak symmetry
- Minimal change in peak widths between 10 and 20mM conc.



Retention and resolution can be manipulated by choice of DFA or FA, and by adding AmmForm

Comparison of DFA and FA for Nucleobase and Nucleoside Separations

Mobile Phase: 95/5 ACN/H$_2$O + (specified combination of acid & AmmForm.)
2.1x100mm 35°C 0.30 mL/min 0.5µL inj. 64-65 bar for all mobile phases

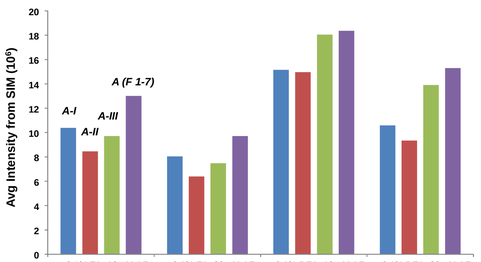


Analytes
1 Thymine
2 2'-Deoxyadenosine
3 Uridine
4 Adenine
5 Adenosine
6 Cytidine

The combination of acidic modifier and AmmForm provides on average higher column efficiency, improved tailing factor, and increased retention. 0.1% DFA and 5mM AmmForm demonstrated the best separation under these conditions.

MS Signal Comparison

- Modest (20%) decrease in intensity with increased [AmmForm]
- The intensities measured for DFA mobile phases were greater than or equal to FA
- There was no ionization suppression for the fluorinated acidic modifier



Conclusions

The selection of appropriate mobile phase modifiers, along with the use of Penta-HILIC columns enabled high performance HILIC separations. This performance was seen across HPLC and LC-MS analyses.

Mobile Phase Considerations:

- AmmForm was shown to be a useful mobile phase modifier for the separations of small basic molecules and peptides. It was shown to be compatible with LC-MS analyses.
- FA and DFA both showed useful characteristic as modifiers. Combinations of each acid with AmmForm provided improvements in separation performance. Both were shown to be compatible with LC-MS analyses.

Stationary Phase Considerations :

- The Penta-HILIC stationary phase was shown to efficiently resolve a complex mixture with good selectivity and high efficiency (>180,000 N/m).
- Penta-HILIC columns provide a high performance option for varying the selectivity in peptide separations, with chromatographic performance comparable to that of RP separations.

Future research will be focused on expanding and evaluating new mobile phase modifiers (and their combinations) for their use in HPLC and LC-MS applications.

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