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Development and Optimization of a HILIC-MS Separation of 17 Free Amino Acids using an XBridge Premier BEH Amide Column

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Abstract

Method development can be an arduous task. While method screening may have a well-defined process associated with it, optimization tasks are less well documented, and often are done on an as-needed basis. Some common parameters that may require optimization include the gradient slope, column temperature, and the mobile phase additive concentration or pH.

In this application note, we describe the development of a HILIC separation of seventeen free amino acids using a stepwise, methodical approach for column and mobile phase pH screening and method optimization. Due to the lack of significant chromophores, mass detection was employed for these analytes, using an ACQUITY QDa Detector. The final method employs not only a water gradient, but also a buffer concentration gradient. Good separation of the isobaric compounds isoleucine and leucine was achieved, as well as acceptable peak shapes for all 17 amino acids.

Benefits

- Baseline separation of isobaric compounds isoleucine and leucine

- Acceptable peak shapes for all 17 amino acids
- Stepwise approach to HILIC method optimization
- Overview of HILIC method screening activities

Introduction

Hydrophilic Interaction Chromatography (HILIC) is a technique specifically designed to separate polar analytes.[1] This technique uses a polar stationary phase and a less polar mobile phase. The stationary phase adsorbs some of the aqueous component of the mobile phase, forming an immobilized aqueous layer into which the analytes may partition. The amount of partitioning depends on the volume of the adsorbed aqueous layer and the chemical properties of the analytes. Ionic and hydrogen-bonding interactions also contribute to retention in HILIC. Unlike reversed-phase chromatography, highly polar analytes have greater retention in HILIC, while less polar compounds have lower retention. Due to the multi-modal retention mechanism present in HILIC, method development is not as straightforward as in reversed-phase chromatography.

Typical method development approaches, developed for reversed-phase assays, are still appropriate for HILIC separations. Column and mobile phase pH screening are very powerful first steps for HILIC method development as the stationary phase can have drastic effects on retention and separation quality. Unlike reversed-phase chromatography though, a strong solvent screening is not typical as water is needed to create the adsorbed aqueous layer on the stationary phase. Alternative solvents to acetonitrile, one of the weakest solvents in HILIC, include methanol, ethanol, and propanol. These polar solvents, while weaker than water in terms of HILIC elution strength will still cause elution to occur and can disrupt the adsorbed aqueous layer on the stationary phase. As such, typical screening parameters are limited to column stationary phase and mobile phase pH. Once the best column and mobile phase pH are selected method optimization can take place. For HILIC separations, optimization can take considerably longer than column screening as there are numerous ways to improve the separation and the intended effects of optimization can be confounded by other factors.

Adjusting the gradient slope, in terms of percent aqueous per column volume, is one step of optimization. Decreasing the gradient slope can lead to more potential interactions between the analytes and stationary phase due to increased time spent on the column. This can be beneficial for separating critical pairs. Another optimization step is adjusting the buffer concentration, which not only impacts the ionic interactions, but can

also affect the volume of the adsorbed aqueous layer and therefore the partitioning mechanism. Adjusting the buffer concentration can affect peak shapes and due to the changes in ionic interactions and partitioning can lead to slightly different selectivity as well. In some cases, optimization can require using a buffer gradient, wherein the buffer concentration is varied along with the aqueous composition as the gradient progresses. This is a common practice in ion exchange chromatography and can be beneficial to improve peak shapes or alter selectivity in HILIC as well.

The work shown here demonstrates the method development and optimization process for the HILIC separation of free amino acids. Using a standard mixture of 17 amino acids, three stationary phases were screened with both low pH and high pH mobile phases. After selection of the best combination of stationary and mobile phases, method optimization was performed using a stepwise, methodical approach. The final method for the assay employed both a water and a buffer gradient, using a pH 3 ammonium formate buffer with an XBridge Premier BEH Amide Column. MS detection was used for this assay as many of the free amino acids do not have significant chromophores for UV detection.

Experimental

Sample Description

An ampoule of the amino acid hydrolysate standard (p/n: WAT088122 < https://www.waters.com/nextgen/global/shop/standards--reagents/wat088122-amino-acid-standard-accq-tag-pico-tag-accq-tag-ultra.html>) was opened and placed on the system for analysis. This standard contains 17 amino acids at concentrations of 2.5 µmol/mL, except glutamic acid (2.6 µmol/mL), isoleucine (2.6 µmol/mL), alanine (2.6 µmol/mL), and cystine (1.27 µmol/mL) in 100% aqueous solution.

LC Conditions

LC system:	ACQUITY UPLC H-Class Plus System with Quaternary Solvent Manager (QSM) with optional solvent select valve, Sample Manager Flow Through Needle (SM-FTN), Column Manager, Column Manager Aux, and QDa mass detector

Detection:	MS full scan (ESI+) SIRs of amino acids determined with final method
Columns:	XBridge Premier BEH Amide Column 2.1 x 50 mm, 2.5 µm (p/n: 186009928) Atlantis™ Premier BEH Z-HILIC Column 2.1 x 50 mm, 2.5 µm (p/n: 186009985) CORTECS™ HILIC Column 2.1 x 50 mm 2.7 µm (p/n: 186007380)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	1.0 µL
Flow rate:	0.15 mL/min
Mobile phase A:	Milli-Q Water
Mobile phase B:	Acetonitrile
Mobile phase D1:	200 mM Ammonium Formate pH 3.0 prepared as outlined in HILIC Primer[1]
Mobile phase D6:	200 mM Ammonium Acetate pH 9.0 prepared as outlined in HILIC Primer[1]
Screening gradient conditions:	Constant 5% Dx was maintained throughout the gradient to ensure consistent additive concentration. Linear gradient of 0–45% A over 11.50 minutes. Hold at 45% A for 3.8 minutes.

	Return to starting conditions of 0% A and hold for 15 minutes. Total run time 31.0 minutes. Optimized gradient details provided in figure captions.

Data Management

Chromatography software:	Empower 3 Feature Release 4

Results and Discussion

The first step in HILIC method development is screening of stationary phases and mobile phase pH values. Care should be taken during this step to use high-pH stable stationary phases when implementing high pH buffer systems. Examples of high-pH stable columns include the BEH Amide and BEH Z-HILIC Columns which utilize a hybrid organic/inorganic base particle. Silica-based columns should not be used at pH values higher than recommended as silica particles are prone to dissolution, leading to column degradation.[2]

The main factor to consider when selecting columns is the bonded ligand and the selectivity differences between different column chemistries. HILIC column selectivity has been the subject of a number of studies.[3] Ideally, the columns selected for method development should span a wide range of selectivity to increase the chance of finding a suitable stationary phase for the separation. For this work, three columns were selected: an XBridge Premier BEH Amide Column, an Atlantis Premier BEH Z-HILIC Column, and a CORTECS HILIC Column. The amide and Z-HILIC phases are based on hybrid particles, allowing them to be used at both high and low pH. The CORTECS HILIC Column, which offers high efficiency due to the solid-core silica particle, is not stable at high pH and therefore was used only at low pH.

Additional consideration should be given to the column hardware selected. The metal oxide layer present on the surface of the metal components in both the system and column hardware can interact with analytes via ionic interactions, leading to non-specific adsorption. This presents as variable and decreased peak area and poor peak shape in the resulting chromatography. Waters MaxPeak High-Performance Surfaces (HPS) hardware, used in MaxPeak Premier Columns, has been proven to mitigate these interactions both in reversed-phase as well as HILIC applications.[4–7] MaxPeak HPS works by utilizing a bonded hybrid organic/inorganic modification

on the surface of the metal.[8] The presence of metal surfaces can confound method development as an analyst will not know if the analyte behavior seen is due to interaction with the stationary phase or with the column hardware. By using MaxPeak Premier Columns, an analyst can be sure that any results seen are purely from the analyte interacting with the mobile and stationary phases and not from other, unpredictable sources.

Based on previously published work, the columns chosen should provide different selectivities for this group of compounds as each has different retention characteristics.[7] The unbonded CORTECS HILIC Column works as a cation exchanger, preferentially retaining basic analytes via ionic interactions. The XBridge Premier BEH Amide Column, being a neutral phase, retains predominantly by partitioning with some weaker ionic interactions taking place as well. Lastly the zwitterionic Atlantis Premier BEH Z-HILIC Column has the greatest adsorbed aqueous volume, and therefore the highest retention via partitioning.[9] Additionally, the two charged groups on the sulfobetaine ligand can contribute to ionic interactions, but also dipole interactions. By testing all three of these stationary phases, the likelihood of finding a suitable separation is increased.

For any new method development activity, it is important to establish goals that must be achieved. When setting these goals, parameters such as resolution, peak shape and retention should be considered. Other parameters may be included based on the intended purpose of the method. For the amino acid separation, given the complex nature of the sample, a resolution requirement was set only for the isobaric compounds isoleucine and leucine. Since these analytes share the same precursor and product ions in a mass spectrometer, it's critical to achieve baseline separation so that they can be identified via retention time. The other amino acids in this mixture have unique masses, making it less critical that baseline separation is achieved. The other parameter that is important for this work is peak shape. Having good peak shape with narrow peaks is important for accurate quantitation and sensitive detection. Ideally the peak shape, as measured by the USP tailing factor, should be between 0.8–1.2 for all amino acids. However, values outside that range may be acceptable on a case-by-case basis.

Figure 1 shows the screening results at high pH using extracted ion chromatograms (EICs) of the 17 amino acids. Savitsky-Golay smoothing was used at a level of 15. On both columns, the isobaric pair of isoleucine/leucine (the starred peaks) was not fully resolved. This would make quantifying these analytes challenging. While not ideal, both columns could be optimized to further improve this separation. The XBridge Premier BEH Amide Column gave slightly better peak shape for the other analytes compared to the results obtained on the Atlantis Premier BEH Z-HILIC Column. This could be due to secondary interactions between the zwitterionic ligand and the amino acids. Additionally, due to the greater adsorbed aqueous volume on the BEH Z-HILIC Column the analytes may be more extensively partitioned into the aqueous layer. This could also cause wider peaks.[9] Low pH mobile

phase conditions may provide better results for this separation. Figure 2 shows the separations of the amino acids on the three low-pH stable stationary phases.

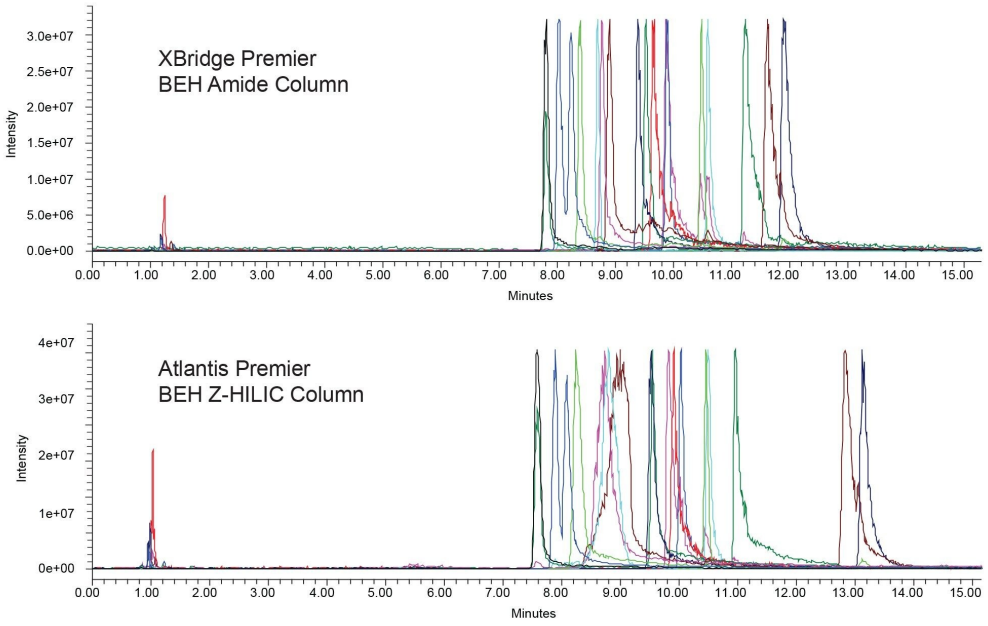

Figure 1. Overlay of sixteen EICs obtained on the indicated columns using a 10 mM ammonium acetate pH 9.0 mobile phase. A linear aqueous gradient of 5–50% over 11.5 minutes with a 4-minute hold at high aqueous content was used. The buffer concentration was maintained at 10 mM throughout the gradient. The tagged peaks are isoleucine and leucine which are isobaric and therefore use the same EIC.

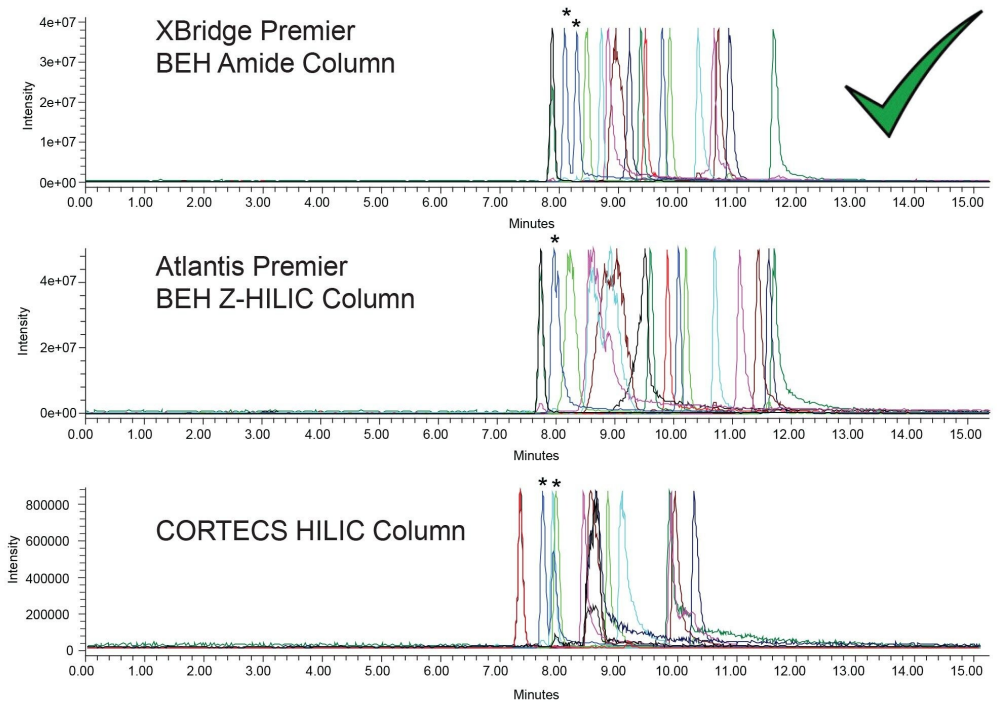

Figure 2. Overlay of sixteen EICs obtained on the indicated columns using a 10 mM ammonium formate pH 3.0 mobile phase. A linear aqueous gradient of 5–50% over 11.5 minutes with a 4-minute hold at high aqueous content was used. The buffer concentration was maintained at 10 mM throughout the gradient. The tagged peaks are isoleucine and leucine which are isobaric and therefore use the same EIC.

Immediately it is apparent that the BEH Z-HILIC Column at low pH is not suitable for this assay using these conditions. Isoleucine and Leucine are almost completely co-eluting and peak shapes for some of the analytes are unacceptable as well. The CORTECS HILIC Column shows reasonable separation of the leucine/isoleucine pair, but poor peak shape and low MS signal for a good portion of the analytes. Additionally, many of the compounds are poorly resolved, which is far from ideal. The BEH Amide Column at low pH shows decent separation for the isobaric compounds, and reasonable peak shape for most of the other amino acids. While still not meeting all criteria, the BEH Amide Column using the low pH mobile phase buffer was selected for optimization. Improvements to peak shape and increasing the resolution of the isobaric compounds can be obtained via optimization.

Optimizing this method is not an easy task due to the complexity of the sample, but by taking the process one

step at a time, an acceptable separation was obtained. First, the gradient steepness was adjusted, specifically reducing the steepness. By making the gradient more shallow, the analytes have more time to interact with the stationary phase, potentially improving the separation quality. Gradient steepness is measured as percent strong solvent, in this case aqueous mobile phase, per column volume. Figure 3 shows the separations achieved using three different gradient slopes. In this case, each separation started at a different aqueous concentration while maintaining the gradient time and ending aqueous concentration.

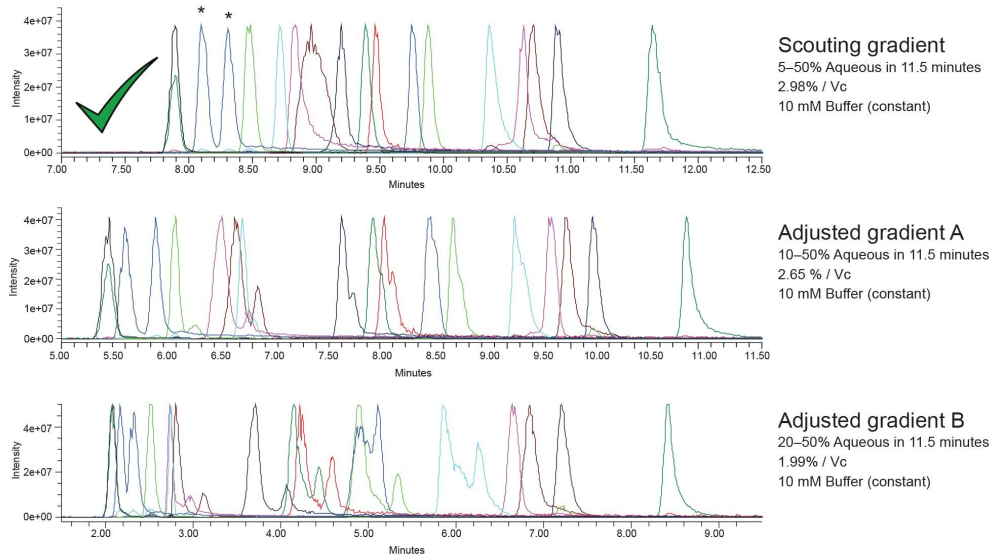

Figure 3. Attempts to improve the amino acids separation using progressively shallower gradient slopes with an XBridge Premier BEH Amide Column using an ammonium formate pH 3.0 buffer at a constant concentration of 10 mM.

As seen, decreasing the gradient slope did not improve the quality of the separation. Quite the opposite in fact, as isoleucine and leucine co-eluted more with shallower slopes, and some peaks developed a second peak at the same mass. Not all analytes are subject to this effect. For example the four latest eluting compounds do not show any secondary peaks forming, while others do. The initial scouting gradient provided the best overall separation for these compounds, and was used in the next step, which is adjusting the buffer concentration.

Typically, adjusting buffer concentration can be done in one of two ways. First, instead of a 10 mM buffer the method could use a different concentration, such as 20 mM while remaining constant throughout the gradient.

However, due to the gradient slope and aqueous gradient of 5–50% the current method is somewhat limited due to potential solubility issues. Instead, the buffer concentration was adjusted another way, which is using a buffer gradient along with the aqueous gradient. This avoids the potential issue of buffer insolubility in high organic mobile phases but still addresses the issue of peak shape or selectivity by affecting the ionic interactions between analyte and stationary phase. Figure 4 shows two different buffer gradients compared to the initial screening gradient which used a constant 10 mM buffer concentration.

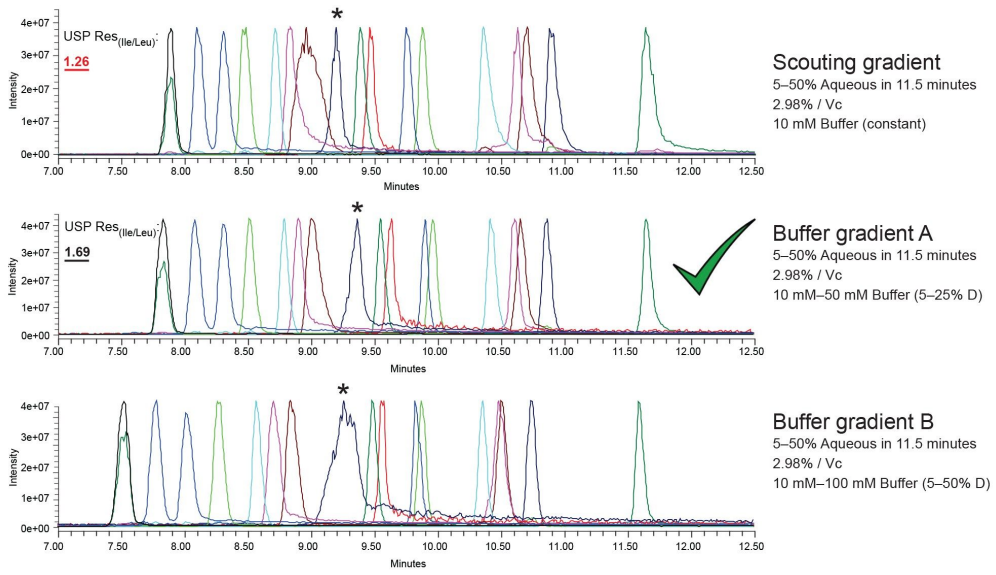

*Figure 4. Buffer gradient comparisons on an XBridge Premier BEH Amide Column. Buffer and mobile phase conditions shown in the figure. The * indicates the alanine peak.*

Using a 10–50 mM gradient buffer improves the resolution of the isoleucine and leucine pair from 1.26 to 1.69, meeting the resolution criterion for this assay. Looking more closely at that separation, peak shapes have been improved for many of the compounds as well. Only one peak showed worse peak shape using the 10–50 mM gradient compared to the constant 10 mM buffer concentration. This is the alanine peak which became slightly wider using the buffer gradient. Conversely the peak eluting directly before alanine, which is proline, is much more symmetrical and narrower when the 10–50 mM buffer gradient was used. This is likely due to the mitigation of ionic interactions between proline and the stationary phase.

A 10–100 mM buffer gradient was also tried, however the peak shape for alanine became considerably worse. The separation of isoleucine and leucine was about the same between the two buffer gradients, but with the better peak shape for alanine, the 10–50 mM buffer was selected for final testing. The final method conditions were tested using replicate injections of the sample and single ion recording (SIR) for the analytes. Scheduled SIRs were used to improve MS signal and a stack plot of the replicate injections is shown in Figure 5.

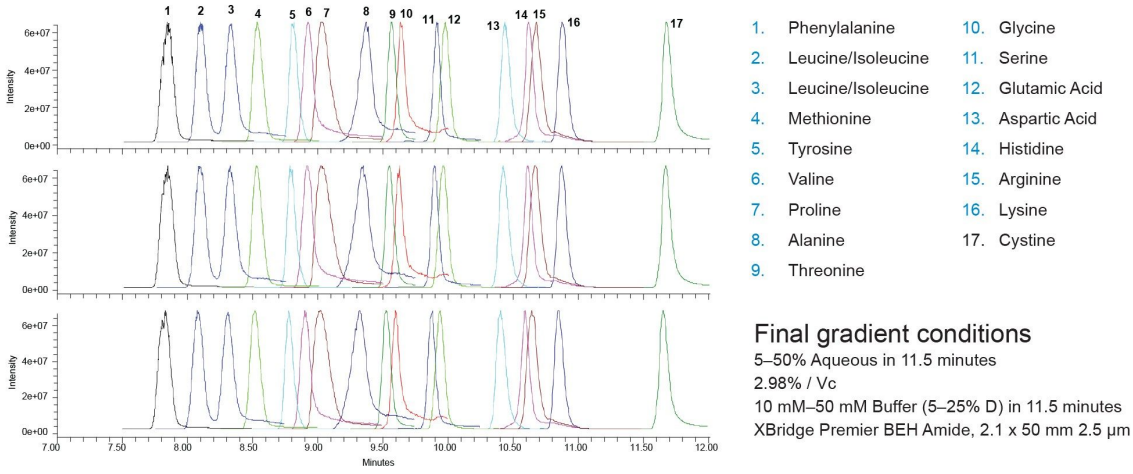

Figure 5. Triplicate injections of the amino acid standard mixture on an XBridge Premier BEH Amide Column using a buffer and aqueous gradient. The buffer concentration varied from 10–50 mM over 11.5 minutes, while the aqueous content was also adjusted from 5–50% over the same time. Scheduled SIRs were used to detect the analytes. 1) phenylalanine, 2/3) isoleucine/leucine, 4) methionine, 5) tyrosine, 6) valine, 7) proline, 8) alanine, 9) threonine, 10) glycine, 11) serine, 12) glutamic acid, 13) aspartic acid, 14) histidine, 15) arginine, 16) lysine, 17) cystine.

The final method meets the criteria set earlier with some exceptions. Baseline separation of isoleucine and leucine was achieved and USP tailing factors were within the target range for most of the compounds. Proline, alanine, glycine, and arginine are the only analytes which have USP tailing factors outside the range of 0.8–1.2, but the obtained values are still less than 2.0 with USP tailing factors for the four compounds at 1.5, 1.5, 1.7, and 1.7 respectively. Based on the results seen during method development, where changing the buffer concentration affects the analytes in different ways, the chosen method is the best compromise for peak shape. This method can be used for future work including moving into a validated workflow or quantifying amino acids in real samples.

Conclusion

A step-by-step method development procedure was shown for free amino acid analysis using HILIC. First, column and mobile phase screening was performed using three different HILIC stationary phases and two mobile phase pH values. The initial scouting results showed that the best results were obtained using an XBridge Premier BEH Amide Column with a pH 3.0 ammonium formate mobile phase buffer. Method optimization was then performed including testing different gradient slopes and buffer concentration gradients. The final method included both an aqueous and buffer gradient which resulted in good separation of the isobaric compounds isoleucine and leucine, as well as reasonable peak shapes for the other fifteen amino acids present in the sample. By approaching HILIC method development in a systematic manner, effective methods can be developed to separate challenging mixtures of polar analytes.

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