Controlling Selectivity on Zwitterionic HILIC Columns by Adjusting pH and Buffer Strength

Petrus Hemstrom[1)], Amos Heckendorf[2)], Wen Jiang[1)], Tobias Jonsson[1)] and Patrik Appelblad[1)]
[1)]EMD Millipore, [2)] The Nest Group

Abstract
Use of ionic interactions, by manipulation of mobile phase pH and buffer strength, alters the selectivity of a zwitterionic, HILIC stationary phase for three similarly charged model compounds, glutamine, α-keto glutarate and glutamic acid.

Introduction
Hydrophilic Interaction Chromatography is quite suitable for discriminating between glutamic acid and other organic acids of similar polar structure. Glutamate (glutamic acid) is abundant in the human body, but particularly in the nervous system and is especially prominent in the human brain where it is the body's most prominent neurotransmitter, the brain's main excitatory neurotransmitter, and also the precursor for GABA, the brain's main inhibitory neurotransmitter. Glutamic acid is valued as a precursor for GABA, a calming brain neurotransmitter known for its ability to cool down anxiety, produce feelings of peace and calm. Thus it has potential as a new approach for anti-anxiety drugs.

Glutamic acid is also used as food flavoring and, as such, there is a need to accurately measure it.

Polar partitioning is the main retention mechanism in HILIC. There are numerous stationary phases with different bonded phase chemistries, most of which carry ionic charges, either deliberately incorporated or from residual silanol groups. The presence of ionic interactions affect HILIC partitioning and it is this mixed mode separation aspect which this paper attempts to explore.
Numerous papers have been published examining the differences between HILIC stationary phases and the effects on retention from altering chromatographic conditions. There is now a consensus that there are four distinct classes of HILIC phases, neutral, anionic (silica), cationic (amine containing phases) and zwitterionic[1].
A charged functional group on a column's surface has an order of magnitude greater free energy of interaction with charged analytes than that of an uncharged (Table1). These electrostatic interactions provide the possibility of changing retention time and controlling selectivity by altering pH and/or buffer strength. The strength of ionic interactions require, however, the addition of high concentrations of salt in order to overcome the electrostatic interactions between charged (anionic or cationic) stationary phases and charged analytes and get reasonable retention.

Truly zwitterionic HILIC stationary phases also provide sites for such electrostatic interactions, but at a much lower magnitude, due to close proximity of ion and counter ion (in balanced proportions) within their functional groups. In the ZIC-HILIC column the distal charge, the sulfonate, will dominate the interaction and the phase will behave as a net cation exchanger[1-3] at buffer concentrations low enough to expose analytes to this charge. The possibility of changing column selectivity is a very powerful tool in a chromatographer's toolbox and in HILIC (especially with zwitterionic phases) it is more easily utilized than in reversed phase chromatography. By changing the buffer strength or pH a similar dramatic effects on selectivity can be achieved as when adding ion-pairing agents in reversed phase, but with the benefit of having incremental control enabling fine-tuning of retention and selectivity within a range of conditions. This is of course only feasible if the charge state of the stationary phase is pH independent. In this note we will show how compounds can be selectively moved to avoid co-elution with other similar compounds by simply adjusting pH and buffer strength.

Interaction	Energy (kcal/mole)
Covalent Interaction	100-300
Ionic Interaction	50-75
Polar Interactions (Hydrogen bonding, dipole-dipole, π-π)	3-7
Non polar inetractions (van der Vaals or dispersion)	1-2

Table 1.

Method

Preparation of a stock solution of buffer at two different pH simplified solvent preparation. Listed buffer concentrations in each experiment refer to final total concentrations. Ammonium formate at pH 3 was prepared using ammonium formate and adjusted with formic acid. The ammonium formate buffer pH 6.5 was used without pH adjustment. A SeQuant® ZIC®-HILIC column, 150 x 2.1 mm (5 µm, 200 Å) was operated at a flow rate of 0.4 mL/min at 30°C, with an eluent of 70% ACN and 30% buffer (v/v) for all experiments. Due to the analytes low UV absorbance detection was with a Sedere Sedex 85 ELSD equipped with a low flow nebulizer cell. Three analytes were chosen for this study. Glutamine (Gln), which is a zwitterionic amino acid, with a hydrophilic side chain containing an amide functionality having pKa values of 2.2 and 9.1, making it exhibit stable state of ionization in the studied pH range. Glutamic acid (Glu), which is an acidic amino acid with a carboxylic side chain having pKa values of 2.2, 4.2 and 9.7. α-Ketoglutarate, which is a dicarboxylic acid with pKa values of 2.4 and 4.4.

Results

Acidic pH & low buffer strength

Protonated "neutral" analytes and ionic betaine sulfonate column surface

At pH 3.0 and 3 mM buffer, the sidechain carboxyls (pKa ~4.2) will be protonated, and thus act as neutral polar groups, whereas the alpha carboxyls of Glu and Gln (pKa 2.2) and α-ketoglutarate (pKa 2.4) will be ionic and repelled by the negatively charged sulfonates of the column. In the amino acids Glu and Gln the negative charge is balanced by the positively charged amino group. Separation of the amino acids relies on the small polarity differences of the side chains: a neutral carboxyl vs. an amide. The more repelled, negatively charged α-ketoglutaric acid has the shortest retention time.

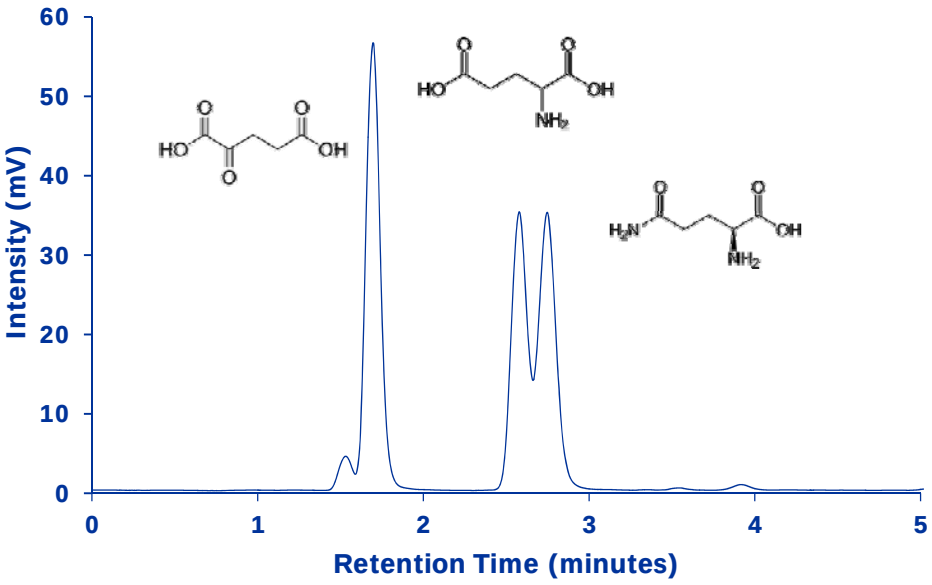

Figure 1. Separation at 10 mM (3 mM final) ammonium formate pH 3.

Results

Neutral pH & low buffer strength

Ionic analytes and ionic betaine sulfonate column surface

At pH 6.5 there is an overall increase in polarity from the, now, all ionized carboxyls; an increased retention for both glutamic and α-ketoglutaric acid, but not for glutamine, could be expected.

However, as the analytes carboxyls are now unprotonated and thus negatively charged, they are now repelled by the negatively charged distal sulfonates on the stationary phase limiting the analytes ability to partition into the water layer on the stationary phase.
The only visible effect of changing the pH is that the glutamic acid is moved to a shorter retention time due to the increased electrostatic repulsion.

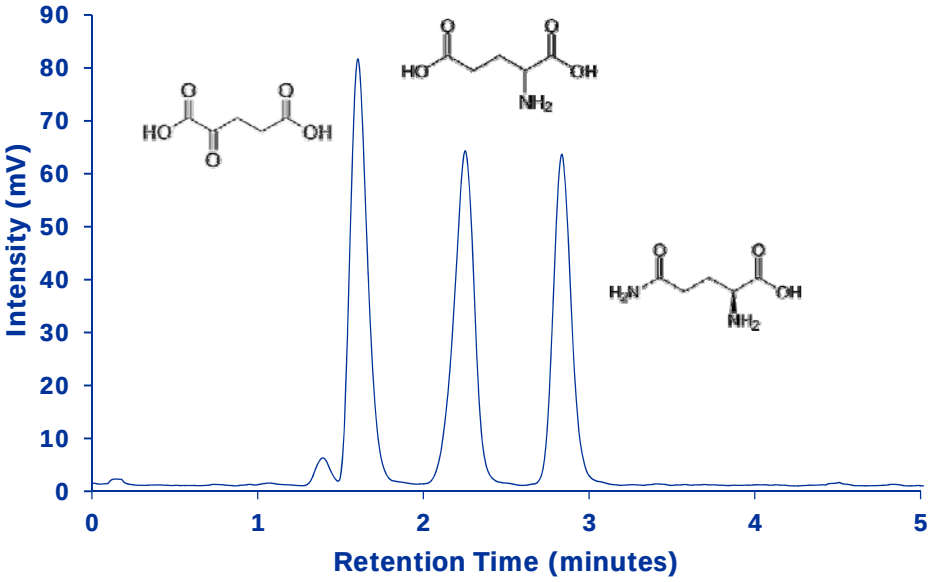

Figure 2. Separation at 10 mM (3 mM final) ammonium formate pH 6.5.

Results

Neutral pH & medium buffer strength

Ionic analytes and "shielded" ionic betaine sulfonate column surface

Increasing the buffer strength to 30 mM will effectively shield the charges on both the column stationary phase and analytes. Again the glutamine is unaffected by the change in eluent composition. None of its functional groups are affected by changing the pH, and due to the close proximity of the charges, their electrostatic interactions with the stationary phase are also unaffected when changing buffer strengths.
The effect on the two acids, especially the α-ketoglutaric acid, is dramatic when increasing the ionic strength. It is deceptive that it has approximately the same retention time in Figures 1 and 2, but here the high hydrophilicity of this doubly negatively charged molecule is allowed to influence its retention so that it is longer retained than the glutamine.
The glutamic acid elutes last, maintaining its relative elution order to α-ketoglutaric acid since it is a more polar molecule.

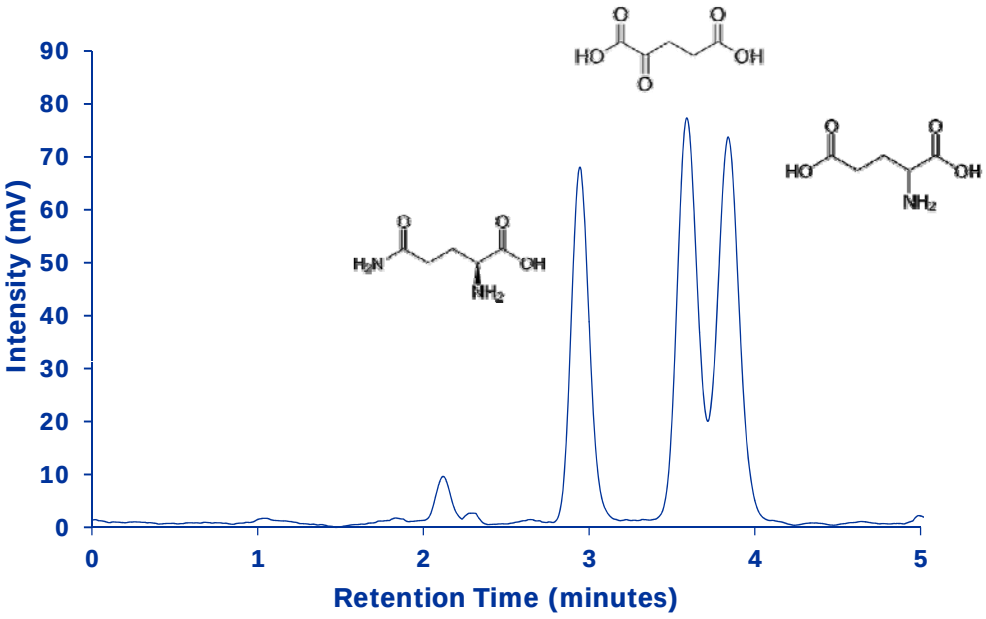

Figure 3. Separation at 100 mM (30 mM final) Ammonium formate pH 6.8, "Shielded", Betaine Sufonate Surface, "Shielded" Carboxyls.

Discussion

This interesting set of examples illustrates the profound effect of coulombic interactions in HILIC partition separations (a.k.a. eHILIC, ERLIC4, ion-pair normal phase5). Coulombic interactions are 10 times more powerful than hydrophilic partitioning forces (hydrogen bonding, dipole-dipole or π-π). In order to manage these interactions, a buffer is required in the mobile phase. It also requires consideration when trying to understand elution profiles, that the ZIC-HILIC column is unaffected by changes in pH, will greatly simplify the interpretation of the results. This separation depends both on the ionic nature of the analytes and their coulombic interactions with the stationary phase, which differs with both eluent pH and ionic strength changes. Resolving the complexity of this separation depends on observing that the retention time for glutamine, was the same under all three mobile phase conditions. Glutamine has no net charge and is thus not affected by changes in the buffer conditions. The only way to change glutamine's retention time would be to change the acetonitrile content in the eluent.

The effect of pH changes on the carboxylic acids are two-fold, but opposing. By deprotonating the acids they become much more hydrophilic, but at low buffer strength they are also strongly repelled from the distal negative charge on the stationary phase. In order to be retained by hydrophilic partitioning the analyte needs to enter the stagnant water layer at the stationary phase surface. If electrostatic repulsion prevents this, retention will be low even though the acid is more hydrophilic. Depending on the ionic strength, deprotonating an acid could give any outcome in terms of retention; shorter, longer or unchanged. This is exemplified with the behavior of the α-ketoglutaric acid which at low buffer strength has a slightly lower retention at pH 6.5 compared to at pH 3, but when the electrostatic repulsion is shielded by a high buffer strength, the retention is much higher at pH 6.5.

Conclusion

While organic content is the most significant factor for adjusting retention in HILIC, the pH and the amount of buffer in the mobile phase can alter elution order of ionic analytes by shielding ionic attraction and/or increasing ionic repulsion. The three experimental conditions (low pH low molarity, neutral pH high molarity, and neutral pH low molarity) with the test mixture employed, enabled manipulation of the elution order. By strictly controlling manufacturing conditions for its production, a stable chemical frame of reference is maintained on the ZIC-HILIC column, offering an excellent platform for exploring separations of a variety of molecules by their polar and/or ionic differences.

References

1. N.P. Dinh, T. Jonsson, and K. Irgum, J. Chromatogr. A, 1218, 2011, 5880-91.
2. Y. Guo, et al., Chromatographia 2007, 66, August (No. 3/4) 223-229.
3. Y. Takegawa, et al., J. Sep. Sci. 2006, 29, 2533 – 2540.
4. A. J. Alpert, Anal. Chem. 2008, 80, 62-76.
5. Wen Ding, et al., Mol. Cell. Proteomics, 2009, 8, September 1, 2170-2185..