Electrostatic Effects in Hydrophilic Interaction Chromatography (HILIC): A Brief Review

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Electrostatic effects are commonly present during separation of charged analytes by HILIC. Such effects can be controlled or suppressed through addition of salt to the mobile phase. In cases where these effects are useful, they can be introduced by operating an ion-exchange column under HILIC conditions. This combination is now widely used in proteomics and can involve either electrostatic attraction or electrostatic repulsion.

Introduction

It is now generally agreed that the basic mechanism of HILIC involves partitioning [1,2]. When a polar stationary phase is eluted with a predominantly organic mobile phase, then a semi-immobilised layer of water forms on the surface. The migration of polar analytes through the column is retarded by their partitioning into that stagnant aqueous layer, an effect called hydrophilic interaction. Other forces can be superimposed upon the hydrophilic interaction. Hydrogen bonding has frequently been cited in this regard. While hydrogen bonding does play a role in some cases [3], most such postulations are not accompanied by evidence to distinguish this from the dipole-dipole interactions that are probably responsible for the generation of the immobilised aqueous layer [1,4]. That leaves electrostatic effects as an important potential contributor to retention in HILIC in addition to the partitioning effects. This review addresses both the inadvertent influence of electrostatic effects and their intentional introduction.

I. Electrostatic effects as a minor mixed-mode effect in HILIC

All silica-based materials currently used for HILIC, and many that are polymer-based, exhibit some degree of electrostatic charge. If an analyte is also charged, then electrostatic interactions will be superimposed upon the hydrophilic interaction. This can result in an increase or decrease in retention time, depending on the relative charges involved. The following principles are handy guidelines:

1) Addition of salt to the mobile phase suppresses electrostatic effects, both attractive and repulsive.

2) Increasing the concentration of organic solvent increases the strength of hydrophilic interaction independently of electrostatic effects.

3) Basic and phosphorylated solutes are the most hydrophilic ones.

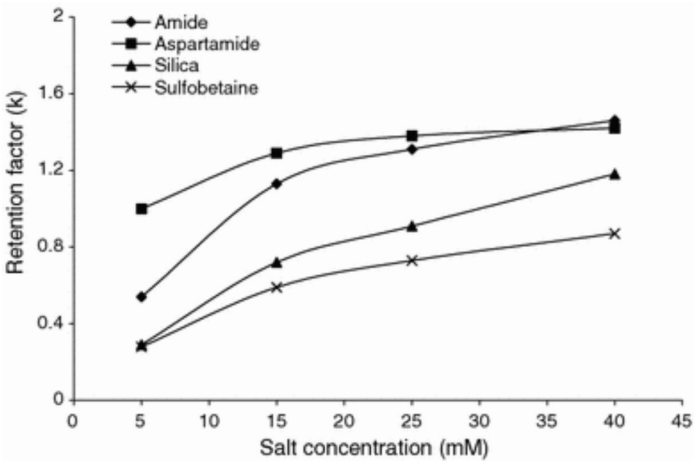

Figure 1. Retention factor of salicyluric acid (negatively charged) on HILIC columns as a function of the concentration of ammonium acetate in the mobile phase.

Columns: a) TSKgel Amide-80, 5µm, 120-Å, 250x4.6 mm; b) PolyHYDROXYETHYL A, 5µm, 100-Å, 200x4.6-mm; c) Atlantis HILIC silica, 5µm, 135-Å, 250x4.6-mm; d) ZIC-HILIC, 5µm, 200-Å, 250x4.6-mm. Mobile phase: ACN/water 80/20, v/v, containing the concentration of ammonium acetate overall as indicated. Flow rate: 1 ml/min.

The eluent pH is another factor that can influence the degree of analyte and surface charge, or even the charge sign. With few exceptions [5], though, the effect of pH on HILIC has been little investigated.

Principle 1 is demonstrated in Figure 1 [6]. Starting with 5 mM ammonium acetate, four different silica-based HILIC stationary phases exhibit some degree of negative (-) charge and so repel a (-) charged analyte. This leads to decreased retention. Increasing the salt concentration suppresses the repulsion and increases the retention time. This effect levels off as the salt concentration reaches the range 20-30 mM, a range high enough to titrate the ionisable groups in the stationary phase and render them electrostatically neutral. The basis of the charge varies with the materials. Silica has abundant charged silanol groups on the surface at pH values > 3. Presumably the amide material (TSK Amide-80®) does so as well. The aspartamide material (PolyHYDROXYETHYL Aspartamide) consists of a neutral polypeptide covalently bound to silica, and so has ionisable N- and C-termini. Accounting for the charge of the sulfobetaine material (ZIC-HILIC®) is not as straightforward. While the ionisable groups in the functional ligands are ostensibly zwitterionic and so neutral overall, the sulphonate groups at the terminus of each ligand appear to be preferentially titrated by an external counterion rather than by the internal amine group in the ligand.

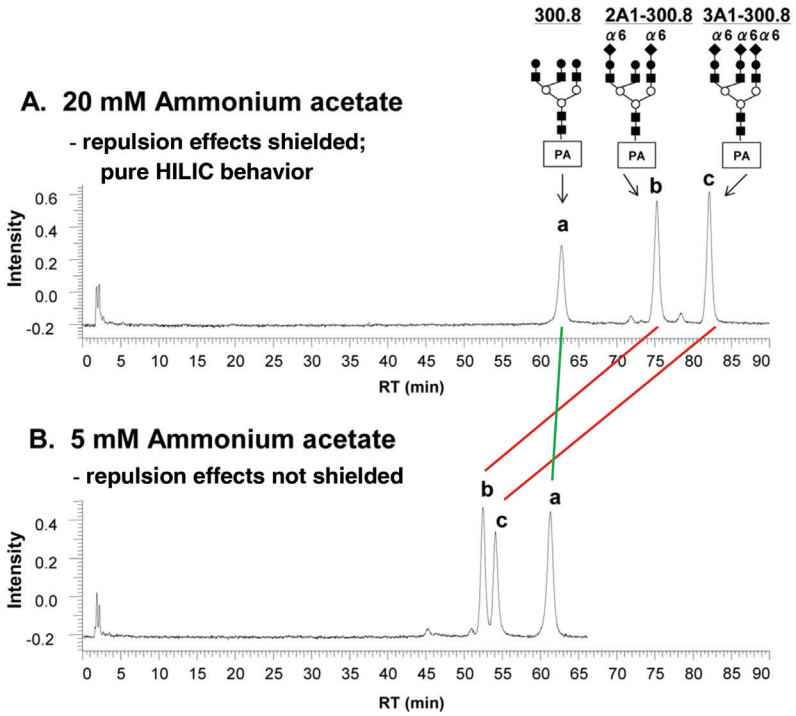

Figure 2. Retention of 2-aminopyridinyl- derivatives of N-linked glycans on a ZIC-HILIC column. Glycans b and c have 2 and 3 sialic acid residues apiece, respectively, while glycan a has none. [TOP] Retention with 20 mM ammonium acetate in the mobile phase. [BOTTOM] Retention with 5 mM ammonium acetate in the mobile phase, a concentration that does not suffice to shield electrostatic repulsion effects. Column: ZIC-HILIC, 3.5µm, 200-Å, 150x2.1-mm. Flow rate: 0.2 ml/min. Gradient: 71.4-60.2% ACN over 120 min., with overall conc. of ammonium acetate as noted. Detection: Fluorescence.

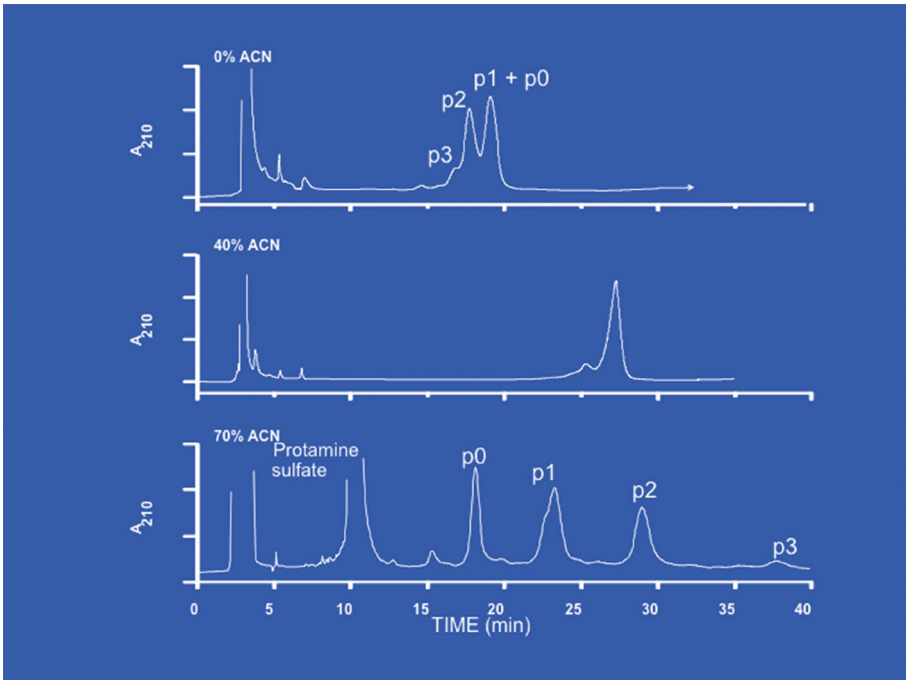

Figure 3. Retention of phosphorylation variants of histone H1.1 from Raji cells on a weak cation-exchange column with [TOP] 0%, [MIDDLE] 40%, or [BOTTOM] 70% ACN in both mobile phases. p0, p1, p2 and p3 denote the number of phosphate groups in the protein. Column: PolyCAT A, 5µm, 300-Å, 200x4.6-mm. Gradient: 0-1 M NaClO4 in 10 mM triethylammonium methylphosphonate, pH 3.0, with %ACN as noted; 0-60% mobile phase B in 5 min, then 60-100% B in 30 min. Flow rate: 1 ml/min. Detection: 210 nm.

Consequently, below 20 mM salt ZIC-HILIC behaves like a high-capacity cation-exchange material [7,8,9]. However, it does behave like a neutral stationary phase above 20 mM salt (Figure 2) [10].

Figure 3 demonstrates principles 2 and 3. This shows the elution of some phosphorylated histone variants from a cation-exchange column with a salt gradient [11]. Histones are basic proteins and are well-retained in cation-exchange chromatography (CEX). Attaching a phosphate group to a histone decreases its net (+) charge, and so the more heavily phosphorylated proteins elute earlier [TOP]. Now, phosphate groups are quite polar, cf. principle 3 above. If 70% ACN is included in both mobile phases [BOTTOM] then a significant amount of hydrophilic interaction is superimposed on top of the electrostatic effects. The phosphate groups then promote retention through hydrophilic interaction more than they decrease it through electrostatic repulsion. As a consequence, the order of elution is inverted. At 40% ACN [MIDDLE], both forces are in balance and the histone phosphorylation variants coelute.

II. Electrostatic attraction superimposed upon hydrophilic interaction

Figure 4 shows the separation of isoforms of histone H4 on a capillary packed with a weak cation-exchange (WCX) material [12]. Detection involved direct elution to a mass spectrometer. Again, histones are basic proteins and so are well-retained in CEX. Acetylation of lysine residues reduces the overall basic charge and so decreases retention (with the more heavily acetylated forms eluting earlier than the forms with little or no acetylation of lysine). In addition, the chromatography starts with 70% ACN in the mobile phase, superimposing a significant degree of hydrophilic interaction upon the electrostatic effects. This renders the stationary phase/ eluent combination capable of distinguishing between modifications that change polarity but not charge, such as methylation of a lysine residue. The boxes associated with each acetylation variant peak list the additional modifications identified among the variants separated within that subset. The gradient involved an increasing concentration of formic acid. This neutralised the carboxyl- groups of the weak cation-exchange material, permitting elution of the proteins in a solvent compatible with mass spectrometry. This combination of chromatography modes is generally called WCX-HILIC. With histones other than H4, the gradient usually involves a decreasing ACN concentration as well, thereby predictably reducing the hydrophilic interaction.

III. Electrostatic repulsion superimposed upon hydrophilic interaction

When there is electrostatic repulsion between a solute and a column material, Figure 1 shows that it can be suppressed with a high concentration of salt. An alternative approach is not to suppress the electrostatic repulsion but to promote

the hydrophilic interaction instead with an increase in the concentration of organic solvent. The two forces can be manipulated independently. This combination is called Electrostatic Repulsion-Hydrophilic Interaction Chromatography, or ERLIC for short [13]. ERLIC permits some separations that would otherwise be difficult if not impossible. Figure 5 compares the isocratic separation of some acidic, neutral, and basic peptides on a neutral HILIC column [TOP] and an anion-exchange column [BOTTOM] in the ERLIC mode. In regular HILIC, the basic peptides elute much later than the other peptides because basic solutes are the most hydrophilic ones (cf. principle 3 above). In ERLIC mode the (+) charge of the anion-exchange column selectively antagonises the retention of the basic peptides, causing them to elute in the same time frame as the other peptides. The basic peptides are still retained because basic compounds are quite hydrophilic, cf. principle 3 above. This method permits the analysis of highly basic, polar peptides that cannot be analysed by other methods. The same principle can be applied to negatively charged analytes such as nucleotides. With a neutral HILIC column, the triphosphonucleotides are far better retained than the mono- and diphosphonucleotides. When a cation-exchange column is used in the HILIC mode, however, then a set of all common nucleotides (AMP, ADP, ATP etc.) is reasonably well resolved isocratically in about 35 minutes [13].

Selectivity in the ERLIC mode is readily manipulated. When the level of hydrophilic interaction is reduced with a gradient of decreasing organic solvent concentration, then at some point it can no longer overcome the electrostatic repulsion and retained analytes are eluted. Selectivity is also quite sensitive to the salt used in the mobile phase. Figure 6 compares the separation of two peptides which differ only in having either an aspartyl- or phosphoserine residue at position 5. Switching from a phosphate to a methylphosphonate salt in the mobile phase selectively enhances retention of the phosphopeptide [14].

At present the most widely used applications of ERLIC involve proteomics. It is a useful alternative to affinity chromatography for isolation of phosphopeptides and glycopeptides, which are selectively retained in ERLIC [14-18]. More recently, as the first dimension of two-dimensional fractionation of complex tryptic digests, it has proven to be superior to cation-exchange (SCX) and the only real rival to high-pH reversed-phase chromatography. Both methods lead to identification of comparable numbers of peptides and can be implemented with conveniently volatile mobile phases. Starting at 85-90% ACN, all tryptic peptides, even basic ones, are retained on an anion-exchange column. A decreasing ACN gradient reduces the hydrophilic interaction while a decreasing pH gradient (e.g., 0.1% acetic acid to 0.1% formic acid) neutralises carboxyl- groups in peptides. Peptides are then eluted in order of decreasing pI value, uniformly distributed throughout the gradient [16,19-21].

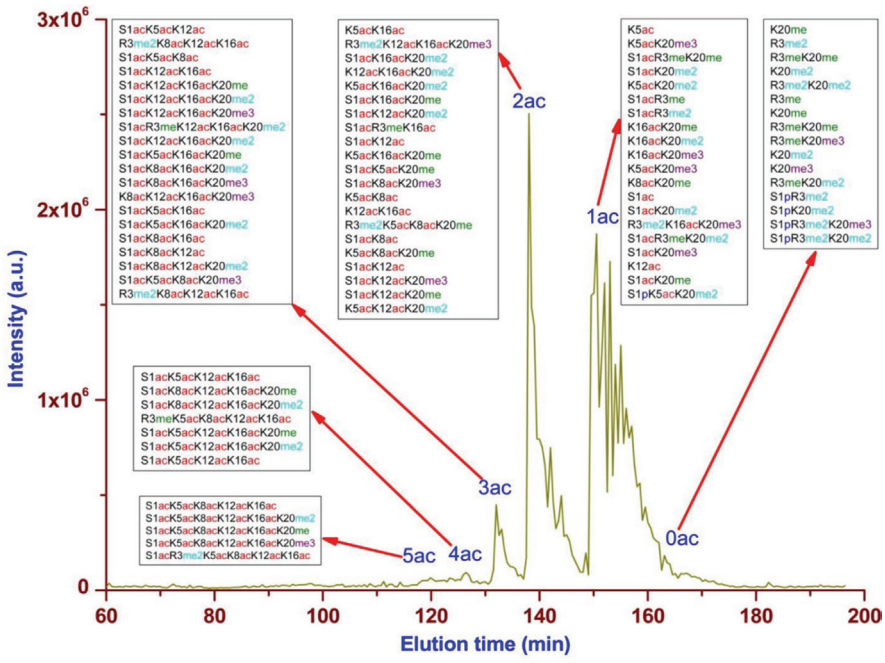

Figure 4. Separation of isoforms of histone H4 from HeLa cells on a weak cation-exchange capillary operated in the HILIC mode (WCX-HILIC). Capillary: 500x0.1 mm; PolyCAT A, 5-µm, 1000-Å. Gradient: 1-8% formic acid in 70% ACN. Detection: ESI-MS/MS.

IV. Orientation Effects

A marked advantage of using an ion-exchange column in the HILIC mode is that charged analytes may migrate through the column with a high degree of orientation [22]. This confers sensitivity to the position of derivatisation; a modification to a peptide at a residue that's remote from the binding site will have less effect on the migration than a modification of a residue that's nearby. Consequently, ERLIC conditions permit the separation of positional variants of phosphopeptides that coelute in HILIC [23].

Conclusions

The intentional combination of electrostatic effects with HILIC permits some separations that would probably not be possible via other methods. This can be implemented with ordinary ion-exchange materials and with conditions compatible with modern instrumental techniques such as mass spectrometry. Accordingly, this combination seems likely to increase steadily in use.

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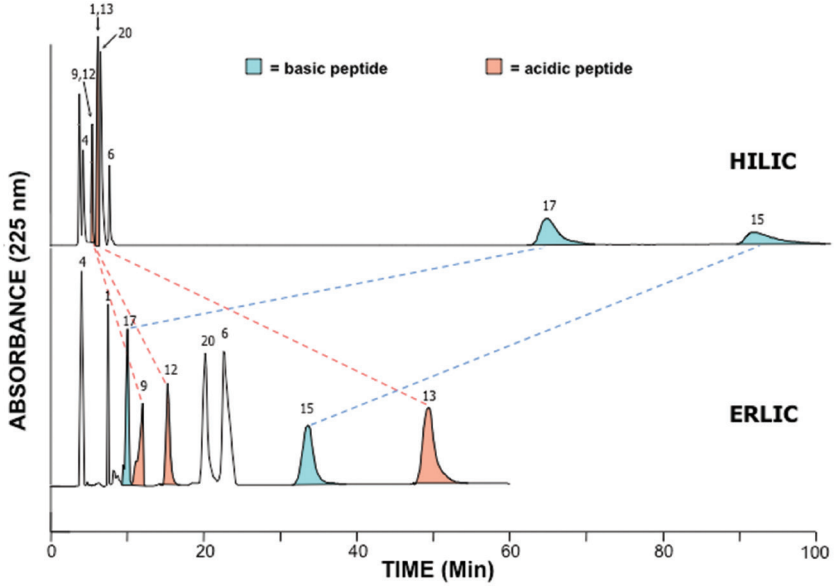

Figure 5. Separation of peptide standards via isocratic elution.

[TOP] A neutral column (PolyHYDROXYETHYL A; 5µm, 300-Å, 200x4.6-mm) operated in the HILIC mode. Mobile phase: 20 mM sodium methylphosphonate, pH 2.0, with 63% ACN; 1 ml/min.

[BOTTOM] An anion-exchange column (PolyWAX LP; 5µm, 300-Å, 200x4.6-mm) operated in the ERLIC mode. Mobile phase: 20 mM sodium methylphosphonate, pH 2.0, with 70% ACN. Flow rate: 1 ml/min. Detection: 225 nm.

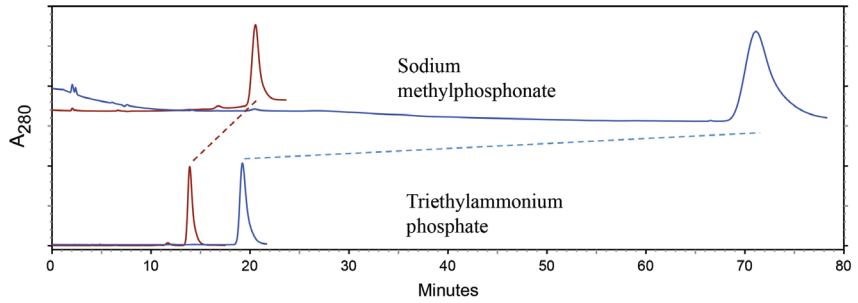

Figure 6. Separation of an acidic and a phosphorylated peptide standard on an anion-exchange column (PolySAX LP, 5µm, 300-Å, 200x4.6-mm) in the HILIC mode using [top] 20 mM sodium methylphosphonate, pH 6.0 or [bottom] 20 mM triethylammonium phosphate, pH 6.0, with 70% ACN. Flow rate: 1 ml/min. Detection: 280 nm.

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