Anal. Chem. **2008,** *80,* 62−76

Electrostatic Repulsion Hydrophilic Interaction Chromatography for Isocratic Separation of Charged Solutes and Selective Isolation of Phosphopeptides

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If an ion-exchange column is eluted with a predominantly organic mobile phase, then solutes can be retained through hydrophilic interaction even if they have the same charge as the stationary phase. This combination is termed electrostatic repulsion-hydrophilic interaction chromatography (ERLIC). With mixtures of solutes that differ greatly in charge, repulsion effects can be exploited to selectively antagonize the retention of the solutes that normally would be the best retained. This permits the isocratic resolution of mixtures that normally require gradients, including peptides, amino acids, and nucleotides. ERLIC affords convenient separations of highly charged peptides that cannot readily be resolved by other means. In addition, phosphopeptides can be isolated selectively from a tryptic digest.

Solutes in a mixture can differ greatly in their properties. In reversed-phase chromatography (RPC), this involves differences in polarity. For ion-exchange chromatography, this involves differences in charge. An elution gradient of some sort is generally used to ensure that all solutes in a mixture elute in the same time frame. This paper introduces an alternative strategy: superimposing a second mode of chromatography that selectively reduces the retention of the solutes that are usually the most strongly retained. The combination used here is ion-exchange and hydrophilic interaction.

The term hydrophilic interaction chromatography (HILIC) was coined in 1990[1] to describe normal-phase chromatography with mobile phases that, typically, are 10−40% aqueous. A sufficiently polar stationary-phase material is more polar than this mobile phase and will retain polar solutes. A model for the retention mechanism postulates partitioning between the dynamic mobile phase and a slow-moving layer of water with which the polar stationary phase is hydrated. The more polar a solute, the more it associates with this stagnant aqueous phase and the later it elutes, a normal-phase direction. HILIC was used for carbohydrate analysis as early as 1975.[2,3] The mechanism of separation was recognized as early as 1967, in the case of Sephadex eluted with a predominantly organic mobile phase.[4] HILIC is useful for analysis of polar solutes in general as RPC is for nonpolar solutes. Since 1990, HILIC has been applied to a wide variety of peptides,[5−10] complex carbohydrates,[11] and some proteins[12−15] and is increasingly being applied to small polar solutes such as pharmaceuticals,[16−17] saponins,[18] urea,[19] aminoglycoside antibiotics,[20] glucosinolates,[21] sugars and glycans,[22−24] folic acid and its metabolites,[25] nicotine and its metabolites,[26] and glycoalkaloids.[27] Yoshida has written a series of papers examining the variables involved in HILIC of peptides.[28−30] Hemström and Irgum have published an

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10.1021/ac070997p CCC: $40.75
Published on Web 11/21/2007

ambitious paper that reviews the entire field and also attempts to ascertain the extent to which partitioning or adsorption account for the separation mechanism.[31] Gradients for elution involve increasing the polarity of the mobile phase, as in regular normal-phase chromatography. Typically this involves decreasing concentrations of organic solvent, although increasing salt concentrations can be used too. Hydrophilic interaction can be superimposed as a mixed mode on an ion-exchange column by running an increasing salt gradient in a mobile phase containing 60−70% organic solvent. These conditions work well to resolve histone variants on a weak cation-exchange column[32−37] and have also been used for chromatography of peptides on a strong cation-exchange column in an extensive series of papers from Robert Hodges' group.[38−40]

Acidic amino acids elute prior to the void volume of a cation-exchange column, since electrostatic repulsion denies them access to the full pore volume of the stationary phase. However, if the mobile phase contains >60% organic solvent, then acidic amino acids are retained almost as well by a polar cation-exchange column as by a polar neutral column.[1] This seeming anomaly reflects the fact that hydrophilic interactions are independent of electrostatic effects. With sufficient organic solvent in the mobile phase, hydrophilic interaction dominates the chromatography. Thus, phosphate groups decrease the retention of basic histone proteins on a cation-exchange column in the absence of organic solvent but lead to a net increase in retention if the mobile phase contains 70% ACN.[32] Under these conditions, the hydrophilic interaction conferred by the phosphate groups is stronger than their electrostatic repulsion by the stationary phase.

This phenomenon has important implications. Basic solutes are normally the best-retained in HILIC, followed by phosphorylated ones.[1] A gradient is necessary if samples contain very basic peptides or nucleotides such as ATP. In extreme cases, a gradient is required with both decreasing organic and increasing salt concentrations.[8] However, in the case of peptides, this could conceivably be unnecessary if an anion-exchange column were used for HILIC. This combination would address the following three extremes of retention.

(1) Late elution of very acidic peptides through electrostatic attraction. This could be moderated by use of mobile phases with pH low enough to uncharge aspartate and glutamate residues, leaving most peptides neutral or basic. In fact, a decreasing pH gradient has been used with anion-exchange cartridges to release trapped acidic peptides[41] and for desalting proteins.[42]

(2) Late elution of very basic peptides through hydrophilic interaction. Electrostatic repulsion by the stationary phase would throw such peptides back into the elution time frame of neutral or moderately acidic peptides. The effect would be analogous to having an immobilized salt gradient.

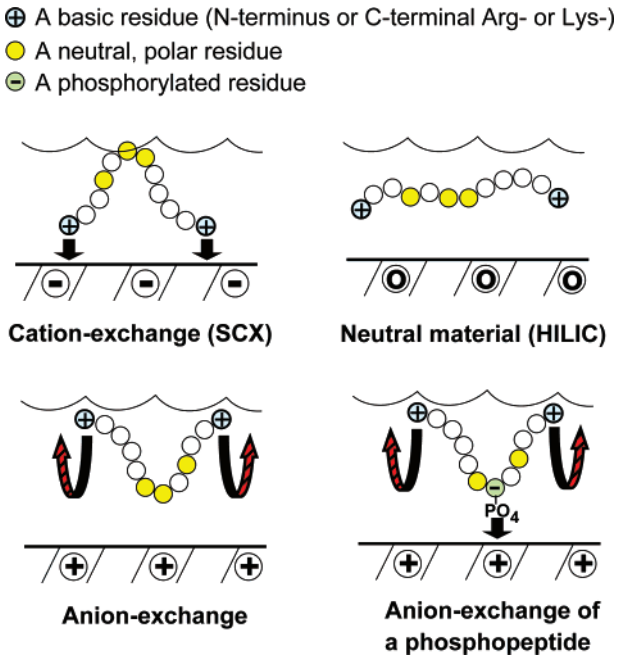

Figure 1. Hypothetical orientation of tryptic peptides on various stationary phases. The basic termini are attracted in cation exchange and repelled in anion exchange.

(3) Elution of basic peptides prior to the void volume through electrostatic repulsion. As with acidic amino acids, in the absence of a high level of organic solvent, basic peptides are excluded from the pore volume of a column of the same charge. This is evident in a recent paper showing elution prior to the void volume of nearly all peptides from a complex tryptic digest run on an anion-exchange material at pH ~2.6 (ref 43, Figure 4H). This is a version of ion exclusion chromatography,[44−46] a technique of limited utility because of its narrow fractionation range. However, one could include enough organic solvent in the mobile phase to generate hydrophilic interaction sufficient for reasonable retention of such peptides. Under these conditions, all peptides in a mixture would be retained through hydrophilic interaction despite being repelled by the stationary phase to some extent (except for neutral peptides). The acronym ERLIC is proposed for this combination, standing for electrostatic repulsion-hydrophilic interaction chromatography. Since the two superimposed modes antagonize each other's extremes of retention, isocratic resolution of heterogeneous peptide mixtures may be practical. Figure 1 displays types of interaction and anticipated orientation of tryptic peptides in ERLIC and other modes of chromatography.

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Table 1. Effect of Salt on Retention of Peptide Standards

peptide standard	peptide category	retention time (min.) in mobile-phase indicated: 20 mM TEAP	50 mM TEAP	20 mM $Na-MePO_4$	50 mM $Na-MePO_4$
1	tryptic	3.5	4.6	2.3	2.8
2	tryptic	2.7	3.2	2.1	2.4
3	tryptic	2.4	2.7	1.8	2.0
4	tryptic	2.3	2.6	1.8	1.9
5	tryptic	2.6	3.1	2.0	2.1
6	tryptic	4.3	5.4	3.4	3.8
7	acidic	3.2	3.2	3.6	3.4
8	acidic	3.2	3.4	3.4	3.2
9	acidic	3.5	3.7	3.9	3.9
11	acidic	3.1	3.2	3.4	3.1
12	acidic	3.5	3.4	5.1	4.0
10	acidic phospho-peptide	10.5	6.0	**64.0**	**24.5**
13	acidic phospho-peptide	6.5	4.7	**19.0**	**11.2**
14	basic	**(>70)**	**(>70)**	1.7	1.9
15	basic	**(>70)**	**(>70)**	2.3	3.4
16	basic	**16.5**	**17.4**	2.1	3.1
17	basic	**6.9**	**22.6**	1.8	2.3

[a] All mobile phases contained 60% ACN and were prepared with stock solution at pH 2.0. Flow rate: 1 mL/min. Column: item 204WX0503 (see Materials and Methods). The void volume was at 2.0 min. Unusually long retention times are listed in boldface type.

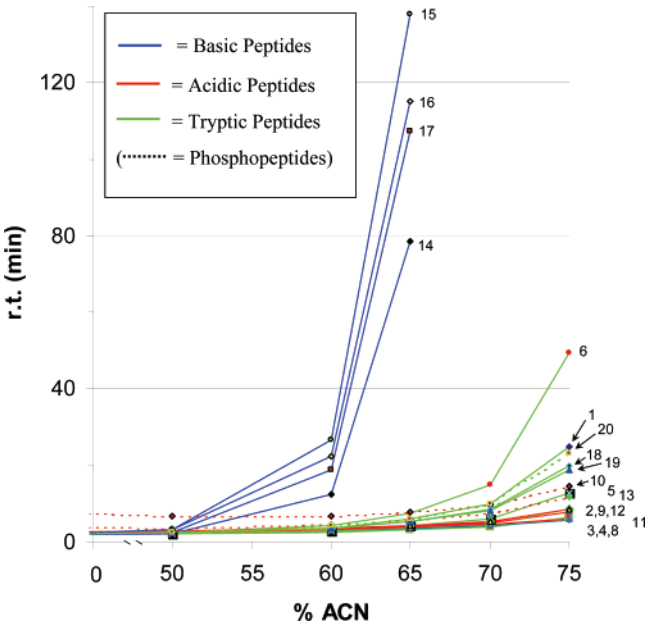

Figure 2. HILIC mode: peptide retention vs % ACN. Peptide standards key: See Methods. Column: Polyhydroxyethyl A, 200 × 4.6 mm; 5 μm, 300 Å. Mobile phase: 20 mM Na-$MePO_4$, pH 2.0, with % ACN indicated. Flow rate: 1.0 mL/min. Discontinuous curves reflect the elution of some solutes outside the time frame of the graph.

Peptides that contained phosphate or sulfate groups would retain some negative charge even at a pH low enough to uncharge Asp and Glu residues. Such peptides would display some electrostatic attraction to the stationary phase used for ERLIC. This would be an asset rather than a liability; numerous applications in biochemistry would benefit from a method permitting the selective isolation of phosphopeptides from a digest. In this ERLIC

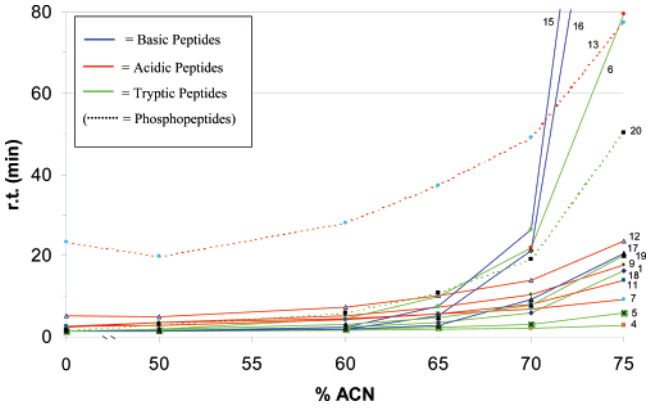

Figure 3. ERLIC mode: peptide retention vs % ACN. Peptide standards key: see Methods. Column: as in Figure 1. Mobile phase and flow rate: as in Figure 2. Discontinuous curves reflect the elution of some solutes outside the time frame of the graph.

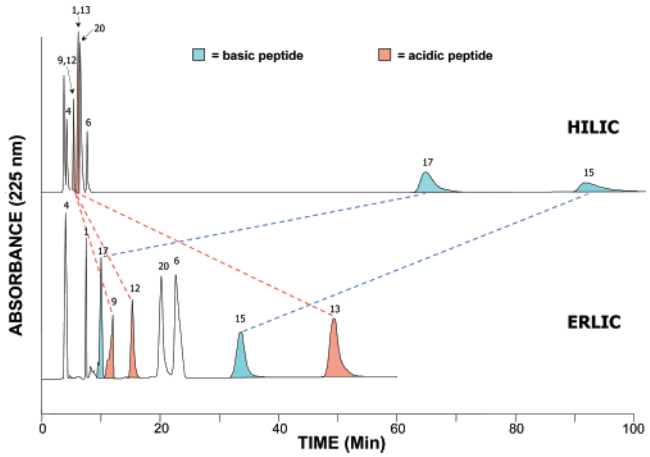

Figure 4. HILIC vs ERLIC separation of peptide standards. Peptide standards key: See Methods. HILIC mode (top). Column: Polyhydroxyethyl A (as in Figure 2). Mobile phase: 20 mM Na-$MePO_4$, pH 2.0, with 63% ACN. Flow rate: 1.0 mL/min. ERLIC mode (bottom). Column: PolyWAX LP (as in Figure 1). Mobile phase: 20 mM Na-$MePO_4$, pH 2.0, with 70% ACN. Flow rate: 1.0 mL/min.

represents an alternative to immobilized metal affinity chromatography and Lewis acids such as titania, zirconia, or alumina.

In addition to peptides, ERLIC could in principle be applied to other solutes with sufficient charge, either positive or negative. This study explores the characteristics and utility of ERLIC as applied to peptides, amino acids, nucleotides, and oligonucleotides.

MATERIALS AND METHODS

HPLC Columns and Equipment. *Columns.* All columns were products of PolyLC Inc. (Columbia, MD), except as noted below, and were made of type 316 stainless steel with titanium frits. PolyWAX LP, a weak anion-exchange material, was used with peptides and amino acids. For peptides, the columns were as follows: (1) 100 × 4.6-mm, 5-μm particle diameter, 300-Å pore diameter (item 104WX0503) or (2) 200 × 4.6 mm, 5 μm, 300 Å (item 204WX0503). For amino acids, the column was 200 × 4.6 mm, 5 μm, 100 Å (item 204WX0501). For ERLIC of nucleotides, a 200 × 4.6-mm column of the strong cation-exchange material Polysulfoethyl aspartamide (Polysulfoethyl A)[47] was used; 5 μm,

(47) Alpert, A. J.; Andrews, P. C. *J. Chromatogr.* **1988**, *443*, 85.

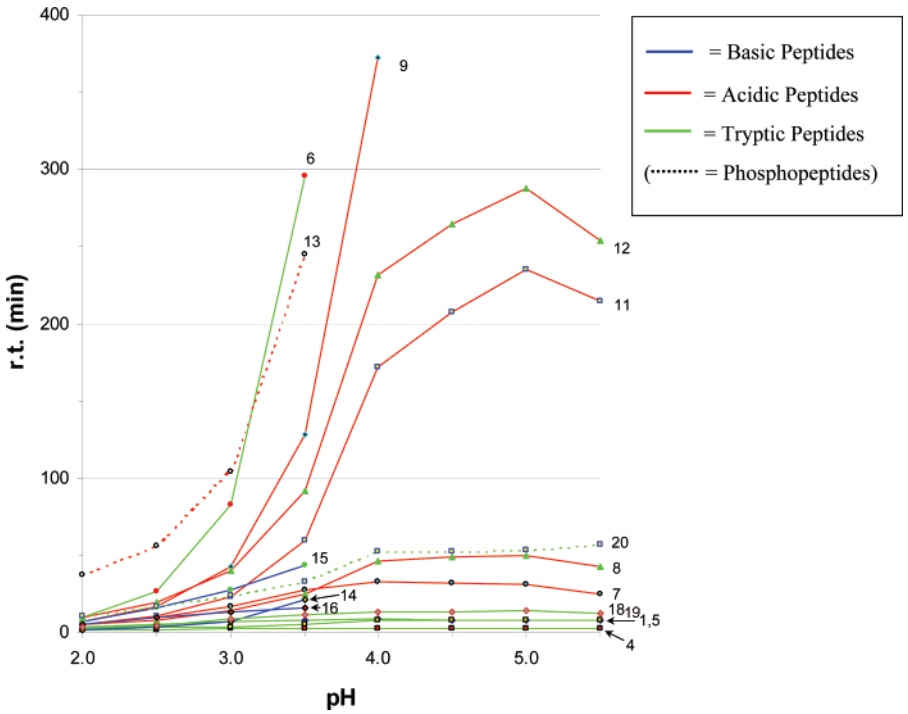

Figure 5. ERLIC mode: peptide retention vs pH. Peptide standards key and column: see Figure 3. Mobile phase: 20 mM Na-MePO$_4$ (pH as noted) with 65% ACN. Flow rate: 1.0 mL/min. Discontinuous curves reflect the elution of some solutes outside the time frame of the graph.

300 Å (item 204SE0503). HILIC data for peptides (Figures 2 and 4), nucleotides, and nucleic acids were obtained with a 200 × 4.6-mm column of Polyhydroxyethyl aspartamide (Polyhydroxyethyl A);[1] 5 μm, 300 Å (item 204HY0503). HILIC data for amino acids were obtained with a 200 × 4.6-mm column of 5-μm, 100-Å Polyhydroxyethyl A (item 204HY0501).

A TSKgel Amide-80 column, 250 × 4.6 mm, 5 μm (item 13071) was obtained from Tosoh Bioscience LLC (Montgomeryville, PA).

Equipment. A Scientific Systems Inc./Lab Alliance (State College, PA) Essence HPLC system was used.

Reagents. Peptide standards 1−20 were purchased from Bachem (Torrance, CA), with the following exceptions: 9 (Sigma Chemical Co., St. Louis, MO); 15, 16 (Peninsula Laboratories, Belmont, CA); and 13, 18−20 (California Peptide Research, Napa, CA). Amino acid, nucleotide, and nucleic acid standards were from Sigma. Phosphoric acid and acetonitrile (ACN) (both HPLC grade) were from Fisher Scientific (Pittsburgh, PA). Triethylamine (99.5%) was from Aldrich Chemical Co. (Milwaukee, WI). Methylphosphonic acid was from Alfa Aesar/ Lancaster Synthesis (Ward Hill, MA). HPLC-grade water was used.

The 0.5 M stock solutions of triethylamine phosphate (TEAP) buffers were prepared as follows: 14.4 g. of 85% phosphoric acid was weighed into a beaker and 150 mL of water added slowly, with stirring. Triethylamine was added (in a hood) until the desired pH was attained. The solution was diluted to 250 mL and filtered (0.45-μm filter). Methylphosphonate stock solutions were prepared using the same procedure, with addition of either triethylamine or NaOH solution. Mobile phases were prepared from water, ACN, and aliquots of the stock solutions. The pH of a mobile phase was neither measured nor adjusted, since ionization constants shift in predominantly organic solution,[48] but was merely designated with the pH of the stock solution used to prepare it.

Peptide Standards. The following model tryptic peptides were used: (1) Thr-Tyr-Ser-Lys; (2) Asp-Leu-Trp-Gln-Lys (uremic pentapeptide); (3) Tyr-Gly-Gly-Phe-Leu-Arg (dynorphin A (1−6), porcine); (4) Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg (Leu-valorphin-Arg); (5) Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg (experimental allergic encephalitogenic peptide); (6) Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys (β-interleukin (163−171), human).

The following acidic peptides were used: (7) Asp-Val; (8) Val-Asp; (9) Glu-Ala-Glu; (10) Asp-Ala-Asp-Glu-(pTyr)-Leu (EGF receptor (988−993), human (phosphorylated); (11) Trp-Ala-Gly-Gly-**Asp**-Ala-Ser-Gly-Glu (DSIP; delta sleep-inducing peptide); (12) [isoAsp^5]-DSIP; (13) [phosphoSer^5]-DSIP.

The following basic peptides were used: (14) ACTH (1−39; human) (6 acidic and 7 basic residues); (15) Arg-Lys-Arg-Ser-Arg-Lys-Glu; (16) Lys-Arg-Gln-His-Pro-Gly-Lys-Arg (TRH precursor peptide); (17) Lys-Pro-Val-Gly-Lys−Lys-Arg−Arg-Pro-Val-Lys-Val-Tyr-Pro.

The following model DSIP-like tryptic peptides were used: (18) Trp-Ala-Gly−Gly-Asp −Ala-Ser-Gly-Lys; (19) [isoAsp^{5}Lys^9]-DSIP. (20) [phosphoSer^{5}Lys^9]-DSIP.

RESULTS

ERLIC of Peptides. (A) Selection of Standards. (1) Tryptic Peptides. Perhaps the most convenient mixture of peptides to analyze by ERLIC would be a tryptic digest. With the exception of those containing His residues or missed cleavages, tryptic peptides contain only two basic groups, the N-terminus and the C-terminal Arg or Lys residue. This would simplify the analysis since no unduly basic peptides would be present. Therefore, a number of tryptic peptides were included in the standards to get

(48) Subirats, X.; Bosch, E.; Rosés, M. *J. Chromatogr., A* **2007**, *1138*, 203.

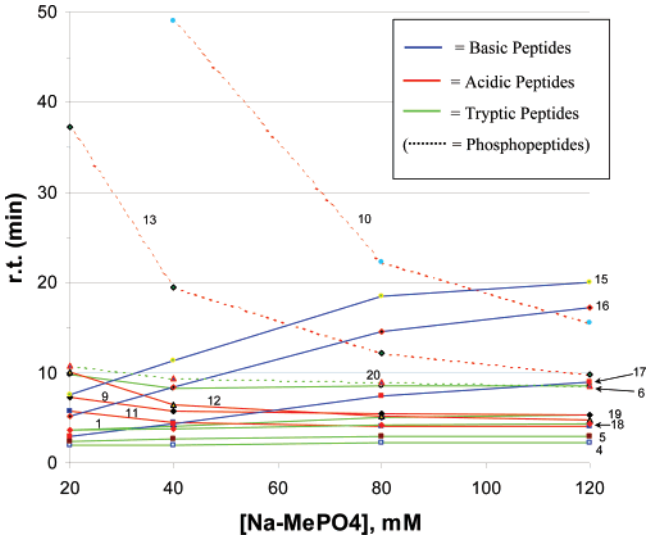

Figure 6. ERLIC mode: peptide retention vs salt concentration. Peptide standards key and column: see Figure 3. Mobile phase: Na-$MePO_4$ (concentration as noted), pH 2.0, with 65% ACN. Discontinuous curves reflect the elution of some solutes outside the time frame of the graph.

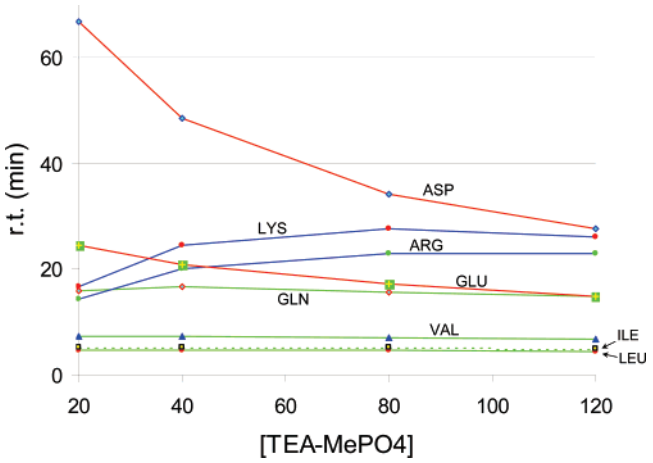

Figure 7. ERLIC of amino acids: retention vs concentration of TEA−$MePO_4$. Column: PolyWAX LP, 200 × 4.6 mm; 5 μm, 100 Å. Mobile phase: TEA−$MePO_4$ (concentration as noted), pH 2.0, with 70% ACN. Flow rate: 1.0 mL/min.

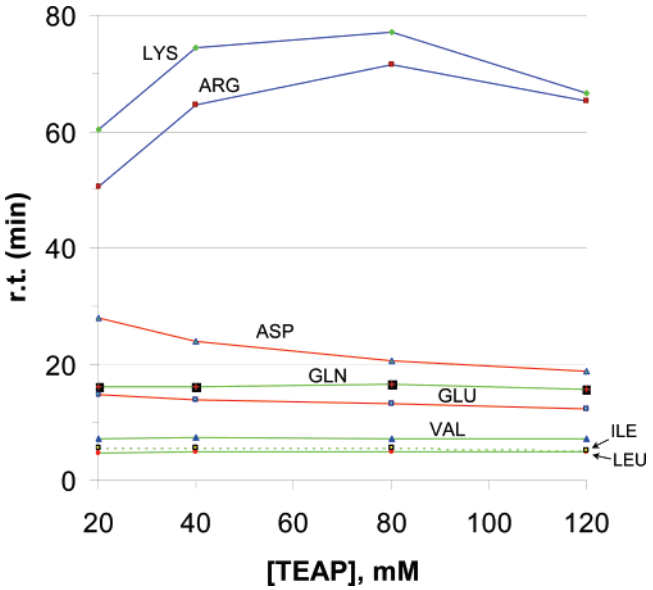

Figure 8. ERLIC of amino acids: retention vs concentration of TEAP. Conditions: Same as Figure 8 but with TEAP substituted for TEA−$MePO_4$.

some idea of the range of elution times that could be expected. Standards 1−5 are ordinary sequences with 0 or 1 acidic residues. Standard 6 is an unusually acidic tryptic sequence. Standards 18−20 are tryptic sequences substituted with an Asp, isoAsp, or phosphoSer residue at one position; this sample set permits an assessment of the effects of these residues on retention.

(2) Acidic Peptides. Standards 7−9 are acidic peptides with no basic groups except for the N-terminus. Standard 10 is an unusually acidic phosphopeptide, as is characteristic of sequences surrounding phosphoTyr residues. Standards 11−13, the DSIP peptides, have the same sequences as standards 18−20 except for substitution of a Glu residue for the C-terminal Lys. This permits the assessment of the effects of replacing an Asp with an isoAsp or phosphoSer residue although in a less controlled fashion than with standards 18−20, since the residue being substituted is not the only acidic residue in the peptide.

(3) Basic Peptides. Standards 15−17 are unusually basic peptides. Standard 14, ACTH, has almost as many acidic as basic residues. This could help in assessing the relative importance of such residues in the overall retention of a peptide.

(B) Selection of Mobile Phases. Table 1 shows a preliminary comparison of retention times with TEAP versus sodium methylphosphonate (Na-$MePO_4$) buffers. With TEAP buffers, retention of acidic phosphopeptides (standards 10 and 13) was not markedly greater than that of other peptides, while retention of basic standards 13−17 was greater than most of the others. This selectivity, characteristic of ordinary HILIC, was the opposite of that desired. By contrast, the selectivity of Na-$MePO_4$ buffers exhibited the characteristics desired of ERLIC, with rapid elution of basic peptides and delayed elution of phosphopeptides. Accordingly, Na-$MePO_4$ buffers were selected for detailed examination of ERLIC of peptides.

(C) Effect of Percent Organic Solvent; HILIC versus ERLIC. Figure 2 shows the effect of percent ACN on the isocratic retention of peptide standards 1−20 in HILIC. Basic peptides (standards 14−17) are by far the best-retained. All the others, including phosphopeptides, elute roughly in the same time frame. Figure 3 shows the same standards under ERLIC conditions. Owing to the electrostatic repulsion, basic peptides now elute in the same time frame as the other peptides. This contrast between HILIC and ERLIC is manifest in Figure 4, which compares chromatograms of a peptide standard set run in both modes. In the HILIC mode, a level of ACN that leads to isocratic elution of basic standards 15 and 17 in less than 100 min affords inadequate retention of the acidic and neutral standards. In the ERLIC mode, the electrostatic repulsion of 15 and 17 now permits the [ACN] to be increased to a level that affords adequate retention and isocratic elution of all standards in this example within 50 min.

Evidently the concentration and pH of the mobile phase in Figure 3 suffices to suppress the ionization of carboxyl groups, since acidic standards 7−9 and 11 elute in the same time frame as the neutral tryptic peptides. The acidic tryptic peptide 6 is retained significantly longer than other tryptic peptides. Judging from Figure 1, this reflects its hydrophilicity as well as its acidity.

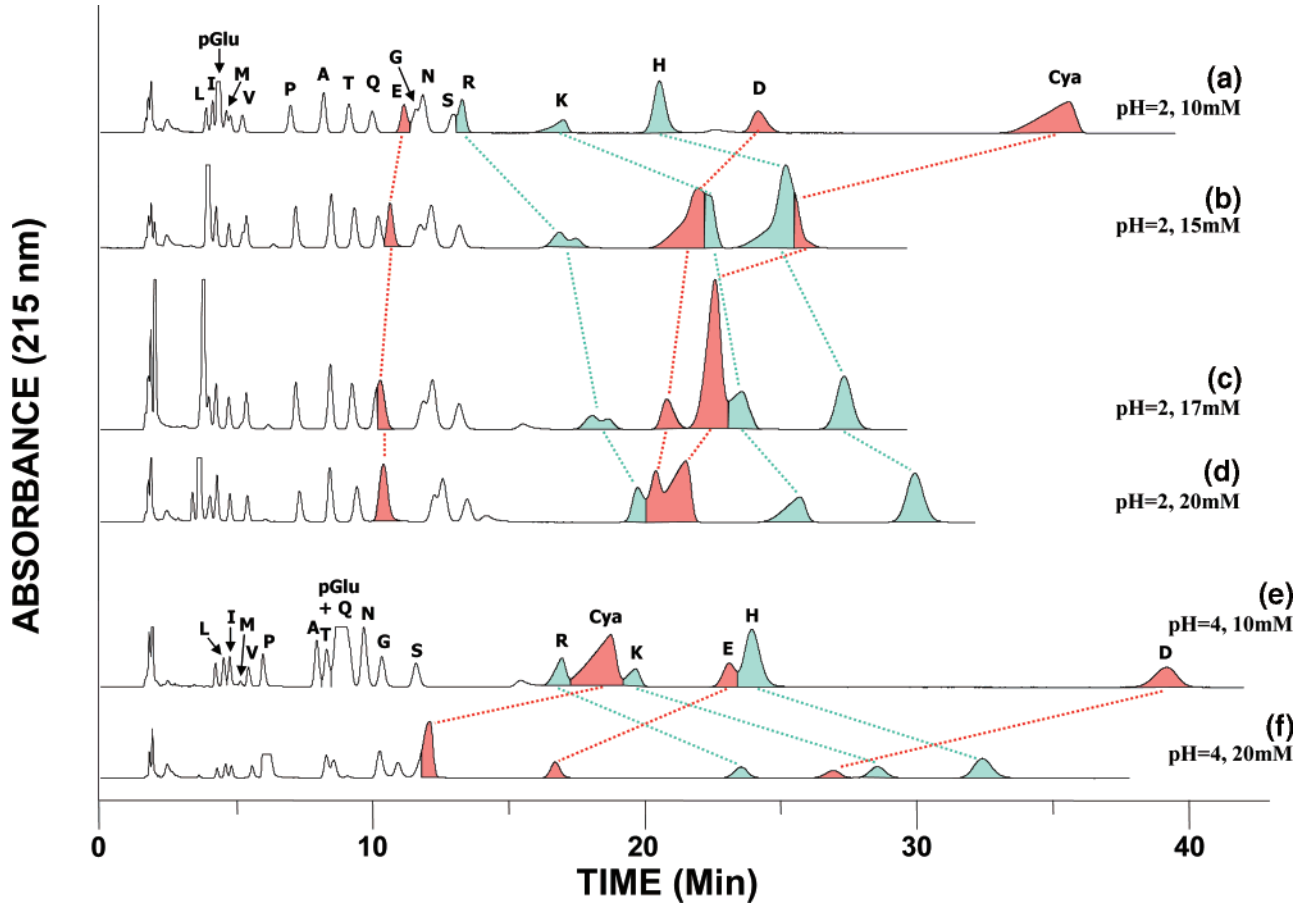

Figure 9. ERLIC of amino acid standards: effect of salt concentration. Column: as in Figure 7. Mobile phase: TEAP (concentration as noted), pH 2.0 (a−d) or 4.0 (e and f), with 65% ACN. Flow rate: 1.0 mL/min. Detection: $A_{215} = 0.04$ AUFS. Sample: 10 μL of amino acids in mobile phase. Cya, cysteic acid. PGlu, pyroglutamic acid. Peaks of basic amino acids are blue while those of acidic amino acids, except for pGlu, are red.

IsoAsp-containing peptides elute somewhat later than their Asp-containing analogues (e.g., 12 vs 11). Between 65−70% ACN, the phosphorylated standard 20 is the last or nearly the last tryptic peptide to elute. Acidic phosphopeptide 13 elutes much later than the other standards under these conditions, while the even more acidic phosphopeptide standard 10 does not elute at all in a reasonable time frame. This emphasizes the importance of the role played by the second basic residue in a tryptic fragment, and the attendant electrostatic repulsion, in assuring elution in a reasonable time frame in ERLIC without the use of high levels of salt. The significant increase in retention of phosphopeptides at high levels of ACN reflects the great hydrophilicity of phosphate groups, which is superimposed on their electrostatic attraction. However, at ACN levels above 70%, hydrophilic interactions become so strong that some basic standards (15 and 16) once again become the best-retained peptides, despite the electrostatic repulsion. This seems to define the window of [ACN] for selective isolation of phosphopeptides from digests. Of course, in tryptic digests with no missed cleavages, no peptides will have large numbers of basic residues unless they are cross-linked or contain His.

Figure 3 suggests that it is possible to set up a well-defined window of isocratic elution for all peptides in a mixture. The width of the window can be adjusted to some extent by varying the percentage of organic solvent. The composition of the peptides is unimportant as long as none are particularly basic or contain phosphate groups.

(D) Effect of pH in ERLIC. Figure 5 shows the effect of pH on retention in ERLIC. Since carboxyl groups are substantially un-ionized at pH 2.0, the best-retained peptides are phosphopeptide 13 and, to a modest extent, tryptic phosphopeptide 20. As carboxyl groups ionize at higher pH values, though, retention comes to reflect the total number of acidic groups of all sorts, and the selectivity for phosphopeptides is lost. These conditions converge upon those of ordinary anion-exchange chromatography. Neutral tryptic peptides are retained almost entirely through hydrophilic interactions. Their retention is little affected by pH as long as the mobile phase contains a reasonable concentration of salt. Retention of acidic peptides reaches a maximum at pH 5.0 and then falls off at higher pH values. This reflects a decrease in the charge density of the weak anion-exchange (WAX) material. Titration curves of suspensions of such materials reveal a continuous increase in charge density from pH 9.5 to pH 5.0.[49]

(E) Effect of Salt Concentration in ERLIC. Figure 6 demonstrates the critical importance of this variable in determining selectivity. Increasing levels of salt shield solutes from all electrostatic effects, both attractive and repulsive, and the selectivity converges on that of HILIC. Thus, retention decreases for

(49) Alpert, A. J.; Regnier, F. E. *J. Chromatogr.* **1979**, *185*, 375.

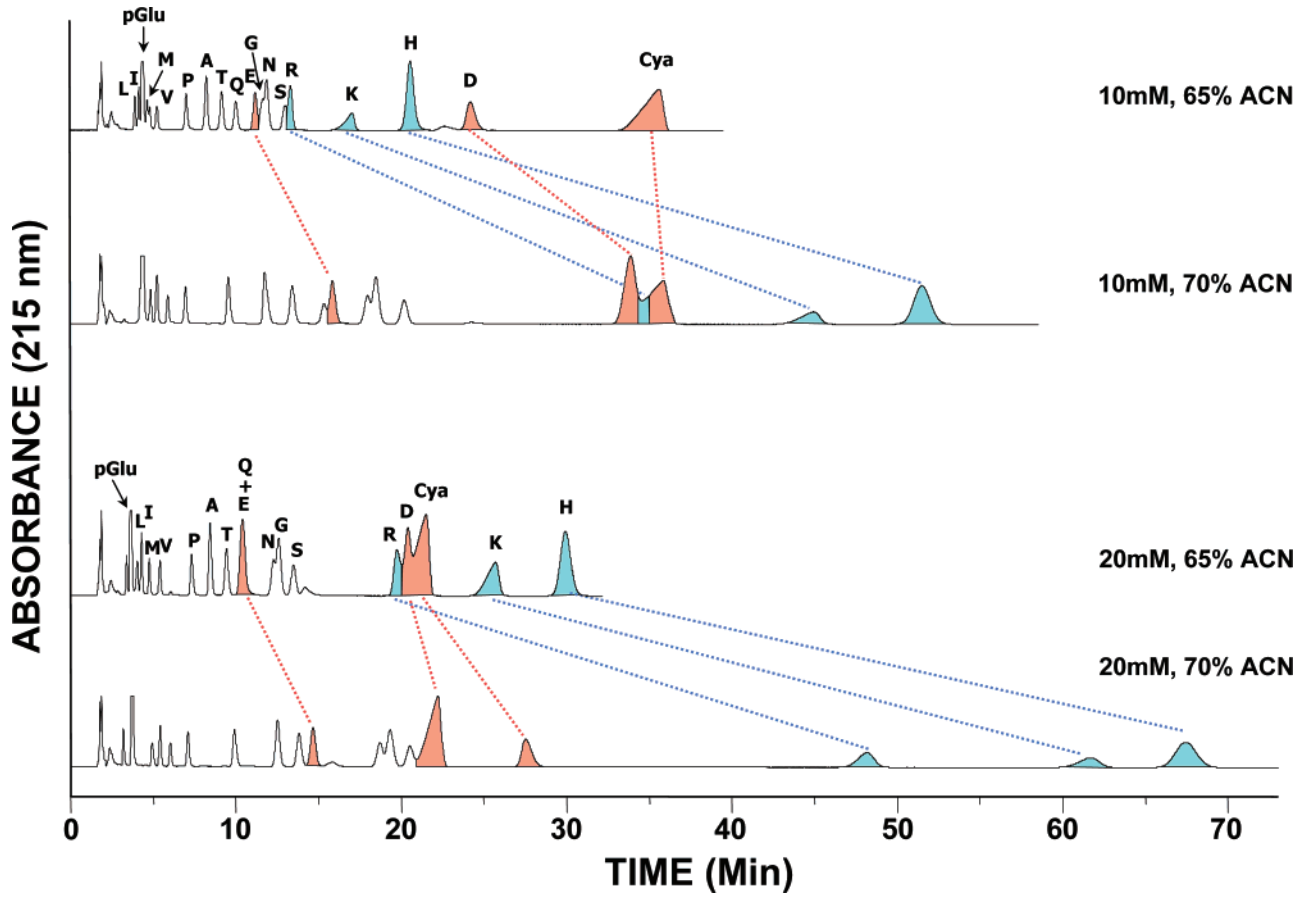

Figure 10. ERLIC of amino acid standards: effect of ACN concentration. Mobile phase: TEAP (pH 2.0) and ACN (concentrations as noted). Other conditions as in Figure 9.

acidic peptides and increases for basic ones, to the point that basic peptides once again become the best-retained at high salt levels. There is a modest increase in retention of neutral tryptic peptides with increasing salt. Presumably this reflects the decreasing repulsion of their N-termini and the basic residues at their C-termini.

These data supplement those in Figure 3 in setting up conditions for a well-defined window of elution of all peptides in a mixture. With enough salt in the mobile phase, even peptides with numerous basic or phosphate groups will elute in a well-defined time frame.

(F) Selective Isolation of Phosphopeptides. The preceding data suggested that peptides with a single phosphate group were likely to be the last or among the last peptides to elute when a tryptic digest was eluted with 20 mM Na-MePO$_4$, pH 2.0, containing 70% ACN. Tryptic peptides with more than one phosphate group proved to require gradient elution. A gradient was selected involving increasing salt and modestly decreasing ACN concentration. The salt chosen was TEAP, which is more effective than Na-MePO$_4$ at eluting phosphopeptides (Table 1). Detailed results are reported elsewhere.[50]

ERLIC of Amino Acids. (A) Effect of Salt Identity and Concentration on Selectivity. The results in Figure 7 compare well with those for peptides in Figure 6; with TEA−MePO$_4$, as with Na-MePO$_4$, there is marked retention of acidic amino acids and comparably early elution of basic ones. Increasing salt concentration suppresses both electrostatic repulsion and attraction, leading to earlier elution of acidic amino acids and later elution of basic ones. There is a slight decrease in retention of neutral amino acids with increasing salt. This reflects the fact that both ERLIC and HILIC are variants of normal-phase chromatography; increasing the polarity of the mobile phase promotes elution. Also, unlike neutral tryptic peptides, neutral amino acids have no marked electrostatic repulsion that would be shielded by the higher salt levels. The results with TEAP (Figure 8) compare well with those for peptides in Table 1; great retention of basic amino acids and comparably weak retention of acidic ones. However, salt concentrations below 20 mM are apparently too low to maintain a counterion layer, or electrical double layer, that effectively screens the underlying stationary phase. The consequence is that solutes are exposed to more of the positive charge of the stationary phase, so basic amino acids are electrostatically repelled more and elute earlier while acidic ones are attracted and elute later. This permits the isocratic elution of both acidic and basic amino acids in the same time frame (Figure 9). The retention times of basic and acidic amino acids are extremely sensitive to the electrolyte concentration within the range of 10−20 mM; higher salt levels shield basic amino acids from electrostatic repulsion and cause them to elute later, while the shielding decreases electrostatic attraction of acidic amino acids and causes them to elute earlier. Retention times of neutral amino acids are little affected in this range. Under these conditions, Phe, Trp, and

(50) Alpert, A. J.; Gygi, S. P.; Li, X.; Ballif, B. A.; Mechtler, K.; Mitulović, G. Submitted for publication.

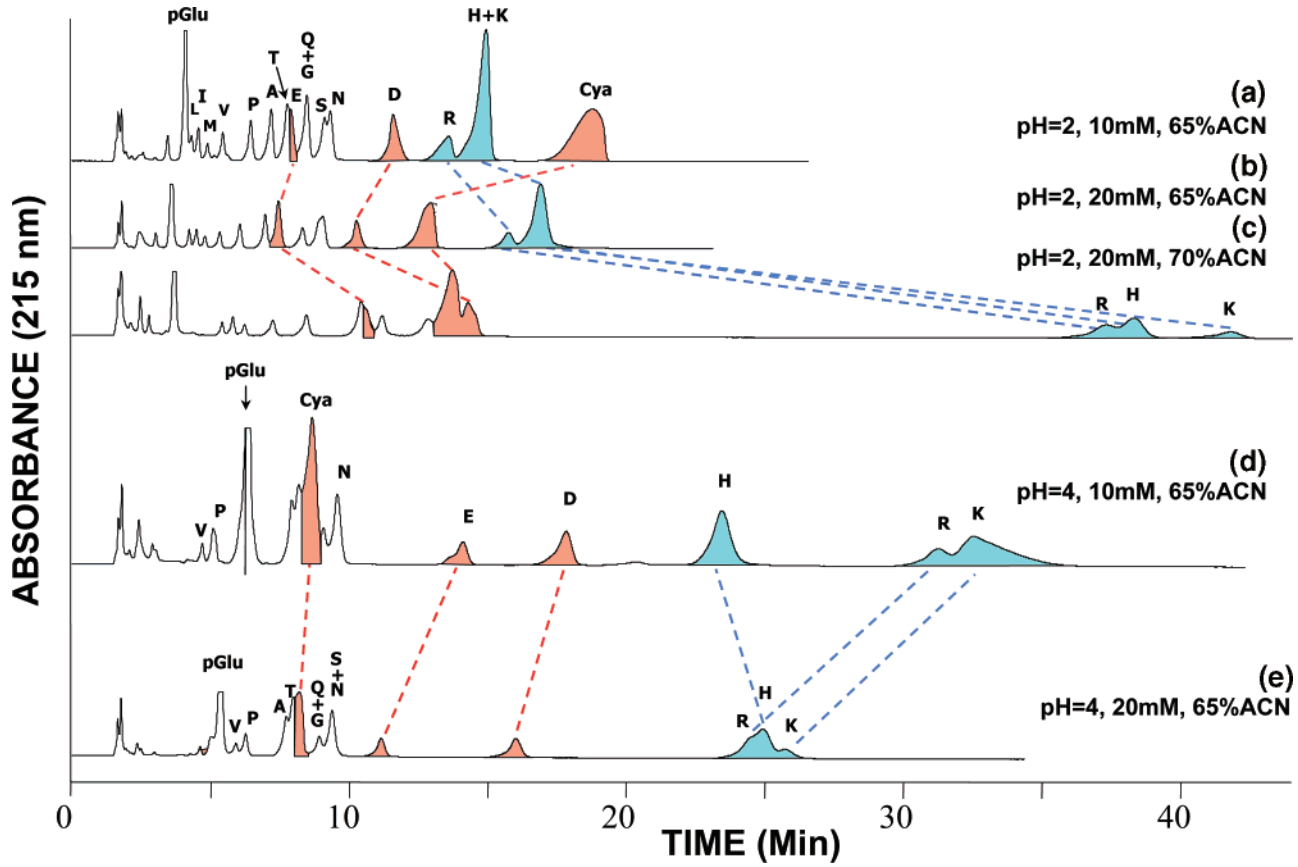

Figure 11. Electrostatic effects on amino acid elution from a Polyhydroxyethyl A column. Column: Polyhydroxyethyl A, 200 × 4.6 mm; 5 μm, 100 Å. Mobile phase: TEAP with ACN (concentrations as noted), pH 2.0 (a–c) or 4.0 (d and e). Other conditions as in Figure 9.

Tyr were incompletely resolved from Leu, Ile, and Val and so were omitted from the mixture. It should be noted that Gln is converted to pyroglutamic acid at pH 2.0, with a half-life of ~24 h for the conversion.

Since the retention of charged amino acids is so sensitive to the salt concentration in the range 10–20 mM, there might be some concern about the robustness of the results. In practice, retention times proved to be quite reproducible under these conditions provided that the columns were equilibrated for at least 1 h following a change to a different salt concentration. This is nearly twice as long as is required with conventional ion-exchange chromatography or HILIC.

Normally Asp would be expected to elute last from an anion-exchange column run in the HILIC mode, reflecting both its charge and the hydrophilic character which exceeds that of Glu and cysteic acid. A pH of 2.0 is low enough to substantially uncharge its functional group, permitting its elution in the same time frame as the other amino acids (unless the wrong salt is used, cf. Figure 7). At pH 4.0, Asp does indeed elute appreciably later than the other amino acids (Figure 9e) unless the electrostatic effects are antagonized by addition of more salt to the mobile phase (Figure 9f). Pyroglutamic acid also has a net negative charge at pH 4.0, and its retention time also decreases significantly (9 to 6 min) when the salt concentration increases from 10 to 20 mM.

When the ACN concentration in the mobile phase is increased from 65 to 70%, retention times of all amino acids increase with the increase in the magnitude of hydrophilic interaction (Figure 10). The most pronounced effect is the increase in retention of the basic amino acids—the most hydrophilic of all[1]—to the point that they no longer elute in the same time frame as the other amino acids even when they and the polar column material bear the same charge. The retention of cysteic acid is notably unaffected by this change in ACN concentration. Cysteic acid, used here as a standard in place of cysteine, appears to be one of the more hydrophobic amino acids[1] whose retention here is due almost entirely to electrostatic attraction. It retains its negative charge even at pH 2.0 ($pK_1 \sim 1.3$). At pH 4.0, the disparity in charge relative to Glu and Asp is appreciably less.

It is instructive to run amino acids on a Polyhydroxyethyl A column under the same conditions. The covalently attached coating is poly(2-hydroxyethyl aspartamide), a neutral polypeptide with free N- and C-termini.[1] Thus, the coating potentially has some positive and negative charge, albeit at a much lower level than does a regular ion-exchange material. At a pH of 4.4, these charges are in balance and the coating is in effect a neutral zwitterion.[51] Above that pH, the net charge is negative; below, positive. At pH 2.0, where the coating has a modest overall positive charge, an increase in the salt concentration in the mobile phase increases retention of basic amino acids and decreases retention of acidic amino acids (Figure 11), as with the PolyWAX LP column. However, at pH 4.0, the coating is near neutrality and an increasing level of salt decreases retention of both basic and acidic amino acids (with the exception of a modest increase in the retention of His). Again, increasing the level of ACN increases hydrophilic interaction and retention for all amino acids, the basic ones in particular (Figure 11c).

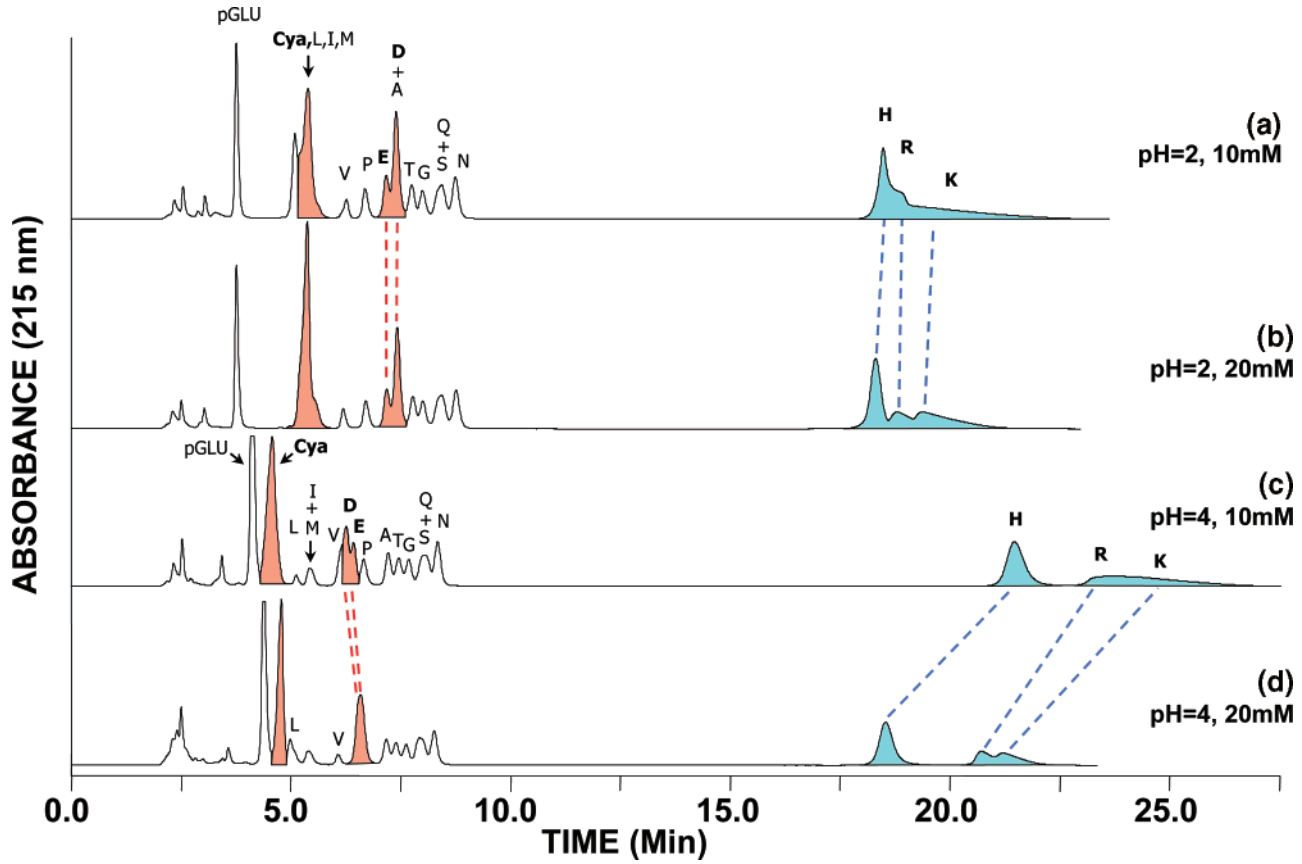

Figure 12. Electrostatic effects on amino acid elution from a TSKgel Amide-80 column. Column: as in Methods. Mobile phase: TEAP (concentrations as noted), pH 2.0 (a and b) or 4.0 (c and d), with 65% ACN. Other conditions as in Figure 9.

The use of nonvolatile salts in HILIC mobile phases is merely a matter of convenience, since salts such as triethylamine phosphate and sodium methylphosphonate permit the use of absorbance detection at low wavelengths and buffer at convenient pH ranges. As with any other essentially neutral stationary phase, Polyhydroxyethyl A can be used with volatile salts or unbuffered acids as electrolyte additives (10, 12−15, 20−23, 25−27) or even with no additive if a solute is not an electrolyte.[24] There are suggestions to the contrary in the literature.[30,52] In order to get a fuller picture of charge effects with various HILIC columns, a TSKgel Amide-80 column was used for amino acids. Figure 12 shows that, at pH 2.0, increasing the salt concentration from 10 to 20 mM does not have much of an effect on the profile. Evidently the material lacks basic groups and is indeed uncharged under these conditions. However, at pH 4.0, the same increase in salt results in a significant decrease in the retention of the basic amino acids and a modest increase in the retention of the acidic amino acids, characteristic of a material with negative charge. Figure 13 compares the PolyWAX LP, Polyhydroxyethyl A and TSKgel Amide-80 columns at pH 4.0. Their charge characteristics at that pH are evident: basic, neutral, and acidic. Guo and Gaiki have also compared Amide-80 with an amino column, ZIC-HILIC (which has a zwitterionic coating derived from sulfobetaine) and uncoated silica for HILIC of polar solutes.[53] While 4-aminosalicylic acid eluted before acetylsalicylic acid from the silica column, it eluted after it from the Amide-80 and ZIC-HILIC columns, suggesting that they had more acidic groups than the silica column. This also suggests that the acidic character of Amide-80 and ZIC-HILIC—modest, to be sure—is a feature of the coatings and not underlying silanols. Increasing the salt concentration from 5 to 20 mM increased the retention of acetylsalicylic acid on all three columns, which Guo and Gaiki correctly speculated involves a weakening of electrostatic repulsion effects by the negatively charged stationary phases. This speculation is examined further in a subsequent paper.[54] One might reasonably conclude that all polar silica-based stationary phases possess some degree of charge under certain conditions and that this does not prevent their convenient use in HILIC.

(B) Kinetics of Counterion Exchange. Initially, amino acid standards were prepared in a solution containing 50 mM TEA−$MePO_4$, pH 2.0, with 70% ACN. When these standards were used for the TEAP concentration study, the basic amino acids afforded increasingly skewed peaks as the concentration of TEAP was decreased in the mobile phases. Figure 14 (bottom) shows an extreme case of this with Arg and 10 mM TEAP. When the Arg standard was prepared with the mobile phase as the solvent, a symmetrical peak was obtained (Figure 14, top). In the bottom trace, the peak at 22 min is probably the elution position of Arg molecules that have retained methylphosphonate as the counterion, while the peak at 42 min is the elution position of Arg

(51) Alpert, A. J. In *Column Handbook for Size Exclusion Chromatography*; Wu, C.-S., Ed.; Academic Press: New York, 1999; pp 249−266 (Figure 8.7).
(52) Higley, T. J.; Yoshida, T. *LCGC Application Notebook*, June 2003; pp 20−21.
(53) Guo, Y.; Gaiki, S. *J. Chromatogr., A* **2005**, *1074*, 71.
(54) Guo, Y.; Srinivasan, S.; Gaiki, S. *Chromatographia* **2007**, *66*, 223.

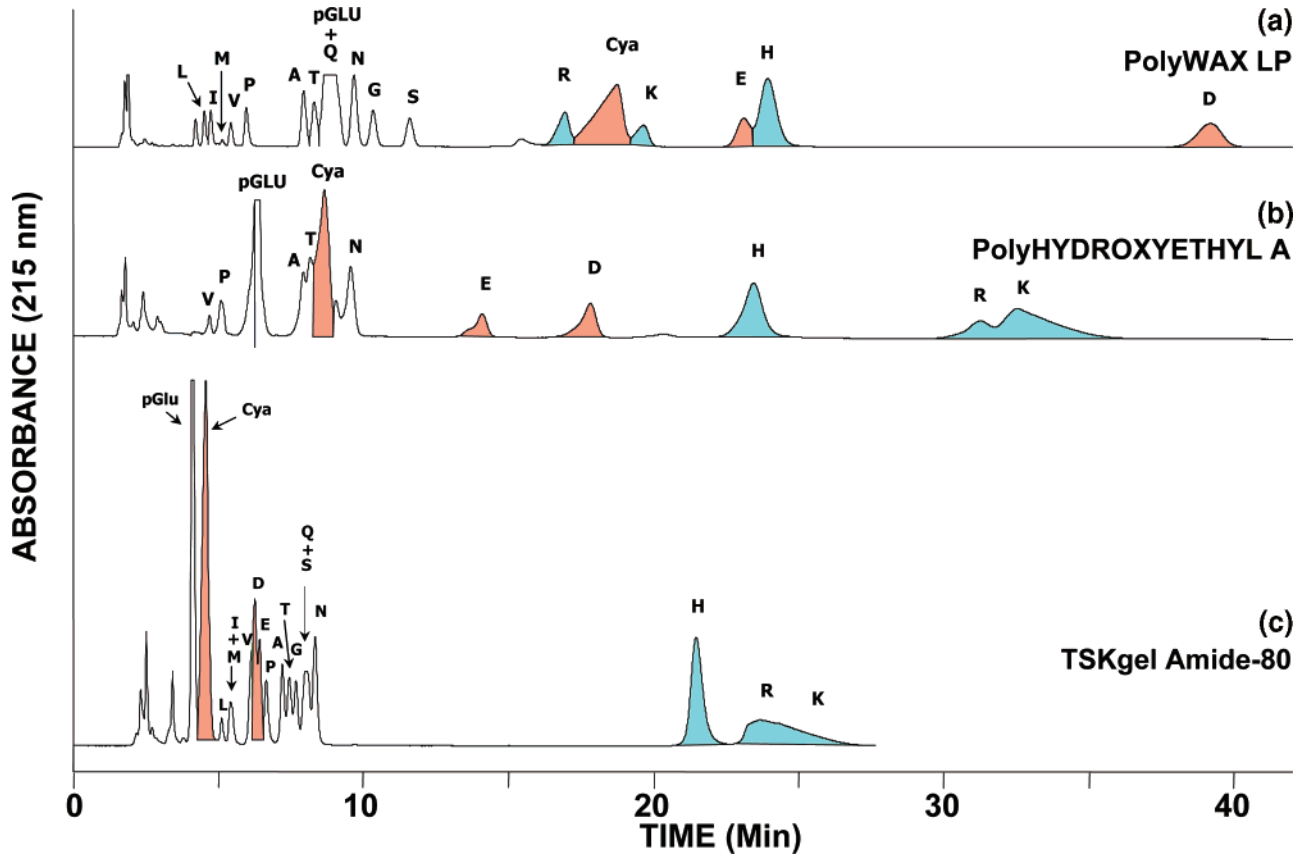

Figure 13. Comparison of amino acid elution from different columns at pH 4.0. Columns: (a) PolyWAX LP (as in Figure 7); (b) Polyhydroxyethyl A (as in Figure 11); (c) TSKgel Amide-80. Mobile phase: 10 mM TEAP, pH 4.0, with 65% ACN. Other conditions as in Figure 9.

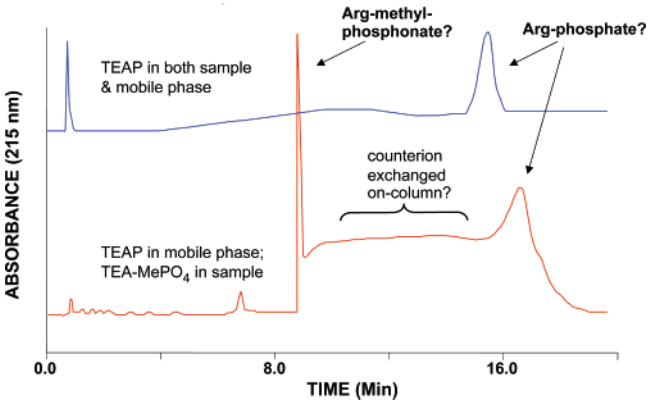

Figure 14. ERLIC of amino acids: effect of counterion on peak shape. Column: see Figure 9. Mobile phase: 10 mM TEAP, pH 2.0, with 70% ACN. Flow rate: 2.0 mL/min. Detection: $A_{215} = 0.16$ AUFS (top) or 0.32 AUFS (bottom). Sample: 5 μL of arginine dissolved in (top) 10 mM TEAP, pH 2.0, with 70% ACN or (bottom) 50 mM TEA–$MePO_4$, pH 2.0, with 70% ACN.

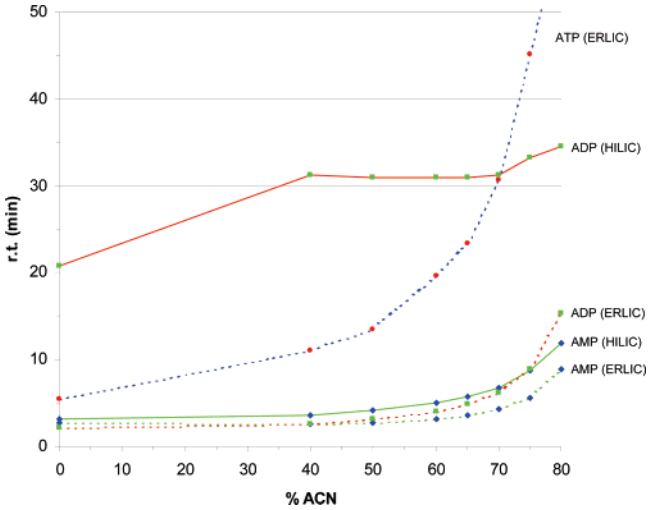

Figure 15. HILIC vs ERLIC of nucleotide standards. Columns: Polyhydroxyethyl A and Polysulfoethyl A, both 200 × 4.6 mm, 5 μm, 300 Å. Mobile phase: 30 mM TEAP, pH 3.0, with % ACN as indicated. Flow rate: 1.0 mL/min.

molecules that have exchanged methylphosphonate for phosphate as the counterion at the beginning of the chromatography. The continuum between these peaks probably reflects the exchange by Arg molecules of methylphosphonate for phosphate at intermediate points during the migration through the column.

These observations demonstrate that counterion exchange can be remarkably slow relative to the time scale of the chromatography. One clear lesson is that samples should be prepared with an appropriate counterion in solution. In addition, the apparent strength of the interaction between solute and counterion sheds light on the mechanism of selectivity (see Discussion).

ERLIC of Nucleotides and Nucleic Acids. (A) HILIC versus ERLIC. Nucleotides and nucleic acids possess negatively charged phosphate groups. Therefore, ERLIC of these compounds was performed with a cation-exchange column. Figure 15 compares the results with HILIC of these compounds on a column of

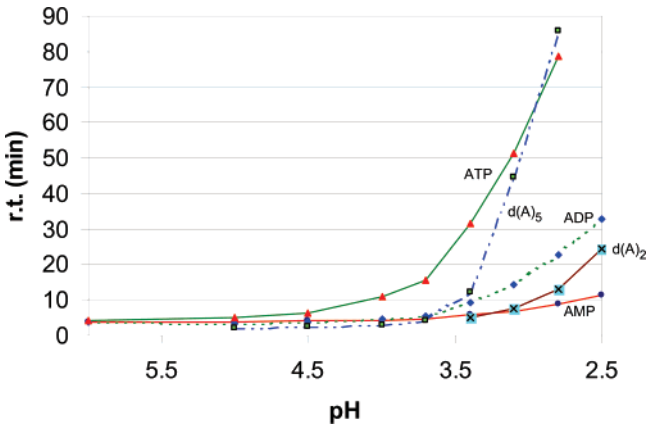

Figure 16. ERLIC of nucleotides and oligonucleotides: Retention vs pH. Column: Polysulfoethyl A (as in Figure 15). Flow rate: 1.0 mL/min. Mobile phase: 30 mM TEAP, pH as indicated, with 80% ACN. The mobile phases were all prepared from stock solutions of 300 mM TEAP at the indicated pH.

a neutral material, Polyhydroxyethyl A. At low concentrations of ACN where hydrophilic interactions are negligeable, ADP elutes earlier than AMP from the cation-exchange column due to its greater electrostatic repulsion. At higher levels of ACN, where hydrophilic interactions with the phosphate groups become significant, their elution order is reversed. One would expect ATP to elute earlier than ADP at low levels of ACN. Its greater retention, seemingly anomalous, is discussed later. With the neutral column, the difference in retention between AMP, ADP, and ATP is much greater, due to the lack of electrostatic repulsion and the great polarity of phosphate groups. This is especially the case here with ADP (ATP did not elute from the neutral column in a reasonable time under these conditions).

(B) Effect of pH in ERLIC. At pH 6, where phosphate groups are beginning to acquire their second negative charge, electrostatic repulsion is so great that no nucleotide or oligonucleotide is retained (Figure 16). Retention increases with decreasing pH, particularly below pH 3.4, where the phosphate groups begin to lose their single negative charge. This effect is especially pronounced with the solutes containing the most phosphates, ATP and $d(A)_5$. With the less-phosphorylated solutes, it is difficult to separate the effect of decreasing negative charge on the phosphate groups from that of the increasing positive charge ($+1 \rightarrow +2$) on the adenine rings (pK_a at 3.6−4.0, depending on the nucleotide[55]).

(C) Effect of Salt Concentration and Identity on Selectivity. Figure 17 shows that the effect of the base on retention in ERLIC is the same as in HILIC:[1] U ~ T < A < G < C. At the ACN level used here, phosphorylation promotes retention in every case. Increasing salt increases the retention of UMP, AMP, and GMP (but not CMP), indicating that electrostatic repulsion is a significant factor in their retention throughout the range. By contrast, the retention of di- and triphosphonucleotides increases to a maximum at 40 mM salt and falls off thereafter. A possible interpretation is that 40 mM salt is sufficient to shield most of the repulsive effects, while higher concentrations shield the electrostatic attraction of the positively charged base for the stationary phase. The mechanism of this effect is discussed later.

(55) Table 8.8, Lange's *Handbook of Chemistry*, 15th ed.; Dean, J. A.. Ed.; McGraw-Hill, Inc.: New York, 1999.

Figure 18 displays the results obtained with TEA−$MePO_4$ substituted for TEAP. There is an increase in sensitivity to the number of phosphate groups at the expense of sensitivity to the base involved. Thus, there is a significant increase in the retention of the triphosphonucleotides relative to the retention of the mono- and diphosphonucleotides; The mechanism of this change is addressed later (see Discussion). TEAP is the better of the two salts with regard to isocratic elution of all the common nucleotides in the same time frame (Figure 19).

DISCUSSION

Some Applications for ERLIC. Using general-purpose isocratic conditions, ERLIC indeed seems to be capable of obtaining separations of electrolyte mixtures that normally would require gradients. The only other mode of chromatography that routinely performs separations isocratically under standardized running conditions is size exclusion chromatography (SEC). The resolution of SEC is limited to the number of peaks that can fit into the range between Vo and Vt. No such limitation pertains to ERLIC; the elution window can be widened merely by increasing the amount of organic solvent in the mobile phase. This affects the selectivity, since polarity effects then assume greater importance compared to electrostatic effects, so the utility of this approach should be assessed on a case-by-case basis. Nonetheless, certain general-purpose running conditions seem to suffice for a wide range of solutes. This should simplify methods development considerably. Not all mixtures will lend themselves to such treatment. No chromatographic method will afford complete separation of all the components in a protein digest that contains over 50 peptides, for example. However, complete separation is not necessary in every case. For example, if a mass spectrometer is used as the detector, it is only necessary to reduce the number of peptides coeluting to an extent that they do not interfere with each others' ionization. In that case, one could use an automatic sample injector to analyze a large number of samples rapidly, injecting each sample after the ERLIC window of elution of the preceding sample. The use of isocratic elution would simplify the equipment needed. ERLIC could be useful for separations performed on a silica wafer or chip, in which many samples might be analyzed simultaneously in numerous channels on a minute scale. Flow rates for such applications would be on the order of nanoliters per minute. It would greatly simplify the equipment needed for such separations as well if they could be performed isocratically. Finally, the advent of bottom-up or shotgun proteomics has increased the demand for alternative ways to fractionate complex mixtures of peptides in multidimensional approaches. ERLIC is a promising complement to current modes of chromatography.

Detection with mass spectrometry or an evaporative light scattering detector will require the development of ERLIC mobile phases based on volatile salts. The pronounced effect of counterions on retention in ERLIC complicates any attempt to substitute volatile salts for nonvolatile salts in the mobile phase. Maintaining a particular combination of selectivity may require careful matching of polarity and steric hindrance of the ions. Ammonium acetate or ammonium propionate may prove to be a suitable substitute for sodium methylphosphonate as long as a pH above 3 is satisfactory for an application, while triethylamine formate may be a satisfactory substitute for triethylamine phosphate. For applications requiring buffering at pH 2.0, one might try am-

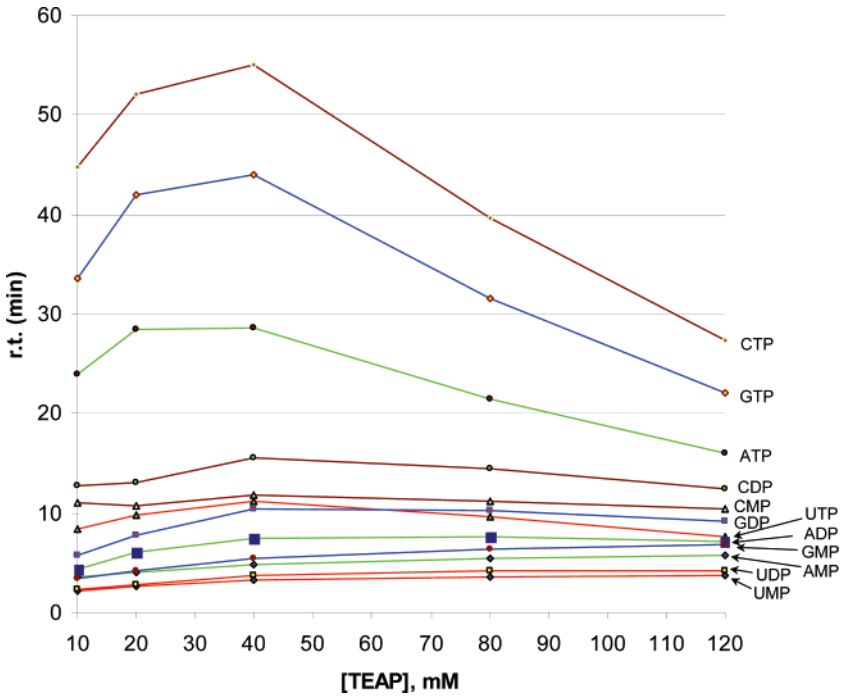

Figure 17. ERLIC of nucleotides: retention vs concentration of TEAP. Column and flow rate: see Figure 16. Mobile phase: TEAP (concentration as indicated), pH 3.0, with 75% ACN.

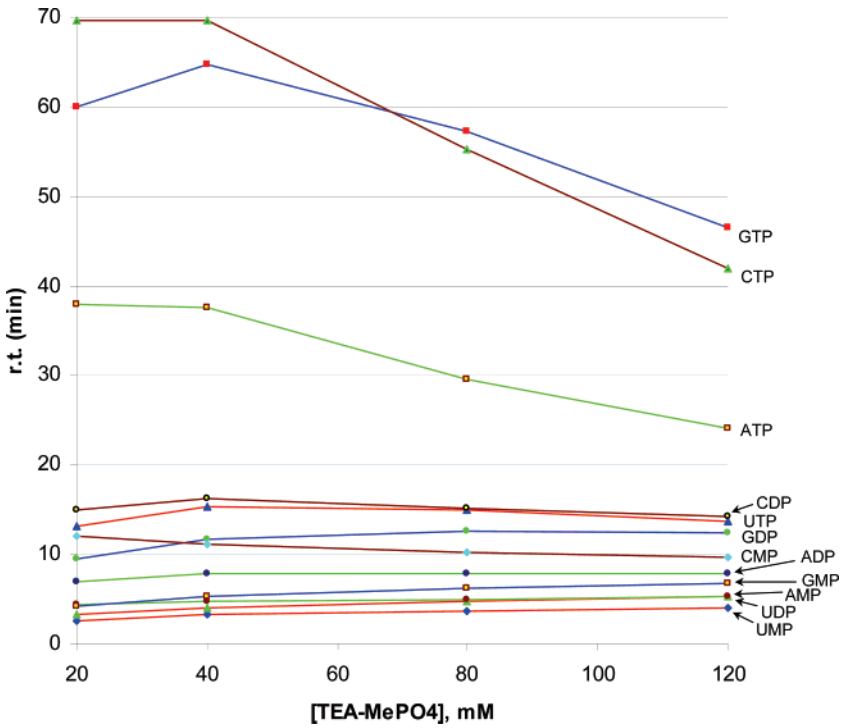

Figure 18. ERLIC of nucleotides: retention vs concentration of TEA−$MePO_4$. Conditions: same as for Figure 17, but with TEA−$MePO_4$ substituted for TEAP.

monium cyanoacetate ($pK_a = 2.46$) or the triethylamine salts of malonic ($pK_1 = 2.8$) or pyruvic ($pK_a = 2.4$) acid if the latter two are sufficiently volatile. Trifluoroacetate forms ion pairs of such hydrophobic character that retention decreases significantly in HILIC (ref 28, Figure 1) and in ERLIC as well (data not shown) and should probably be avoided.

The specific analysis of phosphopeptides is of considerable interest at present for analyses in proteomics, reflecting the increasing evidence that phosphorylation plays a key role in regulation of biological processes. ERLIC of phosphopeptides is treated in depth in a separate paper.[50]

Mechanism of Selectivity Effects. The selection of the salt in ERLIC can have a dramatic effect on selectivity. This can be accounted for if one assumes that these solutes, while small, can nonetheless be oriented in a rigid manner during their migration through the HPLC column. This has already been demonstrated

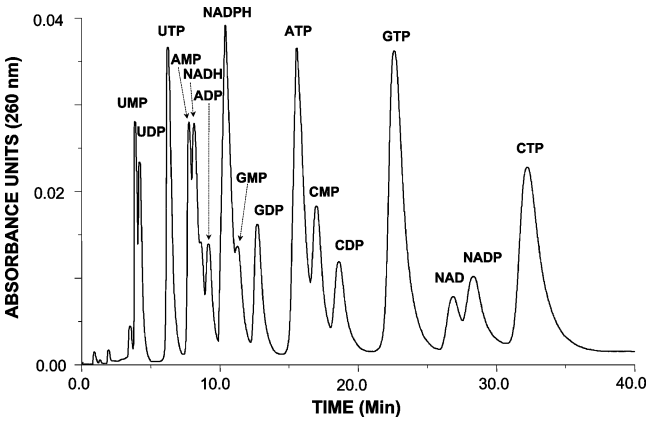

Figure 19. ERLIC of nucleotide standards. Column: see Figure 16. Mobile phase: 80 mM TEAP, pH 3.0, with 84% ACN. Flow rate: 2.0 mL/min. Detection: A_{260} = 0.08 AUFS. Sample: 6 μL of nucleotide standards dissolved in mobile phase.

with disaccharides in HILIC, for example.[11] Figure 20 is a schematic of the orientation of amino acids in ERLIC. With phosphate as the counterion, its potential second negative charge provides a means for the attraction of basic amino acids to the surface. The feasibility of this mechanism is supported by the great stability of such ion pairs shown in Figure 14. The potential for inducing a second accessible negative charge in methylphosphonate ion is significantly less. This would account for the observation that basic amino acids and peptides are better-retained in ERLIC with phosphate than with methylphosphonate as the counterion. By contrast, acidic amino acids would be repelled by the negatively charged layer of phosphate ions on the surface of the stationary phase and hence elute rapidly with TEAP buffers unless the concentration of TEAP is too low to afford complete coverage of the surface. Figure 9 suggests that that is the case below 20 mM TEAP.

Orientation effects with nucleotides appear to be more complicated. Figure 21 is a schematic contrasting the orientation of AMP and ATP. The phosphate group of AMP, being quite hydrophilic, is oriented toward the stationary phase but is repelled by it. Increasing salt concentrations suppress the repulsion and increase the retention of AMP. The nature of the counterion associated with the positively charged base has little influence on retention. With ATP, the repulsion of the three phosphate groups by the stationary phase is so strong that they are oriented away from it. With the base now facing the stationary phase, retention is strongly influenced by the nature of the counterion, as in Figure 17. This inverted orientation also accounts for the retention of ATP on a cation-exchange column in the absence of ACN and hydrophilic interaction (Figure 15). ADP, not having the base oriented so rigidly toward the stationary phase, elutes in the void volume under these conditions. With sufficient ACN, the hydrophilicity of phosphate groups is such that they confer net retention on a molecule whatever their orientation.

A similar rationale can be applied to the orientation of peptides in ERLIC. Basic residues are likely oriented away from the stationary phase, even if they augment the net retention of the peptide. This would enhance selectivity for neutral and acidic residues. Thus, ERLIC should be able to afford separations that would be difficult to obtain in other modes of chromatography, including HILIC. A cautionary note is that ERLIC will not work if a solute does not have an orientation or a domain that is not repelled by the stationary phase. Thus, kanamycin and other aminoglycoside antibiotics elute in the void volume, as does a peptide nucleic acid containing lysine residues on every face (data not shown). A similar peptide nucleic acid with lysine residues on one face only was well-retained.

Electrostatic Repulsion in the Literature. Electrostatic repulsion has been noted in numerous papers in the literature and occasionally exploited. For example, Teichberg et al. performed affinity purification of lectins on columns of the same charge. In the presence of salt, the surface charges were sufficiently shielded that affinity interaction counterbalanced the repulsive forces, but subsequent elution with water eliminated the shielding and the lectins eluted.[56]

Recently there have been some efforts to design stationary phases where electrostatic repulsion plays some role. Nogueira et al. introduced a reversed-phase material in which the hydrophobic ligand terminates with a positively charged aminoquinuclidine group.[57] The material retains peptides through a combination of hydrophobic interaction and electrostatic attraction or repulsion, depending on the charge of the solute. This combination affords selectivity that can be useful on a case-by-case basis but lacks the general utility of ERLIC. For example, a solute with no acidic or hydrophobic groups would not be retained on this material under any circumstances. By contrast, in HILIC or ERLIC, a solute with at least some degree of polarity will be retained if one uses sufficient organic solvent in the mobile phase. In addition, it has yet to be demonstrated with a reversed-phase material that some combination of organic solvent and electrostatic repulsion effects will result in isocratic elution of all solutes in a particular class in a reasonable time frame. Liu et al. developed a coating for HILIC by grafting a cucurbituril to silica.[58] Electrostatic repulsion effects were observed in HILIC of basic alkaloids on this positively charged stationary phase and were clearly explained, although not really exploited.

Of late, one of the more thoroughly studied classes of HILIC materials is silica with a zwitterionic coating. The work of Guo et al. with a sulfobetaine-coated column[53,54] has been mentioned. The sulfonate group is on the end of the ligand while the amine group is embedded in the middle. This column material was developed in the laboratory of Irgum and his colleagues.[31] Recently this group introduced a zwitterionic material with a phosphorylcholine coating: a quaternary amine group on the end of the ligand and a phosphate group embedded in the middle.[59] This is the opposite of the charge arrangements in the sulfobetaine-based materials. It is instructive to inspect their data in light of the principles worked out in the current study. Their coating was determined to have a net negative charge overall. However, there seems to be a trend for the surface amine group to play a more significant role with regard to charge effects as the pH increases. Thus, at pH 7, three angiotensins elute in order of increasing acidity, not increasing hydrophilicity (ref 59, Table 2 and Figure 9). At <70%

(56) Teichberg, V. I.; Aberdam, D.; Erez, U.; Pinelli, E. *J. Biol. Chem.* **1988**, *263*, 14086.
(57) Nogueira, R.; Lämmerhofer, M.; Lindner, W. *J. Chromatogr., A* **2005**, *1089*, 158.
(58) Liu, S.-M.; Xu, L.; Wu, C.-T.; Feng, Y.-Q. *Talanta* **2004**, *64*, 929.
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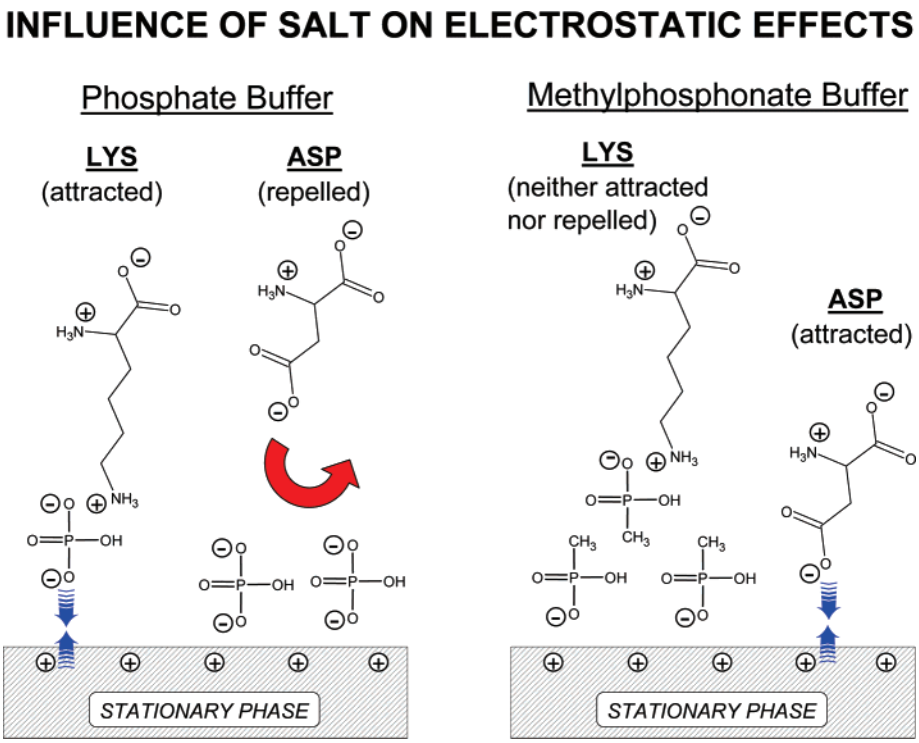

Figure 20. Orientation of amino acids in ERLIC as a function of charge and mobile-phase counterions.

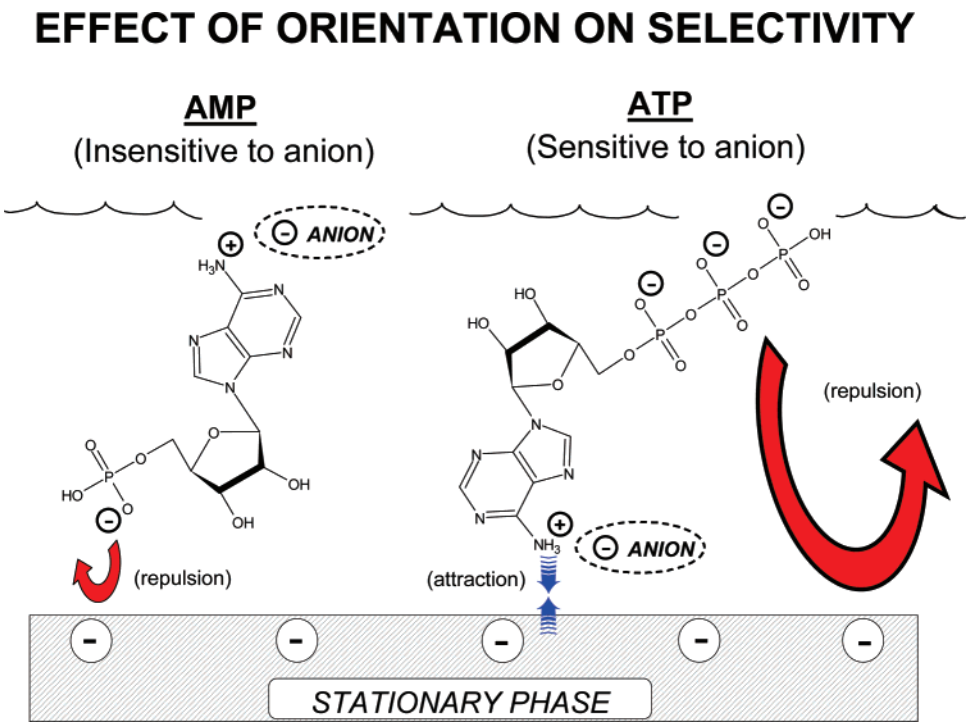

Figure 21. Orientation of nucleotides in ERLIC and its effect on sensitivity to counterions.

ACN, electrostatic repulsion effects are pronounced with either this phosphorylcholine material or with uncoated silica, consistent with the data in the present study. The amine layer on top may be functioning as a permanent electrical double layer atop the phosphate group layer, with any counterions from the mobile phase functioning as an electrical triple layer. The zwitterionic nature of the coating complicates any attempt to explain the role of charge but does not rule it out. Thus, at extremely low salt levels, there is insufficient salt to titrate the charged groups in the coating and a complete triple layer cannot form. The amine groups in the coating interact instead with the embedded phosphate groups in a zwitterionic bond. The coating then manifests its net negative charge and basic peptides are the best retained (ref 59, Figure 7). As the salt level increases, the embedded phosphate groups are titrated and the amine groups are freed to interact with external charged solutes, in this case

via electrostatic repulsion of the basic peptides, whose retention selectively decreases. Coverage appears to be complete around 20 mM salt, consistent with the data in the present study. The data from the present study also lead to the prediction that substitution of TEAP for ammonium acetate in Irgum's study would have resulted in a triple layer with a net negative charge (cf. Figure 20 here) and an increase in retention of basic peptides with salt concentration in the range 4−20 mM rather than the decrease noted with ammonium acetate. Similar effects are evident in the separation of N-glycans on the sulfobetaine (ZIC-HILIC) column.[60] While Takegawa et al. provided a fine explanation of the effect of electrostatic repulsion on retention of sialylated glycans, they had some difficulty accounting for the slight increase in retention of 2-aminopyridine (PA)-derivatized neutral glycans when the ammonium acetate concentration is increased from 5 to 20 mM. At 5 mM salt, one can assume that the sulfonate group in the coating is in a zwitterionic interaction with the embedded amine group. A 20 mM salt concentration suffices to titrate the amine group, freeing the sulfonate group to interact via electrostatic attraction with the positively charged PA group. Presumably a higher concentration of salt would outcompete the PA groups and lead to a decrease in retention again.

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ACKNOWLEDGMENT

The skilled technical assistance of Liya Venzel and Rachel Alpert is acknowledged with thanks, as is the loan of the TSKgel Amide-80 column by Yong Guo (Johnson & Johnson). TSK-Gel is a registered trademark of Tosoh Corp. ZIC-HILIC is a registered trademark of SeQuant AB. Sephadex is a trademark of GE Healthcare.

Received for review May 16, 2007. Accepted September 13, 2007.

AC070997P