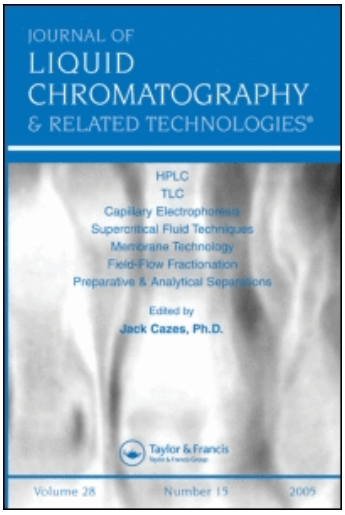

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Liophilic Mobile Phase Additives in Reversed Phase HPLC

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Abstract: The separation of basic compounds can be challenging and the use of inorganic mobile phase additives have been successfully used in chromatographic methods development. About fifteen years ago the role of these additives as ion-interaction agents for selectively adjusting retention of ionic analytes was discovered and the theory of chaotropicity was applied to reversed phase chromatography. In the last ten years the studies of the influence of these counterions on the retention of ionizable analytes and the interaction with the stationary phase in various hydro-organic eluents has expanded our knowledge of this phenomenon.

The general view and understanding of the process have been significantly updated and the use of these ionic additives (liophilic ions) in the mobile phase has become regular practice in the pharmaceutical industry for optimization and fine tuning of complex separations. This paper reviews the latest developments in the field and discusses the modification and expansion of our theoretical understanding of the process. The paper also describes their application in practical separations for a wide variety of analytes, from small molecules to peptides and even chiral separations.

Keywords: Chaotropic, Liophilic, Pharmaceutical, Protonated basic compounds

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INTRODUCTION

Optimization of chromatographic separation for complex mixtures can be accomplished thru modification of the mobile phase by varying type and concentration of organic solvent, mobile phase pH, and type and concentration of ionic additives. Alteration of the retention of basic compounds in their protonated form can present a challenge due to their inherent polarity and consequent early elution in reversed phase HPLC.

While organic eluent composition shows a significant effect on the retention of all analytes in the mixture it has a relatively small effect on the selectivity for components in either the fully ionized or neutral forms. On the other hand, eluent pH could dramatically shift the retention of ionizable analytes and leads to significant selectivity differences. Another approach to modulate changes in retention includes the use of ionic additives that interact with the positively charged basic analytes either in the mobile phase or the stationary phase. This can be accomplished either thru the addition of amphiphilic ions or with the use of liophilic ions. Amphiphilic ions can be loosely categorized as ions that have significant hydrophobic moieties such as long alkyl chains, and behave as ion-interaction reagents below the critical micelle concentration. Liophilic ions are usually small inorganic ions and they possess an important ability for dispersive and electrostatic interactions.

Amphiphilic Ions

The addition of oppositely charged amphiphilic ions, which can interact with the protonated basic compounds either in the mobile phase[1–4] or in the stationary phase via an ion exchange mechanism, have been successfully used to enhance the retention of basic compounds.[1,2,5–8] The enhancement of the retention of basic compounds on reversed phase columns in the presence of amphiphilic ions can be attributed to ion-exchange chromatography, for which a theoretical background has been developed by Horvath,[9] Sokolovski,[10,11] and Stahlberg.[12] It can be characterized as stoichiometric adsorption of ionic species, as well as the adsorption of ions and formation of an electrical double layer.[12] Due to the presence of hydrophobic chains, these anionic additives (amphiphilic ions) tend to be strongly adsorbed by the hydrophobic stationary phase, leading to surface modification of the column, which may not be reversible.[8,13] This may lead to method reproducibility issues especially during method transfer from the development site to production site (quality control).

Liophilic Ions

Liophilic ions are small, usually inorganic ions that have the ability for dispersive type and electrostatic interactions. They are characterized by significant delocalization of their charge, primarily symmetrical, usually in spherical shape, and at the same time they do not have deleterious properties of surfactant agents (surface modification of the bonded phase).[14] Recent developments in application of liophilic ions as mobile phase additives for basic compounds offer additional advantages in variation of selectivity and efficiency. In contrast to the irreversible adsorption of amphiphilic ions on the reversed phase surface, liophilic ions shows relatively weak interactions with the alkyl chains of the bonded phase. In recent years, there has been a heightened interest in the usage of inorganic salts as additives to the mobile phase for the analysis of small basic drugs, proteins, and peptides.

To date, liophilic mobile phase additives have been used extensively for the analysis of basic compounds such as amines, pyridines,[15] ophthalmic drug compounds,[16] beta-blockers,[17] antidepressants,[18] the dansyl-amino acid enantiomers,[19] alkaloids,[20] and antibiotics.[21]

This review article focuses on the influence of liophilic ions as mobile phase additives for the enhancement of chromatographic figures of merit (i.e., retention, peak efficiency, tailing) for basic compounds/peptides in the reversed phase mode.

EFFECT OF LIOPHILIC IONS ON RETENTION OF BASIC ANALYTES

Criteria for Employment of Liophlic Mobile Phase Additives

Selective variation of the protonated basic analytes retention could be achieved by the use of liophilic mobile phase additives. The eluent pH affects the degree of ionization of these basic solutes and the HPLC retention profile in respect to mobile phase pH is a sigmoidal function as shown in Figure 1. Equation (1)[9] describes the effect of mobile phase pH on the retention factor of basic solutes:

$$k = \frac{k_0 + k_1 \cdot ([H^+]/K_{a(BH^+)})}{1 + ([H^+]/K_{a(BH^+)})} \quad (1)$$

where k_0 and k_1 are the retention factors of the neutral and ionized solute, respectively, BH^+ is the protonated basic analyte, K_a is the acid dissociation constant for the base, and $[H^+]$ is the proton concentration.

At a pH 2 units from the analyte pK_a in a particular hydro-organic media, the basic analyte retention is supposed to plateau. According to

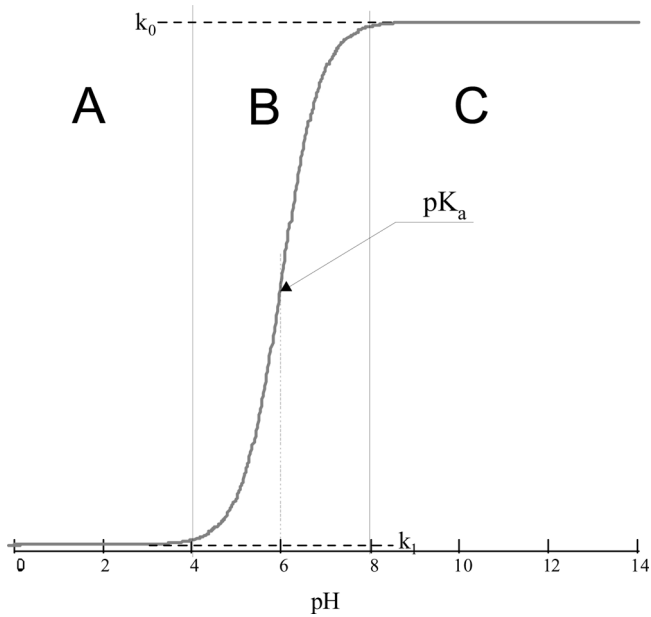
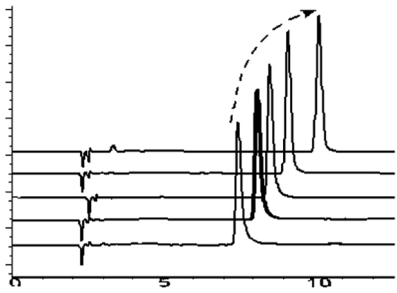


Figure 1. Theoretical curve showing basic analyte retention as a function of mobile phase pH. Region A is where the basic analyte is in its fully protonated form, (BH^+); region B is where both unionized and ionized forms, ($B^+H^+ \Longleftrightarrow BH+$), are present and the pH at the inflection point denotes the pK_a of the compound; region C is where the compound is in its unionized (neutral) form, (B).

the equation (1), analyte retention should not be affected by any variation of the mobile phase pH more than 2 units away from its pK_a. On the other hand, the decrease of the mobile phase pH could only be achieved via variation of the concentration of acidic mobile phase modifier with simultaneous variation of the concentration of the acidic counterions.[22]

Acidic modifiers containing counteranions that are liophilic in nature will interact with the protonated basic analytes. The extent of the ionic interaction is dependent on the concentration of the free counter-anion in the mobile phase, and is not further dependent on the mobile phase pH as far as the analyte is in the fully protonated form. This implies that the increase in retention of the protonated basic analytes may be observed with an increase in concentration of the counter anion by the addition of the acid or salt at a constant pH (i.e., where the basic analyte is in its fully ionized state).

As seen in Figure 2, the retention of pharmaceutical analyte X (pK_a 5) was increased as the concentration of counteranion was increased by addition of sodium perchlorate at a constant pH (pH 2.0). This



Total ClO_4^- [mM]	pH	Rt. (min.)
100	2.0	9.9
89	2.0	
79	2.0	
70	2.0	
55	2.0	7.7

Figure 2. Variation of the retention of basic analyte ($pK_a > 5$) with counteranion concentration [perchlorate]. *Source*: Reprinted with permission from Ref. [23].

modulation of the retention can be a very powerful approach during methods development of basic pharmaceutical compounds.

As it could be seen from comparison of Figures 1 and 2, variation of the mobile phase pH has distinctly different retention variation profiles as compared to the addition of liophilic ions. These two effects are orthogonal to each other to some extent, since concentration of liophilic ions could be varied independently of the mobile phase pH. In paper [24] mutual effect of both of these parameters was studied. The overall picture of modulation of analyte retention as a function of pH and liophilic mobile phase additive concentration is represented in Figure 3.

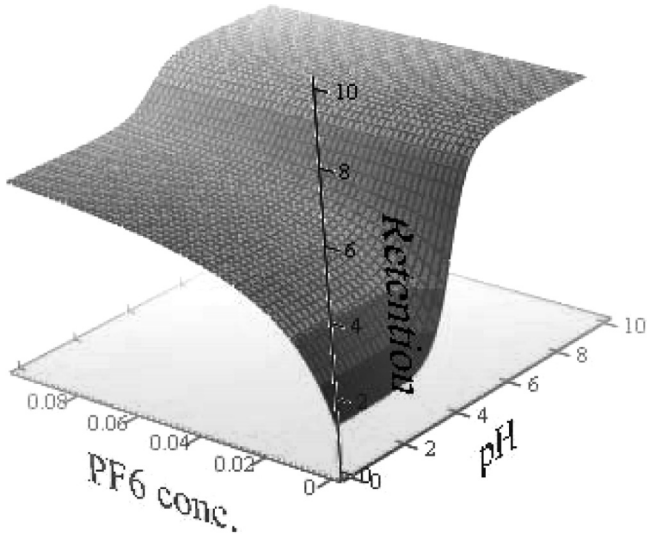


Figure 3. Effect of pH and liophilic ion concentration on basic analyte retention. *Source*: from Ref. [24].

Enhancement of retention of the basic analyte compound with the liophilic mobile phase additive can occur in the pH region, in which the analyte molecule is in its protonated state.

It was shown[15,17] that addition of liophilic anions influence the retention of only cationic species in the sample mixture. In other words, only protonated basic analytes will be affected by the addition of liophilic ions, while for the same analytes in their neutral form their retention generally will not be impacted by addition of liophilic additives. This offers an opportunity for effective variation of chromatographic selectivity for specific compounds.

Ionic Interactions with Liophilic Ions

The influence of liophilic ions on the retention of basic analytes can be best described with three different possible mechanisms:[14]

1. Ion-pairing, which involves the formation of neutral ion-pairs and their retention according to the reversed-phase mechanism.
2. Disruption of the analyte solvation shell. Liophilic counteranions upon ion interaction with analyte disrupt the analyte solvation shell, making the analyte more hydrophobic, thus increasing its retention in RPLC.
3. Adsorption of liophilic counterions on the adsorbent surface. Liophilic ions can adsorb on the hydrophobic stationary phase due to their dispersive interactions ability. This may cause the formation of the charge on the adsorbent surface and corresponding electrostatic interactions between the adsorbed ions and the charged analyte.

It is very likely that all three of these mechanisms coexist with one dominating, depending upon the eluent type and composition and adsorbent surface properties.

Ion-Pairing Vs. Ion Association

The variation of the eluent composition can cause changes in the mobile phase dielectric constant and this can influence the strength of the ionic interactions.[25] At a higher concentration of organic content, there is a greater propensity for ionic interaction between liophilic ion and charged analyte, since the dielectric constant of the medium is decreased.

The stability of ion associated complexes will be more favorable in mobile phases that have a lower dielectric constant as shown in Equation (2). Lower values of the dielectric constant results in greater

attraction forces (***F***) between oppositely charged species at a certain critical distance *d*.

$$F = \frac{q_1 q_2}{3\pi D^2 \varepsilon} \tag{2}$$

Where q_1 and q_2 denote charges of ions, ***D*** is the critical distance between the ions needed for ion-pair formation, and ε is the dielectric constant.

If coulombic forces participate in the formation of ion-pairs, they are formed only if the ions approach each other and the separation of ions is no greater than the critical separation distance (***D***) given by the Bjerrum's Equation (3),[25] shown below:

$$D = z^+ z^- \frac{e^2}{2\varepsilon k T} \tag{3}$$

In Equation (3), z^+ and z^- are the ionic charges, ***e*** is electron charge, ε is dielectric constant, ***k*** is Boltzmann's constant, and ***T*** is the absolute temperature. When the ion separation distance is less than or equal to ***D***, ion-pairing is regarded as taking place. The dielectric constant of a solvent plays a significant role. At the same critical distance (***D***) needed for ion-pair formation, a solvent with a high dielectric constant, such as water ($\varepsilon \sim 80$) will be less favorable for ion-pair formation than with a solvent that has a lower dielectric constant ($\varepsilon < 40$, such as acetonitrile and methanol). Horvath et al. mentioned in his work that changes in organic composition of the mobile phase effect dielectric constant and hence ion-pair formation.[9] Carr and Wang, in their work, also indicated the importance of the value of the dielectric constant of the mobile phase for the ion-pair formation.[26]

Also, the eluent composition in close proximity to the adsorbent surface is different than the bulk mobile phase due to the adsorption of the organic component on the hydrophobic stationary phase surface.[27,28] In this organic enriched region, this environment would further support ion-pair formation.

In recent publications, Guiochon et al. proposed the predominance of the effect of the ion-pairing mechanism on retention of the basic analytes.[29,30,31] Carr et al. examined the retention behavior of small molecules and peptides in their ionized forms as a function of the concentration of acetonitrile in the mobile phase. It was observed, that with the increase of organic concentration as the dielectric constant of the mobile phase decreased, 1) the interactions between the basic analyte in ionized form and the mobile phase counteranions and 2) dynamic ion-exchange contributions was enhanced, which led to an increase in the analyte retention. Carr et al. concluded that the dielectric constant is the major driving force behind the increase in ion-pair stability, which leads to the increased retention time phenomenon.[26]

Carr et al. considered hydration free energies (Gibbs free energy of hydration) of anionic counter ions as the key to understanding their impact on the basic analyte retention. They believe that highly hydrated ions such as $H_2PO_4^-$ (Hydration $\Delta G = -437\,kJ/mole$) have a lesser ability to form ion-pairs compared to less hydrated ions such as acetate (Hydration $\Delta G = -373$ kJ/mole). Also, as a consequence, poorly hydrated ions such as CLO_4^- (Hydration $\Delta G = -214\,kJ/mole$) have a greater tendency to produce stronger ion-pairs and also be adsorbed on non-polar stationary phases.[32]

Effect on Analyte Solvation

In the second process mentioned above in Ionic Interactions with Liophilic Ions, the liophilic mobile phase counteranions upon ion association with protonated basic analytes disrupt the analyte solvation shell, making the analyte more hydrophobic, and as a consequence is increasing retention[15,22] and could be correlated to ions in the Hofmeister series.

The Hofmeister series is a classification of ions in order of their ability to influence the structure of water. Originally the use of these ions, was studied in the field of biochemistry where it was shown that the conformational state and the solvation behavior of proteins and peptides could be affected. The effects of these ions were first determined by Franz Hofmeister, who studied the influence of cations and anions on the solubility of proteins.[33] Hofmeister discovered a series of salts that have an impact on the solubility of proteins and on the stability of their secondary and tertiary structures. One group of salts could be ranked according to their efficiency in precipating proteins (kosmotropes), and a second group could be ranked according to their efficiency in solubilizing proteins (chaotropes), Figure 4.

Ions in solution can also be defined as kosmotropic or chaotropic based on their "water structuring" or "water disrupting" nature, respectively.[35,36] Kosmotropes tend to structure water, such as sulfate, phosphate, magnesium (2^+), lithium (1^+), while chaotropes tend to disrupt water structure, e.g., bromide, iodide, pottassium, caseium (1^+), perchlorate, etc.. In aqueous solution, the presence of certain ions that was found to disrupt the water structure[37] in structured ionic solutions was given the name "chaotropic" ions.[38]

Also a thermodynamic approach, utilizling the Gibbs free energy of hydration, ΔG_{hydr}, can be used to quantify most inorganic and some organic ions as chaotropic or kosmotropic.[39,40] The more negative the free Gibbs energy of hydration, ΔG_{hydr}, the more kosmotropic the salt, due to the increase of water structure around the ion, compared to chaotropic ions.[41] Chaotropic ions disrupt the dynamic hydrogen bond lattices of water, and hence have larger values for ΔG_{hydr} than a

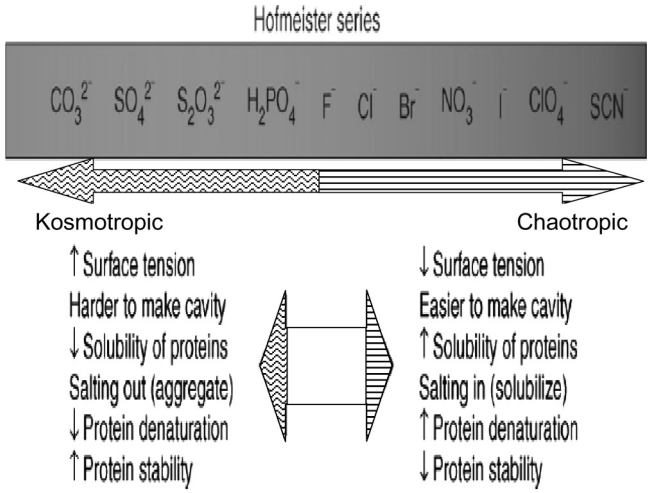


Figure 4. Hofmeister Series. *Source*: Adapted from Ref. [34].

kosmotorpic salt.[39] More recently, computational techniques have been also used to predict and improve the accuracy of the ΔG_{hydr} for the proton[42] and hydroxides,[43] as well as other ions.[44]

The Hofmeister series[45,35] of salts originally used in protein and peptide analysis[36,46] have been applied towards chromatographic analysis of small basic molecules.[15–18,22,32,47–49] The degree of the analyte solvation shell disruption is dependent on the nature of used counterion. The influence of the "chaotropic ions" on the disruption of the basic analyte solvation and ion-interaction with the protonated basic analyte can be essentially related to the increase in ion "hydrophobicity," as a result of charge delocalization and significant polarizability.

Flieger studied the effect of "chaotropic" mobile phase additives on the retention of different alkaloids.[20] He found general agreement of the retention variation with Equation (1) and with the Hofmeister series, for the following sequence of liophilic ions:

$$H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$$

A greater possibility for charge delocalization and higher overall electron density is seen from left to right with a simultaneous increase in the ion symmetry. This leads to an increasing ability of these ions to participate in dispersive interactions.

Chaotropic ions can be considered a subset of liophilic ions. The use of these inorganic mobile phase additives for modulation of the retention of ionic analytes in reversed phase HPLC has lead to the development

of another possible theory of their influence on the chromatographic retention of basic compounds.[15,22,50,51] Protonated basic analytes in water–organic mixtures are highly solvated. The solvation shell suppresses the analyte's ability for hydrophobic interactions with the stationary phase, thus effectively decreasing the analyte's retention. Modulating the disruption of the solvation shell allows for control of the analyte retention. Counter-anions that have a less localized charge, high polarizability, and lower degree of hydration can affect the solvation of basic analytes to different degrees.[52] Upon ion interaction (either in mobile phase or adsorbed on the stationary phase surface) with the solvated protonated basic analyte, leads to the disruption of the analyte solvation shell and increase in the relative hydrophobicity of the analyte or the ion-associated species, which results in an increase in retention of the basic analyte.[14] Kazakevich, LoBrutto, et al. assumed the existence of an equilibrium between solvated and desolvated analyte molecules and counteranions, and this was described mathematically. The Langmuir-type dependence of analyte retention increases with an increase of counterion concentration and has been explained on the basis of solvation-desolvation equilibrium.

The desolvation process of the protonated bases depends on the concentration of free counterion. Some authors emphasize the importance of creating an ion-pair in the mobile phase between the cationic analyte and liophilic anion.[29–31] An ion-pair created in the mobile phase has higher hydrophobicity and has more affinity for the stationary phase and undergoes retention in the form of neutral complexes. Dai and Carr[48] investigated the effect of type of anionic additives (formate, chloride, trifluoroacetate, hexafluorophosphate) on ion-pair formation with basic pharmaceutical compounds. The data obtained by the capillary electrophoresis method (CE) in buffered acetonitrile/water phase, commonly used in chromatography, suggests that the extent of ion-pairing is limited. The influence of the ions on ion-pair formation is ordered as the following: $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$. However, the portion of the analytes present as an ion-pair with anionic additives was estimated to be not greater than 15%.[48]

Disruption of the basic analyte solvation shell theoretically should be possible with interaction of any counteranion used, and the degree of this disruption will be dependent on the type of liophilic anion employed. The influence of liophilic counteranions on analyte desolvation has been established according to their ability to destabilize or bring disorder (chaos) to the structure of water.[10,11]

Adsorbed Ions on Stationary Phase

The ionic interactions of the liophilic ions with protonated analyte should be independent on the proton concentration in the mobile phase,

provided that complete protonation of the basic analyte is achieved. This process shows a ''saturation'' limit, when counteranion concentration is high enough to effectively disrupt the solvation of all analyte molecules. A further increase of counteranion concentration should not produce any noticeable effect on the analyte retention.

Retention of the analyte in completely desolvated form should be independent of the type of counteranion used; however, the experimental results of LoBrutto et al.[23] demonstrated that the use of different counteranions leads to different retention plateaus as shown in Figures 5 and 6. The solvation-desolvation mechanism alone cannot explain the difference in analyte retention in the desolvated form (high counterion concentration) when different counterions are employed.

The enhancement of retention of protonated basic anlaytes with the addition of liophilic anions can also be driven by a ''dynamic ion-exchange'' process as previously described for hydrophobic ion-pairing reagents by Horvath.[4] This model implies that large, weakly hydrated anions such as $PF_6^-, ClO_4^-, CF_3COO^-$ may partition into the adsorbed layer of the organic component of the mobile phase, close to the stationary phase, and create a charged surface capable of ion-exchange. Adsorption of anions on the column varies and could be correlated with the hydration free energy of anions[32,53] and the type and concentration of organic employed.[54,55] Depending on the nature of the liophilic anion, it could be adsorbed to different degrees on the reversed phase packing material. This could provide a suitable rationale in regards to the different retention plateaus observed in Figures 5 and 6 when different anions are employed.

Acetonitrile, when used as an organic component in the mobile phase forms a thick adsorbed layer on the surface of the hydrophobic stationary

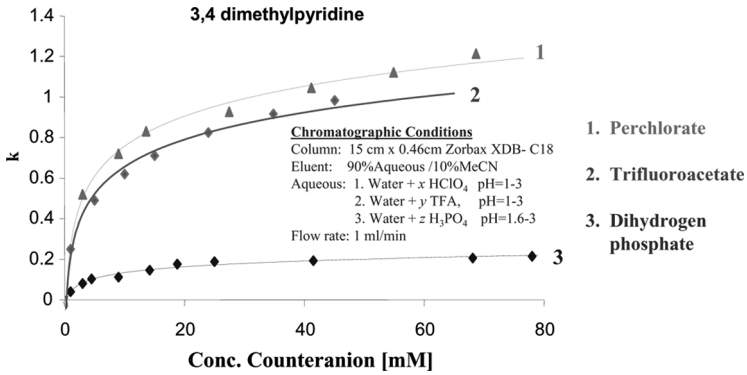


Figure 5. Influence of different counteranions on the retention of 3,4 dimethylpyridine. *Source*: Reprinted with permission from Ref. [23].

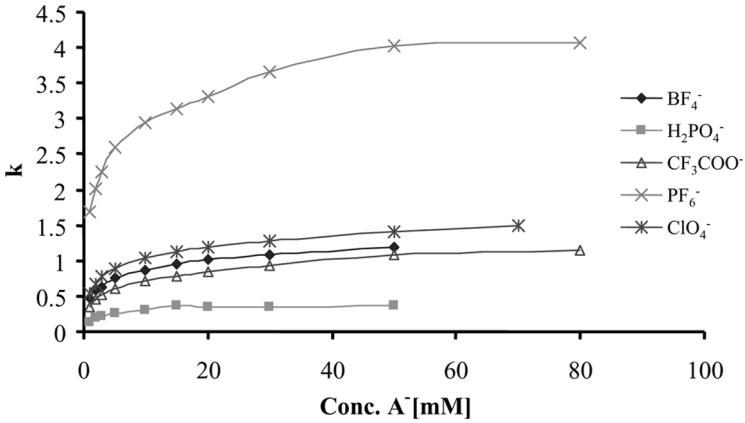


Figure 6. Retention factor variations for acebutolol analyzed with different ionic additives. *Source*: Reprinted with permission from Ref. [17].

phase, while methanol forms a thin monomolecular adsorbed layer on the surface of the stationary phase.[54] The thick multi molecular adsorbed layer of acetonitrile (~14Å) allows for the adsorption of liophilic ions on the stationary phase, adding an electrostatic component to the retention mechanism, while the monomolecular layer of methanol (~2Å) is not suitable for the adsorption of liophilic ions.

Liophilic anions such as BF_4^-, perchlorate, and PF_6^- are retained to different degrees using acetonitrile/water eluents on alkyl- and phenyl-type adsorbents.[55] At all mobile phase conditions with acetonitrile/water, the PF_6^- ion exhibits the greatest retention and is the most liophilic ion in the Hofmeister series. This ion can be characterized as having the highest delocalization of the charge and the highest polarizability, which allows it to interact with acetonitrile. Other anions have similar properties, but their ability for dispersive interactions is lower then PF_6^-. At acetonitrile concentrations up to 20 v/v% acetonitrile, all ions exhibited maximum retention on reversed phase adsorbents.

Acetonitrile adsorbed on top of the bonded phase acted as a suitable phase for ion accumulation. At low organic concentrations (from 0 to 30 v/v% of acetonitrile), the studied ions demonstrated considerable deviation from ideal retention behavior, which resulted in an increase in ion retention with an increase in acetonitrile composition. Considerable absorption of the liophilic anions in this acetonitrile layer creates an electrostatic potential within this adsorbed organic layer. At higher concentrations of organic in the mobile phase, the retention of counteranions is decreased (typical reversed phase retention behavior).

Kazakevich and Snow also studied the adsorption of hexafluorophosphate on reversed phase stationary phases using frontal chromatography. They found that maximum adsorption of PF_6^- occurred at 15% acetonitrile in the mobile phase, and then as organic content increased further, this resulted in an exponential decrease in the PF_6^- adsorption. On the other hand, methanol at all studied concentrations did not show a maximum retention for hexafluorophosphate and did not provide a suitable environment for PF_6^- adsorption. (Figure 7).[56]

Multiple ionic interactions can occur with the liophilic anions and the protonated basic analytes in the reversed phase column. In one process, it could be envisaged that the liophilic anions in the mobile phase (acetonitrile/water) are disrupting the solvation of the analyte (desolvated analyte-anion complex) in the mobile phase and, thus, increasing the apparent analyte hydrophobicity. In another process, since there is an adsorbed layer of acetonitrile (MeCN) on top of the bonded phase in MeCN/Water mobile phases, dispersive interactions could occur between the desolvated analyte-anion complex that partitions into the organic layer and the acetonitrile molecules. Moreover, liophilic anions may also partition in the acetonitrile layer and are consequently adsorbed on the surface of the stationary phase.[55] These liophilic ions have increased solubility in this organic layer due to their ability for dispersive interactions with π-electrons of acetonitrile. Interaction between these liophilic anions in the acetonitrile adsorbed layer and the protonated

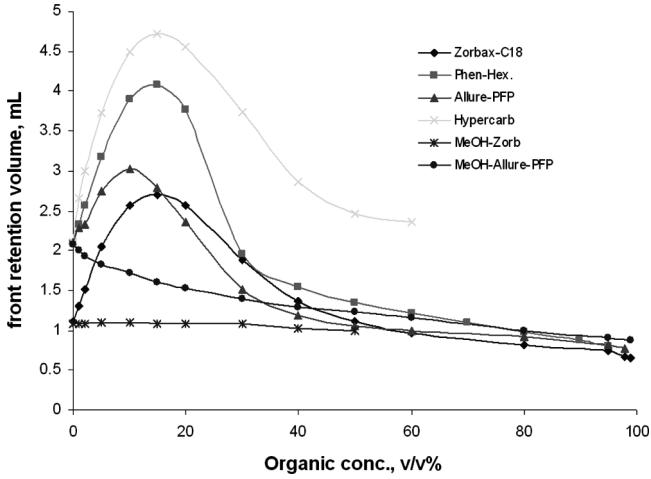


Figure 7. Overlay of the retention volumes of PF_6^- front (0.05 mM concentration of NH_4PF_6 in the solution) measured from acetonitrile/water (all four columns) and methanol/water (Zorbax-C18 and Allure-PFPP columns). *Source*: Reprinted with permission from Ref. [56].

basic analyte can also contribute to the increased retention. Ion-interaction is favored in this organic rich layer due to lower dielectric constant of the MeCN compared to the hydro-organic mixture (Figure 8).

The complex form of the liophilic ions adsorption on the stationary phase, as a function of organic concentration should be also reflected on the retention of basic analytes, and this was experimentally observed (Figure 9[57]). Note, that the analyte relative retention increase is only observed in acetonitrile/water systems where a thick adsorbed organic layer is formed. In methanol/water systems, since methanol only forms monomolecular adsorbed layer, this does not provide a suitable medium for the retention of liophilic ions.

In Figure 10, the effect of different liophilic anions was investigated at three methanol concentrations, 10, 20, and 40% methanol. Further increase of the methanol concentration (above 10%) essentially eliminates the influence of counteranions on the analyte retention for ephedrine and ranitidine. Since, the liophilic ions are not adsorbed on the stationary phase in methanol/water systems, the enhancement of retention is mainly driven by the solvation-desolvation equilibria process in the mobile phase.

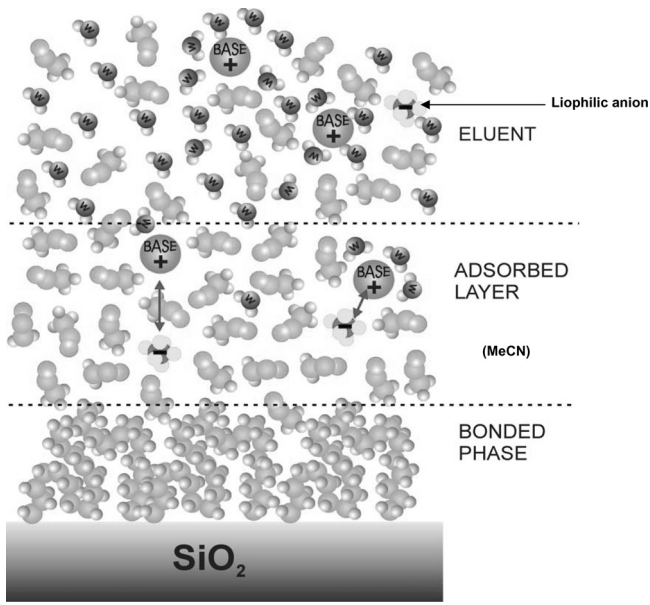


Figure 8. Schematic of the retention mechanism of basic analyte on reversed phase material in water/acetonitrile eluent in the presence of liophilic ions. *Source*: Reprinted with permission from Ref. [14].

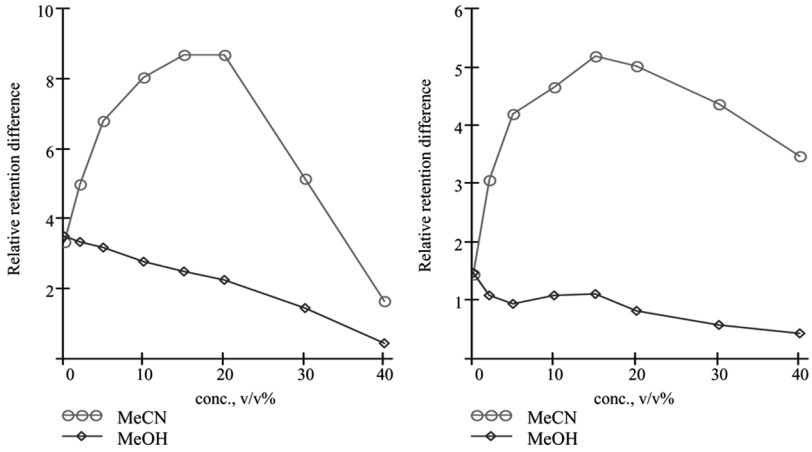


Figure 9. Relative adjusted retention of aniline (PF_6/no-PF_6 ratio) on Allure-PFPP (left) and Zorbax-C_{18} (right) columns from Acetonitrile (circles) and from methanol (diamonds). *Source*: Reprinted with permission from Ref. [57].

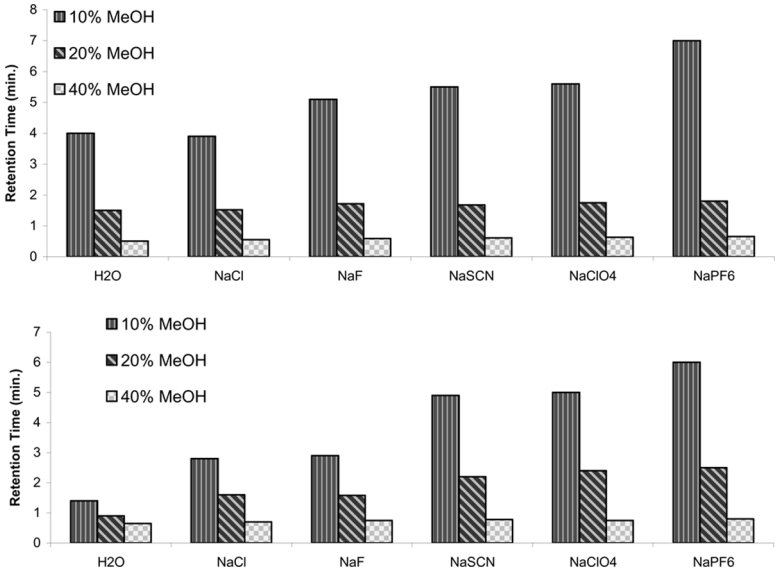


Figure 10. Effect of different type of counteranions at equimolar concentration (10 mM) on the retention of protonated Ranitidine (top) and Ephedrine (bottom) in MeOH/Water mobile phases. Column: Waters Sunfire C_{18} column, 3.5 µm, 50×4.6 mm, Flow rate 1.0 ml/min, Temp. 30^0C, Inj. 5 µL, Detection 254/225 nm, Isocratic, Mobile phase A: 80% Water with 10 mM counteranion, all pHs less than 6.7, Mobile phase B: 10,20,40% MeOH. *Source* from Ref. [58].

PRACTICAL OBSERVATIONS WITH USE OF LIOPHILIC IONS IN CHROMATOGRAPHIC APPLICATIONS

Effect of Type of Liophilic Counteranion

Different counteranions in the mobile phase can affect the chromatographic retention of protonated basic analytes to varying degrees. As the first step towards understanding the mechanism of the counteranion effect, the relative effect of different inorganic ions on the retention of tertiary amines (tertbutylaniline) and pharmaceutical compounds was studied.[58] All the analytes studied had pK_a values greater than 8.4, so they were protonated at neutral and acidic mobile phase pH values and no specific pH adjustment of the mobile phase was needed, and phenol was run as a control. The influence of various anions (F^-, Cl^-, Br^-, SO_4^-, $H_2PO_4^-$, NO_3^-, ClO_4^-, BF_4^-, PF_6^-, SCN^-, CF_3COO^-, CCl_3COO^-, CH_3COO^-, $HCOO^-$) on the model analyte retention were studied (Figure 11) on Sunfire-C_{18} and Luna-C_{18} columns. Acetonitrile/water mobile phases containing 10 mM concentration of the corresponding counteranion (added in the form of salt or acid) was used. Depending on the type of anions used (at equimolar concentration), each anion varied the retention of each protonated analyte to different degrees. Hexafluorophosphate showed the greatest effect on the retention compared to other anions. Similar effects were observed on the Luna-C_{18} column and overall effects were independent of the type of C_{18} bonded phase employed, Luna-C_{18} or Sunfire C_{18}.

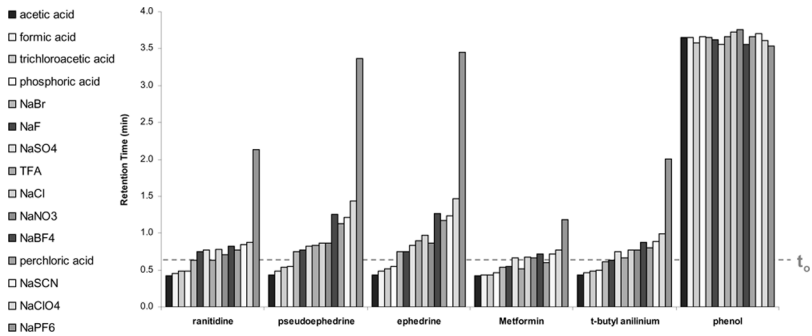


Figure 11. Effect of different type of counteranions at equimolar concentration (10 mM) on protonated basic analyte retention in MeCN/Water mobile phases. Column: Waters Sunfire C_{18} column, 3.5 μm, 50 × 4.6 mm, Flow rate 1.0 ml/min, Temp. 30^0C, Inj. 5 μL, Detection 254/225 nm , Isocratic, Mobile phase A: 80% Water with 10 mM counteranion, all pHs less than 6.7, Mobile phase B: 20% MeCN. *Source*: From Ref. [58].

Flieger[20] studied the type of liophilic mobile phase additive, phosphate, trifuloroacetate, hexafluorophosphate, and perchlorate at a constant pH for different classes of alkaloids. In this study a Zorbax C-18 column was used and the pH of the mobile phase was kept constant at 2.7 (Figure 12). Also, in this evaluation hexafluorophosphate exhibited the greatest changes in retention compared to other mobile phase additives.

Effect of Concentration of Liophilic Mobile Phase Additives on Basic Analyte Apparent Efficiency and Loading Capacity

Ionized basic compounds undergo secondary interactions with underivatized residual free silanols of silica based stationary phases.[51,59,60] This is a very undesirable phenomenon, which may lead to an increase of retention, peak tailing, and hinders accurate quantitation for analytical assays. Carr et al. indicated that the influence of silanophilic interactions would be the greatest between analyte and free residual silanols of the reversed phase silica base at pHs >4. Counter cations (for example, sodium) can interact with ionized silanols and decreases the contribution to retention from ion-exchange of the cationic analyte with ionized silanols.[32] Also, to suppress unwanted effects of silanol interactions, cationic additives to the mobile phases such as hexylamine, octylamine, diethylamine (DEA), triethylamine (TEA), tetrabutylammonium chloride (TBA-Cl)[6,7] may be added to the mobile phase to compete with the protonated basic molecules for the energetic sites (i.e., silanols) on the stationary phase. The use of hydrophobic cationic ions is usually considered as last resort, since these ions may be adsorbed on the surface of the stationary phase permanently. The use of liophilic mobile phase additives provides an alternative approach, for enhancement of peak

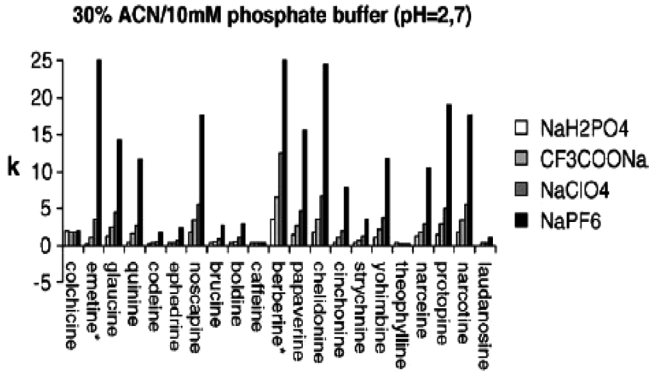


Figure 12. Effect of anionic additive type on the retention of investigated alkaloids. *Source*: Reprinted with permission from Ref. [20].

efficiency and reduction of peak tailing of protonated basic molecules.[16] The liophilic anions may adsorb on the stationary phase and tend to suppress the undesired interactions with highly energetic sites (i.e., residual silanols, active silanols) in the bonded phase. At the same time, liophilic ions, are more advantageous since they can be easily eluted from the reversed phase columns.

Different inorganic counteranions at equimolar concentrations have been noted to lead to an increase in retention and peak symmetry, as well as greater loading capacity of protonated basic compounds. This effect was first shown for the analysis of ophthalmic drugs, substituted pyridines, and aromatic amines[15,16] with the liophilic mobile phase additives. Roberts later observed similar effects[18] in analysis of primary, secondary, and tertiary benzyl amines and antidepressants.

To improve peak symmetry of basic analyte, the sample load on the column can be decreased. At times, however, one needs to inject a large sample size to be able to detect low levels of impurities. The larger sample size may lead to an increase in basic analyte tailing factor and a decrease in peak symmetry. The increase of tailing factor and decrease of peak symmetry could be related to an overloading effect of a small amount of highly energetic adsorption sites on the packing material. Moreover, ion-exchange interactions with these highly energetic sites could lead to slow sorption-desorption of solute molecules on these strong sites, compared to the weak sites leading to a further increase in band tailing.[16,30,31,61–69] Adding liophilic anions to the mobile phase could suppress these undesired secondary interactions with the stationary phase.

Figure 13 illustrates an overlay of labetalol with increasing concentration of 3–31 μg. The mobile phases used consisted of 10 mM dihydrogen phosphate buffer with increasing concentration of perchlorate. The overlays demonstrate a typical pattern with similar peak tails for different analyte loads. This represents a "thermodynamic overload" which occurs when analyte concentration exceeds the linear region of the adsorption isotherm and this isotherm curvature leads to right angled peaks.[28,70,71] The addition of perchlorate anion in the mobile phase suppresses the peak tailing, and as a result, leads to an increase in the peak efficiency.

For basic compounds, LoBrutto et al.[16] demonstrated that an increase of the liophilic counteranion (perchlorate, hexafluorophosphate, and tetrafluoroborate) concentration in the mobile phase led to an increase in apparent efficiency with a concomitant increase in retention. For three basic ophthalmic drug compounds at increasing BF_4^- counteranion concentration from 1 mM to 10 mM, the efficiency (Figure 14 a) was enhanced until it achieved the maximum column efficiency (phenols, neutral markers). Additionally, in Figure 14b, by increasing BF_4^- counteranion concentration, the tailing factor of basic compounds decreased and approached that of the neutral analytes, phenolic compounds.

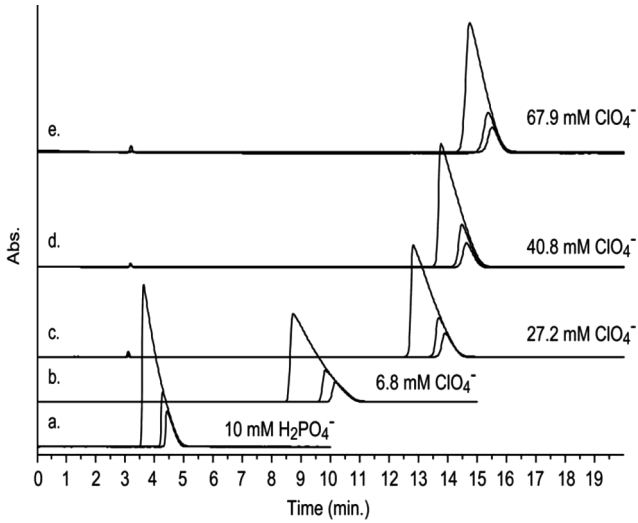


Figure 13. Chromatographic overlays of Labetalol analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion Chromatographic conditions: Column: Zorbax Eclipse XDB-C8, Analyte load: 3.3, 6.5, 31.2 μg , (a) 75%: 0.1 v/v% H_3PO_4: 25% acetonitrile, (b) 75%: 0.05 v/v% $HClO_4$: 25% acetonitrile, (c) 75%: 0.2 v/v% $HClO_4$: 25% acetonitrile, (d) 75%: 0.4 v/v% $HClO_4$: 25% acetonitrile, (e) 75%: 0.5 v/v% $HClO_4$: 25% acetonitrile. *Source*: Reprinted with permission from Ref. [16].

In another study, various counteranions (PF_6^-, ClO_4^-, BF_4^-) at increasing concentration were used for the analysis of a beta-blocker compound (Figure 15). PF_6^- counteranion had the maximum effect on the enhancement of the peak symmetry and at increasing concentration of PF_6^-, the number of theoretical plates of labetolol approached that of the neutral markers (phenols). Other anions such as ClO_4^-, BF_4^- also lead to improvements in the apparent efficiency and a similar trend was observed. Moreover, neutral markers, phenols, showed no considerable changes in retention and efficiency with increased counteranion concentration.[16]

APPLICATIONS OF LIOPHILIC ADDITIVES:

Small Molecules and Peptides

The retention of basic compounds containing primary, secondary, tertiary, and quaternary amines can be altered as a function of the concentration of liophilic mobile phase additives (ClO_4^-, PF_6^-, BF_4^-, $CF_3CO_2^-$)

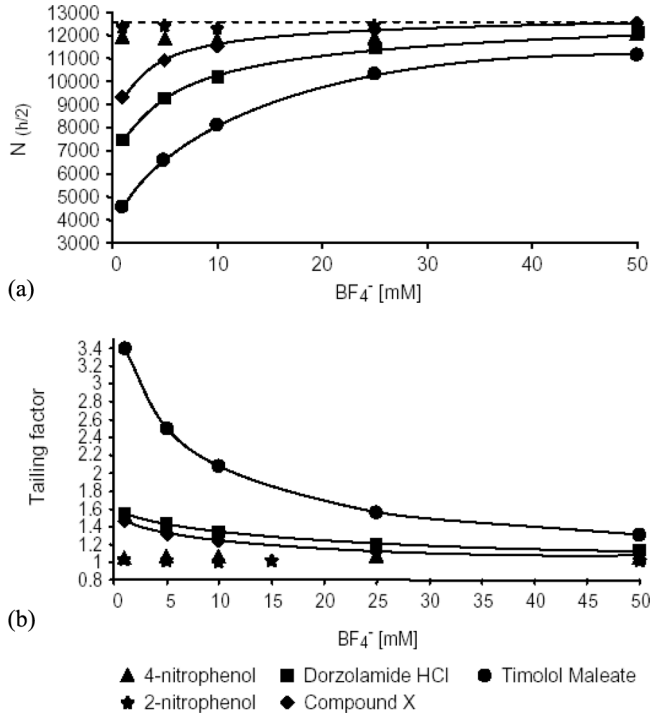


Figure 14. Effect of tetrafluoroborate concentration on analyte apparent efficiency and tailing factor. Column: Zorbax Eclipse XDB-C8, Mobile phase: 0.1 v/v% phosphoric acid + xBF_4 [1 mM – 50 mM]: acetonitrile, Ophthalmic compounds (10% acetonitrile), phenols (25% acetonitrile), (a) N(h/2) vs. tetrafluoroborate concentration. (b) Tailing factor vs. tetrafluoroborate concentration. *Source*: Reprinted with permission from Ref. [16].

at a low pH.[15,17] Barlett et al. showed the application of liophilic mobile phase additive, perchlorate, in their study with dual quaternary amines – paraquat and diquat (contact herbicides). These basic herbicides were difficult to retain using conventional RP-HPLC approaches and upon addition of perchlorate to the mobile phase, an increase of the analyte retention was obtained and a robust method was developed.[72]

Jira and Hashem studied the effect of perchlorate concentration in MeOH/water (pH 3) mobile phases on the retention behavior of beta-blockers on Kromasil C_{18} and C_{18} monolithic columns. On both columns, the increase in $NaClO_4$ concentration led to an increase in retention,[73] and this was independent of the type of stationary phase support.

The selectivity, retention, and elution order of a mixture of acidic, basic, and neutral compounds was modulated to different degrees by

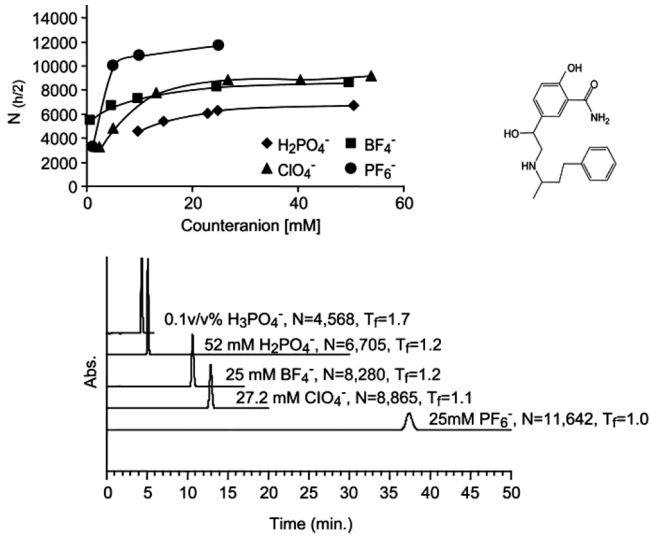


Figure 15. Effect of counteranion type and concentration on analyte retention, peak efficiency, $N_{(h/2)}$, and tailing factor, T_f. Chromatographic conditions: Column: Zorbax Eclipse XDB-C8, Mobile phase: 75% Aqueous: 25% acetonitrile; flow rate: 1.0 ml/min.; temperature: 25°C, Wavelength: 225 nm. *Source*: Reprinted with permission from Ref. [16].

varying the concentration of liophilic mobile phase additive at a constant pH, as shown in Figure 16. The retention of protonated basic compounds, metoprolol and labetalol, increased while the retention of phenol (in its neutral state) remained constant. However, the two acidic compounds are in their ionized state (negatively charged) and with an increase in perchlorate concentration, there was a slight decrease in their retention. This decrease in retention could be attributed to an ion-repulsion phenomena with the negatively charged adsorbed liophilic counter anion. Carr et al. observed the decrease in nitrate retention upon addition of anionic counteranion (20 mM $NaClO_4$) with a low fraction of acetonitrile in the mobile phase. Carr et al. explained this effect by ion exclusion of nitrate from the stationary phase by absorbed perchlorate anion (Figure 17).[26] A decrease in retention was observed for compounds with nitro groups (which contain a partial negative charge) when liophilic mobile phase additive such as hexafluorophosphate was used.[74]

Sodium perchlorate was used as a liophilic additive to enhance the chromatographic selectivity for a forced degradation sample (see Figure 18). Base 1 and Base 2 were known base hydrolysis degradation products. In an effort to increase the retention of Base 1 so that it would elute farther away from the solvent front, perchlorate was added to the

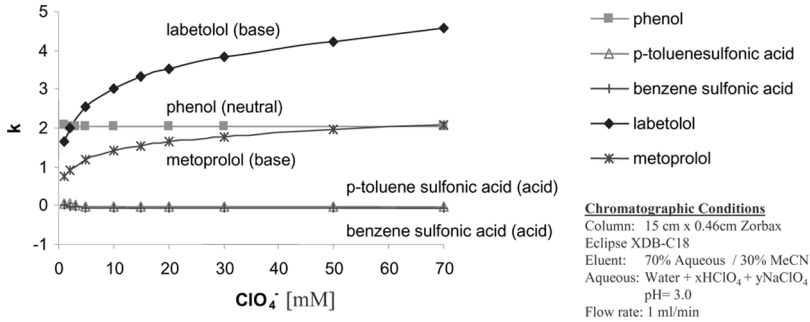


Figure 16. Retention factor of acidic, neutral and basic analytes versus perchlorate concentration. *Source*: Reprinted with permission from Ref. [15].

mobile phase. Upon addition of perchlorate, the retention of basic degradation products (base 1 and base 2) increased, as well as for a new degradation product, base 3. The resolution of "base 3" from the component indicated with an asterisk was significantly improved with the addition of perchlorate in the mobile phase.

The use of liophilic additives (trifluoroacetate and hexafluorphosphate) with different organic modifiers (THF, MeOH, acetonitrile)

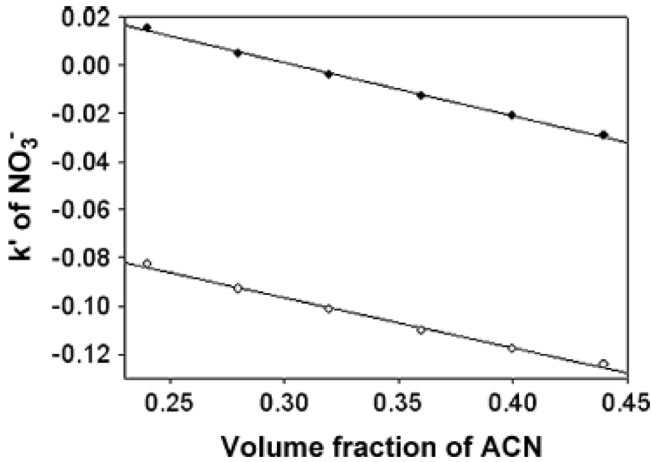


Figure 17. Retention factor of nitrate as a function of % ACN in two different mobile phases. Amount and type of anionic additive in the mobile phase: (filled circles, no perchlorate); (empty circle, 20 mM $NaClO_4$). The average retention time of uracil in the six mobile phases was used to calculate the k' of nitrate. The stationary phase was Ace 5 C_{18} and all mobile phases contained 0.1v/v% formic acid. Other experimental conditions: 1.00 mL/min, 40°C, 210 nm. *Source*: Reprinted with permission from Ref. [26].

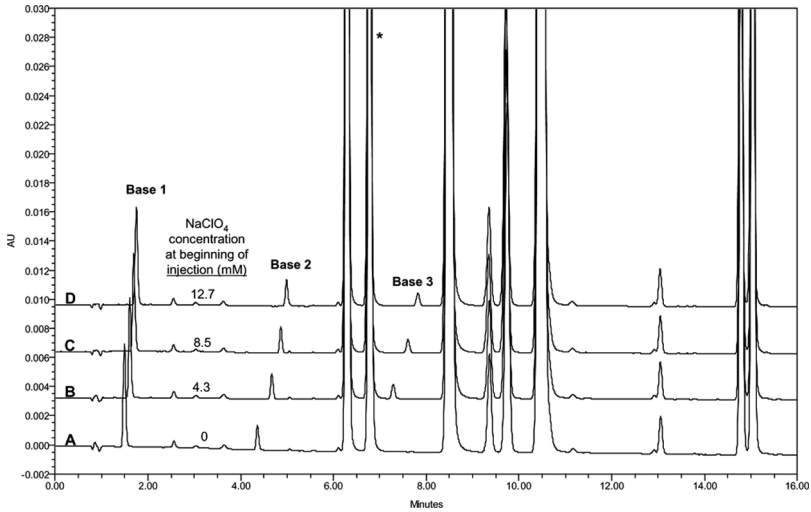


Figure 18. Effect of perchlorate concentration on separation of complex mixture [75]. Column: Waters XBridge C_{18} 3.5 μm, 3.0 × 150 mm, Flow rate 0.75 ml/min, Temp. 30°C, Inj. 5 μL, Initial back Pressure: ~4000 psi, Gradient : Isocratic hold for 1.0 min at 15% B, then over 2 min to 30% B, then over 7 min to 40% B, then over 2 min to 65% B, hold 65% B for 2 min and equilibrate at initial conditions for 5 minutes, Total Run time - 19 min. Mobile phases A) Mobile phase A: 80% H_2O/ 20% ACN/ 0.1% TFA, v/v/v; Mobile phase B: 10% H_2O/ 90% ACN, v/v B) Mobile phase A: 80% H_2O/ 20% ACN/ 0.1% TFA v/v/v, containing 5 mM $NaClO_4$; Mobile phase B: 10% H_2O/ 90% ACN, v/v . C) Mobile phase A: 80% H_2O/ 20% ACN/ 0.1% TFA v/v/v, containing 10 mM $NaClO_4$; Mobile phase B: 10% H_2O/ 90% ACN, v/v; D) Mobile phase A: 80% H_2O/ 20% ACN/ 0.1% TFA v/v/v, containing 15 mM $NaClO_4$; Mobile phase B: 10% H_2O/ 90% ACN, v/v.

was investigated for a series of alkaloid compounds.[76] The optimal separation for the selected alkaloid compounds was obtained with an acetonitrile modifier and hexafluorophosphate additive, Figure 19.

The use of ionic additives in the mobile phase for the separation of peptides/proteins has been used extensively.[77–83] Table 1 lists the additives used for the separation of peptides and proteins.

Hodges et al. studied the effect of perchlorate additive on the separation of model peptides.[84] They also showed that the selectivity of α-helical and random coil peptides can be altered by the use of liophilic ions such as perchlorate, at pH 2. This could be particularly useful when it is undesirable to isolate peptides as their trifluoroacetate salts (additive commonly used for peptide separations).[84]

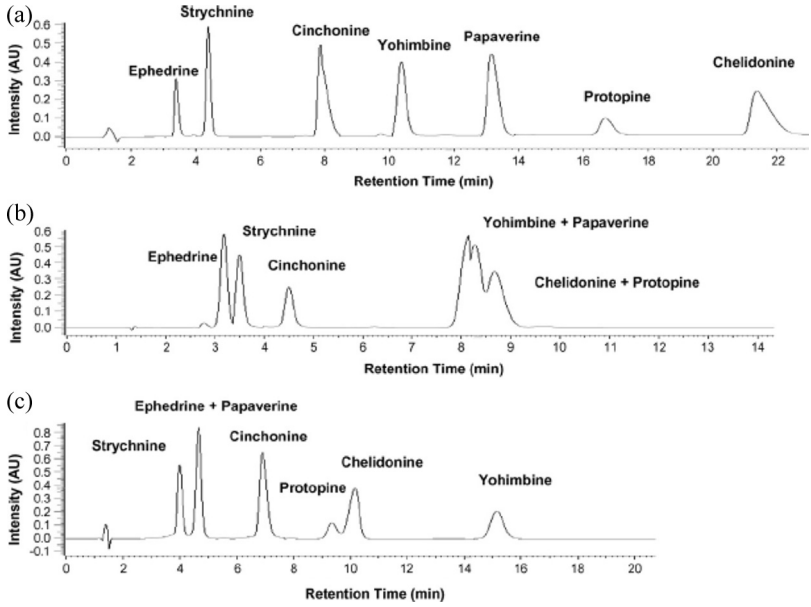


Figure 19. Chromatograms of mixtures of alkaloids obtained by use of different mobile phases, (a) 35% MeCN/10 mM phosphate buffer (pH 2.7) + 30 mM sodium hexafluorophosphate, (b) 40% MeOH/10 mM phosphate buffer (pH 2.7) +30 mM sodium hexafluorophosphate, (c) 25% THF/10 mM phosphate buffer (pH 2.7) + 30 mM sodium hexafluorophosphate. *Source*: Reprinted with permission from Ref. [76].

Peptides have multiple ionizable sites on the same molecule and are protonated at low pH. Consequently, their interactions with liophilic additives will have an impact on their retention.[85] Figure 20 shows the effect of different concentrations of hexafluorophosphate on the separation of hydrophilic lysine and multiply charged peptides. All these species were positively charged at pH 1.8. It was demonstrated that by increasing the concentration of PF_6^- this had an enhanced effect on the retention of molecules with a greater number of positive charges; lysine showed lower retention compared to di-lysine, tri-lysine, and tetra-lysine. Moreover, as demonstrated in Figure 20, the addition of PF_6^- allowed for the resolution of a non-ionized impurity (eluting near the solvent front) from mono-lysine and the multiply charged peptide species.

Multiply charged analytes containing basic moieties such as peptides are prone to excessive tailing and reduced sample loading capacity.[62,65,67] Figure 21 shows an overlay of chromatograms of a model peptide B (at 0.1 μg and 0.3 μg sample load on column) using varying concentrations of perchlorate in the mobile phase. It was

Table 1. Ion pairing reagents used in the analysis of proteins and peptides adapted from [83]

Ionic additive	Concentration in mobile phase	Characteristics of the ionic additive
Perfluorinated carboxylic acids		
Trifluoroacetic acid	0.05–0.5%	Very volatile
Heptafluorobutyric acid	10–50 mM	Volatile
Others: nonafluoropentanoic acid, tridecafluoroheptanoic acid, pentadecafluorooctanoic acid, etc.	10–50 mM	Retention controlled by the alkyl chain-length; volatile
Other acids		
Acetic acid	0.5–1 M	Volatile
Formic acid	20–60%	Volatile; lower resolution and recovery than TFA
Hydrochloric acid	5 mM	Volatile and corrosive
Salts		
Phosphate buffer (pH 2–9)	10–20 mM	Not volatile
Triethylammonium phosphate (pH 4)	125 mM	Not volatile
Formate (ammonium, trialkylammonium or pyridinium salt)	20–200 mM	Volatile
Acetate (ammonium, trialkylammonium or pyridinium salt)	20–200 mM	Volatile
Perchlorate	100 mM	Not volatile
Ammonium bicarbonate (pH 7–.11)	50–100 mM	Volatile
Ammonium sulfate (pH 4)	125 mM	Not volatile
Alkylsulfonates/alkylsulfates (e.g., heptanesulfonate)	10–15 mM	Retention controlled by the alkyl chain-length; not volatile
Teraalkylammonium salts (e.g., tetrabutylammonium salts)	10 mM	Retention controlled by the alkyl chain-length; not volatile
Alkylamines (e.g., triethylamine, dodecylamine, pyridine) (acetate, formate or phosphate)	10 mM	Volatile
Bases		
Morpholine	10 mM	Volatile

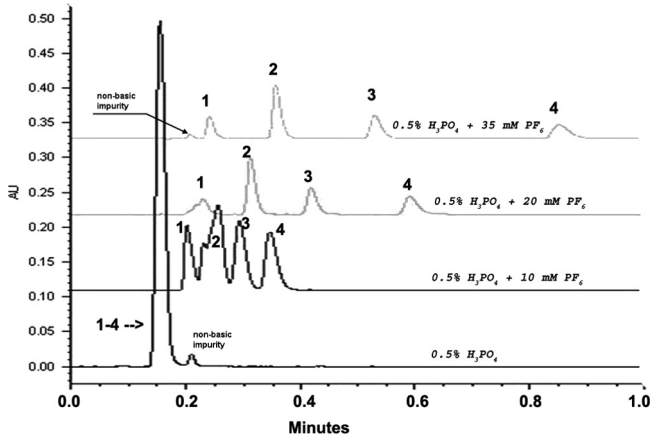


Figure 20. Enhancement of separation selectivity with addition of liophilic additive (KPF_6) Column: Acquity BEH C_{18} 1.7 μm, 2.1 × 50 mm, Flow rate 0.7 ml/min, Temp. 40°C, Inj. 1 μL Conc. 0.5 mg/ml. Run time 1 min. Detection 210 nm. Strong wash: 50/50 ACN/ H_20. Weak: 90/10 H_2O/ACN, Mobile phase A: 0.5% H_3PO_4 pH 1.8 + 0, 10 ,20, 35 mM KPF_6. Mobile phase B: ACN. Starting Pressure: ~ 6800 psi. Isocratic: 85%A. 15%B. 1- lysine. 2- di-lysine. 3- tri-lysine. 4- tetra-lysine. [85]

demonstrated that even a small addition of perchlorate can dramatically increase the number of theoretical plates and decrease the tailing factor for the peptide. Also, analyte load can be increased with use of a liophilic additive. Figure 22 shows the trend of peak efficiency with perchlorate concentration for this peptide B, as well as for the positively charged small molecule labetolol and the neutral molecule butylhydroxybenzoate (BHB). It is shown that in the absence of perchlorate the peptide peak is very broad, exhibiting only 200 theoretical plates. With the addition of 2.5 mM perchlorate ion the peak efficiency increases to approximately 3000 plates; at 5.0 mM perchlorate approximately 7000 plates and at 32.5 mM perchlorate approximately 8000 plates are obtained. Labetolol exhibits a similar effect, though not quite to the same extent since it is a singly charged species. BHB, a neutral molecule, on the other hand is not affected by the addition of perchlorate in the mobile phase.

Also, the addition of the perchlorate ion led to a reduction in the peak tailing for the peptide and labetolol as shown in Figure 23. With multiple charged residues there is a greater propensity for undesired interactions with energetic sites in the bonded phase. The reduction of the peak tailing was more dramatic for the peptide, since the impact of the liophilic additive on the suppression of undesired interactions is magnified.

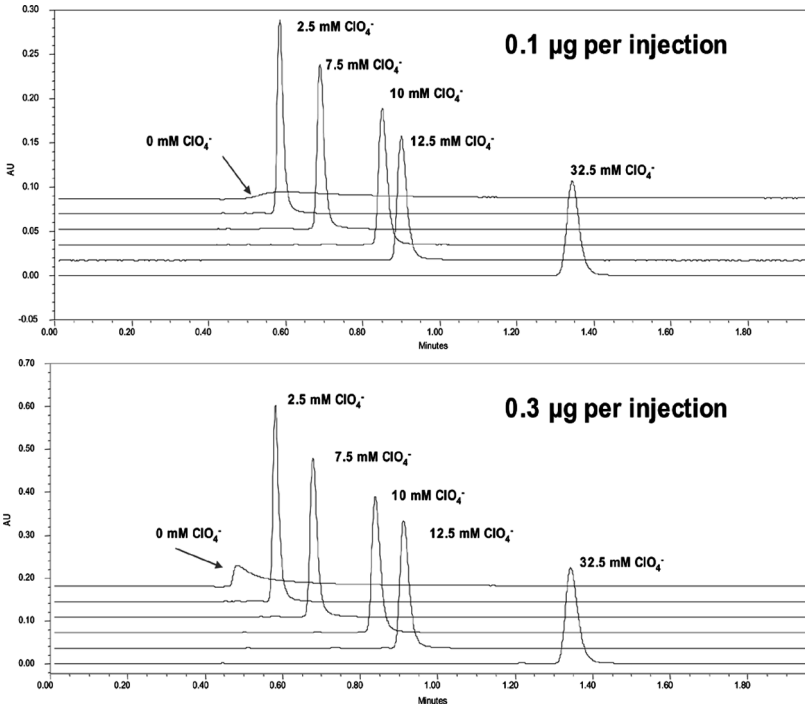


Figure 21. Effect of concentration of liophilic mobile phase additive on retention of peptide B, Conditions: Column: Acquity BEH C_{18} 1.7 um, 2.1 × 100 mm, Flow rate 0.8 ml/min,- Temp. 45°C, Inj. 1 µL , Conc. 0.1 mg/ml, Run time 2 min, Detection 210 nm, Strong wash: 50/50 MeCN/ H_20, Weak 90/10 H_2O/MeCN, Mobile phase A:60%-0.5% H_3PO_4 or 0.05% $HClO_4$ + $NaClO_4$ Mobile phase B: 40% MeCN, Starting Pressure: ~ 8300 psi. *Source*: Reprinted with permission from Ref. [86].

Chiral Separations

Ishikawa and Shibata found that the resolution of propanolol enantiomers could be enhanced by the addition of perchloric acid and/or sodium perchlorate when performing the chiral separation on a Chiralcel OD-R column under reversed phase conditions. Also, the addition of other anions such as PF_6^-, SCN^-, I^-, and BF_4^- and $CCl_3CO_2^-$ gave similar or greater resolution of the enantiomers compared to the separation with a perchlorate mobile phase additive.[52]

Machida et al. found that the use of highly polarizable counteranions as mobile phase additives led to changes in retention and selectivity for alanine-β-napthylamide enantiomers on a crown ether silica based column. A mobile phase containing 15% methanol at a constant mobile

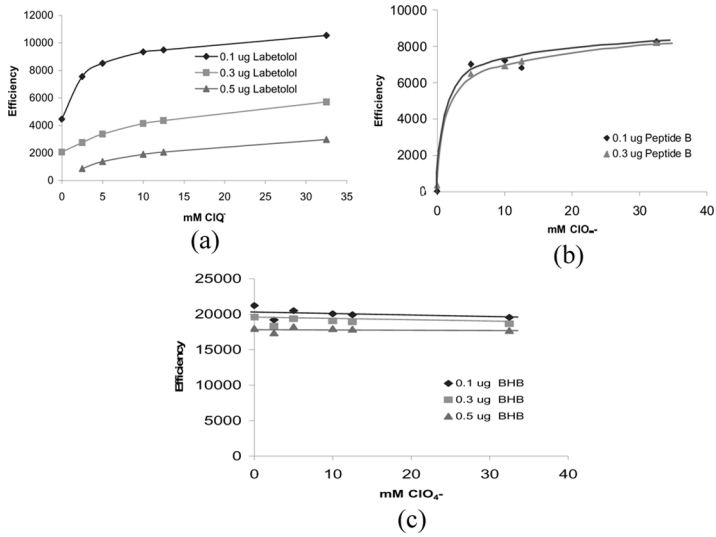


Figure 22. Effect of perchlorate concentration on apparent peak efficiency, N (h/2). A-Labetolol, B- Peptide B, C- butylhydroxybenzoate. *Source*: Reprinted with permission from Ref. [86].

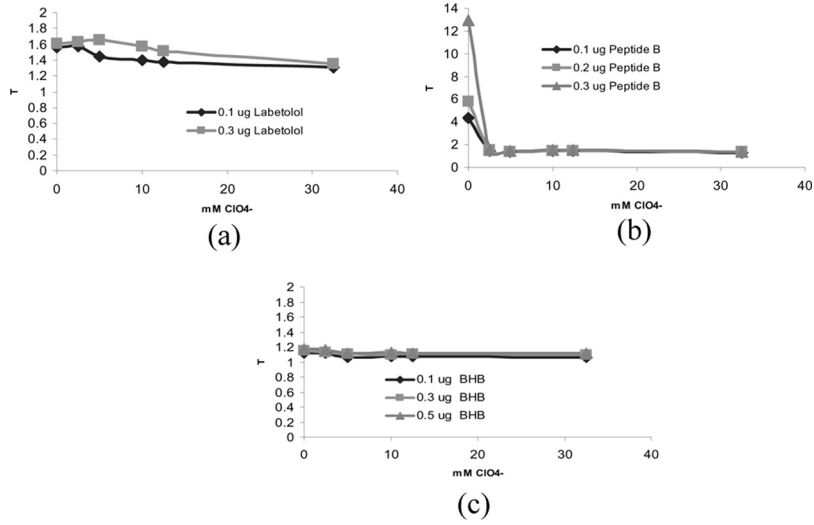


Figure 23. Effect of perchlorate concentration on suppression peak tailing, A-Labetolol, B-peptide B, C- butylhydroxybenzoate. *Source*: Reprinted with permission from Ref. [86].

phase pH of 2 was used with the addition of 500 mM potassium salts of $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, and I^-. The capacity factors and selectivity were the greatest with iodine and the least with dihydrogenphosphate, the most liophilic ion and the weakest liophilic ion, respectively, studied.[47]

Thompson et al. studied the effect of type of counterion for a chiral separation of four optical isomers of aminoindanol on a silica based crown ether column, Crownpak CR (–). They found that the retention for both pairs of enantiomers increased with counteranions that were more polarizable in the following order: $H_2PO_4^- < NO_3^- < CF_3COO^- < ClO_4^-$.[87] The separation factors for both pairs of enantiomers were unchanged and, thus, independent of the nature of the anion.

CONCLUSIONS

The selective increase of the retention of ionized basic pharmaceutical compounds, and small peptides with employment of liophilic mobile phase additives has been demonstrated. The type and concentration of organic modifier employed also influences the interactions of the anions with protonated analytes.

These studies show that judicious selection of inorganic additives would allow for control of the retention and separation selectivity of compounds that contain positively charged basic moieties. Selection of the proper anion can affect the retention to the same degree as changing other chromatographic conditions, and also may lead to improved limit of detection due to decreased background absorbance. Selection of the proper liophilic mobile phase additive can also be considered as a superior alternative to the amphiphilic ion-pairing reagents.

Liophilic mobile phase additives can contribute to secondary equilibria processes in the chromatographic system without irreversible alteration of the surface and significant alteration of the retention of neutral analytes. They enhance dynamic equilibrium between the mobile and stationary phases by disruption of the solvation shell of charged analytes and suppression of interaction with highly energetic adsorption sites, respectively. This allows for flexible alteration of the separation selectivity and retention with an enhancement of apparent efficiency.

The utilization of these counteranions for chromatographic separation is useful as a method development strategy. These liophilic mobile phase modifiers may obviate the necessity for altering column type and/or addition of hydrophobic "ion-pairing" reagents that can irreversibly change the surface of the stationary phase.

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