4

REVERSED-PHASE HPLC

Rosario LoBrutto and Yuri Kazakevich

4.1 INTRODUCTION

Over 25 years ago, Horvath and Melander, in their fundamental work [1], discussed the reason behind the explosive popularity of reversed-phase liquid chromatography (RPLC) for analytical separations. It was estimated that about 80–90% of all analytical separations were performed in RPLC mode, and the authors noted that “the variation of eluent composition alone extends both retention and selectivity in HPLC [high-performance liquid chromatography] over an extremely broad range.” They compared gas chromatography with HPLC, citing “in gas chromatography a plurality of stationary phases has found practical application whereas HPLC tends toward the use of very limited number of columns and optimization of the separation by manipulating the composition of the mobile phase.” To some extent the statement is true even today, except that with introduction of capillary columns in GC today, only a very limited number of stationary phases are used, while in HPLC during the last 25 years of development, thousands of different stationary phases have been introduced. Practically all reversed-phase separations are carried out on stationary phases with chemically modified hydrophobic surfaces. Minor variations in the surface chemistry and geometry can lead to noticeable differences in surface interactions and, as a result, to differences in chromatographic selectivity. Specific stationary-phase properties and their influence on the chromatographic retention, selectivity, and efficiency are discussed in detail in Chapter 3.

HPLC for Pharmaceutical Scientists, Edited by Yuri Kazakevich and Rosario LoBrutto
Copyright © 2007 by John Wiley & Sons, Inc.
Mobile phase (eluent) is by far the major “tool” for the control of analyte retention in RPLC. Variations of the eluent composition, type of organic modifier, pH, and buffer concentration provide the chromatographer with a valuable set of variables for successful development of a separation method.

Mobile-phase pH affects the analyte ionization and thus its apparent hydrophobicity and retention. Most pharmaceutical analytes, API (active pharmaceutical ingredient), in-process intermediates, reaction samples, drug substances, raw materials, drug products, and other types of samples generated during the drug development life cycle are ionizable, and their retention is affected by the mobile-phase pH. At the same time, the pHs of aqueous–organic mixtures are different from the pH of the aqueous component itself. The relationship between measured pH of the aqueous phase and the actual pH of the eluent will be discussed, and approaches on how to correlate the HPLC retention to actual eluent pH will be elaborated. The influence of temperature and type and concentration of organic on analyte and pH modifier ionization and its relation to HPLC retention will also be described.

All the choices the chromatographer has in terms of bonded phase, aqueous phase modifier, and organic modifier can have synergistic effects on the analyte retention and selectivity in reversed-phase chromatography. These parameters will be discussed in this chapter, with specific examples illustrating the power of the selection of the most suitable parameters for control of the analyte retention and selectivity.

4.2 RETENTION IN REVERSED-PHASE HPLC

The basis for the analyte retention in reversed-phase chromatography is the competitive interactions of the analyte and eluent components with the adsorbent surface. The stronger the interactions of the analyte with the surface, the longer its retention. Selectivity or the ability of chromatographic system to discriminate between different analytes is also dependent on differences in the surface interactions of the analytes.

Historically, reversed-phase chromatography could be traced back to the work of Howard and Martin [2], who treated an adsorbent surface (of Kieselgure) with dimethylchlorosilane followed by coating of this nonpolar surface with paraffin oil employing methanol–acetone mixtures as the mobile phase. They treated the retention process as partitioning of the analyte between the mobile phase and paraffin oil, which served as a stationary phase (alkylchlorosilane treatment of the polar surface serves only the purpose of increasing wettability by paraffin oil). For many years the advancement in the developments in HPLC essentially followed the development of phases used for gas chromatography. In the middle of the 1960s, modification of the silica gel surface with hexadecyltrichlorosilane was introduced for GC [3]. Following this, Stewart and Perry [4] suggested that this material would be the best possibility for the advancement of “liquid–liquid” chromatography (the term
RPLC was coined). Later, Majors [5] introduced porous silica microparticles modified with alkylsilanes, a packing material that is almost exclusively used in reversed-phase HPLC today.

This brief historical overview of RPLC development is far from the full description of all significant achievements made in the past; however, the primary goal is to show the path of the development, which was, to a larger extent, in the tail of GC development. Consequently, the models and the descriptions of the retention mechanism were essentially transferred from gas–liquid partition chromatography.

Partitioning describes the transfer of the analyte molecules from one phase into another, where the phase is an isotropic macroscopic object with definite physicochemical characteristics. A monomolecular layer of bonded ligands could not be considered as a phase, although following the terminology widely accepted in the literature the term stationary phase is used to essentially denote a solid surface of immobile packing material in the column.

The retention mechanism in modern RPLC is a superposition of different types of dynamic surface equilibria. Main equilibria governing the analyte retention is the adsorption of the analyte molecule on the surface of packing material. The description of the analyte retention on the basis of this main adsorption equilibrium could be expressed as

\[ V_R = V_0 + SK \]  

where \( V_0 \) is the total volume of the liquid phase in the column (void volume), \( S \) is the adsorbent surface area, and \( K \) is the adsorption equilibrium constant. This expression assumes ideal analyte behavior in the chromatographic system at very low analyte concentration. As follows from equation (4-1), the equilibrium constant, \( K \), has units of length (i.e., volume/m\(^2\)) and, as such, could not be used as a general thermodynamic equilibrium constant (unitless), but rather as a coefficient representing the analyte retention volume per unit of the adsorbent surface (e.g., \( \mu L/m^2 \)). More general expressions and detailed adsorption-based description of the analyte retention in reversed-phase HPLC is given in Chapter 2 of this book.

While dynamic distribution of the analyte between the mobile phase and adsorbent surface is a primary process, there are many secondary processes in the chromatographic system that significantly alter the overall analyte retention and selectivity. Detailed theoretical discussion of the influence of secondary equilibria on the chromatographic retention is also given in Chapter 2.

The analyte nature and its appearance (e.g., ionization state) in the mobile phase are also factors that affect the retention mechanism. Eluent pH influences the analyte ionization equilibrium. Eluent type, composition, and presence of counterions affect the analyte solvation. These equilibria are also secondary processes that influence the analyte retention and selectivity and are of primary concern in the development of the separation methods for most pharmaceutical compounds.
This brief descriptive overview of the reversed-phase process emphasizes the complexity of the retention mechanism and the necessity to consider the influence of different and independent processes on the analyte retention. Since the governing process in the analyte retention is the adsorption equilibrium, the influence of the surface packing material (stationary phase) on the analyte retention in RPLC is described in Section 4.3.

4.3 STATIONARY PHASES FOR RPLC

The introduction of chemically modified stationary phases has had a remarkable impact in the field of liquid chromatography. Successful development and improvement in the technology of manufacturing reproducible bonded layers has revolutionized many chromatographic techniques. Porous silica stationary phases have been modified with ligands of various chemistry and size. The composition and the structure of the bonded organic layer is varied by changing the size of the modifier, specific surface area of the adsorbent, and the bonding density. Chemical bonding of organic ligands with high bonding density on the inner surface of silica pores alters the adsorbent geometry. The effect of surface modification on adsorbent geometric parameters (surface area, pore volume, pore size) has been investigated on several different silica gels [6–8]. It was shown that a decrease in mean pore diameter and in pore volume are associated with the molecular volume of bonded ligands and bonding density. Similar effects were also observed by other researchers [9, 10] Clearly, surface modification has a significant impact on the adsorbent geometry of reversed-phase columns, which will also influence the separation mechanism itself [11]. These effects are discussed in detail in Chapter 3.

Silica-based packing materials dominate in applications for RP separations in the pharmaceutical industry. Hydrophobic surface of these packings typically are made by covalent bonding of organosilanes on the silica surface. This modification involves the reaction of monofunctional alkylidimethylchlorosilanes with the surface silanol groups. Octadecylsilane was the first commercially available silica-based bonded phase and is still the most commonly utilized [12]. Also, alkyl-type ligands of different number of carbon atoms (C1, C4, C8, C12) are often used as well as phases with phenyl functionality; also, polar end-capped, polar embedded phases have been introduced [13–15]. Polar embedded phases provide an additional avenue for potential modification of the chromatographic selectivity, and some of these phases offer an enhancement of retention of polar analytes [16]. These phases can be used with high aqueous mobile phases, even 100% aqueous, without loss of analyte retention that sometimes could be observed for more hydrophobic phases.

Screening several different types of stationary phases during method development for a particular separation is often useful because different columns usually have different selectivity for components in a sample, as can be seen for a forced degradation sample analyzed on three different types of reversed-
phase columns using 0.1 v/v% TFA (Figure 4-1) and phosphate buffer, pH 7 (Figure 4-2) mobile phases. Mobile-phase pH can also provide an alternate means of varying the separation selectivity as well.

Other silica-based phases that are available include phenyl and fluorinated alkyl and phenyl-bonded phases. The phenyl and fluorinated phases offer the potential for $\pi-\pi$ interactions and show different selectivity in comparison to

![Figure 4-1. Effect of column type on selectivity. Mobile phase: Low pH. (A) 0.1 v/v % TFA. (B) 0.1 v/v% TFA in MeCN. Linear gradient from 5% B to 80% B in 40 min, 220 nm. Temperature, 40°C; flow rate, 1.0 mL/min; column dimensions, 150 × 3.0 mm; particle sizes, 3.5μm for Symmetry Shield and Atlantis and 3.0μm for YMC ODS AQ. (Courtesy of Markus Krummen, Novartis Pharmaceuticals.)](image1)

![Figure 4-2. Effect of column type on selectivity. Mobile phase: High pH. (A) 10 mM K$_2$HPO$_4$, pH 7.0. (B) In MeCN, linear gradient from 5% B–80% B in 40 min, 220 nm. Temperature, 40°C; flow rate, 1.0 mL/min; column dimensions, 150 × 3.0 mm; particle sizes, 3.5μm for Symmetry Shield and Atlantis and 3.0μm for YMC ODS AQ. (Courtesy of Markus Krummen, Novartis Pharmaceuticals.)](image2)
the alkylsilane phases [17–21]. The fluorinated phases have shown some size and shape selectivity, particularly for aromatic molecules [22, 23]. Moreover, with phenyl-type phases, selectivity/separation differences could be obtained when methanol or acetonitrile is employed. Acetonitrile is an electron-rich organic modifier, which could modify the π–π interactions between the solute and the aromatic moiety of the stationary phase. Methanol, on the other hand, is a proton donor and does not contain π electrons, and therefore its influence on the analyte retention would be principally different [24–26]. It is generally recognized that the type of organic eluent modifier employed plays a dominant role in separation selectivity, although the mechanism of its influence on the analyte retention still remains a subject of intense investigation.

Most silica-based reversed-phase packing materials have a relatively narrow applicable pH range. Below pH 2, the linkage of the bonded phase to the silica substrate is prone to hydrolytic cleavage. Above pH 7, the silica substrate is prone to dissolution, particularly in aqueous-rich mobile phases. In addition, basic compounds may exhibit peak asymmetry above pH 3 due to secondary interactions between the ionized form of the solute and accessible residual silanols. Some new developments in column chemistry have been adopted to address the issues of limited pH working range and reduction of surface density of silanols. The use of hybrid materials allowed for the introduction of organic bridged silica in which an organic bridge is formed between silicon atoms. Resulting hybrid material have been claimed by vendors to show better pH stability at pHs >7 since Si–C covalent bond is much less prone to hydrolysis than Si–O–Si bonds. However, the stability of phases depends on many factors such as the operating pH, type, and concentration of organic modifier and salt concentration, operating temperature, and operating back-pressure. Another approach to manufacturing hybrid silica (Gemini) was introduced by Phenomenex. A layered hybrid silica is synthesized such that the core of the particle is regular silica and the surface is covered by a layer of organic-embedded silica also lending itself to greater pH stability. These stationary phases are further discussed in Chapter 3.

The narrow pH stability range of silica-based packing materials leads to the continuous search for alternative packings that may provide greater pH stability. The options include polymer-based, zirconia-based, and carbon-based phases. The polymer-based columns include poly(styrene-divinyl benzene) and divinylbenzenemethacrylate. These polymer-based columns tend to be stable in the pH 0–14 range. However, lower efficiencies on these polymeric columns relative to silica-based columns are usually obtained due to slower mass transfer kinetics. These phases are also prone to swelling/shrinking as a function of the mobile-phase composition. Retention and selectivity is based on a combination of hydrophobic and π–π interactions [27]. Zirconia is nearly insoluble at pH 1–14 and is stable at temperatures greater than 150°C. The zirconia surface is positively charged up to pH ~ 8, after which it becomes negatively charged [28]. Surface charge, however, is also influenced by adsorption of mobile-phase anions that are hard Lewis bases. The adsorption of hard Lewis
bases such as phosphate ion results in ion-exchange sites offering different selectivities than silica [29, 30]. A comparison of polybutadiene (PBD)-coated zirconia and octadecylsilane (ODS) phases indicated that ion exchange is the dominant interaction for basic solutes on the PBD phases while hydrophobic interactions dominate on the ODS phases when phosphate is in the mobile phase [31]. Carbon-based columns are chemically stable over pH range 1–14. These phases are very hydrophobic compared to alkylsilane phases and thus are useful for the separation of polar compounds. However, they strongly, sometimes irreversibly, retain very hydrophobic solutes. Graphitized carbon phases are very suited for the separation of positional and conformational isomers, since the majority of their surface is an ideal graphite plane. Porous graphitized carbon consists of multiple graphite microcrystals and thus offers significant difference in the planar interactions for conformational isomers. Intercrystalline dislocations (irregularities in the crystalline structure), on the other hand, are places of higher surface energy and because the whole material is a conductor, they can be chemically active, which reduce column lifetime and should be taken into account if chemically labile compounds should be separated.

4.4 MOBILE PHASES FOR RPLC

Mobile phases commonly used in reversed-phase HPLC are hydro-organic mixtures. The most common reversed-phase organic modifiers include methanol and acetonitrile and/or combinations of these two modifiers. Other mobile-phase modifiers such as tetrahydrofuran, IPA, and DMSO [32] have been also used for minor selectivity adjustment; however, they are not common due to their high backpressure limitations and/or high background UV absorbance.

The concentration of organic modifier in the eluent is the predominant factor that governs the retention of analytes in RPLC. Highly purified solvents (HPLC grade) are recommended in order to minimize contamination of the stationary phase with impurities of the solvents and reduction of the background absorbance if they contain impurities that have UV chromophores >190nm.

Considerations for choice of mobile-phase solvents include compatibility between solvents, solubility of the sample in the eluent, polarity, light transmission, viscosity, stability, and pH. The mobile-phase solvents should be miscible and should not trigger precipitation when they are mixed together. For example, dichloromethane and water are immiscible at most compositions and should not be used as mobile-phase components. Similarly, high concentrations of phosphate buffer should not be used with high levels of acetonitrile because the phosphate will eventually precipitate out, resulting in damage in the pump head and blockage of the column frit. The sample should also be soluble in the mobile phase to avoid precipitation in the column. Light
transmission is an important parameter when using UV detection; see Table 4-1 for UV cutoffs of common reversed-phase organic modifiers.

Solvents with high UV cutoffs such as acetone (UV cutoff 330 nm) and ethyl acetate (UV cutoff 256 nm) cannot be used for analyses at low wavelengths such as 210 nm. Acetonitrile has a very low UV cutoff (<190 nm) and is one of the contributing factors toward its common use as a solvent for reversed-phase separations. Methanol, ethanol, and isopropanol have a UV cutoff of <205 nm, and at higher organic concentrations the mobile phase transmits less light. It is generally recommended to work at wavelengths >210 nm with these solvents. Also the viscosity of the mobile phase plays an important role in the back-pressure generated in the HPLC column (pressure drop). The viscosity is not a linear function and is dependent upon the type and concentration of the organic solvent as well as the operation temperature (Figures 4-3 and 4-4) [33–35]. Also, highly viscous solvents such as methanol and isopropanol can lead to reduced diffusion rates, resulting in peak broadening as well as creating excessively high backpressures in the column. Solvents such as tetrahydrofuran (THF) and other ethers are prone to oxidation to form peroxides. These peroxides can react with the solute or with other mobile-phase components, causing the appearance of spurious peaks.

4.4.1 Eluent Composition and Solvent Strength of the Mobile Phase

HPLC retention is sometimes explained as the result of competitive interactions of the analyte and eluent molecules with the stationary phase. From this point of view the stronger the eluent interactions with the adsorbent surface, the lower the analyte retention, which leads to the term “eluent strength.”

In the development of reversed-phase separation methods the organic part of the eluent is considered the strong solvent. Increasing the fraction of the

<table>
<thead>
<tr>
<th>Solvent</th>
<th>UV Cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>190</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>205</td>
</tr>
<tr>
<td>Methanol</td>
<td>205</td>
</tr>
<tr>
<td>Ethanol</td>
<td>205</td>
</tr>
<tr>
<td>Uninhibited THF</td>
<td>215</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>256</td>
</tr>
<tr>
<td>DMSO</td>
<td>268</td>
</tr>
</tbody>
</table>

*Usually determined as the wavelength at which the absorbance of the neat solvent in a 1-cm cell is equal to 1 AU (absorbance unit) with water used as reference.

bUncommon reversed-phase solvent, may be used in small quantities to adjust selectivity.
Figure 4-3. Viscosity as a function of organic/water composition values obtained from references 33–35.

Figure 4-4. Viscosity as a function of acetonitrile/water composition from 15°C–55°C. Values obtained from references 33–35.
organic solvent increases the solvent strength and allows for elution of the species in a mixture, resulting in smaller analyte retention factors or retention volumes.

Analyte HPLC retention is a competitive process, and in an ideal form assuming only analyte–eluent competition for the stationary phase surface and in the absence of any secondary equilibria, one can write

\[
k = \frac{V_R - V_0}{V_0} = \frac{S}{V_0} K
\]

(4-2)

where \(K\) is a thermodynamic equilibrium constant, which can be expressed as

\[
K = \exp\left( \frac{\Delta G_{\text{analyte}} - \Delta G_{\text{eluent}}}{RT} \right)
\]

(4-3)

where \(\Delta G_{\text{analyte}}\) is the free Gibbs energy of the analyte interaction with adsorbent surface and \(\Delta G_{\text{eluent}}\) is the corresponding free Gibbs energy for eluent. Assuming that the aqueous portion of the reversed-phase eluent is inert and does not interact with the reversed-phase surface, along with using the principle of energetic additivity, one can assume that the free Gibbs energy of the eluent interaction with the stationary phase is proportional to the concentration of organic modifier in the mobile phase.

\[
\Delta G_{\text{eluent}} = \frac{c_{\text{org.}}}{c_{\text{max}}} \cdot \Delta G_{\text{el}}
\]

(4-4)

where \(\Delta G_{\text{el}}\) is the free Gibbs energy of the interaction of neat organic phase with the surface, \(c_{\text{org.}}\) is the current concentration of organic modifier in the mobile phase, and \(c_{\text{max}}\) is molar concentration of neat organic phase. Substituting equations (4-4) and (4-3) into equation (4-2) and taking the logarithm leads to equation (4-5):

\[
\ln(k) = \frac{\Delta G_{\text{analyte}}}{RT} + \ln\left( \frac{S}{V_0} \right) - c_{\text{org.}} \cdot \frac{\Delta G_{\text{el}}}{c_{\text{max}} RT} = A + B c_{\text{org.}}
\]

(4-5)

where \(A = \Delta G_{\text{analyte}} / RT + \ln(S/V_0)\) and \(B = \Delta G_{\text{el}} / c_{\text{max}} RT\) are constants and the logarithm of retention factor is a linear function of the eluent composition. Note that this is only applicable in the absence of secondary equilibria effects, which will be discussed later in this chapter.

Therefore, increasing the concentration of the organic modifier generally leads to an exponential decrease in the analyte retention volume. The general rule of thumb is that for every 10 v/v% increase in organic modifier there is a two- to threefold decrease in the analyte retention factors for analytes with molecular weights of less than 1000 Da. Figure 4-5.
The logarithm of the retention factor ($k$) versus the organic composition is usually taken to be almost linear over a limited range in reversed-phase systems (see Figure 4-6). In the eluent concentration region between 50% and 80% of organic component, the slopes of the retention of homologous compounds were the same for all homologs. However, if a wider organic eluent concentration region is studied as in Figure 4-7, a nonlinear dependence of logarithm of retention factor versus the organic composition is observed.

The dependence of $k$ (retention factor) on the volume percentage of the modifier is a subject of great controversy. One school of thought claims a linear dependence [36, 37], whereas another advocates a quadratic relationship [38, 39] and indicates that deviation from linearity will be more pronounced at high concentrations of the modifier.

Several different theories have been proposed for the description of the influence of the eluent composition on the analyte retention in reversed-phase
HPLC. Probably the very first consistent theory was introduced by Soczewinski [40, 41] for normal-phase separations. He suggested the equation which in the simplified form reads

\[ \ln(k) = \ln(k_2) - S \ln(x) \]  \hspace{1cm} (4-6)

where \( k_2 \) is the hypothetical extrapolated retention factor for the analyte eluted with pure solvent 2 (strongest solvent), \( S \) is an adsorbent surface area, and \( x \) is a molar fraction of the second eluent component. Almost at the same time, Snyder [36] introduced the concept of eluotropic strength essentially on the basis of the correlation with Hildebrand solubility parameter. The influence of eluotropic strength, \( \varepsilon \), of an eluent retention factor was suggested in the form

\[ \ln(k) = \ln(k_w) + A(\varepsilon_w - \varepsilon^0) + \ln C \]  \hspace{1cm} (4-7)

where \( C \) is a complex function of molecular volumes and molecular areas of the eluent components.

Later, Snyder et al. [42] introduced simplified semiempirical equation

\[ \log(k) = \log(k_w) - S \phi \]  \hspace{1cm} (4-8)
where \( k_w \) is the extrapolated analyte retention factor in pure water, \( \phi \) is the volume fraction of the organic eluent modifier, and \( S \) is the slope of this linear function specific for a particular organic modifier used and the nature of the solute (most important is the molecular weight). For small molecules the \( S \) values for methanol and acetonitrile are generally in the range of 3–4 [43], and for biomolecules they are more than 50. It later appears that \( S \) values are not exactly solvent-specific but rather dependent on the type of column used. Horvath and Melander indicated very strong dependence of the \( S \) parameter on the type of the bonded phase [44].

In a simplified form, it is generally accepted that the logarithm of the retention factor shows linear variation with the volume fraction of the eluent composition [45] similar to expression (4-5) above. This statement has to be taken only as a first and very rough approximation, since many deviations from this rule have been reported [46, 47] especially for acetonitrile/water mixtures as shown for \( n \)-hexanol and \( n \)-octanol in Figure 4-8 [47] and phenol and toluene in Figure 4-9.

From a practical point of view, the concept of linearity of the logarithm of the analyte retention factor could be used only for the rough estimation of the eluent composition variation. Also, the curvature of this dependence can show further deviations from linearity if the analyte is changing its ionization state at varying organic composition.

### 4.4.2 Type of Organic Modifier

Mobile-phase strength depends not only on the concentration of the organic modifier, but also on the type of organic modifier used. There were many attempts to create some type of mathematical correlation between the acetonitrile and methanol and THF concentrations which is supposed to result in similar retention of the analytes. More comprehensive estimates of sliding scales of solvent strength of different organic modifiers have been given by Schoenmakers et al. [48, 49] This is known as the same elutropic strength. The solvent strength of the most common organic eluents used at the same volume percentage (v/v%) in reversed-phase chromatography would be: methanol < acetonitrile < tetrahydrofuran. For example, if a similar retention of a neutral compound or an ionizable compound in its fully ionized or neutral state is to be achieved with methanol/water eluent compared to acetonitrile/water eluent on a C18 adsorbent, then an increased concentration of methanol is needed in the mobile phase (about 10 v/v% more of methanol for every 1 v/v% of acetonitrile would be needed for similar elution). If similar retention is to be achieved with acetonitrile/water mobile phase versus THF/water mobile phase on a C18 adsorbent, approximately 10 v/v% more of acetonitrile is needed for every 1 v/v% of THF. Note that these general rules serve only as an approximation because the retention of an analyte in methanol/water versus acetonitrile/water system may be dependent on many parameters lending to different interactions of the analyte with the solvent and/or with the bonded phase.
Figure 4-8. Logarithm of retention factors of $n$-hexanol and $n$-octanol on octadecylsilica at different water/organic compositions. (Reprinted from reference 47, with permission.)

Figure 4-9. Chromatographic conditions column: 15-cm $\times$ 0.46-cm Chromegabond WR-EX C18. Eluent: Buffer/10–80% MeCN. Buffer: 15 mM sodium acetate, pH 4; flow rate, 1 mL/min.
The principal difference in the behavior of acetonitrile and methanol, the most common eluent modifiers, was recently shown [50] where acetonitrile and THF forms a thick multimolecular adsorbed layer on the surface of reversed-phase adsorbent (C1–C18 and phenyl phases), while methanol is adsorbed only in monomolecular fashion. This brings a principal difference in the analyte retention mechanism in these two hydro-organic systems. Different retention mechanisms and their theoretical description are discussed in the Chapter 2.

In a binary eluent system (acetonitrile-water), an adsorbed organic phase with finite thickness and composition different from the bulk mobile phase is preferentially accumulated near the surface of the bonded phase. The organic layer accumulated near the bonded ligands could behave as a liquid stationary phase in reversed-phase HPLC, and it contributes to the overall analyte retention process.

In this scenario, an adsorbed organic layer with a different composition than the bulk mobile phase is formed. An analyte may distribute itself from the bulk eluent into the adsorbed organic layer. This adsorbed organic layer possesses a certain thickness which depends upon the concentration of the organic component in the eluent composition and its adsorption isotherm on the surface of the packing material used. The general retention process is comprised of two processes: (1) the analyte partitions between the bulk eluent and the adsorbed organic layer; (2) the portion of the analyte partitioned into the organic layer is then distributed between this layer and the surface of the modified adsorbent.

Overall analyte retention in acetonitrile/water eluent is the superposition of different processes: partitioning and adsorption. The volume of acetonitrile adsorbed layer is also dependent on the eluent composition (v/v% acetonitrile). This essentially may provide the explanation for the nonlinear behavior of the logarithm of the retention factors as a function of the eluent composition for acetonitrile as opposed to methanol, which forms only monomolecular layer and analyte retention factors generally show linear logarithmic dependence on the eluent composition (v/v% methanol).

### 4.4.3 Selectivity as a Function of Type and Concentration of Organic Composition

Ideally the eluent composition should not affect the selectivity between two species if their ionization state is not changing with an increase in the organic composition see Section 2.14 for details).

Generally the selectivity of neutral components is not affected by changing the organic composition. However, for ionizable components, changing the organic composition may affect changes in the analyte ionization state and lead to changes in selectivity. For example, in all experiments shown in Figure 4-10 the aqueous portion of the mobile phase had a pH of 7, and noticeable
changes in selectivity were observed for critical pairs (i.e., 1,2 and 2,3 and 3,4 . . .) as the organic composition is increased from 30 v/v% acetonitrile to 80 v/v% acetonitrile (Table 4-2). This may be attributed to a change in the pH of the aqueous portion of the mobile phase as well as variation in the analyte ionization state upon the addition of organic component. This change in selectivity for these ionizable species indicates that their degree of ionization varies with the change in organic composition which contribute to the variation in

**Figure 4-10.** (A) 30% MeCN: 70% 20 mM Phosphate, pH 7. (B) 50% MeCN: 50% 20 mM Phosphate, pH 7. (C) 80% MeCN: 20% 20 mM Phosphate, pH 7.

**TABLE 4-2. Effect of Organic Composition on the Chromatographic Selectivity**

<table>
<thead>
<tr>
<th>v/v% MeCN</th>
<th>1, 2</th>
<th>2, 3</th>
<th>3, 4</th>
<th>4, 5</th>
<th>5, 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
<td>1.36</td>
<td>1.36</td>
<td>1.59</td>
<td>1.31</td>
<td>1.86</td>
</tr>
<tr>
<td>50%</td>
<td>1.61</td>
<td>1.18</td>
<td>1.29</td>
<td>1.17</td>
<td>1.62</td>
</tr>
<tr>
<td>80%</td>
<td>1.29</td>
<td>1.00</td>
<td>1.37</td>
<td>1.00</td>
<td>1.21</td>
</tr>
</tbody>
</table>
selectivity. The effect of organic content on changes in mobile-phase pH and analyte ionization will be discussed in Section 4.5.

Variations in the selectivity are sometimes observed with the change in the type of organic modifier due to the specifics of the analyte–solvent interactions (solvation) and the specific adsorption behavior of the organic modifier. In the following example the effect of type and concentration of methanol and acetonitrile modifiers on the retention of acidic, basic, and neutral analytes is discussed.

The separation of four analytes [neutral analyte (toluene), strong basic compound (alprenolol, \( pK_a = 9 \)), weakly basic compound (\( o\)-chloroaniline, \( pK_a = 2.5 \)), and weak acidic compound (phenol, \( pK_a = 10 \))] was performed on a conventional C18 phase using a sodium acetate mobile phase (pH 4.5) with either acetonitrile (Figure 4-11) or methanol (Figure 4-12) as the organic portion of the eluent. The pH of the mobile phase was chosen to be at least two units away from the analytes \( pK_a \)'s in the mixture, such that each of the analytes were analyzed in their fully ionized or neutral states across the range of the organic compositions studied.

In Figures 4-11 and 4-12, linear dependence of the analyte retention is observed in both hydro-organic systems for all analytes in the limited region of the eluent compositions studied. Note that in order to obtain a similar retention factor for each of the analytes using methanol, a higher amount of methanol compared to acetonitrile was necessary (roughly a 10 v/v% increase in methanol content was applied to elute components at similar retention using acetonitrile). However, with the alprenolol (analyzed in its protonated state at pH 4.5) a 20–25 v/v% greater concentration of methanol was required compared to acetonitrile, to elute alprenolol at a similar retention. Also,
differences in selectivity for the compounds in this mixture were observed when the two different organic eluents were used. This demonstrates the “power” of using different types of organic eluent to assist in optimizing the separation selectivity. It is important to note that the slope factor ($S$) given in equation (4-8) must be different for each studied analyte if a selectivity change with change in organic concentration is to be observed between two analytes. Thus, it is possible to optimize both the retention and selectivity by varying the mobile-phase composition in isocratic mode or varying the gradient slope in gradient mode. Varying the gradient slope (change in v/v% organic per unit time) is a very useful approach for adjustment of separation selectivity during reversed-phase HPLC method development.

Under reversed-phase conditions, due to differences in the hydrogen bonding capabilities, polarizability, and different absorption characteristics of acetonitrile, methanol, and THF, the use of different modifiers offer substantial differences in selectivity. As an example, an LC-MS method was required to monitor labeled cortisol/cortisone in the presence of unlabeled cortisone and other possible interfering metabolites (Figure 4-13). Separations using YMC-ODS-AQ column exhibited differences in selectivity that could be further enhanced by using different organic modifiers to separate the eight components of interest (Figure 4-14) [51]. Selectivity differences can also be obtained by using small percentages (<10%) of a third solvent component for more challenging separations. For example, in a water/acetonitrile system the addition of less than 10% THF or methanol in acetonitrile may offer desired selectivity differences.

Changes in selectivity can be obtained by changing the type of stationary phase and the type and concentration of organic for both neutral and
Figure 4-13. Steroids. 1, Cortisol; 2, cortisone; 3, 6β-OHF; 4, 6β-OHE; 5, 20β-DHF; 6, 20β-DHE; 7, prednisolone; 8, prednisone.

Figure 4-14. Selectivity for steroids as a function of organic mobile-phase component. Chromatograms showing the elution order of all eight congeners as a function of organic modifier with 0.1% formic acid as the buffer phase on YMC ODS-AQ column at ambient temperature. (Top panel) 25% acetonitrile, 1.5-mL/min flow rate; (middle panel) 45% methanol, 1.2-mL/min flow rate; (bottom panel) 20% tetrahydrofuran, 1.5-mL/min flow rate. (Reprinted from reference 51, with permission.)
ionizable compounds. However, the pH can have a dramatic effect on the change of the separation selectivity for ionizable compounds. In Section 4.5 the importance for judicious choice and control of pH for the separation of ionizable compounds is discussed.

4.5 pH EFFECT ON HPLC SEPARATIONS

Most pharmaceutical compounds contain ionizable functionalities such as amino, pyridinal, or carboxylic groups. Mobile-phase pH and composition are among the main parameters used to control HPLC retention of most pharmaceutical compounds and to optimize separations. The introduction of new packings that are stable over a wider pH range up to pH 12 allows for a broader applicability of mobile-phase pH as a retention/selectivity adjustment parameter [52, 53]. The pH of the mobile phase has a strong influence on the retention of protolytic solutes and should be controlled in reversed-phase HPLC. Buffers are recommended to control the pH stability of the mobile phase. Common buffers are shown in Table 4-3. Note that the common volatile buffers trifluoroacetate ($pK_a 0.5$), acetate ($pK_a 4.8$), and formate ($pK_a 3.8$) can be used for mass-spectrometric detection; however, they have significant background absorption, depending on their concentration at wavelengths below 220 nm. This usually leads to descending baselines when running gradient separations (since the aqueous portion of the mobile phase is being diluted with the organic and there is a consequent decrease in the background absorbance). It is generally recommended to add the same concentration of acid modifier or salt buffer that is in the aqueous phase to the organic phase to suppress the descending baseline effect (Note: Check the solubility of buffering agents in organic phase). However, even though this leads to a flatter baseline, it still reduces the detection sensitivity because the mobile phase is absorbing at the wavelength of interest (<220 nm).

4.5.1 Mobile-Phase pH. Practical Considerations

The pH specified in analytical methods for pharmaceutical analysis should be that of the aqueous solvent. Note that the addition of organic modifier to aqueous buffer generally results in a shift in mobile-phase pH. The $pK_a$ of the solute is also subject to variation and is dependent on the type and concentration of the organic modifier in the eluent.

Practical Recommendations

The pH of the aqueous portion of the mobile phase must be accurately controlled with a calibrated pH meter. That is, the desired pH of the aqueous portion of the mobile phase must be within the calibrated pH range. The calibration standards should be close in ionic strength and temperature of the buffer solutions that will
be measured. Typical standards include pH 1, pH 2, pH 4, pH 7, and pH 10. Some pH meters allow a three-point calibration curve, and others allow for a two point calibration curve. In any event, the appropriate criteria for the slope of the calibration curve should be met and recorded in a log book and/or notebook and should be calibrated each time prior to use. The pH of the buffer should be controlled within ±0.1 pH units. Additionally, buffers can be made by weighing a known amount of salt of the acid or base to make the buffer and adding, if necessary, a known volume of acid or base to generate the desired pH. Once this is performed, the pH of the aqueous portion of the mobile phase can be measured as an additional precautionary measure.

### TABLE 4-3. Commonly Used Buffers for Reversed-Phase HPLC

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH Range</th>
<th>UV Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA (^a)</td>
<td>0.5</td>
<td>Up to 1.5</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>2.1</td>
<td>1.1–3.1</td>
</tr>
<tr>
<td>Mono/dihydrogen phosphate</td>
<td>7.2</td>
<td>6.2–8.2</td>
</tr>
<tr>
<td></td>
<td>12.3</td>
<td>11.3–13.3</td>
</tr>
<tr>
<td>Formic acid (^a)</td>
<td>3.8</td>
<td>2.8–4.8</td>
</tr>
<tr>
<td>Acetic acid (^a)</td>
<td>4.8</td>
<td>3.8–5.8</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.1</td>
<td>2.1–4.1</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>3.7–5.7</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>4.4–6.4</td>
</tr>
<tr>
<td>Trisamino methane (TRIS)</td>
<td>8.1</td>
<td>7.1–9.1</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2–9.2</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>10.7</td>
<td>9.7–11.7</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>10.8</td>
<td>9.8–11.8</td>
</tr>
<tr>
<td>Ammonium hydroxide (^a)</td>
<td>9.3</td>
<td>8.3–10.3</td>
</tr>
<tr>
<td>Pyrrolidine (^b)</td>
<td>11.3</td>
<td>10.3–12.3</td>
</tr>
<tr>
<td>N-Methyl pyrrolidine (^c)</td>
<td>10.3</td>
<td>9.3–11.3</td>
</tr>
<tr>
<td>Piperidine</td>
<td>11.1</td>
<td>10.1–11.1</td>
</tr>
<tr>
<td>Ammonium acetate (^a)</td>
<td>3.8–5.8 (acetate)</td>
<td>210 nm (10 mM)</td>
</tr>
<tr>
<td>Ammonium bicarbonate (^a)</td>
<td>pK(_1) 6.4</td>
<td>5.4–7.4 (carbonate,1)</td>
</tr>
<tr>
<td></td>
<td>pK(_2) 10.3</td>
<td>9.3–11.3 (carbonate,2)</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>8.3–10.3 (ammonium)</td>
</tr>
</tbody>
</table>

\(^a\) Volatile buffers; these can be used for LC-MS.
\(^b\) Cannot be used in the state of California.
\(^c\) Sometimes purity of this reagent is poor and UV cutoff could be greater.
The impact of the pH in hydro-organic mixtures on the analyte ionization and retention will be thoroughly discussed. The impact of pH on analyte UV absorbance will be discussed in the method development chapter, Chapter 8 (Section 8-6).

4.5.2 Analyte Ionization (Acids, Bases, Zwitterions)

A simple rule for retention in reversed-phase HPLC is that the more hydrophobic the component, the more it is retained. By simply following this rule, one can conclude that any organic ionizable component will have longer retention in its neutral form than in the ionized form. Analyte ionization is a pH-dependent process, so significant effect of the mobile-phase pH on the separation of complex organic mixtures containing basic or acidic components can be expected.

Ionization of the analyte could be expressed by one of the following equilibria:

\[
\text{HA} \leftrightarrow \text{A}^- + \text{H}^+ \quad \text{for acidic components} \quad (4-9a) \\
\text{B} + \text{H}_2\text{O} \leftrightarrow \text{BH}^+ + \text{OH}^- \quad \text{for basic components} \quad (4-9b)
\]

For HA a weak acid the products of the dissociation are hydrogen ion (H\(^+\)) and an anion (A\(^-\)), which is the conjugate base. Equilibrium constants for acids can be written in the following form:

\[
K_a = \frac{[A^-][H^+]}{[AH]} 
\]  

(4-10)

Using the definition for the pH (Henderson–Hasselbalch form), one can rewrite

\[
pK_a = \text{pH} + \log\left(\frac{[AH]}{[A^-]}\right) \quad (4-11)
\]

Similar expressions could be written for bases.

\[
K_b = \frac{[BH^+][OH^-]}{[B]} 
\]  

(4-12)

\[
pK_b = \text{pOH} + \log\left(\frac{[B]}{[BH^+]}\right) \quad (4-13)
\]

Weak acids and bases exist in equilibrium with their ions, and their equilibrium constants \((K_a)\) are small. The position of equilibrium is measured by the equilibrium constant \(K_a \) [equation (4-10)] and \(K_b \) [equation (4-12)]. The dis-
The association constant of water is $10^{-14}$ ($K_w = 10^{-14}$). Since the neutralization reaction of the equimolar quantities of weak acid and base result in the formation of water and salt, the following relationship holds:

$$K_a K_b = K_w$$

(4-14)

The usefulness of relationship (4-14) is that for any base the $K_b$ value can be found from the $K_a$ of its conjugate acid. Conversely, the $K_a$ value of any acid can be found from $K_b$ of its conjugate base.

### 4.5.3 $pK_a$ and $pK_b$ Relationship

Using the $pX$ notation, we commonly speak of the $pK_a$ of an acid and the $pK_b$ of a base, where $pX = -\log X$. Therefore,

$$pK_a = -\log K_a,$$  
$$pK_b = -\log K_b,$$  

(4-15)  
(4-16)

Because $K_a K_b = K_w$, it follows that $pK_a + pK_b = pK_w$. Furthermore, the value of $K_w$ at 25°C is $1.0 \times 10^{-14}$ and so $pK_w = 14.00$ at 25°C.

For any acidic or basic compound the $pK$ values can be expressed as $pK_a$ or $pK_b$; however, in the literature the $pK_a$ values are most often used.

### 4.5.4 Retention of Ionizable Analytes in Reversed-Phase HPLC

Primary equilibrium in the chromatographic system is the analyte distribution between mobile phase and the surface of packing material. If the analyte could be present in the mobile or stationary phase in two or more different forms and there is an equilibria between these forms, this equilibria is usually called "secondary."

Because different forms of analyte usually show different affinity to the stationary phase, secondary equilibria in HPLC column (ionization, solvation, etc.) can have a significant effect on the analyte retention and the peak symmetry. HPLC is a dynamic process, and the kinetics of the secondary equilibria may have an impact on apparent peak efficiency if its kinetics is comparable with the speed of the chromatographic analyte distribution process (kinetics of primary equilibria). The effect of pH of the mobile phase can drive the analyte equilibrium to either extreme (neutral or ionized) for a specific analyte. Concentration and the type of organic modifier affect the overall mobile phase pH and also influence the ionization constants of all ionogenic species dissolved in the mobile phase.

It is generally preferable to use a mobile-phase pH that is at least one unit away from the $pK_a$ values of the analytes in the mixture, so each analyte will be in a predominant single ionization state (>90%) during the chromatography.
graphic run. This essentially suppresses the effect of secondary ionization equilibria on the analyte retention.

Neutral and ionic forms of any analyte have significant differences in their apparent hydrophobicity and thus tend to migrate through the column with different velocity. This causes an instantaneous disturbance of ionic equilibrium in the chromatographic zone microenvironment. The equilibration kinetics of analyte ionic equilibrium has a profound effect on the analyte peak shape. If the kinetics of ionization is slow, the ionic species will tend to move faster than the neutral species, causing a significant broadening of the composite chromatographic zone (kinetics of ionization is in almost every single case faster than the chromatographic kinetics by orders of magnitude).

The kinetics of the ionic equilibration is also dependent on the analyte solvation. The greater the analyte solvation, the slower the equilibration kinetics. Solvation shell restricts the protonation or deprotonation of the analyte. Solvation is also influenced by the eluent ionic strength. With an increase of the concentration of ions in the analyte microenvironment, there is a corresponding decrease in the analyte solvation, thus increasing the ionic equilibration kinetics. The increase of the eluent ionic strength usually improves the analyte peak shape even if the mobile-phase pH is close to the analyte pK_a.

The development of HPLC methods where the mobile-phase pH is close to the analyte pK_a is not recommended because of potential distorted peaks (which can be amended by the increase of the salt or buffer concentration); these methods may not be rugged and will not be easily transferable to other laboratories (manufacturing facility). Any minor variations in the mobile-phase pH in this case can lead to the significant variations in the analyte retention and separation selectivity.

Since the pK_a is a characteristic constant of the specific analyte, from equation (4-11), one can conclude that relative amounts of neutral and ionic forms of the analyte could be easily adjusted by varying the mobile-phase pH. Moreover, if the eluent pH is at least two units away from the component pK_a, more than 99% of the analyte will be in either ionic or neutral form. If the eluent pH is at least one unit away from the component pK_a, more than 90% of the analyte will be in either ionic or neutral form.

In Figure 4-15, a titration curve for aniline is shown such that if the analyte was placed in an acidic medium such as HCl, it would be >99% ionized (0% neutral, bottom plateau), and as the analyte was titrated with a strong base such as NaOH the analyte would become progressively more neutral (0% ionization, top plateau). In Figure 4-16 it can be seen that the analyte in its ionized form shows the lowest retention (low plateau); and as the analyte becomes progressively more neutral, this leads to increased retention. Eventually when the pH of the medium exceeds the 2 units greater than the analyte pK_a, the retention is essentially unchanged (Figure 4-16, top plateau).

4.5.4.1 Basic Compounds. The primary retention dependence of the ionizable analyte versus the mobile-phase pH for basic components will have the form shown in Figure 4-16, assuming that no predominate secondary interac-
tions are occurring with the stationary phase (i.e., interactions with residual silanols).

The retention dependence of basic components on the pH of mobile phase could be subdivided into three regions (Figure 4-16).

A. Fully protonated analyte (cationic form), which shows the lowest retention. The analyte is in the most hydrophilic form. Its interactions with the hydrophobic stationary phase are suppressed. A compound in its ionic form is more hydrophilic, so it tends to have less interaction with hydrophobic stationary phase and also tends to be more solvated.

Figure 4-15. Effect of pH of the ionization of basic analyte.

Figure 4-16. Effect of pH of the retention of basic analyte.
with protic solvents. This may cause the significant decrease in the retention of ionic components. If methanol, a protic solvent, is used as an organic modifier, it can participate in the analyte solvation. The inclusion of the methanol molecules in the analyte solvation shell adds some hydrophobicity to the solvated molecular cluster, and this may lead to the significant distortion of the peak shape. This will be further discussed in Chapter 8.

B. Partial protonation region. Coexistence of two analyte forms (protonated and deprotonated) in the mobile phase in equilibrium may cause poor peak shape and unstable retention. Since analyte in the neutral form has much stronger retention, its molecules tend to interact with the stationary phase stronger and reside there longer. This causes a shift of the ionization equilibrium in the mobile phase with a greater proportion of protonated molecules (more hydrophilic) at the front of chromatographic band. The overall process depends on the superposition of the ionization and adsorption processes and their relative kinetics. Usually, a slight change of the mobile-phase pH greatly shifts the analyte retention in this region.

C. Analyte in its neutral form (the most hydrophobic), which shows the longest retention.

4.5.4.2 Acidic Compounds. Similar retention curves can be obtained for acidic components, but obviously their retention dependence will be the mirror image of that for basic analytes (Figure 4-17).

![Figure 4-17. General retention dependence of acidic analyte on the mobile-phase pH. The inflection point of the curve corresponds to the component pK_a.](image)
These retention profiles could be described by the following equation as a function of eluent pH and analyte pKₐ [54].

\[
k = \frac{k_0 + k_1 \frac{[H^+]}{K_{a[B^+]}}}{1 + \frac{[H^+]}{K_{a[B^+]}}}
\]  

(4-17)

or

\[
k = \frac{k_0 + k_1 10^{(pK_a-pH)}}{1 + 10^{(pK_a-pH)}}
\]  

(4-18)

where for bases \(k_1\) is the limiting retention factor of the protonated form and is represented by the lower plateau in Figure 4-16, and \(k_0\) is the limiting retention factor of the neutral form and is represented by the higher plateau in Figure 4-16. However, for acids \(k_0\) is the retention factor of the anionic form represented by the lower plateau in Figure 4-17, and \(k_1\) is the retention factor of the neutral form represented by the higher plateau in Figure 4-17. For both acids and bases, \(k\) is the retention factor at a given pH and \(k_a\) is the analyte ionization constant.

If compounds are analyzed in region A or C retention variation with mobile-phase pH will be minimal. Methods employing a mobile-phase pH which corresponds to these regions are generally more rugged. On the other hand, each region has its own drawback. Therefore, during the selection of the starting HPLC conditions, one has to account for all possible effects. Some of the drawbacks are discussed below.

**4.5.4.3 Effects in Region A.** Basic analytes show relatively low retention (analyte in its ionic form) and may even elute in the void. The employment of chaotropic additives may be needed to enhance the retention of the protonated basic analytes (see Section 4.10) However, acidic analytes show longer retention times because the acidic analyte would be analyzed in its neutral form.

**4.5.4.4 Effects in Region B.** In this region there is coexistence of appreciable quantities of both ionic and nonionic forms of the analyte. Significant loss of apparent efficiency for both acidic and basic analytes may be present in this region. Peaks broaden and sometimes have a weird shape but are mostly tailing or fronting, depending on the ionic strength of the mobile phase. Very unstable retention is observed, and minor changes in pH or composition of the mobile phase will significantly shift retention. Minor changes of the eluent composition can cause change in selectivity.
4.5.4.5 Effects in Region C. Very long retention for basic analytes thus requires working with high organic concentration of the mobile phase. Acidic components will be in their anionic form at high pH. Organic analytes in their anionic form usually are strongly solvated and may be completely excluded from the pore space of the packing material. This may cause very early elution of the analytes which is usually not adjustable by the eluent composition. Also, silica is soluble at high pH. If the column has some accessible silanols, prolonged operation at high pH may cause steady degradation of the packing material. This brings a loss of the efficiency due to the formation of voids in the column, or steady change of component retention.

4.5.5 Case Studies: Effects of pH on Ionizable Analyte Retention

Knowledge of the $pK_a$ of the analytes in the mixture is very important. Significant changes in retention and even reversals in elution order can be observed. Take, for example, two analytes: one basic and one acidic, both with $pK_a$ values of approximately 4. If a mixture of these two analytes were analyzed at pH 2.3 and pH 6.0, the base would show lower retention at pH 2.3 and higher retention at pH 6.0, and the acidic component would show higher retention at pH 2.3 and lower retention at pH 6.0 (Figure 4-18). Depending on the analytes’ relative hydrophobicity in their ionized form, they may even elute before the void volume, and the component(s) eluting prior to the void volume can and should not be quantified.

Acidic components may be weak acids such as phenolic compounds (8–10) or stronger acids such as carboxylic acids ($pK_a$ 3–4), and the specific analyte $pK_a$ would be dependent on the substitution on the aromatic ring and/or the

![Figure 4-18](image.png)

Figure 4-18. Effect of mobile-phase pH on the retention of ionizable compounds. (Reprinted from reference 55, with permission.)
neighboring groups on the alkyl chain if the ionizable species is not on the aromatic ring. Basic components may also be weak bases such as aniline ($pK_a$ 4.6) or stronger bases such as secondary or tertiary amines ($pK_a$’s 7–10). Figure 4-19 shows theoretical retention factor versus pH curves for these weakly/strongly acidic and weakly/strongly basic compounds. For unknown species in a mixture, the retention can be determined as a function of pH run under isocratic conditions to ascertain if the analytes of interest are acidic or basic in nature.

For a mixture of acidic components (carboxylic acid), decreasing the pH of the aqueous portion of the mobile phase from 9 to 2 led to the enhancement of the retention of the acidic analytes (Figure 4-20). At aqueous mobile-phase

![Figure 4-19](image1.png)

**Figure 4-19.** Theoretical retention versus pH profiles for acidic and basic components. (A) Strong acid and strong base. (B) Weak acid and weak base. (Reprinted from reference 55, with permission.)

![Figure 4-20](image2.png)

**Figure 4-20.** Theoretical retention versus pH profiles for acidic and a zwitterionic component. Chromatographic conditions: Column: 15-cm × 0.46-cm Phoenomenex Luna C18(2), 5μm; 70% 15 mM K$_2$HPO$_4$ adjusted pH 2–9 with phosphoric acid; 30% MeCN; flow rate, 1 mL/min; temperature, 25°C.
pH values greater than and equal to 6, all the acidic components eluted close to the void volume and resolution of all the components was not obtained.

In the pH region from 3.5 to 5, the aqueous pH of the mobile phase was close to that of the analyte $pK_a$ values, which led to a dramatic change in retention and peak skewing. Working in this pH region would not be recommended because small variations in the mobile-phase pH would lead to undesired changes in retention and selectivity. However, as the aqueous pH was changed from 3 to 2, only minor variations in retention were observed for all the components in the mixture. This also led to the optimal selectivity and resolution of all components in the mixture.

The opposite scenario for retention dependence versus pH is observed for basic compounds (Figure 4-21) [56]. In the mixture of basic components shown in Figure 4-21 (pyridinal species), increasing the pH of the aqueous portion of the mobile phase from 1.5 to 9 led to the enhancement of the retention of the basic analytes. At aqueous mobile-phase pH values of 7 and greater, the components exhibited a high retention. However, as the aqueous pH was changed to below 4, the compounds eluted close to the void volume. Generally, it is recommended that very polar bases are analyzed at pHs where the analyte is in its neutral form.

Variation of the mobile-phase pH is a powerful parameter to enhance the chromatographic selectivity and retention for mixtures of basic, acidic, and neutral compounds. Figure 4-22 shows that for neutral analytes (benzamide and flavone) the mobile-phase pH has no effect on the chromatographic retention [57]. However, for the organic acids (hydroxyisophthalic acid and fenoprofen, $pK_a$ 4.5) at pH 2, which is at least 2 pH units below the acid analyte $pK_a$ values, maximum retention is obtained, while at pH 7 and 12 there is a
Figure 4-22. Selectivity differences at pH 2, 7, and 12 on Waters XBridge C18 column. Analytes: 1, doxylamine (base); 2, benzamide (neutral); 3, hydroxyisophthalic acid (acid); 4, doxepine (base); 5, flavone (neutral); 6, 5 fenoprofen (acid). (Reprinted from reference 57, with permission.)
significant decrease in retention for both these acids and hydroxyisophthalic acid is nonretained and elutes in the void. For the bases (doxepin, pKₐ 9.3; doxylamine, pKₐ 9.2) at pH 12, which is at least 2 pH units above their pKₐ values, maximum retention is obtained, and at pH 2 the lowest retention for both basic compounds is obtained.

Zwitterionic components contain both acidic and basic functionalities. Depending on the distance between their pKₐ values, two distinct sigmodial dependencies may be observed, one for the acid and one for the base, and the overall retention dependence is usually in the shape of a bell curve (inverted or upright). These are usually observed when the pKₐ values are greater than 2 units apart. In Figure 4-23 a theoretical curve (bell-shaped) for a zwitterionic compound, 2-amino benzoic acid, is shown. The pKₐ of the basic functionality is 2.1, and that of the acidic functionality is 5.0. On the other hand, if a zwitterionic compound contained an acidic functionality with pKₐ 2 and a basic functionality with pKₐ 5, the bell-shaped curve would be inverted.

The HPLC retention of zwitterionic analytes essentially follows the dependence shown in Figure 4-23. A practical example is demonstrated with Benazepril HCl (SS and SR isomers). Analyte retention was monitored as a function of pH using 10 mM ammonium phosphate buffer at pH 2.1–7.1/acetoniitrile (70/30) on a phenyl-hexyl column (Figure 4-24). The pKₐ values for benazepril in purely aqueous media are 3.7 for the acidic moiety and 4.6 for the basic moiety. However, since Benazepril is being analyzed in a hydroorganic media the analyte ionization can be shifted, depending on the type and percent of organic modifier employed. Bell-shaped retention dependence of both Benazepril diastereomers is clearly visible in Figure 4-24.
There were selectivity differences as a function of pH from 2.1 to 7.1 (pH of the aqueous phase), with concomitant changes in retention. This demonstrates that separations of ionizable compounds in RPLC should be performed where the molecules are in one predominate ionization state(s) to avoid significant changes in retention and selectivity with minor changes in pH.

4.5.6 Mobile-Phase pH

For the separation of ionogenic (ionizable) solutes, the variations of mobile-phase pH can lead to extreme changes in selectivity. The mobile-phase (eluent) pH affects the ionization of ionogenic species and consequently their HPLC retention. However, the pH of the aqueous phase is not equivalent to the pH of the aqueous/organic eluent, and consequently the variation of the mobile-phase composition leads to the variation in pH under both isocratic and gradient conditions [58–60]. Therefore the pH shift of the mobile phase upon the addition of the organic modifier is imperative for a proper description of the
ionogenic analyte retention process and should be accounted in the development of the HPLC separations of such compounds. This will lead to more robust and rugged methods.

### 4.5.6.1 pH Scales in Water–Organic Mixtures

There have been many studies about the determination of pK values of acids [61–65] in acetonitrile/water mixtures. Several models have been used to relate the pK with the solvent composition [66, 67]. Typically, the pH of acidic buffers upon the addition of acetonitrile or methanol increases to higher values, and the pH of basic buffers upon the addition of acetonitrile or methanol decreases to lower values. On the basis of IUPAC rules and recommendations [68, 69], Bosch and co-workers have studied the different pH scales that are employed in pH measurement of hydro-organic mixtures [64]. The three different pH scales are usually considered the \( \text{w} \text{pH} \), \( \text{s} \text{w} \text{pH} \), and \( \text{s} \text{s} \text{pHs} \). The \( \text{w} \text{pH} \) scale is one in which the electrode system is calibrated with aqueous buffers, and the pH of the aqueous portion of the mobile phase is measured prior to the addition of the organic modifier. The \( \text{s} \text{w} \text{pH} \) scale is where the electrode system is calibrated with aqueous buffers, and the pH of the hydro-organic mobile phase is measured after the addition of the organic modifier. The \( \text{s} \text{s} \text{pH} \) has also been described in the literature as the apparent pH or pH\(_{\text{app}}\) [70, 71]. The \( \text{s} \text{s} \text{pH} \) scale is where the electrode system is calibrated with buffer-organic mixtures of the same composition as the mobile phase, and the pH of the hydro-organic mobile phase is measured after the addition of the organic modifier [68, 69]. This sometimes has been referred to in the literature as pH\(_*\) [72]. The s/w and s/s scales could be interconverted by equation (4-19). This equation includes the difference of the liquid junction potentials (\( E_j \)), together with the primary medium effect \(-\log(\gamma_H^w)\) [73]. Espinosa et al. [73] have stated that residual liquid junction potential can be assumed to be negligible if the junction potential of the electrode system in the measurement solution in solvent \( s \) (\( E_s \)) is close to the liquid junction potential in the calibration solution in water (\( E_w \)). Therefore \( \delta = -\log(\gamma_H^w) \) and \( \delta \) is the difference of \( \text{s} \text{pH} \) and \( \text{s} \text{w} \text{pH} \) scales.

\[
\delta = E_j - \log(\gamma_H^w) = \text{s} \text{pH} - \text{s} \text{w} \text{pH} \tag{4-19}
\]

The \( \delta \) is a constant for each mobile-phase composition and type of buffer system employed. The \( \delta \) term determined for various acetonitrile/water and methanol/water mixtures ranging from 10 to 60 v/v% by Roses, Espinosa, and co-workers [73, 74] are shown in Table 4-4 and Table 4-5, respectively.

### TABLE 4-4. Delta Values for Various Acetonitrile/Water Compositions [73, 74]

<table>
<thead>
<tr>
<th>Volume fraction MeCN (( \phi )):</th>
<th>0</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mole fraction:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.04</td>
<td>0.079</td>
<td>0.13</td>
<td>0.186</td>
<td>0.26</td>
<td>0.339</td>
</tr>
<tr>
<td>( \delta ):</td>
<td></td>
<td>-0.01</td>
<td>-0.02</td>
<td>-0.06</td>
<td>-0.13</td>
<td>-0.26</td>
<td>-0.44</td>
</tr>
</tbody>
</table>
An empirical formula representing the variation of the \( \delta \) quantity with mole fraction of acetonitrile (\( \chi \)) from the values in Table 4-4 could be determined using equation (4-20). The dependence of \( \delta \) versus the mole fraction of acetonitrile is shown in Figure 4-25.

\[
\delta = (-3.93)\chi_{\text{MeCN}}^2 + 0.03\chi 
\]  

An empirical formula representing the variation of the \( \delta \) quantity with mole fraction of methanol (\( \chi \)) from the values in Table 4-5 could be determined using equation (4-21). The dependence of delta versus the mole fraction of methanol is shown in Figure 4-26.

\[
\delta = (0.4826)\chi_{\text{MeOH}}^2 + 0.2632\chi 
\]
Similarly, the delta values as a function of any volume composition up to 60 v/v% acetonitrile [i.e., is equivalent to 0.6 volume fraction (φ)] and methanol can be determined using equations (4-22a) and (4-22b). [74]

\[
\delta = \frac{-0.446\phi_{MeCN}^2}{1 - 1.316\phi_{MeCN} + 0.433\phi_{MeCN}^2} \quad (4-22a)
\]

\[
\delta = \frac{0.09\phi_{MeOH} - 0.11\phi_{MeOH}^2}{1 - 3.15\phi_{MeOH} + 3.51\phi_{MeOH}^2 - 1.35\phi_{MeOH}^3} \quad (4-22b)
\]

Note, however, that the difference between \(s_{wpH}\) and \(s_{spH}\) is a constant value for each mobile-phase composition, and the difference between \(s_{wpH}\) and \(s_{spH}\) depends not only on the type and concentration of mobile-phase composition, but also on the particular solution being measured [74–76]. However, these values can serve as estimates for converting from \(s_{wpH}\) to \(s_{spH}\) or \(s_{pK_a}\) to \(s_{spK_a}\).

The authors claim that the \(\delta\) values could be directly used with other electrode systems or by other laboratories, given that the residual liquid junction potential of the respective system is negligible [74–76]. This can be a convenient way to convert from the \(s_{wpH}\) scale to \(s_{spH}\) scale as Espinosa et al. have described [73].

**4.5.6.2 Effect of Organic on Modifier Ionization–pH Shift.** Typically, most reversed-phase HPLC methods use monoprotic or polyprotic acidic buffers. The determination of \(pK\) values of acids in acetonitrile/water mixtures and methanol/water mixtures have been reviewed in the literature [61–65, 67, 77] Several excellent reviews have been published on this topic by Roses and Bosch. [74, 75] The \(s_{spH}\) can be determined directly from \(s_{wpH}\) by the following relationship as shown in equation (4-19).

For example, seven aqueous solutions of 10 mM dipotassium monohydrogen phosphate (adjusted with phosphoric acid) with initial \(s_{wpH}\) (pH 2–9) were prepared in five acetonitrile/water compositions ranging from 10 to 50 v/v% of acetonitrile, and the \(s_{wpH}\) was determined. \(s_{wpH}\) was calculated using equation (4-19), and the final values are shown in Table 4-6. In Figure 4-27 the \(s_{spH}\) values were plotted versus the acetonitrile concentration ranging from 10 to 50 v/v%. It was shown that the \(s_{wpH}\) of the eluent increases with an increase of acetonitrile content. For the buffers that had initial \(s_{wpH}\) values between 2 and 9, the slopes of the plots of \(s_{wpH}\) versus v/v% acetonitrile concentration are essentially independent of the initial aqueous pH with \(R^2 > 0.98\). There is an increase (or upward shift of the pH) of approximately 0.22 pH units for every 10 v/v% of acetonitrile added, indicating a change in the acidic modifier’s dissociation constant (change in the modifier’s \(pK_a\)).

The change in the mobile-phase pH of a particular buffer as a function of the organic compositions will be referred to as the \(pH\) shift in the following sections in this book. For acidic buffers/modifiers, the relative increase in the pH will be dependent upon the type and concentration of acidic modifier and
organic eluent. However, several other typically used acidic buffers such as acetate, dihydrogen phosphate, dihydrogen citrate, hydrogen citrate, and citrate and boric acid show a similar pH shift with an increase of acetonitrile organic modifier. These acids bear a similar trend in increase of the pH with increasing amounts of v/v% acetonitrile. The pH values determined by Espinosa et al. and Subirats et al. in the acetonitrile concentration range from 10 to 60 v/v% are shown in Table 4-7 and correspond to approximately 0.2–0.3 pH units increase per 10 v/v% acetonitrile [64, 78]. A conservative value of 0.2 pH units per 10 v/v% increase in acetonitrile will be used throughout the text to denote the acidic modifier pH shift of the aqueous portion of the mobile phase with the addition of acetonitrile.

The variation of the pKₐ of acidic modifiers with the addition of methanol to the aqueous portion of the mobile phase bears a similar upward trend.

**TABLE 4-6. ³pH Values of 10 mM Monohydrogen Phosphate Buffer Adjusted with Phosphoric Acid in Various MeCN Compositions**

<table>
<thead>
<tr>
<th>v/v% MeCN</th>
<th>³pHᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.09</td>
</tr>
<tr>
<td>10</td>
<td>2.3</td>
</tr>
<tr>
<td>20</td>
<td>2.48</td>
</tr>
<tr>
<td>30</td>
<td>2.65</td>
</tr>
<tr>
<td>40</td>
<td>2.96</td>
</tr>
<tr>
<td>50</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Slope: 0.023, 0.021, 0.024, 0.024, 0.023, 0.022, 0.021

R²: 0.986, 0.988, 0.982, 0.991, 0.988, 0.998, 0.991

ᵃCorrected for delta at each organic composition using δᵦᵥ values from reference 73.

**Figure 4-27.** Effect of concentration of acetonitrile on the pH shift for a 10 mM monohydrogen phosphate buffer.
However, the variation in the positive slope for $pK_a$ values in methanol/water mixtures is smaller than for acetonitrile/water mixtures because methanol is more similar to water. The typical increase in $p$H values of acidic modifiers in methanol/water mixtures is about 0.15 pH units per 10 v/v% methanol.

### 4.5.6.3 Acidic Modifiers: pH Shift and Correlation with Dielectric Constant.

The $pK$ variation of acids is related to changes in the electrostatic interactions upon addition of organic media. pH is the negative log of the concentration of protons that are the result of the acid dissociation (for acidic buffers). With the increase of the content of organic molecules in the solution, the dissociation is decreasing (with the decrease of dielectric constant the stabilization of dissociated ions is decreased), thus increasing the solution pH. As was discussed by Espinosa et al. [79], the pH shift occurs because an increase in organic leads to a change of the dielectric constant of the hydro-organic solution. As the organic content increases, the dielectric constant of the mobile phase decreases. In our studies with a decrease in the dielectric constant of the eluent composition (increasing acetonitrile composition) the $pK_a$ of the dipotassium monohydrogen buffer was observed to increase in a linear fashion at all pHs (Figure 4-28). As the organic content increases, the dielectric con-
stant of the mobile phases decreases. The dielectric constant is expected to influence the position of the equilibrium in ionic secondary chemical equilibria of acidic compounds [80–83]. The solvent has the ability to disperse electrostatic charges via ion–dipole interactions, which is inversely proportional to the dielectric constant of the solvent composition. The lower the dielectric constant, the lower the ionization constant of the acid, $K_a$, and consequently greater $pK_a$ values are obtained.

4.5.6.4 Basic Modifiers: pH Shift. Basic mobile-phase modifiers such as NH$_4^+$/NH$_3$ (pH 9) and BuNH$_3^+$/BuNH$_2$ (pH 10) show a decrease in their $pK_a$ values with increasing organic content [74]. These basic modifiers have an average pH decrease on the order of $-0.05$ to $-0.1$ pH units per 10 v/v% acetonitrile. The minimum of the pH values as a function of acetonitrile composition for basic modifiers is reached at approximately 30–50 v/v% MeCN. Upon further increase in MeCN concentration the pH of the basic modifier will increase. For example, ammonium/ammonia basic modifier pH values in acetonitrile/water mixtures are: 0% MeCN: 9.29, 10% MeCN: 9.27, 20% MeCN: 9.21, 30% MeCN: 9.17, 40% MeCN: 9.19, 50% MeCN: 9.21, 60% MeCN: 9.34 [64]. For BuNH$_3^+$/BuNH$_2$ (pH 10), basic modifier pH values in acetonitrile/water mixtures are: 0% MeCN: 10.00, 20% MeCN: 9.78, 40% MeCN: 9.63, 60% MeCN: 9.79 [64]. For basic modifiers a decrease in pH is also observed with increase of methanol content on the order of 0.1 pH units per 10 v/v% methanol.

4.5.6.5 Amphoteric Buffers: pH Shift. When buffers that contain both ionizable cations and anions such as ammonium acetate or ammonium phosphate are used, the change in the buffer pH (pH shift) is dependent on the pH of the starting buffer. For example, with an ammonium acetate buffer with the
addition of organic modifier, there is an upward pH shift up to \( \text{pH} 6 \) (due to acetate counterion) and a downward pH shift when \( \text{pH} > 7 \) (due to ammonium counterion). These effects are prevalent in both acetonitrile/water and methanol/water systems, as shown in Tables 4-8 and 4-9, respectively. The changes in pH slopes are (a) approximately constant and positive for \( \text{pH} < 

**TABLE 4-8. Calculated \( \text{pH} \) Values of 50mM Ammonium Acetate at Different Acetonitrile/Water Compositions**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>( \text{pH} ) in % MeCN by Volume</th>
<th>Slope per 10 ( \text{v/v}% ) MeCN</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Acetic acid</td>
<td>4.67 4.86 5.08 5.34 5.68 6.04 6.46</td>
<td>0.30 0.981</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>2.67 2.8 2.98 3.16 3.5 3.84 4.23</td>
<td>0.26 0.964</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>3.01 3.15 3.33 3.54 3.86 4.19 4.6</td>
<td>0.26 0.968</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>4.06 4.21 4.43 4.66 5.01 5.33 5.75</td>
<td>0.28 0.977</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>5.07 5.23 5.49 5.74 6.11 6.43 6.88</td>
<td>0.30 0.981</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>6.07 6.24 6.48 6.71 7.05 7.33 7.69</td>
<td>0.27 0.988</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>7.94 7.9 7.85 7.81 7.9 7.97 8.15</td>
<td>-0.04(^a) 0.998(^b)</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>8.94 8.88 8.84 8.76 8.8 8.8 8.87</td>
<td>-0.06(^a) 0.984(^b)</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>9.95 9.88 9.85 9.76 9.8 9.8 9.88</td>
<td>-0.06(^a) 0.968(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All \( \text{pH} \) data were obtained from reference [84], and \( \text{pH} \) values were calculated using \( \delta \) values from reference 73. The pHs were adjusted with formic acid and ammonium hydroxide.

\(^b\) The slope and \( R^2 \) were determined from 0–30\( \text{v/v}\% \) acetonitrile.

**TABLE 4-9. Calculated \( \text{pH} \) Values of 50mM Ammonium Acetate at Different Methanol/Water Compositions**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>( \text{pH} ) in % MeOH by Volume</th>
<th>Slope per 10 ( \text{v/v}% ) MeOH</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Acetic acid</td>
<td>4.76 4.96 5.15 5.36 5.57 5.8 6.03</td>
<td>0.21 0.999</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>2.67 2.8 2.94 3.06 3.22 3.37 3.55</td>
<td>0.15 0.997</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>3.01 3.15 3.24 3.36 3.5 3.65 3.86</td>
<td>0.14 0.986</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>4.06 4.17 4.26 4.38 4.52 4.71 4.92</td>
<td>0.14 0.976</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>5.07 5.16 5.28 5.42 5.6 5.8 6.03</td>
<td>0.16 0.977</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>6.07 6.15 6.26 6.4 6.57 6.75 6.93</td>
<td>0.15 0.983</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>6.96 7.0 7.05 7.05 7.11 7.16 7.25</td>
<td>0.04 0.950</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>7.94 7.9 7.8 7.69 7.63 7.56 7.53</td>
<td>-0.07 0.979</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>8.94 8.89 8.79 8.66 8.56 8.44 8.34</td>
<td>-0.10 0.992</td>
<td></td>
</tr>
<tr>
<td>50 mM Amm. acetate</td>
<td>9.95 9.92 9.79 9.68 9.59 9.47 9.35</td>
<td>-0.10 0.989</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All \( \text{pH} \) data were obtained from reference 84, and \( \text{pH} \) values were calculated using \( \delta \) values from Table 4-5. The pHs were adjusted with formic acid and ammonium hydroxide.
6 where the solution is buffered by the acetic/acetate pair in the solution and (b) constant and negative for \( \text{pH} > 7 \) where the solution is buffered by the ammonium/ammonia pair.

Also, the organic content is expected to influence the dissociation constant of acidic analytes, resulting in an increase in the acidic analyte \( pK_a \) and this could be described as the *acidic analyte \( pK_a \) shift*, which is discussed in Section 4.6. On the other hand, the organic eluent will affect the dissociation of basic analytes in the opposite direction, resulting in a decrease in the basic analyte \( pK_a \), and is discussed in the Section 4.6 as the *basic analyte \( pK_a \) shift*.

### 4.5.7 Analyte Dissociation Constants

The \( pK_a \) is an important physicochemical parameter. The analyte \( pK_a \) values are especially important in regard to pharmacokinetics (ADME—absorption, distribution, metabolism, excretion) of xenobiotics since the \( pK_a \) affects the apparent drug lipophilicity [59]. Potentiometric titrations and spectrophotometric analysis can be used for \( pK_a \) determination; however, if the compound is not pure, is poorly soluble in water, and/or does not have a significant UV chromophore and is in limited quantity, its determination may prove to be challenging.

Dissociation constants of ionizable components can be determined using various methods such as potentiometric titrations [85] CE, NMR, [86] and UV spectrophotometric methods [87]. Potentiometric methods have been used in aqueous and hydro-organic systems; however, these methods usually require a large quantity of pure compound and solubility could be a problem. Potentiometric methods are not selective because if the ionizable impurities in an impure sample of the analyte have a \( pK_a \) similar to that of the analyte, this could interfere with determining the titration endpoint. If the titration endpoint is confounded, then these may lead to erroneous values for the target analyte \( pK_a \).

Liquid chromatography has also been widely used for the determination of dissociation constants [88–92] since it only requires small quantity of compounds, compounds do not need to be pure, and solubility is not a serious concern. However, the effect of an organic eluent modifier on the analyte ionization needs to also be considered. It has been shown that increase of the organic content in hydro-organic mixture leads to suppression of the basic analyte \( pK_a \) and leads to an increase in the acidic analyte \( pK_a \) compared to their potentiometric \( pK_a \) values determined in pure water [74].

Knowledge of \( pK_a \) for the target analyte and related impurities is particularly useful for commencement of method development of HPLC methods for key raw materials, reaction monitoring, and active pharmaceutical ingredients. This practice leads to faster method development, rugged methods, and an accurate description of the analyte retention as a function of pH at varying organic compositions. Relationship of the analyte retention as function of mobile-phase pH (\( \text{pH} \)) is very useful to determine the \( pK_a \) of the particular
analyte in the hydroorganic mixture and can be extrapolated to predict the $pK_a$ of the analyte. Reversed-phase HPLC in isocratic mode can be used for the $pK_a$ determination of new drug compounds.

### 4.5.8 Determination of Chromatographic $pK_a$

The general procedure for the chromatographic determination of the $pK_a$ is to run at least 5 pH experiments isocratically to construct a pH (on the x-axis) versus retention factor (or retention, on the y-axis) plot. The concentration of organic in the mobile phase should be selected to elute the most hydrophilic species (ionized form) with a $k' > 1$. If the compound is acidic, the elution of the fully ionized species will be obtained at 2 pH units greater than the analyte $pK_a$. If the compound is basic, the elution of the fully ionized species will be obtained at 2 pH units less than the analyte $pK_a$. The organic composition chosen must also be able to elute the neutral species within a reasonable retention time (i.e., <30 min). A short column with narrow internal diameter (i.e., 5.0×3.0 mm, using flow rate of 1.5 mL/min) that is stable from $\approx$pH 2–11 should be used for these studies. The mobile phase could be made from 15 mM potassium phosphate, and the pH can be adjusted with either HCl or NaOH from 2 to 11.

If the target analyte is a basic compound, then the lowest pH mobile phase could be run first, to obtain the retention of the ionized species. At least 25 column volumes (1 column volume = $\pi \times$ radius of column$^2 \times$ length of column $\times$ 0.7) should pass through the column in order to obtain stable retention at each pH used. There is no need to run blank injections. Multiple injections of the analyte should be made; and once a stable retention is obtained at a particular pH, the next pH can be evaluated. This is repeated throughout the whole pH range from low pH to high pH. A representative chromatogram overlay at the various pH values is shown in Figure 4-29 for a basic compound (compound M). The retention factor (or retention) is then plotted versus the $\Delta$pH of the mobile phase. A representative plot of the retention dependencies versus the $\Delta$pH of the mobile phase at 30 v/v% acetonitrile compositions is shown in Figure 4-30. Using nonlinear regression analysis software, the $\Delta$pK$_a$ of the analyte can be determined. For the example given in Figure 4-29 the $\Delta$pK$_a$ of compound M at 30 v/v% acetonitrile was determined to be 3.9 (Figure 4-30). Knowing the $\Delta$pK$_a$ of the analyte and the type and concentration of organic modifier used, the $\Delta$pK$_a$ of the analyte can be calculated. For acetonitrile/water systems the $\Delta$pK$_a$ can be calculated by the following empirical formula for basic and acidic compounds:

$$\Delta pK_a = \Delta pK_a + (x\% \text{ organic}) \times B \quad \text{(basic compounds)} \quad (4-23)$$

$$\Delta pK_a = \Delta pK_a - (x\% \text{ organic}) \times A \quad \text{(acidic compounds)} \quad (4-24)$$

where $B = 0.02$ (corresponds to basic analyte $pK_a$ shift per 10 v/v% MeCN) and $A = 0.03$ (corresponds to acidic analyte $pK_a$ shift per 10 v/v% MeCN).
Figure 4-29. Column: Acquity BEH C18 1.7 μm, 2.1*50 mm, flow rate, 0.8 mL/min, temperature, 35°C, injection 2-μL full loop, run time 3–5 min, detection 215 nm. Strong wash: 0.1% NH₄OH 50/50 MeCN/H₂O. Weak wash: 90/10 H₂O/MeCN. Mobile phase A: 15 mM K₂HPO₄ adjusted with HCl. Mobile phase B: MeCN. Starting pressure: ~9000 psi, isocratic 30 v/v% MeCN.

Figure 4-30. Retention versus pH for compound M at 30 v/v% acetonitrile.
The basic and acidic analyte $pK_a$ shift values will be discussed in Section 4.6. Using equation (4-23), the $\tilde{p}K_a$ at 30 v/v\% acetonitrile was estimated to be 4.5. $\tilde{p}K_a = 3.9 + (30 \text{ v/v\% MeCN}) \times 0.02 = 4.5$. Similar pH studies were conducted with 40 and 50 v/v\% MeCN compositions, and the respective $pK_a$ (experimental) and $\tilde{p}K_a$ (predicted) values are shown in Table 4-10. These results agree well with the potentiometric value of 4.4 for this compound M.

### TABLE 4-10. $pK$ Values for Compound M at Various Organic Compositions

<table>
<thead>
<tr>
<th>$pK_a$</th>
<th>30 v/v%</th>
<th>40 v/v%</th>
<th>50 v/v%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tilde{p}K_a$</td>
<td>3.9</td>
<td>3.65</td>
<td>3.5</td>
</tr>
<tr>
<td>Estimated $\tilde{p}K_a$</td>
<td>4.5</td>
<td>4.45</td>
<td>4.5</td>
</tr>
</tbody>
</table>

4.6 EFFECT OF ORGANIC ELUENT COMPOSITION ON ANALYTE IONIZATION

As discussed in Section 4.5.6, the increase of the organic content in hydro-organic mixture leads to suppression of the basic analyte $pK_a$ and to an increase in the acidic analyte $pK_a$. Accounting for the pH shift of the mobile phase and analyte $pK_a$ shift upon the addition of organic modifier is necessary for the chromatographer to analyze the ionogenic samples at their optimal pH values.

In order to avoid any secondary equilibrium effects on the retention of ionogenic analytes, it is preferable to use the mobile-phase pH either two units greater or less than the analyte $pK_a$ in the particular hydro-organic media that is employed. Therefore, one must account for the pH shift of the mobile phase upon the addition of the organic modifier for a proper description of the ionogenic analyte retention process. However, the effect of organic eluent modifier on the analyte ionization needs to also be considered. It has been shown that increase of the organic content in hydro-organic mixture leads to suppression of the basic analyte $pK_a$ and an increase in the acidic analyte $pK_a$ compared to their potentiometric $pK_a$ values determined in pure water [74, 79]. Accounting for the pH shift of modifier in the mobile phase and analyte $pK_a$ shift upon the addition of organic modifier, this will allow the chromatographer to analyze the ionogenic samples at their optimal pH values.

4.6.1 Effect of Organic Modifier on Basic Analyte $pK_a$ Shift

In order for proper description of the basic analyte retention versus the mobile-phase $\tilde{p}$H, the pH shift of the aqueous portion of the mobile phase must be
taken into account. Figure 4-31 is a plot of the retention factor of aniline plotted versus two different pH scales: \( \tilde{w} \text{pH} \) (Figure 4-31, line A) and \( \tilde{a} \text{pH} \) (Figure 4-31, line B). Moreover, a theoretical curve of the retention dependence versus pH of the mobile phase was constructed for aniline, based on its potentiometric \( \text{p}K_a \) of 4.6 in a purely aqueous system (Figure 4-31, line C). The inflection point of the dependence of \( k' \) versus pH corresponds to the analyte \( \text{p}K_a \) at a particular hydro-organic composition. As can be seen, the plot of retention factor versus \( \tilde{w} \text{pH} \) (Figure 4-31, line A) does not correspond to \( \text{p}K_a \) from the theoretical curve (Figure 4-31, line C). The \( \text{p}K_a \) difference between these two curves is actually the combination of two individual shifts occurring in opposing directions: acidic mobile-phase upward pH shift and the basic analyte downward \( \text{p}K_a \) shift. The difference between the \( \tilde{w} \text{pH} \) and \( \tilde{a} \text{pH} \) curve is due to the pH shift of the aqueous portion of the acidic mobile phase which is caused by a change in the dissociation in the acidic buffer in the particular hydro-organic eluent. After the retention factor is plotted versus \( \tilde{a} \text{pH} \) (Figure 4-31, line B), the \( \text{p}K_a \) determined still does not correspond to the \( \text{p}K_a \) from the theoretical curve (Figure 4-31, line C). The difference between the \( \tilde{a} \text{pH} \) curve and the theoretical curve could be attributed to a change of the basic analyte ionization state at a particular hydro-organic composition upon addition of acetonitrile in the mobile phase, and this is denoted as the basic analyte \( \text{p}K_a \) shift.

Figure 4-32 is a plot of the retention factor of aniline versus the \( \tilde{a} \text{pH} \) of the hydro-organic mixture (pH shift of the aqueous portion of the mobile phase is accounted for) from 10 to 50 v/v% MeCN using the values from Table 4-11. In the graph for all organic compositions a sigmoidal dependence of retention factor versus \( \tilde{a} \text{pH} \) is obtained and the plateau regions are the limiting factors for the fully ionized and neutral forms of the analyte. The inflection point of

![Figure 4-31. Retention versus \( \tilde{w} \text{pH} \) and \( \tilde{a} \text{pH} \) for aniline at 50 v/v% MeCN. (15 mM phosphate buffer adjusted with phosphoric acid.) See color plate.](image-url)
the dependence of \( k \) versus \( \text{pH} \) corresponds to the analyte \( pK_a \) at a particular hydro-organic composition.

In Figure 4-33 the analyte \( pK_a \) and \( \text{pK}_a \) is plotted versus 0–50 v/v% MeCN. It is shown that even after correcting for the pH shift of the mobile phase upon addition of organic at each organic composition, the chromatographic \( \text{pK}_a \) at

### TABLE 4-11. Retention Volume of Aniline as a Function of \( \text{pH} \) (10–50 v/v% Acetonitrile)

<table>
<thead>
<tr>
<th>( \text{pH} )</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.62</td>
<td>1.69</td>
<td>2.002</td>
<td>1.587</td>
<td>1.294</td>
<td>2.62</td>
</tr>
<tr>
<td>3.12</td>
<td>2.19</td>
<td>2.043</td>
<td>1.624</td>
<td>1.393</td>
<td>3.12</td>
</tr>
<tr>
<td>3.62</td>
<td>2.69</td>
<td>2.069</td>
<td>1.987</td>
<td>1.645</td>
<td>3.62</td>
</tr>
<tr>
<td>4.12</td>
<td>3.19</td>
<td>2.549</td>
<td>2.182</td>
<td>1.938</td>
<td>4.12</td>
</tr>
<tr>
<td>7.12</td>
<td>6.19</td>
<td>14.64</td>
<td>7.925</td>
<td>6.58</td>
<td>7.12</td>
</tr>
</tbody>
</table>

Figure 4-32. Retention versus \( \text{pH} \) for aniline from 10 to 50 v/v% MeCN.
all of the organic compositions do not correlate to analytes potentiometric pKₐ value determined in the aqueous solvent (pKₐ, 4.6). A decrease of 0.13 pKₐ units per 10% v/v MeCN for aniline was determined (basic analyte pKₐ shift). Similar negative slopes for other monosubstituted aromatic amines were determined (~0.13–0.23 pKₐ units per 10% MeCN) were obtained. (Table 4-12). Linear relationships for pKₐ values in acetonitrile/water mixtures up to

![Figure 4-33. Effect of organic composition on aniline pKₐ shift.](image)

### TABLE 4-12. pKₐ Values of Basic Compounds as a Function of Acetonitrile Composition

<table>
<thead>
<tr>
<th>pKₐ, v/v% MeCN</th>
<th>pKₐ*</th>
<th>pKₐ</th>
<th>pKₐ</th>
<th>pKₐ</th>
<th>pKₐ</th>
<th>pKₐ</th>
<th>Slope</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline, pKₐ, 4.6</td>
<td>4.6</td>
<td>4.49</td>
<td>4.37</td>
<td>4.17</td>
<td>4.07</td>
<td>3.99</td>
<td>-0.013</td>
<td>0.985</td>
</tr>
<tr>
<td>4-Fluoro aniline</td>
<td>4.65</td>
<td>4.49</td>
<td>4.35</td>
<td>4.13</td>
<td>4.01</td>
<td>3.92</td>
<td>-0.015</td>
<td>0.988</td>
</tr>
<tr>
<td>3-Bromo aniline</td>
<td>3.53</td>
<td>3.35</td>
<td>3.14</td>
<td>2.88</td>
<td>2.70</td>
<td>2.37</td>
<td>-0.023</td>
<td>0.992</td>
</tr>
<tr>
<td>3-Chloro aniline</td>
<td>3.52</td>
<td>3.34</td>
<td>3.18</td>
<td>2.92</td>
<td>2.73</td>
<td>2.43</td>
<td>-0.021</td>
<td>0.991</td>
</tr>
<tr>
<td>2-Fluoro aniline</td>
<td>3.2</td>
<td>3.05</td>
<td>2.84</td>
<td>2.59</td>
<td>2.37</td>
<td>-0.019</td>
<td>0.993</td>
<td></td>
</tr>
<tr>
<td>4-Chloro aniline</td>
<td>3.99</td>
<td>3.88</td>
<td>3.66</td>
<td>3.43</td>
<td>3.29</td>
<td>3.15</td>
<td>-0.018</td>
<td>0.989</td>
</tr>
<tr>
<td>3-Fluoro aniline</td>
<td>3.58</td>
<td>3.43</td>
<td>3.25</td>
<td>3.02</td>
<td>2.86</td>
<td>2.64</td>
<td>-0.019</td>
<td>0.996</td>
</tr>
<tr>
<td>4-Bromo aniline</td>
<td>3.88</td>
<td>3.78</td>
<td>3.55</td>
<td>3.32</td>
<td>3.16</td>
<td>3.01</td>
<td>-0.018</td>
<td>0.989</td>
</tr>
</tbody>
</table>

*potentiometric

---

185 REFECT OF ORGANIC ELUENT COMPOSITION
50 v/v% acetonitrile were obtained ($R^2 > 0.98$) (Table 4-12). The downward change in $pK_a$ as a function of the v/v% MeCN between 0 and 50 v/v% MeCN agreed well with the $\delta pK_a$ values determined by Espinosa et al. [93] for aniline, 0.14 $pK_a$ unit decrease per 10 v/v% acetonitrile, and 4-chloro aniline 0.18 $pK_a$ unit decrease per 10 v/v% acetonitrile.

The analyte $pK_a$ shift upon addition of acetonitrile can be estimated by using the slope of this dependence (0.2 $pK_a$ units decrease per 10% MeCN). This will be denoted as the basic analyte $pK_a$ shift for further discussions in the book. The decrease in the analyte $pK_a$ for basic compounds in acetonitrile/water has been attributed to the breaking of the water structure by addition of organic solvent which consequently changes its ionization equilibria [79, 76, 94]. Therefore, specific solvation effects for certain classes of compounds could lead to different slopes of the change in the $pK_a$ as a function of the type and concentration of organic composition. Roses et al. has published parameters for prediction of the slopes and intercepts of the linear correlations between the $\delta pK_a$ values in acetonitrile/water mixtures and the $\delta pK_a$ values in pure water for aliphatic carboxylic acids, aromatic carboxylic acids, phenols, amines, and pyridines [93]. Similar parameters have been determined for this family of compounds for methanol/water mixtures [80]. Using these parameters for each family of compounds for a particular type of organic, the $a_s$ and $b_s$ terms could be determined and the following empirical equation was determined:

$$\delta pK_a = a_s \cdot \bar{p}K_a + b_s$$  \hspace{1cm} (4-25)

This empirical equation could be used to estimate the analyte $\delta pK_a$ values for different classes of acidic and basic compounds in particular acetonitrile/water or methanol/water compositions.

### 4.6.2 Effect of Organic Modifier on Acidic Analyte $pK_a$ Shift

In order for proper description of the acidic analyte retention versus the mobile-phase pH, the pH shift of the aqueous portion of the mobile phase must be taken into account. Plot of the retention factor of 2,4-dihydroxybenzoic acid versus two different pH scales ($\bar{w}$pH (Figure 4-34, line A) and $\bar{s}$pH (Figure 4-34, line B)) is shown in Figure 4-34. A theoretical curve (Figure 4-34, line C) of the retention dependence versus pH of the mobile phase was constructed for 2,4-dihydroxy benzoic acid, based on its potentiometric $pK_a$ of 3.2 in a purely aqueous system. The inflection point of the dependence of $k'$ versus pH corresponds to the analyte $\delta pK_a$ at a particular hydro-organic composition. The difference between the $\bar{w}$pH (Figure 4-34, line A) and the $\bar{s}$pH curve (Figure 4-34, line B) for the acidic analyte is due to the difference between the pH of aqueous portion of the mobile phase ($\bar{w}$pH) and the actual mobile phase pH ($\bar{s}$pH).
However, the $\Delta pK_a$ obtained after correction for the pH shift of the mobile phase (Figure 4-34, line B) does not correspond to $\Delta pK_a$ from the theoretical curve (Figure 4-34, line C). The overall difference between the theoretical curve and $\Delta pH$ curve is due to the $pK_a$ shift of the acidic analyte. The difference between the $\Delta pH$ curve and the theoretical curve is due to a change of the acidic analyte ionization state at a particular hydro-organic composition upon addition of acetonitrile in the mobile phase. In essence, there is a larger $pK_a$ shift for the 2,4-dihydroxybenzoic acid than for the phosphoric acid (used as a buffer).

Dependencies of 2,4-dihydroxybenzoic acid retention factors versus the $\Delta pH$ of the hydro-organic mixture ($pH$ shift of the aqueous portion of the mobile phase is accounted for) at different organic compositions (from 10 to 35 v/v% MeCN) are shown in Figure 4-35. In this graph a sigmoidal dependence of retention factor versus pH is obtained and the plateau regions are the limiting factors for the fully ionized and neutral forms of the analyte. The inflection point of the dependence of $k'$ versus $\Delta pH$ corresponds to the acidic analyte $pK_a$ at a particular hydro-organic composition.

In Figure 4-36 the acidic analyte $\Delta pK_a$ and $\Delta pK_a$ values determined as a function of acetonitrile composition from 10 to 35 v/v% MeCN are shown. It is shown that even after correcting for the pH shift of the mobile phase upon addition of organic, the chromatographic $\Delta pK_a$ values does not correlate to the $pK_a$ that was determined by titration in aqueous solvents, $\Delta pK_a$. An increase of 0.27 $pK_a$ units per 10% v/v MeCN for 2,4-dihydroxybenzoic acid was determined. A similar slope for other mono- and disubstituted aromatic benzoic
acids was determined (∼0.27–0.42 $pK_a$ units per 10% MeCN, Table 4-13). The average upward slope of 0.3 $pK_a$ units upon 10 v/v% addition of MeCN will be denoted as the acidic analyte $pK_a$ shift further in the book.

Also for weakly acidic analytes such as mono- and disubstituted phenols [74, 76], increases of 0.2–0.3 $pK_a$ units per 10 v/v% acetonitrile were obtained: phenol, 0.33 $pK_a$ units; 3,5-dichlorophenol, 0.21 $pK_a$ units; 3-bromophenol, 0.32 $pK_a$ units; 4-chlorophenol, 0.30 $pK_a$ units per 10% acetonitrile.

Figure 4-35. Retention versus $w\text{pH}$ and $s\text{pH}$ for 2,4-dihydroxybenzoic acid from 10 to 35 v/v% MeCN.

Figure 4-36. Effect of organic composition on 2,4-dihydroxybenzoic acid $pK_a$ shift.
Ideally, increasing the concentration of organic in the mobile phase will lead to a decrease in the retention of components in reversed-phase HPLC, along with to a decrease in resolution, while the selectivity should remain constant. The eluent composition should not affect the selectivity between two species if their ionization state is not changing with an increase in the organic composition. However, since the organic eluent does lead to changes in the mobile-phase pH and analyte $pK_a$'s, changes in selectivity may be observed at certain pH values. This may lead to only small changes or no changes in ionizable analyte’s retention with an increase of organic concentration. In an ideal case, the plot of the logarithm of the retention factor versus the acetonitrile composition should give a linear dependence.

In Figure 4-37 the natural logarithm of the retention factor of aniline at different $pH$ values is plotted versus the acetonitrile(buffer eluent composition. Different slopes of retention dependence are obtained at a certain eluent pH versus eluent composition. Comparison of aniline analyzed at $pH \geq 6$, (analyzed in its predominately neutral form) or at $pH 2$, (analyzed in its fully ionized form) at all acetonitrile compositions shows that the logarithmic retention of the neutral and fully ionized species varies linearly with the acetonitrile concentration (Figure 4-37). At both these pH regions the analyte does not change its ionization state with an increase of the acetonitrile composition. However, at $pH 3$ no significant change in retention is observed from 20 to 50 v/v% MeCN. Due to the upward pH shift of the acidic modifier, the $pH$ of the eluent at 20% and 50%, respectively, is 3.4 and 4.0. On the other hand, for the basic analyte due to downward $pK_a$ shift upon increase of the organic concentration from 20% to 50%, the $pK_a$ decreases from 4.4 to 4.0 (values from

---

**TABLE 4-13. $pK_a$ Values of Acidic Compounds as a Function of Acetonitrile Composition**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$pK_a$</th>
<th>$pK_a$</th>
<th>$pK_a$</th>
<th>$pK_a$</th>
<th>$pK_a$</th>
<th>$pK_a$</th>
<th>Slope</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dihydroxybenzoic</td>
<td>3.29</td>
<td>3.50</td>
<td>3.73</td>
<td>3.90</td>
<td>4.06</td>
<td>4.25</td>
<td>0.027</td>
<td>0.986</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>4.20</td>
<td>4.41</td>
<td>4.70</td>
<td>4.87</td>
<td>5.04</td>
<td>5.23</td>
<td>0.030</td>
<td>0.990</td>
</tr>
<tr>
<td>Salicylic</td>
<td>3.00</td>
<td>3.18</td>
<td>3.39</td>
<td>3.53</td>
<td>3.67</td>
<td>3.84</td>
<td>0.024</td>
<td>0.985</td>
</tr>
<tr>
<td>2,4,5-Trimethoxybenzoic</td>
<td>4.24</td>
<td>4.89</td>
<td>5.22</td>
<td>5.40</td>
<td>5.56</td>
<td>5.77</td>
<td>0.042</td>
<td>0.979</td>
</tr>
<tr>
<td>2,3,4-Trimethoxybenzoic</td>
<td>4.24</td>
<td>4.40</td>
<td>4.75</td>
<td>4.94</td>
<td>5.12</td>
<td>5.30</td>
<td>0.031</td>
<td>0.982</td>
</tr>
<tr>
<td>2,3,4-Trihydroxybenzoic</td>
<td>3.30</td>
<td>3.43</td>
<td>3.78</td>
<td>3.95</td>
<td>4.17</td>
<td>4.28</td>
<td>0.030</td>
<td>0.974</td>
</tr>
<tr>
<td>2,5-Dihydroxybenzoic</td>
<td>3.01</td>
<td>3.22</td>
<td>3.35</td>
<td>3.47</td>
<td>3.66</td>
<td>3.84</td>
<td>0.022</td>
<td>0.960</td>
</tr>
<tr>
<td>3,5-Dihydroxybenzoic</td>
<td>3.96</td>
<td>4.27</td>
<td>4.64</td>
<td>4.70</td>
<td>4.85</td>
<td>5.12</td>
<td>0.032</td>
<td>0.987</td>
</tr>
</tbody>
</table>

* potentiometric $pK_a$
Therefore, with an increase of organic concentration at \( \text{pH} \, 3 \), the analyte is being analyzed more progressively in its neutral state. The \( \text{pK}_a \) of aniline at acetonitrile compositions from 10 to 50 v/v\% is shown in Table 4-14. An increase of the acetonitrile concentration in general leads to an exponential decrease of the analyte retention. However, aniline is becoming less ionized upon increase of organic content in the eluent. Therefore, increasing organic content at a certain aqueous \( \text{pH} \) has a supposition of two opposite effects on the overall analyte retention: (1) an increase in analyte retention due to a decrease in analyte ionization since analyte \( \text{pK}_a \) decreases with increase of organic content, which leads to analysis of analyte in a more neutral state, and (2) a decrease in analyte retention due to decreased analyte interaction with the stationary phase, which decreases hydrophobic interaction. This is clearly observed at \( \text{pH} \, 3 \), where no significant change in retention is observed from 20 to 50 v/v\% MeCN and the two effects are in essence compensating each other.

The separation selectivity can be significantly affected as a result of different \( \text{pH} \) shift of different buffers even at the same organic composition. For example, if two buffers are prepared at the same \( \text{pH} \), one using an acidic buffer such as phosphate and another using a basic buffer such as ammonia, both at \( \text{pH} \, 8 \), the separation of a mixture of ionizable components could be different. This could be attributed to the different mobile-phase \( \text{pH} \) after the aqueous is mixed with the organic. Espinosa et al. [64] analyzed \( N,N \)-dimethyl-
benzylamine ($pK_a = 8.8$), 2-nitrophenol ($pK_a = 7.14$), 2,4,6-trimethylpyridine ($pK_a = 7.33$), and 3-bromophenol ($pK_a = 9.00$) on a Waters XTerra MS C18 column using a pH 8 ammonia buffer and a phosphate buffer both containing 60 v/v% acetonitrile. At this acetonitrile composition the pH of the ammonia buffer is estimated to be about 7.7 [84], and the pH of the phosphate buffer is estimated to be about 9.1. Using the phosphate buffer, the basic compound $N,N$-dimethylbenzylamine (compound 4 in Figure 4-38) was more retained (analyzed predominately in its neutral state) than with the ammonia buffer since in the ammonia buffer the analyte was predominately in a more ionized state. On the other hand, the 2-nitrophenol (compound 1 in Figure 4-38) in the phosphate buffer was less retained (analyzed predominately in its ionized state) while in the ammonia buffer it was more retained, since it was being analyzed in a less ionized state.

**4.8 EXAMPLES OF APPLYING pH SHIFT AND ANALYTE $pK_a$ SHIFT RULES**

When developing separation methods for analytes with known $pK_a$ values, determination of the starting mobile-phase pH is highly advisable. This estimation may help to avoid strange analyte retention behavior during further method optimization and variation of the mobile-phase composition. Below we include several examples where the methodology of the combined pH and $pK_a$ shift evaluation is outlined.
Example 1. Putting it All Together: Analyzing a Base in Its Ionized Form. For example, 2,4-dimethylpyridine (base), your target analyte, has a $pK_a$ of 6.7 and the eluent conditions are 50% MeCN and 50% phosphate buffer. What should the pH of the phosphate buffer be in order to obtain the basic analyte in its fully ionized form?

*Step 1.* First account for the downward $pK_a$ shift for the basic analyte upon addition of organic. For every 10 v/v% increase in acetonitrile, the $\Delta pK_a$ of the analyte decreases by 0.2 $pK_a$ units.

*Step 2.* Once this $\Delta pK_a$ is determined, the $\Delta pH$ at which the analyte would be fully ionized needs to be determined. This corresponds to 2 pH units less than the $pK_a$ of 5.7 as shown in step 2 below.

*Step 3.* Then account for the pH shift of the acidic portion of the mobile phase upon addition of acetonitrile. For every 10 v/v% increase in acetonitrile, the pH of the acidic buffer increases by approximately 0.2 pH units. This would correspond to a 1.0 pH unit increase as shown in step 3 below.

*Step 4.* Then determine what the maximum $pH$ of the aqueous portion of the buffer should be prepared at, taking into account the pH shift of the aqueous portion of the mobile phase upon addition of organic as shown in step 4 below. Therefore the optimal pH to analyze this compound would be at aqueous mobile-phase $pH$ of $<2.7$ using isocratic mode. This pH is also applicable for gradient mode separations given that the analyte of interest will elute at 50 v/v% of acetonitrile or less.

\[
\begin{align*}
1: \quad & 6.7 - (5 \times 0.2) = 5.7 \quad \text{Downward analyte } pK_a \text{ shift} \\
2: \quad & 5.7 - 2 = 3.7 \quad \text{ } pK_a \text{ shift} \\
3: \quad & 5 \times 0.2 = 1.0 \quad \text{Upward } pH \text{ shift of the aqueous acidic buffer} \\
4: \quad & 3.7 - 1.0 = 2.7 \quad \text{Max } pH \text{ of the aqueous portion of the mobile phase in order to have analyte in fully ionized form at 50 v/v% MeCN}
\end{align*}
\]

For aromatic amines with $pK_a$ values in the range less than $pK_a < 5$, this presents a problem. Most reversed-phase columns are not stable at the $pH$ below 1.5. This limits the chromatographer to work at either lower organic composition (low upward pH shift of the mobile phase and concurrent lower downward basic analyte $pK_a$ shift) or analysis of basic compounds in their neutral form.

Example 2. Analyzing a Base in its Neutral Form. In this example we will use the same compound as in the example above [2,4-dimethylpyridine (base) with $pK_a$ of 6.7] along with the same eluent conditions (50% MeCN and 50% phosphate buffer). The goal is to calculate pH of the buffer, in order to obtain the basic analyte in its fully neutral form.
**Step 1.** First, account for the downward $pK_a$ shift for the basic analyte upon addition of organic. For every 10 v/v% increase in acetonitrile, the $\Delta pK_a$ of the analyte decreases by 0.2 $pK_a$ units.

**Step 2.** Once this $\Delta pK_a$ is determined, the $\Delta pH$ at which the analyte would be a fully neutral form needs to be determined. This corresponds to 2 pH units greater than the $\Delta pK_a$ of 5.7, as shown in step 2 below.

**Step 3.** Then account for the pH shift of the acidic portion of the mobile phase upon addition of acetonitrile. For every 10 v/v% increase in acetonitrile, the pH of the acidic buffer increases by approximately 0.2 pH units. This would correspond to a 1.0 pH unit increase, as shown in step 3 below.

**Step 4.** Then determine what the minimum $\Delta pH$ of the aqueous portion of the buffer should be prepared at taking into account the pH shift of the aqueous portion of the mobile phase upon addition of organic as shown in step 4 below. Therefore the optimal pH to analyze this compound would be at an aqueous mobile phase pH of $>6.7$.

\[
1: \quad 6.7 - (5 \times 0.2) = 5.7 \quad \text{Downward analyte } pK_a \text{ shift}
\]
\[
2: \quad 5.7 + 2 = 7.7 \quad \Delta pH \text{ at which basic analyte would be fully neutral}
\]
\[
3: \quad 5 \times 0.2 = 1.0 \quad \text{Upward pH shift of the aqueous acidic buffer upon addition of organic (50 v/v% MeCN)}
\]
\[
4: \quad 7.7 - 1.0 = 6.7 \quad \text{Min. $\Delta pH$ of the aqueous portion of the mobile phase in order to have analyte in fully neutral form at 50% v/v% MeCN}
\]

In this scenario as the acetonitrile concentration is raised, as would occur in gradient mode, there is no risk in analyzing this molecule near the $pK_a$. As the organic content increases above 50 v/v%, there is a greater gap between the $\Delta pK_a$ of the basic analyte and the $\Delta pH$ of the mobile phase (only if an acidic buffer is used).

**Example 3. Analyzing an Acid in Its Neutral Form.** For example, if your target is an acidic analyte with a $pK_a$ of 4.0 and the eluent conditions are 40% MeCN and 60% aqueous, what should be the pH of the phosphate buffer in order to obtain the acid analyte in its fully neutral form?

**Step 1.** First account for the upward $pK_a$ shift for the acidic analyte upon addition of organic. For every 10 v/v% increase in acetonitrile the $\Delta pK_a$ of the analyte increases by 0.3 $pK_a$ units.

**Step 2.** Once this $\Delta pK_a$ is determined, the $\Delta pH$ at which the analyte would be fully neutral form needs to be determined. This corresponds to 2 pH units lower than the $\Delta pK_a$ of 5.2, as shown in step 2 below.

**Step 3.** Then account for the pH shift of the acidic portion of the mobile phase upon addition of acetonitrile. For every 10 v/v% increase in acetonitrile the pH of the acidic buffer increases by approximately 0.2 pH units. This would correspond to a 0.8 pH unit increase, as shown in step 3 below.
Step 4. Then determine what the maximum \( \ast pH \) of the aqueous portion of the buffer should be prepared at taking into account the pH shift of the aqueous portion of the mobile phase upon addition of organic, as shown in step 4 below. Therefore the optimal pH to analyze this compound would be at aqueous mobile phase pH of <2.4.

1: \( 4.0 + (4 \times 0.3) = 5.2 \)  
   Upward analyte p\( K_a \) shift

2: \( 5.2 - 2 = 3.2 \)  
   \( \ast pH \) at which acidic analyte would be fully neutral

3: \( 4 \times 0.2 = 0.8 \)  
   Upward pH shift of the aqueous acidic buffer upon addition of organic (40% MeCN)

4: \( 3.2 - 0.8 = 2.4 \)  
   Max. \( \ast pH \) of the aqueous portion of the mobile phase in order to have analyte in fully neutral form at 40% MeCN

Example 4. Zwitterionic Components. Let us go back to Figure 4-24, for the separation of the Benazepril diastereomers on a phenyl-hexyl column, and see if we could have predicted the appropriate \( \ast pH \) to perform the separation just by taking into consideration the analyte p\( K_a \) values and applying the pH shift and p\( K_a \) shift rules. Generally, for every 10 v/v% of acetonitrile there is an upward p\( K_a \) shift of 0.3 for the acidic analyte p\( K_a \) and a downward shift of 0.2 for the basic analyte p\( K_a \). In 30 v/v% acetonitrile the apparent p\( K_a \) for the acidic portion of Benazepril HCl will shift to about \( 3.7 + 0.9 = 4.6 \) (p\( K_a \) upward shift of 0.9), and for the basic site of Benazepril HCl the apparent p\( K_a \) will shift to about \( 4.6 - 0.6 = 4 \) (p\( K_a \) downward shift of 0.6). Generally, at \( \pm 2 \) pH units from each p\( K_a \), the analyte (at each respective ionization center) remains in one predominate ionization state. Therefore in 30 v/v% MeCN, at a mobile phase \( \ast pH \) below \( \ast pH 2.6 \) the acidic functionality is expected to be neutral, and at a mobile phase \( \ast pH \) greater than 6.6 the acidic functionality is expected to be fully ionized. Similarly, in 30 v/v% MeCN, at \( \ast pH \) below 2.0 the basic functionality is expected to be fully protonated and at a mobile phase \( \ast pH \) greater than 6.0 the basic functionality is expected to be predominately neutral. Therefore, taking both ionizable functionalities into consideration at mobile phase \( \ast pH \) values between 2.0 and 6.6, it is expected to see changes in retention due to the change in the ionization states of both the acidic and basic functionalities. Indeed, in the experiment when Benazepril was analyzed at \( \ast pH \) 2.1–6.1 or respectively \( \ast pH \) 2.7–6.7 (ammonium phosphate buffer), significant changes in retention were observed. There were selectivity differences as a function of \( \ast pH \) from 2.7 to 6.7 with concomitant changes in retention. Separations for ionizable compounds in RPLC are to be performed where the molecules are in one predominate ionization state(s) to avoid significant changes in retention with minor changes in pH. For Benazepril, in order to have both ionizable functionalities in one predominate ionization state, at 30 v/v% MeCN the optimal \( \ast pH \) of the mobile phases to run the separation is at \( \leq \ast pH 2.0 \) or \( \ast pH \geq 6.6 \). By applying the p\( K_a \) shift selection rules we would have been able to predict the optimal pH to perform the separation at. These effects of analyte
retention changing as a function of pH should be independent on the type of column employed.

**4.9 EFFECT OF TEMPERATURE ON ANALYTE IONIZATION**

The use of elevated temperatures for the reversed-phase HPLC separation of mixtures has been used primarily for increasing column efficiency or shortening run time [95, 96] and enhancing separation selectivity [97]. Elevated temperatures also increase solute solubility and diffusivity. Column efficiency is also expected to increase with temperature as diffusion rate increases. However, temperature can also affect the dissociation constants of the ionizable components, and this can lead to anomalous retention behavior of these compounds as a function of temperature (i.e., increases in retention with increase in temperature) [98–102]. The pH of a phosphate buffer and acetate buffer are not significantly affected by change in temperature [103–108]. For acidic analytes, depending on the type of acid and its intrinsic properties, the analyte $pK_a$ may not vary as a function of temperature. Phenolic and carboxylic acids $pK_a$'s do not vary significantly with a change of temperature. However, basic analytes may experience greater changes in their ionization constants with increase of the temperature [109]. The weaker an acid, the greater the change in the analyte $pK_a$ (mainly seen for basic compounds) with a change in temperature. Essentially for basic compounds the analyte is being analyzed in its more neutral form with an increase in the temperature and may experience increases in retention at higher analysis temperatures. For example, McCalley found that the retention of nortriptyline at pH 7 and quinine at pH 7 increased as the temperature was increased from 20°C to 60°C. [110] Also, Buckenmaier et al. [111] (Figure 4-39) found that for the bases amitriptyline, benzylamine, nortriptyline, and quinine a continuous retention increase for these compounds from 30°C to 60°C with increasing temperature could be attributed to temperature-dependent $pK_a$ shifts (on the order of $-0.03 \, pK_a$ units/K). However, the retention of the quaternary amine compounds remained constant and/or experienced slight decrease in their retention.

The $pK_a$ of five weak electrolytes of different chemical nature (butylamine, $N,N$-dimethylaniline, phenol, and benzoic acid) in 50% methanol/water at 20–50°C were determined by Castells et al. [108], and the values are shown in Table 4-15. The effect of temperature was the greatest for the basic compound butylamine, and a lesser effect was observed for the weaker bases pyridine and $N,N$-dimethylaniline and the weakly acidic phenol.

Therefore the temperature of the separation should also be taken into consideration when performing method development, especially for basic compounds. Basic compounds that have $pK_a$ values $>6$ usually experience the greatest changes in retention with increase in temperature. The $pK_a$ values of these basic compounds decrease with an increase in temperature, thereby making them more neutral when analyzed at higher temperatures.
Figure 4-39. Effect of temperature on ionizable analyte retention at pH 7.8 using a ODS3V column in temperature range 30–60°C. (1) Benzylamine (pKₐ 8.96, 30°C). (2) BteN (quaternary amine), (3) berberine chloride, (4) quinine (pKₐ 8.3, 30°C) (5) protriptyline (pKₐ 10.0, 30°C) (6) nortriptyline (pKₐ 9.7, 30°C). Flow rate: 1 mL/min. Mobile phase: pH 7.8, acetonitrile-phosphate buffer (40:60, v/v), with ionic strength (I) maintained at 0.05 M. Buffer concentration adjusted to maintain I constant at different temperatures. (Reprinted from reference 111, with permission.)

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>Phenol pKₐ</th>
<th>Butylamine pKₐ</th>
<th>Pyridine pKₐ</th>
<th>N,N-Dimethylaniline pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>11.05</td>
<td>10.05</td>
<td>4.14</td>
<td>4.36</td>
</tr>
<tr>
<td>298</td>
<td>10.97</td>
<td>9.89</td>
<td>4.08</td>
<td>4.28</td>
</tr>
<tr>
<td>303</td>
<td>10.9</td>
<td>9.68</td>
<td>4.02</td>
<td>4.21</td>
</tr>
<tr>
<td>308</td>
<td>10.82</td>
<td>9.53</td>
<td>3.96</td>
<td>4.11</td>
</tr>
<tr>
<td>313</td>
<td>10.73</td>
<td>9.36</td>
<td>3.91</td>
<td>4.03</td>
</tr>
<tr>
<td>318</td>
<td>10.67</td>
<td>9.23</td>
<td>3.85</td>
<td>3.97</td>
</tr>
<tr>
<td>323</td>
<td>10.63</td>
<td>9.1</td>
<td>3.81</td>
<td>3.89</td>
</tr>
</tbody>
</table>

Values from reference 108
4.10 ION-INTERACTION CHROMATOGRAPHY

4.10.1 Introduction

In reversed-phase HPLC with water/organic eluents, ionic interactions always play an important role in regard to analyte retention, solvation, ionic equilibria, and other processes. To some extent, chromatographic effects and practical use of ionic interactions have been discussed in the previous sections of this chapter. In this section the influence of the ionic additives in the mobile phase on the retention of ionic or ionizable analytes will be discussed.

Ion-interaction chromatography is an intermediate between reversed-phase and ion-exchange chromatography. Introduction of amphiphilic and lipophilic ions into the mobile phase causes their adsorption on the hydrophobic surface of packing material with subsequent transformation into a pseudo ion-exchange surface. Ionic interactions with charged analytes can occur in the mobile phase and with counterions that may be adsorbed on the stationary-phase surface.

Amphiphilic ions are usually molecules with relatively long alkyl chain and have a charged group at one end. These substances are surfactants (reason for the name “soap chromatography”) and possess highly localized charge. In the chromatographic system these molecules are accumulated at the interface between the hydrophobic stationary phase and water/organic eluent. They are oriented at the interface so that the charged part of the molecule remains in the eluent and the hydrophobic part (alkyl chain) is adsorbed on the stationary-phase surface. This forms the charged surface, and excessive surface charge should be compensated by the accumulation of the counterions in the mobile phase at the close proximity of the surface (Figure 4-40) forming the corresponding electrical double layer.

Specific effect of amphiphilic ions on the retention of charged analytes was observed by many researchers more than three decades ago [112–115]. Remarkable number of different names was introduced to these methods. The technique has been called “soap chromatography” [113], “solvent-generated ion-exchange” [114], “ion-interaction” [115], and “ion-pair” [116]. Researchers introduced a similar number of different theories for the description of the effect of ionic mobile-phase additives on the retention of charged analytes; essentially, each specific name for this technique corresponds to its own distinct retention theory. Melander and Horvath [116] divided existing theories into two main groups: stoichiometric [113, 114, 117–119] and nonstoichiometric [120–133].

Phenomenologically, two different mechanisms could be envisioned: (a) the formation of the ion pair between the analyte and amphiphilic counterion with subsequent adsorption of this complex on the stationary phase and (b) adsorption of the amphiphilic counterion itself on the stationary phase surface and subsequent retention of charged analyte in essentially an ion-exchange mode. Melander and Horvath [117] concluded that, in reality, probably both mechanisms coexist in the chromatographic system.
4.10.2 Double Layer Theory

The influence of the formation of the electrostatic potential on the adsorbent surface on the retention of a charged analyte could be introduced in an oversimplified form, assuming an ideal partitioning model on a flat surface, where the retention factor is related to the free Gibbs energy as

\[ k_0 = \varphi \exp \left( \frac{\Delta G^0}{RT} \right) \]  \hspace{1cm} (4-26)

In the presence of the double layer the free Gibbs energy of the analyte interaction with the surface will be

\[ \Delta G_i^0 = \Delta G^0 + zF\Psi_0 \]  \hspace{1cm} (4-27)

where \( z \) is the analyte charge and \( \Psi_0 \) is the electrostatic surface potential created by the adsorbed ion-pairing agent.

Stählberg [134, 135] introduced the application of the Gouy–Chapman double layer theory for the retention of small ionic analytes in ion-exchange and ion-pair chromatography (Figure 4-41). The resulting equation for the retention factor is

\[ k = \frac{A}{V_0} \left( \sigma K_{ch} \exp \left( \frac{-zF\Psi_0}{RT} \right) - \int_0^1 \left( \exp \left( \frac{-zF\Psi(x)}{RT} \right) - 1 \right) dx \right) \]  \hspace{1cm} (4-28)

Figure 4-40. Schematic representation of the electrical double layer in reversed-phase ion-pair chromatography. See color plate.
where $K_{ch}$ is the analyte association constant and $\Psi(x)$ is the double layer profile.

Most of these theories have one significant drawback: They are all derived for the flat open adsorbent surface, but HPLC adsorbents are porous materials with average pore size on the level of 100 Å for bare material. After chemical modification of the original silica surface, the effective pore diameter decreases and the properties of electric double layer in the confined space of small pores are significantly different from that on the flat surface.

Detailed and comprehensive discussion of double-layer-based theories of ion-pair and ion-exchange chromatography is given in good review by Stählberg [136].

**Figure 4-41.** A Schematic illustration of the double layer model for ion-exchange chromatography of small ions according to Stählberg. Reprinted with permission from [136].
4.10.3 Ion Pairs

The double layer theory describes the process of ion-exchange and ion-interaction chromatography from the point view of distributed electrostatic field effect on the charged analyte retention. Other approaches have a more stoichiometric character and describes the analyte retention on the basis of the formation of ionic pairs and their subsequent retention on the adsorbent surface.

In 1926, Bjerrum [137] used Debye–Hückel theory to describe ion association and took into account the interaction of ions within a short range. He introduced an ion-pair concept, gave a definition of ion pairs as neutral species formed by electrostatic attraction between oppositely charged ions in solution, and showed how ion-pair formation was dependent on the ions size (radius of ions), solvent (dielectric constant), and temperature.

Later Bjerrum’s theory was supported by the work of Kraus [138], who showed importance of the dielectric constant, and Atherton [139], who demonstrated the existence of ion pairs using electron spin resonance spectroscopy. The formation of ion pairs may be studied by various methods: conductance studies, UV–visible spectrometry, IR spectrophotometry, partition, distribution, or solvent extraction. The lifetime of ion pairs was determined to be at least $10^{-5}$ sec, which is equivalent to about $10^8$ molecular vibrations, demonstrating that ion pairs can be considered as independent species [140]. Today, the ion-pair formation as independent species is widely accepted.

Ion-pair formation is due only to outer-sphere interactions and no chemical bond of any kind is formed. The work of Sadek and Fuoss [141], and later Roberts and Szwarc [142], showed that an ion pair can exist in two forms: as a tight or intimate ion pair, or as a loose or solvent separated ion pair, depending on the nature of solvent–ion interactions. The behavior of the solvent and its affinity for the ion pair can be explained by the solvation theory proposed by Higuchi et al. [143]. They classified ion pairs based on charge accessibility as shown in Figure 4-42. For the first type of ion pair, the cation is large and liophilic except for the positively charged center and the anion would contain an exposed anionic surface (high negative charge per unit area). The anionic portion of this ion pair can be solvated by liophilic molecules having an exposed partial positively charged surface, (dipolar molecules with acidic protons such as phenol; also alcohols). For the second type of ion pair the anion is large and liophilic except for the negatively charged center, and the cation would contain an exposed cationic surface (high positive charge per unit area). The cationic portion of this ion pair can be easily solvated by liophilic molecules having an exposed partial negatively charged surface (these include solvating species that have nucleophilic sites such as ethers, ketones, and amides). The third type of ion pair represents the ion pair with deeply buried charges (no exposed electrically unbalanced surface). In this third case, no solvation would be needed to extract this ion pair by a nonpo-
lar solvent. Higuchi examined the extraction of dextromethorphan hydrobromide pairs into various organic solvent–cyclohexane mixtures and showed that for a chloroform–cyclohexane mixture, the extraction ability exceeded that of either a 1-pentanol–cyclohexane mixture, where the dielectric constants are higher. This observation is very important to note in regard to chromatographic systems. If coulombic forces participate in the formation of ion pairs, then ion pairs are formed only if the ions approach each other and are separated by a critical separation distance \( D \) given by the Bjerrum equation:

\[
D = \frac{z^+z^-e^2}{2\varepsilon kT}
\]  

(4-29)

In equation (4-29) \( z^+ \) and \( z^- \) are the ionic charges, \( e \) is electron charge, \( \varepsilon \) is dielectric constant, \( k \) is Boltzmann’s constant, and \( T \) is the absolute temperature. When the ion separation distance is less than \( D \), 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 (~80) will be less favorable for ion-pair formation compared to a solvent that has a lower dielectric constant (<40, such as acetonitrile and methanol).

On the other hand, Higuchi showed the importance of solvent mediated effects on the formation of ion pairs that include noncoulombic contributions.

Figure 4-42. Classification of ion pairs according to Higuchi et al. (Reprinted from reference 143, with permission).
such as hydrogen bonding and hydrophobic properties of the ions. Therefore, not only does the dielectric constant of the solvent, play a role but other molecular properties of the solvent play an important role in the formation of ion pairs.

Although ion pairing was initially investigated in the field of physical chemistry, the concept was rapidly adopted in colloid chemistry, analytical chemistry, and the pharmaceutical sciences. Higuchi et al. [144] have reported numerous methods for performing extraction of ionized solutes into organic phases in which ions of opposite charge are added to the aqueous phases, resulting in ion pairing between the solute ion and pairing ion. The resulting complex is neutral and poorly hydrated and can be easily transferred to the organic phases. The ion-pair extraction method is widely used in the pharmaceutical and analytical sciences.

In chromatographic practice, the use of classical ion-pairing reagents is usually recommended as a last resort for the separation of very hydrophilic ions that could not be shifted from the void volume by any other means (i.e., change in mobile-phase pH, type of column, change in organic concentration). The reason for careful use of such a powerful method of specific selectivity and retention adjustment is the consequent irreversible adsorption of classical ion-pairing agents on the surface of the reversed-phase adsorbent. The degree of retention of the ionized solutes is directly proportional to the surface charge density generated by the adsorption of the counterion. The amount of ion-pairing reagent adsorbed on the surface at a particular concentration in the mobile phase is dependent on the hydrophobicity of the alkyl portion of the ionpair reagent. The amount of pairing agent adsorbed by the stationary phase from the eluent has been determined using the breakthrough method described by Knox and Hartwick [119]. Once that amount and surface area of stationary phase are determined, the surface concentration (μmol/m²) allows for the calculation of the absorption isotherms. Figure 4-43 shows the adsorption isotherms of a series of alkylsulfates on the surface of reversed-phase adsorbent and shows the column uptake dependence on type and concentration of the reagents. It is observed that for the reagents with small alkyl chains (octyl) the uptake increases but then levels off, indicating that the column is becoming saturated with the reagent.

The retention of the ionized compound is predominately dictated by the uptake of the reagent and the resulting charge on the surface of the column. Figure 4-44 [119] shows the variation of the retention factor of tyrosinamide as a function of the alkylsulfates in the mobile phase.

It has been shown that similar separations may be obtained by reagents of varying hydrophobicity when the reagent concentration in the mobile phase is adjusted to give the same molar uptake by the column (see Figure 4-45). However, this is only true when the solvent ionic strength is kept constant while the mobile phase concentration of the ion pairing ion is increased.

It is logical to expect that with the increase of the concentration of an ion-pairing agent, the retention of oppositely charged analytes increases while
Figure 4-43. Adsorption isotherms of alkylsulfates on Hypersil-ODS from methanol/water (20/80) with 0.02 M phosphate buffer at pH 6.0. (Reprinted from reference 119, with permission.)

Figure 4-44. Capacity factor of tyrosinamide versus concentrations of dodecyl sulfate (upper curve), decyl sulfate (middle curve), and octyl sulfate (lower curve). (Reprinted from reference 119, with permission.)
similarly charged analytes as the ion pairing reagent will elute faster. This indeed has been observed experimentally (Figure 4-46). Figure 4-45 shows the similar retention dependencies of adrenaline retention for different amphiphilic ions adsorbed on the surface of the reversed-phase material, indicating that at the same surface concentration of any amphiphilic ion adsorbed, the retention of basic analyte is the same; thus the retention is dependent on the surface charge density of adsorbed ions. Comparison of Figures 4-46 and 4-45 indicates that the retention of a charged analyte in ion-pairing mode is dependent on the adsorption of ion-pairing ions on the surface of the stationary phase and not on its concentration in the mobile phase.

Same were also observed by Knox in a salt-controlled methanol-aqueous eluent for the analysis of normetadrenaline as a function of octyl, decyl, and lauryl sulfates [119].

In the contrast to the irreversible adsorption of amphiphilic ions on the reversed-phase surface, the liophilic ions shows relatively weak interactions with the alkyl chains of the bonded phase. Liophilic means oil-loving. These liophilic ions are usually small inorganic ions and they possess an important ability for dispersive type interactions. They are (a) characterized by significant delocalization of the charge, (b) primarily symmetrical, (c) usually spherical in shape, and (d) absence in surfactant properties.

The presence of these ions in aqueous solution was found to disrupt the water structure [146]; in other words, they introduce chaos into structured ionic solution that hence are given the name “chaotropic” ions [147]. The effect of chaotropic ions on the disruption of the solvation shell was mainly studied in
the field of biochemistry, where it was shown that they can impact the conformational and the solvation behavior of proteins and peptides [146, 147]. Inorganic ions were arranged according to their ability to disrupt a water solvation shell in the so-called Hofmeiser series [148]. An increase of chaotropicity [149] has a relatively vague phenomenological description, which is essentially related to the increase in hydrophobicity as a result of charge delocalization and significant polarizability. In the sequence

\[ \text{H}_2\text{PO}_4^- < \text{CF}_3\text{COO}^- < \text{BF}_4^- < \text{ClO}_4^- < \text{PF}_6^- \]

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

**Figure 4-46.** Logarithm of the retention of dopamine and 1-benzenesulfonic acid on reversed-phase column as a function of the mobile-phase concentration of ion-pairing additives (pH 2.1). Column: Hypersil-ODS, \( T = 25^\circ \text{C} \); constant ionic strength was maintained by addition of \( \text{NaH}_2\text{PO}_4 \); open circles, butylsulfate; triangles, cyclohexylsulfamic acid; \( \times \), \( d \)-camphor-10-sulfonic acid; half-closed circles, 1-hexanesulfonate; black circles, octansulfonate. (Reprinted from reference 145, with permission.)
4.10.4 Chaotropic Effect

Study of the effect of liophilic ions on the retention of ionic analytes in reversed-phase HPLC has led to the development of yet another possible theory of their influence on the chromatographic retention of basic compounds [150–152]. Ionic analytes in water/organic mixtures are solvated. The solvation shell suppresses the analyte’s ability for hydrophobic interactions with the stationary phase, thus effectively decreasing the analyte’s retention. Controlled disruption of the solvation shell allows for control of the analyte retention. Presence of the counterions in the close proximity to the ionic solvated analyte leads to the disruption of the analyte solvation shell. This effect is known as chaotropic control for the retention of ionic compounds in reversed-phase chromatography. Counteranions that have a less localized charge, high polarizability, and lower degree of hydration show a significant effect on the retention of protonated basic analytes and are known as chaotropic ions. Chaotropic ions change the structure of water in the direction of greater disorder. Therefore, the solvation shell of the basic analytes may be disrupted due to ion interaction with the chaotropic anions.

With the increase of the counteranion concentration, the solvation of the protonated basic analyte decreases. The primary sheath of water molecules around the basic analytes is disrupted, and this decreases the solvation of the basic analyte. The decrease in the analyte solvation increases the analyte hydrophobicity and leads to increased interaction with the hydrophobic stationary phase and increased retention for the basic analytes.

The chaotropic effect is dependent on the concentration of the free counteranion and not the concentration of the protons in solution at pH < basic analyte $pK_a$. This suggests that change in retention of the protonated basic analyte may be observed with the increase in concentration of the counteranion by the addition of a salt at a constant pH as shown in Figure 4-47 for a pharmaceutical compound containing an aromatic amine with a $pK_a$ of 5.

In the example in Figure 4-47, the retention of pharmaceutical analyte X was first altered by decrease of mobile-phase pH (Figure 4-47A), and in the second case (Figure 4-47B) the pH was maintained constant and the concentration of counteranion was increased via addition of its sodium salt. The resulting effect on the retention of basic analyte is strikingly similar if both dependencies are plotted against the concentration of free counteranions of ClO$_4^-$, as shown in Figure 4-48.

Disruption of the basic analyte solvation shell should be possible with practically any counteranion employed, and the degree of this disruption will be dependent on the “chaotropic nature” of the anion. Chaotropic activity of counteranions has been established according to their ability to destabilize or bring disorder (bring chaos) to the structure of water [148, 149].

Even a very low counteranion concentration in the mobile phase will cause significant initial disruption of the solvation shell, thus leading to the significant increase of the analyte retention, while in the high concentration region.
a type of a saturation effect is observed (Figure 4-49). Logically, at high counteranion concentration when all solvation shells are fully disrupted, any further increase of the counteranion concentration should not cause any additional retention increase.

As was shown above, the chaotropic effect is related to the influence of the counteranion of the acidic modifier on the analyte solvation and is independent on the mobile-phase pH, provided that complete protonation of the basic analyte is achieved. Analyte interaction with a counteranion causes a disruption of the analyte solvation shell, thus affecting its hydrophobicity. Increase of the analyte hydrophobicity results in a corresponding increase of retention. 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 does not produce any noticeable effect on the analyte retention.

**Figure 4-47.** Variation of the retention of basic analyte (pK_a > 5) with mobile-phase pH (A) and counteranion concentration (B). (Reprinted from reference 185, with permission.)
4.10.4.1 Chaotropic Model. If the counteranion concentration is low, some analyte molecules have a disrupted solvation shell, and some do not due to the limited amount of counteranions present at any instant within the mobile phase. If we assume an existence of the equilibrium between solvated and desolvated analyte molecules and counteranions, this mechanism could be described mathematically [151].

**Figure 4-48.** Retention of basic analyte ($pK_a > 5$) as a function of ClO$_4^-$ counteranion concentration with variable pH (circles), fixed pH (triangles), and variable pH with phosphate buffer (squares). (Reprinted from reference 185, with permission.)

**Figure 4-49.** Influence of different counteranions on the retention of 3,4-dimethylpyridine. (Reprinted from reference 185, with permission.)
The assumptions for this model are:

1. Analyte concentration in the system is low enough that analyte–analyte interactions could be considered nonexistent.
2. The chromatographic system is in thermodynamic equilibrium.

The analyte solvation–desolvation equilibrium inside the column could be written in the following form:

\[ B_i^+ + A^- \leftrightarrow B^+ \ldots A^- \]  \hspace{1cm} (4-30)

where \( B_i^+ \) is a solvated basic analyte, \( A^- \) is a counteranion, and \( B^+ \ldots A^- \) is the desolvated ion-associated complex. The total amount of analyte injected is \([B]\), analyte in its solvated form is \([B_i^+]\), and analyte in its desolvated form is denoted as \([B^+ \ldots A^-]\), indicating its interaction with counteranions.

The equilibrium constant of reaction (4-30) is

\[ K = \frac{[B^+ \ldots A^-]}{[B_i^+][A^-]} \]  \hspace{1cm} (4-31)

Total analyte amount is equal to the sum of the solvated and desolvated forms of analyte

\[ [B] = [B_i^+] + [B^+ \ldots A^-] \]  \hspace{1cm} (4-32)

The fraction of solvated analyte could be expressed as

\[ \theta = \frac{[B_i^+]}{[B]} \]  \hspace{1cm} (4-33)

The fraction of the desolvated analyte in the mobile phase could be expressed as

\[ 1 - \theta = \frac{[B^+ \ldots A^-]}{[B]} \]  \hspace{1cm} (4-34)

Substituting expressions (4-33) and (4-34) into expression (4-31), we can write an expression for the equilibrium constant:

\[ K = \frac{1 - \theta}{\theta [A^-]} \]  \hspace{1cm} (4-35)

Solving equation (4-35) for \( \theta \) (solvated fraction), we get
Expression (4-36) shows that the solvated fraction of the analyte is dependent on the counteranion concentration and desolvation equilibrium parameter.

Completely solvated analyte has a low retention factor (even if it is equal to 0), which we denote as $k_s$, while the corresponding retention factor for desolvated form is denoted as $k_{us}$.

Assuming that solvation–desolvation equilibrium is fast, we can express the overall retention factor of injected analyte as a sum of the retention factor of solvated form multiplied by the solvated fraction ($\theta$) and the retention factor of the desolvated form multiplied by the desolvated fraction ($1 - \theta$), or

$$k = k_s \cdot \theta + k_{us} \cdot (1 - \theta)$$  \hspace{1cm} (4-37)

Substituting $\theta$ in equation (4-37) from (4-36), we get

$$k = k_s \left( \frac{1}{K[A^-] + 1} \right) - k_{us} \left( \frac{1}{K[A^-] + 1} \right) + k_{us}$$  \hspace{1cm} (4-38)

and the final form can be rewritten as

$$k = \frac{k_s - k_{us}}{K[A^-] + 1} + k_{us}$$  \hspace{1cm} (4-39)

This equation has three parameters: $k_s$ is a “limiting” retention factor for solvated analyte, $k_{us}$ is a “limiting” retention factor for desolvated analyte, and $K$ is a desolvation parameter [151]. The description of the experimental results with function (4-39) is shown in Figure 4-50. Expression (4-39) in principle allows for the calculation of the solvation equilibrium constant from experimental chromatographic data.

**4.10.4.2 Effect of Different Counteranions.** The chaotropic theory was shown to be applicable in many cases where small inorganic ions were used for the alteration of the retention of basic pharmaceutical compounds [153–157]. Equation (4-39) essentially attributes the upper retention limit for completely desolvated analyte to the hydrophobic properties of the analyte alone. In other words, there may be a significantly different concentration needed when different counterions are employed in the eluent for complete desolvation of the analyte. Therefore, the resulting analyte hydrophobicity and thus retention characteristics of analyte in completely desolvated form should be essentially independent on the type of counteranion employed. Experimental results, on the other hand, show that the use of different counterions
leads to the different retention limits of completely desolvated analyte. Figure 4-51 clearly illustrates this effect. This discrepancy could be explained by the presence of two simultaneous processes: the desolvation and ion association (ion pairing). The effect of the counterion concentration on the analyte retention in both processes (desolvation and ion pairing) have Langmuirian shape [156], and overall retention is a superposition of both effects.
**4.10.4.3 Retention of the Counteranions.** Three distinct processes could be envisioned in the effect of chaotropic ions on the retention of basic analytes:

1. Classic ion pairing involves the formation of essentially neutral ion pairs and their retention according to the reversed-phase mechanism.
2. In the chaotropic model, counteranions disrupt the analyte solvation shell, thus increasing its apparent hydrophobicity and retention.
3. Liophilic counteranions are adsorbed on the surface of the stationary phase, thus introducing an electrostatic component into the general hydrophobic analyte retention mechanism.

In their recent papers, Guiochon and co-workers are essentially advocating for the domination of the first process [158–160]. They are explaining the counteranion effect on the basis of the formation of a neutral ionic complex, followed by its adsorption on the hydrophobic stationary phase. Similarity in adsorption behavior of anionic and cationic species is interpreted as a confirmation of their adsorption in the form of neutral complexes.

The retention of ionic components on reversed-phase columns is essentially regarded as ion-pair chromatography, which has been extensively developed by Horvath [161] and Sokolovski [162, 163] in the form of stochiometric adsorption of ionic species and by Ståhlberg in the form of adsorption of ions and formation of an electrical double layer [164].

The adsorption of amphiphilic ions was experimentally confirmed about 30 years ago, while the actual interaction of the small liophilic ions with hydrophobic stationary phase in reversed-phase conditions was found only recently [165].

Most probably all three mechanisms exist while one of them is dominating, depending upon the eluent type, composition, and adsorbent surface properties.

For acetonitrile/water systems it was found that acetonitrile forms thick adsorbed layer on the surface of hydrophobic bonded phase, while methanol adsorption from water formed a classical monomolecular adsorbed layer [166]. The thick adsorbed layer of acetonitrile provides a suitable media for the adsorption of liophilic ions on the stationary phase adding an electrostatic component to the retention mechanism, while monomolecular adsorption of methanol should not significantly affect adsorption of ions.

The study of the retention of chaotropic anions (BF$_4^-$, perchlorate, and PF$_6^-$) was performed using acetonitrile/water eluents on alkyl- and phenyl-type phases with LC–MS detection (electrospray, negative ion mode) [165]. At all mobile-phase conditions with acetonitrile/water PF$_6^-$ ion exhibits the greatest retention, and this is the most liophilic ion in the Hoffmeister series. This ion has the highest degree of charge delocalization and highest polarizability, which facilitates its possible dispersive (or van der Waals) interactions. These properties allow this ion to interact with acetonitrile. Other anions have similar properties, but their ability for dispersive interactions is lower.
then PF₆⁻. At acetonitrile concentrations up to 20 v/v% acetonitrile, all ions exhibit a maximum retention.

General dependence of the analyte retention on the eluent composition in reversed-phase HPLC shows an exponential decay with the increase of the organic modifier concentration. This is usually described in the following form:

\[
\ln(k) = a + xb
\] (4-40)

where \( k \) is a retention factor, \( x \) is the eluent composition, and \( a \) and \( b \) are constants. This relationship has a thermodynamic background because in the partitioning retention model the retention factor is proportional to the distribution equilibrium constant, which in turn is an exponent of the excessive free Gibbs energy of the analyte in the chromatographic system. Excessive free Gibbs energy is the difference of the analyte potential in the stationary phase and its potential in the eluent. This is only true if retention is a result of a single process on the adsorbed surface (e.g., partitioning, or adsorption). If, on the other hand, the retention mechanism is complex, retention dependencies will not adhere to equation (4-40).

The thick acetonitrile layer adsorbed on the bonded phase surface acts as a pseudo-stationary phase, thus making retention in acetonitrile/water systems a superposition of two processes: partitioning into the acetonitrile layer and adsorption on the surface of the bonded phase. Based on the model described in reference 166, analyte retention could be represented in the following form:

\[
V_R(c_{el}) = V_0 + (K_p(c_{el}) - 1)V_{ads} + SK_H K_p(c_{el})
\] (4-41)

where \( V_R(c_{el}) \) is the retention volume of analyte ions as a function of the eluent composition, \( V_0 \) is the void volume, \( K_p(c_{el}) \) is the equilibrium constant for the distribution of the analyte ions between the eluent and adsorbed layer, \( V_{ads}, S \) is the adsorbent surface area, and \( K_H \) is the adsorption equilibrium constant for analyte ions adsorption from neat acetonitrile on the corresponding stationary phase.

Semiempirical expression was derived for the description of the retention of chaotropic counteranions in reversed-phase conditions [165]. Overall expression for the description of the retention dependencies of analyte ions versus eluent composition will have only four unknowns and allow numerical approximation of experimental retention data (shown as a function of the mole fraction of organic eluent component).

\[
V_R(x) = V_0 - V_{ads}(x) + A \cdot \exp\left(\frac{\Delta G_{MeCN} - x\Delta G_{el.}}{RT}\right) \cdot (V_{ads}(x) + SK_H)
\] (4-42)

Essentially equation (4-42) describes the retention volume of the analyte as a sum of the mobile-phase volume \((V_0 - V_{ads})\), assuming that adsorbed
acetonitrile layer is stagnant) and an energetic term that describes analyte (in this case, chaotropic anion) partitioning into the adsorbed layer and its adsorption on the stationary phase surface. Volume of the adsorbed layer on top of the bonded phase is also a function of the acetonitrile concentration in the mobile phase (Figure 4-52).

Coefficient $\Delta G_{el}$ in equation (4-42) has a meaning of energetic span of partitioning constant in the whole concentration region, and it reflects (a) the excessive interactions of studied ions with water and acetonitrile and (b) structural organization of molecules.

The suggested phenomenological model describes the retention of $\text{PF}_6^-$ ions on different reversed-phase columns very well. Average deviation of calculated values from experimentally measured values is on the level of 1%, which confirms that indeed a superposition of several processes govern the retention of liophilic ions in acetonitrile/water systems. Experimental values along with the theoretical curves are shown in Figure 4-53.

The multilayered character of acetonitrile adsorption creates a pseudo-stationary phase of significant volume on the surface, which acts as a suitable phase for the ion accumulation. In the low organic concentration region (from 0 to 20 v/v% of acetonitrile), studied ions show significant deviation from the ideal retention behavior (decrease in ion retention with increase in acetonitrile composition) due to the formation of the acetonitrile layer, and significant adsorption of the chaotropic anions was observed. This creates an electrostatic potential on the surface in which there is an adsorbed acetonitrile layer, which provides an additional retentive force for the enhancement of the retention of protonated basic analytes. When the dielectric constant is lower than 42 [167], this favors the probability of ion pair formation in this organic enriched layer on top of the bonded phase.

However, at high concentration of organic (>25 v/v%) in the mobile phase the retention of counteranions start to decrease, and this is attributed to the

![Figure 4-52. Acetonitrile excess adsorption isotherm from water on Zorbax Eclipse XDB-C8 adsorbent (left); normalized filling of adsorbed layer (right). (Reprinted from reference 165, with permission.)](image-url)
The normal effect of the increase of the organic composition in the mobile phase on the retention of the analyte, which shows an exponential decay. The schematic of the retention mechanism of basic analytes in the presence of lipophilic ions in acetonitrile/water mobile phase is depicted in Figure 4-54. Acetonitrile forms an adsorbed layer where lipophilic ions are soluble due to their ability for dispersive interactions with π-electrons of acetonitrile. The presence of counterions in that layer create additional electrostatic retentive factor for positively charged analyte. The complex form of the lipophilic 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 4-55 [168]). Note that analyte relative retention increase is only observed in acetonitrile/water systems, where a thick adsorbed organic layer is formed, whereas in methanol/water systems, methanol only forms a monomolecular adsorbed layer that does not provide additional capacity for the retention of lipophilic ions. Also, methanol does not have π-electrons, thereby significantly decreasing its ability for dispersive interactions with lipophilic ions.

Hexafluorophosphate retention dependencies similar to the one shown in Figure 4-56 [169] were observed on different stationary phases, but only when acetonitrile was used as an organic eluent component. If acetonitrile was substituted with methanol, the effect of the increase of PF₆⁻ retention with the increase of organic concentration disappears. This indicates that lipophilic ions show strong dispersive interactions with acetonitrile and have little affinity to the hydrophobic absorbent surface—as opposed to the amphiphilic ions, which

![Figure 4-53.](image-url)
**Figure 4-54.** Schematic of the retention mechanism of basic analyte on reversed-phase material in water/acetonitrile eluent in the presence of liophilic ions (PF$_6^-$). See color plate.

**Figure 4-55.** Relative adjusted retention of aniline (PF$_6$/no-PF$_6$ ratio) on Allure-PFPP (left) and Zorbax-C18 (right) columns from acetonitrile (circles) and from methanol (diamonds). (Reprinted from reference 168, with permission.)
show significant and often irreversible adsorption on the surface of the reversed-phase adsorbents regardless of type of organic modifier.

Overall, liophilic ions (usually small ions capable for dispersive interactions) provide a useful means for selective alteration of the retention of basic analytes. Influence of these ions on the column properties is fully reversible, and equilibration requires minimal time (usually less than an hour, or about 10 to 20 column volumes). On the other hand, the mechanism of their effect is very complex and is dependent on the type of organic modifier used and on the concentration applied. Theoretical description and mathematical modeling of this process is a subject for further studies.

4.10.4.4 Effect of the Counteranion Type and Concentration on Peak Efficiency and Asymmetry. Theoretically, a column can generate a certain maximum number of theoretical plates at the optimum flow rate. This number should be independent of the type of the analyte and mobile phase. In reality, any secondary processes, energetic surface heterogeneity, or restrictions in sorption–desorption kinetics in the column will result in the specific decrease of the efficiency for a particular compound.

Increasing the chaotropic counteranion concentration of perchlorate, hexafluorophosphate, and tetrafluoroborate in the mobile phase for basic compounds studied led to an increase in the apparent efficiency of the system until the maximum plate number for the column is achieved [153]. In Figure 4-57A the efficiency for three basic ophthalmic drug compounds increases relatively fast when the concentration of counteranion BF$_4^-$ was increased from 1 mM..

Figure 4-56. Overlay of the retention volumes of PF$_6^-$ front (0.05 mM concentration of NH$_4$PF$_6$ in the solution) on all four columns measured from acetonitrile/water and methanol/water mixtures. (Reprinted from reference 169, with permission.)
to 10 mM. Then upon further increase of the counteranion concentration, the efficiency of the basic compounds increases slowly until it achieves the maximum column efficiency (phenols, neutral markers). Also with an increase of BF$_4^-$ counteranion concentration, the tailing factor of basic compounds decreases and approaches the tailing factor of the neutral analytes, phenolic compounds (Figure 4-57B).

It has been shown that the PF$_6^-$ counteranion has had the greatest effect on the improvement of the peak asymmetry at low concentrations compared to other chaotropic additives. At the highest concentration of counteranions (PF$_6^-$, ClO$_4^-$, BF$_4^-$), the number of plates for most of the basic compounds studied was similar to that of the neutral markers. In contrast, the neutral...
markers, phenols, showed no significant changes in retention and efficiency with increased counteranion concentration.

One of the origins of peak tailing in chromatography can be attributed to energetic surface heterogeneity with overloading of highly energetic adsorption sites \[170–175\]. Moreover, possible ion-exchange types of interactions with these sites could lead to slow sorption–desorption of solute molecules from the strong sites compared to the weak sites, leading to a further increase in band tailing \[176, 177\]. It also has been shown by McCalley and others that basic analyte sample loading may also have an effect on peak efficiency \[170, 178, 179\]. Thus a decrease in sample load has led to the improvement in the efficiency of basic compounds. However, it is sometimes necessary to inject large sample sizes to enable the detection of small impurities with consequent increase in basic analyte tailing factor and decrease in peak symmetry.

However, chaotropic additives can be added to the mobile phase to suppress secondary interactions with the stationary phase. The adsorption of chaotropic counteranions in the adsorbed organic phase on top of the bonded phase can add an electrostatic component to the retention as well as suppressing some undesired secondary interactions leading to peak tailing of protonated basic compounds. The following trend in increase of basic analyte retention factor and decrease of tailing factor was found: \(\text{PF}_6^->\text{ClO}_4^->\text{BF}_4^->\text{H}_2\text{PO}_4^-[153]\).

Figure 4-58 shows an overlay of chromatograms for labetalol with different analyte loads from 1 to 50 \(\mu\)g using a 10 mM dihydrogen phosphate mobile
phase, at increasing perchlorate anion concentrations. These overlays reveal a typical pattern where the peak tails for different analyte loads coincide, indicating a so-called “thermodynamic overload” that occurs when analyte concentration exceeds the linear region on the adsorption isotherm, and this isotherm curvature inevitably leads to right-angled peaks [180–182].

The greater the chaotropic counteranion concentration, the higher the adsorption capacity and the straighter the analyte isotherm, which results in a shorter tail. Excessive electrostatic interactions are relatively weak in the presence of significant amount of counteranions in the mobile phase, and this would lead to the relatively low initial isotherm slope. Electrostatic interactions are relatively long-distance, which would explain relatively high adsorption capacity and the nonexponential shape of the peak tail. With an increase in counteranion concentration at all analyte loadings, an increase in peak efficiency and decrease in peak tailing can be achieved [153].

Increasing the load of basic analytes in order to increase analyte sensitivity can lead to a decrease in apparent peak efficiency and increase in peak tailing. However, if an analysis must be performed at a relatively high sample load, the addition of a chaotropic additive may be employed to increase the apparent peak efficiency and symmetry. Much higher loading capacities could be obtained by operating columns with these mobile-phase additives without substantial deterioration in efficiency.

4.10.4.5 Applications in the Pharmaceutical Industry. Since a great majority of drugs include basic functional groups, HPLC behavior of basic compounds has attracted significant interest [183]. Therefore, reversed-phase HPLC separation of organic bases of different $pK_a$ values is of particular importance in the pharmaceutical industry. It is generally recommended that the chromatographic analysis of basic compounds to be carried at 2 pH units less than the analyte $pK_a$. However, at these conditions the elution of protonated basic compounds may be close to the void volume. Another option might be to analyze these compounds in their neutral form (mobile-phase pH 2 units above the analyte $pK_a$). Note that going to higher pH values might not be feasible due to the pH stability limit of the packing material, or long analysis times might be obtained for the basic analyte in its neutral form. The advantages of employing chaotropic mobile-phase additives at a pH where the basic analyte is in its fully protonated form provides the chromatographer an additional approach to adjust basic analyte retention and chromatographic selectivity without the need of changing type of column, pH, or organic modifier. The retention behavior of basic compounds containing primary, secondary, tertiary, and quaternary amines can be enhanced as a function of the concentration of chaotropic mobile-phase additives ($\text{ClO}_4^-$, $\text{PF}_6^-$, $\text{BF}_4^-$, $\text{CF}_3\text{CO}_2^-$) at a low pH. However, it has also been observed that different inorganic counteranions at equimolar concentrations lead to a concomitant increase in retention as well as peak symmetry and increased loading capacity. This was first observed when the chaotropic approach was implemented for the analysis of substituted
pyridines and aromatic amines and ophthalmic pharmaceutical compounds [184]. Later Roberts et al. [155] also observed similar effects during the analysis of primary, secondary, and tertiary benzyl amines and antidepressants.

The analysis of Dorzolamide HCl at pH 2 with phosphoric acid shows early elution. The addition of hexafluorophosphate to the mobile phase leads to an enhancement of the retention. Figure 4-59 is an overlay of Dorzolamide HCl chromatograms at four increasing PF$_6^-$ concentrations. As the concentration increased, peak tailing decreased, and peak efficiency and analyte retention increased. Figure 4-60 shows the effect of different counteranions on basic analyte retention and peak efficiency. Depending upon the desired selectivity between a neutral component and a charged basic analyte, a particular chaotropic counteranion could be employed.

Moreover, if a method is to be developed with a chaotropic additive that does not have a buffering capacity, a buffer such as phosphate, maybe employed and the increase in retention can be modulated by the addition of the salt of the chaotropic additive as was shown in Figure 4-48. This approach is particularly useful, especially if other ionogenic species are present in the pharmaceutical mixture. The retention of only the protonated basic compounds can be selectively altered by judicious choice of type and concentration of chaotropic mobile-phase additive without any further mobile-phase pH adjustment. A chaotropic approach could be used for the separation of very polar basic compounds as a fast screening method for the resolution of closely eluting basic species without resorting to changing pH, mobile-phase composition, or type of column (Figure 4-61). These methods are especially useful in reaction monitoring where only a few species are present and reaction conversion needs to be determined.
Figure 4-60. 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. (Reprinted from reference 153, with permission.)

Figure 4-61. Chromatographic conditions: Column: Luna C18(2). Mobile phase: 70% aqueous, 30% acetonitrile. pH adjusted to pH 3 with perchloric acid + $x$ mM ClO$_4^-$ adjusted with NaClO$_4$; flow rate, 1.0 mL/min; temperature, 25°C.
The separation selectivity of a mixture of acidic, basic, and neutral compounds can be altered with the addition of chaotropic mobile-phase additives (Figure 4-62). The retention of the basic compounds can be increased by addition of chaotropic counterions in the mobile phase, while the retention of neutral and acidic compounds is generally unaffected. This is particularly useful during the development of impurity profile methods in the pharmaceutical industry where the retention of a polar protonated basic impurity may be adjusted such that adequate separation selectivity is obtained when unionizable, acidic, or basic (in neutral form) impurities in the drug substance are present. In Figure 4-62 the retention of protonated basic compounds, metoprolol and labetalol, increase while the retention of phenol (in its neutral state) remains constant.

The effect of different chaotropic mobile-phase additives can also assist the chromatographer in achieving adequate retention and resolution of critical pairs in complex mixtures [156].

As shown in Figure 4-63 at an equimolar concentration of chaotropic mobile-phase additives, the greatest increases in retention and resolution between critical pairs of components was achieved employing a 30mM PF₆⁻ mobile-phase additive. An increase in retention and an increase in the peak efficiency were obtained, leading to an increase in the resolution of critical pairs of components (A/B and C/D).

The enhancement of retention of very polar basic species is also very important for pharmaceutical applications. For example, Voglibose (VGB) is very hydrophilic; therefore in RPLC even under high aqueous conditions using a mobile phase with 0.1 v/v% of phosphoric acid, it will elute before the void volume. Moreover, VGB has a weak UV chromophore and a very low absorbance at 190 nm. The dose strength for a particular formulation is very low at 0.2 mg, which presents a problem for dissolution in 900-mL vessels, which necessitates the need to either (a) derivatize VGB to produce a suitable
chromophore, (b) use fluorescent or mass spectrometric detection, or (c) use UV detection at 190 nm with a suitable chaotropic modifier in the mobile phase. Due to the weak chromophore of VGB, the use of trifluoroacetic acid as a chaotropic agent is prohibitive since it will have a high background absorbance and will significantly reduce the method sensitivity. However, hexafluorophosphate, the most chaotropic ion in the series, is UV transparent at 190 nm. In Figure 4-64, 50 mM of hexafluorophosphate was added to a 90% 0.1 v/v% phosphoric acid–10% acetonitrile mobile phase, and significant enhancement of the analyte retention was obtained. The VGB peak was eluted and adequately retained and could be properly quantitated.

4.10.4.6 Multiply Charged Species. Liophilic anions were also shown to have an effect on the retention of multiply charged peptides. Mono-, di-, tri-, and tetralysine were analyzed with and without the addition of chaotropic anion in the mobile phase. Mono-, di-, tri-, and tetralysine have two, three, four, and five positively charged residues, respectively.

Lysine and lysine analogs (Figure 4-65) are very polar molecules and typically show early elution on traditional C18 reversed phases. Also, lysine has a weak chromophore, and UV detection below 215 nm is necessary. Detection of lysine may be challenging when TFA is employed since TFA has a UV cutoff of 210 nm. Therefore experiments were performed with the strongest chaotropic agent, hexafluorophosphate, that does not absorb in this UV region. Mobile phases were prepared by keeping the pH constant at 2.1 using 0.1 v/v% H₃PO₄, and the concentration of PF₆⁻ was increased by the addition of KPF₆.
When dihydrogen phosphate up to 20 mM was used as the mobile-phase additive, no significant change in the retention was observed as the concentration of the dihydrogen phosphate ion was increased (Figure 4-66). The more polar peptide, tetralysine, elutes first whereas the more hydrophobic peptide, lysine, elutes last.

Keeping the pH constant at pH 2.1 with phosphoric acid, the concentration of hexafluorophosphate was increased from 0 to 50 mM. With the increase of

![Figure 4-64. Effect of hexafluorophosphate on the VGB retention. Conditions are shown in Figure 4-63.](image)

![Figure 4-65. Structures of lysine analogs.](image)
the hexafluorophosphate concentration, a significant increase in the retention of the lysine analogs was observed (Figure 4-67). Selectivity changes were also observed upon increasing the hexafluorophosphate concentration in the mobile phase. Using 0–2 mM hexafluorophosphate anion, the peptides elute in the order according to their polarity: When employing hexafluorophosphate concentrations greater than 5 mM, the elution order reverses because of enhanced electrostatic interaction between peptides with greater charge density and PF$_6$ anion. This will lead to decreased analyte solvation and consequently increased retention. The employment of chaotropic additives for very polar amino acids can be used as an effective approach to enhance the retention and selectivity of the separation.

4.10.4.7 Chaotropic Mobile-Phase Additives (Concluding Remarks). The effect of small inorganic ions of liophilic nature (chaotropic agents) on the retention of basic analytes in reversed-phase HPLC is, to some extent, similar to the effect of amphiphilic mobile-phase additives (ion-pairing agents). Chaotropic counteranions essentially introduce reversible secondary equilibria in the chromatographic system without irreversible modification of the surface and significant alteration of the retention of neutral analytes. They facilitate mass transfer by disruption of the solvation of charged analytes and introduce weak electrostatic component in the retention process, which allows for flexible alteration of the separation selectivity and enhancement of apparent efficiency.

The use of chaotropic counteranions for a chromatographic separation is beneficial as a method development strategy. These modifiers may replace the need for changing column type and/or addition of hydrophobic “ion-pairing” reagents for the more challenging separations. Further studies are needed to fully elucidate the detailed mechanism of chaotropic mobile-phase additives.

Figure 4-66. Retention of lysine analogs as a function of KH$_2$PO$_4$ concentration. Mobile phase: 30% acetonitrile; 70% 0–20 mM KH$_2$PO$_4$ adjusted to pH 2.1 with H$_3$PO$_4$. Column: Waters Symmetry C18, 150 × 3.0 mm, 3.5 μm; flow rate, 0.4 mL/min; column temperature, 35°C; detection, 210 nm.
Analyte retention in reversed-phase chromatography is a complex process based on superposition of many different and relatively weak interactions. The exposure of the analyte molecules to the significant surface of packing material during their migration through the column enhances the differences in these interactions and thus allows for unique selectivity between otherwise similar analytes.

The main process determining analyte retention is the hydrophobic interactions with the stationary phase and its competition with organic mobile-phase additive. This simplistic description allows for a rough estimation of the analyte retention and, in principle, is applicable only for very ideal systems, like the separation of alkylbenzenes in methanol/water mixtures.

Analyte ionization, solvation, tautomerization, and other processes altering the analyte appearance in the solution adds significant complexity to the

\[ 0.1\% H_3PO_4 \]

\[ 0.1\% H_3PO_4 + 20 \text{ mM PF}_6^- \]

\[ 0.1\% H_3PO_4 + 50 \text{ mM PF}_6^- \]

**Figure 4-67.** Retention of lysine analogs as a function of KPF\(_6\) concentration. Mobile phase: 30% acetonitrile; 70% 0.1 v/v% H\(_3PO_4\) (pH 2.1) concentration of PF\(_6^-\) ions adjusted with KPF\(_6\) (0–50 mM). Column: Waters Symmetry C18, 150 × 3.0 mm, 3.5μm; flow rate, 0.4 mL/min; column temperature, 35°C; detection, 210 nm.
overall retention process. Adsorption of the eluent components and additives on the surface of packing material also influence the analyte migration through the column.

In this chapter we discussed the influence of most known secondary equilibria effects as well as the utilization of the organic eluent component absorption on the surface to describe the analyte retention in reversed-phase HPLC.

RP HPLC is an area of intensive research. Over 5000 papers are published yearly on the theory, development, and practical applications of reversed-phase chromatography. In this chapter we present our vision of the current state of the RP HPLC. We hope that it will be useful for practical chromatographers in their efforts to develop efficient and selective separation methods, and we also hope that it will be encouraging for researchers studying different aspects of HPLC separations.

REFERENCES

REFERENCES


REFERENCES


91. M. Bartolini, C. Bertucci, R. Gotti, V. Tumiatti, A. Cavalli, M. Recanatini, and V. Andrisano, Determination of the dissociation constants (pKa) of basic acetyl-


REFERENCES


REFERENCES


