Reversed Phase Chromatography

Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.
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1. Aims and Objectives

Aims

- To give an overview of the mechanism of Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and explain the basis of the retention mechanism.
- To highlight typical RP-HPLC applications.
- To explain retention order in RP-HPLC and demonstrate the influence of mobile phase composition on retention.
- To explain how the mobile phase composition and constituents might be manipulated to optimize chromatographic separations in RP-HPLC.
- To illustrate the principles which are used to select appropriate stationary phases and column geometry in RP-HPLC.
- To explain how mobile phase pH affects the retention of ionizable compounds in RP-HPLC.

Objectives

At the end of this Section you should be able to:

- To interactively demonstrate how to use mobile phase pH to optimize separations.
- To give practical advice on how to choose buffers for RP-HPLC and how to effectively use the principles of ion-suppression to optimize separations.

The IUPAC Definition of Chromatography

"Chromatography is a physical method of separation, in which the components to be separated are distributed between two phases, one of which is stationary whilst the other moves in a definite direction".

In HPLC the stationary phase is either a solid, porous, surface active material in small particle form, or a liquid which is coated onto micro-particulate beads of an inert solid support (usually silica).

The mobile phase is a liquid that moves through the packed bed of stationary phase in the column under pressure.¹
2. Mechanism of Reversed Phase HPLC

Reversed phase HPLC is characterized by a situation in which the mobile phase used is MORE POLAR than the stationary phase. The name ‘Reversed Phase’ arises as this was the second, (chronologically), mode of chromatography after Normal (or ‘Straight’) phase in which a polar stationary phase is used in conjunction with a less polar mobile phase.

Typical reversed phase stationary phases are hydrophobic and chemically bonded to the surface of a silica support particle (Figure 1). The most commonly used stationary phases are shown (Figure 2). Other support materials and bonded phases are available.

For more information on bonded phases see the CHROMacademy Column Chemistry module:

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=4&fPath=sco4/hplc_2_1.asp

Figure 1: Schematic representation of reversed phase HPLC.

Figure 2: Common reversed phase HPLC stationary phases.
For neutral analytes, the mobile phase consists of water (the more polar component) and an organic modifier that is used to vary the retention of analytes by lowering the polarity of the mobile phase. The most common organic modifiers are shown.

Increasing the water content will repel (‘squeeze’) hydrophobic (non-polar) analytes out of the mobile phase and onto the non-polar stationary phase where they will reside for a time until ‘partitioning’ out into the mobile phase again. Each ‘on-off’ partition is called a ‘Theoretical Plate’.

When ionizable (or ionic) analytes are present, other additives such as buffers or ion pairing reagents can be added to the mobile phase to control retention and reproducibility.

The Chromatogram shown (Figure 3) illustrates the general elution order of hydrophilic and hydrophobic analytes. When working with ionizable analytes the hydrophobicity and, hence, retention characteristics of the analyte will be affected depending on its ionization state (ionized or non-ionized), this will be discussed later in the module.

Figure 3: Representative reversed phase chromatogram detailing analyte retention order based on hydrophobicity or Hydrophilicity.
3. Applications of Reversed Phase HPLC

Reversed-phase HPLC is very widely applicable for small molecule analyses (M.Wt. < 2000 Da). A C$_{18}$ or Octadecylsilane (ODS) column is usually the first choice for method development, although this may differ in certain industries where analytes are chemically very similar (little difference in hydrophobicity).

Reversed phase HPLC successfully separates both polar and nonpolar neutral molecules with molecular weights below 2000 Daltons. Even homologs and benzologs can be baseline separated. Homologs may differ from their counterparts by only one carbon unit (methylene, CH$_2$).

Reversed-phase HPLC can be extended to the separation of weak acids and bases with the addition of mobile phase modifiers and buffers to control ionization of the sample molecules. Stronger acids and bases can be separated using an analog of reversed-phase HPLC - Ion Pair HPLC. Here, an additive is used to bind with the sample ion producing a neutral compound complex for separation. Alternatively stronger acids and bases have been analyzed alongside hydrophobic analytes using mixed-mode chromatography in recent times.

Reversed-phase HPLC is a good choice for peptide and protein separations when short chain alkyl stationary phases are used. Here, analysis of compounds with a molecular weight above 2000 Daltons is possible.

The separation of amines requires more attention, but can be easily accomplished by the use of additives, pH control, or the use of specially treated columns.

Reversed phase HPLC is the first choice for:

- Neutral and non-polar compounds with a molecular weight less than 2000 Da
- Homologs and Benzologs
- Weak acids and bases
- Strong acids and bases (ion pair HPLC)
- Proteins and peptides
More difficult analyses:

- Amines
- Water insoluble compounds

Samples that cannot be easily separated by reversed phase HPLC and the alternative methods that can be used:

- Very hydrophilic compounds – may not have sufficient retention in RP. Use normal phase or Hydrophilic Interaction Liquid Chromatography (HILIC).
- Very hydrophobic compounds – strongly retained under reversed phase conditions and may require the use of non-aqueous conditions. Use non-aqueous reversed phase chromatography (NARP).
- **Achiral** isomers (**stereoisomers, diastereomers, positional isomers** etc.) – there are some cases where these have been separated by reversed phase, however, often reversed phase conditions will require the use of a **cyclodextrin** bonded phase. Normal phase chromatography will work well.
- **Chiral** isomers (**enantiomers**) – reversed or normal phase HPLC.
- Inorganic ions – use mixed mode or ion exchange chromatography.

For more information on:

**HILIC**

[http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=105&fPath=sco105/hilic_1_1_1.asp](http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=105&fPath=sco105/hilic_1_1_1.asp)

**Normal Phase Chromatography**


**Ion Chromatography**

[http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=111&fPath=sco111/ic_1_1_1.asp](http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=111&fPath=sco111/ic_1_1_1.asp)
4. Analyte Retention in Reversed Phase HPLC

The hydrophobicity of an analyte molecule will be the primary indicator as to the retentivity in reversed phase HPLC. Hydrophobicity is often expressed as Log P which is a measure of the way an analyte (in its neutral form) partitions between two immiscible solvents (usually octanol and water) under standard conditions (Equation 1). The higher the value of Log P (between −1 and +1) the more hydrophobic the molecule.

\[
\log P_{\text{oct/wat}} = \log \left( \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{un-ionised}}^{\text{water}}} \right) \quad (1)
\]

Polar analytes interacting with silica surface silanol groups undergo an adsorption type interaction as well as their partitioning behavior – this can lead to detrimental peak shape effects along with an increase in retention time.

The structure of the sample molecules will also give clues as to their elution order. The elution order is governed by the water solubility of the molecule and the carbon content for an analogous series. Some observations governing sample elution order include (again, these assume compounds are of analogous series):

- The less water soluble a sample is, the more retention
- Retention time increases as the number of carbon atoms increase
- Branched-chain compounds elute more rapidly than normal isomers
- Unsaturation decreases retention
- Neutral polar and charged species typically show the least retention followed by acid, then basic compounds all eluting early

The general order of elution is:

Aliphatics > induced dipoles (e.g. CCl₄) > permanent dipoles (e.g. CHCl₃) > weak Lewis bases (esters, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids)

Ionic compounds generally elute with the void volume of the column.
Retention order:

1. Straight chain hydrocarbon – most hydrophobic (C11)
2. Straight chain hydrocarbon – less hydrophobic (C10)
3. Branched n-hydrocarbon (C10)
4. Unsaturated C10 - (more polar due to π electron dipole in the double bond)
5-7. Analytes with functional groups. Ionized analytes will elute fastest of all
5. Retention Order in Reversed Phase HPLC

Interactive exercise on retention order in reversed phase HPLC.

![Chemical Structures]
6. Reversed Phase Mobile Phase Solvents

The mobile phase in reversed phase HPLC usually consists of water/aqueous solution (commonly an aqueous buffer) and an organic modifier. When ionizable compounds are analyzed, buffers and other additives may be present in the aqueous phase to control retention and peak shape.

Chromatographically, in reversed phase HPLC water is the ‘weakest’ solvent. As water is most polar, it repels the hydrophobic analytes into the stationary phase more than any other solvent, and hence retention times are long – this makes it chromatographically ‘weak’. The organic modifier is added (usually only one modifier type at a time for modern chromatography), and as these are less polar, the (hydrophobic) analyte is no longer as strongly repelled into the stationary phase, will spend less time in the stationary phase, and therefore elute earlier. This makes the modifier chromatographically ‘strong’ as it speeds up elution/reduces retention.

As progressively more organic modifier is added to the mobile phase, the analyte retention time will continue to decrease.

The common organic modifiers are detailed below (Figure 1). The Snyder polarity index value is shown which gives a measure of the polarity of the solvents. The $\varepsilon^0$ values are also shown which give a measure of relative elution strength (the values quoted are for elution on C$_{18}$).²

![Figure 5: Typical solvents used in reversed phase HPLC.](image-url)

Solvent selection may be one of the most important parameters in an HPLC separation due to the effect it can have on the selectivity. In fact selectivity may be the most effective tool for optimizing resolution (Figure 6).
The Impact of Selectivity on Resolution

\[
R = \frac{\sqrt{N}}{4} \left( \frac{k}{k + 1} \right) \left( \frac{\alpha - 1}{\alpha} \right)
\]

Figure 6: Impact of selectivity, efficiency, and retention on resolution.

For more information on selectivity, efficiency, and retention see the CHROMacademy Chromatographic parameters module:

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=2&fPath=sco2/hplc_1_2.asp

Changing the organic modifier within a mobile phase can alter the selectivity of the separation as well as the retention characteristics. How would one choose the most appropriate solvent and what are the major considerations (Figure 7)?

Properties of the ideal solvent
- Water-miscible
- Low viscosity
- Low UV detection
- Good solubility properties
- Chemically unreactive

Figure 7: Considerations for solvent choice in reversed phase HPLC.

First of all, the chosen organic solvent must be miscible with water. All of the solvents listed on the previous page are miscible with water.
Solvent miscibility charts can be used to determine if two solvents are miscible (Figure 8).

Figure 8: Solvent miscibility chart.

Download a PDF of the solvent miscibility chart here:

http://www.chromacademy.com/lms/sco5/06-Reversed-Phase-Mobile-Phase-Solvents.html?fChannel=0&fCourse=0&fSco=5&fPath=sco5/06-Reversed-Phase-Mobile-Phase-Solvents.html
Second, a low viscosity mobile phase is favored to reduce dispersion and keep system backpressure low. The use of a low viscosity solvent is preferable (Table 1) due to the lower pressure drop produced at a specific flow rate. It also allows for faster chromatography due to the increased rate of mass transfer.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>UV cut off (nm)</th>
<th>Viscosity (mPa s)</th>
<th>B.P. (°C)</th>
<th>Polarity (P')</th>
<th>Refractive Index&lt;sup&gt;20&lt;/sup&gt;</th>
<th>Dipole&lt;sup&gt;2,5&lt;/sup&gt;</th>
<th>Acidity&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Basicity&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>330</td>
<td>0.306</td>
<td>56.2</td>
<td>5.1</td>
<td>1.359</td>
<td>0.56</td>
<td>0.06</td>
<td>0.38</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>190</td>
<td>0.369</td>
<td>81.6</td>
<td>5.8</td>
<td>1.344</td>
<td>0.60</td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>Chloroform</td>
<td>245</td>
<td>0.542</td>
<td>61.2</td>
<td>4.1</td>
<td>1.443</td>
<td>0.57</td>
<td>0.43</td>
<td>0.00</td>
</tr>
<tr>
<td>DMSO</td>
<td>265</td>
<td>1.987</td>
<td>189</td>
<td>7.2</td>
<td>1.478</td>
<td>0.57</td>
<td>0.00</td>
<td>0.43</td>
</tr>
<tr>
<td>Dioxane</td>
<td>215</td>
<td>1.177</td>
<td>101.2</td>
<td>4.8</td>
<td>1.420</td>
<td>0.60</td>
<td>0.00</td>
<td>0.40</td>
</tr>
<tr>
<td>Ethanol</td>
<td>210</td>
<td>1.074</td>
<td>78.3</td>
<td>5.2</td>
<td>1.361</td>
<td>0.25</td>
<td>0.39</td>
<td>0.36</td>
</tr>
<tr>
<td>Hexane</td>
<td>210</td>
<td>0.300</td>
<td>68.7</td>
<td>0.1</td>
<td>1.357</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IPA</td>
<td>210</td>
<td>2.04</td>
<td>82.4</td>
<td>3.9</td>
<td>1.377</td>
<td>0.24</td>
<td>0.36</td>
<td>0.40</td>
</tr>
<tr>
<td>Methanol</td>
<td>210</td>
<td>0.544</td>
<td>64.7</td>
<td>5.1</td>
<td>1.328</td>
<td>0.28</td>
<td>0.43</td>
<td>0.29</td>
</tr>
<tr>
<td>THF</td>
<td>220</td>
<td>0.456</td>
<td>66.0</td>
<td>4.0</td>
<td>1.407</td>
<td>0.51</td>
<td>0.00</td>
<td>0.49</td>
</tr>
<tr>
<td>Water</td>
<td>191</td>
<td>0.89</td>
<td>100</td>
<td>10.2</td>
<td>1.333</td>
<td>0.45</td>
<td>0.43</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table 1: Properties of common organic solvents. Refractive index at 20 °C. Dipole, acidity, and basicity values are normalized. Some of these values are plotted in the solvent selectivity triangle (Figure 6).<sup>2-4</sup>

It should be noted that the viscosity of mobile phase mixtures, and also therefore instrument pressure, will be markedly higher than the pure solvents (Figure 9 and Table 2). Figure 9 illustrates the situation with different percentages of methanol, acetonitrile, and tetrahydrofuran in aqueous solutions. The viscosity maximum for MeOH/Aq. mixtures is reached at 40% MeOH (1.62 mPa s at 25 °C) which is almost three times the value for MeOH alone. Acetonitrile is often the solvent of choice due to its ability to solubilize many small molecules and its low viscosity. As can be seen from Figure 9, the viscosity of MeCN/Aq. mixtures decreases with increasing amounts of MeCN, with the maximum value being ~0.98 mPa s (20 %B at 25 °C) which is almost half the maximum of MeOH/Aq. It is not uncommon for an instrument to shut down due to overpressuring when employing methanol as the organic solvent during the first injection of gradient method.
Figure 9: Viscosity of reversed phase HPLC solvent mixtures.²

<table>
<thead>
<tr>
<th>Mobile Phase (%)v organic/water</th>
<th>Viscosity (mPa s)</th>
<th>MeOH</th>
<th>THF</th>
<th>MeCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.89</td>
<td>0.89</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.18</td>
<td>1.06</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.40</td>
<td>1.22</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.56</td>
<td>1.34</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>1.62</td>
<td>1.38</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.62</td>
<td>1.43</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1.54</td>
<td>1.21</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>1.36</td>
<td>1.04</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>1.12</td>
<td>0.85</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.84</td>
<td>0.75</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.56</td>
<td>0.46</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Mobile phase viscosity at 25 °C for reversed phase systems.²

The solvent must be stable for long periods of time. This disfavors tetrahydrofuran which, after exposure to air, degrades rapidly, often forming explosive peroxides. Tetrahydrofuran is an interesting solvent in that it is one of the strongest chromatographically and can produce separations in very short times, whilst still being fully miscible with water. However, it does have a relatively high UV cut off (215 nm). Column equilibration can also be slower with THF than with MeOH or MeCN. In the presence of air or oxidizers THF will also form hazardous, explosive peroxide species, which pose both a safety risk and can be reactive towards analytes. Recently THF has also been recently upgraded to carcinogen status by some bodies.

Of course safety is of primary importance and material safety data sheets (MSDS) can be found by using this link

The two remaining mobile phase solvents are methanol and acetonitrile. The use of both solvents usually gives excellent retention characteristics.

When mixing MeOH and aqueous mixtures, each solvent should be weighed or volumetrically measured due to the solvent contraction that occurs upon mixing i.e. 500 mL of water topped up to 1000 mL with MeOH will result in a solution with a MeOH content in excess of 50% by volume.

Care should also be taken if reactive analytes, such as alcohols, aldehydes, or carboxylic acids are being analyzed, as in the presence of MeOH methyl esters can be formed giving rise to erroneous peaks in the chromatogram and quantification errors.

If a UV detector is being used it is important to consider the UV cut off of the mobile phase (organic modifier, buffers, additives etc. Table 1) to ensure that there is no interference with the λ<sub>max</sub> of the analyte.

Once prepared, HPLC mobile phases will have a given shelf life (Table 3). The values shown in Table 3 are a conservative estimate of the usable time period for given mobile phases, therefore, it is important to monitor mobile phase stability periodically and local policies or procedures should take precedence.

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Shelf life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water from water purification system</td>
<td>3 days</td>
</tr>
<tr>
<td>Aqueous solutions (without buffer)</td>
<td>3 days</td>
</tr>
<tr>
<td>Buffer solutions</td>
<td>3 days</td>
</tr>
<tr>
<td>Aqueous solutions with &lt; 15% organic solvent</td>
<td>1 month</td>
</tr>
<tr>
<td>Aqueous solution with &gt; 15% organic solvent</td>
<td>3 months</td>
</tr>
<tr>
<td>Organic solvents</td>
<td>3 months</td>
</tr>
</tbody>
</table>

Table 3: Shelf life of eluent compositions.

Each solvent will interact differently with differing analytes and can be classified by their solvochromatic parameters (Table 1). Dipole character π*, is a measure of the ability of the solvent to interact with a solute via dipolar and polarization forces and will be good for the retention of polarizable analytes. Acidity α, is a measure of the ability of the solvent to act as a hydrogen bond donor towards basic (acceptor) solutes so will be good for the retention of bases. Basicity β, is a measure of the ability of the solvent to act as a hydrogen bond acceptor towards an acidic (donor solute), therefore, it will retain acidic analytes well. These characteristics, along with knowledge of the analyte chemistry, can be used to manipulate analyte elution.

Using solvent solvochromatic properties (α, β, π*) a solvent selectivity triangle can be constructed (Figure 10). This can be useful in optimizing chromatographic selectivity by choosing solvents that have large differences in their properties which will result in large changes in selectivity. When choosing alternative solvents they should come from different areas of the solvent selectivity triangle. For example, if a method uses methanol but this does not produce the required selectivity then using another alcohol will not remedy this, however, using tetrahydrofuran or acetonitrile will result in a change in selectivity. It can be seen from the solvent selectivity triangle that similar solvents will have similar properties and are grouped together; alcohols, esters, benzene derivatives etc.
6.1. Solvent Properties

Listed below are definitions of the properties that will often be reported for solvents. Knowledge of what these properties describe can be useful when selecting solvents for HPLC applications. The values for many of these solvent properties for individual solvents are reported in the tables throughout this module.

**Viscosity** – $\eta$ is given in mPa s at 20 °C. Solvents with a higher viscosity than water ($\eta = 1.00$) are less suitable for HPLC due to the high pressure drop that will be produced.

**UV Cutoff** – The wavelength at which the absorbance of the pure solvent is 1.0, measured in a 1 cm cell with air as the reference (10% transmittance).

**Boiling point** – Solvents with low boiling points are less suitable for HPLC. Produce a danger of vapor bubbles in the HPLC system. Solvent loss can occur during degassing.

**Purity** - HPLC grade solvents and reagents should always be used. Water should be a free solvent; however, high purity water is required for all sample and mobile phase preparation protocols in HPLC. Poor quality solvents, reagents, and water can produce a multitude of chromatographic errors including; altered resolution, ghost peaks, changes in stationary phase chemistry and baseline issues. Possible sources of organic contaminants are from feed water (i.e. tap water), leached from purification media (filters), tubing and containers, bacterial contamination, and potentially, absorption from the atmosphere. For example, alkaline mobile phases are known to absorb polar organics such as formaldehyde, amines, and atmospheric carbon dioxide. Organic contaminants with UV active chromophores can interfere with quantification if they are present in high enough levels. Reactive contaminants may also produce unwanted side reactions with analyte molecules.
Strength - $\varepsilon^o$ defines the elution strength if a solvent when it is used as a mobile phase on a particular stationary phase. It is representative of the adsorption energy of an eluent molecule per unit area of the adsorbent.

Refractive Index – $n_D^{20}$ is given at 20 °C. This will affect the sensitivity of a Refractive Index detector for a particular sample. During gradient elution the composition of the eluent will change and, hence, so will its refractive index. To compensate for the change in refractive index properties a reference wavelength should always be set, where possible, otherwise drifting baselines will occur for UV detectors.

Dipole character - $\pi^*$ is a measure of the ability of the solvent to interact with a solute via dipolar and polarization forces and will be good for the retention of polarizable analytes.

Acidity - $\alpha$ is a measure of the ability of the solvent to act as a hydrogen bond donor towards basic (acceptor) solutes so will be good for the retention of bases.

Basicity - $\beta$ is a measure of the ability of the solvent to act as a hydrogen bond acceptor towards an acidic (donor solute), therefore, it will retain acidic analytes well.

6.2. LC-MS Applications

LC-MS applications require special consideration to optimize the mobile phase and achieve sensitive MS detection of analytes.

Electrospray Ionization (ESI)

The solvent should support ions in solution, i.e. a solvent with some dipole moment. Solvents that are more viscous are less volatile and will reduce sensitivity. A higher percentage of organic modifier gives better sensitivity due to the decreased surface tension and lower solvation energies for polar analytes. Reversed phase solvents are suitable as they are often polar, whereas, normal phase solvents are not.

Atmospheric Pressure Chemical Ionization (APCI)

Most solvents are compatible. It is preferable to have neutral molecules.

Other components of the mobile phase should also be considered. For example non-volatile additives will crystallize and block the ion source so these are best avoided e.g. surfactants such as triton and sodium dodecyl sulphate. Compounds that reduce ionization/ion formation (DMSO, TRIS, glycerol) are also not recommended for use with LC-MS applications. Corrosive reagents such as inorganic acids ($H_2SO_4$, $H_3PO_4$) or alkali metal bases (NaOH, KOH) will damage equipment. Strong ion pair reagents will cause ion suppression (TFA). However, additives such as triethyl- and trimethylamine may enhance ion formation in negative mode.

For more information on selecting HPLC mobile phases see:

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=1&fSco=28&fPath=sco28/hplc_3_1_1.asp

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For more information on mobile phase selection for LC-MS applications see:

7. Mobile Phase Strength and Retention

Retention can be altered by changing the mobile phase strength. Retention values in the range $2 < k < 10$ are desirable. This operating range for $k$ holds well for conventional HPLC ($150 \times 4.6$ mm, 5 µm and 400 bar), however, for high efficiency HPLC, typically with core-shell and sub-2µm columns and UHPLC we can operate over the range $0.5 < k < 5$.

Selectivity ($\alpha$) can be changed by changing mobile phase composition (swap MeOH for MeCN), column stationary phase, and temperature.

Increasing the percentage organic modifier in the mobile phase has a profound effect on analyte retention due to the change in polarity of the mobile phase. An increase in the organic content of the mobile phase of 10% will decrease $k$ for each analyte by a factor of 2 to 3.

The interactive experiment below can be used to investigate the influence of the organic modifier on and HPLC experiment. It is possible to start at a high % organic modifier (to save time as all components will elute very quickly), and then reduce the amount of modifier to obtain the optimum chromatogram. It should be noted that the target is to obtain a suitable resolution of all peaks ($R_s > 1.7$ for all peaks in this instance) in the minimum analysis time.

Make sure ALL modifier concentrations have been investigated before responding to the question below.

Figure 11: Relationship between retention and % organic modifier in reversed phase HPLC.

Solvent strength is the most powerful tool available to alter the resolution between analytes.
Key Learning Points

- Decreasing mobile phase ‘strength’ causes the analyte molecules to be increasingly repelled into the stationary phase.
- As an increasing proportion of analyte molecules are repelled into the stationary phase the analyte band is more strongly retained on the column.
- Mobile phase strength is a key parameter used to adjust retention in reversed phase HPLC.
- Changes in mobile phase strength do NOT generally have a major effect on the selectivity of the separation.
8. Changing the Organic Modifier

If the desired resolution cannot be achieved with the first organic modifier chosen it is possible to change to an alternative solvent. It is important to note that both retention and selectivity will change if the organic:aqueous ratio is altered (Figure 12).

From the separation of neutral chromatographic test compounds shown, it is clear that methanol is the ‘weakest’ solvent chromatographically and that THF is the ‘strongest’. It should also be noted that there are marked differences in the selectivity of each chromatogram.

The selectivity of the separation changes due to the differences in acidity, basicity and polarity of each of the common organic modifiers. Each of the solvents interacts in subtly different ways with the analyte molecules as each analyte has a different chemistry (dipole, acidic, basic, neutral etc.).

A solvent selectivity triangle is shown (Figure 13) on which the properties of common organic solvents are plotted. Comparison of methanol, acetonitrile, and THF shows that THF has the most basic character (proton acceptor) and that acetonitrile has the largest molecular dipole. Proton donor characteristics represent the acidity of the solvent.

![Graphs showing relative solvent strength of some typical reversed phase HPLC solvents.](image-url)
Figure 13: Solvent selectivity triangle.
9. Eluotropic Series

Solvent strength in reversed phase HPLC depends upon the solvent type and amount used in the mobile phase (percentage organic modifier (%B)).

It is possible to interrelate the relative strength of each of the common solvents using an Eluotropic Series. The relative strengths of some common organic solvents are shown in Table 4 on various media. Aluminium oxide or Silica are used for normal phase HPLC and C₁₈ is used for reversed phase HPLC.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>ε° (Al₂O₃)</th>
<th>ε° (C₁₈)</th>
<th>ε° (SiO₂)</th>
<th>Polarity (P')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.56</td>
<td>8.8</td>
<td>0.53</td>
<td>5.1</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.65</td>
<td>3.1</td>
<td>0.52</td>
<td>5.8</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.4</td>
<td>-</td>
<td>0.26</td>
<td>4.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.62</td>
<td>-</td>
<td>-</td>
<td>7.2</td>
</tr>
<tr>
<td>Dioxane</td>
<td>0.56</td>
<td>11.7</td>
<td>0.51</td>
<td>4.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.88</td>
<td>-</td>
<td>-</td>
<td>5.2</td>
</tr>
<tr>
<td>Hexane</td>
<td>0.01</td>
<td>-</td>
<td>0.00</td>
<td>0.1</td>
</tr>
<tr>
<td>IPA</td>
<td>0.82</td>
<td>8.3</td>
<td>0.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.95</td>
<td>1.0</td>
<td>0.7</td>
<td>5.1</td>
</tr>
<tr>
<td>THF</td>
<td>0.45</td>
<td>3.7</td>
<td>0.53</td>
<td>4.0</td>
</tr>
<tr>
<td>Water</td>
<td>Large</td>
<td>-</td>
<td>-</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Table 4: Polarity and elution strength of common HPLC solvents. ε° is a measure of the relative elution strength. P' is the Snyder polarity index value.

Commonly, a nomogram might be used to find equivalent eluotropic strengths between mobile phases that use different organic modifiers (Figure 14). The nomogram relates each solvent using a vertical line to indicate the mobile phase composition (%B), which gives equivalent elution strength - known as an isoeluotropic mobile phase compositions.

Isoeluotropic mobile phases produce separations in approximately the same time frame (measured using retention factor (k) of the last peak); however they may show altered selectivity. This can be very useful when developing or optimizing separations.
The interactive slider bar on the diagram shown can be used to find various isoeluotropic compositions – note that the relationship yields results which are only approximately accurate to within about ±5% B.

Quick rules of thumb

- THF reduces retention of straight chain analytes w.r.t. polarizable cyclic compounds
- THF accelerates the elution of analytes containing methoxy groups
- MeCN reduces retention of esters

The previously studied example of neutral chromatographic standards has now been separated using the isoeluotropic relationship (Figure 15). The final peak in each chromatogram elutes at approximately the same retention time (k 9-10). The most striking selectivity change occurs with peaks 1 & 2 in changing from acetonitrile to methanol or THF; resolution between these peaks is completely lost.

Often, changing the organic component of the mobile phase will change the selectivity and may improve resolution (Figure 16). If the separation id not satisfactory with acetonitrile and water,
methanol and water, or THF and water can be utilized. Another approach is to blend acetonitrile and THF. THF reduces straight chain retention with respect to cyclic compounds. It also accelerates compounds with methoxy groups. Acetonitrile accelerates the elution of compounds containing esters.

Conversely, methanol and acetonitrile or methanol and THF may also be blended as could a quaternary mixture of methanol, acetonitrile, and THF in conjunction with the aqueous component in specific instances. Ternary (two organic solvents with aqueous) and Quaternary (three organic solvents with aqueous) are typically more common when using on automated method development software package such as DryLab, ChromSword etc.

One method for determining the best mobile phase composition is to screen several solvent mixtures using a mobile phase selectivity triangle (Figure 17). This allows the broadest solvent composition to be analyzed with only seven experiments. Initially, a mixture of MeCN/aqueous is used (the percentage of MeCN can be modified to produce the best possible separation within the desired time); changing the organic modifier to MeOH and THF at the same eluotropic strength will give analysis times which are similar, however, with altered selectivity. Ternary (two organic solvents with aqueous) and quaternary (three organic solvents with aqueous) can then also be prepared (Figure 17). The chromatograms produced from these seven runs can be analyzed to determine which mobile phase composition will provide the desired separation, or to identify a region within the mobile phase selectivity triangle where separation is feasible (the solvent composition and strength could then be further modified to obtain the desired chromatography).
Figure 17: Mobile phase selectivity triangle for optimization of reversed phase HPLC separations.

A = acetonitrile/H₂O
M = methanol/H₂O
T = tetrahydrofuran/H₂O
10. Selecting Reversed Phase Columns

The “Column Chemistry” module gives comprehensive information on HPLC column stationary phases. A reminder of the stationary phase properties that are often reported by column manufacturers is shown (Table 5).

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=4&fPath=sco4/hplc_2_1.asp

<table>
<thead>
<tr>
<th>Column</th>
<th>Particle Size (µm)</th>
<th>Pore Size</th>
<th>Surface Area (m² g⁻¹)</th>
<th>Temp. Limits (°C)</th>
<th>pH Range</th>
<th>Endcapping</th>
<th>Carbon Load (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetry C₁₈</td>
<td>3.5, 5</td>
<td>100 Å</td>
<td>-</td>
<td>45</td>
<td>2-8</td>
<td>Yes</td>
<td>19</td>
</tr>
<tr>
<td>Luna C₁₈</td>
<td>3, 5, 10</td>
<td>100 Å</td>
<td>440</td>
<td>-</td>
<td>1.5-10</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Zorbax Eclipse Plus C₁₈</td>
<td>1.8, 3.5, 5.0</td>
<td>95 Å</td>
<td>160</td>
<td>60</td>
<td>2-9</td>
<td>Double</td>
<td>9</td>
</tr>
<tr>
<td>Hypersil BDS C₁₈</td>
<td>3, 5</td>
<td>120 Å</td>
<td>170</td>
<td>-</td>
<td>2-9</td>
<td>Yes</td>
<td>10</td>
</tr>
<tr>
<td>YMC-Pack ODS-A</td>
<td>3, 5</td>
<td>12 nm</td>
<td>330</td>
<td>-</td>
<td>2-7.5</td>
<td>Yes</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 5: C₁₈ column parameters from various manufacturers. 1 nm = 10 Å.

Particle Size

The particle size or particle diameter \(d_p\) is the average diameter of the column packing particles.

Pore Size

The pore diameter chosen is directly related to the hydrodynamic volume of the molecules in the sample. Larger pores allow large molecules to access the bonded phase found within pores for maximum separating ability. Select pore sizes of 150 Å or less for small molecules. Large pores, 300 Å or greater, are used for samples having a molecular weight greater than 2000 Da. As a rule of thumb the pore size should be at least three times the hydrodynamic diameter of the molecule.

Surface Area

The surface area refers to the total area of the solid surface. This is often determined using an accepted measurement technique such as the Brunauer-Emmet-Teller (BET) method, which uses the physical adsorption of nitrogen to measure the surface area. The surface area can have an effect on several chromatographic parameters. High surface area columns may exhibit greater retention, capacity, and resolution. Low surface area packings will generally equilibrate more quickly which can be advantageous in gradient elution.

pH Range

The pH range gives the pH values at which the HPLC column can be used without degradation of the solid support and stationary phase which will ultimately result in deleterious chromatographic results.
Traditional silica has a working range of pH 2.5-7.5. At low pH acidic hydrolysis of the silyl ether linkage between the bonded phase and silica surface will occur, resulting in column bleed (loss of stationary phase), poor peak shape, and loss of efficiency. At high pH the silica surface itself is at risk of basic hydrolysis, sometimes referred to as silica dissolution, whereby, the solid silica support is cleaved apart and fines are created which block the support material pores, interstitial gaps between the particles, and the column outlet frit resulting in the system over pressurizing and shutting down.

**Temperature Limit**

Some column manufacturers will give an operating range or upper temperature limit at which the columns can be used without damaging the stationary phase. Elevated temperatures will also promote basic hydrolysis of the silica support and acidic hydrolysis of the stationary phase.

**Endcapping**

Endcapping is used to remove surface silanol groups on the solid support which can cause unwanted analyte secondary interactions resulting in poor peak shapes; this is particularly evident when analyzing basic, ionizable, and ionic compounds. A small silylating reagent (i.e. trimethylchlorosilane or dichlorodimethylsilane) is reacted with the surface silanol groups to produce, for example, a trimethylsilyl (TMS) capped group (Figure 18).

![Figure 18: Endcapped silica surface.](image)

Fully hydroxylated silica will have a silanol surface concentration of ≈8 μmol/m². Following chemical modification > 4 μmol/m² of these silanols may remain; even with optimum bonding conditions due to steric limitations of the modifying ligands. This indicates that on a molar basis there are more residual silanols remaining than actual modified ligand. This is only a partial solution; however, as not all of the surface silanol groups will be reacted even when using sterically very small ligands and optimized bonding conditions, also the endcapping ligand is prone to hydrolysis especially at low pH.

**Carbon Load**

Carbon load is simply the elemental load of carbon on the support material (which is carbon free in its native form for traditional silica). Hybrid particles such as BEH, XBridge, TriArt and Gemini contain a mixture of inorganic silica and organic groups and will always have a larger background.
carbon content present, even in its native, unmodified state. The carbon load is normally expressed as % carbon. A higher carbon loading will result in greater analyte retention. It should be noted that C18 columns will always have a higher % carbon and columns with different endcapping cannot be subjected to a like to like comparison as the % carbon for the different endcapping groups will differ.

**Particle Size Distribution**

The particle size distribution will give a measure of the distribution of the size of particles used to pack the column. It is desirable to having a narrow particle size distribution as this gives a more homogeneous packing and ultimately more reproducible chromatography. A particle size distribution of $d_p \pm 10\%$ would indicate that the 90% of the particles are between 9-11 µm for a 10 µm average $d_p$ packing.

D10/D90 ratios are often quoted for packing materials as a relative measure of the particle diameter distribution. D10 = particle diameter at 10% of the total size distribution and D90 = particle diameter at 90% of the total size distribution. The closer this value is to unity, the more homogeneous the particle diameter distribution.

**Particle Shape**

Two basic silica particle shapes are available, spherical and irregular (Figure 19). Milling of silica particles followed by sieving to obtain the appropriate particle size and distribution produces irregular particles. Although irregular particles are somewhat less expensive they are known to have poorer efficiency than spherical particles. This is due to the way in which the particles pack into the HPLC column; with irregular particles packing homogeneity is much poorer leading to an exaggeration of eddy diffusion and mass transfer effects.

The use of columns for high flow (pressure) applications and mechanical shock can cause the irregular particles to shear, forming smaller sub-particles known as fines which may migrate and eventually block the outlet frit of the column.

Therefore, spherical particles are generally favored for the reasons outlined above.

![Spherical silica particles](image1.png) ![Irregular silica particles](image2.png)

**Porosity**

The porosity is related to both the pore size and number of pores. The greater the porosity, the less stable the stationary phase. Size exclusion columns have high porosity; thus they often have lower pressure limits than other columns.
Silica Type

Acidic (lone) surface silanol groups give rise to the most pronounced secondary interactions with polar and ionizable analytes (Figure 20). Modern silica is designated as being Type I or A and Type II or B, which primarily describes the nature of the silanol surface. Type A or I silica is ‘high energy’ (non-homogenous) and contains a higher density of lone silanol groups, whereas Type B or II silica is much more homogenous (inter-hydrated) and, therefore, gives rise to much improved peak shapes. In order to create a more uniform (homogeneous) silica surface, manufacturers ensure the silica surface is fully hydroxylated prior to chemical modification. The incorporation of an acid wash step and avoidance of treatments at elevated temperature renders the majority of the surface in the lower energy geminal and bridged (vicinal) confirmation, creating Type II silica.

![Silica particle diagram](image)

**Figure 20**: Surface silanol groups present on the surface of silica particles.

The lone silanols acidity is further increased by the presence of metal ions. The worst offenders being trivalent iron and aluminium. Analytes which are capable of chelating can also interact with the column in this way leading to additional unwanted secondary interactions.

By acid washing the silica to form Type B silica the metal ion impurities are also vastly reduced as is shown in the Table 6. It can be seen that problematic iron and aluminium are present at sub 3 ppm levels. Metal ion contamination these days is rarely resultant from the silica manufacturing process but from contact with the blades used for sub-sieve sizing and non-passivated stainless steel surfaces, i.e. column frits etc.

<table>
<thead>
<tr>
<th>Silica</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Al</th>
<th>Ca</th>
<th>Ti</th>
<th>Fe</th>
<th>Zr</th>
<th>Cu</th>
<th>Cr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type B</td>
<td>10</td>
<td>&lt;3</td>
<td>4</td>
<td>1.5</td>
<td>2</td>
<td>nd</td>
<td>3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1</td>
</tr>
<tr>
<td>Type A</td>
<td>2900</td>
<td>n/a</td>
<td>40</td>
<td>300</td>
<td>38</td>
<td>65</td>
<td>230</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table 6**: Metal ion content of Type A and Type B silica. nd = none detected.

Surface Coverage

Surface coverage gives a better measure of the retentivity/hydrophobicity of the column. This refers to the concentration of stationary phase per unit area bonded to the support and is expressed as µmoles/m².
Figure 21: Silica surface coverage.
11. Reversed Phase HPLC of Ionizable Samples

So far in this section the focus has been on the analysis of neutral samples by reversed phase HPLC. Ionizable analytes present their own particular separation challenges and opportunities.

Before we examine how ionizable samples are typically analyzed, a few fundamental principles should be reviewed.

11.1. pH

The concept of pH is used to indicate the relative acidity or basicity (of a mobile phase in HPLC), and is defined as the negative logarithm of the hydrogen ion concentration in an aqueous solution. Adding an acid or base to a mobile phase will change the solution pH (Figure 22-23).

\[
\text{pH} = -\log [H^+] 
\]

![Figure 22: The link between pH and hydrogen ion concentration in solution](image)

![Figure 23: Effect of addition of acidic and basic modifiers to mobile phase pH](image)
Addition of acids, which are proton donors, will increase the hydrogen ion concentration, thus lowering the pH. Addition of bases, which are proton acceptors, will reduce the hydrogen ion concentration, thus raising the pH.

The mobile phase pH can be used to influence the charge state of ionizable species in solution. The extent of analyte ionization can be used to affect retention and selectivity.

11.2. Acid Base Chemistry

When working with ionizable analytes, it is important to consider the pH of the mobile phase to optimize separation; therefore, knowledge of the analyte is of utmost importance. Knowledge of the functional group chemistry and the associated pKₐ of an analyte will allow for tuning of the pH of the mobile phase.

All acids and bases have a unique “ionization constant” (Kₐ), which specifies the degree to which the species ionizes in aqueous solution. The greater the ionization, the greater the influence of the species on the hydrogen ion concentration, and thus, the “stronger” the acid or base.

For example, a strong acid will completely ionize, creating a high hydrogen ion concentration, and a very low pH in the solution. Conversely, a strong base will completely ionize by removing hydrogen ions from the solution, thus, lowering the hydrogen ion concentration and raising the pH of the solution to a very high value. Weaker acids and bases will ionize to a lesser degree and, therefore, have less effect on changing the pH.

Brønsted was the first to demonstrate the advantage of expressing the ionization of both acids and bases using the same scale. He made an important distinction between strong and weak acids and bases:

- Strong acids and bases are completely ionized over the pH range 0-14
- Weak acids and bases are incompletely ionized within the pH range 0-14

When an acid or base is dissolved in water the equilibria shown are established (Equation 1 and 4). The equilibrium constants (dissociation constants, Kₐ and Kₐ) are given by Equation 2 and 5. The partial acid (or base) dissociation (ionization) constant is defined as the negative logarithm of the equilibrium coefficient of the neutral and charged forms of a compound (Equation 3 and 6). This allows the proportion of neutral and charged species at any pH to be calculated, as well as the basic or acidic properties of the compound to be defined. It is very important to note the extent of ionization at different pH values around the analyte pKₐ.

A strong base will have a large Kₐ and exists in equilibrium with its conjugate acid which is a weak acid with a small Kₐ. The converse is true for acidic species. All acid-base reactions in aqueous solutions can be viewed from the standpoint of the conjugate acid form losing a proton to form the conjugate base. When this is done pKₐ can always be used in calculations and Kₐ or pKₐ is not required, making comparisons of acidic and basic molecules much more facile.
For Acids

\[ HA \rightleftharpoons A^- + H^+ \quad (1) \]

\[ K_a = \frac{[H^+][A^-]}{[HA]} \quad (2) \]

\[ pK_a = -\log_{10}K_a \quad (3) \]

For Bases

\[ B + H_2O \rightleftharpoons HB^+ + OH^- \quad (4) \]

\[ K_b = \frac{[OH^-][HB^+]}{[B]} \quad (5) \]

\[ pK_b = -\log_{10}K_b \quad (6) \]

Remember

- The stronger the acid the smaller the pK_a
- The stronger the base the larger the pK_a

The pH corresponding to the point at which the two forms of the analyte (ionized and non-ionized) are present in equal concentrations (i.e. the analyte is 50% ionized) is the called the pK_a value. Each ionizable functional group on the analyte molecule will have its own pK_a value. Since the pK_a represents an equilibrium value, when the pH is equal to the pK_a the analyte will be rapidly converting between the ionized and non-ionized form, which can result in poor peak shape in HPLC.

As can be seen from Figure 24 the pK_a gives an indication of the strength of the acid or the base (relative to its conjugate acid or base), however, it cannot be decided from the pK_a if the molecule is an acid or a base, the functional groups contained within the molecule must be known. For example, the pK_a of aspirin and diazepam are very similar (3.5 and 3.3 respectively). Aspirin is a weak acid as it contains acidic carboxylic acid groups, while diazepam is a weak base as it contains basic nitrogen functional groups.

![Figure 24: Ibuprofen, aspirin, diazepam, and amphetamine. pK_a values are shown for each molecule. Acidic groups are shown in red and basic groups are shown in blue.](image-url)
Table 7 details the pK\textsubscript{a} range of several common functional groups.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>pK\textsubscript{a} range (H\textsubscript{2}O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic acids</td>
<td>0.65-4.76</td>
</tr>
<tr>
<td>Alcohols</td>
<td>8.4-24.0</td>
</tr>
<tr>
<td>Amines</td>
<td>4.7-38</td>
</tr>
<tr>
<td>Amides</td>
<td>18.2-26.6*</td>
</tr>
<tr>
<td>Imides</td>
<td>8.30-17.9*</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>15-53</td>
</tr>
<tr>
<td>Esters</td>
<td>11-24.5</td>
</tr>
<tr>
<td>Ketones</td>
<td>7.7-32.4*</td>
</tr>
<tr>
<td>Ethers</td>
<td>22.85-49</td>
</tr>
</tbody>
</table>

Table 7: pK\textsubscript{a} ranges for selected functional groups. * = Values in DMSO.\textsuperscript{6}

Each molecule will have a specific pK\textsubscript{a} and there are several resources which detail physicochemical data of known compounds.

**Websites**

- http://www.chemicalize.org
- http://www.chemspider.com
- http://webbook.nist.gov/chemistry

**Books**

- CRC Handbook of Chemistry and Physics\textsuperscript{4}
- Lange’s Handbook of Chemistry\textsuperscript{3}

It is important to realize that the two forms of ionizable analyte molecules give different retention characteristics. The ionized form is much more polar, and its retention in reversed phase HPLC is much lower (shorter retention time (t\textsubscript{R}), smaller retention factor (k)). This behavior is expected, as the more polar analyte has a higher affinity for the mobile phase and moves more quickly through the column. The converse is true of the non-ionized form as it is much more hydrophobic, relative to the ionized form.

Further information on functional group chemistry can be found here:

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=2&fCourse=3&fSco=52&fPath=sco52/psp_C4_AimsObj.asp
12. Controlling the Extent of Ionization

The pH of a solution will influence the charge state of an acidic or basic analyte. For example, addition of an acid to an aqueous solution of a basic analyte will increase the concentration of charged analyte in solution as the hydrogen ion concentration increases. Conversely, raising the pH by addition of a base will increase the concentration of the neutral form of the basic analyte. This principle was first described by Le Chetali and the converse applies to acidic analyte species. Take the example of homovanillic acid shown (Figure 25) – equilibrium between the ionized and non-ionized forms of the analyte will be established according to the solution pH.

Figure 25: The effect of mobile phase pH on extent of ionization for an ionizable analyte in reversed phase HPLC

Key Learning Points

- At pH 3.74 the analyte is in its 50% ionized form - this is equal to the $pK_a$ value of the analyte molecule.
- At approximately pH 5.74 the analyte is 99% ionized - raising the pH further will not significantly affect the degree of ionization (and hence the retention behavior).
- At around pH 1.74 the analyte is almost fully non-ionized (ion-suppressed) - again lowering the pH further will not significantly affect the degree of ionization (and hence the retention behavior).
Adding an acid to a buffered solution of homovanillic acid (i.e. lowering the mobile phase pH), will cause the equilibrium to shift to the left and the analyte will become less ionized (ion suppressed) as the analyte recombines to reduce the effect of the added hydrogen ions (protons) from the acidic species. Adding a base to a buffered solution of homovanillic acid (i.e. increasing the mobile phase pH), will cause the analyte to become more ionized as the solution attempts to regain equilibrium by producing more hydrogen ions to neutralize the added base. The slider bar can be used to investigate the degree of ionization of homovanillic acid in solution at varying pH values (Figure 26).

Figure 26: The effect of mobile phase pH on extent of ionization for an ionizable analyte in reversed phase HPLC.
Homovanillic acid has multiple ionizable groups, each of which has its own pKₐ, the speciation (concentration) of each of the ionized and non-ionized forms of homovanillic acid are shown over the full pH range (Figure 27).

Figure 27: pKₐ values (top), ionized and non-ionized forms (middle), and speciation plot (bottom) for homovanillic acid.

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The extent of ionization of basic molecules is also affected by the addition of acidic or basic species to the mobile phase. Use the slider to investigate the extent of ionization for the basic beta-blocker molecule Atenolol (Figure 28).

Atenolol has two ionizable groups each with its own associated $pK_a$; the speciation (concentration) of each of the ionized and non-ionized forms of atenolol acid are shown over the full pH range (Figure 29).

**Key Learning Points**

- At pH 9.67 the analyte is 50% ionized - this is equal to the $pK_a$ value of the analyte molecule.
- At approximately pH 7.67 the analyte is fully ionized (i.e. is fully protonated) - lowering the pH will not significantly affect the degree of ionization (and hence the retention behavior).
- At around pH 11.67 the analyte is fully non-ionized - again raising the pH will not significantly affect the degree of ionization (and hence the retention behavior).
- It should be noted that the extent of ionization behavior is exactly the opposite to that encountered with acidic species.
Figure 29: pKₐ values (top), ionized and non-ionized forms (middle), and speciation plot (bottom) for atenolol.⁷
13. The 2 pH Rule

The extent of ionization of an analyte molecule against pH has been demonstrated for both acids and bases.

It has been shown that the change in degree of ionization happens over a limited pH range. In fact, because pH and pKₐ scales are logarithmic, it can be shown that at 1 pH unit away from the analyte pKₐ, the change in extent of ionization is approximately 90%. At 2 pH units away from the pKₐ, the change in extent of ionization is approximately 99%, at 3 pH units 99.9% etc. Therefore, a rule of thumb known as the ‘2 pH rule’ is useful in predicting the extent of ionization. The 2pH rule for weak acids and bases is shown here (Figure 30).

![Diagram of the 2 pH Rule](image)

**Figure 30:** Schematic representation of the 2pH Rule describing the effects of mobile phase pH on degree of analyte ionization in solution.
If the pK\textsubscript{a} of an analyte is known the extent of ionization over a specific pH range can be determined and plotted as a speciation plot. Using salicylic acid and methamphetamine as examples the extent of ionization of these compounds is shown over the pH range 0 - 14 (Figures 31 and 32).

Figure 31: pK\textsubscript{a} values (top), ionized and non-ionized forms (middle), and speciation plot (bottom) for salicylic acid.  

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Figure 32: pKa values (top), ionized and non-ionized forms (middle), and speciation plot (bottom) for methamphetamine.© www.chromacademy.com
14. pH vs. Retention in Reversed Phase HPLC

It has been previously shown that there is a relationship between the pH of the mobile phase and the degree of ionization of the analyte molecule. Further, as the degree of ionization also dictates the analyte’s hydrophobicity, it is known that pH can also be used to control retention.

If the pH of the mobile phase matches the $pK_a$ of an analyte (50% ionized/50% non-ionized), would two peaks be present in the chromatogram, one due to the ionized form and the other due to the non-ionized form?

![Interactive exercise on the effect of pH in reversed phase HPLC.](image)

**Figure 33:** Interactive exercise on the effect of pH in reversed phase HPLC.

In reversed phase HPLC hydrophobic analytes are more strongly retained. Ionic analytes are much less hydrophobic (more hydrophilic) when they are in their ionized form and as a result their retention, $k$, will decrease. Analytes in their ionized form will also show secondary interactions with the column (free silanol groups) and with the system which can result in deleterious chromatographic peak shapes; mainly peak tailing.

![Equilibrium for acidic (top) and basic (bottom) species.](image)

**Figure 34:** Equilibrium for acidic (top) and basic (bottom) species.

HA $\rightleftharpoons$ A$^-$ + H$^+$

B + H$^+$ $\rightleftharpoons$ BH$^+$

Hydrophobic (more retained in RP-HPLC) Hydrophilic (less retained in RP-HPLC)
**pH vs Retention in Reverse Phase HPLC**

The aim of this experiment is to investigate the retention behaviour of an acidic analyte over a range of pH values. The selectivity of the separation (and ultimately the minimum resolution) will also be tracked and recorded. Once you have investigated the effect of pH on the separation – choose suitable responses for the question boxes.

1. What is the best pH to carry out the separation of homovanilllic acid from other acidic analytes?

   Select one ▷

2. From the curve of analyte retention vs mobile phase pH – predict the pH of homovanilllic acid.

   Select one ▷

---

[Graph and table below the text]


**pH vs Retention in Reverse Phase HPLC**

The aim of this experiment is to investigate the retention behaviour of an acidic analyte over a range of pH values. The selectivity of the separation (and ultimately the minimum resolution) will also be tracked and recorded.

Once you have investigated the effect of pH on the separation — choose suitable responses for the question boxes:

1. **4. What is the useful pH operating range for improving the selectivity of homovanillic acid?**
   - Select one
   - 1.5
   - Select one

2. **5. From the curves here — pick the shape that would represent the retention vs pH curve for a basic analyte.**
   - Select one

---

**pH**

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity</th>
<th>Tr (min)</th>
<th>Rm (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td></td>
<td>7.12</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* = Peak 4 Homovanillic Acid

---

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15. Basic Analytes and Ion Suppression

Unlike the dissociated (ionized) acids, which when charged, elute rapidly from the column, protonated bases often have long retention times and poor peak shape. This retention behavior is due to the interaction with residual silanol species on the silica surface.

Separations of basic compounds, however, are not were not traditionally carried out under ion suppression conditions as the analyst would have to raise the pH of the mobile phase to produce the neutral molecule. High pH mobile phases can damage traditional silica based RP-HPLC columns (working pH range 2.5-7). However, columns which are specifically designed to operate with high pH conditions (working pH range 1-12) are available allowing the analysis of basic analytes using ion suppression. Traditionally the analysis of weak bases has been carried out at low pH; essentially because the surface silanol species are non-ionized (pKₐ approximately 3.5) and peak tailing is improved (Figure 35).

Figure 35: The effect of mobile phase pH on the extent of surface silanol ionization.

Key Learning Points

- The extent of ionization of the surface silanol groups directly influences the peak shape of basic species - non-ionized silanol groups result in the least peak tailing, whilst the ionized form results in the worst peak shape.
- At pH 3.5 the surface silanol groups are 50% ionized/50% non-ionized.
- Lowering the mobile phase pH causes the acidic silanol groups to become less ionized - below pH 1.5 the silanol groups are approximately 100% non-ionized and peak tailing will be reduced (care should be taken at this extreme mobile phase pH as the bonded phase may be degraded).
- By raising the pH to 5.8 the silanol groups will be 100% ionized and peak tailing will be at its worst with respect to secondary silanol retention.

The unwanted secondary silanol retention may be reduced (or even eliminated) by the addition of a small (sterically), highly surface active base such as triethylamine (TEA), piperazine, N,N',N'-
tetramethylethylenediamine (TMEDA), or dimethyloctylamine (DMOA) (Figure 36). These bases interact with the surface silanol species in preference to the analyte molecule and are called ‘sacrificial bases’. They are added to the mobile phase in sufficient concentration to ensure that the silica surface is fully deactivated at all times.

![Figure 36: Sacrificial bases.](image)

**15.1 Analysis of Basic Analytes at Low and Mid pH**

![Figure 37: Improvement in basic analyte peak shape at low pH due to reduced surface silanol interaction.](image)
15.2 Using Triethylamine as a Sacrificial Base

It is possible to improve the peak shape of basic analytes using a sacrificial base. In this example the amphetamine molecule ($pK_a = 10.01$), exhibits very bad tailing with no triethylamine added ($pK_a = 10.21$), in a mobile phase of pH 7.1. As the concentration of sacrificial base is increased, the peak shape steadily improves. The curve representing peak tailing against TEA concentration in the mobile phase plateaus at approximately 10 mM, indicating almost total coverage of the reactive silanol groups (Figure 38).

There is often a necessity to improve peak asymmetry (tailing) when analyzing bases as many are ionized at pH values used with traditional silica columns. HPLC column manufacturers produce columns which can be used to analyze basic analytes; these columns will either by produced from Type B silica, which has fewer surface active silanols, or will have been endcapped to reduce the number of silanol groups available for the analyte to interact with.

It should be noted that columns are available that can be used at high pH to analyze the ion-suppressed base (non-ionized).

For more information see the CHROMacadey module “Column Chemistry”.

http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=4&fPath=sco4/hplc_2_1.asp
15.3 Applications Using Sacrificial Bases and High pH

The unwanted secondary silanol retention may be reduced (or even eliminated) by the addition of a small (sterically), highly surface active base such as triethylamine (TEA), piperazine, \( N,N',N'-\text{tetramethylethylenediamine} \) (TMEDA), or dimethyloctylamine (DMOA). These bases interact with the surface silanol species in preference to the analyte molecule and are called ‘sacrificial bases’. They are added to the mobile phase in sufficient concentration to ensure that the silica surface is fully deactivated at all times. The interaction of TEA with the silica surface is shown in Figure 39.

The use of both triethylamine and high mobile phase pH has been used to improve peak shape for the separation of antihistamine analytes (Figure 40). At high mobile phase pH values (i.e. 2 pH units above the \( pK_a \) of the analyte) the analyte will be non-ionized (neutral) and there will be no interaction with the silica surface silanol groups, reducing any peak tailing. When a mixture of analytes with varying \( pK_a \) values (i.e. the separation of the antihistamines, Figure 40) are analyzed the use of increased mobile phase pH may not render all analytes neutral, therefore, TEA (or another sacrificial base) can also be used to interact with the surface silanol species and improve peak shape.

The column used in this particular separation has a bidentate ligand structure which acts to protect the silica surface from hydrolysis at high pH. Most manufacturers have columns that can be used at extremes of pH.
Figure 40: Improving peak shape for ionizable analytes using high pH and triethylamine as a mobile phase modifier.

**Column:** ZORBAX Extend-C<sub>18</sub> 4.6 x 150 mm, 5 µm  
**Flow rate:** 1.0 mL/min  
**Temperature:** R.T.  
**Detection:** 254 nm  

Ion pair reagents can be used when other methods such as reversed phase and ion suppression techniques have not been successful. Samples containing both anionic and cationic components have one type ‘masked’ by the ion pair reagent and the other suppressed by pH. This is a useful technique if the \( pK_a \) of sample analytes are not similar.

For example:

- Tetrabutylammonium phosphate \( 'N(C_4H_{9})_4 \) at pH 7.5 forms strong ion pair with acids, the pH suppresses weak base ions (for bases with \( pK_a \sim 5.5 \))
- Alkylsulfonic acids \( SO_3(CH_2)_nCH_3 \) \( (n = 4-7) \) at pH 3.5 forms ion pairs with bases and weak acids (with \( pK_a \sim 5.5 \)) are suppressed by pH
- Trifluoroacetic acid (TFA) forms ion pairs with bases

The mechanisms by which ion pair reagents are thought to operate by are:

1. The analyte is paired in solution with the ion pair reagent and the neutral complex undergoes partition interactions with the stationary phase or
2. The ion pair reagent populates the hydrophobic surface and the analyte molecules undergo an ion exchange reaction. This method is generally accepted as the primary mode of ion pair retention.

Some disadvantages of ion pair reagents are the long equilibration times that are required (> 100 column volumes). Ion pair reagents are notoriously difficult to remove from the column, therefore, it is recommended that a guard column or dedicated column is used for ion pair applications. Ion pair reagents, along with irreversibly modifying the stationary phase, will drastically reduce the column lifetime. Finally, ion pair reagents are not generally suitable for LC-MS work as they will suppress ion formation and reduce sensitivity. There are, however,
alternative approaches that can be used with LC-MS such as HILIC and mixed mode stationary phases.

For further information on:

**Ion Pair Chromatography**


**HILIC and Mixed Mode Chromatography**

[http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=105&fPath=sco105/hilic_1_1_1.asp](http://www.chromacademy.com/frameset-chromacademy.html?fChannel=0&fCourse=0&fSco=105&fPath=sco105/hilic_1_1_1.asp)
16. Buffers for Reversed Phase HPLC

In order to control the retention of weak acids and bases, the pH of the mobile phase must be strictly controlled. This usually involves meticulous preparation and adjustment of the mobile phase to the correct pH. Most analysts will use a buffer to resist small changes in pH that may occur within the HPLC system (i.e. at the head of column when sample diluent and mobile phase mix, or via evaporation of the organic solvent in a pre-mixed mobile phase, ingress of CO₂ into the mobile phase etc.).

The definition of a buffer is a weak acid or base in co-solution with its salt – an example would be acetic acid (the weak acid) and sodium acetate (its salt). A known weight of the salt is usually added to the mobile phase to achieve a known concentration, the weak acid or base is then added to the mobile phase (with stirring), until the desired pH is achieved.

Some common HPLC buffers, their pKₐ, working pH range, and UV cut off are detailed in Table 8.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKₐ</th>
<th>pH range</th>
<th>UV cut off (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>2.1</td>
<td>1.1-3.1</td>
<td>&lt; 200</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>6.2-8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.3</td>
<td>11.3-13.3</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>210 (10 mM)</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>3.7-5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>4.4-6.4</td>
<td></td>
</tr>
<tr>
<td>Carbonate</td>
<td>6.1</td>
<td>5.1-7.1</td>
<td>&lt; 200</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>9.3-11.0</td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210 (10 mM)</td>
</tr>
<tr>
<td>Ammonium bicarbonate</td>
<td>7.6</td>
<td>6.6-8.6</td>
<td>230</td>
</tr>
<tr>
<td>Borate</td>
<td>9.3</td>
<td>8.3-10.3</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 8: Common HPLC buffers.

The compounds detailed Table 9 are not buffers, however, addition of these compounds to a mobile will result in a change in pH. Many of the compounds listed are used to make buffer solutions along with their corresponding salt, acid, or base, they are also used alone to alter the mobile phase pH (TFA in particular) so that surface silanol species will be non-ionized, and finally some will also be used as ion pair reagents.
Table 9: pKₐ values for common HPLC additives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluoroacetic acid</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>7.20</td>
</tr>
<tr>
<td></td>
<td>12.33</td>
</tr>
<tr>
<td>Citric acid</td>
<td>3.13</td>
</tr>
<tr>
<td></td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td>6.40</td>
</tr>
<tr>
<td>Formic acid</td>
<td>3.75</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>4.76</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>4.86</td>
</tr>
<tr>
<td>Carbonic acid</td>
<td>6.35</td>
</tr>
<tr>
<td></td>
<td>10.33</td>
</tr>
<tr>
<td>Tris</td>
<td>8.06</td>
</tr>
<tr>
<td>Boric acid</td>
<td>9.23</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.25</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.78</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>10.21</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>11.27</td>
</tr>
<tr>
<td>Methanesulfonic acid</td>
<td>-1.61</td>
</tr>
</tbody>
</table>

Users of LC-MS require volatile buffers (denoted in Table 1 as #) to avoid fouling of the atmospheric pressure interface and reduced maintenance intervals. The use of trifluoroacetic acid (TFA) is to be avoided for small molecule work due to its ion suppression effects.

A particular buffer is only reliable in the pH ranges given – usually around 1 pH either side of the buffer pKₐ value (note some buffers have more than one ionizable functional group and therefore more than one pKₐ value).

The concentration of the buffer must be adequate, but not excessive. In general, HPLC buffers range in concentration from 25 to 100 mM. Buffers prepared below 10mM can have very little buffering capacity and impact on chromatography, whilst those at high concentration (>50mM) risk precipitation of the salt in the presence of high organic concentration mobile phases (i.e. >60% MeCN), which may damage the internal components of the HPLC system.

16.1 Effect of Buffers on the Analysis of Basic Analytes

Buffers will function correctly unless they are of insufficient concentration or employed at a pH sufficiently away for their pKₐ – outside their operating range. In the top example (Figure 41), the buffer concentration was inadequate. Notice that the peak shapes are broad and retention times are unstable. In the example below, the peak shape has improved when using an appropriate buffer concentration and within its operating range. Typical buffer concentrations range from 25 to 100 mM.
16.2. Buffers and Column Degradation

Buffer type, concentration, and temperature can all affect the column lifetime in HPLC. Ensure all buffers are flushed from the column after use. Citrate may seem to be a more attractive buffer, however, it will attack and cause corrosion of the stainless steel components of the HPLC system.

Figure 42: Effect of buffer on column lifetime and efficiency.
16.3. Buffer Concentration

Reversed phase separation of ionizable analytes is particularly susceptible to changes in pH which is why buffers are used to carefully control and stabilize the pH of the mobile phase.

There are cases where analyte retention in reversed phase HPLC is affected by buffer concentration. These cases are usually confined to situations where there are ion exchange interactions taking place between basic solutes and acidic silanols on the surface of the silica stationary phase support (separation of basic compounds) using reversed phase stationary phases that have significant silanol activity with mobile phase pH > 3. Increasing the concentration of the mobile phase buffer, and thereby increasing the ionic strength of the mobile phase, will sometimes suppress this ion exchange interaction and reduce this ‘secondary retention’ effect.

It should be noted that as the buffer concentration is increased the mobile phase is made more polar (ionic). This can affect analytes in differing ways depending on their chemistry; some analytes may experience reduced retention while some will exhibit slightly increased retention.

The main objective is to use an appropriate buffer (i.e. buffer pK_a vs. mobile phase pH) in the correct concentration to have sufficient buffering capacity to overcome peak shape and retention time irreproducibility.

![Figure 43](attachment:image_url)

**Figure 43:** The effect of mobile phase buffer concentration on selectivity and peak shape in reversed phase HPLC.
Key Learning Points

- Some analytes are susceptible to changes in ionic strength which usually presents as a change in retention time.
- Higher buffer concentrations lead to improved peak shape (better efficiency and asymmetry).
- Below 10 mM the buffer concentration has little effect on the chromatography.
- Ionic strength can critically affect the selectivity of a separation and, therefore, should be optimized and care taken to obtain correct ionic strength when making up buffered mobile phases.

16.4. Buffer Capacity

Buffer capacity is a measure of the efficiency of a buffer to resist small changes in pH. Buffer capacity ($\beta$) can be expressed as the amount of strong acid or base (in gram equivalents) that is required to change 1 liter of a buffer solution by one pH unit (Equation 1).

$$
\beta = \frac{\Delta B}{\Delta pH} \quad (1)
$$

Where:

$\Delta B =$ gram equivalent of strong acid/base to change the pH of 1 liter of buffer solution by 1 pH unit

$\Delta pH =$ the pH change caused by the addition of the strong acid/base

The buffer capacity depends on two factors

1. The ratio of the salt to the acid or base. The buffer capacity is optimal when the ratio is 1:1 i.e. when $pH = pK_a$
2. Total buffer concentration. It will take more acid or base to deplete a 0.5 M buffer than a 0.05 M buffer

The relationship between buffer capacity and concentration is given by the Van Slyke Equation (Equation 2).\(^{10}\)

$$
\beta = 2.3C \frac{K_a[H_3O^+]}{(K_a + [H_3O^+])^2} \quad (2)
$$

Where:

$C =$ the total buffer concentration (i.e. the sum of the molar concentrations of acid and salt)
Figure 44: Buffer capacity for a buffer $pK_a = 7$ as percentage of the maximum buffering capacity.
17. Henderson-Hasselbalch Equation

Lawrence Joseph Henderson wrote an equation, in 1908, describing the use of carbonic acid as a buffer solution. Karl Albert Hasselbalch later re-expressed that formula in logarithmic terms, resulting in the Henderson-Hasselbalch equation.\(^{11,12}\) Hasselbalch was using the formula to study metabolic acidosis.

The Henderson-Hasselbalch equation (Equation 1) is used to calculate the pH of a system using the pK\(_a\) (logarithm of the acid dissociation constant \(K_a\)) of the acidic species in solution. It has many applications and can be used to determine the pH of a solution, calculate the molar ratio of acid and salt required to form a buffer of a particular pH, and give an estimate of the value of the pK\(_a\) of a species if the pH and concentration of the acid and salt components are known.

The Henderson-Hasselbalch equation is derived from the acid dissociation (\(K_a\)) constant as follows:

\[
\begin{align*}
\text{HA} & \quad \xrightarrow{K_a} \quad \text{A}^- + \text{H}^+ \\
K_a &= \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} \\
\log_{10}K_a &= \log_{10}[\text{H}^+] + \log_{10}\frac{[\text{A}^-]}{[\text{HA}]} \\
-pK_a &= -p\text{H} + \log_{10}\frac{[\text{A}^-]}{[\text{HA}]} \\
p\text{H} &= pK_a + \log_{10}\frac{[\text{A}^-]}{[\text{HA}]} \quad (1)
\end{align*}
\]

For buffers:

- [A\(^-\)] = concentration of salt
- [HA] = concentration of acid
18. Buffer Preparation Using the Henderson-Hasselbalch Equation

18.1. Calculating Molar Ratio

Calculate the molar ratio required to prepare an acetate buffer with pH = 5.2 from acetic acid \((pK_a = 4.76)\) and its salt e.g. sodium acetate.

\[
pH = pK_a + \log_{10} \left( \frac{[A^-]}{[HA]} \right)
\]

\[
5.2 = 4.76 + \log_{10} \left( \frac{[A^-]}{[HA]} \right)
\]

\[
5.2 - 4.76 = \log_{10} \left( \frac{[A^-]}{[HA]} \right) = 0.44
\]

\[
\frac{[A^-]}{[HA]} = 10^{0.44}
\]

\[
\frac{[A^-]}{[HA]} = 2.75
\]

18.2. Calculating the Molar Concentration

Calculate the molar concentration of acetic acid and sodium acetate required to produce a buffer with pH = 5.4 and a concentration of 0.05 M.

**Remember:** Total concentration = [acid] + [salt]

First calculate the ratio of \([\text{salt}] / [\text{acid}]\):

\[
pH = pK_a + \log_{10} \left( \frac{[A^-]}{[HA]} \right) \quad (1)
\]

\[
5.4 = 4.76 + \log_{10} \left( \frac{[A^-]}{[HA]} \right)
\]

\[
5.4 - 4.76 = \log_{10} \left( \frac{[A^-]}{[HA]} \right) = 0.64
\]

\[
\frac{[A^-]}{[HA]} = 10^{0.64}
\]

\[
\frac{[A^-]}{[HA]} = 4.37 \quad (2)
\]
Note:
\[
\frac{[A^-]}{[HA]} = 4.37 \quad (2)
\]

Therefore,
\[
\frac{[A^-]}{[HA]} = \frac{4.37}{1}
\]

Hence, \([A^-] = 4.37\) and \([HA] = 1\)

From this the molar concentration of sodium acetate and acetic acid can be calculated:
\[
\frac{[A^-]}{[HA]} = 4.37 \quad (2)
\]

\[
[A^-] = 4.37[HA] \quad (3)
\]

Total concentration = \([A^-] + [HA] = 0.05 \text{ M} \quad (4)\)

Substitute Equation 3 into Equation 4 to give Equation 5
\[
4.37[HA] + [HA] = 0.05
\]

\[
5.37[HA] = 0.05
\]

\[
[HA] = \frac{0.05}{5.37} = 0.0093 \text{ M}
\]

To calculate the concentration of salt \([A^-]\) required substitute the value calculated for \([HA]\) into Equation 3.
\[
[A^-] = 4.37[HA] \quad (3)
\]

\[
[A^-] = 4.37 \times 0.0093
\]

\[
= 0.0407 \text{ M}
\]

18.3. Preparing a Buffer from Stock Solutions

How would you prepare 10 mL of 0.01 M phosphate buffer pH 7.4 from stock solutions of 0.1 M \(\text{KH}_2\text{PO}_4\) and 0.25 M \(\text{K}_2\text{HPO}_4\)? \(pK_a\) of \(\text{KH}_2\text{PO}_4 = 7.2\)

First calculate the ratio of [salt]/[acid]:

\[
pH = pK_a + \log_{10} \frac{[A^-]}{[HA]} \quad (1)
\]
\[ 7.4 = 7.2 + \log_{10}\left(\frac{[A^-]}{[HA]}\right) \]
\[ 7.4 - 7.2 = \log_{10}\left(\frac{[A^-]}{[HA]}\right) = 0.2 \]

\[ \frac{[A^-]}{[HA]} = 10^{0.2} \]

\[ \frac{[A^-]}{[HA]} = 1.58 \ (2) \]

Note:

\[ \frac{[A^-]}{[HA]} = 1.58 \ (2) \]

Therefore,

\[ \frac{[A^-]}{[HA]} = 1.58 \]

Hence, \([A^-] = 1.58\) and \([HA] = 1\)

From this the molar concentration of sodium acetate and acetic acid can be calculated:

\[ \frac{[A^-]}{[HA]} = 1.58 \ (2) \]

\[ [A^-] = 1.58[HA] \ (3) \]

Total concentration = \([A^-] + [HA] = 0.01 \text{ M} \ (4)\]

Substitute Equation 3 into Equation 4 to give Equation 5:

\[ 1.58[HA] + [HA] = 0.01 \]

\[ 2.58[HA] = 0.01 \]

\[ [HA] = \frac{0.01}{2.58} = 0.0039 \text{ M} \]

To calculate the concentration of salt \([A^-]\) required substitute the value calculated for \([HA]\) into Equation 3:

\[ [A^-] = 1.58[HA] \ (3) \]

\[ [A^-] = 1.58 \times 0.0039 \]

\[ = 0.0061 \text{ M} \]
The moles of each component are then calculated:

\[
\text{Moles} = \text{Molarity} \times \text{Liters}
\]

\[
\text{Moles } A^- = 0.0061 \times 0.01 = 6.12 \times 10^{-5} \text{ moles}
\]

\[
\text{Moles } HA = 0.0039 \times 0.01 = 3.87 \times 10^{-5} \text{ moles}
\]

The volume of each stock solution required to make the buffer can then be calculated:

\[
\text{Liters of stock solution} = \frac{\text{moles of buffer component}}{\text{molarity of the stock solution}}
\]

\[
\text{Liters of } A^- = \frac{6.12 \times 10^{-5}}{0.25} = 2.452 \times 10^{-4} \text{ L} = 245 \mu\text{L}
\]

\[
\text{Liters of } HA = \frac{3.87 \times 10^{-5}}{0.10} = 3.869 \times 10^{-4} \text{ L} = 387 \mu\text{L}
\]

Therefore, to prepare this buffer 245 µL of K$_2$HPO$_4$ and 387 µL of KH$_2$PO$_4$ would be accurately measured into a volumetric flask and made up to 10 mL using deionized water. The pH of the buffer would then be checked using a pH meter.

**18.4. Calculating the Masses of Buffer Components**

Calculate the mass of KH$_2$PO$_4$ and K$_2$HPO$_4$ required to make 50 mL of a 0.05 M buffer at pH 7.4.

\[pK_a \; \text{KH}_2\text{PO}_4 = 7.2\]

\[\text{MW KH}_2\text{PO}_4 = 136.086 \text{ g/mol}\]

\[\text{MW K}_2\text{HPO}_4 = 174.2 \text{ g/mol}\]

**Remember:** if your buffer salts are in the hydrated form to use the correct molecular weight.

First calculate the ratio of [salt]/[acid]:

\[
\text{pH} = pK_a + \log_{10} \left( \frac{[A^-]}{[HA]} \right) \quad (1)
\]

\[
7.4 = 7.2 + \log_{10} \left( \frac{[A^-]}{[HA]} \right)
\]

\[
7.4 - 7.2 = \log_{10} \left( \frac{[A^-]}{[HA]} \right) = 0.2
\]
\[
\frac{[A^-]}{[HA]} = 10^{0.2}
\]
\[
\frac{[A^-]}{[HA]} = 1.58 \quad (2)
\]

**Note:**
\[
\frac{[A^-]}{[HA]} = 1.58 \quad (2)
\]

Therefore,
\[
\frac{[A^-]}{[HA]} = \frac{1.58}{1}
\]

Hence, \([A^-] = 1.58\) and \([HA] = 1\)

From this the molar concentration of sodium acetate and acetic acid can be calculated:
\[
\frac{[A^-]}{[HA]} = 1.58 \quad (2)
\]
\[
[A^-] = 1.58[HA] \quad (3)
\]

Total concentration = \([A^-] + [HA] = 0.05 \text{ M} \quad (4)\)

Substitute Equation 3 into Equation 4 to give Equation 5:
\[
1.58[HA] + [HA] = 0.05
\]
\[
2.58[HA] = 0.05
\]
\[
[HA] = \frac{0.05}{2.58} = 0.01934 \text{ M}
\]

To calculate the concentration of salt \([A^-]\) required substitute the value calculated for \([HA]\) into Equation 3:
\[
[A^-] = 1.58[HA] \quad (3)
\]
\[
[A^-] = 1.58 \times 0.01934
\]
\[
= 0.000374 \text{ M}
\]

The moles of each component are then calculated:

\[
\text{Moles} = \text{Molarity} \times \text{Liters}
\]

Moles \(A^- = 0.000374 \times 0.05 = 1.87 \times 10^{-4}\) moles
Moles HA = 0.01934 x 0.05 = 9.67x10^-3 moles

To convert moles to mass

\[
\text{Mass} = \text{Moles} \times \text{Molecular Weight}
\]

\[
\text{Mass } A^- = 1.87 \times 10^{-4} \times 174.2
\]

= 0.0325 g

= 32.5 mg

\[
\text{Mass } HA = 9.67 \times 10^{-3} \times 136.086
\]

= 1.316 g

18.5. Calculating the pH of a Strong Acid Solution

Strong acids completely ionize (dissociate) in solution i.e. one mole of acid HA will dissolve in water to produce one mole of hydrogen (H⁺) ions and one mole of the conjugate base A⁻.

\[
\text{HA}_{(aq)} \rightarrow \text{H}^+_{(aq)} + \text{A}^-_{(aq)}
\]

Some common strong acids are listed in Table 10.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Formula</th>
<th>(pK_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroiodic acid</td>
<td>HI</td>
<td>-9.3</td>
</tr>
<tr>
<td>Hydrobromic acid</td>
<td>HBr</td>
<td>-8.7</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td>HClO₄</td>
<td>-8</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>HCl</td>
<td>-6.3</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>H₂SO₄</td>
<td>-3 ((pK_{a1})) 1.9 ((pK_{a2}))</td>
</tr>
<tr>
<td>Nitric acid</td>
<td>HNO₃</td>
<td>-1.4</td>
</tr>
<tr>
<td>p-Toluenesulfonic acid (organic soluble)</td>
<td>C₇H₆SO₃</td>
<td>-2.8</td>
</tr>
<tr>
<td>Methanesulfonic acid (liquid organic strong acid)</td>
<td>CH₃SO₃</td>
<td>-1.92</td>
</tr>
</tbody>
</table>

Table 10: Strong acids.

Calculating the pH of a strong acid solution is straightforward as it completely dissociates, i.e. for every mole of acids one mole of H⁺ and 1 mole of A⁻ ions will be formed.
Calculate the pH of a 0.1 M HCl solution.

\[
\text{HCl}_{(aq)} \rightarrow \text{H}^+_{(aq)} + \text{Cl}^-_{(aq)}
\]

\[
\text{pH} = -\log[\text{H}^+]
\]

\[
= -\log 0.1
\]

\[
= 1
\]

Calculate the pH of 0.02 M H\textsubscript{2}SO\textsubscript{4}. Note: H\textsubscript{2}SO\textsubscript{4} undergoes two dissociation steps to give 2 equivalents of H\textsuperscript{+} and A\textsuperscript{-} ions.

\[
\text{H}_2\text{SO}_4_{(aq)} \rightarrow \text{H}^+_{(aq)} + \text{HSO}_4^-_{(aq)} \quad \text{1st Dissociation } K_{a1} = \text{very large (strong acid)}
\]

\[
\text{HSO}_4^-_{(aq)} \rightarrow \text{H}^+_{(aq)} + \text{SO}_4^{2-}_{(aq)} \quad \text{2nd Dissociation } K_{a2} = 1.2 \times 10^{-2} \text{ (weak acid)}
\]

\[
\text{pH} = -\log[\text{H}^+]
\]

\[
= -\log 0.04
\]

\[
= 1.4
\]

18.6. Calculating the pH of a Strong Base Solution

Strong bases also fully dissociate in water to give the metal ion (M\textsuperscript{+}) and hydroxide ions (OH\textsuperscript{-}).

\[
\text{MOH}_{(aq)} \rightarrow \text{M}^+_{(aq)} + \text{OH}^-_{(aq)}
\]

Some common strong bases are listed in Table 11.

<table>
<thead>
<tr>
<th>Base</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>NaOH</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>KOH</td>
</tr>
<tr>
<td>Lithium hydroxide</td>
<td>LiOH</td>
</tr>
<tr>
<td>Caesium hydroxide</td>
<td>CsOH</td>
</tr>
<tr>
<td>Calcium hydroxide</td>
<td>Ca(OH)\textsubscript{2}</td>
</tr>
<tr>
<td>Barium hydroxide</td>
<td>Ba(OH)\textsubscript{2}</td>
</tr>
<tr>
<td>Strontium hydroxide</td>
<td>Sr(OH)\textsubscript{2}</td>
</tr>
</tbody>
</table>

Table 11: Strong bases.

Calculating the pH for a strong base solution is not as straightforward as for a strong acid. This is because no H\textsuperscript{+} ions are formed. There are two methods for calculating the pH of a strong base:

1. Using the concentration of [OH\textsuperscript{-}] ions and the relationship

\[
\text{pH} = 14 - \text{pOH}
\]
2. By calculating the concentration of $[H^+]$ ions using the water dissociation constant $K_w$.

The pH value of a solution is directly related to the ratio of the hydrogen ion $[H^+]$ and the hydroxyl ion $[OH^-]$ concentrations. If $[H^+]$ is greater than $[OH^-]$, the solution is acidic and will have a pH value of less than seven. The opposite occurs when $[OH^-]$ exceeds $[H^+]$ and the pH is greater than seven. When $[OH^-]$ is equal to $[H^+]$ the solution will have a pH of seven.

Water dissociates into hydrogen ions ($H^+$) and hydroxide ions ($OH^-$) in aqueous solution and the following equilibria may be used to describe pH:

$$2H_2O(l) \rightleftharpoons H_3O^+(aq) + OH^-(aq)$$
$$H_2O(l) \rightleftharpoons H^+(aq) + OH^-(aq)$$

The equilibrium constant ($K_{eq}$) is:

$$K_{eq} = \frac{[H_3O^+][OH^-]}{[H_2O]^2}$$

Which is equal to:

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]}$$

The concentration of water in a water solution is constant $[H_2O] = 55.5$ M. Therefore, the expression can be simplified to give the equilibrium constant $K_w$ (sometimes referred to as the ionic product of water):

$$K_w = [H_2O]K_{eq} = 55.5 \text{ M} \times K_{eq} = [H^+][OH^-] = 1 \times 10^{-14} \text{ M at } 25^\circ C$$

At $25^\circ C$, $K_w$ remains constant at $1 \times 10^{-14}$ M, therefore, if the concentration of either the hydrogen or hydroxide ions is known, the pH may be calculated.

pH 7 is considered to be neutral as $[H^+] = [OH^-] = 10^{-7}$ M.

**Calculate the pH of a 0.05 M NaOH solution.**

NaOH is a strong base, therefore, it will fully dissociate i.e. 1 mole of NaOH will produce 1 mole of $Na^+$ and 1 mole of $OH^-$ ions.

1. Using the concentration of $[OH^-]$ ions formed.

$$pOH = -\log[OH^-]$$
$$= -\log 0.05$$
$$= 1.3$$

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pH $= 14 - \text{pOH}$

$= 14 - 1.3$

$= 12.7$

2. By calculating the concentration of $[H_+]$ ions using the water dissociation constant $K_w$.

$$K_w = [H^+][OH^-] = 1 \times 10^{-14} \text{ M}$$

$$[H^+]0.05 = 1 \times 10^{-14}$$

$$[H^+] = \frac{1 \times 10^{-14}}{0.05}$$

$= 2 \times 10^{-13} \text{ M}$

$$\text{pH} = -\log[H^+]$$

$= -\log2 \times 10^{-13}$

$= 12.7$

18.7. Calculating the pH of a Weak Acid Solution

Weak acids do not fully dissociate in water. Upon addition to water equilibrium is formed between the acid HA, hydrogen ions H$^+$ and the conjugate base A$^-$. The acid dissociation constant $K_a$ gives a quantitative measurement of the strength of the acid. The larger the value of $K_a$ the more the acid dissociates and, hence, the stronger the acid. Values for $K_a$ can span many orders of magnitude, therefore, a logarithmic measure of $K_a$ is more commonly used in practice, p$K_a$. The larger the p$K_a$ value the weaker the acid.

$$\text{HA}_{(aq)} \rightleftharpoons \text{H}^+_{(aq)} + \text{A}^-_{(aq)}$$

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

$$\text{p}K_a = -\log_{10}K_a$$
Weak acids do not fully dissociate in water making the calculation of pH slightly more complex.

**Calculate the pH of 0.05% formic acid (HCOOH).** \( pK_a = 3.75, \ K_a = 1.78 \times 10^{-4}, \ \rho = 1.22 \text{ gcm}^{-3}, \ MW = 46.025 \text{ gmol}^{-1} \).  

\[
\text{HCOOH}_{(aq)} \rightleftharpoons \text{H}^+_{(aq)} + \text{HCOO}^-_{(aq)}
\]

Firstly the molarity (moles per liter) of the solution must be calculated.

1. Calculate the mass of the solution.

\[
\text{Mass} = \text{volume} \times \text{density} = 1000 \text{ mL} \times 1.22 = 1220 \text{ g}
\]

2. Convert the percent mass of the solution to a decimal.

\[
\text{Mass \%} = 0.05\% = 5 \times 10^{-4}
\]

3. Calculate the number of moles in the solution.

\[
\text{Moles} = \frac{\text{mass} \times \text{mass \%}}{\text{MW}} = \frac{1220 \times 5 \times 10^{-4}}{46.025} = 0.0133 \text{ moles}
\]

4. Calculate the molarity of the solution.

\[
\text{Molarity} = \text{moles per liter} = 0.0133 \text{ M}
\]
To calculate the pH of the solution the concentration of $[H^+]$ ions must be determined; this can be done using the acid dissociation constant $K_a$.

$$K_a = \frac{[H^+][HCOO^-]}{[HCOOH]} = 1.78 \times 10^{-4}$$

Formic acid dissociates to give one $H^+$ ion for every $HCOO^-$ ion, therefore, $[H^+] = [HCOO^-]$.

Let

$x = [H^+]$

$C \cdot x = [HCOOH]$ where $C =$ initial concentration

The expression for $K_a$ can, therefore, be written as follows:

$$K_a = \frac{x \cdot x}{C - x} = \frac{x^2}{C - x}$$

Solve for $x$

$$x^2 = (C - x)K_a$$

$$= CK_a - K_ax$$

$$x^2 + K_ax - CK_a = 0$$

The equation is now in the form of a quadratic equation and can be solved as follows:

$$ax^2 - bx + c = 0$$

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Substitute $a = 1$, $b = K_a = 1.78 \times 10^{-4}$, and $c = CK_a \ (C = 0.0133 \ M)$.

$$x = \frac{-K_a + \sqrt{K_a^2 + 4CK_a}}{2}$$

$$= -1.78 \times 10^{-4} + \sqrt{(1.78 \times 10^{-4})^2 + 4(0.0133)(1.78 \times 10^{-4})}$$

$$= -1.78 \times 10^{-4} + \sqrt{9.50 \times 10^{-6}}$$

$$= -1.78 \times 10^{-4} + 9.50 \times 10^{-6}$$

$$= -1.78 \times 10^{-4} + 9.50 \times 10^{-6}$$
\[
\frac{2.904 \times 10^{-3}}{2} = x = [H^+] = 1.452 \times 10^{-3}
\]
\[
pH = -\log[H^+]
\]
\[
= -\log 1.452 \times 10^{-3}
\]
\[
= 2.84
\]

18.8. Calculating the pH of a Weak Base Solution

When weak bases are react with water to a very small extent when they are dissolved. An equilibrium is established between the unionized form of the base B, hydroxide ions OH\(^-\), and the conjugate acid BH\(^+\). Like acids the equilibrium can be described by a dissociation constant \(K_b\), which is similarly described as a logarithmic value \(pK_b\). Larger values for \(K_b\) indicate that the base is more reactive with water and, hence, it is a stronger base. The lower the value of \(K_b\) the stronger the base.

\[
B\text{(aq)} + H_2O\text{(aq)} \rightleftharpoons BH^+\text{(aq)} + OH^-\text{(aq)}
\]

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

\[
pK_b = -\log K_b
\]

Often values of \(K_a\) and \(pK_a\) are given in relation to bases. The \(K_a\) value relates to the conjugate acid in the following equilibrium (base \(B\), conjugate acid BH).

\[
BH\text{(aq)} \rightleftharpoons B^-\text{(aq)} + H^+\text{(aq)}
\]

\[
K_a = \frac{[H^+][B^-]}{[BH]}
\]

For example, the \(pK_a\) relates to the ammonium ion (NH\(_4^+\), conjugate acid) for ammonia (NH\(_3\), a weak base).

\[
NH_3\text{(aq)} + H_2O\text{(aq)} \rightleftharpoons NH_4^+\text{(aq)} + OH^-\text{(aq)}
\]

The \([OH^-]\) concentration can be calculated as follows:

\[
K_b = \frac{K_w}{K_a} = \frac{[OH^-][NH_4^+]}{[NH_3]}
\]
$K_w$, $pK_a$, $K_a$ and $pK_b$ can also be related as follows:

\[ K_w = K_a K_b = [H^+][OH^-] = 1 \times 10^{-14} \]

\[ pK_a + pK_b = pK_w \]

\[ pK_a + pK_b = 14 \]

<table>
<thead>
<tr>
<th>Base</th>
<th>Formula</th>
<th>$pK_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>NH$_3$</td>
<td>4.75</td>
</tr>
<tr>
<td>Aniline</td>
<td>C$_6$H$_5$NH$_2$</td>
<td>9.13</td>
</tr>
<tr>
<td>Methylamine</td>
<td>CH$_3$NH$_2$</td>
<td>3.36</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>CH$_3$CH$_2$NH$_2$</td>
<td>3.27</td>
</tr>
<tr>
<td>Pyridine</td>
<td>C$_5$H$_5$N</td>
<td>8.77</td>
</tr>
</tbody>
</table>

Table 13: Weak bases.

Calculate the pH of 0.1\% ammonium hydroxide (NH$_4$OH).

\[ pK_b = 4.75, \quad K_b = 1.78 \times 10^{-5}, \quad p = 0.88 \text{ g cm}^{-3}, \quad MW = 35.04 \text{ g mol}^{-1}. \]

Ammonium hydroxide is a solution of ammonia in water, also known as ammonia water, aqueous ammonia, ammoniacal liquor. It can be denoted as NH$_3$(aq) or in relation to its ionic composition [NH$_4$][OH$^-$]. In aqueous solution ammonia reacts with water to give ammonium hydroxide.

\[ \text{NH}_3(\text{aq}) + \text{H}_2\text{O}(\text{aq}) \rightleftharpoons \text{NH}_4^+(\text{aq}) + \text{OH}^-\text{(aq)} \]

Firstly the molarity (moles per liter) of the solution must be calculated.

1. Calculate the mass of the solution.

\[ \text{Mass} = \text{volume} \times \text{density} \]
\[ = 1000 \text{ mL} \times 0.88 \]
\[ = 880 \text{ g} \]

2. Convert the percent mass of the solution to a decimal.

\[ \text{Mass } \% = 0.1\% = 1 \times 10^{-3} \]

3. Calculate the number of moles in the solution.

\[ \text{Moles} = \frac{\text{mass} \times \text{mass } \%}{\text{MW}} \]
\[ = \frac{880 \times 1 \times 10^{-3}}{35.04} \]
\[ = 0.0251 \text{ moles} \]
4. Calculate the molarity of the solution.

Molarity = moles per liter

= 0.0251 M

To calculate the pH of the solution the concentration of \([\text{OH}^-]\) ions must be determined; this can be done using the acid dissociation constant \(K_b\).

\[ K_b = \frac{[\text{NH}_4^+][\text{OH}^-]}{[\text{NH}_3]} = 1.78 \times 10^{-5} \]

The reaction of ammonium with water to give ammonium hydroxide will yield one \(\text{NH}_4^+\) ion for every \(\text{OH}^-\) ion, therefore, \([\text{NH}_4^+] = [\text{OH}^-]\).

Let

\( x = [\text{OH}^-] \)

\( C - x = [\text{NH}_3] \) where \( C \) = initial concentration

The expression for \(K_b\) can, therefore, be written as follows:

\[ K_b = \frac{x \cdot x}{C - x} \]

\[ = \frac{x^2}{C - x} \]

Solve for \(x\)

\[ x^2 = (C - x)K_b \]

\[ = CK_b - K_b x \]

\[ x^2 + K_b x - CK_b = 0 \]

The equation is now in the form of a quadratic equation and can be solved as follows:

\[ ax^2 - bx + c = 0 \]

\[ x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \]

Substitute \( a = 1 \), \( b = K_b = 1.78 \times 10^{-5} \), and \( c = CK_a \) (\( C = 0.0251 \) M).

\[ x = \frac{-K_b + \sqrt{K_b^2 + 4CK_b}}{2} \]
\[
= -1.78 \times 10^{-5} + \sqrt{(1.78 \times 10^{-5})^2 + 4(0.0251)(1.78 \times 10^{-5})} \\
= -1.78 \times 10^{-5} + \frac{\sqrt{1.787 \times 10^{-6}}}{2} \\
= \frac{1.319 \times 10^{-3}}{2} \\
\]
\[x = [\text{OH}^-] = 6.59 \times 10^{-4}\]

As was seen previously with a strong base the pH of the solution can be calculated in two ways.

1. Using the concentration of [OH\(^-\)] ions formed.

\[
p\text{OH} = -\log[\text{OH}^-] \\
= -\log 6.59 \times 10^{-4} \\
= 3.18 \\
pH = 14 - p\text{OH} \\
= 14 - 3.18 \\
= 10.8
\]

2. By calculating the concentration of [H\(^+\)] ions using the water dissociation constant \(K_w\).

\[
K_w = [\text{H}^+][\text{OH}^-] = 1 \times 10^{-14} \text{ M} \\
[\text{H}^+]6.59 \times 10^{-4} = 1 \times 10^{-14} \\
[\text{H}^+] = \frac{1 \times 10^{-14}}{6.59 \times 10^{-4}} \\
= 1.517 \times 10^{-11} \text{ M} \\
pH = -\log[\text{H}^+] \\
= -\log 1.517 \times 10^{-11} \\
= 10.8
19. Getting Started with Ionizable Compounds

A set of suggested starting conditions for the analysis of weakly ionizable compounds is shown (Table 14). Under these conditions it MAY be possible to analyze weak acids and weak bases simultaneously – however, this will depend on finding a suitable organic modifier composition.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td></td>
</tr>
<tr>
<td>Dimensions</td>
<td>4.6 x 150 mm</td>
</tr>
<tr>
<td>Particle size</td>
<td>Traditional packing material 5 or 3.5 µm</td>
</tr>
<tr>
<td>Bonded phase</td>
<td>Core-shell 4 µm C18 or C8</td>
</tr>
<tr>
<td><strong>Mobile Phase</strong></td>
<td></td>
</tr>
<tr>
<td>Solvents</td>
<td>Aqueous buffer/MeCN</td>
</tr>
<tr>
<td>%B</td>
<td>Variable (k = 1-20)</td>
</tr>
<tr>
<td>Buffer</td>
<td>25 mM, pH 3, 5, 7</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1-2 mL/min</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 °C</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>&lt; 50 µL</td>
</tr>
<tr>
<td>Mass</td>
<td>&lt; 25 µg</td>
</tr>
</tbody>
</table>

Table 14. Suggested starting conditions for the analysis of ionizable analytes

The column (C18 or C8) is chosen as it is highly hydrophobic, has a standard particle size, and contains enough phase to indicate the possible quality of the optimized separation, i.e. the analyte will have the opportunity to interact sufficiently with the stationary phase so that selectivity can be judged.

The buffer chosen should be effective at the desired pH. If method development is being carried out for LC-MS a volatile buffer must be used. Carrying out the separation over a range of pH values will help to decide which pH will give the optimum resolution if a mixture of analytes (i.e. neutral, basic, and acid) are being analyzed. This is discussed in more detail in the section Optimizing Separation Selectivity of Ionizable Analytes. At low pH < 3 most weak organic acids will be at least 90% non-ionized, whilst most basic analytes will be fully ionized (protonated), the pH will ensure improved peak shape as silica surface silanol groups will be uncharged.

Temperature is chosen as above ambient to ensure improved efficiency and ease of control. Sample volume and mass are chosen to avoid peak overload. Even if you are working at ‘ambient’ temperatures a thermostated column compartment should always be used, where possible. This will guard against retention time changes caused by the ‘ambient’ temperature changing throughout the day.
20. Optimizing Separation Selectivity of Ionizable Analytes

In reversed phase method development with ionizable compounds, plotting retention \( k \) against pH for the various analytes in the separation can be a very enlightening (Figure 45). This can help even with fairly complex analyte mixtures as can be seen in Figure 45.

![Figure 45: Effect of pH on analyte retention.](image)

**Column:** C\(_{18}\) (300 x 4 mm, 10\( \mu \)m)

**Mobile phase:** 40% methanol/0.025 M phosphate buffer

**Flow rate:** 2 mL/min

**Injection:** 2 µL (1-10 \( \mu \)g of analyte)

**Detector:** UV, 220 nm

There are several observations from the pH vs. \( k \) plot that highlights the usefulness of this approach to optimizing separation selectivity.

- With no indication of peak efficiency, it can be assumed that as lines become closer together the separation (and hence the resolution) between analytes reduces.
- Crossing lines indicate co-elution of the analytes and, therefore, the pH would not suitable for the separation.
- At pH 3 all analytes are separated, although the peaks for some of the analytes are fairly close and good peak efficiency would be essential to ensure the required resolution between all peak pairs. More importantly, the response curves for all analytes are fairly flat – the importance of this will become evident in the next two points.
At pH 5 the peaks are all separated (in terms of selectivity). However the response curve of acetylsalicylic acid is changing with pH which is of great concern for the robustness of the method. Small changes in mobile phase pH, which can be expected, will lead to large changes in analyte retention and, therefore, potential loss of resolution between analytes – which is to be avoided at all costs in method development.

At pH 7 a similar situation is observed with amphetamine and ephedrine which exhibit rapidly changing response curves.

It should also be noted that when deciding on a pH at which to run the separation the column pH working range will need to be considered - even when inside the working range of the column, but close to either the upper or lower limit, column lifetime can be expected to be reduced.

Conclusion – analysis of these compounds (provided peak efficiency is good) at pH 5 should produce a robust separation.

Although there is no indication of peak efficiency or area, theoretical chromatograms of the separations at the various pH values have been generated. It can be seen that as the pH changes the retention and selectivity of the separation are affected. At pH 7 acetylsalicylic acid and sulphanilamide elute very close together, therefore, it could be postulated that if the peaks were not eluted with high efficiency they would overlap resulting in difficulty with integration etc. At pH 9 sulphanilamide and barbitone co-elute.

There are several online and print resources for obtaining physical and chemical data for known compounds including:

**Websites**

- http://www.chemicalize.org
- http://www.chemspider.com
- http://webbook.nist.gov/chemistry

**Books**

- CRC Handbook of Chemistry and Physics
- Lange’s Handbook of Chemistry
21. Effect of Temperature on Reversed Phase HPLC

Temperature can affect retention and selectivity of a separation (Figure 46). An increase in temperature will reduce retention times; this will allow the ability to work at higher flow rates (Figure 47). The ability to work at higher flow rates is attributable to a reduction in viscosity of the mobile phase as the temperature is increased (Figure 48 and Table 15). This reduces back pressure and reduces the effect of the ‘C’ term of the Van Deemter equation, due to the improvement of mass transfer kinetics (i.e. the movement of analyte molecules in and out of the stationary phase pores). Column performance may also increase due to reduced solvent viscosity, system backpressure, and the ability to utilize faster flow rates. The separation factor can either increase or decrease depending on the analyte and, hence, selectivity can be altered, this is particularly prevalent with ionizable analytes as $pK_a$ is temperature dependent.

![Figure 46: The effect of temperature on selectivity.](image)

![Figure 47: Van Deemter curves for various temperatures.](image)
There are of course some disadvantages to working at higher temperatures including; solvent or sample decomposition, increased risk of bubbles in the detector due to increased solvent vapor pressure (this may manifest itself as a rising or erratic baseline, ghost peaks, or incomplete light adsorption). Silica solubility increases with temperature (> 60 °C for chemically bonded phases). Poor reproducibility can also result if thermostating is inadequate. Both pH and pKₐ are temperature dependent which may alter chromatographic parameters. When working at elevated temperatures the flow rate may need to be increased to maintain the optimum linear velocity due to the decrease in solvent viscosity. At increased pressure, the boiling point of a solvent will also increase (Table 16 and Figure 49). Therefore, under HPLC conditions the boiling point of common solvents will be much higher than usual, allowing for column temperatures that exceed the normal boiling point of the solvent to be used. However, to do this a backpressure regulator must be installed at the end of the system, i.e. after the UV detector, to suppress boiling of the mobile phase in the column.
Figure 49: Effect of pressure on boiling point of MeCN and MeOH.

<table>
<thead>
<tr>
<th>Pressure (bar)</th>
<th>Boiling Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>10</td>
<td>186</td>
</tr>
<tr>
<td>50</td>
<td>305</td>
</tr>
<tr>
<td>100</td>
<td>378</td>
</tr>
<tr>
<td>150</td>
<td>430</td>
</tr>
<tr>
<td>200</td>
<td>472</td>
</tr>
<tr>
<td>250</td>
<td>508</td>
</tr>
<tr>
<td>300</td>
<td>541</td>
</tr>
<tr>
<td>350</td>
<td>570</td>
</tr>
<tr>
<td>400</td>
<td>598</td>
</tr>
<tr>
<td>450</td>
<td>624</td>
</tr>
<tr>
<td>500</td>
<td>648</td>
</tr>
<tr>
<td>1000</td>
<td>848</td>
</tr>
</tbody>
</table>

Table 16: Boiling points of common HPLC solvents at various pressures.

A liquid boils when its vapor pressure exceeds the pressure acting on it, therefore, the column pressure must be kept above the vapor pressure of the mobile phase to prevent it from boiling. A back-pressure regulator of approximately 30 bar is usually sufficient to keep typical mobile phases from boiling. The increase in solvent boiling point with pressure results from an increase in the vapor pressure. The change in vapor pressure of a pure substance can be calculated using the Clausius-Clapeyron Equation (Equation 1).
\[ \ln \frac{P_2}{P_1} = \frac{\Delta H_{vap}}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \]  

(1)

Where:

- \( P_1 \) = the partial pressure of the liquid at \( T_1 \)
- \( P_2 \) = the partial pressure of the liquid at \( T_2 \)
- \( \Delta H_{vap} \) = enthalpy of vaporization (kJmol\(^{-1}\))
- \( R \) = gas constant (8.314 JK\(^{-1}\)mol\(^{-1}\))

<table>
<thead>
<tr>
<th>Solvent</th>
<th>( \Delta H_{vap} ) (25 °C) (kJ/mol)</th>
<th>Boiling Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>29.75</td>
<td>81.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>38.56</td>
<td>78.3</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>39.85</td>
<td>82.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>35.21</td>
<td>64.7</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>29.81</td>
<td>66.0</td>
</tr>
<tr>
<td>Water</td>
<td>40.65</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 17: Enthalpy of vaporization (\( \Delta H_{vap} \)) for various solvents. Note: Enthalpies are generally fairly constant with temperature.

There are several resources that can be used if the boiling point of a solvent at different pressures are required.


22. Glossary

**Mobile phase** - The fluid that moves analytes through the HPLC column. In HPLC the mobile phase will interact with both the analyte and the stationary phase, therefore, it can be very influential on the chromatographic separation.

**Polarity** - Bonds within a molecule will be polar due to a difference in electronegativity of the two atoms. The difference in charge produces a dipole (i.e. in HCl). Non-polar bonds will have an electronegativity difference \(< 0.4\) and polar bonds will have an electronegativity difference between 0.4 and 1.7.

Electronegativities Pauling scale

Download a PDF of the periodic table here:


Molecular polarity is a result of the shape and the charge distribution in a molecule. If the arrangement of atoms within the molecule is symmetric the charges will be balanced, therefore, the molecule is non-polar. If there is an asymmetric arrangement of bonds within the molecule the charges will not be balanced and the molecule is polar.
Polar molecules are generally soluble in water because of the polar nature of water (like dissolves like).

**Electronegativity** - Electronegativity was a concept introduced by L. Pauling and describes the tendency of an atom or group to attract electrons (or electron density) towards itself. There are several definitions and values for electronegativity in use, therefore, different values may be seen depending on the scale used. According to Mulliken it is the average of the ionization energy and electron affinity of an atom. The Pauling scale is more commonly used where the dimensionless relative electronegativity differences are defined based on the bond dissociation energies.

**Stationary phase** - Is the substance which is fixed in place within the HPLC column. The stationary phase can be a solid, a bonded, immobilized, or coated phase on a solid support, or a wall coated phase. The stationary phase used will characterize the separation mode i.e. silica gel is used in adsorption chromatography (normal phase) while octadecysilane bonded phase is used in reversed phase chromatography.

**Hydrophobicity** - “Water fearing”. Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules. In terms of HPLC hydrophobic stationary phases will not be compatible with water and hydrophobic molecules will be insoluble in water. Hydrophobic molecules have few polar functional groups and most have high hydrocarbon content (aliphatic and aromatic).

**Hydrophilicity** - “Water loving”. Hydrophilicity is the tendency of a molecule to be solvated by water. In terms of HPLC hydrophilic stationary phases will be fully compatible with water and hydrophilic molecules will be soluble in water. Hydrophilic molecules will contain polar functional groups.

**Lipophilic** - “Fat loving”. Lipophilic molecules have a tendency to dissolve in fat-like (e.g. hydrocarbon) solvents. These molecules will have a high hydrocarbon content.

**Lipophilicity** - Lipophilicity is a measure of the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by the distribution of the molecule within a biphasic system, e.g. partition coefficient in 1-octanol/water.
Lipophobic - “Fat fearing”. Lipophobic molecules will not be soluble in lipids or non-polar solvents.

Theoretical plate - A hypothetical entity which exists within a chromatographic column that is analogous to a fractional distillation. As analytes move through the column they partition between the stationary and mobile phase. Although this is a continuous process a stepwise model is often visualized. One step roughly corresponds to a theoretical plate.

Ionizable - An ionizable molecule can be converted into ions. In terms of HPLC molecules with acidic or basic functional groups are said to be ionizable as a change in pH will result in the production of a charged species.

Ionic - An ionic bond will contain two atoms with very different electronegativities.

Buffer - A buffer is a weak acid or base in co-solution with its salt – an example may be acetic acid (the weak acid) and sodium acetate (its salt). A known weight of the salt is usually added to the mobile phase to achieve a known concentration – the weak acid or base is then added to the mobile phase (with stirring), until the desired pH is achieved.

Ion pairing - Ion pair chromatography is a type of chromatography in which ions in solution can be paired or neutralized and separated as an ion pair on a reversed phase HPLC column. Ion pairing reagents are usually ionic compounds which contain a hydrocarbon chain which impacts a degree of hydrophobicity resulting in retention on a reversed-phase column.

Dalton - An arbitrary unit of mass, being 1/12th the mass of the nuclide of carbon-12, equivalent to 1.657 x 10^{-24} g. Symbol D or Da. Called also atomic mass unit.

Octadecylsilane (ODS, C_{18}) - The most popular reversed phase in HPLC and consists of a C_{18} carbon chain. Octadecylsilane phases are bonded to silica or polymeric packings.

Homolog - Compounds differing regularly by the successive addition of the same chemical group, e.g., by –CH (i.e. ethane (CH\textsubscript{3}CH\textsubscript{3}), propane (CH\textsubscript{3}CH\textsubscript{2}CH\textsubscript{3}), butane (CH\textsubscript{3}(CH\textsubscript{2})\textsubscript{2}CH\textsubscript{3}), pentane (CH\textsubscript{3}(CH\textsubscript{2})\textsubscript{3}CH\textsubscript{3})).

Benzologs - Substances having the same aromatic core structure but with differing substituent groups or the same substituents with differing positional isomerism.

Mixed-mode chromatography - Mixed-mode separations are carried out on a column which can utilize more than one form of interaction between the analyte and stationary phase in order to obtain separation.
**Achiral** - The mirror images of molecules which are achiral can be superimposed on each other. These molecules will often have a plane of symmetry or a center of symmetry.

![Plane of Symmetry](image)

**Stereoisomers** - Molecules which are stereoisomers will have the same atoms (same molecular formula) and connectivity within the molecule, however, they will have different spatial arrangements.

![cis-2-Butene](image) ![trans-2-Butene](image)

**Structural isomers** - Structural isomers will have the same molecular formula, however, the bond connectivity will differ.

![Propanal](image) ![Propanone](image)
**Enantiomer** - Enantiomers are mirror images of each other and non-superimposable.

**Diastereomers** - Unlike enantiomers diastereomers are not mirror images of each other and are non-superimposable. Diastereomers can have different physical properties (i.e. melting or boiling point, density) and different reactivity. They have two or more stereocenters.

**Positional isomers** - Molecules which are positional isomers will have the same carbon skeleton, however, if there are functional groups these will be in different positions.

**Chiral** - The word chiral comes from the Greek work *cheir* which means hand. Chiral molecules cannot be superimposed on their mirror image. Chiral molecules will often contain an asymmetric carbon atom (denoted * in the carvone molecules shown). The two forms of a chiral molecule will often have different properties; taste, biological activity, reactivity etc. A great deal of effort is made by chemists to either synthesize only one of the chiral forms or to separate a single molecule from a mixture of both chiral forms (called a racemic mixture).
Cyclodextrin - Cyclodextrins are a family of compounds which are made up of a cyclic arrangement of sugar molecules.

![β-Cyclodextrin](image)

**Esters**

\[ R^+\text{O}^- R' \]  
R and R' = alkyl or aryl group

**Aldehyde**

\[ R^\text{CH} \]  
R = alkyl or aryl group

**Ketone**

\[ R^\text{CO} R' \]  
R and R' = alkyl or aryl group

**Amine**

- Primary
- Secondary
- Tertiary
- Quarternary

R, R', R'', and R''' = alkyl or aryl group
Alcohol

\[ R^+\text{OH} \]

\( R = \text{alkyl or aryl group} \)

Phenols

\[
\begin{array}{c}
\text{OH} \\
\text{R} \\
\end{array}
\]

\( R = \text{alkyl or aryl group} \)

Carboxylic acid

\[
\begin{array}{c}
\text{O} \\
\text{R} \\
\text{OH} \\
\end{array}
\]

\( R = \text{alkyl or aryl group} \)

**Lewis acid** - Is an electron pair acceptor

\[ A^+ + B^- \rightarrow AB \]

Lewis acid  (electron pair acceptor)  Lewis base  (electron pair donor)

**Lewis base** - Is an electron pair donor

\[ A^+ + B^- \rightarrow AB \]

Lewis acid  (electron pair acceptor)  Lewis base  (electron pair donor)
Resolution ($R_S$) - Resolution is a function of three parameters, retention factor ($k$), selectivity ($\alpha$), and efficiency ($N$) and is defined by the fundamental resolution equation.

$$R_S = \frac{\sqrt{N}}{4} \left( \frac{k}{k + 1} \right) \left( \frac{\alpha - 1}{\alpha} \right)$$

A resolution value of 1.5 or greater between two peaks will ensure that the sample components are baseline separated to a degree at which the area or height of each peak may be accurately measured.

$$R_S = 2 \left( \frac{t_{R2} - t_{R1}}{w_{b1} + w_{b2}} \right) = 1.18 \frac{(t_{R2} - t_{R1})}{w_{1/2(1)} + w_{1/2(2)}}$$

Where:

$t_R$ = peak retention time  
$w$ = peak width at the base  
$w_{1/2}$ = peak width at half the peak height

Retention factor ($k$) - The retention factor is a means of measuring the retention of an analyte on the chromatographic column. The retention factor is equal to the ratio of the retention time of the analyte on the column to the retention time of an unretained compound. The unretained compound has no affinity for the stationary phase and elutes with the solvent front at time $t_0$, which is also known as the hold-up time.

$$k = \frac{(t_R - t_0)}{t_0}$$

Where:

$t_R$ = peak retention time  
$t_0$ = hold-up time (the time required for an unretained compound to be eluted or the time required for one column volume of mobile phase to pass through the column)

Selectivity ($\alpha$) - The selectivity factor is the ability of the chromatographic system to chemically distinguish between sample components. It is measured as the ratio of the retention factors $k$ of the two peaks in question and can be visualized as the distance between the apices of the two peaks. By definition, the selectivity is always greater than one, as when $\alpha$ is equal to one the two peaks are co-eluting.

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

Where:

$t_R$ = peak retention time  
$t_0$ = hold-up time (the time required for an unretained compound to be eluted or the time required for one column volume of mobile phase to pass through the column)
**Efficiency** ($N$) - Efficiency is a measure of the column to produce sharp, well defined peaks. More efficient columns will have more theoretical plates ($N$) and smaller theoretical plate heights ($H$).

\[ N = 16 \left( \frac{t_R}{w} \right)^2 = 5.54 \left( \frac{t_R}{w_{1/2}} \right)^2 \]

Where:

$t_R$ = peak retention time  
$w$ = peak width at the base  
$w_{1/2}$ = peak width at half the peak height

**Hydrodynamic volume** - The hydrodynamic volume of a molecule can be defined as the effective molecular diameter in free solution where the hydrodynamic sphere would be a sphere defined by the molecule as it revolves around its central axis in solution. In size exclusion chromatography (SEC) this can be particularly useful in rationalizing differences in elution order of molecules which have the same molecular weight.

**Trifluoroacetic acid** – a well-known mobile phase additive often used (incorrectly) as a buffer. Due to its very low $pK_a$, the abundant trifluoroacetate ion can form strong ion pairs with positively charged analyte(s), decreasing sensitivity. For this reason, TFA should be avoided in samples with a very low concentration of analyte(s).

**Vapor pressure** – The vapor pressure of a liquid is the equilibrium pressure of a vapor above its liquid (or solid); that is, the pressure of the vapor resulting from evaporation of a liquid (or solid) above a sample of the liquid (or solid) in a closed container. The vapor pressure of a liquid will vary with temperature.
23. References
