

7. Separation Mechanisms

The objective of this section is to:

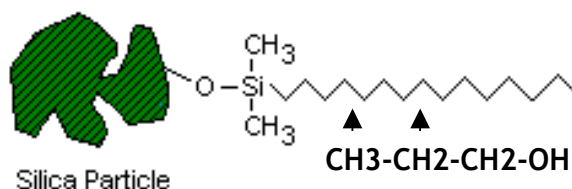
- Describe the types of stationary phase, mobile phase and retention mechanism for:
- Partition (Reversed-phase) chromatography including:
 - Ion suppression and ion pair RP chromatography
 - Adsorption (normal phase) chromatography
 - Ion exchange chromatography
 - Size exclusion chromatography
 - Chiral chromatography
- Discuss practical applications of each of these different modes of chromatography.

7.1 Partition (Reversed-phase) Chromatography

7.1.1 Neutral RP Chromatography

Despite the fact that over 80% of all published HPLC methods are based upon reversed-phase mechanisms, its mode of operation is still not fully understood. In reversed-phase chromatography the retention mechanism is between the column's stationary phase and sample analytes' respective hydrophobic moieties.

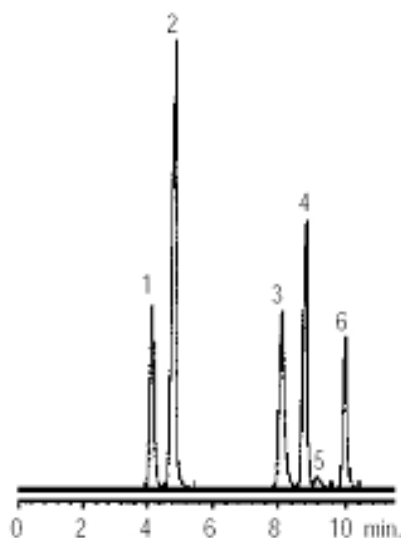
This mechanism is collectively known as "non-specific hydrophobic interactions", and is illustrated in the figure below that shows the propanol analyte binding to the long chain hydrocarbon of the column stationary phase through its hydrophobic saturated carbon chain.



Analyte retention increases with increasing stationary phase carbon chain length. Some typical bonded phases used in reversed-phase HPLC are described below:

Application Examples

The application example shown below is the gradient separation of the Procainamide class of basic drugs by ion suppression reversed-phase chromatography:



Separation conditions:

Sample

Procainamides:

1. Tocainamide
2. Procainamide
3. Quinine
4. Disopyramide
5. Dihydroquinine
6. Diphenhydramine

Stationary phase:

Asahipak ODP-50

Column:

250 x 4.0mm i.d. (5 μ m)

Mobile phase:

Solvent A = Buffer (pH12) 1:19 diluted.

Solvent B = Acetonitrile

Flowrate:

1mL/min

Gradient:

Linear from 10–70% B in 8min

Detection:

UV

Advantages of ion suppression reversed-phase chromatography:

- Low cost.
- Ease of use.
- pH modified reversed-phase conditions.

Limitations of ion suppression reversed-phase chromatography:

- Not suitable for strong acids or bases.
- Not suitable for samples containing both weak acids and weak bases.

In Summary

Ion Suppression Reversed-phase Chromatography

Stationary phase	Non-polar
Mobile phase	Polar, becoming increasingly non-polar
Mobile phase pH modifier	Added to suppress analyte ionisation
Analyte elution order	Polar analytes first

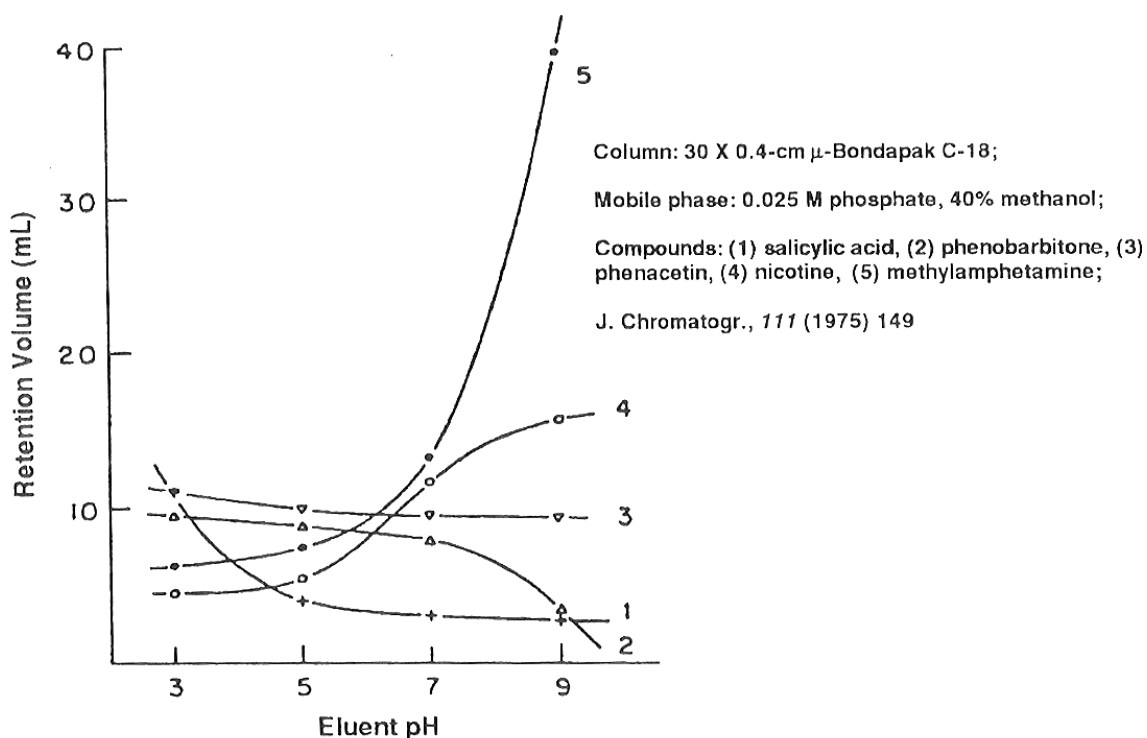
that are easily optimised. Additionally, it helps reduce analyte secondary interactions with residual silanol groups, eliminating excessive retention and improving chromatographic peak shape.

Typical buffer solutions and their effective buffering pH range for basic and acidic compounds are illustrated below:

Ion Suppression Reversed-phase

Basic	Neutral	Acidic
1% NH ₄ OH Acetic Acid NH ₄ Acetate pH 7-9	H ₂ O / Acetonitrile H ₂ O / Methanol	1% KH ₂ PO ₄ pH 5-7

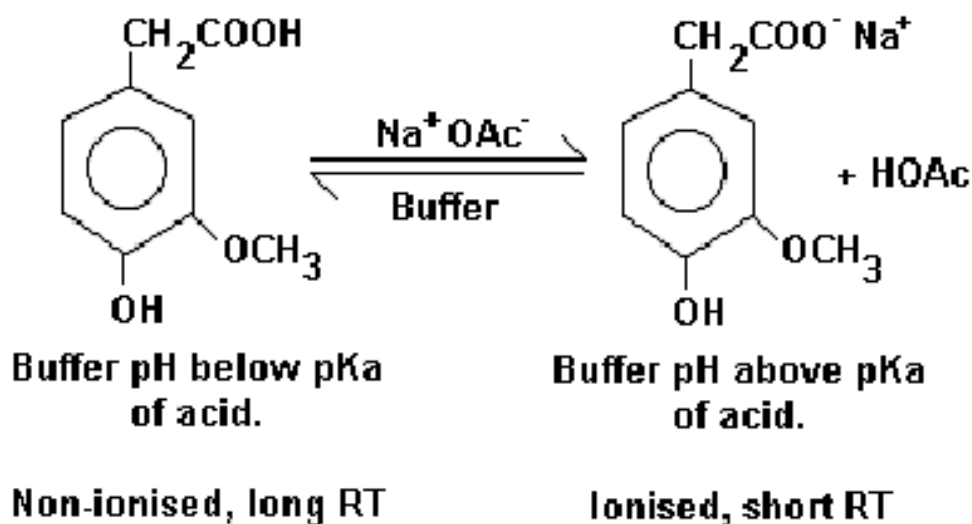
It is interesting to note that adjusting the pH may dramatically alter the selectivity between similar compound classes, so that one analyte is ionised to a greater extent relative to another. This scenario is illustrated below:



7.1.2 Ion Suppression RP Chromatography

Principles of Ionisation

Shown below are the two ionisation states of homovanillic acid. On the left hand side the molecule is in its non-ionised or "ion suppressed" form, while on the right hand side it is in its ionised form. Buffer solutions are used to control whether a molecule is ionised or not.



Ion Suppression

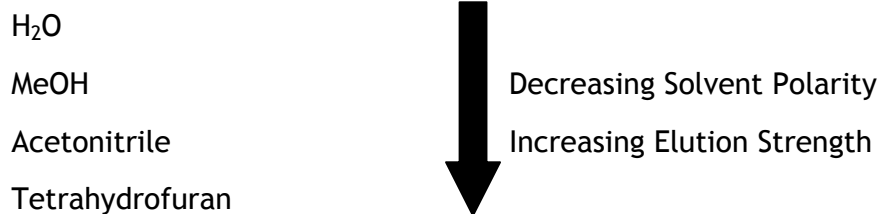
When chromatographing ionisable compounds it is advantageous to have them in their ion suppressed form. Ionised compounds represent highly polar analytes, which under reversed-phase conditions would elute very quickly from the column offering little chance of separation from other ionised analytes.

By using buffer solutions and adjusting the pH to force compounds into their ion suppressed forms, then weak acids and bases may be efficiently chromatographed under reversed-phase conditions.

The pK_a of a molecule can be used to determine the correct pH at which to buffer the mobile phase:

- For organic acids i.e. ethanoic acid $pK_a = 4.75$ the pH is adjusted *below* its pK_a .
- For organic bases i.e. trimethylamine $pK_a = 4.19$ the pH is adjusted *above* its pK_a .

The use of reversed-phase conditions means that the chromatographic separation can be performed in a cost effective way, employing conditions



An empirical approach to fundamental HPLC method development is to initially apply a standard "scouting" gradient run to the sample separation. The purpose of the "scouting" gradient run is to generate an average analyte capacity factor of 5–6.

i.e using a 4.6 x 150mm column (void volume = 1.5mL) with a full-range gradient (5–100% B) and a flowrate of 2mL/min, then a gradient time of approximately 20min will generate the required average capacity factor value of 5.5:

$$t_g = 5.5(k^*) \times 95(\Delta\%B) \times 1.5(V_m) \times 5 / 100 \times 2\text{mL/min}$$
$$t_g = \sim 20\text{min}$$


If the retention time difference (Δt_R) between the first and final eluting chromatographic peaks on division by the total gradient time is ≥ 0.25 then gradient elution is recommended. However, if $\Delta t_R/t_G \leq 0.25$ then an isocratic separation is possible.

Sometimes it is not possible to achieve the chromatographic resolution required using the original choice of organic modifier, and another has to be chosen to alter the selectivity of the separation system. Solvent selection tables are available which show equivalent solvent strengths to assist in such selection. Occasionally a blend of different organic modifiers may be required.

In Summary

Neutral Reversed-phase Chromatography

Stationary phase	Non-polar
Mobile phase	Polar, becoming increasingly non-polar
Analyte elution order	Polar analytes first

$-\text{Si}-(\text{CH}_3)_2 - \text{C}_{18}\text{H}_{37}$	C18 (ODS – Octadecylsilane)		Increasing
$-\text{Si}-(\text{CH}_3)_2 - \text{C}_8\text{H}_{17}$	C8 (Octyl)		Polarity
$-\text{Si}-(\text{CH}_3)_2 - \text{C}_2\text{H}_5$	C2		
$-\text{Si}-(\text{CH}_3)_2 - \text{Ph}$	Phenyl		Decreasing
$-\text{Si}-(\text{CH}_3)_2 - (\text{CH}_2)_3 - \text{CN}$	Cyano		Retention

Stationary phase selection criterion is highly dependent on the particular application and separation aims. In general:

1. C18 (Octyldecylsilane - ODS) Bonded Ligand
The most popular and functional of the available reversed-phase stationary phases.
Particularly useful for the separation of non-polar analyte species.
2. C8 Bonded Ligand
While less non-polar than the C18, it provides similar selectivity while giving appreciably shorter run times.
3. C4 / C3 Bonded Ligand
These shorter-chain hydrocarbons are not as stable or as retentive as their longer chain C18 and C8 counterparts, but provide good separations for a variety of protein and polypeptide analytes.
4. CN (Cyano) Bonded Ligand
Interacts with polar functional groups, allowing its use in both reversed-phase and normal phase chromatography.
5. NH_2 (Amino) Bonded Ligand
Interacts with polar functional groups. Commonly used for the separation of sugars and polysaccharides.
6. Phenyl Bonded Ligand
Exhibits a more polar nature than either the C18 or C8, the π electron clouds providing sites of interaction for a variety of aromatic (ring) analytes.

Mobile phases used in reversed-phase chromatography are polar to ensure analyte retention. Subsequently, the mobile phase is modified by decreasing the proportion of H_2O present, achieved by the addition of an organic solvent modifier such as MeOH or acetonitrile, which causes the retained analyte(s) to elute off the stationary phase. Changing the mobile phase composition in this way is the most effective way of achieving chromatographic resolution, with analyte elution order governed by the H_2O solubility of the molecule and its carbon content.