SECTION 8 Normal-phase & Ion-exchange
Normal-phase chromatography by definition refers to an LC separation in which the stationary phase is more polar than the mobile phase. In that sense, it is the “reverse” of reversed-phase LC. Retention in normal-phase chromatography is based on attraction between polar functional groups on the analyte molecules and polar sites on the column packing.
Samples with Poor RP Retention

- **Hydrophilic, non-ionic compounds**
  - Normal-phase HPLC (silica, cyano, diol, amino)
  - HILIC (poly-aspartate or silica)

- **Very hydrophobic compounds**
  - Non-aqueous RP-HPLC (NARP)
  - Normal-phase HPLC

Some samples show poor reversed-phase retention; the sample may be unretained with water as mobile phase, or strongly retained with methanol or acetonitrile as the mobile phase. In these cases, some other approach is necessary.

Very polar, nonionic molecules are usually unretained with any reversed-phase solvent. However these compounds will then be strongly retained by normal-phase HPLC. There are two kinds of columns for normal-phase HPLC: (a) traditional columns such as silica or polar bonded-phase packings which are used with less polar mobile phases; (b) HILIC columns which are used with water-organic mobile phases (see below).

Very hydrophobic samples can be separated on reversed-phase columns with less polar (nonaqueous) mobile phases such as methylene chloride mixed with methanol or acetonitrile. Sometimes this is called NARP (Non-Aqueous Reversed Phase).

The latter mobile phases are much stronger than water-organic mixtures.

Very hydrophobic samples may also show good retention under normal-phase conditions.
Other Reasons to Use Normal-phase Chromatography

- solubility
- compatibility with workup
- elution order changes
- samples with nonpolar garbage
- samples where structural isomerism is important
- complex samples (multidimensional separation in conjunction with reversed-phase)

As we have already seen, reversed phase columns operate by a mixed mechanism which confers a certain amount of selectivity for structural differences. This versatility has made reversed-phase the first-choice technique in most applications. In many cases, however, normal-phase may be a better choice. Some of the reasons for using normal-phase chromatography are given here.
Normal-Phase HPLC

- Column more polar than mobile phase
- MP strength increases for more polar solvents
- More polar analytes elute last
- Recommended for:
  - carbohydrates, other neutral hydrophilic species, isomers
  - samples were RP was unsuccessful

Normal-phase HPLC uses more polar columns and less polar mobile phases. Sample retention decreases as the mobile phase becomes more polar (the opposite of reversed-phase HPLC). Sample retention order is usually reversed in going from reversed-phase to normal phase HPLC. Because retention is so different, large changes in selectivity often result in going from reversed-phase to normal-phase HPLC.
### Effect of Functional Group Polarity

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Compound</th>
<th>k’</th>
</tr>
</thead>
<tbody>
<tr>
<td>-OCH$_3$</td>
<td>2-methoxynaphthalene</td>
<td>0.6</td>
</tr>
<tr>
<td>-NO$_2$</td>
<td>1-nitronaphthalene</td>
<td>1.8</td>
</tr>
<tr>
<td>-CN</td>
<td>1-cyanonaphthalene</td>
<td>2.7</td>
</tr>
<tr>
<td>-COCH$_3$</td>
<td>1-acetonaphthalene</td>
<td>5.5</td>
</tr>
</tbody>
</table>

As we would expect from the normal-phase mechanism, the order of elution is based primarily on polarity, as evidenced by this table of retention. Simply put, the more polar the functional group, the higher the k’.
Effect of Number of Functional Groups

(1) 2-methoxynaphthalene \( k' = 0.6 \)
(2) 1,7-dimethoxynaphthalene 1.4

(1) 1-nitronaphthalene 1.8
(2) 1,5-dinitronaphthaline 6.1

Similarly, adding polar functional groups substantially increases retention.
Effect of Functional Group Orientation

m-dibromobenzene $k' = 3.8$

p-dibromobenzene 6.9

quinoline $k' = 5.4$

isoquinoline 18.6

1,2,3,4-dibenzanthracene $k' = 0.6$

picene 12.0

Perhaps more surprising is the fact that the orientation of functional groups can make a significant difference in retention. Unlike reversed-phase, normal-phase chromatography is driven by attraction between polar functional groups on the analyte and polar groups on the column surface. Subtle differences in orientation of polar functional on the analyte can affect the way in which they "line up" with the column surface.
Reversed-phase chromatography separates primarily on the basis of hydrophobicity, and provides relatively poor selectivity for isomerism. Neither of the reversed-phase columns shown here can discriminate between isomers of the same carbon number, although both do a good job with homologs.

The normal-phase columns, on the other hand, show relatively more selectivity for isomerism and relatively less (in the case of the alumina column, actually no) selectivity for homologs.

LC/GC, 15, 62S (1997)
Polar sample and solvent molecules interact strongly with polar groups on the column surface. In many cases, this interaction can be highly localized (i.e., "one-to-one"). The orientation of functional groups can have a significant effect on the interaction, with the result that normal-phase chromatography (especially adsorption chromatography) provides a high selectivity for such structural features. Reversed-phase, you will remember, is driven primarily by hydrophobicity. This means that sample-solvent interactions dominate the separation. Because solution phenomena are inherently disordered (liquids have no rigid structure), reversed-phase is inherently not very selective for structural differences between analyte molecules of similar overall polarity.
When carrying out normal-phase HPLC with silica columns, water or methanol is usually added to the mobile phase to suppress strong stationary phase sites (acidic silanols). In the past, with older (less desirable) silica columns, these strong sites resulted in lower plate numbers, peak tailing and changes in sample retention from run to run — due to the effect of traces of water on sample retention.

In these two figures, the effect of added water on retention ($k'$) and column efficiency ($H = \frac{column\ length}{N}$) is shown for a lower-purity silica (Zorbax) vs. a high-purity silica (Rx). The Rx-SIL column shows less retention variation as water is varied, and column efficiency ($H$) is not affected by added water. However, it is necessary to add either water or methanol to the mobile phase to "buffer" the mobile phase against changes in water content and resulting changes in retention.
Why Use Polar Bonded-phase Columns?

- no water “buffering” required
- fewer problems with very polar samples
- very polar contamination easier to remove from column
- easier to use with gradient elution

Polar bonded-phase columns are, on the whole, less selective for structural features such as geometrical isomerism than are adsorption columns. Their many advantages, however, have made them the dominant column type for normal-phase HPLC separations.
Several different kinds of column packing are used for normal-phase HPLC. Silica and alumina were used a lot before 1980, but polar-bonded phases such as cyano, diol and amino are more common now. Column strength varies as: silica > amino > diol > cyano.

Cyano columns are usually the most convenient to use and have fewer problems. These columns are usually used with mobile phases that do not contain water.

Some samples such as sugars are very polar and water soluble. They are not well retained under reversed-phase conditions, but also not very soluble in nonaqueous solvents. Normal-phase columns that can be used with aqueous solvents are preferred for samples of this type. Two such packings are (a) amino and (b) HILIC. We will discuss each of these.
Optimizing Mobile Phase in NP-HPLC

The mobile phase in normal-phase HPLC will be selected for various reasons. In initial experiments, where low-UV detection is desirable, hexane is often used as the A-solvent (weak) and methylene chloride as the B-solvent (strong). A gradient from hexane to methylene chloride can then be used as an initial, exploratory run. It may then be possible to separate the sample with hexane-methylene chloride in either a gradient or isocratic mode.

Solvent optimization can be carried out with the three strong solvents shown in this figure: CH₂Cl₂, MTBE or ethyl acetate. The use of this solvent selectivity triangle is very similar to the way we have described for reversed-phase. CH₂Cl₂ is tried first as the B solvent: %B is adjusted for both retention range 1 < k' < 20) and selectivity. If an adequate separation is obtained, mobile phase optimization is terminated. If further change in selectivity is desired, MTBE and ethyl acetate can be tried as the B-solvent. Blends of two or three of these strong solvents are also possible.
The use of systematic mobile phase optimization requires a solvent-strength nomogram. This figure is a nomogram for silica as column packing. In addition to the preferred solvents for selectivity control (CH$_2$Cl$_2$, tBME = MTBE, and ethyl acetate = EtOAc), THF and IPA are also shown. For more polar samples, hexane can be replaced by dichloromethane as A-solvent for reasons of solvent strength or sample solubility.
Developing a Normal-Phase Separation with a Polar Bonded-Phase Column

1. CN column, 1-100% iPrOH/hexane (220 nm)
2. Adjust %B for 2 < k’ < 10 (1-20)
3. Check for tailing or low plate number
4. Adjust selectivity if necessary
   – fine-tune %B
   – change solvent type
   – mix solvents (?)
   – change column type
5. Fine-tune column conditions

The strategy for normal-phase method development with a polar bonded-phase parallels that for reversed-phase. The initial gradient run allows the isocratic/gradient decision to be made and provides information for preliminary adjustment of k’. If separation is unsuccessful after solvent optimization, a change in column type followed by solvent optimization is often advantageous. Selectivity is more affected by both solvent and column type in normal-phase HPLC vs. reversed-phase, because of the strong interaction of sample molecules with the stationary phase.

Usually polar-bonded phase columns (cyano, diol, amino) will be tried first, but silica is often best for difficult samples and especially isomers.
Normal-phase Example

This simple 2-component separation illustrates the process.

The initial wide-range gradient showed the analytes co-eluting and essentially unretained (note that the merged peaks elute before the sum of $t_0 + t_D$). i_PrOH is too strong for this separation.

A second wide-range gradient using MTBE is more promising. The peaks are retained, and we can see the beginning of a separation.

**Normal-phase Example**

**Separation of phenol (P) and aniline (A) on a cyano bonded-phase column**

- **Column:** 150 x 4.6 mm
- **Flow:** 2 mL/min

Because the two peaks elute close together, we should be able to carry out an isocratic separation. The scouting gradient program suggests 9% strong solvent as a reasonable starting point. The results are not too bad, but k’ values are a bit on the low side, and the resolution could be better. Note that the elution order is opposite from that obtained during the gradient. Decreasing the MTBE concentration increases both k’ and resolution (note that the elution order has reversed back to what we originally saw in the gradient). If necessary, selectivity might be changed further by switching to a different solvent, or a different column.
At the present time, normal-phase HPLC is not used to a great extent for analytical separations. The reason is that this HPLC method has some serious potential problems. Methylene chloride and ethyl acetate absorb UV strongly and cannot be used below 235 nm and 260 nm, respectively. This precludes their use for many samples. By using ACN in place of ethyl acetate (with some CH₂Cl₂ as co-solvent), detection above 235 nm is possible — but this rules out the detection of many samples.

Because of solvent demixing, gradient elution with silica is impractical. However the polar bonded phases can be used in a gradient mode. Solvent volatility in normal-phase HPLC can lead to bubbles in the pump and detector, especially when the room temperature > 25°C.

Plate numbers with normal-phase HPLC are often observed to be lower than expected, but this may be due to problems with the silica. The use of “good” silica for bonded-phase columns may solve this problem.

The need for added water in the mobile phase is quite inconvenient, but this is mainly true for silica.
As mentioned previously, porous graphite columns can combine good isomer selectivity with a reversed-phase mechanism.

LC/GC, 15, 62S (1997)
It has been found that reversed-phase separation using columns with cyclodextrin as the bonded phase gives outstanding separations of many isomeric samples. Cyclodextrin is a cyclic carbohydrate with a cavity in its middle that can accommodate various sample molecules. Usually one isomer will "fit" into the cavity better than another, resulting in a large difference in retention. This is illustrated in this example (Suprofen), for a cyclodextrin column (Cyclobond I from Astec).

Cyclodextrin (CD) exists in three different forms (α, β, γ) which have cavities of different size. α -CD will accommodate sample molecules as large as substituted benzenes, β -CD will allow substituted naphthalenes to enter the cavity, and γ -CD will accommodate even larger molecules. β -CD columns (e.g., Cyclobond I) are generally the best first choice for these isomer separations.

J. Chromatog., 465 (1989) 422
Water-soluble, very polar samples may be best separated under normal-phase conditions using aqueous mobile phases and either amino or HILIC columns. Sugars have traditionally been separated on amino phase columns with ACN/water as mobile phase. This figure shows a typical example. It can also be seen that this is a normal-phase separation, because the retention of maltose decreases as %-ACN decreases.

Note also for the oligosaccharide sample that retention increases in the sequence monosaccharides (1), disaccharides (2), trisaccharides (3), etc. That is, larger saccharides appear to be more polar than smaller saccharides.
This figure plots the retentions of various amino acids vs. %-ACN/water using a HILIC (hydrophilic interaction) column. The structure of this stationary phase is shown and it is seen to be very polar or hydrophilic.

These plots show normal-phase retention for each amino acid, with the more polar amino acids (histidine, arginine, lysine) being most strongly retained, and the nonpolar amino acids (leucine) being weakly retained.

Because of silanols present in C8 or C18 columns, basic samples will sometimes show normal phase retention for %B > 60% under reversed-phase conditions.

HILIC columns were originally developed by Poly-LC (Columbia, MD) and now are available from some other vendors.
Some columns, such as the perfluorinated phases, can show reversed-phase retention at low %-organic and HILIC retention at high %-organic.

Courtesy of R.A. Henry
Fluorophase Mode Change

This Discovery HS F5 fluorophase (Supelco) shows a change in retention mechanism depending on the mobile phase %ACN and compound type. This can be useful for added selectivity options, but also can be confusing if you don’t understand that retention has switched from reversed-phase to HILIC.

Courtesy of R.A. Henry
Fluorophase Shows NP Retention

Fluorophase RP, 5 µm
150 x 4.6 mm
Ammonium formate (pH 3) / ACN
1 mL/min, 230 nm
1. Codeine
2. Lidocaine
3. Cocaine

This fluorinated phase shows normal phase (HILIC) retention – as more organic is added, retention increases.

Courtesy of R.A. Henry
Ion exchange chromatography is based on the use of charged groups permanently attached to the surface of the column packing. Each fixed exchange site (charged group) has associated with it a counterion of opposite charge, provided by the buffer or salt in the mobile phase. Sample ions compete with buffer ions for access to these exchange sites; retention is the result of ionic interaction between the sample ion and the site.
Applications of Ion Exchange

- **strong acids / strong bases**  
  (ion chromatography)

- **biopolymers (using gradients)**

- **weak acids / weak bases**  
  (when ion-pair has problems)

Ion exchange chromatography is the technique of choice for the analysis of strong acid or strong base compounds (it is normally referred to as Ion Chromatography when used in this manner). Ion exchange is also widely used for the analysis of biopolymers (proteins, peptides, nucleic acids), generally using gradients. The technique is less commonly used for the analysis of small-molecule, weak acid or weak base compounds. It has been largely superseded in these applications by ion-pair chromatography, which offers more flexibility.

Ion exchange chromatography for small weak acid / weak base compounds is much more dependent on column chemistry and much less dependent on mobile phase changes than is ion-pair chromatography. This makes the technique more difficult to manipulate and has limited its utility as a general-purpose analytical tool. When an appropriate column can be found or developed, however, the result is often a rugged method that is more trouble-free than an equivalent ion-pair separation.
Classical ion-exchange packings were made from polystyrene-divinylbenzene copolymers derivatized to provide either sulfonic acid or quaternary ammonium functionality. These are generically referred to as strong acid or strong base ion exchangers, respectively. These packings have found limited use in HPLC since they tend to deform at high flow rates and pressures, and to shrink and swell in response to changes in mobile phase ionic strength.
Many ion-exchange HPLC packings provide similar functionality starting with an aromatic bonded-phase silica column. These suffer from the same pH limitations that affect reversed-phase columns.

A wide variety of non-silica based ion exchangers based on hydrophilic polymers is also available. Many of these materials are specially designed for the analysis of biopolymers.
### Ion-exchange Packings

<table>
<thead>
<tr>
<th></th>
<th>strong</th>
<th>weak</th>
</tr>
</thead>
<tbody>
<tr>
<td>cation exchange</td>
<td>- SO(_3^-)</td>
<td>- COO(^-)</td>
</tr>
<tr>
<td>anion exchange</td>
<td>- NR(_3^+)</td>
<td>- NR(_2^+)H(^+)</td>
</tr>
</tbody>
</table>

The charged groups on an ion exchange column are not restricted to sulfonate/quaternary ammonium. Carboxylate or amino functionality results in weak acid / weak base ion exchangers, whose capacity changes with pH.
The ion-exchange capacity of a column packing is the concentration of charged sites in the packing bed. It is variously expressed as milliequivalents per gram of packing or milliequivalents per milliliter of column bed volume. Strong exchangers have essentially constant capacity regardless of pH. Weak exchangers have a capacity which remains relatively constant until the pH approaches the pK\textsubscript{a} of the packing material. In principle, when pH = pK\textsubscript{a}, the capacity is reduced to half its maximum value. ± 1 pH unit on either side of the pK\textsubscript{a} changes the capacity from 10% to 90% of its maximum value.
Controlling Ion-Exchange Chromatography

- column capacity (k')
- mobile phase ionic strength (k')
- pH (α)
- column type (α)
- buffer type (α)
- organic modifiers (α)

Column capacity and mobile phase ionic strength are used primarily to control retention (k'). pH can be used to control retention (e.g., when weak acid or base ion exchangers are used or when the mobile phase contains weak acid or base driving ions), but is more frequently exploited for selectivity control. Assuming similar capacity, changes in column chemistry or mobile phase buffer (salt) are used primarily to control selectivity.
Effect of Column Capacity

Ion-exchange capacities of commercial ion-exchangers vary over several orders of magnitude. Just as an example, cation exchangers range from under 10 µeq/g (Wescan Cation) to about 4 meq/g (BioRad Aminex HPX-87). Generally, a column is chosen to give reasonable k’ values for mobile phase ionic strengths in the 0 - 100 mM range. The chromatogram on the right is a simulation based on published retention data using open-column chromatography normalized to HPLC plate numbers and flow rates. A 0.6M HCl mobile phase is both impractical for conductivity detection and somewhat hard on HPLC equipment! In this example, there is no effect of changing the acid type, only the ionic strength.
Effect of Mobile-phase Ionic Strength

On a given column, retention ($k'$) is controlled primarily by varying the ionic strength of the mobile phase buffer. The higher the ionic strength, the stronger the mobile phase (i.e., the lower the $k'$). In general, there is little selectivity change unless the analyte molecules differ in charge.
In general, the relationship between $k'$ and buffer ionic strength is log/log, with the slope determined by the ratio of analyte charge to buffer charge ($z$). As a result, the selectivity for analytes of similar charge will remain approximately constant as buffer ionic strength changes. Any difference in charge among analytes, however, will be reflected as changes in selectivity.
Effect of pH

Retention behavior as a function of pH for common inorganic anions on a commercially-available resin-based anion exchange column, using 5 mM p-hydroxybenzoate buffers

If weak acid or weak base buffers are used, then the charge on the buffer ion will change with pH. The result can be interesting. Note the shifts in retention seen here for phosphate as the pH increases toward $pK_{a2}$. 
Often, subtle changes in column chemistry can result in major selectivity shifts. In many cases, column manufacturers provide columns that are designed for specific applications. Note the difference in peak spacing on these two columns.
Effect of Salt Type

Cations:

\[
\text{Mg}^{++} > \text{Ca}^{++} > \text{NH}_4^+ > \text{Na}^+ > \text{K}^+
\]

Anions:

\[
\text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{Cl}^- > \text{CH}_3\text{COO}^-
\]

The more tightly bound an ion is to the column packing, the more effective it is as a driving ion. Generally, divalent ions are stronger than monovalent.
Effect of Organic Modifiers

Organic modifiers can change the effective charge on both the column packing and the analytes. In addition, solvation of the stationary phases changes accessibility to the analytes. Finally, organic modifiers can affect secondary retention mechanisms.

Organic modifiers can affect the chromatography in a number of ways:

• By affecting the relative balance of hydrophobicity and ion exchange in mixed-mode separations (probably the most common when dealing with bonded-phase (silica-based ion exchange packings)
• By shifting the pKa of weak acid or weak base analytes (most effective when the mobile phase pH is near the pKa of one or more analytes)
• By shrinking or swelling the stationary phase, thereby changing accessibility for analyte molecules (most common when dealing with resin-based packings).
• By changing the size of the hydration shell around charged groups, thereby changing the effective charge density.

These chromatograms are an example of “ion exclusion” chromatography, in which ions of the same charge as the packing are separated. Such separations often incorporate significant elements of reversed-phase mechanism. The addition of acetonitrile minimizes the reversed-phase contribution, allowing the ion-exclusion mechanism to force earlier elution.
Ion Exchange Method Development

1. Choose anion or cation exchange column (SAX, WAX, SCX, WCX)
   - select buffer pH for sample ionization
   - initial run with 0-1 M NaCl gradient

2. Adjust gradient conditions or select isocratic conditions for $2 < k' < 10$ (1-20)

3. Check for tailing or low plate number

4. Adjust selectivity if required
   - pH
   - salt type
   - added organic
   - column type

5. Fine-tune column conditions

Ion exchange separations are often (but not necessarily) carried out in a gradient mode. The first step is the selection of an appropriate column: anion or cation exchange. For the separation of ionized acids, an anion exchange column is required; the separation of ionized bases requires a cation column. Selectivity can be varied by the choice of a strong vs. weak ion exchanger.

The mobile phase will consist of water, 5-10 mM buffer and a monovalent salt such as NaCl. The buffer pH is adjusted to provide ionization (and retention) of the sample; pH can be further varied as in ion-pair HPLC to control selectivity. The salt concentration is varied to control $k'$ (for isocratic separation). In the case of gradient elution, the salt concentration is increased during the gradient. Other steps in method development are similar to those used for reversed-phase: optimize gradient conditions, vary selectivity, and adjust column conditions.
This column from Sequant (Zic HILIC) has positive and negative charges on the surface of a reversed-phase bonded phase. Zwitter ions are one compound type that can be separated on this column. Alternatively, the mobile phase pH can be adjusted to use ionization control for added selectivity of acids or bases. This is sometimes called a "mixed mode" column.
Effect of pH on Retention and Selectivity

75/25 ACN/NH₄OAc
(17 mM, pH 6.8)

75/25 ACN/NH₄HCO₃
(17 mM, pH 9.6)

Here gentisic, protocatechuic, and isophthalic acid are separated on a Zic pHILIC (polymer base) column.

Courtesy of Sequant
One of the most common applications of ion exchange is not for analytical work, but for sample preparation. Here is a line of polymeric ion exchange solid phase extraction (SPE) phases from Waters. Polymeric supports eliminate some of the unwanted properties of silica, such as limited pH ranges and secondary silanol interactions. For sample prep, it is often best to use a “mixed bed” ion exchanger – that is one that has both ion exchange and reversed-phase characteristics. This tends to be “orthogonal” to the analytical separation, and thus provides better cleanup.

Note that the MCX and MAX phases always carry a charge (strong ion exchangers), whereas WCX and WAX may or may not carry a charge depending on the mobile phase pH.

One line of SPE materials are those from Waters. Many other manufacturers also offer SPE products. For those that maybe are not familiar with Oasis, these are the structures of the sorbents:

- Oasis HLB – hydrophilic-lipophilic balanced co-polymer – reversed-phase retention
- Oasis MCX (Mixed-mode Cation eXchanger)
- Strong sulfonate (-SO3H) groups bonded to Oasis® HLB co-polymer (1 meq/g)
- Oasis MAX (Mixed-mode Anion eXchanger)
- Quaternary amine bonded to Oasis HLB co-polymer (0.25 meq/g)
- Oasis WCX (mixed-mode weak cation exchanger)
- Carboxylic acid bonded to Oasis HLB co-polymer (0.7 meq/g, pKa ~5)
- Oasis WAX (mixed-mode weak anion exchanger)
- Piperazine bonded to Oasis HLB (0.6 meq/g, pKa ~6)

Courtesy of Waters Corp.
In Ion Chromatography (IC), separation and detection are more tightly integrated than in most HPLC techniques. For anion analysis, a low-capacity anion exchange column is used with a weak-acid eluant (usually carbonate/bicarbonate) at moderately high pH. The column effluent is passed through a suppressor which replaces the sodium ions with hydronium.

The buffer thus becomes the weakly conducting carbonic acid, while analytes (such as chloride, nitrate, or sulfate) remain highly conductive. The column capacity and selectivity are optimized within narrow tolerances by the manufacturer for this approach.