7 High Performance Liquid Chromatography

Revision

The following questions cover the important concepts that you should have understood in the introductory chromatography subject.

1. The P in HPLC is often used as an abbreviation for pressure. Explain why.

2. Draw a block diagram of the components of a typical HPLC.

3. How does separation by HPLC differ from that by GLC?

4. What general rules apply to the selection of the two phases in HPLC?

7.1 Injection systems

HPLC instruments typically operate at pump pressures of 2 MPa and higher. This is equivalent to ten times atmospheric pressure. This causes two problems: how to inject the sample against such a pressure, and how to maintain constant pressure without surges and drop-offs.

Direct sample injection, as with a GC, isn't possible: it would be impossible to push the syringe in against such pressure, and no septum could create a seal after the syringe was removed. Therefore, a mechanism for isolating the interior high pressure of the system from the outside world is required. The most common method employs a sample loop, which has a number of outlet points, which are isolated from each other. This is known as a **rheodyne injection system**. Figure 7.1 shows the operation of such a system in the load and inject modes.

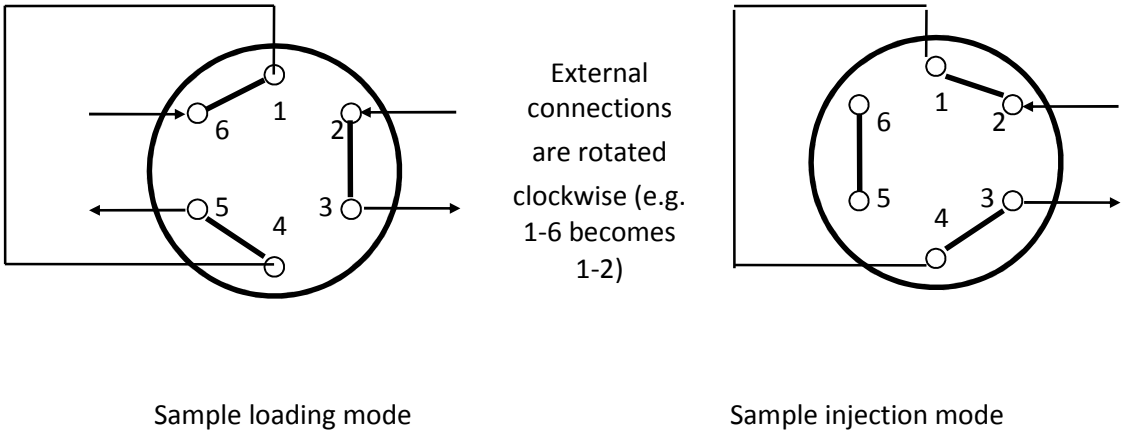


FIGURE 7.1 *Rheodyne sample loop injector (Point 1 - to loop, 2 - from pump, 3 - to column, 4 - from loop, 5 - to waste, 6 - syringe injection)*

The load position of the injector isolates the mobile phase flow to the column from the rest of the system. The sample is injected at point 6, fills through point 1 around the external loop, and any excess is discharged through waste point 5. The internal volume of the loop between points 1 and 6 (in load position) is fixed (typically 20-100 μL for use with packed columns), and thus allows better precision of injection volumes (internal standards are not as important in HPLC as in GC). To inject the sample onto the column, the unit is rotated (clockwise in this case), and the mobile phase is now connected to the sample loop and flushes the sample aliquot onto the column, which is now connected to point 6. The syringe point is isolated still from the high pump pressure.

The rheodyne system lends itself to automation. A robot arm with a tube for drawing in the sample is allied with a carousel turntable which holds the standards and samples. A computer controls the turntable and the delivery tube, through which the solutions are drawn. Autosamplers are becoming a very important tool in analytical laboratories, because of their high efficiency, and the limited operator supervision. Large numbers of samples can be left to be analysed overnight. In the case of an HPLC, remember to have a large store of mobile phase in the reservoir!

7.2 Pumping systems

It was the absence of a satisfactory pump that delayed the development of HPLC instruments until the 1960s because of the high pressures required to deliver useful flow rates. The desirable qualities of an HPLC pumping system are the ability to deliver:

- stable pulse-free flow
- reproducible flow rates
- a range of flow rates from 0.5-5 mL/min
- against very high pressures (up to 100 atm)
- without being affected by different solvents (aqueous and non-aqueous)

CLASS EXERCISE 7.1

Why are these properties desirable?

- *stable pulse-free flow*

- *reproducible flow rates*

- *a range of flow rates*

- *against very high pressures*

- *without being affected by different solvents*

No HPLC pump meets these requirements perfectly, but gradual improvement has meant that each property is at the very least, met sufficiently well to allow proper operation. The most commonly used pump type is known as a **reciprocating piston**.

Its mode of operation is very simple (see Figure 7.2). If we begin with the *piston* in the fully position, then when the cam rotates it will pull the piston out (to the left), this creating a space. This will draw mobile phase through the *inlet check valve*, which is a one-way valve, in the direction shown. The *outlet check valve*, likewise one-way, cannot allow mobile phase already moved through the pump to return. Once the piston reaches the fully out position, the rotating cam will begin to push it back in, thus forcing the liquid in the cavity out the only way it can go: through the outlet check valve.

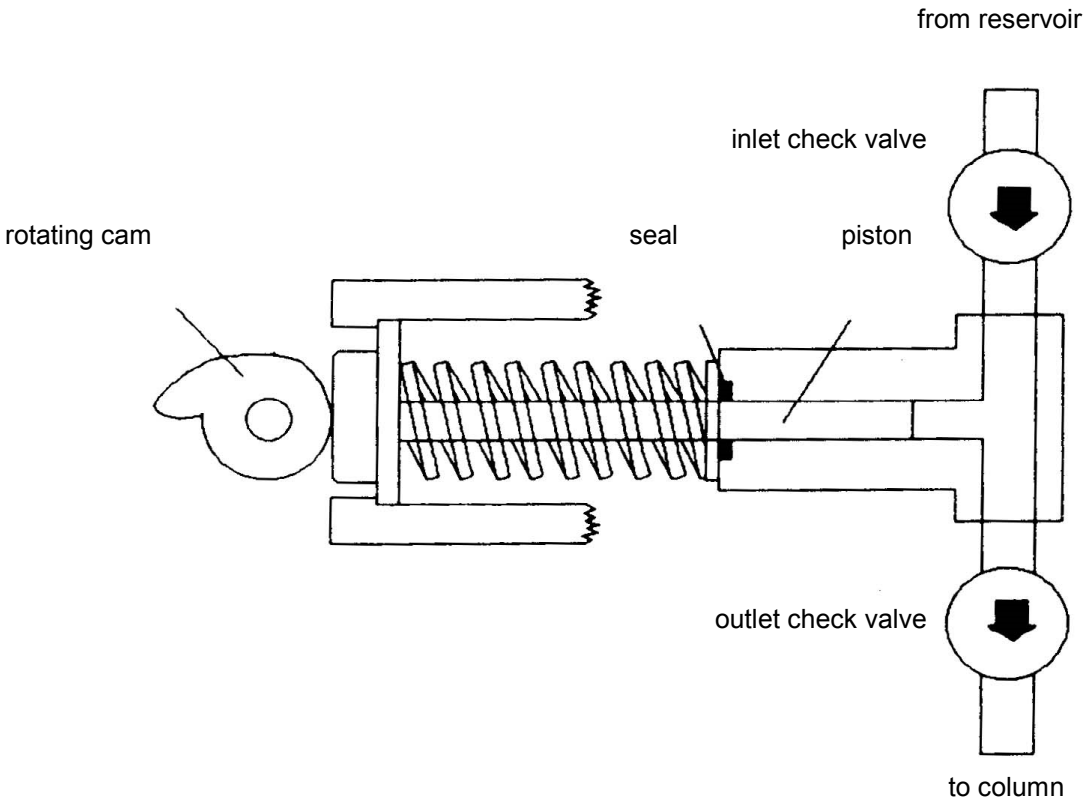


FIGURE 7.2 *A typical single piston HPLC pump (from Ewing,* Analytical Instrumentation Handbook*, Dekker)*

CLASS EXERCISE 7.2

(a) Do you think this pump by itself would create pulse-free flow?

(b) What could be done to fix the problem?

Mobile phase requirements

Mobile phase reservoirs are generally stainless steel or glass vessels. The mobile phase solutions must be **filtered** and **degassed** to remove fine particles which would block the check vales in the pump or the column, and dissolved gases, which may produce bubbles within the column or detector, seriously affecting performance. Further, the reservoirs should be covered to avoid dust fallout.

Solvents should be **HPLC grade**, which is guaranteed not to contain the stabilisers often added to other "pure" solvents. It may not be as pure as AR grade, e.g. HPLC grade hexane may contain some heptane, but it won't have any additives.

7.3 Columns

Columns and connectors

HPLC columns are typically made from stainless steel to withstand the internal pressure and around 15-30 cm in length. They are not coiled because there is no need to save space, and the pressure problems created by coils would be rather worrying. The internal diameter of the column is typically 4-5 mm, but developments in column and packing technology are progressing in parallel with those in GLC. Thus, smaller bore packed columns and capillary columns are now available for HPLC, and are mainly used for HPLC-MS applications where the lesser amount of sample and mobile phase is necessary.

The columns require special fittings at either end to avoid leakages from the high pressures within and to avoid the packing being pushed out. Fine porous glass frits were initially used, but are easily clogged, and difficult remove and clean. They have been replaced by stainless steel gauze. Hand-tightened nuts are used to secure the column to the components at each end. A good seal is achieved by plastic **ferrules**, which the connections seat into. It is vital that the ferrules are not damaged by tightening the nuts too hard.

Packing materials

The development of small-diameter particles as support for the stationary phase was the other stepping stone in the development of successful HPLC. Particle size has been shown to be critical in defining the number of theoretical plates in a column, and therefore its efficiency. Furthermore, the flow rate has less effect on theoretical plate height for smaller particles.

Pellicular materials were the first packing material used in modern HPLC. They consist of glass or plastic beads approximately 40 μm in diameter which are coated with a layer about 1-2 μm thick of silica gel or an ion exchange resin. Their main use these days is in guard columns, and they have been replaced in most applications by **microparticles** (3-10 μm) of silica gel, alumina or certain polymers. Silica is by far the most common packing substance, but does have limitations where the mobile phase is at either end of the pH scale. Figure 7.3 shows the difference between the two types of particles.

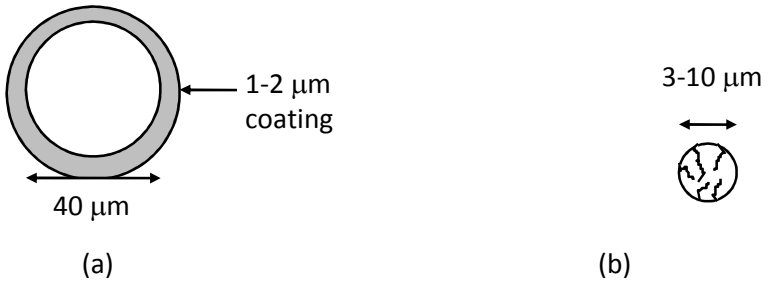


FIGURE 7.3 *HPLC column packing materials (a) pellicular (b) microparticle (not to scale)*

Stationary phases

In early years of HPLC, the liquid stationary phases were physically bonded (adsorbed) onto the porous silica particles. However, significant "bleeding "of the stationary phase was found to occur. Hence, **chemically-bonded** stationary phases are now employed.

This involves chemical reaction of the surface Si-OH (silanol) groups with modifying reagents to yield polar, semi-polar and non-polar functionality. Shorter alkyl chains decrease the hydrophobic nature of the bonded phase, and other groups, such as aromatic rings, amines and alkanols have been used to further increase the polarity, as listed in Table 7.1. Other column types (ion exchange and size exclusion) do not rely on polarity differences for separation. They are discussed in more detail below.

TABLE 7.1 *Some common HPLC stationary phase types*

Name	***Attached Group***	***Polarity***
Reverse phase	C8 to C18	Non-polar
Normal phase	$-(CH_2)_3CN$	Slightly polar
Normal phase	$-(CH_2)_3NH_2$	Polar
Anion exchange	$-NR_3^+$	Ionic
Cation exchange	$-SO_3^-$	Ionic
Size exclusion		No polarity

Guard columns

Because of the cost of HPLC columns, and their ease of clogging by fine particulate matter, the normal practice is to connect a short version (1-2 cm) of the main column between the injection system and the main column. This is a **guard column**, and will catch any particulate matter not removed by ultra-filtration or which has precipitated from the sample as a result of mixing with the mobile phase. A guard column costs $50-100, the main column around $1,000.

7.4 Detectors

Unlike GC, where the detectors (excepting MS) were strange devices, such as thermal conductivity and flame ionisation, HPLC detectors use measurement methods that are familiar in analytical chemical instrumentation, eg UV-VIS, conductivity.

CLASS EXERCISE 7.3

Why can HPLC detectors use devices based on familiar instrumentation?

The requirements for an "ideal" detector are:

- universal response (or selective in a predictable way)
- high sensitivity
- low noise
- wide linear range
- response independent of instrumental variations, such as mobile phase composition, flow rate & temperature
- small internal volume
- flow-through design
- non-destructive
- stable response
- relatively inexpensive

Not asking much are we? No real detector can meet all these, but there is a very wide range of HPLC detectors commercially available. They include:

- UV/VIS
- refractive index
- conductivity
- mass spectrometry
- evaporative light scattering (ELS)
- fluorescence
- infrared
- flame emission
- potentiometric
- polarographic

Table 7.2 compares the characteristics of the more commonly used detectors. The internal workings of the detectors are relatively complex, and will not be covered in these notes.

TABLE 7.2 *Comparison of HPLC detectors*

	Selectivity	***Sensitivity***	***Temp. Sensitivity***	***Gradient elution compatibility***
UV/VIS	Selective	Medium high	Low	Yes
ELS	Universal	High	Low	Yes
RI	Universal	Low	Medium	No
Conductivity	Universal	Medium	High	No
MS	Universal or Selective	Medium high	No	No

Selectivity means there is some adjustment that can be made to pick out specified analytes and ignore others; some UV-VIS detectors are fixed wavelength (most commonly 254 nm) (universal), others allow choice of wavelengths.

CLASS EXERCISE 7.4

Choose a suitable detector for the following analyses:

sugars in grape juice	
fluoride in toothpaste	
determination of FW range of polystyrene plastic	
steroids in blood	

The internal design of HPLC detectors is quite complex and will not be covered in this notes. However, two general aspects – flow-through and internal volume – are important.

Flow through design

Because the flow from the column is continuous, the "sample" cell in which the detector measurement is made, must be flow-through, ie fresh eluant comes in, pushing out that which is already measured. The actual design of such a cell depends on the type of measurement being made.

The simplest cell has two openings, one for the inlet, and one for the outlet. However, where cell pathlength is important for sensitivity (eg UV/VIS, fluorescence, conductivity), the inlet and outlet must as far apart as possible, but without increasing the cell volume. A typical UV detector cell is shown below in Figure 7.4.

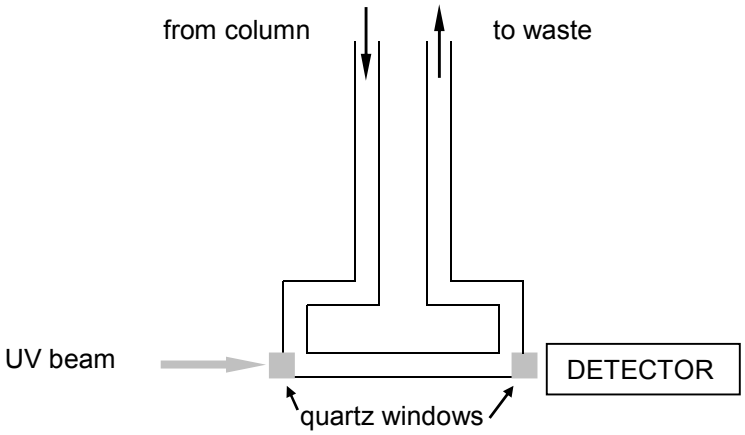


FIGURE 7.4 *Schematic diagram of a flow-through UV detector cell*

Internal volume

The internal volume of the detector is a dead volume where no separation occurs, but which impacts on the ability to the ability to resolve close-together peaks. To illustrate this, let's take an example, which doesn't reflect the typical numbers, but shows the problems that could occur.

Given a typical flow rate of 1 mL/minute, a detector with an internal volume of 1 mL will take 1 minute to fill or to change over the contents, so that any reading from the detector is an average of the eluant from the last minute. Given that two peaks could elute in that minute, their presence would be masked.

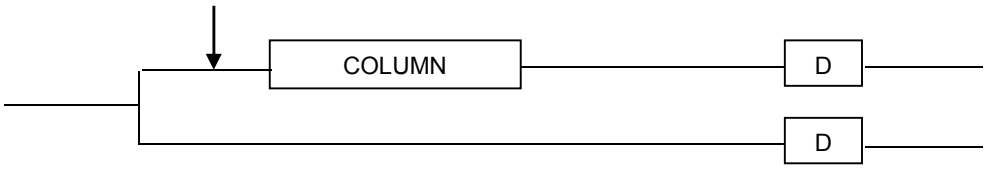
So what volume are we talking about? If we have a changeover time of 1 second, and a maximum flow rate of 3 mL/minute, then the volume required will be no more than 50 μL! In practice, detector cell volumes of less than 10 μL are not uncommon. Imagine a polarographic cell that size!

Solvent response correction in HPLC

Where the mobile phase produces a response at the detector, some means of compensation is required (like a reference beam/cell in spectroscopy). This is because the solvent response will mask small variations due to the low levels of analyte eluting from the column. There are a variety of approaches to this, none of which are entirely satisfactory when coping with gradient elution.

TYPE 1 – SPLIT FLOW BEFORE COLUMN

The mobile phase is split into two flows before the injection port (shown with an arrow), one through the column and the other through a separate path to a reference detector.

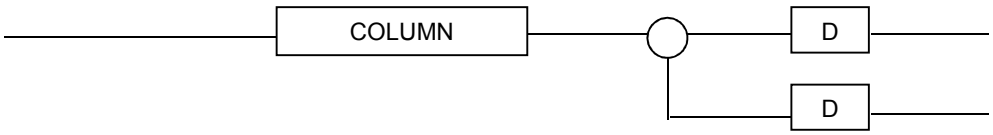


Advantages – copes with changes in solvent response during gradient elution

Disadvantages – two detectors may respond differently, half the flow rate lost

TYPE 2 – FLOW DIVERSION AFTER COLUMN

Here the flow passes through a two-way tap after the column. The flow is not split, but simply can be diverted through either the reference cell or the sample cell. During setup, the mobile phase is directed through the reference cell to fill. Before injection, the flow is returned to the sample cell. The readout subtracts the reference response form the sample.

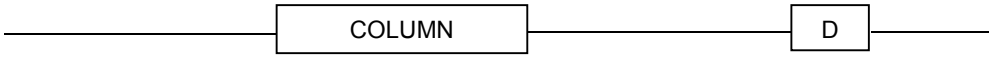


Advantages – no dead time or flow rate problems

Disadvantages – two detectors respond differently, can't cope with changes in response by detector during gradient elution

TYPE 3 – NO SPLITTING

The simplest system, where the single detector is zeroed while only mobile phase is running through (during startup).



Advantages – no detector variation, dead time or flow rate problems

Disadvantages – can only be zeroed initially, can't cope with changes in response by detector during gradient elution

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CLASS EXERCISE 7.5

Which system do the chromatographs used in the practical exercises employ?

7.5 Ion chromatography

In ion-exchange chromatography, mixtures of cations **or** anions (not at the same time) can be separated and analysed by the use of stationary phases, with ionic sites which attract ions of the opposite charge. The mobile phase is usually **an aqueous solution of electrolyte**. Thus, ion chromatography is simply one form of HPLC, with its only feature being special stationary phases. It should not be thought of as a different type of instrumental chromatography.

Stationary phases

Most phases are high molecular weight organic polymers containing large numbers of ionic functional groups. The most common polymeric material is the copolymer of styrene and divinylbenzene, to which cationic or anionic groups have been grafted. As seen in Table 1, **cation** columns most commonly feature sulfonic acid groups (RSO_3H) while **anion** columns employ quaternary amine salts ($RN(CH_3)_3^+$).

The mechanism of ion exchange is based on the equilibria shown in Equations 7.1 (cation exchange) and 7.2 (anion exchange).

$$\text{Polymer-}SO_3^- X^+ + M^+_{(aq)} \longleftrightarrow \text{Polymer-}SO_3^- M^+ + X^+_{(aq)} \qquad \textit{Eqn 7.1}$$

$$\text{Polymer-}N(CH_3)_3^+ X^- + A^-_{(aq)} \longleftrightarrow \text{Polymer-}N(CH_3)_3^+ A^- + X^-_{(aq)} \qquad \textit{Eqn 7.2}$$

The ion attracted to the stationary phase is not bonded permanently to it, and will be released when removed by another ion more attracted to the site or when the distribution equilibrium between the two phases becomes unbalanced. Thus, the basic idea of chromatography holds here, and allows separation of otherwise difficult to analyse mixtures, such as fluoride and nitrate.

Factors affecting ion retention

Separations are based on the fact that attraction to the ion exchange resin is related to the:

- **size of the ion** – the smaller the ion, the less the attraction for similar charge ions,
    - e.g. retention time for $K^+ > NH_4^+ > Na^+ > Li^+$
- **charge on the ion** – the smaller the charge, the less the attraction for similar sized ions
    - e.g. retention time for $Al^{3+} > Ca^{2+} > Na^+$

Similar rules hold for anions, though the retention of nitrite and nitrate is somewhat less predictable, and does tend to vary from one stationary phase to another. Figure 7.5 shows the elution sequence for a mixture of anions.

Mobile phases

Mobile phases used are aqueous, but contain ionic components (known as **modifiers**) and pH buffers included (it should be obvious that these species cannot be of interest as analytes). The main purpose of the modifiers in the mobile phase is to **compete for the resin exchange sites**. Without them, retention times would be extremely long because there would be little reason for the analyte ions to shift from the stationary phase.

Increasing the concentration of the modifiers decreases retention times. Addition of up to 10% of a non-ionic species such as methanol also decreases RTs, though why this should be is not obvious.

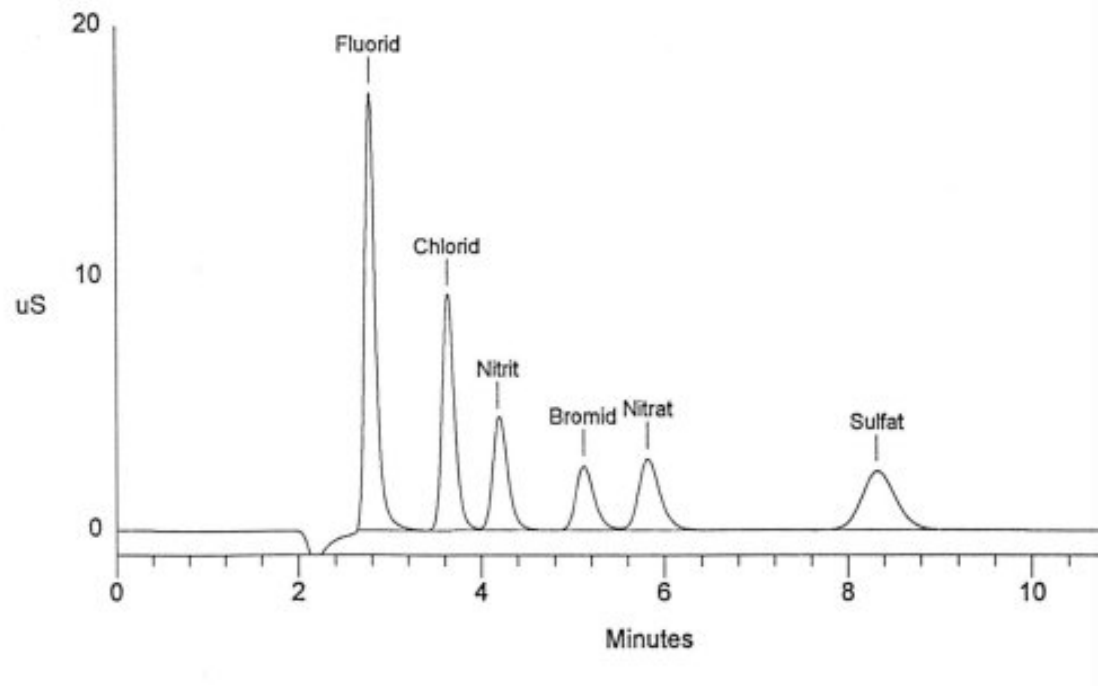


FIGURE 7.5 *Ion chromatogram for a mixture of anions (from www.bayer.com)*

Detection

The most commonly used detector in ion chromatography is based on **conductivity**, being ideal for the measurement of low concentrations of ionic materials. However, the presence in the mobile phase of the modifiers causes a background conductance above which the detector must then be able to see small increases due to the analytes.

Two approaches have been used to compensate for the background conductivity of the mobile phase. The simplest is known as **unsuppressed** and uses low conductivity ionic compounds in the mobile phase. Furthermore, it also employs a reference conductivity cell which stores a small amount of the mobile phase. The detector response to the eluant has the background conductivity subtracted from it.

The **suppressed** mode of ion chromatography employs an acidic or basic mobile phase, and a suppressor column, fitted after the main column, which is an ion-exchange resin of the opposite nature to the main column. To illustrate the function of the suppressor column, consider the example of separation of an anion mixture.

The mobile phase is a carbonate/hydrogen carbonate solution. The suppressor column is a cation exchange resin, loaded with hydrogen ions on the exchange sites. As the eluant passes from the main column into the suppressor column, the sodium ions which are part of the mobile phase exchange on the cation resin, releasing hydrogen ions. These combine with the alkaline mobile phase and are neutralised. The net effect is a loss of background ionic strength before the detector, but still allowing the mobile phase ions to compete for sites in the main column. These processes occurring in the suppressor column are summarised in Equations 3 and 4.

$$\text{Supp-SO}_3\text{H} + \text{Na}^+_{(aq)} \longleftrightarrow \text{Supp-SO}_3^- \text{Na}^+ + \text{H}^+_{(aq)} \qquad \textit{Eqn 3}$$

$$\text{H}^+ + \text{HCO}_3^- \longrightarrow \text{H}_2\text{CO}_3 \qquad \textit{Eqn 4}$$

Suppressed mode is more sensitive, but the extra column increases the dead volume and therefore, band broadening is more of a problem. Furthermore, the suppressor column must be regenerated or replaced on a regular basis (typically 8-10 hours of running time), as the hydrogen ions are used.

7.6 Size exclusion

This form of HPLC is, like ion chromatography, based on a different form of stationary phase. It separates molecules by their **molecular size** as a result of their ability (or lack of it) to passes into the porous structure of the stationary phase. Size exclusion chromatography is unusual in that there is no polarity attraction to the stationary phase, and the mobile phase simply transports the mixture.

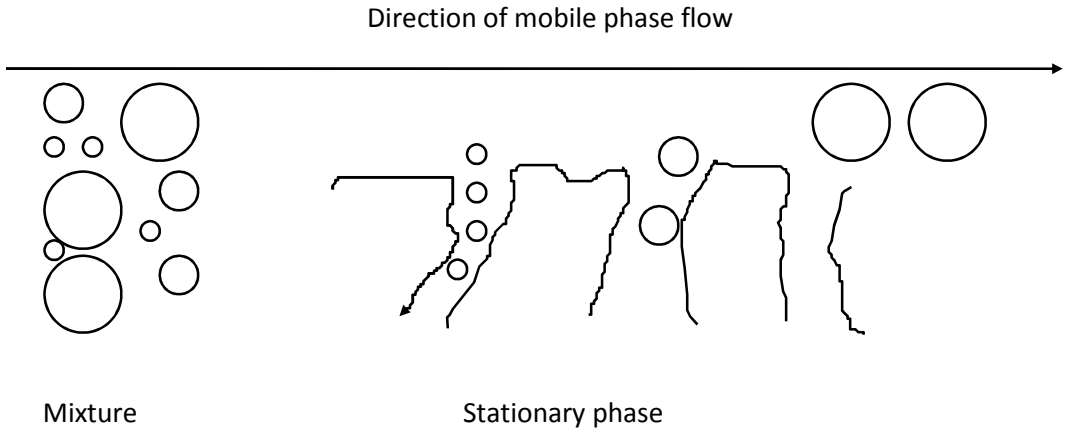
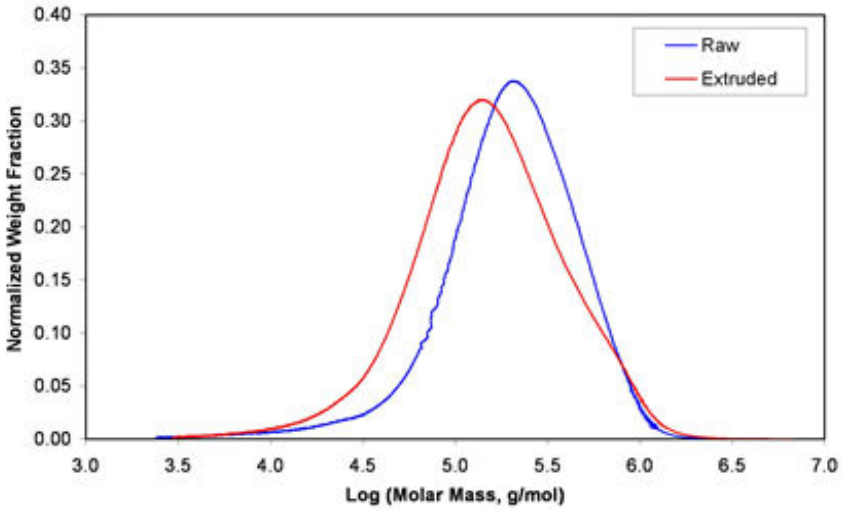


FIGURE 7.6 *Separation mechanism in size exclusion chromatography*

CLASS EXERCISE 7.6

Which compounds would come out first: large or small?

There is a relationship between size and retention volume (measured instead of time), and a calibration graph of compounds of known mass (more readily determined than size) can be constructed. Thus, the mass distribution of materials, such as polymers, proteins and other high FW substances, can be determined. This is important in determining the physical properties of a given material. The chromatogram of a sample of polyester plastic is shown in Figure 7.7.



The mobile phase has no separation function, and hence is only required as a solvent. Detectors used are UV absorption or refractive index.

FIGURE 7.7 *Exclusion chromatogram for polyester sample (from www.polymersolutions.com)*

7.7 Choice of phases

Both the stationary and mobile phases in HPLC play a role in separating the components of a mixture by polarity attractions. The general rule for chromatography is that the samples components must be attracted to the stationary phase for any separation to occur. In HPLC, the range of possible stationary phase types is greater than in GLC: polar through to non-polar, cation and anion exchange and size exclusion. Figure 7.8 summarises the decision-making process for choosing a stationary phase.

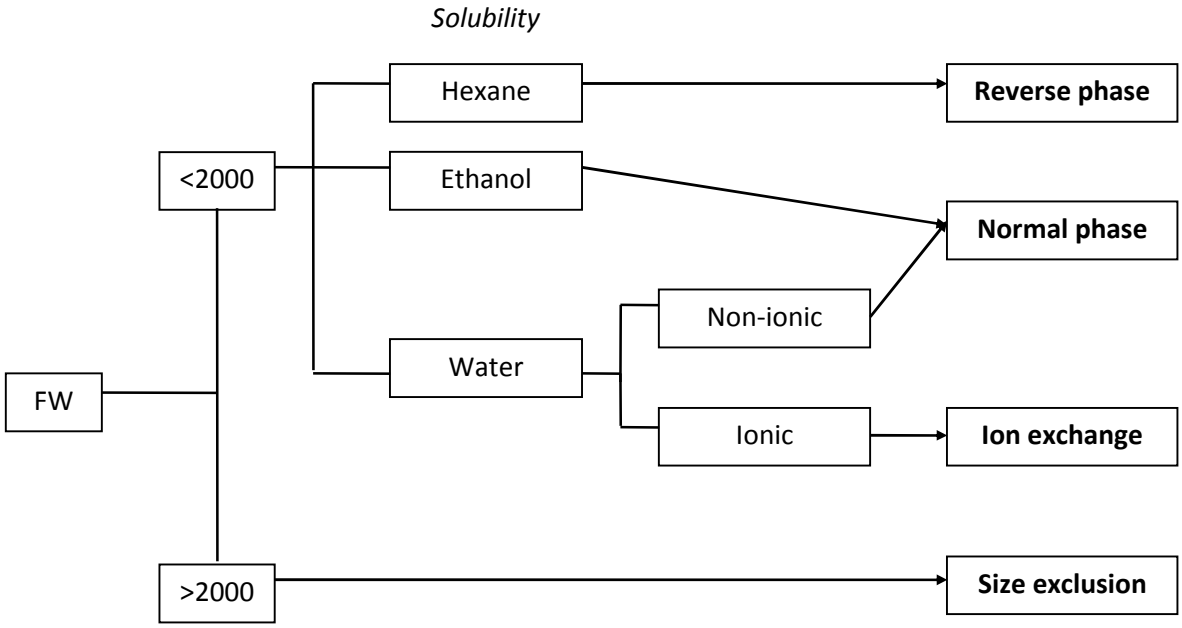


FIGURE 7.8 *Flow diagram for selection of HPLC stationary phase*

CLASS EXERCISE 7.7

Choose a suitable stationary phase for the following analyses:

sugars in grape juice	
fluoride in toothpaste	
determination of FW range of polystyrene plastic	
steroids in blood	

The choice **of mobile phase** is more trial-and-error, though some schemes have been developed to aid in the selection of the optimum mobile phase. The best approach when developing a method is to find a similar sample analysis in the literature (research or manufacturer's information) and to begin there and modify to suit your needs.

The general rule is that the mobile phase (in normal and reverse phase separations) should be of the opposite polarity to the stationary phase, but without making the analytes insoluble or completely unattracted to the mobile phase. Thus, extreme polarity mobile phases (e.g. water and hexane) are rarely used in 100% form. Methanol is a common mobile phase in reversed phase separations, while alkane mixtures with chlorinated hydrocarbons or ethers common in normal phase.

7.8 Elution modes in HPLC

The simplest approach to elution is one mobile phase, known as **isocratic**. This method suits easily separated mixtures, where resolution is satisfactory and retention times are not extreme.

However, as with isothermal GLC, the simple approach is not always sufficient, in this case, where a sample contains compounds of widely different attraction (i.e. polarity) to the stationary phase. The most common approach is to change the composition (and hence the polarity) of the mobile phase during the run. This is primarily used in normal and reverse phase HPLC and is known as **gradient elution**.

Increasing the temperature does decrease RTs, but is not normally done during a run, and is not a significant factor in HPLC. Columns are often in a thermostatted oven to maintain a constant temperature.

CLASS EXERCISE 7.8

Using reverse phase chromatography with ethanol as a mobile phase, a chromatogram with two well-resolved peaks at 2 and 3 minutes retention times also gives a broad peak at 10 minutes. How could this be improved, using gradient elution?

The necessary consequence of gradient elution is that the instrument requires **at least two high pressure pumps**. These draw the individual mobile phases from the different reservoirs. Mixing is done by computer control of the flow rates from each pump in a mixing chamber immediately before the sample injection point. It should be obvious that the two phases must be miscible! The extra pump adds significantly to the cost of the instrument, but allows the separation of more complex mixtures.

Another disadvantage of gradient elution is that the equilibrium conditions inside the column at the end of the run are different to those required at the start of the next. As a consequence, at least five volumes of the starting solvent must be pumped through the column to equilibrate it for the start of the next run.

It also causes problems in terms of changing detector response as the composition of the mobile phase changes. This is covered in an earlier section.

What You Need To Be Able To Do

- define important terminology
- describe the nature and operation of the injection systems commonly employed in HPLC
- list and explain the necessary features of a HPLC pumping system
- explain how a typical HPLC pump operates
- outline the nature of column construction and stationary phase bonding
- list and explain the necessary features of a HPLC detector
- list and compare common HPLC detectors
- outline how solvent response can be compensated for in HPLC
- discuss the techniques of ion and size-exclusion chromatography
- explain the need for the various solution pre-treatment steps
- explain the purpose of gradient elution
- choose suitable phase combinations & detectors for given mixtures

Revision Questions

1. Blood alcohol levels are generally measured by HPLC, rather than GLC, despite the volatility of ethanol. Suggest reasons why this might be the case.
2. In your own words, explain what is happening to the flow of sample and mobile phase in the two settings of the rheodyne injection system.
3. What is the purpose of the guard column?
4. List THREE requirements of an HPLC pump, and explain why they are important.
5. Explain how the piston pump works.
6. How are stationary phases made stationary in modern HPLC columns?
7. Why would GC detectors be unsuitable for HPLC use (except the MS)?
8. What factors affect the order of elution for ions?
9. Why would it be unsuitable for the mobile phase in ion chromatography to be (a) hexane or (b) pure water?
10. What is the main advantage of suppressed-mode ion chromatography? What is different about the system in suppressed mode?
11. Explain how size exclusion chromatography works.
12. What is the meaning of the term “isocratic” in HPLC?
13. What changes to the system would you make given the following chromatograms using a reversed-phase column and ethanol as mobile phase:
    (a) peaks at 6 and 10 minutes
    (b) peaks at 3, 4 and 10 minutes
    (c) overlapping peaks at 2, 2.5 and 3 minutes
14. Compare the characteristics of two of the common employed HPLC detectors.
15. Choose a suitable stationary phase & detector for the following analyses:
    (a) caffeine in tea
    (b) cations in beers
    (c) determination of FW range of polystyrene plastic
16. Why must a HPLC detector be flow-through in design?
17. Why is it necessary that the volume of the measurement cell in an HPLC detector is as small as possible?
18. Describe the solvent correction method employed in the ion chromatograph in our laboratory. Could it cope with gradient elution?

Answers to these questions on following page.

Answers to class exercises can be found in the Powerpoint file provided on the website.

Answers to Revision Questions

Where the answer can be found directly in your notes, a reference to them will be provided.

1. All the non-volatiles in blood would block the column.
2. p7.2
3. p7.6
4. p7.2-3
5. p7.2
6. p7.6
7. They measure properties of gases, not liquids.
8. p7.11
9. (a) no attraction to mobile phase (b) nothing to compete for ion-exchange sites
10. p7.12
11. p7.13
12. p7.15
13. (a) increase flow rate if possible, use a less polar solvent, eg propanone or ethyl ethanoate
    (b) use gradient elution, moving to a less polar solvent after 3 minutes, eg propanone or ethyl ethanoate
    (c) decrease flow rate, use a more polar solvent (eg 50% aqueous ethanol)
14. p7.7
15. (a) reverse-phase or normal-phase (we use reverse), UV
    (b) cation exchange, conductivity
    (c) size exclusion, UV
16. p7.8
17. p7.9
18. Type 3. No