



5. Chromatography

Chromatography is usually introduced as a technique for separating and/or identifying the components in a mixture. The basic principle is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent. It is a powerful method in industry, where it is used on a large scale to separate and purify the intermediates and products in various syntheses.

The theory

There are several different types of chromatography currently in use – *ie* paper chromatography; thin layer chromatography (TLC); gas chromatography (GC); liquid chromatography (LC); high performance liquid chromatography (HPLC); ion exchange chromatography; and gel permeation or gel filtration chromatography.

Basic principles

All chromatographic methods require one static part (the stationary phase) and one moving part (the mobile phase). The techniques rely on one of the following phenomena: adsorption; partition; ion exchange; or molecular exclusion.

Adsorption

Adsorption chromatography was developed first. It has a solid stationary phase and a liquid or gaseous mobile phase. (Plant pigments were separated at the turn of the 20th century by using a calcium carbonate stationary phase and a liquid hydrocarbon mobile phase. The different solutes travelled different distances through the solid, carried along by the solvent.) Each solute has its own equilibrium between adsorption onto the surface of the solid and solubility in the solvent, the least soluble or best adsorbed ones travel more slowly. The result is a separation into bands containing different solutes. Liquid chromatography using a column containing silica gel or alumina is an example of adsorption chromatography (*Fig. 1*).

The solvent that is put into a column is called the eluent, and the liquid that flows out of the end of the column is called the eluate.

Partition

In partition chromatography the stationary phase is a non-volatile liquid which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be separated is carried by a gas or a liquid as the mobile phase. The solutes distribute themselves between the moving and the stationary phases, with the more soluble component in the mobile phase reaching the end of the chromatography column first (*Fig. 2*). Paper chromatography is an example of partition chromatography.

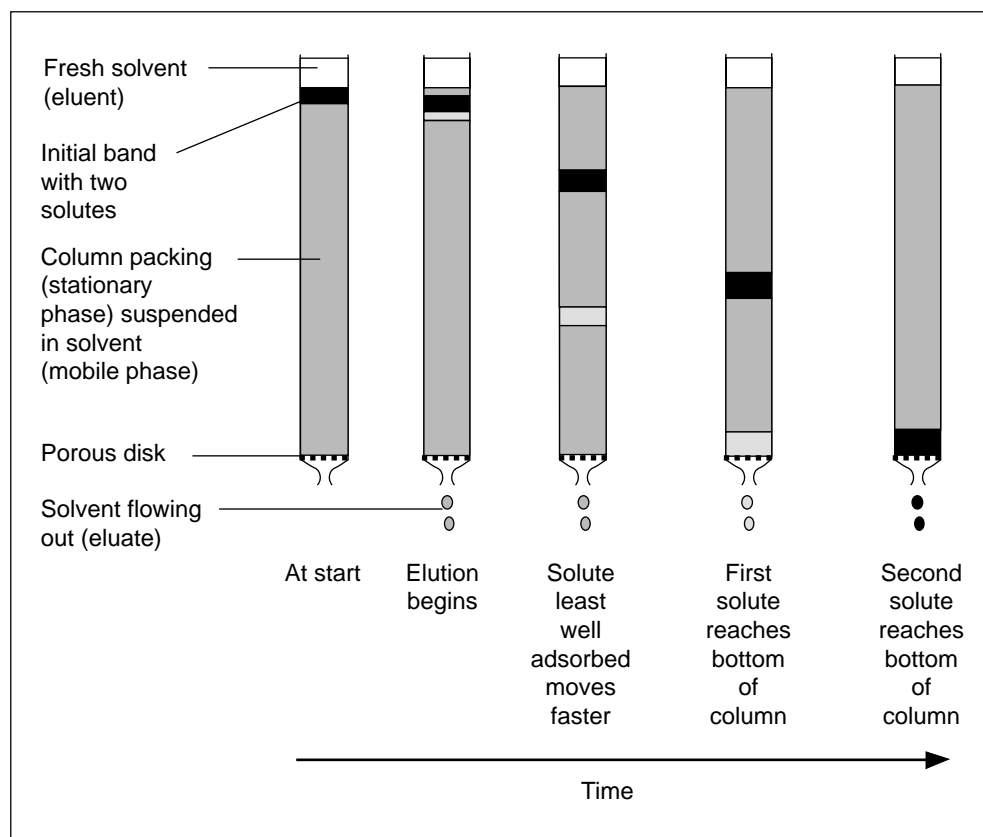


Figure 1 Adsorption chromatography using a column

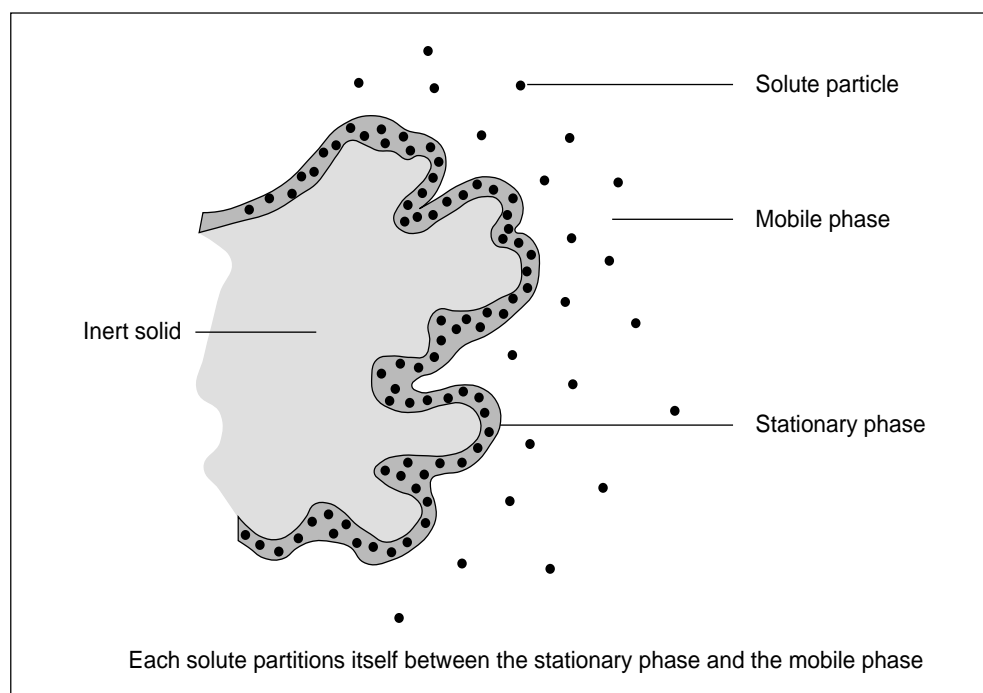


Figure 2 Partition chromatography

Ion exchange

Ion exchange chromatography is similar to partition chromatography in that it has a coated solid as the stationary phase. The coating is referred to as a resin, and has ions (either cations or anions, depending on the resin) covalently bonded to it and ions of the opposite charge are electrostatically bound to the surface. When the mobile phase (always a liquid) is eluted through the resin the electrostatically bound ions are released as other ions are bonded preferentially (*Fig. 3*). Domestic water softeners work on this principle.

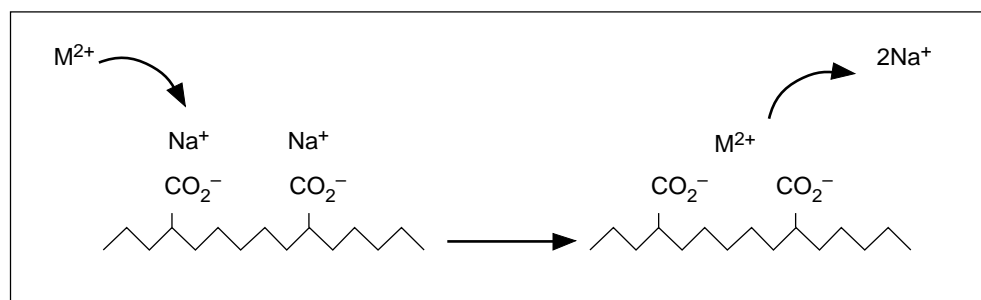


Figure 3 Ion exchange chromatography

Molecular exclusion

Molecular exclusion differs from other types of chromatography in that no equilibrium state is established between the solute and the stationary phase. Instead, the mixture passes as a gas or a liquid through a porous gel. The pore size is designed to allow the large solute particles to pass through uninhibited. The small particles, however, permeate the gel and are slowed down so the smaller the particles, the longer it takes for them to get through the column. Thus separation is according to particle size (*Fig. 4*).

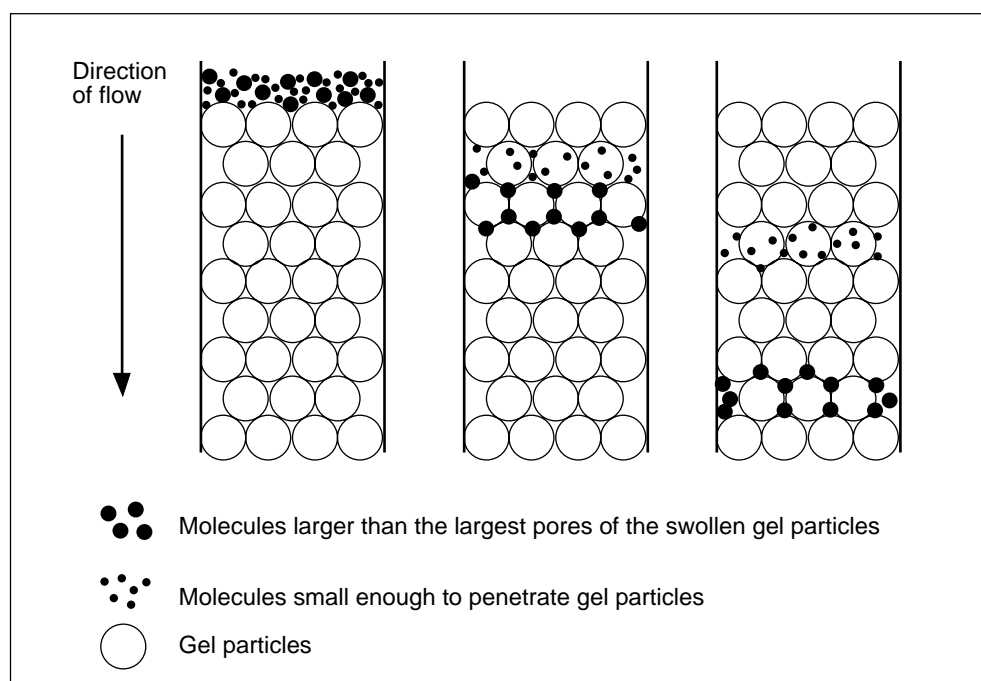


Figure 4 Gel permeation chromatography



Chromatographic techniques

Paper chromatography

This is probably the first, and the simplest, type of chromatography that people meet. A drop of a solution of a mixture of dyes or inks is placed on a piece of chromatography paper and allowed to dry. The mixture separates as the solvent front advances past the mixture. Filter paper and blotting paper are frequently substituted for chromatography paper if precision is not required. Separation is most efficient if the atmosphere is saturated in the solvent vapour (*Fig. 5*).

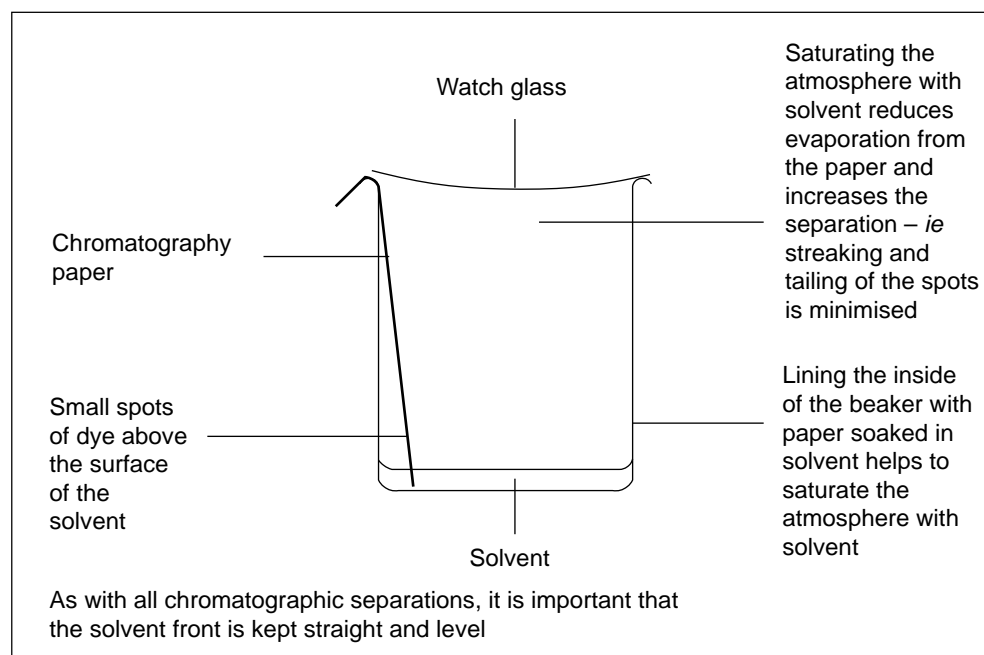


Figure 5 Paper chromatography

Some simple materials that can be separated by using this method are inks from fountain and fibre-tipped pens, food colourings and dyes. The components can be regenerated by dissolving them out of the cut up paper.

The efficiency of the separation can be optimised by trying different solvents, and this remains the way that the best solvents for industrial separations are discovered (some experience and knowledge of different solvent systems is advantageous).

Paper chromatography works by the partition of solutes between water in the paper fibres (stationary phase) and the solvent (mobile phase). Common solvents that are used include pentane, propanone and ethanol. Mixtures of solvents are also used, including aqueous solutions, and solvent systems with a range of polarities can be made. A mixture useful for separating the dyes on Smarties is a 3:1:1 mixture (by volume) of butan-1-ol:ethanol:0.880 ammonia solution.

As each solute distributes itself (equilibrates) between the stationary and the mobile phase, the distance a solute moves is always the same fraction of the distance moved by the solvent. This fraction is variously called the retardation factor or the retention ratio, and is given the symbol R or R_f :

$$\text{Retention ratio} = \frac{\text{distance moved by solute}}{\text{distance moved by solvent}} = R_f$$

So as long as the correct solvent and type of chromatography paper are used, a component can be identified from its retention ratio (*Fig. 6*).

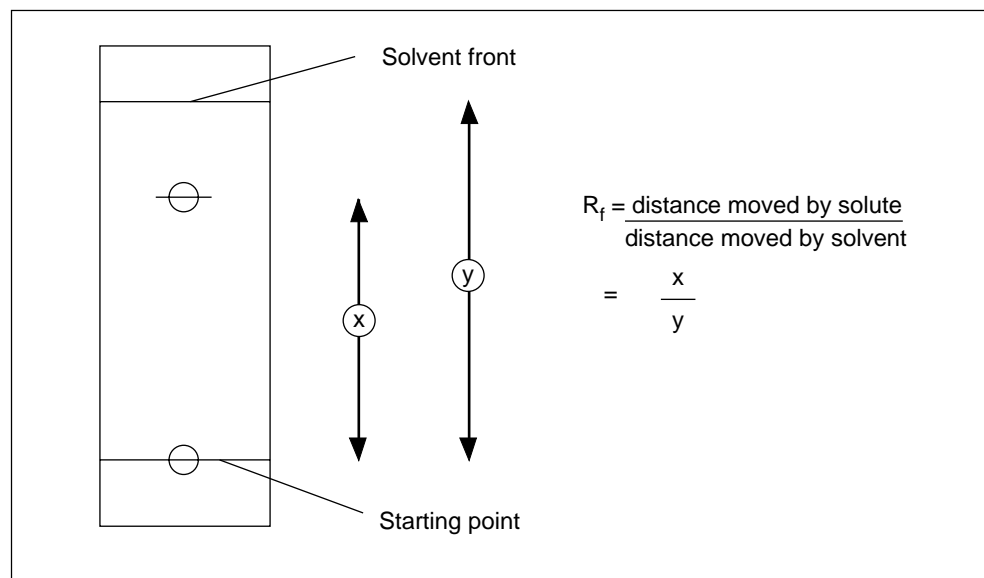


Figure 6 The retention ratio, R_f

It is possible that two solutes have the same R_f values using one solvent, but different values using another solvent (eg this occurs with some amino acids). This means that if a multi component system is not efficiently separated by one solvent the chromatogram can be dried, turned through 90° , and run again using a second solvent, (*Fig. 7*).

Thin layer chromatography (TLC)

Thin layer chromatography is similar to paper chromatography, but the stationary phase is a thin layer of a solid such as alumina or silica supported on an inert base such as glass, aluminum foil or insoluble plastic. The mixture is 'spotted' at the bottom of the TLC plate and allowed to dry. The plate is placed in a closed vessel containing solvent (the mobile phase) so that the liquid level is below the spot.

TLC has advantages over paper chromatography in that its results are more reproducible, and that separations are very efficient because of the much smaller particle size of the stationary phase.

The solvent ascends the plate by capillary action, the liquid filling the spaces between the solid particles. This technique is usually done in a closed vessel to ensure that the atmosphere is saturated with solvent vapour and that evaporation from the plate is minimised before the run is complete. The plate is removed when the solvent front approaches the top of the plate and the position of the solvent front recorded before it is dried (this allows the R_f value to be calculated).

TLC has applications in industry in determining the progress of a reaction by studying the components present; and in separating reaction intermediates. In the latter case a line of the reaction mixture is 'painted' across the TLC plate instead of a single spot, and the line of product after separation is cut out of the plate and dissolved in an appropriate solvent.

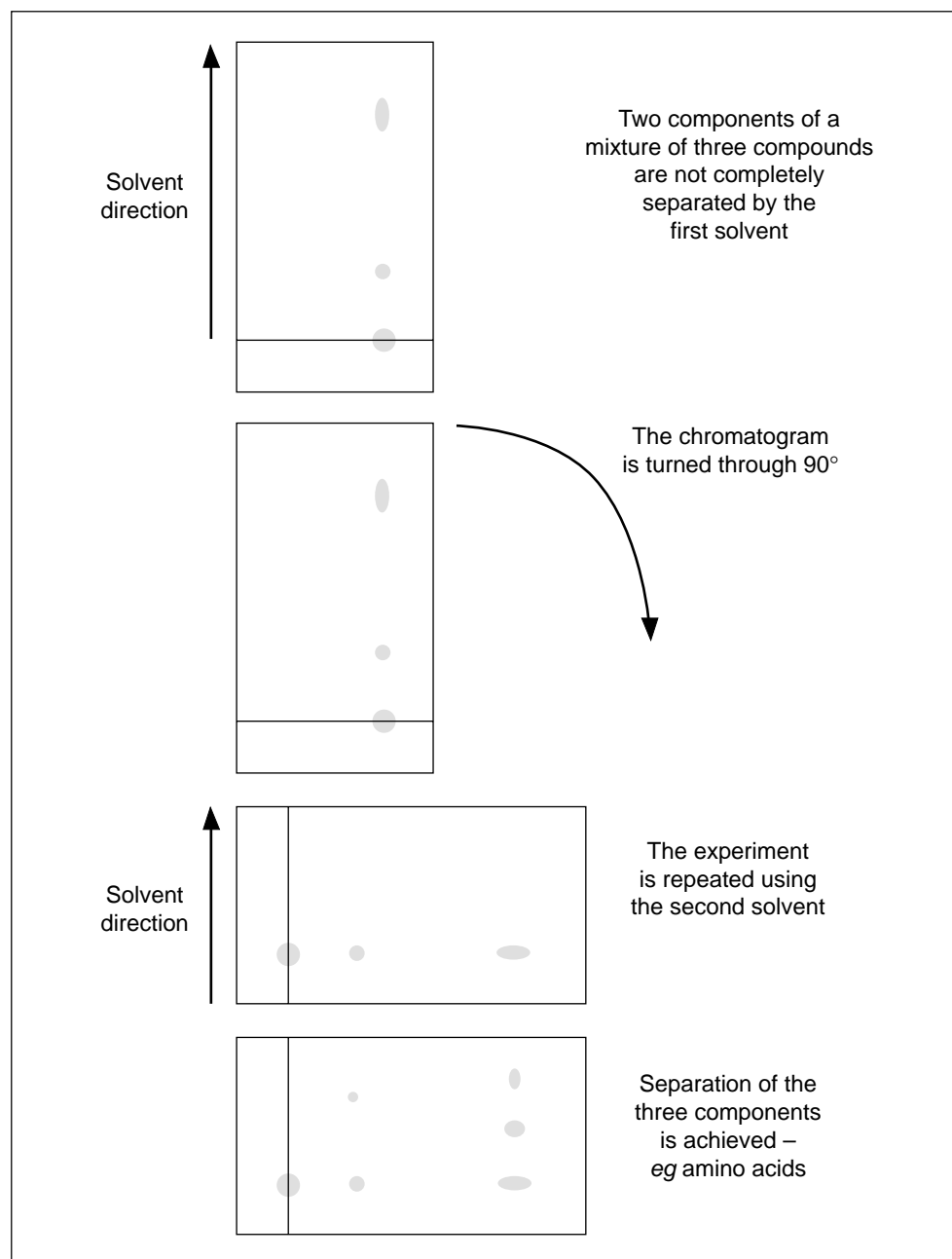


Figure 7 Using two solvents to separate a multi component mixture

Many spots are not visible without the plates being 'developed'. This usually involves spraying with a solution that is reversibly adsorbed or reacts in some way with the solutes. Two examples of developing solutions are iodine in petroleum ether (useful for identifying aromatic compounds, especially those with electron donating groups – eg $C_6H_5NH_2$) and ninhydrin (useful for identifying amino acids). Iodine vapour is also used to develop plates in some cases. Alternatively, specially prepared plates can be used that fluoresce in ultraviolet light. The plates are used in the normal manner, but once dried they are placed under an ultraviolet lamp. Solute spots mask fluorescence on the surface of the plate – ie a dark spot is observed. Some compounds have their own fluorescence which can be used for identification, or

retardation factors can be used to identify known solutes.

Radioactive solutes can be identified on TLC plates by passing the plates under a Geiger counter with a narrow window. A chart recorder plots the count rate as the plate passes under the counter (*Fig. 8*). Accurate quantitative data can be derived by integrating the peaks (this is not shown in the diagram).

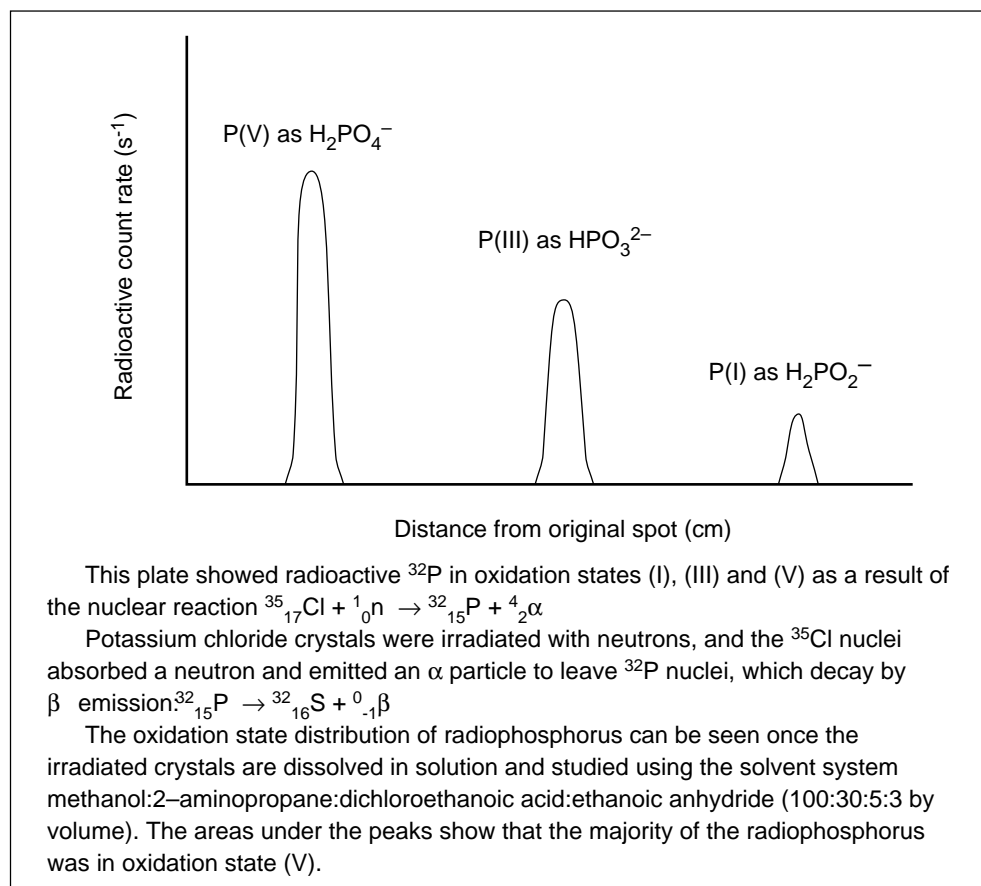
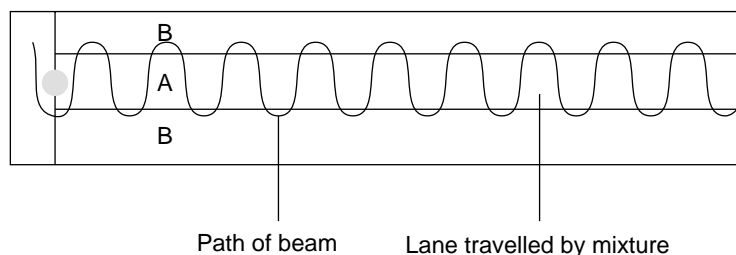


Figure 8 Count rate on a TLC plate

A relatively new method of detecting components on TLC plates is to scan the lane along which a mixture has travelled with a beam of fixed wavelength light. The reflected light from the lane (A) is measured relative to the radiation reflected from outside the lane (B):



The plates can be scanned for ultraviolet/visible absorption; or natural fluorescence. Ultraviolet sensitive plates or already developed plates can also be read.



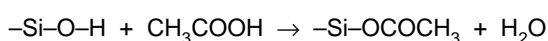
Gas chromatography (GC)

This technique uses a gas as the mobile phase, and the stationary phase can either be a solid or a non-volatile liquid (in which case small inert particles such as diatomaceous earth are coated with the liquid so that a large surface area exists for the solute to equilibrate with). If a solid stationary phase is used the technique is described as gas-solid adsorption chromatography, and if the stationary phase is liquid it is called gas-liquid partition chromatography. The latter is more commonly used, but in both cases the stationary phase is held in a narrow column in an oven and the stationary phase particles are coated onto the inside of the column.

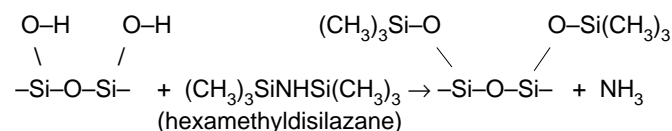
Diatomaceous earth is made from the skeletons of a single-celled non-flowering plant. The skeletons are made of hydrated silica, and are ground to a fine powder of the required particle size.

The advantage of diatomaceous earth is that it has fewer silanol (Si-OH) groups than silica and is less prone to electrostatic attractions (eg hydrogen bonds). The polarity of the OH groups can be reduced or eliminated by esterifying or silanising them.

Esterification with ethanoic acid:



Silanising:



Diatomaceous earth is also known as kieselguhr – the clay Nobel used as the inert base for dynamite.

Practical details

For separation or identification the sample must be either a gas or have an appreciable vapour pressure at the temperature of the column – it does not have to be room temperature. The sample is injected through a self sealing disc (a rubber septum) into a small heated chamber where it is vaporised if necessary (*Fig. 9*). Although the sample must all go into the column as a gas, once it is there the temperature can be below the boiling point of the fractions as long as they have appreciable vapour pressures inside the column. This ensures that all the solutes pass through the column over a reasonable time span. The injector oven is usually 50–100 °C hotter than the start of the column.

The sample is then taken through the column by an inert gas (known as the carrier gas) such as helium or nitrogen which must be dry to avoid interference from water molecules. It can be dried by passing it through anhydrous copper(II) sulphate or self-indicating silica (silica impregnated with cobalt(II) chloride). Unwanted organic solvent vapours can be removed by passing the gas through activated charcoal. The column is coiled so that it will fit into the thermostatically controlled oven.

The temperature of the oven is kept constant for a straightforward separation, but if there are a large number of solutes, or they have similar affinities for the stationary phase relative to the mobile phase, then it is common for the temperature of the

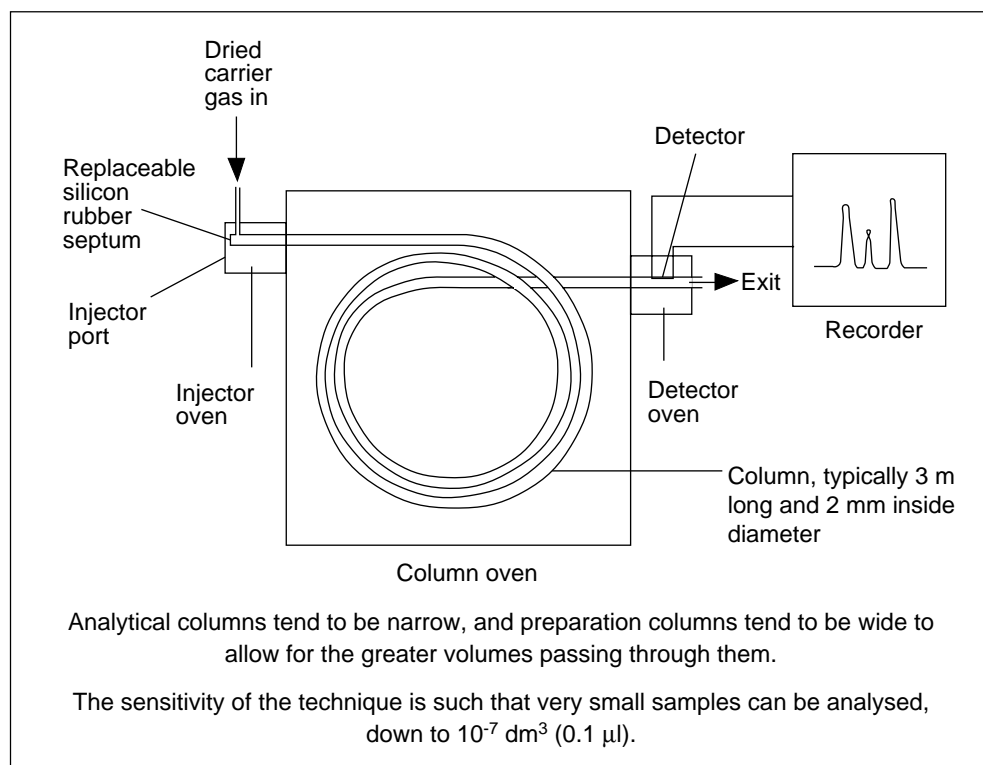


Figure 9 The gas chromatograph

column to be increased gradually over a required range. This is done by using computer control, and gives a better separation if solute boiling points are close, and a faster separation if some components are relatively involatile.

The solutes progress to the end of the column, to a detector. Two types of detector are commonly used: thermal conductivity detectors and flame ionisation detectors. Thermal conductivity detectors respond to changes in the thermal conductivity of the gas leaving the column. A hot tungsten–rhenium filament is kept in an oven set at a given temperature so that all solutes are in the gaseous phase (*Fig. 10*). When the carrier gas – helium, for example, which will have been warmed to the temperature of the detector block – leaves the column it cools the hot filament. However, if a solute emerges with the helium it will cool the filament less (unless the solute is hydrogen, because only hydrogen has a thermal conductivity greater than helium) and the temperature of the filament will rise. Its resistance will then increase, and that can be measured.

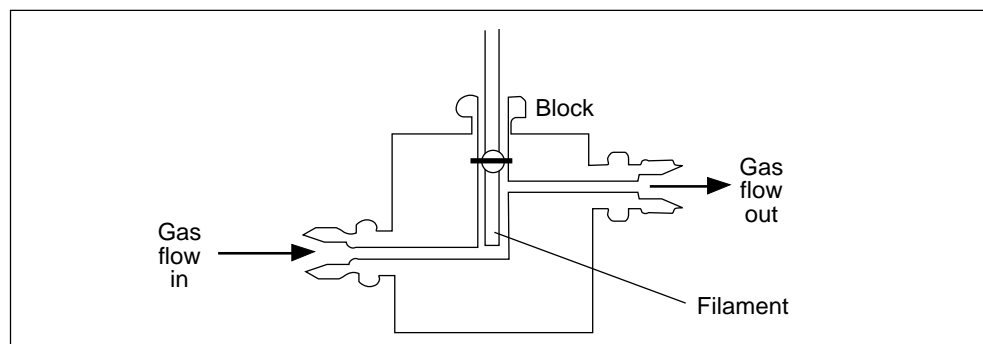


Figure 10 A simple thermal conductivity detector



So that the change in resistance can be monitored directly a second circuit measures the resistance from the pure carrier gas (Fig. 11).

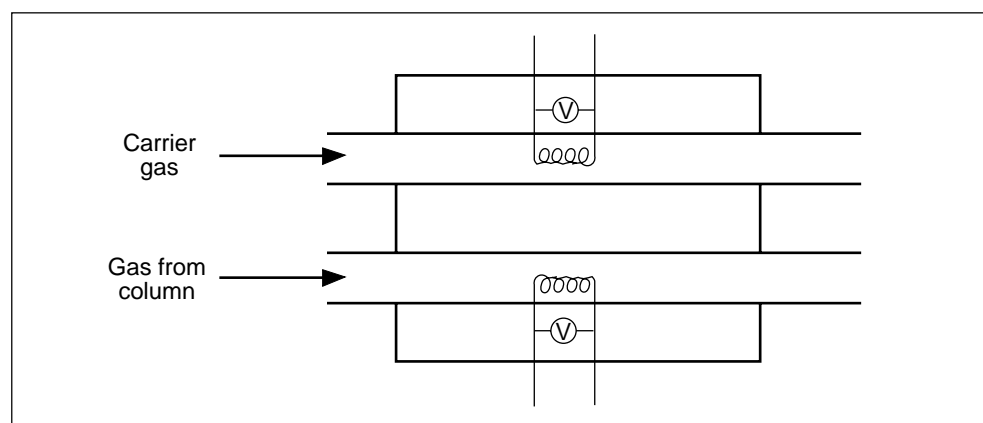


Figure 11 Comparative thermal conductivity detector

The performance of a drug *in vivo* is frequently studied by using GC-MS (see page 22). After giving volunteers a dose of the drug, blood plasma samples are taken at varying intervals, perhaps up to 24 h, and the parent drug and its metabolites are separated and identified. GC-MS has the advantage over other techniques that it is particularly sensitive – eg it can differentiate between the drug and its metabolites when their ultraviolet spectra are very similar.

In reality, most thermal conductivity detectors have four filaments in a Wheatstone bridge arrangement, two filaments in the exit gas from the column and two in the reference gas stream (Fig. 12).

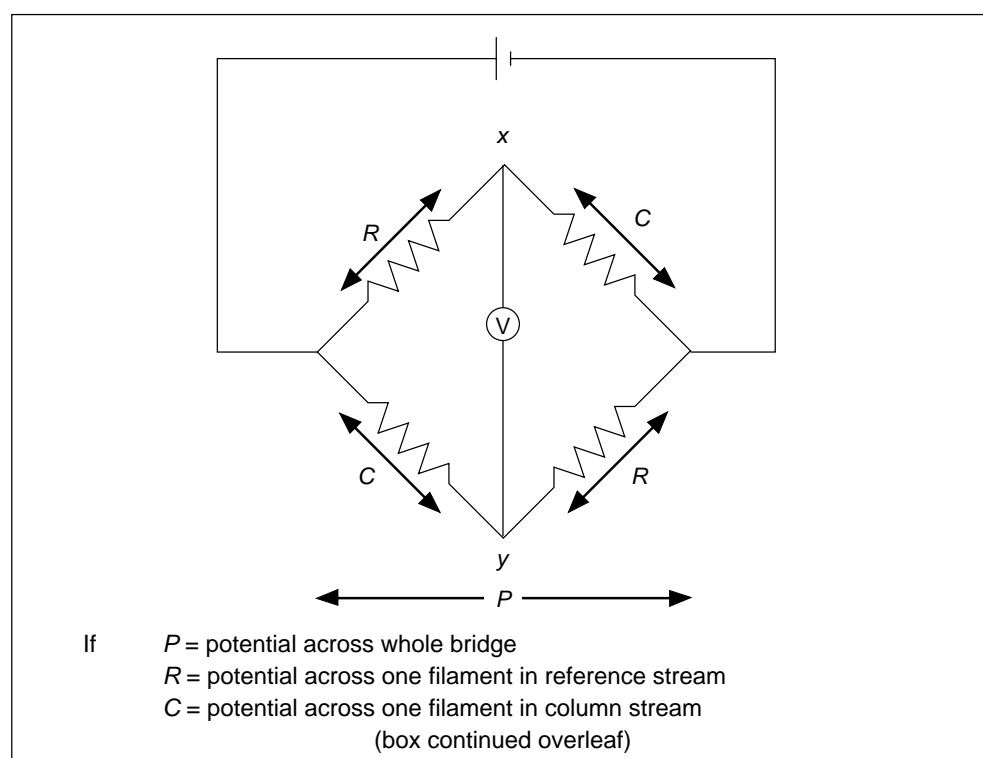


Figure 12 Wheatstone bridge arrangement of filaments

the potential at $x = \frac{R}{C+R} \times P$

potential at $y = \frac{C}{C+R} \times P$

therefore potential between x and y , measured on voltmeter, is

$$x-y = \frac{R-C}{C+R} \times P$$

If the gases in the two sides of the detector are the same, their conductivities will be the same so they will cool the filaments equally and $R-C=0$. The potential between x and y will then be zero. However, if the gases have different compositions then $R-C$ will not be zero and a value will be recorded.

Figure 12 continued

Flame ionisation detection (FID) is particularly useful for detecting organic compounds and this technique is by far and away the most common GC detection system. The gas from the column is mixed with hydrogen and air, and is then burned. Some $\text{CH}\cdot$ radicals, which are formed on combustion, are then oxidised to CHO^+ ions and these ions allow a current to be transmitted via a cathode, the current is then converted to a signal on a chart recorder (*Fig. 13*).

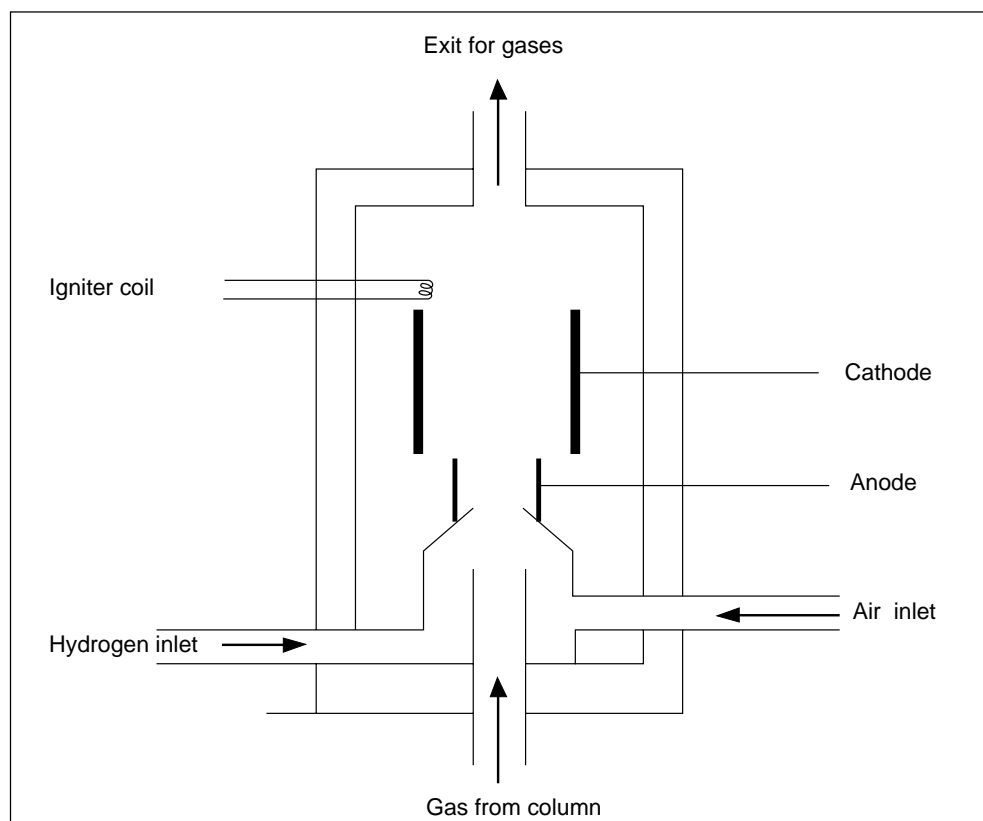


Figure 13 Flame ionisation detector

Although the number of ions produced is small (perhaps one in 10^5 carbon atoms produce an ion) the proportion is constant. The current produced is also proportional



to the number of ions produced, so the total signal on the chart peak is proportional to the amount of that solute in the mixture.

A flame ionisation detector is approximately 1000 times as sensitive as the thermal conductivity detector for organic materials, but is of no use if the solutes do not burn or produce ions. Detectors are available that detect nitrogen and phosphorus as their ions, but these rely on excited rubidium atoms rather than a flame, to produce ions (see Box).

The nitrogen–phosphorus detector (NPD)

The NPD is fundamentally similar to the flame ionisation detector. They both work by forming ions and subsequently detecting them as a minute electrical current. However, a major difference arises in the way the ions are formed.

The eluate (exit gases) of the GC is forced through a jet in the presence of air and hydrogen gas. The mixture passes over the surface of a heated rubidium salt in the form of a bead. The excited rubidium atoms (Rb^*) selectively ionise nitrogen and phosphorus. The ions formed allow a small electric current to flow between two charged surfaces which, under different operating conditions, gives a response to either nitrogen or phosphorus containing compounds, or both (Fig. 14). To differentiate between nitrogen and phosphorus containing compounds the retention time of each solute in the column is used.

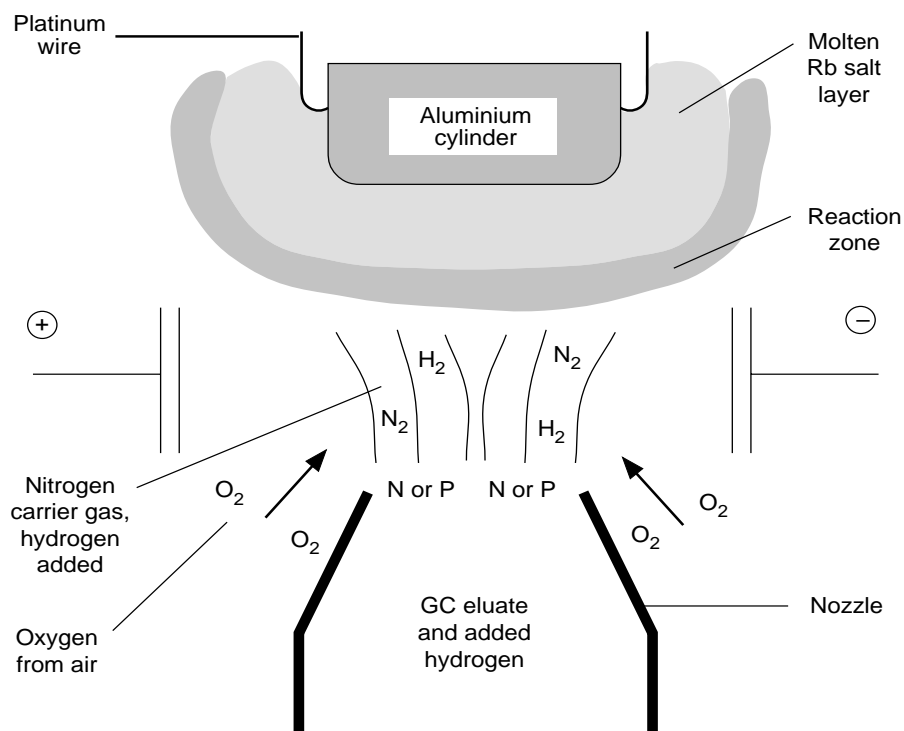


Diagram adapted by courtesy of Hewlett Packard

Figure 14 The nitrogen–phosphorus detector

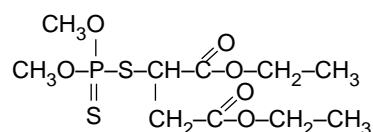
The rubidium bead is mounted on a small aluminium cylinder, and a current is supplied through a platinum wire (the current is a few pico amps). This current heats the bead and excites the rubidium atoms so that they can ionise nitrogen and phosphorus.

The hydrogen is used to maintain the temperature of the bead and consequently only a small flow rate is necessary (Table 1).

Table 1 Typical flow rates in the flame ionisation detector and the nitrogen–phosphorus detector /cm³ min⁻¹

	Air	Hydrogen
FID	300	30
NPD	100	4

The NPD can be set for phosphorus detection to determine very low concentrations of pesticides such as malathion, the active ingredient of Prioderm, a treatment used for killing head lice. The structure of malathion is:



Once a mixture has been separated by GC its components need to be identified. For known substances this can be done from a knowledge of the time it takes for solutes to reach the detector once they have been injected into the column. These are known as retention times and will vary depending on each of the following:

- 1 the flow rate of the carrier gas;
- 2 the temperature of the column;
- 3 the length and diameter of the column;
- 4 the nature of and interactions between the solute and the stationary and mobile phases; and
- 5 the volatility of the solute.

Each material to be identified by GC is run through the column so that its retention time (the time for the components to pass through the column) can be determined. For compounds of completely unknown structure or composition the solutes must be collected individually and then analysed by using another method – eg mass spectrometry.

Liquid chromatography (LC)

Liquid chromatography is similar to gas chromatography but uses a liquid instead of a gaseous mobile phase. The stationary phase is usually an inert solid such as silica gel (SiO₂.xH₂O), alumina (Al₂O₃.xH₂O) or cellulose supported in a glass column (Fig. 15).

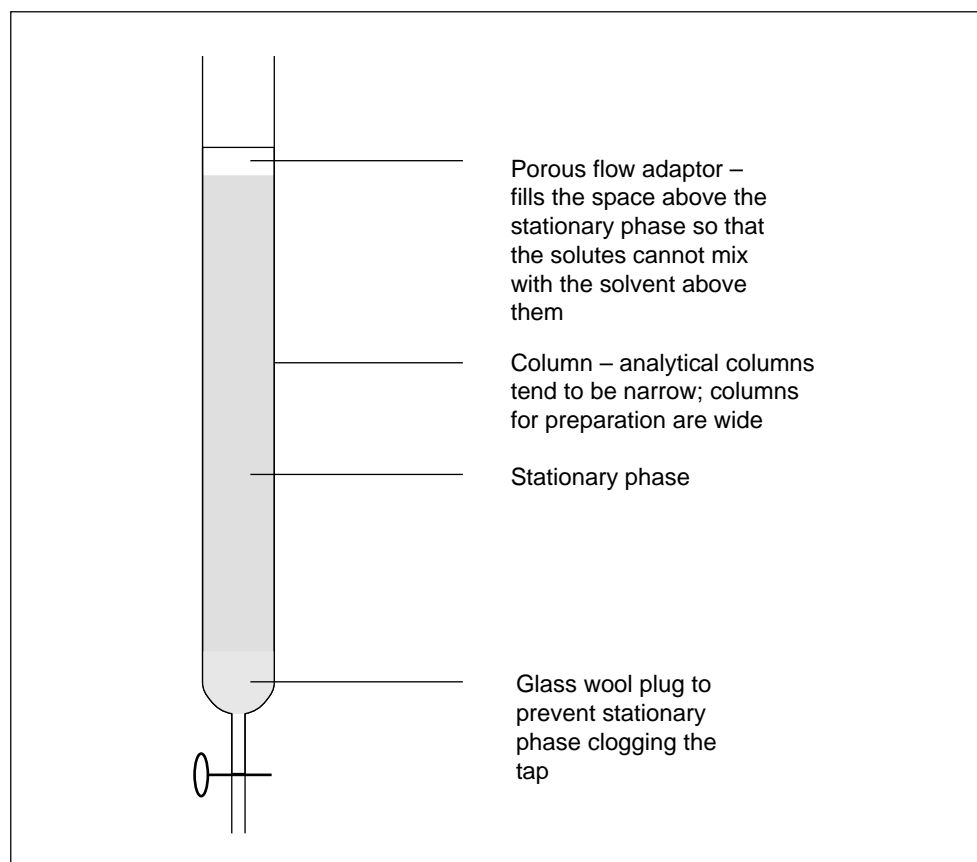
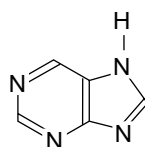


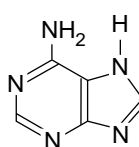
Figure 15 Liquid chromatography column

The adsorbing properties of silica and alumina are reduced if they absorb water, but the reduction is reversed by heating to 200–400 °C. Silica is slightly acidic, and readily adsorbs basic solutes. On the other hand, alumina is slightly basic and strongly adsorbs acidic solutes. Other stationary phases that can be used include magnesia, $\text{MgO} \cdot x\text{H}_2\text{O}$ (good for separating unsaturated organic compounds); and dextran (a polymer of glucose) cross-linked with propan-1,2,3-triol (glycerol, $\text{CH}_2\text{OHCHOHCH}_2\text{OH}$), which is sold as Sephadex and can separate compounds such as purines. Sephadex has the structure shown in *Fig. 16*.

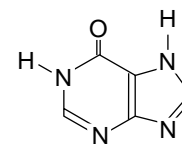
Purines are a class of nitrogen containing bicyclic organic compounds that include adenine and guanine, the bases found in DNA.



Purine



Adenine



Guanine

A wide range of solvents are used in this technique, including hydrocarbons, aromatic compounds, alcohols, ketones, halogenocompounds and esters. A mixture of solvents can also be used. The optimum solvent is chosen by running experiments on a small scale using TLC plates.

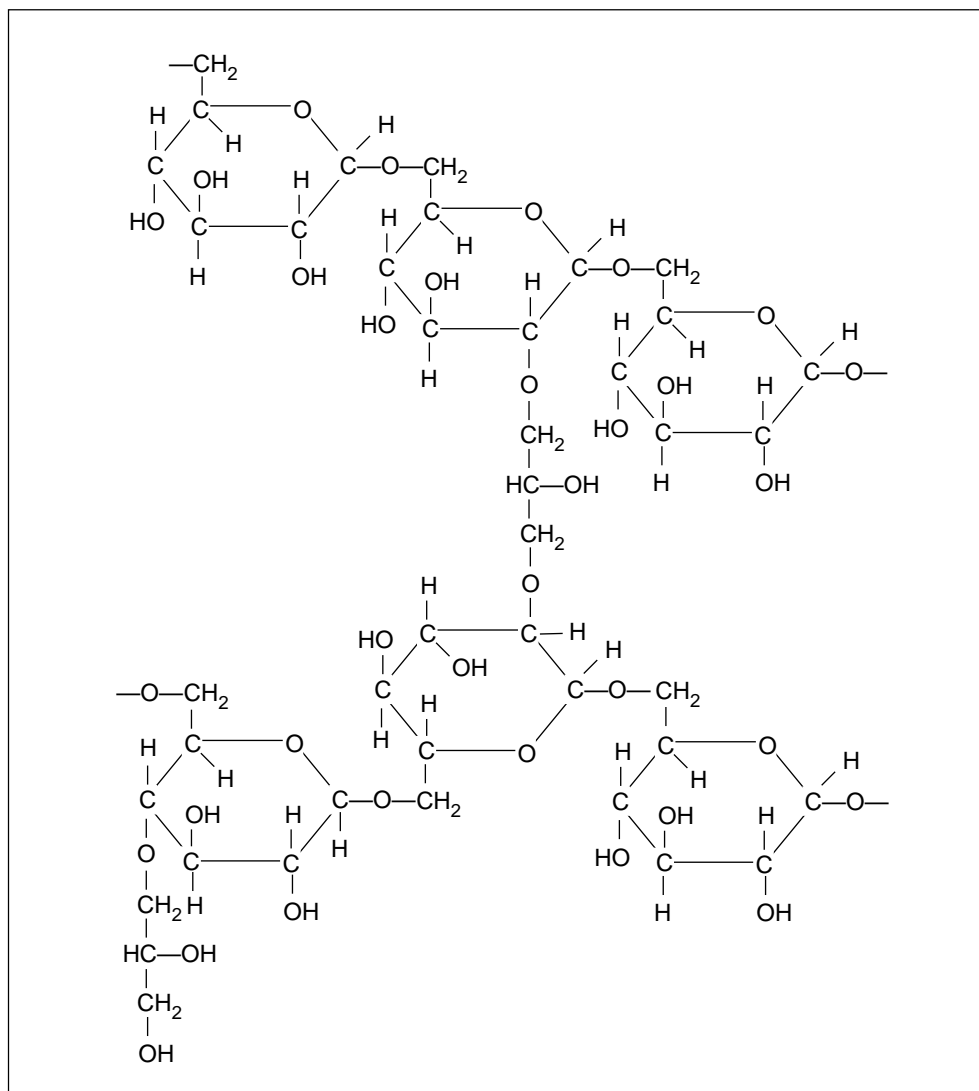


Figure 16 Structure of Sephadex

Practical details

When setting up a liquid chromatography column it is vital that the stationary phase is saturated with solvent, because any air present will interrupt the smooth flow and will result in inefficient or incomplete separation.

An inert material such as ceramic wool is normally inserted to stop any solid from clogging the tap at the bottom of the column. The stationary phase is then added. A common way of separating solutes is to allow the solvent and mixture to descend the column under gravity. For example, alumina can be mixed with a portion of solvent to form a slurry. This is then poured into the column, and any excess solvent is run out of the bottom.

If the column is not saturated with solvent at all times then air bubbles in the column can cause non-uniform separation. The sample is added to the column as a concentrated solution and is absorbed onto the top of the column. The column is then topped up with solvent and the solvent is allowed to flow through.



An alternative technique is 'flash' chromatography. The conditions are similar to those used in liquid chromatography but the solvent is forced through the column at a faster rate by applying pressure from an inert gas such as nitrogen. The alumina or silica can be saturated by pouring solvent over the dry powder in the column, then applying pressure from a nitrogen cylinder.

Once the column is set up, the mixture to be separated is carefully added to the top of the stationary phase and some solvent poured in. A valve is attached to the top of the column and nitrogen is introduced at a pressure of up to $2 \times 10^5 \text{ Nm}^{-2}$ (2 atmospheres). The tap at the bottom of the column is opened and the solvent allowed to pass through. This is collected in tubes and the fractions identified. As the amount of solvent above the stationary phase decreases it can be replaced by closing the tap at the bottom of the column, and releasing the pressure in the column before topping up the head of solvent. The valve is then replaced and the gas supply turned on again before the tap at the bottom of the column is opened.

If the components of the mixture are coloured collecting them is easy. If they are colourless small volumes of the liquid leaving the column (the eluate) can be collected in tubes, and then the solutes identified by another method – eg fluorescence under ultraviolet light, or by TLC.

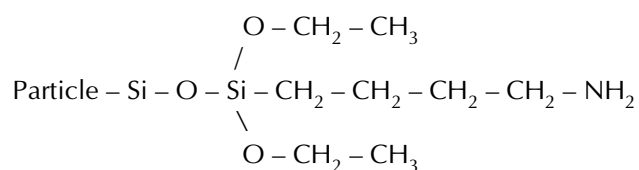
In both liquid chromatography and flash chromatography the solutes can be extracted from the liquid collected (the eluate) by evaporating off the solvent and if necessary they can then be identified by running a simple TLC experiment.

A more elaborate variation on liquid chromatography is high performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC)

The efficiency of a separation increases if the particles in the stationary phase are made smaller. This is because the solute can equilibrate more rapidly between the two phases. However, if the particles are made smaller, capillary action increases and it becomes more difficult to drain the column under gravity. Consequently, a high pressure has to be applied to the solvent to force it through the column. A schematic representation of the process is shown in *Fig. 17*.

The stationary phase normally consists of uniform porous silica particles of diameter 10^{-6} m, the surface pores having a diameter of 10^{-8} – 10^{-9} m. (This gives the solid a very high surface area.) The particles can be bonded with a non-volatile liquid that allows interactions of solutes with different polarities. These liquids are held on the silica particles by covalent bonds – eg the surface of one polar resin has the structure



Interaction is then possible between the lone pair of electrons on the nitrogen atom and the solute molecule.

The stationary phase particles are packed into the HPLC column and are held in place by glass fibres coated with inert alkyl silane molecules. The separation in HPLC is normally so efficient that a long column is not necessary. (If the column was too

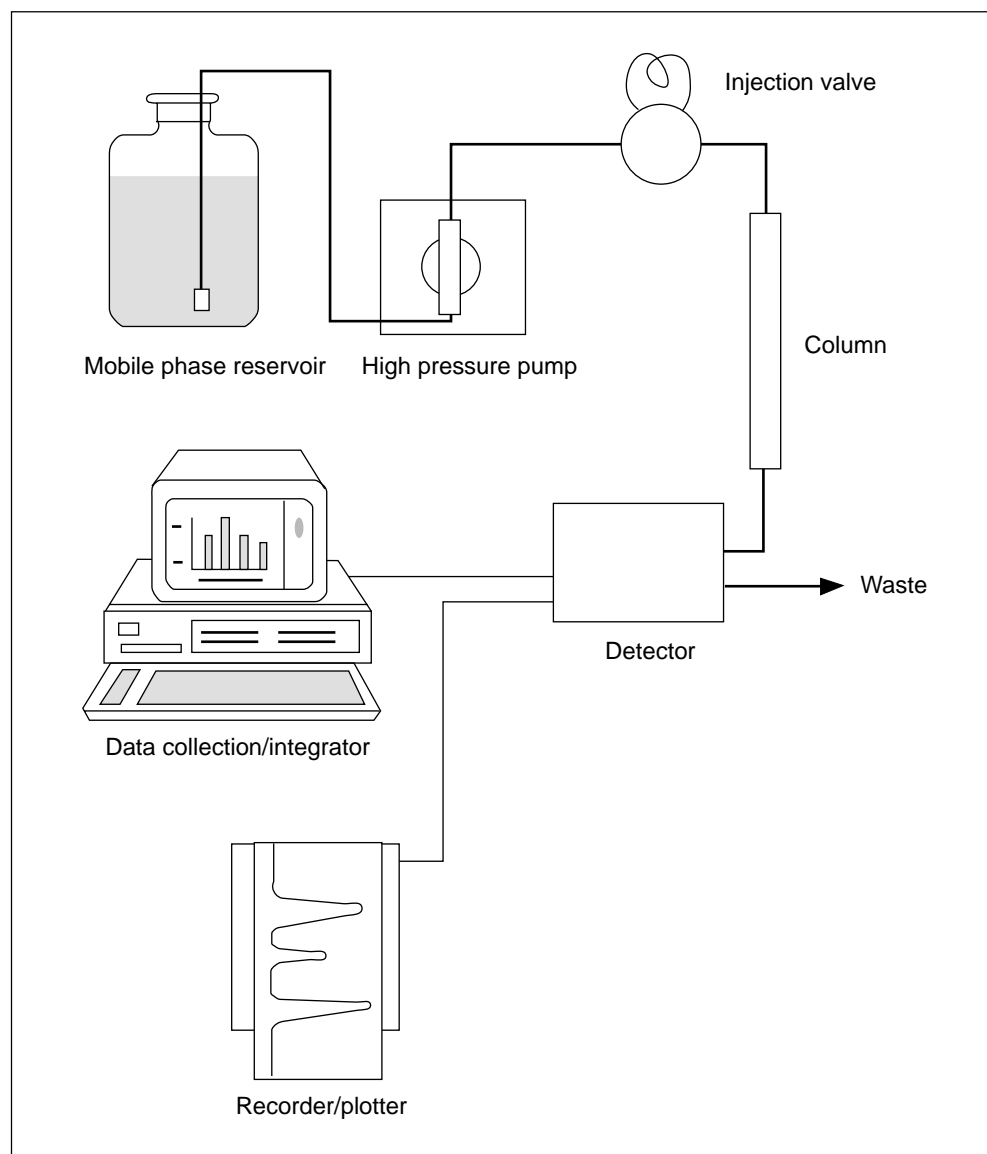


Figure 17 Schematic representation of an HPLC system

long the pressure needed would be excessive.) Columns are typically 10–30 cm long, with an internal diameter of 4 mm.

Reproducibility is essential, and this is only possible if a constant flow rate is maintained. This means that the pump used must be capable of generating a uniform pressure; twin cylinder reciprocating pumps are typical. This type of pump has two chambers with pistons 180° out of phase, and can generate pressures up to 10 MNm⁻² (10 MPa/100 atmospheres). The high pressures involved mean that the instrumentation has to be very strong, and the ‘plumbing’ is usually constructed from stainless steel. The pump and the piping must be inert to the solvent and solutes being passed through them.

The flow rates of HPLC columns are slow – often in the range 0.5–5 cm³ min⁻¹. The volumes of the columns are very small, and this means that the injection of the sample must be very precise and it must be quick without disturbing the solvent flow. Sample volumes are small – 5–20 mm³ is usually sufficient.

The passage of solutes through a GC can be speeded up by increasing the



temperature of the column. The same effect in HPLC is achieved by changing the composition of the mobile phase – *ie* there is a concentration gradient in which the proportion of methanol, say, in a methanol/water system is increased linearly from 10 per cent methanol to 60 per cent methanol, during the separation.

The amounts passing through the column are usually too small to extract from the solvent before identification, so the solutes in solution are analysed as they leave the column. Most compounds separated by HPLC absorb ultraviolet light. The eluate is passed along a small cell so that ultraviolet radiation can be passed through the liquid (*Fig. 18*).

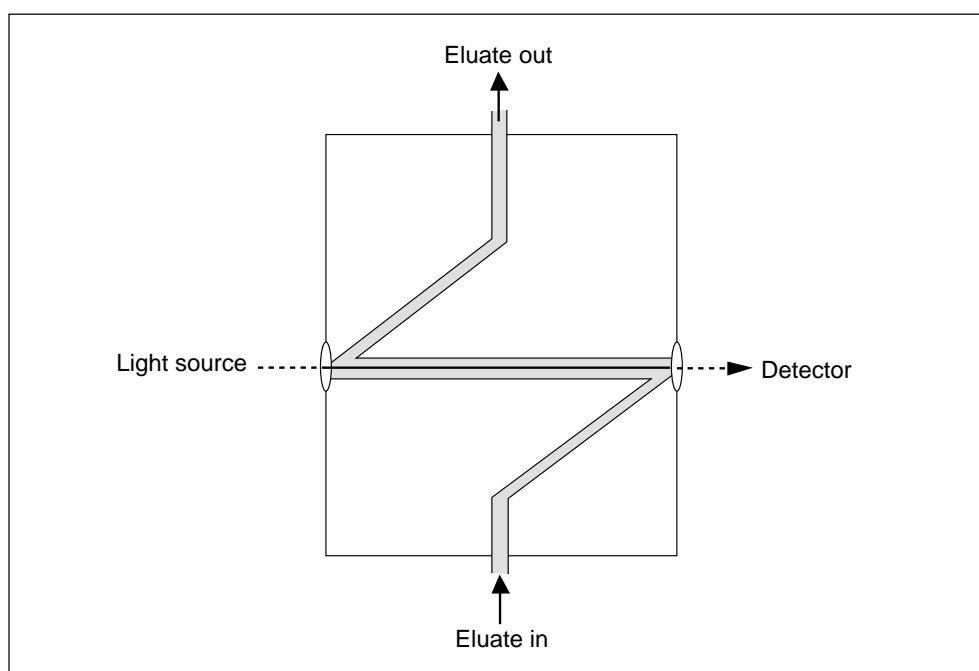


Figure 18 A micro cell for identifying solutes in the liquid leaving an HPLC column

The relative use of HPLC and GC varies from industry to industry and very much depends on the compounds to be separated. Many compounds decompose at the temperatures required for efficient GC separation while HPLC separation can be achieved readily. However, GC is particularly useful in detecting residual solvents in formulations and is also invaluable in looking for degradation products. Amines and acids are not separated well by GC because they tend to be too polar.

Other methods used in conjunction with HPLC for determining the presence of solutes are based on:

- 1 mass spectrometry;
- 2 infrared spectroscopy;
- 3 visible spectroscopy;
- 4 ultraviolet spectroscopy;
- 5 fluorescence spectroscopy;
- 6 conductivity measurement; and
- 7 refractive index measurement.

Whichever method is used it is vital that the volume of liquid used is very small, otherwise the sharpness of the separation peaks will disappear and the resolution of the final chromatogram will be lost.

Once the retention time of a solute has been established for a column using a set of operating conditions, that solute can be identified in a mixture from its retention time (assuming that another component with the same retention time is not also present).

Ion exchange chromatography

Ion exchange chromatography is used to remove ions of one type from a mixture and replace them by ions of another type.

The column is packed with porous beads of a resin that will exchange either cations or anions. There is one type of ion on the surface of the resin and these are released when other ions are bound in their place – eg a basic anion exchange resin might remove nitrate(V) ions (NO_3^-) from a solution and replace them with hydroxide ions (OH^-).

Many of the resins used are based on phenylethene (styrene) polymers with cross-linking via 1,4-bis-ethenylbenzene (divinylbenzene, Fig. 19).

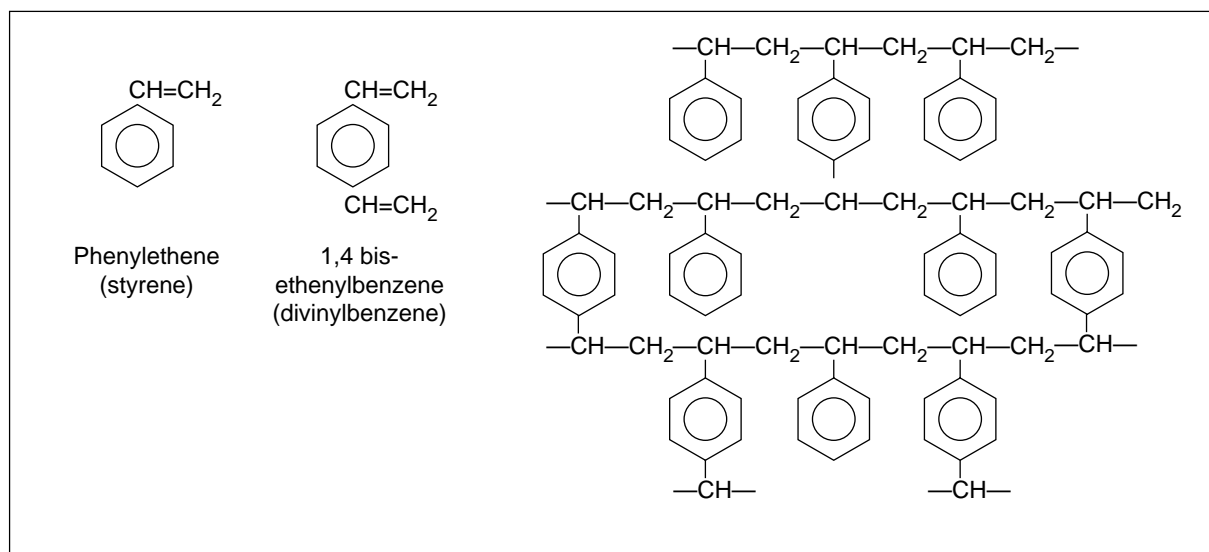
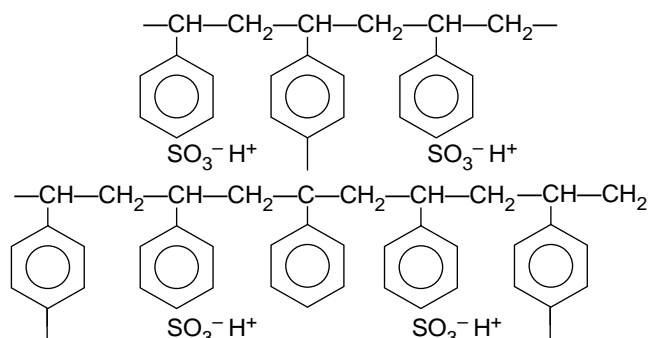


Figure 19 Copolymer of cross-linked styrene–divinylbenzene

If the ion is a quaternary ammonium group the resin is strongly basic (eg $-\text{CH}_2\text{N}(\text{CH}_3)_3^+ \text{OH}^-$) then the resin will selectively remove the ions: $\text{I}^- > \text{NO}_3^- > \text{Br}^- > \text{NO}_2^- > \text{Cl}^- > \text{OH}^- (> \text{F}^-)$, thus liberating hydroxide ions while nitrate(V) ions, for example, are removed. The exchange site can be strongly or weakly acidic or basic depending on the group present. Examples of such groups are shown in Table 2.



The presence of ionic groups bonded to the polymer of phenylethene and 4-ethenylphenylethene provides the ability to exchange ions. For example, the inclusion of $-\text{SO}_3^- \text{H}^+$ groups in the 4-position of phenylethene gives a strongly acidic resin which will selectively remove the ions $\text{Ag}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{K}^+ > \text{NH}_4^+ > \text{Na}^+ > \text{H}^+$. This means that both potassium and sodium ions will be removed from a solution containing both, until all the H^+ ions have been discharged. Then potassium ions from the solution will replace the sodium ions on the column.

Figure 20 A strongly acidic cationic exchange resin

Table 2 Acid/base character of exchange site groups

Character

Strongly acidic	$-\text{SO}_3^- \text{H}^+$
Weakly acidic	$-\text{R}-\text{COO}^- \text{H}^+$
Weakly basic	$-\text{CH}_2\text{NH}(\text{CH}_3)_2^+ \text{Cl}^-$
Strongly basic	$-\text{CH}_2\text{N}(\text{CH}_3)_3^+ \text{OH}^-$

The degree of cross-linking of the copolymer can have a significant effect on the behaviour of the resin, and can change selectivity. The range of cross-linking in commercial resins is approximately 4–12 per cent of the monomer units and the characteristics of resins having low and high degrees of cross-linking can be compared (Table 3).

Table 3 Characteristics of resins having low and high degrees of cross-linking

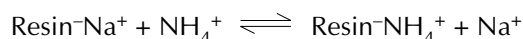
Low degree of cross-linking	High degree of cross-linking
Less rigid	More rigid
More porous	Less porous
Rapid exchange of ions	Slower exchange of ions
Greater swelling in water	Little swelling in water
Lower exchange capacity	Higher exchange capacity
Lower selectivity	Greater selectivity

The selectivity for certain ions can change with the degree of cross-linking because a highly cross-linked resin has smaller pores than one with little cross-linking, and bulky ions cannot get into the smaller pores, so they are not exchanged.



Ion exchange equilibrium

If two ions are competing for the binding sites of a resin an equilibrium will be established. For example, a cation-exchange resin used to remove ammonium ions and replace them with sodium ions would have the equilibrium:



This equilibrium can be described by an equilibrium constant (K) which is called the selectivity coefficient:

$$\text{Selectivity coefficient} = \frac{[\text{Resin}^{-}\text{NH}_4^{+}][\text{Na}^{+}]}{[\text{Resin}^{-}\text{Na}^{+}][\text{NH}_4^{+}]}$$

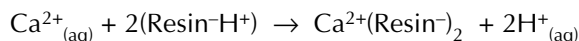
The equilibrium position can be changed in either direction by changing the concentration of ions in the aqueous phase. A column might remove relatively small amounts of ammonium ions almost quantitatively from a solution, but they can be liberated from the column equally efficiently by passing an excess of a concentrated solution of sodium ions through the column. The removal occurs despite the fact that ammonium ions are preferentially bound to the resin, and this principle is useful in regenerating the column. Once a column's exchange capacity is approached, it is regenerated simply by passing a concentrated solution of sodium ions through it. This shifts the equilibrium back to the left hand side.

Under most circumstances absorption and release of ions is effectively quantitative, so it is possible to remove selected ions and determine their concentrations by titration – eg if calcium ions are removed from a sample of hard water and are replaced by hydrogen ions from the acidic form of a resin, then the concentration of hydrogen ions can be determined by titrating the eluate with a standard alkaline solution.

Macromolecules can also be exchanged by using resins with larger pore sizes. The resins are generally derived from proteins or polysaccharides such as cellulose, and the exchange sites are bound to the OH groups of the saccharide units. The sites can be either weakly basic or weakly acidic, with polar covalent groups (eg amines); or they can be strongly acidic or strongly basic, with ionic groups (eg carboxylic or sulphonic acids).

Ion exchange applications

The solubility product of some partially soluble substances can be determined by using exchange methods. For example, a saturated calcium sulphate solution can be passed down a cation exchange column that replaces the calcium ions with hydrogen ions:



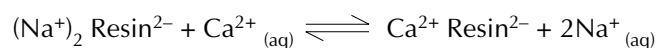
The hydrogen ions liberated can then be determined by titration using a standard sodium hydroxide solution (phenolphthalein as indicator). Once $[\text{Ca}^{2+}]$ has been calculated from $[\text{H}^{+}]$, the solubility product of the salt can be calculated.

By passing a water sample through two columns, one a cationic exchanger and the other an anionic exchanger, it is possible to remove any ionic impurities. Tap water, for example, can be passed through one column where the metal ions present, such as Mg^{2+} and Ca^{2+} , are replaced by H^{+} ; then the anions present, such as F^{-} , Cl^{-} and NO_3^{-} , are replaced by OH^{-} in the second column. The product is deionised water.



The dishwasher

A more sophisticated application of ion exchange resins is the water softener installed in many modern automatic dishwashers. The cleaning efficiency of dishwashers using detergents increases if the water is heated. This causes problems if the water supply has temporary hardness because when it is heated the hydrogencarbonate ions decompose to form carbonate ions and these are deposited on the heating element, reducing its efficiency. Further problems arise if the ions causing hardness are not removed, because inside the dishwasher they can deposit a film on the items that are meant to be cleaned. To overcome these problems calcium and magnesium ions are removed from the water entering the dishwasher by a cationic exchange resin. They are replaced by sodium ions – eg:



The exchange capacity of the resin is regenerated at the start of the next washing cycle by passing saturated sodium chloride (salt) solution through the resin. The high concentration of sodium ions favours the back reaction in the above equilibrium. The unwanted calcium and magnesium ions and the excess sodium chloride are pumped out of the dishwasher during the next drain cycle. It is important that no sodium chloride remains because it is corrosive.

The sodium chloride solution is formed by pumping fresh water through the salt chamber that the user periodically refills. The type of salt put into the dishwasher is important – table salt contains magnesium chloride to keep it free-flowing and the magnesium ions can both cause hardness and interfere with resin regeneration. The small crystal size of table salt also means that it can easily be washed into the resin chamber and cause a blockage. Granular or dendritic salt crystals are recommended to eliminate this risk (minute amounts of potassium hexacyanoferrate(II), $\text{K}_4\text{Fe}(\text{CN})_6$), are added to salt solution to encourage the growth of dendritic crystals).

To minimise unnecessary consumption of salt some dishwashers can be set to take account of the relative hardness of the supply in the area where the dishwasher is used. Hotpoint dishwashers, for example, pump different volumes of salt solution through the resin according to the hardness of the water.

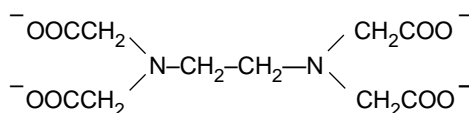
Other uses

Ion exchange resins can be used in HPLC columns, and a variety of such resins exist. It is possible to monitor the solutes leaving the HPLC column by measuring the conductivity of the solution, and extremely small ion concentrations can be determined by using this method. This is routinely done to monitor water purity in environmental analysis. An anionic exchange resin is used to separate ions. The sample to be analysed is added to an aqueous mobile phase containing sodium carbonate and sodium hydrogencarbonate, which is passed through an anionic exchange resin in an HPLC column. The ions to be detected exchange with carbonate ions on the resin surface. The carbonate ion concentration in the mobile phase is high enough to elute them off again, but at different rates. The anions in the sample are recognised by their retention times on the column, and their concentrations are determined from the conductivity of the eluate. Anions typically elute from the column in the order F^- , Cl^- , NO_2^- , Br^- , NO_3^- , PO_4^{3-} , SO_4^{2-} . Concentrations in the order of $10^{-3} \text{ g dm}^{-3}$ ($\text{ppm}/\mu\text{g cm}^{-3}$) are routinely measured. Measuring concentrations down to $10^{-6} \text{ g dm}^{-3}$ is required in the nuclear power industry, where the leak of radioactive contaminants must be detected (and stopped!) immediately.

Many EDTA (ethylenediaminetetraacetate) complexes absorb ultraviolet light, so



transition metal concentrations can be determined by making complexes with EDTA. Alternatively, EDTA can be estimated by adding transition metal ions to its solution. This is done in the food industry, where canned shellfish have added EDTA. Crustacean blood is based on a Cu-haem complex (and not an Fe-haem complex as in humans). On cooking a blue/black colouring appears where the blood was and this colouring spreads into the flesh making it look unattractive. If EDTA is added it competes for the copper ions and complexes them so that discolouration does not occur. To conform to legislation the total EDTA concentration must be within certain limits. Excess copper ions are added to ensure that all the EDTA has complexed as $(\text{Cu}(\text{EDTA}))^{2-}$, and an ion exchange HPLC column is used to separate all the anions present according to their charge. The eluate containing all the EDTA (identified by its retention time on the column) is then passed through a cell where its ultraviolet absorption at 280 nm is measured; and from this measurement the EDTA concentration can be calculated.



EDTA

Gel filtration or gel permeation chromatography

The separation of large molecules, often in biochemical situations, can be achieved in a column which works on the basis of molecular exclusion. The mixture of solutes is carried through the column by a solvent. The stationary phase (the gel) typically consists of particles of a cross-linked polyamide which contains pores. Separation occurs according to molecular size – the larger molecules passing through the column fastest (*Fig. 4*).

Different gels are available that allow the separation of proteins with relative masses ranging from a few hundred to in excess of 10^8 . The greatest resolution is achieved by using very small gel particles, but the flow rate through the column then becomes much slower.

Applications of chromatography

Chromatography is used to separate compounds in reaction mixtures both in the laboratory and on the industrial scale. However, technology has now advanced sufficiently to allow chromatographic techniques to be interfaced directly to other analytical methods. For example, gas chromatographs are routinely linked to mass spectrometers, and HPLC columns are linked to ultraviolet/visible spectrometers.

Determination of caffeine and theobromine

Caffeine is found in both tea (leaf buds and young leaves of *Camellia sinensis*) and coffee (roasted seeds of *Coffea arabica* and *Coffea robusta*). In tea, caffeine constitutes 2–3.5 per cent on a dry leaf basis, though blended teas typically contain 3 per cent caffeine. Roasted coffee beans generally contain 1.1–1.8 per cent caffeine, while instant coffee powders average 3.0 per cent.

Caffeine is widely accepted as being a stimulant, although other physiological effects (such as its diuretic effect) are also observed. Some studies have suggested that there might be a link between caffeine and certain disorders – eg heart disease and kidney malfunction. Some common concerns that people have about caffeine are its stimulant effect and its addictiveness, and this has led to an increased market demand for decaffeinated products. Chlorinated solvents such as trichloroethene



(trichloroethylene) and trichloroethane were often used to extract caffeine but the solvent residues may possibly be more harmful than the caffeine itself. Modern techniques for removing caffeine include using supercritical liquid carbon dioxide as a solvent and reverse osmosis. However, the methods for caffeine removal need careful control to avoid excessive loss of desirable flavour constituents.

Theobromine is a metabolite of caffeine – *ie* it is produced by the biological processes of the body. It also occurs naturally in the cacao beans (*Theobroma cacao*) that are used to make cocoa and chocolate. Approximately 1 per cent of the mass of the cocoa bean is theobromine, a proportion that rises to 2.5 per cent in de-fatted cocoa powder. As this amount is relatively constant it is possible to calculate the cocoa solids content of chocolate products such as confectionery, chocolate coatings, drinking chocolate and ice creams, from their theobromine content.

The chemical structures of caffeine, theobromine (which does not contain bromine) and other related xanthines are shown in *Fig. 22*.

To satisfy legal and labelling requirements it is essential that the amount of caffeine present, both initially and after processing, can be determined accurately. The method described here has the advantage that it can also be used to determine theobromine levels simultaneously.

In the first stage, a known mass of the coffee, tea, or chocolate product is gently boiled with deionised water. The boiling water extraction is essential to ensure complete dissolution of the xanthines, because they are not all readily soluble. The cooled solution is then clarified if necessary, with the aid of Carrez solutions (see Box). These solutions precipitate proteinaceous materials along with any soluble starch components.

There are two Carrez solutions

Carrez Solution 1: 21.9 g of zinc ethanoate dihydrate dissolved in deionised water containing 3.0 g of ethanoic acid, made up to 100 cm³ with deionised water.

Carrez Solution 2: 10.6 g of potassium hexacyanoferrate(II) (ferrocyanide) trihydrate dissolved in deionised water and made up to 100 cm³.

These two solutions are added to the extract in equal proportions to clarify the solution.

The resulting solution is made up to a known volume, mixed thoroughly, and filtered. The clear filtrate remaining is diluted if necessary to obtain xanthine concentrations within a standard calibration range. A schematic plan of the sample preparation is shown in *Fig. 21*.

An aliquot of the sample extract (typically 0.020 cm³/20 μl) is then analysed. It is necessary to determine whether caffeine, theobromine, or both are present, and in what concentrations. The caffeine and theobromine are separated on a 15 cm HPLC column (see Box).

The HPLC column contains a 5×10^{-6} m (particle size) silica based support with its surface chemically modified with bonded C_{18} groups. The solvent system (mobile phase) contains 12 per cent ethanonitrile (acetonitrile) in a 0.5 per cent aqueous solution of ammonium nitrate(V).

Systems such as this one, in which the mobile phase is the more polar, are known as reversed phase chromatography. (In industry reversed phase chromatography is more commonly used than normal phase chromatography.)

In normal phase chromatography the stationary phase is more polar – eg an unmodified silica stationary phase might be used – the surface hydroxy groups giving it its polar nature. Mobile phases used in such separations would tend to be less polar. Some solvents in order of increasing polarity are:

- 1:1 v/v Ethoxyethane:petroleum ether
- 3:1 v/v Ethyl ethanoate:petroleum ether
- 9:1 v/v Dichloromethane:methanol

In normal phase chromatography the least polar solute comes off the column first – in reverse phase chromatography the most polar solute comes off first. In each case the eluting order depends on the strength of the interaction between the solutes and the two phases.

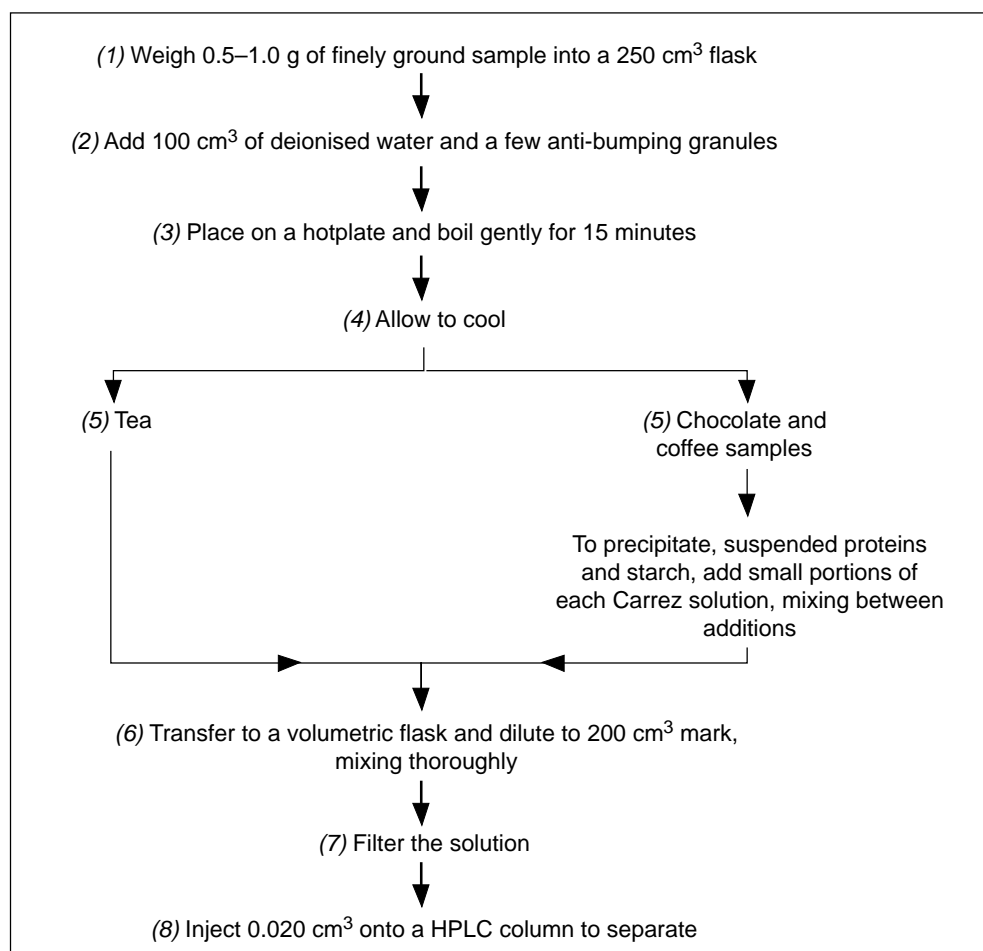


Figure 21 Schematic plan of sample preparation

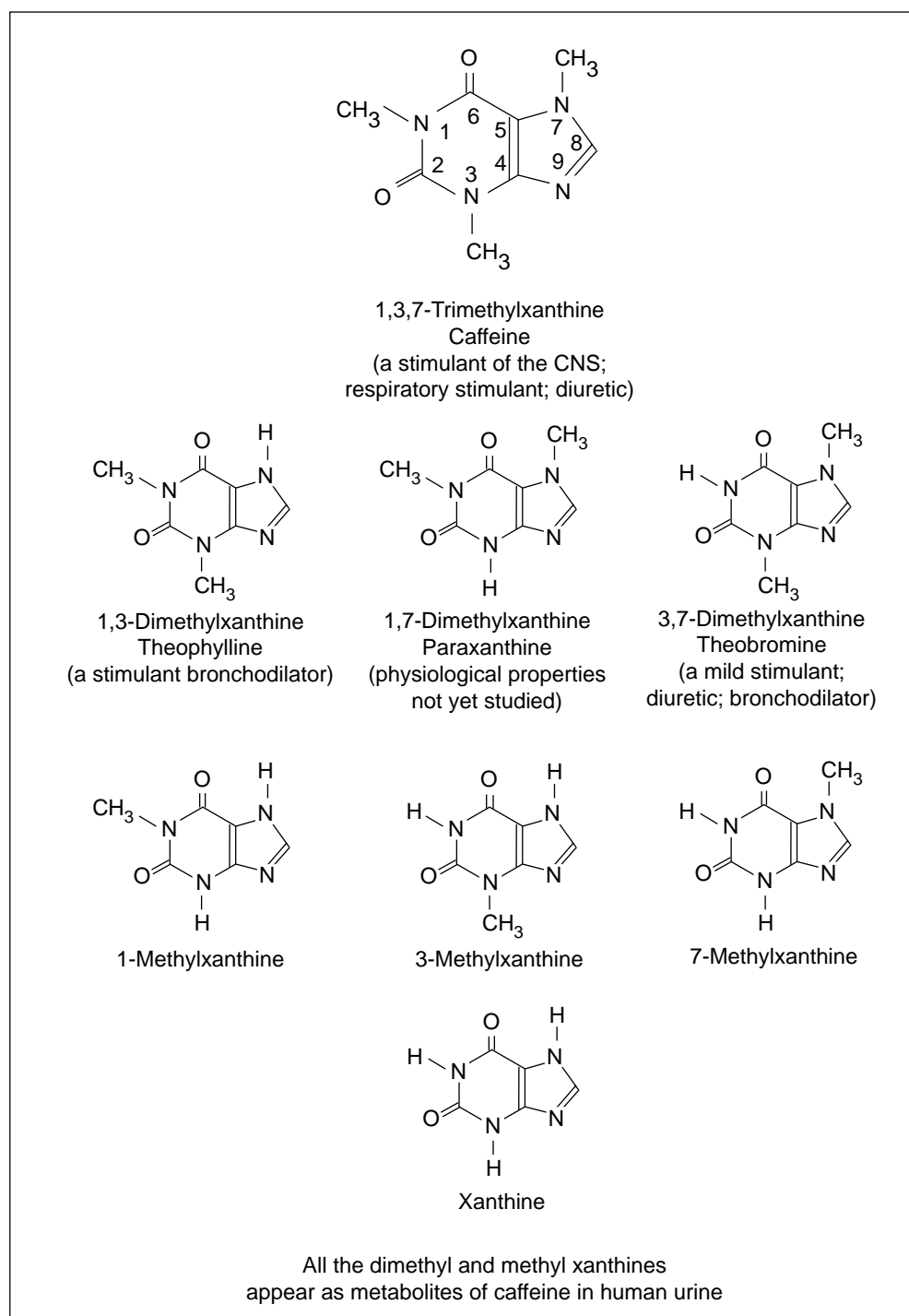


Figure 22 Chemical structures of xanthine and related substances



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The flow rate is typical of HPLC, 1 cm³ per min, and this lends itself nicely to interfacing with ultraviolet absorption detection. The eluate from the HPLC column passes through a 0.008 cm³ (8 μl) flow cell made with ultraviolet transparent quartz windows. A variable wavelength spectrometer is set at 274 nm because both caffeine and theobromine absorb at this wavelength (*Fig. 23*).

The HPLC/UV system is calibrated so that the data emerging from it can be interpreted. Standard solutions of caffeine and theobromine of different concentrations pass through the column and ultraviolet detector so that the retention time of the solutes can be determined as well as the areas of the peaks corresponding to known concentrations (*Fig. 24*).

It is normal to calibrate the instrument by using two standards, and to ensure that the concentration of the solution analysed falls within the range provided by the standards. The standards used are:

	Theobromine μg per cm ³	Caffeine μg per cm ³
Standard 1	10	20
Standard 2	20	40

If the solution being analysed is too concentrated it is simply diluted by an appropriate factor to bring it into the required range. Once the concentrations of theobromine and caffeine have been determined their masses in the boiled solution can be calculated, and hence their percentages by mass.

This method is essentially straightforward and allows the food industry to maintain quality control of its products, as well as researching into the potential of new production methods.

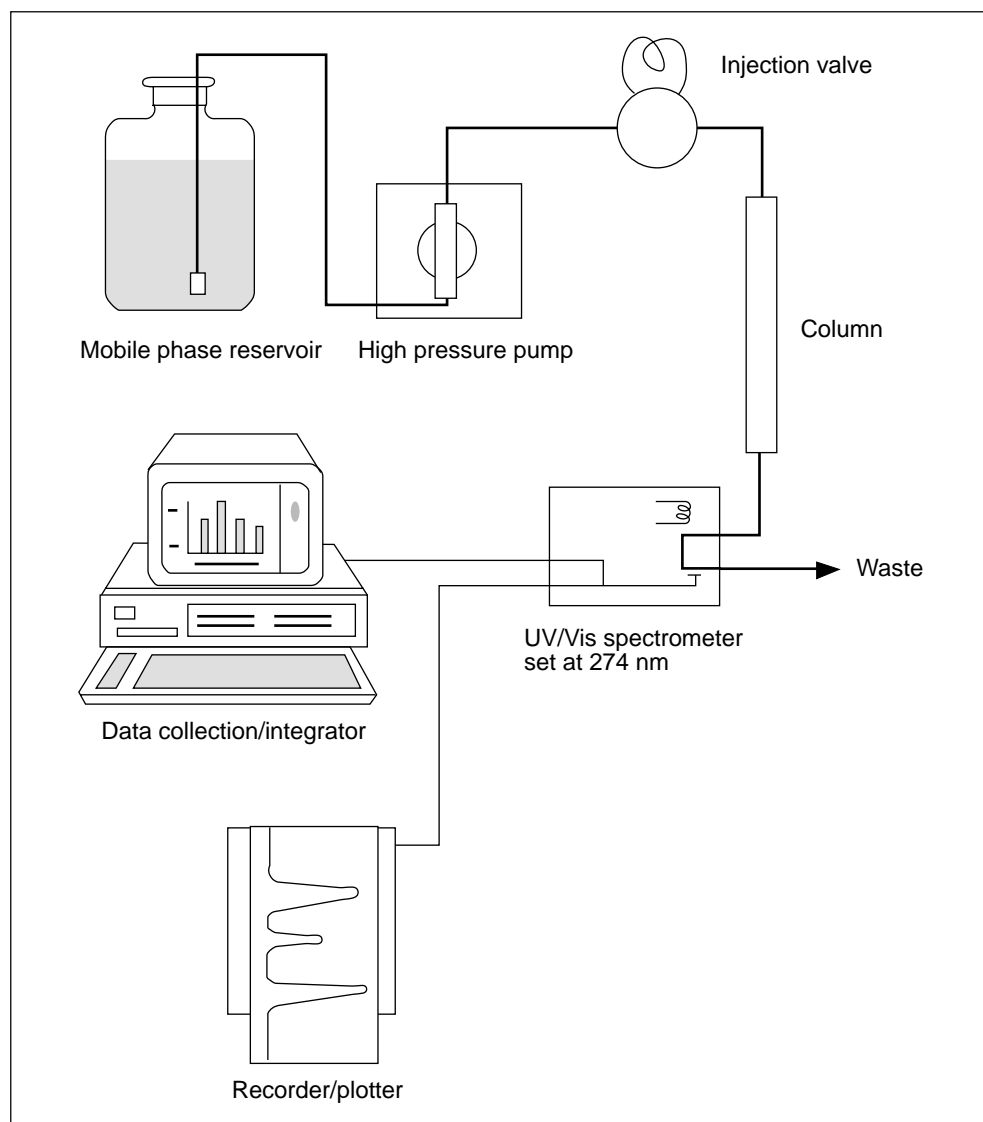


Figure 23 Separation using HPLC and detection using ultraviolet light at 274 nm

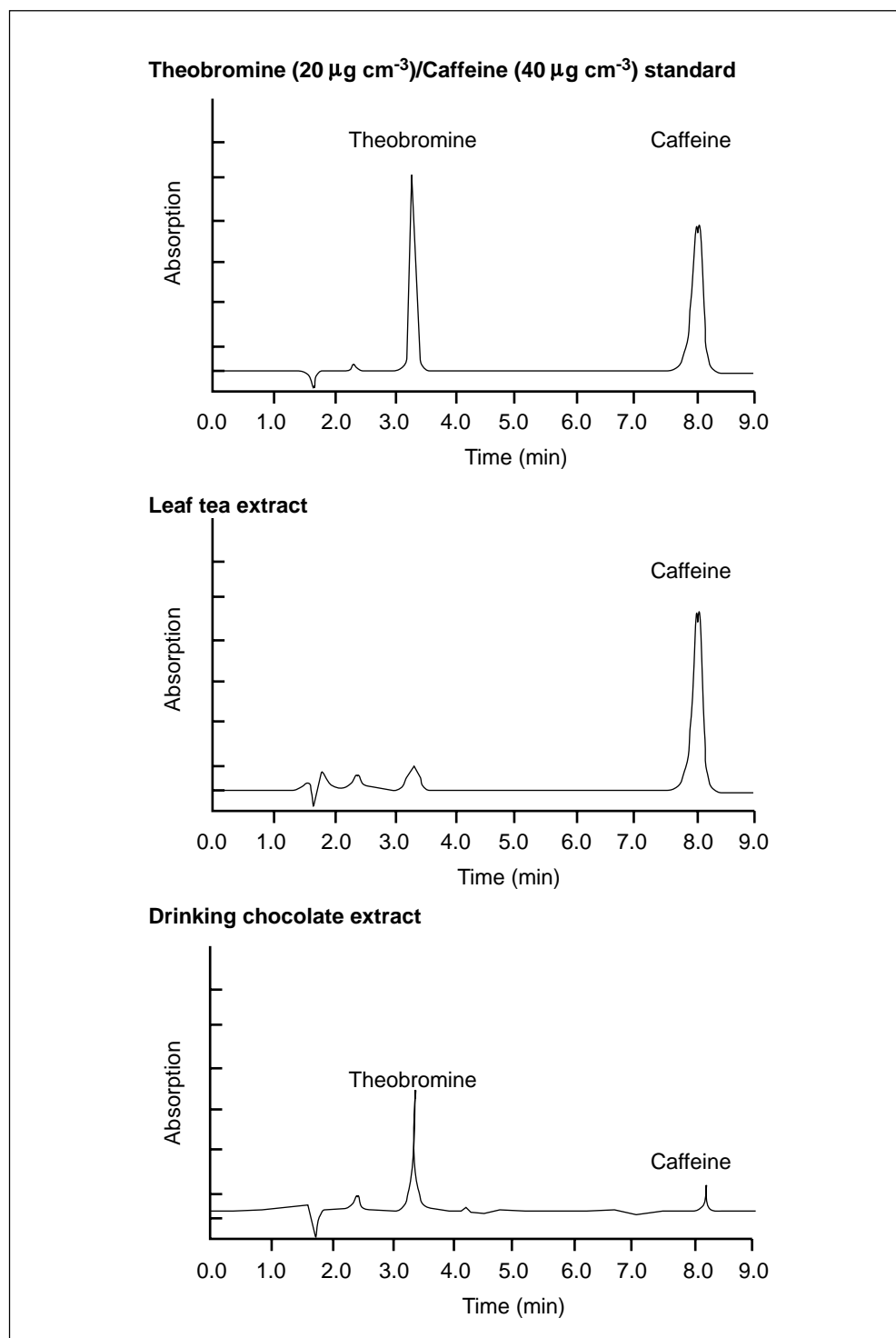


Figure 24 Chromatograms from the analysis of tea and drinking chocolate



Screening for photoallergens

Irritant or allergen?

Some compounds – eg sodium hydroxide – cause irritation if applied to the skin. Each time the irritant is applied a reaction occurs. This is because the chemical affects the skin tissue. Photoirritants also exist, such as 8-methoxypsoralen (the active component of 'giant hogweed'), which cause irritation through a photochemical reaction.

Symptoms of irritancy might be erythema (reddening of the skin), itching or cracking of the skin. The same response is seen if an allergen (a substance causing an allergy) is applied to the skin. However, no response is observed on first exposure – indeed it can take several exposures of low level contact before an allergic reaction is seen. The allergic reaction is a learned response and is not observed in all subjects, whereas irritants cause a reaction in all cases – eg low levels of phenol cause skin irritation in all people, but not everybody shows an allergic reaction to cats.

In some cases allergic reactions occur because a photochemical reaction has taken place – this is known as photoallergy and is not observed on first exposure to the allergen.

To be a photoallergen a chemical must have two properties:

- 1 it must absorb wavelengths of light present in sunlight (if photoallergy is seen these are usually in the ultraviolet region); and
- 2 after absorbing light the chemical must produce a reactive species capable of binding covalently (*ie* irreversibly) to protein molecules in the skin.

Most of the chemicals identified so far as photoallergens in man are germicides and fungicides which were inadvertently used in soaps or medical preparations. These days such chemicals do not pass through screening, so are not marketed.

Photoallergens have a range of structures. A number of them contain halogenated aromatic rings. When ultraviolet light is absorbed halogen radicals (such as Cl^{\bullet} or Br^{\bullet}) are lost. It is the organic radical that remains which binds to skin proteins. Once binding has taken place a conjugate known as a 'complete antigen' is formed.

The complete antigen is recognised as 'foreign' by the Langerhans cells, which leads to the formation of memory cells in the draining lymph nodes. When challenged, the conjugate is recognised by the memory cells which undergo transformation and proliferation. This leads to the release of soluble factors which produce responses such as itching of the skin and is known as a 'learned response'.

Screening

To avoid the possibility of exposing the public to photoallergens it is necessary to test new chemicals before they reach the market place. Testing on humans is regarded as unethical, and traditionally testing has been done on experimental animals. The test material is applied to the backs of the animals. The animals are exposed to simulated sunlight, and the test material is re-applied several weeks later, with further irradiation. If some of the animals show an allergic response the chemical is classed as a photoallergen and it is taken that similar results will be seen on a human population. By using appropriate controls it is possible to tell whether a reaction is due to irritation to the chemical or due to photoallergy.

A new way of testing is to use an *in vitro* method. A model protein, human serum albumin (HSA) extracted from blood, is used to test whether binding of the chemical occurs. HSA is the most abundant protein in human serum, and is also present in the

skin. Binding of the chemical is detected by monitoring changes in the ultraviolet spectrum of the protein.

The technique depends upon two factors:

- 1 separating the protein (together with any substances bound to it) from the remainder of the test chemical; and
- 2 that the ultraviolet absorption spectrum of the conjugate formed between the protein and the test chemical is different from the protein alone.

The ultraviolet spectra of a solution of the protein and the test chemical are taken separately for reference, and then the two components are mixed. The ultraviolet spectrum of the mixture is taken and will usually be the sum of the two individual spectra unless a reaction has taken place. The mixture is then irradiated with an ultraviolet wavelength that is known to be absorbed by the test substance (for a photochemical reaction to occur light must be absorbed, *Fig. 25*).

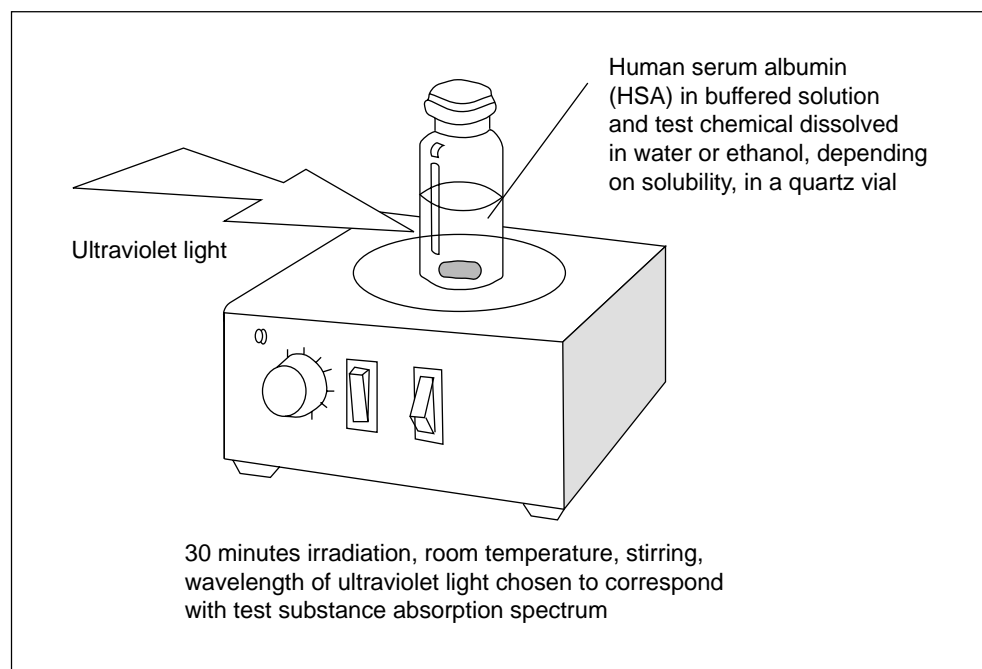


Figure 25 Irradiation of the HSA/test chemical mixture

If the test substance reacts with the protein, the ultraviolet spectrum of the sample will be different after irradiation. If it is the same after irradiation it is likely that binding has not taken place. However, the spectrum could also be different after irradiation because the test chemical has decomposed or because a decomposition product has reacted with the protein. To determine whether or not binding has occurred the protein fraction is separated from the other components of the mixture.

Separation is achieved by using a gel permeation column packed with Sephadex beads. (The principle of gel permeation is given on page 118 and the structure of Sephadex on page 130.) Human serum albumin absorbs radiation at 280 nm, so the eluate is passed through an ultraviolet beam set at this wavelength (*Fig. 26*). The test material can also be detected if it absorbs radiation at this wavelength (*Fig. 27*). The different fractions are identified and collected for spectroscopic analysis.

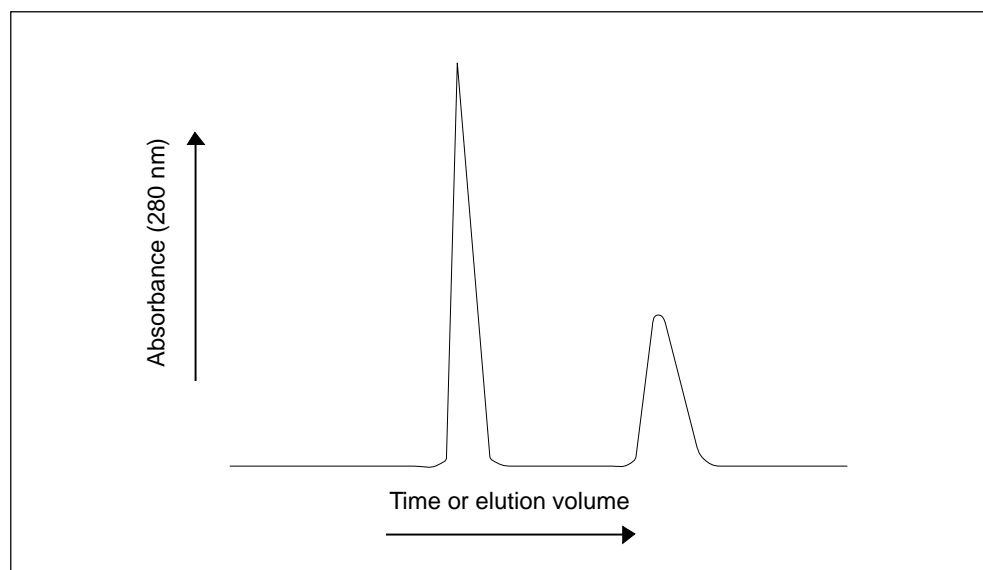


Figure 26 Detection of different fractions using ultraviolet light of wavelength 280 nm

The full spectrum of the protein fraction is then run on a variable wavelength ultraviolet spectrometer (*Fig. 27*) with a diode array detector (*Fig. 32*). If the spectrum is identical to that of HSA alone, it is probable that no binding has occurred. If the spectrum is different it is possible that there has been some binding, though this does not always indicate that the test chemical is a photoallergen. A non-covalent interaction, such as that between hydrophobic groups on the two substances might have occurred or alternatively, the test material might have decomposed when irradiated with ultraviolet light and one of the products may be bound non-covalently to the protein.

To eliminate a false-positive result arising from a non-polar interaction, a less polar solvent can be used to try to separate the molecules. If complete separation results in an ultraviolet spectrum of HSA alone, photochemical binding has not occurred.

To decide whether a photodecomposition product is giving a false-positive result, the test compound alone is irradiated with ultraviolet light before being mixed with HSA. The solution is then separated as before. By mixing the compound with HSA and passing it through the gel permeation column without first irradiating the solution, it is also possible to find out whether binding is taking place without absorbing ultraviolet light. Only if the last two tests give the ultraviolet spectrum of unbound HSA alone is it then possible to conclude that a photochemical reaction has taken place. An overview of the method is given in (*Fig. 29*).

A compound that causes photoallergy is bithionol (bis-(2-hydroxy-3,5-dichlorophenyl) sulphide). This acts as both a fungicide and a germicide. The change observed in the ultraviolet spectrum of HSA when bound to this chemical is shown in *Fig 27*.

Another example of a photoallergen is T_4CS (3,3',4',5-tetrachlorosalicylanilide *Fig. 28*), a germicide, added to toilet soaps in the 1950s and 1960s. A number of people suffered photodermatitis following repeated exposure to soap containing T_4CS , together with exposure to sunlight.

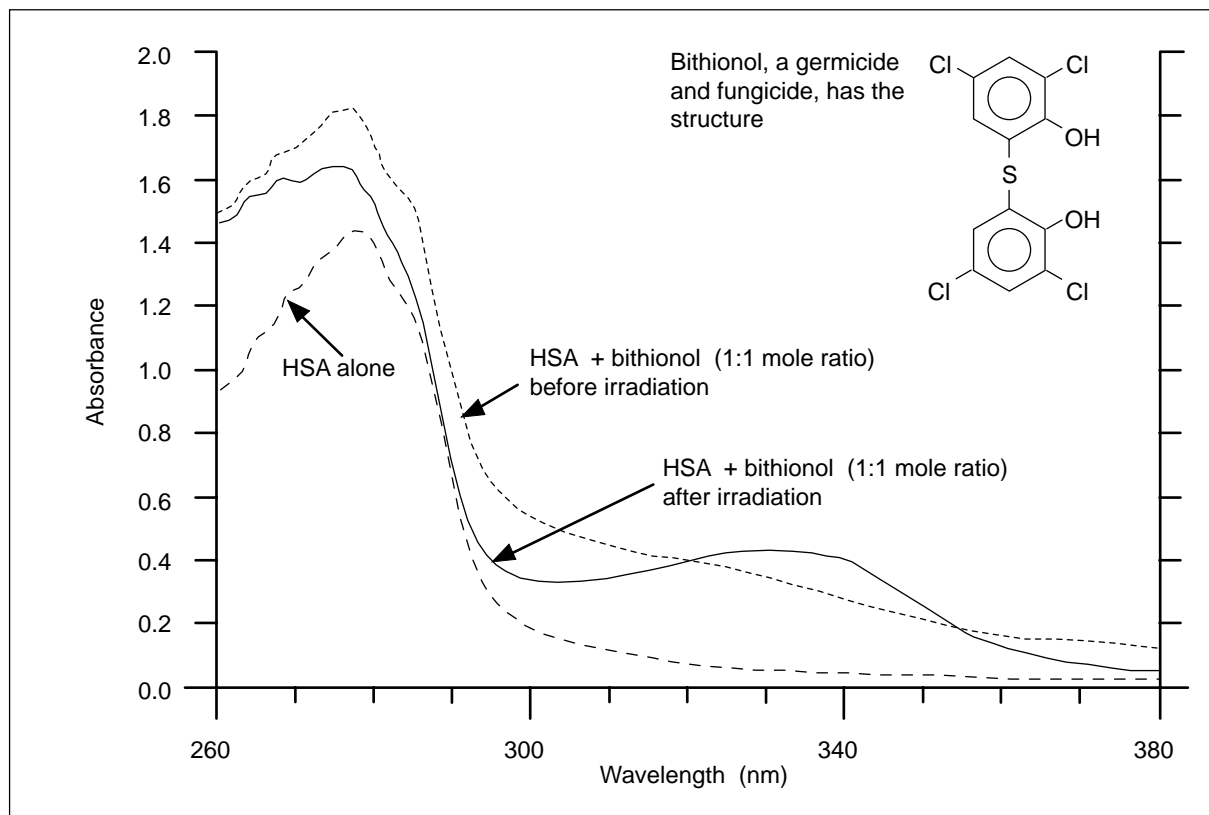


Figure 27 The ultraviolet spectra of HSA and bithionol, a known photoallergen, before and after irradiation with ultraviolet light. (Spectra reproduced with permission R U Pendlington and M D Barratt, *Int. J. Cosmetic Science*, 1990, 12, 91-103, Chapman & Hall)

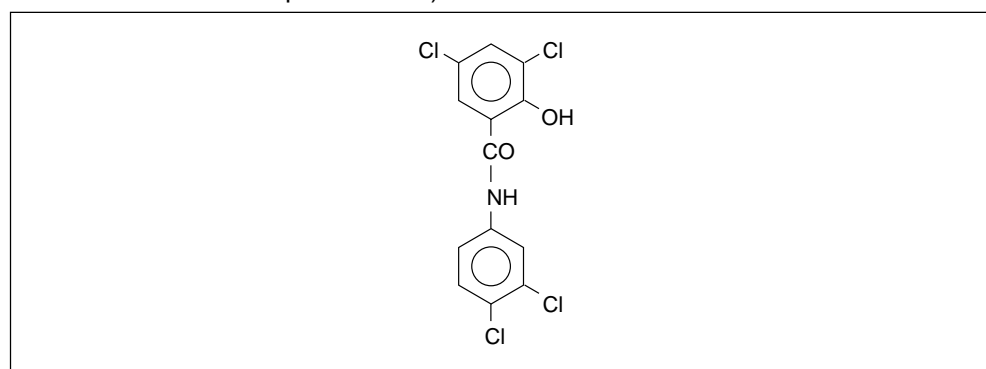


Figure 28 Structure of T₄CS; a germicide

Although testing with HSA is not the same as testing on humans directly, it does have some very significant advantages over tests using guinea pigs:

- 1 it does not involve the use of any animals;
- 2 it is much faster, the whole test routine takes a few days whereas animal tests can take weeks; and
- 3 it is much cheaper.

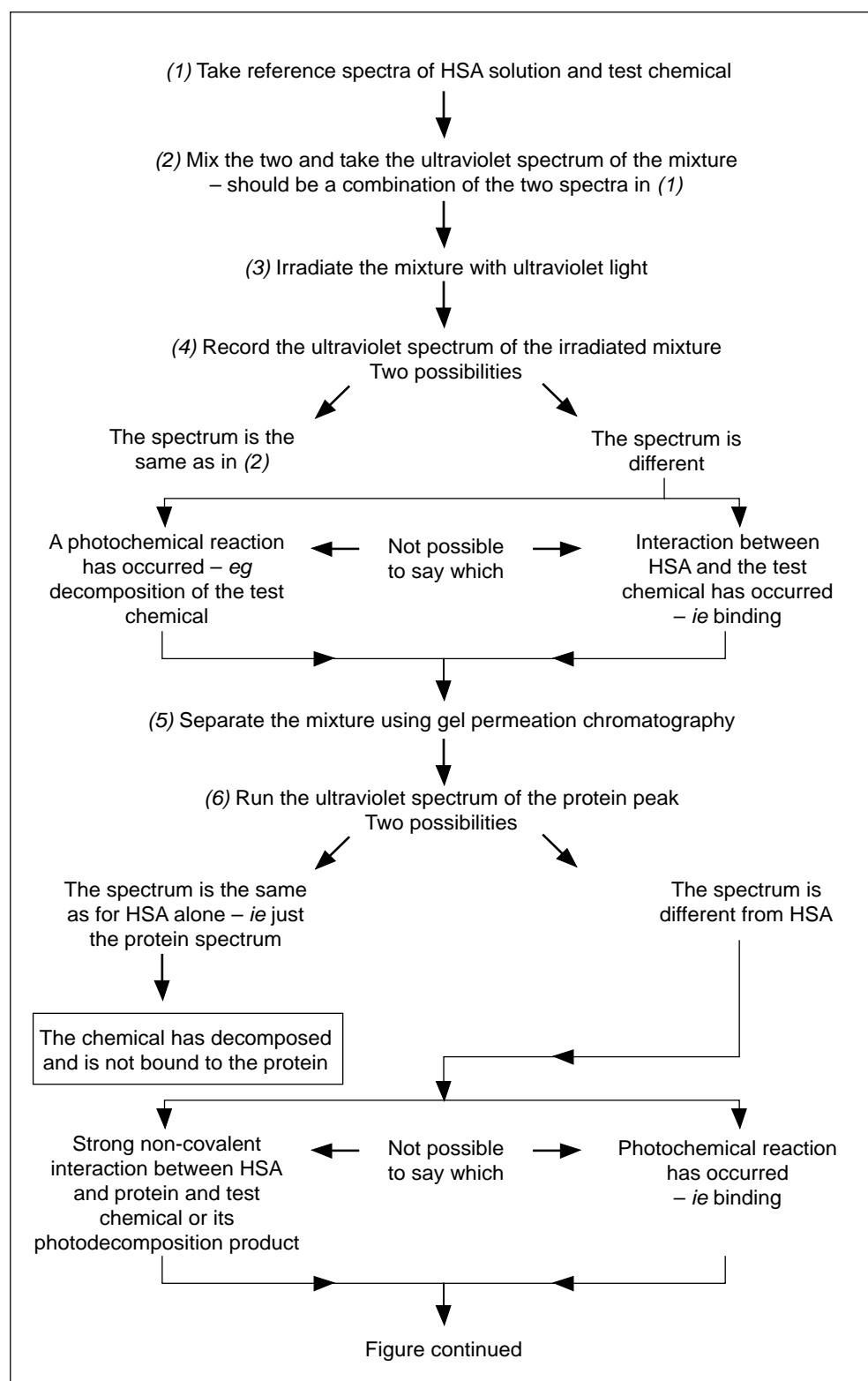


Figure 29 In vitro screening techniques (continued overleaf)

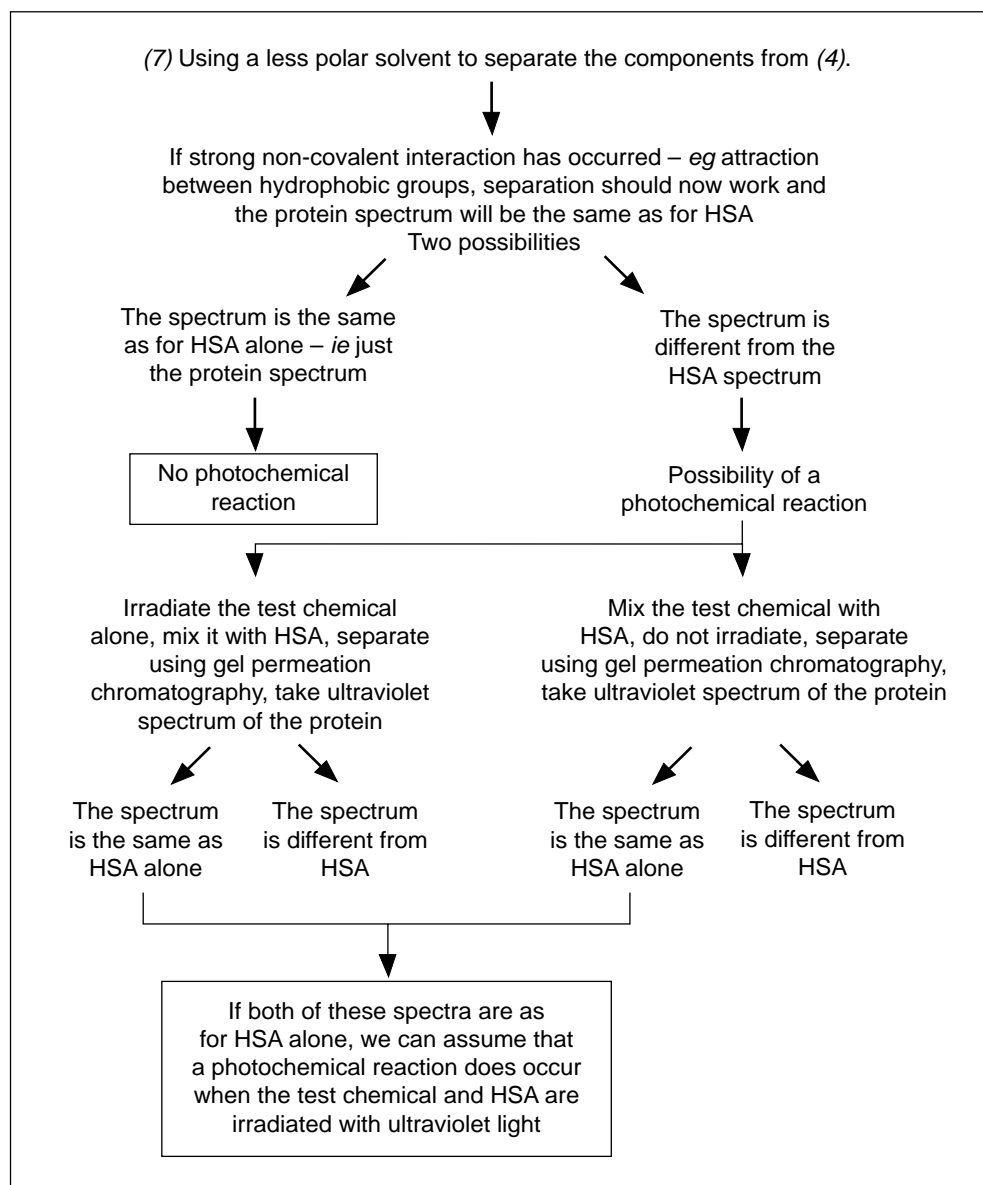


Figure 29 continued In vitro screening technique

Dope testing of horses

The Jockey Club of Great Britain adheres to a number of internationally agreed rules on the use of drugs in horseracing. This ensures that when horses run, they win or lose on their own merits and not because of illegal substances. Approximately 10 per cent of all runners are routinely tested for drugs, 60–70 per cent of which will be race winners. It is the local stewards who decide exactly which horses should be tested, usually after taking advice from a Jockey Club veterinary surgeon. Any horse that performs particularly well, or badly – such as a favourite that is left behind – will usually be tested.

The aim is to have at least one horse from each race tested, though in the case of classic races several runners will be tested. All the UK testing, and some overseas testing is done at the Horseracing Forensic Laboratory in Newmarket. Approximately 0.3 per cent of all domestic tests are positive, but this figure is small compared with



the 2–3 per cent in some countries (although in exceptional cases up to 10 per cent have failed).

If a horse fails a drug test, it is the trainer who is held responsible, and if the horse has been placed it automatically loses the prize money. There are several ways in which a horse can have illicit substances in its body – eg

- 1 through medication. Even if given by a vet to treat a condition, if the substance is not permitted the horse loses the race, the trainer is responsible;
- 2 through negligence. A drug taken orally might be given to the wrong horse; again the trainer is responsible because his stables have not been run efficiently;
- 3 by deliberate doping. This is the most serious scenario, and can be done either to enhance a horse's performance or to slow it down *ie* if it is clear that only two horses stand a realistic chance of winning a race, the favourite might be drugged so that the second favourite can be backed heavily and win; and
- 4 through contaminated feedstuff. Although this might be outside the control of the trainer a horse might lose a race because his feed contained theobromine (which is not allowed) from cocoa wastes.

If a horse fails a drug test the Jockey Club will hold an enquiry. Depending on the circumstances, the trainer might be fined (fines of several thousand pounds have been recorded) or lose his licence if the doping was deliberate. The rules to which the Jockey Club work are those of the sporting body and not criminal law, unless 'nobbling' is involved. In these cases a criminal offence has been committed (fraud) and the police become involved.

In routine testing the Horseracing Forensic Laboratory will look for any substance that might affect a horse's system – to improve or impair performance, or to conceal an injury or illness. These might include diuretics; drugs to fight infection; anabolic steroids that build up muscle fibre and increase the aggression of the horse; sedatives, which can be used to impair a horse's performance and reduce its chances of winning; painkillers and local anaesthetics that allow a horse to run while not really in a fit state to do so; and stimulants that increase a horse's chances of winning.

The presence of these drugs or their metabolites is determined by testing the horse's urine. After a race a veterinary officer's assistant has to wait with the horse until it passes water. The sample collected is split into two portions of approximately 250 cm³. They are kept in two identical sealed bottles which are bar coded so that the testing is anonymous and the analysts cannot be bribed to falsify the results. One portion is tested the day it is received, and the other is frozen and kept. If the tests are negative the second sample is destroyed.

If the tests are positive the trainer and/or owner can elect to have the second sample analysed, so long as they do so within 21 days of being notified that the horse has failed the drug test. The second sample has to be analysed by one of five laboratories – the UK laboratory at Newmarket, or at laboratories in Ireland, France, Germany or Spain. (This is different from human blood alcohol testing, where car drivers are given a sample of their blood and can take it to any certified analyst for independent analysis). To allay any doubts about collusion or falsification of results the owner/trainer is permitted to have a witnessing analyst present when the second sample is tested.

An enormous range of compounds is present in equine urine, and it is first necessary to separate the mixture according to acid/base character (Table 4).

Table 4 Acid/base character and examples of drugs found in horse urine

Character	Examples of types of drug detected in this fraction
Strong acid	Diuretics, anti-inflammatory drugs
Weak acid	Painkillers, anti-inflammatory drugs
Neutral	Diuretics, xanthines (eg caffeine, theobromine)
Basic	Local anaesthetics

Four internal standards are added to a 20 cm³ aliquot of the sample (one for each fraction) so that any foreign substances can be measured against them if necessary. These standards are compounds with structures similar to those of the drugs being tested for, so they will behave in a similar way in the test. The solutes are then totally absorbed on a diatomaceous earth support in a small chromatography column, and a mixture of organic solvents is run through the column (a liquid–liquid extraction). This takes three of the fractions through, and leaves one (strong acids) behind (*Fig. 30*). The acids are eluted off the column by acidifying the organic solvent to suppress the ionisation of the acids and make them soluble.

The solution containing the three remaining fractions is concentrated and separated using solid–liquid chromatography. A buffer/methanol solvent system is used to take two of the fractions through a bonded silica chromatographic cartridge (a small disposable column), and leave one behind (this can be eluted by changing the buffer in the solvent). This is repeated on a different column to separate the two remaining fractions (*Fig. 30*). The four fractions are then treated separately. An overview is given in *Fig. 31*.

The strongly acidic fraction is spotted onto an ultraviolet sensitive TLC plate consisting of silica particles bound to an aluminium support. A dichloroethane/ethanoic acid solvent system is used to separate the components, which show up as bright spots under ultraviolet light. Any spots other than the internal standard are investigated further because they suggest that illegal substances have been administered.

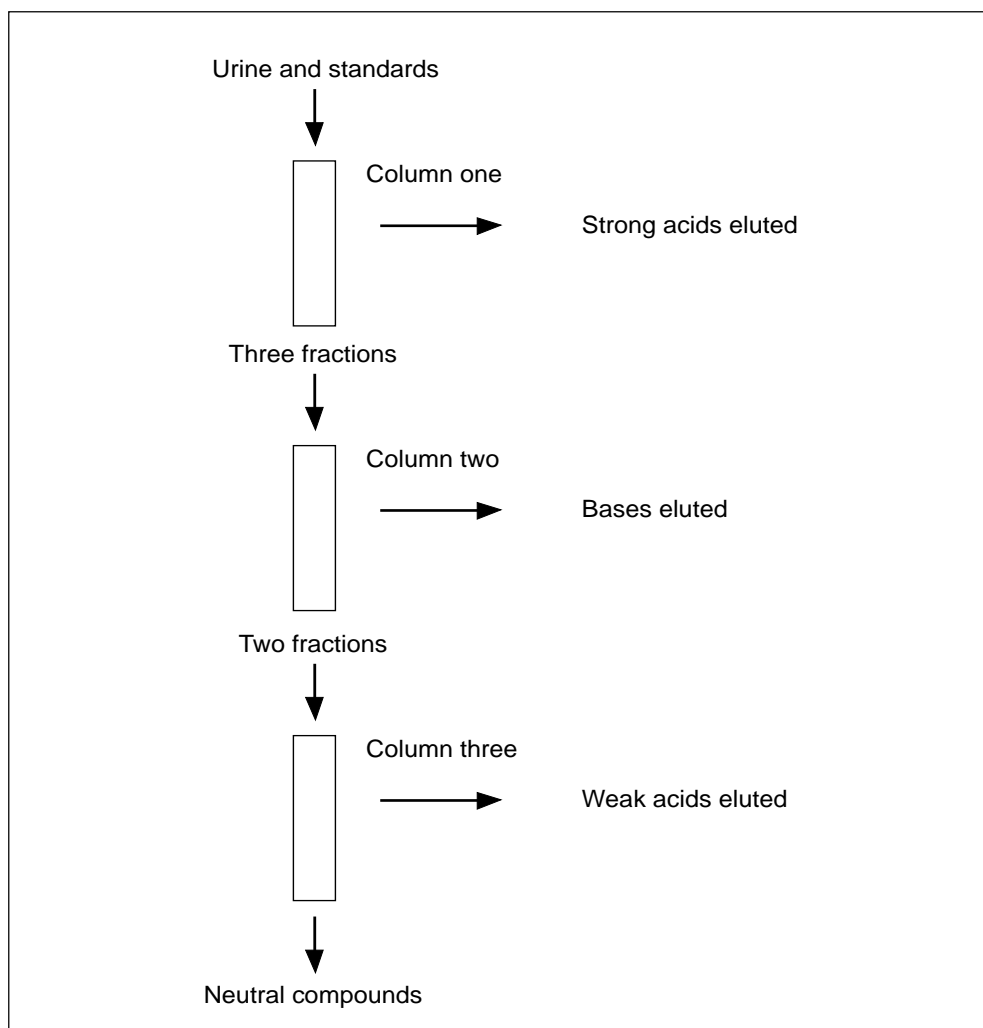


Figure 30 Separation of a sample according to acidity

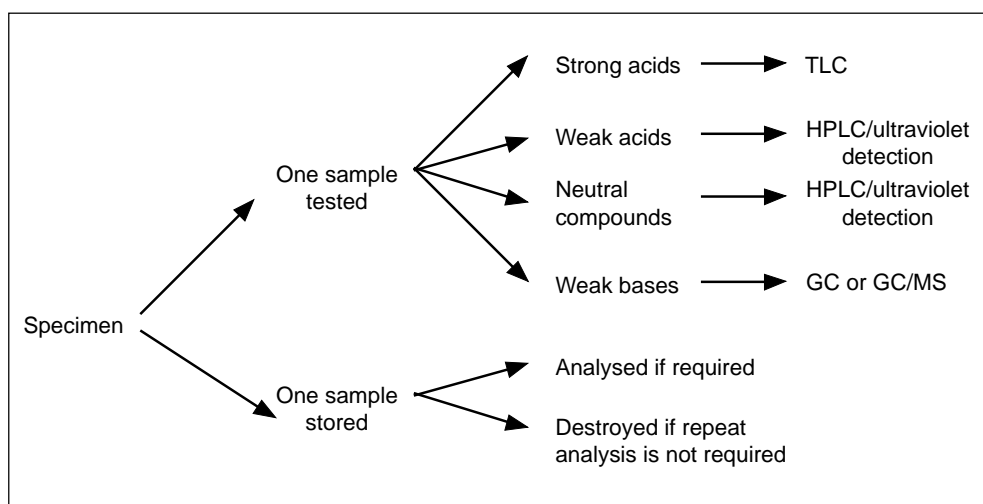


Figure 31 Overview of the analytical techniques used for the dope testing of horses



The weakly acidic and neutral fractions are treated similarly. The 0.02 cm^3 ($20 \mu\text{l}$) portions are injected onto standard octadecylsilane (C_{18}) HPLC columns, and are eluted with a methanol/ethanoic acid solvent. The relative proportions of the two liquids in the solvent are changed during the separation to achieve maximum separation – *ie* there is a concentration gradient. The mixture normally takes 15 min to separate, using a flow rate of $1.5 \text{ cm}^3 \text{ min}^{-1}$. The eluate from the HPLC column passes through a 0.008 cm^3 ($8 \mu\text{l}$) cell and the components are detected from their ultraviolet spectra using a diode array detector (*Fig. 32*). All the data generated are stored on a computer. Once the run is complete each peak is compared with a library of roughly 40 drugs, according to the retention time and ultraviolet spectrum of each peak. Stimulants such as caffeine are frequently detected by using this method. Examples showing HPLC chromatograms of mixtures of some drugs are shown in *Figs. 33* and *34*.

Anything unusual is investigated further. The mass spectrum of a sample of the unknown compound might be obtained first. If any test is positive the whole procedure is repeated by a single person who also runs a blank sample through the system to ensure that there is no doubt about the accuracy of the instrumentation.

The basic compounds are separated by GC. The basic fraction is injected onto a neutral bonded silica GC column by using helium as the carrier gas. (Some compounds are not very volatile, so the column is heated from $70 \text{ }^\circ\text{C}$ to $290 \text{ }^\circ\text{C}$ during the separation.) The presence of any basic solutes is monitored by a nitrogen specific detector (see page 127). The compounds are identified from their retention time on the column and by their mass spectra.

Horses' urine can contain complex water soluble conjugated metabolites, and these conjugates have to be broken down before the drugs, or their metabolites, can be extracted. The conjugated metabolites are hydrolysed by using enzymes before being separated using a solid–liquid chromatography column with a basic organic solvent. Chemically similar compounds are collected, and these are further separated by using GC before their mass spectra are obtained. However, the compounds tend to be too polar to separate efficiently (*ie* – having too many OH groups) so the polarity of the compounds is reduced by making their trimethylsilyl derivatives. The derivatives are then separated by using GC and their mass spectra are recorded.

The mass spectrum of each peak from the GC is stored on computer, however, it is not necessary to interpret all of the peaks of each spectrum. This is because different drugs of a given type have at least one fragment in common. By looking for such peaks it is possible to establish that a tranquilliser or a β -blocker, for example, has been administered. Consequently, five peaks might cover the possible presence of a hundred drugs.

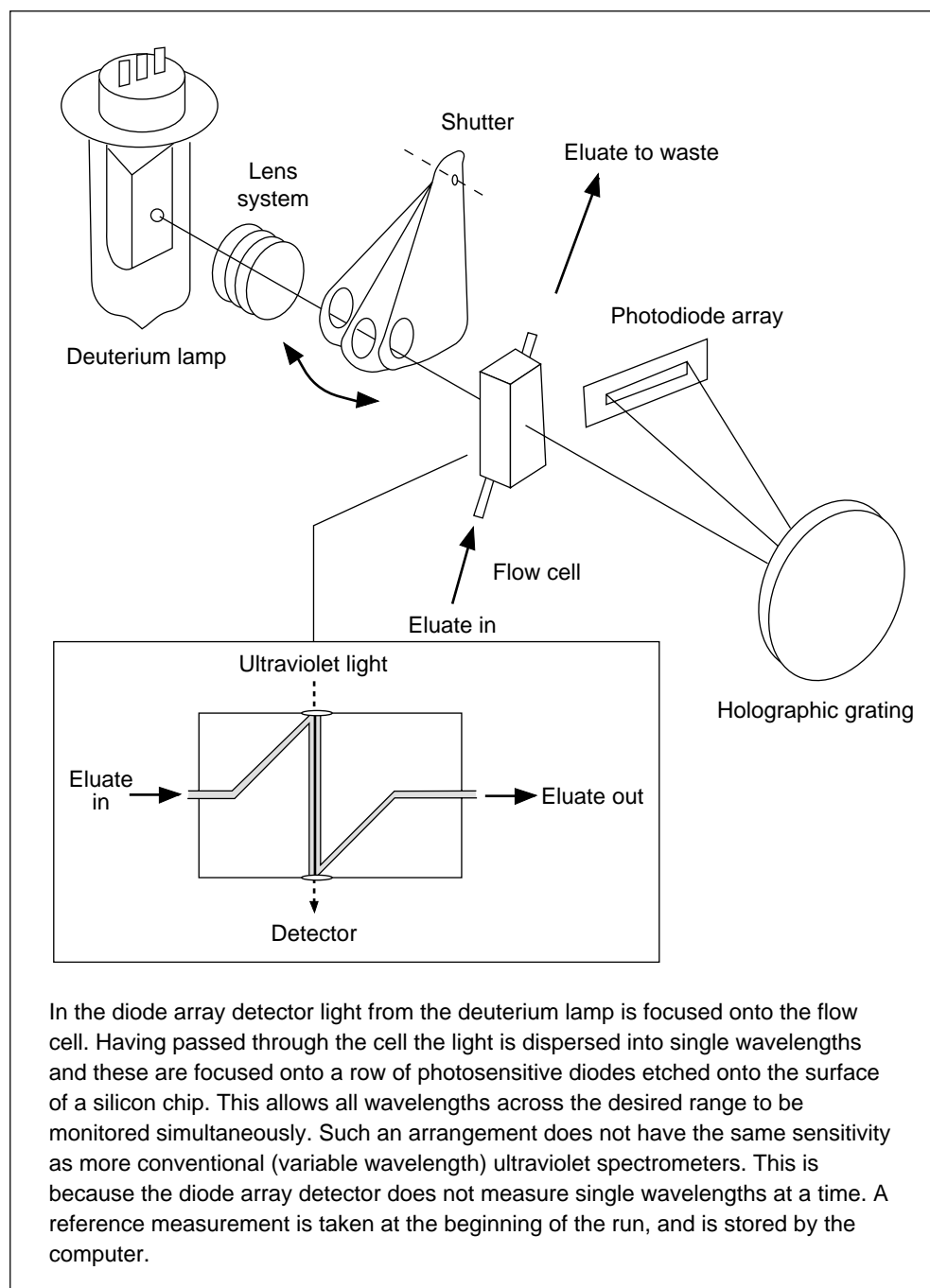


Figure 32 The diode array detector



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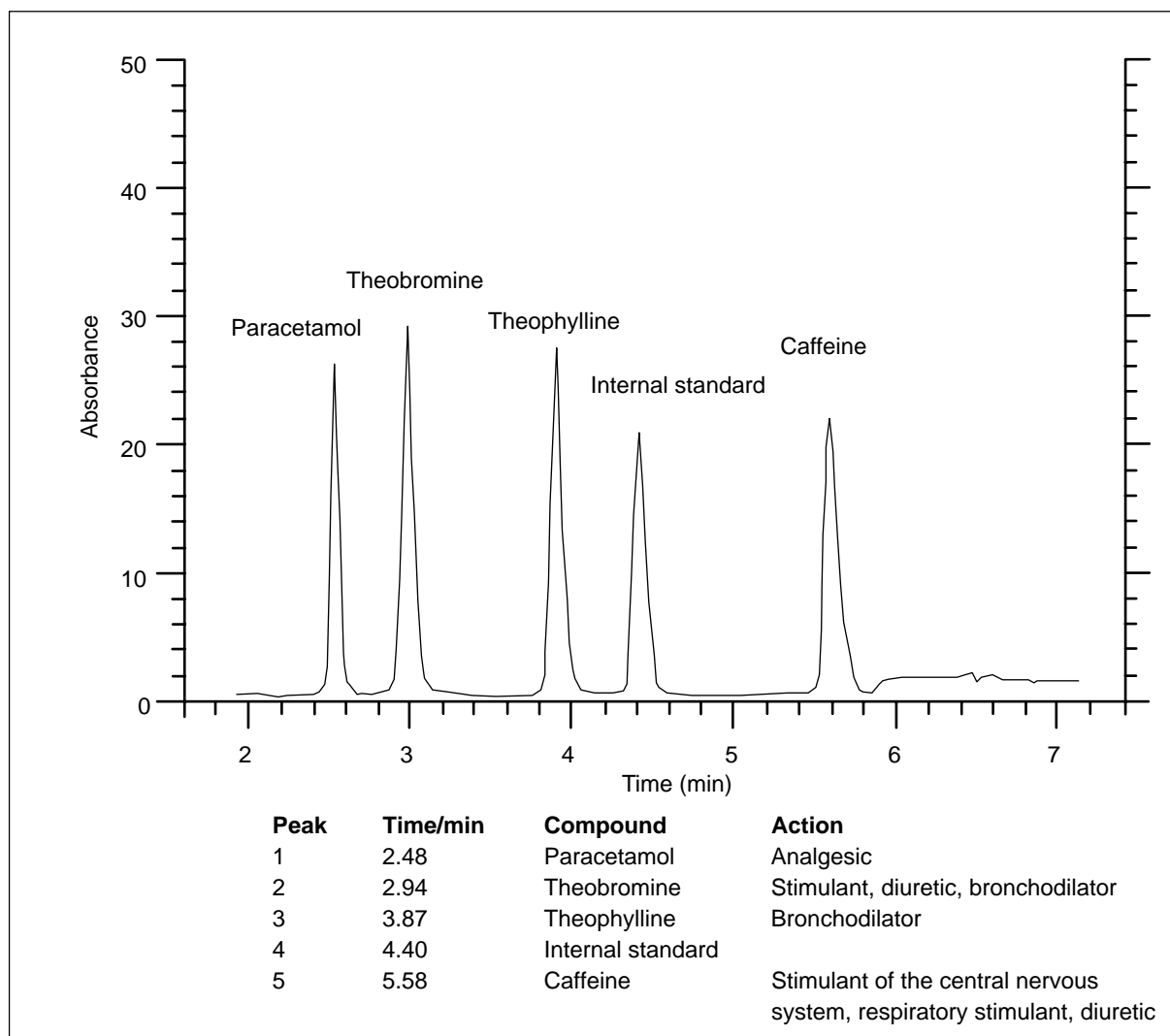


Figure 33 HPLC chromatogram of a mixture of neutral compounds

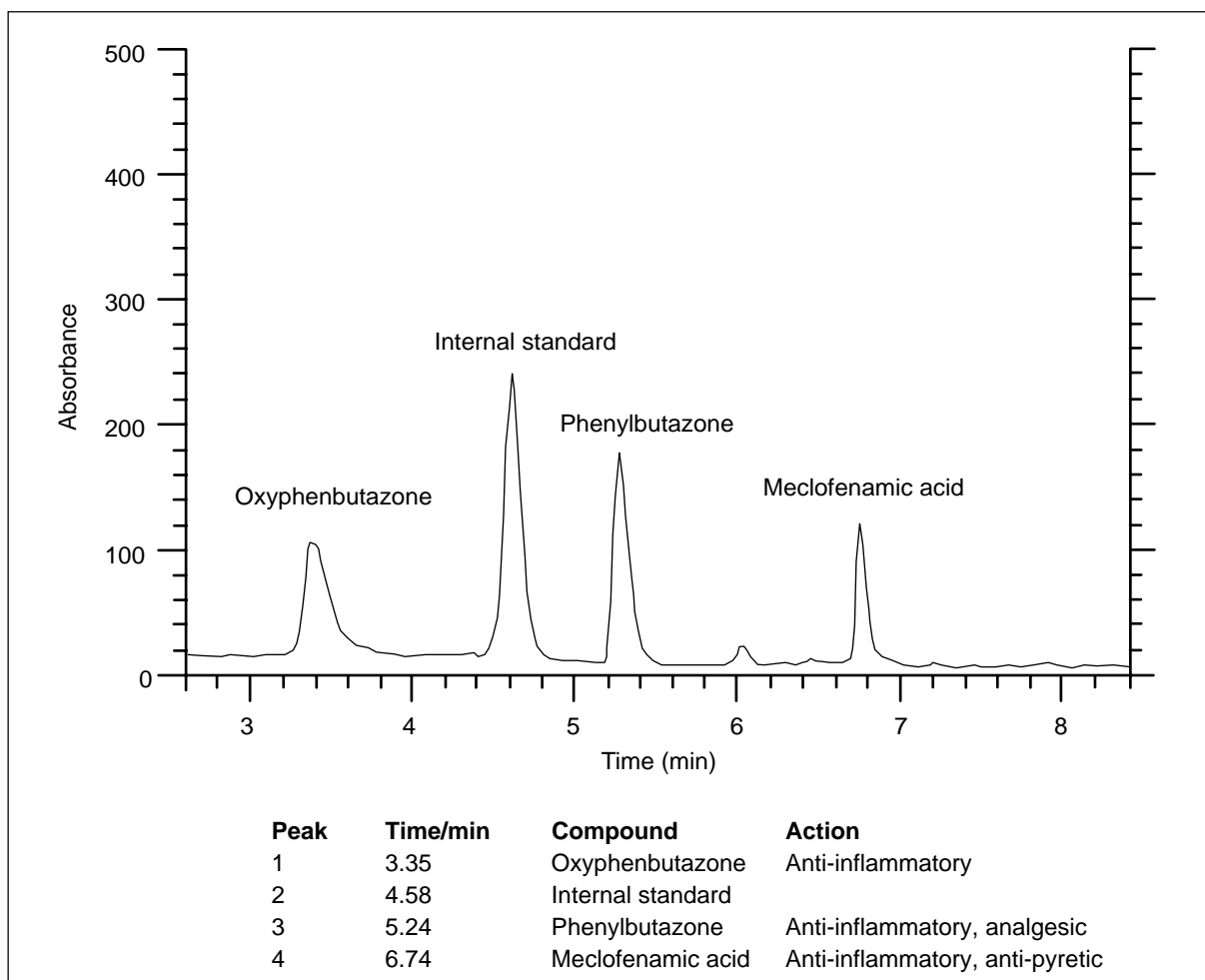


Figure 34 HPLC chromatogram of a mixture of weakly acidic compounds

If the output from the GC is less than $1 \text{ cm}^3 \text{ min}^{-1}$ it can be passed through a heated block before being transferred directly to the mass spectrometer. The heated block ensures that all components in the eluate from the GC are gaseous. The pumping system of the mass spectrometer will then remove enough of the gas to maintain the operating pressure of the spectrometer.

Anabolic steroids are tested for in a rather different way, by using a technique not covered in detail in this book – immunoassay. A sheep or a rabbit is harmlessly and painlessly injected with a form of the drug that is to be tested for. The animal will develop antibodies to the drug, which will appear in its blood shortly afterwards. The antibodies are separated from the blood, and are used as the reagent to react with any of the drug in the horse urine sample. An enzyme- or radio-labelled reaction is usually chosen.

Once in the urine the antibodies will bind to the drug, and the bound complex can be separated. The actual technique used is a little more elaborate. A small labelled sample of the drug is first introduced into the urine, then, when an equal amount of the antibodies is added they will bind exclusively to the labelled drug and all the radioactivity detected will be in the separated complex. However, if a small amount of the drug is already present in the urine some will bind to the antibodies. There will not be sufficient antibodies for all the labelled drug to bind, and once the complex has been separated there will be a residual radioactivity in the urine sample. A strongly positive test will leave a large concentration of the labelled drug in solution, and hence a higher radioactive count rate (*Fig. 35*).

The presence in horse urine of substances not permitted by Jockey Club rules does not automatically mean that an offence has been committed. For example, theobromine and theophylline are metabolites of caffeine. Caffeine is a stimulant and is completely banned. Theobromine is present in chocolate and cocoa residues, so a urine sample will reveal if the horse has been given food containing cocoa residues. As a result, small amounts are permitted. On the other hand, if theophylline is detected there is more of a problem because it is not only a metabolite of caffeine, but is also used in the treatment of asthma. In both cases the trainer can expect a visit from a Jockey Club representative!

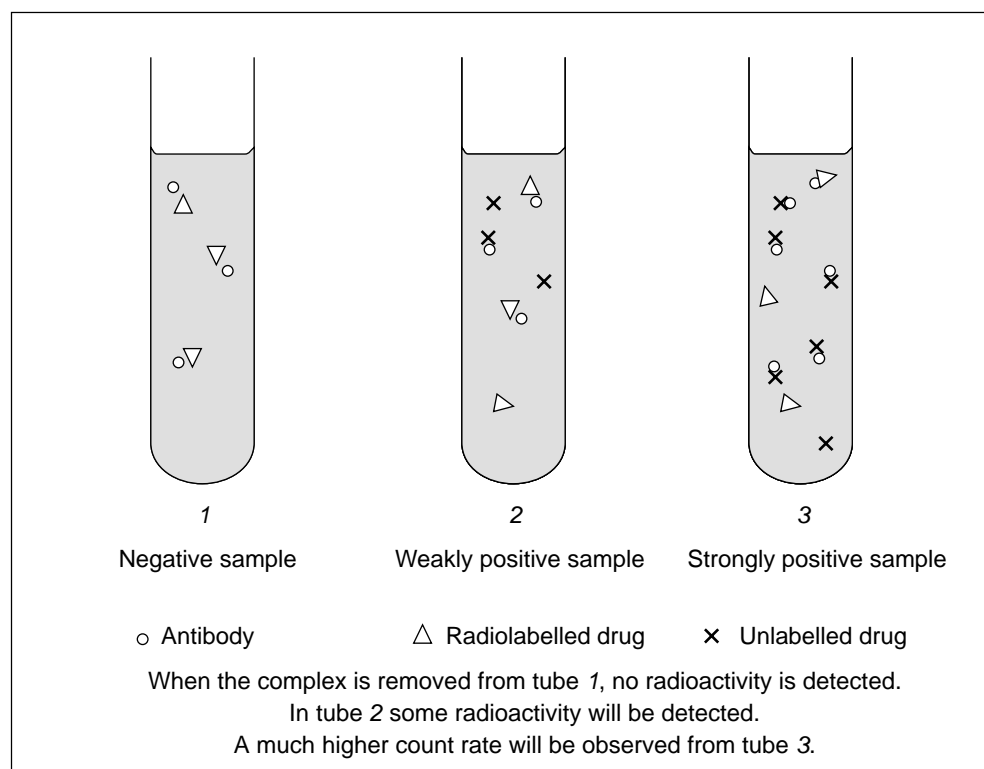
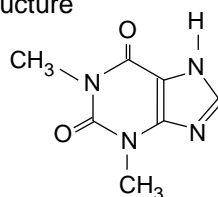


Figure 35 Immunoassay technique

Theophylline has the structure



(continued opposite)



As such, it is similar to caffeine (see page 138, caffeine/theobromine analysis) but with one methyl group replaced by a hydrogen atom.

Theophylline and some of its derivatives are effective bronchodilators so they are frequently used in patients suffering from acute or chronic bronchoconstriction – eg asthmatics.

The compound responsible for the relaxation of smooth (involuntary) muscle in the respiratory system is destroyed by another substance in the body. Theophylline inhibits the action of the destroying substance, enabling the muscle to relax.

Recent trends

Chromatographic techniques have become very important in industry for the purification and separation of intermediates in multi-stage syntheses. (Such separations have to be done in batches rather than in continuous flow.)

In terms of scientific advances, one of the major innovations in the past five years has been the development of efficient columns capable of separating specific chiral compounds from a mixture. They work by the stereospecific adsorption of one enantiomer onto the surface of the stationary phase. The resin contains only one enantiomer, hence its stereoselectivity towards other chiral molecules. The cost of a column for chiral separations can be roughly three times (or even higher) the cost of a standard analytical column.

A similar type of chromatography, affinity chromatography, works on a similar principle – a substrate is covalently bound to a resin, and only enzymes with vacant sites in the correct orientation can interact with these sites.

Research has also shown that graphite-based HPLC packing materials can separate compounds with low or modest polarities, very efficiently. Studies of particle and pore sizes have shown that the surface area of graphite-based materials in HPLC columns can be as high as $1000 \text{ m}^2 \text{ g}^{-1}$. Some classes of compound that have been separated by such columns include:

- 1 aromatic hydrocarbons;
- 2 alkylnaphthalenes;
- 3 methylphenols;
- 4 polychlorinated biphenyls;
- 5 steroids; and
- 6 some amino acids.

By using different solvent systems one area which has been found to have great potential is supercritical fluid chromatography (SFC). A supercritical fluid is one at a temperature and pressure above its critical point. Supercritical carbon dioxide (used to extract caffeine from coffee) in HPLC columns has the solvating properties of a liquid and the transport properties of a gas. Another advantage is that extraction of the solutes from the eluate is easy – the carbon dioxide is simply allowed to evaporate.

However, supercritical carbon dioxide would not be a good solvent for a chromatography column if the eluate were to be analysed by infrared spectroscopy, because of the strong spectral absorption of carbon dioxide which would obscure the absorptions of the eluted compounds. Supercritical xenon has been found to achieve good separation of compounds such as polyaromatic hydrocarbons and if the eluate flows through a microcell no absorption due to xenon is observed. This is a particular advantage if SFC is to be linked with infrared spectroscopy.