Table 3 Amount of effective Fe(II) ions on CR–Fe(II) and mixed valence complex

Complex	*Immobilized ion*		*Surface area* ($m^2\ g^{-1}$)	*K** ($10^3\ atm^{-1}$)	*Effective Fe(II)* ($mmol\ g^{-1}$)	*R*†
	Fe(III) ($mmol\ g^{-1}$)	*Fe(II)* ($mmol\ g^{-1}$)				
CR–Fe(II)	0	2.01	43.1	8.33	0.28	0.14
CR–Fe(II), Fe(III)	1.27	0.44	128.1	10.0	0.12	0.26

*Binding constant of CR–Fe(II)–NO complex formation as determined from Langmuir plots based on eqn [3].
†Efficiency of Fe(II) immobilized on chelate resin determined as the ratio of effective Fe(II) to the amount of immobilized Fe(II). (Toshima N and Asanuma H, *Journal of the Chemical Society, Chemical Communications* 1989: 1075–1076, p. 1076 by the courtesy of Royal Society of Chemistry.)

increase in the surface area, demonstrating that the Fe(III) ions work by making the resin porous. The use of these two metal ions enables efficient NO adsorption.

The polymeric resin as well as the metal complex itself greatly affects the adsorbing ability for the target gas molecules as described in the case of CO, C_2H_4, and NO adsorption. It has been demonstrated clearly that large surface area directly contributes to the rapid adsorption of gaseous molecules. In some cases, the nature of the support dominates the whole adsorption property of the polymer–metal complex rather than the nature of the metal complex. Therefore, further regulation of porosity (e.g. pore size distribution) is quite significant in order to achieve efficient separation of the target gas. Metal complexes are promising compounds for reversible coordination of gaseous molecules so that metal complexes immobilized on polymeric supports with properly regulated porosity will provide further useful materials for efficient gas separation.

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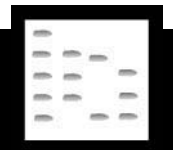
POROUS POLYMERS: LIQUID CHROMATOGRAPHY



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Introduction

Polymers have been used as stationary phases in liquid chromatography (LC) for nearly 40 years. Initially they were used for size exclusion chromatography (SEC), a noninteractive means of separating molecules based on their size. This technique was originally applied to water-soluble macromolecules and is often referred to as gel filtration chromatography (GFC). Gel filtration describes the process particularly well: the stationary phase originally described was a lightly cross-linked agarose in bead form, although other polysaccharides and also acrylamide-based materials are still employed today.

Characteristically such microporous gels are extremely soft and can easily be crushed. They do not possess a fixed pore structure and analytes can diffuse in and out of the aqueous swollen polymer particles. As a consequence column pressures must be minimized; reduced liquid flow rates and wide-bore columns are a necessity, resulting in extremely long run times. By 1964 a new polymeric stationary phase had been introduced based on cross-linked polystyrene. This material had sufficient cross-linker (in this case divinylbenzene) to ensure a high degree of physical strength when compared with earlier microporous

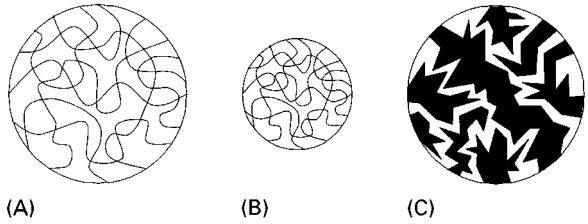

Figure 1 Representation of (A) solvated and (B) nonsolvated microporous beads and (C) a macroporous bead.

particles. The new polymer particles were also made in such a way that the network of pores within the beads was permanent and unaffected by the solvent used. Such particles, which are described as macroporous (**Figure 1**), immediately became useful for the size exclusion separation of organic-soluble macromolecules (or gel permeation chromatography, GPC, as it is commonly known).

Today many types of polymer are employed as stationary phases in chromatography, in both macroporous and microporous forms (**Table 1**). Their use is not restricted to SEC but also covers many aspects of adsorption-based chromatography.

Manufacture of Polymeric Particles

Most polymer-based stationary phases are manufactured as spherical particles, commonly using a suspension polymerization technique. Polystyrene, cross-linked with divinylbenzene (**Figure 2**), is perhaps the commonest form of synthetic polymer used in chromatography at the present time.

The manufacture of poly(styrene-*co*-divinylbenzene) particles, or PS/DVB, involves dispersing a mixture of the monomers including cross-linker and a suitable initiator, all dissolved in an appropriate solvent, into a larger volume of immiscible liquid, known as the continuous phase. Rapid stirring is used to produce the appropriate droplet size, at which point polymerization is triggered; in the case of an organic peroxide or persulfate this is achieved by heating, causing thermal decomposition of the initiator.

Table 1 Comparison between microporous and macroporous particles

Microporous	*Macroporous*
Low cross-link ($< 12\%$)	High cross-link ($> 20\%$)
High swell	Low swell
Variable pore size	Fixed pore size
Poor physical stability	Good physical stability
Low flow/low pressure applications	High flow/high pressure applications
Wide column diameters	Narrow column diameters

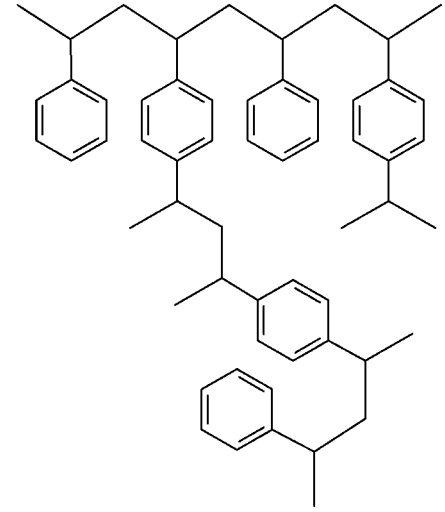

Figure 2 Chemical structure of poly(styrene-*co*-divinylbenzene).

Restricting the amount of cross-linker to less than 12% (by weight) produces a soft microporous particle, whereas using much higher levels of cross-linker ($> 20\%$) produces a rigid particle. In order to introduce the pore structure, the solvent used to dissolve monomers and cross-linker must be a porogen. A porogen acts as a good solvent for the monomers but a poor solvent for the resulting polymer. Once polymerization commences, the growing polymer chain starts to precipitate into small globules, causing phase separation. The globules partially coalesce until all the monomers have been consumed, resulting in polymeric particles containing porogen-filled pores (**Figure 3**).

The particles are then thoroughly washed to remove all traces of porogen and any surface active agents that may also have been used during the manufacturing process. The resultant macroporous particles do not generally have the correct particle size distribution, particularly for high performance applications, and require further refinement by sieving or classification.

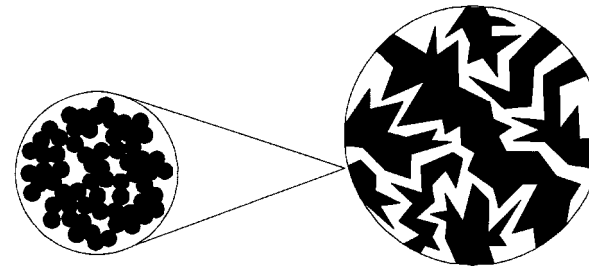

Figure 3 Representation of microspheres formed during suspension polymerization of a macroporous particle.

Size Exclusion Chromatography

The principle of SEC relies on the fact that smaller analytes can penetrate further into the bead, whether they are microporous or macroporous. This increases their residence time so that they elute later from the column. Large macromolecules may be excluded from the pore structure completely, whereas small molecules will totally permeate the accessible pore structure of the beads.

In the manufacture of macroporous particles the choice of porogen and the quantity used enables careful control of the resulting pore size and pore volume. It is thus possible to produce an extensive variety of different products for the analysis of various compounds covering a wide range of molecular weight distributions. Often it is not possible to find an individual column suitable for the analysis of a particular polymer and several different columns employed in series may be required. The actual selection process relies on the accurate calibration of individual columns with highly characterized polymer standards with narrow polydispersity (**Figure 4**).

The development of such techniques has progressed as equipment has been improved and packing materials have evolved. Modern highly efficient stationary phases tend to have a small particle diameter and are packed into relatively narrow bore columns (6–8 mm i.d.) in lengths of 20–60 cm. This has allowed SEC separations to be performed in minutes rather than hours.

Another recent development has been the use of mixed bed columns (**Figure 5**). It was discovered by Yau *et al.* in 1978 that by combining two or more packing materials with different individual pore sizes into the same column it was possible to extend the separation range of such a column, thus greatly simplifying the selection process.

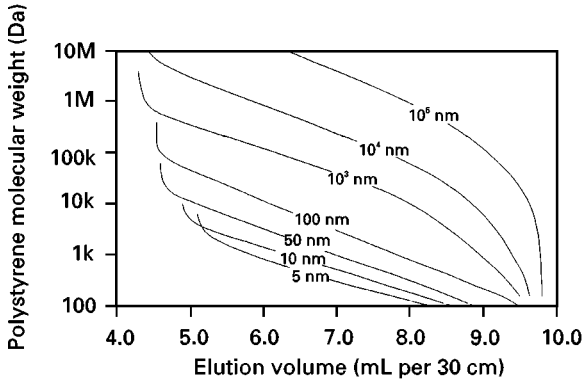

Figure 4 Calibration curves for individual pore sized GPC columns. Column: PLgel, 300 × 7.5 mm, pore size as indicated; eluent: tetrahydrofuran; flow rate: 1.0 mL min^{-1}; detector: refractive index (RI).

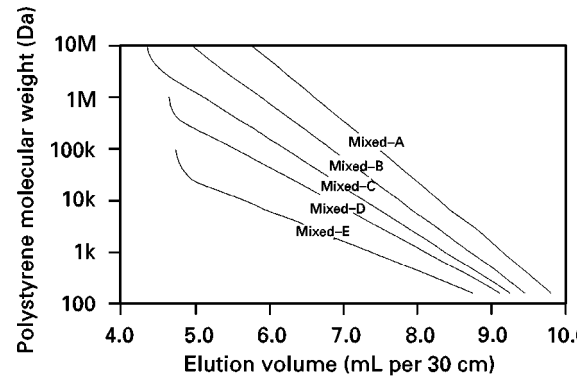

Figure 5 Calibration curves for mixed-bed GPC columns. Conditions as for Figure 4.

While macroporous PS/DVB particles are ideally suited to GPC with organic solvents, such materials are extremely hydrophobic and are not satisfactory for use in SEC separations in an aqueous medium. To eliminate unwanted secondary interactions between the analyte and the stationary phase it becomes necessary either to coat PS/DVB particles with a hydrophilic layer or to move to a completely different polymer system that is inherently aqueous-compatible. Materials produced by both techniques are commercially available, as well as those from silica-based matrices. A polyhydroxyl coating, when applied to PS/DVB particles, can be immobilized by cross-linking it into place utilizing any remaining vinyl groups on the internal and external surfaces of the beads arising from unreacted divinylbenzene. A common alternative polymer system based on methacrylate monomers is also used to produce a range of hydrophilic macroporous particles suitable for GFC.

Reversed-Phase Chromatography

The macroporous polystyrene-based supports used for GPC have also been adapted to reversed-phase chromatography (RPC) applications. The PS/DVB matrix is sufficiently hydrophobic and, coupled with an extremely high internal surface area, this means that analytes partition between the mobile phase and the support in a similar manner to alkyl-bonded silica stationary phases (**Figure 6**).

Some small molecules may still be able to permeate the polymer structure itself despite the extensive cross-linking and so a stronger organic phase may be required. For example, using methanol as an organic phase can lead to excessive retention times unless it is used in the presence of a small amount of a stronger organic eluent such as tetrahydrofuran. Conversely acetonitrile rarely, if ever, presents any problems.

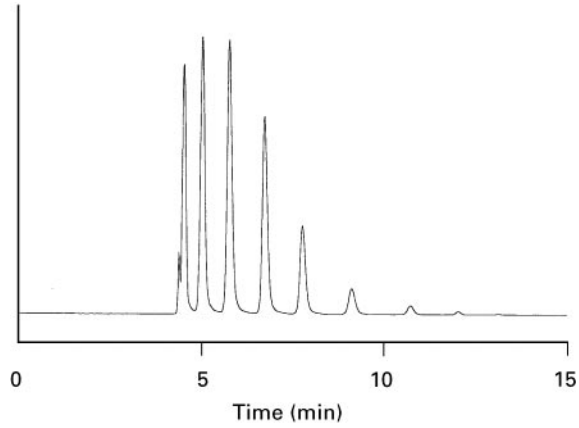

Figure 6 Reversed-phase separation of polyethylene glycol. Column: PLRP-S 10 nm 5 μm, 150 × 4.6 mm; eluent: 40% acetonitrile; flow rate: 0.5 mL min^{-1}; detector: PL-EMD 960 evaporative light-scattering detector.

Given the aromatic electron-rich ring structure of the PS/DVB matrix, it is perhaps unsurprising that π–π interactions are sometimes observed, leading to longer than expected retention times for some compounds. This is in contrast with the acidic nature of silica-based material. Even high density coverage and extensive end-capping cannot completely eliminate residual silanol groups. These are frequently the source of band broadening of basic analytes, usually observed as a severe tailing of the peak.

The absence of any surface functionality or the need to modify the surface of PS/DVB with alkyl ligands results in an extremely inert stationary phase. This property can be exploited, allowing separations to be performed at pH 9 or above where even the most technologically advanced base-deactivated silica matrices will begin to degrade rapidly. In addition it allows sodium hydroxide solution to be used for clean-up and depyrogenation purposes, which is a significant benefit in preparative purification of pharmaceutical products. The properties of silica-based and polymeric reversed-phase materials are compared in **Table 2**.

PS/DVB stationary phases prove amenable to all aspects of conventional RPC including ion pair and ion suppression techniques. **Figure 7** shows the separation of six proteins by reversed-phase ion pair chromatography.

For other forms of RPC, such as hydrophobic interaction chromatography (HIC), PS/DVB particles are too hydrophobic. The technique is principally applied to protein separations and relies on gradient elution profiles that run from high salt concentrations to low salt concentrations. At high salt concentration proteins are forced to interact with hydrophobic sites on the resin and are retained. As the salt concentration is decreased the proteins elute from the column. Polymeric materials suited to this type of application are hydrophilic matrices that have been modified to make them weakly hydrophobic by inclusion of a relatively low level of short chain alkyl groups or phenyl rings.

Table 2 Comparison between silica-based and polymeric reversed-phase materials

Alkyl-bonded silica	*PS/DVB*
pH 2–9	pH 1–14
Dissolution of Si matrix	Matrix not soluble
Loss of alkyl ligand	No bonded phase
Leaching of heavy metal contaminants	No contaminants
Packed bed density ~ 0.6 g mL^{-1}	Packed bed density ~ 0.3 g mL^{-1}

Ion Exchange

Ion exchange materials are frequently derived from polymers owing to the relative ease with which the appropriate ionic functionality can be introduced. Both strong and weak anion and cation exchangers can be formed on polystyrene supports and find use in a wide variety of application areas (**Table 3**).

One difficulty that can occur is due to nonspecific interactions arising from the hydrophobic nature of the PS/DVB backbone. This 'feature' has been exploited in related applications such as ion chromatography and ion exclusion chromatography, but for protein separations this property is less desirable. One solution is to coat the hydrophobic macroporous PS/DVB particle with a hydrophilic layer, in much the same way as for aqueous size exclusion applications described above, prior to introduction of the ionic functionality.

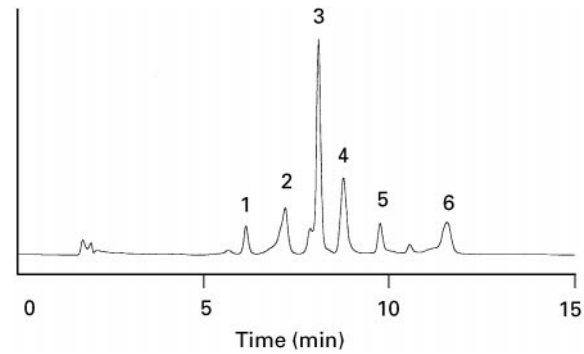

Figure 7 Reversed phase ion pair separation of six proteins. Column: PLRP-S 100 nm 8 μm, 150 × 4.6 mm; eluent: 20–50% aq. acetonitrile with 0.1% trifluoroacetic acid, 0–20 min; flow rate: 1.0 mL min^{-1}; detector: UV, 254 nm. Key: 1, ribonuclease A; 2, cytochrome *c*; 3, lysozyme; 4, bovine serum albumin; 5, myoglobin; 6, ovalbumin.

Table 3 Useful ion exchange functionalities

Type	*Functional group*	*pH range*
Strong cation exchanger	$-SO_3^-$	> 3
Weak cation exchanger	$-CO_2^-$	> 8
Weak anion exchanger	$-NH_3^+$	< 6
Strong anion exchanger	$-NR_3^+$	< 9

When applying a coating technique the risk of pore in-filling remains, which would reduce both the accessibility and ionic capacity towards large biomolecules such as proteins. A very thin uniform layer is required but problems can arise if small areas of polystyrene remain exposed. These will interfere with the separation mechanism and so strict quality control procedures are required to detect such weaknesses. An alternative approach to increase the surface density of the ionic functionality has been to apply a thin layer of microspheres to the surface of the particle, thus significantly increasing the surface area.

Ion Chromatography

Ion chromatography is a particularly important analysis technique, often used for analysis of inorganic ions in water. It covers an enormous range of application areas and may be considered independently from ion-exchange chromatography. Stationary phases for ion chromatography usually possess a very low level of ionic functionality, perhaps 1% that of conventional ion exchange resins. This is so that a very dilute buffer solution can be used as eluent together with a conductivity or similar electrochemical detector in order to achieve the degree of sensitivity required. Even so, several commercial systems require the use of a suppressor column to reduce the background conductivity of the eluent further and so increase detection sensitivity. Indirect UV detection has also been used but can cause difficulties.

Ion Exclusion and Ligand Exchange

Ion exclusion and ligand exchange chromatography are specialized forms of ion exchange, but where many of the columns used are based on low cross-linked microporous PS/DVB gels. The principal application areas for the two column types are the analysis of organic acids and carbohydrates, respectively. Ion exclusion resins are sulfonated polystyrene particles with a cross-link content of around 4–8%. A very high level of sulfonation is used during manufacture, which renders the PS/DVB matrix hydrophilic. Dilute sulfuric acid is commonly used as the eluent in ion exclusion in order to ensure that the organic acids exist in the fully protonated, neutral form. The separation mechanism has some size exclusion properties owing to the soft nature of the resin, but the principal mode of interaction in through hydrogen bonding and ionic interactions. Some reversed-phase-type of hydrophobic interactions may also remain. The separation of organic acids by ion exclusion chromatography is illustrated in **Figure 8**.

Since the stationary phase has such a low level of crosslinker it is usually packed into columns of 7–8 mm i.d. in lengths of up to 30 cm. Even so, the flow rate that can be passed through such columns without generating excessive back-pressure is very low, in the order of 0.5–0.6 mL min^{-1}. The viscosity of water or dilute sulfuric acid is such that the separation is normally carried out at elevated temperature (40–60°C). Some care is needed when performing separations involving certain types of carboxylic acid as it is possible to cause inter- or intramolecular reactions where carboxylic acid groups and hydroxyl groups are both present – dehydration can occur, generating anhydrides and generally interfering with the separation mechanism.

This type of sulfonated polystyrene resin can be further modified to produce media for ligand exchange separations of polysaccharides. By introducing a heavy metal counterion – commonly calcium, lead or sodium – it is possible to introduce a new type of interaction mechanism. The resins still possess size exclusion properties and so the elution order of simple carbohydrates is:

Polysaccharides < oligosaccharides < trisaccharides < disaccharides < monosaccharides < sugar alcohols

The separation of disaccharides, such as maltose and sucrose, and monosaccharides, such as glucose

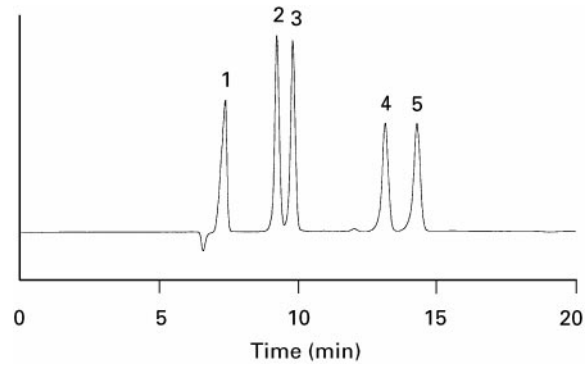

Figure 8 Ion exclusion separation of organic acids. Column: PL Hi-Plex H, 300 × 7.7 mm; eluent: 0.005 mol L^{-1} H_2SO_4; temperature: 55°C; flow rate: 0.6 mL/min; detector: UV, 210 nm. Key: 1, oxalic acid; 2, citric acid; 3, tartaric acid; 4, succinic acid; 5, lactic acid.

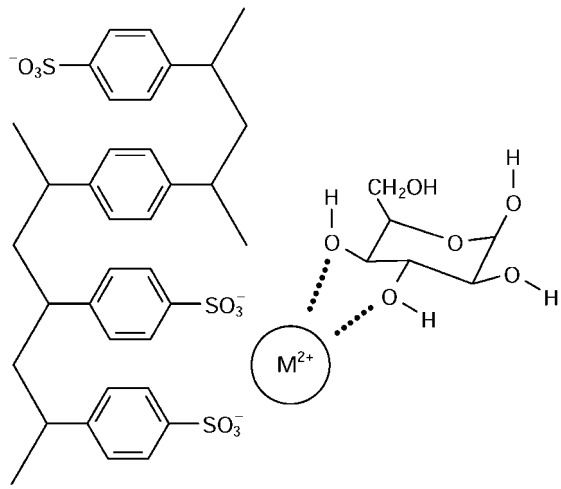

Figure 9 Mechanism of interaction for ligand exchange separation of saccharides.

and fructose, is influenced by the degree of interaction of the numerous hydroxyl groups on the sugar molecules with the metal counterion. Each saccharide molecule has a unique arrangement of axial and equatorial hydroxyl groups, which are able to interact through hydrogen bonding to a greater or lesser extent. The interaction mechanism is illustrated in **Figure 9**.

The separation is further confused by the ability of sugar molecules to exist in an equilibrium state between two anomeric forms (and briefly as the liner molecule). Sugar alcohols are linear and they are able to adopt a flexible conformation, greatly increasing the degree of interaction, and hence retention time, on such columns. **Figure 10** shows the separation of carbohydrates by ligand exchange.

Unlike with ion exclusion materials, it is not possible to use sulfuric acid as an eluent otherwise the heavy metal counterion would be stripped and replaced. It is also important to avoid fouling of individual column types with dissimilar heavy metal ions. The same restrictions on flow rate apply to these column types as to their ion exclusion counterparts but, because the risk of side reactions is removed, the temperature for such analyses can be further increased up to 85°C.

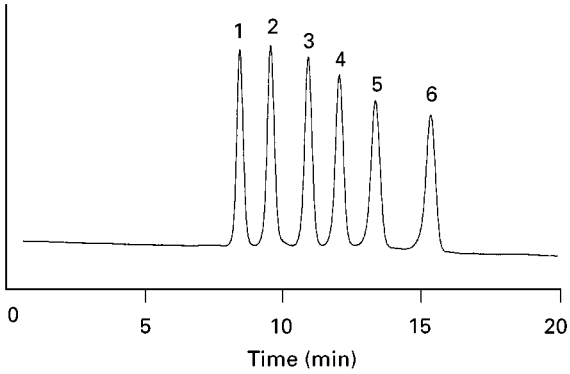

Figure 10 Ligand exchange separation of carbohydrates. Column: PL Hi-Plex Ca, 300 × 7.7 mm; eluent: water; temperature: 85°C; flow rate: 0.6 mL min^{-1}; detector: RI. Key: 1, raffinose; 2, lactose; 3, glucose; 4, galactose; 5, fructose; 6, erythritol.

Affinity Chromatography

Affinity chromatography relies on the introduction of a suitable ligand onto the surface of the resin. Frequently residual hydroxyl functionality is used to react with epichlorohydrin. This generates epoxide groups on the surface of the stationary phase, which can then be used to react with amines on the ligand molecule where appropriate.

As with other separations performed in predominantly aqueous media, it is important to eliminate nonspecific binding. For this reason PS/DVB materials are not particularly suitable unless coated with a hydrophilic film. Methacrylate resins (as described above) can be manufactured incorporating the epoxide functionality *in situ*; otherwise agarose-type materials are perhaps the most common.

Other Unusual Polymeric Supports

A number of unique polymer-based materials have been introduced in recent years to enable unusual separations to be performed. Many of these materials are covered more extensively elsewhere in this encyclopedia and so are not covered in detail here.

Chiral separations can be considered to be a form of affinity chromatography – the ligand chosen is often chiral itself or otherwise possesses an unusual affinity for another chiral molecule. A further development of this process has been to incorporate a ligand as a template molecule into the monomer mixture during the polymerization process. The ligand is removed during washing and clean-up of polymer particle but a molecular imprint remains that can increase the retention time for these molecules during actual analyses.

Pirkle supports are unusual in the respect that the outer surface of the bead contains a different functionality to the inner surface of the macropores. This is usually achieved by modifying the chemistry of the bead as a whole, but then the outer surface of the bead is further modified using a polymeric reagent or enzyme that is excluded from the pore structure. For very large molecules such as plasmids or DNA, which may be unable to penetrate even the largest pores of polymeric media, separations are still possible by using solid particles. The surface area in comparison

to a macroporous molecule is fractional but by using very small particle diameters it can be increased somewhat to enable a separation to take place. The small particle size results in an extremely high backpressure, and so column lengths are greatly reduced.

In a move away from particles it has become possible to polymerize the monomer/porogen mixture within the column itself, generating the pore structure in much the same way as particles but resulting in a monolith structure – a rigid polymeric cylinder containing through pores as well as diffusive pores. Columns containing such structures are now commercially available.

See also: **II/Chromatography: Liquid:** Mechanisms: Ion Chromatography; Mechanisms: Reversed Phases; Mechanisms: Size Exclusion Chromatography. **III/Carbohydrates:** Liquid Chromatography.

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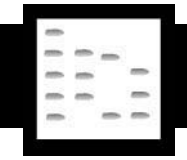
PORPHYRINS: LIQUID CHROMATOGRAPHY



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Introduction

Porphyrins are cyclic tetrapyrrolic compounds (**Figure 1**) occurring widely in nature. They are, except for protoporphyrin, the oxidized by-product of the porphyrinogens (hexahydroporphyrins) which are the intermediates in the pathways of haem and chlorophyll biosynthesis.

The analysis and separation of porphyrins not only is important in the fields of chemistry and biochemistry of this important group of tetrapyrrolic pigments but is also valuable in the biochemical diagnosis of human porphyrias, a group of diseases associated with abnormal haem biosynthesis and consequently the overproduction of haem precursors. Since some of the enzymes of the haem pathway are sensitive to certain toxic chemicals, analysis of porphyrin excretion patterns may provide a sensitive indicator of exposure to these toxic chemicals which often results in characteristic and diagnostic metabolic alterations of the pathway.

Figure 2 shows the structures of some of the most commonly analysed naturally occurring porphyrins. High performance liquid chromatography (HPLC) is the best technique for the separation of these and other porphyrins. The resolution achieved by HPLC is far superior to other methods, including thin-layer chromatography and capillary electrophoresis (CE).

The HPLC separation of porphyrins, their important metal complexes and hexahydroporphyrins (por-

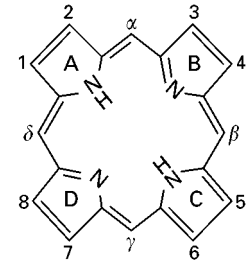

Figure 1 Structure of porphyrin macrocycle (Fischer's numbering system). The four pyrrole rings are designated A, B, C and D. The β-positions, which are usually substituted with acetic acid (Ac), propionic acid (Pr), methyl (Me), ethyl (Et) and vinyl (V) groups, are numbered 1–8. The four methine bridges or meso-positions are denoted α, β, γ and δ.