Centrifugation: Analytical Centrifugation. **Chromatography:** Protein Separation. **III/Enzymes:** Liquid Chromatography. **Proteins:** Capillary Electrophoresis; Centrifugation; Crystallization; Electrophoresis; Glycoproteins: Liquid Chromatography; High-Speed Countercurrent Chromatography; Ion Exchange; Metalloproteins: Chromatography. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins.**

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Liquid Chromatography

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Introduction

Enzymes find applications in food, pharmaceutical and biochemical industries. They are found in combination with other macromolecules or various small molecules. Their uses require identification and purification of the enzymes. The nature, quality, and quantity of the desired enzyme are determined by its intended use. For example, the food industry needs enzymes in large quantities and the pharmaceutical industry requires ultra pure enzymes. High-performance liquid chromatography (HPLC) is widely used for the separation or purification of enzymes on a preparative or analytical scale. It is also used for the analysis of the enzymatic activity.

Properties of Proteins and Practical Implications

All enzymes are proteins. All proteins are macromolecules with molecular weights ranging from hundreds to several thousands. The sequence of amino acid in a protein is specific and this gives each protein unique properties. A protein with just amino acids as a building block is a simple protein; those that contain additional units, such as a nucleic acid, a lipid or a metal etc., are called conjugated proteins.

There are 20 naturally occurring amino acids found in proteins that vary in structure; thus, it is the amino acid sequence and composition that determine the properties of enzymes. Two distinct properties are the size and polarity of the protein, which are important factors for understanding their separation. The size of a protein depends upon the number of amino acid units in the protein, whereas the polarity depends on the hydrophilic and hydrophobic units present.

A protein molecule contains one of the three groups: uncharged polar, potentially positively charged (basic side chain) or a potentially negatively charged (acidic side chain). These side chains are normally ionizable and this leads to proteins having characteristic isoelectric points. Since there are other issues governing the protein molecule, such as size, shape and nature of the solution (pH), the overall net charge and polarity depends upon the combination of these factors. In general, the chromatographic processes associated with the properties of enzymes can be summed up as indicated in **Table 1**.

Table 1 Chromatographic processes associated with enzyme properties

Enzyme property	*Chromatographic method*
Net charge	Ion-exchange chromatogrphy
Size	Size-exclusion chromatography
Substrate affinity and conformation	Affinity chromatography
Polarity	Reversed-phase chromatography and hydrophobic-interaction chromatography

High-Performance Liquid Chromatographic Techniques

Size-Exclusion Chromatography

Size-exclusive chromatography (SEC) is primarily used as a first step in purification when molecules

differ significantly in size. This technique is used extensively in biochemistry for fractionation and molecular weight determination of proteins and enzymes. The basis of separation in SEC, as the name suggests, is the size of the molecules to be separated. Spherical beads made of a cross-linked gel of a polymer such as silica, agarose, or polyacrylamide are used as column packings. Small molecules can enter all pores and elute with a characteristic volume equivalent to the column hold-up volume. Large molecules are excluded from those pores with a smaller cross-section than the solute and elute in a smaller volume than the small molecules. Consequently, molecules passing through the column separate on the basis of their size and elute in order of decreasing molecular weight.

The resolution depends on gel bead size, pore size, column size, sample size, and flow rate of the mobile phase. Low flow rates of the mobile phase, long and narrow columns, and small gel bead sizes give the highest resolution. The pore size of gel beads is designed according to the size of the molecules of interest.

The nature of the mobile phase in SEC is very important in enzymatic separation as the protein (enzyme) conformation can be changed due to solvent polarity, pH, ionic strength, and salt concentration. Conformational change can change the whole chromatographic behaviour of an enzyme. In SEC, solute–stationary phase interaction is completely prohibited for better separation. However, the mobile phase may produce (ionic or hydrophobic or both) solute–stationary phase interactions. It is necessary to design an ideal mobile phase for chromatography to avoid the above problems. Most enzymes are stable in the pH range of 5–8. The desired pH of mobile phase in SEC is obtained by using buffers suitable for both the enzyme to be analysed and the stationary phase. Tris(hydroxymethyl)aminomethane salt solution or phosphate buffers are widely used for enzymes. Denaturing solvents are sometimes also employed for the chromatographic separation of enzymes. Protein denaturants, detergents in mobile phase, change the original proteins to random coil confirmations.

Organic solvents such as acetonitrile can be used for SEC of enzymes. Using acetonitrile is very advantageous because it can be evaporated after elution to concentrate the enzyme solution. Acetonitrile is an ideal organic solvent if a UV detection method is used. However, the solubility of enzymes in acetonitrile solution limits its use in the mobile phase.

A prepacked column of cross-linked methacrylate gel (Ultrahydrogel from Waters) was used for preliminary isolation of fractions of peptidoglutaminase (PGase) from *Bacillus circulan* cell extract (1). PGase sample loading was 2–30 mg in 0.05 M sodium phosphate buffer (pH 8.0) and the eluent was 0.05 M sodium phosphate at flow rate of 0.3 mL min^{-1} (**Figure 1**). The pooled PGase peak from multiple injections was then further purified by anion-exchange chromatography.

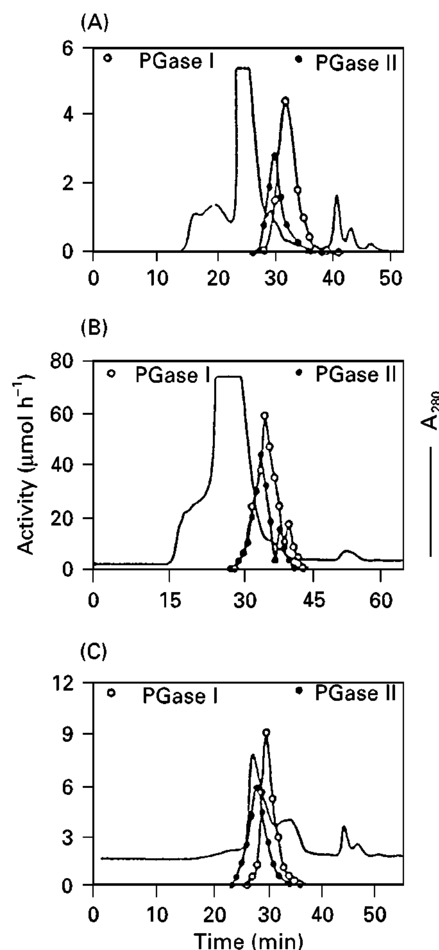

Figure 1 Gel permeation of *Bacillus circulans* proteins on acrylate gel (300 × 7.8 mm) at flow-rate of 0.5 mL min^{-1} using 2 mg (A) and 20 mg (B) of cell extract proteins. Pooled active peaks (0.1 mg) from (B) were reinjected (C). Reproduced from Hamada JS (1995) *Journal of Chromatography A* 702, 163–172, with permission from Elsevier Science.

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is a widely used technique for enzyme purification because of the net charge characteristics of enzymes. In IEC, the charged functional groups are covalently bound to the solid surface of the matrix. Cellulose, silica or styrene-divinylbenzene is used as a matrix. Cation exchanger resins contain immobilized negatively charged functional groups (i.e. RSO_3^-, RCO_2^-, RPO_4^- and RO^-) and anion exchangers contain immobilized positively

charged functional groups (i.e. quaternary ammonium groups). Resins containing sulfonyl and quaternary ammonium groups are strong ion exchangers and ionize at any pH, whereas weak ion exchangers containing functional groups like carboxyl and secondary or primary amines ionize within a certain range of pHs. When an ionized solute passes through an ion exchange column, the sample ions adsorb and displace counter ions on the surface. The adsorption process is reversible and adsorbed ions are eluted by a salt solution. Using a suitable mobile phase regenerates the resins.

The choice of the appropriate ion exchange resin for a particular enzyme separation depends on the isoelectric point of the enzyme to be separated. The isoelectric point of any molecule is the pH value at which the molecule has no net charge, i.e. an equal number of negative and positive charges. Enzymes are acidic at a pH above the isoelectric point and anion-exchange resins are used for separation of such enzymes. Similarly, cation-exchange resins are used for basic enzymes.

Column packing material, particle size and pore diameter of the support, column length, mobile phase, and temperature are some important parameters which affect separation of enzymes in IEC. In a column with smaller packing particle sizes, large enzyme molecules diffuse at a slower rate and this results in enhanced resolution and lower elution time. Most of the surface area of a support is confined within the pores and the diameter of pores affects the penetration of enzyme molecules into the column matrix and hence the mass transfer and loading capacity. Pore diameters of ≈ 300 Å are ideal for most enzymes with molecular weight up to 100 000, providing loading capacity and good resolution. Larger pore diameters are needed for higher molecular weight enzymes. The column length is not an important factor in the resolution of enzymes in IEC. Using short columns has many advantages, e.g. concentrated eluents, lower pressure and lower column cost. Lower loading capacity is a disadvantage of using a short column.

The pH, ionic strength and salt composition of mobile phase are also important factors in IEC separation of enzymes. Aqueous organic solvents are used as mobile phases. The amount of organic solvent in the mobile phase is determined by trial and error and depends completely on the nature of the molecules to be separated. Excessive organic solvent should be avoided because it can destroy the stability of enzyme molecules. The isoelectric point (pI) of enzymes determines the type of column used for separation (discussed earlier) as well as the pH of the mobile phase. The net charge on enzyme molecules depends on the pH of the solvent. Ionization of ammonium groups occurs at any pH below the isoelectric point (pI) and contributes a positive charge on the enzyme. Similarly, a negative charge on the enzyme is obtained by a pH above its pI. The retention on ion exchange columns therefore depends on the net charge carried by the enzyme molecule to be separated and the pH of the mobile phase is chosen accordingly. The pH of the mobile phase should be slightly above the pI of the enzymes to be separated for anion-exchange columns and vice versa for cation-exchange columns. The nature of displacing counterion in the salt used also affects the enzyme retention on the column. Higher valent ions are stronger displacers than lower valent ions and thus give lower retention. The smaller size of ions also favours lower retention if the charge on the counterion is the same. Gradient elution based on ionic strength variation or pH changes is used in IEC. Chromatographic separation of most enzymes is carried out at a low temperature to preserve enzyme stability.

The quaternary methylamine resin from Waters (Accel Plus QMA) has been used for the separation of peptidoglutaminase from *B. circulam* cell extract. Enzyme load was 1.0 mg in 20 μL of 0.02 M phosphate buffer (pH 8.0) and the eluent used was 0.05 M sodium phosphate buffer and 0.1–0.8 M KCl at a flow rate of 0.5 mL min^{-1} for analytical separation and 1.5–10.0 mL min^{-1} for preparative separation (**Figure 2**).

Reversed-Phase Chromatography

Reverse-phase chromatography (RPC) is the most popular chromatographic method for the purification, separation, and analysis of the biological molecules because of its high resolution and ease of handling. Column packings are usually prepared from silica particles and hydrophobic long-chain alkylsilyl ligands. n-Butyl (C4), n-octyl (C8), n-octadecyl (C18), and alkylphenyl groups are used for separating enzymes. The nonhydrophobic molecules in the sample do not strongly interact with the hydrophobic stationary phase of the column and elute earlier, while hydrophobic molecules in the sample interact with the hydrophobic stationary phase of the column and elute later.

The column packing material, particle size and pore diameter of support, column dimension, mobile phase, and length of hydrophobic ligands, determine the effectiveness of an RPC procedure. Silica is the most widely used support because of its mechanical stability, efficiency, and ability to be bonded with hydrophobic ligands. However, silica supports are not stable under basic conditions ($pH > 8$). Small

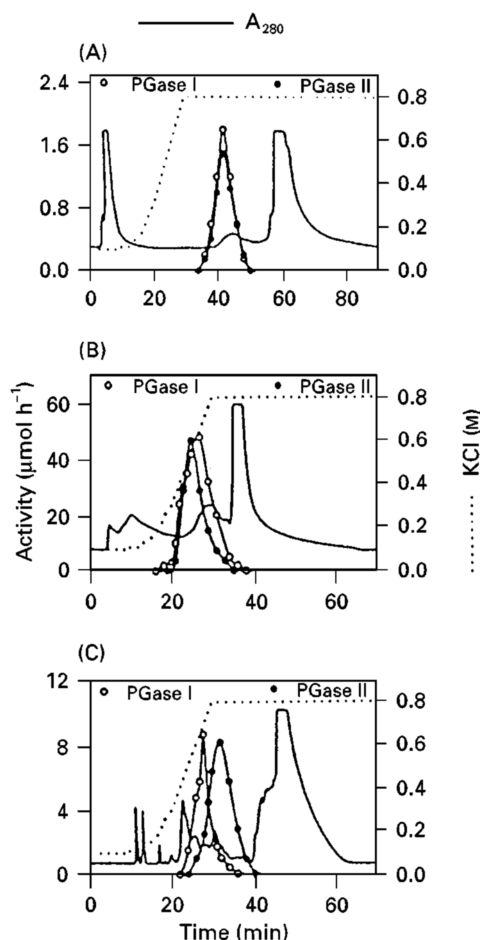

Figure 2 Anion-exchange separation of *Bacillus circulans* proteins using (A) QM anion exchange (150 × 3.9 mm) column at 1.0 mg load and 0.5 mL min^{-1} flow rate, (B) 150 × 19 mm QM anion exchange column at 30 mg load and 5.0 mL min^{-1} flow rate, and (C) DEAE anion exchange (150 × 21.5 mm) column at 5 mg load and 5 mL min^{-1} flow rate. Reproduced from Hamada JS (1995) *Journal of Chromatography A* 702, 163–172, with permission from Elsevier Science.

support particles favour higher resolution, but high column backpressures and the large size of enzyme molecules to be separated do not favour the smaller particle sizes. A particle size of ≈ 20 μm is optimal for RPC column for enzymatic HPLC. A pore size of 300 Å is the most commonly available size in commercial RPC columns. For large enzyme molecules use of a large pore size (≈ 1000 or greater) is suggested to avoid any diffusional problems.

The hydrophobicity of n-alkyl group attached to the silica decreases with decreasing chain length of n-alkyl group (C18 > C8 > C4 > C2). Smaller n-alkyl group chains are favoured for highly hydrophobic samples and vice versa. More hydrophobic columns (e.g. C18) require a stronger mobile phase (higher amount of organic solvent). Organic solvents such as acetonitrile, methanol or isopropanol and ion-pairing agents or buffers are added to the mobile phase to achieve reasonable retention times. The effectiveness of these organic modifiers depends on solvent polarity and increases with decrease in polarity. Isopropanol is a very good solvent for highly hydrophobic enzymes and methanol is better for hydrophilic enzymes. Acetonitrile is the most suitable organic modifier because it has intermediate polarity, low viscosity, and low UV adsorption. It is volatile and can be easily removed from the eluent. The function of added solvents is to decrease the interaction between the stationary phase and highly hydrophobic molecules and thus reduce the retention time. Ion-pairing agents or buffers set the eluent pH and interact with the enzyme to enhance the separation. Trifluoroacetic acid (TFA) is widely used as an ion pairing agent. Buffers such as phosphate or hydrochloric acid are also used. The mobile phase for RPC typically consists of water, organic solvent, and trifluoroacetic acid (0.1%) or phosphoric acid.

Capillary columns packed with nonporous (pellicular) supports have been used for fast separation of enzymes or proteins at high temperatures and at high flow rates. Packed capillary RP-HPLC columns have several advantages over conventional columns – fast separation, reduction in solvent usage and the ability to work with small samples. The mass transfer between the stationary phase and the mobile phase is fast with pellicular packings because the diffusional distances in the stationary phase are short owing to limited chromatographic interaction at the outer surface. Capillary columns are stable at higher temperatures and at higher pressures because of the solid, fluid-impervious core of the micropellicular packing. Rapid mass transfer resulting from the pellicular configuration and higher temperature is mainly responsible for the fast separation of enzymes. Higher temperatures may not be appropriate for the stability of some enzymes.

Fast separation of a mixture of four proteins was performed in 6 s at 120°C on a 3 cm column packed with 2 μm pellicular ODS-silica (**Figure 3**).

The biological activity of enzymes is sometimes lost due to high backpressures, mobile phase (low pH and organic modifiers) and a strong hydrophobic stationary phase. Hydrophobic interaction chromatography (HIC) has less harsh chromatographic conditions than RPC and can be used to preserve the biological activity of enzymes.

Hydrophobic Interaction Chromatography

The basis of separation in hydrophobic interaction chromatography (HIC) is the same as for RPC. These methods differ in the properties of the mobile and stationary phases. HIC is carried out with an aqueous

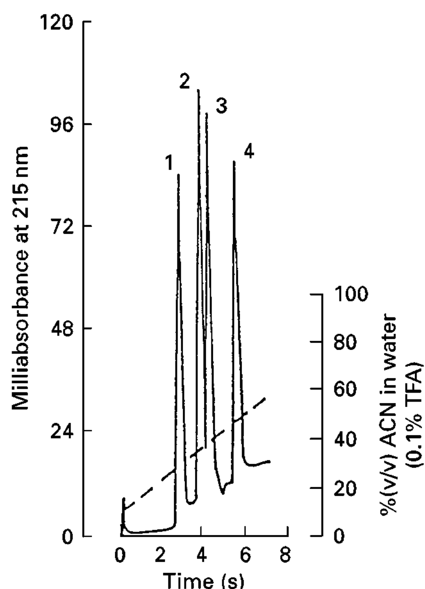

Figure 3 Fast separation of standard mixture of proteins: 1 = ribonuclease A; 2 = cytochrome *c*; 3 = lysozyme; 4 = lactoglobulin B. Column (30 × 4.6 mm) packed with 2 μm pellicular ODS-silica; 12 s linear gradient from 10 to 90% (v/v) acetonitrile (ACN) in water containing 0.1% trifluoroacetic acid (TFA), temperature = 120°C, flow rate = 5 mL min^{-1} and column inlet pressure = 240 bar. Reproduced from Chen H and Horvath CS (1995) *Journal of Chromatography A*, 705, 3–20, with permission from Elsevier Science.

solution of higher salt concentration at neutral conditions and uses a weak hydrophobic stationary phase. The higher concentration of salt in the mobile phase enhances the binding between enzymes and weakens the hydrophobic stationary phase.

Most separation variables in HIC behave in the same way as in RPC but the nature of the mobile phases and stationary phases differs in these two HPLC methods. The HIC performs separation under nondenaturing conditions whereas RPC denatures enzymes during separation because of the mobile phase conditions (organic solvent and highly acidic) and the highly hydrophobic stationary phase. The mobile phase in HIC is neutral and nonorganic and protects enzymes from denaturation. Salts such as sodium or potassium phosphate are added into the mobile phase to buffer it at pH ≈ 7. The bonded phase in HPHIC is an aryl or smaller alkyl ($n < 5$) group, weak hydrophobic ligands, attached to silica support.

The purification of *Chromobacterium viscosum* lipase has been studied using hydrophobic interaction chromatography. The stationary phase was prepared by covalent immobilization of polyethylene glycol on Sepharose gel (Sepharose CL-6B, Pharmacia). The extent of lipase was affected by the salt used and increases with increasing ionic strength in the eluent buffer and with higher pH value. The best recovery of lipase was observed when potassium phosphate was used as a salt compared to NaCl, Na_2SO_4 and $(NH_4)_2SO_4$ (**Figure 4**).

Affinity Chromatography

The basis of affinity chromatography (AC) is the selective adsorption of the molecule to be separated

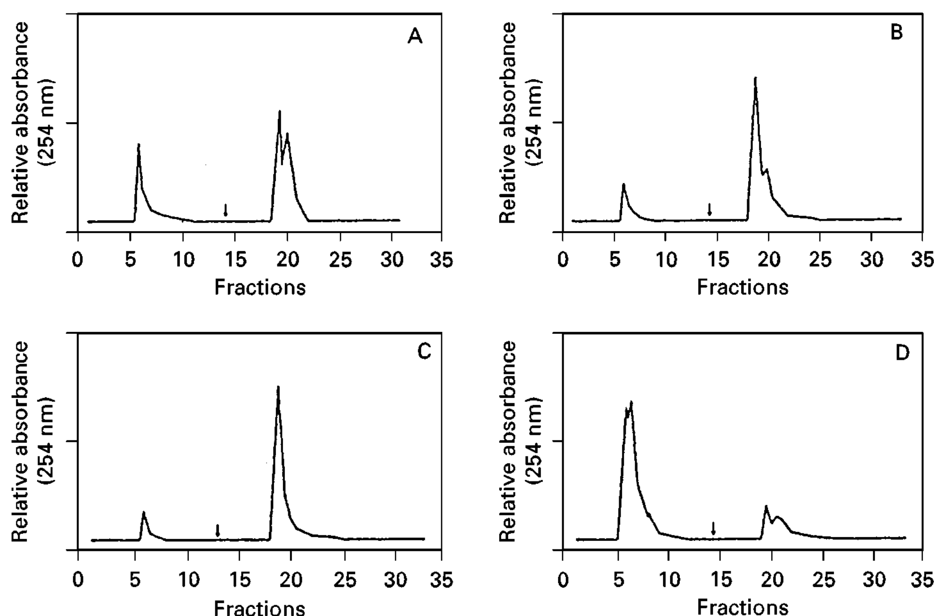

Figure 4 HIC on PEG 10 000-Sepharose CL-6B column. Buffer: (A) 15% (w/w) K_3PO_4; (B) 20% (w/v) $(NH_4)_2SO_4$; (C) 15% (w/v) Na_2SO_4; (D) 4 M NaCl in 10 mM phosphate (pH 7). Desorption with 10 mM phosphate buffer (pH 7). From Queiroz JA, Garcia FAP and Cabral JMS (1996) *Journal of Chromatography A* 734, 213–219, with permission.

from mixture on the matrix of the column. A specific ligand for a specific biological molecule is chosen and it is covalently bound to the matrix of the column. For example, a ligand such as adenosine will bind only enzyme adenosine deaminase and not any other molecules. When a mixture is applied to the affinity chromatography column then the molecule that has specific affinity for the ligand will stay in the column and all other unbound molecules will migrate through the column. The interaction between adsorbed molecule and ligand is reversible. Changing the pH or other conditions of the mobile phase can desorb the bound molecule. A molecule that has more affinity for the ligand than the bound molecule can be included in the mobile phase to elute the desired bound molecule. This chromatographic method is carried out under nondenaturing conditions during the separation of enzymes or proteins.

The type of ligand and its support, state of mobile phase at each stage of separation and flow rate determine the resolution in AC. Ligands can be specific for a molecule or a group of molecules. The desired ligand must be highly specific for enzyme molecule(s) to be separated, be stable under applied conditions, have reversible binding with applied sample and possess an appropriate functional group to couple with the support. Cross-linked agarose or other pressure-stable polymer is used as a support for the ligand.

Buffers at each step of separation must be nondenaturing to maintain specificity of ligand and eluting enzyme(s). Low pH buffers are employed in the desorption step to break the solute–ligand interaction. Specific desorbing agents, which compete with adsorbed solute molecule(s) for the same binding site, are sometimes also used. The flow rate of mobile phase also affects the retention time and peak shape.

Alhama *et al.* have applied AC technique for the purification of glutathione reductase and glucose-6-phosphate dehydrogenase from cell-free extract of baker's yeast, fish liver, and rabbit hemolysates with high recovery. They used an epoxy-activated silica column derivatized with the ligand 8-[(6-aminohexyl)amino]-2′-phosphoadenosine-5′-diphosphoribose. The bound ligand concentration was 11.4 μmol g^{-1} of dry silica and the loading capacity was 2–3 mg of glutathione reductase.

Hydroxyapatite High-Performance Liquid Chromatography

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is a form of calcium phosphate which has been used, in particular, as a packing material for enzymes and proteins separations. The basis of separation is ionic interactions between amine groups of the enzymes and phosphate groups on the surface of the hydroxyapatite and, also, calcium coordination complex formation between calcium groups in the hydroxyapatite and carboxyl groups in enzymes. Low concentration phosphate buffers are used to elute acidic and neutral enzymes and high concentration buffers are used to elute basic enzymes.

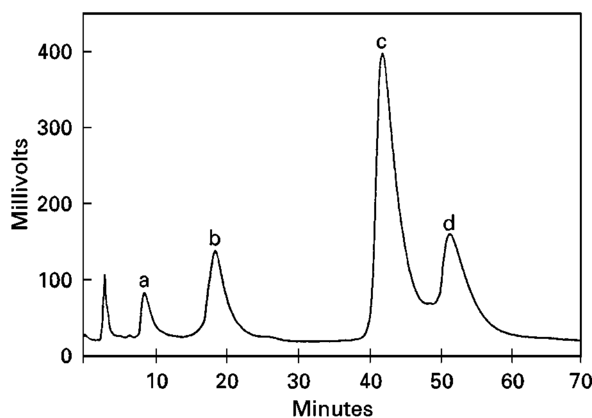

Figure 5 Separation of a protein mixture: a = transferrin, b = myoglobin, c = lysozyme, and d = cytochrome *c*. Packing: Nucleosil 1000-5 DIOL CaP-HA 2.5% (100 × 6 mm). Linear gradient of sodium phosphate (pH 6.8), 1–350 mM (60 min); flow rate = 1 mL min^{-1}, temperature = 25°C. Reproduced from Bruno G, Gasparrini F, Misiti D, Arrigonimartelli E, and Bronzetti M (1990) *Journal of Chromatography A* 504, 319–333, with permission from Elsevier Science.

A protein mixture containing transferrin, myoglobin, lysozyme, and cytochrome *c* was separated using hydroxyapatite as a support. Protein solution (10–50 μL; 1 μg μL^{-1} protein) was loaded onto the column and eluted with a linear gradient of sodium phosphate buffer (pH 6.8) (**Figure 5**).

Perfusion Chromatography

Perfusion chromatography is a new chromatographic technique, introduced by Afeyan and coworkers in 1989–1991, for reducing resistance to stagnant mobile phase mass transfer in liquid chromatography. It may be used for both rapid analysis and preparative chromatography of large molecules such as enzymes. A new chromatographic packing material (POROS, Perseptive Biosystems) which contains two sets of interconnecting bimodal pores has been employed in perfusion chromatography. The members of one pore set having a mean diameter in the range 6000–8000Å are called throughpores. The high surface area needed for adequate sample capacity is achieved by the smaller diffusive pores ($d_{pore} \approx 1000$ Å). The mobile phase flows through the through-pores. In this manner, solutes enter the interior of the particles convectively by the through-pores and then diffuse into the diffusive pores.

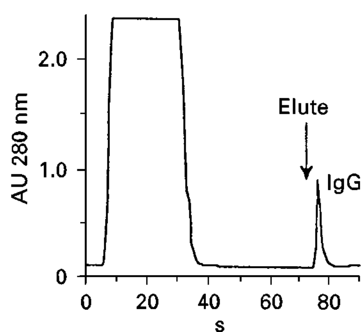

Figure 6 Separations of hybridoma cell cultural supernatant on protein A POROS M. 0.5 mL injection; 30 × 2.1 mm column; 10 mM phosphate pH 7.4, 0.15 M NaCl; elution with 0.3 M acetic acid (2%, v/v), 0.3 M $MgCl_2$; 2 mL min^{-1} flow-rate. Reproduced from Afeyan NB, Fulton SP and Regnier FE (1991) *Journal of Chromatography A* 544, 267–279, with permission from Elsevier Science.

POROS-based chromatographic packing material can be used in any chromatographic mode such as ion exchange, reversed-phase, hydrophobic interaction, and affinity chromatography. These supports separate proteins rapidly compared with conventional HPLC using higher mobile phase velocities.

The separation of immunoglobulin G(IgG) from hybridoma cell culture supernatant has been completed in 80 s using a POROS protein A (aldehyde-coupled) column. The sample was loaded in a 0.1 M phosphate buffer pH 7.4 with 0.15 M NaCl and eluted with 0.3 M acetic acid (2%, v/v) with 0.3 M $MgCl_2$. The column was loaded with 0.5 mL of hybridoma cell culture supernatant with the flow rate of 2 mL min^{-1} (**Figure 6**).

Assay of Enzymatic Activity

An important aspect of the separation of enzymes by HPLC is the assay of enzyme activity. Enzyme-catalysed reactions can be monitored spectrophotometrically and many of the substrates or products absorb visible or UV light. It allows determination of the progress of a reaction by direct and continuous monitoring. While other methods of discontinous assay focus on monitoring one of the compounds of the reaction, the HPLC technique offers the simultaneous determination of several substrates of the reaction. This method is probably the best as it offers a complete mass balance of the reaction being analysed.

Detectors for Enzyme Analysis

Ultraviolet-visible (UV-vis) detectors are the most commonly encountered detectors in enzyme analysis because enzymes are UV-active and UV detectors are simple to use and relatively inexpensive. The analysis is also nondestructive and hence suitable for preparative work. Furthermore the solvents best suited for liquid chromatography are transparent to UV-vis. Refractive index detectors are nondestructive, concentration sensitive and universal in that they respond to virtually all compounds with the proper choice of mobile phase but are of low sensitivity. The specificity of analysis of enzymes by fluorescence detection arises because many parameters related to the fluorescence intensity can be exploited. However, this technique requires the use of very selective regents that react with specific functional groups of the analyte to produce fluorescent derivatives.

Future Trends

HPLC will continue to be the important tool for separation of enzymes. The new capillary columns packed with nonporous support and microsporous support in perfusion chromatography will be helpful in fast analysis of enzymes or proteins. Separation is faster and more selective when HPLC is carried out at higher temperatures. A heat exchanger, which can bring the eluent rapidly to column temperature, will increase separation reliability at higher temperatures. Conventionally, HPLC is used for the analytical separation as well as for preparative separation of enzymes. Discontinuity of the HPLC process and the dilution of the products after elution are two major disadvantages. The simulated moving bed (SMB) technique can make HPLC a continuous process. A column packing material should be designed for a higher sample loading and for fast HPLC. Thus, a large-scale separation should be fully automated and continuously operating, loading samples, collecting fractions, regenerating the column and with various fail-safe devices to protect the column and product.

See also: **II/Affinity Separation:** Theory and Development of Affinity Chromatography. **Chromatography: Liquid:** Mechanisms: Ion Chromatography; Mechanisms: Reversed Phases; Mechanisms: Size Exclusion Chromatography. **III/Peptides and Proteins:** Liquid Chromatography.

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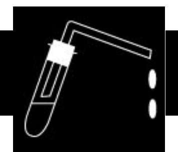
ESSENTIAL OILS



Distillation

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Introduction

Essential oils are generally understood to be volatile compounds which are freely soluble in alcohol, ether and vegetable and mineral oils and are usually assumed to be the result of distillation or a steam-stripping process. The use and processing of essential oils began in the East more than 2500 years ago. The process of distillation, which is the technical basis of the essential oil industry, was also conceived and first employed in the Orient, especially in Egypt, Persia and India. Turpentine and camphor appear to be the first documented essential oils prepared by distillation in Greece by Herodotus (484–425 BC).

The use of essential oils in ancient times consisted of preparing ointments by mixing oils from flowers with fatty oils; this was done by placing flowers and roots with the oil in glass bottles which were then allowed to sit for periods of time. Sometimes the flowers or roots were macerated with wine before the fatty oil was added, and the product obtained by digestion filtered and boiled down to a thicker consistency.

Medieval alchemists laboured for many years to extract from materials found in nature what they called the *quinta essentia* or the fifth essence. They believed that a combination of earth, air, fire and water existed in some form or other from which quintessential materials could be extracted from some plants. These quintessential extracts derived from plants were believed to be remedies for a wide variety of diseases.

The production and use of essential oils did not become widespread until the second half of the 16th century. In 1507, Hieronymus Brunschwig's book on distillation, *Liber De Arte Distillandi*, described distillation techniques for four essential oils, namely, turpentine (known since antiquity), juniper wood, rosemary and spike.

Before the ninth century it was still widely believed that most essential oils had strong curative properties. Therefore it was chiefly pharmacists who developed and improved methods of distillation for the recovery and purification of natural essential oils.

Eventually, with the development of the fields of medicine and pharmacology and the dispelling of some medicinal myths, the use of essential oils in pharmaceutical products lost importance and their use became restricted to perfumes, beverages and foodstuffs.

Applications of Essential Oils

Attractive aromas which leave a pleasant memory association are used as marketing devices to sell edible and cosmetic products, including unlikely materials such as detergents. The producer is counting on the consumer preferring a product that left a pleasant aromatic memory.

Current specific uses of essential oils are to add flavour to foodstuffs and beverages and to scent perfumes, lotions, soaps, detergents and household cleaners. For example, *d*-limonine from citrus peel is a very strong solvent and it is used in a wide variety of cleaning products. Essential oils are a major part of carbonated beverage flavourings; the most common flavours include lemon, lime, orange, cassia, cinnamon and nutmeg. Essential oils are also used to flavour many foods such as sweets and candies, cookies, snacks and chewing gum.

The field of aromatherapy constitutes a small part of the essential oils industry but it is a fast-growing area and requires a wide variety of essences. Not much scientific work has been done in this area to support any of the medicinal and psychosomatic