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High Performance Liquid Chromatography: Principles and Methods in Biotechnology

Edited by

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Preface

During the last decade basic and applied research in the fields of molecular biology, biochemistry, and biotechnology have proceeded at a very rapid pace. The availability of synthetic oligodeoxyribonucleotides, the development of monoclonal antibody technology, as well as the invention of the polymerase chain reaction have greatly contributed to recent advances in the diagnosis and treatment of cancer, the detection of genetic and infectious diseases in humans, biotherapeutic monitoring, and the detection of pathogens of environmental importance.

Characterization, analysis, and purification of biomolecules such as proteins, oligosaccharides, and nucleic acids are of utmost importance in all bioscience applications. The use of recombinant microorganisms by the pharmaceutical industry has created very stringent purity requirements for the pharmaceuticals. HPLC lends itself to the separation of thermally labile, biologically active molecules and has become one of the major analytical tools in the life science laboratory.

A number of recently published books addressed advanced HPLC developments in biotechnology applications, specifically for protein purification and analysis. Rather than focusing on the advanced HPLC developments, this book—*High Performance Liquid Chromatography. Principles and Methods in Biotechnology*—intends to cover major theoretical and practical aspects of HPLC to provide a bioresearcher, who is already using or will be using HPLC in the future, with better understanding of the principles, advantages, and limitations of the technique. The book describes critical aspects of HPLC such as: general column theory, sample preparation, application-driven selection of stationary phases and columns available for bioseparations, detection and identification of biomolecules, and general applications to protein, oligosaccharide, and nucleic acid analyses. In addition, the book covers theory and practice of preparative-scale HPLC. For comparison purposes, it also includes an overview of bioseparations that are not based on HPLC.

High Performance Liquid Chromatography: Principles and Methods in

Biotechnology. is written by HPLC experts, who are actively working in the biotechnology field, for molecular biologists, biochemists, and biotechnologists, who are practicing or will be practicing HPLC to purify, characterize, and quantitate biomolecules such as proteins, oligosaccharides, and nucleic acids. The book should also be useful for analysts and chromatographers who want to understand the intricacies of HPLC as they are related to the separations specific for the biomolecules.

Elena Katz

Norwalk, CT

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CHAPTER 1

INTRODUCTION TO CHROMATOGRAPHY IN BIOTECHNOLOGY

Colin F. Simpson

1. Introduction

In the late seventies, the first companies exploiting biotechnological methods for producing novel compounds were established. The industry now has a multimillion dollar turnover and has had an enormous impact on the production of new drugs and methods of synthesis, since the use of enzymes produces specific products with few side reactions. The ability to monitor the purity and/or composition of the products has placed considerable strain on analytical chemistry and separation science in particular. Indeed, virtually all methods of separation have been applied to solving the problem of assessing the purity of the products formed. The two principal and powerful methodologies used for assessing purity and/or composition are the chromatographic and electrophoretic techniques. Since the start of biotechnological methods of synthesis, considerable research has been undertaken in improving the separation methods available which has had a significant impact on separation science in general. The methodologies which have been developed during this period, such as the high efficiency analytical methods of capillary electrophoresis and packed capillary chromatography, have become accepted as separation techniques.

In this chapter the characteristics of the various relevant chromatographic techniques will be reviewed. All forms of chromatography have been applied to the difficult problem of the separation of complex mixtures of materials, which can be thermally labile, or catalytically rearranged on the surface of the chromatographic supports or irreversibly adsorbed on the stationary phase.

2. Chromatography of Biopolymers

It would be useful to consider briefly any problems which can be experienced in performing liquid chromatography of biopolymers. The theory and practice of liquid chromatography for biomolecules is the same as that for the separation of small molecules. The selectivity of the separations is the same as or better than for small molecules, but the resulting column efficiency is relatively low because of the slow diffusion characteristics of large molecules, their relatively slow kinetics of adsorption/desorption and the secondary equilibria possible.

The high selectivity allows the use of gradient elution, which can offset the low column efficiencies experienced, indeed it is common practice to use gradients in this type of separation, which has the added advantage of increasing the peak capacity. Elevated pressures with high flow rates can bring about denaturation of proteins through the high shear forces experienced in packed columns and narrow bore connecting tubes and further, the presence of the column packing itself can lead to surface denaturation and/or irreversible adsorption. In spite of these deleterious effects, LC has been used very successfully for biopolymer separations both for analytical and preparative purposes.

3. Liquid Chromatography Methodology

Not all of the liquid chromatographic methods of separation can be applied to the separation of biomolecules and care must be employed in the choice of method.

The principal methods used are given in Table 1.

Table 1

Chromatographic methods Used in Biomolecule Separations

Method	Interaction	Application
Size Exclusion (SEC)	Separation by Molecular Size, No Molecular Interactions	Peptides and Proteins Polysaccharides
Ion Exchange (IEC)	Electrostatic Interactions Buffers	Proteins and Peptides Nucleic Acids Polysaccharides
Hydrophobic Interaction (HIC)	Dispersive Interactions Salt Gradients	Proteins Polysaccharides
Reversed Phase (RPC)	Dispersive Interactions Aqueous/organic	Proteins, Peptides Amino Acids Nucleic Acids Proteins, Enzymes
Affinity (AF)	Biospecific (very strong)	Proteins and Enzymes
Metal Chelate Interaction (MCIC)	Specific Metal Binding	Amino Acids Proteins
Chromatofocusing	pI	Peptides, Proteins

4. Equipment for Biopolymer Separations

In principle, any liquid chromatograph can be used for biopolymer separations, all that is needed is a mobile phase delivery system, sample introduction device, columns with appropriate packing and a detector, usually a variable wavelength ultra-violet system capable of operating down to 200 nm. The electrochemical detector can provide additional information. The description of a general liquid chromatographic system is given in Chapter 2 and specific detection systems are discussed in Chapter 4. While equipment of this nature may be used satisfactorily, for some

analytes the stainless steel construction of the various parts of a conventional liquid chromatograph may interact with the solute molecules, bringing about degradation or adsorption. To prevent this occurrence, the wetted parts of the chromatograph and column have been constructed of titanium and this equipment is available from several manufacturers. Pharmacia market Fast Protein Liquid Chromatographs (FLC), designed to perform low-pressure chromatography, where all wetted parts are made out of either glass or PTFE.

5. The Nature of Biomaterials

5.1. Proteins and Peptides

Proteins and polypeptides are linear condensation polymers of different amino acid residues (about 20 in man) in which the individual amino acids are linked by the peptide bond; the differences between proteins lie in the nature of the side-chains, the number and nature of the amino acids present and the cross-linking which can occur through the presence of two or more molecules of cysteine in the protein. Proteins always are associated with at least one sugar residue. Proteins and polypeptides differ in that a protein has physiological function while a peptide does not but may be considered to act as a trigger. A further distinction lies in the stable secondary structure which can be formed by proteins; polypeptides do not form conformationally stable secondary structures and because of this may be more difficult to separate as discrete bands.

Thus proteins may be considered as having surface regions which are apolar with electrostatic groups associated with them; the number and sign depending on the pH of the environment. It is these polar regions which may be exploited in obtaining a separation by RPC or HIC alternatively, the charged groups may be exploited in IEC to obtain a separation.

5.2. Nucleic Acids

Nucleic acids, nucleotides, nucleosides and related materials are the fundamental genetic material of living organisms. They occur as long chain

polymers consisting of a limited number of bases; adenine, guanine and cytosine, thymine or uracil. DNA contains thymine, in RNA the thymine is replaced by uracil. The bases are associated with phosphoric acid and sugar molecules, ribose (RNA) and deoxyribose (DNA). The bases and corresponding nucleosides and nucleotides are given in Table 2.

Table 2

The Bases and Corresponding Nucleosides and Nucleotides

Bases	**Nucleosides (Bases + Sugar)**	**Nucleotides (Bases+Sugar +Phosphate)**
Adenine	Adenosine	AMP, ADP, ATP
Guanine	Guanosine	GMP, GDP, GTP
Cytosine	Cytidine	CMP, CDP, CTP
Thymine	Thymidine	TMP, TDP, TTP
Uracil	Uridine	UMP, UDP, UTP

Hydrolysis of these polymers can occur in two ways: (1) total hydrolysis, in which the polymer is totally fragmented into its components or (2) partial hydrolysis, where the products are in the form of nucleosides, nucleotides and various oligonucleotides. Nucleic acids occur in various spatial arrangements in DNA, such as linear, single stranded polymers or circular and supercoiled DNA. Over the past decade and more, various HPLC and slab gel electrophoresis methods have been developed for the separation of these complex, molecules and fairly recently capillary gel electrophoresis has been applied with a high degree of success (*e.g.* in the human genome project).

5.3. Polysaccharides

Polysaccharides are condensation products of (principally) the hexoses; glucose and fructose. In contrast to the polynucleotides and proteins, polysaccharides form branched chains at various positions on the pyranose

or furanose rings, although for the linear polysaccharide cellulose, the linkage is in the 1:4 position, and this linkage point dominates in all the polymers except when branching occurs and highly complex mixtures of differing degrees of branching are produced. Starch for example consists of two species, amylose (20%) and amylopectin (80%). Amylose is a linear condensation product of glucose but the polymer contains the maltose substructure. Its molecular weight can range between 150,000 and 600,000 Daltons. Amylopectin is formed from glucose but forms branched chains with branching occurring every 20–25 glucose units. Although its molecular weight runs into millions it is more soluble in water than amylose. It will be clear that even in these simple products the number of possible isomers can be very large, hence the difficulty of obtaining a separation is high.

In addition to these oligomers, nature also forms oligomers from modified glucose units, usually by replacing one of the hydroxyl groups by an amino group. These are called glycosylamines if the amino group is attached to the anomeric carbon atom or amino deoxy sugars if attached elsewhere. The glycosylamines are present in the nucleic acids as indicated above. For example, ribonucleic acid is a polymer of repeating nucleotides which are in effect substituted glycosylamines.

It will be clear from the above very limited account that the problem of separation of substances of this type can be severe. Nevertheless, application of the various operating methods of the liquid chromatographic systems outlined below has provided means whereby at least partial answers have been provided. Clearly the ability to provide separations across the complete range of biotechnological products will require the utmost ingenuity on the part of the separation scientist.

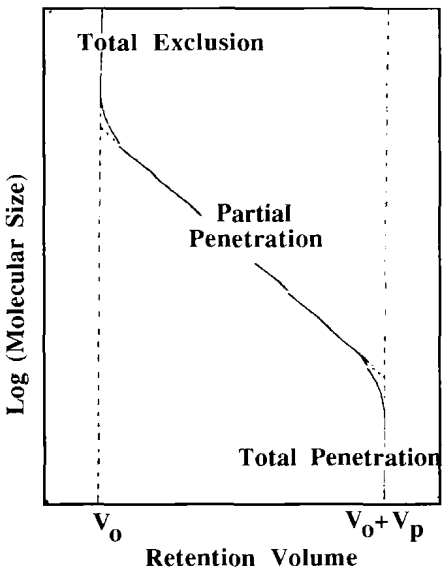
6. Liquid Chromatography and Biomaterials

6.1. Size Exclusion Chromatography (SEC)

Size exclusion chromatography, in the ideal case, is a non-interactive form of chromatography in which separation is effected through differences in the molecular size of the sample components. The separation medium is a

range of porous solid, *e.g.* cross-linked polystyrene, porous silica, porous glass or hydrophilic polymer gel (which have various pore size distributions see Chapter 3), and the pores of these materials are filled with the mobile phase which, in effect, acts as the 'stationary phase'.

The solute molecules can sample the various pores to differing extents depending on their molecular dimensions. Those molecules capable of penetrating all the pores are said to be totally included, those which do not penetrate any of the pores are said to be totally excluded. Thus there is a finite window in which separation can occur and the range of molecular weights a given material can accommodate is determined by the exclusion limits of the packing.



The slope of the line between the limits of total exclusion and total penetration controls the component capacity and the resolution possible.

Figure 1 Theoretical Curve for the Size Exclusion Separation Mechanism Showing the Limits of Total Exclusion and Total Permeation

A size exclusion packing with exclusion limits 5,000 – 50,000 implies that the packing will be able to discriminate between molecules whose molecular weights range between these limits, but molecules with molecular weights above or below these limits will not be separated and will be co-eluted. This concept is shown in Figure 1.

Thus exclusion by SEC occurs between two defined elution volumes (V_i), the interstitial void volume and (V_p) the total volume of stationary phase contained within the pores of the packing. The retention in SEC is given by equation (1).

$$V_r = V_i + K_{GPC} V_p \qquad (1)$$

Where, (V_r) is the retention volume of the solute,
(K_{GPC}) is the distribution coefficient in SEC and ranges, between 0 (totally excluded) and 1 (totally included).

In contrast to synthetic polymers which can cover a wide range of molecular weights, *i.e.* a molecular weight distribution, natural biopolymers exist as discrete entities and elute from SE columns as single peaks. The separation capacity of an SE column depends upon the number of single species it is possible to fit into the separation window. This is a function of two factors, the efficiency of the column and the pore volume/pore size distribution of the packing (which controls the slope of the line in Figure 1). Column efficiency is expressed in terms of the number of theoretical plates developed by the column and is given by:

$$N = 5.545\left(\frac{V_r}{w_{(0.5)}}\right)^2 \qquad (2)$$

where (N) is the number of theoretical plates,
(V_r) is the retention volume,
($w_{(0.5)}$) is the peak width at half height.