Journal of Chromatography, 418 (1987) 3-26
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3726

REVIEW

GENERAL STRATEGIES IN THE SEPARATION OF PROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

MILTON T.W. HEARN

Department of Biochemistry, Monash University, Clayton, Victoria 3168 (Australia)

(Received April 7th, 1987)

CONTENTS

1. Introduction 3
2. Separation parameters in protein purification 5
  2.1. Bulk properties 5
  2.2. Chromatographic modes 6
  2.3. Combination of separation parameters 10
  2.4. Interactive stationary phases 11
  2.5. Retention behaviour 12
  2.6. Gradient elution 14
  2.7. Recovery of bioactivity 14
  2.8. Retention mechanisms 15
  2.9. Multi-zoning 16
  2.10. Resolution optimization 18
  2.11. Positive-negative affinity mode 19
  2.12. Batch methods 19
  2.13. Mixed-bed separation 20
3. Conclusion 21
4. Acknowledgements 23
5. Summary 23
References 23

1. INTRODUCTION

Over the past decade many challenges have arisen in biopolymer separation as a consequence of advances in molecular biology, protein engineering, recombinant DNA methods and cell culture technologies. Central to these developments has been the redefinition by governmental regulatory agencies of the require-

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

ments for the quality control of peptides and proteins intended to be used as therapeutic products. With the availability of many proteins through the advent of genetic engineering techniques, the criteria for establishing protein purity are currently undergoing substantial re-examination. The potential now exists, due to the emergence of rapid, high-resolution chromatographic and electrophoretic techniques, to address systematically the quality control of peptides and proteins at analytical levels hitherto not feasible and also to develop new strategies for the purification of a specific peptide or protein from complex mixtures.

The attainment of very high purities for peptides or proteins can only be achieved by the application of several high-resolution separation techniques. Integral to the definition of the final purity of a protein or peptide are the detection limits set for the analysis of contaminants. Different analytical separation and detection methods are required depending on whether these contaminants are proteinaceous, nucleic acids, lipids or polysaccharides. Even with the most sensitive analytical methods based on polyacrylamide gel electrophoresis (PAGE) with silver or gold staining, detection of contaminants under the most favourable staining conditions down to 5–10 ppm represent currently feasible limits [1–5]. With some enzyme-linked immunoassays it is possible to improve the limits of detectability by a further order of magnitude [6,7]. However, further significant improvement is required if the detection limits for biological contaminants are to approach the ppb ($\leqslant$ng/g) level now made mandatory by public and governmental concern with therapeutic proteins.

In order to purify a biopolymer to near homogeneity and satisfy the stringent limits of contaminant detection, a battery of sophisticated analytical and preparative separation techniques are required by the biochemist. For these reasons it is not surprising that extensive research is currently under way to refine and extend existing chromatographic and electrophoretic procedures in order to allow improved resolution and recovery. In this article, some of the options currently available for protein separation by high-resolution chromatographic methods are examined. The article's theme has been deliberately developed as a general overview with the biologist, who has only recently initiated an interest in modern separation techniques, kept in mind. In the other chapters of this Special Volume, various application areas of high-resolution chromatographic methods in the life sciences are summarised in detail.

Nearly all modern high-performance liquid chromatographic (HPLC) methods lend themselves to the requirements of either analytical separation or scale-up preparative separation. In the research laboratory setting the advantages of electrophoretic procedures, judiciously used in a purification procedure or in the case of capillary zone electrophoresis for high-sensitivity analysis, should not be overlooked. By combining the potentials of the various separation techniques, it is now feasible to achieve purification factors between 100 000 and 500 000 for bioactive substances present in only trace amounts in biological fluids. The recent purification of the gonadal protein hormone, inhibin, is typical of these current possibilities. A 58 000-Da form of inhibin has been purified [8–10] from follicular fluid to near homogeneity by a five-stage purification procedure involving gel-permeation chromatography, weak hydrophobic-interaction chromatography

under low pH conditions, reversed-phase HPLC and preparative sodium dodecyl sulphate (SDS) PAGE in conjunction with electrophoretic elution of the bioactive protein zone. The 58 000-Da protein was recovered with a purification factor of 150 000 in sufficient microgram quantities to allow partial amino-terminal sequencing of the A- and B-subunit polypeptide chains, thus leading to the subsequent cloning and full nucleotide sequence analysis of the corresponding cDNA species.

The above study is representative of many other research investigations related to the isolation and characterisation of the myriad of biological substances now of interest in life science research. Importantly, this study on the purification of inhibin embodied the five basic tenents of modern purification approaches. These principles are: (1) combine separation techniques (and associated analytical methods) into rational hierarchial schemes which amplify at each stage known biological or chemical properties of the protein, such as affinity for the stationary phase, so that selectivity differences are optimised; (2) use in the early stages of fractionation simple techniques and progressively increase the level of sophistication in the resolving power; (3) work fast and minimise as much as is possible the manipulative handling of the samples; (4) keep flexibility in the purification strategy so that the unanticipated result can be capitalised upon; (5) anticipate the level of abundance and recovery of the desired protein on the basis of a rigorous assessment of the known biology of the substance. In the following sections of this review different aspects of the separation parameters involved in protein purification will be examined and recommendations made on how they can be successfully linked at either the micropreparative or the macropreparative levels into an integrated purification strategy.

2. SEPARATION PARAMETERS IN PROTEIN PURIFICATION

2.1. Bulk properties

The chemical structure and the surface topography of a peptide or a protein are the two key parameters around which most separation skills must be developed. Table 1 lists factors known to control chromatographic stability and resolution of peptides and proteins. Classical fractionation methods which manipulate either the temperature stability or solubility of a protein tend not to provide high-resolution discrimination except in very specialised cases, e.g. thermal denaturation of high-molecular-weight proteins during the recovery of low-molecular-weight bioactive peptides. Despite this low level of resolving power, exploitation of solubility product differences remains the routine method for the initial fractionation of many proteins in the research laboratory, and still forms the basis of the commercial biorecovery of most therapeutic proteins, i.e. plasma proteins, insulin from tissue extracts.

Several options are available for the manipulation of protein solubility and include techniques based on salt precipitation (ammonium sulphate, sodium sulphate, etc.), organic solvent precipitation (typically ethanol), organic polymer precipitation (polyethylene glycols or polyvinylpyrrolidone), isoelectric precip-

TABLE 1

FACTORS CONTROLLING CHROMATOGRAPHIC STABILITY OF PROTEINS

Mobile phase	Stationary phase
1. Organic solvents	1. Ligand composition
2. pH	2. Ligand density
3. Metal ions	3. Surface heterogeneity
4. Chaotropic reagents	4. Surface area
5. Oxidising or reducing reagents	5. Pore diameter distribution
6. Temperature	
7. Buffer composition	
9. Ionic strength	
10. Loading concentration	

itation by pH gradation or extraction/partitioning in aqueous–aqueous or aqueous–non-aqueous two-phase liquid–liquid systems. All of these procedures take advantage of solute hydration effects and a bulk property of the solute such as its ability to form a finite or infinite intermolecular network or aggregate under a particular set of ionic strength or solvent dielectric conditions. Although these processes clearly involve the participation of solution chemical equilibria, and hence have the potential to allow modulation of separation selectivity or zone broadening processes, the interaction of solvent molecules or ions with biopolymers has until recently been explored largely by conventional methods in terms of differences of the induced physical characteristics of the solutes. For example, the differential migration of biopolymers in gravitational or thermal fields (the basis of all centrifugation procedures, thermal denaturation methods or thermal field flow fractionation) largely reflects the physical characteristics of the solute in terms of its molecular mass, hydrodynamic volume, state of self-aggregation or association with other biopolymers. Differences in size and shape of the biopolymers, which reflect molecular mass and sequence differences, also form the basis of ultrafiltration. SDS-PAGE and gel-permeation chromatographic separations. Again, such separation procedures largely reflect a bulk property of a biopolymer in terms of its average molecular mass, average Stokes radius, etc. Although such bulk properties are often taken to imply a fixed physical property of a biopolymer, in fact all biomacromolecules undergo dynamic changes in shape, self-association and mobility in response to variations in the surrounding liquid environment. In most cases these environmental changes affect the chemical potential of the biopolymer and involve reversible solution equilibria processes. Knowledge of solute–solvent interactions and their optimisation thus represents one of the essential requirements behind the development of general strategies for biopolymer separation, based on either non-interactive or interactive chromatographic media.

2.2. Chromatographic modes

In recent years, a large amount of developmental effort has been expending in transferring knowledge gained with chemically modified soft polymeric gels such

TABLE 2

CRITERIA FOR THE SELECTION OF CHROMATOGRAPHIC MEDIA

1.	Chemical and physical stability
2.	Particle uniformity
3.	Mechanical strength and resistance to deformation
4.	Hydrophilicity and wettability
5.	Sterilisability
6.	Cost
7.	Reproducibility between batches
8.	High capacity
9.	High resolution or selectivity
10.	High mass and biological recoveries
11.	High product throughput
12.	Potential for good manufacturing practice scale-up

as the cross-linked dextrans, agaroses or acrylate copolymers into the selection and chemical modification of mechanically more robust stationary phases with narrow particle and pore size distributions. Many of the criteria listed in Table 2 apply equally to analytical separations as they do to large-scale preparative separations. Clearly in the latter case the issues of column productivity, in terms of kilograms of product resolved at a defined purity level per unit time per unit cost of operating the overall separation system, and the potential of the purification approach to satisfy good manufacturing practice, scale-up procedures, and thus to meet governmental regulatory agency guidelines, are of major importance in industrial application of protein purification methods. It has been appreciated for many years that the so-called non-interactive modes of separation do not exhibit the same level of resolution as adsorption techniques. The most successful separation techniques are those capable of probing the topography of a biopolymer and, in particular, the asymmetry of coulombic charge or hydrophobicity on the biopolymer surface through selective interaction between an immobilised ligand on the surface of the stationary phase and the biopolymer in question. A number of separation techniques are capable of resolving biopolymers on the basis of differences in net charge. These include zone electrophoresis, isotachophoresis and most importantly ion-exchange chromatography. Under conditions in which a transient or static pH condition can be generated, such that the pH at a point within the separation system corresponds to the isoelectric point, p*I*, of the protein of interest, further extensions of the net charge separation approach are found in chromatofocusing and isoelectrofocusing.

Just as ion-exchange chromatographic separations take advantage of the net charge and charge distribution on the surface of the biopolymer, hydrophobic-interaction chromatography exploits the accessibility and surface distribution of lipophilic or non-polar residues. Because of its historical origin the term hydrophobic-interaction chromatography is frequently attributed to separations affected by a decreasing salt concentration whilst the term reversed-phase chromatography has become identified with separations involving an increasing concentration of organic solvent in the eluent. However, the underlying

physicochemical basis for both these separation methods is common and is largely a result of incremental changes in the microscopic surface tension associated with the solute–solvent–stationary phase interaction [11–15].

Further examples of separation techniques which exploit the asymmetric distribution of amino residues at the surface of folded proteins, for example access to exposed histidine residues or a coordination site of a metal ion cofactor, include ligand-exchange chromatography and other various forms of chelate affinity chromatography. Similar regioselective discrimination is also observed with hydroxyapatite chromatography and with group-specific affinity chromatography such as dye affinity and borate affinity as well as other forms of ligand interactions based on generic biological ligands (i.e. biotin–avidin system, protein A–immunoglobulin G system, nicotinamide-adenine dinucleotide–oxidoreductase systems, oligosaccharide–lectin systems).

The final group of separation parameters, and the ones which give the highest selectivity, are methods which exploit functional properties of a biopolymer such as a specific ligand binding site, antigenicity or a structural element such as a lipid-binding amphiphatic non-polar domain in lipoproteins or subunit contact regions of multimeric protein complexes. With appropriate immobilisation chemistries and ligand choice, biospecific affinity chromatography and immunoaffinity chromatography both have the potential to generate separation peak capacities more than two orders of magnitude greater than observed with adsorption methods based on simple chemical ligands such as those typically employed for ion-exchange or reversed-phase chromatography. Table 3 summarises examples of the separation parameters used in protein purification and the ranges of purification factors which can be expected in typical single-stage procedures.

Proper utilisation of the specificity inherent in biological phenomena can form the basis for very elegant immunoaffinity separations with, for example, monoclonal antibodies or biospecific affinity separation with the appropriate biological ligand. The recent purification [16] of the murine transferrin receptor is illustrative of the latter case. This receptor specifically interacts with the iron-binding protein transferrin and is an integral membrane glycoprotein located on the surface of all proliferating cells. The murine transferrin receptor is a glycoprotein existing as a dimer composed of two similar or identical polypeptide chains (relative molecular weight, $M_r = 95\,000$) joined by disulphide bonds. Small quantities (200 pmol) of the murine transferrin receptor were purified to homogeneity from NS-1 myeloma cells (total protein 100 g) with a total purification factor of 200 000. The key step in the purification hinged on the changing affinity of transferrin, when depleted of iron by a pH step, for its receptor. In this sense the immobilised transferrin affinity chromatography method functioned as a retrometal chelate support. In the presence of iron (III) the immobilised transferrin bound strongly to its corresponding receptor at pH 5.0. By simply changing the pH of the eluent to 2, the iron (III) dissociated from the transferrin resulting in a conformational change in the ligand protein and the concommitant dissociation of the receptor protein from the immobilised biological ligand. Crucial to the success of this purification method was the investigators' ability to mimic with laboratory procedures what nature does continuously. The ability to exploit at different stages of

TABLE 3

SEPARATION PARAMETERS USED IN PROTEIN PURIFICATION

Parameter	Process	Typical purification factor range*
Temperature stability	Heat denaturation	2–20
Solubility	Salt precipitation	2–20
	Solvent precipitation	2–15
	Polymer precipitation	2–15
	Isoelectric precipitation	5–20
	Partitioning in aqueous two-phase system	5–20
Size and shape	Gel permeation	2–20
	Ultrafiltration	2–5
	Gel electrophoresis	2–10
Net charge	Free electrophoresis	2–5
	Zone electrophoresis	2–5
	Isotachophoresis	2–10
	Ion-exchange chromatography	2–40
Isoelectric point	Isoelectric focusing	2–40
	Chromatofocusing	2–10
Hydrophobicity	Hydrophobic-interaction chromatography	2–30
	Reversed-phase chromatography	2–200
Function	Bioaffinity chromatography	50–10 000
Antigenicity	Immunosorption (e.g. monoclonal antibodies)	20–10 000
Carbohydrate content	Lectin affinity chromatography	2–10
Content of free sulphydryl groups	Covalent chromatography	2–10
Exposed histidine	Metal chelate affinity chromatography	2–20
Exposed metal ion	Chelate affinity chromatography	2–10
Other	Hydroxyapatite chromatography	2–10
	Dye affinity chromatography	2–40

*Data compiled from refs. 52, 124, 125.

a purification procedure the known cellular biochemistry of a protein can be readily appreciated as a crucial component behind many successful biospecific affinity chromatographic purification attempts. Other recent examples, where information gained from studies on the cellular biology have proved fundamental to the purification of a very potent bioactive protein, include the heparin-binding growth factors [17–20], the insulin receptor [21,22] and the epidermal growth factor receptor [23], and a variety of peptide and protein hormones including the haemopoietic growth factors [24], epidermal growth factor [25], platelet-derived growth factor [26,27], angiogenin [28], β-transforming growth factor [29,30], insulin-like growth factor 1 [31], interleukin 2 [32], nerve growth factor [33], colony-stimulating factor 1 [34], interleukin 3 [35], colony-stimulat-

ing factor 2 [36], α-transforming growth factor [37], pro-opiomelanocortin (POMC) peptides [38], gonadotropin-releasing hormone [39], cholecystokinin [40], follicle stimulating hormone (FSH) releasing protein [41,42] and neurophysins [43].

2.3. Combination of separation parameters

Because of the inherent requirements for high resolution in biopolymer purification, it is routine to utilise combinations of all of the separation parameters listed in Table 3 at different stages in the process. Irrespective of whether the peptide or protein components of interest are to be isolated from a natural tissue source or from genetically engineered cell types, the early stages of biorecovery invariably involve a combination of cell distintegration and clarification techniques. Modest purification factors (of the order of 2–10 fold) can be achieved at this stage by appropriate choice of cell disruption, homogenization and clarification methods. In the case of recombinant proteins, expressed in prokaryotic cells often as refractile bodies, the use of low-spread centrifugation leads to a recovered precipitate considerably enriched in the protein of interest. Similarly the judicious use of appropriate detergents can greatly enhance solubilities of particular components, not only in the case of hydrophobic membrane proteins, but also for more polar proteins which, due to their low abundance, may self-aggregate or associate with other substances. Even at these initial stages of biorecovery the issue of separation selectivity is clearly important. Although such methods as cell distintegration, homogenisation, clarification by filtering aids, etc. have relatively poor discrimination power for proteins in the same molecular mass range, the important benefit of these early stages is that they are capable of removing other classes of biopolymers and macromolecules, such as lipid, polysaccharide or nucleic acid components. For the successful purification of a natural peptide or protein, the correct choice of the tissue is a crucial prerequisite. In the case of eukaryotic organisms the total number of different proteins, if all permutations associated with protein–protein macromolecular structures, phenotypic heterogeneity and protein–lipid, protein–nucleic acid or protein–lipid structures are taken into account, is probably in the range 90–100 million. In a particular tissue all of these combinations will not be present in equal abundance, due to inherent controls over cellular expression and processing. Nevertheless, isolation studies for biologically important proteins present in low abundance routinely require purification factors in excess of 10^5 before a component can be considered to be in "in near homogeneous" form. Equally challenging, of course, is the assessment of residual contaminants which in many cases require detection at levels below 100 ppb. Similar requirements for the correct choice of cell type and expression vector also apply to the early stages of biorecovery of proteins available by recombinant DNA techniques.

The subfractionation of a cell supernatant usually requires initial stages of precipitation, batch adsorption techniques and/or ultrafiltration. Phase partitioning and in particular affinity partitioning methods with immobilized ligands in an aqueous two-phase system are increasingly finding application as unit operations in the recovery of commodity proteins such as industrial enzymes. The

resolving power of these initial fractionation stages again tends to be relatively modest with a purification factor between 2 and 20 being the norm for more abundant proteins such as bulk enzymes [44–48] of bacterial or yeast origin, and some plasma proteins, e.g. albumin [49,50], of commercial interest.

2.4. Interactive stationary phases

A full knowledge of the mechanistic processes underlying biopolymer separation selectivity and the kinetics of solute transport under the separation conditions is an ideal scenario rarely attainable in practice. Much of the research effort associated with the development of new chromatographic separation media, the introduction of improved preparative electrophoretic methods, and the application of additional principles such as magnetised bed extraction or field flow fractionation, has nevertheless addressed the same questions central to the physicochemical nature of biopolymer separation selectivity and biopolymer kinetics. Particularly with adsorptive chromatographic systems the molecular dynamics associated with multi-site interaction of biopolymers with the stationary phase control not only the retention and zone broadening behaviour but also the mass and bioactivity recovery.

For several practical reasons, e.g. cost or difficulties with column regenerability, high-resolution purification methods are usually not brought into play with sub 10-μm microparticulate adsorption media of narrow particle diameter (d_p) distributions and narrow pore size (P_d) distributions until clarification is complete and partial fractionation has been carried out. Considerable activity is now under way at both academic and industrial centres, exploring different options for improving stationary phase characteristics to allow enhanced separation selectivity and improved kinetics with mesoparticulate media ($d_p > 30$ μm) in preparative separations of biopolymers. Because interactive stationary phases have the potential to probe the topography of a biopolymer and in particular surface accessible regions or binding sites unique to the protein of interest, purification strategies based on a rational mix of ion-exchange chromatography, affinity chromatography and hydrophobic-interaction chromatography represent the core methods for high-resolution separation. Exploitation of the interplay between hydrophobic and coulombic interactive phenomena forms the basis of the so-called mixed-mode chromatographic procedures. For example, under appropriately chosen eluent conditions proteins can be efficiently separated on stationary phases with immobilised coulombic ligands with hydrophobic selectivity [51,52], i.e. in order of increasing hydrophobicity under conditions of decreasing displacing salt concentration from high ionic strengths typically $\mu = 3$–5 down to $\mu = 0.5$. Similarly, under appropriately chosen solvent conditions hydrophobic supports such as *n*-alkylsilicas can be induced to exhibit polar phase selectivity with peptides and proteins eluting in order of increasing polarity [53–56]. As a consequence, retrogradients based on eluents of high organic solvent content (90%) to lower organic solvent (50%) can be used [57,58] to separate hydrophobic peptides and proteins on reversed-phase packing materials with polar phase selectivity.

2.5. Retention behaviour

The composite interplay between size exclusion phenomena, solvophobic and coulombic interaction processes is a feature of all current chromatographic stationary phases. Depending on the magnitudes of these retention dependencies retention behaviour in interactive systems can be formalised in terms of the summation of the corresponding size exclusion, hydrophobic and coulombic–polar contributions to the overall retention process. Which of these terms makes the greatest overall contributions to retention of the biopolymers depends not only on the permeability, ligand composition and ligand density of the stationary phase, but also on the mobile phase characteristics in terms of water content, pH, ionic strength, organic solvent content, the buffer composition and whether such additives as ion-pairing reagents, organic dissociating reagents or surfactants are present in the eluent.

The general form of the retention relationship in adsorption HPLC methods can be written in terms of the empirical expression

$$k' = \rho_{sec}k'_{sec} + \rho_r k'_r + \rho_c k'_c + \rho_h k'_h$$

$$k' = \rho_{sec}k'_{sec} + \rho_r k'_0 e^{-S\xi} + \rho_p k'_p e^{-D(1-\xi)}$$

where $\rho_{sec}k'_{sec}$ corresponds to the size exclusion term, $\rho_r k'_r = \rho_r k'_0 e^{-S\xi}$ to the solvophobic term and $\rho_h k'_h + \rho_c k'_c = \rho_p k'_p e^{-D(1-\xi)}$ to the polar coulombic term for different mole fraction values, ξ, of the solvent or ionic modifier (for more formal mechanistic and non-mechanistic treatments of biopolymer retention in adsorption HPLC see refs. 59–65). The coefficients S and D correspond to solute-specific parameters and are related to the slope of the plots of the logarithmic capacity factor for a particular biopolymer versus the reciprocal logarithmic concentration of organic solvent modifier in the case of reversed-phase separations, or versus reciprocal logarithmic concentration of displacing ion in the case of hydrophobic interaction and coulombic separations, whilst the k'_0 and k'_p correspond to the solute capacity factors in neat water.

Depending on the magnitude of the S, D, k'_0 and k'_p parameters a variety of solute retention versus mobile phase elutropic strengths scenarios can be calculated. Fig. 1 represents four limiting cases of such retention dependencies. Case (a) is typified by shallow log k' versus ξ (or log $-1/[c]$) dependencies with small log k' values at ξ (or $[c] = 0$) and represents a commonly observed situation with small polar peptides separated under reversed-phase or ion-exchange HPLC conditions [66–70]. Case (b) which again exhibits shallow log k' versus ξ (or log $1/[c]$) dependencies but with large values of log k'_0 is more representative of situations found with middle molecular mass but very hydrophobic peptides under some reversed-phase conditions; in affinity displacement ion exchange or substrate analogue displacement elution in affinity chromatography where the substrate analogue or displacing species is again typically of low molecular mass [71–73]. Some examples of peptide displacement chromatography correspond also to this case. Case (c) represents a typical scenario for polypeptide and globular protein purification in reversed-phase and hydrophobic-interaction tech-

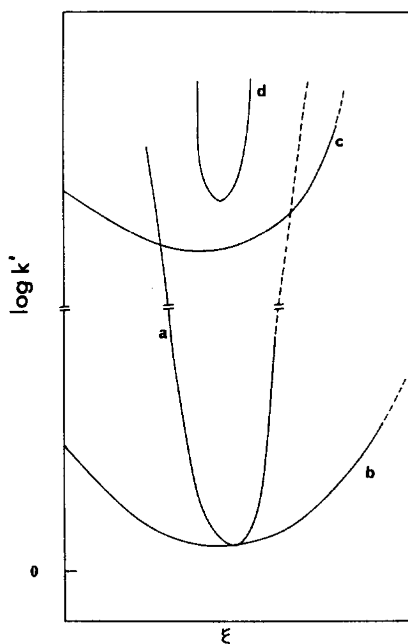


Fig. 1. Schematic representations of the retention dependencies for peptides or proteins chromatographed on mixed-mode support media. The figure illustrates four case histories for the dependency of the logarithmic capacity factor (log k') on the mole fraction, ξ (or concentration, [c]) of the displacing species. As the contact area associated with the solute–ligand interaction increases the slopes of the log k' versus ξ plots increases resulting in a narrowing of the elution window over which the solute will desorb under isocratic (and often gradient) elution conditions.

niques and with most polymer- and silica-based anion- and cation-exchange HPLC stationary phases [74–80]. From practical considerations the limiting chromatographic conditions are frequently chosen such that the minima of the plot of log k' versus ξ (or log $1/[c]$) correspond to k' values equal to or less than unity. Typically this criterion is easier to achieve in ion-exchange than reversed-phase separations. In situations associated with the purification of large globular proteins or hydrophobic proteins such retention behaviour has not been observed with reversed-phase or ion-exchange HPLC. In these cases retention dependencies approaching case (d) are experienced. From the point of view of a generalised purification strategy it is desirable to select chromatographic conditions in which the retention dependencies approximate case (a) or case (c) rather than cases (b) and (d) where clearly the affinity of the solute for the stationary phase is too high, the elution window for desportion too narrow, the solute solubility parameters of the protein too low and the mass (or bioactivity) recovery potentially impaired. However, from a selectivity point of view such situations should not necessarily be excluded out of hand. Exploitation of the potential offered by the case (b) and case (d) scenario has proved very useful for the removal of undesirable contaminants during the purification of a number of therapeutic proteins, for example the removal of trace components of Hageman factor and asso-

ciated plasminogen activator – prekallikrein proteins from therapeutic-grade human immunoglobulins based on a tandem dye-affinity – anion-exchange chromatographic method [81].

2.6. Gradient elution

Because of the pronounced dependencies of retention and zone broadening phenomena on chromatographic conditions, a behaviour which reflects the magnitude of the distribution coefficients and the complexity of the retention kinetics established between the bipolymer and the stationary phase, the most commonly adopted method for elution of biopolymers from adsorptive media involves gradient or step elution procedures. Such conditions take advantage of the severity of the log k' versus ξ (or log $1/[c]$) dependencies but do not necessarily address the important requirements of desorption kinetics and conformational dynamics of the solute. Although optimisation of chromatographic resolution with low-molecular-mass solutes is now a mature area of the separation sciences [82,83], similar endeavours with biomacromolecules are still relatively in their infancy. However, important progress has recently been made in the application of gradient elution theory which allows gradient retention data for peptides and proteins to be predicted from the corresponding retention data with the same solute in isocratic systems and vice versa [82–86]. Furthermore, it is feasible in circumstances of regular retention and recovery behaviour with for example peptides and small globular proteins to apply data derived from small-scale or analytical experiments as normalised integrals of the elution volume, column performance, etc., to the scale-up of the chromatographic bed configuration and the choice of the physical characteristics of the separation media [87–90].

2.7. Recovery of bioactivity

With low-molecular-mass solutes, the conventional approach to purification has been based on scale-up extensions of analytical column systems which allow very high resolution through optimisation of chromatographic selectivity and zone bandwidth. When similar methods are applied to proteins, their biological activity may be lost. Inherent to all bipolymer purification strategies is the question "how will the purified biopolymer be used?" If the task involves purification solely for the purposes of subsequent primary structure determination then the requirements of adequate control over bioactivity are not necessarily relevant. Obviously, in the case of a new or partially characterised protein the recovery of the component with high mass and bioactivity balance is essential.

Similarly, in preparative approaches where subsequent biological uses are contemplated it is mandatory that the design of the separation system specifically addresses the issue of recovery of bioactivity. By proper attention to the physicochemical and biological consequences of the dynamic behaviour of the solute in bulk solution and at liquid–solid interfaces the criterion of high recovery of bioactivity can usually be satisfied. Where conformational requirements impinge on a purification strategy then other data, gained from time course solution spectroscopic measurements, on-line chromatographic measurements (e.g. refs.

91–93) and from evaluation of biological/immunological activity profiles (e.g. refs. 94 and 95) in response to changes in separation variables in batch experiments are essential prerequisites.

The major challenge here is to obtain sufficient information to allow a proper understanding of the factors controlling the stability of the biopolymer structure during the chromatographic distribution process such that high mass and high bioactivity recovery can be achieved on elution. System residency effects, the nature of the binding heterogeneity associated with the overall distribution process and the participation of entropic effects associated with solute binding or permeation through the stationary phase internal surfaces are all important parameters in preparative HPLC separations if proteins are to be recovered in bioactive form. If these parameters are to be adequately included in the chromatographic optimisation process, then clearly quantitative structure–retention relationships must be developed.

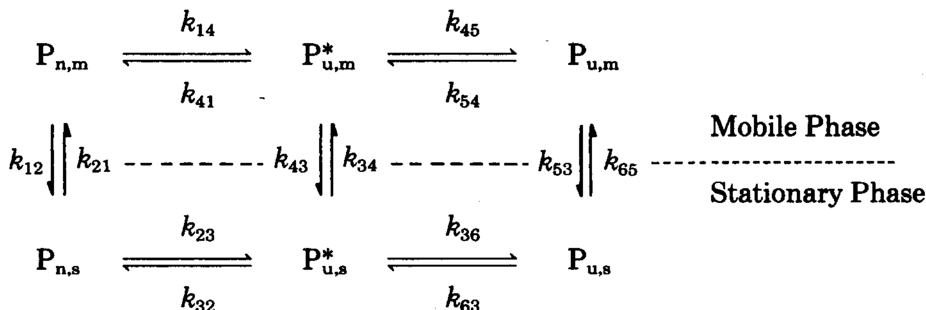
2.8. Retention mechanisms

Such mechanistic approaches based on stochastic prediction models require an extensive data base before adequate response function and factor design analyses can be carried out. Ultimate selection of the optimal chromatographic strategy will hinge very much on the ability of the response function approaches to iterate structure–function data into the overall chromatographic optimisation scheme. From a chromatographic point of view the assessment of the quality of the separation in response to a change in a chromatographic variable, such as the mobile phase composition, stationary phase particle diameter or column configuration, or alternatively a solute variable such as net charge or charge distribution, can be based on evaluation of the system peak capacity and system productivity in terms of bioactive mass throughput per unit of time. Since the peak capacity (PC) depends on both the relative selectivity and bandwidth, i.e. for a chromatographic system with an average resolution R_s of 1, peak capacity can be defined as $PC = (t_g - t_0)/4\sigma_t$, where t_g is the solute retention time, t_0 is the column dead time and σ_t the average standard deviation of the peak, optimisation of peak capacity must of necessity take into account knowledge of kinetic behaviour associated with conformational or secondary chemical equilibria mediated by the stationary phase surface or alternatively components in the mobile phase. Biopolymer conformational interconversion associated with unfolding and refolding pathways thus represents a unique set of resolution challenges, from both theoretical and experimental aspects not experienced with low-molecular-mass, conformationally rigid solutes.

Although at present the empirical recipe, for reasons of practical expediency, dominates most purification studies with peptides and proteins using HPLC or electrophoretic systems, the trend is already evident for more systematic approaches based on computer-aided analysis of retention and kinetic data in terms of different mechanistic models for biopolymer retention in high-resolution adsorption chromatography. Preliminary approaches to the classification of retention and kinetic data in terms of different mechanistic pathways have already been described [96,97] for reversed-phase and ion-exchange HPLC of a number

of enzymes and globular proteins. The ability of modern HPLC techniques to yield quantitative data on rate constants for protein folding and unfolding transitions as well as to resolve conformers with relaxation half-times ${}^{\tau}t > 10$ s has important ramifications in the selection of a purification procedure.

For example, if a protein were to undergo a two-stage interconversion in both the mobile phase and at the stationary phase surface, then a retention cycle represents a process of distribution of the protein in its native form, P_n, and two unfolded forms, P_u^* and P_u, between the two chromatographic phases can be writ,ten as shown below:



Based on such a distribution cycle, a number of different retention mechanistic pathways can be envisaged. Table 4 identifies some of these interconversion possibilities. Whether the retention mechanism of a particular biosolute can be described in terms of these or other retention models will depend on the respective rate constants for the various distribution and interconversion pathways. The impact of these kinetic processes escalates rapidly in multi-step purification procedures where the disastrous effect of low repetitive yields can result in unacceptable purification productivities. For example, if the average yield per step in a ten-step purification method was 60% (a relatively favourable situation), then the overall yield would be only 0.6%. Should the average yield per step drop to 30% due to partial denaturation, then the overall recovery reaches the disastrous value of 0.0006%, i.e. 1000 times as much raw material would have to be processed to yield the same mass of purified protein.

2.9. Multi-zoning

As is evident from Table 1 a large variety of mobile phase and stationary phase factors can influence the chromatographic stability and recovery of proteins. The effect of most of the mobile phase characteristics, such as the nature and concentration of organic solvent or ionic additives, the temperature or the pH can be ascertained very readily from batch test tube pilot experiments. However, more subtle mobile phase effects, such as the influence of loading concentration on the stability of the protein or the influence of other protein components at the solid–liquid interface, are much harder to assess. Similarly, the influence of many stationary phase variables, such as ligand composition, ligand density, surface heterogeneity, surface area and pore diameter distribution, can be ascertained

TABLE 4

CLASSIFICATION OF SEVERAL DIFFERENT RETENTION PATHWAYS IN BIOPOLYMER ADSORPTION CHROMATOGRAPHY

This classification is based on the $P_n > P_u^* > P_u$ interconversion. More complexed branched interconversions can be similarly described using the notation $(\text{isobi})_{br}$. The classification of the pathways when the solute, in its various conformational forms, binds to the same class of ligand can be noted as an 'ordered' pathway. When binding of the solute to a heterogeneous stationary phase surface occurs the notation of a 'random' pathway is used. In this manner, an N-dimensional retention network can be identified for the different conformational/secondary equilibrium processes which proteins and other biopolymers undergo at adsorptive interfaces.

1. Uni-uni pathway: typified by simple distribution process and kinetics, narrow bandwidth with high mass and biological recovery; may be characterised by very rapid interconversion kinetics as the solute transverses the chromatographic bed as a single, averaged structure.

2. Isouni-uni pathway: typified by solvent induced solute unfolding-refolding phenomena in the mobile phase with both solute species binding to the stationary phase with the same distribution coefficient; single elution zone with high mass recovery possible but time-dependent loss of biological activity in the mobile phase evident.

3. Uni-isouni pathway: typified by ligand induced solute unfolding-refolding phenomena at the stationary phase surface; single elution zone but time-dependent loss of mass and bioactivity possible when $k_{23} > k_{32}$.

4. Isouni-isouni pathway: typified by solvent and ligand induced solute unfolding-refolding phenomena in both phases; may be characterised by impaired mass recovery of two species, i.e. native and non-native, with time-dependent loss of bioactivity when k_{14}, $k_{23} \gg k_{41}$, k_{32}.

5. Isobi-uni pathway: typified by solvent induced, time-dependent biphasic unfolding-refolding of solute in the mobile phase but stabilisation of structure by the ligand surface if $k_{12} > k_{14}$; charcterised by high mass recovery, elution of a single zone with apparent half-life for loss of bioactivity larger than in the mobile phase alone.

6. Uni-isobi pathway: typified by ligand-induced, time-dependent biphasic unfolding-refolding of the solute at the stationary phase surface with further destabilisation of structure; characterised by time-dependent loss of mass and activity.

7. Isobi-uni pathway: typified by mobile induced and ligand induced biphasic unfolding-refolding of the solute: characterised by the time dependent loss of mass and bioactivity with the emergence of a second, often latter eluting, inactive zone of non-active solute if k_{12}, $k_{43} \gg k_{21}$, k_{34}.

from small-scale batch experiments. However, it is clear that the behaviour of many proteins in such static batch systems can vary significantly from that observed in dynamic systems as usually employed in column chromatography. This behaviour is not only related to issues of different accessibility of the biosolute to the stationary phase surface area and hence different loading capacities but also involves the complex relationships between diffusion kinetics and adsorption kinetics in the overall mass transport phenomenon. Conformational

reordering of a protein structure can occur in both the mobile phase and stationary phase and leads to multi-zoning of a component into active and/or inactive zones. Other phenomena, however, can also lead to multi-zoning of a biopolymer with adsorptive chromatographic stationary phases. Probably the easiest of these phenomena to remedy is the so-called split peak breakthrough effect, very often seen in bioaffinity chromatography [98,100] and to lesser extents in ion-exchange and hydrophobic-interaction chromatography [89,93]. This effect is manifested as a non-retained (or weakly retained) peak and a retained peak with the bound/free ratio dependent on the solute's diffusion kinetics and adsorption kinetics. The amount of protein in the breakthrough zone is influenced by the flow-rate, stationary phase nominal pore diameter and ligand density, and the injection volume. This effect can be circumvented by the choice of a lower flow-rate, the selection of stationary phases with better surface area–ligand accessibility characteristics for the particular protein of interest and more appropriate loading volumes and concentrations. A second type of multi-zoning phenomena is associated with non-linear isotherm behaviour due to matrix heterogeneity and non-uniformity of the ligand distribution over the stationary phase surface. This effect is very noticeable between virgin and conditioned columns and is problematic during the first few cycles of use of a particular column at preparative loadings. At the micropreparative level this effect can lead to catastrophic results where irreproducible recoveries may occur. Other forms of multi-zoning, associated with slow equilibria between the monomeric form and higher oligomeric forms of the protein, also are known to affect resolution and recovery.

2.10. Resolution optimization

Since multiple chromatographic steps are the norm in protein purification strategies, the stage at which a particular chromatographic selectivity mode is employed requires careful planning. Previous experience with the systematic optimisation of resolution for low-molecular-mass solutes based on the solvent selectivity triangle concept can be used as a basis for multi-step chromatographic optimisation involved with the purification of peptides and proteins. Resolution contour plots for each of the peak zones can be obtained from the experimental data obtained with different binary/ternary mobile phase combinations under either isocratic or gradient elution conditions. By integrating this information with data on bioactive contour profiles, derived for example from on-line manipulation of spectroscopic data accumulated with multi-channel or photodiode array spectrometers such as second derivative spectra [91–93,101], it is feasible to explore a variety of separation variables with single-column or multi-column systems. Importantly, these approaches offer considerable potential for the optimisation of resolution of very complex mixtures of proteins using the same stationary phase operating under different elution conditions. Such methods have been widely used as multi-dimensional techniques in reversed-phase HPLC of peptides and proteins for a number of years [66,67]. Integral to this approach has been the application of mobile phases of different composition, notably different ion-pairing systems.

Similar procedures are equally pertinent to ion-exchange HPLC separations with ions of different solvated radius and electronegativity [51,79]. For example, in a recent study [94] the separation and purification of the isohormones of lutropin, follicotropin and thyrotropin was based on the sequential use of size-exclusion chromatography, preparative isoelectrofocusing and anion exchange, exploiting the selectivity advantages of the latter step with ions of different displacing characteristics. Two observations of general applicability to protein fractionation by ion-exchange HPLC are evident in this study. Firstly, by employing ion-exchange supports after a preparative isoelectrofocusing stage protein components with the same p*I* value can be resolved by taking advantage of the Donnan effect on ionisation equilibria within the micro-environment of the stationary phase and the ability of the coulombic ligand to act as a molecular probe for the asymmetric distribution of charge on the protein surface. Secondly, by utilising mobile phases of different ion compositions multidimensional separation strategies can be readily automated and efficiently carried out with tandem columns packed with the same ion exchanger. The potential for resolution optimisation exploiting ions of different electronegativity and solvation stage along the Hoffmeister series has been utilised extensively in salting-in and salting-out phenomena with biopolymers. The availability of rapid, high-resolution ion exchangers and hydrophobic-interaction media will lead to further development of this potential into much more predictive capabilities for purification of specific proteins in complex mixtures.

2.11. Positive–negative affinity mode

One avenue of current research which offers considerable versatility with tandem columns is the so-called positive–negative affinity mode. In this strategy, the chemical characteristics of the immobilised ligand are so selected that components of interest are either absorbed or not absorbed by sequential or tandem columns [96,102]. Such approaches are particularly suited to group-specific affinity, metal chelate systems and also form the basis of the immobilised dye affinity approaches. Typical of this approach has been the integration of dye affinity and ion-exchange methods as positive–negative modes into an automated protocol for the preparation of immunoglobulins from Cohn fractions, which has lead to the development of general strategies for the purification of other plasma proteins [81].

2.12. Batch methods

With batch adsorption methods the effective peak capacity may be only between 2 and 10 per stage. Intercalation of batch methods with gradient elution techniques, where peak capacities in excess of 200 can be realised, has many desirable features for biopolymer fractionation. Tandem batch methods when used at an early stage of a purification scheme frequently improve overall recoveries. Because greatly decreased protein masses are loaded onto chromatographic beds following batch fractionation, their use allows substantially higher peak capacities to be

achieved at subsequent chromatographic stages. Batch methods are readily incorporated into a purification strategy using both chemically modified silica-based and polymer-based media. Such a batch approach has recently been employed in the purification of amino-terminal truncated variants of the basic fibroblast growth factor where a three-stage method based on cation exchange, dye-ligand affinity and heparin affinity fractionation was employed [17] with a minimum of handling procedures and without the necessity of concentration or desalting prior to the next fractionation stage. Similar tandem strategies based on the sequential use of different immobilised dye ligands have been applied for the purification of nucleotide-dependent oxido-reductases, phosphokinases and glycolytic enzymes [102–108]. In the case of the dye affinity systems the participation of multiple retention mechanisms is again evident, with the features of both cation exchange and hydrophobic interaction particularly noticeable under neutral or weakly acidic pH conditions. For the purification of hormonal peptides from endocrine tissues a number of tandem column approaches have been proposed based on cartridges or columns packed with chemically modified silicas [38,54,58,66,67,97].

2.13. Mixed-bed separation

Whether a particular stationary phase functions in a single elution mode is clearly not an essential requirement for its successful application in high-resolution purification of biomacromolecules. The logical extension of mixed-mode interactions is, of course, biospecific chromatography and its immunological counterpart, immunoaffinity chromatography, where the composite interplay of coulombic, hydrogen-bonding and hydrophobic forces determines the magnitude and nature of the association and dissociation phenomena. As currently manufactured all micro- and mesoparticulate chromatographic media, irrespective of the nature of the ligand or the chemical functionality of the matrix, exhibit separation features characterised by these composite phenomena. Exploitation of secondary retention capabilities of a stationary phase should not thus be discounted out of hand since it may potentially provide the solution to difficult separation tasks. For example, mixed chromatographic beds containing hydrophobic-interaction and ion-exchange media have been successfully used [109–112] for the purification of a variety of proteins under conditions where the relative selectivity was significantly different from that observed with a single type of stationary phase. Extensions of this mixed-bed approach underly the recent further development of multimodal or mixed-ligand separation media and salt-promoted adsorption media [112–118]. In the case of hydrophobic adsorption phenomena, the salt-mediated changes in retention are largely entropically driven with changes in the associated water structure or bound ions providing a mechanism to either stabilise or destabilise the three-dimensional structures of proteins. Although there is useful information at hand on the effects of different salt species on protein conformation in bulk solution [119–121] as noted before, the systematic extension of these studies to adsorption chromatography with micro- and mesoparticulate HPLC media requires substantial investigation before it reaches a similar level of predictive maturity. For example, the salting-in or salting-out

behaviour of chaotropic salts can be quantitatively described in terms of the empirical Setchenow equation such that,

$$\log(\text{solubility}) = C - S^* \times (\text{concentration of salt})$$

where S^* is the so-called Setchenow constant, which is a characteristic of the salt and protein in question, whilst C is a system constant. Although the form of the empirical Setchenow equation is remarkably similar to the empirical retention equation used to describe reversed-phase, hydrophobic-interaction and ion-exchange chromatography, namely $\log k' = \log k'_0 - S\xi$, the demonstration of a direct physical relationship in terms of mechanistic pathways between precipitation and adsorption parameters S^* and S has yet to be firmly made. Such studies will certainly be a fruitful avenue of research over the next several years because of their impact on preparative biopolymer separation by chromatographic methods.

3. CONCLUSION

Since a purification strategy for a protein is predicted by the ease or difficulty of resolving to a predefined level of purity the desired component from other substances, many of which may exhibit similar separation selectivities at the different fractionation stages and may be present in different abundance levels, for high-resolution purification procedures to be carried out it is self evident that rapid, multi-stage high-recovery methods must be utilised. In order to minimise losses and improve productivity, on-line evaluation of each of the recovery stages is an important objective if the overall optimisation and automation of the individual unit operations is to be achieved. For these steps to be properly integrated detailed assessment of the fractionation data using computer-aided methods for factor analysis are required at each stage to monitor the separation selectivity and biorecovery contours of the purification procedure. The development of new generations of on-line detectors capable of monitoring these structure–function–retention characteristics of biopolymers represents a pressing challenge for spectroscopists. In preparative chromatography, improved methods to evaluate the relationship between mass loadability to productivity, i.e. the product throughput in terms of grams (or kilograms) of bioactive protein per cycle per currency unit are also relevant. The early recognition of unacceptable conditional effects associated with time-dependent denaturation or degradation of the protein in question or with secondary equilibria such as protein aggregation also will have an important impact on the outcome of a purification approach. Although non-specific proteolytic degradation can be minimised by the use of generic inhibitors for serine or thiol proteases, such procedures may not achieve the inhibition of much more specific degradative enzymes present in trace amounts and packaged in co-purifying cell debris, lipid membrane micelles or adsorptively bound to the protein. Careful inspection of the analytical data generated during the purification strategy, e.g. SDS-PAGE or analytical HPLC data, can, however, give early warning of such difficulties and suggest likely remedies. For example, the use of a multiple "tea bag" affinity chromatographic approach for the removal

of cell proteases, anti-toxins, nucleic acid fragments or particles can be particularly effective at the initial stages of a purification procedure.

A routine approach to preparative scale-up adopted at this and other laboratories has been to optimise separation selectivity with a particular type of stationary phase under conditions which are known not to lead to significant loss of bioactivity and then progressively increase the sample load until a change in relative retention of approximately 5% occurs. A totally different approach to selectivity optimisation is deliberately to introduce into the protein structure a unique purification handle. Such methods form the basis of most substrate bioaffinity chromatographic procedures including bioaffinity elution in the ion-exchange mode. A recent development which has arisen as a logical extension of recombinant DNA technology is to introduce into the protein of interest a fusion peptide which exhibits a unique binding characteristic for a defined type of stationary phase. The concept behind this approach is relatively simple. Certain amino acids by virtue of their intrinsic hydrophobicity or polarity, when clustered together provide a direct binding region or handle for interaction of the fusion protein with either a hydrophobic or coulombic ligand immobilized onto a polymeric matrix. The recently described polyarginine insert [122] into urogastrone is representative of this approach. The incorporation of such fusion handles into a biosynthetic protein changes its p*I*, polarity and solubility properties such that it can be more readily resolved on reversed-phase or ion-exchange media from other protein components.

If the amino acid sequence corresponding to the fusion cluster represents a portion of the binding domain of another functional protein, e.g. the protein A subunit domain, then the specificity of this biomimetic handle can be exploited by interaction with its complimentary bio-specific ligand, e.g. IgG, immobilised as a biospecific chromatographic support. The recent application [123] of this approach with a protein A domain fusion and generic IgG immunoaffinity columns in the production of several biosynthetic proteins including insulin-like growth factors has documented their feasibility.

Although conceptually attractive the fusion synthesis approach is not without some difficulties. For example, the fusion handle must subsequently be removed chemically, for example by cyanogen bromide cleavage, or enzymatically with carboxypeptidases or with specific endopeptidases. The prospect of success for these cleavage processes will depend markedly on the primary amino acid sequence of the protein in question. With further work on the development of reagents which allow more selective cleavage of proteins at a unique processing site, and the use of cell lines with improved stability and product secretory capabilities the use of the fusion purification approach should find wide application, particularly with therapeutic products where high stringency requirements apply to their purity. These and other challenges in the high-resolution separation of biopolymers will certainly be met over the next decade if the current level of research activity in the field of biochemical separation is a reliable indicator of future developments.

4. ACKNOWLEDGEMENTS

The support of the National Health and Medical Research Council of Australia, the Australian University Grants Commission and Monash University Special Research Grants are gratefully acknowledged.

5. SUMMARY

General fractionation strategies for the high-resolution purification of proteins are described. The impact of different separation parameters and resolution optimisation approaches with tandem-based systems on retention and recovery behaviour is reviewed. Procedures for the successful linkage of different chromatographic steps into a preferred sequence of operations are discussed in terms of the underlying principles and modus operandi of high-performanc liquid chromatographic purification of proteins and related biomacromolecules.

REFERENCES

1 I.B. Holbrook and A.G. Laver, Anal. Biochem., 75 (1976) 634.
2 C.R. Merril, D. Goldman and M.L. van Keuren, Electrophoresis, 3 (1982) 17.
3 G. Berson, Anal. Biochem., 134 (1983) 230.
4 D.C. Ocks, E.H.M. Conkey and D.W. Sammons, Electrophoresis, 2 (1981) 304.
5 E.W. Willoughby and A. Lambert, Anal. Biochem., 130 (1983) 353.
6 G. Bers and D. Garfin, Biotechniques, 3 (1985) 276.
7 V.C.W. Jsang, G.E. Bers and K. Hancock, in T.T. Ngo and H.M. Lenhoff (Editors), Enzyme-Mediated Immunoassay, Plenum Press, New York, 1986, p. 265.
8 R.G. Forage, J.M. Ring, R.W. Brown, B.V. McInerney, G.S. Cobon, R.P. Gregson, D.M. Robertson, F.J. Morgan, R.E.H. Wettenhall, J.K. Findlay, H.G. Burger, M.T.W. Hearn and D.M. de Kretser, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 3091.
9 D.M. Robertson, M.L. Foulds, L. Leversha, F.J. Morgan, M.T.W. Hearn, H.G. Burger, R.E.H. Wettenhall and D.M. de Kretser, Biochem. Biophys. Res. Commun., 126 (1985) 220.
10 L.J. Leversha, D.M. Robertson, F.L. de Vos, F.J. Morgan, M.T.W. Hearn, R.E.H. Wettenhall, J.K. Findlay, H.G. Burger and D.M. de Kretser, J. Endocrinol., 13 (1987) 1.
11 W.R. Melander and Cs. Horváth, Arch. Biochem. Biophys., 183 (1977) 393.
12 W.R. Melander, D. Corradini and Cs. Horváth, J. Chromatogr., 317 (1984) 67.
13 M.T.W. Hearn and B. Grego, J. Chromatogr., 203 (1981) 349.
14 J.L. Fausnaugh and F.E. Regnier, J. Chromatogr., 359 (1986) 131.
15 O. Sinanoglu, in B. Pullman (Editor), Molecular Associations in Biology, Academic Press, New York, 1968, pp 427–445.
16 I.R. van Driel, P.A. Stearne, B. Grego, R.J. Simpson and J.W. Goding, J. Immunol., 133 (1984) 3220.
17 J. Bertolini and M.T.W. Hearn, Mol. Cell. Endocr., in press.
18 M.T.W. Hearn, M. Guthridge and J. Bertolini, J. Chromatogr., 397 (1987) 371.
19 F. Ensch, A. Baird, N. Ling, N. Meno and D. Gospodarowicz, Proc. Natl. Acad. Sci., U.S.A., 82 (1985) 6057.
20 Y. Shing, J. Feldman, R. Sullivan, C. Butterfield, J. Murray and M. Klagsbrun, Science (Washington, D.C.), 223 (1984) 1296.
21 A. Ullrich, J.R. Bell, E.Y. Chen, R. Herrara, L.M. Petruzzelli, R.J. Dull, A. Gray, L. Coussens, Y.C. Liao, M. Tsubokawa, A. Mason, P.H. Seeburg, C. Grunfeld, O.M. Rosen and J. Ramachandran, Nature (London), 313 (1985) 756.
22 P.F. Pilch and M.P. Czech, J. Biol. Chem., 255 (1980) 1722.

23 J. Downward, Y. Yarden, E. Mayes, J.G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schessinger and M.D. Waterfield, Nature (London), 307 (1984) 521.
24 A.D. Whetton and T.M. Dexter, Trends Biochem. Sci., 11 (1986) 207.
25 M. Das, Rev. Cytol, 78 (1982) 233.
26 M.D. Waterfield, G.T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.H. Heldin, J.S. Huang, and T.F. Deuel, Nature (London), 304 (1983) 35.
27 C.N. Chesterman, T. Walker, B. Grego, K. Chamberlain, M.T.W. Hearn and F.J. Morgan, Biochem. Biophys. Res. Commun., 116 (1983) 809.
28 R. Shapiro, J.F. Riordan and B.L. Vallee, Biochemistry, 25 (1986) 3527.
29 A.B. Roberts, M.A. Anzana, C.A. Meyers, J. Wideman, R. Blacher, V.C. Pan, S. Stein, S.R. Lehrman, L.C. Smith, L.C. Lamb and M. Sporn, Biochemistry, 22 (1983) 5692.
30 C.A. Frolik, L.L. Dart, C.A. Meyers, D.M. Smith and M.B. Sporn, Proc. Natl. Acad. Sci. U.S.A., 80 (1983) 3676.
31 E. Rinderknecht and R.E. Humbel, J. Biol. Chem., 253 (1978) 2769.
32 R.J. Robb, R.M. Kutny and V. Chowdry, Proc. Natl. Acad. Sci. U.S.A., 80 (1983) 5990.
33 R. James and R.A. Bradshaw, Ann. Rev. Biochem., 53 (1984) 259.
34 N.A. Nicola, D. Metcalf, M. Matsumoto and G.R. Johnston, J. Biol. Chem., 258 (1983) 9017.
35 I. Clark-Lewis and J.W. Schrader, J. Immunol., 127 (1981) 1941.
36 D. Metcalf, Science (Washington, D.C.), 229 (1985) 16.
37 R. Derynck, J. Cell Biochem., 32 (1986) 293.
38 H.P.J. Bennett, J. Chromatogr., 359 (1986) 383.
39 P.H. Seeburg and J.P. Adelman, Nature (London), 311 (1984) 666.
40 J.R. Reeve, V. Eysselein, J.H. Walsh, C.M. Ben-Avram and J.E. Shively, J. Biol. Chem., 261 (1986) 16392.
41 W. Vale, J. Rivier, J. Vaughan, R. McClintock, A. Corrigan, W. Woo, D. Karr and J. Spiess, Nature (London), 321 (1986) 776.
42 D.M. Robertson and M.T.W. Hearn, Mol. Cell. Endocrinol., in press.
43 T. Kanmera and I.M. Chaiken, J. Biol. Chem., 260 (1985) 8474.
44 G. Koperschläger, G. Lorenz and E. Usbeck, J. Chromatogr., 259 (1983) 97.
45 M.R. Kula, G. Johansson and A.F. Buckmann, Biochem. Soc. Trans., 7 (1979) 1.
46 A.F. Buckmann, M. Morr and G. Johansson, Makromol. Chem., 182 (1981) 1379.
47 B. Mattiasson and T.G.I. Ling, J. Chromatogr., 376 (1986) 235.
48 G. Johansson, G. Kopperschläger and P.A. Albertsson, Eur. J. Biochem., 131 (1983) 589.
49 Y.L. Hao, K.G. Ingham and M. Wicherhauser, in J.M. Curling (Editor), Methods of Protein Fractionation, Academic Press, New York, 1980, p. 57.
50 G. Kopperschläger and G. Birkenmeier, J. Chromatogr., 376 (1986) 141.
51 M.T.W. Hearn and A.N. Hodder, Chromatographia, submitted for publication.
52 M.T.W. Hearn in J.C. Janson (Editor), High Resolution Protein Purification, VCH, Deerfield Beach, FL, in press.
53 M.T.W. Hearn and B. Grego, J. Chromatogr., 218 (1981) 497.
54 M.T.W. Hearn, Adv. Chromatogr., 20 (1982) 1.
55 M.T.W. Hearn and B. Grego, J. Chromatogr., 255 (1983) 125.
56 M.T.W. Hearn and B. Grego, J. Chromatogr., 266 (1983) 75.
57 B. Grego, E.C. Nice and R.J. Simpson, submitted for publication.
58 M.T.W. Hearn and M.I. Aguilar, Proc. Aust. Biochem. Soc., 18 (1986) 93.
59 L.R. Snyder, in Cs. Horváth (Editor), High Performance Liquid Chromatography - Advances and Perspectives. Vol. 1, Academic Press, New York, 1980, p. 208.
60 M.A. Stadalius, M.A. Quarry and L.R. Snyder, J. Chromatogr., 327 (1985) 93.
61 M.T.W. Hearn, A.N. Hodder and M.-I. Aguilar, J. Chromatogr., 327 (1985) 47.
62 R.R. Drager and F.E. Regnier, J. Chromatogr., 359 (1986) 147.
63 X. Geng and F.E. Regnier, J. Chromatogr., 296 (1984) 15.
64 M.T.W. Hearn and M.I. Aguilar, J. Chromatogr., 397 (1987) 47.
65 S.-L. Wu, A. Figueroa and B.L. Karger, J. Chromatogr., 371 (1986) 3.
66 M.T.W. Hearn, in Cs. Horváth (Editor), High Performance Liquid Chromatography - Advances and Perspectives, Academic Press, New York, Vol. 3, 1983, p. 87.

67 M.T.W. Hearn (Editor), Ion-Pair Chromatography, Marcel Dekker, New York, 1985, pp. 1–300.
68 M.J. O'Hare and E.C. Nice, J. Chromatogr., 171 (1979) 209.
69 I. Molnár and Cs. Horváth, J. Chromatogr., 142 (1977) 623.
70 M.I. Aguilar, A.N. Hodder and M.T.W. Hearn, J. Chromatogr., 327 (1985) 115.
71 R.W. Stout, S.I. Sivakoff, R.D. Ricker and L.R. Snyder, J. Chromatogr., 353 (1986) 439.
72 W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, J. Chromatogr., 266 (1983) 3.
73 K.M. Gooding and M.N. Schmuck, J. Chromatogr., 296 (1984) 321.
74 K. Benedek, S. Dong and B.L. Karger, J. Chromatogr., 317 (1984) 227.
75 L.A. Kennedy, W. Kopaciewicz and F.E. Regnier, J. Chromatogr., 359 (1986) 73.
76 R. Janzen, K.K. Unger, H. Gieshe, J.N. Kinkel and M.T.W. Hearn, J. Chromatogr., 397 (1987) 91.
77 S.Y.M. Lau, A.K. Taneja and R.S. Hodges, J. Chromatogr., 317 (1984) 129.
78 J.L. Glajch, M.A. Quarry, J.F. Vasta and L.R. Snyder, Anal. Chem., 58 (1986) 280.
79 M.T.W. Hearn and A.N. Hodder, Biochemistry, submitted for publication.
80 C.T. Mant and R.S. Hodges, J. Chromatogr., 327 (1985) 147.
81 M.T.W. Hearn, P. Marley and J.R. Davies, Int. Pat. PCT/AV 86-200118.
82 P.J. Schoenmakers, Optimisation of Chromatographic Selectivity, Elsevier, Amsterdam, 1986.
83 J.J. Kirkland and J.L. Glajch, J. Chromatogr., 255 (1983) 27.
84 M.A. Stadalius, H.S. Gold and L.R. Snyder, J. Chromatogr., 327 (1985) 27.
85 M.A. Quarry, R.L. Grob and L.R. Snyder, J. Chromatogr., 285 (1984) 1.
86 M.T.W. Hearn and M.I. Aguilar, J. Chromatogr., in press.
87 J.E. Eble, R.L. Grob, P.E. Antle and L.R. Snyder, J. Chromatogr., 384 (1987) 25.
88 H. Poppe and J. Kraak, J. Chromatogr., 255 (1983) 395.
89 F.L. de Vos, D.M. Robertson and M.T.W. Hearn, J. Chromatogr., 392 (1987) 17.
90 J. Frenz and Cs. Horváth, AIChE J., 31 (1985) 400.
91 G.E. Katzenstein, S.A. Vrona, R.J. Wechsler, B.L. Steadman, R.V. Lewis and C.R. Middaugh, Proc. Natl. Acad. Sci., U.S.A., 83 (1986) 4268.
92 B. Grego, E.C. Nice and R.J. Simpson, J. Chromatogr., 352 (1986) 359.
93 M.T.W. Hearn and M.I. Aguilar, submitted for publication.
94 R.C. Johnston, P.G. Stanton, D.M. Robertson and M.T.W. Hearn, J. Chromatogr., 397 (1987) 389.
95 R. Janzen, K.K. Unger, H. Giesche, J.N. Kinkel and M.T.W. Hearn, J. Chromatogr., 397 (1987) 81.
96 M.T.W. Hearn, in C.J. King and J.D. Navratil (Editors), Chemical Separations, Litarvan Press, Arvada, CO, 1986, pp. 77–98.
97 D.B. Wetlaufer and M.R. Koenigbauer, J. Chromatogr., 359 (1986) 55.
98 D.S. Hage, R.R. Walters and H.W. Heathcote, Anal. Chem., 58 (1986) 274.
99 M.T.W. Hearn, J. Chromatogr., 376 (1986) 245.
100 P.J. Hogg and D.J. Winzor, Arch. Biochem. Biophys., 240 (1985) 70.
101 S.-L. Wu, K. Benedek and B.L. Karger, J. Chromatogr., 359 (1986) 3.
102 R.K. Scopes, J. Chromatogr., 376 (1986) 131.
103 S.K. Roy and A.H. Nishikawa, Biotechnol. Bioeng., 21 (1979) 775.
104 C.R. Lowe, S.J. Burton, J.C. Pearson, Y.D. Clonis and V. Stead, J. Chromatogr., 376 (1986) 121.
105 K.D. Kulbe and R. Schuer, Anal. Biochem., 93 (1979) 46.
106 M.D. Scawen, P.M. Hammond, M.J. Corner and A. Atkinson, Anal. Biochem., 132 (1983) 413.
107 A. Cordes and M.-R. Kula, J. Chromatogr., 376 (1986) 375.
108 C.R. Lowe and J.C. Pearson, Methods Enzymol., 104 (1984) 97.
109 Z. El Rassi and Cs. Horváth, J. Chromatogr., 359 (1986) 255.
110 J. Porath, B. Olin and B. Granstand, Arch. Biochem. Biophys., 255 (1983) 543.
111 E. Bollin and E. Sulkowski, J. Gen. Virol., 52 (1981) 227.
112 M.T.W. Hearn and J.R. Davies, in preparation.
113 L.A. Kennedy, W. Kopaciewicz and F.E. Regnier, J. Chromatogr., 359 (1986) 73.
114 A.J. Alpert, J. Chromatogr., 359 (1986) 85.
115 Z. El Rassi and Cs. Horváth, Chromatographia, 19 (1984) 9.

116 J.-P. Chang, Z. El Rassi and Cs. Horváth, J. Chromatogr., 319 (1985) 396.
117 F. Maisano, M. Belew and J. Porath, J. Chromatogr., 321 (1985) 305.
118 J. Porath, F. Maisano and M. Belew, FEBS Lett., 185 (1985) 306.
119 J. Porath, J. Chromatogr., 376 (1986) 331.
120 R. Arakawa and S.N. Timasheff, Biochemistry, 21 (1982) 6545.
121 T. Arakawa and S.N. Timasheff, Biochemistry, 23 (1984) 5912.
122 J.C. Smith and S. Brewer, Gene, 32 (1984) 321.
123 B. Nilsson, L. Abrahmsen and M. Vhlen, EMBO J., 4 (1985) 1075.
124 M.T.W. Hearn, in J.A. Asenjo (Editor), Downstream Processing and Biotechnology, Marcel Dekker, New York, in press.
125 J. Bonnerjean, S. Oh, M. Hoare and P. Dunhill, Biotechnology, 4 (1986) 954.