# Reversed Phase Chromatography

Principles and Methods

Amersham
Biosciences

Reversed Phase
Chromatography

Contents

Chapter 1

Introduction

Adsorption chromatography depends on the chemical interactions between solute molecules and specifically designed ligands chemically grafted to a chromatography matrix. Over the years, many different types of ligands have been immobilised to chromatography supports for biomolecule purification, exploiting a variety of biochemical properties ranging from electronic charge to biological affinity. An important addition to the range of adsorption techniques for preparative chromatography of biomolecules has been reversed phase chromatography in which the binding of mobile phase solute to an immobilised n-alkyl hydrocarbon or aromatic ligand occurs via hydrophobic interaction.

Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography with excellent recovery and resolution. In addition, the use of ion pairing modifiers in the mobile phase allows reversed phase chromatography of charged solutes such as fully deprotected oligonucleotides and hydrophilic peptides. Preparative reversed phase chromatography has found applications ranging from micropurification of protein fragments for sequencing (1) to process scale purification of recombinant protein products (2).

This handbook is intended to serve as an introduction to the principles and applications of reversed phase chromatography of biomolecules and as a practical guide to the reversed phase chromatographic media available from Amersham Biosciences. Among the topics included are an introductory chapter on the mechanism of reversed phase chromatography followed by chapters on product descriptions, applications, media handling techniques and ordering information. The scope of the information contained in this handbook will be limited to preparative reversed phase chromatography dealing specifically with the purification of proteins, peptides and nucleic acids.

Theory of reversed phase chromatography

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phase. The actual nature of the hydrophobic binding interaction itself is a matter of heated debate (3) but the conventional wisdom assumes the binding interaction to be the result of a favourable entropy effect. The initial mobile phase binding conditions used in reversed phase chromatography are primarily aqueous which indicates a high degree of organised water structure surrounding both the solute molecule and the immobilised ligand. As solute binds to the immobilised hydrophobic ligand, the hydrophobic area exposed to the solvent is minimised. Therefore, the degree of organised water structure is diminished with a corresponding favourable increase in system entropy. In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e. solute and ligand, to associate (4).
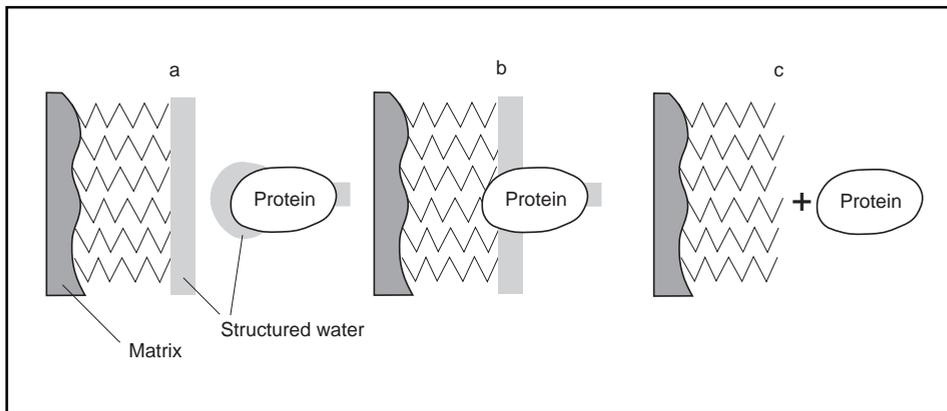
Fig. 1. Interaction of a solute with a typical reversed phase medium. Water adjacent to hydrophobic regions is postulated to be more highly ordered than the bulk water. Part of this 'structured' water is displaced when the hydrophobic regions interact leading to an increase in the overall entropy of the system.

Reversed phase chromatography is an adsorptive process by experimental design, which relies on a partitioning mechanism to effect separation. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase. Initially, experimental conditions are designed to favour adsorption of the solute from the mobile phase to the stationary phase. Subsequently, the mobile phase composition is modified to favour desorption of the solute from the stationary phase back into the mobile phase. In this case, adsorption is considered the extreme equilibrium state where the distribution of solute molecules is essentially 100% in the stationary phase. Conversely, desorption is an extreme equilibrium state where the solute is essentially 100% distributed in the mobile phase.
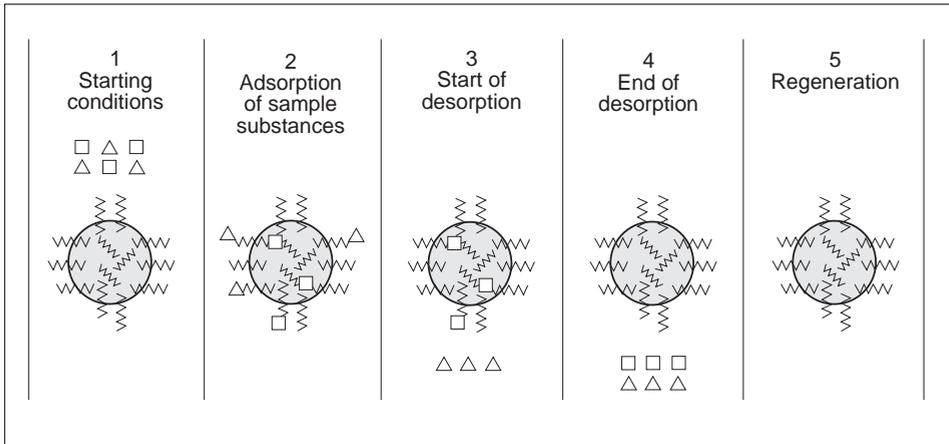
Fig. 2. Principle of reversed phase chromatography with gradient elution.

Reversed phase chromatography of biomolecules generally uses gradient elution instead of isocratic elution. While biomolecules strongly adsorb to the surface of a reversed phase matrix under aqueous conditions, they desorb from the matrix within a very narrow window of organic modifier concentration. Along with these high molecular weight biomolecules with their unique adsorption properties, the typical biological sample usually contains a broad mixture of biomolecules with a correspondingly diverse range of adsorption affinities. The only practical method for reversed phase separation of complex biological samples, therefore, is gradient elution (5).

In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. The majority of reversed phase separation experiments are performed in several fundamental steps as illustrated in Figure 2.

The first step in the chromatographic process is to equilibrate the column packed with the reversed phase medium under suitable initial mobile phase conditions of pH, ionic strength and polarity (mobile phase hydrophobicity). The polarity of the mobile phase is controlled by adding organic modifiers such as acetonitrile. Ion-pairing agents, such as trifluoroacetic acid, may also be appropriate. The polarity of the initial mobile phase (usually referred to as mobile phase A) must be low enough to dissolve the partially hydrophobic solute yet high enough to ensure binding of the solute to the reversed phase chromatographic matrix.

In the second step, the sample containing the solutes to be separated is applied. Ideally, the sample is dissolved in the same mobile phase used to equilibrate the chromatographic bed. The sample is applied to the column at a flow rate where optimum binding will occur. Once the sample is applied, the chromatographic bed is washed further with mobile phase A in order to remove any unbound and unwanted solute molecules.

Bound solutes are next desorbed from the reversed phase medium by adjusting the polarity of the mobile phase so that the bound solute molecules will sequentially desorb and elute from the column. In reversed phase chromatography this usually involves decreasing the polarity of the mobile phase by increasing the percentage of organic modifier in the mobile phase. This is accomplished by maintaining a high concentration of organic modifier in the final mobile phase (mobile phase B). Generally, the pH of the initial and final mobile phase solutions remains the same. The gradual decrease in mobile phase polarity (increasing mobile phase hydrophobicity) is achieved by an increasing linear gradient from 100% initial mobile phase A containing little or no organic modifier to 100% (or less) mobile phase B containing a higher concentration of organic modifier. The bound solutes desorb from the reversed phase medium according to their individual hydrophobicities.

The fourth step in the process involves removing substances not previously desorbed. This is generally accomplished by changing mobile phase B to near 100% organic modifier in order to ensure complete removal of all bound substances prior to re-using the column.

The fifth step is re-equilibration of the chromatographic medium from 100% mobile phase B back to the initial mobile phase conditions.

Separation in reversed phase chromatography is due to the different binding properties of the solutes present in the sample as a result of the differences in their hydrophobic properties. The degree of solute molecule binding to the reversed phase medium can be controlled by manipulating the hydrophobic properties of the initial mobile phase. Although the hydrophobicity of a solute molecule is difficult to quantitate, the separation of solutes that vary only slightly in their hydrophobic properties is readily achieved. Because of its excellent resolving power, reversed phase chromatography is an indispensable technique for the high performance separation of complex biomolecules.

Typically, a reversed phase separation is initially achieved using a broad range gradient from 100% mobile phase A to 100% mobile phase B. The amount of organic modifier in both the initial and final mobile phases can also vary greatly. However, routine percentages of organic modifier are 5% or less in mobile phase A and 95% or more in mobile phase B.

The technique of reversed phase chromatography allows great flexibility in separation conditions so that the researcher can choose to bind the solute of interest, allowing the contaminants to pass unretarded through the column, or to bind the contaminants, allowing the desired solute to pass freely. Generally, it is more appropriate to bind the solute of interest because the desorbed solute elutes from the chromatographic medium in a concentrated state. Additionally, since binding under the initial mobile phase conditions is complete, the starting concentration of desired solute in the sample solution is not critical allowing dilute samples to be applied to the column.

The specific conditions under which solutes bind to the reversed phase medium will be discussed in the appropriate sections in greater detail.

Ionic binding may sometimes occur due to ionically charged impurities immobilised on the reversed phase chromatographic medium. The combination of hydrophobic and ionic binding effects is referred to as mixed-mode retention behaviour. Ionic interactions can be minimised by judiciously selecting mobile phase conditions and by choosing reversed phase media which are commercially produced with high batch-to-batch reproducibility and stringent quality control methods.

The matrix

Critical parameters that describe reversed phase media are the chemical composition of the base matrix, particle size of the bead, the type of immobilised ligand, the ligand density on the surface of the bead, the capping chemistry used (if any) and the pore size of the bead.

A reversed phase chromatography medium consists of hydrophobic ligands chemically grafted to a porous, insoluble beaded matrix. The matrix must be both chemically and mechanically stable. The base matrix for the commercially available reversed phase media is generally composed of silica or a synthetic organic polymer such as polystyrene. Figure 3 shows a silica surface with hydrophobic ligands.

Fig. 3. Some typical structures on the surface of a silica-based reversed phase medium. The hydrophobic octadecyl group is one of the most common ligands.
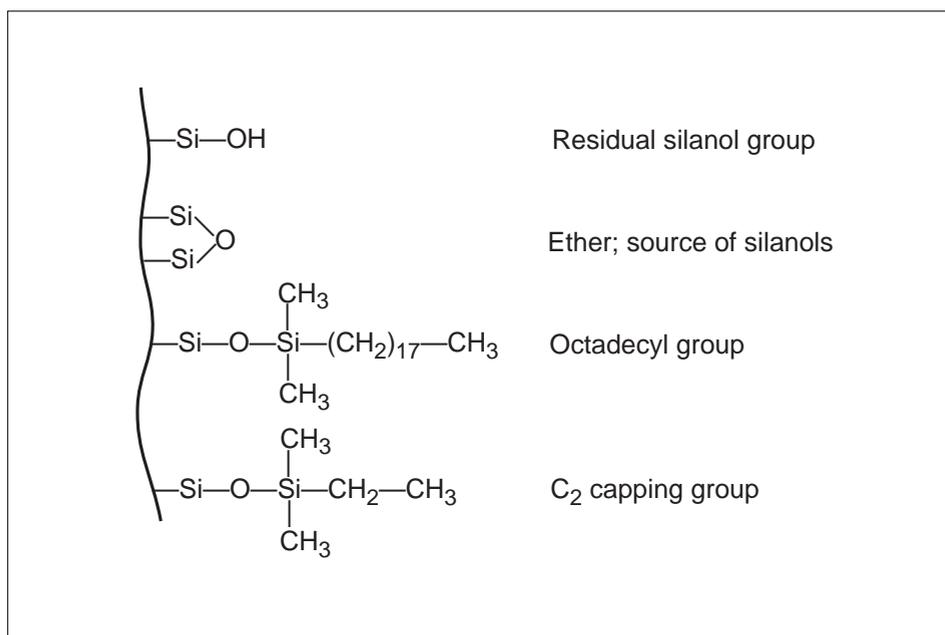
Silica was the first polymer used as the base matrix for reversed phase chromatography media. Reversed phase media were originally developed for the purification of small organic molecules and then later for the purification of low molecular weight, chemically synthesised peptides. Silica is produced as porous beads which are chemically stable at low pH and in the organic solvents typically used for reversed phase chromatography. The combination of porosity and physical stability is important since it allows media to be prepared which have useful loading capacities and high efficiencies. It is worth noting that, although the selectivity of silica-based media is largely controlled by the properties of the ligand and the mobile phase composition, different processes for producing silica-based matrices will also give media with different patterns of separation. The chemistry of the silica gel allows simple derivatisation with ligands of various carbon chain lengths. The carbon content, and the surface density and distribution of the immobilised ligands can be controlled during the synthesis.

The primary disadvantage of silica as a base matrix for reversed phase media is its chemical instability in aqueous solutions at high pH. The silica gel matrix can actually dissolve at high pH, and most silica gels are not recommended for prolonged exposure above pH 7.5.

Synthetic organic polymers, e.g. beaded polystyrene, are also available as reversed phase media. Polystyrene has traditionally found uses as a solid support in peptide synthesis and as a base matrix for cation exchange media used for separation of amino acids in automated analysers. The greatest advantage of polystyrene media is their excellent chemical stability, particularly under strongly acidic or basic conditions. Unlike silica gels, polystyrene is stable at all pH values in the range of 1 to 12. Reversed phase separations using polystyrene-based media can be performed above pH 7.5 and, therefore, greater retention selectivity can be achieved as there is more control over the degree of solute ionisation.
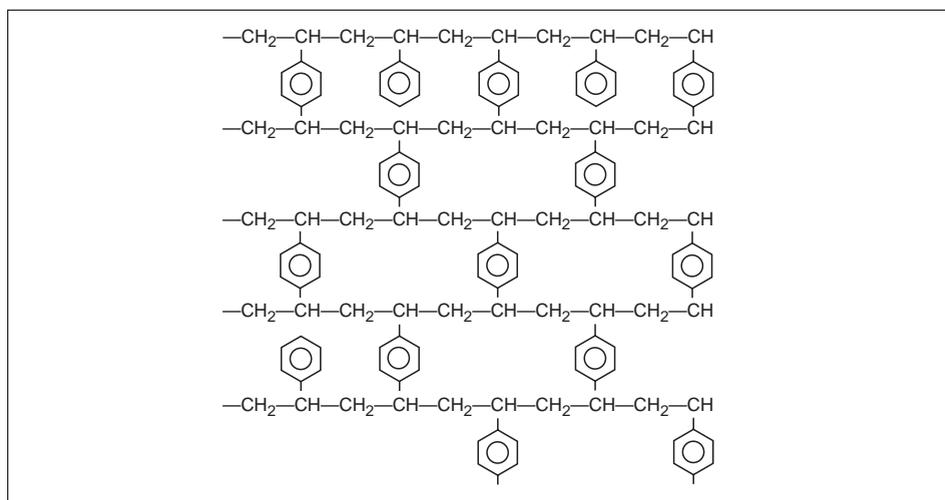
Fig. 4. Partial structure of a polystyrene-based reversed phase medium.

Fig. 5. Reverse phase media with wide pores allow the most efficient transfer of large solute molecules between the mobile and the stationary phases.

The surface of the polystyrene bead is itself strongly hydrophobic and, therefore, when left underivatised unlike silica gels that have hydrophobic ligands grafted to a hydrophilic surface.

The porosity of the reversed phase beads is a crucial factor in determining the available capacity for solute binding by the medium. Note that this is not the capacity factor (k´) but the actual binding capacity of the medium itself. Media with pore sizes of approximately 100 Å are used predominately for small organic molecules and peptides. Media with pore sizes of 300 Å or greater can be used in the purification of recombinant peptides and proteins that can withstand the stringent conditions of reversed phase chromatography.

The ligands

The selectivity of the reversed phase medium is predominantly a function of the type of ligand grafted to the surface of the medium. Generally speaking, linear hydrocarbon chains (n-alkyl groups) are the most popular ligands used in reversed phase applications. Some typical hydrocarbon ligands are shown in Figure 6.

Fig. 6. Typical n-alkyl hydrocarbon ligands. (A) Two-carbon capping group, (B) Octyl ligand, (C) Octadecyl ligand.

Although it is not possible to predict theoretically which ligand will be best for a particular application, a good rule of thumb is: the more hydrophobic the molecule to be purified, the less hydrophobic the ligand needs to be. The more hydrophilic molecules tend to require strongly hydrophobic immobilised ligands in order to achieve sufficient binding for separation. Typically, chemically synthesised peptides and oligonucleotides are efficiently purified on the more hydrophobic C18 ligands. Proteins and recombinant peptides, because of their size, behave as hydrophobic molecules and most often bind very strongly to C18 ligands. They are usually better separated on C8 ligands. The less hydrophobic eight carbon alkyl chain is less disruptive to the protein and peptide structures since lower concentrations of organic solvent are required for elution. In addition to ligand structure, the density of the immobilised hydrocarbon ligands on the silica surface also influences the selectivity shown by reversed phase media. Therefore, reproducible chemical derivatisation of the silica surface is critical for efficient reversed phase chromatography with consistent batch-to-batch selectivity.

The hydrocarbon ligands are generally coupled to the silica gel via silanol groups on the silica surface using chlorotrialkylsilane reagents. Not all of the silanol groups will be substituted in this coupling reaction. The C18 and C8 reagents are large and bulky so that steric hindrance often prevents complete derivatisation of all the available silanol groups. The residual silanol groups are believed to be responsible for the deleterious mixed mode ion exchange effects often present during reversed phase separation of biomolecules. In order to reduce these damaging side effects, the residual silanol groups are reacted with smaller alkylsilane reagents (chlorotrimethyl- and chlorotriethylsilanes) where steric effects do not interfere with complete coverage of the silanol groups remaining on the surface of the silica gel. This process is referred to as "end-capping". The extent of end-capping also affects selectivity, so reproducibility in the capping step is critical for a well behaved reversed phase medium. The derivatisation reaction is shown in Figure 7.

Fig. 7. Substitution of silica with octadecyl chains by reaction with monochlorodimethyloctadecylsilane.
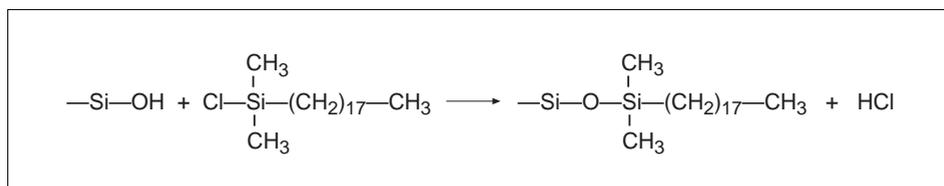
The particle size of the bead, as measured by its diameter, has important consequences for the size of the chromatographic bed which can be usefully packed, and for the efficiency of the separation. The larger particle size media are generally used for large scale preparative and process applications due to their increased capacity and lower pressure requirements at high flow rates. Small scale preparative and analytical separations use smaller particles since separation efficiency, i.e. peak width, is directly related to particle size (see section on

Fig. 8. Determination of the resolution between two peaks.

$$R_s = \frac{v_2 - v_1}{(w_2 + w_1)/2}$$
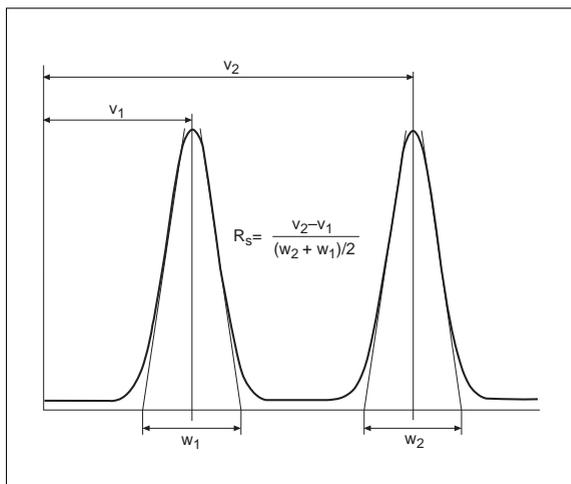
efficiency and selectivity). Analytical and small scale preparative applications are usually performed with 3 and 5 μm beads while larger scale preparative applications (pilot and process scale) are usually performed with particle sizes of 15 μm and greater. Micropreparative and small scale preparative work can be accomplished using particle sizes of 3 μm since the limited capacity of the small columns packed with these media is not a severe problem when only small quantities of material are purified.

Resolution in reversed phase chromatography
Resolution

Adequate resolution and recovery of purified biological material is the ultimate goal for reversed phase preparative chromatography. Resolution, Rs, is generally defined as the distance between the centres of two eluting peaks as measured by retention time or volume divided by the average width of the respective peaks (Fig. 8). For example, an Rs value of 1.0 indicates 98% purity has been achieved (assuming 98% peak recovery). Baseline resolution between two well formed peaks indicates 100% purity and requires an Rs value greater than 1.5 (Fig. 9).

Calculating Rs is the simplest method for quantitating the actual separation achieved between two solute molecules. This simple relationship can be expanded to demonstrate the connection between resolution and three fundamental parameters of a chromatographic separation. The parameters have been derived from chromatographic models based on isocratic elution but are still appropriate when used to describe their effects on resolution when discussing gradient elution (consider a continuous linear gradient elution to be a series of small isocratic elution steps). The parameters that contribute to peak resolution are column selectivity, column efficiency and the column retention factor.
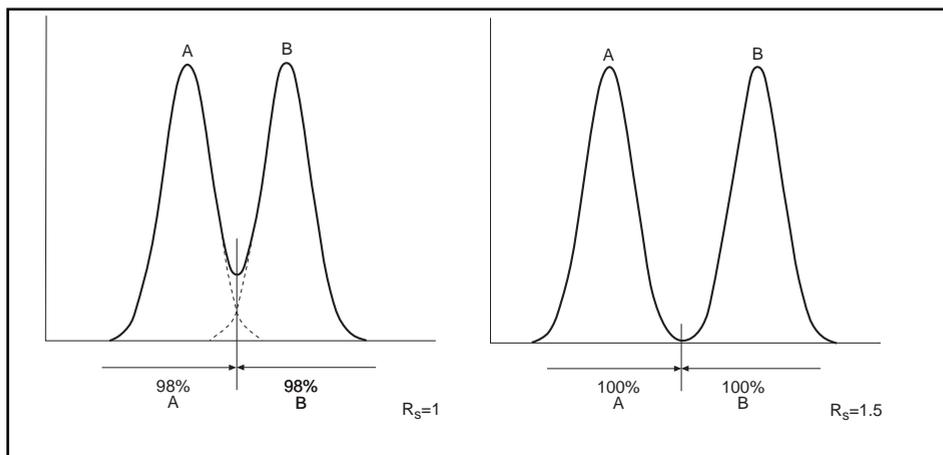
Fig. 9. Separation results with different resolution.

$$Rs = \frac{1}{4} \frac{(\alpha - 1)}{\alpha} \left(\sqrt{N} \right) \frac{k'}{1 + k}$$

Resolution Rs is a function of selectivity α, efficiency (number of theoretical plates N) and the average retention factor, k', for peaks 1 and 2.

Capacity factor

The capacity factor, k', is related to the retention time and is a reflection of the proportion of time a particular solute resides in the stationary phase as opposed to the mobile phase. Long retention times result in large values of k'. The capacity factor is not the same as the available binding capacity which refers to the mass of the solute that a specified amount of medium is capable of binding under defined conditions. The capacity factor, k', can be calculated for every peak defined in a chromatogram, using the following equations.

$$\text{Capacity factor} = k' = \frac{\text{moles of solute in stationary phase}}{\text{moles of solute in mobile phase}}$$

$$k' = \frac{T_R - T_O}{T_O} \quad \frac{V_R - V_O}{V_O}$$

where T_R and V_R are the retention time and retention volume, respectively, of the solute, and T_o and V_o the retention time and retention volume, respectively, of an unretarded solute.

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Fig. 10. Hypothetical chromatogram. V_O=void volume, V_C=total volume, V_1, V_2, and V_3 are the elution (retention) volumes of peaks 1, 2 and 3, respectively.
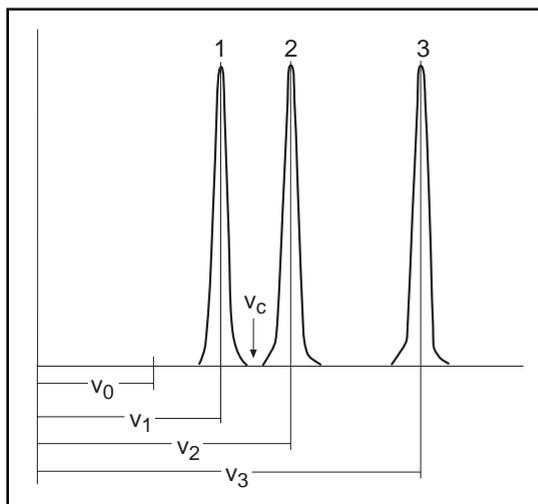
In the resolution equation previously described, the k´ value is the average of the capacity factors of the two peaks being resolved. Unlike non-adsorptive chromatographic methods (e.g. gel filtration), reversed phase chromatography can have very high capacity factors. This is because the experimental conditions can be chosen to result in peak retention times greatly in excess of the total column volume.

Efficiency (N)

The efficiency of a packed column is expressed by the number of theoretical plates, N. N is a dimensionless number and reflects the kinetics of the chromatographic retention mechanism. Efficiency depends primarily on the physical properties of the chromatographic medium together with the chromatography column and system dimensions. The efficiency can be altered by changing the particle size, the column length, or the flow rate. The expression "number of theoretical plates" is an archaic term carried over from the theoretical comparison of a chromatography column to a distillation apparatus. The greater the number of theoretical plates a column has, the greater its efficiency and correspondingly, the higher the resolution which can be achieved. The column efficiency (N) can be determined empirically using the equation below based upon the zone broadening that occurs when a solute molecule is eluted from the column (Fig. 11).

The number of theoretical plates, N, is given by

$$N = 5.54 \ (V_1/W_{1/2})^2$$

where V_1 is the retention volume of the peak and $W_{1/2}$ is the peak width (volume) at half peak height.
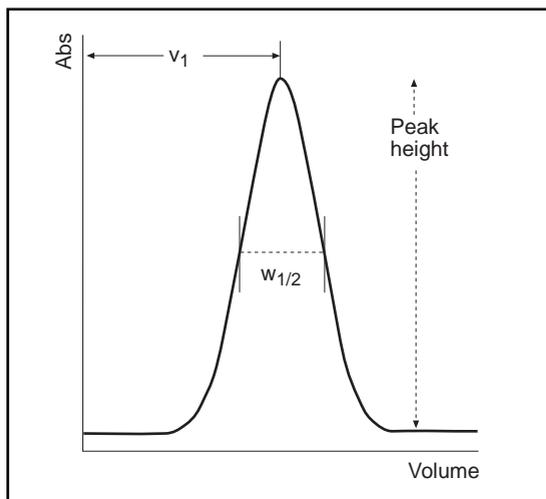
Fig. 11. Measurements for determining column efficiency. V_1 is the retention volume of the peak and $W_{1/2}$ is the peak width (volume) at half peak height.

The number of theoretical plates is sometimes reported as plates per metre of column length (N/L).

The height equivalent to a theoretical plate, H, is given by

$$H = L/N$$

where L is the column length and N is the number of theoretical plates.

Any parameter change that increases N will also increase Rs. The relationship between the two is defined by the square root of N. For example, an increase in N from 100 to 625 will improve the resolution by a factor of 2.5, rather than 6.25. The main contribution to column efficiency (N) is particle size and the efficacy of the column packing procedure.
It should be noted that the smaller the particle size, the more difficult it is to pack an efficient column. This is the reason why reversed phase media with particle sizes less than 10 μm are commercially available only in pre-packed formats.

Selectivity

Selectivity (α) is equivalent to the relative retention of the solute peaks and, unlike efficiency, depends strongly on the chemical properties of the chromatography medium.

The selectivity, α, for two peaks is given by

$$\alpha = k_2{'}/k_1{'} = V_2 - V_0/V_1 - V_0 = V_2/V_1$$

where V_1 and V_2 are the retention volumes, and $k_2{'}/k_1{'}$ are the capacity factors, for peaks 1 and 2 respectively, and V_0 is the void volume of the column.

Selectivity is affected by the surface chemistry of the reversed phase medium, the nature and composition of the mobile phase, and the gradient shape.

Fig. 12. Selectivity comparison between different silica based media at pH 2.0 and pH 6.5. A mixture of closely related angiotensin peptides was used as sample. (Work by Amersham Biosciences AB, Uppsala, Sweden.)

1. Val4-Ile7-AT III (RVYVHPI)
2. Ile7-AT III (RVYIHPI)
3. Val4-AT III (RVYVHPF)
4. Sar1-Leuß-AT II (Sar-RVYIHPL)
   (Sar=sarcosine, N-methylglycine)
5. AT III (RVYIHPF)
6. AT II (DRVYIHPF)
7. des-Asp1-AT I (RVYIHPLFHL)
8. AT I (DRVYIHPFHL)

Columns:
a) and e) Sephasil Protein C4 5 µm 4.6/100
b) and f) Sephasil Peptide C8 5 µm 4.6/100
c) and g) Sephasil Peptide C18 5 µm 4.6/100
d) and h) µRPC C2/C18 ST 4.6/100

Eluent A (pH 2): 0.065% TFA in distilled water
Eluent B (pH 2): 0.05% TFA, 75% acetonitrile
Eluent A (pH 6.5): 10 mM phosphate
Eluent B (pH 6.5): 10 mM phosphate, 75% acetonitrile
Flow: 1 ml/min
System: ÄKTApurifier
Gradient: 5–95% B in 20 column volumes

Fig. 13. The effect of selectivity and efficiency on resolution.

Both high column efficiency and good selectivity are important to overall resolution. However, changing the selectivity in a chromatographic experiment is easier than changing the efficiency. Selectivity can be changed by changing easily modified conditions like mobile phase composition or gradient shape.

Binding capacity

The available binding capacity of a reversed phase medium is a quantitative measure of its ability to adsorb solute molecules under static conditions. The dynamic binding capacity is a measure of the available binding capacity at a specific flow rate. Both values are extremely important for preparative work.

The amount of solute which will bind to a medium is proportional to the concentration of immobilised ligand on the medium and also depends on the type of solute molecule being adsorbed to the medium. The available and dynamic binding capacities depend on the specific chemical and physical properties of the solute molecule, the properties of the reversed phase medium (porosity, etc.) and the experimental conditions during binding.

The porosity of the bead is an important factor which influences binding capacity. The entire hydrophobic surface of macroporous media is available for binding solute. Large solute molecules (i.e. high molecular weight) may be excluded from media of smaller pore size and only a small fraction of the whole hydrophobic surface will be used. When maximum binding capacity is required, a medium with pores large enough to allow all the molecules of interest to enter freely must be used.

Critical parameters in reversed phase chromatography

Column length

The resolution of high molecular weight biomolecules in reversed phase separations is less sensitive to column length than is the resolution of small organic molecules. Proteins, large peptides and nucleic acids may be purified effectively on short columns and increasing column length does not improve resolution significantly. The resolution of small peptides (including some peptide digests) may sometimes be improved by increasing column length. For example, the number of peaks detected when a tryptic digest of carboxamidomethylated transferrin was fractionated by RPC increased from 87 on a 5 cm long column to 115 on a 15 cm long column and 121 on a 25 cm long column (6).

The partition coefficients of high molecular weight solutes are very sensitive to small changes in mobile phase composition and hence large molecules desorb in a very narrow range of organic modifier concentration. The retention behaviour of large molecules may be considered to be governed by an on/off mechanism (i.e. a large change in partition coefficient) which is insensitive to column length. When small changes in organic modifier concentration result in small changes in the partition coefficient, longer column lengths increase resolution.

The use of gradient elution further reduces the significance of column length for the resolution of large biomolecules by reversed phase chromatography. Gradients are required since most biological samples are complex mixtures of molecules that vary greatly in their adsorption to the reversed phase medium. Due to this variety of adsorption affinities, the mobile phase must have a broad range of eluting power to ensure elution of all the bound solute molecules. Under these conditions, especially with moderate to steep gradient slopes, column length is not a critical factor with regard to resolution.

Flow rate

Flow rate is expected to be an important factor for resolution of small molecules, including small peptides and protein digests, in reversed phase separations.

However, reversed phase chromatography of larger biomolecules, such as proteins and recombinantly produced peptides, appears to be insensitive to flow rate. In fact, low flow rates, typically used with long columns, may actually decrease resolution due to increased longitudinal diffusion of the solute molecules as they traverse the length of the column.

The flow rate used during the loading of the sample solution is especially significant in large scale preparative reversed phase chromatography, although not critical during analytical experiments. Dynamic binding capacity will vary depending on the flow rate used during sample loading. When scaling up a purification, the dynamic binding capacity should be determined in order to

assess the optimum flow rate for loading the sample. Dynamic binding capacity is a property of the gel that reflects the kinetics of the solute binding process. The efficiency of this step can have enormous consequences for the results of a large scale preparative purification.

Temperature

Temperature can have a profound effect on reversed phase chromatography, especially for low molecular weight solutes such as short peptides and oligonucleotides. The viscosity of the mobile phase used in reversed phase chromatography decreases with increasing column temperature. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process, decreasing solvent viscosity generally leads to more efficient mass transfer and, therefore, higher resolution. Increasing the temperature of a reversed phase column is particularly effective for low molecular weight solutes since they are suitably stable at the elevated temperatures.

Mobile phase

In many cases, the colloquial term used for the mobile phases in reversed phase chromatography is "buffer". However, there is little buffering capacity in the mobile phase solutions since they usually contain strong acids at low pH with large concentrations of organic solvents. Adequate buffering capacity should be maintained when working closer to physiological conditions.

Organic solvent

The organic solvent (modifier) is added to lower the polarity of the aqueous mobile phase. The lower the polarity of the mobile phase, the greater its eluting strength in reversed phase chromatography. Although a large variety of organic solvents can be used in reversed phase chromatography, in practice only a few are routinely employed. The two most widely used organic modifiers are acetonitrile and methanol, although acetonitrile is the more popular choice. Isopropanol (2-propanol) can be employed because of its strong eluting properties, but is limited by its high viscosity which results in lower column efficiencies and higher back-pressures. Both acetonitrile and methanol are less viscous than isopropanol.

All three solvents are essentially UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors. Acetonitrile is used almost exclusively when separating peptides. Most peptides only absorb at low wavelengths in the ultra-violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths.

The retention, or capacity factor (k´), for a given solute is a function of the mobile phase polarity. The elution order can be affected by changing the type of organic modifier or by the addition of ion pairing agents. Changes in elution order are most pronounced for proteins that are denatured in organic solvents. Denaturation of the protein can result in a change in its hydrophobicity.

<div align="center">Ion suppression</div>

The retention of peptides and proteins in reversed phase chromatography can be modified by mobile phase pH since these particular solutes contain ionisable groups. The degree of ionisation will depend on the pH of the mobile phase. The stability of silica-based reversed phase media dictates that the operating pH of the mobile phase should be below pH 7.5. The amino groups contained in peptides and proteins are charged below pH 7.5. The carboxylic acid groups, however, are neutralised as the pH is decreased. The mobile phase used in reversed phase chromatography is generally prepared with strong acids such as trifluoroacetic acid (TFA) or ortho-phosphoric acid. These acids maintain a low pH environment and suppress the ionisation of the acidic groups in the solute molecules. Varying the concentration of strong acid components in the mobile phase can change the ionisation of the solutes and, therefore, their retention behaviour.

The major benefit of ion suppression in reversed phase chromatography is the elimination of mixed mode retention effects due to ionisable silanol groups remaining on the silica gel surface. The effect of mixed mode retention is increased retention times with significant peak broadening.

Fig. 14. Typical effects of mixed-mode retention. Peaks are broader and skewed, and retention time increases.

(A) Reversed phase chromatography
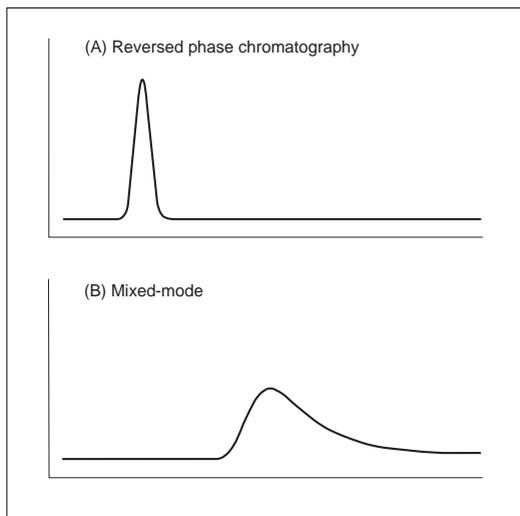
(B) Mixed-mode

Mixed mode retention results from an ion exchange interaction between negatively charged silanol groups exposed on the surface of the silica and the positively charged amino groups on the solute molecules. Silanol groups on the surface of silica-based media can arise from two primary sources. The first is due to inadequate end-capping procedures during the manufacture of the gel. It is critical to choose a manufacturer that produces a gel with reproducibly low mixed mode retention effects, since these artefact can affect resolution.

The other source of surface silanol groups is column ageing. The silica gel surface is continually eroded during the life of the column, resulting in exposed silanol groups and progressive deterioration in column performance. Prolonged exposure to aqueous solutions can accelerate column ageing.

The low pH environment (usually less than pH 3.0) of typical reversed phase mobile phases suppresses the ionisation of these surface silanol groups so that the mixed mode retention effect is diminished.

Ion suppression is not necessary when dealing with reversed phase media based on polystyrene or other synthetic organic polymers. Polystyrene media are stable between pH 1-12 and do not exhibit the mixed mode retention effects that silica gels do with mobile phases at high pH.

Ion pairing agents

The retention times of solutes such as proteins, peptides and oligonucleotides can be modified by adding ion pairing agents to the mobile phase. Ion pairing agents bind to the solute by ionic interactions, which results in the modification of the solute hydrophobicity. Examples of ion pairing agents are shown in chapter 3.
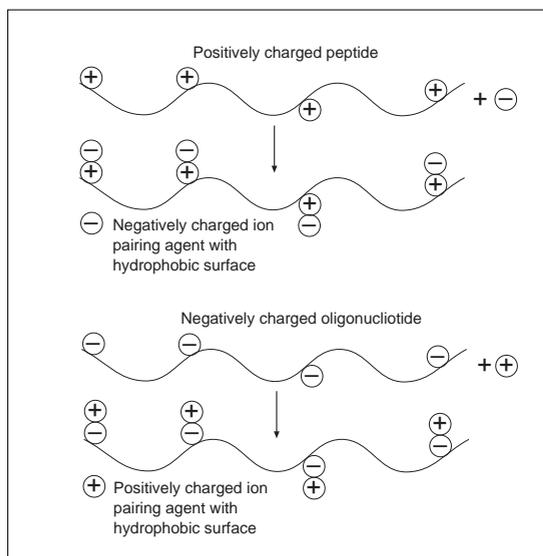
Fig. 15. Ion pair formation with (A) anionic or (B) cationic ion pairing agents.

Both anionic and cationic ion pairing agents are used depending on the ionic character of the solute molecule and the pH of the mobile phase. For example, a typical ion pairing agent for peptides at pH less than 3.5 is trifluoroacetic acid. The ion pairing agent used with oligonucleotides, which contain a negative charge at neutral to high pH, is typically triethylamine.

In some cases the addition of ion pairing agents to the mobile phase is an absolute requirement for binding of the solute to the reversed phase medium. For example, retention of deprotected synthetic oligonucleotides, i.e. without the trityl protecting group attached, requires triethylamine in the mobile phase. The same is true for hydrophilic peptides where binding is negligible in the absence of a suitable ion pairing agent such as trifluoroacetic acid.

The concentration of ion pairing agents in the mobile phases is generally in the range 0.1 - 0.3%. Potential problems include possible absorbance of UV light by the ion pairing agent and changes in extinction coefficient with concentration of organic modifier. This can result in either ascending or descending baselines during gradient elution.

Gradient elution

Gradient elution is the method of choice when performing preparative reversed phase chromatography of biomolecules. The typical gradients for preparative reversed phase chromatography of proteins and peptides are linear and binary, i.e. involving two mobile phases. Convex and concave gradients are used occasionally for analytical purposes particularly when dealing with multi-component samples requiring extra resolution either at the beginning or at the end of the gradient.

The concentration of organic solvent is lower in the initial mobile phase (mobile phase A) than it is in the final mobile phase (mobile phase B). The gradient then, regardless of the absolute change in percent organic modifier, always proceeds from a condition of high polarity (high aqueous content, low concentration of organic modifier) to low polarity (lower aqueous content, higher concentration of organic modifier).

Gradient shape (combinations of linear gradient and isocratic conditions), gradient slope and gradient volume are all important considerations in reversed phase chromatography. Typically, when first performing a reversed phase separation of a complex sample, a broad gradient is used for initial screening in order to determine the optimum gradient shape.

After the initial screening is completed, the gradient shape may adjusted to optimise the separation of the desired components. This is usually accomplished by decreasing the gradient slope where the desired component elutes and increasing it before and after. The choice of gradient slope will depend on how

closely the contaminants elute to the target molecule. Generally, decreasing gradient slope increases resolution. However, peak volume and retention time increase with decreasing gradient slope. Shallow gradients with short columns are generally optimal for high molecular weight biomolecules.

Gradient slopes are generally reported as change in percent B per unit time (%B/min.) or per unit volume (%B/ml). When programming a chromatography system in time mode, it is important to remember that changes in flow rate will affect gradient slope and, therefore, resolution.

Resolution is also affected by the total gradient volume (gradient time x flow rate). Although the optimum value must be determined empirically, a good rule of thumb is to begin with a gradient volume that is approximately ten to twenty times the column volume. The slope can then be increased or decreased in order to optimise resolution.

Mode of use

Desalting

Desalting is a routine laboratory procedure in which low molecular weight contaminants are separated from the desired higher molecular weight biomolecules. The procedure is sometimes simply referred to as buffer exchange. Non-chromatographic techniques for buffer exchange include ultra-filtration and dialysis.

Desalting is used in the laboratory primarily for sample preparation, e.g. desalting fractions obtained by other methods such as ion exchange chromatography. Size exclusion chromatography (gel filtration) with Sephadex™ G-25 is commonly used for desalting proteins and nucleic acids, and Sephadex G-10 is used for desalting small peptides. Size exclusion chromatography is a valuable method for desalting due to its simplicity and gentleness, although it suffers from the unwanted side effect of sample dilution.

Proteins, peptides and oligonucleotide samples can be conveniently desalted using reversed phase chromatography. When desalting samples using reversed phase techniques, the samples can be recovered and reconstituted into small volumes thereby avoiding the sample dilution effects of gel filtration.

The sample is passed through a small reversed phase column where it binds and concentrates on the reversed phase medium. Unlike gel filtration, reversed phase is an adsorption technique and sample volume is not limited. Reversed phase chromatography columns can concentrate large volumes of dilute samples at the same time as desalting them.

After the entire sample has been processed, the bound solute is eluted using a small volume of low polarity mobile phase, typically acetonitrile. If the solvent is volatile, as acetonitrile is, it can then be removed by evaporation and the sample residue re-suspended in the desired volume of new buffer.
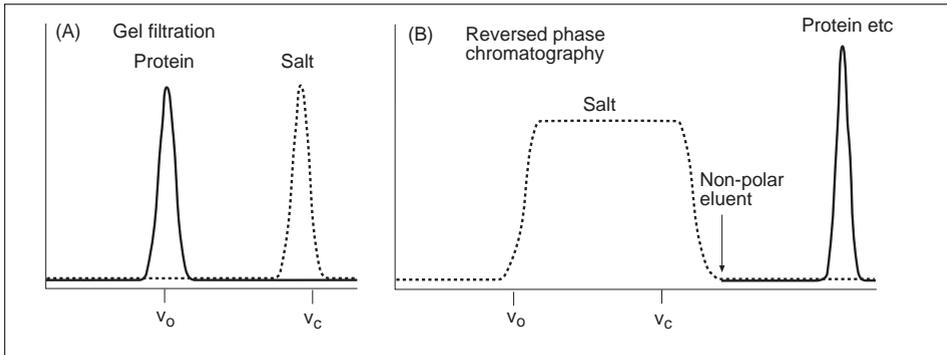
Fig. 16. Desalting by (A) gel filtration and (B) reversed phase chromatography. The large molecules elute first in gel filtration; the salt elutes without changing the eluent. The salt elutes first in reversed phase chromatography; a less-polar eluent is needed to elute proteins and other molecules which are retained on the column.

High resolution separations

Reversed phase chromatography is most typically used as a high resolution technique, where its inherent robustness is especially advantageous. However, certain applications push the resolving power of the reversed phase technique to its limit. These tend to be in the intermediate stages of preparative applications or when isolating structurally similar components from a complex mixture. Examples include isolation of specific peptides from enzymatic digests or purification of oligonucleotides from a complicated mixture of oligonucleotide contaminants. In these cases, a great many peaks must be resolved from each other and recovered in sufficient amounts for further analysis. Reversed phase media of very small particle size, typically 3 and 5 μm beads, are usually required together with painstaking attention given to details such as column temperature, gradient slope and mobile phase composition. When dealing with smaller solutes, such as short oligonucleotides, digested protein fragments and short peptides, the optimisation of other factors such as flow rate and column length may also be necessary in order to maximise resolution.

Large scale preparative purification

The large scale purification of biomolecules such as synthetic oligonucleotides and peptides, and recombinant peptides and proteins by reversed phase chromatography requires both high resolution separation together with the ability to scale up the purification. In these cases, the purification is optimised using a small particle reversed phase medium and then scaled up accordingly using a medium with similar selectivity but with a larger particle size. The techniques of scale up used with reversed phase chromatography are similar to those used with other chromatographic techniques such as ion exchange. Specific examples of preparative, large scale reversed phase purification of biomolecules are shown in chapter 4.
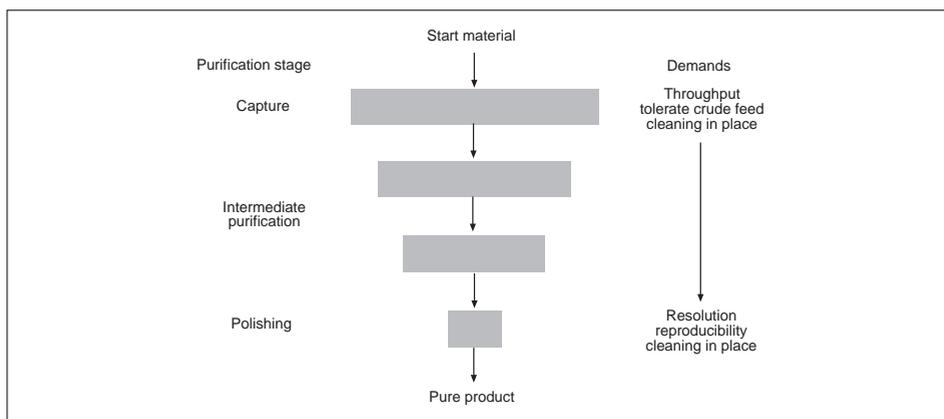
Fig. 17. Stages in a purification scheme.

Stages in a purification scheme

Once the source for a biomolecule has been determined, whether microbial, chemical, natural or other, and the starting material has been produced in sufficiently large quantities, the desired substance must then be purified from contaminants present in the crude sample. There are essentially three functional stages in the purification of a biomolecule from a crude preparation or extract. These are referred to as Capture, Intermediate Purification and Polishing. The suitability of any separation technique, including reversed phase chromatography, at any stage of purification will always depend on the specific sample, the specific separation problem at hand and the intended use of the purified material.

Capture

Capture is the first step in the purification procedure. At this stage, the sample volume is usually at its largest and the sample may contain particulates or viscous materials. The purpose of the capture step is to isolate, concentrate and stabilise the target molecule from the crude preparation rapidly, and with good recovery. The capture step is not expected to be highly resolving but is required to isolate the molecule of interest from contaminating substances that are dissimilar to the desired molecule. The capture step may be considered as a group separation rather than as a high resolution purification. Consequently, time, capacity and recovery are more important than resolution in a successful capture step. Reversed phase chromatography is a suitable method for the capture of synthetic peptides and synthetic oligonucleotides. However, it is usually less suitable for capture of peptides and proteins from biological sources. This is because of the presence of lipids and other highly hydrophobic solutes which bind strongly, reduce the dynamic capacity for the molecule of interest, and can be difficult to remove from the column. Additionally, the small particle size of most reversed phase media requires particulates to be removed from the sample to prevent the column from clogging. Ion exchange chromatography and hydrophobic interaction chromatography using bead diameters greater than 90 μm are better suited for capture in these instances.

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Fig. 18. Steps during capture.

Apply sample   Elute unbound sugars, salts etc   Elute peptides, proteins etc
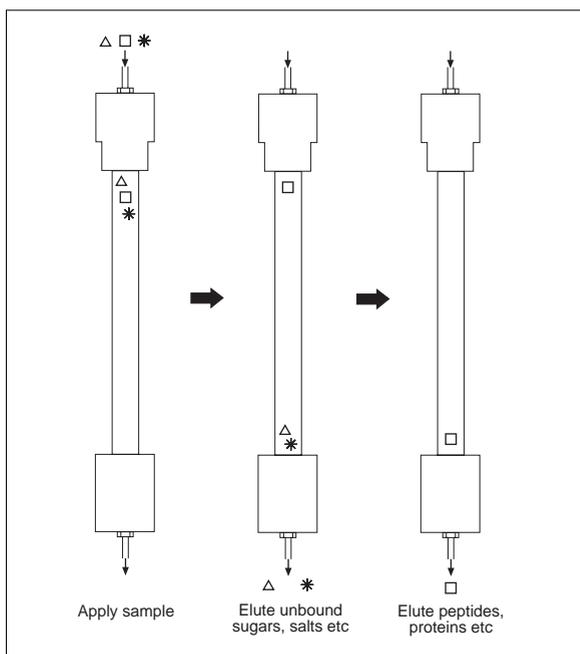
Intermediate stages

In the intermediate purification phase the focus is to separate the target molecule from most of the bulk impurities such as other proteins, peptides, nucleic acids, endotoxins and viruses. An ability to resolve similar components is of increased importance since contaminants at this stage are often similar to the target molecule in terms of functional or structural properties. The critical requirements are recovery and resolution. Reversed phase chromatography is a suitable technique for this stage of the purification because of the high resolution that can be achieved.

Polishing

Polishing is the final step in the preparation of a pure product. The polishing step is used to remove trace contaminants and impurities. The purified biomolecule should be in a form suitable for its intended use. Contaminants at the polishing stage are often very similar to the target molecule. Typical contaminants may include "conformers" and structural variants of the target molecule. Structural variants can include dimers, oligomers, aggregates, oxidised amino acids, protease-clipped molecules, desamidated amino acids etc. Other micro-heterogeneities may also occur. In process related applications, polishing also removes final traces of leachables, endotoxins, viruses etc.

The goals of the polishing stage might be product purity of 100% in less than two steps with a recovery of greater than 99%. Polishing can be performed using size exclusion, especially when dimers and aggregates must be removed. However, when dealing with slight structural variants and micro-heterogeneities, reversed phase chromatography with its excellent resolving power is the method of choice.

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Product Guide

Reversed phase media from Amersham Biosciences provide a broad range of selectivity for different applications for use at analytical, laboratory and production scale.
Table 1 below reviews briefly the main characteristics of the media together with their application suitability.

Media	Medium	Particle size (approx)	Applications
SOURCE™ 5RPC	Polystyrene/divinyl-benzene	5 µm mono-sized	High resolution analysis and small scale purification. Alternative selectivity to silica, especially for separations performed at high pH. Ideal for recombinant and synthetic peptides and oligonucleotides.
SOURCE 15RPC	Polystyrene/divinyl-benzene	15 µm mono-sized	Preparative purification of proteins, peptides and oligonucleotides. Alternative selectivity to silica, especially for separations performed at high pH. Excellent pressure/flow characteristics.
SOURCE 30RPC	Polystyrene/divinyl-benzene	30 µm mono-sized	Large scale purification of proteins, peptides and oligonucleotides. Alternative selectivity to silica, especially for separations performed at high pH. Excellent pressure/flow characteristics.
µRPC C2/C18	Silica	3 µm	High efficiency media, for peptide mapping, analysis and micropurification.
Sephasil™ Protein C4 Sephasil Peptide C8 Sephasil Peptide C18	Silica	5 µm	High resolution analysis and purification. Suitable for recombinant and synthetic peptides.
Sephasil Protein C4 Sephasil Peptide C8 Sephasil Peptide C18	Silica	12 µm	Preparative purification of peptides, proteins and oligonucleotides.
Sephasil C8 Sephasil C18	Silica	5 µm	SMART™ system pre-packed columns. Micropurification and analysis.

Table 1

SOURCE RPC
Product Description

SOURCE RPC media are designed for analytical and preparative chromatography of synthetic peptides, oligonucleotides and proteins. SOURCE RPC is based on rigid, monosized, polystyrene/divinyl benzene beads (Fig. 19) that give rapid, reproducible, high capacity separations with excellent resolution at high flow rates.

SOURCE RPC is a useful alternative to RPC matrices based on silica, especially for separations which must be performed at high pH or when different selectivity or higher capacity are required.
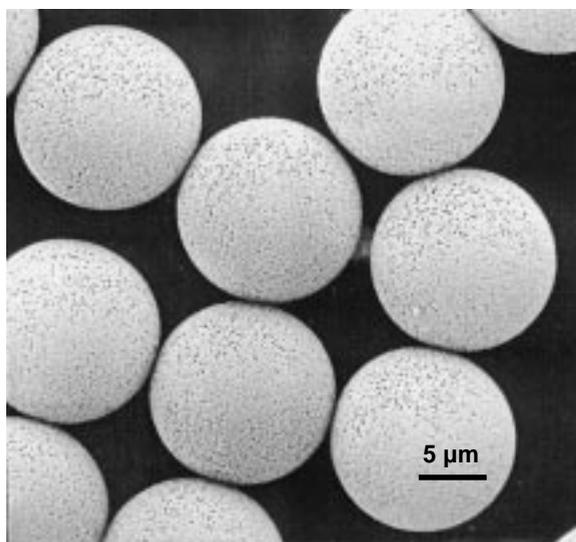
Fig. 19. Scanning electron micrograph of SOURCE 15RPC. Note the uniform size distribution.

The pore size distribution, batch-to-batch reproducibility (Fig. 20) and excellent scalability (Fig 21) of SOURCE RPC ensure outstanding chromatographic properties at any scale of operation.

Sample:	(Ile7) angiotensin III (0.5 mg/ml)
	(Val4) angiotensin III (0.5 mg/ml)
	Angiotensin III (0.5 mg/ml)
	Angiotensin I (0.5 mg/ml)
	25 µl applied.
Column:	RESOURCE™ RPC, 1 ml (i.d. 6.4, length 30 mm)
Eluent A:	0.1% TFA in water
Eluent B:	0.1% TFA, 60% acetonitrile in water
Gradient:	15-65% B in 20 min
Flow:	1 ml/min

Column:	SOURCE 30RPC, 10 mm i.d. x 300 mm column (24 ml)
	200 mm i.d. x 300 mm column (10 l)
Sample:	Mixture of Angiotensin II, Ribonuclease A and Insulin
Sample load:	0.064 mg/ml medium, total load
Solution A:	0.1% TFA/0.05 M NaCl
Solution B:	0.1% TFA/60% n-propanol
Flow:	150 cm/h
Gradient:	20–70% B, 5 column volumes (cv)
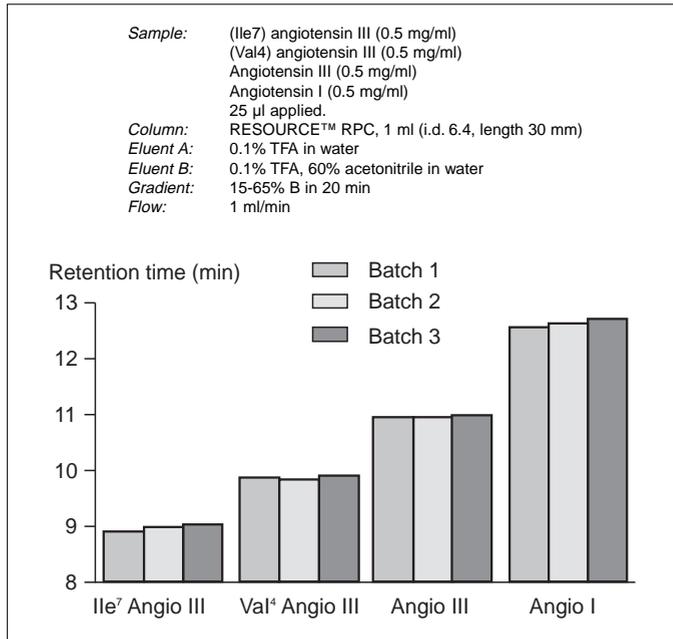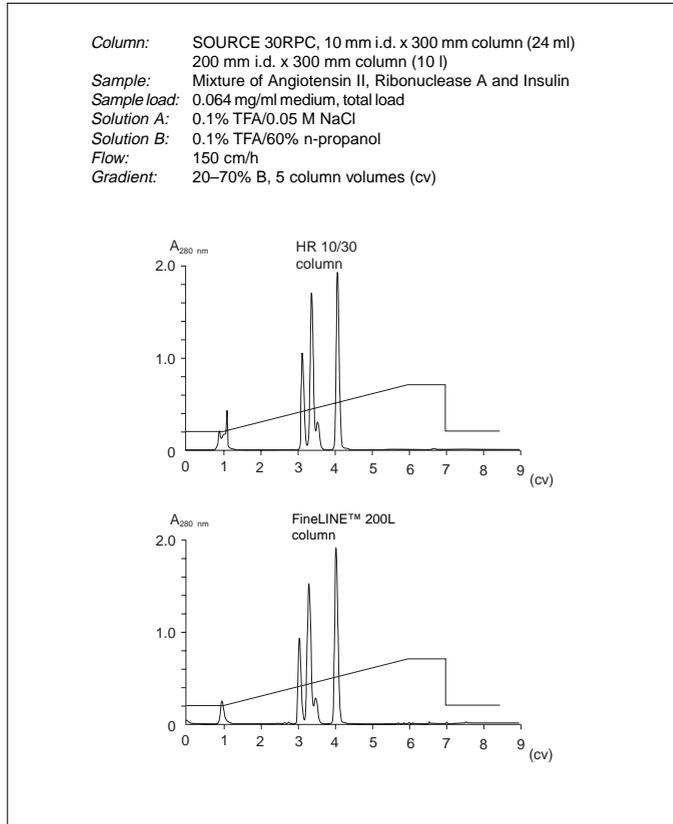
Characteristics of SOURCE RPC are shown in Table 2.

	SOURCE 5RPC	SOURCE 15RPC	SOURCE 30RPC
Base matrix and stationary phase	Polystyrene/divinyl benzene	Polystyrene/divinyl benzene	Polystyrene/divinyl benzene
Particle size	5 µm	15 µm	30 µm
Particle size distribution	monosized	monosized	monosized
Typical separation flow velocity (cm/hr)	100 -480	200 - 900	100 - 1 000
pH stability (operational)	1 - 12	1 - 12	1 - 12
pH stability (cleaning range)	1 - 14	1 - 14	1 - 14
Dynamic binding capacity (per ml medium at 300 cm/h)	~ 80 mg bacitracin/ml	~ 10 mg BSA/ml ~ 50 mg insulin/ml	~ 14 mg BSA/ml ~ 72 mg insulin/ml

Table 2.

High Chemical Stability

SOURCE RPC has an operating range between pH 1 - 12 allowing a free choice for running conditions. Since peptide solubility is frequently pH dependent, successful separations of some peptides may require conditions at high pH. SOURCE RPC therefore offers much greater pH stability and flexibility than silica based reversed phase matrices.

Figure 22 demonstrates the chemical stability of SOURCE 30RPC, showing the separation of angiotensins before and after incubation of the medium for one week at 40°C in 1M HCl and, similarly, in 1M NaOH.

The extremely high pH tolerance (1 - 14) gives full flexibility for frequent cleaning procedures improving both media lifetime and overall economy at every scale of application. Figure 23 shows results from a SOURCE 5RPC 4.6/150 column on which a thousand runs were performed, including four cleaning-in-place steps with 1 M NaOH and 1.0 M HCl, during a 21 days cycle. The resolution and retention times remained unchanged.

Column:	SOURCE 30RPC, 5 mm i.d. x 50 mm column (1 ml)
Sample:	Mixture of (Ile^7) Angiotensin III, (Val^4) Angiotensin III,
	Angiotensin III and Angiotensin II
Sample load:	0.13 mg/ml media of each peptide
Solution A:	0.1% TFA
Solution B:	0.1% TFA/60% Acetonitrile
Flow:	300 cm/h
Gradient:	15–65% B, 20 column volumes (cv)
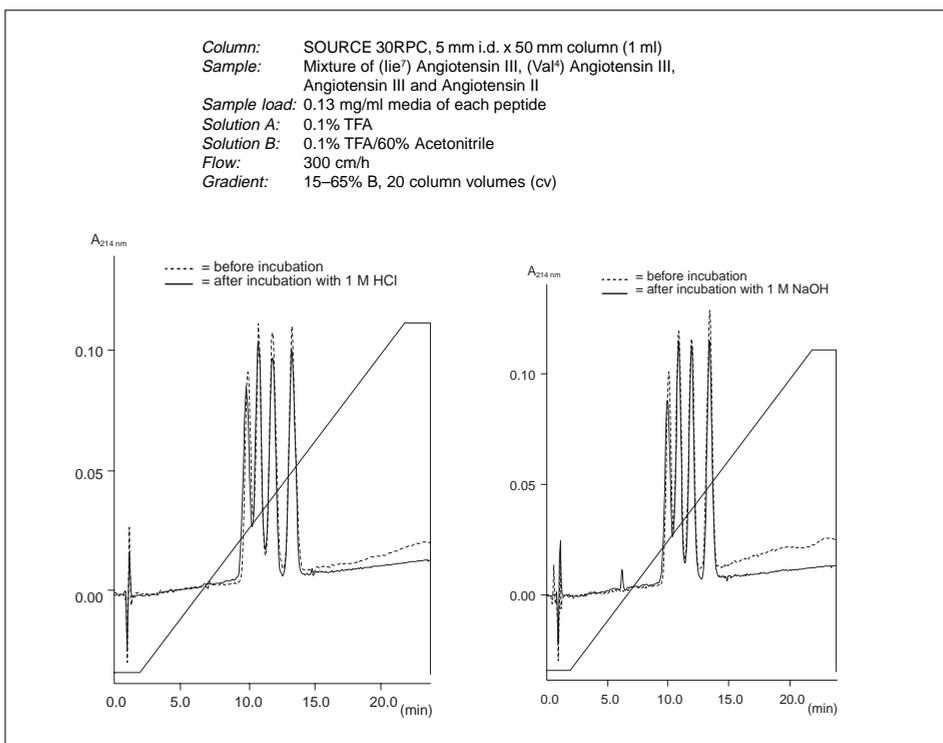
Fig. 22. Separation of model protein mixture on SOURCE 30RPC before and after incubation for one week at 40 °C in 1 M HCl, and similarly, in 1 M NaOH.

Column:	SOURCE 5RPC ST 4.6/150
System:	ÄKTA™explorer 10S system
Sample mixture:	1. (Ile^7) Angiotensin III
	2. (Val^4)Angiotensin III
	3. Angiotensin III
	4. Angiotensin I
Sample concentration:	0.1 mg/ml of each peptide
Sample volume:	20 µl
Eluent A:	20 mM Boric acid/NaOH, pH 10.0
Eluent B:	Acetonitrile
Flow:	1.0 ml/min
Gradient:	5–35% B over 15 minutes (6 CV)
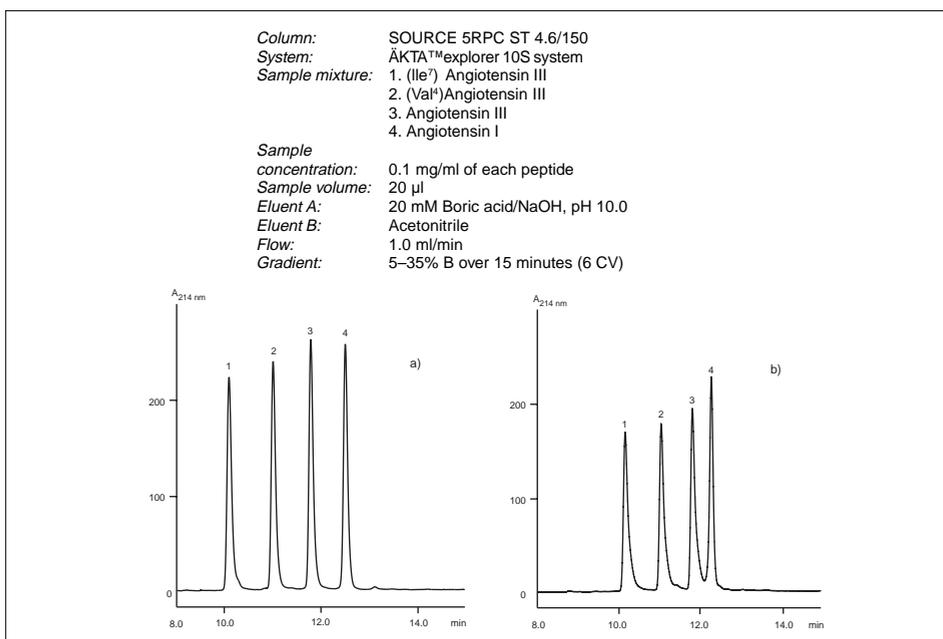
Fig. 23a and 23b. Separation of peptides on SOURCE 5RPC ST 4.6/150. **Figure a** shows the first injection of the peptide mixture and **Figure b** the 1000th injection. CIP with 1 M HCl and 1 M NaOH was performed after 275, 400, 600 and 800 runs.

SOURCE RPC media and FineLINE™, HR, RESOURCE RPC and ST columns are resistant to all solvents commonly used in reversed phase chromatography, such as 0.1% TFA in water and 0.1% TFA in acetonitrile. SOURCE RPC is resistant to other organic solvents such as methanol, isopropanol, ethanol, acetic acid, and tetrahydrofuran. Due to the inert aromatic/hydrocarbon structure of the polystyrene/divinylbenzene matrix, SOURCE RPC is stable to more disruptive chemical reagents, such as 6 M guanidine hydrochloride and 0.1% SDS.

Excellent Flow/ Pressure Characteristics

The uniform bead size and spherical shape of SOURCE RPC beads give stable densely packed beds with excellent flow properties unlike media with a wide range of particle sizes. The low operating back-pressure generated by SOURCE RPC allows higher flow rates to be used during separations and cleaning procedures while still giving excellent resolution, as demonstrated in Figure 24 which shows the performance maintained by SOURCE 30RPC even at high flow rates.

Column: SOURCE 30RPC, 10 mm i.d. x 100 mm column (8 ml)
Sample: Mixture of Ribonuclease A, Insulin and Albumin
Sample load: 1 mg/ml medium, total load
Solution A: 0.1% TFA
Solution B: 0.1% TFA/60% Acetonitrile
Flow: 150 and 600 cm/h
Gradient: 20–80% B, 20 column volumes (cv)
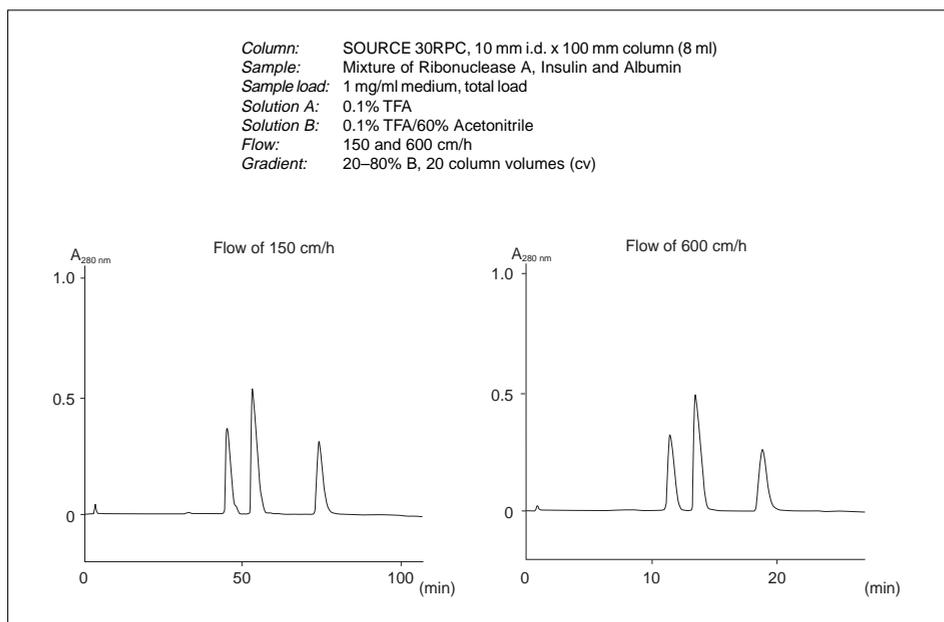
Fig. 24. The influence of increasing flow velocity on resolution.

Actual pressure values generated during a run will depend upon the solvent used and the operating temperature.

Figure 25 shows pressure versus flow curves with several solvents for RESOURCE RPC columns, packed with SOURCE 15RPC, and FineLINE columns, packed with SOURCE 30RPC.
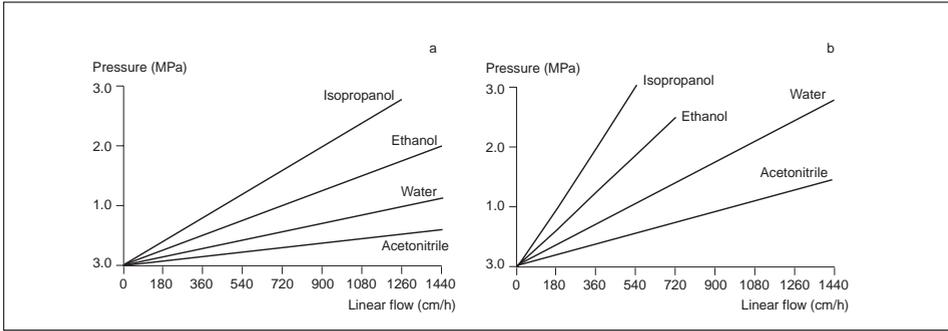
Fig. 25a. Pressure:flow curves for (a) RESOURCE RPC, 1 ml and (b) RESOURCE RPC, 3 ml with various organic solvents and water. (Work by Amersham Biosciences AB, Lillestrøm, Norway.)
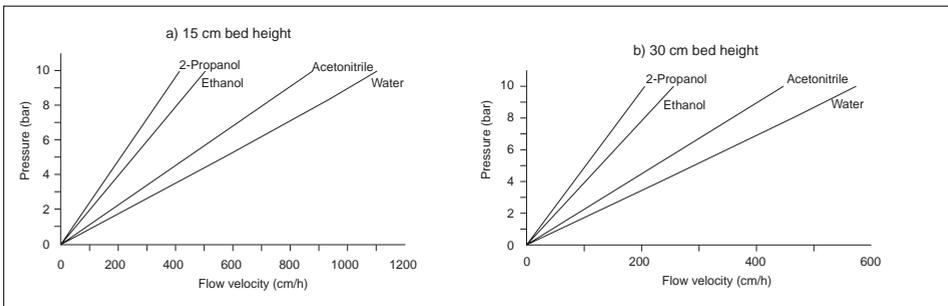
Fig. 25b. Pressure/flow characteristics of SOURCE 30RPC in various organic solvents and water at room temperature. The pressure/flow velocity data were determined in a FineLINE column with a) 15 cm and b) 30 cm bed height.

High Capacity

The controlled uniform pore size distribution in SOURCE RPC is responsible for the high capacities obtained for peptides, proteins and oligonucleotides. The dynamic binding capacity of SOURCE 15RPC is illustrated in Figure 26 while Figure 27 shows an example of the performance maintained by SOURCE 30RPC even with high sample loads.

Fig. 26. Dynamic binding capacity of SOURCE 15RPC. (Work by Amersham Biosciences AB, Lillestrøm, Norway.)
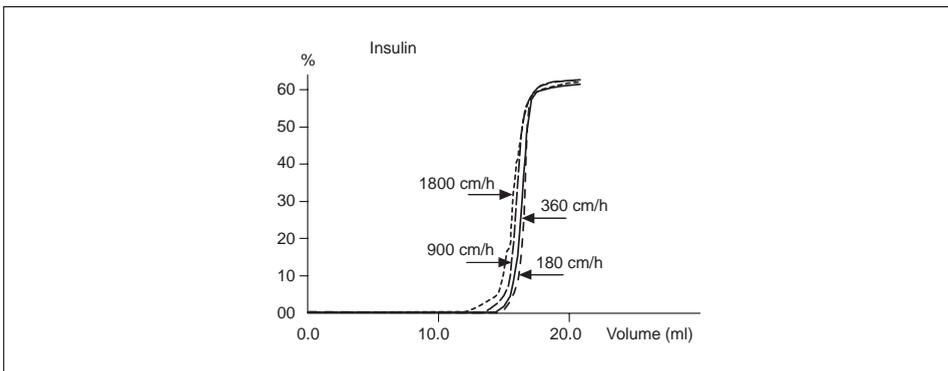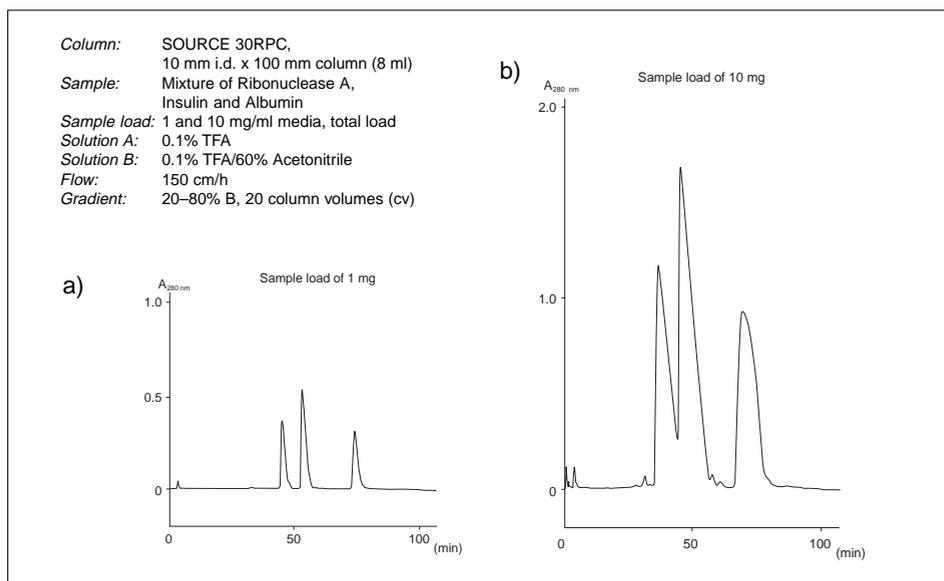
Fig 27. The influence of increasing sample load on resolution.

Availability

SOURCE 15RPC and SOURCE 30RPC are available in 10 ml, 200 ml, 500 ml, 1 litre and 5 litre pack sizes and should be packed in HR or FineLINE columns according to the scale of the separation.

SOURCE 15RPC is also supplied pre-packed in PEEK or stainless steel ST columns: RESOURCE RPC 1 ml is recommended for rapid screening experiments where as RESOURCE RPC 3 ml and SOURCE 15RPC 4.6/100 are better suited for applications where higher resolution is necessary.

SOURCE 5RPC is supplied in stainless steel 4.6/150 columns and is recommended for small scale or analytical separations which require the higher resolution which can be achieved using the smaller 5 µm bead size.
All pre-packed columns are fully compatible with ÄKTAdesign and other high performance liquid chromatography systems. Ordering information is shown in Section 7.

Table 3. Pre-packed SOURCE RPC columns

Column	Dimensions (i.d. x bed height) mm	Recom- mended flow (ml/min)	Efficiency N/m	Maximum flow (ml/min)	Maximum operating pressure (MPa, bar, psi)
RESOURCE 1 ml	6.4/30	1.0 - 5.0	> 12 000	10	3, 30, 435
RESOURCE 3 ml	6.4/ 100	1.0 - 5.0	> 12 000	10	3, 30, 435
SOURCE 15RPC ST 4.6/100	4.6/100	0.5 - 2.5	> 20 000	5.0	4, 40, 580
SOURCE 5RPC ST4.6/150	4.6/100	1.0	> 60 000	1.5	40, 400, 5800

μRPC C2/C18
Product Description

μRPC C2/C18 is a porous microparticulate silica (3 μm) to which C2 and C18 alkyl chains have been covalently bonded. μRPC C2/C18 is ideally suited for peptide mapping, analysis and micropurification. The extremely small particle size ensures high efficiency and excellent resolution of complex samples, as shown in Figure 28.

Fig. 28. Separation of a tryptic digest of equine and bovine cytochrome c on μRPC C2/C18 SC 2.1/10.

System: SMART™ System
Column: μRPC C2/C18 SC 2.1/10
Sample: Equine and bovine cytochrome c digested with trypsin
Buffers: A. 0.15% trifluoroacetic acid (TFA) in water
B. 0.14% TFA in acetonitrile/water, 60/40
Flow: 250 μl/min
Gradient: 0% B for 2 min
0–33% B for 20 min
33–55% B for 42 min
55–100% B for 5 min
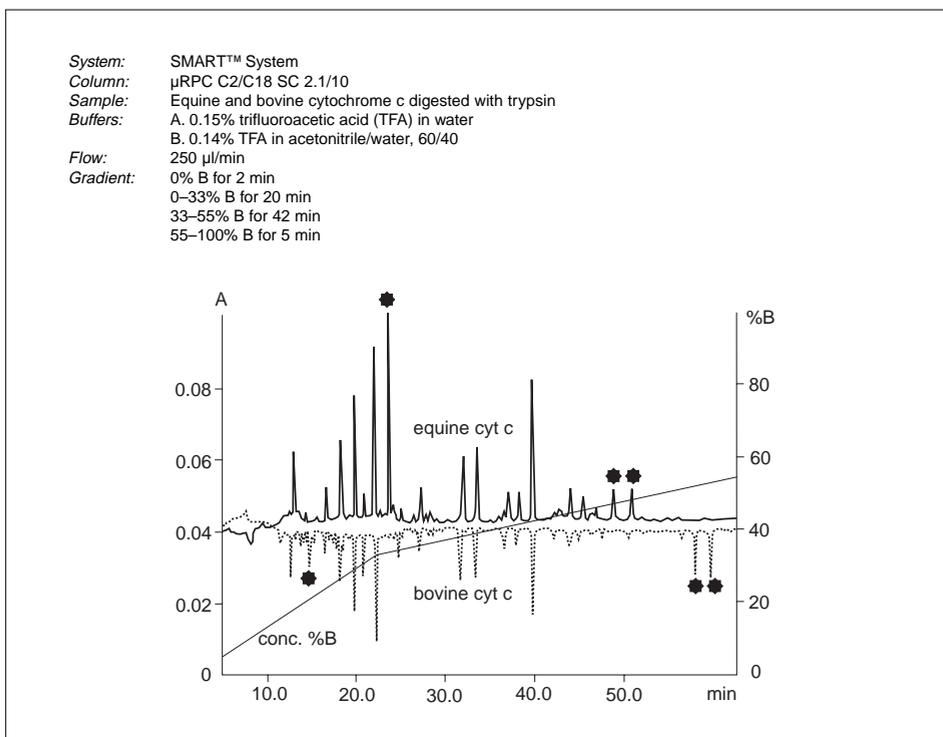
μRPC C2/C18 is supplied in 2 pre-packed column formats. μRPC C2/C18 PC 3.2/3 can be used at relatively high flow rates in many micro-preparative applications. The longer bed height of μRPC C2/C18 SC 2.1/10 produces extremely high resolution of complex mixtures where long, shallow elution gradients are often used. Characteristics of these columns are shown in Table 4.

	μRPC C2/C18 PC 3.2/3 (glass column)	μRPC C2/C18 SC 2.1/10 (stainless steel column)
Dimensions i.d. x bed height (mm)	3.2 x 30	2.1 x 100
Efficiency (N/m)	>90 000	>100 000
Operational pressure limit (MPa, bar,psi)	15, 150, 2250	25, 250,3625
Pore size	120 Å	120 Å
Particle size	3 μm	3 μm
Recommended flow (ml/min)	0.01 - 1.2	0.01 - 0.25
Practical loading capacity (μg protein/peptide/column)	0.2 - 500	0.01 - 500

Table 4.

Chemical and Physical Stability

μRPC C2/C18 can be used with aqueous and organic solvents miscible in water in the pH range 2 - 8. As with all silica based media extended exposure to pH extremes should be avoided as the matrix begins to degrade at pH values greater than 7 - 8 and less than 2 - 3. SOURCE 5RPC media should be chosen whenever separation conditions demand pH conditions to be above pH 8.0.

Additives such as guanidine hydrochloride, urea, formic acid (< 60%) and detergents may be used with μRPC C2/C18. Both columns may be operated over the temperature range of 4 - 40 °C up to the maximum pressures shown in Table 4.

Flow/Pressure Characteristics

Higher flow rates than those specified in Table 4 are possible with low viscosity solvents, but the integrity of the packing may be compromised if the pressure limit is exceeded.

Capacity

The maximum capacity of peptides for the μRPC C2/C18 PC 3.2/3 column is approximately 1 - 3 mg and for μRPC C2/C18 SC 2.1/10 approximately 1 - 2 mg. However, to minimise the risk of losing unbound material in the flow-through fractions or losing resolution, lower and more practical loading ranges are recommended. The detection limit for one peak may be below 1 ng under optimal conditions.

Both columns are designed specifically for use with SMART System. They can be connected to ÄKTApurifier or other high performance chromatography systems via a Precision Column Holder (Code No. 17-1455-01). Ordering information is shown in Section 7.

Sephasil Protein/Sephasil Peptide
Product Description

Sephasil are porous silica-based media giving excellent resolution and offer alternative selectivities compared to SOURCE RPC media. Carefully controlled production conditions ensure batch-to-batch reproducibility for consistent performance in both analytical and process scale applications.

Sephasil Protein and Sephasil Peptide are available with three different selectivities C4, C8 and C18. Sephasil Protein C4 is based on a wide-pore 300Å silica that makes it particularly suitable for proteins, whereas Sephasil Peptide is based on a 100Å silica which is more suitable for smaller biomolecules. Sephasil 5 µm media are recommended for high resolution analysis and purification and Sephasil 12 µm media for preparative purification of peptides, proteins or oligonucleotides. Characteristics of Sephasil pre-packed columns are shown in Table 5.

Column (bonded phase particle size dimensions i.d. mm/bed height mm)	Pore size (Å)	Specific pore volume (ml/g)	Maximum operating pressure* (MPa, bar, psi)	Efficiency (N/m)	Recom- mended flow (ml/min)
Sephasil Protein C4 5 µm ST 4.6/100	300	0.6	25, 250,3625	>70 000	0.5 - 2.0
Sephasil Peptide C8 5 µm ST 4.6/100	100	0.7	"	"	"
Sephasil Peptide C18 5 µm ST 4.6/100	100	0.7	"	"	"
Sephasil Protein C4 5 µm ST 4.6/250	300	0.6	25, 250,3625	>70 000	"
Sephasil Peptide C8 5 µm ST 4.6/250	100	0.7	"	"	"
Sephasil Peptide C18 5 µm ST 4.6/250	100	0.7	"	"	"
Sephasil Protein C4 12 µm ST 4.6/250	300	0.6	25, 250,3625	>40 000	0.5 - 2.0
Sephasil Peptide C8 12 µm ST 4.6/250	100	0.7	"	"	"
Sephasil Peptide C18 12 µm ST 4.6/250	100	0.7	"	"	"
Sephasil Protein C4 12 µm ST 10/250	300	0.6	25, 250,3625	>40 000	2 - 8
Sephasil Peptide C8 12 µm ST 10/250	100	0.7	"	"	"
Sephasil Peptide C18 12 µm ST 10/250	100	0.7	"	"	"
Sephasil Protein C4 12 µm ST 20/250	300	0.6	25, 250,3625	>40 000	5 - 20
Sephasil Peptide C8 12 µm ST 20/250	100	0.7	"	"	"
Sephasil Peptide C18 12 µm ST 20/250	100	0.7	"	"	"
Sephasil Protein C4 12 µm ST 50/250	300	0.6	14, 140, 2000	>40 000	20 - 60
Sephasil Peptide C8 12 µm ST 50/250	100	0.7	"	"	"
Sephasil Peptide C18 12 µm ST 50/250	100	0.7	"	"	"

*refers to pressure above which bed compression may begin
Table 5.

Chemical and Physical Stability

The chemical and physical stability of Sephasil media ensure consistent performance. Sephasil is resistant to all solvents commonly used in reverse phased chromatography with an operational pH range of pH 2 - 8. As with all silica based media, extended exposure to pH extremes should be avoided as the matrix begins to degrade at pH values greater than 7 - 8 and less than 2 - 3. SOURCE RPC media should be considered as an alternative if the separation conditions demand pH conditions to be above pH 7.5.

Additives such as guanidine hydrochloride, urea, formic acid (< 60%) and detergents may be used. Columns may be used at pressures up to 25 MPa over a temperature range of 4 - 70 °C.

Flow /Pressure Characteristics

The flow/pressure characteristics of Sephasil Protein and Sephasil Peptide columns are shown in Table 5. The final pressure values generated during a run will depend upon the solvent used, the operating temperature as well as the bed height of the column. Similarly, higher flow rates may be possible with low viscosity solvents, but the integrity of the medium may be compromised if the pressure limit is exceeded.

Availability

Sephasil Protein and Sephasil Peptide are available in the pre-packed columns shown in Table 5 and in 100g and 1kg pack sizes. Sephasil columns are fully compatible with ÄKTAdesign and other high performance chromatography systems. Ordering information is shown in Section 7.

3. Methods

The following sections will discuss the practical steps involved in planning and implementing a reversed phase separation. Critical aspects of selecting the appropriate stationary phase, mobile phase and gradient conditions are discussed. Practical considerations of sample preparation, solvent handling, and potential pitfalls which may be encountered will also be presented.

Choice of separation medium

The proper choice of reversed phase medium is critical for the success of a particular application. This choice should be based on the following criteria:

1) The unique requirements of the application, including scale and mobile phase conditions.
2) The molecular weight, or size of the sample components.
3) The hydrophobicities of the sample components.
4) The class of sample components.

Unique requirements of the application

Resolution

It is essential to choose a medium which will yield the required resolution for a given purification. Applications involving fractionation of multi-component samples, such as peptide mapping, require extremely high resolution. Preparative reversed phase applications, such as the purification of synthetic peptides, are more concerned with throughput, and resolution is routinely traded off against both speed and capacity.

Resolution in reversed phase chromatography depends on both the selectivity and the efficiency of the column. Selectivity for a specific separation depends on the nature of the immobilised hydrophobic ligand, the chemistry used to derivatise the silica matrix and chromatographic conditions used, such as the composition of the mobile phase.

The maximum efficency of a reversed phase column depends on the process used to pack the column and the particle size of the medium. The smallest particles, e.g. μRPC C2/C18, give the highest efficiency followed by the larger 5 μm media, such as SOURCE 5RPC, Sephasil Protein 5 μm and Sephasil Peptide 5 μm. Lower efficiency is seen with larger 12 μm, 15μm and 30 μm particles such as Sephasil Protein 12 μm, Sephasil Peptide 12 μm, SOURCE 15RPC and SOURCE 30 RPC.

Scale of the purification

For micro-purification narrow bore columns packed with 3 or 5 μm particles should be used e.g. μRPC C2/C18 or SMART columns Sephasil C8 and Sephasil C18. The ligands C2/C18, C8 and C18 will offer significantly different selectivities from which to choose.

For typical laboratory scale purification 5 μm media can be selected e.g. SOURCE 5RPC, Sephasil Protein 5 μm and Sephasil Peptide 5 μm. It may be more suitable to use 12 μm media for particularly crude samples or SOURCE 15RPC media, if a high pH or alternative selectivity to silica is required for a successful separation.

Media with larger particle sizes should always be chosen for pilot and process scale purification, using their equivalent smaller particle size media for method scouting prior to scale up e.g. RESOURCE RPC pre-packed columns exhibit similar selectivity to SOURCE 30RPC.

Mobile phase conditions

Choice of reversed phase matrix may also be influenced by the composition of the mobile phase. If the stability or solubility of the sample components dictates the use of a specific solvent system, then the stability of the base matrix in that solvent needs to be considered. For example, when the reversed phase chromatography will involve mobile phases with pH above 7.5, a polystyrene-based matrix, such as SOURCE RPC, should be used.

Throughput and scaleability

The amount of sample material that can be processed within a defined time (i.e. throughput) is determined by such properties as capacity, flow characteristics and the size of the column the medium is packed into. SOURCE 15RPC, SOURCE 30RPC, Sephasil Protein 12 μm and Sephasil Peptide 12 μm have been optimised for throughput in large scale preparative chromatography with easy scale up under high performance conditions.

Molecular weight of the sample components

The accessibility of the sample components to the immobilised hydrophobic ligands will determine the available capacity of the reversed phase medium for a specific biomolecule. Accessibility depends greatly on the pore size and the pore volume of the bead. Reversed phase media with large pores will typically give better capacity and resolution with larger biomolecules than media with small pores. Generally, pore sizes of 300 Å or larger are recommended for the separation of proteins. Pore sizes less than 300 Å are recommended for the separation of peptides and oligonucleotides. The pore sizes and capacities for the reversed phase media supplied by Amersham Biosciences are given in Chapter 2.

Hydrophobicity of the sample components

Unlike other chromatographic techniques, such as ion exchange and size exclusion chromatography, it is difficult, if not impossible, to predict the retention of biomolecules in reversed phase chromatography. Attempts to predict retention on the basis of hydrophobicity factors derived from studies of standard peptides have been the subject of several studies. One which provides an algorithm which has been shown to mimic quite closely the retention behaviour of protein samples subjected to proteolytic digestion has been described by Sakamoto (7). The general benefit from such studies has been an improved understanding of the binding process. The important parameters affecting the retention of a peptide appear to be a combination of the amino acid sequence of the peptide together with any secondary structure, such as a-helices and b-pleated sheets, that the peptide may possess. The situation for proteins is further complicated by their tertiary structure.

The selection of a reversed phase medium must, therefore, be made empirically, depending on the nature of the biomolecule components in the sample. Some prediction of resolution can be made based on the hydrophobicity of the sample relative to the immobilised ligand, i.e. the more hydrophobic the sample, the less hydrophobic the immobilised ligand should be. Consequently, a medium with C8 ligands, is generally recommended for preparative purification of more hydro-phobic biomolecules than those purified by the corresponding C18 medium.

Choice of reversed phase matrix may also be influenced by the composition of the mobile phase. When the reversed phase chromatography will involve mobile phases with pH above 7.5, a polystyrene-based matrix, such as SOURCE RPC, should be used. When the stability of the sample components dictates the use of a specific solvent system, then the stability of the base matrix in that solvent needs to be considered.

Class of sample components

Reversed phase chromatography is used for purification of many classes of biomolecules.

Whilst the conditions of reversed phase chromatography usually have no harmful effects on the chemical integrity of oligonucleotides and peptides, the question of the stability and biological activity of proteins must be considered carefully. Polypeptide interactions with a hydrophobic surface and organic solvents generally leads to some loss of tertiary structure. The loss in tertiary structure may then give rise to different conformational states for a given biomolecule (8) and each of these states may interact differently with the reversed phase medium.

The widespread use of reversed phase chromatography for the large-scale purification of recombinant and synthetic proteins and peptides, such as insulin, growth hormone, growth factors and many others, indicates, however, that problems caused by denaturation can often be overcome. Loss of structure and, consequently, loss of activity can be minimised by proper treatment. The kinetics

be reversed by transferring the protein to conditions under which its native structure is favoured. Note that complex enzymes and multi-component proteins are more likely to lose activity than small peptides or highly stabilised and cross-linked proteins. However, when a protein or peptide is purified for primary structure determination, denaturation is not a problem unless precipitation occurs and reversed phase chromatography has found widespread use in preparing pure biomolecules for subsequent sequencing.

Choice of mobile phase

The mobile phase in reversed phase chromatography of biomolecules generally contains a "buffer" component, an organic modifier and, often, an ion pairing agent added to the mobile phase to affect selectivity.

All solvents, buffering salts, ion pairing agents, as well as the water used to prepare the mobile phases, must be of high chemical purity and should be free of any metal ions. Solvents, salts and water should be labelled as "HPLC grade" to ensure sufficient purity. Chemical purity is important in preparative reversed phase chromatography, since any contaminants in the mobile phase may affect the chromatography by producing unwanted extra peaks, ghost peaks, and may contaminate the purified biomolecule.

The organic solvent

The organic solvent is added to the mobile phase to lower its polarity. Two solvents with different polarity, such as an aqueous low pH solution and acetonitrile, may be mixed together resulting in a solvent with polarity intermediate between those of the original components. The lower the polarity of the solvent mixture, the higher its eluting power in reversed phase chromatography. There is a large number of water miscible organic solvents that can be used in reversed phase chromatography but few of them are used in practice. The properties of some typical organic solvents used in reversed phase chromatography are shown in Table 6.

Table 6. Solvents used in reversed phase chromatography.

Solvent	Boiling point (°C)	UV cut-off* (nm)	Viscosity (cP at 20 °C)	Comments
Acetonitrile	82	190	0.36	More powerful denaturant than alcohols. Toxic.
Ethanol	78	210	1.20	
Methanol	65	205	0.60	
1-propanol (n-propanol)	98	210	2.26	Viscous
2-propanol (iso-propanol)	82	210	2.30	Viscous
Water	100	<190	1.00	

Absorbance is approx. 1 for HPLC-grade solvent at this wavelength.

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Acetonitrile and methanol are the most commonly used since both have low viscosity (even when mixed in aqueous solution) and are UV transparent. 2-Propanol has the advantage of lower polarity and therefore higher eluting strength. It is UV transparent and is excellent for cleaning the reversed phase column. However, use of 2-propanol as the organic modifier results in high viscosity mobile phases. High viscosity mobile phases are undesirable since they result in poor mass transport of solute between the mobile and stationary phases and high back-pressure even at moderate to low flow rates. Figure 29 shows the change in pressure during gradient formation due to the viscosity effects of various solvents.

Fig. 29. Variation of pressure during elution with a gradient from 100% water to 100% organic solvent at constant flow. Note that the pressure increases by ca. 50% from the starting value (pure water) during elution with methanol. The increase in the case of acetonitrile is much smaller, ca. 10%. (Work by Amersham Biosciences AB, Uppsala, Sweden.)


```
Column:   C18 silica, 10 mm (i.d. 4.6 mm, length 250 mm).
Eluent A: 100% water
Eluent B: (A) 100% acetonitrile
          (B) 100% methanol
Gradient: 0-100% B in 10 min
          100% B for 2 min
          100-0% B in 3 min
          0% B for 2.5 min
Flow:     1.75 ml/min
```
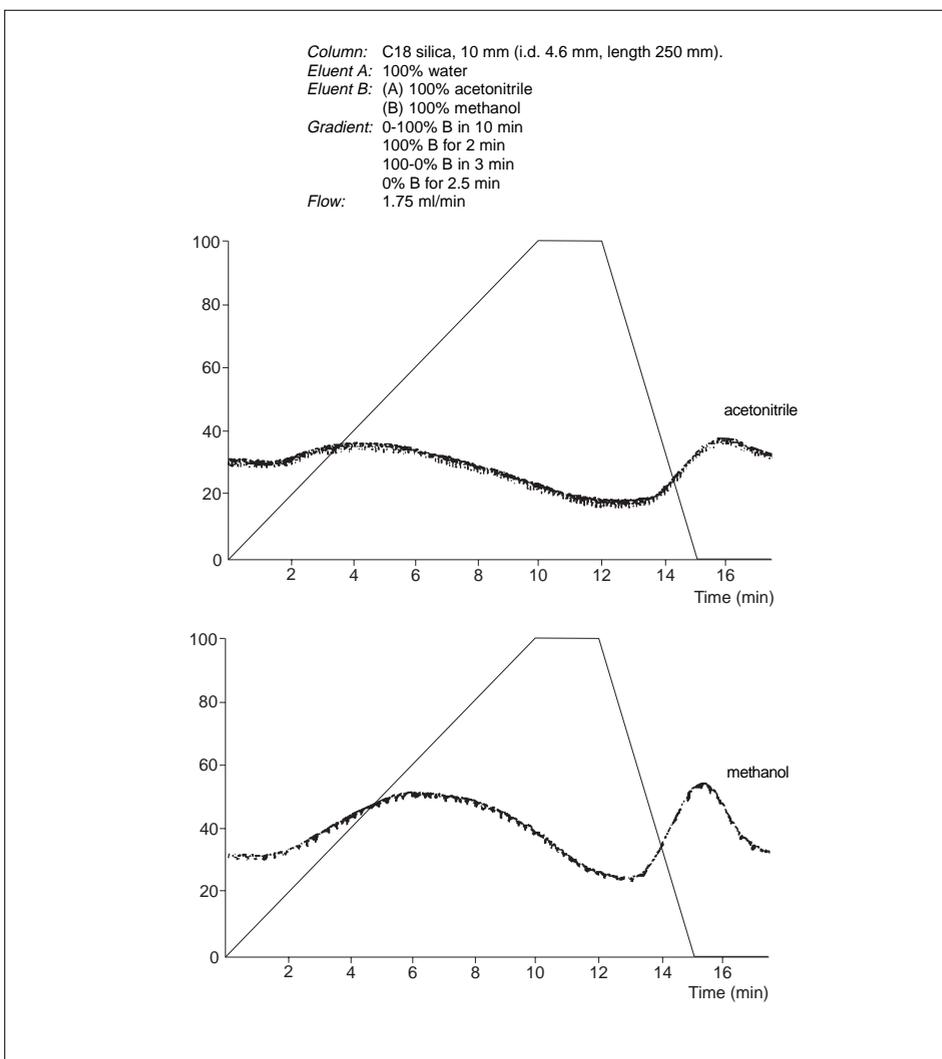
Only high quality organic solvents should be used in reversed phase chromatography. HPLC grade solvents are desirable since small particulates that can clog reversed phase columns have been removed. They provide adequate chemical purity with good transparency to low wavelength UV light. Organic solvent in the eluting buffer is generally removed from the recovered biomolecule by evaporation and residual organic contaminants, such as acrylic acid, can be damaging to the biological integrity of the recovered sample. UV transparency is especially important since many reversed phase separations are monitored below 220 nm for optimum detection sensitivity (e.g. peptides, proteins lacking significant amounts of Trp or Tyr, etc.). The UV cut-off value for an organic solvent used in reversed phase chromatography should ideally be below 210 nm. This will provide better baseline stability when running gradients in which the content of organic modifier is varied.

pH

Reversed phase separations are most often performed at low pH values, generally between pH 2 - 4. The low pH results in good solubility of the sample components and ion suppression, not only of acidic groups on the sample molecules, but also of residual silanol groups on the silica matrix. Acids such as trifluoroacetic acid, heptafluorobutyric acid and ortho-phosphoric acid in the concentration range of 0.05 - 0.1% or 50 - 100 mM are commonly used.

Mobile phases containing ammonium acetate or phosphate salts are suitable for use at pH's closer to neutrality. Note that phosphate buffers are not volatile.

Polystyrene-based reversed phase matrices, such as SOURCE RPC, allow reversed phase chromatography to be performed routinely at pHs well above neutral. The advantage of performing chromatography at these elevated pH values include increased control of selectivity and, in some cases, improved solubility and yield of active sample components. Basic peptides often tail during elution from reversed phase columns at low pH. Better resolution of basic peptides is often achieved above pH 8.

The acids, bases or buffering salts used to control pH must also be transparent to UV below 220 nm and must be soluble under the low polarity conditions of the mobile phases used in reversed phase chromatography.

It is convenient, but not essential, that the buffer salts and acids used to prepare reversed phase mobile phases are volatile. Volatile buffer components can be removed from the eluted sample by evaporation along with the organic component. If non-volatile salts are used in the mobile phase, they must be separated from the recovered sample by an additional desalting step.

Sample: Angiotensin II (0.25 mg/ml), Angiotensin III (0.25 mg/ml)
Column: RESOURCE RPC 3 ml (i.d. 6.4 mm, length 100 mm).
Eluent A: (A) 0.1% TFA in water, pH 2
(B) NaOH 10 mM in water, pH 12
Eluent B: A) 0.1% TFA, 60% acetonitrile in water
(B) NaOH 10 mM, 60% acetonitrile in water
Gradient: 10-65% B in 10 min
**Flow:** 2 ml/min
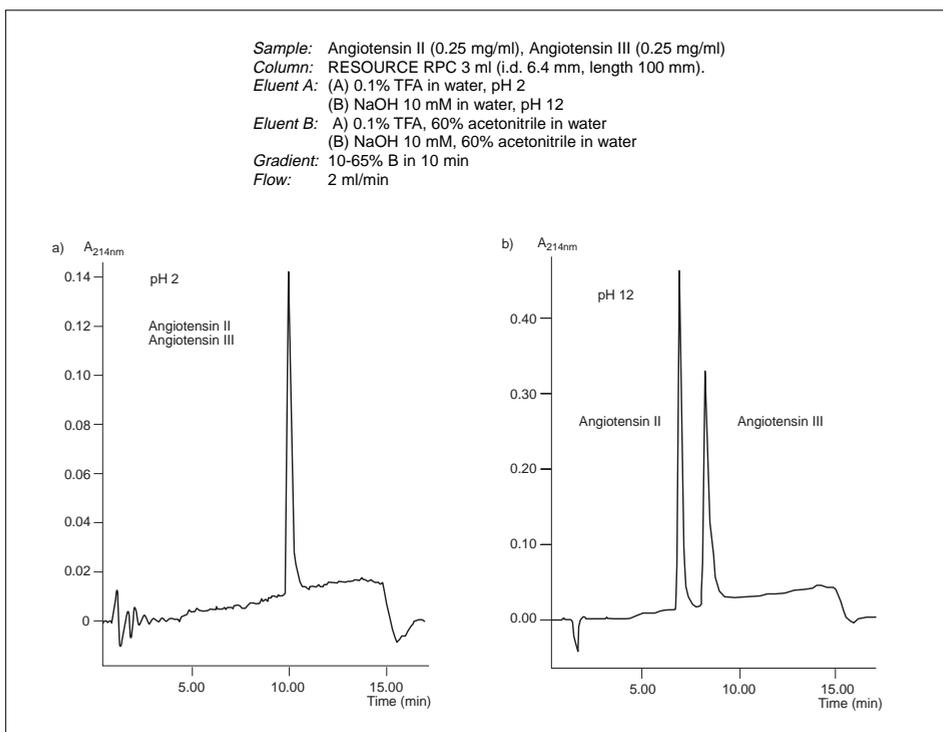
Fig. 30. Separation of angiotensin II and angiotensin III at a) pH 2 and b) pH 12. The selectivity is changed significantly by changing the pH. (Work by Amersham Biosciences AB, Uppsala, Sweden.)

Table 7. Examples of mobile phases for use at different pH.

Buffer	Approximate pH	Comments
Hydrochloric acid	2 - 3	
Phosphoric acid	2 - 3	Non-volatile.
Trifluoroacetic acid (TFA)	2 - 3	
Triethylammonium phosphate (TEAP)	6	Non-volatile.
Ammonium acetate	6 - 7	
Sodium hydroxide	12	Non-volatile. Only with stationary phases based on organic polymers.

Ion pairing agents

The third component usually added to the mobile phase is an ion pairing agent. As described in Chapter 1, ion pairing agents are thought to bind by ionic interaction to the solute molecules to increase the hydrophobicity of the solute molecule and change selectivity. Aside from the fact that some reversed phase separations (such as purification of synthetic oligonucleotides) absolutely require the use of ion pairing agents, their greatest advantage is in affecting selectivity
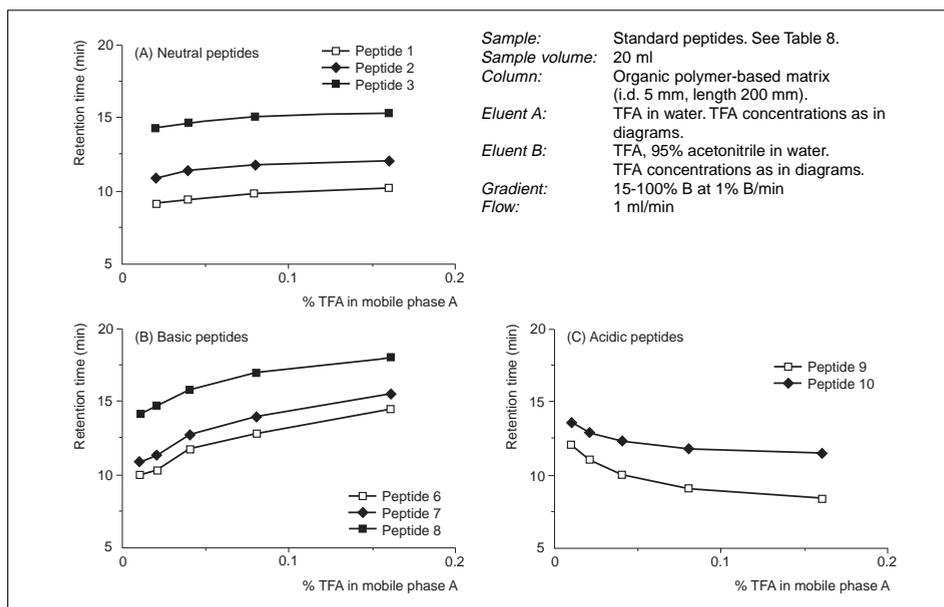
Fig. 31. Variation of retention time with concentration of the anionic ion pairing agent (TFA) for (A) neutral, (B) basic and (C) acidic peptides. (Work by Amersham Biosciences Inc., Piscataway, NJ, USA.)

thereby improving the chances for complete resolution of sample components. The retention behaviour of the sample components may be affected by both the type and concentration of ion pairing agent used. The effects of varying the concentration of trifluoroacetic acid on peptide retention time depend on the type of peptide (neutral, acidic or basic) (Fig. 31).

Table 8. Standard neutral, basic and acidic peptides used for Fig. 31.

Peptide	Type	Amino acid sequence										
1	Neutral	Trp	Val	Pro	Thr	Asn	Val	Gly	Ser	Glu	Ala	Phe
2	Neutral	Glu	His	Trp	Ala	Tyr	Gly	Leu	Gln	Pro	Gly	
3	Neutral	Glu	Ala	Asp	Pro	Asn	Lys	Phe	Tyr	Gly	Leu	Met
6	Basic	Tyr	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	
7	Basic	Asn	Arg	Val	Tyr	Val	His	Pro	Phe	Asn	Leu	
8	Basic	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Gly	Leu	Met	
9	Acidic	Lys	Gly	Asp	Glu	Glu	Ser	Leu	Leu	Ala		
10	Acidic	Trp	Ala	Gly	Gly	Asp	Ala	Ser	Gly	Glu		

Some ion pairing agents, such as trifluoroacetic acid, also act to maintain the pH of the mobile phase. Typical concentration ranges for ion pairing agents in the mobile phase are 0.01% to 0.1% or between 10 - 100 mM. Some common anionic and cationic ion pairing agents are shown in Table 9.

Table 9. Ion pairing agents.

Ion pairing agent	Formula of pairing ion	Comments
Anionic		
Trifluoroacetic acid (TFA)	CF_3COO^-	Low UV-absorbance. Volatile, low pH,
Pentafluoroproprionic acid (PFPA)	$CF_3CF_2COO^-$	More hydrophobic than TFA. Volatile, low pH
Heptafluorobutyric acid (HFBA)	$CF_3CF_2CF_2COO^-$	More hydrophobic than TFA. Volatile, low pH
Ammonium acetate	CH_3COO^-	
Phosphoric acid	$H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}	Less hydrophobic than TFA.
Cationic		
Tetramethylammonium chloride	$^+N(CH_3)_4$	
Tetrabutylammonium chloride	$^+N(C_4H_9)_4$	
Triethylamine	$NH^+(C_2H_5)_3$	

Again, the requirements for the ion pairing agent are that it is sufficiently pure, it is UV transparent below 220 nm and it is soluble under the low polarity conditions of the mobile phase. Additionally, volatile ion pairing agents ensure that sample recovery will not require a separate desalting step.

Sample preparation

The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually effect the separation so long as the volume of the sample loaded is small compared to the column volume. The only effect when large sample volumes are applied may be an extra peak or two eluting in the void volume after sample injection. In situations where the sample is soluble in mobile phase A (or a reasonable facsimile thereof) and the sample component of interest will bind to the column, the volume of the sample usually does not have any effect on the subsequent chromatography. Since reversed phase chromatography is an adsorption technique, a large volume of dilute sample can be concentrated and purified in a single step.

It is recommended that the sample is used directly after solubilization to minimise any undesirable side reactions such as oxidation of the sample components. All samples should be centrifuged at 10,000 g for 10 min prior to injection of sample or, alternatively, filtered through a 0.22 μm (or 0.45 μm) sterile filter. Be sure to choose a solvent resistant filter if the sample solution contains organic modifier.

For particularly dirty samples, desalting through a large particle version of a reversed phase or a size exclusion medium can be done to remove gross contaminants that may foul a more valuable reversed phase column. Some columns have a replaceable, in line "guard" column that prevents dirty samples from fouling the main body of the reversed phase column.

It is important to maintain sample solubility throughout the loading process in order to avoid precipitation of the sample on the column. Sample precipitation can be avoided by not overloading the column and by ensuring that the sample is sufficiently soluble in the sample solvent prior to injection on the column.

Mobile phase preparation

As stated before, all solvents and additives used to prepare the mobile phases should be of HPLC grade or, when not available, of the highest purity grade. The solvents used to prepare mobile phases should also be de-gassed in a sonic bath for 10 - 15 minutes to prevent gas formation under elution conditions. Alternatively, they may be de-gassed under vacuum with magnetic stirring or by sparging with helium.

All mobile phases to which solids have been added should be filtered through 0.22 µm filters before use to prevent particles from clogging the reversed phase column.

When adding volatile ion pairing agents to the mobile phases (e.g. trifluoroacetic acid), the ion pairing agent should be added after de-gassing is complete. In this way, the concentration of ion pairing agent is not altered by the de-gassing process.

Storage of mobile phases

A general rule is to use freshly prepared mobile phases in reversed phase chromatography. If the solvents have to be stored, it is important that the reservoirs are closed to avoid changes in the solvent composition caused by evaporation. Aqueous solutions at neutral pH should not be stored for more than 2 - 3 days because of the risk of microbial growth. For long term storage, store all solvents covered at 4 °C. Cold solvents must be allowed to come to running temperature and then be de-gassed before use. This will reduce the risk of bubble formation in the system.

Solvent disposal

Unlike the aqueous salt solutions used as mobile phases for other types of biomolecule chromatography, reversed phase chromatography employs organic solvents in addition to aqueous buffers. The mobile phases must be handled as toxic chemical waste and disposed of properly. This includes methanol, which itself is not a significant chemical pollutant but will probably contain additives such as TFA, HFBA, etc. Under process conditions where large volumes of organic solvents are used to prepare mobile phases, it is generally more economical to recycle waste mobile phase.

Skin exposure to organic solvents should be avoided, especially with acetonitrile, as should breathing of any fumes. It is advisable to work with these solvents under a fume hood when preparing mobile phases. The same precautions should be exercised when dealing with organic amines such as triethylamine (TEA) which is used as an ion pairing agent at neutral and basic pH.

Extreme care must be followed when dealing with mineral acids, such as hydrochloric acid, ortho-phosphoric acid, etc. and perfluorinated organic acids such as TFA, PFPA and HFBA. These acids can cause severe skin burns and inhalation of fumes should be avoided. These substances should be handled with the utmost caution and always within the secure confines of a well ventilated fume hood. Be sure to wear adequate protection such as gloves, goggles and a lab coat.

Detection

It is essential to use reagents and solvents of high purity to ensure minimum detection limits for optimum sensitivity. All organic solvents and many additives, such as ion pairing agents, absorb in the UV range and the detection limit is related to the wavelength (9). Detection below 220 nm is necessary when separating short peptides that lack aromatic amino acid residues such as Trp and Tyr. Synthetic oligonucleotides absorb in the region 250 - 260 nm and proteins absorb at 280 nm. Detection difficulties are generally manifest when separating short peptides since they require the shortest wavelengths for detection. The solvents and additives recommended in this manual have been chosen on the basis of providing optimal separation in combination with low background absorbance (see Tables 6, 7, 9).

Ghosting

A common problem caused by poor quality mobile phase components is a phenomenon referred to as "ghosting". Trace levels of organic impurities in the mobile phases can bind to the reversed phase medium and concentrate during the equilibration and sample loading steps. Upon elution of the column with organic modifier, the bound organic contaminants desorb and appear in the chromatogram as unknown, or "ghost" peaks. The size of a ghost peak will usually depend on the equilibration time and the level of organic impurities in the mobile phase.

Ghosting may also be caused by incomplete elution of sample components in a previous run. A blank gradient, with no sample, should be run as a check, especially if subsequent runs are to be performed with high sensitivity detection.

Mobile phase balancing

When reversed phase columns are eluted with a gradient in the mobile phase, it is frequently noticed that the baseline drifts. During a typical run, where the pro-portion of mobile phase B in the gradient increases, the baseline can progressively increase or decrease in an approximately linear fashion (see, for example Fig. 30).

The drifting baseline may originate from an ion pairing agent (or strong acid component) or an organic modifier that absorbs significantly at the wavelength used to monitor the chromatography (10).

The background absorbance of the initial mobile phase A is corrected for during the equilibration of the column. As the run is executed, the chemical environment of the mobile phase changes dramatically as the proportion of organic component is steadily increased. The absorption properties of the buffer components can change as the solvent characteristics of the eluting mobile phase change during progress of the gradient. If this happens there will be a gradual increase (or decrease) in the UV-absorption of the mobile phase as the gradient forms and a drifting baseline. It is interesting to note that the progress of the gradient is usually represented by the electronic signal from the two mixing pumps, but the baseline drift is a better reflection of the actual "chemical" gradient being produced.

A drifting baseline can be compensated by using different concentrations of UV-absorbing ion pairing agents (or buffer acids) in mobile phases A and B. In this way, the "concentrations" with respect to UV-absorption properties are balanced, and the baseline should approximate a straight line.

Because of batch-to-batch variations in the absorption properties of the components of the mobile phases and other differences between the conditions in different runs, it is not practical to give hard and fast recommendations for particular solvent systems. For gradients from TFA in water to TFA in acetonitrile it will usually be found that the concentration of TFA in acetonitrile will be need to be 10-30% lower than in water. However, the balanced concentrations of UV-absorbing components should be determined empirically. The difference in concentration of ion pairing agent between the two mobile phases is generally not large enough to affect the chromatography adversely.

Column conditioning

Reversed phase columns should be "conditioned" for first time use, after long term storage or when mobile phase conditions are changed significantly. The mobile phases used in the conditioning should be the same as those used in the subsequent chromatography. The general procedure for conditioning most reversed phase columns is as follows:

1)   Wash the column with approximately 3 column volumes of mobile phase B at a low to moderate flow rate appropriate for the particular column.

2)   Run a 2 - 3 column volume linear gradient from 100% mobile phase B to 100% mobile phase A at same flow rate as above.

3)   Equilibrate the column with at least 5 column volumes of mobile phase A. Continue equilibration until all monitor signals are stable.

4)   Every time the mobile phase system is changed, a blank run should be performed to check for any artifacts that might appear due to UV-absorbing impurities in the mobile phase. Again, return to 100% A and equilibrate to a stable baseline prior to sample injection.

Elution conditions

Reversed phase separations can be achieved using either a stepwise or a continuous gradient to elute sample components. Step gradients (i.e. a series of isocratic elutions at different % B) are useful for applications such as desalting, but for separations requiring high resolution, a linear, continuous gradient is required.

Step gradients are also ideal when performing process scale applications providing the desired resolution can be obtained, as less complex instrumentation is required to generate step gradients. Additionally, step gradients can be generated more reproducibly than linear gradients.

The ideal gradient shape and volume must be empirically determined for a particular separation. Generally, the sample to be purified is chromatographed using a broad range linear gradient to determine where the molecule of interest will elute. The initial conditions usually consist of mobile phase A containing 10% or less organic modifier and mobile phase B containing 90% or more organic modifier. The initial gradient runs from 0% B to 100% B over 10 - 30 column volumes. At a flow of 1 ml/min with a 1 ml column, this corresponds to a gradient slope between 3 - 10% B/min. A blank gradient is usually run prior to injecting sample in order to detect any baseline disturbances resulting from the column or impurities originating in the mobile phase.

Gradients can be measured either in volume mode or in time mode. In reversed phase chromatography, changes in flow (at constant gradient slope) appear to have little effect on resolution (Fig. 24). At constant flow, however, gradient slope has a significant effect on column resolution.

After the initial gradient has been run, the resolution of the peaks is evaluated to see whether improvement is needed or not. As shown previously, resolution can be improved by adjusting the components of the mobile phase but resolution can also be improved by using a shallower gradient (Fig. 32). This can be achieved either by increasing the gradient time or volume or by using a segmented gradient (Fig. 33).

When only one of the sample components is of interest, as in a large scale peptide or protein purification, the segmented technique is more economical with respect to both time and mobile phase consumption. Shallow gradients are especially useful in increasing the resolution of protein separations since the retention times for large protein molecules are particularly sensitive to subtle changes in mobile phase polarity.

Sample: Synthetic peptide: NVILTKPEVSEGTEVTVK
Column: Sephasil Peptide C18 5 µm ST 4.6/100
Eluent A: 0.06% trifluoroacetic acid (TFA), pH 2.5
Eluent B: 84% acetonitrile in 0.055% TFA
Gradient: (A) 0 - 60% (B) 20 - 35%
Flow: 1 ml/min
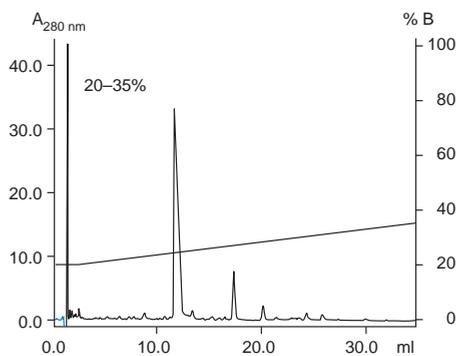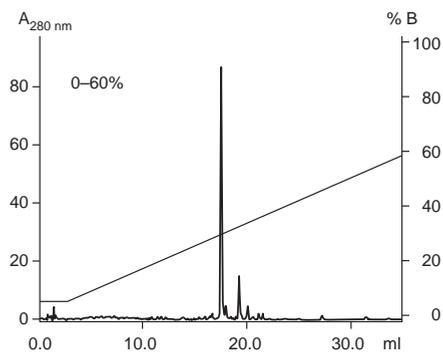
Fig. 32. Effect of gradient slope on resolution of a synthetic peptide at constant flow. (Work by Amersham Biosciences AB, Uppsala, Sweden)

Fig. 33. Schematic chromatograms showing effects expected in a segmented gradient compared with a linear gradient. Where the gradient is steeper (segments A and C), resolution is lost. Where the gradient is shallower (segment B), resolution is improved.
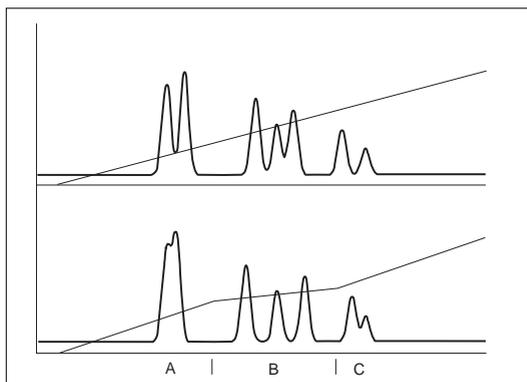
Column re-equilibration

Irrespective of the final concentration of the gradient used for the separation, the column should always be equilibrated with several column volumes of 100% mobile phase B to remove any remaining sample components. If the separation gradient is completed at lower level than 100% B, a gradient up to 100% B should be run, followed by equilibration at 100% B. The column is then re-equilibrated with mobile phase A, preferably in a gradient to avoid any risk of damaging the column packing by a too abrupt change in the composition of the mobile phase.

Column cleaning

Periodic column cleaning is recommended, especially when dealing with samples containing particulate matter and any column fouling contaminants. Increased back-pressure, loss of resolution or discoloration at the top of the bed (for glass columns) are all signs that the column may need to be cleaned. It should be noted that with silica-based reversed phase media, loss of resolution due to peak broadening may also indicate the presence of silanol groups on the silica gel surface or a head space in the column caused by dissolution of the matrix during frequent use above pH 7. Silanol groups can also form as a natural process of gel ageing during prolonged exposure to aqueous solvents during the normal course of the column lifetime. Poor resolution from the effect of exposed silanol groups can be minimised by ion suppression using low pH mobile phases.

A common procedure for cleaning a typical reversed phase column is as follows:

1) Equilibrate the column at a low flow rate with several column volumes of mobile phase A containing 0.1% TFA in water.
2) Run a gradient (approximately 20 - 30 column volumes) from 100% A to 100% mobile phase B where mobile phase B is 0.1% TFA in 2-propanol.
3) Equilibrate the column at 100% B for several column volumes and then bring the column back to 100% A using another gradient.

4) Equilibrate the column with several column volumes of mobile phase A before equilibrating the column with the initial mobile phase to be used in the subsequent chromatography. If the new mobile phase A is significantly different from 0.1% TFA in water then the column should be introduced to the new mobile phase A using a linear gradient.

Custom packed columns, such as those used in large scale and process applications, can also be cleaned with the medium in place since the reversed phase matrix (whether silica- or polystyrene-based) does not significantly shrink or swell with solvent changes.

SOURCE RPC can be cleaned using more aggressive chemical agents due to the extreme stability of the polystyrene-based matrix. A very effective cleaning procedure is equilibration with several column volumes of sodium hydroxide solution up to 0.5 M. Sodium hydroxide is an extremely effective cleaning agent and the ability to use it for cleaning is a major advantage of using SOURCE 15RPC or SOURCE 30RPC for large scale work.

Column storage

Reversed phase media based on silica gel should not be stored in aqueous solution. This is due to the inherent instability of silica under aqueous conditions. Silica-based reversed phase columns and media are usually stored in a pure organic solvent such as methanol (acetonitrile is not recommended) free from TFA or any other additives. Polystyrene-based reversed phase media can be store in either pure methanol or 20% ethanol.

Chapter 4

Applications

Reversed phase chromatography has proven itself to be an indispensable technique in the purification of biomolecules. The technique was originally developed in the 1960s for the separation of small organic molecules. In recent years, with the advent of high performance media and instrumentation, reversed phase chromatography has been applied to the purification of biomolecules such as peptides, proteins and oligonucleotides. Reversed phase chromatography has proven so successful for biomolecule purification in the research laboratory that it is now routinely applied for process scale purification of synthetic peptides, and recombinant peptides and proteins.

The examples illustrated in the following section have been obtained from the published literature and from work in the laboratories at Amersham Biosciences.

Designing a biochemical purification

Except for a few specific applications, reversed phase chromatography is rarely used by itself in a biochemical purification. Reversed phase is usually combined with other chromatography techniques such as gel filtration and ion exchange chromatography. The choice of the additional techniques along with the sequence in which they are employed is critical to a successful purification.

At the beginning of a purification protocol sample loading, sample dilution and impurity contamination are usually at their highest. At this stage, high capacity, low resolution techniques that also result in sample concentration are usually employed. At later stages in the protocol, higher and higher resolution techniques are utilised. The choice of a general protocol depends on the type of biomolecule being purified as well as its source.

From the point of view of reversed phase chromatography, the biomolecules that we have so far focused on in this booklet may be grouped as follows:

- Naturally occurring peptides and proteins
- Recombinant peptides and proteins
- Chemically synthesised peptides
- Protein fragments from enzyme digests
- Chemically synthesised oligonucleotides

All these groups of biomolecules differ in several ways which prove important in their purification. Examples of the use of reversed phase chromatography in solving some typical purification problems are presented in the following pages, together with some general considerations for designing successful purification strategies.

Naturally occurring peptides and proteins

Peptides and proteins are purified from their natural sources for many reasons. Due to the complexity of the starting material, their purification usually requires a series of steps using several complementary chromatography techniques (see Protein Purification Handbook Code no. 18-1132-29 for guidelines on purification strategies).

Peptides and proteins often occur naturally in very small quantities and large amounts of starting material are usually required in order to get a reasonable amount of pure material. Several different separation techniques are applied in sequence to achieve a very high degree of purity.

During the capture phase the main points are to reduce volume and eliminate the majority of gross contaminants while the later steps remove the components with similar characteristics.

Early chromatographic steps could include ion exchange and gel filtration, using low to medium performance media. The final purification step would then employ high performance reversed phase chromatography, combined if necessary with high performance gel filtration. The purity throughout the various stages of the purification can be monitored using electrophoresis on PhastSystem™ .

There are several points to consider when using reversed phase chromatography to purify molecules from complex biological sources. Firstly, if a protein or peptide is to be used in studies of its function or, for example, structure-activity relationships, then it will be important to ensure that its biological activity is maintained throughout the purification procedure. This is not a critical problem with peptides and small proteins, but larger proteins tend to denature under reversed phase conditions. The proper choice of reversed phase medium and elution conditions, including time of exposure to potentially denaturing conditions, must be optimised in order to maintain biological integrity.

The presence of proteases may also present difficulties when dealing with polypeptides from natural sources. It is advisable to work rapidly at the initial stages of purification in order to minimise the contact time the desired peptide or protein will have with the protease. If proteases prove to be an intractable problem, specific chemical protease inhibitors may be added to the buffers.

A third problem common with materials from natural sources is aggregation. Fortunately, the low polarity mobile phases used in reversed phase chromatography are often good solvents for just those polypeptides which are poorly soluble in aqueous buffer solutions at physiological pH. Soluble aggregates which do cause problems can be separated from the corresponding monomers by gel filtration.

Purification of platelet-derived growth factor (PDGF)

Reversed phase chromatography was used for the rapid purification of dimeric Platelet-Derived Growth Factor (PDGF, Mr 30 000). In the initial method, cation exchange chromatography was combined with a second purification step using reversed phase chromatography. Further studies showed that the ion exchange step could be omitted to give a simple and rapid one-step method.

A freeze/thaw extract of pig blood platelets was clarified by centrifugation and the supernatant was dialysed overnight against 30 volumes of 10 mM sodium phosphate, pH 7.4. After further centrifugation, the clear extract was applied to a RESOURCE RPC 3 ml column. The column was eluted with a gradient from TFA (0.1%) in water to TFA (0.1%) in acetonitrile (Fig. 34). PDGF was purified to apparent homogeneity in a single run.

Fig. 34. Rapid purification of Platelet-derived Growth Factor (PDGF) from freeze/thaw extract of porcine blood platelets by reversed phase chromatography.
(Work by Amersham Biosciences AB, Lillestrøm, Norway.)

Sample:	Platelet derived growth factor (Porcine blood platelet extract containing 10 mg/ml protein). 100 µl applied.
Column:	RESOURCE RPC 3 ml (6.4 x 100 mm)
Eluent A:	0.1% TFA in water
Eluent B:	0.1% TFA in acetonitrile
Gradient:	4 ml 0% B, 4-48 ml, 0-60% B
Flow:	2 ml/min (370 cm/h)
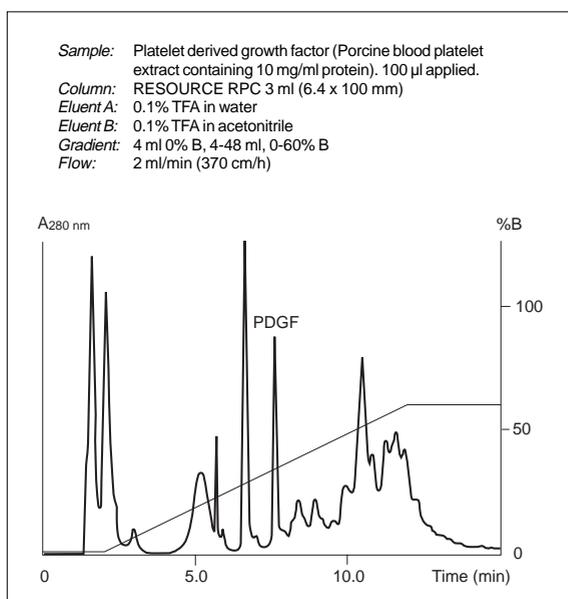
Trace enrichment

Since naturally occurring peptides are typically present in very low concentrations in biological sources, the use of reversed phase techniques for trace enrichment from large volumes of dilute solution is of special interest. For example, a narrow-bore column with a total bed volume of 240 µl (µRPC C2/C18 PC 3.2/3) can easily concentrate proteins from over 40 times as much (10 ml) of a dilute sample (Fig. 35). The solutes concentrated on the column can then be separated in the usual way, in this case by a linear gradient of acetonitrile concentration.

Note that sample volumes differing 100-fold gave very similar results (Fig. 35 a and b).

| Sample: | Ribonuclease A (10 µg), Cytochrome C (2.5 µg), Albumin (10 µg), Catalase (10 µg) |
Sample volume:	(A) 100 µl, (B) 10 ml
Column:	µRPC C2/C18 PC 3.2/3 (i.d. 3.2 mm, length 30 mm)
Eluent A:	0.1% TFA in water
Eluent B:	0.1% TFA in acetonitrile
Gradient:	0-60% B in 10 min
Flow:	300 µl/min
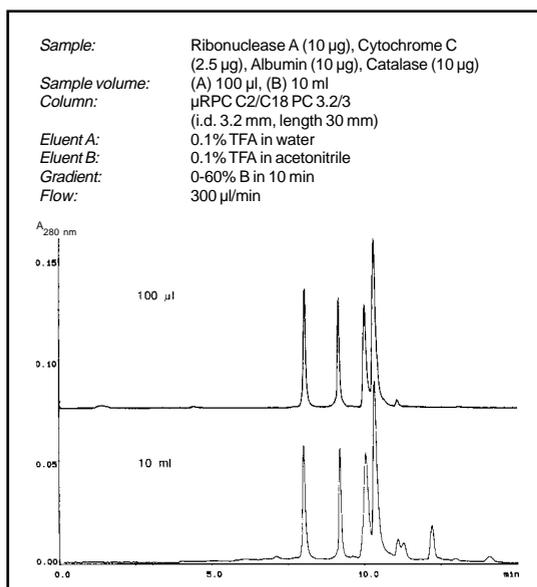
Fig. 35. Trace enrichment of standard proteins followed by gradient elution on a narrow-bore reversed phase column, (i.d. 3.2 mm, length 30 mm). Sample volume (A) 100 µl, (B) 10 ml. (Work by Amersham Biosciences AB, Uppsala, Sweden.)

Purification of cholecystokinin-58 (CCK-58) from pig intestine

This purification shows that, by selection of appropriate techniques and manipulation of elution conditions, a pure product can be obtained from very crude material after only a few chromatographic steps.

In this example cation exchange chromatography (HiLoad™ 26/10 SP Sepharose™) was used for the initial capture of an ethanol-precipitated extract of intestinal peptides. Fractions which showed bio-activity were collected and purified further using an HR 16/10 column packed with SOURCE 15RPC. Figure 36 a shows the result of the reversed phase purification on one of the fractions. CCK-58, the molecule of interest, was present in region 11. The run was repeated several times and the bioactive material corresponding to region 11 was combined and lyophilised. The next purification step was carried out on a Sephasil Peptide 5 µm ST 4.6/100 column which gave an alternative selectivity to SOURCE RPC. As can be seen in Figure 36 b the bioactivity was found under the major peak of the chromatogram. This elution used an aqueous methanol gradient elution. For further purification the same column was used again but with an aqueous acetonitrile gradient. The peptides eluted from the final purification step (Figure 36 c) were shown to be pure by capillary zone electrophoresis, MALDI TOF mass spectrometry and amino acid composition analysis (11).

System: ÄKTA purifier
Column: HR 16/10 column packed with SOURCE 15RPC
Sample: 50 ml of fraction 7 from a cation exchange step
Eluent A: 0.1% TFA in water
Eluent B: 0.1% TFA in acetonitrile/water
Gradient: 0% B for 2 column volumes (CV),
    0–20% B for 1 CV,
    20–55% B for 11CV, 55–100% B for 1 CV,
    100% B for 2 CV
Flow: 6 ml/min
Bioassay: Guinea pig gall bladder concentration *in vivo*

a)
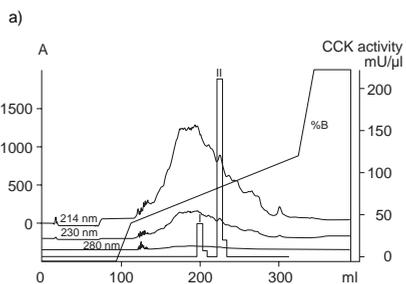
System: ÄKTA purifier
Column: Sephasil Peptide C18 5 µm ST 4.6/100
Sample: 450 µg of dry peptide material from
    bioactivity region II
Eluent A: 0.1% TFA in water
Eluent B: 0.1% TFA in methanol
Gradient: 60% B for 2 column volumes (CV),
    60–81% B for 20 CV,
    83–100% B for 2 CV,
    100% B for 2 CV
Flow: 6 ml/min
Bioassay: Guinea pig gall bladder concentration *in vivo*

b)

System: ÄKTA purifier
Column: Sephasil Peptide C18 5 µm ST 4.6/100
Sample: Fraction 13 from the chromatography step b
Eluent A: 0.1% TFA in water
Eluent B: 0.1% TFA in 90% acetonitrile/water
Gradient: 30% B for 2 column volumes (CV),
    30–48% B for 16 CV,
    48–100% B for 2 CV, 100% B for 2 CV
Flow: 0.6 ml/min
Bioassay: Guinea pig gall bladder concentration *in vivo*

c)
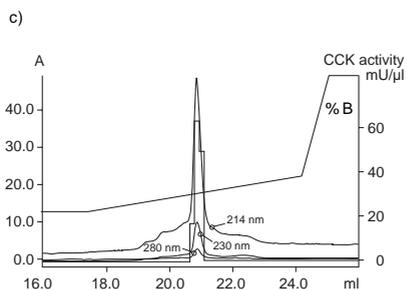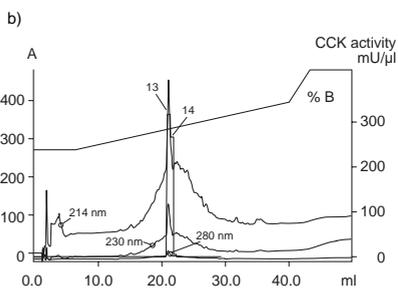
Fig. 36. RPC steps from the purification of cholecystokinin.

Recombinant peptides and proteins

Some of the problems associated with isolating peptides and proteins from their native sources can be overcome by producing them through recombinant techniques. Peptides and proteins produced in this way are used for basic research, diagnostic and therapeutic purposes.

Recombinant peptides and proteins can be expressed by vectors in different hosts, e.g. *E. coli,* yeast, mammalian cells or viruses. A vector (plasmid or chromosome) is the vehicle used to transport the gene coding for the polypeptide into the host cell. The cell reproduction mechanism then produces many copies of the desired recombinant DNA molecule or clone. The cells containing the correct clone are grown and the gene which has been introduced is expressed to provide a continuous supply of the desired peptide or protein.

The expressed peptide or protein may be found either in the culture medium or inside the cells. Peptides or proteins which are secreted into the culture medium are relatively simple to purify (12) and are generally not as susceptible to proteases. Polypeptides which are retained inside the cells are more difficult to purify since they must first be released by lysis (13). If the concentration of polypeptide within the cell is too high, precipitation can occur resulting in insoluble inclusion bodies. These inclusion bodies must be dissolved using a chaotropic agent such as guanidine hydrochloride (6 M) and the polypeptide carefully renatured (14). When refolding methods exist, it can be advantageous to produce proteins via inclusion bodies as only centrifugation, washing and solubilisation are necessary to obtain a relatively pure starting material (15). High purity can then be achieved through the addition of a single polishing step.

The purification of extracellular recombinant peptides and polypeptides is usually complicated by large starting volumes requiring an initial capture step to concentrate the sample. This inconvenience can be overcome by introducing a specific terminal sequence, such as protein A (16), glutathione transferase (17) or polyamino acids, at the plasmid level which allows the polypeptide to be purified by affinity chromatography. After initial purification and concentration, followed if necessary by chemical or enzymatic removal of the affinity handle, the recombinant polypeptide may then be finally purified by reversed phase chromatography.

Intracellular polypeptides must be released by cell lysis (18, 19) before the initial purification can begin. After removal of cellular debris, the initial capture steps aim at removing endotoxins, nucleic acids and any remaining cell debris or potentially coagulating substances such as lipids. As in the case of secreted polypeptides, initial purification is facilitated by use of an affinity handle. If an affinity handle cannot be used then other concentrating chromatographic techniques, such as ion exchange, must be used in the initial capture steps. Reversed phase chromatography, sometimes in combination with high resolution gel filtration, is used in the final polishing steps to produce a homogeneous polypeptide.

The purification of recombinant polypeptides can be monitored by techniques like analytical reversed phase chromatography, amino acid analysis, peptide mapping, bioassay or polyacrylamide electrophoresis.

Process purification of inclusion bodies

As previously discussed, relatively pure starting material can be produced by expressing a recombinant protein as insoluble inclusion bodies, as long as a satisfactory solubilisation and refolding protocol is available. High purity can then be achieved by a single polishing step. Since reversed phase chromatography is known to be a particularly efficient means of removing endotoxins it would appear to be especially suitable as a polishing step for proteins which are to be used in biological systems.

However, until recently, only silica based media were available for large scale purification of inclusion bodies. These media have low binding capacity, are easily fouled and almost impossible to regenerate. High back pressures are generated which lead to longer separation times and, consequently, lower recoveries. These limitations have now been overcome by the use of SOURCE 30RPC, a media specifically designed for large scale purification. The 30 µm monosized beads of SOURCE 30RPC not only generate very low back pressures, but give high recoveries and are easily sanitised. An example of the use of SOURCE 30RPC for large scale purification of inclusion bodies can be found in Downstream Vol. 28, p.14 -17, 1998 Code no: 18-1132-72, Amersham Biosciences.

Purification of recombinant human epidermal growth factor

A protocol was developed for purifying recombinant human Epidermal Growth Factor (rhEGF), Mr ca. 6 000, expressed as an extracellular product by Saccharomyces cerevisiae. The purification procedure involved a capture step followed by hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow (high sub) and ion exchange chromatography on Q Sepharose High Performance.

The first two steps were efficient for purification and gave good recovery. However, the product pool after ion exchange chromatography still contained small amounts of impurities that were unresolved from the main product. These stubborn product variants were removed in a polishing step using reversed phase chromatography on SOURCE 15RPC (Fig. 37). A small scale experiment (Fig. 39 a) showed that elution with a gradient of acetonitrile concentration (1.3% B/min) resulted in a product which gave a single peak in analytical reversed phase chromatography on C2/C18 silica.

a
Sample: 2.14 ml (0.34 mg EGF) partially purified rhEGF.
Column: RESOURCE RPC, 3 ml (i.d. 6.4 mm, length 100 mm)
Eluent A: 0.05% TFA, 5% acetonitrile in water
Eluent B: 0.05% TFA, 80% acetonitrile in water
Gradient: 1.3% B/min
Flow: 1.6 ml/min; 300 cm/h

b
Sample: 62.5 ml (10 mg EGF) partially purified rhEGF.
Column: SOURCE 15RPC (i.d. 35 mm, length 100 mm)
Eluent A: 0.05% TFA, 5% acetonitrile in water
Eluent B: 0.05% TFA, 80% acetonitrile in water
Gradient: 1.3% B/min
Flow: 50 ml/min; 300 cm/h

Fig. 37. Preparative purification of rhEGF on SOURCE 15RPC at different scales. Sample load (a) 0.34 mg EGF, (b) 10 mg EGF. (Work by Amersham Biosciences AB, Uppsala, Sweden.)

The result obtained on the 3 ml column (RESOURCE RPC, 3 ml) could be scaled up directly by a factor of 30 (Fig. 37 b). A column with a larger diameter was used to accommodate the larger volume of medium required for the increased mass of sample. Increasing column length to increase the column volume is inefficient since both the separation time and the operating pressure are increased with little or no increase in resolution. The volumetric flow (ml/min) was increased in proportional to the increase in column cross-sectional area to compensate for the increased column volume. In this way the flow velocity (cm/h) and the separation time were held constant.

The differences between the results obtained at the two scales were negligible. The recovery was 92% and the recovered material was shown to be homogeneous using analytical reversed phase chromatography.

Chemically synthesised peptides

Synthesis of peptides containing fewer than 20 amino acids is now a routine laboratory procedure. Peptides up to 100 amino acid residues can be synthesised, but with more difficulty.

Essentially two different chemistries are used for peptide synthesis, but both procedures result in similar major contaminants in the final product. These contaminants include peptides with amino acid deletions, peptides with truncated amino acid sequences and peptides with modified amino acid residues. Small organic molecules, such as phenol and thiols, are also present, resulting from the removal of the synthesised peptide from its solid support.

Synthetic peptides of less than 20 amino acid residues can often be purified to the required level by a single reversed phase separation. To monitor purity an analytical reversed phase medium is used (with a different selectivity compared to the medium used for purification), usually in combination with mass spectrometry.

Reversed phase media should be selected according to the selectivity for the target peptide and also according to the scale of purification. For microgram quantities a 5µm medium will give good separation (see Figure 38) whereas larger quantities may require 15µm or 30 µm media to achieve sufficient capacity and speed for an efficient purification.

Other purification techniques, such as ion exchange, may be used in combination with reversed phase for the purification of larger synthetic peptides.

Purification of a phosphorylated PDGF α-receptor derived peptide

Peptide pY574α is an 18 amino acid residue phosphorylated peptide constituting part of the intracellular domain of the PDGF α-receptor. The peptide was designed and synthesised for use as a ligand for subsequent affinity purification of potential signal transduction proteins present in cell lysates.

Figure 40 shows the single reversed phase separation used to achieve required purity. PY574α is readily soluble under alkaline conditions, but poorly soluble under acidic conditions. This eliminates the possibility of using silica gels because of their incompatability with alkaline conditions. SOURCE RPC was therefore selected to allow a high yield purification under alkaline conditions. The 5 µm medium SOURCE 5RPC gave sufficient capacity, speed and resolution (20).
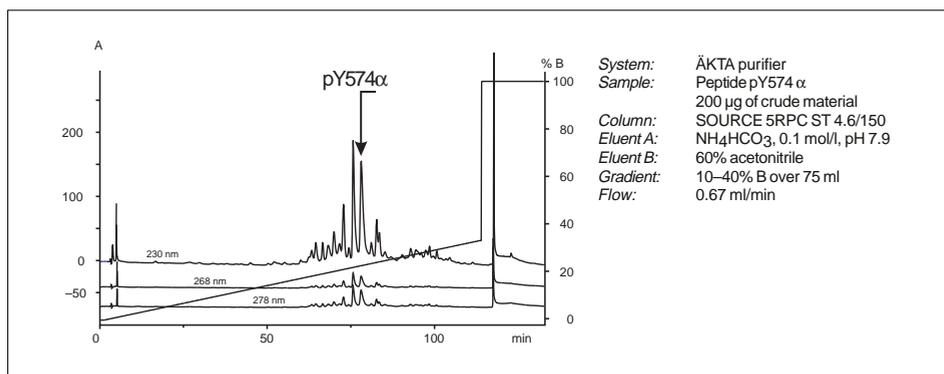
Fig. 38. Purification of the PDGF α -receptor derived peptide using SOURCE 5RPC ST4.6/150 at pH 7.9.

Structural characterisation of a 165 kDa protein

In order to analyse its structure, a native 165 kDa protein was subjected to tryptic digestion. If cleavage according to the specificity of trypsin was complete, the digest was expected to contain more than 150 fragments. As shown in Figure 39, this very large number of peptides was separated on a µRPC C2/C18 ST 4.6/100 column using a shallow gradient (21).

The separation was monitored at 215 nm, 254 nm and 280 nm. The 215 nm is specific for the peptide bond, thus revealing all eluted peptides. The other two wavelengths are useful for monitoring the aromatic amino acid residues. By use of peak absorbance ratio calculations fragments containing phenylalanine, tyrosine and tryptophan can be specifically detected which is a significant help during subsequent structural analysis of the collected fragments (22).
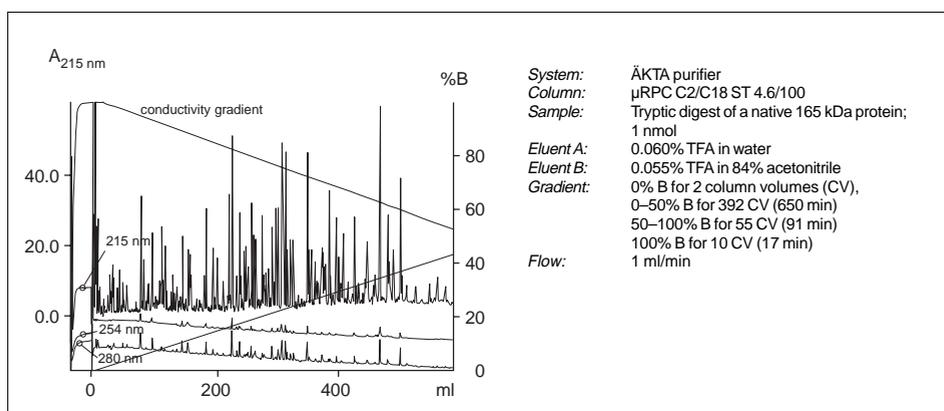

Fig.39. Separation of a tryptic digest of a native 165 kDa protein using ÄKTApurifier and µRPC C2/C18 3 µm ST 4.6/100

Protein fragments from enzyme digests
## Protein characterisation at the micro-scale

Reversed phase chromatography plays a central role in a cluster of techniques (Fig. 42), including polyacrylamide gel electrophoresis (PAGE), mass spectrometry and micro-scale high performance LC, of increasing importance in high sensitivity characterisation of proteins (23, 24, 25, 26, 27)
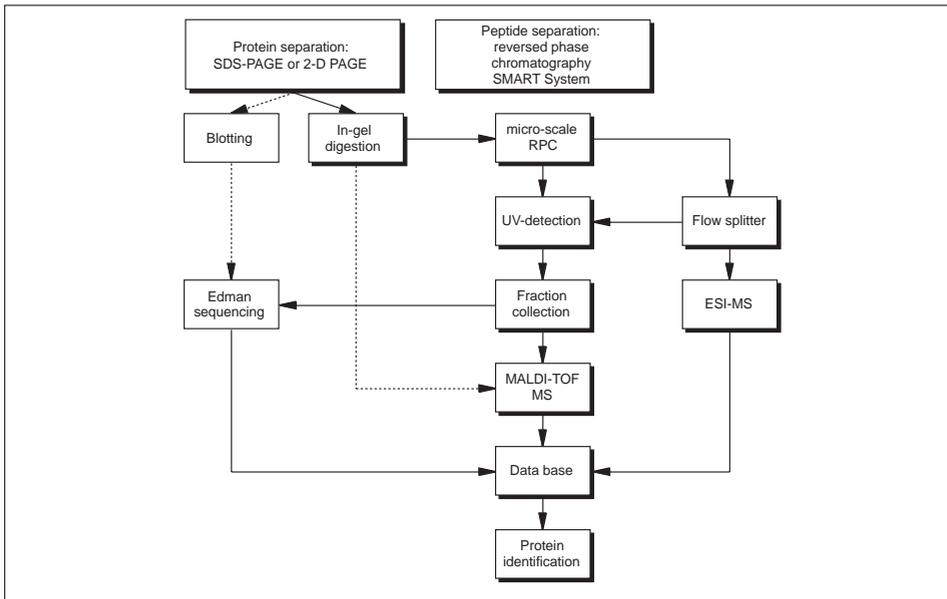
**Fig. 40.** Techniques for high sensitivity characterisation of proteins.

Many gene products are first isolated in very small amounts as bands or spots in SDS-PAGE or 2D-PAGE. Micro-scale reversed phase chromatography allows partial amino acid sequences, either terminal or internal, to be obtained for these proteins, thereby permitting their subsequent identification by matching against very large protein structure data-bases (28, 29).

Although partial N-terminal sequences can sometimes be obtained from blotted proteins, in-gel digestion of the protein to produce characteristic peptide fragments may be more generally applicable. The combination of in-gel protein digestion (30, 31, 32, 33) with micro-scale high performance reversed phase purification of the fragments, followed by Edman sequence analysis and sequence matching against protein sequence data bases is highly efficient. Hellman and Gonez (34) were able to identify human glial fibrillary protein (hGFAP) after electrophoretic separation of glioma cell proteins even when the polyacrylamide gel had been dried onto paper for storage. The peptides from the in-gel digest were extracted and subsequently fractionated by micro-scale high performance reversed phase chromatography using SMART System (Amersham Biosciences) (Fig. 41).
The peptide in fraction 22 (Fig. 42) was sequenced.

67

Sample: Protein digested with trypsin in situ in an SDS-PAGE gel, extracted with acetonitrile (60%) and redissolved in eluent A
Column: μRPC C2/C18 SC 2.1/10 (i.d. 2.1 mm, length 100 mm)
Eluent A: 0.065% TFA in water
Eluent B: 0.05% TFA in acetonitrile
Gradient: 1% B for 6 min
1-41% B in 80 min
41% B for 10 min
41-81% B in 10 min
Flow: 0.1 ml/min
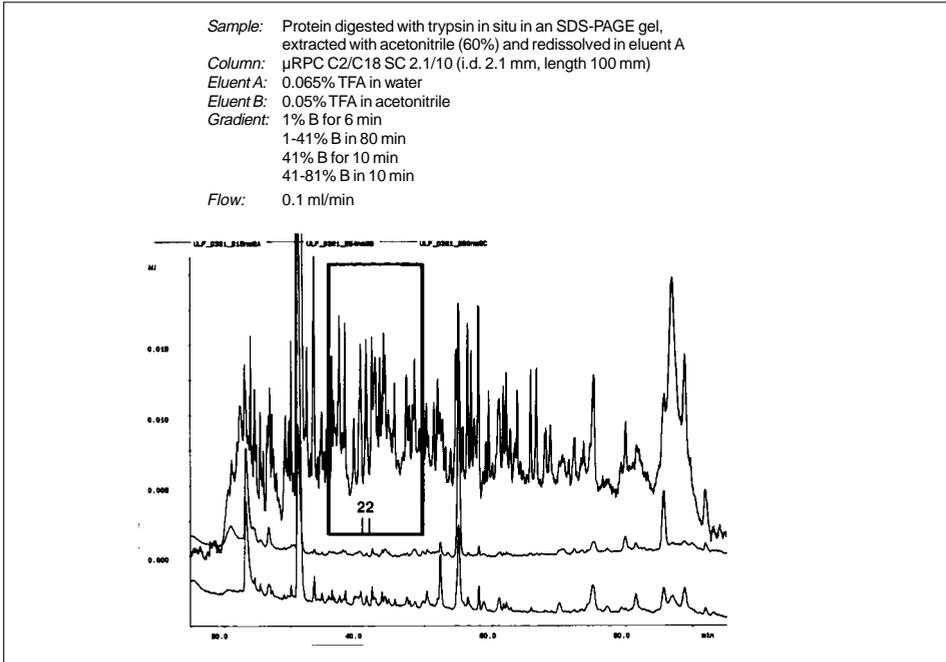
Fig. 41. Fractionation of peptides obtained by in-gel digestion of a single band from an electrophoretic separation of glioma cell proteins. (SMART Bulletin hGFAP (1993). Hellman, U., Gonez, J.)

Three matches were obtained in a search of a protein sequence data base and, since only one of the matches corresponded to a protein of human origin, the protein could be identified as hGFAP.
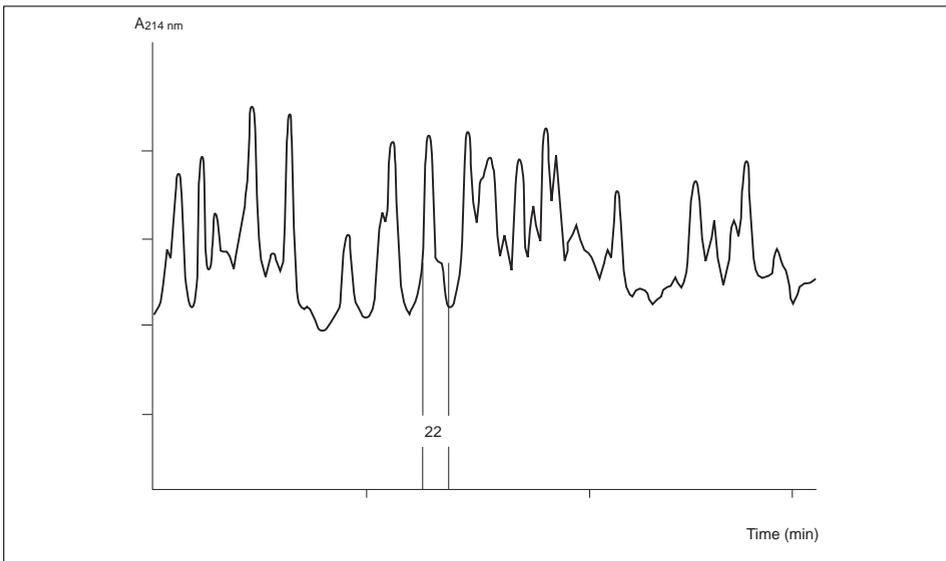
Fig. 42. Enlargement of the indicated part of Figure 43. The peptide in fraction 22 was sequenced. (SMART Bulletin hGFAP (1993). Hellman, U., Gonez, J.)

Protein identification by LC-MS

Developments in mass spectrometry now allow highly accurate estimates of the masses of peptides to be made after separation by reversed phase chromatography. The column eluent may be split so that part is delivered to an Electrospray-Ionisation Mass Spectrometer (ESI-MS) for on-line analysis (35), while the major part of the eluent is collected in a fraction collector for off-line analysis, including amino acid sequencing and Matrix-Assisted Laser-Desorption-Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Reversed phase chromatography coupled to ESI-MS provides high precision data for protein identification by matching against a protein-fragment data-base.

In studies of two forms of apolipoprotein A, Apo A1 and Apo A1-M, Renlund et al. (36) have given an elegant demonstration of how structural modifications of a protein can be elucidated by the high resolving power of reversed phase chromatography in combination with ESI-MS. Apo A1 is the major protein component of plasma high density lipoproteins (HDL) and plays an important role in the reverse transport of cholesterol from tissues (37). High plasma levels of Apo A1 are associated with a low incidence of coronary artery disease. Apolipoprotein A1-Milano (Apo A1-M) is a genetic variant of normal Apo A1 (38), characterised by the replacement of Arg at position 173 by Cys. In vitro studies have indicated a threefold increase in the cholesterol transporting ability of Apo A1-M. The carriers of Apo A1-M have quite low levels of HDL, but they do not show any signs of arteriosclerosis.

Apolipoprotein A1 was purified from normal human plasma. Apo A1-M was produced in an excreting *E. coli* system and isolated using conventional LC techniques followed by reversed phase HPLC. Both proteins were digested with Lys-C specific endopeptidase, and the peptides were separated on a μRPC C2/C18 SC 2.1/10 column connected to SMART System (Amersham Biosciences) equipped with a Flow Splitter. The flow split ratio (MS flow/Total flow) was regulated to give a flow to the electrospray mass spectrometer (VG Platform, Fisons, Manchester, UK) in the range 5-10 μl/min.

Figure 43 shows a separation of 400 pmol each of Apo A1-M and Apo A1. The most significant difference between these chromatograms is in the retention of the last peak, indicated by K15A1-M and K15A1 respectively. The ESI mass spectra of the peptides eluted in these peaks are shown in Figures 44 and 45. The mass difference between the two fragments is 52.92 Da, which deviates by 0.18 Da from the mass difference, 53.10, between Arg and Cys. This would indicate, with an error in the mass estimation less than $4 \times 10^{-3}\%$, that an Arg should be substituted with a Cys in Apo A1-M.

Samples:	Upper: Lys-C endopeptidase digest of Apo A1-M (400 pmol)
	Lower: Lys-C endopeptidase digest of Apo A1 (400 pmol)
Column:	µRPC C2/C18 SC 2.1/10 (i.d. 2.1 mm, length 100 mm)
Eluent A:	0.25% pentafluoropropionic acid (PFPA) in water
Eluent B:	0.25% PFPA in acetonitrile
Gradient:	10-60% B in 200 min
Flow:	25 µl/min
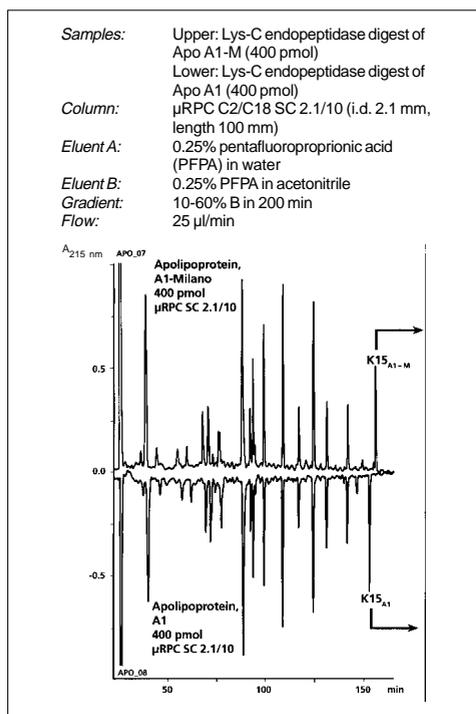
Fig. 43. Reversed projection of separations of Lys-C endopeptidase digests of Apo_{A1}-M (upper) and Apo_{A1} (bottom) by reversed phase chromatography. Renlund, S., Wadensten, H., Persson, P. et al. (36).
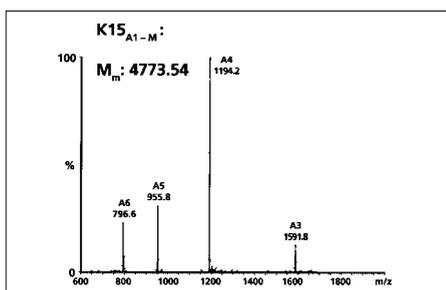
Fig. 44. ESI-MS mass spectrum obtained from peptide material from peak $K15_{A1}$-M in Fig. 45. M_m for $K15_{A1}$-M was 4773.54. See the text for details. Renlund, S., Wadensten, H., Persson, P. et al. (36).
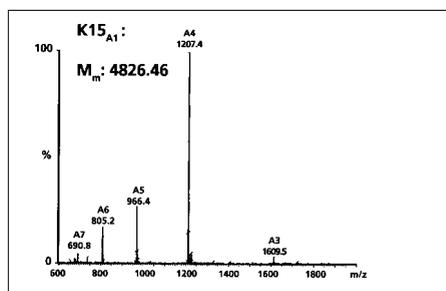
Fig. 45. ESI-MS mass spectrum obtained from peptide material from peak $K15_{A1}$ in Fig. 45. M_m for $K15_{A1}$ was 4826.46. See the text for details. Renlund, S., Wadensten, H., Persson, P. et al. (36).

Chemically synthesised oligonucleotides

Automated solid phase synthesis is a commonly used procedure for preparing oligonucleotides 20 to 30 bases long for use as DNA probes and templates for PCR (39) reactions. Much longer oligonucleotides, 100 bases or more, are also synthesised.

The main contaminants are truncated sequences together with smaller amounts of oligonucleotides which contain modified bases.

After gel filtration to remove small organic contaminants which are produced during chemical cleavage of the oligonucleotide from its solid support, the desired oligonucleotide can generally be purified to sufficient purity by a single pass through a reversed phase column.

Separation of the complete sequence from incomplete sequences is simplified if the purification is performed with the 5´ trityl protecting group still attached as none of the truncated sequences produced as side products during the synthesis contain the protecting group. The large hydrophobic trityl group contains three benzene ring structures and causes the complete sequence to elute significantly later than the truncated sequences (Fig. 46).
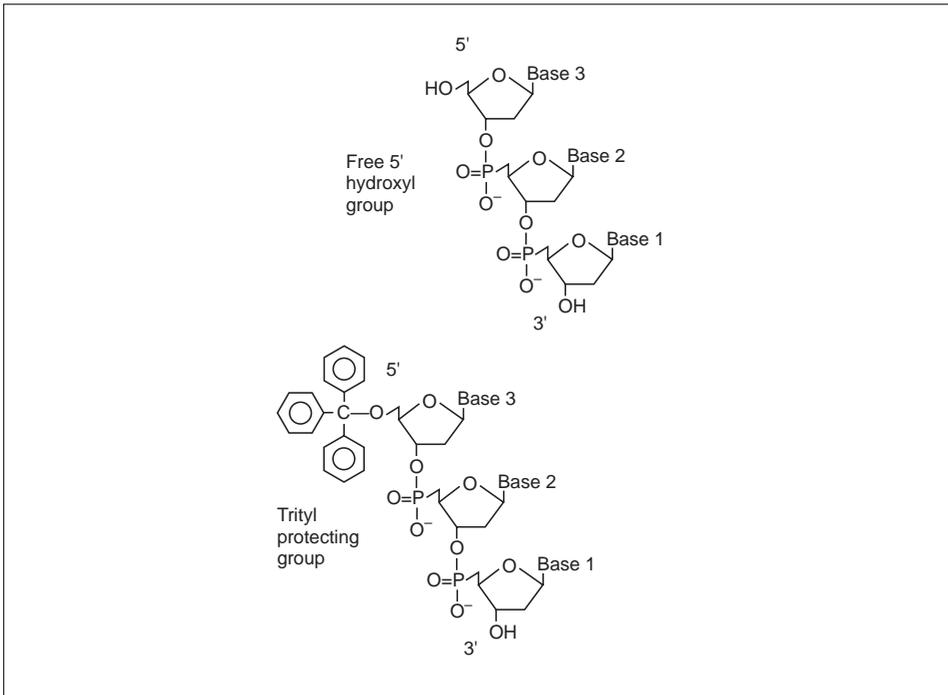
Fig. 46. Partial structure of a synthetic oligonucleotide with the trityl protecting group (A) off and (B) on.

The separation is carried out close to neutral pH with triethylammonium acetate as the ion pairing agent and a linear gradient of increasing acetonitrile concentration. Purification of a typical synthetic oligonucleotide (5´ DMTr-T TCT AGC TCA ACC GGT CAA) at different scales is shown in Figure 47.

Sample: Purification of crude synthetic 19-base oligonucleotide, trityl on, by reversed phase chromatography at different sample loads. (A) 65 µg, (B) 650 µg, (C) 6500 µg
Column: RESOURCE RPC 1 ml
Eluent A: Triethylamine acetate (0.1 M, pH 7.0) in water
Eluent B: 100% acetonitrile
Gradient: 5% B for 5 min
5-40% B in 20 min
Flow: 1 ml/min
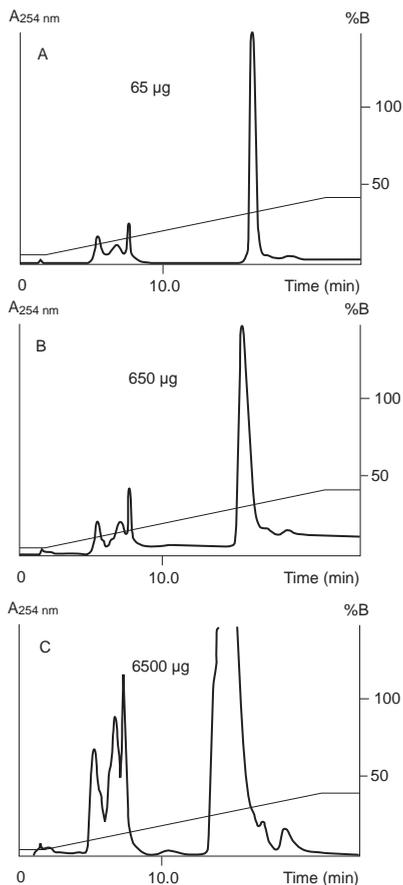
Fig. 47. Reversed phase purification of a synthetic 19-base oligonucleotide with the trityl protecting group on. (Work by Amersham Biosciences AB, Uppsala, Sweden.)

Due to the excellent resolving power of the reversed phase technique, additional purification steps are generally not required.

Chapter 5

Fault finding chart

Symptom	Cause	Remedy
Column is clogged	Column filter is clogged.	Replace the filter. Always filter samples and mobile phase before use.
	Presence of particulates, lipoproteins or protein aggregates.	Clean or replace the column. Prior to chromatography, precipitate with 10% Dextran Sulphate or 3% PVP.
	Precipitation of proteins in the column caused by removal of stabilising agents during fractionation.	Clean and regenerate the column. Modify the eluent to maintain stability.
No flow through the column	No flow from pump.	Check the pump and system for leaks. Make sure the injector valve is in the correct position.
	End-piece or adapter or tubing is blocked.	Remove and clean, if possible.
Reduced or poor flow through the column	Bed surface blocked by precipitated proteins.	Clean the column by recommended procedures or change the pre-column.
	Bed is compressed.	Replacing the column may be necessary.
Back pressure increases during a run or during successive runs	Precipitation of sample in the column and/or at the top of the bed.	Clean the column and exchange or clean the filter or clean/replace the pre-column. Alternatively, replace any additives initially used to solubilize the sample so long as they are compatible with the reversed phase medium.

Symptom	Cause	Remedy
Back pressure increases during a run or during successive runs	Turbid sample has been applied to the column.	Improve sample solubility by increasing the concentration of organic modifier in the initial mobile phase. Alternatively, adjust the pH of the initial mobile phase to increase sample solubility.
	Column filter is clogged.	Replace the filter. Always filter samples and mobile phase before use.
Sample does not elute in the organic solvent gradient	Unsuitable pH.	Adjust the pH so that the sample is not denatured.
	Final concentration of organic modifier in the gradient is too low.	Increase the concentration of organic modifier in mobile phase B.
	Eluting power of the organic modifier is too low.	Switch to a reversed phase column with a less hydrophobic immobilised ligand. Alternatively, change to an organic modifier with more efficient elution properties.
Sample components elute in the equilibrium phase	Sample not hydrophobic enough to adsorb to column.	Add or increase the concentration of ion pairing agent. Alternatively, switch to a column with a more hydrophobic immobilised ligand or change to an organic modifier with less efficient elution properties.
	Unsuitable pH.	Adjust the pH so that the sample binds.
	Impurities adsorbed to the column.	Clean the column by recommended procedures.

Symptom	Cause	Remedy
	Concentration of organic modifier in the initial mobile phase is too high.	Decrease the concentration of organic modifier.
	Column is not properly equilibrated.	Repeat or prolong the equilibration step.
Resolution is less than expected	Gradient slope is too steep.	Use a shallower gradient or a plateau in the gradient.
	Poor selectivity.	Add or adjust the concentration of ion pairing agent.
	Detector cell volume is too big.	Change the flow cell.
	Column is poorly packed.	Check packing by doing a plate count and re-pack or replace the column if necessary.
	Proteins or lipids have precipitated on the column.	Clean and regenerate the column. Change the eluent to maintain stability.
	Column was overloaded.	Clean and regenerate the column. Decrease the sample load.
	Sample has not been filtered.	Filter the sample before applying it to the column.
	Protein in the sample has aggregated and bound strongly to the medium.	Increase organic content of initial mobile phase.
	Mixed mode retention behaviour due to surface silanols on the silica gel.	Lower the pH to suppress ionisation of silanol groups or replace the column.
	Large mixing spaces in or after column.	Reduce all post column volumes.
	Flow rate is too high.	Run the separation at a lower flow rate (not a problem with proteins).
	Column ageing.	Adjust mobile phase pH to suppress ionisation of surface silanols or replace the pre-column. If necessary, replace the column.

Symptom	Cause	Remedy
Leading or very rounded peaks	Column is poorly packed.	Check packing by doing a plate count and re-pack or replace the column if necessary.
	Column needs regeneration.	Clean and regenerate the column. Replace the pre-column or column if necessary.
	Column was overloaded.	Clean and regenerate the column. Decrease the sample load.
Tailing peaks	Precipitation of sample in the column and/or at the top of the bed.	Clean the column by recommended procedures and replace the top filter. Alternatively, replace the pre-column.
	Sample too viscous.	Reduce sample concentration.
Previous elution profile cannot be reproduced	Proteins or lipids have precipitated on the column.	Clean and regenerate the column. Change the eluent to maintain stability.
	Incomplete column equilibration.	Equilibrate until the baseline is stable (5 - 10 column volumes).
	Sample has not been filtered.	Filter the sample before applying it to the column.
	Incorrect mobile phase pH or loss of organic modifier by evaporation.	Prepare fresh mobile phase.
	Sample has altered during storage.	Prepare fresh sample.
Low recovery of activity while normal recovery of sample mass	Sample components denatured or inactivated in the mobile phase.	Determine the pH and organic solvent stability of the sample. Decrease the separation time in order to limit exposure of sample components to mobile phase. Alternatively, use a reversed phase medium with a less hydrophobic ligand requiring milder elution

Symptom	Cause	Remedy
		conditions. Alternatively, change the organic modifier.
	Enzyme sample separated from cofactor or similar.	Test by pooling fractions and repeating the assay.
Sample amount in the eluted fractions is much less than expected	Sample has been degraded by proteases or nucleases.	Add inhibitors or minimise separation time.
	Sample precipitates.	Decrease the sample load or change mobile phase conditions to maintain stability.
	Basic proteins adsorbed to column by ionic retention.	Increase pH of mobile phase or add/adjust ion pairing agent concent-ration in the mobile phase.
	Sample adsorbed to filter during preparation.	Use a different type of filter.
More activity is recovered than was applied to the column	Removal of inhibitors during separation.	Replace if necessary.
	Different assay conditions have been used before and after the chromatographic step.	Use the same assay conditions for all the assays in your purification scheme.
Peaks too small	Sensitivity range incorrectly set on the detector or recorder.	Adjust.
	Excessive zone broadening.	Check the column packing by doing a plate count. Re-pack if necessary. Broad peaks may be due to column ageing. Adjust mobile phase pH to suppress ionisation of surface silanols or replace the pre-column. If necessary, replace the column.

Symptom	Cause	Remedy
Peaks to small	Sample absorbs poorly at the chosen wavelength.	Monitor at a different wavelength.
Bubbles in the bed	Column packed or stored at cool temperature and then warmed up.	Small bubbles can often be removed by passing well de-gassed mobile phase upwards through the column. The column may need to be re-packed. Take special care if mobile phases are used after storage in a refrigerator or cold room. Do not allow column to warm up
Cracks in the bed	Large air leak in the column.	Check all connections for leaks. Re-pack the column.
Distorted bands as the sample runs into the bed	Net in upper adapter is clogged or damaged.	Dismantle the adapter, clean or replace the net.
	Particles in eluent or sample.	Filter or centrifuge the sample. Protect eluents from dust.
	Air bubble trapped at the top of the column or in the inlet adapter.	Re-install the adapter (if present) taking care to avoid air bubbles.
Distorted bands as the sample passes down the bed	Column is poorly packed.	Re-pack the column. Be careful not to pack at excessively high pressures.
Negative peaks at solvent front	Refractive index effects.	Make sure sample is dissolved in initial mobile phase.
Strange peaks in the chromatogram	Impurities in mobile phase.	Use high quality reagents.
Peaks on blank gradients	Organic impurities in water used to prepare mobile phase A.	Use high quality reagents. Pre-filter water through large particle reversed phase medium before preparing the mobile phase if necessary.

Symptom	Cause	Remedy
	Incomplete elution from previous run.	Wash the column according to the recommended method.
Spikes in the chromatogram	Air bubble trapped in the UV cell.	Use de-gassed solutions.
UV baseline rises with gradient	Eluents A and B absorb differently at the chosen wavelength.	Adjust the concentrations of mobile phase components which absorb at the chosen wavelength or monitor at a different wavelength.
	Impurities in mobile phase.	Use high quality reagents.
Retention time of same sample component increases over time	Mixed mode retention behaviour due to surface silanols on the silica gel.	Lower the pH to suppress ionisation of silanol groups or replace the column.
Peak width increases over time	Mixed mode retention behaviour due to surface silanols on the silica gel.	Lower the pH to suppress ionisation of silanol groups or replace the column.
Excessive baseline noise	UV absorption by component of mobile phase.	Monitor at different UV wavelength or reduce concentration of UV absorbing component (usually the ion pairing agent). If organic modifier is absorbing, change to one with a lower UV cut-off.
	Impurities in mobile phase.	Use high quality reagents.

Chapter 6

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Chapter 7

Ordering Information

Product	Column material	Quantity/Pack size	Code no.
SOURCE 5 RPC			
SOURCE 5RPC ST 4.6/150	Steel	1	17-5116-01
SOURCE 15 RPC			
RESOURCE RPC,1 ml	PEEK	1	17-1181-01
RESOURCE RPC, 3 ml	PEEK	1	17-1182-01
SOURCE 15RPC ST 4.6/100	Steel	1	17-5068-01
SOURCE 15RPC	-	10 ml	17-0727-20
SOURCE 15RPC	-	200 ml	17-0727-02
SOURCE 15RPC	-	500 ml	17-0727-03
SOURCE 15RPC	-	1 litre	17-0727-04
SOURCE 15RPC	-	5 litre	17-0727-05
SOURCE 30 RPC			
SOURCE 30RPC	-	10 ml	17-5120-20
SOURCE 30RPC	-	200 ml	17-5120-02
SOURCE 30RPC	-	500 ml	17-5120-03
SOURCE 30RPC	-	1 litre	17-5120-04
SOURCE 30RPC	-	5 litre	17-5120-05
Sephasil Protein			
Sephasil Protein C4 5 µm ST 4.6/100	Steel	1	17-6000-24
Sephasil Protein C4 5 µm ST 4.6/250	Steel	1	17-6000-21
Sephasil Protein C4 12 µm ST 4.6/250	Steel	1	17-6000-27
Sephasil Protein C4 12 µm ST 10/250	Steel	1	17-6000-57*
Sephasil Protein C4 12 µm ST 20/250	Steel	1	17-6000-58*
Sephasil Protein C4 12 µm ST 50/250	Steel	1	17-6000-59*
Sephasil Protein C4 12 µm	-	100 g	17-6000-51*
Sephasil Protein C4 12 µm	-	1 kg	17-6000-52*
Sephasil Peptide			
Sephasil Peptide C8 5 µm ST 4.6/100	Steel	1	17-6000-25
Sephasil Peptide C8 5 µm ST 4.6/250	Steel	1	17-6000-22
Sephasil Peptide C18 5 µm ST 4.6/100	Steel	1	17-6000-26
Sephasil Peptide C18 5 µm ST 4.6/250	Steel	1	17-6000-23
Sephasil Peptide C8 12 µm ST 4.6/250	Steel	1	17-6000-28
Sephasil Peptide C8 12 µm ST 10/250	Steel	1	17-6000-60*
Sephasil Peptide C8 12 µm ST 20/250	Steel	1	17-6000-61*
Sephasil Peptide C8 12 µm ST 50/250	Steel	1	17-6000-62*
Sephasil Peptide C8 12 µm	-	100 g	17-6000-53*
Sephasil Peptide C8 12 µm	-	1 kg	17-6000-54*
Sephasil Peptide C18 12 µm ST 4.6/250	Steel	1	17-6000-29
Sephasil Peptide C18 12 µm ST 10/250	Steel	1	17-6000-63*
Sephasil Peptide C18 12 µm ST 20/250	Steel	1	17-6000-64*
Sephasil Peptide C18 12 µm ST 50/250	Steel	1	17-6000-65*
Sephasil Peptide C18 12 µm	-	100 g	17-6000-55*
Sephasil Peptide C18 12 µm	-	1 kg	17-6000-56*
µRPC			
µRPC C2/C18 PC 3.2/3#	Glass	1	17-0703-01
µRPC C2/C18 SC 2.1/10#	Steel	1	17-0704-01
µRPC C2/C18 ST 4.6/100	Steel	1	17-5057-01
Sephasil			
Sephasil C8 SC 2.1/10##	Steel	1	17-0769-01
Sephasil C18 SC 2.1/10##	Steel	1	17-0904-01

*available on request

#PC and SC columns are designed for SMART System. Precision Column Holder (17-1455-01) is required for attachment to ÄKTAdesign systems or other HPLC systems.

Sephasil C8 and Sephasil C18 media available only in pre-packed columns for SMART System

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