HPLC of Peptides and Proteins

High-performance liquid chromatography (HPLC) is an essential tool for the purification and characterization of biomacromolecules. The choice of the chromatographic method and the type of high-performance equipment are determined by the molecular nature of the investigated molecules and the aim of the research. There are eight basic modes of HPLC currently in use for peptide and protein analysis and purification, namely size-exclusion chromatography (HP-SEC), ion exchange chromatography (HP-IEX), normal phase chromatography (HP-NPC), hydrophobic interaction chromatography (HP-HIC), reversed-phase chromatography (RP-HPLC), hydrophilic interaction chromatography (HP-HILIC), immobilized metal ion affinity chromatography (HP-IMAC), and biospecific/biomimetic affinity chromatography (HP-BAC), and a number of subsets of these chromatographic modes, e.g., mixed mode chromatography (HP-MMC), charge transfer chromatography (HP-CTC), or ligand-exchange chromatography (HP-LEC).

In terms of usage, versatility, and flexibility, RP-HPLC techniques dominate the application world with peptides and proteins at the analytical- and laboratory-scale preparative levels. All of these various chromatographic modes can be operated under isocratic or gradient elution conditions, and in analytical or preparative situations. They all have common start-up procedures, which are outlined in the initial sections of this unit, and specific standard conditions that are detailed for the major modes and which represent starting points for further method development. As an example for appropriate method development, the RP-HPLC mode is described in greater detail and discussed according to four possible intended purposes, i.e.: (1) the purification of one component out of a natural or synthesized sample; (2) the simultaneous purification of several components; (3) the desalting of proteins or polypeptides obtained from other purification procedures; and (4) the characterization of the physicochemical properties of peptides or proteins. They all require a good understanding of the underlying common principles of polypeptide-ligand interaction. The basics of these principles are touched upon with references to further reading. Finally, a short section of this unit is dedicated to troubleshooting; however, many of the “check-back” confirmatory procedures implicit to sound operational practices and the identification of suitable alternatives for the separation strategy are included in the section on method development.

THE PROPERTIES OF PEPTIDES AND PROTEINS AND THEIR IMPLICATIONS FOR HPLC METHOD DEVELOPMENT

Peptides and proteins are a class of molecules containing amino acids as the fundamental units. The chemical organization (i.e., the primary structure or amino acid sequence) and the folded structure (i.e., the secondary, tertiary and quaternary structure) are the essential features of a polypeptide or protein, around which a chromatographic separation can be designed. Two sets of factors must be considered. The first relates to the structural properties of the amino acid entities themselves; the second relates to the chemical and physical attributes of the separation system per se.

Biophysical Properties of Peptides and Proteins

The 20 naturally occurring l-α-amino acids found in peptides and proteins vary dramatically in terms of the properties of the side chain or R-groups. Table 8.7.1 lists some of the fundamental properties of the common l-α-amino acids found in peptides and proteins. This chemical diversity becomes even greater in circumstances where some of these side chains have been post-translationally modified with carbohydrates or lipid moieties. The side-chains are generally classified according to their polarity (e.g., non-polar or hydrophobic versus polar or hydrophilic). The polar side chains are divided into three groups: uncharged polar, positively charged or basic, and negatively charged or acidic side chains. Peptides and proteins generally contain several ionizable basic and acidic functionalities. They therefore typically exhibit characteristic isoelectric points with the overall net charge and polarity in aqueous solution varying with pH, solvent composition and temperature. Cyclic peptides without ionizable side chains will have zero net charge, and they represent an exceptional subgroup.

The number and distribution of charged groups will influence the polarizability and ionization status of a peptide or protein, as well as...
as the microscopic and global hydrophobicity. These important factors ultimately determine the selection of the optimal separation conditions for the resolution of peptide and protein mixtures. Table 8.7.1 can be used to evaluate the impact of amino acid composition on retention behavior. For example, this information can be used to direct the choice of eluent composition or the gradient range in RP-HPLC; to assess the impact on retention of amino acid substitution or deletion with small peptides; or alternatively to guide the identification of peptide fragments derived from tryptic digestion of proteins for further sequence analysis.

### Parameters of the Mobile Phase/Stationary Phase

These parameters directly impact on the molecular properties of the polypeptide or protein during liquid chromatographic separations, and are listed in Table 8.7.2. In solution, a polypeptide or protein can, in principle, explore a relatively large array of conformational space. For small peptides (up to \( \sim 15 \) amino acid residues) a defined secondary structure (\( \alpha \)-helical, \( \beta \)-sheet or \( \beta \)-turn motif) is generally absent. With increasing polypeptide chain length, depending on the nature of the amino acid sequence, specific regions/domains of a polypeptide or protein can adopt preferred secondary, tertiary or quaternary structures. In aqueous solutions this folding, which internalizes the hydrophobic residues and thus stabilizes the
polypeptide structure, becomes a significant feature of peptides and proteins for chromatographic separations. A critical factor in the selection of an HPLC procedure is that the choice of experimental conditions will inevitably cause perturbations of the conformational status of these biomacromolecules. Although polypeptide and protein conformational stability can be manipulated in a number of ways (e.g., mobile and stationary phase composition, temperature) in HPLC, in most cases an integrated biophysical experimental strategy—including 1H 2-dimensional NMR (UNIT 17.5), Fourier transformed infrared (FTIR), ESI-MS (UNIT 16.1), or circular dichroism–optical rotatory dispersion (CD-ORD) spectroscopy—is required in order to determine the secondary and higher-order structure of a polypeptide or protein in solution or in the presence of specific ligands. Availability of such instrumentation is not mandatory, but the quality of the interpretation of the experimental results will become more substantial when additional results are independently obtained with such spectrometric procedures to confirm the participation of conformational or self-self aggregation effects with peptides or proteins under HPLC conditions.

**DETECTION OF PEPTIDES AND PROTEINS IN HPLC**

The peptide bond absorbs strongly in the far-ultraviolet (UV) region of the spectrum (\( \sim \lambda = 205 \) to 215 nm). Hence UV detection is the most widely used method for detection of peptides and proteins in HPLC (Table 8.7.3). Besides absorbing in the far-UV range, the aromatic amino acid residues (and to some extent cysteine) also absorb light above 250 nm. Knowledge of the UV spectra, in particular the extinction coefficients of the non-overlapping absorption minima of these amino acids, allows, in conjunction with UV-diode array detection (DAD) and second derivative or difference UV-spectroscopy, verification of peak purity and determination of the aromatic amino acid content.

<table>
<thead>
<tr>
<th>Group</th>
<th>Wavelength (nm)</th>
<th>Log ( \varepsilon_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide bond</td>
<td>190-210</td>
<td>2.0-3.8</td>
</tr>
<tr>
<td>His</td>
<td>211</td>
<td>3.8</td>
</tr>
<tr>
<td>Cys</td>
<td>250</td>
<td>2.5</td>
</tr>
<tr>
<td>Trp</td>
<td>280</td>
<td>3.7</td>
</tr>
<tr>
<td>Tyr</td>
<td>274</td>
<td>3.1</td>
</tr>
<tr>
<td>Phe</td>
<td>257</td>
<td>2.3</td>
</tr>
</tbody>
</table>

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**Table 8.7.2** Chemical and Physical Factors of the Chromatographic System that Contribute to the Variation in the Resolution and Recovery of Polypeptides, Proteins and Other Biomacromolecules in HPLC Systems (Hearn, 2000a)

<table>
<thead>
<tr>
<th>Mobile phase contributions</th>
<th>Stationary phase contributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic solvents</td>
<td>Ligand composition</td>
</tr>
<tr>
<td>pH</td>
<td>Ligand density</td>
</tr>
<tr>
<td>Metal ions</td>
<td>Surface heterogeneity</td>
</tr>
<tr>
<td>Chaotropic reagents</td>
<td>Surface area</td>
</tr>
<tr>
<td>Oxidizing or reducing reagents</td>
<td>Pore diameter</td>
</tr>
<tr>
<td>Temperature</td>
<td>Pore diameter distribution</td>
</tr>
<tr>
<td>Buffer composition</td>
<td>Particle size</td>
</tr>
<tr>
<td>Ionic strength</td>
<td>Particle size distribution</td>
</tr>
<tr>
<td>Loading concentration and volume</td>
<td>Particle compressibility</td>
</tr>
</tbody>
</table>

---

**Table 8.7.3** Relevant Absorption Bands and Extinction Coefficients in Proteins (Campbell and Dwek, 1984)

<table>
<thead>
<tr>
<th>Group</th>
<th>Wavelength (nm)</th>
<th>Log ( \varepsilon_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Tyr</td>
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<td>3.1</td>
</tr>
<tr>
<td>Phe</td>
<td>257</td>
<td>2.3</td>
</tr>
</tbody>
</table>
acid content of peptides and proteins. Moreover, the knowledge of the relative UV/VIS absorbancy of a peptide or protein is therefore crucial, since the choice of detection wavelength of peptides and proteins in RP-HPLC (and in the other HPLC modes) depends on the different UV cutoffs of the eluents used (Table 8.7.4). The common use of $\lambda = 215$ nm as the preferred detection wavelength for most analytical reversed-phase applications (and for those of other HPLC modes) with peptides and proteins is a good compromise between detection sensitivity and potential detection interference due to buffer absorption. However, wavelengths between 230 and 280 nm are frequently employed in preparative applications, where the use of more sensitive detection wavelengths could result in overloading of the detector response (usually above an absorbance value of 2.0 to 2.5 AU).

**START-UP PROCEDURES**

Correct selection of these first important steps may take more time than the ultimate experimental procedure if a high-performance separation of high resolution, robustness, and reproducibility is to be achieved. They require good planning and thorough work. The following details are representative of the types of equipment, materials, chemicals, and experimental protocols that can be routinely required for isocratic or gradient elution HPLC.

**Sample**
Peptide or protein sample (kept at 4°C if not used)

**Apparatus**
Pump module
Mixing chamber
Spectrophotometer with analytical or preparative flow cell
Injection valve
Analytical (10 to 100 µl) or preparative (500 to 1000 µl) sample loop
Column oven or thermostated column coolant-jacket coupled to recirculating cooler
Autosampler (optional)
Computer, printer, and software, e.g., Beckman, System Gold; Hewlett-Packard HP-1090A liquid chromatograph, or Waters 600/486 HPLC system with attendant data management systems and system automation controllers

**Chemicals**
Acetonitrile (HPLC grade)
Methanol (HPLC grade)
Acetone (HPLC grade)
Thiourea or sodium nitrate
Milli-Q water

**Glassware**
Two 1-liter eluent bottles
Two 1-liter measuring cylinders
10-ml measuring cylinder
Waste bottle
All glassware coming into contact with sample before and during analysis should be rinsed three times with Milli-Q water

**Mobile phase filtration facility**
Vacuum pump
1-liter reservoir
Support base with glass frit and integral vacuum connection
Funnel
Clamp
47-mm membrane filter (0.2 µm PTFE)

**Gases**
Helium
Nitrogen (for autosampler)

**Columns**
See respective sections below

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**Table 8.7.4** UV Cutoff Values of Different Organic Solvents in RP-HPLC

<table>
<thead>
<tr>
<th>Eluent</th>
<th>UV cutoff (nm)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>205</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>188</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>205</td>
</tr>
</tbody>
</table>

$^a$Wavelength at which the absorbance of a 1-cm-long cell filled with the solvent was 1.0, measured against water as reference.
HPLC peptide standards
See respective sections below

Tools
Screwdrivers:
\(\frac{1}{8}\)-in. and \(\frac{3}{4}\)-in. flat-head screwdrivers
Phillips screwdriver no. 2
Wrenches:
12-in. adjustable wrench (for compressed gas tank regulator)
Three open-end wrenches (two \(\frac{1}{4}\)-in. \(\times\) \(\frac{3}{16}\)-in.; one \(\frac{1}{2}\)-in. \(\times\) \(\frac{1}{16}\)-in.) for fittings columns and valves
Two long-jaw needle-nose pliers
Two bastard files
Two hex key (Allen) wrench sets (metric and nonmetric)
Tweezers
Pump seal insertion tool (if required)
Inner reamer
Teflon tape
Flashlight
Magnetic pick-up tool

Logbook
All steps must be documented to facilitate troubleshooting and reproduction

Spare parts
Zero dead volume union
Ferrules (steel, rhodyne) \(\frac{1}{16}\)-in. short and long (for rhodyne valves)
Bushing nuts
One-piece PEEK fitting
Tubing (steel, PEEK)
Column frits
In-line filter
Inlet filter
Fuses

Miscellaneous
Graduated 25-µl Hamilton glass syringe
1-ml syringe with truncated needle
Conical vials for autosampler
Laboratory coat
Gloves
Safety glasses
Stopwatch

PREPARATION OF THE SAMPLE
The following considerations are relevant to the preparation of samples containing peptides and proteins.

1. Dissolve the sample with half the target volume of eluent A (weak mobile phase). If the sample is not soluble, a small amount of eluent B (strong mobile phase) may be added (typically <25% of total volume). Small amounts of strong mobile phase may cause pre-elution of the sample depending on the sample loop size, column dimensions, and starting mobile phase composition of the gradient.

2. Inspect the sample for clearness and filter through a 0.2 µm PTFE filter if insoluble, opalescent, or solid particles are present. Alternatively, centrifuge the sample using the supernatant for injection. Samples that are not fully dissolved should not be injected, since they can block injector and column.

3. Store sample if not in use at 4°C or −20°C depending on the planned storage time and usage. Peptides and protein samples can degrade at room temperature. Avoid repeated freeze-thawing of the sample. Rather, prepare small aliquots of the peptide or protein sample, which are kept at −20°C and are used for one task.

PREPARATION OF THE MOBILE PHASE
The following considerations are relevant to the preparation of mobile phases to be used with samples containing peptides and proteins.

1. Prepare 1 liter each of eluent A (weak mobile phase) and B (strong mobile phase).

2. Mix each eluent either by stirring with a magnetic “flea” or by shaking a stoppered cylinder (time depends on volume).

3. Filter the eluents, first A, then B, through a 0.2 µm PTFE filter. Filtering of eluents increases the column lifetime and contributes to degassing.

4. Close unused eluent bottles with a stopper to avoid evaporation of the organic solvent.

SETTING UP THE HPLC INSTRUMENT
The following steps can be done either with or without a column connected to the HPLC instrumentation.

1. Switch on gas supply, nitrogen for the operation of the autosampler (if available), and helium for the degassing of the eluents.

2. Degas the eluents for 10 min at a rate of 100 ml/min, then reduce to a rate of 20 ml/min, which may be maintained throughout the experiments. Avoid excessive sparging since this will change the eluent composition.

3. Switch the detector lamp on in order to warm it up.
PRIMING OF THE PUMPS AND LOW PRESSURE LINES WITH ELUENTS

The following steps can be done either with or without a column connected to the HPLC instrumentation.

1. Open purge valve.
2. Set the pump at 100% buffer A and a flow rate of 1 ml/min. Under these settings, the eluent will bypass the column and travel directly to the waste fluid container (all wastes are collected for appropriate disposal).
3. Prime all tubing lines by switching the selector to the “prime line” position or by opening an additional valve which has an outlet to attach a syringe (see manufacturers’ handbook for specific details).
4. Draw (via the opened valve) 20 ml out of the eluent bottle with the syringe. Close the valve and discharge eluent from syringe in waste.
5. Prime all tubing lines again by drawing 10 ml in syringe, but keep the eluent in the syringe.
6. Switch selector to the “prime pump” position and push eluent gently through the pump.
7. Repeat the last four steps with 100% buffer B.
8. Put the selector in the “operate” position, close the valve, and remove the syringe from instrument.
9. Close the purge valve (now the eluent will travel through the column). This procedure should expel air bubbles from the pump heads and replace the previous eluent (~20 ml) in the solvent lines.

PREPARATION OF THE HPLC SYSTEM

The considerations below are relevant to the preparation of the HPLC system for use with samples containing peptides and proteins. The following steps can be done either with or without a column connected to the HPLC instrumentation.

1. Test the pump delivery system with eluents comprising 100% A and 100% B each for 5 min at a flow rate of 1 ml/min, collecting the eluent into a 10 ml cylinder. Some HPLC systems allow on-line pump diagnostics (e.g., Beckman System Gold). This procedure tests the reliability of the inlet and outlet valve, which may be blocked or not closing properly, e.g., due to salt from previous eluents. Some instrument systems allow the removal of the valve, which then can be sonicated in methanol for 15 min (care must be taken not to mix up the inlet and outlet valves, which often cannot be visually distinguished).
2. Flush the HPLC system with the eluent (e.g., 50% A and 50% B) and monitor the detector baseline. If spikes occur after 15 min of flushing in the absence of the column, the pressure in the detector cell is too low, leading to an outgassing of air and cycling bubble formation. Use the back-pressure restrictor (alternatively a restriction capillary) on the detector outlet to slightly, and very carefully, enhance the back-pressure. Every piece of capillary must be checked for blockage beforehand by connecting to a pump, thus bypassing the column and detector, since the detector cell is very sensitive to high pressure (consult manufacturer’s handbook for details).
3. Flush the needle port with the eluent B using a truncated 1-ml syringe in systems with manual injector. This procedure removes sample residues from previous injections.
4. Flush the Hamilton glass syringe (for manual injection) with methanol, then water to rinse it.
5. Flush the sample loop (in the load position) with three times the sample loop volume with eluent B using a Hamilton glass syringe.

THE GRADIENT DELAY (DWELL VOLUME) OF THE HPLC SYSTEM

The following steps must be done without a column connected to the HPLC instrumentation.

1. Connect the injector directly to the detector with union piece (zero length column).
2. Prepare a special eluent A and B, 200 ml each:
   - Eluent A: acetonitrile
   - Eluent B: acetonitrile/0.2% acetone.
3. Run a gradient of 10% to 90% B in 10 min at a flow rate of 2.0 ml/min. Detection is carried out at 254 nm. The measured value of the dwell volume can be influenced by the injection technique. If after the injection the valve remains in the inject position, the dwell volume will include the volume of the sample loop; if the valve is put back in load position, the dwell volume will not. This effect can produce errors with sample loops >100 µl. The same consideration is valid if the sample loop is exchanged from an analytical separation (e.g., a sample loop of 50 µl) to a semipreparative separation (e.g., a sample loop of ≥500 µl) on the same column.
4. Determine the gradient delay and present the results graphically in a format similar to that shown as Figure 8.7.1.
The dwell volume, \( V_D \), is the volume of eluent from the pump heads to the column inlet (including the mixing chamber volume). The dwell volume values range from 2 to 7 ml; autosamplers in particular make a large contribution to the delay volume. It should be determined with an accuracy of ±0.5 ml. The profile can be used for diagnostic purposes, since the volume accuracy of the pump delivery is also monitored.

Knowledge of the gradient delay is essential for method development, since it allows the accurate calculation of the \( S \) and \( k_0 \) values (Ghrist and Snyder, 1988a; Hearn, 1991a). Its determination is particularly important when establishing segmented gradients (since various errors can accumulate here), and when an established HPLC method is transferred from one instrument to another instrument.

**CONNECTING THE COLUMN**

The following considerations are relevant to the preparation of the HPLC column for use with samples containing peptides and proteins.

1. Flush the column with eluent B with the inlet connected to the injector and the outlet facing the waste collector (5 min at 1 ml/min for analytical columns). This procedure removes air that may have been trapped and replaces the storage buffer.
2. Connect the column outlet to the detector. Start the flow with 0.5 ml/min and slowly increase the flow rate to 1 ml/min.
3. Equilibrate the column first with eluent B until a stable baseline is reached or alternatively with 10 column volumes (~15 min for analytical column at 1 ml/min) and then with eluent A again with 10 column volumes. The pressure should be monitored and documented for each eluent since it can be used for diagnostic purposes.

**PROGRAMMING THE HPLC INSTRUMENT**

The following considerations are relevant to the programming of the HPLC instrument for use with samples containing peptides and proteins.

1. Program, according to the manufacturer’s handbook, the pump, the detector, the integration module, and the autosampler. Test the method with a test run before leaving the instrument alone.
2. Program a shutdown method for overnight runs, which will switch off the lamp and pump. This approach prolongs lamp life and saves eluents.

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**Figure 8.7.1** Graphical illustration of the approach employed to determine the gradient delay volume, \( D_v \). In this figure the gradient profile is illustrated for a defined flow rate (2 ml/min), with the gradient profile recorded from 10% to 90% buffer B at a specified wavelength such as 254 nm, as described in the text with acetonitrile-water-acetone mixtures.
INJECTING THE SAMPLE

The following considerations are relevant to the injection of the samples containing peptides and proteins onto the HPLC column.

1. Switch the sample loop to the load position and rinse with eluent A. This procedure
   removes eluent B (which may be present from rinsing the loop or previous runs). Failure to do
   so can cause pre-elution of the peptide or protein sample, particularly in conjunction with
   partially filled sample loops.

2. Load the sample slowly into the loop avoiding air bubbles. Do not squirt the sample
   into the loop too fast as it will end up in the waste.

3. Inject the sample by switching the valve swiftly into the inject position. If the switching
   is done too slowly, the pumps might shut down because the pressure limit is exceeded, as the
   valve is blocked in the intermediate switching position.

TESTING THE FUNCTIONAL HPLC SYSTEM

The following considerations are relevant to the testing of the HPLC system for use with
samples containing peptides and proteins.

1. Produce a blank run (inject eluent A) and run a gradient from 100% eluent A to 100%
   eluent B under the same conditions as intended for the peptide or protein sample. Repeat if
   peaks occur. This procedure cleans the column of peptides and proteins from previous separations, which have not been removed by the flushing process.

2. Measure the dead volume of the column with thiourea or sodium nitrate (or any other
   noninteractive solute).

3. Test the column performance with a gradient run and an appropriate test mixture (see
   below for details). First, this test allows the evaluation of the column bed integrity (low
   integrity will be associated with split, fronting or tailing peaks) and column performance (in
   terms of plate numbers). Second, this test allows, if repeated at regular intervals, the monitor-
   ing of the performance during the lifetime of a column, and the assessment of batch-to-
   batch differences of column fillings.

LOGBOOKS

Record keeping is essential for liquid chromatography system maintenance and confirmation/substantiation of the experimental results. Three types of logbooks are recommended, the system logbook, the column logbook, and the experiment/assay logbook (Dolan and Snyder, 1989).

System Logbook

This logbook should contain information on:

1. The module identification (brand, model, serial number, purchase data, warranty
   information) for the entire liquid chromatography (LC) system: injector, autosampler, pump(s), detector, software, column.

2. Module replacements, maintenance records and upgrades.

3. Reference chromatograms and operating parameters.


5. Column replacement (cross-reference to column logbook).

Column Logbook

This logbook should contain information including a summary of use, column life in months and number and type of samples, cause of failure, and suggestions for extending life. For each column this would be tabulated as follows.

1. Date column first used.

2. Specification: mobile phase flow rate, plate number, peak shape, dead volume over the lifetime of the column.

3. Performance of new column (validated with a test mixture).

4. Record of use (instrument, operator, number and type of samples).

5. Storage information.

6. Maintenance performed (type of back-flush protocols, frit replacement, etc.).

7. Revaluation of column performance.

8. Cause of failure.

Experiment/Assay Logbook

This logbook should contain information on:

1. Equipment configuration.

2. Operating condition(s).

3. Mobile phase recipe(s) (literature references if available).

4. Sample pre-treatment method(s) (literature references if available).

5. Assay procedure(s) (literature references if available).

6. Data analysis procedure(s).

In the following sections, illustrative examples of standard operating protocols for the major modes of HPLC are described based on
the instrumental system validation procedures described above.

**STANDARD OPERATING CONDITIONS FOR HP-SEC**

The separation of peptides and proteins by high-performance size-exclusion chromatography (HP-SEC) is based on the concept that molecules of different sizes (hydrodynamic volume, Stoke’s radius) permeate to different extents into porous SEC separation media and thus exhibit different permeation coefficients according to differences in their molecular weights (Regnier, 1983). However, many SEC materials are slightly hydrophobic or can weakly act as ion exchangers. These properties lead to nonideal behavior (specifically electrostatic or hydrophobic interactions between the peptide or protein and the matrix). This feature is not necessarily a disadvantage, since mixed-mode selectivities can be achieved, but can be suppressed by the addition of a salt at a reasonably high ionic strength, i.e., ≥100 mM, to the mobile phase (Mant et al., 1987).

**Chromatographic Conditions**

Column: e.g., TSK-250 (10 µm, 300 Å, 300-mm length × 7.5-mm i.d.)
Sample size: <2 mg peptide/protein
Sample loop size: 20 to 200 µl
Isocratic elution
Eluent A: 50 mM KH₂PO₄, pH 6.5, 0.1 M KCl
Flow rate: 0.5 ml/min
Detection: 214 nm
Temperature: room temperature
Peptide standards for column testing as described, e.g., in Mant and Hodges (1991a)

Method development in the HP-SEC of peptides and proteins can be performed via the following steps:

1. Select sorbent of the most appropriate average pore size, packed into a column of suitable length.
2. Check for “ideal” and “non-ideal” retention effects.
3. Optimize plate number (adjust the flow rate or change to a column of different length).

An example of HP-SEC for the separation of peptides and proteins is illustrated in Figure 8.7.2. In this example, the resolution of the 50S ribosomal proteins from *Thermus aquaticus* was achieved on a tandem TSK-250 column (75-mm length × 7.5-mm i.d. and 300-mm length × 7.5-mm i.d.) at a flow rate of 0.5 ml/min with a 50 mM (NH₄)₂SO₄, 20 mM NaH₂PO₄ buffer system, pH 5.0.

![Figure 8.7.2](image-url)

**Figure 8.7.2** Illustrative example of the use of HP-SEC in peptide and protein separation and analysis. In this example the resolution of 50S ribosomal proteins from *Thermus aquaticus* in 2% acetic acid (Molnar et al., 1989b) was achieved with a tandem TSK-250 column (75-mm length × 7.5-mm i.d. and 300-mm length × 7.5-mm i.d.) using a flow rate of 0.5 ml/min and a 50 mM (NH₄)₂SO₄, 20 mM NaH₂PO₄, pH 5.0, with detection at 205 nm.
STANDARD OPERATING CONDITIONS FOR HP-NPC

Chromatographic systems in which the stationary phase is more polar than the mobile phase were developed at the beginning of the modern era of liquid chromatography and were known under the acronym NPC (“normal phase” liquid chromatography). In contrast to RPC with immobilized n-alkyl ligands, where the interaction of solute and stationary phase is based on solvophobic phenomena, the interaction in NP-LC is based on adsorption. The retention behavior of peptides and proteins in NP-LC is often described in terms of the classical concepts of multisite displacement and site occupancy theory (Snyder, 1970). Today, NP-HPLC is mainly used for the separation of polyaromatic hydrocarbons (PAHs), heteroaromatic compounds, nucleotides, nucleosides, etc., and much less frequently for protected synthetic peptides, deprotected small peptides in the “flash chromatographic mode,” and protected amino acid derivatives used in peptide synthesis (Ballschmiter and Wossner, 1998).

Originally, normal phase chromatography was limited to unmodified silica columns. Recent work has, however, utilized polar bonded phases such as amino (−NH₂), cyano (−CN), or diol (−COHCOH⁻) coated sorbents. Chromatography on such modified normal phase packing materials is also known as polar bonded phase chromatography (PBPC), which is used for the separation of peptides (Yoshida, 1998) and proteins (Buchholz et al., 1982). Today, one of the main applications of modified normal phase silica materials is in HPLC-integrated solid phase extraction procedures (SPE; Papadoyannis et al., 1995). These types of sorbents, particularly when used as precolumn packing materials in LC-LC column switching settings in conjunction with restricted access sorbents materials (RAM), allow multiple injections of untreated complex biological samples such as hemolyzed blood, plasma serum, fermentation broth, cell tissue homogenates, etc., for the isolation of bioactive peptides. Typically, with RAM materials, hydrophilic, electroneutral diol groups are immobilized onto the outer surface of spherical particles. This layer prevents nonspecific interactions between the support matrix and protein(s) or other high-molecular-weight biomolecules, which are thus excluded from the interior regions of the particle and elute as nonretained components. The inner surfaces of the porous RAM particles are, however, chemically modified with n-alkyl ligands, which are only freely accessible for low molecular analytes, such as peptides. As a consequence, significant enrichment or partial resolution of peptide analytes can be achieved.

Chromatographic Conditions

Column: e.g., diol-phase, aminopropyl-phase, cyano-phase column, 250-mm length × 4.6-mm i.d.
Sample size: <2 mg peptide/protein
Sample loop size: 20 to 200 µl
Linear A→B gradient
Eluent A: 0.1% TFA in water
Eluent B: 0.1% TFA in 20% acetonitrile/80% water (v/v)
Gradient range and time: 0% to 100% eluent B in 60 min
Flow rate: 1 ml/min
Detection: 214 nm
Temperature: room temperature

An example of the use of HP-NPC for the separation of peptides and proteins is illustrated in Figure 8.7.3. In this example, the resolution of ferritin, bovine serum albumin, chymotrypsinogen, cytochrome c, and alanine was achieved on a LiChrosorb Diol column (250-mm length × 16-mm i.d.) at flow rate of 2.5 ml/min and a 0.1 M phosphate buffer system, pH 5.0 (Buchholz et al., 1982).

STANDARD OPERATING CONDITIONS FOR HP-HIC

As in reversed-phase chromatography, the hydrophobic interaction between the peptide or protein and the nonpolar ligands immobilized onto the surface of the sorbent represents the dominant effect in hydrophobic interaction chromatography (Fausnaugh et al., 1984; Fausnaugh and Regnier, 1986; Gooding et al., 1986; Wu et al., 1986a,b; Melander et al., 1989; Antia et al., 1995; Hearn, 2000b). In both techniques, peptides and proteins are eluted by lowering the surface tension of the mobile phase. However, in HP-HIC, this is achieved with a decreasing salt concentration, i.e., by increasing the water content of the eluent, in contrast to RP-HPLC where the decrease in surface tension of the eluent is achieved through an increase in the organic solvent content of the mobile phase. As such, the minimum surface tension reached in the HP-HIC of polypeptides or proteins with binary water-salt systems corresponds to the surface tension of pure water, i.e., 78 dynes/cm. These differences between RP-HPLC and HP-HIC have fundamental effects on the recovery of proteins in bioactive state, as well as on the selectivity of the system. Typically, kosmot-
ropic (anti-chaotropic) salts, i.e., ammonium sulfate, sodium sulfate, or magnesium chloride of high molal surface tension increment, are to be preferred for HP-HIC applications with polypeptides and proteins. In HP-HIC, non-polar ligands with lower hydrophobicity and lower ligand density (∼1/10th that of RP-HPLC sorbents) are employed. HP-HIC sorbents should be selected on the basis of the critical hydrophobicity concept (Hearn, 2000a,b). In combination with nondenaturing mobile phases, proteins, in particular, can potentially be eluted in their native conformation from HP-HIC sorbents.

**Chromatographic Conditions**

Column: e.g., TSK Phenyl column, 75-mm length × 7.5-mm i.d., 10 µm
Sample size: <2 mg peptide/protein
Sample loop size: 20 to 200 µl
Linear A→B gradient is the preferred method with “hold” options

Eluent A: 0.1 M NaH$_2$PO$_4$, 2.0 M (NH$_4$)$_2$SO$_4$, pH 7.0
Eluent B: 0.1 M NaH$_2$PO$_4$, pH 7.0
Gradient rate: 5% eluent B/min
Gradient range and time: 0 to 100% B in 20 min
Flow rate: 1 ml/min
Detection: 214 nm
Temperature: room temperature

The method development in HP-HIC can be performed in the following steps:

1. Select type of salt (antichaotropic) and concentration range, taking into account the concentration of the salt that is required to reach saturation, and then keep the maximum concentration below this value by ∼20%.
2. Optimize the gradient conditions (gradient run time, starting and final mobile phase composition).
3. Optimize the band spacing (pH, organic solvent).
4. Optimize the column conditions (flow, column length).

**Figure 8.7.3** Illustrative example of the use of HP-NPC in peptide and protein separation and analysis. In this example the resolution of ferritin ($M_r$, 364,600), bovine serum albumin ($M_r$, 67,000), chymotrypsinogen ($M_r$, 25,000), cytochrome c ($M_r$, 12,500), and alanine ($M_r$, 121; Buchholz et al., 1982) was achieved on a LiChrospher Diol column (250-mm length × 16-mm i.d.) at a flow rate of 2.5 ml/min and a 0.1 M phosphate buffer system, pH 5.0. In this specific case, size exclusion effects dominated the global resolution, with polar phase effects contributing to the individual selectivities of these proteins, as assessed from the “nonideality” of the log $M_r$ versus $V_e$ plots.
Examples of the use of HP-HIC with proteins are described in Fausnaugh et al. (1984); Fausnaugh and Regnier (1986); Gooding et al. (1986); Melander et al. (1989); Wu et al. (1986a, b); Antia et al. (1995); and Hearn (2000a, b). Figure 8.7.4 illustrates the resolution of cytochrome c (1), ribonuclease (2), lysozyme (3), bovine serum albumin (4), ovalbumin (5), \( \alpha \)-chymotrypsin (7), and myoglobin (8) on a Butyl-G3000 SW column (150-mm length \( \times \) 6-mm i.d., 10 \( \mu \)m) with a 60-min linear gradient from 0% to 100% eluent B (eluent A: 1.5 M ammonium sulfate, 0.1 M phosphate buffer, pH 6.0; eluent B: 0.1 M phosphate buffer, pH 6.0).

**STANDARD OPERATING CONDITIONS FOR HP-IEX**

Peptides and proteins can be eluted in ion exchange chromatography by either isocratic or gradient elution (Chang et al., 1976; Kopaciewicz and Regnier, 1983a, 1986; Regnier, 1984; Kopaciewicz et al., 1985; Hearn et al., 1988; Heinitz et al., 1988; Hodder et al., 1990). Gradient elution is usually performed with a linear A \( \rightarrow \) B gradient of a salt such as sodium or potassium chloride in phosphate buffer. The retention of peptides and proteins on ion-exchange sorbents arises from electrostatic interactions between the ionized surface of the solute and the charged surface of the sorbent. For peptide and protein separations, the use of a strong cation exchanger has a considerable advantage over other varieties of ion-exchanger (Mant and Hodges, 1985), since the column can retain its negatively charged character over the whole range from acidic to neutral pH. Both weak and strong cation exchangers, e.g., based on carboxymethyl or sulfonopropyl ligands, as well as weak and strong anion exchangers, e.g., dimethylamino or quaternary ammonium ligands, are available and can be applied to the HP-IEX of peptides and proteins.
With peptides and proteins, at neutral pH, the side-chain carboxyl groups of the acidic amino acid residues—glutamic acid and aspartic acid—are completely ionized. Below pH 3.0 they are almost completely protonated. A change of pH therefore allows the retention of peptides and proteins to be varied according to the net charge of these biosolute(s). Even when the most dominant effect in IEX is electrostatic, in many cases the participation of a mixed mode separation, whereby hydrophobic interaction contributes, cannot be excluded (Burke et al., 1989). This is not necessarily a disadvantage, since it permits selectivity modulation with complex mixtures of peptides and proteins. Hydrophobic interactions can be suppressed by adding a nonpolar solvent, such as acetonitrile, or a nonionic detergent, such as Brij-25, to the mobile phase.

**Chromatographic Conditions**

Column: e.g., strong cation exchanger with sulfonate functionality (5 µm, 300 Å, 75-mm length × 7.5-mm i.d.)

Sample size: <2 mg peptide/protein

Sample loop size: 20 to 200 µl

Linear A→B gradient

Eluent A: 5 mM KH₂PO₄, pH between 3.0 and 7.0

Eluent B: 5 mM KH₂PO₄/0.5 M KCl, pH between 3.0 and 7.0

Gradient rate: 3.3% eluent B/min

Gradient range and time: 0% to 100% eluent B in 30 min

Flow rate: 1 ml/min

Detection: 214 nm

Temperature: room temperature

Peptide standards for column testing as described, e.g., in Mant and Hodges (1991a)

The method development in HP-IEX can be performed in the following steps:

1. Select type of anion or cation exchanger.

2. Optimize gradient conditions (gradient run time, starting and final mobile phase composition).

3. Optimize band spacing (pH, salt type).

4. Optimize column conditions (flow, column length).

Examples of the use of HP-IEX with proteins are described in Chang et al. (1976); Regnier (1984); Kopaciewicz et al. (1985); Kopaciewicz and Regnier (1986); Hearn et al. (1988); Heinitz et al. (1988); Burke et al. (1989); Hodder et al. (1990); Mant and Hodges (1985, 1991b); and Hearn (2000a). Figure 8.7.5 illustrates the use of HP-IEC for the resolution under gradient elution conditions from 0% to 100% B (eluent A: 0.1 M Tris-Cl, pH 7.5: Eluent B: 0.1 M Tris-Cl/0.2 M NaCl, pH 7.5) of ovalbumin isoforms on a TSK-GEL IEX-545 DEAE SIL column (150-mm length × 6-mm i.d.) using a 90-min linear gradient at a flow rate of 1.0 ml/min.

**Figure 8.7.5** Illustrative example of the use of HP-IEX in peptide and protein separation and analysis. In this example the resolution of ovalbumin isoforms (Kato et al., 1982), FrIa and FrIb, was achieved on a TSK-GEL IEX-545 DEAE SIL column (150-mm length × 6-mm i.d.) using a 90 min linear gradient at a flow rate of 1.0 ml/min from 0-100% B (eluent A: 0.1 M Tris-Cl, pH 7.5: eluent B: to 0.1 M Tris-Cl, 0.2 M NaCl, pH 7.5).
Peptides can be separated on strong hydrophilic materials, i.e., poly(2-hydroxyethylaspartamide) silica (Alpert, 1990), whereby their selectivity changes with the concentration of the organic modifier. At high organic modifier concentrations (i.e., >55\% \text{-} \text{propanol}), the solute is retained on the column. The solute is subsequently eluted with a decreasing gradient of organic modifier, whereby with low organic modifier concentrations the separation of the solutes is governed by molecular sieving effects. HP-HILIC allows specifically the evaluation of highly polar compounds, which cannot be retained on traditional reversed-phase stationary phases. To date a variety of HP-HILIC sorbent packings, including amide-, poly(2-hydroxyethyl)-aspartamide-, cyclodextrin-, or teicoplanin-derivatized stationary phases are available (Strege, 1998; Risley and Strege, 2000). HP-HILIC can be applied in a variety of challenging separation tasks, e.g., the separation of glycopeptides (Zhang and Wang, 1998) or the desalting of electroeluted proteins from SDS-PAGE systems (Jeno et al., 1993). In this mixed-mode hydrophilic interaction/cation exchange chromatographic mode (Mant et al., 1998a,b; Litowski et al., 1999), peptides are usually subjected to linear, decreasing salt gradients in the presence of high levels of organic modifier. The following example is representative of the conditions that can be employed in the HP-HILIC of peptides and small proteins.

**Chromatographic Conditions**

Column: e.g., HILIC PolySulfoethyl A sorbent with a poly(2-sulfonethylaspartimide) functionality (5 µm, 300 Å, 200-mm length × 1-mm i.d.)

Sample size: <2 mg peptide/protein

Sample loop size: 20 to 200 µl

Linear A→B gradient

Eluent A: 20 mM triethylammonium phosphate/80\% acetonitrile, pH 3.0

Eluent B: eluent A with 400 mM NaClO₄, pH 3.0.

Gradient rate: 2.5\% B/min

Gradient range and time: 0\% to 100\% B in 90 min

Flow rate: 1 ml/min

Detection: 214 nm

Temperature: 30°C

Method development in the HP-HILIC of peptides and proteins can be performed via the following steps:

1. Select sorbent of the most appropriate average pore size, packed into a column of suitable length.

2. Check for “ideal” and “non-ideal” retention effects.

3. Optimize selectivity through the changes in the composition or concentration of the organic solvent and kosmotropic/chaotropic salt additives.

4. Optimize plate number (by adjusting the flow rate, or changing to a column of different length).

Examples of the use of HP-HILIC with peptide standards for column testing are described in Mant and Hodges (1991b). In Figure 8.7.6 is shown the separation of the tryptic glycopeptides of Asn97 for recombinant human interferon γ (Zhang and Wang, 1998) on a polyhydroxyethylaspartamide column (150-mm length × 1-mm i.d.) using a flow rate of 50 µl/min. In this example, a 60-min gradient from 100\% A (85\% acetonitrile, 15\% water, 10 mM triethylamine) to 100\% B (20\% acetonitrile, 15\% water, 10 mM triethylamine, 25 mM sodium perchlorate), pH 6.0 was employed.

**STANDARD OPERATING CONDITIONS FOR HP-IMAC**

Immobilized metal-chelate affinity chromatography (IMAC) exploits the affinities of the side chain moieties of specific surface accessible amino acids in peptides and proteins for the coordination sites of immobilized transition metal ions (Porath et al., 1975; Zachariou et al., 1993). The majority of investigations employed tri- or tetradentate ligands, such as iminodiacetic acid (IDA), nitrotriacetic acid (NTA), tris-(carboxymethyl)ethylenediamine (TED), O-phosphoserine (OPS) or carboxy-methylaspartic acid (CMA) (Zachariou et al., 1993). Novel immobilized chelate systems, such as 2,6-diaminomethylpyridine or cis and trans carboxymethylproline, have shown binding properties different from the IMAC behavior of other chelating ligands (Hearn et al., 1997; Chaouk and Hearn, 1999). These techniques, in conjunction with soft gel matrices, have been predominantly applied for the preparative purification of globular proteins. Procedures to immobilize the IMAC ligand at the surface of silica supports, based on classical ligand exchange principles, have permitted useful guidelines to be developed for the design of HP-IMAC applications for peptides and proteins at the analytical level (Wirth and Hearn, 1993). However, for the majority of the possible HP-IMAC applications with peptides or proteins
no standard chromatographic conditions exist. Rather, different elution regimes have tended to be employed with HP-IMAC systems, based on the selection of either empirical step- or gradient-elution protocols involving changes in pH, buffer composition, or the concentration of salts or another competitive binding reagent such as imidazole or malonic acid. Various applications of different IMAC systems for the separation of peptides and proteins have recently been reviewed elsewhere (Porath et al., 1975; Jeno et al., 1993; Mant et al., 1998a,b; Litowski et al., 1999; Hearn, 2000a). The following example is representative of the conditions that can be employed in the HP-IMAC of peptides and small globular proteins.

**Chromatographic Conditions**

Column: e.g., HP-IMAC sorbent with a Cu$^{2+}$ ion chelated to immobilized iminodiacetic acid functionality, i.e., IDA-Cu(II) TSK gel chelate-SPW column (5 µm, 300 Å, 100-mm length × 4.6-mm i.d. or 75-mm length × 8-mm i.d.)

Sample size: <1 mg peptide/protein

Sample loop size: 20 to 200 µl

Linear A→B gradient

Eluent A: 50 mM acetic acid/50 mM MES/50 mM HEPES/80 mM Na$_2$SO$_4$/2 × 10$^{-6}$ M Cu$^{2+}$, pH 8.0; or 1 mM imidazole/20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0

Eluent B: 50 mM acetic acid/50 mM MES/50 mM HEPES/50 mM ammonium acetate/2 × 10$^{-6}$ M Cu$^{2+}$, pH 5.5; or 20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0 with 20 mM imidazole gradient

Gradient rate: 5% B/min

Gradient range and time: 0% to 100% eluent B in 20 min

Flow rate: 1 ml/min

Detection: 280 nm

Temperature: 25°C

Method development in the HP-IMAC of peptides and proteins can be performed via the following steps:

1. Select sorbent of the most appropriate average pore-size, packed into a column of suitable length.

2. Check for “ideal” and “non-ideal” retention effects.
3. Optimize selectivity through the changes in the type of chelating ligand and metal ion, i.e., whether borderline, hard or soft metal ion, the pH, the type and concentration of the buffer used at the loading and washing stages, and finally at the elution stage.

4. Optimize plate number (by adjusting the flow rate or changing to a column of different length).

Examples of the use of HP-IMAC with proteins are described in Porath et al. (1975); Wirth and Hearn (1993); Zachariou et al. (1993); Jiang et al. (1998); Porath (1988); and Hearn (2000a). Figure 8.7.7 illustrates the use of HP-IMAC for the resolution of human gastrin-I (24), human GIP (29), Trp(for)-human GIP (30), human big gastrin (35), human GIP (37), porcine GIP (38), and bovine GIP (39) (Yip et al., 1989) was achieved on an IDA-Cu(II) TSK gel chelate-5PW column (75-mm length \( \times \) 8-mm i.d.) with a flow rate of 1 ml/min equilibrated with a 1 mM imidazole, 20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0, and eluted with an 1-20 mM imidazole gradient (dotted line).

**Figure 8.7.7** Illustrative example of the use of HP-IMAC in peptide and protein separation and analysis. In this example the resolution of human gastrin-I (24), human GIP(21-42) (29), Trp(for)-human GIP(21-42) (30), human big gastrin (35), human GIP (37), porcine GIP (38), and bovine GIP (39) (Yip et al., 1989) was achieved on an IDA-Cu(II) TSK gel chelate-5PW column (75-mm length \( \times \) 8-mm i.d.) with a flow rate of 1 ml/min equilibrated with a 1 mM imidazole, 20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.0 and eluted with a 1 to 20 mM imidazole gradient (dotted line).

**STANDARD OPERATING CONDITIONS FOR HP-BAC**

During the last 25 years, biospecific affinity chromatography (BAC) had evolved as a well established method for analytical separation (Porath, 1988), as well as the preparative scale purification of biopolymers of complex biological origin. Additionally, HP-BAC is a useful technique to investigate peptide-protein and protein-protein interactions. In HP-BAC, the separation is based on the propensity for biorecognition between the protein or peptide and its naturally occurring ligand (or suitable mimic), in particular, on its relative binding affinity for a specific ligand. The ligand should have a high specificity for the protein or peptide, should bind to the protein or peptide reversibly with rapid on-and-off kinetics, and
should be chemically stable under elution conditions. The introduction of HP-BAC dramatically increased the speed of affinity chromatographic separation of proteins, including sample loading and column regeneration (Ohlson et al., 1978). However, since the specificity and the selectivity of the HP-BAC both are very high, the range of applications is, paradoxically, much more limited than the other modes of HPLC. Every separation ideally demands its own ligand type, ligand density, column configuration, and particle type in order to enable the optimal purification of a particular component. Consequently, there are no “standard” HP-BAC conditions due to the broad range of ligand types. Some ligands are specific for a particular class of protein; however, other ligands can be multifunctional and bind to a number of related molecules. Currently, commercially available HP-BAC columns are supplied as activated supports (epoxy-, N-hydroxysuccinimido-, tresyl-, hydroxylpropyl-) ready for coupling appropriate ligands (via accessible SH, OH, or NH₂ groups) or alternatively as preformed matrices with commonly used ligands already attached (e.g., protein A). The following example is representative of the conditions that can be employed in the HP-BAC with peptides and small globular proteins.

**Chromatographic Conditions**

**Column**: custom designed, i.e., glycidoxypropyl-activated silica such as epoxy-activated LiChrosorb containing a suitable immobilized ligand functionality (10 µm, 10000 Å, 100-mm length × 4.6-mm i.d.)

**Sample size**: <2 mg peptide/protein

**Sample loop size**: 20 to 200 µl

**Linear gradient or step elution**

Eluent for equilibration: non-denaturing conditions with adequate buffer capacity

Eluent for desorption: non-denaturing conditions of different pH to the equilibration conditions, with adequate buffer capacity and appropriate competitive species to affect efficient dissociation of the affinant-affinate cognate interaction

**Gradient rate**: according to affinity of interaction, HP-BAC sorbent characteristics, and flow rate

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![Figure 8.7.8](image-url)  
**Figure 8.7.8** Illustrative example of the use of HP-BAC in peptide and protein separation and analysis. In this example, the enzyme activity is shown for rabbit muscle lactate dehydrogenase (LDH) (Anspach et al., 1988) separated on a Procion Red MXSB immobilized non-porous silica column (40-mm length × 6-mm i.d.) using a flow rate of 1 ml/min and step elution (eluent A: 10 mM phosphate buffer, pH 8.0: eluent B: 0.5 M sodium chloride in 10 mM phosphate buffer, pH 8.0).
Flow rate: 0.5 to 1 ml/min
Detection: 214 to 280 nm
Temperature: 4°C

In Figure 8.7.8 an example is shown of the use of HP-BAC for the resolution of rabbit muscle lactate dehydrogenase (LDH) on a Procion Red MX5B dye immobilized onto nonporous silica column (40-mm length × 6-mm i.d.) using a flow rate of 1 ml/min and step elution with eluent A: 10 mM phosphate buffer, pH 8.0, and eluent B: 0.5 M sodium chloride in 10 mM phosphate buffer, pH 8.0.

**STANDARD OPERATING CONDITIONS FOR RP-HPLC**

As noted above, RP-HPLC procedures currently represent the majority of applications for peptide analysis and purification, and over 80% of all analytical studies with proteins. The dominant effect in reversed-phase chromatography is a hydrophobic interaction between the nonpolar amino acid residues of peptides or proteins and the nonpolar ligands, typically immobilized onto the surface of a spherical, porous silica particle (Hearn, 2001), although nonpolar polymeric sorbents derived, e.g., from cross-linked polystyrene-divinylbenzene, can also be employed. In this technique, isocratic elution, step elution, or gradient elution modes can be utilized to purify peptides and proteins. Besides the requirement for an organic solvent to be used as a surface tension modifier, ion-pair reagents (Mant and Hodges, 1991c) are utilized at low pH (e.g., pH 2.1) to suppress silanophilic interactions between free silanol groups on the silica surface and basic amino acid residues. Silica-based packing materials of 3 to 10 µm average particle diameter and ≥300 Å pore size, with n-butyl, n-octyl, or n-octadecyl ligands, are widely used.

**Chromatographic Conditions**

**Chemicals**

HPLC-grade acetonitrile, 2-propanol, methanol, or other suitable organic solvents with UV transparency down to 210 nm
Trifluoroacetic acid (TFA)
NaH₂PO₄ or other suitable salts
H₃PO₄
Milli-Q water or equivalent

TFA employed for protein sequencing may not be suitable because it can contain antioxidants.

**Preparing the mobile phase**

1. Prepare eluent A and B, e.g.: eluent A: 0.1% TFA in water and eluent B: 0.09% TFA in 60% acetonitrile/40% water (v/v).

The volumes of organic solvent and water are measured in two different cylinders and then combined, because of the volume contraction upon mixing, which may be up to 30 ml per liter of prepared solvent (depending on the nature of the solvent). Failure to do so can lead to substantial errors in mobile phase composition.

To compensate for the baseline shift in gradient elution (because organic components absorb more light at low wavelengths) when working with water/organic eluents, the amount of ion pair reagent (TFA, H₃PO₄) in eluent B is usually decreased by 10% to 15% in comparison with eluent A, yielding a flat baseline (Dolan, 1991).

It is imperative that eluents be prepared in the fume hood. TFA is extremely corrosive; laboratory coat, gloves, and protective glasses must be worn.

2. Mix the solvent either by stirring with a Teflon-coated magnetic flea or by shaking a stoppered cylinder.

Parafilm should not be used, under any circumstances, to cover the eluents, since the organic solvents in combination with acidic ion-pair reagent components in the eluents will dissolve components in the Parafilm, yielding extra peaks in the chromatogram.

**Testing the RP-HPLC stationary phase**

The following considerations are relevant to the evaluation of HPLC stationary phases for use with samples containing peptides and proteins.

1. Test the column performance with a gradient run and a test mixture: RP-HPLC test mixture example: 0.15% (w/v) dimethylphthalate, 0.15% (w/v) diethylphthalate, 0.01% (w/v) diphenyl, 0.03% (w/v) O-terphenyl, 0.32% (w/v) dioctylphthalate in methanol.

2. Peptide standards for column testing are described, for example, in Mant and Hodges (1991b).

**Peptide purification by RP-HPLC**

In addition to the reagents and procedures described in the preceding section the following additional materials are required:

Chemicals: acetonitrile, trifluoroacetic acid (TFA)
Purification of peptides derived from Solid-Phase Peptide Synthesis (SPPS) can be performed in the following steps.

1. Separation of the crude peptide (~100 µg) with an analytical RP-HPLC procedure allows the assessment of the sample in terms of purity, peak profile and elution conditions. Appropriate equipment/conditions are as follows:
   - Column: e.g., C-4, C-8, C-18, etc. (5 µm, 300 Å, 150-mm length × 4.6-mm i.d.)
   - Sample size: <2 mg peptide/protein
   - Sample loop size: 20 to 200 µl
   - Linear A→B gradient
   - Eluent A: 0.9% aqueous TFA
   - Eluent B: 0.1% TFA in acetonitrile/water
   - Gradient rate: 1% B/min
   - For example, for 60% ACN/water: gradient range and time: 0% to 100% eluent B in 60 min
   - Flow rate: 1.0 ml/min
   - Detection: 214 nm
   - Temperature: room temperature

   An example of the analytical use of RP-HPLC in peptide analysis is shown in Figure 8.7.9. In this case, resolution of the crude N-acetyl lipocortin-I[2-26] product from solid phase peptide synthesis (panel A), and the purified product (panel B) was achieved with a TSK-ODS-120 T column (150-mm length x 4.6-mm i.d., 120 Å, 5 µm, end-capped) using a flow rate of 1 ml/min and a 60 min gradient from 100% A (0.1% TFA in water) to 100% B (90% acetonitrile 0.09% TFA), pH 2.1. (A) Crude peptide; (B) purified peptide.

Conventional Chromatographic Separations

8.7.19
Eluent A: 0.9% aqueous TFA
Eluent B: 0.1% TFA in acetonitrile/water
Gradient rate: 0.66% eluent B/min
For example for 60% ACN/water: gradient range and time: 0% to 100% eluent B in 90 min
Flow rate: 7.5 ml/min
Detection: 254 nm
Temperature: room temperature

The preparative use of RP-HPLC in peptide purification is shown in Figure 8.7.10. In this example, the isolation of the synthetic N-acetyl lipocortin-1[2-26]—Ac-AMVSEFLKQAW-FIENEEQYVQTVCNH2—(70 mg) from the crude mixture obtained from solid phase peptide synthesis using a TSK-ODS-120 T column (300-mm length × 21.5-mm i.d., 120 Å, 10 µm, end-capped) using a flow rate of 7.5 ml/min and a 135-min gradient from 25% B to 100% B (A: 0.1% TFA in water, B: 60% acetonitrile 0.09% TFA), pH 2.1.

In order to avoid detector response overloading effects, usually wavelengths from 230 to 280 nm are chosen. However, small amounts of chemical scavengers (used during the SPPS procedures) present in the crude peptide solution can absorb very strongly in this wavelength range. It may be worthwhile to sacrifice up to 1 mg of sample and to perform a preparative separation with detection at 214 nm in order to unambiguously determine the retention time of the main peptide product.

3. Collection of HPLC-fractions (3 to 7.5 ml).
4. Analysis of aliquots (30 to 50 µl) of the collected fraction with an analytical RP-HPLC (see step 4). A blank, the crude peptide solution, the fraction of interest and the 2 fractions before and 2 fractions after are typically analyzed.
5. Freeze drying of selected fraction.
6. Carry out off-line or on-line the ES-MS of purest fraction.
DESALTING OF PEPTIDE AND PROTEIN MIXTURES BY RP-HPLC TECHNIQUES

RP-HPLC can be utilized to desalt peptide or protein samples derived from extraction procedures or from previous HP-HIC, HP-IEC, HP-IMAC, HP-HILIC, or HP-BAC separations. Peptide or protein solutions are injected onto a small RP-HPLC column. An aqueous buffer is used to elute the salts, while the peptides or proteins are concentrated on the top of the column. After elution of the salts, monitored by UV detection, the peptides or proteins are eluted with water-acetonitrile or water 2-propanol mobile phases. The loading capacity of an analytical column (100- to 300-mm length × 4-mm i.d.) is typically ~8 mg, while the loading capacity for a semi-preparative column (30-mm length × 16-mm i.d.) is ~34 mg (Pohl and Kamp, 1987).

In addition to the reagents and procedures described in the preceding sections the following materials, reagents and conditions are required:

**Chromatographic Conditions**

Chemicals: acetonitrile, 2-propanol, trifluoroacetic acid (TFA)

Column: e.g., C-4, C-8, C-18, etc. (10 µm, 300 Å, 300-mm length × 21.5-mm i.d.)

Sample size: 8 mg peptide or protein sample

Sample loop size: 1 ml

Step elution

Eluent A: 0.1% aqueous TFA

Eluent B: 0.1% TFA in acetonitrile or 2-propanol

Elution conditions: 100% eluent A for 3 min, then 100% eluent B for 3 min

Flow rate: 2.5 ml/min

Detection: 230 nm

Temperature: room temperature

Illustrative of the use of RP-HPLC in the desalting of peptide and protein samples isolated by other techniques is the example shown in Figure 8.7.11 for the step elution of a 50S ribosomal protein sample derived from *Thermus aquaticus* preparation on a C-4 column (40-mm length × 4-mm i.d., 300 Å, 5 µm) using a flow rate of 2.5 ml/min.

**Figure 8.7.11** Illustrative example of the use of RP-HPLC in the desalting of peptide and protein samples isolated by other techniques. In this example, the 50S ribosomal protein from *Thermus aquaticus* on a C-4 column (40-mm length × 4-mm i.d., 300 Å, 5 µm) were desalted at a flow rate of 2.5 ml/min using a step elution protocol with 3 min elution with eluent A (0.1% TFA in water) and 3 min elution with eluent B (0.1% TFA in 2-propanol), pH 2.1. Abbreviations: S, salt; P, protein; A, time of injection.
Figure 8.7.12  Diagram of column switching unit employed in multi-dimensional HPLC. Abbreviations: D, detector; P₁,₂, two-pump module for gradient elution mode; P₃, pump equilibrates offline SEC column, while RPC gradient is performed; V₁, injection valve; V₂ and V₃, switching valves. The black field in the switching valve position symbolizes the joined valve positions. In the depicted position, the SEC and the RPC column are connected.

Table 8.7.5  Valve Positions and Pump Activities During the Four Steps in LC-LC Coupling

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<tr>
<th>Step</th>
<th>Event</th>
<th>HP-SEC</th>
<th>Start LC-LC</th>
<th>End LC-LC</th>
<th>RP-HPLC</th>
</tr>
</thead>
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<td>On</td>
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</tr>
<tr>
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<tr>
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<td>Mode</td>
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<td>Isocratic</td>
<td>Isocratic</td>
<td>Gradient</td>
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<tr>
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<td>Pump 2</td>
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<tr>
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<td>Pump 3</td>
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</tbody>
</table>

Figure 8.7.13  (At right) Illustrative example of the use of multimodal HPLC techniques for the separation and analysis of peptide and protein samples. (A) RP-HPLC resolution of the 50S ribosomal proteins from *Thermus aquaticus* in 2% acetic acid (Molnar et al., 1989b) on a C8 column (250-mm length × 4.6-mm i.d.) using a flow rate of 1.5 ml/min and a 30 min gradient from 10% to 100% eluent B [eluent A: 125 mM NaH₂PO₄, 125 mM H₃PO₄; eluent B: acetonitrile: 250 mM NaH₂PO₄, 250 mM H₃PO₄ (50:50), buffer system, pH 2.1]. (B) HP-SEC separation of the 50S ribosomal proteins from *Thermus aquaticus* in 2% acetic acid on a tandem TSK-250 column (75-mm length × 7.5-mm i.d. and 300-mm length × 7.5-mm i.d.) using a flow rate of 1.5 ml/min with an isocratic separation with 10% B (eluents as described above) with a fraction cut out and directed on to the reversed-phase column. (C) RP-HPLC separation of the fraction of the 50S ribosomal proteins from *Thermus aquaticus* in 2% acetic acid obtained on-line from the size-exclusion chromatography on a C8 column (250-mm length × 4.6-mm i.d.) using a flow rate of 1.5 ml/min and a 30 min gradient from 10% to 100% B (eluents as described above). Proteins 6, 9, and 10, which were coeluted with other proteins in chromatogram A, could now be obtained in relatively pure form for further investigations.
Figure 8.7.13  (See legend on facing page.)
MULTIMODAL HPLC: COLUMN SWITCHING

Electronically controlled multimodal HPLC is a valuable technique (Kopaciewicz and Regnier, 1983b; Hulpke and Werthmann, 1986), which allows automated fractionation and sample cleanup. In addition to the reagents and procedures described in the preceding sections, the following materials, reagents, and conditions are required for such experiments: (1) an autosampler with two rhodyne 7010-080 valves, third pump (optional), and (2) columns (e.g., for HP-SEC and RP-HPLC).

Automated fractionation, for example, with a HP-SEC-RP-HPLC coupling, can be performed with the setup shown in Figure 8.7.12. The valves V₂ and V₃ allow the on-line or off-line position of the HP-SEC and RP-HPLC column to be independently controlled. To establish an electronically controlled automated fractionation method, the following steps are needed:

1. Establish a suitable method for HP-SEC-RP-HPLC, whereby the HP-SEC is performed in the isocratic mode and the RP-HPLC is performed in the gradient elution mode, and the HP-SEC eluent and the eluent A of the RP-HPLC are identical.
2. Perform a HP-SEC run (the RP-HPLC column is off-line).
3. Perform a RP-HPLC run (the HP-SEC column is off-line).
4. Determine the valve switching times.
5. Equilibrate both columns with eluent A (with pump P₁), with both columns connected.
6. Switch the RP-HPLC column off-line.
7. Perform a HP-SEC run according to step 1 (see Table 8.7.5).
8. Switch to LC-LC coupling according to step 2 (see Table 8.7.5). The fraction is collected at the inlet of the RP-HPLC column.
9. Switch back to the HP-SEC only mode according to step 3 (see Table 8.7.5). The fraction collection on the RP-HPLC column is stopped. The sample stays on the column (no flow) until the HP-SEC column run is completed.

<table>
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<tr>
<th>Task</th>
<th>Purification of one component</th>
<th>Purification of several components simultaneously</th>
<th>Desalting of peptide or protein sample</th>
<th>Characterization of physico-chemical properties</th>
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<tr>
<td>Example</td>
<td>Peptides from solid phase peptide synthesis, enzymatic digestion of a protein</td>
<td>Peptide from tryptic digests, ribosomal proteins, proteins from biological extracts</td>
<td>Fractions from IEX or HIC</td>
<td>Thermodynamic parameters of solute/ligand interaction</td>
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<table>
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<th>Preparative scale</th>
<th>Measuring of ( t_0 )</th>
<th>Measuring of ( t_D )</th>
<th>Column testing</th>
<th>A value</th>
<th>X value</th>
<th>Y value</th>
<th>Extra-column band broadening (σ)</th>
<th>Regression analysis</th>
<th>Optimization procedures</th>
<th>Computer simulation</th>
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<td>Yes</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
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</table>

Table 8.7.6 Methodological Requirements of the Different Tasks in RP-HPLC
10. After completion of the HP-SEC run, the HP-SEC column is switched off-line, but flushed with buffer by pump 3, while a RP-HPLC gradient elution run is performed according to step 4 (see Table 8.7.5).

Illustrative of the use of multimodal HPLC techniques for the separation and analysis of peptide and protein samples is the example shown in Figure 8.7.13, panels A to C, for the separation of closely related 50S ribosomal proteins from *Thermus aquaticus* under RP-HPLC, HP-SEC, and again RP-HPLC protocols under different elution conditions to achieve optimal resolution of specific proteins in high purity for further investigations.

**METHOD DEVELOPMENT IN RP-HPLC**

For the separation of the diverse components of a sample containing peptides or proteins of unknown composition, usually a model is first developed which aims to create separation conditions that result in different retention times of the various components. Currently no algorithm can predict with absolute fidelity, on the basis of the amino acid sequence, the separation behavior of peptides or proteins. In many cases, the nature of the components is not known anyway. Moreover, changes in retention and peak shape will always occur when sample overload or volume overload conditions prevail. There are, however, empirical concepts that describe the retention behavior of peptides and proteins with a ligand in the presence of different solvent combinations. The most commonly adapted concepts are based on the solvophobic theory (Horvath et al., 1976, 1977) and the linear solvent strength theory (Snyder, 1980). These concepts allow the development of fast, robust, and cost-effective separation methods (Boysen et al., 1998).

Various aspects of these theoretical approaches can be used to reach different aims with a specific separation (Table 8.7.6). These aims may be:

a. the purification of one component, or alternatively several components simultaneously;
b. the desalting of a peptide or protein sample;
c. the characterization of physico-chemical properties of peptides and proteins in hydrophobic environments;
d. examination of the unfolding behavior of proteins under different sorbent or mobile phase conditions;
e. determination of linear free energy dependencies between different members of a peptide analog family; selection and optimization of different elution protocols;
f. examination of the effects of different hydrogen bonding solvents on the retention behavior of peptides or proteins;
g. the generation of a large variety of empirical data that permits different sorbent types to be validated or batch-to-batch variations characterized. The requirements, in terms of preliminary measurements, for these optimization procedures and data analyses are outlined in Table 8.7.6.

**SYSTEMATIC APPROACH TO METHOD DEVELOPMENT**

The quality of a separation is determined by the resolution of individual peak zones (Hearn, 1991a). Hence, method development is always an optimization of the resolution, with the trade-off either being the speed or the capacity (sample size) of the separation. The resolution of adjacent peak zones can be defined as:

\[
R_s = \frac{(t_1 - t_2)}{(1/2)(\omega_1 - \omega_2)}
\]

Equation 8.7.1

Here, \(t_1\) and \(t_2\) are the retention times, while \(\omega_1\) and \(\omega_2\) are the peak widths of two adjacent peaks. The baseline separation (an overlap of the peaks less than 1%) is given by definition as \(R_s = 1.5\). The resolution of two peak zones depends on a large number of factors, including effects that influence the peak symmetry but also on the ratio of the peak areas. \(R_s\) values are easily estimated using tables and graphs (Snyder et al., 1988). In a chromatogram, every peak pair has a different \(R_s\) value. In developing a very high-resolution analytical separation of a complex mixture of peptide or protein components, method development always focuses on the least well resolved peak pair. If conditions can be developed to ensure that this “critical peak pair” is well resolved, then all other peaks are also well resolved. However, the peaks that constitute this critical pair can change as a consequence of changes in the experimental conditions. For preparative separations, method development always focuses on the peak of interest and the two adjacent contaminant peaks. In this case, all other peaks can be viewed as superfluous, and directed to the waste. Optimization of the resolution of the peak of interest from the adjacent peaks has to
take into account the sample size and the relative abundances of the three components that form the basis of the separation task.

In gradient elution, the resolution also depends on the plate number $N$, the selectivity $\alpha$, and the capacity factor $k$, all of which can be experimentally influenced through systematic changes in individual chromatographic parameters. In this case, resolution is determined from:

$$R_s = \left(\frac{1}{4}\right)N^\frac{1}{2}(\alpha - 1)\left[\frac{k}{1+k}\right]$$

**Equation 8.7.2**

The plate number, $N$, is the band broadening of the peak zone caused by the column and is a measure of the column performance. The selectivity $\alpha$ describes the selectivity of a chromatographic system for a defined peak pair and is the ratio between the $k$ value of the second peak zone and the $k$ value of the first peak zone. The capacity factor $k$ is a dimensionless parameter of the retention in gradient elution. Its calculation from gradient elution data will be described further below.

In contrast to the isocratic elution, in a gradient elution system $N$, $\alpha$, and $k$ are the median values for these variables (Hearn, 1991b), since they change during the separation as the shape or duration of the gradient changes. The plate number $N$ has no influence on the selectivity or the retention (except for conditions of temperature change). The selectivity $\alpha$ and the capacity factor $k$ have only a minor influence on $N$. While $N$ and $\alpha$ change only slightly during the solute migration through the column, the $k$ value can change by a factor of 10 or more. The best chromatographic separation is achieved within a $k$ value range between 1 and 20. Although the resolution is mainly influenced by the mobile phase variables $\alpha$ and $k$, and nearly independent of $N$ for a given column, an optimization strategy should start, from a logistical point of view, with the optimization of the stationary phase, because many ab initio choices (like column dimensions, choice of ligand, etc.) are determined by the overall strategy (e.g., analytical versus preparative separations). It should be noted, from a theoretical point of view, that optimization of the stationary phase is often performed last (as in some computer simulation programs) when an established analytical method is up-scaled to a preparative method. Typically, a particular RP-HPLC sorbent will be selected empirically as the starting point for the separation. Rarely do most investigators go to the trouble of optimizing the surface chemistries of their own sorbents, although a number of notable exceptions exist. Based on these considerations and in view of the implication of Equation 8.7.2, the optimization can be performed in three modules in the following order: (1) the optimization of the plate number $N$, then (2) optimization of the selectivity $\alpha$, and finally, (3) optimization of the $k$ values.

**Optimization Module 1: The Stationary Phase**

The optimization of the peak efficiency, expressed as the theoretical plate number, $N$, requires an independent optimization of each of the contributing factors that influence the band broadening of the peak zones due to column and the extra-column effects. With a particular sorbent (ligand type, particle size, and pore size) and column configuration, this can be achieved through optimization of the linear velocity (flow rate), the temperature, the detector time constant, and the column packing characteristics, as well as by minimizing extra-column effects, e.g., by using zero–dead volume tubing and connectors. The temperature of the column and the eluents should be constant ($\pm 0.1^\circ$C) using a thermostatically controlled system in order to facilitate the reproducible determination of the various column parameters and to ensure reproducibility in the resolution.

The theoretical plate numbers measured on the same column are smaller for proteins than for small peptides. This difference in peak width behavior between peptides and proteins can be explained directly from the Knox equation (Knox and Scott, 1983; Freebairn and Knox, 1984), if the $N$ values are expressed as reduced plate heights, i.e., $h = L/Nd_p$, where $L$ is the column length, and $d_p$ is the average particle diameter of the sorbent. According to the Knox equation, $h$ is related to the reduced mobile phase velocity, $v$, through the relationship $h = Av^{0.33} + Bv^{-1} + C$, where $v = ud_p/D_m$, $u$ is the linear velocity, and $A$, $B$, and $C$ are constants that describe the packing and solute transport characteristics of a particular sorbent in a column. Observations of reduced plate heights ($h$ values) in the range $3 < h < 15$ for small peptides with reversed-phase sorbents of average particle size of 3 to 10 $\mu$m, and for proteins and other high molecular weight macromolecules where $h$ values are $> 50$, have frequently been made, confirming that the Knox equation can be approximated (Snyder et al.,
within the narrow range of 0.8 ≥ HPLC sorbents of large pore diameters (e.g., range 10,000 to 80,000, separated using RP-
for proteins with molecular weights within the peptide or protein in the bulk mobile phase.

The following parameters can be used to assess column and extra column effects:
The A value of the column (a term that reflects the quality of the packing of the sorbent in the column) can be determined according to the procedures described in Stadalius et al. (1987). The A values for well packed columns fall within the limits of 0.5 ≤ A ≤ 1. An A value equal to one corresponds to a column containing a theoretically ideal bed of particles in a dodecahedral close-packed arrangement (Stout et al., 1983).
The X value (the column volume of mobile phase outside the pores of the particles) can be calculated according to the procedures described in Stadalius et al. (1987).
The average Y value (an estimate of the diffusional restriction of proteins within the pores of the sorbent) can be calculated according to the procedures described in Stadalius et al. (1987). When no restricted diffusion occurs, the value of Y, which corresponds to the ratio (Dp/Dm) is equal to one. Here, Dp is the diffusion constant of the protein within the pores of the particle and Dm is the diffusion coefficient of the peptide or protein in the bulk mobile phase.
For proteins with molecular weights within the range 10,000 to 80,000, separated using RP-HPLC sorbents of large pore diameters (e.g., ≥30 nm or larger), the Y values typically fall within the narrow range of 0.8 ± 0.1, indicating that limited restricted diffusion can occur, but that the Dp/Dm ratio is largely independent of the molecular size and weight of the examined proteins. However, with particles of smaller pore size, such as ≤10 nm, a larger range of Y values (e.g., 0.05 ≤ Y ≤ 0.80) will occur with a significant dependency of the Y value on the molecular characteristics of the test proteins.
The measurement of the extra-column band broadening σ (which describes the decrease in column performance due to instrumental design) can be determined with a “zero-length column” that connects the injector with a sample loop of 1 µl directly to the detector (Freebairn and Knox, 1984; Hupe et al., 1984). Its value can be calculated based on the calculated peak asymmetry values according to the procedures described in Stadalius et al. (1987).
The extra-column band broadening or volume dispersion effects caused by the injector, tubing, and detector influence the performance of the column and should be less than 10% of the plate number of the column. The extra-column band broadening expressed as peak standard deviation should therefore be less than a third of the band broadening due to the characteristics of the packed column, e.g., as given by:

\[ \sigma_{\text{col}} = \frac{V}{\sqrt{N}} \text{ with } \sigma_{\text{di}} \leq (1/3) \sigma_{\text{col}} \]

Equation 8.7.3

Hence, the LC system should be designed so as to have minimal extra-column band broadening. Care should be taken to attach fittings in such a way as to avoid unnecessary void volumes and to choose tubing with the smallest possible inner diameter, as well as to keep the volume of the detector cell low.
For a standard analytical column, the maximum extra-column band broadening can be therefore be calculated as:

\[ \sigma_{\text{col}} = \frac{1900 \mu l}{\sqrt{10000}} = 19 \mu l \text{ with } \sigma_{\text{di}} \leq 6.3 \mu l \]

Equation 8.7.4

In order to obtain this value, the detector cell volume must be 5 µl or less.
The flow rate to achieve the minimum plate height, H, for a column can be taken from the literature or experimentally determined according to procedures described in Stadalius et al. (1987).

Optimization Module 2: The Mobile Phase Composition
Change in selectivity of the separation with peptides and proteins is the most effective way to influence resolution. This is mainly achieved by changing the chemical nature or concentration of the organic solvent modifier (acetonitrile, methanol, isopropanol, etc.) or the choice of an ion pair reagent (Hancock et al., 1978).

Choice of the organic solvent modifier
A good starting point is the solvent selectivity triangle approach. Here, solvents are classified according to their relative dipole moment, basicity, or acidity in a triangle. Blends of three different solvents, plus water to provide an appropriate K range, are selected to differ as much as possible in their polar interactions. This selection permits the solvent combinations to mimic the selectivity that is possible for any given solvent, and confines the boundaries of the triangle (Snyder, 1974, 1978). At the
Figure 8.7.14  (A) Plots of logarithmic capacity factor $\log k'$ versus the volume fraction of organic solvent, $\varphi$, for 9 different proteins, denoted 7 to 15, obtained from Thermus aquaticus on a C18 RP-HPLC column. As evident from this plot, the intersection of two plots represents complete overlap of the peak zones at the specific gradient run times $t_G$ 90 and 270 min. (Legend continues on next page.)
same time, these solvents must be totally miscible with each other and with water. Three solvents that best meet these requirements are methanol, acetonitrile, and tetrahydrofuran. Four-solvent mobile phase optimization using three organic solvents and water provides a significant control over $\alpha$ values in reversed-phase HPLC. If different organic solvents are used, the different eluotropic strengths (Schoenmakers et al., 1979; Patel and Jefferies, 1987) must be considered in order to elute the sample in the appropriate $k$ range.

**Choice of ion pair reagent**

The retention of peptides can be influenced by the presence of ion pair reagents (Horvath et al., 1977; Hearn et al., 1979; Goldberg et al., 1984; Mant and Hodges, 1991c). The ion pair reagents interact with the ionized groups of the peptides: anionic counterions (e.g., hexanesulfonic acid, orthophosphoric acid, TFA, HFBA) interact with basic residues (arginine, lysine, histidine) of a peptide as well as with the protonated $N$ terminus; cationic counterions (e.g., triethylammonium, tetrabutyl-ammonium) interact with ionized acidic residues (glutamic and aspartic acid, tyrosine and cysteic acid) as well as ionized free C-terminal carboxylic groups. The actual effect on retention depends strongly on the hydrophobicity of the ion-pair reagent and the number of oppositely charged groups on the peptide. Typically, the retention of peptides and proteins in the presence of ion pairing reagents follow asymptotic dependencies on the concentration of the different ion pairing reagent.

Once the selectivity parameter is fixed due to the *initial* choices of the mobile and stationary phase, the further optimization should concentrate on resolution optimization via achieving the most appropriate $k$ value for the different peptides or proteins in the mixture.

### Optimization Module 3: The Gradient Conditions

For resolution optimization, the strategy takes advantage of the relationship between the gradient retention time of a protein (expressed as the median capacity factor, $k$) and the median volume fraction of the organic solvent modifier $\phi$, in regular RP-HPLC systems based on the concepts of the linear-solvent-strength theory.

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<th>Parameter</th>
<th>$S$ and log $k$</th>
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</tr>
<tr>
<td>Flow rate $F$ (ml/min)</td>
<td>—</td>
<td>Yes</td>
</tr>
<tr>
<td>Column plate number $N$</td>
<td>—</td>
<td>Yes</td>
</tr>
<tr>
<td>Retention times $t_{g1}$ for all peaks</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Retention times $t_{g2}$ for all peaks</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 8.7.14 *(Continued) (B)* Plots of the resolution $R_S$ (resolution taken as the difference in retention times, $\Delta t_g$, for two adjacent peaks) versus the gradient run time $t_G$ of these 9 different proteins from *Thermus aquaticus*. These data represent the blueprint for generation of the relative resolution map (RRM). Since the plotted values of the resolution are absolute values, negative resolution values of peaks that change their elution order are depicted as broken lines. *(C)* RRM is shown for the nine different proteins from *Thermus aquaticus*, depicting only the critical peak pairs including their change in retention order. The resolution optimum for this specific set of chromatographic conditions occurs at $t_G = 150$ min.

**Table 8.7.7** Parameters Required for the Calculation of $\ln k_0$ and $S$ Values and for the Manual Optimization and Optimization with DryLabG/plus

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**Conventional Chromatographic Separations**

8.7.29
HPLC of Peptides and Proteins

8.7.30

(Horvath et al., 1976; Snyder, 1980; Hearn and Grego, 1983), such that:

$$\ln k = \ln k_0 - S\bar{\phi}$$

**Equation 8.7.5**

where \(k_0\) is the capacity factor of the solute in the absence of the organic solvent modifier, and \(S\) is the slope of the plot of \(\ln k\) versus \(\bar{\phi}\). The values of \(\ln k_0\) and \(S\) can be calculated by linear regression analysis. The underlying principles of an intuitively performed optimization and manually achieved optimization (using Excel spreadsheets, for example, to calculate the \(\ln k_0\) and \(S\) values), or, alternatively, optimization via computer simulation software (e.g., Simplex methods, multivariate factor analysis programs, DryLab G/plus, etc.) are essentially the same. However, the outcomes result in different levels of precision. Two representative approaches are collectively outlined below.

Resolution \(R_S\) of peak zones is optimized through adjustment of \(k\) by successive change of the parameters \(t_G\) and \(\Delta \phi\) in the gradient elution mode according to the following steps:

- **Initial experiments**;
- **Peak tracking and assignment of the peaks**;
- **Calculation of \(\ln k\) and \(S\) values from initial chromatograms**;
- **Optimization of gradient run time \(t_G\) over the whole gradient range**;
- **Determination of new gradient range**;
- **Calculation of new gradient retention times \(t_G\)**;
- **Change of gradient shape (optional)**;
- **Verification of results**.

**Initial experiments**

In initial experiments, the peptide or protein sample is separated under two linear gradient conditions differing by a factor of 3 in their gradient run times \(t_G\) (all other chromatographic parameters being held unchanged; Dolan et al., 1989) to obtain the RP-HPLC retention times of each peptide or protein. Irrespective of what optimization strategy will be used, it is advisable to separate any sample with at least two different gradient run times, in order to identify overlapping peaks. For optimization of the gradient shape and to achieve maximum resolution between adjacent peak zones, the ability to determine the retention times of the peptides or proteins and to classify the parameters that reflect contributions from the mobile phase composition and column dimensions (Table 8.7.7) is essential (Ghrist and Snyder, 1988a,b; Ghrist et al., 1988). The determination of the volume, \(V_m\), is useful (Studalns et al., 1984); determination of dead volume and gradient delay are essential (Snyder, 1980). With these compiled input parameters, various algorithms including DryLab G/plus can generate the relative resolution map (RRM), based on calculation of the corresponding \(S\) values and \(k_0\) values for each component. If no DryLab G/plus is available, the resolution information can be extracted directly from the vertical distances of the individual \(R_S\) versus \(t_G\) plots, whereby a cross-over of lines reflects a peak overlap as shown in Figure 8.7.14A,B,C. Over a small range of \(\bar{\phi}\) values, the \(S\) values and \(k_0\) values for an individual peptide or protein will remain essentially constant. It is assumed that no significant conformational change of the individual peptides or proteins occurs as a consequence of the differences in column residency time and gradient time (Purcell et al., 1993). The typical retention behavior of the peptides and proteins is depicted in Figure 8.7.14 as plots of \(\ln k\) versus \(\bar{\phi}\). These plots become nonparallel, and are frequently intersecting, as the mobile phase composition is varied.

**Choice of starting conditions**

The \(S\) values of peptides and proteins can be empirically correlated with their molecular weights (Snyder et al., 1983), based on a correlation of \(S = 0.48(MW)^{0.44}\). Thus \(S\) values, estimated with this equation or, alternatively, values of similar peptides or proteins taken from the literature, can be used to calculate a reasonable starting point for the initial experiments according to the following equation:

$$t_G = \frac{V_m \times \Delta \phi \times S \times k}{0.87 \times F}$$

**Equation 8.7.6**

The required gradient run time, \(t_G\), for a separation of peptides and proteins with expected \(S\) values of around 20 can be calculated for a gradient of 0% to 100% (60% ACN) at a flow rate of 1 ml/min and the ideal \(k\) value of 5 as follows:

$$t_G = \frac{1.9 \times 0.6 \times 20 \times 5}{0.87 \times 1 \text{ ml/min}} = 131 \text{ min}$$

**Equation 8.7.7**

Based on this calculation, the initial gradients from 0% to 100% eluent B for gradient times of 1 and 3 hr duration can be selected.

On the other hand, availability of the \(S\) values, derived from two gradient elution RP-
HPLC experiments, can be used as an analytical criterion early in the separation of the target peptide or protein from other components in soluble extracts of biological sources, to distinguish low- from high-molecular-weight molecules and, e.g., to exclude peptide fragments participating in the optimization process, without having to resort to SDS-PAGE experiments. The RP-HPLC behavior of small peptides with molecular weights from 300 to 1000 are approximately correlated (Hearn and Grego, 1981; Snyder, 1990) to S values between 3 and 10. For medium-molecular-weight globular proteins, S values above 20 are expected (Aguilar et al., 1985). As an example, cytochrome c, with a molecular weight of ~12,000, has an S value of 28.8 on a Nucleosil C-18 column (Stadalius et al., 1984).

### Calculation of ln k₀ and S values

The retention times tₙ₁ and tₙ₂ for a peptide or protein solute separated under conditions of two different gradient run times (tₙ₁ and tₙ₂, whereby tₙ₁ < tₙ₂) can be given by the following equations (Quarry et al., 1986; Ghrist et al., 1988):

\[
t_{n1} = \left(\frac{t_0}{b_1}\right) \log \left(2.3k_0 b_1\right) + t_0 + t_d
\]

Equation 8.7.8

\[
t_{n2} = \left(\frac{t_0}{b_2}\right) \log \left(2.3k_0 b_2\right) + t_0 + t_d
\]

Equation 8.7.9

where:

\[
\frac{b_1}{b_2} = \frac{t_{n2}}{t_{n1}} = \beta
\]

Equation 8.7.10

Here, tₙ₁, tₙ₂ are the gradient run time values of tₙ for two different gradient runs, resulting in different values of b (b₁, b₂) and tₙ (tₙ₁, tₙ₂) for a single solute; tₙ₁, tₙ₂ are the gradient retention times for a single solute in two different gradient runs; b₁, b₂ are the gradient steepness parameters for a single solute and two gradient runs differing only in their gradient times. Steep gradients correspond to large b values and small k₀ values; k₀ is the solute capacity factor at the initial mobile phase composition; β is the ratio of tₙ₂ and tₙ₁ which is equivalent to the ratio of b₁ and b₂; t₀ is the column dead time; and tᵩ is the gradient delay time.

For peptides and proteins there is an explicit solution (Stadalius et al., 1984; Quarry et al., 1986) for b and k₀, namely:

\[
b_1 = \frac{t_0 \log \beta}{t_{n1} - \left(\frac{t_{n2}}{\beta}\right) + (t_0 + t_d)\left(1 - \frac{\beta}{\beta}\right)}
\]

Equation 8.7.11

\[
\log k_0 = \left(\frac{b_1}{t_0}\right)(t_{n1} - t_0 - t_d) - \log \left(2.3b_1\right)
\]

Equation 8.7.12

From the knowledge of b and k₀ the values of k and ϕ can be calculated (Snyder, 1980; Hearn, 1991b):

\[
\bar{k} = \frac{1}{1.15b_1}
\]

Equation 8.7.13

\[
\bar{\phi} = \left[\frac{t_{n1} - t_0 - t_d - \left(\frac{t_0}{b_1}\right)\log 2}{t_{n1}^0}\right]
\]

Equation 8.7.14

Here, k is the value of k’ (capacity factor) for a solute when it reaches the column midpoint during elution; ϕ is the volume fraction of solvent in the mobile phase; Δϕ is the change in ϕ for the mobile phase during the gradient elution (Δϕ = 1 for a 0% to 100% gradient); ϕ is the effective value of ϕ during gradient elution and the value of ϕ at band center when the band is at the midpoint of column, and ϕₐ is the normalized gradient time with ϕₐ = βtₙ₁/Δϕ.

By linear regression analysis, using k and ϕ, the S value (empirically related to the hydrophobic contact area between solute and ligand) can be derived from the slope of the log k versus ϕ plots, and ln k₀ (empirically related to the affinity of the solute towards the ligand) as the y-intercept (Horvath et al., 1976):

\[
S = \frac{\left(\ln k_0 - \ln \bar{k}\right)}{\bar{\phi}}
\]

Equation 8.7.15

### Peak tracking and assignment of the peaks

Complex chromatograms that result from reversed-phase gradient elution can often exhibit changes in peak order when the gradient steepness is changed. Before ln k₀ and S values...
are calculated, or computer simulation is used, the peaks from the two initial runs need to be correctly assigned. Several approaches to peak tracking have been described, using algorithms based on relative retention and peak areas (Glajch et al., 1986; Lankmayr et al., 1989; Molnar et al., 1989a), or alternatively, based on diode-array detection (Berridge, 1986; Strasters et al., 1989).

The assignment of peaks can be done in the following steps:
1. Integrate the chromatograms (using the integration software of the HPLC system or alternatively an integrator) of the initial runs and correct integration due to baseline drift or other instrumental causes where necessary.
2. Print out both chromatograms including the peak area percent reports.
3. Number all relevant peaks in the chromatogram with the better resolution. Relevant peaks have, e.g., an area >0.5% of the overall peak area.
4. Assign the peaks according to their peak areas allowing a reasonable elution window.

The total peak areas, \( A_T1 \) and \( A_T2 \) for the initial runs 1 and 2 (whereby \( t_{G1} < t_{G2} \)) are determined, as:

\[
A_T = \sum_{i=1}^{n} A_i
\]

**Equation 8.7.16**

and their ratio is calculated:

\[
R_T = \frac{A_{T2}}{A_{T1}}
\]

**Equation 8.7.17**

Consequently the ratio of the peak areas, \( A_{1i} \) and \( A_{2i} \) of a baseline separated single component in the initial runs 1 and 2, respectively, is:

\[
R_i = \frac{A_{1i}}{A_{2i}}
\]

**Equation 8.7.18**

If the peaks are correctly assigned, \( R_T = R_i \); difficulties arise when peaks partially or completely overlap. If \( R_T < R_i \) and the peak in run 2 is composed of a single component, the peaks are wrongly matched. If \( R_T > R_i \) then the peaks might be correctly matched, but the peak area in run 1 could be enlarged by a hidden additional peak. In order to resolve these difficulties, the deviation (\( \%R_T \)) of \( R_i \) from \( R_T \) can be calculated and taken as a measure of the likelihood that a peak assignment hypothesis is correct:

\[
\%R_T = \frac{100R_T - 100}{R_T}
\]

**Equation 8.7.19**

**Optimization of the gradient time, \( t_G \), over the whole gradient range**

The capacity factor \( \bar{k} \) is a linear function of the gradient run time \( t_G \) if \( \Delta \phi \) is kept constant. Hence:

\[
\bar{k} = \frac{0.87F}{t_G \times \Delta \phi \times S} = \text{constant} = C
\]

**Equation 8.7.20**

The optimized gradient run time \( t_{GRRM} \) can be obtained from the RRM or alternatively, from the plot of \( R_S \) versus \( t_G \) (see Fig. 8.7.14, panels A to C), and yields for each peptide or protein the new values of \( \bar{k}_{\text{new}} \) by \( t_{GRRM} \) being multiplied by \( C \):

\[
C t_{GRRM} = \bar{k}_{\text{new}}
\]

**Equation 8.7.21**

**Determination of the new gradient range**

If the gradient run time \( t_{GRRM} \) is changed in relation to \( \Delta \phi \) with \( \rho_{G1} = \text{constant} \), the \( \bar{k} \) values do not change, as can be seen from the following equation:

\[
t_{G1}^0 = \frac{t_{GRRM}}{\Delta \phi} \frac{V_S \times S \times \bar{k}}{0.87 \times F}
\]

**Equation 8.7.22**

where:

\[
\Delta \phi_{\text{opt}} = \frac{t_{G0}^{\text{opt}}}{t_{G1}^0}
\]

**Equation 8.7.23**

and the retention time \( t_G \) of the first peak is \( >\left( t_0 + \Delta t_{G0} \right) \) and the retention time \( t_G \) of the last peak is \(<\Delta t_{G\text{opt}}\).

**Calculation of the new gradient retention times \( t_G \)**

Based on the knowledge of the \( S \) and the ln \( k_0 \) values, new gradient retention times can then be calculated.

**Change of gradient shape (optional).** A multisegmented gradient should only be performed when the gradient delay has been meas-
ured. With multisegmented gradients, an error in the gradient delay will reoccur at the beginning and at the end of each gradient step. In addition, the effect of $V_{\text{mix}}$ (which can be determined according to the procedures described in Ghrist et al., 1988), which modifies the composition of the gradient at the start and end (rounding of the gradient shape), can lead to deviation of the experimentally determined from the predicted “ideal” retention times in DryLab G/plus simulations.

**Verification of the results.** After completion of the optimization process, the simulated chromatographic separation can now be verified experimentally using the predicted chromatographic conditions.

**DETERMINATION OF THERMODYNAMIC PARAMETERS ASSOCIATED WITH PEPTIDE OR PROTEIN INTERACTIONS WITH IMMOBILIZED LIGANDS**

Isocratic elution can be utilized to determine thermodynamic parameters associated with the interaction of a peptide or protein with an immobilized ligand in all of the various HPLC modes. Thus, the enthalpy, $\Delta H^{0}_{\text{assoc}}$, the entropy, $\Delta S^{0}_{\text{assoc}}$, and the heat capacity, $\Delta C^{0}_{p}$, of the association of a peptide or protein interacting with immobilized nonpolar ligand in RP-HPLC can be evaluated from the dependency of ln $k'$ on $T$, i.e., from the van’t Hoff plots (Melander et al., 1984; Haidacher et al., 1996; Vailaya and Horvath, 1996; Boysen et al., 1999; Hearn and Zhao, 1999; Hearn, 2001), and from the Boltzmann-Helmholtz expression:

$$\ln k' = \frac{-\Delta H^{0}_{\text{assoc}}}{RT} + \frac{\Delta S^{0}_{\text{assoc}}}{R} + \ln \Phi$$

**Equation 8.7.24**

In order to derive this fundamental information, in addition to the procedures and reagents described in the sections above, the following materials and conditions are required:

- **Two 5-liter flasks**
- Peptide or protein (purity >95%) sample at a concentration of 1 mg/ml in a suitable buffer

1. Measure the dead volume $t_0$ of the system as described above.

2. Determine the isocratic conditions at which the peptides or proteins elute in a $k'$ range of 2 to 10. Typically, these mobile phase conditions will encompass only a narrow range of values, e.g., 22% to 27% acetonitrile/water ($v/v$)/0.1% TFA for a RP-HPLC separation, and must be determined by running the sample under different eluent conditions.

3. Prepare stock solutions: e.g., 5 liters each of eluents of appropriate compositions that are $\pm 2\%$ above and 2% below the determined elution range (per the example above 20% and 30% v/v acetonitrile/water/0.1% TFA).

4. Manually mix final buffer in 1% steps from the two stock solutions, according to mixing tables (e.g., Tables for the Laboratory, Merck). Anticipate different eluent consumption for different mobile phase compositions.

5. Inject the sample in order to determine the retention times of the peptide or protein in the temperature range of $5^\circ$ to $65^\circ$C (or higher depending on column specifications) at different isocratic eluent compositions in $5^\circ$C increments, allowing no more than $0.5^\circ$C column temperature fluctuation, measuring each data point at least twice.

**Table 8.7.8** Troubleshooting Strategy and Resulting Spare Part Policy

<table>
<thead>
<tr>
<th>Troubleshooting strategy</th>
<th>Spare part strategy</th>
<th>Parts replaced</th>
</tr>
</thead>
</table>
| Preventive maintenance           | Have always available and change frequently | Precolumn
|                                  |                                      | Guard column
|                                  |                                      | Column frits
|                                  |                                      | In-line filter
|                                  |                                      | Inlet filter
| Anticipation of problems         | Have available as backup parts        | Column
| Wait until complete breakdown   | Purchase as needed                    | Fitting and tubing valves
|                                  |                                      | Lamps
|                                  |                                      | Circuit boards
|                                  |                                      | Pump heads
|                                  |                                      | Fuses

Conventional Chromatographic Separations

8.7.33
6. Establish the van’t Hoff plots and fit the data to a polynomial equation using linear regression analysis as described in Boysen et al. (1999).

7. Use the derived coefficients to calculate the thermodynamic parameters, the enthalpy $\Delta H_{\text{assoc}}^0$ of the association, the entropy $\Delta S_{\text{assoc}}^0$ of the association, and the heat capacity $\Delta C_p^0$ exemplified for a second-order approximation of the solute-ligand interaction by the following equations (Boysen et al., 1999):

$$\ln k' = b_{(0)} + \frac{b_{(1)}}{T} + \frac{b_{(2)}}{T^2} + \ln \Phi$$

Equation 8.7.25

$$\Delta H_{\text{assoc}}^0 = -R \left[ b_{(1)} + \frac{2b_{(2)}}{T} \right]$$

Equation 8.7.26

$$\Delta S_{\text{assoc}}^0 = R \left[ b_{(0)} - \frac{b_{(2)}}{T^2} \right]$$

Equation 8.7.27

$$\Delta C_p^0 = R \left[ \frac{2b_{(2)}}{T^2} \right]$$

Equation 8.7.28

The physical basis and applications of this approach to characterize peptide- or protein-ligand interactions have been extensively described in a variety of papers and reviews (Hearn, 2000a,b,c).

**TROUBLESHOOTING**

There are three approaches to troubleshooting, which are usually practiced in combination: (1) preventive maintenance; (2) anticipation of problems during use by small signs of malfunction; and (3) complacency until complete breakdown.

The choice of the troubleshooting approach ultimately affects the spare part strategy and general instrument management and maintenance approach (see Table 8.7.8). As a consequence, the concept of preventative maintenance addresses both the hardware and software, as well as the selection of sorbent characteristics, mobile phase composition, and sample preparation.

**Preventive Maintenance**

By practicing preventive maintenance, the downtime of HPLC instrumentation used in peptide and protein separation can be substantially minimized.

As a general principle, use clean buffer and reagents, use proper sample preparation techniques, keep air out of the LC system, check the system for leaks, remove all unwanted substances (sample, buffer components) from the system at the end of each day’s work, and be aware of how the system works under normal conditions.

**Mobile phase**

Filter all mobile phases except pure HPLC-grade solvents through 0.2-µm filters. During filtration, avoid contamination that might result from the use, e.g., of dirty glassware or rubber stoppers. Degas all solvents thoroughly. Ensure that all fittings on the low-pressure side of the HPLC pump are tight, so that air is not drawn in. Add sodium azide (10,000 ppm) to the mobile phase in order to stop microbial growth, particularly in phosphate and acetate buffers, if they are stored at room temperature for longer than one day.

Use a solvent inlet line filter (usually 10 µm porosity) in order to prevent particulate matter entering the pump and to act as a weight to keep the inlet line at the bottom of the solvent reservoir. Cap reservoir loosely to prevent dust from entering the mobile phase and to allow air influx. Avoid cross-contamination of buffer, discard stale mobile phases, and clean the eluent reservoir regularly.

**Stationary phase**

Ensure compatibility of the sample solvent with the mobile phase. Incompatibility of the sample buffer with the mobile phase can cause a precipitation of sample in the pores of the column packing.

Ensure compatibility of the mobile phase components with each other. Mobile-phase strength effects can cause precipitation of eluent components, such as salts, inside the column, resulting in column blockage or a packing void.

Ensure compatibility of the column with the mobile phase. The set of guidelines supplied with the column reveal the pressure and temperature limit at which pH range the column can be used (the wrong pH can cause loss of bonded phase with many silica-based sorbents) and which solvents are compatible with the stationary phase (an incompatible solvent can
shrink or swell column particles with the polymeric-type of sorbents).

Use in-line filter or guard columns. For SEC and other chromatographic modes with impure samples, the use of an 0.5-µm in-line filter is recommended in order to prevent blockage of the column inlet frit. A guard column, preferably packed with the same material as the separation sorbent, prevents a blockage of the column inlet frit and deterioration of the column performance. The integrity of the guard column must be checked regularly, and it must be exchanged when necessary, since a poor guard column can adversely affect column performance.

Avoid pressure shocks. Pressure shocks can be minimized by using a pulse damper and by avoiding drastic flow rate changes.

Avoid exceeding the pressure limit by using the upper limit switches of the pump in the method program. Do not use default values. Select the upper limit of pressure according to the back-pressure of a new column with an associated method (depending on the viscosity of the mobile phase, it could be the mid-gradient pressure when using water-organic solvents, e.g., 1500 psi) and add a margin of 1000 psi in order to avoid stopping of overnight runs, etc. (yielding a pressure limit of, e.g., 2500 psi).

Wash the column after use. All columns should be flushed with 10 column volumes of either an organic solvent/water mixture or water with 10,000 ppm sodium azide to suppress microbial growth.

Store the column, when not in use, in the appropriate solvent (see manufacturers’ instructions)—either in >10% organic solvent or with sodium azide when organic solvents have to be avoided. The mobile phase in which the column is stored should not contain any salts, acids, or bases.

**Fittings and tubing**

Assemble the fittings so that the tubing contacts the bottom of the fitting body. Tighten the fitting sufficiently to prevent leaks, but not so much as to distort the ferrule. In order to minimize the extra column band broadening, all tubing on the high-pressure side from the injector to the detector (excluding detector outlet) should be 0.01-in. and as short as convenient. Larger i.d. tubing (e.g., 0.020-in.) is suitable for connecting the pump with the injector. The extra column band broadening can be measured with a zero length column.

**Manual injector**

Filter all samples. Avoid pressure values above 5000 psi. Do not use syringes with sharp needles (e.g., from gas chromatography). Maintain adequate gas pressure for air- or nitrogen-operated valves. Flush injector at the end of each day’s work. If a leaking valve requires the replacement of a damaged seal, rebuild the valve using the kit and instructions supplied by valve vendor. Finally, store the injector in a nonbuffered mobile phase when not in use.

**Pump(s)**

Avoid air bubbles entering pump. If an air bubble arises in the pump see discussion of Priming of the Pumps and Low-Pressure Lines with Eluents, above.

Always flush the HPLC after use to prevent salt deposits at the pump valves. If blocked see Preparing the HPLC System, above.

Flush behind the pump seal at the end of each day’s LC operation, first with 5 to 10 ml water, then with 5 to 10 ml methanol or isopropanol in order to remove the water. Many LC systems have a flushing port. By design, the pump seal does not seal completely around the piston. Crystalline buffer residues may act as an abrasive on the pump seal when restarted, damaging the seal with possible scratching of the piston. Store pumps in nonbuffered mobile phase or pure organic solvent.

**Detector(s)**

Keep the detector cell clean by washing it at the end of each day’s work with the strong mobile phase. Do not perform acid washing on a routine basis. Switch the detector lamp off when not in use for the next 2 hr or longer. Avoid frequent “on and off” switching.

**Recorder, printer and data systems**

Replace ink when recorder ink begins to fade. Lift the pen from the paper and cap it, when recorder is not in use. Check paper supply before HPLC instrument is started and before leaving the HPLC instrument unattended. Ensure that adequate disk storage space exists within the computer for unattended runs.

**Anticipation of Problems**

With this strategy parts are replaced when minor signs of malfunction occur.
Troubleshooting after HPLC Breakdown

The first aim of troubleshooting is the reduction of HPLC instrument down time. Equally important is the prevention of further damage to the HPLC system, which includes the consequences of troubleshooting. It is therefore crucial to identify the point at which help is needed or whether an event is a case for the manufacturer’s service (as most electronic problems are). Before calling the service engineer, the following sources of help can be utilized if a problem occurs:

- Operation manual
- Internal laboratory records, including the logbooks
- Troubleshooting guides
- Other people in the laboratory
- Manufacturer’s technical support line.

If technical support from the manufacturer is needed, agree on precise appointment times, be there in order to learn from the support personnel, or alternatively, to challenge them. Brief them thoroughly, and have all of the activities recorded for further reference. Check on warranty times of parts and on proper invoicing.

With analytical thinking, and the following good practices, most of the problems can be solved:

- Describe the problem in as unprejudiced a way as possible.
- Observation of a problem should be confirmed twice before taking further action.
- Operate the instrument under reference conditions, if possible.
- Take notes of every change you make while trying to solve the problem.
- Label all removed parts.
- Make only one change at a time.
- Only use replacement parts that have been proven not to be faulty or worn out.
- Change a part back, if it did not resolve the problem. This does not apply if a risk exists to cause further damage to parts when they are replaced, if parts are inexpensive, if reinstallation creates the risk of damaging a module, or if parts are scheduled for replacement anyway.
- Document problem solution.

TERMINOLOGY

In this chapter, the nomenclature follows that proposed for the chromatographic sciences by the IUPAC recommendations (Ettre, 1994).

SUMMARY

In this overview, a variety of protocols and instrumental considerations have been introduced, and various factors involved in the correct selection of experimental procedures have been described. This information represents a starting point for good laboratory practices for the resolution of complex mixtures of peptides and proteins by HPLC methods—not the ultimate solution. As a consequence, the various sections have been prepared with the novice practitioners in mind, rather than the expert chromatographic scientist or peptide/protein chemist with advanced chromatographic skills. These introductory sections should thus facilitate the successful development by biologists and other life scientists with little formal training in the modern separation sciences, but who nevertheless face numerous experimental challenges requiring the high-resolution separation of complex mixtures of peptides and proteins by HPLC techniques.

LITERATURE CITED


KEY REFERENCES

Dolan and Snyder, 1989. See above.

An excellent practical manual.


An advanced text for experienced scientists.


A comprehensive text encompassing most applications.


A detailed survey of application and theory.


A comprehensive survey of applications.


A general guide to HPLC.

Snyder et al., 1988. See above.

An excellent advanced text.


A useful guide to modern chromatography.

Contributed by Reinhard I. Boysen and Milton T.W. Hearn
Monash University
Victoria, Australia