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# HPLC of Peptides and Proteins

## *Methods and Protocols*

*Edited by*

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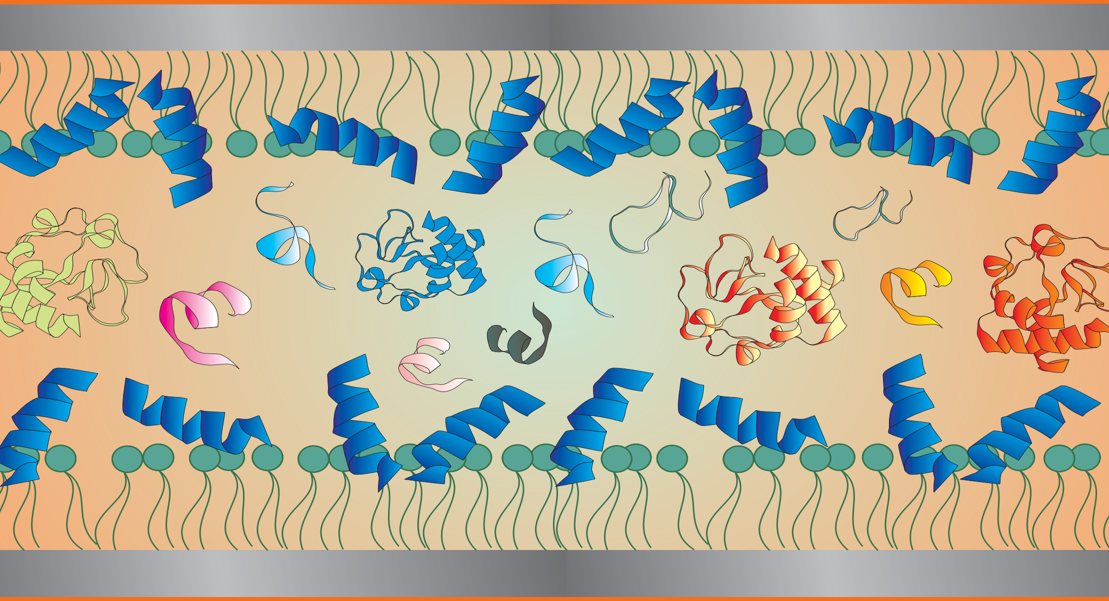

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## Multidimensional HPLC Purification of Proteins

**Edouard C. Nice and Marie-Isabel Aguilar**

### 1. Introduction

Purification of trace compounds (e.g., growth factors, receptors) from bulk biological samples typically requires high purification factors to achieve purification to homogeneity ***(1–9)***. The sequential use of microcolumns of varying selectivity, assuming that they have good recovery characteristics, allows very high purification factors to be achieved. As described in Chapter 11, the use of short narrow-bore (2.1 mm id), microbore (1 mm id), or capillary (<1 mm id) columns allows the recovery of purified proteins and peptides in reduced volume at increased concentration compared with larger bore columns. Indeed, as shown in **Table 1**, it can be calculated that purification factors far in excess of those achieved by two-dimensional polyacrylamide gel electrophoresis can be readily achieved using multidimensional purification protocols, with the added advantage that the purified sample is in a form directly compatible with downstream analysis. Successful micropreparative HPLC requires minimal losses of both mass and biological activity during the chromatographic purification and other associated nonchromatographic sample manipulation (e.g., sample dilution, pH adjustment, storage, chemical manipulation) which we refer to as micromanipulation ***(3,6,7)***. By maintaining high overall recovery throughout the procedure, it is possible to take a sample though successive chromatographic steps (i.e., multidimensional purification) and still have sufficient material for structural and/or biological analysis. This chapter illustrates the potential of short micropreparative columns with different selectivity by the sequential use of size exclusion, anion-exchange and reversed-phase (RP) columns for the final purification of a sample of murine epidermal growth factor (mEGF) isolated from mouse salivary glands ***(4)***. By using the columns in this sequence,

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**Table 1**
**Potential Peak Resolution in Multidimensional HPLC**

| Chromatographic mode | Peak capacity[a] | Cumulative resolution |
|---|---|---|
| Hydrophobic interaction | 50 | 50 |
| Size exclusion | 10 | 500 |
| Ion-exchange | 50 | 25,000 |
| Reversed phase | 100 | 2,500,000 |

[a] Peak capacity is defined as the number of peaks that can be theoretically resolved in a typical chromatographic run.

it is possible to reinject the recovered sample without prior dilution because the buffers used for size exclusion and anion-exchange stages are compatible with subsequent trace enrichment onto the anion exchange and RP columns. Thus by careful consideration of the order in which columns are used in a multidimensional purification scheme, the need to manipulate the sample between stages can be avoided, or minimised, thereby minimising sample handling and dilution and, hence, improving overall recoveries. The differing selectivities obtainable with each column used in the protocol described in this chapter resulted in purification of the mEGF from minor contaminants at each stage.

## 2. Materials

### *2.1. Equipment*

1. Pharmacia SMART system equipped with automatic fraction collection. This method can also be performed with other instruments with appropriate features as outlined in Chapter 11.
2. Solvent filtration apparatus equipped with a 0.22-µm Teflon filter.
3. Sample filters, 0.22-µm porosity.

### *2.2. Buffers*

1. Size exclusion chromatography.
   a. 1% w/v $NH_4HCO_3$ / 0.02% v/v Tween-20 (*see* **Note 1**).
2. Anion-exchange chromatography.
   a. Buffer A: 20 m*M* Tris-HCl (pH 7.5).
   b. Buffer B: 1 *M* NaCl in 20 m*M* Tris-HCl (pH 7.5).
3. RP chromatography.
   a. Buffer A: 0.15% (v/v) aqueous trifluoroacetic acid (TFA).
   b. Buffer B: 60% acetonitrile (HPLC grade) / 40% water (Milli-Q) containing 0.125% (v/v) aqueous TFA (*see* **Note 2**).

### 2.3. Columns

1. Superose 12PC 3.2/30 (300 × 3.2 mm id) (Amersham Biosciences).
2. Mono-Q PC 1.6/5 anion-exchange column (50 × 1.6 mm id) (Amersham Biosciences).
3. Brownlee RP-300 (30 × 2.1 mm id) RP column (Applied Biosystems).

## 3. Methods

### 3.1. Sample Preparation

Dissolve sample in 100 µL of Buffer A (*see* **Note 3**). If there is some undissolved material, centrifuge the sample in an Eppendorf tube (*see* **Note 4**).

### 3.2. Solvent Preparation

Filter all solvents through a 0.22-µm filter before use. This removes particulates that could block solvent lines or the column and also serves to degas the solvent. If the HPLC instrument is not installed with on-line degassing capability, check with your instrument requirements to assess whether further degassing is required.

### 3.3. Size-Exclusion Chromatography

1. Connect the Superose 12 column to the solvent delivery system according to the HPLC system requirements and equilibrate using 1% w/v $NH_4HCO_3$ / 0.02% w/v Tween-20 at a flow rate of 100 µL/min, a column temperature of 25°C and detection at 215 nm.
2. Once a stable baseline is obtained, inject 4.5 µg of mEGF (750 pmol) (either manually or via an automatic injector).
3. Collect the main eluting protein peak for injection onto the micropreparative anion-exchange column (*see* **Note 4**).

### 3.4. Anion-Exchange Chromatography

1. Connect the Mono-Q column to the solvent delivery system according to the HPLC system requirements and equilibrate using Buffer A at a flow rate of 100 µL/min, a column temperature of 25°C and detection at 215 nm.
2. After controlled equilibration (*see* **Note 5**), inject the fraction from the size exclusion column onto the anion-exchange column (either manually or via an automatic injector).
3. Elute the sample with a linear gradient from 0–100% Buffer B over 50 min.
4. Collect the main eluting protein peak for injection onto the micropreparative RP column.

### 3.5. RP Chromatography

1. Connect the RP column to the solvent delivery system according to the HPLC system requirements and equilibrate using Buffer A at a flow rate of 2 mL/min, a column temperature of 25°C and detection at 215 nm.

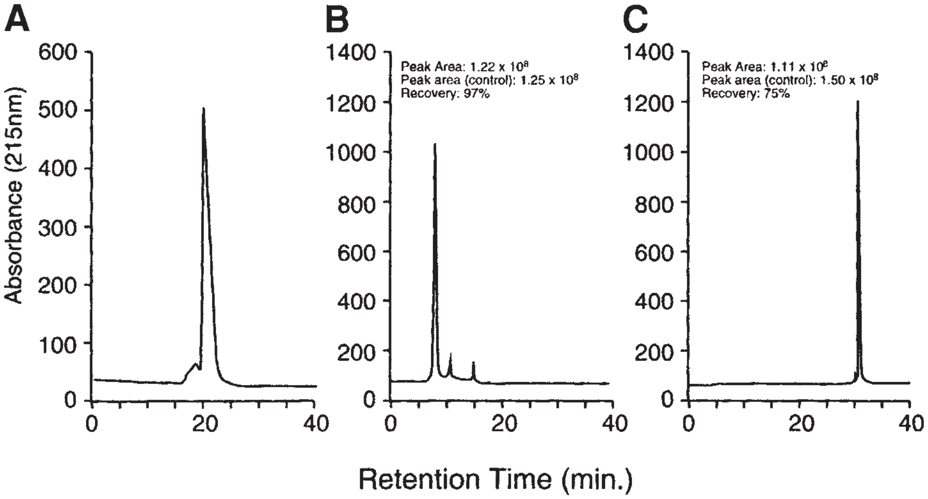

Fig. 1. Mulitdimensional micropreparative HPLC of mEGF. (**A**) Size exclusion chromatography. (**B**) Anion-exchange chromatography. (**C**) RP chromatography. The operating conditions for each system are given in the text. Reproduced with permission from **ref.** ***4*** © 1993 John Wiley & Sons.

2. After controlled equilibration, reduce the flow rate to 100 µL/min and inject the fraction from the anion-exchange column onto the reversed phase column (either manually or via an automatic injector).
3. Elute the sample with a linear gradient from 0–100% Buffer B over 60 min.
4. Collect the main eluting protein peak.

Following micropurification, the sample is suitable for a number of downstream manipulations such as bioassays, microsequencing, mass spectrometry or protein interaction studies using optical biosensors *(8–15)*. **Figure 1** shows typical chromatograms obtained using the protocols outlined earlier for isolation of mEGF. The integrated peak area (Panels **A**, **B**, and **C**) and the calculated recovery between stages (Panels **B** and **C**) are indicated in the figure. Using the conditions defined, the mEGF should elute at a retention time close to those shown. However, as outlined in Chapters 2–5, there are a number of operating parameters that can be changed in order to manipulate the resolution of peptide and protein mixtures in size exclusion, ion-exchange, and RP HPLC.

## 4. Notes

1. Tween-20 helps prevent sample losses by nonspecific adsorption. The bicarbonate buffer is a volatile buffer system BUT is not compatible with many silica-based columns.

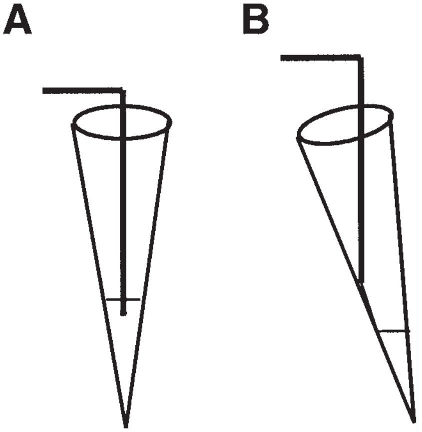

Fig. 2. Split-drop methods for manual fraction collection in micropreparative chromatography.

2. The slight differences in TFA concentration help minimize baseline drift: in fact, the TFA can be carefully titrated into the solvents, using the detector to see the baseline differences at 0 and 100% B until the solvents are balanced.

   For size exclusion chromatography, any buffer can be used. Typically, the sample is made up in 1% w/v $NH_4HCO_3$, but other buffers can be used since the sample will be buffer-exchanged in the column. For downstream bioassays, isotonic saline or PBS are particularly useful. If BIAcore assays are being used, then the BIAcore running buffer can be used (typicaly HEPES) to eliminate refractive index changes ***(15)***.
3. Eppendorf tubes are ideal and show low nonspecific losses compared with some other brands. Avoid filters for clarifying samples as the large surface areas can cause significant sample loss when working at low levels.
4. With the SMART system, fraction collection can be performed automatically. If fractions are collected manually on other instruments, allowance must be made for the dead volume between the detector and the outlet. When collecting fractions manually, to reduce sample crossover bewteen fractions use the split drop methods obtained holding the collection tube either just under the surface (*see* **Fig. 2**) or against the side wall of the tube. When operating at low flow rates the volume of a single drop (typically 5–10 µL) becomes significant.
5. When using gradient elution methods, it is important that the same volume of solvent is used for equilibration between each run. Also, the “first run” syndrome (where a couple of runs are often required before reproducible chromatograms are observed) can be avoided by leaving the column in 100% buffer B and re-equilibrating from there, using the defined volume of solvent.

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