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Scale-up of recombinant protein purification by hydrophobic interaction chromatography

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ABSTRACT

The scale-up of hydrophobic interaction chromatography is described. Human recombinant superoxide dismutase was used as a model. The scale-up was performed by keeping the height to diameter (H/D) ratio of the column constant. The success of scale-up was evaluated by reversed-phase high-performance liquid chromatography of the eluted material. The wrong H/D ratio causes decreased resolution.

INTRODUCTION

There are several strategies for scaling up preparative and process chromatography. Gareil *et al.* [1] reported increasing the throughput by overloading the column. Both volume and concentration overload are applicable. Changing the column geometry is another approach, which is often used in pilot-scale and industrial chromatography [2]. The guidelines for the scale-up of chromatography are relatively simple. The bed height, linear flow-rate, sample concentration, ratio of gradient volume and total column volume are held constant and only the column diameter is increased. The sample load and the volumetric flow-rate must be increased by the factor $(D_1/D_0)^2$, where D_0 is the original diameter and D_1 is the diameter of the larger column. However, some chromatographic rules must be considered to ensure that the entire process is scaled up efficiently. The sample distribution system at the column inlet and the bottom of the column and the plumbing of the entire system are important features. Care must be taken to ensure that laminar flow is achieved throughout the system to avoid additional band spreading by turbulent flow. Another approach involves a complete change of the chromatographic system. The fluid stream is changed from axial flow to radial flow [3].

In the early days of preparative chromatography for protein purification, scale-up was achieved by enlarging the column with a constant height-to-diameter ratio [4]. Many complicated equations have been advocated for this type of scale-up of process chromatography. However, for gel filtration a simple means of obtaining the same elution pattern with increasing scale of operations was found to depend on maintaining dynamic similarity. Dynamic similarity is obtained by arranging both large and small columns to be the same with respect to H/D and $HV\rho/\mu$, where H = column height, D = column diameter, V = velocity (flow-rate/cross-sectional area of the column), ρ = liquid density and μ = viscosity.

In this paper scale-up of hydrophobic interaction chromatography (HIC) is described following the concept of constant H/D ratio, but without maintaining dynamic similarity. Human recombinant superoxide dismutase was used as a model. The pre-purified protein was subjected to HIC on Phenyl Sepharose Fast Flow, using a linear descending salt gradient. The purification was monitored by re-

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versed-phase high-performance liquid chromatography (RP-HPLC).

EXPERIMENTAL

Starting material

Escherichia coli broth [JM 105, expressing recombinant human copper–zinc superoxide dismutase (SOD)] according to Bayer *et al.* [5] was used. In the laboratory-scale experiments 10-l fermenters were used, whereas in the pilot-scale work the material was produced in a 100-l fermenter in the fully automated pilot plant of the Institute of Applied Microbiology. No difference was observed in the composition of the harvested culture broth between the laboratory- and pilot-scale experiments.

Homogenization

The chilled *E. coli* cells were homogenized using a two-valve high-pressure homogenizer (SHL 05) from Brahn and Lübbe (Hamburg, Germany). The homogenization valve was diamond coated. The pressure drop at the first valve was from 1000 to 150 bar and at the second valve from 150 bar to atmospheric pressure. The flux was 50 l/h. The culture broth was delivered to the homogenizer under a 4-bar pressure. Cell breakage was between 95% and 99%. Less than 0.001% of viable cells could be detected after homogenization.

Flocculation

Flocculation was carried out as described previously [6] with charged pellicular flocculents (BPA 1050; Rohm and Haas, Philadelphia, PA, USA). Flocculent was added to the *E. coli* homogenate. The final flocculent concentration was 3000 ppm. After flocculation, the clarified supernatant was filtered through 0.8- and 0.2-μm filters connected in series.

Chromatographic media for hydrophobic interaction chromatography

Phenyl Sepharose Fast Flow High Sub from Pharmacia–LKB (Uppsala, Sweden) was used for HIC. The degree of substitution is *ca.* 40 μmol of phenyl groups per millilitre of swollen gel. The mean particle size is 90 μm and the bead-size range is 45–165 μm. The gel is incompressible over a wide pressure (flow-rate) range. These data were obtained from the manufacturer.

Chromatographic columns

Two different types of columns, a 0.7-l column (P 90 X 250) from Amicon (Stonehouse, UK) and a 20-l column (BPG 200) from Pharmacia–LKB were used. The columns were connected to either a Biopilot or a Bioprocess system from Pharmacia–LKB. Instead of the original 0.8 mm I.D. tubing of the Biopilot system, 2.0 mm I.D. tubing was used. The Bioprocess system was equipped with 1/4-in. tubing.

Packing of columns

Packing of columns was carried out in a manner slightly different to the manufacturer's recommendation. About 250 ml (P 90 X 250 column) and 1 l (BPG 200 column) of 20% ethanol were poured into the column, followed by the gel slurry. The gel was allowed to settle, then the adaptor was fitted to the column and 20% ethanol was percolated through the column at a flow-rate of 250 cm/h. The adaptor was lowered stepwise and fixed in a position 1–2 mm above the packed gel. The packed column was stored in 20% ethanol at room temperature.

Chromatographic conditions

The column was operated in an upward direction. As equilibration buffer, 25 m*M* Tris buffer (pH 7.5) (titrated with 25% HCl) treated with ammonium sulphate to 60% saturation was used. The starting material was a prepurified protein solution (*E. coli* homogenate was flocculated), which was treated with solid ammonium sulphate to 60% saturation. The filtered solution was applied to the column. The unbound material was washed out with equilibration buffer. A linear descending ammonium sulphate gradient was used for elution. The gradient was formed by mixing 60% saturated ammonium sulphate solution which was diluted with 25 m*M* Tris buffer (pH 7.5) to a conductivity of 200 mS/cm at 20°C and 25 m*M* Tris buffer titrated with ammonium sulphate to a conductivity of 120 mS/cm at 20°C. The gradient volume corresponded to ten total column volumes (v_t). The column was regenerated with 25 m*M* Tris buffer (pH 7.5), cleaned with 0.1 *M* NaOH and stored in 20% ethanol. Loading and washing were carried out at a flow-rate of 200 cm/h and elution at a flow-rate of 40 cm/h.

Metal chelate chromatography (MCC)

Final purification was performed by copper-chelate chromatography, using Chelating Sepharose Fast Flow, as described by Bayer *et al.* [5]. Briefly, the column was activated with 50 m*M* $CuSO_4$. After activation, unbound copper was washed out with 20 m*M* potassium phosphate buffer (pH 6.4). After loading, unbound proteins were washed out with equilibration buffer. SOD was eluted with 100 m*M* citric acid buffer, made up by titrating trisodium citrate to pH 5.0 with 25% HCl. After elution, the column was stripped with one column volume each of 25 m*M* EDTA and 1 *M* acetic acid. The column was operated at a flow-rate of 40 cm/h. Activation and stripping were performed at a flow-rate of 90 cm/h.

Superoxide dismutase

SOD was determined by enzyme-linked immunosorbent assay (ELISA) according to the method of Porstmann *et al.* [7] and by the activity assay of Steindl *et al.* [8].

Protein and purity

Protein was determined according to Bradford [9]. The method was modified for microtitre plates and carried out according to the supplier's instructions (Bio-Rad Labs., Richmond, CA, USA).

Purity was monitored by RP-HPLC on a 5-μm, 300-Å C_{18}, column (125 mm × 4.6 mm I.D.). Eluent A was 0.1% aqueous trifluoroacetic acid (TFA); Eluent B was 0.1% TFA in acetonitrile. The UV absorbance was monitored at 214 nm.

RESULTS

Binding capacity and elution conditions

After prepurification of the starting material, the protein solution was filtered through 0.8- and 0.2-μm filters. The maximum loading capacity was determined with a 75-ml column. An excess of protein solution was pumped through the column and the breakthrough of SOD was determined. The maximum load capacity without any loss of the material in the breakthrough was 30 mg of SOD per millilitre of gel (Table I). Elution was optimized, but complete elution with sufficient purity at a defined single step could not be obtained (Table II). The problem was circumvented by applying a linear gradient for elution. Under these conditions (10 v_t gradient volume), SOD was always eluted in a conductivity window of 170 and 150 mS/cm with a yield higher than 80% (Fig. 1A and B).

Resolution

Resolution was improved by varying the height of the laboratory-scale column. At maximum load capacity the minimum *H/D* ratio for obtaining sufficient resolution is 5. This is a prerequisite for the next step of obtaining 99.9% pure SOD in the final purification step by metal chelate chromatography. This unconventional approach was used because the aim of the purification process was to obtain pure protein, and in order to construct an optimum continuous process from several individual steps, it is necessary for each step to fit in with the previous one.

TABLE I

PROTEIN LOAD OF PHENYL SEPHAROSE FAST FLOW

Column size (ml)	Protein load (g)	Protein per ml of gel (mg)	Specific load (v_t)	Volume (ml)	C_o[b] (ng/ml)
75[a]	2.25	30	36	2700	0.83
700	17.71	25.3	30	21 000	0.84
20 000	263	13.15	13.25	265 000	0.99

[a] The 75-ml column was used only for determining the dynamic capacity.

[b] C_0 is the protein concentration of the material loaded on to the column.

TABLE II

SUMMARY OF RECOVERY OF SOD BY STEPWISE ELUTION FROM PHENYL SEPHAROSE FAST FLOW

Experiment No.	Conductivity of elution buffer at 20°C (mS/cm)	Yield of SOD per elution step (%)
1	180	40
	160	30
2	160	5
	130	59
3	160	5
	110	70

In Fig. 2 the effects of sub-optimum and optimum H/D ratios on the purification are compared. RP-HPLC of the eluted peaks shows more hydrophobic proteins in relation to SOD under sub-optimum conditions. The resolving power of the next step was too low to remove the bulk of the hydrophobic proteins. Under optimum conditions (H/D = 5), the final purification (MCC) yields a pure protein. The two peaks in RP-HPLC, the small one in front of the large one, are characteristic of human copper–zinc superoxide dismutase, depending on the buffer system used. This is because SOD shows some microheterogeneity [10]. Under optimum conditions (H/D = 5) the eluate from HIC contained

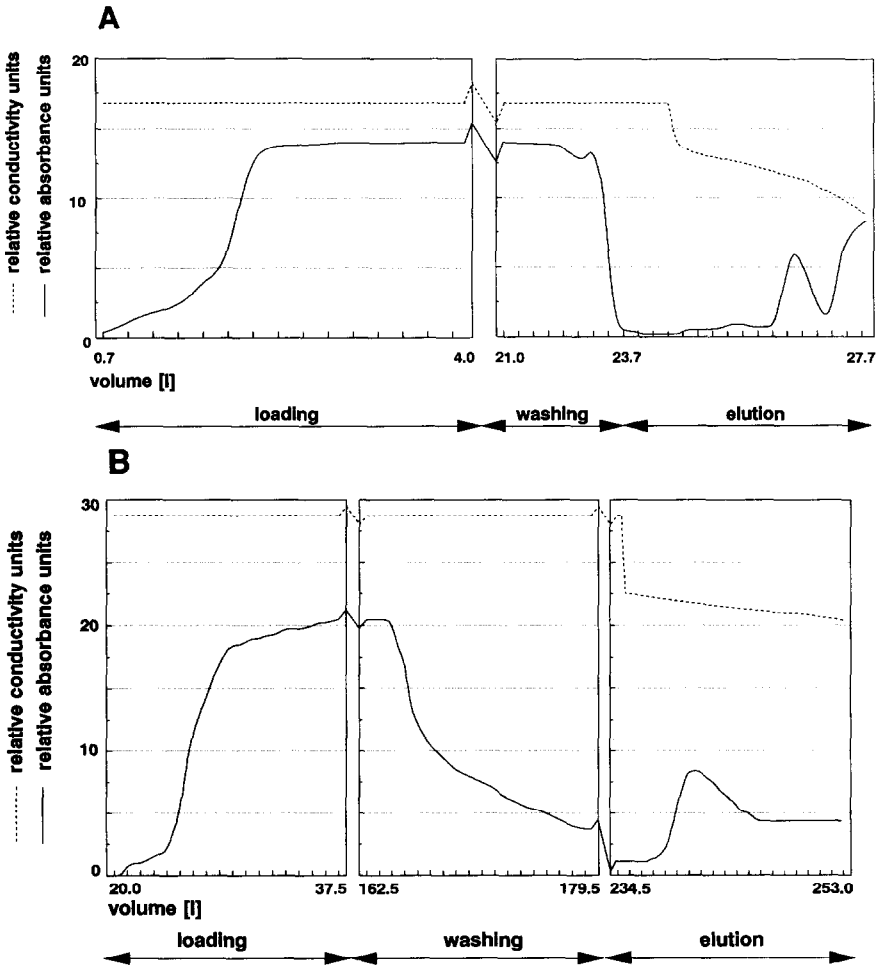

Fig. 1. HIC of SOD with a descending linear salt gradient. The gradient was prepared from (A) an ammonium sulphate buffer of 200 mS/cm at 20°C and (B) an ammonium sulphate buffer of 120 mS/cm. (A) Laboratory-scale experiment; (B) pilot-scale experiment. In both instances, the gradient volume corresponded to 10 v_t.

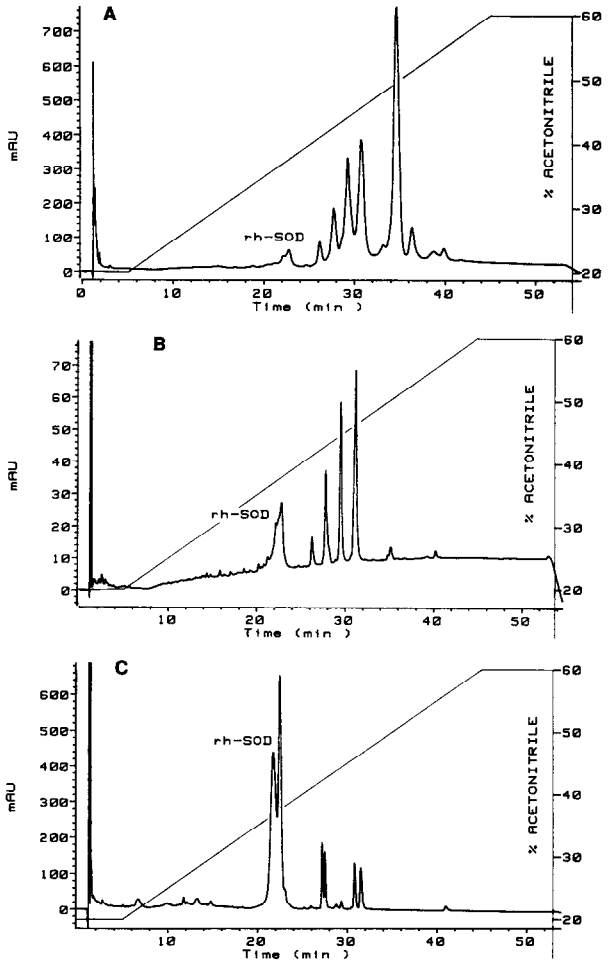

Fig. 2. RP-HPLC of the eluted fraction from Phenyl Sepharose Fast Flow with different H/D ratios. H/D = (A) 1.1; (B) 2.1; (C) 5.7.

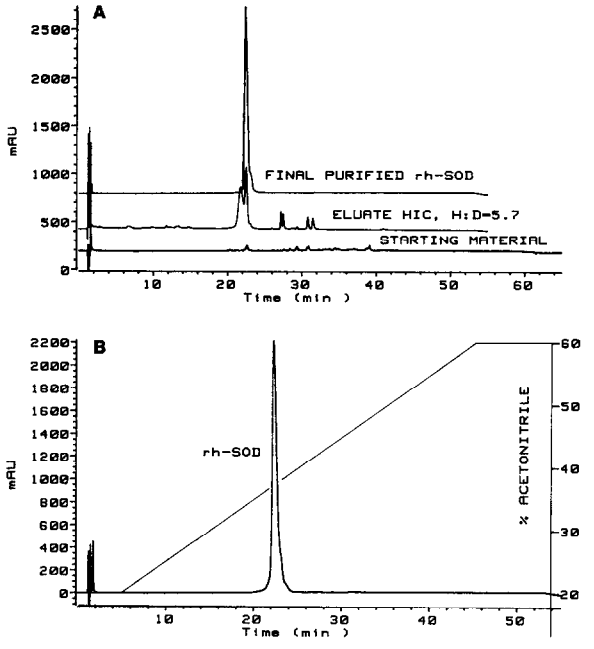

Fig. 3. RP-HPLC of final purified SOD (pretreated *E. coli* broth, HIC and MCC) with (A) a sub-optimum H/D column at the HIC step and (B) an optimum H/D column at the HIC step.

SOD in excess. A small fraction of hydrophobic proteins can still be detected. The resolving power of MCC is strong enough to remove these substances (Fig. 3A and B).

Scale-up of HIC

The 0.7-l column was enlarged by a factor of 28. A 20-l Phenyl Sepharose Fast Flow column was used. A protein solution which corresponds to 100 l of *E. coli* broth could be processed in a single run. In Fig. 4 the RP-HPLC traces for the 0.7- and the 20-l column eluates are shown. For both column sizes the ratios of SOD and hydrophobic proteins

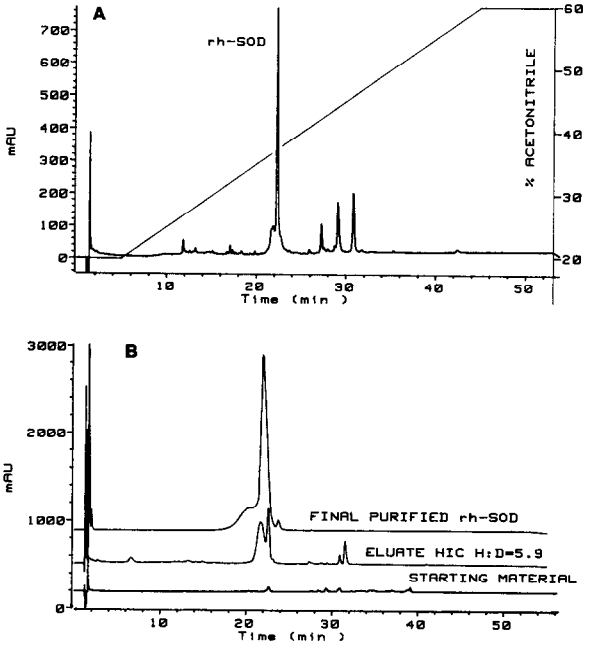

Fig. 4. RP-HPLC of pretreated *E. coli* homogenate eluate from HIC and final purified material of MCC. (A) A 0.7-l column was used in the laboratory-scale experiment and (B) a 20-l column in the pilot-scale experiment.

were in the same range. The final purification yielded homogeneous material. In Table II the protein load and volumetric load of the scales studied are summarized.

DISCUSSION

The objective of this work was to investigate the scale-up of a purification process. The process consisted of flocculation, HIC and MCC. The critical step in the scale-up was the HIC.

As shown in Fig. 4, the chromatographic purification of HIC could be scaled up successfully. Experiments were performed to find an optimum system (Table II and Figs. 2 and 3).

To reduce the column size and mobile phase volume for large-scale work, maximally loaded columns are desirable. The dynamic capacity is not completely used in order to save material. Sample application is stopped when product (SOD) can be detected in the flow-through. After optimization of the load capacity, the elution conditions were optimized.

According to theory, elution should be carried out at the minimum mobile phase modifier (MPM) concentration where elution is accomplished. The MPM concept, described by Velayudhan and Horváth [11], generalizes the mobile phase composition (acetonitrile, salts, etc.) that promotes elution. At higher MPM concentrations more tightly bound proteins are also desorbed. This material contaminates the product. Optimum elution is obtained only with a small window of MPM concentration. We could not find an MPM concentration at which SOD was quantitatively eluted in a single step in appropriate purity. To avoid losses of product by incomplete desorption, a descending linear gradient [12] was used for the subsequent experiments in stead of a descending step gradient [13]. To make the elution conditions as reproducible as possible, the ammonium sulphate concentration was controlled by conductivity at a given temperature (20°C). The Tris buffer was titrated with ammonium sulphate to give a defined conductivity. This approach led to very reproducible elution. Small variations in temperature, load, etc., led only to a shift in the peak position, but complete elution was still obtained. The large buffer volumes necessary to produce a linear gradient compared with stepwise elution are regarded as a disadvantage in process chromatography. In our opinion, this disadvantage is compensated by complete elution.

An incomplete distribution over the gel surface at the column inlet may also contribute to losses in resolution. The effects of both perturbation propagated by centres of disturbances and incomplete distribution of fluid may be one explanation why scale-up at constant H/D is necessary. HIC in general is more sensitive than ion-exchange chromatography or affinity chromatography to external changes. Several workers have described a partial unfolding during adsorption [14]. Unfolding increases with increasing residence time of the protein in a column [15]. By the scale-up concept of keeping H/D constant, unfolding of proteins could be likely, because the residence time of a protein in the column also increases with increasing size. Therefore, band spreading should occur. This event causes decreases in resolution. Nevertheless, we did not observe significant decreases in resolution in the large-scale experiments (Fig. 4). Hjertén *et al.* [16] could not find an influence of residence time on resolution. They pointed out that "the residence time of proteins on the column has no observable influence on the appearance of the chromatograms". Fausnaugh and Regnier [17] assumed that the alteration of the retention behaviour of proteins may be caused in some instances by the salt composition, which is responsible for the alteration of protein structure. SOD is regarded as one of the most stable proteins described so far. This unusual high stability may explain why the resolution does not decrease when the residence time is increased.

In our particular case, separation of SOD from hydrophobic proteins is of great importance for obtaining a pure product. We assume that these proteins are heat-shock proteins from *E. coli*. Some representatives of the *ca.* 40 heat-shock proteins have accessible histidines on the surface. They are able to interact with the chelated copper in the column. If these proteins are present in excess, they cannot be separated from SOD by subsequent MCC.

Scale-up of process chromatography cannot be performed apart from the other purification steps. Scale-up of HIC by keeping H/D constant led to the same product purity as observed in the laboratory-scale experiments.

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REFERENCES

1 P. Gareil, L. Personnaz, J. P. Feraud and M. Caude, *J. Chromatogr.*, 192 (1980) 53–74.
2 G. K. Sofer and L. E. Nyström, *Process Chromatography (a Practical Guide)*, Academic Press, London, 1989, p. 55.
3 A. Jungbauer, F. Unterluggauer, K. Uhl, A. Buchacher, F. Steindl, D. Pettauer and E. Wenisch, *Biotechnol. Bioeng.*, 32 (1988) 326–333.
4 S. E. Charm and C. C. Matteo, *Methods Enzymol.*, 22 (1971) 476–478.
5 K. Bayer, A. Jungbauer, W. Kramer, H. Lettner, W. Schönhofer, M. Skias, F. Steindl, N. Taferner and K. Uhl, *BioEng.*, 6 (1990) 24–30.
6 K. Vorauer-Uhl, M. Skias, A. Trkola, W. Schönhofer, F. Unterluggauer, P. Schulz and A. Jungbauer, *Biosep.*, in press.
7 T. Porstmann, R. Wietschke, H. Schmechta, R. Grunow, B. Porstmann, R. Bleiber, M. Pergande, S. Stachat and R. von Baehr, *Clin. Chim. Acta*, 171 (1988) 1–10.
8 F. Steindl, A. Jungbauer, K. Vorauer-Uhl, M. Skias and W. Schönhofer, *Anal. Biochem.*, submitted for publication.
9 M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
10 A. Jungbauer, C. Tauer, E. Wenisch, K. Uhl, J. Brunner, M. Purtscher, F. Steindl and A. Buchacher, *J. Chromatogr.*, 512 (1990) 157–163.
11 A. Velayudhan and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 13–29.
12 D. L. Gooding, M. N. Schmuck, M. N. Nowlan and K. Gooding, *J. Chromatogr.*, 359 (1986) 331–337.
13 R. C. Friedberg and S. V. Pizzo, *Prep. Biochem.*, 18 (1988) 303–320.
14 A. F. Drake, M. A. Fung and C. F. Simpson, *J. Chromatogr.*, 476 (1989) 159–165.
15 S. Wu, A. Figueroa and B. L. Karger, *J. Chromatogr.*, 371 (1986) 3–27.
16 S. Hjertén, K. Yao, K. Erikson and B. Johansson, *J. Chromatogr.*, 359 (1986) 99–109.
17 J. L. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 131–146.