Journal of Chromatography, 556 (1991) 363–372
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 284

Chromatographic characterization of ion exchangers for high-performance liquid chromatography of proteins

I. Chromatographic determination of loading capacity for low- and high-molecular mass anions[a]

D. BENTROP and H. ENGELHARDT*
Angewandte Physikalische Chemie, Universität des Saarlandes, D-6600 Saarbrücken (Germany)

ABSTRACT

The capacity of silica-based anion exchangers was determined by three independent methods (potentiometric titration, calculation from elemental analysis data and frontal analysis with sodium nitrate). All three methods gave almost identical results. Breakthrough experiments with sodium nitrate were carried out at different concentrations and flow-rates. The capacity of a large-pore ion exchanger for macromolecular anions such as chondroitin sulphate and bovine serum albumin (BSA) was also determined by frontal analysis. The capacity is much lower than that for small anions and is influenced by the irreversible adsorption of sample ions onto the stationary phase. The capacity for the linear polyanion of chondroitin sulphate was independent of its concentration in the carrier solution (within the range 1–10 g/l). For both biological samples the binding capacity depends on the eluent velocity; the capacity for chondroitin sulphate clearly increases with decreasing flow-rate, whereas globular BSA shows little dependence of capacity on flow-rate. The influence of band spreading on the determination of binding capacity by frontal analysis is discussed.

INTRODUCTION

The number of stationary phases available for the ion-exchange chromatography of proteins is abundant and still growing [1]. However, there is a remarkable lack of pragmatic chromatographic criteria for the characterization of these media. As individual proteins show different chromatographic behaviours it does not seem to be possible to develop a simple, generally applicable test procedure such as that described for reversed-phase chromatography [2]. Therefore, a number of chromatographic techniques have been used to obtain practically useful information on the interactions of proteins with ion exchangers.

[a] Part of this work was presented at the *14th International Symposium on Column Liquid Chromatography, Boston, MA, May 20–25, 1990.*

0021-9673/91/$03.50

One of the most important properties of ion exchangers in terms of their analytical and preparative applications is their exchange capacity, which can be measured easily and reliably by frontal chromatography [3,4]. There are several papers dealing with the determination and/or theoretical modelling of adsorption isotherms of model proteins on ion exchangers [5–9]. Some workers [10–12] investigating the protein binding capacities of ion exchangers found a decrease in capacity with increasing flow-rate, which was explained by factors such as mass transfer resistances and hindered diffusion of the macromolecules in the pores because of their low molecular diffusion coefficients. As these workers determined the binding capacity via the breakthrough volume or from the sample load volume at which the sample concentration of the effluent, monitored by UV absorbance, is 10 or 20% of the feed stream, there might be another explanation for the observed decrease of this capacity with increasing flow-rate. (The knowledge of this binding capacity is important for practical purposes in preparative chromatography; usually, it is smaller than the "true" capacity that has to be determined via the retention volume of the protein front). Fig. 1 is a schematic representation of band spreading in elution and frontal chromatography. As can be easily seen, the breakthrough volume (*i.e.* capacity) decreases with increasing flow-rate because of increasing band spreading, which is especially significant for proteins as a result of their small diffusion coefficients.

There is an ongoing contradictory discussion on the flow-rate dependence of protein loading capacity, even when it is calculated via the retention volume of the breakthrough curve. Recently, Blanco *et al.* [13] investigated the adsorption of β-lactoglobulin onto a weakly hydrophobic surface. They measured the adsorbed amount of protein via the retention volume of the protein front and found the capacity to be independent of the flow-rate. However, Huang *et al.* [9], who studied the adsorption of chicken albumin onto TSK-DEAE 5 PW anion exchanger at pH 8.6, found a dramatic decrease of the adsorbed amount of protein at linear velocities above 1.3 mm/s, although they determined the capacity via the retention volume of the front.

This paper discusses, from a practical point of view, the binding capacities of a

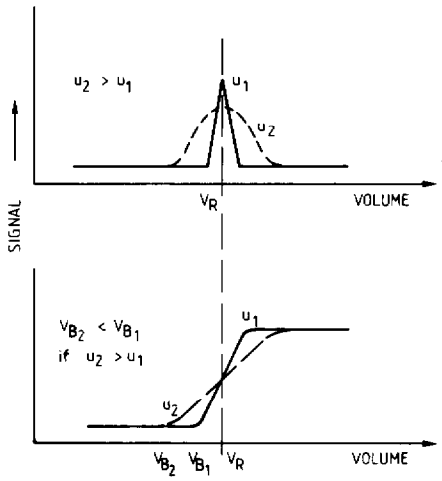

Fig. 1. Schematic representation of band spreading in elution and frontal chromatography. Symbols: u = linear velocity; V_R = retention volume; V_B = breakthrough volume.

silica-based, polymer-encapsulated anion exchanger for low- and high-molecular mass solutes and its dependence on the flow-rate used during the breakthrough measurement. A simple measure for the accessibility of exchange sites on the chromatographic surface for macromolecules can be obtained by comparing their capacity with that for nitrate.

EXPERIMENTAL

Chromatographic instrumentation

Breakthrough measurements were carried out with a modular high-performance liquid chromatography (HPLC) system consisting of a low-pressure eluent selection valve (Rheodyne, Berkeley, CA, USA), a Model 64 HPLC pump from Knauer (Berlin, Germany) or Model 2150 from LKB (Bromma, Sweden), a pulsation dampener (Orlita, Giessen, Germany), a modified Model 7125 sample injector (Rheodyne) without a sample loop, a laboratory-made UV detector (254 nm, flow cell volume, 8 μl) or a Model R401 refractive index detector from Waters Assoc. (Milford, MA, USA) and a strip-chart recorder. All tubings and connections downstream of the injector valve were kept as small as possible.

Materials

Sodium nitrate and the other inorganic chemicals used throughout this study (NaCl, LiCl, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, H_3PO_4) were of analytical-reagent grade from either Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). HPLC-grade water was delivered by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Bovine serum albumin (BSA) (fraction V) was purchased from J. T. Baker (Phillipsburg, NJ, USA) and Sigma (St. Louis, MO, USA) and the sodium salt of chondroitin sulphate (puriss) was obtained from Serva (Heidelberg, Germany). All chemicals were used as received, without further purification. Sodium azide (Merck) was added to the solutions of chondroitin sulphate and BSA at a concentration of 10 mg/l to prevent microbial growth.

Columns

The polymer-encapsulated strong anion exchanger with quaternary ammonium

TABLE I

PROPERTIES OF THE STRONG ANION EXCHANGERS USED FOR THE BREAKTHROUGH EXPERIMENTS DESCRIBED IN THIS STUDY

Property	SAX100	SAX300
Particle size (μm)	10	7
Surface area (m^2/g)	320	100
Pore size (nm)	10	30
Carbon content (% w/w)	13.60	4.56
Nitrogen content (% w/w)	2.63	0.78
Capacity (μmol/g)		
from nitrogen content	939	279
by potentiometric titration	899	253

groups (SAX) which was used throughout this study was synthesized according to the procedure described in ref. 14. The characteristic properties of the ion exchangers based on either LiChrosorb Si100 (SAX100) or Nucleosil 300-7 (SAX300) are summarized in Table I. The stationary phases were slurry-packed into stainless-steel columns 25 or 15 cm in length and 0.41 cm I.D.

Procedures

The column loading capacities for sodium nitrate, chondroitin sulphate and BSA were determined using frontal chromatography at different flow-rates and sample concentrations. The employed measurement procedure was discontinuous and similar to that described by Kopaciewicz *et al.* [10].

The column was equilibrated with carrier solution [water for nitrate and chondroitin sulphate, 20 m*M* sodium phosphate buffer (pH 7.5) for BSA] at 2 ml/min. After switching the injector valve to the load position the pumping system was purged with sample solution. Thereafter, the valve was switched to the inject position so that the pump delivered the sample solution to the column. The flow-rates were measured volumetrically during each frontal analysis run. After each breakthrough measurement the ion-exchange column was washed with a solution of sodium chloride (0.5 *M* and 2 *M* for nitrate and chondroitin sulphate, respectively) and re-equilibrated with water. After every loading of BSA the column was washed with 50 m*M* phosphoric acid and 0.5 *M* sodium chloride before re-equilibration with the buffer.

The ion-exchange capacity for nitrate was calculated from the retention volume of its almost symmetrical breakthrough curves, whereas for chondroitin sulphate and BSA the breakthrough point of the curve was used for the determination of binding capacity [15].

RESULTS AND DISCUSSION

Exchange capacity for nitrate

The capacity of the ion exchanger SAX100 for low-molecular-mass anions was determined by frontal analysis with sodium nitrate at different nitrate concentrations and flow-rates, which corresponded to linear velocities up to 7.1 mm/s.

Surprisingly, the binding capacity for nitrate depended on its concentration in the mobile phase. Fig. 2 shows the exchange isotherm of nitrate, illustrating a decrease of capacity with increasing nitrate concentration. Chemistry does not offer an obvious explanation for this behaviour. It is not due to a lack of stability of the stationary phase (data not shown), but it may have been caused by a systematic error linked to the discontinuous experimental technique and to the fact that the retention times of the nitrate fronts were shorter at higher nitrate concentrations. Consequently, the data at high concentrations are less reliable. Linear regression of the isotherm data gives a straight line which intercepts the y-axis at a capacity of 879 μmol/g. This extrapolation to a nitrate concentration of zero yields a capacity value that is close to the result of 899 μmol/g obtained by potentiometric titration and to the capacity of 939 μmol/g calculated from nitrogen content. Thus, frontal analysis with low-molecular mass ions is a suitable method for the determination of the capacity of ion-exchange columns without unpacking the column or the destruction of the stationary phase.

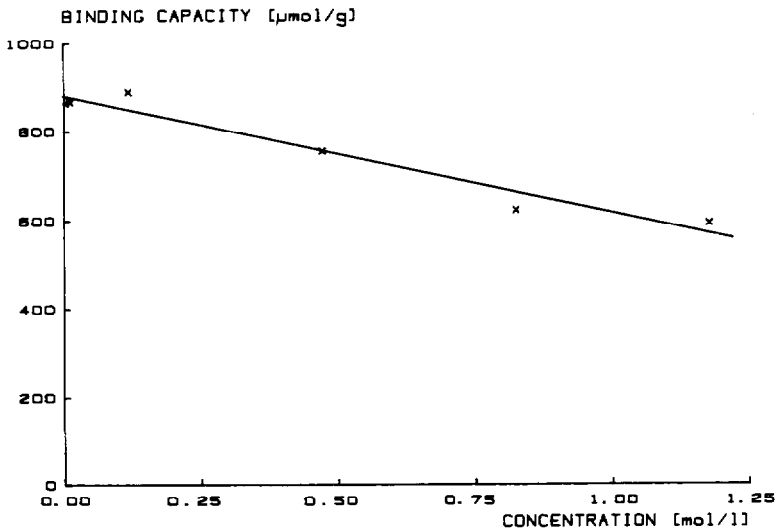

Fig. 2. Exchange isotherm of sodium nitrate on SAX100. Flow-rate, 1.0 ml/min.

Fig. 3. shows a plot of the nitrate binding capacity *versus* flow-rate for two nitrate concentrations. Within the range of experimental error, the capacity is independent of the mobile phase flow-rate, indicating that the nitrate ions can penetrate all the pores and that there are not mass transfer resistances in this instance.

The polymer-encapsulated ion exchanger SAX300 was synthesized on a wide-pore silica column (Nucleosil 300-7) to evaluate its capacity for biological macromolecules. Its actual capacity was also determined by three independent methods that gave almost identical results: (1) calculation from nitrogen content, 279 μmol/g; (2) potentiometric titration, 253 μmol/g; and (3) frontal analysis with sodium nitrate 252 μmol/g. Again, the nitrate binding capacity was independent of the flow-rate.

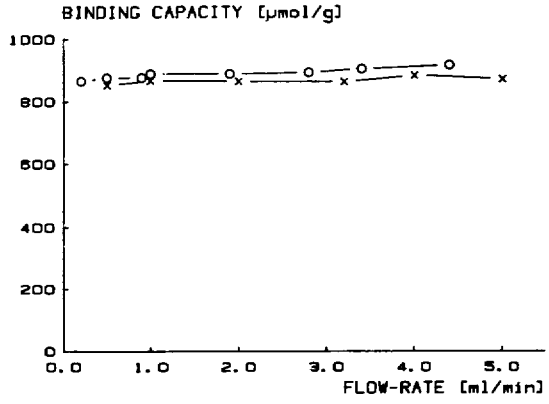

Fig. 3. Plot of nitrate binding capacity *versus* flow-rate for concentrations of 0.012 (×) and 0.118 mol/l (o). Stationary phase, SAX100.

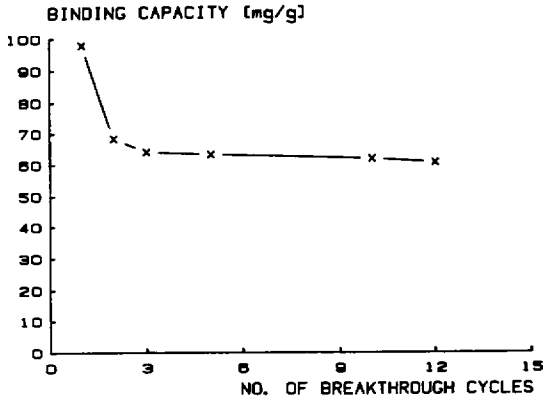

Fig. 4. Influence of the number of breakthrough cycles on the binding capacity of SAX300 for BSA. Flow-rate, 0.5 ml/min. Mobile phase, 4.87 g/l BSA in carrier solution.

Binding capacity for bovine serum albumin

During the first breakthrough cycles the SAX300 irreversibly adsorbed a considerable amount of BSA. Fig. 4 shows a *ca.* 35% loss of protein binding capacity until the most active sites of the stationary phase were saturated with protein after the first three cycles. The irreversible adsorption of such a large amount of BSA (34 mg/g) may have been caused by hydrophobic interactions of protein molecules with the polymeric ion exchanger (which is known to exert a certain hydrophobicity [14]) and their subsequent denaturation on the chromatographic surface.

The irreversible binding of BSA is also well reflected by a 10% reduction of the nitrate binding capacity and an increase of the carbon content from 4.56 to 5.12% (w/w). A calculation of the irreversibly adsorbed amount of BSA via the increase of the nitrogen content by 0.39% (w/w) and the nitrogen content of the BSA used [15.4% (w/w)] yields a value of 25 mg/g, which is in good agreement with that from frontal analysis data.

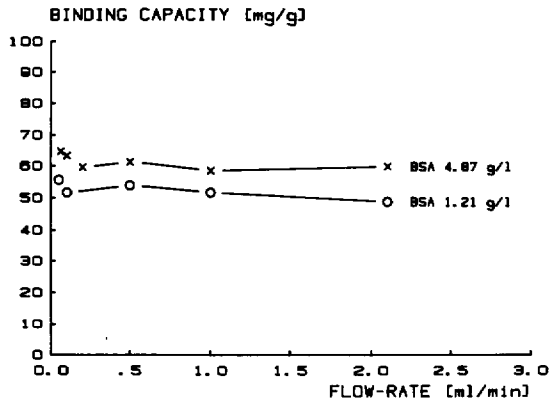

Fig. 5. Plot of BSA binding capacity *versus* flow-rate for concentrations of 4.87 and 1.21 g/l BSA in carrier solution. Stationary phase, SAX300.

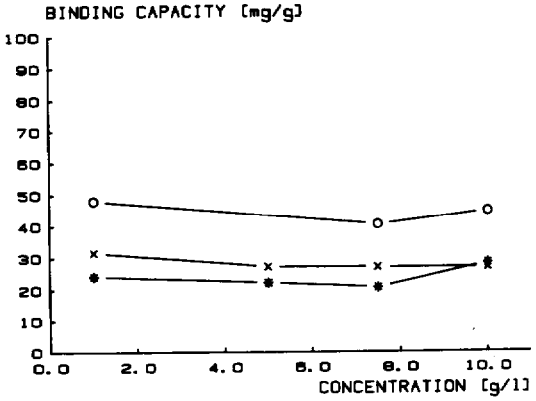

Fig. 6. Influence of mobile phase flow-rate on the exchange isotherm of the sodium salt of chondroitin sulphate. Flow-rates: o = 0.1; × = 0.5; * = 2 ml/min. Stationary phase, SAX300.

Fig. 5 shows the dependence of the capacity for BSA on the flow-rate, which was measured only after the irreversible adsorption had occurred. The linear velocities of the mobile phase were in the range 0.1–3.6 mm/s. Within this range, the BSA capacity was almost independent of the flow-rate; there was only a slight increase of capacity at flow-rates below 0.2 ml/min. Thus the mass transport of BSA was not significantly hindered; this is not surprising as BSA is a fast-diffusing transport protein [16].

Frontal analysis with the sodium salt of chondroitin sulphate

The linear polyanion of chondroitin sulphate also underwent irreversible adsorption onto the polymer-encapsulated anion exchanger SAX300. The blocking of exchange sites by this irreversible binding led to a 14% reduction of the capacity for nitrate, which can be considered as a minor change in the stationary phase properties

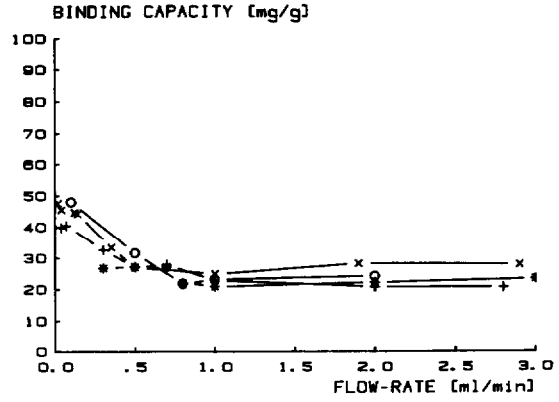

Fig. 7. Plot of binding capacity *versus* flow-rate for different concentrations of the sodium salt of chondroitin sulphate. Concentrations: o = 1.0; * = 5.0; + = 7.5; × = 10.0 g/l. Stationary phase, SAX300.

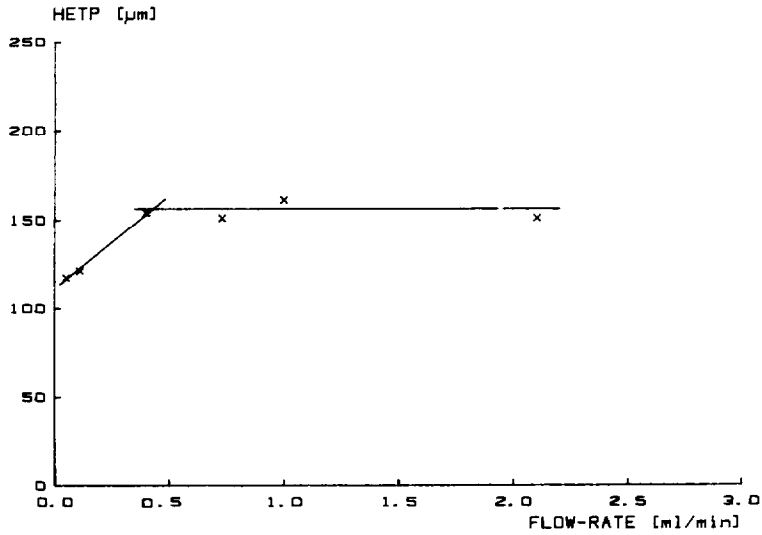

Fig. 8. Plot of HETP *versus* flow-rate for the sodium salt of chondroitin sulphate. Mobile phase, aqueous solution of sodium chloride (1 mol/l). Stationary phase, SAX300 (15 cm column).

compared with the conversion of a silica-based DEAE anion exchanger to a cation exchanger by the adsorption of chondroitin sulphate described by Liao *et al.* [17].

Frontal experiments with chondroitin sulphate after the saturation of the irreversibly adsorbing sites revealed that the capacity was independent of the sample concentration (in the range 1–10 g/l) in the carrier solution (Fig. 6). In contrast, the binding of chondroitin sulphate was strongly influenced by the flow-rate. Fig. 7 shows that there was a significant increase in the binding capacity at flow-rates below 0.8 ml/min (1.4 mm/s linear velocity), leading to a doubling of the capacity at flow-rates below 0.1 ml/min (0.2 mm/s linear velocity). This behaviour can be explained by a restricted mass transfer and slow adsorption kinetics, which are also reflected by asymmetric breakthrough curves that do not allow an unequivocal judgement as to whether equilibrium (*i.e.* complete saturation of the stationary phase or a plateau of the breakthough curve, respectively) has been reached. Anspach *et al.* [18] attribute this phenomenon to the rearrangement of adsorbed molecules on the chromatographic surface.

The increase in capacity at low flow-rates can also be correlated with the height equivalent to a theoretical plate (HETP) *versus* flow-rate curve of chondroitin sulphate (Fig. 8). In principle, the shape of this graph is typical for polymeric solutes [19] that do not necessarily show a minimum in the HETP *versus* flow-rate plot. There is a significant decrease in band spreading at flow-rates below 0.5 ml/min which corresponds well to the flow-rate dependence of the binding capacity calculated from the breakthrough point (cf. Figs 1 and 7). A comparison of the capacity *versus* flow-rate plots for BSA and chondroitin sulphate reveals that the diffusion of the linear polyanion into the pores is much more restricted than that of globular BSA, probably due to the high number of electrostatic interactions that are possible between chondroitin sulphate and the anion exchanger.

Comparison of exchange capacities

The capacity of the wide-pore anion exchanger SAX300 for low-molecular mass anions such as nitrate is 250 μmol/g. Its capacity for macromolecular anions is much lower because high-molecular mass anions cover or block a large number of exchange sites. The maximum exchange capacity for chondroitin sulphate is 0.87 μmol/g, which means that each adsorbed anion of chondroitin sulphate with a molecular mass of approximately 50 000 and two acidic groups per monomer unit covers about 290 quaternary ammonium groups. Thus, it can be assumed that there is an approximately stoichiometric ion exchange between the stationary phase and the (calculated) *ca.* 220 negative charges of a linear chondroitin sulphate anion.

The maximum capacity for globular BSA is 0.93 μmol/g. At this maximum load each BSA molecule covers or blocks 270 exchange sites, although its negative net charge at pH 7.5 is only *ca.* 20 [20][a]. This low binding capacity for BSA can be attributed to a certain inaccessibility of the exchange sites on the chromatographic surface for the negative charges of BSA that are likely to be spread all over the protein molecule.

CONCLUSIONS

Frontal analysis is a fast and reliable technique for the determination of the binding capacities of ion-exchange columns for both low and high molecular weight solutes.

For low-molecular mass ions the exchange capacity is independent of the flow-rate, whereas the capacity for macromolecular samples is influenced by chromatographic band spreading, which increases strongly with increasing eluent velocity due to the small diffusion coefficients of macromolecules. For mainly practical reasons, it is not possible to work with high-molecular mass solutes at the optimum linear velocity where the contribution of mass transfer resistance to peak broadening can be neglected. Consequently, the flow-rate dependence of the binding capacity has to be measured individually for each sample and each stationary phase according to specific differences in the type and number of interactions.

REFERENCES

1 R. E. Majors, *LC · GC Int.*, 3 (1990) 10.
2 H. Engelhardt and M. Jungheim, *Chromatographia*, 29 (1990) 59.
3 J. R. Conder and C. L. Young, *Physicochemical Measurement by Gas Chromatography*, Wiley, Chichester, 1979, Ch. 9.
4 A. W. J. de Jong, J. C. Kraak, H. Poppe and F. Nooitgedacht, *J. Chromatogr.*, 193 (1980) 181.
5 J. X. Huang and Cs. Horváth, *J. Chromatogr.*, 406 (1987) 285.
6 R. D. Whitley, J. M. Brown, N. P. Karajgikar and N. H. L. Wang, *J. Chromatogr.*, 483 (1989) 263.
7 G. L. Skidmore, B. J. Horstmann and H. A. Chase, *J. Chromatogr.*, 498 (1990) 113.
8 G. L. Skidmore and H. A. Chase, *J. Chromatogr.*, 505 (1990) 329.
9 J. X. Huang, J. Schudel and G. Guiochon, *J. Chromatogr.*, 504 (1990) 335.
10 W. Kopaciewicz, S. Fulton and S. Y. Lee, *J. Chromatogr.*, 409 (1987) 111.
11 J. A. J. Schutyser, T. J. W. Buser, D. van Olden and T. Overeem, *J. Liq. Chromatogr.*, 10 (1987) 2151.

[a] In ref. 20, the value of net charge is given for human serum albumin (HSA), but there is hardly any difference in the amino acid composition of BSA and HSA [21].

12 A. M. Tsai, D. Englert and E. E. Graham, J. Chromatogr., 504 (1990) 89.
13 R. Blanco, A. Arai, N. Grinberg, D. M. Yarmush and B. L. Karger, *J. Chromatogr.*, 482 (1989) 1.
14 H. Engelhardt, H. Löw, W. Eberhardt and M. Mauss, *Chromatographia*, 27 (1989) 535.
15 I. Mazsaroff, R. Bischoff, P. A. Tice and F. E. Regnier, *J. Chromatogr.*, 437 (1988) 429.
16 A. L. Lehninger, *Biochemistry*, Worth, New York, 1970, p. 137.
17 A. W. Liao, Z. ElRassi, D. M. LeMaster and Cs. Horváth, *Chromatographia*, 24 (1987) 881.
18 F. B. Anspach, A. Johnston, H.-J. Wirth, K. K. Unger and M. T. W. Hearn, *J. Chromatogr.*, 499 (1990) 103.
19 P. Wichner, *Ph. D. Thesis*, University of the Saarland, Saarbrücken, 1985.
20 *The Merck Index*, Merck, Rahway, NJ, 1983, p. 1217.
21 F. W. Putnam, in H. Neurath (Editor), *The Proteins – Composition, Structure and Function*, Academic Press, New York, 1965, p. 189.