ELSEVIER

Journal of Chromatography A, 852 (1999) 25–30

JOURNAL OF CHROMATOGRAPHY A

Short communication

Retention behavior of very large biomolecules in ion-exchange chromatography

Shuichi Yamamoto[a,*], Eiji Miyagawa[b]

[a]*Department of Chemical Engineering, Yamaguchi University, Tokiwadai, Ube 755-8611, Japan*
[b]*Ube Research Laboratory, Fujirebio Inc., Ube 759, Japan*

Abstract

Hepatitis B virus surface antigen (HBsAg) particles were efficiently adsorbed (retained) on a Sulfate–cellulose (S–C) bead column, and then desorbed with sodium chloride solutions (0.5–3.0 *M*). The HBsAg particles were not efficiently retained onto either sulfopropyl–agarose (SP–A) or quaternary amine–agarose (Q–A) at pH 4.5, 6 and 8. The size-exclusion curve showed that proteins of molecular mass higher than ca. 20 000 cannot penetrate into the pores of S–C beads. The dynamic binding capacity (DBC) values of lysozyme (ca. 7 mg/ml-gel) and of γ-globulin (ca. 3 mg/ml gel) for S–C did not depend on the flow velocity while the DBC of γ-globulin for SP–A decreased sharply with an increase in flow velocity. These results indicated that very large molecules are adsorbed only onto the surface of S–C, which resulted in fast adsorption–desorption rates although the equilibrium adsorption capacity is lower than conventional porous gel beads. Because of the rapid adsorption rate, the DBC values of γ-globulin for S–C at high flow-rate regions are similar to those for SP–A. Bovine serum albumin was not adsorbed onto S–C. As this can not be explained by a simple electrostatic interaction mechanism, molecular recognition of S–C might be different from the agarose-based ion-exchange beads.

Keywords: Hepatitis B virus; Stationary phases, LC; Binding studies; Antigens; Proteins

1. Introduction

Ion-exchange chromatography (IEC) is an efficient method for purifying various biomolecules, and widely used not only in laboratory but also in production processes [1–6]. IEC can also be used for the separation of much larger biomolecules than small or medium-size proteins (molecular mass up to 60 000). Such large biomolecules include immunoglobulins, plasmid DNAs and viruses.

In contrast to a large number of publications on preparative protein chromatography, theoretical and experimental investigations on preparative chromatography of large biomolecules such as large proteins (globulins), plasmid DNAs, polypeptides and viruses are still not adequate. It is known that protein adsorption capacity on most ion-exchange gels decreases drastically when the molecular mass is over 100 000 [1,4,5]. Among such large biomolecules virus separation by chromatography is becoming more important as various types of virus applications are now available such as hosts for gene therapy [7–8] as well as vaccines [9].

There are several strategies for purifying viruses and other large biomolecules by chromatography. For example, size-exclusion chromatography of viruses can be performed with packing media (gel beads) having very large pores [9]. However, it is

*Corresponding author. Fax: +81-836-35-9933.
E-mail address: shu-yama@po.cc.yamaguchi-u.ac.jp (S. Yamamoto)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00594-4

uncertain if such large-pore gels are also suited for ion-exchange or other affinity-based separations. Cellulose-based beads having a sulfate ether group (Cellufine–Sulfate) are known to be efficient media for virus separations [10]. Our experiments with hepatitis B virus surface antigen (HBsAg) particles also showed better separation performance compared with conventional ion-exchange agarose gel beads for protein separation (Fig. 1). In order to understand this efficient separation, both the molecular recognition and the mass transfer mechanisms must be investigated. The purpose of this paper is to examine the latter mechanism (mass transfer). The size-exclusion curve and the plate height (HETP) under non-binding conditions were measured. The dynamic binding capacities of several proteins were determined. As a comparison, anion- and cation-exchange agarose gel beads were also used for these experiments. On the basis of these experimental data the mass transfer rates for large molecules were discussed.

2. Experimental

Bovine serum albumin, human-γ-globulin, lysozyme, ribonuclease A were purchased from Sigma (USA). Vitamin B12 and other reagents were obtained from Wako (Osaka, Japan).

HBsAg particles were prepared from cultured

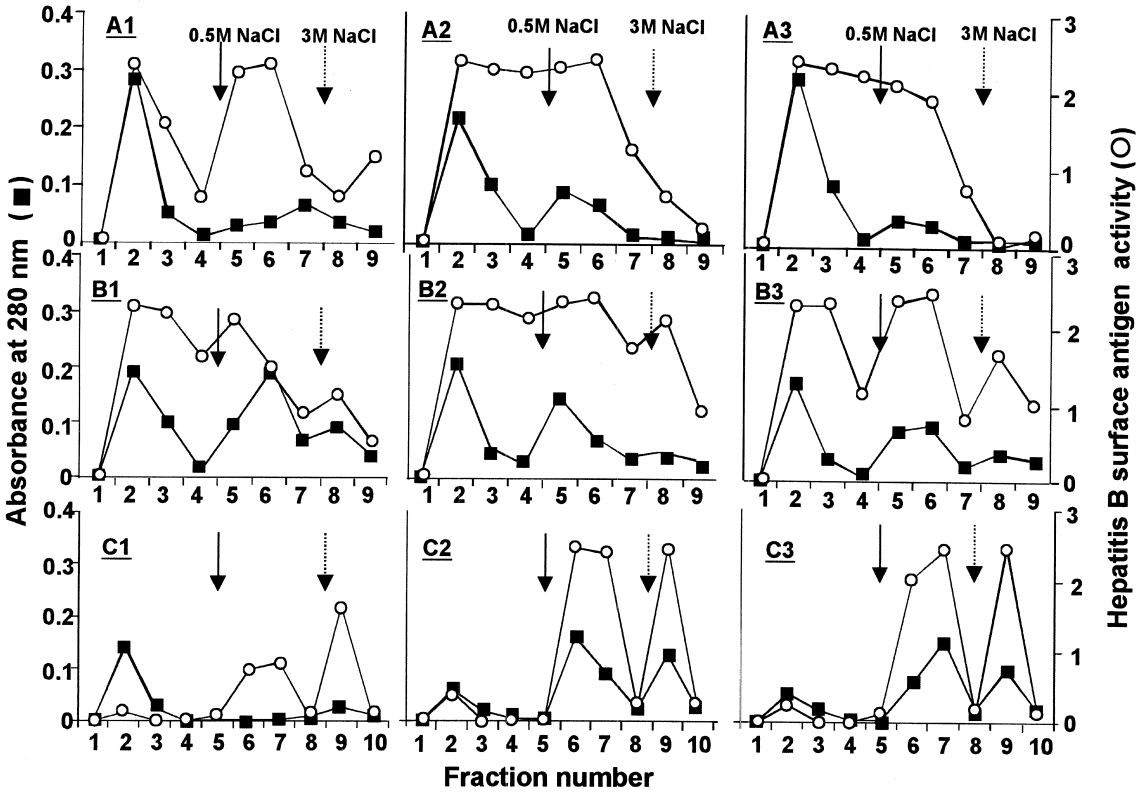

Fig. 1. Hepatitis B virus surface antigen (HBsAg) particle separation by ion-exchange chromatography columns. Column (bed) size 2×1.6 cm I.D. Sample: 2 ml HBsAg particle solution, flow-rate 1 ml/min. Fraction volume=4 ml. A1–A3: SP–Sepharose FF (SP–A), B1–B3: Q–Sepharose FF (Q–A), C1–C3:Cellufine–Sulfate (S–C). A1,B1,C1: 50 m*M* acetate buffer (pH 4.5), A2,B2,C2: 50 m*M* phosphate buffer (pH 6.0), A3,B3,C3: 50 m*M* Tris–HCl buffer (pH 8.0). Arrows indicate the change of the buffer containing a specified sodium chloride concentration. HBs Antigen activities (○) were determined by enzyme-linked immunosorbent assay (ELISA) (see text). Note that the total area for absorbance at 280 nm and for the ELISA activities varied from run to run. This may be due to the experimental error of the ELISA assay and irreversible adsorption.

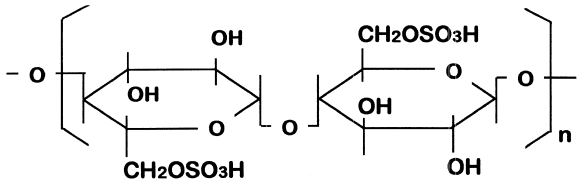

Fig. 2. Structure of sulfated cellulose beads, Sulfated–Cellufine.

supernatants of PLC/PRF/5 human liver carcinoma cell line (the Alexander cell). HBsAg was assayed by a commercial enzyme immuno assay kit (FRELISA II HBsAg, Fujirebio, Tokyo, Japan).

Cellufine–Sulfate m (Chisso, Tokyo, Japan) is a spherical cellulose bead (particle diameter 44–105 μm) having a sulfate ester group (8 μ*M*/ml) (hereafter called S–C) [10]. Its partial structure is shown in Fig. 2. SP– and Q–Sepharose FF ion-exchange gels (Amersham Pharmacia Biotech, Uppsala, Sweden) are 6% cross-linked spherical agarose gel beads having a sulphopropyl group (SP–) and a quaternary amine group (Q–) [4]. They are abbreviated as SP–FF (or SP–A) and Q–FF (or Q–A). These beads were packed into plastic columns (15 cm×9 mm I.D.) for size-exclusion and adsorption studies (5 cm×9 mm I.D. for SP–FF). Vitamin B_{12}(M_r=1356), standard globular proteins from gel filtration calibration kit (Amersham Pharmacia Biotech) and acetone were used for the size-exclusion experiment under non-binding conditions (10 m*M* phosphate pH 8 containing 0.5 *M* NaCl).

A fully automated liquid chromatography (LC) system (Prosys workstation, Beckman, Fullerton, USA) was used for the size exclusion and the adsorption studies with standard proteins. A simple LC setup (Pharmacia) with an open column (2 cm× 16 mm I.D.) was used for HBsAg separation experiments. The column experiments were done at 298±1 K. The details of chromatographic conditions are shown in the figure captions.

3. Results and discussion

Fig. 1 shows HBsAg particle separation results by cation-exchange agarose (SP–A), anion-exchange agarose (Q–A) and sulfated cellulose (S–C) media. HBsAg particles were efficiently adsorbed (retained) on, and subsequently desorbed with 0.5 and 3.0 *M* NaCl buffer solutions from the S–C column at pH 4.5, 6 and 8.

On the other hand, remarkable leakages of HBsAg activities during the sample charge indicate that HBsAg particles are not fully retained on the agarose-based IEC columns especially at pH 6 and 8 for SP–A, and at pH 4.5 and 6 for Q–A. It may be possible to adsorb and desorb HBsAg particles at pH 4.5 for SP–A, and at pH 8 for Q–A. The amount of HBsAg charged, ca. 0.35 mg/ml media, is below the overloading conditions [3–5].

These results indicate that the interaction (molecular recognition) of HBsAg with Sulfate–Cellulofine is stronger than that with Sepharose IEC gels. However, the mass transfer mechanism must also be examined as the adsorption and desorption performance is strongly influenced by slow mass transfer rates of large molecules in the gel beads, which may be also part of the reason for poor performance of agarose-based IEC columns. Sepharose FF gels (SP–FF and Q–FF) are 6% cross-linked agarose gel beads designed for protein separation. The pore size is large enough for most proteins [1,3,4,11]. However, the dynamic adsorption capacity (DBC) of proteins is known to decrease drastically with increasing protein molecular mass from ca.70 000 (albumin) to 160 000 (globulin) [1,4,5]. This indicates that very slow mass transfer (diffusion) rates in the pores govern the dynamic adsorption capacity. HBsAg particles are much larger than most proteins, and known to be ca. 20 nm in diameter [12,13]. It is easily expected that the diffusion rate of such a large particle in the pore of the agarose bead is very low. The molecular diffusion coefficient D_m of HBsAg at 298 K is calculated with its diameter as ca. $2\cdot10^{-11}$ m^2/s by the equation proposed by Tyn and Gusek [14]. When the obstruction factor in the pore $\gamma_s=D_s/D_m$ is assumed to be $\gamma_s=K/4=0.1/4=0.025$ [3,15], the pore (stationary phase) diffusivity D_s becomes ca. $5\cdot10^{-13}$ m^2/s (The distribution coefficient *K* was estimated as ca. 0.1 from our size exclusion curve measurements. See Fig. 3). This value is much lower than estimated D_s value ($1\cdot10^{-11}$ m^2/s) for a medium size protein such as bovine serum albumin (BSA, M_r 68 000).

The pore size of S–C beads is claimed to be so small that proteins are excluded and only very small

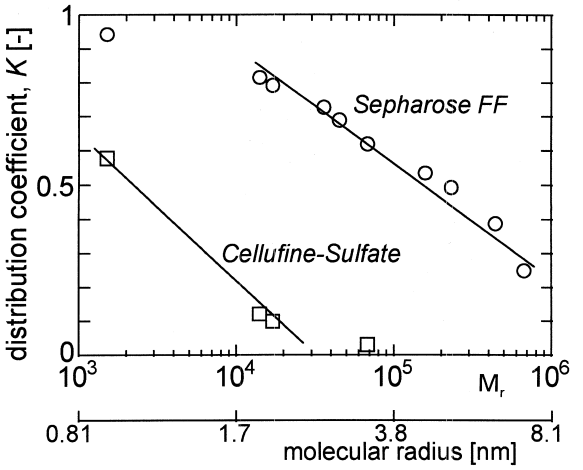

Fig. 3. Size-exclusion curves (distribution coefficient K vs. molecular mass M_r at non-binding conditions. K was determined from the retention volume V_R as $K=(V_R-V_0)/(V_t-V_0)$ where V_t= total bed volume, V_0=void volume determined from the V_R of Dextran T2000. This K is equivalent to $K_{av.}$ in the literature [1]. The experiments were carried out with 10 mM phosphate buffer (pH 8) containing 0.5 M NaCl as the mobile phase (isocratic). Column size: 15×0.9 cm I.D. The molecular radius was calculated by 0.081 $M_r^{1/3}$ [15]. Vitamin B_{12}(M_r=1356) and standard globular proteins from gel filtration calibration kit (Amersham Pharmacia Biotech) were used. The data for acetone were not shown in the figure.

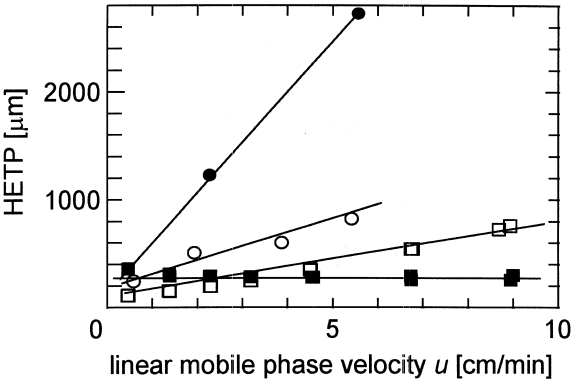

Fig. 4. HETP as a function of linear mobile phase velocity, u. HETP=$Z(w_v/V_R)^2/8$ where Z=column (bed) length, w_v=peak width in ml at $C=0.3679C_{max}$ (C_{max}=maximum peak height, C=peak height). $u=F/(A_cV_o/V_t)$ where F=volumetric flow-rate, A_c=cross sectional area, V_0=void volume and V_t=total bed volume. The other conditions are the same in Fig. 3. ● ovalbumin on SP–Sepharose FF, ○ vitamin B_{12} on SP–Sepharose FF, □ acetone on Cellufine–Sulfate, ■ bovine serum albumin on Cellufine–Sulfate.

molecules are able to diffuse into the pores [10]. If this is true, only the surface of the cellulose bead is accessible for HBsAg particles. In order to confirm this the size exclusion curve under non-binding conditions was determined with several standard globular proteins and small molecules (Fig. 3). The K value of acetone was 0.65, which indicates the bead is highly crosslinked so that even a very small molecule can not utilize the intraparticle (pore) volume fully. A small protein like ribonuclease (M_r 18 000) was almost excluded as the distribution coefficient (K) was ca. 0.08. BSA (M_r 68 000) was completely excluded ($K<0.02$). When the molecule is excluded, the plate height (HETP) does not depend on the flow-rate [3,16]. This can be confirmed in Fig. 4 where the HETP values of BSA are constant and the values of acetone increase with the flow velocity. These findings indicate that proteins which are excluded adsorb only on the surface of S–C bead under binding conditions at relatively high adsorption/desorption rates.

As we did not have enough amounts of HBsAg particles for dynamic binding capacity measurements, γ-globulin (M_r 150 000) was chosen as a model protein. Lysozyme and bovine serum albumin were also used for comparison. As shown in Fig. 5, the adsorption (DBC) of lysozyme (ca.7 mg/ml gel) and of γ-globulin (ca. 3 mg/ml gel) on S–C did not depend on the flow velocity while the DBC of γ-globulin for SP–A decreased markedly with an increase in flow velocity because of slow diffusion in the pores of the gel beads [3,17,18]. These results indicate that proteins adsorb only onto the surface of S–C, which can explain fast adsorption–desorption rates although the equilibrium capacity is much lower than conventional porous agarose ion-exchange beads (for example, 100–150 mg lysozyme/ml gel) [1,4]. Because of this rapid adsorption rate, the DBC values of γ-globulin for S–C at high flow-rate regions are similar to those for SP–A. Breakthrough curves as a function of flow velocity are shown in Fig. 6. Advantages and disadvantages of the use of the surface layer of ion-exchange resins were already discussed in detail [19,20]. Even with a macroporous resin initial adsorption rates are high as they are controlled by mass transfer to the outermost subparticles [17].

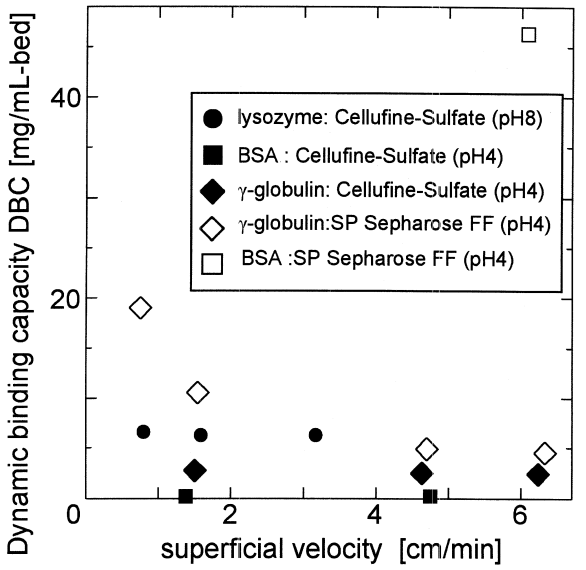

Fig. 5. Dynamic binding capacity (DBC) as a function of superficial velocity. DBC=$C_0(V_B-V_0)/V_t$ where C_0=protein (feed) concentration,V_B=breakthrough volume at the relative concentration=10%, V_0=void volume and V_t=total bed volume. Mobile phase=10 m*M* phosphate buffer containing 0.03 *M* NaCl (pH 8) or 20 m*M* sodium acetate buffer containing 0.03 *M* NaCl (pH 4). Column (packed bed) size: 15 cm×0.9 cm I.D. (Cellufine–Sulfate, S–C); 5×0.9 cm I.D., (SP–Sepharose FF, SP–FF). ● Lysozyme on S–C pH8, ■ bovine serum albumin (BSA) on S–C pH4, ♦ γ-globulin on S–C pH 4, □ BSA on SP–FF pH 4, γ-globulin on SP–FF pH 4.

BSA was not adsorbed onto S–C. As this can not be explained by a simple electrostatic interaction mechanism, molecular recognition of S–C might be different from the agarose-based ion-exchange beads. Polysaccharide sulfate (heparinoid) is known to have some specific affinity to such biomolecules as lipoproteins and some viruses. This may explain the biological recognition mechanism of sulfated–cellulose beads (see Fig. 1).

In conclusion, only the sulfate groups on the surface of Cellufine–Sulfate bead (cellulose bead having a sulfate ether group) is accessible for large molecules (M_r>70 000). This is unfavorable in terms of the total adsorption capacity but is favorable for relatively rapid adsorption–desorption rates of large molecules such as viruses and virus-like particles. There is a biological recognition (biospecific affinity) mechanism between polysaccharide sulfate (heparinoid) and some biological products, which may also play an important role in retention of virus and virus-like particles.

Acknowledgements

This work was supported by a Grant-in-Aid for scientific research on Priority Areas (No. 296) (Grant No. 10145241) and (C2, No. 10650746) from

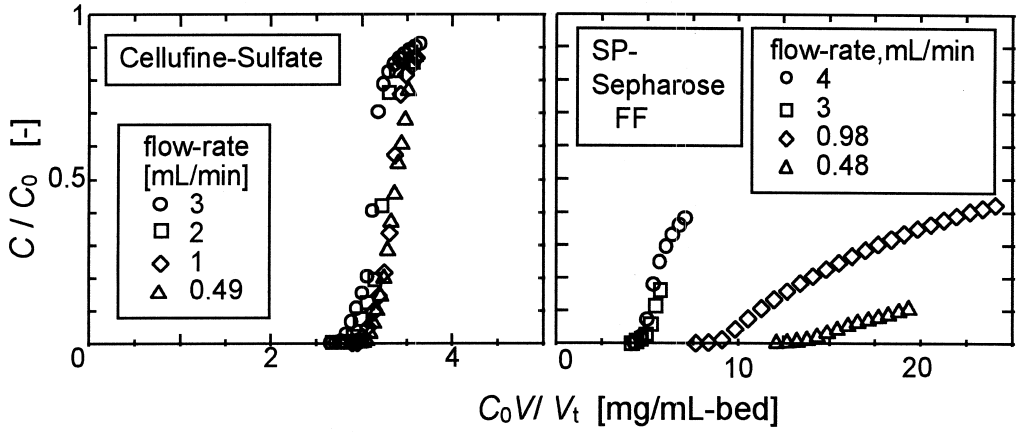

Fig. 6. Breakthrough curves of γ-globulin for the S–C column and for the SP–FF column at pH 4. C_0=initial protein (γ-globulin) concentration=1 mg/ml for the S–C column and C_0=0.5 mg/ml for the SP–FF column. V_t=bed volume. Column (packed bed) size: 15×0.9 cm I.D. (Cellufine–Sulfate, S–C); 5×0.9 cm I.D. (SP–Sepharose FF, SP–FF).

the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] E. Karlsson, L. Ryden, J. Brewer, in: J.-C. Janson, L. Ryder (Eds.), Protein Purification, 2nd ed, Wiley–VCH, New York, 1998, pp. 145–205.
[2] G. Sofer, L. Hagel, Handbook of Process Chromatography, Academic Press, San Diego, CA, 1997.
[3] S. Yamamoto, K. Nakanishi, R. Matsuno, Ion-Exchange Chromatography of Proteins, Marcel Dekker, New York, 1988.
[4] Ion-Exchange Chromatography – Principles and Methods, 3rd ed, Pharmacia Biotech, 1991.
[5] R.K. Scopes, Protein Purification, 2nd ed, Springer, New York, 1987.
[6] F. Regnier, Methods Enzymol. 104 (1984) 170.
[7] F. Blanche (Ed.), Downstream, Vol. 27, Amersham Pharmacia Biotech, 1998, p. 16.
[8] Anonymous, Downstream, Vol. 27, Amersham Pharmacia Biotech, 1998, p. 125.
[9] A. Foriers, B. Rombaut, A. Boeye, J. Chromatogr. 498 (1990) 105.
[10] Sulfate–Cellulofine Application Notes, Chisso, Tokyo, 1989.
[11] J.-C. Janson, J.-A. Jonsson, in: J.-C. Janson, L. Ryden (Eds.), Protein Purification, Wiley–VCH, New York, 1998, pp. 43–78.
[12] K. Koike, E. Yoshida, K. Katagiri, M. Katayanagi, M. Oda, H. Tsunoo, K. Yaginuma, M. Kobayashi, Jpn. J. Cancer Res. (Gann) 78 (1987) 1341.
[13] M. Belew, M. Yafang, L. Bin, J. Berglof, J.-C. Janson, Bioseparation 1 (1991) 397.
[14] M.T. Tyn, T.W. Gusek, Biotech. Bioeng. 35 (1990) 327.
[15] L. Hagel, in: J.-C. Janson, L. Ryden (Eds.), Protein Purification, 2nd ed, Wiley–VCH, New York, 1998, pp. 79–143.
[16] S. Yamamoto, M. Nomura, Y. Sano, J. Chromatogr. 394 (1987) 363.
[17] L.E. Weaver, G. Carta, Biotechnol. Prog. 12 (1996) 342.
[18] H.A. Chase, J. Chromatogr. 297 (1984) 179.
[19] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 1974.
[20] Cs. Horváth, Ion Exchange Solvent Extract 5 (1973) 207–260.