Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 1092 (2005) 89–100
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Generic method for systematic phase selection and method development of biochromatographic processes
Part I. Selection of a suitable cation-exchanger for the purification of a pharmaceutical protein

Martin Lohrmann[a,c,*], Michael Schulte[b], Jochen Strube[c]

[a] Department of Biochemical and Chemical Engineering, University of Dortmund, 44221 Dortmund, Germany
[b] Merck KGaA, 64271 Darmstadt, Germany
[c] Bayer Technology Services GmbH, PT-PT-CEM, B310, 51368 Leverkusen, Germany

Available online 15 June 2005

Abstract

Even if the first protein therapeutics are now for more than 20 years on the market the selection of suitable adsorbents for the preparative downstream processing (DSP) of these biomolecules as well as the method development towards process conditions are still based mainly on ‘trial and error’. Therefore, theses processes are not perfectly efficient, but indeed very time consuming and laborious. In this study a novel systematic method is introduced to find a suitable adsorbent (not necessarily the best one) with appropriate separation parameters for a specific separation with reduced effort. Following this strategy, the adsorbents must first be packed into columns under preparative conditions and then characterized completely with regard to, e.g. pressure drop, k'-values, plate heights (HETP curves), selectivity and capacity by using test substances, which are similar in their characteristics (molecular mass, size, charge distribution, hydrophobicity) to the target proteins. With the database once determined, a preselection of most suitable adsorbents including separation parameters is made regarding chromatographic and also economical properties. After this, preparative experiments must be conducted with a reduced number of adsorbents to figure out the individual influence of side components. This approach is demonstrated for the separation of an exemplary industrial protein mixture using cation-exchange chromatography (CEX). Characterization of different weak CEX-adsorbents is illustrated. After comparing these phases with each other, a first preselection and a prediction of suitable adsorbents is made. In the following preparative separation conditions (load, velocity, gradient) are determined for the preparative separations using the database and results of some additional experiments. The final comparison of separation performance in preparative scale confirms this selection and so the applicability of the new method.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Phase screening; Phase selection; Biochromatography; Pharmaceutical protein; Preparative chromatography; Ion-exchange chromatography

1. Introduction

Purification of biotechnological peptide or protein drugs in the downstream process is made mainly with a sequence of different chromatographic steps in order to get the required high purity. To obtain economic purification processes suitable adsorbents have to be selected under technical as well as economical considerations. This selection has to be fixed during process development. The final quality of biological products, used for clinical trials is defined by the process due to the inability of analytical procedures to fully characterize the product. A later process optimization including the change of the adsorbent or new process parameters is generally not accepted by the authorities.

To select suitable adsorbents for the separation of biomolecules, different approaches exist. Comparable to phase selection to separate small molecules or enantiomeres [1–6], retention models are used to predict the retention of proteins. The parameters of these models are determined

* Corresponding author. Tel.: +49 214 30 74149; fax: +49 214 30 81554.
E-mail address: martin.lohrmann@bayertechnology.com (M. Lohrmann).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.05.067

empirically [7–9] or alternatively they are predicted with QSRR (quantitative structure-retention relationship) models. For instance Mazza et al. described the surfaces of two cation-exchangers with different molecular descriptors obtained from crystal structure in such a good way, that they can predict the retention times as well as selectivity reversals for different proteins [10,11].

Also retention maps are used for selection of suitable adsorbents [12,13]. The disadvantage of all these approaches is that phase selection is only based on resolution under analytical conditions. But for an economical process, the chromatographic behavior under preparative conditions is decisive, which cannot be estimated well from analytical results. Therefore, adsorbents must be tested absolutely under overloaded conditions and evaluated in regard to technical and economical aspects.

But due to time pressure in process development (to reduce the time to market), only a few adsorbents may be tested. Otherwise, testing of a larger number of different adsorbents requires enormous efforts. Preparative experiments take place mainly under standardized conditions, which are not suitable for all adsorbents. Phase selection is therefore often only based on the separation performance parameters (purity, yield). Due to this time consuming procedure, often only one adsorbent with separation parameters is found, with which the desired separation may be obtained. But from the simple observation that the separation will work technically it cannot be estimated that the process will work under economical considerations too.

To improve this situation, a new generic and systematic method is presented. With this approach the selection of an economically suitable adsorbent out of a large number of different choices is also feasible. In addition, the effort is reduced in comparison to the common "trial and error" methods. Especially, the number of expensive preparative experiments, consuming time, material and analytic resources is reduced drastically.

The applicability of this new systematic method is presented for the purification of an industrial protein mixture using a cation-exchange adsorbent. To show the generic applicability of this method for the purification of any biological product, another example will be shown in a second publication for the reversed phase chromatographic purification of a peptide drug.

2. New method for systematic phase selection

The new method for systematic phase selection can be divided into the following steps:

- qualification of adsorbents for their use in process chromatography;
- packing of adsorbents;
- selection of test substances;
- characterization of the adsorbents with test substances;
- preselection of adsorbents and definition of preparative separation parameters;
- preparative experiments with the real feed mixture;
- final selection of 1–2 adsorbents.

Adsorbents can only be used in process chromatography, if they are produced reproducibly in large batches (≥100 l) at acceptable costs. For each chromatographic separation task, there is a variety of adsorbents with different matrices and different ligands available [14,15]. To achieve the process optimization goal in a reasonable time frame, only a limited number of around six adsorbents with different features (matrix, ligand, pore and particle structure, supplier) is examined.

Subsequently, the adsorbents are packed in laboratory scale columns. The packed beds in these columns must correspond to the beds of process scale columns to allow a later scale up. Therefore, equal packing methods have to be applied. Especially for compressible materials the influence of the wall effect for different column diameters has to be taken into consideration (see Section 3.3.1).

Test substances are selected in a way, that the characteristics, e.g. size, hydrophobicity and charge distribution on the surface, which influence the chromatographic separation the most, are comparable to those of the target protein. With these test substances the adsorbents are characterized completely. Pressure drop, capacity factors, van Deemter curves, selectivity for the test substances, dynamic capacities or adsorption isotherms are measured, to obtain the complete database. Some data are available for several cation-exchanger resins in the literature [16–21]. More references can be found at [16]. But not all characteristic parameters and all adsorbents were always determined. Furthermore, the experimental conditions (pH, conductivity, velocity, buffer, quality of test substances, method to determine the binding capacities) differ in the literature and therefore the data from two different publications are often not comparable.

For process chromatography phase selection can only be carried out by conducting preparative experiments with the real feed mixture of the target protein to consider the influence of the side components. To reduce the number of these expensive experiments to a minimum, a preselection of suitable adsorbents with regard to their chromatographic and economic properties is accomplished with the once determined database. Therefore, only those adsorbents are tested furthermore, which have a sufficient selectivity for the test substances. Using the previously determined database and results of isocratic binding strength measurements for the target protein reasonable separation parameters (load, velocity, gradient) can be defined. Determining the loading of the single adsorbents the following points have to be considered:

- differences between the test substances and the target compound, e.g. size of the molecule [22–24];
- differences in feed conditions, e.g. purity of the feed mixture [25];

- as well as differences in chromatographic conditions such as pH or buffer concentration [26–28].

After this first set of experiments, a theoretical productivity can be calculated for each adsorbent. The process economy of the separation is then calculated taking into consideration the product dilution or concentration, which can be estimated based on the plate height. A ranking of the suitable adsorbents is set up based on this evaluation.

To check the results from the evaluation study, preparative experiments with the feed mixture are then only made for the most suitable adsorbents using the same velocity and load (In this case the productivity is set to be constant.). These experimental conditions are selected in such a way, that the process guarantees a sufficient high productivity and high economy. As soon as the required purity is obtained by one or several adsorbents, a technically suitable material is found. The final selection of one specific adsorbent is then made by comparison of the separation performance and by estimation of the optimization potential.

3. Experimental

3.1. Materials

Six different weak cation-exchange adsorbents (C1–C6) were purchased from several well-known manufactures. The mean particle sizes for the different adsorbents were in the range of 40–120 μm. Resin C6 had a bimodal pore structure. All other adsorbents were monomodal with a mean pore diameter between 40 and 100 nm (Table 1).

For pulse experiments lysozyme (L6876) was ordered from Sigma Aldrich (Taufkirchen, Germany) and for capacity measurements lysozyme (62971) was purchased from Fluka (Buchs, Switzerland). Bayer Healthcare (Elberfeld, Germany) kindly provided Aprotinin and the target protein mixture. The Properties of test proteins are given in Table 2.

Table 1
Properties of the weak cation-exchangers, mean values given by the manufactures

Resin	Matrix	Particle size (μm)	Mean pore diameter (nm)
C1	Dextran	40–120	Not given
C2	Methacrylate	60	60–80
C3	Agarose	90	40
C4	Methacrylate	65	100
C5	Methacrylate	50	100
C6	Silica	50	Bimodal

Table 2
Properties of test proteins

Protein	p*I*	Molecular mass (kDa)
Lysozyme	11	15
Aprotinin	10.5	6.5

Sodium dihydrogenphosphate dihydrate (106345), disodium hydrogenphosphate anhydrous (106586), sodium chloride (106404) and sodium hydroxide were of analytical reagent grade and were purchased from Merck (Darmstadt, Germany). For the analytics of the target protein mixture triethylamine was ordered from Bio-Rad Laboratories (Munich, Germany), ammoniumsulfate from Fluka and acetonitril from Merck.

3.2. Instrumentation

For flowrates up to 10 ml/min an Agilent 1100 Series LC system (Palo Alto, CA, USA) was used as the chromatographic system. Injection was done via an autosampler equipped with different sample loops (up to 1.5 ml). For higher flowrates a system consisting of two preparative pumps (M305/306) from Gilson (WI, USA) was used. The injection was made with a six-port valve (Model 7725) from Rheodyne (Rohnert Park, CA, USA).

UV detection by use of a multiwavelength detector of the Agilent 1100 Series was used in both system configurations. To control the buffer composition, a conductivity and a pH monitor (pH/C 900, Pharmacia Biotech, Uppsala, Sweden) was placed behind the UV detector. For preparative experiments the fraction collector of the Agilent 1100 Series was added and placed behind pH monitor.

For fraction analysis a second Agilent 1100 Series LC system was used. The autosampler was additionally temperature controlled.

To compensate for differences in extra column volume and residence volume of both system configurations, correction factors were defined, so that the test results of both systems could be compared with each other.

3.3. Methods

All experiments for characterization of the adsorbents were performed under similar conditions, which means that column dimensions, temperature, buffer, buffer concentration, pH, conductivity and gradients were kept constant. Independent of the different methods used for characterization, some common similarities may be registered. In all experiments columns were first equilibrated with at least 10–15 column volumes of the running buffer of the next experiment. Within a test series (especially during capacity determinations) test substances out of one batch were used for all experiments. Substances were dissolved in the equilibration buffer. Control of the concentration was done with a UV spectrometer (Cary 50) from Varian (Palo Alto, CA, USA).

In pulse experiments the injection volume was kept constant. The concentration had to be adapted due to different extinction coefficients. The wavelength was set to 280 nm and was adapted during capacity determinations, so that the maximum signal was still in the linear range of the detector. All experiments took place at room temperature.

The pHs were adjusted after adding salt by use of NaOH. All buffers and solvents were filtered through a 0.2 μm filter (Pall Gelman Labaratory, Michigan, USA) prior to use.

3.3.1. Packing

The ion-exchanger resins were packed into glass columns (Superformance, Götec Labortechnik, Mühltal, Germany) with an inner diameter of 16 mm to a bed height of 10 cm according to the packing procedures recommended by the resin manufactures. For the compressible media, smaller compression factors were attained in these columns than those later used in the process scale columns. These lower compressions are caused by wall effects, which are first irrelevant above a diameter/length ratio of 2 [29]. Therefore, beds were additionally compressed manually at the end of the packing, until the typical compressions for process columns recommended by the resin manufactures were achieved. This is the only possible way to use the results of lapscale experiments to evaluate the chromatographic behavior of the adsorbent in a process column.

3.3.2. Pressure drop

A precision manometer (Leo 2, 0–30 bar, accuracy 0.1%, Keller, Winterthur, Switzerland) was installed between the pump and the column to measure the pressure drop accurately. Pressure drop curves were determined for a 20 mM phosphate buffer at pH 6.0. The velocity was increased in small steps until the compressible media showed an additional compression (headspace). Rigid materials were tested at velocities up to 1250 cm/h. Higher velocities are today not relevant in production.

3.3.3. Plate height determinations

Since in biochromatography elution is done mainly with a linear salt gradient, plate heights for lysozyme and aprotinin were also determined for such a gradient [30]. Buffer A was a 20 mM phosphate buffer. Buffer B consisted additionally of 2 M sodium chloride. Both buffers were adjusted to pH 6.0. A volume of 1 ml of protein solution was injected into the test column. Due to large differences of the extinction coefficients, the concentration of aprotinin was set to 2.5 g/l and for lysozyme a concentration of 1 g/l was chosen. For the elution of the proteins the ion strength of the buffer was enhanced. During 20 column volumes the sodium chloride concentration was raised from 0 to 2 M. To avoid destroying of the adsorbents in these experiments, the velocity was limited to 150 cm/h for adsorbent C1 and for other adsorbents to 600 respectively 800 cm/h.

For Gaussian peaks, the plate numbers N were calculated by using the retention time t_R and the peak width $w_{1/2}$ at half peak height:

$$N = 5.54\,\frac{t_R^2}{w_{1/2}^2} \tag{1}$$

For peaks with symmetry factors greater than 1.3, an approximation equation for asymmetrical peaks was used. The plate number was estimated as following [31]:

$$N = 41.7\,\frac{(t_R/(A+B))^2}{B/A + 1.25} \tag{2}$$

Length A and B were measured at 10% height of the peak maximum. The height of a theoretical plate H is then calculated with the column length L_{column}:

$$H = \frac{L_{column}}{N} \tag{3}$$

3.3.4. Capacity factors

Capacity factors for the two test substances were calculated based on pulse experiments for determination of HETP curves as described previously. Due to large differences in the porosity of the different adsorbents (data not shown), it is not very suitable to calculate capacity factors for gradient elution with the standard equation:

$$k' = \frac{V_R - V_0}{V_0} \tag{4}$$

V_R is elution volume and V_0 is representing the column dead volume. To describe the binding strength and herewith the necessary elution volume, the capacity factor k'_{El} was defined with the column volume V_{column} as following:

$$k'_{El} = \frac{V_R}{V_{Säule}} \tag{5}$$

For the selection of suitable gradients for the preparative experiments, isocratic retention measurements as a function of sodium chloride concentration with product component were additionally performed. A volume of 500 μl of the protein solution with a concentration of 8 g/l was injected each time. The sodium chloride concentration was varied between 0.1 and 1 M depending on the resin. These experiments were also performed at pH 6.0.

3.3.5. Capacity determinations

Stationary as well as dynamic capacities at 10% breakthrough for lysozyme were all determined by frontal chromatography. The protein was dissolved in a saltfree 20 mM phosphate buffer, pH 9.0 with a concentration of 10 g/l. For dynamic capacity measurements, the velocity was varied between 70 and 1200 cm/h. For stationary capacity determinations the experiment was stopped at a breakthrough between 90 and 94% – to save time and material. Breakthrough curves were then extrapolated to 100% breakthrough assuming a constant slope.

Additionally dynamic capacities (10% breakthrough) were measured for the target protein and for lysozyme at a higher ionic strength to determine a suitable loading for the preparative experiments. The velocity in these experiments was set to 70 cm/h. The protein was dissolved in loading buffer (90 mM phosphate, pH 9.0) with a concentration of 8 g/l.

In all experiments calibration curves, which convert detector signal to protein concentrations were used, to find exactly the point of desired breakthrough. The amount of protein adsorbed onto the media was obtained from a mass balance. To reduce the extra column effects, the column was directly placed behind the pump and all tubes were shortened to a minimum. The remaining extra column volume was negligible. Alteration of the mobile phase composition within the pores was only considered for determination of stationary capacity.

After each determination of capacity a cleaning in place (CIP) procedure took place to guarantee a complete desorption. First, the column was washed with a high salt concentration (1–2 M NaCl) until the detector signal indicated no longer any eluting protein. There after, the column was washed at a low velocity with 0.2–0.5 M NaOH (2–5 CV) and then stored in sodium hydroxide for a longer period (∼1 h). After washing with the equilibration buffer, a pulse experiment was carried out to control the cleaning procedure. The results (retention time, symmetry, plate number) were compared with data obtained before loading. In case of substantial differences the cleaning procedure was repeated.

To confirm that the decrease of dynamic capacities at high velocities did not refer to aging of the adsorbents or an incomplete desorption, the first experiment was repeated on each column. Beside these two runs, the order of experiment was randomized.

4. Result and discussion

4.1. Characterization

4.1.1. Pressure drop

The pressure drop curves for the different adsorbents are shown in Fig. 1. All adsorbents show a linear relationship between pressure drop and velocity until they reach their pressure limits. Due to different mean particle diameters as well as especially different compression factors, big differences for pressure drops of the single adsorbents can be observed. Adsorbent C2 with the highest compression shows the largest pressure drop at all velocities. In contrast, the adsorbent C3 with the largest average particle size and adsorbent C1 with the lowest compression factor have the lowest pressure drops, which are 50–70 percent lower than for adsorbent C2. It is worthwhile mentioning that these adsorbents have also the lowest pressure stability, which is in detail shown for adsorbent C1, which could only be tested at small velocities. Additionally this rather traditional adsorbent shrinks and swells strongly depending on pH and ionic strength of the buffer. Therefore the maximum velocity must be reduced furthermore at other buffers.

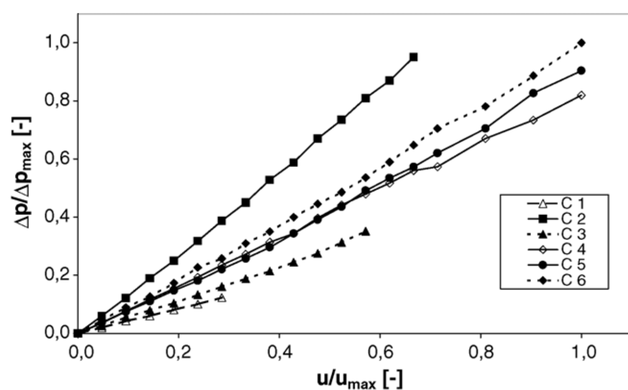

Fig. 1. Normalized pressure/flow curves for a 20 mM phosphate buffer (pH 6.0) at a bed height of 10 cm.

4.1.2. HETP curves

HETP values for lysozyme and aprotinin were calculated based on results from pulse experiments and are presented in Fig. 2 as a function of velocity. First of all, it is remarkable, that at the lowest velocity adsorbent C5 shows for both proteins a far higher plate height compared to all other adsorbents. A repetition of packing of this adsorbent showed the same results. All adsorbents show a linear slope of the HETP curve for lysozyme and aprotinin. Material C1 has the highest slope for both proteins. This soft, non-pressure stable adsorbent shows a limitation of mass transfer even at low velocities. Only the adsorbent C5 exhibits also a stronger increase of band broadening. The other four tested adsorbents show very similar curves. Only for aprotinin there are small differences. Adsorbent C5, which might exhibit additional convective transport due to its bimodal pore structure, has the lowest values for this protein. In general these differences are rather small, because all adsorbents have a good accessibility to their pores for this small protein and consequently a fast diffusion.

4.1.3. Capacity factors

Capacity factors k'_{El} for lysozyme and aprotinin were calculated based on results from pulse experiments and are shown in Fig. 3 as a function of velocity. Lysozyme is very strongly bound to all adsorbents due to the big differences between its isoelectric point (11) and the buffer pH (6). This protein shows the strongest binding on adsorbent C5. For the chosen salt gradient the elution occurs at a salt concentration of 1.2 M. The second highest desorption concentration (700 mM) for lysozyme is required on adsorbent C6. For all other adsorbents, a sodium chloride concentration of 500 mM is sufficient for complete desorption. For aprotinin qualitatively similar results are obtained. Adsorbents C5 and C6 also require the highest, whereas adsorbent C2 requires the lowest salt concentration for a complete elution. In this case the differences are not so pronounced. The required salt concentration varies only between 500 and 900 mM. With exception of adsorbent C5 all resins bind aprotinin stronger than lysozyme.

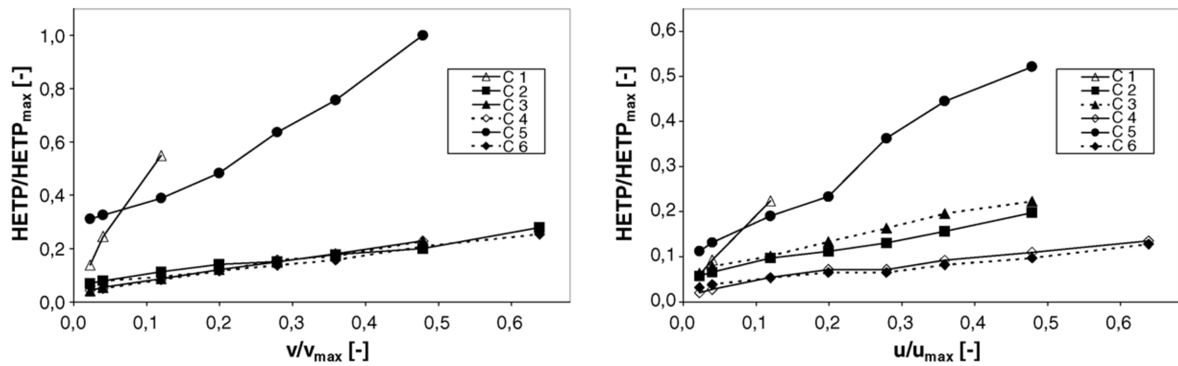

Fig. 2. Normalized HETP curves of lysozyme (left) and aprotinin (right). Van Deemter curves were determined by applying a 1 ml pulse of 1 mg/ml, respectively, 2.5 mg/ml protein solution in 20 CVs linear gradient from 0 to 2 M NaCl in a 20 mM phosphate buffer at pH 6.0.

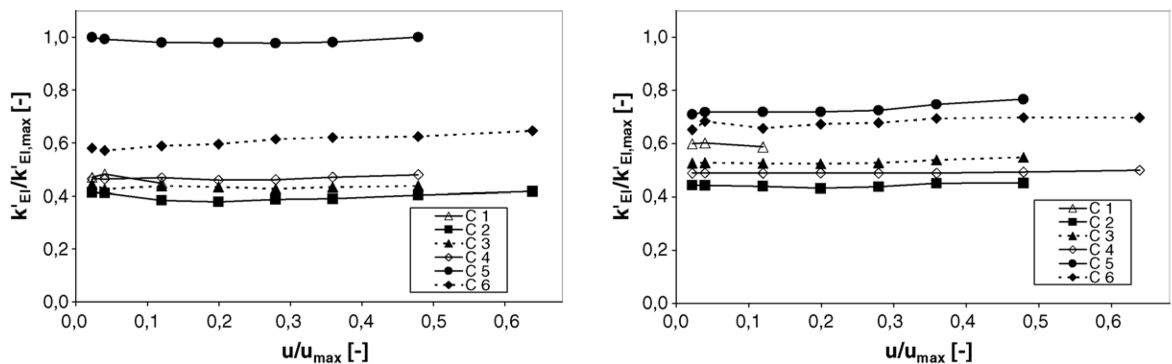

Fig. 3. Normalized elution volumes for the protein lysozyme (left) and aprotinin (right). k'_{El} values were determined by applying a 1 ml pulse of 1 mg/ml, respectively, 2.5 mg/ml protein solution in 20 CVs linear gradient from 0 to 2 M NaCl in a 20 mM phosphate buffer at pH 6.0.

4.1.4. Capacity determinations

Dynamic capacities for a saltfree lysozyme solution, which were calculated based on breakthrough curves, are shown in Fig. 4 as a function of velocity.

Large differences can be seen between the single adsorbents concerning the value for dynamic capacity, and especially, the dependency of velocity. Adsorbent C1 has at the lowest velocity the highest capacity (145 g/l_{CV}). Similar capacities are obtained only for adsorbents C3 and C6. All other adsorbents have far lower capacities, which are half or in case of adsorbent C5 2/3 lower. The capacity decreases at higher velocities are the largest for soft materials, since diffusive mass transport is limited strongly at higher velocities. In case of adsorbent C1, this limitation causes a decrease in capacity of 50% at an increase of velocity up to 250 cm/h. Up to this conditions adsorbents C3 has still a constant capacity. But a further increase in velocity then also results in a significant decrease in capacity for this adsorbent. At a velocity of 650 cm/h the capacity drops to 50%. For adsorbents C6, the capacity remains nearly constant over the whole tested velocity range. This confirms, that there is an additional convective transport due to bimodal pore structure. Therefore, the capacity only drops by 12% at increased velocity from 70 to 1000 cm/h. Consequently, this adsorbent has the by far highest capacity in the medium velocity range. It is remarkable, that capacities of the other adsorbents are also quite constant over a broad velocity range, but on a far lower level. A transport limitation due to a too low diffusion is recognized first at a velocity higher than 500 cm/h.

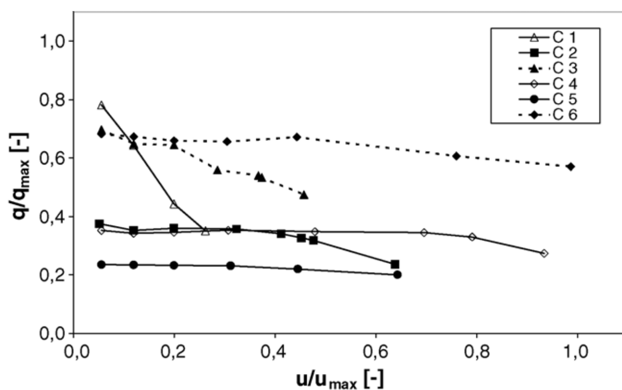

Fig. 4. Normalized dynamic capacities for lysozyme at 10% breakthrough. Capacities were determined for a 10 g/l protein solution in a saltfree 20 mM phosphate buffer (pH 9.0) at a column height of 10 cm. Relative capacities are based on the stationary capacity of adsorbent C1.

There are only minor differences between the stationary capacities, which are shown in Fig. 5, and the dynamic capacities (10% breakthrough) at the lowest velocity tested. Due to these small differences, all binding sites inside the pores must be well accessible for the relatively small lysozyme

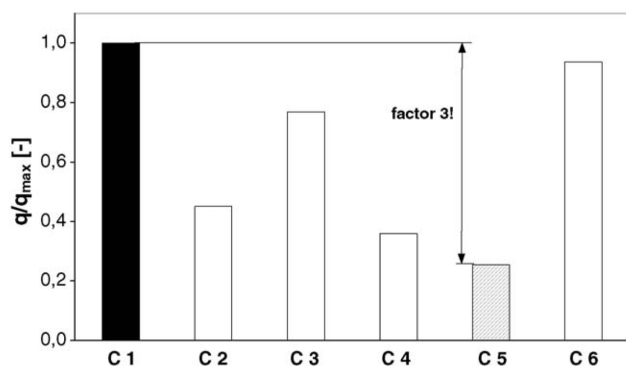

Fig. 5. Normalized stationary capacities for lysozyme. Data were determined for a 10 g/l protein solution in a saltfree 20 mM phosphate buffer at pH 9.0 by integration of breakthrough curves. Experiments were stopped at a breakthrough of 90–94% and then extrapolated up to 100% assuming a constant slope.

molecule. Only adsorbent C6 shows a different behavior. Its stationary capacity is approximately 1/3 higher, due to a very slow breakthrough curve. Therefore, also small pores must exist, in which the protein can first diffuse at higher residence times.

Repeated experiments at the end of each test series confirmed the first measured dynamic capacities (±10%) and therewith the reproducibility of the results. The results for dynamic capacities for the target protein and for lysozyme at higher ion strength are presented in the next chapter.

4.2. Preparative experiments

4.2.1. Separation problem

A cation-exchange step in a multi step purification process of an industrial protein mixture was optimized by application of the new methodology. In this process the adsorbent C1, which had been so far used in process development, was not able to separate sufficiently between a side component and the product component. This problem is shown in Fig. 6, which shows the elution curves of both components. The peak of the side component is nearly completely situated below the peak

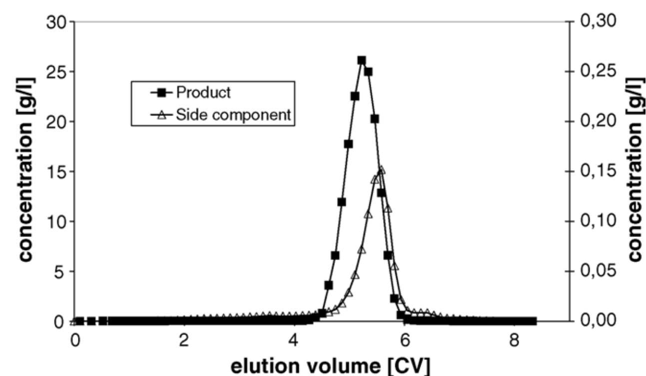

Fig. 6. Elution profiles of target product protein and side component for adsorbent C1 at high loading. For desorption, a 9.2 CVs linear gradient from 0.2 to 0.65 M NaCl at pH 6.0 (no buffering) was used.

of the product component. Therewith, the complete removal of the side component is related to a high yield loss of the product.

Therefore, a new adsorbent with appropriate separation parameters had to be found in only a few preparative experiments.

4.2.2. Phase selection and definition of separation parameters

Preselection of suitable adsorbents and definition of the separation parameters for preparative experiments were made based on the results of the adsorbent characterization previously described. Due to the characteristics of the product component, aprotinin was chosen as characteristic test substance. All necessary and important data of the target protein mixture are listed in Table 3. Besides the economic aspects (capacity, velocity) especially the chromatographic parameters capacity factor and selectivity had to be considered for the phase selection. Due to high buffer concentration in the feed mixture (conductivity = 11 mS/cm), a negative influence of additional ions had to be considered. The influence is reduced, the higher the capacity factor k'_{El} for a given adsorbent is. Adsorbents with small k'_{El} values have under this conditions distinctly far lower capacities than under low ionic strength conditions, which were used for the adsorbent characterization. A high selectivity was an additional prerequisite due to minor differences between the target product and the side component (difference of three amino acids). Therefore, it was assumed, that a complete separation of this side component is not possible with those adsorbents, which did not show a sufficient selectivity for the both test proteins lysozyme and aprotinin.

Under these pre-assumptions, adsorbents were ranked as shown in Table 4, whereby the chromatographic parameters k'_{El} and selectivity were higher rated than all other parameters.

The results of the isocratic retention measurements of the target product protein as a function of sodium chloride concentration are illustrated in Fig. 7. These data are used for the definition of the separation gradients. The curves increase exponentially below a certain salt concentration, which is different for each individual adsorbent. There are mayor differences in the value of the concentrations as well as in slope of these curves. Adsorbent C2 need the lowest salt concentration for elution and has the highest slope, so that there is no retention for the target product component above salt concentrations larger of 250 mM. On the other hand, adsorbent C5 needs the highest salt concentration for elution and exhibits the lowest slope. Even at a concentration of 650 mM the protein is retarded on this adsorbent. Curves of all other adsorbents are in between the curves of C2 and C5.

Table 3
Properties of the industrial protein mixture

c_{product} (g/l)	Purity (%)	Feed buffer
8	98	90 mM phosphate

Table 4
Qualitative comparison of adsorbents (+++: very good; −−−: very bad) and the deduced adsorbent ranking for the specific separation problem

Resin	Capacity	k'	Velocity			Dilution	Selectivity (test proteins)	Ranking
			Δp	HETP	Cap.			
C1	+++	+	–	−−	−−	0	−	–
C2	0	−	−	0	+	0	−	4
C3	++	0	+	0	−	0	++	2
C4	0	−	0	+	+	0	−−	5
C5	−	++	0	−	+	−	++	3
C6	++	+	++	+	++	0	0	1

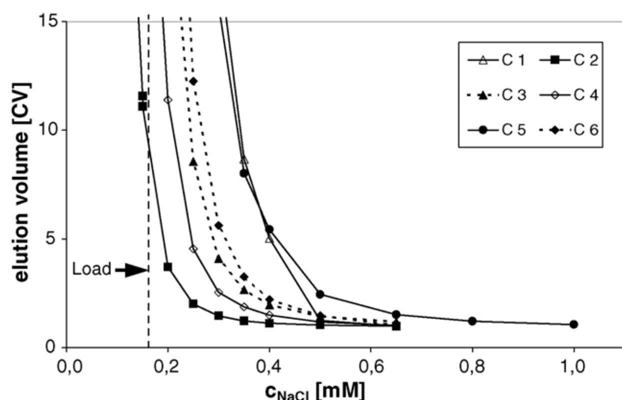

Fig. 7. Isocratic binding strength measurements of the product protein as a function of sodium chloride concentration at pH 6.0. Curves were determined by applying a 500 μl pulse of 8 mg/ml protein solution. Calculation of elution volume at peak maximum.

Due to these large differences, it was absolutely necessary to define an individual separation gradient for each adsorbent. It had to be ensured, that during loading a complete adsorption ($k' \geq 15$), during washing no desorption ($k' \geq 15$) and at the end of gradient a complete desorption ($k < 1$) takes place. Thus, gradients as shown in Table 5 were chosen. The gradient volume in each experiment was 9.2 column volumes.

In addition to the gradient formation, a suitable loading had to be defined for the preparative experiments. This was achieved by using data from the database and only a few additional experiments. First main characteristics of the protein as well as the loading conditions were compared between the test system and the target protein system, as shown in Table 6. The target proteins had similar isoelectric points in both systems. These isoelectric points are far apart from the pH of the loading buffer, allowing a good adsorption under these conditions. In addition there the size differences of the proteins are only small and thus negligible.

Table 5
Chosen gradients for the preparative experiments

Resin	$c_{NaCl,start}$ (mM)	$c_{NaCl,end}$ (mM)
C1	0.2	0.65
C2, C4	0.12	0.35
C3	0.12	0.5
C5	0.12 (0.2)	(0.65) 1
C6	0.2	0.65

The most important factor is the big difference in phosphate buffer concentration between both systems, which had to be considered. This difference had a mayor influence for the adsorption on adsorbent C2, whereas for adsorbent C5 the influence of higher ionic strength was not important (see Fig. 7). Therefore, the dynamic capacities for lysozyme were determined additionally for these two adsorbents (C2, C5) as well as for adsorbent C3 for the loading buffer composition and the feed concentration of the target protein mixture. The results for all dynamic capacity determinations as well as the percent changes between the different test series are shown for these three adsorbents in Table 7. Relative capacities are based on the capacity of adsorbent C3 for lysozyme and 20 mM phosphate buffer. The capacities decrease drastically for all adsorbents with higher phosphate concentrations and lower protein concentrations. As expected, the decrease of the capacity for adsorbent C2 with nearly 70% is by far larger as for adsorbent C5, where the dynamic capacity is reduced only by 40%. A decrease in capacity of the other adsorbents could be roughly estimated based on these two extremes. This was confirmed by the data for adsorbent C3, which percent capacity decrease is in between adsorbent C2 and C5.

Up to this point, the influence of ionic strength on the adsorption was only determined for lysozyme, although the more characteristic test substance for the target protein was aprotinin. Therefore, the influence of the buffer concentration had to be compared between the two test proteins. This was made by comparison of the capacity factors k'_{El} shown in Fig. 3. For adsorbent C5, the capacity factor is larger for lysozyme, for adsorbent C2 the factors are nearly the same and for adsorbent C3 the capacity factor of aprotinin is a little bit larger than for lysozyme. Therefore, it can be assumed, that the capacity for the target product is smaller for adsorbent

Table 6
Comparison between the industrial separation problem and the test system

Characteristic	Separation problem	Test system
Protein		
p*I*	10.5	11.0
MM_r (kDa)	6.5	15
Feed		
Purity (%)	97	100
Concentration (g/l)	8.0	10.0
Buffer	90 mM phosphate	20 mM phosphate
pH	6.0	6.0

Table 7
Comparison between capacities for the target product protein and lysozyme

Resin	Lysozyme, 20 mM phosphate (10 g/l)	Lysozyme, 90 mM phosphate (8 g/l)		Product component, 90 mM phosphate (8 g/l)	
	Relative cap. (%)	Relative cap. (%)	± (%)	Relative cap. (%)	± (%)
C2	58	19	−68	20	+5
C5	39	23	−40	17	−25
C3	100	35	−65	37	+7

Relative capacities are based on the capacity of adsorbent C3 for lysozyme and 20 mM phosphate buffer.

C5, nearly constant for adsorbent C2 and a little bit higher for adsorbent C3. These assumptions were confirmed as it can be seen in the third column of Table 7. These results are based on a capacity comparison of lysozyme and the target product, both determined for 90 mM phosphate buffer. Additionally, dynamic capacities for the target product of all adsorbents are presented in Fig. 8.

By using the values of Table 7 a suitable load was defined. The same data could be defined, by using the values from Fig. 8, but the target product is generally not available in sufficient amounts for capacity determinations during early process development.

4.2.3. Results of preparative experiments

The load, which has been defined in the previous chapter, was lower than the load, which is normally used in the industrial process to be optimized. Therefore, it was first tested, if a better separation could be realized with adsorbent C1 at lower loading. Unfortunately the elution curves in Fig. 9 show, that the elution profiles of both proteins are still overlapping at the lower loading, which means a separation is not possible. Therefore, adsorbent C1 is not suitable for a separation even at lower loading.

There after, the other adsorbents were tested in preparative scale based on the ranking made in the previous chapter using the separation parameters (gradient, load, velocity) defined there. First, the adsorbent C6 was tested. The elution curves are presented in Fig. 10. In comparison to adsorbent C1, the retention times of both components were shifted to shorter times. The resolution between the compounds is a lot better,

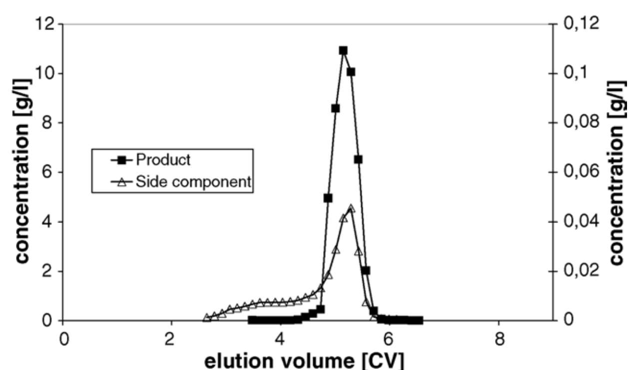

Fig. 9. Elution profiles of target product protein and side component for adsorbent C1 at medium loading. For desorption, a 9.2 CVs linear gradient from 0.2 to 0.65 M NaCl at pH 6.0 (no buffering) was used.

but for a nearly complete isolation of the side component, high yield losses would have to be accepted with this adsorbent. The separation on this adsorbent was not yet good enough, so that in the following experiments the next adsorbent in ranking (C3) was examined.

For the elution of proteins on this adsorbent, a linear salt gradient from 0.12 to 0.5 M NaCl was used. The result of the separation is demonstrated with the elution curves in Fig. 11. With this adsorbent, the side component could be separated nearly completely without yield losses of the target protein. Adsorbent C3 shows therefore the best option for the separation task. A small disadvantage is still, that the peaks elute over a broad salt range, so that the product concentration, which were observed for the two previously tested

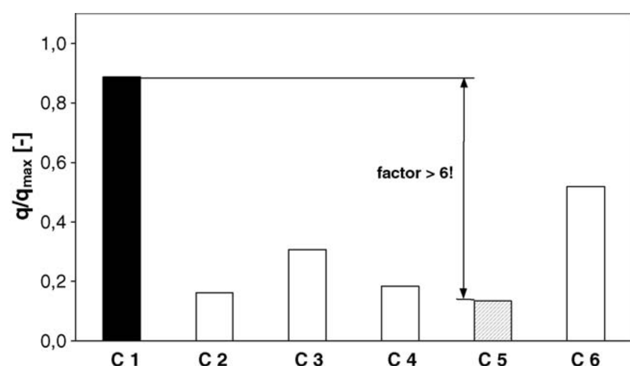

Fig. 8. Dynamic capacities for the target product component at 10% breakthrough for a velocity of 70 cm/h. Binding capacities were determined for a 8 g/l protein solution in a 90 mM phosphate buffer at a column height of 10 cm.

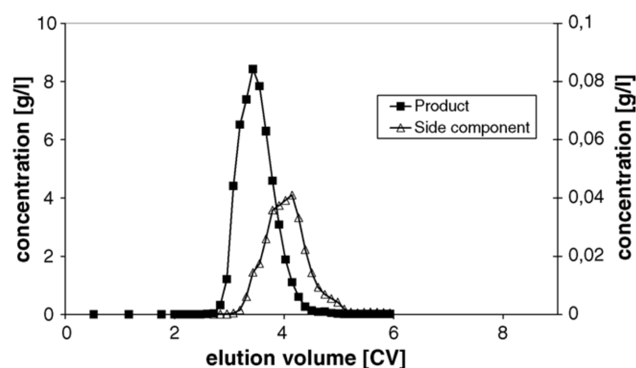

Fig. 10. Elution profiles of target product protein and side component for adsorbent C6. For desorption, a 9.2 CVs linear gradient from 0.2 to 0.65 M NaCl at pH 6.0 (no buffering) was used.

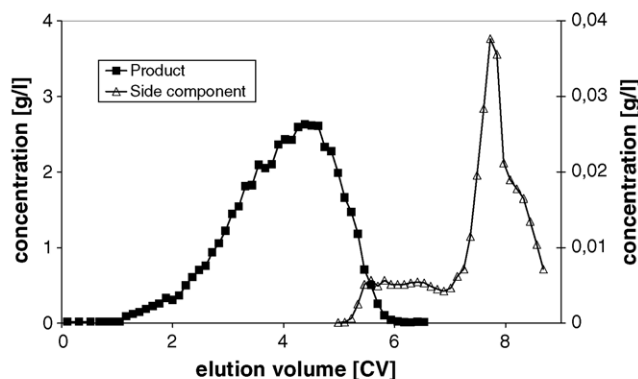

Fig. 11. Elution profiles of target product protein and side component for adsorbent C3. For desorption, a 9.2 CVs linear gradient from 0.12 to 0.5 M NaCl at pH 6.0 (no buffering) was used.

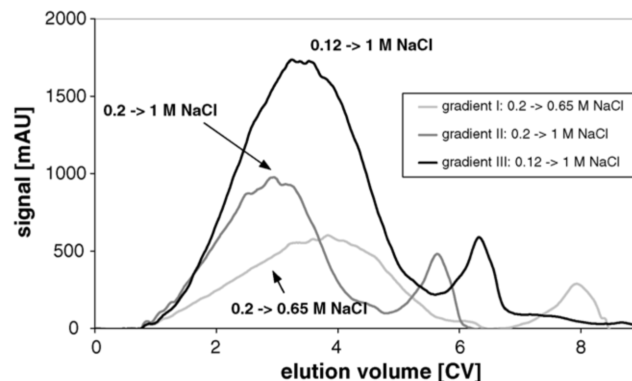

Fig. 12. Chromatograms (elution) of adsorbent C5 for three different gradients.

adsorbents could not be achieved. The average concentration with approximately 1.5 g/l is still sufficiently high.

For economical evaluation of the preparative experiments, yield losses have to be considered. Not only yield losses during elution due to an insufficient separation performance, but also during loading, washing and regeneration must be measured and calculated. In addition, the recoveries for all components have to be controlled to check the quality of the washing procedures, which are critical for the lifetime of the adsorbent. The different yield losses and recoveries for the single adsorbents are summarized in Table 8. Minimal yield losses of less than 2%, which are negligible, are found during the washing step of adsorbent C3. The recovery for all experiments is in the range between 83 and 90%. Since further preparative experiments had recoveries in this range, the difference to 100% stems from inaccuracies of the extinction coefficient and the calibration curve. Therefore, for all adsorbents a complete recovery was assumed.

By applying the new proposed methodology adsorbent C3 was identified as a suitable material with which the separation task could be optimized under consideration of economical aspects.

To confirm the quality of the ranking, additional preparative experiments with other adsorbents were carried out. This is not necessarily required, if it is the task of the study to only select a suitable and not the best adsorbent for the purification of the target protein mixture.

Another experiment with adsorbent C5 followed. For this adsorbent an individual gradient had to be optimized. First, a gradient from 0.2 to 0.65 M NaCl (gradient I) was used in the industrial process. Fig. 12 presents a chromatogram of the elution. It can be already concluded from the size of the peak areas, that under these conditions only a minor part of product will be desorbed during elution. The reasons for this effect are losses during washing as well as an incomplete desorption during elution. Therefore, in a second test the final concentration of the gradient (gradient II) was increased up to 1 M NaCl. The chromatogram for these conditions is also shown in Fig. 12. It can be directly seen, that the peak areas are much larger. The retention time of the peak maximum is shorter and a significant concentration increase has been achieved due to the larger slope of the gradient. A complete desorption was received in this experiment, but the yield losses of 21% during washing are still too high. The yield losses mainly occur during the washing step, in which the ionic strength is increased to the start concentration of the gradient. In a third preparative experiment another gradient (gradient III) was therefore applied, for which the salt concentration increased from 0.12 to 1 M NaCl. The chromatogram is shown in Fig. 12. The further increase of the peak area indicates smaller yield losses during the washing steps. Additionally a further elution concentration is also observed. Therefore, this run was fractionated and the fractions were analyzed. The elution curves of the two components are additionally illustrated in Fig. 13. A good separation between the two components can be seen.

Table 8
Yield losses and recoveries for the adsorbents C1, C3 and C6

Resin	Yield losses (%)			Recovery (%)	
	Load	Wash	Regeneration	Product	Side component
C1	<0.2	0.2	1	83.3	90.3
C6	<0.2	<0.2	1.2	87.2	90.3
C3	0.2	1.5	0.8	83.1	89.7

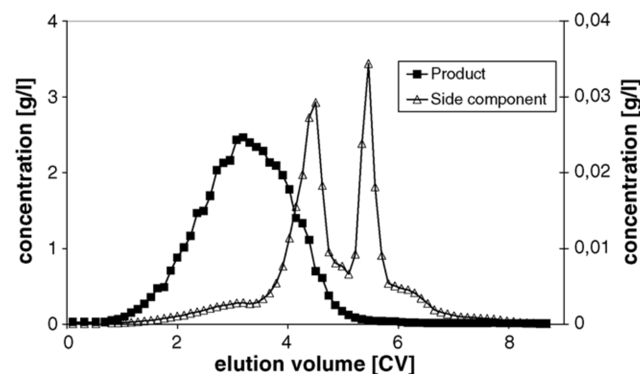

Fig. 13. Elution profiles of target product protein and side component for adsorbent C5. For desorption, a 9.2 CVs linear gradient from 0.12 to 1 M NaCl at pH 6.0 (no buffering) was used.

Table 9
Yield losses and recoveries for the adsorbents C2 and C5

Resin	Yield losses (%)			Recovery (%)	
	Load	Wash	Regeneration	Product	Side component
C5 (gradient III)	1.4	11.0	0.8	82	94
C2	61		–	–	–

The double peak indicates, that this adsorbent also separates the side component, which normally consists of two components. Unfortunately, the yield losses during washing are still too high with 11% (see Table 9). A further decrease of the start concentration was not possible due to given feed and loading conditions.

Due to these losses and, in comparison to adsorbent C3, a worse separation performance as well as higher dilution adsorbent C5 is less suitable for this process. Besides this the optimization potential is minimal. Due to the high slope of the HETP curve, an increase of velocity would quickly result in a reduced separation performance. Also the load, which is now 38% of the dynamic capacity, cannot be increased significantly. This relative high loading causes the yield losses during washing, although Fig. 7 shows very large capacity factors under these conditions. Due to the high relative load, the peak is deformed in such a way, that the beginning of the elution is shifted to far shorter times.

For further conformation of the adsorbent ranking another preparative experiment with adsorbent C2 was conducted. The gradient was started at 0.12 M NaCl, which is the lowest possible concentration due to boundary feed conditions. As expected from Fig. 7, this adsorbent is under the existing conditions not suitable for the process due to too high yield losses during the washing step (Table 9). For testing the separation performance of this adsorbent, the feed had to be diluted or even desalted. This would decrease the productivity of the whole downstream process due to longer loading times or due to the implementation of an additional desalting step. Testing of adsorbent C4 under preparative scale was not necessary, because this adsorbent has a comparable dynamic capacity and similar elution behavior as adsorbent C2 and is therefore not very suitable under the given conditions.

5. Conclusions

A new generic phase selection methodology for the downstream processing of biotechnological drugs was presented. With this methodology a suitable adsorbent with appropriate separation parameters for the purification of an industrial target protein mixture was identified. After characterization of the adsorbents with test substances and following ranking preparative experiments had to be carried out for the examined adsorbents. The comparison of the separation performance at preparative scale shows a good correlation for the previously achieved adsorbents ranking. Therefore, it is sufficient to carry out the expensive preparative experiments only with the two or three best-suited adsorbents. This drastic reduction of cost and efforts is the biggest advantage of the new methodology in comparison to the normally used empirical approach, even though at the beginning additional work for the determination of the adsorbent database has once to be invested. Furthermore, the preparative experiments in the presented example clearly showed the necessity to select an individual gradient for each adsorbent. The use of only one standard gradient for all adsorbents leads to sub-optimal results, as it was shown for adsorbent C5.

With new processes optimized by applying this methodology, the existing database will improve, therefore the quality of the ranking will advance and consequently the effort for phase selection will be further reduced. Furthermore, new adsorbents can be characterized by the standard procedure, thereafter classified in the database and then ranked for a specific separation problem. The applicability of this methodology is only limited, if no characteristic test substances can be found in the database, which match the target protein of the preparative separation task. Then adsorbents must be first characterized with new test substances to refine the database. After numerous applications of this methodology for different separation tasks this limitation will be minimized further.

In the chosen example a good correlation between the selectivity for the two test proteins at analytical scale and the selectivity for the two target proteins, which were similar in their properties at preparative scale was found. This confirms the assumption made in the ranking of the adsorbents.

6. Nomenclature

A, B	symmetry factors (determined at 10% height of peak maximum)
H, HETP	plate height (height equivalent of a theoretical plate)
k', k'_{El}	capacity factor
L_{column}	column length
M_r	molecular mass
N	plate number
p, Δp	pressure, pressure drop
q	capacity
t_R	retention time
u	velocity
V_0	death volume of column
V_R	retention volume
V_{column}	column volume
$w_{1/2}$	peak width at half height
Y	yield

Acknowledgements

This work was done during my dissertation at the university of Dortmund in cooperation with Bayer Technology Services, Leverkusen and Merck KGaA, Darmstadt.

References

[1] S.V. Galushko, J. Chromatogr. 552 (1991) 91.
[2] S.V. Galushko, Chromatographia 36 (1993) 39.
[3] S.V. Galushko, A.A. Kamenschuk, G.L. Pit, J. Chromatogr. A 660 (1994) 47.
[4] S.V. Galushko, A.A. Kamenschuk, G.L. Pit, Am. Lab. 3 (1995) 421.
[5] I. Molnar, J. Chromatogr. A 965 (2002) 175.
[6] C. Roussel, P. Piras, Pure Appl. Chem. 65 (1993) 235.
[7] E. Hallgren, F. Kalan, D. Farnan, C. Horvath, J. Stahlberg, J. Chromatogr. A 877 (2000) 13.
[8] C.M. Roth, K.K. Unger, A.M. Lenhoff, J. Chromatogr. A 726 (1996) 45.
[9] W. Kopaciewics, M.A. Rounds, J. Fausnaugh, F.E. Regnier, J. Chromatogr. A 266 (1983) 3.
[10] C.B. Mazza, N. Sukumar, C.M. Breneman, S.M. Cramer, Anal. Chem. 73 (2001) 5457.
[11] C.B. Mazza, C.E. Whitehead, C.M. Breneman, S.M. Cramer, Chromatographia 56 (2002) 147.
[12] D. Karlsson, N. Jakobsson, K.J. Brink, A. Axelsson, B. Nilsson, J. Chromatogr. A 1033 (2004) 71.
[13] Amersham Pharmacia Biotech, Ion Exchange Chromatography: Principles and Methods, Amersham Pharmacia Biotech, Uppsala, Schweden, 1999.
[14] M. Kastner, Protein Liquid Chromatography, Elsevier Science B.V., Amsterdam, 2000.
[15] E. Boschetti, J. Chromatogr. A 658 (1994) 207.
[16] A. Staby, M.-B. Sand, R.G. Hansen, J.H. Jacobsen, L.A. Andersen, M. Gerstenberg, U.K. Bruus, J. Chromatogr. A 1034 (2004) 85.
[17] P. DePhillips, A.M. Lenhoff, J. Chromatogr. A 883 (2000) 39.
[18] P. DePhillips, A.M. Lenhoff, J. Chromatogr. A 933 (2001) 57.
[19] P.R. Levison, C. Mumford, M. Streater, A. Brandt-Nielsen, N.D. Pathirana, S.E. Badger, J. Chromatogr. A 760 (1997) 151.
[20] D.C. Nash, H. Chase, J. Chromatogr. A 807 (1998) 185.
[21] C. Chang, A.M. Lenhoff, J. Chromatogr. A 827 (1998) 281.
[22] A.K. Hunter, G. Carta, J. Chromatogr. A 971 (2002) 105.
[23] G. Garke, R. Hartmann, N. Papamichael, W.-D. Decker, F.B. Anspach, Sep. Sci. Technol. 34 (1999) 2521.
[24] R.K. Scopes, Protein Purification: Principles and Practice, Springer-Verlag, Berlin, 1994.
[25] A. Staby, N. Johansen, H. Wahlstrom, I. Mollerup, J. Chromatogr. A 827 (1998) 311.
[26] A.K. Hunter, G. Carta, J. Chromatogr. A 930 (2001) 79.
[27] J.C. Bosma, J.A. Wesselingh, AIChE J. 44 (1998) 2399.
[28] M. Rendueles de la Vega, C. Chenou, J.M. Loureiro, A.E. Rodrigues, Biochem. Eng. J. 1 (1998) 11.
[29] J.J. Stickel, A. Fotopoulos, Biotechnol. Prog. 17 (2001) 744.
[30] S. Yamamoto, Biotechnol. Bioeng. 48 (1995) 444.
[31] J.P. Foley, J.G. Dorsey, Anal. Chem. 55 (1983) 730.