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Improved performance of protein separation by continuous annular chromatography in the size-exclusion mode

Günter Iberer[a,b], Horst Schwinn[a], Djuro Josić[a], Alois Jungbauer[b], Andrea Buchacher[a,*]

[a]*Octapharma Pharmazeutika Produktionsges mbH, Oberlaaerstrasse 235, A-1100 Vienna, Austria*

[b]*Institute of Applied Microbiology, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria*

Abstract

In size-exclusion chromatography (SEC), proteins and peptides are separated according to their molecular size in solution. SEC is especially useful as an effective fractionation step to separate a vast amount of impurities from the components of interest and/or as final step for the separation of purified proteins from their aggregates, in a so-called polishing step. However, the throughput in SEC is low compared to other chromatographic processes as good resolution can be achieved only with a limited feed volume (i.e., maximal approximately 5% of the column volume can be loaded). This limitation opposed widespread application of conventional SEC in industry despite its excellent separation potential. Therefore a continuous separation process (namely preparative continuous annular chromatography) was developed and compared to a conventional SEC system both using Superdex 200 prep grade as sorbent. An immunoglobulin G sample with a high content of aggregates was chosen as a model protein solution. The influence of the feed flow-rate, eluent flow-rate and rotation rate on the separation efficiency was investigated. The height equivalent to a theoretical plate was lower for preparative continuous annular chromatography which could be explained by reduced extra column band broadening. The packing quality was proved to be identical for both systems. The productivity of conventional batch SEC was lower compared to continuous SEC, consequently buffer consumption was higher in batch mode. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Continuous annular chromatography; Annular chromatography; Preparative chromatography; Size-exclusion chromatography; Height equivalent to a theoretical plate; Proteins; Immunoglobulins

1. Introduction

Size-exclusion chromatography (SEC) is one efficient method for the purification of proteins derived from human plasma [1]. SEC is primarily suited for the final phase in a separation process, the so-called polishing step [2]. This method allows the separation of biologicals according to their respective sizes. SEC provides an important information about intermolecular interactions and the physical state of proteins in solution. Therefore SEC is widely used for analytical purposes. Its suitability for preparative isolation of biopolymers is limited by the low volume capacity of the SEC columns. These limitations prevented wide application of SEC for industrial separation processes, despite its separation efficiency. To improve the productivity different continuous separation techniques such as moving bed systems, simulated moving bed (SMB) system [3], parallel operating systems and carrousel chromatography [4] and more recently annular chromatography

*Corresponding author. Tel.: +43-1-61032-241; fax: +43-1-61032-285.

E-mail address: andrea.buchacher@octapharma.at (A. Buchacher).

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were developed. The principle of continuous annular chromatography (CAC) has been known since the 1950s [5]. This technique allows separation in a really continuous mode. A further advantage is that CAC is suitable for all chromatographic techniques and resins which are already known for conventional batch chromatography. CAC has been successfully applied for the separation of metals [6], sugars [7], and amino acids [8]. In the field of protein purification several approaches using CAC were carried out [9–11]. Also for SEC some applications of preparative CAC (P-CAC) are described in the literature [12–15]. The system was originally developed at the Oak Ridge National Laboratory [16]. Further mechanical improvements led to the current system of the P-CAC, manufactured by Prior Separation Technology (Götzis, Austria). The sorbent is packed in the space between two concentric cylinders, the so-called annulus. The annulus is the rotating unit of the P-CAC system, the feed and eluent application and the collecting port are stationary. The eluent is uniformly distributed from the head of the column over the whole area, while the sample is applied onto the rotating sorbent at a fixed point by a feed nozzle. The different compounds of the sample move downwards forming helical bands. The shape of the moving zone depends on the velocity of the eluent, the rotating speed of the sorbent and the distribution coefficient of the component between fluid and adsorbent phases depending on the size of the components in SEC mode. At the bottom of the P-CAC system the different components are collected at different angle distances from the point of feed application.

The height equivalent to a theoretical plate (HETP) is considered to be one representative measurement for the performance of the separation [17]. It can be described by the fundamental relationship between the peak variance and the retention volume of the solute injected to a chromatographic column. In comparing HETP studies it is necessary to determine the influence of the extra column effects which contribute to peak broadening [18]. The influence of the extra column effects is much bigger for small than for large columns [19]. This is also important for the upscaling and downscaling of chromatographic processes. In annular chromatography it is difficult to determine these extra column effects, because it is not possible to run these systems without sorbent or in a bypass mode. Therefore annular columns were packed with different bed heights and the HETP was determined. By extrapolation to a bed length of zero the extra column effects were estimated.

Recently we showed that continuous separation of immunoglobulin G (IgG) polymers from monomers is possible by annular chromatography in the SEC mode [13]. In this paper we want to focus on the comparison of the batch system with a continuous one by calculating representative parameters as HETP and productivity. The HETP was determined in dependence of different flow-rates, column heights and applied sample volumes. In the particular separation problem a low HETP is important to efficiently separate polymers from dimers and monomers, because one quality criteria of intravenous IgG preparations is the polymer content. According to the requirements of the European Pharmacopoeia, the polymer content of IgG preparations for intravenous use must not exceed 3%. The goal is to keep the polymer content as low as possible while obtaining the highest possible throughput. The productivity of SEC is low compared to adsorption chromatography due to the low loadability of SEC gels. A continuous system should achieve a higher productivity. Therefore the productivity of both systems was calculated and compared.

We used a batch of IgG containing artificially high amounts of polymers as model protein and Superdex 200 as sorbent packed in batch and P-CAC columns.

2. Theory

SEC experiments were performed using different flow-rates, sample volumes and different column bed heights to compare both techniques with regard to the extra column effects, the packing and the separation efficiency. The abscissa of chromatograms obtained by P-CAC were transformed from elution angle to elution volume using Eq. (1):

$$V_E = \frac{\alpha}{\omega} \cdot F \quad (1)$$

where V_E is the elution volume, α is the elution angle, ω is the rotation rate and F is the total

volumetric flow-rate. To apply the same amount of sample volume per sorbent volume a dimensionless sample volume (V'_F) was calculated for batch columns in Eq. (2) and for P-CAC in Eq. (3):

$$V'_F = \frac{V_F}{V_t} \tag{2}$$

$$V'_F = \frac{\frac{360}{\omega} \cdot 60 \times F_F}{V_t} \tag{3}$$

In Eqs. (2) and (3) V_F is the sample feed volume, V_t is the volume of the packed bed and F_F is the feed flow-rate. The chromatograms were fitted with the exponentially modified Gauss (EMG), function which is a chromatography model describing tailed peaks [20]. The different peak moments were calculated using the EMG function as fitting function. Eq. (4) represents the general formula for the calculation of the peak moments:

$$M_n = \frac{\int_0^\infty t^n c(t)\mathrm{d}t}{\int_0^\infty c(t)\mathrm{d}t} \tag{4}$$

where M_n is the nth statistical moment and $c(t)$ is the concentration of the solute at time t at the detector. The first peak moment (M_1) is the retention time measured at the centre of the gravity. It is different from the retention time when the peak is unsymmetrical. The second peak moment (M_2) is equivalent to the variance of the peak profile, which is the sum of the variances contributed by the different parts of the instrument system. The first peak moment was plotted against the volume of the packed bed (V_t) [19]. At the intercept of the regression with the ordinate of this plot the extra column volume (V_{ex}) can be seen. The slope of the regression of this plot gives a hint about the void volume. The good agreement of the regression line with the experimental data indicates a high reproducibility in column packing.

The number of the theoretical plates is a measure for column efficiency. The number of plates (N) can be calculated with Eq. (5):

$$N = \frac{M_1^2}{M_2} \tag{5}$$

The HETP is than found by using Eq. (6):

$$\mathrm{HETP} = \frac{L}{N} \tag{6}$$

where L is the column length. The resolution was calculated using Eq. (7):

$$R = \frac{2 \cdot (M_{1,2} - M_{1,1})}{(W_{B1} + W_{B2})} \tag{7}$$

R is the resolution, $M_{1,2}$ is the first peak moment of the second peak, $M_{1,1}$ is the first peak moment of the first peak, W_{B1} is the base width of the first peak and W_{B2} is the base width of the second peak.

A calculation for the productivity (P) for batch chromatography was developed by Yamamoto et al. [21]. Buchacher et al. [13] showed that this equation is also valid for annular chromatography. The productivity (P) is defined as:

$$P = \frac{Q_R C_0 V_F}{V_t t_c} \tag{8}$$

where Q_R is the recovery rate, V_t the volume of the packed bed and t_c the cycle time. The recovery rate (Q_R) was defined as throughput of protein at a certain purity, V_F the sample feed volume, C_0 the initial concentration of protein, and m_c the amount of pure protein:

$$Q_R = \frac{m_c}{V_F C_0} \tag{9}$$

The cycle time t_c was defined as the time required to perform one cycle including equilibration, but excluding the time for regeneration.

For comparison of productivity between batch and continuous operation V_t was kept equal for P-CAC and batch columns and also the amount of sample per sorbent volume was equal (Table 1). The process time in batch chromatography is calculated as:

$$t_{c,1} = \frac{V_E + V_F}{F} \tag{10}$$

with V_E as eluent volume. The process time for n cycles is calculated as:

$$t_{c,n} = t_{c,1} n \tag{11}$$

Table 1
Data for the calculation of the productivity and buffer consumption for batch column and P-CAC

Parameter	Batch system	P-CAC
Recovery rate	0.85	0.85
Column volume (l)	2.7	2.7
Column diameter (cm)	7.5	Does not apply
Annulus inner diameter (cm)	Does not apply	13
Annulus outer diameter (cm)	Does not apply	15
Height (cm)	61.5	61.5
Total flow-rate (ml/min)	44	44
Linear velocity (cm/h)	60	60
Rotation rate (degrees/h)	Does not apply	1100
Sample volume (% V_t)	3	3
Feed flow-rate (ml/min)	Does not apply	4.12
Eluent flow-rate (ml/min)	Does not apply	39.9
Feed concentration (mg/ml)	2	2
Time for the first cycle (min)	45	60
Time for n cycles (min)	$45\times n$	$60+[(19.7\times(n-1)]$

The process time in batch chromatography does not change from cycle to cycle. The equilibration, application and elution of the sample takes always the same time. Therefore the process time increases from cycle to cycle with the same time span, the summing up of all cycle times being linear.

The process time for the first cycle in annular chromatography ($t'_{c,1}$) takes longer that the following cycles since only in the first cycle the time for equilibration and elution of the sample is included. From that time the sample is applied and eluted continuously. That means that for the further cycles only the time for application of the sample counts because the elution is performed simultaneously. Therefore the sum of the process times increases hyperbolically.

The process time was calculated from the elution volume of solute including the time for the sample application using Eq. (12):

$$t'_{c,1} = \frac{V_E}{F} + \frac{V_F}{F_F} \tag{12}$$

Calculating the process time for n cycles ($t'_{c,n}$) following equation is obtained:

$$t'_{c,n} = t'_{c,1} + \frac{V_F}{F_F} \cdot (n-1) \tag{13}$$

The buffer consumption (V_B) in batch mode is calculated as:

$$V_B = (t_{c,n} F_e) - V_F \tag{14}$$

In annular chromatography the buffer consumption (V'_B) is:

$$V'_B = t'_{c,n} F_e \tag{15}$$

3. Materials and methods

3.1. Proteins and chemicals

An intravenous immunoglobulin (IVIG) concentrate produced in the Research and Development (R&D) Laboratory of Octapharma Pharmazeutika was used as feed. This sample was purposely produced for R&D investigations and not for therapeutic use, denoted as laboratory IgG concentrate. The concentrate exhibited a high polymer content in order to investigate the full potential of the SEC separation. It was diluted from 50 to 2 mg/ml with the eluent buffer in all experiments. Prior to chromatography, the solution was filtered through a 0.2-μm low protein-binding filter membrane (Nalgene, Rochester, NY, USA). All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). The columns were packed with Superdex 200 prep grade, purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) using packing equipment.

3.2. Size-exclusion chromatography in the batch mode

An Äkta system (Amersham Pharmacia Biotech) was used as chromatography system for batch experiments. The XK 16 glass columns (Amersham Pharmacia Biotech) were packed with Superdex 200 prep grade with different bed heights: 21.0, 41.0 and 61.5 cm, respectively. Batch experiments were performed at different flow-rates (15, 30, 45, 60, 75 cm/h) and sample volumes (0.4, 1, 3, 5% V_t). As eluent buffer 40 m*M* Na_2HPO_4/NaH_2PO_4, containing 0.2 *M* NaCl was used. For the calculation of the retention time, the peaks were approximated by an EMG function and the first and second peak moments were determined using the computer software Peak Fit (SPSS, Chicago, IL, USA).

3.3. Size-exclusion chromatography in preparative continuous annular chromatography

The P-CAC unit was constructed by Prior Separation Technology. A schematic drawing of the apparatus used for the continuous separation has been described by Buchacher et al. [13] and is shown in Fig. 1. The P-CAC system consists of two concentric cylinders forming an annulus into which the stationary phase is packed. The outer cylinder had a diameter of 15 cm and the inner one a diameter of 13 cm, resulting in an annulus width of 1 cm. The upper part of the outer cylinder is constructed of glass and the lower part of polypropylene. The inner cylinder is made of polypropylene and is shorter than the outer one leaving a head space at the top. Both cylinders are closed by a head made of polyether ether ketone (PEEK) through which the eluent and feed streams are inserted. The feed stream is applied at the top of the gel bed through a fixed feed nozzle, whose tip is covered with a layer of glass beads to avoid fluid mixing. At the bottom of the unit the two cylinders are attached to a stainless steel plate which contains 90 exit holes covered by a nylon filter (11 μm). The bottom of the rotating column is connected to a fixed PTFE slipring which also contains 90 exit ports connected to a short section of Tygon tubing (Norton Performance Plastic, Akron, OH, USA). The exit ports are evenly distributed at 90 intervals along the annulus. The Tygon tubes are fixed in a special

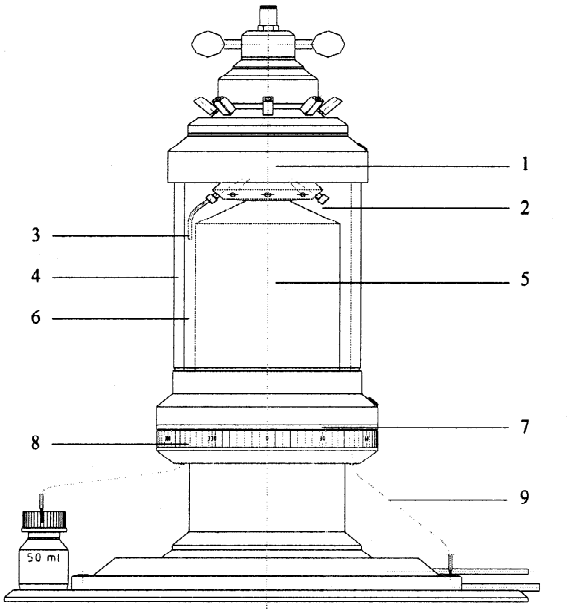

Fig. 1. Schematic drawing of the annular chromatograph (obtained from Prior Separations). 1: Head, 2: head space, 3: feed nozzle, 4: outer cylinder, 5: inner cylinder, 6: annulus, 7: stainless steel plate (with 90 exit holes), 8: fixed PTFE slipring (with 90 exit ports), 9: Tygon tubing.

mounting plate. The column was packed with Superdex 200 prep grade at the same bed heights as the batch columns: 21.0, 41.0 and 61.5 cm column bed height. These dimensions resulted in bed volumes of approximately 0.9, 1.8 and 2.7 l gel. The height of the upper layer consisting of glass beads was 2.6 cm.

As an eluent buffer, 40 m*M* Na_2HPO_4/NaH_2PO_4, containing 0.2 *M* NaCl, pH 6.0 was used. The eluent was delivered to the column by a P-6000 pump and the feed by a P-500 pump (both from Amersham Pharmacia Biotech).

P-CAC experiments were performed with different flow-rates (15, 30, 45, 60, 75 cm/h) and sample volumes (0.4, 1, 3, 5% V_t). After each run the gel was regenerated using 1 *M* NaCl, 50% acetic acid and 1 *M* NaOH, each solution applied for 1 h by the feed nozzle with a flow-rate of 2.5 ml/min and a rotation rate of 600°/h.

3.4. Absorbance measurements for the fractions from P-CAC

The effluent stream from the bottom of the

annulus was collected through 90 outlets. One effluent tube corresponds to 4° of the circumference. Samples were transferred to UV transmissible microtiterplates (Costar, Corning, NY, USA) and absorbance was measured at 280 nm with a spectrophotometer from Molecular Devices (Sunnyvale, CA, USA). The UV data were transferred to an MS Excel spreadsheet (Microsoft, Redmond, WA, USA) or a Sigma Plot (SPSS) and then the elution profile was constructed.

4. Results and discussion

In the process industry there is a need for effective methods for separation of biologicals which preserve the integrity of the molecules. SEC is one efficient method that allows the separation of biologicals under mild conditions according to their sizes. However, its productivity is low compared to adsorption chromatography since the sample volume is normally limited to a maximum of 5% of the column volume. The productivity of a real continuous system should be higher than that of a batch system. Systematic studies on the performance and productivity of annular chromatography are not available. The modelling of an annular chromatograph has been reported by Byers et al. [22]. Sisson et al. [12] demonstrated that the plate theory, as adapted to CAC, is well suited to predict experimental results.

Here we tried to compare the performance of size-exclusion chromatography performed in batch chromatography and annular chromatography. Experiments under different chromatographic conditions were carried out to study the different effects of the flow-rate and sample volume on the separation of IgG. The IgG concentrate was diluted 1:25 with elution buffer. Sample dilution was necessary to operate in the linear range of the UV detector of the chromatography system. The P-CAC rotation rate was optimized in such a way that the beginning and the end of the elution profile of IgG did not overlap. The whole 360° were used for the separation in order to obtain the highest possible sorbent utilization. For each flow-rate the optimization of the rotation rate was performed with the largest sample volume (5% V_t). The highest possible rotation rate was selected to maximize the zone width. In this case the accuracy of the collection of the different fractions at the bottom of the P-CAC system could be improved. The rotation rate did not influence the calculation of the HETP and extra column effects, because the elution angle was transformed to the elution volume using Eq. (1).

Typical elution profiles of IgG are shown from batch columns in Fig. 2A and from P-CAC in Fig. 2B. The IgG sample was separated in a polymer, dimer and monomer fraction. The shape of the peaks eluted from P-CAC are not that sharp as from batch columns since the recording of the absorbance is not automatically. As described in the Material and methods section the effluent from all 90 outlets is collected when the system has reached steady state

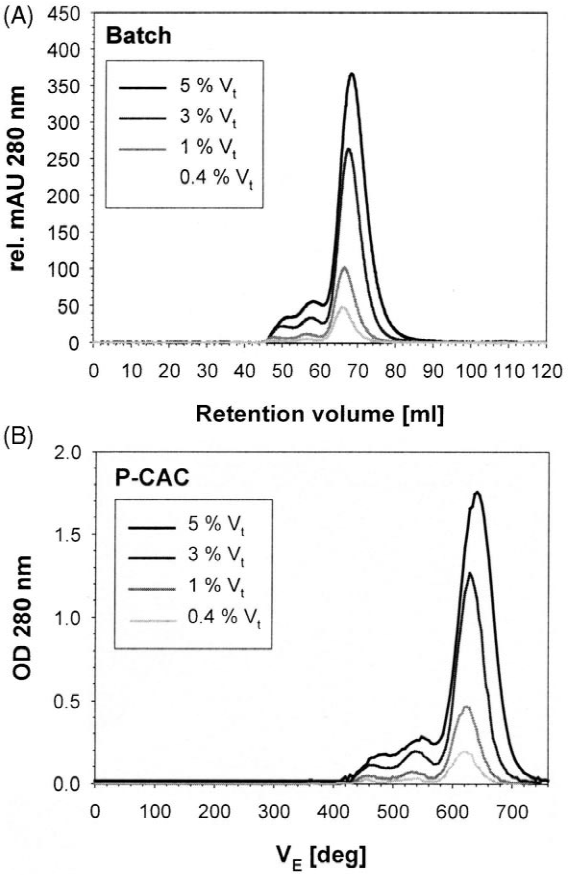

Fig. 2. Chromatograms of IgG samples obtained from batch column (A) and P-CAC (B) at different sample volumes. Bed length was 61.5 cm, linear velocity 45 cm/h and for P-CAC rotation rate was 826°/h.

conditions. The manual testing of the optical density by a spectrophotometer gave less smooth elution profiles. For relative feed corresponding up to 1% of the total column volume (V_t) baseline separation was achieved. The elution profile of IgG using P-CAC shows that for the IgG components two rotations are necessary to migrate from the feed point to the bottom of the column. Therefore the scale of the abscissa was extended to 720° (Fig. 2B) which is equivalent to two rotations. Application of such a high rotation rate to achieve a good separation, made it impossible to introduce a regeneration step simultaneously. This would lead to an overlapping zone of the regenerating solvent with the IgG fractions and consequently protein precipitation and clogging of the sorbent would occur.

To examine the quality of packing, columns with different column heights were packed under identical conditions and then pulse response experiments were performed. The first peak moment was used to determine V_E. Plots of V_E versus V_t are a simple measure for the quality of the packing of different columns according to Kaltenbrunner et al. [19]. These plots for batch columns and P-CAC and are shown in Fig. 3A and B. A linear relationship of V_E with V_t was observed, indicating a high reproducibility of packing. The slope of the regression line equals the void fraction and the intercept of the regression line with the ordinate represents the extra column volume. The slope of the regression line from the P-CAC and the batch columns are almost identical. Therefore we concluded that the packing quality of the P-CAC and the batch columns were the same. The extra column volume of the batch system was approximately 0.4 ml which is very small and was in good agreement with the dead volumes of the pipes and valves of the chromatography system. On the P-CAC system the extra column volume is negligibly small.

The HETP was calculated for P-CAC and batch columns according to Eq. (6) from the Theory section. The effect of the flow-rate and the sample volume on the HETP was studied by experiments performed with different sample volumes and flow-rates. In Fig. 4A–C the HETP was plotted against the flow-rate using different sample volumes and also different column heights. The HETP is lower for P-CAC than for batch columns. We explained this by

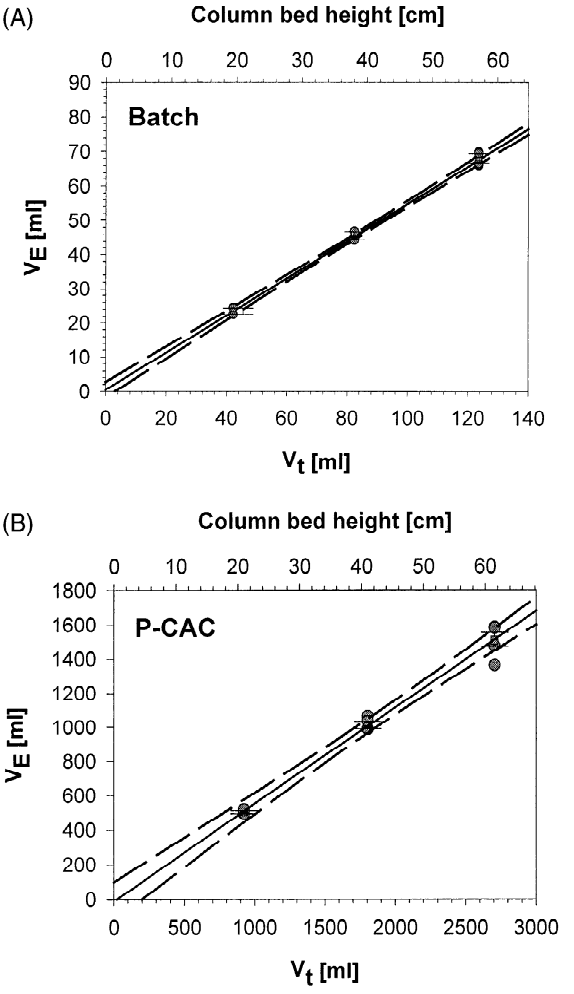

Fig. 3. Plot of the elution volume (V_E) against the total volume of the packed bed (V_t) of the batch columns (A) and of P-CAC (B). The full line represents the regression and the dashed lines indicates the confidence interval (99%).

reduced extra column band spreading of the P-CAC system. The top part is constructed in a way that the feed is directly introduced onto the sorbent. Dilution of the sample by a hold-up volume does not take place. At the bottom the solute is directly collected without passing a space, where it can be retained. In batch chromatography two times, at the bottom and at the top of the column, the zones are spread in the column adaptors. Such a wash out follows an exponential characteristic described by Sternberg et al. [23].

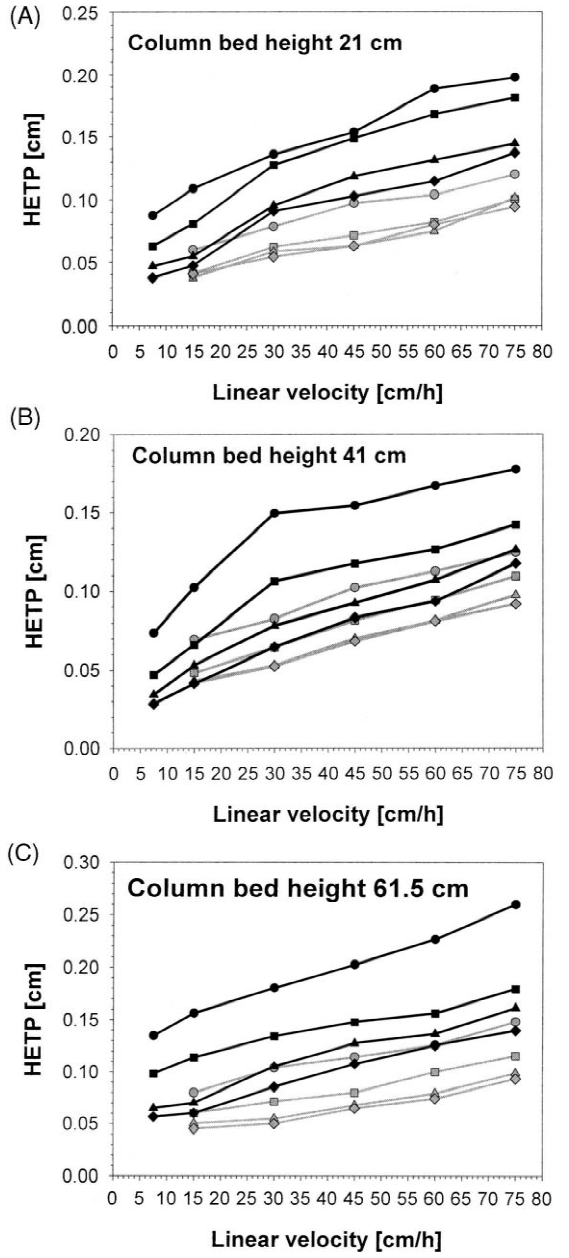

Fig. 4. Performance in batch chromatography and P-CAC; column height 21 cm (A), column height 41 cm (B) and column height 61.5 cm (C). The black lines represent the batch column and the grey lines represent the P-CAC. Sample volume: 5% V_t (●), 3% V_t (■), 1% V_t (▲), 0.4% V_t (♦).

To get a parameter for the selectivity of SEC, the resolution was calculated according to Eq. (7). The resolution describes the separation of two different components. In SEC chromatography the resolution decreases with increasing sample volume and also with increasing flow-rate [24]. In annular chromatography a further parameter, the rotation rate, influences the separation behaviour. We determined the resolution between the dimer and the monomer of IgG at different rotation rates and a constant dimensionless sample volume. The resolution is independent of the rotation rate (Fig. 5). The elution angles of the peaks (Fig. 6), the differences in retention times of the two components and the peak width (Fig. 7) are increasing linearly with increasing rotation rate, resulting in a constant resolution (Fig. 5). Below a rotation rate of 200°/h it was impossible to determine the resolution. This is due to the technical construction of the P-CAC system. At a rotation rate below 200°/h the whole sample elutes within 50°. Since there is an outlet only every 4° it is impossible to construct an accurate elution profile and therefore the resolution could not be calculated.

Productivity and buffer consumption are very important parameters for optimized preparative chromatographic processes. The productivity of P-CAC and batch columns were calculated as described in the Theory section. In Table 1, the values used for calculation are shown. The recovery rate was 0.85

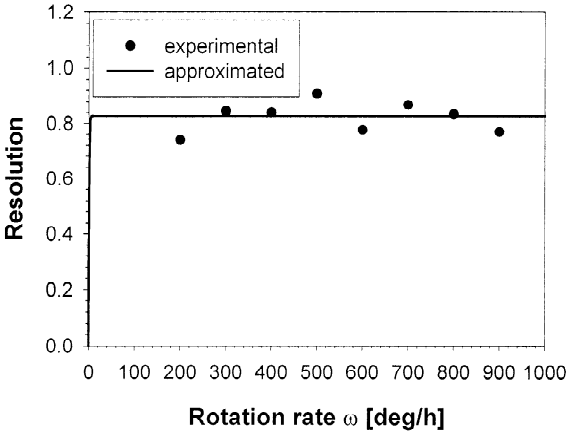

Fig. 5. Influence of the rotation rate (ω) on the resolution of P-CAC. The closed symbols (●) represent the experimental datapoints and the black line represents the approximated resolution according to the experimental datapoints.

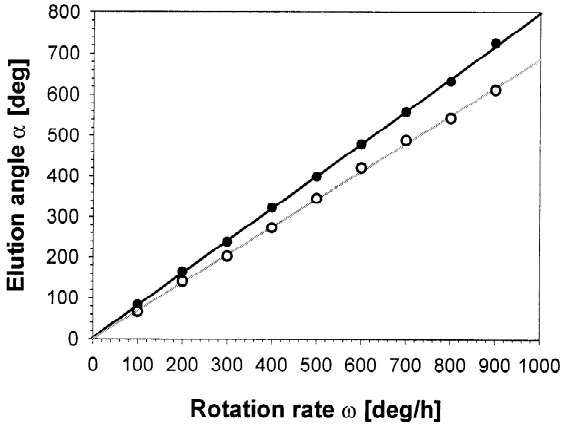

Fig. 6. Influence of the rotation rate (ω) on the elution angle (α) of the monomer and dimer peaks. The closed (●) and open (○) symbols represent the experimental data of the monomer and dimer peaks, respectively, and the full black and grey line represent the regressed data.

calculated according to Eq. (9). The column volume, the bed length and the separation conditions were the same for both systems. For the calculations a diameter of 7.5 cm was assumed for the batch column to achieve the same sorbent volume and bed height as for the P-CAC system. Fig. 8 shows the productivity and buffer consumption of the P-CAC system compared to batch mode. The time for regeneration was not included in the calculation of productivity. For the batch system the productivity remains constant

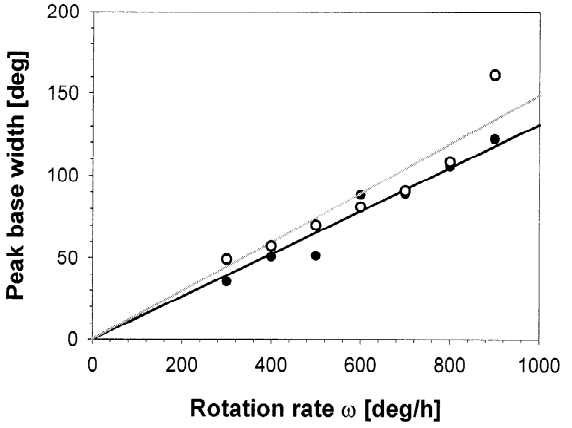

Fig. 7. Influence of rotation rate (ω) on the base widths of the peaks. Closed (●) and open (○) symbols represent the experimental data of the monomer peak and dimer peak respectively, the full black and grey line represents the regressed data.

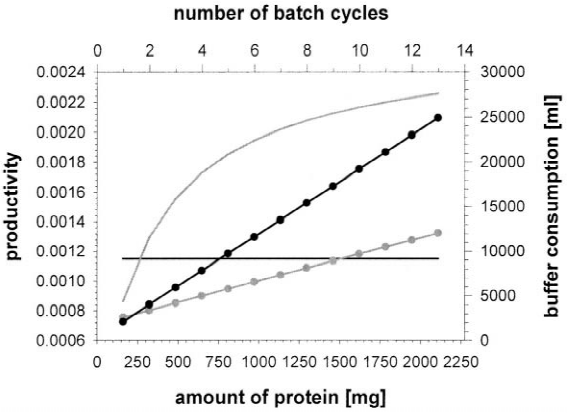

Fig. 8. Comparison of productivity in batch mode and continuous mode. The black line represents the productivity of the batch column and the grey one the P-CAC. The grey line with the grey symbols shows the buffer consumption of the P-CAC and the black one the batch column.

whereas for P-CAC productivity increases from cycle to cycle. As a result the buffer consumption of P-CAC is lower than the batch column. Only for the first cycle the productivity of the batch column is higher than the P-CAC. This can be explained by the time which is necessary to apply the same amount of feed on the P-CAC system.

5. Conclusion

We demonstrated that the performance of continuous operation in the size-exclusion mode in a P-CAC system measured as the HETP is superior compared to the batch mode. The packing quality can be definitely excluded as a reason for the better performance, since in both operation modes the same bed voidage was obtained. The better performance is explained by the reduced extra column effects.

6. Nomenclature

C_0	Sample concentration (mg/ml)
$c(t)$	Concentration of the solute at time t at the detector
F	Total volumetric flow-rate (ml/min)
F_e	Eluent flow-rate (ml/min)
F_F	Feed flow-rate (ml/min)

L	Column length (cm)
m_c	Amount of pure protein (mg)
M_n	n^{th} statistical moment
$M_{1,1}$	First peak moment first peak
$M_{1,2}$	First peak moment second peak
M_2	Second peak moment
N	Number of theoretical plates
P	Productivity
Q_R	Recovery rate
R	Resolution
t_c	Cycle time (min)
$t_{c,1}$	Time for the first cycle of batch column (min)
$t_{c,n}$	Time for n cycles of batch column (min)
$t'_{c,1}$	Time for the first cycle of P-CAC (min)
$t'_{c,n}$	Time for n cycles of P-CAC (min)
V_E	Elution volume (ml)
V_{ex}	Extra column volume (ml)
V_F	Sample volume (ml)
V'_F	Dimensionless sample volume
V_t	Column volume (ml)
W_{B1}	Base width of the first peak
W_{B2}	Base width of the second peak

Greek symbols

α	Elution angle (degrees)
ω	Rotation rate (degrees/h)

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