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Continuous purification of a clotting factor IX concentrate and continuous regeneration by preparative annular chromatography

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Abstract

Preparative continuous annular chromatography, a method to separate proteins in a truly continuous manner, was investigated in an industrial environment. Plasma-derived clotting factor IX concentrate was used as model protein. Separation of vitronectin, a common impurity in commercial available factor IX concentrates, from factor IX was studied and compared to conventional packed bed chromatography in batch mode. As sorbent, Toyopearl DEAE 650M was used. Regeneration was performed simultaneously with the purification of factor IX in continuous mode. All required parameters applied for preparative annular chromatography such as feed flow-rate and elution flow-rate were first estimated from experiments on conventional batch columns. Then preparative annular chromatography and conventional packed beds were compared regarding enrichment, purity and productivity. Three different process scenarios, the optimal batch process, the preparative annular chromatography process and the batch process equivalent to the preparative annular chromatography process were investigated. The productivity of the optimal batch process was higher than that of the preparative annular chromatography and batch process equivalent to the preparative annular chromatography process. Therefore the throughput could not be increased by the use of the continuous chromatographic system.

Keywords: Continuous annular chromatography; Preparative chromatography; Factor IX; Proteins; Vitronectin

1. Introduction

Liquid chromatography is one of the most important techniques for the purification of proteins intended for therapeutic use. Often only with a chromatographic separation process can the required product purity be achieved. Many efforts have been made to increase the throughput of chromatographic separation processes. The most common chromatographic processes are scaled up by increasing the column diameter, once optimal column height has been estimated. Also repetitive or cycling operation on large diameter columns are used to increase the throughput [1]. A completely different way is obtained by true continuous operations. This is advantageous especially when other continuous steps are coupled to the downstream processing. There are two main principles to perform a chromatographic separation in a continuous mode. The first is the simulating moving bed (SMB) process [2] which is already known since the 1960s and applied in large scale purification processes [3]. The second one is annular chromatography which was conceptualised by Martin [4] in 1949. In principle annular chromatography can be imagined as a circular array of an infinite number

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of chromatography tubes which are rotating around a fixed point. Hypothetical infinite small chromatography columns are operated in sequence. Preparative continuous annular chromatography (P-CAC) has been used for size-exclusion [5,6], ion-exchange [7–11], displacement [12] and affinity chromatography [13]. Industrial large scale applications of P-CAC have not been reported. Continuous concentration of recombinant proteins from perfusion cell culture have been described by Vogel et al. using an autoclavable P-CAC prototype [14].

It is easier to transfer an existing separation performed in batch wise operation to continuous mode using P-CAC. Only the transformation of the time axis into an angular axis as described by Wankat [15] is required and no further optimisation to find the optimal operation parameters is necessary. In contrast to SMB the separation of multicomponent feedstocks is possible with P-CAC [16]. Also the necessary equipment and the control system for the P-CAC is not as complicated as for SMB systems. By using the P-CAC only the annular chromatograph and the pumps have to be controlled whereas the operation of an SMB process requires the shifting of more than one column connected to each other by a great number of valves. In addition, in operating SMB more process parameters than in conventional chromatography have to be optimized.

The separation of human factor IX concentrate was investigated and productivity was compared to conventional packed bed chromatography. Human clotting factor IX is one of the proteins in the clotting cascade which plays an important role for hemostasis. It is a vitamin K-dependent multidomain glycoprotein synthesized in the liver. Normally the factor IX concentration in the human plasma is about 5 μg/ml. In patients suffering from haemophilia B, active factor IX is missing [17,18] and has to be substituted by infusion. Factor IX has a theoretical molecular mass of 46,548.2. The apparent molecular mass is higher due to posttranslational modifications such as γ-carboxylation [19], glycosylation [20,21] and phosphorylation [22]. From sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), size exclusion chromatography and sedimentation equilibrium analysis, an apparent molecular mass between 55,000 and 71,000 was estimated [23–26]. The isoelectric point of the isoforms range from 4.0 to 4.6 [27–33]. Due to the low abundance of factor IX in plasma and the presence of a large number of other proteins in plasma, appropriate purity is only achieved by a process consisting of multiple purification steps. All industrial high-purity plasma-derived factor IX concentrates are obtained by chromatographic purification from cryo-poor plasma. In most cases, production processes involve the use of the following affinity ligands: heparin, murine anti-human factor IX antibodies, metal chelate or sulfated dextran [34]. Processes usually involve capture of the factor IX complex (containing factors II, VII, X, IX, protein C, proteins S and other proteins from cryo-poor plasma) on DEAE-Sephadex A-50 or DEAE-cellulose [35]. The DEAE-Sephadex eluate is then further purified on DEAE and an immobilized-heparin column [36,37]. Despite the high level of purification of currently available products, vitronectin has been identified as an accompanying protein in several preparations of factor IX from human plasma. It is a glycoprotein with a molecular mass of ~78,000 and with an isoelectric point in the range of 4.75–5.25. The concentration of vitronectin in human plasma is between 0.2 and 0.4 mg/ml and 2–8 μg/IU factor IX in the final preparations [38]. Vitronectin can be separated from factor IX concentrates by size exclusion chromatography [39] or by anion-exchange chromatography on CIM (Convective Interaction Media) monolithic columns [40].

In this paper the separation of human clotting factor IX from vitronectin on anion-exchange resin Toyopearl DEAE 650M was investigated. The process was optimised on a batch column and then transformed to the P-CAC. Due to the arrangement of the feed nozzles in the head of the P-CAC system, a direct transformation was impossible and a new process was designed to meet the requirements of the construction of the P-CAC system. Therefore also the transformation back from the P-CAC to the batchwise operation was necessary to obtain comparable operating conditions. Thus three different scenarios for this separation were investigated: the optimal batch process, the P-CAC operation and the batch process equivalent to the P-CAC process.

2. Theory

In order to keep the contact time of the feed and

the other process solutions with the sorbent constant, a transformation from the time-dependent process cycle of a conventional batch column to the angular-dependent process cycle of the P-CAC as described by Wankat [15] was applied:

$$t = \frac{\theta}{\omega} \tag{1}$$

In Eq. (1) t is the time, θ is the angular position and ω is the rotation rate. The optimised batch process (Fig. 1A) was transformed with Eq. (1) to the P-CAC (Fig. 1B). In order to apply the same volumes of feed, elution buffers and regeneration solutions (NaOH and Tris–HCl buffer), per volume sorbent Eq. (2) for batch columns and Eq. (3) for P-CAC were used, leading to a dimensionless feed volume (V'_F) for both systems:

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

in P-CAC V'_F is calculated as:

$$V'_F = \frac{\frac{360}{\omega} \times 60 \times F_1}{V_t} \tag{3}$$

In Eqs. (2) and (3) V_F is the feed volume, V_t is the volume of the packed bed and F_1 is the overall feed flow-rate. Eqs. (2) and (3) can be also applied to the other solutions, which are pumped through the inlet nozzles of the P-CAC, such as the elution buffers and the regeneration solutions. In Eq. (2) only V_F must be replaced by the volume of the elution buffers (V_{E1}, V_{E2}), or the volume of the regeneration solutions (V_{Tris}, V_{NaOH}). In Eq. (3) F_1 has to be changed to the flow-rate of the elution buffers (F_2, F_3), or the flow-rate of the regeneration solutions (F_4, F_5). Using this procedure all flow-rates of solutions applied through feed nozzles to the P-CAC such as the feed and the different elution buffers etc., are determined.

For the calculation of the linear superficial velocity for the batch column (u_{Batch}) Eq. (4) was used:

$$u_{Batch} = \frac{60 F_{Batch}}{A_{Batch}} \tag{4}$$

In Eq. (4) F_{Batch} is the flow-rate and A_{Batch} is the superficial area of the batch column. The total flow-rate of all buffers and liquids delivered through the inlet nozzles is termed as $\Sigma_{i=1}^{n} F_i$. $\Sigma_{i=1}^{n} F_i$ is the sum of all flow-rates of all solutions which were applied through the inlet nozzles to the P-CAC, F_1 is the flow-rate of feed solution, F_2 elution buffer 1, F_3 elution buffer 2, F_4 regeneration solution 1 and F_5 regeneration solution 2.

The eluent flow-rate for P-CAC to obtain the same linear velocity as on the batch column is given by Eq. (5):

$$F_e = \frac{u_{Batch} A_{P\text{-}CAC}}{60} - \left(\sum_{i=1}^{n} F_i \right) \tag{5}$$

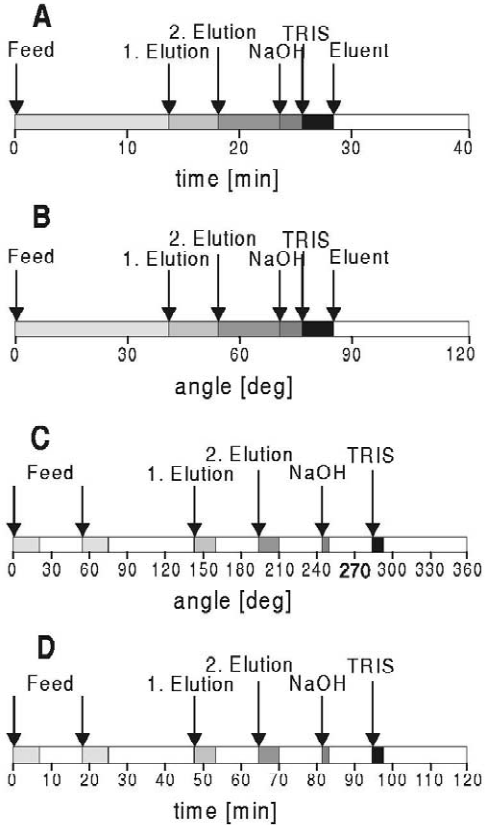

Fig. 1. Process cycles for the different scenarios: (A) optimized batch process, (B) transformation of the optimized batch process to the theoretical P-CAC process, (C) real P-CAC process, (D) batch process equivalent to the P-CAC process. Bars represent the feed solution, represents the first elution buffer, represents the second elution buffer, represents the first regeneration solution, represents the second regeneration solution and the represents the eluent.

where $A_{\text{P-CAC}}$ is the superficial area of the P-CAC and $\Sigma_{i=1}^{n} F_i$ is the sum of all flow-rates from all solutions which were applied through feed nozzles to the sorbent in the P-CAC. Then the linear superficial velocity in the P-CAC ($u_{\text{P-CAC}}$) is obtained by rearrangement of Eq. (5) and the linear superficial velocity of the P-CAC is identical to the linear superficial velocity of the batch chromatography:

$$u_{\text{P-CAC}} = \frac{60 F_{\text{P-CAC}}}{A_{\text{P-CAC}}} \tag{6}$$

with $F_{\text{P-CAC}}$ the total flow-rate of the P-CAC which is given by the sum of F_e and $\Sigma_{i=1}^{n} F_i$.

In order to transform identical P-CAC conditions to batch conditions we use Eqs. (1), (3) and (6). The problem is the transformation of the eluent zone between the inlet nozzles. To apply the equivalent fraction of eluent buffer to the batch column the following approximation was derived. The ratio (X) between the eluent flow-rate and the total flow-rate is given by Eq. (7):

$$X = \frac{F_e}{F_{\text{P-CAC}}} \tag{7}$$

The angular position of the different feed nozzles (θ_n) is shown in Fig. 2. Δt is the time required for the rotation of the bed between the inlet nozzles ($\Delta\theta_n = \theta_{n+1} - \theta_n$). Inserting this term in Eq. (1) yields Eq. (8):

$$\Delta t_n = \frac{\Delta\theta_n}{\omega} \tag{8}$$

When P-CAC is transformed back to batchwise operation to identical conditions, the additional time required for application of the eluent buffer is determined with Eq. (9):

$$t_{B,n} = X\Delta t_n \tag{9}$$

By application of Eq. (9) for all zones the identical process for batch column was achieved (Fig. 1D).

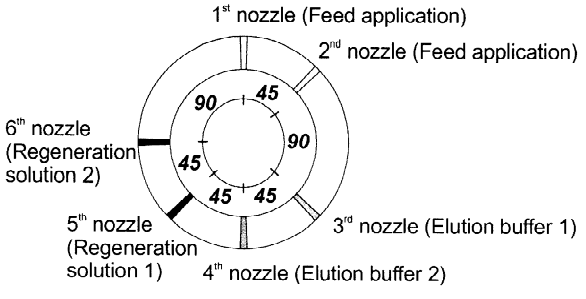

Fig. 2. Arrangement of the inlet nozzles of the P-CAC. The inner circle shows the $\Delta\theta_n$ values for the separation of factor IX.

The productivity (P) for batch and continuous chromatography was calculated according to Yamamoto et al. [41] who have described this relationship for batch chromatography. Buchacher et al. [6] showed that this equation is also valid for annular chromatography:

$$P = \frac{Q_R C_0 V_F}{V_t t_c} \tag{10}$$

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:

$$Q_R = \frac{m_c}{V_F C_0} \tag{11}$$

The cycle time t_c was determined for the time required to perform one cycle including equilibration and regeneration which is equivalent to one rotation using the P-CAC.

For comparison of productivity between batch and continuous operation, all parameters were equal, such as sample amount per sorbent volume and gradient volume per sorbent volume (Table 1).

The process time in batch chromatography is calculated as:

$$t_{c,1} = \frac{V_e + V_F + V_{E1} + V_{E2} + V_{\text{NaOH}} + V_{\text{Tris}}}{F_{\text{Batch}}} \tag{12}$$

with V_e the eluent volume, V_F the feed volume, V_{E1} and V_{E2} are the volumes of the elution buffers and V_{NaOH} and V_{Tris} are the volumes of the regeneration solutions. The process time for n cycles is calculated as:

$$t_{c,n} = t_{c,1} n \tag{13}$$

The process time for one cycle in annular chromatography ($t'_{c,1}$) was calculated from the time which was necessary to proceed the same dimensionless

Table 1
Process related parameters used for separation of factor IX in the three different processes

Parameter	Notation	Optimised batch process	Real P-CAC process	Batch process equivalent to P-CAC
Column volume (ml)	V_t	22.1	483.8	22.1
Column diameter (cm)	d	1.6		1.6
Annulus I.D. (cm)	R_{in}		13	
Annulus O.D. (cm)	R_{out}		15	
Height (cm)	h	11	11	11
Superficial velocity (cm/h)	u	120	120	120
Superficial area (cm^2)	A	2.01	43.98	2.01
Total flow rate (ml/min)	F	4.02	87.96	4.02
Rotation rate (degrees/h)	ω		180	
Load volume (% V_t)	V_F	2.50	$2.50 \cong 10.1$ ml/min	2.50
Eluent 1 volume (% V_t)	V_{E1}	1.00	$1.00 \cong 4.0$ ml/min	1.00
Eluent 2 volume (% V_t)	V_{E2}	1.00	$1.00 \cong 4.0$ ml/min	1.00
NaOH volume (% V_t)	V_{NaOH}	0.37	$0.37 \cong 1.5$ ml/min	0.37
Tris volume (% V_t)	V_{Tris}	0.50	$0.50 \cong 2.0$ ml/min	0.50
Time for one cycle (min)	$t_{c,1}$	40	120	120
Time for n cycles (min)	$t_{c,n}$	$40 \times n$	$120 \times n$	$120 \times n$

feed volume as in batchwise operation. The time therefore is equivalent to the time which is necessary for one rotation. The process time in annular chromatography was only determined in steady state of the P-CAC. The time to achieve this steady state was not included in the calculation. This would lead to a lower productivity for the P-CAC:

$$t'_{c,1} = \frac{V_F}{F_F} \quad (14)$$

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

$$t'_{c,n} = t'_{c,1} n \quad (15)$$

3. Materials and methods

3.1. Proteins and chemicals

A plasma-derived factor IX solution out of the production process of Octapharma (Vienna, Austria) was used as feed. Since this sample was from an intermediate step of the production process it contained some protein impurities, mainly vitronectin. Before chromatography the sample was diafiltrated against 10 volumes of running buffer in order to achieve the desired low conductivity for ion-exchange chromatography (IEC) experiments. The factor IX concentration after diafiltration was adjusted to ~50 IU factor IX per ml sample. Ultrafiltration was carried out with a Miniscale Omega membrane made of PES from PALL Filtron (Frankfurt, Germany) with a nominal molecular mass cut off of 10,000. All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). The columns were packed with Toyopearl DEAE 650M, purchased from Tosoh Biosep (Stuttgart, Germany).

3.2. Ion-exchange chromatography (IEC) in batch mode

An Äkta system (Amersham Biosciences, Uppsala, Sweden) was used as chromatography system for batch experiments. The XK 16 glass column (Amersham Biosciences) was packed with Toyopearl DEAE 650M gel to a bed height of 11.0 cm. For the optimisation of the step gradient and for the choice of buffer composition a smaller column (HR 5/50, diameter 5 mm, height 50 mm) was chosen to save starting material. The factor IX concentration in the starting material was about 50 IU factor IX per ml solution and the flow-rate was 120 cm/h. The sample volume was 2.5 ml of factor IX solution per ml sorbent. As equilibration buffer a 50 m*M* Tris–HCl buffer pH 7.0 was used. For the first elution step 200 m*M* NaCl and 1 *M* NaCl for the second elution step were added to the equilibration buffer. For

regeneration, a 1 *M* NaOH solution (regeneration solution 1) and for the adjustment of the pH, 1 *M* Tris–HCl buffer pH 7.0 (regeneration solution 2) was used. Detailed elution volumes can be seen in Table 1.

3.3. Ion-exchange chromatography in preparative continuous annular chromatography

The P-CAC unit was constructed by Prior Separation Technology (Götzis, Austria). A schematic drawing of the apparatus used for the continuous separation used here has been described by Buchacher et al. [6]. The annular column was packed with Toyopearl DEAE 650M gel at a column bed height of 11.0 cm. These dimensions resulted in a bed volume of ~0.5 l gel. The upper layer consisting of glass beads was 5.0 cm high. The rotation rate was 180 degrees per hour.

Eluent buffer, elution buffers 1 and 2, and regeneration solutions were the same as in the batch column. The eluent was delivered to the column by a P-6000 pump and the feed by two P-50 pumps. The elution buffers and the regeneration solutions were pumped by P-500 pumps (all from Amersham Biosciences).

P-CAC experiments were performed at a linear superficial velocity of 120 cm/h and the sample volume was the same as in the batch column (Table 1). The arrangement of the feed nozzles on the head of the P-CAC can be seen from Fig. 2. The feed nozzles need a special design because the column was 20 cm high but only packed to a height of 11 cm with sorbent. To apply the solutions 1 cm over the top of the packed bed the feed nozzles need a length of 10 cm and were stabilised to prevent twisting with two teflon rings. The sample, introduced through two feed nozzles, bound on the sorbent until the sorbent has rotated to the position of the first eluent nozzle where the first gradient solution was applied. Here the factor IX is eluted, at the next feed nozzle the second step gradient eluted the other proteins, mainly vitronectin. Through the next feed nozzle NaOH is introduced for regeneration of the sorbent and at the last feed nozzle a buffer is introduced to adjust the pH again to 7.

3.4. Determination of factor IX activity and protein

Factor IX activity was determined in a one-stage coagulation assay using an Amelung KC4A coagulometer from AVL (Vienna, Austria) or alternatively with an ACL 300 apparatus from Instrumentation Laboratory (Vienna, Austria). The assay was performed by mixing factor IX-deficient plasma with diluted sample, in the presence of lipid extract and kaolin as activator (all reagents from Instrumentation Laboratory, Lexington, MA, USA). Coagulation was triggered by adding $CaCl_2$ and the time required for a clot to form was measured.

The protein in the sample was determined using a protein determination kit from Pierce (Rockford, IL, USA). Bovine serum albumin also from Pierce was used as a standard.

3.5. Analytical size-exclusion chromatography (SEC)

In analytical size-exclusion HPLC, samples were separated by a TSK 3000 SW column (8.0×600 mm) from Tosoh Biosep. The column was connected to an LC 1100 system from Agilent Technologies (Vienna, Austria). As running buffer, a phosphate buffered saline (0.025 mol of $NaH_2PO_4 \cdot 2H_2O$, 0.0125 mol of $NaH_2PO_4 \cdot H_2O$ and 0.2 mol of NaCl per 1 l of distilled water) was used. The flow-rate was 0.5 ml/min. Absorbance was measured at 280 nm. For identification of the different pools, standards of factor IX and vitronectin (both purified in our laboratory) were used.

3.6. SDS–PAGE and Western blotting

SDS–PAGE was performed according to the method of Laemmli [42]. Sample preparation included the addition of reducing sample buffer and boiling for 10 min. Then 3 μg protein for SDS–PAGE and 1 μg protein per lane for blotting were applied. The electrophoresis chamber, blot module and the 4–20% precast polyacrylamide gradient gels were from Novex (San Diego, CA, USA). The precision protein standard was from Bio-Rad Labs (Hercules, CA, USA). Gels were stained for 60 min with Coomassie Brillant Blue and destained with water or electro-

blotted for 60 min onto a 0.2 μm nitrocellulose membrane from Schleicher & Schuell (Dassel, Germany) using blotting buffer. As antibodies against factor IX and vitronectin, mouse mAB from Sigma (St. Louis, MO, USA) were used. The respective secondary antibodies were alkaline phosphatase conjugated and also obtained from Sigma. Bands were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates purchased from Sigma.

3.7. Absorbance measurements for the fractions from P-CAC

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

4. Results and discussion

Here we wanted to investigate the transformation of a batchwise operation of factor IX purification as a model system to continuous chromatography. The starting situation was an optimised batch process (Fig. 1A) where the feed volume was equivalent to 2.5 column volumes. Also the volumes of the elution buffers and regeneration solutions were given by the batch column. The target was to operate this separation process with annular chromatography and to enable the comparison between the conventional batch chromatography and the P-CAC. First the purification of factor IX was optimized in small scale laboratory columns. A factor IX solution was used as starting material. Then the process was scaled up to an XK 16 column packed to a bed height of 11.0 cm. An XK 16 column was chosen to obtain nearly the same geometric conditions with the batch column (diameter 16 mm) as on the P-CAC (annulus 10 mm). The transformation from the time-dependent batch operation (Fig. 1A) to the angular-dependent P-CAC process (Fig. 1B) described by Eq. (1) was used.

With the P-CAC it was not possible to perform a direct transformation into a continuous separation (Fig. 1B), because the inlet nozzles of the P-CAC are fixed (Fig. 2). Another problem was the application of the feed solution. It was not possible to apply the feed solution with high feed flow-rates through one feed nozzle, because then the feed solution was not able to penetrate into the sorbent leading to mixing of feed solution and running buffer in the head space of the P-CAC. For better visualisation of this effect the feed solution was exchanged against a blue dextrane solution and the feed flow-rate was kept constant to obtain the same feed volume of 2.5 column volumes. Also the other hydrodynamic properties (flow-rates of eluent, elution buffers, regeneration solutions and the rotation rate of the P-CAC) were identical. Mixing of the blue dextrane solution with the eluent buffer, showing real sample distribution in the head space of the P-CAC, was observed (Fig. 3A). Therefore the application of the feed solution had to take place through two feed nozzles (Fig. 3B) to reach the correct transformed feed flow-rate. Then mixing in the head space could be avoided. It became evident from the experiments with blue dextrane as feed solution, that the feed flow-rate is limited. When the feed flow-rate exceeds a certain level the feed will also migrate into the head space. On the other hand, using batch columns there exists no limitations by the hydrodynamic behaviour of the fluids. In batch columns the feed is forced to penetrate into the packed bed. There the limitations would be the viscosity of the eluent leading to viscous fingering [43]. At linear velocities suited for P-CAC, viscous fingering was never observed. All these adjustments resulted in the process cycle for the P-CAC schematically shown in Fig. 1C. This new angular process cycle was also transformed back to a batchwise operation to enable the comparison of the P-CAC with the batchwise operation under the same conditions (Fig. 1D). This process is further called a batch process equivalent to the P-CAC process.

The chromatogram observed with the optimized batch process is shown in Fig. 4A. The first peak with elution buffer 1 contains the majority of factor

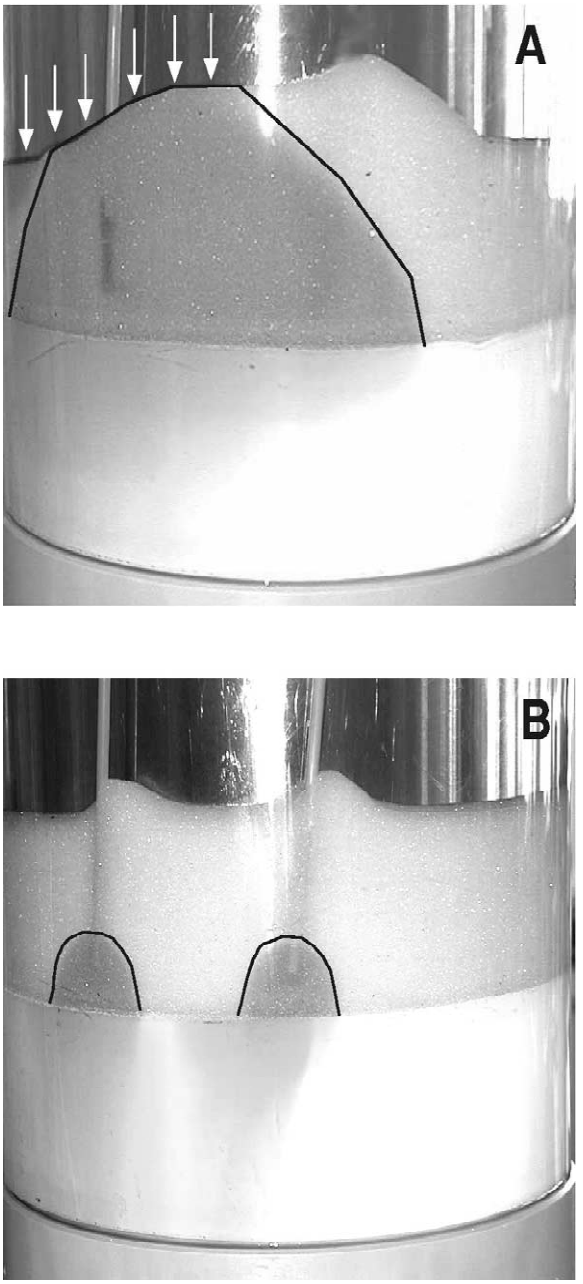

Fig. 3. Observation of the sample distribution using one feed nozzle (A) or two feed nozzles (B). For better visualisation blue dextrane was chosen as sample solution. The black line represents the front of blue dextrane distribution. In (A) the white arrows show the mixing zone of blue dextrane with eluent buffer.

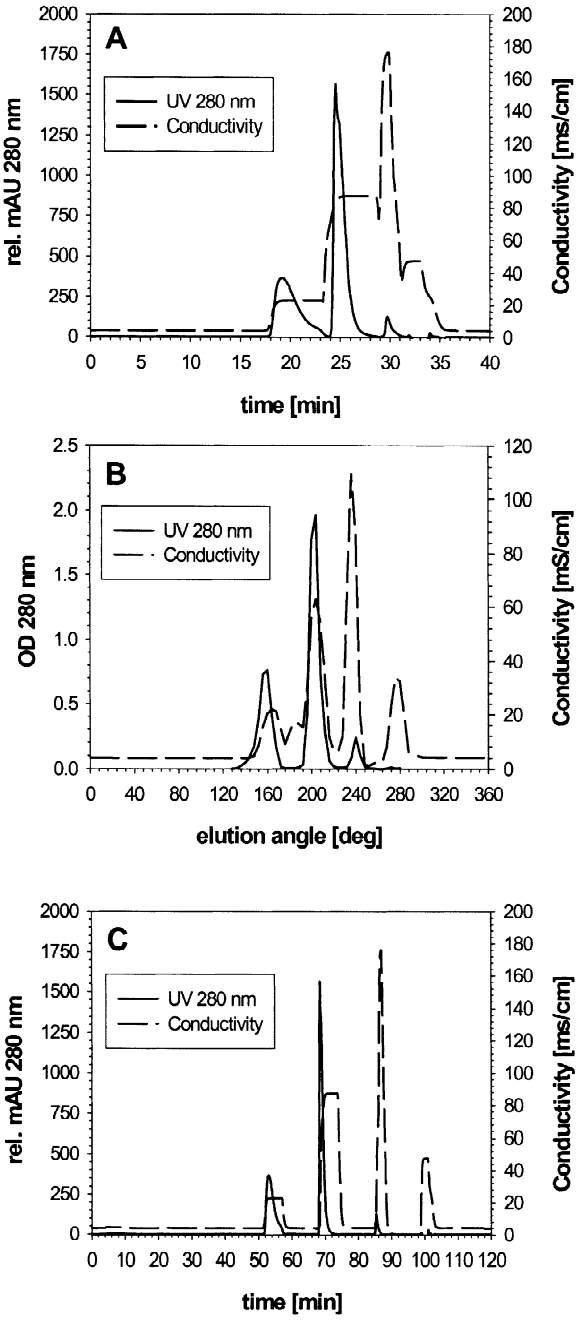

Fig. 4. Elution profile of the different scenarios: (A) optimised batch process, (B) real P-CAC process and (C) batch process equivalent to the real P-CAC process.

IX and the second peak eluted with elution buffer 2 contains vitronectin. The elution profile of the P-CAC process is shown in Fig. 4B. Since the continuous and the optimised batch process is not identical the continuous process was transferred back to a batch operation. The chromatogram of the batch process equivalent to the P-CAC process is shown in Fig. 4C. The separation time of the optimised batch process is three times smaller than from the P-CAC and batch process equivalent to the P-CAC. The higher separation time is mainly due to the design of the arrangement of the feed nozzles in the current P-CAC system. With the current design of the P-CAC the feed nozzles are fixed, so adjusting of the feed nozzles position was not possible. However the profile of the optimised batch process could never be achieved using the P-CAC. This situation is due to the hydrodynamic flow properties of the P-CAC. With the current P-CAC it is impossible to apply 2.5 column volumes feed solution, 2 column volumes elution buffers and 0.8 column volumes regeneration solutions, if applying only 2 column volumes of elution buffer at the same time. At such high flow-rates the solutions applied through the feed nozzles would never be able to penetrate into the sorbent, leading to a mixture of all solutions in the head space as shown by the experiments using blue dextrane (Fig. 3A). Therefore a certain level of eluent flow-rate has always to be set to force the fluids, coming out from the feed nozzles, into the sorbent.

The flow-rates for the eluent of the P-CAC and the solutions applied through the feed nozzles were calculated as described by Eqs. (3) and (5). The elution profile of the separation using the P-CAC is shown in Fig. 4B. In continuous ion-exchange chromatography, sample application, elution, regeneration and adjustment of the pH are performed simultaneously. Different conductivity levels have to be achieved in IEC to enable the binding and elution of the different sample components. To ensure that the protein of interest can bind continuously to the sorbent, the conductivity and pH level had to be decreased again after regeneration. In Fig. 5 the conductivity and pH profile of the P-CAC is shown. The pH was increased up to 12 during the regeneration. Therefore an efficient continuous regeneration of the sorbent was achieved. Also the pH and conductivity level is decreased again when the sample was applied to the sorbent. For the adjustment of the pH, a buffer with a high buffering capacity (1 *M* Tris–HCl buffer pH 7.0) was introduced between the NaOH and the feed application zone through a feed nozzle.

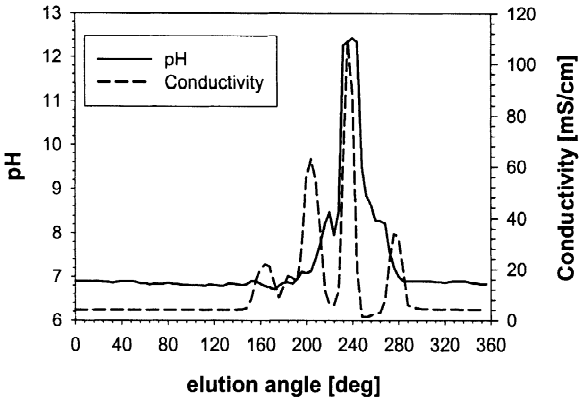

Fig. 5. pH and conductivity profile of the P-CAC.

The different fractions were analysed by analytical SEC. For the identification, standards of factor IX and vitronectin were used. The elution profiles of the different peaks are shown in Fig. 6. Vitronectin eluted in the void fraction and factor IX eluted at 32 min run time. A good separation of factor IX and vitronectin for the P-CAC and the batch column is confirmed by analytical SEC.

The collected peaks were further analyzed by SDS–PAGE under reducing conditions (Fig. 7A) and Western blotting with mAB against factor IX (Fig. 7B) and vitronectin, respectively (Fig. 7C). In the SDS–PAGE under reducing conditions the first peak migrates as a broad band with an apparent molecular mass of ~70,000. This band is factor IX as shown in Fig. 7B. The second peak contained material migrating as a double band with an apparent molecular mass of 65,000 and 75,000. These two bands were assigned to vitronectin in human plasma as shown in Fig. 7C. These results were confirmed by the Western blots against factor IX (Fig. 7B) and vitronectin (Fig. 7C). The major fraction of factor IX appears in the first peak and only a small amount in the second and nearly all the vitronectin is in the second peak. These results corroborate the results obtained by the analytical SEC (Fig. 6). Between the different process scenarios (optimised batch, P-CAC process and

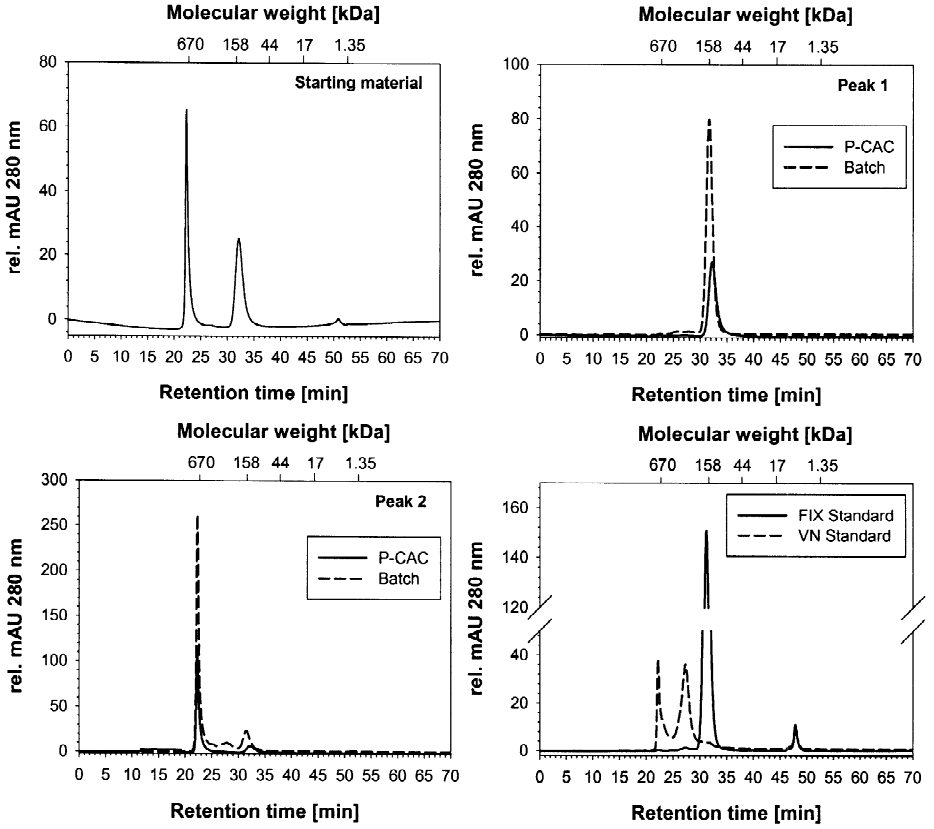

Fig. 6. Analytical SEC of the starting material (upper left chromatogram), the peak 1 (upper right), the peak 2 (lower left) and the standard (lower right) of the factor IX–vitronectin separation for the P-CAC (———) and the batch column (- - -).

batch process equivalent to the P-CAC) the same purity levels assessed by SDS–PAGE (Fig. 7A), Western blotting (Fig. 7B and C) and SEC (Fig. 6) could be observed.

The recovery of factor IX was determined for all process scenarios. After having reached the steady state conditions using the P-CAC the factor IX and vitronectin fractions were collected. The applied feed volume was also measured in the same time interval. The same was done with the batch processes. The recovery of the batch processes was higher than that of the P-CAC and also the obtained factor IX concentration was higher (Table 2). The enrichment of factor IX given as a concentration factor calculated from factor IX concentration over the process and the specific activity as a measure of the purity of the product is shown in Table 3. The concentration factor obtained by P-CAC was lower compared to the batch processes. The specific activity is in the same range as the batch processes indicating that the same purity level was reached. The overlay of the elution profiles of the P-CAC process with the batch equivalent to the P-CAC process (Fig. 8) showed that the elution peaks using the P-CAC are much broader than that of the batch process. The slope of the salt front in P-CAC is shallower than in the batchwise operation. This is due to mixing of the elution buffer with the eluent buffer in the layer of glass beads covering the ion-exchanger bed. The width of the eluted protein in ion-exchange chromatography is directly related to the steepness of the salt front [44]. Previously we have compared size-exclusion chromatography in batch and continuous mode. The width in P-CAC and batch were identical [45]. It seems that the mixing of elution buffers is very critical in IEC, whereas it is negligible in

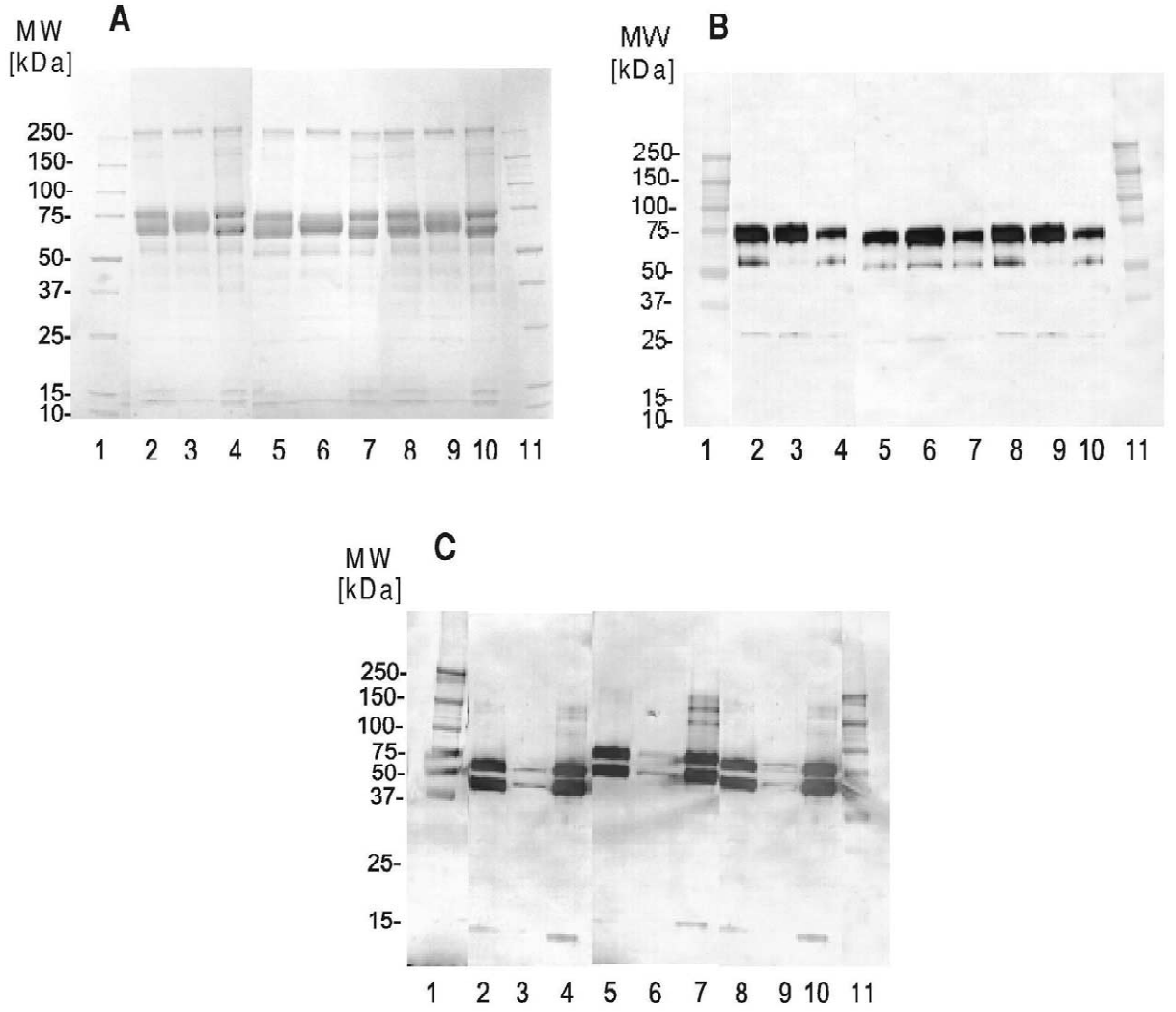

Fig. 7. SDS–PAGE (A) and immunoblots against factor IX (B) and vitronectin (C) of pools of separation of factor IX from vitronectin of the three different scenarios under reducing conditions. Optimal batch process, lanes 2–4; real P-CAC process, lanes 5–7; batch process equivalent to the P-CAC process, lanes 8–10. The following samples were applied: 1, marker; 2, starting material; 3, factor IX pool; 4, vitronectin pool; 5, starting material; 6, factor IX pool; 7, vitronectin pool; 8, starting material; 9, factor IX pool; 10, vitronectin pool; 11, marker.

size-exclusion chromatography. Another reason for increased peak width in P-CAC could be the influence of the circumferential diffusion [46]. A significant contribution of the circumferential diffusion to the overall dispersion process could not be observed [47].

For industrial separation an important parameter is productivity. The productivity of the three different process scenarios was calculated according to Eq. (10). The parameters for the calculation of the productivity were obtained from Tables 1 and 2. The applied feed solution per volume of sorbent was constant for all process scenarios. Therefore it was possible to compare the three different scenarios (Fig. 1A, C and D) under the same separation conditions. The lifetime of the sorbent was not included in the calculation of the productivity. For the calculation of the productivity of the P-CAC only

Table 2
Recovery of factor IX after separation according to the 3 different scenarios

Fractions	Mass (g)	Factor IX activity (IU factor IX/ml)	Total factor IX activity (IU factor IX)	Recovery (%)
Optimised batch process				
Start	55.3	53.7	2969.91	100.0
Factor IX pool	23.1	114.5	2644.95	89.1
VN pool	15.7	22.4	351.68	11.8
NaOH pool	7.8	0.0	0.00	0.0
Total				100.9
Real P-CAC process				
Start	80.3	56.1	4504.80	100.0
Factor IX pool	57.9	52.5	3039.80	67.5
VN pool	62.6	14.0	876.40	19.5
NaOH pool	17.7	0.0	0.00	0.0
Total				87.0
Batch process equivalent to the P-CAC				
Start	55.3	61.4	3395.42	100.0
Factor IX pool	23.1	139.3	3217.83	94.8
VN pool	15.7	27.9	438.03	12.9
NaOH pool	7.8	0.0	0.0	0.0
Total				107.6

Table 3
Enrichment of factor IX and productivity after separation according to the 3 different scenarios

Fractions	Optimised batch process	Real P-CAC process	Batch process equivalent to the P-CAC
Concentration factor			
Starting material (IU factor IX/ml)53.756.161.4			
Factor IX pool (IU factor IX/ml)	114.5	52.2	139.3
Concentration factor	2.1	0.9	2.3
Specific activity			
Starting material (IU factor IX/mg protein)	105.3	106.9	117.6
Factor IX pool (IU factor IX/mg protein)	229	262.5	293
Productivity (IU factor IX/ml sorbent/min)	2.99	0.79	1.22

the steady state was considered. If the time which is necessary to achieve the steady state is also considered, the productivity would slightly decrease. At sufficiently long process times, which is normal for continuous processes, the influence of the time to achieve the steady state is negligible. In Table 3 the productivity of the three different scenarios are shown. The productivity of the P-CAC is the lowest of all scenarios due to the lower recovery of factor IX. Theoretically the productivity of the P-CAC process should be the same as the batch process equivalent to the P-CAC process. One reason for a lower one was the broader salt front.

5. Conclusion

Continuous separation of factor IX from vitronec-

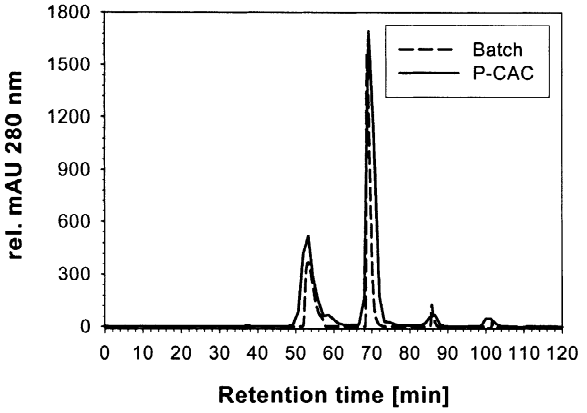

Fig. 8. Comparison of the elution profile of the real P-CAC process (———) with the batch process equivalent to the P-CAC process (- - -).

tin was possible by P-CAC. This was demonstrated through analysis of the eluted fractions. Simultaneous separation and regeneration using the P-CAC was achieved. The application of the feed solution is only possible up to a certain range of the feed flow-rate without mixing with the eluent buffer in the head space of the P-CAC, depending on the ratio of the feed flow-rate to the eluent flow-rate. Using this equipment and protein model the productivity of the continuous system in the steady state applied for adsorption chromatography is not higher than that in a conventional batch column.

6. Nomenclature

A_{Batch}	superficial area of the batch column (cm^2)
$A_{\text{P-CAC}}$	superficial area of the P-CAC (cm^2)
C_0	sample concentration (mg/ml)
d	diameter (cm)
$\Sigma_{i=1}^{n} F_i$	sum of all flow-rates from all solutions applied through the feed nozzles of the P-CAC (ml/min)
F	total volumetric flow-rate (ml/min)
F_{Batch}	total volumetric flow-rate batch column (ml/min)
F_e	eluent flow-rate (ml/min)
$F_{\text{P-CAC}}$	total volumetric flow-rate P-CAC (ml/min)
F_1	overall feed flow-rate (ml/min)
F_2	flow-rate for the first elution buffer (ml/min)
F_3^2	flow-rate for the second elution buffer (ml/min)
F_4	flow-rate for the NaOH solution (ml/min)
F_5	flow-rate for the Tris solution (ml/min)
m_c	amount of pure protein (mg)
n	number of cycles
P	productivity (IU factor IX/ml sorbent/min)
Q_R	recovery rate
R_{in}	annulus inner diameter (cm)
R_{out}	annulus outer diameter (dm)
Δt	time to move from one feed nozzle to the next (min)
t_B	time batch column (min)
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)
u_{Batch}	linear superficial velocity of the batch column (cm/h)
$u_{\text{P-CAC}}$	linear superficial velocity of the P-CAC (cm/h)
V_{E1}	volume of the first elution buffer (ml)
V_{E2}	volume of the second elution buffer (ml)
V_{NaOH}	volume of NaOH (ml)
V_{Tris}	volume of Tris (ml)
V_F	sample volume (ml)
V'_F	dimensionless sample volume
V_t	column volume (ml)
X	ratio between eluent flow-rate and the sum of all flow-rates applied to the P-CAC

Greek symbols

α	elution angle (degrees)
θ	angular position (degrees)
ω	rotation rate (degrees/h)

Abbreviations

AC	annular chromatography
CAC	continuous annular chromatography
CIM	convective interaction media
C.V.	column volume (ml)
DEAE	diethylaminoethyl
Factor IX	clotting factor IX
IEC	ion-exchange chromatography
P-CAC	preparative continuous annular chromatography
PES	poly ether sulfone
SEC	size-exclusion chromatography

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