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Review

Continuous annular chromatography

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Abstract

The principle of continuous annular chromatography (CAC) has been known for several decades. CAC is a continuous chromatographic mode, which lends itself to the separation of multi-component mixtures as well as of bi-component ones. In CAC, the mobile and stationary phases move in a crosscurrent fashion, which allows transformation of the typical one-dimensional batch column separation into a continuous two-dimensional one. With the exception of linear gradient elution, all chromatographic modes have at present been applied in CAC. This review focuses on the capacity of CAC for preparative bioseparation. The historical developments and the predecessors of modern CAC are briefly summarized. The state-of-the-art in the theoretical prediction and simulation of CAC separations is discussed, followed by an overview of current CAC instrumentation and example applications, especially for the isolation of proteins and other bio(macro)-molecules. In this context, issues of scale up as well as method development and transfer from batch to continuous CAC columns are discussed using recent bioseparation efforts as pertinent examples.

Keywords: Reviews; Continuous annular chromatography; Annular chromatography; Preparative chromatography; Proteins; DNA

Contents

1. Introduction and historical background

Liquid chromatography is often the only choice for the separation of complex multi-component mixtures, e.g. the typical feeds found in industrial

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biotechnology, because the method is universally applicable, versatile and it has high-resolution capabilities. Complex biomolecules (proteins, antibodies, nucleic acids etc.) can be isolated using a number of interaction modi ranging from electrostatic and hydrophobic interactions (ion-exchange, reversed-phase and hydrophobic interaction chromatography) to specific biological interaction (affinity chromatography). If necessary several orthogonal separation principles can be used in series. In addition, the relative simple separation by size (size exclusion chromatography, gel filtration) is often applied. Performing a chromatographic separation is also aided by the ever increasing selection of dedicated chromatographic media that are commercially available.

At preparative scale, however, the capacity/loadability and thus the throughput of the column frequently present a limiting factor for chromatographic operations. The idea of combining the adaptation of the chromatographic separation process to large-scale with the introduction of continuous feed injection and product withdrawal, has therefore been discussed for more than 50 years as a means to make chromatography more competitive in the industrial sector. In 1949 Martin [1] imagined an annular chromatographic system able to render continuous chromatography, which should in principle be applicable to most chromatographic operation modes known at that time. Concomitantly, he also envisaged an alternative to the annular column in the form of a circular array of columns. This should not make an essential difference to the operation of the system, but would perhaps present less practical difficulty. In the same year, and according to his own statement independently from Martin, Svensson [2] started to build exactly such an apparatus for continuous chromatographic separations, which he presented in 1955. The apparatus consisted of 36 column tubes (11 mm I.D.) arranged in a large, slowly rotating circle. Along with this assembly of columns rotates a specially designed liquid flow distributor, which ensures equal eluent flow (forced by gravity) into each of the columns, while preventing liquid from running between the columns. Contrary to the column arrangement and the eluent distributor, the sample feed point and the fraction collector arrangement are fixed in space. Chromatographically separated components can therefore be continuously collected from defined compartments of the collector.

Fig. 1 elucidates this principle in a more schematic manner, which also may serve to explain the principle of continuous annular chromatography (CAC) in general. The feed is continuously introduced from a fixed inlet point to an array of ''batch'' columns that slowly rotate past it. All columns are continuously flushed with the eluent. As a given column passes the injection point, a certain feed volume is injected and the separation starts along the column axis (hence the crosscurrent move of the liquid and the solid phases). After a given time the separated substances appear at the column outlet. Depending on the elution time, this will be the case after the column has rotated and hence moved a certain distance (angle) in relation to the injection point. This elution angle is therefore fixed in relation to the injection point and—given a reproducible column performance—the separated components can be collected at their fixed exit angles. If one goes from a

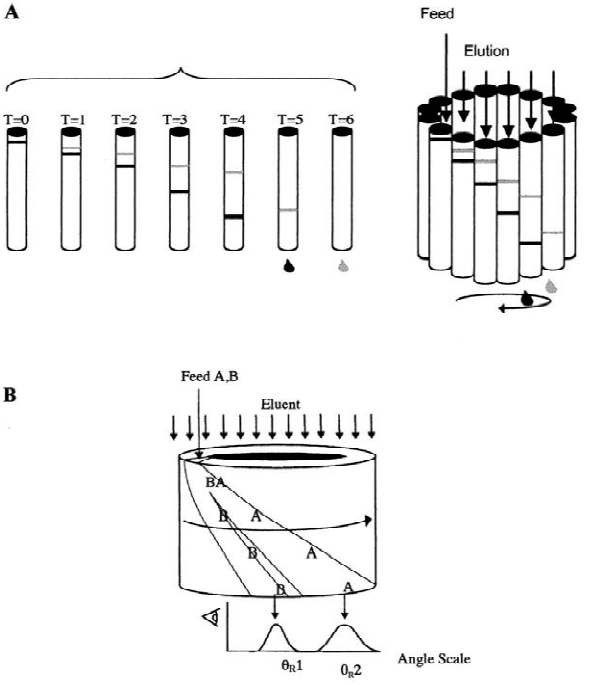

Fig. 1. Schematic presentation of a separation by continuous annular chromatography. (A) Column-based separation, (B) annular column.

set of columns to a single annular column, the same mechanism applies. The overall impression in this case is the resolution of the feed components into a number of helical bands, which sometimes leads to the assumption of a radial component in the direction of the separation. However, just as in conventional batch chromatography, the separation in CAC occurs strictly in the axial direction. Some diffusion in the radial direction may occur in the annular column, although in typical protein separations this does not contribute significantly to the observed band broadening.

One challenging prerequisite for a successful multi-component separation with a multiple column-based continuous chromatographic system was already discussed by Sevensson et al. [2]. This is the necessity for all columns to have identical flow resistances. While uniform column packing in an annular cylinder column was no problem according to the authors, they could not achieve it in the case of the 36 individual columns. That might be the reason why in their paper they reported 1 month's satisfactory uninterrupted working of the apparatus but not its application to any practical separation problem. The only successful application to date reported for a circular column array CAC system is the continuous gas-chromatographic separation of a mixture of organic solvents at preparative scale communicated by Dinelli et al. [3]. Their gas–liquid chromatography unit consisted of 100 columns (1.2 m×6 mm I.D.) arranged in a rotating carousel. Carrier gas passes through a head into all columns, except the one column, which is at a given time receiving the vaporized feed. For the separation of a mixture of six organic solvents the overall efficiency of this unit was the same as that of a single analytical column.

2. Instrumental design and operation principles

The first true continuous annular chromatograph was constructed by Fox et al. [4,5]. The device consisted of a rotating annulus with an outer diameter (O.D.) of 294 mm, a width of 9.6 mm, and a height of 30 cm. In this original CAC apparatus the feed was pumped continuously through a nozzle at a fixed position above the adsorbent bed. A specially developed leveling plow kept the bed surface absolutely flat during rotation. Eluent was filled in the upper part of the annulus, and a uniform, gravity-forced eluent flow through the entire CAC column was achieved by the simple means of keeping the eluent level constant. As time progressed, stationary helical component bands developed from the feed point to the foot of the column, whose slopes depended upon the eluent velocity, the rotational speed, and the distribution coefficient (retention factors) of the components between the fluid and adsorbent phases.

At steady-state, the component bands formed regular helices between the feed sector at the top of the bed and the individual fixed exit points (100) at the bottom of the annular bed, where the separated components were continuously recovered. As long as the conditions remained constant, the angular displacement of each component band from the feed point also remained constant. Thus, this rotating annular chromatograph was a truly continuous, steady-state process that retained the ability for multi-component separations and the flexibility, which is typical for chromatography in general. Two examples of isocratic biomolecule separation have been reported with the apparatus [6], namely the separation of protein, salt, lactose, and riboflavin from skim milk using Sephadex G-25 as chromatographic phase and the separation of myoglobin from hemoglobin in beef heart extracts on Sephadex G-75 (1.2 l bed volume). In the latter case, myoglobin was separated at 0.4 ml/min feed flow-rate, 15 ml/min eluent flow-rate and a rotation speed of 180°/h. The protein was recovered with 97% purity. In terms of product quality, the CAC result was comparable to a separation of a (discontinuous) batch column (8.3× 30.0 cm, 1.6 l) available in the laboratory, but according to the authors it was superior in regard to time and effort.

In 1976, Scott et al. [7] at the Oak Ridge National Laboratory (ORNL), USA, introduced the first prototype of a gas-pressurized CAC device (Fig. 2). It was hoped that the capability to work under high pressure would allow advantage to be taken of the recently developed high-resolution separation media. The inlet distributor head of the ORNL-CAC system allows access of one feed and up to six eluent lines, while a gas line provides the constant pressure

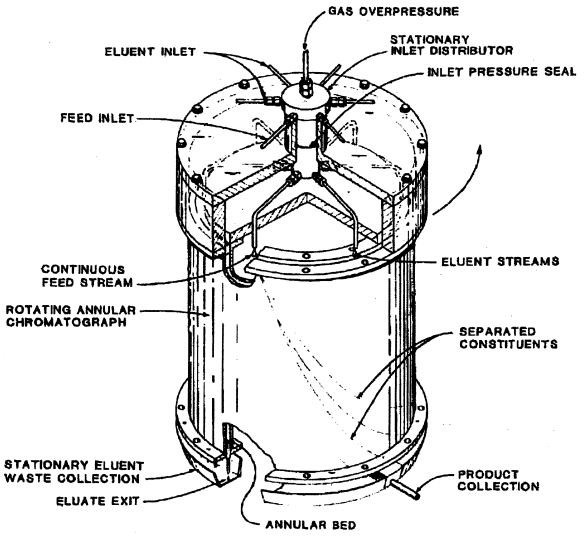

Fig. 2. Gas pressurized continuous annular chromatograph as designed by Scott et al. (from Ref. [7] with permission).

required for forcing the liquid through the bed. Forty-eight spacers are placed on the top and at the bottom of the annular column at equal distance. In this way, (step) gradient elution over annular segments becomes in principle possible by using the spacers at the top. The spacers at the bottom of the annular column prevent backmixing of the eluates, a problem which could not be solved by Svensson et al. [2]. An additional advantage of the ORNL-CAC system was the possibility to analyze the eluates by an in-line monitoring device. Reported applications of the ORNL-CAC system are the isocratic separation of Ni/Co complexes using the Dowex 50W-X8 ion-exchanger and the separation of Blue Dextran 2000 and cobalt ions by size-exclusion chromatography with Sephadex G-25 as separation medium.

In order to improve the chromatographic performance and the data consistency, Scott's prototype was subsequently re-designed by Canon and Sisson [8]. The inlet was completely changed in order to give more flexibility to the operation of the system, e.g. by allowing gradient elution. A layer of glass beads was put on top of the column bed in order to allow the feed band to spread until its velocity matches the eluent velocity and thus to overcome the problem of defining the initial bandwidth of the feed. The glass bead layer also considerably reduced the danger of forcing gas bubbles through the bed. The concomitant change of the exit ports' design solved the problem of circumferential mixing. The separation of Co/Ni complexes on the Dowex 50W-X8 resin in a pH gradient using a CAC device with 28.4 cm outer diameter, 1.25 cm annulus width, 46 cm bed height, and 180 exit ports was taken as a performance test. In addition, a larger CAC model was constructed [9], the cross-sectional area of which was increased by a factor of 7 (59.7 cm O.D., 4.45 cm annulus width, identical bed height). Four different eluents could be applied simultaneously to this CAC apparatus. Performance tests were run for metal separations (Cu–Ni–Co, Fe–Al, Zr–Hf) and it was concluded that the (annular) cross-sectional area could serve as a suitable, initial scale-up factor.

In batch chromatography the resolution usually increases when either a longer column or a smaller sample volume (concentration) or a lower eluent flow-rate is used. The latter at least as long as the flow-rate does not become so low as to allow molecular diffusion of the analytes to become a significant contribution to zone broadening. Bergovich et al. [10–12] pioneered the investigation of column and operation parameters on the performance of a CAC column. In particular these authors investigated the effect of the annulus width on the performance in metal ion separation. CAC instruments with 8.9 to 27.9 cm outer diameters and annuli widths between 0.64 and 5.08 cm were built. In all cases, the performance, the efficiency, and the resolution were identical as long as the eluent velocity was kept constant. The upper limit for the annulus width is determined by the appearance of flow inhomogeneities and problems caused by analyte diffusion. For an extreme CAC apparatus with an outer diameter of 27.9 cm and an annulus width of 11.43 cm (corresponding to more than 80% of the available cross-sectional area), a new feed system and bottom plate had to be designed in order to guarantee uniform flow distribution and thus similar results under comparable operational conditions [11]. In all reported cases, the experimentally obtained continuous chromatograms for the separation of the metal ions corresponded well to the predictions made by the plate theory [10,12,13]. The possibility to scale-up the separations to a pilot scale CAC unit (44.5 cm O.D., 3.18 cm annulus width) has been

demonstrated by the same authors [11]. A generic discussion of the effect of the operating conditions on the CAC profiles can also be found in the review by Uretschläger and Jungbauer [14].

At present, CAC instruments are commercially available under the name P-CAC from Prior Separation Technology (Götzis, Austria). The principle difference of the P-CAC to the units developed at Oak Ridge National Laboratory is the design of the distribution head. Instead of using gas pressure, the entire head is flooded with the main eluent at defined pressure provided by a pump [15]. Several relevant separation applications have been demonstrated using this type of CAC device, some of which will be discussed below [16–22].

Alternatively to rotating the annulus in a CAC unit it is also possible to keep the annulus fixed while rotating the inlet head and the fraction collector simultaneously. Dunnill and Lilly [23] presented such a prototype in 1972, which was designed as part of a fully continuous enzyme isolation process. Although they found the principle of CAC easy to apply, they did not succeed in constructing a prototype which could comply to industrial standards in terms of reliability, robustness, and long-term stability. In 1987 Goto and Goto [24] reported on the construction of a similar, albeit more robust unit (16 cm O.D., 1 cm annulus width), and demonstrated the feasibility of using this apparatus for the separation of NaCl and methanol by size-exclusion chromatography (Dowex 50W-X8 resin). Further applications were the separations of amino acids [25,26] and proteins [27].

3. Theoretical considerations

Predicting the elution profiles, and changes thereof upon changes of the operational parameters, is an important tool in the set-up and optimization of chromatographic separations. In continuous annular chromatography the space and time coordinates typically used in the modeling of conventional batch chromatographic separation are replaced by the space and annular displacement coordinates of the rotating bed. Two-dimensional, steady-state separations in CAC are therefore analogous to one-dimensional, time-dependent separations in conventional fixed-bed elution chromatography [28]. Assuming an isothermal, steady-state separation process with uniform concentration and fluid profiles in the radial direction and local equilibrium between fluid and solid phase, the mass balance describing the development of the concentration profiles for a component i in an annular chromatograph can be formulated by a coupled set of N_c two-dimensional convection diffusion equations [29]. The general form of these equations is given by Eq. (1):

$$\omega\frac{\partial c_i}{\partial\theta} + \omega F\frac{\partial q_i}{\partial\theta} + u\frac{\partial c_i}{\partial z} = D_z\frac{\partial^2 c_i}{\partial z^2} + D_\theta\frac{\partial^2 c_i}{\partial\theta^2} \text{ in } \Omega; \quad i = 1, N_c \tag{1}$$

The solid-phase concentration q_i and the liquid phase concentration c_i are related through the adsorption isotherm. The coefficients D_z and D_θ quantify band broadening by dispersion and diffusion in the axial (z) and the angular (θ) directions, respectively. The phase ratio F is given by $F = (1 - \varepsilon)/\varepsilon$ with ε being the porosity of the column. ω corresponds to the rotation velocity, and u is the axial flow velocity. If angular dispersion is neglected ($D_\theta = 0$), the continuity Eq. (1) is a term by term equivalent transformation ($t_R \rightarrow \alpha_R/\omega$, with α_R the elution angle) of the corresponding unsteady state equation for conventional elution chromatography [28].

If typical CAC conditions apply, i.e. no eluate backmixing, continuity of the outgoing flow, and periodicity for the angular direction, the numerical solution of Eq. (1) with the assumption of nonlinear isotherms (Langmuir-type) for a two-component mixture can be used to demonstrate possible effects of a change in D_θ on the peak shape. Seidel-Morgenstern et al. [29] simulated, e.g., the effect of D_θ on peak height, elution region and peak shape. They found that when D_θ is increased the peak maxima decrease, the elution regions (peak width) increase, and the peak shapes become more symmetrical. In addition, their simulations predict that an increase of the rotation rate increases and shifts the elution range of the components. Concomitantly, smaller peak maxima, broader outflow intervals and in a certain range better separations should manifest themselves under these conditions. Increas-

ing the feed's concentration, on the other hand, should lead to more asymmetric peaks with maxima at smaller elution angles. Stronger adsorption of the components serves to increase the elution angles and to reduce the peak heights, while a more pronounced non-linearity of the adsorption isotherm reduces the elution angle. All of these effects correspond exactly to similar phenomena known from conventional fixed-bed chromatography.

Howard et al. [30] used a linear two-film mass transfer model to simulate the CAC elution profiles of a sugar mixture where the fluid-particle mass transfer rate Eq. (2) is written in terms of a global interphase mass transfer coefficient $k_o a$:

$$\omega(1-\varepsilon)\frac{\partial q_i}{\partial \theta} = k_o a\left(c_i - \frac{q_i}{K}\right) \quad (2)$$

where K is the distribution coefficient of a given substance between the stationary and the mobile phase.

Under the assumptions that (i) the feed arc is infinitely small, (ii) the number of transfer units n ($n = k_0 az/u$) is greater than five, (iii) axial and angular dispersion are negligible, (iv) the adsorption equilibrium is described by a linear isotherm, and (v) concentration and fluid velocity gradients in the radial direction can be ignored, an approximate analytical solution can be derived for the chromatographic response $c(z, \tau)$ in an isocratic CAC operation for appropriate boundary conditions, see Eq. (3):

$$c_i(z,\tau) = \frac{QI}{2\pi^{0.5}}\left\{\frac{(k_o a)^2}{u^3 z\tau[(1-\varepsilon)K]^3}\right\}^{0.25} \times \exp\left\{-\left[\sqrt{\frac{k_o az}{u}} - \sqrt{\frac{k_o a\tau}{(1-\varepsilon)K}}\right]^2\right\} \quad (3)$$

where

$$\tau = \frac{\alpha_R}{\omega} - \frac{\varepsilon z}{u}$$

and

$$QI = \frac{c_F u Q_F}{Q_T}\cdot\frac{360^\circ}{\omega}$$

QI is the quantity of solute injected with the feed mixture per unit cross-sectional area, c_F is the solute concentration in the feed mixture, Q_F is the feed flow-rate, and Q_T is the total flow-rate of fluid through the annular bed.

The authors subsequently attempted to predict the isocratic CAC elution profiles of a glucose–fructose model mixture assuming that the time required for establishing the equilibration is much longer than the time required for the separation. For this, K, n and ε (and thus $k_o a$) were determined from fixed-bed chromatographic experiments. Comparison of the theoretical results with the experimental ones obtained during an isocratic CAC separation of the sugar mixture at low feed concentration and modest column loading, showed acceptable agreement. In particular, the separation was achieved using a calcium loaded cation-exchange adsorbent, namely Dowex 50W-X8 (particle diameter 50–60 μm), in a continuous annular chromatograph (27.9 cm O.D., 1.27 cm annulus width) at 66.7 ml/min eluent flow, 1.0 ml/min feed flow and 240°/h rotation rate. For this particular separation the angular dispersion could indeed be neglected, as the peak spreading was largely determined by intraparticle diffusion as the major resistance to mass transfer. The comparison between numerical and experimental results confirmed expected trends in the profiles upon changes of the operation parameters. The peak position shifted to higher elution angles and the peak broadened when the rotation rate was increased. In the range of 0.5 to 2.5 ml/min (under these conditions the feed may be considered as infinitesimal), the feed flow-rate had no effect on the resolution, while increasing the rotation speed of the CAC column did increase the resolution. Increasing the axial flow-rate of the liquid phase or the feed concentration had a detrimental effect on the resolution at least under experimental conditions. The calculations, on the other hand, predicted no change in resolution with increasing feed concentration.

Qualitatively, it was observed that under the given operating conditions the experimentally determined resolution of the fructose–glucose mixture with the CAC column was about 20% lower than the value calculated based on the parameters obtained from the fixed bed experiments. The deviation between experiment and calculations was attributed to the presence of calcium ions in the eluent and to a generically greater dispersion in the CAC system compared to the fixed bed column. Possible reasons for the latter phenomenon, whose extent can be characterized by a determination of the Peclet num-

ber as a function of the Reynolds number, are the annulus packing method and putative wall effects. Recently, Bart et al. [31] as well as Wolfgang et al. [32] repeated these experiments and calculations for a modified sugar mixture consisting of fructose, mannitol and sorbitol under nearly identical experimental conditions. Using Dowex 50W-X8 as column packing material, they found an excellent agreement between the measured CAC profiles and those predicted by Eq. (3) under a number of operation conditions.

It can thus be stated with some confidence that for molecules, which are linearly and non-competitively adsorbed, and for cases where axial and angular dispersion can be neglected, CAC elution profiles can be calculated in the simplified manner described above, and particularly by Eq. (3). At high loading the feed occupies a significant sector of circumference (according to Ref. [33] this is the case for a feed sector occupying more than 6 degrees) and Eq. (3) no longer applies. In this case, the analytical solution derived for the periodic application of a finite feed volume to a chromatographic annulus under the assumption of negligible axial and angular dispersion can be used [33]. This approach was, e.g., used to model the separation of industrial sugar feedstocks under overloading conditions (feed sector of up to 55 degrees). Good agreement was found between the experimental results and the predictions based on the linear adsorption model, although the experimental peaks were higher than expected from calculations based on parameters determined from fixed bed experiments. Interestingly and in spite of the overall similarity of the applied experimental conditions, the global mass transfer coefficient $k_o a$ for fructose, derived in this series of publications [30–33] from fixed bed experiments showed significant differences. The value given by Byers et al. [33] is, e.g., 61% lower than the one determined by Howard et al. [30].

Linear chromatographic separations are rather insensitive to the exact nature of the mass transfer mechanism assumed in the model [34]. In the case of large molecules such as proteins and porous resins, intraparticle diffusion usually presents the main resistance to mass transfer. For instance, in the case of a CAC column packed with S-Sepharose and used for the isocratic separation of a mixture of bovine serum albumin (BSA), hemoglobin and cytochrome *c* at modest sample loading and under essentially linear adsorption conditions ($[Na^+] > 100$ m*M*), the separation could be adequately described by a particle diffusion model, whose linear equilibrium constants as well as the values for the diffusivities and the mass transfer parameters were obtained from fixed bed experiments. The calculations also showed a nearly ideal behavior of the CAC unit, indicating the virtual absence of hydrodynamic and extracolumn non-idealities.

The extension of step elution to (nearly) linear equilibrium systems has been published by Carta et al. [35]. For predicting the CAC performance in the separation of Fe^{3+} and Cr^{3+} ions on Dowex 50W-X8 applying a step gradient of increasing ammonium sulfate concentration, an additional conservation term for the eluent had to be included into the continuity equation. Numerical solution of the continuous step elution experiment showed—in good agreement with the experimental results—that the ammonium sulfate concentration is the most important variable. As the ammonium sulfate concentration increases, the dispersion coefficients decrease rapidly. As a result the peaks are sharpened and exit the annulus at a smaller elution angle than in the corresponding isocratic experiment, albeit at the cost of an over-all reduced resolution.

Bloomingburg and Carta [36] developed a model, which by numerical solution of the differential mass balance equations accurately predicted the elution profile of BSA and hemoglobin in a continuous NaCl step gradient elution separation (pH 6.5) using a CAC column packed with a cation-exchanger material (S-Sepharose). The model is based on the assumptions that (i) BSA is not adsorbed to the resin and is retained only in the pore volume, while concomitantly being unaffected in its progress along the column axis by the presence of salt and hemoglobin; (ii) NaCl is in large molar excess, is retained only in the pore volume, and its propagation through the column bed is not affected by the presence of the proteins; (iii) hemoglobin is adsorbed by the resin and its progress through the column beds hence depends on the local salt concentration; (iv) the intraparticle mass transport of all compounds can be described by the linear driving force approximation, and (v) axial and angular dispersion can be neg-

lected. Equilibrium constants (Langmuir type) and mass transfer parameters were determined by independent experiments.

A simple mathematical equilibrium stage model describing approximately the continuous displacement separation of a dilute mixture of three amino acids by NaOH using a cation-exchange resin has been developed by De Carli et al. [37]. When the number of stages N was set to be equal to 50 and assuming constant selectivity values and a sharp displacer front, this model predicted concentration and pH profiles, which were in general agreement to the experimental (batch column and CAC) results. Based on their results the authors stated that under such conditions CAC suffers little or no additional peak dispersion relative to conventional batch chromatography.

Continuous annular size-exclusion chromatography can qualitatively be predicted by using the plate theory (e.g. Ref. [38]), developed already by Scott et al. [7]. As demonstrated for the separation of Blue Dextran from $CoCl_2$ using Sephadex G15, experimental and predicted results are in very good agreement when the theoretical plate height is well known. More stringent mathematical models for continuous annular size-exclusion chromatography were published by Dalvie et al. [39] and Hashimoto et al. [40].

Another model, this time considering the separation of two amino acids by ion-exchange chromatography, was proposed by Kitakawa et al. [41]. This model considers also the dissociation of the amino acids and the eluent buffer composition. In addition, Nernst–Planck-type intraparticle ionic transport equations, and a nonlinear ion-exchange equilibrium term based on ion-exchange selectivity are used.

4. Use and application of continuous annular chromatography

Several examples for the separation of biological molecules by continuous annular chromatography have been published, see Table 1 for a non-exhaustive list. These applications demonstrate that CAC is a viable option for continuous separation even of complex feed-streams. From the viewpoint of bioprocess development, one of the most attractive possible applications of the CAC approach seems to be for the processing of large volumes of complex, low titer product streams, e.g. in molecular or cellular biotechnology. Especially when interfaced to a continuous production process, CAC may considerably contribute to a reduction of the scale of the production facility. Below a number of practical aspects of CAC application in such (bio)separations are discussed.

4.1. The annular column

Bed instabilities are known to be a problem in large-scale preparative chromatography columns. Already Giddings [42] recognized that many industrial-scale packed columns exhibit non-uniformities in flow at large diameters and that theoretically by using a rotating annular column with the same total cross-sectional area and bed height as a fixed column, but with an annulus width small enough so that no flow non-uniformities occur, a process can be scaled up without loss of resolution. In a recent investigation of plasmid DNA purification from clarified bacterial lysates by CAC, Giovannini and Freitag [21] determined the plate height H of the CAC system in comparison to an analytical column of similar width (column diameter vs. annulus width) as well as to a preparative column of similar cross-sectional area and volume under otherwise identical conditions. It was found that the preparative column (5 cm diameter) consistently had lower plate heights than either the analytical one (inner diameter 0.4 cm) or the CAC (annulus width 0.5 cm). This was ascribed to the wall effect. The wall effect, has a dual effect on the column bed. It stabilizes the bed, but it is also known to decrease the packing density (and consequently the plate number) over a distance of up to 30–50 times the particle diameter from the wall [43]. Because 40 μm particles were used in the above-mentioned study, this adverse effect of the wall exerted an influence over 50% of the analytical column bed (and of the annular system) compared to only 5% in the case of the preparative column. A similar decrease of the negative effect of the wall on the column efficiency could be expected during a scale up (increase in annulus widths) of the CAC. In this case the volume of the CAC column could be considerably increased without encountering bed

Table 1
Overview of published biomolecule (pressurized) CAC separations

Substrate	Separation mode	Elution mode	Ref.
Sugars			
Fructose/glucose/sucrose	Ca^{2+} coordination	Isocratic	[30]
Fructose/glucose/sucrose	Ca^{2+} coordination	Isocratic	[33]
Sucrose (beet molasses)	Na^{+} coordination	Isocratic	[52]
Fructoligosaccharides	Na^{+} coordination	Isocratic	[53]
Fructose/mannitol/sorbitol	Ca^{2+} coordination	Isocratic	[31]
Fructose/mannitol/sorbitol	Ca^{2+} coordination	Isocratic	[32]
Amino acids			
Glutamic acid/valine/leucine	Displacement	Isocratic	[37]
Aspartic acid/glutamic acid/glycine	IEC	Isocratic	[25]
Glutamic acid/glycine/valine	IEC	Isocratic/step elution	[26]
Glutamic acid/valine	IEC	Isocratic	[49]
Glutamic acid/valine	IEC	Isocratic	[41]
Glutamic acid/valine	IEC	Isocratic	[47]
Glutamic acid/valine/leucine	IEC	Step elution	[48]
Proteins			
BSA/hemoglobin/cytochrome *c*	IEC	Isocratic	[34]
Myoglobin/hemoglobin	IEC	Step elution	[27]
BSA/hemoglobin	IEC	Step elution	[36]
BSA/salt	SEC	–	[54]
Lipase	SEC	–	[55]
BSA/myoglobin/vitamin B12	SEC	–	[40]
IgG (CHO cell culture supernatant)	Affinity and hydroxyapatite	Step elution	[20]
BSA, IgG	SEC	–	[16]
GFP (yeast cell culture supernatant)	SEC	–	[17]
IgG/IgG polymers	SEC	–	[18,19]
Whey proteins/STI	Displacement	Isocratic	[22]
Nucleic acids			
Plasmid DNA (bacterial lysate)	Hydroxyapatite	Step elution	[21]

instabilities, which would already occur in a conventional preparative column of similar bed height and volume.

A second observation made by Giovannini and Freitag in their study [21] concerned the relationship between the plate height and the rotation speed of the column. As shown in Fig. 3, at a given flow-rate the column efficiency increases with the rotation speed until for a speed of ~520°/h a similar plate height is measured as for the analytical column. Since the chromatographic events in the CAC and the batch column are identical, this behavior was attributed to an artifact caused by the design of the P-CAC fraction collection system. Whereas the outflow of the batch column is continuously monitored, in CAC the ''chromatogram'' is represented by the (steady state) composition of the column's 90 outlets. As a result, each outlet represents an elution angle of 4° over which the concentrations are averaged. Especially at low rotation speed, where a given peak elutes only over a few outlets, this contributes considerably to peak broadening. At higher rotation speeds, the approximation of the true peak shape becomes better and the broadening effect is less pronounced and the efficiency hence approaches that of a conventional column of similar bed width.

A prerequisite for any high-performance separation by CAC is the uniform packing of the annular column. Experience with the commercially available P-CAC system shows that uniform packing can be usually achieved by slurry packing at maximum rotation rate (5000°/h for the P-CAC) and compres-

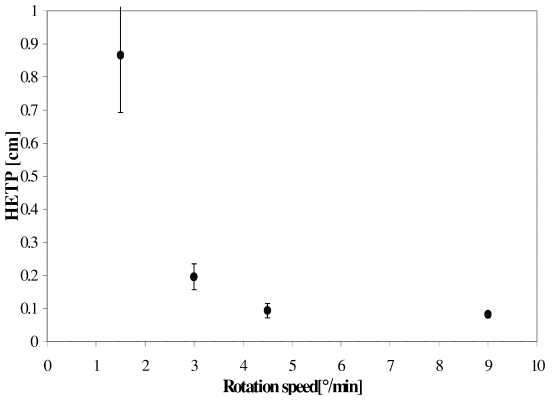

Fig. 3. HETP as a function of the rotation speed for the P-CAC (●) and a small batch column of similar width at the same loading factor. The volumetric flow-rate (■) and the linear flow-rate (▲) were kept constant in all cases. P-CAC conditions were as follows: feed flow-rate (2% acetone in 10 m*M* phosphate buffer pH 6), 0.2 ml/min; eluent flow-rate, 35.85 ml/min; rotation rates, 90°/h (LF=0.102), 180°/h (to LF=0.051), 270°/h (LF=0.034), 540°/h (LF=0.017). Batch column conditions were as follows: volumetric flow-rate, 0.24 ml/min (■); linear flow-rate, 1.6 cm/min (▲); injected volumes, 52.8 μl (LF=0.017), 105.6 μl (LF=0.034), 160.8 μl (LF=0.051), 319.2 μl (LF=0.102) (from Ref. [20] with permission).

sing the bed to height at maximum main eluent flow [22]. Since in CAC the effluent is collected over a certain angular region usually represented by several outlets, minor stochastical deviation of the angular displacement at constant rotation rate must be expected due to variations of the hydrodynamical resistances of the exit ports [44]. True inhomogeneities in the annular bed (''bed warping'') are easily recognizable, e.g. by the fact that at constant rotation rate the elution angle of an inert tracer shifts by more than 20°.

4.2. Method transfer and scale up

Since the chromatographic events are identical in batch and continuous annular chromatography, and since an annular column can be expected to be of similar efficiency as an analytical column with a diameter similar to the annulus width, the transfer of a method developed using such an analytical column to the CAC system should be easy. In principle, a simple relationship exists between the retention time (t_R) in batch chromatography and the rotation speed ω and the elution angle α_R in CAC [28]. Assuming an isothermal, steady-state separation process with uniform concentration and fluid profiles in the radial direction and local equilibrium between fluid and solid phase, we expect:

$$\alpha_R = t_R \omega \tag{4}$$

Using this approach Giovannini and Freitag [20] found—taking the isolation of a recombinant antibody from CHO cell culture supernatants by recombinant (r) Protein A and hydroxyapatite chromatography as an example—that the direct transfer of a method developed for a 3-ml batch column to a 500-ml P-CAC is indeed possible. Concomitantly, the compatibility of the CAC with complex, realistic feed streams, step gradient elution, and continuous-column regeneration was demonstrated for the first time in this paper. Similar yields (between 75 and 85% in case of the rProtein A columns) and purification factors (50 and 52 for the rProtein A CAC and batch columns, respectively) were obtained and 99% of the nucleic acids were removed from the product by both columns. A direct comparison showed, however, that the chromatograms recorded for the CAC and the batch column were not identical. Instead the peaks were shifted in the CAC separation.

In a second application, namely the isolation of plasmid DNA from clarified *E. coli* lysates by hydroxyapatite chromatography [21], the dimensionless loading factor (LF) concept was developed as a concept for more direct method transfer. The LF, known from batch column separations, where it is used to set the injected sample volume in correlation to the total column volume and whenever columns of different sizes are to be compared, is given by Eq. (5):

$$\mathrm{LF}\ [-] = \frac{Q_T\ [\mathrm{cm^3\ min^{-1}}]\ t_I\ [\mathrm{min}]}{H\ [\mathrm{cm}]\ S\ [\mathrm{cm^2}]} \tag{5}$$

For CAC the authors proposed the following equation:

$$\mathrm{LF}\ [-] = \frac{Q_F\ [\mathrm{cm^3\ min^{-1}}]\ 360\ [^\circ]}{H\ [\mathrm{cm}]\ S\ [\mathrm{cm^2}]\ \omega\ [^\circ\ \mathrm{min^{-1}}]} \tag{6}$$

with t_I being the time required for injection, H the bed height, and S the cross-sectional area.

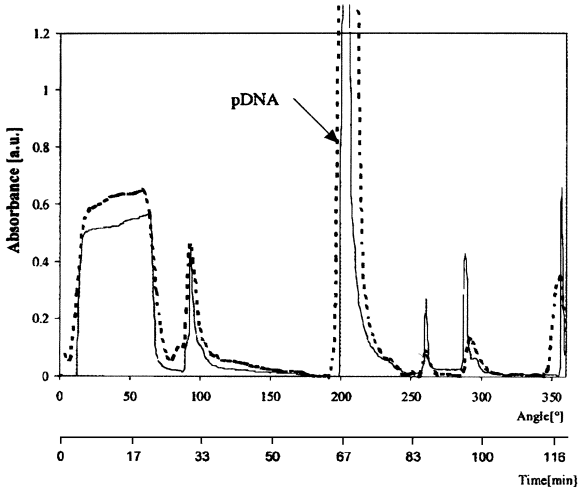

Fig. 4. Overlay of the CAC (dashed line) separation and the separation on the small batch column (continuous line) of a plasmid DNA (pDNA) containing clarified bacterial lysate (from Ref. [21] with permission).

Taking into account that in the P-CAC system (pressure limit 3 bar) feed, step eluents, and CIP agents are introduced from fixed inlets so that in-between steps are filled with main eluent, the calculation of LF for each step can be used for the direct transfer of a given separation method from batch to CAC column or vice versa. Fig. 4 shows the overlay of a CAC (15 cm O.D.; 0.5 cm annulus width; 21.5 cm bed height) separation of plasmid DNA from a clarified lysate and the one performed on an analytical column (250×4 mm). The conditions for the optimized CAC system are given in Table 2. The general applicability of the loading factor concept most recently has also been demonstrated by the transfer of a protein separation in the displacement mode from the batch column to the P-CAC [22]. Given the fact that displacement chromatography is inherently sensitive to even minor changes in the chromatographic conditions, this successful transfer can be considered a major argument for the conclusion that by using the loading factor concept a separation developed using a small (few ml) batch column can be transferred to the P-CAC and yield similar results in terms of product yield and quality.

The LF concept thus allows the successful scale-up from a 3-ml analytical batch column to a 500-ml CAC system. Further scale-up by increasing the feed

Table 2
Plasmid DNA separation from clarified lysate; optimized conditions for a batch column (250×4 mm) and for the CAC system (15 cm O.D.; 0.5 cm annulus width) [21]

Batch column	
Feed injection	0–5.87 min
160 m*M* phosphate pH 6	–13.58 min
10 m*M* phosphate pH 6	–16.76 min
400 m*M* phosphate pH 6	–22.63 min
10 m*M* phosphate pH 6	–24.20 min
CIP (0.5 *M* NaOH)	–27.16 min
10 m*M* phosphate pH 6	–35.37 min
Regeneration (400 m*M* phosphate pH 6)	–36.53 min
10 m*M* phosphate pH 6	–40.00 min
CAC	
Feed rate	15.0 ml/min
First eluent (160 m*M* phosphate pH 6)	19.0 ml/min
Second eluent (400 m*M* phosphate pH 6)	15.0 ml/min
CIP (0.5 *M* NaOH)	7.5 ml/min
Regeneration (400 m*M* phosphate pH 6)	3.0 ml/min
Main eluent (10 m*M* phosphate pH 6)	41.5 ml/min
Rotation rate	540/h
Column flow	101.5 ml/min
	4.46 cm/min
	0.207 column volumes/min
Bed height	215 mm

concentration is limited by the viscosity difference between feed and eluent. As a rule of thumb, a viscosity factor of 2 is the limit before flow inhomogeneities and ''viscous fingering'' occur [33]. In CAC, scale-up is also possible via the annulus width (see above for a discussion of this factor on the bed stability) but this requires a suitable inlet design [10]. In addition, while it should be possible to approximate the minimum geometry (annulus radius and width, bed height) of an annular chromatograph for the separation of the components, for the CIP and adsorbent regeneration steps the reaction constants must be known in order to determine the limiting factor for the final optimized geometry [16].

4.3. Performance, productivity, resolution and throughput

It has been argued based on purely theoretical considerations, e.g. by Seidel-Morgenstern and co-workers [45,46], that in terms of production rate and eluent consumption annular chromatography should not differ from conventional (non-continuous) chromatography. In their comparison a mathematical model was used by the authors to optimize these two parameters for a minimal purity of the collected fractions of 99% and at minimum recovery of 95%.

From a more practical point of view, Buchacher and co-workers [18,19] investigated productivity and buffer consumption for the separation of monomeric IgG from its polymers by batch and continuous annular size-exclusion chromatography using Superdex 200 to pack the annular column and without including continuous CIP and regeneration steps. They found that when setting the recovery rate at 85%, the cycle time, which is an inverse measure of productivity, was higher for the batch than for the annular chromatography after the first cycle. In batch chromatography a new cycle typically begins only when the preceding one has been completely finished. In this case, the cycle time is constant for all cycles. In annular chromatography only the time for further application of sample counts, because the elution is performed simultaneously. From the second cycle onwards, annular size-exclusion chromatography therefore has an advantage in terms of productivity and consequently in terms of buffer consumption compared to conventional batch chromatography.

Up to a point, resolution can be increased in chromatography by reducing the liquid phase flow-rate (higher efficiencies, lower plate heights). However, under such conditions the throughput is also reduced. Kitakawa et al. [47] developed a partial recycling operation mode for CAC, which allows the complete separation of a binary mixture while keeping the throughput high. Fukumura et al. [48] extended this method to the successful separation of a ternary amino acid mixture by step elution in combination with a partial recycling operation.

The principle of the partial recycling operation mode is illustrated in Fig. 5a. A chromatographic system is used, in which the eluent flow is not controlled by pressure but by a multichannel peristaltic pump for variable eluent withdrawal [49]. Let's assume that a binary mixture, where component A is less retained by the adsorbent than component B, elutes partially separated with an overlap of X. Product streams containing pure A and B, respectively, are directly collected, while the eluate from the overlap zone is split into two fractions. In fraction A + B component A is in excess over component B and in fraction B + A component B is in excess over component A. Sample A + B is again injected into inlet nozzles placed after the initial feed inlet nozzle, while B + A is reinjected via an inlet nozzle placed before that of the initial feed nozzle. In this way, a complete separation of the binary mixture can be achieved as demonstrated for the case of a mixture of glutamic acid and valine (Fig. 5b). Such a steady state operation with partial recycling, which leads to complete separation, cannot be carried out in the conventional column chromatographic operation, which is intrinsically an unsteady state process. It should, however, be interesting to compare the performance of such a CAC system with a continuous separation of a binary mixture by simulated moving bed (SMB [50]) but also by the recently developed closed-loop recycling with periodic intra-profile injection (CLRPIPI [51]) method. CLRPIPI has been developed for the separation of binary mixtures. The process is repetitive and a steady state is reached where highly purified fractions can be collected. However, contrary to SMA and CAC, CLRPIPI is not continuous.

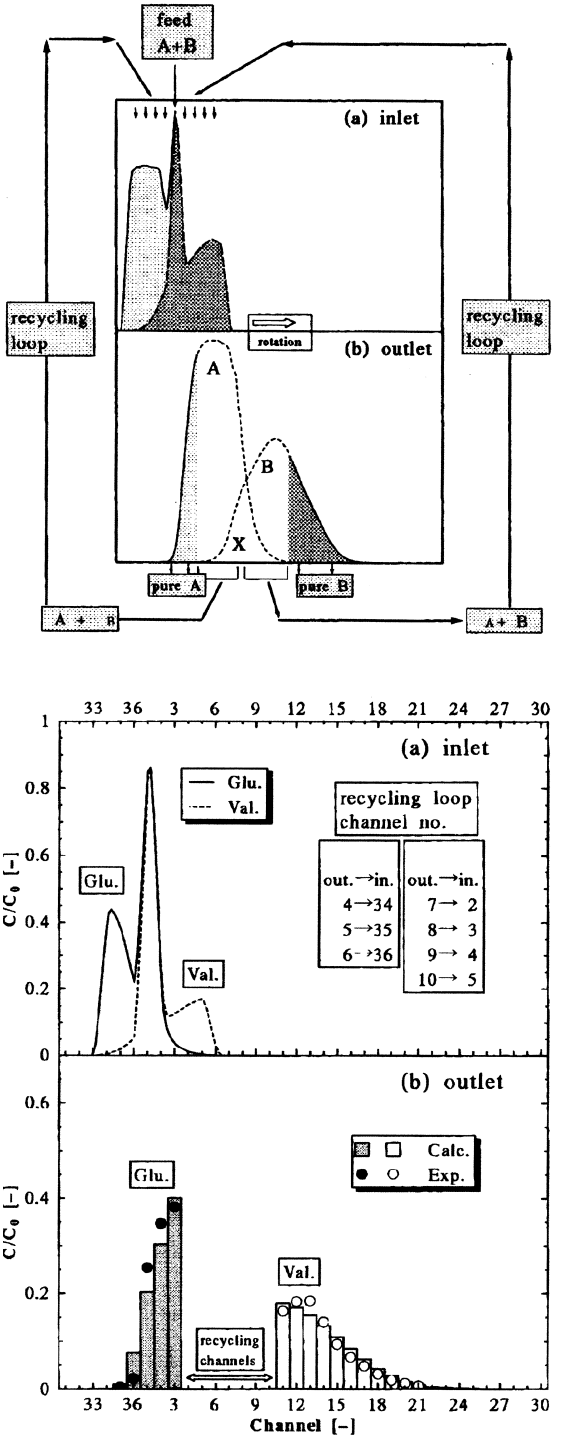

Fig. 5. (a) Schematic presentation of the concept of CAC with partial recycling; (b) elution profiles of amino acids in CAC with partial recycling (from Ref. [47] with permission).

5. Perspectives

The advances in continuous annular chromatography in recent years clearly demonstrate that this method performs, for several separation modi, at least as well as conventional batch chromatography. The tools for convenient method transfer from a batch to the continuous column or vice versa and for numerical profile prediction are available. The potential of CAC lies clearly in its continuous character. To our knowledge, Bayer AG is currently the only company developing CAC for a relevant bioseparation process: the concentration of continuously produced recombinant factor VIII. This, and hopefully also other developments, will show whether the integration of CAC up- or downstream in a continuous process has economical advantages over conventional chromatography. The additional determining factor for the success of CAC will be the development of reliable CAC devices allowing a reproducible and truly continuous, long-term separation using chromatographic beds at industrial size.

6. Nomenclature

BSA	bovine serum albumin
CAC	continuous annular chromatography
CHO	Chinese hamster ovary
CIP	clean in place
CLRPIPI	closed-loop recycling with periodic intra-profile injection
GFP	green fluorescent protein
HETP	height equivalent to a theoretical plate
IEC	ion exchange chromatography
IgG	immunoglobulin G
ORNL	Oak Ridge National Laboratory
P-CAC	preparative continuous annular chromatography
SEC	size-exclusion chromatography
SMB	simulated moving bed
c_F	solute concentration in the feed mixture
c_i	liquid phase concentration
D_z, D_θ	band broadening by dispersion and diffusion in the axial (z) and the angular (θ) direction
F	phase ratio
H	bed height

I.D.	inner diameter
K	distribution coefficient of a given substance between the stationary and the mobile phase
$k_o a$	global interphase mass transfer coefficient
LF	loading factor
n	number of transfer units ($n = k_o a z/u$)
N	number of stages/plates
O.D.	outer diameter
Q_F	feed flow-rate
q_i	solid-phase concentration
QI	quantity of solute injected with the feed mixture per unit cross-sectional area
Q_T	total flow-rate of fluid through the annular bed
S	cross-sectional area
t_I	time required for injection
t_R	retention time
u	axial flow velocity
z	axial direction
α_R	elution angle
ε	column porosity
ω	column rotation velocity
θ	annular direction

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