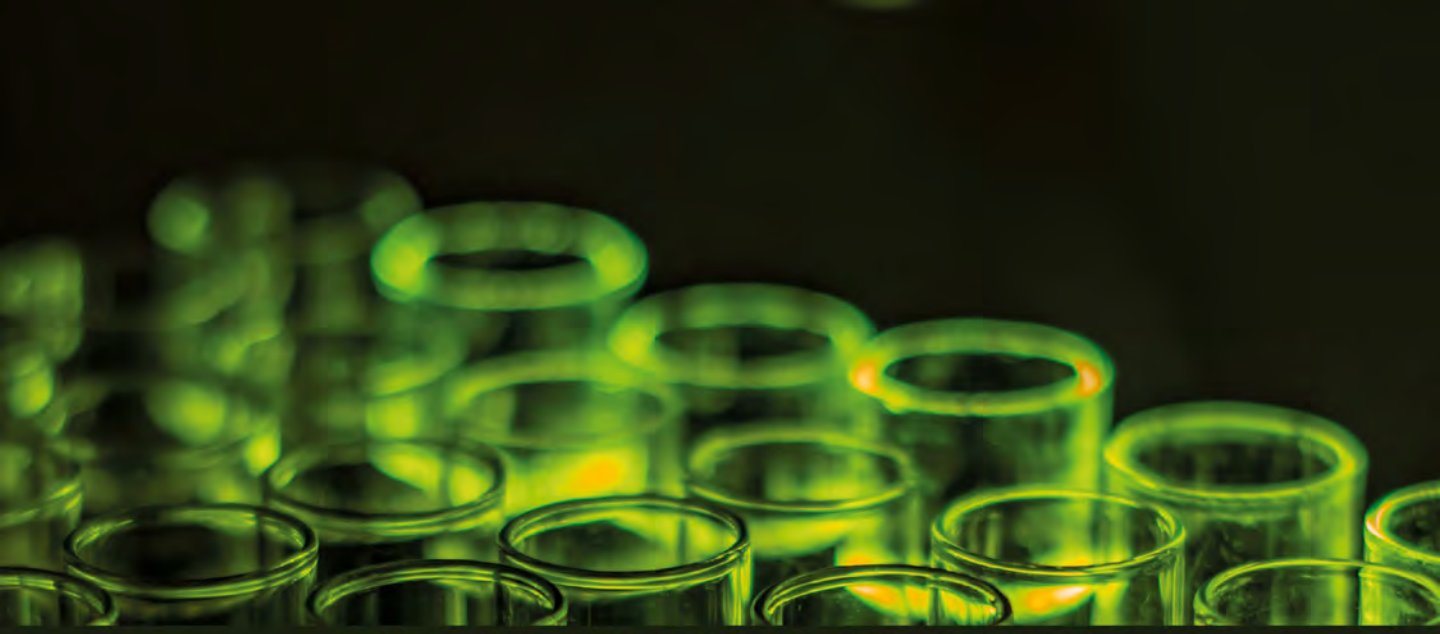

HANDBOOK OF SEPARATION SCIENCE

Colin F. Poole, Series Editor

ION-EXCHANGE CHROMATOGRAPHY AND RELATED TECHNIQUES

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CHAPTER

26

Continuous ion-exchange chromatography for protein polishing and enrichment

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OUTLINE

1 Introduction

Proteins have demonstrated considerable efficacy in the treatment of a variety of complex diseases owing to their high specificity and affinity for the target, reduced toxicity, and extended pharmacokinetics. Despite their potential and promise, the challenges associated with process development and manufacturing need to be considered. The advances in upstream bioprocesses, particularly the use of continuous bioreactors, are likely to replace conventional batch bioreactors, thus shifting the focus to downstream separation and purification. To meet the stringent

purity criteria of the downstream process, one or more polishing steps are necessary. The most versatile and flexible polishing technique is ion-exchange chromatography (IEC). Based on the electrostatic interaction between a charged protein and an oppositely charged chromatographic resin, IEC can provide a high-resolution separation with high loading capacity. However, sometimes it is difficult for complex mixtures to obtain a high-resolution separation to yield a considerable amount of pure target compound using traditional batch IEC.

2 Limits of batch chromatography

Generally, polishing chromatography is conducted as a batch process that utilizes a single chromatographic column to separate target product from impurities. The similarities between the product and product-related impurities often result in overlapping peaks and a low resolution. This overlapping region contains a considerable amount of product, especially for complex mixtures with many product-related impurities. Discarding the overlapping region completely would improve purity, but at the expense of a reduced yield. Conversely, broadening the collection window would increase yield, but decrease purity. This purity-yield trade-off is the primary limitation of batch chromatography, which can be visualized by a vector projection of Pareto curves in the purity-yield plane as seen in Fig. 1 with three-dimensional space coordinates productivity, yield, and purity. Note that the productivity in this review is defined by time.

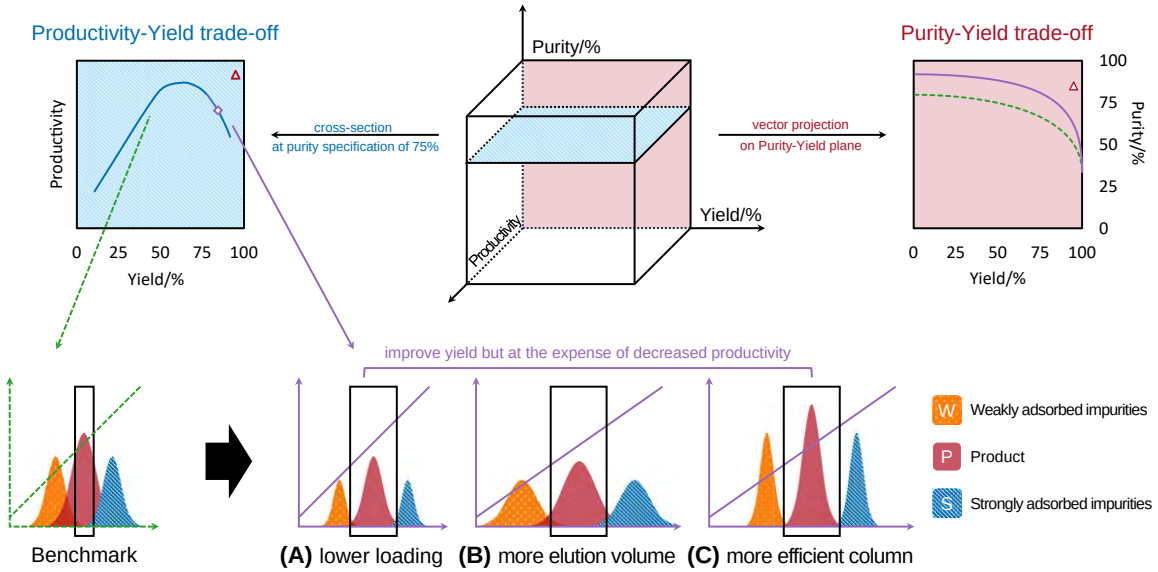

FIG. 1 Productivity-yield trade-off (cross-section on the *left*) and purity-yield trade-off (vector projection on the *right*) of batch chromatography. Solutions for surmounting purity-yield trade-off include continuous modes (*triangle* points) and traditional methods at expense of decreased productivity, lower loading (A), more elution volume (B), and more efficient column (C).

The most popular methods for surmounting the purity-yield trade-off include: (1) decreasing the protein loading in Fig. 1A, which results in lower product concentration; (2) increasing the elution volume in Fig. 1B, which needs more eluent and operation time; (3) using a more efficient column with smaller particles in Fig. 1C, which increases the back pressures and reduces the allowable velocity. All these solutions can improve yield, but at the expense of decreased productivity leading to a productivity-yield trade-off.

3 Continuous chromatography for protein purification

The utilization of continuous chromatography has emerged as a viable solution to address the trade-offs associated with batch chromatography. This approach offers the advantage of simultaneously achieving enhanced yield and higher purity, and increasing productivity at fixed purity specifications due to the utilization of the interval time. In most cases, modes for continuous operation can also save fresh eluent and improve resin utilization.

3.1 Rotating chromatography

The easiest way of transforming from batch to continuous separation is rotating chromatography. Depending on where the setup rotates, this method is divided into two modes: column switching chromatography with a rotated carrousel and annular chromatography with rotated columns.

3.1.1 Column switching chromatography

Column switching chromatography is a form of rotating chromatography that utilizes two or more columns mounted on a carrousel with interconnecting valves as shown in Fig. 2B. This method is used for relatively simple separations, where either the impurities or the target component can be strongly bound to the stationary phase while the other is easily eluted. In this process, the feed is pumped through a column until a breakthrough of the target component is observed. The injection is then switched to a second column, while the first one is desorbed by introducing an eluent from a second pump. The initial eluent conditions are readjusted before the first column is again used for adsorption.

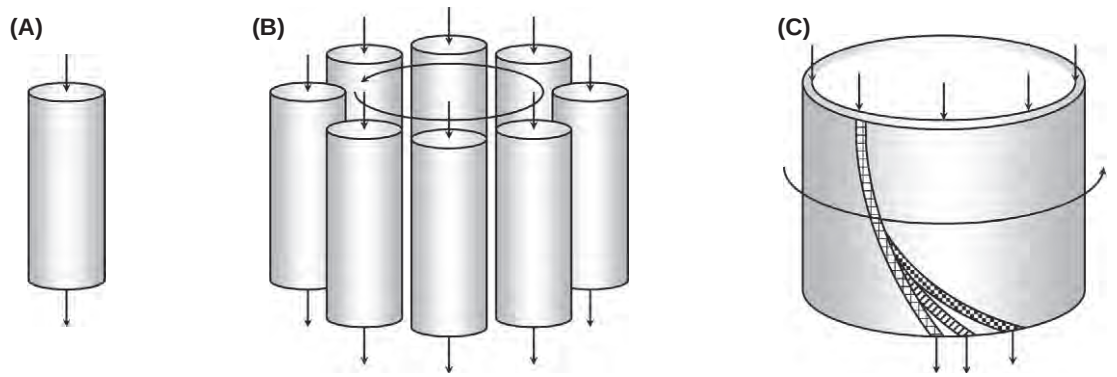

FIG. 2 An overview of batch chromatography (A), column switching chromatography (B), and annular chromatography (C).

3.1.2 Annular chromatography

The other rotating chromatography for continuous separation of complex mixtures is the annular chromatography developed in 1949. In this process as illustrated in Fig. 2C, the stationary phase is positioned within the annular space of two concentric cylinders, which rotate around a central axis. The feed mixture is constantly supplied to the top of the bed, and the eluent is injected evenly across the annulus. The feeding and fraction collector remain stationary, while the annular column rotates gently creating a cross-current movement of solid and liquid phases. The components of the feed mixture migrate in helical bands downward through the bed and are separated into several product streams, which elute at different angles at the bottom of the bed. Annular chromatography can be conducted in gradient conditions to improve multicomponent separations by introducing eluents with different solvent strengths. This continuous process offers high productivity and resolution and can be used to purify large quantities of material in a short time. However, its main disadvantage is the difficulty in packing efficient columns. This is due to peak broadening caused by angular rotation, axial, and tangential dispersions.

3.2 Simulated moving bed

In comparison with the two chromatographic techniques operated in a concurrent mode (also referred to as cross-current), the counter-current operation can provide a more efficient separation. All counter-current chromatography systems involve the solid phase and liquid phase moving in opposite directions. However, it is unfeasible to move the solid phase in most cases. Consequently, some technical solutions must be employed to simulate the movement of the solid phase.

Simulated moving bed (SMB) is one of the most well-established techniques operated in counter-current mode. It involves the switching of inlet and outlet valves of columns connected through a series of valves to simulate the movement of solid phase. This operation was developed in 1950 as the first continuous chromatography apparatus operated in counter-current mode. Subsequently, SMB has been the subject of extensive research. More information on improved SMB is available in the textbook by Kaspereit and Schmidt-Traub [1].

3.2.1 Classical simulated moving bed

Fig. 3A illustrates a conventional SMB with four zones: one to feed, one to separate the binary mixture under isocratic conditions, one to regenerate the adsorbent, and one to regenerate the eluent. The mobile phase flows through the columns of the fixed bed in a single direction. To achieve counter-current flow of both phases, the columns are periodically moved upstream in the direction opposite to the liquid flow. However, all ports are shifted in the direction of the liquid flow-through valves. The process is thus named after the periodic switching of valves that simulates a genuine counter-current movement of the two phases. A cycle is completed when the number of switching events or shifting intervals is equal to the number of columns in the system, and all external streams have returned to their initial positions.

3.2.2 Gradient simulated moving bed

The classical SMB presents a major disadvantage in its isocratic operation. To counter this, the gradient SMB was proposed. It utilizes a two-step gradient to adjust the solvent strength of components as shown in Fig. 3B. This technique proves invaluable in improving process performance and could offer a high-resolution separation that would not be achievable through isocratic conditions. Additionally, it was applied to address the challenge of finding suitable

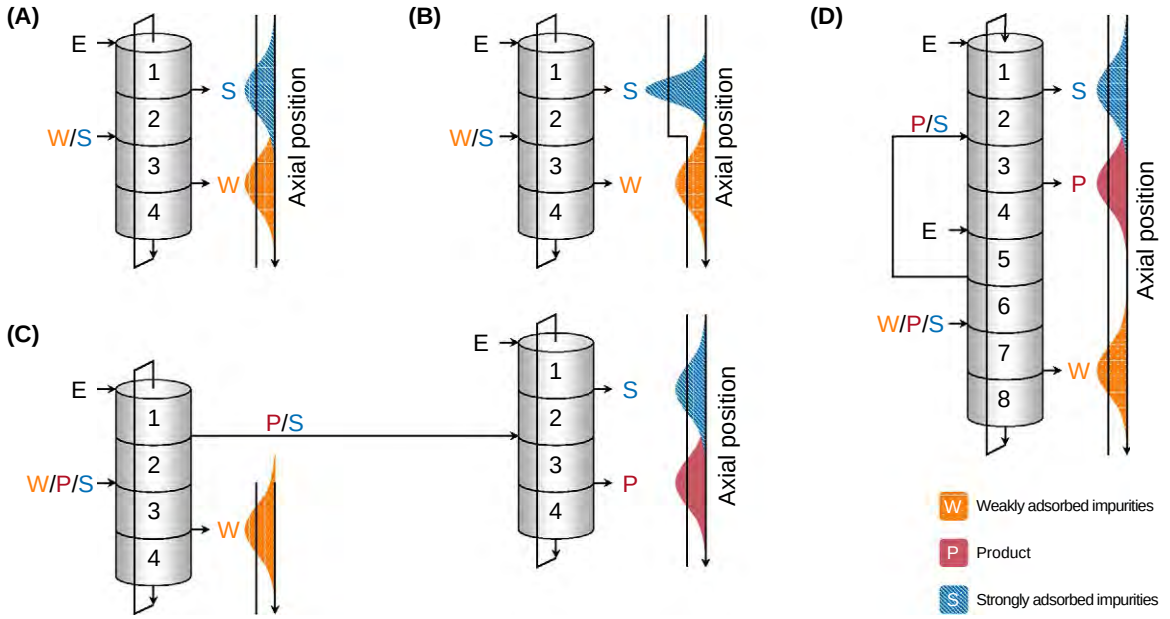

FIG. 3 An overview of classical four-zone SMB (A), gradient SMB (B), two-units-in-series SMB for ternary separation (C), and eight-zone SMB interconnected for ternary separation (D). *W*, weakly adsorbed impurities; *P*, product; *S*, strongly adsorbed impurities; *E*, eluent.

liquid and solid phases for isocratic SMB, where only the first component was well adsorbed.

3.2.3 *Simulated moving bed for ternary separation*

In the polishing step for protein purification, normally two variants need to be removed from a purified protein. The classical SMB is limited due to its two outlets ports for binary separations.

To facilitate its applications in ternary mixtures, various improvements have been made. One approach is the two-units-in-series SMB as shown in Fig. 3C. In this setup, the component is isolated from the other two components in the first unit before transferring into the next unit for further separation. It is not necessary for the chromatographic conditions of the first unit to be consistent with those of the second unit. This inconsistency can be conducive to isolation.

An alternative approach to the in-series unit is to increase the number of zones and recirculate partially separated streams internally, for example, the eight-zone interconnected unit in Fig. 3D. This interconnected unit necessitates partial solvent removal between the units to balance the dilution of the product delivered by the upstream SMB unit. Additionally, a restrictive design aspect of such processes is the identical switching time applied to all zones.

3.3 Multicolumn continuous chromatography

The aforementioned SMB improvements can be concluded as follows: (1) the softening of the trade-offs of batch conditions; (2) the use of gradient elution for improved resolution; (3) the availability of more operating modes or more columns for ternary separation; and (4) the

possibility of different chromatographic conditions for different columns. However, these improvements also have some drawbacks: (1) the inability of gradient SMB for ternary separation; and (2) the limited design due to the identical switching time.

With the advances in technology, there are now several state-of-the-art multicolumn continuous chromatography systems that combine the features of those improvements and overcome the limitations of SMB.

3.3.1 Multicolumn counter-current solvent gradient purification

Based on the aforementioned SMB improvements, a novel continuous chromatography known as multicolumn counter-current solvent gradient purification (MCSGP) was introduced for ternary separation [2].

In batch chromatography, the side fractions are typically discarded as waste. The side fractions contain: (1) the product (termed P) containing weakly adsorbed impurities (termed W) eluting before P; (2) the strongly adsorbed impurities (termed S) eluting after P; (3) a considerable amount of P. However, MCSGP is capable of internally recirculating the side portions, thus providing a remarkable pure product in comparison with batch chromatography. Note that W or S refers to a group of impurities with analogous chromatographic behavior, rather than a single species.

The initial draft of MCSGP consisted of six columns, each of which was completed through individual steps necessary for purification. However, due to the varying time intervals required to complete each step, some steps can be combined and performed in one column. After multiple iterations and optimization, MCSGP was eventually simplified into a two-column mode, referred to as the twin-column MCSGP [3].

The twin-column MCSGP operates in two modes: interconnected (I) and batch (B), which are determined by the position of the inlet and outlet column valves. This process achieves counter-current flow of the stationary and mobile phases, like SMB, by periodically reversing the direction of the liquid flow upstream of the column. A schematic representation of the twin-column MCSGP is presented in Fig. 4, where Column 2 is loaded with the effluent from Column 1 in the first switch and vice versa in the second switch. Depending on the operational mode and characteristic time, the MCSGP cycle can be divided into four distinct parts:

I1: Column 1 loaded with W/P/S is upstream of preequilibrated Column 2. As the eluent (E) 's solvent strength increases, W/P from the outlet of Column 1 are introduced into Column 2 and mixed with a second eluent stream of lower solvent strength for inline dilution (D).
B1: Columns 1 and 2 are operated in parallel, with feed (F) introduced into Column 2 and P eluted from Column 1.
I2: Similar to I1, but the overlapping region for recirculation is P/S;
B2: The eluent and regeneration (R) buffer are used to yield pure W and S from Columns 2 and 1, respectively.

Following the exchange of positions between the two columns, an MCSGP switch is completed. Upon repeating the four steps and the two columns returning to their original configurations, an MCSGP cycle is concluded. It can be deduced that one cycle is composed of two switches.

After several cycles, the chromatography system reaches a steady state, characterized by nearly identical UV profiles and highly comparable purity and yield from the fraction collected at each switch. The loading entering the connected column for each switch is composed of three parts: W/P, P/S, and the fresh feed, which can be calculated using mass conservation.

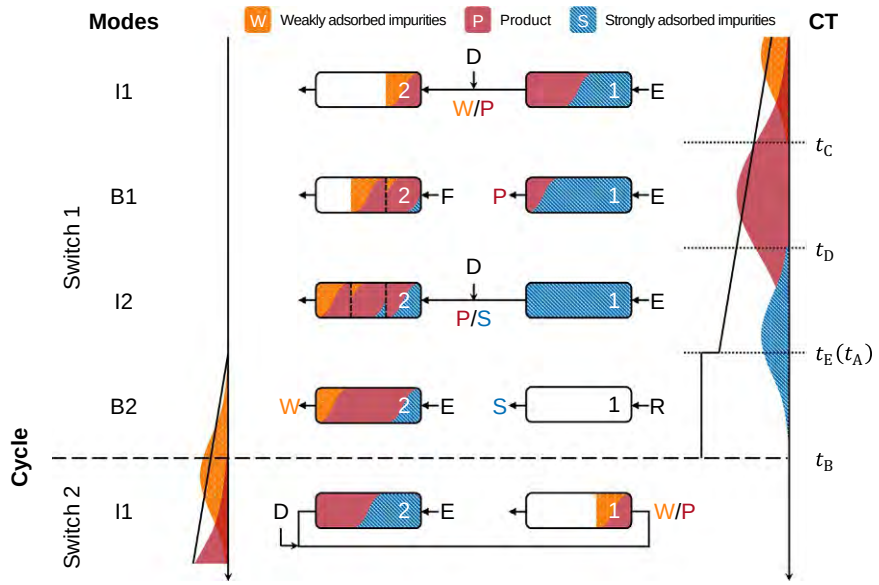

FIG. 4 Principle of multicolumn counter-current solvent gradient purification (MCSGP) in a twin-column mode. *I*, interconnected; *B*, batch; *W*, weakly adsorbed impurities; *P*, product; *S*, strongly adsorbed impurities; *D*, inline dilution; *E*, eluent; *F*, feed; *R*, regeneration buffer; *CT*, characteristic times.

The MCSGP process combines the SMB improvements while avoiding the limitations and granting greater flexibility of process design. For example, the implementation of linear gradient conditions can provide a higher resolution of co-eluting components. The linear gradient conditions and counter-current flow separation resolve the purity-yield trade-off associated with batch conditions. Furthermore, the twin-column setup for ternary separation is simpler than for SMB, yet the column properties must be identical. Krattli et al. [4] demonstrated an extension for quaternary separation by introducing more columns and fractions, though they concluded that more than five fractions are impractical due to the pressure drop.

3.3.2 *Two-column batch-to-batch recirculation process*

The steps B1 and B2 in MCSGP require time for loading and regeneration, respectively, because one column must wait for the other to finish. This phenomenon is referred to as column synchronization. To overcome this time-consuming limitation, Nilsson's group [5] developed the two-column batch-to-batch recirculation process as presented in Fig. 5. This new mode is similar to MCSGP, with the only difference being the loading order. In MCSGP, W/P, the fresh feed, and P/S are loaded in order. But in this new mode, W/P, P/S, and the fresh feed are loaded. As we can see, the loading orders of recirculated species (W/P and P/S) are interconnected. This provides a distinct advantage that the new setup can run in an integrated column sequence as any batch setup, such as in a two coupled ÄKTA system. Strube's group [6] similarly identified the issue of column synchronization in MCSGP. To address this, they developed a unit named continuous twin-column chromatography (CTCC), which employs the same loading sequence as Nilsson's group but achieves it by storing the fractions W/P and P/S in additional tanks.

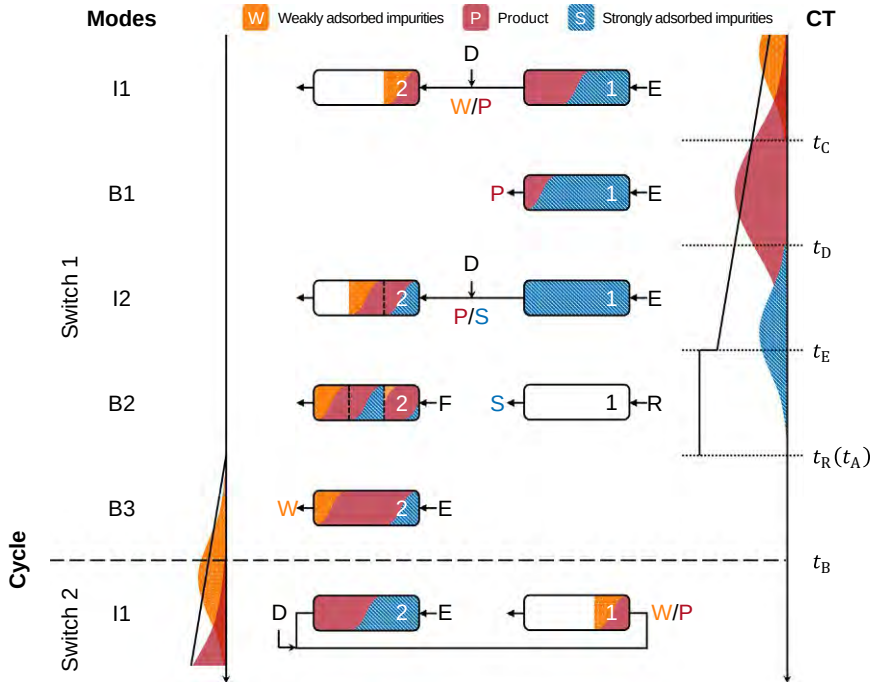

FIG. 5 Principle of two-column batch-to-batch recirculation process step. *I*, interconnected; *B*, batch; *W*, weakly adsorbed impurities; *P*, product; *S*, strongly adsorbed impurities; *D*, inline dilution; *E*, eluent; *F*, feed; *R*, regeneration buffer; *CT*, characteristic times.

3.3.3 *N-rich*

In contrast to the previously established methods designed for the cyclic steady state to obtain product with uniform concentration and purity, N-rich aims to enrich target compounds (commonly referred to as product-related impurities), while other compounds in the mixture are depleted [7–10]. As illustrated in Fig. 6, N-rich operates on a similar concept as MCSGP. Bigelow et al. [7] conducted the first N-rich process in an MCSGP unit, recognizing it as a part of the full MCSGP process. The only difference between MCSGP and N-rich is that the recirculated region in N-rich contains species eluting earlier or later than the target compound in MCSGP. This enables the target impurities to be accumulated and enriched after each cycle. At the end of the cyclic phase, the highly enriched impurities are eluted in one step with high purity and concentration, thus reducing the number of fractions for analysis and eliminating the need for upconcentration steps. An alternative variation of N-rich is the twin-column recycling chromatography with a solvent gradient (TCRC-SG) proposed by Wei et al. [11–13]. By manipulating operational conditions, TCRC-SG can be employed for both enrichment of impurity components similar to N-rich [11,12], and multicomponent separations akin to MCSGP [13].

The classical chromatographic technique for isolating and enriching impurities is to pool the fractions containing impurities from multiple high-performance liquid chromatography

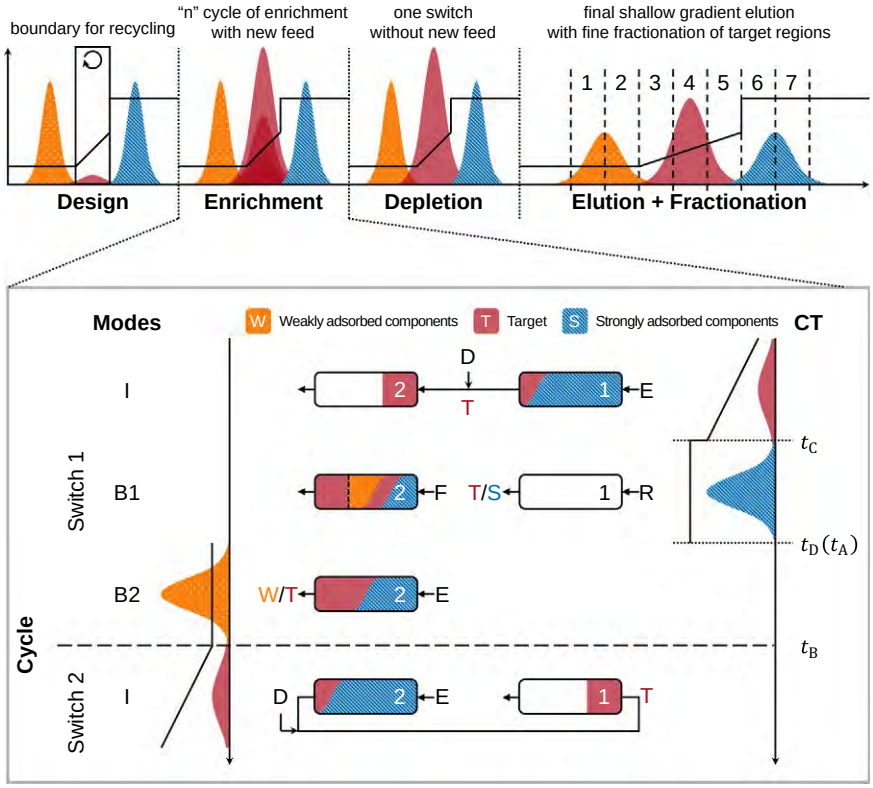

FIG. 6 Principle of N-rich process. *I*, interconnected; *B*, batch; *T*, target compound; *D*, inline dilution; *E*, eluent; *F*, feed; *R*, regeneration buffer; *CT*, characteristic times.

runs until sufficient material has been accumulated. This process is often laborious and results in a productivity-yield trade-off. In contrast, N-rich provides a novel and convenient method for producing target compounds in large quantities required for further detailed studies.

3.3.4 *Flow2*

Recently, frontal chromatography has been proposed as an alternative to the aforementioned bind-elute modes due to its high productivity and straightforward operation. In this process, loading is continuously fed into the column beyond its binding capacity, while the effluent is collected as products.

Morbidelli's group has proposed a new twin-column counter-current frontal chromatography, Flow2 [14,15]. In this setup, the binding components can be classified into W and P. Fig. 7 illustrates a cycle of the Flow2 process:

I1: In a two-column-in-series configuration, the sample provided in a quantity sufficient to exceed the binding capacity of both columns is introduced via the inlet of Column 1. The excess sample that cannot be bound with Column 1 is then adsorbed in Column 2.
I2: The injection of a wash buffer into Column 1 pushes the residual sample in its pore space into Column 2, where it is mixed with a second stream of eluent of lower solvent

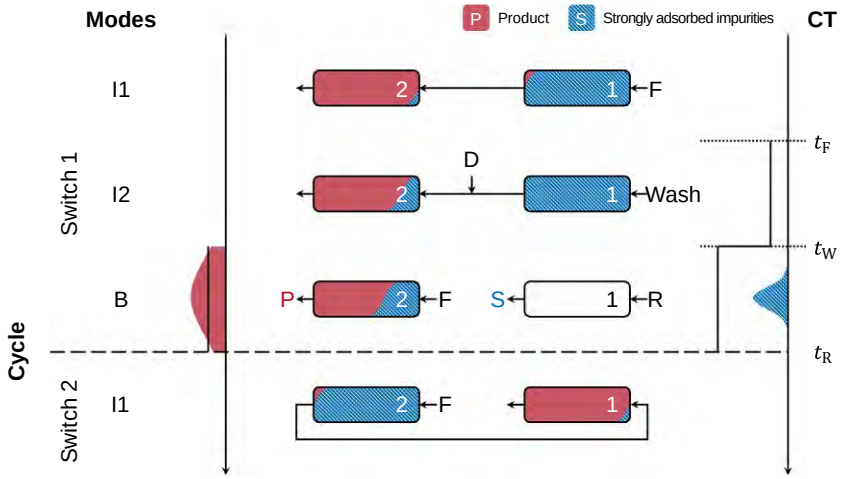

FIG. 7 Principle of Flow2 process. *I*, interconnected; *B*, batch; *P*, product; *D*, inline dilution; *F*, feed; *R*, regeneration buffer; *CT*, characteristic times.

strength for inline dilution, thus minimizing sample loss.
B: The columns are operated in parallel, with Column 1 regenerated in the same manner as in the batch process, and Column 2 loaded with fresh feed.

The exchange of positions between two columns completes a Flow2 switch. Upon returning to their original configurations, a Flow2 cycle is concluded. One cycle is composed of two switches. After several cycles, the chromatography system reaches a steady state with comparable purity, yield, and a negligible mass balance error between the feed and eluted protein.

Compared to batch chromatography, Flow2 demonstrates a wider design space of loading conditions in terms of concentration, composition, and flow rate, providing beneficial alleviation of the productivity-yield trade-off. Additionally, the process displays increased process robustness, particularly against the loading variations. Furthermore, the inline dilution provides a wash buffer with lower solvent strength, thus broadening the design space and displaying high yields without compromising purity (softening the purity-yield trade-off).

Nevertheless, due to the inherent limitations of frontal chromatography, Flow2 is only applicable in scenarios in which the purity requirement is low and the product has weaker adsorbability than other compounds, such as the polishing step of monoclonal antibodies (mAbs).

3.3.5 *Multicolumn self-displacement chromatography*

The Flow2 mode needs a target product with the weakest adsorbability, which is often unfeasible in practice. As an alternative, the displacement mode may be employed if there are compounds with weaker binding affinity than the target product. In the classical IEC process, protein binding to the medium is often recovered by the displacement with counter-ions or appropriate chemical displacers.

A multicolumn self-displacement continuous chromatography with the sample as displacer was proposed by Lenhoff's group [16,17] to overcome the limitations of batch chromatography and reduce the overlap of neighboring variants in a ternary mixture. Their setup includes two-, three-, and four-column configurations. The two-column operation is conceptually

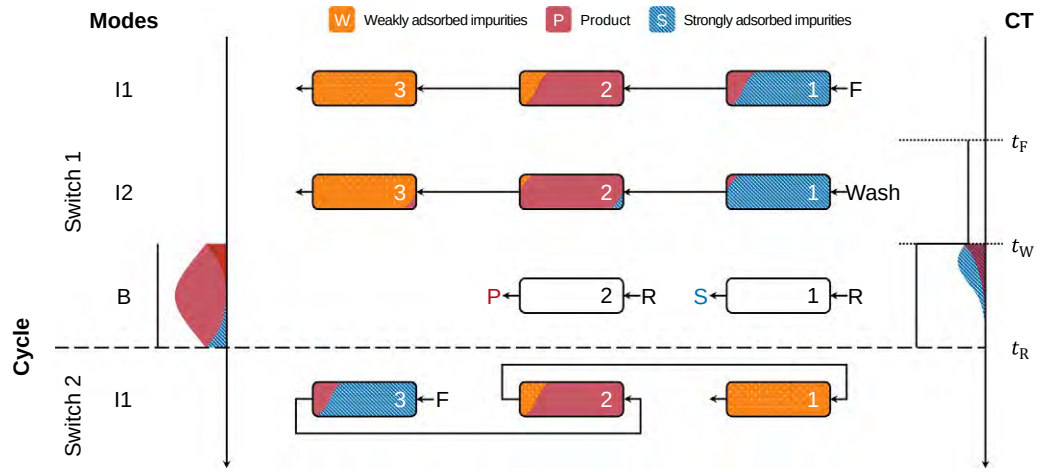

FIG. 8 Principle of three-column displacement chromatography. *I*, interconnected; *B*, batch; *P*, product; *S*, strongly adsorbed impurities; *F*, feed; *R*, regeneration buffer; *CT*, characteristic times.

similar to Flow2, but with different displacers and target compounds.

The most general setup is a three-column configuration as shown in Fig. 8, which incorporates interconnected and batch modes in a cyclical fashion:

I1: In a three-column-in-series configuration, the sample provided in a quantity sufficient to exceed the binding capacity of all columns is introduced via the inlet to Column 1. The excess sample that cannot be bound by Columns 1 and 2 is then adsorbed onto Column 3. W, P, and S are retained in Columns 3, 2, and 1, respectively, despite their broad overlapping zones.
I2: The injection of a wash buffer into Column 1 pushes the residual sample in the pore space of Columns 1 and 2 onto Column 3, thus minimizing sample loss.
B: The columns are operated in parallel, with the eluent being used to separately recover the bound samples from Columns 1 and 2.

After the regeneration of Columns 2 and 3, Columns 1 and 3 exchange position for the next switch. Upon returning to their original configurations, a cycle is concluded, thus demonstrating that one cycle is composed of two switches. After the first switch, the loading is reduced from the capacity of three columns to that of the first two columns, and the system will remain in this configuration until the desired purity of P is achieved.

This mode, combining displacement and recirculation, allows P and S from the last column to be recirculated, thus improving yield and mitigating the purity-yield trade-off. This technique selectively retains strongly binding impurities as products while significantly increasing resin utilization in contrast to Flow2, which yields weakly adsorbed compounds as products. However, the primary design difficulty of self-displacement chromatography in ternary separation is determining the resin capacity for each compound.

3.4 Comparison of different continuous modes

The similarities and disparities between various continuous chromatographic techniques are compared in Table 1. The continuous operation can reduce the processing time and thereby increases process productivity at fixed purity specifications, surmounting the productivity-yield trade-off. Of all the chromatography

TABLE 1 Comparison of different continuous modes.

Continuous chromatography	Continuous level	Flow direction	Number of columns	Number of purified components	Column identity	Alleviated trade-off	Resin type
Column switching	Fully	CRO, COU	2+	2+	Yes	Pr-Y	CEX, AEX, RP, HI, SE
Annular	Fully	CRO	1	2+	–	Pr-Y	CEX, AEX, RP, HI, SE
Isocratic SMB	Semi	COU	2+	2	Yes	Pr-Y	CEX, AEX, RP, HI, SE
Gradient SMB	Semi	COU	2+	2	Yes	Pr-Y	CEX, AEX, RP, HI, SE
In-series SMB for ternary separation	Semi	COU	4+	3+	No	Pr-Y	CEX, AEX, RP, HI, SE
Eight-zone SMB for ternary separation	Semi	COU	4+	3	Yes	Pr-Y	CEX, AEX, RP, HI, SE
MCSGP	Semi	COU	2, 3, 4, 6, 8	3, 4	Yes	Pr-Y (partial), Pu-Y	CEX, AEX, RP, HI
Batch-to-batch recirculation	Semi	COU	2	3	Yes	Pr-Y, Pu-Y	CEX
N-rich	Semi	COU	2	1, 2	Yes	Pr-Y, Pu-Y	CEX, AEX, RP, HI
Flow2	Semi	COU	2	1	Yes	Pr-Y, Pu-Y	CEX
Multicolumn self-displacement	Semi	COU	2, 3, 4	1, 2, 3	No	Pr-Y, Pu-Y	CEX, AEX, RP

SMB, simulated moving bed; *MCSGP*, multicolumn counter-current solvent gradient purification; *CRO*, cross-current; *COU*, counter-current; *Pr-Y*, productivity-yield trade-off; *Pu-Y*, purity-yield trade-off. *CEX*, cation exchange; *AEX*, anion exchange; *RP*, reversed phase; *HI*, hydrophobic interaction; *SE*, size exclusion.

techniques considered so far, only rotating chromatography (e.g., column switching and annular chromatography) is operated in a fully continuous mode, while others are pseudocontinuous (semicontinuous) operations. To achieve continuous operation, all processes require a minimum of two columns except for annular chromatography. Generally, specific chromatographic modes are developed for the system to separate a given number of compounds. By introducing more columns, the modes can be extended for samples with more components, for example, two-units-in-series SMB for ternary separations and three-units-in-series for quaternary separations, as well as three-column self-displacement chromatography for binary separation and four columns for ternary separations.

The overlapping regions internally recirculated into the next column can alleviate the purity-yield trade-off by increasing purity and yield. In all chromatographic modes with internal recirculation, only N-rich can be used to recirculate and enrich the product-related

impurities. In the SMB processes, the recirculated stream is mixed with fresh feed, while an inline dilution buffer with less solvent is used in multicolumn continuous chromatography, except for the sample-displacement mode.

Due to significant improvements, continuous chromatography has been used with ion-exchange resins, as well as other resins, making it a viable alternative to batch chromatography. Despite this, continuous chromatography still has a long way to go before it completely replaces the batch mode. This is because batch processes remain inherently simpler with more degrees of freedom, and allow fast process design [18]. In contrast, the process design for continuous chromatography is challenging due to the numerous operating parameters involved.

4 Process design for continuous purification

The different continuous chromatographic modes necessitate the design of distinct operating parameters. For those modes featuring internal recirculation, the recirculating window is a critical factor for process performance (e.g., purity, yield, productivity, and buffer consumption). For those modes with an inline dilution stream, dilution factors should be considered. The compound entering the following column often contains a buffer with high solvent strength, leading to direct elution from the column. To prevent this, it should be diluted with a buffer of low solvent strength. The calculation of both dilution factors and the recirculating window is linked to the determination of characteristic times, which is essential for the transfer of a chromatographic method from batch to continuous, as well as the alternation of columns between interconnected and batch states. Other operating parameters similar to batch chromatography, such as loading conditions and the gradient slope, should also be considered. To this end, both experiment-based and model-based process design approaches have been proposed to identify the optimal operating parameters.

4.1 Experiment-based design

Experiment-based design, referred to as "purely empirical design" by Muller-Spath et al. [19], is a well-established approach for process design of continuous chromatography, such as MCSGP [2] and N-rich [8], involving a batch gradient selected to be transferred to the unit and reproduced within the process.

The user-friendliness of this approach obviates the need for knowledge of the adsorption isotherms and dispersive processes (e.g., mass transfer and axial dispersion) of the system components. However, the batch experiments for design must be conducted in two identical columns, which will be applied for continuous operation. This requirement, coupled with the complex dynamics, cyclic operation, and numerous influential parameters, might cause the experiment-based design of continuous chromatography difficult or even impossible. Recently, Jing et al. proposed an improved process design for MCSGP, called reMCSGP [20]. This approach involves the redesign of operating conditions based on the chromatogram obtained from the first MCSGP run, deviating from the conventional reliance solely on batch chromatograms. It is undeniable that operating parameters obtained from the experiment-based design must be fine-tuned experimentally, and are not absolutely optimal. Consequently, much deeper and more exhaustive analysis like the model-based design procedure is needed [21].

4.2 Model-based design

Process modeling of continuous chromatography using mechanistic models [22–24] is an effective approach to identify the optimal operating parameters.

Among the aforementioned five multicolumn continuous chromatographic techniques, only full process modeling of the two-column batch-to-batch recirculation process has been established [5]. Robust models are not yet available for other modes, at least not for all stages. Persson et al. [5] combined the equilibrium dispersive model with the stoichiometric displacement model to describe the elution behavior of the two-column batch-to-batch recirculation process, following model calibration in a batch experiment (yield of 45.4% at given purity specifications). After 20 cycles, the model predicted a yield of 93.6%, which alleviated the purity-yield trade-off for batch conditions, despite a slight discrepancy from the experimental result of 78.8%. The validated model was then used to find optimal dilution factors, associated with the characteristic times. After optimization, the productivity after 10 cycles increased to 1.5 times the original at the same purity requirements without compromising yield, which softened the productivity-yield trade-off. This strategy was beneficial for accelerating the design process and for identifying optimum operating parameters. Despite this, it was still based on the well-known theory of nonlinear chromatography in a single-column batch mode, thus limiting its further applicability.

The triangle theory, a simplified mechanistic model of chromatography that disregards mass transfer and axial dispersion effects, has been widely used for process design of SMB and extended to MCSGP. Steinebach et al. [25] applied it to describe the batch chromatogram for experiment-based MSCGP process design, and the impact of zone lengths (e.g., I1, B1, I2, calculated by characteristic times) on process performance. The results of the mechanistic model (88.0% purity and 99.7% yield) were comparable to those of the simplified model (94.0% purity and 99.7% yield) with a minor difference. They attributed this deviation to the neglected mass transfer. Notably, this method offers an optimal design space rather than a design point.

Data-driven or hybrid models have potential in the design of batch chromatography and can be introduced to continuous processes. Data-driven models like machine learning techniques can be utilized to control the operation (referred to as model predictive control), reject disturbances, and maintain the product specifications with reduced buffer consumption. It cannot only surmount trade-offs but facilitate automation and digitalization.

5 Applications for protein polishing using ion-exchange

5.1 Monoclonal antibodies

Monoclonal antibodies, mAbs, are a class of biomolecules composed of various isomers and aggregates, which must be separated to guarantee a high-quality product that meets drug specifications. The antibodies have been successfully purified and enriched using MCSGP, N-rich [7,8], Flow2 [14,15], and multi-column self-displacement chromatography [16,17]. Table 2 provides a fair comparison between batch chromatography and MCSGP. The term "fair" implies that the comparison is conducted under identical conditions, which is achieved using equivalent column lengths [26], identical purity specifications, and comparable yield requirements. The Pareto curve, as depicted in Fig. 1, is a better choice for comparison. From this table, it is evident that MCSGP has the potential to improve yield at fixed purity specifications and increase productivity when the number of columns is two or three, thus alleviating two trade-offs associated with batch modes. Additionally, Muller-Spath et al. [27] suggested that MCSGP can produce mAbs with higher bioactivity by removing low-activity variants compared to batch chromatography.

For the removal of antibody aggregates, Vogg et al. [15] developed a Flow2 unit with more flexible flow rates, which resulted in a

TABLE 2 Applications of ion-exchange MCSGP for protein polishing.

			Batch			Continuous			
Compound	**Number of columns**	**Number of cycles**	**Pu (%)**	**Y (%)**	**Pr (g/L/h)**	**Pu (%)**	**Y (%)**	**Pr (g/L/h)**	**References**
mAbs	6	12	<80	<10	–	93	93	0.36	Muller-Spath et al. [19]
mAbs	3	60	<80	<10	–	95	100	–	Grossmann et al. [28]
Bevacizumab	4	8	80	41	0.11	80	94	0.33	Muller-Spath et al. [27]
Trastuzumab	3	6	90	21	0.03	89	83	0.12	Muller-Spath et al. [27]
Cetuximab	3	7	49	5	0.002	67	75	0.027	Muller-Spath et al. [27]
Cetuximab	4	6	90	49	0.023	90	81	0.031	Krattli et al. [4]
Cetuximab	4	6	90	34	0.017	90	65	0.043	Krattli et al. [4]
PEGylated lysozyme	3	45	95	44	0.3	95	85	0.9	Ingold et al. [29]
mAbs	2	5	92	85	1.8	92	94	2.6	Steinebach et al. [26]
PEGylated lysozyme	2	5.5	84	80	4.21	84	93	4.30	Kim et al. [30]
PEGylated lysozyme	2	4.5	98	73	1.12	98	79	1.32	Kim et al. [31]
Cytochrome C	2	20	99	45	–	99	79	–	Persson et al. [5]

A number of cycles include the startup phase. *Pu*, purity; *Y*, yield; *Pr*, productivity.

doubling of productivity compared with the batch process for the same total column lengths. This unit was able to achieve a yield of 80% with a purity specification of 98%. Additionally, three-column self-displacement chromatography was used to separate mAbs variants to obtain a yield of 82% and a purity of 91%, given an initial purity of 65%. However, there is no available information comparing the performance of continuous and batch modes in Flow2 and multicolumn self-displacement.

A single continuous unit, as discussed aforementioned, is not sufficient to meet the requirements of continuous manufacturing of commercial mAbs products. To address this, Steinebach et al. [32] reported an end-to-end integrated continuous process for the continuous production of commercial mAbs products at the laboratory scale. This setup incorporated a continuous cultivation, a continuous two-column capture process, a virus inactivation step, a twin-column MCSGP, and a batch-wise flow-through polishing step and was capable of stable operation with uniform cycles.

5.2 PEGylated proteins

PEGylation is used to prolong the in vivo circulation half-life of proteins. This process results in a mixture of conjugates with varying degrees of PEGylation, each of which is composed of multiple positional isomers. To improve the selectivity of the reaction, Ingold et al. [29]

introduced a reactive MCSGP unit. The integration of the PEGylation reaction and separation steps is the basis for the development of highly efficient PEGylation technologies. Kim et al. [30] found that the yield and productivity of mono-PEGylated proteins were significantly increased compared to the batch process at the same purity requirements. To further demonstrate the economic advantages of the MCSGP unit, Kim et al. [31] conducted a cost analysis to predict the cost of goods in different manufacturing scenarios compared to the standard batch process. The case study revealed a near fourfold increase in productivity and a 35% decrease in solvent consumption when the MCSGP operation was implemented for the purification of PEGylated proteins.

6 Conclusion

Continuous chromatography is a powerful tool for protein purification, offering several advantages over batch chromatography. These advantages include higher yield, purity, productivity, and lower buffer consumption, which surmount the purity-yield and productivity-yield trade-offs associated with batch chromatography. Different types of continuous chromatography, such as rotating chromatography, SMB, and multicolumn continuous chromatography, have been developed to meet the needs of different applications. Their performance is heavily dependent on process design. Both experiment-based and model-based methods have been applied for this purpose with the latter demonstrating more potential in the foreseeable future.

Continuous IEC has been applied in many laboratory-scale and industrial-scale studies of biopharmaceuticals, such as mAbs and PEGylated proteins. As a promising technology, further applications of continuous chromatography for improved protein separations are expected.

References

[1] M. Kaspereit, H. Schmidt-Traub, Process concepts*, in: H. Schmidt-Traub, M. Schulte, A. Seidel-Morgenstern (Eds.), Preparative Chromatography, third ed., Wiley, Germany, 2020, pp. 251–310, https://doi.org/10.1002/9783527816347.ch5.

[2] L. Aumann, M. Morbidelli, A continuous multicolumn countercurrent solvent gradient purification (MCSGP) process, Biotechnol. Bioeng. 98 (5) (2007) 1043–1055, https://doi.org/10.1002/bit.21527.

[3] C. De Luca, S. Felletti, G. Lievore, T. Chenet, M. Morbidelli, M. Sponchioni, A. Cavazzini, M. Catani, Modern trends in downstream processing of biotherapeutics through continuous chromatography: the potential of Multicolumn Countercurrent Solvent Gradient Purification, Trends Analyt. Chem. 132 (2020) 116051, https://doi.org/10.1016/j.trac.2020.116051.

[4] M. Krattli, T. Muller-Spath, M. Morbidelli, Multifraction separation in countercurrent chromatography (MCSGP), Biotechnol. Bioeng. 110 (9) (2013) 2436–2444, https://doi.org/10.1002/bit.24901.

[5] O. Persson, N. Andersson, B. Nilsson, Design of two-column batch-to-batch recirculation to enhance performance in ion-exchange chromatography, J. Chromatogr. A 1531 (2018) 112–121, https://doi.org/10.1016/j.chroma.2017.11.035.

[6] S. Zobel-Roos, F. Vetter, D. Scheps, M. Pfeiffer, M. Gunne, O. Boscheinen, J. Strube, Digital twin based design and experimental validation of a continuous peptide polishing step, Processes 11 (5) (2023) 1401, https://doi.org/10.3390/pr11051401.

[7] E. Bigelow, Y.-L. Song, J. Chen, M. Holstein, Y.-P. Huang, L. Duhamel, K. Stone, R. Furman, Z.-J. Li, S. Ghose, Using continuous chromatography methodology to achieve high-productivity and high-purity enrichment of charge variants for analytical characterization, J. Chromatogr. A 1643 (2021) 462008, https://doi.org/10.1016/j.chroma.2021.462008.

[8] S.-Y. Jing, C. Shi, H.-Y. Leong, J.J. Yuan, D. Gao, H.-B. Wang, S.-J. Yao, D.-Q. Lin, A novel twin-column continuous chromatography approach for separation and enrichment of monoclonal antibody charge variants, Eng. Life Sci. 21 (6) (2021) 382–391, https://doi.org/10.1002/elsc.202000094.

[9] G. Lievore, R. Weldon, M. Catani, A. Cavazzini, T. Muller-Spath, Enrichment and recovery of oligonucleotide impurities by N-rich twin-column continuous chromatography, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1209 (2022) 123439, https://doi.org/10.1016/j.jchromb.2022.123439.

[10] R. Weldon, T. Muller-Spath, Enrichment and purification of peptide impurities using twin-column

continuous chromatography, J. Chromatogr. A 1667 (2022) 462894, https://doi.org/10.1016/j.chroma.2022.462894.

[11] F. Wei, Y. Hu, Y.-X. Zhao, Y.-X. Wu, G.-X. Jin, Purification and concentration of minor impurity in the bulk drug by step-gradient twin-column recycling chromatography, Ind. Eng. Chem. Res. 62 (15) (2023) 6241–6250, https://doi.org/10.1021/acs.iecr.3c00328.

[12] F. Wei, Z.-W. Yang, Y.-X. Zhao, Q. Wang, A twin-column recycling chromatography with solvent gradient for reinforcing the isolation of minor impurities, AIChE J. 65 (2) (2019) 702–711. 10.1002/aic.16444.

[13] F. Wei, J.-R. Sang, Y.-X. Zhao, Theoretical study of twin-column recycling chromatography with a solvent-gradient for preparative binary separations, J. Chromatogr. A 1651 (2021) 462306, https://doi.org/10.1016/j.chroma.2021.462306.

[14] C. Shi, Q.-L. Zhang, S. Vogg, D.-Q. Lin, M. Sponchioni, M. Morbidelli, Analysis and optimal design of batch and two-column continuous chromatographic frontal processes for monoclonal antibody purification, Biotechnol. Bioeng. 118 (9) (2021) 3420–3434, https://doi.org/10.1002/bit.27763.

[15] S. Vogg, T. Muller-Spath, M. Morbidelli, Design space and robustness analysis of batch and counter-current frontal chromatography processes for the removal of antibody aggregates, J. Chromatogr. A 1619 (2020) 460943, https://doi.org/10.1016/j.chroma.2020.460943.

[16] O. Khanal, V. Kumar, A.M. Lenhoff, Displacement to separate host-cell proteins and aggregates in cation-exchange chromatography of monoclonal antibodies, Biotechnol. Bioeng. 118 (1) (2021) 164–174, https://doi.org/10.1002/bit.27559.

[17] O. Khanal, V. Kumar, K. Westerberg, F. Schlegel, A.M. Lenhoff, Multi-column displacement chromatography for separation of charge variants of monoclonal antibodies, J. Chromatogr. A 1586 (2019) 40–51, https://doi.org/10.1016/j.chroma.2018.11.074.

[18] F. Steinebach, T. Muller-Spath, M. Morbidelli, Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production, Biotechnol. J. 11 (9) (2016) 1126–1141, https://doi.org/10.1002/biot.201500354.

[19] T. Muller-Spath, L. Aumann, L. Melter, G. Strohlein, M. Morbidelli, Chromatographic separation of three monoclonal antibody variants using multicolumn countercurrent solvent gradient purification (MCSGP), Biotechnol. Bioeng. 100 (6) (2008) 1166–1177, https://doi.org/10.1002/bit.21843.

[20] S.-Y. Jing, C. Shi, D. Gao, H.-B. Wang, S.-J. Yao, D.-Q. Lin, Improved process design for monoclonal antibody charge variants separation with multicolumn countercurrent solvent gradient purification, J. Chromatogr. A (2023) 464292, https://doi.org/10.1016/j.chroma.2023.464292.

[21] D.-Q. Lin, Q.-L. Zhang, S.-J. Yao, Model-assisted approaches for continuous chromatography: current situation and challenges, J. Chromatogr. A 1637 (2021) 461855, https://doi.org/10.1016/j.chroma.2020.461855.

[22] Y.-C. Chen, S.-J. Yao, D.-Q. Lin, Parameter-by-parameter method for steric mass action model of ion exchange chromatography: theoretical considerations and experimental verification, J. Chromatogr. A 1680 (2022) 463418, https://doi.org/10.1016/j.chroma.2022.463418.

[23] Y.-C. Chen, S.-J. Yao, D.-Q. Lin, Parameter-by-parameter method for steric mass action model of ion exchange chromatography: simplified estimation for steric shielding factor, J. Chromatogr. A 1687 (2023) 463655, https://doi.org/10.1016/j.chroma.2022.463655.

[24] Y.-C. Chen, X.-Y. Chen, Z.-Y. Lin, S.-J. Yao, D.-Q. Lin, Practical teaching of modeling tools for ion-exchange chromatography: a case study, J. Chem. Educ. (2023), https://doi.org/10.1021/acs.jchemed.3c00439. In press.

[25] F. Steinebach, M. Krattli, G. Storti, M. Morbidelli, Equilibrium theory based design space for the multicolumn countercurrent solvent gradient purification process, Ind. Eng. Chem. Res. 56 (45) (2017) 13483–13490, https://doi.org/10.1021/acs.iecr.7b00569.

[26] F. Steinebach, N. Ulmer, L. Decker, L. Aumann, M. Morbidelli, Experimental design of a twin-column countercurrent gradient purification process, J. Chromatogr. A 1492 (2017) 19–26, https://doi.org/10.1016/j.chroma.2017.02.049.

[27] T. Muller-Spath, M. Krattli, L. Aumann, G. Strohlein, M. Morbidelli, Increasing the activity of monoclonal antibody therapeutics by continuous chromatography (MCSGP), Biotechnol. Bioeng. 107 (4) (2010) 652–662, https://doi.org/10.1002/bit.22843.

[28] C. Grossmann, G. Strohlein, M. Morari, M. Morbidelli, Optimizing model predictive control of the chromatographic multi-column solvent gradient purification (MCSGP) process, J. Process Control 20 (5) (2010) 618–629, https://doi.org/10.1016/j.jprocont.2010.02.013.

[29] O. Ingold, D. Pfister, M. Morbidelli, A reactive continuous chromatographic process for protein PEGylation, React. Chem. Eng. 1 (2) (2016) 218–228, https://doi.org/10.1039/c5re00055f.

[30] T.K. Kim, C. Botti, J. Angelo, X.K. Xu, S. Ghose, Z.J. Li, M. Morbidelli, M. Sponchioni, Experimental design of the multicolumn countercurrent solvent gradient purification (MCSGP) unit for the separation of PEGylated proteins, Ind. Eng. Chem. Res. 60 (29) (2021) 10764–10776, https://doi.org/10.1021/acs.iecr.1c01345.

[31] T.K. Kim, B. Sechi, J.J.R. Conde, J. Angelo, X. Xu, S. Ghose, M. Morbidelli, M. Sponchioni, Design and economic investigation of a multicolumn countercurrent solvent gradient purification unit for the separation of an industrially relevant PEGylated protein, J. Chromatogr. A 1681 (2022) 463487, https://doi.org/10.1016/j.chroma.2022.463487.

[32] F. Steinebach, N. Ulmer, M. Wolf, L. Decker, V. Schneider, R. Walchli, D. Karst, J. Souquet, M. Morbidelli, Design and operation of a continuous integrated monoclonal antibody production process, Biotechnol. Prog. 33 (5) (2017) 1303–1313, https://doi.org/10.1002/btpr.2522.