From batch to continuous chromatographic purification of a therapeutic peptide through Multicolumn Countercurrent Solvent Gradient Purification (MCSGP)

From batch to continuous chromatographic purification of a therapeutic peptide through multicolumn countercurrent solvent gradient purification

Chiara De Luca[a], Simona Felletti[a], Giulio Lievore[a], Alessandro Buratti[a], Sebastian Vogg[b], Massimo Morbidelli[b], Alberto Cavazzini[a], Martina Catani[a,*], Marco Macis[c], Antonio Ricci[c,*], Walter Cabri[c,d]

[a] Dept. of Chemistry and Pharmaceutical Sciences, University of Ferrara, via L. Borsari 46, 44121 Ferrara, Italy
[b] Institute for Chemical and Bioengineering, ETH Zurich, Vladimir-Prelog-Weg 1, 8093 Zurich, Switzerland
[c] Fresenius Kabi iPSUM, via San Leonardo 23, 45010, Villadose, Rovigo, Italy
[d] Department of Chemistry "G. Ciamician", University of Bologna, via Selmi 2, 40126, Bologna, Italy

ARTICLE INFO

ABSTRACT

A twin-column Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) process has been developed for the purification of a therapeutic peptide, glucagon, from a crude synthetic mixture. This semi-continuous process uses two identical columns operating either in interconnected or in batch mode, thus enabling the internal recycle of the portions of the eluting stream which do not comply with purity specifications. Because of this feature, which actually results in the simulated countercurrent movement of the stationary phase with respect to the mobile one, the yield-purity trade-off typical of traditional batch preparative chromatography can be alleviated. Moreover, the purification process can be completely automatized.

Aim of this work is to present a simple procedure for the development of the MCSGP process based on a single batch experiment, in the case of a therapeutic peptide of industrial relevance. This allowed to recover roughly 90% of the injected glucagon in a purified pool with a purity of about 90%.

A comparison between the performance of the MCSGP process and the classical single column batch process indicates that percentage increase in the recovery of target product is +23% when transferring the method from batch conditions to MCSGP, with an unchanged purity of around 89%. This improvement comes at the expenses of a reduction of about 38% in productivity.

1. Introduction

Over the last decades, the interest towards small proteins and peptides in the pharmaceutical field has dramatically increased. This trend has been driven by the introduction of novel synthetic strategies, based on amino acid modifications and incorporation of other moieties, that allow to modulate not only the pharmacokinetic properties of these biomolecules but also their specificity towards a particular target. As a result, peptides currently find many applications as antimicrobials, antioxidants, anti-hypertensives, anticoagulants, but also as appetite regulators and functional foods, to name but a few [1–4].

The industrial production of therapeutic peptides is mainly carried out by means of solid phase synthesis. This technique consists in the addition of one amino acid at a time at the end of the growing chain tied to an insoluble resin, in a cycle of deprotection-wash-coupling-wash. However, since it is not possible to perform an intermediate purification, many impurities (for example diastereoisomeric products, incomplete protected sequences, wrong amino acid insertions, oxidations, reductions, etc.) coming from incomplete or side-reactions can be generated [5,6]. The target peptide needs to be isolated from these impurities in order to meet the strict purity specifications required for pharmaceuticals, therefore one or more purification steps are necessary. This point of the downstream process is often the bottleneck in terms of time and costs in the whole production of synthetic peptides [7–

*

https://doi.org/10.1016/j.chroma.2020.461304

10]. Liquid chromatography on a preparative scale is the most employed method for the industrial purification of peptides [5,6,11–15]. However, the presence of impurities chemically similar to the target represents a critical issue during the purification process since their chromatographic peaks can overlap both in the front and in the tail of the peak of the target [16]. As a consequence, batch processes generally lead to a severe trade-off between purity and yield. Indeed, by enlarging the product pool window and including the overlapping regions, purity is reduced but yield can be improved; on the contrary, if a very narrow product window is taken purity increases at the expense of yield. This problem is intrinsic of single-column batch preparative chromatography [6,16].

The yield-purity trade-off can be overcome by employing continuous (or semi-continuous) countercurrent purification techniques [16,17], where two (or more) identical columns, properly connected through several switching valves, are operated both in batch and in interconnected mode, alternatively. As in other countercurrent techniques, the movement of the stationary phase in the opposite direction as that of the mobile one is not real but it is simulated through the switching valves, that change the connection between the inlets and the outlets of the columns and hence the path accessible to the mobile phase. This leads to considerable advantages in terms of yield of purification [18]. Moreover, the continuous internal recycling of partially unresolved component into the system allows to completely automatize the purification process [19,20]. Among the continuous techniques, Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is particularly suitable for the purification of complex mixtures containing product-related impurities co-eluting with the target [6]. Indeed, MCSGP can be applied to ternary separations, such as the case of a target that almost coelutes with more weakly adsorbed (from here on called W) and more strongly adsorbed (from here on called S) impurities, in the front and in the tail, respectively [7,21]. Moreover, the great advantage of MCSGP over other continuous purification techniques (such as Simulated Moving Bed) is that it can handle separation problems where a gradient of modifier is required [9,22–25]. This is the case of purification of large biomolecules, whose retention is strongly affected by the organic modifier concentration [24,26,27]. In particular, when a large number of product-related impurities is present, shallow gradients should be used in order to increase the selectivity of the separation [28].

In its first arrangement, MCSGP was realised with 6 columns [29,30]. Later on, this unit has been more and more simplified to three [31] and finally to only two columns [6], which clearly results in a decreased complexity from the point of view of tubing and valves.

Briefly, the operating principle of twin-column MCSGP is the following: two identical columns (with the same stationary phase as that of the design batch) carry out four different tasks in parallel but shifted of half a cycle, as illustrated schematically in Fig. 1. In particular, the portion of the peak satisfying the purity requirement is collected from the upstream column during every cycle (zone 6); on the contrary, the unresolved side portions of the peak (front and tail), that are contaminated with impurites but contain a large amount of peptide, are recycled into the downstream column (zones 5 and 7). Then the columns exchange position. In this way, it is possible to avoid the product loss that would occur if the overlapping fractions were discarded. Note that in order to inject the same quantity of peptide cycle after cycle, a certain amount of fresh feed needs to be injected into the downstream column receiving the overlapping fractions [6,14]. All the operations will be further described in the Theory section.

In this work, the MCSGP process is applied to the purification of a synthetic crude mixture of glucagon, a linear polypeptide hormone consisting of 29 amino acidic residues (MW = 3485 Da) excreted by the pancreatic α-cells. This is the principal hyperglycemic hormone, acting as a counterbalance to insulin.

The objective of this work is to illustrate, using an industrially relevant purification process, how to quickly design a MCSGP process from a single column chromatogram, without any specific need of process performance optimization. A comparison between the performances of batch (single column) and MCSGP (two columns) processes is also presented.

2. Theory

2.1. MCSGP principles

The starting point to design an MCSGP process is the definition of a design batch chromatogram, through which parameters such as loading, gradient slope and regeneration procedure are defined. This chromatogram must be optimized in order to meet purity requirements at least in some portion of the main peak. Generally, a single column process is made up of four parts: equilibration, load, elution and stripping (that is, washing with high organic modifier concentration to remove whatever chemical is still inside the column). In turn, the elution of the feed can be divided in 5 steps: elution of W, overlapping region containing W/P, window where the product P is pure, overlapping region containing P/S, elution of S. The overlapping windows are undoubtedly contaminated but contain a large quantity of target product; therefore, they cannot be wasted but need to be recovered, in order to obtain a satisfactory process yield. In batch processes, this is performed through an external recycle, with waste of time and risk of errors made by the operator. The MCSGP technique allows for the automation of this step, which is performed inside the unit continuously, with no interruption in time.

When the process is transferred from batch to continuous chromatography, the columns work alternately either in batch or in interconnected manner. Let's consider Fig. 1, which represents schematically the case where column-1 is in the upstream position and column-2 is in the downstream position; this means that what comes out from column-1 can be recycled in column-2. The gradient program starts in column-1 and W impurities begin to elute (zone 4); this stream is wasted. In the meanwhile, column-2 undergoes the stripping and equilibration phases (zone 8), to get ready to receive the feed. During this step the columns work in batch mode. As soon as the overlapping region W/P starts eluting from column-1, the valves exchange configuration and the eluate enters column-2 (zones 5 and 1 respectively). This is the recycling of W/P which happens in interconnected mode. Then during the Product Elution Window (PEW), the pure peptide eluting from column-1 is collected (zone 6), while column-2 is loaded with fresh feed to compensate the loss of product leaving column-1 (zone 2, batch mode). Next, P/S window is recycled into column-2 (zone 3) after leaving column-1 (zone 7, interconnected mode). At this point, the gradient can be performed in column-2, which has been completely loaded, and column-1 is stripped to remove S. At this point, the columns exchange position (column-1 is now in downstream position and vice versa) and this represents the end of the switch; after 2 switches, when the columns come back to the initial configuration, a cycle is completed [12,32,33]. Hence, each column is loaded and eluted once per cycle. It must be highlighted that the loading step is subdivided in three steps: uptake of W/P, injection of fresh feed, uptake of P/S. If all the operating conditions, such as load amount, gradient slope, etc., are kept constant from batch to MCSGP, then the only degrees of freedom characterizing the continuous process are the values of the characteristic elution times or elution column volumes, which define the five zones of the elution [14]. Particularly, the elution of W starts at the beginning of the gradient, in correspondence with the time t_A, and it

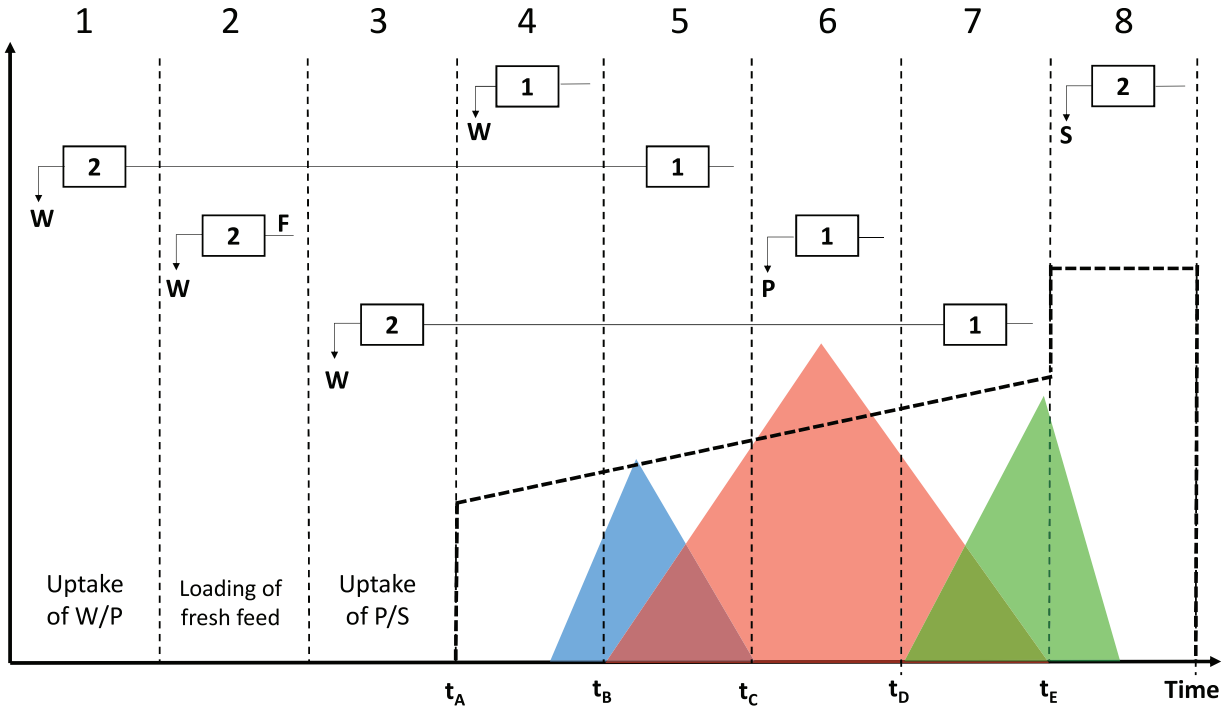

Fig. 1. Schematic illustration of a batch chromatogram and the corresponding tasks within a switch where column-1 is in the upstream position. The blue (green) peak corresponds to the weak (strong) impurities, while the red peak is the target product. Five intervals are identified where different fractions are eluting: W (t_A to t_B), overlapping of W and P (t_B to t_C), P (t_C to t_D), overlapping of P and S (t_D to t_E) and S, during the stripping. t_B, t_C, t_D and t_E are the characteristic switching times of the MCSGP process. t_A is the time where the gradient starts. Note that the loading of the target in column-2 happens in three steps: during the recycling of W/P, the loading of fresh feed and the recycling of P/S. The linear gradient of the modifier is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ends at the time t_B, where W/P begins to elute from the upstream to the downstream column. The PEW extends from t_C to t_D, at the beginning of the elution of P/S, which on the other side ends at t_E. Each of these characteristic times, if multiplied for the elution flow rate, leads to a characteristic elution volume (CV_B, CV_C, CV_D, CV_E). t_A, on the other hand, indicates the time where the gradient starts (see Fig. 1).

It is noteworthy that the overlapping regions need to be diluted with a compensation buffer before being loaded to the next column. Indeed, these regions elute during the gradient and this implies that the concentration of modifier is continuously increasing during this period. Dilution is therefore necessary to allow their adsorption on the stationary phase. Usually, the first recycle stream (W/P) is diluted so as to reach the modifier concentration value at the beginning of the elution of W/P, which allows the product to adsorb and the weak impurities to start moving. On the other hand, the P/S recycling stream is diluted to the modifier concentration value at the beginning of the gradient, in order to adsorb both the product and the strong impurities.

2.2. Performance parameters

In order to quantify the performance of a purification process, either in batch or in continuous, some suitable parameters need to be introduced. These are calculated from the HPLC chromatogram of a fraction (in the case of a batch) or of a pool (in the case of MCSGP). The most important parameter is the purity of the target compound, which must satisfy the strict requirements imposed. It is defined as the ratio of the area of the target peak in the product pool (or fraction) to the total area in the product pool or fraction (sum of the areas of the target and impurities), measured in analytical conditions:

$$\text{Purity} = \frac{\text{area}_{\text{target}}}{\text{area}_{\text{tot}}} \times 100 \quad (1)$$

Process recovery, otherwise called yield, is the ratio between the mass of the target recovered within purity specifications ($m_{target\ recovered}$) and the mass loaded in the feed ($m_{target\ loaded}$):

$$\text{Recovery} = \frac{\text{m}_{\text{target recovered}}}{\text{m}_{\text{target loaded}}} \times 100 \quad (2)$$

Finally, productivity is defined as the mass of the target recovered within purity specifications, divided by the corresponding operation time (t_{run}) and the total volume of stationary phase, measured in CVs (i.e., the geometrical volume of the column):

$$\text{Productivity} = \frac{\text{m}_{\text{target recovered}}}{\text{t}_{\text{run}} \times \text{ CV}} \quad (3)$$

In case of an MCSGP run, these parameters are calculated per cycle, for example t_{run} is the duration of a cycle (which, in the case of batch processes, represents the total duration of a purification run). It must be also taken into account that in MCSGP two columns are used, therefore the total volume of the stationary phase, that is the CV value in the denominator of Eq. (3), is twice the volume of one single column. Moreover, in MCSGP, the performance parameters are typically computed as the mean of the values obtained for all the cycles that are at the steady-state, which in practice is defined as the condition reached when the UV profiles of two consecutive cycles can be completely superimposed.

By fractionating the chromatogram eluting from a single column, it is possible to estimate the performance parameters corresponding to different sizes of the overall target collection fraction. Thus, starting from the purest fraction (which also exhibits the lowest recovery) and successively including neighboring fractions, the values for purity and yield corresponding to various hypothetical batch pool can be measured. As stated above, the broader

the pool, the lower the purity, and moreover the higher the recovery. These pairs of values when reported on a recovery versus purity plot describe a so called pareto curve, which characterises the performance of this specific single column process, by defining for each purity value what is the maximum recovery that can be achieved. For the MCSGP process instead, for each operation (that is, for each set of characteristic times) a single pair of purity and recovery values is obtained. This is calculated as the average parameters of the pools at the steady-state. With reference to the same plot mentioned above, the point belonging to the Pareto of the specific MCSGP process considered can be compared to the Pareto corresponding to the single column process. It is clear that points closer to the upper-right corner of the plot correspond to better process performances, meaning that at the same purity the recovery is higher.

3. Experimental section

3.1. The crude mixture

Glucagon has been synthesized by Fresenius Kabi iPSUM (Villadose, Rovigo, Italy) through Solid Phase Synthesis. The peptide constitutes 30% on weigh of the crude mixture, and has a chromatographic purity of 55%. In this research, only the first step of the purification process was investigated, where the purity requirement is rather low, around 85–90%. A second step is then needed to achieve the required purity specifications.

The crude mixture was dissolved in a solution of 20% acetonitrile (ACN) and 0.01% trifluoroacetic acid (TFA), having a pH=1.8. The feed was prepared at 1 g/l and stored for no more than two days, in order to avoid gelation, which is very common for glucagon, particularly at high concentration or under vigorous stirring [34].

3.2. Preparative separations

The columns used were three Daisogel-SP-120-10-C8-Bio, functionalised with C8 chains. The pore size was 120 Å, the particle size 10 µm and the internal diameter 4.6 mm. The column used for the batch was 25 cm long and the two columns for MCSGP were each 15 cm long. The columns are completely identical apart for the length.

To perform both the single column and the MCSGP runs, a ContiChrom CUBE Combined instrument has been used (ChromaCon/YMC, Zurich, Switzerland) equipped with two UV detectors and a Foxy R1 fraction collector. The wavelength was set at 280 nm.

The buffers used for the linear gradient were two mixtures of aqueous and organic solvents. In particular, mobile phase A (MP-A) was 80% ammonium acetate 20 mM in water and 20% ACN, whereas mobile phase B (MP-B) was 60% ammonium acetate 20 mM in water and 40% ACN. The duration of every step is expressed in eluted column volumes, CVs. The feed is loaded at 3 mL/min with a concentration of 10 mg/mL_{column}, corresponding for the batch to 10 CVs of feed with a concentration of 1 g/L. On the contrary, only a certain percentage of these 10 CVs is injected in each switch in the MCSGP, as decided for each operation from time to time, based on the time windows chosen. Basically, the amount of fresh feed to be injected for each switch in MCSGP corresponds to the difference between the mass loaded in batch and the mass of the target product contained in the recycling windows. The mass to inject is then equal to the target product wasted in W + wasted in S + collected in the window P in the batch process (see Fig. 2).

After the loading, 2 CVs are used to wash the column with 35% MP-B, at 2.5 mL/min. Now the column can undergo the gradient,

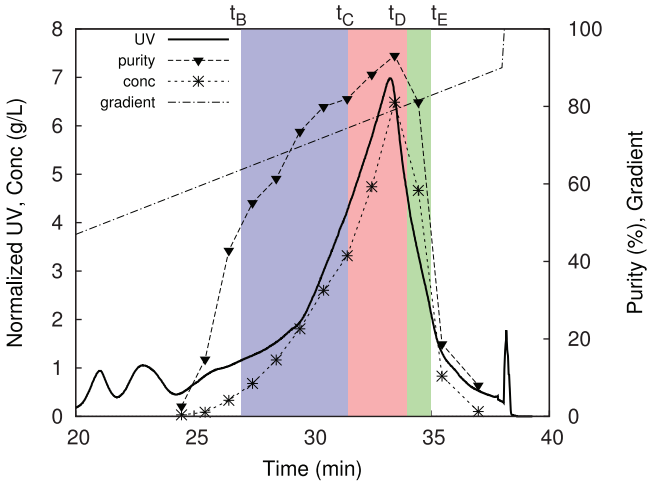

Fig. 2. Scheme of the design batch chromatogram together with the linear gradient of the modifier concentration, the purity profile and target product concentration profile. The values have been obtained by analyzing the fractions collected at every minute at the outlet of the 15 cm column. Moreover, the intervals for the recycling of W/P (blue) and P/S (green) and the collection of the target product (red) chosen for designing the MCSGP process and the relative switching times (in the top of the graphic) are highligthed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which lasts for 11 CVs and goes from 35 to 90% of MP-B, at a rather low flow rate (1.2 mL/min). At the end of the elution, a stripping step is required to wash the column and remove all the strongly adsorbed impurities; for this, a buffer made of 90% ACN and 10% ammonium acetate 20 mM is used for 3 CVs at 3 mL/min.

An MCSGP process is basically composed of three steps: i) firstly, there is a so-called "Start-Up" step, where the same quantity of feed as in the batch run is loaded on the first column; ii) then the cycles start; and iii) finally a so-called "Shut-Down" is performed, where no feed is injected, and the columns are cleaned to end the process.

As compensation buffer, MP-A, containing a very low concentration of ACN, was used for intercolumn online dilution.

Fractions were collected every 60 s for the batch process, while for MCSGP one pool was collected for every switch (two pools per cycle). It was observed that steady-state conditions were usually achieved after two or three switches; the whole process was run for 5 cycles (10 switches) in total.

3.3. Analytics

Every fraction, pool and feed have been analysed in HPLC on an Agilent 1100 (Agilent, Santa Clara, CA, USA) according to the method reported by USP. A 150 × 3.0 mm ACE 3 C_{18} column packed with 3 µm particles was used. Mobile phases were a phosphate buffer solution at pH = 2.7 (MP_A) and a mixture of water/acetonitrile 60:40%(v/v) (MP_B). Gradient program run from 39 to 88% MP_B in 4 min, followed by 1 min of isocratic at 88% MP_B. Then initial conditions (39% MP_B) were restored in 1 min. Detection wavelenght was set at 214 nm. Injection volume was 15 μL. Calibration was performed using samples with known concentration of pure peptide, ranging from 0.05 to 2 g/L.

4. Results and discussion

4.1. Design batch chromatogram

As a preliminary study, several batch experiments have been run with different gradient conditions, that is, different slope and

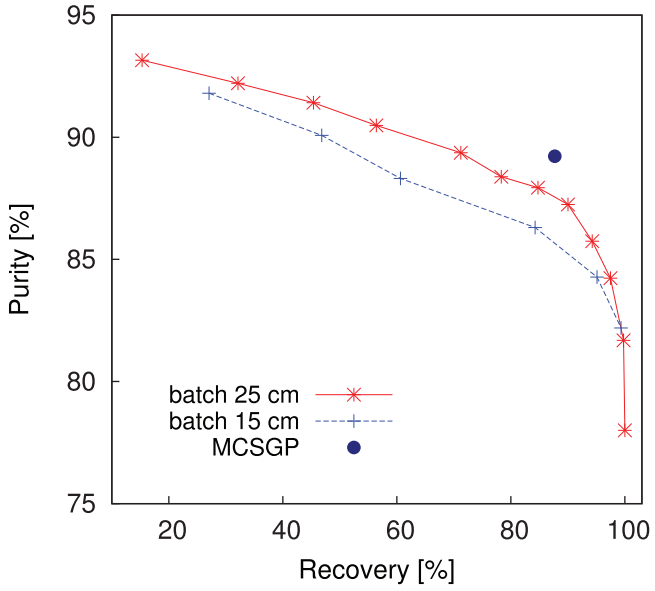

Fig. 3. Pareto curves corresponding to two batch runs using a 15cm column (blue crosses) and a 25cm column (red stars). The single point refers to the MCSGP operation. Note that the productivity values are different for the different operating conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Switching times and injection volume per switch for the MCSGP process.

t_B (min)	t_C (min)	t_D (min)	t_E (min)	V_{inj} (CV)
27	31.5	33	34	5.7

initial and final modifier concentrations, using the 25 cm long column. Among these, the conditions showing the least steep Pareto curve (i.e., where the purity decreases less dramatically with increasing recovery) has been chosen as the design batch chromatogram. In the case under examination, the gradient described in Section 3.2 has been found. This leads to the chromatogram shown in Fig. 2, where the values of purity and target product concentration in the various fractions are indicated, together with the UV signal. In the purest fraction, meaning with the narrowest collection window, the purity is around 93%, while, on the other hand, the recovery is only 15%. By enlarging this window, purity decreases and yield improves, as it is illustrated on the Pareto curve in Fig. 3. The same method has also been run on the short column (15 cm). As expected, the Pareto curve of the longer column lies above that of the 15 cm one, meaning that, for the same recovery values, higher purities can be obtained on the 25 cm column. Note that the performance obtained in batch conditions on the 25 cm long column is used as reference for a fair comparison between batch and continuous runs.

4.2. MCSGP

The MCSGP method has been set up starting from the design batch chromatogram run on the 15 cm column, which has been used to select the times t_B to t_E and then to define the recycling and collection windows. Several MCSGP runs have been performed with different sets of switching times, while all the other parameters have been kept constant. The choice of these times strongly affects the MCSGP process and even a difference of half a minute can change the outcome of purification. The best set of operating times is reported in Table 1 while the corresponding collection and recycling windows are illustrated in Fig. 2. These points correspond

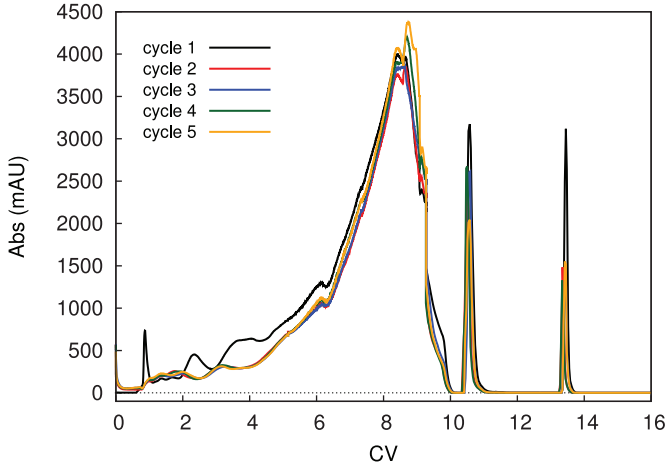

Fig. 4. Overlap of the UV signals measured at the outlet of one column of the MCSGP unit during 5 different cycles. The sharp peaks on the right correspond to the strip and re-equilibration of the column after the overlapping fraction P/S has been recycled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

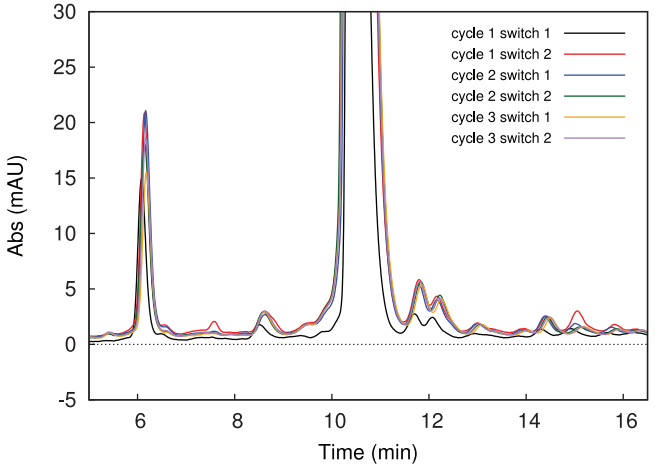

Fig. 5. Analytical HPLC chromatograms of the product fractions collected during the first six switches after the start-up of the MCSGP unit. It appears that, except for switch 1 in the first cycle, all the other chromatograms overlap, indicating that steady-state conditions have been achieved. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to an hypothetical batch pool (betwen $t_C = 31.5$ min and $t_D = 33$ min) on the 15 cm column with a purity of 89% and recovery of 53% (meaning that 53% of the injected peptide is recovered in the target product collection window). The remaining mass of the injected peptide is distributed in the other fractions as follows: 32% in W/P, 11% in P/S and 4% is wasted in weak and strong.

In order to keep the loading constant, 57% of the batch loading has been reinjected at every switch in MCSGP, taking into account the fact that 43% (32% + 11%) of the peptide is already present into the downstream column after the recycling of W/P and P/S. After one cycle, steady-state conditions were already reached. The profiles of the 5 cycles during MCSGP are shown in Fig. 4. As it can be seen, the first cycle is rather different from the others, while the remaining four are well overlapped, suggesting that steady-state conditions are already achieved after the first cycle. Also the analytical chromatograms of the first three cycles (six switches) are compared in Fig. 5. Also here it is possible to note that only the first switch differs significantly from the others.

With these operating conditions, the average target product purity in the MCSGP pools resulted to be 89.2% with a recovery of

Table 2
Performance of the batch and the MCSGP purification of glucagon using a 25 cm column and two 15 cm columns, respectively.

	Batch (25 cm)	MCSGP (2 × 15 cm)
Purity (%)	89.3	89.2
Recovery (%)	71.2	87.7
Productivity (g/L/h)	9.9	6.1

87.7% (see Table 2). For a fair comparison of process performance, the Pareto curve has been measured also using a 25 cm column, with a volume (CV = 4.2 mL) similar to the total volume of the two 15 cm columns used in the MCSGP unit (CV = 2 * 2.5 = 5 mL). Of course, the same stationary phase was chosen for the comparison. For a similar purity as that of the MCSGP, a recovery value of 71.2% has been obtained in batch. This means that MCSGP, with this particular set of switching times, allows to increase the recovery of about 16% (from 71.2% to 87.7%). This difference corresponds to a percentage increase in the target product recovery of about +23% in the MCSGP with respect to the batch process. It is important to point out that when the target product is very expensive (as it is the case of glucagon) any improvement in recovery leads to economic benefits when the target product is particularly expensive.

Another relevant parameter in evaluating the performance of a purification process is productivity. In order to compare the two processes, we considered for the batch process, at the numerator, the mass of the fraction of the design chromatogram having the same purity as the MCSGP operation. The duration of the batch run was 39 min while the duration of a cycle in MCSGP was 64 min. Accordingly, as reported in Table 2, the value of the productivity is lower for MCSGP by about 38%. This is due to the fact that MCSGP requires the use of lower flow rates during the interconnected steps than that used for the batch for the entire elution period. However, in case of very expensive products, such as glucagon or other biotherapeutics, it is preferable to maximize the recovery, in order to diminish the amount of waste product, with respect of productivity. In any case, the increase in recovery more than compensates the loss in productivity.

5. Conclusions

In this work, a simple procedure to design an MCSGP process for the purification of an industrial synthetic glucagon crude mixture starting from a single batch column experiment has been presented. The MCSGP performance strictly depends on the set of switching times that are chosen to define the collection and recycling windows, whereas all the other experimental parameters (mobile phases, gradient slope and duration, loading, etc.) are the same as those used in the single column run. At the same purity of 89%, the gain in the target molecule recovery was found to be +23% in the MCSGP in comparison with the value found for the batch process.

This increase in yield was not sufficient to compensate the still better productivity of the batch, calculated exclusively as the gram of purified peptide collected in the pool per run and per mL of stationary phase. However, in terms of overall process performance, this loss is more than compensated by the increase in recovery. Moreover, it must be noted that once the operational conditions for the MCSGP process have been defined, the purification process can be completely automatized and very large amount of feed can be continuously purified cyle after cycle, with no human intervention.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Italian University and Scientific Research Ministry (grant PRIN2017Y2PAB8 003, title:“Cutting edge analytical chemistry methodologies and bio-tools to boost precision medicine in hormone-related diseases”). The authors also acknowledge Fresenius Kabi iPSUM for the kind donation of glucagon and ChromaCon YMC (Zurich, Switzerland) for technical support. Dr. Marco Carmosino from the University of Ferrara is also acknowledged for technical support.

References

[1] R.J.S. de Castro, H.H. Sato, Biologically active peptides: processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries, Food Res. Int. 74 (2015) 185–198.
[2] R. Khalaf, N. Forrer, G. Buffolino, A. Butté, M. Morbidelli, Model-based description of peptide retention on doped reversed-phase media, J. Chromatogr. A 1407 (2015) 169–175.
[3] D. Agyei, C.M. Ongkudon, C.Y. Wei, A.S. Chan, M.K. Danquah, Bioprocess challenges to the isolation and purification of bioactive peptides, Food Bioprod. Proc. 98 (2016) 244–256.
[4] R. Khalaf, N. Forrer, G. Buffolino, D. Gétaz, S. Bernardi, A. Butté, M. Morbidelli, Doping reversed-phase media for improved peptide purification, J. Chromatogr. A 1397 (2015) 11–18.
[5] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, Liquid chromatography-mass spectrometry approach for the characterization and purification of crude synthetic peptide hormones, Anal. Bioanal. Chem. 377 (2003) 306–315.
[6] T. Müller-Späth, G. Ströhlein, O. Lyngberg, D. Maclean, Enabling high purities and yields in therapeutic peptide purification using multicolumn countercurrent solvent gradient purification, Chem. Today 31 (2013) 56–60.
[7] L. Aumann, M. Morbidelli, B. Schenkel, G. Ströhlein, Protein peptide purification using the multicolumn countercurrent solvent gradient purification (MCSGP) process, Biopharm. Intern. 22 (2009) 46–53.
[8] A. Tarafder, L. Aumann, M. Morbidelli, The role of ion-pairing in peak deformations in overloaded reversed-phase chromatography of peptides, J. Chromatogr. A 1217 (2010) 7065–7073.
[9] C. Grossman, G. Ströhlein, M. Morari, M. Morbidelli, Optimizing model predictive control of the chromatographic multicolumn solvent gradient purification (MCSGP) process, J. Proc. Control 20 (2010) 618–629.
[10] G. Ströhlein, L. Aumann, T. Müller-Späth, A. Tarafder, M. Morbidelli, The multicolumn countercurrent solvent gradient purification process-a continuous chromatographic process for monoclonal antibodies without using protein a, Biopharm. Intern. 22 (2007) 42–48.
[11] S. Bernardi, D. Gétaz, N. Forrer, M. Morbidelli, Modeling of mixed-mode chromatography of peptides, J. Chromatogr. A 1283 (2013) 46–52.
[12] 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.
[13] D. Gétaz, G. Stroehlein, A. Butté, M. Morbidelli, Model-based design of peptide chromatographic purification processes, J. Chromatogr. A 1284 (2013) 69–79.
[14] S. Vogg, N. Ulmer, J. Souquet, H. Broly, M. Morbidelli, Experimental evaluation of the impact of intrinsic process parameters on the performance of a continuous chromatographic polishing unit (MCSGP), Biotech. J. 14 (2019).
[15] E.J. Close, J.R. Salm, D.G. Bracewell, E. Sorensen, Modelling of industrial biopharmaceutical multicomponent chromatography, Chem. Eng. Res. Des. 92 (2014) 1304–1314.
[16] F. Steinebach, T. Müller-Späth, M. Morbidelli, Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production, Biotech. J. 11 (2016) 1126–1141.
[17] , Preparative Chromatography, H. Schmidt-Traub, M. Schulte, A. Seidel-Morgenstern (Eds.), Wiley-VCH, 2020.
[18] G. Subramanian, Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH, 2014.
[19] G. Carta, A. Jungbauer, Protein Chromatography. Process Development and Scale-Up, WILEY-VCH, 2010.
[20] D. Baur, M. Angarita, T. Müller-Späth, F. Steinebach, M. Morbidelli, Comparison of batch and continuous multi-column protein a capture processes by oprimal design, Biotech. J. 11 (2016) 920–931.
[21] D. Pfister, L. Nicoud, M. Morbidelli, Continuous biopharmaceutical processes - chromatography, Bioconjugation and Protein Stability, Cambridge University Press, 2018.
[22] W. Jin, P.C. Wankat, Two-zone SMB process for binary separation, Ind. Eng. Chem. Res. 44 (2005) 1565–1575.

[23] A.L. Zydney, Continuous downstream processing for high value biological products: a review, Biotech. Bioeng. 113 (2016) 465–475.
[24] G. Ströhlein, L. Aumann, M. Mazzotti, M. Morbidelli, A continuous, counter-current multi-column chromatographic process incorporating modifier gradients for ternary separations, J. Chromatogr. A 1126 (2006) 338–346.
[25] T. Müller-Späth, L. Aumann, L. Melter, G. Ströhlein, M. Morbidelli, Chromatographic separation of three monoclonal antibody variants using multicolumn countercurrent solvent gradient purification (MCSGP), Biotech. Bioeng. 100 (2008) 1166–1177.
[26] N. Marchetti, F. Dondi, A. Felinger, R. Guerrini, S. Salvadori, A. Cavazzini, Modeling of overloaded gradient elution of nociceptin/orphanin FQ in revesed-phase liquid chromatography, J. Chromatogr. A 1079 (2005) 162–172.
[27] D. Åsberg, M. Leśko, T. Leek, J. Samuelsson, K. Kaczmarski, T. Fornstedt, Estimation of nonlinear adsorption isotherms in gradient elution rp-lc of peptides in the presence of an adsorbing additive, Chromatographia 80 (2017) 961–966.
[28] L. Aumann, A. Butté, M. Morbidelli, K. Büscher, B. Schenkel, Modeling of the chromatographic solvent gradient reversed phase purification of a multicomponent poypeptide mixture, Sep. Sci. Tech. 43 (2008) 1310–1337.
[29] L. Aumann, M. Morbidelli, A continuous multicolumn countercurrent solvent gradient purification (MCSGP) process, Biotech. Bioeng. 98 (2007) 1043–1055.
[30] L. Aumann, G. Ströhlein, M. Morbidelli, Parametric study of a 6-column countercurrent solvent gradient purification (MCSGP) unit, Biotech. Bioeng. 98 (2007) 1029–1042.
[31] M. Krättli, T. Müller-Späth, M. Morbidelli, Multifraction separation in countercurrent chromatography, Biotech. Bioeng. 110 (2013) 2436–2444.
[32] M. Krättli, F. Steinebach, M. Morbidelli, Online control of the twin-column countercurrent solvent gradient process for biochromatography, J. Chromatogr. A 1293 (2013) 51–59.
[33] M.M. Papathanasiou, S. Avraamidou, R. Oberdieck, A. Mantalaris, F. Steinebach, M. Morbidelli, T. Müller-Späth, E.N. Pistikopoulos, Advanced control strategies for the multicolumn countercurrent solvent gradient purification process, Amer. Inst. Chem. Eng. J. 62 (2016) 2341–2357.
[34] K.L. De Jong, B. Incledon, C.M. Yip, M.R. DeFelippis, Amyloid fibrils of glucagon characterized by high-resolution atomic force microscopy, Biophys. J. 91 (2006) 1905–1914.