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Enabling high purities and yields in therapeutic peptide purification using multicolumn countercurrent solvent gradient purification

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KEYWORDS

Countercurrent chromatography; Multicolumn countercurrent solvent gradient process; multicolumn chromatography; MCSGP; Peptide purification.

ABSTRACT

A therapeutic peptide was purified using multicolumn countercurrent solvent gradient purification (MCSGP) chromatography and its performance was compared to the performance of an optimized batch process in terms of yield, purity, productivity and solvent consumption. MCSGP simultaneously achieved high yield and purity while batch chromatography had to trade-off between these performance parameters. Using a single step preparative batch chromatography, product with a purity exceeding 97.0% could only be obtained with a yield lower than approximately 75%. For a final purity of 98.7% MCSGP showed a four-fold yield improvement (19% to 94%), a 10-fold increase in productivity (from 3 g/L/h to 30 g/L/h) and a decrease of the solvent consumption by 70% (from 3.5 L/g to 1.0 L/g).The results also showed the importance of gradient selection to obtain MCSGP parameters delivering a constant impurity profile from cycle-to-cycle.

INTRODUCTION

Today, many therapeutic peptides for pharmaceutical applications are produced by chemical solid phase synthesis. Since this technique involves the execution of many chemical steps without the ability to conduct intermediate purification, the crude product may contain many impurities which are closely related to the main product, such as omission of one amino acid or stereoisomers at one or more chiral centers. Reverse phase chromatography offers a powerful and scalable purification technology that is capable of separating the product from related impurities at a reasonable throughput. However some impurities have very similar properties to the product, and the resolution of the product and the impurities becomes worse when the load is increased. Generally, under preparative conditions, overlaps of the product and closely eluting impurities are observed in the peak front and in the tail. Product pools including the overlapping regions may not fulfill the purity specifications, and may require a narrower pooling range. By narrowing the pooling range and discarding the product in the overlapping regions, the product yield is reduced. This situation where high purity can only be obtained at the cost of yield and vice-versa is called yield-purity trade-off and is intrinsic to batch chromatography.

In order to improve on standard chromatography the MCSGP process (multicolumn countercurrent solvent gradient purification) was developed. The MCSGP process features internal recycling and re-separation of the overlapping fractions in order to recover the product contained therein. The capability of MCSGP to recover the product contained in overlapping fractions allows for the use of conditions that lead to improved throughput but also enhanced overlapping. These conditions include the use of shorter columns and/or larger particles, increased load and steeper gradients. Since its invention in 2005 (1), the MCSGP process has been experimentally verified with decreasing number of columns and hardware effort over the years, decreasing costs and risk of failure. Initially, a six-column process was demonstrated for peptide purification using reversed phase (2). Subsequently, three- and four column systems were introduced and used for the purification of peptides using reversed phase chromatography (3) and of monoclonal antibodies (mAbs) (4, 5) and mAb variants (6-8). Recently, a twin-column process was introduced and used for the purification of bispecific antibodies (9). Also the online control of the twin-column process has been described (10).

It is important to note that reducing the number of columns increases the flexibility of the process by carrying out process tasks in a sequential manner rather than in a parallel manner: Since, in MCSGP, all columns need to switch positions at the same time, carrying out tasks in parallel with more columns can lead to the situation where one or more columns have to wait until the process task of the column with the longest task has been completed. If tasks are carried out sequentially with fewer columns, this situation occurs less frequently since more switching intervals are available to complete the same number of tasks. The applicability to different separation challenges (peptides, mAbs etc) is not limited by reducing the number of columns but is actually improved. The concept of internal recycling for the simultaneous achievement of high yield and purity remains the same.

The process principle of MCSGP is shown below for a twin-column setup in Figure 1.

The schematic chromatogram at the bottom of Figure 1 represents a batch chromatogram that has been divided into different sections (vertical dashed lines) according to the

tasks that are carried out in the batch chromatography run (equilibration, feeding, washing, elution, cleaning) and according to the elution order of the chromatogram (elution of weakly adsorbing impurities W, elution of the overlapping part W/P (W and the product P), elution of pure P, elution of the overlapping part of P/S and elution of "pure" S, the strongly adsorbing impurities. In the MCSGP process these individual tasks correspond to unique flow paths in the system and are carried out analogously in pairs in the twin column setup. Thus the process tasks of the single column batch process and the MCSGP process are analogous and it is possible to derive the operating parameters for MCSGP from the batch operating parameters and the corresponding chromatogram. Briefly, the operating parameters are computed such that the elution volumes and the gradient start and end concentrations of the respective zones are the same in the batch and the MCSGP process.

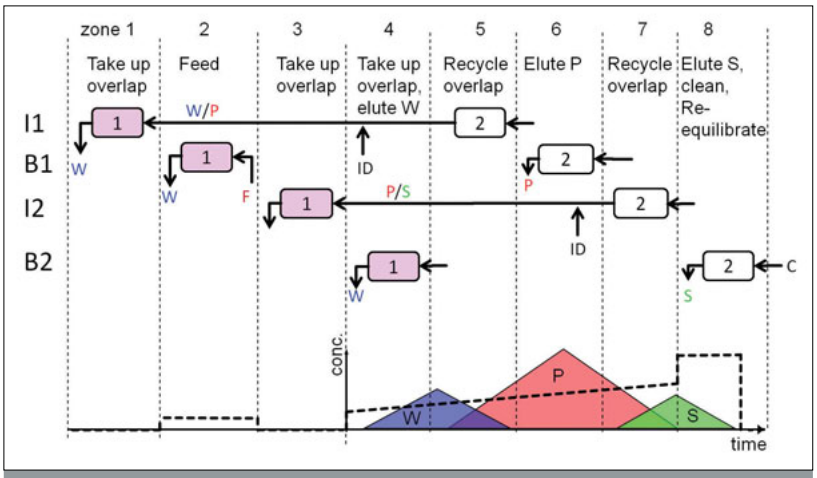

Figure 1. Schematic illustration of the twin-column MCSGP process principle (1st switch). The dashed vertical lines separate the different MCSGP process tasks corresponding to the zones of the schematic batch chromatogram shown in the lower part of the figure. Phases I1, B1, I2, B2 are carried out sequentially.

A complete cycle of a twin-column MCSGP process comprises two "switches" with four pairs of tasks each (I1, B1, I2, B2) as illustrated in Figure 1.

The phases in each switch are identical; the difference is only in the column position: In the first switch, column 1 is downstream of column 2 while in the second switch (not shown in Figure 1) column 2 is downstream of column 1. The four phases comprise the following tasks:

- Phase I1: The overlapping part W/P is eluted from the upstream column, and internally recycled into the downstream column (zones 5 and 1). In between the columns, the stream is diluted inline with solvent to re-adsorb P (and overlapping W) in the downstream column. At the end of phase I1, pure product is ready for elution at the outlet of the upstream column (zone 5).
- Phase B1: Pure P is eluted and collected from the column in zone 5 (column 2 in Figure 1), keeping the overlapping part P/S and S in the column. At the same time, fresh feed is injected into the column in zone 2.
- Phase I2: The overlapping part P/S is eluted from the upstream column, and internally recycled into the downstream column (zones 7 and 3). In between the columns, the stream is diluted inline with solvent to re-adsorb P in the downstream column. At the end of the step, all remaining P has been eluted from the upstream column and only S is left in the upstream column.
- Phase B2: The column in zone 8 (column 2 in Figure 1) is cleaned to remove S and re-equilibrated. At the same time, W is eluted from the other column in zone 4.

After having completed these tasks, the columns switch positions and in the next phase I1 (not shown in Figure 1), column 2 is in the downstream position (zone 1) and column 1 is in the upstream position (zone 5). At the beginning of this I1 phase, column 2 is cleaned and re-equilibrated and ready for uptake of the W/P fraction from column 1. After having completed B1, I2, and B2 for the second time the columns are returning to their original positions and one cycle has been completed. At this point column 1 is clean and ready or uptake of W/P of the column 2 in phase I1 (as shown in Figure 1).

The data shown in this study were obtained using a three-column MCSGP process since at the time of the project execution the twin-column process had not yet been developed. However, since the separation power of the twin column process is superior to the three-column process (since W/P and P/S recycling are carried out in independent interconnected phases) it is reasonable to expect that the performance in terms of yield/ purity and productivity would improve using twin-column versus three-column MCSGP.

In contrast to the 3-column process, the twin-column process features an independent recycling of W/P and P/S which improves the flexibility in terms of the recycling phase durations at the cost of slightly increased solvent consumption.

As in other countercurrent chromatographic processes, in practice in MCSGP the column movement is simulated by connecting and disconnecting column inlets and outlets through valve switching and not by physical movement of the columns.

In order to compare MCSGP and batch processes, performance parameters are defined below.

The process yield *Y* is defined as the ratio of the product mass recovered in the product pool m_{pool} and the product mass contained in the feed m_{feed}, within one cycle. In MCSGP chromatography, the yield is measured during cyclic steady state, i.e. when the process has started up and the UV profiles do not change anymore from cycle to cycle (typically 2-3 cycles):

$$Y = \frac{m_{pool}}{m_{feed}} \qquad \text{(equation 1)}$$

The mass balance closure *mb* is defined as the ratio of the product mass leaving the system m_{out} including all fractions and the product mass entering the system via the feed within the same time period (m_{feed}). In batch and MCSGP chromatography (in cyclic steady state) the mass balance closure is typically referred to one cycle:

$$mb = \frac{m_{out}}{m_{feed}} \qquad \text{(equation 2)}$$

The purity P is defined by the area ratio of the peptide product peak, A_{pool}, and the total peak area, A_{total}, and is given in percent [%]. The areas are extracted from the analytical chromatograms of pooled fractions:

$$P = \frac{A_{pool}}{A_{total}} \qquad \text{(equation 3)}$$

The load *L* is defined as the ratio between product mass in the feed and the total bed volume V_{col} (in MCSGP including all columns), measured within the same time period (typically one cycle):

$$L = \frac{m_{feed}}{V_{col}} \qquad \text{(equation 4)}$$

The productivity *Prod* is defined as the ratio of the product mass contained in the product pool, m_{pool}, of one cycle, the cycle duration t_{cycle} and the total stationary phase volume V_{col}:

$$Prod = \frac{m_{pool}}{t_{cycle} \cdot V_{col}} = \frac{L}{t_{cycle}} \cdot Y \qquad \text{(equation 5)}$$

The solvent consumption *SC* is defined as the ratio of the solvent volume consumed V_{solv} and the mass obtained in the product pool m_{pool}, measured within the same time period (typically one cycle):

$$SC = \frac{V_{solv}}{m_{pool}} \qquad \text{(equation 6)}$$

MATERIALS AND METHODS

Crude peptide and purified reference materials were obtained from Bristol-Myers Squibb (New Brunswick NJ). The peptide load solution for chromatography was prepared by dissolving 5.0 g crude sample (purity 66.1%) in 1L sample solvent (5 vol % acetonitrile in water with 7.4 g/L ammonium acetate). The sample was filtered through 3 μm and 0.45 μm filters and the pH was adjusted to pH 4.0 using glacial acetic acid.

For the determination of concentration and impurity content, analytical HPLC was performed using a 4.6 x 150 mm SymmetryShield RP8, 3.5 μm column (Waters, Milford, MA, USA) with the following solvents (percentages given as volume %): Solvent A: 80.0% de-ionized water, 20.0% acetonitrile, 2.07g perfluorooctanoic acid (PFOA); Solvent B: 20.0% de-ionized water, 80.0% acetonitrile, 2.07g PFOA. The pH was not adjusted. The flow rate was 0.8 mL/min, the column and buffers were maintained at 60°C, the maximum pressure was set to 300 bar and UV absorption at 215 and 280 nm was recorded. A gradient was run from 47%B to 77%B over 30 min. Afterwards the column was cleaned for 4 min with 100%B and re-equilibrated at 47%B for 11 min.

The minimum required preparative product pool purity was 96.0% as determined by the analytical method described above.

Preparative batch purification used a Kromasil C18 10 μm, 100 Å, 0.46 x 25 cm column. MCSGP chromatography used Kromasil C18 10 μm, 100 Å, 0.78 x 5 cm columns. The following solvents were used for all preparative runs (volume %): Solvent A: 100 mM ammonium acetate in de-ionized water with 3.0% acetonitrile, Solvent B: 100 mM ammonium acetate in de-ionized water with 25.0% acetonitrile. The pH was adjusted with glacial acetic acid to 4.7. For the column cleaning step, a solvent containing 5% de-ionized water and 95% acetonitrile (pH not adjusted) was used.

Analytical HPLC was performed using an Agilent HP 1100 instrument (Agilent, Santa Clara, CA, USA) with integrated diode array UV detector. Preparative batch gradient elutions and MCSGP experiments were carried out using Contichrom equipment (ChromaCon, Zurich, Switzerland). The Contichrom UV detectors were set to monitor the A_{215} signal.

The preparative batch experiment was run according to the method reported in Table 1 at a flow rate of 1.0 mL/min and at 25°C.

The MCSGP operating parameters for the optimized MCSGP run 3 are listed in Table 2. The batch phase B

Step	Duration [min]	Column volumes	Concentration [%B]
Equilibration	8.3	2.0	0-0
Feed	43.7	10.5	Feed
Wash	12.5	3.0	5-5
P elution gradient 1	12.5	3.0	5-40
P elution gradient 2	37.4	9.0	40-60
P elution gradient 3	12.5	3.0	60-100
Strip	12.5	3.0	CIP
Re-Equilibration	8.3	2.0	0-0

Table 1. Gradient conditions for preparative batch purification.

that had a total duration of 9 min was divided into two sub-parts with durations of 6 min and 3 min, respectively: Feeding and washing (6 + 3 min), P-elution (9 min), S-elution and re-equilibration (6 + 3 min) were carried out in parallel. The MCSGP process was run over 6 cycles.

RESULTS

The chromatogram for the preparative purification of the peptide is shown in Figure 2. The preparative batch gradient eluate was fractionated and the fractions were analyzed. Both purity evaluations at 215 nm and 280 nm showed that product fulfilling the purity specification can be withdrawn in the interval between 79 min and 90 min.

The MCSGP operating parameters were derived from the batch chromatogram shown in Figure 2. As in the batch run, the zones of the MCSGP process that are relevant for the separation are those that correspond to the actual (gradient) elution (zones 4-7 in Figure 1). In these zones the solvent gradient is run. The other zones are dedicated to recycling uptake, to feeding and washing (zones 1-3 in Figure 1) and to the cleaning and re-equilibration of the columns (zone 8).

The first part of the bi-linear gradient in the batch separation process was a gradient from 5 to 40%, run in 3.0 column volumes at a flow rate of 1.0 mL/min. This gradient was reproduced in the MCSGP process for elution of W

Step	Phase	Duration [min]	CV [-]	Concentration [%B]
W/P recycle	I	7	3.2	0-38.5
W/P inline dil	I	7	6.3	0-0
P elution	B	9	2.9	39.0-55.0
Feed	B	6	7.6	Feed
wash	B	3	2.0	0-0
P/S recycle	I	7	1.5	55.0-40.0
P/S inline dil	I	7	n.a.	n.a.
S elution	B	6	4.1	solv C
Re-equilibration	B	3	2.0	0-0
W elution	B	9	n.a.	n.a.

Table 2. MCSGP operating parameters for run 3 for the 3-column MCSGP setup.

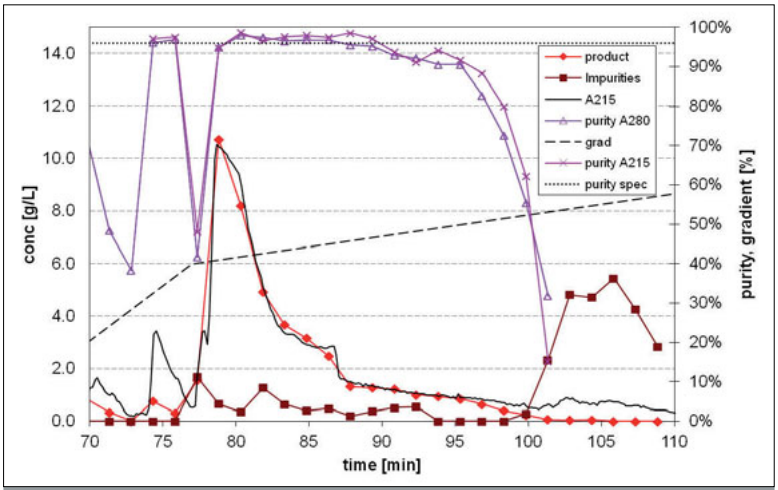

Figure 2. Optimized batch gradient chromatogram. The concentrations of the product and the impurities are shown together with the scaled A215 signal (left y-axis). Purities determined at 215 and 280 nm and the gradient concentration are shown on the right y-axis.

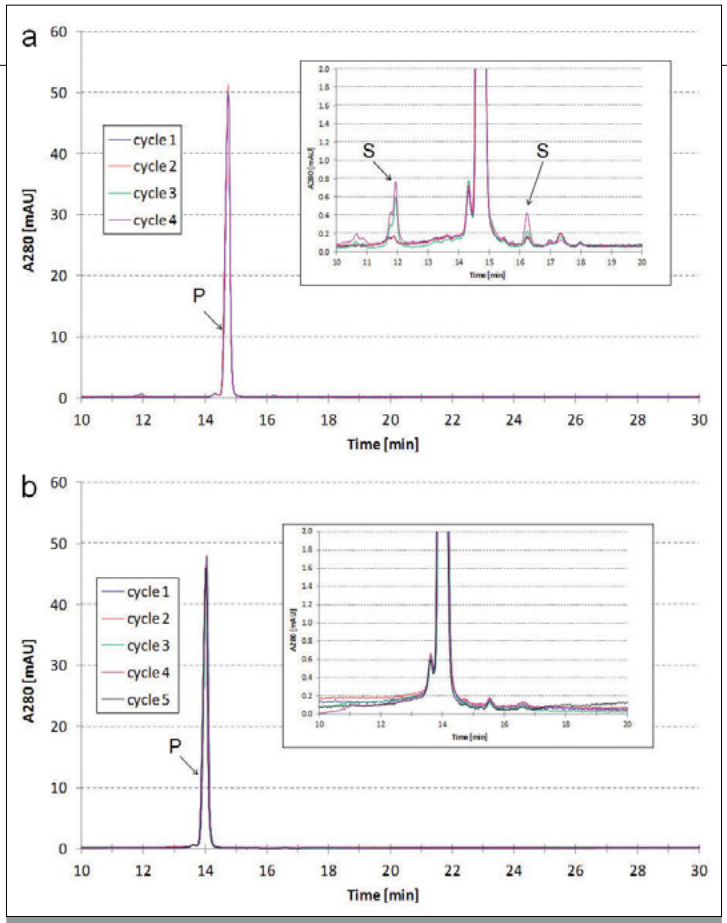

Figure 3. Overlay of analytical chromatograms (A280) of product samples taken at: a) Four steady state cycles (cycles 2-5) of MCSGP run 2, (isocratic operation for P/S recycling in zone 7); and b) Five steady state cycles (cycles 2-6) of MCSGP run 3 (negative gradient for P/S recycling in zone 7). The rectangles in the figure show magnifications of the chromatograms. "S" indicates accumulating impurities and "P" the product.

and W/P recycling (zones 4 and 5 in Figure 1). The second part of the bi-linear gradient was the product elution gradient (40-60% in 9.0 column volumes) that was reproduced in the MCSGP process for product elution and P/S recycling (zones 6 and 7 in Figure 1). In the batch gradient elution and in the initial MCSGP operating conditions, the gradient in zone 7 was a continuation of the gradient corresponding to zone 6 with the same slope, thus resulting in the overall bi-linear gradient described earlier (MCSGP run 1).

However, when analyzing the product pools of subsequent cycles of MCSGP run 1 it was observed that the overall amount of S impurities in the product pool increased over time, indicating an accumulation of these impurities within the system. In order to avoid this accumulation, the gradient used for S/P recycling (zone 7) was modified, while the first linear gradient (extracted from the batch gradient and corresponding to zones 4 and 5), was left unchanged since the removal of W impurities was sufficient. The gradient used for product elution was also not changed (zone 6). In order to retard and remove the S impurities, the slope of the gradient corresponding to P/S elution (zone 7) was initially changed to zero (isocratic operation) and subsequently to a negative slope. The results of this optimization are shown in Figure 3. Figure 3-a shows the isocratic case where an accumulation of S impurities can be observed while Figure 3-b shows a constant impurity profile over the cycles without S accumulation, confirming the successful gradient optimization. Note that one S impurity group is strongly adsorbing on the preparative stationary phase but early eluting in the analytical method.

Figure 4 shows the results of MCSGP run 3 in terms of yield, purity and concentration curves as a function of the cycle number. Yield, purity and concentration values refer to the product withdrawn over one cycle (one product collection from each column per cycle) from the P-outlet corresponding to zone 6. The "loss in W" and "loss in S" in Figure 4 refer to the product that is lost in the outlets corresponding to zones 1-4 (W) and zone 8 (S), respectively, and are below 3.0% each in most cases. The fact that the values are constant from the third cycle onwards indicates that the process has reached cyclic steady state.

The performance data yield, purity, productivity, product concentration and solvent consumption were calculated for batch and MCSGP chromatography and are summarized in Table 3. These data refer to the batch run shown in Figure 2, to the MCSGP run 3 (Figure 4) and to MCSGP runs with similar operating parameters that were carried out to demonstrate process robustness (runs 4 and 5). For MCSGP, the purity and yield refer to the last cycle of the respective run. From the data in Table 3 it can be seen that the maximum purity that can be reached is equal for the batch and MCSGP processes. The mass balance values for all runs are close to 100%. In the case of MCSGP, where the mass balance is calculated using analysis of all outlet streams, this implies that no product is accumulating within the system and that the process has reached cyclic steady state. With respect to the productivity, it can be seen that with batch chromatography a value of 14 g/L/h is obtained for a purity of 96.4%. For a purity that is similar to that obtained with MCSGP (ca. 98.5%), the productivity of the batch process is about 3 g/L/h. Thus, with MCSGP, the productivity is increased about 10-fold reaching around 30 g/L/h.

The solvent consumption of the batch process is between 0.7 L/g (low purity/ high yield pool) and 3.5 L/g (high purity / low yield pool). For MCSGP, the solvent consumption is 1.0 L/g.

A graphical overview of the performance data in terms of yield and purity is provided in Figure 5. Hypothetical batch

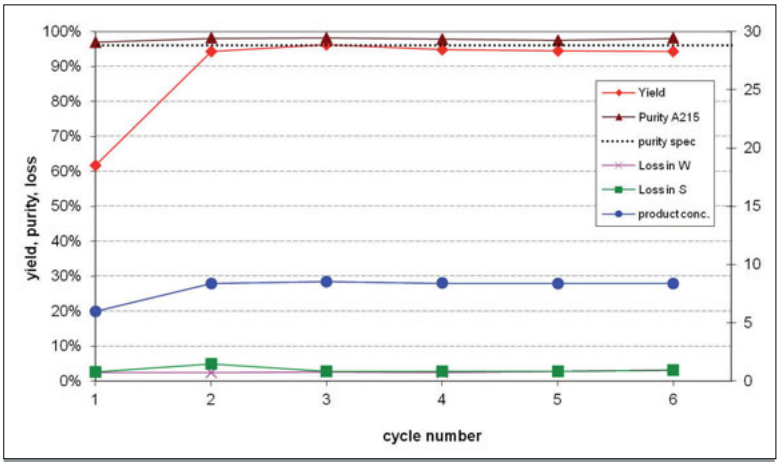

Figure 4. Representative performance from MCSGP run 3. Average yield, purity, product concentration and product losses are shown as a function of the cycle number. The purity specification (96.0%) is represented by a horizontal dotted line.

Batch	Purity	Yield	mb	Prod	Load	SC	conc P
	[%]	[%]	[%]	[g/L/h]	[g/L]	[L/g]	[g/L]
High purity pool	98.7	19.3	105.0	3	37	3.5	8.2
High yield pool	96.4	93.5	105.0	14	37	0.7	3.3

MCSGP	Purity	Yield	mb	Prod	Load	SC	conc P
	[%]	[%]	[%]	[g/L/h]	[g/L]	[L/g]	[g/L]
Run 3	98.7	94.3	100.6	28	21	1.0	8.4
Run 4	98.4	92.3	99.9	30	20	0.9	8.4
Run 5	98.4	94.7	100.5	31	22	0.9	9.3

Table 3. Summary of performance data of batch and MCSGP chromatography

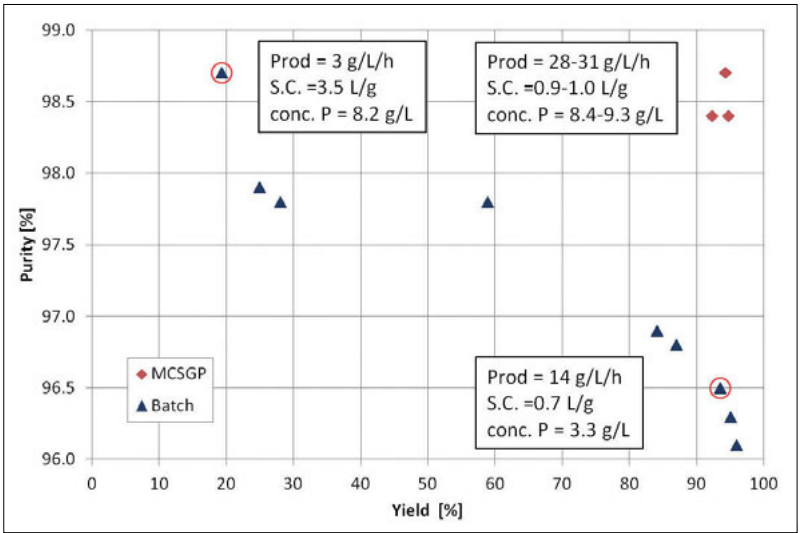

Figure 5. Overview of the performance data of batch and MCSGP chromatography. The triangles indicate batch pools of varying purity and yield. The circles indicate the batch pools with approximately the same yield or the same purity as the MCSGP runs (diamonds). Detailed information for selected batch pools (circled) with approximately the same yield or the same purity as the MCSGP runs is shown in the boxes next to the circles. Detailed information on MCSGP is shown next to the diamonds.

pool yield and purity values were calculated from batch fraction analyses by starting with the fraction of the highest purity and successively including neighboring fractions. The red circles indicate the two batch data points listed in Table 3 which have approximately either the same purity or the same yield values as the MCSGP runs (shown in the upper right corner of the chart). The figure shows that with MCSGP it is possible to produce the target peptide with high purity and high yield simultaneously, while batch chromatography is limited by a yield/purity trade-off. The product pool concentration of batch chromatography is 3.3 g/L (low purity/ high yield pool) and 8.2 g/L (high purity / low yield pool), respectively. The product concentrations of the MCSGP pools are between 8.4 and 9.3 g/L.

CONCLUSION

The MCSGP process showed significant advantages over the batch process for the purification of the investigated therapeutic peptide in terms of productivity, yield and solvent consumption at comparable purity. Compared to the batch process, the yield could be quadrupled and the productivity could be increased by a factor of 10 for the same high purity using the three-column setup employed in this work. The solvent consumption could be reduced by 70%. These findings are in line with the improvements observed by Grill et al. when comparing simulated moving bed (SMB) and batch chromatography for binary separations of enantiomers (11). The advantages include a productivity increase of approximately 10-fold and a solvent consumption reduction of 85%. In both cases, SMB for binary separations and MCSGP for ternary separations, the improvements can be attributed to the countercurrent principle and the internal recycling features of the processes. These allow for use of short columns with low resolution but high throughput, as the material contained in the overlapping parts of product and impurities is recovered.

The operating parameters of the MCSGP processes can be easily adjusted to parameters for MCSGP with a different column number. Additional improvements may be anticipated by using a twin-column MCSGP process that provides greater flexibility through independent recycling of the two product-containing overlapping regions, significantly reduced hardware requirements and a more simple operation.

The use of traditional hardware including pumps and valves makes MCSGP a scalable process solution and the improvements in terms of throughput and solvent consumptions can lead to significant cost savings on a larger scale.

REFERENCES

1. Aumann L, Morbidelli M. WO/2006/116886
2. Aumann L., Morbidelli M., Biotechnol. Bioeng. **98(5)**, 1043–1055 (2007)
3. Aumann L., Morbidelli M., Biotechnol. Bioeng. **99(3)**,728–733 (2008)
4. Müller-Späth T., Aumann L., Morbidelli M., Sep. Sci. Technol. **44(1)**, 1–26 (2009)
5. Müller-Späth T., Aumann L., et al., Biotechnol. Bioeng. **107(6)**, 974-984 (2010)
6. Müller-Späth T., Aumann L., et al., Biotechnol. Bioeng. **100(6)**,1166-1177 (2008)
7. Müller-Späth T., Krattli M., et al., Biotechnol. Bioeng. **107(4)**, 652–662 (2010)
8. Krättli M., Müller-Späth T., Morbidelli M., Biotechnol. Bioeng., **110(9)**, 2436–2444 (2013)
9. Müller-Späth T., Ulmer N., et al., BioProcess Int., **11(5)**, 36–45 (2013)
10. Krättli M., Steinebach F., Morbidelli M., J. Chromatogr., A, **1293**, 51-59 (2013)
11. Grill C.M., Miller L., Yan T.Q., J Chromatogr A., **1026(1-2)**, 101-108(2004)