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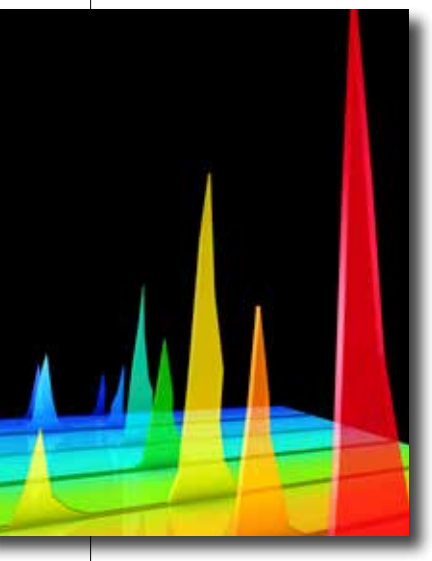

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# Size-Exclusion Chromatography for Preparative Purification of Biomolecules

**Andreas Stein, Anja Heinen-Kreuzig, André Kiesewetter, and Sebastian Schwarm**

## ABSTRACT

High-resolution size-exclusion chromatography (SEC) is not often used at large scales compared with other chromatography modes because of the restrictions in sample size and flow rate. In this article, the authors provide application data to support the use of SEC beyond small-scale operations, using the tentacle resin Fractogel EMD BioSEC (S). The separation of 20 and 40 kDa proteins was illustrated in a case study, in which flow rate and sample size were varied to examine the operational limits with regard to productivity. In a second application, efficient aggregate removal in monoclonal antibody purification was demonstrated. With SEC as the second step after Protein A affinity capture, the monomer with a purity of 99.5% was obtained. Purity and yield in both applications were determined by model-based peak deconvolution. For the antibody purification, the productivity of SEC was compared with the productivity using cation-exchange chromatography (CEX). In a third section, the pressure flow behavior of Fractogel EMD BioSEC (S) was characterized on pilot scale. These data are key for designing an SEC operation with regard to step time, target bed height, and appropriate column hardware.

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In size-exclusion chromatography (SEC), molecules are separated according to the differences in size and structure as they penetrate differentially into the pores of the stationary phase. Two separation modes can be distinguished. In group separation, a group of small molecules is separated from a group of large molecules, such as in buffer exchange. High-resolution fractionation is carried out in fractionation mode, where molecules of similar size are separated from each other. Some important parameters that influence the resolution in SEC are bed height, flow rate, sample volume, particle size, and particle pore size. As the separation challenge in group separation is usually low, this mode can be operated relatively fast and with relatively high throughput. Short bed heights of 10–20 cm are usually sufficient. Linear flow rates of 100 to 250 cm/h can be applied and sample volumes may reach up to 30% of the column volume (CV). The fractionation mode requires higher separation efficiency, resulting in long columns, low flow rates, and small sample volumes. A bed height of 60 cm has shown to be adequate for a multitude of applications. Bed heights above 60 cm and up to 120 cm can be operated with the appropriate resins and col-

umn hardware and have been implemented in individual cases (1–5). SEC continues to be used in biopharmaceutical areas, including the purification of adenovirus (6, 7), lentivirus (8), influenza virus (9, 10), antibody fragment vaccine against HIV1 (11), plasmid (12), or viral clearance (13).

In the current work, the authors used Fractogel EMD BioSEC (S), a methacrylate-based resin with a narrow particle size distribution of 20–40 µm, which is designed for chromatographic applications up to production scale for high-resolution fractionation. First, the impact of sample volume and flow rate on the separation of 20 and 40 kDa proteins was examined. Secondly, efficient aggregate removal was demonstrated in purification of a monoclonal antibody (mAb). Yield and purity were calculated from the chromatograms using a numerical method. In a third part, the pressure flow behavior was characterized at pilot scale. For the antibody purification, the productivities of SEC and of cation-exchange chromatography (CEX) were compared.

## MATERIALS AND METHODS

Conalbumin and ovalbumin were from Sigma (Steinheim, Germany) and chymotrypsinogen A was from Serva (Heidelberg, Germany). Trypsin inhibitor (from soybean), all other chemicals, and the Fractogel EMD resins were purchased from Merck KGaA (Darmstadt, Germany). Monoclonal antibodies mAb01 and mAb02 were from in-house feedstocks. Column hardware (Superformance 600-16, Superformance 600-26, Superformance 1000-50, Super Compact 240-5) was obtained from Goetec-Labortechnik (Bickenbach, Germany). Column packing and preparative separation runs were performed on an ÄKTAexplorer 100 chromatographic system (GE Healthcare, Uppsala, Sweden) with UV detection at 280 nm. Analytical SEC high-performance liquid chromatography (HPLC) was conducted on a Chromaster HPLC system (VWR–Hitachi, Darmstadt, Germany) using a G3000SWXL column (Tosoh Bioscience GmbH, Griesheim, Germany).

### High-resolution fractionation

Mixtures of two proteins with molecular weights of approximately 20 kDa and 40 kDa were separated using a column of 60 x 1.6 cm inner diameter (i.d.) (packed to 20% compression). Sample volume and flow rate were varied. Each molecular weight (Mw) was represented by two proteins with different isoelectric points (pI). The rationale was to look at potential protein–protein interaction. Proteins were used at a concentration of 5 mg/mL of each protein.

| | Molecular weight (kDa) | Isoelectric points (pI) |
|---|---|---|
| ovalbumin | 45 | 4.6 |
| conalbumin | 40 | 6.9–7.0 |
| chymotrypsinogen A | 25 | 9.1 |
| trypsin inhibitor | 21.5 | 4.5 |

Sample buffer and eluent was 20 mM sodium phosphate plus 0.3 M sodium chloride (NaCl), pH 7.2. The resolution was calculated using Unicorn software version 5.3.1 from GE Healthcare (Uppsala, Sweden).

For antibody purification with SEC employing a column of 52.5 x 2.6 cm i.d. (packed to 18% compression), the starting material was a Protein A affinity chromatography elution pool containing mAb01 at a concentration of 12.8 mg/mL with 6.2% of aggregates adjusted to pH 7. Isocratic elution was performed using 20 mM sodium phosphate buffer plus 0.15 M NaCl, pH 7.2.

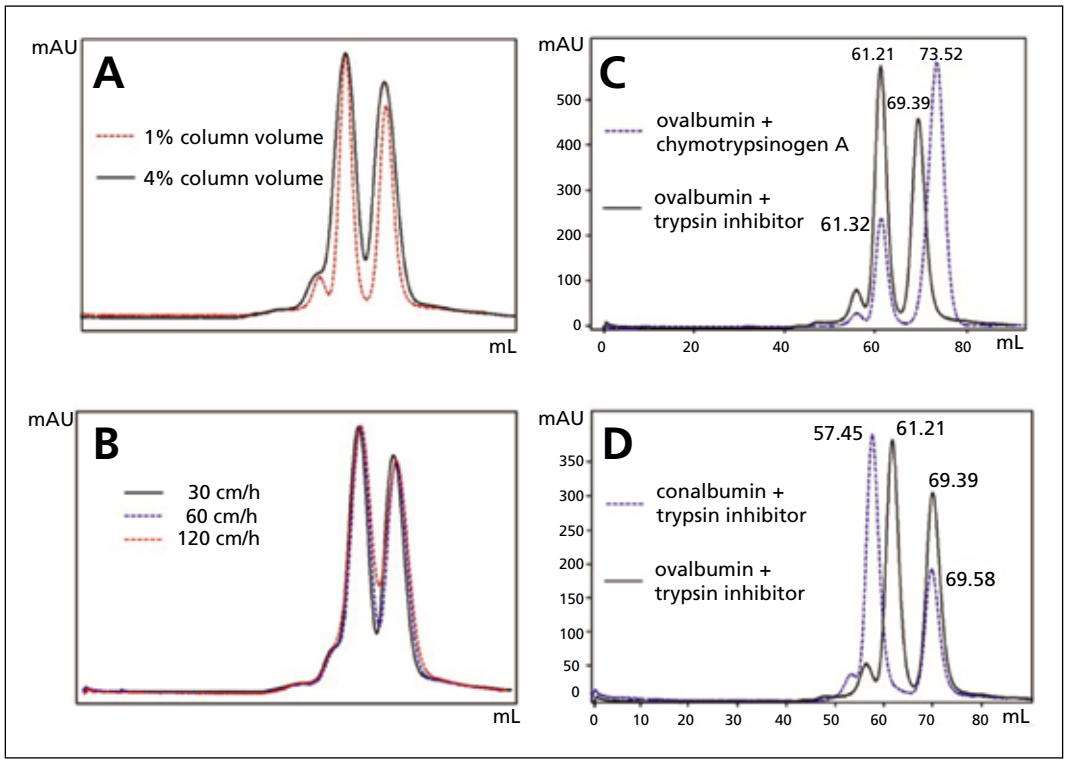
### Peak deconvolution using the EMG model

Preparative chromatographic separations are usually evaluated by quantifying target compounds and overlapping impurities in appropriate fractions employing analytical techniques. As an alternative, the mathematical separation of the recorded signals exists, known as inverse convolution or deconvolution. The exponentially modified Gaussian (EMG) model (14, 15), a convolution of a Gaussian profile with an exponential decay function, is one of the preferred models in chromatography as it may describe a variety of peaks (16, 17). It was applied in this work using PeakFIT V4.11 (Systat Software Inc., London, UK). The cut point for fractions was set to the minimum between both peaks.

### CEX for antibody purification

Fractogel EMD SO3- (M), packed to 20-cm bed height and 12% compression in a 0.5-

**Figure 1:** The influence of sample volume (A), flow rate (B), and protein species (C + D) on the high-resolution fractionation of 20 kDa and 40 kDa proteins (chromatograms). A + B: main peak 1 = ovalbumin, main peak 2 = trypsin inhibitor. A: linear flow rate = 30 cm/h. B: load = 4% column volume (CV). C: main peak 1 = ovalbumin. D: main peak 2 = trypsin inhibitor. C + D: sample volume = 1% CV, linear flow rate = 30 cm/h. mAU = milli absorbance unit.




cm i.d. column, was equilibrated with 50 mM acetic acid buffer containing 24 mM NaCl, pH 5.0, conductivity 5.5 mS/cm. The starting material was a Protein A chromatography elution pool conditioned to pH 5.0 and 5.5 mS/cm containing mAb02 with a concentration of 4.9 mg/mL and 2.5% aggregates. The column was loaded to 80 mg mAb02/mL CV. Elution buffer was 50 mM acetic acid + 1 M NaCl, pH 5.0 and a 0–50% linear gradient over 20 CV was applied. Aggregates were quantified by analytical SEC HPLC (18). Productivity was calculated as the amount of protein recovered per column volume and per processing time.

## RESULTS AND DISCUSSION

### High-resolution fractionation

Mixtures of two proteins with molecular weights of approximately 20 kDa and 40 kDa were separated using Fractogel EMD BioSEC (S) in a laboratory-scale column. Sample volume and flow rate were varied by up to a factor of four. The chromatograms of these runs are provided in **Figure 1,** and the performance as defined by resolution, purity, and yield is summarized in **Table I. Figure 2** illustrates an example for peak deconvolution. Increasing the sample volume from 1% to 4% CV at a low linear flow rate of 30 cm/h resulted in a minor loss in purity and yield of up to 1.5%. Doubling the flow rate at a load of 4% CV did not change performance. Doubling the flow rate again to 120 cm/h caused an additional drop in purity and yield of up to approximately 4%. The total decrease in purity and yield when changing from 1% CV and 30 cm/h to 4% CV and 120 cm/h was up to about 6%. This change of process conditions would correspond to a 15-fold increase in productivity. For many applications, such a large gain in productivity may easily overcompensate the small losses in purity and yield.

ALL FIGURES ARE COURTESY OF THE AUTHORS.

**Table I:** High-resolution fractionation of 20 kDa and 40 kDa proteins. The influence of three different parameters, namely sample volume, flow rate, and protein species on fractionation is described in the table. "con" is conalbumin, "chymo" is chymotrypsinogen A, "ov" is ovalbumin, "tryps" is trypsin inhibitor, and "CV" is column volume.

| Run no. | Parameters varied | Resolution | Purity (%) | | Yield (%) | |
|---|---|---|---|---|---|---|
| | | | main peak 1 | main peak 2 | main peak 1 | main peak 2 |
| #a | ov/tryps mixture load = 1% CV flow rate = 30 cm/h | 1.60 | 99.94 | 98.56 | 99.15 | 99.96 |
| #b | ov/tryps mixture load = 4% CV flow rate = 30 cm/h | 1.13 | 98.48 | 98.07 | 98.59 | 98.36 |
| #c | ov/tryps mixture load = 4% CV flow rate = 60 cm/h | 1.11 | 98.41 | 97.69 | 98.32 | 98.28 |
| #d | ov/tryps mixture load = 4% CV flow rate = 120 cm/h | 0.97 | 94.45 | 96.99 | 97.71 | 93.80 |
| #e | ov/chymo mixture load = 1% CV flow rate = 30 cm/h | 2.20 | 100.00 | 100.00 | 100.00 | 100.00 |
| #f | con/tryps mixture load = 1% CV flow rate = 30 cm/h | 2.52 | 100.00 | 100.00 | 100.00 | 100.00 |

**Figure 2:** Deconvolution example from the high-resolution fractionation of 20 kDa and 40 kDa proteins. Separation of ovalbumin und trypsin inhibitor at a linear flow rate of 60 cm/h, sample volume = 4% column volume (CV) (Run #c). EMG is exponentially modified Gaussian.

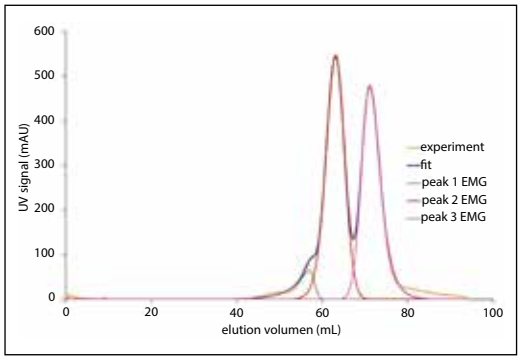



**Figure 1** also reveals that no protein–protein interaction occurred. For the separation of ovalbumin (negatively charged) from chymotrypsinogen A (positively charged) and from trypsin inhibitor (negatively charged), the elution volume of ovalbumin was almost constant, regardless of the different charges of the respective protein partner (see **Figure 1C**). The same held true for the mixtures of trypsin inhibitor (negatively charged) with ovalbumin (negatively charged) or with conalbumin (neutral, no charge) where the elution volume of trypsin inhibitor remained nearly the same (see **Figure 1D**). This observation is consistent with the expectation that the elevated salt concentration in the mobile phase would suppress electrostatic interactions.

For both protein pairs of similar size (ovalbumin/conalbumin and chymotrypsinogen A/trypsin inhibitor), a partial resolution was recognized, although the differences in molecular weight were very small. Both pairs displayed an unexpected, reversed retention order with the smaller protein eluting first. Because the hydrodynamic radius of molecules is essential for their elution order, this behavior is likely to reflect deviations from the ideal globular shape.

For Fractogel EMD BioSEC (S), a novel and additional size-dependent separation effect has been described. This entropic interaction is related to the brush-like hydrophilic graft polymer (19) and contributes to the broad fractionation range and high resolution obtained with the tentacle SEC resin (20).

**Figure 3:** Pressure flow curves of Fractogel EMD BioSEC (S). Recorded in 150 mM NaCl. Resin packed in Superformance 1000–50 column to 18% compression.

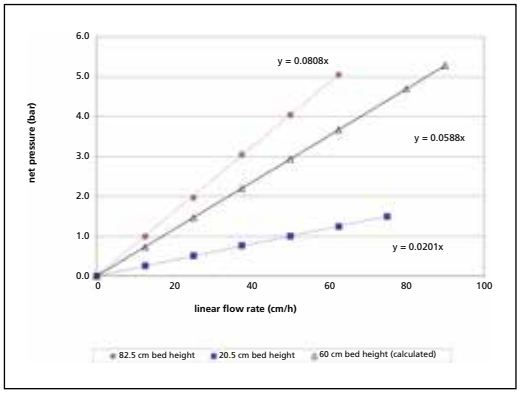
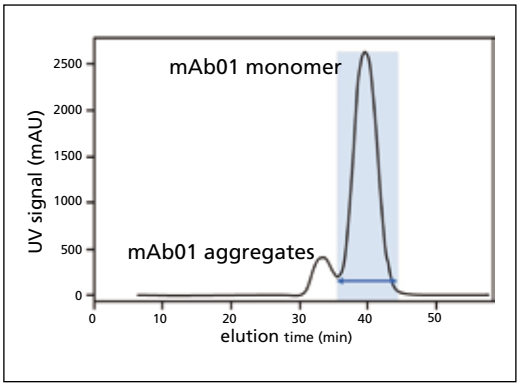



**Figure 4:** Separation of monoclonal antibody monomer and aggregates on Fractogel EMD BioSEC (S). The monomer pool as indicated by the arrow contained a residual aggregate level of 0.6%.




### Pressure flow characteristics

**Figure 3** presents the pressure flow curves of Fractogel EMD BioSEC (S) measured for the bed heights of 20.5 cm and 82.5 cm. The curves are linear across the measured window, which reflects a stable gel bed. A linear correlation between pressure drop and flow rate is characteristic for noncompressible beds of rigid particles (21). For the polymer-based semi-rigid Fractogel resins, linearity still extends over a relatively wide range. Conventional carbohydrate-based soft gels, in contrast, exhibit a much narrower linear range. The factor of four between the short and long bed is exactly mirrored by the slopes of the pressure flow curves. This proportional relationship allows prediction of the pressure flow behavior for any bed height and, as such, greatly facilitates step design. The pressure flow data for 60-cm bed height were calculated using the slope of the curve for 82.5-cm bed height. The diagram demonstrates that columns of 5-cm i.d. packed to a bed height of 60 cm can be operated at linear flow rates of up to approximately 80 cm/h, when using eluents with a viscosity equivalent to water. For larger columns with an i.d. of 10 cm and a bed height of 60 cm, the same slope was reported, but the curve was only measured up to a flow rate of 50 cm/h (20). Care must be taken not to exceed the pressure limits supplied by the manufacturer of the column hardware.

### Productivities

For mAb purification, the chromatogram is shown in **Figure 4**. Characteristic run parameters and the calculated productivities are compiled in **Table II**. Run #1 represents the single experimental SEC separation. Runs #2 through #5 are virtual (theoretical) runs through which specified changes of parameters for the resulting productivities were calculated. For the experimental run, purity was 99.4% and yield was 99.5% as derived from deconvolution. Purity was independently determined by analytical SEC HPLC and resulted in an almost identical 99.5%. For this run, a productivity of 0.159 g/(L x h) was obtained. To gain further insight into how a change of run parameters would influence step productivity, the virtual runs #2 through #5 were created, in which run parameters were set to their limits. Parameters were changed under the assumption that purity and yield would remain constant. The model protein work above adds credibility to this assumption because yield and purity remained fairly constant over a four-fold range of loading and at least a two-fold range of flow. The sample volume was increased from an initial 3.2% CV to 5.0% (run #2), which is at the upper end of a range of 2–6% and was verified for the fractionation mode (22). The linear flow rate was increased to its maximum of 80 cm/h as defined by the pressure flow characteristics of the resin (run #3), valid for 60-cm bed height and small pilot scale (columns of 5-cm i.d.). The sample concentration was increased from the initial 12.8 mg/mL to a virtual 60 mg/mL (run #4), based on the

**Table II:** Description of antibody purification runs and productivity calculation.

| Run number | Resin | Comment | Column volume, CV (mL) (200 x 5 mm i.d.) (525 x 26 mm i.d.) | Load (mg mAb/mL CV) (mL sample/mL CV) | Load SEC (mg mAb/mL CV) | Sample concentration SEC (mg/mL) | mg mAb in eluate pool/mL CV | Equilibration | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Volume (CV) | Linear flow rate (cm/h) | Step time (min) |
| #1 | FG BioSEC (S) | Initial conditions | 278.7 | 0.032 | 0.413 | 12.8 | 0.411 | 2 | 60 | 105.0 |
| #2 | FG BioSEC (S) | Sample volume increased | 278.7 | **0.050** | 0.640 | 12.8 | 0.637 | 2 | 60 | 105.0 |
| #3 | FG BioSEC (S) | Flow rate set to maximum | 278.7 | 0.032 | 0.413 | 12.8 | 0.411 | 2 | **80** | 78.8 |
| #4 | FG BioSEC (S) | Sample concentration increased | 278.7 | 0.032 | 1.938 | **60.0** | 1.928 | 2 | 60 | 105.0 |
| #5 | FG BioSEC (S) | All changes combined | 278.7 | **0.050** | 3.000 | **60.0** | 2.985 | 2 | **80** | 78.8 |
| #6 | FG SO3- (M) | Aggregates in pool ≤ 1.5 %, yield = 89 % | 3.93 | 80 | n.a. | n.a. | 71.2 | 2 | 200 | 12.0 |
| #7 | FG SO3- (M) | Aggregates in pool ≤ 0.5 %, yield = 63 % | 3.93 | 80 | n.a. | n.a. | 50.4 | 2 | 200 | 12.0 |

| Run number | Load | | | Wash | | | Elution + strip | | | Re-equilibration | | | Cycle time (h) | Productivity (g/(L x h) | Relative productivity CEX versus SEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Volume (CV) | Linear flow rate (cm/h) | Step time (min) | Volume (CV) | Linear flow rate (cm/h) | Step time (min) | Volume (CV) | Linear flow rate (cm/h) | Step time (min) | Volume (CV) | Linear flow rate (cm/h) | Step time (min) | | | |
| #1 | 0.032 | 30 | 3 | n.a. | n.a. | n.a. | 0.444 | 30 | 47 | n.a. | n.a. | n.a. | 2.6 | 0.159 | 99 |
| #2 | **0.050** | 30 | 5 | n.a. | n.a. | n.a. | 0.444 | 30 | 47 | n.a. | n.a. | n.a. | 2.6 | 0.244 | 65 |
| #3 | 0.032 | **80** | 1 | n.a. | n.a. | n.a. | 0.444 | **80** | 17 | n.a. | n.a. | n.a. | 1.6 | 0.253 | 63 |
| #4 | 0.032 | 30 | 3 | n.a. | n.a. | n.a. | 0.444 | 30 | 47 | n.a. | n.a. | n.a. | 2.6 | 0.746 | 21 |
| #5 | **0.050** | **80** | 2 | n.a. | n.a. | n.a. | 0.444 | **80** | 17 | n.a. | n.a. | n.a. | 1.6 | 1.824 | 9 |
| #6 | 16.8 | 200 | 101 | 6.5 | 200 | 39 | 16.7 | 200 | 100 | 3 | 200 | 18 | 4.5 | 15.83 | n.a. |
| #7 | 16.8 | 200 | 101 | 6.5 | 200 | 39 | 16.7 | 200 | 100 | 3 | 200 | 18 | 4.5 | 11.20 | n.a. |

FG = Fractogel EMD, i.d. = inner diameter, CEC = cation exchange chromatography, mAb = monoclonal antibody, n.a. = not applicable. Lettering in blue refers to CEC only. Area in grey denotes theoretical runs. No experiments were conducted. Highlighted in yellow are parameters which were changed in the individual rows. Yield for all SEC runs was or was set 99.5 %.

observation of viscosity effects at sample concentrations exceeding 75 mg protein/mL (22). In run #5, all parameter changes were combined. **Table II** elucidates the incremental changes in productivity associated with the change of run parameters. The maximum productivity was about 1.8 g/(L x h), which was an eleven-fold increase compared with the initial, non-optimized state. This value is well aligned with literature, where 0.13 to 0.6 g/(L x h) and an anticipated 1 g/(L x h) for a 30 µm preparative grade resin material have been reported (22–24). Productivity in SEC can be increased considerably when operated as simulated moving bed process (12, 25, 26) or using pulsed-fed (23). When comparing CEX as conducted in this work (run #6) with SEC, ion exchange was 99 times more productive than non-optimized SEC, and still nine times more than the optimized case. This observation confirms the

assessment of SEC as a low-productivity operation, while at the same time putting it into perspective to CEX. SEC has its strength in challenging separations where target specifications may not be reached with other chromatography types or may only be reached at the expense of low yield. Such a case is exemplified by the antibody purification using CEX. A low aggregate level of ≤0.5% could only be achieved at a yield of 63% (run #7), which is usually not acceptable as a step yield. For ion exchangers operated in bind and elute mode, productivities from 10–20 g/(L x h) for antibodies (27, 28) and 0.035 to 240 g/(L x h) for other proteins have been described (29, 30). The severity of a separation may require that operational parameters are restricted below a resin's inherent maximum potential.

## CONCLUSION

For preparative SEC using the tentacle resin Fractogel EMD BioSEC (S), the influence of sample volume and flow rate on high-resolution protein fractionation was examined. The authors demonstrated that a load of up to 4% of the column volume and a relatively high linear flow rate of up to 120 cm/h using a laboratory-scale column was feasible to achieve high purities and yields of >90%. For small pilot scale, flow rates of up to 80 cm/h could be realized with a bed height of 60 cm. For the separation of antibody monomer from aggregates, productivity was maximized to 1–2 g/(L x h) when the relevant process parameters were pushed to their limits in virtual separations. The authors confirmed that the productivity of SEC was considerably lower compared to CEX and may still be only one-tenth, even with optimized conditions. The strength of SEC, however, is its generic separation according to molecule size. This advantage should allow successful purification for whole families of molecules as long as their size remains similar, regardless of whether these molecules vary in charge or hydrophobic properties.

## REFERENCES


1. A.J. Crooks, et al., *J Chromatogr.* 502, 59–68 (1990).
2. D. Josic, et al., *J. Chromatogr. A.* 796, 289–298 (1998).
3. A. Navdaev, et al., *J. Biol. Chem.* 276 (24) 20882–20889 (2001).
4. J. Polgár, et al., *J. Biol. Chem.* 272 (21) 13576–13583 (1997).
5. A. Schütz, et al., *Eur. J. Biochem.* 270, 2322–2331 (2003).
6. E. Burova and E. Ioffe, *Gene Ther.* 12, S5–S17 (2005).
7. M.N. Eglon, et al., *J. Gene Med.* 11, 978–989 (2009).
8. V. Slepushkin, N. Chang, and R. Cohen, *Bioprocess J.* 2 (5) 89–95 (2003).
9. B. Kalbfuss, et al., *J. Chromatogr. B. Analyt Technol Biomed Life Sci.* 873 (1) 102–12 (2008).
10. B. Kalbfuss, et al., *Biotechnol. Bioeng.* 96 (5) 932–944 (2007).
11. J.S. Gach, et al., *J Biotechnol.* 128 (4) 735–746 (2007).
12. G. Paredes, et al., *Chem. Eng. Technol.* 28, 1335–1345 (2005).
13. D.A. Horneman, et al., *AIChE J.* 53 (6), 1441–1449 (2007).
14. P.J. Naish and S. Hartwell, *Chromatographia* 26 (1) 285–296 (1988).
15. *Quantification in LC and GC. A practical guide to good chromatographic data.* H.J. Kuss and S. Kromidas, Eds. (Weinheim, Wiley-VCH, 2009).
16. J.R. Torres-Lapasió, et al., *Anal. Chem.* 69 (18) 3822–3831 (1997).
17. V.B. Di Marco and G.G. Bombi, *J. Chromatogr. A.* 931 (1–2) 1–30 (2001).
18. A. Stein and A. Kiesewetter, *J. Chromatogr. B.* 848, 151–158 (2007).
19. P. Pang, et al., *Biotechnol. Bioeng.* 90 (1) 1–13 (2005).
20. L.R. Jacob and L. Britsch, "Size exclusion chromatography on Fractogel EMD BioSEC," in *Column Handbook for Size Exclusion Chromatography*, C.S. Wu, Ed. (Academic Press, San Diego, CA, 1999).
21. J.J. Stickel and A. Fotopoulos, *Biotechnol. Prog.* 17 (4) 744–751 (2001).
22. G. Jagschies, G.K. Sofer, and L. Hagel, "Separation Technologies," in *Handbook of Process Chromatography: Development, Manufacturing, Validation and Economics* (Academic Press, Amsterdam, Oxford, San Diego, 2nd ed., 2008), pp. 81–126.
23. Y. Chen, S. Su, and J. Leong, *Process Biochem.* 45 (9) 1570–1576 (2010).
24. A. Kale and M. Cheryan, *Process Biochem.* 44 (4) 481–485 (2009).
25. P. Nestola, et al., *J. Chromatogr. A.* 1347, 111–121 (2014).
26. M. Wellhoefer, *J. Chromatogr A.* 1337, 48–56 (2014).
27. M. Degerman, et al., *J. Chromatogr. A.* 1113, 92–100 (2006).
28. D. Karlsson, et al., *J. Chromatogr. A.* 1055, 29–39 (2004).
29. K.A. Barnthouse, et al., *J. Biotechnol.* 66, 125–136 (1998).
30. H.C.S. Chenette, et al., *J. Membrane Sci.* 423–424, 43–52 (2012). ◆