Efficient Aggregate Removal from Impure Pharmaceutical Active Antibodies

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Polishing with membrane chromatography (MC) has achieved acceptance as state-of-the-art technology for charged impurities. Traditionally, anion-exchange (AEX) and cation-exchange (CEX) membrane chromatography have been used to remove charged contaminants such as host-cell proteins (HCPs), recombinant DNA, protein A, endotoxins, and viruses. In monoclonal antibody (MAb) processes, polishing steps usually follow a protein A affinity column step. In some cases, CEX capture is applied, either with at least one AEX or a combined AEX and CEX step. The latter may be replaced by a hydrophobic-interaction chromatography (HIC) step. Ceramic hydroxyapatite is also used, though less frequently.

Hydrophobic antibody aggregates formed during MAb manufacturing are frequent process-related impurities that must be removed during downstream processing because they can cause loss of activity as well as toxicity and immunogenicity. Because of their toxic potential, such aggregates can cause an unwanted response or even overreaction of a patient's immune system (anaphylaxis).

Typically, product aggregate levels are monitored using size-exclusion chromatography (SEC). Removal of aggregates from a protein solution, however, is typically performed using HIC because monomeric proteins display less hydrophobicity than aggregates do. Because they form at lower concentrations, flow-through mode is most favorable for modern MC, which is primarily driven by volume rather than mass capacity. This is reasonable because a flow-through approach significantly reduces buffer consumption and allows application of disposable devices. Until recently, however, HIC has been applied only in a bead/column format and bind-and-elute mode. Trace contaminants can be efficiently removed, particularly HCPs, recombinant DNA, leached protein A, and product-related impurities such as soluble aggregates.

To make use of membrane capabilities for high flow rates and convective flow, Sartorius Stedim Biotech addressed the limitation of conventional beads and developed a hydrophobic membrane adsorber carrying a phenyl ligand to efficiently remove product aggregates (**1**). The novel phenyl membrane adsorber has proven useful for aggregate removal in a MAb purification process.

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Table 1: Examples for reduction of aggregate levels in one step during downstream processing

	From (%)	To (%)
Protein 1 (non-IgG)	15.0 %	≤1.0 %
Protein 2 (non-IgG)	30.0 %	≤0.1 %
Protein 3 (IgG)	6.0 %	0.8 %
Protein 4 (IgG)	7.0 %	1.0 %

Development of the HIC Membrane

Flow rate and diffusion limitations with packed-bed resins can lengthen process times, which may increase the risk of protein unfolding and denaturation, leading to product loss (**2**). The developer's intention was to create a hydrophobic adsorber that shows hydrophobic interaction at high salt concentrations but keeps mass transfer limitation as small as possible.

Product Focus: Proteins (antibodies)

Process Focus: Downstream processing

Who Should Read: Process development engineers, analysts

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Level: Intermediate

That would circumvent a number of disadvantages seen with traditional resins.

The new macroporous phenyl membrane adsorber has a pore size of >3 µm with a recommended flow rate of five bed volumes per minute. Binding sites for proteins are accessible by convection rather than diffusion. That minimizes the effect of decreased binding capacity at high flow rates (**3**). The mechanism for

Table 2: Ammonium sulfate concentrations (mmol/L) applied in twelve semichromatographic batch experiments

Condition	Equilibration, Loading, and Washing	Elution 1	Elution 2	Elution 3	Elution 4
1	0	0	0	0	0
2	50	25	0	0	0
3	75	50	25	0	0
4	100	75	50	25	0
5	125	100	75	50	0
6	150	100	50	25	0
7	200	150	100	50	0
8	300	200	75	25	0
9	400	200	100	50	0
10	600	300	150	50	0
11	800	400	150	50	0
12	1000	500	200	75	0

Table 3: Buffers and chromatographic parameters applied in laboratory-scale experiment for aggregate removal (transfer from batch to dynamic conditions)

Step	Buffer	Volume (mL)	Flow Rate (mL/min)
Equilibration	50 mmol/L sodium phosphate buffer at pH 7.0 with 480 mmol/L ammonium sulfate (78.8 mS/cm)	27	5
Load	30.9 mg MAb in 50 mmol/L sodium phosphate buffer at pH 7.0 with 480 mmol/L ammonium sulfate (78.8 mS/cm)	10	5
Washing	50 mmol/L sodium phosphate buffer at pH 7.0 with 480 mmol/L ammonium sulfate (78.8 mS/cm)	9	5
Elution 1	50 mmol/L sodium phosphate buffer at pH 7.0 with 430 mmol/L ammonium sulfate (70.5 mS/cm)	9	5
Elution 2	50 mmol/L sodium phosphate buffer at pH 7.0 with 330 mmol/L ammonium sulfate (57.5 mS/cm)	9	5
Elution 3	50 mmol/L sodium phosphate buffer at pH 7.0 with 230 mmol/L ammonium sulfate (43.6 mS/cm)	9	5
Elution 4	50 mmol/L sodium phosphate buffer at pH 7.0 (6.03 mS/cm)	9	5

Table 4: Buffers and chromatographic parameters applied in laboratory-scale experiment for aggregate removal (optimized dynamic conditions)

Step	Buffer	Volume (mL)	Flow Rate (mL/min)
Equilibration	50 mmol/L sodium phosphate buffer at pH 7.0 with 430 mmol/L ammonium sulfate (70.5 mS/cm)	27	5
Load	31.4 mg MAb in 50 mmol/L sodium phosphate buffer at pH 7.0 with 480 mmol/L ammonium sulfate (78.8 mS/cm)	22	5
Washing	50 mmol/L sodium phosphate buffer at pH 7.0 with 430 mmol/L ammonium sulfate (70.5 mS/cm)	29	5
Regeneration 1	50 mmol/L sodium phosphate buffer at pH 7.0 (6.03 mS/cm)	15	5
Regeneration 2	20% isopropanol	15	5
Regeneration 3	Purified water	40	5
Storage	20% ethanol	12	5

capturing hydrophobic target molecules is defined by interactions between the hydrophobic surfaces of proteins and the adsorber. A number of hydrophobic spots on each protein are open for interaction with the hydrophobic matrix at high salt concentrations.

Membrane Matrix: A second-generation membrane was developed that displays a porous structure to enhance surface accessibility. Structure and pore size of the base membrane drives permeability, accessibility, and binding capacity of this membrane (**4**). To exclude grafting processes (as known from traditional adsorbers), the HIC ligand was directly attached to cross-linked and reinforced cellulose. Binding capacity at high salt concentrations was almost equal to that of conventional beads, to which selectivity is similar when the membrane is loaded with protein mixtures (**3**).

Ligand: HIC separates and purifies biomolecules based on differences in their hydrophobicity. Half of a protein surface may be accessible for hydrophobic interactions. In this case, the strength of interaction depends on a sufficient number of exposed hydrophobic groups and on membrane ligand type and density. Sample properties, temperature, type, and pH influence the binding process, as do concentrations of salt and additives. The main development reason for choosing the phenyl ligand in this membrane adsorber was its capability to remove product-derived hydrophobic impurities and contaminants during MAb production. The ligand also displayed high selectivity and ≤20 mg MAb/mL dynamic binding capacity, making it a good compromise for polishing IgG in bind-and-elute operations (**3**).

Table 5: Summary of yields and monomer content in fractions collected from chromatographic run with adapted conditions

Step (fraction)	Mass (mg)	Yield (%)	Monomer (%)
Load	31.4	100	94.5
Flow through (F2)	21.6	68.7	99.7
Wash (F3)	4.90	15.6	99.4
Wash (F4)	0.69	2.2	97.7
Wash (F5)	0.38	1.2	97.7
Regeneration 1 (F6)	1.66	5.3	48.8
Regeneration 2, isopropranol (F7)	2.18	6.9	25.8
Regeneration 3, purified water (F8)	0.49	1.6	—

Figure 1: Procedure conducted in batch experiments to determine the best conditions for aggregate removal in 96-well format

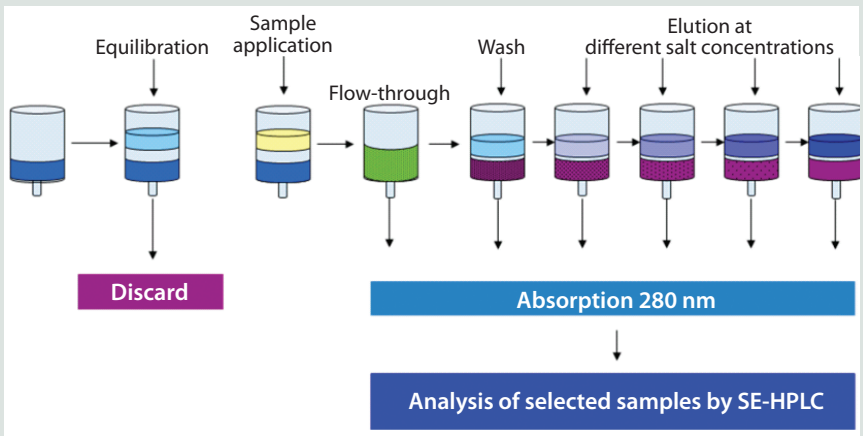
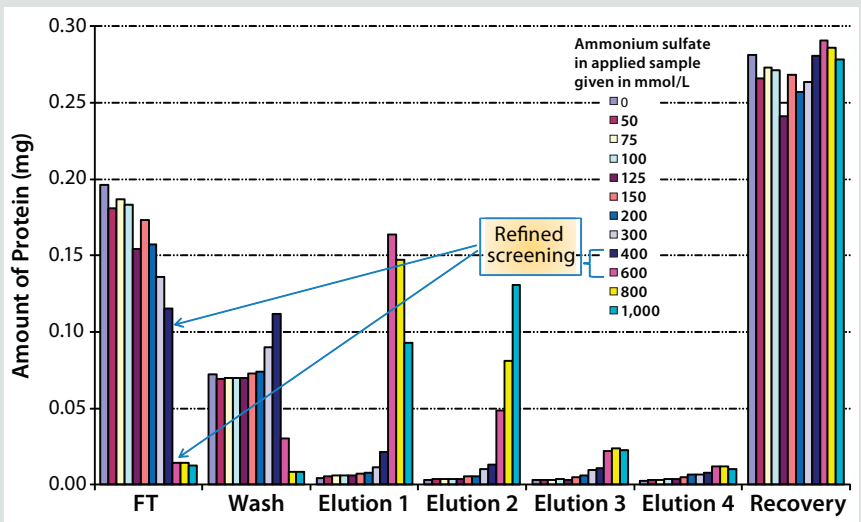


Figure 2: First batch experiment to determine optimal conditions for aggregate removal (0.28 mg MAb/well)



Formats

Process development times can be drastically reduced when high-throughput tools are applied to test different conditions with limited material in a short time. For screening different salt concentrations on this phenyl membrane, we assembled 12 strips of eight wells in a 96-well plate to evaluate aggregate removal from a MAb. Each well was equipped with three membrane layers. To further correlate our findings with a scalable device, we used a nanocapsule with 3 mL (110 cm^2). In such a capsule, the membrane is rolled up to form a cylinder with a membrane bed height of 8 mm (equivalent to 30 membrane layers). The capsule forms a down-scale base for larger capsules in the Sartobind product line of 150 mL (0.55 m^2) up to 5 L (18.2 m^2). When used in flow-through mode, membrane capsules can be designed much smaller than columns, which reduces buffer consumption ≤95% and process times ≤75% (**5**).

The nature of a protein determines its sensitivity to aggregate formation. Aggregates decrease product quality

and stability. Low-pH conditions often used for virus inactivation induce aggregate formation, as does elution at high concentrations from a chromatography column. Other factors include mechanical stress, elevated temperatures, irradiation, and lengthy storage. During MAb purification,

Figure 3: Results for conditions in the refined semichromatographic batch experiment (0.71 mg MAb/well)

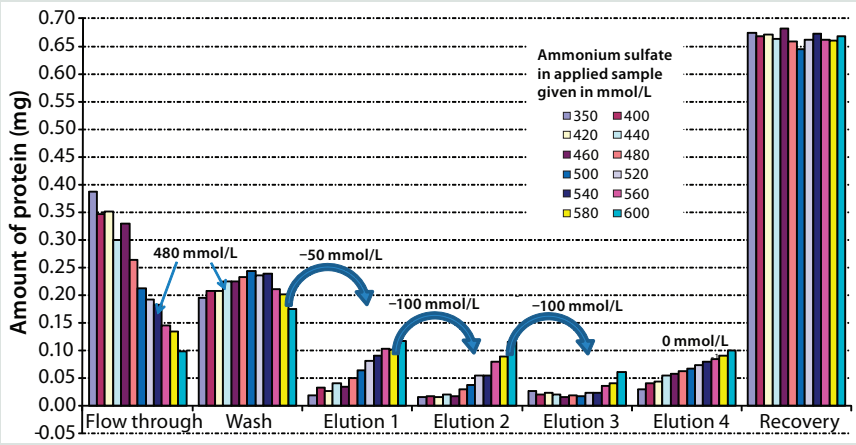


Figure 4: Aggregate levels in best-condition pools loaded at 480 mmol/L ammonium sulfate (no fragments detectable)

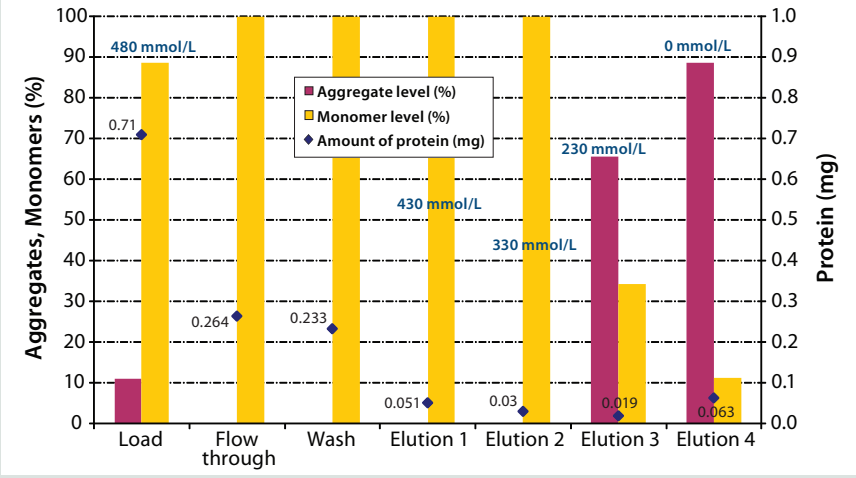
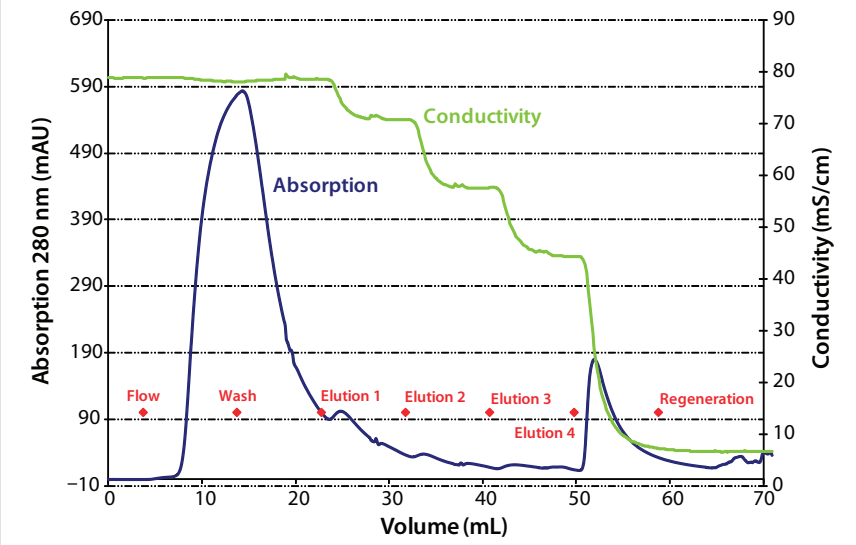


Figure 5: Absorption and conductivity profile of a laboratory-scale run using a 3-mL Sartobind phenyl nanocapsule



high-molecular aggregates are found in concentrations of 0.5–15% in harvested cell-culture fluid (6) and must be reduced typically below 1%.

For in-process control, soluble and insoluble aggregates need to be distinguished. Size-exclusion HPLC and field-flow fractionation are common methods for measuring the level of soluble aggregates present in a protein solution. Insoluble aggregates are determined by measuring turbidity. Because monomers and product aggregates differ in their physicochemical properties (e.g., hydrophobicity), significant depletion is possible in a single processing step (Table 1).

Material and Methods

We divided our experimental set-up into two parts (Figure 1): first a screening experiment for testing in 96-well plates to define the optimal conditions for aggregate removal and second, the transformation of top conditions on the membrane adsorber at laboratory scale.

For the 96-well format, we used a set of 12 eight-well strips with a Sartobind phenyl membrane. Centrifugation forces started the flow. In this semichromatographic mode, we tested each condition with four repeats. Hence, 12 conditions (semichromatograms) were tested with one 96-well plate. After equilibration, we applied protein to the phenyl membrane. We collected the flow-through pool, wash pool, and pools of the four elution steps in 96-well plates. In all steps, we used 200 µL of 50 mmol/L sodium phosphate buffer (pH 7.0) with different ammonium sulfate concentrations (Table 2). A plate was obtained for each pool of a semichromatographic step at different applied conditions. These plates were analyzed by absorption at 280 nm in a microplate reader.

We then repeated the batch experiment with refined conditions. Ammonium sulfate concentrations were 350, 400, 420, 430, 440, 460, 480, 500, 520, 540, 560, 580, and 600 mmol/L, as used in the subsequent washing step. We then reduced the concentration by about 50 mmol/L in elution 1, about 100 mmol/L in elution 2, about 100 mmol/L in elution 3, and finally

Figure 6: Analyzing levels of monomers, aggregates, and fragments by size-exclusion HPLC; protein concentrations are depicted as diamonds (dark blue).

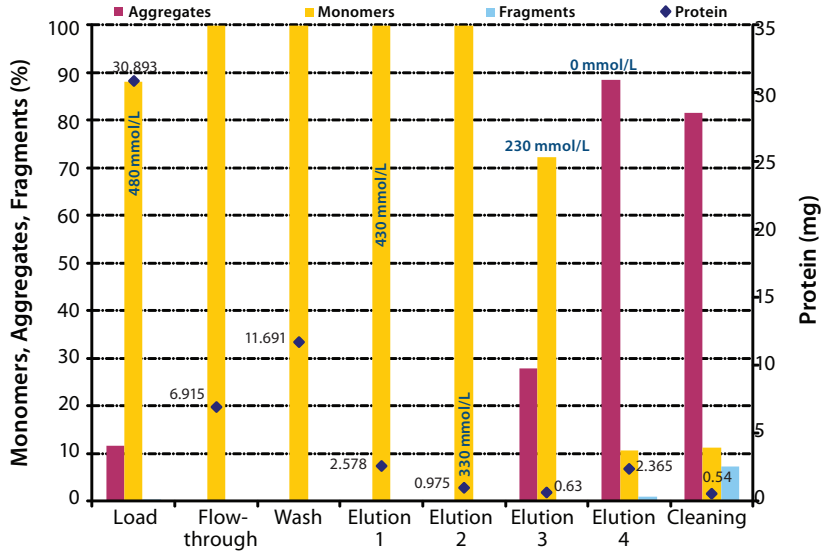


Figure 7: Absorption (blue) and conductivity (green) profiles of the chromatographic run with adapted conditions

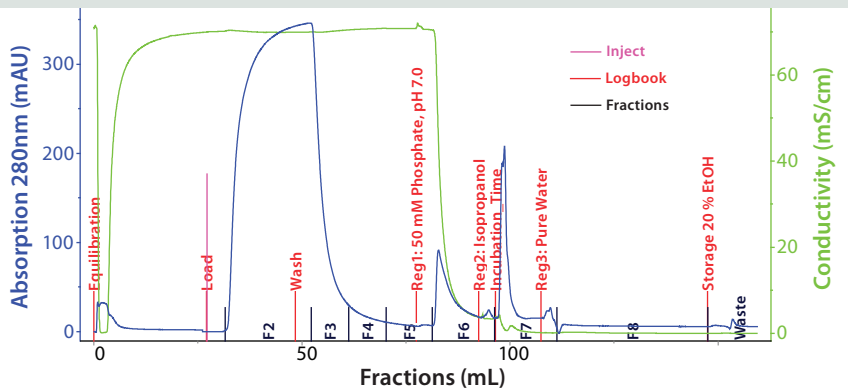
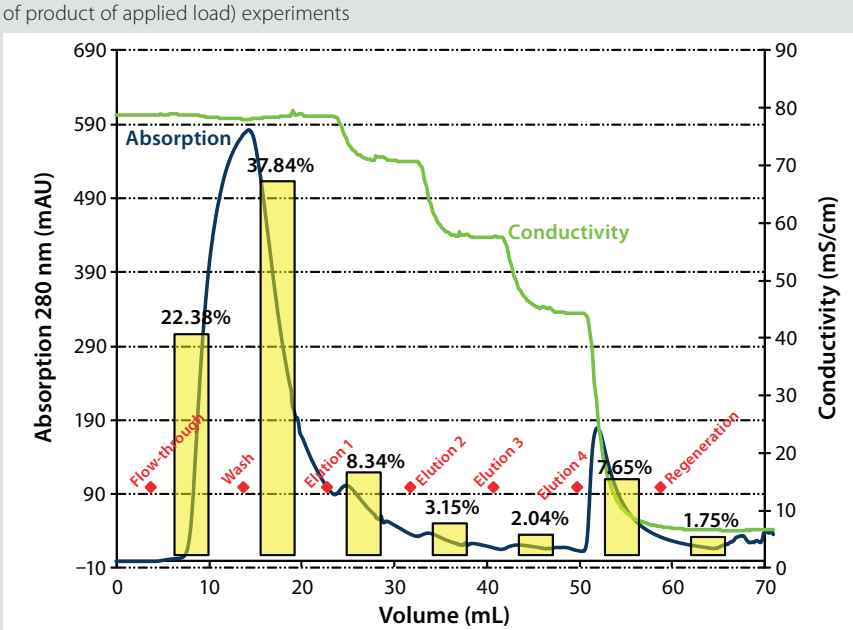
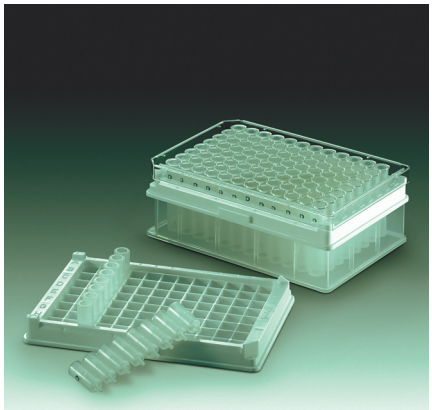


Figure 8: Comparing laboratory scale (dark blue line = UV profile) and batch (yellow bars = percent of product of applied load) experiments





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to 0 mmol/L in elution 4 for each condition. Samples obtained at the most promising conditions were selected and analyzed with size-exclusion HPLC.

Best-hit conditions were transferred to a 3-mL Sartobind phenyl nanocapsule. This chromatographic run was performed using an ÄKTA Explorer 100 system from GE Healthcare (www.gelifesciences.com). Table 3 summarizes the chromatographic parameters, and Table 4 summarizes conditions applied in the herewith developed chromatographic run.

Results and Discussion

In the batch experiment, we determined the optimal ammonium sulfate concentration for binding and elution of MAb monomers. Figure 2 shows results of the first batch experiment. Protein concentration in the flow-through pool dropped sharply between 400 and 600 mmol/L ammonium sulfate, so optimal conditions for product flow-through are located between those two concentrations. Considering the concentrations in the elution steps, the monomer starts to elute at 300–500 mmol/L ammonium sulfate.

We conducted a refined batch experiment to analyze the gap for flow-through of monomers and adsorption of aggregates. We used 350–600 mmol/L ammonium sulfate concentrations in protein load applied to the membrane. Figure 3 shows our results. We selected samples of fractions with the most promising conditions expected and analyzed them with size-exclusion HPLC. Figure 4 shows the result of the best hit (starting with 480 mmol/L ammonium sulfate in the load material). Under those conditions, product passed the membrane, and retained aggregates began to elute at 230 mmol/L ammonium sulfate. Thus we obtained a clear separation of monomers and aggregates.

Conditions applied in batch mode were then transferred to a 3-mL Sartobind phenyl nanocapsule (Figure 5). Figure 6 shows aggregate levels determined with size exclusion HPLC. The load applied to the membrane adsorber contained 11.6% aggregates, 88.1% monomer, and 0.3% fragments. In the flow-through, postload wash, and elution 1 and 2 pools, we obtained 100% monomers. Aggregates eluted at 230 mmol/L ammonium sulfate. The aggregate level was significantly reduced with loading conditions at 480 mmol/L ammonium sulfate in 50 mmol/L sodium phosphate buffer at pH 7.0, including a postload wash with 480 mmol/L ammonium sulfate in 50 mmol/L sodium phosphate buffer at pH 7.0. We recovered 85% of the product.

Conditions were further adapted, and another run was performed to show application for production. Figure 7 shows the absorption profile at 280 nm with the conductivity profile. Table 5 summarizes yields and monomer content in fractions collected from the chromatographic run with adapted conditions. Product recovery was 100%. The yield obtained for a product pool of fractions F2–F4 was 86%, which corresponds to a 91% yield of monomeric product. We implemented the regeneration steps applied here to detect total product recovery.

In summary, comparison of the batch experiment (static conditions) and laboratory-scale experiment (dynamic conditions) gave comparable results (Figure 8). So the optimal conditions for a phenyl membrane adsorber can be selected quickly in a 96-well format, allowing not only the most rapid determination of the aggregate removal step within one or two days, but also saving limited protein materials by using a minimal amount. Subsequently, this process can be transferred easily to the capsules at laboratory scale and further adapted to conditions suitable for an economic production step. Capsules display a higher throughput per bed volume than columns and require a smaller footprint. They also allow for easy handling and can reduce validation costs when used as disposables.

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