Tools and solutions for separation of charged mAb variants

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A biosimilar is an almost identical version of an originator product, but to attain regulatory approval, a comparable quality to the reference product in terms of efficacy, purity, and safety should be demonstrated. Biomolecules, however, exhibit high structural complexity and are often sensitive to alterations in the manufacturing process. Although having access to the originator product, a follow-on manufacturer does not have access to the original cell clone and method used for production of the reference. This work aims to demonstrate different strategies to alter the distribution of charge variants of a monoclonal antibody (mAb) in a downstream polishing step. Based on ion exchange and multimodal chromatography, these strategies include displacement chromatography as well as selection of mAb binding conditions where separation of charged variants are optimized with regards to both yield and resolution.

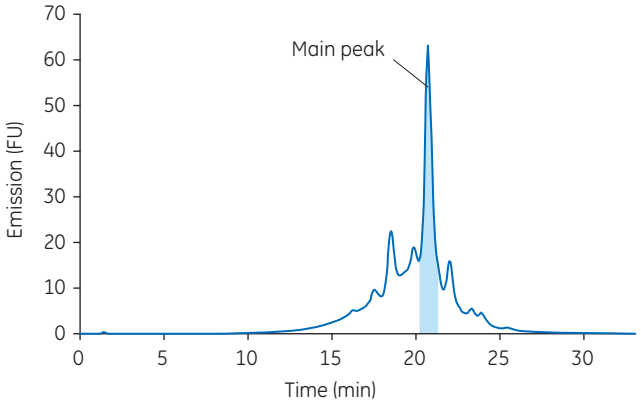

Fig 1. Chromatogram from analytical cation exchange chromatography showing the finger print of a mAb after the Protein A capture step. Variants eluting early are defined as acidic, whereas variants eluting later are defined as alkaline.

Introduction

The homology of the antibody Fc region allows almost all antibodies to be purified using a standard approach, and most commercially approved manufacturing processes utilize Protein A capture as the initial step in downstream purification of mAbs. The distribution of mAb charge variants, however, can differ for a specific mAb and is depending on the selected cell clone as well as on culture conditions (medium and supplements), culture technique (batch, fed-batch, or perfusion), and culture time. Figure 1 shows the fingerprint of a mAb after the Protein A capture step.

There are several strategies that can be used to separate charged mAb variants from the main mAb. Design-of-experiments (DoE) is used to identify or screen input parameters that could affect the process output. DoE techniques enable us to learn about process behavior by running a number of experiments, where a maximum amount of information is obtained in a minimum number of runs. High-throughput screening tools, such as 96-well filter plates and mini-column units prefilled with chromatography resin, facilitate the use of a DoE approach. In this work, 96-well filter plates were used to determine resin binding capacity. Conditions that optimize separation of charge variants were further explored in column experiments. Set criteria for this work were a more than 50% clearance of acidic mAb variants at a mAb recovery of above 50%.

Materials and methods

Sample

The target mAb (pI 8.4) was produced in Chinese hamster ovary cells grown in HyClone™ ActiPro™ medium supplemented with daily additions of 4% HyClone Cell Boost™ 7a and 0.4% Cell Boost 7b (of starting culture volume). Cells were harvested after 13–14 days of culture, and produced mAb was captured from clarified cell culture harvest using MabSelect SuRe™ Protein A affinity resin packed in a chromatography column. Bound mAbs were eluted with 100 mM sodium acetate (NaOAc), pH 3.5. Concentration of mAb in the pooled fractions was about 20–25 g/L.

Resins

Resins evaluated were Capto™ S ImpAct and Capto SP ImpRes cation exchange resins as well as the multimodal Capto MMC ImpRes cation exchange resin.

Plate experiments

PreDictor™ 96-well filter plates were used for screening of static binding capacity (SBC). Each well filled with resin was washed three times with 50 mM NaOAc/50 mM sodium phosphate, pH 5–8 + 0–300 mM sodium chloride (NaCl). A 200 µL sample of mAb (3 g/L) in 50 mM NaOAc/50 mM sodium phosphate, pH 5–8 + 0–300 mM NaCl, was applied to each well. The plate was incubated for 1 h in room temperature (RT) at 1100 rpm, before centrifuged for 2 min at 500 RCF in RT. Flowthrough was collected in a UV-plate, and analyzed at 280 and 305 nm. Static binding capacity was determined as the amount (g) of bound mAb per volume (L) of resin.

Column experiments

Tricorn™ 5/50 or 5/100 columns, packed with resin to a bed height of 5 or 10 cm (column volume [CV] = 1 or 2 mL), were used for evaluation of resin performance in separation of charge variants. A sample of mAb in Buffer A was applied to the column, the column was washed, and bound protein was eluted using a gradient of Buffer B. Different elution pH gradients were employed. Fractions of 0.5 mL were collected.

Analysis of charge and main mAb variants in eluted fractions

Charge variant distribution was determined by cation exchange chromatography on a ProPac WCX-10 2 × 250 mm protein column (Thermo Fisher Scientific). A 60 µg sample was applied to the column, equilibrated with 10 mM sodium phosphate, 10 mM Tris, pH 6.5 (Buffer A), at a flow rate of 300 µL/min. A pH gradient was applied at a rate of 10%/min of 10 mM sodium phosphate, 10 mM Tris, pH 9.5 (Buffer B) up to 50%, after which the rate was changed to 3.33%/min up to 100% of Buffer B. Fractions were analyzed at 295 nm/350 nm (extinction/emission) using a fluorescence detector.

Results and discussion

Plate experiments

Screening plates are convenient tools for data collection and identification of usable conditions. When designing a binding experiment, however, there are a few things to consider. Figure 2 depicts an isotherm. To correctly perform a binding study, an excess of protein needs to be used to secure that all binding sites are given the opportunity to bind to the protein under the given condition (e.g., a specific conductivity and pH). When equilibrium has been established, only the given conditions (not lack of protein or incubation time) should reflect the differences in binding capacity between conditions evaluated. Hence, static binding capacity experiments are performed using a low resin volume, typically 2–6 µL, and a protein concentration sufficiently high to allow 50% to remain in the surrounding solution at equilibrium.

Figure 3 shows contour plots for binding capacity of Capto MMC ImpRes and Capto S ImpAct. As shown, binding capacity was high at low pH, even at rather high salt

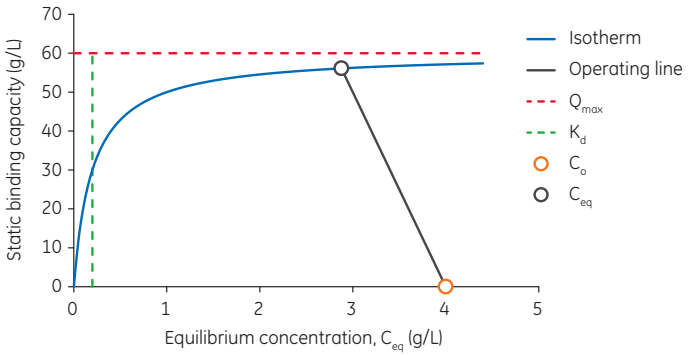

Fig 2. Isotherm showing the main features necessary to know for determination of static binding capacity of a resin. Q_{max} = maximum capacity, C_{eq} = protein concentration in the surrounding liquid at equilibrium, K_d = dissociation constant, C_0 = initial protein concentration. The slope of the operating line shows the ratio between the total liquid volume in the well and the volume of the resin.

PreDictor plate:	Capto MMC ImpRes, 6 µL Capto S ImpAct, 2 µL
Wash:	3 × 200 µL 50 mM NaOAc/50 mM sodium phosphate pH 5–8 in NaCl 0–300 mM
Sample application:	40 µL mAb (15 g/L) in 160 µL 50 mM NaOAc/ 50 mM sodium phosphate, pH 5–8 + 0–300 mM NaCl
Incubation:	60 min in RT, at 1100 rpm vortex
Evacuation:	Centrifugation at 500 RCF for 2 min in RT
Collection plate:	UV-plate
Analysis:	Spectrophotometrically at 280 and 305 nm

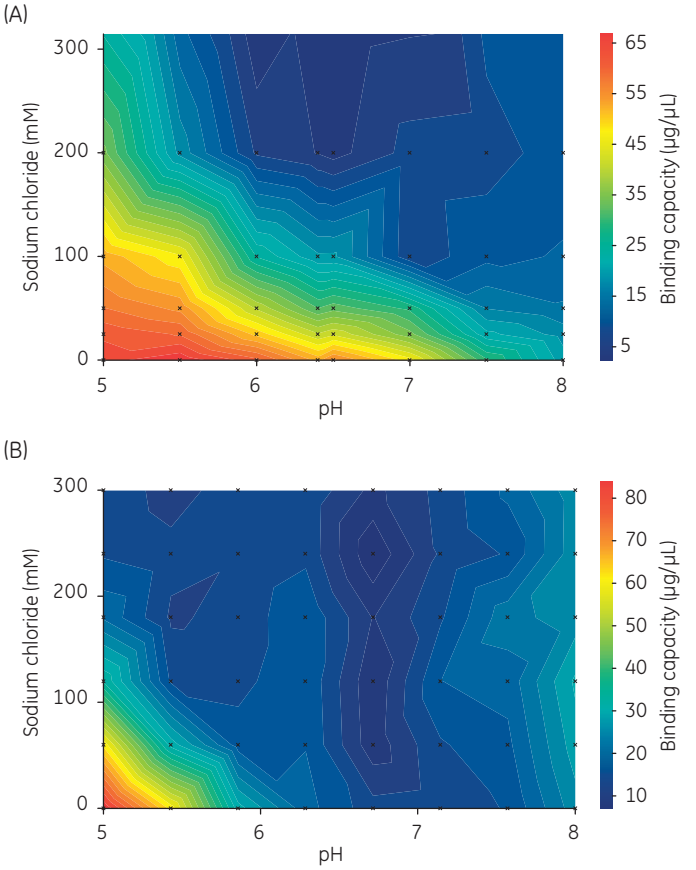

Fig 3. Binding capacity of (A) Capto MMC ImpRes and (B) Capto S ImpAct for analyzed mAb when pH and conductivity are varied. Colors indicate a relative scale for the binding capacity of the resin, where red indicates a high binding capacity and blue a low binding capacity.

concentrations. As the contour plot represents an average binding capacity for all sample components, the most promising area to investigate further is in between conditions promoting mAb binding and conditions preventing mAb binding. Given the studied ranges of the conditions tested, the contour plots indicate that elution using a pH gradient would be more suitable than a salt gradient. This finding was also confirmed in column experiments (data not shown).

Column experiments

The performance of Capto S ImpAct, Capto SP ImpRes, and Capto MMC ImpRes columns operated in bind-elute mode, employing pH gradient elution, were compared regarding separation of mAb charge variants. As shown in Figure 4, selectivity and elution volume differ between the different resins due to their different ligands, ligand concentration, particle size, and particle porosity. In general, a traditional ion exchange resin is the first choice as this type of resins usually provides a better recovery and smaller peak volume. A multimodal resin is the choice when the sample contains high salt concentrations or when an ordinary ion exchange resin shows poor resolution. As demonstrated for Capto SP ImpRes, altering pH at elution start can improve resolution. Figure 5 depicts separation of charged mAb variants in an elution pH gradient from 6.3 to 8.2 on Capto SP ImpRes.

Column:	Tricorn 5/100
Resin:	Capto S ImpAct Capto SP ImpRes Capto MMC ImpRes
Sample:	6 mL mAb (2.7 g/L) in Buffer A
Sample load:	8.1 g mAb/L resin
Flow rate:	0.25 mL/min (8 min residence time)
Buffer A:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 5
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 9
Wash:	10 CV of Buffer A
Elution:	Gradient, 0%–100% Buffer B in 10 CV (pH 5–9)

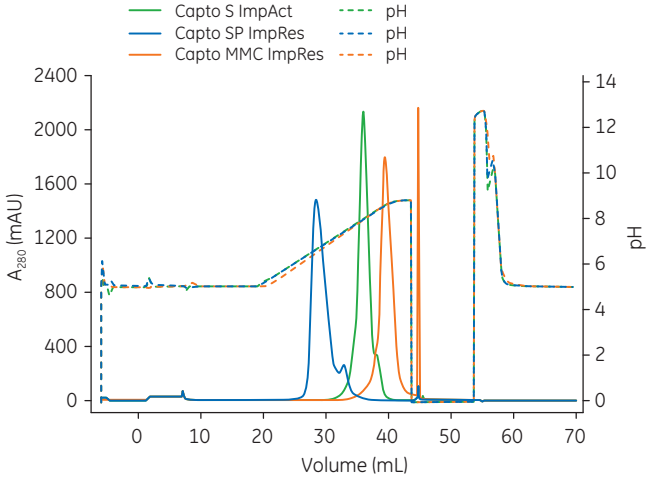

Fig 4. Separation of charged mAb variants on Capto S ImpAct, Capto SP ImpRes, and Capto MMC ImpRes, employing pH gradient elution.

Column:	Tricorn 5/100
Resin:	Capto SP ImpRes
Sample:	1 mL mAb (2 g/L) in Buffer A
Sample load:	1 g mAb/L resin
Flow rate:	0.5 mL/min (4 min residence time)
Buffer A:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 5
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 9
Wash:	10 CV of Buffer A
Elution (blue):	Gradient elution, 35%–100% Buffer B in 10 CV (pH 6.1–9)
Elution (orange):	Gradient elution, 45%–100% Buffer B in 10 CV (pH 6.4–9)
Elution (green):	Gradient elution, 55%–100% Buffer B in 10 CV (pH 6.7–9)

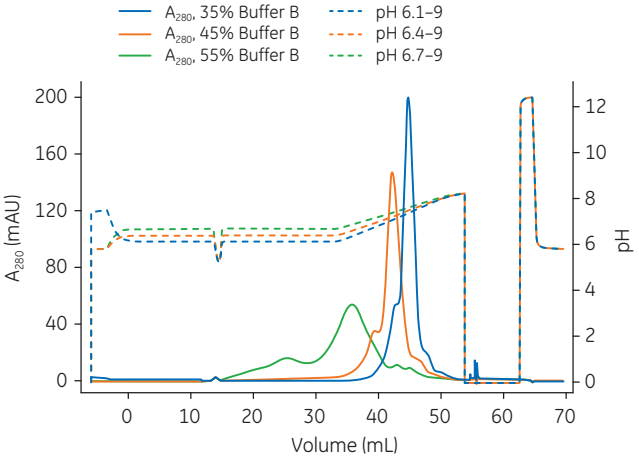

Fig 5. Separation of charged mAb variants on Capto SP ImpRes, employing different elution pH gradients.

In some cases, it is possible to separate one or more impurities from the target by choosing conditions under which one of the species is directed into the flowthrough. For example, on a cation exchange resin, the acid variants will elute earlier, and by choosing the right pH (i.e., a pH that promotes binding of the main variants but not the acid variants), it is possible to collect the unwanted acidic variants in the flowthrough. Figure 6 shows separation of charged mAb variants in a pH gradient from 7.1 to 8.6 on Capto MMC ImpRes. Analysis of the collected mAb-containing fractions showed that a substantial separation was achieved, with the larger part of the acidic charge variants in the flowthrough.

Capto S ImpAct and Capto MMC ImpRes were selected for further investigations due to their higher binding capacities compared with Capto SP ImpRes. A head-to-head comparison at a load close to breakthrough indicated that Capto S ImpAct provides an improved resolution over Capto MMC ImpRes (Fig 7). To further investigate the impact of load on the resolution of Capto S ImpAct, a scouting with three different loads was performed, and fractions corresponding to the shaded areas in Figure 8 were pooled and analyzed. Results summarized in Table 1 show that an improved purity, defined as a higher main-to-acidic ratio, was achieved at a higher load. When using an overload of sample, there is a possible risk of a loss in yield.

Column:	Tricorn 5/100
Resin:	Capto MMC ImpRes
Sample:	1 mL mAb (6 g/L) in Buffer A
Sample load:	3 g mAb/L resin
Flow rate:	0.25 mL/min (8 min residence time)
Buffer A:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 7.1 + 50 mM NaCl
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 8.6 + 50 mM NaCl
Wash:	10 CV of Buffer A
Elution:	Gradient, 0%–100% Buffer B in 10 CV (pH 7.1–8.6)

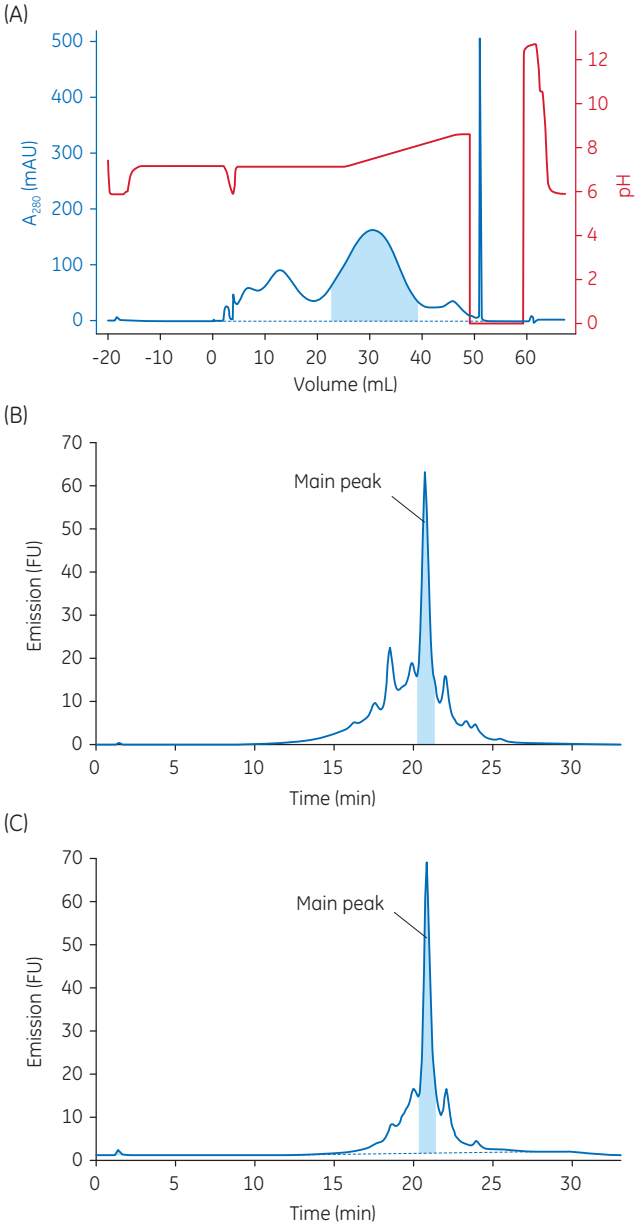

Fig 6. (A) Separation of charged mAb variants on Capto MMC ImpRes, employing elution pH gradient from 7.1 to 8.6. (B) Fingerprint of the mAb sample. (C) Analysis of collected mAb-containing fractions (blue area in A) from Capto MMC ImpRes.

Column:	Tricorn 5/50
Resin:	Capto S ImpAct and Capto MMC ImpRes
Sample:	4.4–6.6 mL mAb (9 g/L) in equilibration buffer
Sample load:	40–60 g mAb/L resin
Flow rate:	0.25 mL/min (4 min residence time)
Buffer A:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 5.0
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 8.8
Equilibration:	10 CV of 50% Buffer B in Buffer A (pH 6.8)
Wash:	10 CV of Buffer A
Elution:	Gradient, 50%–100% Buffer B in 10 CV (pH 6.8–8.8)

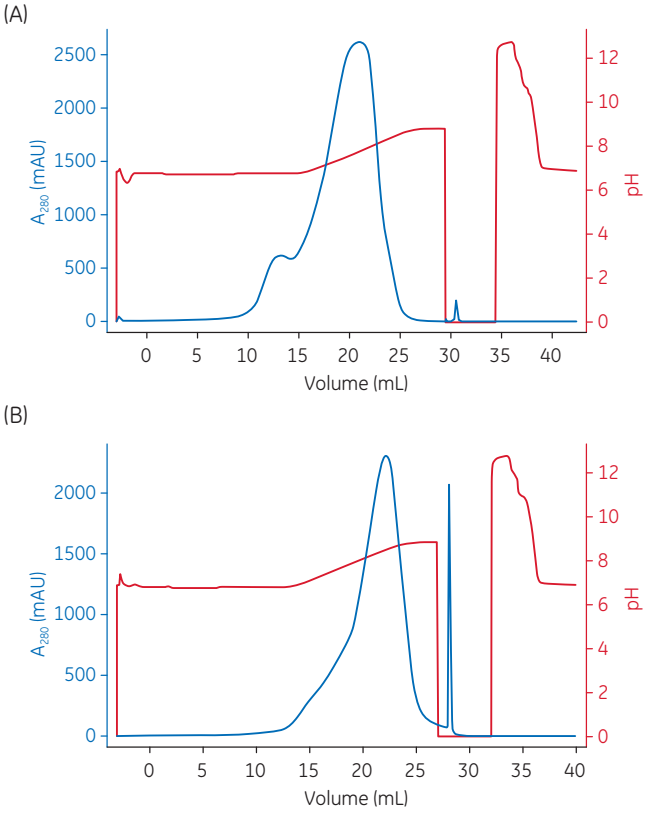

Fig 7. Comparison of the resolution of (A) Capto S ImpAct and (B) Capto MMC ImpRes at a load close to breakthrough.

Column:	Tricorn 5/50
Resin:	Capto S ImpAct
Sample:	2.0–6.4 mL mAb (15.8 g/L) in equilibration buffer
Sample load:	31–101 g mAb/L resin
Flow rate:	0.25 mL/min (4 min residence time)
Buffer A:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 5.0
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 8.8
Equilibration:	10 CV of 50% Buffer B in Buffer A (pH 6.8)
Wash:	10 CV of Buffer A
Elution:	Gradient, 50%–100% Buffer B in 10 CV (pH 6.8–8.8)

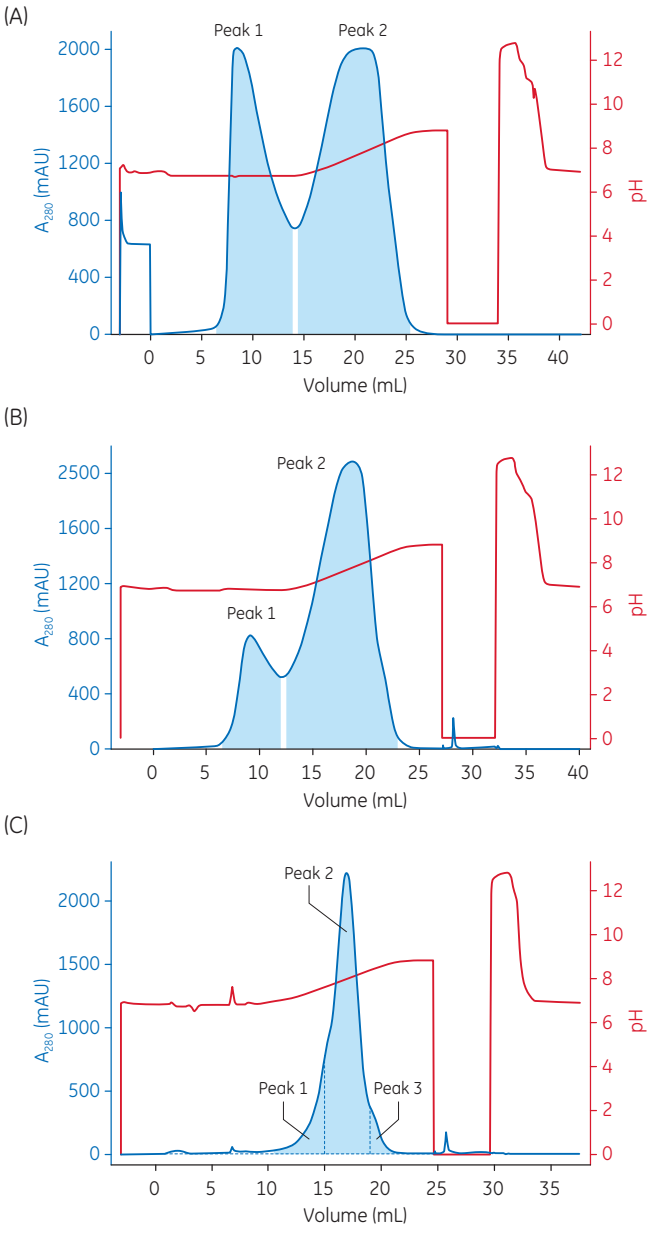

Fig 8. Comparison of the resolution of Capto S ImpAct at a load of (A) 101 mg, (B) 71 mg, and (C) 31 mg.

Table 1. mAb chare variant distribution at different sample loads on Capto S ImpAct (see Fig 8)

Load	Peak	Retention main variant (mL)	Conc. acidic variants (%)	Conc. main variants (%)	Conc. alkaline variants (%)
Start sample		21.128	47.044	38.206	14.8
101 mg	1	21.178	75.662	21.116	3.222
	2	21.152	32.263	46.66	21.077
71 mg	1	21.472	93.1	5.432	1.468
	2	21.152	39.921	43.304	16.775
31 mg	1	21.383	98.353	0.996	0.996
	2	21.181	42.172	44.006	13.822
	3	21.471	32.459	9.78	57.76

Displacement chromatography

Capto S ImpAct was further investigated by displacement chromatography, where sample is continuously applied to the column that is eventually saturated with sample components. When analyzing the collected fractions, it can be observed that the presence of the different mAb charge variants and their concentrations vary during the sample load phase. As shown in Figure 9A, there was a breakthrough of acidic variants after approximately 36 mL (97 g/L). The breakthrough of main variants occurred after 54 mL applied sample, corresponding to 145.8 mg mAb. Figure 9B shows a magnification of the part of the chromatogram in Figure 9A where the breakthrough of the acidic variants starts. Figure 9C shows an overlay of chromatograms from analysis of the marked fractions in Figure 9B.

Altogether, for Capto S ImpAct, the combination of pH gradient elution and high load seemed to increase the main-to-acidic ratio. A DoE approach was used to find conditions where high purity could be achieved also at high recovery. DoE allows obtaining a maximized amout of information about the process, using a minimal number of experiments. Table 2 shows results from the DoE study, using pH and load as input parameters and mAb recovery and clearance of acidic charge variants as responses. The important factors and quality of the model are displayed in Figures 10 and 11. As shown for both mAb recovery and clearance of acidic charge variants in Figure 11, the model validity is low and even negative, which is a consequence of the small differences between the replications of the center point experiments.

Column:	Tricorn 5/50
Resin:	Capto S ImpAct
Sample:	70 mL mAb (6 g/L) in Buffer A
Flow rate:	0.25 mL/min
Buffer A:	20 mM sodium phosphate, pH 5
Buffer B:	10 mM of citrate, 10 mM sodium phosphate, 10 mM Tris, pH 9
Wash:	10 CV of Buffer A
Elution:	Gradient elution, 0%–100% Buffer B in 10 CV

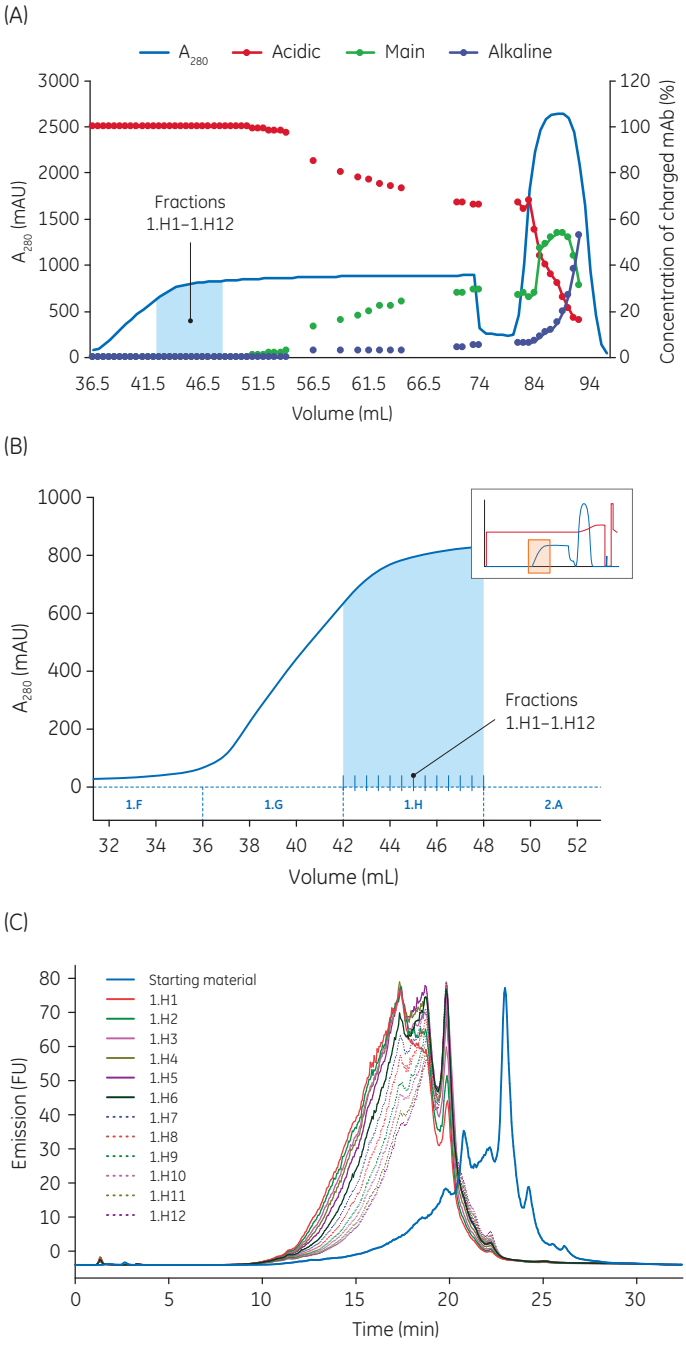

Fig 9. Separation of mAb on Capto S ImpAct: (A) mAb charge variant distribution, (B) magnification of area for fractions 1.H1–1.H12, and (C) overlay of analyses of fractions 1.H1–1.H12 by analytical cation exchange chromatography.

Table 2. Results from the DoE, where pH and load were input parameters and mAb recovery and clearance of acidic mAb variants were responses

Experiment no	pH	Load (g/L)	Recovery (main + alkaline) (%)	Clearance (acidic) (%)
1	7	70	102.36	0.5
2	7.5	70	68.93	77.76
3	7	140	104.33	37.59
4	7.5	140	56.53	89.39
5	7	105	97.89	14.32
6	7.5	105	75.15	86.7
7	7.25	64.8	105.52	22.69
8	7.25	145.2	96.29	73.62
9	7.25	105	107.82	53.76
10	7.25	105	107.77	51.36
11	7.25	105	106.93	51.86

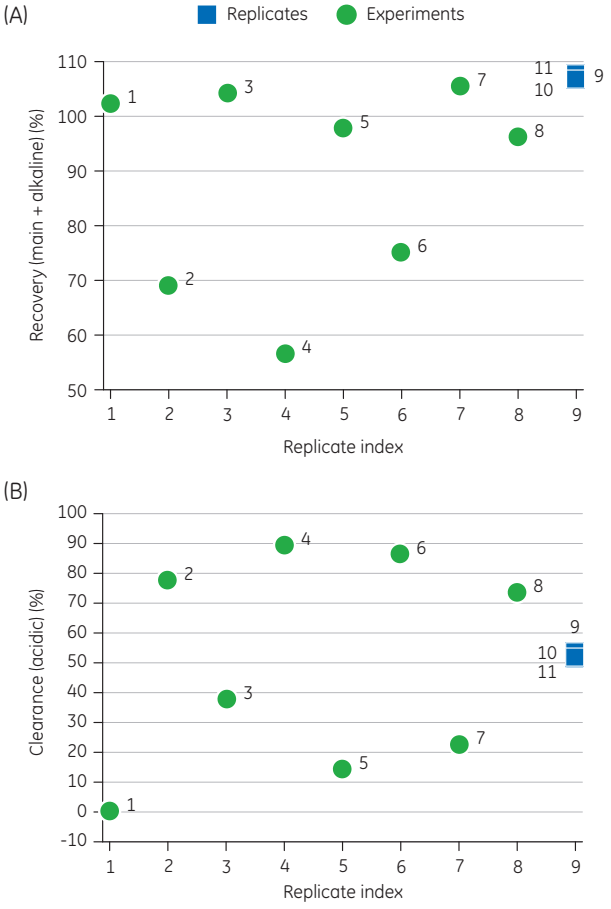

Fig 10. Replicate plots for the two responses, (A) mAb recovery and (B) clearance of acidic mAb variants.

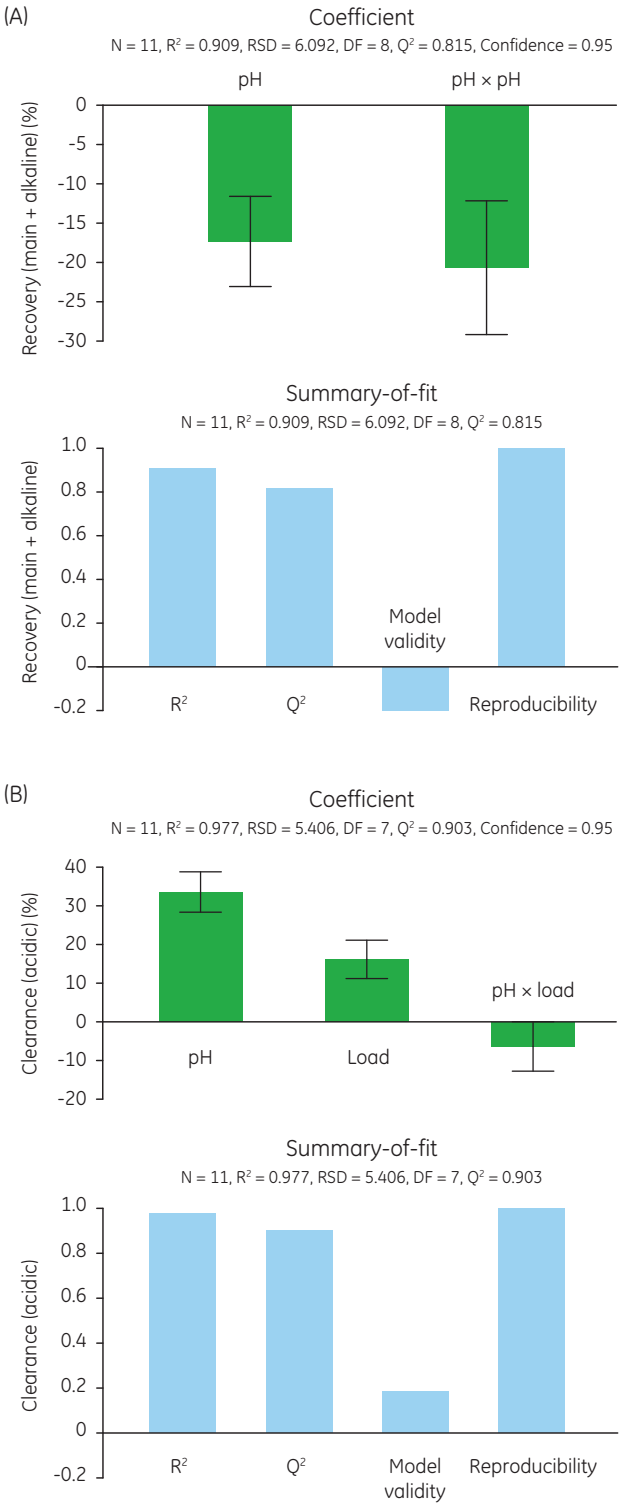

Fig 11. Coefficient and summary-of-fit plots (part of the DoE software) for (A) mAb recovery and (B) clearance of acidic mAb variants.

Overall, the models were good, with Q^2 values of > 0.8 The sweet spot plot in Figure 12 indicates a rather large window of operation (WoO). To determine the robustness of the WoO, uncertainties in factor settings were considered by running a Monte Carlo simulation. The precision of the factor settings for the Monte Carlo simulation was ± 0.05 and ± 5 g/L for pH and load, respectively. The result is shown in Figure 13. More information on DoE study setups can be found from the Handbook: Design of Experiments in Protein Production and Purification (1).

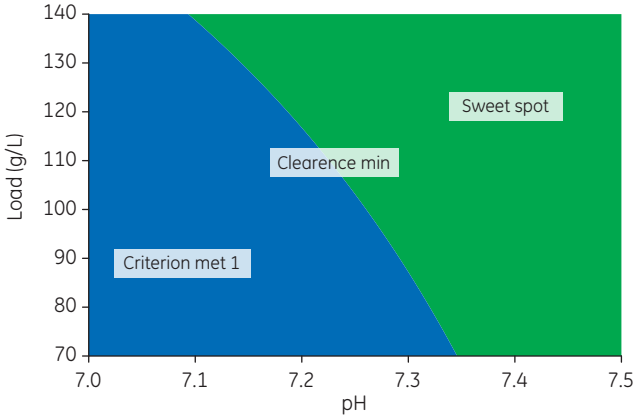

Fig 12. Sweet spot plot showing the area (green) where the criteria > 50% mAb recovery and > 50% clearance of acidic mAb variants are fulfilled. Blue area indicates that only one of the criteria is fulfilled.

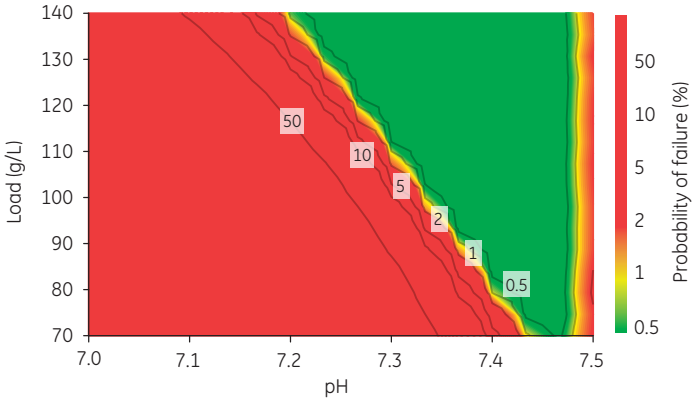

Fig 13. Monte Carlo simulation showing the probability of failure against the set criteria > 50% mAb recovery and > 50% clearance of acidic mAb variants. The precision of the pH and load was 0.05 and 5 g/L, respectively. Monte Carlo simulation is not included in the UNICORN software package and, hence, a separate software tool is required.

Conclusion

There are several tools and solutions available to separate charged mAb variants from the main mAb pool. This work presents an evaluation of traditional as well as multimodal ion exchange chromatography in the separation of main mAb from its charged variants. High-throughput screening tools and displacement chromatography were used to find conditions optimal for binding of the main mAb, while preventing binding of unwanted acidic variants. With suitable tools and solutions, it is possible to balance the ratio of charged mAb variants to mimic the fingerprint of an originator product. For the specific mAb used in this study, the presented techniques enabled development of a one-step purification protocol that fulfills the set criteria of reaching a reduction in acidic variants of above 50% with a recovery of more than 50%. Although the presented techniques are used for a specific model mAb, the methodology is general and can be applied for all mAb molecules.

Reference

1. Handbook: Design of Experiments in Protein Production and Purification, GE Healthcare, 29103850, Edition AA (2014).

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