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Statistical Approach to IgG Binding on a Strong Cation Exchanger

A Case Study of Optimization

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Among biopharmaceuticals, monoclonal antibodies (MAbs) are most widely used as immunodiagnostic and therapeutic products. More than 20 MAbs are approved as pharmaceuticals, and more than 200 are in different stages of development (1, 2). The emergence of novel therapeutic MAbs suggests an exponential increase in their demand — and therefore production. A recent review of MAb facilities and their production capacity identifies bottlenecks in downstream processes resulting from advances in cell culture/fermentation and new options for overall improvement (3). Downstream processes are challenged to improve throughput over their current status as the MAb titers approach 5 g/L (4). To keep up with demands and advances in upstream processes, four to six times the current downstream capacity or throughput may be needed. Selective and cost-effective purification processes are therefore required.

Product Focus: Recombinant proteins

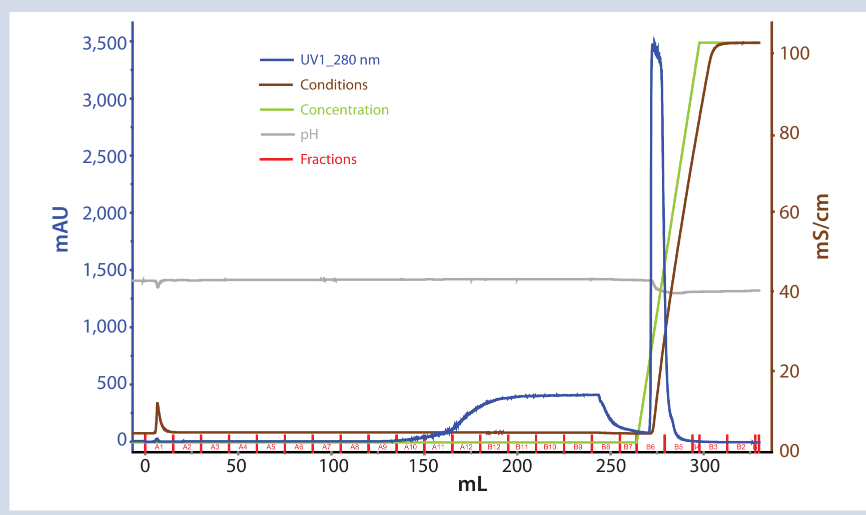
Process Focus: Downstream processing

Who Should Read: Process engineers, project managers, QA/QC, analytical personnel

Keywords: DOE (design-of-experiments), optimization, chromatography, cation exchange, binding capacity

Level: Intermediate

Figure 1: Typical binding and elution profiles for IgG on PolyCSX



Chromatography is a major technique used in almost all purification processes for various therapeutic biomolecules. Of all factors determining throughput in a chromatographic process, the most closely examined should be media capacity, mobile-phase linear velocity, and selectivity of chromatography media. Variables such as flow rate, pH, and conductivity play a major role in the binding capacities of proteins on different chromatographic media. However, finding conditions for maximum dynamic binding capacity (DBC) by changing one parameter at a time in a series of experiments is often time-consuming because of interaction among variables.

Our study represents an attempt to use a simple approach to understand different parameters that affect chromatographic processes and meet challenges posed by complex separations and high-throughput requirements. We demonstrate that statistical tools such as design of experiments (DOE) and regression analysis are useful in determining optimum conditions for loading a selected protein using a minimum number of experiments. We used simple DOE methodology and studied a strong cation exchanger, PolyCSX medium from Mallinckrodt Baker (www.mallbaker.com), to show how several interacting factors are important in determining DBC of large proteins such as immunoglobulin G (IgG).

Our purpose was to closely examine the properties of PolyCSX medium and how they can be used to meet today's challenge of purifying proteins from complex matrices at a high throughput. This study also demonstrates a flexibility in certain operating parameters of the medium for meeting desired conditions of each process and protein to be purified. The statistical model we obtained through DOE and regression helps to ascertain maximum IgG capacity and optimize throughput while meeting purity requirements.

Experimental

The chromatographic medium used in these experiments is BAKERBOND PolyCSX (PN 7587, Mallinckrodt Baker, Inc.), which is primarily a strong cation exchanger with weak cation and anion exchange sites. All separations and binding capacity measurements were performed with it packed in a 4.7-mL column (0.78-cm × 10 cm) using an Äkta Explorer chromatographic system from GE Healthcare (www.gehealthcare.com). Rabbit IgG, hemoglobin, lactoferrin, and ovalbumin were obtained from Sigma Aldrich (www.sigmaaldrich.com). We used 2-(n-morpholino)ethane sulfonic acid (MES) and other analytical-grade salts and solvents from Mallinckrodt Baker, Inc.

For capacity studies, we loaded Rabbit IgG (2 mg/mL) in a suitable buffer onto the column at different flow rates. For the binding buffer (50 mM MES) required pH and conductivity were adjusted with 1 M NaOH and sodium chloride respectively. The elution buffer consisted of the binding buffer with 1 M NaCl added. We determined the actual concentrations of IgG after filtration at different pH and conductivity using the bicinchoninic acid protein assay (BCA) method (5, 6). Loading buffer conductivities were adjusted with sodium chloride.

Typical binding capacity determination is a four-step process: equilibrating a column with five times its volume (CV) of an appropriate loading buffer, loading the column with IgG in the loading buffer at selected velocity and conditions, washing the column with 5 CV loading buffer, and eluting the column with elution buffer containing 1 M NaCl. We calculated recovery values by analyzing all fractions using the BCA method. DBCs were determined at 10% peak height of maximum absorption at 280 nm.

We performed two different sets of loading experiments. The first is based on initial screening conditions generated by Minitab factorial designs (www.minitab.com) to determine significant main effects. The second study involves regression analysis to elucidate the interaction among several variables. For our initial screening factorial DOE study, we chose two levels of pH, flow rate, and conductivity with one central point for a total of nine experiments at extreme conditions. For our type of chromatographic medium, we used pH values of 4.0 and 6.0, with the central point being pH 5.0. Table 1 lists other experimental conditions. In addition to DOE studies, we examined selectivity of the chromatographic media and effect of linear

Table 1: Screening of DOE parameters

Run	Flow Rate (cm/hr)	pH at 25 °C	Conductivity (mS/cm)
1	102	4.0	1.4
2	636	6.0	18.9
3	102	6.0	18.9
4	636	4.0	1.4
5	102	6.0	1.4
6	636	6.0	1.4
7	102	4.0	18.9
8	636	4.0	18.9
9	369	5.0	10.2

Figure 2: Interaction plot among pH, velocity, and conductivity

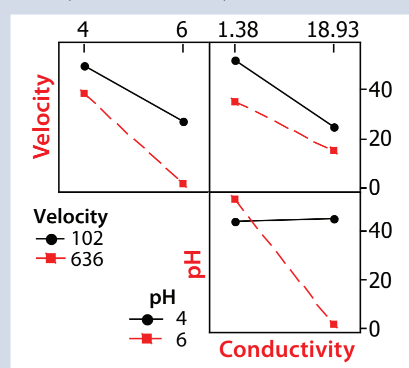


Table 2: Proteins and their respective pI values used in this separation

Protein	Source	pI
Ovalbumin	Chicken egg	~ 4.7
IgG	Rabbit	~ 7.0
Hemoglobin	Rat	~ 7.5
Lactoferrin	Bovine milk	~ 8.7

Figure 3: Effect of pH and conductivity on dynamic binding capacity

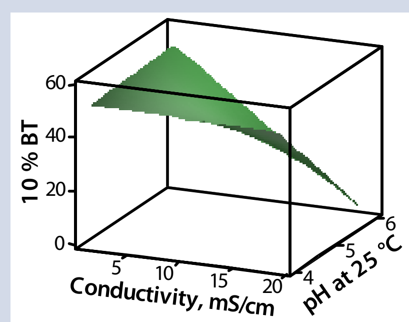


Figure 4: Binding capacity of IgG for different flow rates at pH 5.6

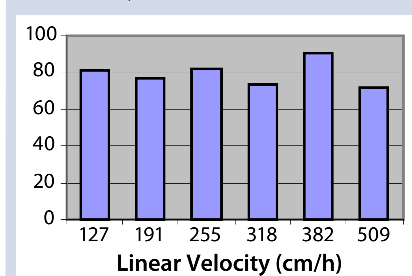


Figure 5: Surface ligand structure of PolyCSX illustrates mixed-mode functionality for enhanced selectivity

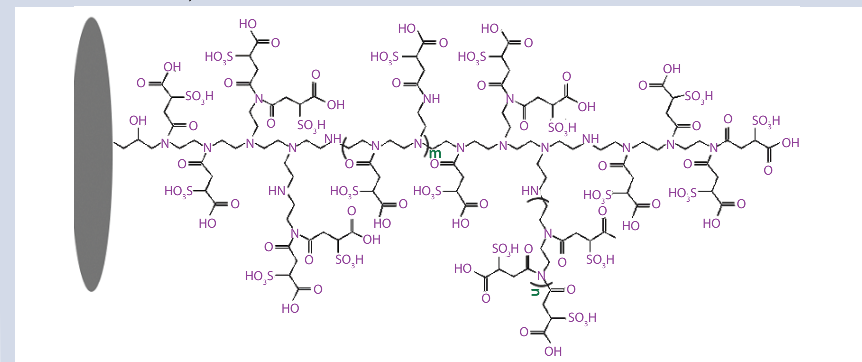


Table 3: Dynamic binding capacities obtained in the DOE screening study

Run	Flow Rate (cm/hr)	pH at 25 °C	Conductivity (mS/cm)	10% Breakthrough (mg/mL)
1	102	4	1.4	52.0
4	636	4	1.4	35.3
7	102	4	18.9	48.5
8	636	4	18.9	41.5
9	369	5	10.2	40.2
5	102	6	1.4	52.7
3	102	6	18.9	1.5
6	636	6	1.4	49.7
2	636	6	18.9	1.1

Figure 6: Interaction plot for 10% BT at six different pH values

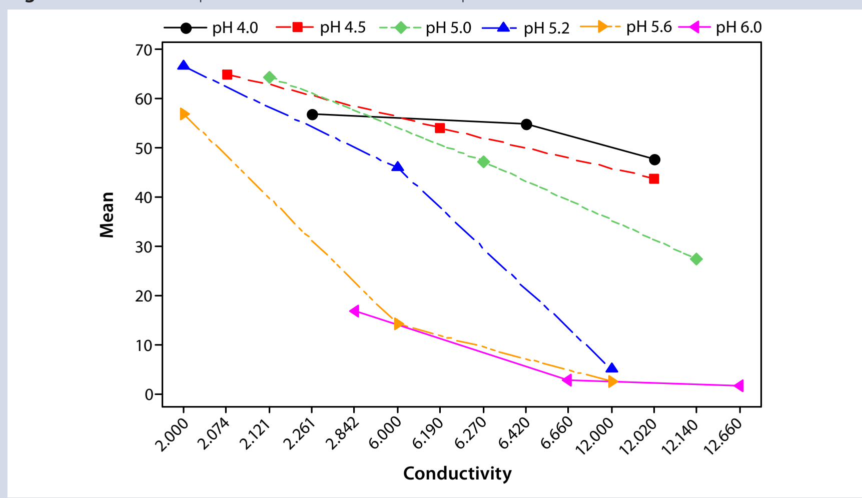


Table 4a: Statistical analysis (estimated effects and coefficients for 10% BT in coded units); S = 3.69129; R-Sq = 99.33%; R-Sq(adj) = 97.32%

Term	Effect	Coef	SE Coef	T	P
Constant		35.46	1.564	22.68	0.002
Flow rate, cm/h	–7.56	–3.78	1.698	–2.23	0.156
pH at 25 °C	–18.86	–9.43	1.698	–5.56	0.031
Conductivity, mS/cm	–23.66	–11.83	1.798	–6.58	0.022
Flow rate, cm/hr*pH at 25 °C	4.25	2.12	1.698	1.25	0.338
Flow rate, cm/hr*Conductivity, mS/cm	3.70	1.85	1.798	1.03	0.412
pH at 25 °C*Conductivity, mS/cm	–25.03	–12.52	1.798	–6.96	0.020

Table 4b: Statistical analysis (analysis of variance for 10% BT in coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	3,032.90	2,362.90	787.635	57.81	0.017
2-Way Interactions	3	1,002.74	1,002.74	334.248	24.53	0.039
Residual Error	2	27.25	27.25	13.626		
Lack of Fit	1	27.21	27.21	27.206	604.58	0.026
Pure Error	1	0.04	0.04	0.045		
Total	**8**	**4,062.89**				

velocity on separation using a mixture of proteins that have similar pI values (Table 2).

Results and Discussion

The 10% breakthrough point was determined at 280 nm, and Figure 1 shows typical binding and elution profiles of IgG on PolyCSX. Table 3 shows DBCs for the DOE study.

Figure 1 also shows a typical binding and elution profile of IgG on PolyCSX medium packed in a 4.7-mL column (0.78 cm × 10 cm) at 400 cm/h linear velocity. The 10% breakthrough point is 66.30 mg/mL, determined at 280 nm with a pH of 5.2 and 2.0 ms/cm conductivity. We attribute the steep slope of this binding curve to the fast mass transfer of IgG in CSX. All results obtained in the DOE study for DBC were modeled and analyzed using Minitab software (Table 4). The obtained R-Sq (adj) value of 97.3% indicates the high validity of this model (Table 5).

Significant DOE Results: The P value for flow rate is 0.156, which is higher than the typically chosen a-level of 0.05, indicating that linear velocity of up to 636 cm/h does not significantly affect the binding capacity of PolyCSX medium. However, P values for pH and conductivity are 0.031 and 0.022, respectively, both lower than 0.05, which indicates that they can significantly affect the dynamic binding capacity of IgG.

We also analyzed P values for both the main effects and two-way interactions between different factors. The only significant interaction at the chosen α-level of 0.05 is between pH and conductivity (P = 0.020). This is also reflected by the P value of 0.039 in our variance analysis. For example, the capacity of 48.5 mg/mL and 41.5 mg/mL at 102 cm/h and 636 cm/h (pH 4.0 and conductivity 19 mS/cm), respectively, indicate no significant effect of flow rate and operating feasibility at high conductivities. The interaction plot in Figure 2 clearly demonstrates that DBC at pH 4.0 is almost independent of conductivity. However, there is a sharp drop in capacity at pH 6.0. To use higher salt concentrations, operation at lower pH levels (5.0 or less) would provide the highest breakthrough capacities. However, the effect of such conditions on selectivities must be considered (Figure 3).

Our DOE analysis indicates that DBC is largely independent of linear velocity. We demonstrated this further by measuring DBC of IgG at several flow rates under the same conditions (pH 5.6 and conductivity 0.8 mS/cm). DBC remained essentially the same at all flow rates (Figure 4). Less than 10% difference was observed from a velocity of 100 to over 600 cm/h. We attribute this to excellent mass transfer because of a well-defined pore structure on PolyCSX medium, to high ligand density (0.4–0.5 meq/mL), and to extensive surface ligand coverage because of the polymeric nature of PEI spacers (Figure 5).

Because linear velocity has only a minimum effect on DBC, we carried out extended DOE experiments with only two variables: pH at six levels and conductivity ranging 2.0–12.7 mS/cm at a flow rate of ~400 cm/h. Table 5 gives the results along with recovery values we calculated by determining protein concentration in each fraction using the BCA protein assay method.

Our results established a fairly good model with the R-Sq (adj.) = 75.8%. As expected, both pH and conductivity are critical — as shown by P values of <0.05. Figure 6 shows interaction of conductivity at six different pH levels. The interaction plot clearly indicates that PolyCSX medium is quite tolerant to high salt concentrations and can be operated with high conductivities at a lower pH. The medium offers highest dynamic binding capacities at a pH that is two units lower than the pI of rabbit IgG (7.0), even at a conductivity of 19 mS/cm. To assess the effect of pH on DBC at low conductivity, we examined DBCs between pH 4 and 6 at 2 mS/cm. The

Table 5: Extended DOE study conditions and results

Run	pH at 25 °C	Conductivity (mS/cm)	10% Breakthrough (mg/mL)	Recovery
1	4.0	2.3	56.63	100.1%
2	4.0	6.4	54.81	99.7%
3	4.0	12.0	47.52	100.6%
4	4.5	2.1	64.80	97.1%
5	4.5	6.2	53.91	102.5%
6	4.5	12.0	43.59	101.6%
7	5.0	2.1	64.24	101.2%
8	5.0	6.3	47.07	104.1%
9	5.0	12.1	27.18	102.7%
10	5.2	2.0	66.30	97.5%
11	5.2	6.0	45.78	97.1%
12	5.2	12.0	4.84	98.3%
13	5.6	2.0	56.69	95.5%
14	5.6	6.0	13.93	98.6%
15	5.6	12.0	2.28	104.1%
16	6.0	2.8	16.49	100.6%
17	6.0	6.7	2.58	105.1%
18	6.0	12.7	1.33	101.6%

The regression equation is
10% BT = 177 – 23.1 pH – 3.34 Conductivity:

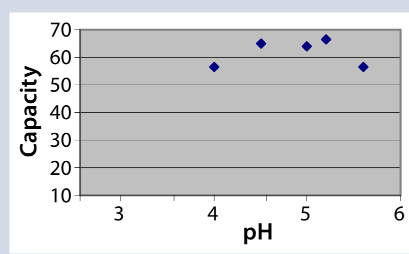
Predictor	Coef	SE Coef	T	P
Constant	176.63	21.85	8.08	0.00
pH	–23.060	4.211	–5.48	0.00
Conductivity	–3.3402	0.6835	–4.89	0.00

S = 11.8363; R-Sq = 78.6%; R-Sq(adj) = 75.8%

Analysis of Variance:

Source	DF	SS	MS	F	P
Regression	2	7725.9	3863.0	27.57	0.00
Residual Error	15	2101.5	140.1		
Total	17	9827.4			

Figure 7: Effect of pH on binding capacity at 2 mS/cm



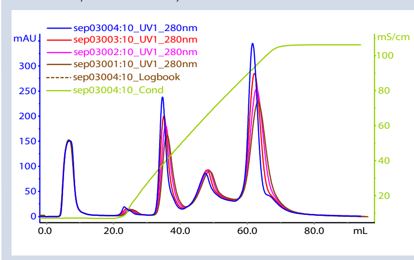
results in Figure 7 show only a minor effect at low conductivities.

As mentioned, throughput is a function of both DBC and selectivity. To assess the separation performance of PolyCSX medium, we separated a mixture of proteins with similar pI under the following conditions:

- Buffer A: 137 mM H0Ac/TEA, 32.9 mM NaCl, pH 4.7
- Buffer B: 1.0 M NaCl in Buffer A, gradient 0–100% B at 10 CV
- Sample: 1.5 mg/mL ovalbumin, 0.9 mg/mL IgG, 1.8 mg/mL hemoglobin, 1.8 mg/mL lactoferrin in buffer A (sample size 3.6 mL)
- Flow rate: 204 cm/hr, 408 cm/hr, 611 cm/hr, and 815 cm/hr.

The results of that experiment (Figure 8) show high resolution between proteins of similar pI, even at higher flow rates.

Figure 8: Separation of proteins with similar pI values at different flow rates (blue = 1.6 mL/min, 204 cm/hr; red = 3.2 mL/min, 408 cm/hr; pink = 4.8 mL/min, 611 cm/hr; brown = 6.4 mL/min, 815 cm/hr)



The unique selectivity and capacity independent of flow rate are also attributed to the surface chemistry of PolyCSX medium. Appropriately cross-linked synthetic polymethacrylate spherical beads (for high mechanical strength and rigidity) enable operation at a very high linear velocities. Polyethylenimine (PEI), used as a spacer to provide hydrophilic interaction with proteins, is bonded to the cross-linked polymethacrylate core. Subsequently, PEI is modified with carboxylic acid and sulfonic acid groups, giving it mixed-mode functionality. Beads have an average particle size of 35 µm and a median pore diameter of 550Å for access of proteins in >10^6 Da.

A Useful Model

We have used DOE along with regression analysis to optimize and understand the parameters affecting breakthrough capacity of new mixed-mode cation-exchange resins. This approach can be used to understand process parameters by conducting a minimum amount of experiments. Our data demonstrate that a mixed-mode polymeric ion exchanger, PolyCSX medium, maintains high dynamic binding capacities at high linear velocities and high conductivities for certain pH values. As such, it can be used in the design of modern downstream processes where high throughput is needed. The ability to maintain high dynamic capacities even at high linear velocities is intriguing and can be helpful in downstream process optimization. Tolerance of high salt content at low pH values is another attribute that can help in developing robust purification processes. The ion-exchange medium employed in our experiments can perform at a wide range of conditions and therefore be a valuable purification tool in high-performance platforms.

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