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Purification of an Fc-fusion biologic: Clearance of multiple product related impurities by hydrophobic interaction chromatography

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Abstract

An hydrophobic interaction chromatography step was developed for the large-scale production of an Fc-fusion biologic. Two abundant product-related impurities were separated from the active monomer using a Butyl resin and a simple step-wash and step-elution strategy. Capacity and resolution of the HIC step was optimal when sodium sulfate was employed as the lyotropic salt and pore size of the Butyl resin was 750 Å. Factorial analysis identified critical parameters for the Butyl chromatography and an operating window capable of delivering high product quality and yield over a broad column loading range.

Keywords: Hydrophobic interaction chromatography; Butyl; Fc-fusion; Biologic; Manufacturing

1. Introduction

Hydrophobic interaction chromatography (HIC) is a useful technique for large-scale purification of recombinant proteins [1–9]. It exploits the reversible interaction of protein hydrophobic patches with the hydrophobic ligand of a chromatography sorbent under mild conditions in the presence of a lyotropic salt [10–14]. Key considerations for the development of a manufacturing scale-purification process are efficiency, robustness, cost of goods and waste management. Furthermore, recent improvements in protein titers achieved using mammalian cell expression systems have increased the demand for high capacity unit operations, including chromatography steps that minimize column cycling and maximize throughput [15–19]. In accordance with regulatory guidance, the design space of a biologics manufacturing process is routinely explored by experiments performed according to factorial design. Process specifications are thus set based on modeling and statistical analysis of data [20–23]. This approach minimizes the number of experiments required while building an accurate model of the process and establishing quality by design.

Here we describe the development of a preparative HIC step appropriate for manufacturing scale production, in which two major product-related impurities were separated from an antibody Fc-fusion protein by the application of simple step gradients. The feed for the HIC step (neutralized eluate from a protein A affinity capture step) consisted of the product, termed active monomer (61% of the total), two related monomeric, disulfide-scrambled species, termed Inactive-1 (5%) and Inactive-2 (13%), dimer and higher molecular weight forms (termed aggregate; 21%). The target for the HIC step was to reduce the level of Inactive-1 and Inactive-2 to less than 1% of the total protein with a product yield acceptable for a manufacturing scale production (>50%). As the product was expressed in mammalian cells, reduction of host cell protein and DNA impurities was also required of the step. Furthermore, the HIC step followed a protein A affinity chromatography step, and therefore clearance of protein A leachate was sought. In addition, HIC chromatography may also be effective for the clearance of 20 nm, non-enveloped viruses that, unlike enveloped viruses, are not chemically inactivated (by low pH or detergent) in other processing operations. Here, the capacity of the HIC step was optimized by resin and salt screening experiments while critical chromatography parameters were identified by a factorial design approach. The conditions chosen for the HIC chromatography are discussed in the context of process robustness, economy and waste management.

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2. Experimental

2.1. Laboratory scale chromatography

Chromatography was performed using an AKTA Explorer 10 (10 mm path-length flow cell) with Unicorn software (GE Healthcare). Columns were 0.5 cm (Pharmacia HR5/5), 0.66 cm (Omnifit) or 2.6 cm (GE Healthcare) in diameter. Feedstock for HIC chromatography was clarified chinese hamster ovary cell culture medium purified by protein A affinity chromatography, stored at 4 °C. HIC load material was adjusted to ambient temperate (22–24 °C) prior to the addition of load adjustment solution at ambient temperature with gentle stirring. Load adjustment volume was 165% or 100% with adjustment solution containing 4.0 M NaCl or 1.2 M Na_2SO_4, respectively. Column load materials were filtered (0.22 μM, Millipak 20 or Millex-GV) immediately prior to the start of each chromatography experiment. Chromatography was performed at ambient temperature (22–24 °C) and the column was incubated at 24 °C using an ECHOtherm Chiller/Heater (#C030; Torrey Pines Scientific, CA). Tosoh Bioscience HIC resins were defined prior to use by washing once with water then twice with HIC equilibration buffer. Resins were packed in HIC equilibration buffer. Resins employed were Butyl 650 M or 750 M (Tosoh Bioscience), SOURCE 30PHE or Phenyl Sepharose 6 Fast Flow Low Sub (GE Healthcare).

2.2. Analysis of column intermediates by high pressure liquid chromatography (HPLC)

HPLC analysis was performed using a Waters 600S controller, 717plus autosampler and Millennium software. Analysis of intermediates for the level of Inactive-1/Inactive-2/active monomer by HIC–HPLC was performed using an Applied Biosystems HP2/20 column (2.1 × 30, #1-4522-12) with a salt gradient elution strategy operated at a flow rate of 1.0 mL/min. Analysis of intermediates for high molecular weight/monomeric/low molecular weight forms by size exclusion HPLC (SE-HPLC) was performed under isocratic conditions using a Tosoh Biosciences G3000SW_{XL} analytical column with a TSK_{gel} guard column (#08543). Measurement of Absorbance at 280 nm was performed using a Synergy 2 Platereader (Biotek Instruments Inc., VT) and UV transparent 96-well plates (#3635, Corning).

2.3. Statistical analysis

Design of experiments and statistical analysis was performed using JMP software version 6.0 (SAS). To identify critical parameters for the Butyl HIC step, a three-factor, two-level full factorial screening design with two center points was employed with full resolution of main effects and two-way interactions (10 chromatography experiments in total). The factors examined were column load amount (18 g/L and 26 g/L), wash (0.46 M and 0.54 M) and elution step (0.17 M and 0.23 M) sodium sulfate concentration. The responses measured were the level of Inactive-1 and Inactive-2 in the eluate, the yield of active monomer and the amount of protein in the wash fraction. The regression analysis was performed using a standard least squares fitting personality.

2.4. Analysis of process-related impurities in Butyl intermediates

Host cell protein (HCP) was measured using custom antibodies generated against mammalian HCPs and enzyme linked immunosorbent assay (ELISA) format utilizing electrochemiluminescence (ECL) for detection. Host cell DNA was detected using a quantitative polymerase chain reaction assay and custom primers homologous to mammalian DNA sequences. Protein A leachate was detected by ELISA using antibodies directed against recombinant protein A.

3. Results and discussion

A purification process was sought capable of separating an Fc-fusion drug candidate from two related but inactive forms at manufacturing scale. During the initial characterization of the drug candidate its purification was achieved by a protein A affinity capture, to concentrate the feedstock, followed by a hydrophobic interaction chromatography (HIC) step to separate active product from the two related inactive species (termed Inactive-1 and Inactive-2). However, the initial HIC step employed a resin with poor pressure-flow characteristics and a continuous gradient elution strategy combined with fractionation to achieve the required separation (not shown), both of which were considered undesirable for large-scale production. To identify a purification strategy appropriate for drug manufacturing nine HIC resins obtained from four different vendors were screened for pressure-flow characteristics compatible with large-scale production (for example, backpressure less than 0.3 MPa at a linear flow velocity of 100 cm/h) and separation of the active and inactive forms via a simple step gradient-wash and step-elution strategy. In this initial screen (not shown) a Butyl sorbent was identified that resolved the product-related species into three fractions (Fig. 1). Using a step gradient strategy, Inactive-1 was recovered in an intermediate wash fraction while active monomer and aggregate were each recovered in the elution fraction. Surprisingly, Inactive-2 remained bound during the elution step and was removed by stripping the column with water. Quantitation of product-related species present in the process intermediates was performed using two different high pressure liquid chromatography (HPLC) assays (Fig. 2). A conventional size exclusion HPLC assay was employed to quantitate the amount of aggregate and monomeric species, while a custom HIC–HPLC assay, capable of resolving the monomeric active and inactive species, was additionally employed to provide an overall estimate of active monomer yield.

Sodium chloride at 1.6 M supported a dynamic binding capacity of 8.0 g of protein per liter of the Butyl resin. In contrast, ammonium sulfate supported a higher binding capacity but with poor resolution of protein species (not shown). However, ongoing improvements to the fermentation process increased the feedstock titer from an initial level of 300 mg/L in early

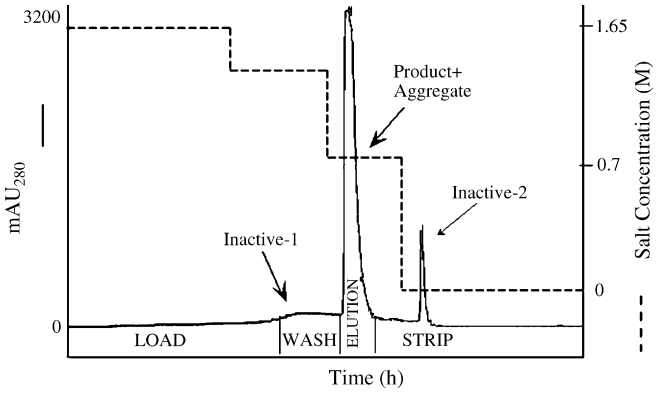

Fig. 1. Chromatogram of Butyl step from early development. Product-related impurities were separated by a step-wash and step-elution strategy. The distribution of the various species is indicated, except that a portion of the active monomer product was recovered in the wash fraction in addition to Inactive-1. Protein was bound to the column in the presence of 1.65 M NaCl. The wash and elution solutions contained 1.55 M and 0.7 M NaCl, respectively. The final column strip was with water. Solid line, absorbance at 280 nm; dashed line, NaCl concentration.

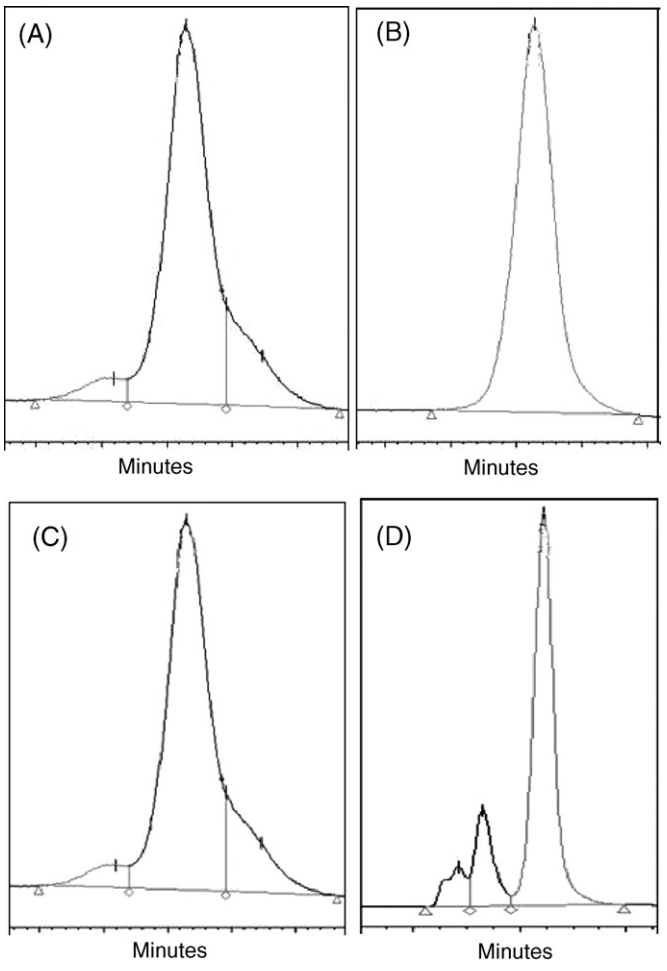

Fig. 2. Analysis of product-related impurities in Butyl load and eluate material. (A and B) Chromatograms generated by HIC–HPLC analysis of Butyl intermediates. (C and D) Chromatograms generated by SE-HPLC analysis of Butyl intermediates. (A and C) Analysis of Butyl load material. (B and D) Analysis of Butyl eluate material. (A) Peak 1 (earliest-eluting peak) corresponds to the Inactive-1 species. Peak 2 (main peak) comprises both active monomer and aggregated material. Peak 3 (late-eluting peak) corresponds to the Inactive-2 species. (B) Main peak comprises active monomer and aggregate (note that the peaks corresponding to Inactive-1 and Inactive-2 are absent). (C) Early-eluting peaks (three in total) correspond to high molecular weight material and dimer. Main peak comprises active monomer and Inactive-2. Shoulder lagging main peak corresponds to Inactive-1. Late-eluting minor peak corresponds to low molecular weight material. (D) Same as C except main peak corresponds to active monomer alone; note that the shoulder lagging the main peak (corresponding to Inactive-1) is absent. *Y*-axes: absorbance units.

development to 900 mg/L, requiring a corresponding increase in downstream capacity to avoid excessive column cycling in manufacturing. This prompted a second screen of the HIC operating conditions in which multiple lyotropic salts and chromatography resins were tested for dynamic binding capacity, resolution and yield. Two HIC resins were identified that were capable of resolving Inactive-1 and Inactive-2 from active monomer and aggregate while supporting a binding capacity of >20 g/L of resin (Table 1). Of these, one resin (bearing a phenyl ligand) was eliminated due to its bead size of 30 μm, which was considered undesirable for manufacturing scale production due to suboptimal pressure-flow characteristics. Thus, it was expected that the 30 μm bead size would limit the maximum flow rate and column bed height achievable, reducing process capacity and throughput. The second resin was similar to the Butyl sorbent identified during initial development but with a larger pore size (750 Å) designed by the manufacturer to provide improved capacity for antibody-like molecules. This resin displayed favorable pressure-flow characteristics (typical backpressure 0.3 MPa at a flow rate of 125 cm/h) with bed height at 20 cm. Sodium sulfate was chosen as the lyotropic salt for the Butyl chromatography. Unlike NaCl it supported a dynamic binding capacity of 28 g/L of resin (10% breakthrough) and maintained resolution of the Inactive-1 and Inactive-2 species from the product and aggregate. In contrast, high capacity but poor resolution was achieved using ammonium sulfate or potassium phosphate as the binding salt (not shown). Sodium sulfate (Na_2SO_4) offers a number of advantages for large-scale production, including low cost (comparable to NaCl), and favorable waste management requirements, in particular eliminating the presence of chloride or ammonium ions in the column effluent. Moreover, due to its position in the Hofmeister series Na_2SO_4 can promote strong protein-binding to HIC resins when present at a relatively low solution concentration. For the Butyl chromatography described here (Table 1), Na_2SO_4 supported a dynamic binding capacity of 23 g/L (80% of the 10% breakthrough capacity) when present in the column feed at a concentration of 600 mM. Nevertheless, Na_2SO_4 presents some disadvantages, including a maximum solubility in aqueous solution of 1.3 M, which may limit the maximum concentration used to promote protein binding in HIC, especially when minimization of load volume is required. For example, in the present case a binding Na_2SO_4 concentration of 600 mM was desired requiring the addition of an equal volume of load-adjustment solution containing 1.2 M Na_2SO_4 to the Butyl feedstock prior to column loading. Moreover, the solubility of Na_2SO_4 is reduced by the presence of other salts, such as those commonly used to control solution pH, and is strongly influenced by temperature which may restrict its use to ambient processes.

Table 1
Dynamic binding capacity and resolution provided by different HIC resins

	Chromatography resin			
	Butyl resin-1	Butyl resin-2	Phenyl resin-1	Phenyl resin-2
Mean pore size (Å)	650	750	340	350
Mean bead size (μm)	65	65	30	90
Binding salt	Dynamic binding capacity (mg/mL of resin)			
NaCl (1.6 M)	10[a]	N.D.	<2.0	0
Na_2SO_4 (0.6 M)	>16	28[a]	>23	>8
Binding salt	Resolution of Inactive-1/Inactive-2/active monomer product			
Na_2SO_4 (0.6 M)	Yes	Yes	Yes	No

[a] Dynamic binding capacity at 10% breakthrough.

The optimized Butyl chromatography was designed to remove Inactive-1 from the product via a wash step in which the Na_2SO_4 concentration was stepped-down from 600 mM to 500 mM and wash volume was limited to three column volumes (CVs). Product (and aggregate) was subsequently recovered by eluting the column with 200 mM Na_2SO_4. While this strategy resulted in complete clearance of Inactive-1, the separation of Inactive-1 and product during the wash step was incomplete, such that the wash and elution peaks were poorly resolved and product was lost in the wash fraction (Fig. 3A). To simplify collection of the eluate fraction, an alternative gradient elution strategy was tested to separate Inactive-1 from the other protein species while simultaneously resolving the wash and elution peaks (Fig. 3B). In this approach the Na_2SO_4 concentration was stepped-down at the start of the wash step but, in contrast to the step-wash strategy, this was followed by the immediate application of a positive salt gradient during which the Na_2SO_4 concentration was gradually returned to the loading concentration over the course of four-CVs. As before, the product (plus aggregate) was recovered by subsequent step-elution while Inactive-2 was stripped from the column with water. The gradient-wash strategy cleared Inactive-1 with an efficiency comparable to that of the step-wash approach, even though Na_2SO_4 concentration was returned to the column loading condition by the end of the wash. This may occur because the gradient approach accentuates differences in the retardation of different species by the HIC column. Thus, the gradient may permit the less hydrophobic species (Inactive-1) to migrate through the column at a high rate for a greater portion of the 4-CV wash relative to the more hydrophobic forms, which are expected to undergo stronger retardation earlier in the gradient as the Na_2SO_4 concentration is returned to the loading concentration.

A screening study employing a factorial design of experiments approach was performed to examine the effect of three parameters – column load, wash Na_2SO_4 concentration and elution Na_2SO_4 concentration – on the removal of impurities during the Butyl chromatography (Table 2). These factors (and their levels) were chosen based on the theoretical considerations for the

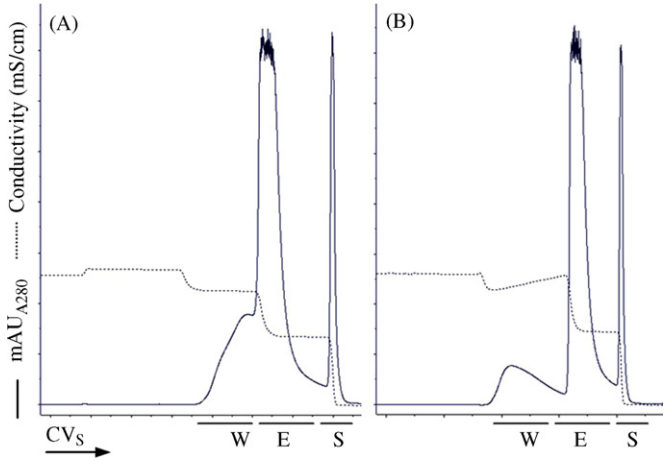

Fig. 3. Resolution of HIC wash and elution peaks by a gradient-wash strategy. (A) Low-resolution step-wash strategy. Material was bound to the column in the presence of 0.5 M Na_2SO_4. The column was washed (2.5 CVs) and eluted (2.5 CVs) with solutions containing 0.4 M and 0.21 M Na_2SO_4, respectively and stripped with water (2.5 CVs). The eluate fraction contained 1.1% Inactive-1 while Inactive-2 was undetectable. Total yield in eluate was 75%. (B) High resolution gradient-wash strategy. Binding, elution and strip conditions as in A. Following column loading, the Na_2SO_4 concentration was stepped-down initially to 0.4 M, then a positive gradient was applied immediately in which the Na_2SO_4 concentration was returned to 0.5 M over 4.0 CVs. The eluate contained 1.2% Inactive-1 while Inctive-2 was undetectable. Total yield in eluate was 70%. (A and B) Bed height 19 cm; load 8.0 mg/mL; flow velocity 120 cm/h. Solid line (mAU$_{280}$), milliabsorbance units at 280 nm; dotted line, conductivity (mS/cm); CVs, solution volume in column volumes; W, wash step; E, elution; S, strip.

Table 2
Factorial analysis of Butyl chromatography[a]

Factors		
Load (mg/mL of resin)	Wash [Na_2SO_4] (mM)	Elution [Na_2SO_4] (mM)
26	460	230
26	540	230
22	500	200
18	460	230
18	540	170
26	460	170
18	540	230
18	460	170
26	540	170
22	500	200

[a] Experiments described in the legend to Fig. 4.

Table 3
Factorial analysis of Butyl chromatography: statistical analysis of responses[a]

Statistical analysis	Parameter and range	Response			
		Active monomer yield (%)	Inactive-1 in eluate (%)	Inactive-2 in eluate (%)	Material in wash (%)
R^2	Multiple regression model with two-way interactions	0.88	0.92	0.81	0.98
$P > \rho$	Load amount	0.0190	0.0064	0.9225	<0.0001
	Wash [Na_2SO_4]	0.0045	0.0064	0.4191	<0.0001
	Elution [Na_2SO_4]	0.2684	0.5971	0.0061	0.1640
	Load amount × wash [Na_2SO_4]	0.6849	0.0064	0.7850	0.6306

[a] Individual parameters and total regression were analyzed using the student's *t*-test while two-way parameter interactions were analyzed using the ANOVA *t*-test. A *P* value of <0.05 was considered to be statistically significant.

mechanism of HIC, prior factorial analysis of similar HIC resins and preliminary screening experiments that indicated a strong effect of the parameters on Butyl performance and eliminated other factors that displayed insignificant effects (not described). Examination of the data using statistical analysis software (see Section 2) revealed a strong effect of column load and Na_2SO_4 concentration on the clearance of Inactive-1 during the wash step, which in turn was correlated with total material recovered in the wash fraction (Table 3). Higher column load and lower wash salt concentration each led to a more efficient clearance of Inactive-1 but a greater loss of total protein in the wash fraction and therefore a lower yield of product in the eluate (Fig. 4). This was consistent with the incomplete resolution of Inactive-1 and product achieved by the step-wash strategy. In contrast, the amount of Inactive-2 present in the eluate was strongly correlated with the third factor tested, Na_2SO_4 concentration in the eluant (Table 4). High Na_2SO_4 concentration in the elution solution improved clearance of Inactive-2 because it promoted retention of Inactive-2 on the column during elution of product. Thus Inactive-2 behaves as a more hydrophobic species than the active monomer permitting its differential retention on the column in the presence of an appropriate elution salt concentration. Surprisingly, aggregated material displayed a lower hydrophobicity than Inactive-2 during the Butyl chromatography, as it was recovered with active monomer in the elution fraction prior to stripping of Inactive-2 from the column with

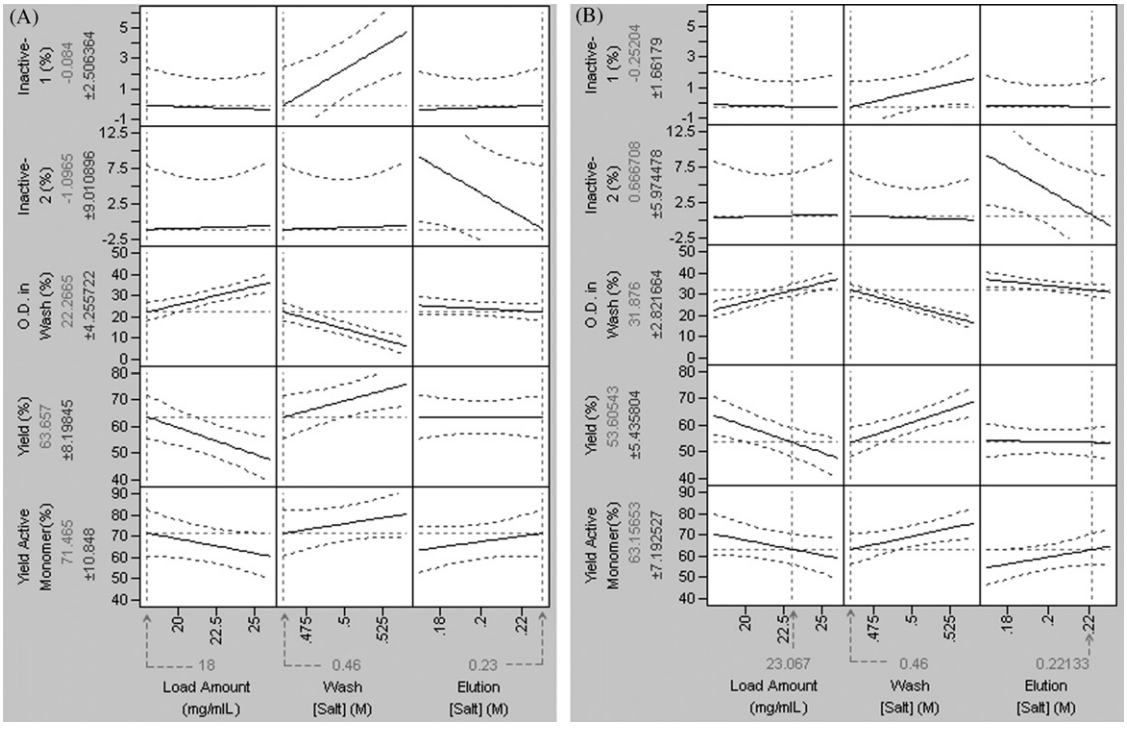

Fig. 4. Factorial analysis of Butyl chromatography–prediction traces. *X*-axis; factors with ranges described in Table 2; *Y*-axis, the range of four responses is shown. Solid horizontal line, current predicted value of each *Y* variable for current values of *X* variables with 95% confidence interval for the predicted values (accompanying broken lines). Vertical dotted line, *current value* of *X* variable. (A) *X* variables are set at column load 18 mg/mL, wash [Na_2SO_4] 0.46 M and elution [Na_2SO_4] 0.23 M as additionally indicated by the dashed arrows. (B) *X* variables are set at column load 23 mg/mL, wash [Na_2SO_4] 0.46 M and elution [Na_2SO_4] 0.22 M. Columns were operated with identical equilibration strip and regeneration conditions, feedstock, bed height (19 cm) flow velocity (125 cm/h) and temperature (24 °C). Feedstock was bound at 0.6 M Na_2SO_4 and the column was stripped and regenerated with water and 1.0N NaOH, respectively. Wash and elution volumes were 3.0 and 1.1 CVs, respectively. Column intermediates were analyzed for protein concentration and the level of product-related impurities as described in the text.

water. The model generated by the factorial analysis predicted that, at a wash Na_2SO_4 concentration of 460 mM, Inactive-1 could be cleared efficiently (undetectable in the elution fraction) even when column loading was at the low end of the range (18 g/L of resin) and only 22% of total protein was lost in the wash fraction (Fig. 4A). Based on these data, the Butyl column loading range was set to 18–24 g/L. At a column load of 23 g/L, the maximum loss of material in the wash and minimum recovery of active monomer in the eluate was expected to be 33% and 62%, respectively, while minimum yield was expected to be 54% (Fig. 4B). Furthermore the model predicted that a high elution Na_2SO_4 concentration (220–230 mM) could be employed to clear Inactive-2 efficiently without strongly impacting product yield (Fig. 4A and B). Contour plots of the relevant parameters provided a visualization of an appropriate operating window for the Butyl chromatography (Fig. 5) and limits of failure, thus identifying a limited design space for the purification.

The predictive value of the model was confirmed by scale-up of the Butyl step to a 2.6 cm diameter column (Fig. 6). In close agreement with predictions from the model (Fig. 4B), the yield of total material obtained in the Butyl eluate was 59% while yield of the active monomer species was 69% when the column was loaded to 23 g/L of resin. Moreover, the Inactive-1 and Inactive-2 species were undetectable in the Butyl eluate fraction as expected. As observed in small-scale experiments, aggregated material was not separated from the product (not shown). These confirmatory results supported the conclusion that the simple linear model generated by the screening design of experiments was reliable in predicting critical effects on the

Table 4
Clearance of process related impurities by Butyl chromatography[a]

Butyl intermediate	HCP[b] (ppm)[c]	DNA[d] (ppb)[e]	ProA[f] (ppm)
Butyl load	1846	22.9	9.0
Butyl eluate	120	<1.7[g]	1.5
Rf[h]	1.2	>1.1	0.8

[a] Data from the experiment described in Fig. 6.
[b] Host cell protein.
[c] Parts per million.
[d] Host cell DNA.
[e] Parts per billion.
[f] Protein A leachate (from prior column step).
[g] Below the lowest level of quantitation for the assay.
[h] Log_{10} reduction factor.

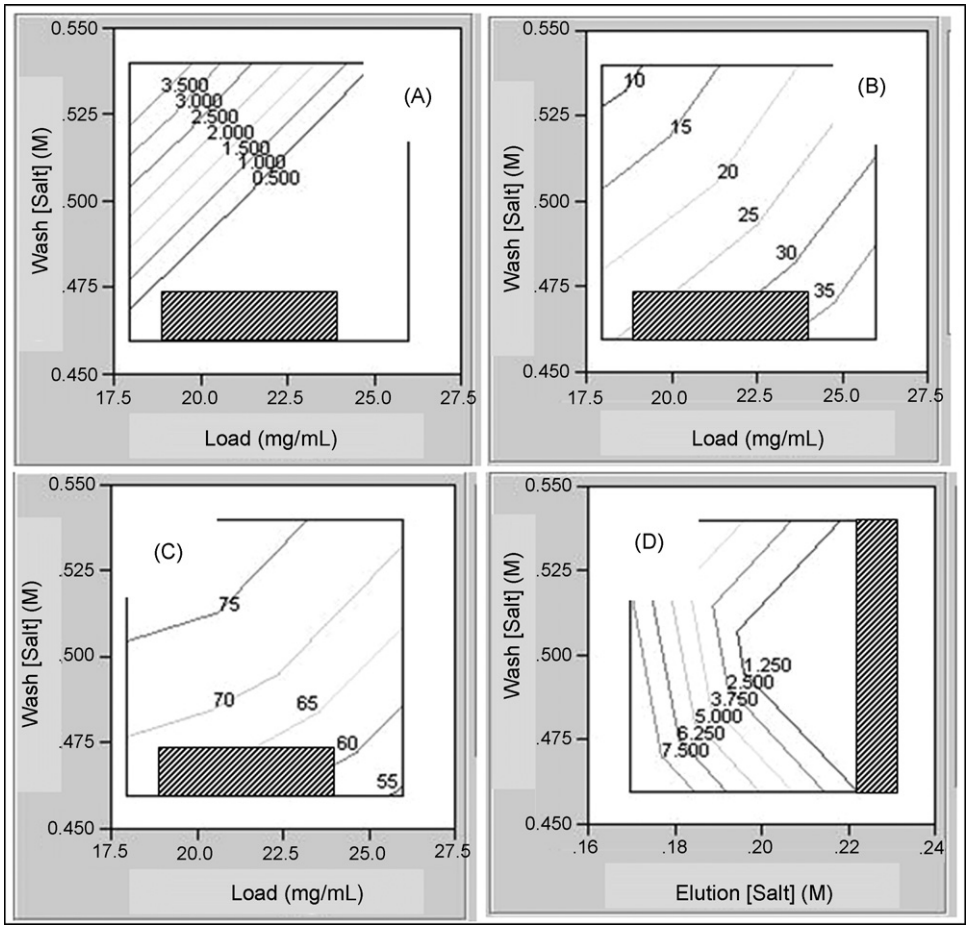

Fig. 5. Optimal operating window of the Butyl chromatography step. (A–C) Predicted level of Inactive-1 in the Butyl eluate fraction, total material in the wash fraction and yield of active monomer in the eluate fraction, respectively, as a function of column load and wash [Na_2SO_4]. (D) Predicted level of inactive-2 in the Butyl eluate fraction as a function wash and elution [Na_2SO_4]. Hatched box, predicted range of conditions under which optimal removal of product-related impurities is achieved by the Butyl chromatography. Contour plots were generated using JMP software. Factor ranges are indicated on the *X*- and *Y*-axes while response levels are indicated by the contour labels. Data are from the experiments described in Fig. 4 and Table 2.

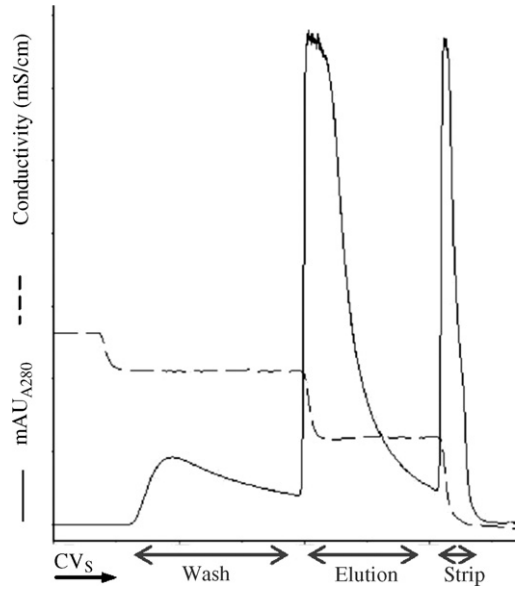

Fig. 6. Scale-up of Butyl chromatography performed under optimized conditions. The Butyl column (diameter 2.6 cm, bed height 20 cm) was operated under the conditions described in Fig. 4 and loaded to a capacity of 23 g/L of resin. Elution Na_2SO_4 concentration was 0.22 M. Load material contained aggregate, Inactive-1 and Inactive-2 at 23%, 5% and 14%, respectively, while the eluate contained active monomer at 78% and aggregate at 22%. Solid line (mAU_{280}), milliabsorbance units at 280 nm; dashed line, conductivity (mS/cm); CVs, solution volume in column volumes.

Butyl chromatography. Nevertheless, it is likely that the predictive capacity of the model would be improved through the analysis of the critical factors at multiple (three or four) different levels and by the inclusion of a greater number of center point conditions in the experimental design. In addition, the scale-up experiment revealed that the Butyl step was capable of providing clearance of three process related impurities, host cell protein, DNA and protein A leachate (Table 4).

Finally, three different lots of the Butyl resin were tested for performance consistency. Three separate columns were packed to a comparable bed height and operated with a common feedstock and chromatography solutions and to emphasize possible differences in the resin lots the columns were loaded at the high end of the range (23 mg/mL). Under these conditions Inactive-1 and Inactive-2 were undetectable in the eluate fractions obtained from each of the resin lots (not shown). Nevertheless, the amount of material recovered in the wash and elution fractions was different for the three lots tested (Table 5). Because removal of Inactive-1 is critically related to the amount of material recovered in the Butyl wash fraction, which is turn critically effected by the column load amount (see Figs. 4 and 5) it was concluded that a resin lot use-test would be required to guarantee efficient Inative-1 clearance over the designated load range of 18–24 g/L. Thus it was envisaged that, in some circumstances, the Butyl column loading range or the sodium sulfate concentration in the wash solution might require adjustment in order to achieve the desired clearance of product-related impurities by a particular resin lot. This is undesirable for biologics manufacturing in which solution lot records must be subjected to quality control and where blending of different resin lots may be necessary to achieve a packed bed of sufficient volume for large-scale production. Therefore, the resin lot variability observed in the current study represented a lack of robustness in the Butyl chromatography step.

Table 5
Butyl resin lot variability[a]

Resin lot #	Wash Material recovered (%)	Eluate Yield of active monomer (%)
60BUMC502G	21	61
60BUMC504G	19	62
60BUMC503G	16	67

[a] Experiments were performed as described in Fig. 5. Na_2SO_4 concentration in the load, wash and elution fractions was 0.6 M, 0.46 M and 0.22 M, respectively.

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