

Behavior of weakly adsorbing protein impurities in flow-through ion-exchange chromatography

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Abstract

Flow-through ion-exchange chromatography is frequently used in polishing biotherapeutics, but the factors that contribute to impurity persistence are incompletely understood. A large number of dilute impurities may be encountered that exhibit physicochemical diversity, making the flow-through separation performance highly sensitive to process conditions. The analysis presented in this work develops two novel correlations that offer transferable insights into the chromatographic behavior of weakly adsorbing impurities. The first, based on column simulations and validated experimentally, delineates the relative contributions of thermodynamic, transport, and geometric properties in dictating the initial breakthrough volume of dilute species. The Graetz number for mass transfer was found to generalize the transport contributions, enabling estimation of a threshold in the equilibrium constant below which impurity persistence is expected. Impurity adsorption equilibria are needed to use this correlation, but such data are not typically available. The

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second relationship presented in this work may be used to reduce the experimental burden of estimating adsorption equilibria as a function of ionic strength. A correlation between stoichiometric displacement model parameters was found by consolidating isocratic retention data for over 200 protein-pH-resin combinations from the extant literature. Coupled with Yamamoto’s analysis of linear gradient elution data, this correlation may be used to estimate retentivity approximately from a single experimental measurement, which could prove useful in predicting host-cell protein chromatographic behavior.

Keywords: Flow-through, Ion-exchange, Impurity, Clearance, Breakthrough, Stoichiometric displacement model

1. Introduction

The biopharmaceutical market has grown substantially over the past few decades, with over 300 biologics having received regulatory approval and hundreds more in development pipelines [1, 2]. Monoclonal antibodies (mAbs) comprise the majority of these biologics, partly because platform purification processes have enabled their rapid development [3, 4]. One stage in a typical platform process is polishing, where trace impurities such as host-cell proteins (HCPs) are removed prior to product formulation using one or more chromatographic operations [5]. Despite their low concentrations, some of these impurities may pose a risk to therapeutic safety and stability, and polishing operations are designed to remove them completely [6, 7].

Polishing may be performed using ion-exchange (IEX), hydrophobic interaction or multimodal resins. Since the majority of secreted HCPs are more

14 acidic than the typical mAb [8], anion-exchange (AEX) resins are more natu-
15 rally suited to flow-through operations [9], in which impurities are intended to
16 adsorb to the resin, but they may be used in bind-and-elute mode as well [10].
17 The inverse is true of cation-exchange (CEX) stationary phases. The flow-
18 through operational mode offers the advantage of relatively high throughput;
19 as a heuristic, 1 g of mAb usually requires 1 ml of resin in flow-through IEX
20 [11]. For mAb concentrations on the order of 20 mg/ml, this corresponds to
21 an apparent processing capacity on the order of 50 column volumes (CV).
22 Such large capacities can enable high productivity operations with relatively
23 small columns. If convective media are used instead of resin particles, there
24 is the potential to increase productivity beyond the limitations inherently
25 associated with packed column flow rate constraints [12][12, 13]. These pro-
26 cess intensification benefits have led to an increasing consideration of flow-
27 through operations for applications outside of polishing, particularly in the
28 development of continuous downstream processes [11, 14, 15].

29 Despite the utility of flow-through IEX steps, relatively few studies have
30 systematically investigated their development and limitations. This is partly
31 because impurity behavior is essential to understanding flow-through separa-
32 tions, but it is difficult to interrogate experimentally. Total HCP concentra-
33 tions encountered in mAb polishing are usually on the order of 1000 ppm [16],
34 rendering in-line detection and quantification infeasible [17, 18]. Hundreds
35 of HCPs may be present, and their biophysical diversity leads to heteroge-
36 neous chromatographic behavior. Although process conditions are tuned to
37 maximize HCP adsorption, they are generally less conducive to strong reten-
38 tion than in bind-and-elute mode. This may make impurity clearance highly

39 sensitive to the mobile phase composition and the resin.

40 Coupled with the plethora of available resins, the high sensitivity of sep-
41 aration performance typically necessitates screening studies during process
42 development. Commercial IEX resins are available that differ in particle
43 size, pore size and morphology, base matrix, ligand chemistry, ionic capac-
44 ity, and surface functionalization [19, 20]. Screening studies to determine
45 the best resin among several alternatives can provide valuable application-
46 specific data, and perhaps furnish general resin retentivity heuristics [21], but
47 they offer limited transferable insights into individual HCP chromatographic
48 retention and capacity. Column modeling has therefore been suggested as a
49 complementary technique to elucidate the factors that contribute to impurity
50 persistence [17, 18]. A better understanding of this phenomenon could po-
51 tentially improve flow-through process design, expedite development, reduce
52 costs, and increase consistency with quality by design principles. It could also
53 help direct future *in silico* optimization efforts, which to date have focused
54 primarily on product molecules rather than impurities.

55 Two HCP persistence mechanisms have been hypothesized for flow-through
56 processes: product association and weak adsorption [5]. The relative im-
57 portance of these mechanisms remains unclear. Product association is ex-
58 pected to vary mechanistically with the therapeutic molecule and the impu-
59 rity species, which may hinder a general understanding of the phenomenon.
60 However, it may be tractable to analyze generally the breakthrough of weakly
61 adsorbing impurities. Two attributes of the flow-through process simplify the
62 analysis, namely that the process is isocratic, and that the adsorption equi-
63 lbria of weakly adsorbing species are expected to be approximately linear

64 and independent. Assuming HCP concentrations on the order of 1000 ppm,
65 column capacities on the order of 100 mg/ml, and the load heuristic of 1 g
66 mAb/ml of column, only $\sim 1\%$ of the column is expected to saturate with
67 HCPs. The majority of the column is therefore available for weakly retained
68 HCPs to adsorb with negligible competition for the IEX surface.

69 This work focuses on developing transferable insights into the break-
70 through of weakly adsorbing impurities in flow-through IEX. We placed em-
71 phasis on understanding contributions to the initial breakthrough volume, as
72 the goal of flow-through polishing is the complete removal of trace impuri-
73 ties. To maintain generalizability across diverse sets of HCPs, we simulated
74 the chromatographic behavior of dilute species with a variety of transport
75 and thermodynamic properties. Simulated initial breakthrough volumes were
76 analytically related to the Graetz number for mass transfer, and this rela-
77 tionship was validated experimentally. Transport and thermodynamic pa-
78 rameters need to be estimated to use this relationship, but HCP adsorption
79 equilibrium constants are usually unknown. To gain a better understanding
80 of IEX adsorption equilibria, we consolidated dilute isocratic retention data
81 from the extant literature on model species. From these data, a correlation
82 was observed between stoichiometric displacement model (SDM) parameters
83 that are commonly used to relate the adsorption equilibrium constant to
84 ionic strength [22]. This thermodynamic correlation was corroborated by
85 linear gradient elution data, suggesting a way to estimate SDM parameters
86 approximately from one experimental measurement. These relationships pro-
87 vide novel insights into properties that contribute to flow-through impurity
88 clearance.

2. Theory and simulation

2.1. Column chromatography model

The 1D general rate model of column chromatography was used in this work, which describes the transport of solutes within the column interstitial volume as [20, 23, 24]:

$$\frac{\partial c}{\partial t} + v \frac{\partial c}{\partial z} = D_{ax} \frac{\partial^2 c}{\partial z^2} - \frac{3}{r_p} \left(\frac{1 - \varepsilon_c}{\varepsilon_c} \right) k_f \left(c - c_p|_{r=r_p} \right) \quad (1a)$$

with Danckwerts' boundary conditions:

$$v (c_{in} - c|_{z=0}) = -D_{ax} \frac{\partial c}{\partial z} \Big|_{z=0} \quad (1b)$$

$$\frac{\partial c}{\partial z} \Big|_{z=L_{col}} = 0 \quad (1c)$$

where c is the solute concentration in the interstitial volume, c_p is the solute concentration in the resin bead pore space, t is time, z is the column axial coordinate, r is the resin bead radial coordinate, c_{in} is the column inlet concentration, L_{col} is the column length, r_p is the resin bead radius, v is the interstitial velocity, D_{ax} is the axial dispersion coefficient, k_f is the film mass transfer coefficient, and ε_c is the **column porosity** **interstitial column porosity (i.e., the bed void fraction)**. Solute transport within the resin beads is described by:

$$\varepsilon_p \frac{\partial c_p}{\partial t} + (1 - \varepsilon_p) \frac{\partial q}{\partial t} = \varepsilon_p D_p \left(\frac{\partial^2 c_p}{\partial r^2} + \frac{1}{r} \frac{\partial c_p}{\partial r} \right) + (1 - \varepsilon_p) D_s \left(\frac{\partial^2 q}{\partial r^2} + \frac{1}{r} \frac{\partial q}{\partial r} \right) \quad (2a)$$

with boundary conditions of spherical symmetry at the bead center and a mass balance at the bead edge, respectively:

$$\frac{\partial c_p}{\partial r} \Big|_{r=0} = 0, \quad \frac{\partial q}{\partial r} \Big|_{r=0} = 0 \quad (2b)$$

106

$$\varepsilon_p D_p \frac{\partial c_p}{\partial r} \Big|_{r=r_p} + (1 - \varepsilon_p) D_s \frac{\partial q}{\partial r} \Big|_{r=r_p} = k_f \left(c - c_p \Big|_{r=r_p} \right) \quad (2c)$$

107 where q is the adsorbed solute concentration, ε_p is the resin bead porosity
 108 accessible to the solute, D_p is pore diffusivity, and D_s is surface diffusivity.
 109 Typically, the relevant initial conditions are:

$$c|_{t=0} = 0, \quad c_p|_{t=0} = 0, \quad q|_{t=0} = 0 \quad (3)$$

110 An isotherm relating q to c_p is needed to complete the column model,
 111 along with a description of the adsorption kinetics. Since adsorption and
 112 desorption steps are usually much faster than the other transport processes,
 113 instantaneous equilibration was assumed in this work. [The Although a linear](#)
 114 [isotherm was assumed for comparison with experimental data \(section 2.2\), in](#)
 115 [general the](#) Langmuir isotherm was used:

$$q = q_{max} \left(\frac{K_L c_p}{K_L c_p + 1} \right) \quad (4)$$

116 where q_{max} is the resin saturation capacity and K_L is a thermodynamic pa-
 117 rameter (equal to the ratio of the adsorption and desorption rate constants,
 118 k_a/k_d). The adsorption equilibrium constant, K_{eq} , is defined by the initial
 119 isotherm slope, which for the Langmuir model is:

$$K_{eq} \equiv \lim_{c_p \rightarrow 0} \frac{q}{c_p} = q_{max} K_L \quad (5)$$

120 Dimensions of volume concentration were used for q in this work (as opposed
 121 to excess surface concentration), making K_{eq} a dimensionless parameter.

122 Column model equations were solved with the Chromatography Analysis
 123 and Design Toolkit (CADET, versions 4.1.0 and 4.2.0) [25].

124 2.2. Simulations for analyzing breakthrough volumes

125 To ascertain qualitative differences between the breakthrough of concen-
 126 trated and dilute species, simulations were performed for single-component
 127 systems with load concentrations between 10 mg/ml and 1 $\mu\text{g}/\text{ml}$ with q_{max}
 128 fixed at 100 mg/ml of packed column, K_{eq} varied from 1 to 10000, and trans-
 129 port parameters selected from representative calibrations to model species.
 130 Exploratory simulations were then performed for a load concentration of 1
 131 $\mu\text{g}/\text{ml}$ at variable v , D_p , L_{col} , and r_p . D_{ax} was estimated as a function of
 132 v from correlations for beds packed with solid particles [26], using a dilute
 133 solution diffusivity of $7.5 \times 10^{-11} \text{ m}^2/\text{s}$ to represent general protein behavior
 134 [27]. Film mass transfer was assumed **to be relatively fast** **not to be rate-limiting**,
 135 and k_f was **consequently** set to $1 \times 10^{-3} \text{ m/s}$ **for to remove this degree of free-**
 136 **dom from** all simulations. D_s was estimated as a function of K_{eq} using results
 137 reported for a mAb of the form $D_s = aK_{eq}^b$, where the power law **coefficient co-**
 138 **efficients** $a = 1.66 \times 10^{-12} \text{ m}^2/\text{s}$ and $b = -0.24$ [28]. **All simulation parameters**
 139 **are summarized in Supplementary Table ??.**

140 Results from these simulations were correlated to relate breakthrough
 141 volume with system parameters, making use of an effective intraparticle dif-
 142 fusivity (D_{eff}) to describe the combined effects of pore and surface diffusion
 143 [29]:

$$D_{eff} \equiv D_p + \left(\frac{1 - \varepsilon_p}{\varepsilon_p} \right) K_{eq} D_s \quad (6)$$

144 This expression may be derived from Equation 2a by assuming instantaneous
 145 equilibration and linear chromatographic conditions, as given by:

$$q = K_{eq} c_p \quad (7)$$

146 A linear isotherm was used for comparing simulations with experimental
 147 data, and the extra-column volume, which consisted of valves and 0.75 mm
 148 ID tubing, was described as a continuously stirred tank reactor (CSTR) in
 149 series with a dispersed plug flow reactor (PFR) [30]. The fraction of the
 150 extra-column volume allocated to the CSTR was fit as a function of flow
 151 rate, and the PFR axial dispersion coefficient was set to $1 \times 10^{-12} \text{ m}^2/\text{s}$.

152 2.3. Models for ion-exchange adsorption equilibria

153 A number of models have been proposed to relate protein IEX adsorp-
 154 tion equilibria to solution conditions, with perhaps the simplest and most
 155 commonly cited being the stoichiometric displacement model (SDM), which
 156 describes adsorption as a strict ion-exchange process [22]. From the law of
 157 mass action, the adsorption equilibrium constant is related to ionic strength,
 158 I , as:

$$K_{eq} = \alpha I^{-\nu} \quad (8)$$

159 where α is a measure of adsorption strength and ν is the protein charac-
 160 teristic charge, which represents the stoichiometry of ion exchange. These
 161 parameters are commonly estimated by fitting isocratic retention data at
 162 different ionic strengths or regressing linear gradient elution data according
 163 to Yamamoto’s method [31]. The fundamental equation of ideal linear chro-
 164 matography predicts that retention factors should also exhibit a power law
 165 dependence on ionic strength [23]:

$$k' = \phi K_{eq} = \phi \alpha I^{-\nu} \quad (9)$$

166 where $k' = (V_R - V_0)/V_0$ is the retention factor for the solute and solution
 167 conditions of interest, V_R is the retention volume, and V_0 is the flow-through

168 **retention** volume under non-adsorbing conditions. For dimensionless K_{eq} ,
 169 $\phi = (1 - \varepsilon_t)/\varepsilon_t$ is a dimensionless phase ratio where $\varepsilon_t = \varepsilon_c + (1 - \varepsilon_c)\varepsilon_p$ $\varepsilon_t =$
 170 $\varepsilon_c + (1 - \varepsilon_c)\varepsilon_p = V_0/V_{column}$ represents the total column porosity.

171 More sophisticated colloidal models that are based on statistical thermo-
 172 dynamics have also been proposed for estimating K_{eq} from the free energy
 173 change of molecular adsorption, ΔF , using some form of the general expres-
 174 sion [32]:

$$K_{eq}(I, \text{pH}) = \int_{\Omega} \int_{z_0}^{\infty} (e^{-\Delta F(z, \Omega, I, \text{pH})/k_B T} - 1) dz d\Omega \quad (10)$$

175 where Ω represents the protein orientation with respect to the resin and z is
 176 the separation distance. Previous models have used continuum electrostatics
 177 and van der Waals equations to estimate ΔF , often making use of mean-field
 178 approximations [33, 34]. Notably, the K_{eq} estimates from these models are
 179 quite sensitive to ΔF due to the Boltzmann weighting.

180 **3. Materials and methods**

181 *3.1. Chemicals and buffers*

182 Sodium hydroxide, hydrochloric acid, sodium chloride, monobasic sodium
 183 phosphate, dibasic sodium phosphate, acetic acid, sodium acetate, boric
 184 acid, and sodium bicarbonate were purchased from Fisher (Fair Lawn, NJ).
 185 Sodium carbonate, ethanolamine, and 2000 kDa blue dextran were pur-
 186 chased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) and
 187 dimethylformamide were purchased from Thermo Scientific (Rockford, IL).
 188 All chemicals were used without any further purification.

189 Buffer solutions were prepared to the desired constituent concentrations
190 at room temperature with deionized water from an EMD Millipore Milli-Q
191 system ($> 18.2 \text{ M}\Omega \text{ cm}$). Low and high ionic strength buffers were prepared
192 with 0 and 1 M added NaCl, respectively. Buffer pH and conductivity were
193 measured with a Cole-Parmer PC200 meter, and pH adjustments were made
194 with concentrated sodium hydroxide and hydrochloric acid solutions. Prior
195 to use, buffer solutions were filtered with Fisher $0.2 \mu\text{m}$ aPES membranes
196 (Pittsburgh, PA).

197 *3.2. Proteins*

198 Lyophilized hen egg white lysozyme was purchased from Sigma (St. Louis,
199 MO). A mAb sample was obtained from the protein A eluate pool of a
200 manufacturing process at Bristol Myers Squibb (Devens, MA). The mAb
201 was supplied at 35 mg/ml and stored at -80°C . It was thawed prior to use
202 and exchanged into the desired buffer using a GE Sephadex G25 desalting
203 column, and protein solution concentrations were measured with a Thermo
204 Scientific NanoDrop Lite spectrophotometer.

205 *3.3. Resins and chromatography equipment*

206 All chromatographic measurements were performed using an Amersham
207 Biosciences Äkta Explorer equipped with a P-960 sample pump, fraction
208 collector, 10 mm UV flow cell, and in-line pH and conductivity meters. SP
209 Sepharose FF, a strong cation-exchanger with a nominal particle diameter of
210 $90 \mu\text{m}$, was purchased from GE Healthcare (Uppsala, Sweden). It was ex-
211 changed three times into a pH 7.0 (25 mM sodium phosphate) buffer by
212 centrifugation and decantation prior to slurry packing (50%) into a $0.5 \times$

213 10 cm Waters AP chromatography column to a final bed volume of 2.0 ml.
214 Column packing was validated at pH 7.0 by injecting a 100 μ l pulse of a 1 M
215 NaCl buffer and verifying that the conductivity trace asymmetry fell within
216 commonly accepted limits [19].

217 3.4. *Linear gradient elution*

218 The retention of lysozyme on SP Sepharose FF was measured with lin-
219 ear gradient elution at pH 5.0, 7.0, and 9.0 (in 75 mM sodium acetate, 25
220 mM sodium phosphate, and 25 mM ethanolamine buffers, respectively) at a
221 superficial velocity of 300 cm/h. Feed solutions were prepared by dissolving
222 lysozyme in the appropriate low ionic strength buffer at \sim 20 mg/ml, and
223 lyophilized excipients were removed with three buffer exchange cycles using
224 EMD Millipore Amicon centrifugal filter units (Cork, Ireland). Lysozyme
225 solutions were filtered using Thermo Scientific 0.2 μ m PVDF membranes
226 (Rockwood, TN) and diluted to \leq 10 mg/ml prior to use. The SP Sepharose
227 FF column was equilibrated in the relevant low ionic strength buffer for 5
228 **column volumes (CV)**, and gradient elution from 0 to 1 M NaCl began
229 shortly after a 100 μ l sample injection. The gradient elution volume was
230 varied from 10 to 50 CV in increments of 10 CV, and the conductivity at
231 peak elution was determined. This was used with a correlation for the in-
232 strument's conductivity response to estimate the peak elution ionic strength.
233 Columns were regenerated at least once every five runs via 15 minutes of
234 contact with 0.5 M NaOH.

235 *3.5. Breakthrough measurements during isocratic elution*

236 To validate corresponding simulation results, frontal loading chromatog-
237 raphy was performed with dilute lysozyme solutions on SP Sepharose FF
238 at pH 7.0 (20 mM sodium phosphate) under conditions of weak to moder-
239 ate retention. The requisite ionic strength for such retention was estimated
240 from linear gradient elution measurements and tuned empirically to target
241 a breakthrough volume of ~ 10 CV. A feedstock of 10 $\mu\text{g}/\text{ml}$ lysozyme was
242 used to mimic the low impurity concentrations encountered in flow-through
243 polishing, and outlet concentrations were estimated from the absorbance at
244 215 nm with a computed extinction coefficient [35]. At such low protein con-
245 centrations, appreciable variability in component separation was observed
246 between replicate feedstock preparations. For this reason, feedstock batches
247 were prepared at the desired ionic strength in sufficient volume to service
248 the entire set of measurements, and a batch exhibiting minimal component
249 separation was selected for use. For each measurement, a 10 CV equilibra-
250 tion period preceded a 20 CV load through the Äkta's sample pump, after
251 which the column was washed as described previously. This was performed
252 at superficial velocities ranging from 30 to 305 cm/h in a randomized order,
253 and comparable breakthrough measurements were made under non-adsorbing
254 conditions (at high ionic strength).

255 To qualitatively validate simulated trends under conditions of greater
256 relevance to flow-through polishing, breakthrough behavior was also demon-
257 strated with fluorescently labelled lysozyme in the presence of a mAb. Lysozyme
258 was conjugated with FITC according to the manufacturer's protocol, produc-
259 ing a complex product mixture with different label ratios and conjugation

260 sites. A fraction of the conjugation products exhibiting relatively homo-
261 geneous chromatographic behavior was required to effectively represent the
262 breakthrough of an individual impurity. The conjugation product mixture
263 was therefore subjected to repeated fractionation on SP Sepharose FF at
264 pH 9.5 (20 mM sodium carbonate) using shallow ionic strength steps during
265 elution. Average label ratios and FITC-lysozyme concentrations in the col-
266 lected fractions were estimated from the absorbance profiles at 280 and 495
267 nm. Once a sufficiently homogeneous fraction was obtained, it was spiked
268 to $\sim 10 \mu\text{g/ml}$ in a mAb solution at pH 9.5, where the mAb was observed
269 to have negligible retention on SP Sepharose FF. As with lysozyme break-
270 through measurements, an ionic strength was identified to achieve weak to
271 moderate FITC-lysozyme retention, and breakthrough profiles were acquired
272 over a range of superficial velocities in randomized order. The mAb concen-
273 tration was dilute due to material constraints, and the eluate absorbance at
274 495 nm was used to distinguish FITC-lysozyme breakthrough from the mAb
275 flow-through.

276 *3.6. Parameter estimation for test simulations*

277 The breakthrough of lysozyme fed in dilute solution on SP Sepharose FF
278 was simulated in CADET as described in Section 2.2. Sequential parameter
279 estimation was used to reduce the dimensionality of the optimization space
280 when fitting the experimental data. Duplicate column bypass profiles were
281 acquired with lysozyme at each flow rate used in breakthrough measurements.
282 These were fit with a trust region optimization algorithm interfaced by the
283 `Scipy least_squares` function to determine the extra-column volume (which
284 is invariant with flow rate) and the fraction of that volume allocated to the

285 CSTR in the system model (which varies with flow rate). Triplicate blue
 286 dextran pulses, monitored with the absorbance at 280 nm, were then passed
 287 through the column to measure ε_c . Peak tailing was observed, which is
 288 attributable to size heterogeneity in the blue dextran tracer. For this reason,
 289 the average peak mode was used instead of the average first moment to
 290 estimate ε_c .

291 Duplicate lysozyme pulses under non-adsorbing conditions (high ionic
 292 strength) were then passed through the column at each flow rate. Retention
 293 volumes under non-adsorbing conditions were computed from the first mo-
 294 ment of the absorbance profiles and used to estimate ε_p . These profiles were
 295 then fit to estimate D_p (which is invariant with flow rate) and D_{ax} (which
 296 varies with flow rate). An evolutionary algorithm interfaced by the **Scipy**
 297 **differential_evolution** function was used to estimate D_p and a coarse
 298 dependence of D_{ax} on flow rate. The estimated value of D_p was retained,
 299 and the coarse D_{ax} estimates were refined using the **least_squares** func-
 300 tion. Without further refinement, the estimated parameters were validated
 301 by modeling dilute lysozyme breakthrough profiles under non-adsorbing con-
 302 ditions (at high ionic strength). Dilute breakthrough profiles under adsorbing
 303 conditions (at lower ionic strength) were then simulated by fitting K_{eq} and
 304 D_s (which are invariant with flow rate) using the **differential_evolution**
 305 function.

306 4. Results and discussion

307 4.1. Breakthrough volume correlation

308 In the context of polishing operations, the value of flow-through IEX is its
309 ability to completely remove as many trace HCPs as possible from a mAb so-
310 lution. Challenges inherent in this task may not be readily apparent because
311 it is difficult to study the chromatographic behavior of dilute solutes em-
312 pirically. A column simulation was therefore used to investigate differences
313 between the breakthrough of overloaded products and dilute impurities. An
314 initial set of single-component simulations were performed at different feed
315 concentrations and K_{eq} values. Transport and geometric parameters were
316 held fixed, and a Langmuir isotherm with constant $q_{max} = 100$ mg/ml of
317 column was used. Results for extreme 10 mg/ml and 1 μ g/ml feed concentra-
318 tions are juxtaposed in Figure 1, and analogous plots for intermediate corresponding
319 to separation factors that are $\ll 1$ (highly favorable) and ~ 1 (near-linear), re-
320 spectively [36], while results for intermediate feed concentrations are shown in
321 Supplementary Figure ??.

322 As may be expected for the system with a 10 mg/ml feed , retention in-
323 creased (separation factor $\ll 1$), retention increases with K_{eq} until the column
324 saturatedsaturates, and breakthrough profiles became become sharper as column
325 saturation was is approached. This self-sharpening behavior did does not occur
326 for the 1 μ g/ml feed (near-linear conditions) over the volume scale simulated,
327 revealing dilute solute breakthrough profiles to become more diffuse as reten-
328 tion increases due to the essentially linear isotherm. An order-of-magnitude
329 difference can be observed between the load volumes corresponding to the
330 initial breakthrough and the inflection point in the more strongly retained

331 profiles. This illustrates an appreciable challenge; even if trace impurities ex-
 332 hibit strong to moderate retention, their diffuse distribution on the column
 333 may lead to relatively early breakthrough and preclude adequate clearance.

334 To better understand this challenge, the load volume corresponding to
 335 1% breakthrough was identified and plotted against K_{eq} (Figure 2). A cou-
 336 ple of trends may be observed. When column saturation is not approached,
 337 the breakthrough volume appears to scale linearly with K_{eq} . An ideal limit on
 338 A limiting estimate of the K_{eq} dependence may be roughly estimated obtained as
 339 $CV_{breakthrough} = \varepsilon_t + (1 - \varepsilon_t)K_{eq}$ based on the fundamental equation of ideal
 340 linear chromatography. As shown, the slope of the dilute solute series dif-
 341 fers markedly from the ideal limit, due largely to finite transport rates that
 342 limit the system's approach to equilibrium. The discrepancy between the ob-
 343 served and ideal behavior therefore suggests that transport rate optimization
 344 may provide a useful means of improving impurity clearance in flow-through
 345 processes.

346 Further simulations showed The simulation data show that the slope of the $CV_{1\%}$
 347 vs. K_{eq} curve for dilute solutes varies with transport and geometric param-
 348 eters, and the curve approaches an intercept between ε_c and ε_t at $K_{eq} = 0$. This
 349 observation indicates that the initial breakthrough volume may be approxi-
 350 mately described as:

$$CV_{breakthrough1\%} = \varepsilon_t + (1 - \varepsilon_t)K_{eq}f_{1\%} \quad (11)$$

351 where $f_{\varepsilon_c} < \varepsilon < \varepsilon_t$ and $f_{1\%}$ is a function of transport parameters that
 352 modifies the breakthrough volume dependence on K_{eq} , with $0 \leq f \leq 10 \leq$
 353 $f_{1\%} \leq 1$. The form of this relationship has two implications: it suggests
 354 that thermodynamic and transport contributions to impurity breakthrough

may be independent, and that transport contributions may be described by a single factor, which can presumably be expressed generally in terms of a dimensionless group.

Based on this rationale, a second set of simulations were performed for a 1 $\mu\text{g}/\text{ml}$ feed. A with a variety of thermodynamic, transport, and geometric parameters were used, and f . $f_{1\%}$ was computed from the results by rearranging Equation 11. However, and substituting ε_c for ε , because ε_c was used in place of the ε_t intercept when computing f from Equation 11 because this was observed to better generally describe the simulated breakthrough volumes better than ε_t in the limit of $K_{eq} \rightarrow 0$. Based on ; this may be attributable to finite transport rates limiting the solute exploration of intraparticle void volumes. As was done for the dynamic binding capacity correlation presented by Chen et al. [37], f $f_{1\%}$ is plotted in Figure 3 against the dimensionless group:

$$Gz_{eff} = \frac{vd_{part}^2}{D_{eff}L_{col}} = Péc_{p-eff} \frac{d_{part}}{L_{col}}$$

which represents a Graetz number for mass transfer, written as a product of two dimensionless subgroups. The first is :

$$Gz_{eff} = \frac{vd_{part}^2}{D_{eff}L_{col}} \quad (12)$$

This is often written as $Gz_{eff} = (d_{part}/L_{col}) Péc_{p-eff}$, where the particle Péclet number, which represents the ratio of convective to diffusive transport rates $Péc_{p-eff} = vd_{part}/D_{eff}$ represents a ratio of characteristic times for diffusive and convective transport on the length scale of the resin particle, when D_{eff} is used to describe the diffusional transport. The second subgroup is . However, it is more directly meaningful as a ratio of characteristic length scales, namely the resin particle diameter and the column length the characteristic times for intraparticle diffusion, d_{part}^2/D_{eff} , and convection along the column, L_{col}/v .

Figure 3 indicates that Equation 11 with a modified intercept may be written more specifically as:

$$CV_{\%} = \varepsilon_c + (1 - \varepsilon_t)K_{eq}f_{\%} \left(\frac{vd_{part}^2}{D_{eff}L_{col}}Gz_{eff} \right) \quad (13)$$

where $CV_{\%}$ is the load volume at a given percent breakthrough threshold, and $f_{\%}$ is the corresponding function of transport and geometric parameters. Shown are the Graetz number. Rearranging Equation 13 reveals this function to be approximately the ratio of dynamic to static binding capacities under linear adsorption conditions when $CV_{\%} \gg \varepsilon_c$. Figure 3 shows two series for illustrative breakthrough thresholds, but any arbitrary threshold could be selected. Some noise is apparent, but it is largely attributable to the approximation that $CV_{\%}$ is invariant with transport parameters when $K_{eq} = 0$. Within the noise, it is clear that $CV_{\%}$ decays monotonically with the value of the Graetz number.

To maximize the load volume before breakthrough, which is approximated by $CV_{1\%}$, the Graetz number should be minimized. Unfortunately, doing so comes at a cost to throughput if v is reduced or an increase in column pressure drop if d_{part} is decreased or L_{col} is increased. This correlation does therefore therefore does not suggest a free way of improving flow-through processing. What it does quantify, however, is the inherent balance between separation performance and throughput. Notably, the initial breakthrough volume decays much more quickly with the Graetz number than the intermediate breakthrough volume. The benefit of optimizing the system geometry and transport rates is expected to be only marginal for typical process conditions, which correspond to Graetz numbers on the flat part of the 1% breakthrough curve in Figure 3. However, if the Graetz number could be decreased below ~ 5 , the benefit would become much more pronounced.

402 An interesting subtlety that is implicit in this correlation is the effect
 403 of thermodynamics on intraparticle transport rates, which is described by
 404 the dependence of D_{eff} on K_{eq} . D_{eff} captures the combined effects of pore
 405 and surface diffusion in the dilute solute limit by describing the effective
 406 intraparticle diffusivity as a weighted sum, where K_{eq} weights the relative
 407 importance of surface diffusion. IEX surface diffusivities have been shown
 408 to follow a power law of the form $D_s = aK_{eq}^b$, where [28], where the empirical
 409 power law coefficients a and b are expected to be on the order of D_p and -0.5 ,
 410 respectively, with $b < 0$ [28][38, 39]. This leads to competing effects: increasing
 411 K_{eq} decreases the surface diffusivity but simultaneously increases the driving
 412 force for surface diffusion, with the effect that dominates being determined
 413 by whether the power law exponent $b > -1$. If this is the case, increasing K_{eq}
 414 leads to an increase in D_{eff} and a reduction in the Graetz number, meaning
 415 that both thermodynamic and transport contributions to delaying impurity
 416 breakthrough are improved simultaneously.

417 Equation 13 also enables estimation of the problematic K_{eq} threshold
 418 below which impurities will persist via weak adsorption. To gain a sense for
 419 the threshold's order of magnitude, an illustrative calculation was performed
 420 with a spline fit to the $f_{1\%}$ curve shown in Figure 3. Results for a 20 cm
 421 column with a 6 minute residence time are shown in Figure 4 as a function
 422 of load volume using $\varepsilon_c = 0.35$, $\varepsilon_p = 0.5$, $d_{part} = 50 \mu\text{m}$, $D_p = 10^{-11} \text{ m}^2/\text{s}$,
 423 and D_s estimated from the power law discussed previously. The results show
 424 that, for load volumes on the order of 100 CV, HCPs with $K_{eq} < \sim 400$ are
 425 liable to break through before the end of loading. This would be equivalent to
 426 a retention factor of ~ 200 for an input feed pulse under identical conditions.

4.2. Validation of the breakthrough volume correlation

To validate the breakthrough volume correlation that was developed from simulation results, frontal loading chromatography was performed using dilute feeds as described in Section 3.5. Using a highly pure feedstock was found to be essential for demonstrating the behavior of individual species in the dilute limit. Various model proteins were tested with AEX and CEX resins, but only lysozyme was found to be readily prepared in sufficient purity. It was therefore used with SP Sepharose FF, despite the fact that most mAb flow-through purification processes are performed with AEX resins. Figure 5 shows breakthrough profiles acquired by loading lysozyme onto SP Sepharose FF at 10 $\mu\text{g}/\text{ml}$ under high and low ionic strength conditions. The flow rate was varied to change the value of the Graetz number, and an increase in breakthrough volume was observed at lower flow rates. Equation 13 indicates that transport parameters should have a more pronounced effect on the initial breakthrough volume when retention is stronger, due to the K_{eq} weighting of $f\%$. This is consistent with the observed behavior at low ionic strength, where K_{eq} is higher. Column models with fit parameters showed excellent agreement at high ionic strength. Although not perfect, the agreement was also quantitatively close at low ionic strength. Discrepancies from simulation under these conditions may be attributable to feedstock component separation, as well as inaccuracies in describing extra-column effects with the simplified model of a CSTR in series with a PFR, which are more pronounced at low flow rates.

The breakthrough volume correlation was also qualitatively validated using FITC-lysozyme in the presence of a mAb. As with lysozyme measurements, the flow rate was varied to change the value of the correlation variable

under conditions of weak FITC-lysozyme retention. Figure 6 shows breakthrough profiles that were computed from absorbance measurements at 495 and 280 nm, using the average FITC-lysozyme label ratio estimated during fractionation of the conjugation products. Appreciable measurement noise is apparent in the FITC-lysozyme profiles due to the low load concentration of $\sim 10 \mu\text{g/ml}$. Nonetheless, the observed trend is consistent with the previous results: using lower flow rates delayed the onset of FITC-lysozyme breakthrough. This was not modeled explicitly due to the multicomponent nature of the FITC-lysozyme conjugation products, but it does support the breakthrough volume correlation qualitatively.

4.3. Correlation of SDM parameters

A knowledge of phenomenological properties is required to estimate breakthrough volumes using Equation 13, specifically values of D_p , D_s , and K_{eq} . However, such information is typically unknown for impurities such as HCPs on IEX resins. Methods of estimating these properties could therefore be useful, and some heuristics already exist for estimating the two intraparticle diffusivities. For instance, D_s is typically an order of magnitude smaller than D_p under the relevant conditions of weak adsorption, and D_p should theoretically scale with the free solution diffusivity, D_0 , according to [19]:

$$D_p = \frac{\varepsilon_p \psi_p}{\tau_p} D_0 \quad (14)$$

where ψ_p and τ_p are the diffusional hindrance coefficient and the resin tortuosity factor, respectively. If a putative weakly adsorbing HCP has been identified, its molar mass may be used with the Stokes-Einstein equation or correlations to estimate D_0 [27]. Appreciable uncertainty surrounds the value

475 of ψ_p/τ_p , but 0.2 appears to be representative of its order of magnitude [40].
 476 Assuming a value like this enables rough estimation of D_p and D_s based on
 477 protein mass.

478 This leaves the estimation of K_{eq} , which varies with the solution condi-
 479 tions, as the main obstacle to applying the breakthrough volume correlation
 480 in practice. It is expected that K_{eq} will follow a power law in ionic strength
 481 of the SDM form, but the dependence on pH is less well defined. Several elec-
 482 trostatics models have been proposed for describing this behavior, but doing
 483 so predictively remains an open problem. In the absence of such predictive
 484 tools, insights may be drawn from experimental data on the retention of
 485 model proteins. To facilitate observation of system-independent trends, iso-
 486 cratic k' measurements at different ionic strengths were consolidated from the
 487 extant literature on 230 protein-pH-resin combinations, as shown in Supple-
 488 mentary Figure ?? and the accompanying spreadsheet [21, 30, 41–48]. These
 489 data were regressed according to Equation 9 to extract the SDM parameter
 490 ν and the quasi-SDM parameter $\phi\alpha$, which are plotted against each other in
 491 Figure 7 and observed to follow a significant intrinsically linear correlation
 492 (as determined by regressor t-tests).

493 This previously unobserved correlation is expected to be a consequence of
 494 adsorption thermodynamics rather than resin morphology, so the true rela-
 495 tionship represented in Figure 7 is suspected to be between ν and α . Inverse
 496 size exclusion chromatography data are unavailable for the majority of the
 497 resins studied, which precludes estimation of ϕ values that are specific to
 498 protein-resin pairs. However, all phase ratios are expected to be of compara-
 499 ble magnitude, and α may span several orders of magnitude, so the inclusion

500 of ϕ is not expected to introduce much noise in the correlation. The fact
 501 that $\phi\alpha$ spans several orders of magnitude makes the prediction interval ap-
 502 preciablely broad in terms of absolute values, and explaining some of the noise
 503 with additional factors would be advantageous. The ion-exchange type was
 504 tested for this purpose, and a significant difference was found between the
 505 correlations for AEX and CEX isocratic data (by including an interaction
 506 with the categorical variable). The mechanisms underlying this difference
 507 are not understood.

508 The utility of these relationships lies in their ability to remove one degree
 509 of freedom from the analysis of retention data. A series of measurements
 510 are usually performed to estimate SDM parameters, either from isocratic
 511 retention at different ionic strengths or elution peak ionic strength under
 512 linear gradient salt elutions of different volumes. The identified correlation
 513 could potentially enable the estimation of SDM parameters from only one
 514 experimental measurement. This concept is illustrated in Figure 8, which
 515 shows linear gradient elution data for lysozyme on SP Sepharose FF, collected
 516 as part of this study, plotted in the regression space for Yamamoto’s GH
 517 analysis [31]. Juxtaposed with these data are predictions from the correlation
 518 of isocratic CEX SDM parameters. Close agreement is observed between
 519 predictions and experiment at each of the pH values tested. One point in each
 520 series would be sufficient to estimate roughly the value of ν , and therefore
 521 that of $\phi\alpha$ when using the correlation. However, because the uncertainty in
 522 $\phi\alpha$ is with respect to its order of magnitude, the absolute value of its estimate
 523 needs to be interpreted with caution.

524 The linear gradient elution data were regressed according to Yamamoto’s

method, and the SDM parameters are plotted in Figure 7. Analogous CEX
gradient elution data for a mAb and its aggregates that had relatively large
SDM parameters were also obtained from the literature and included in Figure 7 [49]. Both sets of gradient elution data are consistent with the correlation of isocratic CEX data within the 95% prediction interval. This confirms the correlation’s utility in removing a degree of freedom from the analysis of retention data. Unfortunately, it does not eliminate the need for some experimental data in estimating K_{eq} , but it may reduce the burden of doing so. As proteomic techniques advance, it may become possible to quantitatively measure HCP retention. This would foreseeably be an expensive operation, and it may be essential to minimize the number of requisite measurements. The SDM parameter correlation may be useful in such an application.

From an intuitive perspective, the essential features of this correlation appear to be broadly consistent with theory. If the characteristic charge indeed represents the number of attractive point charge interactions between the protein and the IEX resin, it seems reasonable to hypothesize that increasing this number at constant ionic strength would result in an approximately proportional increase in the adsorption free energy. This would result in an order-of-magnitude increase in K_{eq} , which at the unit concentration of ionic strength is equivalent to α ; such an argument may rationalize the positive and log-linear correlation between ν and $\phi\alpha$. The reality is necessarily more complex, however, as the adsorption free energy would theoretically be a function of the pH, resin, protein, and the protein’s adsorbed orientation. These are incorporated in a statistical thermodynamic calculation of K_{eq} in Equation 10, albeit implicitly with respect to the resin and protein species.

Perhaps the dependence on all four variables can be generalized approximately by the characteristic charge, but this would require evaluation and proof using a rigorous biophysical model. Developing that proof, or at least exhibiting behavior that is consistent with the empirical SDM parameter correlation (Figure 7), may provide a good validity test for molecular adsorption models.

5. Conclusions

Unlike concentrated solutes, the approximately linear chromatographic behavior of dilute impurities can lead to diffuse breakthrough. This can make it challenging to remove flow-through impurities completely, but it also enables breakthrough volumes to be analytically related to phenomenological properties. Specifically, the Graetz number can describe transport contributions to dilute solute breakthrough volumes generally when an effective intraparticle diffusivity is used. Knowledge of this relationship enables a problematic equilibrium constant threshold to be estimated, which is on the order of 400 for typical process conditions. However, for this to be used in practice, the IEX adsorption equilibria of impurities of interest must be known. The correlation of SDM model parameters may be useful in estimating such data approximately from one linear gradient elution measurement, and it may serve as a validity test for molecular adsorption models as well.

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574 **Declaration of competing interest**

575 The authors declare that they have no known competing financial inter-
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578 **CRedit authorship contribution statement**

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588 **Supplementary material**

589 Supplementary material is associated with this article.

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