

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

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

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

with Danckwerts' boundary conditions:

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

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

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 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:

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

105

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

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

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

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

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

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

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

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

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

123 2.2. Simulations for analyzing breakthrough volumes

124 To ascertain qualitative differences between the breakthrough of concen-
 125 trated and dilute species, simulations were performed for single-component
 126 systems with load concentrations between 10 mg/ml and 1 $\mu\text{g}/\text{ml}$ with q_{max}
 127 fixed at 100 mg/ml of packed column, K_{eq} varied from 1 to 10000, and trans-
 128 port parameters selected from representative calibrations to model species.
 129 Exploratory simulations were then performed for a load concentration of 1
 130 $\mu\text{g}/\text{ml}$ at variable v , D_p , L_{col} , and r_p . D_{ax} was estimated as a function of
 131 v from correlations for beds packed with solid particles [26], using a dilute
 132 solution diffusivity of $7.5 \times 10^{-11} \text{ m}^2/\text{s}$ to represent general protein behav-
 133 ior [27]. Film mass transfer was assumed not to be rate-limiting, and k_f
 134 was consequently set to $1 \times 10^{-3} \text{ m/s}$ to remove this degree of freedom from
 135 all simulations. D_s was estimated as a function of K_{eq} using results re-
 136 ported for a mAb of the form $D_s = aK_{eq}^b$, where the power law coefficients
 137 $a = 1.66 \times 10^{-12} \text{ m}^2/\text{s}$ and $b = -0.24$ [28]. All simulation parameters are
 138 summarized in Supplementary Table S1.

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

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

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

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

145 A linear isotherm was used for comparing simulations with experimental
 146 data, and the extra-column volume, which consisted of valves and 0.75 mm
 147 ID tubing, was described as a continuously stirred tank reactor (CSTR) in
 148 series with a dispersed plug flow reactor (PFR) [30]. The fraction of the
 149 extra-column volume allocated to the CSTR was fit as a function of flow
 150 rate, and the PFR axial dispersion coefficient was set to 1×10^{-12} m²/s.

151 2.3. Models for ion-exchange adsorption equilibria

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

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

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

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

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

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

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

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

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

179 **3. Materials and methods**

180 *3.1. Chemicals and buffers*

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

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

196 *3.2. Proteins*

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

204 *3.3. Resins and chromatography equipment*

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

212 10 cm Waters AP chromatography column to a final bed volume of 2.0 ml.
213 Column packing was validated at pH 7.0 by injecting a 100 μ l pulse of a 1 M
214 NaCl buffer and verifying that the conductivity trace asymmetry fell within
215 commonly accepted limits [19].

216 3.4. *Linear gradient elution*

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

234 3.5. Breakthrough measurements during isocratic elution

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

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

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

275 3.6. *Parameter estimation for test simulations*

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

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

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

305 4. Results and discussion

306 4.1. Breakthrough volume correlation

307 In the context of polishing operations, the value of flow-through IEX
308 is its ability to completely remove as many trace HCPs as possible from a
309 mAb solution. Challenges inherent in this task may not be readily apparent
310 because it is difficult to study the chromatographic behavior of dilute solutes
311 empirically. A column simulation was therefore used to investigate differences
312 between the breakthrough of overloaded products and dilute impurities. An
313 initial set of single-component simulations were performed at different feed
314 concentrations and K_{eq} values. Transport and geometric parameters were
315 held fixed, and a Langmuir isotherm with constant $q_{max} = 100$ mg/ml of
316 column was used. Results for 10 mg/ml and 1 μ g/ml feed concentrations are
317 juxtaposed in Figure 1, corresponding to separation factors that are $\ll 1$
318 (highly favorable) and ~ 1 (near-linear), respectively [36], while results for
319 intermediate feed concentrations are shown in Supplementary Figure S1.

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

retention, their diffuse distribution on the column may lead to relatively early breakthrough and preclude adequate clearance.

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

The simulation data show that the slope of the $CV_{1\%}$ vs. K_{eq} curve for dilute solutes varies with transport and geometric parameters, and the curve approaches an intercept between ε_c and ε_t at $K_{eq} = 0$. This observation indicates that the initial breakthrough volume may be approximately described as:

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

where $\varepsilon_c < \varepsilon < \varepsilon_t$ and $f_{1\%}$ is a function of transport parameters that modifies the breakthrough volume dependence on K_{eq} , with $0 \leq f_{1\%} \leq 1$. The form of this relationship has two implications: it suggests 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 with a variety of thermodynamic, transport, and geometric parameters. $f_{1\%}$ was computed from the results by rearranging Equation 11 and substituting ε_c for ε , because ε_c was observed to generally describe the simulated breakthrough volumes better than ε_t in the limit of $K_{eq} \rightarrow 0$; 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], $f_{1\%}$ is plotted in Figure 3 against a Graetz number for mass transfer:

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

This is often written as $Gz_{eff} = (d_{part}/L_{col})P\acute{e}_{p-eff}$, where the particle Péclet number $P\acute{e}_{p-eff} = vd_{part}/D_{eff}$ represents a ratio of characteristic times for diffusive and convective transport on the length scale of the resin particle. However, Gz_{eff} is more directly meaningful as a ratio of 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 may be written more specifically as:

$$CV_{\%} = \varepsilon_c + (1 - \varepsilon_t)K_{eq}f_{\%}(Gz_{eff}) \quad (13)$$

where $CV_{\%}$ is the load volume at a given percent breakthrough threshold, and $f_{\%}$ is the corresponding function of 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

377 attributable to the approximation that $CV_{\%}$ is invariant with transport pa-
 378 rameters when $K_{eq} = 0$. Within the noise, it is clear that $CV_{\%}$ decays
 379 monotonically with the value of the Graetz number.

380 To maximize the load volume before breakthrough, which is approxi-
 381 mated by $CV_{1\%}$, the Graetz number should be minimized. Unfortunately,
 382 doing so comes at a cost to throughput if v is reduced or an increase in col-
 383 umn pressure drop if d_{part} is decreased or L_{col} is increased. This correlation
 384 therefore does not suggest a free way of improving flow-through processing.
 385 What it does quantify, however, is the inherent balance between separa-
 386 tion performance and throughput. Notably, the initial breakthrough volume
 387 decays much more quickly with the Graetz number than the intermediate
 388 breakthrough volume. The benefit of optimizing the system geometry and
 389 transport rates is expected to be only marginal for typical process conditions,
 390 which correspond to Graetz numbers on the flat part of the 1% breakthrough
 391 curve in Figure 3. However, if the Graetz number could be decreased below
 392 ~ 5 , the benefit would become much more pronounced.

393 An interesting subtlety that is implicit in this correlation is the effect
 394 of thermodynamics on intraparticle transport rates, which is described by
 395 the dependence of D_{eff} on K_{eq} . D_{eff} captures the combined effects of pore
 396 and surface diffusion in the dilute solute limit by describing the effective
 397 intraparticle diffusivity as a weighted sum, where K_{eq} weights the relative
 398 importance of surface diffusion. IEX surface diffusivities have been shown to
 399 follow a power law of the form $D_s = aK_{eq}^b$ [28], where the empirical power
 400 law coefficients a and b are expected to be on the order of D_p and -0.5 ,
 401 respectively, with $b < 0$ [38, 39]. This leads to competing effects: increasing

402 K_{eq} decreases the surface diffusivity but simultaneously increases the driving
 403 force for surface diffusion, with the effect that dominates being determined by
 404 whether $b > -1$. If this is the case, increasing K_{eq} leads to an increase in D_{eff}
 405 and a reduction in the Graetz number, meaning that both thermodynamic
 406 and transport contributions to delaying impurity breakthrough are improved
 407 simultaneously.

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

418 4.2. Validation of the breakthrough volume correlation

419 To validate the breakthrough volume correlation that was developed from
 420 simulation results, frontal loading chromatography was performed as de-
 421 scribed in Section 3.5. Using a highly pure feedstock was found to be essential
 422 for demonstrating the behavior of individual species in the dilute limit. Vari-
 423 ous model proteins were tested with AEX and CEX resins, but only lysozyme
 424 was found to be readily prepared in sufficient purity. It was therefore used
 425 with SP Sepharose FF, despite the fact that most mAb flow-through purifica-
 426 tion processes are performed with AEX resins. Figure 5 shows breakthrough

427 profiles acquired by loading lysozyme onto SP Sepharose FF at 10 $\mu\text{g}/\text{ml}$
 428 under high and low ionic strength conditions. The flow rate was varied to
 429 change the value of the Graetz number, and an increase in breakthrough vol-
 430 ume was observed at lower flow rates. Equation 13 indicates that transport
 431 parameters should have a more pronounced effect on the initial breakthrough
 432 volume when retention is stronger, due to the K_{eq} weighting of $f\%$. This is
 433 consistent with the observed behavior at low ionic strength, where K_{eq} is
 434 higher. Column models with fit parameters showed excellent agreement at
 435 high ionic strength. Although not perfect, the agreement was also quantita-
 436 tively close at low ionic strength. Discrepancies from simulation under these
 437 conditions may be attributable to feedstock component separation, as well as
 438 inaccuracies in describing extra-column effects with the simplified model of
 439 a CSTR in series with a PFR, which are more pronounced at low flow rates.

440 The breakthrough volume correlation was also qualitatively validated us-
 441 ing FITC-lysozyme in the presence of a mAb. As with lysozyme measure-
 442 ments, the flow rate was varied to change the value of the correlation variable
 443 under conditions of weak FITC-lysozyme retention. Figure 6 shows break-
 444 through profiles that were computed from absorbance measurements at 495
 445 and 280 nm, using the average FITC-lysozyme label ratio estimated during
 446 fractionation of the conjugation products. Appreciable measurement noise
 447 is apparent in the FITC-lysozyme profiles due to the low load concentra-
 448 tion of $\sim 10 \mu\text{g}/\text{ml}$. Nonetheless, the observed trend is consistent with the
 449 previous results: using lower flow rates delayed the onset of FITC-lysozyme
 450 breakthrough. This was not modeled explicitly due to the multicomponent
 451 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 of ψ_p/τ_p , but 0.2 appears to be representative of its order of magnitude [40]. Assuming a value like this enables rough estimation of D_p and D_s based on protein mass.

This leaves the estimation of K_{eq} , which varies with the solution conditions, as the main obstacle to applying the breakthrough volume correlation in practice. It is expected that K_{eq} will follow a power law in ionic strength of the SDM form, but the dependence on pH is less well defined. Several electrostatics models have been proposed for describing this behavior, but doing so predictively remains an open problem. In the absence of such predictive

475 tools, insights may be drawn from experimental data on the retention of
 476 model proteins. To facilitate observation of system-independent trends, iso-
 477 cratic k' measurements at different ionic strengths were consolidated from the
 478 extant literature on 230 protein-pH-resin combinations, as shown in Supple-
 479 mentary Figure S2 and the accompanying spreadsheet [21, 30, 41–48]. These
 480 data were regressed according to Equation 9 to extract the SDM parameter
 481 ν and the quasi-SDM parameter $\phi\alpha$, which are plotted against each other in
 482 Figure 7 and observed to follow a significant intrinsically linear correlation
 483 (as determined by regressor t-tests).

484 This previously unobserved correlation is expected to be a consequence of
 485 adsorption thermodynamics rather than resin morphology, so the true rela-
 486 tionship represented in Figure 7 is suspected to be between ν and α . Inverse
 487 size exclusion chromatography data are unavailable for the majority of the
 488 resins studied, which precludes estimation of ϕ values that are specific to
 489 protein-resin pairs. However, all phase ratios are expected to be of compara-
 490 ble magnitude, and α may span several orders of magnitude, so the inclusion
 491 of ϕ is not expected to introduce much noise in the correlation. The fact
 492 that $\phi\alpha$ spans several orders of magnitude makes the prediction interval ap-
 493 preciably broad in terms of absolute values, and explaining some of the noise
 494 with additional factors would be advantageous. The ion-exchange type was
 495 tested for this purpose, and a significant difference was found between the
 496 correlations for AEX and CEX isocratic data (by including an interaction
 497 with the categorical variable). The mechanisms underlying this difference
 498 are not understood.

499 The utility of these relationships lies in their ability to remove one degree

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

515 The linear gradient elution data were regressed according to Yamamoto’s
 516 method, and the SDM parameters are plotted in Figure 7. Analogous CEX
 517 gradient elution data for a mAb and its aggregates that had relatively large
 518 SDM parameters were also obtained from the literature and included in Fig-
 519 ure 7 [49]. Both sets of gradient elution data are consistent with the correla-
 520 tion of isocratic CEX data within the 95% prediction interval. This confirms
 521 the correlation’s utility in removing a degree of freedom from the analysis of
 522 retention data. Unfortunately, it does not eliminate the need for some ex-
 523 perimental data in estimating K_{eq} , but it may reduce the burden of doing so.
 524 As proteomic techniques advance, it may become possible to quantitatively

525 measure HCP retention. This would foreseeably be an expensive operation,
526 and it may be essential to minimize the number of requisite measurements.
527 The SDM parameter correlation may be useful in such an application.

528 From an intuitive perspective, the essential features of this correlation ap-
529 pear to be broadly consistent with theory. If the characteristic charge indeed
530 represents the number of attractive point charge interactions between the
531 protein and the IEX resin, it seems reasonable to hypothesize that increas-
532 ing this number at constant ionic strength would result in an approximately
533 proportional increase in the adsorption free energy. This would result in an
534 order-of-magnitude increase in K_{eq} , which at the unit concentration of ionic
535 strength is equivalent to α ; such an argument may rationalize the positive
536 and log-linear correlation between ν and $\phi\alpha$. The reality is necessarily more
537 complex, however, as the adsorption free energy would theoretically be a
538 function of the pH, resin, protein, and the protein's adsorbed orientation.
539 These are incorporated in a statistical thermodynamic calculation of K_{eq} in
540 Equation 10, albeit implicitly with respect to the resin and protein species.
541 Perhaps the dependence on all four variables can be generalized approxi-
542 mately by the characteristic charge, but this would require evaluation and
543 proof using a rigorous biophysical model. Developing that proof, or at least
544 exhibiting behavior that is consistent with the empirical SDM parameter cor-
545 relation (Figure 7), may provide a good validity test for molecular adsorption
546 models.

547 **5. Conclusions**

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

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565 **Declaration of competing interest**

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

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579 **Supplementary material**

580 Supplementary material is associated with this article.

581 **References**

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