

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

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

Despite the utility of flow-through IEX steps, relatively few studies have systematically investigated their development and limitations. This is partly because impurity behavior is essential to understanding flow-through separations, but it is difficult to interrogate experimentally. Total HCP concentrations encountered in mAb polishing are usually on the order of 1000 ppm [16], rendering in-line detection and quantification infeasible [17, 18]. Hundreds of HCPs may be present, and their biophysical diversity leads to heterogeneous chromatographic behavior. Although process conditions are tuned to maximize HCP adsorption, they are generally less conducive to strong retention 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),
 115 in 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 v
 132 from correlations for beds packed with solid particles [26], using a dilute solu-
 133 tion diffusivity of $7.5 \times 10^{-11} \text{ m}^2/\text{s}$ to represent general protein behavior [27].
 134 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
 136 freedom from all simulations. D_s was estimated as a function of K_{eq} us-
 137 ing results reported for a mAb of the form $D_s = aK_{eq}^b$, where the power
 138 law ~~coefficient~~ coefficients $a = 1.66 \times 10^{-12} \text{ m}^2/\text{s}$ and $b = -0.24$ [28]. All
 139 simulation parameters are summarized in Supplementary Table S1.

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 1×10^{-12} m²/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 ~~$\varepsilon_t = \varepsilon_c + (1 - \varepsilon_c)\varepsilon_p$~~
 170 ~~$\varepsilon_t = \varepsilon_c + (1 - \varepsilon_c)\varepsilon_p = V_0/V_{column}$~~ 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
210 of $90 \mu\text{m}$, was purchased from GE Healthcare (Uppsala, Sweden). It was
211 exchanged 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 ~~CV~~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 concen-
318 trations are juxtaposed in Figure 1, ~~and analogous plots for intermediate~~
319 corresponding to separation factors that are $\ll 1$ (highly favorable) and ~ 1
320 (near-linear), respectively [36], while results for intermediate feed concentra-
321 tions are shown in Supplementary Figure S1.

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

331 strongly retained profiles. This illustrates an appreciable challenge; even if
 332 trace impurities exhibit strong to moderate retention, their diffuse distribu-
 333 tion on the column may lead to relatively early breakthrough and preclude
 334 adequate clearance.

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

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

$$CV_{\underline{breakthrough}1\%} = \varepsilon_{\underline{t}} + (1 - \varepsilon_t)K_{eq}f_{\underline{1\%}} \quad (11)$$

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

modynamic 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 $f_{1\%}$~~ 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], $f_{1\%}$ 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:

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

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 separa-
tion performance and throughput. Notably, the initial breakthrough volume

399 decays much more quickly with the Graetz number than the intermediate
400 breakthrough volume. The benefit of optimizing the system geometry and
401 transport rates is expected to be only marginal for typical process conditions,
402 which correspond to Graetz numbers on the flat part of the 1% breakthrough
403 curve in Figure 3. However, if the Graetz number could be decreased below
404 ~ 5 , the benefit would become much more pronounced.

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

420 Equation 13 also enables estimation of the problematic K_{eq} threshold
421 below which impurities will persist via weak adsorption. To gain a sense for
422 the threshold's order of magnitude, an illustrative calculation was performed
423 with a spline fit to the $f_{1\%}$ curve shown in Figure 3. Results for a 20 cm

column with a 6 minute residence time are shown in Figure 4 as a function of load volume using $\varepsilon_c = 0.35$, $\varepsilon_p = 0.5$, $d_{part} = 50 \mu\text{m}$, $D_p = 10^{-11} \text{ m}^2/\text{s}$, and D_s estimated from the power law discussed previously. The results show that, for load volumes on the order of 100 CV, HCPs with $K_{eq} < \sim 400$ are liable to break through before the end of loading. This would be equivalent to 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 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 tools, insights may be drawn from experimental data on the retention of model proteins. To facilitate observation of system-independent trends, isocratic k' measurements at different ionic strengths were consolidated from the extant literature on 230 protein-pH-resin combinations, as shown in Supplementary Figure S2 [and the accompanying spreadsheet](#) [21, 30, 41–48]. These data were regressed according to Equation 9 to extract the SDM parameter ν and the quasi-SDM parameter $\phi\alpha$, which are plotted against each other in Figure 7 and observed to follow a significant intrinsically linear correlation (as determined by regressor t-tests).

This previously unobserved correlation is expected to be a consequence of

498 adsorption thermodynamics rather than resin morphology, so the true rela-
 499 tionship represented in Figure 7 is suspected to be between ν and α . Inverse
 500 size exclusion chromatography data are unavailable for the majority of the
 501 resins studied, which precludes estimation of ϕ values that are specific to
 502 protein-resin pairs. However, all phase ratios are expected to be of compara-
 503 ble magnitude, and α may span several orders of magnitude, so the inclusion
 504 of ϕ is not expected to introduce much noise in the correlation. The fact
 505 that $\phi\alpha$ spans several orders of magnitude makes the prediction interval ap-
 506 preciable broad in terms of absolute values, and explaining some of the noise
 507 with additional factors would be advantageous. The ion-exchange type was
 508 tested for this purpose, and a significant difference was found between the
 509 correlations for AEX and CEX isocratic data (by including an interaction
 510 with the categorical variable). The mechanisms underlying this difference
 511 are not understood.

512 The utility of these relationships lies in their ability to remove one degree
 513 of freedom from the analysis of retention data. A series of measurements
 514 are usually performed to estimate SDM parameters, either from isocratic
 515 retention at different ionic strengths or elution peak ionic strength under
 516 linear gradient salt elutions of different volumes. The identified correlation
 517 could potentially enable the estimation of SDM parameters from only one
 518 experimental measurement. This concept is illustrated in Figure 8, which
 519 shows linear gradient elution data for lysozyme on SP Sepharose FF, collected
 520 as part of this study, plotted in the regression space for Yamamoto’s GH
 521 analysis [31]. Juxtaposed with these data are predictions from the correlation
 522 of isocratic CEX SDM parameters. Close agreement is observed between

523 predictions and experiment at each of the pH values tested. One point in each
524 series would be sufficient to estimate roughly the value of ν , and therefore
525 that of $\phi\alpha$ when using the correlation. However, because the uncertainty in
526 $\phi\alpha$ is with respect to its order of magnitude, the absolute value of its estimate
527 needs to be interpreted with caution.

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

541 From an intuitive perspective, the essential features of this correlation ap-
542 pear to be broadly consistent with theory. If the characteristic charge indeed
543 represents the number of attractive point charge interactions between the
544 protein and the IEX resin, it seems reasonable to hypothesize that increas-
545 ing this number at constant ionic strength would result in an approximately
546 proportional increase in the adsorption free energy. This would result in an
547 order-of-magnitude increase in K_{eq} , which at the unit concentration of ionic

548 strength is equivalent to α ; such an argument may rationalize the positive
 549 and log-linear correlation between ν and $\phi\alpha$. The reality is necessarily more
 550 complex, however, as the adsorption free energy would theoretically be a
 551 function of the pH, resin, protein, and the protein's adsorbed orientation.
 552 These are incorporated in a statistical thermodynamic calculation of K_{eq} in
 553 Equation 10, albeit implicitly with respect to the resin and protein species.
 554 Perhaps the dependence on all four variables can be generalized approxi-
 555 mately by the characteristic charge, but this would require evaluation and
 556 proof using a rigorous biophysical model. Developing that proof, or at least
 557 exhibiting behavior that is consistent with the empirical SDM parameter cor-
 558 relation (Figure 7), may provide a good validity test for molecular adsorption
 559 models.

560 5. Conclusions

561 Unlike concentrated solutes, the approximately linear chromatographic
 562 behavior of dilute impurities can lead to diffuse breakthrough. This can
 563 make it challenging to remove flow-through impurities completely, but it
 564 also enables breakthrough volumes to be analytically related to phenomeno-
 565 logical properties. Specifically, the Graetz number can describe transport
 566 contributions to dilute solute breakthrough volumes generally when an effec-
 567 tive intraparticle diffusivity is used. Knowledge of this relationship enables
 568 a problematic equilibrium constant threshold to be estimated, which is on
 569 the order of 400 for typical process conditions. However, for this to be used
 570 in practice, the IEX adsorption equilibria of impurities of interest must be
 571 known. The correlation of SDM model parameters may be useful in estimat-

572 ing such data approximately from one linear gradient elution measurement,
573 and it may serve as a validity test for molecular adsorption models as well.

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578 **Declaration of competing interest**

579 The authors declare that they have no known competing financial inter-
580 ests or personal relationships that could have appeared to influence the work
581 reported in this paper.

582 **CRedit authorship contribution statement**

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592 **Supplementary material**

593 Supplementary material is associated with this article.

594 **References**

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