

Behavior of weakly adsorbing 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 column porosity (also known as 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 des-
 111 orption steps are usually much faster than the other transport processes, in-
 112 stantaneous equilibration was assumed in this work. The Langmuir isotherm
 113 was used:

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

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

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

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

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

122 2.2. Simulations for analyzing breakthrough volumes

123 To ascertain qualitative differences between the breakthrough of concen-
 124 trated and dilute species, simulations were performed for single-component
 125 systems with load concentrations between 10 mg/ml and 1 $\mu\text{g}/\text{ml}$ with q_{max}
 126 fixed at 100 mg/ml of packed column, K_{eq} varied from 1 to 10000, and trans-
 127 port parameters selected from representative calibrations to model species.
 128 Exploratory simulations were then performed for a load concentration of 1
 129 $\mu\text{g}/\text{ml}$ at variable v , D_p , L_{col} , and r_p . D_{ax} was estimated as a function of
 130 v from correlations for beds packed with solid particles [26], using a dilute
 131 solution diffusivity of $7.5 \times 10^{-11} \text{ m}^2/\text{s}$ to represent general protein behav-
 132 ior [27]. Film mass transfer was assumed to never be rate-limiting, and k_f
 133 was set to $1 \times 10^{-3} \text{ m/s}$ to essentially remove this degree of freedom from
 134 all simulations. D_s was estimated as a function of K_{eq} using results re-
 135 ported for a mAb of the form $D_s = aK_{eq}^b$, where the power law coefficient
 136 $a = 1.66 \times 10^{-12} \text{ m}^2/\text{s}$ and $b = -0.24$ [28]. All simulation parameters are
 137 summarized in Supplementary Table S1.

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

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

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

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

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

150 2.3. Models for ion-exchange adsorption equilibria

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

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

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

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

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

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

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

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

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

178 **3. Materials and methods**

179 *3.1. Chemicals and buffers*

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

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

195 3.2. *Proteins*

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

203 3.3. *Resins and chromatography equipment*

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

211 10 cm Waters AP chromatography column to a final bed volume of 2.0 ml.
212 Column packing was validated at pH 7.0 by injecting a 100 μ l pulse of a 1 M
213 NaCl buffer and verifying that the conductivity trace asymmetry fell within
214 commonly accepted limits [19].

215 3.4. *Linear gradient elution*

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

233 *3.5. Breakthrough measurements during isocratic elution*

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

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

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

274 *3.6. Parameter estimation for test simulations*

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

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

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

304 4. Results and discussion

305 4.1. Breakthrough volume correlation

306 In the context of polishing operations, the value of flow-through IEX is its
307 ability to completely remove as many trace HCPs as possible from a mAb so-
308 lution. Challenges inherent in this task may not be readily apparent because
309 it is difficult to study the chromatographic behavior of dilute solutes em-
310 pirically. A column simulation was therefore used to investigate differences
311 between the breakthrough of overloaded products and dilute impurities. An
312 initial set of single-component simulations were performed at different feed
313 concentrations and K_{eq} values. Transport and geometric parameters were
314 held fixed, and a Langmuir isotherm with constant $q_{max} = 100$ mg/ml of
315 column was used. Results for extreme feed concentrations are juxtaposed
316 in Figure 1, and analogous plots for intermediate concentrations are shown
317 in Supplementary Figure S1. As may be expected for the system with a 10
318 mg/ml feed, retention increased with K_{eq} until the column saturated, and
319 breakthrough profiles became sharper as column saturation was approached.
320 This self-sharpening behavior did not occur for the 1 μ g/ml feed over the vol-
321 ume scale simulated, revealing dilute solute breakthrough profiles to become
322 more diffuse as retention increases due to the essentially linear isotherm. An
323 order-of-magnitude difference can be observed between the load volumes cor-
324 responding to the initial breakthrough and the inflection point in the more
325 strongly retained profiles. This illustrates an appreciable challenge; even if
326 trace impurities exhibit strong to moderate retention, their diffuse distribu-
327 tion on the column may lead to relatively early breakthrough and preclude
328 adequate clearance.

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

340 Further simulations showed that the slope of the $CV_{1\%}$ vs. K_{eq} curve for
 341 dilute solutes varies with transport and geometric parameters. This obser-
 342 vation indicates that the initial breakthrough volume may be approximately
 343 described as:

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

344 where $f_{1\%}$ is a function of transport parameters that modifies the break-
 345 through volume dependence on K_{eq} , with $0 \leq f_{1\%} \leq 1$. The form of this
 346 relationship has two implications: it suggests that thermodynamic and trans-
 347 port contributions to impurity breakthrough may be independent, and that
 348 transport contributions may be described by a single factor, which can pre-
 349 sumably be expressed generally in terms of a dimensionless group.

350 Based on this rationale, a second set of simulations were performed for
 351 a 1 $\mu\text{g}/\text{ml}$ feed. A variety of thermodynamic, transport, and geometric pa-
 352 rameters were used, and $f_{1\%}$ was computed from the results by rearranging

Equation 11. However, ε_c was used in place of the ε_t intercept when computing $f_{1\%}$ from Equation 11 because it was observed to better describe the simulated breakthrough volumes in the limit of $K_{eq} \rightarrow 0$, which may be attributable to finite transport rates limiting the solute exploration of intraparticle void volumes. Based on the dynamic binding capacity correlation presented by Chen et al. [36], $f_{1\%}$ is plotted in Figure 3 against a Graetz number for mass transfer:

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

which represents the ratio of characteristic time scales for intraparticle diffusion and axial convection. This may be alternatively written as $Gz_{eff} = (d_{part}/L_{col}) Péc_{p-eff}$, where the particle Péclet number $Péc_{p-eff} = vd_{part}/D_{eff}$ represents a ratio of convective to diffusive transport rates on the length scale of the resin particle.

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

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

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

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

387 An interesting subtlety that is implicit in this correlation is the effect
 388 of thermodynamics on intraparticle transport rates, which is described by
 389 the dependence of D_{eff} on K_{eq} . D_{eff} captures the combined effects of pore
 390 and surface diffusion in the dilute solute limit by describing the effective
 391 intraparticle diffusivity as a weighted sum, where K_{eq} weights the relative
 392 importance of surface diffusion. IEX surface diffusivities have been shown to
 393 follow a power law of the form $D_s = aK_{eq}^b$ [28], where the empirical power
 394 law coefficients a and b are expected to be on the order of D_p and -0.5 ,
 395 respectively, with $b < 0$ [37, 38]. This leads to competing effects: increasing
 396 K_{eq} decreases the surface diffusivity but simultaneously increases the driving
 397 force for surface diffusion, with the effect that dominates being determined by
 398 whether $b > -1$. If this is the case, increasing K_{eq} leads to an increase in D_{eff}

and a reduction in the Graetz number, meaning that both thermodynamic and transport contributions to delaying impurity breakthrough are improved simultaneously.

Equation 13 also enables estimation of the problematic K_{eq} threshold below which impurities will persist via weak adsorption. To gain a sense for the threshold’s order of magnitude, an illustrative calculation was performed 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 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 commercially available 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 [39]. 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

471 model proteins. To facilitate observation of system-independent trends, iso-
 472 cratic k' measurements at different ionic strengths were consolidated from the
 473 extant literature on 230 protein-pH-resin combinations, as shown in Supple-
 474 mentary Figure S2 and Table S2 [21, 30, 40–47]. These data were regressed
 475 according to Equation 9 to extract the SDM parameter ν and the quasi-
 476 SDM parameter $\phi\alpha$, which are plotted against each other in Figure 7 and
 477 observed to follow a significant intrinsically linear correlation (as determined
 478 by regressor t-tests).

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

494 The utility of these relationships lies in their ability to remove one degree
 495 of freedom from the analysis of retention data. A series of measurements

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

The linear gradient elution data were regressed according to Yamamoto’s method, and the SDM parameters are plotted in Figure 7. Analogous CEX gradient elution data for a mAb and its aggregates that had relatively large SDM parameters were also obtained from the literature and included in Figure 7 [48]. Both sets of gradient elution data are consistent with the correlation of isocratic CEX data within the 95% prediction interval. This confirms the correlation’s utility in removing a degree of freedom from the analysis of retention data. Unfortunately, it does not eliminate the need for some experimental data in estimating K_{eq} , but it may reduce the burden of doing so. As proteomic techniques advance, it may become possible to quantitatively measure HCP retention. This would foreseeably be an expensive operation,

521 and it may be essential to minimize the number of requisite measurements.
522 The SDM parameter correlation may be useful in such an application.

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

542 5. Conclusions

543 Unlike concentrated solutes, the approximately linear chromatographic
544 behavior of dilute impurities can lead to diffuse breakthrough. This can

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

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560 **Declaration of competing interest**

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

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574 Supplementary material

575 Supplementary material is associated with this article.

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