**Response to reviewers' comments – JCA-21-1423**

We thank the reviewers for their careful and thorough reading of the manuscript and for their constructive comments. Their insights have identified points that we did not make sufficiently clear in our original presentation; we have sought to address these in the revision. The reviews are reproduced verbatim below in blue and a response to each point is provided in black.

***Reviewer #1***

The authors investigated the separation behavior of proteins in flow-through chromatography. Lysozyme was chosen as a model impurity protein for the product monoclonal antibody. Models (methods) were developed for the process design. The paper is interesting and informative. However, I recommend the revision of the manuscript so that the reader can understand and use the proposed methods easily. My comments/questions are shown below.  
  
1) Most likely figure numbers were not properly converted when the PDF file was created (Fig ??)   
So I assumed the figure number based on the content of the text.

We apologize for this oversight. Although we checked the PDF document prepared by the Elsevier submission site, we apparently overlooked the LaTeX compilation omission that left the figure numbers indeterminate. We will check this more specifically on resubmission.

2) Unfortunately, Journal of Chromatography A does not allow the list of symbols. So it is essential to define and describe the mathematical symbols precisely. As you know, this is sometimes difficult as several different technical terms are used for describing a symbol. For example, epsilonC is defined as the column porosity, which is also called bed porosity or bed void fraction.

We have added a couple of clarifying qualifications, including the void fraction suggested by the reviewer and emphasizing the interstitial character of the quantity (p. 6).

3) L162-163 : V0 is the flow-through volume under non-adsorbing conditions.  
It is better to describe "V0 is the retention volume under non-adsorbing conditions."  
It is also helpful if epsilon\_t=V0/(total column volume) is shown.

We have renamed "the retention volume under non-adsorbing conditions" (pp. 9-10), and we also added to the in-line equation where is introduced (p. 10).

4) L204 SP Sepharose FF: nominal particle diameter should be given.

The nominal particle diameter of SP Sepharose FF has been added (p. 11).

5) L222 CV should be defined. This is somewhat confusing as CV is not dimensionless in this paragraph. The gradient elution volume was varied from 10 to 50 CV (L223). So CV seems the column volume [mL]. However, CV is dimensionless in Fig.1 and in Eqs.

CV was actually defined in the manuscript before this point (line 22), but the definition is now restated for clarity on p. 12. To address the second part of this comment, we note that "CV" is intended to represent a dimensionless measure of volume both in this paragraph and elsewhere in the manuscript. So the statement that the "gradient elution volume was varied from 10 to 50 CV" (previous line 223) signifies that the reported gradient elution volume was made dimensionless via normalization by the column volume. Although this nomenclature can raise questions, we believe it is used with sufficient frequency in the extant literature to be understood generally.

6) L328 Here, CVbreakthrough is dimensionless. The subscript breakthrough is ambiguous as CV1% or CV% also appears in the following parts.

As indicated, "CV" represents a dimensionless measure of volume everywhere in the manuscript. However, we have altered subscripts in Equation 11 to remain consistent with the 1% breakthrough discussion.

7) L339 The physical meaning of f is not clear. I am wondering if f is defined by the y-axis of Fig.3, (CV%-epsilon\_c)/[Keq(1-epsilon\_t)].

The introduction of the variable has been updated to be consistent with Equation 11, and the axes of Figure 3 have been updated for clarity. We did previously explain that " was computed from the result by rearranging Equation 11" (p. 17-18), but we hope that our modification of Figure 3 and the updated notation further clarifies this.

8) Eq.(12) : Graetz number Gz =(Dh/L)ReSc was originally introduced for describing the phenomena for a flow in a tube. It is of course possible to re-define Gz for the packed bed. However, for example, the particle Pe number is not used for a dimensionless group (udp/Dm), which is commonly used as the reduced velocity.  
So I recommend to describe "Graetz number like dimensionless group" (vdp^2/DeffLcol).

We describe as "a Graetz number for mass transfer", which is an established dimensionless group in the transport literature. However, to improve clarity and provide a better physical interpretation, we have modified Equation 12 and the surrounding text to emphasize the direct physical origins of the Graetz number as a ratio of meaningful characteristic times for transport. In so doing we have in fact de-emphasized the use of the particle Peclet number, which, although widely used, has less physical significance in the present context.

9) Eq.(13) : f%(vdp^2/DeffLcol) is most likely the value of y-axis in Fig.3 as a function of (vdp^2/DeffLcol).

This is the same issue raised by the reviewer’s comment 7, which we have sought to address by making all notation consistent with the discussion of 1% breakthrough and modifying the axis labels of Figure 3.

10) Fig. 1: This figure illustrates how the breakthrough curve shape changes with 1/(1+KLcp), which is sometimes called separation factor [ Eq. 16-33 Perry's Handbook 7th ed.] So for (A), Keq=10 when KL=10 and 1/(1+KLcp)=0.01 (highly favorable). For (B) almost all curves are linear isotherms. For example, Keq=990 when KL=10 and 1/(1+KLcp)=0.9 (almost linear). Other parameters used for simulations should be given.

The purpose of Figure 1 is to illustrate simply qualitative differences between the breakthrough of concentrated and dilute solutes. However, we agree with the reviewer's use of separation factors to characterize this phenomenon and have included such characterization in the text (p. 16). We have also added Supplementary Table S1 to summarize all simulation parameters, and we direct the reader’s attention to this with a sentence on p. 8, as well as brief statements in the captions of Figures 1-3.

11) Fig. 2: It is difficult to understand the meaning of two horizontal curves.  
I wonder if Keq>2000 is practically possible.

The horizontal lines represent the hypothetical load volumes required to saturate the column in the absence of transport limitations; the caption of Figure 2 has been revised to clarify this interpretation. Values of up to and beyond 10000 are certainly encountered in IEX chromatography, but the reviewer's reference to "practically possible" may refer to measurement of under isocratic conditions, in which case we are in agreement.

12) Fig. 3: The y-axis (CV%-epsilon\_c)/[Keq(1-epsilon\_t)] is approximately equal to (breakthrough volume)/(equilibrium volume). There is no explanation on this in the text.

The reviewer is generally correct, albeit with a couple of caveats, in this physical characterization and we agree that it is useful to include it; we have therefore added a mention of it on p. 18.

13) Fig. 5: (A) is for approximately linear isotherms whereas (B) is for non-linear isotherms.

As we indicated in the caption, Figure 5 depicts differences between “(A) non-adsorbing (high ionic strength) conditions and (B) adsorbing (low ionic strength) conditions at different superficial velocities.” As described on pp. 13 and 20-21, a dilute solute was used at both of these conditions, such that the isotherm should be approximately linear. We therefore respectfully disagree that the conditions represented by Figure 5 (B) reflect a nonlinear isotherm.

14) Fig. 8: Parameter values for three curves should be given.

We have added the fit and parameters that were obtained by regressing the data in Figure 8 to the figure's caption, along with the parameters corresponding to the dashed grey correlation lines of closest fit.

***Reviewer #2***

In this work the behaviour of weakly adsorbing impurities in flow-through chromatography on ion exchangers is analysed using correlations based on thermodynamic and mass transport parameters as well as adsorption equilibria as a function of ionic strength derived from isocratic retention and linear gradient elution data. The work and the suggested methodology represents an elegant and interesting method to estimate the breakthrough behaviour of host cell proteins in flow through chromatography.

Some points should be addressed here and included in a revision of the paper:  
- A weak point is that the experimental verification was performed using a highly unlikely purification scenario. Lysozyme has an isoelectric point of 11. As the author mentioned in the introduction most host cell proteins are acidic. In their examples conditions are chosen where mAb is not bound to the cation exchanger, like most other host cell proteins would not do at these binding conditions. In essence such a scenario does not make much sense for practical purposes.

We thank the reviewer for raising this point. We tried using several model proteins to demonstrate the breakthrough volume correlation with AEX resins but we found that, in the dilute limit, such a demonstration requires extremely pure feedstocks. Lysozyme was the only protein that we found to be commercially available in sufficient purity or sufficiently purifiable in our own lab to enable the demonstration of dilute single-component behavior. This is why our data are for lysozyme in CEX despite the fact that flow-through CEX is not a likely purification scenario for mAb processing. We have added the following statement on pp. 20-21 to clarify this:

“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.”

- line 133: film mass transfer was set to 1x10-3 m s-1. This is an extremely fast film mass transfer. I wonder if this is justified, especially for the experiments with low velocities.

We had acknowledged in the original manuscript that "film mass transfer was assumed to be relatively fast". In our experience, film mass transfer is very rarely rate-limiting, so we used a high value to essentially remove one degree of freedom from the simulations. We have modified the text to clarify this rationale (p. 8) as follows:

“Film mass transfer was assumed not to be rate-limiting, and was consequently set to m/s to remove this degree of freedom from all simulations.”

- line 348: Why do the authors suppose that when using eps-c instead of eps-t the fit was better? Please comment. I assume eps-t should represent the total porosity (bed porosity), it is not defined in the text

We have expanded the definition of , on p. 10 in the original, to accommodate Reviewer 1’s suggestion to include the ratio /. We believe that the improvement observed when using the intercept in place of may be attributable to finite transport rates limiting the solute exploration of intraparticle void volumes; we have added a statement to this effect on p. 18. In response to Reviewer 1's comments we have also expanded the discussion of the use of the two porosities more generally (p. 6)

-line 387.: more information on the significance and the range of exponents b and a should be provided in the discussion

As the dependence on is still a relatively novel finding, there is little literature from which to discuss the physical significance or customary ranges of the power law parameters and , but we have modified the discussion on pp. 19-20 to emphasize that they have been used as empirical fitting parameters. We have also provided orders of magnitude that we expect for these parameters, and we have added two relevant references (38 and 39) to support this discussion.

- Supplementary Figure S2 as it is presented is just a collection of lines without information beyond the fact that retention data can be fitted using the model for linear gradient elution. Data for resins, proteins, equilibrium constants etc. must be provided in a Table.

We agree with the reviewer's suggestion that publication of tabulated data would be useful, despite the fact that we have consolidated it from the literature by digitizing plots, which decreases the data precision. We have added a spreadsheet to the Supplementary Information to make these data accessible. With this addition, we feel that Supplementary Figure S2 not only demonstrates the fitting process, but it also shows the scope and qualitative trends of the consolidated data, albeit without legend entries indicating protein-pH combinations.

***Reviewer #3***

The manuscript JCA-21-1423 entitled »Behavior of weakly adsorbing impurities in flow-through ion-exchange chromatography« submitted to Journal of Chromatography A by Herman et al. provides an in-depth analysis of weakly adsorbing impurities. This is extremely important topic considering increased number of processes operating in a flow-through mode implementing, in many cases also convection based media. The manuscript contains novel very interesting data and results. Besides, it is well structured and clearly written. Therefore I do recommend it for publication after some minor modifications.

There was recent publication from Trnovec et al. (doi: 10.1016/j.chroma.2019.460518) investigating experimentally HCP break-through on membranes, also implementing Yamamoto's model. It would be interesting to include this data in analysis.

We thank the reviewer for making us aware of this work - we have added a reference to this article (13) in the introduction. However, we feel that the inclusion of Trnovec et al.’s data in this manuscript would be misguided, as we have primarily sought to consolidate isocratic retention factor data in this work, not linear gradient elution data (which have been produced by multiple studies). Doing so would perhaps necessitate an expansion of the manuscript’s scope, which would certainly be interesting, but we feel that it is unnecessary for communicating the essential features of our analysis.

It would be useful many to add short paragraph at the end of manuscript with protocol how to implement described approach described in industry.

We agree that a statement of how the approach and results presented here can be used in practice would be informative to the reader. We have, in fact, sought to do that in the last part of the Conclusions section, where the key quantitative information is the estimate of the threshold value of *Keq* for which premature breakthrough might be expected. In order not to make unrealistic claims, however, we also discuss there the challenges of determining *Keq* values for an appreciable number of impurities. Nevertheless, we hope that the overall mechanistic analysis presented in this manuscript may be used to better understand the chromatographic behavior of flow-through impurities generally.

Also, there was probably problem with formation of PDF since many figure numbers are missing in the text (e.g. Figure ??). Please verify.

Yes, as noted in our response to Reviewer 1, we experienced a LaTeX compilation problem that removed figure numbers inadvertently, and this has been addressed.