**Effect of flow behavior in extra-column volumes on the retention pattern of proteins in small columns**

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**Highlights**

* Deformation of protein peaks in extra-column volume (ECV) was indicated
* ECV effect stems from radial velocity distribution and slow protein diffusivity
* ECV effect influences on the protein retention on a small column
* Mathematical model was used to predict the extra-column volume effect

**1. Introduction**

Columns with very small volumes, i.e., maximum 1 mL, are often used in the development stage of protein chromatography to estimate operating parameters for large stale operations at minimal material consumption. Small columns are exploited in high throughput experiments to acquire a huge number of chromatographic elution data that are subjected to statistical analysis to determine the bounds for the process operating window as well as characterize the process dynamics and provide optimum of operating conditions with respect to the process performance. However, reduction in the column size causes an increase in the ratio of the extra-column volume (ECV) in the workstation to the column volume. Therefore, band broadening in ECV can significantly affect the separation efficiency. In this study, the experimental and theoretical analysis of deformation of band profiles in ECV was performed, and its influence on the retention pattern of proteins in a small chromatographic column was quantified [1].

**2. Methods**

A few model proteins were used for the elution experiments: lysozyme, LYZ; bovine serum albumin, BSA; monoclonal immunoglobulin, IgG4; fibroblast growth factor, FGF2. Additionally, blue dextran as a representative of large macromolecules, and acetone as a representative of small-molecule compounds, were used. The column was packed with a cation exchange resin UNOsphere S (Bio-Rad Laboratories, Hercules, CA, USA, particle diameter 80 μm). The mobile phase was a phosphate buffer pH = 7 free of salt or with 1M NaCl in the solution. Two injection systems were used: a superloop and an injection loop capillary.

**3. Results and discussion**

The effect of the presence of ECV on shape of band profiles resulted from non-uniform velocity distribution in the radial direction and slow diffusivity of proteins. The phenomenon vanished for a small molecule compound, and it was enhanced with increasing molecular weight of the model compound. The difference in flow behavior of the macromolecule and small-molecule compounds caused them to migrate with different velocities in ECV, which resulted in partial separation of their bands. To describe the elution profiles in ECV and in the column, a mathematical model was used, which accounted for nonideality of the flow pattern. The model reproduced accurately band profiles of macromolecules within a range of relatively low velocities, typical however for protein chromatography.

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|  | Fig. 1. **Experimental band profiles and the corresponding model simu-lations for small-volume injections for** proteins and acetone. A) in ECV; B) in the column. |
| Fig. 2. Band profiles in ECV | Fig. 3. Band profiles at the column outlet |
| Experimental band profiles **and the corresponding model simulations** for large-volume injections. A) Fronts of breakthrough curves for the proteins and acetone injected through the loop capillary; B) comparison of the IgG bands for the injections through the loop capillary and the superloop. |

Typical experimental data and the model simulations for small pulse injections recorded in the chromatographic system with and without presence of the column are shown in Figures 1A and 1B respectively. The protein peaks are characterized by deformations which enhance with increase in molecular weight of the protein. Band profiles obtained for large injections are shown in Figs 2 and 3. They exhibit strong asymmetry of the fronts with a slow concentration transition in their upper part. The courses of the concentration decay in the desorption parts of the band profiles obtained by injections using the loop capillary and the superloop differ markedly. In case of the superloop injections, peak tailing is markedly reduced compared to the loop capillary injections. This stems from the differences in the elution pattern of proteins in both injections systems.

**4. Conclusions**

The ECV effect contributed strongly to the elution behavior of macromolecules in the small column. Peak deformation was observed for small-volume injections and asymmetry of breakthrough curve fronts for large-volume injections. In the latter case, the system performance was higher for the superloop injections compared to the loop capillary injections. Neglecting the EVC effect may result in wrong interpretation of the protein retention mechanism.

**References**

[1] W. Marek, D. Sauer, A. Dürauer, A. Jungbauer, W. Piątkowski, D. Antos, J. Chromatogr. A, 1566 (2018), 89–101.

[2] K. Baran, W. K. Marek, W. Piątkowski, D. Antos, *Effect of flow behavior in extra-column volumes on the retention pattern of proteins in small columns*, J. Chromatogr. A, submitted.

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