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ORIGINAL

Flow-Reversal Experiments with Macromolecules to Measure Column End Efficiency and Bed Heterogeneity

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Abstract

A flow-reversal method combined with peak parking has been introduced recently to determine the band broadening occurring at the two respective column ends and in the bed of the packing material. Flow-reversal has a peak compression effect, therefore, the peaks observed are always narrower and more symmetrical than the peaks obtained without reversing the flow. This phenomenon can originate from the compensation of the multipath dispersion effects. In the present study, peak parking and flow-reversal measurements were extended to macromolecules and carried out with human insulin. We observed that the peaks of insulin are always narrower with reversed flow than without reversing the flow, and the compression effect can be significantly larger than it is for small molecules. The contributions of the column inlet and outlet to the total band variance have been characterized.

Keywords Peak parking · Arrested flow · Axial heterogeneity · Column end structure · Flow-reversal

Introduction

In liquid chromatography, flow-reversal—also known as bi-directional flow—measurements have been used for various reasons, for instance for the determination of the local plate-height, for the elution of strongly retained impurities, or for the local characterization of the chemical modification of the stationary phase [1–5].

Recently, Lambert et al. used the peak parking method to measure the effective diffusion coefficient (D_{eff}) for the estimation of longitudinal band variance combined with the flow-reversal method with an unretained compound (thiourea) to characterize the sample band broadening in the chromatographic column and for showing the differences between the two respective column ends [6].

In the aforementioned study, it was observed that flow-reversal has a peak compression effect, therefore, the peaks detected after reversing the flow are always narrower and more symmetrical than the peaks obtained without reversing the flow. The origin of this phenomenon is the velocity biases observed in packed columns.

Wong et al. [7] presented results of experiments with downward slurry-packed chromatography columns to measure the axial heterogeneity of the packed bed. They found, that the most poorly packed region of the bed was the column inlet section, irrespective of the bed length.

Gritti et al. [8] designed a custom-made low-dispersion system made of two small-volume optical detection cells placed immediately before and after the column. They applied the flow-reversal technique to determine radial and axial structural heterogeneities of packed chromatographic beds. A rather significant peak refocusing was observed when reversing the flow direction which confirms the existence of long-range velocity biases in packed chromatographic columns.

Gritti and Gilar studied the impact of frit dispersion [9]. They found that in gradient elution chromatography, the outlet frit has a serious limitation on the achieved efficiency.

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In the present study, peak parking and flow-reversal measurements were carried out with a macromolecule, human insulin. A small molecule, such as thiourea can diffuse easily from a fast flow channel to a slow one, however, proteins usually have small diffusivity, thus a different behavior may be expected. When macromolecules are used for peak parking and flow-reversal experiments, increased parking time and rather small flow-rate should be used to study the axial heterogeneity of packed columns.

Theory

Band variance is defined and used in various units in separation science. The second central moment (μ_2) of the peaks (band variance) can be measured on time scale, which yields the temporal variance (σ_t^2). The longitudinal or spatial variance (σ_z^2) determines the band spreading in the column. It can be calculated from the σ_t^2. The extra-column contributions are usually characterized with the volumetric variance (σ_V^2), which is calculated for a peak as

$$\sigma_V^2 = \sigma_t^2 \, F_V^2 \tag{1}$$

It should be noted that the band variances must be corrected for the effect of the extra-column band broadening before the calculations of the flow-reversal experiments. We want to confirm this theory with molecules larger than thiourea, therefore, we used insulin during our measurements. The various contributions to the observed band broadening are thus written as

$$\sigma_{t/V}^2 = \sigma_{\text{system}}^2 + \sigma_{\text{park}}^2 + \sigma_{\text{end}}^2 + \sigma_{\text{bed}}^2 \tag{2}$$

where σ_{park}^2 is the band broadening originating from the diffusion during the peak parking period, σ_{end}^2 comes from the inhomogeneity and from the mixing effect at the column ends while σ_{bed}^2 comes from the broadening of the peak as it passes through the homogeneous column bed. When the moments of the recorded peaks are corrected for the extra-column broadening, we get the following relationship

$$\sigma_{t/V}^2 = \sigma_{\text{park}}^2 + \sigma_{\text{end}}^2 + \sigma_{\text{bed}}^2 \tag{3}$$

As the analyte migrates in the column, its band variance increases linearly with the distance traveled. The longitudinal variance is expressed as

$$\sigma_{\text{bed}}^2 = Hz \tag{4}$$

The increase of the longitudinal band variance during the parking period (t_{park}) can be expressed as follows:

$$\sigma_{\text{park}}^2 = 2D_{\text{eff}} t_{\text{park}} \tag{5}$$

where D_{eff} is the effective diffusion coefficient. When the observed band variance is plotted against the penetration distance, a straight line is obtained, and the local plate height of the homogeneous packing bed is determined from the slope of that line. The intercept of the line gives the combined contributions of peak parking and column ends [6].

$$\sigma_{z,\text{app}}^2 = \sigma_{z,\text{end,app}}^2 + 2D_{\text{eff}} t_{\text{park}} + 2Hz \tag{6}$$

Experimental

Instrument and Chemicals

Chromatographic analyses were performed with a Shimadzu UFLC XR equipment. The system consisted of solvent delivery units, an automsampler, a column oven, a degasser and an UV-VIS detector (at 220 nm), and a computer data station with LCsolution software. In this study, a reversed phase Kinetex (C_{18}) column (4.6 × 100 mm, packed with 2.6 µm core-shell particles) was tested, which was provided by Phenomenex (Torrence, CA, USA). During the measurements, the mobile phase was a mixture of acetonitrile and water, both HPLC grade (VWR International), furthermore, trifluoroacetic acid (Sigma Aldrich) additive was used.

The human insulin samples were prepared from analytical standard purchased from Sigma-Aldrich. For the peak parking and flow-reversal experiments, 65% acetonitrile and 35% water with 0.1% trifluoroacetic acid was used as eluent. The flow-rate was set to 0.05 mL/min, and the column was thermostated at 45 °C . The injection volume was 1.0 µL, while the concentration of the insulin sample was 0.5 mg/mL.

Taylor–Aris Disperson of Macromolecules

Since the molecular diffusivity of insulin is about an order of magnitude smaller than that of small molecules, we had to decrease the flow-rate significantly (from 0.2 mL/min to 0.05 mL/min), to observe Taylor–Aris dispersion [10, 11] of insulin in the connecting capillaries. When the flow-rate is too high, the insulin molecules will have no time sample the entire cross-section of the tubings, thus the extra-column band broadening will be much larger than it should be. The following equation limits the flow velocity when Taylor dispersion should be observed [12]:

$$t_{\text{capillary}} < \frac{d^2}{D_m} \tag{7}$$

where $t_{\text{capillary}}$ is the residence time of the analyte in the capillaries, d the diameter of the capillaries and D_m the molecular diffusion coefficient [12–14]. On the Shimadzu equipment, the inner diameter of the capillaries is $d = 0.18$ mm,

thus $t_{capillary} < 110$ s should hold. Therefore, we established a $F_v = 0.05$ mL/min flow-rate with a 120 s measured extra-column residence time.

Furthermore, longer parking time periods should be used for peak parking and flow-reversal experiments too. When using insulin, the parking time was at least 60 min, and if larger proteins (for instance BSA) are used, the parking time should be still further increased.

It should also be noted that the principles of spreading of a sample peak in a long straight open tube is studied here. As the tubing becomes shorter than 30 theoretical plates, though, the eluted peak turns into non-gaussian and the theory for long tubes will not apply [15].

Peak Parking Experiments

Knox and McLaren [16] introduced the peak parking (PP) method, also known as arrested flow method. After the injection of the analyte, the eluent is pumped under isocratic conditions until the sample reaches the middle of the column. Then, the mobile phase flow is arrested for a time called parking time (t_{park}), during which the sample band diffuses in the axial direction of the column. When t_{park} has passed, the elution is resumed until the sample leaves the column.

From the band broadening occurring during t_{park}, the effective axial diffusion coefficient can be calculated. If we plot σ_z^2—which is derived from μ_2—against t_{park}, a linear correlation is observed. The slope of this straight line gives D_{eff} based on Eq. 5 [17, 18].

The Flow-Reversal Method

The flow-reversal method has been used to test the bed heterogeneity at the column ends. The method includes the stopping of the mobile phase flow when an unretained marker has migrated a given penetration distance into the column, and the reversal of the column under the parking time when the flow is arrested. When the flow has been restarted, the sample elutes at the same column end where it entered the column. Due to the specificity of the column packing procedure, one can suppose that the inlet and the outlet ends of the columns represent a different structure and efficiency, hence experiments were performed to test either the column inlet or the outlet, and the σ_{end}^2 variance was determined based on Eq. 6 [6].

Calculations

The second central moments of the observed peaks were used for the calculations of variances. The temporal second moments (σ_t^2) were determined by fitting an exponentially modified Gaussian (EMG) peak to the experimentally recorded peaks. The moments were corrected for the extra-column effect ($\sigma_{system}^2 = 500\ \mu L^2$), which was determined by replacing the column with a zero dead-volume union. The variances obtain during the flow-reversal experiments were expressed in μL^2 (σ_V^2).

Results and Discussion

Molecular Diffusivity of Insulin

There are several empirical equations to estimate the molecular diffusivity of molecules. For globular proteins surrounded with a hydrate shell, the correlation derived by Young et al. can be used [19]:

$$D_m = 8.34 \times 10^{-8} \frac{T}{\eta M_w^{1/3}} \tag{8}$$

where T is the absolute temperature, η the viscosity of the mobile phase expressed in cP and M_w is the molecular weight of insulin. For human insulin, this value is $D_m = 2.94 \times 10^{-6}$ cm^2/s under the current experimental conditions.

Measurements of D_{eff} with Peak Parking Method

The peak parking method permits the measurement the effective molecular diffusion coefficient. After the insulin is injected into the column, the sample zone is arrested in the column, and during the parking time insulin is let free to diffuse. While small molecules, such as thiourea need a short time (1, 2, 5, 10 min) to diffuse during peak parking experiments, insulin needs much more time to diffuse to a similar extent. Thus, longer parking time periods must be used for both peak parking and flow-reversal experiments. When we work with long parking times (60, 120, 180, 240 and 300 min) as we can see in Fig. 1, the band broadening of the peaks increases linearly with increasing parking time.

Using the peak parking method, the effective diffusion coefficient of insulin was determined from the slope of the straight line plotted in Fig. 2: $D_{eff} = 1.6 \times 10^{-6}$ cm^2/s. From this value, we calculated $\sigma_{park}^2 = 50.3\ \mu L^2$ for $t_{park} = 60$ min.

Flow-Reversal Experiments

The column heterogeneity was tested at the respective column ends with flow-reversal. After insulin was injected and the sample zone migrated a given distance into the column (4, 5, 6, or 7 cm distance from the column inlet or outlet, respectively) the flow of the mobile phase was stopped for 60 min. When the pressure has relaxed, the column was reversed and after a 60-min parking time the flow was restarted and the sample zone eluted at the same end as it entered the column.

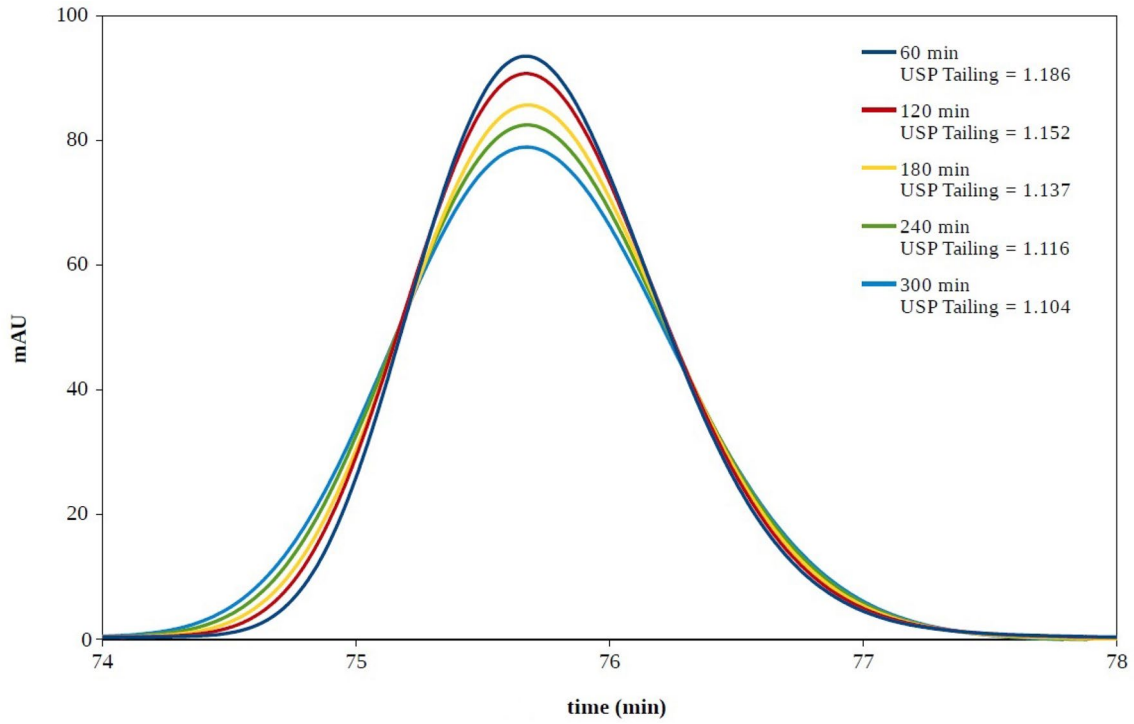

Fig. 1 Peak parking experiments. The peak shapes of insulin observed with the elution in backward flow direction including 60, 120, 180, 240 and 300 min arrested flow using water:acetonitrile 65:35% (v/v) as mobile phase at 0.05 mL/min flow-rate. The peaks are normalized to 60 min parking time

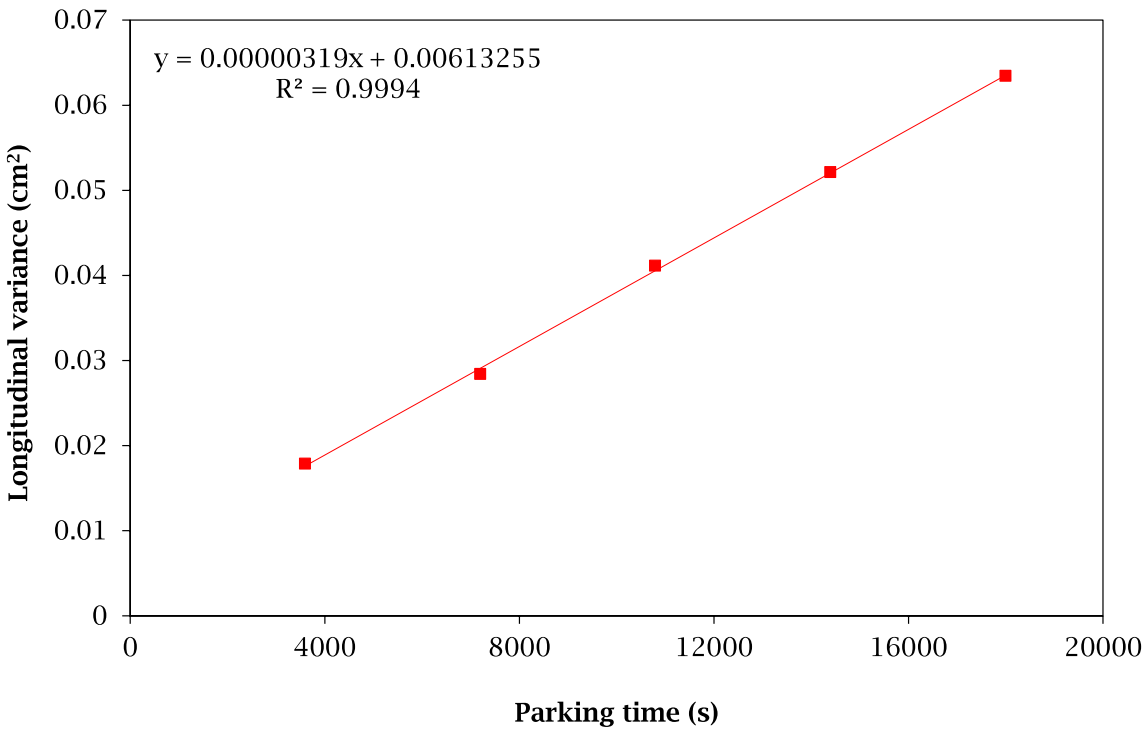

Fig. 2 The change of the longitudinal variance of the insulin peak with increasing parking time. The slope of the fitted straight line gives $2D_{\text{eff}}$

As it can be seen in Fig. 3, with flow-reversal method we noticed slightly narrower and more symmetrical peaks than without reversing the flow and including $t_{park} = 60$ min arrested flow in both cases. Although both peaks are fairly symmetrical in Fig. 3, the USP tailing factor is slightly smaller when the flow is reversed. The peak compression effect due to flow-reversal seems to be small in Fig. 3, but one should note that the relatively large extra-column peak broadening contribution of the equipment ($\sigma^2_{V,system} = 500\ \mu L^2$) makes the comparison difficult.

The band compression effect of flow-reversal becomes obvious in Fig. 4 where the results have been corrected for the system contribution. If the flow is stopped when the sample zone migrated 5 cm into the column, the observed variances are much smaller if the flow is reversed after the parking time. For instance, when the column inlet is investigated, the variance is $\sigma^2_V = 139.9\ \mu L^2$ if the flow is simply resumed after $t_{park} = 60$ min parking. The variance drops to $\sigma^2_V = 106.7\ \mu L^2$ if the flow is reversed before resuming it. In a similar manner, when the column outlet is studied, the variance is $\sigma^2_V = 110.6\ \mu L^2$ if the flow is simply resumed after parking and it drops to $\sigma^2_V = 75.6\ \mu L^2$ if the flow is reversed before resuming it. These numbers yield 24–32% focusing effect due to flow-reversal for insulin.

The intrinsic, local plate height for the homogenous column bed was determined from the slope of the fitted straight lines in Fig. 4 using Eq. 3. We found that $H_{forward} = 7.4\ \mu m$ is larger than $H_{backward} = 5.3\ \mu m$. That difference creates a slightly better efficiency when the column is used in the flow direction opposite to what the manufacturer suggests.

From the intercepts of the lines in Fig. 4, we calculated the variances for the column inlet ($\sigma^2_{V,end,in}$) and outlet ($\sigma^2_{V,end,out}$). The results are summarized in Table 1 as well. There is a difference between the inlet and outlet variances; the inlet end variance is 10 μL^2 larger than the outlet end variance. That difference demonstrates the effect of the column end structure and the difference in packing quality near the respective frits.

When looking at the overall variance, one can state that the homogenous packed bed gives one third and the two column ends the other two thirds of the total band broadening.

Conclusion

The insulin peaks observed after flow-reversal are definitely narrower and more symmetrical than the peaks obtained without reversing the flow. For small molecules, the band compression effect was found to be less [6, 8]. The molecular diffusivity of macromolecules is much smaller than that

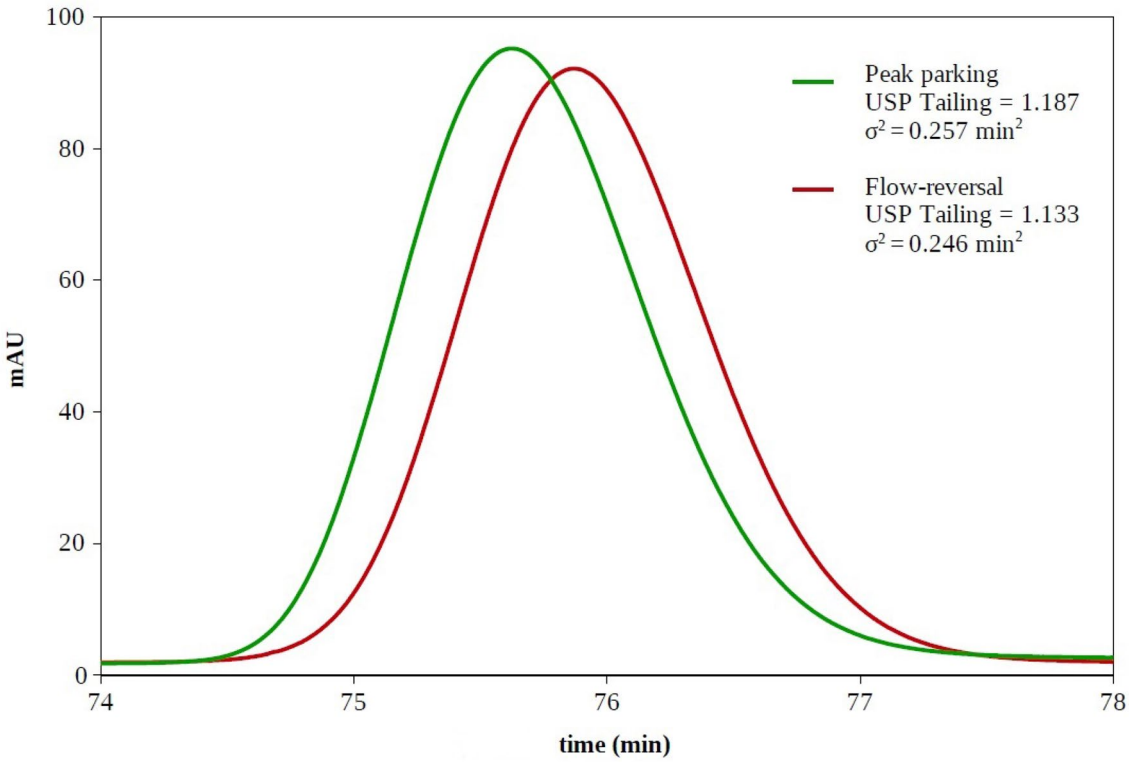

Fig. 3 Comparison of the peak shapes of insulin observed with and without flow-reversal, including 60 min arrested flow in both cases

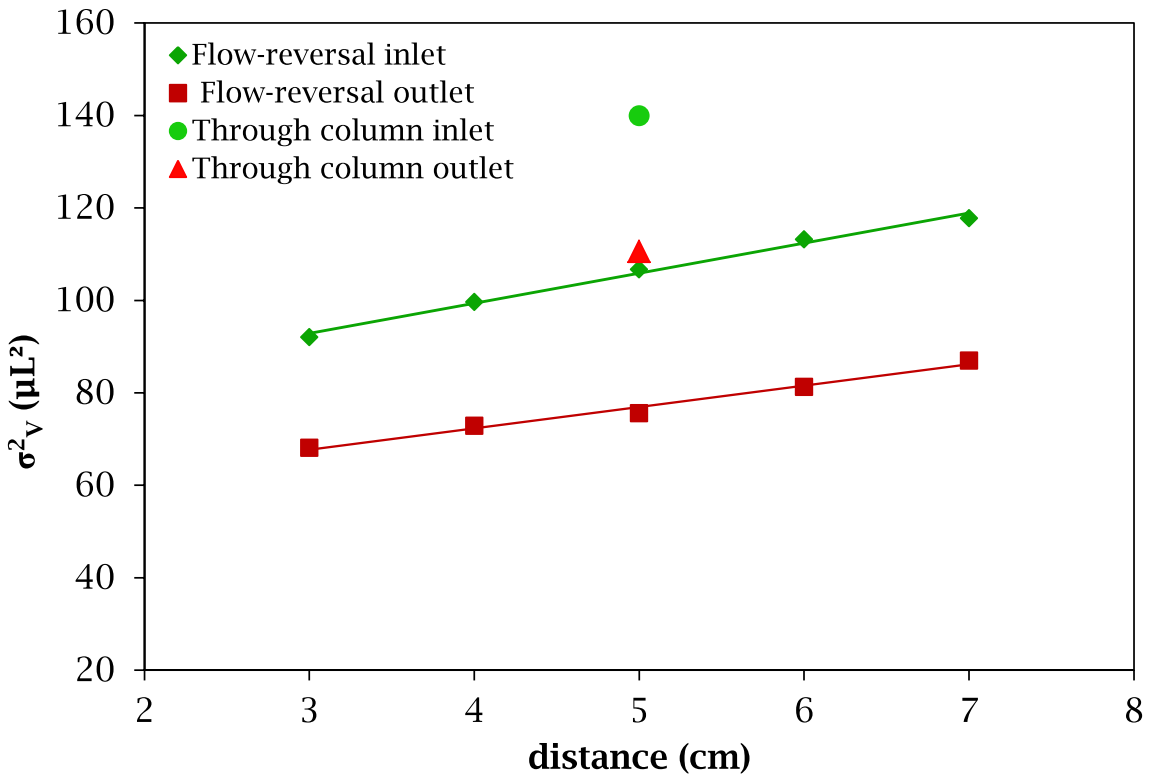

Fig. 4 Volumetric variances obtained with flow-reversal when the sample band was stopped at 3, 4, 5, 6, 7 cm distance from the column inlet or outlet, respectively. In each case, the flow was arrested for 60 min

Table 1 Volumetric variances obtained for the various band broadening contributions

	Inlet	Outlet
σ^2_{total}	639.9 µL^2	610.6 µL^2
σ^2_{column}	89.9 µL^2	60.6 µL^2
$\sigma^2_{V,\text{end}}$	28.5 µL^2	18.6 µL^2
$\sigma^2_{V,\text{bed}}$	32.6 µL^2	23.1 µL^2
H	7.4 µm	5.3 µm

$\sigma^2_{V,\text{park}} = 50.3$ µL
$\sigma^2_{V,\text{system}} = 500$ µL

of small molecules. Thus, the radial heterogeneity of the packed bed influences in a lesser extent their migration and consequently a larger band compression effect can observed for macromolecules when the flow is reversed.

With the flow-reversal experiment, we could show the differences between the two respective column ends and also between the local plate heights of the homogeneous column bed when using in forward or backward direction after injecting macromolecules.

For the investigated Kinetex column, the local plate height is somewhat smaller (5.3 µm) when we use the column against the recommended flow direction (7.4 µm) and the inlet of the column (fitting, frits and column end structure) has a larger contribution to band variance than the outlet of the column. Most probably that difference is due to the column packing procedure, since at the vicinity of the respective column ends the particle density and structure must be different. Furthermore, the band broadening caused by the two column ends is larger than the band broadening in the homogenous bed.

Finally, due to the Taylor–Aris dispersion, the experimental conditions should be significantly altered compared to the studies with small molecules, thus the experiments are much lengthier than with the thiourea or other unretained markers with small molecular weights.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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