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Short communication

The determination of the plate count for large molecules under reversed-phase gradient conditions

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ABSTRACT

The true performance of HPLC columns in the gradient separation of macromolecules can be assessed by measuring the true plate count, if we know the retention factor of the analyte at the point of elution. We are demonstrating in this short communication how this can accomplished in a straightforward fashion. The procedure used here is a significant simplification over previous approaches, and enables the chromatographer to do so without complex algebra or additional experiments.

1. Introduction

In isocratic reversed-phase (RP) chromatography of small molecules, it is rather straightforward to measure the theoretical plate height and the plate count. For large molecules, the retention factor under isocratic conditions is difficult to control in RP separation. This is due to the rather steep dependence of the retention factor on solvent composition. The problems worsen with increasing molecular weight of the analyte. It is still possible to measure isocratic retention times and plate counts for insulin (MW 5808) [1], but this task becomes impossible for much larger proteins. Also, this is not a problem for other techniques, such as ion-exchange [2], hydrophobic-interaction [3], or for techniques without retention, such as size-exclusion chromatography [4].

Due to the fact that an isocratic determination of plate height and plate count in RP chromatography is at least very troublesome, if not impossible, for very large molecules, most authors decided to measure the column performance under conditions where the analyte is not retained on the column (e.g. [5]). As an alternative, one can resort to model calculations [6]. However, one would like to get true plate height information under conditions of retention in RP chromatography, since this is the essence of the interaction of the analyte with the packing material. Such data will enable us to compare the mass transfer properties of different stationary phases to each other.

A procedure for such a determination was outlined in Ref. [7], and our current publication is an expansion and significant refinement of the original idea. It avoids the calculation of the protein-specific slope of the logarithmic retention factor versus the solvent composition from a non-linear equation, and gives a simple and direct view of the evolution of peak width and protein plate height with retention.

2. Theory

The theoretical plate height H is defined as the variance of the peak σ^2 (in length units) at column outlet divided by the column length L:

$$H = \frac{\sigma^2}{L} \tag{1a}$$

Thus the plate count is

$$N = \frac{L}{H} = \frac{L^2}{\sigma^2} \tag{1b}$$

The chromatogram is recorded in time units, and the peak standard deviation in length units can be calculated from the peak standard deviation in time units σ_t, if the migration velocity of the peak at the column outlet u_m is known:

$$\sigma = \sigma_t \cdot u_m \tag{2}$$

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This migration velocity depends on the true retention factor at the point of elution k_e:

$$u_m = \frac{u_0}{k_e + 1} = \frac{L}{t_0} \cdot \frac{1}{k_e + 1} \quad (3)$$

We substitute Eqs. (2) and (3) into Eq. (1b) to obtain:

$$N = \frac{t_0^2}{\sigma_t^2} \cdot (k_e + 1)^2 \quad (4)$$

The elution time of a peak and its peak width in time units can be translated into volume units simply by multiplication with the flow rate F:

$$V_0 = t_0 \cdot F \quad (5a)$$

$$\sigma_v = \sigma_t \cdot F \quad (5b)$$

$$N = \frac{V_0^2}{\sigma_v^2} \cdot (k_e + 1)^2 \quad (5c)$$

where σ_v is the peak standard deviation in volume units and V_0 is the retention volume of the protein under unretained conditions.

We note that the peak widths and the elution time or volume under conditions of no retention are readily measurable. For the determination of the true plate count under gradient conditions, the knowledge of the retention factor at the point of elution, k_e is missing. This value, however, is accessible from the theory of gradient elution, and this will be shown in the next few lines.

For a linear RP gradient, the (true) retention factor at the point of elution k_e is [8,9]:

$$k_e = \frac{k_0}{G \cdot k_0 + 1} \quad (6)$$

where k_0 is the retention factor of the analyte at the beginning of the gradient, and G is the generalized gradient slope. For large molecules such as proteins (and a few other conditions) k_0 is typically very large, and the equation for k_e simplifies to:

$$k_e = \frac{1}{G} \quad (7)$$

The generalized gradient slope depends on the execution of our gradient:

$$G = B \cdot \Delta c \frac{t_0}{t_g} = B \cdot S \quad (8)$$

Δc is the difference in the solvent composition over the gradient, t_0 is the retention time of an unretained peak, t_g is the gradient run time, and B is the slope of the relationship between the natural logarithm of the retention factor of the analyte and the solvent composition. We program the gradient parameters $S = \Delta c \cdot t_0/t_g$, but we do not know the factor B, which is compound specific.

With Eqs. (7), (8) and (5c), we can write down the relationship between the gradient conditions and the measured peak width:

$$\sigma_v = \frac{V_0}{\sqrt{N}} \cdot \left(\frac{1}{B \cdot S} + 1\right) \quad (9a)$$

$$\sigma_v = \frac{V_0}{\sqrt{N}} \cdot \frac{1}{B \cdot S} + \frac{V_0}{\sqrt{N}} \quad (9b)$$

Therefore, if we plot the peak standard deviation (in volume units) versus the inverse of the known gradient slope, we will get a straight line with an intercept of $V_0/\sqrt{N}$ and a slope of $V_0/\sqrt{N} \cdot 1/B$. Dividing the first value by the second value gives us the protein-specific value B, whose knowledge in turn permits a calculation of the true protein plate count as a function of the gradient execution G via Eq. (10):

$$N = \frac{V_0^2}{\sigma_v^2} \cdot \left(\frac{1}{G} + 1\right)^2 \quad (10)$$

Eq. (9) is a simplification over procedures used by us in the past [7] or published in textbooks [8]. It permits a direct observation of the evolution of the peak width without an independent determination of the protein-specific value B from separate observations/experiments and a non-linear equation. We believe that this straightforward procedure is valuable to the practitioner.

3. Experimental

The column used was an ACQUITY UPLC® BEH300 C_4, 2.1 mm × 50 mm 1.7 μm column from Waters Corporation. This packing has been designed for protein separations under UPLC conditions, using a Waters UPLC instrument with TUV detection at 210 nm. Gradients with slopes of 3.0%, 2.25%, 1.5%, 1.0%, 0.5%, 0.25%, 0.125% and 0.0625% change in acetonitrile composition per column volume were performed at 0.2 mL/min. The mobile phase contained 0.1% TFA. The proteins, myoglobin, MW 16700, and enolase, MW 46800 were obtained from Sigma. The injection volume was 3.3 μL, resulting in a mass load of ~1.6 μg, which avoided any visible overload. The peak width was measured at 10% of the height of the peaks and translated into the standard deviation.

4. Results and discussion

A plot of the dependence of the peak standard deviation on the experimental gradient slope S is shown for the two proteins myoglobin and enolase for a large range of gradients in Fig. 1. To be specific, the peak standard deviation is plotted versus the inverse of the gradient slope S (defined in Section 2). The slopes and the intercept were determined, and the values of the protein-specific slope value B were calculated from these data as outlined in Section 2 (Eq. (9)). We found that the values for the slopes B were 82 for myoglobin and 160 for enolase, in agreement with the change in molecular weight. The value is expected to be larger, i.e. the slope steeper, with increasing molecular weight of the protein, and the molecular weight of enolase is much higher than that of myoglobin. A typical value of B for a small molecule tends to be around 10. Thus the results for the proteins match our expectation.

With the now known values for B, we can calculate the generalized gradient slope G from Eq. (8). Finally, the plate counts for all individual measurements are determined from Eq. (10) with these values for the generalized gradient slope. A plot of the plate count as a function of the generalized gradient slope is shown in Fig. 2. It is observed that the plate count is best for rather steep gradients, and it declines for flatter gradients to values around 1000 for myoglobin and a bit less for enolase. This demonstrates the importance of gradient steepness on the separation performance in gradients.

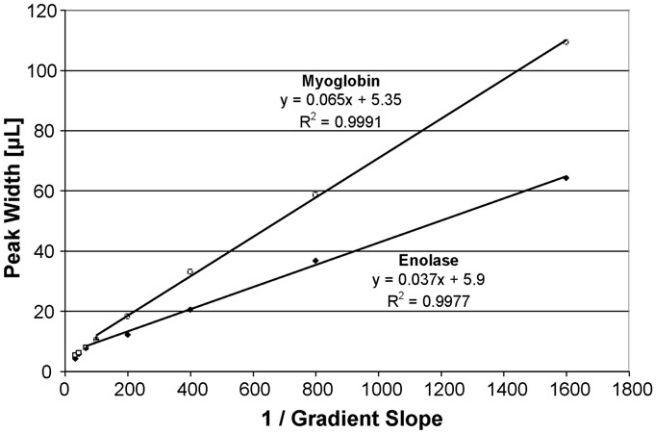

Fig. 1. Plot of volume standard deviation versus the inverse of the gradient slope.

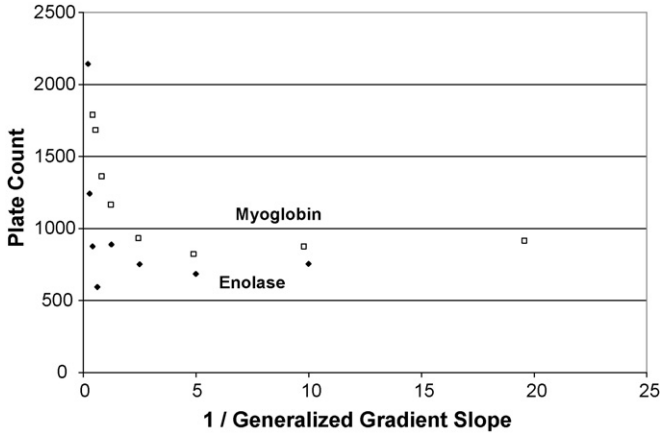

Fig. 2. Plate count versus the inverse of the generalized gradient slope for two proteins.

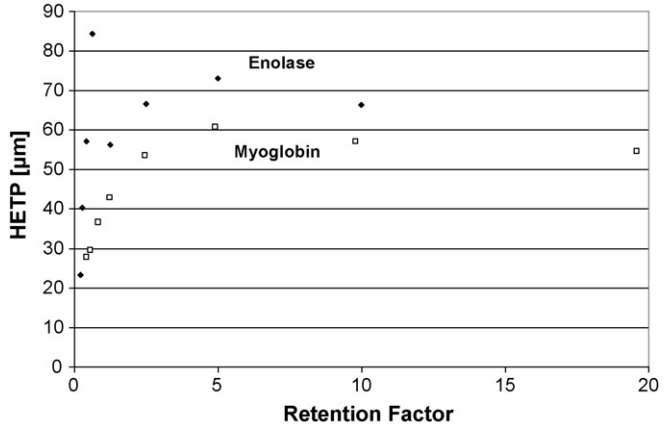

Fig. 3. HETP as a function of the retention factor at the point of elution for two proteins.

It is not clear if the improvement is due to band compression with steeper gradients [10] or due to the improvement in mass transfer.

A still better understanding is obtained, if we plot the HETP (following Eq. (1b)) as a function of the retention factor at the point of elution (Eq. (7)) in Fig. 3. We see a low plate height at low retention, and a deterioration of the plate height at intermediate retention. There is also a slight improvement again of the plate height at further increased retention. Considering that our data were obtained under conditions of dominance of the mass transfer term of the H/u plot, this is exactly what one expects from chromatographic theory: it suggests that higher retention is associated with a decreased mass transfer performance, but that this behavior improves again at very large retention factors, and this is what we see in Fig. 3. Therefore we attribute the pattern of the plate height changes with retention to the mass transfer properties of the packing.

The small deviations from linearity in Fig. 1 are likely to be a function of band compression. Attempts to explain such small curvature using more complex equations [11] did not succeed.

5. Conclusions

We have demonstrated a simple procedure that permits the calculation of a true protein plate count under gradient conditions in RP chromatography. We have also shown how the plate count changes as a function of the gradient execution. The procedure outlined here will enable a proper evaluation and comparison of the mass transfer properties of different RP packings, together with aspects of band compression as a function of the gradient execution.

References

[1] F. Gritti, G. Guiochon, J. Chromatogr. A 1216 (2009) 6124.
[2] T. Ishihara, T. Kadoya, S. Yamamoto, J. Chromatogr. A 1162 (2007) 34.
[3] B.C.S. Toa, A.M. Lenhoff, J. Chromatogr. A 1205 (2008) 46.
[4] R. Groh, I. Halász, Anal. Chem. 53 (1991) 1325.
[5] N. Afeyan, N. Gordon, I. Mazsaroff, L. Varady, S. Fulton, Y. Yang, F. Regnier, J. Chromatogr. 519 (1990) 1.
[6] A.E. Rodrigues, Z.P. Lu, J.M. Loureiro, G. Carta, J. Chromatogr. A 653 (1993) 189.
[7] U.D. Neue, HPLC-Columns—Theory, Technology, and Practice, Wiley-VCH, 1997.
[8] L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution, Wiley, 2007.
[9] U.D. Neue, J. Chromatogr. A 1079 (2005) 153.
[10] U.D. Neue, D.H. Marchand, L.R. Snyder, J. Chromatogr. A 1111 (2006) 32.
[11] U.D. Neue, Chromatographia 63 (2006) S45.