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Note

Effect of sample volume on peak width in high-performance gel filtration chromatography

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In traditional gel filtration chromatography, sample volumes of 1–2% of the total bed volume are commonly recommended[1]. However, we found that this general rule was not applicable to high-performance gel filtration, *i.e.*, when using columns packed with 30- or 10-μm materials.

Fortunately, the effect of sample volume on peak width may be readily derived from the theory of peak dispersion in liquid chromatography. The aim of this work was to test the applicability of the theoretical relationship and to provide a rational basis for the estimation of sample volume effects for a given set of experimental conditions in high-performance gel filtration chromatography.

THEORETICAL

The dispersion of a solute zone in a liquid chromatography system is generally described by[2]

$$\sigma^2_{tot} = \sigma^2_{injector} + \sigma^2_{column} + \sigma^2_{detector} + \sigma^2_{tubings} + \sigma^2_{connectors} \quad (1)$$

The contributions from the detector, tubing and connectors are, with the use of high-performance instrumentation, very small (*i.e.*, $\leqslant 5\%$) compared with the total peak width in gel filtration and the equation may be reduced to

$$\sigma^2_{tot} \approx \sigma^2_{injector} + \sigma^2_{column} \quad (2)$$

The peak dispersion caused by the injector can be expressed in terms of the injection volume, V_s, by $\sigma^2_{injector} = V_s^2/K$, where the factor K has a theoretical value of 12 as derived from the standard deviation of a rectangular distribution. However, the standard deviation of the zone will increase, owing to dispersion of the solute caused by the laminar flow profile, when the sample is injected with a completely filled loop. With this injection technique K is found experimentally to be close to 5[2–4]. This, together with the definition of plate number, *i.e.*, $N = (V_e/\sigma)^2$ yields

$$\sigma^2_{tot} \approx V_s^2 K^{-1} + V_e^2 N^{-1} \quad (3)$$

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and

$$\frac{\sigma^2_{injector}}{\sigma^2_{tot}} = \frac{1}{1 + [(V_e/V_s)^2 K/N]} \quad (4)$$

The contribution of sample volume to peak width can therefore be calculated from the elution volume and plate count of the solute of interest and an experimentally determined value of K (*e.g.*, we found $K = 5$ to be in excellent agreement with the experimental data for our injection device). From eqn. 4, it can be concluded that the effect of sample volume on peak width is reduced by using an appropriate injection technique (*i.e.*, yielding a large value of K[4]) and by using columns with large diameters and/or lengths to yield a large elution volume. It is also seen that the effect of sample volume is first noted on columns with high plate counts and thus the inherently high resolution properties of analytical columns are sacrificed when large sample volumes are applied.

As the plate counts of present high-performance gel filtration columns are approximately four times of those of traditional columns (*i.e.*, 10 000 plates per 30 cm and 2500 plates per 70 cm), eqn. 4 yields an approximate value for the sample volume on these columns of 0.5% of the bed volume. This sample volume would contribute 20% to the total peak width, as can be seen from Fig. 1.

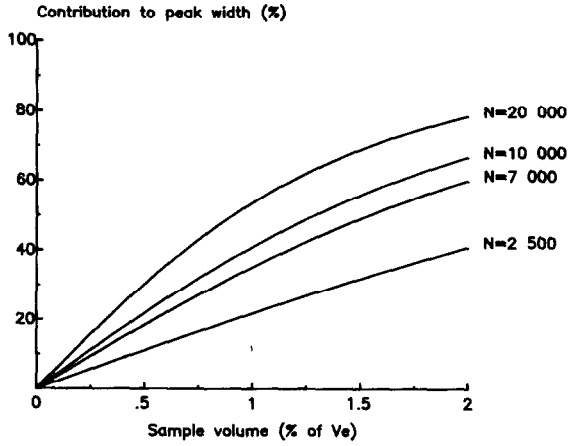

Fig. 1. Contribution of sample volume to peak width for columns of different efficiencies. Graphs calculated from eqn. 4 with $K = 5$.

EXPERIMENTAL

Superose 6B (30 μm) (Pharmacia) was packed in 500 × 10 mm and 500 × 16 mm glass columns as described earlier[5]. Superose 6 (13 μm) (Pharmacia) was packed in a 500 × 10 mm glass column, according to a proprietary procedure developed in the author's laboratory for this type of material. The packing procedures yielded efficient columns (*i.e.*, with a reduced plate height of 2) as judged from the plate count calculated from the half-height peak width of cytidine of 7000 and 20 000

plates per column for Superose 6B and Superose 6, respectively. The average particle sizes, d_p, expressed as the median of the volume-size distribution[5], were found to be 33 and 13 μm, respectively.

The effect of sample volume was tested by applying a mixture of thyroglobulin (0.5 mg) (Pharmacia), bovine serum albumin (0.8 mg) (Pharmacia), β-lactoglobulin (0.25 mg) (Sigma), myoglobin (0.1 mg) (Sigma), cytochrome *c* (0.1 mg) (Sigma) and cytidine (0.01 mg) (Merck) prepared in various volumes of buffer solution (0.05 *M* sodium phosphate buffer in 0.15 *M* sodium chloride adjusted to pH 7.0). The buffer was delivered from a high-precision pump (P-500), the sample was injected with a valve (V-7) equipped with loops of different volumes, the effluent was continuously monitored at 280 nm (UV-1, HR 10 cell) and the signal was traced with a recorder (REC 481). All instruments were obtained from Pharmacia.

The peak dispersion was calculated from the peak width at half-height and the sample application point taken when half the sample volume had been injected.

RESULTS AND DISCUSSION

Some of the separations of the protein mixture are shown in Fig. 2, together with a run on a traditional gel filtration medium. The impact of particle size, in addition to sample volume, on the resolution can be readily seen. The nominal linear flow-rate was adjusted to 2.5, 10 and 38 cm/h for the 110-, 33- and 13-μm material, respectively.

The influence of the sample volume on column efficiency, expressed as the reduced plate height, $h = \mathrm{HETP}/d_p$, was found to be in accordance with eqn. 3 with the value of K set to 5, as shown in Fig. 3. The high efficiency of analytical columns is seen to be significantly reduced by sample volumes exceeding 1 ml.

The zone broadening is also affected by the sample molecular weight[5], and this will yield different values of σ_{column} for various solutes. The effect is illustrated in Fig. 4, which shows the peak width (*i.e.*, 4σ) for different solutes as a function of sample volume.

The fit of the experimental data to the equations are acceptable in most instances for sample volumes of up to 2 ml. Above this limit an overestimation of the sample volume effect is obtained. This may be due to the fact that the injection profile of large sample volumes will be closer to a plug and hence the value of K will be between 5 and 12. The experimental data obtained in this study were in accord with a value of K of 6 and 7 for sample volumes of 3 and 4 ml, respectively.

Figs. 3 and 4 illustrate that eqn. 3 is very useful for the prediction of peak widths at different sample volumes, provided that the values of K and N are known. If this information is unavailable, it seems reasonable to assume a value of K close to 5 when using a completely filled sample loop, and to calculate a value of σ_{column} from $h = 2$ for an efficient column. Such predictions are necessary when designing an optimal analytical system where extra-column effects are to be avoided or a preparative system when large sample volumes are to be applied. As an example it may be calculated that running a 4-ml sample in a 500 × 16 mm bed of 30-μm material will yield the same total peak width as running four 1-ml samples in a 300 × 10 mm bed of 10-μm material in the same total time. This may also be predicted from Fig. 4.

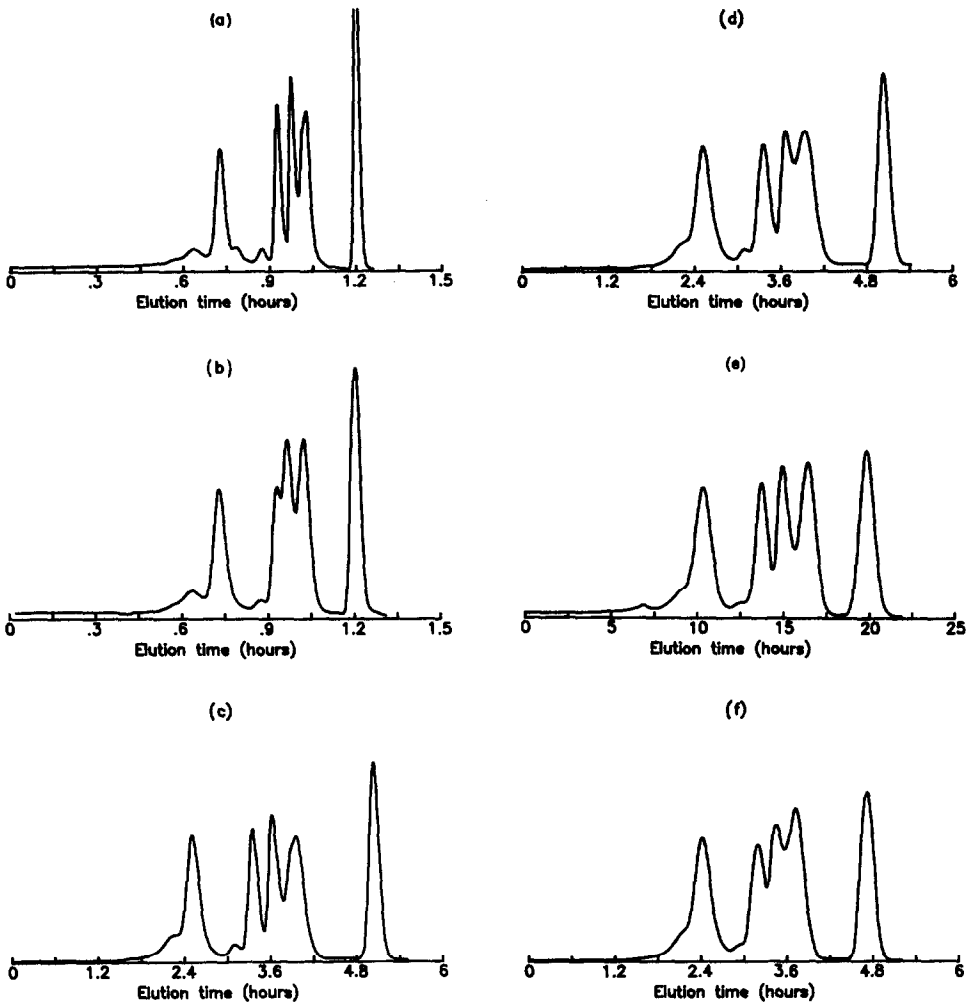

Fig. 2. Effect of particle size and sample volume on the resolution of a protein mixture. Sample: thyroglobulin + bovine serum albumin + β-lactoglobulin + myoglobin + cytochrome *c* + cytidine. Conditions: (a) 50-μl and (b) 1000-μl sample on Superose 6 (13 μm) in a 500 × 10 mm column eluted at 38 cm/h; (c) 100-μl and (d) 1000-μl sample on Superose 6B (33 μm) in a 530 × 10 mm column eluted at 10 cm/h; (e) 1000-μl sample on Sepharose CL 6B (110 μm) in a 510 × 16 mm column eluted at 2.5 cm/h; (f) 4000-μl sample on Superose 6B (33 μm) in a 510 × 16 mm column eluted at 10 cm/h.

Hence, in order to retain the high efficiency of analytical columns for gel filtration chromatography, the sample volume should be minimized and generally not exceed 0.5% of the total bed volume. In preparative purifications of large sample volumes, the use of 30-μm bulk material allowing for the preparation of columns with optional dimensions seems favourable.

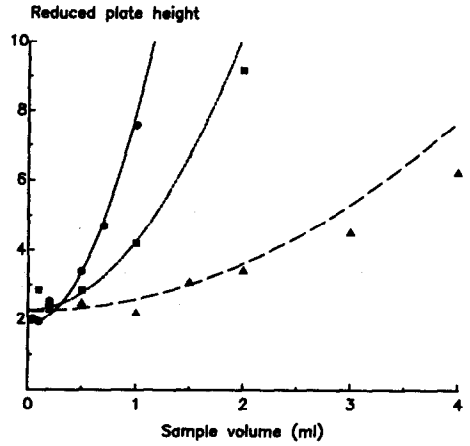

Fig. 3. Effect of sample volume on the reduced plate height of cytidine. Columns: (●) Superose 6, 500 × 10 mm; (■) Superose 6B, 530 × 10 mm; (▲) Superose 6B, 510 × 16 mm. Conditions as in Fig. 2. Curves calculated from $h = (\sigma_{tot}/V_e)^2 L/d_p$ and eqns. 2 and 3 with $K = 5$ and $\sigma_{column} = 250$, 480 and 1150 μl for the three columns. The points represent experimental data.

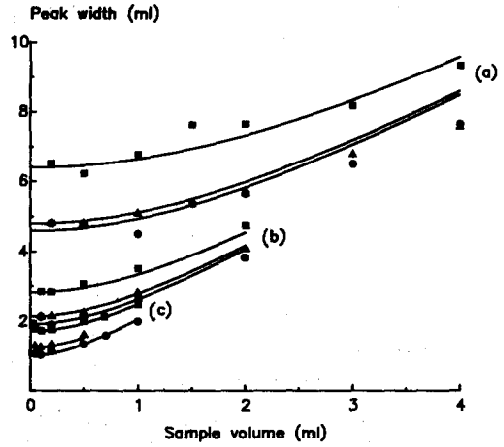

Fig. 4. Peak width ($4\sigma_{tot}$) as a function of sample volume for various solutes. Solutes: (■) thyroglobulin; (▲) bovine serum albumin; (●) cytidine. Columns: (a) Superose 6B, 510 × 16 mm; (b) Superose 6B, 530 × 10 mm; (c) Superose 6, 500 × 10 mm. Curves calculated from eqns. 2 and 3 with $K = 5$ and σ_{column} = 1.60, 1.20 and 1.15 ml in (a), 0.705, 0.535 and 0.480 ml in (b) and 0.430, 0.310 and 0.250 ml in (c).

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