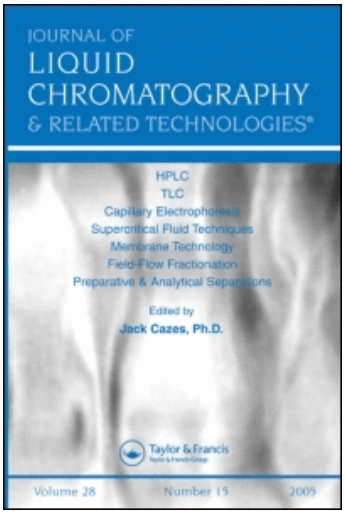

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Direct Examination of Elution Nonequilibrium in Liquid Chromatography by Laser-Induced Fluorescence

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DIRECT EXAMINATION OF ELUTION NONEQUILIBRIUM IN LIQUID CHROMATOGRAPHY BY LASER-INDUCED FLUORESCENCE

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ABSTRACT

Nonequilibrium interactions, which are known to influence broadening throughout the column, become very important at the exit of the chromatographic column. Theoretical predictions, derived herein, show a substantial increase in length variance and decrease in concentration when the solute is detected off-column. The discrepancy between on- and off-column values for variance and concentration is predicted to increase markedly with capacity factor. Experimental investigation of elution nonequilibrium is accomplished by measuring the length variance and concentration for a homologous series of model solutes both on- and off-column by laser-induced fluorescence. Excellent agreement of experimental measurement with theoretical predictions is seen for solutes with capacity factors ranging from one to nine. These results have interesting implications for the general elution problem in chromatographic separations.

INTRODUCTION

In simplified chromatographic theory, separations are generally modelled as an equilibrium process. In reality, however,

the transfer of solute molecules between the mobile and stationary phases is rarely instantaneous. Due to the finite rate of exchange, solute molecules in the mobile phase will travel some distance along the column before transfer occurs, while molecules in the stationary phase are fixed. Consequently, the solute zone profile in the mobile phase is slightly in advance of that in the stationary phase. This discrepancy results in broadening of the solute zone, due only to nonequilibrium processes (1).

Although this phenomenon occurs throughout the column, it reaches an extreme at the exit of the chromatographic column. In this region, the effective rate of solute transfer from mobile to stationary phase, which is a finite value on-column, becomes zero off-column where no stationary phase is present. As the zone elutes from the column, the front portion must travel at the mobile-phase velocity, while the back portion continues at the slower mean zone velocity. Thus, the solute zone passes from a region where it is retained (on-column) to a nonretentive region (off-column) at a rate dictated not only by the linear velocity but also by the capacity factor. This elution nonequilibrium results in an increase in the length variance and a decrease in maximum concentration of the solute zone detected off-column.

THEORY

Some insight into the possible magnitude of this elution effect can be gained by applying the nonequilibrium approach of Giddings (1). For a solute zone migrating from the column to a point an infinitesimal distance off-column, the time variance (σ_T^2) on- and off-column can be assumed to be equal.

$$(\sigma_T^2)_{OFF} = (\sigma_T^2)_{ON} \qquad [1]$$

By definition, the length variance (σ_L^2) is proportional to the time variance:

$$\sigma_L^2 = \sigma_T^2 \; U^2 = \sigma_T^2 \; [u/(1 + k)]^2 \qquad [2]$$

where k is the solute capacity factor, and U and u are the mean zone velocity and mobile-phase velocity, respectively. The on- and off-column length variance can then be expressed by substitution of this relationship in Equation [1].

$$(\sigma_L^2)_{OFF} = (\sigma_L^2)_{ON} \; (u_{OFF}/u_{ON})^2 \; (1 + k)^2 \qquad [3]$$

Because the volumetric flowrate (F) is constant on- and off-column, the mobile-phase velocities are related by

$$F = \pi \; r_{ON}^2 \; u_{ON} \; \varepsilon_T = \pi \; r_{OFF}^2 \; u_{OFF} \qquad [4]$$

where r is the tube radius, and ε_T is the total porosity of the packed bed (on-column), which is unity for an open tube (off-column). Thus, the corresponding length variances on- and off-column are given by the following expression:

$$(\sigma_L^2)_{OFF} = (\sigma_L^2)_{ON} \; (r_{ON}^2/r_{OFF}^2)^2 \; \varepsilon_T^2 \; (1 + k)^2 \qquad [5]$$

According to this relationship, two distinct factors determine the change in length variance upon solute elution. Dispersion arising from the increase or decrease in solute zone volume is reflected in the radial and porosity terms. More importantly, a substantial increase in length variance measured off-column is predicted as a function of capacity factor, due solely to elution nonequilibrium.

The effect of this increase in length variance on solute concentration (C) can be derived assuming a Gaussian zone profile, where

$$C = (1/\sigma_L \; (2\pi)^{1/2}) \; \text{EXP} \; [-1/2 \; ((x - \mu)/\sigma_L)^2] \qquad [6]$$

In this normalized form, x represents the length displacement along the column with a zone center at μ. At the concentration maximum of the zone ($x = \mu$), the ratio of concentrations off- and on-column is given by

$$C_{OFF}/C_{ON} = (\sigma_L)_{ON}/(\sigma_L)_{OFF} \quad [7]$$

Direct substitution of Equation [5] gives an expression for maximum concentrations present on and off the chromatographic column.

$$C_{OFF} = C_{ON} \left[r_{OFF}^2/r_{ON}^2\right] \left[1/\varepsilon_T\right] \left[1/(1 + k)\right] \quad [8]$$

Thus, an increase in off-column variance yields a concomitant decrease in concentration due solely to this nonequilibrium effect.

These theoretical predictions are shown schematically in Figure 1, which illustrates the movement and dispersion of solute zones along an open-tubular column of length L. In this illustration, the zone variance on-column was calculated to increase with capacity factor according to Golay theory (2),

$$\frac{\sigma_L^2}{L} = \frac{2\,D_m}{u} + \frac{(1 + 6k + 11k^2)\,r^2\,u}{24\,(1+k)^2\,D_m} \quad [9]$$

normalized to a nonretained solute. The off-column variance was subsequently determined utilizing Equation [5] for an open-tubular column with no change in radius. During the transition from on- to off-column, the nonretained solute exhibits no change in velocity or zone characteristics, whereas the retained solutes are substantially affected. For a solute with a capacity factor of ten, the length variance is predicted to increase more than one hundred-fold while the concentration at the zone maximum is decreased more than ten-fold. This effect appears to be a substantial contribution to the general elution problem in chromatographic separations.

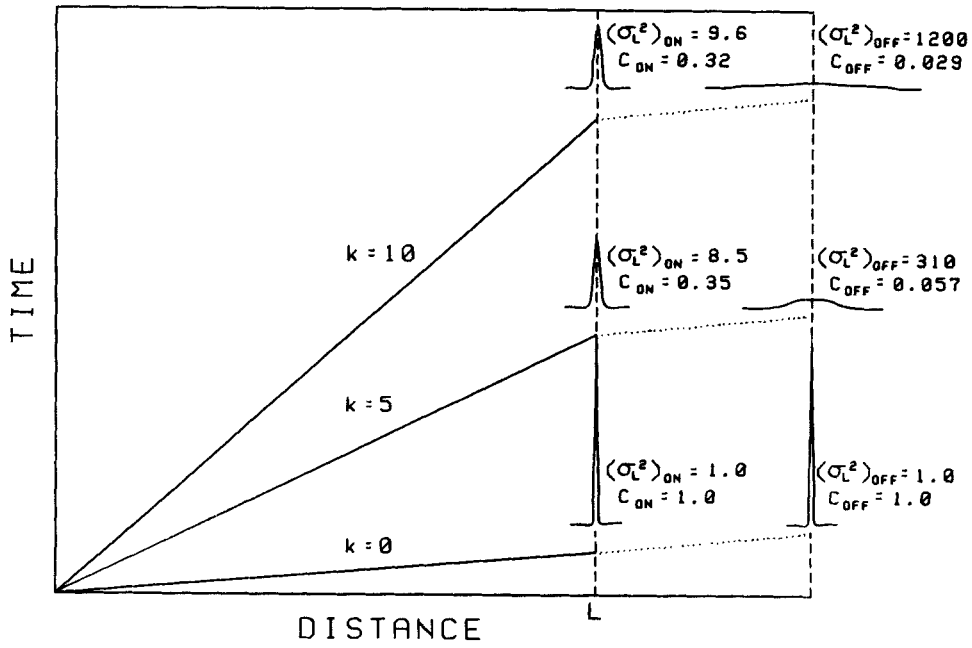

FIGURE 1: Predicted effect of elution nonequilibrium on solute zone variance (σ_L^2) and concentration (C) as a function of capacity factor (k).

In this paper, systematic investigation of elution nonequilibrium is accomplished by detecting solute zones sequentially on- and off-column. Practical application of this experimental scheme requires that both the column body and the packing material or stationary phase do not interfere with the detection technique of interest. This criterion is satisfied by a very limited number of analytical techniques among which laser-induced fluorimetry appears to be the most promising (3-5). The feasibility of fluorescence detection directly on the chromatographic column has been recently demonstrated using both packed and open-tubular microcolumns fabricated from optically transparent fused-silica capillaries (6-9). Fluorescence detection makes possible the sequential detection of solute zones

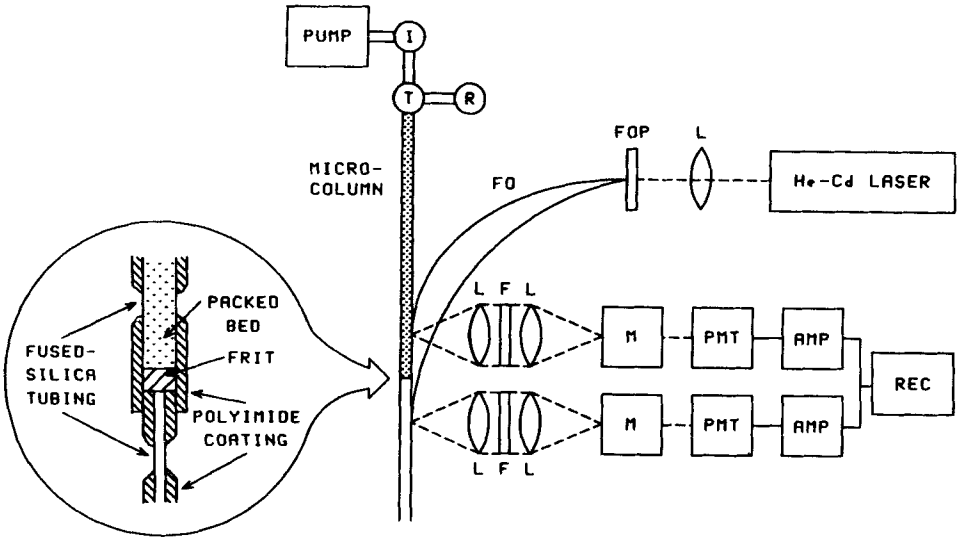

FIGURE 2: Schematic diagram of liquid chromatographic system with sequential fluorescence detection. I=injection valve, T=splitting tee, R=restricting capillary, FOP=fiber optic positioner, FO=fiber optic, L=lens, F=filter, M=monochromator, PMT= photomultiplier tube, AMP=amplifier/current-to-voltage converter, REC=chart recorder.

on- and off-column, thus allowing the direct examination of this elution nonequilibrium effect.

EXPERIMENTAL METHODS

Analytical Methodology. Saturated fatty acids (Sigma) were derivatized with 4-bromomethyl-7-methoxycoumarin (Sigma), as described previously (10). An anhydrous mixture of sodium sulfate (J.T. Baker) and potassium bicarbonate (MCB Reagents) were combined with dibenzo-18-crown-6 (Aldrich) in a slurry formed using dry acetone (Burdick and Jackson). An aliquot of 10^{-3} M stock solution containing *n*-$C_{12:0}$ to *n*-$C_{24:0}$ fatty acids

was added along with the coumarin reagent. The reaction was allowed to proceed in the dark at 50 °C for 2.5 hours, with intermittent stirring.

Fluorescence excitation and emission spectra of the coumarin reagent were obtained with a grating fluorimeter (Perkin-Elmer, Model LS5). The methanol (Burdick and Jackson) and *n*-decane (J.T. Baker) solvents utilized for the spectra showed no discernible background fluorescence.

Chromatographic System. Solvent delivery was accomplished with a dual-syringe micropump (Brownlee Labs, MPLC Model MG). Sample introduction utilized a 1.0 μL injection valve (Valco, CI4W1). The eluent was then split between the microcolumn and a restricting capillary (1:30).

The packed microcolumn was prepared using a fused-silica capillary (Hewlett-Packard) of 0.220 mm inner diameter (i.d.) and 91 cm length, terminated by a porous teflon frit (11), and followed by a 0.0524 mm i.d. capillary (Polymicro Technologies). The column was packed with a slurry of 5 μm spherical octadecylsilica packing material (Brownlee Labs, RP-18), as described previously (12). The total porosity (ε_T) of the packed column was determined to be 0.53 utilizing the well-known relationship described by Bristow and Knox (13),

$$\varepsilon_T = t_0 \, F/\pi \, r^2 \, L \qquad [10]$$

where t_0 is the elution time of a nonretained solute, and all other parameters are as previously defined.

Laser Fluorescence Detectors. As illustrated in Figure 2, a helium-cadmium laser (Omnichrome, Model 3112-10S) was used as the excitation source in two identical detector blocks. The laser radiation (10-25 mW, 325 nm) was focused onto a 100 μm UV-grade optical fiber which transmitted the incident light to the column. Fluorescent emission was collected in a 90-degree, coplanar

geometry utilizing a 500 μm optical fiber. The emitted radiation was filtered to minimize stray light, focused on the entrance slit of a monochromator at 420 nm (Instruments SA, Model H1061), and detected with a photomultiplier tube (Hamamatsu, R1463). The resulting photocurrent was amplified, converted to voltage, and finally displayed on a chart recorder (Linear, Model 585).

The two identical detector blocks are mobile and may be placed at any position along the column. For this study, the first detector was located on the packed bed approximately 2.9 cm before the end frit, while the second detector was 2.3 cm after the column exit.

Calculations. The method of statistical moments (14) was utilized to provide the most accurate determination of the solute zone variance. Statistical moments may be defined as

$$M_0 = \Sigma\, f(t)\, dt \qquad \text{zeroth moment}$$

$$M_1 = \Sigma\, t\, f(t)\, dt/M_0 \qquad \text{first moment} \qquad [11]$$

$$M_2 = \Sigma\, (t - M_1)^2\, f(t)\, dt/M_0 \qquad \text{second moment}$$

where f(t) represents the concentration as a function of time, measured from the detector response. Physically, the moments express the area (M_0), centroid or retention time (M_1), and variance (M_2) of the solute zone, respectively. Statistical moments (M_0 - M_2) were calculated manually by finite summation of 40 to 60 data points which were evenly distributed across the zone profile.

RESULTS AND DISCUSSION

Detection of solute zones in chromatography is commonly performed after the exit of the chromatographic column. It is presumed that post-column detection accurately reflects zone characteristics occurring on the column. Although widely

accepted, this assumption appears to be incorrect due, at least in part, to nonequilibrium effects within the solute zone when eluting from the column (6). Theoretically, this nonequilibrium effect can result in a substantial increase in length variance (Equation [5]) and decrease in concentration (Equation [8]) of the solute zone as it elutes from the column.

In previous studies, this effect has been described as an apparent enhancement of the fluorescence signal when detection is performed on the chromatographic column (6,15-17). Using a simple steady-state model, Guthrie and Jorgenson (6) derived an expression, whereby on-column detection should have better sensitivity than post-column detection by a factor of (1+k). Takeuchi and Yeung (15) described the influence of mobile- and stationary-phase environment on fluorescence intensity for the on- and off-column cases. Although these factors have been discussed previously, the influence of nonequilibrium at the exit of the chromatographic column has been largely ignored.

A systematic investigation is attempted here by simultaneously detecting on- and off-column, as shown in Figure 2. In this detection scheme, a single solute zone is measured directly on a packed chromatographic column and then again immediately after the zone exits the column. By detecting the same solute as it traverses the exit region of the column, direct experimental measurement of the change in the zone profile is possible.

In addition, careful selection of model solutes is important for detailed examination of nonequilibrium phenomena. Saturated fatty acids (n-$C_{12:0}$ to n-$C_{24:0}$) derivatized with 4-bromomethyl-7-methoxycoumarin (10) are utilized in this study for a number of reasons. First, this homologous series of solutes is chromatographically well-behaved, showing symmetrical peak shape and an ideal logarithmic dependence of retention on alkyl chain length. Second, these solutes exhibit a wide range of capacity factors under isocratic conditions (k = 0.9 to 8.9 in pure methanol), as shown in Figure 3. Finally, the fluorescence

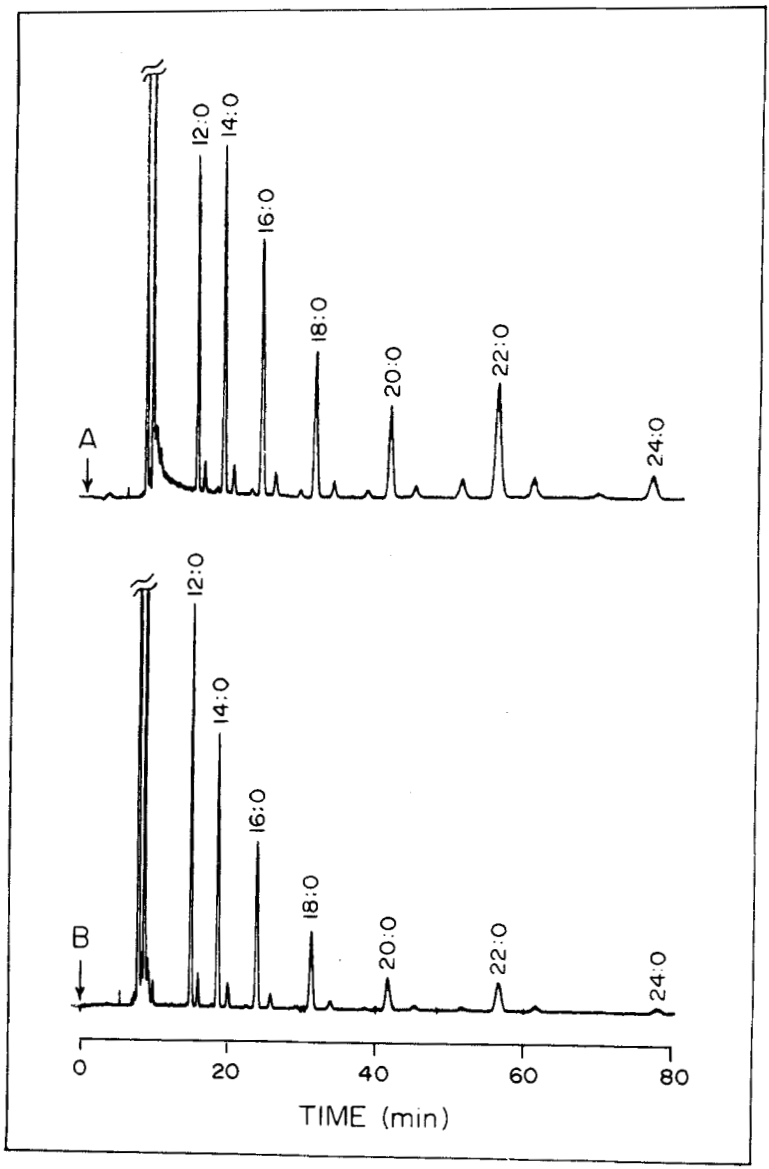

FIGURE 3: Chromatograms of *n*-$C_{12:0}$ to *n*-$C_{24:0}$ fatty acids derivatized with 4-bromomethyl-7-methoxycoumarin. Chromatogram A: On-column detection, 2.9 cm before frit, 200 nA full-scale. Chromatogram B: Off-column detection, 2.3 cm after frit, 20 nA full-scale. Column: 91 cm x 0.220 mm fused silica packed with Spheri-5 RP-18. Mobile phase: Methanol, F=1.45 µL/min. Fluorescence detection: λ_{EX}=325 nm, λ_{EM}=420 nm, PMT=-900 V.

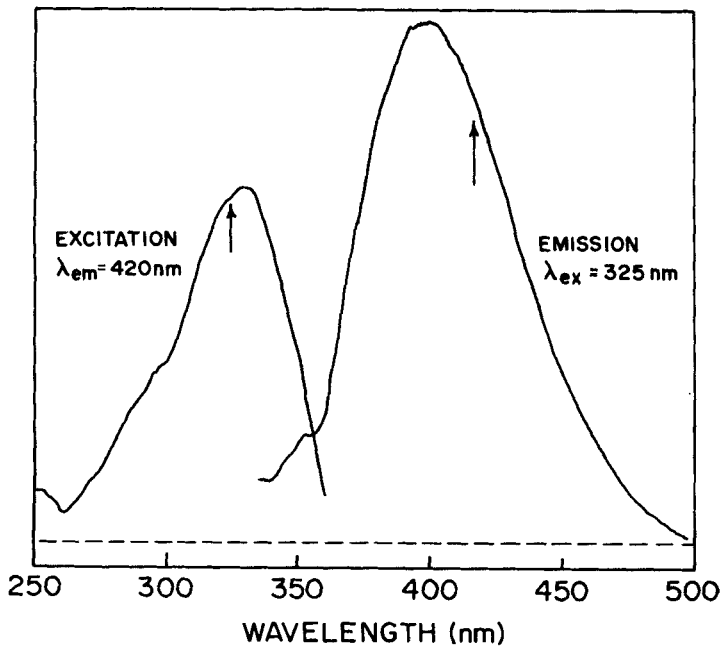

FIGURE 4: Fluorescence excitation and emission spectra of 4-bromomethyl-7-methoxycoumarin in methanol (——) and *n*-decane (---).

characteristics of these derivatives indicate favorable intensity in the methanol mobile phase, while no apparent fluorescence is detected when *n*-decane is used to mimic the stationary phase (Figure 4). This appears to be due to lack of solubility of the polar coumarin molecule in nonpolar media. It is hypothesized that the nonpolar alkyl chain of the fatty acid is retained in the stationary phase, while the fluorescent coumarin label remains in the mobile phase. Thus, the residence of the label in the mobile phase combined with isocratic mobile-phase composition allow elution nonequilibrium to be isolated from any change in fluorescence intensity due to environmental factors.

TABLE 1

Retention and Variance of Fatty Acid Derivatives by Simultaneous On- and Off-Column Detection

C#	k	σ_T^2 (s²) ON	σ_T^2 (s²) OFF	σ_L^2 (cm²) ON	σ_L^2 (cm²) OFF
12	0.9	90.9	89.4	0.348	139
14	1.4	135	139	0.338	216
16	2.0	229	227	0.353	354
18	3.0	403	400	0.363	623
20	4.3	646	708	0.327	1100
22	6.2	1320	1190	0.361	1850
24	8.9	2300	2090	0.333	3260

Representative determinations of time and length variance for both on- and off-column detection of the model solutes are summarized in Table 1.

The fatty acid derivatives, listed here by carbon number, are separated using a pure methanol mobile phase at a linear velocity of 0.12 cm/s. The time variance (σ_T^2) of each solute zone profile is evaluated both on- and off-column directly from the second statistical moment (M_2). Length variance values are subsequently determined by using Equation [2]. For this calculation, the on-column zone velocity (U) is determined using the first moment (M_1) for each solute zone together with the distance between the injector and on-column detector. The off-column zone velocity (u) is constant for all solutes, and is derived from the volumetric flowrate using Equation [4].

As can be seen in Table 1, time variance values for a single solute zone measured on- and off-column are approximately equal. Consequently, extra-column contributions to variance between the two detectors appear to be negligible. The observed equality of time variances confirms the basic assumption (Equation [1]) used to derive theoretical expressions relating on- and off-column length variance and concentration (Equations [5] and [8]).

In contrast to the time variances, the length variances determined for a single solute zone are not constant on- and off-column. Moreover, the length variances evaluated on-column remain small and relatively constant with increasing capacity factor, while those measured off-column increase drastically. These results imply that elution nonequilibrium may be a predominant contribution to retention-dependent sources of dispersion. Thus, the solute zone broadening commonly ascribed to the general elution problem may not arise from the column proper, as previously thought, but may occur as the solute elutes from the chromatographic column.

To investigate the magnitude of this elution nonequilibrium effect, the ratio of off- to on-column length variances is calculated as a function of capacity factor for the data in Table 1. These experimentally determined length variance ratios are compared with theoretical predictions in Figure 5. A linear relationship is observed between the length variance ratio and the function of capacity factor $(1+k)^2$, as predicted by Equation [5]. Excellent agreement is seen between experimentally determined and theoretically predicted length variance ratios over a wide range of capacity factors. In addition, similar experimental results for mobile-phase linear velocities ranging from 0.06 to 0.13 cm/s are summarized in Figure 5. No explicit dependence of length variance ratios on mobile-phase velocity is observed, as expected from Equation [5]. These results indicate that elution nonequilibrium contributes significantly to solute zone dispersion in a manner which is consistent with theoretical considerations.

In concurrent studies, the influence of elution nonequilibrium on solute zone concentration has been examined with the same experimental data. The concentration is measured directly from the maximum photocurrent of the fluorescence signal both on- and off-column. The experimental concentration ratios, shown in Figure 6, correlate well with theoretical predictions over a wide range of capacity factors. These concentration ratios are linearly dependent on the function $1/(1+k)$, as predicted by

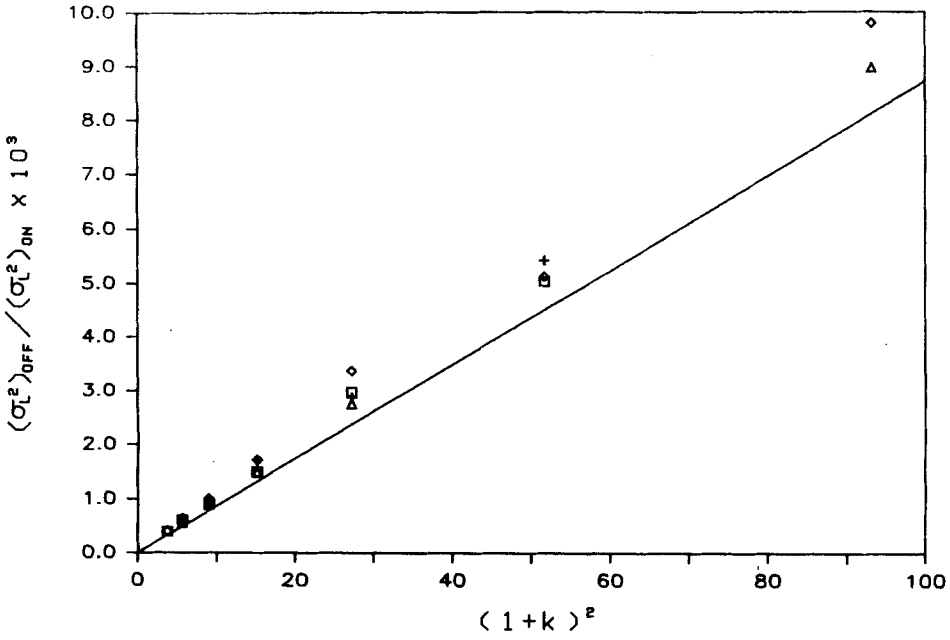

FIGURE 5: Ratio of the length variance measured on- and off-column *vs.* $(1+k)^2$. Theoretical prediction (——) by Equation [5] utilizing measured r_{ON}=0.220 mm, r_{OFF}=0.0524 mm, and ε_T=0.53. Experimental measurements at various linear velocities (□) 0.06 cm/s, (+) 0.09 cm/s, (◊) 0.12 cm/s, (△) 0.13 cm/s.

Equation [8]. No apparent dependence of the concentration ratio on mobile-phase velocity is theoretically predicted or experimentally observed. The results presented herein clearly demonstrate that detection performed after the exit of the chromatographic column has a detrimental effect on both the column efficiency and solute detectability.

CONCLUSIONS

The extent of broadening which occurs at the column exit due to nonequilibrium interactions can be described theoretically.

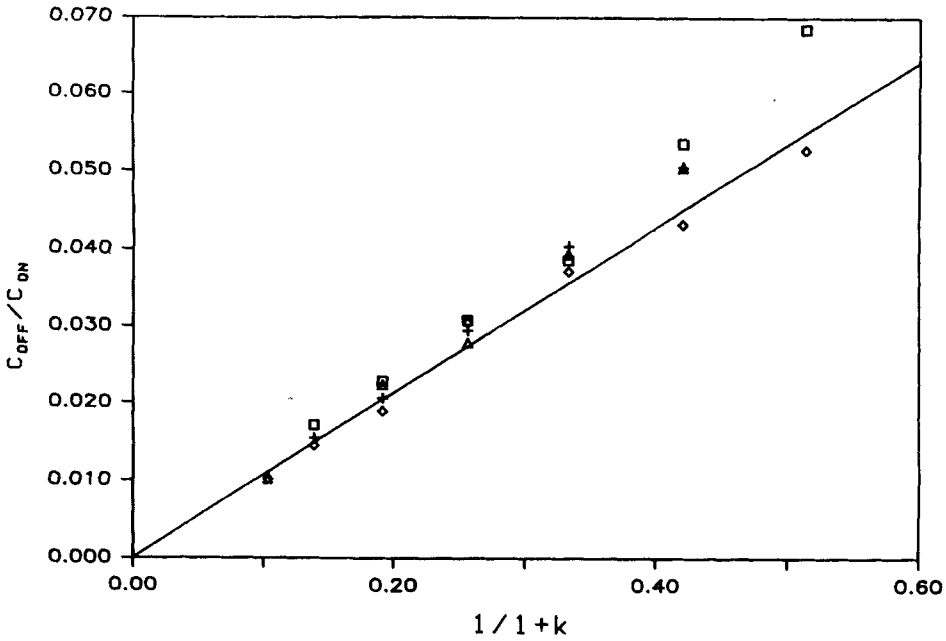

FIGURE 6: Ratio of maximum concentration measured on- and off-column *vs.* (1/1+k). Theoretical predictions (——) by Equation [8] utilizing measured r_{ON}=0.220 mm, r_{OFF}=0.0524 mm, and ε_T=0.53. Experimental measurements at various linear velocities (□) 0.06 cm/s, (+) 0.09 cm/s, (◊) 0.12 cm/s, (△) 0.13 cm/s.

An increase in length variance proportional to $(1+k)^2$ and concomitant decrease in concentration proportional to (1/1+k) is predicted from nonequilibrium considerations. Sequential measurements of length variance on- and off-column show excellent agreement with theoretical predictions for capacity factors ranging from one to nine. Concentration ratios, measured from the maximum photocurrent on- and off-column, are also in good agreement with predictions which assume a Gaussian zone profile. Thus, both theoretical and experimental results indicate that off-column detection does not accurately reflect the on-column characteristics of the solute zone. Although experimental

investigations described here are limited to liquid chromatography, these results may have direct implications for gas and supercritical fluid chromatography as well.

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