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ZONE BROADENING DUE TO SAMPLE INJECTION IN CAPILLARY ZONE ELECTROPHORESIS

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SUMMARY

A new source of zone broadening in capillary zone electrophoresis (CZE) associated with electrokinetic and hydrostatic injection techniques has been identified. The actual insertion of the capillary into or withdrawal of the capillary from the sample solution results in the extraneous injection of sample into the capillary. Examination of the sample plug resulting from this extraneous injection indicates that its length can exceed the maximum value permitted to realize the high separation efficiency of CZE. Experiments show that this mode of injection is present even under conditions where hydrostatic flow should occur from the capillary back into the sample vial.

INTRODUCTION

Capillary zone electrophoresis (CZE) is fast becoming a popular separation technique due to its inherently high separation efficiency in terms of plate numbers. This promise of high efficiency has spurred a large number papers on applications of CZE.

Surprisingly few studies can be found in the literature on the effect of operating conditions on CZE efficiency. Martin and co-workers[1,2] discussed cases where the plug flow is distorted by the walls of the capillary. Lukacs and Jorgenson[3] experimentally examined the effect of capillary inner diameter, capillary length, and solute concentration on the plate number. Sepaniak and co-workers[4] experimentally investigated the effects of sample injection, applied voltage, column dimensions and buffer concentration on the efficiency in micellar electrokinetic capillary chromatography[5]. Knox and Grant[6] as well as Grushka *et al.*[7] derived an expression for the mass transfer contribution to the plate height due to temperature effects arising from Joule heating. Walbroehl[8] has examined theoretically some of the band broadening effects in CZE.

Many experimental parameters can adversely affect solute zone broadening in CZE; these include hydrostatic flow superimposed on electroporetic mobility, radial temperature gradients in the capillary, and solute adsorption onto the capillary wall.

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This communication deals specifically with zone broadening associated with an artifact of sample introduction.

Ideally, the sample should be introduced into the CZE capillary as an infinitely narrow zone. The two most popular sample introduction techniques are electrokinetic and hydrostatic injection. In the former method, one end of the capillary is removed from the buffer reservoir and immersed in a buffer containing the sample and an electrode from the power supply. Voltage is applied momentarily across the capillary, during which time the sample is "pumped" into the capillary by electrophoretic and/or electroosmotic migration. The voltage is then turned off and the capillary end is moved from the sample vial back to pure buffer. The voltage is turned on again and the electrophoretic analysis begins.

In the second injection method, one end of the capillary is removed from the buffer reservoir and inserted into a sample solution which is elevated above the capillary outlet. Hydrostatic pressure causes the sample to siphon into the capillary. After a predetermined length of time, the capillary is returned to the buffer reservoir and the analysis proceeds as in the previous case. Although there are other methods of sample introduction (see, for example, refs. 9 and 10), the preceding two techniques are used most frequently because of their simplicity.

Some information on the effect of the injection process on CZE efficiency can be found in the literature[4,8,9]. In the electrokinetic injection, the sample is assumed to enter the capillary as a square plug. According to Sternberg[11], the contribution of a plug injection to the total height equivalent to theoretical plate (HETP) is:

$$\sigma_{inj}^2 = \frac{l_{inj}^2}{12} \tag{1}$$

where σ_{inj}^2 is the variance due to the injection and l_{inj} indicates the length of the plug. This equation permits the calculation of the acceptable length of the injected sample plug for a given loss in efficiency. Using the Sternberg expression in eqn. 1 and the following plate equation[12] for CZE

$$H = \frac{2D}{U} \tag{2}$$

the injection length can be approximated as:

$$l_{inj} = (24DEt)^{\frac{1}{2}} \tag{3}$$

where D is the solute's diffusion coefficient, U is the solute's velocity, t is the elution time, and E is the acceptable increase in H relative to the theoretical minimum HETP of the system (from eqn. 2). For example, for protein as a solute (diffusion coefficient of $1 \cdot 10^{-6}$ cm^2/s), the theory predicts that at a migration velocity of 0.1 cm/s, the minimum plate height should be 0.2 μm. If the increase in HETP due to the injection is to be less than 10% of this HETP value (*i.e.*, E = 0.1), then for a 10-min analysis time, the length of the injection plug should be less than 380 μm. For a smaller molecule having a diffusion coefficient of $1 \cdot 10^{-5}$ cm^2/s and identical experimental conditions as

above, the allowed injection plug length for a 10% decrease in efficiency is about 1.2 mm. These calculations are based on the assumption that eqn. 2 defines the plate height of the CZE system. Eqn. 2 is pertinent when diffusion is the major band-broadening mechanism in the CZE separation; if other mechanisms are active, then eqn. 2 does not strictly apply and the stringency of the injection plug lengths is somewhat alleviated relative to the values calculated from eqn. 3.

The realization that the injection plug should be less than 400 μm for a large solute molecule, coupled with the fact that measured efficiencies in CZE are routinely well below the theoretical values, prompted us to examine carefully the injection process. Our studies revealed that when a capillary is inserted into the sample vial, a volume of sample penetrates into the capillary even under experimental conditions where hydrostatic flow is from the capillary back into the sample vial. We describe here some experiments which demonstrate this mode of unwanted, but ubiquitous, injection. To the best of our knowledge, the existence of this source of zone broadening has not been previously reported.

EXPERIMENTAL

Instrumentation

A detailed description of the electrophoretic system is given elsewhere[13]. In the present system, the power supply was changed to a Glassman Series EH high-voltage unit. The dimensions of the fused-silica capillary were 110 cm length; 60 cm separation distance; 50 μm I.D.; 375 μm O.D.

Penetration of liquid into the capillary was observed with a Micromaster Model E microscope (Fisher Scientific, King of Prussia, PA, U.S.A.). For these experiments, the CZE capillary was mounted on a glass microscope slide and the glass tube (1 mm I.D.) containing the dye was held in a micromanipulator designed in-house from optical bench parts. An x–y–z translation stage was used to manipulate the dye tube into contact with the capillary, which was filled with 150 m*M* Na_2HPO_4 (pH 8).

Chemicals

Phenol was used as the solute for the electrophoresis runs. The buffer in the capillary was a pH 6 phosphate buffer prepared by dissolving 150 mmol of NaH_2PO_4 in 100 ml of water and adjusting the pH with concentrated NaOH. The dye used in the injection study was bromophenol blue dissolved at a concentration of 20 mg/ml in pH 8 phosphate buffer.

Procedure

The sample injection was accomplished using the electrokinetic or the hydrostatic method. To study the sample penetration into the capillary, the liquid in the sample vial was placed 2 cm below the level in the reservoir at the other end of the capillary.

RESULTS AND DISCUSSION

Allowable injection plug length

Eqn. 3 in the Introduction allows us to examine the allowable injection length as

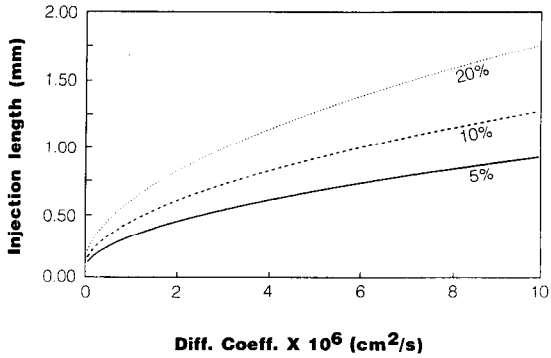

Fig. 1. The allowed injecton plug length as a function of the solute's diffusion coefficient. The analysis time was assumed to be 10 min. Each line corresponds to a different allowable loss in the efficiency (increasing H).

a function of the diffusion coefficient (or molecular weight) of the solute and of the elution time from the system. Fig. 1 shows plots of injection length as a function of the solute's diffusion coefficient for specified losses in separation efficiency. A 10-min analysis time was assumed in the calculations. Three different cases are given: 5%, 10% and 20% loss in efficiency. Not surprisingly, the restriction on the injection plug length is rather stringent for large molecules (small diffusion coefficients); this arises primarily because the small D for these solutes imparts a high inherent efficiency (small H, large N) to the CZE system for these solutes.

Fig. 2 shows the injection length as a function of the analysis time. In this case, the diffusion coefficient was constant at $1 \cdot 10^{-6}$ cm^2/s. Again, each of the curves depicts the behavior for a different acceptable loss in efficiency. In terms of injection length requirements, a longer analysis time appears to be more advantageous, primarily because band broadening due to diffusion during the separation becomes more significant and thus reduces the stringency of the injection process.

Extraneous injection

The preceding discussion indicates that the injection procedure should be controlled tightly in terms of injection voltages and times to avoid peak broadening. In

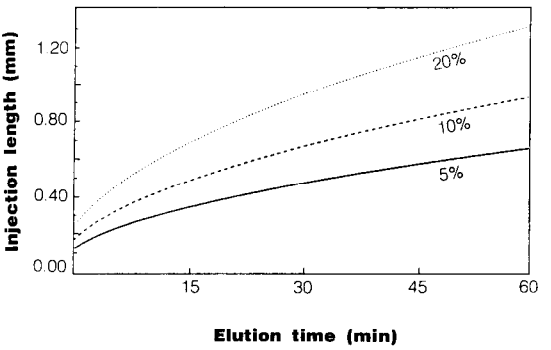

Fig. 2. The allowed injection plug length as a function of the analysis time. The diffusion coefficient was taken as $1 \cdot 10^{-6}$ cm^2/s. Each line corresponds to a different allowable loss in the efficiency.

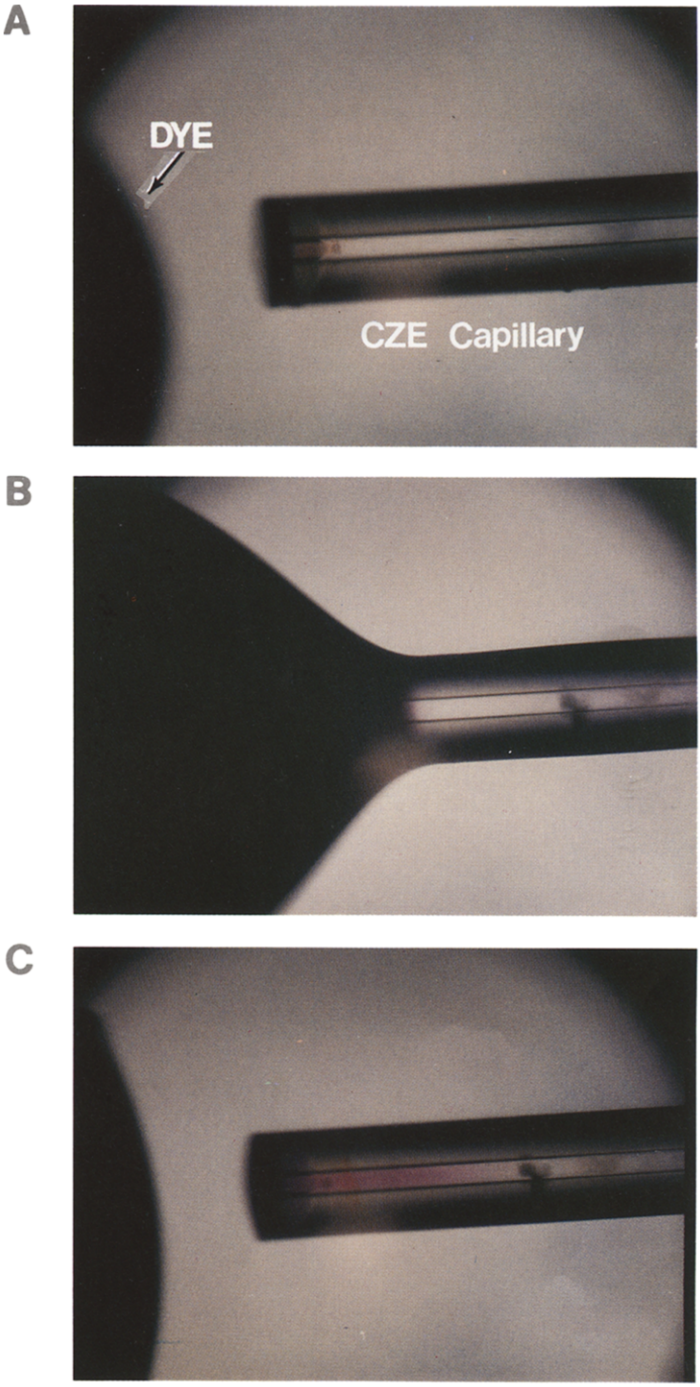

Fig. 3. (A) The CZE capillary (on the right) before contact with the dye pool (on the left). (B) The CZE capillary touching the dye pool. (C) After contact between the capillary and dye. The dye inside the CZE capillary is clearly visible.

our studies it soon became apparent that a additional source of peak broadening in which the sample enters the capillary in an uncontrolled manner is associated with the injection process.

Fig. 3A shows a photograph of a CZE capillary (on the right of the photograph) just before touching a larger tube containing a dye solution (on the left). The other end of the CZE capillary (1 m length) was elevated 5 cm relative to the end inserted into the dye. Fig. 3B shows a photograph of the CZE capillary touching the dye in the other tube. As a result of surface tension, there is considerable wetting of the CZE capillary by the dye. Fig. 3C shows the capillary after contact with the dye was broken. This sequence of photographs reveals that some dye solution penetrated into the CZE capillary. The length of the dye zone in the capillary is about 700 μm. This plug size can cause a serious deterioration in the system performance in the case of large solutes, such as proteins (see Fig. 1).

Frequently, the sample penetrates into the capillary not only upon withdrawal

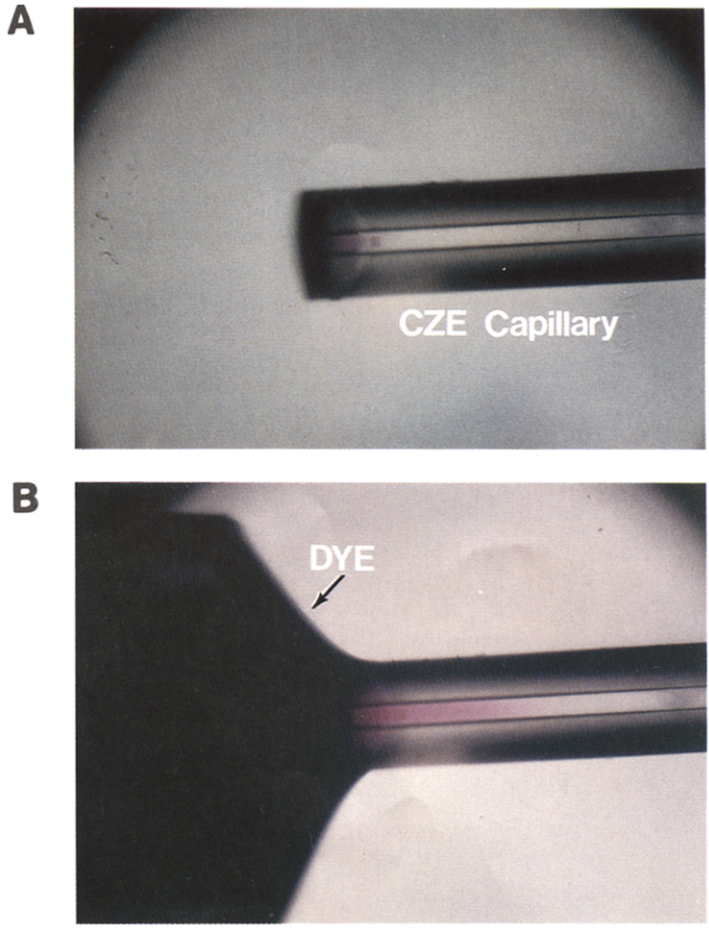

Fig. 4. (A) The CZE capillary (on the right) before contact with the dye pool. (B) The CZE capillary immediately after touching the dye pool. The dye inside the CZE capillary is clearly visible.

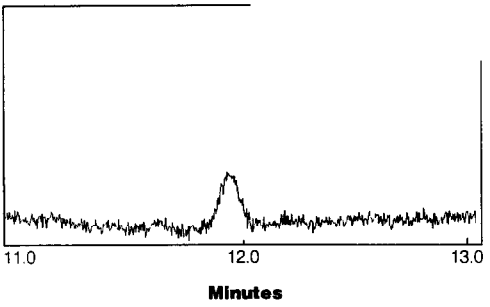

Fig. 5. An electropherogram resulting from an extraneous injection.

from the dye solution, but also upon contact. Fig. 4 shows a photograph of a CZE capillary immediately after contact with the dye. The dye inside the CZE capillary (on the right) is evident. The length of the dye zone in the capillary is again about 700 μm. The injection end of the capillary was lowered 5 cm relative to the elution end of the capillary. Similar results were obtained when the elution end of the CZE capillary was at the same height as the injection end.

These photographs demonstrate that the actual insertion (or withdrawal) of the capillary into (from) the sample vial can cause an extraneous injection of solute into a CZE capillary. Fig. 5 shows an electropherogram showing a phenol peak resulting from such an extraneous injection. In this case, the liquid level in the sample vial was about 2 cm lower than that in the buffer vial at the elution end of the capillary. In addition, the capillary was left in the sample vial for 2 min, during which time hydrostatic flow should have been in the direction from the capillary back into the sample vial. Still, enough sample entered the capillary to result in a peak. The peak is quite broad, yielding a plate number which is much less (48% of N_{theor}) than the theoretical value.

This experiment was repeated several times for several insertion intervals: 20, 30, 60, 120 and 300 s. Though experiments at each insertion interval resulted in a peak, no clear trends, either with respect to peak height or HETP values, could be found. The lack of a clear pattern in the peak heights or widths is due to the random nature of sample penetration because, in any given experiment, insertion, withdrawal, or both actions can result in sample penetration into the capillary. This mode of extraneous injection will be superimposed on the electrokinetic or hydrostatic injections resulting in total injection plug sizes which can be unacceptable in terms of plate heights.

Three mechanisms may be responsible for the extraneous sample injection: (a) insertion of the capillary into the sample solution may displace a small volume of the sample into the capillary; (b) viscosity, surface tension, and/or density differences between the sample solution and the buffer could cause a convective outflow of some buffer and subsequent inflow of sample into the CZE capillary; (c) diffusion of the solute into the capillary may occur. Initial experiments indicate that density differences between the sample and the buffer play a major part in this extraneous injection. This undesired mode of injection is rather ubiquitous, and it may be operative under a wide range of experimental conditions without operator awareness. Further research is now underway to elucidate the exact cause of sample penetration, as well as to develop alternative approaches to sample introduction into CZE systems.

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