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Volume-load capacity in fast-gradient liquid chromatography Effect of sample solvent composition and injection volume on chromatographic performance

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Abstract

We studied the effects of sample solvent composition and injection volumes on the chromatographic performance of ODS-bonded silica columns under fast-gradient running conditions. Chromatographic performance is compromised as a function of both sample injection volume and sample solvent strength, with earlier-eluting analytes being much more affected than later-eluting ones. In general, when injecting samples dissolved in a strong solvent, performance was improved by diluting the strong injection solvent and injecting a proportionally larger volume. Volume loading capacity can be increased by using a longer column, or by using a column of equivalent length, but with a larger inner diameter. Data also suggest that sample solvent strength, not viscosity, is responsible for the noted effects.

Keywords: Gradient elution; Injection volume; Sample solvent strength; Loading capacity; Retention factor; Efficiency

1. Introduction

Modern chromatographic practice is dominated by two contradictory goals. One trend is toward improved chromatographic performance – high resolution and reproducibility – while the continuing push towards high-throughput separations often sacrifices those goals for the sake of reduced analysis time. The stringent requirements in quality assurance/quality control laboratories for batch-to-batch reproducibility, base-silica purity, and column efficiency are compromised in the numerous other laboratories that analyze combinatorial libraries or have open-access systems. The sample concentration and sample solvent strength is usually of little concern to these users whose main concern in acquiring purity or structural information as quickly as possible. The chromatographic system is taken to its limits by running short columns at very high mobile phase velocities, with steep gradients, and little if any sample preparation. Under such conditions chromatography is necessarily compromised. However, the use of mass spectrometers may compensate to some extent for the loss of chromatographic performance, as mass spectrometry (MS) can readily discriminate between even totally co-eluting peaks. While there are numerous published works that have examined and characterized chromatographic performance under ''ideal'' conditions of mobile phase composition, sample preparation, etc., many analysts now must work under situations that are far from ideal. What are the chromatographic problems that may be faced under such situations, and are there solutions?

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The vast numbers of potential drug candidates and the need to identify their metabolites has led to the need to increase sample throughput in all aspects of analysis, from sample preparation through liquid chromatography (LC)–MS analysis. While solid-phase extraction (SPE) can generate clean samples, even for complex matrices such as serum, and thus may simplify the chromatographic separation and identification of metabolites, it tends to be a time-consuming process and the extraction and recovery steps often take longer than the high-performance liquid chromatography (HPLC) analysis [1]. ''Crashing'' samples has evolved as a simple and fast alternative to SPE. In this method, a ratio, usually 2:1, of acetonitrile is added to a serum mixture to precipitate the proteins, which are then separated by centrifugation. Although both techniques are effective in removing the proteins that may interfere with subsequent HPLC analysis, the analyst is left with the choice of either injecting a sample high in acetonitrile content, or else going through the time-consuming process of evaporation and reconstitution in buffer.

Tseng and Rogers [4] found that isomers of dihydroxybenzene, when dissolved and injected in mobile phase (pure methanol), generated single symmetrical peaks, but split peaks resulted when the samples were dissolved in water. Similarly, many authors have noted peak distortion and peak splitting effects when analytes were injected in solvents other than the mobile phase [1–3,5–7]. Several works have postulated that the differences in the sample solvent strength and the mobile phase solvent strength accounted for the peak distortions and peak splitting [3,8–10]. However, in a study of band distortion in size-exclusion chromatography (SEC) as a function of sample solvent injection, Czok et al. [2] used sample solvents of varying glycerol content to determine that sample solvent viscosity, not strength, could account for distortion effects. While Czok et al.'s work was focused on SEC, other works seemed to confirm these effects in reversed-phase systems as well. Zapata and Garrida [11] found that chlorophylls gave a single peak when injected in methanol–water (95:5), but gave split peaks when injected in an equivalent solvent strength mixture of acetone–water (63:31). Further investigations using reversed-phase systems have demonstrated the viscous fingering phenomenon when using sample solvent of greater or lesser viscosity than that of the mobile phase [12,13]. In addition, Plante et al. [14] have even visualized this ''viscous fingering'' effect in SEC using magnetic resonance imaging.

The detrimental effects of injecting samples high in organic content, and therefore of differing viscosity and strength than typical reversed-phase mobile phases, may be further magnified by the trend of gradient elution using short columns ($<$50 mm) packed with small particles. Such high-efficiency separations generate peaks with very small peak volumes. While these types of analyses can generate incredibly fast and highly efficient separations, the drawback is that the small peak volumes may be very sensitive to the differences in sample and mobile phase viscosity and/or strength [15]. In addition, the small internal volumes of such columns, particularly in 2.0 mm I.D. formats, would seem to make them especially sensitive when large injection volumes are needed.

While the previously cited works show that viscous fingering may be the cause of band distortions when injecting samples high in organic content, the studies have been primarily made under isocratic conditions and have tended towards the use of longer columns (250×4.6 mm). The studies that have used gradient analyses have focused primarily on the topic of on-column focusing [16,17] or on mass overload rather than volume overload [18,19]. Thus, the goal of this present work was to expand upon the previous studies of band distortion as a function of sample solvent composition and injection volume, with an emphasis on high-throughput HPLC using short columns, steep gradients, and high linear velocities. We also offer practical solutions for situations when chromatographic analyses are performed under conditions far from ideal as a result of time constraints related to sample preparation.

2. Experimental

2.1. Equipment

The HPLC system used was a HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a HP 1100 in-line degasser (part No.

G1322A), a HP 1100 autosampler (part No. G1313A), a HP 1100 column thermostat set to 30°C (part No. 1316A), a HP 1100 binary pump (part No. G1312A), and a HP 1100 diode-array detector equipped with an 8-μl volume flow cell with a 6-mm path length (part No. G1315A). Absorbance was monitored at 254 nm, and extraneous connective tubing was kept to a minimum (0.007 in. I.D.; 1 in.=2.54 cm) to reduce extra-column volume effects. HP ChemStation (version 6.03) was used for data acquisition and analysis.

2.2. Columns and reagents

All chromatography was performed using Luna 3 μm C_{18}(2) (Phenomenex, Torrance, CA, USA) packed into various column dimensions (50×2.0 mm, 30×2.0 mm, 20×2.0 mm, and 20×4.0 mm). Column efficiencies ranged between 90 000 and 110 000 plates m^{-1} for each of the columns using naphthalene as the probe under isocratic running conditions (65% acetonitrile in water, 1 ml min^{-1} flow-rate). Acetonitrile and water were HPLC grade (EM Science, Gibbstown, NJ, USA). All reagents were of the highest possible purity and were purchased from Sigma–Aldrich (St. Louis, MO, USA). In several of the figures (Figs. 5–9), column efficiencies have been expressed as apparent efficiency (plates per column) and the unusually high values reflect peak compression due to the gradient.

2.3. Sample preparation

The goal of this study was to examine the effect of sample solvent composition and injection volume on the chromatographic performance of small volume columns. Thus, it was important to eliminate any mass loading effects from interfering with the results. To accomplish this, it was necessary to maintain equivalent mass loads of analytes across a broad (1–100 μl) injection volume range. A stock solution containing thiourea (32 mg ml^{-1}), caffeine (40 mg ml^{-1}), phenol (16 μl ml^{-1}), acetophenone (24 μl ml^{-1}), dimethylphthalate (64 μl ml^{-1}), and valerophenone (32 μl ml^{-1}) was prepared in sample solvents consisting of the following concentrations of acetonitrile: 25, 33, 50, 66, 75 or 100%. For each of these six sample solvent compositions, serial dilutions were made to accommodate injection volumes from 1 to 100 μl while maintaining equivalent mass loads in the linear range for the columns (less than 1 μg g^{-1} of sorbent) (10 μl of stock solution+990 μl each sample solvent). Three injections were made at each volume/concentration point and the mean value was used for data analysis.

2.4. HPLC running conditions

Solvent A consisted of water with 0.1% (v/v) formic acid, and solvent B was 0.1% (v/v) formic acid in acetonitrile. The binary pump delivered a linear gradient from 95% A to 95% B in a time period such that the retention factors of the probes were equivalent on all four columns (3, 3, 4.5 and 7.5 min gradient times when using the 20×2.0 mm, 20×4.0 mm, 30×2.0 mm, and 50×2.0 mm columns, respectively). The flow-rate was 1 ml min^{-1} for the 2.0 mm I.D. columns, and 4 ml min^{-1} for the 4.0 mm I.D. column (linear velocity of 0.3 cm s^{-1}). A 10-column volume re-equilibration period followed each injection. The V_0 was determined using thiourea, and retention factors for the test probes ranged from 5 (caffeine) to over 20 (valerophenone).

3. Results and discussion

The effect of sample solvent composition on peak shape is illustrated in Fig. 1. When caffeine is injected in a relatively weak solvent (33% acetonitrile), the peak is eluted with symmetrical shape. As the percentage of acetonitrile in the sample solvent is increased to 66 and 100%, the peak gradually becomes distorted and exhibits a preceding bulge. Castells and Castells [12] found similar results when injecting phenol dissolved in acetonitrile–water (75:25) into a mobile phase of methanol–water (78:22). Likewise, Hoffman et al. [9] also noted peak distortion when injecting benzyl alcohol dissolved in different acetonitrile–water mixtures into a mobile phase of methanol–water (20:80).

According to the viscous fingering theory, an unstable boundary between the injection solvent and the mobile phase causes distortions in the analyte bands that elute in temporal proximity to the injection solvent plug [2,12,13]. In this case, one could

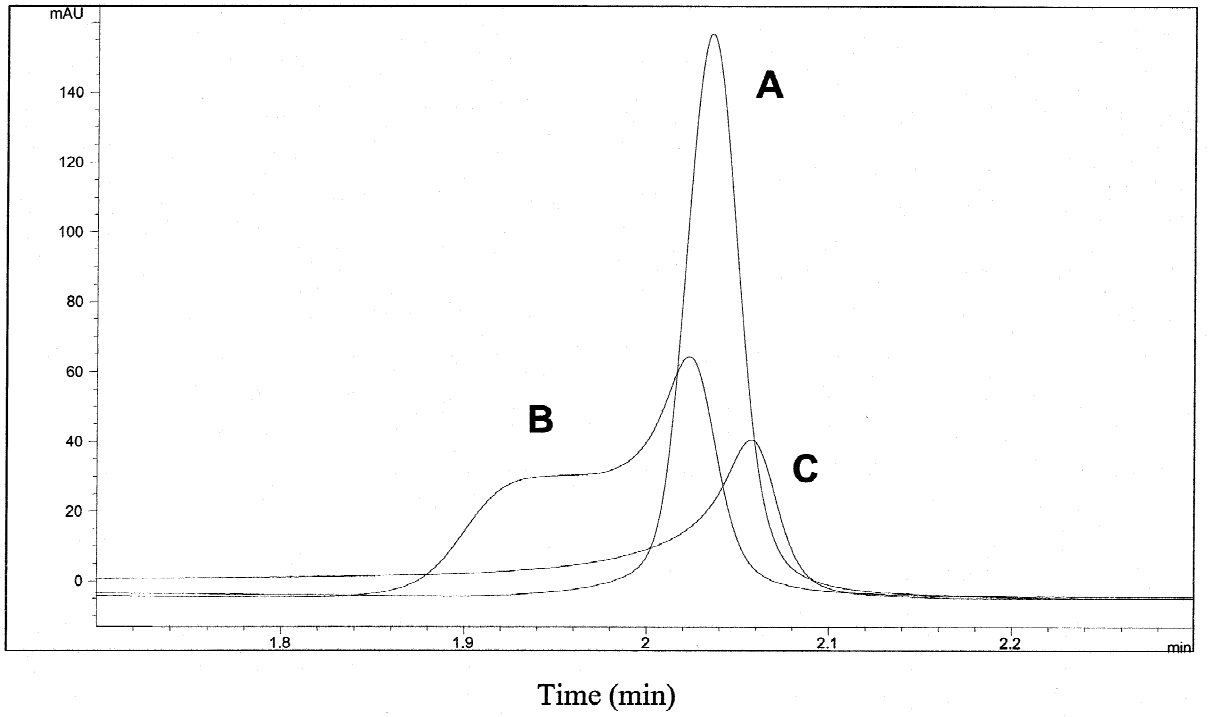

Fig. 1. Elution profiles for caffeine dissolved in (A) 33% acetonitrile (B) 66% acetonitrile and (C) 100% acetonitrile. Caffeine at 0.75 mg ml^{-1}, 4 μl injection volume. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

envision the low-viscosity injection solvent extruding forward into the more viscous mobile phase, resulting in the early elution of a fraction of the analytes which elute close to the injection solvent band [12,13]. Alternatively, the peak distortions may be due to the increase in injection solvent strength relative to the mobile phase [3,8,9]. Presumably, a strong injection solvent can disrupt the distribution equilibrium between the analyte and stationary phase, resulting in some of the analyte being carried through with the injection plug [9]. Whether such band distortions are due to the differences in the viscosity or solvent strength of the injection solvent and mobile phase is still unclear, as in each of these cases there are differences in both possible variables. What is clear is that the plug of strong sample solvent in its transition through the column induces a momentary local disturbance that may distort the analyte bands.

To investigate whether the injection solvent viscosity or solvent strength was responsible for the peak distortions, a high-viscosity liquid (polyethylene glycol, PEG-600) was added to the sample solvent (66% acetonitrile) to compensate for the decrease in viscosity. Dissolving the sample mixture in 66% acetonitrile containing increasing ratios of PEG-600 did not improve peak shape (Fig. 2, data for water–acetonitrile–PEG-600, 1:2:2, only shown). Although the viscosity of the sample solvent mixture was greatly increased, there was almost no change in peak shapes for the test analytes. Other viscous polymers, such as Tween-80 and glycerol, also gave no significant improvement in peak shape. These findings seem to indicate that sample solvent strength and not viscosity, is the cause of peak distortion. Although Czok et al. [2] did state that differences in injection solvent and mobile phase viscosity could cause peak distortions due to viscous fingering, they were quick to note that such effects were much more pronounced using large-bore columns that are typical of size-exclusion chromatography (e.g., 7.8 mm I.D.), as opposed to microbore columns. Thus, the

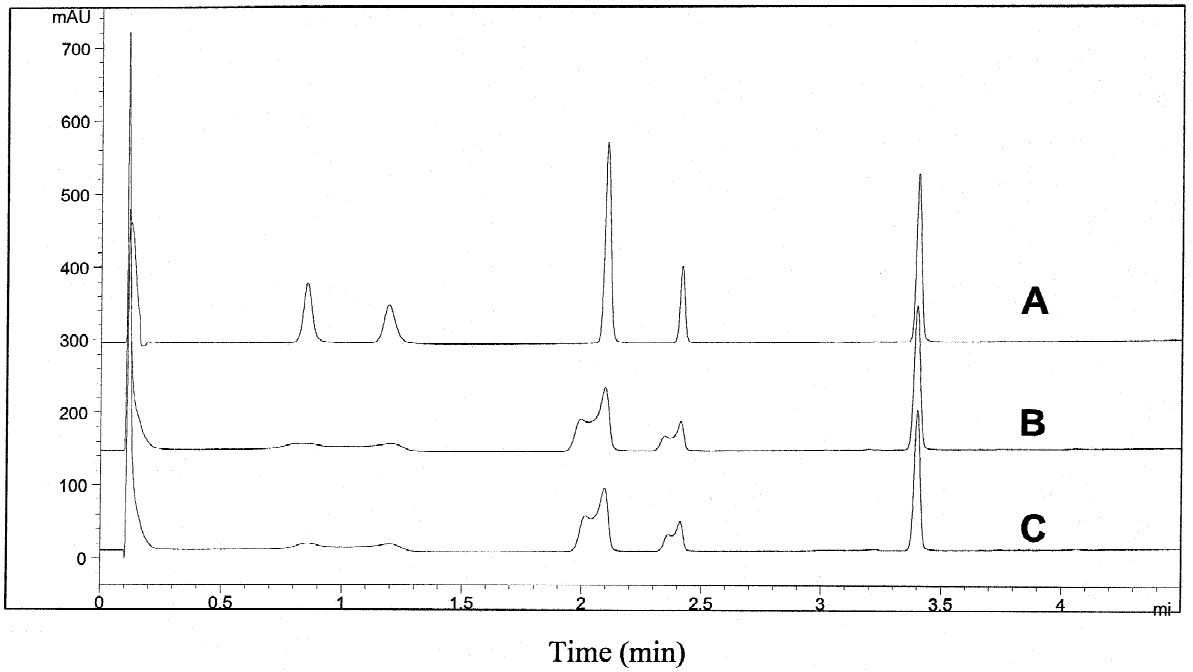

Fig. 2. Effect of adding PEG-600 to compensate for decrease in viscosity of injection solvent. (A) Sample mixture dissolved in water. (B) Sample mixture dissolved in 66% acetonitrile. (C) Sample mixture dissolved in water–acetonitrile–PEG-600 (1:2:2). Column: Luna 3 μm C_{18}(2) 30×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 4.5 min at a flow-rate of 1 ml min^{-1}. Injection volume: 20 μl.

findings of this study, using 2.0 mm I.D. columns, lend further support to solvent strength, rather than viscosity, being the root of the peak distortion effect.

As is apparent from the overlaid chromatograms in Fig. 3, the injection volume at a given injection solvent composition can also have a drastic effect on chromatographic performance.

In 100% acetonitrile, there is no distortion in the peak shape of caffeine when 1 μl is injected, but peak distortion increases as injection volume increases to 4 μl. Kachik et al. [6] found peak splitting for carotenoids when increasing the injection volume of hexane or THF into a methanol–acetonitrile–methylene chloride mobile phase from 50 to 100 μl. Zapata and Garrido [11] also found the extent of peak distortion for chlorophylls dependent upon the volume injected.

It has been stated that volume overloading can contribute to poor performance through band broadening effects [15] and that stronger solvents will have more of a negative effect than weaker solvents [20]. The explanation of the latter effect may be a disruption in the equilibrium distribution of analytes between the stationary phase and the mobile phase. For instance, we would expect that, upon injection of 10 μl of sample in 100% acetonitrile, the equilibrium distribution of analyte would be shifted greatly towards the solvent plug rather than the stationary phase. Alternatively, injection in a 25% acetonitrile would have less of an affect on the equilibrium distribution of the analyte between mobile phase and stationary phase.

The noted increase in band distortion with increasing injection volume probably has to do with the rates of dilution, or diffusional relaxation, of the injection plug. Since diffusional relaxation will be occurring at the external boundaries of the solvent band, we would expect that broad, large-volume solvent bands would take longer to be diffused by the mobile phase than thin, small-volume bands since it would take longer for the core of the band to be affected. Thus, we can expect to see peak

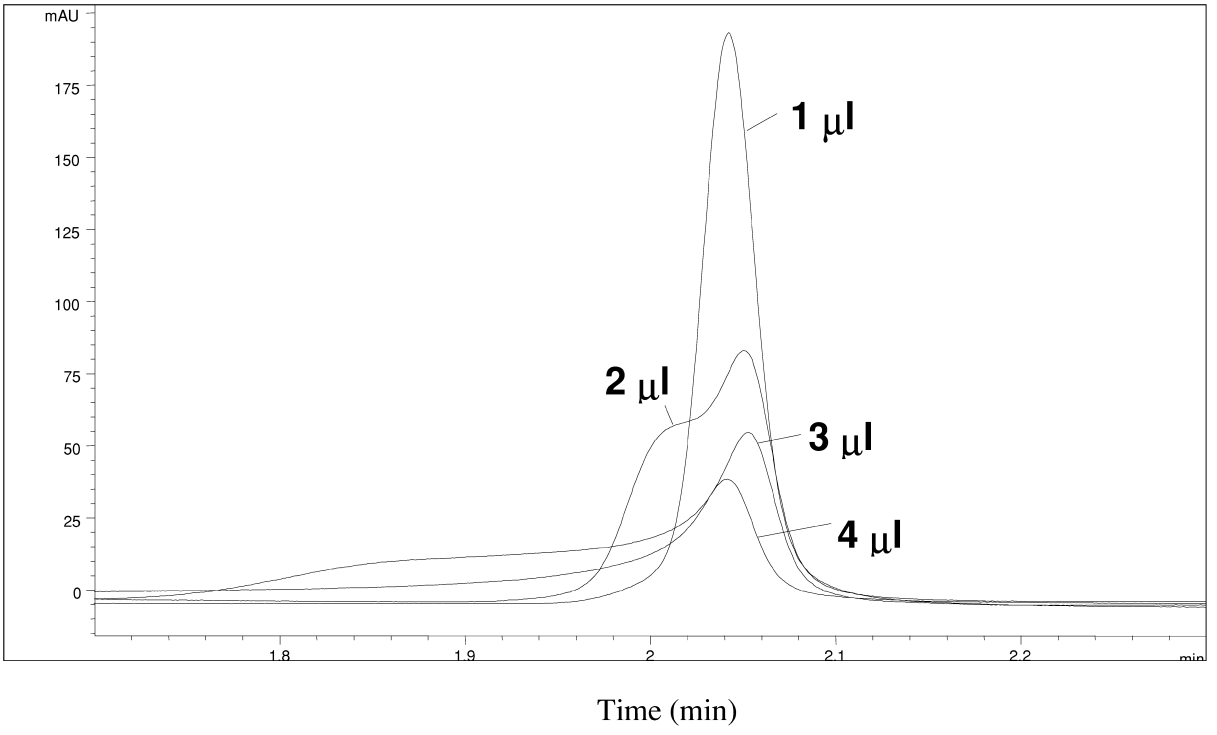

Fig. 3. Effect of sample injection volume for a given injection solvent composition. Caffeine at 0. 75 mg ml^{-1} in 100% acetonitrile. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

distortion as a function of the relative solvent strength of the injection solvent and of the volume injected for a given solvent composition. This explanation is supported by the fact that, for weaker sample solvents, a larger volume is needed before peak distortion becomes evident (e.g., when injecting a sample solvent of 25% acetonitrile, there is no distortion in the caffeine peak until 15 μl is injected, data not shown).

In addition, due to the temporal dependence of the sample solvent dispersion, one can expect that later-eluting compounds will be less affected than earlier-eluting analytes [6,9,17]. As is evident from Fig. 4, early eluting analytes (low *k*), such as caffeine, are distorted at low (1 μl) injection volumes of strong sample solvent (100% acetonitrile). However, later eluting analytes (large *k*) are able to withstand successively larger injection volumes before peak distortion is evident. Presumably, more strongly-retained analytes are not affected at low injection volumes because, by the time that the analyte band elutes from the column, the strong sample solvent plug has been diffused by the mobile phase and so cannot distort the eluting analyte band.

Thus, it seems that the peak distortion effects noted in this and other studies are due to a complex interaction of sample solvent composition, injection volume, and analyte retention factor. Graphically, this complex relationship can be depicted using three-dimensional mesh plots which show column efficiency (plates per column) (*z*-axis) as a function of the retention factor of the test probes (*y*-axis) and the injection volume (*x*-axis) at a given injection solvent composition (Figs. 5–7 for 100, 66 and 33% acetonitrile, respectively). In these graphs, column efficiency has been stated as ''apparent efficiency'' since the values obtained during gradient runs do not accurately reflect true plate heights due to compres-

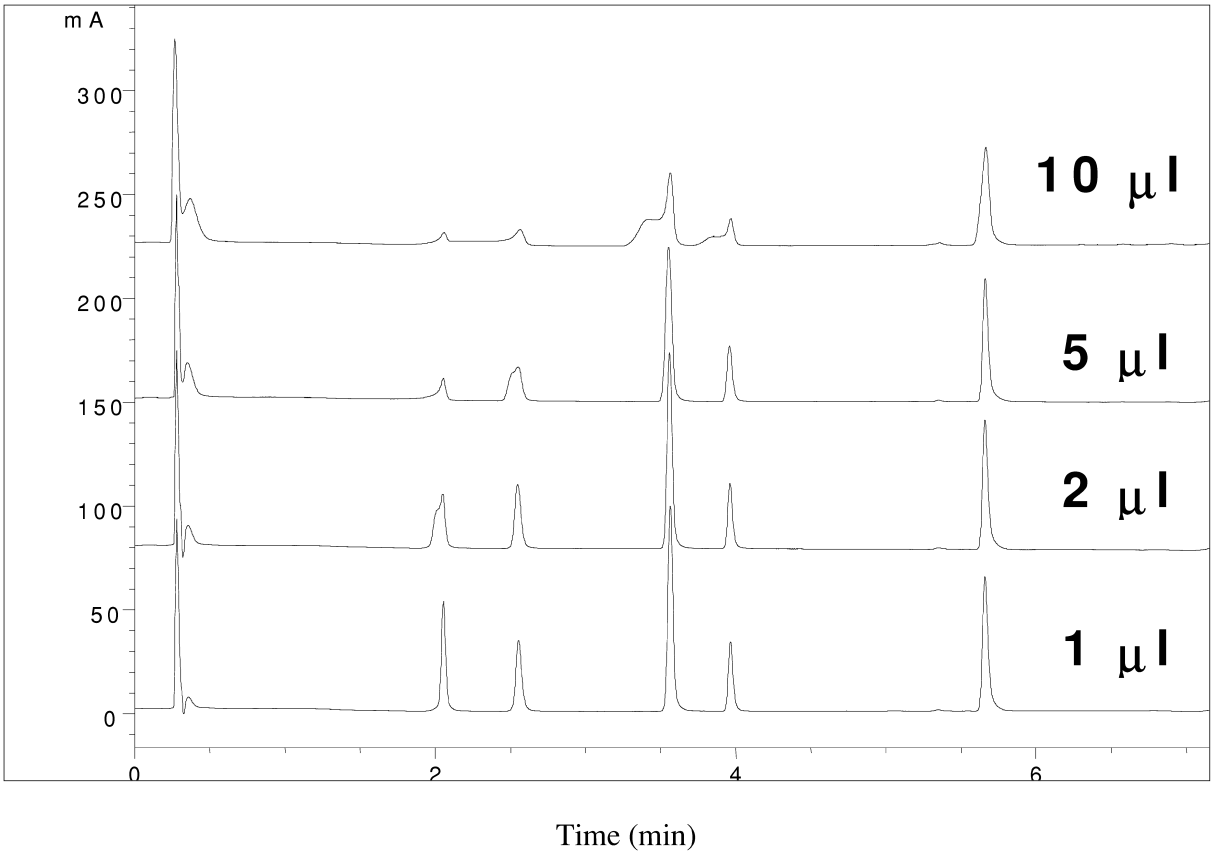

Fig. 4. The effect of injection volume and analyte retention factor on the extent of peak distortion. Sample mixture in 100% acetonitrile. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

sion of the peaks during gradient analyses. Several trends are made clear in these figures. (1) Later-eluting analytes (high *k* values) are able to withstand a larger injection volume than earlier-eluting peaks at each sample solvent composition. (2) When the strongest sample solvent is injected (100% acetonitrile), peak shapes deteriorate rapidly as indicated by the drastic drop in apparent efficiency with increasing injection volume. As the injection solvent is weakened (66% acetonitrile in Fig. 6 and 33% acetonitrile in Fig. 7), it is clear that column performance is maintained over a larger range of injection volumes and *k* values, with practically no decrease in performance for later-eluting analytes. From a practical point of view, these graphs indicate that analysts can maintain peak shapes by either using weaker sample solvents (e.g., diluting a given sample), or by adjusting the gradient such that the target analyte will be eluted with a larger *k* value.

However, in such instances when that is not possible, due to the presence of co-eluting analytes or other mitigating factors, we examined other possible solutions to the peak distortion problems associated with injecting strong solvents onto low-volume columns. One possible solution, as stated previously, is to inject a weaker sample solvent. For the analyst, this would mean taking a sample dissolved in a strong solvent (e.g., 66% acetonitrile), diluting it with a weak solvent or mobile phase (1:1 dilution), and then injecting a proportionally larger

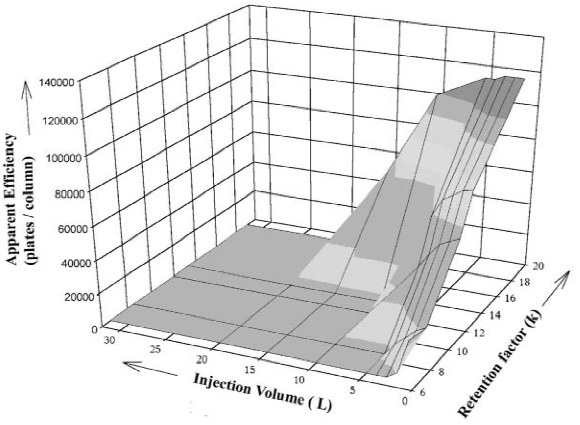

Fig. 5. Interaction between injection volume, analyte retention factor and apparent efficiency (plates per column). Samples solvent 100% acetonitrile. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

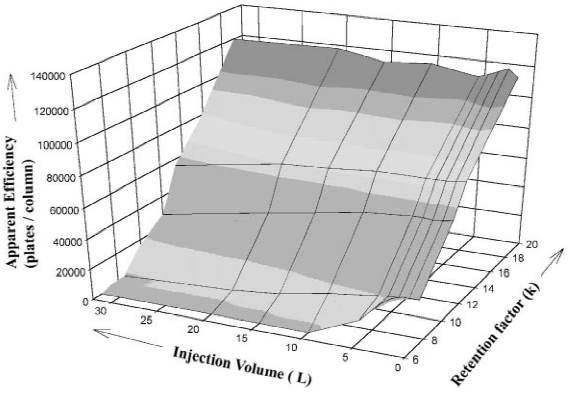

Fig. 7. Interaction between injection volume, analyte retention factor and column efficiency (plates per column). Samples solvent 33% acetonitrile. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

volume to maintain an equivalent mass load (20 μl in 33% acetonitrile as opposed to 10 μl in 66% acetonitrile). As shown in Fig. 8a and b, the effectiveness of such a technique may depend on the injection volume needed to obtain a suitable (based on qualitative or quantitative needs) mass load. At low injection volumes (<10 μl on a 30×2.0 mm, Fig. 8a), diluting a strong sample solvent and then injecting a larger volume appears to have little effect on chromatographic performance, presumably due to the fact that small volumes of solvent, generating relatively narrow solvent bands in the column, will be rapidly dispersed by the surrounding mobile phase. However, when larger sample volumes have to be injected (>10 μl, Fig. 8b) and solvent dispersion is slower, there is a significant improvement in chromatographic performance when a stronger sample solvent is diluted and a proportionally larger volume is injected.

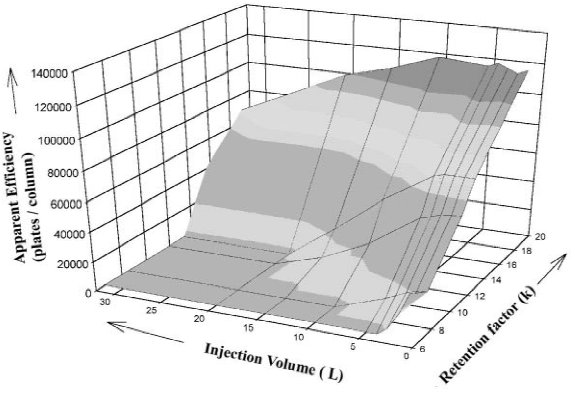

Fig. 6. Interaction between injection volume, analyte retention factor and apparent efficiency (plates per column). Samples solvent 66% acetonitrile. Column: Luna 3 μm C_{18}(2) 50×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 7.5 min at a flow-rate of 1 ml min^{-1}.

Increasing either the column length or inner diameter increases the relative loading capacity of the column overall (Fig. 9). As column length is increased (Fig. 9a), the volume loadability seems to increase. However, these results are somewhat misleading since, when the graphs are normalized to column length (not shown), the three lines overlap. This implies that, in an absolute sense, all three column lengths are degraded equally by the same volume load (e.g., a 20 μl injection volume will cause a 10% decrease in efficiency on each column). However, in a relative sense, since a longer column will have a higher plate count to begin with, it can withstand a greater decrease in performance before peak shape is completely distorted. When increasing column inner diameter (Fig. 9b), there is clearly an absolute increase in volume loadability.

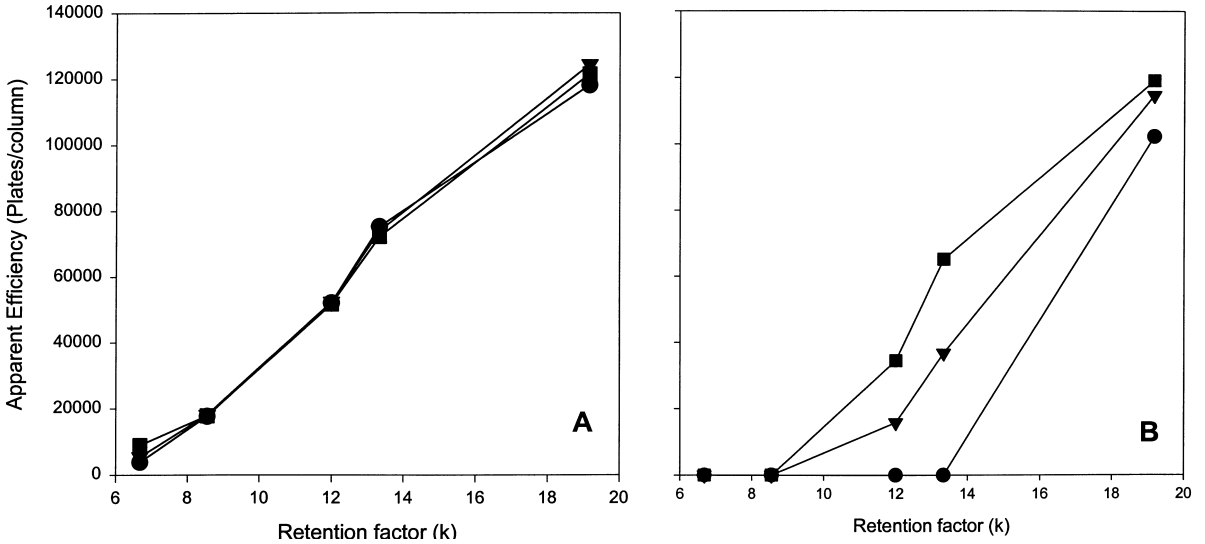

Fig. 8. The effectiveness of diluting a strong sample solvent and injecting a proportionally larger volume. (a) (●) 2 μl in 100% acetonitrile, (▼) 4 μl in 50% acetonitrile, and (■) 10 μl in 25% acetonitrile. (b) (●) 10 μl in 100% acetonitrile, (▼) 20 μl in 50% acetonitrile, and (■) 40 μl in 25% acetonitrile. Column: Luna 3 μm $C_{18}(2)$ 30×2.0 mm. Mobile phase: gradient 5 to 95% acetonitrile (with 0.1% formic acid) in 4.5 min at a flow-rate of 1 ml min^{-1}.

4. Conclusions

Chromatographic performance can be compromised as a function of the composition of the sample solvent and the amount injected. The data indicate that differences in sample solvent strength rather than viscosity are to account for the noted loss of performance when injecting samples which are dis-

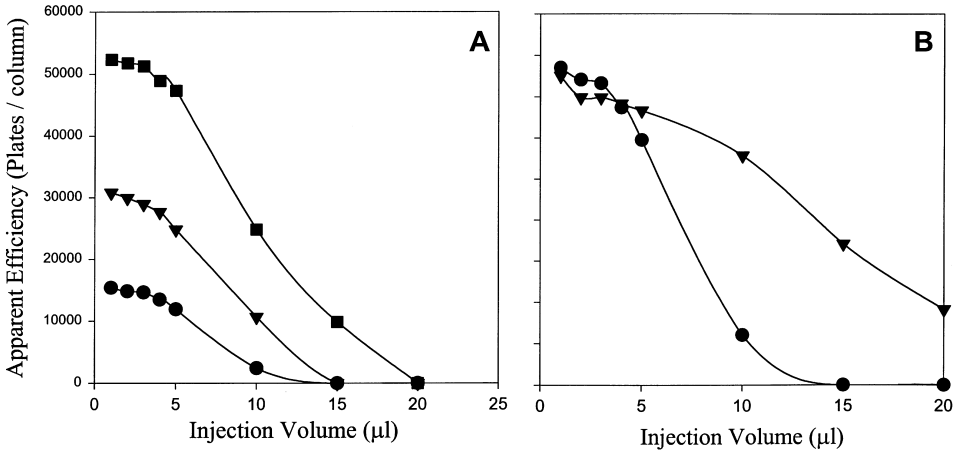

Fig. 9. Effect of changing column length and inner diameter on chromatographic performance (plates per column). (a) (●) 20×2.0 mm, (▼) 30×2.0 mm, and (■) 50×2.0 mm. (b) (●) 20×2.0 mm and (▼) 20×4.0 mm. Acetophenone (24 μl ml^{-1}) in 66% acetonitrile. Gradients and flow-rates adjusted so that analytes gave same retention factors on each column.

solved in strong solvents such as acetonitrile. This extent of peak distortion as a result of injecting solvents which differ in strength from the mobile phase is the result of a complex interaction between solvent strength, injection volume, and analyte retention factor. When injecting large volumes of strong solvents (>10 μl), chromatographic performance can be improved by diluting the strong sample solvent and injecting a proportionally larger volume to maintain an equivalent mass load. However, at low injection (<10 μl) volumes, there seems to be no significant improvement in chromatographic performance when diluting strong sample solvents.

If dilution of the sample is not an option, several parameters can be adjusted to improve performance: (1) adjust the gradient so that target analyte elutes with larger *k* values and so are less influenced by the early-eluting solvent band; (2) use a longer column and adjust the gradient to maintain the separation time; (3) switch to a column with a larger inner diameter and adjust gradient appropriately to maintain the separation.

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