APPLICATIONS

The Impact of Sample Solvent Composition and Injection Volume on Chromatographic Performance and Loading

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Introduction

Modern laboratories today are under pressure to improve chromatographic performance through high resolution and reproducibility; however due to increasing throughput, separations often sacrifice these goals for the sake of faster analysis times. In this tech note we study the effects of sample solvent concentration and injection volumes on the chromatographic performance of C18 bonded porous silica columns under gradient conditions. We explore the chromatographic problems which are faced under such situations and the solutions available[1].

Solid phase extraction (SPE) has been widely adapted for generating cleaner sample extracts of complex matrices such as serum. The drawback to such a technique however is it relies on extraction in high levels of organic solvent and leaves the chromatographer with the time consuming process of drying down the eluent to reconstitute it in a more appropriate injection solvent for the subsequent HPLC method. If analytes are injected directly on to the HPLC column in the strong eluting solvents typical in SPE methods peak distortion and splitting are observed.

Various authors have noted peak splitting and distortion when an injection solvent other than mobile phase is used however the origin of the distortion and splitting has been the subject of discussion. Several publications cite solvent strength[2-5] as the reason for the observed peak shape and others the solvent viscosity[6]. It was also found these effects were further magnified by the use of smaller columns in gradient elution mode.

Preparative chromatographers also face problems due to mobile phase – injection solvent mis-match. Much of this stems from the strong solvents required within prep chromatography to solubilize highly concentrated samples for purification and DMSO is commonly used. At column dilution can be used to relieve some of the effects seen where weaker solvents and smaller injection volumes cannot be used.

Experimental Conditions

System

Agilent HP 1100 HPLC system with an inline degasser (G1322A), autosampler (G1322A) and a HP 1100 column thermostat set to 30 °C (1316A), a HP 1100 binary pump (G1312A), and a HP 1100 DAD equipped with a 6-mm path length (G1315A). Absorbance was monitored at 254 nm and extraneous connective tubing was kept to a minimum (0.007 in I.D) to reduce extra-column volume effects. HP ChemStation (v 6.03) was used for data acquisition and analysis.

Materials

Acetonitrile, HPLC grade, by EM Science, Gibbstown, NJ, USA. Water, HPLC grade, by EM Science, Gibbstown, NJ, USA. Thiourea, Caffeine, Phenol, Acetophenone, Dimethylphthalate, Valerophenone, Formic Acid.

Sample Preparation

The goal of the study was to examine the effect of sample solvent composition and injection volume on chromatographic performance so it was important to eliminate mass loading effects.

A stock solution of Thiourea (32 mg/mL) Caffeine (40 mg/mL) Phenol (16µL/mL) Acetophenone (24µL/mL) Dimethylphthalate (64 µL/mL) Valerophenone (32 µL/mL) was prepared in acetonitrile at 6 different concentrations (25, 33, 50, 66, 75, 100 %). Using each of these 6 samples serial dilutions were made to accommodate appropriate injection volumes from 1-100µL to maintain equivalent mass load. Three injections were made for each volume and concentration point and the mean average used for data analysis.

HPLC Running Conditions

All chromatography was performed using the Luna C18(2) 3 µm sorbent packed into 4 different column dimensions (50 x 2.0 mm, 30 x 2.0 mm, 20 x 2.0 mm and 20 x 4.0 mm)

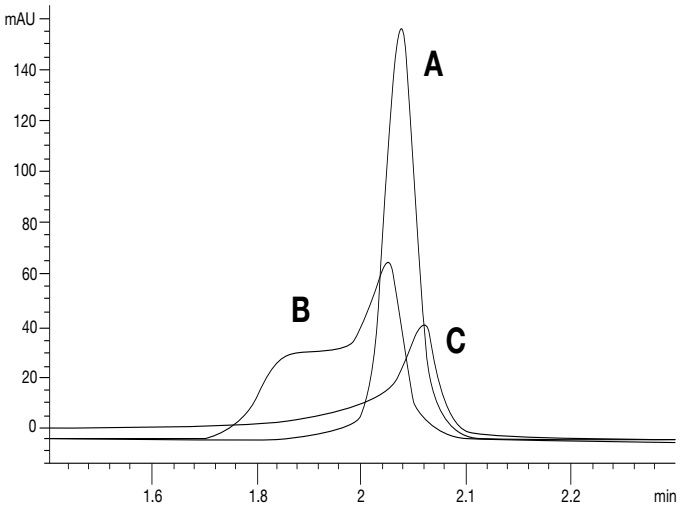
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile
Gradient: A/B (95:5) to A/B (5:95) over 3 minutes for the 20 mm columns, 4.5 minutes for the 30 mm columns and 7.5minutes for the 50 mm columns.
Flow Rate: 1 mL/min for the 2.0 mm ID and 4 mL/min for the 4.0 mm ID columns to maintain a linear velocity of 0.3 cm/s
Detection: DAD @ 254 nm

Results and Discussion

This work demonstrated the effect of diluent strength and sample volume on chromatographic performance.

In a moderately weak solvent (33 % acetonitrile) caffeine is eluted with symmetrical peak shape (A), as the diluent strength is increased (from 66 % (B) to 100 % (C) a gradual distortion is observed (**Fig. 1**)[1]

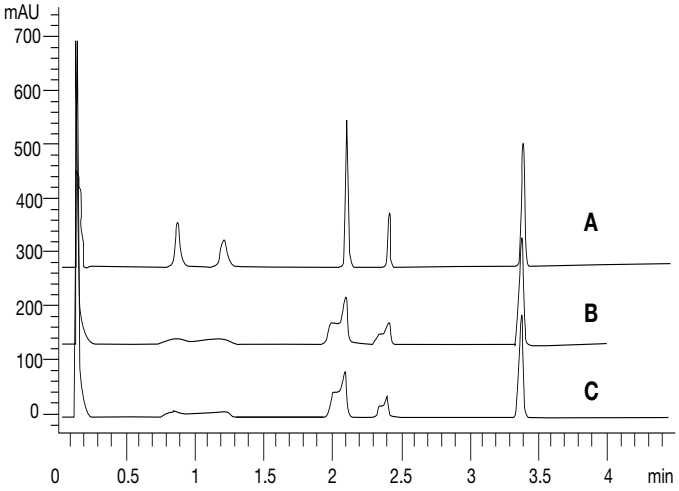
Fig 1. Column Luna C18(2) 3µm 50 x 2.0 mm, injection volume 4 µL



To ascertain whether the nature of this distortion was due to solvent strength or solvent viscosity a high viscosity liquid (PEG-600) was added to B (66 % acetonitrile) to counter the decrease in viscosity through the increase in acetonitrile. No improvement in peak shape was observed suggesting the distortion in peak shape is a direct result of an increase in solvent strength and not through a change in the viscosity of the injecting solvent.

This data is displayed in **Fig. 2** and clearly shows there is little different in traces B and C which were both run using a 66 % acetonitrile diluent (compared to trace A in 100 % water) If the effect seen in **Fig. 1** was a result of viscosity we would have seen a difference in the results for B and C when the PEG-600 was added to C and this was not evident.

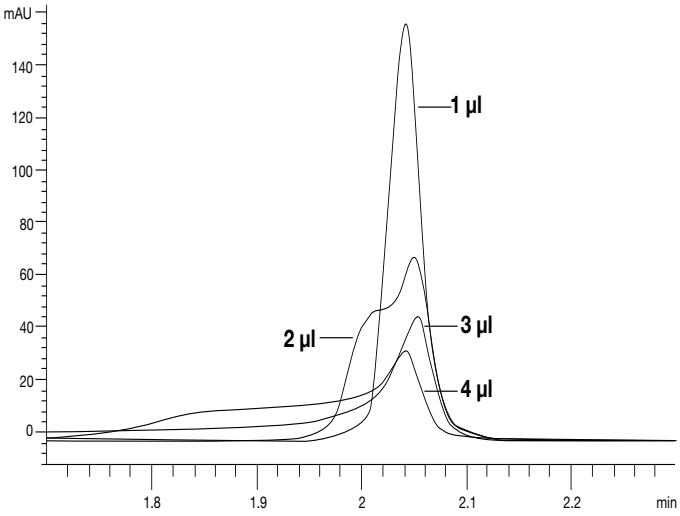
Fig 2. Column – Luna C18(2) 3 µm 30 x 2.0 mm, injection volume 20 µL



(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)

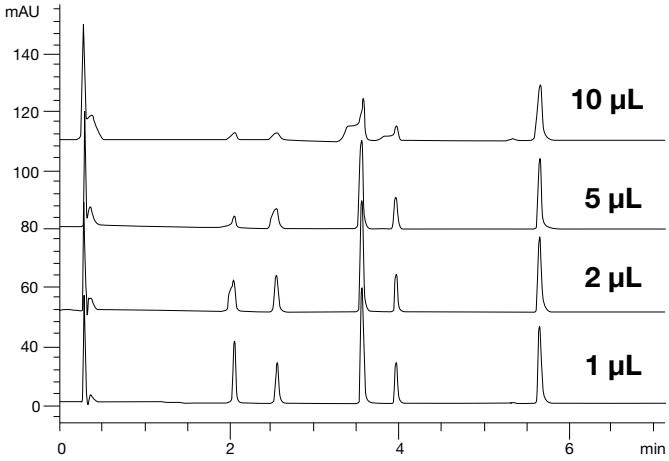
Injection volume can also have a marked effect on peak shape (**Fig. 3**) When injecting caffeine in 100 % acetonitrile at a volume of 1 µL there is no marked effect on the symmetry of the peak however as the volume increase peak shape gradually distorts.

Fig 3. Column – Luna C18(2) 3 µm 50 x 2.0 mm, Caffeine at 7.5 mg/mL in 100 % acetonitrile



Band broadening through volume overload is seen to a greater extent when a strong injection solvent is used which results in a disruption to the equilibrium of the distribution between the analyte and stationary phase. This effect is greater for early eluting peaks. Later eluting peaks are more able to withstand successively larger injection volumes before the peak distortion is observed (**Fig. 4**).

Fig 4. Column Luna C18(2) 3 µm 50 x 2.0 mm, injection solvent 100 % acetonitrile



Through the use of 3-dimensional mesh plots (not shown) several trends become clear. Later eluting analytes are able to withstand larger injection volumes than the early eluting peaks at the same sample composition. A rapid drop in efficiency is observed with increasing sample volume when the strongest diluent (100 % acetonitrile) is used. This is improved as the diluent is weakened thus column performance maintained over a larger range of injection volumes

In summary analysts can maintain peak shape by either using a weaker strength diluent or adjusting the gradient such that the analyte will have a higher k value.

For preparative chromatography the challenges are different. Higher sample concentrations and large injection volumes are necessary and typically this results in DMSO and other strong organic solvents being used to solubilize the sample. The recommendations for preparative separations are much the same analytical applications and where possible should match the starting conditions of the method however in reality this does not always prove possible and preparative chromatographers may have to use at column dilution[7] to alleviate some of the effects from the constraints of the injection solvent.

Conclusions

The composition of the sample diluent is critical to the success of the chromatographic method. Chromatographic performance can be compromised if the sample strength is too high or the injection volume too great. Solvent strength appears to account for this loss of performance rather than viscosity of the sample and is a result of a complex relationship between solvent strength, injection volume and analyte retention factor.

Where dilution of your sample is not an option other parameters can be adjust to improve the performance of the method

1. Adjusting the gradient to increase the retention of your target analyte (K) later eluting peaks are less effected by injection in strong solvents

2. Use a longer column to increase retention time

3. Switch to a column with a larger internal diameter to allow for greater volume loading and adjust the gradient to maintain separation

In a preparative environment where it is not possible to match the mobile phase composition to the diluent due to solubility concerns a chromatographer may want to use at-column-dilution to reduce this effect.[7] At-column dilution delivers the organic solvent to the column in the same path as the injector which introduces the sample. This stream is combined with the aqueous portion immediately before the column which reduces the distortion and peak tailing when using solvents such as DMSO as a diluent. Alternatively a pre-column installed at the head of column filled with silica particles of 20 µm-60 µm can also be used to reduce effect of strong injection solvents by disrupting the effect of this diluent as it reaches the pre-column at the head of the preparative system[8]. In effect the pre-column acts as a mixing chamber between sample injection and mobile phase prior to reaching the HPLC column.

References

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Luna® Ordering Information

3 µm Minibore, MidBore™, and Analytical Columns (mm)						SecurityGuard Cartridges (mm)	
Phases	50 x 2.0	50 x 3.0	150 x 3.0	50 x 4.6	100 x 4.6	4 x 2.0*	4 x 3.0*
						10pk	10pk
C18(2)	00B-4251-B0	00B-4251-Y0	00F-4251-Y0	00B-4251-E0	00D-4251-E0	AJ0-4286	AJ0-4287
						for ID: 2.0-3.0 mm	3.2-8.0 mm

5 µm Minibore, MidBore™, and Analytical Columns (mm)							SecurityGuard Cartridges (mm)	
Phases	50 x 2.0	150 x 3.0	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 2.0*	4 x 3.0*
							10pk	10pk
C18(2)	00B-4252-B0	00F-4252-Y0	00B-4252-E0	00D-4252-E0	00F-4252-E0	00G-4252-E0	AJ0-4286	AJ0-4287
							for ID: 2.0-3.0 mm	3.2-8.0 mm

5 µm Semi-Prep Columns (mm)		SecurityGuard Cartridges (mm)
Phases	250 x 10	10 x 10***
		3/pk
C18(2)	00G-4252-N0	AJ0-7221
		for ID: 9-16 mm

5 µm Analytical and Semi-Prep Columns (mm)				SecurityGuard Cartridges (mm)	
Phases	100 x 4.6	150 x 4.6	250 x 4.6	4 x 3.0*	10 x 10‡
C18(2)	00D-4252-E0	00F-4252-E0	00G-4252-E0	AJ0-4287	AJ0-7221
				3.2 - 8.0 mm	9 - 16 mm

5 µm Axia Packed Preparative Columns (mm) (cont'd)				SecurityGuard Cartridges (mm)
Phases	50 x 30	100 x 30	250 x 30	15 x 30 ◊
C18(2)	00B-4252-U0-AX	00D-4252-U0-AX	00G-4252-U0-AX	AJ0-8301
				for ID: 30 - 49 mm

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000
* SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282
‡ SemiPrep SecurityGuard Cartridges require holder, Part No.: AJ0-9281
** PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8223
◆ PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8277

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