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Characterization of a 2.6 μm Kinetex porous shell hydrophilic interaction liquid chromatography column in supercritical fluid chromatography with a comparison to 3 μm totally porous silica

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ABSTRACT

The first systematic study of the performance of a porous shell, hydrophylic interaction liquid chromatography (HILIC) column in supercritical fluid chromatography (SFC) is presented. Observed efficiency on 2.6-μm porous shell particles exceeded all reports using UHPLC on 100-mm long columns packed with <2-μm totally porous particles. A Kinetex 4.6 × 150 mm, 2.6 μm HILIC column significantly outperformed a 3 μm Luna totally porous silica of the same length and diameter. A 17 component, low molecular weight test mix, consisting of a range of small drug-like molecules was separated isocratically on each column, with similar selectivity, but the porous shell column required ½ the time (≈2 min vs. 4 min), with almost 50% higher efficiency. Even little retained compounds ($k < 0.5$) exhibited more than 30,000 plates under some conditions. Reduced plate heights were higher than previously reported on porous shell particles in both HILIC and rHPLC, with the lowest value of 1.62. Significant fronting was sometimes observed. The cause of the fronting was not determined. The least symmetrical peaks showed the highest apparent efficiency. Pressure drop at optimum velocity (2.5 ml/min) and low modifier concentrations was <60 bar, and only exceeded 250 bar at near double optimum flow and 65% modifier. Peak widths were mostly just over 0.01 min (20 Hz) wide. There was a loss of efficiency when the injection volume was increased. The chromatograph was shown to have extremely low extra-column dispersion, on the order of 5–10 μL^2, which is also the lowest reported in an SFC, in spite of using standard components. This is likely due to turbulent flow in the tubing and fittings.

1. Introduction

1.1. Sub-2 μm particles for high speed HPLC

After stagnating for more than a decade, column development for high performance liquid chromatography (HPLC) has recently seen dramatic changes. The most obvious change has been in the proliferation in the use of sub-2 μm particles and the chromatographic hardware necessary to use them, now being re-characterized as ultra-high performance liquid chromatography (UHPLC). Such particles promise a 10-fold decrease in analysis time, or a significant increase in total efficiency compared to 5 μm particles. However, the price is a dramatic increase in system pressure requirements. Pump pressure requirements have increased from 400 bar to 600 bar, to 1200 bar, and continue to rise. This shift has threatened to make the vast majority of installed HPLC's obsolete, and has prompted a search for alternative technology.

The resistance to mass transfer by small particles results in heating of the mobile phase [1–5]. Radial temperature gradients, caused by resistive heating, have been shown to cause losses in efficiency [3], which, in turn, dictates the use of smaller, i.e. 2.1 mm ID columns, to minimize these radial temperature effects. On such columns, flow rates of <0.25–0.5 ml/min are common. Pressure drops approaching 1000 bar are common [6–9]. Mixing volumes, or gradient delay volumes need to decrease 10×, since flow rates are lower, and runs are faster. Run times are up to 10 times faster, and gradients must be 10 times steeper. Injection volumes must drop perhaps 10×, yet maintain precision. Some autosampler/software systems impose a >2 min wait between the end of one run and the injection of the next run. Autosampler and software delays between injections should be <50% of the total analysis cycle time, or result in unnecessarily long delays between short chromatographic runs. The required detector cell volume (<1 μL), and path length, become extremely small. Detector signal filtering must decrease (10×), causing electronic noise to increase 2.7×, based simply on the laws of physics. With the extremely low variance of peaks produced by such columns, the chromatograph must, subsequently, have extremely low extra-column dispersion.

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1.2. Porous shell particles for high efficiency separations

A relatively recent, alternative, approach [10] to sub-2 μm particles, yielding higher efficiency separations, has involved the use of porous shell particles, which have found rapid acceptance in HPLC, and even more recently in UHPLC. A hard, non-porous center particle is coated by a thick film of totally porous silica. Unlike pellicular packings from 20+ years ago, where the porous layer was thin, the porous layer of these new packings is a significant fraction of the total particle diameter. Efficiency is related to the diffusion path length, which is the porous shell thickness, not the total particle diameter. A common particle size is ≈2.6 μm, with approximately 2/3rds taken up by the porous shell. The column pressure drop is dictated by the particle diameter squared (d_p^2). Obviously, 2.6 μm particles will have a lower pressure drop compared to 1.7 or 1.8 μm particles, yet produce more plates/meter. Lower pressure drops should mean lower resistive heating and somewhat relax the requirement for smaller column inner diameters. In porous shell packings, the cost of higher efficiency and lower pressure drops, may be in loadability, which is proportional to the total surface area of the column.

High HPLC efficiencies have been generated in relatively short times, with relatively low pressure drops, using porous shell particles. Gritti and Guiochon [11] reported reduced plate heights of 1.0–1.3, which was a new "record" in LC. These numbers were obtained after independently measuring, then subtracting the extra-column contributors to plate height, which appeared to encompass approximately 20% of the total dispersion observed.

The porous shell, 2.6 μm particles, were shown to out-perform 1.7 μm particles in UHPLC, in terms of efficiency, yet generated relatively modest pressure drops. Others have produced reduced plate heights as low as 1.2 in HPLC using the Kinetex 2.6 μm particles [12]. There have been fairly extensive theoretical analyses [13,14] of these particles.

McCalley [15] reported reduced plate eights of 1.5 with a porous shell column used in HILIC mode with a mobile phase consisting of 90% acetonitrile, 10% aqueous formic acid mixtures.

Obviously, a column packed with porous shell particles could theoretically be half as long as a totally porous column of the same particle diameter, with twice the speed, but with 1/2 the pressure drop. Similarly, a column packed with the porous shell particles could be half as long as a totally porous column of ½ the particle diameter and produce the same efficiency, in the same time, but with 1/4th the pressure drop.

1.3. SFC for high speed separations

Supercritical fluid chromatography (SFC) is yet another approach to achieving very high efficiency in very short times, with lower pressure drops, while still using a solvating mobile phase. In HPLC, the emphasis is, necessarily, on limiting the diffusion distance, assuming the diffusion coefficients are fixed. The shorter the diffusion distance, the higher the optimum linear velocity. Totally porous sub 2 μm particles limit the diffusion distance by making the particles smaller. Porous shell particles limit the diffusion distance, by decreasing the total diffusion distance to the thickness of the porous shell. In SFC, the improvement in speed is based on both the diffusion distance and the difference in the diffusion coefficients in CO_2 based mobile phases, compared to the diffusion coefficients in water, organic solvents, or mixtures of the two.

Generally, in SFC, the mobile phase consists mostly of highly compressed CO_2. Since there are no strong intermolecular interactions (the fluid is a gas at atmospheric conditions), the diffusion coefficients and mobile phase viscosities in SFC remain up to an order of magnitude higher [16–20] and lower [21,22], respectively, compared the mostly organic solvent/aqueous based mobile phases used in reversed phase or HILIC. Since the diffusion coefficients are usually 3–5× larger in SFC, molecules can diffuse 3–5 times further in the same time, compared to HPLC. This means that particles can be 3–5 times bigger and still produce the same speed as in HPLC (or UHPLC).

On the same sized particles, SFC should always produce similar efficiency, significantly faster, with significantly lower pressure drops, compared to any form of HPLC. Unfortunately most published separations in SFC have been performed on 5 μm particles, largely obscuring the very real advantages of SFC over HPLC or even UHPLC.

In a recent report [23], very fast separations were demonstrated using 1.8 μm totally porous particles in SFC, simultaneously exhibiting superior speed to UHPLC, with 1/5th to 1/8th the pressure drops. The low pressure drops appear to make it possible to achieve high speeds, with high efficiencies, using mostly conventional 400 bar HPLC equipment.

Porous shell particles potentially offer a major increase in efficiency at any given particle diameter, compared to totally porous particles. Adapting such particles to SFC should allow the same efficiency as in HPLC, while decreasing analysis time by 3–5 times, plus decreasing pressure drops. Surprisingly, there is almost no mention in the chromatographic literature of the use of porous shell particles in SFC. In fact, a single chromatogram using a porous shell column appeared in a review of SFC columns [24]. There are no systematic studies on the performance of porous shell particles in SFC.

Most column manufacturers only sell some version of C_{18} in porous shell format, and have not developed normal phase versions of such particles, although a HILIC phase is available.

In this report, a 2.6 μm porous shell HILIC column was evaluated in SFC. The manufacturer did not explicitly state the nature of the phase but it appears to act as bare silica. It was used because it was the only commercially available porous shell column compatible with SFC. Efficiency was measured as a function of flow rate at several modifier concentrations. The dispersion of a slightly modified HPLC was evaluated to determine if extra-column effects affect measured plate heights. Pressure drops with and without the column were measured. A diverse range of small drug like molecules were separated and compared to a conventional silica column. A preliminary study of solute loadability was presented.

2. Experimental

2.1. Instrumentation

The chromatograph consisted of a Fusion™ A5 SFC conversion module (Aurora SFC Systems, Inc., Redwood City, CA) with an Agilent Technologies (Waldbron, Germany) HPLC. The HPLC consisted of: a Model 1200SL binary (high pressure mixing) pump, solvent cabinet, wellplate autosampler, thermostated column compartment (TCC), Model 1200C diode array detector (80 Hz) with a 1.7 μL, 6 mm path, 400 bar flow cell, a Model 1100 degasser, and a standard ChemStation. The low pressure active inlet check valves of the Agilent pump were replaced with ruby ball, sapphire seat passive dual check valves, with a return spring on one of the balls, and a large filter frit.

The autosampler used is normally plumbed as "broken loop", where the syringe, needle and a transfer line are all under system pressure during a run. This style of autosampler is incompatible with SFC since, when switched to the load position, the compressed mobile phase in the syringe and transfer line, decompress and are vented to atmosphere. Subsequently, the empty syringe cavitates and cannot pick up samples, or must be filled with a large volume of a solvent stronger than the mobile phase, destroying peak shapes of early eluting solutes in the next run.

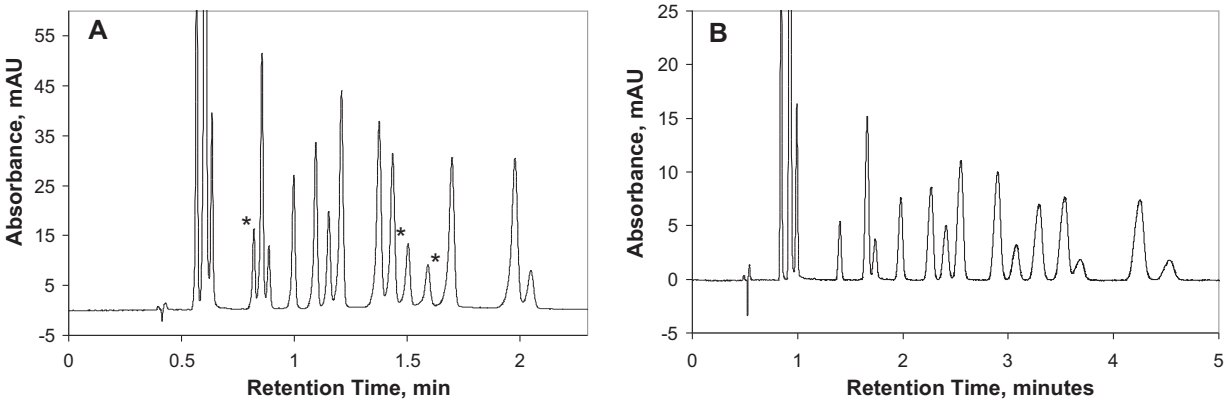

Fig. 1. Separation of a 17 component mix: top: Kinetex 4.6 × 150 mm, 2.6 μm HILIC column, bottom: Luna 4.6× 150 mm, 3 μm bare silica column. Conditions: 3.5 ml/min, 15% methanol in CO_2, 175 bar column outlet pressure, 50 °C. See Table 1 for compound identities. Asterisk's denote peak reversals.

The injection valve was converted to be used in an "external loop" mode. The 2 groove rotor used in the standard Agilent injection valve, was replaced by a 3 groove rotor. The various positions on the valve were replumbed. A length of 10 cm long, 0.0127 cm i.d. stainless steel tubing was used as the external loop between ports 2 and 5. The pump and column were connected to ports 1 and 6. The needle and waste were connected to ports 3 and 4. An injector program was substituted for the standard injection sequence. The injection program and modifications have been documented elsewhere [23,25].

Both heat exchangers (HX's) in the TCC were used; one HX to preheat the mobile phase before entering the column and the other HX to match the fluid temperature to the detector cell temperature (post column thermal conditioning). One HX is reported as having a 3 μL volume and the other a 6 μL volume. A zero dead volume connector and a 10 cm piece of 0.125 mm ID tubing connected the detector tubing to the HX.

The SFC module pre-compressed the CO_2 before it reached the left hand (A side) HPLC pump. Consequently, the compressibility of the left (A-side) CO_2 HPLC metering pump was set to zero. There is no chiller on this metering pump. The compressibility of the modifier pump (B-side) was set to 130×10^{-6}/bar, for use with degassed methanol.

The flow rate, modifier concentration, column temperature, injector and, detector settings etc., were controlled by the standard Agilent ChemStation. The column outlet pressure was controlled by a small software add-on in the ChemStation graphical user interface (GUI).

2.2. Columns

The columns were 4.6 × 150 mm, 2.6 μm Kinetex HILIC, and 4.6 × 150 mm, 3 μm Luna silica, both kindly donated by Phenomenex, Torrence, CA. Both were packed in a non-aqueous mobile phase for shipment. They were washed for 15 min with pure CO_2, followed by 15 min with 40% methanol in CO_2.

2.3. Chemicals

"Beverage grade" CO_2 was purchased from Terry's Supply Co., Sarasota, FL in 50 pound steel cylinders without a DIP tube. Methanol was Omnisolve grade from SECO, Aston, PA. Aceamidophenol, caffeine, cortisone, flurbiprofen, hydrocortisone, ibuprofen, ketoprofen, naproxen, prednisilone, prednisone, sulfadimethoxine, sulfamethizole, sulfamethoxazole, sulfaquinoxaline, theobromine, theophyline, thymine, uracil, and warfarin (all > 98% pure) were purchased from Sigma Aldrich, St. Louis, MO, and used as received.

3. Results and discussion

3.1. Comparing selectivity of Kinetex HILIC and luna silica

A diverse group of 17 small drug-like molecules was separated on both a Kinetex porous shell HILIC and a Luna totally porous silica column, under the same isocratic conditions, as shown in Fig. 1. The compounds, listed in Table 1, were selected from a list, often used to test column selectivity [23,25]. Most are, or have been, widely used pharmaceuticals. They represent hydroxysteroids, non-steroidal anti-inflammatories, sulfonamides and xanthenes.

Each solute was individually injected under a number of different isocratic chromatographic conditions. Retention time reproducibility was shown to be 0.1–0.3% (n = 5–10). Mixtures representing each family of compounds were also injected. Finally the complete mix was injected under the same sets of experimental conditions. The selectivity of the two columns was compared, as shown in Fig. 1.

Each different solute family behaved differently to changes in physical parameters, making isocratic optimization tricky. The over-all resolution of the 17 component mix, which was not quite baseline resolved at 15%, was significantly degraded at either 14%

Table 1
Elution order of the 17 component test mix on the Kinetex 4.6 × 150 mm, 2.6 μm HILIC porous shell column and on the Luna 4.6 × 150 mm, 3 μm silica column. Note the 3 apparent peak reversals between columns indicated in bold. Conditions: 3.5 ml/min, 15% methanol, 175 bar outlet pressure, 50 °C.

Kinetex HILIC	Luna silica
1. Flurbiprofen	Flurbiprofen
2. Naproxen	Naproxen
3. Ketoprofen	Ketoprofen
5. Warfarin	Warfarin
6. Theophyline	Theophyline
7. Caffeine	Caffeine
9. Thymine	Thymine
10. Uracil	**Cortisone**
11. Cortisone	**Uracil**
12. Prednisone	**Prednisone**
13. Aceamidophenol	**Aceamidophenol**
14. Hydrocortisone	**Sulfamethizole**
15. Sulfamethizole	**Hydrocortisone**
16. Prednisilone	**Sulfadimethoxine**
17. Sulfadimethoxine	**Prednisilone**
18. Sulfaquinolaline	**Sulfaquinolaline**
19. Sulfamethizide	Sulfamethizide

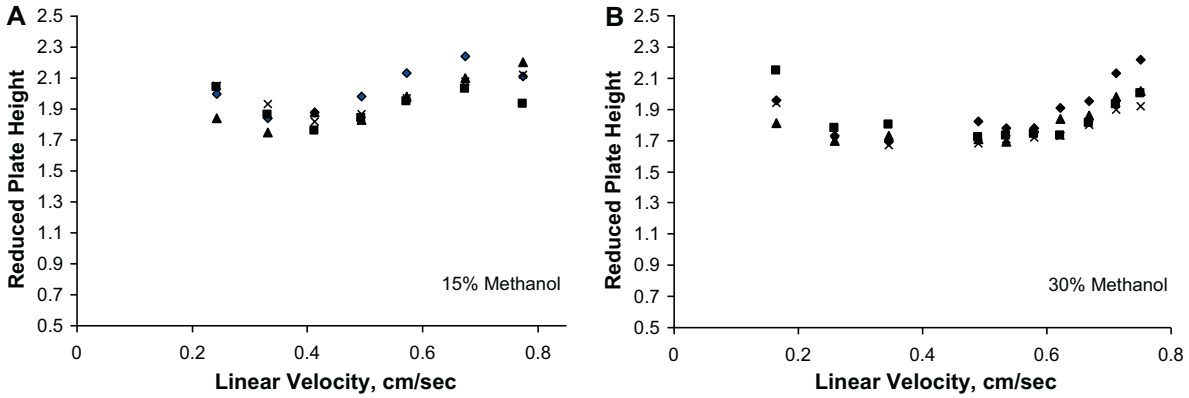

Fig. 2. Plots of reduced plate height vs. average linear velocity at top: 15% methanol, bottom: 30% methanol. Other conditions 150 bar outlet pressure, 50 °C. Column: Kinetex porous shell 4.6 × 150 mm, 2.6 μm HILIC.

or 16% modifier. Increasing the pressure tended to improve the separation of several pairs.

The selectivity of the two columns was only slightly different. The HILIC column appears to act essentially as a bare silica column, but with slight differences. The asterisks in Fig. 1, and the bold characters in Table 1, point out the main differences. When running individual standards, uracil and cortisone appeared to reverse retention order on the two columns. However, on comparing the chromatograms in Fig. 1, the relative peak heights suggest this reversal does not happen when the mixed standard is injected. The other two peak reversals are obvious.

3.2. "Apparent" "average" linear velocity

Apparent average linear velocity was used to generate pseudo-Van Deemter plots. "Apparent", since the first perturbation of the baseline after injection was assumed to be due to "unretained" sample solvent. Non-retention was not necessarily true. "Average" since there was a pressure drop from the column inlet to its outlet that results in a significant drop in density and increase in linear velocity along the length of the column. Further, the fluid was heated from just under ambient temperature near the pump to 50 °C just before the column, significantly decreasing the mobile phase density. It is unknown whether there is a temperature increase along the length of the column due to resistive heating, as in HPLC, or a temperature decrease due to the expansion of the fluid, or a combination of the two. Thus, the convention of "average" linear velocity, used in gas chromatography, was used here in SFC.

3.3. Efficiency

Reduced plate height was plotted against the apparent, average linear velocity. The column efficiency was measured with a test mix of naproxen, thymine, hydrocortisone, and sulfamethizide. Each of the 4 solutes, representing significantly different compound types, with rather low molecular weights, exhibited similar high efficiencies. The Luna 3 μm totally porous column generally exhibited ≈23,000 plates, or approximately 92% of theoretical, with an average reduced plate height of 2.17.

Representative plots of reduce plate height vs. average linear velocity, for the Kinetex column at two different modifier concentrations, are presented in Fig. 2. Reduced plate heights were only as low as 1.67, somewhat higher than reported by the manufacturer, or McCalley in HILIC [15] and significantly higher than reported by Gritti and Guiochon [11] in rHPLC on a C18 Kinetex column with similar 2.6 μm particles. The curves were flat but somewhat irreproducible. To achieve the highest efficiencies, small injection volumes (0.1–0.5 μL) were required.

The Kinetex porous shell column showed significantly higher efficiency, in half the time, compared to the totally porous Luna silica column with a similar particle size, as shown in Fig. 1. The highest efficiency observed exceeded 35,000 plates. The shorter retention time on the porous shell column was probably due to the lower total surface area on that column compared to the totally porous column. The increased efficiency, compared to the totally porous packing allowed better resolution of the mix in spite of the shorter run time.

3.4. Flow rate considerations

The optimum flow rate was found between 2 and 2.5 ml/min (as set at the pump). However, using raw pump flow rates in SFC can be deceptive. The fluids remain highly compressible. The density of the fluid changes significantly between the pump and the column. At a column head pressure of 400 bar and an ambient temperature of 20 °C, the density of CO_2 in the pump would be 1.021 g/cm^3. If the CO_2 is heated to 50 °C in the column compartment, the density at the head of the column (<400b) drops to <0.921 g/cm^3 and the local volumetric flow rate at the head of the column expands 11%, compared to at the pump (2.22 vs. 2 ml/min). If the fluid exits the column at 150 bar, and (nominally) 50 °C, the density would be 0.698 g/cm^3, and the local volumetric flow rate would be >2.9 ml/min. In an even more extreme case, the outlet could be at 80 bar and 50° where local density would be 0.219 g/cm^{3,} and the local flow rate would increase 4.66× to 9.3 ml/min, with the pump flow rate still set to 2 ml/min.

Generally the efficiency of the Kinetex column improved slightly at higher modifier concentrations and higher outlet pressures. All the test solutes produced 26,000 to 30,000 plates at 4.25 ml/min which is 1.7–2.1× optimum velocity.

Even the least retained compounds showed remarkably high efficiencies, and reduced plate heights, compared to totally porous particles of similar size (2.6 μm vs. 3 μm). The retention times, partition factor, symmetry factor, and efficiency of all 17 solutes, under one set of (non-optimal) conditions, on the Kinetex column are presented in Table 2.

3.5. Effect of k on N

In standard HPLC, the partition factor, k, is usually kept above 5–7 to avoid losses in efficiency. In the present work, k seldom exceeded 4 and values below 0.5 were common. Almost no correlation between efficiency and k, was found, as shown in Fig. 3. There was almost no change in efficiency above $k = 1$, and only a small effect below, when the injection volume was kept very low (≈0.1 μL). The k of naproxen, the earliest eluting peak of

Table 2
Retention times, partition factor, symmetry and efficiency of the 17 component mix. Conditions 3.5 ml/min, 15% MeOH in CO_2, 175 bar, 50 °C, 1.7 μL flow cell, all tubing 0.005 in. i.d. Filter set at >0.01 min. Column 2.6 μm d_p, 4.6 × 150 mm Kinetex HILIC. Apparent column hold-up time 0.414 min. Apparent average linear velocity 0.605 cm/s.

Compound	t_R, min	k	Symm	N, plates
Flurbiprofen	0.567	0.370	1.09	23273
Naproxen	0.605	0.461	1.07	24136
Ketoprofen	0.635	0.534	1.00	23638
Warfarin	0.821	0.983	1.24	26786
Theophyline	0.857	1.070	1.22	27847
Caffeine	0.888	1.145	1.11	27325
Thymine	0.998	1.411	1.21	29161
Uracil	1.096	1.647	1.33	27978
Cortisone	0.153	1.785	1.19	28374
Prednisone	1.209	1.920	1.33	27756
Aceamidophenol	1.376	2.324	1.33	24176
Hydrocortisone	1.436	2.469	1.26	27642
Sulfamethizole	1.504	2.633	1.37	27550
Prednisilone	1.592	2.693	1.45	25344
sulfadimethoxine	1.699	3.104	1.47	28681
Sulfaquinolaline	1.978	3.778	1.38	28663
Sulfamethizide	2.049	3.949	1.30	28412

the 4 component mix, never exceeded 0.5, yet it produced an optimum efficiency of over 31,000 plates at 15% modifier and 33,000 plates at 30% modifier (uncorrected reduced plate heights of 1.86–1.75). In the context of regular chromatographic theory, using totally porous particles, this behavior is odd.

3.6. Asymmetric peaks with the highest efficiency

All the test solutes eluted from either column with no sign of tailing. To the contrary, on the Kinetex column most of the peaks fronted considerably, despite being significantly narrower than those on the totally porous silica column. Diluting the standard 20 fold, failed to improve peak shapes, largely eliminating overload as a possible source for the fronting. A few solutes did not front, suggesting the fronting was not due to poor packing.

The symmetry factor of peaks in the Kinetex chromatogram in Fig. 1, ranged from 1.00 to 1.47, as shown in Table 2. Under some conditions the symmetry was significantly worse. In this work, the worst fronting peaks tended to produce the highest efficiencies. Sulfamethizole produced the highest observed efficiency of 35,500 plates using 30% methanol (not shown), which yielded an uncorrected reduced plate height of ≈1.63, despite exhibiting among the worst symmetries. There appears to be almost no correlation between the symmetry factor, and efficiency, as shown in Fig. 4.

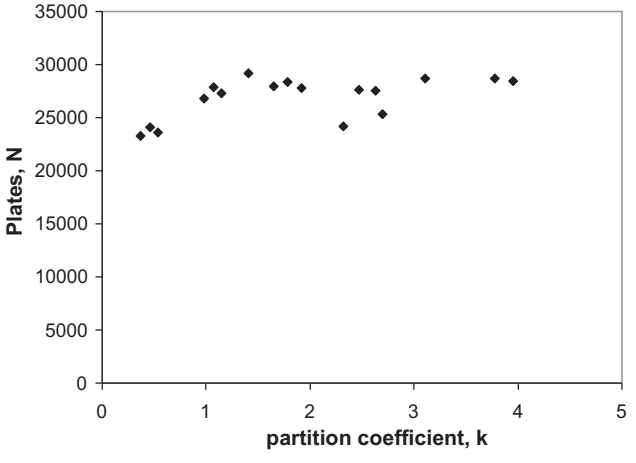

Fig. 3. Plot of efficiency, in plates, as a function of partition coefficients, k, of various solutes in the 17 component mix. Note that there is almost no correlation. Conditions: 3.5 ml/min, 15% methanol in CO_2, 175 bar outlet pressure, 50 °C. Column: Kinetex 4.6 × 150 mm, 2.6 μm porous shell HILIC.

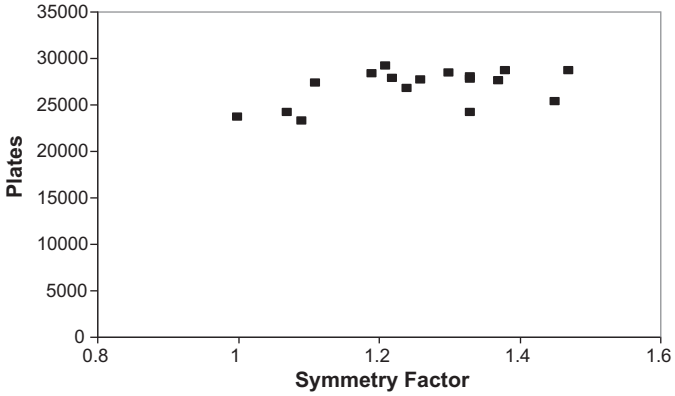

Fig. 4. Plot of efficiency in plates vs. peak symmetry, as reported by the ChemStation software. Again, there is almost no correlation between fronting and loss of efficiency. Conditions: 3.5 ml/min, 15% methanol in CO_2, 175 bar outlet pressure, 50 °C. Column Kinetex 4.6 × 150 mm, 2.6 μm porous shell HILIC.

One would expect a decrease in efficiency with increasing asymmetry, but in Fig. 4 there was virtually no slope, with a slight decrease at conditions where one would expect an increase and the highest efficiency. The fronting implied that the full efficiency of the column was not being observed.

All things considered, the Kinetex column appeared to exhibit less efficiency in SFC compared to use in normal HILIC operation [15]. This may, or may not, be due to the significant fronting observed. On the other hand, efficiencies and speed were high compared to conventional totally porous particles.

3.7. Extra-column plumbing

The system performance was somewhat unexpected (fronting peaks, with high efficiency). The maximum efficiency observed did not match either those in the manufacturers literature or those reported in HPLC or HILIC. It was, therefore, decided to try to separate out any extra-column effects from total system performance, although the large column ID, and length, made this unlikely.

The column was removed and replaced by a zero dead volume (ZDV) fitting. Partial loop injections were performed. All the external tubing was 0.005", or 0.125 mm ID. Both the 3 μL and 6 μL heat exchangers (HX) in the thermostated column compartment were part of the flow path. Inner diameter of the tubing in the HX's are reported as 0.007", or 0.178 mm making them 12 and 24 cm long. The injection valve was connected to the top left fitting of the right hand HX with a 20 cm piece of tubing. The bottom right of the HX fitting was connected to the zero dead volume (ZDV) fitting with a 10 cm piece of tubing. The ZDV was connected to the upper right fitting of the left hand HX with a 20 cm piece of tubing. The lower left fitting of the HX was connected to another ZDV with a 20 cm piece of tubing. The inlet tubing for the detector cell (20 cm) was connected to this second ZDV. Total tubing length, including the HX's, was ≈1.26 m.The calculated volume of the tubing was 11 μL, The collective volume of the fittings was calculated as 0.35 μL, the total reported volume of the 2 HX's was 9 μL. The cell volume was 1.7 μL. The total system volume between the injection valve and the detector outlet was ≈22 μL. The electronic filter was set to <0.0025 min or 80Hx, the fastest available with this detector.

At 2.5–4.5 ml/min (unmixed, at the pumps), the cumulative hold-up time of the injection valve, tubing, heat exchangers, and detector cell should be 0.009–0.005 min, or 0.524–0.293 s, after the valve turns. The time required to rotate the valve was not

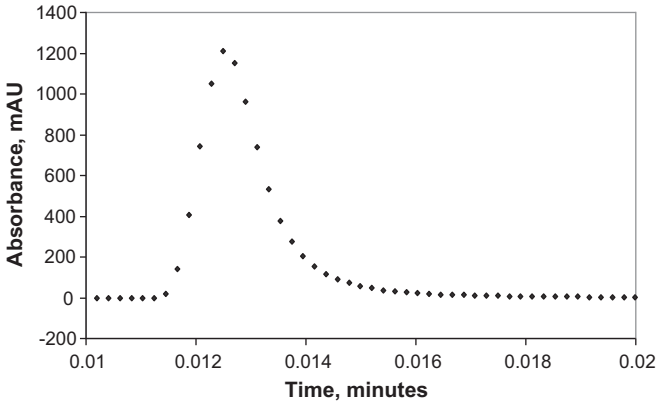

Fig. 5. Detector response to an injection with the column removed and replaced by a zero dead volume fitting. Conditions: 4.5 ml/min, 15% methanol, 150 bar outlet pressure, 50 °C. Detector response time set to 80 Hz.

reported by the HPLC manufacturer but the valve manufacturer lists several different rotation times for different actuators as 0.1–0.25 s. Thus, the total hold up time should be between ≈0.4 and 0.8 s. The expected drop in density through the system should decrease the hold-up time. The experimental hold-up times were 0.012–0.018 min, or 1.08–0.72 s, which is slightly slower than expected. One should probably conclude that the timing is imprecise at the scale of these experiments.

3.7.1. Extra-column variance

The peak widths at half height, without a column, were reported by the control software as between 0.003 and 0.0012 min (0.18–0.072 s) at 2.5 and 4.5 ml/min, respectively. A representative peak is shown in Fig. 5, with the discrete data points displayed. At 80 Hz, the detector was collecting one data point every 0.0125 s. The fastest of these peaks are just within the acceptable data rate of the system.

Since $w_{1/2} = 2.35\sigma$, then σ_t (in time) = 0.031–0.077 s. If the extra-column σ_v (in volume) = $F \times \sigma_t$, then σ_v = 2.3–3.2 μL. The extra column (ec) variance, based on width at half height was calculated as:

$$\sigma_v^2 = \sigma_{ec}^2 = 5.3 - 10.2\ \mu L^2$$

across the range of flow rates and compositions explored. This was a fairly remarkable finding, rivaling, or even exceeding the dispersion of many of the advanced UHPLC systems available, yet being produced by a much less extreme set of equipment. There was no attempt to shorten tubes, decrease inner diameters or decrease the size of the flow cell or the heat exchangers. This finding implies that the flow is non-laminar, and transitional or fully turbulent, which would be a significant advantage for SFC (see Section 3.7.5).

3.7.2. Symmetry-mixing chamber variance only

The bands (without a column installed) *tail* fairly significantly as indicated in Fig. 5. This is a clear indication of mixing chamber behavior. A mixing chamber superimposes an exponential decay on the standard Gaussian distribution. Mixing chambers sharpen the leading edge and broaden out the trailing edge (peaks tail). A mixing chamber is a volume with an inner diameter larger than the inlet tube feeding it. The time constant for a mixing chamber is its volume divided by the flow rate. The variance is the square of the time constant. Obviously, the detector cell, the fittings on the ends of the HX's and the ZDV unions all act as small mixing chambers. Collectively, they are a sub-component of the total system variance.

Each of the fittings was assumed to have a through hole 0.25 mm ID, 1 mm long. The volume of each is calculated as 0.07 μL. Each time constant would be <0.002 s.

Superficially, the most obvious single mixing chamber was the UV detector flow cell. With a volume of 1.7 μL and a path length of 0.6 mm, the inner diameter was 0.3 mm. The tube through which the fluid entered was 0.127 mm ID.

The symmetry factor reported by the ChemStation was used to calculate the variance of the combined mixing chambers. The Agilent Chemstation does not unambiguously state how the symmetry was calculated. One of two different approaches were used depending on conditions. However, it is not reported which was used in any specific chromatogram (!). In one approach, verticals are drawn at the inflection points and the apex, dividing the peak into 4 areas and 4 time segments. The heights at the inflection point are weighted with the peak height at the apex. These are all used to calculate a "pseudomoment", through a series of algebraic equations (see Agilent Chemstation users manual). However, if one or both of the inflection points cannot be found, a much simpler approach is used, which is essentially A/B where A is the *area* of the peak from the beginning of the peak to the apex, and B is the area from the apex to the end of the peak. If one assumes the second approach was used, one can calculate the variance of the components causing the tailing, based solely on the reported symmetry and the nominal flow rate.

The reported symmetry factors, for the peaks without a column installed, ranged from 0.39 to 0.53. Assuming the widths in front of and to the rear of the apex are proportional to the corresponding areas, the reported peak widths at half height can be used to estimate extra-column variance caused by mixing chamber behavior. Solving the simultaneous equations for A and B:

$$A + B = w_{1/2}$$

$$\frac{A}{B} = \text{Symm.}$$

Then using the equation:

$$\sigma^2 = \frac{F^2(B-A)^2}{5.54}$$

where F is the (instrumentally set at the pump) flow rate, in μL/min, B is the area of the peak between a vertical, dropped from the apex, to the back of the peak. A is the area of the peak from the front to the vertical.

The resulting extra-column variances, due only to mixing chambers, were remarkably low:

$$\sigma_{mix} = 0.72\ \mu L^2 - 1.9\ \mu L^2$$

These results probably represent a minimum value, since the fluid expands after the pump and the value of the "flow rate" is ambiguous.

In many cases, the calculated partial widths were narrower than the sampling rate. The flow cell time constant is 0.041 s at 2.5 ml/min and 0.023 s at 4.5 ml/min. At the acquisition frequency used <2 to <4 data points were collected during the residence time of any one molecule in the detector cell.

Such small variances suggest that normal mixing under laminar flow is NOT occurring and also implies the fluid is mostly in the turbulent flow region in the fittings.

3.7.3. Peak fidelity

The peaks, with the column installed, were on the order of 0.01–0.08 min (0.6–4.8 s) wide, at half height (2.35σ), depending on retention and flow rate. The fastest peaks with the smallest variance that would be most affected by extra-column effects, exhibited

$\sigma_t = 0.0043$ min, $\sigma_v = 15$ μL. Minimum total system variance was =/> 222 μL^2. The measured extra-column variance (based on half height measurements) was =/< 10.2 μL^2.

A concept called peak fidelity allows the calculation of the fraction of the actual column efficiency that can be observed with any system with a known extra-column variance:

$$\left[\left[\frac{1}{\phi^2}\right] - 1\right] = \frac{{\sigma_{ec}}^2}{{\sigma_{col}}^2}$$

where ϕ is peak fidelity, the fraction of the actual column efficiency observed. For the present case:

$$\frac{{\sigma_{ec}}^2}{{\sigma_{col}}^2} = \frac{< 10.2\ \mu L^2}{> 222\ \mu L^2} =< 0.0459$$

and

$$\phi => 0.978$$

Thus, with the low level of extra-column band broadening observed, the system should still be capable of demonstrating >97.8% of the inherent performance of the column. Therefore, extra-column effects should not have any significant effect on observed efficiency. It appears that there is no correction necessary, or appropriate, for extra-column band broadening.

3.7.4. Viscosity of CO_2/MeOH mixtures

Literature values [26] for the viscosity of CO_2/MeOH mixtures and H_2O/MeOH mixtures [27] are compared in Fig. 10. At 20% modifier, the viscosity of CO_2/MeOH is 16.6× lower than the viscosity of H_2O/MeOH. At the same flow rate the pressure drop would be proportionally lower in SFC. At 3–5 times the flow the pressure drop will remain at <1/5th the pressure drop using H_2O/MeOH.

3.7.5. Reynolds number estimation in the tubing

The Reynolds number (Re) is an indication as to whether flow is laminar or turbulent. Turbulent flow is desirable since it breaks up a significant part of extra-column band broadening. A Re value below 2300 indicates probable laminar flow. A Re value above 4000 indicates probable turbulent flow. A simple equation for the Reynolds number is:

$$Re = \frac{u d_h}{V}$$

where u is the fluid velocity in m/s, d_h is the hydraulic distance, here tube ID in meters, and v is the kinematic viscosity, in m^2/s. The viscosity of CO_2/MeOH (at 40 °C) varies between 0.068 and 0.1903×10^{-6} m^2/s between pure CO_2 and 0.5 mole fraction CO_2. At 15% modifier, 40 °C the viscosity is 0.102×10^{-6} m^2/s. Viscosity should drop approximately 15% with an increase in temperature of 10 °C–50 °C.

Most of the tubing was 0.005 in. (0.127 mm) ID. The optimum flow rate was 2–2.5 ml/min. When the pump delivered 2.5 ml/min (at the pump), the density dropped to 0.698 at 150 bar and 50 °C and volumetric flow in the tubing increased approximately 25%. The velocity in the tube (at 50 °C, 150 bar) would be > 4.1 m/s. The Reynolds number would be 5100, which indicated turbulent flow.

In measuring the extra-column variance, in Section 3.7.1, the flow was set to 2.5 and 4.5 ml/min. The variance was found to be lower (5 μL^2 compared to 10.2 μL^2) at the higher flow rate, further suggesting more turbulent conditions at the higher flow rate.

Such Re calculations are far from definitive. Much, but not all, of the tubing was in the oven. Some of the tubing was significantly colder causing higher viscosity. There was a pressure drop across the tubing, creating a velocity gradient. Some of the tubing will be at a higher pressure, lower velocity, lower Re. Finally, the viscosity measurements of mixtures of CO_2 with methanol were not taken under identical conditions to those used experimentally although the densities are similar. At higher modifier concentrations, lower temperature, or lower flow rates, the Re numbers should decrease, and make the flow more laminar.

These results suggest several instrumental advantages of SFC over HPLC using aqueous based mobile phases that have not been adequately stressed in the past. In SFC, the solute binary diffusion coefficients are typically 3–5 times higher, compared to aqueous based fluids. This requires higher linear velocities (flows) to reach optimum efficiency. Any minor mixing chambers are minimized by the much higher flow rates (the time constant is volume divided by flow rate).

In some cases in SFC, the linear velocity in the tubes appears to be such that turbulent or transitional flow occurs. This further decreases extra-column effects and allows the use of larger ID, longer connecting tubes without creating extra-column band broadening. These results suggest the use of larger columns (3–4.6 mm ID) will exhibit lower extra-column effects than smaller ID columns, operated at lower flow rates.

3.8. Column void volume

Nominal void volume using totally porous silica would be in the vicinity of 1.75 ml in this column. However, the use of particles with a large porous shell obviously decreases this volume somewhat.

At 3.5 ml/min, 15% methanol at 150 bar, 50 °C, the empirical column hold-up time was 0.414 min, which corresponds to a nominal column void volume of 1.45 ml (based on volumetric flow at the pump). There is an underlying assumption that the first perturbation of the baseline are caused by unretained mobile phase components. It has already been pointed out that the fluid expands after leaving the pump. While the pressure/density behavior of pure carbon dioxide is well documented, there is much less information available for binary mixtures. Thus, it is impossible to determine the actual "void volume" of the column without additional data on the pressure and temperature profile along the column and precise pressure/temperature/density measurements of the mobile phase compositions used.

3.9. Pressure drop in tubing and fittings

The pressure drop across the system with the column replaced by a ZDV union was found to be significant (up to 75 bar), a function of flow rate, and, to a lesser extent, composition, as shown in Fig. 6. Such large pressure drops in the tubing and fittings were somewhat surprising. The column is normally installed roughly in the middle of this pressure drop. Therefore, the precise pressure at the head and outlet of the column is generally not known, complicating calculations such as density gradients, or thermal effects along the column.

3.10. System pressure drop

The pressure, measured between the HPLC pump, and the back pressure regulator, with the Kinetex column installed, was recorded as a function of methanol concentration at 4 ml/min., as shown in Fig. 7. The optimum flow rate was previously found to be between 2 and 2.5 ml/min making 4 ml/min nearly double optimum. With the column outlet pressure set at 150 bar, the head pressure only exceeded 400 bar above approximately 65% methanol. This exceeds the modifier concentration requirements for all but the fastest screening gradients. The pressure drops reported in Fig. 7, included the pressure drop in the tubing. In some cases, the pressure drop in the tubing approached half the total pressure drop measured.

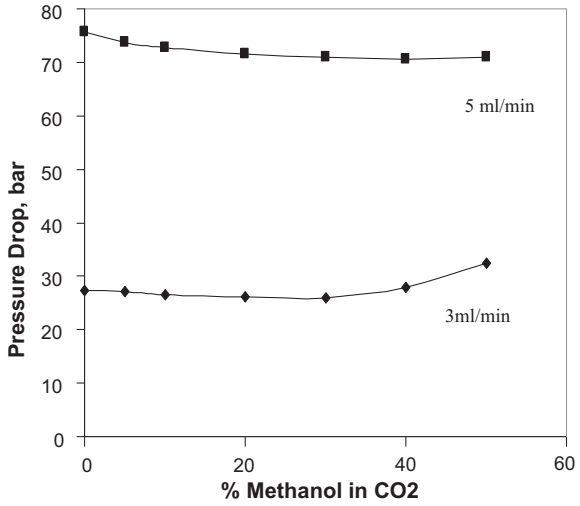

Fig. 6. . Extra-column pressure drop in the system at 3 and 5 ml/min, 0–50% modifier. Optimum is between 2 and 2.5 ml/min, so all these results are super-optimum. Between the injection valve and the detector there were 90 cm of 0.005 in. ID tubing, plus both the column compartment heat exchangers, the 1.7 μL flow cell, and part of the valve.

The flow rate was also varied at a constant 10% methanol, as shown in Fig. 8. Even at the maximum flow of the pump (5 ml/min) column head pressure never exceeded 370 bar. The checkout HPLC chromatogram sent with the column listed the column pressure drop at 1.6 ml/min, 90% acetonitrile as 208 bar. In Fig. 8, the pressure drop at 10% methanol, 3.2 ml/min is approximately 100 bar. The pressure drop in this SFC example was, thus, less than half that of HILIC, at double the flow rate on this column.

Near the optimum flow rate, the column (and tubing) pressure drop seldom exceeded 100 bar under typical conditions of temperature, outlet pressure and composition. The results reported in Figs. 7 and 8 indicate such columns can be used over the full range of SFC conditions, with some older 400 bar equipment.

3.11. Loadability

The efficiency observed was strongly dependent on the volume injected. The external loop employed had an internal volume

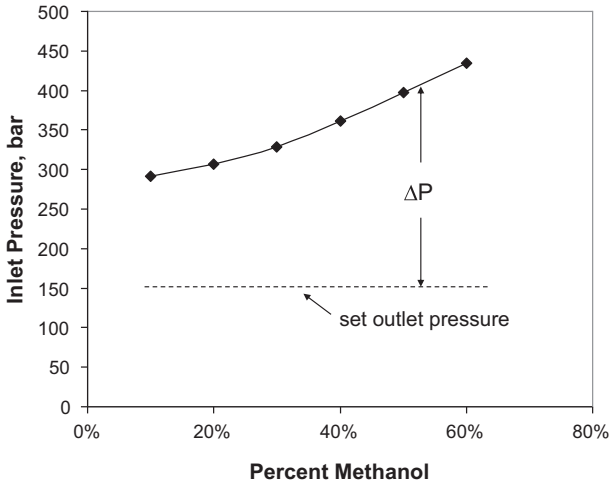

Fig. 7. Effect of modifier concentration on the pressure drop across a 4.6 × 150 mm Kinetex 2.6 μm HILIC column, with 4.0 ml/min of MeOH/CO_2, 150 bar outlet, 50 °C. The pressure drop includes the drop across the tubing, which accounts for as much as half the total pressure drop.

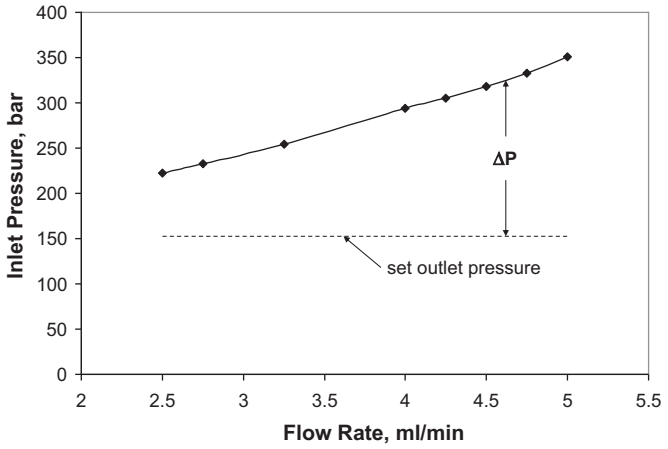

Fig. 8. Effect of flow rate on the pressure drop across a Kinetex 4.6 × 150 mm 2.6 μm HILIC column in SFC. Methanol concentration 10%, 150 bar outlet, 50 °C.

of only 1.27 μL. The transfer line from the needle to the valve, a through hole in the valve rotor and one groove in the rotor is in-line with the sample loop during loading. The volumes of these components must be accounted for when the syringe delivers metered amounts of sample. Partial loop injections were required. Small amounts of sample were bracketed by air bubbles in the loop, to center the sample in the loop. This arrangement yielded rather poor area reproducibility.

The injection volume was increased in 0.1 μL increments, starting at 0.5 μL and the observed efficiency was plotted, as shown in Fig. 9. The efficiency, as measured in plates, showed a continuous modest decrease with a slope of approximately.

(-) 400plates/0.1 μL injected. These findings are preliminary and require further development with a different style of autosampler.

3.12. UV sensitivity

In the past, the most serious deficiency of SFC was poor UV–vis sensitivity. Higher efficiency and speed are of little use if sensitivity is inadequate. The chromatographic conditions were changed to make the run shorter and peaks narrower to fully test the

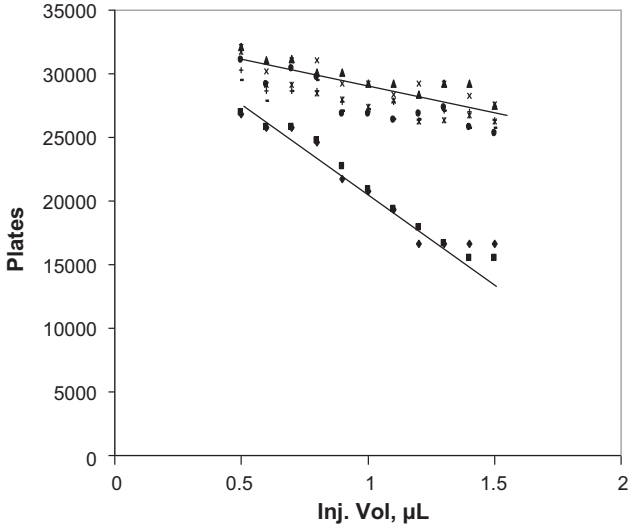

Fig. 9. Plot of efficiency vs. injection volume. Efficiency appears to be affected by the size of the sample injection through a sample solvent effect. The lower curve is for naproxen which had a $k < 0.5$ and was most affected by increased polar solvent volume.

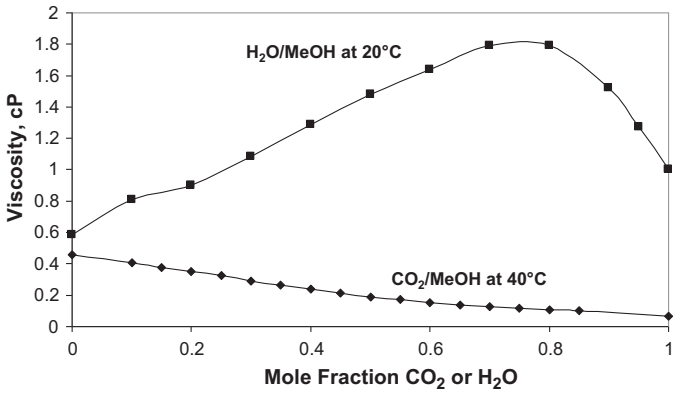

Fig. 10. Literature values for the viscosity of CO_2/MeOH [26], and H_2O/MeOH [27] mixtures at 40 °C and 20 °C, respectively.

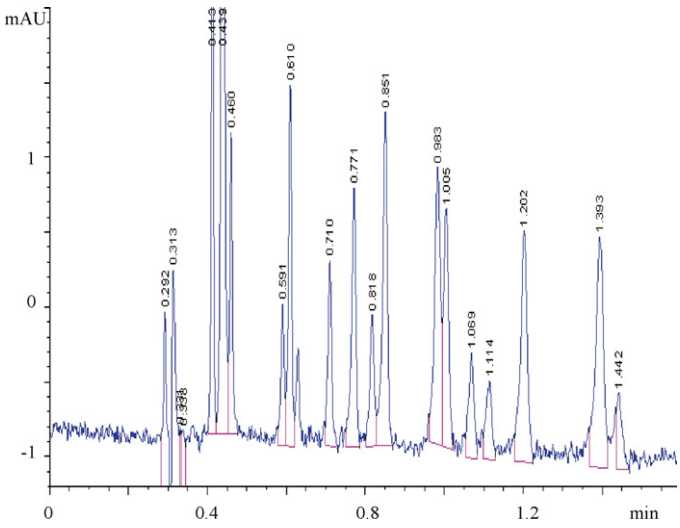

Fig. 11. High speed chromatogram of the 17 component mix, collected with the UV detector filter set to 80 Hz. Some peaks are as narrow as 0.005 min, peak to peak noise is < 0.1 mAU indicating the system could quantitate a peak representing 0.1% of an on-scale parent peak, with a signal to noise of 10:1, even at the fastest data rate allowed. Conditions: 15% methanol in CO_2, 4.75 ml/min, 150 bar outlet, 50 °C. 4.6 × 150 mm 2.6 μm Kinetex HILIC column.

chromatographs performance. The detector bandwidth was 80 Hz. A chromatogram of the 17 component mix at 15% methanol, 4.75 ml/min, 50 °C, and 150 bar outlet pressure is presented in Fig. 11. The narrowest peaks are approximately 0.005 min wide at half height. The peak to peak noise =/< 0.1 mAU, even at 80 Hz. This suggests one could quantify a very fast peak representing 0.1% of an on-scale parent peak, with a signal to noise ratio >10.

3.13. Thermal effects

The resistance to flow by small particles tends to cause resistive heating in HPLC [3–5]. In SFC, the much lower pressure drops, plus the potential for significant adiabatic expansion, are likely to cause far smaller thermal gradients. However, thermal radial gradients may be causing the lower efficiency, and distorted peaks observed here, as compared to the manufacturers report and others [11,15]. Further work with smaller ID columns may clarify these issues.

4. Conclusions

Porous shell particles should be widely used in SFC, since they have been shown to produce high efficiencies with short analysis times. The porous shell 2.6 μm Kinetex particles significantly outperformed totally porous Luna 3 μm silica particles. Uncorrected reduced plate heights as low as 1.62 were observed, compared to < 2.2 on the Luna silica column. Similar separations of a 17 component mix were achieved on both columns but, the Kinetex column produced the separation in half the time, with better resolution. Compared to 1.8 μm totally porous particles [23] (3 × 100 mm), the Kinetex column produced much higher efficiency, with lower pressure drops, but in longer time.

Many of the peaks fronted on the Kinetex column. Efficiency did not appear to be related to the degree of fronting. The source of this fronting was not discovered. The column did not deliver efficiency as high as reported in HPLC, where several groups have reported reduced plate heights from 1.0 to 1.5.

The extra-column variance was found to be remarkably low (5–10 μL^2), despite long lengths of tubing, multiple fittings and a relatively large detector flow cell (1.7 μL). The results imply the mobile phase is exhibiting turbulent flow in connecting tubing, which would minimize extra-column effects.

Near the optimum flow rate of 2–2.5 ml/min, the system pressure drop seldom exceeded 100 bar, with a significant portion due to the tubing. Even at 2× optimum velocity, and 65% modifier, the column head pressure only slightly exceeded 400 bar. This work could have mostly been performed using older 400 bar equipment, with 20 Hz detectors. However, the higher data rates used allowed the low dispersion of the chromatograph to be estimated.

In UHPLC radial thermal gradients caused by resistance to flow dictate the use of small ID columns, smaller ID tubing, smaller flow cells, smaller injection valve ports, etc. In SFC this does not appear to be the case and larger ID columns may be used without deleterious effects, using standard 400 bar equipment. In fact the onset of turbulent flow in the tubing and fittings at higher flows suggests larger ID columns should yield superior efficiency.

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References

[1] I. Halĭasz, R. Endele, J. Asshauer, J. Chromatogr. 112 (1975) 37.
[2] C. Horvĭath, H.J. Lin, J. Chromatogr. 149 (1978) 43.
[3] A. de Villiers, H. Lauer, R. Szucs, S. Goodall, P. Sandra, J. Chromatogr. A 1113 (2006) 84.
[4] F. Lestremau, A. de Villiers, F. Lynen, A. Cooper, R. Szucs, P. Sandra, J. Chromatogr. A 1138 (2007) 120.
[5] A. de Villiers, D. Cabooter, F. Lynenc, G. Desmet, P. Sandra, J. Chromatogr. A 1216 (2009) 3270.
[6] O. Lacina, O. Urbanova, J. Poustka, J. Hajslova, J. Chromatogr. A 1217 (2010) 648.
[7] H.G. Gika, G. Theodoridis, J. Extance, A.M. Edge, I.D. Wilson, J. Chromatogr. B 871 (2008) 279.
[8] D. Guillarme, D.Y.-T. Nguyen, S. Rudaz, J.-L. Veuthey, Eur. J. Pharm. Biopharm. 66 (2007) 475.
[9] M. Ibañez, C. Guerrero, J.V. Sancho, F. Hernández, J. Chromatogr. A 1216 (2009) 2529.
[10] J.J. Kirkland, F.A. Truszkowski, C.H. Dilks Jr., G.S. Engel, J. Chromatogr. A 890 (2000) 3.
[11] F. Gritti, G. Guiochon, J. Chromatogr. A 1217 (2010) 1604.
[12] E. Olah, S. Fekete, J. Kefete, K. Ganzler, J. Chromatogr. A 1217 (2010) 3642.
[13] F. Gritti, A. Felinger, G. Guiochon, J. Chromatogr. A 1136 (2006) 57.
[14] F. Gritti, A. Cavazzini, N. Marchetti, G. Guiochon, J. Chromatogr. A 1157 (2007) 289.
[15] D.V. McCalley, J. Chromatogr. A 1193 (2008) 85.
[16] C. Pizarro, O. Suárez-Iglesias, I. Medina, J.L. Bueno, J. Supercrit. Fluids 48 (2009) 1.
[17] S.A. Smith, V. Shenai, M.A. Matthews, J. Supercrit. Fluids 3 (1990) 175.
[18] T. Funazukuri, C.Y. Kong, S. Kagei, J. Supercrit. Fluids 27 (2003) 85.
[19] C. Mantell, M. Rodrĭguez, E. Martĭnez de la Ossa, J. Supercrit. Fluids 29 (2004) 165.
[20] T. Funazukuri, C.Y. Kong, S. Kagei, J. Supercrit. Fluids 38 (2006) 201.

[21] K. Stephan, K. Lucas, Viscosity of Dense Fluids, Plenum Press, New York, 1979.
[22] E. Heidaryan, T. Hatami, M. Rahimi, J. Moghadasi, J. Supercrit. Fluids 56 (2011) 144.
[23] T.A. Berger, Chromatographia 72 (2010) 596.
[24] T.A. Berger, B.K. Berger, R.E. Majors, LC-GC North America 1 (May) (2010).
[25] T.A. Berger, B.K. Berger, J. Chromatogr. A. 1218 (2011) 2320.
[26] R. Sih, F. Dehlghani, N.R. Foster, J. Supercrit. Fluids 41 (2007) 148.
[27] B. Gonzalez, N. Calvar, E. Gomez, A. Dominguez, J. Chem. Thermo. 39 (2007) 1578.