Performance to burn? Re-evaluating the choice of acetonitrile as the platform solvent for analytical HPLC

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Acetonitrile is by far the preferred organic solvent for use in Reversed Phase High Performance Liquid Chromatography (RP-HPLC) today, owing to an excellent combination of physical properties of this solvent that are favorable for chromatography (viscosity, UV absorbance cutoff, boiling point). In recent years, improvements in both instrument and stationary phase technologies have greatly enhanced the performance of routine laboratory HPLC, leading to an excess of performance for many users. In this study we re-examine the choice of acetonitrile as the platform organic solvent for HPLC, addressing the question of whether greener solvents such as ethanol could potentially replace acetonitrile in some instances.

Introduction

Reversed Phase High Performance Liquid Chromatography (RP-HPLC) is the most extensively utilized analytical technique in the pharmaceutical industry today, being employed in nearly every stage of the discovery, development and manufacturing of new drug products.[1] Consequently, a pharma company may have well over a thousand HPLC instruments in operation, and while the amount of waste generated by an individual instrument is small (~ 0.5 L/day), the cumulative waste volume is substantial.[2] We have previously described opportunities for greening analytical HPLC in pharma by adopting microflow HPLC technology,[3] which effectively reduces solvent consumption and waste generation by more than a factor of 100, and by adopting Supercritical Fluid Chromatography (SFC)[4] in which inexpensive and readily available pressurized carbon dioxide replaces toxic, flammable, expensive, and difficult to dispose petrochemical-derived hydrocarbons. Yang has recently reviewed the use of high temperature to perform subcritical water chromatography that requires no organic co-solvent whatsoever.[5] In this study we investigate opportunities for greening analytical RP-HPLC using existing equipment, by switching to more environmentally acceptable solvents.

By way of introduction, a brief overview of RP-HPLC may be in order.[6] A schematic view of an idealized HPLC instrument is shown in Fig. 1. In the typical setup, precision high pressure pumps are used to deliver two solvents (often acetonitrile and water) that are thoroughly mixed in a downstream mixer. The flow rates of the two solvent delivery pumps are often varied during the course of the chromatographic run, with a gradient of increasing acetonitrile over the course of the experiment being typical. A small volume of sample is introduced to the system through a sample injector, which generally has the capability to automatically inject samples from vials or microplates. Following injection, the sample mixture is carried

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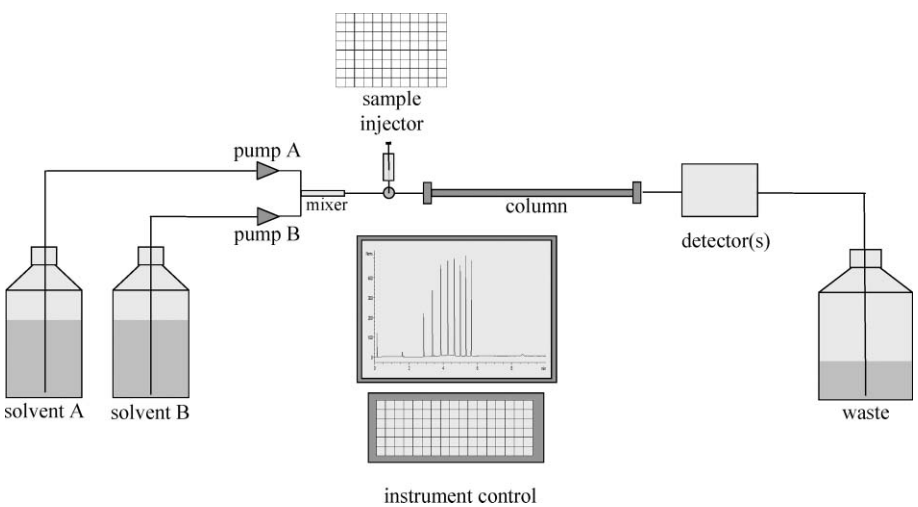

Fig. 1 Schematic representation of an analytical HPLC instrument.

to the column, where differential partition of the sample components between the stationary phase and mobile phase gives rise to chromatographic separation, with components that are more strongly retained by the stationary phase eluting last from the column. As the analytes elute from the column, they pass through a detector (typically a UV absorbance monitor) for visualization and quantitation. Waste solvent from the system is collected and disposed as organic waste.

Recent years have seen a number of technical advances that have improved the HPLC performance. At the instrument level, what amounts to a series of incremental plumbing improvements have reduced extracolumn volume (the volume between the injector and the column inlet, and the volume between the column outlet and the detector). These improvements, along with improvements in UV detector flow cell design, have led to decreased dispersion, and consequently sharper chromatographic peaks.[7] Similarly, improved lower volume mixing technologies have led to decreased gradient dwell time (the time required for a change in flow composition at the pumps to show up at the head of the column), with consequent improvement in the speed of gradient chromatographic experiments.[8]

More important has been a veritable revolution in HPLC column packing materials that has led to dramatic improvements in peak efficiency (sharpness). As a general rule, the smaller the particles packed into the chromatography column, the better the chromatographic efficiency. In the early days of HPLC, now some 35 years ago, columns were packed with large, irregular silica particles, but soon converged on uniform spherical 5 micron inner diameter (i.d.) particles as a *de facto* industry standard for many years.[9] Recent years have seen a dramatic shift to smaller particle size, with particle diameters below 2 microns emerging as something of a new preferred industry standard.[10] In general, the backpressure generated by flowing through a column at a given flow rate increases as the particles within the column decrease. Consequently, the shift to small particle column technology has been accompanied by a shift to greater operating pressures for analytical HPLC instrumentation. Older HPLC instruments generally have a backpressure limit of ~6,000 pounds per square inch (psi), while most new products have a limit of in excess of 10,000 psi.[11] Recent introduction of fused core particle technology, in which tiny particles are fused to a solid core, affords a material with the chromatographic efficiency characteristics of the smaller particle columns without the accompanying high backpressure.[12] In combination, these improvements in both instruments and stationary phase technologies have greatly increased the performance of routine

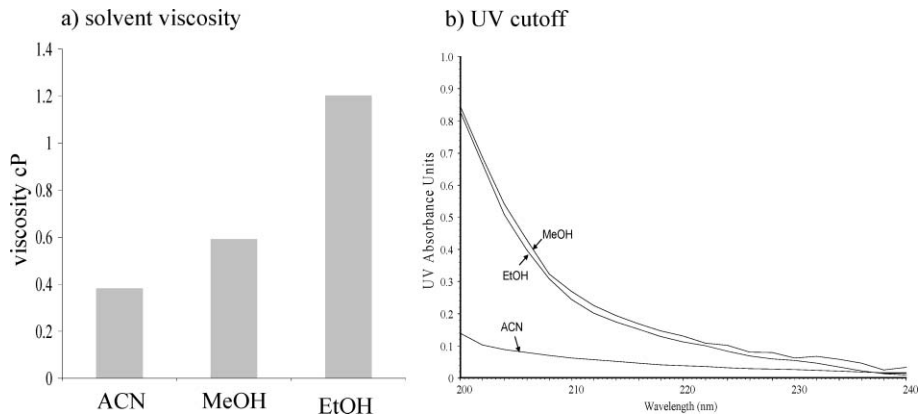

Fig. 2 Comparison of physical properties illustrates why acetonitrile is currently the preferred organic solvent for use in RP-HPLC. a) low viscosity of acetonitrile enables higher flow rate at a given backpressure, resulting in faster chromatography. b) decreased UV absorbance of acetonitrile at lower wavelengths enables increased signal to noise and greater sensitivity for quantifying analytes at lower wavelengths.

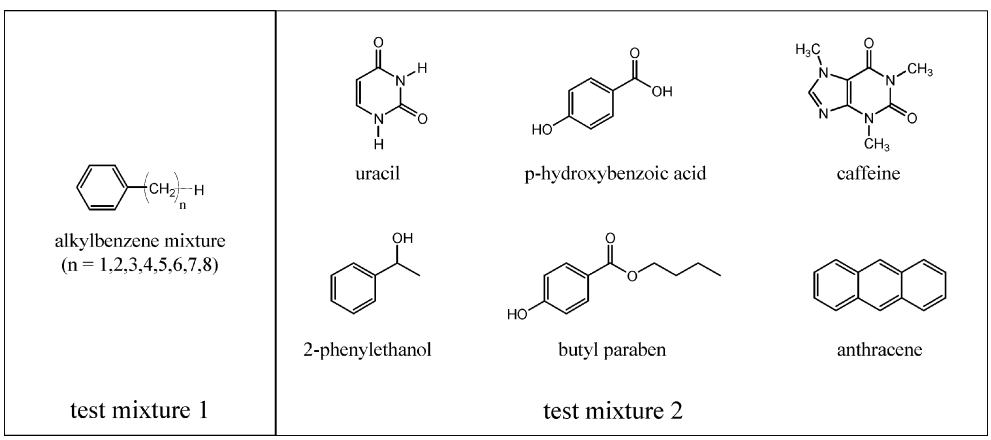

Fig. 3 Tests analytes used in the study 1. Test mixture 1 consists of a homologous series of alkylbenzenes, while test mixture 2 consists of a mixture of compounds from different functional group classes, which more closely approximates the types of molecules encountered in pharmaceutical discovery and development.

laboratory HPLC instrument, leading to performance that exceeds needs for many applications users.

Acetonitrile is by far the preferred organic solvent used in RP-HPLC today, owing largely to the combination of physical properties of this solvent that are exceptionally well suited for HPLC (Fig. 2).[6] Acetonitrile is produced as a coincidental byproduct in the manufacture of acrylonitrile, which is used in the production of plastics and resins. A recent downturn in plastics production has resulted in severe shortages and escalating costs for acetonitrile, negatively impacting chromatographers on a worldwide basis.[13] As the chromatography community addresses this situation, it may be a good time to consider greener replacements for acetonitrile in RP-HPLC. Owing to the toxic properties of acetonitrile, aqueous waste streams containing acetonitrile are typically disposed as chemical waste. While methanol is less toxic than acetonitrile, methanol-containing streams are also typically treated as waste. A more environmentally friendly and easily renewable solvent such as ethanol[14,15] could allow for reduced environmental impact of waste solvent disposal, with direct sewering of aqueous waste streams potentially possible in some cases. Ethanol has recently been advocated as an environmentally friendly co-solvent for the RP-HPLC analysis of cosmetics.[16] In this study we re-examine the choice of acetonitrile as the platform organic solvent for HPLC, addressing the question of whether greener solvents such as ethanol could potentially replace acetonitrile in some instances.

Experimental section

Chemicals

All solvents used in the study were HPLC grade or better. Acetonitrile and water were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Ethanol was obtained from Pharmco-Aaper

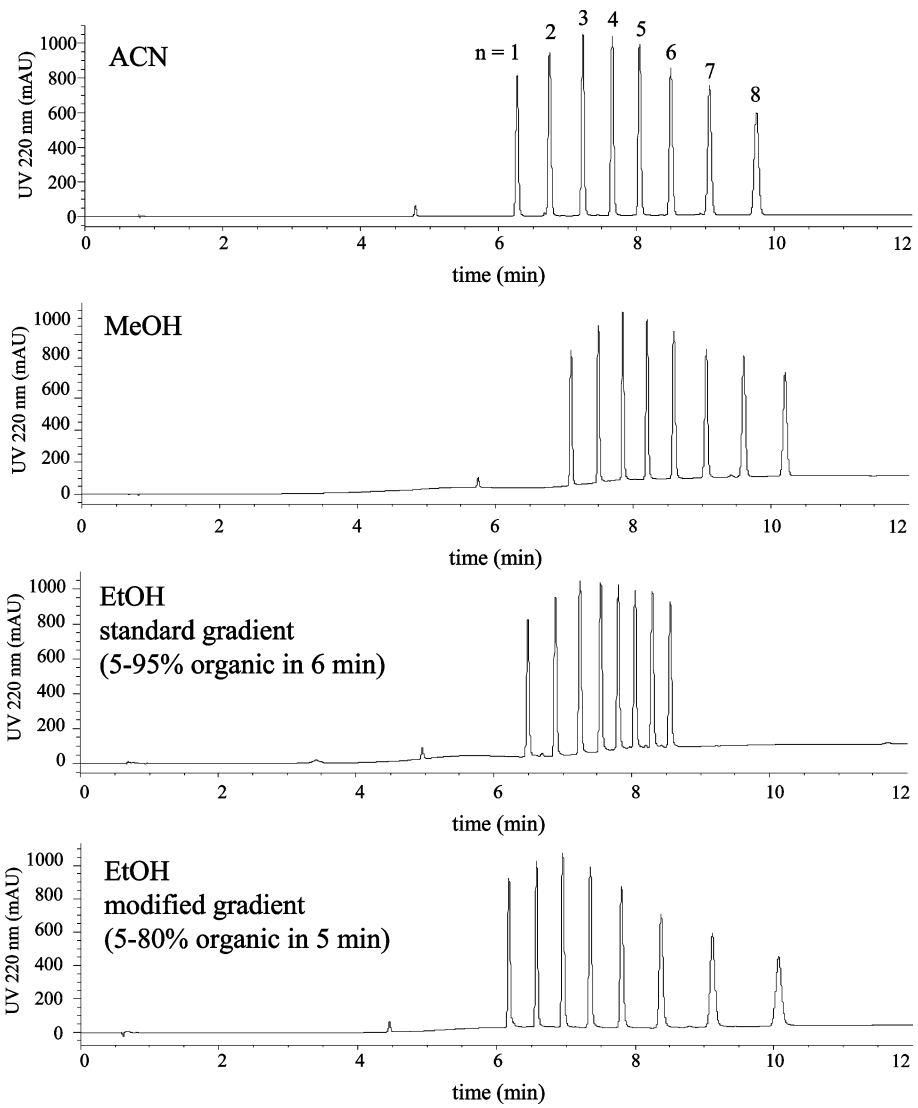

Fig. 4 Separation of test mixture 1 (homologous series of alkylbenzenes) by RP-HPLC using the organic modifiers a) ACN, b) MeOH, or c) EtOH. Conditions: Column = Eclipse Plus C_{18}, 1.8 μ particles, 4.6 mm i.d. × 50 mm, temperature = 25 °C, flow rate = 0.8 mL/min, detection = UV 220 nm, aqueous = 0.1% H_3PO_4, gradient = 5–95% organic in 6 min, with 6 min hold, d) Modified gradient conditions (5–80% in 5 min, with 7 min. hold) allows tuning of elution window for components of the mix.

(Brookfield, CT). O-Phosphoric acid 85% was obtained from Fisher Scientific (Fairlawn, NJ). Components of Text Mix 1 and 2 were obtained from Fisher Scientific (Fairlawn, NJ), Sigma-Aldrich, Inc. (St. Louis, MO), or Acros Organics Morris Plains, NJ).

Columns

The Eclipse Plus C_{18}, 50 × 4.6 mm, 1.8 micron column was obtained from Agilent Technologies (Palo Alto, CA) and the Ascentis Express C_{18}, 50 × 4.6 mm, 2.7 micron column was obtained from Supelco Analytical (Bellefonte, PA).

Instrumentation

The HPLC instrument used in this study was an Agilent 1100 series HPLC system equipped with a quaternary pump and a photo diode array detector (Agilent Technologies, Paolo Alto, CA). Chromatographic conditions are as reported in the figures. A Spectromax M5 Microplate Specrophotometer (Molecular Devices, Sunnyvale, CA) was used for the measurement of UV spectra, along with a quartz 96-well flat-bottomed microplate (Hellma, Plainview, NY)..

Results and discussion

Characterization of the performance of different RP-HPLC columns and instrument setups has been intensively studied for decades.[17] For the purpose of this study, we opted to investigate the separation of two different analyte test mixtures (Fig. 3) under a variety of conditions. Test mixture 1 consists of a homologous series of alkylbenzenes, the members of which differ in bulk hydrophobicity depending on the length of a hydrocarbon tail. As differences in hydrophobicity are the dominant factor controlling retention in RP-HPLC, this test mixture is well suited to evaluating performance. However the lack of more complex functional groups in these analytes make the mixture highly idealized, and poorly representative of 'real world' analytes that can sometimes exhibit broadened

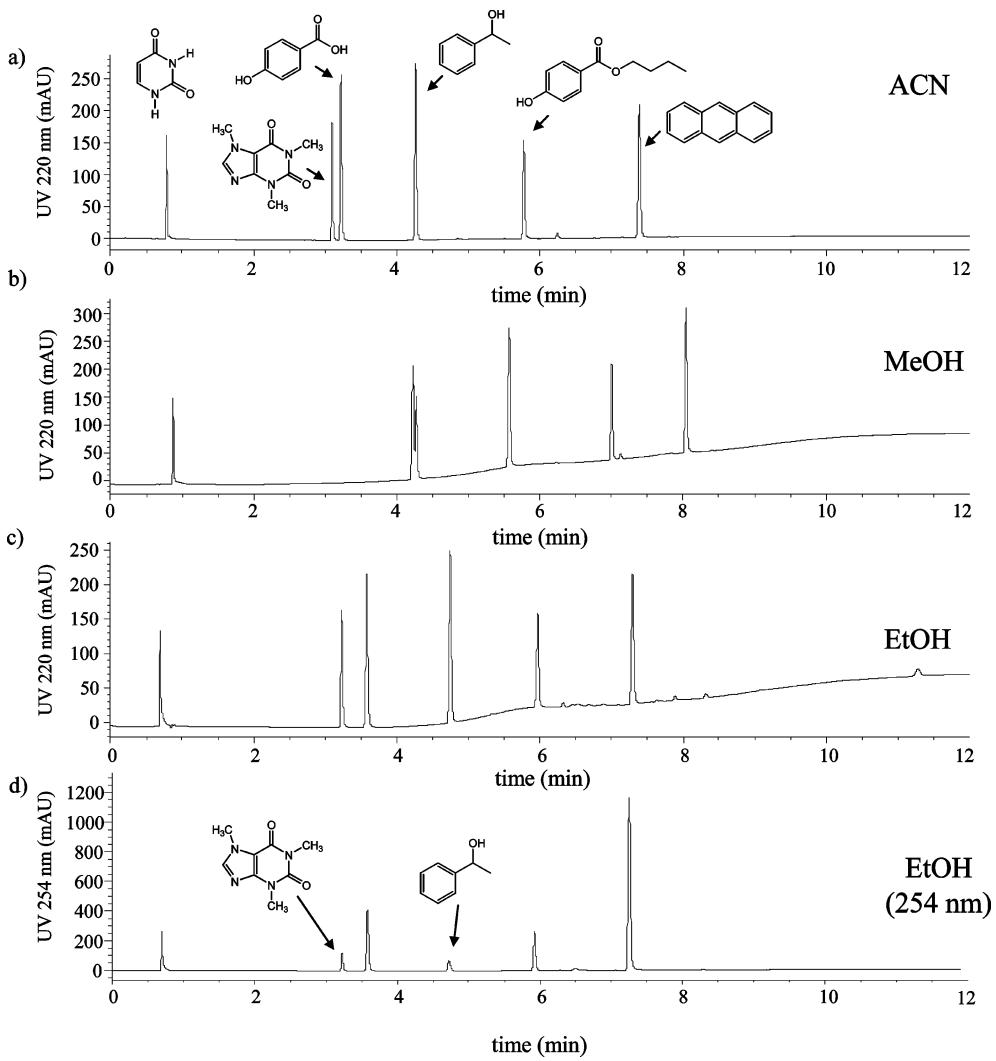

Fig. 5 Separation of test mixture 2 by RP-HPLC using three different organic modifiers a) ACN, b) MeOH, or c) EtOH. Conditions: Column = Eclipse Plus C_{18}, 1.8 μ particles, 4.6 mm i.d. × 50 mm, flow rate = 0.8 mL/min, temperature = 25 °C, detection = UV 220 nm, aqueous = 0.1% H_3PO_4, gradient = 5–95% organic in 6 min, with 6 min hold d) Change in the detection wavelength from 220 nm to 254 nm reduces problem of rising baseline, but greatly decreases the signal for the poorly absorbing 2-phenylethanol and caffeine components.

or distorted chromatographic peaks stemming from secondary interactions.[18] To address this issue, we also included test group 2 in the study, which contains an assortment of compounds from a number of functional group classes, more nearly approximating the types of molecules that would be routinely evaluated in pharmaceutical discovery and development.

Chromatograms showing the separation of the components of test mixture 1 using standard gradient conditions (0.8 mL/min, 5–95% organic in 6 min, with 6 min hold) with ACN, MeOH, and EtOH based eluents on a sub 2 micron particle reversed phase column (Agilent Eclipse Plus C_{18}, 1.8 μ particles, 4.6 mm i.d. × 50 mm) are shown in Fig. 4. The elution of all peaks in the MeOH chromatogram (Fig 4b) are shifted to longer elution times, reflecting the slightly poorer eluting strength in RP-HPLC of methanol, relative to acetonitrile or ethanol. The peaks in the EtOH chromatogram (Fig 4c) are substantially compressed relative to the other two solvents, beginning to approach the point where peak overlap would begin to occur. Again, this reflects the greater hydrophobicity and hence greater eluting strength of EtOH, relative to the other two solvents. It is possible to adjust the gradient conditions to compensate for this difference, by using a modified gradient (5–80% EtOH in 5 min.) as illustrated in Fig. 4d.

Chromatograms showing the separation of the components of test mixture 2, with the three solvent conditions, are shown in Fig. 5. Again, small differences in eluting strength of the three solvents can be observed, for example, in the differences in retention of anthracene. The caffeine and *p*-hydroxybenzoic acid components are best resolved with EtOH, barely resolved with ACN, and partially overlapping with MeOH. Such slight differences in retention and resolution resulting from changes in eluent composition are commonly observed, with sometimes one solvent, sometimes another being preferred. Therefore, generalizations about preferred eluents based on this one example would not be justified. The more important point of interest from this study is that the peaks are generally well resolved, and nicely shaped with all three solvent systems.

Interestingly, a rising baseline can be seen in the chromatograms with the alcoholic solvents (Fig. 5b, 5c). These chromatograms were collected with UV detection at 220 nm, where the alcohol solvents still have a significant UV absorbance, as illustrated in Fig. 1b. As a consequence, the alcoholic solvents are poorly suited for operation at the lower detection wavelength of 210 nm that is often used for ACN-based RP-HPLC, particularly when gradient elution is used. The problem of rising baseline from residual UV absorbance of the alcoholic solvents is especially problematic in the gradient elution and low level quantitation of analytes that have poor UV chromophores. Fig. 5d shows the same chromatogram as 5c at the higher observe wavelength of 254 nm. While the baseline rise in the latter part of the chromatogram is suppressed, analytes such as 2-phenylethanol and caffeine, which have poor UV chromophores, are poorly detected.

The chromatograms shown in Figs. 4 and 5 were collected at a flow rate of 0.8 mL/min to enable comparison between the three eluents. However, RP-HPLC separations using this same column with ACN-based eluents are typically performed at higher flow rates. The 0.8 mL/min flow rate used in the previous example is near the maximum flow for the ethanol based effluents that will remain below the instrument pressure limit of 6,000 psi during

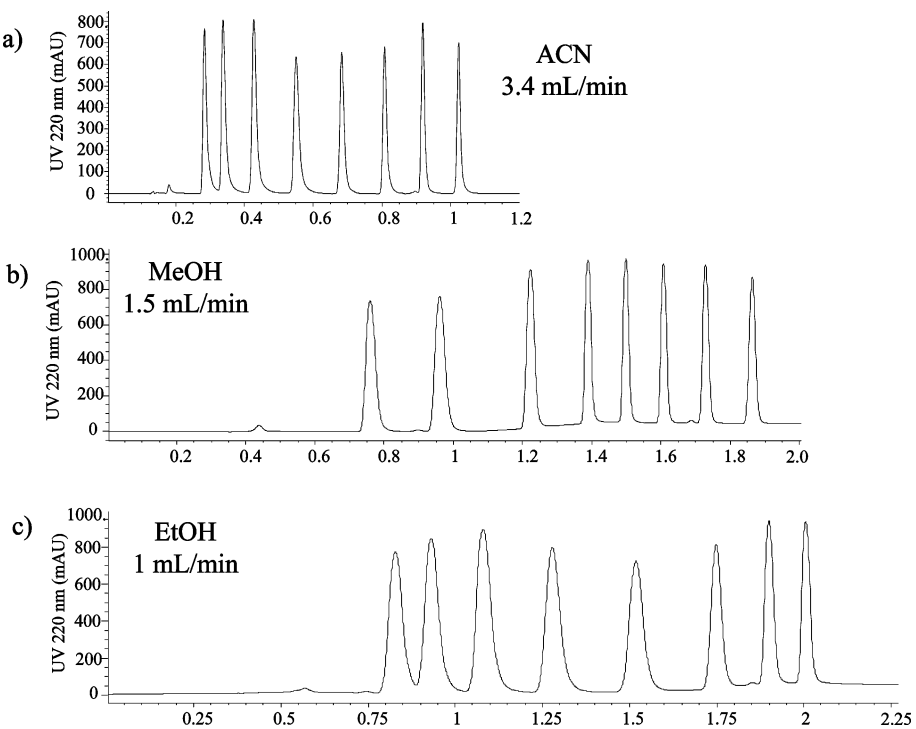

Fig. 6 Separation of alkylbenzenes test mixture with fast gradient at pressure limiting flow rates, using three different organic modifiers. Conditions: Column = Eclipse Plus C_{18}, 1. 8 μ particles, 4.6 mm i.d. × 50 mm, temperature = 25 °C, flow rates as noted in chromatograms, aqueous 0.1% H_3PO_4, detection = UV 220 nm, gradient = 80–100% organic in 0.5 min.

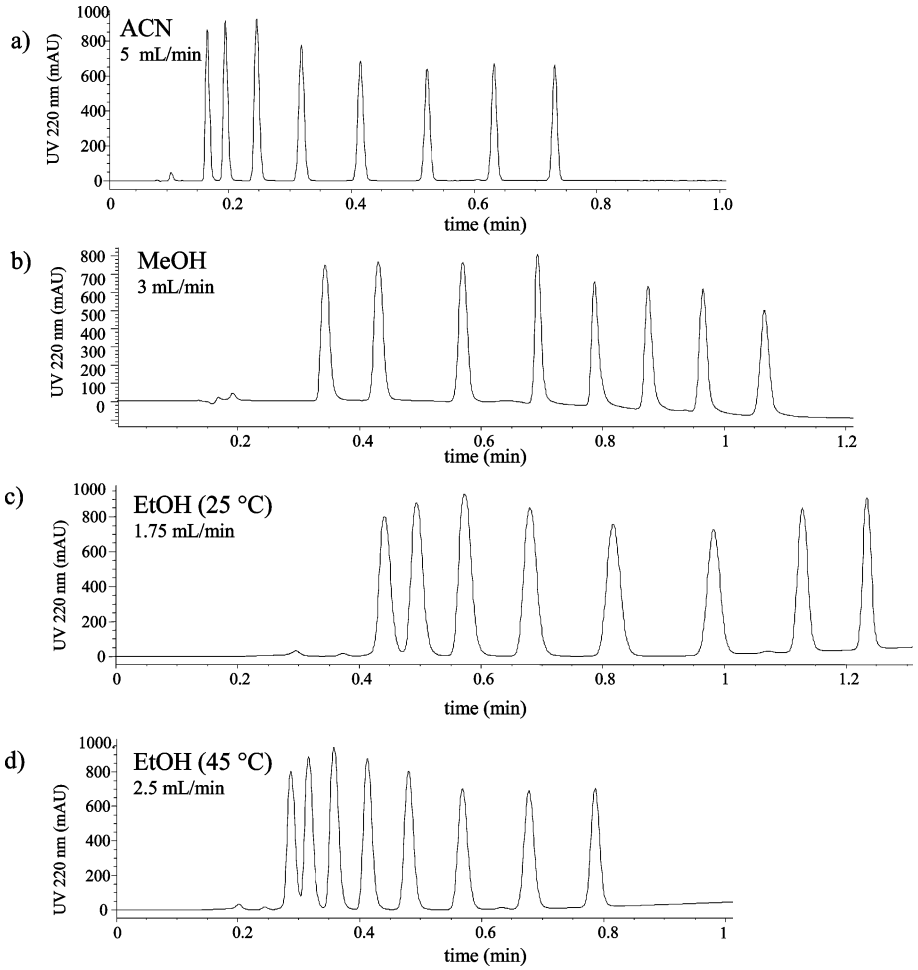

Fig. 7 Separation of alkylbenzene test mixture with fast gradient at pressure limiting flow rates, using three different organic modifiers. Conditions: Column = Ascentis Express C_{18}, flow rates as noted in chromatograms, temperature = 25 °C, except as noted, aqueous 0.1% H_3PO_4, detection = UV 220 nm, gradient = 80–100% organic in 0.5 min. d) high temperature chromatography often allows greater flow and faster chromatography.

the course of the gradient elution. Using this same criterion, much higher limiting flow rates for MeOH and especially ACN are possible.

To a first approximation, faster flow rates mean faster chromatography, although there are some limits in this regard relating to the resistance to mass transfer at high flow rates. Chromatograms showing the separation of the alkylbenzene test mixture 1 with a fast gradient (80–100% organic modifier in 0.5 min) for the three solvents, each operating near the 6,000 psi pressure limit, are shown in Fig. 6. Clearly, while the use of EtOH eluent can lead to separation, the same result can be obtained with MeOH, or especially ACN in less time and with better peak shape. Thus, high throughput analysis laboratories with high levels of instrument utilization may understandably find the switch from acetonitrile unacceptable. On the other hand, the difference in performance between the three solvents is much less than one might expect *a priori*, suggesting that the greener solvent alternative of ethanol may be suitable in some instances.

One of the most exciting recent developments in HPLC column technology in recent years has been the introduction of fused core particle technology, in which tiny particles are fused to a solid core, affording a material with the chromatographic efficiency characteristics of the smaller particle columns without the accompanying high backpressure. Fig. 7 illustrates the separation of the alkylbenzenes test mixture 1 components on a fused core particle column (Supelco Ascentis Express C_{18}, 2.7 μ particles, 4.6 mm i.d. × 50 mm) using the same fast gradient used in Fig. 6, again using pressure-limiting flow rates for the 6,000 psi system. Increased speed relative to the conventional sub 2 micron column technology is evident, particularly for the alcohol solvents, where substantially higher flow is possible. The acetonitrile flow rate used in this example (5 mL/min) is actually at the upper limit of what is allowed by this instrument, and consequently, the run time for this example is slightly longer than what would be possible at even higher flow rates. This general problem can easily be circumvented by the use of smaller diameter columns, which require proportionally smaller flow rates to achieve the same linear velocity. For example, the same chromatography observed with a 4.6 mm i.d. column operating at 5 mL/min could be obtained with a 3 mm i.d. column operating at 2.13 mL/min.

Carrying out chromatography at elevated temperatures is one way to reduce the solvent viscosity and resulting backpressure

at higher flow rates.[19] Increasing temperature can also lead to changes in analyte adsorption, which in combination with the ability to operate at higher flow rates, often leads to faster chromatography that can be used for high throughput analysis. Fig. 7d shows separation of the alkylbenzenes test mixture at 45 °C using the EtOH based mobile phase, where a pressure-limiting flow rate of 2.5 mL/min is now possible.

Conclusion

The examples presented in this study are highly idealized, but serve to illustrate the point that while acetonitrile undoubtedly delivers outstanding performance as an HPLC solvent, greener alternatives such as ethanol perform reasonably well, and may be suitable as acetonitrile replacements in many instances. For those few laboratories engaged in high throughput analysis studies with very high levels of instrument utilization, the performance advantage of acetonitrile may mean that fewer instruments are required to run the same number of samples in a given amount of time. However, many laboratories would be able to accommodate the slightly longer analysis times afforded by the use of ethanol by having instruments run a bit later into the night. For laboratories that specialize in the analysis of compounds with poor UV chromophores – for example, lipids, fats and esters, the poorer UV cutoff of ethanol may make the switch from acetonitrile unacceptable. On the other hand, laboratories that specialize in the analysis of compounds with good UV chromophores–for example, aromatic hydrocarbons, pigments and dyes, and most drugs, may be able to make the switch to ethanol with little disruption. In addition, as mass-spectrometry continues to supplant UV as the preferred detection technique for HPLC, the higher UV cutoff of the alcohol solvents may become less important over time.

Many analysts are uncomfortable with the idea of giving up **any** performance, preferring to have as much firepower as possible available for handling whatever scenario may arise. While justifiable in some settings, the fact remains that every bit of excess performance delivered by modern HPLC technology is probably not needed in most analytical settings, and may be contributing to the ever escalating cost and complexity of carrying out simple analyses, which is becoming increasingly difficult to justify in today's economically constrained environment. As the cost of acetonitrile and the resulting cost in waste disposal continue to escalate, the decreased cost of the ethanol alternative becomes increasingly compelling.

An additional advantage of the use of ethanol as an HPLC solvent stems from the universal availability of this solvent. Recent increasing use of ethanol as a fuel suggests that the cost and quality of ethanol should continue to improve in coming years. Finally, the decreased environmental impact of ethanol *vs.* acetonitrile waste streams is another important consideration arguing in favor of adoption of this greener platform solvent for HPLC. With recent trends in miniaturization and simplification of HPLC technology, a future can be envisioned in which HPLC instrumentation becomes truly ubiquitous, being routinely used for on-site environmental monitoring in sensitive environments, in the offices of doctors, dentists, by farmers and food processors, and even within the home.[20] The use of a greener solvent such as ethanol would be strongly preferred for such applications.

Acknowledgements

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