A SYSTEMATIC APPROACH FOR ACHIRAL METHODS DEVELOPMENT IN CONVERGENCE CHROMATOGRAPHY

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Convergence chromatography (CC) is a chromatographic technique that provides alternative selectivity to reversed-phase LC while reducing solvent consumption. Using supercritical carbon dioxide as the primary mobile phase, mobile-phase viscosity is decreased, allowing for high chromatographic efficiencies and throughput, compared with traditional HPLC. The numerous modes of interaction between the stationary phase and solute can make methods development both complex and time consuming for the first-time user. With a systematic method development approach, the use of CO_2 mobile phases provide the analytical LC chemist with new separation tools to use in challenging separations.

Historically, the adoption of supercritical fluid chromatography suffered from irreproducible results, relatively poor instrument reliability, and a minimum of industry expertise. CC aims to address those issues by incorporating technological advancements in LC developed over the last decade. These advancements increase instrument robustness, practical use results, and ease-of-use implementation.

As supporting documentation regarding applicability, workflow, and strategy associated with CC, this white paper describes a methods development protocol that relies, for achiral methods development, on Waters ACQUITY UPC^{2}® Technology and sub-2-µm column chemistries.

INTRODUCTION

Convergence chromatography uses compressed carbon dioxide as its primary mobile phase. Though based on the principles of supercritical fluid chromatography, CC relies on the ACQUITY UPC^2 System and sub-2-µm column chemistries.

Methods development using supercritical fluid chromatography has been documented and described since the 1980s.[1] As in any chromatographic technique, method development strategies often begin with a screening protocol, typically the evaluation of a set of conditions, including mobile phases, on various stationary phases.[2,3] Stationary phases and conditions are selected to provide differences in selectivity and retentivity while permitting high throughput, to reduce screening time.

The practical considerations associated with CC differ from those of reversed-phase liquid chromatography. Both aim to provide an optimum or desired separation in as few runs as possible, yet the conditions that most significantly affect chromatography can vary. For example, in CC, highly basic compounds commonly undergo undesired secondary interactions with the stationary phase. In such cases, adding modifiers containing basic additives is necessary to minimize such interactions and thus improve peak shape.[4,5] In CC, an additional chromatographic tool available for adjusting chromatography is density. Changes in pressure and temperature affect the density of CO_2 and thus its solvating power, which influences chromatographic behavior.[1] For that reason and for others, CC methods development requires special consideration.

To render method development in CC more effective, a systematic approach to achiral method development was developed, the initial steps evaluating the properties of the analyte of interest. On the basis of these properties, chromatography variables of column type, organic modifiers, and additives are screened for their affect on peak shape and selectivity. Columns may include RPLC, NPLC, HILIC, and mixed-mode stationary phases, allowing for a "stationary-phase-agnostic" approach. When combined with sub-2-µm column chemistries and mass-spectrometric detection, this strategy achieves an efficient and effective approach for CC method development. Combining the selectivity enhancements of a stationary-phase-agnostic approach, the high efficiencies of sub-2-um particle size columns and the specificity of MS detection permits the confident analysis of a wide range of compounds.

EXPERIMENTAL

Sample description

Analytes possessing a wide range of both chemical and physical properties[6] were selected for evaluation. These samples included acids, bases, and neutrals. In addition, compounds with a range of octanol-water partition coefficients (logP) were represented (Table 1).[6] The compounds, all soluble in methanol, were grouped according to their physical and chemical properties.

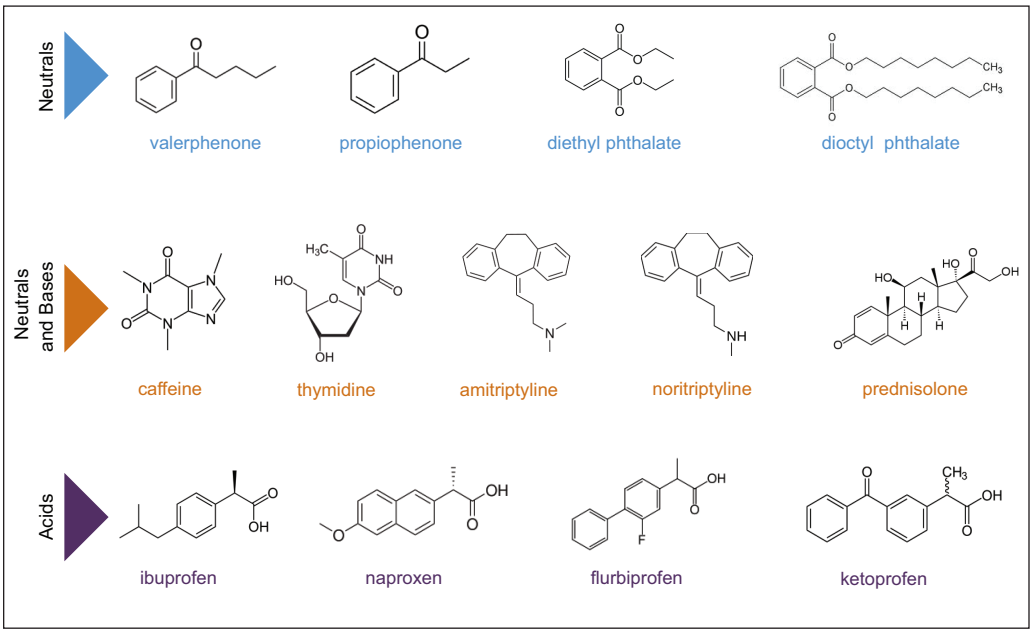
Analyte	MW	Log p (ACD)
amitriptyline	277.4	4.41
caffeine	194.2	-0.63
diethyl phthalate	222.24	2.71
dioctyl phthalate	390.56	8.52
flurbiprofen	244.3	3.66
ibuprofen	206.3	3.5
ketoprofen	254.3	2.91
naproxen	230.3	2.88
nortriptyline	263.4	3.97
prednisolone	360.4	1.64
propiophenone	134.18	2.18
thymidine	242.23	-0.85
valerphenone	162.23	3.2

Table 1. List of compounds, MW, and logP values. lopP values were calculated using Advanced Chemistry Design (ACD).

Standards were obtained from Sigma-Aldrich, unless otherwise specified. Stock solutions were prepared in 2-propanol at concentrations of 2 mg/mL. Samples were sonicated for 10 minutes, to ensure their complete dissolution. The stock solutions were combined, to prepare working samples at concentrations of 0.2 mg/mL. The diluent was 90:10 heptane/2-propanol.

Method conditions

LC conditions	
System	ACQUITY UPC^2 with ACQUITY UPC^2 Photodiode Array (PDA) detector
Wavelength	254 nm or 220 nm: compensated 350-450 nm
ABPR	2000 psi
Column temperature	50 °C
Flow rate	2.0 mL/min
Gradient	Non polar compounds: 2–40% modifier in 5 minutes Polar compounds: 5–40% modifier in 5 minutes
Injection volume	1 µL
Sample diluent	9:1 Heptane/2-propanol
Columns (3.0 x 100 mm):	ACQUITY UPC^2 BEH, 130Å, 1.7 µm ACQUITY UPC^2 HSS C_{18} SB, 100Å, 1.8 µm ACQUITY UPC^2 BEH 2-Ethylpyridine, 130Å, 1.7 µm ACQUITY UPC^2 CSH Fluoro-Phenyl, 130Å, 1.7 µm
Mobile phase A:	CO_2
Mobile phase B:	B1: methanol B2: 1:1 methanol/acetonitrile B3: 15 mM ammonium formate with 2% HCOOH in methanol B4: 20 mM citric acid in methanol
MS conditions	
MS system	Single quadrupole detector (SQD) with MS splitter
Ionization mode	ESI+, ESI -
Acquisition range	50-500 *m/z*
Capillary voltage	2 kV
Cone voltage	30 V
Make up flow	0.2% NH_4OH in methanol at 0.4 mL/min
Desolvation gas	900 L/hr nitrogen
Desolvation temperature	450 °C



RESULTS AND DISCUSSION

Methodology

A screening protocol based on chromatographic attributes and other critical factors was developed. Accordingly, initial screening occurs on a pre-selected set of columns and modifiers (Figure 1). The BEH column was chosen to address a wide range of polarity, and the BEH 2-EP column was chosen because of the alkaline pKa of the stationary phase. Two organic solvents were chosen on the basis of their protic and aprotic properties. These choices were hypothesized to provide a fair assessment for the next steps of method development. If insufficient retention is observed, then additional, nonpolar stationary phases are tested. If adequate retention is observed, the separation is evaluated for peak shape, retention, and selectivity.

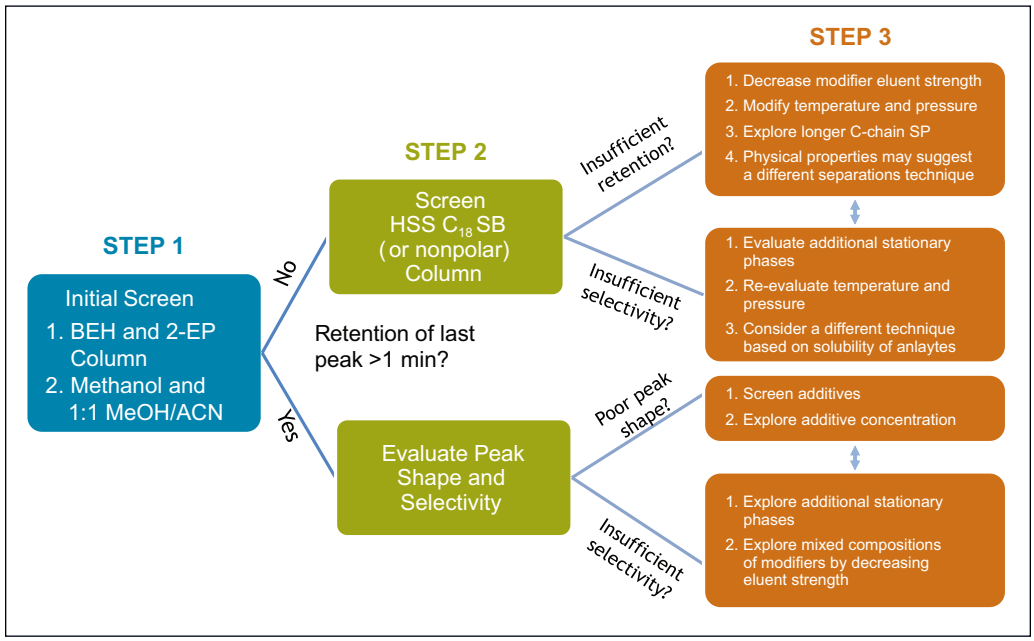


Figure 1. Method development screening protocol for supercritical fluid chromatography (SFC).

Additives have been found to affect secondary interactions.[4,5] Thus they are used to influence peak shape. Various stationary phases are used to address issues of retentivity and selectivity, and parameters such as gradient slope, temperature, and pressure are used to optimize and fine tune a separation.

As with any screening protocol, criteria for success must be established. For this study, acceptance criteria for the critical pair were specified as USP resolution >1.5 and USP tailing ≤1.0. The systematic protocol addresses one critical factor at a time, to simplify the method development decision points for CC separations.

Method development for neutral compounds

As a technique derived from normal-phase chromatography, CC has proved successful for analyzing nonpolar compounds.[7,6] This class of compounds includes phenones and phthalates, which are typically used and monitored in the pharmaceutical, food, environmental, and chemical materials industries. A set of phthalates was screened according to the protocol previously described. The initial screening of methanol and 1:1 methanol/acetonitrile with the two selected stationary phases yielded greater retentivity with the modifier of weaker elution strength (1:1 methanol/acetonitrile), as shown in Figure 2. Nevertheless, the phenones (valerphenone and propiophenone) co-eluted in all separations.

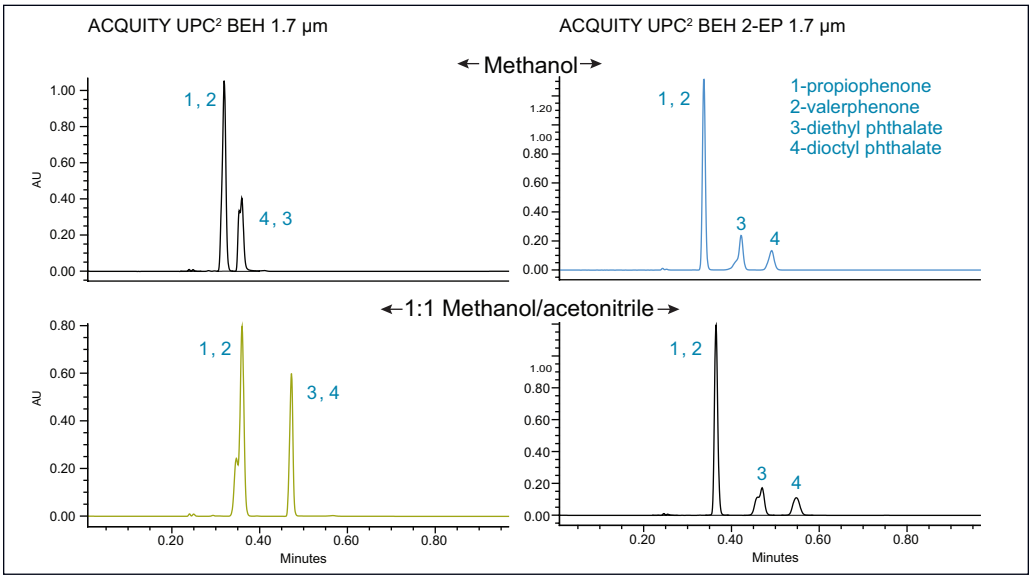


Figure 2. Initial column screening of nonpolar compounds and evaluation of stationary phase.

Based on the workflow and initial screening results, a nonpolar stationary phase (ACQUITY UPC^2 HSS C_{18} SB Column) was subsequently evaluated. The hydrophobic interaction between the stationary phase and the analyte produced greater retentivity of the neutral phthalate compounds. Yet separation of the phenolic compounds (valerphenone and Ipropiophenone) remained relatively poor, as evidenced by a co-elution occurring near the system's void volume (Figures 3A and 3B). The similar structural attributes of these compounds, rather than the stearic-retention mechanisms, appeared to dominate the retentivity.

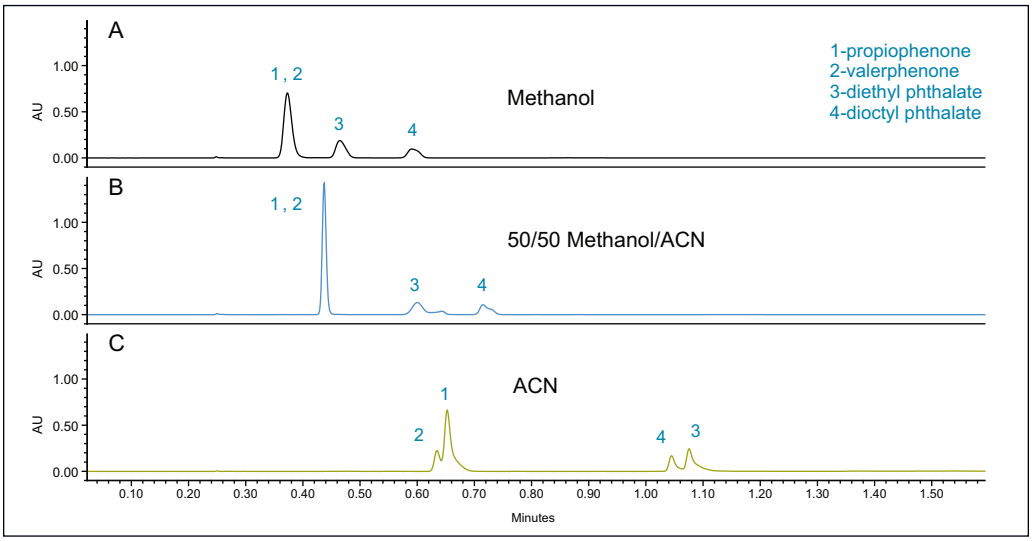


Figure 3. Modifer screening results performed on the ACQUITY HSS C_{18} SB column.

To increase retentivity, and to improve resolution of the critical pair, the next step of the methods development protocol evaluated decreasing modifier eluent strengths. The ACQUITY UPC^2 HSS C_{18} SB Column was then screened using a weaker modifier. Acetonitrile was used in place of methanol, resulting in partial resolution of the phenones as well as changes in selectivity for the phthalates (Figure 3c).

To further improve the separation, the gradient slope was evaluated. The gradient was manipulated by adjusting the modifier percentage according to the elution of the analytes. The overlay of percent modifier and the chromatographic trace (Figure 4a) showed that the most retained analyte eluted at approximately 8% modifier, whereas the critical pair eluted at approximately 4% modifier. Given the

low percent modifier required for elution of the phenones, an isocratic separation was explored to obtain greater retention and resolution. Isocratic separations of 1% to 2% modifier yielded greater retention of the phenones (Figure 4b – 4c), but no significant improvement in resolution was observed.

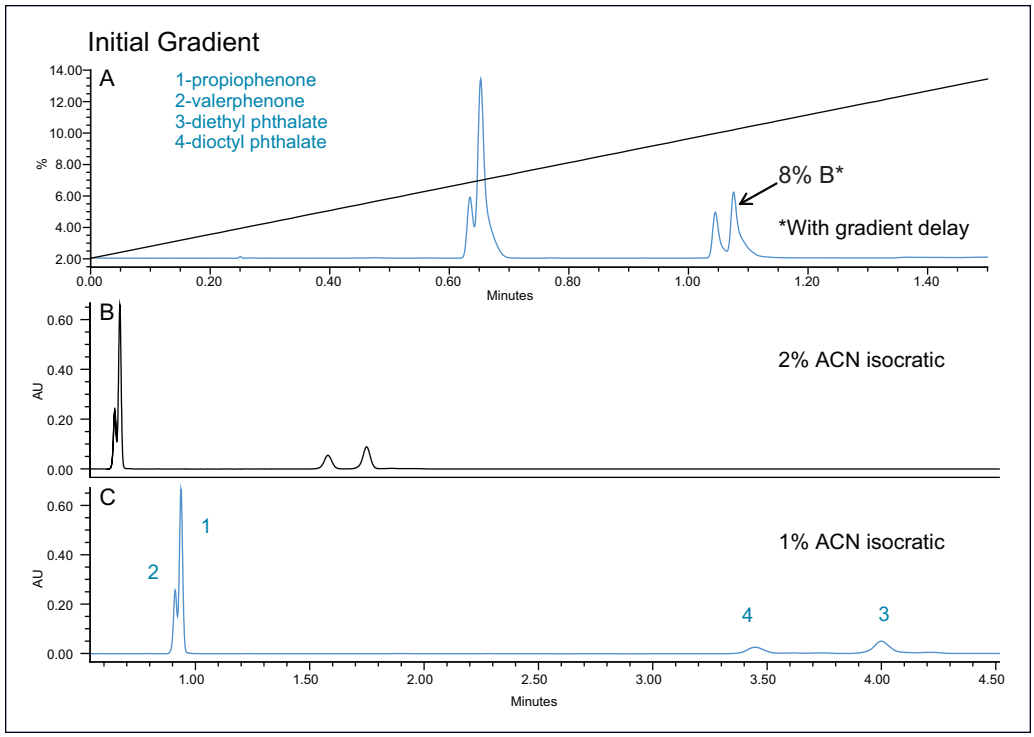


Figure 4. Evaluation of gradient in method development of nonpolar compounds.

The column temperature and the system's back pressure were then explored. The isocratic separation was tested from 30–50 °C and from 2000–3000 psi of applied back pressure. Lower temperatures decreased retention of the phthalates (Figure 5) while significantly improving the resolution of the critical pair (USP resolution from 0.85 to 1.36).

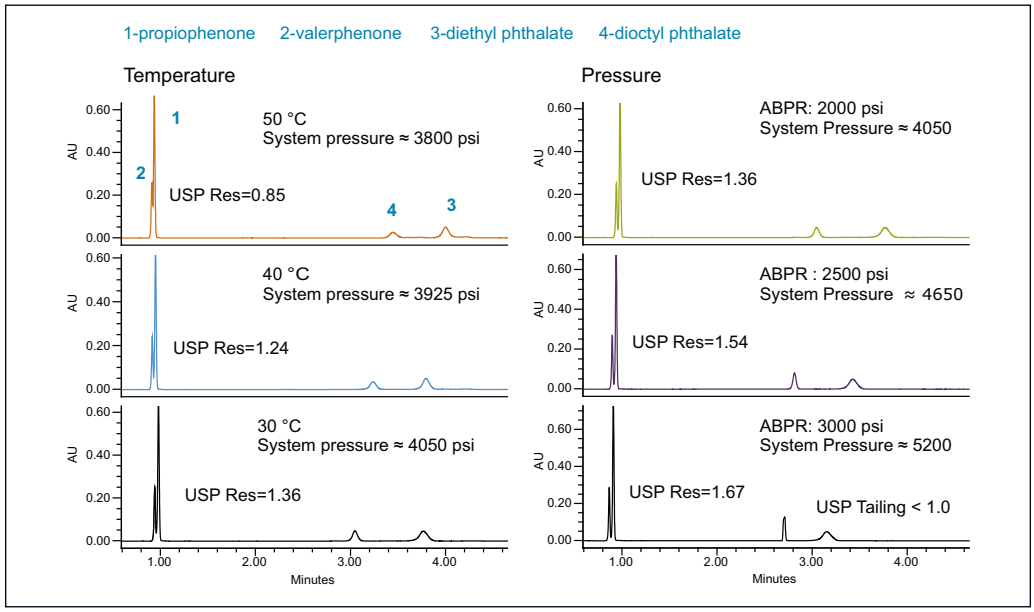


Figure 5. Evaluation of temperature and pressure in method development of nonpolar compounds.

Further improvement in resolution was achieved by increasing the applied back pressure from 2000 to 3000 psi. The final separation met the established criteria (USP resolution >1.5 and USP tailing >1.0). Mass spectrometry (MS) confirmed the elution order of the peaks in the final methodology (Figure 6).

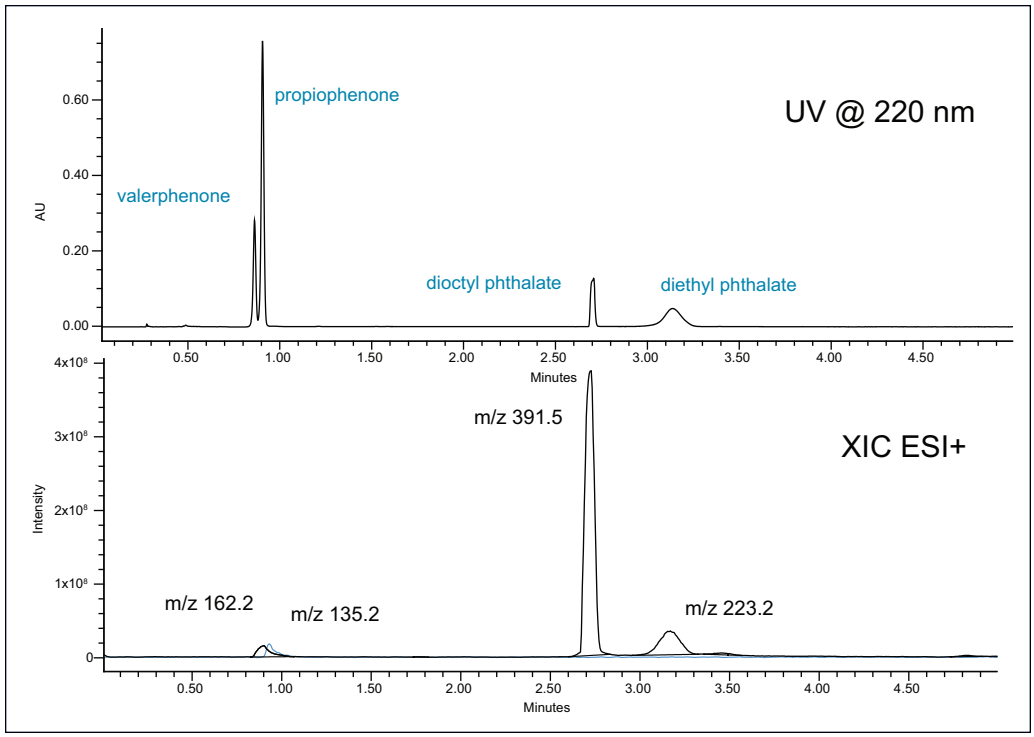


Figure 6. Comparison of UV and mass spectrum chromatograms of the nonpolar constituents.

Method development for basic compounds

Screening with different additives is a frequently used tool in SFC method development for polar compounds.[5,11,12] However, the chemical properties of the analyte greatly influence which method development variables affect the chromatography. For instance, in SFC, bases typically undergo secondary interactions with the stationary phase. Spiking the modifier with alkaline additives can reduce these interactions while only minimally affecting the peak shape of the acidic and neutral analytes.

The protocol (step 1) was applied to a set of samples containing both neutrals (prednisolone, caffeine, and thymidine) and bases (amitriptyline and nortriptyline). Co-elutions indicated that neither of the modifiers provided adequate peak shape for some of the alkaline analytes. Given the poor peak shape of these analytes, retention times were difficult to discern by UV alone, so they were confirmed by MS.

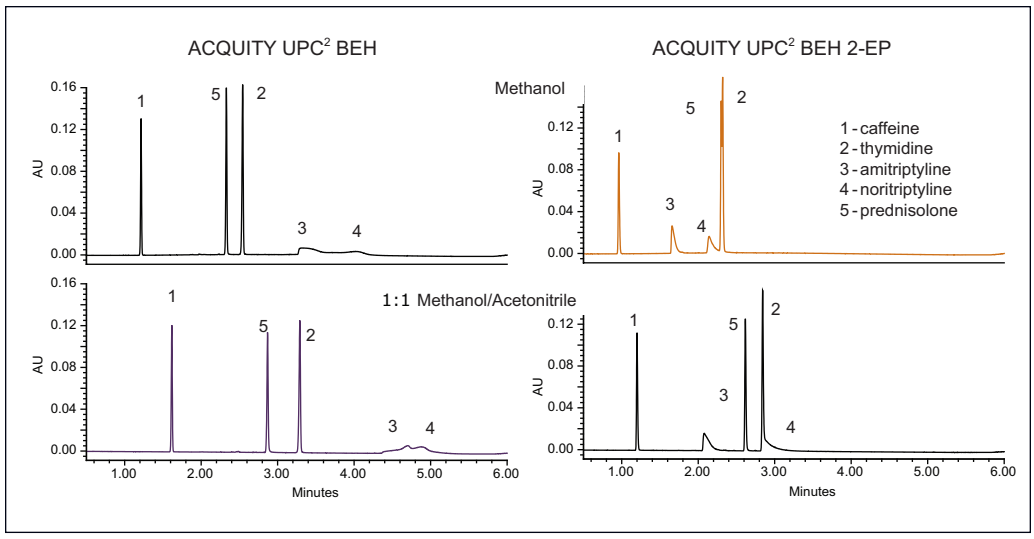


Figure 7. Protocol Step 1 results for the neutral/bases mixture.

Because of the poor peak shape of the basic analytes, protocol step 2 in the systematic method development approach investigated modifiers spiked with alkaline additives (Figure 7).

A wide variety of acidic and alkaline additives have been used in SFC separations. The additives and modifiers in this experiment were chosen to provide an adequate peak shape for the greatest number of analytes while also being MS-compatible. The modifiers selected were 0.2% ammonium hydroxide and 15 mM ammonium formate, the latter with 2% formic acid. Though ammonium hydroxide (NH_4OH) is a common additive used in SFC,[4,13] ammonium salts combined with an acidic mobile phase have recently exhibited promising results.[14] The effect of both additive combinations on the peak shape and retention times of neutral compounds proved minimal (Figure 8).

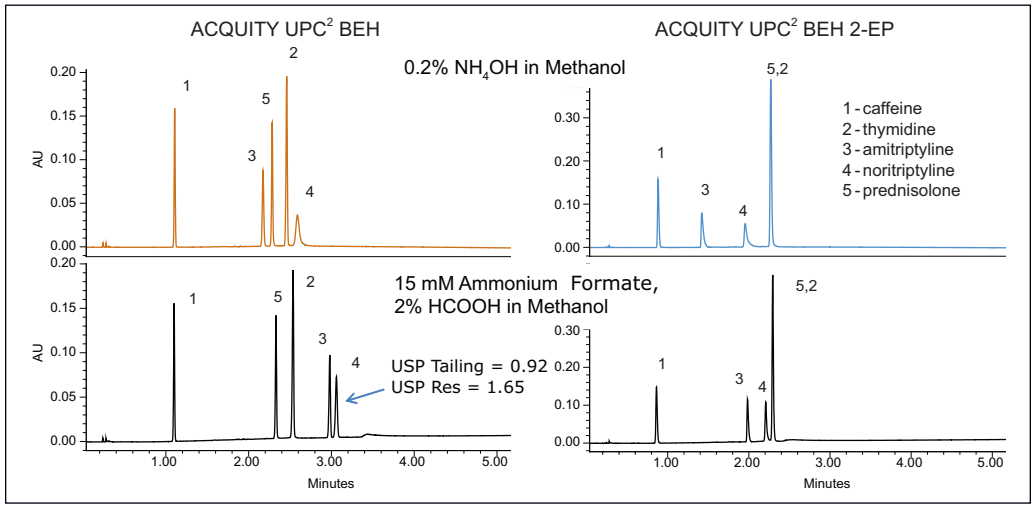


Figure 8. Protocol Step 2 results for the bases mixture. Screening additives for bases.

Nevertheless, the peak shape of the bases improved. USP tailing was reduced to <1.0, and retention times were earlier. Both results are consistent with a reduction in ionic interactions between the solute and the stationary phase. The BEH column chemistry with the salt/acid modifier resulted in the lowest USP tailing value (0.92) while meeting the USP resolution criteria of >2.0. As in previous examples, the elution order of the analytes was confirmed by MS (Figure 9).

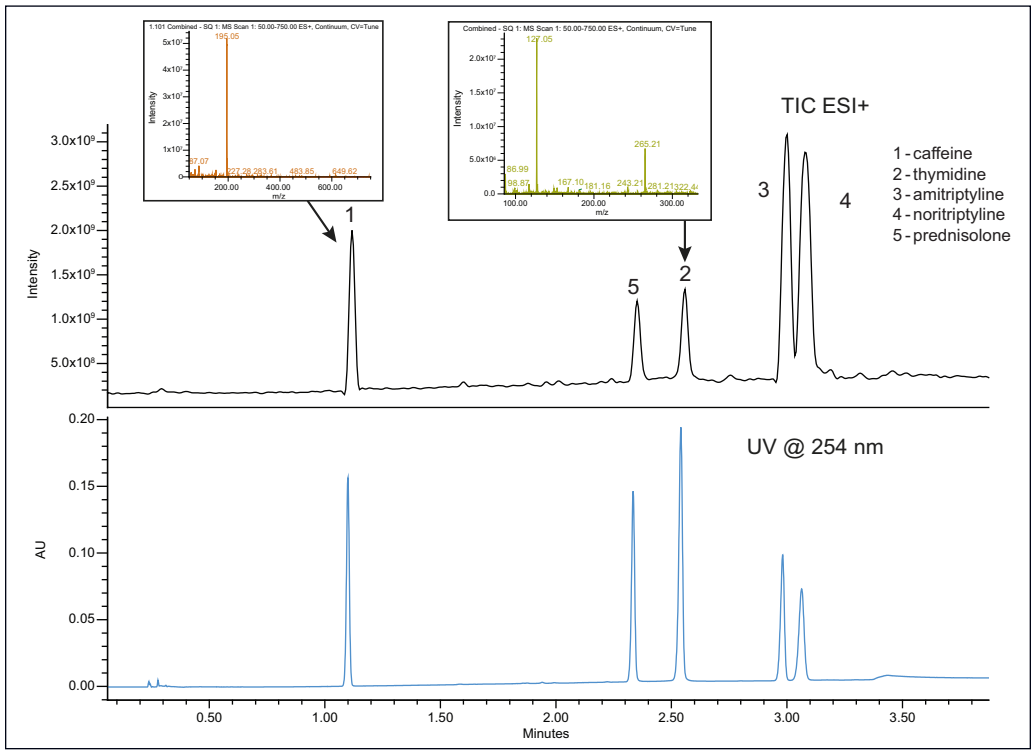


Figure 9. Method development results for bases: UV and mass spectrum chromatograms.

Method development for acidic compounds

Ideally, a comprehensive method-development strategy encompasses compounds whose range of chemical and physical properties is broad. Yet, as mentioned previously, secondary interactions in SFC between charged species (acids and bases) and stationary phases often result in poor peak shape and delayed retention. As with bases, the peak shape of acidic analytes is best improved by the use of additives.[11,15,16] The challenge for this particular class of compounds lies in choosing additives that provide optimum peak shape and MS compatibility.

A set of non-steroidal, anti-inflammatory drugs (NSAIDs) were tested using the screening protocol (Figure 1). Unlike the screening of earlier samples, the effect of the stationary phase on the analytes' peak shape was pronounced. The ACQUITY UPC^2 BEH 2-EP Column provided the lowest USP tailing and the greatest sensitivity for the NSAIDs (Figure 10). The improved peak shape of the acids on the ethylpyridine stationary phase is consistent with the reduction in ionic interactions typical of that stationary phase.[17] Screening with 1:1 methanol/acetonitrile resulted in longer retention and increased tailing. Analysis of the results revealed that retention times, in what can be described as the polar retention region of the chromatogram, were adequate (>1 minute for the last peak), but the peak symmetry failed to fall within the standard criterion for USP tailing (<1.5).

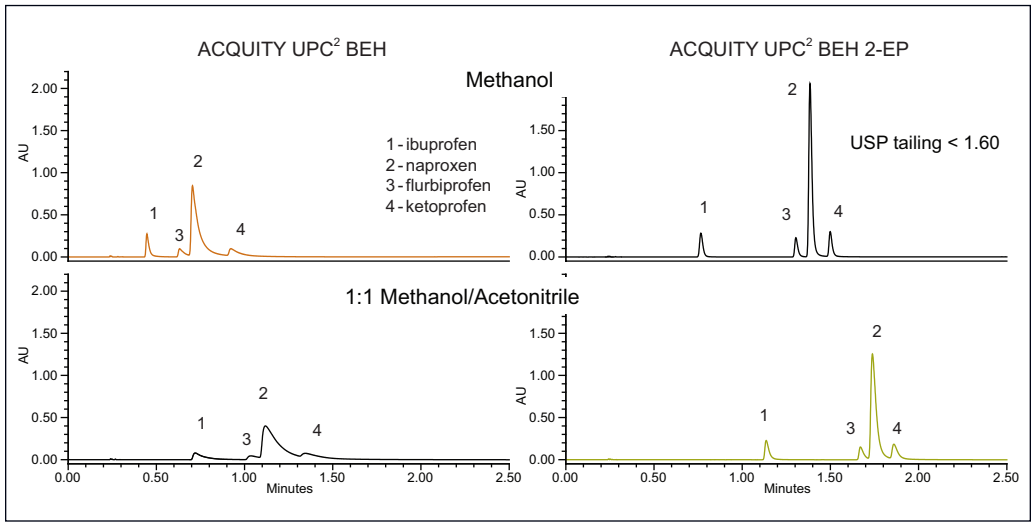


Figure 10. Initial column screening of acidic non-steroidal anti-inflammatories (NSAIDs).

To improve peak shape, additional chemical factors were evaluated. A set of acidic additives were screened for their ability to reduce ionic interactions between the acidic analytes and the stationary phase. Mobile phases containing 2% formic acid, 20 mM citric acid, and 0.2% heptafluorobutyric acid (HFBA) and screened on both the ACQUITY UPC^2 BEH and the ACQUITY UPC^2 BEH 2-EP Columns showed differences in selectivity and peak shape (Figure 11).

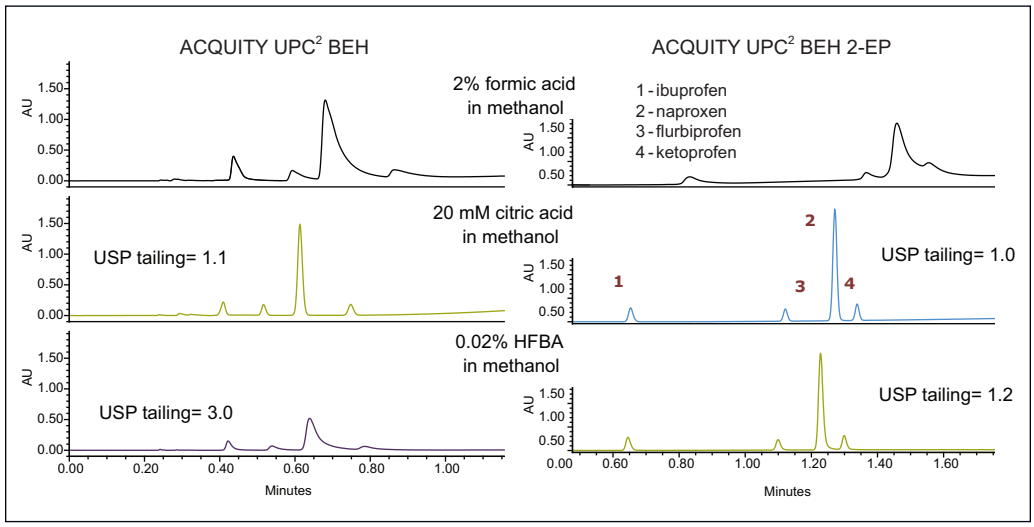


Figure 11. Screening of additives for non-steroidal anti-inflammatories (NSAIDs).

Formic acid resulted in no significant improvement in peak shape, whereas citric acid reduced USP tailing factors for all analytes. The addition of heptafluorobutyric acid to the mobile phase reduced tailing on the BEH 2-EP column, but it did not significantly affect the peak shape on the BEH column. Although the separation using citric acid met the established criteria, additional efforts were made to find conditions that were more MS-compatible.

Additional additives explored the combination of TFA (0.05%) and water for the separation of the NSAIDs. Testing the additive combination on the BEH 2-EP column reduced peak tailing (USP tailing <2.0) (Figure 12).

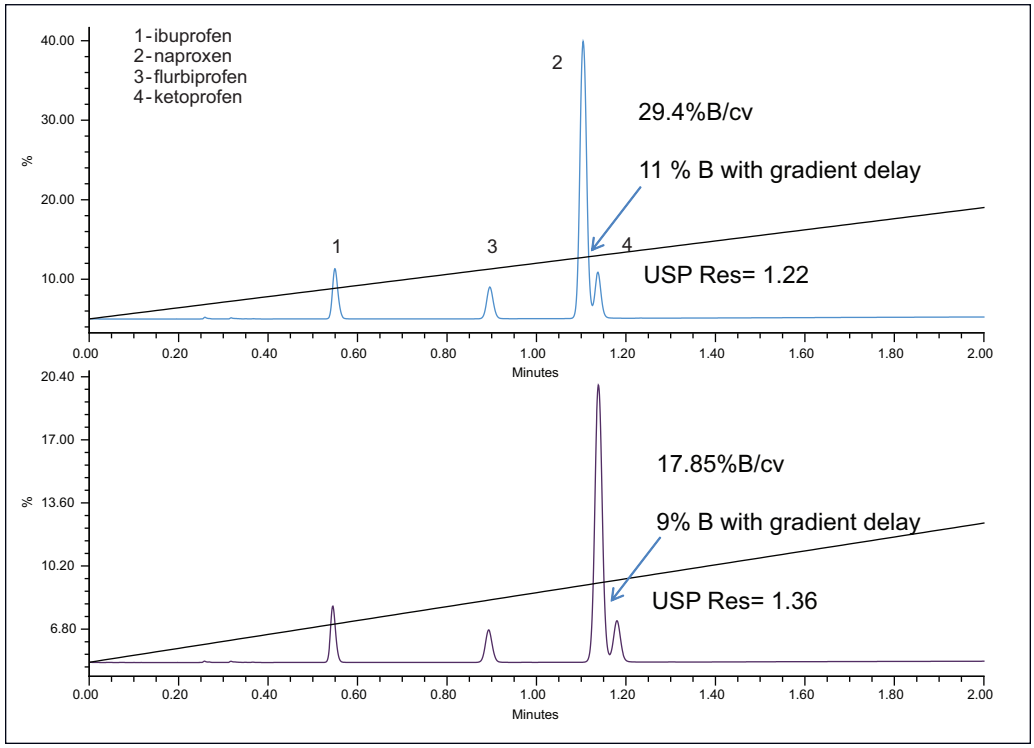


Figure 12. Evaluation of gradient for method development of NSAIDs. Modifier of 0.05% TFA/2% water in methanol.

Differences in selectivity, however, resulted in poor (<2.0) resolution of the naproxen and ketoprofen. In an attempt to improve resolution of the critical pair, the physical conditions of the separation were adjusted, including the gradient and temperature. The initial gradient (5% B to 40% B, in 5 minutes) produced a change in B of 29.4% per column volume. Analysis of the resultant chromatogram showed elution of the last peak occurring at approximately 12% B. To improve resolution of the critical pair, a shallower gradient was evaluated. The new gradient produced a 17.85% change in B per column volume (5% B to 20% B in 4 minutes) while maintaining the initial conditions. The results showed a minimal effect on the resolution of the critical pair with no significant change in USP resolution or retention (Figure 12). The effect of temperature on the chromatography was greater (Figure 13). Increasing the column temperature from 40 °C to 50 °C increased the USP resolution of naproxen and ketoprofen from 1.36 to 2.55 without a significant accompanying change in peak tailing, meeting method criteria.

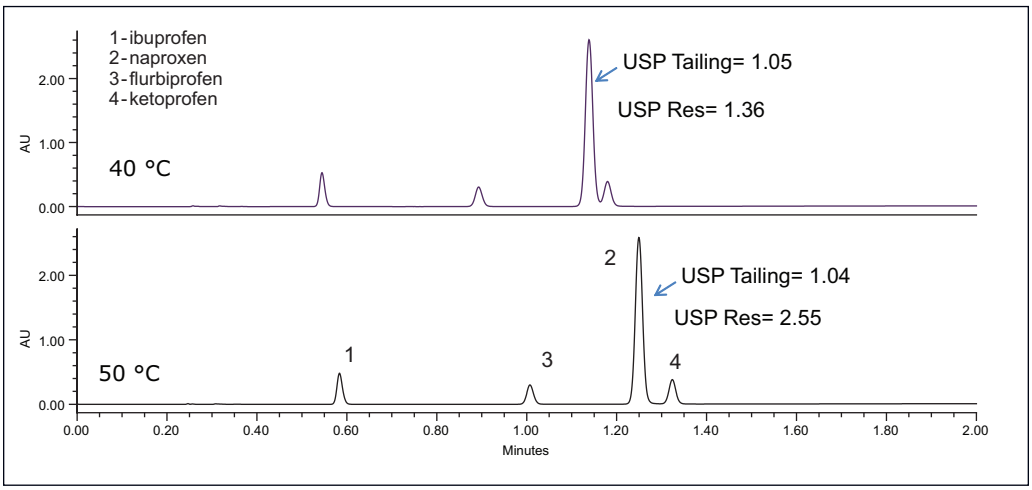


Figure 13. Evaluation of temperature in method development of acidic NSAIDs. Modifier of 0.05% TFA/2% water in methanol.

CONCLUSIONS

Convergence chromatography is a chromatographic technique that uses a CO_2 mobile phase to provide alternative selectivity to traditional reversed-phase HPLC while reducing solvent consumption.

The method development protocol proposed here outlines a systematic approach. The results speak to the practicality of that approach and provide some insight on the chromatographic behavior associated with the technique. CC separations can be manipulated using a range of variables including stationary phases, organic modifiers, and physical factors. The effect of each method variable, chemical or physical, on the chromatographic separation can vary (Table 3). Whereas chemical factors influence selectivity and retentivity, physical parameters affect efficiency and retentivity.

	Efficiency	Retentivity	Selectivity
Stationary phase		■	■
Mobile phase		■	■
Additive	■	■	■
Column length	■	■	
Flow rate	■	■	
Particle size	■		
Temperature		■	■
Pressure		■	

Table 2.

Considering these factors, a systematic approach to methods development that manipulates selectivity, retentivity, and efficiency was developed. To improve peak shape, stationary phases and combinations of modifiers and additives were first explored, while retentivity was manipulated by changing CO_2 density, using pressure and temperature. This systematic approach to method development can serve a variety of compounds that exhibit different chemical properties.

Acknowledgments

Helene Boiteux, Stephane Dubant, Magdalena Abad

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