

## Method Development for the Separation of Sulfated Estrogens by UltraPerformance Convergence Chromatography (UPC<sup>2</sup>)

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### Abstract

In this study, method development for the application of UltraPerformance Convergence Chromatography (UPC<sup>2</sup>) for the separation of 10 structurally similar sulfated estrogens is explored.

### Benefits

- Analysis of sulfated estrogens in their native form, thus reducing sample preparation and analysis by >90% compared to traditional methods
- Does not require hazardous chemicals typical for sample derivatization prior to GC analysis
- Full resolution of all isobaric compound pairs

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### Introduction

The chromatographic analysis of steroids and steroid derivatives often presents a difficult challenge due to their structural similarities. Even with the inclusion of mass spectrometric detection, due to the isobaric

nature of many of these compounds, chromatographic resolution is often required. One such group of compounds is the conjugated estrogens. Because free steroids have limited aqueous solubility, they are often found in a biologically conjugated form, which in mammals can be as sulfates or glucuronates. The resulting ester formed with the negatively charged hydrophilic side groups increase their aqueous solubility, and thus their bioavailability. The sulfated estrogens are frequently used as hormone replacement therapy for post-menopausal women, to provide both symptomatic relief from vasomotor symptoms (hot flashes) and prevent osteoporosis.<sup>1,2,3</sup> They have also been used in the treatment of hormone deficiencies in younger women.<sup>4</sup> Because of these treatment regimes, there is great interest in the development and characterization of therapeutic preparations of the sulfated estrogens. Typical methods employing gas chromatography (GC) involve lengthy sample preparation and derivatization steps, and results in inadequate resolution for one isobaric pair of compounds. Additional methods have been developed utilizing LC-MS yielding a relatively complex method requiring approximately 90 minutes for analysis. In this study, method development for the application of UltraPerformance Convergence Chromatography (UPC<sup>2</sup>) for the separation of 10 structurally similar sulfated estrogens is explored (Figure 1).

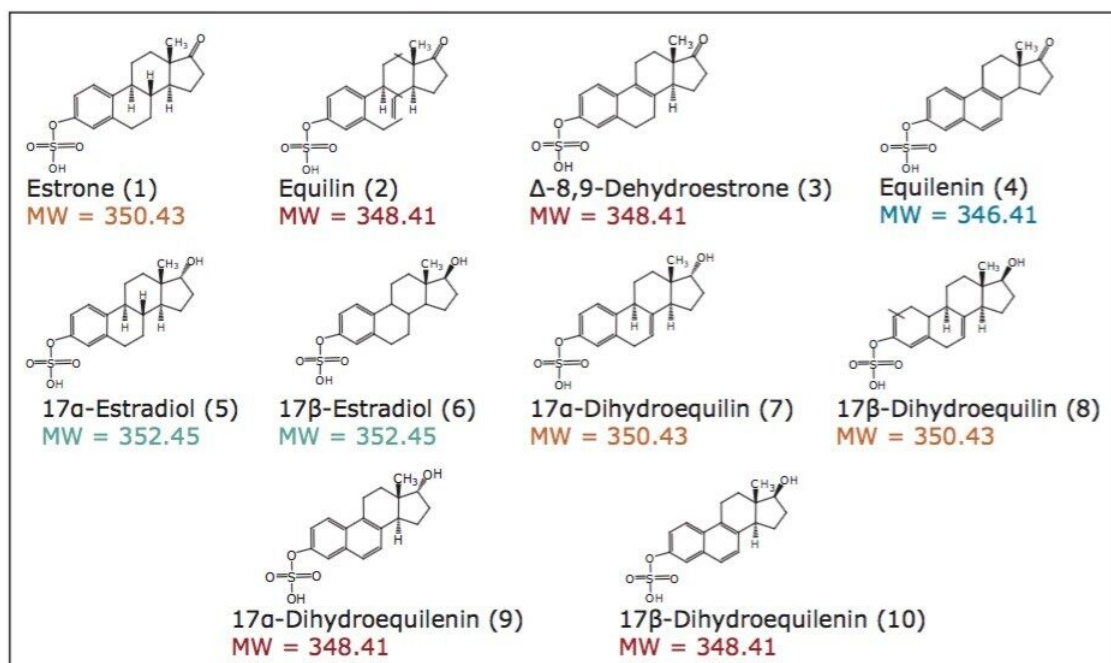


Figure 1. Structures of ten sulfated estrogens. Compounds with the same color font for molecular weight indicate that they are isobaric. The numbers assigned to each compound are used for peak identification in all figures.

## Experimental

### Sample preparation

Stock solutions were prepared from individual sulfated estrogen samples using methanol (MeOH), ethanol (EtOH), and 2-propanol (IPA) as diluents. The stock solutions were used to prepare the final sample mixture, at the concentrations shown in Table 1, using ethanol as a diluent.

Peak #	Sulfated estrogens	Molecular weight	Stocks		Sample mix (in EtOH) Final conc. (mg/mL)
			Conc. (mg/mL)	Diluent	
1	Estrone	350.43	3	30:70 IPA/EtOH	0.4
2	Equilin	348.41	5	EtOH	0.4
3	$\Delta$ -8,9-Dehydroestrone	348.41	3	EtOH	0.4
4	Equilenin	346.41	3	40:60 MeOH/EtOH	0.05
5	17 $\alpha$ -Estradiol	352.45	10	EtOH	0.4
6	17 $\beta$ -Estradiol	352.45	10	EtOH	0.4
7	17 $\alpha$ -Dihydroequilin	350.43	5	EtOH	0.4
8	17 $\beta$ -Dihydroequilin	350.43	10	EtOH	0.4
9	17 $\alpha$ -Dihydroequilenin	348.41	10	EtOH	0.05
10	17 $\beta$ -Dihydroequilenin	348.41	10	EtOH	0.05

Table 1. Stock and sample preparation.

### UPC<sup>2</sup> conditions

System:	ACQUITY UPC <sup>2</sup> with PDA detector
Column:	ACQUITY UPC <sup>2</sup> BEH 2-Ethylpyridine 1.7 $\mu$ m, 2.1 x 150 mm (p/n 186006579)
Mobile phase A:	CO <sub>2</sub> (tank, medical grade)
Mobile phase B:	Methanol with additional additives, as specified for each chromatogram
Column temp.:	20 °C to 50 °C

## UPC<sup>2</sup> conditions

ABPR:	1700 to 4100 psi
UV detection:	220 nm (compensated 380 to 480 nm) [40 pts/s]
Injection volume:	1.5 µL
Strong needle wash:	Methanol
Weak needle wash:	2-Propanol
Seal wash:	Methanol
Gradient conditions:	Shown in individual chromatograms
Vials:	LCMS Certified Maximum Recovery Vials

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## Results and Discussion

The current USP method for the characterization of conjugated estrogens utilizes GC analysis of the derivatized estrogens.<sup>5</sup> Prior to the derivatization of the estrogens, the conjugated estrogen must be treated to cleave the sulfate group using a sulfatase enzyme. This process takes approximately two hours in multiple steps involving shaking, sonication, water baths, centrifuging, buffers, pH adjustments, and filtration. The derivatization step requires another 25 minutes with the addition of pyridine, bis(trimethylsilyl)trifluoroacetamide, and trimethylchlorosilane. The sample is then analyzed by a 35-minute GC-FID method. As shown in Figure 2, even under these conditions with a total sample preparation and analysis time of approximately three hours, two of the compounds are not completely resolved.

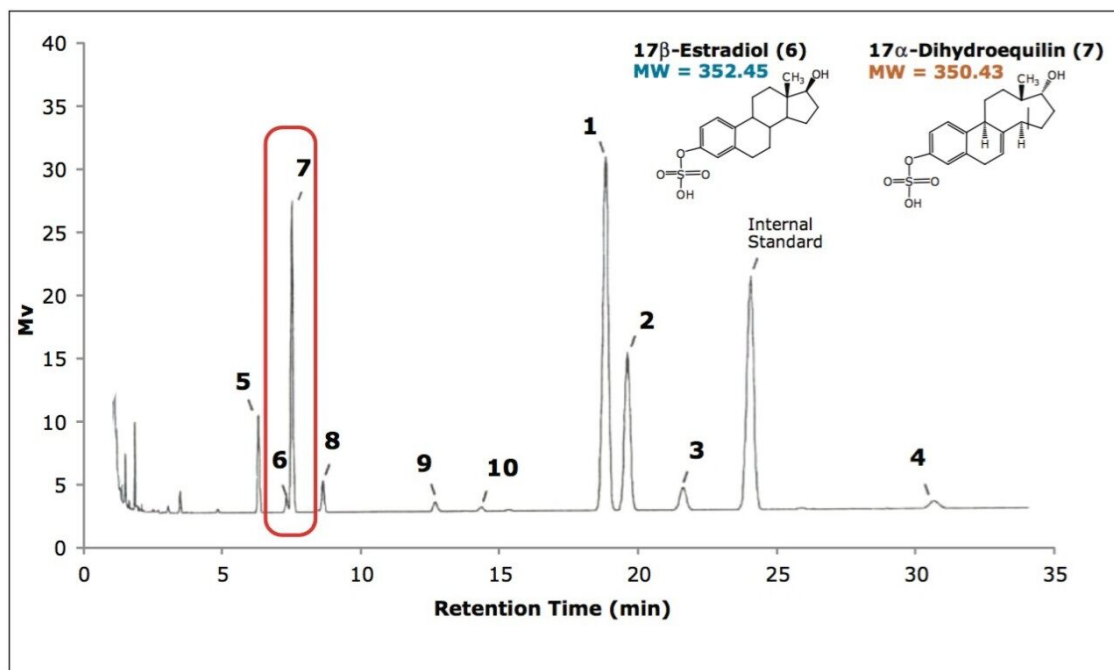


Figure 2. GC-FID separation of ten estrogens using the USP method for conjugated estrogens. Sample preparation includes cleavage of the sulfate group from the conjugated estrogen prior to chemical derivatization of the sample. Total sample preparation time is greater than 2.5 hours. Two of the compounds, peaks 6 and 7 (circled in red) are not completely resolved.

Preparation of the sample for UPC<sup>2</sup> analysis eliminates the need for sulfate removal followed by derivatization, thereby providing a significantly faster and easier analysis than GC, and involves simple dilution of the sulfated estrogens in the appropriate diluents (Table 1). For the current application, the goal was to separate the 10 sulfated estrogens with resolution values (RS) greater than 1.5 with UV detection. To achieve this, the method development for the separation was performed following a stepwise procedure (Figure 3) to explore the effect of various stationary phases, modifiers, additives, pressures, temperatures, and gradient conditions. After the evaluation of each set of parameters, the optimum results were used to move forward to the next set of experiments. With the exception of evaluating the effects due to variations in pressures (either through variation of the pressure directly or through variation of the flow rate), the steps for method development using UPC<sup>2</sup> technology are not significantly different from those used for conventional LC techniques.

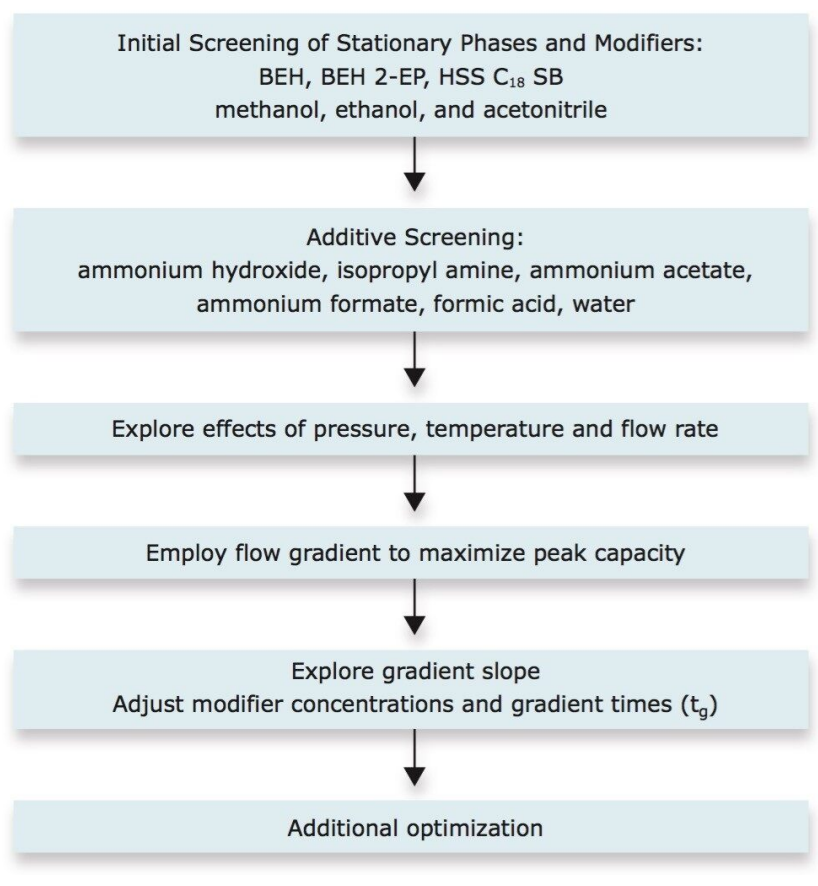


Figure 3. Method development process for the UPC<sup>2</sup> separation of the sulfated estrogen mixture.

Preliminary method screening was performed using different combinations of stationary phase and mobile phase modifier with the addition of a modifier additive. Additives have been shown to play a critical role in the retention mechanisms governing separations with liquid CO<sub>2</sub>, offering several hypotheses regarding their function including: suppression of ionization, formation of ion pairs, coverage of stationary phase active sites, or altering the polarity of the stationary and/or mobile phases. For the current example, without the addition of basic, acidic, or salt additives to the mobile phase modifier, the charged estrogen sulfates are not eluted from the column (data not shown). Initial evaluations utilized 10 mM ammonium acetate (AmOAc) with 5% water as additives in methanol (MeOH), ethanol (EtOH), or acetonitrile (ACN). The results from this screening on the ACQUITY UPC<sup>2</sup> BEH, BEH 2-Ethylpyridine, and HSS C<sub>18</sub> SB Columns are shown in Figure 4.

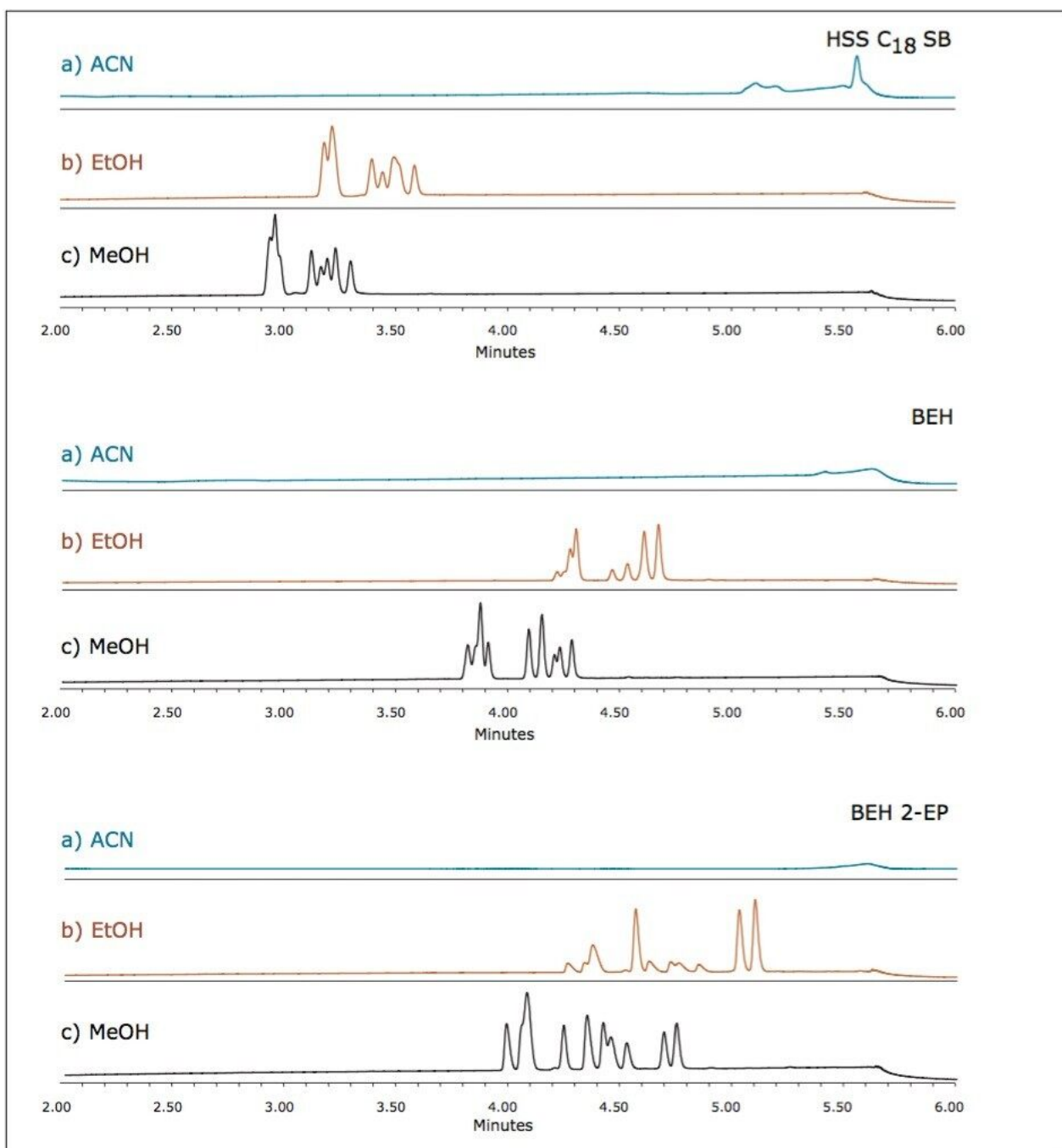


Figure 4. Screening of the sulfated estrogen mixture on 3.0 x 100 mm ACQUITY UPC<sup>2</sup> columns: 1.8  $\mu$ m HSS C<sub>18</sub> SB (top), 1.7  $\mu$ m BEH (middle), and 1.7  $\mu$ m BEH 2-Ethylpyridine (bottom), using ACN (a), EtOH (b), and MeOH (c) mobile phase modifiers. In each case, 10 mM of ammonium acetate (AmOAc) and 5% water were added to the modifier. A five-minute gradient from 5% to 50% modifier was run at 1.3 mL/min and 50 °C. The ABPR settings were 1600 psi for the HSS C<sub>18</sub> SB column and 2175 psi for the BEH and BEH 2-EP columns.

In each case, using acetonitrile as the modifier, the estrogenic compounds did not elute under the conditions studied. Conversely, methanol seemed to have the greatest ability to provide the best resolution between the compounds. Based on these evaluations, it was surmised that the optimum separation of the analytes would be achieved on the BEH 2-Ethylpyridine column, using methanol as the mobile phase modifier. All additional method development focused on this combination of column chemistry and modifier, with further exploration of additives, pressures, temperatures, flow rates, and gradient slopes.

### Effect of additives to the modifier

In the previous example, the use of a modifier additive was necessary for the charged analytes to elute from the UPC<sup>2</sup> Column. Using ammonium acetate in water as the additive, the optimum separation was obtained on the BEH 2-EP column with methanol as the modifier. The choice of additive can have a significant impact on the retention and resolution, so various additives were next explored. Due to the analyte retention window observed for the column screening example, the gradient slope for these evaluations was decreased by using a smaller range of modifier concentration, with a gradient from 9% to 40% modifier. All other parameters remained the same. Figure 5 shows the impact of the various additives on the separation.



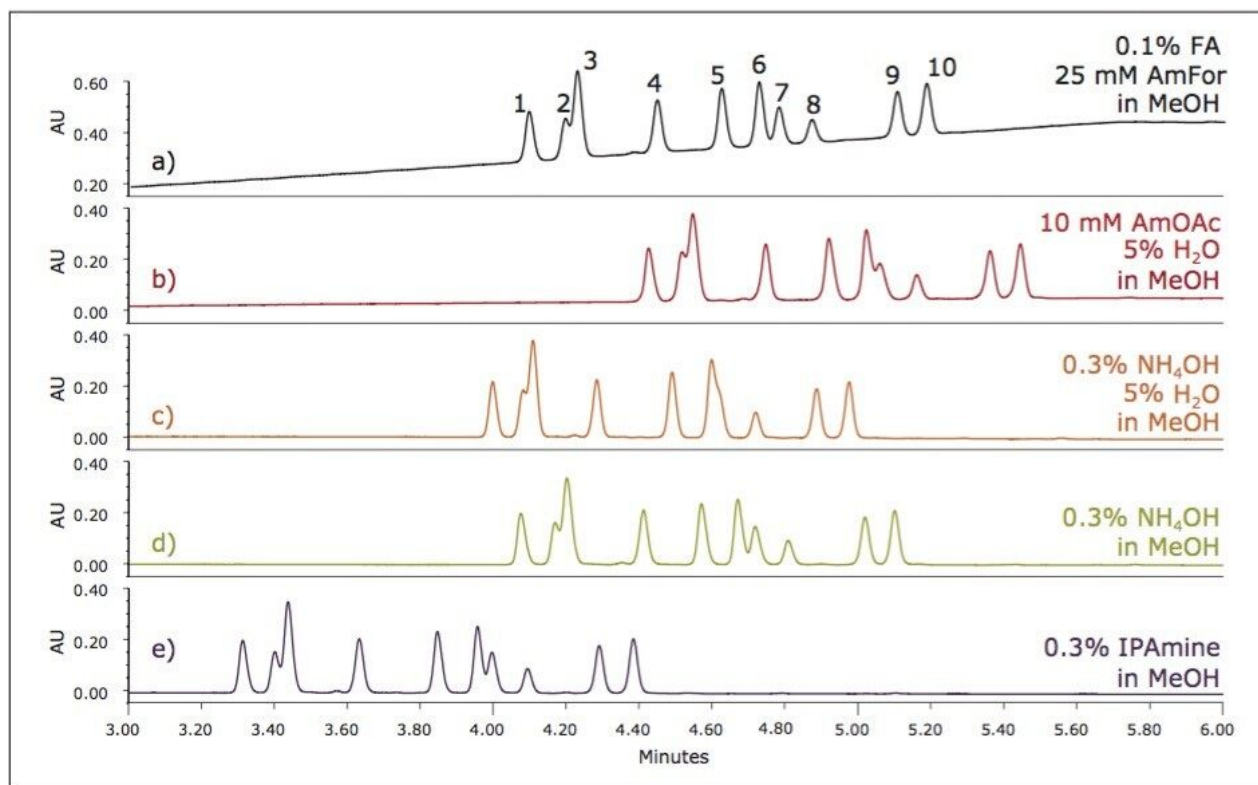


Figure 5. Screening of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7 µm, 3.0 x 100 mm Column using a methanol modifier with the following additives: (a) 0.1% formic acid and 25 mM ammonium formate, (b) 10 mM ammonium acetate and 5% water, (c) 0.3% ammonium hydroxide and 5% water, (d) 0.3% ammonium hydroxide, and (e) 0.3% isopropyl amine. For each experiment, a five-minute gradient from 9% to 40% modifier was run at 1.3 mL/min and 50 °C. The ABPR setting was 2175 psi.

In each of the examples with the various additives, the elution order remains the same. Only the retention times and resolution is impacted by the choice of additive. The separations in Figure 5 show two sets of critical pairs for resolution; peaks 2 and 3, and peaks 6 and 7. Based on the resolution for these two pairs, there are multiple additives that may be acceptable with further optimization. The choice of the set of conditions to move forward with may depend on the ultimate goals of the separation. If this final method was intended for mass spectrometric detection, optimization may have focused on either the ammonium hydroxide or the formic acid/ ammonium formate additives, due to their compatibility with the detection technique. Using UV detection for the current evaluation, the isopropyl amine (IPAmine) additive was selected for further optimization.

## Pressure effects

It is well known that separations using liquid CO<sub>2</sub> as a mobile phase have analyte retention factors that are greatly influenced by the density of the mobile phase. Because of the compressibility of liquid CO<sub>2</sub>, the density can change significantly with changes in pressure, resulting in retention factors decreasing with increasing mobile phase density (pressure). In addition, the selectivity and resolution of individual analytes may be impacted as they respond differently to the changes in mobile phase density. Using isopropyl amine as the additive, the effects of pressure were studied by varying the pressure setting for the Automated Back Pressure Regulator (ABPR) on the ACQUITY UPC<sup>2</sup> System, with pressures ranging from 1700 to 3200 psi. The results are shown in Figure 6.

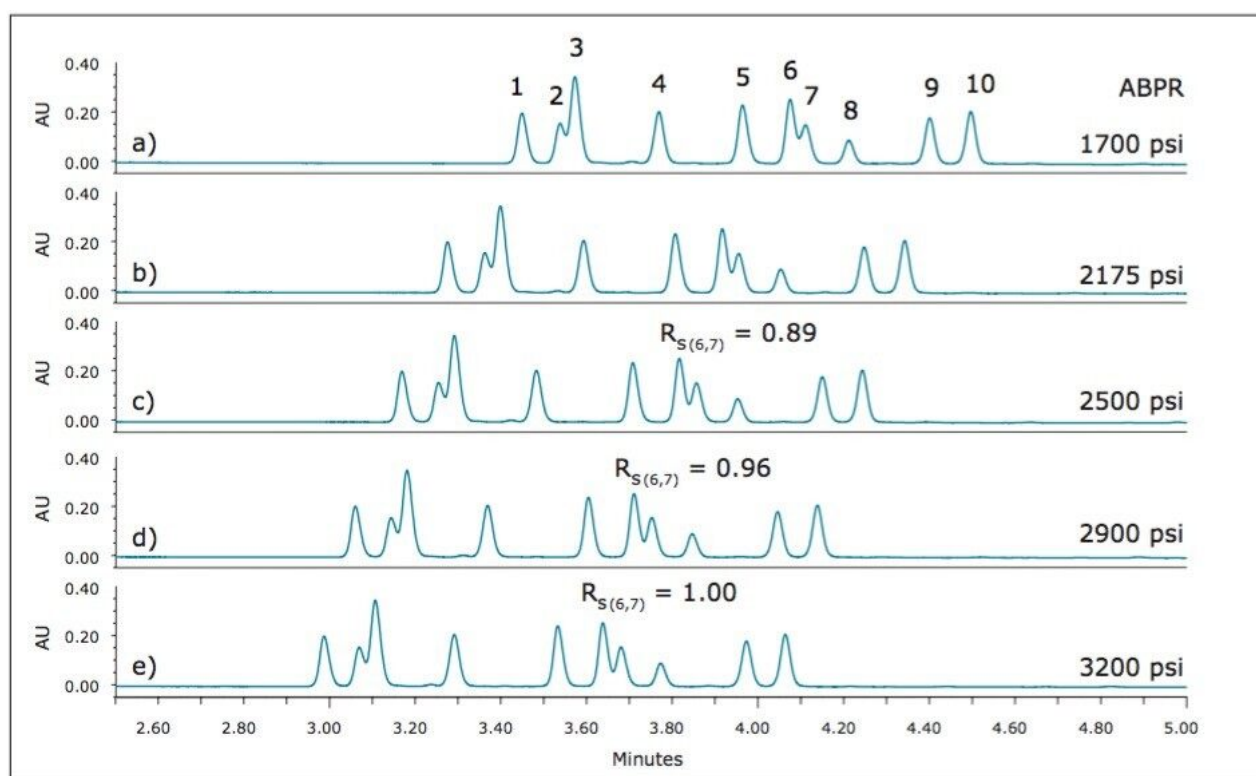


Figure 6. Screening of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine with variable ABPR settings as follows: (a) 1700 psi, (b) 2175 psi, (c) 2500 psi, (d) 2900 psi, and (e) 3200 psi. A five-minute gradient from 9% to 40% modifier was run at 1.3 mL/min and 50 °C.

With attention to the two critical pairs, Figure 6 shows that for this set of analytes, the resolution of peaks 2 and 3 is not significantly impacted by changes in pressure, while for peaks 6 and 7, the resolution is improved slightly at the higher pressure, higher density conditions. The greatest resolution for peaks 6 and 7 ( $R_{S(6,7)} = 1.00$ ) was observed with an ABPR setting of 3200 psi.

## Temperature effects

Temperature can also be a useful tool to influence selectivity and resolution. Using the highest ABPR setting of 3200 psi, further evaluations were performed at 40 °C and 30 °C, as shown in Figure 7.

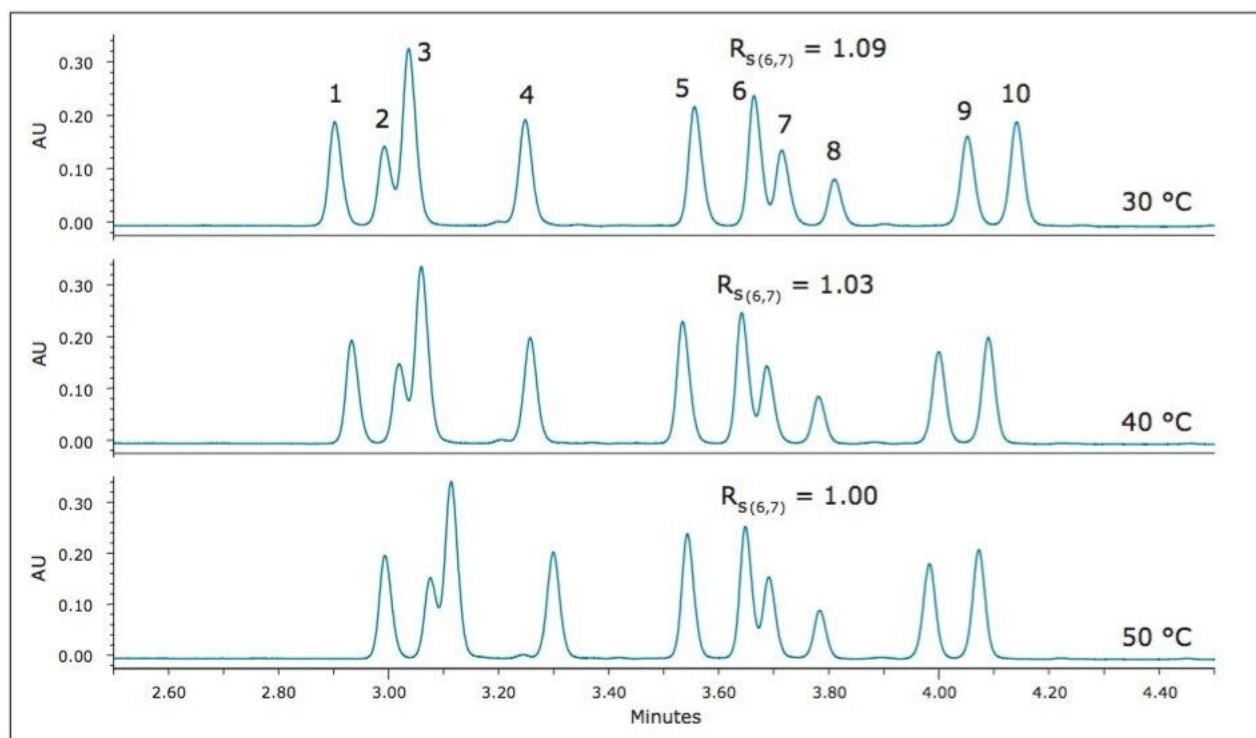


Figure 7. Screening of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine at various temperatures including: 30 °C (top), 40 °C (middle), and 50 °C (bottom). A five-minute gradient from 9% to 40% modifier was run at 1.3 mL/min with an ABPR setting of 3200 psi.

Again, focusing on the critical pairs, it can be seen that the decrease in temperature results in the increase in resolution for both critical pairs. The optimum separation for these analytes is achieved at the lowest temperature evaluated, 30 °C.

## Flow rate effects

Because of the co-dependent relationship between flow rate and pressure, exploring the effects of flow rate requires pressure to be considered as a parameter. In the earlier evaluation of pressure effects, the best separations were achieved at the higher pressures. Because of this, as the various flow rates were explored, the ABPR pressure setting was adjusted to maintain the highest possible pressures during the separation

within the specifications of the ACQUITY UPC<sup>2</sup> System. In addition, the gradient times ( $t_g$ ) were adjusted based on the flow rate used so that the number of column volumes was the same for each separation (13.9 column volumes), thus the  $t_g$  was increased for lower flow rates, while the  $t_g$  was decreased for the faster flow rates. The separations at flow rates from 0.8 mL/min to 1.9 mL/min are shown in Figure 8.

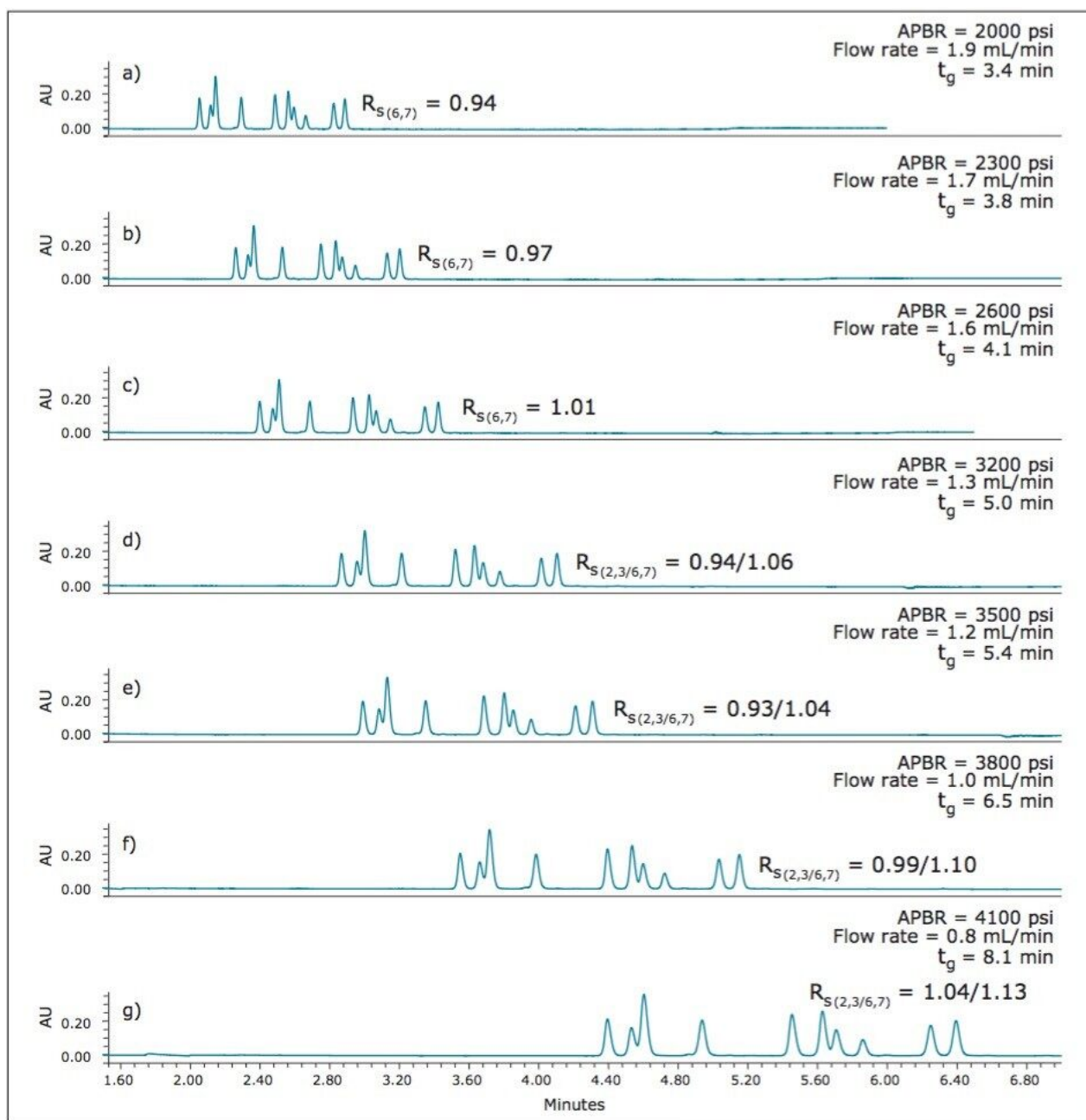


Figure 8. Screening the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine at variable ABPR settings. At each pressure setting, the flow rate was adjusted to the fastest flow possible within the pressure specifications of the ACQUITY UPC<sup>2</sup> System. In addition, the gradient time ( $t_g$ ) was adjusted for each flow rate to maintain the same gradient volume (13.9 column volumes) for each separation. The settings for ABPR, flow rate, and  $t_g$  are as follows: (a) 2000 psi, 1.9 mL/min, 3.4 min, (b) 2300 psi, 1.7 mL/min, 3.8 min, (c) 2600 psi, 1.6 mL/min, 4.1 min, (d) 3200 psi, 1.3 mL/min, 5.0 min, (e) 3500 psi, 1.2 mL/min, 5.4 min, (f) 3800 psi, 1.0 mL/min, 6.5 min, and (g) 4100 psi, 0.8

mL/min, 8.1 min. The gradient was 9% to 40% modifier at 30 °C.

For each set of conditions, the resolution values for the critical pairs were recorded when possible, as shown in Table 2. The highest resolution for both pairs was obtained at the lowest flow rate evaluated (0.8 mL/min), using an ABPR setting of 4100 psi, and a gradient time of 8.1 minutes.

ABPR (psi)	Flow rate (mL/min)	Maximum system pressure (psi)	t <sub>g</sub> (min)	R <sub>S(2,3)</sub>	R <sub>S(6,7)</sub>
2000	1.9	5723	3.4	-	0.94
2300	1.7	5638	3.8	-	0.97
2600	1.6	5751	4.1	-	1.01
3200	1.3	5791	5.0	0.94	1.06
3500	1.2	5915	5.4	0.93	1.04
3800	1.0	5806	6.5	0.99	1.10
4100	0.8	5716	8.1	1.04	1.13

Table 2. Resolution of critical peak pairs as a function of flow rate.

## Flow gradients

Throughout the preliminary screening of these compounds under a variety of conditions, including various pressure and flow rate settings, it was consistently observed that the best separations were obtained at conditions with the highest pressures. However, as the gradient transitions to higher modifier concentrations, the increased mobile phase viscosity results in higher system pressures leading to system over-pressure shut-down above 6000 psi. In order to stay within the pressure specifications of the system, flow rates lower than the optimum linear velocity are required, resulting in a lower efficiency separation with lower peak capacities. To achieve the fastest separations with the greatest peak capacity, reverse flow-gradients can be used to maintain the system pressure at a nearly constant level near the upper specification for the system. As the concentration percentage of organic modifier is increased, the flow rate is decreased. This can be seen in the gradient table shown in Figure 10 (bottom) for the separation of the sulfated estrogens using methanol as the modifier with 0.3% isopropyl amine (IPAm) as the additive. As the percent modifier transitions from 9% to 40%, the flow rate is decreased from 1.3 mL/min to 0.8 mL/min, the maximum flow rates that were determined empirically at each concentration of mobile phase modifier. Figure 9 compares the separation at constant flow with that using a flow gradient, with the resulting system pressure traces shown in orange. For the flow gradient, the system pressure is nearly constant throughout the gradient



retention window. The use of the flow gradient enables collection of data at flow rates as close to the optimum linear velocity as possible, within the pressure specifications of the system, maximizing the overall peak capacity of the separation.

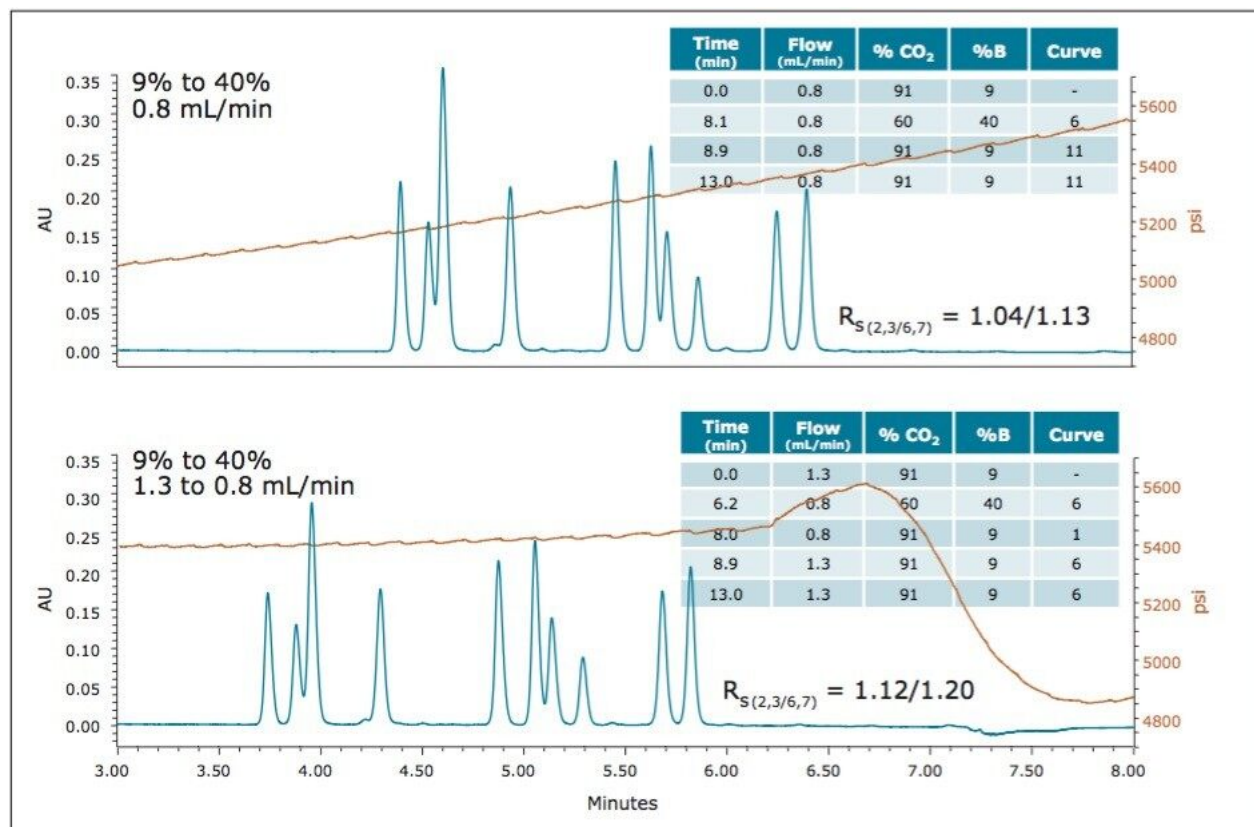


Figure 9. Separation of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine with a constant flow rate of 0.8 mL/min (top), and employing a flow gradient from 1.3 to 0.8 mL/min (bottom). For the flow gradient, the gradient time ( $t_g$ ) was adjusted to maintain the same gradient volume (13.9 column volumes) as in the constant flow example. For both experiments, the gradient was 9% to 40% modifier at 30 °C with an ABPR setting of 4100 psi. The gradient tables along with the system pressure traces for each separation are shown in orange.

## Gradient slope

The slope of the gradient can also have significant impact on the separation. The slope can be explored by altering either the beginning and ending modifier concentrations, or by increasing or decreasing the gradient time ( $t_g$ ). Figure 10 illustrates the effect of altering the starting and ending modifier concentration for the gradient, while holding the gradient time constant. The optimum resolution for the critical pairs is obtained with the shallowest gradient evaluated (8% to 33% modifier). Using these starting and ending modifier

concentration values for the gradient, further evaluation of the gradient slope was explored by altering the length of the gradient ( $t_g$ ). Figure 11 shows the effect of increasing and decreasing the slope of the gradient by altering the length of the gradient, or the number of column volumes used during the gradient separation.

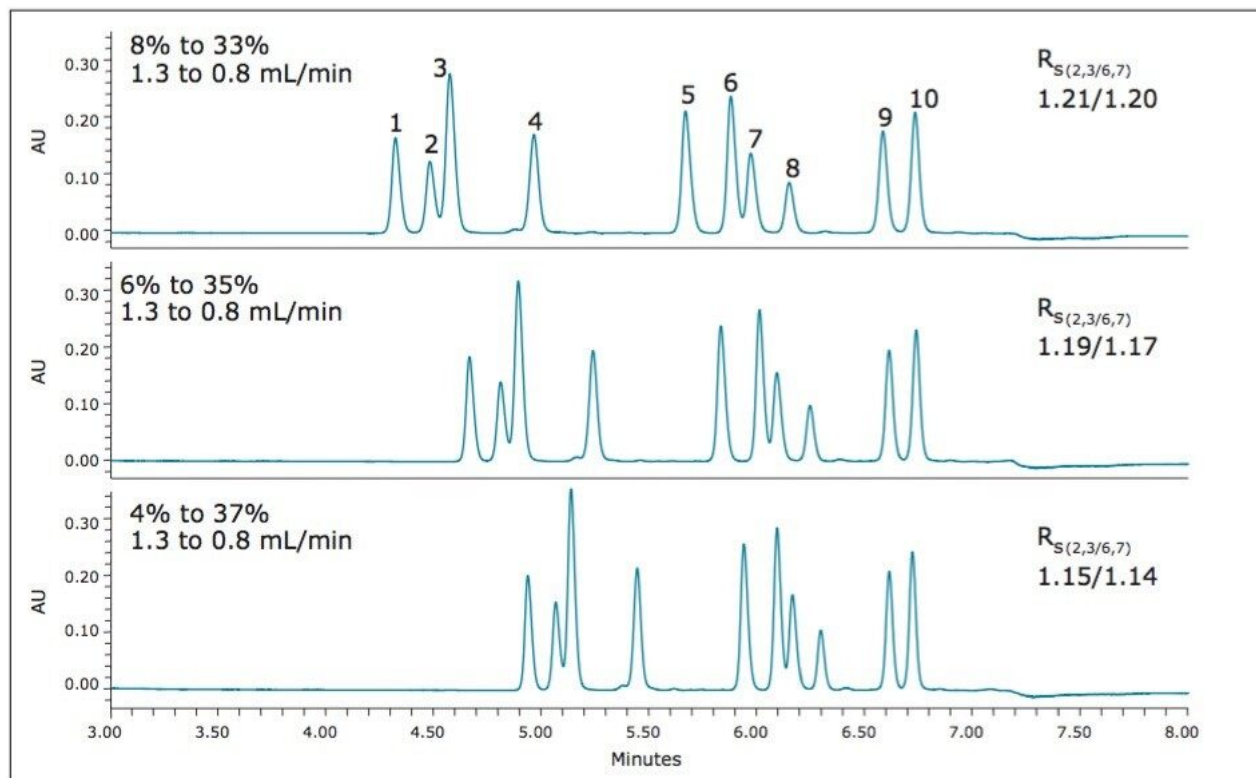


Figure 10. Separation of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine employing a flow gradient from 1.3 to 0.8 mL/min. For each example, the gradient slope was adjusted by changing the beginning and ending modifier concentrations: 8% to 33% (top), 6% to 35% (middle), and 4% to 37% (bottom). For each experiment, the gradient time ( $t_g$ ) was 6.2 minutes at 30 °C with an ABPR setting of 4100 psi.

The greatest resolution of the critical pairs is again obtained with the shallowest gradient (8% to 33% modifier in 12 minutes or ~2% per minute), as shown in Figure 11.



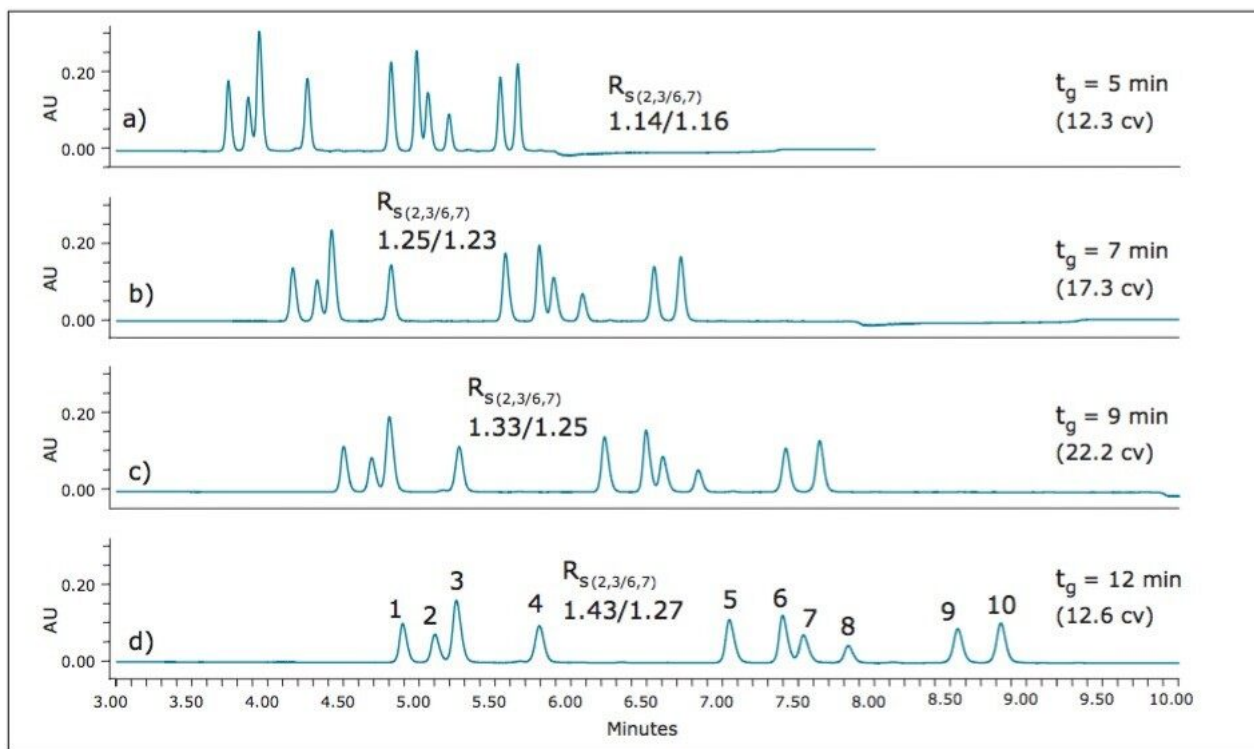


Figure 11. Separation of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine employing a flow gradient from 1.4 to 0.9 mL/min, with 8% to 33% modifier. For each example, the gradient slope was adjusted by changing the gradient time ( $t_g$ ) as follows: (a) five minutes, (b) seven minutes, (c) nine minutes, and (d) 12 minutes. Each chromatogram was collected at 30 °C with an ABPR setting of 4100 psi. The gradient volumes were 12.3, 17.3, 22.2, and 29.6 column volumes, respectively.

### Additional optimization

The goal of this method development was to obtain resolution values >1.5 for each of the analytes. While the best separation in the previous example approaches this criteria, additional optimization was still required. During the temperature evaluations, the optimum separation was achieved at 30 °C, but lower temperatures were not explored. For additional optimization, lower temperatures were evaluated with an additional adjustment to decrease the slope of the gradient. Temperatures of 20 °C, 10 °C, and 5 °C were evaluated with a 12-minute gradient from 8% to 27% modifier (~1.6% per minute). The results of these evaluations are shown in Figure 12.

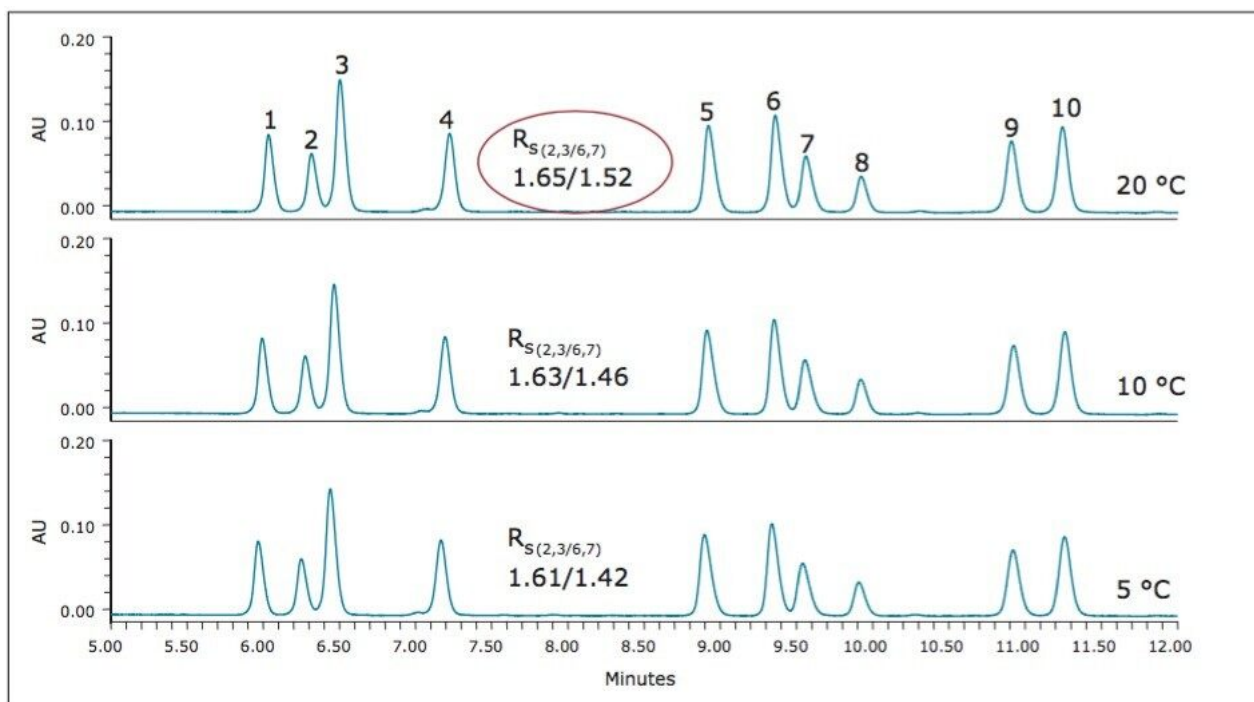


Figure 12. Separation of the estrogen mixture on the ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with 0.3% isopropyl amine , using a 12-minute gradient from 8% to 27% modifier, employing a flow gradient from 1.4 to 0.9 mL/min. The temperatures were 20 °C (top), 10 °C (middle), and 5 °C (bottom). Each chromatogram was collected with an ABPR setting of 4100 psi. The separation at 20 °C meets the resolution criteria required for the current separation.

Figure 12 shows that the goals for this separation,  $R_s > 1.5$  for all analytes, can be achieved at 20 °C, using the shallower gradient of 8% to 27% modifier in 12 minutes.

### Alternative separations

During the initial evaluation of additives, based on the goals set forth for this method, isopropyl amine was chosen for additional optimization. If the method incorporated mass spectrometry, optimization may have been focused on another additive, either the ammonium hydroxide or the formic acid/ammonium formate additive because of their increased compatibility with the mass spectrometry system. Purely as an exploratory exercise, these two additives were re-evaluated with the current optimized method. Based on the increased retention observed during the initial additive evaluations (Figure 5), the modifier concentrations were increased slightly (3%), while keeping all other parameters constant, including the gradient slope. Figure 13 shows the optimum separation achieved with the isopropyl amine additive, using the optimized gradient from 8% to 27% modifier, along with the separations of the two alternative modifiers, utilizing a

gradient from 11% to 30% modifier.

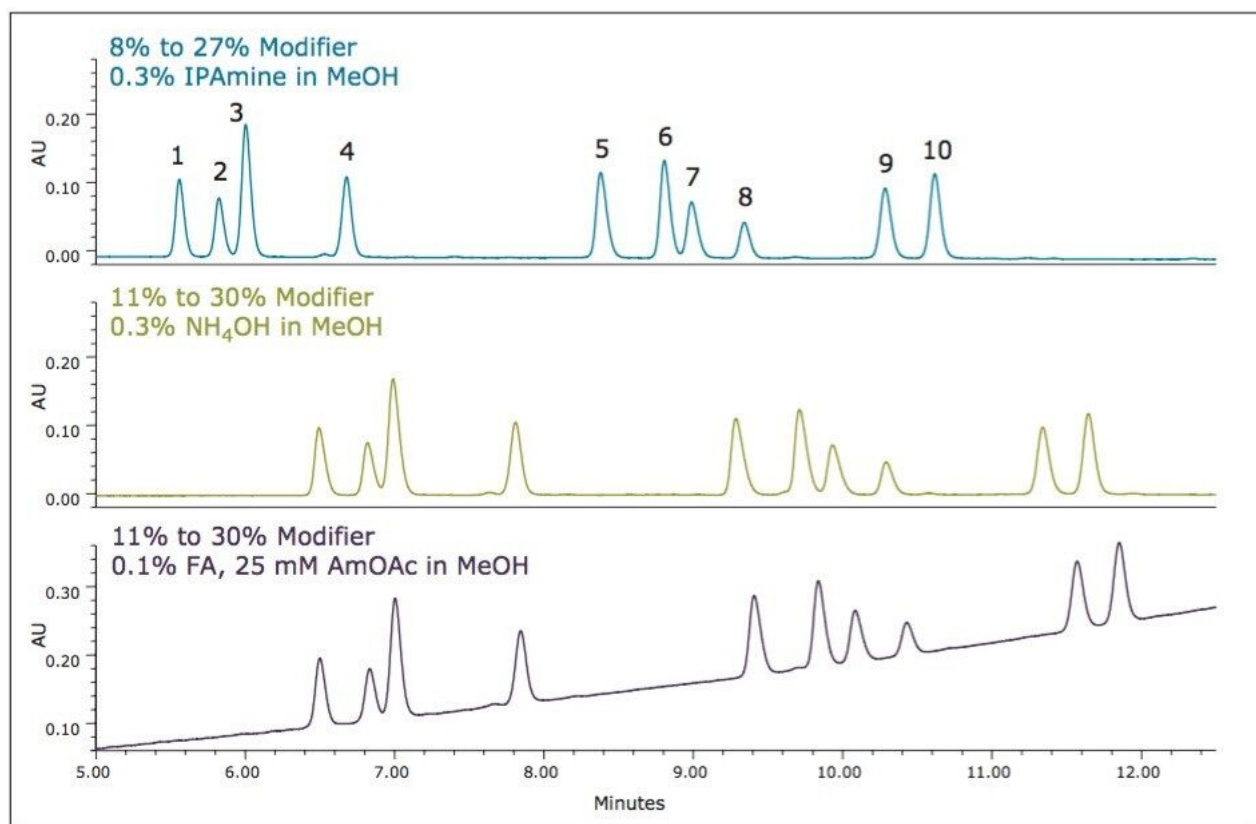


Figure 13. Separation of the sulfated estrogen mixture on an ACQUITY UPC<sup>2</sup> BEH 2-EP 1.7  $\mu$ m, 3.0 x 100 mm Column using a methanol modifier with various additives; 0.3% isopropyl amine (top), 0.3% ammonium hydroxide (middle), and 0.1% formic acid with 25 mM ammonium acetate (bottom). For each separation, 12-minute gradients with equal slopes were used as follows: 8% to 27% (top), and 11% to 30% (middle and bottom). Each separation also utilized a flow gradient from 1.4 to 0.9 mL/min. The data was collected at a temperature of 20 °C with an ABPR setting of 4100 psi.

This exercise demonstrates the versatility of convergence chromatography, offering flexibility for altering conditions to accommodate different detection methods.

## Conclusion

UPC<sup>2</sup> method development for sulfated estrogens involved several critical parameters for an optimal

separation, including column chemistry, choice of modifier, additive, pressure, temperature, flow rate, and gradient slope. Although the choice of additive had minimal impact on overall selectivity, the presence of the additive is critical for elution of these charged analytes, and can impact the peak shape and resolution for the analytes. The importance of pressure cannot be understated, and the application of reverse flow-gradients to maintain the highest possible flow rates and greatest efficiencies can be extremely beneficial, while staying within the pressure limitations of the system.

The application of this technology to the separation of sulfated estrogens represents a dramatic improvement over conventional gas chromatographic methods, with greater than 90% reduction in overall analysis time for the synthetic mixture of 10 sulfated estrogens. This is mostly due to the improvement in workflow provided by the elimination of sample desulfation and derivatization requirements prior to analysis. The reduction in the complexity and time requirements for the sample preparation yields increased laboratory efficiency with less opportunity for compounded errors. As demonstrated in this study, the ability of UPC<sup>2</sup> technology to separate compounds with very similar structures make it well suited for the analysis of steroid and steroid-related compounds.

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