

# HPLC-UV Method Development for Baseline Resolution of 17 Cannabinoids

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## Introduction

The global cannabis industry is growing rapidly, with many countries and US states adding regulatory frameworks for medical and recreational cannabis programs. Quality control is an essential component in protecting health and safety of the consumer in this emerging market and there is increasing demand upon cannabis testing laboratories for analytical determination of multiple cannabinoids.

Current regulations concerning potency vary by jurisdiction but usually require testing for the active forms of THC and CBD. In addition to those, many require testing for the acid forms, THCA and CBDA, along with other cannabinoids like CBG, CBGA, THCV, CBC and CBN. As regulations evolve, and as research interests in minor cannabinoids expand, it is important to have robust analytical methods in place that are capable of meeting those needs. Herein, the baseline resolution of 17 cannabinoids by high performance liquid chromatography (HPLC) with UV detection is described. Chromatographic method development was performed with particular attention to speed of analysis and a means for changing selectivity to improve resolution of critical pairs, as needed.

## Experimental

Figure 1 shows the 17 cannabinoids and their structures that were used in this work. 1 mg/mL standards were acquired from Cerilliant, and 50  $\mu$ L of each were combined as received to create the test mixture. Final concentration of each cannabinoid was approximately 59  $\mu$ g/mL in 53:47 methanol:acetonitrile.

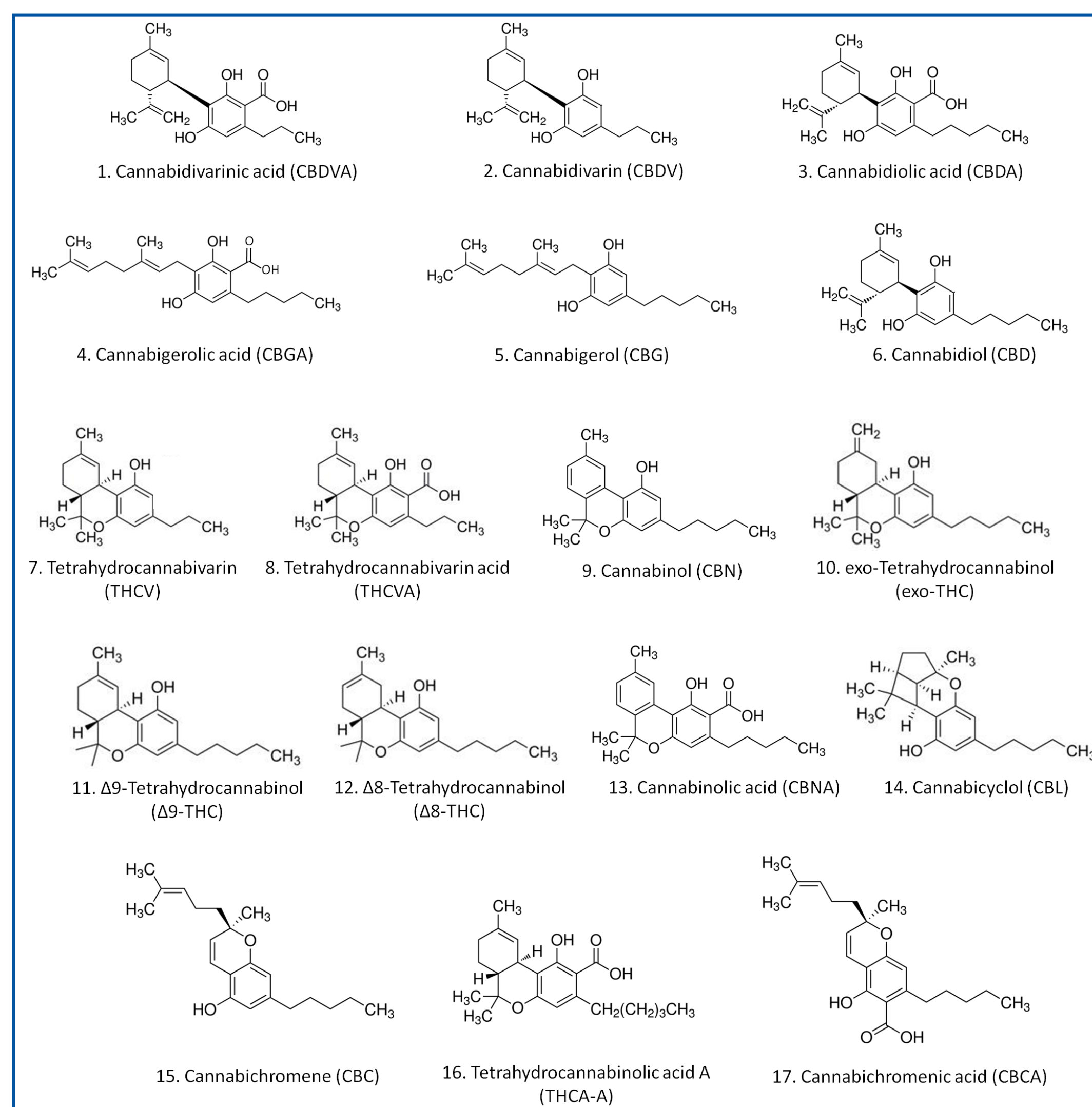


Figure 1: Structures of cannabinoids

Method development was performed using an Evoke™ C18 15 cm x 4.6 mm, 3  $\mu$ m column from Regis Technologies, Inc. Aqueous mobile phase conditions were screened with different organic modifiers (methanol and acetonitrile) under both isocratic and gradient modes of operation. Acid additives (formic, TFA) were also investigated. Table 1 lists the analytical conditions that gave the best results (i.e. the most baseline resolved peaks) and served as the foundation for further method development.

Column:	Evoke™ C18; 15 cm x 4.6 mm; 3 $\mu$ m (Cat.#1-760208-300)									
Instrument:	Shimadzu Nexera									
Mobile phase A:	Water + 0.1% formic acid (+ ammonium formate concentration specified with chromatogram)									
Mobile phase B:	Acetonitrile + 0.1% formic acid									
Gradient:	<table border="1"> <thead> <tr> <th>Time (min.)</th> <th>Flow (mL/min)</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>2.0</td> <td>75</td> </tr> <tr> <td>15.00</td> <td>2.0</td> <td>90</td> </tr> </tbody> </table>	Time (min.)	Flow (mL/min)	%B	0.00	2.0	75	15.00	2.0	90
Time (min.)	Flow (mL/min)	%B								
0.00	2.0	75								
15.00	2.0	90								
Oven Temp.:	30°C									
Inj. Vol.:	5 $\mu$ L									
Detection:	228 nm									

Table 1: HPLC-UV conditions

## Results

### Initial Results: 0 mM Ammonium Formate

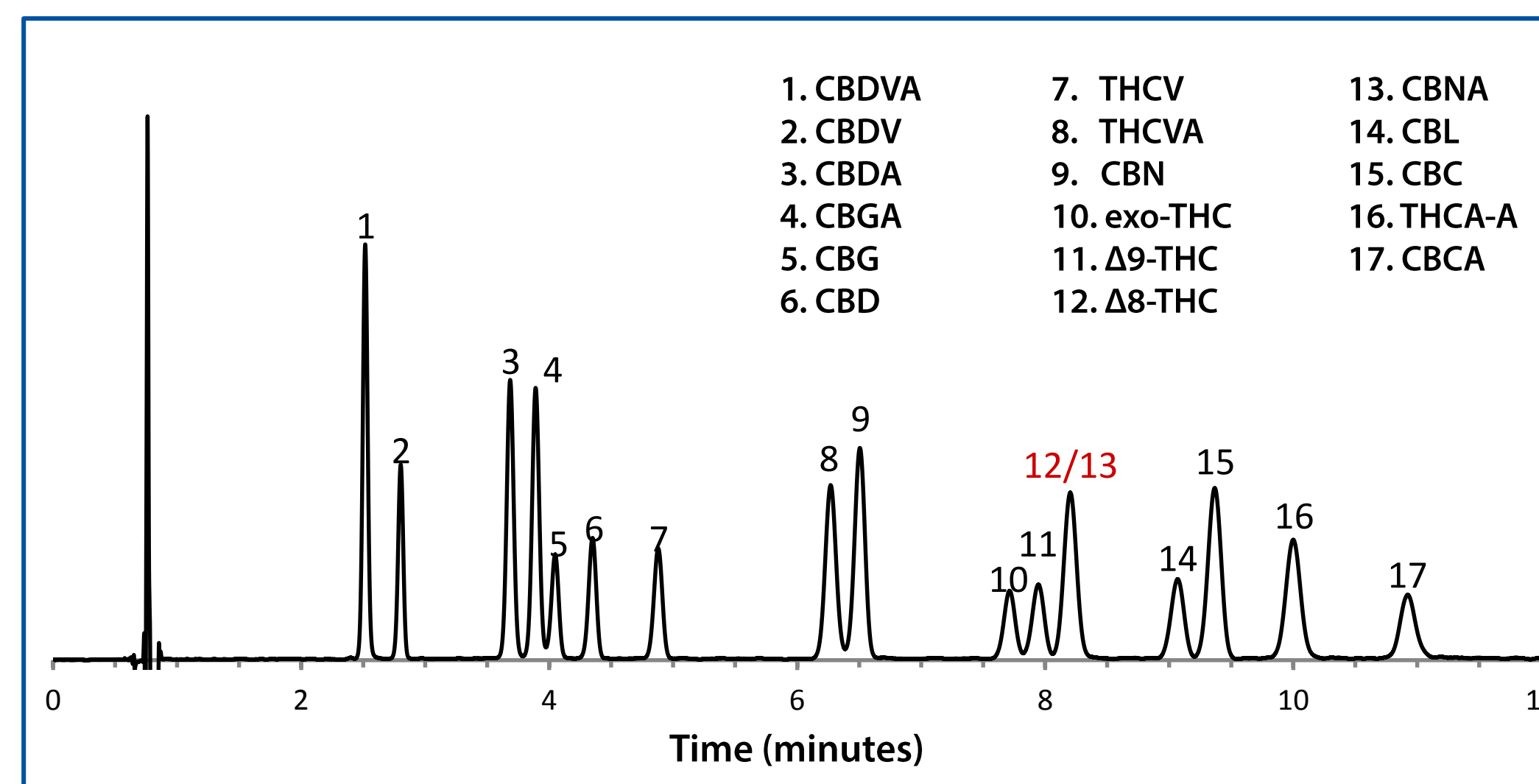


Figure 2: Separation of 17 cannabinoids using the conditions listed in Table 1 and 0 mM ammonium formate; co-elution of  $\Delta$ 8-THC and CBNA

Figure 2 shows the separation of the 17 cannabinoid test mixture using the conditions listed in Table 1. Near-baseline resolution is achieved for each component peak with the exceptional coelution of  $\Delta$ 8-THC and CBNA at 8.20 minutes. Adjustments to gradient steepness and flow rate did not affect the resolution of these two peaks.

### Effect of Mobile Phase Ionic Strength

The effect of ionic strength on the cannabinoid separation was investigated by adding ammonium formate to Mobile Phase A in concentrations ranging between 5 and 10 mM. It should be noted that, since ammonium formate was added to only the aqueous portion, the total ionic strength changes throughout the course of the gradient. Nevertheless, with sufficient re-equilibration time, results are reproducible.

### 5 mM Ammonium Formate

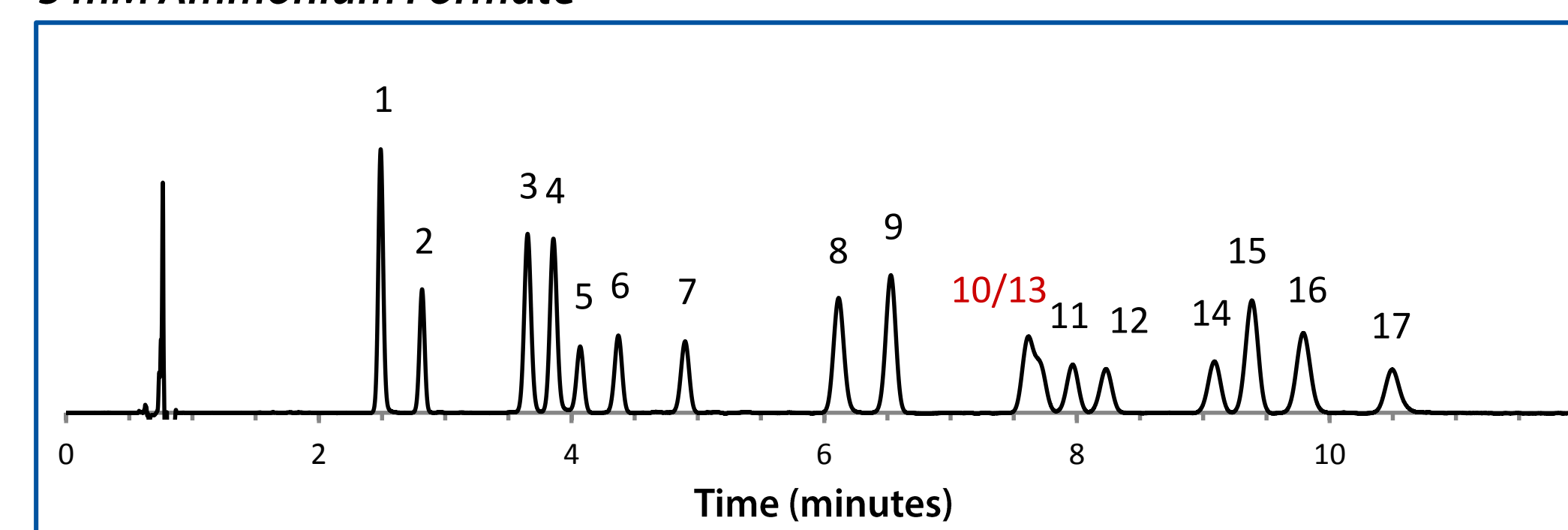


Figure 3: Separation of 17 cannabinoids using the conditions listed in Table 1 and 5 mM ammonium formate; co-elution of exo-THC and CBNA

### 10 mM Ammonium Formate

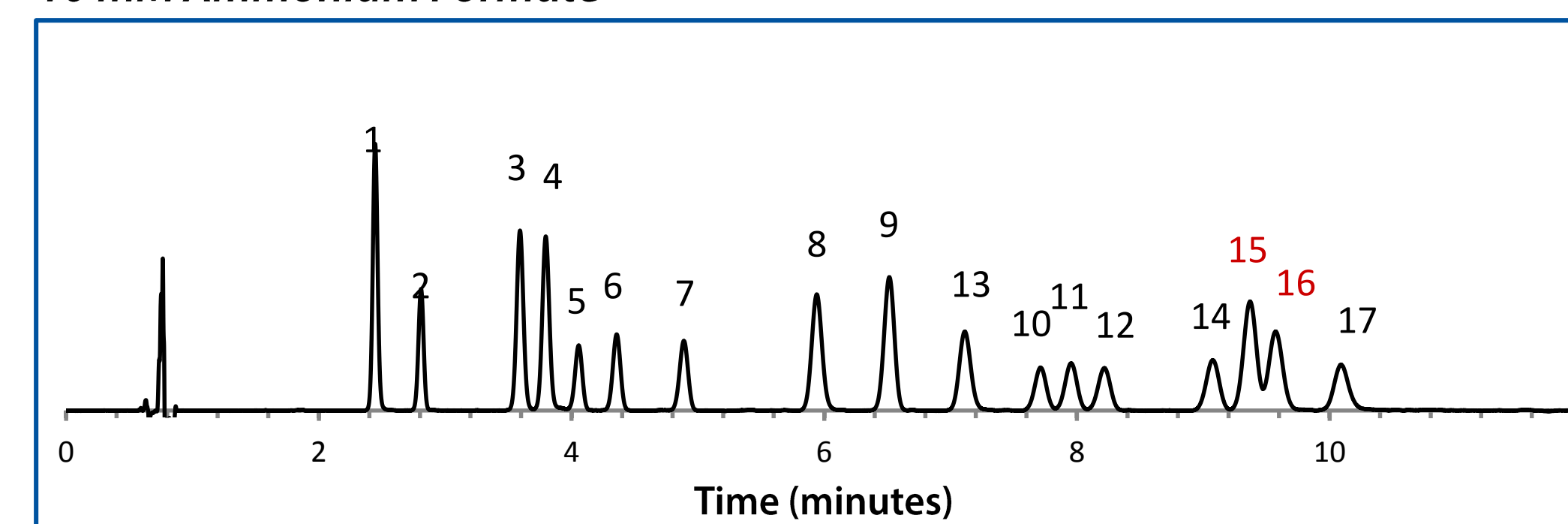


Figure 4: Separation of 17 cannabinoids using the conditions listed in Table 1 and 10 mM ammonium formate; co-elution of CBC and THCA-A

### 7.5 mM Ammonium Formate

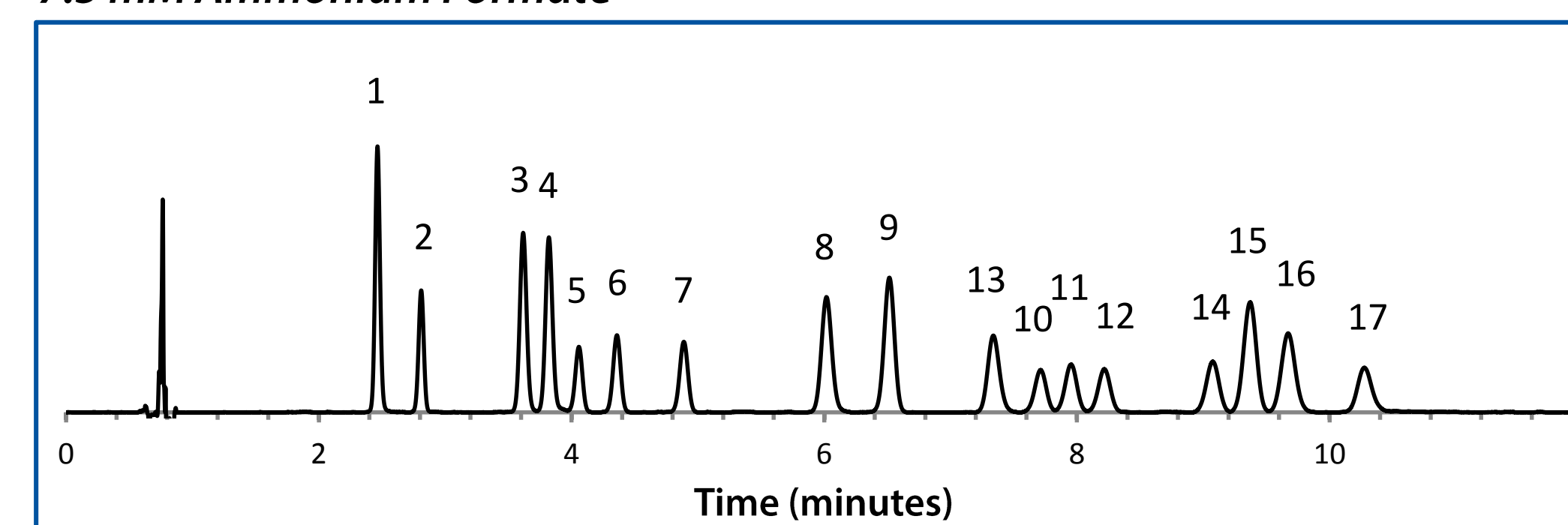


Figure 5: Separation of 17 cannabinoids using the conditions listed in Table 1 and 7.5 mM ammonium formate; baseline resolution of all components

The addition of ammonium formate to Mobile Phase A results in retention shifts of the carboxylated cannabinoids while the decarboxylated species are unaffected. With 5 mM ammonium formate, CBNA co-elutes with exo-THC, an impurity formed in the synthesis of  $\Delta$ 9-THC (Fig. 3). With 10 mM, the retention of THCA-A is shifted and is inadequately resolved from CBC (Fig. 4). 7.5 mM ammonium formate was found to provide baseline resolution of all 17 cannabinoids (Fig. 5).

## Results and Discussion

### UHPLC Method Transfer

Provided that UHPLC instrumentation with sufficiently low dispersion volume is available, the method used for the separation shown in Figure 5 can be transferred to a 10 cm x 2.1 mm, 1.8  $\mu$ m column to achieve faster analysis times. Table 2 outlines the UHPLC-UV conditions used to generate the chromatographic data shown in Figure 6.

Column:	Evoke™ C18; 10 cm x 2.1 mm; 1.8 $\mu$ m (Cat.#1-760107-300)									
Instrument:	Shimadzu Nexera									
Mobile phase A:	Water + 0.1% formic acid + 7.5 mM ammonium formate									
Mobile phase B:	Acetonitrile + 0.1% formic acid									
Gradient:	<table border="1"> <thead> <tr> <th>Time (min.)</th> <th>Flow (mL/min)</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>0.6</td> <td>75</td> </tr> <tr> <td>8.00</td> <td>0.6</td> <td>90</td> </tr> </tbody> </table>	Time (min.)	Flow (mL/min)	%B	0.00	0.6	75	8.00	0.6	90
Time (min.)	Flow (mL/min)	%B								
0.00	0.6	75								
8.00	0.6	90								
Oven Temp.:	30°C									
Inj. Vol.:	1 $\mu$ L									
Detection:	228 nm									

Table 2: UHPLC-UV conditions

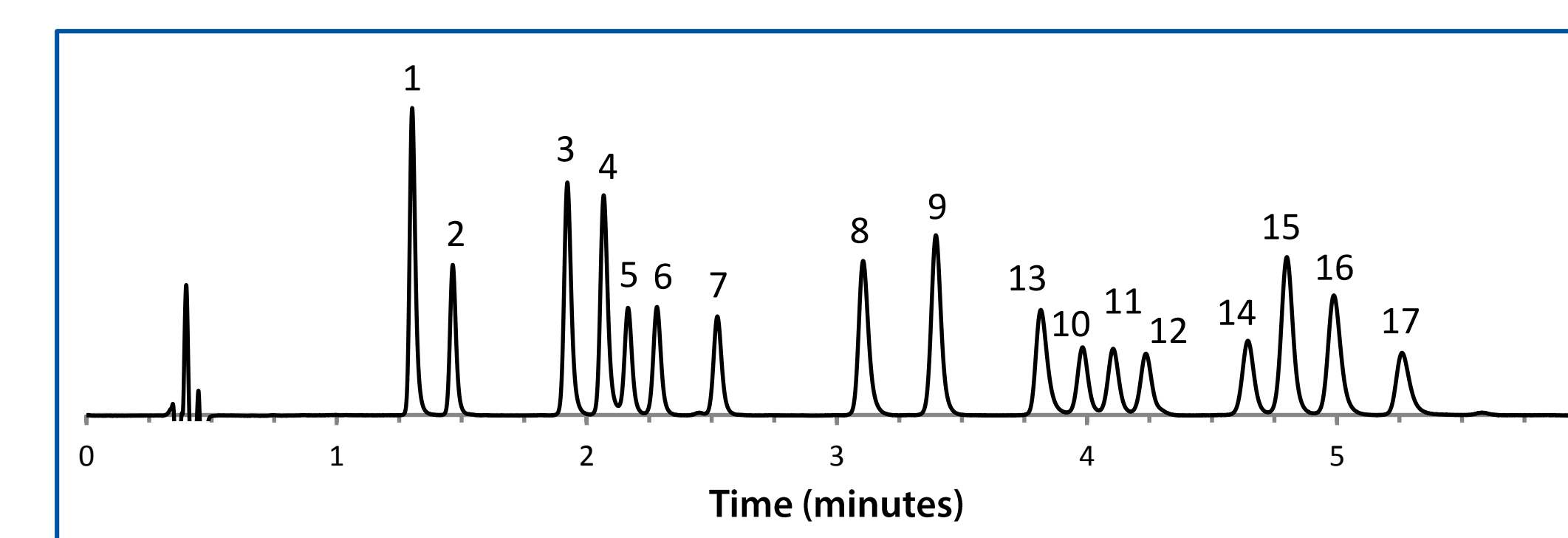


Figure 6: Separation of 17 cannabinoids using the conditions listed in Table 2

### Improving Resolution of Critical Pairs through Selectivity

In certain assays, analysts may be especially concerned with the resolution of key critical pairs, such as in cases where one component is far more abundant than the other. In the gradient separation shown in Figure 6, the resolution ( $R_s$ ) between  $\Delta$ 9-THC and  $\Delta$ 8-THC is 1.63. Often, it is possible to improve resolution by lowering the steepness of the gradient by moving to isocratic analysis or by changing flow rate. In the case of  $\Delta$ 9-THC and  $\Delta$ 8-THC, the greatest effect is observed by changing the composition of Mobile Phase B.

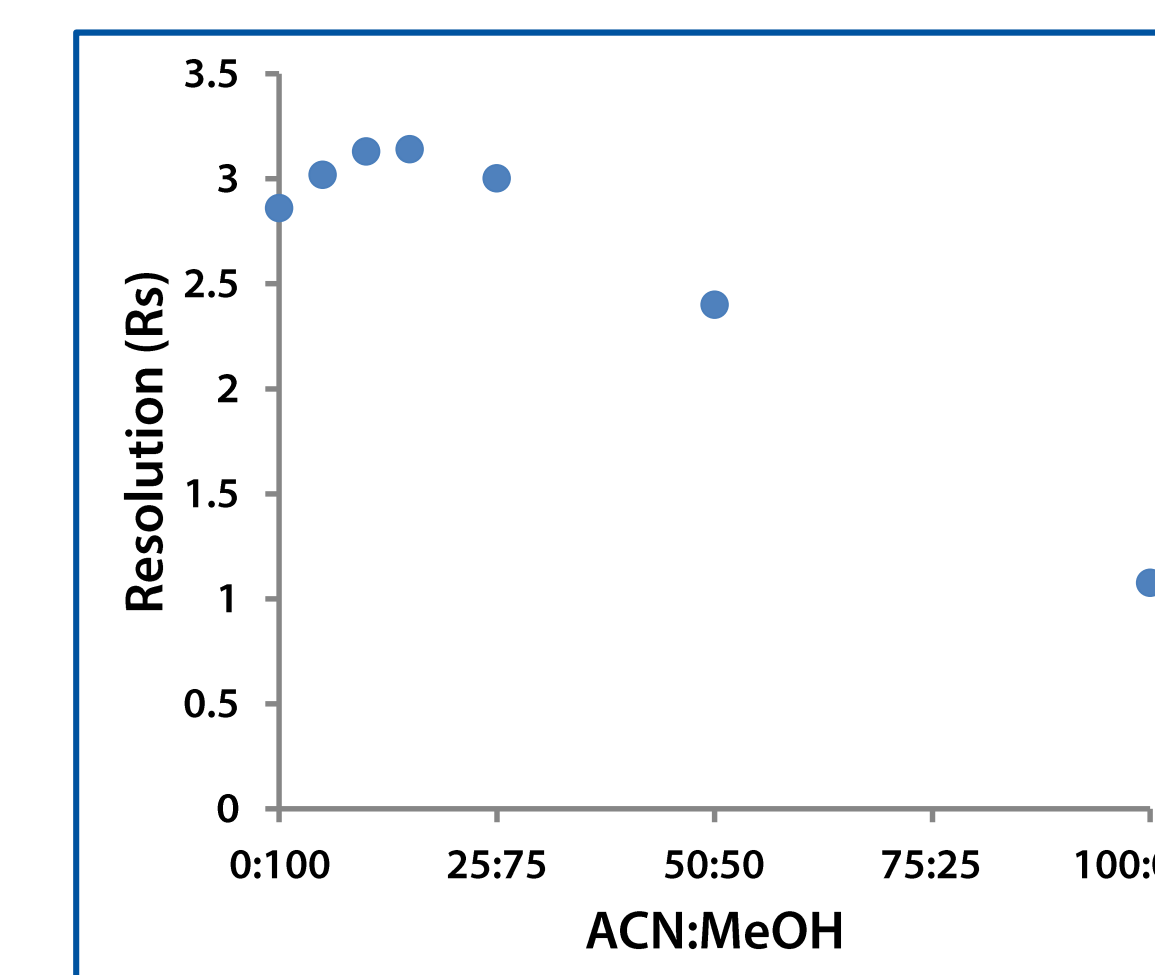


Figure 7: Resolution ( $R_s$ ) of  $\Delta$ 9-THC and  $\Delta$ 8-THC as a function of Mobile Phase B composition. MPA:MPB = 10:90; Flow = 1.5 mL/min.

Figure 7 plots the effect of varying the composition of Mobile Phase B on the isocratic resolution of 1:1  $\Delta$ 9-THC :  $\Delta$ 8-THC. All separations were performed with MPA:MPB = 10:90. When MPB = 100% acetonitrile,  $R_s$  = 1.08. With MPB = 100% methanol,  $R_s$  = 2.86. Maximum resolution ( $R_s$  = 3.14) was observed when Mobile Phase B consisted of a 15:85 mixture of acetonitrile:methanol. (Note: An isocratic separation using 15:85 water:methanol achieved  $R_s$  = 3.72, but analysis time was nearly double.)

## Summary and Conclusions

HPLC method development toward the baseline resolution of 17 cannabinoids using an Evoke™ C18 15 cm x 4.6 mm, 3  $\mu$ m column is shown. Initial mobile phases were selected after generic screening runs based on achievement of the most baseline resolved peaks for the 17 cannabinoid test mixture. The addition of ammonium formate to Mobile Phase A provided a means to shift retentions of the acidic forms relative to the decarboxylated species. The concentration was optimized to allow for baseline resolution of all 17 cannabinoids. The method was transferred to UHPLC conditions using an Evoke C18™ 10 cm x 2.1 mm, 1.8  $\mu$ m column, and analysis time was effectively cut in half.

In situations where additional resolution between specific critical pairs is desired, adjustments to mobile phase composition may prove helpful. While acetonitrile served best in the gradient separation of the 17 cannabinoids, the resolution between  $\Delta$ 9-THC and  $\Delta$ 8-THC was improved by changing to an isocratic method using methanol as the organic modifier. Combinations of methanol and acetonitrile were shown to improve the resolution further.