HPLC Method Development for Baseline Resolution of Seventeen Cannabinoids

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The global cannabis industry is growing rapidly, with many countries and US states adding regulatory frameworks for medical and recreational programs. Quality control is an essential component in protecting the 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 surrounding potency vary by jurisdiction, but usually require testing for the active forms of THC and CBD. In addition, many require testing for the acid forms, THCA and CBDA, along with other cannabinoids like CBG, CBGA, THCV, CBC, CBL, 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.

The preferred technique for quantifying cannabinoids is HPLC (High Pressure Liquid Chromatography) with detection by UV (Ultraviolet) or MS (Mass Spectrometry). In general, all approaches to HPLC method development look to balance several elements, among which are the ultimate goals of the analysis, resolution of target compounds and potential interferences, speed, and assay robustness. Upon evaluating the molecules of interest in terms of their charges, polarities, and other functionalities, chromatographic method developers turn their focus to column and solvent selection, pH conditions, buffer selection and concentration, temperature, etc. Specific approaches can differ depending upon the primary goals of a separation. For example, if comprehensive characterization of a complex sample is desired, approaches to maximizing overall separation at the expense of analysis time may be acceptable. If, on the other hand, resolution of only a particular critical pair is required, speed and selectivity (for the crucial pair) may be the primary focus.

With these concerns in mind, we set out to develop an HPLC method capable of fully resolving 17 cannabinoids in a minimal amount of time. Additionally, a second objective concerning the resolution of a specific critical pair of THC isomers (Δ8-THC and Δ9-THC) was explored.

Seventeen analytical reference cannabinoid standards (1 mg/mL) were acquired from Cerilliant (Round Rock, TX, USA) and combined to a final component concentration of approximately 59 µg/mL in 53:47 methanol:acetonitrile. The mixture was composed of Δ8-tetrahydrocannabinol (Δ8-THC), Δ9-tetrahydrocannabinol (Δ9-THC), cannabichromene (CBC), cannabichromenic acid (CBCA), cannabicyclol (CBL), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabidivarin (CBDV), cannabidivarinic acid (CBDVA), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN), cannabinolic acid (CBNA), exo-tetrahydrocannabinol (exo-THC), tetrahydrocannabinolic acid A (THCA-A), tetrahydrocannabivarin (THCV), and tetrahydrocannabivarinic acid (THCVA).

Column:	Evoke C18; 15 cm x 4.6 mm; 3 µm	
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	
Flow:	2.0 mL/min	
Gradient:	Time (min.)	%B
	0.00	75
	15.00	90
Oven Temp:	30° C	
Inj. Vol:	5 µL	
Detection:	228 nm	

Table 1 – Chromatographic conditions used in the development of the method to separate 17 cannabinoid analytical reference standards.

Chromatographic method development was performed on a Shimadzu Nexera (Kyoto, Japan) using an Evoke C18, 15 cm x 4.6 mm column, packed with 3 µm fully porous particles from Regis Technologies, Inc. (Morton Grove, IL, USA). Reversed-phase conditions were screened using different organic modifiers (methanol and acetonitrile) in both isocratic and gradient modes of operation. Acid additives (formic acid and trifluoroacetic acid) were also investigated and found important in achieving adequate retention and maintaining the peak shape of carboxylated species (e.g. CBCA, CBDA, etc.). The conditions that resulted in the most baseline resolved peaks and served as the foundation for further method development are listed in Table 1.

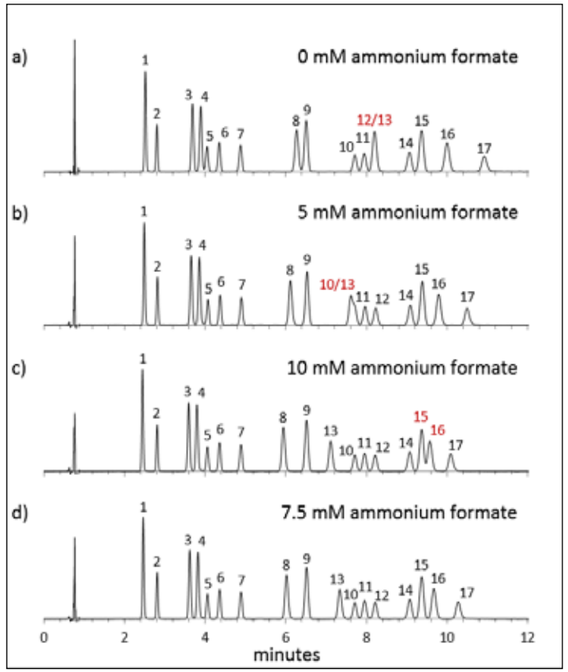


Figure 1 – Effect of the addition of ammonium formate to mobile phase A. a) No ammonium formate added. b) 5 mM ammonium formate added. c) 10 mM ammonium formate added. d) 7.5 mM ammonium formate added. Additional chromatographic conditions listed in Table 1.

Figure 1a shows the baseline-subtracted chromatogram for the separation of the 17 cannabinoid test mixture using the conditions listed in Table 1. Baseline resolution is achieved for each of the component peaks with the exceptions of CBGA and CBG (Rs = 1.40), THCVA and CBN (Rs = 1.42), and the coelution of Δ8-THC and CBNA at 8.20 minutes. In efforts to improve the resolution of these pairs, the effect of adding ammonium formate to mobile phase A in concentrations

ranging between 5 and 10 mM was investigated. The addition of ammonium formate to formic acid mobile phases increases the ionic strength as well as slightly raises the pH.

As shown in Figure 1, the addition of ammonium formate to mobile phase A resulted in reduced retention of the carboxylated cannabinoids while the decarboxylated species remain unaffected, thus baseline-resolving CBGA/CBG and THCVA/CBN. With 5 mM ammonium formate, the retention time of CBNA is shifted to 7.63 minutes and co-elutes with exo-THC, an impurity formed in the synthesis of Δ9-THC (Fig. 1b). By increasing the concentration to 10 mM ammonium formate, the retention of CBNA is shifted, causing it to elute earlier than the THC isomers, but THCA-A is shifted into co-eluting with CBC (Fig. 1c). An intermediate concentration of 7.5 mM ammonium formate was found to provide baseline resolution of all 17 cannabinoids in the test mixture (Fig. 1d).

With typical re-equilibration time, run-to-run results were found to be reproducible. Nevertheless, it should be noted that since ammonium formate is added to only the aqueous component of the mobile phase, the total ionic strength changes throughout the gradient runtime. For example, when 7.5 mM ammonium formate in mobile phase A is used in the gradient listed in Table 1, the total concentration on the column changes from 1.875 mM to 0.75 mM over the course of the 15-minute run. Attempts to maintain a constant concentration by adding an intermediate concentration of salt to both mobile phases A and B resulted in unfavorable retention time shifts at either the early portion or the latter portion of the chromatographic run. Thus, the concurrent gradients in eluotropic strength and pH/ionic strength synergistically serve to provide the separation shown in Figure 1d.

In some assays, analysts are concerned with improving the resolution of certain critical pairs. This may be especially true in cases where one component is far more abundant than the other. In the gradient separations shown in Figure 1, the resolutions between Δ9-THC and Δ8-THC are approximately 1.50. These isomers are neutral, and their retentions are largely unaffected by changes in mobile phase pH or ionic strength. Often, it is possible to improve resolution by running an isocratic analysis and by reducing eluent strength. In the case of Δ9-THC and Δ8-THC, the greatest effect is observed by changing the composition of mobile phase B.

Figure 2 plots the effect of varying the percentage and composition of mobile phase B (MPB) on the isocratic

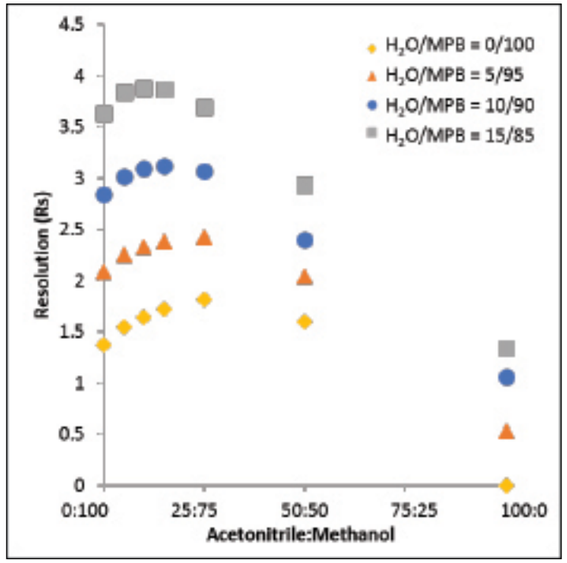
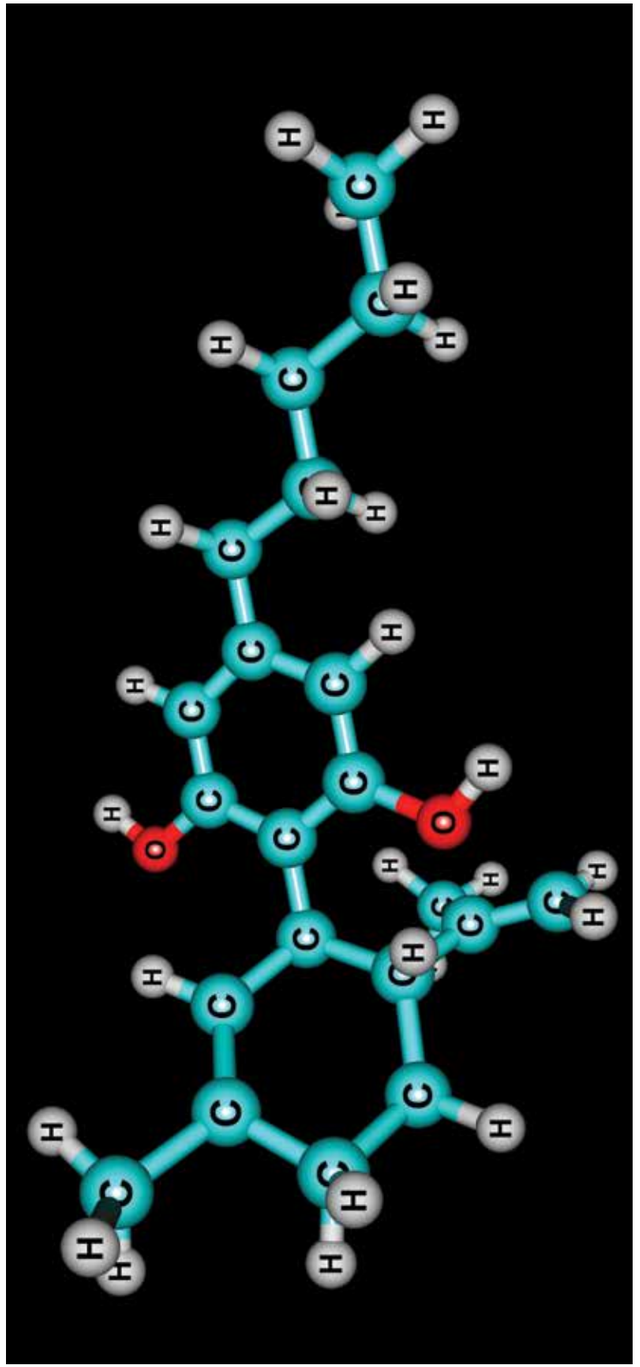


Figure 2 – The effect of the percentage and composition of mobile phase B (MPB) on the resolution of Δ9-THC and Δ8-THC. A blended organic modifier results in better resolution than pure methanol or pure acetonitrile. Evoke C18, 15 cm x 4.6 mm, 3 µm, 1.5 mL/min.

resolution of 1:2 Δ9-THC:Δ8-THC using the same Evoke C18, 15 cm x 4.6 mm column. Consider the analysis when performed with H_2O/MPB = 10/90. The resolution of Δ9-THC and Δ8-THC is 1.06 when MPB = 100% acetonitrile. When MPB = 100% methanol, the resolution is 2.84. Maximum resolution (Rs = 3.12) is observed when MPB = 15:85 acetonitrile:methanol. That relatively minor improvement in resolution afforded by the blended MPB might suggest pure methanol to be the preferred organic modifier for this analysis, especially given the convenience of using a single solvent over pre-mixing a blend of acetonitrile:methanol or investing in alternative pumping instrumentation (e.g. quaternary pumps). With complex samples, though, care must be taken to observe how a desired change in selectivity can affect other analytes in the separation.

A brief example serves to illustrate that several parameters should be considered when developing a chromatographic method for the resolution of complex samples involving key critical pairs. Consider again the separation of 1:2 Δ9-THC:Δ8-THC in the presence of CBL. In Figure 2, it can be seen that the resolution of the THC isomers is superior with pure methanol than with pure acetonitrile as the organic modifier.



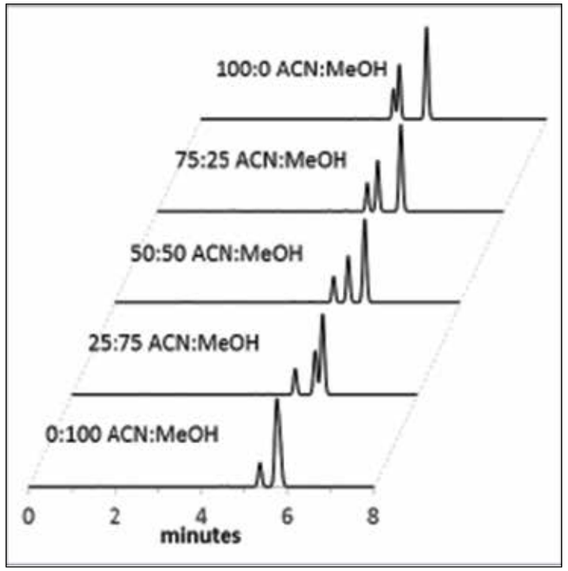


Figure 3 – Separation of 1:2:3 Δ9-THC:Δ8-THC:CBL. An organic modifier of pure methanol results in the co-elution of Δ8-THC and CBL while pure acetonitrile results in incomplete resolution of the THC isomers. A 50:50 blend of acetonitrile:methanol resolves all three analytes. Evoke C18, 15 cm x 4.6 mm, 3 µm, 1.5 mL/min, H2O/MPB = 10/90.

As shown in Figure 3, though, if CBL is present, it co-elutes with Δ8-THC in H_2O/methanol = 10/90. CBL elutes well away from the critical pair if pure acetonitrile is used, but the THC isomers are insufficiently resolved (Rs = 1.06). A 50:50 blend of acetonitrile:methanol provides good resolution, with Rs > 2.5 for both pairs. So, while binary mobile phase systems are very common in reversed-phase HPLC separations, ternary mobile phases can provide access to unique selectivity.

To recap, we developed an HPLC method that fully resolves 17 cannabinoids by using screening runs that altered concentrations of organic and acid modifiers and provided the foundation for further development. The addition of ammonium formate to mobile phase A gave a means to shift the retentions of the carboxylated species relative to the neutral ones, and an optimized concentration allowed for the baseline resolution of all cannabinoids in the test mixture. In addition, the use of a ternary mobile phase system (water, methanol, acetonitrile) was shown to improve the resolution of THC isomers while permitting the flexibility to avoid potential interferences.