Gradient HPLC-UV Method for Cannabinoid Profiling

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The global cannabis industry is growing rapidly, with many countries and US states adding regulatory frameworks for medical and recreational cannabis programs [1,2]. 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 along with potential contaminants such as pesticides, mycotoxins, heavy metals, etc. Current regulations surrounding potency vary by jurisdiction, but usually require testing for the active forms of tetrahydrocannabinol (THC) and cannabidiol (CBD). In addition, many require testing for the acid forms, tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), along with other cannabinoids like cannabigerol (CBG), cannabigerolic acid (CBGA), tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabicyclol (CBL), and cannabinol (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.

Historically, gas chromatography/mass spectrometry (GC/MS) has been used for the separation and quantification of cannabinoids and other compounds of interest in cannabis analysis. With GC, though, care must be taken to avoid decarboxylation of acidic species during the heated injection. High pressure liquid chromatography (HPLC) methods permit analysts to eschew many sample preparation and derivatisation steps and have become the preferred approaches to cannabis potency analysis [3,4,5].

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.

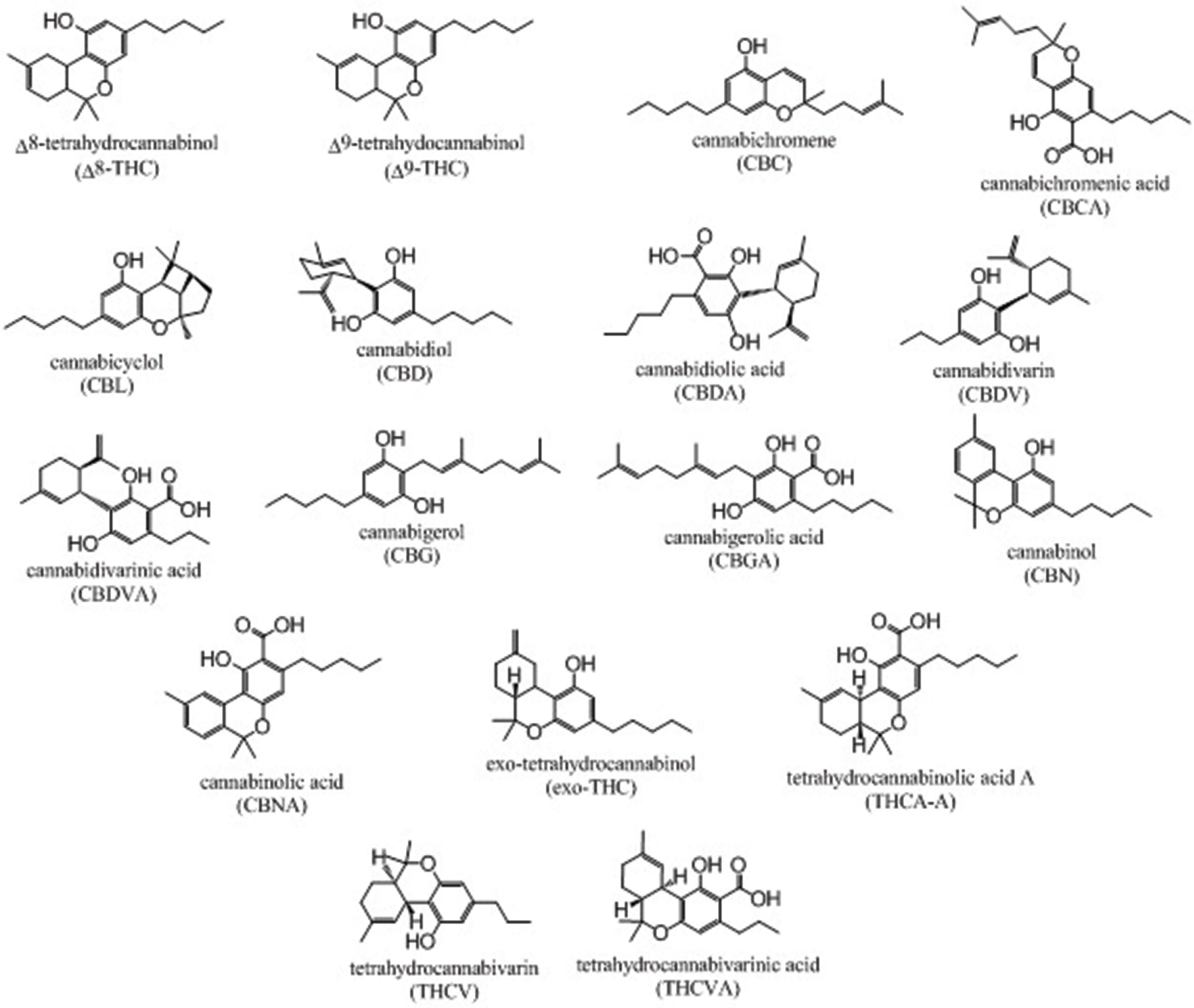

Figure 1: Molecular structures of the 17 cannabinoids separated in this application.

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 characterisation of a complex sample is desired, approaches to maximising 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 improved 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). The molecular structures of these cannabinoids are shown in Figure 1.

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 good peak shape for the 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.

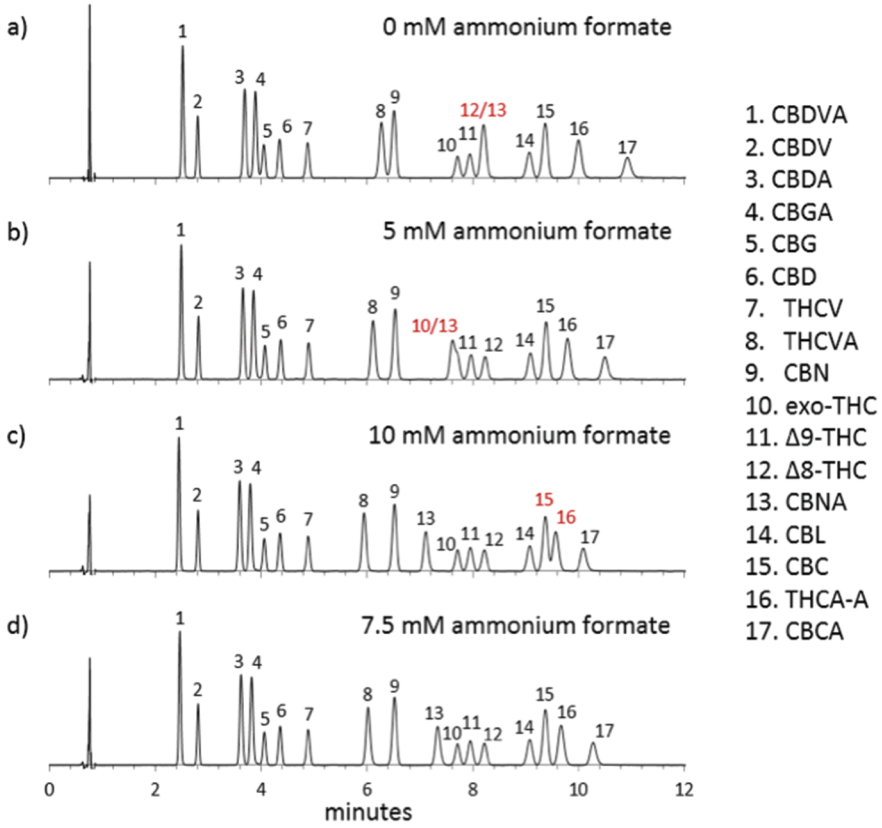

Figure 2. Effect of the addition of ammonium formate to mobile phase A. a) No ammonium formate added. b) 5 mM ammonium formate added. c) 10 mM ammonium formate added. d) 7.5 mM ammonium formate added. Additional chromatographic conditions listed in Table 1.

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

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	

Figure 2a 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 an effort to improve the resolution of these pairs, the effect of adding ammonium formate, the ammonium salt of formic acid, 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 [6,7]. With 0.1% formic acid and ammonium formate concentrations of 0 mM, 5 mM, and 10 mM, the pH values of mobile phase A were measured to be 2.7, 3.1, and 3.5, respectively.

As shown in Figure 2, the addition of ammonium formate to mobile phase A results 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

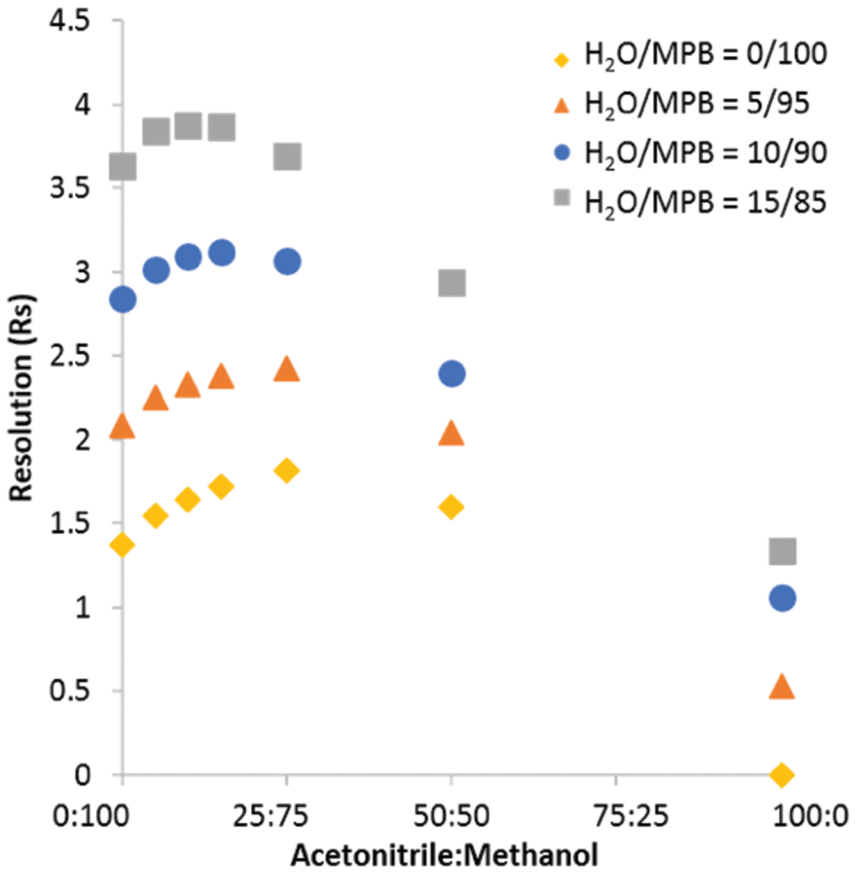

Figure 3. The effect of the percentage and composition of mobile phase B (MPB) on the resolution of Δ9-THC and Δ8-THC. A blended organic modifier results in better resolution than pure methanol or pure acetonitrile. Evoke C18, 15 cm x 4.6 mm, 3 µm, 1.5 mL/min.

Table 2: Retention time of each acidic cannabinoid as a function of the ammonium formate concentration in MPA. Additional chromatographic conditions listed in Table 1.

	Retention time (min)			
	0 mM	**5 mM**	**7.5 mM**	**10 mM**
CBDVA	2.52	2.49	2.46	2.45
CBDA	3.69	3.65	3.61	3.59
CBGA	3.89	3.86	3.82	3.80
THCVA	6.27	6.11	6.02	5.94
CBNA	8.20	7.61	7.38	7.11
THCA-A	10.00	9.79	9.68	9.57
CBCA	10.92	10.50	10.38	10.09

Table 3: Conditions used to investigate the retention of CBNA as a function of pH and ammonium formate concentration. Additional chromatographic conditions (flow, gradient, etc.) listed in Table 1.

Condition #	**[HCOONH4]**	**pH of mobile phase A**	**Rt of CBNA**
1	0 mM	2.7 (0.1% formic acid)	8.31
2	5 mM	3.1 (0.1% formic acid)	7.76
3	10 mM	3.5 (0.1% formic acid)	7.34
4	10 mM	6.6 (no formic acid)	1.64
5	10 mM	3.1 (pH adjusted)	7.36
6	10 mM	2.8 (pH adjusted)	7.52

formate, the retention time of CBNA is shifted to 7.63 minutes and coelutes with exo-THC, an impurity formed in the synthesis of Δ9-THC (Figure 2b). By increasing the concentration to 10 mM ammonium formate, the retention of CBNA is further shifted, causing it to elute earlier than the THC isomers, but THCA-A is shifted into coeluting with CBC (Figure 2c). An intermediate concentration of 7.5 mM ammonium formate was found to provide baseline resolution of all 17 cannabinoids in the test mixture (Figure 2d). Table 2 shows how the retention times of the acidic cannabinoids change when the concentration of ammonium formate buffer in mobile phase A is varied.

The interplay of buffer concentration and pH was further investigated with respect to the retention time of one of the carboxylated species, CBNA. The conditions are outlined in Table 3. In the first three cases, mobile phase A was prepared with 0.1% formic acid and ammonium formate concentration of 0 mM, 5 mM, and 10 mM. The unadjusted pH values were measured as 2.7, 3.1, and 3.5, respectively. As described above, retention of CBNA decreased with increased buffer concentration (8.31 min, 7.76 min, and 7.34 min). In the fourth case, 10 mM ammonium formate was used in mobile phase A and no formic acid was used in either mobile phase A or B. CBNA is ionised under these conditions, and its retention was reduced to 1.64 minutes. In the fifth case, 10 mM ammonium formate was used and pH was adjusted with formic acid to a value of 3.1 in order to match the pH of mobile phase A when prepared with 0.1% formic acid and 5 mM ammonium formate, and the retention time of CBNA was 7.36 minutes. In the final case, 10 mM ammonium formate was adjusted to a pH value of 2.8, and the retention time was 7.52 minutes. Thus, it can be seen that the retention of the carboxylated, ionisable cannabinoids is a complex function of eluotropic strength, pH (and the corresponding protonation state of the analyte), and buffer concentration/ionic strength.

It should be noted that since ammonium formate is added only to 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. Nevertheless, with approximately 5 minute re-equilibration, run-to-run results were found to be reproducible. With real world samples, such as plant extracts, matrix effects may prove to be a concern. Although not determined

here, the gradient method may permit some flexibility to build in a weaker solvent hold prior to the 75-90% MPB gradient in order to clear matrix interferences. Analysts must also assess whether other endogenous cannabis compounds, such as terpenes and terpenoids, potentially interfere with identification of cannabinoids.

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 2, 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 with various ratios of acetonitrile and methanol.

Acetonitrile and methanol are two of the most common organic modifiers used in reversed-phase HPLC, and many studies have detailed the differing and often complementary selectivities that they provide. Fundamental understandings of the solute-mobile phase, solute-stationary phase, and stationary phase-mobile phase molecular interactions can inform the strategies used in HPLC method development [8]. For instance, it has been noted that, depending on the modifier used and how it has partitioned or adsorbed into the stationary phase, differences in hydrophobicity, hydrogen-bonding, and dipole-type interactions can be observed [9,10]. When developing methods and selecting appropriate mobile phases, it can be useful to consult Snyder's solvent selectivity triangle, which plots solvents according to their acidic, basic, and dipolar properties [11,12,13]. Solvents that feature one of those properties more prominently than the other two can be readily identified from the plot. For example, methanol has acidic properties, and acetonitrile has dipole properties. Since they are miscible, they can be mixed in any ratio to achieve intermediate or new solvent properties.

Figure 3 plots the effect of varying the percentage and composition of mobile phase B (MPB) on the isocratic 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 cannabicyclol (CBL). In Figure 3 it can be seen that the resolution of the THC isomers is superior with pure methanol than with pure acetonitrile as the organic modifier. As shown in Figure 4, though, if CBL is present, it coelutes 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 selectivities based on the combination of acidic, basic, and dipolar properties of the mobile phases used.

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 optimised 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.

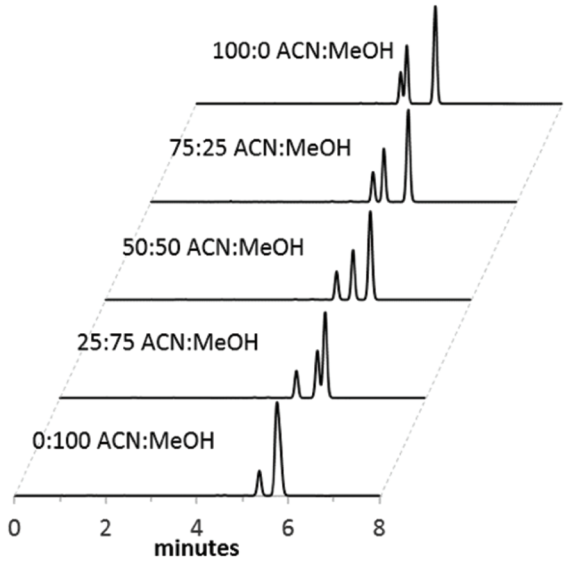

Figure 4: Separation of 1:2:3 Δ9-THC:Δ8-THC:CBL. An organic modifier of pure methanol results in the coelution of Δ8-THC and CBL while pure acetonitrile results in incomplete resolution of the THC isomers. A 50:50 blend of acetonitrile:methanol resolves all three analytes. Evoke C18, 15 cm x 4.6 mm, 3 µm, 1.5 mL/min, H_2O/MPB = 10/90.

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First published in Chromatography Today Volume 12 Issue 3 August/September 2019