Part 4: A New Hope

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Hey everyone, and welcome back to the cannabis method development series. So far in our quest to separate the twelve cannabinoids, we have messed around with gradient rates, mobile phase modifiers, and flow rates, yet have only been able to successfully resolve 11 of the 12 target compounds. Up until now all the work we have done has been using the same column – a 2.6 µm C18 100 x 4.6 mm column. So, it's high time we started looking at some

Effect of Column Length

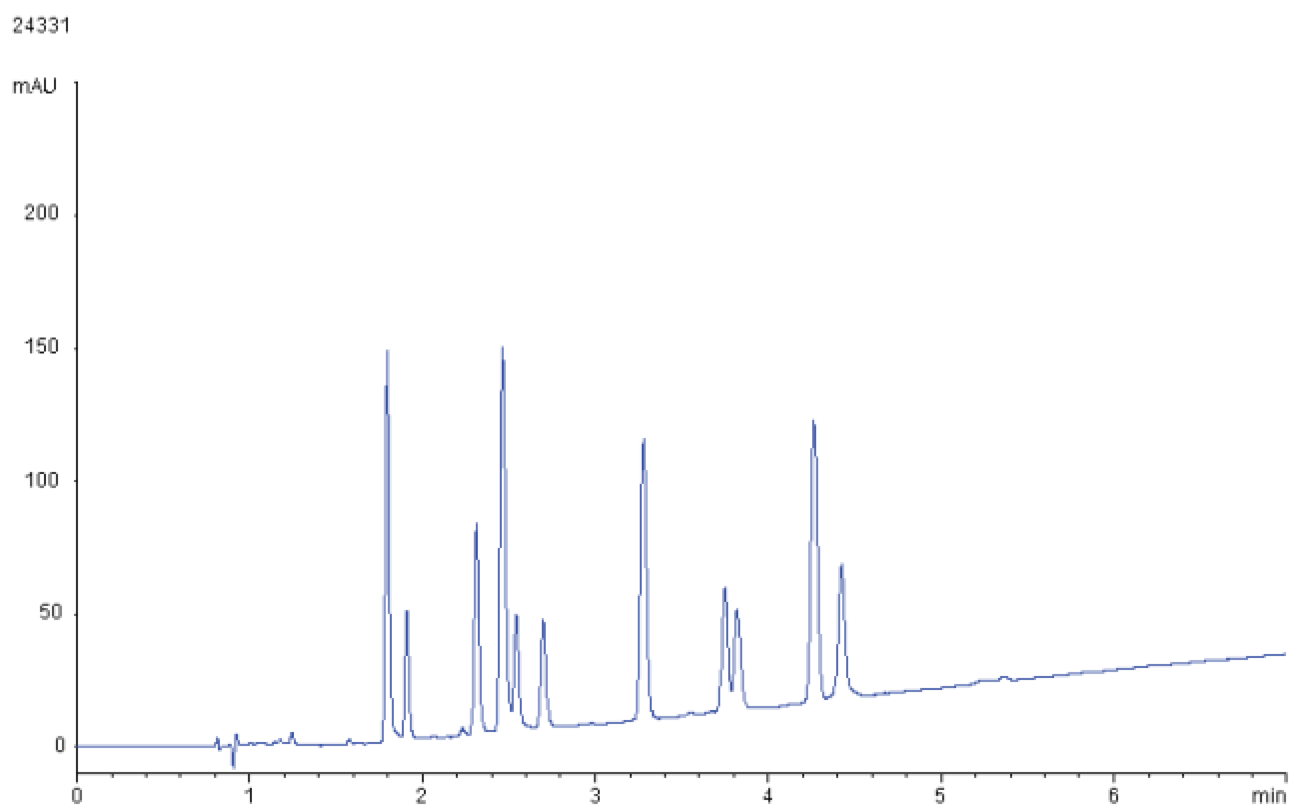
Changing column length can be a very simple and effective way to increase resolving power. All things being held equal, the efficiency of a column will be directly proportional to its length – double the length and double the efficiency. However, resolution – which is what we really care about – is only proportional to the **square root of the change in efficiency**. *So, if we double the column length, we increase our resolving power by a factor of about 40%. Still, in a case like this, every little bit helps, so let's see what happens when we move to a longer column.*

As you can see from **Figure 1a**, moving from our original 100 x 4.6 mm column to a 150 x 4.6 mm column, we are finally able to partially resolve our mystery 12th peak – you can see it as a shoulder of the 4th peak, indicated by the red arrow in **Figure 1b**. Please note, that we changed the column length without changing anything else – both columns were run using the same 7 min gradient from 75-100% B at a flow rate of 1 mL/min. In many cases, when changing column length, we will adjust the gradient profile in proportion to the new

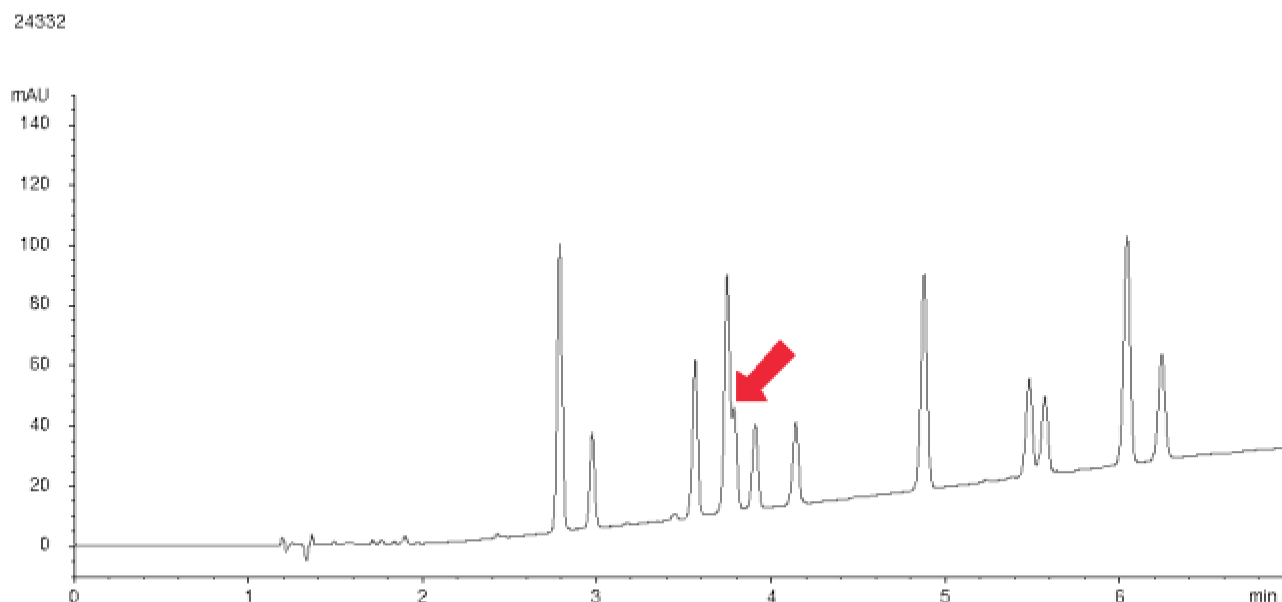
length. After we did this we found that it did provide significant improvement in resolution, but created a longer run time. Thus, we chose to stick with the original 7-minute gradient for the sake of a faster analysis time.

Figure 1. Effect of increasing column length on cannabinoids separation.

a. 100 x 4.6 mm



b. 150 x 4.6 mm



If moving to a 150 x 4.6 mm column could provide partial separation of that critical 12th peak, logically you should be able to further increase the length to a 250 x 4.6 mm to provide more resolution. Unfortunately, this was not a viable option for us due to wanting to use a standard LC system and needing to stay below ~300 Bar, which created large pressure generated by a 250 x 4.6 mm column packed with 2.6 µm particles. If we had access to a system with a higher pressure limit, then certainly that would be a viable route to pursue (as long as we were also OK with the increase in run time). However, we are limited on pressure and can't go beyond a 150 mm length, so let's try some **alternative stationary phase** chemistries to see if we can find a phase that can fully resolve the 12th peak (without concurrently causing a different set of peaks to co-elute).

Effect of Different Stationary Phases

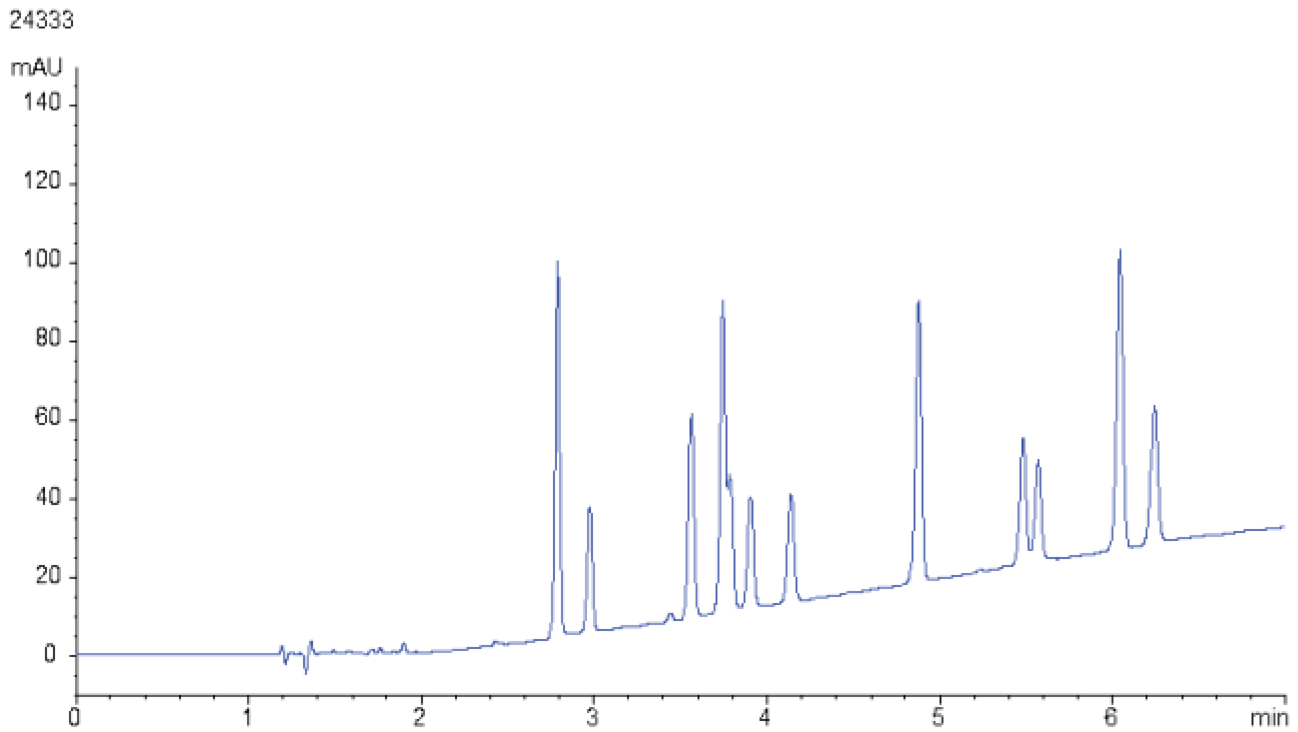
Figure 2 contains the representative chromatograms for five different stationary phases. All the columns were 150 x 4.6 mm and were operated using the same running conditions (75 – 100% B over 7 min at 1 mL/min; 0.1% TFA in water and acetonitrile).

For this application, we moved from the traditional Kinetex® 2.6 µm C18 column (**Fig. 2a**) to the Kinetex 2.6 µm XB-C18 (**Fig. 2b**). We saw a significant improvement in the resolution of the critical pair shown with the red arrow. Then we used the Kinetex 2.6 µm Polar C18 (another C18 with a modified surface chemistry to provide an alternative selectivity to a traditional C18) there was an increase in resolution (**Fig. 2c**). That same Polar C18 chemistry applied to a fully porous 3 µm column (Luna® Omega 3 µm Polar C18) provided similar resolution of the first critical pair, but we lost some resolution in a later-eluting pair shown by the blue arrow (**Fig. 2d**).

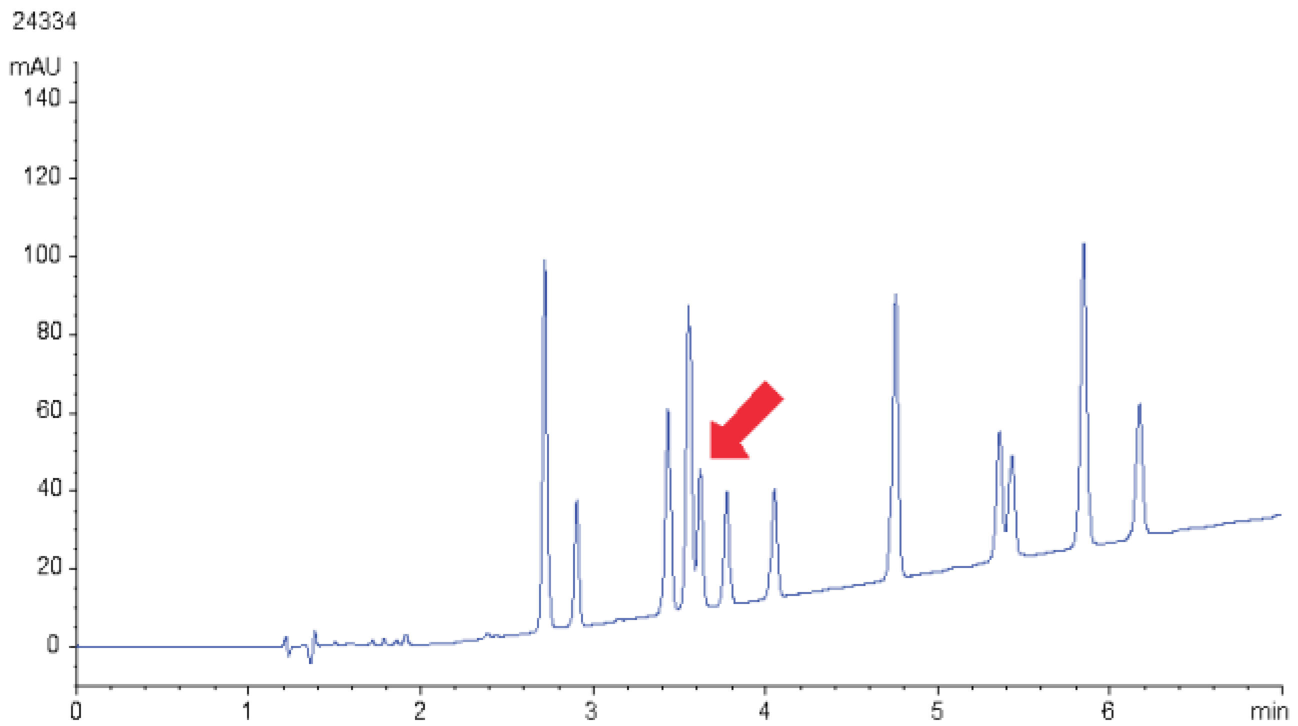
Lastly, we also evaluated a non-C18 phase (Kinetex 2.6 µm F5; **Fig. 2e**) to see if a column with a completely orthogonal selectivity to a C18 might provide better results. While the Kinetex F5 was able to provide some separation of that critical pair, under these conditions all of the peaks eluted in a very short time frame and had extensive co-elution. After some time trying to optimize the mobile phase with this column, we were not able to achieve separation comparable to the C18 columns. We discovered that a C18 phase of some variety is required to achieve the separation of this specific set of closely-related cannabinoids.

Figure 2. Effect of different stationary phases on cannabinoids separation.

a. Kinetex 2.6 µm C18



b. Kinetex 2.6 µm XB-C18



c. Kinetex 2.6 µm Polar C18

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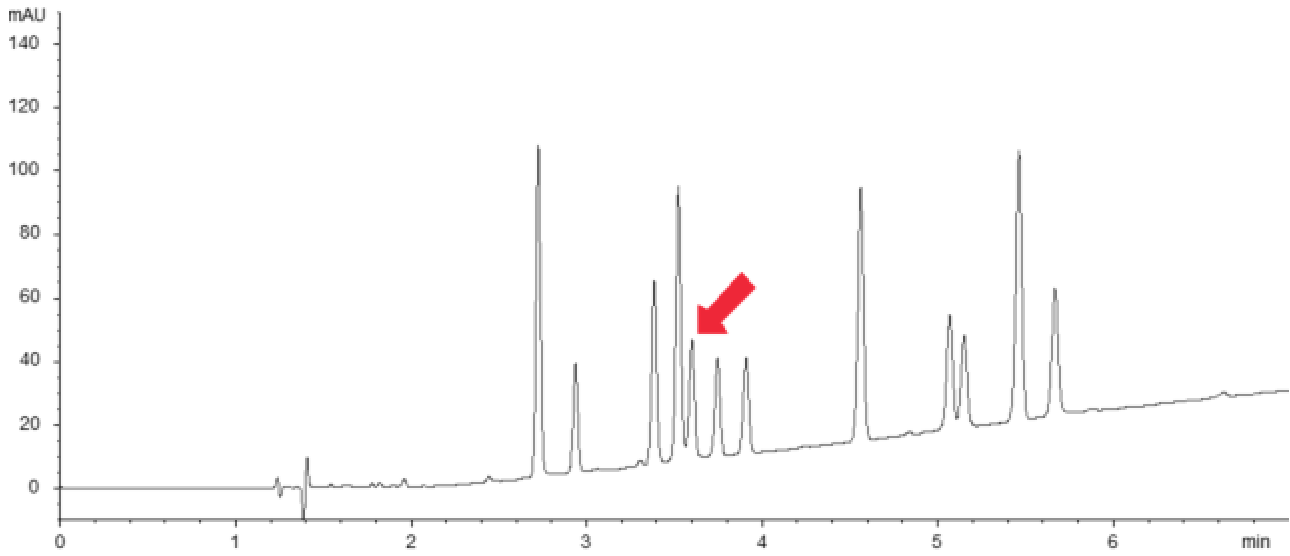
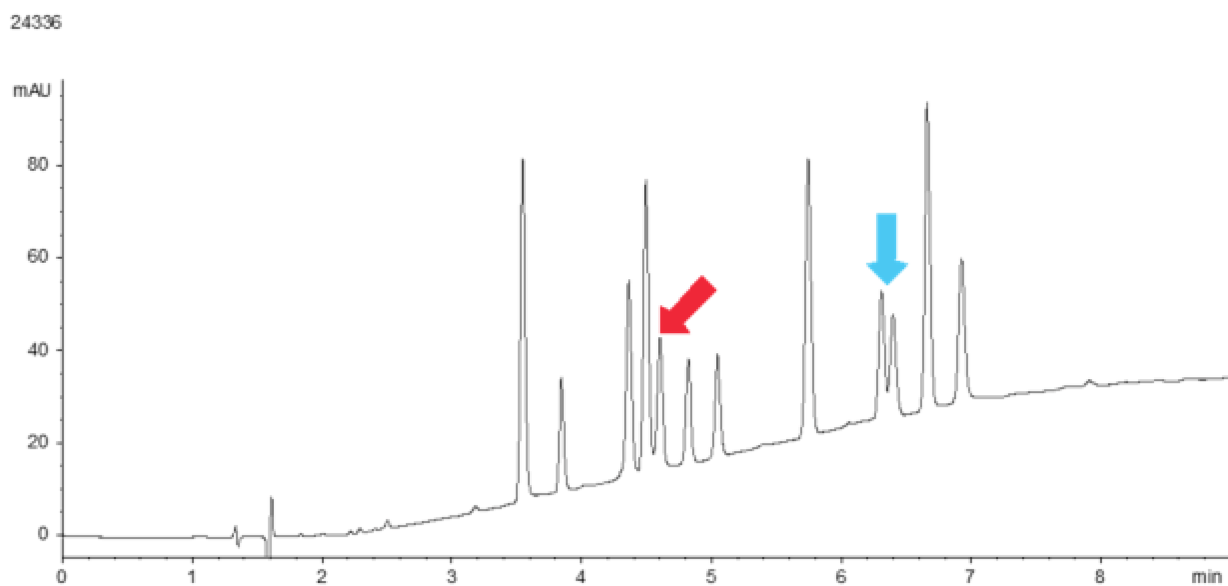


d. Luna Omega 3 µm Polar C18



e. Kinetex 2.6 µm F5

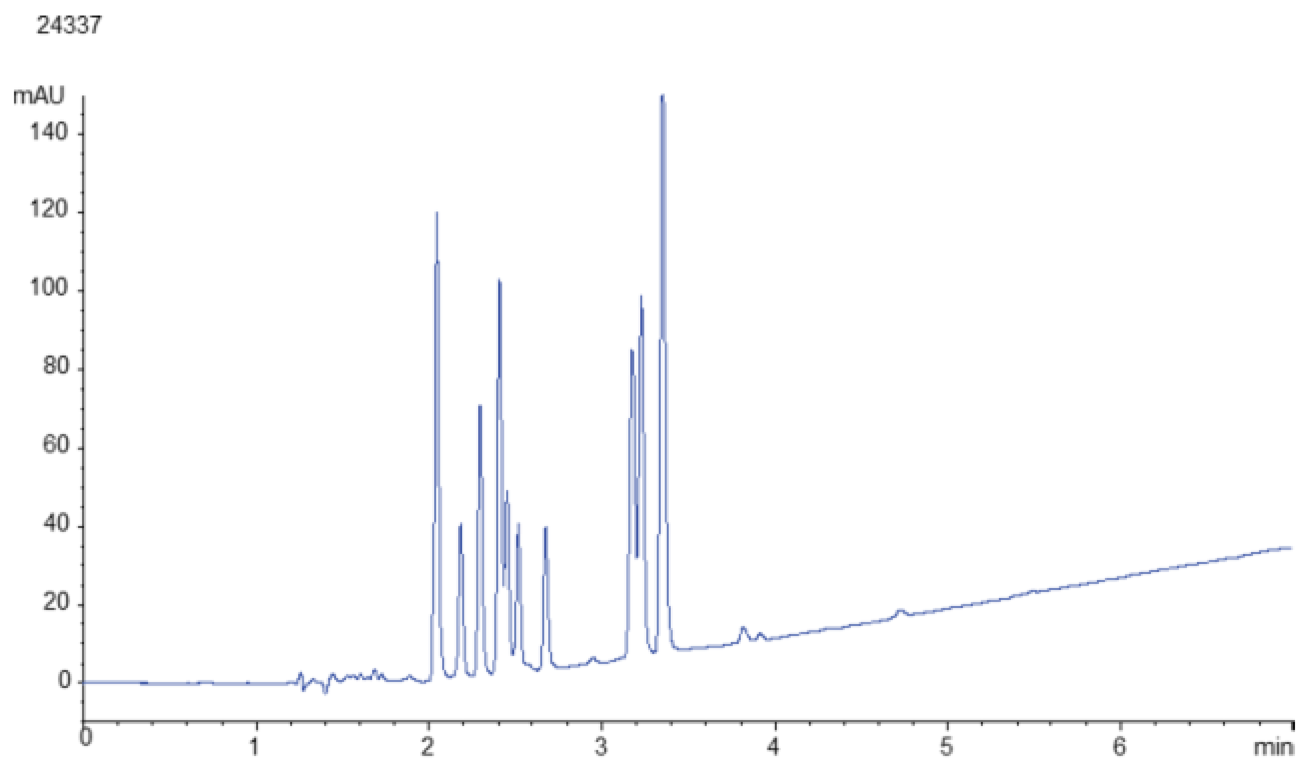


Table 1. Tabulated results for critical resolution using different stationary phases.

Phase	Resolution of Critical Pair
Kinetex 2.6 µm C18	0.72
Kinetex 2.6 µm XB-C18	1.13
Kinetex 2.6 µm Polar C18	1.42
Luna Omega 3 µm Polar C18	1.43
Kinetex 2.6 µm F5	0.92

As we can see from the chromatograms and the quantitated resolution values in Table 1, it appears that moving to a Kinetex 2.6 µm Polar C18 phase is going to give us the best performance for our complex mixture of 12 analytes.

However, we can always do a little more fine-tuning and optimization. In the next article, we will discuss our results from playing around with temperature and looking at the use of methanol instead of acetonitrile with our new stationary phase selection.

Now it's back to the lab!

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