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응용 자료

Kenneth D. Berthelette, Jamie Kalwood, Jonathan E. Turner, Kim Haynes

Waters Corporation

Abstract

The approach taken when performing method development can have a profound impact on the results and the time needed to reach said results. The use of method development software with analytical quality by design (AQbD) approaches can produce robust methods but are limited by both access to the software and user proficiency. Other approaches require more manual intervention to select the correct conditions, interpret the results, and make decisions based on the data. Employing a systematic screening protocol, which utilizes a structured decision-making process, alleviates the pressure on the analyst to select the best set of conditions and streamlines the method development process.

In the work shown, a systematic screening protocol was used to develop a method for the analysis of a mixture of nine structurally similar spice cannabinoids. The final method conditions were realized after only three days of development and optimization. It utilized an XBridge™ Premier BEH Shield RP18 Column and basic modified acetonitrile mobile phases. Good separation and detection were achieved for all nine analytes in both UV and MS detection, and three batches of material were tested to show batch-to-batch reproducibility of the separation.

Benefits

- Baseline separation of nine analytes using an XBridge Premier BEH Shield RP18 2.5 µm Column
- Fast Method development of a mixture of structurally similar compounds in three days
- Reduced decision-making time by employing a streamlined systematic screening approach

- Reproducibility tested with three batches of stationary phase packed at different sites

Introduction

Method development can be a challenging endeavor, even for expert chromatographers. Not only must an analyst find appropriate method conditions for separating their analytes, which can be challenging on its own, but often they need to keep track of how those conditions were determined and selected for future reference and documentation purposes. For laboratories where multiple method development activities happen concurrently, having different analysts develop methods can lead to different overall approaches. This makes tracking the activities even more difficult; in that situation it can be beneficial to implement a standardized protocol for all new method development work. This may include screening certain column chemistries, as well as certain mobile phase conditions. By standardizing method development across a lab, the process that yielded the final method conditions is better understood and can be easily documented. Additionally, having a standardized protocol allows less experienced users to perform method development, freeing up the experts to handle more challenging separations.

A variety of method development approaches exist, including software assisted AQbD and full factorial screening. Full factorial screening is designed to test each combination of the three critical parameters for HPLC methods: stationary phase chemistry, strong solvent, and mobile phase pH. This is the most comprehensive approach but does require not only creation of all the different mobile phases, but also considerable analyst and instrument time. Given a panel of four stationary phases, a full factorial approach would require 16 runs at a minimum, not including running replicates of each condition. This can take a significant amount of time to analyze and interpret once the data has been collected. AQbD approaches are generally faster than full factorials as only some of the critical parameters are tested. Typically employing design of experiments (DOE), the software assisted AQbD approach models the critical parameters for the separation and suggests the most robust area for method development. While this may be good for robustness testing or validation, AQbD does not account for different stationary phases, and often requires specialized training to run the software.

A more streamlined approach is the systematic screening protocol. Unlike full factorial, the systematic protocol first assesses the effects of mobile phase pH on the retention of the analytes. After at least two analytical runs, an analyst can easily determine the pH that provides the best retention for their analytes and then proceed to column and strong solvent screening. For a typical panel of four stationary phases, the systematic screening

protocol can provide the best method conditions after 10 injections, not including replicates, saving time over the full factorial approach. In this application, the systematic screening protocol was applied to develop a method to separate nine, structurally similar, spice cannabinoid compounds. To ensure the most accurate results are obtained, all columns used in this application note employ MaxPeak Premier High-Performance Surfaces (HPS) technology. This technology is specifically designed to mitigate secondary interactions between metal surfaces and the analytes and has been shown to improve overall separation quality.[1–2]

The final method achieves baseline separation of all nine components, with good detection using both UV and MS detection. After development, a simple reproducibility experiment was performed using three batches of the stationary phase packed in two different sites on different days. Good results seen across the three batches indicate good reproducibility of the method and is the first step towards method validation.

Experimental

Sample Description

Two separate mixtures of spice cannabinoids were obtained from Cerilliant as 100 µg/mL stock standards. 100 µL of both Spice Cannabinoids Mix (S-038) and Spice Cannabinoids Mix 2 (S-041) were combined and diluted with 800 µL Milli-Q water to a final analyte concentration of 10 µg/mL. Final sample composition for injection was 80:20 water:methanol.

LC Conditions

LC system:	ACQUITY™ UPLC H-Class Plus System with Quaternary Solvent Manager (QSM) with optional solvent select valve, Sample Manager Flow Through Needle (SM-FTN), Column Manager, Column Manager Aux, and QDa mass detector
Detection:	UV @ 220 nm (unless noted) MS full scan and SIRs used for peak tracking

Columns:	All columns are 2.1 x 50 mm with 2.5 µm particles XBridge Premier BEH C_{18} Column (p/n: 186009827) XSelect™ Premier CSH Phenyl-Hexyl Column (p/n: 186009879) XBridge Premier BEH Shield RP18 Column (p/n: 186009914) Atlantis™ Premier BEH C_{18} AX Column (p/n: 186009390)
Column temperature:	30 °C
Sample temperature:	10 °C
Injection volume:	1.0 µL
Flow rate:	0.7 mL/min
Mobile phase A:	Milli-Q Water
Mobile phase B:	Acetonitrile
Mobile phase C:	Methanol
Mobile phase D:	0.1% Formic Acid (D1) or 200 mM Ammonium Hydroxide (D2)
Gradient conditions:	Constant 5% D was maintained throughout the gradient to ensure consistent additive concentration. Linear gradient of 5–95% B/C over 4.90 minutes. Hold at 95% B/C for 0.82 minutes. Return to starting conditions of 5% B/C and hold

for 1.6 minutes. Total run time 7.37 minutes. Optimized gradient details provided in figure captions.

Data Management

Chromatography software: Empower™ 3 Feature Release 4

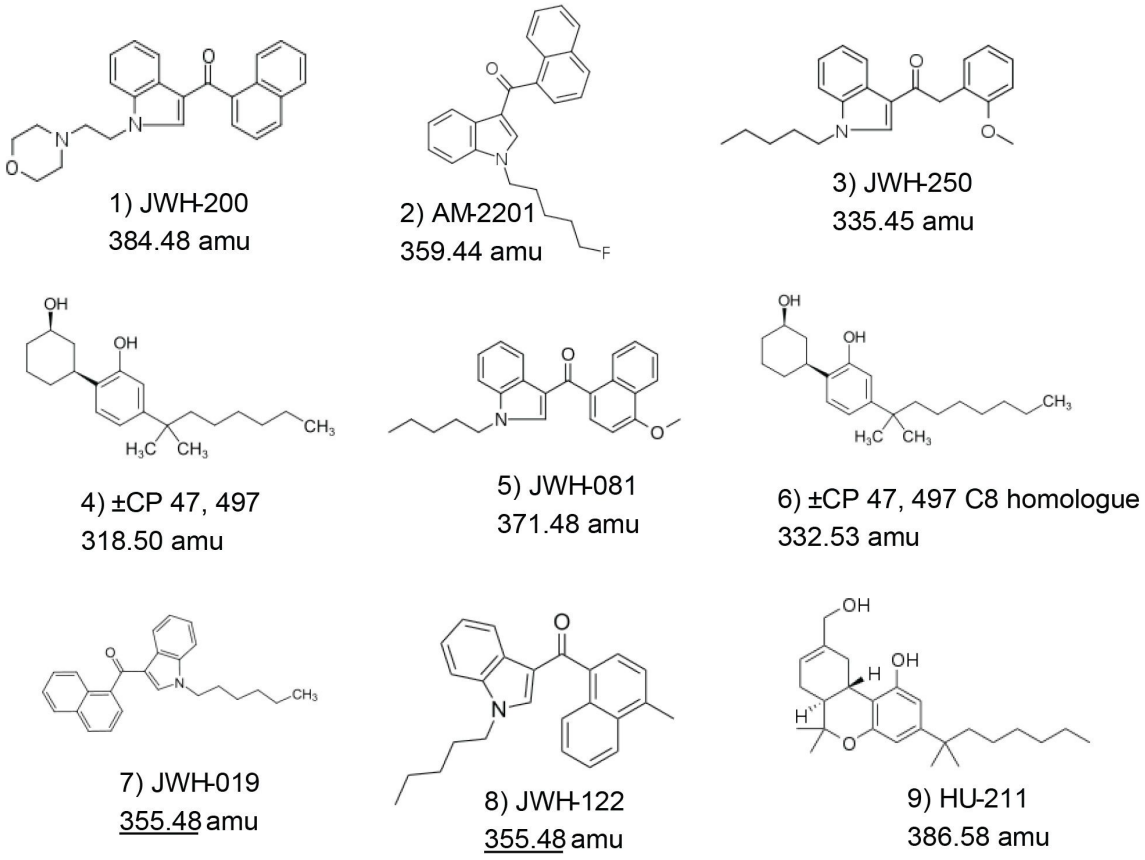

Figure 1. Chemical structures of analytes present in sample mixture. Note isobaric compounds JWH-019 (7) and JWH-122 (8).

Results and Discussion

Using a systematic screening approach to method development starts with setting minimum allowable criteria for the final method and defining the system that will be used. The criteria can be as broad or as specific as the laboratory requires. Typically, criteria such as USP resolution or tailing factors are used, however, some analyses may require other parameters to be met. These criteria are not only a goal for the method but are also a "stopping point" to let the analyst know when a method has been created that meets the needs of the analysis. Over-development of a method can waste time when an already sufficient method exists. Worse yet, without specific criteria, a method may be developed for weeks only to find that the final version yields similar results as the first few attempts. In the work shown, USP resolution values must be >1.5 for all compounds, and USP tailing factors must be between 0.8 and 1.2. For this work, an ACQUITY UPLC H-Class Plus with Quaternary Solvent Manager (QSM) and optional solvent select valve was used to maximize the solvent versatility of the system, limiting the need to pre-mix mobile phase additives and strong solvents. The QSM with solvent select valve allows mixing of all 4 solvent lines (A, B, C, D) with line D having up to six mobile phases to choose from (D1–D6).

Additionally, and in an effort to get the best possible separation, all the columns tested used MaxPeak Premier High-Performance Surfaces (HPS) technology. This technology mitigates secondary interactions between the metal surfaces of the column and the analytes of interest. Without knowing whether the probes of interest will interact with the exposed metals, using MaxPeak Premier HPS Columns is a safe starting point that will eliminate the possibility of those interactions, eliminating any doubt in the final separation.

Keeping these criteria in mind, we employed the systematic screening protocol outlined in previous applications.[3–6] The first step of the systematic screening protocol involves assessing the retention of the analytes at high pH and low pH and determining the conditions that provide the best retention. For this step, it is important to select a column that is stable at high pH, like the XBridge Premier BEH C_{18} Column. Unlike the final method requirements set earlier, in this step, only retention is assessed. The conditions which provide the best overall retention for the compounds of interest will be selected for the next step. Figure 2 shows the chromatography from the pH scouting.

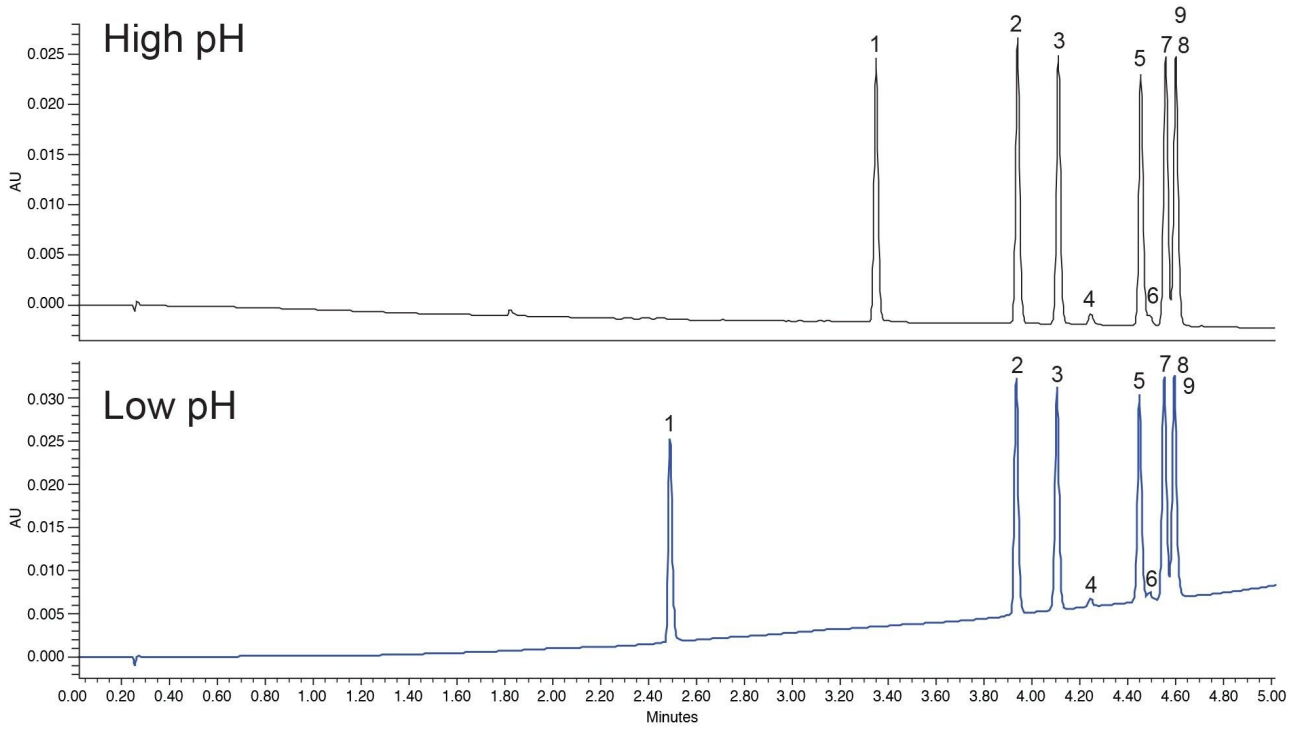

Figure 2. Rapid pH scouting results using the XBridge Premier BEH C_{18} Column using UV detection at 254 nm. Component IDs listed in Figure 1.

Both high and low pH show good retention for the analytes, with none retaining too early in the chromatogram. Components 2–9 seem unaffected by pH changing, indicating that these probes are neutral or the change in pH was not enough to change their charge state. Only component 1, JWH-200, showed a change in retention between the two pHs. With a pKa of ~7, the tertiary nitrogen present in JWH-200 can either be charged, which occurs at low pH, or neutral at high pH. This is confirmed by the fact that at low pH the compound is less retained. The other compounds present in this mixture, contain polyaromatic rings with heterocyclic nitrogen and are neutral at most pHs.

Following the systematic screening protocol, and assessing the results of overall retention, high pH is selected for the next step. While low pH would still be appropriate due to the mixture being tested and the initial results, the first step of the protocol is to assess retention only, not overall separation performance. Once high pH mobile phases are selected, the next step must be considered, which includes strong solvent and column stationary phase screening. At high pH, only hybrid stationary phases can be used. Silica stationary phases are not rated at

high pH due to the likelihood of silica dissolution and decreasing column performance.[7]

For this work, the four columns selected for solvent screening were the XBridge Premier BEH C_{18} Column, the XSelect Premier CSH Phenyl-Hexyl Column, the XBridge Premier BEH Shield RP18 Column, and the Atlantis Premier BEH C_{18}-AX Column. As previously mentioned, the XBridge Premier BEH C_{18} Column is a great starting point for high pH analyses due to its stability at high pH. Additionally, this phase is among the most common and most successful phases. The XSelect Premier CSH Phenyl-Hexyl Column also employs a hybrid particle allowing its use at high pH; however, this base particle has a slight positive charge applied during manufacturing, which can provide some selectivity benefits compared to the BEH base particle. Additionally, the phenyl-hexyl ring can provide unique retention mechanisms for analytes with aromatic rings due to π-π interactions with the stationary phase. The XBridge Premier BEH Shield RP18 Column is similar to the C_{18} stationary phase, however, the Shield RP18 functional group employs an embedded carbamate group. which can provide some selectivity benefits compared to the straight chain C_{18}. Lastly, the Atlantis Premier BEH C_{18}-AX Column is a low ligand density C_{18} stationary phase with an anion exchange functional group bonded to a BEH hybrid particle. The lower ligand density C_{18} allows more interaction with the base particle, and the anion exchange groups on the surface of the particle provide potential for unique selectivity. The results of methanol screening using these four phases is shown in Figure 3. UV detection was switched to 220 nm for screening and optimization, as the compounds show better absorbance at that wavelength, and no baseline shifts are detected with high pH mobile phases.

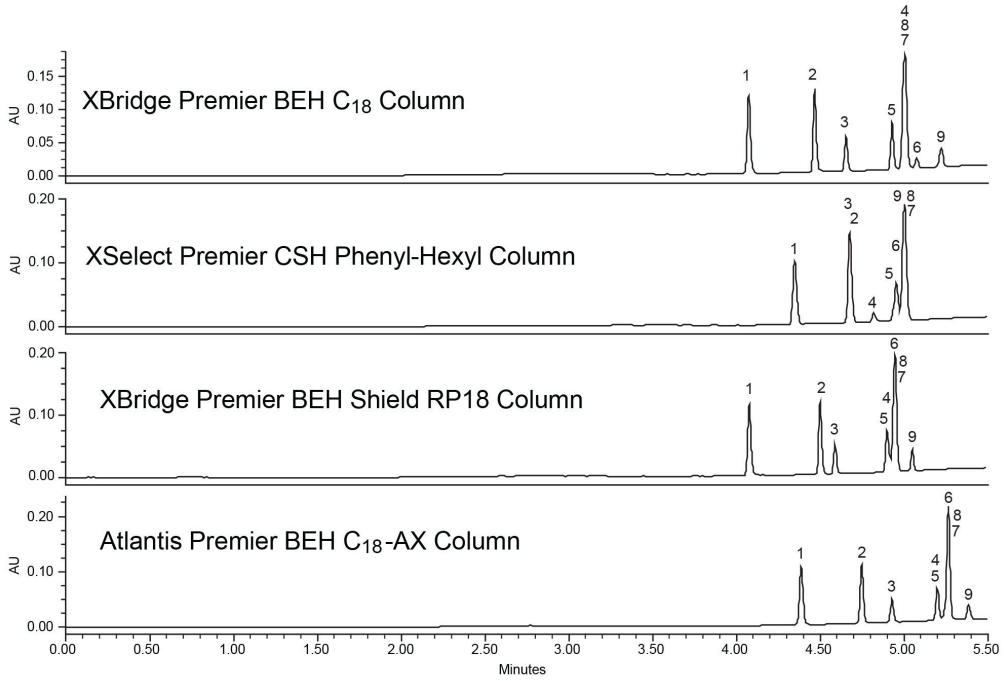

Figure 3. Chromatograms of column and solvent screening using methanol at UV 220 nm. Components IDs listed in Figure 1.

The chromatography created using methanol mobile phases is less than ideal. Each stationary phase shows poor separation of the nine components, with heavy co-elution of at least two components. Having complete co-elution of multiple components, as seen on the XBridge Premier BEH C_{18} Column, does not allow for easy method development. Optimization of these conditions to separate the components would be quite the challenge and may not be possible. Thankfully, the screening step of the systematic screening protocol also tests acetonitrile mobile phases on the selected columns. These chromatograms are shown in Figure 4.

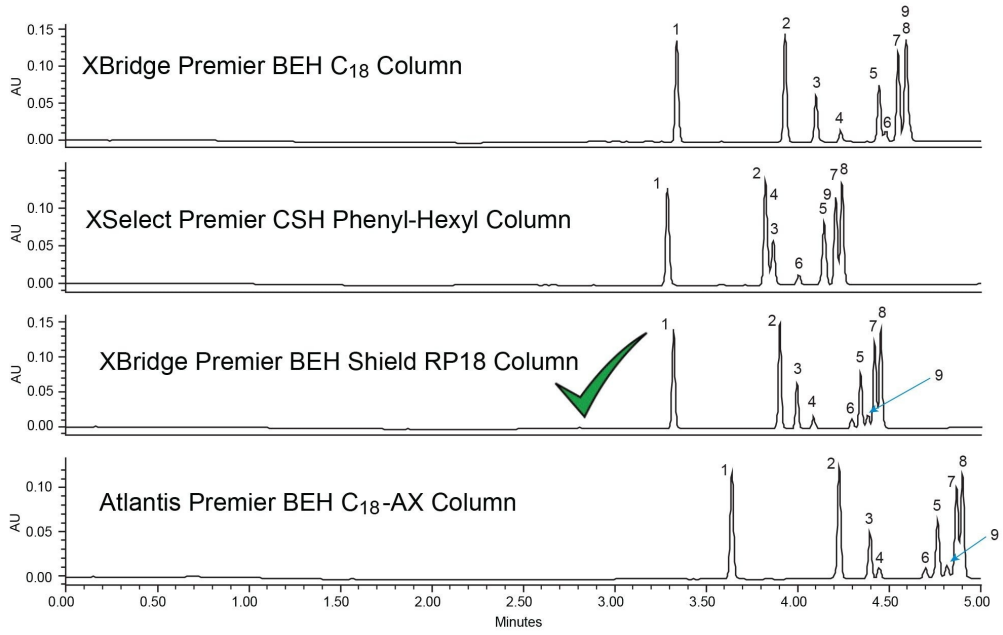

Figure 4. Chromatograms of column and solvent screening using acetonitrile at UV 220 nm. Components IDs listed in Figure 1.

The results obtained using acetonitrile were better than those with methanol. Across the four stationary phases screened, there was less co-elution of the compounds and better overall separation. In fact, of the four stationary phases tested with acetonitrile mobile phases, two could at least partially separate all nine components. The Shield RP18, and C_{18}-AX stationary phases still had partial co-elution of the peaks, but the components could still be seen as discrete peaks. The Phenyl-Hexyl stationary phase only showed seven discreet peaks, meaning at least two peaks were "hidden" as complete co-elutes with other peaks. The C_{18} phase showed eight discreet peaks, with component nine co-eluting with component eight. Looking more closely at the two semi-successful separations, and keeping the separation criteria specified earlier in mind, the XBridge Premier BEH Shield RP18 Column provided the best separation in terms of USP resolution for the components. The separation of components seven and eight, which are isobaric, were the best on the Shield RP18 phase. Additionally, the separation on the Shield RP18 phase was slightly less retained, providing more opportunities for method optimization.

Method optimization can include, but is not limited to, changes in gradient slope, adjusting column temperature, adjusting mobile phase pH by small amounts, and using a longer or shorter column configuration to either

improve separation or shorten run times. These steps were taken for the sample and the final method conditions are shown in Figure 5.

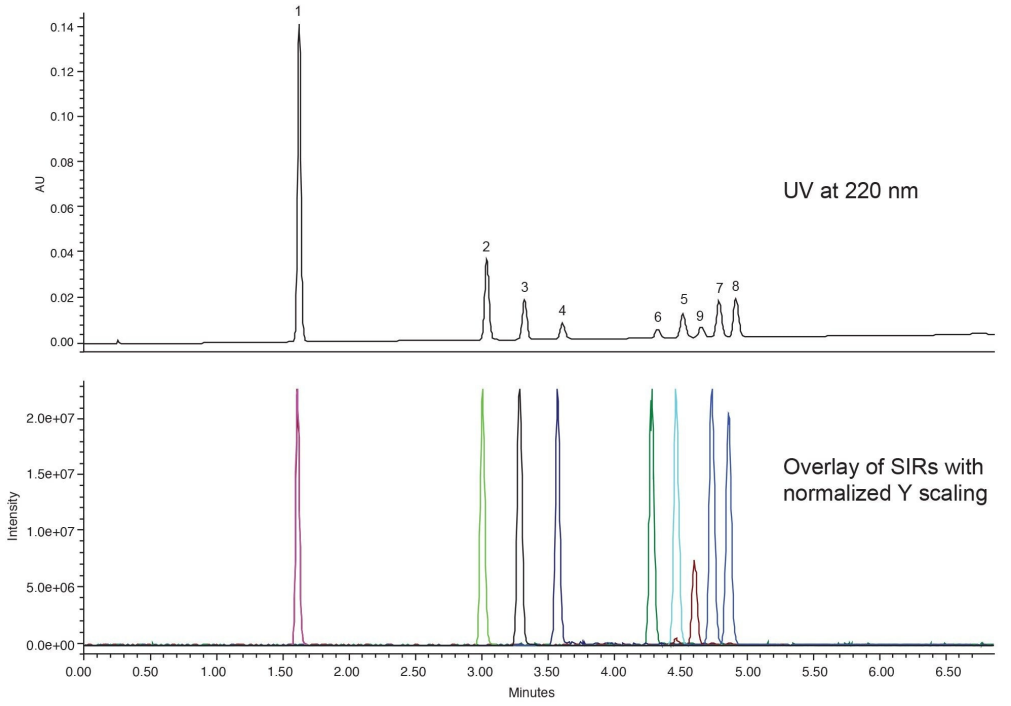

Figure 5. Final separation method for spice cannabinoid mixture using both UV and MS detection. XBridge Premier BEH Shield RP18, 2.5 µm, 2.1 x 100 mm Column. Constant 5% of D6 to maintain mobile phase pH was used. A linear 50–80% acetonitrile gradient over 6.52 minutes (gradient slope of 1.50%/V_c) was used with a total run time of 11.46 minutes. Component IDs listed in Figure 1.

The optimized method conditions show baseline separation of all nine components, with good peak shape and detection in both UV and MS detectors. Selected Ion Recording (SIR) of the nine components was performed in ESI+ mode, except for components four and six, which only ionized in ESI- mode. Peak intensity differences between the optimized testing conditions and screening methods are due to analyte stability and degradation. A fresh sample was prepared prior to batch-to-batch testing. Even before reproducibility testing was performed, the separation shown was acceptable and meets all the separation criteria set as part of the method development approach. Column reproducibility was outside of scope of method development and typically

performed as a part of method validation. Figure 6 shows the results of batch-to-batch testing of three different XBridge BEH Shield RP18 stationary phase lots, packed in two different sites. Batches 131 and 136 were packed in Wexford, Ireland, while batch 132 was packed in Milford, Massachusetts. All three columns used different lots of column hardware and were packed on different days by different technicians.

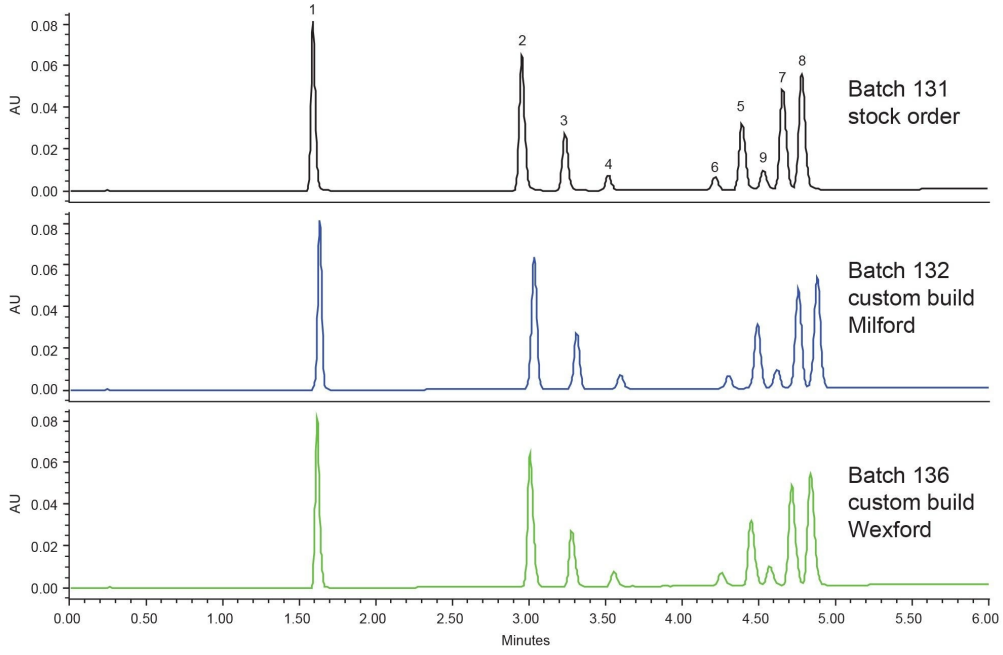

Figure 6. Batch-to-batch testing of XBridge BEH Shield RP18 stationary phase lots for the optimized method conditions of the analysis of nine spice cannabinoids. Data shown is 220 nm UV data. Component IDs are listed in Figure 1.

The separation performance across the three columns were comparable. All separation criteria set as a part of method development were still met with the lowest USP resolution being 1.52. USP tailing results were also within our limits. Using the systematic screening protocol to method development allows even a challenging mixture like spice cannabinoids to be separated. In three days, a method to separate all nine compounds was developed, and even tested for reproducibility, using three different batches of stationary phase. Using a streamlined process like the systematic screening protocol can quicken the method development process and reduce the ambiguity in the development procedure. By combining this process with the reliability and reproducibility of MaxPeak Premier Columns, analysts can eliminate doubt in their separations and produce

quality results they can rely on for all of their future workflows.

Conclusion

A step-by-step approach can be a critical asset for method development activities. Not only does having a standardized protocol ensure that all analysts are developing methods in the same fashion, but it can also reduce overall method development time if designed appropriately. The systematic screening protocol outlined in this application note does not require specialized software or training and can streamline the process of method development. A systematic screening protocol first examines the effects of pH on retention, before moving on to a column and strong solvent screen. By first assessing pH, the selected attribute can be locked in, eliminating it from further activities and impacting the selection of stationary phases to use in subsequent steps.

The systematic screening protocol was used to develop a method for the analysis of a mixture of nine structurally similar spice cannabinoids. Using the systematic protocol, a final method using acetonitrile, ammonium hydroxide, and an XBridge Premier BEH Shield RP18 Column was developed after just three days. Three batches of material, packed at two different sites on different days, were used to separate the compounds using the final method conditions and all provided comparable results. By utilizing the systematic screening protocol, coupled with the reliability of MaxPeak Premier Columns, a suitable separation was obtained and could be used in a variety of workflows including quantitation or identification of an unknown sample.

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