

## Method Development of Ten Antibiotic Compounds Using a Systematic Screening Protocol on an ACQUITY Arc With UV and QDa Detection

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### Abstract

Developing new LC and LC-MS methods can be challenging regardless of analyst experience. Without dedicated protocols, an analyst may not know where to start or the steps to take to develop a new method. Some method development protocols include the screening of multiple stationary phases, different mobile phase additives, different pH values, and even different strong solvents. The complexity of method development is compounded by the approach taken to method development, in which different analysts may approach the task differently adding uncertainty and potentially leading to different end results.

The work shown here highlights the benefits of a method development protocol based on a tiered screening approach, and its use towards developing a method to separate ten antibiotic compounds. Due to the nature of the compounds being tested, both UV and MS detection is required as one of them lacks a UV chromophore. An ACQUITY Arc System equipped with both a PDA detector and an ACQUITY QDa Mass Detector was used to develop the method. Using the tiered approach, first the pH of the mobile phase was tested and assessed to determine which performed better. Once locked into a mobile phase pH, a more traditional column screening experiment was performed with columns compatible with the determined mobile phase. During column screening both acetonitrile and methanol phases were tested and a suitable separation was determined. A final

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method, suitable for both LC-UV and LC-MS activities, was developed within two business days, highlighting the speed that a dedicated method development protocol can have.

## Benefits

- Perform method development activities quickly using a tiered screening protocol
  - Track peaks during method development using the ACQUITY QDa without the need to run individual standards
  - Detect compounds without UV chromophores using the ACQUITY QDa as an orthogonal detection technique
  - Achieve baseline separation of ten generic antibiotic compounds
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## Introduction

Strategic method development processes can significantly improve the speed and efficiency of method development activities. The most common approaches to method development can be broken into four categories: One Factor at a Time (OFAT), Full Factorial Screening (FFS), Tiered Screening (TS), and Analytical Quality by Design (AQbD). Each have their own strengths and weaknesses including the need for experienced analysts, or access to different types of software. OFAT requires a highly experienced analyst to interpret the results and determine what other conditions to try. This approach can be quite lengthy if the sample is particularly complex, or if the analyst is not familiar with method development techniques. FFS is the most comprehensive approach as every combination of stationary phase, mobile phase pH and mobile phase strong solvent are tested.<sup>1-2</sup> Because every aspect of the separation is probed, data collection and data interpretation can be time-consuming. Recently, AQbD approaches have gained significant traction but require special software such as Fusion or DryLab.<sup>3-5</sup> This software requires some training; however, the end results are a robust and well-developed method. By using a highly statistical approach to method development, AQbD generates highly robust data. During development the robustness of the method is probed to ensure that slight changes will not cause drastically different results. By having robust methods, the need for re-work, re-validation, and failed methods is reduced. Additionally, method validation can be performed more quickly if the robustness has already been tested and proven to be sufficient. Finally, the TS approach balances the speed of OFAT with the comprehensive testing of FFS.<sup>6</sup> In a tiered screen, method development activities are performed step-wise, with a decision step after each experimental screen. One variable is tested at a time, which is then “locked” in for

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subsequent testing of additional variables.

To demonstrate the use of a tiered screening approach, a mixture of ten antibiotic compounds including amoxicillin, clavulanic acid, piperacillin, tazobactam, ampicillin, oxacillin, cloxacillin, dicloxacillin, azithromycin, and cefalexin was created. These compounds include some of the most commonly prescribed antibiotics in the United States,<sup>7</sup> as well as two compounds coming off patent within the next few years, piperacillin and tazobactam. Four different stationary phases were tested in order to separate these compounds, as well as two strong solvents and two mobile phase pH additives. The final method was selected from the Tiered Screening approach on the second day of testing, without the need for further optimization to the separation.

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## Experimental

### Sample Description

Ten antibiotics were purchased from Sigma Aldrich and diluted in 90:10 water:acetonitrile to the following concentrations: amoxicillin, cefalexin, ampicillin, oxacillin, cloxacillin, dicloxacillin, piperacillin, and tazobactam at 95 µg/mL, azithromycin at 40 µg/mL, and clavulanic acid at 200 µg/mL.

### Method Conditions

#### LC Conditions

LC systems:	ACQUITY Arc with a 2998 PDA Detector and ACQUITY QDa Mass Detector
Detection:	UV @ 215 nm, Absorbance-MBF SIR of Antibiotic Compounds
Vials:	LCMS Certified Clear Glass Vial 2 mL (p/n: 600000751CV)
Column(s):	XSelect CSH C <sub>18</sub> Column, 3.0 x 50 mm, 2.5

µm (p/n: 186006105)

XSelect CSH Phenyl-Hexyl Column, 3.0 x 50  
mm, 2.5 µm (p/n: 186006129)

XSelect CSH Fluoro-Phenyl Column, 3.0 x  
50 mm, 2.5 µm (p/n: 186006117)

XSelect HSS C<sub>18</sub> SB Column, 3.0 x 50 mm,  
2.5 µm (p/n: 186006165)

Column temp.: 30 °C

Sample temp.: 10 °C

Injection volume: 3 µL

Flow rate: 0.85 mL/min

Mobile phase A: Water with either 0.1% formic acid (~pH 3)  
or 10 mM ammonium hydroxide (~pH 10)

Mobile phase B: Acetonitrile with either 0.1% formic acid or  
10 mM ammonium hydroxide

Mobile phase C: Methanol with either 0.1% formic acid or 10  
mM ammonium hydroxide

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B/C	Curve
0.00	0.85	95	5	6
8.22	0.85	5	95	6
8.72	0.85	5	95	6
8.73	0.85	95	5	6
12.00	0.85	95	5	6

## MS Conditions

MS system:	ACQUITY QDa Mass Detector
Ionization mode:	SIR, ESI+ mode
Acquisition range:	175 <i>m/z</i> –550 <i>m/z</i> (SIRs collected as described in Figure 1)
Capillary voltage:	1.5 kV
Cone voltage:	15 V

## Data Management

Chromatography software:	Empower 3 Feature Release 4
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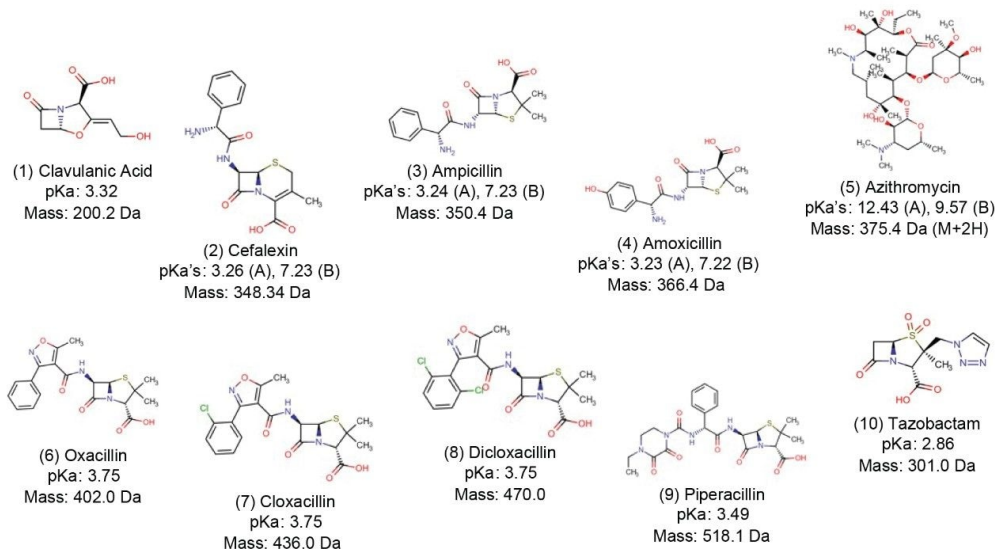


Figure 1. Chemical structures of the ten antibiotics present in the sample mixture. Values shown for pKa were calculated by the ChemAxon Software, Chemicalize, and are listed as strongest acid (A) and strongest base (B), where applicable. All unlabeled pKa's are acidic pKa's unless noted. Masses listed are the charged (M+H) masses used to detect the component via SIRs unless noted.

## Results and Discussion

In a tiered screen (TS), different "levels" or "tiers" of method development experiments are performed. Typically, each "tier" tests one or two parameters, *i.e.* mobile phase pH, or stationary phase chemistry. After each set of experiments, a decision must be made on how to proceed given the results obtained. For the work presented, the Tiered Screen used is shown in Figure 2. In the first "tier" mobile phase pH is tested and the results examined for retention of the test probes. From this data, the mobile phase pH is selected for subsequent testing. This step-wise approach allows an analyst to test conditions that are appropriate and applicable for the assay.

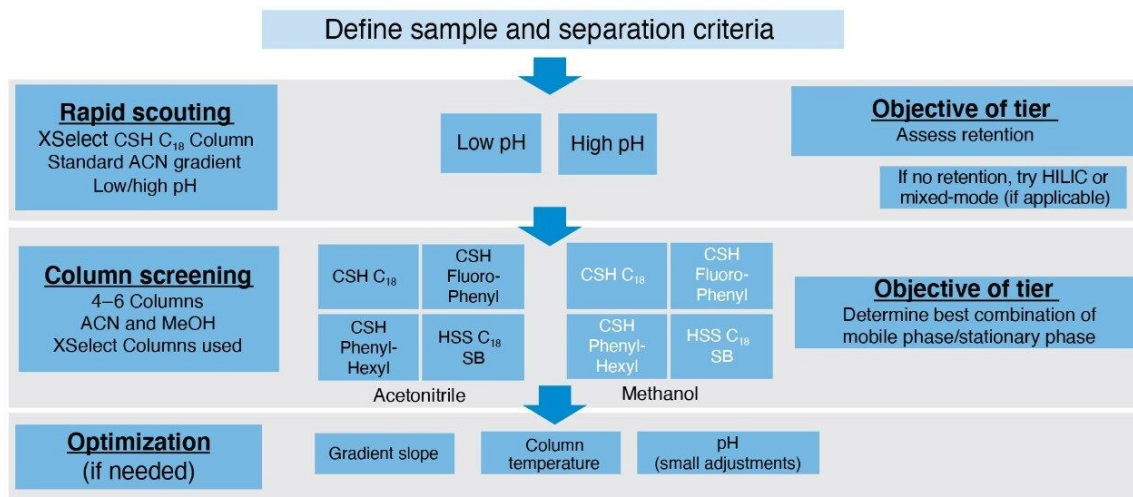


Figure 2. Tiered screening protocol used to develop a method to separate ten antibiotic compounds.

The first step in method development is to define the sample and separation criteria, which will allow for a clear stopping point during development. Examples of separation criteria can include, but are not limited to, achieving certain USP resolution values for closely eluting peaks or having good peak shape. For the separation of the antibiotics three goals must be achieved to have an acceptable method. First, all the compounds must be separated with USP resolution of >1.5, which is generally recognized as baseline resolution. Second, the compounds must have good peak shape with peak symmetry as close to 1 as possible. Lastly, the compounds must be retained.

Once the criteria is set for a separation, the first step of the Tiered Screen can be performed. This step, often called Rapid Scouting, is performed using both low pH and high pH mobile phase additives to assess the retention of the probes. During this step, if neither high nor low pH can achieve sufficient retention, alternative chromatographic techniques such as mixed mode chromatography or hydrophilic interaction liquid chromatography (HILIC) may be needed. In order to perform Rapid Scouting, a stationary phase that is compatible with both high and low pH mobile phases is required. In this example, an XSelect CSH C<sub>18</sub> Column was selected for Rapid Scouting. This column, which is packed with a hybrid organosilica stationary phase, is stable from pH 1–11 and thus suitable for scouting experiments. Figure 3 shows the results of the Rapid Scouting experiments for the ten antibiotics using the XSelect CSH C<sub>18</sub> Column with an acetonitrile mobile phase.

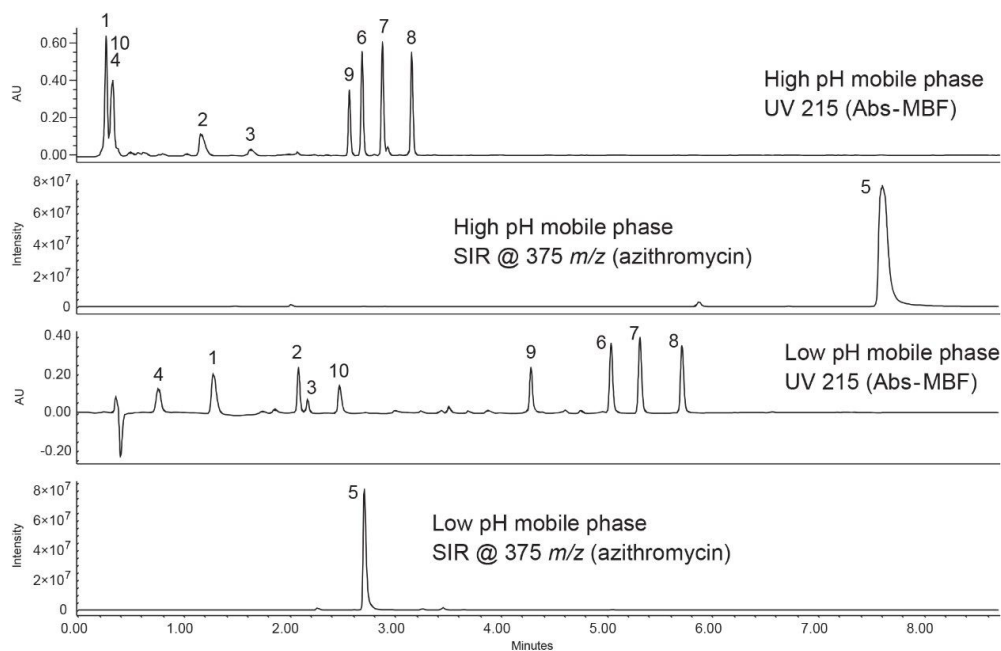


Figure 3. UV chromatograms of ten antibiotics using an XSelect CSH C<sub>18</sub> Column with high pH (top) and low pH (bottom) mobile phase additives and SIRs of azithromycin (a compound with a chromophore). 1) clavulanic acid, 2) cefalexin, 3) ampicillin, 4) amoxicillin, 5) azithromycin, 6) oxacillin, 7) cloxacillin, 8) dicloxacillin, 9) piperacillin, and 10) tazobactam.

UV detection was used for the analysis of all compounds except azithromycin, a compound without a chromophore that was instead evaluated with MS detection. Absorbance-MBF was used to reduce the background noise of formic acid at the 215 nm wavelength. From Figure 3, selectivity differences between high pH and low pH mobile phase additives can be observed. The most noticeable difference is for azithromycin (5), which elutes before 3 minutes at low pH and at almost 8 minutes at high pH. At low pH, azithromycin is doubly charged at the amine groups, but at high pH, the amines are no longer charged and the compound is neutral, leading to higher retentivity. Azithromycin also changes ionization efficiency between the two pHs. At high pH Azithromycin is better ionized, but is a wider peak, while at low pH the signal intensity is approximately the same albeit with a narrower peak. Tazobactam is partially charged at low pH and is retained well, eluting at ~2.4 minutes. At high pH, tazobactam is fully charged and is therefore more polar and less retained. Similar trends are seen for most of these compounds, wherein their retention at high pH is less than at low pH. While this trend is



primarily due to the charge state of the compounds, it is also partially impacted by the mixed mode capabilities of the XSelect CSH C<sub>18</sub> Column.

The XSelect CSH C<sub>18</sub> Column uses a hybrid base particle designed to include a partial positive charge. This modification improves the peak shape of basic compounds at low pH, and provides a mixed-mode interaction with acidic probes. At low pH the anionic exchange functionality of the stationary phase helps retain the acidic probes, whereas at high pH the anion-exchange functionality is reduced. This, combined with the acidic or basic functionality of the analytes, contributes to the lower retention seen for compounds like oxacillin (6) at high pH. At low pH, eight of the ten compounds are fully resolved with good retention and good peak shape. Thus, for the separation of antibiotics, the low pH mobile phase condition was chosen. In other cases, the high pH mobile phase may be more beneficial, *i.e.* the quantification of azithromycin.

In the next step of tiered screening, the selected mobile phase pH is locked in, and a panel of appropriate columns is screened using both acetonitrile and methanol mobile phases. Figures 4 and 5 show the separation of the antibiotics on four selected columns using acetonitrile and methanol mobile phases respectively. Only the 215 nm UV channel is shown, but the retention of azithromycin is marked by the placement of the component number (5).

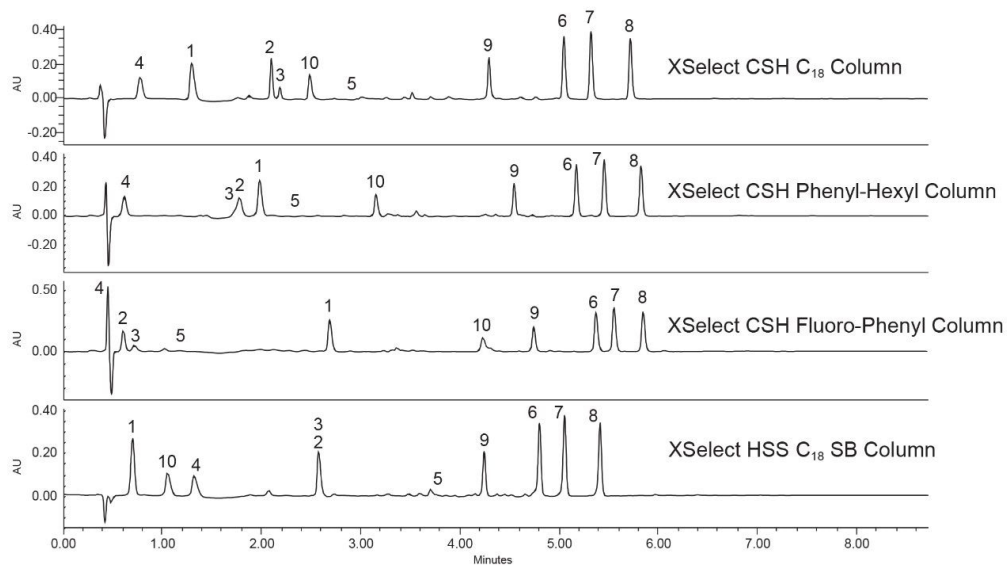
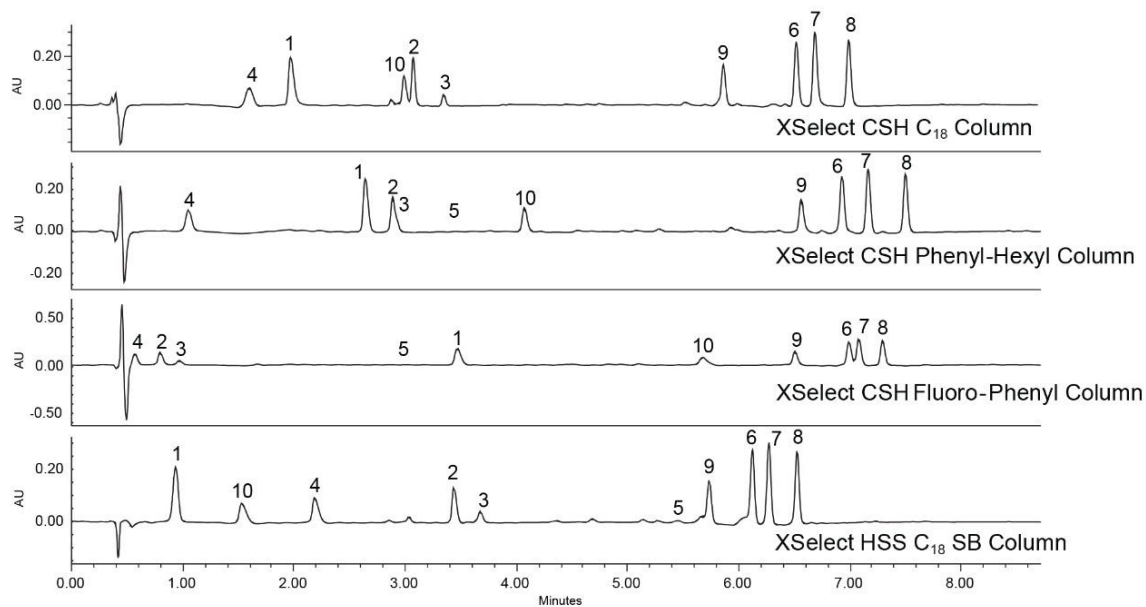


Figure 4. Separation of ten antibiotics on four stationary phases using acetonitrile as the strong solvent. 1) clavulanic acid, 2) cefalexin, 3) ampicillin, 4) amoxicillin, 5) azithromycin, 6) oxacillin, 7) cloxacillin, 8) dicloxacillin, 9) piperacillin, and 10) tazobactam.



*Figure 5. Separation of ten antibiotics on four stationary phases using methanol as the strong solvent. 1) clavulanic acid, 2) cefalexin, 3) ampicillin, 4) amoxicillin, 5) azithromycin, 6) oxacillin, 7) cloxacillin, 8) dicloxacillin, 9) piperacillin, and 10) tazobactam.*

From these figures, it can be seen that the separation of components 2 and 3 (cefalexin and ampicillin) is difficult. Both the XSelect CSH Fluoro-Phenyl Column and XSelect CSH C<sub>18</sub> are able to separate the two compounds. The Fluoro-Phenyl phase yields poor retention for amoxicillin, and lower overall retention for cefalexin, ampicillin and azithromycin however. This lower retention could be due to the basic functionality of these probes at low pH and their interaction with the positive charge on the base particle. This is seen to a lesser extent on the C<sub>18</sub> and Phenyl-Hexyl phases. The XSelect HSS C<sub>18</sub> SB Column provides the best retention for cefalexin, ampicillin, and amoxicillin highlighting the fact that the base particle is playing a significant role in the retention of these compounds. Using acetonitrile as the strong solvent meets the criteria set at the beginning of method development only when the XSelect CSH C<sub>18</sub> Column is used. The lowest USP resolution value obtained is 1.92 for the critical pair of cefalexin and ampicillin while also having good peak shape and retention for the compounds. The XSelect CSH Fluoro-Phenyl Phase is a close second as it achieves good separation (>1.5 USP resolution for all probes) but poor retention. Examining these same columns with methanol mobile phases may produce an even better separation than seen with acetonitrile.

Methanol mobile phases show different results than what was seen with acetonitrile. While increased retention is

expected with methanol, selectivity differences may not always be seen. However, when a Phenyl-Hexyl stationary phase like the XSelect CSH Phenyl-Hexyl is used, methanol as the strong solvent can provide some unique selectivity differences. Comparing the separations on the XSelect CSH Phenyl-Hexyl Column using methanol and acetonitrile an elution order change is noticed. In an acetonitrile mobile phase, clavulanic acid (1), cefalexin (2), and ampicillin (3) elute in the sequence 3,2,1. However with methanol the elution order changes to 1,2,3. This change in selectivity is due to secondary interactions between the compounds and the stationary phase. Both cefalexin and ampicillin have phenyl rings that can interact via pi-pi interactions with the Phenyl-Hexyl stationary phase when methanol is used.

Using the methanol mobile phase, the XSelect CSH C<sub>18</sub> Column, and XSelect HSS C<sub>18</sub> SB Column are both able to retain and separate all of the compounds, whereas co-elution of peaks can be seen when using the other two columns. The XSelect CSH C<sub>18</sub> Column gives slight co-elution, USP resolution of 1.38, for components 10 and 2 (tazobactam and cefalexin) that could potentially be improved in the optimization step. The XSelect HSS C<sub>18</sub> SB, while able to retain and separate all compounds, produces poor peak shape for components 9, 6, and 10. These peak shapes are not seen in blank injections and are likely caused by interactions with the stationary phase. Taking both the separations performed with acetonitrile and the ones performed with methanol into account a final set of test conditions can be selected. For these compounds, the use of an XSelect CSH C<sub>18</sub> Column at low pH with an acetonitrile mobile phase produces the best separation. The same column with methanol could also be used but would require optimization to better separate the critical pairs. Since the use of acetonitrile and the XSelect CSH C<sub>18</sub> Column achieves all set criteria, optimization is not need for this separation. Figure 6 shows the individual SIRS for the ten compounds using the final method conditions.

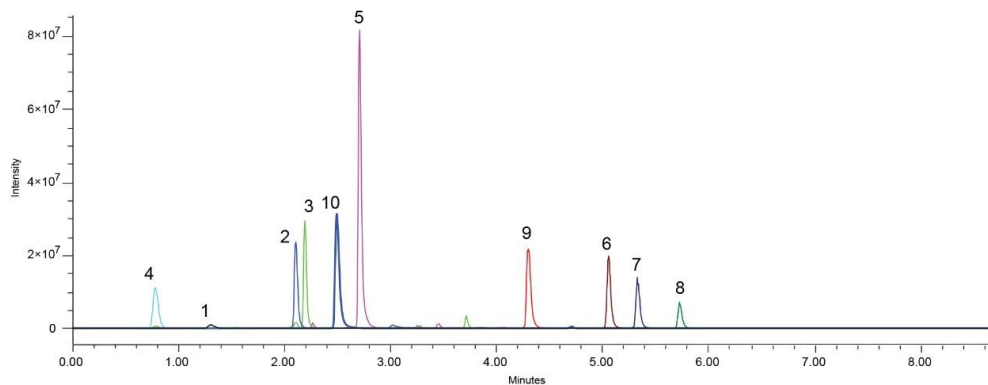


Figure 6. Overlay of ten SIR channels for ten antibiotic compounds separated on an XSelect CSH C<sub>18</sub> Column 3.0 x 50 mm, 2.5 μm. Formic acid modified water and acetonitrile mobile phase at 0.85 mL/min. Gradient conditions from 5–95% acetonitrile in 8.22 min. 1) clavulanic acid, 2) cefalexin, 3) ampicillin, 4) amoxicillin, 5) azithromycin, 6) oxacillin, 7) cloxacillin, 8) dicloxacillin, 9) piperacillin, and 10) tazobactam.

The Tiered Screening approach to method development balances speed with comprehensive testing of the sample. Here, the rapid scouting took place on the first day, allowing for proper column equilibration when switching between the high and low pH mobile phases. The column screening experiments were then set up to run overnight and into the following day. In the end, even with a 12-minute run time, the final separation was developed in just under two days.

## Conclusion

Using a systematic screening protocol for method development employs multiple “tiers” to the process. With each “tier” a set of conditions are tested to determine if either provides a better result. Then those conditions are “locked” and used for subsequent tests. To demonstrate the effectiveness of this method development protocol a mixture of ten commonly prescribed antibiotics was used. The mixture was baseline separated with good retention and peak shape using an XSelect CSH C<sub>18</sub> Column with an acetonitrile mobile phase and the low pH mobile phase additive formic acid. Use of both UV and MS detection allowed for all compounds to be tracked during method development. The developed method could now be validated for further use at the discretion of

the analyst or transferred to another lab. By having a dedicated method development protocol in place, like the systematic screening protocol shown here, new methods can be developed quickly and effectively even by a novice or intermediate analyst.

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720007352, August 2021



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