A Complete Solution to Perform a Systematic Screening Protocol for LC Method Development

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INTRODUCTION

In the analytical lab, a number of strategies can be used for reversed-phase liquid-chromatographic (RPLC) method development. Many labs perform method development by evaluating one factor at a time. While this approach often consists of a results-guided, step-by-step sequence, it can be time-consuming. It may be straightforward to implement, yet it often fails to provide a robust method because of the limited experimental space explored. Other labs approach method development using a statistics-based approach that aligns with quality-by-design (QbD) principles.[1,2] QbD is a more comprehensive approach in that experiments are statistically designed to explore multiple variables using the methodology known as design of experiments (DOE). QbD approaches can require specialized software platforms to evaluate data. These approaches can, in turn, require further investment and additional analyst training. A third option employed by many labs is systematic method development, which consists of a predefined experimental setup and an ordered protocol.[3-5] Systematic method development is a controlled process in which the screening data can be collected and analyzed within a single run.

The approaches to systematic method development have been well-documented and described,[6] and many of these approaches rely considerably on the efficacy of instrumentation and the chromatography data systems (CDS). Method development instrument manufacturers have invested in improving the ease-of-use, robustness, and versatility of LC instrumentation and detection. For example, current instrumentation enables the analyst to set up for unattended screening of multiple variables (columns and mobile phases). UltraPerformance LC™ (UPLC™), can be combined with low-dispersion systems and detectors, resulting in greater resolving capabilities. With advances in detection, including the ACQUITY™ QDa™ Detector, this system can be used with multi-detection for peak tracking and detection of co-elutions. In addition, the acquisition software can provide additional information through the use of customizable features such as reporting, processing, and interpretation of data.

This white paper describes a synergistic, systematic protocol that takes advantage of UPLC instrumentation, sub-2-µm column chemistries, and Empower™ 3 Software for a streamlined method development approach. The instrumentation used for this systematic approach consists of the ACQUITY UPLC H-Class PLUS System with a quaternary pump fitted with a solvent-select valve, column manager, low-dispersion detectors (including the ACQUITY UPLC PDA Detector), and the ACQUITY QDa Mass Detector. Combined with sub-2-µm columns and Empower 3 Software, this system provides the analyst the means to perform systematic method development for a range of compounds.

OVERVIEW

For the analytical chemist, chromatographic method development represents a considerable investment of time. Yet a workflow that considers the chemical and physical factors affecting chromatographic resolution increases the ability to develop a reliable separation. Incorporating these considerations into a clear, guided strategy minimizes any delays caused by repeat or circular testing, and it provides the analyst an organized approach for developing streamlined methods.

The workflow for systematic method development follows a series of steps, each with a clear objective. Figure 1 illustrates the steps for a systematic protocol. Also shown are the conditions, the columns, mobile phase, and other variables. Each step is designed to provide the analyst with the knowledge he or she needs to ensure that key parameters affecting selectivity and resolution of a separation are evaluated. The end goal is to develop a robust method by adopting a systematic approach.

To achieve these goals, we must consider the principles of chromatographic separations. As the fundamental resolution equation shows, efficiency, selectivity, and retention affect the resolution of two peaks, and they do so to varying degrees.

$$Rs = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k}{k+1}$$

Efficiency | Selectivity | Retention factor

Thus, to affect resolution, we must increase efficiency while taking advantage of chemical factors (retention and selectivity). The former requires optimized instrumentation and stationary phases for highest efficiency and reduced dispersion, whereas the latter can be explored more efficiently using a systematic protocol that prioritizes those parameters.

Step 1: Define Criteria

A method development exercise should include a set of predefined criteria and objectives. The objectives can be numerous and varied, including sensitivity and linearity, throughput and turnaround time, sample preparation, cost, transferability, required operator skill, etc. Additional goals can include assay-suitability criteria (USP resolution and USP tailing factors) or other factors such as the total number of peaks. Using a CDS capable of evaluating these objectives and criteria, as well as automatically generating the corresponding numerical values, reduces the time required to process and evaluate the data.

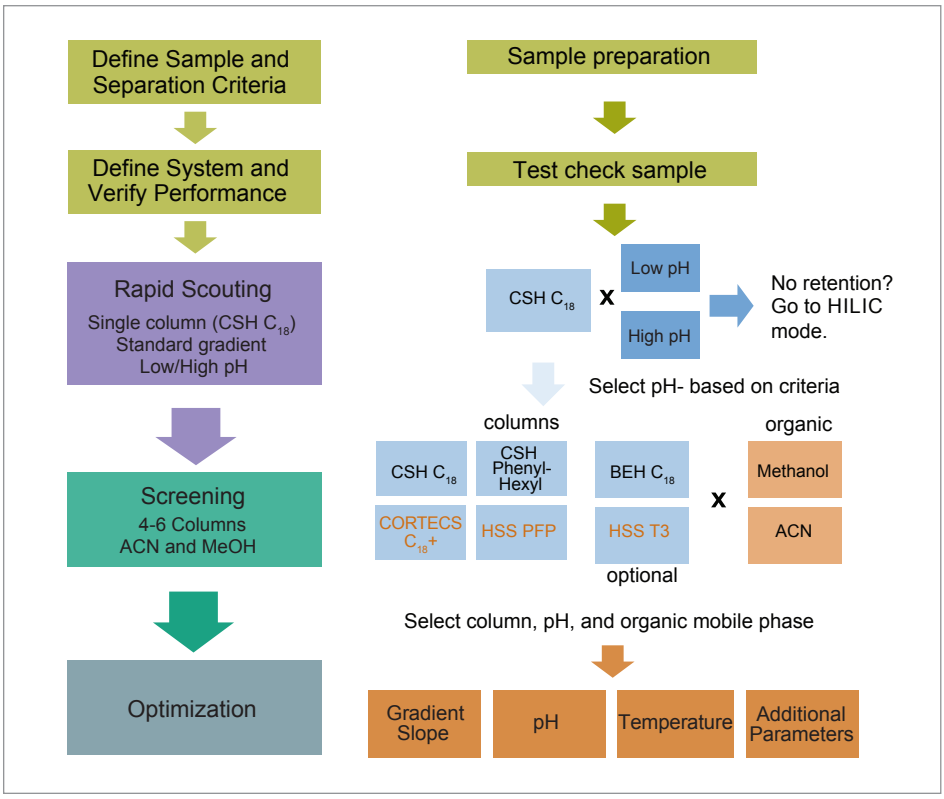


Figure 1. Steps and conditions for a systematic method development workflow.

Step 2: Define System and Verify Performance

SYSTEM SET-UP

Performing method development can be laborious. Often, the choice of instrument and CDS can impact the development process or subsequent transfer of the method. Instrumentation that is incompatible with the analyst's workflow can make method development even more cumbersome. The lack of appropriate maintenance can lead to instrument downtime, decreasing the laboratory's productivity. For these reasons, UPLC Systems, which include a quaternary solvent manager and offer the option to manage multiple columns, allow the greatest selectivity space to be explored. UPLC Systems also provide the efficiency benefits of low-dispersion flow paths, detectors capable of rapid sampling of the column effluent, and the productivity benefits of Automated System Qualification Testing protocols and quality control reference material for system performance verification. The choice of injector can also affect method development. With flow-through-needle injectors, such as the Sample Manager FTN-H, the entire gradient passes through the needle and sample loop. Thus, all of the sample transfers to the column, reducing the potential for carryover phenomena. The flow-through-needle design allows for injection-volume flexibility without the need to reconfigure the injector. Upper injection volume on the ACQUITY UPLC H-Class PLUS System is listed in the specification sheet as 1.0 mL with optional extension loop and syringes. This enables the method developer to quickly change the amount of sample on column when needed to explore sensitivity or changes in column configuration.

In addition to the solvent and sample delivery system, careful consideration must be given to the detection technique. A single detection technique may fail to provide enough information to ensure the detection of all sample components. Nor may it identify non-homogeneous or co-eluting peaks, provide information that helps identify a novel or unknown component, or track peaks. The routine use of orthogonal detectors, such as UV and mass-based detectors, can provide greater assurance that all of a sample's components are detected and resolved. Incorporation of a mass detector, such as the ACQUITY QDa Detector, facilitates this process and reduces the need to verify analyte retention times using individual standards. The availability of other detection techniques (evaporative light scattering, fluorescence) provides additional means for detecting a wide range of compounds.

The CDS is also an important consideration. A CDS such as Empower 3 Software provides multiple tools that are important for method development including instrument control, injection sequencing, and function coordination. In addition, Empower 3 Software utilizes a relational database to catalog, store, retrieve, and protect data in a compliant environment. Custom or user-created calculations and input fields offer additional functionality, as do view filters, for organizing data, and batch processing and reporting, for streamlined data analysis. Empower 3 Software combines these attributes to provide simple measurements of method-suitability criteria to evaluate each separation. Furthermore, by providing a single window for reviewing mass and UV information simultaneously, it simplifies data evaluation.

Combining the features and capabilities of versatile, newer instruments, columns, and software provides the analyst the means to utilize an optimally defined system to facilitate higher-quality, more efficient method development (Table 1). The method development process is streamlined using software and hardware that facilitate automation.

LC conditions	
System:	ACQUITY UPLC H-Class PLUS with PDA with Column Manager
Seal wash and purge lines:	90:10 water:acetonitrile
Wash line:	50:50 water:acetonitrile
Column temperature:	40 °C
Flow rate	0.6 mL/min
Injection volume:	1–5 µL
Sample diluent:	Methanol
Wavelength range:	210–500 nm
Resolution:	1.2 nm
Sampling rate:	10 pts/s
Filter constant:	Normal
Columns:	ACQUITY UPLC CSH C_{18}, 1.7 µm, 2.1 x 50 mm CORTECS UPLC C_{18}+, 1.6 µm, 2.1 x 50 mm ACQUITY UPLC CSH Phenyl-Hexyl, 1.7 µm, 2.1 x50 mm ACQUITY UPLC HSS PFP, 1.7 µm, 2.1 x 50 mm
Solvents:	Line A: 125 mM formic acid in water Line B: 125 mM ammonium hydroxide in water Line C: MilliQ water Line D1: Acetonitrile Line D2: Methanol
Gradient:	Low pH: 10% A, 85% C, 5% D to 10% A, 0% C, 90% D in 5 minutes High pH: 10% B, 85% C, 5% D to 10% B, 0% C, 90% D in 5 minutes
Chromatography Data Software:	Empower 3 FR2
MS conditions	
MS system:	ACQUITY QDa Detector
Ionization mode:	ESI+, ESI-
Acquisition range:	100 –1250 *m/z*
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temperature:	600 °C
Data collection:	Centroid
Sampling rate:	5 points/sec

Table 1. System and conditions for systematic method development.

SYSTEM CHECK

The investment in time and resources for method development can be significant. Testing should be performed on a system that was evaluated to ensure it is operating to specifications. The evaluation can be accomplished by running a known set of components or a quality-control reference material (QC Reference Material), preferably that comprises a set of analytes that elute across the gradient and that can verify the performance of the column and system. Thus when changes in retention time or peak-shape occur, the chromatography can be reviewed for evidence of physical or chemical abnormalities in either the system or column.

As illustrated in Figure 2, an ACQUITY QDa QC Reference Material standard (p/n 186007345) was tested on an ACQUITY UPLC H-Class PLUS System that includes an ACQUITY QDa Detector, to evaluate the performance of the system and column. As compared with earlier analyses, the resultant UV and extracted-ion chromatogram (XIC), met the expected retention times and peak shapes. Yet a later check, or benchmark, of the system produced broadened peaks, lower sensitivity, and a drop in system pressure (Figure 2, bottom chromatogram). Troubleshooting revealed an improper or leaking column connection. Check samples that serve as reference standards provide a tool to benchmark a system's performance before developing methods.

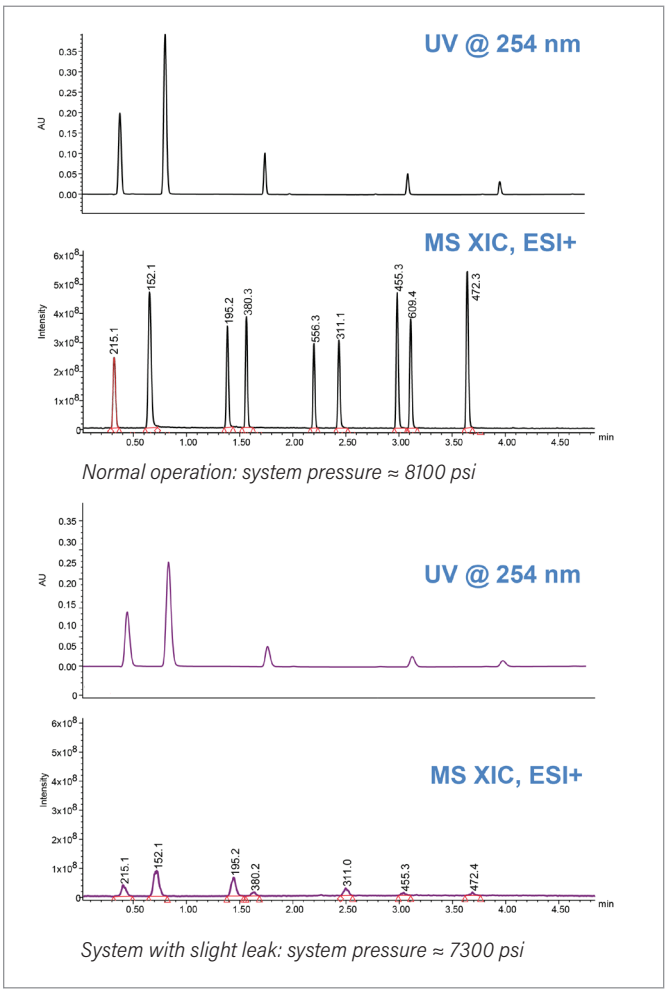


Figure 2. Verification of system performance using a check standard ensures a properly working instrument and provides the analytical chemist confidence in the results. In this example, poor or loss of sensitivity and increased peak widths of the check standard were caused by a leak in the system.

Step 3: Rapid Scouting

An efficient method development protocol reduces duplication. It is structured to obtain informative and meaningful data at each step of the process. For the method development protocol we have outlined an initial rapid-scouting step, at high and low pH. This provides key information that can streamline the method development process. The use of mobile phases of high pH to dramatically affect selectivity in reverse phase LC (RP-LC) has been documented.[8] Operating at high pH is made possible by column packings that are stable at high pH.[7] With this added tool, a single sample can be analyzed at high and low pH using the same column.

IMPACT OF MOBILE PHASE pH

In RPLC, retention is driven by the interaction between the analyte, stationary phase, and mobile phase. The separation is determined by partitioning of the sample between the hydrophobic (nonpolar) stationary phase and the hydrophilic (more polar) mobile phase. When the ionization state of the compound changes, the retention time is affected by differences in the partitioning of the compound between the nonpolar stationary phase and the polar mobile phase. For example, at low pH (<3) the weak acid is fully un-ionized, resulting in greater interaction with the nonpolar stationary phase and greater retention (Figure 3). At high pH (>8), the same effect occurs for bases, resulting in greater retention. In the middle pH range, many acids and bases undergo fractional charging, with less pronounced changes in retention. Thus, analysis at high and low pH can determine if the sample contains acids and/or bases that would preclude retention at the low or high pH.

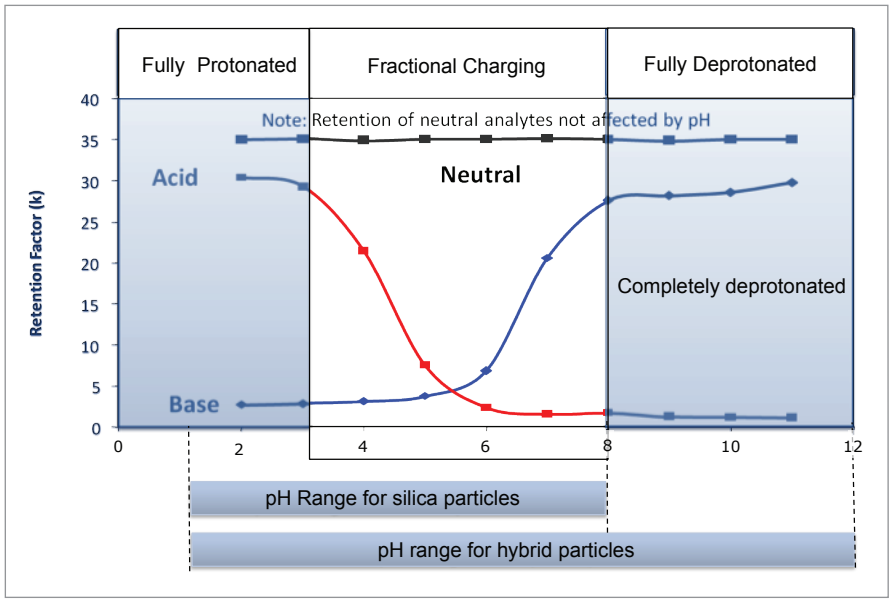


Figure 3. Reversed-phase retention map illustrating the effect of pH. Ionizable analytes are most retained in their un-ionized state. Neutrals do not have an ionizable functionality and are, therefore, unaffected by pH.

This set of experiments also allows the classification of samples into five categories (strong acids, weak acids, strong bases, weak bases, and neutrals), which will define subsequent experiments. If no retention is observed at either high or low pH, the analytes are, most likely, strong acids or bases, making retention by reversed-phase chromatography more challenging and likely requiring additional considerations. While the use of ion-pairing reagents in the mobile phase or sample diluent can be considered, hydrophilic-interaction chromatography (HILIC) may also provide greater retention and the desired separation for the polar analytes.[9,10] If both low and high pH give adequate retention with different selectivity, then both should be subsequently evaluated. If both give adequate retention with no change in selectivity, the sample may be devoid of weak acids and bases and additional development might omit pH control.

COLUMN SELECTION FOR pH RAPID SCOUTING

To screen at both low and high pH, the stationary phase must be compatible with the mobile-phase conditions. Silica-based stationary phases are not chemically stable at high pH (>8), owing to dissolution of the silica particles.[11] Hybrid particle technologies are more inert to these effects and can be used with high-pH mobile phases.[7] The lower surface-silanol concentrations of these particles can result in differences, compared with silica, in selectivity and peak shape. To further improve peak shape of basic compounds, a positive surface charge can be applied to the surface of the BEH base particle.[12] The positive charge applied to the particle (CSH™) provides improved peak shape for basic analytes under low-ionic-strength acidic conditions (0.1% formic acid), a suitable choice for rapid scouting at both low and high pH.

INSTRUMENTATION TO FACILITATE RAPID SCOUTING

In addition to the column, the instrument configuration can be selected to facilitate the rapid-scouting step. The quaternary pump of the ACQUITY UPLC H-Class PLUS System allows the unattended screening of both low-and high-pH mobile phases. Referred to as Auto•Blend, this ternary or quaternary mixing approach enables blending of stock solutions to produce the desired mobile phase. For example, pH can be altered simply by varying the proportion or amount of acid or base in the mobile phase. For the rapid-scouting step, both low-and high-pH mobile phases can be screened with solvents from four bottles: a stock acid (125 mM formic acid), a stock base (125 mM ammonium hydroxide), water, and acetonitrile (Figure 4), facilitating rapid scouting.

RAPID SCOUTING FOR AN API AND RELATED COMPOUNDS

Combining these various components with the protocol, rapid scouting was conducted for the active pharmaceutical ingredient (API) ondansetron and its related compounds (Figure 5). Analysis at low and high pH reveals differences in retention and selectivity. Separation of all of the analytes is achieved at low pH, while at high pH, the bases (1, 2, and 3) have shifted retention significantly but moved as a block. While the elution order of the bases is different at high pH, the spacing among the bases is approximately the same, indicating that these analytes respond differently to the large pH shift. For these analytes, therefore, exploring the pH range in smaller increments might lead to differences in selectivities.

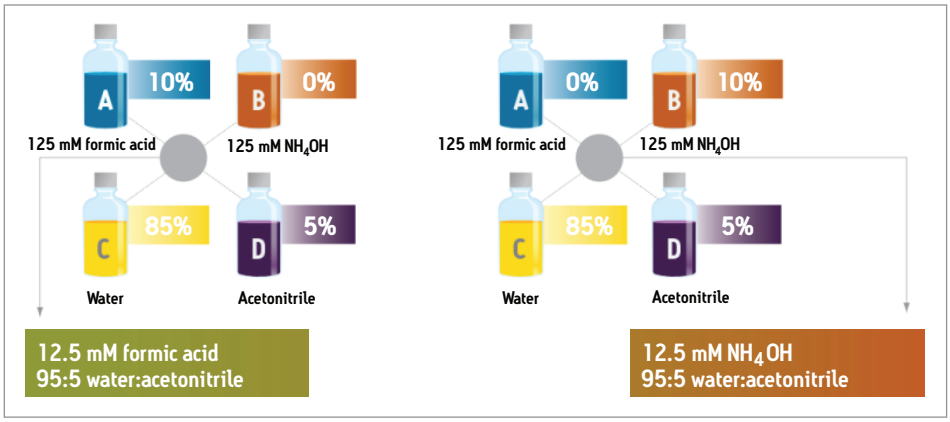
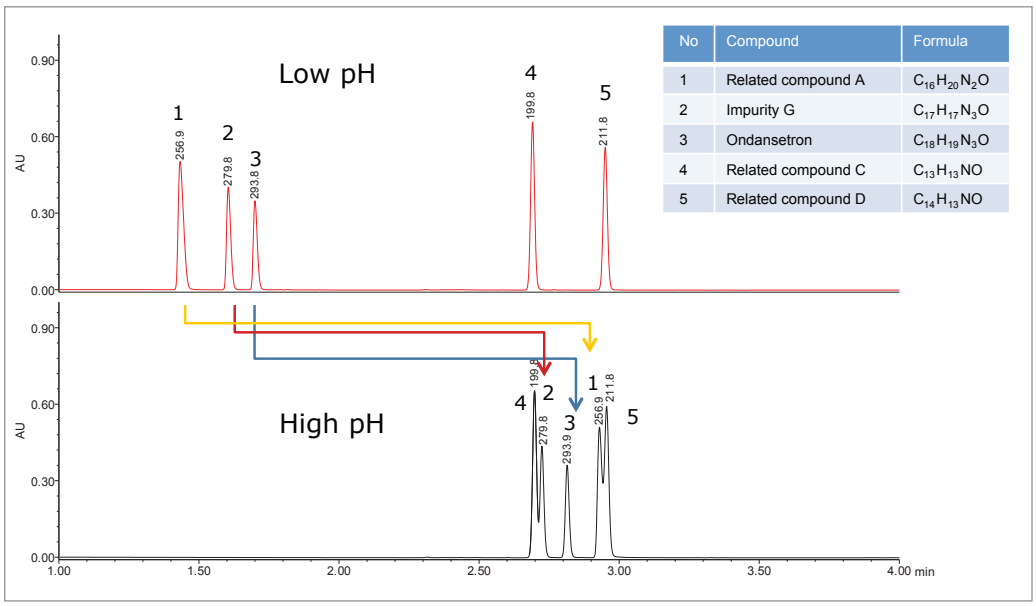


Figure 4. Quaternary mixing using Auto•Blend™ enables automated formulation of mobile phase by combining stock buffers and pure solvents in varying ratios.



No	Compound	Formula
1	Related compound A	$C_{16}H_{20}N_2O$
2	Impurity G	$C_{17}H_{17}N_3O$
3	Ondansetron	$C_{18}H_{19}N_3O$
4	Related compound C	$C_{13}H_{13}NO$
5	Related compound D	$C_{14}H_{13}NO$

Figure 5. Rapid scouting of ondanestron and related impurities allows for determination of appropriate conditions for further testing. Analysis at low-mobile-phase pH provides greater resolution. Streamlined software allows for scoring of runs based on suitable criteria.

USING A CDS TO REVIEW SCOUTING RESULTS

To help the analyst evaluate the data, the CDS can provide numerous tools. In the above-mentioned example, peak tracking can be performed more easily using the mass label of each peak, which represents the most intense ion at the peak apex (Figure 5). In addition, the analysis of multiple streams of data is simplified by a single window that includes both chromatographic and spectral views (Figure 6). The chromatographic display (lower portion of window) includes the UV chromatogram, the total-ion chromatogram (TIC), and the extracted-ion chromatogram (XIC) for the separation. The XIC, which is automatically generated, is composed of the extracted base peak ion from the TIC for each of the integrated UV peaks. The spectral data for each peak are displayed at the upper portion of the screen and show both the UV and mass-spectral information. This display provides a combined view of mass and UV chromatograms and spectral information, providing the analyst ways to track peaks and determine co-elutions.

The CDS can also provide the means to assist the analyst in determining the presence of co-eluting peaks. As shown in the rapid scouting of orange extract at high pH (Figure 7), the asymmetrical peak shape of peak 3 indicates two partially resolved analytes. The analyst can investigate further by evaluating the spectral information at the leading, apex, and trailing segments of the peak (inset). Specifically, the UV and mass spectra of different segments of a single peak help the analyst determine homogeneity of the peak and the presence of co-elutions. Evaluation of the spectra of peak 3 show different ratios of the most prominent ions (*m/z* 403.1 and 343.0) at the peak's apex, leading, and trailing segments. This view – the purity view – suggests the presence of two analytes instead of a single component, an indication that would prompt further investigation. The comprehensive view enables the analyst to perform peak tracking and obtain information on peak purity.

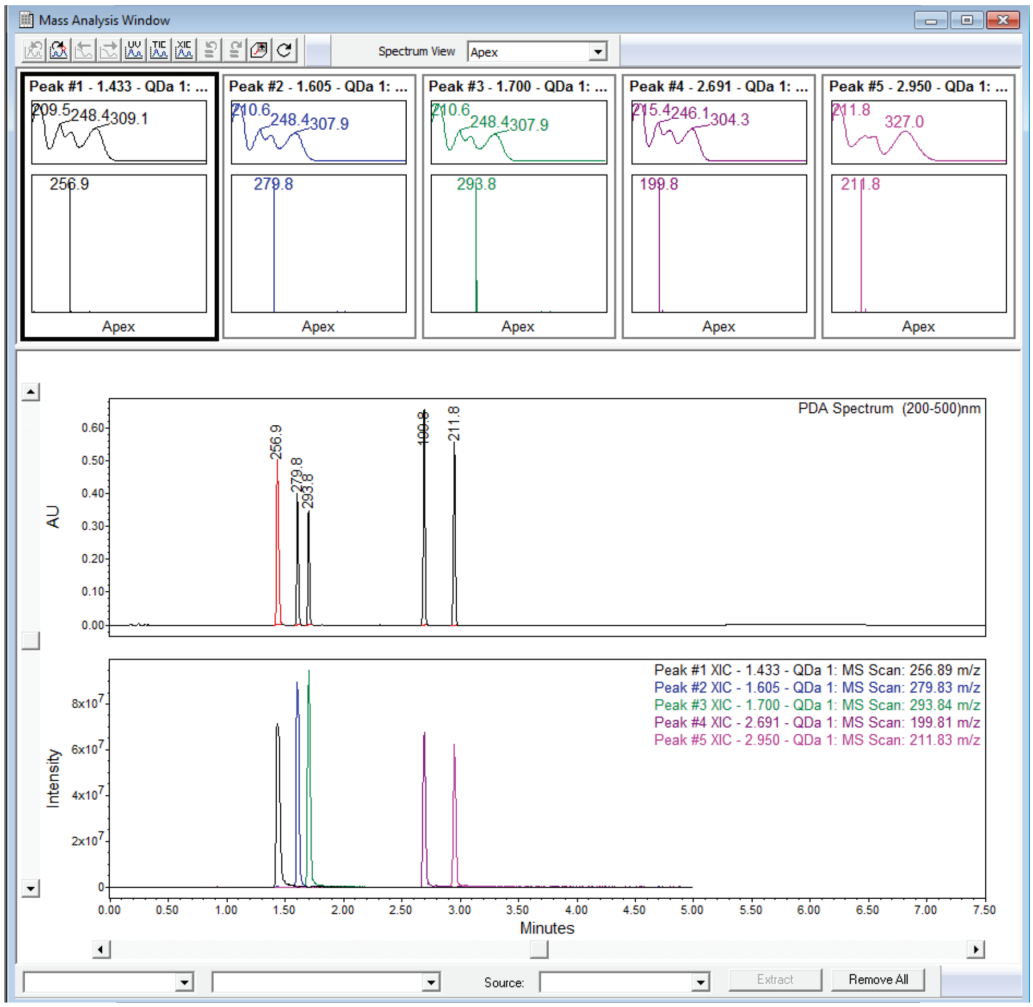


Figure 6. CDS displays combined view of mass and UV chromatograms and spectra, providing analysts information for peak tracking and determining co-elutions.

Step 4: Screening

After evaluation of the scouting results, additional method development may be necessary for greater resolution, for alternative selectivity, to correct poor peak shape, or to address any other critical attribute in need of rectifying. Therefore, a more thorough screening can be performed with columns and strong solvent that can provide alternative selectivity. As described in the resolution equation, the factors of selectivity, efficiency, and retention all influence resolution. The choice of column, too, can affect those parameters. For example, separations with increased efficiencies can be achieved with sub-2-µm particles. Nevertheless, one of the most powerful means of improving resolution in a separation is to alter selectivity, achievable by using stationary phases with various ligands and base particles, which cover a wide range of selectivity. For example, selectivity differences can be observed using Ethylene Bridged Hybrid (BEH Technology™) particles or High Strength Silica (HSS) particles, the latter having higher mechanical stability than typical high-pore-volume silica columns.[13]

Apart from selectivity differences caused by the base particle, the stationary phase also can affect selectivity. Extensive studies have documented differences in selectivity through π-π interactions with phenyl columns or through the use of embedded polar phases, among others.[8] The stationary phases selected must also be compatible with the selected mobile phase, pH, and temperature. Given these considerations, the columns used for the screening step should include those that provide a wide range of selectivity both at low and high pH (Figure 1).

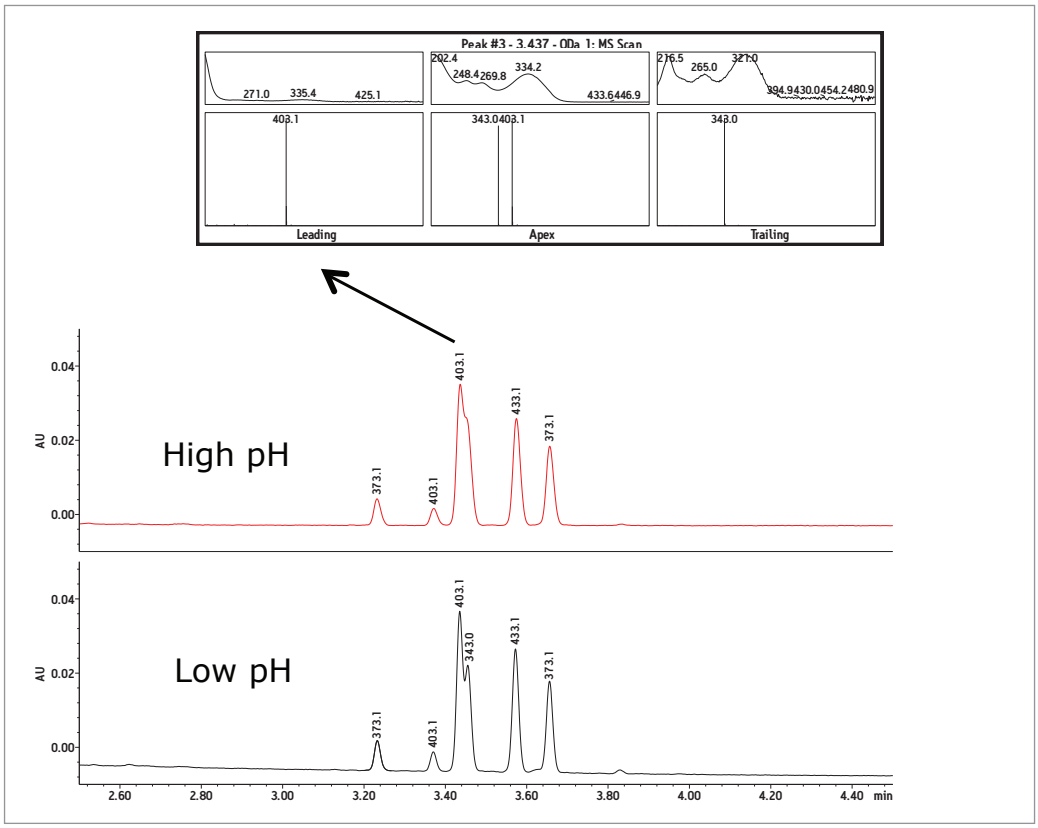


Figure 7. Rapid scouting of orange extract. Peak purity view allows the analyst to gather information about peak purity and possible co-elutions. In this example, two partially resolved analytes are observed in the rapid scouting of orange extract at high pH. The peak purity view in the mass analysis window reveals varying ion ratios of 403.1 and 343.0 at the leading, apex, and trailing segments of the peak, indicating the presence of two unresolved analytes

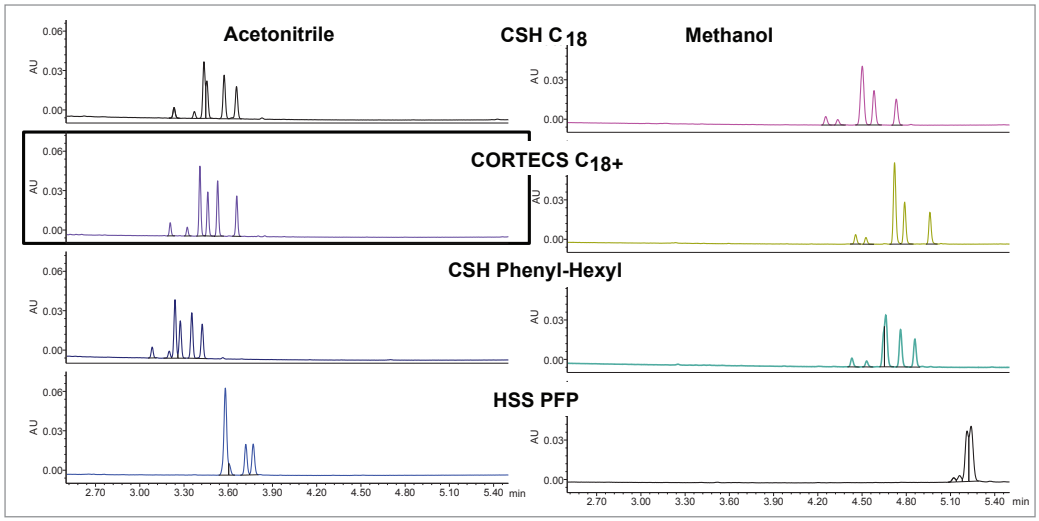


Figure 8. Following the initial scouting of an orange extract sample (Figure 7), Step 2 or Screening was performed. This step was carried out using four columns and two strong solvents at low pH. The highlighted chromatogram represents the conditions chosen for optimization (see Figure 9).

INSTRUMENTATION TO FACILITATE SCREENING

To screen these multiple columns in an unattended fashion, the analyst must consider column management. A multi-column compartment, as available on the ACQUITY UPLC H-Class PLUS System, is capable of independently switching columns using integrated valves. In addition, each column can be independently temperature-controlled, giving users the flexibility to select a variety of column chemistries regardless of their operational specifications. Column compartments capable of holding multiple columns provide flexibility and eliminate the need for manually changing columns between analyses.

In the screening step, adjustments to selectivity can also be made by altering the strong solvent. A quaternary pump fitted with a solvent-select valve allows the unattended screening of both low-and high-pH and strong solvent combinations. By combining multiple columns and both protic and aprotic strong solvents (methanol and acetonitrile, respectively), a wide selectivity space can be evaluated.

SCREENING OF COLUMNS AND STRONG SOLVENTS

To illustrate this screening approach, flavonoids present in orange extract were evaluated. It is important to note that, because the screening step is an extension of the rapid-scouting step, to eliminate duplication, the CSH C_{18} Column requires evaluation only with methanol, as a strong solvent. The scouting step at low and high pH for this set of compounds (Figure 7) produced minimal changes in selectivity and retention, suggesting the class of compounds is neutral or, possibly, slightly acidic. Given the improved resolution of the critical pair, screening was performed under low-pH conditions.

The various columns and strong solvents produced very different retention and selectivity (Figure 8). Greater retention on all columns was observed with methanol, as compared with acetonitrile. This is expected since methanol, which can undergo polar-polar interactions with solutes, is a weaker solvent in reversed-phase systems. The separations on both C_{18} stationary phases were similar. Nevertheless, the superficially-porous particle (CORTECS C_{18}+ Column) provided slightly greater efficiencies and separation of the critical pair. The phenyl-hexyl stationary phase provides interaction through both phenyl pi pi interactions and the alkyl chain. Therefore, for this set of analytes the CSH phenyl-hexyl column produced lower retention with selectivity similar to that of the BEH C_{18} stationary phase. Lastly, the pentafluorophenyl stationary phase (HSS PFP) column, which undergoes pi-pi interactions with Lewis bases, is often used for basic compounds that are difficult to retain. In this example, the PFP interacted with the neutral and acidic solutes for greater retention; however, selectivity differences resulted in less overall resolution.

SCORING REPORT

Empower 3 SOFTWARE

Sample Set ID: 1954, 8451, 9465, 8828, 8387 Run Time: 7.5 Minutes
Result Set ID: 6388 Injection Volume: 5.00 uL
Processed Channel Descr.: PDA 315.0 nm (200-500)nm, PDA 260.0 nm (200-500)nm

	Injection Id	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Lowest Rs	Min k*	RT of Last Peak
1	8526	CORTECS C_{18}+	ACN	Low pH	6	5	6	2.27	9.69	3.66
2	8407	CSH C_{18}	ACN	Low pH	6	4	4	0.45	9.78	3.66
3	8610	CSH PH	MeOH	Low pH	6	3	4	2.23	13.77	4.86
4	8440	CSH C_{18}	ACN	High pH	6	2	4	1.50	9.77	3.66
5	8673	CORTECS C_{18}+	MeOH	Low pH	5	4	5	2.19	13.85	4.96
6	8741	CSH C_{18}	MeOH	Low pH	5	3	5	1.94	13.18	4.73
7	2084	CSH PH	ACN	Low pH	5	1		0.82	9.74	3.41
8	9486	HSS PFP	MeOH	Low pH	4	0		0.31	16.03	5.23
9	8871	HSS PFP	ACN	Low pH	4	0	2	1.51	10.93	3.77

Figure 9. Automated CDS reporting allows the analyst to determine appropriate column and strong solvent, for continued method development, in an unbiased fashion.

USING A CDS TO REVIEW AND REPORT SCREENING RESULTS

To evaluate the data, a CDS such as Empower 3 Software can enable automated measurement of method suitability criteria. The linkages in the relational database provide the ability to rank and display any combination of fields according to system-suitability criteria, as determined by the analyst's needs.[14,15]

Some examples of the types of custom fields that can be created include peak-result and sample-level fields. Furthermore, with a CDS platform that contains customizable processing and reporting, the analytical chemist can evaluate the data in an automated fashion (Figure 9).[14] This flexibility allows the results or report for an analysis to be automatically generated and tailored to place priority on the attributes most critical to the analyst. In this report separation-criteria values are automatically calculated from values in the predefined custom fields. Ranking is sequential and is based on the order of fields selected in the table view. Multiple parameters are evaluated only if there are ties in those evaluated earlier. The analyst can then review the results to ensure the appropriate selection criteria are chosen.

To illustrate these features, the screening results of orange extract were processed and ranked by pre-established criteria (Figure 9) including the greatest total number of peaks, total number of peaks with USP Resolution greater than 2.0, and total number of peaks with USP tailing less than 1.5. For this particular example, multiple separations produced the same number of peaks – that is, six. However, the CORTECS C_{18}+ Column, with acetonitrile as the strong solvent, produced the greatest number of peaks, five in all, in which USP resolution was greater than 2.0. According to the criteria we selected, these conditions sufficed for the next step, optimization.

STEP 5: OPTIMIZATION

When necessary, optimization of the chromatographic separation typically requires additional analyses beyond pH, column, and solvent screening. In such cases, however, the experimental design varies from analysis to analysis, depending on the method requirements. Goals of method development might include completeness of the separation, certainty of peak identification, sensitivity, and quantitative properties. Additional requirements might include separation criteria such as resolution, peak purity, analysis time, or tailing factors. To achieve these goals, numerous means are available for optimization. They include column temperature, column dimensions, flow rate, pH, gradient slope, and gradient time.

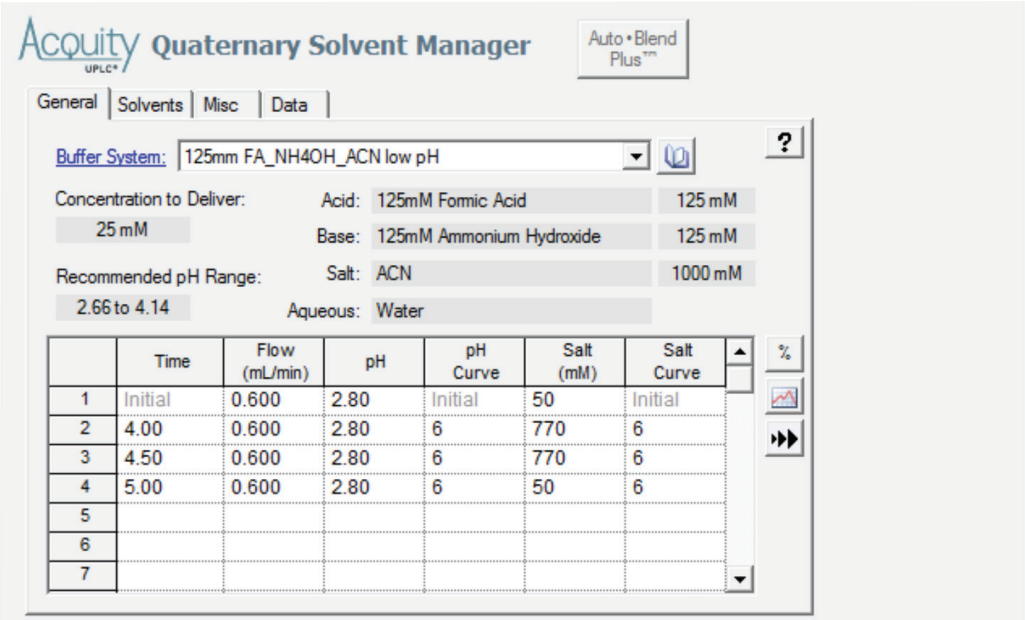


Figure 10. Automation of instrument control software allows for pH of mobile phase to be programmed directly into the gradient table using Auto•Blend Plus. The proportion of acid and base for a specific pH are based on empirical table or pKa of acid/base combination. The salt column is used for the organic or strong solvent and reflects the percent delivered multiplied by a factor of 10 (50 mM = 5%).

USING AUTO•BLEND PLUS FOR OPTIMIZATION OF MOBILE PHASE pH

Manipulating many of these variables is fairly straightforward, but evaluating the effect of slight changes in mobile-phase pH can be laborious. Preparing multiple mobile phases at different pH levels can be tedious and time-consuming. Nevertheless, slight changes in mobile-phase pH can significantly affect selectivity, retention, and sensitivity, particularly for fractionally charged analytes (Figure 3). Thus, to develop a robust method, mobile-phase pH should be evaluated.

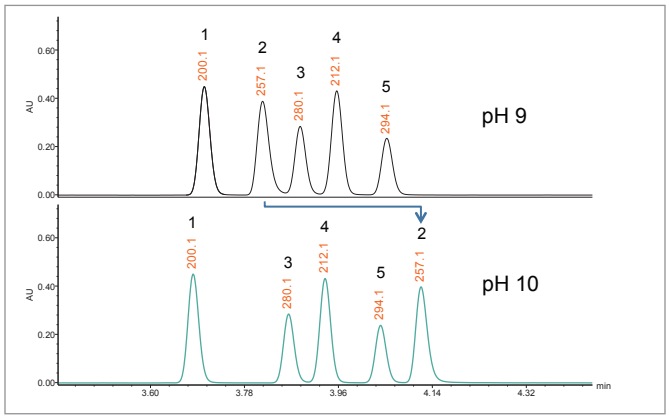


Figure 11. Effect of mobile phase pH on separation of ondansetron (peak 5) and related impurities. Changes in mobile phase pH produce changes in retentivity and selectivity for this class of compounds. In this example a change of 1 pH unit resulted in peak 2, shifting retention 0.4 minutes to latest eluting peak.

Using the Auto•Blend Plus™ feature of the ACQUITY UPLC H-Class PLUS System, analysts can use specifically designed software to program methods to control pH (Figure 10). This approach to controlling pH provides flexibility. It allows the analyst to use any buffer to meet the needs of the sample and method and to create appropriate mixtures of buffers for an extended pH range. According to the programmed value, the ratio of acid and base are determined, either from an empirical table or the pKa of the acid/base combination. This determination enables the analyst to evaluate small changes in pH in an unattended fashion, a useful tool for method development, particularly in the case of ionizable compounds.

In Figure 11, Auto•Blend Plus is used to evaluate the separation of ondansetron and related impurities. As discussed above (see "rapid scouting"), the bases shifted retention and changed selectivity with a large, pH shift. Exploring the pH range in smaller increments was postulated to lead to a wide range of differences in selectivities. In fact, using Auto•Blend Plus, a change of one pH unit (9 to 10) was found to dramatically effect selectivity. Peak 2 (*m/z* 257.1) switched selectivity from the second eluting peak to the last eluting peak. This example illustrates the effect that a change of one pH unit can have on the retention and electivity. In addition, the online generation of mobile phase from pure solvents and concentrated stocks means fewer measurements in preparation.

EVALUATING OPTIMZATION RESULTS FOR FINAL SEPARATION

In addition to pH, a wide range of variables (temperature, gradient, flow rate, etc.) may be explored. The analyst can evaluate the effect of each of these variables with the aid of tools in the CDS. As described earlier, after the data has been processed, the automated report method may be customized, placing greater weight on key attributes. Figure 12 shows the report for optimizing the separation of flavonoids in an orange extract. In this example, our goals included complete separation of the flavonoids, USP resolution greater than 2.0 for all peaks, and a retention time of less than 3.0 minutes for all analytes.

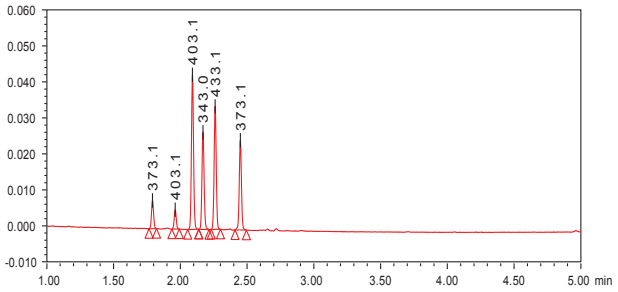
The sample was evaluated under varying column temperatures and gradient slopes (not shown). The final adjustments were made to the run time (Figure 12 report). The final method conditions were specified as 35 °C, a gradient slope of 2.3% B change per column volume, and a run time of 5 minutes.

To provide assurance that all the flavonoids were fully resolved, each peak within the separation was evaluated by both UV and mass spectra. Comparison of the UV and mass spectra (Figure 13a) at the leading, apex, and tailing portions of the peak indicate its homogeneity. Each portion of the peak contained the same ions as well as the same UV spectra. Peak purity was also confirmed by the Empower 3 Software's PDA Peak Purity algorithm (Figure 13b). As shown for peak 3 in the final method, the PDA peak-purity analysis shows the purity angle (green line) is lower than the purity threshold (blue line), indicating homogeneity of the peak. Therefore, method development for this sample is complete.

SCORING REPORT

Sample Set ID: 9082, 9123 — Run Time: 8.0, 9.0 Minutes
Result Set ID: — Injection Volume:5.00 uL
Processed Channel Descr.: PDA 315.0 nm (200-500)nm

	Injection Id	Column	Run Time	Gradient slope (% per min)	Total Peaks	Total Peaks Rs>=1.5	Lowest Rs	Min k'	RT of Last Peak
1	9221	CORTECS C_{18}+	5 min	2.3	6	5	2.66	4.97	2.45
2	9107	CORTECS C_{18}+	7 min	2.3	6	5	2.57	11.98	4.50



Peaks Table
Injection Id: 9221

	Injection Id	Column	Strong Solvent	pH	Retention Time (min)	Area	Height	USP Res	USP Res (HH)	USP Tailing	Base Peak (m/z)
1	9221	CORTECS C_{18}+	ACN	Low pH	1.792	8638	7855			1.043	373.09
2	9221	CORTECS C_{18}+	ACN	Low pH	1.962	5725	5348	5.82	5.95	1.042	403.11
3	9221	CORTECS C_{18}+	ACN	Low pH	2.091	47398	42972	4.45	4.54	1.055	403.11
4	9221	CORTECS C_{18}+	ACN	Low pH	2.170	30711	27031	2.66	2.71	1.092	343.03
5	9221	CORTECS C_{18}+	ACN	Low pH	2.261	37749	34163	3.08	3.14	1.020	433.12
6	9221	CORTECS C_{18}+	ACN	Low pH	2.450	28897	24977	6.34	6.46	1.019	373.09

Figure 12. Optimization report for separation of flavonoids in orange extract. Flexible reporting allows the analyst to select variables and criteria for desired separation conditions.

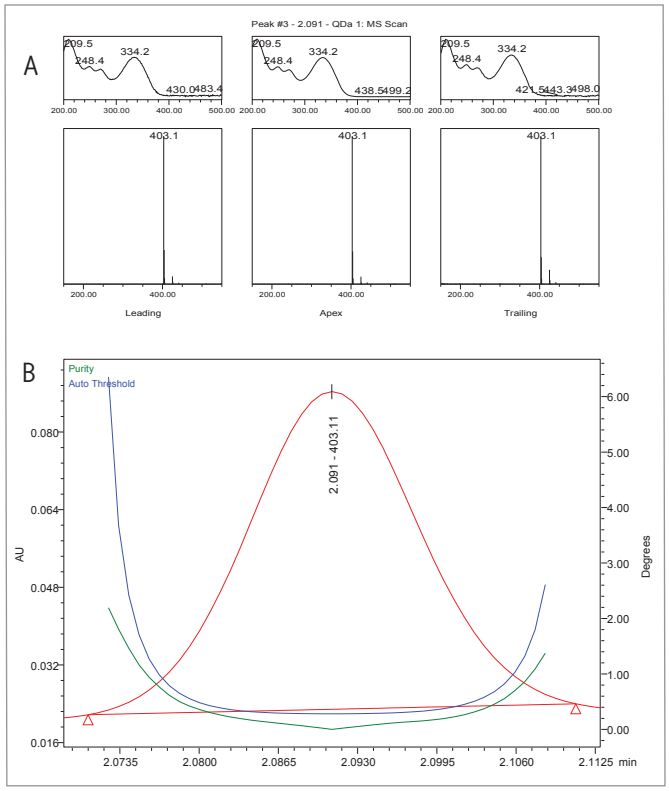


Figure 13. Peak purity view in Empower 3 software. Optimized separation of orange extract, peak 2. (a) Combined mass and UV spectral-data view of peak 2 in mass analysis window. Comparison of leading, apex, and trailing portions of peak indicate peak homogeneity. (b) PDA peak purity view of max plot indicates homogeneity of peak by UV.

CONCLUSIONS

Method development often requires a significant amount of resources both in labor and instrumentation. A thoughtfully chosen, synergistic system of columns, instrumentation, software, and mobile phases enables a streamlined, automated process for method development and the following tools for the method developer:

- A low-dispersion system, for improved throughput and higher resolution and sensitivity
- An automated system for screening multiple columns, mobile phases, and temperatures
- A quaternary mixing system to enable blending of stock buffers as well as the ability to program directly in units of pH
- A flow-through needle auto sampler to reduce carryover and ensure the entire sample reaches the column
- Column stationary phases that enable performing both high- and low-pH screening on a single column
- A wide range of available detectors to ensure complete sample characterization and detection of co-elutions
- An acquisition and processing-software platform that provides automated peak detection, data analysis, and reporting, all of which provide an analyst the means to readily evaluate data quality
- Customizable CDS calculations and reports, to aid in evaluating sample and separation criteria

The complete system solution equips the analytical chemist with the tools to develop a more robust, reliable, and reproducible separation. In doing so, it instills greater confidence that a method will provide consistent, accurate results and an increased opportunity for success in method validation.[16]

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