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Application News

Supercritical Fluid Chromatography System Nexera™ UC
Software for Efficient Method Development LabSolutions™ MD

Efficient Optimization of Separation Conditions for Synthetic Peptide Using Super Critical Fluid Chromatography

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User Benefits

- Supercritical fluid chromatography (SFC) can be applied to hydrophilic peptide analysis.
- Efficient workflow of optimizing separation conditions using SFC can be provided by LabSolutions MD.
- The mobile phase blending function enables automated preparation of mobile phases, contributing to the improvement of efficiency and reproducibility.

■ Introduction

Supercritical fluid chromatography (SFC) has advantages of shorter analysis time than liquid chromatography (HPLC) due to the large diffusion coefficient and low viscosity of carbon dioxide used as the mobile phase, and it is superior in separating structurally similar substances. SFC has been developed as an analytical method for chiral compounds, but in recent years its application has expanded to separation and analysis of achiral compounds. SFC is excellent for the separation and analysis of hydrophobic compounds based on the hydrophobic nature of carbon dioxide, but by adding a highly polar organic solvent (modifier) such as methanol to the mobile phase, SFC can also be used for the analysis of hydrophilic compounds.

Peptides, a typical hydrophilic compound, are generally analyzed using reversed-phase HPLC, but SFC is also available; since SFC shows different retention behavior from LC, it can be expected to provide satisfactory separation that cannot be done using LC.

In this article, Automated workflows in screening" and "optimization" phases using a standard peptides mixture as a simulated sample, using the supercritical fluid chromatograph Nexera UC and the analytical method development support software LabSolutions MD.

■ How to select SFC column

Column scouting is important for optimizing separation conditions because the same stationary phase often shows different retention behavior in SFC and HPLC, and it is difficult to estimate SFC retention from existing HPLC data.

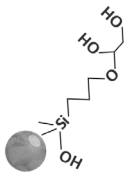
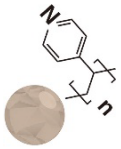
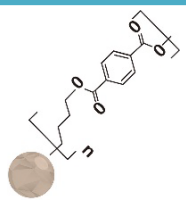
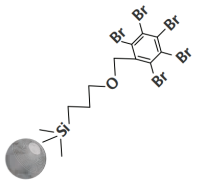
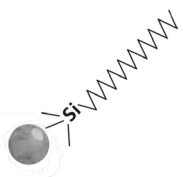
Therefore, to employ a different type of stationary phase could be effective to change separation selectivity. The "six-column set", a package of SFC columns (Shim-pack™ UC series) of different separation selectivities, is the best choice for the first step of optimizing separation conditions. Column types and features are shown in Table 1.

■ Column screening

In the screening phase, the optimal combination of column and modifier (including acidic or basic additives) could be a significant impact on retention and separation. In this article, the optimal combination of column and modifier was determined through the modifier screening including acid-base additives, following the selection of an appropriate column for the separation in the column screening phase.

In the column screening, analyses were conducted using the "six-column set" (Table 1) of different retention selectivities and methanol, a highly polar organic solvent suitable for the analysis of hydrophilic compounds, as modifier. The analytical conditions are shown in Table 2.

Table 1 Contents and features of six-column set

	Shim-pack UC-Diol Ⅱ	Shim-pack UC-Sil Ⅱ	Shim-pack UC-PolyVP
Chemistry			
Feature	• Normal phase separation • Suppressed non-specific interaction	• Excellent retention for basic compounds and recognition of steric structure	• Good peak shape without acidic or basic additive in mobile phase
	Shim-pack UC-PolyBT	**Shim-pack UC-PBr**	**Shim-pack UC-ODS**
Chemistry			
Feature	• Excellent recognition for aromatic compounds owing to π-π interaction	• Improved separation for shortly retained compounds in C18 column	• Reversed phase separation • Hydrophobic retention

LabSolutions MD can automatically generate an analytical batch under a variety of conditions combining various parameters such as columns and modifiers correctly. In addition, the modifiers ((1) in Fig. 1) and the columns ((2) in Fig. 1) can be automatically switched using flow change-over valves. Furthermore, the composition of the modifier can be automatically prepared using the mobile phase blending function.

In the analytical batch creation, the mobile phase that contains the selected composition of modifier is automatically prepared by just selecting the modifier if the blending ratio is set in advance. This is effective for preventing preparation errors as well as great reduction of manual preparation work.

Nexera UC supports up to seven different modifiers and their automatic blending by the mobile phase blending function. In addition, up to twelve columns can be automatically switched for the consecutive analyses, providing comprehensive searching for analytical conditions under a variety of situations.

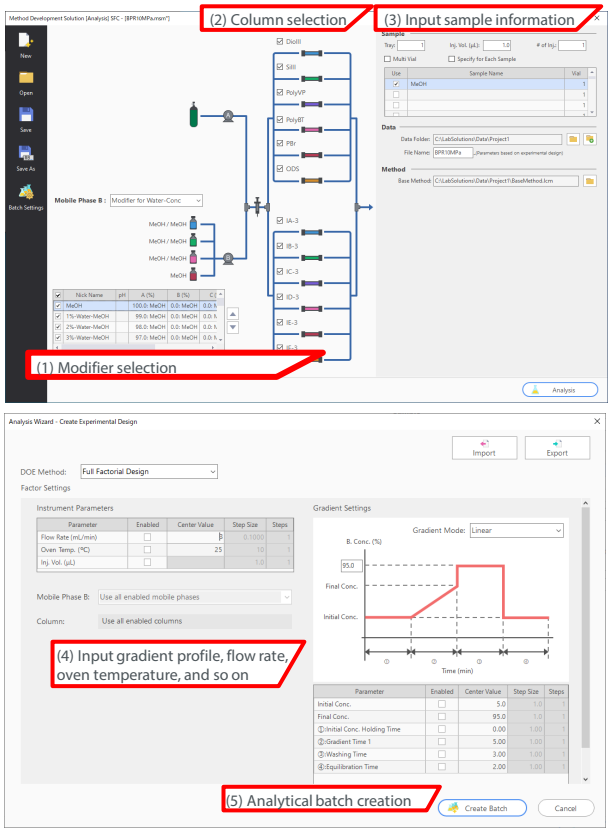


Fig.1 Analytical batch creation screen

Table 2 Column screening conditions

System	:	Nexera UC
Column 1	:	Shim-pack UC-SIL II *1
Column 2	:	Shim-pack UC-Diol II*2
Column 3	:	Shim-pack UC-PolyVP*3
Column 4	:	Shim-pack UC-PolyBT*4
Column 5	:	Shim-pack UC-PBr*5
Column 6	:	Shim-pack UC-ODS*6
		(250 mm × 4.6 mm I.D., 5 µm for all columns)
Mobile phases A	:	CO_2
Mobile phases B	:	Methanol
Flow rate	:	2.5 mL/min
Time program	:	B.Conc. 5%(0 min) → 75% (8-10 min) → 5% (10-12 min)
Column temp.	:	25 ℃
BPR pressure	:	10 MPa
Column temp.	:	50 ℃
Injection volume	:	2 µL (1000mg/L for all compounds)
Compounds	:	(A) lopinavir, (B) ritonavir, (C) angiotensin I, (D) angiotensin II, (E) insulin, (F) daptomycin, (G) pneumocandin B0
Sample solvent	:	Dimethyl sulfoxide (DMSO)
Detection	:	220 nm (SPD-M40, high-pressure flow cell)

*1 P/N：227-32607-02, *2 P/N：227-32606-02
*3 P/N：227-32509-12, *4 P/N：227-32503-12
*5 P/N：227-32602-02, *6 P/N：227-32608-05

■ Quick searching for optimal conditions from screening results

The screening results for the six columns are shown in Fig. 2. Since the screening process produces as many chromatograms as the number of conditions tried, it is essential to evaluate which conditions provide the desired separation, which requires a great deal of knowledge of chromatography as well as a great deal of labor. LabSolutions MD can quantitatively evaluate and rank the separations under respective conditions using the following equation1, allowing anyone to search for optimal conditions quickly and easily without depending on the intuition and the experience of HPLC expat.

(Evaluation value) = P × (Rs1 + Rs2 + ... + RsP-1) (Equation 1)

The evaluation value is calculated as the product of the number of detected peaks (P) and the sum of the resolutions (Rs). Fi. 3 shows the results of the evaluation values in descending order obtained from the mobile phase and the column screenings. It is also possibles to rank the columns by parameters such as minimal resolution and number of detected peaks.

The best evaluation values were obtained when Shim-pack UC-PolyVP was employed as the column shown in the red frame. However, the number of detected peaks (6) was less than that of compounds in the sample (7), and several detected peaks did not have excellent peak shapes. Therefore, we improved the separation and peak shapes by adding acids or bases into the modifier. Additional screening analyses were conducted using methanol with six additives ((1) 0.1% formic acid, (2) 20 mmol/L ammonium formate, (3) 0.1% acetic acid, (4) 20 mmol/L ammonium acetate, (5) 0.1% TFA, and (6) 20 mmol/L ammonium TFA) as the modifiers in the mobile phase.

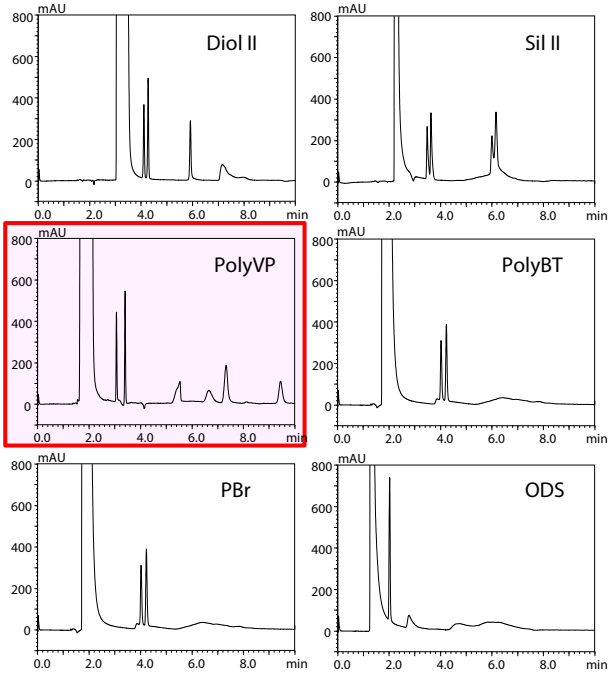


Fig. 2 Chromatograms obtained through column screening

	Response			
Column Nick Name	Minimum Resolution	Peak Count	Separated Peak Count	Evaluation Value
PolyVP	2.02	6	6	120.778
SilII	1.416	5	3	62.147
DiolII	0.835	4	0	28.97
PBr	1.647	3	2	19.942
PolyBT	2.194	2	2	4.388
ODS	0	1	0	0

Fig. 3 Ranking for respective conditions

■ Additional screening analysis results using acidic and basic additives

Additional screening analyses using modifiers with six different additives ((1) 0.1% formic acid, (2) 20 mmol/L ammonium formate, (3) 0.1% acetic acid, (4) 20 mmol/L ammonium acetate, (5) 0.1% TFA, and (6) 20 mmol/L TFA ammonium salt) showed no improvement in separation or peak shape compared to those without the acidic and basic additives (Fig. 4).

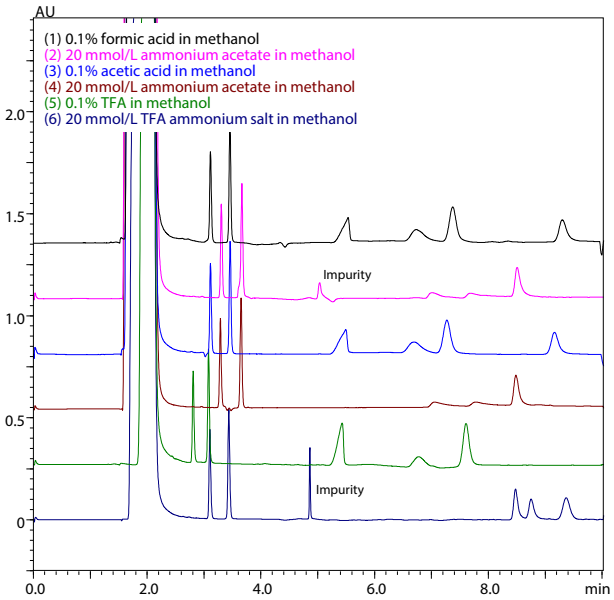


Fig.4 Chromatograms obtained through additional screening with additives

■ Effect of adding water to modifier

In SFC analysis of hydrophilic compounds, water is sometimes added as a modifier in addition to acidic or basic additives, and small difference in the ratio of water in the modifier can greatly affect separation and peak shape. Water cannot be used as a modifier as it is since carbon dioxide, the main mobile phase component of SFC, is not miscible with water. However, it can be added in trace amounts to organic solvent modifiers that are miscible with water.

We confirmed whether the addition of water improved separation and peak shape for following two modifiers. One was “0.1% acetic acid in methanol”, which obtained the highest evaluation score in the post run data analyses of additional screening analysis with acidic and basic additives using LabSolutions MD. The other was “0.1% TFA in methanol”, which often showed good separation and peak shape in peptide analysis in reversed phase HPLC. In SFC analysis, it is known that a column temperature affects separation and peak shape as in HPLC analysis, so the column oven temperature oven was also evaluated as well.

In the optimization phase, the analysis was conducted by varying the ratio of water added to 0.1% acetate acid in methanol and 0.1% TFA in methanol at 0, 1, 2, 3, 4, and 5% (six levels) and the column oven temperature at 25, 45, and 65 ℃ (three levels), respectively.

Chromatograms obtained by varying the water addition ratio at a column oven temperature of 25 ° C are shown in Fig. 5 to Fig.8. Focusing on the resolution of angiotensin I and angiotensin II, it was found that the resolution was increased with the increase in the amount of added water in both cases of 0.1% acetic acid in methanol and 0.1% TFA in methanol were used as modifiers.

Since Nexera UC can automatically prepare mobile phase using the mobile phase blending function, the analyses can be performed with excellent repeatability even under conditions in which small difference in the addition ratio of water may affect the separation.

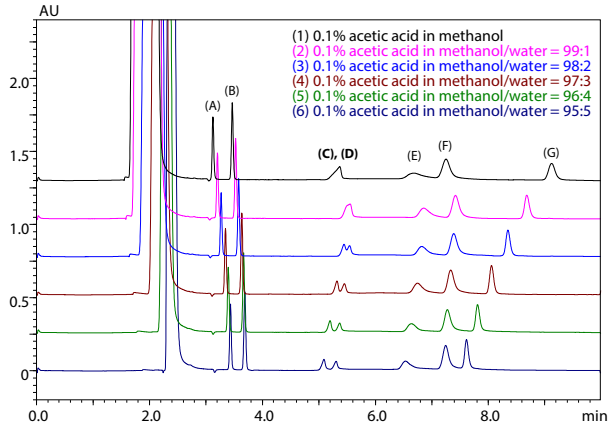


Fig. 5 Chromatograms obtained in different water addition ratios
Modifier: 0.1% Acetic acid in methanol

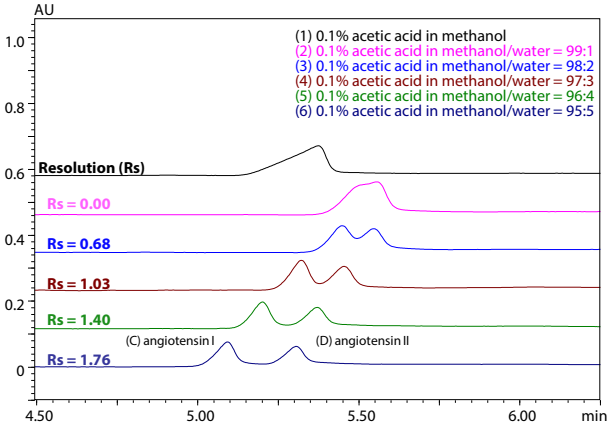


Fig. 6 Enlarged chromatograms of Fig. 5 focusing on separations of (C) angiotensin I and (D) angiotensin II with respective resolutions

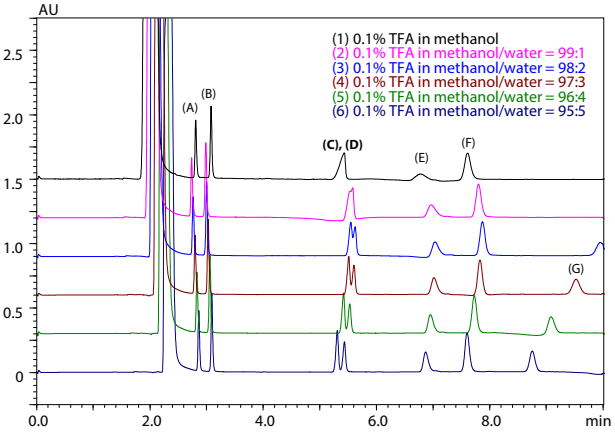


Fig. 7 Chromatograms obtained in different water addition ratios
Modifier: 0.1% TFA in methanol

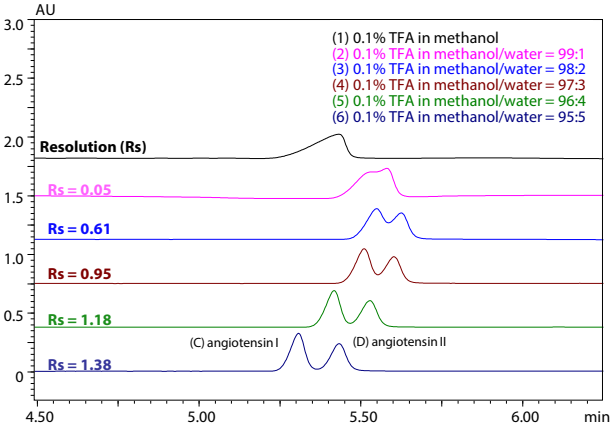


Fig. 8 Enlarged chromatograms of Fig. 7 focusing on separations of (C) angiotensin I and (D) angiotensin II with respective resolutions

■ Visual evaluation of resolution and peak shape by design space

LabSolutions MD can visualize the effect of the variations of analytical parameters on separation as a design space. The design space of the resolution of angiotensin I and angiotensin II when 0.1% acetic acid in methanol was used as modifier is shown in Fig. 9, in which the vertical axis represents the ratio of water to modifier and the horizontal axis represents the column oven temperature. The design space drawing shows that the higher the addition ration of water to the modifier and the higher column oven temperature provided better separation.

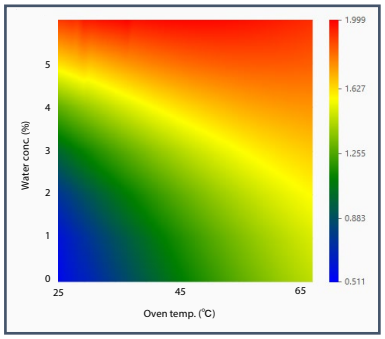


Fig. 9 Design space for the resolution of (C) angiotensin I and (D) angiotensin II

Overlaid multiple design spaces also provides efficient searching for optimal conditions that meet multiple criteria. For example, in order to search for conditions with good peak shape and separation of peaks of interest, the minimal resolution was defined as 1.2 or higher and the allowable tailing factor interval was from 0.8 to 1.8 at the 10% height of each peak, and regions that met these criteria were searched by overlaying design spaces (Fig. 10). The colored regions in this figure don't meet both criteria simultaneously, and the other regions (green line hatched) are those that meet the criteria.

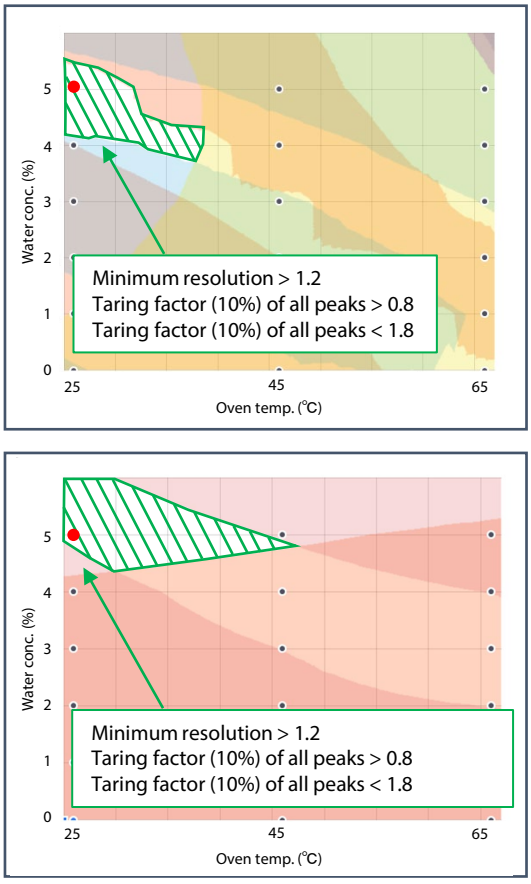


Fig. 10 Searching for optimized conditions using overlaid design space (1)
Upper: 0.1% acetic acid in methanol Lower: 0.1% TFA in methanol

The best results were obtained when the water addition ratio was 5% and the column oven temperature was 25 °C (red circle in Fig. 10) for both 0.1% acetic acid in methanol and 0.1% TFA in methanol modifiers.

In addition, the regions that met following criteria were searched for using overlaid design spaces Fig. 11.

- Minimal resolution was 1.2 or higher.
- Allowable tailing factor interval was from 0.8 to 1.2 (more severe than that in previous searching) at the 10% height of each peak.

When 0.1% acetic acid in methanol is used, there is no region that meet the criterion. On the other hand, when 0.1% TFA in methanol is used, the conditions that the water addition ratio is 5% and the column oven temperature is 25 °C (red circle in Fig. 11) meet both criteria, and a better peak shape is obtained. The chromatogram obtained under these optimal conditions is shown in Fig. 12.

As shown in the figure, LabSolutions MD enables easy and quick searching for conditions that meet desired criteria for multiple peaks by overlaying design spaces.

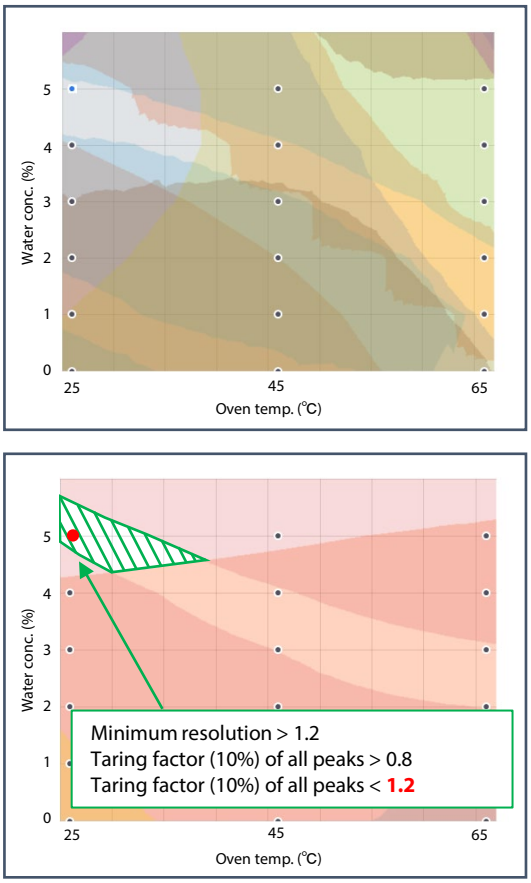


Fig. 11 Searching for optimized conditions using overlaid design space (2)
Upper: 0.1% acetic acid in methanol Lower: 0.1% TFA in methanol

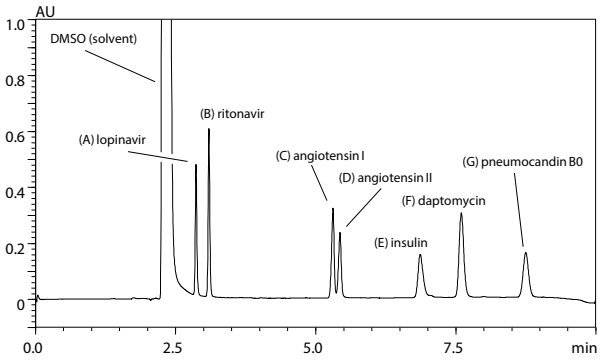


Fig. 12 Optimized chromatogram using 0.1% TFA in methanol
Water addition ratio: 5% Column oven temperature: 25 °C

■ Conclution

Supercritical fluid chromatography (SFC) can be applied to the analysis of hydrophilic compounds such as peptides using appropriate selecting of column, modifier, and additive. In the analysis of peptide mixtures, the addition of water to the modifier greatly improved separation and peak shape. On the other hand, the searching for optimal separation conditions involves comprehensive analysis of column, modifier, and additive is very time-consuming due to creation of many analytical batches huge amount of post run data processing. LabSolutions MD provides automatic analytical batch creation and mobile phase preparation and in terms of analysis, it is possible to rank the chromatographic separations and efficient searching for optimal conditions using design space. This streamlines the workflow of SFC method development.

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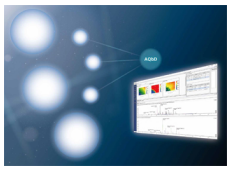

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