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Application News

LabSolutions™ MD : Software for Efficient Method Development based on Analytical Quality by Design

Efficient Method Development for Synthetic Peptide and Related Impurities

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User Benefits

- LabSolutions MD improves the efficiency of the entire workflow for method development of synthetic peptide and impurities.
- Screening for multiple mobile phases and columns can be automated using mobile phase and column switching valves.
- Molecular weights of peptides and related impurities can be estimated and accurately tracked with LCMS-2050, a single quadrupole mass spectrometer.

■ Introduction

Peptide therapeutics, characterized by specific amino acid sequences crucial to their function, can be synthesized chemically, similar to small molecule drugs. The production of synthetic peptides involves multiple steps, including deprotection, activation, coupling, and the cleavage of the final sequence from the solid support. Impurities, such as those resulting from premature chain termination or missing amino acids, can affect the safety and efficacy of the final product. Therefore, these impurities must be separated by liquid chromatography (LC). In LC analysis, selecting the appropriate mobile phase and column across a wide range of combinations is critical for achieving optimal separation, as it significantly impacts the separation. However, since the separation pattern varies depending on the peptide chain lengths, amino acid compositions, and presence of modifications, optimizing separation for each peptide sequence is time-intensive. This study describes how to efficiently find the best separation conditions for peptides and related impurities utilizing LabSolutions MD, a dedicated software for supporting method development, through both screening and optimization phases.

■ Target Sample

A target peptide and five related impurities with different sequences were used as a model sample of a synthetic peptide (Table 1). Full length peptide (FLP : beta-Melanotropin), deletion sequences of p.A1del, p.A1_E2del, p.A1_K3del, and p.A1_D5del as shorter length of products, and Met(O2) (methionine in FLP is oxidized to methionine sulfone) were prepared as a sample mixture.

Table 1 Sequences of the Target Peptide and Related Impurities

Name	Sequence
FLP	AEKKDEGPYRMEHFRWGSPPKD
p.A1del	EKKDEGPYRMEHFRWGSPPKD
p.A1_E2del	KKDEGPYRMEHFRWGSPPKD
p.A1_K3del	KDEGPYRMEHFRWGSPPKD
p.A1_D5del	EGPYRMEHFRWGSPPKD
Met(O2)	AEKKDEGPYR{Met(O2)}EHFRWGSPPKD

Note : Met(O2) = methionine sulfone

■ Mobile Phases and Columns Screening

In the screening phase (analytical conditions : Table 2), the optimal combination of mobile phase and column was investigated. For the mobile phases, four aqueous solutions (0.1% TFA, 0.1% formic acid, 10 mmol/L ammonium formate (pH 4.0), and 10 mmol/L ammonium acetate (pH 5.0)), and three organic solvents (acetonitrile, methanol, and a 1 : 1 mixture of acetonitrile/methanol) were evaluated. For the columns, six columns with different stationary phases and pore sizes were tested. A comprehensive analysis schedule was created with a total of 72 (4 x 3 x 6) patterns of these mobile phases and columns to explore the optimal combination. LabSolutions MD can quickly and easily create an analysis schedule with different parameters such as mobile phase compositions and columns (steps (1) to (5) in Fig. 1). In addition, the mobile phases ((1) in Fig. 1) and the columns ((2) in Fig. 1) can be switched automatically using flow change valves, respectively. Furthermore, mobile phase blending function automatically prepares the mobile phases with the different mixture ratios of acetonitrile and methanol by simply clicking the desired mobile phases (step (1) in Fig. 1). This significantly reduces the amount of manual work and prevents human errors during preparation.

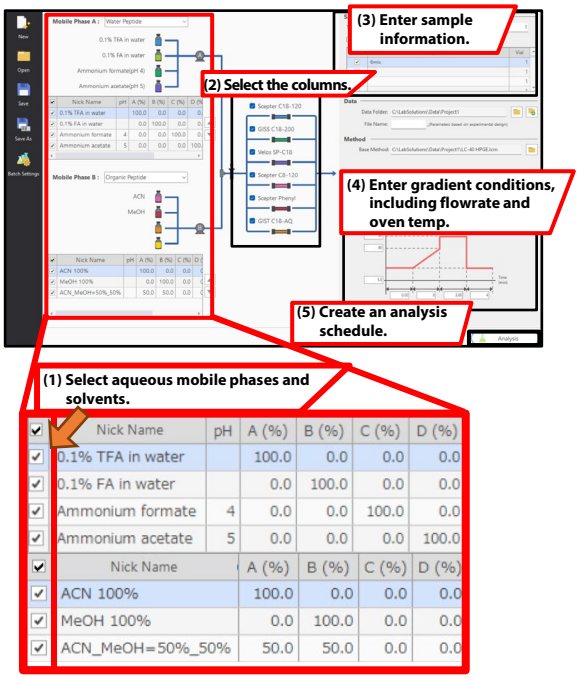

Fig. 1 Steps for Creating Analysis Schedule

Table 2 Analytical Conditions for Mobile Phases and Columns Screening

System	: Nexera™ X3 (Method Scouting System)
Column 1	: Shim-pack Scepter™ C18-120 *1
Column 2	: Shim-pack™ GISS C18 *2
Column 3	: Shim-pack Velox™ SP-C18 *3
Column 4	: Shim-pack Scepter C8-120 *4
Column 5	: Shim-pack Scepter Phenyl-120 *5
Column 6	: Shim-pack GIST-HP C18-AQ *6
	(100 mm × 3.0 mm I.D., 1.9 µm : column 1, 2, 4~6)
	(100 mm × 3.0 mm I.D., 1.8 µm : column 3)
Temperature	: 40 °C
Injection volume	: 2 µL (FLP : 1000 mg/L, other impurities : 100 mg/L)
Sample solvent	: Water
Mobile phases	
Pump A – Line A	: 0.1% TFA (Trifluoroacetic acid) in water
– Line B	: 0.1% formic acid in water
– Line C	: 10 mmol/L ammonium formate (pH 4) in water
– Line D	: 10 mmol/L ammonium acetate (pH 5) in water
Pump B – Line A	: Acetonitrile
– Line B	: Methanol
Flow rate	: 0.5 mL/min
Time program (%B)	: 10 % (0 min) →60 % (10 min)
	→10 % (10.01-15 min)
Detection	: 220 nm (SPD-M40, UHPLC cell)
System	: LCMS-2050
Ionization	: ESI/APCI (DUIS™), positive mode
Mode	: SCAN (*m/z* 300-2000)
Nebulizing gas	: 2.0 L/min (N_2)
Drying gas	: 5.0 L/min (N_2)
Heating gas	: 7.0 L/min (N_2)
DL temp.	: 200 °C
Desolvation temp.	: 450 °C
Interface Voltage	: 1.0 kV

*1 P/N：227-31013-03, *2 P/N：227-30049-02
*3 P/N：227-32002-02, *4 P/N：227-31034-03
*5 P/N：227-31064-03, *6 P/N：227-30808-02
(Shimadzu GLC product number)

■ Results of Mobile Phases and Columns Screening

Chromatograms acquired under different conditions of mobile phases (aqueous mobile phases : 0.1% TFA, 0.1% formic acid, 10 mmol/L ammonium formate, 10 mmol/L ammonium acetate / organic solvents : 100% acetonitrile, 100% methanol) on each column are shown in Fig. 2-7.

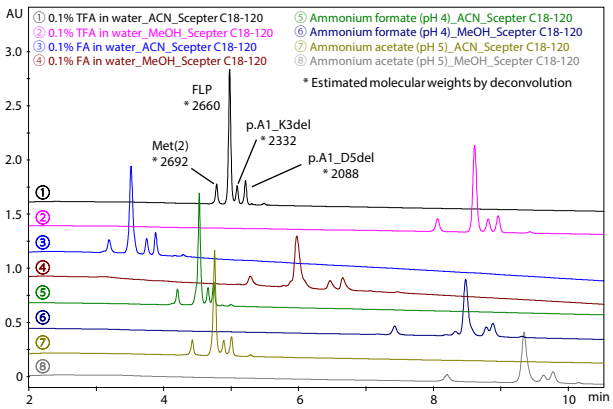

Fig. 2 Chromatograms Acquired by Scepter C18-120

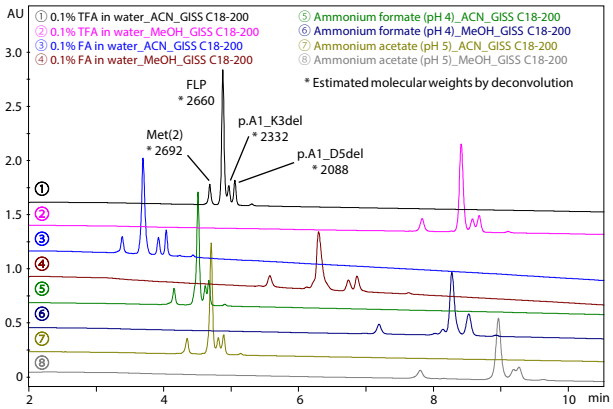

Fig. 3 Chromatograms Acquired by GISS C18-200

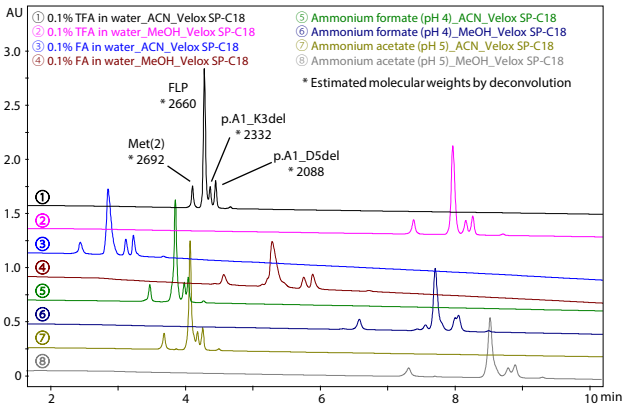

Fig. 4 Chromatograms Acquired by Velox SP-C18

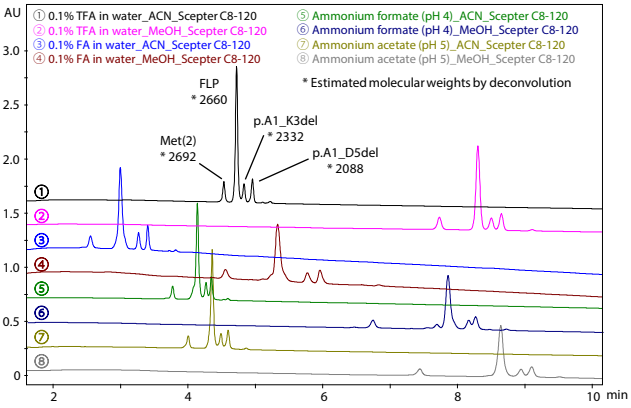

Fig. 5 Chromatograms Acquired by Scepter C8-120

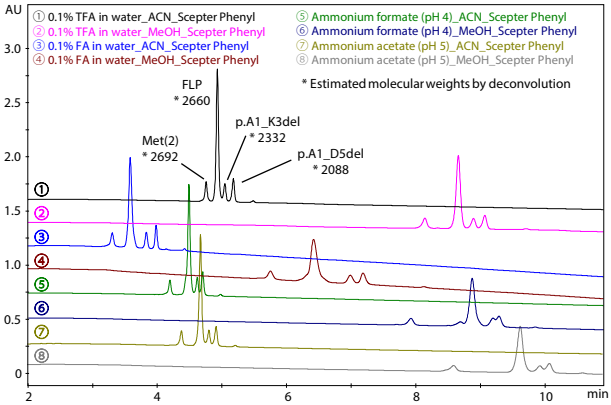

Fig. 6 Chromatograms Acquired by Scepter Phenyl

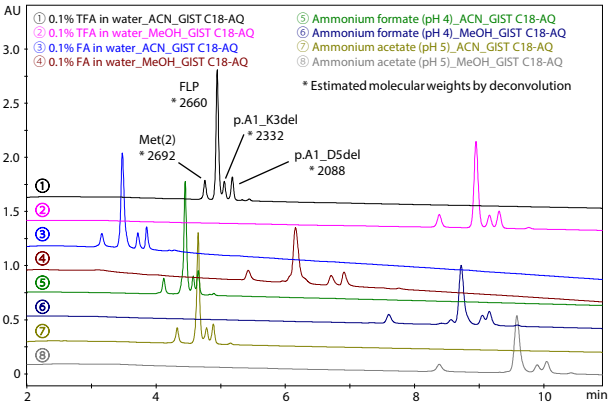

Fig. 7 Chromatograms Acquired by GIST C18-AQ

The results of mobile phases and columns screening indicated that the type of aqueous solution and the composition of organic solvent have a significant effect on separation of FLP and related impurities. Additionally, LabSolutions MD can calculate molecular weights (estimated by deconvolution of MS spectrum acquired by LCMS-2050) for each peak, facilitating the confirmation of synthesized compounds and the estimation of molecular weights for unknown impurities.

■ Quickly Find Optimal Condition

Many chromatograms are generated based on the number of conditions considered in the screening phase, and a certain level of chromatographic knowledge, along with significant effort, is required to evaluate which condition provides the desired separation. LabSolutions MD can quickly and easily find optimal condition using equation (Eq. 1) below to quantitatively evaluate the separation.

$$(\text{Evaluation Value}) = P \times (Rs_1 + Rs_2 + \ldots + Rs_{P-1}) \qquad \text{(Eq. 1)}$$

Evaluation Value is calculated as the number of peaks detected (P) multiplied by the sum of resolution factor (Rs) for all peaks. Fig. 8 shows Evaluation Value obtained through mobile phase screening and listed in the order from the highest to the lowest. The top three chromatograms with the highest evaluation values are shown in Fig. 9. The best chromatogram was obtained under the mobile phase composition of 0.1% formic acid and methanol using Scepter C8-120 column. However, in the conditions with the second and third highest evaluation values, p.A1_E2del was also detected, and a certain level of separation was achieved. Therefore, further investigation was conducted with different column oven temperatures (40, 50, 60, 70, and 80 °C) for these top three conditions.

MPA Nick Name	MPB Nick Name	Column Nick Name	Evaluation Value
0.1% FA in water	MeOH	Scepter-C8-120	26.889
Ammonium acetate (pH 5)	ACN_MeOH=50_50	Velox SP-C18	26.328
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	26.073
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter-C8-120	25.948
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter C18-120	25.792
Ammonium formate (pH 4)	ACN_MeOH=50_50	Scepter Phenyl-120	25.575
Ammonium formate (pH 4)	ACN_MeOH=50_50	GIST-C18-AQ	25.502
Ammonium formate (pH 4)	MeOH	Scepter C18-120	25.318
Ammonium formate (pH 4)	ACN	Scepter-C8-120	24.890
Ammonium formate (pH 4)	ACN	Scepter Phenyl-120	24.860

Fig. 8 Ranking of Each Condition by Evaluation Value
(Top 10 Chromatograms Listed from the Highest to the Lowest)

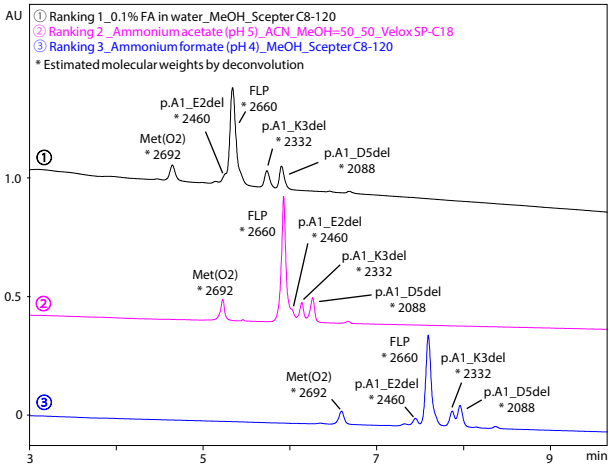

Fig. 9 Chromatograms of the Top Three Evaluation Values

■ Results of Column Oven Temperature Screening

Chromatograms obtained by varying the column oven temperatures at 40, 50, 60, 70, and 80 °C for the top three chromatograms (Fig. 9) are shown in Fig. 10-12, respectively. Higher temperatures improved separation in all conditions, indicating that column oven temperature is effective in improving the separation. In addition, it was confirmed that increasing the column oven temperature improves the resolution between p.A1del and FLP, which are the most difficult to separate. Fig. 13 shows the resolution results between p.A1del and FLP, listed in the order from the highest to the lowest. The highest resolution between p.A1del and FLP was observed with the mobile phase composition of 0.1% formic acid and methanol using Scepter C8-120 column (chromatogram ① in Fig. 10).

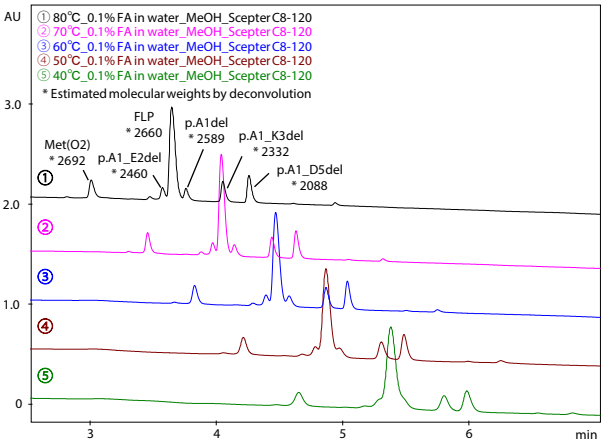

Fig. 10 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(0.1% formic acid/ MeOH / Scepter C8-120)

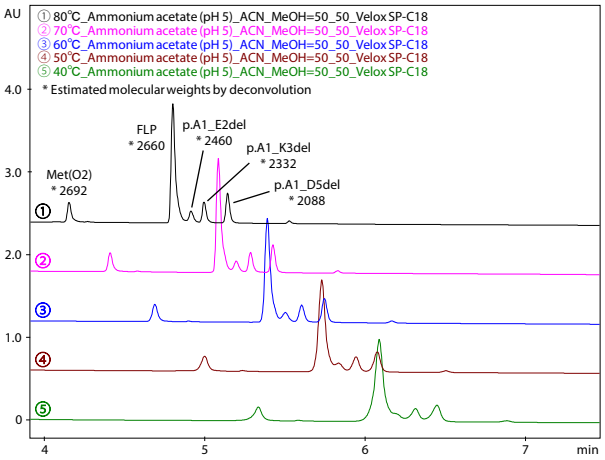

Fig. 11 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(10 mmol/L ammonium acetate / ACN : MeOH=50:50 / Velox SP-C18)

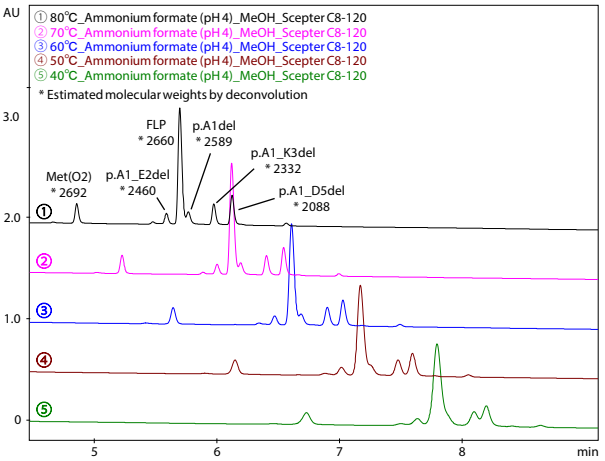

Fig. 12 Chromatogram with Different Column Oven Temperatures
80 °C(①), 70 °C(②), 60 °C(③), 50 °C(④), 40 °C(⑤)
(10 mmol/L ammonium formate / MeOH / Scepter C8-120)

MPA Nick Name	MPB Nick Name	Column Nick Name	Oven Temp	Resolution (p.A1del)
0.1% FA in water	MeOH	Scepter-C8-120	80	1.492
0.1% FA in water	MeOH	Scepter-C8-120	70	1.396
0.1% FA in water	MeOH	Scepter-C8-120	60	1.238
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	80	1.175
Ammonium formate (pH 4)	MeOH	Scepter-C8-120	70	1.172

Fig. 13 Ranking by Resolution between p.A1del and FLP
(Top 5 Chromatograms Listed from the Highest to the Lowest)

Next, the optimization phase was conducted to further improve separation and robustness by adjusting LC parameters, such as gradient conditions and flow rate.

■ Optimization Phase

Based on the optimal condition (aqueous solution : 0.1% formic acid , organic solvent : methanol, column : Scepter C8-120, column oven temperature : 80 °C), initial gradient concentration (5, 10, and 15% : Fig. 14), gradient time (5, 10, and 15 min : Fig. 14), and flow rate (0.5, 0.6, and 0.7 mL/min), were optimized to further improve the separation of FLP and related impurities. The obtained chromatograms are shown in Fig. 15-17. Higher initial gradient concentration and longer gradient time tended to improve the resolution between neighboring peaks, while the flow rate had a smaller effect on resolution. Then, peak tracking was performed for FLP and related impurities to visualize the resolution through design spaces.

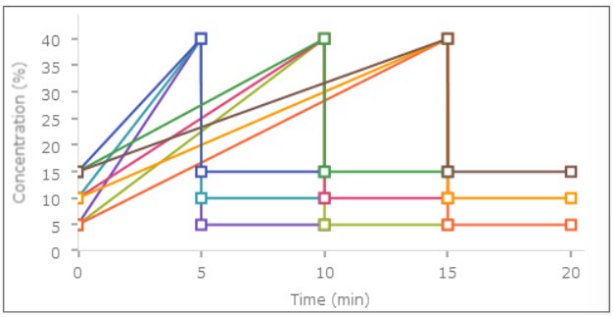

Fig. 14 Optimization of Gradient Conditions

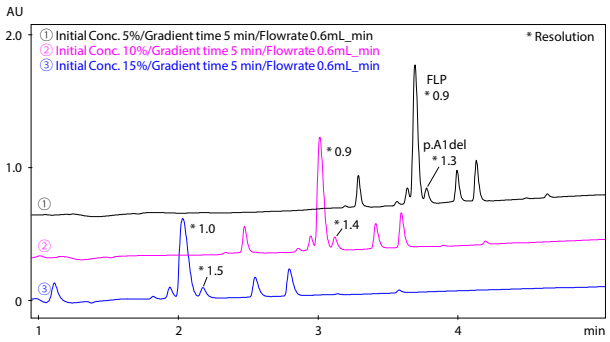

Fig. 15 Chromatograms with Different Initial Concentration
5%(①) , 10%(②) , 15%(③)

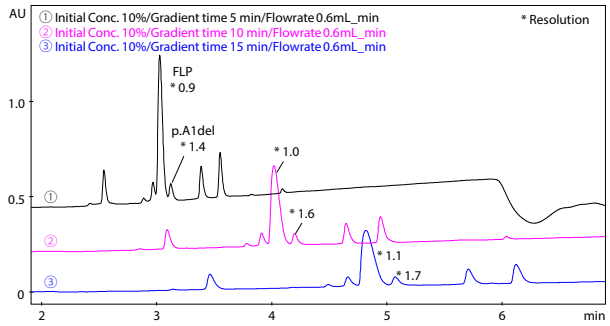

Fig. 16 Chromatograms with Different Gradient Time
5 min(①) , 10 min(②) , 15 min(③)

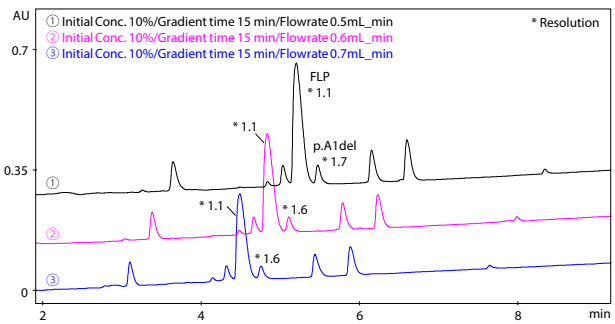

Fig. 17 Chromatograms with Different Flow Rate
0.5 mL/min(①) , 0.6 mL/min (②) , 0.7 mL/min (③)

■ Automated Peak Tracking by LCMS-2050

LC chromatograms obtained with a gradient time of 5 min, flow rate of 0.6 mL/min, and initial concentration of 5 % and 15 %, along with molecular weights for FLP and impurities, are shown in Fig. 18. The UV spectra for each impurity are displayed in Fig. 19. The similarity between UV spectra of Met(O2), p.A1del, p.A1_E2del, p.A1_K3del, and p.A1_D5del is greater than 0.99, suggesting that peak tracking based on UV spectra would be challenging. In contrast, LabSolutions MD enables peak tracking based on molecular weights acquired by LCMS-2050, facilitating accurate identification of compounds with similar UV spectra (Fig. 18). The estimated molecular weights for each compound show small mass errors compared to the theoretical values (Table 3), which can be used to confirm the molecular weights of known compounds as well as to approximate the molecular weights of unknown impurities.

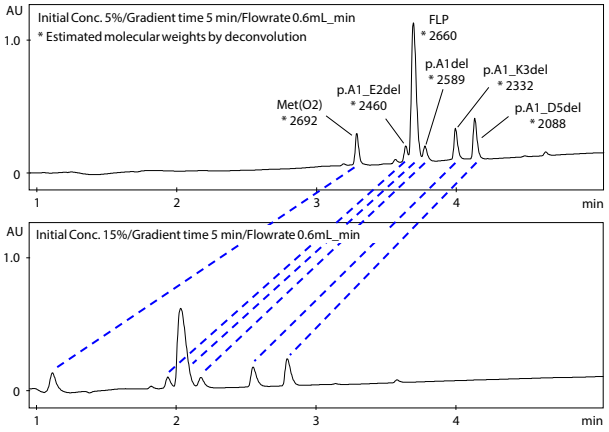

Fig. 18 LC Chromatograms at a Gradient Time of 5 min, Flow Rate of 0.6 mL/min, and Initial Concentration of 5 % (Upper) and 15 % (Lower)
(Dashed lines indicate tracking based on molecular weights.)

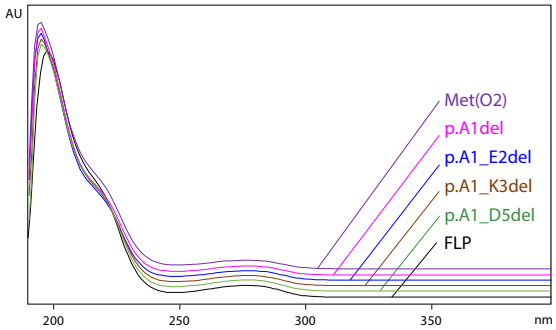

Fig. 19 UV Spectra of FLP and Impurities

Table 3 Estimated / Theoretical Molecular Weight of FLP and Impurities

Name	Estimated Molecular Weight	Theoretical Molecular Weight
FLP	2660	2660
p.A1del	2589	2589
p.A1_E2del	2460	2460
p.A1_K3del	2332	2332
p.A1_D5del	2088	2089
Met(O2)	2692	2692

Next, by visualizing resolution of FLP and each impurity with design spaces, the optimal condition that provides the best resolution and robustness was identified.

■ Design Space Evaluation for Optimal Condition

Design spaces of resolution of FLP and each impurity were shown (Fig. 20). The vertical axis represents gradient time, while the horizontal axis represents initial concentration. The red region indicates higher resolution, and the blue region indicates lower resolution. By visualizing the resolution through design spaces, it became evident that higher initial concentrations and longer gradient times improve the resolution of each peak.

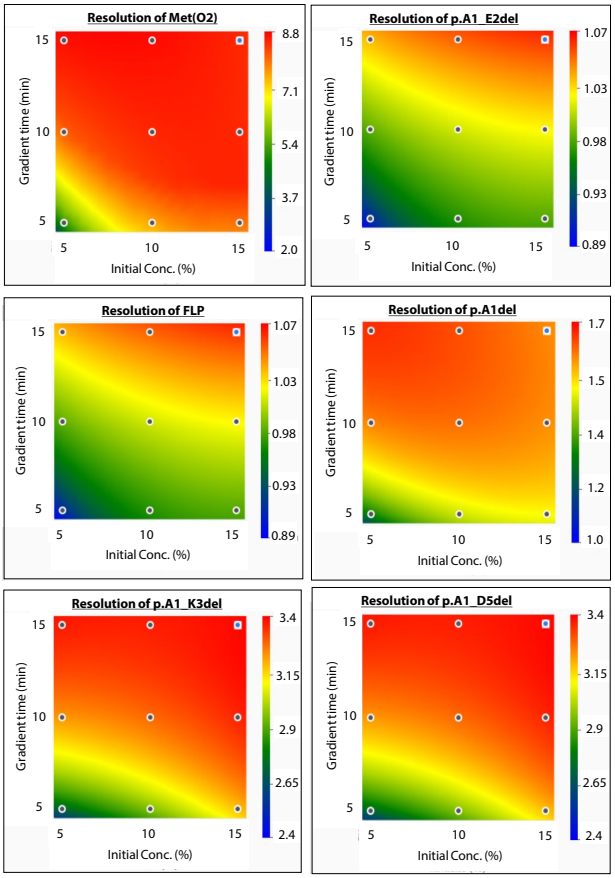

Fig. 20 Design Space for Resolution of FLP and Impurities
(Flow rate : 0.6 mL/min)

LabSolutions MD can automatically search for the optimal conditions that meet multiple criteria by overlaying design spaces. For example, Fig. 21 shows the area of the analytical conditions that satisfy the following requirements : resolution of FLP and p.A1del > 1.5, resolution of FLP and p.A1_E2del > 1.0, retention time of p.A1_D5del < 6 min to reduce analysis time, and retention time of Met(O2) > 3 min for proper retention. The region enclosed by the orange line is where the resolution of FLP and p.A1del < 1.5, the region enclosed by the pink line is where the resolution of FLP and p.A1_E2del < 1.0, the region enclosed by the yellow line is where the retention time of p.A1_D5del > 6 min, and the region enclosed by the brown line is where the retention time of Met(O2) < 3 min. Point A (red circle) in the remaining region (shown by the black hatching), represents the optimal conditions (initial concentration : 9%, gradient time : 11.5 min, flow rate : 0.6 mL/min) that satisfy all the criteria. By overlaying design spaces, the desired conditions can be easily identified.

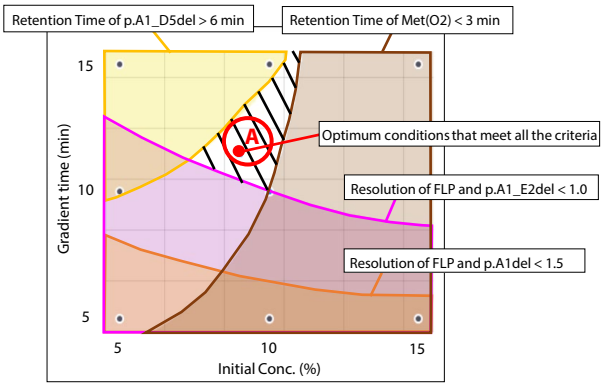

Fig. 21 Overlay of Design Spaces of Resolution and Retention Time

■ Chromatogram at Optimal Condition

The chromatogram obtained at optimal conditions (point A) is shown in Fig. 22. It shows that the resolution of FLP and p.A1del > 1.5, resolution of FLP and p.A1_E2del > 1.0, retention time of p.A1_D5del < 6 min, and retention time of Met(O2) > 3 min, which successfully satisfies the optimization criteria. By utilizing design spaces, the desired conditions can be easily identified without relying on chromatography experience.

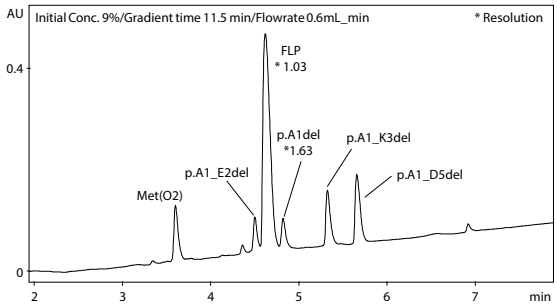

Fig. 22 Chromatogram at Optimal Conditions
(aqueous solution : 0.1% formic acid / organic solvent : MeOH / column : Scepter C8-120 / column oven temperature : 80 °C)

■ Conclusion

The separation patterns of synthetic peptides vary depending on mobile phase composition, column type, and various LC parameters, such as gradient conditions, column oven temperature, and flow rate. Separation behavior can also differ based on peptide structure, including length, amino acid composition, and the presence of modifications. Therefore, it is necessary to optimize the separation for each peptide sequence individually. However, optimizing analytical conditions through numerous analyses and data processing can be time-consuming. LabSolutions MD automates the entire workflow, including generating analysis schedules, preparing mobile phases, and processing the data, thanks to functionalities such as automated peak tracking, ranking of chromatograms, and design space visualization. This article introduced a case where the optimal separation conditions for synthetic peptides were efficiently identified through screening and optimization phases. LabSolutions MD also offers fully automated gradient optimization by AI algorithm to meet user-defined criteria. For more details, please refer to the application news, "Automatic Optimization of Gradient Conditions by AI Algorithm on Synthetic Peptide and Impurities : 01-00814".

01-00780A-EN First Edition: Oct. 2024
Revision A: Dec. 2024

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