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Application News

Software for Efficient Method Development Based on AQbD LabSolutions™ MD
Liquid Chromatograph Mass Spectrometer LCMS-2050, LCMS-9050

Efficient Method Development for Separation of Capped mRNA Fragments

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User Benefits

- LabSolutions MD can improve the efficiency of method development for 5' capped mRNA fragments and related impurities.
- The LCMS-2050 single quadrupole mass spectrometer and the LCMS-9050 quadrupole time-of-flight mass spectrometer enable confirmation of molecular weight.
- The LabSolutions Insight™ Biologics analysis software enables analysis of modifications and impurities specified by the user.

■ Introduction

There has been increased attention on new drug discovery modalities for mRNA because of its efficacy in COVID-19 vaccines. Currently authorized mRNA vaccines are synthesized using *in vitro* transcription to add a Cap-0 (m7GpppR-) or Cap-1 structure (m7GpppRm-) on the 5' end. When the cap structure is added by the post-transfer capping method, related impurities (pppR-, ppR-, and GpppR-) are generated by that process[1]. Cap structures contribute to mRNA recognition, efficiency of translation, and stability of mRNA in cells, making 5' cap structure analysis an important element of quality control.

For LC separation, one commonly used mode is reversed-phase ion-pair chromatography. The separation patterns obtained with this mode can vary depending on the concentration of the ion-pair reagent and the composition of the organic solvent. In addition, the separation behavior can differ depending on the length of products, nucleobases, and modifications. Therefore, it is important to optimize the separation parameters for each oligonucleotide sequence. This article describes how to efficiently achieve the optimal peak separation for mRNA fragments and related impurities by utilizing LabSolutions MD, which is dedicated software for supporting method development, together with LC/MS identification results.

■ Samples

Given that mRNA molecules are large, LC/MS analysis is typically done by analyzing fragments generated by cleavage enzyme reactions. In this study, the model sample included Cap-0 and Cap-1 structures on mRNA molecules with 36 bases obtained by *in vitro* transcription using plasmid DNA as a template. Their related impurities (pppR- and GpppR-) were also provided for analysis. GpppR- exists in equilibrium with ppR- in the presence of a vaccinia mRNA capping enzyme (Fig. 1)[1].

■ Screening of Mobile Phases and Columns

For the first screening (analytical conditions in Table 1), the parameters that have a large effect on separation, such as the ion-pair reagents (DIPEA, TEA, DBA, and HA) were considered. The concentration of the HFIP was 100 mmol/L, the concentration of the ion-pair reagent was 10 mmol/L, and the ratio of acetonitrile in the organic mobile phase was 100 %. An LCMS-9050 system was used to confirm the molecular weight of compounds for detected peaks (analytical conditions in Table 2).

For the second screening (analytical conditions in Table 3), two different levels of column pore size (120 and 300 Å), the four HA concentration levels that gave the best separation in the first screening (5, 10, 15, and 20 mmol/L), and five different acetonitrile ratio levels (0, 25, 50, 75, and 100 % in organic solvent) were evaluated, for a total of 40 patterns (2 × 4 × 5), with the aim of finding the combination that results in the optimal separation of target mRNA fragments and impurities.

Table 1 Analytical Conditions for 1st Screening

System:	Nexera™ XS inert (Method Scouting System)
Column:	Shim-pack Scepter™ Claris C18-120 (100 mm × 2.1 mm I.D., 1.9 µm)*1
Temperature:	60 °C
Injection Volume:	1 µL
Mobile Phases:	
Pump A – Line A:	100 mmol/L HFIP*2 and 10 mmol/L DIPEA*3 in water
– Line B:	100 mmol/L HFIP and 10 mmol/L TEA*4 in water
– Line C:	100 mmol/L HFIP and 10 mmol/L DBA*5 in water
– Line D:	100 mmol/L HFIP and 10 mmol/L HA*6 in water
Pump B – Line A:	Acetonitrile
Flowrate:	0.3 mL/min
Time Program (%B):	2 % (0-2 min) → 30 % (22 min) → 90 % (23-24 min) → 2 % (24.1-30 min)
Detection:	260 nm (SPD-M40, UHPLC inert cell)

*1: P/N 227-31210-02
*2: 1,1,1,3,3,3-hexafluoro-2-propanol
*3: N,N-Diisopropylethylamine
*4: Triethylamine
*5: Dibutylamine
*6: Hexylamine

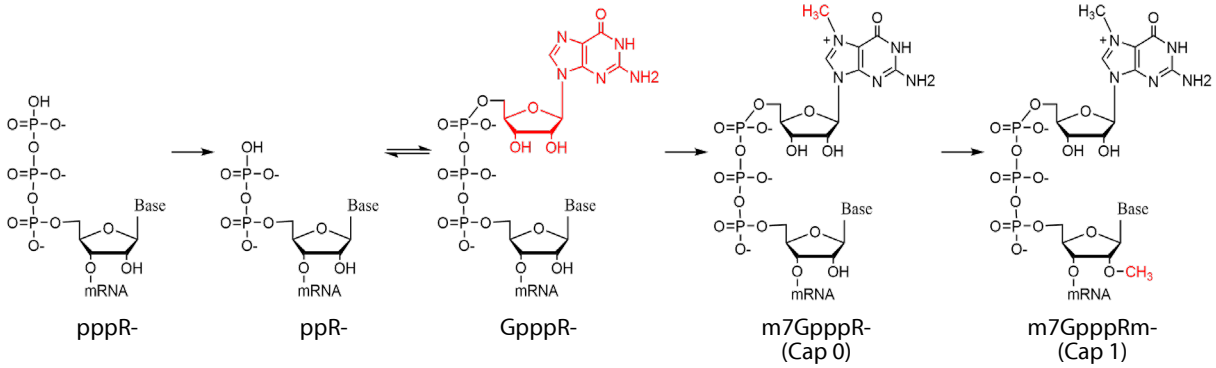

Fig. 1 mRNA Post-Transfer Capping Process

Table 2 Analytical Conditions for LCMS-9050

Ionization:	ESI negative
Mode:	MS *m/z* 600-2000
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
Heating Gas Flow:	10.0 L/min
Interface Temp.:	250 °C
DL Temp.:	250 °C
Block Heater Temp.:	400 °C

Table 3 Analytical Conditions for 2nd Screening

System:	Nexera XS inert (Method Scouting System)
Column – 1:	Shim-pack Scepter Claris C18-120 (100 mm × 2.1 mm I.D., 1.9 µm)
– 2:	Shim-pack Scepter Claris C18-300 (100 mm × 2.1 mm I.D., 1.9 µm)*7
Temperature:	60 °C
Injection Volume:	1 µL
Mobile Phases:	
Pump A – Line A:	100 mmol/L HFIP and 20 mmol/L HA in water
– Line B:	100 mmol/L HFIP in water
Pump B – Line A:	Acetonitrile
– Line B:	Methanol
Flowrate:	0.3 mL/min
Time Program (%B):	2 % (0-2 min) → 60 % (22 min) → 90 % (23-24 min) → 2 % (24.1-30 min)
Detection:	260 nm (SPD-M40, UHPLC inert cell)

*7: P/N 227-31209-02

LabSolutions MD can quickly and easily generate analysis schedules by setting the parameters, such as several types of mobile phases and the column oven temperature (steps (1) to (5) in Fig. 2). In addition, mobile phase blending functionality can automatically prepare mobile phases with different concentrations of the ion-pair reagent, as well as mixture ratios of acetonitrile and methanol, by simply clicking the mobile phases to use for automated screening (step (1) in Fig. 2). This significantly reduces the amount of work and human errors involved in manual preparation.

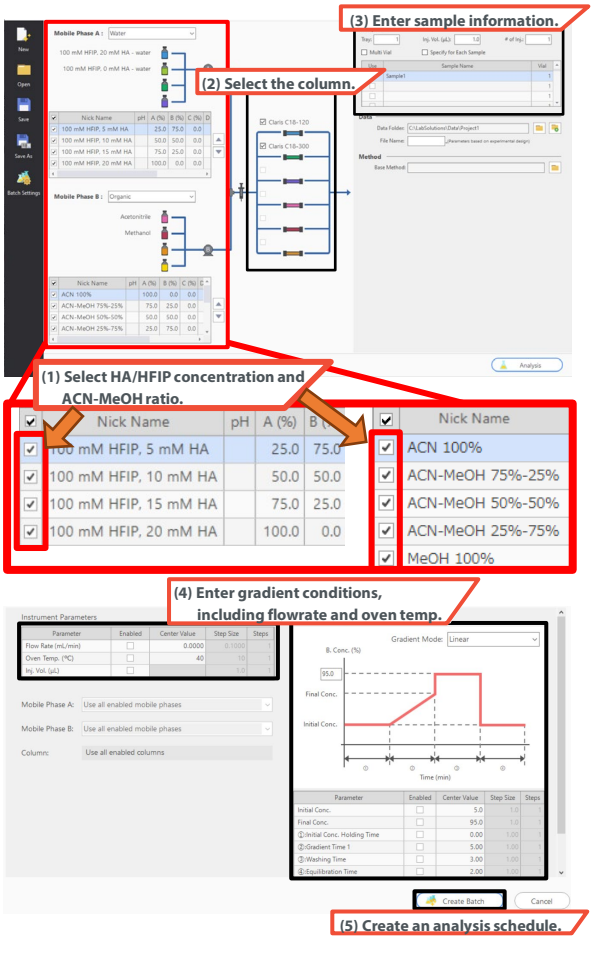

Fig. 2 Steps for Creating Analysis Schedule

■ Results of Screening

Chromatograms from the first screening, measured under different ion-pair reagent conditions, are shown in Fig. 3. When DIPEA or TEA was added to the mobile phase, each mRNA fragment coeluted into a single peak, whereas when DBA or HA was used, multiple peaks were detected.

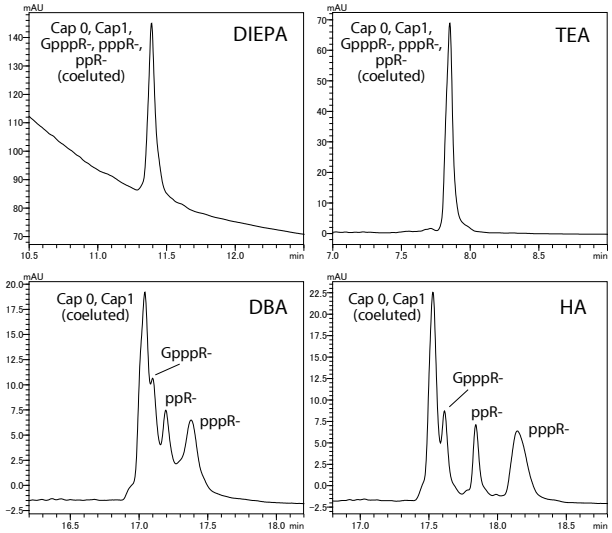

Fig. 3 Chromatograms Obtained from 1st Screening

The detected peaks were identified using an LCMS-9050 high-resolution mass spectrometer and LabSolutions Insight Biologics data analysis software. The LCMS-9050, which can obtain precise mass information makes it possible to confirm the molecular weights with high accuracy, even when compounds with similar molecular weight are coeluted. As an example, Fig. 4 shows the component chromatograms (based on MS1 spectra and generated by combining signals from different valences and isotopes) obtained using DBA or HA mobile phase conditions. The Target Modifications tab was also used to select the anticipated impurities. In addition to pre-registered nucleobases, linkers, ribose, and modifications, Insight Biologics can also search for molecular information added by the user in the Parameter Configuration window.

For both conditions, Cap-0 and Cap-1 components were co-eluted, followed by GpppR-, ppR-, and pppR-. The LCMS-9050 was able to distinguish and identify the 14 Da difference, which is the smallest mass difference between the mRNA fragments. The separation of Cap-0 and Cap-1 components was better when using the DBA condition than the HA condition, but the separation of Cap-1 and GpppR- was better with the HA condition. In the following optimization, the HA condition with good separation of Cap-1 and GpppR- was adopted to prioritize the separation of fragments with the cap structures (Cap-0 and Cap-1) and related impurities to be optimized.

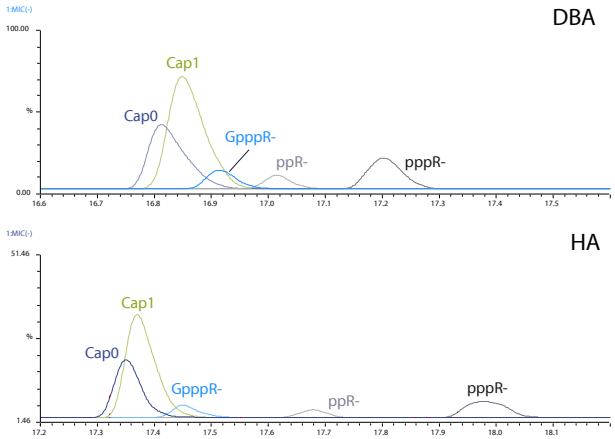

Fig. 4 Component Chromatograms Obtained by LCMS-9050 Analysis (Top: DBA Condition; Bottom: HA Condition)

For the second screening, chromatograms were measured with columns of different pore size, different concentrations of HA, and different ratios of acetonitrile in the organic mobile phase. As an example, the chromatograms obtained by varying the ratio of acetonitrile, using the C18-300 column, and adding 20 mmol/L HA are shown in Fig. 5. The resolution of Peak 1 (Cap-0, Cap-1) and Peak 2 (GpppR-) was best when the acetonitrile ratio was 75 % (Fig. 5 (4)).

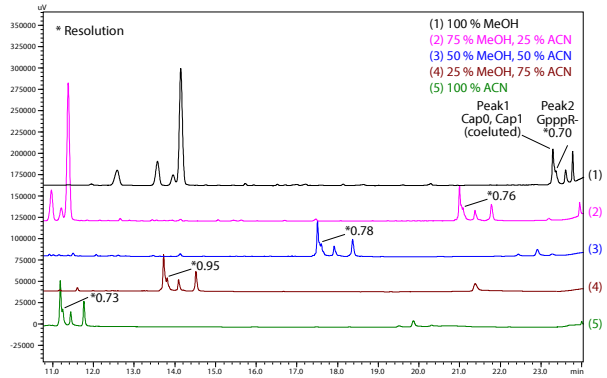

Fig. 5 Chromatograms Obtained with Different Ratio of Acetonitrile
(Column: C18-300, HA Conc.: 20 mmol/L)

Fig. 6 shows the chromatograms measured with the acetonitrile ratio 75 % that resulted in the best resolution for Peak 1 (Cap-0 and Cap-1) and Peak 2 (GpppR-) in Fig. 5, while varying the column pore size and the added HA concentration. Separation improved the most using a 300 Å column pore size and adding a 20 mmol/L concentration of HA (Fig. 6 (8)).

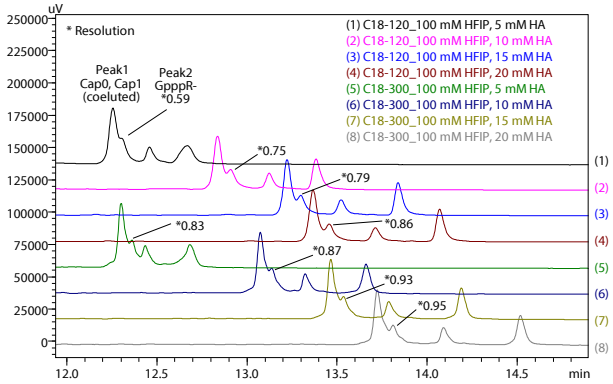

Fig. 6 Chromatograms Obtained with Different Pore Size and HA Concentration (Acetonitrile Ratio: 75 %)

■ Quickly Find Optimal Conditions

Because screening generates as many chromatograms as the number of analyses scheduled, they must be evaluated to determine which one is optimal. Checking all chromatograms manually is tedious and time-consuming.

However, LabSolutions MD can quickly and easily find optimal conditions by ranking each condition based on the number of detected peaks, the resolution level, and other information.

Focusing on the optimization of the separation of fragments with cap structures (Cap-0 or Cap-1) and their related impurities, the resolution of Peak 1 (Cap-0 and Cap-1) and Peak 2 (GpppR-) was evaluated. The conditions that resulted in the highest resolution was the C18-300 column, 20 mmol/L HA concentration, and a 75 % acetonitrile ratio (Fig. 7 and 8). Next, for the optimization phase, the column oven temperature and gradient program parameters were considered to further improve the separation.

			Compound#2 (peak2)
MPA Nick Name	MPB Nick Name	Column Nick Nam	Resolution
100mMHFIP,20mMHA	25%MeOH_75%ACN	Claris C18-300	0.949
100mMHFIP,15mMHA	25%MeOH_75%ACN	Claris C18-300	0.934
100mMHFIP,5mMHA	50%MeOH_50%ACN	Claris C18-300	0.907
100mMHFIP,10mMHA	25%MeOH_75%ACN	Claris C18-300	0.872
100mMHFIP,20mMHA	25%MeOH_75%ACN	Claris C18-120	0.864
100mMHFIP,5mMHA	MeOH100%	Claris C18-300	0.858
100mMHFIP,15mMHA	MeOH100%	Claris C18-300	0.858
100mMHFIP,10mMHA	MeOH100%	Claris C18-300	0.857
100mMHFIP,5mMHA	75%MeOH_25%ACN	Claris C18-300	0.831
100mMHFIP,5mMHA	25%MeOH_75%ACN	Claris C18-300	0.825

Fig. 7 Condition Settings Ranked by Resolution (Top 10)

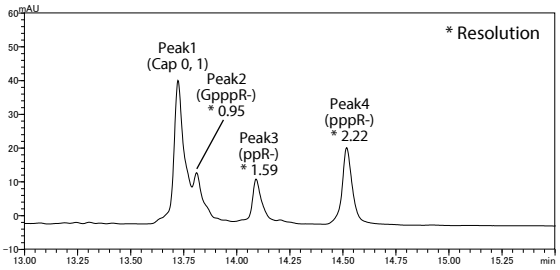

Fig. 8 Chromatogram of Highest Resolution
(Enlargement of Chromatogram in Fig. 6 (8))

■ Optimization Phase

Based on the optimal mobile phase and column conditions determined from the screening phase, analytical conditions were further optimized for the separation of mRNA fragments by changing the gradient time (20, 25, and 30 min), initial concentration of the gradient program (2, 11, and 20 %), and column oven temperature (40, 50, and 60 °C). The obtained chromatograms are shown in Figs. 9 to 11. The results show that the longer the gradient time, the higher the initial concentration, and the lower the column oven temperature are, the better the resolution of Peak 1 (Cap-0 and Cap-1) and Peak 2 (GpppR-).

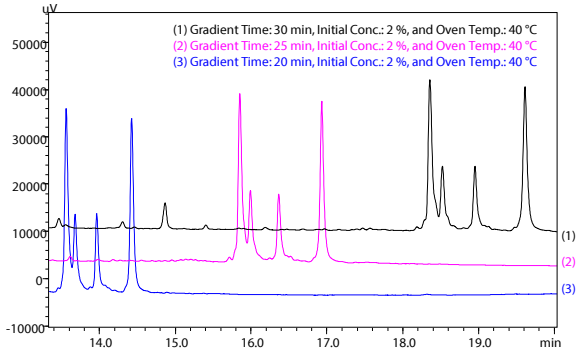

Fig. 9 Chromatograms with Different Gradient Times

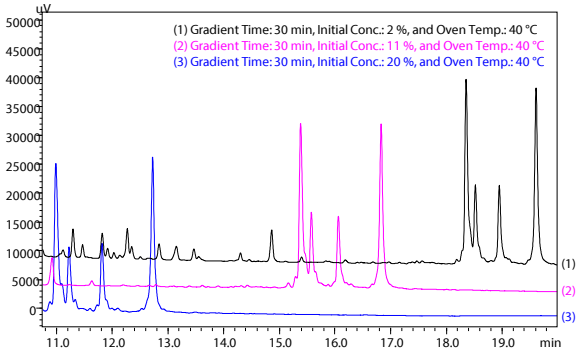

Fig. 10 Chromatograms with Different Initial Concentrations

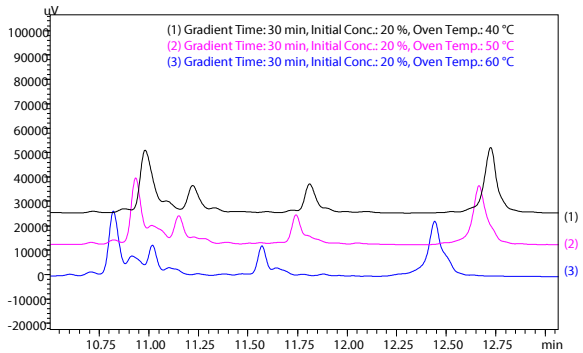

Fig. 11 Chromatograms with Different Column Oven Temperatures

■ Determining Optimal Conditions by Design Space Evaluation

To visualize the optimal conditions, a design space was created based on results from evaluating various LC parameters. In order to prioritize the separation of Peak 1 (Cap-0 and Cap-1) and Peak 2 (GpppR-), the peak-to-valley ratios of Peak 1 and Peak 2 were visualized (Fig. 12). In Fig. 12, the vertical line shows gradient time or initial concentration, the horizontal line shows column oven temperature. The red region indicates larger peak valley ratios and the blue region indicates smaller peak valley ratios. By visualizing peak-to-valley ratios with a design space, it became evident that the longer the gradient time, the higher the initial concentration, and the lower the column oven temperature are, the better the separation of Peak 1 and Peak 2 becomes. Finally, the optimal analytical conditions with a gradient time of 30 minutes, initial gradient concentration of 20 %, and a column oven temperature of 40 °C, yielded values of 7.77 and 1.80 for the peak-to-valley ratio and resolution of Peaks 1 and 2, respectively.

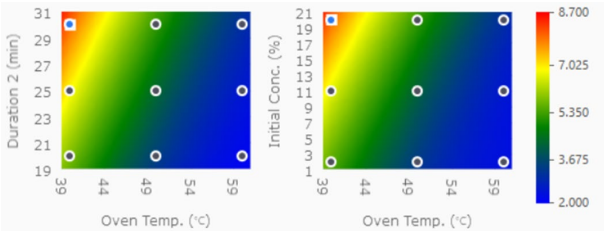

Fig. 12 Design Space for Peak to Valley Ratio of Peak1 and Peak2
(Left: With 20 % Initial Concentration; Right: With 30 min Gradient Time)

■ Identification by LC/MS with Optimal Separation Conditions

As shown in Fig. 3, the LCMS-9050 quadrupole time-of-flight mass spectrometer, which can obtain precise mass information, is suitable for cases where it is unclear which compound the detected peak corresponds to, such as in the early phase of analytical method development. On the other hand, the LCMS-2050 single quadrupole mass spectrometer, which is easy to use, is useful for identifying compounds after the analytical method is established. The LCMS-2050 is equipped with a heated DUIS™ ion source for ionization, which combines the advantages of both ESI and APCI methods. It enables user-friendly operability that is similar to LC systems. Unlike quadrupole time-of-flight mass spectrometers, single quadrupole mass spectrometers cannot obtain MS/MS or accurate mass information, but they do allow simple confirmation of molecular weight.

Molecular weight was confirmed using the LCMS-2050 system under the optimal LC conditions determined by design space evaluation. The analytical conditions are shown in Table 4, and the component chromatograms are shown in Fig. 13, which shows that sufficient separation of Cap-0, Cap-1, and GpppR- was achieved. Complete separation of Cap-0 and Cap-1 was not possible, but target mRNA fragments could be identified using the LCMS-2050.

Table 4 Analytical Conditions for LCMS-2050

System:	LCMS-2050
Ionization:	DUIS negative
Mode:	SCAN (*m/z* 550 - 2000)
Nebulizing Gas:	2.0 L/min
Drying Gas:	5.0 L/min
Heating Gas:	7.0 L/min
DL Temp.:	200 °C
Desolvation Temp.:	450 °C
Interface Voltage:	-2.0 kV

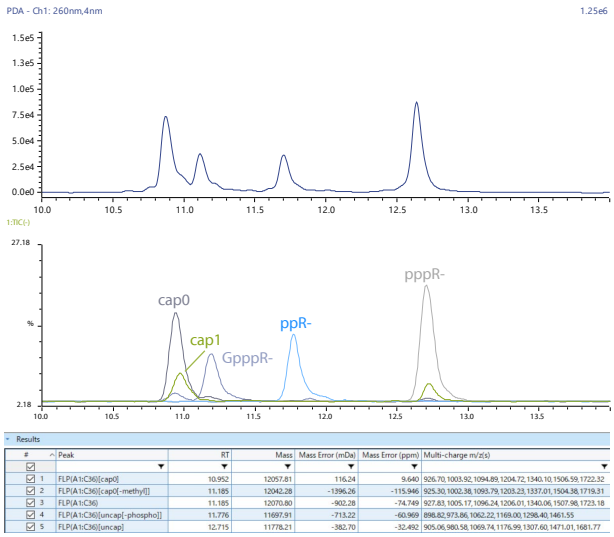

Fig. 13 LCMS-2050 Measurement Results
Top: UV and Component Chromatograms; Bottom: Identification Results

■ Conclusion

The separation pattern of oligonucleotides differ depending on the concentration of ion-pair reagents in the aqueous mobile phase, the ratio of acetonitrile in the organic mobile phase, and column pore size, in addition to column oven temperature and the gradient program. The separation behavior can differ depending on the structure of the oligonucleotide, such as the length, nucleobase, and the presence of modifications. Therefore, the separation must be individually optimized for each sequence of oligonucleotide. On the other hand, the number of analyses and data processing needed for the optimization of analytical conditions is a time-consuming challenge. LabSolutions MD can automate the entire workflow, including the generation of an analysis schedule, mobile phase preparation, and data processing thanks to specific functionalities, such as for ranking chromatograms by criteria values (ex. resolution), and for design space evaluation.

In combination with the LCMS-2050 single quadrupole mass spectrometer or the LCMS-9050 quadrupole time-of-flight mass spectrometer, it confirm the molecular weight of compounds. The LCMS-9050 can be used to obtain accurate mass information, for example, when the information about a compound contained in a sample is not clear in the early phase of analytical method development, whereas the easy usability of the LCMS-2050 is useful for quality control analysis after the method is established.

Reference

1. A. Ramanathan, G. B. Robb, S. H. Chan, mRNA capping: biological functions and applications, *Nucleic Acids Res.* 2016, 44, 7511-26

Related Applications

1. An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software
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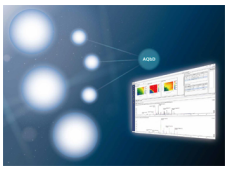

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