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Application News

High Performance Liquid Chromatograph Nexera™ XS inert

# Oligonucleotides analysis by Ion Exchange Chromatography and Effects of pH Changes in the Mobile Phase on Separation

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## User Benefits

- Short chain oligonucleotides can be separated based on the base unit.
- Oligonucleotides can be separated from impurities such as protecting groups used in the chemical synthesis process.
- By controlling pH changes in the mobile phase, robust analytical results can be obtained.

## ■ Introduction

Nucleic acid drugs, such as antisense oligonucleotides, exert their efficacy by interacting with target genes inside and outside cells. Unlike conventional small molecule drugs, they are capable of targeting disease causes at the genetic level and are attracting attention as a next-generation drug. Nucleic acid drugs are mainly produced through chemical synthesis, but the synthesis process also produces many impurities such as shorter length components and protecting groups, so proper separation and purification of the target oligonucleotide is required.

In this article, we introduce an analytical method for the separation of oligonucleotides of different length by ion-exchange chromatography, assuming that shorter length components are impurities derived from the synthesis process. In order to achieve optimal analytical performances an inert UHPLC system was used. The Nexera XS inert, which is designed to suppress the adsorption of metal-coordinating compounds containing phosphate groups. We also report the effect of changes in mobile phase pH on analytical results.

## ■ Reagents

The sequence of the oligonucleotide to be analyzed is shown in Table 1. Target oligonucleotides in 20 mer and 4 sequences that were deleted from n-1 to n-4 on the 3' terminus of target were prepared as impurities derived from the synthesis. All of them were unmodified single-stranded DNA and synthesized by a solid phase synthesis (HPLC purification). These 5 sequences were diluted to 5 µmol/L with water, and an oligonucleotide mixture was prepared.

## ■ Analytical Conditions

In ion-exchange chromatography, the samples are separated and eluted using a mobile phase of varying salt concentration or pH. In this analysis, we used an aqueous solution of sodium hydroxide that contain sodium perchlorate, which has high elution strength, for a mobile phase (Table 2).

In ion-exchange chromatography, the separation is based on the number of phosphate groups in the oligonucleotide, i.e., the difference in negative charge. Therefore, generally, shorter oligonucleotides are eluting first. Figure 1 shows a chromatogram of a five-sequence oligonucleotide mixture. Each oligonucleotide was separated by their length.

## ■ Reproducibility

Table 3 shows the relative standard deviations (% RSD, n = 6) of the retention time and area of the 16 - 20 mer oligonucleotide mixture shown in Table 1. For both parameters the RSD% was less than 1%.

Table 1 Oligonucleotides

| | Sequence (5 '→ 3') | Length (mer) |
|---|---|---|
| 1 | TCTTGGTTACATGAAA | 16 |
| 2 | TCTTGGTTACATGAAAT | 17 |
| 3 | TCTTGGTTACATGAAATC | 18 |
| 4 | TCTTGGTTACATGAAATCC | 19 |
| 5 | TCTTGGTTACATGAAATCCC | 20 |

Table 2 Analysis Conditions

| | | |
|---|---|---|
| System | : | Nexera XS inert |
| Column | : | Shim-pack™ Bio IEX Q-NP (100 mm x 4.6 mm I.D., 5 µm) *1 |
| Mobile phase A | : | 10 mmol/L NaOH |
| Mobile phase B | : | 10 mmol/L NaOH containing 1 mol/L $NaClO_4$ |
| Flow rate | : | 0.8 mL/min |
| Time program (B Conc.) | : | 25 ~ 32.5% (0 -15 min) → 100% (15 -20 min) → 25% (20 -25 min) |
| Column temp. | : | 30 °C |
| Injection volume | : | 4 µL |
| Detection | : | UV 260 nm (SPD-M40), Standard cell |
| Vial | : | Shimadzu 1.1 mL sample vial *2 |

*1 P/N: 227 -31003 - 03, *2 P/N: 228 -21283 -91

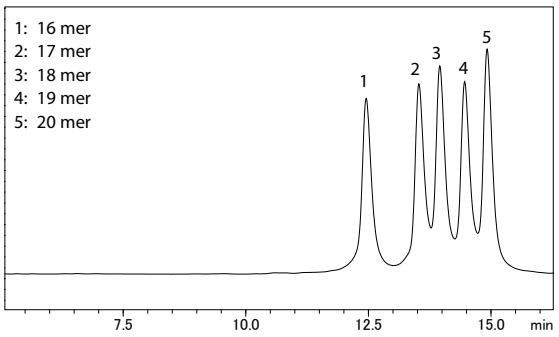


Figure 1 Chromatogram of oligonucleotides mixture

Table 3 Relative standard deviation (% RSD) of each component (n = 6)

| Length (mer) | Retention time | Area |
|---|---|---|
| 16 | 0.138 | 0.224 |
| 17 | 0.105 | 0.335 |
| 18 | 0.098 | 0.494 |
| 19 | 0.085 | 0.161 |
| 20 | 0.075 | 0.307 |

## ■ Analysis of an oligonucleotides mix containing impurities

A mixture of five oligonucleotides, at 5 µmol/L concentration each, was prepared (four of them were HPLC-purified while 1 was only desalted) and compared with the mixture of all HPLC-purified nucleotides(Figure 2). The target oligonucleotides were completely separated from impurities such as free protecting groups and shorter length oligonucleotides.

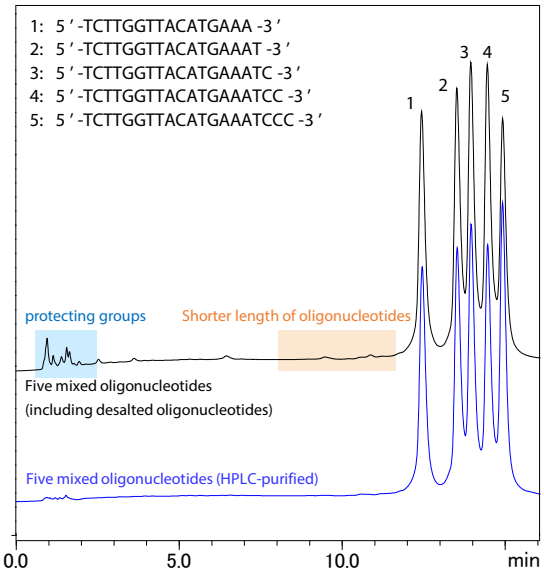


Figure 2 Chromatograms of the oligonucleotide mixture containing impurities

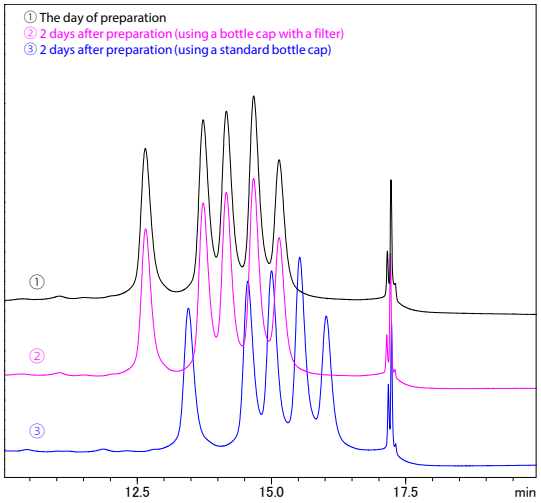


Figure 3 Chromatograms of the oligonucleotide mixture analyzed on the day of preparation and 2 days after.

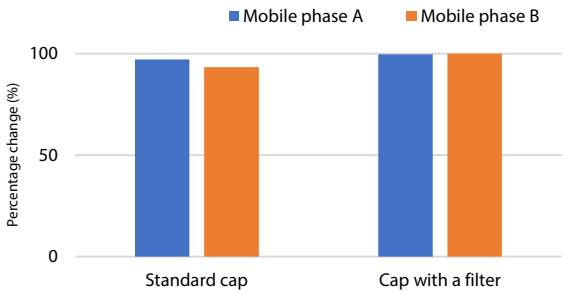


Figure 4. Rate of change in pH after 2 days of mobile phase preparation compared to the day of mobile phase preparation

## ■ Effect of pH Change in the Mobile Phase on Retention Time

As described above, when oligonucleotides are analyzed using ion exchange chromatography, the samples are separated and eluted using a salt concentration gradient. It is well known that, in the case of a mobile phase with high pH (basic), the absorption of carbon dioxide from the air can change the pH level. For ion-exchange chromatography even a slight change in the pH can have a significant impact on the analytical results (the separation mechanism is based on the charge difference of the analytes). For this reason, it is important to prevent any changes in the pH of mobile phases in order to obtain stable analytical performances.

We compared a standard mobile phase bottle cap and a cap with a filter that suppresses evaporation of the solvent. These analyses using each cap for the bottle mobile phase were performed on the day of preparation and after two days (The conditions are same as shown in Table 2). The chromatograms of the mixed oligonucleotides sample are shown in Figure 3. Figure 4 shows the rates of pH change from the day of preparation in both mobile phases.

When using a standard mobile phase bottle cap, the retention time increased by an average of 8% (③) as the pH changed compared to that on the day of preparation of the mobile phase (①), whereas when using a cap with a filter, the retention time did not change substantially (②). The use of a cap with a filter suppressed the change in the pH of the mobile phase, resulting in stable analysis results.

## ■ Summary

This article introduces an example of oligonucleotide analysis by ion exchange chromatography using Nexera XS inert and Shim-pack Bio IEX. In this analysis, we achieved reproducible separation of the desired oligonucleotide from impurities such as protecting groups used during chemical synthesis and oligonucleotides with different chain lengths generated by incomplete synthesis. Since the change in pH of the mobile phase affects the retention time of analysis, pH control of the mobile phase is important for stable analysis. Thanks to the use of inert UHPLC system, it was possible to obtain stable analytical results.



01-00227-EN First Edition: Feb. 2022

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