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Technical Report

Retention Time Repeatability in Peptide Mapping under Shallow Gradient Conditions Using Nexera X4

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Abstract:

Peptide mapping with an HPLC system is an important quality evaluation test for confirming the primary structures of proteins. After enzymatic digestion of proteins, peptides are separated using a reversed-phase column or similar techniques. Because the number of target peaks is extremely large, the analysis requires a prolonged run time and shallow gradient conditions to ensure adequate separation. However, to achieve high retention time repeatability under these conditions, very precise and stable solvent delivery performance is required. This article describes the evaluation of retention time repeatability in peptide mapping of protein digests using the Nexera X4 system.

Keywords: Nexera X4, Peptide, Peptide mapping, Shallow gradient

1. Background

Typically, when checking the quality of proteins used in biopharmaceuticals, the proteins are digested with enzymes and then peptide mapping is used to analyze the resulting peptide fragments. Because peptide mapping requires separating and analyzing such a wide diversity of peptide types at once, the analysis time becomes long and shallow gradient conditions are typically used. Under such shallow gradient conditions, the solvent delivery unit must provide extremely accurate and stable solvent delivery performance to ensure retention time repeatability.

2. Nexera X4 Features

The Nexera X4 is Shimadzu's next-generation ultra high performance liquid chromatograph (UHPLC) system based on technologies cultivated for previous Nexera series models. Due to its cutting-edge fluid control technology, the Nexera X4 offers outstanding solvent delivery stability even for analyses that require especially accurate delivery, such as shallow gradient analysis.

Figure 1: Nexera X4 System

3. Advanced Solvent Delivery Algorithm Reduces Pulsation

The LC-40B X4 is a binary gradient pump with 4 independently actuated plungers and a new pressure feedback mechanism. By actuating each plunger independently to optimize the timing of mobile phase suctioning/discharging actions, the pump significantly reduces pulsation originating from the pump. In general, retention times tend to fluctuate especially when the solvent B concentration is extremely low or the gradient is shallow, but the LC-40B X4 pump achieves outstanding repeatability.

Figure 2: LC-40B X4 Solvent Delivery Unit Installed in the Nexera X4 (Left: Exterior; Right: Internal Configuration)

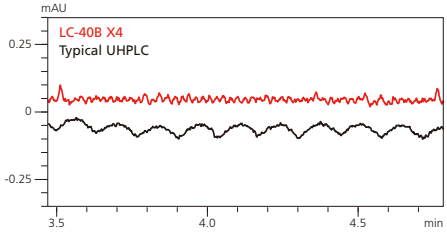

Figure 3: Reduction in Baseline Fluctuation due to the LC-40B X4

4. PDA Detector Reduces Refractive Index Effect

For peptide mapping with a shallow gradient, low concentration peptides must be analyzed in the short-wavelength region. However, for HPLC gradient analysis, changes in the refractive index of solvents in different mobile phase mixtures can cause baseline fluctuation (refractive index effect). The Nexera X4 is equipped with an SPD-M40 X4 PDA detector that features a newly designed capillary cell that reduces the refractive index effect to achieve a stable baseline.

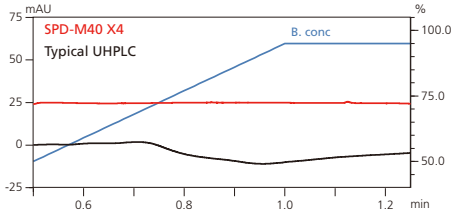

Figure 4: Improved Baseline Stability due to SPD-M40 X4 Refractive Index Effect Reduction

1 Analytical & Measuring Instruments Division

5. Analysis

5-1. Preparing Peptide Fragments for Analysis

In this article, myoglobin (SIGMA, code M0630) was digested with trypsin and the resulting digestion products were used as samples for analysis. Figure 5 shows the pretreatment procedure. Analytical samples were prepared by adding 160 µL of LC mobile phase A to the acquired samples.

17 mg/mL Myoglobin(horse) in water 2 µL
← 0.1 mol/L NH_4HCO_3 pH8.0 30 µL
← 0.61 mg/mL Trypsin in 0.1 mol/L NH_4HCO_3 pH8.0 1 µL
Incubate at 37 ˚C for 20 hours
← HCOOH 2 µL
Sample (Tryptic digests of Myoglobin)

Figure 5: Sample Pretreatment Procedure

5-2. Analytical Conditions

Table 1 shows the analytical conditions. A Shim-pack Scepter series column was used due to its outstanding durability over a wide range of pH levels and temperatures.

Table 1: Analytical Conditions

System	: Nexera X4
Column	: Shim-pack Scepter C18* (100 mm×2.1 mm I.D., 1.9 µm)
Temperature	: 65 ˚C
Injection volume	: 10 µL (Sample loop 15 µL)
Mobile phases	: A 0.1 % Trifluoro acetic acid in Water B 0.1 % Trifluoro acetic acid in Acetonitrile
Flow rate	: 0.2 ml/min (MR-20 µL mixer)
Time program	: B conc. 1 % (0-3 min)→50 % (88 min)→90 % (90-100 min) →1 % (102-130 min)
Detection	: 214 nm

* 227-31012-05

5-3. Analytical Results

Figure 6 shows the chromatograms obtained. Using a shallow gradient, the chromatograms show that a large number of peptide fragments were separated from the myoglobin digest. In addition, an overlay of the chromatograms shows the stability of peak patterns and indicates good retention time repeatability.

5-4. Evaluation of Retention Time Repeatability

17 representative peaks were selected from the obtained chromatograms to evaluate retention time repeatability. For comparison, a typical UHPLC system was also used to acquire data under the same analytical conditions. These results are summarized in Figure 7. The data confirmed superior retention time repeatability during non-retained elution, isocratic elution, and shallow gradient elution compared with a typical UHPLC system.

6. Conclusions

- The LC-40B X4 solvent delivery unit achieves stable solvent delivery and baseline stability even under ultra-high pressure conditions, thanks to its 4 independently actuated plungers and pressure feedback mechanism.
- SPD-M40 X4 detector features a newly designed capillary cell and optical system that reduce the refractive index effect during gradient analysis.
- Nexera X4 include superior gradient control technology that achieves excellent retention time repeatability for peptide mapping with a shallow gradient.

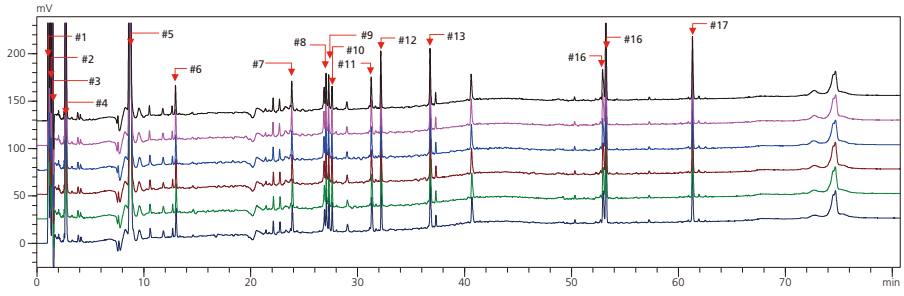

Figure 6: Chromatograms of Myoglobin Digestion Products Measured with the Nexera X4

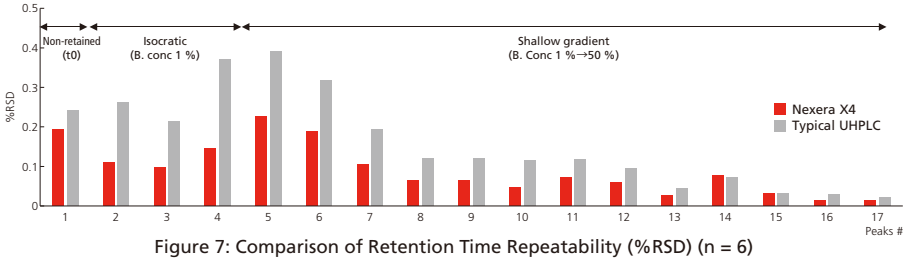

Figure 7: Comparison of Retention Time Repeatability (%RSD) (n = 6)

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