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Application News

High Performance Liquid Chromatograph Nexera™ XR/RF-20AXS

# High-Speed Simultaneous Analysis of Amino Acids by Pre-column Derivatization Using Automatic Pretreatment Function

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## User Benefits

- Analysis of 20 proteinogenic amino acids can be performed in 26 minutes per cycle.
- Pre-column derivatization analysis of amino acids can be easily performed by using the automatic pretreatment function of Nexera XR.
- Nexera XR can also be used to analyze analytes besides amino acids.

## ■ Introduction

Amino acid analysis is required in various fields, including the development of food and drugs. Post-column derivatization method is commonly used for amino acid analysis with high performance liquid chromatography (HPLC), and Shimadzu also uses post-column derivatization method for Amino Acid Analysis System. However, it is difficult to realize high-speed analysis due to the characteristics of the column. The pre-column derivatization method, in which amino acids are derivatized before entering the column, enables high-speed analysis and simple derivatization procedure in comparison with the post-column derivatization method by automating the derivatization procedures.

This article introduces an optimized analytical conditions of amino acids analysis by the automated pre-column derivatization method using HPLC (Nexera XR).

## ■ Automatic Pre-column Derivatization

Nexera XR is equipped with an automatic pretreatment function including sample dilution and reagent addition. For this study, we set the system to automatically mix the sample and derivatization reagents in the autosampler needle. *O*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) are well-known derivatization reagents that can react rapidly with amino acids at room temperature. Therefore, pre-column derivatization can be performed automatically using the automatic pretreatment function, reducing the time and labor taken in comparison with manual derivatization.

In addition, the consumption of samples and reagents can be minimized, and the setting of reaction vials required in the conventional method is not necessary because the derivatization reaction takes place in the autosampler needle.

Fig. 1 shows a scheme of pre-column amino acid derivatization reactions, and Fig. 2 shows a chromatogram acquired through the analysis of 20 proteinogenic amino acids.

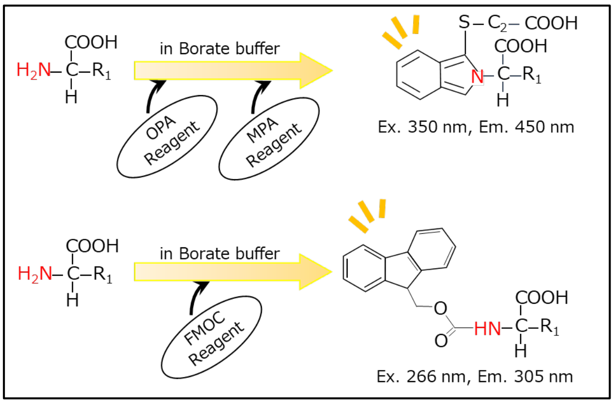
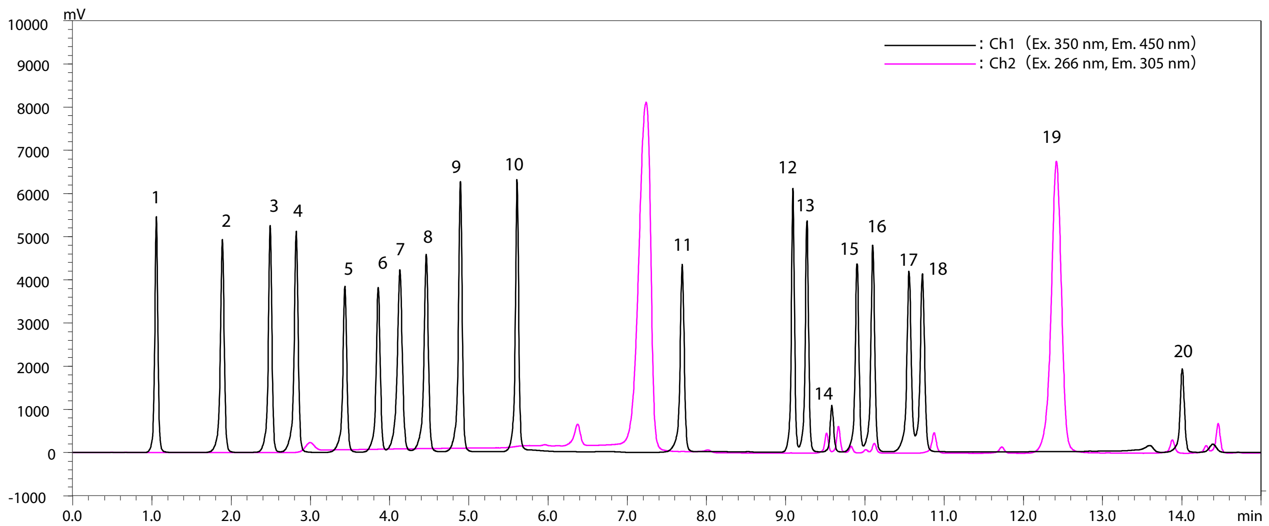



Fig. 1 Scheme of Pre-column Amino Acid Derivatization Reactions
Top: Reaction with OPA Reagent,
Bottom: Reaction with FMOC Reagent
(In this article, FMOC reagent was used for the derivatization of proline.)




1, Aspartic Acid 2, Glutamic Acid 3, Asparagine 4, Serine 5, Glutamine 6, Histidine 7, Glycine 8, Threonine 9, Arginine 10, Alanine 11, Tyrosine 12, Methionine 13, Valine 14, Cystine 15, Tryptophan 16, Phenylalanine 17, Isoleucine 18, Leucine 19, Proline 20, Lysine

Fig. 2 Simultaneous Analysis of 20 Proteinogenic Amino Acids (100 µmol/L each)

## ■ Analytical Conditions

Table 1 shows the analytical conditions, and Table 2 shows the time program.

Table 1 Analytical Conditions

| | |
|---|---|
| Column | : Shim-pack™ XR-ODSII*1<br>100 mm x 3.0 mmI.D., 2.2 µm |
| Mode | : Low pressure gradient |
| Mobile phase | : A) 20 mmol/L (Sodium) acetate buffer (pH 6)<br>B) Water/Acetonitrile=100:900<br>C) 20 mmol/L (Sodium) acetate buffer (pH 5) containing 0.5 mmol/L EDTA-2Na |
| Flow rate | : 1.0 mL/min |
| Column temperature | : 40 °C |
| Injection volume | : 1 µL |
| Sample cooler | : 4 °C |
| Detection | : Ch1) Ex. 350 nm, Em. 450 nm<br>Ch2) Ex. 266 nm, Em. 305 nm<br>(RF-20AXS , cell temperature 25 °C) |

*1: P/N 228-41624-92

Table 2 Analytical Conditions (Preparation of Mobile Phases)

- Mobile Phase A
  Add 2.67 g of sodium acetate trihydrate and 41 µL of acetic acid into 1000 mL of ultrapure water.
- Mobile Phase B
  Add 100 mL of ultrapure water into 900 mL of acetonitrile.
- Mobile Phase C
  Add 0.19 g of EDTA-2Na, 2.03 g of sodium acetate trihydrate and 308 µL of acetic acid into 1000 mL of ultrapure water.

Table 3 Time Program

| Time (min) | A.conc | B.conc | C.conc |
|---|---|---|---|
| 0 | 95 | 5 | 0 |
| 0.2 | 93 | 7 | 0 |
| 1 | 93 | 7 | 0 |
| 4 | 87 | 13 | 0 |
| 5 | 0 | 15 | 85 |
| 7.5 | 0 | 30 | 70 |
| 12 | 0 | 35 | 65 |
| 14 | 0 | 45 | 55 |
| 14.01 | 0 | 95 | 5 |
| 17 | 0 | 95 | 5 |
| 17.01 | 95 | 5 | 0 |
| 19.5 | 95 | 5 | 0 |

## ■ Calibration Curve

Linearity ($r^2$, contribution ratio) of calibration curves for all the amino acids were 0.999 or greater in the concentration range of 1, 5, 12.5, 25, and 100 µmol/L (Table 4). And it also shows the repeatability of peak area for 25 µmol/L standard solution (n= 6) .

Table 4 Linearity and Repeatability of Peak Area

| | $r^2$ | Area (%RSD) |
|---|---|---|
| Asp | 0.9999 | 1.62 |
| Glu | 0.9994 | 1.19 |
| Asn | 0.9991 | 1.22 |
| Ser | 0.9991 | 1.21 |
| Gln | 0.9992 | 1.13 |
| His | 0.9990 | 1.08 |
| Gly | 0.9993 | 1.14 |
| Thr | 0.9993 | 1.11 |
| Arg | 0.9990 | 0.97 |
| Ala | 0.9993 | 1.10 |
| Tyr | 0.9992 | 1.09 |
| Met | 0.9994 | 1.11 |
| Val | 0.9994 | 1.18 |
| Cystine | 0.9996 | 1.30 |
| Trp | 0.9994 | 1.19 |
| Phe | 0.9994 | 1.22 |
| Ile | 0.9993 | 1.15 |
| Leu | 0.9993 | 1.19 |
| Pro | 0.9998 | 4.93 |
| Lys | 0.9993 | 1.16 |

## ■ Analyses of Samples

Fig. 3 shows the chromatograms of samples. Sample were filtered through a 0.22 micrometer filter, diluted 100 times with 10 mmol/L hydrochloric acid solution, and subjected to analysis.

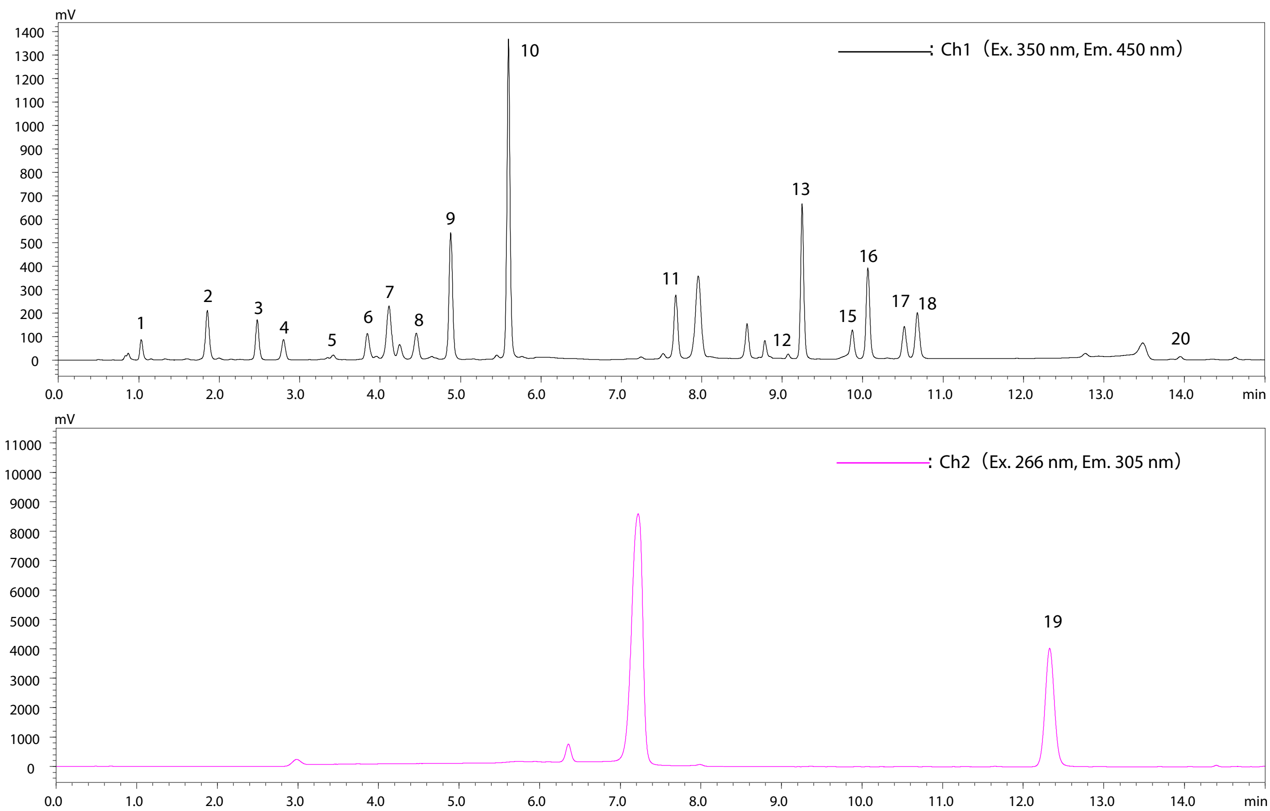


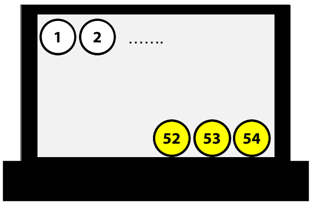

Fig. 3 Chromatograms of Beer
(Top: The result in Ch 1, Bottom: The result in Ch 2. For peak numbers, see Fig. 2.)

## ■ Setting of Automatic Pre-column Derivatization

Table 5 shows the preparation of derivatization reagents used in this article. Fig. 4 shows an example of the locations of derivatization reagents vials in the autosampler.

Table 5 Preparation of Derivatization Reagents

- Mercaptopropionic acid Reagent (MPA Reagent)
  Add 10 µL of 3-mercaptopropionic acid into 10 mL of 0.1 mol/L borate buffer.
- OPA Reagent
  Add 0.3 mL of ethanol into 10 mg of *o*-phthalaldehyde and dissolve completely. Then add 0.7 mL of 0.1 mol/L borate buffer and 4 mL of ultrapure water.
- MPA/OPA Solution
  Mix 600 µL of MPA Reagent and 300 µL OPA Reagent.
- FMOC Reagent
  Dissolve 10 mg of 9–fluorenylmethyl chloroformate into 100 mL of acetonitrile.
- Phosphoric acid aqueous solution
  Add 0.5 mL of phosphoric acid (85%) into 100 mL of ultrapure water.




| Vial | Reagent |
|---|---|
| 52 | MPA/OPA Solution |
| 53 | FMOC Reagent |
| 54 | Phosphoric acid aqueous solution |

Fig. 4 Locations of Derivatization Reagents Vials in the Autosampler (1.5 mL Sample Plate)

Fig. 5 shows the flowchart of the automated pre-column derivatization used in this article. To run these operations, the pretreatment program is set as shown in Table 6 in the "pretreatment program mode" of the autosampler. The program shown here assumes the case in which the respective derivatization reagents vials are placed in the autosampler as shown in Fig. 4.

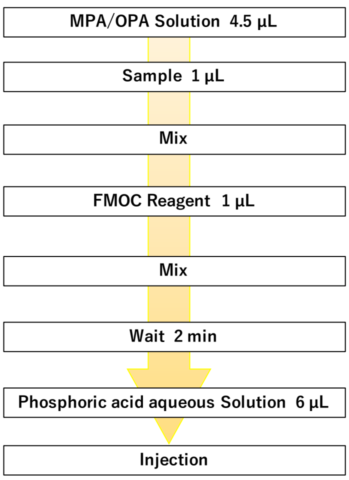



Fig. 5 Flowchart of Automated Pre-column Derivatization

Table 6 Details of Pretreatment Program

| | Page1 |
|---|---|
| 1 | a1=4.5 |
| 2 | a2=1 |
| 3 | a3=1 |
| 4 | a4=5.0 |
| 5 | vial.n 1,52 |
| 6 | air.a 5.5,ss |
| 7 | n.strk ns |
| 8 | aspir a1,ss |
| 9 | d.rinse |
| 10 | vial.n rn,sn |
| 11 | n.strk ns |
| 12 | aspir a2,ss |
| 13 | air.a 1.0,ss |
| 14 | d.rinse |
| 15 | n.drain |
| 16 | for a5=1,10 |
| 17 | aspir a4,5.0 |
| 18 | disp a4,5.0 |
| 19 | next a5 |
| 20 | d.rinse |
| 21 | wait 0.5 |
| 22 | vial.n 1,53 |
| 23 | n.strk ns |
| 24 | aspir a3,ss |
| 25 | air.a 2.0,ss |
| 26 | d.rinse |
| 27 | n.drain |
| 28 | for a5=1,40 |
| 29 | aspir a4,5.0 |
| 30 | disp a4,5.0 |
| 31 | next a5 |
| 32 | wait 2.0 |
| 33 | goto f2 |
| 34 | end |

| | Page2 |
|---|---|
| 1 | vial.n 1,54 |
| 2 | n.strk ns |
| 3 | aspir 6.0,ss |
| 4 | n.drain |
| 5 | d.rinse |
| 6 | inj.p |
| 7 | s.inj |
| 8 | purge.ml mv,rs |
| 9 | purge.rp rv,rs |
| 10 | end |

## ■ Conclusion

This article introduced an example of high-speed analysis of 20 proteinogenic amino acids by pre-column derivatization using the automatic pretreatment function of Nexera XR. More stable analysis can be performed since the automatic derivatization provide constant reaction time in comparison with manual derivatization. Nexera XR is a highly versatile instrument which can analyze analytes besides amino acids, therefore high operating rates can be expected.

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