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

Application News

Pre-column Amino Acid Analysis of Hydrolyzed Alternative Proteins

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User Benefits

- Very easy amino acid analysis can be performed because of automated derivatization involving complicated processes.
- Samples treated through three different hydrolyses (hydrochloric acid hydrolysis, alkaline hydrolysis, and performic acid oxidation) can be analyzed using a same HPLC instrument.
- Two different analytical methods can be employed using the same column, the same reaction reagents.

■ Introduction

Hydrochloric acid hydrolysis is commonly used as a pretreatment when analyzing amino acids constituting proteins and peptides. During hydrochloric acid hydrolysis, asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively. Tryptophan and the sulfur-containing amino acids methionine, cysteine, and cystine are decomposed in the process, making accurate determination impossible. To achieve accurate determination, alkaline hydrolysis is used in the determination of tryptophan. For methionine, cysteine, and cystine, performic acid treatment oxidizes methionine to methionine sulfone and cysteine and cystine to cysteic acid. Hydrochloric acid hydrolysis is then used to determine methionine sulfone and cysteic acid, respectively.

In this article, Analysis of alternative proteins[1)] hydrolyzed by a pre-column derivatization method using Nexera XR, high performance liquid chromatograph, is presented.

Table 1 Free amino acids generated by hydrolysis

Proteinogenic amino acid	Hydrolysis	Target amino acid	No.
Aspartic Acid	Acid hydrolysis	Aspartic Acid	1
Asparagine			
Glutamic Acid		Glutamic Acid	2
Glutamine			
Serine		Serine	3
Histidine		Histidine	4
Glycine		Glycine	5
Threonine		Threonine	6
Arginine		Arginine	7
Alanine		Alanine	8
Tyrosine		Tyrosine	9
Valine		Valine	11
Phenylalanine		Phenylalanine	14
Isoleucine		Isoleucine	15
Leucine		Leucine	16
Proline		Proline	17
Lysine		Lysine	18
Tryptophan	Alkaline hydrolysis	Tryptophan	13
Cysteine	Performic acid oxidation + Acid hydrolysis	Cysteic acid	19
Cystine			
Methionine		Methionine Sulfone	20

■ Analyses of standard solutions

Chromatograms of mixed standard solutions of amino acids produced by hydrolysis (Table 1) are shown in Fig. 1 and Fig. 2.

To quantitate hydrolyzed amino acids, two sets of analytical conditions are required: one for amino acids obtained by hydrochloric acid hydrolysis and alkaline hydrolysis (hereinafter referred to as analytical conditions 1), and the other for amino acids obtained by performic acid oxidation and hydrochloric acid hydrolysis (hereinafter referred to as analytical conditions 2). In the pre-column amino acid analysis described in this article, samples and the derivatization reagents are automatically mixed in the needle of the autosampler using the automatic pretreatment function that is originally equipped with Nexera XR. Analytical conditions 1 are shown in Tables 2 and Table 3, and the derivatization flow is shown in Fig. 3. Analytical conditions 2 are shown in Table 4 and Table 5, and the derivatization flow is shown in Fig. 4. Please refer to Application News 01-00441-EN[2)] for the detailed pretreatment program settings.

Both sets of analytical conditions use the partly same derivatization reagents and the sample preparation solution. Respective preparation methods are shown in Table 6.

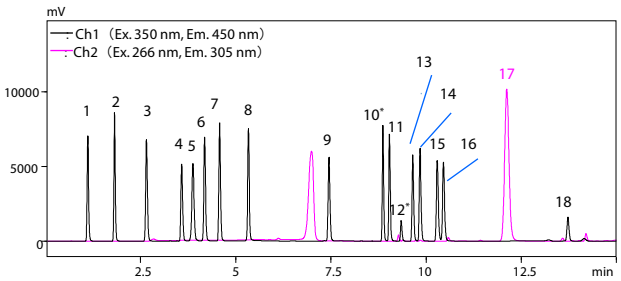

Fig.1 Chromatogram of eighteen standard amino acids mixture (each 100 µmol/L) (Analytical conditions 1)

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

* are not amino acids generated by hydrolysis.

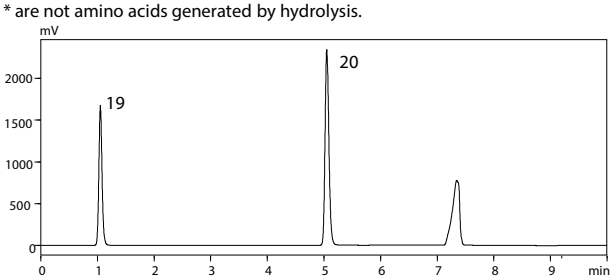

Fig.2 Chromatogram of cysteic acid and methionine sulfone (each 100 µmol/L) (Analytical conditions 2)

19 Cysteic acid, 20 Methionine Sulfone

Table 2 Analytical conditions 1

System	: Nexera XR
Column	: Shim-pack™ XR-ODSII*1 (100 mm × 3.0 mm I.D., 2.2 µm)
Mode	: Low pressure gradient
Mobile phase	: A) 20 mmol/L (Sodium) acetate buffer (pH 6) *2 B) Water/Acetonitrile = 1:9 C) 20 mmol/L (Sodium) acetate buffer (pH 5) containing 0.5 mmol/L EDTA-2Na *3
Flow rate	: 1.0 mL/min
Column temp.	: 40 ℃
Injection volume	: 1 µL
Sample cooler	: 4 ℃
Detection	: Fluorescence detector (Cell temp. : 25 ℃) Ch1) Ex. 350 nm, Em. 450 nm Ch2) Ex. 266 nm, Em. 305 nm
Vial	: SHIMADZU LabTotal™ for LC 1.5 mL, Glass*4

*1 P/N 228-41624-92
*2 Mobile Phase A :
Add 2.67 g of sodium acetate trihydrate and 41 µL of acetic acid into 1000 mL of ultrapure water.
*3 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.
*4 P/N 227-34001-01

Table 3 Gradient profile (Analytical conditions 1)

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

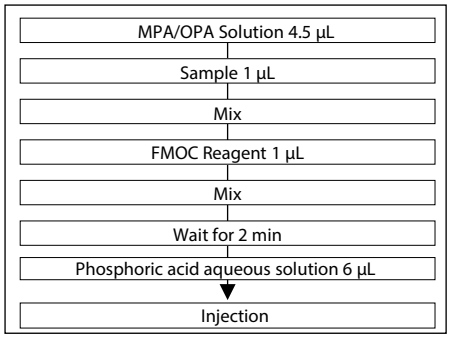

Fig.3 Derivatization procedure using automatic pretreatment function (Analytical conditions 1)

Table 4 Analytical conditions 2

System	: Nexera XR
Column	: Shim-pack XR-ODSII (100 mm × 3.0 mm I.D., 2.2 µm)
Mode	: Low pressure gradient
Mobile phase	: A) 20 mmol/L (Sodium) citrate buffer (pH 4.6)*1 B) Water/Acetonitrile = 1:9
Flow rate	: 1.0 mL/min
Column temp.	: 40 ℃
Injection volume	: 1 µL
Sample cooler	: 4 ℃
Detection	: Fluorescence detector (Cell temp. : 25 ℃) Ex. 350 nm, Em. 450 nm

*1 Mobile Phase A :
Add 2.1 g of citric acid monohydrate and 2.94 g of trisodium citrate dihydrate into 1000 mL of pure water.

Table 5 Gradient profile (Analytical conditions 2)

Time (min)	A.conc	B.conc
0	85	15
5	78	22
6	78	22
6.01	0	100
8	0	100
8.01	85	15
10	85	15

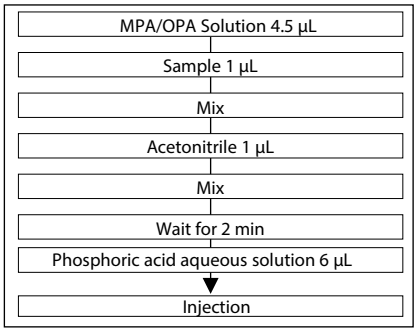

Fig.4 Derivatization procedure using automatic pretreatment function (Analytical conditions 2)

Table 6 Preparations of derivatization reagents and sample preparation solution

- 0.1 mol/L Borate buffer
  Add 0.62 g of boric acid and 0.20 g of sodium hydroxide into 100 mL of ultrapure water.
- 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 into 100 mL of pure water.
- 10 mmol/L HCl (The sample preparation solution)
  Add 4.35 mL of hydrochloric acid into 500 mL of ultrapure water. Then dilute this solution 10-fold with ultrapure water.

■ Linearity

Please refer to Application News 01-00441-EN for the linearities of calibration curves of amino acids except for methionine sulfone and cysteic acid.

This article mentions the linearities of methionine sulfone and cysteic acid. The linearities (coefficient of determination, r^2) calculated using concentrations of 2.5, 5, 10, 25, 50, and 100 µmol/L were more than 0.999 for both, as shown in Table 7.

Table 7 Coefficients of determination of calibration curves

Compound	Linearity (r^2)
Cysteic acid	0.99996
Methionine Sulfone	0.99931

■ Analyses of alternative proteins

The chromatograms of cricket powder and soybean meat after hydrochloric acid hydrolysis shown in Fig. 5 are shown in Fig. 6 and Fig. 7. Fig. 9 and Fig. 10 show the chromatograms of the two samples after alkaline hydrolysis as shown in Fig. 8. Fig 12 and Fig. 13 show the chromatograms of the two samples after perform performic acid oxidation and hydrochloric acid hydrolysis as shown in Fig. 11.

In the repeatability test, the analyses were repeated six times, and the relative standard deviation of respective peak areas were evaluated. The results of reproducibility test are shown in Table 8.

Real sample analyses after hydrochloric acid hydrolysis

Sample 5 mg
- 6 mol/L HCl (500 µL)
  Seal vessel under reduced pressure
  Stand at 110 ℃, 22 hours
  Evaporate to dryness under nitrogen atmosphere
- Water (500 µL)
  Filtration (0.45 µm, 14000 rpm×5 minutes)

Dilute 100-fold with 10 mmol/L HCl

HPLC

Fig.5 Sample pretreatment (hydrochloric acid hydrolysis)

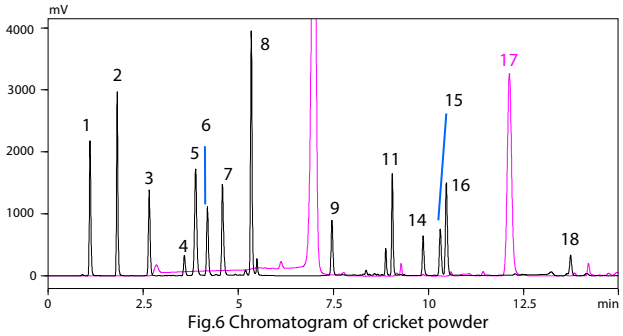

Fig.6 Chromatogram of cricket powder
(hydrochloric acid hydrolysis)

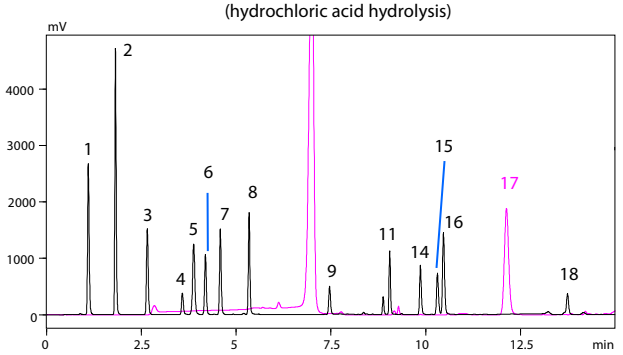

Fig.7 Chromatogram of soybean meat
(hydrochloric acid hydrolysis)

Real sample analyses after alkaline hydrolysis

Sample 5 mg
- 4.2 mol/L NaOH (500 µL)
- 2,2-Thiodiethanol (100 µL)
  Seal vessel under reduced pressure
  Stand at 110 ℃, 22 hours
- Neutralization with 6 mol/L HCl

5 mL - Water

Dilute 5-fold with 10 mmol/L HCl

Filtration (0.22 µm filter)

HPLC

Fig.8 Sample pretreatment (alkaline hydrolysis)

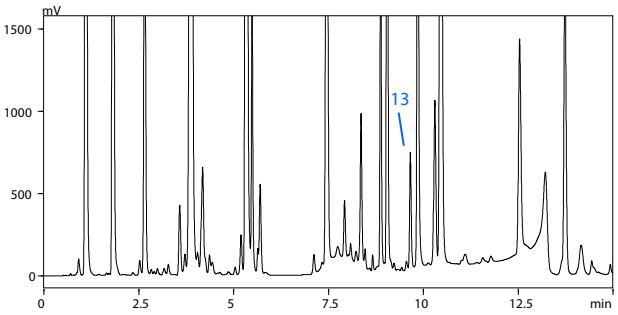

Fig.9 Chromatogram of cricket powder (alkaline hydrolysis)

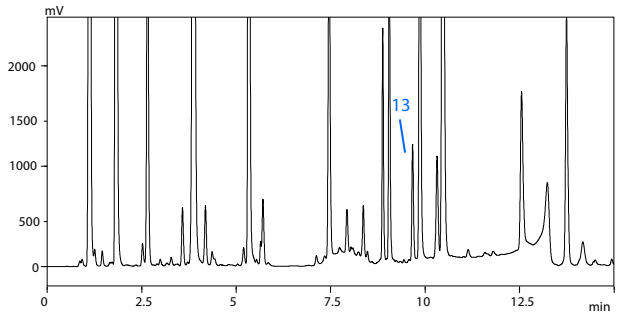

Fig.10 Chromatogram of soybean meat (alkaline hydrolysis)

Real sample analyses after performic acid oxidation・hydrochloric acid hydrolysis

Sample 5 mg
- Performic acid reagent*1(400 µL)
  Store in ice overnight
- Hydrobromic acid (150 µL)
  Evaporate to dryness under nitrogen atmosphere
- 6 mol/L HCl (500 µL)
  Seal vessel under reduced pressure
  Stand at 110 ℃ for 22 hours
  Evaporate to dryness under nitrogen atmosphere
- Water (500 µL)
  Filtration (0.45 µm, 14000 rpm×5 minutes)

Dilute 20-fold with 10 mmol/L HCl

HPLC

Fig.11 Sample pretreatment
(performic acid oxidation・hydrochloric acid hydrolysis)

*1: Mix 9 mL of formic acid and 1 mL of 30% hydrogen peroxide. Stand at room temperature for 1 hour.

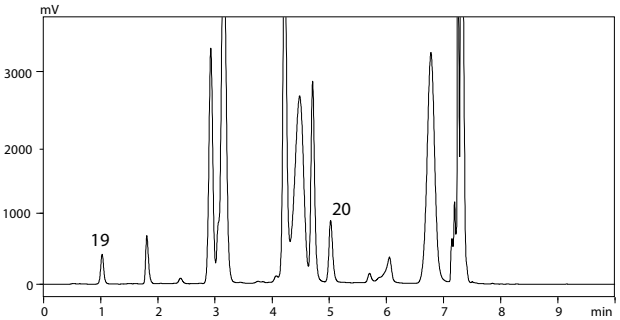

Fig. 12 Chromatogram of cricket powder
(performic acid oxidation・hydrochloric acid hydrolysis)

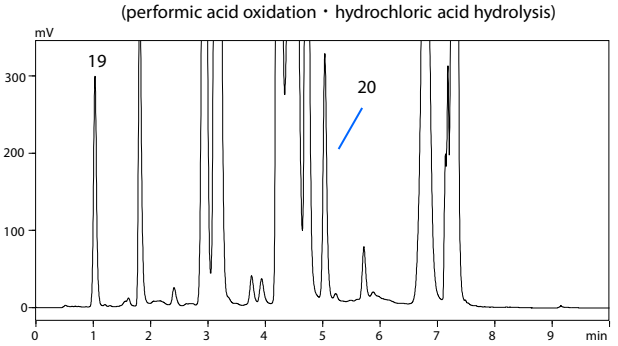

Fig.13 Chromatogram of soybean meat
(performic acid oxidation・hydrochloric acid hydrolysis)

Table 8 Results of peak area repeatabilities （%RSD, n=6）

Target amino acid	Area repeatability	
	Cricket powder	Soybean meat
Aspartic Acid	1.58	1.74
Glutamic Acid	1.58	1.79
Serine	1.99	1.81
Histidine	1.72	2.02
Glycine	2.12	1.57
Threonine	1.60	1.80
Arginine	1.52	1.89
Alanine	1.69	1.67
Tyrosine	1.29	1.86
Valine	1.40	1.98
Phenylalanine	1.30	1.82
Isoleucine	1.37	1.85
Leucine	1.46	1.74
Proline	4.43	2.92
Lysine	2.26	2.09
Tryptophan	2.42	4.56
Cysteic acid	1.00	2.69
Methionine Sulfone	0.92	3.27

■ Conclusion

In this article, amino acid analysis using the automatic pretreatment function has been introduced. Manual derivatization operations were not required, derivatization was executed automatically prior HPLC analysis. In addition, the two different sets of analytical conditions employed the same column, same derivatization reagents, and same mobile phase B, which can be expected to reduce labor and instrument downtime.

The two types of HPLC analysis provided comprehensive determinations of understanding of amino acid contents and would enable indication of nutrition indices such as amino acid score[3].

<References>

1) Good Food Institute, “What are alternative proteins?”, Defining alternative proteins | GFI
2) High-Speed Simultaneous Analysis of Amino Acids by Pre-column Derivatization Using Automatic Pretreatment Function, 01-00441-EN
3) 農林水産省 日本食品標準成分表 2020年版 （八訂）アミノ酸成分表編 (Standard table of food composition in Japan 2020 8th edition amino acid composition table, Ministry of Agriculture, Forestry and Fisheries)

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