

## Application News

High Performance Liquid Chromatograph LC-2050C/ RF-20Axs

### Pre-column Amino Acid Analysis of Hydrolyzed Pet Food

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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 pet food hydrolyzed by a pre-column derivatization method using LC-2050C, integrated high performance liquid chromatograph, is presented.

#### ■ 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 LC-2050 C. Analytical conditions 1 are shown in Tables 2 to 4. Analytical conditions 2 are shown in Tables 5 to 7. Please refer to Application News 01-00709-EN<sup>1)</sup> 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 8.

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	Cysteic Acid	19
Cystine			
Methionine	Acid hydrolysis	Methionine Sulfone	20

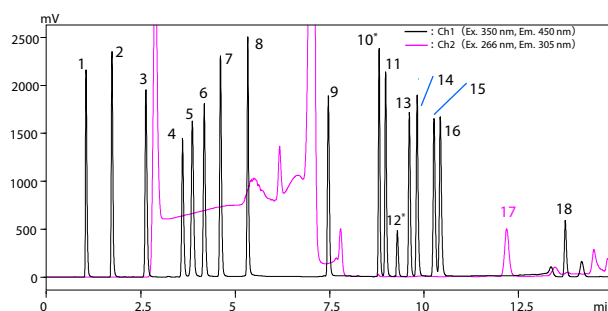


Fig.1 Chromatogram of eighteen standard amino acids mixture (each 25 µmol/L) (Analytical conditions 1)

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.

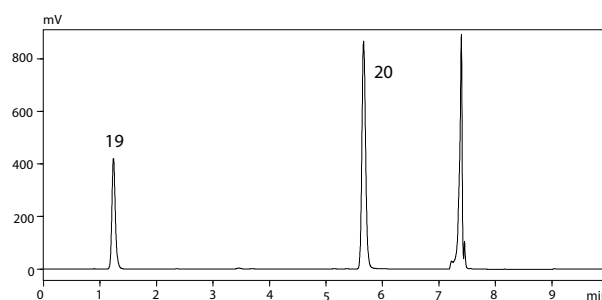


Fig.2 Chromatogram of cysteic acid and methionine sulfone (each 100 µmol/L) (Analytical conditions 2)

19 Cysteic Acid, 20 Methionine Sulfone

Table 2 Analytical conditions 1

System	: LC-2050C <sup>*1</sup>
Column	: Shim-pack™ XR-ODSII <sup>*2</sup> (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) <sup>*3</sup> B) Water/Acetonitrile = 1:9 C) 20 mmol/L (Sodium) acetate buffer (pH 5) containing 0.5 mmol/L EDTA-2Na <sup>*4</sup>
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Injection volume	: 1 μL
Sample cooler	: 4 °C
Detection	: Fluorescence detector (Cell temp. : 25 °C) Ch1) Ex. 350 nm, Em. 450 nm Ch2) Ex. 266 nm, Em. 305 nm
Vial	: SHIMADZU LabTotal™ for LC 1.5 mL, Glass <sup>*5</sup>

\*1 Applicable for LC-2050C 3D

\*2 P/N 228-41624-92

\*3 Mobile Phase A :

Add 2.67 g of sodium acetate trihydrate and 41 μL of acetic acid into 1000 mL of pure water.

\*4 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 pure water.

\*5 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

Table 4 Allocations and addition volumes of derivatization reagents (Analytical conditions 1)

Reagent	Injection volume (μL)
MPA/OPA Solution	9.0
FMOc Solution	0.5
Phosphoric acid solution	5.0

Table 5 Analytical conditions 2

System	: LC-2050C
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) <sup>*1</sup> B) Water/Acetonitrile = 1:9
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Injection volume	: 1 μL
Sample cooler	: 4 °C
Detection	: Fluorescence detector (Cell temp. : 25 °C) : Ex. 350 nm, Em. 450 nm
Vial	: SHIMADZU LabTotal for LC 1.5 mL, Glass

\*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 6 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

Table 7 Allocations and addition volumes of derivatization reagents (Analytical conditions 2)

Reagent	Injection volume(μL)
MPA/OPA Solution	15.0
Acetonitrile	0.5
Phosphoric acid solution	5.0

Table 8 How to prepare 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 300 μL of MPA Reagent and 600 μL OPA Reagent.
- FMOc Reagent  
Dissolve 10 mg of 9-fluorenylmethyl chloroformate into 50 mL of acetonitrile.
- Phosphoric acid aqueous solution  
Add 0.5 mL of phosphoric acid into 100 mL of ultrapure 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

This article mentions the linearities of amino 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 9.

Please refer to Application News 01-00709-EN for the linearities of calibration curves of methionine sulfone and cysteine acid.

Table 9 Coefficients of determination

Target amino acid	Linearity ( $r^2$ )
Asparatic Acid	0.999953
Glutamic Acid	0.999992
Serine	0.999980
Histidine	0.999959
Glycine	0.999991
Threonine	0.999977
Arginine	0.999973
Alanine	0.999967
Tyrosine	0.999918
Valine	0.999996
Phenylalanine	0.999985
Isoleucine	0.999983
Leucine	0.999975
Proline	0.999358
Lysine	0.999874
Tryptophan	0.999997

## ■ Analyses of pet food

The chromatograms of pet food after hydrochloric acid hydrolysis shown in Fig. 3 are shown in Fig. 4. Fig. 6 shows the chromatograms of the sample after alkaline hydrolysis as shown in Fig. 5. Fig. 8 shows the chromatograms of the sample after perform performic acid oxidation and hydrochloric acid hydrolysis as shown in Fig. 7.

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 10.

### Real sample analyses after hydrochloric acid hydrolysis

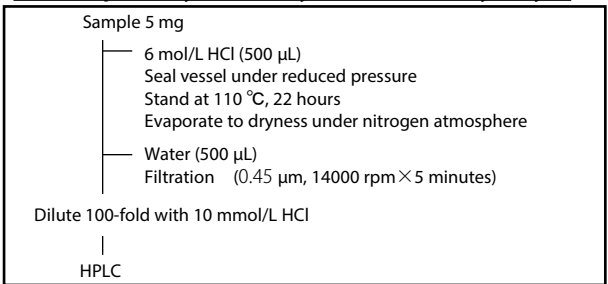


Fig.3 Sample pretreatment (hydrochloric acid hydrolysis)

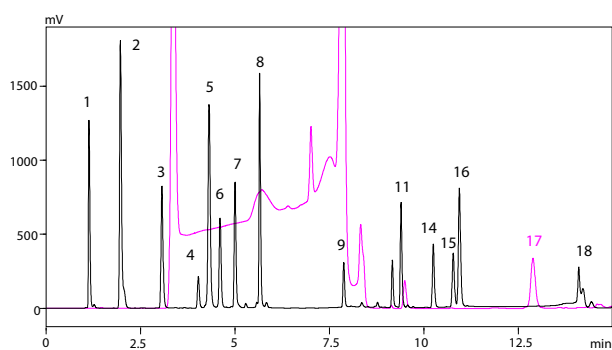


Fig.4 Chromatogram of pet food (hydrochloric acid hydrolysis)

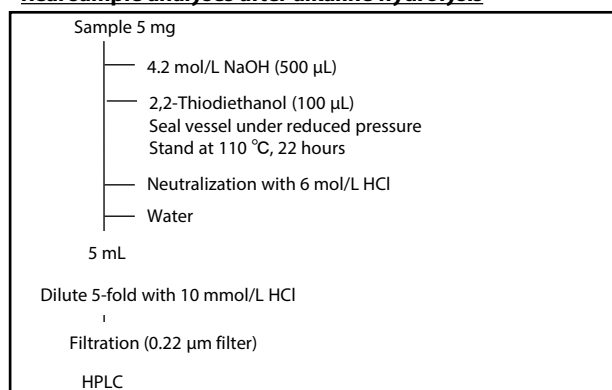
**Real sample analyses after alkaline hydrolysis**

Fig.5 Sample pretreatment (alkaline hydrolysis)

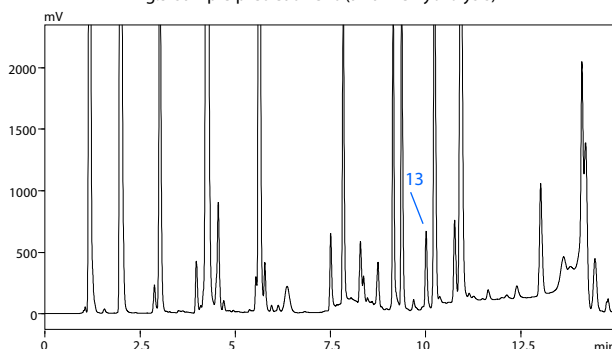


Fig.6 Chromatogram of pet food (alkaline hydrolysis)

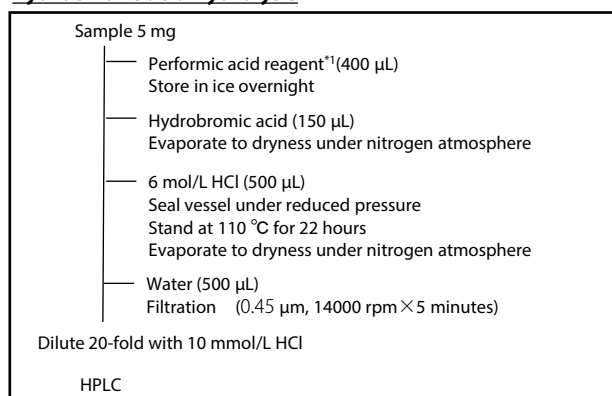
**Real sample analyses after performic acid oxidation • hydrochloric acid hydrolysis**

Fig.7 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.

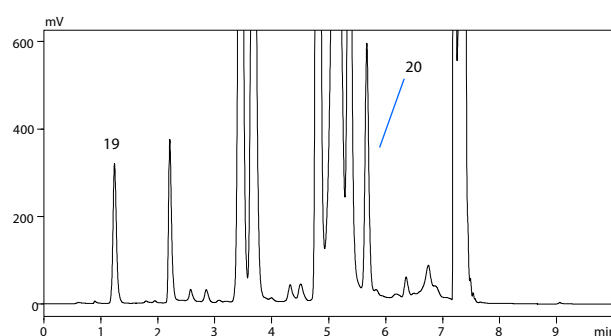
Fig.8 Chromatogram of pet food  
(performic acid oxidation • hydrochloric acid hydrolysis)

Table 10 Results of peak area repeatabilities (%RSD, n=6)

Target amino acid	Area repeatability
Aspartic Acid	0.81
Glutamic Acid	0.58
Serine	0.60
Histidine	0.57
Glycine	0.93
Threonine	0.55
Arginine	0.67
Alanine	0.61
Tyrosine	0.66
Valine	0.66
Phenylalanine	0.65
Isoleucine	0.56
Leucine	0.59
Proline	4.08
Lysine	2.29
Tryptophan	1.46
Cysteic acid	4.36
Methionine Sulfone	4.06

**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<sup>2)</sup>. This analytical method is expected to contribute to product development that meets the health-oriented needs of pets.

## &lt;References&gt;

- 1) Analysis of Methionine Sulfone and Cysteic Acid Using Automated Pretreatment Functions, 01-00709-EN
- 2) Standard table of food composition in Japan 2020 8th edition amino acid composition table, Ministry of Agriculture, Forestry and Fisheries

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