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Application News

No. L529A

High Performance Liquid Chromatography

Simultaneous Analysis of Amino Acids Using Automatic Pretreatment Function of Prominence™-i Integrated LC System

The analysis of amino acids is necessary in various fields such as the fields of food and pharmaceuticals. A method often used in the analysis of amino acids with HPLC is post-column derivatization, and Shimadzu amino acid analysis system also uses the method. However, post-column derivatization is difficult to speed up due to the characteristics of the column which is used.

Previously, in Application News No. L432A, we introduced the rapid pre-column derivatization method using a UHPLC system for amino acid analysis. This article introduces even easier and convenient method of amino acid analysis using an automated pre-column derivatization method which can be used with integrated HPLC system.

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Automated Pre-Column Derivatization

The Prominence-i (LC-2030C) is equipped with automatic pretreatment functions in injection unit as standard. Using the co-injection function, solutions can be aspirated sequentially from multiple vials and mixed within the needle. Well-known derivatization reagents for amino acids are *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC), both of which react rapidly with amino acids at room temperature. Use of this function allows derivatization to be performed automatically within the needle.

Fig. 1 shows the setting details and Fig. 2 shows the operation flow. As indicated, settings are simple even for operations involving sequential aspiration of solutions.

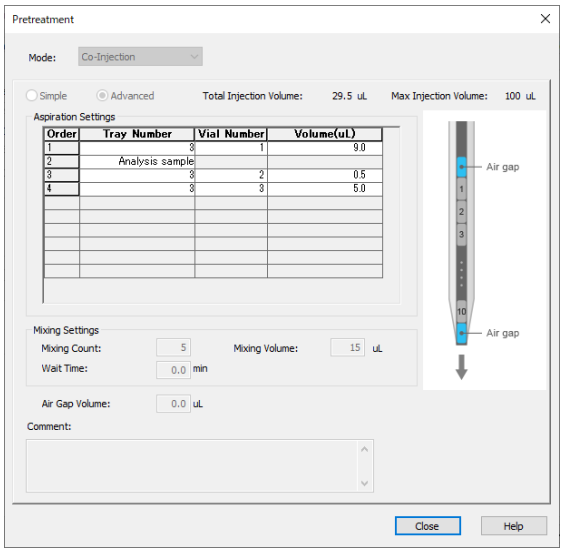


Fig. 1 **Prominence-i (LC-2030C) Pretreatment (Co-Injection) Setting Window**

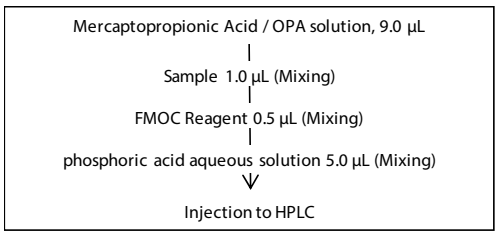


Fig. 2 **Automated Pre-Column Derivatization by Prominence-i**

Table 1 **Derivatization Reagents**

- Mercaptopropionic Acid 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 pure water.
- Mercaptopropionic Acid / OPA solution
  Mix 300 µL of Mercaptopropionic Acid Reagent and 600 µL OPA Reagent.
- FMOC Reagent
  Add 10 mg of 9 – fluorenylmethl chloroformate into 50 mL of acetonitrile.
- Mobile phase A : 20 mmol/L Sodium acetate buffer (pH6)
  Add 2.67 g of sodium acetate trihydrate and 41 µL of acetic acid into 1000 mL of pure water.
- Mobile phase B : Water/Acetonitrile = 100/900
- Mobile phase C : 20 mmol/mL Sodium acetate buffer (pH5) containing 0.5 mmol/L EDTA-2Na
  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.
- phosphoric acid aqueous solution
  Add 0.5 mL of phosphoric acid into 100 mL of pure water.

Analysis Results

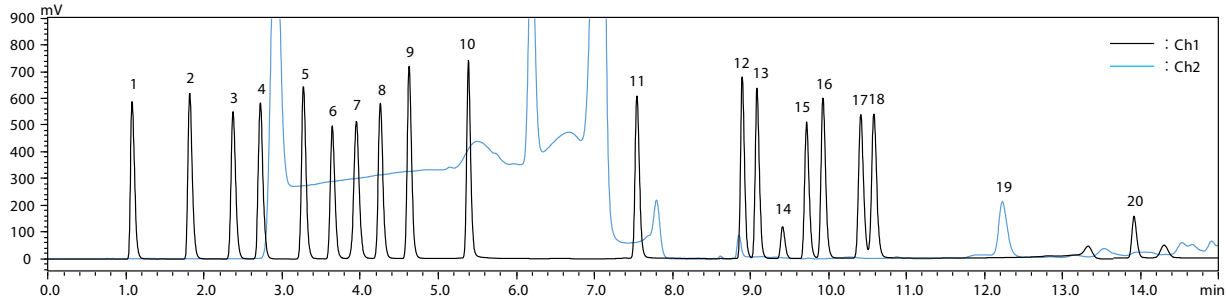
The analytical conditions are listed in Tables 2 and 3. The chromatograms obtained from the analysis of 20 proteinogenic amino acids are shown in Fig. 3.

Table 2 **Analytical Conditions**

Column	:	Shim-pack™ XR-ODSII 100 mmL. × 3.0 mmI.D., 2.2 µm
Mode	:	Low pressure gradient
Mobile phase	:	See the table 1
Flow rate	:	1.0 mL/min
Column temp.	:	40 °C
Injection volume	:	1 µL
Detection	:	Fluorescence detector Ch1) Ex. 350 nm, Em. 450 nm Ch2) Ex. 266 nm, Em. 305 nm

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



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. 3 **Simultaneous Analysis of 20 Proteinogenic Amino Acids (12.5 pmol/µL Each)**

Linearity and Repeatability

For each of the amino acids, we evaluated the linearity (r^2: coefficient of determination) using concentrations of 0.25, 1.25, 2.5, 12.5, and 25 pmol/µL (except for proline (Pro) for which 2.5, 12.5, 25, 62.5, and 125 pmol/µL were used). We also evaluated the area repeatability at 12.5 pmol/µL through repeated analyses (n=6). The resulting values are listed in Table 4.

Table 4 **Linearity and Area Repeatability**

	Linearity (r^2)	Area (%RSD)		Linearity (r^2)	Area (%RSD)
Asp	0.9999978	0.597	Tyr	0.9999841	0.699
Glu	0.999984	0.567	Met	0.9999028	0.660
Asn	0.9999949	0.537	Val	0.9999785	0.627
Ser	0.9999009	0.522	Cystine	0.9999784	0.520
Gln	0.9999862	0.592	Trp	0.9999718	0.607
His	0.9999564	0.706	Phe	0.9999847	0.818
Gly	0.9999155	0.446	Ile	0.9999703	0.633
Thr	0.9998575	0.660	Leu	0.999986	0.902
Arg	0.9997389	0.660	Pro	0.99935	4.768
Ala	0.9994783	0.470	Lys	0.9994412	1.914

Analysis of Other Amino Acids

There are many amino acids which are said to contribute to health in addition to the 20 proteinogenic amino acids. A simultaneous analysis of 4 amino acids which are particularly gaining attention in recent years—citrulline, taurine, theanine, and γ-aminobutyric acid (GABA)—together with the predescribed 20 amino acids is shown in Fig. 4.

Analysis of Actual Samples

An example analysis of beer is shown in Fig. 5. The sample was diluted with10 mmol/L hydrochloric acid by a factor of 10, filtered through a filter with a pore size of 0.22 µm and then analyzed. The analytical conditions listed in Tables 2 and 3 were used.

As described, amino acids can be analyzed easily through pre-column derivatization using the automatic pretreatment function of Prominence-i. Since the derivatization is performed within the needle, the required sample and reagent volumes can be minimized. In addition, all reactants are injected into the column resulting in a highly sensitive analysis. Compared with the conventional method using an empty vial for derivatization reaction, this new method eliminates the need to prepare additional vials for the reaction, thereby simplifies analysis preparations as well.

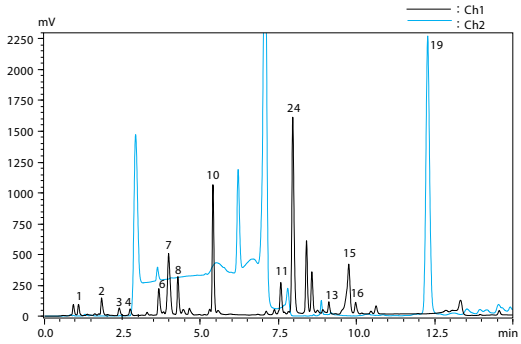
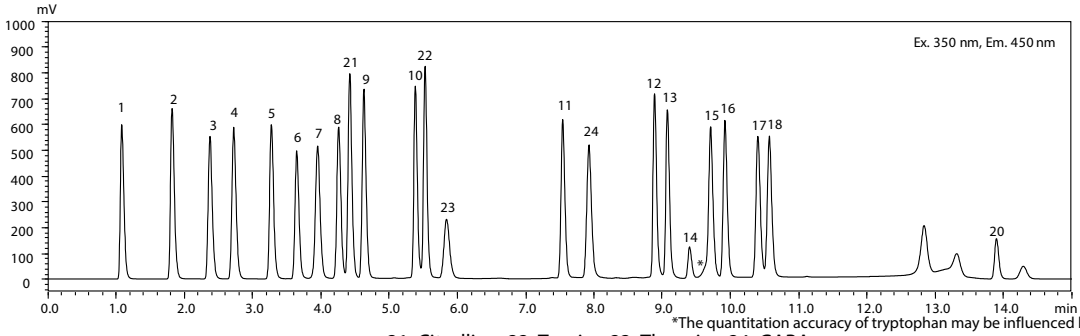


Fig. 5 **Chromatograms of Beer (Peak numbers and corresponding amino acids are as indicated in Figs. 3 and 4.)**



*The quantitation accuracy of tryptophan may be influenced by GABA content.

21, Citrulline, 22, Taurine 23, Theanine 24, GABA

Fig. 4 **Analysis of 23 Amino Acids Including Functional Amino Acids (12.5 pmol/µL Each)**

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First Edition: Jan. 2019
Second Edition: Jan. 2020

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