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Application News

No. L579

High Performance Liquid Chromatography

Selection of Detector in Analysis of Amino Acids Using Pre-column Derivatization Method

Most amino acids, except for some of the aromatic amino acids, only have ultraviolet (UV) absorption at short wavelength(200 nm – 210 nm). In general, there are two methods to analyze amino acids with HPLC. One is called pre-column derivatization method that derivatize amino acids before column. The other is called post-column derivatization method that derivatize amino acids after column. Pre-column derivatization method is widely used because it can be easily transferred to UHPLC analysis using reversed phase columns.

Furthermore, there are two main methods of detecting derivatized amino acids: UV detection and fluorescence detection. Since fluorescence detection directly detects the light energy emitted from fluorescent substances, it is highly selective and sensitive.

This article introduces an analysis of amino acids with pre-column derivatization method using a UV detector, comparison of sensitivity between UV detection and fluorescence detection, and the effects of sample solvents in pre-column derivatization of amino acid. Pre-column derivatization was performed automatically using the co-injection function of the integrated HPLC system i-Series introduced in L529B.

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Automatic Pre-column Derivatization

i-Series is equipped with automatic pretreatment functions in autosampler as standard. The derivatization of amino acids was performed using the co-injection function. Derivatization reagents for amino acids were *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC), both of which react rapidly with amino acids at room temperature.

Use of this function provide automatic derivatization within the needle. Fig. 1 shows the screen capture of pretreatment function window on LabSolutions™. Table 1 shows the derivatization reagents and mobile phases. Table 2 and Table 3 show the analytical conditions and time program, respectively. For UV detection, 350 nm and 266 nm, the excitation wavelengths for fluorescence detection, were used.

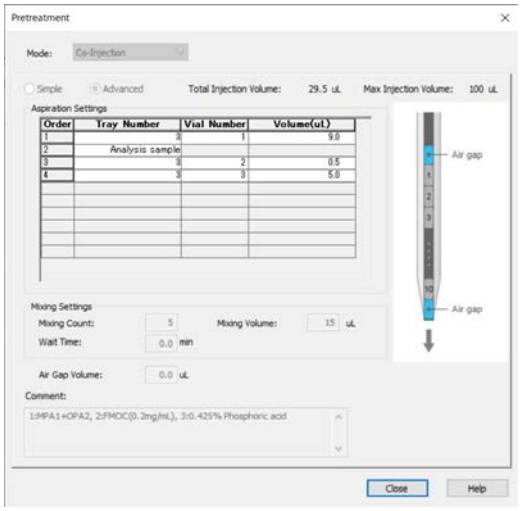


Fig. 1 Screen Capture of Pretreatment Function Window (Co-Injection)

Table 1 Derivatization Reagents and Mobile Phases

- **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 ultrapure water.
- **Mercaptopropionic Acid / OPA solution**
  Mix 300 µL of Mercaptopropionic Acid Reagent and 600 µL of OPA Reagent.
- **FMOC Reagent**
  Add 10 mg of 9-fluorenylmethyl chloroformate into 50 mL of acetonitrile.
- **Mobile Phase A: 20 mmol/L Sodium acetate buffer (pH 6)**
  Add 2.67 g of sodium acetate trihydrate and 41 µL of acetic acid into 1000 mL of ultrapure water.
- **Mobile Phase B: Water/Acetonitrile = 100 : 900**
  Add 100 mL of ultrapure water into 900 mL of acetonitrile.
- **Mobile Phase C: 20 mmol/L Sodium acetate buffer (pH 5) 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 ultrapure water.
- **Phosphoric Acid Aqueous Solution**
  Add 0.5 mL of phosphoric acid into 100 mL of ultrapure water.

Table 2 Analytical Conditions

System	: i-Series (LC-2050C 3D)
Column	: Shim-pack™ XR-ODSII (100 mm × 3.0 mm I.D., 2.2 µm)*1
Vial	: SHIMADZU LabTotal™ Vial for LC 1.5 mL, Glass*2
Mobile phase	: See the table1
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Injection volume	: 1 µL
Detection	: ①Ch1) 350 nm, Ch2) 266 nm (UV) ②Ch1) Ex. 350 nm, Em. 450 nm Ch2) Ex. 266 nm, Em. 305 nm (RF-20AXS)

*1: P/N 228-41624-92, *2: P/N 227-34001-01

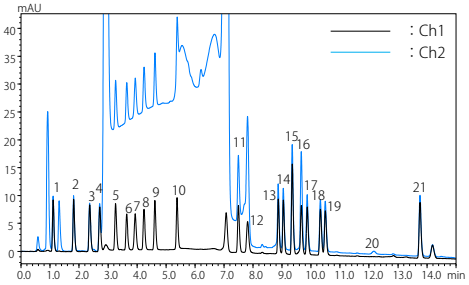
Table 3 Time Program

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

Analysis Using UV Detector

The chromatograms of 21 amino acids standard solution (125 µmol/L), consisting of proteinogenic amino acids and γ-aminobutyric acid (GABA) as a functional compound, using the UV detector are shown in Fig. 2.

The chromatogram of sports drink (UV detection) is shown in Fig. 3. When the concentrations of the target amino acids are high enough, the UV detector can be applied.



GABA may affect the quantitative accuracy of tryptophan.

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, GABA 13, Methionine 14, Valine
15, Cystine 16, Tryptophan 17, Phenylalanine 18, Isoleucine
19, Leucine 20, Proline 21, Lysine

Fig. 2 Chromatograms of 125 µmol/L Amino Acids Standard Solution (UV Detection)

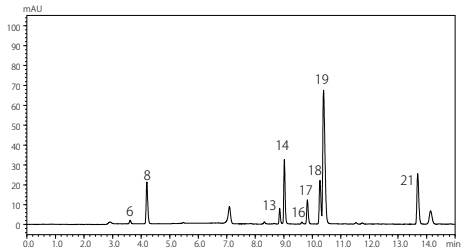


Fig. 3 Chromatogram of Sports Drink (UV detection, Ch1) (For the peak numbers, see Fig. 2)

Analysis Using Fluorescence Detector

The chromatograms of 25 µmol/L 21 amino acids standard solution using fluorescence detector, are shown in Fig. 4. Furthermore, Table 4 shows the relative sensitivity of fluorescence detection based on the UV detection through amino acids standard analysis. These values were calculated by dividing the limit of detection (LOD) of UV detector by the LOD of fluorescence detector. Although it depends on the type of amino acids, fluorescence detection is approximately 200 times more sensitive than UV detection.

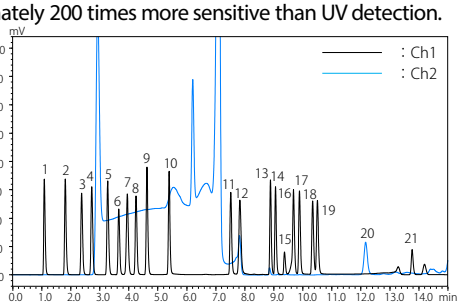


Fig. 4 Chromatograms of 25 µmol/L Amino Acids Standard Solution (Fluorescence Detection) (For the peak numbers, see Fig. 2)

Table 4 Relative Sensitivity of Fluorescence Detection based on UV Detection

Compound	Relative sensitivity of fluorescence detection	Compound	Relative sensitivity of fluorescence detection
Asp	225	Tyr	206
Glu	219	GABA	210
Asn	206	Met	199
Ser	222	Val	193
Gln	230	$(Cys)_2$	28
His	199	Trp	200
Gly	236	Phe	208
Thr	219	Ile	189
Arg	240	Leu	201
Ala	226	Pro	35
		Lys	49

Effect of Sample Solvents

Derivatization of amino acids by OPA is known to have different reaction yield depending on the pH of reaction environment. Therefore, it is important to make the identical pH condition of sample to that of standard solution as much as possible. The solvents of amino acids standard solutions are often 100 mmol/L hydrochloric acid aqueous solution.

Fig. 5 shows the results of tomato juice diluted 1000 times with different concentrations of hydrochloric acid aqueous solutions. This graph represents the relative area values of amino acids obtained by diluting the sample with 100 mmol/L hydrochloric acid aqueous solution, where the area values of each amino acid in the sample diluted with 10 mmol/L hydrochloric acid aqueous solution are defined as 100%. Even in the same sample, the difference in reaction yield results in different peak areas due to different sample solvents. The highly sensitive fluorescence detector allows the samples to be sufficiently diluted, making it easy to achieve the final solvents pH equivalent to the standard solutions.

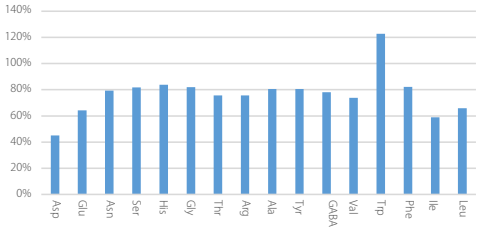


Fig. 5 Relative Area Values of Amino Acids in Tomato Juice Diluted with 100 mmol/L Hydrochloric Acid Aqueous Solution (The area values of amino acids when diluted with 10 mmol/L hydrochloric acid aqueous solution were defined as 100%)

Conclusion

In this article, the pre-column derivatization analysis of amino acids using the UV detector was firstly introduced. When the concentrations of the target amino acids are high enough, the UV detector can also be applied. Next, the sensitivity of UV detection and fluorescence detection were compared. For the 21 amino acids mentioned above, fluorescence detection was 200 times more sensitive than UV detection. The fluorescence detector is recommended when highly sensitive analysis is required. For the analysis of amino acids using pre-column derivatization method, it is important to make the identical pH condition of sample to that of standard solution as much as possible. Since fluorescence detection is highly sensitive, it is easy to dilute the solvents of samples to get closer to the solvents of standard solutions. It is useful when the solvents composition of the samples differ greatly from the standard solutions.

i-Series is equipped with either one UV or PDA detector as standard and can be used for analysis of amino acids using pre-column derivatization method. If highly sensitive analysis is required, additional fluorescence detector can be installed.

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