

UHPLC Method for Sensitive Automatic Analysis of Thirty-Seven D/L-Amino Acids and for Liquor Profiling

Natsuki Iwata

User Benefits

- ◆ Enables high-sensitivity simultaneous fast analysis of thirty-seven D/L-amino acids using simple operations.
- ◆ Good reproducibility of analytical results can be achieved by keeping the derivatization reaction time constant.
- ◆ Statistical analysis software enables easy multivariate analysis of multiple samples.

■ Introduction

Recent advances in analytical techniques have revealed the presence of D-amino acids in fermented foods and biological samples and clarified their role in the taste, preservation, and aroma of foods, and as potential disease biomarkers. Therefore, the demand for D/L separation of amino acids is increasing. Because D-amino acids are only present in trace amounts in fermented foods and biological systems, unlike L-amino acids, it is necessary to determine the concentration of each D-amino acid by separating them from the high concentrations of L-amino acids.

A comprehensive method for quantitating the thirty-seven D/L-amino acids was previously reported¹⁾, in which two chiral derivatizing reagents were employed to provide different diastereomers for each amino acid, with the instrument automatically switching between two separation methods to analyze each sample twice.

This article introduces an improved method that employs a single chiral derivatizing reagent to enable simultaneous analysis of D/L-amino acids in a shorter time.

■ Fluorescence Derivatization of D/L-Amino Acid Diastereomers

N-isobutryl-L-cysteine (NIBC) was used for chiral derivatization. Fluorescence derivatization of D/L-amino acid diastereomers was performed by reaction with *o*-phthalaldehyde (OPA) in the presence of NIBC.

■ D/L-Amino Acid Analysis by UHPLC

In general, LC/MS or multi-dimensional LC is used to analyze D/L-amino acids by HPLC, because it is difficult to separate proteinogenic D/L-amino acids with a single separation mode. However, it is known that LC/MS is susceptible to matrix effects and less quantitative than HPLC. It is also known that the multi-dimensional LC method requires a long analysis time and very complicated HPLC setup. Therefore, a simple operational method that provides good separation for D/L-amino acids in a short time is needed.

For food analysis, a small particle column is used to achieve appropriate separation of the small amounts of D-amino acids from the large amounts of L-amino acids and co-existing contaminants. A UHPLC system (Nexera™ X3) was employed in this study due to the increased system pressure caused by the use of a small particle column.

■ Method for Automatic High-Sensitivity Analysis of D/L-Amino Acids

Thirty-seven D/L-amino acids were targeted (excluding D/L-prolines from proteinogenic amino acids).

Table 1 shows the analytical conditions, and Fig. 1 shows the process flow diagram of the HPLC configuration for automated analysis. As in [Application News No. L592](#), the mobile phases were prepared automatically using the mobile phase blending function of the solvent delivery pump. D/L-amino acids were derivatized using the automatic pretreatment function of the autosampler. The mobile phase blending function provided the solutions to deliver at the specified blending ratio just by setting the organic solvents at the ports of the low-pressure gradient kit integrated into the pump. The labor and the working time were reduced by this function. In addition, the automatic pretreatment function is described below. The OPA/NIBC derivatizing reagents and the target samples were set in the autosampler. This derivatization process was completed within the injection needle and the derivatized diastereomers were introduced into the column without any exposure to the external air (Table 2 and Table 3). For reference, a schematic diagram of the autosampler rack is shown in Fig. 2. On the other hand, automation of the sample-preparation step is essential due to OPA derivatization products beginning to degrade immediately after reaction initiation. The elapsed time from starting the derivatization to injection to HPLC was kept constant using the pretreatment function, resulting in good reproducibility of analysis results. In addition, the cost of consumables was reduced because vials were not required for derivatization. The automatic operations reduced the labor and approximately an hour of the working time in comparison with manual operations (Table 4). The analysis described in this article can be implemented with a simple instrument configuration, as shown in Fig. 1. Furthermore, optimization of the automated pre-column derivatization procedure enabled detection with higher sensitivity than conventional methods²⁾.

Table 1 Analytical Conditions

System:	Nexera X3
Column:	CERI L-column 3 C18 (150 mm × 2.1 mm I.D., 2.0 μm) using pre-column filter
Flowrate:	0.22 mL/min
Mobile Phase:	Pump A 10 mmol/L (Sodium) phosphate buffer (pH 6.9) Pump B b) Acetonitrile c) Methanol b/c = 15:85 using mobile phase blending function
Time Program:	8 % B (0 - 7.27 min) → 27 % B (18.18 - 30.00 min) → 36 % B (32.00 min) → 41 % B (40.91 min) → 46 % B (54.55 min) → 50 % B (58.55 min) → 52 % B (65.28 min) → 80 % B (66.19 - 74.36 min) → 8 % B (75.27-87 min)
Column Temp.:	20 °C
Injection Volume:	1 μL
Vial:	SHIMADZU LabTotal for LC 1.5 mL, Glass*1
Detection:	RF-20AXS, Ex: 338 nm, Em: 455 nm (semimicro cell)

*1: P/N: 227-34001-01

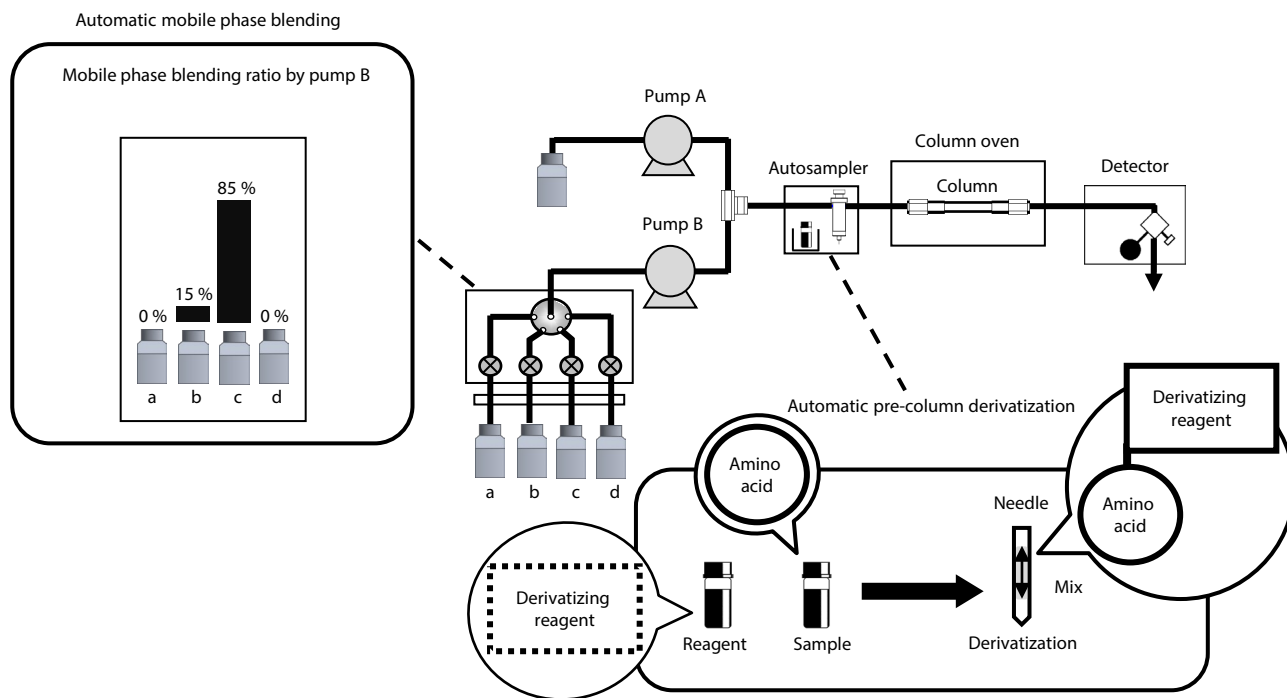


Fig. 1 Schematic Diagram of Automated System with Mobile Phase Blending and Automatic Pre-column Derivatization

Table 2 Preparation of Derivatizing Reagents

0.1 mol/L Borate Buffer (pH 9.1):	Add 0.62 g boric acid and 0.20 g of sodium hydroxide to 100 mL of ultrapure water, and dissolve completely.
OPA Reagent:	Add 0.3 mL of ethanol to 10 mg of o-phthalaldehyde and dissolve completely. Then add 0.7 mL of 0.1 mol/L borate buffer (pH 9.1) and 4 mL of ultrapure water.
NIBC Solution:	Add 10 mg of N-isobutryl-L-cysteine to 10 mL of 0.1 mol/L borate buffer (pH 9.1).
OPA/NIBC Solutions:	Mix equal volumes of OPA reagent and NIBC solution.

Table 3 Automatic Pretreatment Program with Autosampler (When OPA/NIBC Solution Vial is Placed in Tray No. 3, Vial No. 54)

(a) File 1

Line	Command
1	av=0.0
2	air.a 6.0,ss
3	s.aspir 3,54,2.0
4	s.aspir m,sn,iv
5	air.a 0.1,ss
6	call f2
7	wait 1.5
8	s.load
9	s.inj
10	posttr
11	end

(b) File 2

Line	Command
1	n.drain
2	for a1=1,10
3	aspir 5.5,ss
4	disp 5.5,ss
5	next a1
6	end

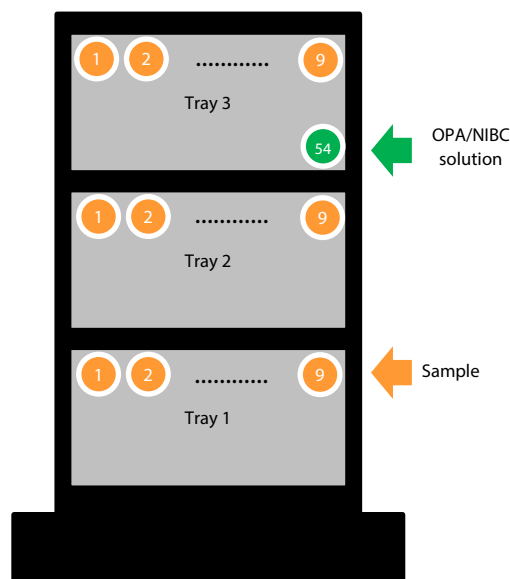


Fig. 2 Schematic Diagram of Autosampler Rack (OPA/NIBC Solution Vial: Tray No. 3, Vial No. 54)

Table 4 Comparison of Working Time between Automatic and Manual Operations when 20 Samples were Analyzed

		Automatic	Manual
Mobile phase preparation	Buffer	5 min	
	Organic solvent	○ 0 min Note: Mobile phase blending function	× 5 min
Derivatizing reagent preparation		10 min	
Derivatization	○ 0 min Note: Automatic pre-column derivatization	× 50 min	
Total	○ 15 min	× 70 min	

■ Analysis of D/L-Amino Acids Standard Solution

A chromatogram of a standard solution of D/L-amino acids (1 $\mu\text{mol/L}$ each) is shown in Fig. 3. The thirty-seven D/L-amino acids were separated in approximately 66 minutes.

■ Calibration Curve

The linearities of the calibration curves for the thirty-seven D/L-amino acids were good. Each contribution ratio (r^2) was 0.999 or greater (Table 5).

■ Repeatability

The relative standard deviations (%RSD) of the peak areas based on six repeated analyses of a standard solution of D/L-amino acids (0.1 $\mu\text{mol/L}$ each and 5 $\mu\text{mol/L}$ each) were 1.6 % or less and 0.8 % or less, respectively. Repeatability was satisfactory by keeping the time from derivatization to the start of analysis constant.

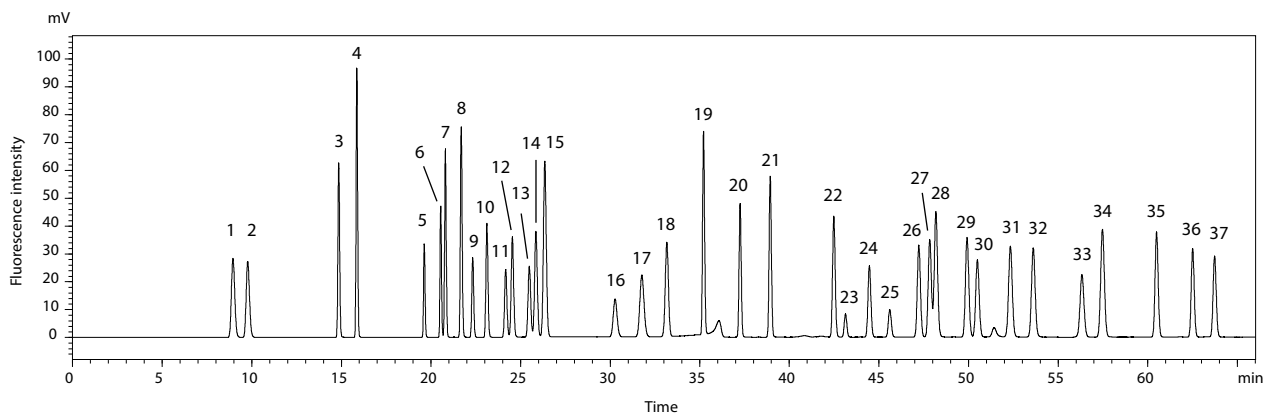


Fig. 3 Chromatogram for Mixed Standard Solution (1 $\mu\text{mol/L}$ each)

Table 5 Concentration Ranges of Calibration Curves and Coefficients of Determination (r^2)

No.	Compound	Conc. Range ($\mu\text{mol/L}$)	r^2	No.	Compound	Conc. Range ($\mu\text{mol/L}$)	r^2
1	L-Asp	0.5-50	0.9997	20	L-Tyr	1-50	0.9996
2	D-Asp	0.01-2	0.9999	21	D-Tyr	0.01-2	0.9999
3	L-Glu	1-50	0.9996	22	L-Val	1-50	0.9996
4	D-Glu	0.01-2	0.9999	23	L-(Cys) ₂	0.05-10	0.9999
5	L-Asn	0.5-50	0.9997	24	L-Met	0.01-5	0.9999
6	D-Asn	0.05-5	0.9999	25	D-(Cys) ₂	0.02-2	0.9999
7	L-Ser	0.2-50	0.9997	26	L-Trp	0.5-50	0.9996
8	D-Ser	0.01-5	0.9999	27	D-Met	0.01-5	0.9999
9	L-Gln	1-50	0.9997	28	D-Val	0.01-2	0.9999
10	D-Gln	0.1-5	0.9999	29	L-Ile	0.2-50	0.9997
11	L-His	1-50	0.9996	30	L-Phe	1-50	0.9996
12	L-Thr	1-50	0.9996	31	D-Trp	0.01-2	0.9999
13	D-His	0.1-10	0.9999	32	D-Phe	0.01-1	0.9999
14	D-Thr	0.01-5	0.9999	33	L-Leu	1-50	0.9996
15	Gly	1-50	0.9997	34	D-Ile	0.01-2	0.9999
16	L-Arg	1-50	0.9996	35	D-Leu	0.01-2	0.9999
17	D-Arg	0.01-2	0.9999	36	L-Lys	0.5-50	0.9997
18	L-Ala	1-50	0.9996	37	D-Lys	0.01-2	0.9999
19	D-Ala	0.01-5	0.9999				

Application to Liquor Samples

Two kinds of beer (Beer A and B), sake, red wine, and white wine were used as samples. Beer A, Beer B, red wine, and white wine were diluted ten-fold with 10 mmol/L hydrochloric acid and then passed through 0.2 μm membrane filters. The sake was diluted twenty-fold with 10 mmol/L hydrochloric acid and then passed through a 0.2 μm membrane filter.

As shown in Fig. 4, twenty-five to twenty-eight amino acids were separated and detected in five liquor samples. Table 6 shows the D/L-amino acids contained in the liquor samples obtained from four repeated analyses. It should be noted that the concentrations of some L-amino acids were outside of the quantitation ranges. The overall ratio of D-amino acid to D/L-amino acid (%D) was found to be 6% or less in all samples.

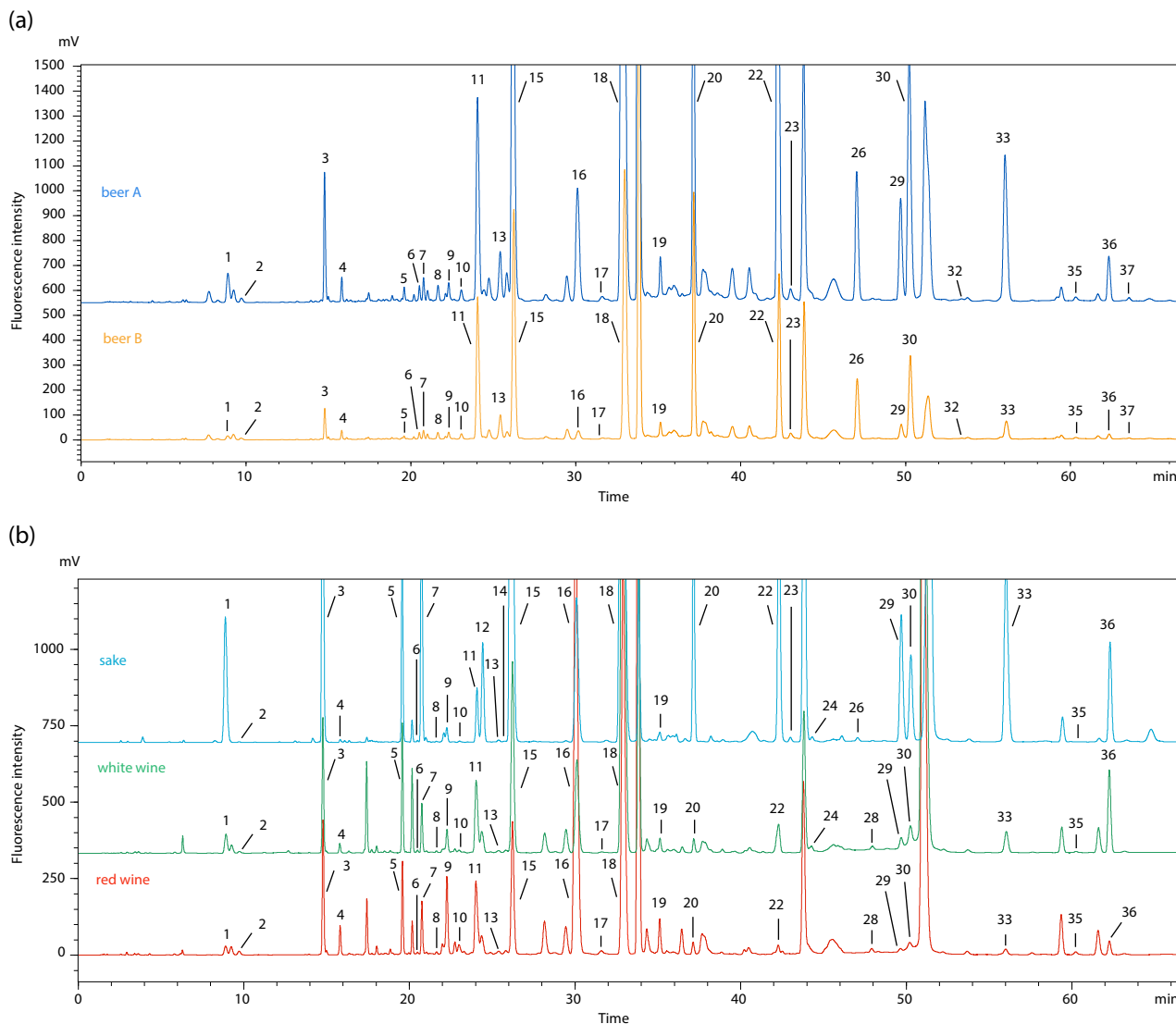


Fig. 4 Chromatograms of Liquor Samples
(a) Beer A and Beer B, (b) Sake, White Wine, and Red Wine

Table 6 Contents of D/L-Amino Acids in Liquor Samples*2,*3 (μmol/L, n = 4)
(a) Beer A and Beer B, (b) Sake, Red Wine, and White Wine

(a)

	Beer A			Beer B			Sake		
	D	L	%D*5	D	L	%D*5	D	L	%D*5
Asp	6.9 (0.4)	38.9 (0.4)	15.0	3.3 (1.2)	9.5 (0.9)	25.6	2.0 (3.6)	311.7 (2.9)	0.6
Glu	11.6 (0.3)	92.1 (0.3)	11.2	4.9 (1.1)	27.1 (1.3)	15.4	2.6 (1.1)	779.6 (1.3)	0.3
Asn	16.4 (0.4)	21.3 (0.6)	43.6	8.8 (1.1)	7.8 (0.7)	53.1	3.7 (1.4)	502.8 (1.1)	0.7
Ser	11.0 (0.5)	17.2 (0.3)	39.0	5.6 (1.1)	8.6 (1.2)	39.4	1.5 (1.3)	439.7 (0.9)	0.3
Gln	15.7 (0.5)	33.1 (0.5)	32.1	8.5 (1.2)	16.1 (0.7)	34.6	3.0 (0.6)	46.5 (1.3)	6.1
His	94.1 (0.4)	432.5 (0.4)	17.9	52.8 (1.2)	327.6 (1.2)	13.9	6.3 (1.3)	169.9 (1.6)	3.3
Thr	n.d.	n.d.		n.d.	n.d.		1.3 (1.8)	214.4 (2.1)	0.1
Gly		439.9 (0.4)			180.5 (1.2)			1498.3*4 (0.9)	
Arg	9.4 (1.2)	336.3 (0.4)	2.7	2.8 (1.5)	30.0 (1.2)	8.6	n.d.	796.5 (1.2)	
Ala	24.4 (0.5)	1228.0*4 (0.6)	1.9	10.1 (1.2)	447.8 (1.2)	2.2	11.8 (0.8)	2762.5*4 (0.9)	0.4
Tyr	n.d.	443.1 (0.4)		n.d.	251.8 (1.2)		n.d.	407.4 (1.1)	
Val	n.d.	620.6*4 (0.3)		n.d.	183.9 (1.2)		n.d.	538.9 (1.1)	
Met	n.d.	n.d.		n.d.	n.d.		n.d.	8.0 (0.9)	
(Cys) ₂	n.d.	68.1 (0.4)		n.d.	35.4 (1.1)		n.d.	48.1 (1.6)	
Trp	n.d.	170.4 (0.3)		n.d.	89.6 (1.1)		n.d.	12.9 (0.8)	
Ile	n.d.	120.4 (0.3)		n.d.	20.9 (1.1)		n.d.	273.5 (1.1)	
Phe	1.6 (1.6)	398.1 (0.3)	0.4	0.7 (1.5)	148.3 (1.3)	0.5	n.d.	240.0 (1.1)	
Leu	4.9 (1.6)	296.1 (0.4)	1.6	2.3 (1.8)	36.2 (1.2)	5.9	0.4 (1.5)	646.6 (1.0)	0.1
Lys	6.2 (1.8)	63.2 (0.2)	8.9	2.1 (3.2)	8.7 (0.9)	19.1	n.d.	259.8 (1.1)	
Total	202.1	4819.2	4.0	101.9	1830	5.3	32.5	9957.1	0.3

(b)

	Red wine			White wine		
	D	L	%D*5	D	L	%D*5
Asp	6.0 (0.8)	14.1 (0.3)	30.0	2.4 (0.3)	22.8 (0.3)	9.6
Glu	12.5 (0.4)	81.8 (0.4)	13.3	3.9 (0.7)	76.0 (0.6)	4.9
Asn	2.5 (0.5)	104.6 (0.4)	2.3	3.3 (1.0)	140.8 (1.0)	2.3
Ser	1.5 (0.8)	30.3 (0.4)	4.7	1.2 (1.4)	28.4 (1.1)	4.1
Gln	13.6 (0.9)	114.1 (0.6)	10.6	3.8 (1.3)	36.2 (0.9)	9.6
His	5.9 (0.4)	131.6 (0.5)	4.3	4.4 (1.1)	130.8 (1.2)	3.3
Thr	n.d.	n.d.		n.d.	n.d.	
Gly		77.0 (0.6)			110.5 (1.4)	
Arg	7.1 (0.3)	1260.8*4 (0.5)	0.6	2.2 (1.2)	234.7 (1.2)	0.9
Ala	17.8 (0.8)	497.8 (0.5)	3.5	6.5 (1.5)	891.4*4 (1.1)	0.7
Tyr	n.d.	12.6 (0.4)		n.d.	13.4 (0.8)	
Val	3.1 (0.4)	11.3 (0.9)	21.7	2.8 (0.3)	36.2 (0.7)	7.2
Met	n.d.	n.d.		n.d.	4.4 (0.9)	
(Cys) ₂	n.d.	n.d.		n.d.	n.d.	
Trp	n.d.	n.d.		n.d.	n.d.	
Ile	n.d.	3.9 (0.4)		n.d.	9.8 (1.1)	
Phe	n.d.	10.5 (0.7)		n.d.	24.2 (2.0)	
Leu	3.2 (0.9)	11.9 (0.3)	21.0	1.5 (1.0)	36.7 (1.1)	3.9
Lys	n.d.	17.0 (0.5)		n.d.	97.8 (1.1)	
Total	73.2	2379.1	3.0	32.0	1894.1	1.7

*2: n.d. indicates "not detected." *3: Numbers in parentheses indicate %RSDs.

*4: Concentrations of some L-amino acids were outside of the quantitation ranges.

*5: Overall ratio of D-amino acid to D/L-amino acid.

Liquor Profiling

Principal component analysis (PCA) was performed using the statistical analysis software eMSTAT Solution to determine the content of each compound in liquor samples obtained from four repeated analyses (Fig. 5). As a result of the PCA, Beer A and Beer B were plotted close together on the score plot, as well as the red and white wines (Fig. 5a). It was also found that sake showed different characteristics from the other liquor samples. The loading plot suggested that the two kinds of beers and the red and white wines contained relatively larger amounts of D-isomers, whereas the sake contained relatively larger amounts of L-isomers (Fig. 5b). Specifically, L-Trp, D-His, and D-Lys contributed significantly to the two kinds of beers, D-Val contributed to the red and white wines, and L-Thr contributed to sake. The first principal component (PC1) reflects differences among the liquor types, while the second principal component (PC2) suggests that each liquor is characterized by differences in amino acid stereoisomer composition (D-isomers versus L-isomers).

Conclusion

A method was developed for simultaneous separation of OPA/NIBC diastereomers for thirty-seven D/L-amino acids using a simple UHPLC system in approximately half the time of the conventional methods. By optimizing the derivatization reaction, all compounds were determined sensitively, accurately, and precisely. Separation of the target compounds from the contaminants was good in the liquor samples when evaluated using real liquor samples. It is expected to be easily applicable to D/L-amino acid profiling in food and beverage samples without using expensive MS detection or complicated multi-dimensional HPLC configurations as long as samples are prepared properly. Moreover, D/L-amino acid profiling could be used to research and develop foods containing D-amino acids intended to help to maintain and improve the intestinal environment, and could help in the early detection of diseases and determining optimal treatment strategies through biomarker discovery.

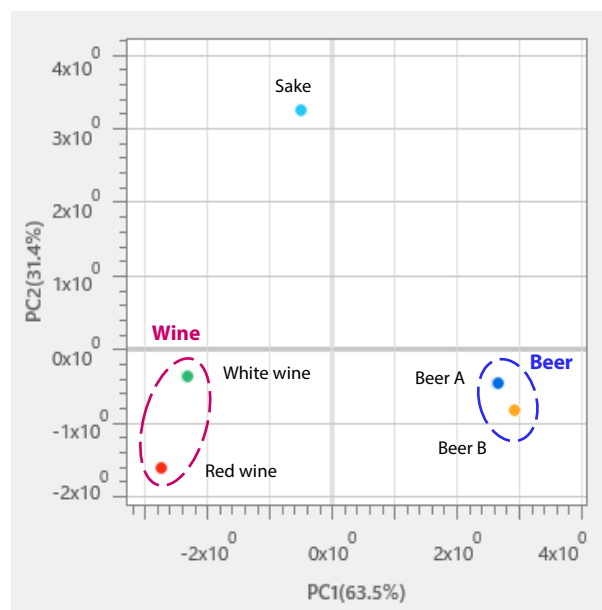
References

- 1) Iwata N., Watabe Y., Horie S., and Hayakawa Y., *Chromatography*, **42**, 133-141 (2021)
- 2) Iwata N. and Kobayashi M., *Chromatography*, **45**, 63-72 (2024)

Related Applications

1. Automated Analysis of Thirty-seven D/L-amino Acids using Liquid Chromatography with Fluorescence Detection and Its Application to Liquor Samples, [Application News No. L592](#)

(a) Score Plot



(b) Loading Plot

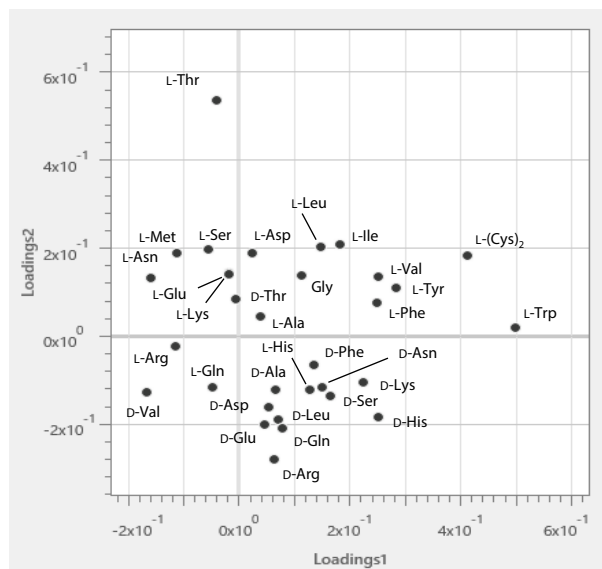


Fig. 5 Principal Component Analysis Result (n = 4)
(a) Score Plot, (b) Loading Plot

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