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Development of Sensitive and Simultaneous Determination Method for Thirty-Seven D/L-Amino Acids by Automatic Pre-column Derivatization with Chiral Thiol Using UHPLC

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1. Introduction

Recently, the role of D-amino acids in the taste, preservation, and flavor of foods and as disease biomarkers has become known, and the demand for D/L separation of amino acids is increasing. LC/MS analysis is susceptible to matrix effects and less quantitative than HPLC with ultraviolet or fluorescence detection. Multi-dimensional LC analysis requires a long analysis time and very complicated HPLC setup. Therefore, a simple operational method that provides good D/L-amino acid separation in a short time is required. A complementary determination method for 37 proteinogenic D/L-amino acids excluding proline by analyzing each sample twice while automatically switching between two separation methods using two derivatizing reagents with chiral structures, o-phthalaldehyde (OPA)/*N*-acetyl-L-cysteine (NAC) and OPA/*N*-isobutyryl-L-cysteine (NIBC), was previously developed. [1] In this study, separation of 37 OPA/NIBC-derivatized fluorescent diastereomers of D/L-amino acids with higher hydrophobicity under a single analytical method by reversed phase chromatography was investigated. Then automated analytical procedures including the derivatization and mobile phase preparation were developed after optimizing analytical conditions.

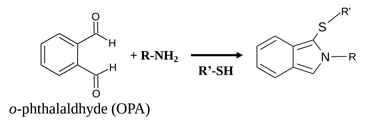
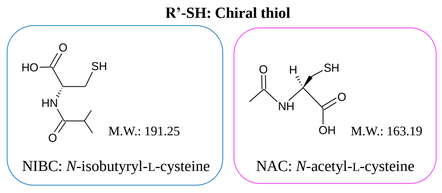


Figure 1. OPA fluorescence derivatization.



2. Experimental

2-1. Sample preparations and analytical conditions

Two kinds of beer (beer A and B), sake, red wine, and white wine were used as samples. Beer A and beer B were ale type (top fermenting) and lager type (bottom fermenting), respectively. Beer A, beer B, red wine, and white wine were diluted ten-fold (twenty-fold for sake) with 10 mmol/L hydrochloric acid and then passed through 0.2 µm PTFE membrane filters.

Fluorescence derivatization of the diastereomers was performed by the reaction with OPA under NIBC condition. A simple UHPLC equipped with fluorescence detector (Shimadzu Corporation) was used for determination for 37 D/L-amino acids (Fig. 2) and eMSTAT Solution (Shimadzu Corporation) was used for principal component analysis (PCA).

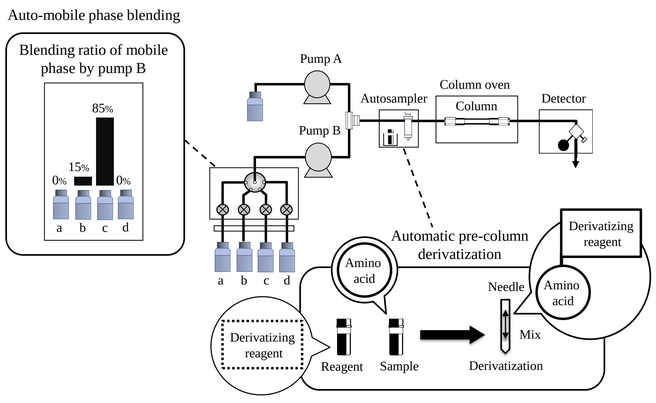


Figure 2. Flow diagram of automated HPLC setup.

Table 2 Analytical conditions.

System	: Nexera^{TM} X3
Column	: CERI L-column3 C18 (150 mm × 2.1 mm I.D., 2.0 µm) using pre-column filter
Flow rate	: 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	: Gradient elution
Column temp.	: 20 ˚C
Injection volume	: 1 µL
Detection	: FL Ex: 338 nm, Em: 455 nm

Table 3 Overview of automatic pre-column derivatization.

1.	OPA/ NIBC solution 2 µL
2.	Sample 1 µL
3.	Mix
4.	Wait 1.5 minutes
5.	Injection

Table 1 Preparation of derivatizing reagent.

1. 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 (pH 9.1) and 4 mL of ultrapure water.
2. NIBC solution
   Add 10 mg of *N*-isobutyryl-L-cysteine into 10 mL of 0.1 mol/L borate buffer (pH 9.1).
3. OPA/ NIBC solution
   Mix equal volume of OPA reagent and NIBC solution.

3. Results and discussion

3-1. Optimization of derivatization reaction

The optimum combination concentration about OPA reagent and NIBC solution was examined using a standard solution of D/L-amino acids (1 µmol/L each). The concentrations of the combinations were six conditions. The fluorescence intensities of D/L-Asp increased with increasing OPA concentrations under constant NIBC concentrations. On the other hand, the fluorescence intensities of D/L-Asp decreased with increasing NIBC concentrations under constant OPA concentrations. The combination of 3 mg/mL for OPA and 1 mg/mL for NIBC increased the column loading pressure. Therefore, the combination of 2 mg/mL for OPA and 1 mg/mL for NIBC was adopted as the optimum concentration (Fig. 3).

To promote the derivatization reaction and obtain good repeatability, a constant waiting time after mixing the OPA/NIBC solution and the sample within the injection needle for autosampler was introduced into the pretreatment program. Waiting time was compared to five conditions. As a result, since the peak area was constant in the case of 1.5 minutes or more, the waiting time was set to 1.5 minutes (Fig. 4).

Comparison
OPA : 1, 2, 3 mg/mL, and NIBC : 1, 2 mg/mL
▼
Optimization
OPA 2 mg/mL, NIBC 1 mg/mL

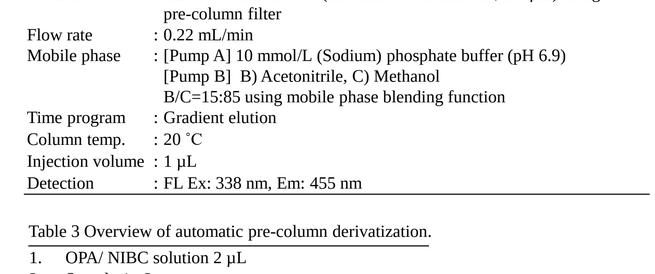


Figure 3. Fluorescence intensity of D/L-Asp (n=3) by concentration ratio of OPA reagent and NIBC solution.

Comparison
0, 0.5, 1, 1.5, and 2 min.
▼
Optimization
1.5 min.

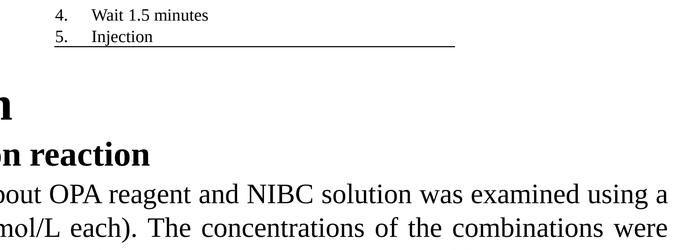


Figure 4. Peak area of D/L-Asp (n=3) by waiting time.

3-2. Analysis of a standard solution of 37 D/L-amino acids

Although the previously reported method with two switching conditions required 120 minutes for separation per sample [1], it was possible to separate the 37 diastereomers of D/L-amino acids in a single analysis within 66 minutes by increasing the methanol ratio of the mobile phase and setting the column temperature to 20 ºC (Fig. 5).

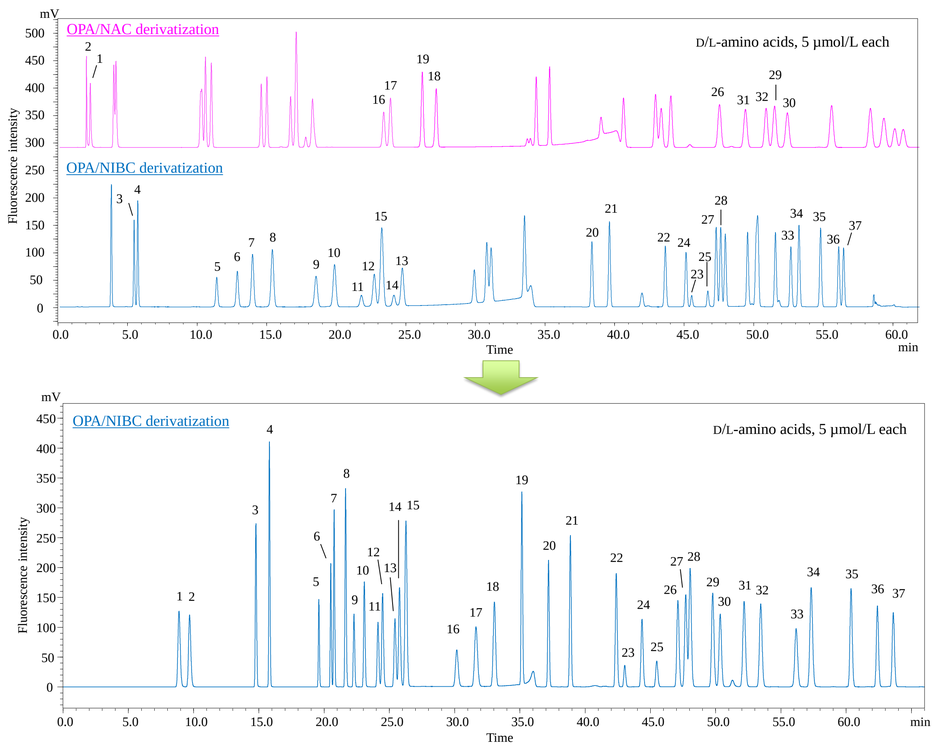


Figure 5. Chromatograms of a standard solution of D/L-amino acids (5 µmol/L each).

Table 4 Target compounds.

1	L-Asp	20	L-Tyr
2	D-Asp	21	D-Tyr
3	L-Glu	22	L-Val
4	D-Glu	23	L-$(Cys)_2$
5	L-Asn	24	L-Met
6	D-Asn	25	D-$(Cys)_2$
7	L-Ser	26	L-Trp
8	D-Ser	27	D-Met
9	L-Gln	28	D-Val
10	D-Gln	29	L-Ile
11	L-His	30	L-Phe
12	L-Thr	31	D-Trp
13	D-His	32	D-Phe
14	D-Thr	33	L-Leu
15	Gly	34	D-Ile
16	L-Arg	35	D-Leu
17	D-Arg	36	L-Lys
18	L-Ala	37	D-Lys
19	D-Ala		

Pink : OPA/NAC derivatization
Blue : OPA/NIBC derivatization

3-3. Method validation

The relative standard deviations (%RSD, n=6) of the peak areas of a standard mixture of D/L-amino acids (0.1 µmol/L each) were 1.6% or less. Good repeatability were achieved thanks to the automatic pre-column derivatization introduced to keep the derivatization time constant. The linearities of the calibration curves of the 37 D/L-amino acids were good enough and the coefficient of determination ratio r^2 were 0.999 or greater. Beer B was used for recovery testing. Beer B was spiked with the standard solution of 31 D/L-amino acids to give a final concentration of 1 µmol/L each, except for six amino acids with a final concentration of 10 µmol/L each. Then six samples were pretreated simultaneously according to the pretreatment protocol. As a result, the recovery rates were 84.9-108.6%, and their %RSDs were 0.8-9.5%.

3.4 Application to liquor samples

As shown in Fig. 6, 25 to 28 amino acids were separated and detected in five liquor samples. The overall ratio of D-amino acid to D/L-amino acid (%D) was found to be 6% or less in all samples.

Our determination results of D/L abundance ratios of specific amino acids (Table 5) were close to those of the previous studies using HPLC [2] and GC/MS [3] for various real sample analyses.

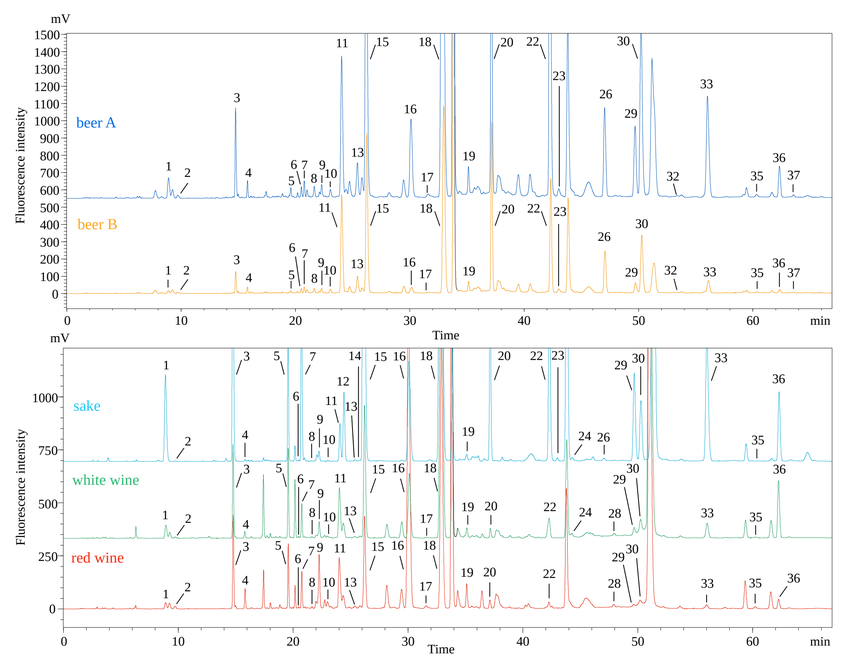


Figure 6. Chromatograms of OPA/NIBC-derivatized diastereomers in liquor samples.

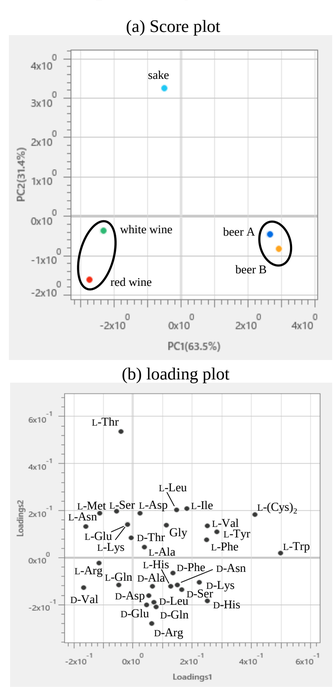


Figure 7. Results of the PCA (n=4).

Table 5. Contents of D/L-amino acids in liquor samples*1,2 (µmol/L, n=4).

	beer A			beer B			sake			red wine			white wine		
	D	L	%D*4	D	L	%D*4	D	L	%D*4	D	L	%D*4	D	L	%D*4
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	6.0 (0.8)	14.1 (0.3)	30.0	2.4 (0.3)	22.8 (0.3)	9.6
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	12.5 (0.4)	81.8 (0.4)	13.3	3.9 (0.7)	76.0 (0.6)	4.9
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	2.5 (0.5)	104.6 (0.4)	2.3	3.3 (1.0)	140.8 (1.0)	2.3
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	1.5 (0.8)	30.3 (0.4)	4.7	1.2 (1.4)	28.4 (1.1)	4.1
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	13.6 (0.9)	114.1 (0.6)	10.6	3.8 (1.3)	36.2 (0.9)	9.6
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	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.		1.3 (1.8)	214.4 (2.1)	0.1	n.d.	n.d.		n.d.	n.d.	
Gly		439.9 (0.4)			180.5 (1.2)			1498.3*3 (0.9)			77.0 (0.6)			110.5 (1.4)	
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)		7.1 (0.3)	1260.8*3 (0.5)	0.6	2.2 (1.2)	234.7 (1.2)	0.9
Ala	24.4 (0.5)	1228.0*3 (0.6)	1.9	10.1 (1.2)	447.8 (1.2)	2.2	11.8 (0.8)	2762.5*3 (0.9)	0.4	17.8 (0.8)	497.8 (0.5)	3.5	6.5 (1.5)	891.4*3 (1.1)	0.7
Tyr	n.d.	443.1 (0.4)		n.d.	251.8 (1.2)		n.d.	407.4 (1.1)		n.d.	12.6 (0.4)		n.d.	13.4 (0.8)	
Val	n.d.	620.6*3 (0.3)		n.d.	183.9 (1.2)		n.d.	538.9 (1.1)		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.	n.d.		n.d.	8.0 (0.9)		n.d.	n.d.		n.d.	4.4 (0.9)	
$(Cys)_2$	n.d.	68.1 (0.4)		n.d.	35.4 (1.1)		n.d.	48.1 (1.6)		n.d.	n.d.		n.d.	n.d.	
Trp	n.d.	170.4 (0.3)		n.d.	89.6 (1.1)		n.d.	12.9 (0.8)		n.d.	n.d.		n.d.	n.d.	
Ile	n.d.	120.4 (0.3)		n.d.	20.9 (1.1)		n.d.	273.5 (1.1)		n.d.	3.9 (0.4)		n.d.	9.8 (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)		n.d.	10.5 (0.7)		n.d.	24.2 (2.0)	
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	3.2 (0.9)	11.9 (0.3)	21.0	1.5 (1.0)	36.7 (1.1)	3.9
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)		n.d.	17.0 (0.5)		n.d.	97.8 (1.1)	
Total	202.1	4819.2	4.0	101.9	1830.0	5.3	32.5	9957.1	0.3	73.2	2379.1	3.0	32.0	1894.1	1.7

*1: n.d. : not detected. *2: Numbers in parentheses indicate %RSDs. *3: Concentrations of some L-amino acids were out of the quantification ranges. *4: Overall ratio of D-amino acid to D/L-amino acid.

PCA was performed using the content of each compound (n=4) in liquor samples (Fig. 7). As a result of the PCA, beer A and beer B were plotted close together on the score plot as well as red and white wines. It was also found that sake showed different characteristics from these liquor samples. The loading plot showed that two kinds of beers and red and white wines contained many D-isomers and sake contained many L-isomers. In particular, L-Trp, D-His, and D-Lys contributed significantly to two kinds of beers, D-Val to red and white wines, and L-Thr to sake. It was suggested that the first principal component (PC1) showed differences in the types of liquor, and the second principal component (PC2) showed differences in the isomers.

4. Conclusions

- ✓ A method for simultaneous separation of 37 OPA/NIBC-derivatized D/L-amino acids using a simple UHPLC system in approximately half the time of the conventional methods was developed.
- ✓ Optimizing the derivatization reaction, sensitive, accurate, and precise determination were achieved for all compounds.
- ✓ The target compounds were good separated from the contaminants in real liquor samples.
- ✓ The established method has the potential to be applied to liquor profiling.
- ✓ Just a simple and generally used gradient UHPLC setup with an autosampler affords D/L-amino acid determination in a short time without using expensive MS detection or complicated multi-dimensional HPLC setup.

References

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[2] Jin, D.; Miyahara, T.; Oe, T.; Toyo'oka, T. *Anal. Biochem.* **1999**, *269*, 124-132.
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