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High Performance Liquid Chromatograph Nexera™ X3 RF-20AXS

Application News

No. L592

Automated Analysis of Thirty-seven D/L-amino Acids using Liquid Chromatography with Fluorescence Detection and Its Application to Liquor Samples

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User Benefits

- Separation and determination of thirty-seven D/L-amino acids in a short time with simple operation are possible.
- The automatic operation reduces the labor and more than an hour of the working time per 20 samples consecutive analyses.
- The elapsed time of the derivatization reaction is kept constant, resulting in good reproducibility of determination.

■ Introduction

Amino acids have D/L enantiomers that have asymmetric centers in their molecular structures except for glycine. In contrast to L-amino acids, there has been limited studies of D-amino acids. The role of D-amino acids in the palatability, preservability, and the aroma of food and foodstuff has been largely unknown until recently. However, fermented foods and biological samples are known to contain small amounts of D-amino acids in addition to large amounts of L-amino acids. Therefore, the demand for D/L separation of amino acids is increasing.

This article introduces the analyses of fluorescent diastereomers of the proteinogenic D/L-amino acids using derivatizing reagents with chiral structures. In addition, it also introduces the automated analysis including derivatization.

■ Derivatization for chiral amines

Two chiral thiols, *N*-acetyl-L-cysteine (NAC) and *N*-isobutyryl-L-cysteine (NIBC), were used for derivatization. Fluorescence derivatization of diastereomers of D/L-amino acids was performed by the reaction with *o*-phthalaldehyde (OPA) under NAC or NIBC.

■ LC analysis of D/L-amino acids

In general, LC/MS or multi-dimensional LC is used for D/L-amino acids analyses by HPLC, because it is difficult to separate proteinogenic D/L-amino acids in 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 required.

In food analysis, a small particle column is used because of the appropriate separation of small amount of D-amino acids from large amount of L-amino acids and co-existing contaminants. Therefore, Nexera X3 with 130 MPa pressure tolerance was used because the system pressure became high.

■ Analytical Conditions and Automated Analysis

The target compounds are thirty-seven D/L-amino acids excluding D/L-proline from the proteinogenic amino acids (Table 1). The background colors of red and blue indicate OPA/NAC and OPA/NIBC derivatized amino acids, respectively.

Figure 1 shows the flow diagram of the HPLC setup for automated analysis. In this study, the mobile phases were prepared automatically using the mobile phase blending function of the solvent delivery pump, and two sets of analytical conditions were switched automatically. D/L-amino acids were derivatized using the automatic pretreatment function of the autosampler. The differences between the two sets of analytical conditions shown in Table 2 are the blending ratio of organic solvents and the gradient profile. The mobile phase blending function provides the solutions to deliver at the specified blending ratio just by setting the organic solvents and ultrapure water in the ports of the low-pressure gradient kit integrated into the pump. The labor and the working time required for the mobile phase preparation and the mobile phase replacement accompanied with switching the analytical conditions were reduced by this function.

In addition, the automatic pretreatment function is described below. The derivatizing reagents of OPA/NAC and OPA/NIBC 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 outside (Table 3 and Table 4). Figure 2 shows the setup screen of the pretreatment program on the workstation LabSolutions™. The elapsed time from starting the derivatization to injection to HPLC was kept constant using the pretreatment function, resulting in good reproducibility of determination. In addition, the consumable cost was reduced because the vials for derivatization were not required.

Table 5 shows the comparison of the required working time of the automatic and the manual operations when 20 samples were analyzed. The automatic operations reduced the labor and more than an hour of the working time in comparison with the manual operation.

Table 1 List of Target Compounds

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

* Background color; Red: OPA/NAC derivatized amino acids, Blue: OPA/NIBC derivatized amino acids

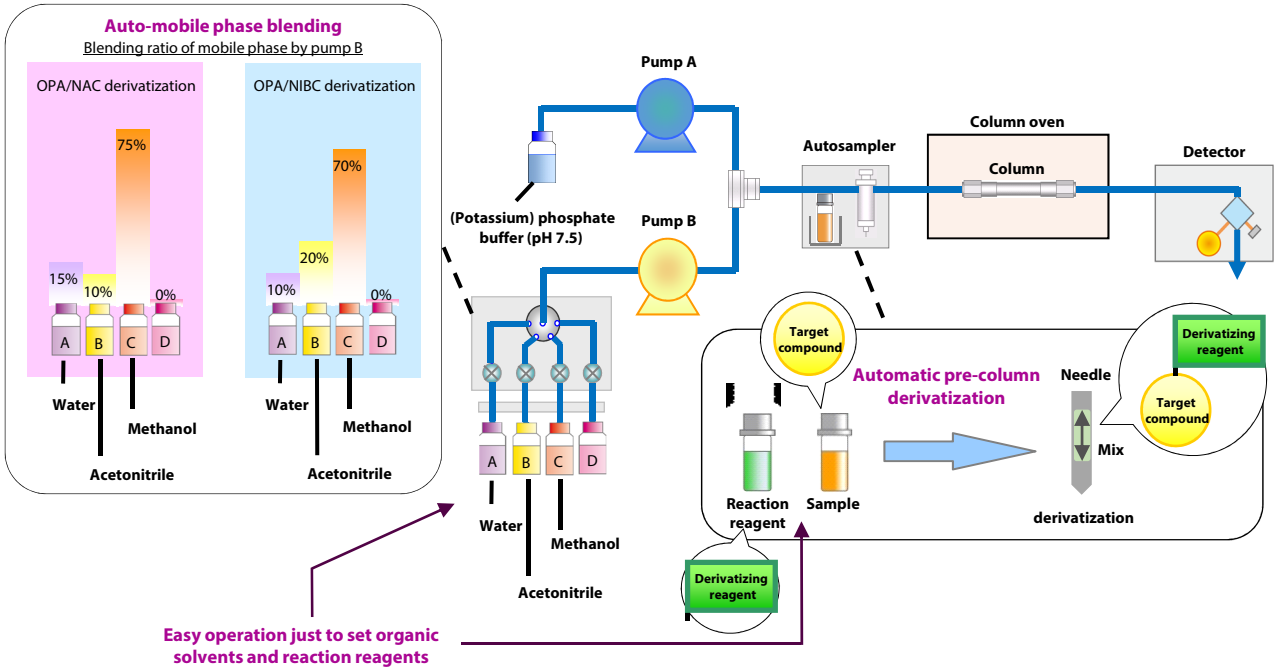


Fig. 1 Flow Diagram of the HPLC Setup for Automated Analysis

Table 2 Analytical Conditions

		<OPA/NAC derivatization>	<OPA/NIBC derivatization>
System	:	Nexera X3	
Column	:	Shim-pack Scepter™ 1.9 µm C8＊1 (150 mm x 3.0 mm I.D., 1.9 µm)	Shim-pack Scepter 1.9 µm C8 (150 mm x 3.0 mm I.D., 1.9 µm)
Flow rate	:	0.6 mL/min	0.6 mL/min
Mobile phase	:	<Pump A> 10 mmol/L (potassium) phosphate buffer (pH 7.5)※ <Pump B> mobile phase blending A) Water B) Acetonitrile C) Methanol **A/B/C=15:10:75**	<Pump A> 10 mmol/L (potassium) phosphate buffer (pH 7.5) <Pump B> mobile phase blending A) Water B) Acetonitrile C) Methanol **A/B/C=10:20:70**
Time program	:	**4%B (0-3 min)→11%B (13 min)→14%B (22 min)→ 25%B (30 min)→30%B (35 min)→41%B (61 min)→ 80%B (61.01-63 min)→4%B (63.01-67 min)**	**10%B (0 min)→15%B (3-15 min)→20%B (25 min)→ 52%B (57 min)→80%B (57.01-59 min)→10%B (59.01-63 min)**
Column temp.	:	35 ˚C	35 ˚C
Injection volume	:	1 µL	1 µL
Vial	:	SHIMADZU LabTotal™ for LC 1.5 mL, Glass＊2	SHIMADZU LabTotal for LC 1.5 mL, Glass
Detection (FL)	:	Ex: 350 nm, Em: 450 nm (RF-20AXS)	Ex: 350 nm, Em: 450 nm (RF-20AXS)

＊1 P/N: 227-31034-04, ＊2 P/N: 227-34001-01
※ Add 0.68 g of potassium dihydrogen phosphate and 2.61 g of dipotassium hydrogen phosphate into 2000 mL of ultrapure water, and dissolve completely.

Table 3 Outline of Automatic Pre-column Derivatization with Autosampler

<OPA/NAC derivatization>	<OPA/NIBC derivatization>
① Aspirate OPA/NAC solution 4 µL	① Aspirate OPA/NIBC solution 4 µL
② Aspirate sample 1 µL	② Aspirate sample 1 µL
③ Mix	③ Mix
④ Inject to HPLC	④ Inject to HPLC

Table 4 Preparation of Derivatizing Reagents

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.
0.1 mol/L borate buffer	Add 0.62 g boric acid and 0.20 g of sodium hydroxide into 100 mL of ultrapure water, and dissolve completely.
NAC solution	Add 20 mg of *N*-acetyl-L-cysteine into 10 mL of 0.1 mol/L borate buffer.
NIBC solution	Add 20 mg of *N*-isobutyryl-L-cysteine into 10 mL of 0.1 mol/L borate buffer.
OPA/NAC solution	Mix equal volume of OPA reagent and NAC solution.
OPA/NIBC solution	Mix equal volume of OPA reagent and NIBC solution.

Table 5 Comparison of the Work Time between Automatic and Manual Operations when 20 Samples were Analyzed

		Automatic		Manual	
Mobile phase preparation	Buffer	5 minutes			
	Organic solvent	○	**0 minutes ※Mobile phase blending function**	×	10 minutes
Mobile phase replacement accompanied with switching analytical conditions		○	**0 minutes ※Mobile phase blending function**	×	10 minutes
Derivatizing reagent preparation		10 minutes			
Derivatization		○	**0 minutes ※Automatic pre-column derivatization**	×	50 minutes
Total		○	**15 minutes**	×	85 minutes

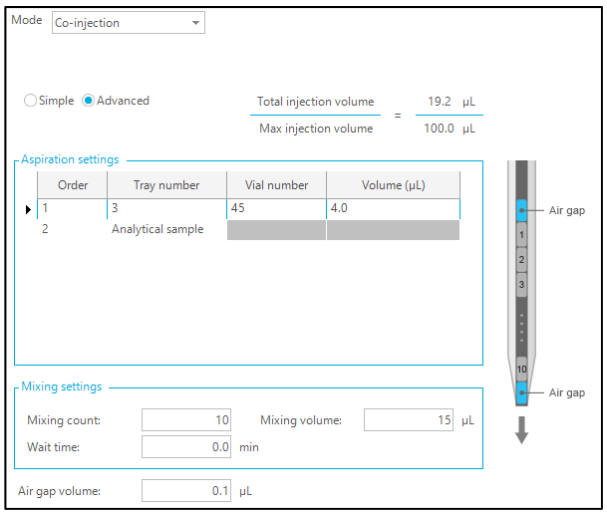


Fig. 2 Setup Screen of Pretreatment Program

■ Analysis of a Standard solution of D/L-Amino acids

Figure 3 shows a chromatograms of a standard solution of D/L-amino acids (5 µmol/L each). The thirty-seven D/L-amino acids were separated complementarily in a total of approximately 120 minutes using two chiral thiols.

■ Reproducibility

The relative standard deviations (%RSD) of the retention times and the peak areas based on six repeated analyses of a standard solution of D/L-amino acids (2 µmol/L each) were 0.1 % or less and 1.5 % or less, respectively (Table 6).

■ Calibration Curve

The linearities of the calibration curves of the thirty-seven D/L-amino acids were good. Each contribution ratio r^2 was 0.999 or greater (Fig. 4 and Table 7).

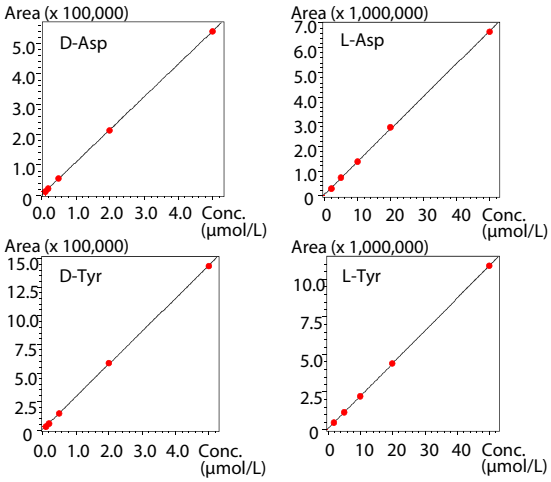


Fig. 4 Calibration Curves

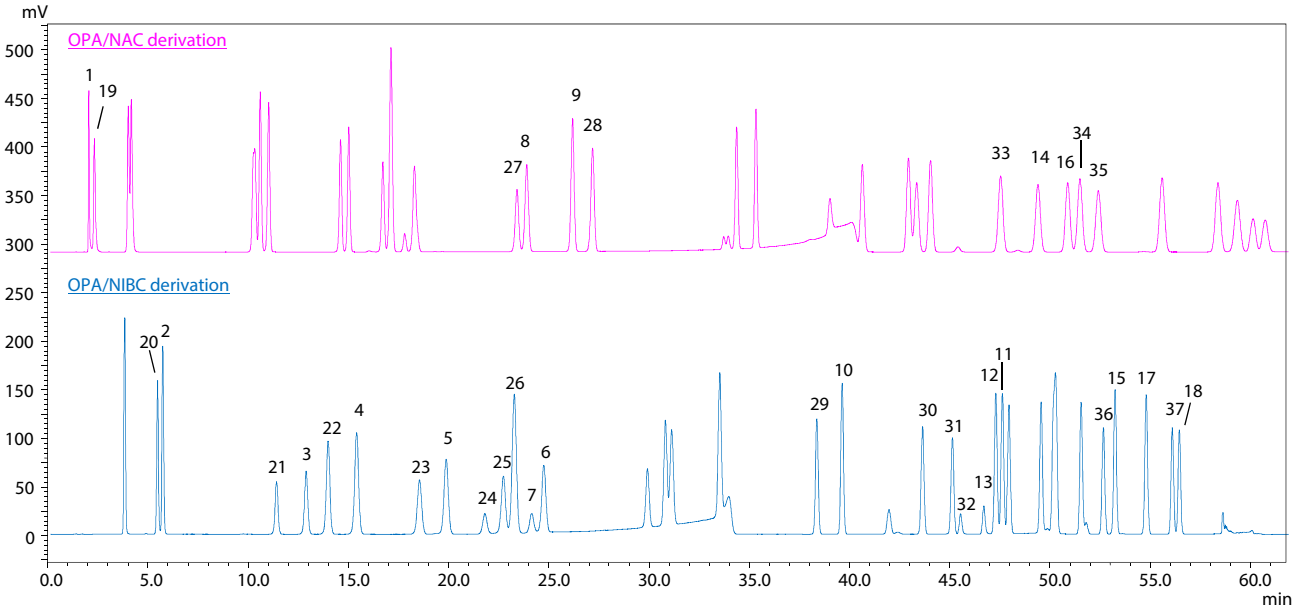


Fig.3 Chromatograms of Standard Solution of D/L-amino Acids (5 µmol/L for each).

Table 6. Reproducibility (% RSD, n = 6)

	Compound	Retention time	Peak area		Compound	Retention time	Peak area		Compound	Retention time	Peak area
1	D-Asp	0.23	1.50	14	D-Trp	0.05	0.42	27	L-Arg	0.02	0.18
2	D-Glu	0.09	0.47	15	D-Ile	0.02	0.38	28	L-Ala	0.03	0.54
3	D-Asn	0.09	0.29	16	D-Phe	0.06	0.51	29	L-Tyr	0.02	0.28
4	D-Ser	0.10	0.19	17	D-Leu	0.02	0.30	30	L-Val	0.01	0.37
5	D-Gln	0.09	0.26	18	D-Lys	0.02	0.50	31	L-Met	0.01	0.35
6	D-His	0.07	0.40	19	L-Asp	0.35	1.32	32	L - $(Cys)_2$	0.02	0.62
7	D-Thr	0.06	0.25	20	L-Glu	0.09	0.44	33	L-Trp	0.05	0.44
8	D-Arg	0.02	0.19	21	L-Asn	0.09	0.26	34	L-Ile	0.06	0.72
9	D-Ala	0.03	0.49	22	L-Ser	0.10	0.19	35	L-Phe	0.06	0.52
10	D-Tyr	0.01	0.28	23	L-Gln	0.10	0.29	36	L-Leu	0.02	0.31
11	D-Val	0.02	0.62	24	L-His	0.06	0.40	37	L-Lys	0.02	0.43
12	D-Met	0.02	0.35	25	L-Thr	0.07	0.35				
13	D - $(Cys)_2$	0.02	0.38	26	Gly	0.07	0.41				

* Background color; Red: OPA/NAC derivatized amino acids, Blue: OPA/NIBC derivatized amino acids

Table 7 Concentration Range of Calibration Curve and Contribution Ratio (r^2)

Compound		Conc. range (μ mol/L)	r^2	Compound		Conc. range (μ mol/L)	r^2
1	D-Asp	0.1-5	0.99997	19	L-Asp	2-50	0.99968
2	D-Glu	0.1-5	0.99986	20	L-Glu	2-50	0.99999
3	D-Asn	0.1-5	0.99992	21	L-Asn	2-50	0.99999
4	D-Ser	0.1-5	0.99997	22	L-Ser	0.5-20	0.99996
5	D-Gln	0.1-5	0.99997	23	L-Gln	0.5-20	0.99995
6	D-His	0.2-50	0.99995	24	L-His	0.2-100	0.99991
7	D-Thr	0.1-5	1.00000	25	L-Thr	0.1-10	0.99957
				26	Gly	0.5-100	0.99996
8	D-Arg	0.1-20	0.99994	27	L-Arg	2-100	0.99993
9	D-Ala	0.1-5	0.99997	28	L-Ala	5-100	0.99951
10	D-Tyr	0.1-5	0.99993	29	L-Tyr	2-50	0.99998
11	D-Val	0.1-2	1.00000	30	L-Val	2-50	0.99998
12	D-Met	0.1-5	0.99999	31	L-Met	0.1-5	0.99999
13	D - $(Cys)_2$	0.1-5	0.99993	32	L - $(Cys)_2$	2-50	0.99995
14	D-Trp	0.1-5	0.99996	33	L-Trp	2-50	0.99994
15	D-Ile	0.1-5	0.99990	34	L-Ile	0.5-20	0.99987
16	D-Phe	0.1-5	0.99997	35	L-Phe	2-50	0.99991
17	D-Leu	0.1-5	0.99996	36	L-Leu	2-50	0.99999
18	D-Lys	0.1-5	0.99996	37	L-Lys	0.5-20	0.99993

* Background color; Red: OPA/NAC derivatized amino acids, Blue: OPA/NIBC derivatized amino acids

■ Application to Liquor samples

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

D-aspartic acid, D-glutamic acid, D-serine, D-histidine, D-alanine and D-leucine were contained in all the five liquor samples used in this study. D-glutamine and D-tryptophan were found only in red wine and white wine. D-phenylalanine was found only in the two kinds of beer (Fig. 5 and Fig. 6). The content of D-amino acid in beer A was about two times larger than that in beer B. On the other hand, the ratio of D-amino acids to D/L-amino acids in beer B was about two times larger than that in beer A. In addition, it was confirmed that the amount of D-isomer was very small compared to that of L-isomer (Fig. 7 and Fig. 8).

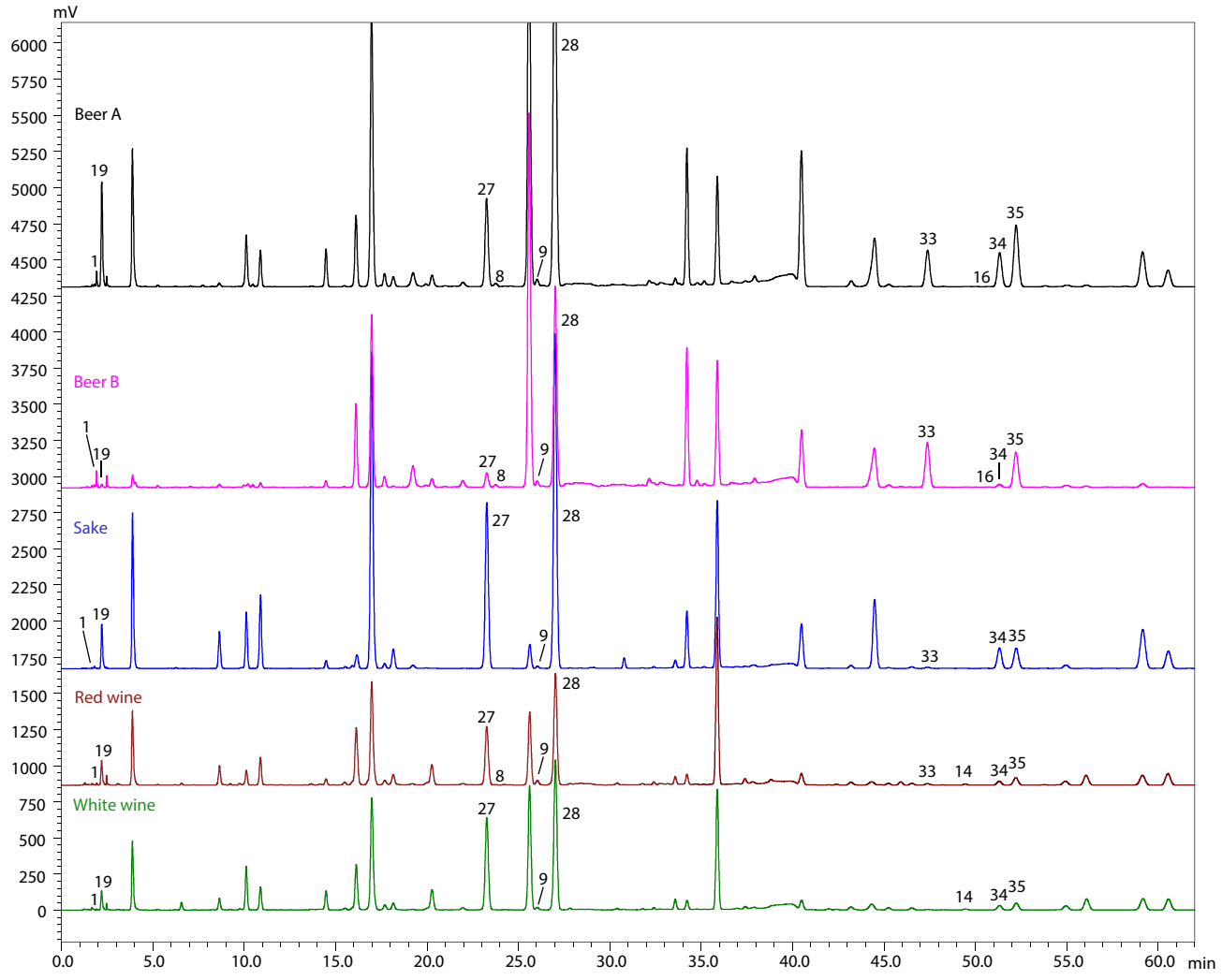


Fig. 5 Chromatograms of Liquor Samples (OPA/NAC derivatization)

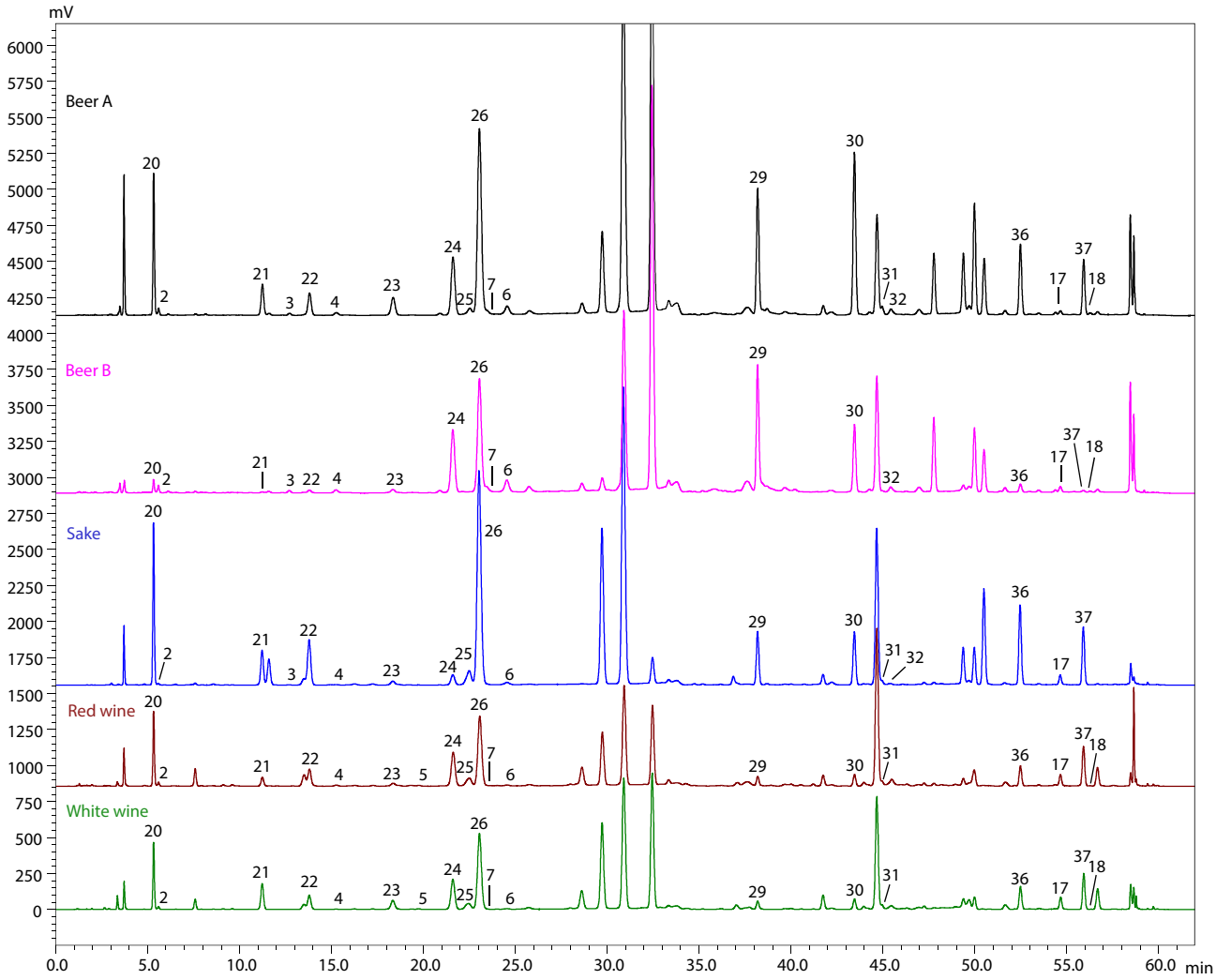


Fig. 6 Chromatograms of Liquor Samples (OPA/NIBC derivatization)

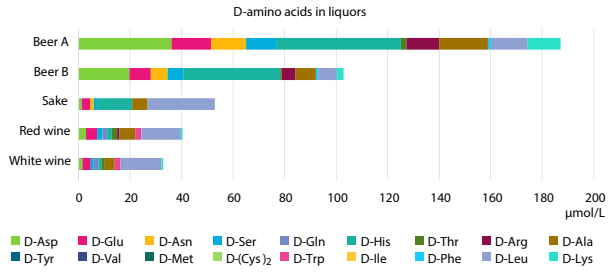
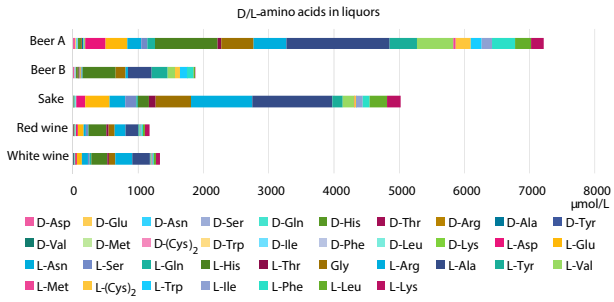


Fig. 7 Contents of D-Amino acid in Liquor Samples



∗ Concentrations of some L-amino acids were out of the quantification ranges

Fig. 8 Contents of D/L-Amino Acid in Liquor Samples

■ Conclusion

The two independent HPLC methods were designed to obtain complementary separation for the two sets of fluorescent diastereomers of the proteinogenic D/L-amino acids. Complete determination of thirty-seven D/L-amino acids was able to be done with two automated procedures of the derivatization and the sequential method switching analysis.

Therefore, the analysis was executed and the result was achieved with a simple HPLC configuration without a mass spectrometer or complex multi-dimensional HPLC setup.

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