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Liquid Chromatograph Mass Spectrometer LCMS™-2050

Application News

Chiral Amino Acid Analysis Using a Single Quadrupole Mass Spectrometer

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User Benefits

- Separation with a chiral column enables direct analysis of chiral amino acids without pretreatment by derivatization.
- By using a high-performance single quadrupole LC-MS system, even trace quantities of D-amino acids can be detected in foods and cosmetics.

■ Introduction

Of the 20 types of amino acids included in proteins, optical isomers exist for all but glycine. Most naturally occurring amino acids are L-amino acids, but advancements in analytical technology have revealed the presence of D-amino acids in many organisms. It is also becoming evident that D-amino acids have different physiological functions than L-amino acids. Consequently, D-amino acids perform attracted attention in basic research related to their physiological and medical functions and for applications in the fields of food, health, and beauty.

This Application News article describes using a single quadrupole LC-MS system with a crown ether-type chiral column to analyze chiral amino acids, which enables rapid analysis of chiral amino acids without derivatization pretreatment. If a comprehensive analysis of all hydrophilic metabolites that includes amino acids is required, refer also to Application News No. 01-00334-EN, which describes a useful technique for such analysis.

■ Samples and Pretreatment

The following were used as samples: 5 types of commercially marketed table vinegar (4 types of black vinegar and 1 type of grain vinegar), 4 types of lactic acid bacteria beverage (including 1 type of fermented milk), 10 types of beer (including 1 type of non-alcoholic beer), and 1 type of lotion.

To pretreat the vinegar and beer samples, 100 µL of 100 ppm DL-alanine-d4 (an internal standard) and 800 µL of the mobile phase were added to 100 µL of the samples. After stirring, the mixtures were separated by centrifuging (15 minutes at 15,000 rpm), and the supernatant was used as the measurement samples.

To pretreat the lactic acid bacteria beverage and lotion samples, 350 µL of methanol and 50 µL of ultrapure water were added to 100 µL of the samples, stirred, and centrifuged (15 minutes at 15,000 rpm). In addition, 360 µL of chloroform and 180 µL of ultrapure water were added to 360 µL of the resulting supernatant and stirred and centrifuged (15 minutes at 15,000 rpm). Then 25 µL of 100 ppm DL-alanine-d4 (an internal standard) and 175 µL of the mobile phase were added to 50 µL of the collected supernatant and stirred for use as measurement samples.

■ Instruments and Analysis Conditions

Analysis was performed with a Nexera series HPLC system that was combined with an LCMS-2050 LC-MS system (Fig. 1). Although compact, the LCMS-2050 single quadrupole mass spectrometer offers easy operability and outstanding performance. Equipped with a DUIS™ heated ion source, which provides the benefits of both ESI and APCI, it can analyze masses ranging from *m/z* 2 to 2000. That makes it well suited for simultaneously analyzing all metabolites, including amino acids, as described in Application News No. 01-00334-EN.

The analysis condition settings for HPLC and MS are shown in Table 1. The analysis conditions for LCMS-2050 was configured based on the analysis conditions in the LC/MS/MS Method Package for D/L Amino Acids. Using a crown ether-type chiral column enables the chiral separation of all amino acids except proline, which is a secondary amine.

Fig. 1 Nexera™ and LCMS™-2050 Systems

Twenty types of amino acids contained in proteins were targeted.

Table 1 Analysis Conditions

HPLC Conditions (Nexera XR)	
Column:	CROWNPAK CR-I(+) or CR-I(-) (150 mm × 3.0 mm I.D., 5 µm)
Mobile Phases:	Acetonitrile/ethanol/water/TFA=80/15/5/0.5
Flow Rate:	0.4 mL/min
Mode:	Isocratic elution
Column Temp.:	30 °C
Injection Volume:	1 µL
MS Conditions (LCMS-2050)	
Ionization:	ESI/APCI (DUIS), Positive mode
Mode:	SIM (20 events)
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	5.0 L/min
Desolvation Temp.:	500 °C
DL Temp.:	150 °C

■ Analysis of Standard Solution

A standard solution containing a mixture of 18 types of L-amino acids, 18 types of D-amino acids, glycine, and D/L-proline was analyzed to check the linearity, reproducibility, and limit of detection (LOD) of the method. The calibration curve concentration range, coefficient of determination (r^2), relative standard deviation (%RSD for n = 4) of peak areas at the minimum calibration curve concentration, and LOD (at concentration with S/N = 3) values are shown in Table 2. Although the LOD values were worse by about a factor of 10 than they would be with a triple quadrupole LC-MS system, the results show that the sensitivity for most components was as high or higher than if using a fluorescence detector.

Table 2 Linearity, Reproducibility, and Limit of Detection

Compounds	CROWNPAK CR-I(+) Range (ppb)	r^2 value	RSD of peak area (%)	LOD (ppb)	CROWNPAK CR-I(-) Range (ppb)	r^2 value	RSD of peak area (%)	LOD (ppb)
D-Ala	500 - 5000	0.9999	8.0	180.8	500 - 5000	0.9998	3.3	588.2
D-Arg	5 - 5000	0.9918	10.8	3.0	5 - 5000	0.9931	11.2	4.5
D-Asn	5 - 5000	0.9961	6.9	3.2	5 - 5000	0.9994	3.5	3.1
D-Asp	50 - 5000	0.9954	4.6	11.8	50 - 5000	0.9999	1.1	26.4
D-Cys	10 - 5000	0.9998	7.6	10.8	50 - 5000	0.9957	2.7	15.2
D-Gln	5 - 5000	0.9984	9.0	6.3	5 - 5000	0.9999	8.3	11.4
D-Glu	5 - 5000	0.9992	7.5	2.0	5 - 5000	0.9893	8.9	3.2
D-His	10 - 5000	0.9994	8.8	8.8	5 - 5000	0.9995	10.0	8.0
D-Ile	5 - 5000	0.9997	8.4	5.8	10 - 5000	0.9999	5.6	13.8
D-Leu	5 - 5000	0.9997	5.3	6.2	50 - 5000	0.9997	6.1	33.6
D-Met	5 - 5000	0.9996	3.8	1.9	5 - 5000	0.9997	8.9	3.9
D-Phe	5 - 5000	0.9982	6.9	11.2	50 - 5000	0.9988	4.9	80.5
D-Ser	50 - 5000	0.9992	6.2	27.1	10 - 5000	0.9996	4.7	11.2
D-Thr	5 - 5000	0.9991	5.0	8.6	5 - 5000	0.9973	4.6	7.6
D-Trp	5 - 5000	0.9996	5.3	1.5	5 - 5000	0.9972	1.6	4.2
D-Tyr	5 - 5000	0.9954	9.7	8.5	50 - 5000	0.9991	4.6	28.5
D-Val	50 - 5000	0.9999	8.9	24.5	50 - 5000	0.9999	4.4	10.7
L-Ala	500 - 5000	0.9999	1.7	319.5	500 - 5000	0.9999	3.6	469.9
L-Arg	5 - 5000	0.9928	7.4	2.7	5 - 5000	0.9951	6.0	3.6
L-Asn	5 - 5000	0.9994	7.9	3.4	5 - 5000	0.9971	6.5	2.9
L-Asp	50 - 5000	0.9996	6.8	18.4	50 - 5000	0.9996	6.9	18.6
L-Cys	10 - 5000	0.9971	10.3	11.4	5 - 5000	0.9995	7.2	4.8
L-Glu	10 - 5000	0.9998	4.8	6.7	5 - 5000	0.9999	3.0	17.6
L-His	10 - 5000	0.9957	9.5	9.0	5 - 5000	0.9988	1.9	2.1
L-Ile	10 - 5000	0.9995	5.8	6.4	5 - 5000	0.9999	5.3	3.0
L-Leu	50 - 5000	0.9999	2.6	11.1	5 - 5000	0.9998	11.9	7.1
L-Lys	50 - 5000	0.9998	5.2	23.6	5 - 5000	0.9998	2.9	8.1
L-Met	10 - 5000	0.9997	6.4	4.7	5 - 5000	0.9991	3.2	2.0
L-Phe	50 - 5000	0.9994	4.9	19.8	50 - 5000	0.9991	8.6	62.8
L-Ser	50 - 5000	0.9986	5.2	29.6	10 - 5000	0.9997	3.9	13.3
L-Thr	10 - 5000	0.9985	7.4	7.9	5 - 5000	0.9999	5.4	6.8
L-Trp	5 - 5000	0.9974	5.9	1.9	5 - 5000	0.9999	6.7	3.1
L-Tyr	50 - 5000	0.9983	4.8	13.8	10 - 5000	0.9965	8.9	15.4
L-Val	50 - 5000	0.9998	4.9	26.3	50 - 5000	0.9999	4.9	11.1
D/L-Pro	5 - 5000	0.9993	2.8	12.6	10 - 5000	0.9998	8.1	10.3
Gly	500 - 5000	0.9999	3.7	171.0	100 - 5000	0.9991	5.5	108.0

■ Analysis of Vinegars

The analysis of the black and grain vinegars detected 15 types of D-amino acids, 16 types of L-amino acids, glycine, and D/L-proline. The number of D-amino acid and L-amino acid components detected from each sample is indicated in Table 3. Multiple D-amino acids were detected in black vinegar that were not detected in grain vinegar, such as D-alanine and D-phenylalanine.

Table 3 Number of Components Detected (Table Vinegar)

	Black Vinegar A	Black Vinegar B	Black Vinegar C	Black Vinegar D	Grain Vinegar A
D-Amino Acids	14	13	13	14	8
L-Amino Acids	16	16	16	16	16

The D-amino acid concentrations in each sample are indicated in Fig. 2. Black vinegars C (fermented/aged for 1 year or more) and D (fermented/aged for 5 years) that were fermented/aged for long periods contained higher D-amino acid concentrations than black vinegars A and B. In particular, they contained very high concentrations of D-alanine (200 ppm or more) and had a 20 % or higher D-amino acid ratio (ratio of D-amino acids to all L and D-amino acids) (Fig. 3). The higher number of D-amino acids in black vinegars C and D was presumably produced by lactic acid bacteria during the long fermentation/aging process.

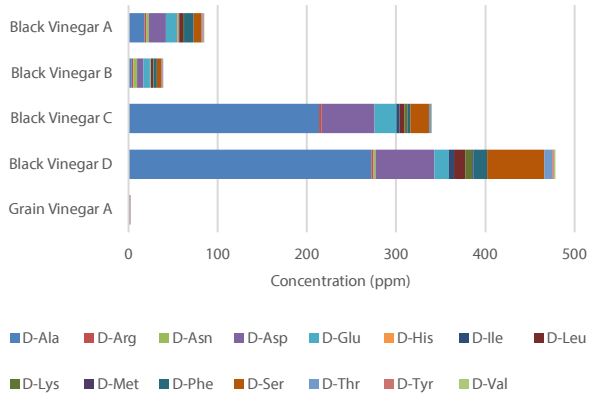

Fig. 2 Concentration of D-Amino Acids in Table Vinegar

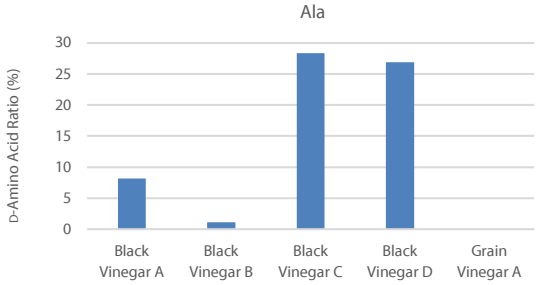

Fig. 3 Ratio of D-Amino Acids (Ala) in Table Vinegar

■ Analysis of Lactic Acid Bacteria Beverages

In the analysis of lactic acid bacteria beverages and fermented milk, 5 types of D-amino acids, 15 types of L-amino acids, and D/L-proline were detected. D-serine was only detected in fermented milk A. The number of D-amino acid and L-amino acid components detected from respective samples is indicated in Table 4.

Table 4 Number of Components Detected (Lactic Acid Bacteria Beverages)

	Lactic Acid Bacteria Beverage A	Lactic Acid Bacteria Beverage B	Lactic Acid Bacteria Beverage C	Fermented Milk A
D-Amino Acids	3	3	3	4
L-Amino Acids	15	15	15	14

The concentrations of D and L-amino acids in the respective sample extract solutions are shown in Figs. 4 and 5. Although lactic acid bacteria beverages B and C were made by the same manufacturer, they contained different numbers of the same lactic acid bacteria. There was not a large difference in the D-amino acid concentrations, but there was a large difference in their L-amino acid concentrations because C contained more lactic acid bacteria.

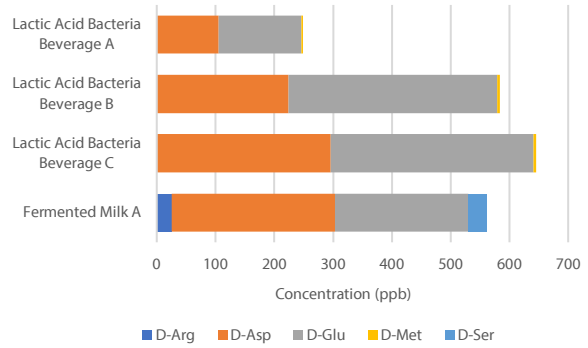

Fig. 4 Concentration of D-Amino Acids in Lactic Acid Bacteria Beverage Extracts

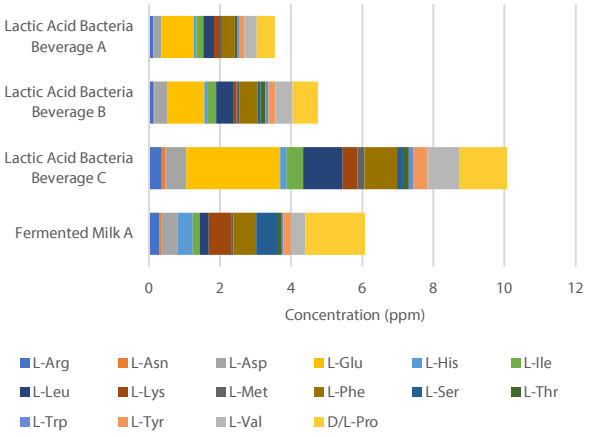

Fig. 5 Concentration of L-Amino Acids in Lactic Acid Bacteria Beverage Extract

■ Analysis of Beers

Analysis of beer (9 types) and non-alcoholic beer (1 type) detected 11 types of D-amino acids, 18 types of L-amino acids, glycine, and D/L-proline. The number of D-amino acid and L-amino acid components detected from respective samples is indicated in Table 5. Only 2 types of D-amino acids were detected in the non-alcoholic beer A, which was very different from beer A-I.

The concentration values for each component were used for principal component analysis using eMSTAT Solution ver. 2.0 software. The corresponding scores and loading plots are shown in Fig. 6. Beers A and F were made by major beer manufacturers, and their similar score plot positions show they had similar characteristics. Beer D, which is plotted toward the right, had very different characteristics compared to the other beers. It contained large amounts of D and L-amino acids. Beer D and other beers plotted toward the right of the score plot were made by dry hopping, which involves adding hops during the fermentation process. This suggest that the first principal component (PC1) axis is related to dry hopping.

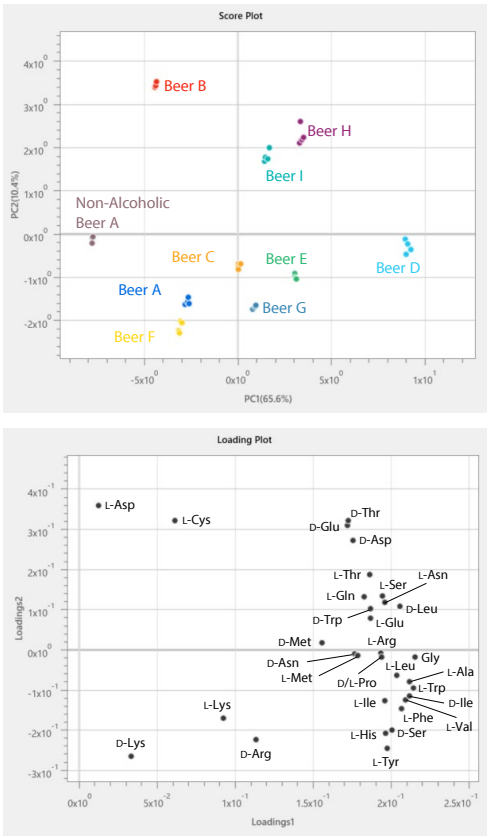

Fig. 6 Principal Component Analysis Results

Measuring the ratios of D-amino acids in each beer revealed that nearly 20 % of some amino acids, such as isoleucine, were D-amino acids. The D-amino acid ratios in beer I are shown in Fig. 7.

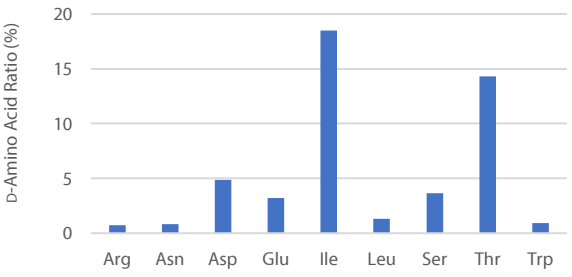

Fig. 7 D-Amino Acid Ratios in Beer I

Table 5 Number of Components Detected (Beer)

	Beer A	Beer B	Beer C	Beer D	Beer E	Beer F	Beer G	Beer H	Beer I	Non-Alcoholic Beer A
D-Amino Acids	11	6	8	11	10	10	10	11	9	2
L-Amino Acids	18	18	18	18	18	18	18	18	18	16

■ Analysis of Lotion

Analysis of the lotion extract solution detected D-glutamate, D-alanine, and D-methionine. The mass chromatograms for the respective components obtained with the CROWNPAK CR-I(+) column are shown in Fig. 8. In addition, good retention time and peak area reproducibility results were obtained, as shown in Table 6.

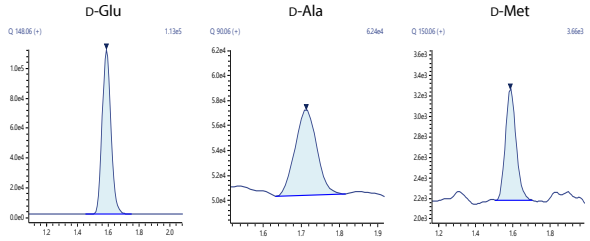

Fig. 8 Mass Chromatograms (Lotion Extract)

Table 6 Reproducibility

Compounds	%RSD (n=4)	
	Retention time	Peak area
D-Glu	0.08	4.2
D-Ala	0.17	4.5
D-Met	0.13	3.0

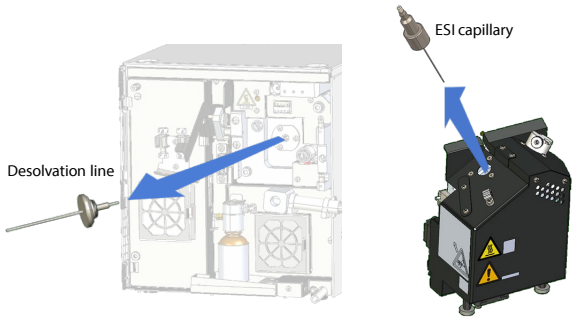

Fig. 9 Desolvation Line and ESI Capillary Replacement

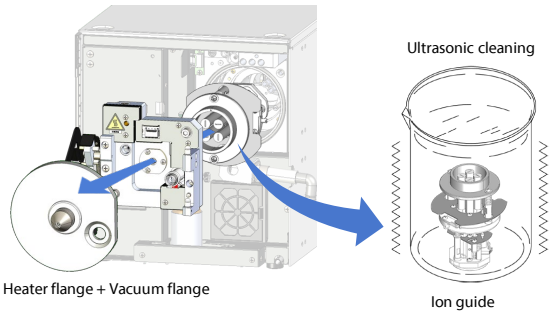

Fig. 10 Maintenance of Lens System

■ Simple Maintenance

If a mobile phase containing TFA is used, TFA will remain in the system even after the mobile phase is changed. So it requires particular care due to higher background levels and inhibition of ionization, as well as other effects from ionizing the TFA and possible effects on subsequent analyses. To eliminate the effects from TFA-derived ions, flow channels need to be adequately cleaned, and parts that come into contact with the mobile phase need to be replaced in the HPLC unit, and the lens system in the MS unit must be cleaned. The area around the interface of the LCMS-2050 is designed so it can be maintained easily. The desolvation line (DL), which delivers samples into the vacuum chamber, and the ESI capillary can be replaced without tools and without releasing the vacuum (Fig. 9). The Qarray and multi-pole ion guides in the lens system can be removed easily through the front of the instrument for immediate washing (Fig. 10). Thus, the LCMS-2050 offers extremely easy maintenance and can also be used for other applications besides the chiral amino acid analysis method described here.

■ Conclusion

This article describes using a chiral column and a single quadrupole LC-MS system to analyze chiral amino acids. The technique enables direct analysis of chiral amino acids without derivatization pretreatment. Although sensitivity is lower than a triple quadrupole LC-MS system, it offers equivalent or higher sensitivity than a quadrupole time-of-flight LC-MS system or a fluorescence detector. So while it is not appropriate for analyzing ultra-trace concentrations of chiral amino acids, it is well suited to analyzing foods and other samples that contain large amounts of chiral amino acids. It also offers good throughput, with the ability to analyze samples within 15 minutes, even if that involves switching between (+) and (-) columns to separate components from contaminants in the samples. This also makes it well suited for analyzing large quantities of samples. Widespread adoption of the described techniques for chiral amino acid analysis can be expected to lead to continuous advancements in the R&D of foods, cosmetics, and other samples.

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. eL592
2. Analysis of Chiral Amino Acids within Fermented Beverages Utilizing a Column Switching System Application News No. eC156
3. Food Metabolomics of Alcoholic Beverage Using Single-Quadrupole Mass Spectrometer Application News No. 01-00334-EN

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