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High-throughput comprehensive analysis of trace D- and L- amino acids using extra-facile chiral separation and column switching

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Introduction

Amino acids (except for glycine) have a chiral carbon atom adjacent to the carboxyl group and form two enantiomers that are mirror images of each other. L-Amino acids are present in the body as a component of proteins and nutrients in large quantities. On the other hand, D-Amino acids are extremely low, but it is drawing attention in various fields such as ingredient analysis of fermented foods, physiological function analysis in the cranial nervous system and biomarker search, as well as health and beauty. Here we developed the method that be possible to analyze chiral amino acids with high sensitivity in just 10 minutes using a chiral column without derivatization and confirmed ratio of L/D amino acids in fermented foods.

Methods

Separation was achieved within 10 min using CROWNPAK CR-I(+) / CR-I(-) (3 mmI.D. x 150 mmL, 5µm, DAICEL corp.) maintained at 20°C on a HPLC system (Prominence, Nexera X2, Shimadzu corporation, Kyoto, Japan). Data acquisition was performed on triple quadrupole mass spectrometer LCMS-8050 / 8060 (Shimadzu Corporation, Kyoto, Japan). The mobile phase consisted of a mixture of acetonitrile, ethanol, water and TFA (80/15/5/0.5) and the flow rate was set to 0.6 mL/min in isocratic condition.
We investigated automatically analysis system using valve switching unit (Figure 2) for continuously analyzing CROWNPAK CR-I(+) and CR-I(-). A mixture of 22 amino acids was diluted to working concentrations in mobile phase. The fermented food samples treated with liquid-liquid extraction by water, methanol and chloroform *[1] (Figure 1).

Table 1 Analytical condition

HPLC (Prominence / Nexera X2)	
Mobile Phase	: ACN/EtOH/H2O/TFA = 80/15/5/0.5
Column	: CROWNPAK CR-I(+) / CR-I(-) (3 mmI.D. x 150 mmL., 5 µm, DAICEL corp.)
Flow Rate	: 0.6 mL/min
Column Temperature	: 20 °C / 25 °C
Injection Volume	: 1 µL
MS (LCMS-8050 / 8060)	
Probe position	: + 3 mm
Ionization	: ESI positive
Nebulizing Gas Flow	: 3.0 L/min
Drying Gas Flow	: 15 L/min
Heating Gas Flow	: 5.0 L/min
Interface Temperature	: 250 °C
DL Temperature	: 250 °C
HB Temperature	: 300 °C

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Mix 100 µL of sample, 350 µL of MeOH and 50 µL of water

↓

Mix with vortex and centrifugation at 10,000 rpm for 10 min at 4 °C

↓

Mix 360 µL of supernatant, 180 µL of water and 360 µL of chloroform

↓

Mix with vortex and centrifugation at 10,000 rpm for 10 min at 4 °C

↓

Mix 50 µL of supernatant with 200 µL of mobile phase.

Figure 1 Pretreatment protocol

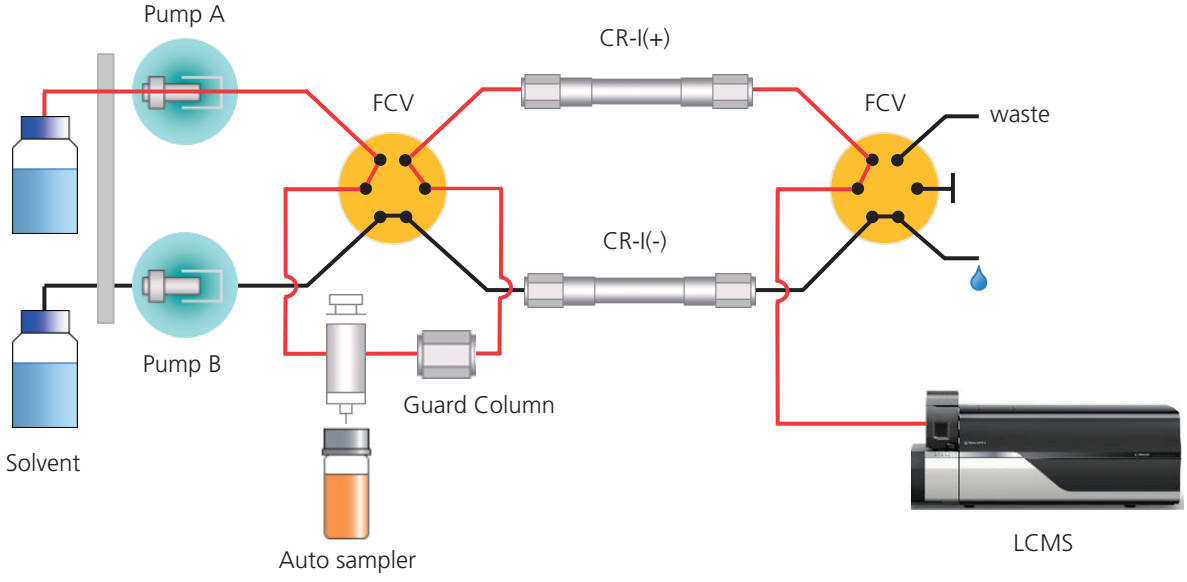

Figure 2 Chiral amino acid analysis system using valve switching unit

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Result

Table 2 Ratio of D/L amino acids in black vinegars and yogurts

	Vinegar A		Vinegar B		Vinegar C		Vinegar D		Vinegar E	
	Area	ratio of D/L	Area	ratio of D/L	Area	ratio of D/L	Area	ratio of D/L	Area	ratio of D/L
D-Ala	7127	3.8%	54094	20.5%	26505	15.5%	140959	164.0%	37900	40.2%
L-Ala	187083		263547		171483		85940		94190	
D-Arg	23703	0.6%	81626	2.4%	106896	1.7%	81779	6.9%	95602	36.5%
L-Arg	3945110		3353883		6214029		1192614		262060	
D-Asn	7047	1.3%	11213	3.4%	13135	3.0%	60836	43.2%	3209	16.5%
L-Asn	547867		333152		433012		140872		19416	
D-Asp	6934	1.5%	7086	2.3%	8248	2.2%	47149	38.1%	2441	15.3%
L-Asp	476730		302901		370152		123860		16003	
D-Cys	(N.D.)	-	(N.D.)	-	(N.D.)	-	(N.D.)	-	(N.D.)	-
L-Cys	(N.D.)		(N.D.)		(N.D.)		(N.D.)		(N.D.)	
D-Gln	4153	56.1%	5013	128.1%	5738	17.3%	4743	0.6%	5157	19.8%
L-Gln	7399		3912		33155		856603		26021	
D-Glu	11658	0.7%	36502	2.2%	7575	1.1%	412572	4091.1%	163715	5069.6%
L-Glu	1635202		1675657		713130		10085		3229	
Gly	2375		6382		3163		957		1106	
D-His	(N.D.)	-	(N.D.)	-	(N.D.)	-	(N.D.)	-	9030	5.2%
L-His	351973		410895		232228		839834		175326	
D-Ile	1262	0.3%	(N.D.)	-	1861	0.6%	1428	0.8%	1366	1.0%
L-Ile	392041		580580		330869		176626		130832	
D-allo-Ile	1816	50.3%	(N.D.)	-	2519	136.9%	2225	59.4%	1247	39.3%
L-allo-Ile	3612		4357		1840		3744		3172	
D-Leu	3255	0.5%	4698	0.5%	4198	0.9%	4042	1.0%	(N.D.)	-
L-Leu	691108		1031536		493487		403567		132923	
D-Lys	13921	1.4%	4446	0.4%	28009	5.1%	1151264	73.5%	24797	3.5%
L-Lys	965688		1220610		548517		1565451		698677	
D-Met	(N.D.)	-	(N.D.)	-	(N.D.)	-	463	0.9%	(N.D.)	-
L-Met	22647		48753		13151		54490		(N.D.)	
D-Phe	2738	0.4%	3587	0.7%	3634	0.9%	1600	0.5%	1799	1.5%
L-Phe	746758		549410		419561		313615		117732	
DL-Pro	301069		683984		549718		2094819		888155	
D-Ser	10568	9.3%	8036	7.5%	4653	8.5%	14619	14.4%	8332	29.3%
L-Ser	113543		106729		54472		101651		28395	
D-Thr	2646	1.7%	4374	2.3%	2036	1.2%	1711	1.5%	3314	4.6%
L-Thr	159723		193429		170581		112074		71653	
D-allo-Thr	1973	91.6%	3538	120.7%	1297	66.6%	1973	42.5%	1020	23.7%
L-allo-Thr	2153		2932		1946		4647		4294	
D-Trp	2098	23.2%	2195	39.1%	4159	39.6%	3039	1.9%	1879	13.3%
L-Trp	9045		5609		10506		155899		14086	
D-Tyr	7314	1.7%	2495	0.8%	4026	1.4%	4882	2.1%	5876	107.4%
L-Tyr	437963		314522		297401		230926		5470	
D-Val	3046	0.5%	3186	0.4%	3613	0.9%	1241	0.4%	1277	0.9%
L-Val	573054		870777		387972		285792		148323	

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Figure 3 Ratio of 7 D/L amino acids in black vinegars and yogurts.
Blue bar is D amino acids and red bar is L amino acids.

Table 2 shows the ratio of D/L amino acids in black vinegars and yogurts. All sample contained D-amino acids. In 2 yogurts, D-Glu was included 40 times larger than L-Glu. Figure 3 shows the ratio of 7 D/L amino acids found in relatively large amounts of D-form in black vinegars and yogurts. D-Ala is exists in all sample. 2 yogurts contained D-Glu, but L-Glu was not contained. Especially, Yogurt D includes large amount of D-Ala and D-Lys.

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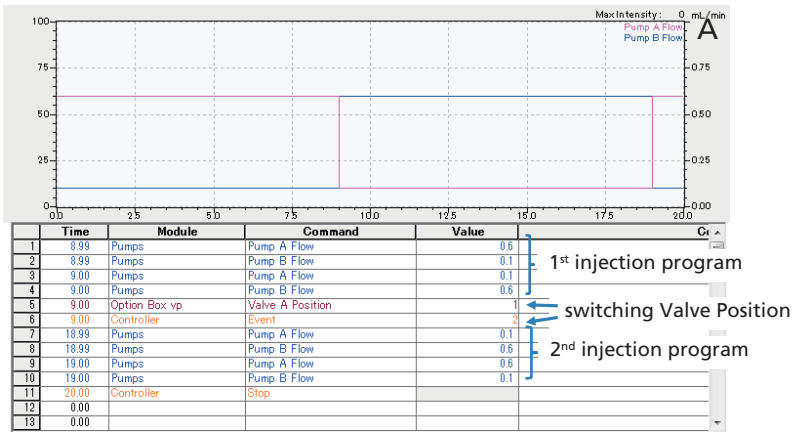
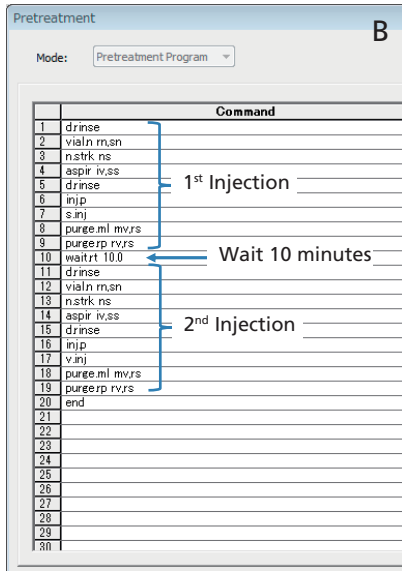


	Time	Module	Command	Value
1	8.99	Pumps	Pump A Flow	0.6
2	8.99	Pumps	Pump B Flow	0.1
3	9.00	Pumps	Pump A Flow	0.1
4	9.00	Pumps	Pump B Flow	0.6
5	9.00	Option Box vp	Valve A Position	1
6	9.00	Controller	Event	2
7	18.99	Pumps	Pump A Flow	0.1
8	18.99	Pumps	Pump B Flow	0.6
9	19.00	Pumps	Pump A Flow	0.6
10	19.00	Pumps	Pump B Flow	0.1
11	20.00	Controller	Stop	
12	0.00			
13	0.00			



	Command
1	drinse
2	vialn rn,sn
3	n.strk ns
4	aspir iv,ss
5	drinse
6	inj.p
7	s.inj
8	purge.ml mv,rs
9	purgerp rv,rs
10	waitrt 10.0
11	drinse
12	vialn rn,sn
13	n.strk ns
14	aspir iv,ss
15	drinse
16	inj.p
17	v.inj
18	purge.ml mv,rs
19	purgerp rv,rs
20	end
21	
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30	

Figure 4 (A) LC Time Program
(B) Pretreatment Program (SIL-30AC)

Valve position of 2 FCV units and flow rate can change by LC Time Program in 9 minutes (Figure 4A). In 19 minutes, flow rate changes initial conditions. Using Pretreatment Program (SIL-30AC), we can inject sample twice during one analysis (Figure 4B). It is possible to quantify and identify all D/L amino acids in one data file. And it is clear that 2 sample injected from same vial. The method using these programs can support continuous analysis in batch analysis.

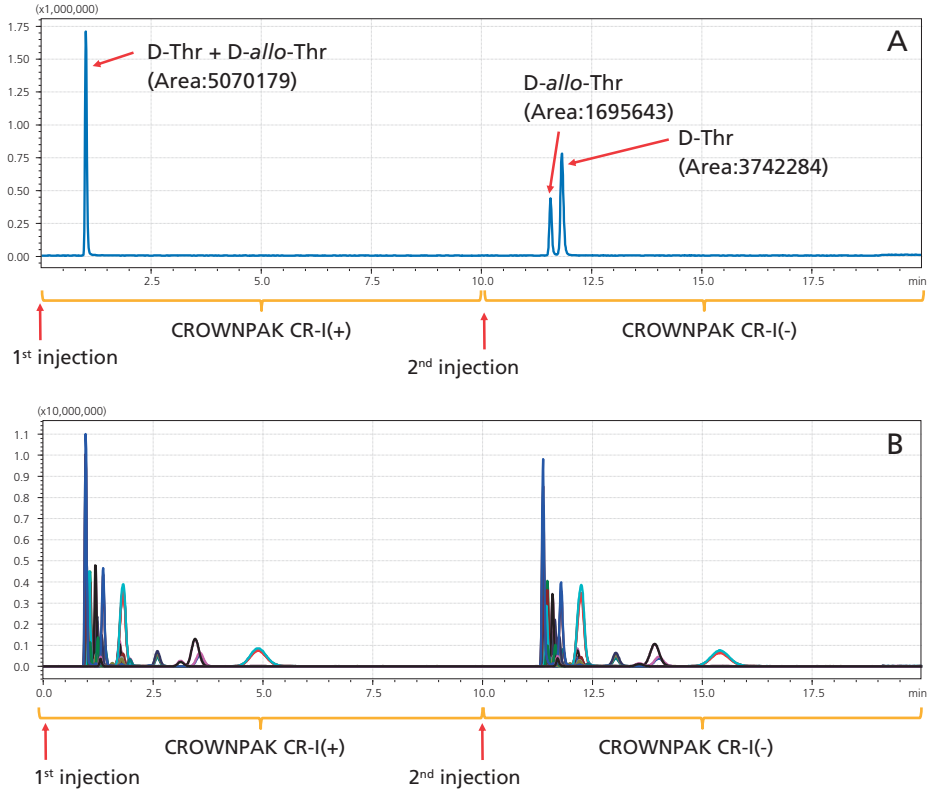

Figure 5 (A) MRM chromatogram of D-Threonine and D-*allo*-Threonine
(B) MRM chromatogram of 22 D/L amino acids.

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Thr and *allo*-Thr have very similar physicochemical properties. Therefore, almost the same MRM transition was obtained in the triple quadrupole-type mass spectrometer and it could not be possible to separate because it co-eluted. However even when co-eluting at the same retention time in CR-I(+), separation could be confirmed by switching to CR-I(-) (Figure 5A). A mixture of 22 amino acids can be detected with sufficient sensitivity using this method (Figure 5B). The analysis results using 2 columns could be compiled in one data.

Conclusions

- The automatically analysis system using valve switching is useful for the D/L amino acids analysis by LC-MS/MS.
- Using LC Time Program and Pretreatment Program of SIL-30AC, the system can support high-throughput comprehensive analysis of D/L amino acids.

Reference

*[1] Nakano,Y., Konya,Y., Taniguchi,M., Fukusaki,E., Journal of Bioscience and Bioengineering, 123, 134-138 (2017)

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