High-Speed Separation of Amino Acids using Pre-column Derivatization by Extreme Liquid Chromatography (X-LC™)

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SUMMARY

We developed ultra-high-speed automated amino acid analysis system using an X-LC™ system with a 2.0 mm I.D. x 50 mm L column packed with 2.0-µm dia. packing material. We have successfully reduced the original analysis time from 38 min to 7 min. This is an improvement by a factor of more than 5. Pre-column derivatization with OPA (orthophthalic aldehyde) was performed automatically by using the new JASCO Model X-LC™ 3059AS auto-sampler.

INTRODUCTION

Amino acid analysis is becoming more important in a variety of application fields, ranging from food analysis to protein science. A number of separation and detection methods are currently used. Among them, a combination of pre-column derivatization with OPA (orthophthalic aldehyde) and separation on a C18 column with fluorescence detection is generally preferred due to the simplicity and high sensitivity of the method [1-6].

A typical conventional method uses a 4.6 mm I.D. x 150 / 250 mm L column packed with 5-µm dia. packing material that requires approximately 38 min to complete the analysis.

EXPERIMENTAL

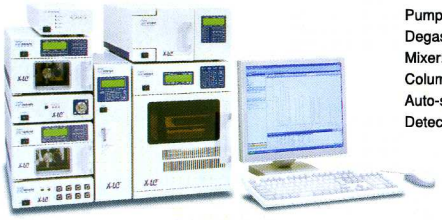

Figure 1. X-LC™ system

Pump:	X-LC™ 3185PU x 2
Degasser:	X-LC™ 3080DG
Mixer:	X-LC™ 3080MX
Column oven:	X-LC™ 3067CO
Auto-sampler:	X-LC™ 3059AS
Detector:	X-LC™ 3120FP

Automated Pre-column Derivatization System The derivatization of amino acids was performed using an auto-sampler program. The procedure for derivatization is described in the text. Figure 2 shows the flow diagram of the auto-sampler.

Procedure for Automated Pre-column Derivatization

1) Wash needle
2) Draw reagent and sample with air segmentation
3) Eject to reaction vial
4) Mix sample and reagent by air bubbling
5) Wash needle
6) Pause to allow reaction to complete
7) Draw derivatized sample
8) Injection onto column

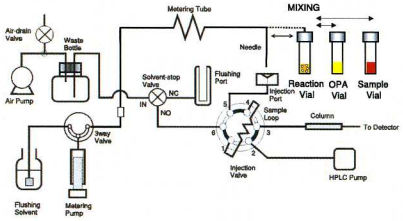

Figure 2. Flow diagram of the auto-sampler (X-LC™ 3059AS)

(1) Optimization of Derivatization Conditions

Peak areas of 6 amino acids were plotted against reaction times as shown in Figure 3. The derivatives of glycine and lysine showed appreciable decay of fluorescence intensity when reaction times became more than 300 seconds. In addition, the lysine derivative gave a very low response compared with the derivatives of other amino acids. This phenomenon was also observed by Jones [1]. We selected the reaction time of 30 seconds shown by a blue bar.

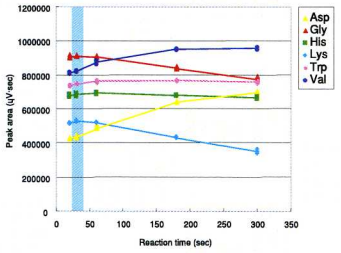

Figure 3. Effect of reaction time on peak area

Conditions of the Pre-column Derivatization

Sample: Amino acid standard solution, type H + Cysteic acid + Trp (20 pmol/µL each)
Derivatization reagent:
0.4 M borate buffer (pH9.0)/OPA (1% MeOH solution)/2-mercaptoethanol (1/0.5/0.01)
2-mercaptoethanol was purchased from Pierce, IL, USA. All other chemicals were from Wako Pure Chemicals, Osaka, Japan.

Auto-sampler reaction conditions:

sample volume:	100µL
reagent volume:	20µL
reaction time:	30sec
mixing times:	2
injection volume:	1µL

We followed the derivatization conditions described in reference[1] except for retention time.

(2) Optimization of Separation Conditions

As shown in Figure 4, pH greatly affected the separation of histidine and serine. We selected pH 5.8.

The change in the column temperature greatly influenced separations shown in orange, green and blue in Figure 5. We set the column temperature to 40°C.

Optimized conditions

Eluent A: 1.0M citrate buffer (pH5.8) 3.5mL in 1L of H_2O
Eluent B: 1.0M citrate buffer (pH5.8) 3.5mL in 1L of $CH_3CN/C_2H_5OH/H_2O$ (30/30/40)
Gradient condition:1cycle 10min
A:B = 90:10 → 90:10 → 72:28 → 72:28 → 42:58 → 42:58 → 23:77 → 0:100 → 0:100 → 90:10
0.2min 2.2min 2.5min 4.6min 5.0min 6.1min 6.15min 7.0min 7.05min
Flow rate: 0.6mL/min
Column: X-PressPak V-C18 (2-µm dia., 2.0mm I.D. x 50mm)
Column temperature: 40°C
Detection: Fluorescence; Ex 345nm, Em 455nm; Gain x100

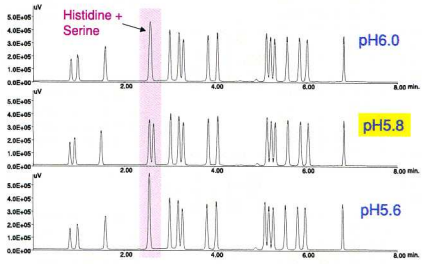

Figure 4. Effect of eluent pH on separation of histidine and serine

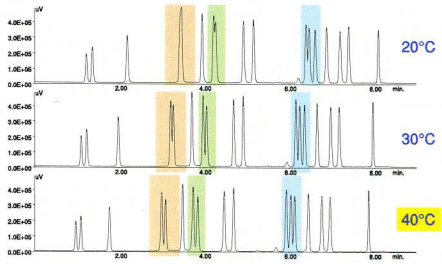

Figure 5. Effect of column temperature on separation of several amino acids

RESULTS AND DISCUSSION

This analysis was performed under the optimized conditions as shown in Figure 6. The blue line indicates gradient profile (B%).

Figure 7 compares X-LC™ with conventional HPLC. High-speed analysis became possible by using X-LC™; the total analysis time was reduced to 1/5. In addition, it is remarkable that the amount of solvent consumption was reduced to only 1/10.

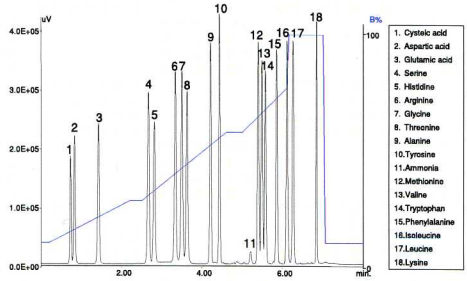

Figure 6. Analysis of standard mixture of 18 amino acids (20pmol/µL each)

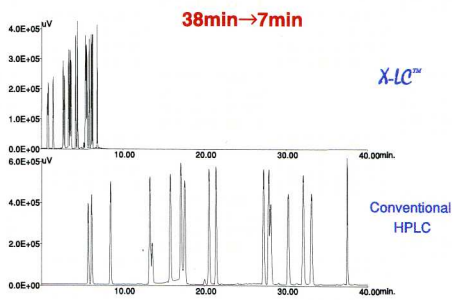

Figure 7. Comparison between X-LC™ and conventional HPLC

(1) Precision of the Analysis

Reproducibility of Retention Time and Peak Area Reproducibilities of the retention times and peak areas were calculated by the results of ten consecutive analyses of 8 amino acid standards (20pmol each) as shown in Table 1. The relative standard deviations were between 0.048 and 0.429% for the retention times, and between 0.546 and 1.90% for the peak areas. Excellent reproducibilities of the retention times and peak areas were observed.

Linear Dynamic Range and Detection Limit Figure 8 indicates linear dynamic range for arbitrary chosen amino acid derivatives. Correlation coefficients (r) were also calculated by using the results of analyses of 7 amino acid derivatives ranging from 0.2 to 20pmol. Excellent linearity was observed.

Highly sensitive detection was realized by the use of the fluorescence detector; the detection limit (S/N=3) was between 21 and 49 fmol/μL as shown in Table 2.

Table 1. Relative standard deviation (%RSD) for retention time and peak area

	%RSD	
	Rt	Peak area
Cys-SO$_3$	0.423	1.806
Asp	0.429	1.899
Glu	0.338	1.047
Ser	0.161	0.604
His	0.164	0.701
Arg	0.149	0.683
Gly	0.114	0.869
Thr	0.104	0.859
Ala	0.080	0.584
Tyr	0.074	0.782
NH$_3$	0.071	2.290
Met	0.061	0.584
Val	0.060	0.998
Trp	0.060	0.546
Phe	0.058	0.651
Ile	0.054	0.911
Leu	0.055	0.601
Lys	0.048	0.743

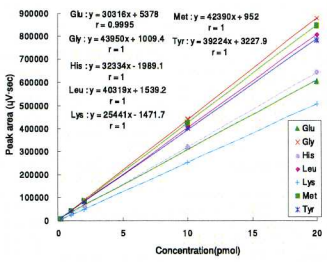

Figure 8. Linear dynamic ranges for 7 amino acid derivatives

Table 2. Detection limits for 18 amino acid derivatives

Amino acid	S/N=3
	fmol/μL
Cys-SO$_3$	48.7
Asp	40.9
Glu	37.3
Ser	30.5
His	37.1
Arg	27.2
Gly	27.3
Thr	30.4
Ala	23.7
Tyr	20.9
NH$_3$	434.4
Met	23.6
Val	25.7
Trp	27.1
Phe	24.4
Ile	23.5
Leu	23.4
Lys	21.6

(2) Application to Food Analysis

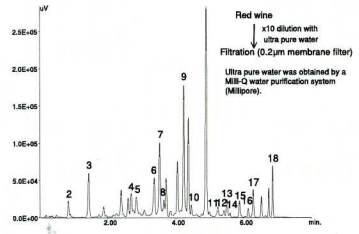

Figure 9. Analysis of amino acids in red wine

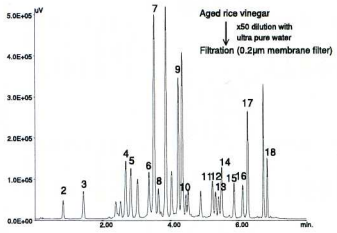

Figure 10. Analysis of amino acids in aged rice vinegar

Aged rice vinegar contains various amino acids and minerals. Seventeen amino acids were detected.

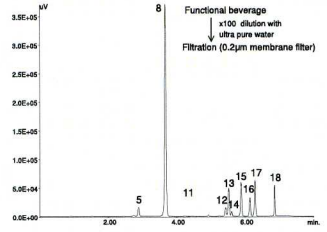

Figure 11. Analysis of amino acids in functional beverage

This beverage contained 9 essential amino acids, i.e., His, Thr, Met, Val, Trp, Phe, Ile, Leu and Lys.

Analysis of Other Amino Acids Figure 12 shows the chromatogram of standard mixture of 21 amino acids containing GABA (γ-aminobutyric acid). GABA is an important inhibitory neurotransmitter in the central nervous system and essential for brain metabolism and function. Asparagine and glutamine are contained in this standard sample besides GABA. The analysis conditions are the same as shown in Figure 6.

Figure 13 shows the chromatogram of amino acids in white wine containing GABA. The label of this wine said that it contained lychee juice that is known as good source of GABA. The analysis conditions are the same as shown in Figure 6.

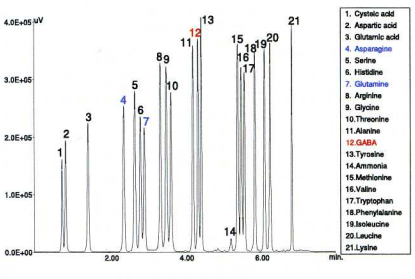

Figure 12. Analysis of standard mixture of 21 amino acids containing GABA (20pmol/μL each)

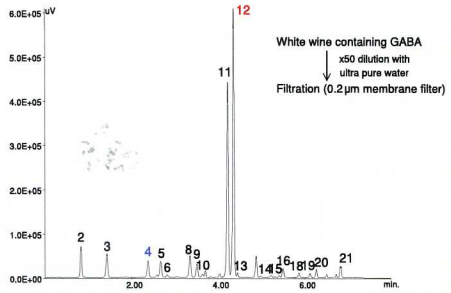

Figure 13. Analysis of amino acids in white wine containing GABA

Analysis of Theanine Theanine is an amino acid which is a derivative of glutamine. It is found almost exclusively in tea plants and it produces a feeling of relaxation. Theanine has a sweet and *umami* taste and shows the highest correlation to the quality of Japanese green tea. Figure 14 shows the chromatogram of standard mixture of 18 amino acids containing theanine. The analysis condition is different from that in Figure 6. The standard mixture contained two internal standards, AABA and norvaline. AABA was used as an internal standard for the analysis of green tea, but norvaline is suitable for the analysis of black tea. Because it is difficult to separate impurity peaks from AABA.

Figure 15 shows the chromatogram of amino acids of green tea. It should be noted that the green tea contained a large amount of theanine, and it was the major component of amino acids.

Analysis Condition (Theanine)

This analysis condition is aimed at the analysis of amino acids containing theanine in tea. This analysis operates in a gradient elution mode with a binary ethanol-citrate buffer mobile phase system.

Eluent A: 1.0M citrate buffer (pH6.0) 3.5mL in 1L of C_2H_5OH/H_2O (12/88)

Eluent B: 1.0M citrate buffer (pH6.0) 3.5mL in 1L of C_2H_5OH/H_2O (50/50)

Gradient condition: 1cycle 14min

A:B = 100:0 → 0:85 → 0:85 → 100:0

9.0min 0.5min 0.05min

Flow rate: 0.4mL/min

Column: X-PressPak V-C18 (2-μm dia., 2.0mm I.D. x 50mm)

Column temperature: 40°C

Detection: Fluorescence; Ex 340nm,Em 450nm; Gain x100

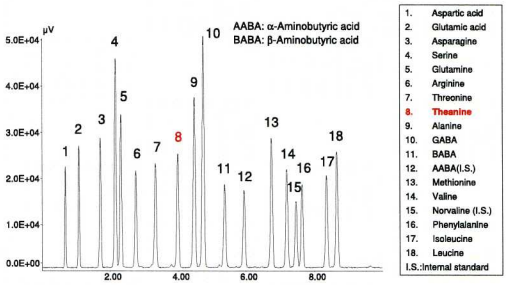

Figure 14. Analysis of standard mixture of 18 amino acids containing theanine (0.4mg/L each)

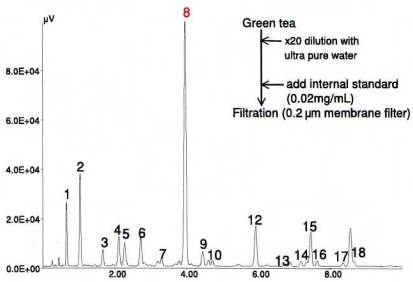

Figure 15. Analysis of amino acids in green tea

CONCLUSION

(1) Ultra-High-Speed Analysis
Total analysis time: ca 1/5 (38min→7min)

(2) Reduction of Solvent Consumption
Amount ratio: ca 1/10 (60mL→6mL)

(3) Highly Sensitive Detection
Detection limit (S/N=3): 21 - 49 fmol/ μL

(4) Excellent Reproducibility (n=10)
RSD%: 0.05 - 0.43% (retention time), 0.58 - 1.90% (peak area)

(5) Applicable to various Food Analyses

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