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Principles and Applications of the Prominence Amino Acid Analysis System

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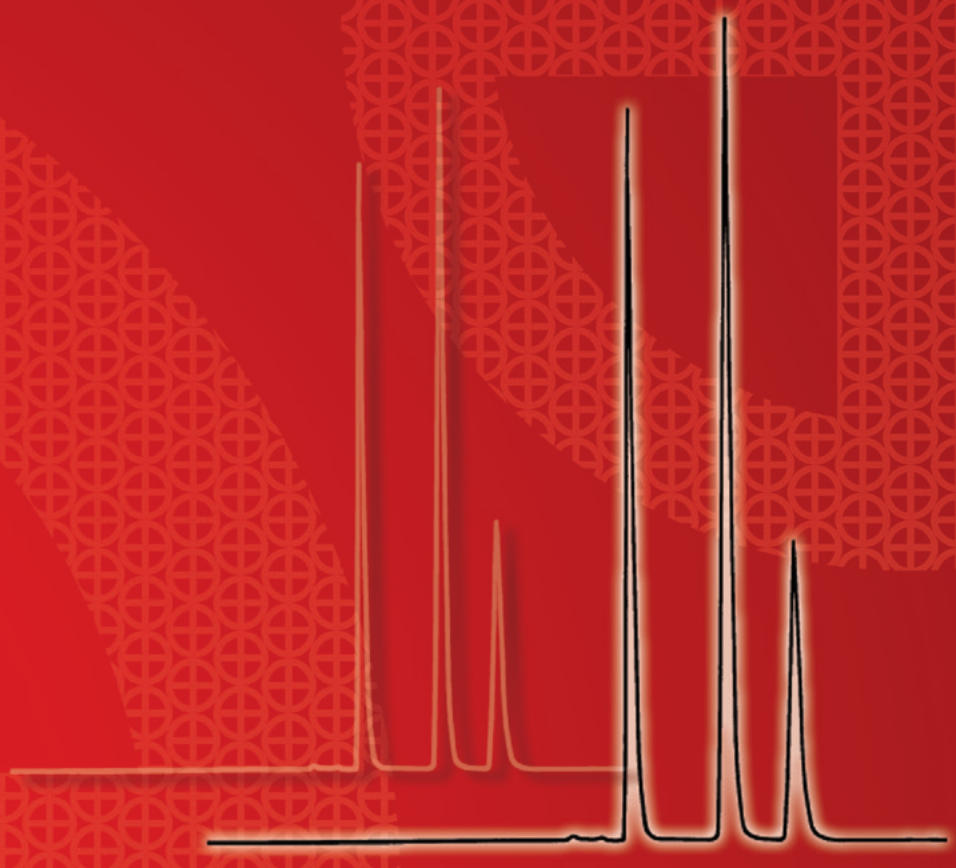

Principles and Applications of the Prominence Amino Acid Analysis System

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1. Introduction

The amino acid analyzer[1] was developed in the latter half of the 1950s by Moore, Stein and others. It performed separation of amino acids using the combination of a cation exchange column and a stepwise gradient that switched several of mobile phase. It achieved simultaneous analysis of amino acids by conducting post-column derivatization detection using a ninhydrin reagent as the reaction solution.

On the other hand, high-performance liquid chromatography (HPLC) appeared at the end of the 1960s and rapidly spread to a wide variety of fields as a revolutionary method of separation analysis. Various investigations were conducted using an amino acid fluorescent derivatization method[2] for amino acid analysis that utilized reactions between an ortho-phthalaldehyde (OPA) reagent and primary amines. In the latter half of the 1970s, Shimadzu created an HPLC amino acid analysis system incorporating the post-column derivatization method using this OPA reagent. In addition, to simultaneously detect the secondary amino acid proline, a "non-switching flow" method was proposed[3] that added an oxidizing agent (sodium hydrochloride) continuously to the column eluate to establish a high-sensitivity amino acid analysis system.

This report introduces the principles of the Prominence Amino Acid Analysis System and applications in the field of food products. This system is based on Shimadzu's many years of expertise in amino acid analysis.

Table 1 Abbreviation for Amino Acids

Amino acid	Symbol		Amino acid	Symbol	
Glycine	Gly	G	Aspartic acid	Asp	D
Alanine	Ala	A	Asparagine	Asn	N
Valine	Val	V	Glutamic acid	Glu	E
Leucine	Leu	L	Glutamine	Gln	Q
Isoleucine	Ile	I	Arginine	Arg	R
Serine	Ser	S	Lysine	Lys	K
Threonine	Thr	T	Histidine	His	H
Tyrosine	Tyr	Y	Phenylalanine	Phe	F
Cysteine	Cys	C	Tryptophan	Trp	W
Cystine	$(Cys)_2$	-	Proline	Pro	P
Methionine	Met	M			

2. Principles of Amino Acid Analysis

2.1 Cation Exchange Mode for Amino Acid Separation

Several separation modes can be selected for HPLC amino acid analysis. The Prominence Amino Acid Analysis System uses the cation exchange mode in the same way as an amino acid analyzer, as it conducts post-column fluorescent derivatization detection after directly separating the amino acids. The amino acids are loaded into the cation exchange column along with a weakly acidic citric acid buffer solution, which is the primary mobile phase. They are then separated by gradient elution that increases the concentration ratio of the basic citric acid buffer solution, which is the secondary mobile phase.

At this time, it is necessary to select the cation exchange column counterions according to the amino acid components to be subjected to analysis. The "Shim-pack Amino-Na" sodium-type column was used to analyze the 17 amino acid components comprising a protein with the Prominence Amino Acid Analysis System. The "Shim-pack Amino-Li" lithium-type column was used for the analysis of 38 free amino acid components.

2.2 Fluorescence Detection with OPA Post-column Derivatization

As the ultraviolet absorption of the aromatic amino acids (such as phenylalanine, tyrosine, etc.) originates in the benzene ring, they can be detected at 250 to 280 nm. However, the fatty amino acids have an ultraviolet absorption based on the carboxyl group in the 200 to 210 nm range, making detection with a direct absorbance detector rather limited. Consequently, a "derivatization detection method" is used to detect amino acids after reacting them with reagents to convert them to ultraviolet-visible-absorbent or fluorescent substances. This type of derivatization can employ a "pre-column derivatization method" that conducts the reaction before introducing the sample into the instrument or a "post-column derivatization method" that conducts the reaction after separation in the column. The Prominence Amino Acid Analysis System employs a post-column fluorescent derivatization detection system that is easily automated and offers outstanding reproducibility.

Fig. 1 shows the formula for the amino acid fluorescent derivatization reaction with an OPA reagent.

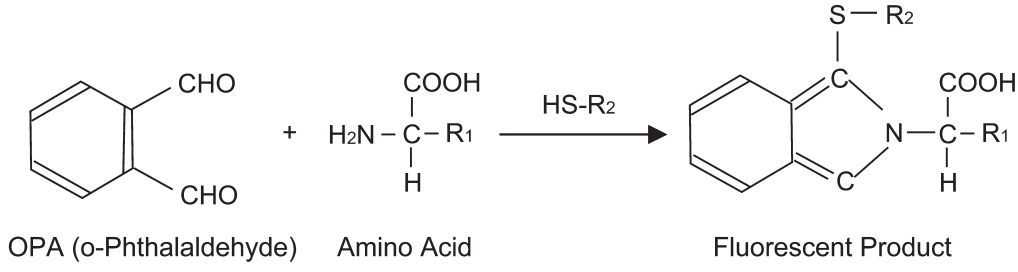


Fig. 1 Reaction of Amino Acid with *o*-Phthalaldehyde

The primary amino acids eluted from the column sequentially react with the continuously added OPA reagent, and are converted to fluorescent derivatives that are detected. However, as the secondary amino acids, such as proline, do not directly react with the OPA, sodium hypochlorite solution is added continuously as an oxidizing reagent, continuously immediately prior to mixing with the OPA to convert the secondary amino

acids into primary amines that do react with OPA. A compound with a -SH group is essential for this reaction between OPA and amino acids (primary amino group) and, generally, 2-mercaptoethanol ($SHCH_2CH_2OH$) or ethanethiol (SH CH_2CH_3) is used. However, as both these reagents have the characteristic foul odor of -SH compounds in liquid form, there are problems in handling them when preparing the reaction solution. This method also has the disadvantage of inadequate proline detection sensitivity.

As a substitute for these reagents, Shimadzu discovered that *N*-acetyl-L-cysteine (Fig. 2), an odorless white crystal, greatly increases the sensitivity for proline and introduced it as the -SH compound for the OPA reaction. Fig. 3 shows a schematic diagram comparing the fluorescent intensity (peak height) of each amino acid using *N*-acetyl-L-cysteine and 2-mercaptoethanol. (It also shows the results of increasing OPA concentration with 2-mercaptoethanol.) These results show that *N*-acetyl-L-cysteine significantly enhances the peak intensity of proline and achieves the same sensitivity levels for other amino acids.

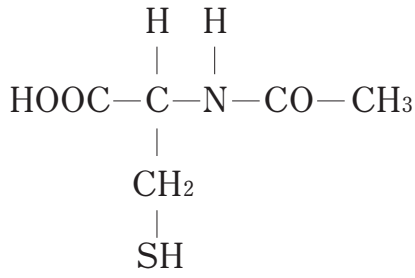


Fig. 2 *N*- Acetyl-L-cysteine

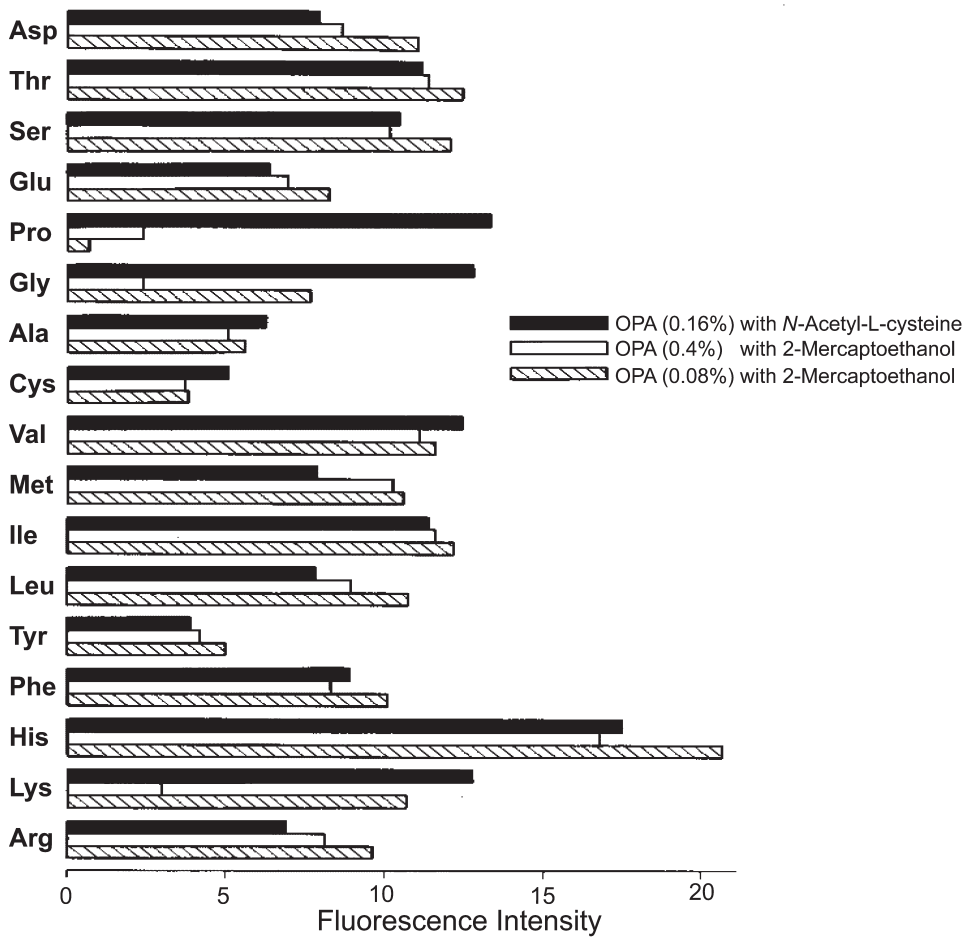


Fig. 3 Comparison of Fluorescence Intensity between *N*- Acetyl-L-cysteine and 2-Mercaptoethanol

3. The Prominence Amino Acid Analysis System

3.1 Equipment Configuration

The Shimadzu HPLC Prominence Amino Acid Analysis System comprises an LC-20AB mobile phase delivery unit, FCV-11ALS flow-switching valve, SIL-20AC autosampler, CTO-20AC column oven, RF-10A$_{XL}$ fluorescence detector, CBM-20A system controller, two LC-20AD reaction reagent delivery units (or a PRR-2A peristaltic pump), two* DGU-20A$_3$ degassers, and an amino acid analysis tubing kit. In addition, an LCsolution workstation is used for data processing and equipment control. Fig. 4 shows the flow diagram for this system.

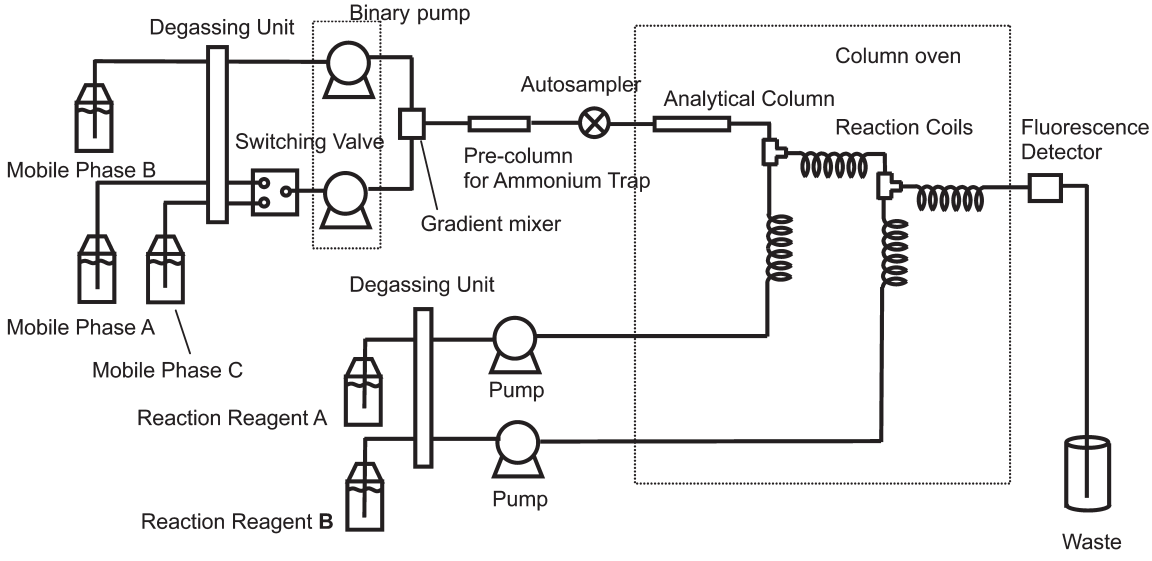


Fig. 4 Flow Diagram of the Prominence Amino Acid Analysis System

3.2 Analytical Conditions and the Analysis Kit

The analytical conditions for the Prominence Amino Acid Analysis System are determined according to the objectives of the analysis. Three types of conditions — "high-separation conditions," "high-speed conditions," and "tryptophan simultaneous conditions" — with different gradient programs and flow rates are available for protein hydrolysis amino acid analysis using the Shim-pack Amino-Na column (hereinafter "Na-Type"). Standard conditions for free amino acid 38-component analysis using the Shim-pack Amino-Li column (hereinafter "Li-Type") are also provided, meaning a total of four kinds of analytical conditions are provided.

The mobile phase and reaction solutions used for analysis are available as the "Amino Acid Analysis Kit," which eliminates the effort required to prepare the mobile phases and reaction solutions. (However, the preparation and addition of sodium hypochlorite to create reaction solution A is still required.)

Table 2 and Table 3 show the analytical conditions for the Na-Type analysis and Li-Type analysis, respectively.

* One DGU-20A$_5$ degasser if the PRR-2A peristaltic pump is used.

Table 2 Analytical Conditions for Na-Type

<Separation>	
Analytical Column	: Shim-pack Amino-Na (100 mmL. x 6.0 mmI.D.)
Pre-column	: Shim-pack ISC-30Na (50 mmL. x 4.0 mmI.D.)
Mobile Phase	: Mobile Phase Kit-Na, gradient elution*
	A : Sodium citrate buffer
	B : Sodium citrate buffer containing boric acid
	C : Sodium hydroxide solution
Flow Rate	: 0.4 - 0.6 mL/min*
Column Temp.	: 60°C
<Detection>	
Reaction Reagent	: Reaction Reagent Kit
	A : Sodium hypochlorite in carbonate buffer
	B : OPA and *N*-Acetyl-L-cysteine in carbonate buffer
Flow Rate	: 0.2 mL/min each
Reaction Temp.	: 60°C
Detection	: Fluorescence Ex.350 nm, Em.450 nm

Table 3 Analytical Conditions for Li-Type

<Separation>	
Analytical Column	: Shim-pack Amino-Li (100 mmL. x 6.0 mmI.D.)
Pre-column	: Shim-pack ISC-30Li (50 mmL. x 4.0 mmI.D.)
Mobile Phase	: Mobile Phase Kit-Li, gradient elution
	A : Lithium citrate buffer
	B : Lithium citrate buffer containing boric acid
	C : Lithium hydroxide solution
Flow Rate	: 0.6 mL/min
Column Temp.	: 39°C
<Detection>	
Reaction Reagent	: Reaction Reagent Kit, the same as shown in table 2
Flow Rate	: 0.2 mL/min each
Reaction Temp.	: 39°C
Detection	: Fluorescence Ex.350 nm, Em.450 nm

* The gradient programs and flow rates differ for the “high-separation conditions,” “high-speed conditions” and “tryptophan simultaneous conditions.”

4. Standard Amino Acid Analysis

4.1 Simultaneous Analysis with the Na-Type Analytical Conditions

Most proteins separate into 17 amino acids when subjected to hydrolysis by hydrochloric acid or some other reagent. The standard method for such protein hydrolysis amino acid analysis uses a "Shim-pack Amino-Na" sodium-type column and the Na-Type high-separation conditions. Fig. 5 shows an analysis example of 17 amino acid components under these conditions (each 0.1 nmol/μL, 10 μL injected volume).

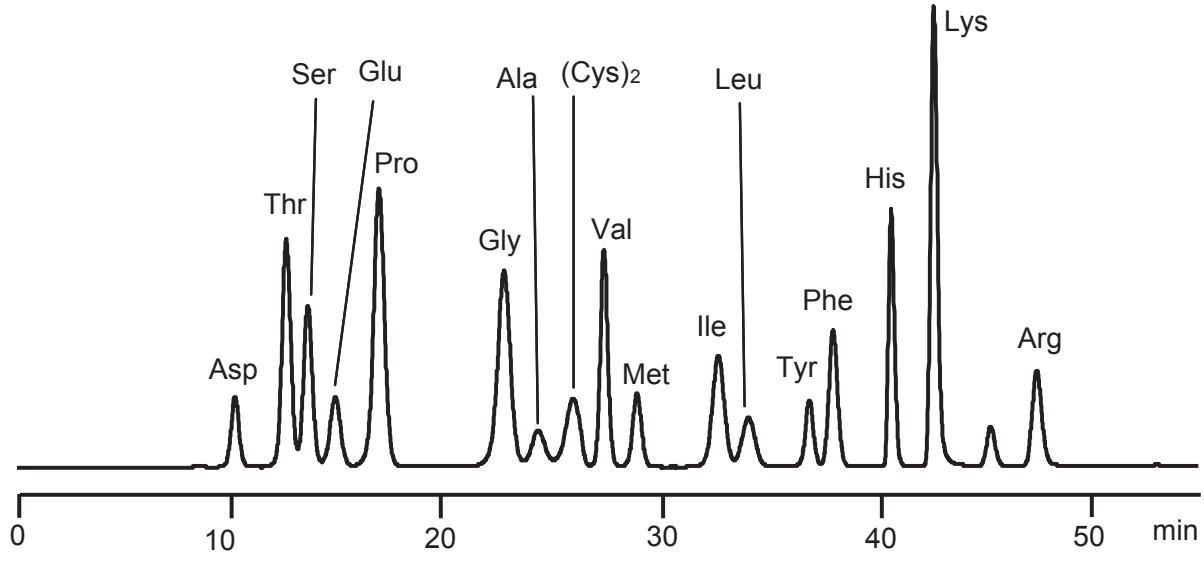


Fig. 5 Chromatogram of a Standard Mixture of 17 Amino Acids
− High-Separation Analysis Mode (Na)

To analyze 18 amino acids including tryptophan, the tryptophan simultaneous conditions are used and tryptophan is eluted between histidine and lysine. Fig. 6 shows an example of the analysis of 18 amino acids using the tryptophan simultaneous conditions (each 0.1 nmol/μL, 10 μL injected volume).

In addition, the analysis period under high-separation conditions is 62 minutes, including elution, washing and re-equilibration, but can be shortened to 45 minutes by using the high-speed conditions. Fig. 7 shows an example of analysis using the high-speed conditions.

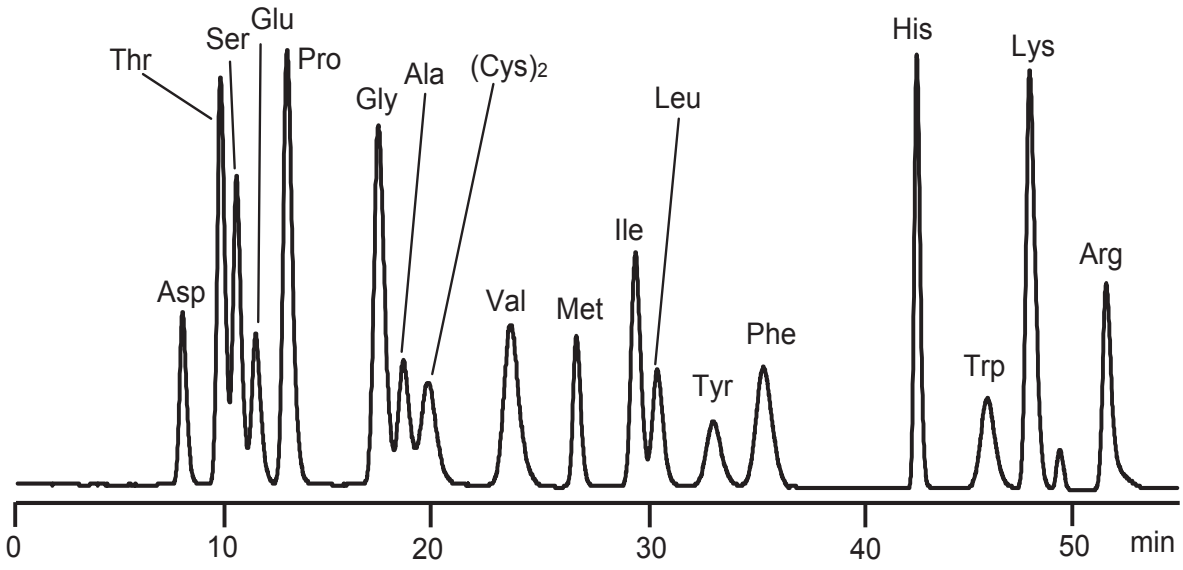


Fig. 6 Chromatogram of a Standard Mixture of 18 Amino Acids Including Tryptophan − Simultaneous Tryptophan Analysis Mode (Na)

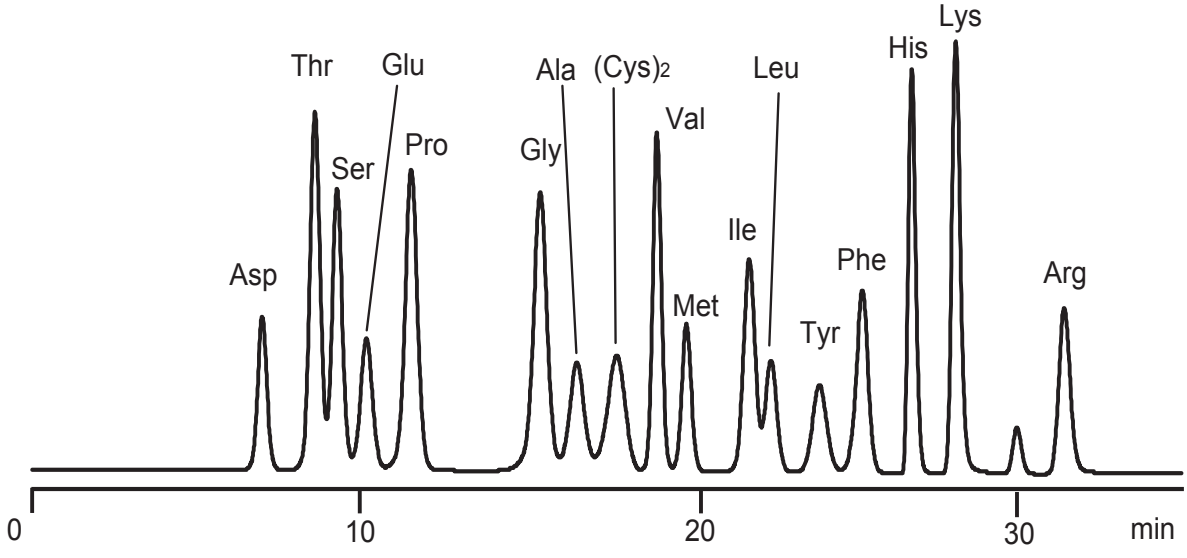


Fig. 7 Chromatogram of a Standard Mixture of 17 Amino Acids − High-Speed Analysis Mode (Na)

4.2 Simultaneous Analysis with the Li-Type Analytical Conditions

In addition to the basic amino acids, natural substances and substances related to living organisms contain a large number of amino acids and related substances, such as amino acids whose residues have been partially modified and substances with structures analogous to amino acids. Simultaneous analysis of these substances is in demand, not simply to confirm the amino acids that comprise proteins, but also for a wide range of applications in such areas as food product quality control and medical and biochemical fields.

The Shim-pack Amino-Li is a lithium-type cation exchange column that is used for the analysis of free amino acids. This column has a selectivity that differs from the sodium-type column because the counterions of the ion exchange base are lithium. It enables the simultaneous analysis of amino acids and related substances. Fig. 8 shows an example of the analysis of amino acids and 38 related substances under Li-Type analytical conditions.

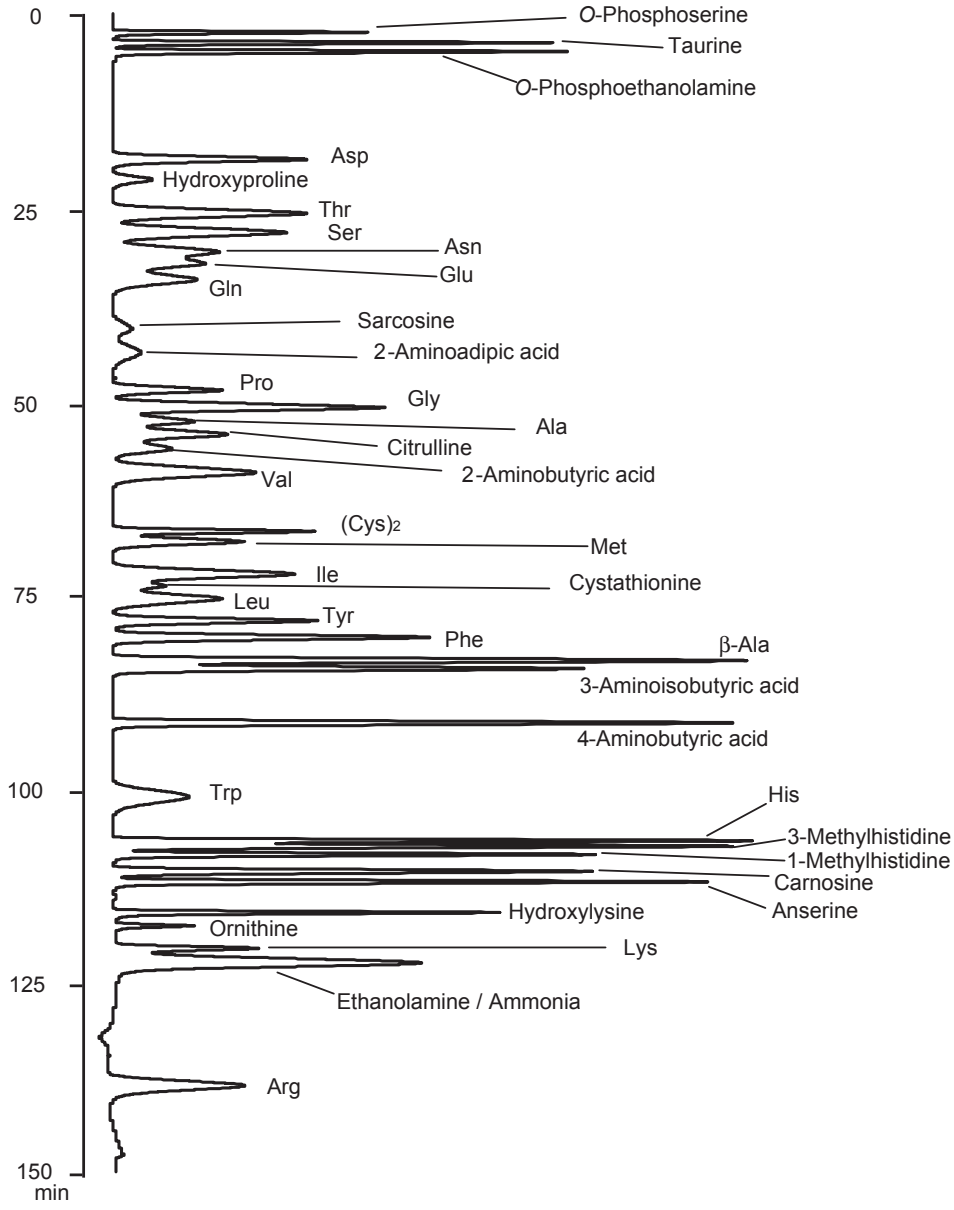


Fig. 8 Chromatogram of a Standard Mixture of 38 Amino Acids − Standard Analysis Mode (Li)

5. Applications in the Analysis of Food Products

Amino acids are very important target substances in food product analysis and they are widely analyzed as an indicator of quality and flavor. For the analysis of amino acids in food products, the proteins may be hydrolyzed and the constituent amino acids analyzed, or the free amino acids may be analyzed directly. The sections below explain the general protein hydrolysis method and the deproteinization method required for free amino acid analysis, and introduce some application examples.

5.1 Hydrolysis Methods

Some representative hydrolysis methods are presented below. After the decomposition products obtained from the respective hydrolysis methods have been decompressed and dried or neutralized, they are diluted in a citric acid buffer solution (pH 2.2) to the concentration level of the analyzed components to produce the analysis samples.

5.1.1 Hydrochloric Acid Hydrolysis Method

Several methods[5] are used, but the typical method is to add 6 mol/L hydrochloric acid, seal the sample in a tube and hydrolyze it for 22 to 72 hours at 110°C. However, as some amino acids are affected by this method, there are cases where it is desirable to control the hydrolysis time or to use a different method. In particular, it is known that tryptophan, cysteine and methionine are easily affected by this method.

5.1.2 Alkaline Hydrolysis Method

As tryptophan readily decomposes in an acidic atmosphere, hydrolysis is conducted for 16 to 24 hours at 110°C with an alkaline solution using a 4 mol/L sodium hydroxide aqueous solution. Tryptophan is quantitatively hydrolyzed by this method, but as arginine and serine are degraded, the method is mainly used for hydrolysis when analyzing tryptophan.

5.1.3 Performic Acid Oxidation and Hydrochloric Acid Hydrolysis Method

Performic acid (5:95 vol% mixture of 30% hydrogen peroxide and 90% formic acid) is used for the quantitation of cysteine, cystine and methionine. Cysteine and cystine are converted to cysteic acid and methionine is converted to methionine sulfone. Subsequently, the hydrochloric acid hydrolysis process described above achieves a high recovery rate for these amino acids.

5.2 Deproteinization Methods

5.2.1 Organic Solvent Method

An acetonitrile or ethanol organic solvent that is compatible with water is added to a sample at a ratio of about 1:1 or 2:1 vol%. After the protein denatures and is immobilized in the organic solvent, the insoluble material is removed by centrifuging or filtering. The polymer substrate of the analysis column used in this amino acid analysis system should not be subjected to large amounts of organic solvent, so the sample injection volume must be restricted to the minimum required.

5.2.2 Acidic Solution Method

Generally, an acidic aqueous solution with a high denaturing effect, such as 1 mol/L perchloric acid aqueous solution, 5% trichloroacetic acid aqueous solution, or 5% tungstic acid aqueous solution, is used as the acidic solution. A method is available that uses sulfosalicylic acid as a strong acid with a high denaturing action. However, as sulfosalicylic acid is fluorescently detected and background peaks appear under the conditions used in this system, this method is not considered suitable. Further, if the pH of the injected sample solution is low due to the effects of the acidic aqueous solution, pH fluctuations in the column result in fluctuations of the retention time. Consequently, during deproteinization pretreatment using an acidic aqueous solution, mobile phase C solution must be added to the sample after deproteinization, and the pH adjusted to between 2 and 3, the same as mobile phase A.

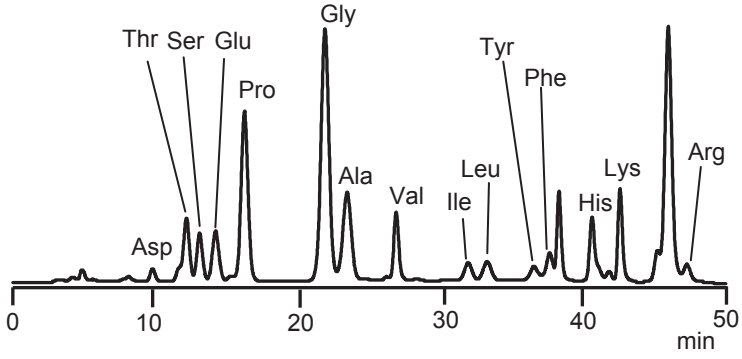
5.2.3 Ultra-filtration Membrane Method

This is a method of removing substances with a molecular weight above a certain limit. The low-molecular-weight free amino acids pass through the ultra-filtration membrane but the proteins are eliminated. Several methods are used, including syringe pressurization or centrifuging, to pass the sample through the ultrafiltration membrane. Dialysis achieves almost the same results as ultrafiltration.

5.3 Application Examples using Na-Type Conditions

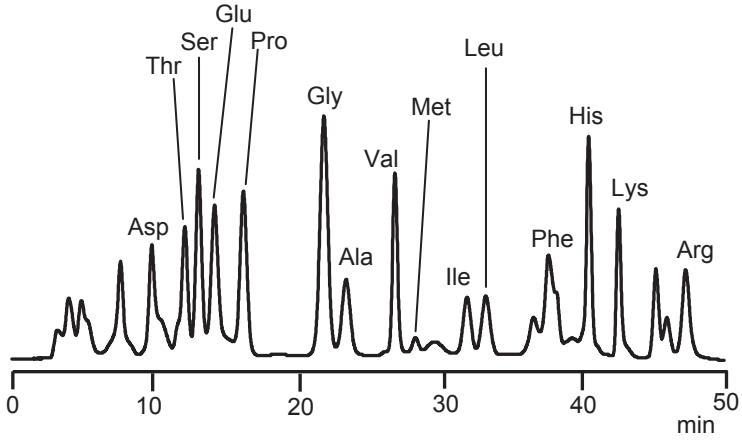
As described in 4.1 Simultaneous Analysis with the Na-Type Analytical Conditions, the Na-Type conditions are the analytical conditions used for separating 17 types of amino acids (18, if tryptophan is included) obtained by hydrolyzing proteins. If a sample contains mainly these 17 types of amino acids, these Na-Type conditions can be applied not only to protein hydrolysis but also to the measurement of free amino acids in food products and pharmaceuticals. However, if the sample also contains other amino acids, the use of Li-Type conditions is recommended, as there are cases where some amino acids are not separated.

Examples of the analysis of free amino acids in sake (Fig. 9), mirin (sweetened sake used for cooking) (Fig. 10), and soy sauce (Fig. 11) are shown below using the Na-Type conditions. As some peaks are inadequately separated, it is advisable to verify the validity of the quantitative values by comparing the results with analysis using the Li-Type conditions.



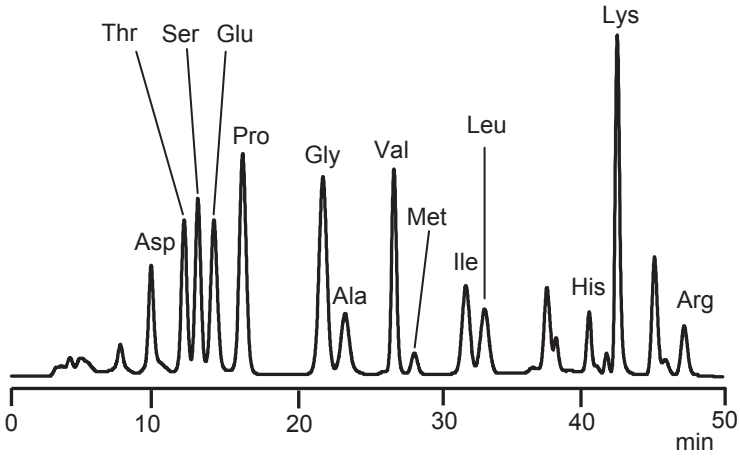
Sample preparation
100μL of Sake
↓
10 times dilution
with Na-Type sample diluent
↓
Filtration
through 0.45μm membrane filter
↓
10μL injected

Fig. 9 Chromatogram of Sake − High-Separation Analysis Mode (Na)



Sample preparation
100μL of Mirin
↓
10 times dilution
with Na-Type sample diluent
↓
Filtration
through 0.45μm membrane filter
↓
10μL injected

Fig. 10 Chromatogram of Mirin − High-Separation Analysis Mode (Na)



Sample preparation
100μL of Soy Source
↓
200 times dilution
with Na-Type sample diluent
↓
Filtration
through 0.45μm membrane filter
↓
10μL injected

Fig. 11 Chromatogram of Soy Source − High-Separation Analysis Mode (Na)

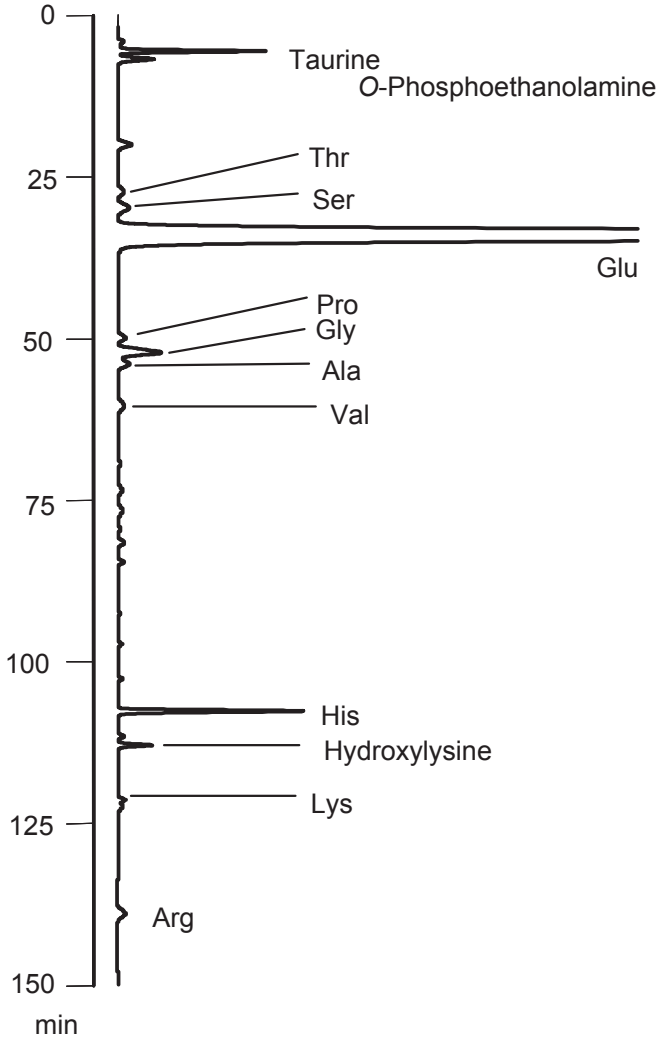
5.4 Application Examples Using Li-Type Conditions

As described in 4.2 Simultaneous Analysis with the Li-Type Analytical Conditions, the Li-Type analytical conditions were established to simultaneously analyze a large variety of naturally occurring amino acids. While the Li-Type conditions significantly surpass the Na-Type in respect of the separation of amino acids, the analysis time is longer.

The Li-Type should be selected for the analysis of amino acids occurring in a free state in food products. However, when the analysis time is restricted, the previously described Na-Type may be selected.

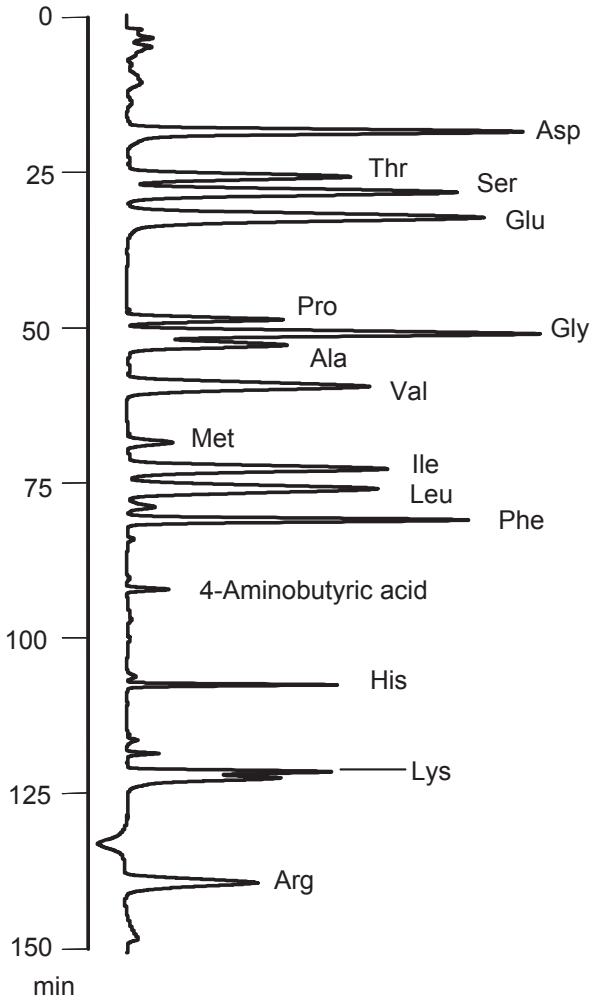
The problem with analyzing free amino acids is the pretreatment included when proteins coexist in the sample. Since proteins can cause problems, such as clogging of the columns, perform analysis after removing proteins from the sample by following the method described in 5.2 Deproteinization Methods.

Examples of the analysis of powdered soup stock (Fig. 12), miso (Fig. 13) and collagen tablets (a dietary supplement, Fig. 14) are shown below using the Li-Type conditions.



Sample preparation
0.5g of seasoning powder
in 10mL of 0.1 mol/L HCl
↓
Sonication (15 min.)
↓
Centrifugation at 3,000 rpm (15 min.)
↓
10μL of the supernatant
with 900μL of Li-Type sample diluent
↓
Filtration through 0.45μm membrane filter
↓
10μL injected

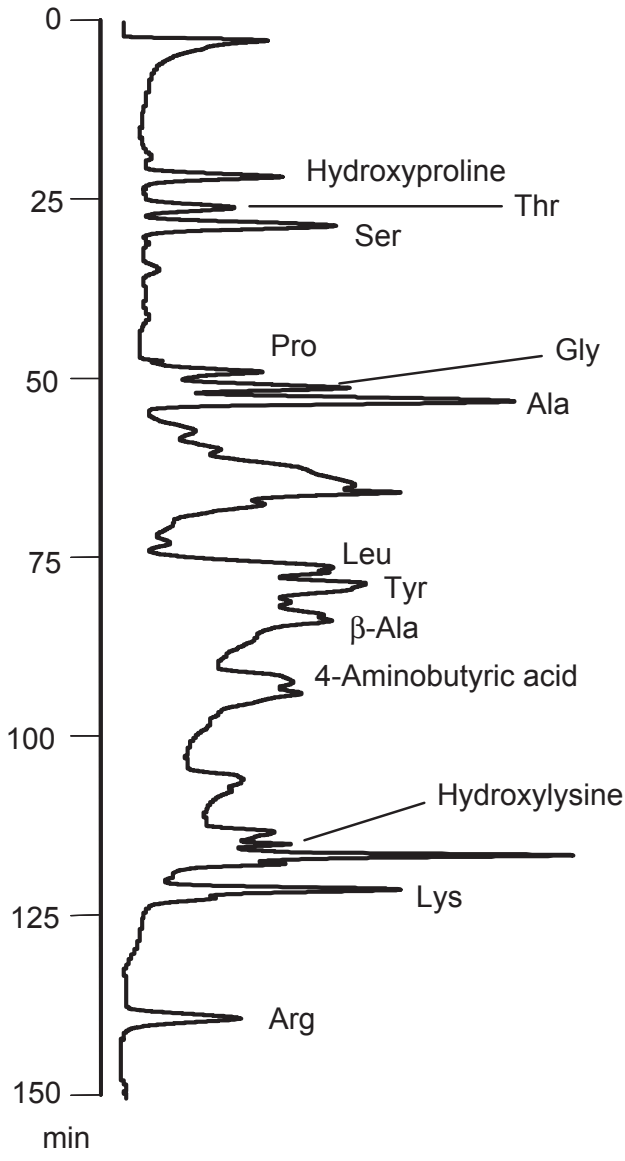
Fig. 12 Chromatogram of Seasoning Powder − Standard Analysis Mode (Li)



Sample preparation

1.0g of Miso
in 10mL of 5% trichloroacetic acid
↓
Stirred using vortex mixer (1 min.)
↓
Centrifugation at 10,000 rpm (10 min.)
↓
700μL of the supernatant
with 700μL of Li-Type sample diluent
↓
Filtration through 0.45μm membrane filter
↓
10μL injected

Fig. 13 Chromatogram of Miso − Standard Analysis Mode (Li)



Sample preparation

Collagen supplement tablet

↓

0.5g of pulverized tablet in 0.1mol/L HCl

↓

Centrifugation at 10,000 rpm (10 min.)

↓

Sonication (15 min.)

Centrifugation at 3,000 rpm (15 min.)

↓

Ultra-filtration of the supernatant

(Molecular cut-off 10,000)

↓

Centrifugation at 15,000 rpm (10 min.)

↓

100μL of the supernatant

with 900μL of Li-Type sample diluent

↓

Filtration

through 0.45μm membrane filter

↓

10μL injected

Fig. 14 Chromatogram of Collagen Tablet (Dietary Supplement) − Standard Analysis Mode (Li)

6. Conclusions

As described above, the Prominence Amino Acid Analysis System is an automatic analysis system using post-column fluorescent derivatization detection that adopts Shimadzu's original OPA/N-acetyl-L-cysteine reagent. It offers high sensitivity and selectivity for the analysis of amino acids. In addition, the Prominence Amino Acid Analysis System standardizes the optimum analytical conditions according to the objective and offers the mobile phases and reagents in kit form to achieve the same level of convenience as a dedicated amino acid analyzer.

Amino acid analysis will continue to hold an important position in such fields as food products, natural products, and pharmaceuticals in the future. Shimadzu is striving to further improve the systems and analytical conditions to achieve even higher analysis efficiency.

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3) Y. Ishida, T. Fujita, K. Asai: J. Chromatogr., 204, 143 (1981)
4) Japanese Patent No. 1567849 "Amino Acid Analysis Methods and Equipment."
5) Hiroshi Tsugita: *Science and Living Organisms* 26, 343 (1988), and others

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