SHEET NO. 67

SUBJECT: HIGH RESOLUTION ANALYSIS METHOD FOR SAMPLES OXIDIZED BY PERFORMIC ACID

INSTRUMENT: HITACHI MODEL L-8800 AMINO ACID ANALYZER

1. INTRODUCTION

In analysis of protein composing amino acids with an amino acid analyzer, hydrolysis is carried out as a pretreatment. However, an ideal condition have yet to be established for cutting off all peptide links quantitatively and besides, protecting all amino acids from fracture during hydrolysis. Therefore, it has been attempted to select a decomposition method suitable for a specific analyte or to integrate the values obtained by decomposition under some different conditions. With the most general hydrolysis at 110 °C and for 24 hours using 6 mol/L hydrochloric acid, cysteine (Cys) and methionine (Met) cannot be recovered in a high ratio. Therefore, such amino acids are quantitated after conversion into cysteric acid ($CySO_3H$) and methionine sulfone (MetSON) by oxidation with performic acid.
However, conversion into MetSON enhances acidity. As a result, elution becomes faster and the peak of MetSON appears before that of aspartic acid (Asp) so as to cause overlapping using the conventional protein hydrolyzate analysis method. Thus, there is a problem of degraded quantitation accuracy.
Recently, an analysis method of 21 standard amino acid constituents has been developed, in which separation of MetSON and Asp is better than before and an internal standard substance norleucine (Nle) is added in order to exactly analyze a sample oxidised by performic acid.

Also, for allowing both measurements of a performic sample oxidised by performic acid and a general sample hydrolyzed with hydrochloric acid just by exchange of analysis programs, an analysis method of 18 hydrolyzed standard constituents has been generated depending on use of the same column and eluent.
In addition, an accelerated analysis method also with the same column and eluent has been devised for assaying lysine (Lys), histidine (His) and arginine (Arg) which are basic amino acids slow in elution.
These 3 analysis methods using the same column and eluent are introduced here. They will make the instrument applicable to various samples with no need for column exchange.

2. ANALYTICAL CONDITIONS

Column	:	#2620M 4.6 × 60 mm L
Ammonia trap column	:	#2650 4.6 × 80 mm L
Eluent	:	L-8500 PH-Kit (B1 prepared by adding 9 g of citric acid monohydrate, 2 g of NaCl and 20 mL of ethanol to 1 L of PH-1)

3. ANALYTICAL EXAMPLES

3.1 Analysis of 21 Standard Amino Acids by Adding Methionine Sulfone, Cysteric Acid and Norleucine

For improving separation of MetSON and Asp (eluted at about 8 minutes), citric acid is added to PH-1. And for improving the separation of Gly to Phe (eluted within a time zone from 16 to 31 minutes), which will be otherwise degraded due to addition of citric acid, sodium chloride and ethanol are added.

Further, from about 20-minute point, B4 and B1 are flowed in mixed state to reverse the elution order of NH_3 and Lys in the usual internal standard method, thereby ensuring a wider separation of NH_3 and later amino acids.
(For PH-1 and B2 to B5, the L-8500 PH-Kit is used.)

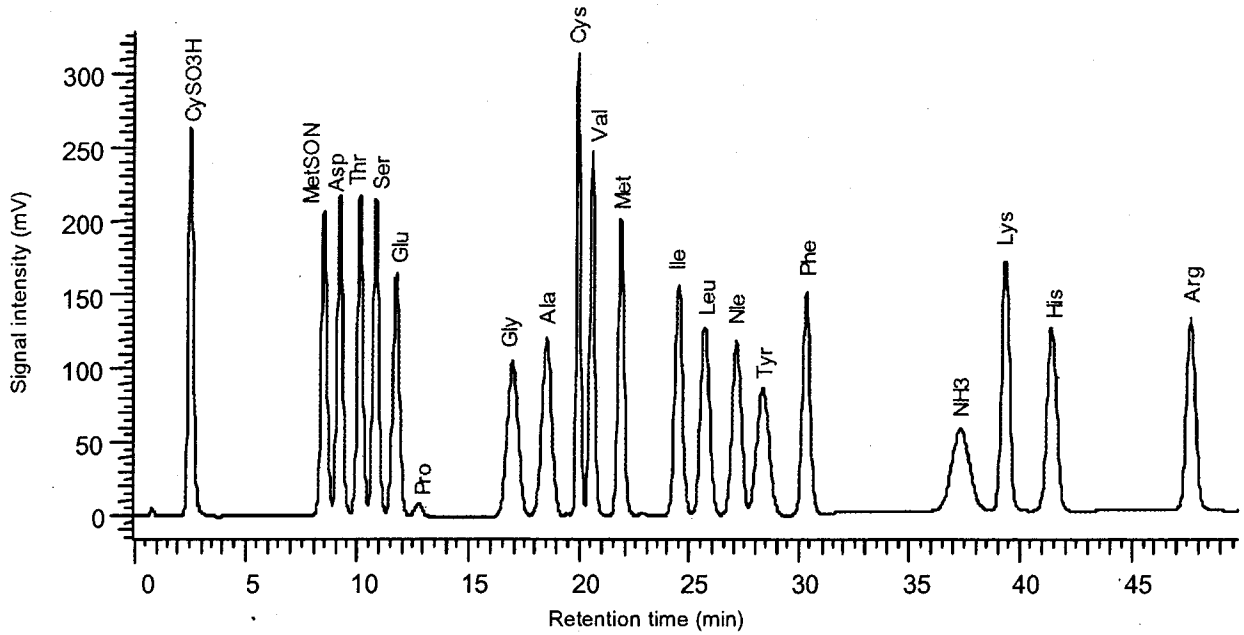

Fig. 1 Chromatogram of 21 Amino Acids in Mixture Standard Sample (5 nmol)

Gradient Program

Time (min)	%B1	%B2	%B3	%B4	%B5	Temperature (°C)	Flow rate 1 (mL/min)	%R1	%R2	%R3	Flow rate 2 (mL/min)
0.0	100.0	0.0	0.0	0.0	0.0	60	0.250	50.0	50.0	0.0	0.200
1.5	100.0	0.0	0.0	0.0	0.0						
1.6	0.0	100.0	0.0	0.0	0.0						
8.5	0.0	100.0	0.0	0.0	0.0						
8.6	0.0	0.0	100.0	0.0	0.0						
20.4	0.0	0.0	100.0	0.0	0.0						
20.5	40.0	0.0	0.0	60.0	0.0						
23.0						50					
27.0						70					
36.4	40.0	0.0	0.0	60.0	0.0						
36.5	0.0	0.0	0.0	100.0	0.0						
41.9	0.0	0.0	0.0	100.0	0.0						
42.0	0.0	0.0	0.0	0.0	100.0						
47.9								50.0	50.0	0.0	
48.0								0.0	0.0	100.0	
48.4	0.0	0.0	0.0	0.0	100.0						
48.5	0.0	100.0	0.0	0.0	0.0						
49.4	0.0	100.0	0.0	0.0	0.0						
49.5	100.0	0.0	0.0	0.0	0.0	60					
50.4								0.0	0.0	100.0	
50.5								50.0	50.0	0.0	
70.0	100.0	0.0	0.0	0.0	0.0						

3.2 Analysis of 18 Hydrolyzed Standard Amino Acids

A program has been generated for analyzing 18 hydrolyzed standard amino acids with the same column and eluent as those for the analysis of 21 amino acids in the previous page. The elution order is the same as in the usual internal standard method and analysis time is 10 minutes shorter than in the aforementioned 21-component analysis.

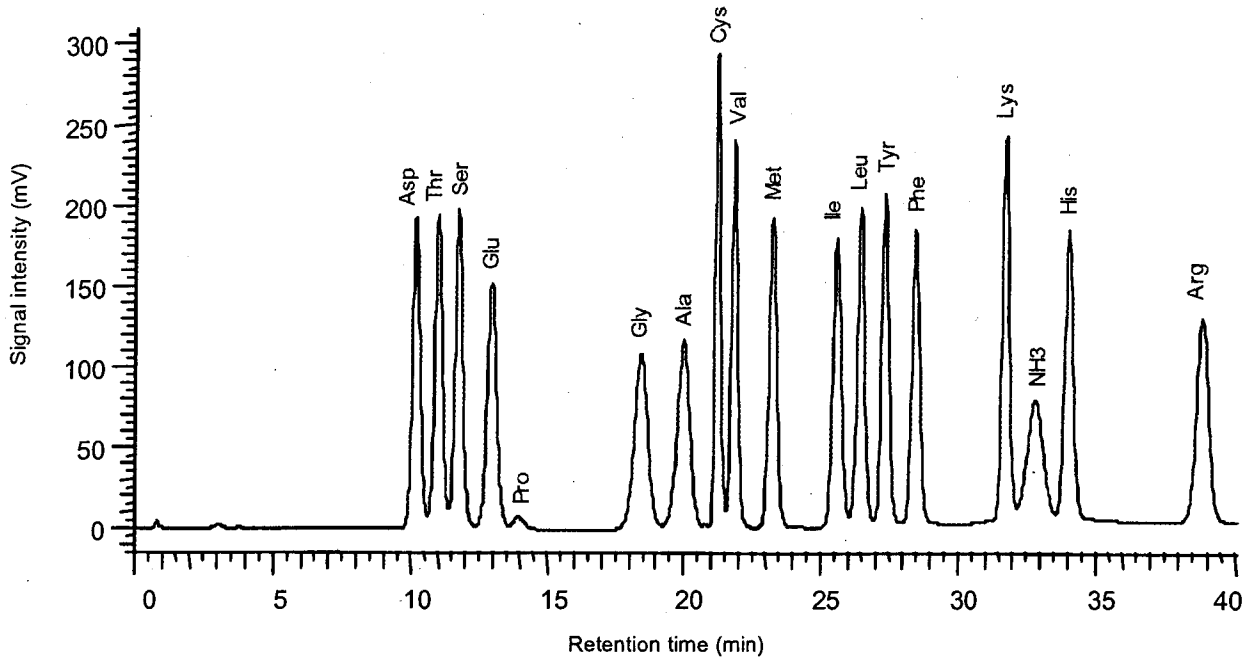

Fig. 2 Chromatogram of Standard Mixture (5 nmol) for Protein Hydrolyzate Analysis

Gradient Program

Time (min)	%B1	%B2	%B3	%B4	%B5	Temperature (°C)	Flow rate 1 (mL/min)	%R1	%R2	%R3	Flow rate 2 (mL/min)
0.0	100.0	0.0	0.0	0.0	0.0	57	0.250	50.0	50.0	0.0	0.200
1.5	100.0	0.0	0.0	0.0	0.0						
1.6	0.0	100.0	0.0	0.0	0.0						
7.5	0.0	100.0	0.0	0.0	0.0						
7.6	0.0	0.0	100.0	0.0	0.0						
17.5	0.0	0.0	100.0	0.0	0.0						
17.6	0.0	0.0	0.0	100.0	0.0						
23.0						70					
33.5	0.0	0.0	0.0	100.0	0.0						
33.6	0.0	0.0	0.0	0.0	100.0						
37.0								50.0	50.0	0.0	
37.1								0.0	0.0	100.0	
38.0	0.0	0.0	0.0	0.0	100.0						
38.1	0.0	100.0	0.0	0.0	0.0						
39.0	0.0	100.0	0.0	0.0	0.0						
39.1	100.0	0.0	0.0	0.0	0.0						
41.0						60					
42.0								0.0	0.0	100.0	
42.1								50.0	50.0	100.0	
60.0	100.0	0.0	0.0	0.0	0.0						

3.3 Accelerated Analysis of Basic Amino Acids

In case only basic components are desired to be quantitated, the above-mentioned analysis methods will take an analysis time as long as 40 or 50 minutes, because the analyte components will be eluted in the later phase.

Hence, an accelerated analysis program has been prepared so that the analysis time can be shortened to about 20 minutes (cycle time 35 minutes) by flowing B2 and B4 in mixed state.
(For B2 and B4, PH-2 and PH-4 in the L-8500 PH-Kit are used.)

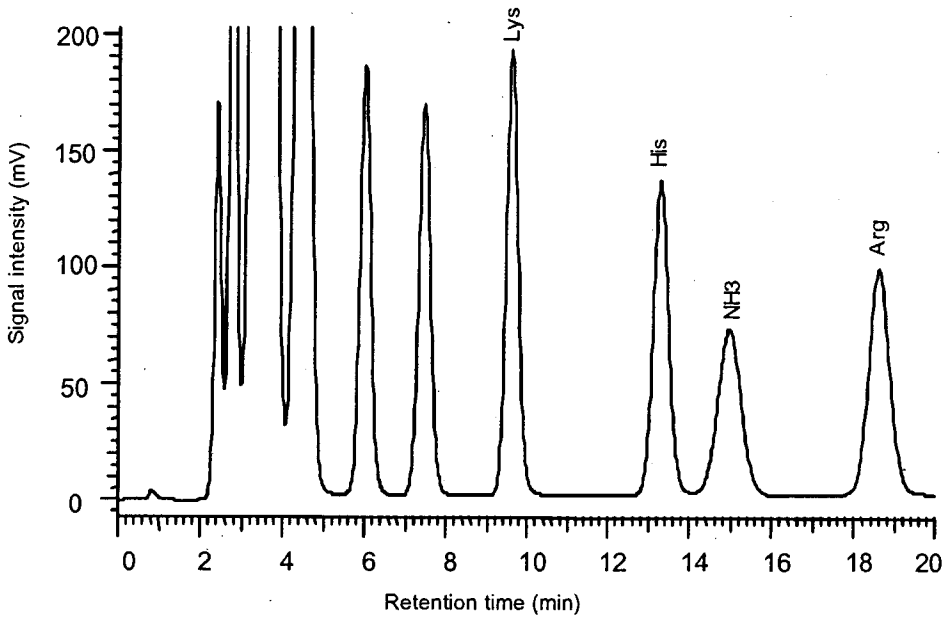

Fig. 3 Accelerated Analysis of Basic Amino Acids (5 nmol)

Gradient Program

Time (min)	%B1	%B2	%B3	%B4	%B5	Temperature (°C)	Flow rate 1 (mL/min)	%R1	%R2	%R3	Flow rate 2 (mL/min)
0.0	0.0	20.0	0.0	80.0	0.0	70	0.250	50.0	50.0	0.0	0.200
13.0	0.0	20.0	0.0	80.0	0.0						
13.1	0.0	0.0	0.0	0.0	100.0						
19.0	0.0	0.0	0.0	0.0	100.0						
19.1	0.0	20.0	0.0	80.0	0.0						
35.0	0.0	20.0	0.0	80.0	0.0						

Keywords: L-8800, Methionine sulfone, Cysteric acid, Norleucine, Performic oxidation
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Printed in Japan TM-M (LT)