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Determination of amino acids by pre-column fluorescence derivatization with 1-methoxycarbonylindolizine-3,5-dicarbaldehyde

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Abstract

The fluorescence intensity of 15 amino acids and ammonia as their 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA) derivatives was investigated over the pH range 2–8. At pH 2–6, most derivatives showed strong fluorescence at 482 nm (emission); the intensity decreased with increasing pH (pH 6–8) when exposed to 291 and 414 nm light. The time course of the derivatization of the amino acids and their retention behavior on an ODS column in high-performance liquid chromatography (HPLC) were also investigated. Derivatization of all amino acids – except for histidine, threonine and ammonia – was completed within 15 min and was relatively stable for 2 h. Using reversed-phase HPLC equipped with a fluorescence detector (λ_{ex}=414 nm, λ_{em}=482 nm), a 15-component amino acid and ammonia standard mixture was analyzed on an ODS column with a 60-min sample turnover using binary gradient elution with 20 m*M* ammonium dihydrogen phosphate (adjusted to pH 2.6 with phosphoric acid) in the presence of 10 m*M* 1-octanesulfonic acid as an ion-pairing reagent and acetonitrile. The precision of the method is less than 2% (peak area) and 0.5% (retention time) at the 100 μM (166 pmol/injection) level. Linearity was established over the concentration range 0.5–100 μM of each derivative. The detection limits range from 0.2 fmol for leucine, isoleucine and phenylalanine to 200 fmol for methionine; however, proline, tyrosine and tryptophan did not respond at all to this method. In order to determine the accuracy, the data on the composition of protein hydrolysates (soybean and human hair) obtained with the present method are compared with those obtained with an amino acid analyzer.

Keywords: Derivatization, LC; Amino acids; 1-Methoxycarbonylindolizine-3,5-dicarbaldehyde

1. Introduction

Over the last 20 years, a considerable number of investigations have dealt with the development of methods for the analysis of amino acids. Some of them present the development of fast analysis methods. In a recent report [1], 21 amino acids were separated as their phenylisothiocyanate derivatives within only 6 min by high-performance liquid chromatography (HPLC) on an ODS column. The run-time of this separation is more than 10 to 20 times shorter than that of the classical method using ion-exchange chromatography and ninhydrin as the post-column labeling reagent [2]. Another paper showed improvement of the detection limit. In order to enhance the detection sensitivity, fluorescence de-

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tection is preferred to photometric detection. For example, the *o*-phthalaldehyde/2-mercaptoethanol reagent (abbreviated OPA[1]) [3–5] and fluorescamine [6] are representative fluorescence derivatization reagents for amino acid analysis used in many reagents so far developed. Udenfriend et al. [6] were the first to describe the use of fluorescamine, which reacts rapidly and quantitatively with primary amines to form highly fluorescent products. OPA has been introduced for post-column fluorogenic detection of amino acids in conventional amino acid analyzers. Benson and Hare [4] compared ninhydrin with those reagents and showed that fluorescamine is five times more sensitive than ninhydrin and OPA is five to ten times more sensitive than fluorescamine. However, as these reagents are labile, they are restricted to post-column labeling methods. Recently, Cohen and Michaud described an excellent pre-labeling reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [7], which is as sensitive as OPA. However, based on the florescence profiles of AQC and AQC-amino acids, AQC can probably not be used in the post-column labelling method as the fluorescence profiles of the reagent and the derivatives overlapped around the emission wavelength at 400 nm. While amino acid analysis using derivatizing reagents is a mature analytical method, other reagents, such as 1-fluoro-2,4-dinitrobenzene (DNP-F) [8], 1-dimethylaminopthalene-5-sulfonyl chloride (dansyl-Cl) [9] and 9-fluorenylmethyl chloroformate (Fmoc-Cl) [10], also have several disadvantages with respect to specificity, significant interference caused by the reagent, sensitivity, quantitation and accuracy. In a previous paper [11], we reported the synthesis of an indolizine derivative containing heteroaromatic N-rings, 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA), and investigated its reactivity toward primary amines using high-performance capillary electrophoresis (HPCE). IDA easily reacted with amino acids with a primary amine under alkaline conditions (pH 10) and 50% ethanol at room temperature for 15 min in the dark; IDA-amino acid derivatives possess strong fluorescence (λ_{ex}=414 nm, λ_{em}=482 nm) and absorption (λ_{max}=409 nm). The fluorescence or absorption spectra did not overlap and did not interfere with that of the reagent. Furthermore, IDA derivatives have a two or three times stronger fluorescence and absorption compared with that of OPA and ninhydrin, respectively. Therefore, the reagent has a high sensitivity for pre- and/or post-column labeling methods for amino acid analysis using HPLC.

In the present paper, we investigate the fluorescence characteristics of individual amino acid and ammonia derivatives and the derivatization time course. The retention behavior of each amino acid derivative on an ODS column was studied and the IDA derivatization pre-column optimized. A comparison with present methods used for the determination of the composition of soybean protein and human hair protein hydrolysate is also described.

2. Experimental

2.1. Reagents and materials

The reagents used were of HPLC grade or the highest grade commercially available. All aqueous solutions were prepared using water purified with a Milli-Q purified system (Millipore). Dried IDA reagent (10 μg) was prepared and used in the present study. IDA was dissolved in ethyl acetate by sonication to make a 0.100 mg/ml solution. A 100-μl portion (10 μg; 0.043 μmol) was transferred to a 0.6-ml microcentrifuge (polypropylene) tube. The solvent was carefully evaporated to dryness by placing the tubes in a desiccator under reduced pressure. The reagent tubes were then tightly capped and stored until use in a refrigerator in the dark. The derivatization procedure was started by addition of the reaction buffer and the sample solution into an IDA reagent tube.

The reaction buffer was prepared by mixing equal volumes of ethyl alcohol and 20 m*M* phosphate–borate buffer (equal volumes of 20 m*M* sodium dihydrogenphosphate and 20 m*M* sodium tetraborate

[1] Abbreviations used: OPA, *o*-phthalaldehyde/2-mercaptoethanol reagent; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; DNP-F, 1-fluoro-2,4-dinitrobenzene; dansyl-Cl, 1-dimethylaminopthalene-5-sulfonyl chloride; Fmoc-Cl, 9-fluorenylmethyl chloroformate.

were mixed, and the pH was adjusted to 10 by adding 1 *M* sodium hydroxide).

Each of the individual amino acids (Takara Kohsan, Tokyo, Japan) and ammonium chlorides (Wako, Osaka, Japan) was dissolved in water (0.1 *M* hydrochloric acid was used in case of cystine and tyrosine) to make a 2.15 m*M* solution.

A 1-ml sample of the amino acids mixture (Type H; Wako) was diluted with water to 50 ml (100 μM). These solutions were stored in a refrigerator prior to use.

2.2. Apparatus

For measurement of the fluorescence emission intensity, a Hitachi F-3010 fluorescence spectrometer with a 1-cm quartz cell was employed. The HPLC system consisted of two intelligent PU-980 pumps (Jasco, Tokyo, Japan) and gradient-mode elution was used. Samples were loaded with a Rheodyne syringe loading valve (Rheodyne, CA, USA) provided with a 10-μl sample loop. The analytical column was an Asahipak ODP-50 column (150×6.0 mm I.D.; Asahi, Tokyo, Japan). The column was maintained at 40°C in a CO-965 column oven (Jasco). Column effluents were monitored with a 821-FP fluorescence detector (Jasco). The excitation and emission wavelengths were set at 414 nm and 482 nm, respectively. All mobile phases were degassed through a DG-980-51 on-line degasser (Jasco). The flow-rate was 1.0 ml/min. All chromatographic data were printed with the intelligent integrator 807-IT (Jasco).

2.3. HPLC separation

A 10-μl sample of derivatized solution was directly injected onto the ODS column, and derivatives were separated using binary gradient elution. Eluent A was ammonium dihydrogen phosphate buffer (10, 20 and 30 m*M*) containing various concentrations of sodium 1-octanesulfonic acid (0, 5, 10, 15 and 20 m*M*) as an ion-pairing reagent. The pH of the buffer was adjusted in the range of 2.6–4.0 with phosphoric acid, and in the range of 5.0–7.0 with 25% aqueous ammonia solution. Eluent B was acetonitrile. Gradient elution programs are listed in Table 1.

Table 1
Gradient elution program for HPLC separation

Program I			Program II		
Time	Eluent (%)[a]		Time	Eluent (%)[a]	
(min)	A	B	(min)	A	B
0	93	7	0	93	7
35	80	20	40	80	20
40	50	50	50	70	30
			55	50	50
45	93	7	60	93	7

[a] Eluent A is ammonium dihydrogen phosphate buffer. Eluent B is acetonitrile.

2.4. Derivatization procedure

Reaction buffer (100 μl) was added into a dry reagent tube containing 10 μg (0.043 μmol) of IDA, and the solution was sonicated for 30 s. After addition of 20 μl of amino acid solution, the reaction mixture was mixed well and left at room temperature for 15 min in the dark.

2.5. Measurement of fluorescence intensity

Each 20-μl sample of individual standard amino acid and ammonium chloride solutions (2.15 μmol/ml) was derivatized as described above. Then 20 μl of the derivatized solution was transferred to the 1-cm quartz cell containing 3 ml of water, which was shaken after tightly sealing the cell with Sealon Film (Fuji Photo Film, Tokyo, Japan). The quartz cell was placed into the fluorescence spectrophotometer, and the fluorescence intensity was measured.

2.6. Hydrolysis procedure

An amount of 10 mg of soybean protein (Soya-Farm, Nishinn Seiyu, Tokyo, Japan) or of human hair (cut into about 1-mm segments) was suspended with 10 ml of 6 *M* hydrochloric acid in a 50-ml glass ampule. The ampule was heated at 110°C for 20 h after sealing under vacuum. Then a 1-ml portion of hydrolyzed solution was evaporated to dryness. The brownish solid was dissolved in 1 ml of 0.1 *M* hydrochloric acid and diluted to 10 ml with water.

Then the mixture was filtered with a DISMIC-13cp syringe filter unit (Adovantec, Tokyo, Japan). The filtrate of hydrolyzed solution of soybean protein was further diluted 10-fold with water.

3. Results and discussion

3.1. Effect of pH on fluorescence intensity

The effect of pH on the fluorescence intensity of the 15 individual amino acids and ammonia derivatives were investigated for the pH range 2–8 (Fig. 1). The fluorescence emission intensity at 482 nm was measured by irradiation of the light at 292 nm (Fig. 1A) and 414 nm (Fig. 1B). With the exception of aspartic acid, glutamic acid, glycine, methionine and ammonia, the maximum fluorescence intensities were around pH 4–6, and subsequently the fluorescence decreased with increasing pH. In general, the emission intensity of most of the derivatives excited by 414 nm light was stronger than that at 291 nm. The intensity in acidic to neutral buffer was stronger than that in basic medium (data not shown). Thus, 414 nm as the excitation wavelength and acidic solvents were found to be satisfactory for fluorimetric determinations. In addition, the unreacted reagent did not fluoresce when an excitation wavelength of 414 nm was used.

The mechanism of the change in fluorescence intensity in media of different pH is not clear because the structure of the IDA-amino acid derivatives is not known.

3.2. Reaction time course of amino acid derivative

The reaction time courses for each amino acid and the reagent were traced by measurement of the peak areas of the derivatives separated by HPLC at 0.5, 5, 10, 15, 30, 60 and 120 min after the start of the derivatization reaction by adding the standard amino acids and ammonia mixture into the IDA solution in the tube. The reaction profiles for the 15 derivatives and ammonia are shown in Fig. 2. From these results, it is clear that derivatization of most of the amino acids was completed within 15 min and that

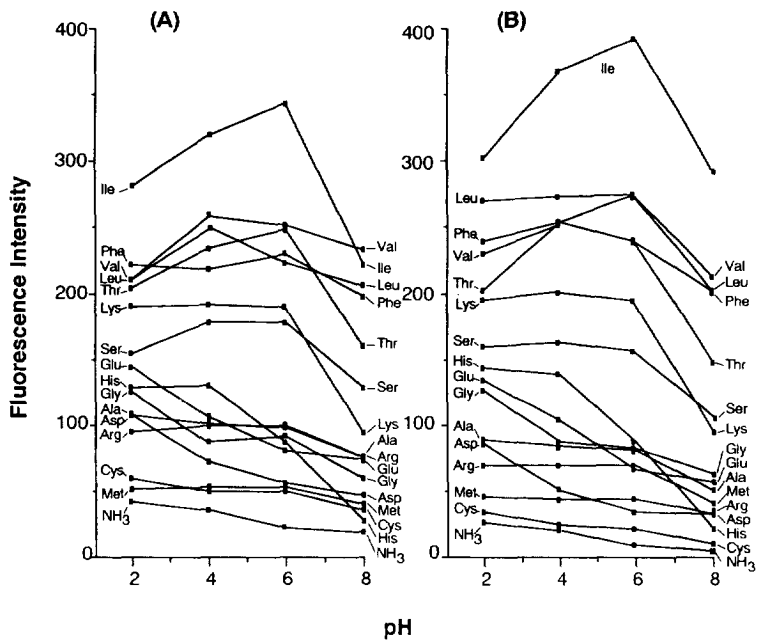

Fig. 1. Effect of pH on fluorescence intensity of IDA derivatives. The concentration of each derivative was 2.4 nmol/ml of buffer (pH 2–8). Fluorescence intensity was measured at 482 nm when derivative was excited at (A) 291 nm and (B) 414 nm, respectively.

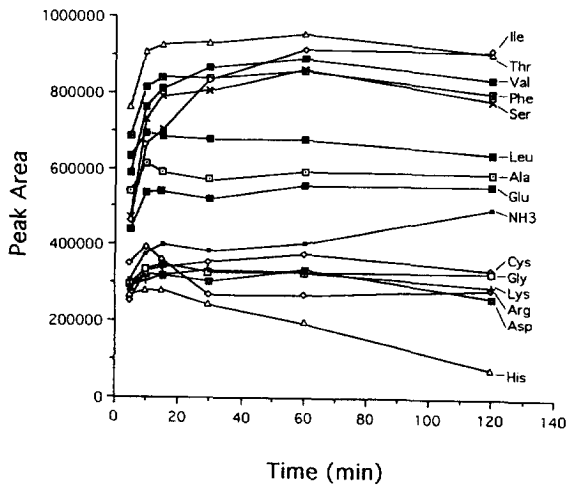

Fig. 2. Plots of peak area of the derivatized analyte versus time. Each value was obtained from HPLC separation after IDA derivatization of the standard solution. For HPLC separation, 20 m*M* ammonium dihydrogen phosphate buffer (adjusted to pH 2.6 with phosphoric acid) in the presence of 10 m*M* sodium 1-octanesulfonic acid as eluent A and acetonitrile as eluent B was employed. Gradient elution program I (see Table 1) was used; the other HPLC conditions are as described in Section 2.

the derivatives are relatively stable for 2 h. However, histidine shows a decreasing fluorescence while ammonia and threonine show an increase in fluorescence.

3.3. Optimization of HPLC separation and retention behavior

In order to separate the derivatives on the ODS column, the derivatized molecules with an amino or carboxyl group should not dissociate into ions. Ionization of the carboxyl group was suppressed by using a medium with a low pH. Furthermore, using a solvent with a low pH is advantageous with respect to the fluorescence intensity of the derivatives. Thus it is clear that an acidic solvent should be used for the HPLC mobile phase. Amino groups were ion-paired with 1-octanesulfonic acid. Optimization of the HPLC separation and retention behavior was studied after derivatization of the standard amino acid/ammonia mixture. The effects were investigated by changing eluent A using gradient elution program I. The elution of the derivatives was expressed as retention time, not as capacity factor because the gradient elution mode was employed.

The effect of the pH on the retention time was investigated over the pH range 2.6–7.0 by keeping the buffer concentration at 20 m*M* with 10 m*M* sodium 1-octanesulfonic acid. From Fig. 3 it can be seen that the retention times of all amino acids derivatives – except for ammonia – decreased with increasing pH, while the elution time of ammonia was nearly independent of pH. The elution order of some of the derivatives changed with pH (pH 2.6 to 4). From these studies, an optimal pH of 2.6 was chosen.

On the other hand, when basic buffer (pH 10) was used as eluent A, most of the derivatives were not detected because they did not fluoresce (data not shown).

The effect of the concentration of the ion-pairing reagent on the separation was examined at concentrations of 0, 5, 10, 15 and 20 m*M* in 20 m*M* buffer at pH 2.6. Fig. 4 shows that the elution order of derivatives changed only little, but the ammonia and cystine derivatives were delayed at increasing concentrations. As the result of this study, the optimized concentration of the ion-pairing reagent was 10 to 15 m*M*.

With the pH of the buffer and the sodium 1-octanesulfonic acid concentration were fixed at 2.6

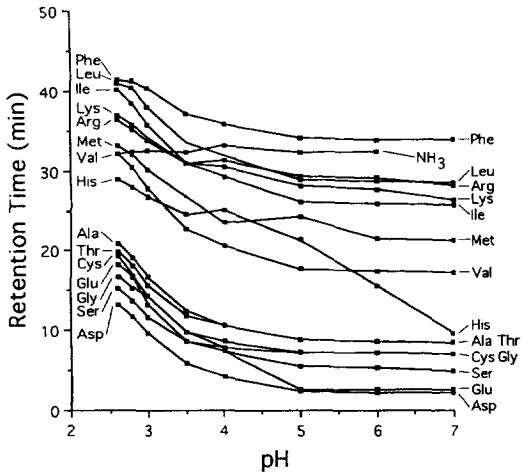

Fig. 3. Effect of pH on the HPLC separation of derivatives. The pH of 20 m*M* ammonium dihydrogen phosphate buffer containing 10 m*M* sodium 1-octanesulfonic acid of eluent A was changed. Other HPLC conditions as in Fig. 2.

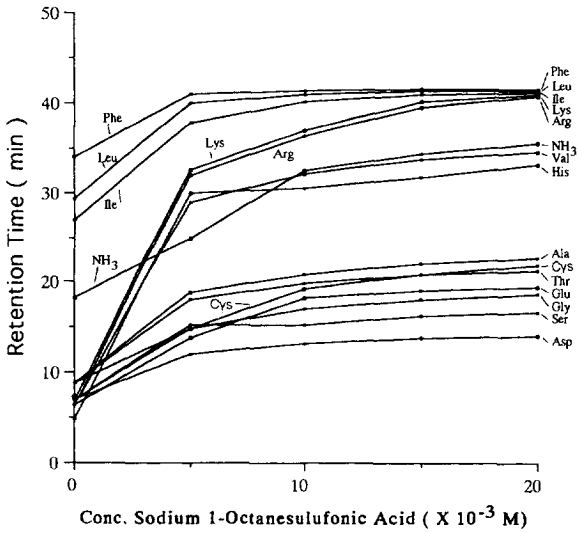

Fig. 4. Effect of sodium 1-octanesulfonic acid as an ion-pairing reagent. The concentration of ion-pairing reagent in 20 m*M* ammonium dihydrogen phosphate buffer at pH 2.6 of eluent A was changed. Other HPLC conditions as in Fig. 2.

and 20 m*M*, respectively, buffer concentrations of 10, 20 and 30 m*M* were studied. Fig. 5 shows the profile of retention time versus buffer concentration.

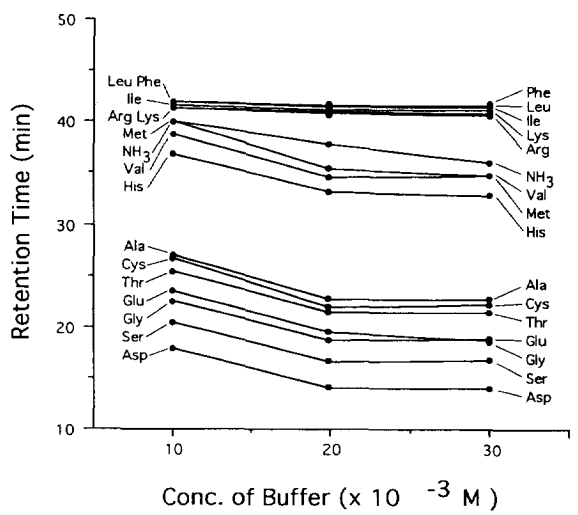

Fig. 5. Effect of buffer concentration on retention time. The pH of the buffer and the sodium 1-octanesulfonic acid concentration in eluent A were fixed at 2.6 and 20 m*M*, respectively. The concentration of ammonium dihydrogen phosphate buffer was changed. Other HPLC conditions as in Fig. 2.

In general, the retention time increased gradually with increasing buffer concentration. The elution order of the IDA-amino acids changed only slightly. In contrast, cystine and ammonia were fast and delayed with increasing buffer concentration, respectively. Fig. 6 shows a typical chromatogram of the 15 amino acid and ammonia derivatives, indicating satisfactory separation. Although the assay was performed within 60 min, this is not short in comparison with other recently used methods. If the other high-resolution columns were optimized, it might be possible to shorten the run-time.

3.4. Analytical reproducibility

The analytical reproducibility was investigated from a series of five identical derivatizations of the standard amino acid and ammonia mixture at 100 μM prior to each injection with HPLC running gradient elution program II. Table 2 shows the

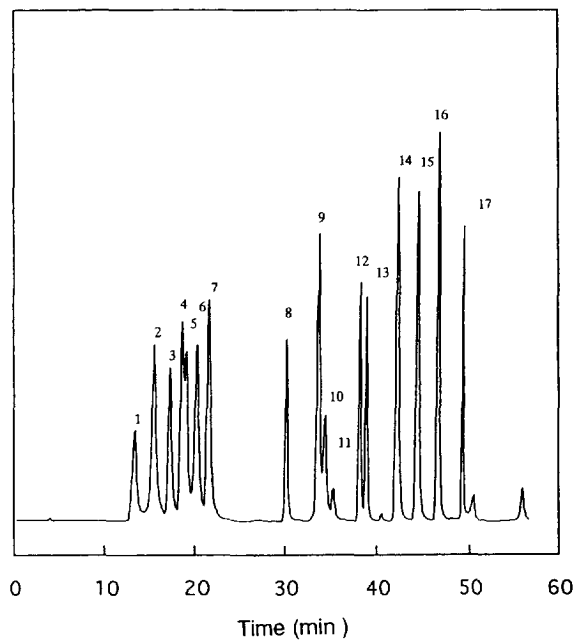

Fig. 6. Typical HPLC chromatogram of the standard mixture as 15-component amino acid and ammonia derivatives obtained by using gradient elution program II. Other HPLC conditions as in Fig. 2 Peaks: 1=Asp, 2=Ser, 3=Gly, 4=Glu, 5=Cys, 6=Thr, 7=Ala, 8=His, 9=Val, 10=NH_3, 11=Met, 12=Arg, 13=Lys, 14=Ile, 15=Leu, 16=Phe, 17=unknown from IDA.

Table 2
Reproducibility of peak-area response and retention time

Amino acid	C.V. (%, $n=5$)	
	Peak area	Retention time
Alanine (Ala)	1.64	0.35
Arginine (Arg)	0.86	0.26
Asparagine (Asp)	0.13	0.24
Cystine (Cys)	1.22	0.24
Glutamic acid (Glu)	0.67	0.35
Glycine (Gly)	0.98	0.30
Histidine (His)	0.15	0.12
Isoleucine (Ile)	1.78	0.14
Leucine (Leu)	0.83	0.12
Lysine (Lys)	1.68	0.23
Methionine (Met)	1.46	0.45
Phenylalanine (Phe)	1.12	0.14
Serine (Ser)	1.34	0.32
Threonine (Thr)	1.57	0.39
Valine (Val)	1.23	1.69
Ammonia (NH_3)	1.02	1.77

Concentration of each amino acid and ammonia, 100 μM (166 pmol/injection).

results of the reproducibility of peak-area response and retention time for each derivative. For all amino acid and ammonia derivatives, the average peak area and retention time showed coefficients of variation (C.V.) ($n=5$) less than 2 and 0.5%, respectively. The methods using OPA [5] and AQC [7] for derivatization gave C.V.s less than 1% (peak height) and 1.60% (peak area), respectively. Thus, the reproducibility of this method is relatively good.

3.5. Linearity, peak-response factor and detection limit

The standard amino acid and ammonia mixture over the concentration range 0.1 μM (0.17 pmol) to 500 μM (833 pmol) was derivatized according to the method described above and analyzed by HPLC separation using gradient elution program II. The linearity of the peak area response was calculated by the least squares regression method for $y = ax + b$, where y is the peak area and x is the concentration of each derivative. The linearity showed a good correlation factor (r) in the concentration range between 0.5 μM (0.833 pmol) and 100 μM (166 pmol). In the case of ammonia, no good relationship between peak-area response and concentrations less than 10 μM (1.66 pmol) was observed. The peak-area response was normalized to alanine at the 100 μM (166 pmol) level, and detection limits were obtained from a signal-to-noise ratio (S/N) of 3. These parameters are listed in Table 3. A trace of ammonia derivative was detected when a sample without ammonia was analyzed. In Table 3, no detection limit of ammonia is given. On the other hand, the derivatization time course of ammonia (see Fig. 4) showed an increasing line. This presumably corresponds to the formation of small amounts of ammonia during derivatization or to decomposition of the derivatives. Based on these data, the detection limits range from 0.2 fmol for leucine, isoleucine and phenylalanine to 200 fmol for methionine. For AQC [7] the detection limits range from 40 fmol for phenylalanine to 800 fmol for cystine. Thus, the method allows detection at the femtomole level, which is one of the highest sensitivities reported to date for amino acid analysis.

3.6. Comparison of the present method and the classical method

To determine the accuracy of the present method, aliquots of soybean and human hair protein hydrolysates were analyzed by the present method and the classical amino acid analyzer. The currently available automated amino acid analyzer (Jasco amino acid analyzer, Tokyo, Japan) was equipped with an ion-exchange column (AApak Na II-S, Jasco, Tokyo) and with post-column labeling units for derivatization with OPA. A comparison of the data obtained is shown in Table 4. The data obtained with the present method are approximately the same as those from the authentic method using the currently available automated amino acid analyzer.

With the present method, as well as with OPA as derivatizing reagent, secondary amino acids (proline and oxyproline) could not be determined. However, using an oxidation process (e.g. with sodium hypochlorite, which is commonly used to open the proline ring), these amino acids could be detected.

Table 3
Linearity of peak-area response, detection limit and peak response factor

Amino acid	Linearity[a] (0.83–166 pmol/injection)			Detection limit (fmol/injection)	Peak response factor
	a	*b*	*r*	(*S/N*=3)	(*n*=5)[b]
Alanine (Ala)	12 358	4553	0.9999	1	1.000
Arginine (Arg)	10 930	7356	0.9995	1	0.941
Asparagine (Asp)	6746	−1461	0.9999	1	0.523
Cystine (Cys)	7265	9972	0.9999	1	0.622
Glutamic acid (Glu)	9928	13 003	0.9999	1	0.825
Glycine (Gly)	8577	12 523	0.9999	1	0.698
Histidine (His)	7747	−22 151	0.9994	3	0.590
Isoleucine (Ile)	14 545	54 273	0.9996	0.2	1.291
Leucine (Leu)	13 116	25 985	0.9999	0.2	1.141
Lysine (Lys)	9798	14 344	0.9999	1	0.923
Methionine (Met)	1481	9433	0.9986	200	0.128
Phenylalanine (Phe)	14 704	47 215	0.9996	0.2	1.213
Serine (Ser)	11 542	4624	0.9999	1	0.935
Threonine (Thr)	9345	28 242	0.9997	1	0.774
Valine (Val)	15 944	−7162	0.9999	2	1.269
Ammonia (NH_3)[c]	4857	2079	0.9788	–	0.408

[a] y (peak area)=ax(amino acid concentration)+b, r=correlation factor.
[b] Normalized to alanine.
[c] Concentrations of NH_3 used are 20 μM (33 pmol/injection) to 100 μM (166 pmol/injection).

4. Conclusion

IDA derivatives show strong fluorescence at 482 nm when excited at 292 and 414 nm. Since excitation at the latter wavelength gives stronger fluorescence in acidic to neutral media, this was employed throughout. The amino acids analysis presented here uses pre-column labeling with IDA prior to ion-

Table 4
Composition of hydrolyzed soybean and human hair protein

Amino acid	Soybean protein		Human hair	
	Method I[a]	Method II[a]	Method I[a]	Method II[a]
Alanine (Ala)	965.4	973.1	42.4	44.4
Arginine (Arg)	801.5	804.8	54.9	57.3
Asparagine (Asp)	1819.5	1827.9	56.4	57.3
Cystine (Cys)	13.6	12.8	2.9	2.5
Glutamic acid (Glu)	3331.8	3284.4	108.7	110.1
Glycine (Gly)	1203.4	1218.4	51.9	56.5
Histidine (His)	347.2	333.8	9.8	8.0
Isoleucine (Ile)	701.5	703.1	22.9	24.1
Leucine (Leu)	1252.6	1258.3	56.3	60.0
Lysine (Lys)	724.6	737.7	22.0	24.6
Methionine (Met)	151.2	150.5	4.5	3.1
Phenylalanine (Phe)	627.4	629.1	14.9	14.0
Serine (Ser)	1151.1	1157.2	104.4	110.1
Threonine (Thr)	695.3	689.2	69.7	68.9
Valine (Val)	827.1	840.4	45.3	49.5
Ammonia (NH_3)	2753.8	2625.9	179.8	168.2

[a] Method I is the present method; method II is the amino acid analyzer.

paired reversed-phase HPLC systems equipped with a fluorescence detector. The major advantages of this method are its extremely high sensitivity and simplicity. Although the present method cannot be applied to secondary amines and some amino acids, a femtomole level of detection can be attained even without performing a clean-up step prior to HPLC analysis. This detection limit presents one of the highest sensitivities reported to date for amino acid analysis. This method is applicable to the analysis of amino acids in protein hydrolysates and biological fluids.

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