Review

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# Amino acid analysis of sub-picomolar amounts of proteins by precolumn fluorescence derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

**Akiko Masuda, Naoshi Dohmae***

*Biomolecular Characterization Team, Riken Advanced Science Institute 2-1 Hirosawa, Wako, Japan.*

**Summary** **Amino acid analysis (AAA) method is the most accurate methodology for absolute quantification of proteins. The conventional postcolumn method employing ninhydrin labeling of amino acids, which is adopted in automatic amino acid analyzer, is limited by low sensitivity. Therefore, a highly sensitive AAA method is required to confirm the data obtained from mass spectrometry or N-terminal sequence analysis. To increase the sensitivity of AAA, an analytical method based on precolumn derivatization with fluorescent 6-aminoquinolyl-carbamyl (AQC) reagent and separation of the AQC-amino acid derivatives by ion-pair chromatography using a reversed-phase column is reported herein. The sensitive analysis of low abundance proteins requires strict prevention of environmental contamination. In this review, we provide a protocol for high sensitivity amino acid analysis and show that the amino acid composition of bovine serum albumin below 100 ng, *i.e.*, 1.5 pmol, determined using the presented method, matched with the theoretical composition in with low standard deviations. These results suggest that the current AAA method is potentially applicable for highly sensitive analysis as a complement to mass spectrometry-based proteomics.**

***Keywords:*** Highly sensitive amino acid analysis, protein quantification

## 1. Introduction

Amino acid analysis (AAA) is a classical analytical method that is essential for the absolute quantification of peptides and proteins. The quantitative accuracy of the method has rendered it useful for compositional analysis of proteins and biological materials, as well as for protein identification (*1*) and for confirmation of the data obtained from Edman degradation or mass spectrometry (*2*). Since the development of an automated amino acid analyzer by Moore and Stein in the mid-1950s (*3*), the range of application of AAA has been expanded to cover many fields such as protein science, pharmacology, physiology, and food chemistry. The currently employed amino acid analyzer remains unchanged in principle and operates based on a postcolumn derivatization method in which amino acids are separated on an ion-exchange column followed by derivatization with ninhydrin for detection of the amino acids.

The disadvantages of the postcolumn method include its low sensitivity, resulting from the need for visible absorbance detection of ninhydrin adducts, and the requirement for a long analysis time using ion-exchange chromatography. Currently, commercial auto-analyzers using ninhydrin detection require as much as several tens of micrograms of proteins (at least 2 nmol of each amino acid) and an analysis time of 2 h for compositional analysis. Fluorescent *o*-phthaldialdehyde (OPA) has been developed as an alternative postcolumn reagent, the use of which resulted in an order of magnitude increase in sensitivity (*4*). One major limitation of this methodology, however, has been that OPA reacts only with primary amines; consequently, the amino acids proline and hydroxyproline cannot be detected directly with OPA in the absence of an oxidizing agent such as hypochlorite under alkaline

**Address correspondence to:*
Dr. Naoshi Dohmae, Biomolecular Characterization Team, Riken Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
e-mail: dohmae@riken.jp

conditions (*5*).

As another derivatization alternative, the precolumn derivatization method in which amino acids are derivatized with chromophores or fluorophores before application to the column has been developed. Because the hydrophobicity of the amino acids is enhanced by the derivatization, the derivatized amino acids can be separated on a reversed-phase column, resulting in faster analysis and higher sensitivity than the conventional ion-exchange method. In 1984, a new precolumn method was reported, with a 1-pmol sensitivity, using phenylisothiocyanate (PITC). PITC reacts with amino acids to yield phenylthiocarbamyl (PTC) amino acids capable of UV absorbance (*6*). Furthermore, precolumn derivatization has enabled the sub-picomolar quantitation of amino acids with the development of fluorescent reagents such as OPA, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), 9-fluorenylmethyl-chloroformate (FMOC-Cl), and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (See details in Section 2.2.). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection of pre-labeled amino acids has been also reported including pre-labeling by amine-reactive isobaric tagging reagents that include different mass group of stable isotope (*7*,*8*), by *p*-*N*,*N*,*N*-trimethylammonioanilyl-*N*'-hydroxysuccinimidyl carbamate iodide (*9*), and by AQC (*10*).

There are some approaches without any derivatization of amino acids for MS detection coupled to capillary electrophoresis (*11*) or LC using a pentafluorophenylpropyl-bonded silica column (*12*) or a porous graphitic carbon column (*13*).

Such highly sensitive AAA described above can complement MS-based proteomics of low abundance proteins. This review provides an overview of the methodology using AQC required for highly sensitive amino acid analysis of small quantities of samples while maintaining precise quantitative performance.

## 2. Quantitation of acid hydrolysates of protein

### 2.1. *Acid hydrolysis*

Proteins or peptides have to be completely hydrolyzed to yield free amino acids prior to AAA. Among the many potential methods of protein hydrolysis, such as the use of a strong acid as hydrochloric or methanesulfonic acid (*14*), a strong base such as sodium hydroxide (*15*), or enzymes (*16*), hydrolysis using hydrochloric acid (HCl) is currently universally applied because HCl can cleave peptide bonds completely and can easily be removed from hydrolysates by evaporation. Because constant boiling HCl retains the same content of HCl in the gas-phase and liquid-phase, protein samples can be hydrolyzed either by adding HCl directly to the samples (liquid-phase hydrolysis) or by distillation of HCl *in situ* and exposing the samples to gaseous HCl (gas-phase hydrolysis). In the case of highly sensitive analysis of small amounts of samples, gas-phase hydrolysis is preferred to prevent contamination from HCl.

Recently, we proposed an innovative hydrolysis method using cation exchange resin as solid acid catalyst, in which the sulfonate group of the resin hydrolyzes the peptide bonds (*17*). Hydrolysis of adsorbed proteins is achieved simply by heating the column packed with the resin after injection of proteins onto the column. Even though successful automation of the hydrolysis process was achieved using the packed catalyst, the manual protocol employing conventional hydrolysis by gas-phase HCl is explained in this review.

### 2.2. *Precolumn derivatization with fluorescent reagent*

As mentioned in the Introduction, AAA based on precolumn derivatization with fluorophores became a popular alternative to the postcolumn method. Sub-picomolar sensitivity has been realized by using fluorescent reagents and by carrying out reversed-phase chromatography that prevents dilution of peaks. NBD-F (*18*) and FMOC-Cl (*19*) are two examples of fluorescent derivatization reagents for amino acids. In general, the pre-column derivatization reagent is added in large excess. In the case where the reagent itself emits fluorescence, such as NBD-F and FMOC-Cl, the excess reagent has to be removed prior to chromatographic analysis because the fluorescence of the reagent interferes with detection of the amino acids. On the other hand, the use of OPA as a fluorescent derivatization reagent (*20*) precludes the need for reagent removal given that OPA only emits if it forms an adduct with an amino acid. OPA, however, has significant disadvantages in that it reacts only with primary amines in the absence of oxidants as described in the previous section, and it forms unstable adducts with amino acids. The instability of the OPA-amino acid adducts makes the use of OPA unsuitable for precolumn derivatization because the derivatized amino acids must be separated on a column after derivatization. For our purposes AQC was selected as a derivatization reagent (*17*). This reagent reacts with primary and secondary amines including proline and hydroxyproline to produce stable derivatives and excess reagent is immediately hydrolyzed to yield 6-aminoquinoline (AMQ) (*21*). Although AMQ has the same excitation maximum as the AQC-derivatives, the emission maximum of AMQ is different from that of AQC-derivatives. The difference in the emission maxima allows for the selective detection of the AQC-derivatives in the presence of a large excess of AMQ. Removal of the excess reagent prior to injection onto the reversed-phase column thus becomes unnecessary. We summarized features of these fluorescent reagents in Table 1.

**Table 1. Comparison of fluorescent reagents for precolumn derivatization**

| Fluorescent reagent | Reactivity with secondary amines | Stability of amino acid adducts | Removement of excess reagent prior to chromatographic analysis |
|---|---|---|---|
| AQC | Yes | 1 week at *r.t.* | Unnecessary |
| NBD-F | Yes | Unstable | Necessary |
| FMOC-Cl | Yes | 30 h at *r.t.* | Necessary |
| OPA | No[a] | Unstable | Unnecessary |

[a] OPA reacts with secondary amines in the presence of an oxidizing agent. Abbreviations: AQC, 6-aminoquinolyl-carbamyl; NBD-F, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole; FMOC-Cl, 9-fluorenylmethyl-chloroformate; OPA, *o*-phthaldialdehyde.

### 2.3. *Chromatographic analysis*

In general, fluorescence intensity is stronger in organic solvents than in water due to fluorescence quenching by water molecules (*21*). Consequently, under the water-organic solvent gradient conditions employed in reversed-phase chromatography, the AQC-derivatives eluted at longer times, *i.e.*, the more hydrophobic amino acids, show higher intensity in a chromatogram. This difference in the intensity leads to a different response factor for each amino acid. Shindo *et al*. have developed an ion-pair chromatographic technique (*22*) that enhances their hydrophobicity of AQC-derivatives of hydrophilic amino acids and produces a similar response factor for all of the amino acids. This method is slightly modified in the current technique, as described in the Protocols section.

### 2.4. *Quantification of acid hydrolysates of protein*

A portion of the AQC-amino acids was separated on a reversed-phase column and the detected peaks in the chromatogram were calibrated with those of a standard solution of amino acids that contains 17 amino acids other than Trp, Asn, and Gln. Under the conditions of the conventional acidic hydrolysis, Asn and Gln are completely hydrolyzed to Asp and Glu, Trp is completely destroyed, Met is partially oxidized, Cys cannot be directly determined, and cystine is partially destroyed. We quantified Cys as half-cystine, Asp as the sum of Asp and Asn, and Glu as the sum of Glu and Gln. Usually, norvaline is added to a protein solution prior to hydrolysis as an internal standard for corrections of physical or chemical losses and variations of amino acids during hydrolysis. For labile amino acids, Ser and Thr, or for slow cleavage amino acids, Ile and Val, time-course hydrolysis is often employed. By extrapolating the observed amounts of Ser and Thr to zero-hour hydrolysis time, the starting amounts of these amino acids can be determined. In the case of Ile and Val, the observed amounts will reach a plateau as the hydrolysis time advances and the value at the plateau is regarded as the true amount of these amino acids (*23,24*).

There are two methods for calculating the amount of protein from the quantified amino acids. In one method, the protein amount is computed by adding all the values of multiplication of the observed amount of each amino acid by its molecular weight. Although the amount of protein can be determined without information on the amino acid sequence of the proteins, amino acids destroyed during hydrolysis cannot be considered. In the second method, the protein amount is calculated by dividing the observed amount of each amino acid by its theoretical residue number calculated from the amino acid sequence of the protein. Under ideal conditions, the obtained protein amount is the same when calculated from each amino acid. Because some amino acids are, however, partially or completely destroyed as described above, and incomplete bond cleavage between Ile-Val, Val-Ile, Val-Val, and Ile-Ile are known (*25,26*), well-recovered amino acids are chosen to quantify the amount of protein. The protein amount was determined herein by averaging the protein contents calculated from the recovery of Ala, Phe, and Leu.

### 2.5. *Compositional analysis of bovine serum albumin*

Bovine serum albumin (BSA, 500 ng) was hydrolyzed, and two portions (45 ng and 100 ng) of the BSA hydrolysates were analyzed. The amino acid compositions of the respective hydrolysates are listed in Table 2. In both experiments, three samples were independently analyzed, and the standard deviation of each mean was calculated as $(\Sigma(\text{experimental value} - \text{average value})^2/2))^{1/2}$. The sequence of BSA reported in the Swiss-Prot database with accession number P02769 [25-607] was used to obtain the theoretical BSA composition, which was calculated by considering the conversion from Asn/Gln to Asp/Glu and the complete destruction of Trp.

As listed in Table 2, the obtained amino acid composition was in agreement with the theoretical composition, except that cystine was partially destroyed. In our laboratory, the setting of samples in the reaction vessel is carried out in a $N_2$-saturated hood and the reaction vessel is degassed sufficiently to prevent the oxidation of amino acids. As listed in Table 2, Met was quantitatively recovered in our system.

### 2.6. *Response linearity*

It is important to demonstrate response linearity in the rage of analytical interest for quantification. There are

two factors that affect the linearity: (*i*) the linearity of the photomultiplier tube (PMT) response of the fluorescence detector and (*ii*) the reaction linearity of the AQC reagent.

The PMT response depends on the electric voltage that is applied to the PMT. To evaluate the linearity of the PMT response, various amounts of Arg, in the range from 10 fmol to 5 nmol, were derivatized. The area under the Arg peaks was plotted against the injected amount as shown in Figure 1A. Under the experimental conditions used in the reviewed method, a linear response of the PMT was obtained in the Arg concentration range of 100 fmol to 500 pmol. Next, various amounts of amino acids standards were derivatized with the same amount of AQC, in the range from 50 pmol to 1 nmol, corresponding to total amino acid contents of 850 pmol to 17 nmol. A portion of the derivatized standard was injected in order not to exceed the linearity range of PMT. As shown in Figure 1B, linearity was maintained up to a concentration of 14 nmol of amino acids; at increasing concentrations of amino acids, the amount of AQC reagent was insufficient for complete derivatization and linearity was lost.

**Table 2. Amino acid compositions after gas-phase acid hydrolysis of BSA at 110°C for 20 h**

| Amino acid[a] | Theoretical composition (%) | Estimated composition (%)[b] | |
|---|---|---|---|
| | | 45 ng of BSA | 100 ng of BSA |
| Arg (R) | 4.0 | 4.0 ± 0.03 | 4.1 ± 0.04 |
| His (H) | 2.8 | 3.0 ± 0.01 | 2.9 ± 0.00 |
| Ser (S) | 4.8 | 5.0 ± 0.07 | 4.8 ± 0.05 |
| Gly (G) | 2.8 | 3.0 ± 0.01 | 3.0 ± 0.05 |
| Thr (T) | 5.7 | 5.8 ± 0.08 | 5.9 ± 0.04 |
| Pro (P) | 4.8 | 5.0 ± 0.17 | 5.0 ± 0.12 |
| Ala (A) | 8.1 | 8.1 ± 0.02 | 8.2 ± 0.04 |
| Asp (D) | 9.5 | 9.5 ± 0.11 | 9.2 ± 0.05 |
| Glu (E) | 14 | 15 ± 0.10 | 15 ± 0.04 |
| Tyr (Y) | 3.4 | 3.3 ± 0.04 | 3.3 ± 0.02 |
| Val (V) | 6.2 | 6.1 ± 0.04 | 6.1 ± 0.01 |
| Met (M) | 0.69 | 0.74 ± 0.01 | 0.76 ± 0.02 |
| Lys (K) | 10 | 10 ± 0.10 | 10 ± 0.04 |
| Ile (I) | 2.4 | 2.3 ± 0.02 | 2.4 ± 0.02 |
| Leu (L) | 10 | 11 ± 0.13 | 11 ± 0.04 |
| Cys (C)[c] | 6.0 | 3.7 ± 0.09 | 3.6 ± 0.01 |
| Phe (F) | 4.6 | 4.7 ± 0.03 | 4.7 ± 0.02 |

[a] Letters in parenthesis are one-letter codes of amino acids. [b] Values listed are the means and standard deviations of three independent experiments (See text for experimental conditions). [c] Cysteine was quantified as half-cystine.

## 3. Materials

For the analysis of small amounts of samples, a clean hood and polymer gloves are needed in order to prevent contamination during sample preparation.

### 3.1. *Reagents and apparatuses in a clean hood*

1) 6 mm × 32 mm borosilicate glass tubes (Crimp Top Vials, P/N: 03-CVG, Chromacol, UK) for samples and 27.75 mm × 70 mm borosilicate glass vials (P/N: 224832, Wheaton, NJ USA) as hydrolysis vessels (*See Note 1*).
2) Mininert valves (No.SC-24, P/N: 10130, Pierce, IL USA) for gas phase hydrolysis.
3) Centrifugal concentrator (Micro Vac MV-100, TOMY Seiko Co., Ltd., Tokyo, Japan) (*See Note 2*).
4) Heat block bath (Thermo Alumi bath ALB-121, Scinics Co., Tokyo, Japan) with an aluminum block possessing 28 mm diameter and 100 mm depth holes and an aluminum block cover.
5) Constant boiling HCl prepared from purchased HCl (P/N: 086-03925, Wako Pure Chemical Industries, Ltd., Osaka, Japan).
6) Crystalline phenol (P/N: 162-17361, Wako) to prevent halogenation of Tyr (*27*).
7) Clean forceps and a spatula, pipettes and disposable tips.
8) Vortex mixer (N-20M, Nissin Scientific Co.,

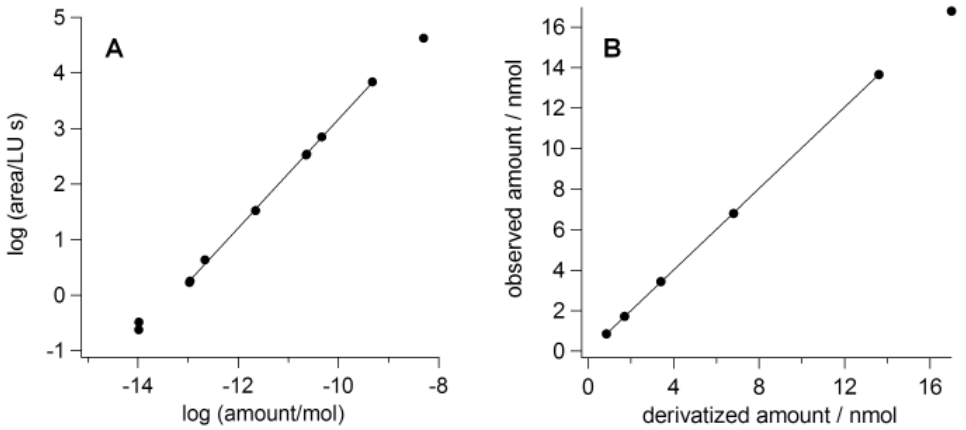


**Figure 1. Linearity of the analysis: Detector response and AQC reagent reactivity. (A)** Linearity of photomultiplier response. Peak areas of various amounts of arginine were plotted against injected amount. **(B)** Linearity of reactivity of AQC reagent. Observed total amino acids of standard solutions were plotted against those of standard solution derivatized with same amount of AQC reagent.

Tokyo, Japan).

9) Pre-cut polyethylene snap cap, 8 mm, for the Chromacol glass tube (P/N: 8-PEC1X, Chromacol).
10) Derivatization reagent (AQC powder dissolved in acetonitrile to provide a 3 mg/mL solution ca. 10 mM): AQC was included in a commercial kit (AccQ Fluor Reagent Kit, P/N: WAT052880, Waters Co., MA, USA) or was synthesized according to the literature (*14*). Briefly, 6-aminoquinoline (1.5 g, 10 mmol, P/N: 275581, Sigma-Aldrich Co., MO, USA) dissolved in 50 mL of dry acetonitrile (P/N: 013-15545, Wako) was added dropwise to a refluxing solution of di(*N*-succinimidyl)carbonate (3 g, 12 mmol, P/N: 43720, Sigma-Aldrich) in 100 mL of dry acetonitrile. The resulting crystals were filtered, washed, and recrystallized from dry acetonitrile and the synthesized AQC was stored in a dry place (*10*).
11) 0.2 M borate buffer (pH 8.8) made by weighing 0.76 g of sodium tetraborate (P/N: 71999-250G, Sigma-Aldrich) in a pyrolyzed glass vial and adding 9 mL of MilliQ water with heating for dissolution. The solution was then titrated to pH 8.8 with 6 N HCl, and MilliQ water is added to increase the weight of the solution to 10 g.
12) 10 pmol/μL amino acid standard solution, diluted from the commercial mixed standard stock solution (amino acid standard H containing 17 amino acids (2.5 mmol/L each except for 1.25 mmol/L of cystine) other than Trp, Asn, and Gln, P/N: 20088, Pierce).
13) 20 mM HCl: Mix 3 μL of constant boiling HCl with 997 μL of MilliQ water.

*Note 1*. Glassware was set in a deep, glass Petri dish and heated to 550°C for 3 h in a Muffle furnace to pyrolyze organic substances.
*Note 2*. The concentrator is custom-made for acid resistance.

3.2. *Chromatographic analysis*

1) Acetonitrile (HPLC grade, P/N: 01031-2B, Kanto Chemical Co., Inc., Tokyo, Japan).
2) Tetrabutylammonium bromide (TBA-Br, P/N: 207-04335, Wako) as ion-pair reagent.
3) Phosphate for chromatography: sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, P/N: 106346) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, P/N: 106580) purchased from Merck KGaA, Darmstadt, Germany.
4) Elution buffer A: 95% of 30 mM phosphate buffer (pH 7.3) containing 5 mM TBA-Br and 5% acetonitrile (HPLC grade), elution buffer B: 50% of 30 mM phosphate buffer (pH 7.3) and 50% acetonitrile (HPLC grade).
5) Column: InertSustain C18HP, 3 μm, 3.0 mm × 250 mm, (P/N: 5020-14426, GL Sciences Inc., Tokyo Japan) with pre-column filter A-701 (Upchurch Scientific, WA, USA).
6) Agilent 1200 series HPLC system equipped with degassers (G1379B), binary pump (G1312B), an autosampler (well-plate sampler, G1367A or standard sampler, G1329B), a column oven (G1316B), a diode-array detector (G1315C) with flow cell of 1.7-μL volume, 6-mm cell path length, and a fluorescence detector (excitation at 250 nm and emission at 395 nm, G1312A) with flow cell of 8-μL volume. Control of the HPLC and data treatment was done using ChemStation (Agilent Technologies, Inc., Santa Clara, CA, USA) software.

3.3. *Other reagents and apparatuses*

1) Bovine serum albumin (BSA, A7638) was purchased from Sigma-Aldrich. BSA solution was prepared by dissolving BSA in MilliQ water.
2) Heat bath for derivatization at 55°C with an aluminum block possessing 6 mm diameter holes (DRI-BLOCK DB-1L, M & S Instruments Inc., Osaka Japan).
3) Electronic balance (ER-182A, minimum weight = 0.01 mg and FX-3000, minimum weight = 0.01 g, A & D Co., Ltd., Tokyo, Japan).
4) Static eliminator (AD1683, A & D) for weighing samples.
5) Ultrasonic cleaner (AU-80C, Aiwa Medical Industry Co., Ltd., Tokyo, Japan) for dissolving AQC in acetonitrile.
6) Rotary vacuum pump (GCD-051X, ULVAC Inc., Kanagawa, Japan) for centrifugal concentrator with trap in Dewar vessel including liquid nitrogen.

**4. Protocols**

4.1. *Sample preparation*

In order to obtain accurate compositions using AAA, protein samples must be purified. It is also recommended that substances that may interfere with derivatization, such as salts or detergents, be removed if possible in the case of the precolumn method.

1) Weigh the purified proteins by the combined use of the microelectronic balance and the static eliminator.
2) Dissolve the protein in MilliQ water to provide a sample solution with a typical concentration in the range of 0.2-1 μg/μL.

4.2. *Hydrolysis*

1) Use a pipette to place the protein sample solution (typically less than 2 μg) or amino acid standard solution (typically containing 50 pmol of each amino acid) into a clean 6 mm × 32 mm glass tube

containing 50 pmol of norvaline as an internal standard. Prepare a clean glass tube containing 50 pmol of norvaline only as a control blank of the hydrolysis.

2) Evaporate the protein solution, the standard solution, or blank control to dryness using the centrifugal concentrator with the rotary vacuum pump.
3) Place the sample tube into the glass vial containing 200 μL of constant-boiling HCl and a piece of phenol crystal (ca. 1-2 mg).
4) Seal the vial after evacuation for a few minutes, by using the Mininert valve.
5) Hydrolyze the sample at 110°C for 20 h in the heat bath.
6) Remove the vial from the heat bath and allow it to cool. Open the vial and remove the tubes with the forceps. Wipe the outside of the tubes with a Kimwipe. Remove excess HCl using the centrifugal concentrator with the rotary vacuum pump to prevent interference with the subsequent derivatization.

*Note*: The procedural steps from 3 to 5 are preferably performed in a glove box, in an inert nitrogen atmosphere, given that atmospheric oxidation of amino acids results in poor quantitation.

**Table 3. Elution program for separation of AQC-amino acids**

| Time (min) | Concentration of buffer B (%) |
|---|---|
| 0 | 2 |
| 3 | 7.3 |
| 44 | 60 |
| 45 | 99 |
| 47 | 99 |
| 48 | 2 |
| 62 | 2 |

See text for details of buffer composition and flow rate.

4.3. *Derivatization*

4.3.1. *Calibration standard*

1) Place 5 μL of 10 pmol/μL standard, norvaline, and carboxymethylcysteine solutions into a clean 6 mm × 32 mm glass tube; evaporate to dryness and add 10 μL of 20 mM HCl.
2) Add 30 μL of 0.2 M borate buffer and mix.
3) Add 10 μL of a 3 mg/mL AQC solution, vortex immediately after the addition.
4) Seal the tube using aluminum foil and the pre-cut polyethylene snap cap.
5) Heat the vial in the block heater at 55°C for 10 min.

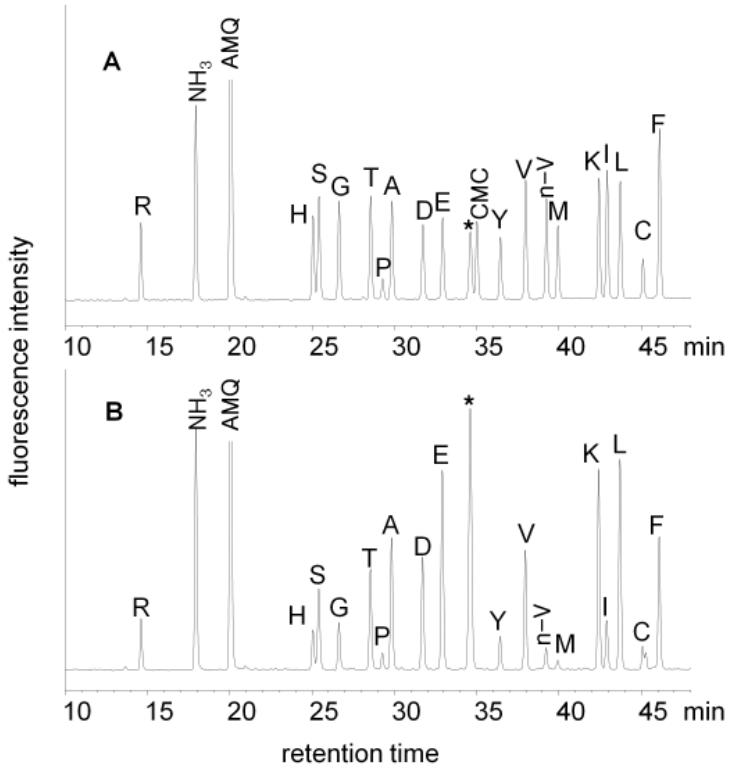


**Figure 2. Chromatogram of AQC-amino acids of standard and BSA hydrolysate. (A)** Chromatogram of 5 pmol each of the AQC-amino acid standard. **(B)** Chromatogram of 150 fmol of hydrolyzed BSA. The expansions of the one-letter abbreviations of the AQC-amino acids are indicated in Table 1. Peak labeled C is cystine. Abbreviations: $NH_3$, ammonium; AMQ, 6-aminoquinoline; CMC, *S*-carboxymethylcysteine; n-V, norvaline. Peak with an asterisk is unknown chemical peak.

4.3.2. *Control blank for derivatization*

1) Place 5 μL of 10 pmol/μL norvaline in a clean glass tube. Add 5 μL of 20 mM HCl.
2) Add 30 μL of 0.2 M borate buffer and mix.
3) Add 10 μL of AQC solution and vortex.
4) Seal the tube using aluminum foil and the pre-cut polyethylene snap cap.
5) Heat the vial in the block heater for 10 min at 55°C.

4.3.3. *Sample hydrolysis*

1) Add 10 μL of 20 mM HCl to the sample tube and vortex.
2) Add 30 μL of borate buffer and vortex.
3) Add 10 μL of AQC solution and vortex.
4) Seal the tube with aluminum foil and the pre-cut polyethylene snap cap.
5) Heat the vial in the block heater for 10 min at 55°C.

4.4. *Chromatographic analysis*

1) Analyze AQC-amino acids using the elution program shown in Table 3. Set flow rate at 0.4 mL/min and column temperature to 42°C (*See Note*). Entire separation is completed in 47 min as shown in Figure 2. Including column re-equilibration, the total run time for a typical analysis is 62 min.
2) Injection volume of sample solution, standard or blank control solution: typically 5 μL.

   *Note*: Using the 3.0 mm × 250 mm (3 μm)-column under these conditions, the system pressure is about 190 bar.

4.5. *Quantitation of amino acids*

1) Evaluate the amounts of 17 amino acids in the protein sample by calculating the ratio of the peak-height of an amino acid in the sample to that of the same amino acid in the standard.
2) Calculate the percentage composition of the amino acids by dividing the amount of each residue by the total amount of amino acids and multiplying by 100.

## 5. Discussion

Highly sensitive AAA of low abundance proteins can be achieved by using fluorescent reagent, AQC, for derivatization of amino acids. For the high sensitivity AAA, prevention of environmental contamination is critical to obtain a precise result. Especially, sample-handling procedures before proteins are hydrolyzed to their component amino acids require extra attention because the amino acids of contaminant proteins cannot be distinguished from those of the sample protein. In this review, we provided the protocol for the high sensitivity AAA. Using the protocol, the amino acid composition of BSA less than 100 ng, *i.e.*, 1.5 pmol, was determined and the composition was matched the theoretical composition with low standard deviations.

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