Quantitative Determination of Total Amino Acids Based on Surface-Enhanced Raman Scattering and Ninhydrin Derivatization

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In the present study, we propose a simple and sensitive method for the determination of total amino acids without any separation steps. The procedure described here is based on the ninhydrin derivatization reaction with amino acids, followed by surface-enhanced Raman scattering (SERS) measurements of the producing mixtures. A good linear correlation of excess ninhydrin SERS signals and the log values of the total amino acids concentrations is obtained; the detection limit of the method is $4.3 \times 10^{-9}$ mol L$^{-1}$. The derivatization reaction is reliable and the whole experimental procedure is very simple. The sensitivity of the proposed protocol allows quantitative analysis of total amino acids at picomole levels without any separation procedures. On the basis of the conventional ninhydrin reaction, we put forward a simple SERS method for determining the total amino acids concentrations with high sensitivity, which is a promising way for routine detection.

Keywords Amino acids, ninhydrin, SERS, determination

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phenylalaine (Phe), lysine (Lys), histidine (His), tyrosine (Tyr), tryptophan (Try), proline (Pro) and cysteine (Cys) were purchased from Sigma-Aldrich Co., Ltd. Acetic acid, sodium acetate, HCl, ethyl alcohol and all other chemicals were from Beijing Chemical Co., Ltd. All chemicals were analytical-grade reagents and used without further purification. Ultrapure water (18.25 MΩ cm) was used throughout the experiments.

**Apparatus**

UV-Vis extinction spectra were measured with a UV-3600 spectrophotometer (Shimadzu). The SEM image was measured on a JEOL JSM-6700F field-emission scanning electron microscope (FE-SEM) operating at 5.0 kV. Raman spectra were obtained using a Jobin Yvon/HORIBA LabRam ARAMIS Raman spectrometer equipped with an integral BX 41 confocal microscope. The radiations from an air-cooled internal HeNe laser (633 nm) and an external cavity diode laser (785 nm) were used as the excitation sources. Moreover, a Renishaw Raman System Model 1000 spectrometer with the 532 nm excitation wavelength was used. The exposure time was set at 30 s with one accumulation.

**Preparations of self-assembled Ag NPs film**

Ag NPs were prepared according to the method of Lee and Meisel.25 Briefly, 4 mL of a trisodium citrate solution (1%, w/v) was added to 200 mL of a silver nitrate (10−3 mol L−1) aqueous solution at a slightly boiling state, and then the temperature was kept at around 85°C for 40 min to give a grey-green color.

Glass slides were ultrasonically rinsed in order by ultrapure water, ethyl alcohol, acetone, chloroform and then rinsed reversely for 10 min each. They were then hydroxylated by 30% H2O2 and 98% H2SO4 (3:7, v/v), boiling for 30 min and rinsed by water. The resulting hydroxylated slides were then soaked in a 0.5% PDDA solution for 60 min and rinsed with water. Finally, the PDDA-coated slides were immersed in Ag NPs for 4 h, rinsed by water and dried by N2. Thus, a self-assembled Ag NPs film was prepared.

**Preparations of solution**

First, 0.01 mol L−1 stock solutions of the nine amino acids were prepared by 0.12 mol L−1 HCl, respectively. A standard amino acids mixture of 9 × 10−3 mol L−1 was then prepared by putting the above nine 0.01 mol L−1 stock solutions (5 mL for each amino acid) into a 50-mL volumetric flask and diluting with 0.12 mol L−1 HCl. All other standard amino acids mixtures with lower concentrations were diluted with water. Ninhydrin of 4% was prepared by dissolving 2 g of ninhydrin solid into a 50-mL volumetric flask and diluting with water. Water was used as the blank sample.

**SERS-based ninhydrin derivatization**

Working reagent: a 4% ninhydrin solution + water + acetate buffer (4 mol L−1, pH 5.29) (2:1:1, v/v/v). The working reagent was treated with N2 for 15 min before being mixed with standard amino acids mixtures (1:4, v/v). Then, the mixed solutions were heated in boiling water for 30 min; 2.5 μL of the producing solutions were dropped onto Ag NPs films for SERS measurements after natural drying. Then, 1 mL of ethyl alcohol was added into each of the producing solutions at a volume of 5 mL for UV-Vis measurements.

**Results and Discussion**

Ag nanomaterials are most commonly used SERS-active substrates; they have wide applications in analytical fields.26 The prepared Ag NPs were uniform, and provide a high amplification of SERS signals. The UV-Vis extinction spectrum of Ag NPs and an SEM image of the assembled Ag NPs film (inset) are shown in Fig. S2 (Supporting Information). The Ag NPs colloid has the maximum absorption at 424 nm, and the SEM image showed that the NPs were mainly spherical. Herein, we assembled Ag NPs with negative charges onto PDDA-monolayer coated glass slides to form a self-assembled Ag NPs monolayer film as SERS-active substrates for the detection of total amino acids.

Since the development of the photometric ninhydrin method, the method has been further modified and combined with some separation techniques, such as thin-layer chromatography, capillary electrophoresis, gas chromatography and high-pressure liquid chromatography for individual amino acids.4,9 However, the ninhydrin method still has the disadvantage of limited sensitivity. Continual advances have been made by fluorescence detection of amino acids based on reactions with various derivatization reagents including OPA,5 phenyl isothiocyanate (PITC),27 6-aminohydroxysuccinimidyl-carbamate (AQC).28 These fluorescence methods have ultrahigh sensitivity, but they generally need an additional separation procedure.

The ninhydrin method that we adapted here is conventional and reliable for total amino acids quantification. In a weak acidic medium, ninhydrin reacts with α-amino acids to produce RP involving a nucleophilic-type displacement of an OH group of ninhydrin hydrate by a nonprotonated amino group.29 The ninhydrin reagent is excess in the derivatization step. We measured the UV-Vis extinction spectra of the diluted derivative solutions from a blank sample, nine amino acids and the standard amino acids mixture (9 × 10−3 mol L−1), respectively. As shown in Fig. 1, the derivative solutions of individual amino acid and their mixture have the absorption of 570 nm basically. Cys and Pro have an absorption of about 440 nm due to their distinctive structures compared with the other amino acids mentioned here. Pro belongs to imino acid and reacts with ninhydrin in an entirely different way: the imino acid that results
from decarboxylation condenses directly with ninhydrin to form a yellow end-product. The shifted extinction spectrum of Cys is due to a competitive nucleophilic displacement of the sulfhydryl and amino group of Cys on ninhydrin. It seems that one reacts and that the other is protected by cyclisation.\textsuperscript{29,30} In contrast, no absorptions at 570 and 440 nm were observed in the UV-Vis extinction spectrum of the blank sample.

In order to choose an optimal excitation wavelength under our experimental conditions, 532, 633 and 785 nm excitation wavelengths were employed in the Raman characterization of the derivative solutions from nine amino acids. We took Phe and His as examples. The excitation wavelength-dependent Raman spectra of derivative solutions for Phe and His are shown in Fig. 2. The peak at 880 cm\(^{-1}\) belongs to ethyl alcohol that we added. Distinct characterization peaks of the producing derivative solutions were found to appear at 661 and 789 cm\(^{-1}\). It can be seen from Fig. 2 that the spectra obtained at 633 nm are of high quality, and its intensity is stronger compared with those at 532 and 785 nm. No available peaks, except for a peak from ethyl alcohol were obtained at 785 nm for nine amino acids. The signal-to-noise ratio of the spectra obtained at 532 nm is rather low. The spectra at 532 nm have been baselined before plotting the figure. The original spectra at 532 nm laser line have a strong fluorescence background, by which their Raman signals may be covered. According to the Raman results of the derivative solutions from nine amino acids with excitation wavelengths of 532, 633 and 785 nm, it can be concluded that 633 nm is the optimal excitation wavelength. Therefore, 633 nm was chosen as the excitation wavelength for further Raman/SERS measurements and quantitative analysis.

The Raman spectra of the derivative solutions from individual amino acids are shown in Fig. 3. The spectra were baselined and normalized by an ethyl alcohol band at 880 cm\(^{-1}\). Clear characteristic bands at 661 and 789 cm\(^{-1}\) were observed except for Pro and Cys due to different products resulting from their distinct structures, which also correspond to their UV-Vis results.

The resulting derivative solutions from individual amino acids were dropped onto the self-assembled Ag NPs film. The SERS measurements were conducted with the 633 nm excitation wavelength when the solutions were dried naturally. The SERS spectra of the derivative solutions from the nine amino acids are
shown in Fig. 4. Similarly, strong bands at 661 and 789 cm\(^{-1}\) were observed from Gly, Phe, Lys, His, Glu, Try and Tyr. The integrated intensity of Pro is relatively low compared with the above seven amino acids. As for the SERS spectrum of the derivative solution from Cys, many different peaks appeared compared with those from other amino acids. The relative-intensity ratio between 661 and 789 cm\(^{-1}\) becomes larger than that of the Raman spectra in Fig. 3. The tiny differences between the Raman and SERS spectra may originate from the fact that the product RP is difficult to adsorb on the Ag substrates. RP has a C=N double bond to link two ninhydrin molecules, resulting in a kind of approximate planar structure. In addition, other groups of RP are also difficult to adsorb the substrates (see Fig. S1). The peaks at 661 and 789 cm\(^{-1}\) are assigned to the out-of-plane bending vibration of C=O.31 According to the SERS selection rules,32 we infer that RP tends to lie on the Ag film.

On the basis of the Raman and SERS characterization, we examined the SERS spectra of the derivative solutions from the standard amino acids mixtures in different concentrations. Representative concentration-dependent SERS spectra of the derivative solutions from the standard amino acids mixtures are shown in Fig. 5(A). The SERS intensities of the characteristic bands at 661 and 789 cm\(^{-1}\) from RP products were found to decrease gradually while reducing the standard amino acids mixture concentrations, the enhanced bands include 661 and 789 cm–1; the reduced bands include 1081, 1163, 1229, 1385, 1498, 1560, 1596 and 1651 cm–1. We assigned the band at 1651 cm–1 to the stretching vibration of C=O from unreacted ninhydrin after derivatization. When the concentration of the standard amino acids mixture was below 9 \times 10^{-9} mol L^{-1}, the SERS intensity of excess ninhydrin in the products was not detected. However, it is noted that the intensity of band at 1651 cm–1 shows a decreasing trend along with an increase of the concentration of the standard amino acid mixture. Therefore, the SERS intensity at 1651 cm–1 \textit{versus} the log values of standard amino acid mixtures concentrations. Each error bar indicates the standard deviation of the SERS intensity. Excitation wavelength: 633 nm.

![Fig. 5](image)

### Table 1 Recovery test of the standard curve

<table>
<thead>
<tr>
<th>True concentration/mol L(^{-1})</th>
<th>Recovery, %</th>
<th>RSD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 \times 10^{-9}</td>
<td>97.8</td>
<td>14</td>
</tr>
<tr>
<td>1.0 \times 10^{-7}</td>
<td>97.0</td>
<td>10</td>
</tr>
<tr>
<td>5.0 \times 10^{-5}</td>
<td>101.1</td>
<td>7.5</td>
</tr>
</tbody>
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9 \times 10^{-9} – 9 \times 10^{-5} mol L\(^{-1}\) (Y = -1133.9093 – 464.952X, R^2 = 0.9964). The limit of detection (LOD) is calculated to be 4.3 \times 10^{-9} mol L\(^{-1}\) (3\(\sigma\)). The LOD of this study is comparable to the detectable minimum, 2.5 picomoles for the determination of total amino acids by fluorescence without the separation of amino acids.30 In addition, the standard deviation of the SERS intensity for each concentration is rather small (see the error bar in Fig. 5(B)), indicating the reproducibility of the measurements. Moreover, the recovery test of the method also proves its accuracy and precision (Table 1).

### Conclusions

In conclusion, a SERS quantitative method for total amino acids based on the conventional ninhydrin derivatization without separation steps has been established with 633 nm excitation. The whole detection process is simple, including the ninhydrin derivatization with total amino acids and SERS measurements on self-assembled Ag NPs substrates. The results demonstrated the SERS intensity of excess ninhydrin in the producing derivative solutions from the standard amino acids mixture shows a good linear relationship with the log value of total amino acids concentration. Moreover, the LOD of the method reaches 4.3 \times 10^{-9} mol L\(^{-1}\), which is as sensitive as the detectable minimum for determination of total amino acids by fluorescence. Therefore, we have explored a simple and sensitive method for total amino acids without any separation process based on the ninhydrin reaction and the SERS technique. The simple experimental operation and high sensitivity make the study a promising method for routine total amino acids quantification.
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Supporting Information

The Supporting Information provides the reaction mechanism between ninhydrin and primary amino acids and the characterization of Ag NPs. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References