



Supporting Information

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Direct Identification of Tryptophan in a Mixture of Amino Acids by Naked Eye: a New Color Reaction of Tryptophan

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General experimental

All commercially available reagents and solvents were of at least analytical grade, and used as received. L-Tryptophan and other amino acids were purchased from Sigma, ninhydrin from Merck, and indole from Beijing Chemical Company. Hydrochloric acid (37%), formic acid (88%), and indole-3-propanic acid (IPA) were purchased from Beijing Chemical Plant. Reduced glutathione was obtained from Amresco Company, and egg white albumin from Fluka. A purified hydrochloric acid by distillation was also used for comparison. Deionized and distilled water was used throughout. L-Tryptophan (25 mM) and indole (25 mM) solutions were prepared in water. IPA (50 mM) solution was prepared in methanol-water (40:60, v/v). The absorption spectra were measured with a Techcomp UV-8500 spectrophotometer (Shanghai, China). A model HI-98128 pH-meter (Hanna Instruments Inc., USA) was used for pH measurements. Unless otherwise stated, HPLC analyses were performed on a HiQ sil C18W (4.6×200 mm) column using a Jasco HPLC system consisting of a PU-2086-plus pump and a UV-2075-plus detector (Tokyo, Japan) at 280 nm with methanol-water (50:50, v/v) solution as eluent. Electrospray ionization mass spectra were recorded with a Shimadzu LCMS-2010 (Japan).

Acid dependence of color reaction of tryptophan: Reactions of tryptophan in various acids, such as HCl, H₂SO₄, and H₃PO₄, were examined and compared under the same condition. The experimental results showed that, in all of the tested acid solutions, tryptophan produces a yellow color with a little different and broad absorption band around 350-500 nm (Figure S1), suggesting that H⁺ may be responsible for the yellow color reaction. Figure S2 depicts such a case in HCl media, together with the effect of HCl concentration on the absorption spectra. As can be seen, the absorbance around 500-700 nm is also increased with the increase of HCl concentration. However, only in HCl media the addition of formic acid can induce the appearance of the violet-blue color

(Figure S3), indicating that the violet-blue color reaction of tryptophan must have something to do with the existence of chloride ion.^[1]

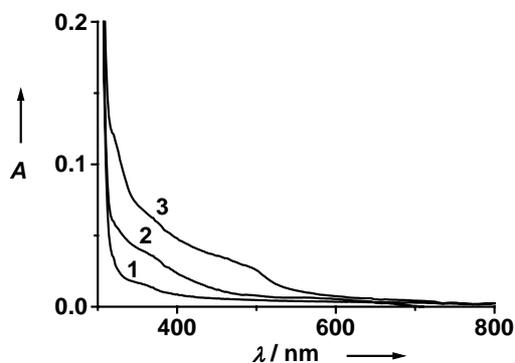


Figure S1. Absorption spectra of tryptophan (5 mM) in different acids: 1) 2 M H₃PO₄, 2) 6 M HCl, 3) 3 M H₂SO₄. The spectra were obtained by heating tryptophan in different acid media at 50 °C for 5 h, cooling the reaction solutions to room temperature, and then measuring the solutions against water.

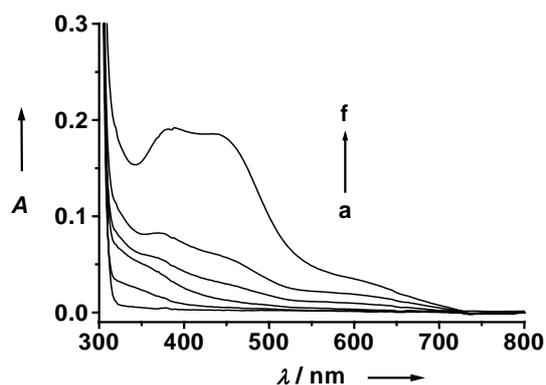


Figure S2. Absorption spectral changes of tryptophan (5 mM) in water with varied HCl concentrations: a) 0, b) 2.4, c) 4.8, d) 6.0, e) 7.2 and f) 9.6 M HCl. The spectra were obtained by heating tryptophan in the above solutions at 50 °C for 5 h, cooling the reaction solutions to room temperature, and then measuring them against water blank.

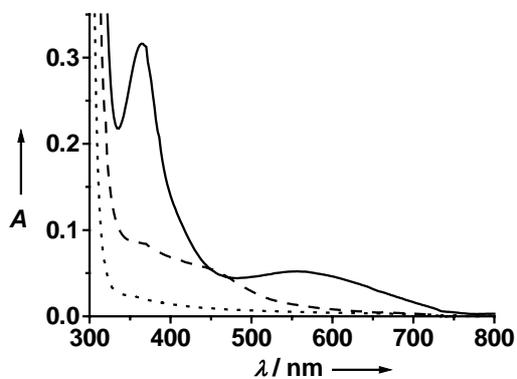


Figure S3. Absorption spectra of tryptophan (5 mM) after treated at 50 °C for 5 h in an aqueous solution of 18% formic acid containing 6 M HCl (solid), 3 M H₂SO₄ (dashed), and 2 M H₃PO₄ (dotted), respectively.

It is known that tryptophan is unstable in hot aqueous HCl due to its autoxidation and decomposition.^[2,3] In order to study the violet-blue color reaction and avoid the possible interferences from degradation products, a concentration of 6 M HCl and a heating time of 5 h at 50 °C were chosen for the present reaction, since under these conditions no obvious degradation of tryptophan was detected by HPLC analysis (Figure S4) and the color reaction became slow (Figure S5). At the same time, a distinct color change can be observed by naked eye. It should be pointed out that commercial hydrochloric acid might contain impurities of oxidizing agents (e.g., ferric chloride). To test the influence of the impurities, the color reaction was also performed in several commercial hydrochloric acids and a purified hydrochloric acid, but the results showed no apparent difference.

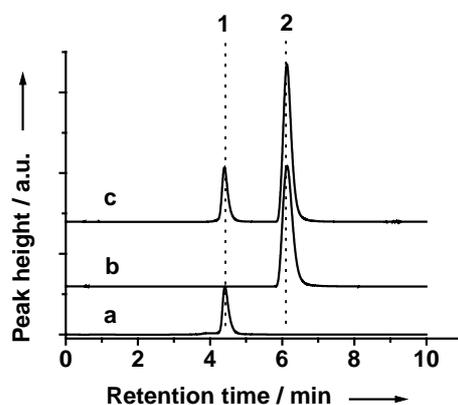


Figure S4. HPLC chromatograms of (a) HCl solution, (b) tryptophan (0.5 mM) in water, and (c) tryptophan (0.5 mM) in HCl solution after treatment at 50 °C for 5 h. Peaks 1 and 2 denote an unidentified product from column and tryptophan, respectively. HPLC condition: methanol-water eluent (50/50, v/v), flow rate 0.5 mL/min, UV detection at 280 nm.

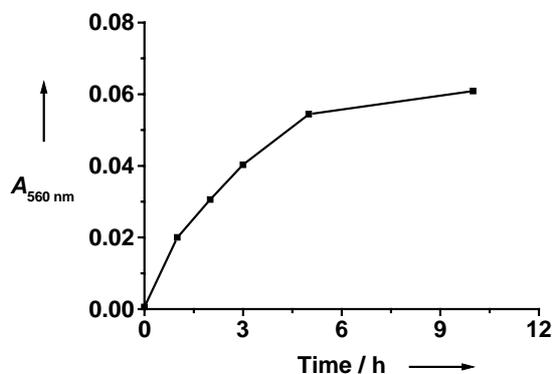


Figure S5. Absorbance changes of tryptophan (5 mM) at 560 nm in the mixed solution containing 18% formic acid and 6 M HCl with different reaction times at 50 °C.

Figure S6 shows the absorption spectral change of tryptophan with its concentration. It is seen that by monitoring the absorbance at about 364 or 560 nm, quantitative estimation of tryptophan content in samples might be achieved, though sensitivity is not high.

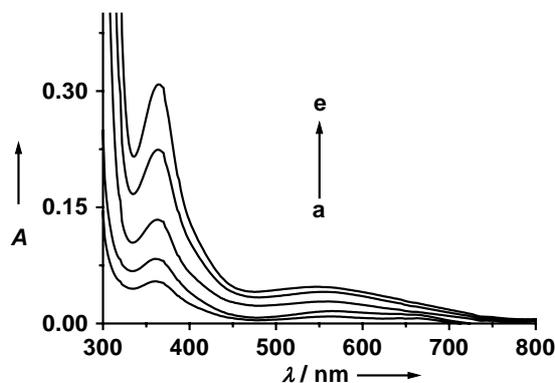


Figure S6. Absorption spectra of tryptophan at different concentrations: a) 0.125 mM, b) 0.25 mM, c) 2 mM, d) 4 mM, and e) 7.5 mM, after treatment at 50 °C for 5 h in an aqueous solution containing 18% formic acid and 6 M HCl.

The specificity of the reaction with tryptophan: To assess the specificity of the reaction, 20 standard amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) were tested in parallel under the same condition. The results are shown in Figures S7-S10.

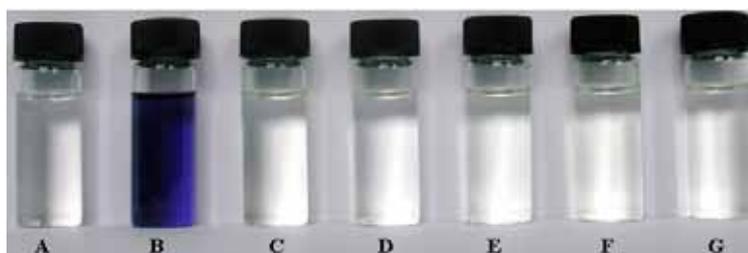


Figure S7. A picture of color reactions of several amino acids in HCOOH–HCl media. A) Reagent blank (reaction solution of 18% formic acid and 6 M HCl); B) L-tryptophan; C) L-leucine; D) L-isoleucine; E) L-glutamic acid; F) L-aspartic acid; G) L-asparagine. The concentration of each of the amino acids was 20 mM. The color reactions (A – G) were carried out at room temperature (25 °C) for 7 days.

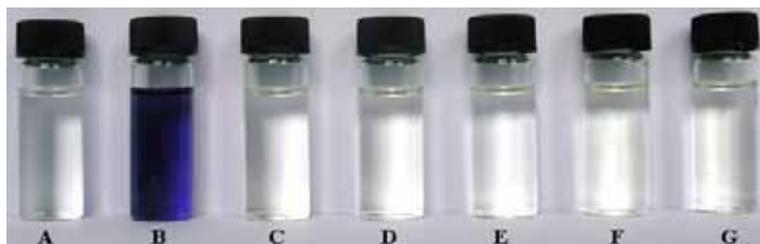


Figure S8. A picture of color reactions of several amino acids in HCOOH–HCl media. A) Reagent blank (reaction solution of 18% formic acid and 6 M HCl); B) L-tryptophan; C) DL-lysine; D) DL-valine; E) DL-threonine; F) glycine; G) L-cysteine. The concentration of each of the amino acids was 20 mM. The color reactions (A – G) were carried out at room temperature (25 °C) for 7 days.

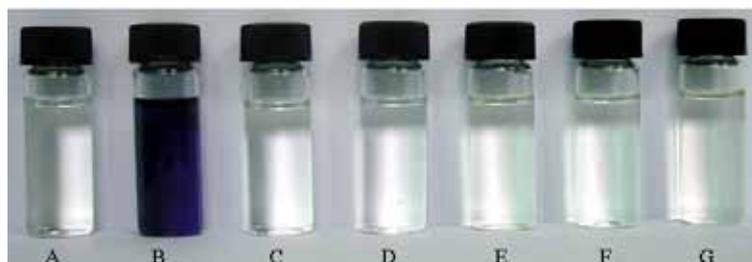


Figure S9. A picture of color reactions of several amino acids in HCOOH–HCl media. A) Reagent blank (reaction solution of 18% formic acid and 6 M HCl); B) L-tryptophan; C) L-alanine; D) L-arginine; E) L-glutamine; F) L-histidine; G) DL-methionine. The concentration of each of the amino acids was 20 mM. The color reactions (A – G) were carried out at room temperature (25 °C) for 7 days.

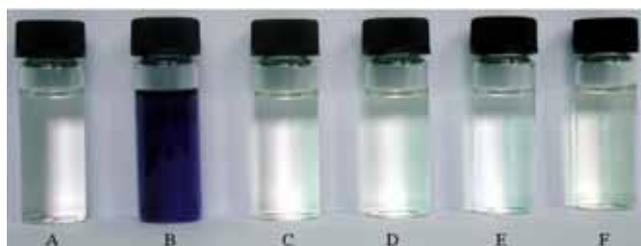


Figure S10. A picture of color reactions of several amino acids in HCOOH–HCl media. A) Reagent blank (reaction solution of 18% formic acid and 6 M HCl); B) L-tryptophan; C) L-phenylalanine; D) L-proline; E) DL-serine; F) L-tyrosine. The concentration of each of the amino acids was 20 mM. The color reactions (A – F) were carried out at room temperature (25 °C) for 7 days.

Comparative experiments with indole and IPA: It was found that the reaction phenomena of indole and IPA resembled that of tryptophan. Namely, in formic acid media both indole and IPA did not exhibit any color, while in HCl media they indeed gave a yellow color, which may be ascribed to the formation of dimeric or trimeric derivatives.^[4,5] Thus, the above observed yellow color from tryptophan in acid solutions may also arise from its oligomerization. However, in HCOOH–HCl media these three compounds (tryptophan, indole and IPA) produced different

colors (Figure S11), which may result from the different substituents at the 3-position in indole ring.

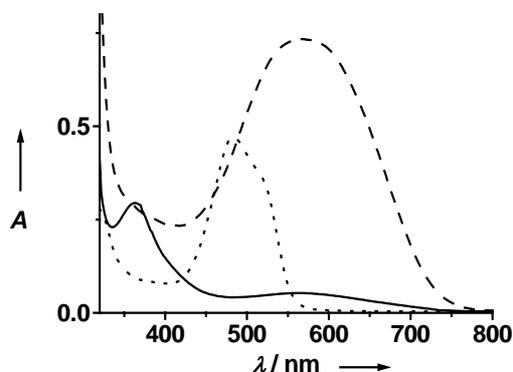


Figure S11. Absorbance spectra of tryptophan (5 mM, solid), IPA (5 mM, dashed) and indole (0.1 mM, dotted) after treatment in the solution containing 18% formic acid and 6 M HCl at 50 °C for 5 h.

Analysis of reaction products by HPLC and mass spectrometry: HPLC technique was attempted to separate the colored product. It was found that two chromatographic peaks appeared at 6.03 and 7.12 min (Figure S12), which were characterized to be tryptophan and 1-formyltryptophan, respectively, by absorption spectra^[6] (recorded with the on-the-fly spectral scanning mode of HPLC, Figure S13) and ESI mass spectra (Figure S14). Nevertheless, the expected blue pigment was not found. Perhaps under this test condition such a compound is unstable and easy to decompose.

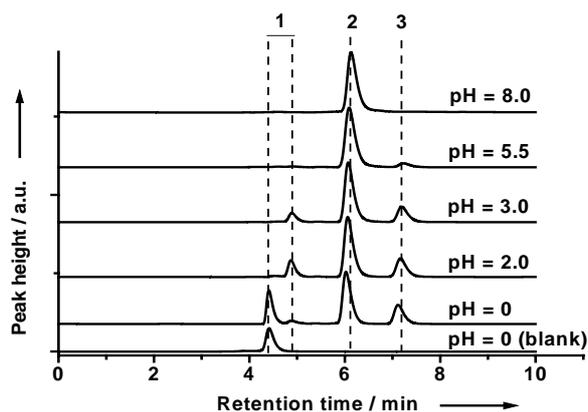


Figure S12. HPLC chromatograms of reaction products of tryptophan (0.5 mM) after the reaction solution was adjusted to pH = 0, 2.0, 3.0, 5.5, and 8.0 with NaOH. The HCl solution alone (1 M, pH = 0) was also tested as a blank. The assignment of the peaks: (1) 4.41–4.89 min, unidentified products from column in the presence of HCl; (2) 6.03 min, tryptophan; (3) 7.12 min, 1-formyltryptophan.

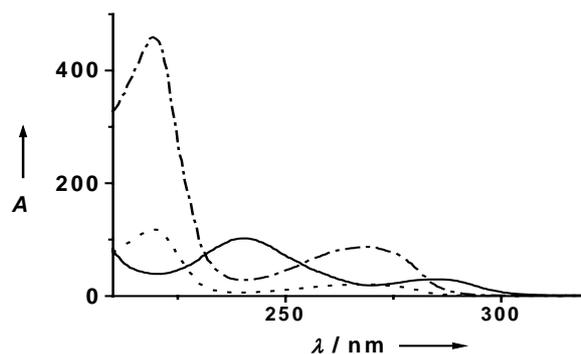


Figure S13. The absorption spectra recorded by the on-the-fly spectral scanning mode of HPLC with UV-2075-plus detector. Standard tryptophan (having a retention time of 6.03 min, dotted); the separated product with retention time of 6.03 min by HPLC (dash-dotted); and the separated product with retention time of 7.12 min by HPLC (solid).

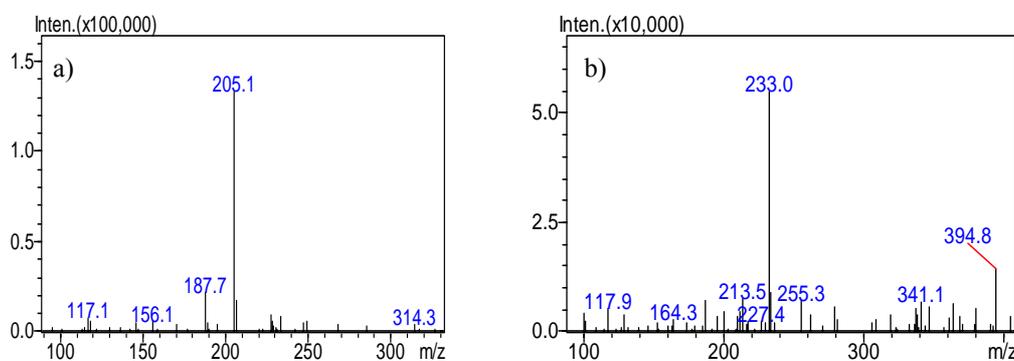


Figure S14. Positive ESI mass spectra of (a) the separated reaction product with the retention time of 6.03 min; and (b) the separated reaction product with the retention time of 7.12 min.

The reaction solution was then directly subjected to ESI-MS analysis. As shown in Figure S15, in addition to tryptophan (m/z 205 $[M+H]^+$) and 1-formyltryptophan (m/z 233 $[M+H]^+$), two more products (m/z 463 and m/z 491) with higher molecular weights are detected, which may result from the formation of compounds with a large conjugation structure.

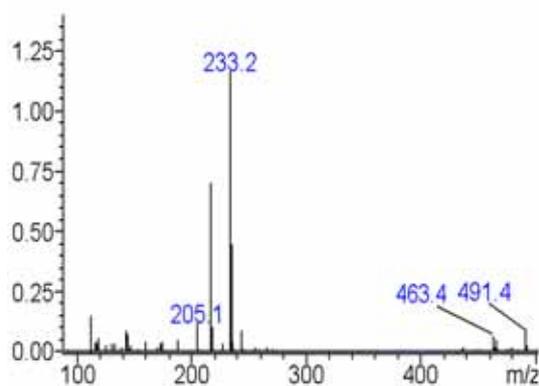


Figure S15. Positive ESI mass spectrum of reaction products of tryptophan in HCOOH-HCl media.

Acid-base equilibrium test: As shown in Figure S16, the absorption maximum at about 560 nm gradually decreased and eventually vanished with the increase of pH. Reversely, after the reaction solutions were acidified back to strong acid media and incubated at 50 °C overnight, the absorption spectra of the solutions were mostly retrieved (see the inset of Figure S16). Furthermore, the recovery behaviors of absorption spectra of indole and IPA resembled that of tryptophan (Figure S17).

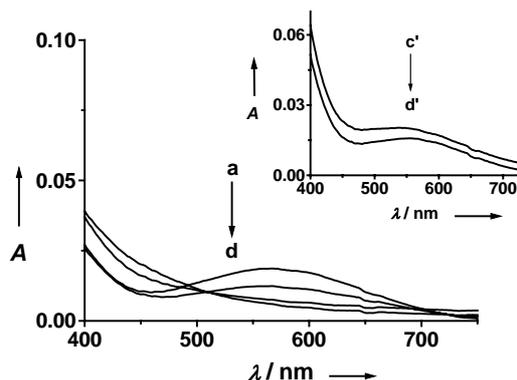


Figure S16. Spectral changes of reaction solutions of tryptophan (1 mM) after adjusted with NaOH to different pH values: a) 0, b) 3.0, c) 5.5, and d) 9.5. The reaction was conducted at 50 °C for 5 h in HCOOH–HCl media, and the spectra were obtained by measuring the reaction solutions against water. The inset shows the spectral recovery of the above reaction solutions of pH 5.5 (c') and 9.5 (d') after acidifying them to pH < 0 with HCl and incubating at 50 °C overnight.

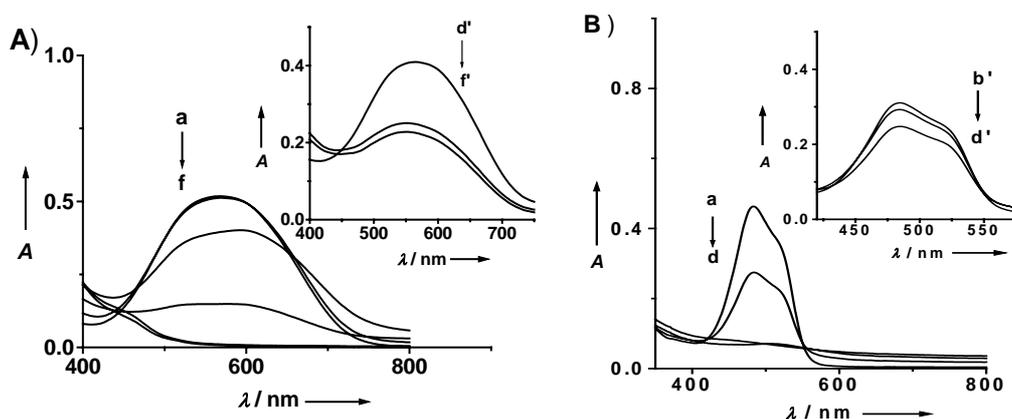


Figure S17. A) Spectral changes of reaction solutions of IPA (1 mM) after adjusted with NaOH to different pH values: a) 0, b) 3.0, c) 5.5, d) 8.0, e) 9.5 and f) 12. The inset shows the spectral recovery of the above reaction solutions of pH 8.0 (d'), 9.5 (e') and 12.0 (f') after acidifying them to pH < 0 with HCl but no incubating at 50 °C. B) Spectral changes of reaction solutions of indole (0.1 mM) after adjusted with NaOH to different pH values: a) 0, b) 3.0, c) 5.5, and d) 9.5. The inset shows the spectral recovery of the above reaction solutions of pH 3.0 (b'), 5.5 (c') and 9.5 (d') after acidifying them to pH < 0 with HCl but no incubating at 50 °C. All of the reactions were conducted at 50 °C for 5 h in HCOOH–HCl media, and the spectra were obtained by measuring the reaction solutions against water.

Comparative study of the present method with other methods: The results of the comparative study with other known approaches,^[7] especially with the acid ninhydrin method, are described as follows. In the acid ninhydrin method, the reaction of tryptophan with ninhydrin produces an unstable yellow color (about 10% loss within 24 h; see Figure S18). The yellow color may result from the formation of a yellow product 2,3,4,5-tetrahydro- β -carboline.^[8] It is interesting to note that a weak absorption band around 500-600 nm (Figure S18) is produced in the acid ninhydrin system, which might be ascribed to the reaction of tryptophan with the “HCOOH–HCl” solution. But this phenomenon was not mentioned in the previous studies.

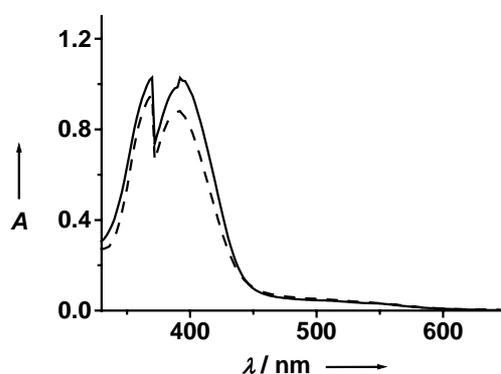


Figure S18. Absorption spectral change with time of the reaction solution of tryptophan with the acid ninhydrin. The spectra were recorded after the reaction solution stood at room temperature for 0.5 h (solid) and 24 h (dashed), respectively. The reaction solution was obtained by treating tryptophan with ninhydrin in the HCOOH–HCl (3/2, v/v) media in a boiling water bath for 10 min, and then diluting the reaction mixture with ethanol (95%). The final concentrations of tryptophan and ninhydrin in the reaction solution were 50 μ M and 3.1 mg/mL, respectively.

The acid ninhydrin method is fast and sensitive, but is inconvenient and unselective. For example, since both hydrochloric acid and formic acid at 100 °C are extremely volatile, the reaction needs to be carried out in a tube covered with metal caps or glass marbles.^[7a] Furthermore, ninhydrin itself has an absorption at about 350 nm, therefore the amount of ninhydrin added has to be controlled strictly; otherwise a large error in absorbance measured at 390 nm would be produced.

More unfortunately, because ninhydrin can react with various α -amino acids,^[8] some amino acids such as cysteine and tyrosine have been observed to interfere with the determination of tryptophan.^[9] As shown in Figure S19, cysteine displays a pink color (bottle C) with two absorption peaks at about 364 and 558 nm (the red curve in Figure S20), respectively, and tyrosine gives a light yellow color (bottle D) with an absorption peak at 374 nm (the blue curve in Figure S20). The spectra of these two amino acids overlap with that (the cyan curve in Figure S20) of tryptophan in

the ultraviolet region, thereby interfering the determination of tryptophan (especially in the sample with a high molar ratio of cysteine or tyrosine to tryptophan). The difference between the cyan curve and the black curve in Figure S20 clearly shows the interference of cysteine and tyrosine with the tryptophan determination. Besides, when tryptophan, cysteine and tyrosine are mixed in equimolar amounts, a red color (bottle E), instead of the yellow color (bottle B) characteristic of tryptophan, is generated. This indicates that, in the presence of other amino acids such as cysteine and tyrosine, tryptophan cannot be identified directly with the acid ninhydrin method by naked eye.



Figure S19. A picture of color reactions of tryptophan, cysteine and tyrosine with the acid ninhydrin reagent. A) The acid ninhydrin reagent blank; B) L-tryptophan; C) L-cysteine; D) L-tyrosine; E) the above three amino acids mixture. The reaction was conducted by treating the three amino acids (each 50 μM) with ninhydrin as described in the caption of Figure S18.

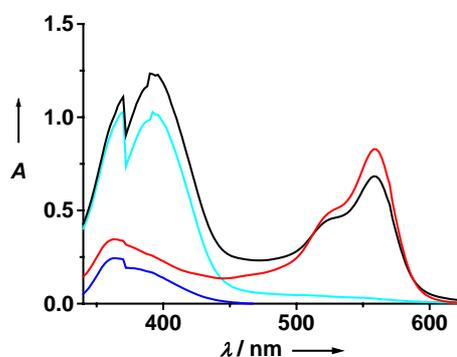


Figure S20. Absorption spectra of the reaction solutions of tryptophan, cysteine and tyrosine with the acid ninhydrin reagent. Tryptophan (cyan); cysteine (red); tyrosine (blue); and the three amino acids mixture (black). The color solutions were obtained by reacting the three amino acids (each 50 μM) with ninhydrin as described in the caption of Figure S18. The spectra were recorded against the reagent blank.

The reaction of tryptophan with *p*-dimethylaminobenzaldehyde and sodium nitrite in sulfuric acid has been widely used for tryptophan detection. However, in this method intense light, chloride ions, and oxidizing and reducing agents in low concentrations interfere with the tryptophan determination. Therefore, this method is inconvenient and is limited in accuracy.^[10]

Compared with the approaches discussed above, the present method based on the violet-blue color reaction of tryptophan in the media of HCOOH and HCl is highly selective and simple.

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