

Analytical Methods

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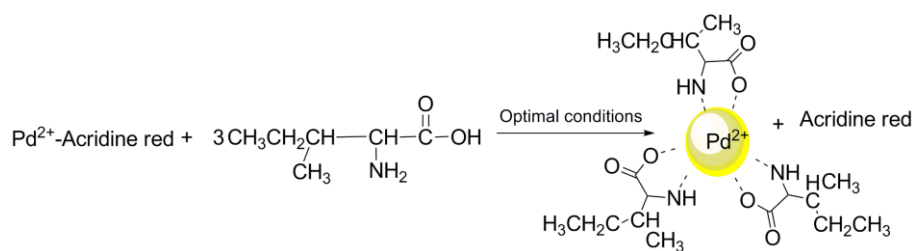
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1 Graphic abstract:



Study of the interaction between laevorotatory-isoleucine and a composite fluorescent probe of Pd (II)

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Abstract

In the Kolthoff buffer solution (pH=10.7), fluorescence intensity of Acridine red, which is quenched by Pd²⁺ can be enhanced when adding a certain amount of laevorotatory-isoleucine (L-isoleucine). Thereby, we establish a fluorescence spectrometry method for the detection of L-isoleucine. When the excitation slit width is 5nm and emission slit width is 10nm, we get the following results: the linear regression equation: $F = -33.884 + 468.07C$ (mg/L); correlation coefficient: $R = 0.9984$, linear range: 0.125~1.375mg/L; the detection limit: 0.00027 mg/L. Besides, we calculate the association constants of Pd²⁺-Acridine red ($k_1 = 1.62 \times 10^2$) and Pd²⁺-isoleucine ($k_2 = 7.04 \times 10^2$) by the method of spectrophotometric.

Key words: Analysis; Acridine red; Determination; Fluorescence; Fluorescent probe; Laevorotatory-isoleucine

1. Introduction

L-Isoleucine is one of the essential amino acids, which must be obtained from food, and it almost exists in all kinds of proteins. L-isoleucine is mainly used for the preparation of infusion and oral solution. Besides, it is involved in the metabolic regulation of thymus, spleen and pituitary gland, which is the General Command of thyroid and gonads. L-isoleucine is also used as a branched chain amino acid for the preparation of special type of amino acid infusion to provide treatment for liver and kidney. Those infusions and oral solutions that using L-isoleucine as the main material has significant effect on the treatment of various liver diseases such as liver Ann syrup and liver wake oral Liquid. If our bodies lack L-isoleucine, it will lead to a poor appetite, bad physique, anemia, and other problems. So, it is very important for our bodies and its consumption shows a sharp increasing, too.¹⁻³

It is found that our daily demand for L-isoleucine is about 0.7g. Excessive intake will cause antagonism with L-leucine. So, it requires maintaining the balance of the amino acids in our bodies. Therefore, it becomes very important for the quantity control of L-isoleucine in food and medicines. Until now, there exists limited methods for the determination of L-isoleucine, such as automatic amino acid analyzer, high performance liquid chromatography, gas chromatography or enzymatic. These methods have detected many kinds of amino acids precisely. Nevertheless, those methods haven't solve a few problems, such as complex pre-treatment of the sample, long determination time, the analytical instruments and the analysis cost are too expensive. In our experiment, we first studied methods of detecting L-isoleucine domestic and abroad^{4,11} and then established a new method for the determination of L-isoleucine by using a complex formed by Pd²⁺ and Acridine red as a fluorescent probe. By optimizing pre-processing method of samples and experimental conditions, a rapid, simple and accurate method for the determination of L-isoleucine is established.

2. Experimental

2.1. Reagents used in the experiment

1 PdCl₂ solution (1.0×10⁻³ mol/L), Acridine red solution (2.5×10⁻³ mol/L), Pd²⁺-
2 Acridine red solution (1.0×10⁻⁴ mol/L); cationic surfactant Cetyltrimethylammonium
3 Bromide (CTMAB, 1.0×10⁻² mol/L); Cetylpyridinium Bromide (CPB, 1.0×10⁻³ mol/L)
4 and an anionic surfactant Sodium Dodecyl Sulfate (SDS, 1.0×10⁻³ mol/L); Sodium
5 Lauryl Sulfate (SLS, 1.0×10⁻³ mol/L, Shanghai reagent Factory) aqueous solution
6 (1.0×10⁻³ mol/L); Non-ionic surfactant Tween-80 (1.0×10⁻² mol/L); OP solution (1 g/L,
7 Qingming Chemical Co., Ltd.); Na₃CO₃ solution (0.05 mol/L); Na₄BO₇ · 10H₂O
8 solution (0.05 mol/L); Series of Kolthoff buffer solution (pH=10.0~11.8,
9 Na₃CO₃-Na₄BO₇ · 10H₂O mixed solution); NaCl aqueous solution (0.1 mol/L);
10 Methanol, ethanol, acetone and acetonitrile (Beijing Chemical Works); 732[#] sodium
11 cation exchange resin (Tianjin Kermel Chemical Reagent Co., Ltd); NH₄Cl (0.5
12 mol/L)

13 Reagents used are of analytical grade. The water used in the experiment is
14 redistilled water.

15 16 2.2. Apparatus used in the experiment

17
18 FP-6500 fluorescence spectrometer (JASCO Corporation, Japan); TU-1900-double
19 beam UV-visible spectrophotometer (Beijing Analysis Apparatus Factory); PHS-3C
20 digital pH meter (Hangzhou Dongxing Instrument Equipment Factory); KQ-218
21 ultrasonic cleaner (Kun Shan Ultrasonic Instruments Co., Ltd.); Thermostatic bath
22 (Shanghai Heng Ping scientific Instrument Co.).

23 24 2.3. Analytical method

25
26 Add 0.7 mL Kolthoff buffer solution (pH=10.7), 0.9 mL CTMAB solution
27 (1.0×10⁻² mol/L), 0.6 mL Pd²⁺-Acridine red solution to a series of 10 mL volumetric
28 flasks; take one of the bottles as blank. Then, add different amounts of L-isoleucine
29 solution (25 mg/L) to the rest flasks, shake a few minutes to make them fully react and
30 leave them for 120 minutes. Then, irradiate them with exciting light whose
31 wavelength is 539 nm. Write down the fluorescence intensity (F) at 560 nm.
32 Meanwhile, write down the blank's fluorescence intensity (F₀) under the same
33 condition, and get the difference. (ΔF, ΔF=F-F₀).

34 35 2.4. Sample treatment¹²⁻¹⁵

36
37 Two bottles of Amino Acid Compound Injections (18AA) are bought from two
38 different hospitals in Xinxiang City. Take the original solution of 10.00 mL to 732[#]
39 cation exchange resin, and elute it with 0.5 mol/L ammonium chloride solution under
40 the flow rate of 3 drops/min. Collect 855-865 mL of eluent. Take eluent of 1.00 mL and
41 pour it into a 100 mL volumetric flask, and dilute with redistilled water to the mark
42 and for testing.

43 44 3. Results and discussion

45 46 3.1. Absorption spectrum

47
48 The absorption spectrum of Acridine red (curve 1), Pd²⁺-Acridine red (curve 2),
49 Pd²⁺-Acridine red-L-isoleucine (0.5 mg/L, curve 3) and Pd²⁺-Acridine
50 red-L-isoleucine (1.0 mg/L, curve 4) are presented in Fig. 1. As shown in this figure, in
51 the Kolthoff buffer solution (pH=10.7), the Acridine red solution appears red and its
52 maximum absorption peak is at 534 nm. After adding appropriate volume of Pd²⁺, the
53 solution appears slight purple and that the maximum absorption peak has a red shift to
54 539 nm. Moreover the intensity of it becomes weak sharply. Then after adding
55 L-isoleucine, the value of UV absorbance is enhanced. It indicates there are reactions
56 not only between Acridine red and Pd²⁺ but also between Pd²⁺-Acridine red and
57 L-isoleucine.

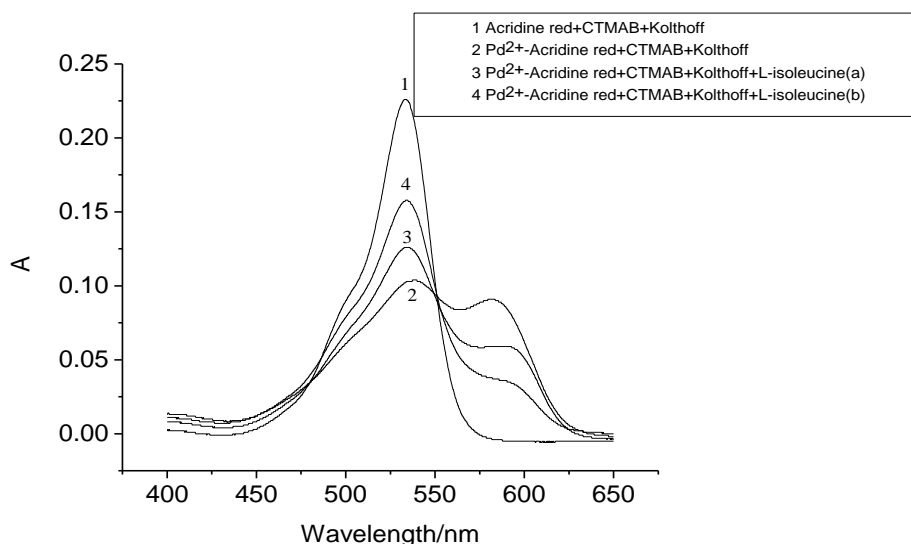


Fig.1 System of Absorb spectra diagram of Acridine red, Pd²⁺-Acridine red and Pd²⁺-Acridine red-L-isoleucine. λ_{\max} (Acridine red)/nm 535 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$), λ_{\max} (Pd²⁺-Acridine red)/nm 539 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$)

[Acridine red]: 6×10^{-6} mol/L. [Pd²⁺-Acridine red]: 6×10^{-6} mol/L. pH=10.7. [L-isoleucine (a), (b)]: 0.5 mg/L, 1 mg/L. [Kolthoff]: 0.7 mL. [CTMAB]: 9×10^{-4} mol/L

3.2. Fluorescence spectrum

As is shown in Fig. 2, in the Kolthoff buffer solution (pH=10.7), the maximum excitation wavelength (λ_{ex}) and the maximum emission wavelength (λ_{em}) of Pd²⁺-Acridine red is 539 nm and 560 nm (curve 1). When adding a certain amount of L-isoleucine, its fluorescence intensity increases with the increasing of the concentration of L-isoleucine (curve 2, 3), and the degree of the enhancement shows a good liner relationship along with the increasing of L-isoleucine in a certain range. So, the system can be used for the determination of L-isoleucine.

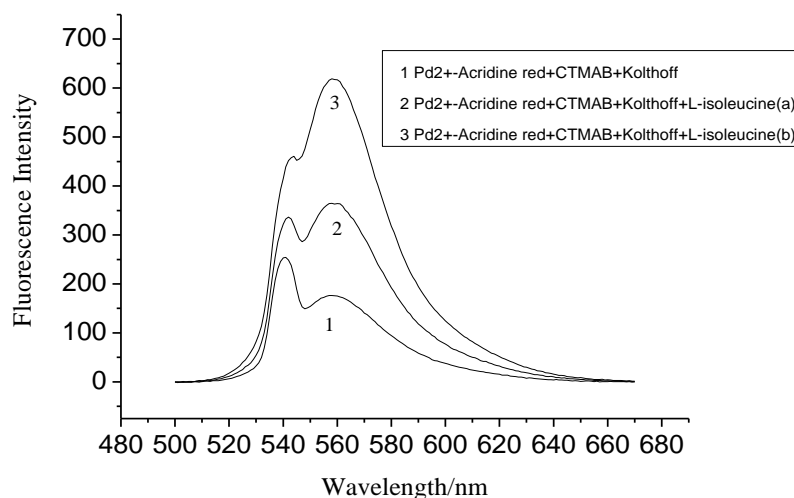


Fig.2 System of fluorescence spectra diagram of Pd²⁺-Acridine red and Pd²⁺-Acridine red-L-isoleucine.

[Pd²⁺-Acridine red]: 6×10^{-6} mol/L. pH=10.7. [L-isoleucine (a), (b)]: 0.5 mg/L, 1 mg/L.
[Kolthoff]: 0.7 mL. [CTMAB]: 9×10^{-4} mol/L

3.3. Optimization of the reaction system and the reaction conditions

(1) Optimization of the reaction system

Select different types of surfactants (cationic surfactant CTMAB and CPB, anionic surfactant SLS and SDS, nonionic surfactant OP and Tween-80), and then form the systems of Pd²⁺-Acridine red-CTMAB, Pd²⁺-Acridine red-CPB, Pd²⁺-Acridine red-SLS, Pd²⁺-Acridine red-SDS, Pd²⁺-Acridine red-OP and Pd²⁺-Acridine red-Tween-80 for comparative. The results show that CTMAB has a sensitizing effect on Pd²⁺-Acridine red system. Therefore, we select Pd²⁺-Acridine red-CTMAB system in this study.

(2) Optimization of buffer solution and pH

Pd²⁺-Acridine red shows strong fluorescence in alkaline condition but dissociates in acidic condition. Therefore, we choose a series of buffer solution (pH=10.7) for testing (Michaelis, B-R, Kolthoff, Sørensen). The results indicate that ΔF has its maximum in the Kolthoff buffer solution and the sensitivity of the system is the highest too. So, we choose the Kolthoff solution in this experiment. Then, a series of Kolthoff buffer solutions with the pH ranging from 10.0 to 11.8 are selected for testing. The results in SI.1 show that the response value increases significantly with the increasing of pH, but when the pH is higher than 10.6, value of ΔF decreases slightly first and then it has a sharp decrease (The reason is probably that the probe dissociates when pH is beyond 11.2 or lower than 10.6). Considering the sensitivity and stability, we select pH=10.7 in this study.

(3) Optimization of the concentration of fluorescent probe

In the selected buffer solution and pH, with the increasing of the fluorescent probe (SI.2), value of ΔF increases dramatically at first but decreases rapidly when the concentration of it is higher than 6×10^{-6} mol/L (we speculate the reason for this phenomenon may be when the concentration of fluorescent probe is higher than 6×10^{-6} mol/L, fluorescent probe itself can form dimer or polymersomes, thus, it makes it difficult to dissociate). So, we choose 6×10^{-6} mol/L of fluorescent probe in our study.

(4) Optimization of the concentration of CTMAB

Results in SI.3 show that with the increasing of CTMAB, the response value increases at first and then becomes stable. But the response value decreases dramatically when its concentration is a little lower than 8×10^{-4} mol/L, so we choose 9×10^{-4} mol/L as the optimal concentration of CTMAB in this test. The reason is probably that it has achieved its critical micelle concentration when its concentration is 8×10^{-4} mol/L, so the response value has little change when its concentration keeps increasing.

(5) Optimization of the dosage of the buffer solution

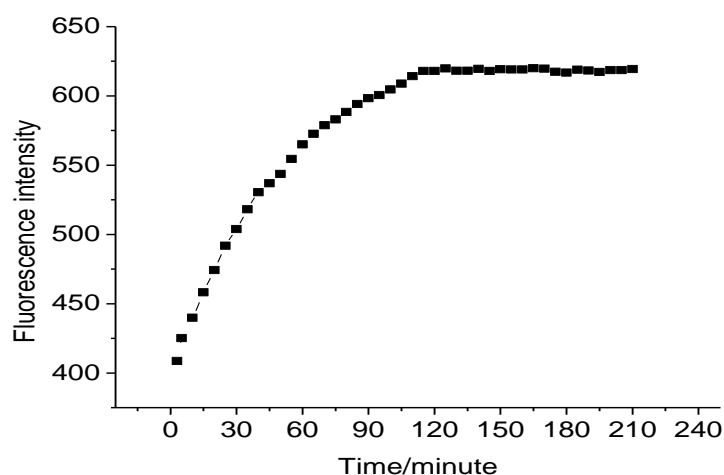
As is shown in SI.4, with the increasing of the dosage of buffer solution, the response value increases sharply at first and then stays stable. ΔF reaches its maximum when the dosage of the buffer solution is 0.6 mL, but, when the dosage is lower than 0.6 mL, value of ΔF reduces suddenly. So, we choose 0.7 mL as the optimal concentration of the buffer solution.

(6) Optimization of the order of addition of the reagents

In accordance with the principles of permutations and combinations, we investigate how the 24 different orders of addition of the four different reagents mentioned above influence the fluorescence intensity. It shows that ΔF reaches its maximum and the system stays stable when the reagents are added in the following order: Kolthoff \rightarrow CTMAB \rightarrow Pd²⁺-Acridine red \rightarrow L-isoleucine. So, we choose the order of addition of the reagents is as follows in this study: Kolthoff \rightarrow CTMAB \rightarrow Pd²⁺-Acridine red \rightarrow L-isoleucine.

(7) Optimization of reaction time

In the optimized conditions, fluorescence intensity of the system increases gradually as time goes by (Fig.3), and the system becomes stable after 115 minutes and the response value has little change within 2.5 hours. Thus, the fluorescence intensity is measured after 120 minutes in this study; the specific reason will be explained in mechanism. Then, we investigate how the temperature influences the system, and it shows that ΔF decreases gradually along with the increasing of temperature (the reason may be: with the increasing of temperature, Pd²⁺-Acridine red becomes more and more unstable, and then begins to dissociate, which makes the response value decreases). Therefore, this experiment is done at room temperature. We can also do the experiment at low temperature if necessary.



$$r = \frac{\Delta c}{\Delta t} = 6.6 \times 10^{-10} \text{ mol L}^{-1} \cdot \text{min}^{-1} = 8.7 \times 10^{-5} \text{ mg} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$$

Fig.3 Influence of reaction time on the system and the average reaction rate

[Pd²⁺-Acridine red]: 6×10^{-6} mol/L. pH=10.7. [CTMAB]: 9×10^{-4} mol/L. [Kolthoff]: 0.7mL. [L-isoleucine]: 1.0mg/L

(8) Influence of ionic intensity

We studied the effect of ionic strength on the system by adding different amounts of NaCl (0.1mol/L), and the results are shown in SI.5. It has a sensitizing effect on system when the content of NaCl is within 8~14%, but the sensitizing effect is limited. Therefore, we choose not to add the NaCl in this experiment, and not to change the ion strength of the system. But we may consider adding a certain amount of NaCl to change the ionic strength if necessary.

(9) Influence of organic solvents on system

We investigate the effect of common organic solvents on the system by adding methanol (SI.6a), ethanol (SI.6b), acetone (SI.6c), acetonitrile (SI.6d) to the system, as is shown in SI.6, with the addition of the above organic reagents, the response values decrease sharply, so we choose not to add organic solvents to this system. The reason may be that the organic solvents can make the probe dissociate, which cause the response value deceases.

3.4. Working curve

In the optimal experimental conditions, we get the working curve (Fig.4) according to the method 2.3. When the excitation slit width is 5nm and emission slit width is 10nm, we get the following results: linear regression equation: $F = -34.39 + 468.1C$ (mg/L); correlation coefficient: $R = 0.9986$; linear range: 0.125~1.375mg/L; detection limit: 0.00027mg/L. We measure 11 times on the content of 0.75mg/L of standard solution by our method (Fig.5); the average value is 0.756mg/L; the relative standard deviation is 4.2%.

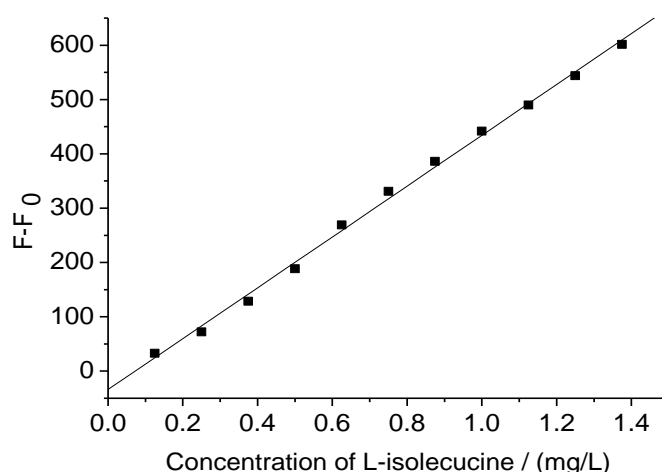


Fig.4 Working curve

[Pd²⁺-Acridine red]: $6 \times 10^{-6} \text{ mol L}^{-1}$. pH=10.7. [CTMAB]: $9 \times 10^{-4} \text{ mol/L}$. [Kolthoff]: 0.7mL. [L-isoleucine]: 0.125, 0.250, 0.375, 0.500, 0.625, 0.750, 0.875, 1.000, 1.125, 1.25, 1.375mg/L

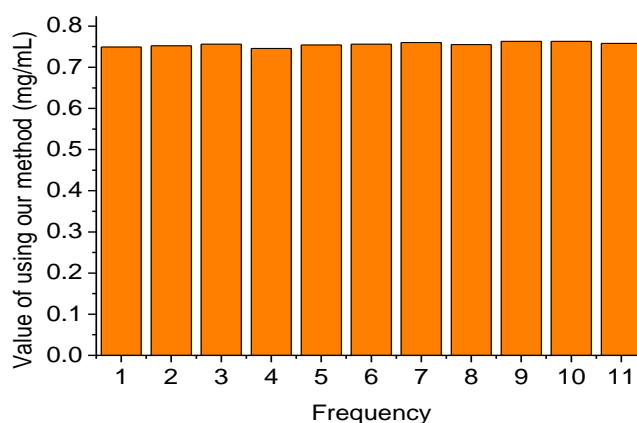


Fig.5 Determination of precision

[Pd²⁺-Acridine red]: 6×10^{-6} mol/L. pH=10.7. [CTMAB]: 9×10^{-4} mol/L. [Kolthoff]: 0.7mL. [L- isoleucine]: 0.750mg/L

3.5. Influence of coexistences

Under the optimal experimental conditions, we examine the effect of the some common coexisting ions and compounds in the determination of L-isoleucine in 18AA. When the relative error is within $\pm 5\%$ and concentration of L-isoleucine is 1.0mg/L, the results in Table 1 show that most of the coexistences in 18AA are more than the maximum allowable. So, the samples are passed through a cation exchange column before measuring.

Table 1 Influence of coexistences

Coexisting substance	Concentration (mg/L)	Relative error (%)	Coexisting substance	Concentration (mg/L)	Relative error (%)
Cl ⁻	70	+4.2	NH ₄ ⁺	0.5	+5.6
Na ⁺	4.6	-5.1	starch	200	-4.1
Ca ²⁺	40	+4.5	lactose	800	+4.8
Mg ²⁺	0.8	-4.1	L- proline	0.0035	-4.8
SO ₄ ²⁻	45	+4.8	L- glucose	110	+4.9
Fe ³⁺	12	-5.5	L- maltose	70	+4.2
Cu ²⁺	45	+4.8	L- valine	0.1	-4.1
D-L- cystine	0.2	+4.8	L- glutamate	0.48	+4.9
L- leucine	0.08	+5.1	D-L- methionine	0.005	+4.6
sucrose	65	-4.6	L- lysine	0.38	+4.5
L-phenylalanine	0.2	-5.3	sorbitol	500	+5.8
Sodium bisulfite	0.18	+4.1	L-tyrosine	0.66	+4.7
L-arginine	0.5	+4.3	L- glycine	0.6	+4.9
L- aspartic acid	0.2	+4.4			

3.6. Ratio of Pd²⁺ and Acridine red and the association constant of Pd²⁺-Acridine red

Reaction between Acridine red and Pd²⁺ form a complex finally. We calculated the ratio of Pd²⁺ and Acridine red and the association constant of Pd²⁺-Acridine red by the method of Spectrophotometric (SI.7a), we find the molar ratio of the two is 1:1 and

the association constant of Pd^{2+} -Acridine: $k_1=1.62 \times 10^2$.

3.7. Ratio of Pd^{2+} and L-isoleucine and the association constant of Pd^{2+} -L-isoleucine

Reaction between L-isoleucine and Pd^{2+} form a complex finally. We calculated the ratio of Pd^{2+} and L-isoleucine and the association constant of Pd^{2+} -L-isoleucine by the method of Spectrophotometric (SI.7b), we find the molar ratio of Pd^{2+} and L-isoleucine is 1:3 and the association constant of Pd^{2+} -L-isoleucine: $k_2=7.04 \times 10^2$.

3.8. Study of mechanism

What we can find from 2.1 is that Pd^{2+} can make the fluorescence intensity of Acridine red quench. When adding a certain amount of L-isoleucine, what we can find from 2.5 and 2.6 is that $k_2 > k_1$, which means that L-isoleucine and Pd^{2+} can form a more stable complex than Acridine red and Pd^{2+} , and Acridine red is dissociated from Pd^{2+} -Acridine red, then the fluorescence intensity of the system is enhanced, and the rate of the enhancement shows a good linear relationship along with the increasing of L-isoleucine within a certain range (the schematic diagram is shown in Fig.6). Moreover, the changing of the color of the system can also support this mechanism (Acridine red itself is red, the color becomes purple after adding Pd^{2+} , and then it becomes the original red when adding a certain amount of L-isoleucine (Fig.7)).

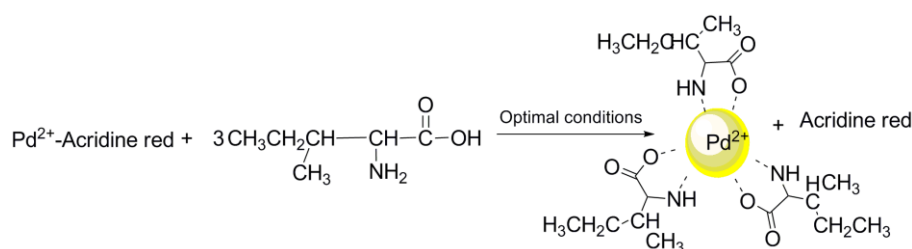


Fig.6 Schematic diagram



Fig.7 Different colour when concentration of L-isoleucine is different

[Pd^{2+} -Acridine red]: $6 \times 10^{-6} \text{ mol L}^{-1}$. pH=10.7. [CTMAB]: $9 \times 10^{-4} \text{ mol/L}$. [Kolthoff]: 0.7mL. [L-isoleucine]: 0, 0.250, 0.500, 1.00mg/L (From left to right)

In addition, we can find that the system becomes stable after 115 minutes. So, we can calculate the average reaction rate from the dates (Fig.3). What can be seen from the average reaction rate is that the average reaction rate is very slow, which also explains why the system becomes stable after 115 minutes.

4. Application

4.1. Determination of samples

We test the blank and samples according to method 2.3 in the optimal experimental conditions, and each sample are tested for 4 times. Besides, the results are compared with the national standard. The detail dates are shown in Table 2.

Table 2 Results of sample determination using our method and Standard content

	Sample 1	Sample 2
Measured values using our method (mg/mL)	36.325、 35.637、 35.913、 35.277	35.115、 34.853、 35.969、 35.789
Average value using our method (mg/mL)	35.788	35.432
National Standard (mg/mL)	35.2	35.2
RSD%	2.3	1.7

The results in Table 2 show that there exists no significant difference between our method and National Standard, and the relative standard deviation is allowable. Therefore, we have established a reliable method for the determination of L-isoleucine.

4.2. Results of recovery of standard addition

Take 0.3mL of samples for testing and then add 0.1, 0.15, 0.20mL standard solution of L-isoleucine (25mg/L); each sample is measured 6 times, and the recovery results are shown in Table 3.

Table 3 Results of recovery

Amount of added standard solution (mg/L)	Average concentration of recovery (mg/L)	Average recovery rate (% , n=6)	RSD (% , n=6)
0.25	0.267	106.8%	2.08%
0.375	0.396	105.6%	1.85%
0.5	0.572	114.4%	3.83%

Results in Table 3 show that the recovery rate is 105.6%~114.4%, and the relative standard deviation is 1.85%~3.83%, which also shows that our method is reliable.

5. Conclusion

1 This study establishes a new method for the determination of L-isoleucine by using
2 a new fluorescent probe and the mechanism is reasonable too. The method has the
3 following advantages: high sensitivity, using a new fluorescence probe, low detection
4 limit, rapid detection, less amount of sample, simple operation and so on. Moreover,
5 we determine the actual samples successfully, and the results are compared with the
6 national standard, showing that our method is reliable and reproducible for the
7 determination of L-isoleucine in 18AA. This method can also be used for the quantity
8 control of L-isoleucine in food, medicine, cosmetics and so on. Thus, we provide a
9 new and reliable method for the detection of L-isoleucine. It can also
10 provide some references for the later research of amino acids.

11 12 **References**

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