Study on Fluorescence Spectra of Thiamine and Riboflavin

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Abstract: This paper presents the intrinsic fluorescence characteristics of vitamin B1 and vitamin B2 with 3D fluorescence Spectrophotometer. Three strong fluorescence areas of vitamin B2 locate at λex/λem=270/525nm, 370/525nm and 450/525nm and one fluorescence areas of vitamin B1 locates at λex/λem=370/460nm were found. The influence of pH of solution are also discussed, and with the PARAFAC algorithm, 9 vitamin B2 and vitamin B1 mixed solutions are successfully decomposed, and the emission profiles, excitation profiles, central wavelengths and the concentration of the two components were retrieved precisely through about 10 iteration times.

Introduction

The fluorescence of a folded protein or bio-aerosol is a mixture of the fluorescence from individual aromatic component and coenzyme. Riboflavin, known as vitamin B2, is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. As such, vitamin B2 is required for a wide variety of cellular processes. Vitamin B2 plays a key role in energy metabolism, and is required for the metabolism of fats, ketone bodies, carbohydrates, and proteins [1]. Thiamine, known as vitamin B1, is one of 8 B vitamins, is used in many different body functions and deficiencies may have far reaching effects on the body, yet very little of this vitamin is stored in the body, and depletion of this vitamin can happen within 14 days. B vitamins are water-soluble and of great importance for the health of people, they can not be synthesized within the body, but be obtained from daily food, so the content measurement of B vitamins in the food is necessary for food security and research.

PARAFAC, a three way-decomposition method, has been found to be very useful in identifying the independent spectra of different types of fluorophores [2]. Compared to its predecessor, Principal Component Analysis (PCA) technique, PARAFAC provides both a quantitative and qualitative model of the data and separates the complex signal measured into its individual underlying fluorescent phenomena with specific excitation and emission spectra. It can track even small variations in EEM datasets by separating several independent groups of fluorophores from the overlapped components with a high resolution, so it is commonly used technique to monitor the mixed fluorescence EEMs. On the other hand, the weakness of PARAFAC model may include the assumption of the independence among the estimated components in the model, and potential inclusion of one or more poorly estimated components, which may significantly affect the spectra and scores of all other components [3].

Some previous studies reported that PARAFAC was applied to resolve the mixed and

overlapped spectra of vitamin B1, B2 and B6 [4], accordingly, in this paper, discussed not only the fluorescence spectra of riboflavin and thiamine individually, but also the overlap of fluorescence spectra of vitamin B1 and vitamin B2 at the Ex/Em area with wavelength of 300~550/370~610nm, by means of Parallel factor analysis (PARAFAC) method.

Experimental Section

instruments and reagents

The Molecular ΣH2O ultra pure water machine (Shanghai Molecular Co. Ltd) was used to generate the ultra purified water, UPW whose pH value is 5.4. The vitamin B2 mother liquid were compounded with Riboflavin from Amresco co.Ltd whose purity greater than or equal to 98%, and the vitamin B1 from Sigma co.Ltd with purity greater than or equal to 99%. All reagents and materials were weighed with Mettler Toledo precise electronic balance, and dissolved with Briton Robson Buffer with different pH values (1.95, 5.4, 5.66, 8.0 and 11.92).

The solvents used in the experiments include 0.2mol/L Na_2HPO_4 buffer, 0.1mol/L $C_6H_8O_7{\cdot}H_2O$ buffer, Na_2HPO_4 and $C_6H_8O_7{\cdot}H_2O$ mixed buffer, 1% $K_3[Fe(CN)_6]$ buffer, 2mol/L NaOH buffer and 3mol/L H_2SO_4 buffer. 0.2mol/L Na_2HPO_4 buffer was compounded by putting 28.4g sodium phosphte dibasic anhydrous (Na_2HPO_4, Tianjin guangfu chemical research institute, China) into 1000mL UPW. 0.1mol/L $C_6H_8O_7{\cdot}H_2O$ buffer was compounded by put 21.01g citric acid monohydrate($C_6H_8O_7{\cdot}H_2O$, Sinopharm Chemical Reagent Co.Ltd) into 1000mL UPW. Na_2HPO_4 and $C_6H_8O_7{\cdot}H_2O$ mixed buffer was compounded by mixing 0.2mol/L Na_2HPO_4 buffer and $C_6H_8O_7{\cdot}H_2O$ buffer at the ration of 6:1; 1% $K_3[Fe(CN)_6]$ buffer was compounded by put 1g Potassium hexacyanoferrate buffer(Tianjin guangfu chemical research institute) into 100mL UPW, and was diluted by mixing with 3mL 2mol/L NaOH buffer to 10mL.

The vitamin B2 and B1 mother liquid concentration are 10mg/L respectively. The vitamin B2 and vitamin B1 mother reagent solutions were transferred through DragonLab whole disinfection manual single channel adjustable liquid shifter and dilute to working solutions of different concentrations. All reagents were of analytical grade, all solutions and put in amber glass bottles and stored in a refrigerator (4˚C) because of the light sensitivity of vitamin B2.

3D fluorescence intensity measurements were carried out on an F-7000 FL spectrophotometer (Hitachi High-Technologies Corporation, Japan).

instrument settings and experiment procedure

500ul Briton Robson Buffers with different pH values and mother liquids of different volumes were injected into the 10ml test tubes, and diluted with purified water to form the working liquids and background liquids.

For the fluorescence EEM measurements of vitamin B2, the spectrophotometer excitation wavelength ranged from 200.0nm, to 550.0nm, emission wavelength ranged from 450.0nm to 650.0nm, scan speed was set at 12000nm/min with excitation and emission sampling interval of 10.0 nm, excitation and emission slit of 5.0nm, the PMT voltage was set at 700 V. Accordingly, for fluorescence EEM of thiamine, the excitation wavelength ranged from 300.0nm, to 400.0nm, emission wavelength ranged from 350.0nm to 550.0nm. All experiments were performed at room temperature at 25˚C.

The 1st level and 2nd level Rayleigh scattering, Raman scattering and other background components within the fluorescence signals were corrected for the following analysis.

Multi-components discrimination using PARAFAC method

Based on the tri-linear decomposition theory, the parallel factor analysis(PARAFAC) method is a kind of mathematical model implemented through alternating least squares algorithm, which is widely applied to analyze three-dimensional or multi-dimensional data, to decompose *N*-dimensional data to the *N* load matrixes.

The measured fluorescence spectrum EEM data is a $I \times J \times K$ matrix, in which, *I* indicates the number of the samples, while *J* and *K* are the number of excitation wavelengths and emission wavelengths of samples respectively. Using Parallel Factor decomposition model, the fluorescence spectrum data matrix can be decomposed to score matrix *A*, load matrix *B* and *C*. The decomposition model can be represented as

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad , \quad i=1, 2, \ldots, I, \quad j=1, 2, \ldots, J, k=1, 2, \ldots, K \qquad (1)$$

where, x_{ijk} is the fluorescence intensity of sample *i* at excitation wavelength *j* and emission wavelength *k*, *F* is the column number of load matrix, or the number of factors, e_{ijk} is the residual element, a_{if} , b_{jf} , c_{kf} are the elements in load matrix *A*, *B* and *C* respectively. The algorithm will be aborted until convergence of the PARAFAC model, that is, the minimum loss function $f_{SSR} = \sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K} e^2_{ijk} < 10^{-6}$.

In this study, PARAFAC modeling was performed using the MATLAB 7.0 code. The appropriate number of components was determined primarily based on the three diagnostic tools including residual analysis, core consistency and visual inspection of spectral shapes of each component, which are widely used by other similar studies. The components extracted by PARAFAC represent groups of the organic components that exhibit similar fluorescence properties. The component scores indicate the relative concentration of the groups, not the actual concentration of a particular material/fluorophore. However, it is typically assumed that the scores are proportional to the concentrations of the different components [5, 6].

Fluorescence EEM Characteristics of Thiamine and Riboflavin

Intrinsic fluorescence EEM characteristics of riboflavin and thiamine

For riboflavin/vitamin B2, there are three strong fluorescence areas, whose center locate at λex/λem=270/525nm, 370/525nm and 450/525nm respectively, and the emission wavelength ranges from about 500nm to 600nm, as shown in Figure 1. The fluorescence intensity excited by 270nm excitation wavelength is much stronger than that by 370nm and 450nm, the ratio of fluorescence intensity is 1:0.41:0.25 approximately.

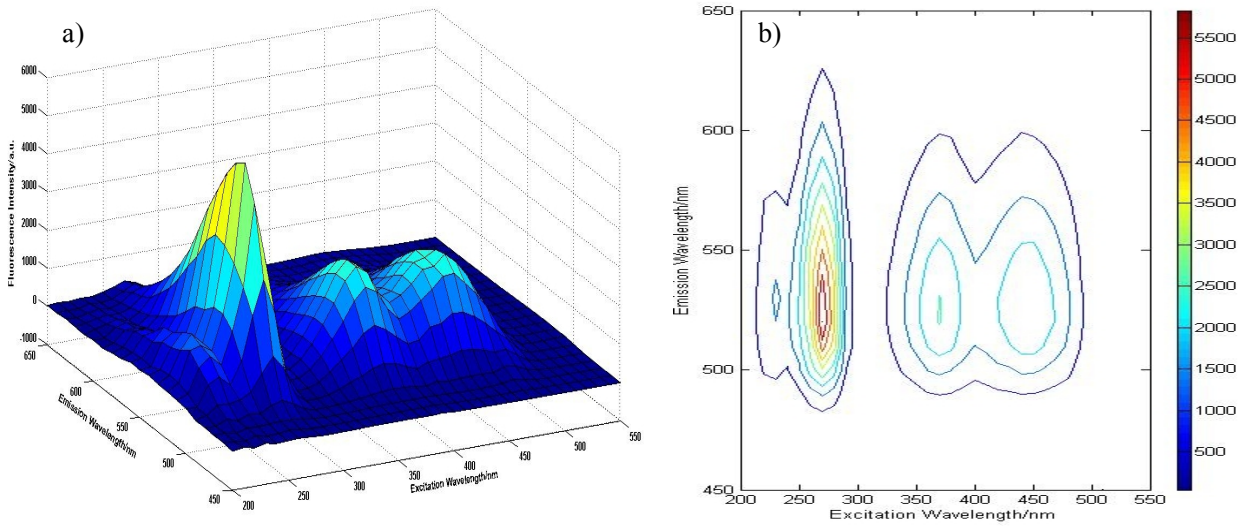

Figure1. Fluorescence intensity distribution of riboflavin@200ug/L

Accordingly, the Thiamine/vitamin b1 has only one strong fluorescence areas, whose center locate at λex/λem=370/460nm, the excitation wavelength ranges from about 320nm to 400nm, and the emission wavelength ranges from about 370nm to 550nm, as shown in Figure 2.

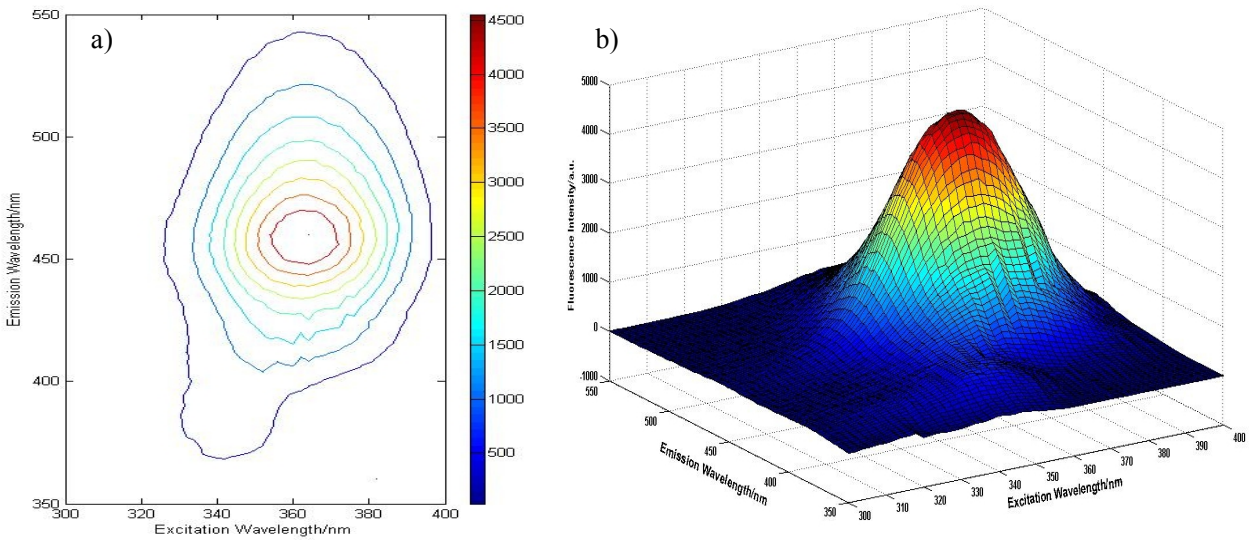

Figure2. Fluorescence intensity distribution of thiamine@75ug/L

The fluorescence efficiency of riboflavin is about two times stronger than that of thiamine for the same solvent.

Affection of pH to the fluorescence emission intensity distribution of riboflavin

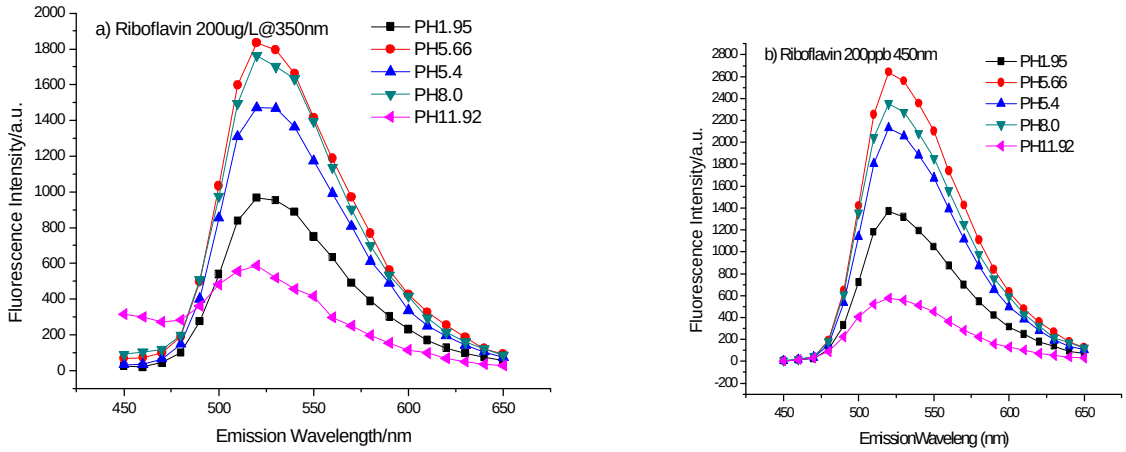

Figure3. Fluorescence intensity distribution of riboflavin@350nm and 450nm affected by pH value of the resolvent.

When resolved in strong alkaline and strong acid solution, the structure of vb2 molecular is distorted, so the fluorescence intensity declines sharply compared to the weak alkaline and weak acid solution, and if resolved in weak alkaline and weak acid solution, the fluorescence intensity are much stronger(figure3).

Analysis of Fluorescence Spectra of Riboflavin and Thiamine Mixed Solutions

Instrument settings

Firstly thiamine and riboflavin mother liquids of different concentrations were put into 10mL numbered test tubes, and then 1mL Na_2HPO_4 and $C_6H_8O_7{\cdot}H_2O$ mixed buffer and 0.22ml $K_3[Fe(CN)_6]$ buffer were added into the test tubes. The pHs of mixed solutions were then adjusted to neutral with added 3mol/L H2SO4 and UPW. Lastly, the mixed solutions were laid stationary for 10 minutes for the following measurements.

For the fluorescence EEM Matrixes decompose of the mixed solution of riboflavin and thiamine, the spectrophotometer excitation wavelength ranged from 300.0nm, to 550.0nm, emission wavelength ranged from 390.0nm to 610.0nm, scan speed was set at 12000nm/min with excitation and emission sampling interval of 5.0 nm, excitation and emission slit of 10.0nm, the PMT voltage was set at 700 V.

3D fluorescence spectrogram of thiamine and riboflavin mixed solutions

Fluorescence EEM intensity of thiamine and riboflavin mixed solutions of different concentrations (listed in table1) are shown in Figure4 (a) ~ (f). The two separated fluorescence EEM peaks at 370nm/460nm and 470nm/520nm are obviously when the thiamine's concentration is low at 10ug/mL, and with the concentration rising of thiamine from 10ug/mL to 25ug/mL and 50ug/mL, the two peaks merged together.

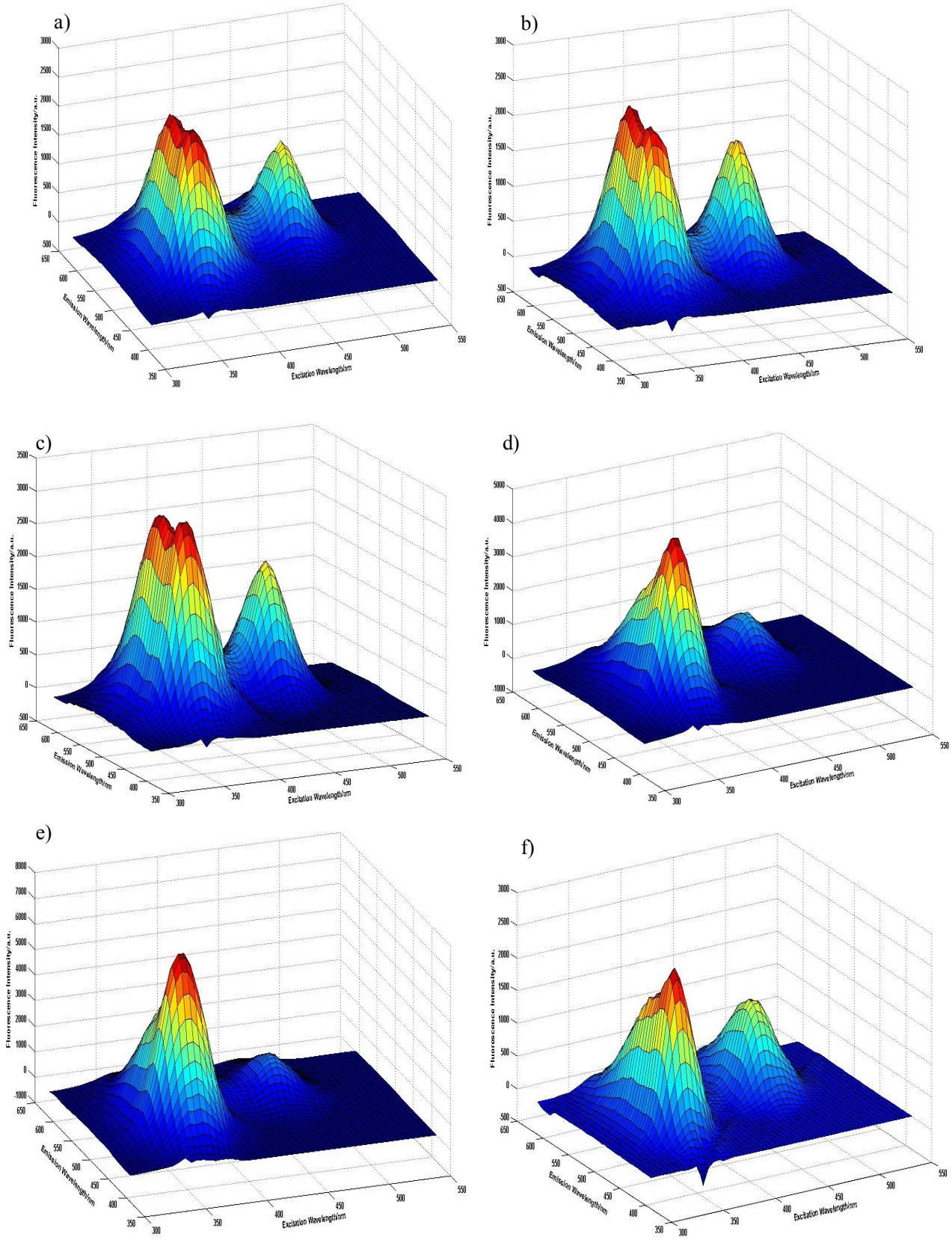

Figure4. Fluorescence EEM intensity distribution of riboflavin and thiamine mixed solutions of different concentrations.

Results retrieved by PARAFAC algorithm

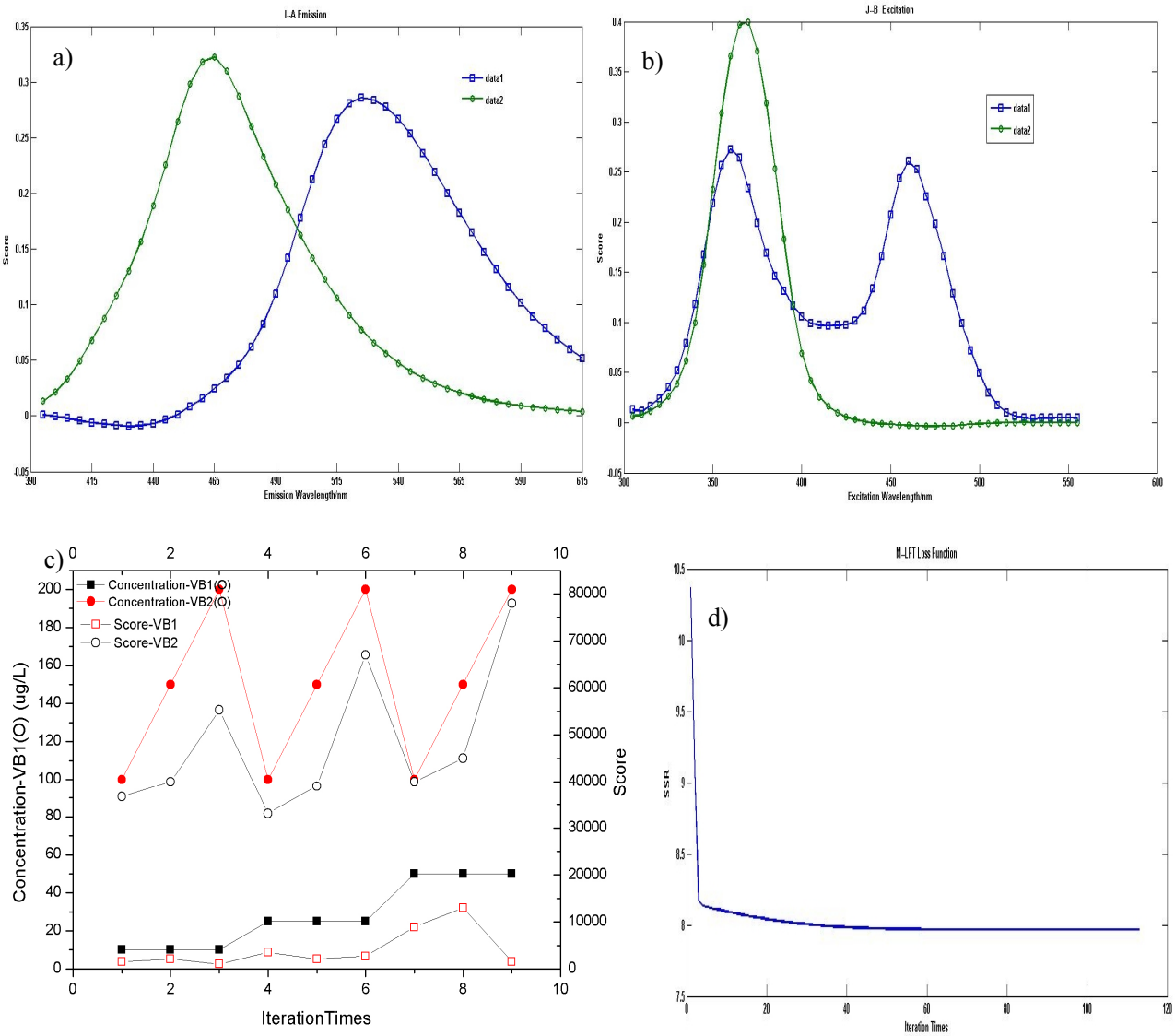

Figure5. Excitation and emission spectral profile retrieved by PARAFAC algorithm (a)Emission spectra, (b) Excitation spectra and (c) concentrations of original solutions and retrieved (d) SSR and iteration number

The emission spectra retrieved by PARAFAC are shown in Figure 5(a), the blue curve with block symbol (data1) indicates the retrieved emission profile of riboflavin and the green curve with circle symbol (sample2) indicates the retrieved emission profile of thiamine. From figure 5(a) and figure 1, figure 2, one can see that the retrieved emission profile and central wavelength of riboflavin and thiamine are coincident with their real emission profiles very well.

The excitation spectra retrieved by PARAFAC are show in Figure 5(b), the blue curve with block symbol (sample1) indicates the excitation profile of riboflavin and the green curve with circle symbol (sample2) indicates the excitation profile of thiamine. From figure 5(b) and figure 1, it can be seen that the retrieved excitation profile and central wavelength of riboflavin are coincident with its real excitation profile, the two strong excitation wavelength at 370nm and 450nm are obvious. And for the retrieved excitation profile and central wavelength of thiamine, the single excitation wavelength peak is also clear and coincident with the real excitation wavelength profile.

The good linear correlations of original and retrieved concentrations of thiamine and riboflavin can be observed in Figure 5(c). From Figure 5(d) one can see that SSR of PARAFAC logarithms

decreases quickly and sharply at the beginning of the iteration times, the SSR is stable when iteration times≥5.

Table 1. Analytical concentrations of riboflavin and thiamine

Sample	Component			
	Thiamine		Riboflavin	
	Original (ug/L)	Retrieved score	Original(ug/L)	Retrieved score
a)	10	1500	100	36701
b)	10	2100	150	40010
c)	10	1000	200	55310
d)	25	3500	100	33090
e)	25	2100	150	39010
f)	25	2700	200	67001
g)	50	8900	100	40010
h)	50	13000	150	45010
i)	50	1520	200	78001

Conclusions

The fluorescence of a protein or bio-aerosol or bio-agent is a mixture of the fluorescence from individual aromatic residues and coenzyme. Using fluorescence Spectrophotometer, the intrinsic fluorescent characteristics of vitamin B1 and vitamin B2 are measured with solutions of different pH and discussed. Vitamin B2 and vitamin B1 mixed solutions are successfully decomposed and resolved by PARAFAC algorithm. The retrieved emission profiles, excitation profiles, central wavelengths and the concentration of the two components are coincident precisely with real emission profiles, excitation profiles, central wavelength of each component.

Acknowledgments

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