Synchronous Fluorescence as a Rapid Method for the Simultaneous Determination of Folic Acid and Riboflavin in Nutritional Beverages

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ABSTRACT: A rapid synchronous spectrofluorimetric method was first developed for the simultaneous determination of folic acid and riboflavin in nutrimental beverages. Folic acid could be detected by using H_2O_2 plus Cu(II) as oxidation system to produce pterine-6-carboxylic acid, which had strong fluorescence in aqueous solution, and riboflavin itself was obviously fluorescent. Various operational parameters were thoroughly discussed in terms of their effects on the fluorescence signals, including instrumental parameters, concentration of the oxidation system, and pH. Under optimum conditions, the calibration curves were linear in the ranges of 100−250 μg/L for folic acid and 1−250 μg/L for riboflavin, and the detection limits were 2.0 and 0.014 μg/L, respectively. In addition, this method was applied to the determination of folic acid and riboflavin in nutrimental beverages with satisfactory results.

KEYWORDS: synchronous fluorescence, folic acid, riboflavin, nutrimental beverage

INTRODUCTION

Folic acid and riboflavin are essential to the development and normal growth of human bodies, life, and good health by regulating metabolism and circadian enginery (Figure 1). Folic acid is crucial for nucleotide synthesis, cell division, and gene expression. At the same time, folic acid is critically important for normal fetal development during pregnancy, and fetal neural tube defects can be caused due to its deficiency in the first 3 months of pregnancy.[1] As a part of flavin coenzyme, riboflavin is important to our metabolic function, which can be converted to flavin mononucleotide and flavin adenine dinucleotide. Riboflavin can not be synthesized in the human body. Long-term dietary deprivation may cause a variety of clinical abnormalitiesd including degeneration of the nervous system. This will not only impair energy metabolism but also harm our health and impact our life.[2,3] Multivitamins always exist simultaneously in vegetables, foods, pharmaceutical dosages, and drinks, etc. As important water-soluble vitamins, folic acid and riboflavin always coexist in nutrimental beverages. Hence, simultaneous determination of folic acid and riboflavin in nutritional beverages will be of realistic significance.

High-performance liquid chromatography (HPLC),[4−6] capillary electrophoresis,[7,8] and fluorescence spectrometry[9,10] were used to detect folic acid and riboflavin. However, the analytical procedures of the above methods had the following disadvantages: the organic solvent is toxid; the procedures are time-consuming; and a preseparation step to remove matrix interferences is required. For this aim a simple and accurate method that allowed the determination of both species at different concentrations was in great demand. Synchronous fluorescence spectrometry was first introduced by Lloyd,[11] who achieved measurement at a constant-wavelength difference ($\Delta\lambda$) between the emission and excitation in the whole measurement process. There are several advantages for the synchronous spectra, such as spectral simplification, spectral bandwidth reduction, and the avoidance of Rayleigh scattering effects.[12,13] Compared with conventional fluorescence
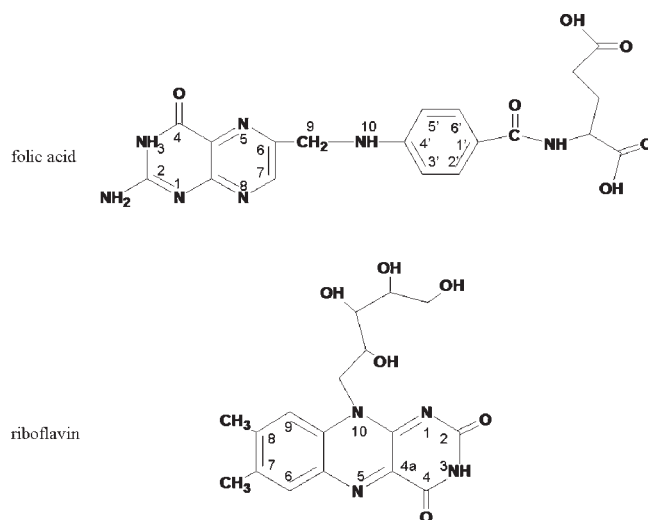

Figure 1. Chemical structures of folic acid (a) and riboflavin (b) with numbered atoms.

spectrometry, synchronous fluorescence was more appropriate for the analysis of multicomponent samples.

The synchronous fluorescence had been mostly applied to detecting multicomponent polycyclic aromatic hydrocarbons.[14,15] It was also applied to pharmaceutical dosages,[16,17] protein, and amino acid.[18−20] Furthermore, relative efforts had been explored for the determination of water-soluble vitamins. For instance, synchronous fluorescence was used to analyze the characteristics of thiamin, riboflavin, and pyridoxine, and the method showed the influence of micellar bis(2-ethylhexysulfosuccinate) sodium

salt on the fluorescent intensity of the three vitamins.[21] Thiamin, riboflavin, and pyridoxine were studied for the first time adopting a fiber-optic sensor system, and it was successful in analyzing their contents in complex vitamin B tablets.[22] The fluorescence emission spectra, synchronous fluorescence spectra, and derivative synchronous fluorescence spectra of thiamin, riboflavin, and pyridoxine were studied, and the derivative synchronous fluorescence was used to detect the vitamins in real samples.[23]

In this work, a synchronous fluorescence method was adopted as a powerful technique for the simultaneous determination of folic acid and riboflavin without any separation. This method was also selective and had been applied to the simultaneous determination of folic acid and riboflavin in nutrimental beverage samples with satisfactory results.

■ EXPERIMENTAL PROCEDURES

Chemicals. A 25 mg/L standard stock solution of folic acid was prepared by dissolving 12.5 mg of folic acid in water, and a small quantity of 0.3 mol/L NaOH solution was added to promote dissolution in a 0.5 L volumetric flask, whereas a 25 mg/L standard stock solution of riboflavin was prepared by dissolving 12.5 mg of riboflavin with 0.02 mol/L acetic acid solution in a 0.5 L volumetric flask. The vitamin standard stock solutions were stored at 4 °C in the dark. Working standard solutions were obtained by stepwise dilution with deionized water. The buffer solutions were prepared by mixing citric acid (0.1 mol/L) and disodium hydrogen phosphate (0.2 mol/L) in different ratios and adjusting the mixed solution to pH 2.2−6.0 with citric acid (0.1 mol/L). A 1 mol/L solution of H_2O_2 was obtained daily by dissolving 30% H_2O_2 (v/v). A 0.1 mol/L solution of Cu(II) was prepared by dissolving $CuCl_2 \cdot 2H_2O$ with water. All reagents were of analytical reagent grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Double-deionized water from a Milli-Q system was used throughout the experiments. All containers were treated with 10% HNO_3 for at least 24 h, rinsed with deionized water, and dried at room temperature before usage.

All samples were measured immediately after the bottle had been opened followed by degassing by ultrasonic device for enough time to drive the air away. A fresh portion of each beverage was measured every time, and all of the measurements were repeated three times in random order.

Apparatus. An F-4500 spectrofluorometer (Hitachi, Japan) equipped with a 1 cm quartz cell was used to measure the fluorescence and synchronous spectra. The slit widths of excitation and emission were both kept at 10 nm, a scan speed of 240 nm/min, a PMT voltage of 700 V, and a response time of auto. A model pHS-25 pH-meter (Shanghai, China) was employed for the measurement of pH. A KQ118 ultrasonic launder (Kunshan, China) was used to drive the air away from the samples. The HPLC apparatus (Agilent 1200) with PDA detector was used for the validation of both vitamins. The chromatographic conditions were the following: flow rate of mobile phase, 1.0 mL/min; UV detection wavelength, 267 nm; sampling volume, 20 μL; column temperature, 25 °C; analytical column (TOSOH), TSK gel ODS-80Ts, 5 μm, 150 mm × 4.6 mm; mobile phase, 40% methanol and 60% water. All of the experiments were carried out at room temperature.

General Procedure. To a 25 mL brown volumetric flask were added different quantities of folic acid and/or riboflavin, 2.5 mL of 1 mol/L H_2O_2, and 2.5 mL of 0.1 mol/L Cu(II), respectively. Then the mixture was diluted to final volume with a buffer solution of pH 5.0 and thorough shaking. Synchronous fluorescence spectra were obtained by scanning from 330 to 550 nm at constant-wavelength difference of $\Delta\lambda = 60$ nm for different samples. The synchronous fluorescence
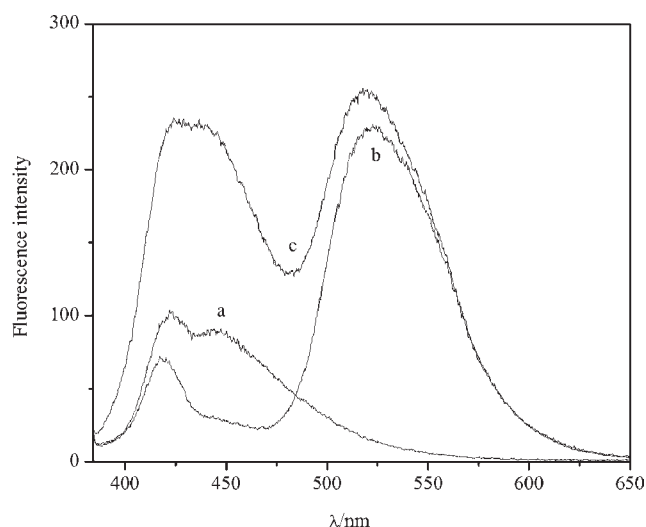

Figure 2. Fluorescence spectra of folic acid (a), riboflavin (b), and their mixture (c). All samples were measured in citric acid/disodium hydrogen phosphate buffer (pH 5.0). The concentrations of folic acid and riboflavin were both 100 μg/L. The excitation wavelength was 367 nm.
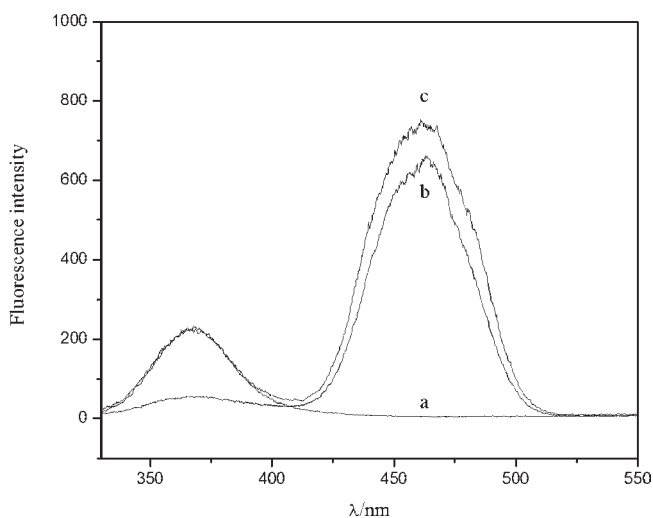

Figure 3. Synchronous fluorescence spectra of folic acid (a), riboflavin (b), and their mixture (c) with wavelength interval of 60 nm.

intensities were measured at the maximum of each component (for folic acid $\lambda = 371$ nm and for riboflavin $\lambda = 460$ nm). Deionized water was chosen as the blank solution and subjected to the sample operation as described above, and the blank values were determined. The determined values were obtained after subtraction of the blank value.

■ RESULTS AND DISCUSSION

Spectral Characteristics of Folic Acid and Riboflavin. According to the general procedure, the fluorescence spectra of folic acid, riboflavin, and their mixture were measured. As shown in Figure 2, the fluorescence emission spectra of folic acid (a) and riboflavin (b) were located at 433 and 520 nm, respectively. It was noticeable that the fluorescence emission spectra of the two vitamins (c) were overlapped, and it was difficult to identify individual bands. Figure 3 shows synchronous fluorescence spectra of folic acid (a), riboflavin (b), and their mixture (c).

The spectral bandwidth of riboflavin and folic acid became narrower, and their mixture was well separated (for folic acid $\lambda = 371$ nm and for riboflavin $\lambda = 460$ nm) in synchronous fluorescence mode. Therefore, it was possible to detect the two vitamins simultaneously.

Selection of Optimum Wavelength Interval. The synchronous fluorescence technique was widely used in quantitative analysis, in which both excitation and emission monochromators were scanned simultaneously by keeping a constant wavelength difference between them. Hence, $\Delta\lambda$ was an important instrumental parameter, which could directly influence the shape, bandwidth, and intensity of the synchronous fluorescence spectra. To find the optimum $\Delta\lambda$ for the simultaneous determination of the two vitamins, a wide range of $\Delta\lambda$ from 10 to 120 nm at an interval of 10 nm was examined by synchronous fluorescence scan. When $\Delta\lambda$ was lower than 20 nm or higher than 110 nm, the shape of its peak was poor. When $\Delta\lambda$ was from 20 to 110 nm, the synchronous fluorescence spectra of the two vitamins were obtained, and synchronous fluorescence intensity was strongest with high sensitivity at $\Delta\lambda$ of 60 nm. Consequently, the wavelength interval 60 nm was selected as the optimized wavelength interval for the assay to minimize spectral interference without loss of sensitivity.

Selection of Oxidation System and Optimum Concentration. The folic acid molecule itself scarcely fluoresced, but it could be decomposed and oxidized into pterine-6-carboxylic acid by appropriate oxidation system, leading to a strong fluorescent enhancement. Consequently, the selection of oxidation system was vital to fluorescence intensity and could directly affect the determination of folic acid. The fluorescence intensity of folic acid was tested by several oxidants, including $K_3[Fe(CN)_6]$, $KMnO_4$, $K_2S_2O_8$, and H_2O_2. It was found that the fluorescence intensity was so weak that it cannot be detected with the alkaline $K_3[Fe(CN)_6]$. Compared with other oxidants ($KMnO_4$, $K_2S_2O_8$, H_2O_2), folic acid with the H_2O_2 oxidation system in the presence of Cu(II) exhibited the maximum fluorescence value. Hence, H_2O_2 with added Cu(II) was employed as the oxidation system in the experiment.

The effect of H_2O_2 concentration on the synchronous fluorescence spectra was investigated with 0.01 mol/L Cu(II). It is demonstrated in Figure 4 that the fluorescence intensity of folic acid enhanced rapidly with increasing H_2O_2 concentration up to 0.05 mol/L, and then became increasingly slow in the concentration range of 0.05–0.25 mol/L. This phenomenon could be explained because folic acid consisted of pteridine and aminobenzoyl moieties. The pteridine moiety had a finer fluorescent property, because its fluorescence was depressed through intramolecular electron transfer from the aminobenzoyl moiety, which caused folic acid itself to weakly fluorescence. However, under the H_2O_2 circumstance, the unstable C—N bond between pteridine and aminobenzoyl moieties was broken, and pteridine was oxidized into pterine-6-carboxylic acid, which had strong fluorescence.[24,25] However, the fluorescence intensity of riboflavin directly decreased with increasing concentration of H_2O_2. This was because the C(4a)=N(5) double bond of riboflavin fractured to yield the single bond in the presence of H_2O_2. The conjugate nature of riboflavin was lessened, which resulted in decline of fluorescence intensity.[26] With consideration to the signal intensity both of the folic acid and riboflavin, 0.1 mol/L H_2O_2 was selected as the suitable value in this assay.

Folic acid can be decomposed to produce fluorescence spectra by H_2O_2, and the fluorescence intensity in the presence of Cu(II)
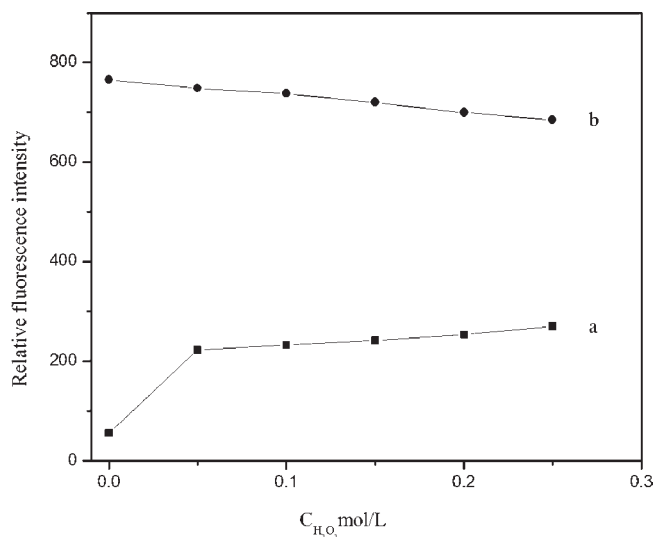

Figure 4. Effect of H_2O_2 concentration on the synchronous fluorescence intensity of folic acid (a) and riboflavin (b).
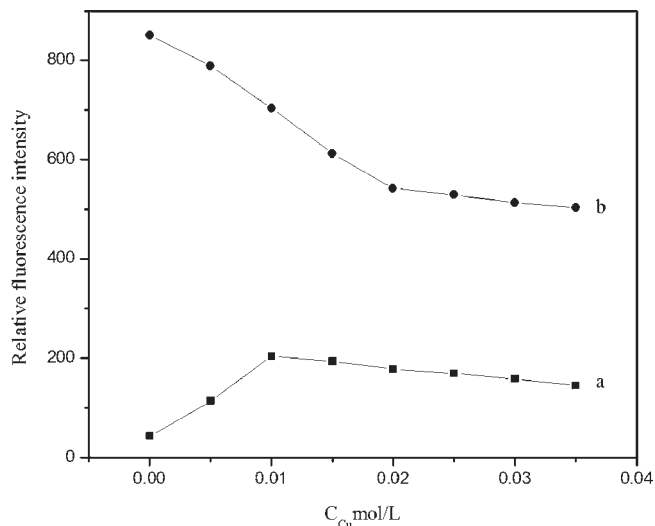

Figure 5. Effect of Cu(II) concentration on the synchronous fluorescence intensity of folic acid (a) and riboflavin (b).

was clearly higher than that without Cu(II). Different concentration solutions of Cu(II) in 0.1 mol/L H_2O_2 were prepared to study its effect on the fluorescence intensity. As indicated in Figure 5, the fluorescence intensity of folic acid enhanced with increasing concentration of Cu(II), reached its maximum when the concentration of Cu(II) was 0.01 mol/L, and then weakened with increasing concentration of Cu(II). This could be explained as due to the formation of reactive intermediates such as the Cu(I)—hydroperoxo complex [Cu(I)OOH] during the reaction of H_2O_2 with Cu(II), and the reactive intermediates accelerated the formation of pterine-6-carboxylic acid to produce the stronger fluorescence.[27] On the contrary, redundant Cu(II) could bind to pterine-6-carboxylic acid in the N(5)—C(4a)—C(4)—O(4) chelate site to form the O(4)—Cu(II)—N(5) chelate ring, which destroyed the conjugation and quenched the fluorescence of pterine-6-carboxylic acid.[28] At the same time, the fluorescence intensity of riboflavin reduced throughout the
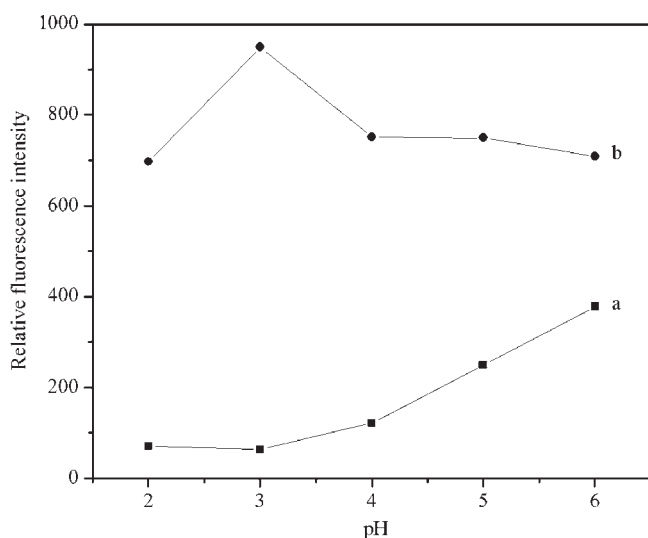
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Figure 6. Effect of pH on the synchronous fluorescence intensity of folic acid (a) and riboflavin (b).

Figure 7. Effect of time on the synchronous fluorescence intensity of folic acid (a) and riboflavin (b).

increase in Cu(II) concentration. This may be attributed to the fact that in the presence of H_2O_2 and Cu(II), hydroxylated riboflavin was formed and conjugation was lost, decreasing the light absorption of riboflavin. Hence, the fluorescence intensity of riboflavin decreased.[29] For further experiments, 0.01 mol/L Cu(II) was chosen.

Effect of pH. The influence of solution pH on the synchronous fluorescence intensity was studied by mixing citric acid and disodium hydrogen phosphate in different ratios. In this work, blue flocculation occurred in the vitamin solution when the pH was >7.0. Hence, the pH range of 2.2−6.0 was chosen. As shown in Figure 6, the fluorescence intensity was enhanced over the pH range of 2.2−6.0 for folic acid. This was due to the effect of ionic strength on the oxidizing reaction and the fluorometric behavior of the product.[10] At first, the fluorescence intensity of riboflavin increased as the pH rose and reached the maximum at pH 3.0. Afterward, it decreased with increasing pH. This phenomenon was caused by riboflavin's existence in the neutral form when the pH was 3.0 and the fluorescence quantum yield was highest.[30] Even though the fluorescence intensity of riboflavin was sufficiently high at this pH, the intensity of folic acid was extraordinarily low. Hence, a pH of 5.0 was employed for further work.

Effect of Time. The effect of time was studied on the synchronous fluorescence of the vitamins. The sample solution was placed in a 1 cm quartz absorption cell at room temperature, and its fluorescence intensity was measured by scanning simultaneously at constant wavelength difference of $\Delta\lambda = 60$ nm with time. As shown in Figure 7, the synchronous fluorescence intensity remained stable for at least 30 min.

Effect of Foreign Species. To assess the effects of foreign substances on the synchronous fluorescent procedure described above, a series of various metal ions and vitamins that may be potential interferences in nutritional beverage samples had been added into a standard solution containing folic acid (100 μg/L) and riboflavin (100 μg/L) and the synchronous fluorescence was tested. The tolerance level was defined as a relative error not exceeding ±5% in the determination of the analytes. The results of tolerable maximum concentration are presented in Table 1, which shows that most of the vitamins and metal ions did not
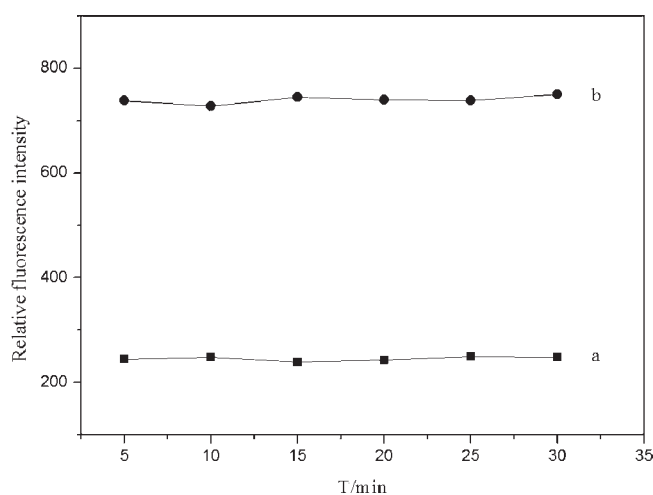
Table 1. Tolerable Concentration with Respect to Folic Acid and Riboflavin for Some Interfering Species

foreign species	folic acid (mg/L)	riboflavin (mg/L)
VB_1, VB_3, VC	2	1
VB_5	2	2
VB_6	4	1
Cr^{6+}	10	6
Pd^{2+}	15	5
Co^{2+}	0.4	6
Fe^{3+}	6	6
Zn^{2+}	6	3
Ni^{2+}	3	7
Al^{3+}	20	5
Mn^{2+}	50	9
Ca^{2+}	70	5

interfere with the determination of folic acid and riboflavin. Thus, this method had good selectivity and may also be useful to detect folic acid and riboflavin in vegetables, foods, and pharmaceutical dosages.

Analytical Parameters of the Procedure. As mentioned above, the calibration graphs for the determination of folic acid and riboflavin were obtained under the experimental optimum conditions. It was found that the linear relationships between the synchronous fluorescence intensity and the concentration of folic acid and riboflavin were in the ranges of 100−250 and 1−250 μg/L, respectively. The regression equations of the calibration curves for folic acid and riboflavin were $\Delta F = 2.11C + 15.38$ $(C, \mu g/L)$ and $\Delta F = 7.71C + 6.98$ $(C, \mu g/L)$ with correlation coefficients of $R^2 = 0.9977$ and $R^2 = 0.9995$, respectively. The repeatability of the method was determined by using a series of 11 standard samples, and the relative standard deviations for folic acid and riboflavin were 4.12 and 0.57% at the concentration of 100 μg/L. The detection limits of folic acid and riboflavin were 2.0 and 0.014 μg/L by calculation with the equation LOD = $3\sigma/s$, where σ is the standard deviation of

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Table 2. Analytical Results of Nutrimental Beverage Samples ($n = 3$)

sample	vitamin	original (mg/L)	added (mg/L)	found (mg/L)	recovery (%)	found by HPLC (mg/L)
A	folic acid	0.514 ± 0.008	0.500	1.025 ± 0.002	102.20	0.508 ± 0.012
	riboflavin	2.936 ± 0.030	3.000	5.850 ± 0.004	97.13	2.942 ± 0.025
B	folic acid	0.385 ± 0.005	0.400	0.776 ± 0.002	97.75	0.390 ± 0.013
	riboflavin	0.236 ± 0.006	0.250	0.481 ± 0.001	98.00	0.239 ± 0.007

the blank measurements ($n = 11$) and s is the slope of the calibration graph.

Analytical Application to Nutritional Beverage Samples. The established method was employed to assay the water-soluble vitamin content in two kinds of commercial nutritional beverages. The samples were appropriately diluted with double-deionized water, and their synchronous fluorescence spectra were obtained under the experimental conditions. The accuracy of the proposed method was checked by performing recovery tests. The recoveries ranged from 97.13 to 102.20%, which demonstrated the reliability of the proposed system for simultaneous monitoring of folic acid and riboflavin (Table 2). The method for the determination of vitamins was also validated by HPLC. The starting contents of folic acid and riboflavin were found to be in good agreement with the obtained values by HPLC. Hence, the proposed method is accurate and reliable.

In conclusion, a constant-wavelength synchronous fluorescence method for the simultaneous determination of folic acid and riboflavin in nutrimental beverages has been developed. By using H_2O_2 plus Cu(II) as the oxidation system, the two vitamins could be detected simultaneously. Interference study indicated that the method had good selectivity. The limit of detection was low, and the relationship between synchronous fluorescence intensity and concentrations of folic acid and riboflavin in aqueous solution was linear. Moreover, further work will combine the synchronous fluorescence method with a flow injection technique to minimize sample/reagent consumption and waste generation as well as enhance the sampling frequency.

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