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Novel spectrofluorimetric method for the determination of thiamine with iron(III) tetrasulfonatophthalocyanine as a catalyst

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A sensitive, selective and rapid spectrofluorimetric method is proposed for the determination of thiamine by using mimetic enzyme iron(III) tetrasulfonatophthalocyanine (FeTSPc) as a catalyst for the oxidation reaction between thiamine and hydrogen peroxide. It is based on the oxidation of thiamine in alkaline medium to give an intensively fluorescent compound, which has an excitation wavelength of 375 nm and an emission wavelength of 440 nm. The determination was found to be activated by fluorogenic substrates with a *p*-hydroxyphenyl structure such as L-tyrosine, tyramine and *p*-hydroxyphenylpropionic acid. Under optimum conditions, the responses for thiamine were linear from 1.0×10^{-8} to 1.0×10^{-4} mol L^{-1}, with a detection limit of 4.3×10^{-9} mol L^{-1}. The relative standard deviation was 2.2% for 2.0×10^{-7} mol L^{-1} thiamine ($n = 6$). The activation of the *p*-hydroxyphenyl substrates, the effects of some experimental conditions and the influence of foreign substances were investigated. The potential application of the method was tested by selectively determining thiamine in commercial vitamin B_1, vitamin B complex and rice.

Thiamine (vitamin B_1) is a natural nutrient present in many foods. It is a biologically and pharmaceutically important compound, essential for carbohydrate metabolism, maintenance of neural activity and prevention of beri beri. Since its discovery and isolation, there have been numerous reports on the determination of thiamine by microbial,[1,2] chromatographic,[3,4] spectrophotometric,[5–9] spectrofluorimetric,[10–15] chemiluminescence,[16] potentiometric[17–19] and reaction rate[20] methods. Among these methods, spectrofluorimetry and spectrophotometry are most often used. Some spectrophotometric methods for the determination of thiamine often require a previous derivatization for measurement in the visible region since measurement in the UV region is subjected to serious limitations imposed by the extensive spectral overlap of the vitamin B compounds. Other spectrophotometric methods rely on an appropriate previous oxidation or even on UV photodegradation.[5–9] A recent improvement made to the UV spectrophotometric method involving solid-phase spectrophotometry (SPS) with a combination of the solid-phase preconcentration of thiamine with direct spectrophotometric determination shows interesting features such as an improvement in selectivity and a significant increase in sensitivity.[21] However, the SPS method requires an ion-exchange procedure for preconcentration, which complicates the analytical procedure.

Spectrofluorimetric methods are usually more sensitive and selective than spectrophotometric methods. Thiamine is a non-fluorescent compound, but it can be converted into an intensely fluorescent thiochrome derivative (TC) in two ways: one is based on the oxidation of thiamine by appropriate oxidants (an ordinary fluorimetry) the other on the photooxidation of thiamine by UV irradiation (photochemical fluorimetry). Compared with ordinary fluorimetry, the photochemical fluorimetric method has the advantages of high sensitivity and no impurities introduced because of the use of a photochemical ‘reagent’; however, one has to change the wavelength of the excitation monochromator frequently during the measurement in order to acquire radiation of one wavelength to induce the photochemical reaction and another wavelength as the excitation radiation for fluorescence measurement.[14] For contrast, the ordinary fluorimetric method depends mainly on the choice of appropriate oxidants. Reports show that the oxidation of thiamine to TC is always accompanied by the simultaneous formation of thiamine disulfide (TDS), a condensation product of two thiamine molecules,[15] and the ratio of TC to TDS is affected by the pH,[22] solvent[23] and specific oxidizing agents.[24] So far, several oxidants including the previously used potassium hexacyanoferrate(III),[25] $KMnO_4$ and MnO_2,[26] cyanogen bromide,[27] H_2O_2 and I_2,[28] Hg(II),[15] Cu(II)[12] and Co(II)[13] have been developed for the fluorimetric determination of thiamine. Among them, $KMnO_4$, H_2O_2 and I_2 are said to favor the production of non-fluorescent TDS.[29] However, reports also indicate that the oxidation of thiamine by H_2O_2 under the catalysis of horseradish peroxidase (HRP) at pH 8.5 leads to a >95% yield of TC, which shows strong fluorescence at 440 nm with excitation at 375 nm.[30]

In our experiments, it was found that the interaction of thiamine with H_2O_2 could lead to a high yield of TC by using iron(III) tetrasulfonatophthalocyanine (FeTSPc) (Fig. 1) as a mimetic enzyme instead of HRP and the reaction could be activated by some compounds with a *p*-hydroxyphenyl struc-

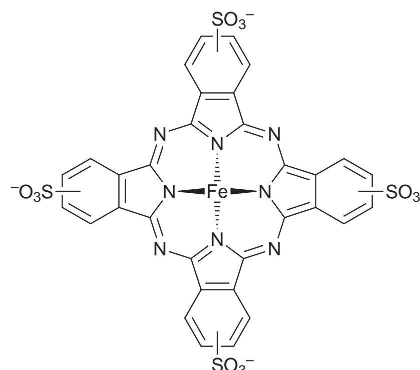

Fig. 1 Structure of FeTSPc.

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ture. Based on this reaction a novel, selective and highly sensitive spectrofluorimetric method is proposed for the determination of thiamine. The method was applied to the determination of thiamine in pharmaceutical preparations, and the results show that it is simple, fast and feasible.

Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensities were measured with a Hitachi (Tokyo, Japan) Model 650 10S spectrofluorimeter with a 10 mm silica cell. The excitation and emission bandwidths were set at 5 nm throughout the experiment.

Reagents and materials

Thiamine hydrochloride (biochemical reagent) stock standard solution of 0.01 mol L^{-1} was prepared in water and was stable for several weeks when kept refrigerated. Working standard solutions were prepared daily by dilution with water. FeTSPc was synthesized and purified as described,[31] and a stock standard solution of 0.001 mol L^{-1} was prepared; it was stable at room temperature for several months. An H_2O_2 stock standard solution (0.1 mol L^{-1}) was prepared from commercially available H_2O_2 (30% v/v) and standardized by titration with $KMnO_4$ standard solution. L-Tyrosine and tyramine were dissolved in 0.1 mol L^{-1} NaOH solution to give stock standard solutions of 0.01 mol L^{-1}. *p*-HPPA (0.01 mol L^{-1}) was dissolved in water. A buffer solution of pH 10.8 was prepared by mixing Na_2CO_3 (0.1 mol L^{-1}) and $NaHCO_3$ (0.1 mol L^{-1}) solutions in a volume ratio of 9 : 1. All chemicals, except where indicated otherwise, were of analytical-reagent grade or better and doubly de-ionized water was used throughout.

Thiamine determination

To a set of 10 mL tubes containing 1.0 mL of Na_2CO_3–$NaHCO_3$ buffer, 0.20 mL of 0.01 mol L^{-1} H_2O_2, 0.05 mL of 0.001 mol L^{-1} FeTSPc, 1.0 mL of 0.01 mol L^{-1} L-tyrosine and various volumes of thiamine standard solutions were added in that order and diluted to volume with water. The mixture was allowed to stand at room temperature (25 °C) for 2 min, then the fluorescence intensity was measured at an excitation wavelength of 375 nm and an emission wavelength of 440 nm.

Preparation of sample solution

Ten tablets of the commercial vitamin B_1 tablets which contained a nominal amount of 10.0 mg were powdered and dissolved in 0.01 mol L^{-1} HCl and then filtered. The filtrate and washings were collected in a 1000 mL calibrated flask and diluted to volume with water. An aliquot of vitamin B_1 solution was diluted by a factor of 200 for analysis.

Ten tablets of vitamin B complex which had a nominal amount of 3.0 mg of vitamin B_1 were treated in the same way as vitamin B_1 tablets. An aliquot of the solution was diluted by a factor of 100 for analysis.

Thiamine was extracted from polished rice in the following way. A 10.0 g amount of rice flour and 10 mL of 1.0 mol L^{-1} hydrochloride acid were placed in a sealed tube, followed by the digestion at 120 °C for 12 h. The cooled hydrolysate was adjusted to pH 7.0 by addition of NaOH solution, then centrifuged and washed to remove the insoluble materials. The supernatant and washings were diluted to 50 ml. A 0.2 ml volume of the solution was used directly for analysis.

Results and discussion

Spectral characteristics

Oxidation of thiamine by H_2O_2 under the catalysis of FeTSPc gave a high yield of thiochrome, which showed an excitation maximum at 375 nm and an emission maximum at 440 nm. Our experiments indicated that the application of a substrate with a *p*-hydroxyphenyl structure as an activator to the thiamine–FeTSPc–H_2O_2 system led to much higher yields of thiochrome. The fluorescence spectra of the activated and non-activated systems are shown in Fig. 2.

In Na_2CO_3–$NaHCO_3$ buffer solution of pH 10.8, which favored the determination of thiamine, a substrate with a *p*-hydroxyphenyl structure such as L-tyrosine was subjected to oxidation by H_2O_2 to give a fluorescent product, dityrosine, under the catalysis of FeTSPc, which showed strong fluorecence at 410 nm with excitation at 325 nm (see Fig. 2). As can be seen from Fig. 2, in the tyrosine-activated system the emission spectrum of dityrosine overlapped with the excitation spectrum of thiochrome, and so did the excitation spectra of dityrosine and thiochrome, but the fluorescence of dityrosine at 410 nm was too low to be detected when the excitation wavelength was set at 375 nm, hence the determination of thiamine did not suffer interference.

Activation of *p*-hydroxyphenyl structure substrates

Our experimental results showed that substrates with a *p*-hydroxyphenyl structure such as L-tyrosine, *p*-HPPA and tyramine could be used as activators for the oxidation of thiamine to thiochrome by H_2O_2 in the presence of FeTSPc, the final fluorescence of the thiamine–FeTSPc–H_2O_2 system being significantly enhanced by the addition of such substrates. In order to establish the mechanism of the activation, L-tyrosine was taken as an example. As mentioned above, the fluorescence of dityrosine at 410 nm was too low when the excitation was set at 375 nm, so the possibility of the final fluorescence being enhanced by energy transfer from dityrosine to thiochrome could be excluded. Therefore, further investigations were made and it was found that the extent of fluorescence enhancement closely depended on the order of addition of the reagents, and especially on the time interval between adding tyrosine and thiamine; a suitable sequence of adding the reagents for the

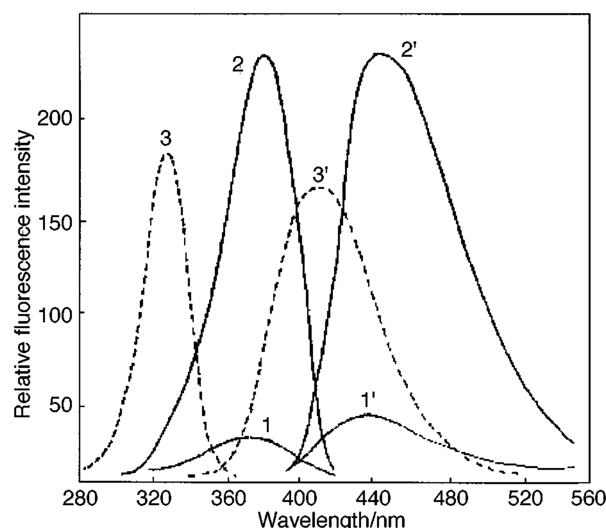

Fig. 2 Fluorescence excitation and emission spectra: 1, 1′, H_2O_2–FeTSPc–thiamine; 2, 2′, H_2O_2–FeTSPc–tyrosine–thiamine; 3, 3′, H_2O_2–FeTSPc–tyrosine. FeTSPc, 5.0 × 10^{-6} mol L^{-1}; H_2O_2, 2.0 × 10^{-4} mol L^{-1}; thiamine, 1.0 × 10^{-6} mol L^{-1}; L-tyrosine, 1.0 × 10^{-3} mol L^{-1}.

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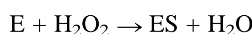
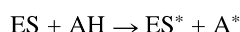
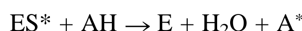
determination of thiamine was buffer, H_2O_2, FeTSPc, *p*-hydroxyphenyl substrate and thiamine. Fig. 3 shows the dependence of fluorescence intensity on the time interval between mixing the first four reagents (buffer, H_2O_2, FeTSPc and tysosine) and thiamine. Obviously, maximum fluorescence enhancement occurred when tyrosine and thiamine were added simultaneously to the mixture of buffer, H_2O_2 and FeTSPc, and no enhancement was observed when the time interval exceeded 30 min. Owing to the fact that in the reaction time range 30–50 min the fluorescence of the tyrosine–H_2O_2–FeTSPc system (λ_{ex} 325 nm, λ_{em} 410 nm) was maximum and constant (see Fig. 4), which means that the formation reaction of dityrosine has reached equilibrium, it could be inferred that dityrosine did not cause any activation of the oxidation of thiamine to fluorescent thiochrome. Similarly, we also found that only a small fluorescence enhancement was obtained when thiamine was added prior to tyrosine. This might imply that tyrosine itself also did not play the role in the activation of thiamine oxidation. Based on the above observations, it is considered that the intermediate in the reaction between tyrosine and H_2O_2 under the catalysis of FeTSPc may serve as the activator for the oxidation of thiamine. According to the possible mechanism reported for mimetic peroxidase-catalyzed fluorogenic reactions between *p*-hydroxyphenyl substrates and H_2O_2:[32]



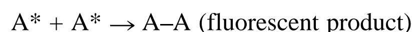
$$E + H_2O_2 \rightarrow ES + H_2O$$



$$ES + AH \rightarrow ES^* + A^*$$



$$ES^* + AH \rightarrow E + H_2O + A^*$$



$$A^* + A^* \rightarrow A\text{–}A \text{ (fluorescent product)}$$

where E is the mimetic enzyme, AH is the fluorogenic substrate and ES and ES* are the enzyme–substrate complex and its radical, respectively, formed in the reaction. Therefore, it was deduced that either ES* or A* might play the role of activator in thiamine oxidation.

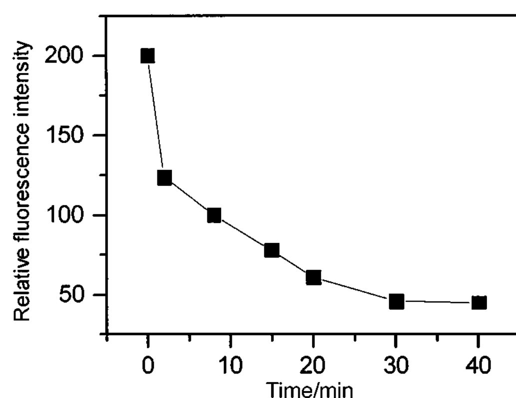


Fig. 3 Dependence of fluorescence intensity on the time interval between mixing the first four reagents and adding thiamine. L-Tyrosine, 1.0×10^{-3} mol L^{-1}; FeTSPc, 5.0×10^{-6} mol L^{-1}; H_2O_2, 2.0×10^{-4} mol L^{-1}; thiamine, 1.0×10^{-6} mol L^{-1}.

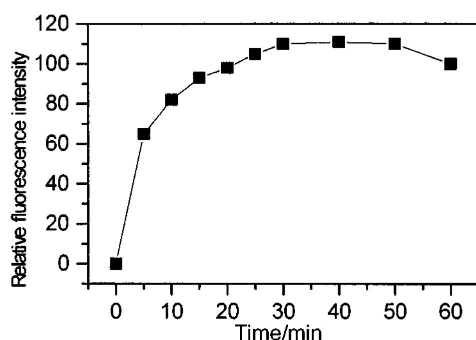


Fig. 4 Kinetic profile of the H_2O_2–FeTSPc–tyrosine reaction system. FeTSPc, 5.0×10^{-6} mol L^{-1}; H_2O_2, 2.0×10^{-4} mol L^{-1}; L-tyrosine, 1.0×10^{-3} mol L^{-1}.

Catalytic effect of FeTSPc

FeTSPc played an important role in the sensitive determination of thiamine. First, the fluorescence of the non-activated thiamine oxidation system was low without the catalysis of FeTSPc. Second, the activation of *p*-hydroxyphenyl substrates could not be induced in the absence of FeTSPc since the formation of a radical intermediate in the reaction between tyrosine and H_2O_2 relied on the involvement of FeTSPc. Fig. 5 shows the kinetic curves for the L-tyrosine–thiamine–H_2O_2 system in the presence of different amounts of FeTSPc. It can be seen that in the absence of FeTSPc, much lower fluorescence was obtained even in the presence of L-tyrosine activator, and a longer time was also needed for the reaction to reach equilibrium. Also, it can be seen that the fluorescence enhancement was increased with increase in the amount of FeTSPc, and maximum and constant fluorescence was reached when the amount of FeTSPc was in the range 3.0×10^{-6}–8.0×10^{-6} mol L^{-1}. Furthermore, only 2 min were needed for the FeTSPc-catalyzed system to reach reaction equilibrium.

Optimization of experimental parameters

Effect of pH and buffer. The FeTSPc-catalyzed reaction is dependent not only on the pH but also on the specific buffer used. This behavior is similar to that of HRP. The effects of pH and buffers on the reaction were studied. Three kinds of buffer systems, glycine–NaOH, Na_2CO_3–$NaHCO_3$ and $Na_2B_2O_4$–NaOH, were tested. The results showed that the fluorescence increased with increase in pH and reached a maximum in the pH range 10.0–10.8, and Na_2CO_3–$NaHCO_3$ buffer solution was found to be the most suitable of the three tested.

Optimum amounts of reagents. The maximum fluorescence was obtained when the final concentrations of FeTSPc and H_2O_2 were in the ranges 3.0×10^{-6}–8.0×10^{-6} mol L^{-1} and 1.0×10^{-4}–3.0×10^{-4} mol L^{-1}, respectively. Therefore, 0.05 mL of 0.001 mol L^{-1} FeTSPc and 0.20 ml of 0.10 mol L^{-1} H_2O_2 were adopted throughout in thiamine determinations.

The optimum amount of tyrosine was also studied. The fluorescence first increased with increasing the amount of tyrosine and then leveled off at a final concentration of 1.0×10^{-3} mol L^{-1}, as shown in Fig. 6, but the increase in background fluorescence was negligible. Therefore, 1.0 mL of 0.01 mol L^{-1} tyrosine was adopted.

Reaction time and temperature. As shown in Fig. 7, the fluorescence of the H_2O_2–FeTSPc–tyrosine–thiamine system

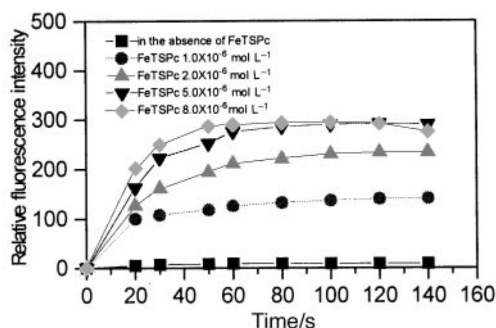


Fig. 5 Kinetic curves for the catalyzed and non-catalyzed systems. L-Tyrosine, 1.0×10^{-3} mol L^{-1}; H_2O_2, 2.0×10^{-4} mol L^{-1}; thiamine, 1.0×10^{-6} mol L^{-1}.

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reached a maximum and remained constant in the reaction time range 90–150 s, and the background fluorescence was almost constant and negligible; therefore, 2 min after mixing the reagents was chosen as the time to measure the fluorescence.

This reaction was shown to be temperature dependent. Maximum and constant fluorescence was obtained over the range of 15–35 °C. Room temperature was therefore adopted.

Analytical performance

The calibration graphs obtained for the determination of thiamine in activated and non-activated systems are listed in Table 1. The relative standard deviation was 2.1% for 2.0 × 10^{-7} mol L^{-1} thiamine (n = 6).

Effect of foreign substances

Interference from foreign substances was tested by analyzing a standard solution of thiamine (5.0 × 10^{-7} mol L^{-1}). The tolerances to some relevant substances are reported in Table 2. Metal ions such as Mn^{2+} and Cu^{2+} may serve as an additional catalyst for the oxidation of thiamine by H_2O_2 and thus interfere with the determination, but the interference can be eliminated by adding EDTA. Also, it should be noted that interference by tyrosine may occur when the proposed tyrosine-activated method is applied to the determination of thiamine in samples containing tyrosine. However, as shown in Fig. 6, constant fluorescence was obtained when the amounts of tyrosine were in the range 1.0 × 10^{-3}–2.0 × 10^{-3} mol L^{-1}, that is, the possible interference of tyrosine present in samples could be neglected when the total concentration of tyrosine is in the range 1.0 × 10^{-3}–2.0 × 10^{-3} mol L^{-1}. Actually, the tyrosine content in most samples was far below 1.0 × 10^{-3} mol L^{-1}, so it is unlikely to cause interference with thiamine determination.

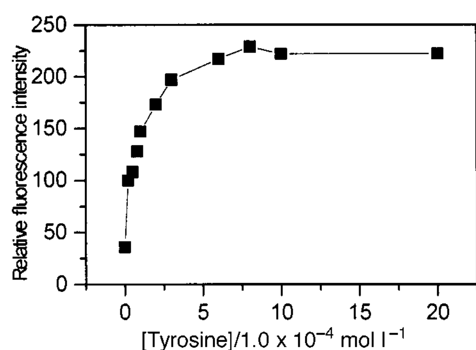

Fig. 6 Effect of the amount of tyrosine on the fluorescence intensity of the H_2O_2–FeTSPc–tyrosine–thiamine system. FeTSPc, 5.0 × 10^{-6} mol L^{-1}; H_2O_2, 2.0 × 10^{-4} mol L^{-1}; thiamine, 1.0 × 10^{-6} mol L^{-1}.

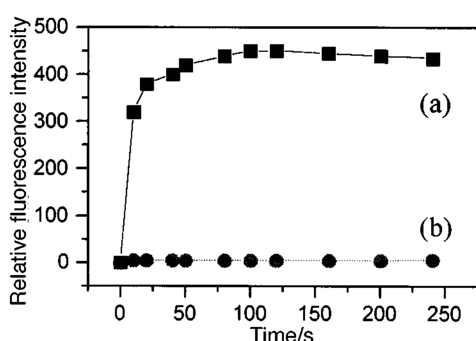

Fig. 7 Kinetic behavior of the systems (a) H_2O_2–FeTSPc–tyrosine–thiamine and (b) H_2O_2–FeTSPc–tyrosine. FeTSPc, 5.0 × 10^{-6} mol L^{-1}; H_2O_2, 2.0 × 10^{-4} mol L^{-1}; thiamine, 1.0 × 10^{-6} mol L^{-1}; L-tyrosine, 1.0 × 10^{-3} mol L^{-1}.

Table 1 Analytical performance of the proposed method

	Linear range/mol L^{-1}	LOD/mol L^{-1}	Correlation coefficient
Activated system	1.0 × 10^{-8}–1.0 × 10^{-5}	4.3 × 10^{-9}	0.997
	5.0 × 10^{-6}–1.0 × 10^{-4}		0.998
Non-activated system	8.0 × 10^{-8}–2.0 × 10^{-5}	2.5 × 10^{-8}	0.997
	1.0 × 10^{-5}–1.0 × 10^{-4}		0.999

Applications

Determination of vitamin B_1 in pharmaceutical tablets. The application of the method to the determination of thiamine in commercial vitamin B complex and vitamin B_1 tablets was tested. The accuracy of the method was investigated by performing recovery tests on standard additions in real samples (Table 3). Although the amounts of thiamine obtained by this method were slightly lower than that claimed by the manufacturers, they were still reasonably in agreement with the nominal values. The difference may be due to the time on the shelf of the tablets. The recovery experiments gave satisfactory results.

Table 2 Tolerance of foreign substances

Substance	Molar ratio to thiamine
Mg^{2+}, Ca^{2+}	1000
Zn^{2+}	500
Al^{3+}, Fe^{2+}	200
Pb^{2+}	100
Ag^{+}	5
Cu^{2+}	1
Mn^{2+}	0.25
Glycine, glutamic acid	1000
Pyridoxine	100
Nicotimamide	100
Riboflavin	2
Vitamin C	5
Vitamin B_{12}	0.03

Table 3 Determination of thiamine in vitamin B complex and vitamin B tablets

Samples	Proposed method/mg[a]	Claimed/mg	Thiamine added/ mol L^{-1}	Recovery (%)
Vitamin B_1 tablet	9.11	10.0	5.0 × 10^{-7}	95.7
			1.0 × 10^{-6}	104.1
			2.0 × 10^{-6}	94.4
Vitamin B complex	2.90	3.0	5.0 × 10^{-7}	95.4
			1.0 × 10^{-6}	102.2
			2.0 × 10^{-6}	96.0

[a] Mean of six determinations.

Table 4 Determination of vitamin B_1 in polished rice from northeast China

Thiamine added/ 10^{-7} mol L^{-1}	Thiamine found/ 10^{-7} mol L^{-1}	Recovery (%)
0	3.07[a]	–
0.5	3.59	102
1.0	4.04	97
2.0	5.02	95

[a] Content of thiamine after the sample solution (10 g in 50 ml) had been diluted 50-fold.

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Determination of vitamin B_1 in rice. In order to eliminate the interference of metal ions (*e.g.*, Mn^{2+}) from rice, 0.3 mL of 0.2 mol L^{-1} EDTA was added prior to the addition of tyrosine. Vitamin B_1 was determined by the standard additions method and results are given in Table 4.

Conclusion

The proposed method for the determination of thiamine is simple, rapid, selective and sensitive and has a wide linear range, and should be useful in routine pharmaceutical determinations. Its coupling with FIA is under investigation.

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