The Use of Crude Extract of Kohlrabi (*Brassica oleracea gongylodes*) as a Source of Peroxidase in the Spectrofluorimetric Determination of Thiamine

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Received August 12, 2006

The crude extract of kohlrabi (*Brassica oleracea gongylodes*) was prepared as a rich source of peroxidase and its enzymatic and specific activities in the presence of cross-linked polyvinylpyrrolidone as a stabilizer were determined. This extract was used to catalyze the oxidation of thiamine in the presence of hydrogen peroxide to thiochrome at pH 8.0. Thiochrome shows strong fluorescence at 436 nm with excitation at 370 nm. Based on the obtained results, a sensitive and simple spectrofluorimetric method was developed for the determination of thiamine. In the optimum conditions, the calibration graph was linear from 2×10^{-7} to 1×10^{-4} mol L^{-1}, with a detection limit of 6.2×10^{-8} mol L^{-1}. The relative standard deviation (RSD) was 1.2% for 5×10^{-6} mol L^{-1} thiamine. The method was successfully applied to the determination of thiamine in vitamin B1 and vitamin B complex tablets and vitamin B complex syrup.

Key Words : Thiamine, Spectrofluorimetry, Peroxidase, Kohlrabi, Crude extract

Introduction

Thiamine (vitamin B1) is one of the water-soluble B-group vitamins essential for carbohydrate metabolism, maintenance of normal neural activity and prevention of beriberi. People usually obtain the nutrient from natural and fortified foods. When needed, the vitamin can also be obtained from various pharmaceutical preparations.

Several analytical methods have been proposed for the determination of thiamine including spectrophotometry,[1,2] spectrofluorimetry,[3-5] chemiluminescence,[6,7] chromatography,[8,9] voltammetry[10] and kinetic methods.[11] The most widely used method for the assay of thiamine is the spectrofluorimetric method, which involves the oxidation of thiamine to form fluorescent thiochrome (TC). This reaction is always accompanied by the simultaneous formation of non-fluorescent compound, thiamine disulfide (TDS). It is known that the ratio of TC to TDS is affected by the pH, solvent and specific oxidizing agent.[12] So far several oxidants including hexacyanoferrate(III), $KMnO_4$ and MnO_2,[13] cyanogen bromide,[14] H_2O_2, I_2 and Hg(II),[12] Cu(II)[15] and Co(II)[16] have been used for fluorimetric determination of thiamine. Among them, $KMnO_4$, I_2, and H_2O_2 (in alkaline solution) have been shown to favor the production of non-fluorescent TDS. Hexacyanoferrate in the absence of solvent extraction is not an appropriate oxidant and cyanogens bromide and Hg(II) are highly toxic and would cause problems as regards environmental pollution. On the other hand, it has been reported that the oxidation of thiamine by H_2O_2 under the catalysis of horseradish peroxidase at pH 8.5 leads to a greater than 95% yield of TC.[17] Based on this reaction a fiber optic sensor has been developed for the determination of H_2O_2.

Peroxidase (POx, EC 1.11.1.7) is a hem-containing enzyme that is extensively distributed in plants, animals and microorganisms. The main source of POx for commercial production is the roots of horseradish. Therefore it is usually known as horseradish peroxidase. This enzyme utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds.[18] Although enzymes are widely used in biochemistry and clinical chemistry, they are expensive and their solutions are not stable. Therefore, the study of potential alternatives is one of the interesting trends in analytical biochemistry. Various metal-porphine derivatives have been used as mimetic enzymes instead of POx.[19] Chen *et al.*[20] have utilized iron(III) tetrasulfonatophthalocyanine as a catalyst for the oxidation of thiamine by H_2O_2. In recent years the use of plant tissue materials and crude extract of various vegetables as novel biocatalysts has also received considerable interest for replacing isolated enzymes.[21-25] The use of such biological materials is very attractive because of their simplicity, high stability, very low cost and fewer cofactor requirements in comparison with the pure enzymes. Vieira and Fatibello-Filho[26] have used the crude extract of zucchini as a source of POx for the spectrophotometric determination of H_2O_2. They have applied an insoluble polyvinylpyrrolidone for stabilizing the extract.

We have found that the crude extract of kohlrabi (*Brassica oleracea gongylodes*) is a rich source of POx and has higher enzymatic activity than zucchini.[27] In the present work, we have utilized this extract as a source of POx for the oxidation of thiamine by H_2O_2. Based on this reaction, a very simple and sensitive method is proposed for the determination of thiamine in pharmaceutical samples.

Experimental

Apparatus. Fluorescence spectra and intensity measurements were made on a Shimadzu RF-540 spectrofluorimeter equipped with a 150 W xenon lamp, using 1.0 cm quartz cell. Slit widths of both monochromators were set at 5 nm. All measurements were performed at 40 °C by use of a

thermostated cell holder and thermostatically controlled water bath. A Metrohm model 654 pH meter was used for pH measurements. A Hettich model EBA centrifuge was used in preparation of crude extract of kohlrabi.

Reagents. All reagents used were of analytical reagent grade. Doubly distilled water was used throughout. Thiamine hydrochloride was purchased from Merck and its stock standard solution of 0.01 mol L^{-1} was prepared in water (with addition of a few drops of concentrated HCl) and kept in refrigerator. Working standard solutions were prepared daily by proper dilution with water. An H_2O_2 stock standard solution (0.1 mol L^{-1}) was prepared from 30% H_2O_2 solution and standardized by titration with $KMnO_4$. Working standard solution (5×10^{-3} mol L^{-1}) was prepared daily by proper dilution with water. Polyvinylpyrrolidone cross-linked was purchased from Acros Chemical Company and purified as described in the literature.[23] Sodium azide was purchased from Merck and a 1% (w/v) solution was prepared in water.

A variety of healthy kohlrabi obtained from a local producer, were washed, hand-peeled and frozen in a refrigerator.

Preparation of kohlrabi crude extract. A piece of the frozen peeled kohlrabi was finely chopped and 25 g of it was mixed with 100 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.5) and 7.5 g of polyvinylpyrrolidone. The mixture was stirred for 5 min at 4-7 °C and rapidly filtered through a layer of cheesecloth and centrifuged at 3000 rpm for 15 min at this temperature. The supernatant was deoxygenated by Ar bubbling and stored at 4 °C in a refrigerator. In this condition the solution was stable for at least two months. This solution was utilized as enzymatic source.

Peroxidase activity and total protein determinations. Peroxidase activity present in the crude extract of kohlrabi was determined by measuring an increase in absorbance at 510 nm resulting from the reaction of 4-aminoantipyrine and phenol with hydrogen peroxide.[28] The crude extract was diluted 10 times and 0.1 mL of the resulted solution was mixed with 1.4 mL of mixture solution, containing 0.0025 mol L^{-1} 4-aminoantipyrine and 0.17 mol L^{-1} phenol, and 1.5 mL of 0.0017 mol L^{-1} H_2O_2 solution. The increase in absorbance at 510 nm and 25 °C was recorded for 5 min. One activity unit is defined as the amount of enzyme that causes an increase of 0.001 absorbance per minute under the conditions described above.

Total protein concentration was determined by the Lowry method[29] using bovine serum albumin as a standard.

General procedure for the determination of thiamine. To a set of 10 mL volumetric flasks containing various volumes of thiamine standard solutions, 1 mL 5×10^{-3} mol L^{-1} H_2O_2, 2 mL 0.5 mol L^{-1} phosphate buffer (pH 8.0) and 0.6 mL crude extract (with activity of 9610 unit) were added and diluted to the volume with water. The final concentration of thiamine in these solutions should be in the range of 2×10^{-7} to 1×10^{-4} mol L^{-1}. The solutions were thermostated at 40 °C for 40 min. Exactly after this time, 0.5 mL 1% sodium azide solution was added to stop the reaction and the fluorescence intensity was measured at an excitation wavelength of 370 nm and an emission wavelength of 436 nm.

Sample preparation. Four commercial vitamin B1 preparations were analyzed: vitamin B1 tablet (Tehran Chimie, Iran) with nominal content of 100 mg, vitamin B1 tablet (Darou Pakhsh, Iran) with nominal content of 300 mg, vitamin B complex tablet (Exir Pharmaceutical company, Iran) which had a nominal amounts of 5 mg vitamin B1, 2 mg vitamin B2, 2 mg vitamin B6 and 20 mg nicotinamide and vitamin B complex syrup which had a nominal amounts of 5 mg vitamin B1, 2 mg vitamin B2, 2 mg vitamin B6, 20 mg nicotinamide and 3 mg dexpanthenol in 5 mL syrup.

Five tablets of vitamin B1 or vitamin B complex were weighed in order to find the average mass of each tablet. Then the tablets were powdered and mixed. A portion of 20.0 mg of this powder was accurately weighed and dissolved in 0.01 mol L^{-1} HCl and then filtered into 100 mL volumetric flask. The residue was washed several times with water and solution was diluted to the mark. An appropriate aliquot of this solution was used for analysis.

3.0 mL of vitamin B complex syrup was dissolved in 0.01 mol L^{-1} HCl solution and diluted to 100 mL in a volumetric flask. A suitable aliquot of this solution was used for analysis.

Results and Discussion

Preliminary investigations revealed that the crude extract of kohlrabi is a rich source of POx. The peroxidase activity and total protein concentration of the crude extract, prepared as described in the Experimental section, was found to be 9610 unit and 1.50 mg mL^{-1}, respectively. Therefore the specific activity of the extract would be 6400 unit mg^{-1}. In order to compare enzymatic activities, the crude extract of zucchini was also prepared by the same procedure and its specific activity was found to be 1690 unit mg^{-1}. These results indicate the merit of kohlrabi crude extract as an enzymatic source.

The use of cross-linked polyvinylpyrrolidone to remove natural phenolic compounds from the solution in the preparation of the crude extract of kohlrabi led to substantial increase in enzyme activity and storage time. The enzyme activity of the crude extract obtained using this insoluble polymer did not vary for at least two months when deoxygenated and stored at 4 °C. Study of the effect of various parameters on the activity of crude extract and optimization of extraction procedure have been reported elsewhere.[27]

Preliminary works also showed that the crude extract of kohlrabi could catalyze the oxidation of thiamine by H_2O_2 to thiochrome, which has an excitation maximum of 370 nm and an emission maximum of 436 nm. The fluorescence spectra of catalyzed and non-catalyzed systems are shown in Figure 1.

Effect of pH and buffer. The oxidation reaction of thiamine by H_2O_2, using the crude extract of kohlrabi as a source of POx, is dependent on the pH and also on the specific buffer used. The effects of pH and buffer on the reaction were studied. Four kinds of buffer system, phosphate, carbonate, borate and ammonical were tested. In the presence of phosphate buffer solution the highest fluorescence signal

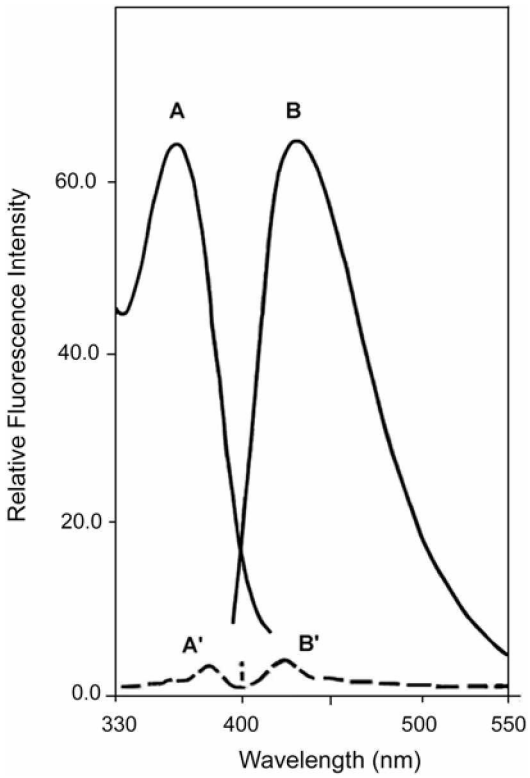

Figure 1. Fluorescence excitation (A,A') and emission (B,B') spectra of thiamine- H_2O_2 system in the presence of 576 unit mL^{-1} kohlrabi crude extract (A,B) and in the absence of crude extract (A',B'). pH = 8.0, [thiamine] = 1.5×10^{-5} mol L^{-1}, [H_2O_2] = 5×10^{-4} mol L^{-1}, T = 40 °C, incubation time = 40 min, [sodium azide] = 0.05%, λ_{ex} = 370 nm, λ_{em} = 436 nm.

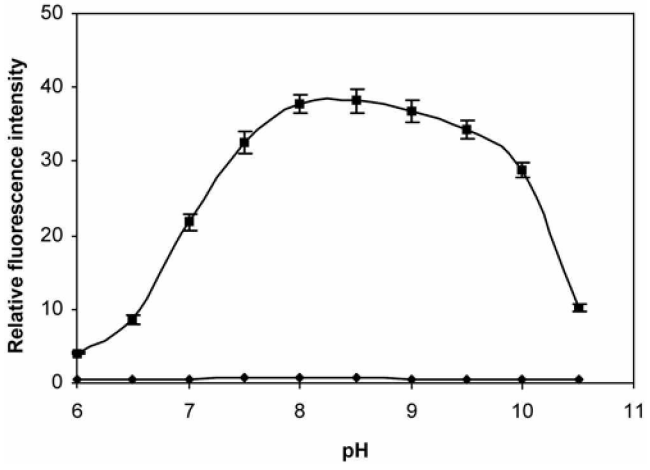

Figure 2. Effect of the pH on the fluorescence intensity of thiamine-H_2O_2-kohlrabi crude extract (■) and H_2O_2-kohlrabi crude extract (●) systems. [thiamine] = 1×10^{-5} mol L^{-1}, [The crude extract] = 480 unit mL^{-1}. Other conditions are as Fig. 1.

was obtained. Figure 2 shows the effect of pH on the fluorescence signal that was studied by using phosphate buffers. As can be seen, the fluorescence intensity reaches a maximum in the pH range 8.0-8.5. The effect of buffer concentration was also studied. The fluorescence increased by increasing the concentration and reaches a maximum at

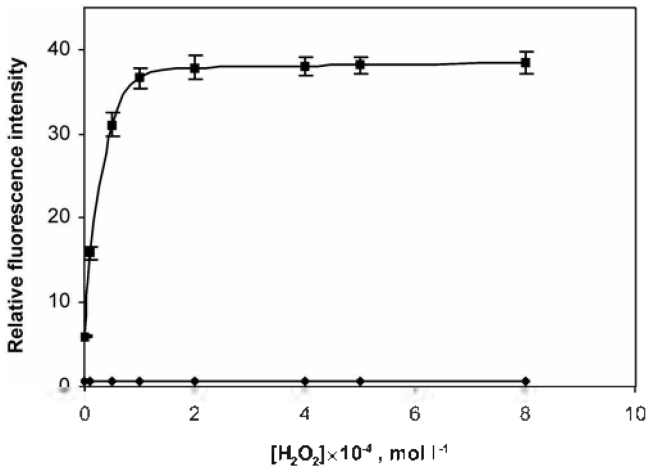

Figure 3. Effect of H_2O_2 concentration on the fluorescence intensity of thiamine-H_2O_2-kohlrabi crude extract (■) and H_2O_2-kohlrabi crude extract (●) systems. [thiamine] = 1×10^{-5} mol L^{-1}, [The crude extract] = 480 unit mL^{-1}. Other conditions are as Fig. 1.

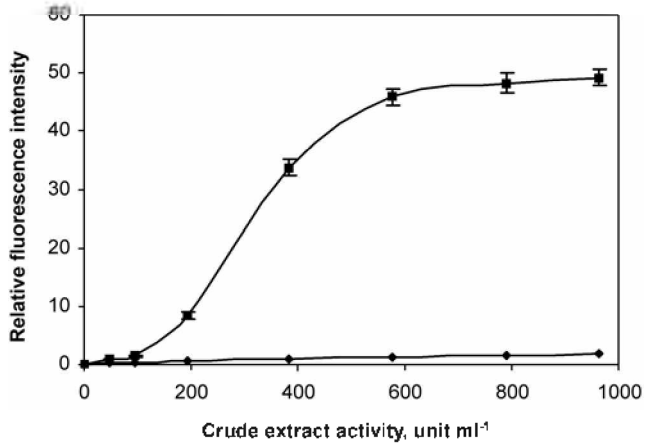

Figure 4. Effect of the crude extract concentration on the fluorescence intensity of thiamine-H_2O_2-kohlrabi crude extract (■) and H_2O_2-kohlrabi crude extract (●) systems. [thiamine] = 1×10^{-5} mol L^{-1}. Other conditions are as Fig. 1.

0.1 mol L^{-1}. Therefore, pH 8.0 phosphate buffer with concentration of 0.1 mol L^{-1} was chosen for pH adjustment.

Effect of H_2O_2 concentration and enzyme activity. Figure 3 shows the effect of H_2O_2 concentration on the fluorescence signal. As can be seen the signal reaches a maximum when the final concentration of H_2O_2 is in the range 2×10^{-4}-8×10^{-4} mol L^{-1}. Therefore 1 mL of 5×10^{-4} mol L^{-1} H_2O_2 was adopted throughout in thiamine determination.

The effect of POx activity from 48 to 960 unit mL^{-1} on the fluorescence intensity was investigated. As can be seen from Figure 4, the signal increases with increase in enzyme activity up to about 700 unit mL^{-1}. However since the blank signal is also gradually increased by increasing the enzyme activity, so 576 unit mL^{-1} was selected as suitable amount of the enzyme. This activity can be achieved by adding 0.6 mL of the crude extract to a 10 mL calibrated flask.

Effect of time and temperature. Figure 5 shows the kinetic curves for thiamine-H_2O_2-POx system at various

Table 1. Comparison of the Analytical characteristics of the proposed method with those of other methods for the determination of thiamine

Method	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	Reference
Spectrofluorimetry by using Iron(III) tetrasulfonatophthalocyanine	1.0×10^{-8} - 1.0×10^{-4}	4.3×10^{-9}	20
Spectrofluorimetry by using Hg(II)	2.0×10^{-7} - 7.0×10^{-6}	3.4×10^{-8}	12
Spectrofluorimetry by using Cu(II)	8.9×10^{-7} - 1.8×10^{-5}	5.0×10^{-7}	15
Solid phase Spectrophotometry	1.8×10^{-6} - 5.6×10^{-5}	4.7×10^{-7}	2
This method	2.0×10^{-7} - 1.0×10^{-4}	6.2×10^{-8}	–

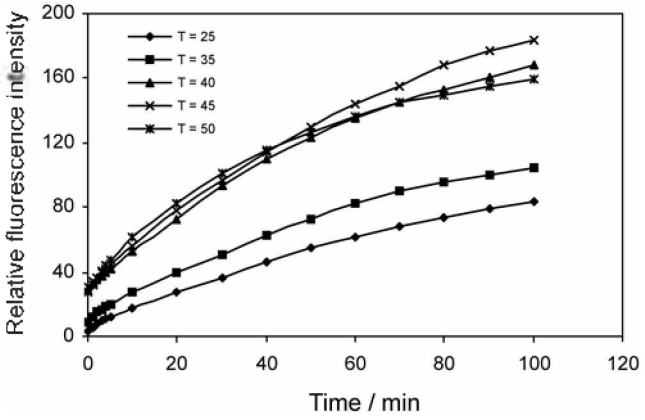

Figure 5. Kinetic curves for thiamine-H_2O_2-kohlrabi crude extract system at various temperatures. [thiamine] = 1×10^{-5} mol L^{-1}. Other conditions are as Fig. 1.

temperatures. As the temperature raises the rate of reaction is increased and reaches an almost constant value in the range of 40-50 °C. However, in all cases the reaction does not reach equilibrium before two hours that is too long for analytical process. Therefore, we decided to use sodium azide that is known to inhibit POx,[30] to stop the reaction at an appropriate time. When the reaction mixtures are thermostated at 40 °C for 40 min, the fluorescence signal is high enough for sensitive analysis. It was found that 0.05% sodium azide was sufficient to stop the reaction.

Analytical figures of merit. By using the optimum conditions described above, a spectrofluorimetric method was developed for the determination of thiamine. The calibration graph (n = 14) was found to be linear in the range 2×10^{-7} - 1×10^{-4} mol L^{-1} and its equation was $F = 3.68 (\pm 0.036) \times 10^6 C + 4.546 (\pm 1.34)$, where F is fluorescence intensity and C is thiamine concentration in mol L^{-1}. The correlation coefficient (r) was 0.9994. The detection limit calculated as $3S_b/m$ (where S_b is standard deviation of the blank and m is slope of the calibration graph) was found to be 6.2×10^{-8} mol L^{-1}.

In order to study the precision of the method, a series of nine standard solutions of 5×10^{-6} mol L^{-1} thiamine were measured in the same day. By applying the IUPAC definition, the relative error and the relative standard deviation (RSD) were 0.6 and 1.2%, respectively.

Table 1 compares the analytical characteristics of the proposed method with some published methods for the determination of thiamine. As can be seen, our proposed method has comparable or better detection limit than the methods studied except for the one that used a synthetic catalyst as the mimetic enzyme. But the preparation of crude extract is much simpler than the synthesis of this mimetic enzyme.

Table 2. Tolerance limit of foreign substances on the determination of 5×10^{-6} mol L^{-1} thiamine

Substance	Molar ratio to thiamine
Na^+, K^+, Mg^{2-}	1000
Zn^{2-}, Ag^-, Ca^{2-}	100
Mn^{2-}	50
Al^{3+}	20
Pb^{2+}	3
Fe^{2+}	2
Cu^{2+}	0.2
Glutamic acid, Glycine, Nicotinamide	1000
Vitamin C	10
Pyridoxine (B6)	5
Vitamin B12	1
Riboflavin (B2)	0.8

Table 3. Determination of thiamine in pharmaceutical preparations

Samples	Thiamine content (mg)[a]	Thiamine found (mg)[a,b]	Thiamine added (mol L^{-1})[c]	Recovery (%)[b]
Vitamin B1 Tablet	100	99.0 ± 0.6	1.0×10^{-5}	103 ± 1.4
			1.5×10^{-5}	102 ± 1.1
			2.0×10^{-5}	99.3 ± 0.8
Vitamin B1 Tablet	300	308 ± 5	1.0×10^{-5}	104 ± 2.1
			2.0×10^{-5}	100.3 ± 0.7
			3.0×10^{-5}	100 ± 1.2
Vitamin B complex Tablet	5.0	5.1 ± 0.1	2.0×10^{-6}	99.0 ± 0.9
			3.0×10^{-6}	101 ± 1.3
			4.0×10^{-6}	102 ± 1.9
Vitamin B complex Syrup	5.0	5.12 ± 0.08	2.0×10^{-6}	104 ± 1.1
			3.0×10^{-6}	102.5 ± 0.8
			4.0×10^{-6}	101 ± 1.5

[a]mg per tablet or mg per 5 ml syrup. [b]Averages of three determinations ± standard deviation. [c]The given values are the concentrations in the final solutions.

Study of interferences. The effects of foreign substances on the determination of 5×10^{-6} mol L^{-1} thiamine were examined. A substance was considered to interfere when the variation in fluorescence signal was more than 5%. The results are shown in Table 2. The tolerance level for other water-soluble vitamins allows the proposed method to be

used to determine the thiamine content in vitamin B complex preparations.

Applications. The proposed method was applied to the determination of thiamine in four pharmaceutical samples. The results are shown in Table 3. Statistical analysis of these results showed that there are no significant differences between experimental values and those reported by the manufacturing laboratories. It should be noted that concentrations of other ingredients present in vitamin B complex tablet and syrup are lower than their tolerance limits. Therefore, as the obtained results confirm, there are no interferences from these compounds.

Recovery experiments on pharmaceutical preparations spiked with different amounts of thiamine were also carried out. As can be seen from Table 2, the obtained recoveries are between 99 and 104%.

Conclusions

The crude extract of kohlrabi is an inexpensive and stable source of peroxidase and can be used in the spectrofluorimetric determination of thiamine. The proposed method is sensitive and has a wide linear range and was successfully applied to the determination of thiamine content of vitamin B complex preparations.

Acknowledgement. The authors acknowledge financial support offered by the Research Affairs of the University of Tabriz.

References

1. Ozdemir, D.; Dinc, E. *Chem. Pharm. Bull.* **2004**, *52*, 810.
2. Barrales, P. O.; de Cordova, M. L. F.; Diaz, A. M. *Anal. Chim. Acta* **1998**, *376*, 227.
3. Perez-Ruiz, T.; Martinez-Lozano, C.; Sanz, A.; Guillen, A. *J. Pharm. Biomed. Anal.* **2004**, *34*, 551.
4. Vinas, P.; Lopez-Erroz, C.; Cerdan, F. J.; Campillo, N.; Hernandez-Cordoba, M. *Michrochim. Acta* **2000**, *134*, 83.
5. Chen, H. W.; Zhu, J. P.; Cao, X. X.; Fang, Q. J. *Analyst* **1998**, *123*, 1017.
6. Du, J. X.; Li, Y. H.; Lu, J. R. *Talanta* **2002**, *57*, 661.
7. Song, Zh.; Hou, S. *J. Pharm. Biomed. Anal.* **2002**, *28*, 683.
8. Bohrer, D.; do Nascimento, P. C.; Ramirez, A. G.; Mendonca, J. K. A.; de Carvalho, L. M.; Pomblum, S. C. G. *Microchem. J.* **2004**, *78*, 71.
9. Li, K. *Biomed. Chromatogr.* **2002**, *16*, 504.
10. Wan, Q. J.; Yang, N. J.; Ye, Y. K. *Anal. Sci.* **2002**, *18*, 413.
11. Ryan, M. A.; Ingle, J. D. *Anal. Chem.* **1980**, *52*, 2177.
12. Martinez-Lozano, C.; Perez-Ruiz, T.; Tamas, V.; Albellan, C. *Analyst* **1990**, *115*, 217.
13. Dyke, S. F. *The Chemistry of Vitamins*; Interscience: New York, 1965.
14. Fujiwara, M.; Matsui, K. *Anal. Chem.* **1953**, *25*, 810.
15. Perez-Ruiz, T.; Martinez-Lozano, C.; Tamas, V.; Ibarra, I. *Talanta* **1992**, *39*, 907.
16. Jie, N. Q.; Yang, J. H.; Zhan, Z. G. *Anal. Lett.* **1993**, *26*, 2283.
17. Li, J. Z.; Zhang, Z. J.; Li, L. *Talanta* **1994**, *41*, 1999.
18. Veitch, N. C. *Phytochemistry* **2004**, *65*, 249.
19. Iwada, A.; Mifune, M.; Hazawa, T.; Mukuno, T.; Oda, J.; Motohashi, N.; Saito, Y. *Anal. Sci.* **1999**, *15*, 841.
20. Chen, Q. Y.; Li, D. H.; Yang, H. H.; Zhu, Q. Z.; Zhang, H.; Xu, J. G. *Analyst* **1999**, *124*, 771.
21. Rechnitz, G. A.; Ho, M. Y. *J. Biothechnol.* **1990**, *15*, 201.
22. Uchiyama, S.; Suzuki, S. *Anal. Chim. Acta* **1992**, *261*, 361.
23. Fatibello-Filho, O.; Vieira, I. da C. *Analyst* **1997**, *122*, 345.
24. Fatibello-Filho, O.; Vieira, I. da C. *Anal. Chim. Acta* **1997**, *354*, 51.
25. Fatibello-Filho, O.; Vieira, I. da C. *Anal. Chim. Acta* **1998**, *363*, 111.
26. Vieira, I. da C.; Fatibello-Filho, O. *Analyst* **1998**, *123*, 1809.
27. Manzoori, J. L.; Amjadi, M.; Orooji, M. *Anal. Sci.* **2006**, *22*, 1201.
28. Trinder, P. *Ann. Clin. Biochem.* **1966**, *6*, 24.
29. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
30. Zollner, H. *Handbook of Enzyme Inhibitors*, 2nd Ed., Wiley-VCH: New York, 1993; Part A, pp 367-368.