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Indirect Competitive Immunoassay for Detection of Vitamin B_2 in Foods and Pharmaceuticals

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Supporting Information

ABSTRACT: An indirect immunoassay for the determination of vitamin B_2 in food samples and vitamin tablets was developed. A carbodiimide-modified active ester method was used to synthesize the immunogen for vitamin B_2. The coupling ratio of vitamin B_2 to carrier protein in immunogen was 19.98:1. The titer of the polyclonal antibody was 1:64000, and the antibody showed high specificity in the presence of vitamin B_2 photolytic products and other B group vitamins. The immunoassay showed detection limits (LODs) of 1.07 ng/mL in PBS, 24.6 ng/g in vitamin drink, and 0.50 mg/kg in milk powder. Recovery was 99.58−110.91% in milk powder and 70.20−100.5% in vitamin drink. Vitamin B_2 samples were analyzed by high-pressure liquid chromatography (HPLC) and the immunoassay, and results showed good agreement. Finally, this method was applied to detect vitamin B_2 in commercial milk powder and vitamin tablets, and the detected amount correlated well with the labeled amount.

KEYWORDS: *vitamin B_2, polyclonal antibody, immunoassay, pharmaceutical formulations*

INTRODUCTION

Vitamin B_2 (riboflavin) is an important member of the water-soluble vitamin family. It has been recognized that vitamin B_2 plays a pivotal role in many physiological activities of cells such as electron transfer processes in the respiratory chain.[1] Recent reports also link it to nucleic acid repair processes and cell apoptosis.[2] Inadequate dietary intake may lead to skin and mucosal disorders, including angular cheilitis and anemia. In addition, vitamin B_2 deficiency may also result in sensitivity to sunlight, glossitis, and seborrheic dermatitis.[3] Therefore, vitamin B_2 becomes a very important part in vitamin-fortified food because the human organism cannot synthesize it.

The Recommended Daily Allowance (RDA) for vitamin B_2 in China is similar to that in the United States. Recommendations for daily vitamin B_2 intake with pregnancy and lactation are 1.6 mg (U.S.)[4] and 1.7 mg (China). For infants, the RDA is 0.3−0.4 mg/day, and for children it is 0.6−1.2 mg/day. In March 2012, China updated the regulation "Hygienic Standard for the Use of Nutritional Fortification Substances in Foods" (GB 14880-94) to version GB 14880-2012. In the new standard, which was formally implemented from January 1, 2013, vitamin B_2 fortification needs to be 8−14 mg/kg in infant food and 4−22 mg/kg in pregnancy formulations. Thus, analysis of vitamin B_2 in nutrient foods for quality control is necessary and important.

To date, analytical methods for vitamin B_2 determination include microbiological assay,[5] spectrophotometric,[6,7] HPLC (LOD = 30 ng/mL),[3,8] fluorescence (LOD = 3 ng/mL),[9−11] chemiluminescence,[12] and electrochemical methods (LOD = 9.1 ng/L).[13,14] Instrumental detections usually require expensive apparatus and skilled operators. There are also commercial kits using microbiological methods; however, the operation is time-consuming. For example, incubation time needs to be 44 h (LOD = 0.4 mg/kg) for VitaFastVitamin B_2 microbiological vitamin test kits (R-Biopharm AG, Darmstadt, Germany). As described above, the present methods are disadvantageous in requiring time-consuming operation and expensive instruments and are not suitable for on-site testing. As an ideal method for rapid determination, immunoassay methods have been developed for several vitamins, including vitamins D_2 and D_3,[15,16] folic acid,[17,18] and vitamin B_{12}.[19,20] The immunoassay established on the basis of antibody−antigen interaction is specific and rapid. It requires only common instrumentation and can be sensitive. As far as we know, there is no published report about immunoassay development for vitamin B_2. In this study, an indirect enzyme-linked immunoassay (ELISA) was developed by producing a sensitive and specific polyclonal antibody against vitamin B_2. The described method was comparable in vitamin B_2 detection with a traditional HPLC method and could be used as a screening test of vitamin B_2 in milk and pharmaceutical formulations.[21]

MATERIALS AND METHODS

Materials and Apparatus. Bovine serum albumin (BSA), ovalbumin (OVA), ethylenediamine, 4-dimethylaminopyridine (DMAP), *N*,*N*′-carbonyldiimidazole (CDI), 1-(3-dimethylamino-propyl)ethylcarbodiimide hydrochloride (EDC·HCl), *N*,*N*-dimethyl-

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Figure 1. Synthesis of immunogen (cBSA−vitamin B_2).

formamide (DMF), Freund's complete adjuvant (FA), incomplete adjuvant (iFA), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit IgG labeled with horseradish peroxidase (HRP), 3,3′5,5′-tetramethylbenzidine (TMB), and urea hydrogen peroxide were from Sangon Biotech Co. (Shanghai, China). Lumichrome is from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Vitamins B_2, B_1, B_5, B_6, B_8, B_9, B_{12}, and PP (B_3, niacin), lumiflavine, and trinitrobenzenesulfonic acid (TNBS) were from Sigma-Aldrich. Vitamin B_2 tablets and compound vitamin B tablets were from Tianjin LiSheng Pharmaceutical Co. Ltd. (Tianjin, China). Nestlé milk powders were purchased from a local supermarket (Tianjin, China).

The buffers used in this work were (1) PBS solution, sodium phosphate-buffered saline (pH 7.4) containing 0.138 mol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, and 7 mmol/LNa_2HPO_4; (2) coating buffer (pH 9.6), 0.015 mol/L Na_2CO_3 and 0.035 mol/L $NaHCO_3$ in distilled water; (3) blocking buffer, 10 g/L OVA in PBS with addition of 0.5 mL/L Tween 20; (4) washing buffer (PBST), PBS buffer containing 0.5 mL/L Tween 20; (5) substrate buffer, TMB solution (1 mmol/L TMB mixed with an equal volume of 1 mmol/L urea hydrogen peroxide citrate buffer, pH 5.5); and (6) enzymatic stopping solution, 2 mol/L hydrochloric acid.

ELISA signal was measured by enzyme immunoassay microplate reader model 680 from Bio-Rad Laboratories (Hercules, CA, USA). Polystyrene microtiter plates (96-well) were from Jet Biofiltration Products Co., Ltd. (Beijing, China). UV−vis spectra of conjugates were obtained with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Preparation of Cationized BSA (cBSA) and Cationized OVA (cOVA). To convert carboxylic acid groups in BSA into primary amino groups, BSA was treated with an excess of ethylenediamine as described previously.[22] For this purpose, 15 mL of ethylenediamine was slowly added into 30 mL of PBS (0.1 M, pH 7.4) at 4 °C. Then, 30 mL of 12 N HCl was added to keep the mixture neutral. One gram of BSA and 0.369 g of EDC were added under stirring followed by incubation for 4 h at room temperature. The mixture was dialyzed against PBS (0.1 M, pH 7.4) for 3 days and then against distilled water for another 3 days. The solution in the dialysis bag was lyophilized and stored at −20 °C before use. The cOVA was prepared in the same way.

Preparation of Immunogen and Coating Antigen. Immunogen cBSA−vitamin B_2 and coating antigen cOVA−vitamin B_2 were prepared by a carbodiimide-modified active ester method (Figure 1).[23] In this procedure, 19.4 mg of vitamin B_2, 25.0 mg of CDI, and 0.6 mg of DMAP were dissolved in 4 mL of DMF with stirring for 12 h at room temperature. The mixture was then slowly added into 4 mL of PBS (0.1 M, pH 7.4) containing 100 mg of cBSA and incubated for 8 h at room temperature. The mixture was dialyzed against PBS (0.1 M, pH 7.4) for 3 days and then against distilled water for another 3 days. The dialyzing medium was changed every 12 h, and the solution in the dialysis bag was lyophilized. Coating antigen (cOVA−vitamin B_2) was prepared and purified according to a similar method.

The conjugation ratio of hapten to carrier protein was measured by trinitrobenzenesulfonic acid (TNBS) assay.[24] To 1 mL of 100 μg protein solution (cBSA, cBSA−vitamin B_2, cOVA or cOVA−vitamin B_2), 1 mL of $NaHCO_3$ (4%, pH 8.5) and 1 mL of 0.01% TNBS solution were added. The reaction was kept at 42 °C for 2 h, and 1 mL of 10% SDS with 0.5 mL of 1 N HCl was added. UV absorbance of the mixture was read at 335 nm to build a standard curve with BSA after blank corrections. The conjugation ratio was calculated on the basis of the decrease of UV absorbance in different samples.

Production of Polyclonal Antibodies for Vitamin B_2. Animal treatments were conducted in accordance with the guidelines of the Chinese Association for Laboratory Animal Sciences. Two healthy male New Zealand white rabbits (2.0 kg) were immunized to generate the polyclonal antibody against vitamin B_2. Before immunization, 1 mL of blood was taken from each rabbit and used as the negative group. For the initial immunization, 0.5 mg of immunogen in 0.5 mL of saline were emulsified with 0.5 mL of FA and subcutaneously injected at multiple sites on the backs of the rabbits. For subsequent immunizations, the amount of immunogen was decreased by half using iFA instead of FA. The immunizations were carried out every 2 weeks. At the fifth immunization, 0.25 mg of immunogen was dissolved in 0.5 mL of saline and injected. One week later, all rabbits were exsanguinated by heart puncture under general anesthetic. The blood was allowed to clot for 2 h at room temperature, overnight at 4 °C, and then centrifuged at 8000*g* for 10 min. The supernatant liquor from the centrifuge tube was collected and served as the antiserum for the next experiments.

Immunoassay Procedure and Evaluation. *Titer Determination.* A 96-well polystyrene microtiter plate was coated with 100 μL/well of the coating antigen (2 μg/mL) and incubated at 37 °C for 2 h. After the excess coating buffer was removed, blocking buffer was added (250 μL/well) and the wells were washed with PBST after 2 h of incubation at 37 °C. The antiserum was serially diluted (1:4000, 1:8000, 1:16000, 1:32000, 1:64000, and 1:128000) and then added at 100 μL/well, followed by incubation at 37 °C for 0.5 h. The wells were then washed three times using 250 μL of PBST. Then, 100 μL of HRP-conjugated goat anti-rabbit IgG (1:2000 in PBS) was added and incubated for 0.5 h. After washing, 100 μL of TMB substrate solution was added. After 10 min, the enzymatic reaction between HRP and TMB was immediately stopped by 100 μL of enzymatic stopping solution, and the absorbance was measured at 450 nm with a microplate reader. The titer of the antibody was calculated as the dilution factor of the antiserum with absorbance value twice that for the negative group.

Sensitivity. The sensitivity of the method was estimated by an indirect competitive ELISA method. The determination was performed in a fashion basically the same as indicated above, except that after the blocking buffer was removed, 50 μL of diluted antibody (1:2000) was added to each well, and then 50 μL of analyte samples was added and mixed to incubate at 37 °C for 30 min. ELISA result was expressed as B/B_0, where B_0 is the absorbance value of blank sample and B is the absorbance value of the sample with different concentrations of vitamin B_2. The competitive curve was generated by B/B_0 versus vitamin B_2 level. The IC_{50} value was calculated according to the midpoint of the curve by Origin 8.5 (OriginLab, USA).

Specificity. The specificity of the antibody was investigated by cross-reactivity (CR) experiments. Two photolytic products and several water-soluble vitamins were tested, including vitamins B_1, B_5, and B_6, biotin, folic acid, vitamin PP, and vitamin B_{12}. Cross-reactivity was expressed as percentage ratio of IC_{50} value based on 100% response of vitamin B_2 and calculated as follows:

$$\mathrm{CR}\,(\%) = (\mathrm{IC_{50}\ of\ vitamin\ B_2/IC_{50}\ of\ cross\text{-}reacting\ compound}) \times 100\%$$

Comparison with HPLC Method. Standard solutions of vitamin B_2 were prepared and detected by HPLC method with an Inert Sustain C18 column (150 × 4.6 mm i.d., 5 μm, Shimadzu-GL, Shanghai, China). A volume of 20 μL of standard or sample solution was injected into an Ultimate 3000 liquid chromatography system (Dionex,

Sunnyvale, CA, USA). Mobile phase was a mixture of 35% methanol in double-distilled water. Flow rate was 1.0 mL/min with detection at 267 nm. Peak areas of the standard solutions were plotted against vitamin B_2 concentration, and the resulting standard curve was used to determine vitamin B_2 concentrations in PBS.

ELISA Application in Authentic Foods and Pharmaceuticals. Milk powder and vitamin drink were purchased from a local supermarket. Vitamin drink was diluted 10-fold with PBS. Two kinds of milk powder were used. One is milk powder with no vitamin B_2 on the label, used as unfortified sample to measure matrix effect. Another is infant milk powder that has been already spiked with vitamin B_2 and, therefore, used as the positive food product. Milk powder (1 g) was dissolved in distilled water (100 mL) and centrifuged at 8000*g* for 10 min. The fat layer was discarded, and the upper liquid was diluted different times with PBS. For unfortified sample, the upper liquid was directly analyzed without further dilution; for the positive group, the upper liquid was diluted by 50 times before analysis.

Twenty unfortified samples of vitamin drink and milk powder were determined to calculate the limit of detection in each matrix. Recovery and coefficients of variation (CVs) were determined at fortified levels of 5, 25, and 50 ng/mL.

Commercial infant milk powder, vitamin B_2 tablet, and compound vitamin B tablet were analyzed by ELISA. One vitamin B_2 tablet was dissolved in distilled water (500 mL) and then diluted 1000 times to 10 ng/mL (vitamin B_2). The compound vitamin B tablet was handled in a similar way, and the final concentration of vitamin B_2 was 10 ng/mL. Then ELISA was performed as described above. The detected level was calculated to compare with the labeled amount.

■ RESULTS AND DISCUSSION

Verification of Immunogen. Before coupling with vitamin B_2, carrier proteins were activated by ethylenediamine first, so that carboxylic acid groups can be converted into primary amino groups and conjugate with the hydroxyl group in vitamin B_2 by a carbodiimide-modified active ester method (Figure 1).

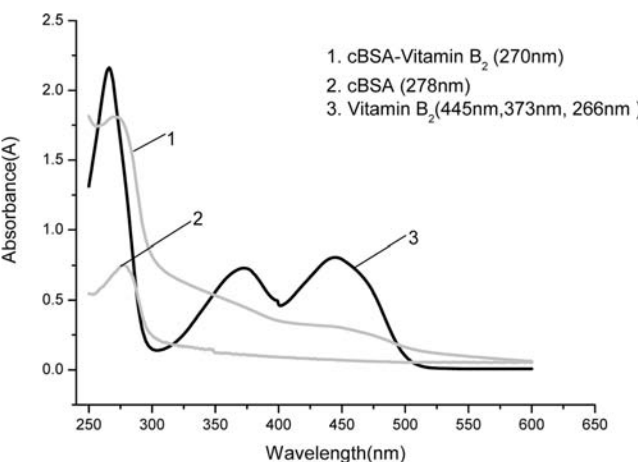

Figure 2. UV spectra of vitamin B_2 (30 μg/mL), cationized bovine serum albumin (cBSA, 1 mg/mL), and the immunogen (cBSA–vitamin B_2, 2.4 mg/mL).

The conjugation was then investigated by UV analysis. In UV spectra (Figure 2), cBSA showed a characteristic absorption peak at 278 nm, whereas cBSA–vitamin B_2 has the peak at 270 nm. A significant blue shift was observed in the UV absorption peak of cBSA–vitamin B_2, which is probably due to vitamin B_2 (λ_{max} = 266 nm) coupling. In the trinitrobenzenesulfonic acid assay, ε-amino groups in carrier proteins could react with TNBS reagent. Absorbance (B) in 335 nm was in good linear relationship with the amount (m) of BSA (50–200 μg range) in the sample ($B = 0.001m - 0.0052$, $R^2 = 0.992$). Assuming that BSA and OVA have 59 and 20 TNBS reactive amino groups, respectively,[25] the numbers of ε-amino groups in cBSA, immunogen (cBSA–vitamin B_2), cOVA, and coating antigen (cOVA-vitamin B_2) could be calculated. The conjugation ratio of hapten to carrier proteins can be accordingly obtained. As shown in Table 1, both carrier proteins have more ε-amino groups after activatation using ethylenediamine, and conjugation ratios are 19.98:1 and 10.26:1 for cBSA–vitamin B_2 and cOVA–vitamin B_2, respectively.

Table 1. Coupling Ratio of Immunogen (cBSA–Vitamin B_2) and Coating Antigen (cOVA–Vitamin B_2)

sample	ε-amino groups	coupling ratio[a]
BSA	59[b]	
cBSA	113.6	
cBSA–vitamin B_2	93.92	19.98:1
OVA	20[b]	
cOVA	62.71	
cOVA–vitamin B_2	54.45	10.26:1

[a]Coupling ratio is the molar ratio of hapten to carrier protein. [b]The number of ε-amino groups in BSA and OVA has been demonstrated previously.[25]

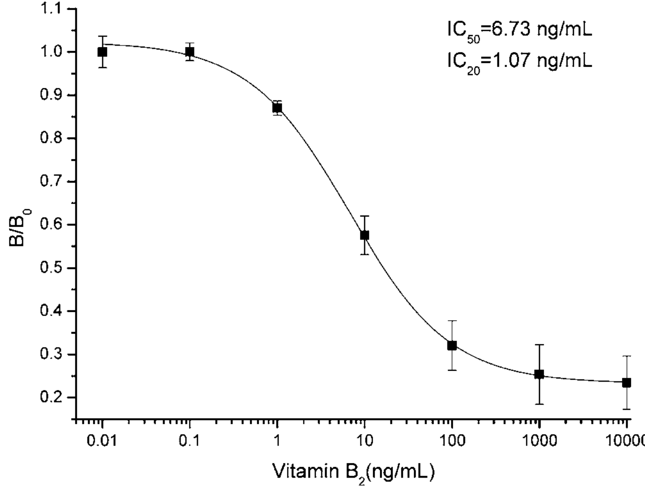

Figure 3. Inhibition curve of vitamin B_2 according to the proposed ELISA method (n = 3).

Table 2. Accuracy and Precision of Vitamin B_2 Detection in PBS by ELISA (n = 4)

added amount (ng/mL)	day	measured amount (ng/mL)	recovery (%)	intra-assay CV (%)	interassay CV (%)
5	1	5.07 ± 0.26	101.45	5.07	11.63
	2	5.25 ± 0.28	105.15	5.33	
	3	5.62 ± 0.60	112.40	10.73	
25	1	24.63 ± 0.86	98.52	3.49	5.17
	2	24.40 ± 1.17	97.63	4.78	
	3	24.98 ± 1.41	99.92	5.63	
50	1	51.70 ± 2.05	103.40	3.96	9.90
	2	50.61 ± 1.43	101.22	2.82	
	3	47.44 ± 4.19	94.89	8.83	

Table 3. Cross-Reactivity of ELISA to Related Compounds and Vitamins

Analyte	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
Vitamin B_2		6.72	100
Lumichrome		1026.7	0.66
Lumiflavine		1444.9	0.46
Vitamin B_1 (Thiamine)		>100000	<0.01
Vitamin B_5 (Pantothenic acid)		>100000	<0.01
Vitamin B_6 (Pyridoxine)		>100000	<0.01
Vitamin B_8 (Biotin)		>100000	<0.01
Vitamin B_9 (Folic acid)		>100000	<0.01
Vitamin PP (Niacin)		>100000	<0.01
Vitamin B_{12}		>100000	<0.01

Immunoassay Evaluation. This is the first report of antibody production and ELISA development against vitamin B_2. In the checkerboard experiment, the optimal concentration of coating antigen was found to be 2 μg/mL, and the dilution factor of antibody was 1:2000. The titer of the antibody was calculated as the dilution factor of the positive group with the absorbance value twice that for negative group. Finally, the titer was determined to be 1:64000.

Before evaluation of method sensitivity, we optimized the ELISA test with several variables, including buffer solution with

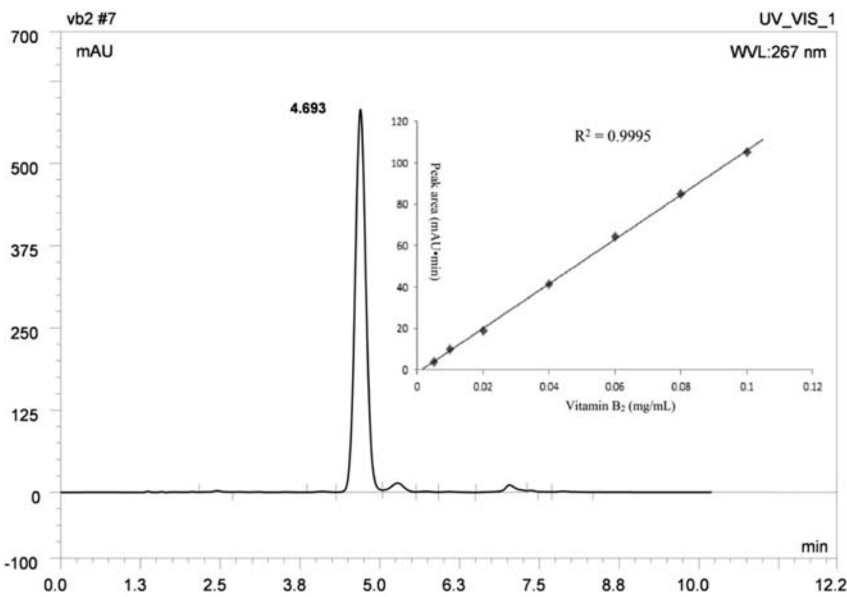

Figure 4. Representative HPLC chromatogram of vitamin B_2 and linear standard curve of the HPLC method (inset).

Table 4. Analysis of Vitamin B_2 by ELISA in Comparison with the HPLC Method (n = 3)

	ELISA		HPLC	
added (μg/mL)	measured (μg/mL)	recovery (%)	measured (μg/mL)	recovery (%)
14	15.50 ± 0.61	110.71	13.04 ± 0.32	93.14
50	51.84 ± 3.28	103.68	46.99 ± 0.80	93.98
90	88.28 ± 4.46	98.09	88.67 ± 0.17	98.52

different ionic strengths and pH values and incubation time. Finally, we selected PBS (pH 7.4) as the buffer. Incubation time for antibody with analyte was 30 min (detailed procedure and results are included in the Supporting Information). Fifty microliters of vitamin B_2 standard solution in PBS (0.01, 0.1, 1, 10, 100, 1000, 10000 ng/mL) was analyzed. As shown in Figure 3, the antibody showed significant binding with vitamin B_2, resulting in a typical inhibition curve. The vitamin B_2 concentration leading to 50% binding decrease (50% B/B_0, IC_{50}) was calculated to be 6.73 ng/mL. The limit of detection (LOD) defined as IC_{20} value was 1.07 ng/mL. The obtained B/B_0 values (y) showed good linear relationship with logarithmic vitamin B_2 concentration (x) in 1−80 ng/mL ($y = -0.3550x + 0.9442$, $R^2 = 0.9942$, $n = 4$). The results showed that the proposed method is sensitive for the detection of vitamin B_2, being even more sensitive than the traditional fluorescence detection system (3 ng/mL).[9]

For precision assessment, vitamin B_2 standard solutions (5, 25, and 50 ng/mL) were detected four times per day and three different days to calculate intra-assay and interassay variations. As shown in Table 2, the intra-assay coefficient of variation (CV) ranged from 2.82 to 10.73% in three days, and the interassay CV was <11.63%. It is certified that the ELISA method was stable and credible.

The specificity of the antibody should be considered the most important characteristic for an immunoassay. The cross-reaction of vitamin B_2 antibody was evaluated using two vitamin B_2 photolytic products, lumiflavine and lumichrome, and seven commonly used vitamin B compounds in this work. The result in Table 3 showed that the vitamin B_2 antibody did not react with the other vitamin B compounds (<0.01%) and cross-reacted little with the photolytic products (CR < 1.0%), which was acceptable in the analysis.

The performance of the ELISA method was compared with a confirmatory HPLC method for vitamin B_2 analysis. As indicated in Figure 4, vitamin B_2 could be detected at 4.69 min. The inset shows the linear relationship of peak area with vitamin B_2 concentration in buffer ($R^2 = 0.9995$) in the range of 0.005−0.1 mg/mL. The LOD of the HPLC method was measured to be 1.503 ng/mL, which is a little higher than that of the ELISA method (LOD = 1.07 ng/mL). Three vitamin B_2 samples (14, 50, and 90 μg/mL) were tested by ELISA and HPLC methods, respectively. The results obtained by ELISA

Table 5. Recovery and Coefficient of Variation (CV) for Vitamin B_2 Determination in Food Products by ELISA

		interassay			intra-assay		
sample	spiked (ng/mL)	detected (ng/mL)	recovery (%)	CV (%)	detected (ng/mL)	recovery (%)	CV (%)
milk powder	5	5.20 ± 0.20	103.95	3.92	5.10 ± 0.59	102.07	11.62
	25	26.31 ± 2.25	105.22	8.57	27.72 ± 2.88	110.91	10.37
	50	49.79 ± 3.38	99.58	6.79	52.29 ± 4.88	104.58	9.33
vitamin drink	5	3.50 ± 0.22	70.20	6.40	3.72 ± 0.34	74.40	9.01
	25	21.86 ± 1.28	87.43	5.84	24.54 ± 2.56	98.16	10.43
	50	49.415 ± 5.15	98.83	10.43	50.27 ± 5.61	100.54	11.16

Table 6. Determination of Vitamin B_2 in Authentic Samples ($n = 5$)

vitamin B_2 product	labeled amount	found amount	recovery (%)	qualified?
milk powder	≥12 mg/kg	15.50 ± 0.47 mg/kg	not applicable	yes
vitamin B_2 tablet	5 mg/tablet	5.16 ± 0.54 mg/tablet	103.2	yes
compound vitamin B tablet	1.5 mg/tablet	1.68 ± 0.19 mg/tablet	112.3	yes

and HPLC agreed well with each other (Table 4) with a satisfactory coefficient of 0.9998.

ELISA Application in Authentic Foods and Pharmaceuticals. It is reported that milk is the most common food group contributing to the total vitamin B_2 intake (14.5−16.0%), so milk powder was selected as the matrix, and vitamin drink was also studied. The two matrices were treated as described above and detected by using the proposed method.

When food samples are used to evaluate ELISA performance, a LOD value should be known, to determine the fortification amount of analyte in food samples. A LOD value was calculated as the mean detected vitamin B_2 concentration in 20 unfortified samples plus 3 times the standard deviation (SD) (average + 3SD). As a result, the LOD for blank milk powder was 5.02 (1.96 + (3 × 1.02) ng/mL), whereas the LOD for vitamin drink was 2.46 (1.22 + (3 × 0.41) ng/mL), both higher than that in buffer (1.07 ng/mL). Presented as weight unit in original products, LOD values are 0.5 mg/kg for milk powder and 24.6 ng/g for vitamin drink. The matrices were spiked with final vitamin B_2 concentrations of 5, 25, and 50 ng/mL and tested four times on one day and on three different days. As shown in Table 5, analytical recoveries of vitamin B_2 were 99.58−103.95% in milk powder and 70.20−98.83% in vitamin drink.

Infant milk powder and two kinds of vitamin tablets were chosen for the ELISA analysis. As shown in Table 6, the detected amount of vitamin B_2 in vitamin tablets and milk powder by ELISA method correlated well with the labeled amount, indicating that the proposed method could be a satisfactory way for vitamin B_2 quality control in authentic samples.

This work presents a sensitive and specific high-throughput detection method for vitamin B_2 determination in foods and pharmaceutical formulations. The limit of detection reaches 1.07 ng/mL in PBS, 24.6 ng/g in vitamin drink, and 0.50 mg/kg in milk powder. The recovery rates and CVs of this method in milk powder and vitamin tablets are satisfactory. The compatibility between the immunoassay and HPLC method showed the potential of the ELISA method as an ideal screening tool for testing a large number of food samples before validation of positive samples by instrumental analysis.

ASSOCIATED CONTENT

Supporting Information

Procedure and results (one table and one figure) for ELISA optimization in buffer and incubation time. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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