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Disposable Sensor for Measurement of Vitamin B_2 in Nutritional Premix, Cereal, and Milk Powder

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Methodologies for the determination of vitamin B_2 in food matrixes and a premix using simple sample conditioning steps coupled with a convenient and cheap electrochemical sensing device are presented. Electrochemical analysis based on differential pulse voltammetry (DPV) coupled to carbon electrodes gave a well-defined reduction peak at −0.42 V versus a Ag/AgCl quasi-reference electrode. Using a straightforward sample preparation step, vitamin B_2 can be measured successfully in a nutritional premix and food products. Standard additions of riboflavin were used to confirm the analyte concentrations and to provide precision data.

KEYWORDS: Vitamin B_2; voltammetry; screen-printed sensor; nutritional premix; corn flake cereal; milk powder

INTRODUCTION

A problem for companies manufacturing nutritionally fortified foods or foods intended as slimming aids is the need to monitor and control the levels of micronutrients (premixes) added during food production, to comply with labeling, quality, and safety requirements. Nutritional components such as vitamins are known to degrade during preparation, preservation, and storage. These problems arise through breakdowns caused by variation in the environmental conditions, e.g. pH, humidity, and temperature. With premix sometimes added at sufficient "overage" (e.g. 2−4 times the actual recommended daily allowance in the cereal manufacturing industry) to account for the level of nutrient loss occurring during processing and storage of the product, there is an urgent need for simple, rapid procedures for the quantitative measurement of micronutrients in breakfast cereals, milk products, and premixes (*1*, *2*).

Various analytical techniques have been used to determine micronutrients such as vitamin B_2 (riboflavin). These include chemiluminescence (*3*), high-pressure liquid chromatography (HPLC) coupled to fluorometric (*4*−*6*) or ultraviolet (UV) (*7*, *8*) detection, electrochemical methods such as voltammetry (*9*−*13*), biosensors based on the principle of surface plasmon resonance (*14*), fluorescence (*15*), and spectrophotometry (*16*). HPLC with optical detection is widely used for quantitation of vitamin B_2 in food samples (*14*) because of its ability to effect rapid separation and its high sensitivity of detection (*7*, *13*). The high cost of the required instrumentation is, however, a disadvantage. Voltammetry, on the other hand, offers a viable alternative due to the availability of sensitive but inexpensive instrumentation with low power requirements (*13*).

In this work, simple sample conditioning procedures coupled to a voltammetric sensor are described for the detection of vitamin B_2 in a corn flake cereal as well as milk powder and nutritional premix. This involves the use of low cost disposable screen-printed sensors incorporating a three-electrode system (carbon working electrode, silver/silver chloride quasi-reference electrode, and carbon counter electrode).

EXPERIMENTAL PROCEDURES

Equipment and Reagents. Voltammetric experiments were performed using screen-printed sensors and an electrochemical potentiostat workstation (Autolab PGSTAT10, Eco Chemie B.V., Utrecht, Netherlands) with PC software control (GPES 4.9, Eco Chemie). An edge connector (Maplin, Milton Keynes) was used to complete electrical connection to the potentiostat. An HP 8452A diode array spectrophotometer was used for visible absorbance measurements.

All reagents were of analytical grade. Riboflavin, dibasic sodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$), potassium chloride, acetic acid, copper(II) sulfate pentahydrate, hydrochloric acid, sucrose, starch, magnesium oxide heavy, ferrous sulfate, folic acid, pyridoxine hydrochloride (vitamin B_6), cyanocobalamin (vitamin B_{12}), thiamine hydrochloride, and nicotinamide were obtained from Sigma-Aldrich (Dorset, U.K.). Deionized water was obtained from an Elgastat Option 3 water purification system (Elga Ltd, U.K.).

Samples. The samples used for this study included a vitamin B complex premix, a dietetic milk powder reference sample, and a corn flake cereal.

Sensor Fabrication. Electrodes (1 cm × 4.5 cm) were printed in-house with a DEK 1760RS semiautomatic screen-printer (Printing Machines, Weymouth, U.K.) using graphite-carbon (ED5000, Electrapolymers & Chemicals Ltd, Tonbridge, U.K.), silver/silver chloride, and dielectric inks (C61003P7 and D60202D1, GEM Ltd, Pontypool, U.K.). The multistage printing process involved the sequential deposition of the inks onto the PVC substrate material (Cadillac Plastic, Swindon, U.K.) in controlled patterns and thicknesses. The carbon conductive tracks were printed first onto PVC substrate (440 μM thick)

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and cured (55 °C, 3 h). Subsequently, dielectric was printed over the carbon conductive tracks, leaving openings that allow electrical contact with the measurement circuit at one end and the sample solution at the other end. The layer was dried (55 °C, 16 h) before printing a silver/silver chloride pad positioned appropriately over one of the carbon tracks.

Procedure for Electrochemical Study of Vitamin B_2. *Riboflavin Stock Solution.* The concentration dependence of vitamin B_2 was examined using a stock solution prepared from a weighed amount of riboflavin. The riboflavin stock solution was prepared in a pH 6 acetate-phosphate/KCl buffer (50 mM acetic acid, 50 mM $Na_2HPO_4 \cdot 7H_2O$, and 50 mM KCl) and sonicated in an ultrasonic bath (F5100b, Decon Laboratory Ltd, England) for 20 min. The solution was protected from photodegradation by covering its container with aluminum foil.

A molar extinction coefficient (ϵ) value of 11 100 M^{-1} cm^{-1} at 445 nm was determined on the basis of the slope relating measured absorbances versus calculated vitamin B_2 concentrations for test solutions prepared from riboflavin stock. A value of 12 500 M^{-1} cm^{-1} was reported in the literature (*17*). Spectrophotometric measurements of subsequent stock solutions were carried out to verify the concentration of vitamin B_2 from the measured absorbance values.

Sample Preparation for Vitamin B Complex Premix. For the determination of vitamin B2 in the nutritional premix sample, an amount between 5 and 9 mg was rigorously weighed and dissolved in 30 mL of buffer solution. The solution was sonicated in an ultrasonic bath for 10 min. Samples for measurement were prepared by pipetting 0.5 mL of the premix sample solution into a cuvette filled with 1.5 mL of acetate-phosphate/KCl buffer solution. For quantification, standard additions of different volumes of the riboflavin stock solution were added to the premix−buffer solution and differential pulse voltammetry (DPV) measurement was carried out.

Sample Preparation for Dietetic Milk Powder. The sample preparation procedure developed for the milk powder samples involved precise weighing of between 1 and 3 g of milk powder sample and suspending it in 20 mL of 0.1 M HCl solution. The suspension was mixed for ~3 min to homogenize it, and 7.84 g of ammonium sulfate was added to precipitate the proteinous components in the milk powder suspension. The suspensions were spin mixed for 3 min and then centrifuged (Denley BS400, England) for another 15 min at 3000 rpm. Filtration was then carried out with a 0.2 μm syringe filter (Minisart-plus 17823 K, Vivascience AG, Germany) to obtain the solution for vitamin B_2 analysis. Samples for measurement were prepared by pipetting 2 mL of the sample solution into a cuvette containing 2 mL of acetate-phosphate/KCl buffer solution. For quantification, standard additions of different volumes of the riboflavin stock solution were added to the sample−buffer solution and DPV measurement was carried out.

Sample Preparation for Corn Flake Cereal. The sample preparation procedure for the corn flake cereal involved precise weighing of between 1.7 and 2.0 g of ground sample and suspending it in 10 mL of deionized water. The suspension was mixed for ~3 min to homogenize it, and 3.92 g of ammonium sulfate was added to precipitate out the proteinous components present. The suspensions were spin mixed for 3 min and centrifuged for another 10 min at 3000 rpm. Filtration was carried out with a 0.2 μm syringe filter to obtain the extracted vitamin B_2 solution. To quantify the vitamin B_2 in the cereal, three standard additions of different concentrations of riboflavin were made to the cereal before the extraction. The determination of vitamin B_2 in the corn flakes sample extract was made by adding 2 mL of the supernatant solutions to 2 mL of the acetate-phosphate buffer/KCl (total volume = 4 mL).

Voltammetric Measurements. DPV measurements with screen-printed sensors were carried out using the following voltammetric parameters: modulation amplitude = 50 mV; modulation time = 0.05 s; interval time = 0.25 s; step size = 2 mV. Measurements were recorded between −0.1 V and −0.8 V (unless otherwise stated) using the screen-printed sensor. The concentration of vitamin B_2 in the sample was quantified using the standard additions method. The samples were not deoxygenated prior to measurement.

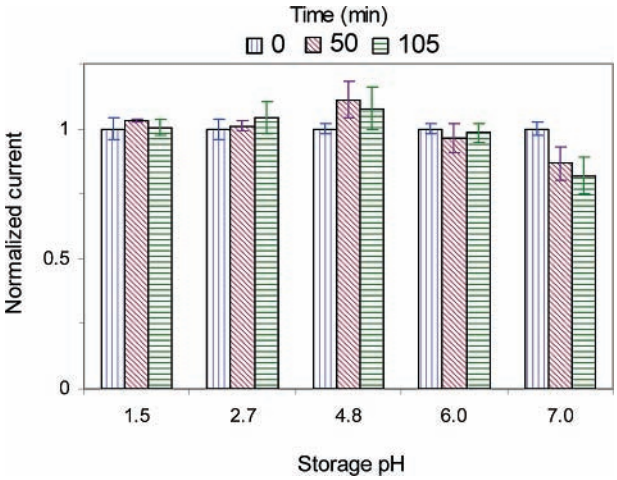

Figure 1. Normalized plot obtained for current versus pH for riboflavin prepared in different dissolution mediums prior to electrochemical measurements using 0.05 M acetate-phosphate/KCl buffer. Initial potential = –0.1 V, final potential = –0.8 V, modulation amplitude = 50 mV, modulation time = 0.05 s, interval time = 0.25 s, step size = 2 mV. The error bars represent the standard deviation of three independent measurements.

RESULTS AND DISCUSSION

Stability Studies of Vitamin B_2 at Different pH Values. This study was carried out to check whether the stability of riboflavin in solution was affected by the pH of the medium in which it was dissolved prior to electrochemical measurements. Sonication (20 min) was used to prepare riboflavin in a range of aqueous solutions: 0.1 M hydrochloric acid; 0.1 M acetic acid; pH 4.8 buffer—prepared from 0.1 M acetic acid by addition of sodium acetate to give the desired pH; pH 6 buffer—prepared by mixing equal volumes of 50 mM acetic acid and 50 mM sodium phosphate dibasic; and pH 7 buffer—prepared by mixing equal volumes of 50 mM sodium phosphate mono- and dibasic. A small volume of each stock solution was subsequently diluted with pH 6 acetate-phosphate/KCl buffer for electrochemical measurement. The measurements were carried out at 0, 50, and 105 min intervals (**Figure 1**). The plot suggests that riboflavin (vitamin B_2) is stable in acid media ($1.5 \leq pH \leq 6$) over the studied time period. However, at pH above 6, the electrochemical response decreased due to degradation of the riboflavin. An acetate-phosphate buffer containing KCl (pH 6.0, $I = 0.05$ M) was used for dissolving riboflavin and as supporting electrolyte in subsequent studies.

Analytical Curve and Reproducibility. It was important that the calibration data covered the concentration range expected for food products and premixes. A calibration curve for vitamin B_2 was prepared on the basis of DPV measurements by using standard solutions of riboflavin made by taking different volumes of the stock solution and diluting with pH 6 acetate-phosphate/KCl buffer in a cuvette. The peak currents obtained from DPV measurements were used to construct a calibration graph that was linear ($r^2 = 0.995$) in the 1−23 μg mL^{-1} concentration range ($Ip/\mu A = \{0.067 \pm 0.001\}$ [vitamin $B_2/\mu g$ mL^{-1}] + 0.057 ± 0.021). The detection limit (S/N = 3) was 0.9 μg mL^{-1}.

To evaluate the reproducibility of the electrode response, a series of 10 successive measurements of 4 μg mL^{-1} riboflavin were done. Between each voltammetric measurement, the electrode was exchanged for a new one. A mean peak response of 0.37 μA, with a range of 0.35−0.39 μA and a relative standard deviation (RSD) of 5.1%, was observed.

Table 1. Interference Study Involving 2 μg mL^{-1} (5 μM) Riboflavin in Acetate/Phosphate Buffer

interfering species	conc ratio[a] (vitamin B_2/interferent)	increase in current (%)
copper	1:0.6	+110
copper	1:7	+345
iron	1:8	−8
iron	1:80	−15
magnesium	1:103	−6
folic acid	1:0.2	−10
nicotinamide	1:25	−7
pyridoxine HCl	1:2	−17
cyanocobalamin	1:0.2	−15
thiamine HCl	1:1	−8

[a] Calculated on a mole per unit volume basis.

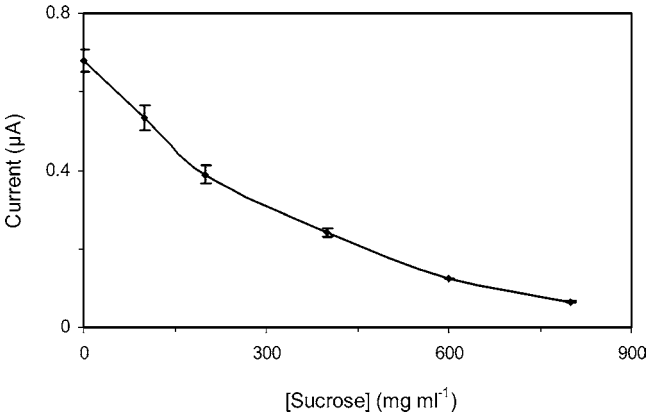

Figure 2. Effect of different amounts of sucrose on the DPV response of 5 μg mL^{-1} riboflavin (vitamin B_2) in 0.05 M acetate-phosphate/KCl buffer (pH 6). Other experimental conditions are as in Figure 1.

Interference Studies. The influence of commonly used excipients and additives in food products and premixes was tested under the optimized conditions with a riboflavin standard solution of 2 μg mL^{-1} (5 μM). **Table 1** shows the effect of the various interferents including minerals and other types of vitamins. Significant interference was observed from the presence of copper, which resulted in the enhancement of the voltammetric signal for vitamin B_2. Work is in progress to understand the mechanism for this enhancement effect, which could be a plausible way of improving the vitamin B_2 response and, hence, method sensitivity.

The influence of sugar concentration on the DPV response of 5 μg mL^{-1} riboflavin is depicted in **Figure 2**. The peak current for the vitamin B_2 response was found to decrease to 40% in the presence of about 200 mg mL^{-1} sucrose, and it was almost suppressed at sucrose concentrations higher than 500 mg mL^{-1}. This interference is likely to be due to the increase of the viscosity of the solution with increasing sugar concentration, which results in a lower rate of diffusion of the analyte toward the electrode surface and, hence, a decrease of the peak current. A similar effect on the DPV response of vitamin B_2 was observed for starch (not shown). The matrix effect caused by sugar or starch was overcome using the method of standard addition.

Determination of Vitamin B_2 in a Premix, a Milk Powder, and a Cereal Product. To verify the proposed method, the determination of vitamin B_2 in a nutritional premix sample, a dietetic milk powder sample, and a corn flake cereal sample was carried out using the method of standard additions described in the Experimental Procedures. The DPV responses and the standard addition plot for a typical determination are shown in

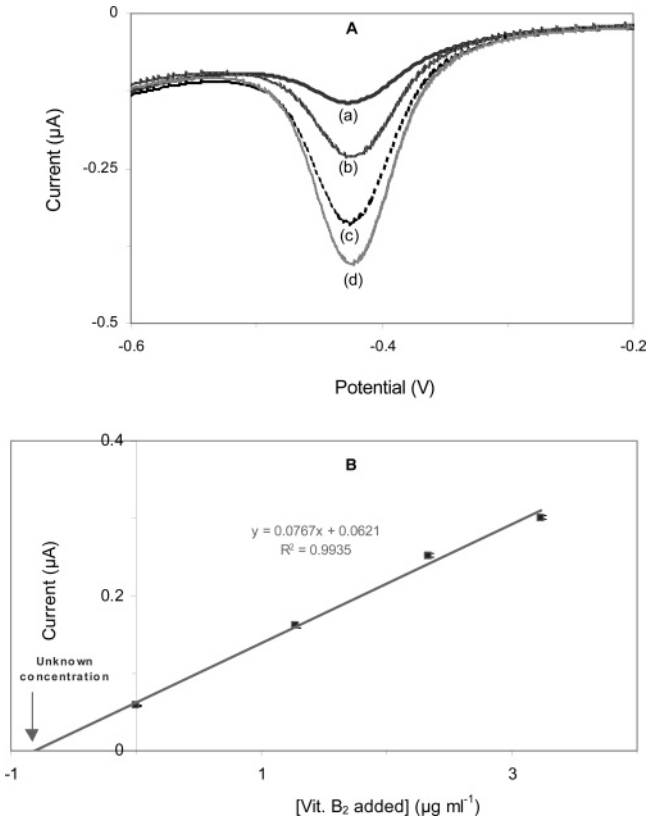

Figure 3. Typical voltammograms (**A**) and standard addition plot (**B**) for quantifying vitamin B_2 in milk sample extract: (a) sample; (b, c, and d) sample + increasing standard spikes (3.4, 6.2, and 8.6 μM). Volume of standard spikes: 200, 400, and 600 μL. Other experimental conditions are as in Figure 1.

Table 2. Analytical Data Obtained for Vitamin B_2 in Fortified Samples

sample	proposed method[a] (mg g^{-1})	ref method[b] (mg g^{-1})
vitamin B complex premix	84 ± 8[c]	81 ± 8[c]
dietetic milk powder	0.0132 ± 0.001[c]	0.0134 ± 0.001[d]
corn flake cereal	0.0133 ± 0.002[c]	0.0143 ± 0.001[c]

[a] DPV measurement with a disposable screen-printed sensor. [b] HPLC–UV. Data supplied by the manufacturing companies. [c] Mean of three replicate measurements ± standard deviation. [d] Mean obtained from collaborative studies ± standard deviation.

Figure 3, while the collected determination results are shown in **Table 2**. The t-test analyses performed on the vitamin B_2 concentration values obtained by both methods demonstrated that results generated by the proposed method were comparable to those of the reference method (HPLC−UV detection) and do not show significant bias at the 95% confidence level. This is also confirmed by the plot in **Figure 4**, where the difference between the obtained measurement data and the expected measurement data for vitamin B_2 in vitamin B complex premix, corn flake cereal, and milk powder is plotted against the mean (*18*, *19*). A mean difference of 0.004 μg mL^{-1} between the data sets was found. The best-fit regression line (not shown) over the concentration range examined (0.3−6.9 μg mL^{-1}) for the obtained values (y) versus expected values (x) was $y = 0.978x + 0.0491$ ($r^2 = 0.99$, $n = 28$), indicating a strong correlation between the two data sets.

The reproducibility (RSD) of the results obtained with the proposed method was between 6.9 and 10% for the three different samples analyzed. As well as standard addition

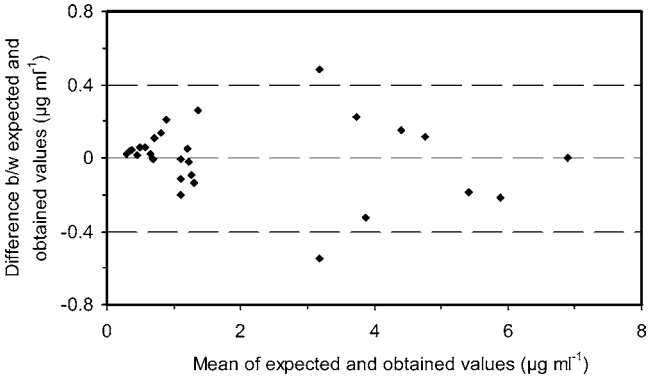

Figure 4. Difference plot between obtained and expected results for the measurement of vitamin B_2 in nutritional premix, milk powder, and corn flake cereal.

measurements, the determination of vitamin B_2 in the premix and milk powder samples could be more simply deduced by reference to a calibration curve of the peak current versus concentration of vitamin B_2. This is because the slope values obtained for the determination of vitamin B_2 in vitamin B complex premix (0.073 ± 0.007 μA mL μg^{-1}) and in the milk powder sample (0.076 ± 0.006 μA mL μg^{-1}) using the standard addition method were close to the slope obtained for model solutions of riboflavin (0.067 ± 0.001 μA mL μg^{-1}).

Conclusion. Determination of vitamin B_2 in nutritional premix and food products using a straightforward sample preparation step was demonstrated. Experimental results show that precise and accurate data can be obtained. The successful application of the method to three different matrixes could make it desirable as a routine method in food or vitamin laboratories.

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