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Enhancement by Copper(II) of the Voltammetric Signal of Vitamin B_2 Applied to Its Determination in Breakfast Cereals

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Addition of copper(II) to breakfast cereal samples was shown to significantly enhance the analytical signal obtained by electrochemical reduction of vitamin B_2 using linear sweep voltammetry on disposable carbon electrodes. The enhancement was observed only when dissolved oxygen was present. In model solutions the analytical signal was linear in the concentration range 6−150 ng/mL with a calculated limit of detection of 5 ng/mL (S/N = 3). This compared favorably with earlier work using a similar measurement approach—but in the absence of copper—in which the limit of detection was calculated to be 900 ng/mL. The effects of potential interferents commonly found in cereals were examined. In addition to signal attenuation by both sugar and starch (already reported), folic acid was found to increase (+6%) and iron to decrease (−11%) the analytical signal when present in the maximum concentration ratios, with respect to vitamin B_2, that are normally found in breakfast cereals. Nevertheless, the simplicity of the approach was potentially attractive for near-line quality control applications in manufacturing. The utility of the measurement approach was demonstrated by the addition of excess copper(II) sulfate to determine vitamin B_2 in aqueous extracts of breakfast cereals. The results agreed well with those provided by the cereal manufacturer who used an established HPLC method.

KEYWORDS: Vitamin B_2; linear sweep voltammetry; screen-printed carbon electrode; copper(II) ions; breakfast cereals

INTRODUCTION

Vitamin B_2 is an important micronutrient that is commonly added to baby foods, breakfast cereals, fruit drinks, and vitamin-enriched milk products—and is widely used in vitamin supplements. As a consequence, food manufacturers have a need for cost-effective analytical methods that do not require complex instrumentation but are suited to use in routine process control laboratories.

Commonly used methods for determining vitamin B_2 are based on microbiological assay (*1*−*3*) or HPLC (*4*−*8*), but there are a plethora of other techniques reported in the scientific literature. We have previously described the use of low-cost, single-use electrodes for the determination of vitamin B_2 in nutritional premix, cereal, and milk powder using a standard addition method (*9*). In that work, it was reported that copper (commonly found in fortified foods) was a potentially serious interferent because it affected the analytical vitamin B_2 signal. This paper describes how this effect was turned to an advantage by adding excess copper(II) to samples to provide enhanced analytical signals—enabling simpler electrochemical techniques (and equipment) to be used to determine vitamin B_2 with improved sensitivity over the previously reported procedures. The potential utility of the approach was demonstrated using breakfast cereals.

EXPERIMENTAL PROCEDURES

Sensor Fabrication. Sensors (1 × 4.5 cm) consisting of carbon working and counter electrodes together with a silver/silver chloride quasi-reference electrode were printed in-house with a DEK 1760RS semiautomatic screen-printer (Printing Machines, Weymouth, U.K.). The multistage printing process involved the sequential deposition of carbon (D2) dielectric (D2) and silver/silver chloride (P7) inks (Gwent Electronic Materials Ltd., Pontypool, U.K.) onto a PVC substrate (Cadillac Plastic, Swindon, U.K.) in controlled patterns and thicknesses. A similar printing process was described in the previous paper (*9*), but a different carbon ink was employed in the work reported here because it was more electrochemically active.

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Equipment and Reagents. Voltammetric experiments were performed using an electrochemical potentiostat workstation (Autolab PGSTAT30, Eco Chemie B.V., Utrecht, The Netherlands) with PC software control (GPES 4.9, Eco Chemie).

All reagents were of analytical grade. Riboflavin (98%), dibasic sodium phosphate heptahydrate, sodium phosphate monobasic monohydrate, potassium chloride, acetic acid, copper(II) sulfate pentahydrate, and sucrose were obtained from Sigma-Aldrich (Poole, U.K.). Maize starch was obtained from Fisher Scientific (Loughborough, U.K.). Deionized water was obtained from an Elgastat Option 3 water purification system (Elga Ltd., High Wycombe, U.K.).

Acetate−phosphate buffer, pH 6.0, was prepared by mixing volumes of acetic acid (100 mM) and dibasic sodium phosphate (100 mM). Acetate−phosphate buffer, pH 8.0, was prepared by dropwise addition of acetic acid (100 mM) to dibasic sodium phosphate (50 mL, 100 mM) and dilution of the resulting solution to 100 mL with water. Potassium chloride (50 mM) was included in all buffers.

Samples. Breakfast cereals were from Kellogg U.K. Ltd. and were provided as ground materials sealed in foil packs.

Procedure for Electrochemical Study of Vitamin B_2. *Vitamin B_2 Stock Solution.* Vitamin B_2 stock solutions were prepared weekly in pH 6.0 buffer and sonicated in an ultrasonic bath (20 min, F5100b, Decon Laboratory Ltd., Hove, U.K.) to ensure complete dissolution. Solutions were protected from photodegradation by covering the containers with aluminum foil and refrigerating (4 °C).

Sample Preparation. Breakfast cereal was prepared by suspending 0.5−1.0 g of ground sample in 10 mL of deionized water. The suspension was mixed vigorously (3 min) to homogenize, and then ammonium sulfate (∼4 g) was added to precipitate out the protein components; the suspensions were spin mixed (3 min) and then centrifuged (10 min, 3000 rpm, BS400, Denley Ltd., Heckmondwicke, U.K.). The supernatant was filtered (0.2 μm syringe filter, Minisart-plus 17823K, Vivascience AG, Goettingen, Germany) to obtain a clear extract solution. For quantification, three standard additions of known concentrations of vitamin B_2 were made to the cereal before the extraction to carry the standard additions through the extraction process.

Voltammetric Measurements. Preliminary measurements were made with standard solutions in a variety of buffer systems; details are provided in the text. To 0.1 mL of each sample extract was added 3.65 mL of pH 8.0 buffer and 0.25 mL of an aqueous copper(II) sulfate (1.83 mM). To make a measurement, a sensor unit was dipped into the sample solution and was connected to the electrochemical workstation. Linear sweep voltammetric measurements were made using a scan rate of 50 mV s^{-1} and a step potential of 1.0 mV. Unless otherwise indicated, scans were carried out from −0.1 to −1.0 V. All potentials are quoted with respect to the screen-printed silver/silver chloride quasi-reference electrode.

Deoxygenation of the solutions was not carried out because the enhancement occurred only in the presence of dissolved oxygen. The concentration of vitamin B_2 in the sample was calculated using standard addition samples described above.

RESULTS AND DISCUSSION

Voltammetry of Vitamin B_2. The linear sweep voltammetric (LSV) peak height in the presence of copper(II) ions was used as the measure of vitamin B_2. The peak increased proportionately with the concentration of vitamin B_2 (**Figure 1**). No further increase in the analytical signal was obtained by "accumulating" over a period of time at a suitably poised or open circuit measurement electrode potential. This indicated that the observed enhancement was not due to a typical surface accumulation process. Sparging of solutions with oxygen-free nitrogen reduced (∼50%) the observed enhancement, but extended sparging with helium effectively eliminated the enhancement. Furthermore, the enhanced signal was not obtained using either a copper-plated carbon working electrode or a "pure" copper working electrode. The mechanism resulting in the observed enhancement is the subject of continuing investigation, but it appears that the presence of both oxygen and copper(II) is necessary to provide the observed signal enhancement. The effect might be due to a catalytic process with reduced vitamin B_2 being reoxidized by oxygen.

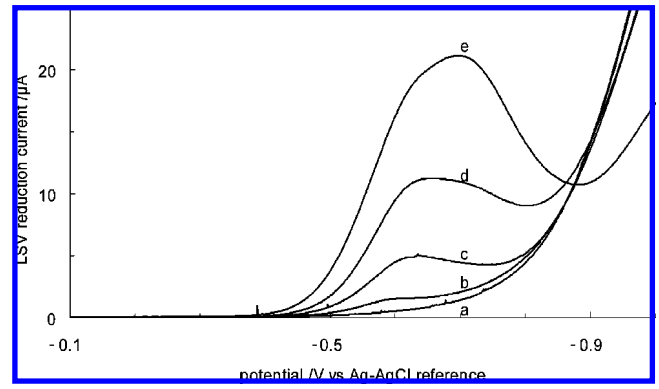

Figure 1. Linear sweep voltammetry (LSV) of vitamin B_2 in pH 8.0 buffer at a screen-printed carbon electrode: (a) buffer + 100 μM copper(II) sulfate; (b) a + 10 nM vitamin B_2; (c) a + 50 nM vitamin B_2; (d) a + 130 nM vitamin B_2; (e) a + 250 nM vitamin B_2.

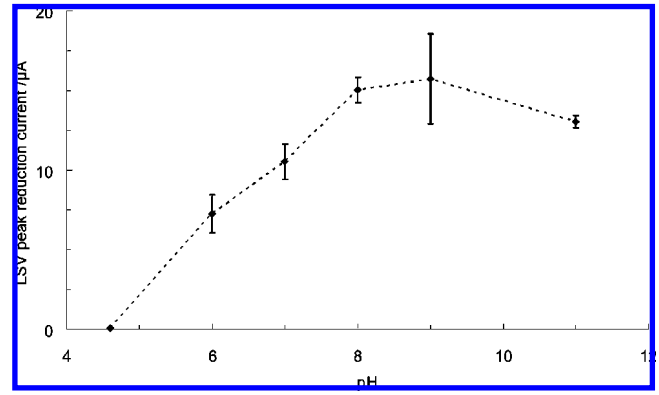

Figure 2. Effect of pH on the voltammetric response of 300 nM vitamin B_2. Buffers: (pH 4.6) monobasic phosphate; (pH 7) monobasic and dibasic phosphate; (pH 9) dibasic phosphate; (pH 11) dibasic phosphate and sodium hydroxide. Other experimental details were as given in the text.

Effect of pH on the Voltammetric Response of Vitamin B_2. The peak currents and potentials of vitamin B_2 were strongly influenced by the pH of the measurement solution. The peak potential moved toward more negative values as the pH was increased. The peak current increased rapidly in the pH range of 4−8 and leveled out above pH 8 (**Figure 2**). The vitamin was unstable in increasingly alkaline solutions; thus, pH 8.0 buffer was used as the preferred medium for subsequent electrochemical measurements.

Influence of Copper Concentration on the Voltammetric Response of Vitamin B_2. Copper(II) concentration was varied between 5 and 470 μM in the presence of 19, 74, and 298 ng/mL (respectively, 50, 200, and 800 μM) vitamin B_2 to optimize the analytical conditions. At pH 8.0, the vitamin B_2 signal increased rapidly with the concentration of copper(II) until it reached a plateau (**Figure 3**). The signal was independent of copper(II) concentration above 50 μM, at which concentration the maximum signal was obtained. For optimized sample measurements, copper(II) sulfate was added so as to give a final concentration of 100 μM.

Direct Determination of Vitamin B_2 in Standard Solutions. For standard solutions, a linear relationship was obtained between the concentration of vitamin B_2 and the electrochemical signal in the range of 6−150 ng/mL. The correlation coefficient

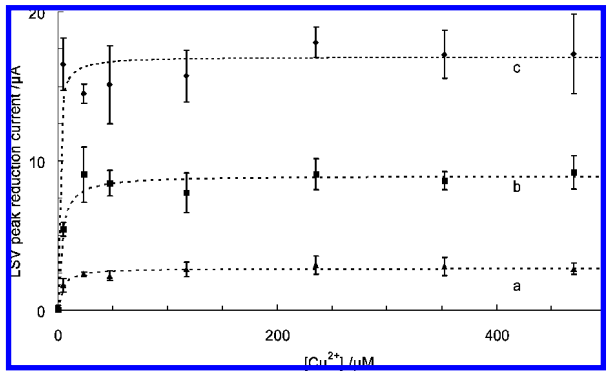

Figure 3. Dependence of vitamin B_2 peak current on copper(II) concentration. Phosphate−acetate buffer, pH 8.0; [vitamin B_2] (a) 50 nM; (b) 200 nM; (c) 800 nM. Other experimental conditions were as for **Figure 1**.

Table 1. Measured Interferences from Typical Micronutrients Found in Breakfast Cereals

compound	concn ratio used (compound/vitamin B_2)[a]	variation in current[b] (%)	typical concn ratios in breakfast cereals[a,c]
folic acid	0.1	+6	0.07−0.1
iron	10	−2	40−249
	300	−11	
magnesium[d]	103	−2	1000
nicotinamide	25	+5	3.3
thiamin	33	+9	1−1.3
vitamin B_6	20	−1	2−2.8
vitamin B_{12}	0.01	−1	0.00018
	16	−100	

[a] Concentration of vitamin B_2 was 75 ng/mL in acetate−phosphate buffer. Concentration ratios were calculated on a molar basis. [b] Mean of four replicate measurements. [c] Typical values are quoted for a range of U.K. breakfast cereals. (Typical concentrations of riboflavin in U.S. breakfast cereals can be found in the USDA National Nutrient Database for Standard Reference, release 18; http://www.nal.usda.gov/fnic/foodcomp-/Data/SR18/nutrlist/sr18w405.pdf, January 2008). [d] Magnesium greatly exceeded the solubility limit even at the concentration used.

was 0.994 ($n = 18$), and the regression equation was as follows: peak height/μA = (0.013 ± 0.001)[vitamin B_2 in ng/mL] + (0.15 ± 0.16), with a limit of detection of 5 ng/mL (3 times the standard deviation of the intercept divided by the slope). This was significantly better than the limit of detection in the absence of copper(II) ions, which was calculated to be 900 ng/mL (*9*).

Twelve successive measurements, using a new sensor unit for each determination, of a 19 ng/mL vitamin B_2 solution gave a relative standard deviation of 6.5%.

Interference Studies. In earlier work (*9*) it was shown that both sucrose and starch diminished electrochemical signals due to the increased viscosity of solutions and the consequent reduced flux of vitamin to the electrode surface. The revised approach to measurement described here was similarly affected by viscosity, and therefore it was necessary still to use a standard addition approach rather than a direct calibration method for determinations.

To evaluate the selectivity of this method further, possible specific interferences from other additives were examined under optimized conditions with a 75 ng/mL vitamin B_2 standard solution (**Table 1**). When present in the maximum concentration ratios, with respect to vitamin B_2, that are reported to be found in typical breakfast cereal products, magnesium, nicotinamide, thiamin, vitamin B_6, and vitamin B_{12} had negligible influence on the vitamin B_2 signal, whereas both iron (−11%) and folic acid (+6%) were potentially significant.

Table 2. Analytical Data for Vitamin B_2 in Fortified Breakfast Cereals

sample	no. of sample measurements	slope/μA (μg/mL)$^{-1}$	measured value/mg (100 g)$^{-1}$	ref value[a]/mg (100 g)$^{-1}$
Cornflakes	7	45 ± 5	1.31 ± 0.10	1.40 ± 0.10
Cocopops	3	72 ± 5	1.42 ± 0.10	1.40 ± 0.10
Rice Krispies	4	85 ± 8	1.47 ± 0.14	1.40 ± 0.10
Honey Loops	3	66 ± 3	1.81 ± 0.28	1.80 ± 0.10

[a] Reference values were obtained by the manufacturer using an established HPLC-UV method. When applicable, values are quoted as the mean ± a single standard deviation.

Determination of Vitamin B_2 in Breakfast Cereal. The proposed method was applied to the determination of vitamin B_2 in breakfast cereals using the method of standard additions. **Table 2** shows that good agreement was obtained between the measured value and the data obtained by the manufacturer using an established HPLC method. Thus, the potential interference identified from copper in fortified food products (*9*) was turned to advantage by providing an excess concentration of copper(II) ions to give enhanced analytical signals for the determination of vitamin B_2 at low concentrations (linear range of 6−150 ng/mL). The simple techniques and equipment make the procedure potentially attractive for quality control applications for which conventional analytical techniques are not always available or appropriate.

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