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Determination of Cobalamin in Chlorella Food by Cation Exchange Column and Graphite Furnace Atomic Absorption Spectrometry

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ABSTRACT

A method for the rapid determination of cobalamin (Vitamin B_{12}) in chlorella food was proposed. This method utilized a strong cation exchange column to remove free cobalt (II), and the cobalamins were quantitatively determined by measuring total cobalt in the column effluent with graphite furnace atomic absorption spectrometry (GFAAS). Under optimized conditions, the method detection limit was 24 μg /100 g. The method was validated by spiking cobalamin standards in different chlorella food, recoveries from spike preparations ranged from 98.3 to 99.5%, with coefficients of variation of 1.2~4.9%. The results show the suitability of the proposed method for determining vitamin B_{12} in chlorella food.

Key words: cobalamin, vitamin B_{12}, chlorella food, graphite furnace atomic absorption spectrometry (GFAAS), method detection limit

INTRODUCTION

Cobalamin (Vitamin B_{12}), the only vitamin that contains transition element, plays an important role in protein synthesis and energy metabolism. The common symptoms of deficiency of cobalamin include pernicious anemia and neurological disorders[1-2].

Chlorella is categorized as health supplement in Japan[3]. Dietary research[4] showed that algae and/or chlorella are potential sources of vitamin B_{12} for vegetarians. Kittaka-Katsura *et al.*[5] identified that the purified compound from chlorella by hot water extraction was the active form of vitamin B_{12} (cyanocobamide). Although the bioavailabillity of the chlorella to human remains to be determined, the development of analytical measurement technique is necessary. Microbiological assay[6-7] for measuring cobalamin is thought to be with high sensitivity but extremely time-consuming[8]. Moreover, when use microbiological method in chlorella sample with high content of cobalamin, sample dilution to proper concentration range is always a problem in order to fit in to the standard curve. The major goal of this work is to develop a simple, rapid and reliable analytical method for determining cobalamin in chlorella food.

In this study, cobalamin was first extracted with water by ultrasonic extraction process. Cation exchange column was utilized to remove inorganic free cobalt (II). The effluent that contains organic cobalt form is measured by graphite furnace atomic absorption spectrometer (GFAAS). The amount of cobalt in column effluent was then calculated to obtain the total amount of cobalamin.

The evaluation of the proposed method was first investigated by analyzing chlorella powder and compared the result with that obtained by AOAC 952.20 official method. Paired *t*-test was used to check if there is any significant difference between these two results. Furthermore, the recoveries and relative standard deviations of cobalt in 4 different spiked chlorella samples were used to monitor the accuracy and suitability of the proposed method.

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MATERIALS AND METHODS

I. *Apparatus*

(I) Perkin-Elmer 5100 atomic absorption spectrometer with the Zeeman background correction, equipped with a HGA-600 graphite tube atomizer and an AS-60 autosampler.
(II) Elma model transsonic digitals ultrasonic device (Elma, Singen, Germany).
(III) Strong cation exchange cartridge [LiChrolut SCX (40-63 μm), 500 mg, 3 mL] was obtained from Merck (Darmstadt, Germany).

II. *Reagents*

(I) Stock solution of cobalt (II) — 1000 mg/L (Merck, Darmstadt, Germany).
(II) Nitric acid — 65% (Suprapur, Merck, Darmstadt, Germany).
(III) Cyanocobalamin, purity > 99% (Sigma, St. Louis, MO, USA).
(IV) Certified reference materials: Dogfish muscle and liver (DOLT-2) and Lobster hepatopancreas (TORT-2) were purchased from the National Research Council,

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Canada (NRCC, Canada).
(V) Ultrapure deionized water (18 mΩ) was purified using a Milli-Q purification system (Millipore, Milford, MA, USA). In all analytical procedures such water was used.
(VI) Palladium-modifier for graphite furnace AAS — 10 g/L (Merck, Darmstadt, Germany).
(VII) Magnesium nitrate hexahydrate (Suprapur, Merck, Darmstadt, Germany).

III. *Sample Preparation*

Chlorella food (tablet or powder) was homogenized and weighed accurately (ca 1.0 g) in a plastic centrifuge tube. After 30 mL of water was added, extraction was carried out in a ultrasonic device at 50°C for 30 min, followed by centrifuging at 5000 rpm for 10 min. Removed the supernatant solution and repeated the extraction procedure for three times, the combined extracts were then evaporated under vacumn to dryness. The dried extract was dissolved and diluted to 10 mL with water.

A strong cation cartridge was first conditioned with 2 mL of water. Proper amount (≤ 5 mL) of extract was applied to the cartridge, and the column was eluted and washed with 1~2 mL of water afterwards. Collected all the effluent in a 10-mL volumetric flask, added 1 mL of concentrated HNO_3 and diluted to the mark with water. Graphite furnace atomic absorption spectrometer was then used to determine the cobalt content in effluent.

IV. *Preparation of Standard Solution*

Standard solutions containing 1~40 ng/mL cobalt were all freshly prepared by appropriate dilution of a stock solution (1000 mg/L) in 0.1 mol/L HNO_3.

V. *Sample Analysis*

A modifier was prepared by mixing 30 mg palladium nitrate and 100 mg $Mg(NO_3)_2$ and diluted to 50 mL with water. A hollow cathode lamp of cobalt operating at 15 mA and a spectral bandwidth of 0.2 nm were used for cobalt determination at the wavelength of 242.5 nm. A typical furnace heating program for the GFAAS measurements of cobalt is given in Table 1. The linear calibration curve of peak area versus concentration of cobalt was constructed and the cobalt concentration of sample was converted to cobalamin by a multiplying factor of 23 (the cobalt to cobalamin ratio is 4.3%).

RESULTS AND DISCUSSION

I. *Method Validation for Cobalt Determination by GFAAS*

To validate the analytical method of cobalt determination by GFAAS, two certified reference materials (CRMs) were chosen and analyzed. The results in Table 2 show that they are in excellent agreement with the certified values.

II. *Instrument and Method Detection Limit*

According to the definition of the International Union for Pure and Applied Chemistry (IUPAC), the detection limit is 3 times the standard deviation of the measurement of the blank divided by the slope of the calibration curve[9]. Seven reagent blanks were examined with the same preparation process to generate the detection limit. The detection limit of instrument for cobalt is 0.5 ng/mL (i.e. 12 ng/mL of vitamin B_{12}). Considering the mass of sample portion and number of dilutions[10], the method detection limit for vitamin B_{12} was 24 μg /100 g (12 ng/mL × 10 mL × 10 mL / 5 mL / 1 g) for a typical sample mass of 1.0 g.

III. *Extraction Solvent and Matrix Effect*

Since vitamin B_{12} can dissolve easily in water and alcohol, they were chosen as possible candidates for extraction solvent in this proposed method. Experimental results showed that lots of pigments and impurities were found in the extract when alcohol was used as extraction solvent. With high content of impurities, the cobalt in the extract was impossible to determine directly by GFAAS. Therefore water was utilized as extraction solvent in this method.

In order to further study the matrix effect of the water extract on the analytical signal for cobalt, we performed the method of standard additions (MOSA) to estimate the possibility of interference[11]. The slopes of calibration curves with and without standard additions were compared to identify the possible interferences from the matrix. Each curve was constructed from five points and each point

Table 1. Furnace heating program for the GFAAS measurements of cobalt

Step	Temperature (°C)	Ramp (sec)	Hold (sec)	Internal flow (mL/min)
Drying	110	1	40	300
Drying	120	10	50	300
Ashing	1600	1	30	300
Cooling	20	0	5	0
Cleaning	2700	1	5	300

Table 2. Analytical results of cobalt content for NRCC DOLT-2 and TORT-2

	Co content (mg/kg)	
Certified reference material	Determined value[a]	Certified value[a]
DOLT-2 (n = 6)	0.24 ± 0.01	0.24 ± 0.05
TORT-2 (n = 9)	0.55 ± 0.03	0.51 ± 0.09

[a]Mean ± standard deviation.

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represents the mean of two replicate measurements. As shown in Figure 1, the slopes for both calibration curves were quite similar. The proportional error is 102.7% by calculating the ratio of the slopes between the calibration curves with and without standard additions. The close agreement of the slopes strongly suggests that the matrix does not interfere the cobalt determination.

IV. *The Capacity of Cation Exchange Resin*

The cation exchange resin has the ability to exchange positive ions in the stationary phase with positive ions in solution, and its exchange ability is dependent on the valance of ions. Generally speaking, cation exchange resin has relatively higher bond strengths and affinities for divalent metal ions compared to other cations.

According to previous research[12], the major divalent metal ions in chlorella food are calcium, magnesium, iron, zinc, copper and manganese. The concentration ranges are calcium from 1000 to 10000 ppm, magnesium from 1000 to 6000 ppm, iron from 400 to 5000 ppm, zinc and copper from 1 to 40 ppm, and manganese from 20 to 300 ppm. Under the described condition, different content of cobalt and cobalamin were spiked to the solution containing much higher content of above-mentioned divalent cations (i.e. 20000 ppm of calcium, magnesium and iron, 1000 ppm of zinc, copper and manganese), then recoveries determined by GFAAS were used as indexes to evaluate the ion exchange capacity.

The results in Table 3 demonstrate that even in the presence of high levels of various divalent cations, cation exchange resin can still separate free cobalt ion (II) and cobalamin effectively. Since the sample solution took only 3~5 min to pass through a cation exchange column, we suggested that two exchange columns can be connected in series to assure that all the cobalt ions have been removed.

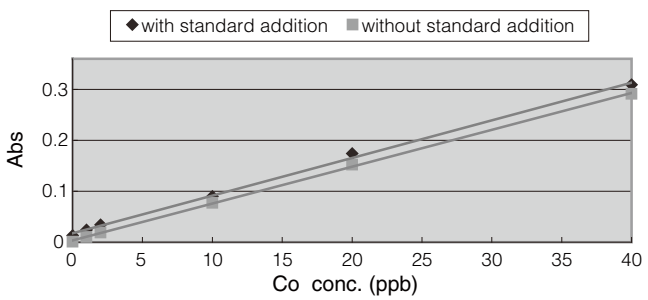

Figure 1. The comparison of calibration curves with and without standard additions.

V. *Comparative Analysis*

In order to make sure that all of the organic form of cobalt in effluent is cobalamin, the proposed method was parallelly compared with a microbiological assay[6] for the vitamin B_{12} content in chlorella powder. Moreover, the analytical results were statistically analyzed by ANOVA. Results show (Table 4) that there is no significant difference ($p > 0.05$) between these two methods. Therefore, it confirms that all cobalt compounds in the effluent should be cobalamin. This has further illustrated the feasibility of the proposed method for determination of vitamin B_{12} in chlorella food.

VI. *Method Validation and Suitable Range*

In order to further evaluate the accuracy of the proposed method, recoveries were determined by spiking cobalamin into four different chlorella samples with different vitamin B_{12} content. Table 5 shows that recoveries from spike preparations ranged from 98.3% to 99.5%,

Table 3. Recoveries of different levels of cobalt and cobalamin passing through cation exchange column in the presence of divalent cation with Ca^{+2}, Mg^{+2} and Fe^{+2} at concentration up to 20000 ppm, and Cu^{+2}, Mn^{+2} and Zn^{+2} up to 1000 ppm

Co (II) amount (ng)	Cobalamin amount (ng)	Recovery of cobalamin (%)
5000	5000	103.70
4000	10000	97.94
3000	20000	98.45
2000	50000	97.15
1000	250000	97.90

Table 4. Determination of vitamin B_{12} in chlorella powder by two different experimental approach

Method	Vitamin B_{12} content[a] (μg/100 g) (n = 5)
AOAC 952.20	997.5 ± 0.4
Proposed method	972.8 ± 0.2

[a]Mean ± standard deviation.

Table 5. Recoveries of vitamin B_{12} for spiked chlorella food

Sample[a]	Vitamin B_{12} (μg/100g)	Added (μg/100g)	Measured value (μg/100g)	Recovery (%)	C.V. (%)
Chlorella food A (n = 4)	972.8	1294	2250	98.27	2.43
Chlorella food B (n = 4)	771.9	1014	1781	99.52	2.44
Chlorella food C (n = 4)	91.7	136.5	227.1	99.17	4.90
Chlorella food D (n = 3)	47.0	33.0	79.8	99.36	1.21

[a]Sample A and C are chlorella powders; sample B and D are chlorella tablets.

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Table 6. Values and uncertainties in vitamin B_{12} analysis

	Description	Value	Standard uncertainty	Relative standard uncertainty *u(x)/x*
C, u (x_0,y)	Content of cobalt in the solution	16.89014	0.195057	0.011548544
W_S	Influence of mass	1.8522	0.4093×10^{-3}	0.000220963
V_1	Dilution Factor (volume of the flask)	25	0.03443	0.0013772
p	Purity of cobalt	1001	2	0.001998
rep	Repeatability	1	0.01744719	0.0174472

with coefficients of variation of 1.2~4.9%. Once again, it demonstrates the suitability of the proposed method for the vitamin B_{12} determination in chlorella food.

VII. *Measurement Uncertainty*

One particular chlorella food was selected as test sample for measurement uncertainty calculation according to Eurachem/Citac Guide[13], and the result was 530.9 ± 22.3 μg /100 g. (The different uncertainty contributions are given in Table 6)

CONCLUSIONS

Microbiological assay is regarded as an effective technology with high sensitivity for analysis of vitamin B_{12} in foods. However, it takes at least 3 days for each analytical run. The proposed method can be done within only one day without compromising the accuracy. In addition, chlorella contains rich vitamin B_{12} content with broad concentration range. It is hard to estimate the dilution factor accurately when using microbiological method and therefore lots of time is wasted due to trials and errors. Moreover, high number of dilution factor may potentially generate operational errors. This paper describes a simple, rapid and reliable method for the determination of cobalamin in chlorella food. Hopefully in the future, it will be further enhanced and improved for vitamin B_{12} determination in other kinds of food products.

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