

Effects of Microwave Heating on the Loss of Vitamin B₁₂ in Foods

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To clarify the effects of microwave heating on the loss of vitamin B₁₂ in foods, raw beef, pork, and milk were treated by microwave heating and then their vitamin B₁₂ contents were determined according to a chemiluminescent vitamin B₁₂ assay with hog intrinsic factor. Appreciable loss (~30–40%) of vitamin B₁₂ occurred in the foods during microwave heating due to the degradation of vitamin B₁₂ molecule by microwave heating. When hydroxo vitamin B₁₂, which predominates in foods, was treated by microwave heating and then analyzed by silica gel 60 thin layer chromatography, two vitamin B₁₂ degradation products were found. One of the compounds with a *R_f* of 0.16 was purified and partially characterized. The vitamin B₁₂ degradation product did not show any biological activity in the growth of a vitamin B₁₂ requiring microorganism, *Euglena gracilis* Z, and was not bound to hog intrinsic factor, a mammalian vitamin B₁₂ binding protein. Intravenous administration of the compound (1 μg/day) for 7 days to rats showed that the compound neither has toxicity nor acts as a vitamin B₁₂ antagonist in mammals. These results indicate that the conversion of vitamin B₁₂ to the inactive vitamin B₁₂ degradation products occurs in foods during microwave heating.

Keywords: Vitamin B₁₂; degradation; microwave heating; intrinsic factor; rat

INTRODUCTION

A deficiency of vitamin B₁₂ (B₁₂) can lead to the human disease pernicious anemia. Foods contain various B₁₂ analogues with different β-ligands; especially, methyl vitamin B₁₂ (MeB₁₂) and 5'-deoxyadenosyl vitamin B₁₂ (AdoB₁₂), which function as coenzymes of methionine synthase (EC 2.1.1.13) involved in methionine biosynthesis, and methylmalonyl-CoA mutase (EC 5.4.99.2), which is involved in amino acid and odd-chain fatty acid metabolisms in mammals, respectively (Schneider and Stroiński, 1987).

Cyano vitamin B₁₂ (CN-B₁₂), used in medicine, does not occur in raw foods, which contain predominantly hydroxo vitamin B₁₂ (OH-B₁₂). Even if the coenzyme B₁₂ form occurs, it would be photolyzed to form OH-B₁₂ by light (Schneider, 1987). Bennink and Ono (1982) have reported appreciable loss of B₁₂ during cooking of raw beef, but it is unclear why such loss occurs.

Microwave ovens are widely used for cooking and food processing. Extensive studies (Cross and Fung, 1982; Hoffman and Zabik, 1985) have shown equal or better retention of some vitamins (B₁, B₂, B₆, C, and folic acid) after microwave heating compared with conventional heating. There is, however, little information on how much loss of B₁₂ occurs in foods during microwave heating. Here the effects of microwave heating on the loss of B₁₂ in foods are described, and the loss of B₁₂ due to its conversion to biologically inactive B₁₂ degradation products by microwave heating is demonstrated.

MATERIALS AND METHODS

Materials. OH-B₁₂, CN-B₁₂, AdoB₁₂, and MeB₁₂ were obtained from Sigma Chemical Co. (St. Louis, MO). Thin layer chromatography (TLC) plates (silica gel 60) were obtained from Merck (Darmstadt, Germany). A reversed-phase high-pressure liquid chromatography (HPLC) column (Wakosil-II 5C18RS, Ø4.6 × 150 mm; particle size 5 μm) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Raw beef and pork and pasteurized cow's milk were obtained from a local market in Kochi, Japan.

Microwave Heating of Foods. Ten grams of raw beef (or pork) was homogenized in 50 mL of distilled water at room temperature using a universal homogenizer (Nihon Seiki Seisakusho, Co., Tokyo, Japan). Each of the homogenates and pasteurized cow's milk (100 g) were placed into a glass dish suitable for microwave cooking and treated for 6 min by microwave heating. The microwave oven used was a Funai (Tokyo, Japan) Model MO57-6A. The full power was 500 W, and the frequency of radiation was 2450 MHz. Beef and pork homogenates and cow's milk without microwave treatment were used as controls.

Cow's milk (100 g) was also treated for 30 min in a boiling water bath and used as a boiled sample. Temperatures of the boiled and microwave-treated milk were measured during the experimental time course by a Asea Fiber 1110 thermometer (Vasteras, Sweden).

Extraction and Assay of B₁₂. Total B₁₂ was extracted from the control, boiled, or microwave-treated foods by boiling with KCN in acidic pH (Frenkel et al., 1980). The extraction procedures were done in a Dalton (Tokyo, Japan) draught chamber. Ten milliliters of 0.5 mol/L acetate buffer, pH 4.8, and 20 mg of KCN were added to the beef and pork homogenates, which were boiled for 30 min at 98 °C in the dark. These homogenates were centrifuged at 10000g for 10 min. The supernatant was used as the sample for the B₁₂ assay.

In the case of milk, 100 mL of 0.2 mol/L acetate buffer, pH 4.8, and 20 mg of KCN were added to the control, boiled, or microwave-treated milk, boiled for 30 min at 98 °C in the dark,

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and then centrifuged at 10000g for 10 min. The supernatant was used as the sample for the B₁₂ assay.

B₁₂ was assayed by a fully automated chemiluminescent ACS-180 B₁₂ analyzer (Chiron Diagnostics, East Walpole, MA) according to the manufacturer's instruction. The B₁₂ extract was directly applied to the analyzer and the B₁₂ concentration was automatically determined.

Microwave Heating of Authentic OH-B₁₂. Authentic OH-B₁₂ used in the experiments was purified by silica gel 60 TLC with 1-butanol/2-propanol/water (10:7:10) as a solvent, since the OH-B₁₂ reagent (≈98% pure) contained a small amount of an impurity (R_f value = 0.27). The red compound with R_f of 0.03 (OH-B₁₂ fraction) was collected, dissolved in an appropriate amount of the solvent, and extracted three times from the silica gels. The combined extracts were evaporated to dryness and dissolved in a small amount of distilled water. The concentration of the purified OH-B₁₂ was determined on the basis of a molecular extinction coefficient for aqua vitamin B₁₂ of 8.5×10^3 (mol/L)⁻¹ cm⁻¹ at 527 nm in water. Aqua vitamin B₁₂ is the conjugated acid form of OH-B₁₂. Aqua and hydroxo vitamin B₁₂ are interconvertible in solution depending on pH (below pH 8.0, aqua vitamin B₁₂ tends to predominate over OH-B₁₂ at equilibrium) (Smith et al., 1962). Two milliliters of 0.1 mmol/L purified OH-B₁₂ solution was treated for 6 min in the dark by microwave heating with the Funai microwave oven at 500 W. The OH-B₁₂ solution was also treated for 30 min in a boiling water bath in the dark. Each treated solution was evaporated to dryness and dissolved in a small amount of distilled water. The concentrated solution was spotted quantitatively several times in an identical site on the silica gel 60 TLC plate and developed with 1-butanol/2-propanol/water (10:7:10) as a solvent in the dark. Authentic OH-B₁₂, CN-B₁₂, AdoB₁₂, and MeB₁₂ (all at 10 mmol/L) were used as standards in TLC analysis.

The TLC plates developed were dried at room temperature in the dark and analyzed spectrophotometrically with a Shimadzu (Kyoto, Japan) dual-wavelength TLC scanner (CS-930) and data recorder (DR-2). B₁₂ was monitored by measurement of absorbance at 527 nm. The amounts of the degradation products formed from OH-B₁₂ were expressed as percentage against total B₁₂ peak area detected in a lane of the TLC plate.

Isolation of B₁₂ Degradation Product. When the purified OH-B₁₂ was treated by microwave heating and analyzed by silica gel 60 TLC, two B₁₂ degradation products with R_f of 0.16 ($18.2 \pm 0.6\%$ of total B₁₂) and R_f of 0.27 ($4.2 \pm 0.3\%$) were found. The B₁₂ degradation product with R_f of 0.16 was isolated as follows. The purified OH-B₁₂ solution was treated by microwave heating for 6 min in the dark. The treated solution was evaporated to dryness and dissolved in a small amount of distilled water. The concentrated solution was put on the silica gel 60 TLC plates and developed with 1-butanol/2-propanol/water (10:7:10) as a solvent in the dark. The plates were dried at a room temperature, and the red spot with R_f of 0.16 was collected in the dark. The collected TLC silica gels were dissolved in an appropriate amount of the solvent, and the B₁₂ degradation product was extracted from the silica gels three times. The combined extracts were evaporated to dryness and dissolved in a small amount of distilled water. The compound obtained was further purified by the silica gel 60 TLC under the same conditions.

The B₁₂ degradation product was further purified by HPLC using a Shimadzu HPLC apparatus (LC-6A pump, SPD-6A spectrophotometer, C-R6A Chromatopac). The sample was put on a reversed-phase HPLC column (Wakosil-II 5C18RS, $\varnothing 4.6 \times 150$ mm; particle size 5 μ m) equilibrated with 5% (v/v) methanol solution containing 1% (v/v) acetic acid at 35 °C. The flow rate was 1 mL/min. The B₁₂ degradation product was eluted at 40 mL of a linear gradient (5–99%, v/v) of methanol in the same solution, monitored by measuring absorbance at 275 nm, and collected at 1.0 mL with a Bio-Rad (Hercules, CA) fraction collector (Model 2110). The B₁₂ degradation product was separated in two peaks with retention times of 15.0 (identical retention time to authentic OH-B₁₂) and 19.0 min. The peak with retention time of 19.0 min was pooled,

evaporated to dryness, and dissolved in a small amount of distilled water. The purified compound was assayed by using the same HPLC system with OH-B₁₂ as a standard.

The purified B₁₂ degradation product gave a single red spot with R_f values of 0.01, 0.14, 0.09, 0.04, 0.14, and 0.60 on analytical silica gel 60 TLC with various solvent systems [1-butanol/2-propanol/water (10:7:10); 2-butanol/acetic acid (99:1); 2-butanol/acetic acid/water (127:1:50); 2-butanol/28% NH₄OH/water (50:7:18); 2-propanol/28% NH₄OH/water (7:1:2), respectively].

Biological Activity of the Purified B₁₂ Degradation Product in *Euglena gracilis* Z. *E. gracilis* Z was normally cultured for 16 days at 25 °C with illumination ($50 \mu\text{E m}^{-2} \text{s}^{-1}$) in a propionate-supplemented (6.6 g/L medium) Cramer–Myers medium (CN-B₁₂, 10 $\mu\text{g/L}$ medium) (Watanabe et al., 1996). Authentic OH-B₁₂ (10 $\mu\text{g/L}$) or the purified B₁₂ degradation product (10 $\mu\text{g/L}$) with R_f of 0.16 or both were added aseptically to a culture (150 mL) of *E. gracilis* Z in the Cramer–Myers medium without CN-B₁₂. *Euglena* culture (3.0 mL) was sampled every 3 days, and cell growth was estimated by measuring absorbance at 660 nm with a Shimadzu UV-1600 spectrophotometer.

Intravenous Administration of the Purified B₁₂ Degradation Product in Rats. Eight male Wistar rats (5 weeks old) were obtained from Kiwa Laboratory Animals Co. Ltd. (Wakayama, Japan). B₁₂-deprived diet contained 400 g (per kg of diet) of soybean protein (Fuji Oil Ltd., Osaka, Japan), 438 g of glucose anhydrous (Nacalai Tesque, Ltd., Kyoto, Japan), 100 g of soybean oil (Nacalai), 50 g of mineral mixture, 5 g of *dl*-methionine (Nacalai), 5 g of B₁₂-free vitamin mixture, and 2 g of choline chloride (Nacalai) as described previously (Watanabe et al., 1991). The rats were fed the B₁₂-deprived diet a week before experiments. The 6-week-old rats were housed in individual metabolic cages at 24 °C in a room with a 12 h light/dark cycle. They had free access to the B₁₂ deprived diet and tap water during experiments. The 6-week-old rats (188.4 ± 12.5 g) were injected intravenously with 1.0 mL (1 μg) of the B₁₂ degradation product–saline (1 $\mu\text{g/mL}$) every 24 h for 7 days. Rats injected with only 1.0 mL of saline under the same conditions were used as controls.

The urine of the 7-day control and B₁₂ degradation product treated rats in individual metabolic cages was sampled for 24 h. Livers and blood were obtained from the rats killed by decapitation under diethyl ether anesthesia. Livers were washed with saline and weighed. A portion (wet weight, 1 g) of the livers was immediately homogenized (using a glass homogenizer with a Teflon pestle) in ~4 volumes of 10 mmol/L acetate buffer, pH 4.8. Extraction and assay of total B₁₂ from the liver homogenate and blood was done as described above.

Urinary methylmalonic acid was assayed by using HPLC as described previously (Toyoshima et al., 1994).

Statistics. Statistical analysis was performed using GB-STAT 5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). One-way repeated-measures ANOVA was used, with post-hoc two-tailed Dunnett's test. In the case of body weight gains of the 7-day control and B₁₂ degradation product-treated rats, a nonparametric test (Mann–Whitney U-test) was used. Differences were considered significant if $P < 0.01$. Values in the text are mean \pm SD.

RESULTS AND DISCUSSION

To determine how much loss of biologically active B₁₂ occurred in foods during microwave heating, raw beef and pork and pasteurized cow's milk were treated for 6 min with a microwave oven. The total B₁₂ was extracted and assayed with the chemiluminescent method using hog intrinsic factor, a mammalian B₁₂ binding protein (Table 1). The control (without the microwave treatment) samples of beef, pork, and milk contained 2.56 ± 0.03 , 0.64 ± 0.02 , and $0.27 \pm 0.01 \mu\text{g}$ of B₁₂/100 g of wet weight, respectively; these values are identical to previously published values (Schneider, 1987). The concen-

Table 1. Effect of Microwave Heating on Vitamin B₁₂ Concentration in Some Foods

	B ₁₂ concn ^a (μg/100 g of wet wt)		
	beef	pork	milk
control	2.56 ± 0.03	0.64 ± 0.02	0.27 ± 0.01
microwave	2.13 ± 0.13*	0.55 ± 0.01*	0.14 ± 0.02*

^a Beef and pork homogenates and cow's milk were treated with or without (control) microwave heating for 6 min. All values represent means ± SD (*n* = 5). *Significantly different from control (without the microwave heating), *P* < 0.01.

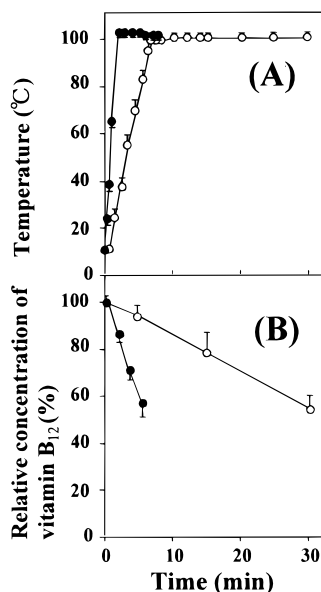


Figure 1. Effect of treatment times on concentration of cow's milk B₁₂ by microwave heating. Cow's milk was treated by boiling at 100 °C (control) or by microwave heating. B₁₂ was extracted from both treated milk samples and assayed as described in the text. Data represent mean ± SD (*n* = 4). (A) Temperature: control (○); microwave treatment (●). (B) Relative B₁₂ concentration: control (○); microwave treatment (●). The relative B₁₂ concentration was expressed as percentage against the B₁₂ concentration of milk without the boiling and microwave heating.

tration of B₁₂ in these foods was reduced significantly by the microwave heating. These results suggest that an appreciable loss (~30–40%) of B₁₂ occurs in foods during the 6 min microwave heating, which is a lengthy heating period for reheating of foods but a likely time period for food processing and cooking.

Cow's milk is an excellent source of B₁₂, since 1.0 L of cow's milk (2 μg of B₁₂) is sufficient to satisfy the demands of daily intake for humans (Resources Council, Science and Technology Agency, 1995). Figure 1 shows the effect of microwave heating and boiling times on the loss of B₁₂ in cow's milk. The loss of B₁₂ increased with an increase in the treatment times for the boiled milk, as well as in the microwave-treated milk. The rate of the B₁₂ loss in the microwave-treated milk was greater than that in the boiled milk after the temperature of milk reached ≈100 °C. The amount of B₁₂ loss in the 6 min microwave-treated milk sample did not differ from that in the 30 min boiled milk.

To determine whether the loss of B₁₂ in the microwave-treated foods is derived from the conversion of B₁₂ to some inactive B₁₂ degradation products, OH-B₁₂, which predominates in foods, was analyzed with silica gel 60 TLC when treated by microwave heating for 6 min (Figure 2). The treated OH-B₁₂ was separated into three red spots [major compound **I** with *R_f* of 0.03;

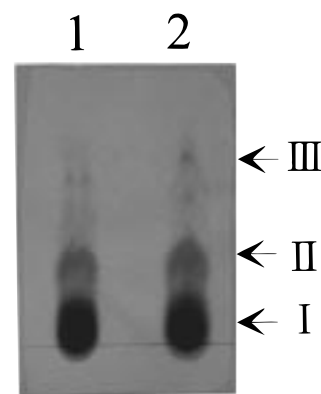


Figure 2. Formation of degradation products from purified hydroxy vitamin B₁₂ by microwave heating. Two milliliters of 0.1 mmol/L purified OH-B₁₂ was treated in the dark by boiling or microwave heating as described in the text. (Lane 1) OH-B₁₂ solution with the 30 min boiling treatment. (Lane 2) OH-B₁₂ solution with the 6 min microwave treatment. The data are representative of TLC patterns from five independent experiments.

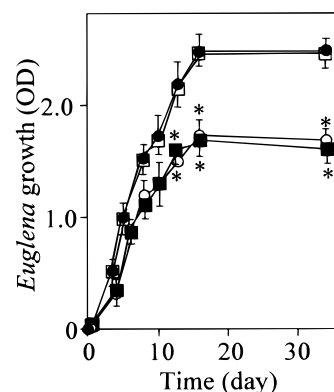


Figure 3. Effect of the B₁₂ degradation product (compound **II**) formed by microwave heating on growth of *E. gracilis* Z. Authentic OH-B₁₂ or the isolated compound **II** (10 μg/L) or both were added aseptically to a *Euglena* culture. *E. gracilis* Z was cultured as described in the text. The figure plots *Euglena* cell growth in the culture supplemented with (●) or without (○) OH-B₁₂ and with compound **II** (■) and OH-B₁₂ plus compound **II** (□). Data represent mean ± SD (*n* = 3). *Significantly different from control (OH-B₁₂ supplemented *Euglena* cells), *P* < 0.01.

Table 2. Binding of Compound II to Hog Intrinsic Factor, a Mammalian Vitamin B₁₂ Binding Protein

	B ₁₂ concn ^a (pg/mL)
OH-B ₁₂	786 ± 31.8
compound II	56 ± 6.2*

^a Each solution (800 pg/mL) of authentic OH-B₁₂ and purified compound **II** was assayed by the chemiluminescent vitamin B₁₂ assay system using hog intrinsic factor. All values represent means ± SD (*n* = 3). *Significantly different from authentic OH-B₁₂, *P* < 0.01.

identical *R_f* of intact OH-B₁₂, and minor compound **II** (18.2 ± 0.6%) with *R_f* of 0.16 and compound **III** (4.2 ± 0.3%) with *R_f* of 0.27]. These results strongly suggest that the loss of B₁₂ caused by microwave heating in foods is due to the conversion of B₁₂ to B₁₂ degradation products.

To determine the biological activity of the purified compound **II** in a *E. gracilis* Z (a B₁₂ requiring microorganism) culture, which is used for the bioassay for B₁₂ in biological samples (Ross, 1952), authentic OH-B₁₂ or the purified compound **II** or both were added aseptically

Table 3. Effects of Intravenous Administration of Compound II on Body Weight Gain, Urinary Methylmalonic Acid Excretion, and Plasma and Hepatic Vitamin B₁₂ Concentrations in Rats^a

	body wt gain (g/week)	liver wt (g)	urinary methylmalonic acid excretion (μmol/day)	vitamin B ₁₂ concn	
				plasma (pg/mL)	liver (ng/total liver)
control	28.33 ± 0.58	7.99 ± 0.68	ND	396.3 ± 74.5	700.8 ± 96.6
compound II	16.93 ± 5.96	7.12 ± 0.65	0.14 ± 0.21	406.2 ± 80.9	759.0 ± 140.8

^a All values represent means ± SD (*n* = 4). ND represents not detectable.

to a 0 time B₁₂ deficient culture (Figure 3). The growth of *E. gracilis* Z was increased significantly by the addition of OH-B₁₂ (control) or OH-B₁₂ plus compound II, while in the 32-day cultures supplemented only with compound II, *Euglena* cell growth was inhibited up to ~70% of the control culture; an identical growth curve was also obtained in the B₁₂ deficient culture. Addition of compound II did not cause further growth inhibition of *Euglena* cells. These results indicate that compound II is inactive in *E. gracilis* Z but does not act as a B₁₂ antagonist. *Euglena* cells have been unable to utilize B₁₂ analogues lacking the α-ligand (the cobalt-coordinated nucleotide) for cell growth (Watanabe et al., 1992), and *Euglena* B₁₂ binding protein has shown an absolute requirement for the α-ligand and intact (*b*)-propionamide side chain of B₁₂ molecule in B₁₂ binding (Watanabe et al., 1993).

Intrinsic factor, which is involved in the intestinal absorption of B₁₂ (Seetharam and Alpers, 1982), recognizes the structure of the B₁₂ molecule more strictly than the *Euglena* B₁₂ binding proteins (Watanabe et al., 1993). When authentic OH-B₁₂ and compound II (an identical amount, 800 pg/mL) were assayed with the chemiluminescent B₁₂ assay system using hog intrinsic factor (Table 2), the amount of compound II was estimated to be ~7.1% of that of OH-B₁₂. The results indicate that the intrinsic factor cannot bind compound II, which would not be absorbed in mammalian intestine by the intrinsic factor-mediated system.

To determine whether compound II has an acute toxicity in mammals, effects of intravenous administration of the compound on body weight gain, plasma and hepatic B₁₂ concentrations, and urinary methylmalonic acid excretion as an index of B₁₂ deficiency were studied in rats (Table 3). The 6-week-old rats were injected with 1 μg of compound II every 24 h for 7 days. The administration of compound II did not affect liver weights or plasma and hepatic B₁₂ concentrations in the rats. The urinary methylmalonic acid excretion (0.14 ± 0.21 μmol/day) became detectable in the compound II treated rats, but it was not significantly different from the control. Severely B₁₂ deficient rats have been reported to excrete 214.3 ± 115.2 μmol of methylmalonic acid/day in urine (Watanabe et al., 1991). The body weight gain of the 7-day compound II treated rats was reduced relative to the control, but the differences between them are not significant. These results indicate that compound II neither has toxicity nor acts as a B₁₂ antagonist in mammals within the experiment time course examined.

In our preliminary experiments, the ¹H NMR spectrum of compound II was measured on a JEOL GX-270 at 270 MHz in D₂O (δ = 4.71 ppm; internal reference). The ¹H NMR spectrum of authentic CN-B₁₂ showed the presence of three aromatic protons assigned to the dimethylbenzimidazolyl moiety: δ_H 6.36 (1H, s), 6.94 (1H, s), and 7.13 (1H, s) ppm. The spectra of compound II showed the lack of these signals in the low-field

region. The signals due to ribose anomeric proton at δ_H 6.22 (1H, d, *J* = 3.1 Hz) and H-10 proton δ_H 5.95 (1H, s) disappeared, and the signals of the sugar moiety and methyl groups were also changed. These findings show that compound II may be due to the elimination of the base portion and the skeletal alterations by microwave heating. The NMR studies support the observation that compound II is inactive in *Euglena* cells and mammals. Structural information on compound III with *R_f* of 0.27 is not available because a purified sample was not obtained for NMR study.

These results indicate that biologically inactive B₁₂ degradation products are formed in foods by microwave heating. Bennink and Ono (1982) have reported that there is a 27–33% loss of B₁₂ during cooking of foods. They have assayed the B₁₂ content of raw and cooked beefs by a radioisotope dilution method with hog intrinsic factor. Our result that the intrinsic factor could not bind compound II indicates that the compound is unable to be assayed according to this method. The loss of B₁₂ during cooking as reported by Bennink and Ono would be due to the conversion of B₁₂ to biologically inactive B₁₂ degradation products.

ABBREVIATIONS USED

AdoB₁₂, 5'-deoxyadenosyl vitamin B₁₂; B₁₂, vitamin B₁₂; CN-B₁₂, cyano vitamin B₁₂; HPLC, high-pressure liquid chromatography; MeB₁₂, methyl vitamin B₁₂; OH-B₁₂, hydroxo vitamin B₁₂; TLC, thin layer chromatography.

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