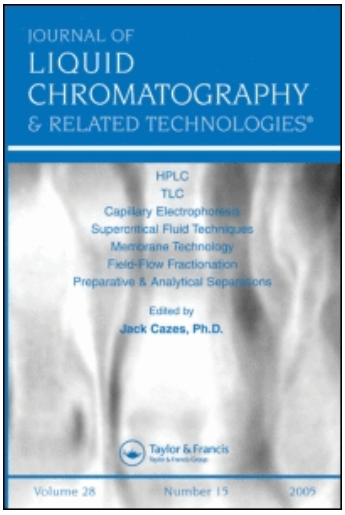

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TLC SEPARATION AND ANALYSIS OF VITAMIN B_{12} AND RELATED COMPOUNDS IN FOOD

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TLC SEPARATION AND ANALYSIS OF VITAMIN B_{12} AND RELATED COMPOUNDS IN FOOD

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ABSTRACT

To evaluate why differences between the vitamin B_{12} contents determined by both microbiological and intrinsic factor-chemiluminescence B_{12} assay methods occur in some edible shellfish and algal foods, or how much loss of B_{12} occurs in food during microwave heating, some B_{12}-compounds and their degradation products formed during microwave heating were purified and characterized using silica gel 60 thin layer chromatography.

Although dried green and purple lavers (nori), some algal health foods, and most shellfish contained considerable amounts of true B_{12}, pseudovitamin B_{12}, an inactive B_{12}-compound, predominated in spirulina tablets.

Significant loss of B_{12} occurred in foods during microwave heating due to the conversion of B_{12} to inactive B_{12} degradation products.

These results indicate that thin-layer chromatography has great advantages (simplicity, flexibility, speed, and relative

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inexpensiveness) for the separation and analysis of B_{12} compounds in foods.

Key Words: Bioavailability; Cobalamin; Corrinoid; Food purification; Vitamin B_{12}

INTRODUCTION

Vitamin B_{12} (B_{12} or CN-B_{12}) is synthesized only in certain bacteria. Usual dietary sources of B_{12} are animal food products (meat, milk, egg, and shellfish), but not plant food products.[1] Some plant foods, edible seaweeds and microalgae, however, contain large amounts of B_{12}, which appears to be inactive B_{12} compounds so that they may not be bioavailable in mammals.[2] Foods contain various B_{12}-compounds with different upper ligands (L) (Fig. 1); especially, MeB_{12} and AdoB_{12} funcion as coenzymes of methionine synthase (EC 2.1.1.13) involved in methionine biosynthesis, and of methylmolonyl-CoA mutase (EC 5.4.99.2) involved in amino acid and odd-chain fatty acid metabolism in mammals, respectively.[3]

Historically, B_{12} contents of foods have been determined by bioassay with B_{12}-requiring microorganisms; *Lactobacillus delbrueckii* subsp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC 7830 has been used widely.[4] Radioisotope dilution assay method with radio-labeled B_{12} and hog intrinsic factor (IF), the most specific B_{12}-binding protein, has been also used for the determination of B_{12} contents in foods.[5,6] Recently, a chemiluminescence (acridinium ester)-labeled B_{12} derivative has been devised instead of a radioactive label. A fully automated chemiluminescence B_{12} analyzer (Chiron Diagnostics, East Walpole, MA) with the acridinium ester-labeled B_{12} derivative and IF has been commercialized. B_{12} has been assayed in foods by the IF-chemiluminescence method, which was compared with the *Lactobacillus* microbiological method.[7] In shellfish and spirulina (*Sprirulina* sp) tablets (an algal health food), the values determined by the microbiological method were about 6–8-fold greater than the values determined by the IF-chemiluminescence method, although there was good similarity between the values by the two methods in other foods.[7] *L. delbruekii* ATCC 7830 used for the determination of B_{12} in foods cannot utilize cobinamide (lower-ligand-free corrinoids), but the B_{12}-compounds inactive for human as well as intact B_{12}, and both deoxyribosides and deoxynucleotides also may substitute B_{12}.[4] To evaluate why such differences between the values determined by both microbiological and IF-chemiluminescence methods occur in these foods, B_{12}-compounds should be purified and characterized.

This review summarizes our work on the purification and characterization of B_{12}-compounds found in various foods and B_{12} degradation compounds formed by cooking and/or food processing, using TLC as a powerful separation and analytical tool.

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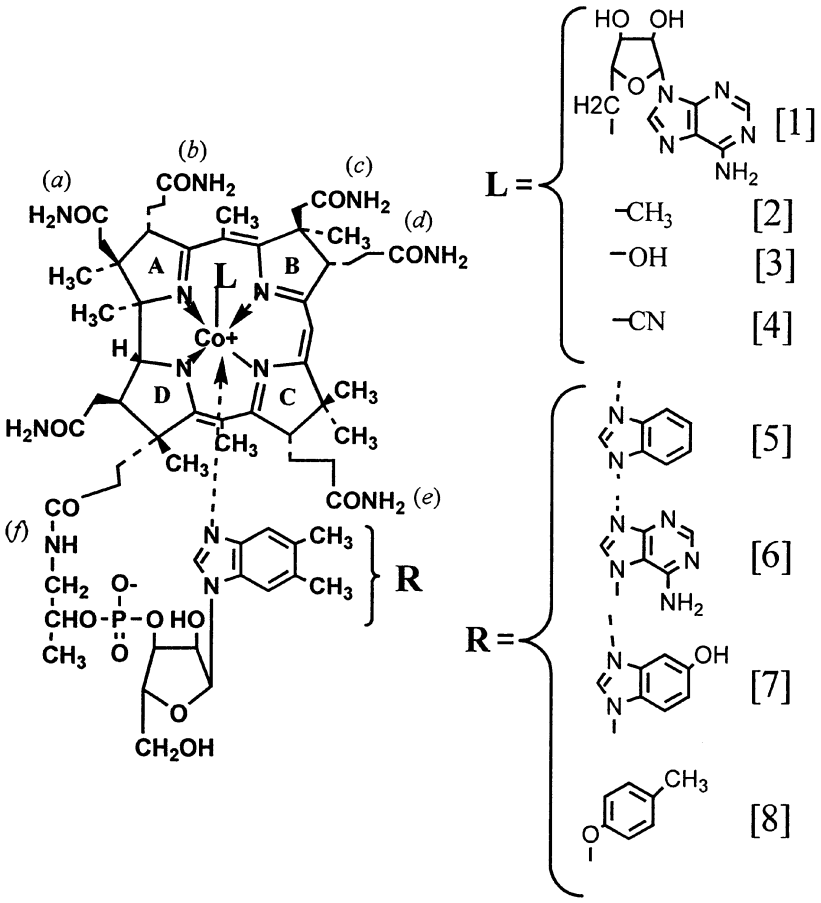

Figure 1. Structural formula of B_{12} and partial structures of B_{12}-compounds. The partial structures of B_{12}-compounds show only those portions of the molecule that differ from B_{12}. 1, AdoB_{12}; 2, MeB_{12}; 3, OH-B_{12}; 4, CN-B_{12} or B_{12}; 5, benzimidazolyl cyanocobamide; 6, pseudovitamin B_{12}; 7, 5-hydroxybenzimidazolyl cyanocobamide; 8, *p*-cresolyl cyanocobamide.

SEPARATION AND ANALYSIS OF VITAMIN B_{12} DEGRADATION PRODUCTS DURING COOKING OR PROCESSING OF FOOD

Microwave ovens are widely used for cooking and food processing. Extensive studies[8,9] have shown equal or better retention of some vitamins

(B_1, B_2, B_6, C, and folic acid) after microwave heating, compared with conventional heating. Bennink and Ono[10] have reported appreciable loss of B_{12} during cooking of raw beef, but it is unclear why such loss occurs. There is little information on how much loss of B_{12} occurs in foods during microwave heating.

Cow's milk is an excellent source of B_{12} (2 μg of B_{12}/L).[11] The loss of B_{12} increased with an increase in the treatment times for the boiled milk, as well as in the microwave-treated milk. The rate of the B_{12} loss in the microwave-treated milk was greater than that in the boiled milk after the temperature of milk reached about 100°C (Fig. 2).[12] The amount of B_{12} loss in the 6 min-microwave-treated milk sample did not differ from that in the 30 min-boiled milk.

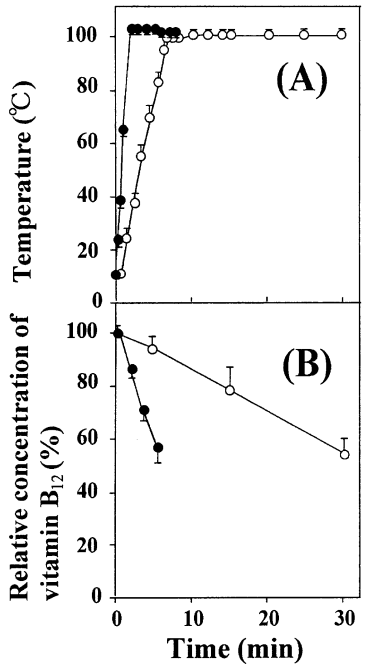

Figure 2. Effects of treatment times on concentration of cow's milk B_{12} by microwave heating. Cow's milk was treated by boiling at 100°C (control) or by microwave heating. B_{12} was extracted from both treated milk samples and assayed. Data represent mean ± SD ($n = 4$). (A) Temperature: control (○); microwave heating (●). (B) Relative B_{12} concentration: control (○); microwave treatment (●). The relative B_{12} concentration was expressed as percentage against the B_{12} concentration of milk without the boiled and microwave heating. (Adapted, with permission, from Ref. [12]. Copyright 1998 American Chemical Society).

To determine whether the loss of B_{12} in the microwave-treated foods is derived from the conversion of B_{12} to some inactive B_{12} degradation products, OH-B_{12}, which predominates in food,[13] was analyzed with silica gel 60 TLC when treated by microwave heating for 6 min (Fig. 3).[12] Authentic OH-B_{12} used in the experiments was purified by silica gel 60 TLC with 1-butanol/2-propanol/water (10 : 7 : 10 v/v) as a solvent, since the OH-B_{12} reagent (about 98% pure) contained a small amount of an impurity (a R_f value = 0.27). The treated OH-B_{12} was separated into three red spots [major compound I with a R_f of 0.03; identical R_f of intact OH-B_{12}, and minor compound II (about 18.2%) with a R_f of 0.16 and compound III (4.2%) with a R_f of 0.27].

After OH-B_{12} was treated by microwave heating for 6 min, OH-B_{12} degradation products formed were separated by a silica gel 60 column chromatography. The column eluate was fractionated into fractions I–V as indicated in Fig. 4.[14] OH-B_{12} was tightly bound to the top of the gels in this solvent system. When fraction I–V were analyzed by silica gel 60 TLC, the OH-B_{12} degradation products with a R_f of 0.27 and 0.16 predominated in fractions I–II and III–IV, respectively. A novel OH-B_{12} degradation product with a R_f of 0.12 was recovered in fraction V and has not been separable from intact OH-B_{12} by the TLC system.

The OH-B_{12} degradation products with a R_f of 0.12 and 0.16 were further purified to homogeneity by silica gel 60 TLC and C18 reversed-phase HPLC.[12,14] The ^{1}H-NMR spectra of the OH-B_{12} degradation products show

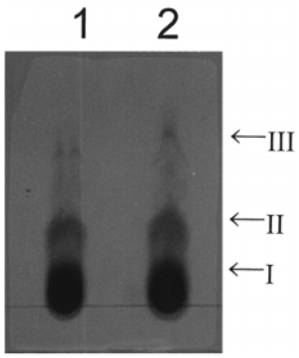

Figure 3. Formation of degradation products from purified OH-B_{12} by microwave heating. Two milliliters of 0.1 mmol/L purified OH-B_{12} was treated in the dark by boiling or microwave heating. (Lane 1) OH-B_{12} solution with the 30 min boiling treatment. (Lane 2) OH-B_{12} solution with the 6 min-microwave treatment. The data are representative of TLC patterns from five independent experiments. (Adapted, with permission, from Ref. [12]. Copyright 1998 American Chemical Society).

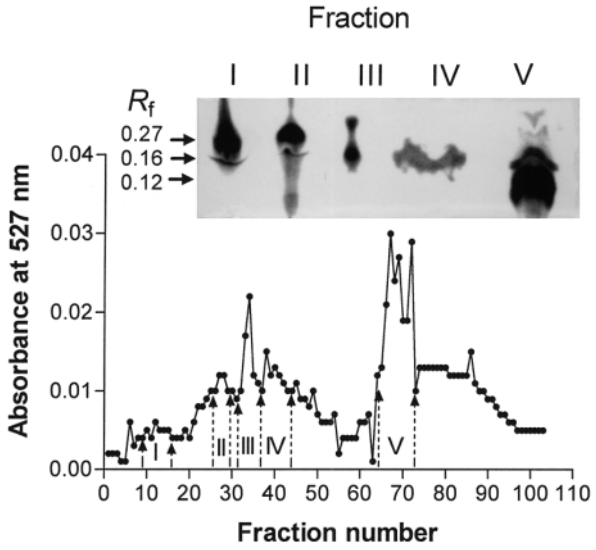

Figure 4. Elution profile of OH-B_{12} treated by microwave heating for 6 min during silica gel 60 column chromatography. Fifty milliliters of the treated OH-B_{12} solution (5 mmol/L) was evaporated to dryness and dissolved in a small amount of 1-butanol/2-propanol/water (10 : 7 : 10 v/v) as a solvent. The concentrated solution was put on a column (1.4 × 15.0 cm) of silica gel 60 equilibrated with the same solvent and eluted at 4.0 mL with a fraction collector. Fractions I–V were pooled, evaporated to dryness, dissolved with a small amount of distilled water, and analyzed with silica gel 60 TLC. Inset represents the mobile pattern of the OH-B_{12} degradation products of fractions I–V on the TLC plate. Data are a typical one of five experiments. (Adapted, with permission, from Ref. [14]. Copyright 1998 American Chemical Society).

that the degradation product with a R_f of 0.12 is a B_{12}-compound with the lower ligand structure changed slightly, but that with a R_f of 0.16 is a B_{12} compound without the base portion in the lower ligand. Although the degradation product with an R_f of 0.12 has about 13 and 23% biological activity of authentic B_{12} in hog IF (a mammalian B_{12}-binding protein) and *L. delbruekii* ATCC 7830, respectively, the product with an R_f of 0.16 did not show any biological activity. Intravenous administration of both purified degradation products to rats indicates that the compounds are not toxic. These results indicate that the conversion of B_{12} to these inactive B_{12} degradation products occurs in food during microwave heating (Fig. 5).

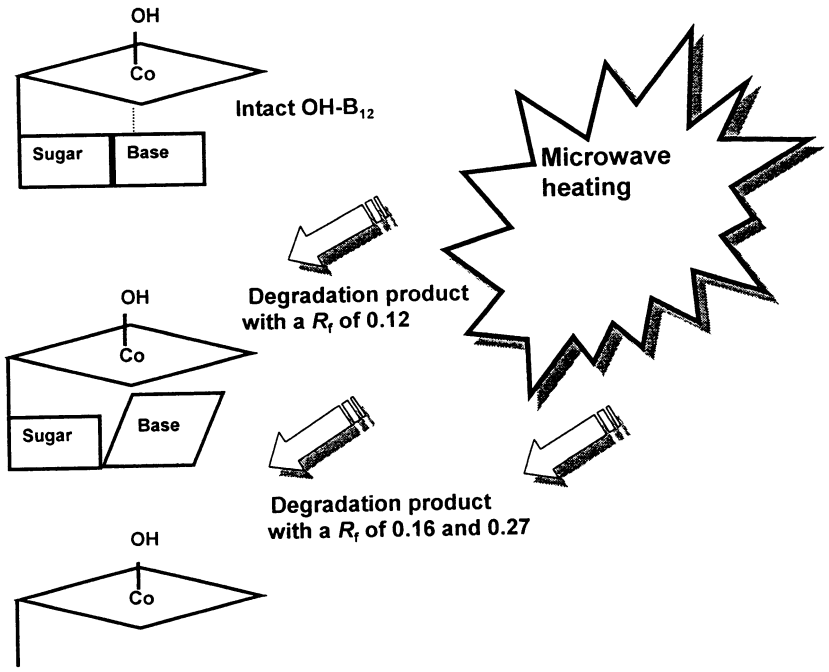

Figure 5. OH-B_{12} degradation compounds formed by microwave heating.

SEPARATION AND ANALYSIS OF VITAMIN B_{12} DEGRADATION PRODUCTS DURING STORAGE OF MULTI-VITAMIN-MINERAL SUPPLEMENTS

In the multi-vitamin-mineral food supplements containing B_{12}, appreciable loss of biologically active B_{12} occurs, since B_{12} is converted to inactive B_{12}-compounds by the addition of substantial amounts of vitamin C (C) in the presence of copper.[1] Some of the inactive B_{12}-compounds have been reported to block B_{12} metabolism in mammalian cells.[15] The destruction of B_{12} is probably concerned with radicals generated by C in the presence of copper.

As shown in Fig. 6, C alone or metal ion (Cu^{2+}) alone did not decompose B_{12}. However, B_{12} was destroyed significantly by mixing both C and Cu^{2+} (C–Cu^{2+} system).[16] Many B_{12} degradation compounds (ladder-like red colored spots) were separated from the B_{12} treated with the C–Cu^{2+} system by silica gel 60 TLC so that we could not find main products among the B_{12} degradation compounds or identify any chemical properties of the compounds.

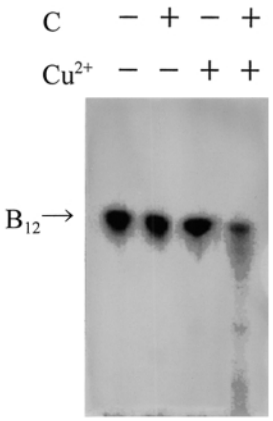

Figure 6. Silica gel 60 TLC of B_{12} treated with C in the presence or absence of copper. The reaction mixture contained 0.5 mmol/L B_{12}, 10 mmol/L C, and 1 mmol/L $CuCl_2$, and left for a week at 25°C in the dark. An aliquot of the mixture was quantitatively put on a silica gel 60 TLC plate, which was developed with 2-propanol/NH_4OH (30%)/water (7 : 1 : 2 v/v) as a solvent at room temperature in the dark. Data represent a typical TLC pattern of the B_{12} treated by the C–Cu^{2+} system from seven experiments. C, vitamin C; Cu^{2+}, $CuCl_2$; −, absence; +, presence. (Adapted, with permission, from Ref. [16]. Copyright 1997 Japan Society for Bioscience, Biotechnology, and Agrochemistry).

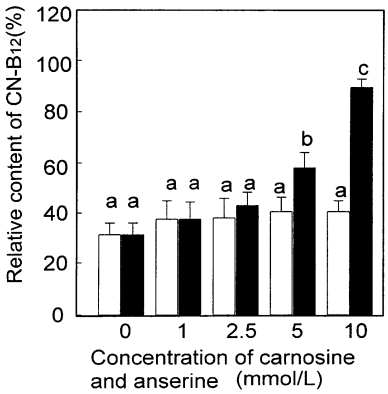

Figure 7. Effects of concentrations of carnosine and anserine on the degradation of B_{12} by C in the presence of copper. Relative content of B_{12} is expressed percentage of the control (without the C–Cu^{2+} treatment). ■, carnosine; □, anserine. All values represent mean ± SD ($n = 5$). Different letters denote significant differences ($p < 0.05$). (Adapted, with permission, from Ref. [16]. Copyright 1997 Japan Society for Bioscience, Biotechnology, and Agrochemistry).

Carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-3-methyl- L-histidine) are present in high concentrations in animal muscles,[17] some of which are used as foodstuffs (beef, pork, and chicken).[18] Many biochemical studies have indicated that carnosine and anserine function as antioxidants in mammalian cells.[19] The extent of the destruction of B_{12} by the C–Cu^{2+} system was estimated to be about 70% of the control (without the treatment) by TLC scanning (Fig. 7).[17] The destruction of B_{12} was reduced significantly by the addition of 10 mmol/L carnosine or anserine; carnosine repressed the B_{12} destruction more effectively under the conditions than anserine did. In protecting B_{12} from destruction by the C–Cu^{2+} system, carnosine would act as antioxidant or chelator for copper (or both).

SEPARATION AND ANALYSIS OF VITAMIN B_{12} AND RELATED COMPOUNDS IN FOOD

Edible Shellfish

Various shellfish are available as food items. The shellfish which siphon large quantities of B_{12}-synthesizing microorganisms in the sea are known to be excellent sources of B_{12}.[1,11] The microorganisms can synthesize various B_{12}-compounds (including inactive B_{12}-compounds for humans). If most of the B_{12} found in shellfish are inactive B_{12}-compounds, they may not be bioavailable in humans.

B_{12} contents of various edible shellfish were determined by both *L. delbruekii* ATCC 7830 microbiological and IF-chemiluminescence methods. The values determined by the microbiological method were 1.2–19.8-fold (M/C ratio) greater in the shellfish than the values determined by the IF-chemiluminescence method.[7,20] To clarify why such differences in the B_{12} contents determined by both methods occur, some B_{12}-compounds were purified from edible shellfish, i.e., oyster (M/C, 1.5), mussel (M/C, 1.2), and short-necked clam (M/C, 2.7), and partially characterized.[20] The silica gel 60 TLC and C18-HPLC patterns of each red-colored B_{12}-compound (M/C, 1.0–1.2) purified from these shellfish were identical to those of authentic B_{12} (Fig. 8). Although the higher values in the determination of B_{12} by the microbiological method may be due to occurrence of B_{12}-substitutive compounds (probably deoxyribosides and/or deoxynucleotides), the edible shellfish would be excellent B_{12} sources judging from the values ($\geq 6\,\mu g/100\,g$) determined by the IF-chemiluminescence method.

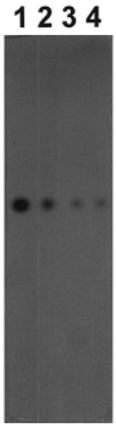

Figure 8. Silica gel 60 TLC patterns of the purified shellfish B_{12}-compounds. Each final purified preparation (2 μL) was spotted on the silica gel TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7 : 1 : 2 v/v) at room temperature in the dark. 1, authentic B_{12}; 2, oyster; 3, short-necked clam; 4, mussel. Data present a typical migration pattern of the purified B_{12}-compounds on the TLC form three experiments.

Edible Seaweed

Various types of seaweed are available as food items. Seaweed is known to be rich in vitamins and minerals as well as dietary fiber.[21] Most of the B_{12} found in seaweed has been reported to be inactive B_{12}-compounds, so that they may not be bioavailable in mammals.[2] Dried lavers (nori) appear to be most widely eaten in the world among the seaweed, and they have been reported to contain substantial amounts of B_{12}.[11] Thus, the bioavailability of the laver B_{12} in mammals is not well understood. Purification and characterization of B_{12}-compounds from the dried lavers have been studied.

B_{12} concentrations of dried green (*Enteromorpha* sp.) and purple (*Porphyra* sp.) lavers (nori) were determined by both *L. delbruekii* ATCC 7830 microbiological and IF-chemiluminescence methods.[22] The values (69.2 and 25.1 μg/100 g of dried green and purple lavers, respectively) determined by using the microbiological method were identical to those found by using the IF-chemiluminescence method in both dried lavers. Occurrence of B_{12}-coenzymes in these dried lavers was determined with silica gel 60 TLC. The dried green laver contained four known types of biologically active B_{12}-compounds (approximately OH-B_{12}, 45%; CN-B_{12}, 35%; AdoB_{12}, 6%; and MeB_{12}, 0.2%), and the non-coenzyme forms (OH and CN forms) of B_{12} predominated (Fig. 9).[22] Most

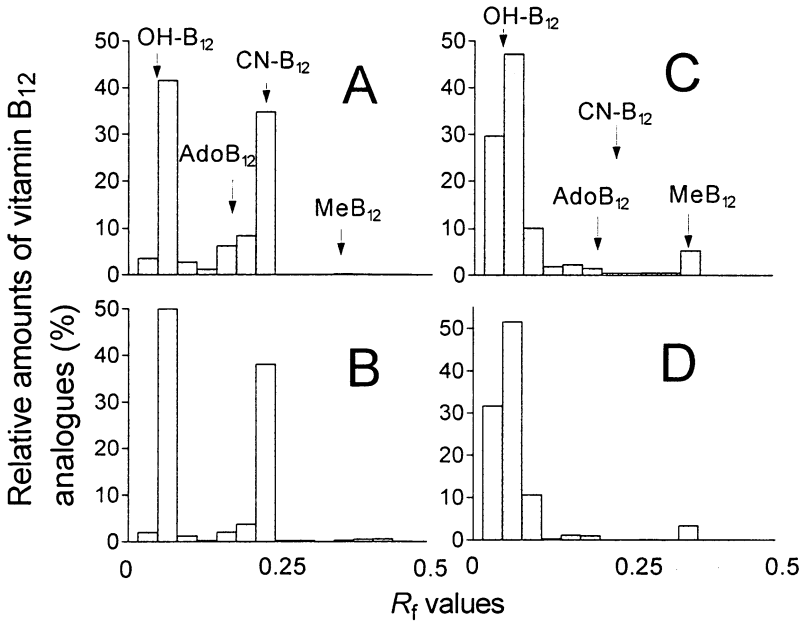

Figure 9. Silica gel 60 TLC analysis of B_{12}-compounds of dried green and purple lavers. The green laver B_{12}-compounds were determined according to the IF-chemiluminescence B_{12} assay (A) an microbiological B_{12} assay (B) methods. The purple laver B_{12}-compounds were determined according to the IF-chemiluminescence B_{12} assay (C) and microbiological B_{12} assay (D) methods. The R_f values of authentic OH-B_{12}, AdoB$_{12}$, CN-B_{12}, and MeB$_{12}$ on this TLC system were 0.03, 0.20, 0.33, and 0.36, respectively. Data present a typical migration pattern of B_{12}-compounds on the TLC from three experiments. (Adapted, with permission, from Ref. [22]. Copyright 1999 American Chemical Society).

B_{12} (about 80%) determined by both B_{12} assay methods in the dried purple laver were recovered in the OH-B_{12} fraction.

To evaluate whether the B_{12} detected in the edible purple laver, *Porphyra yezoensis*, is true B_{12} or inactive B_{12}-compound, a B_{12} compound was purified from the lyophilized purple laver, and partially characterized.[23] The silica gel 60 TLC and C18-HPLC patterns of the purified pink-colored compound were identical to those of authentic B_{12}, but not to those of B_{12}-compounds inactive for humans: identical results have been obtained in the dried green laver (*Enteromorpha prolifera*).[24]

To establish the bioavailability of the dried purple laver in mammals, the feeding experiments of the purple laver-supplemented diet to 9-week-old

Table 1. B_{12} Concentration of Spirulina Tablets

	B_{12} Concentration (μg/100 g Dry Weight)		
	Claim on Bottle[a]	Microbiological Assay	Chemiluminescence Assay
A	233	244.3 ± 3.3	8.3 ± 0.1
B	100–250	127.2 ± 6.6	6.2 ± 0.1
C	None	147.5 ± 19.3	17.4 ± 0.2

All values obtained represent mean ± SEM ($n = 4$).
[a]Determined by the microbiological assay.
(Adapted, with permission, from Ref. [30]. Copyright © 1999 American Chemical Society).

B_{12}-deficient rats was conducted.[25] When the 9-week-old B_{12}-deficient rats, which excreted substantial amounts of methylmalonic acid (71.7 ± 20.2 μmol/day) in urine, were fed the diet supplemented with the dried laver (10 μg of B_{12}/diet) for 20 days, urinary methylmalonic acid excretion (as an index of B_{12} deficiency) became undetectable and hepatic B_{12} (especially AdoB_{12}) levels were significantly increased. These results indicate that B_{12} in the dried purple laver is

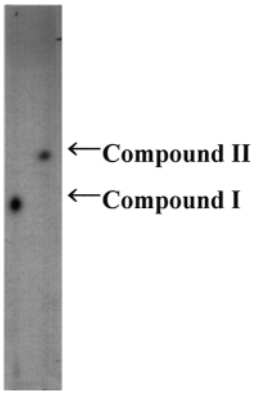

Figure 10. Silica gel 60 TLC pattern of the purified spirulina B_{12}-compounds. Each final purified preparation (2 μL) was spotted on the silica gel TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7 : 1 : 2 v/v) at room temperature in the dark. Data present a typical migration pattern of the purified B_{12}-compounds on the TLC from three experiments. (Adapted, with permission, from Ref. [30]. Copyright 1999 American Chemical Society).

Table 2. R_f Values and Retention Times of the Purified Spirulina B_{12}-Compounds, Authentic B_{12}, and Cyanocobamides on TLC and HPLC

	Spirulina Compound I	Spirulina Compound II	B_{12}	Benzimidazolyl Cyanocobamide	5-Hydroxybenzimidazolyl Cyanocobamide	Pseudovitamin B_{12}	*p*-Cresolyl Cyanocobamide
Silica gel 60 TLC[a]							
Solvent I	0.14	0.23	0.23	0.18	0.20	0.14	0.38
Solvent II	0.42	0.56	0.56	0.52	0.47	0.42	0.62
C18 reversed-phase HPLC[b]							
Isocratic	12.8	15.6	15.6	12.2	11.8	12.8	> 30
Gradient	18.4	19.0	19.0	18.3	18.2	18.4	26.1

[a]Solvent I: 1-butanol/2-propanol/water (10 : 7 : 10 v/v), Solvent II: 2-propanol/NH_4OH (28%)/water (7 : 1 : 2 v/v).
[b]Isocratic: 20% (v/v) methanol solution containing 1% (v/v) acetic acid, Gradient: a linear gradient of methanol (5–70%, v/v) in 1% (v/v) acetic acid solution.
(Adapted, with permission, from Ref. 30. Copyright © 1999 American Chemical Society).

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bioavailable to rats. These results suggest that the biological active B_{12} compounds from the dried lavers are also active in humans.

Edible Microalgae as Human Food Supplements

A health food fad involves tablets of *Spirulina* sp., blue-green algae. Many preclinical studies suggest that *Spirulina* cells have alleged therapeutic properties.[26–28] Spirulina tablets also contain large amounts of B_{12}, and can contribute to human B_{12} needs, especially for vegetarians. Several studies, however, have showed that spirulina B_{12} may not be bioavailable in mammals.[2,6,29] There is, however, no information available on the detailed chemical properties of the spirulina B_{12}-compounds.

B_{12} concentration of algal health food, spirulina tablets, was determined by both *L. delbruekii* ATCC 7830 microbiological and IF-chemiluminescence methods. The values determined by the microbiological method were about 6–9 fold greater in the spirulina tablets than the values determined by the IF-chemiluminescence method (Table 1).[30] To evaluate whether the B_{12} found in the spirulina tablets is true B_{12} or inactive B_{12}-compound, B_{12}-compounds (major and minor) were purified from the spirulina tablets and partially characterized.[30] The major (83%) and minor (17%) compounds were identified as pseudo-B_{12} and B_{12}, respectively (Fig. 10), judging from data of TLC (Table 2), C18-HPLC, ^{1}H-NMR spectroscopy, ultraviolet-visible spectroscopy, and biological activity. Pseudo-B_{12} appears to be inactive for human pernicious anaemia,[31] but not to act as a B_{12}-antogonist in mammals.[30] The results suggest that spirulina tablets are not suitable for use as a B_{12} source, especially in vegetarians. From other algal health foods, *Chlorella* (unpublished work), *Dunaliella*,[32] coccolithophorid alga,[33] some B_{12} compounds have been also purified and characterized using silica gel 60 TLC.

Our studies indicate that TLC has great advantages (simplicity, flexibility, speed, and relative inexpensiveness) for the separation and analysis of B_{12} compounds in foods.

ABBREVIATIONS

AdoB_{12}, adenosylcobalamin or adenosyl-vitamin B_{12}; B_{12} or CN-B_{12}, cyanocobalamin or vitamin B_{12}; MeB_{12}, methylcobalamin or methyl-vitamin B_{12}; OH-B_{12}, hydroxocobalamin or hydroxo-vitamin B_{12}.

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