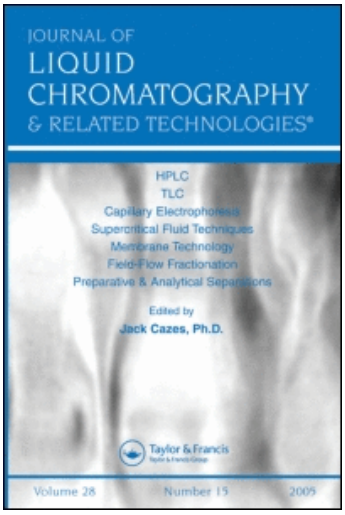

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TLC-Bioautogram Analysis of Vitamin B_{12} Compounds from Boiled and Dried Japanese Anchovy (*Engraulis japonica*) Products

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Abstract: Vitamin B_{12} contents of boiled and dried Japanese anchovy (*Engraulis japonica*) products (mild- and semi-dried white bait, and dried whole), which are commercially available in Japan, contained about $3.8 \sim 4.6$ and 44.6 μg of vitamin B_{12} per 100 g weight. TLC-bioautogram analysis indicated that the vitamin B_{12}-activity found in each anchovy product was given as a single spot whose R_f value (0.56) was identical to that of authentic vitamin B_{12}. A red-tint compound was further purified to homogeneity from the dried whole product and partially characterized. TLC and HPLC patterns of the purified compound were identical to those of authentic vitamin B_{12}. These results indicate that the dried whole Japanese anchovy would be an excellent source of vitamin B_{12} for humans.

Keywords: Boiled and dried fish, *Engraulis japonica*, Japanese anchovy, TLC, Vitamin B_{12}

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INTRODUCTION

Vitamin B_{12} (B_{12}) compounds are synthesized only by certain prokaryotic microorganisms (bacteria and archaea).[1] The B_{12} compounds synthesized by bacteria are concentrated mainly in the bodies of higher predatory animals in the natural food chain system. The usual dietary sources of B_{12} are animal food products (i.e., meat, milk, egg, fish, and shellfish).[2] Japanese people obtain most (~84%) of daily B_{12} intake from both fish and shellfish,[3] and enjoy certain boiled and dried Japanese anchovy **(***Engraulis japonica***)** products as a popular side dish. Boiling and drying processes of food, however, may cause both loss and destruction of B_{12}. Indeed, a portion of B_{12} found in raw food has been reported to be converted into some harmful B_{12} analogues during the drying process.[4]

There is little information available on whether the boiled and dried Japanese anchovy products contain true B_{12} or the harmful B_{12} analogues. In this study, we analyzed B_{12} compounds in three-kinds of boiled and dried anchovy products, using TLC as the tool for separation and analysis.

EXPERIMENTAL

Materials

Vitamin B_{12} and a reversed-phase high performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, $\varphi 4.6 \times 150$ mm; particle size, 5 μm) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cosmosil 140C18-OPN was obtained from Nacalai Tesque (Kyoto, Japan). A B_{12} assay medium for *Lactobacillus delbrueckii* subspecies *lactis* (formerly *L. leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin layer chromatography (TLC) aluminium sheets were obtained from Merck (Darmstadt, Germany). Amberlite XAD-4 was obtained from Japan Organo Co. (Tokyo, Japan). All other reagents used were of the highest purity commercially available. The boiled and dried Japanese anchovy products were provided from local markets in Tottori- and Kochi-prefectures, Japan.

A visible spectrophotometer (Ultrospec 10 pro, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used for measuring the turbidity of *L. delbreuckii* test cultures in the microbiological B_{12} assay method. A Shimadzu (Kyoto, Japan) UV-visible spectrophotometer (UV-160A) was used for spectral analysis of the purified B_{12} compound.

Methods

Extraction and Assay of Vitamin B_{12} in the Boiled and Dried Japanese Anchovy Products

After about 30 g of each sample of the boiled and dried Japanese anchovy products were homogenized with a mixer (TML160, Tescom & Co., Ltd, Tokyo, Japan), a portion (2 g) of each homogenate was used for the sample. Total B_{12} was extracted with boiling at acidic pH range and assayed by the microbiological method with *L. delbrueckii* ATCC 7830 according to the method described in the Japanese Standard Tables of Food Composition.[5] Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (known as an alkali-resistant factor) as well as B_{12}, the amount of true B_{12} was calculated by subtracting the values of the alkali-resistant factor from the values of total B_{12}.

Bioautogram of Vitamin B_{12} Compounds with Vitamin B_{12}-Dependent *Escherichia Coli* 215

Bioautograms of B_{12} compounds were done according to the method of the reference cited.[6] Two μL of the B_{12} extract prepared above and authentic B_{12} (cyanocobalamin, 10 μg/L) were spotted onto the silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at room temperature (25°C). After the TLC sheet was dried, agar containing basal medium and pre-cultured *E. coli* 215 was overlaid and then incubated at 30°C for 20 h. After being sprayed with a methanol solution of 2,3,5-triphenyltetrazolium salt on the gel plate, B_{12} compounds were visualized as red in color, indicating *E. coli* growth.

Purification of Vitamin B_{12} Compound from the Boiled and Dried Japanese Anchovy Product "Niboshi"

About 1.0 kg of the boiled and dried Japanese anchovy product "Niboshi" were homogenized with an MB-911 Magnum Blender (Hamilton Beach Commercial, USA) and added to 4 L of 0.57 mmol/L acetate buffer, pH 4.8, containing 0.05% (w/v) KCN. B_{12} compounds were extracted from the solution by boiling for 30 min at 98°C in the dark. The extraction procedures were done in a Dalton (Tokyo, Japan) draught chamber with a fume hood. The boiled solution was cooled to room temperature (25°C), filtered through a bleached cloth, and centrifuged at $10{,}000 \times g$ for 10 min. The supernatant fraction was put onto a column (5.0×40.0 cm) of Amberlite XAD-4 resin which had been washed with 5 L of methanol and equilibrated with distilled water. After the column was washed with 3 L of distilled water, B_{12} compounds were

eluted with 2.0 L of 80% (v/v) ethanol. The 80% (v/v) ethanol eluate was pooled, evaporated to dryness under reduced pressure, and dissolved in 20 mL of distilled water. The solution was centrifuged at $10{,}000 \times g$ for 5 min to remove insoluble materials, and then placed onto a column (24×150 mm) of Cosmosil 140C18-OPN (Nacalai Tesque) which had been washed with 75% (v/v) ethanol and equilibrated with distilled water. The B_{12} compounds were eluted with a stepwise gradient (0, 10, 20, 30, and 80% v/v) of ethanol. These five fractions were separately evaporated to dryness under reduced pressure and dissolved with a small amount of distilled water. The 10 and 20%-eluates were combined and fractionated with Cosmosil column chromatography under the same conditions. The 20% (v/v)-ethanol fraction was concentrated under reduced pressure and then purified with silica gel 60 TLC, which was developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as the solvent in the dark at room temperature (25°C). A spot with red-tint on the dried TLC sheet was collected, extracted with 80% (v/v) methanol, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was purified by HPLC using a Shimadzu HPLC apparatus (LC-6A Pump, SPD-6A Spectrophotometer, CTO-6A column oven, C-R6A Chromatopac). The sample (100 μL) was put onto a reversed-phase HPLC column (Wakosil-II 5C18RS, φ4.6 × 150 mm; particle size, 5 μm) equilibrated with 20% (v/v) methanol containing 1% (v/v) acetic acid at 35°C. The flow rate was 1.0 mL/min. The compound with the red-tint was isocratically eluted with the same solution, monitored by measuring absorbance at 278 nm, and collected at 1.0 mL with a Bio-Rad Laboratories fraction collector (model 2110). The fractions with the red-tint were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by HPLC under the same conditions. The peak fraction of the eluate with the red-tint was evaporated to dryness under reduced pressure, and dissolved in 100 μL of distilled water, and used as a purified B_{12} compound.

Analytical TLC and HPLC

Concentrated solutions (2 μL each) of the purified compound and authentic B_{12} were spotted on silica gel 60 TLC sheets and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) and 1-butanol/2-propanol/water (10:7:10 v/v) as solvents I and II, respectively, in the dark at room temperature (25°C).

In the case of HPLC, the diluted solutions (20 μL each) of the purified compound and authentic B_{12} were analyzed with the reversed-phase HPLC column (Wakosil-II 5C18RS, φ4.6 × 150 mm; particle size, 5-μm) using the Shimadzu HPLC apparatus. The B_{12} compounds were

isocratically eluted with 20% (v/v) methanol containing 1% (v/v) acetic acid at 35°C, and monitored by measuring the absorbance at 278 nm. The retention times of these compounds were determined at a flow rate of 1.0 mL/min.

Ultraviolet-Visible Spectra

The purified preparation was dissolved in 1.0 mL of distilled water. The spectra of the purified compound and authentic B_{12} (100 mg/L) were measured with a Shimadzu spectrophotometer (UV-160A) at room temperature (25°C). Quartz cuvettes (1.0 mL, $d = 1$ cm) were used.

RESULTS AND DISCUSSION

B_{12} contents of three-kinds of the boiled and dried products of Japanese anchovy (Fig. 1) were determined with the microbiological B_{12} assay method as described in the Standard Tables of Food Composition in Japan.[5] Mild- and semi-dried white bait products contained considerable amounts ($3.8 \sim 4.6\,\mu g/100\,g$) of B_{12}, while substantial amounts ($44.6\,\mu g/100\,g$) of B_{12} were found in the dried whole product (Table 1). These B_{12} contents were similar to the values that are described in the Standard Tables of Food Composition in Japan.[5]

To evaluate whether the B_{12}-activity detected in the boiled and dried anchovy products by the microbiological assay method is derived from true B_{12} or not, each B_{12} extract of the anchovy products was analyzed with B_{12}-dependent *E. coli* 215 bioautogram after being separated by silica gel 60 TLC (Fig. 2). The B_{12}-activity found in each of the boiled and dried products was given as a single spot, whose R_f value (0.56) was identical to that of authentic B_{12}.

To identify the B_{12}-active compound found in the boiled and dried anchovy products, a B_{12} compound was purified from the dried whole product "Niboshi" (No. 3 in Fig. 1) and then characterized.

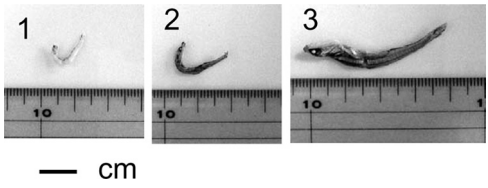

Figure 1. Boiled and dried anchovy products tested in the experiments. (1) white bait, mild-dried; (2) white bait, semi-dried; (3) whole, dried.

Table 1. Vitamin B_{12} contents of the boiled and dried Japanese anchovy products

Sardines (μg/100 g)	Vitamin B_{12} contents	Reference[1]
White bait		
Mild-dried	4.6 ± 0.9 ($n = 8$)	4.3
Semi-dried	3.8 ± 2.5 ($n = 8$)	6.3
Whole dried	44.6 ± 10.8 ($n = 4$)	41.3

[1]The values are described in the standard tables of food composition in Japan.

The red-tinted compound was easily purified with silica gel 60 TLC and reversed-phase HPLC. The ultraviolet-visible spectrum of the purified compound showed a typical absorption of cobalt-containing corrinoid compound (Fig. 3a); it is identical to that of authentic B_{12} (Fig. 3b).

The purified compound and authentic B_{12} were analyzed by silica gel 60 TLC and reversed-phase HPLC. The R_f values (0.56 and 0.09 in solvent I and II, respectively) of the purified compound were identical to the values of authentic B_{12}, of which the retention time (11.4 min) was also identical to that of the purified compound in reversed-phase HPLC (Data not shown). These results indicate that the boiled and dried anchovy product "Niboshi" contains true B_{12}, but not inactive or harmful B_{12} compounds.

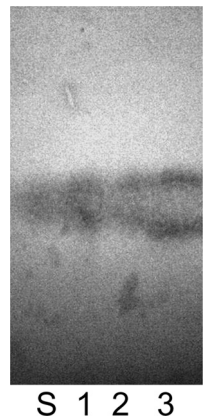

Figure 2. Silica gel 60 TLC patterns of the B_{12} extracts of the boiled and dried anchovy products. (S) Authentic B_{12}; (1) white bait, mild-dried; (2) white bait, semi-dried; (3) whole, dried. Data are typical bioautogram patterns in three independent experiments.

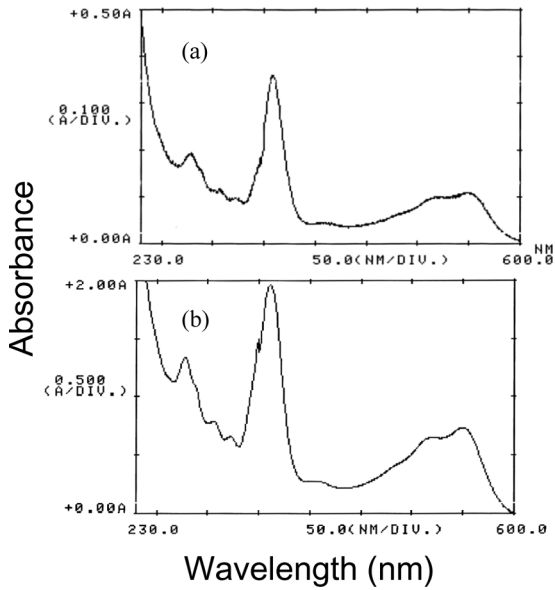

Figure 3. Ultraviolet-visible spectra of the compound purified from the boiled and dried anchovy product "Niboshi" (a) and authentic B_{12} (b). A portion of the purified preparation was dissolved in 1.0 mL of distilled water. The spectra were measured with a Shimadzu spectrophotometer (UV-160A) at room temperature. Data are typical spectra in three independent experiments.

These results presented here suggest that the boiled and dried Japanese anchovy products, especially the dried whole "Niboshi", would be excellent sources of B_{12} for humans.

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