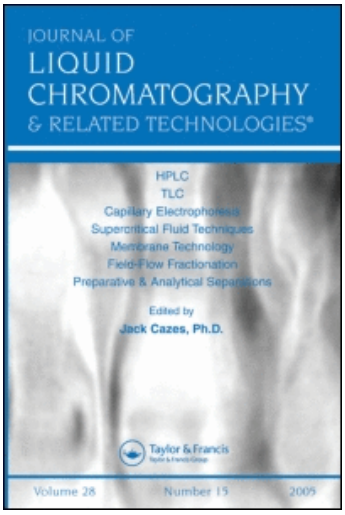

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

TLC Analysis of a Corrinoid Compound from Dark Muscle of the Yellowfin Tuna (*Thunnus albacares*)

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To cite this Article Nishioka, Michiko , Tanioka, Yuri , Miyamoto, Emi , Enomoto, Toshiki and Watanabe, Fumio(2007) 'TLC Analysis of a Corrinoid Compound from Dark Muscle of the Yellowfin Tuna (*Thunnus albacares*)', Journal of Liquid Chromatography & Related Technologies, 30: 15, 2245 — 2252
To link to this Article: DOI: 10.1080/10826070701451605
URL: http://dx.doi.org/10.1080/10826070701451605

Journal of Liquid Chromatography & Related Technologies®, 30: 2245–2252, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701451605

TLC Analysis of a Corrinoid Compound from Dark Muscle of the Yellowfin Tuna (*Thunnus albacares*)

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Abstract: A significant amount of vitamin B12 (52.9 $\pm$ 8.9 μg/100 g) was found in the dark muscle of the yellowfin tuna (*Thunnus albacares*), in comparison to that of the light muscle. A corrinoid compound was purified to homogeneity from the dark muscle and partially characterized. TLC and HPLC patterns of the purified corrinoid compound were identical to those of authentic vitamin B12. These results indicate that dark muscle of the yellowfin tuna would be an excellent source of vitamin B12 for humans and aminals.

Keywords: Yellowfin tuna, *Thunnus albacares*, Dark muscle, HPLC, TLC, Vitamin B12

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INTRODUCTION

Vitamin B12 (B12) compounds can be synthesized only by certain prokaryotic microorganisms (bacteria and/or archaea).[1] Various forms of B12 compounds with different base moieties in the lower ligand occur in nature. Although some of them are not active for humans, they are assayed by the method authorized for food analysis. For example, the edible cyanobacterium *Spirulina* sp. contains substantial amounts of pseudovitamin B12 which is inactive for humans.[2] The salted and fermented fish product, fish sauce, contains a considerable amount of an inactive or unidentified corrinoid compound.[3]

The B12 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 B12 are animal food products (i.e., meat, milk, egg, fish, and shellfish).[4] Considering the food chain system, the meats of larger-sized fish (*e.g.*, the yellowfin tuna) would be expected to contain high amounts of B12. The white meat (light muscle) of tuna is a popular food item for people in various countries. While dark meat (dark muscle) of tuna is not usually used as food item for humans, it is used mainly as a source of pet food for dogs and cats. Although our preliminary study has indicated that dark muscle of tuna contains substantial amounts of B12, it is unclear whether this muscle contains true B12 or an inactive corrinoid compound.

In this study, we describe the purification and characterization of a corrinoid compound from the dark muscle of the yellowfin tuna using TLC as the tool for separation and analysis.

EXPERIMENTAL

Materials

B12 and a reversed-phase high performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, $\phi 4.6 \times 150$ mm; particle size, 5 μm) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cosmosil 140C180-OPN was obtained from Nacalai Tesque (Kyoto, Japan). A B12 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 tested samples of dark muscle of the yellowfin tuna (*Thunnus albacares*) were provided from local markets in both Ishikawa- 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 B12 assay method. A Shimadzu (Kyoto, Japan) UV-visible spectrophotometer (UV-1600) was used for spectral analysis of the purified corrinoid compound.

Methods

Extraction and Assay of Vitamin B12 in Dark Muscle of the Yellowfin Tuna

After about 50 g of each sample of dark muscle of the yellowfin tuna were homogenized with a mixer (MX-X51-H, National, Osaka, Japan), a portion (2 g) of each homogenate was used for the sample. Total B12 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 B12, the amount of true B12 was calculated by subtracting the values of the alkali-resistant factor from the values of total B12.

Bioautography of Corrinoid Compounds with Vitamin B12-Dependent *Escherichia coli* 215

Bioautograpy of corrinoid compounds was done according to the modified method of the reference cited.[6] Two μL of the B12 extract prepared above and authentic B12 (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, corrinoid compounds were visualized as red in color, indicating *E. coli* growth.

Purification of Corrinoid Compound from Dark Muscle of the Yellowfin Tuna

About 500 g of dark muscles of the yellowfin tuna were homogenized with an MB-911 Magnum Blender (Hamilton Beach Commercial, USA) and added to 4 L of 10 mmol/L acetate buffer, pH 4.8, containing 10 mmol/L KCN. Corrinoid compound was 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) and centrifuged at 10,000 × *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, the corrinoid compound was 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 50 mL of distilled water. The solution was placed on 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 corrinoid compound was 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. Each concentrated solution was purified by silica gel 60 TLC, which was developed with 2-propanol/NH_4OH (28%)/water (7:1:2, v/v) as the solvent in 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 on 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 corrinoid compound.

Analytical TLC and HPLC

Concentrated solutions (2 μL each) of the purified compound and authentic B12 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 B12 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 corrinoids 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 corrinoids were determined at a flow rate of 1.0 mL/min.

Figure 1. Silica gel 60 TLC patterns of the B12 extract of dark muscle of the yellowfin tuna. Authentic B12 (1) and B12 extract of dark muscle of the tuna (2) were analyzed by *E. coli* 215 bioautography.

Ultraviolet-Visible Spectrum

The purified preparation was dissolved in 0.1 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-16000) at room temperature (25°C). Super micro quartz cuvettes (0.1 mL, $d = 1$ cm) were used.

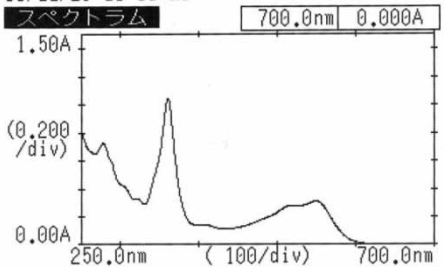

Figure 2. Ultraviolet-visible spectrum of the purified compound. A portion of the purified preparation was dissolved in 0.1 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, super-micro quartz cuvettes (0.1 mL, $d = 1$ cm) being used.

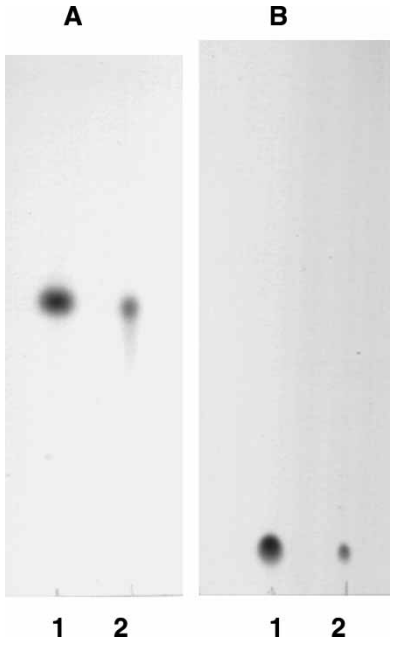

Figure 3. Silica gel TLC patterns of the compound purified from dark muscle of the yellowfin tuna and authentic B12. Concentrated solutions (2 μL) of the authentic B12 (1) and the purified compound (2) were spotted on silica gel 60 TLC sheets and developed with solvent I (A) and II (B).

RESULTS AND DISCUSSION

A substantial amount (52.9 ± 8.9 μg/100 g) of B12 was found in dark muscle of the yellowfin tuna ($n = 5$) in comparison to that of the light muscle whose B12 content (5.8 μg/100 g) is described in the Standard Tables of Food Composition in Japan.[5] To evaluate whether the B12 activity detected in the dark muscle by the microbiological assay method is derived from true B12 or not, the B12 extract of the dark muscle was analyzed with B12-dependent *E. coli* 215 bioautography after being separated by silica gel 60 TLC (Fig. 1). The B12-activity found in dark muscle of the yellowfin tuna was given as a single spot, whose *R*f value (0.56) was identical to that of authentic B12.

To identify the B12-active compound found in the dark muscle, a B12 compound was purified and characterized. The spot with the red-tint was found only in the 20% (v/v) ethanol fraction during Cosmosil 140 C18 OPN column chromatography in the purification steps. The red-tint compound was easily purified with silica gel 60 TLC and reversed-phase HPLC.

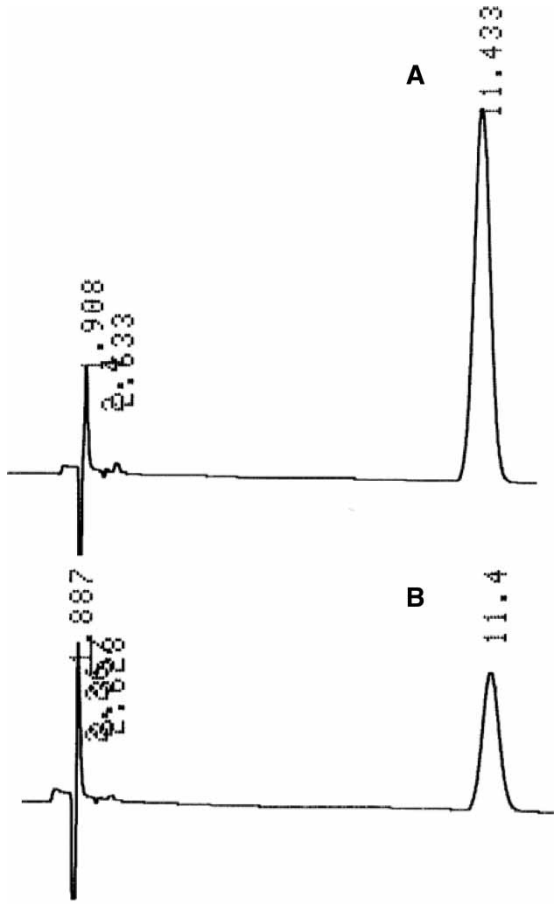

Figure 4. Reversed-phase HPLC patterns of the compound purified from the dark muscles of tuna and authentic B12. Diluted solutions (20 μL) of the authentic B12 (A) and the purified compound (B) were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions described in the text.

The ultraviolet-visible spectrum of the purified compound showed a typical absorption of cobalt-containing corrinoid compound (Fig. 2); λmax nm (absorption) was at 550.0 (0.312), 521.0 (0.281), 361.0 (1.048), and 278.5 (0.727).

The purified compound and authentic B12 were analyzed by silica gel 60 TLC and reversed-phase HPLC. As shown in Fig. 3, 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 B12, of which the retention time (11.4 min) was also identical to that of the purified compound in reversed-phase HPLC (Fig. 4). These results indicate that dark muscle of the yellowfin tuna contains substantial amounts of true B12.

The results presented here indicate that dark muscle of the yellowfin tuna would be an excellent source of B12 for both humans and animals.

ACKNOWLEDGMENT

This study was supported in part by a grant for Comprehensive Research on Cardiovascular Diseases from Ministry of Health, Labor and Welfare of Japan (to F. W.).

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Received November 4, 2006
Accepted November 29, 2006
Manuscript 6104D