Food and Nutrition Sciences, 2014, 5, 1326-1332
Published Online August 2014 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2014.514144

Determination of Vitamin B_{12} in Chinese Black Tea Leaves

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Received 25 May 2014; revised 28 June 2014; accepted 13 July 2014

Abstract

We determined vitamin B_{12} content of Chinese black tea leaves using a microbiological assay based on *Lactobacillus delbrueckii* ATCC 7830. Trace levels (0.25 - 0.69 μg/100g dry weight) of vitamin B_{12} were detected in Pu'er, Fu, and Brick tea leaves. However, vitamin B_{12} content (0.06 - 1.37 μg/100g dry weight) of Ryubao tea leaves significantly varied. To determine whether Chinese black tea leaves contain vitamin B_{12} or other corrinoid compounds that are inactive in humans, corrinoid compounds were purified from Ryubao tea by an immunoaffinity column chromatography and vitamin B_{12} was identified by liquid chromatography-electrospray ionization/tandem mass spectrometry. Vitamin B_{12} content in the tea drink prepared from Ryubao tea leaves was very low (0.8 ng/100mL). Our results indicate that Chinese black tea is usually not a good source of B_{12}, although Ryubao tea leaves with the highest B_{12} content may be utilized as a source of this vitamin for vegetarians.

Keywords

Chinese Black Tea Leaves, Cobalamin, Ryubao Tea, Vitamin B_{12}

1. Introduction

Tea is the second highest consumed nonalcoholic beverage worldwide, and an important dietary source of flavonoid compounds [1]. Although these tea polyphenols possess therapeutic properties, including anti-cardiovascular and anti-cancer effects *in vitro* and *in vivo*, epidemiological and clinical studies suggest an association with moderately reducing the risk of chronic diseases [2]. Chinese tea is generally divided into at least three

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How to cite this paper: Teng, F., Bito, T., Takenaka, S., Yabuta, Y. and Watanabe, F. (2014) Determination of Vitamin B12 in Chinese Black Tea Leaves. *Food and Nutrition Sciences*, **5**, 1326-1332. http://dx.doi.org/10.4236/fns.2014.514144

categories on the basis of different production methods: non-fermented (green tea), semi-fermented (oolong tea), and fully fermented (black tea) [3]. Among these, only black tea leaves are withered, rolled, and fermented with bacteria [3]. Biologically active compounds containing anti-oxidative [4], anti-mutagenic [5], and anti-hypertriacylglycerolemia [6] properties have been observed in black tea leaves.

Vitamin B_{12} (B_{12}) is synthesized by certain bacteria and is mainly concentrated in the bodies of higher predatory organisms in the natural food chain system [7]. In daily life, we mainly ingest B_{12} from animal-derived foods (fish, shellfish, meat, and eggs). Thus, strict vegetarians have a greater risk of developing B_{12} deficiency compared with non-vegetarians [8]. The major symptoms of B_{12} deficiency are neuropathy and megaloblastic anemia [9]. Thus, we need to identify plant foods that contain high levels of B_{12} to prevent vegetarians from developing B_{12} deficiency.

Although plant-derived foods generally contain zero or trace B_{12}, Kittaka-Katsura *et al.* [10] demonstrated that the Japanese black tea leaf Batabata-cha contains approximately 0.5 μg of B_{12} per 100 g of dried tea leaves, which is bioavailable in mammals. In addition, they determined that B_{12} content of two types of Chinese black tea leaves [11] is similar to that of Batabata-cha. However, B_{12} content in various types of Chinese black tea leaves and the identification of B_{12} compounds in Chinese black tea leaves as "true" B_{12} or inactive corrinoid compounds in humans remain to be established.

Here we describe the characterization of B_{12} compounds from various Chinese fermented black tea leaves using thin-layer chromatography—*Escherichia coli* 215 bioautography and liquid chromatography-electrospray ionization/tandem mass spectrometry (LC/ESI-MS/MS).

2. Materials and Methods

2.1. Materials

Authentic B_{12} was obtained from Sigma (St. Louis, Missouri, USA). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were purchased from Merck (Darmstadt, Germany). All other reagents were high-grade and commercially available. Various types of Chinese black tea leaves (Pu'er, Ryubao, Fu, and Brick) were purchased from local markets in Japan (**Figure 1**).

2.2. Extraction and Assay of B_{12} from Chinese Black Tea Leaf Samples

Each sample (5 g) of dried Chinese black tea leaves was homogenized in a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). A portion (2.0 g) of the homogenate was used as the test sample. Total B_{12} compounds were extracted by boiling at pH 4.8 in the presence of 4.0×10^{-4} % KCN and assayed using a microbiological B_{12} assay based on *L. delbrueckii* ATCC 7830, according to the method described in the Standard Tables of Food Composition in Japan [12]. *L. delbrueckii* ATCC 7830 utilizes deoxyribosides, deoxyribonucleotides (known as alkali-resistant factor), and B_{12}.

The correct B_{12} values were calculated by subtracting results for the alkali-resistant factor from those for total B_{12}. Tea from 3-g Ryubao leaves (sample H) with the highest B_{12} content among those tested was extracted for 5 min with 150 mL of boiling water. After cooling down to 40˚C, the extract was used as a tea drink, and B_{12} was extracted from 50 mL of liquid using the above-mentioned method.

2.3. Bioautography of Corrinoid Compounds Using B_{12}-Dependent *E. coli* 215

Bioautography of corrinoid compounds was performed as previously described [13]. B_{12} extract (50 mL) prepared as described above was partially purified and concentrated using Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, USA) prewashed with 5 mL of 75% (v/v) ethanol and equilibrated with 5 mL of distilled water. The C18 cartridge was washed with 5 mL of distilled water, and B_{12} compounds were eluted using 2 mL of 75% (v/v) ethanol. The eluate was evaporated in a centrifugal concentrator (Integrated SpeedVac® System ISS110; Savant Instruments Inc., NY, USA) and the residual fraction was dissolved in 2.0 mL of distilled water. Concentrated B_{12} extracts (1 μL) and authentic B_{12} and pseudo B_{12} (50 μg/L each) were spotted onto the silica gel 60 TLC sheet and developed in the dark using 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) at room temperature (25˚C). After drying, the TLC sheet was overlaid with agar containing a basal medium and precultured *E. coli* 215, and incubated at 37˚C for 20 h. The gel plate was sprayed with methanol solution containing 2, 3, 5-triphenyltetrazolium salt and B_{12} compounds were visualized as red, indicating *E. coli* growth.

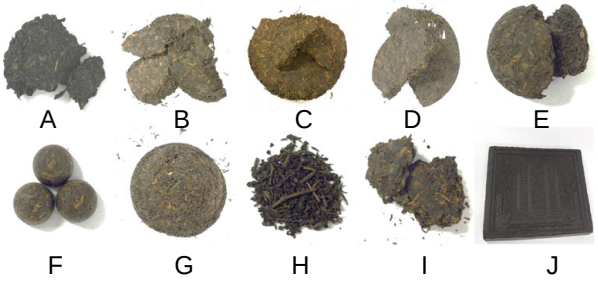

Figure 1. Types of Chinese black tea leaves. Pu'er tea (samples A - G), Ryubao tea (sample H), Fu tea (sample I), and Brick tea (sample J) leaves were used in this study.

2.4. Liquid Chromatography-Electrospray Ionization/Tandem Mass Spectrometry Analysis

Sample H (10 g) containing high levels of B_{12} was suspended in 500 mL of distilled water and homogenized in a mixer (TML 160). The homogenate was added to 50 mL of 0.57 mol/L acetic buffer (pH 4.5) with 0.05 g KCN and boiled for 30 min to extract B_{12} compounds. Extraction procedures were performed in a draught chamber (Dalton Co., Tokyo, Japan). The boiled suspension was centrifuged at 5000× *g* for 10 min. An aliquot (approximately 200 mL) of the supernatant was placed in Sep-pak Vac 20 cc (5 g) C18 cartridges (Waters Corp.) prewashed with 75% (v/v) ethanol and equilibrated with distilled water. The C18 cartridges were washed with 30 mL of distilled water and B_{12} compounds were eluted using 30 mL of 75% (v/v) ethanol. The remaining supernatant was treated in the same manner. Combined eluates were evaporated to dryness under reduced pressure, and the residual fraction was dissolved in 5.0 mL of distilled water and centrifuged at 10,000 × *g* for 10 min to remove any insoluble material. The supernatant fraction was loaded onto EASI-EXTRACT Vitamin B_{12} Immunoaffinity Column (P80) [R-Biopharm AG, Darmstadt, Germany], and corrinoids were purified according to the manufacturer's recommended protocol. B_{12} compounds, pseudo B_{12}, and B_{12} were dissolved in 0.1% (v/v) acetic acid and filtered using a Nanosep MF centrifual device (0.4 μm, Pall Corp., Tokyo, JAPAN) to separate small particles. Aliquots (2 μL) of filtrate were analyzed using LCMS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, JAPAN). Each purified corrinoid was injected into an InertSustain column (3 μm, 2.0 × 100 mm, GL Science, Tokyo, JAPAN) and equilibrated with 85% solvent A [0.1% (v/v) acetic acid)] and 15% solvent B (100% methanol) at 40˚C. Corrinoid compounds were eluted using a linear gradient of methanol (15% solvent B for 0 - 5 min, increasing the concentration from 15% to 90% solvent B for 5 - 11 min, followed by decreasing the concentration from 90% to 15% solvent B for 11 - 15 min) at a flow rate of 0.2 mL/min. ESI conditions were determined by injecting pseudo B_{12} or B_{12} into the MS detector to ascertain the optimum parameters for detecting the parent B_{12} compound and daughter ions. ESI-MS was operated in the positive ion mode with argon as collision gas. Pseudo B_{12} (*m/z* 672.777) and B_{12} (*m/z* 678.292) as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

3. Results and Discussion

3.1. Vitamin B_{12} Contents

B_{12} levels were assayed in 10 Chinese black tea leaves that are commercially available worldwide using the microbiological B_{12} assay method based on *L. delbrueckii* ATCC 7830 (**Table 1**). Traces (0.25 - 0.69 μg/100g dry weight) of the corrected B_{12} were observed in Pu'er, Fu, and Brick tea leaves. However, Ryubao tea leaves (sample H) contained the highest B_{12} content (1.37 μg/100g dry weight), which is similar to that previously reported [11]. To further clarify whether Ryubao tea leaves generally contain high levels of B_{12}, we determined the B_{12} content of the other Ryubao leaf samples. As shown in **Table 2**, the corrected B_{12} content of various Ryubao tea leaves varied (0.06 - 1.37 μg/100g dry weight), and their mean value was calculated as approximately 0.69 μg of B_{12}, which is only slightly higher than that for Pu'er tea leaves (approximately 0.49 μg/100g dry weight). High levels (0.61 - 2.02 μg B_{12} equivalent/100 g dry weight) of the alkali-resistant factor were detected in all tested Chinese black tea leaves.

3.2. *E. coli* 215 Bioautography Analysis

B_{12} compounds identified in Chinese black tea leaf samples A - J were analyzed using the *E. coli* 215 bioautogram after separation using silica gel 60 TLC (**Figure 2**). The Ryubao tea leaf extract (sample H) produced a

Table 1. Vitamin B_{12} content of various types of Chinese black tea leaves.

	Apparent B_{12}	Alkali-resistant factor	Corrected B_{12}
	(μg/100g dry weight)		
Pu'er tea leaves			
Sample A	1.94	1.32	0.62
Sample B	1.75	1.5	0.25
Sample C	2.55	1.86	0.69
Sample D	1.53	1.05	0.48
Sample E	2.35	1.77	0.58
Sample F	1.52	1.07	0.45
Sample G	2.42	2.02	0.40
Ryubao tea leaves			
Sample H	2.94	1.57	1.37
Fu tea leaves			
Sample I	1.87	1.30	0.57
Brick tea leaves			
Sample J	0.74	0.61	0.13
Mean ± SD	1.96 ± 0.63	1.41 ± 0.43	0.55 ± 0.33

*Total B_{12} compounds were extracted from a portion (2.0 g) of each type of black leaf homogenate by boiling at pH 4.5 in the presence of 4.0×10^{-4} % KCN and assayed using the *Lactobacillus delbrueckii* ATCC 7830 microbiological assay. *L. delbrueckii* ATCC 7830 utilizes deoxyribosides, deoxyribonucleotides (alkali-resistant factor), and B_{12}. Correct B_{12} values were calculated by subtracting the results for the alkali-resistant factor from those for total B_{12} concentration. The B_{12} assay was performed in triplicate.

Table 2. Vitamin B_{12} content of various types of Ryubao tea leaves.

	Apparent B_{12}	Alkali-resistant factor	Corrected B_{12}
	(μg/100g dry weight)		
Sample H	2.94	1.57	1.37
Sample K	1.57	1.23	0.34
Sample L	2.89	1.90	0.99
Sample M	1.68	1.62	0.06
Mean ± SD	2.27 ± 0.75	1.58 ± 0.27	0.69 ± 0.60

*Total B_{12} compounds were extracted from a portion (2.0 g) of Ryubao tea leaf homogenates by boiling at pH 4.5 in the presence of 4.0×10^{-4} % KCN, and assayed using *Lactobacillus delbrueckii* ATCC 7830 microbiological assay. Correct B_{12} values were calculated by subtracting the results for the alkali-resistant factor from those for total B_{12} concentration. The B_{12} assay was performed in triplicate.

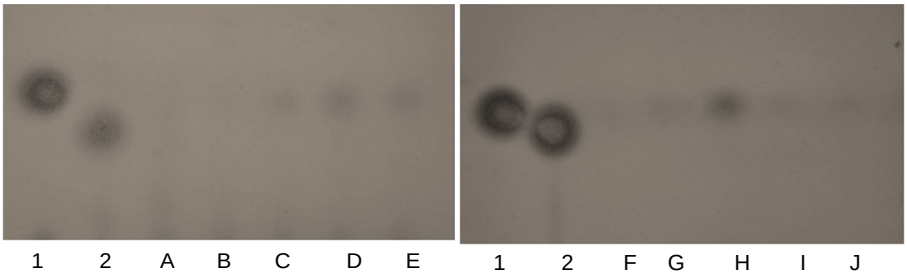

Figure 2. *Escherichia coli* 215 bioautogram analysis of B_{12} compounds detected in various black tea leaf sample. Authentic B_{12} (1), pseudo B_{12} (2), and concentrated extracts of various black tea leaf samples A - J. Typical bioautograms from three independent experiments are presented.

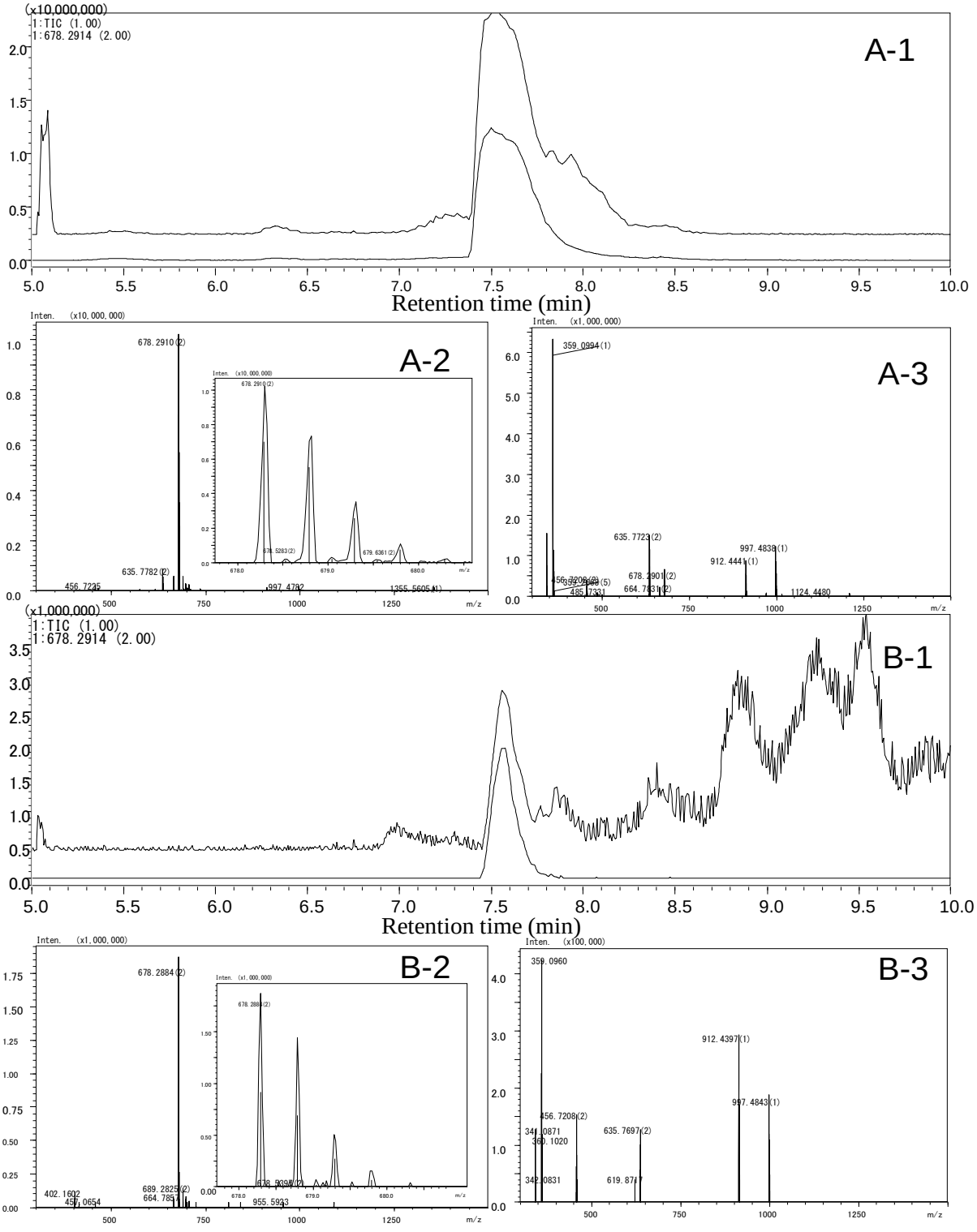

Figure 3. LC/ESI-MS/MS chromatograms of authentic B_{12} and the B_{12} compounds purified from black tea leaf sample H. B_{12} compounds were analyzed using the LCMS-IT-TOF system (Shimadzu). Panels (A-1) and (B-1) show total ion chromatograms and those (*m/z* 678.2914) of authentic B_{12} and B_{12} compounds purified from sample H. Mass spectra of authentic B_{12} and purified B_{12} compounds at 7.5 min are shown in panels (A-2) and (B-2), respectively (magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel). MS/MS spectra for the peak of authentic B_{12} at *m/z* 678.2910 and that of purified B_{12} compounds at *m/z* 678.2884 are shown in panels (A-3) and (B-3), respectively.

single, clear spot with an R_f value identical to that of authentic B_{12}. Indistinct spots with the R_f value identical to that of authentic B_{12} were detected in samples C, D, E, G, and I. The remaining samples showed no spot because of their lower B_{12} contents.

3.3. LC/ESI-MS/MS Analysis

To more precisely identify the corrinoid compounds present in Chinese black tea leaves, corrinoids were purified from the Ryubao tea leaf extract (sample H) containing high B_{12} content and identified using LC/ESI-MS/MS (**Figure 3**). Authentic B_{12} was eluted as a peak with a retention time of 7.5 min. The mass spectrum of authentic B_{12} primarily comprised a doubly charged ion with *m/z* 678.2910 $[M + 2H]^{2+}$ (**Figure 3(A-1)** and **Figure 3(A-2)**). MS/MS spectra revealed a predominant monovalent ion with *m/z* 359.0994, which was largely attributable to the nucleotide moiety of B_{12} (**Figure 3(A-3)**). The corrinoid purified from the Ryubao tea leaf sample H was eluted as several ion peaks, indicating the presence of impurities. The mass spectrum of the main peak with *m/z* 687.2914 had a retention time of 7.5 min in the purified sample (**Figure 3(B-1)** and **Figure 3(B-2)**). The MS/MS spectrum of the purified compound with a monovalent ion with *m/z* 359.0960 was identical to that of authentic B_{12} (**Figure 3(A-3)** and **Figure 3(B-3)**). These results indicate that the Ryubao tea leaf sample H contained authentic B_{12} but not pseudo B_{12} which is inactive in humans.

B_{12} content in the tea drink prepared from the Ryubao tea leaf sample H was 0.8 ng/100mL of black tea. Therefore, consumption of approximately 300 L of this tea would provide the recommended dietary allowance for adults (2.4 μg/day) [14] [15], although ingestion of such large quantities of tea on a daily basis is not recommended. Notably, Kittaka-Katsura *et al.* [10] demonstrated that administration of the Japanese black tea drink (B_{12} content, approximately 2.0 ng/100mL) considerably improves B_{12} status in B_{12}-deficient rats. Considering these earlier observations and our present findings, we propose that Ryubao tea leaves containing significantly levels of B_{12} can be utilized as a source of vitamin B_{12} for vegetarians.

Acknowledgements

This work was supported by JSPSKAKENHI Grant number 25450168 (FW).

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