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ORIGINAL ARTICLE

Chemistry and Biochemistry

Determination and characterization of vitamin B_{12} compounds in edible sea snails, ivory shell *Babylonia japonica* and turban shell *Turdo Batillus cornutus*

Fei Teng[1] · Yuri Tanioka[2] · Natsumi Hamaguchi[3] · Tomohiro Bito[1] · Shigeo Takenaka[4] · Yukinori Yabuta[1,3] · Fumio Watanabe[1,3]

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Abstract In this study, we characterized and quantified vitamin B_{12} compounds in popular edible snails *Babylonia japonica* and *Turdo Batillus cornutus* using a microbiological assay based on *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830. The meat and viscera of *B. japonica* contained 27.2 ± 9.1 and 92.8 ± 25.8 µg of vitamin B_{12} per 100 g, respectively. However, the meat and viscera of *T. cornutus* contained extremely low amounts of vitamin B_{12} (3.0 ± 1.5 and 15.1 ± 8.3 µg of vitamin B_{12} per 100 g, respectively). We identified the vitamin B_{12} compounds from the edible portions (meat and viscera) of *B. japonica* and *T. cornutus* using liquid chromatography–electrospray ionization/tandem mass spectrometry. We found that *B. japonica* contained substantial amounts of true vitamin B_{12}, while pseudovitamin B_{12} was the predominant corrinoid in *T. cornutus*. These results indicate that the meat and viscera of *B. japonica* are excellent sources of vitamin B_{12} for humans.

Keywords Edible sea snails · Ivory shell · Pseudovitamin B_{12} · Turban shell · Vitamin B_{12}

✉ Fumio Watanabe
watanabe@muses.tottori-u.ac.jp

1 Division of Applied Bioresources Chemistry, United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8553, Japan

2 Department of Nutrition, Junior College of Tokyo University of Agriculture, Setagayaku 156-8502, Japan

3 Department of Agricultural, Biological and Environmental Sciences, School of Agriculture, Tottori University, Tottori 680-8553, Japan

4 Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan

Introduction

Vitamin B_{12} compounds are synthesized only by certain bacteria and are concentrated mainly in the bodies of higher predators in the natural food chain. The usual dietary sources of vitamin B_{12} are animal products (i.e., meat, milk, egg, fish, and shellfish) [1]. The Japanese obtain most (approximately 84 %) of their daily vitamin B_{12} intake from fish and shellfish [2]. Shellfish siphon large quantities of vitamin B_{12}-synthesizing bacteria from seawater and freshwater and are excellent sources of vitamin B_{12} (>10 µg/100 g wet weight) [1, 3]. However, these vitamin B_{12}-synthesizing bacteria can also synthesize other corrinoids that contain a different base moiety in the lower ligand of the molecule [4].

Our previous studies indicated that vitamin B_{12} levels were significantly higher in edible bivalves (approximately 60 µg/100 g wet weight) than in edible snails (approximately 20 µg/100 g wet weight) [5]. The corrinoid compounds purified from most edible bivalves (clams, oysters, mussels, etc.) have been identified as 'true' vitamin B_{12} [6]. In the edible sea snail abalone, vitamin B_{12} and pseudovitamin B_{12} (Coβ-cyano-N7-adeninyl-cobamide, an inactive corrinoid for humans; as shown in Fig. 1) were observed to be the major and minor corrinoid compounds, respectively [5]. However, there is little information available on the vitamin B_{12} compounds of other edible sea snails. Among the edible sea snails, ivory shell *Babylonia japonica* and turban shell *Turdo Batillus cornutus* are the most popular food items in Japan. Their meat and viscera are edible because high levels of vitamin B_{12} accumulate in the viscera of shellfish [5]. If these popular snails contain a large amount of "true" vitamin B_{12}, they would be good sources of vitamin B_{12} in humans.

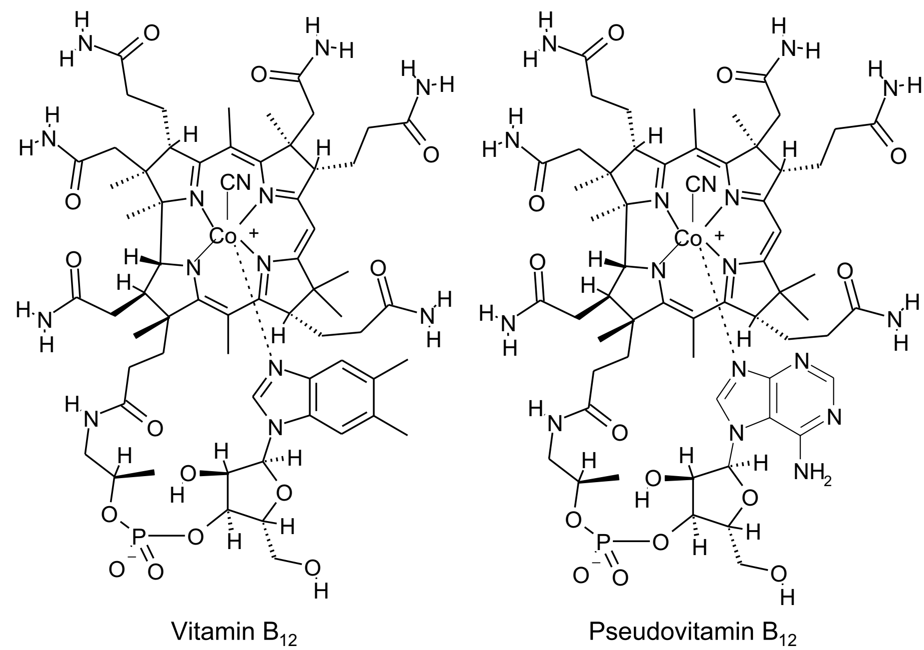

Fig. 1 Structures of vitamin B_{12} and pseudovitamin B_{12}

In this study, we identified vitamin B_{12} compounds from the edible portions (meat and viscera) of *B. japonica* and *T. cornutus* using liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS). We found that *B. japonica* contains substantial amounts of "true" vitamin B_{12}, while pseudovitamin B_{12} was the predominant corrinoid in *T. cornutus*. These results indicate that the meat and viscera of *B. japonica* are excellent sources of vitamin B_{12} for humans.

Materials and methods

Materials

Vitamin B_{12} (cyanocobalamin) was obtained from Sigma-Aldrich (St Louis, Missouri, USA). Pseudovitamin B_{12} that had been purified from *Aphanizomenon flos-aquae* and identified with proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy [7] was used in this study. A vitamin B_{12} assay medium based on *Lactobacillus delbrueckii* (formerly *L. leichmannii*) ATCC 7830 was obtained from Nissui (Tokyo, Japan). Raw *B. japonica* and *T. cornutus* were purchased from local markets in Tottori prefecture, Japan.

Extraction and assay of vitamin B_{12} from edible snails

After the shells were removed from *B. japonica* and *T. cornutus*, their edible portions (meat and viscera) were sampled. Each sample was homogenized using a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). An aliquot (2.0 g) of the homogenate was used as the sample for the vitamin B_{12} assay. Vitamin B_{12} compounds were extracted from each sample by boiling for 30 min under acidic conditions (pH 4.5) and then assayed using a microbiological method based on *L. delbrueckii* ATCC 7830, according to a previously described method [4]. The extraction procedures were done in a Dalton (Tokyo, Japan) draft chamber in the dark.

Because *L. delbrueckii* ATCC 7830 can utilize deoxyribosides and deoxyribonucleotides (known to be an alkali-resistant factor) as well as vitamin B_{12}, the correct vitamin B_{12} values were calculated by subtracting the values for the alkali-resistant factor from the total vitamin B_{12} values.

Identification of sea snail vitamin B_{12} compounds by LC/ESI–MS/MS

Each vitamin B_{12} extract (40 mL) was partially purified and concentrated using a Sep-Pak® Plus C18 cartridge (Waters Corp., MA, USA), as described previously [4]. The eluate was evaporated to dryness under reduced pressure, and then was dissolved in 3 mL of distilled water and centrifuged at $10{,}000\times g$ for 10 min to remove insoluble material. The supernatant fraction was loaded onto an immunoaffinity column [EASI-EXTRACT® vitamin B_{12} immunoaffinity column (P80), R-Biopharm AG, Darmstadt, Germany], and were purified according to the manufacturer's protocol. The purified vitamin B_{12} compounds were dissolved in 0.1-% (v/v) acetic acid and filtered through a Nanosep MF centrifuge device (0.4 µm; Pall Corp., Tokyo, Japan) to remove small particles. An aliquot (2 µL) of the filtrate was analyzed using an LC–MS ion trap-time-of-flight (IT−TOF) system coupled to an ultra-fast LC system (Shimadzu,

Table 1 Vitamin B_{12} content of edible sea snails

	Amount of vitamin B_{12} compounds			Body weight[a] (g)
	Muscle (μg/100 g)	Viscera (μg/100 g)	Whole body[a] (μg/one body)	
Ivory shell *Babylonia japonica* ($n = 5$)	27.2 ± 9.1	92.8 ± 25.8	4.7 ± 0.7	11.0 ± 0.9
Turban shell *TurdoBatillus cornutus* ($n = 5$)	3.0 ± 1.5	15.1 ± 8.3	1.7 ± 0.6	20.1 ± 4.0

Data represent the values per wet weight

[a] except their shells

Kyoto, Japan). Each purified corrinoid was injected into an inert-sustain column (3 μm, 2.0 × 100 mm; GL Science, Tokyo, Japan) equilibrated with 85 % of solvent A [0.1-% (v/v) acetic acid] and 15 % of solvent B (100-% methanol) at 40 °C. Corrinoids were eluted using a linear gradient of methanol (15 % of solvent B for 0–5 min, 15–90 % of solvent B for 5–11 min, and 90–15 % of solvent B for 11–15 min). The flow rate was 0.2 mL/min. ESI conditions were determined by injecting the corrinoids into the MS detector, thereby identifying the optimum parameters for detecting parent and daughter ions of vitamin B_{12} compounds. The ESI–MS system was operated in a positive ion mode, and argon was used as the collision gas. The identities of pseudovitamin B_{12} (*m/z* 672.7749) and vitamin B_{12} (*m/z* 678.2914) as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

Results

Vitamin B_{12} content of edible sea snails

We analyzed the vitamin B_{12} content of the edible sea snails of the ivory-shelled *B. japonica* and the turban-shelled *T. cornutus*, which are commonly consumed in Japan, using the *L. delbrueckii* ATCC 7830 microbiological assay method (Table 1). The viscera of *B. japonica* contained a substantial amount of vitamin B_{12} (approximately 92.8 μg/100 g wet weight); 3.4 times greater than that of the meat (approximately 27.2 μg/100 g wet weight). The meat and viscera of *T. cornutus* contained significantly lower amounts of vitamin B_{12} (approximately 3.0 and 15.1 μg/100 g wet weight, respectively). These results indicated that high levels of vitamin B_{12} accumulate in the viscera of these edible sea snails. The vitamin B_{12} content (approximately 4.7 μg) per whole body of *B. japonica* was 2.8 times greater than that of *T. cornutus*. The vitamin B_{12} contents determined in our analysis are significantly higher than those [4.3 and 1.3 μg of vitamin B_{12} per 100 g of edible portion (without shell and viscera) of ivory shell and turban shell, respectively] described in the *Standard Tables of Food Composition in Japan 2010* [8].

Identification of corrinoid compounds from edible sea snails using LC/ESI–MS/MS analysis

Edible snail extracts were purified using a vitamin B_{12} immunoaffinity column and then analyzed using LC/ESI–MS/MS. Authentic pseudovitamin B_{12} and vitamin B_{12} were eluted as peaks with retention times of 7.4 and 7.5 min, respectively (Fig. 2a, d, respectively). The mass spectrum of authentic pseudovitamin B_{12} indicated that a doubly-charged ion with an *m/z* of 672.7769 $[M + 2H]^{2+}$ was prominent (Fig. 2b). The exact mass calculated from its formula ($C_{59}H_{83}CoN_{17}O_{14}P$) was 1343.5375 and the isotope distribution data showed that pseudovitamin B_{12} was the major doubly-charged ion under the LC/ESI–MS conditions used in our analyses. For authentic vitamin B_{12}, which has an exact mass of 1354.5674 ($C_{63}H_{88}CoN_{14}O_{14}P$), a doubly-charged ion with an *m/z* of 678.2883 $[M + 2H]^{2+}$ was prominent (Fig. 2e). The MS/MS spectra of authentic pseudovitamin B_{12} and vitamin B_{12} indicated that their dominant ions at *m/z* 348.0695 and *m/z* 359.0984, respectively, were attributable to the nucleotide moiety of each corrinoid compound (Fig. 2c, f). The corrinoids purified from the meat of *B. japonica* were eluted as an ion peak with *m/z* 678.2914 at a retention time of 7.5 min. The mass spectrum showed that a doubly-charged ion was formed at *m/z* 678.2928 (Fig. 3a, b). The MS/MS spectrum of the compound was identical to that of vitamin B_{12} (Fig. 3c). Identical spectral data were obtained for the corrinoids purified from *B. japonica* viscera (Fig. 3d–f). These results indicate that vitamin B_{12} is the predominant corrinoid compound in *B. japonica*. The compounds purified from *T. cornutus* meat eluted as several total ion peaks, indicating that impurities remained. The ion peaks of *m/z* 672.7749 and *m/z* 678.2914 due to pseudovitamin B_{12} and vitamin B_{12}, respectively, were also found (Fig. 4a). Their retention times of 7.3 and 7.4 min, respectively, were similar to those of authentic pseudovitamin B_{12} (retention time of 7.4 min) and vitamin B_{12} (retention time of 7.5 min). Such slight differences in retention times may be due to the existence of impurities in the purified compounds. The mass spectra of the materials eluting at retention times of 7.3 and 7.4 min showed doubly-charged ions at *m/z* 672.7764 (Fig. 4b) and *m/z*

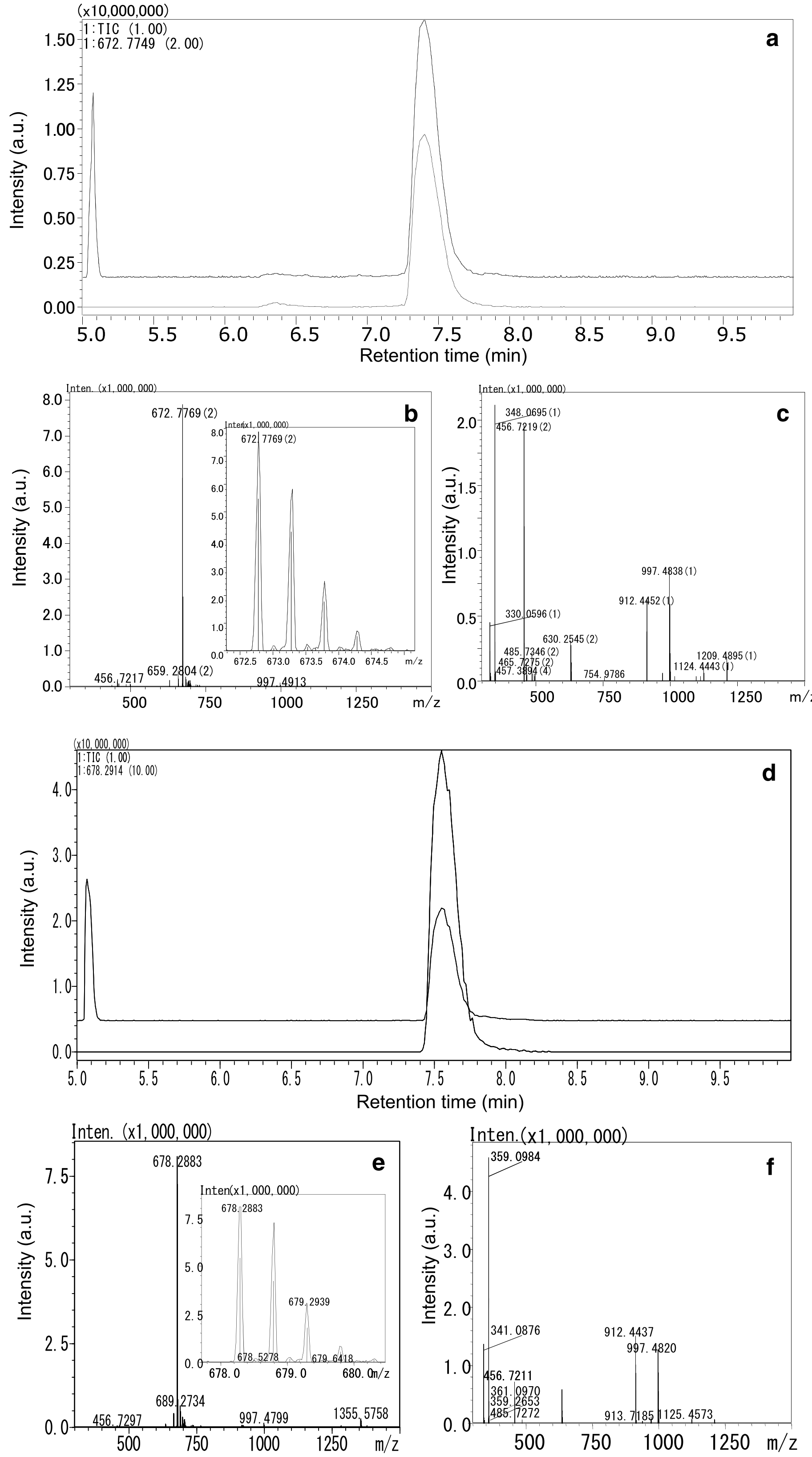

Fig. 2 LC/ESI–MS/MS chromatograms of authentic pseudovitamin B_{12} and vitamin B_{12}. Pseudovitamin B_{12} and vitamin B_{12} were analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. **a**, **d** Total ion chromatograms (TICs) of authentic pseudovitamin B_{12} and vitamin B_{12}, respectively. **b**, **e** Mass spectra of the ion peaks from pseudovitamin B_{12} (*inserts* magnified mass spectra from *m/z* 672 to 675) and vitamin B_{12} (*inserts* magnified mass spectra from *m/z* 678 to 680), respectively. **c**, **f** MS/MS spectra of the peaks of pseudovitamin B_{12} and vitamin B_{12}, respectively

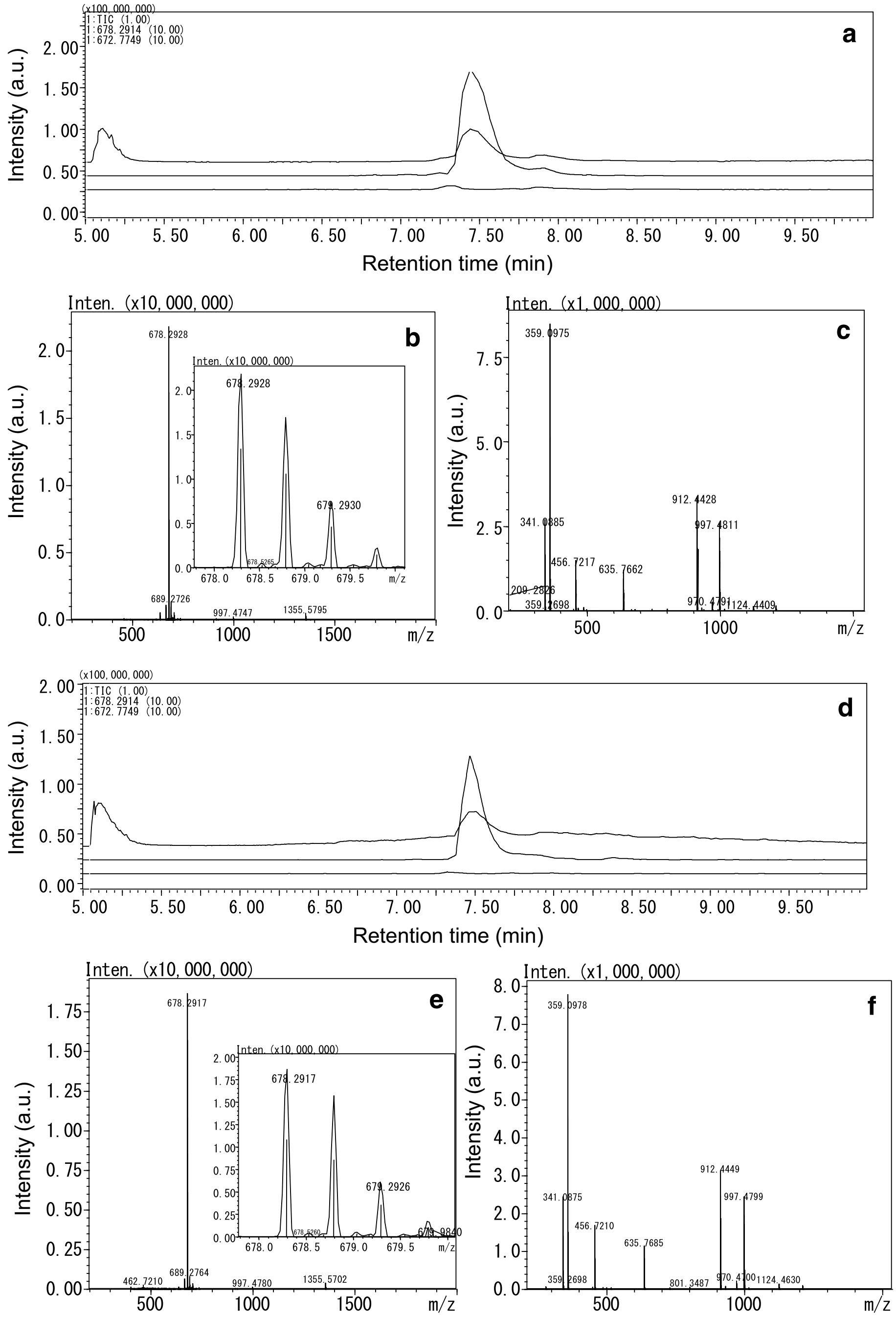

Fig. 3 LC/ESI–MS/MS chromatograms of the vitamin B_{12} compounds purified from the meat and viscera of *B. japonica*. **a**, **d** TICs and ion chromatograms for *m/z* 678.2914 (×10) and 672.7749 (×10) of the vitamin B_{12} compounds purified from the meat and viscera of *B. japonica*, respectively. **b**, **e** Mass spectra of the ion peaks of the meat and visceral vitamin B_{12} compounds at retention times of 7.5 min (*inserts* magnified mass spectrum from *m/z* 678 to 680), respectively. **c**, **f** MS/MS spectra for the peaks of the muscle and visceral vitamin B_{12} compounds at *m/z* 678.2928 and at *m/z* 678.2917, respectively

678.2856 (Fig. 4d), respectively. The MS/MS spectra of these compounds were identical to those of pseudovitamin B_{12} (Fig. 4c) and vitamin B_{12} (Fig. 4e). Similar results were obtained with the visceral sample, but no vitamin B_{12} was detected (Fig. 4f–h). These results indicate that pseudovitamin B_{12} is the predominant corrinoid compound in *T. cornutus*. Similar results were reported in abalone [5]. This result indicated that *T. cornutus* would not be a suitable source of vitamin B_{12}.

Discussion

The vitamin B_{12} content of foods were determined using the *L. delbrueckii* ATCC 7830 bioassay method. Our previous studies showed that the observed correlation rate between the values determined by the *L. delbrueckii* ATCC 7830 bioassay and intrinsic factor (the most specific vitamin B_{12}-binding protein)-based chemiluminescence method is excellent, except for foods containing substantial amounts of pseudovitamin B_{12} [9]. These results indicated

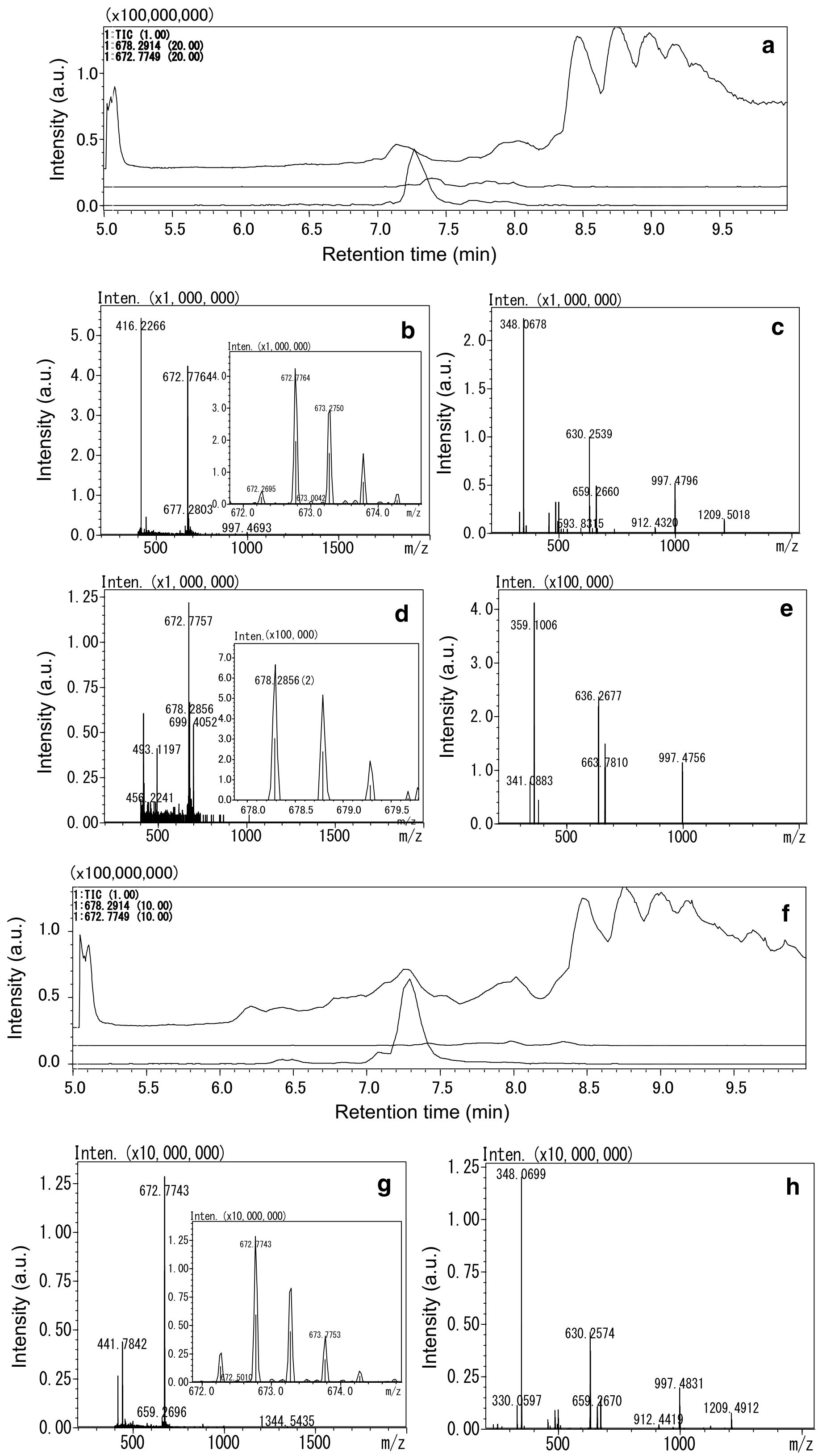

Fig. 4 LC/ESI–MS/MS chromatograms of the vitamin B_{12} compounds purified from the meat and viscera of *T. cornutus*. **a**, **f** TICs and ion chromatograms for *m/z* 678.2914 (×10 and ×20) and 672.7749 (×10 and ×20) of the vitamin B_{12} compounds purified from the meat and viscera of *T. cornutus*, respectively. **b**, **d** Mass spectra of the ion peaks of the meat vitamin B_{12} compounds at retention times of 7.3 min (*inserts* magnified mass spectrum from *m/z* 672 to 675) and 7.4 min (*inserts* magnified mass spectrum from *m/z* 678 to 680), respectively. **c**, **e** MS/MS spectra for the peaks of the muscle vitamin B_{12} compounds at *m/z* 672.7764 and at *m/z* 678.2856, respectively. **g** Mass spectrum of the ion peak of the visceral vitamin B_{12} compounds at a retention time of 7.3 min (*inserts* magnified mass spectrum from *m/z* 672 to 675). **h** MS/MS spectrum for the peak of the visceral vitamin B_{12} compound at *m/z* 672.7743

that *L. delbrueckii* ATCC 7830 utilizes pseudovitamin B_{12} as well as vitamin B_{12}. Thus, pseudovitamin B_{12} found in the edible portions of turban shells was determined as vitamin B_{12} using the *L. delbrueckii* ATCC 7830 bioassay.

The differences in content and vitamin B_{12} compounds between these edible sea snails is dependent on their dietary habitats, because *B. japonica* and *T. cornutus* are carnivorous and herbivorous sea snails, respectively. Vitamin B_{12} is synthesized only by certain bacteria and is concentrated mainly in the bodies of higher predators in the natural food chain. The usual dietary sources of vitamin B_{12} are animal-derived products but not plant-derived products [1]. The vitamin B_{12} content of tengusa *Gelidium pacificum Okamura* and wakame *Undaria pinnatifida*, the foods of *T. cornutus*, are very low (0.2 − 0.5 μg/100 g dry weight) [8]. Yamada et al., [10] demonstrated that wakame predominantly contained certain vitamin B_{12} analogues. Moreover, various blue-green algae (cyanobacteria) contain substantial amounts of pseudovitamin B_{12} [1].

The consumption of one whole body (meat and viscera, approximately 11 g) of *B. japonica*, which contains a considerably high vitamin B_{12} level (approximately 4.7 μg), could supply the entire recommended dietary allowance for an adult (2.4 μg/day) [11]. Our unpublished studies indicated that the edible portions (without shell and viscera) of other carnivorous sea snails, e.g., whelks *Buccinum striatissium* and *Neptunea intersculpta*, also contain considerable amounts of vitamin B_{12} [10.13 ± 3.33 (n = 5) and 28.72 ± 5.48 (n = 5) μg/100 g, respectively]. The results presented here indicate that these edible carnivorous sea snails would be excellent sources of vitamin B_{12} for humans.

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