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ORIGINAL ARTICLE

Chemistry and Biochemistry

Determination and characterization of vitamin B_{12} in the muscles and head innards of edible shrimp

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Abstract

We determined the vitamin B_{12} content in both the muscles and head innards of various shrimp species [*Argis lar* (Owen, 1839); Togezako shrimp, *Argis toyamaensis* (Yokoya, 1933); *Pandalopsis japonica*, Balss, 1914; *Pandalus eous* Makarov, 1935] using a microbiological assay based on *Lactobacillus delbrueckii* subsp. *lactis* ATCC7830. Approximately 2–4 µg vitamin B_{12}/100 g wet weight—a considerable amount—was detected in shrimp muscles. The shrimp head innards contained significantly higher levels of vitamin B_{12} (~ 12–33 µg/100 g wet weight). Commercially available shrimp-innard products contained ~ 30 µg vitamin B_{12}/100 g wet weight. We purified vitamin B_{12} compounds from the extracts of shrimp muscles and head innards using an immunoaffinity column. The muscle extract contained only one corrinoid compound, which was identified as vitamin B_{12} using liquid chromatography–electrospray ionization/tandem mass spectrometry, whereas the shrimp head innards contained three corrinoid compounds, which included large amounts of vitamin B_{12} and two smaller amounts of vitamin B_{12}-*d*-monocarboxylic acid and tentatively identified vitamin B_{12} dicarboxylic acids.

Keywords *Argis lar* · *Argis toyamaensis* · *Pandalopsis japonica* · *Pandalus eous* · Vitamin B_{12}

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Introduction

Vitamin B_{12} (B_{12}), also known as cobalamin, is a water-soluble vitamin that is one of a group of compounds containing a corrin ring. The lower ligand of B_{12} is attached to the cobalt-coordinated corrin ring through the nucleotide loop containing 5,6-dimethylbenzimidazole as a base (Watanabe and Bito 2016). B_{12} is synthesized by specific archaea and bacteria but not by animals or plants (Watanabe et al. 2014). The synthesized B_{12} is accumulated mainly in higher predatory animals through the natural food chain; therefore, animal-derived foods contain considerable amounts of B_{12} (Watanabe and Bito 2018a, b). In particular, fish and shellfish are reported to be important nutritional sources of B_{12} for humans (Bourre and Paquotte 2008; Scheers et al. 2014). Thaumarchaeota and cyanobacteria have recently been identified as the major authentic B_{12} and pseudovitamin B_{12} (pseudo-B_{12}) producers, respectively, in the oceans (Heal et al. 2017). Pseudo-B_{12} is inactive in humans because it carries adenine in place of 5,6-dimethylbenzimidazole in the nucleotide moiety of the molecule (Watanabe et al. 1999).

To maintain an adequate supply of B_{12} for the general population, more detailed information on biologically active B_{12} compounds in seafoods is warranted. Therefore, we have purified and identified corrinoid compounds from various seafood species (Bito et al. 2018). Although most of the meats from the fish and shellfish that we tested contained B_{12} but not pseudo-B_{12} (Bito et al. 2018), pseudo-B_{12} was detected in the edible portion of some sea snails (Teng et al. 2015).

Shrimp is one of the most consumed seafoods worldwide, and both aquaculture-produced and wild-caught shrimp are used to supply seafood products for human consumption (Butcherine et al. 2019). Shrimp is a good source of several types of nutrients, such as amino acids, polyunsaturated fats, and minerals (Kaymaci and Altun 2016); however, the detailed characteristics of B_{12} have not been elucidated in this edible seafood. Although microbiological assays have determined that there is ~ 2 μg B_{12}/100 g wet weight in the raw muscle of edible shrimp (Ministry of Education, Culture, Sports, Science and Technology 2010), information on the B_{12} content in the other edible portions, such as the shrimp head innards, are lacking; shrimp head innards are used to make shrimp sauces and other products in Asian countries. Moreover, it is unclear whether both shrimp muscles and head innards contain pseudo-B_{12}.

In the present study, we determined the B_{12} contents of the shrimp head innards and muscles in four species of shrimp and purified and characterized corrinoid compounds from both of these edible portions using liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS). To the best of our knowledge, this is the first study to demonstrate that shrimp head innards contain substantial amounts of B_{12} plus two unidentified B_{12} compounds, which might be inactive for humans.

Materials and methods

Materials

The cyanocobalamin that was used as B_{12} in this study was purchased from Sigma-Aldrich (St. Louis, MO). B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids were prepared and their concentrations were determined on the basis of $\varepsilon_{361} = 28.06 \times 10^3$ M^{-1} cm^{-1} as described previously (Watanabe et al. 1992). The revised designation of these B_{12} acid compounds were used according to Anton et al. (1980). *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 was purchased from ATCC (Manassas, VA). A B_{12} assay medium based on *L. delbrueckii* subsp. *lactis* ATCC 7830 was purchased from Nissui (Tokyo). Four types of fresh shrimp (Alaskan pink shrimp, *Pandalus eous* Makarov, 1935; Morotoge shrimp, *Pandalopsis japonica* Balss, 1914; Kuro shrimp, *Argis lar* [Owen, 1839], and Togezako shrimp, *Argis toyamaensis* [Yokoya, 1933]) were purchased from local markets in Tottori, Japan. Products made from shrimp head innards were purchased from various seafood markets in Japan.

Extraction and assay of B_{12} in shrimp muscles and head innards

B_{12} was assayed using a microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830, which has been adopted by the *Standard Tables of Food Composition in Japan* (Watanabe and Bito 2018a, b). After removing the shells from the fresh samples, the muscles and head innards were separated. Each muscle (2.0 g) or innard sample (1.0 g) was homogenized using a mortar and pestle and then added to 40 ml distilled water, 10 ml of 0.57 M acetic acid buffer (pH 4.5), and 0.4 ml of 0.05% (w/v) potassium cyanide (KCN). Total B_{12} was extracted by boiling the solution for 30 min in a draft chamber. After cooling, 0.6 ml of 10% (w/v) metaphosphoric acid was added to the B_{12} extract and the vessel was filled to 100 ml with distilled water. The prepared extract was filtrated through a 150-mm-diameter Whatman filter paper (GE Healthcare UK, UK). The filtrate was then divided into two portions of 25 ml each, the pH of one portion adjusted to 6.0, and the vessel filled to 50 ml with distilled water to use as extract A for the total B_{12} assays. The pH of the remaining portion was adjusted to 11 and the portion autoclaved (MC-23; ALP, Tokyo) at 121 °C for 30 min. After cooling, the pH of the solution was readjusted to 6.0, and the vessel was filled to 50 ml with distilled water to use as extract B to determine the alkali-resistant factor.

A 2.5-ml assay mixture was created containing 0.01 ml of extract A, B, or standard B_{12} solution (0, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 μg/l B_{12}) and 1.25 ml B_{12} basal medium for the assay (Nissui) prepared according to the manufacturer's protocol, and 1.24 ml distilled water. This mixture was placed in polypropylene tubes (13 × 100 mm; Bio-Rad Laboratories, Hercules, CA), vigorously shaken to mix it, and autoclaved at 121 °C for 5 min. After *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 were precultured in a B_{12} inoculum broth (Nissui) for 18 h, the bacterial cells were washed several times and diluted with sterile saline. The diluted bacteria solution was aseptically added to each assay mixture and allowed to stand for 16–18 h at 37 °C. To estimate bacterial growth, the turbidity of each assay mixture was measured at 600 nm using the a ultraviolet–visible (UV-Vis) spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan).

Lactobacillus delbrueckii subsp. *lactis* ATCC 7830 requires B_{12} as an essential nutrient, but exhibits nucleotide and deoxyribonucleotide activity, which is known to be an alkali-resistant factor, as well as B_{12}. The correct B_{12} values were calculated by subtracting the values for the

alkali-resistant factor from the total B_{12} values. The recovery rate of B_{12} from the extraction was calculated to be 105% after adding a known amount of authentic B_{12} to a sample.

Analysis of B_{12} compounds purified from the muscles or head innards of shrimp using LC/ESI–MS/MS

After ~ 30 g muscle or ~ 2 g head innards from Alaskan pink shrimp and Kuro shrimp, respectively, was homogenized using a mortar and pestle, the B_{12} compounds were extracted by boiling at pH 4.5 in the presence of KCN, as described above. The B_{12} compounds were partially purified from the extract using Sep-Pak Vac 20 cc (5 g) C18 cartridges (Waters, Milford, MA) that were washed with 20 ml 75% (v/v) ethanol and then equilibrated with 20 ml Milli-Q water (Merck Millipore, Burlington, MA). The extract was filtered through a 150-mm-diameter Whatman filter paper (GE Healthcare) and loaded onto the C18 cartridge, which was washed with 25 ml Milli-Q water (Merck Millipore). The B_{12} compounds were eluted with 10 ml of 75% (v/v) ethanol. The eluate was evaporated to dryness under reduced pressure using the Integrated SpeedVac System ISS110 centrifugal concentrator (Savant Instruments, Holbrook, NY). The residue was dissolved in 1 ml Milli-Q water (Merck Millipore) and loaded into an EASI-EXTRACT vitamin B_{12} immunoaffinity column (P80; R-Biopharm, Darmstadt, Germany), and the B_{12} compounds were purified according to the manufacturer's instructions. The purified B_{12} compounds were dissolved in Milli-Q water (Merck Millipore) and filtered through a Millex-LH membrane filter (Merck Millipore). Aliquots (5 µl) of the filtrate were analyzed using an ACQUITY UPLC H-Class Xevo G2-SQT (Waters). Each purified sample was injected into a 3-µm 2.1 × 100-mm InertSustain column (GL Science, Tokyo) equilibrated with 85% solvent A [0.1% (v/v) acetic acid] and 15% solvent B (methanol) at 40 °C. The B_{12} compounds were eluted using a linear gradient of methanol (15% solvent B for 0–5 min, 15–90% solvent B for 5–11 min, and 90–15% solvent B for 11–15 min) at a flow rate 0.2 ml/min. The identification of authentic B_{12} (*m/z* 678.2914 representing $[M+2H]^{2+}$; retention time 9.55 min) was confirmed by comparing the observed molecular ions and their retention times.

To determine the relative content (percent) of B_{12}, B_{12}-*d*-monocarboxylic acid, and tentatively identified B_{12}-dicarboxylic acids in the head innards of both shrimp, the absorbance was measured at 361 nm. The relative content of each peak area against total peak area (i.e., the sum of the peak area of B_{12} and B_{12}-acid compounds) was calculated. The recovery rate of B_{12} from the extraction and purification was calculated to be 102% after adding a known amount of authentic B_{12} to a sample.

High-performance liquid chromatography analysis of B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids and unidentified B_{12} compounds found in the head innards of Alaskan pink shrimp *P. eous*

B_{12} compounds were purified from the head innard extract from the Alaskan pink shrimp using the B_{12} immunoaffinity column as described above. The purified compounds were dissolved in 80 µl Milli-Q water (Merck Millipore), filtered through a Millex-LH membrane (Merck Millipore), and loaded into the Shimadzu HPLC system (SPD-10AV UV–Vis detector, SCL-10A VP system controller, DGU-$20A_3$ degasser, LC-10Ai liquid chromatograph, and CTO-20A column oven). A 35-µl aliquot of the purified compounds was loaded into a reversed-phase high-performance liquid chromatography column (φ 4.6 × 150 mm; Wakosil-II 5C18RS; FUJIFILM Wako Pure Chemical, Osaka, Japan), isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C, and monitored by measuring the absorbance at 361 nm. The flow rate was 1 ml/min. Authentic B_{12} and B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids (each 1 µg) were analyzed under the same conditions.

Results

The B_{12} contents of the muscles and head innards of four species of shrimp were determined using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC 7830 (Table 1). The shrimp muscles contained ~ 2.4–4.3 µg B_{12}/100 g wet weight, the values of which were identical to those described in the *Standard Tables of Food Composition in Japan* (Ministry of Education, Culture, Sports, Science and Technology 2010) but approximately eight times greater in the head innards than in the muscles. Shrimp-innard products A and B also contained ~ 28.5–30.1 µg B_{12}/100 g wet weight. The muscles, head innards, and whole bodies (except the shells) of the shrimp tested contained ~ 0.2, 0.3, and 0.5 µg B_{12}/fresh total body weight (g), respectively. These results suggest that shrimp is a source of B_{12} for humans.

To evaluate whether the muscles and head innards of Alaskan pink shrimp contain B_{12} or an inactive corrinoid, such as pseudo-B_{12}, corrinoids were purified using an immunoaffinity column and analyzed using LC/ESI–MS/MS. Authentic B_{12} eluted as one ion peak with a retention time of 9.6 min (Fig. 1a). The mass spectrum of authentic B_{12} indicated that a doubly charged ion with an *m/z* of 678.2894 $[M+2H]^{2+}$ was prominent (Fig. 1b). The exact mass calculated from its formula of $C_{63}H_{88}CoN_{14}O_{14}P$ was 1354.5674 g/mol, and the isotope-distribution data showed that B_{12} was the major doubly charged ion under LC/ESI–MS/MS conditions. The MS/MS spectrum of authentic

Table 1 Vitamin B_{12} content in the muscles and head innards of raw shrimp and products from shrimp head innards

	Vitamin B_{12} content				
	(μg/100 g wet weight)		(μg/total fresh body)		
	Muscles	Innards	Muscles	Innards	Whole body
Alaskan pink shrimp *Pandalus eous*	2.49 ± 0.24	28.24 ± 5.74	0.21 ± 0.04	0.36 ± 0.02	0.57 ± 0.06
Morotoge shrimp *Pandalopsis japonica*	2.61 ± 0.29	21.44 ± 4.52	0.18 ± 0.05	0.22 ± 0.06	0.40 ± 0.11
Kuro shrimp *Argis lar*	4.12 ± 0.53	33.21 ± 5.70	0.29 ± 0.07	0.41 ± 0.09	0.69 ± 0.16
Togezako shrimp *Argis toyamaensis*	4.33 ± 0.72	12.48 ± 4.75	0.30 ± 0.03	0.14 ± 0.07	0.44 ± 0.10
Shrimp head innards product A	–	30.06 ± 4.34			
Shrimp head innards product B	–	28.48 ± 6.66			

Data are presented as mean ± SD of three independent experiments ($n = 3$)

B_{12} indicated that its singly charged ions at *m/z* 359.1007 and *m/z* 997.4782 were attributable to the nucleotide and corrin ring moieties, respectively (Fig. 1c). The compounds purified from the muscles of Alaskan pink shrimp eluted as one ion peak with a retention time of 9.5 min (Fig. 2a), and its mass spectrum primarily comprised a doubly charged ion with *m/z* 678.2894 $[M+2H]^{2+}$ (Fig. 2b). The MS/MS spectrum of the purified compounds with singly charged ions at *m/z* 359.1007 and *m/z* 997.4782 were identical to those of authentic B_{12} (Fig. 2c).

The corrinoid compounds purified from the head innards of Alaskan pink shrimp eluted as three ion peaks with retention times of 9.4, 9.8, and 10.0 min (Fig. 3a), respectively. The MS spectrum of the ion peak with a retention time of 9.4 min showed that the doubly charged B_{12} ion was formed at *m/z* 678.2894 $[M+2H]^{2+}$ (Fig. 3b). The MS/MS spectrum of the latter peak was identical to that of authentic B_{12} (Fig. 3e). The MS spectra of the ion peaks with a retention time of 9.8 min (compound A) and 10.0 min (compound B) showed that a major doubly charged ion was formed at *m/z* 678.7849 $[M+2H]^{2+}$ and 679.2751 $[M+2H]^{2+}$, respectively (Fig. 3c, d). The MS/MS spectra of compounds A and B with a singly charged ion at *m/z* 359.1007 were identical to that of authentic B_{12}; however, the MS/MS spectra of compounds A and B with singly charged ions at *m/z* 998.4603 and *m/z* 999.4493, respectively, were not identical to that of authentic B_{12} at *m/z* 997.4782 (Fig. 3f, g). These results suggest that the head innards of Alaskan pink shrimp contained B_{12} and two B_{12} compounds, A and B, which were B_{12} compounds with different corrin ring moieties that have one and two additional hydrogen atoms, respectively (Figs. 2, 3). Similar results were obtained for the other shrimp tested.

The chemical structure of B_{12} and the molecular masses of the unidentified B_{12} compounds were used to predict their identity. Compound A was hypothesized to be B_{12}-monocarboxylic acid while compound B was predicted to be B_{12}-dicarboxylic acid (Fig. 4). Immunoaffinity chromatography was used to purify corrinoids from head innard extract obtained from Alaskan pink shrimp. This was followed by analysis using a C18-reversed phase HPLC column. HPLC studies showed that B_{12}, unidentified compound A and unidentified B compound eluted as three separate peaks with retention times of 9.8, 16.2, and 18.3 min, respectively (Fig. 5a). HPLC analysis of known forms of B_{12} under the same conditions showed that authentic B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids eluted as single peaks with retention times of 9.7, 13.5, 16.2, and 20.0 min, respectively (Fig. 5b–e). The retention time of B_{12}-*d*-monocarboxylic acid was identical to that of compound A (retention time of 16.2 min). When B_{12}-*d*-monocarboxylic acid was analyzed using LC/ESI–MS/MS, it eluted as an ion peak with a retention time of 9.6 min (Fig. 6a). This was similar to be the retention time observed for compound A (retention time of 9.8 min) (Fig. 3a). Thus, MS and MS/MS spectra of B_{12}-*d*-monocarboxylic acid (Fig. 6b, c) were identical to that of compound A (Fig. 3c, f).

However, the identity of compound B could not be established as the preparation methods for B_{12}-*bd*-, -*be*-, and -*de*-dicarboxylic acids have not been reported. At present, compound B can be hypothetically identified as one of these B_{12}-dicarboxlyic acids.

To determine the relative B_{12} content (%), B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxlyic acid in the shrimp head innards of Alaskan pink shrimp and Kuro shrimp were selected because of their high B_{12} contents compared with those of the other shrimp tested. The relative B_{12} content (%) of each peak area against the total peak area (i.e., the sum of the peak area of B_{12} and unidentified compounds) was calculated by measuring the absorbance at 361 nm (Table 2). Approximately 65% of the B_{12} compounds identified in the head innards of Alaskan pink shrimp and 25% in the case of Kuro shrimp were derived from B_{12}-monocarboxylic and -dicarboxylic acids. The total B_{12} content in shrimp head innard products A and B contained ~ 22% these B_{12} acid compounds.

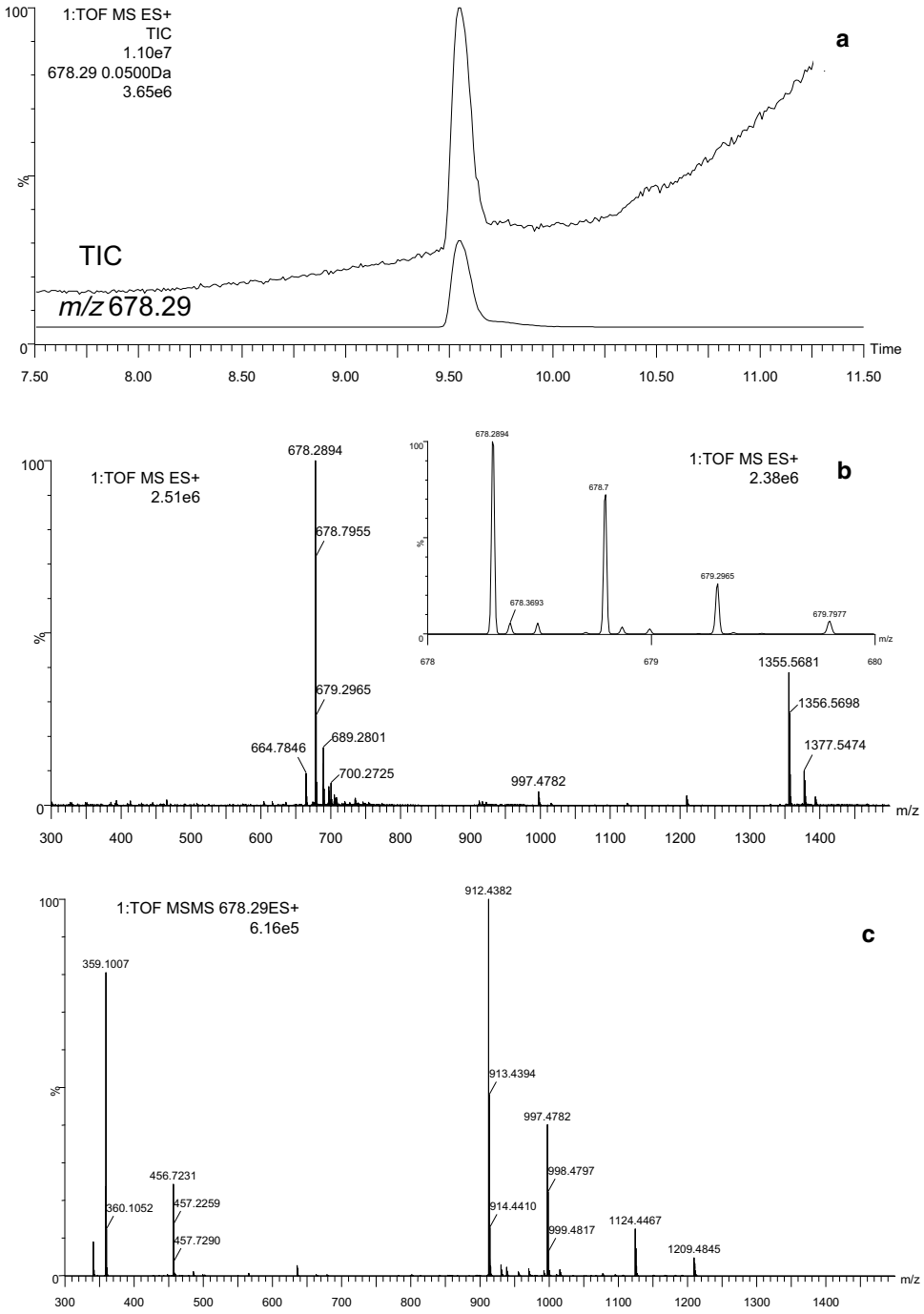


Fig. 1 Liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS) chromatograms of authentic B_{12}. **a** Total ion chromatogram (*TIC*) and mass chromatogram of authentic B_{12} (*m/z* 678.29). **b** Mass spectrum of authentic B_{12} (insert magnified spectrum from *m/z* 678 to 680). **c** MS/MS spectrum of the peak of authentic B_{12} at *m/z* 678.2894

The ability of B_{12}-*d*-monocarboxylic acid to show B_{12} activity was studied using the standard microbiological assay with *L. delbrueckii* subsp. *lactis* ATCC 7830. In particular, the effect of various doses of B_{12} and B_{12}-*d*-monocarboxylic acid (0.01–0.05 pmol) on the growth of the lactic acid bacterium was studied (Fig. 7). At a concentration of 0.05 pmol, the activity shown by B_{12}-*d*-monocarboxylic acid was only 6% of that displayed by the authentic B_{12}. These results suggest that B_{12}-*d*-monocarboxylic acid hardly functions as B_{12} in this bacterium.

Discussion

Although shrimp is one of the most consumed seafoods worldwide and is a good source of several nutrients (Kaymaci and Altun 2016), the detailed characteristics of

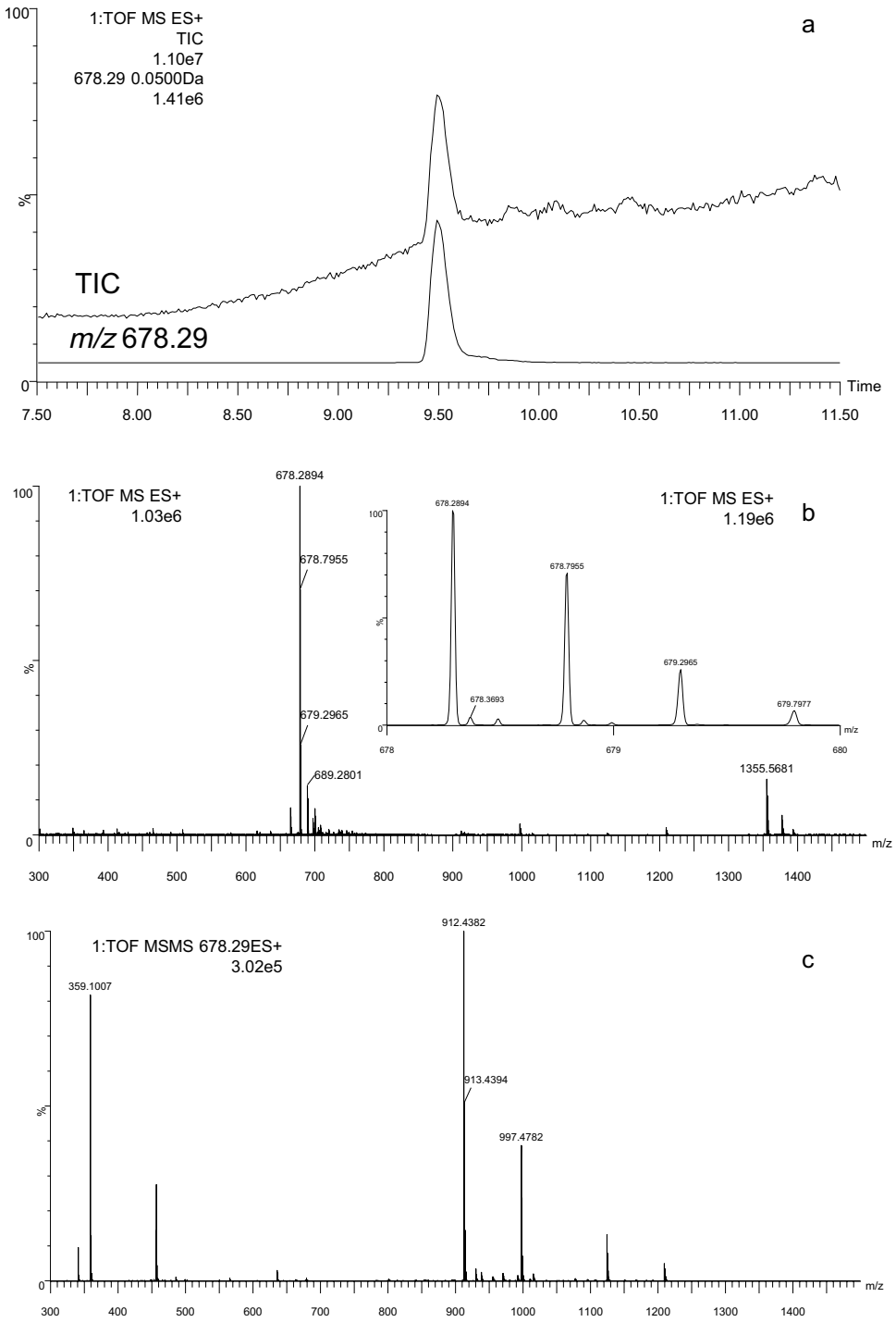


Fig. 2 LC/ESI–MS/MS chromatograms of corrinoid compounds purified from muscles of *Pandalus eous* Makarov, 1935. **a** TIC and mass chromatogram of the corrinoid compounds (*m/z* 678.29). **b** Mass spectrum of the corrinoid compounds at 9.49 min (insert magnified spectrum from *m/z* 678 to 680). **c** MS/MS spectrum of the peak of the corrinoid compounds at *m/z* 678.2894. For abbreviations, see Fig. 1

B_{12} have not been elucidated in this seafood. Thus, we determined the B_{12} content in both the muscles and head innards of various shrimp species using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC7830. As shown in Table 1, approximately 2–4 μg B_{12}/100 g wet weight was detected in shrimp muscles. The shrimp head innards contained significantly higher levels of B_{12} (~ 12–33 μg/100 g wet weight). Consumption of approximately eight to 14 shrimp muscles, six to seven shrimp head innards, and three to six whole shrimp bodies could supply the recommended dietary allowance of B_{12} for adults (2.4 μg/days) (Institute of Medicine 1998), which suggests that shrimp is a suitable source of B_{12} for humans.

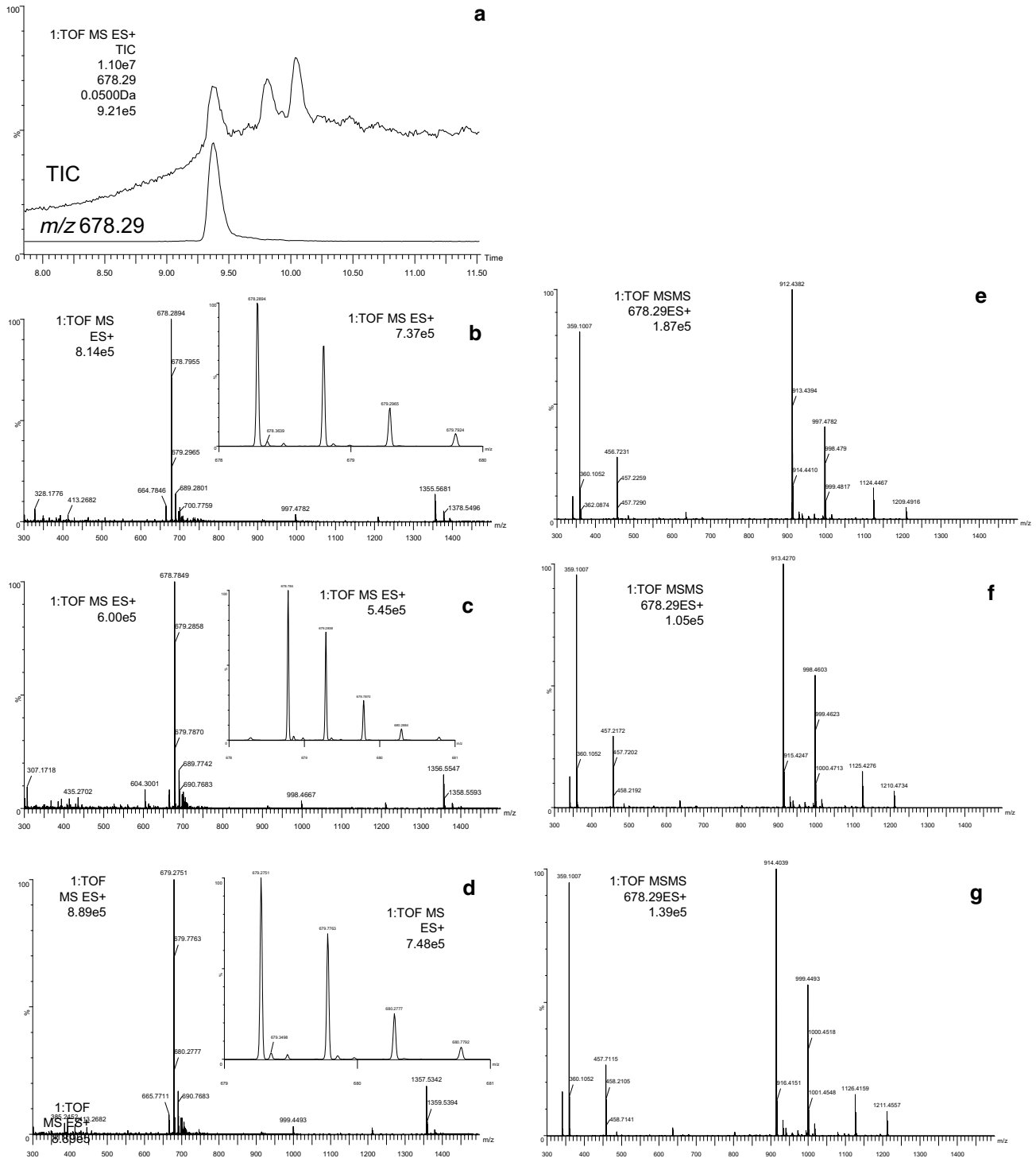
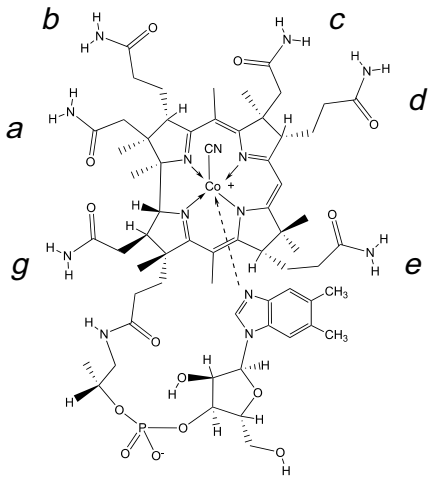
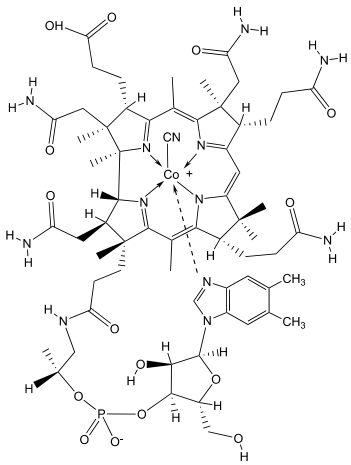


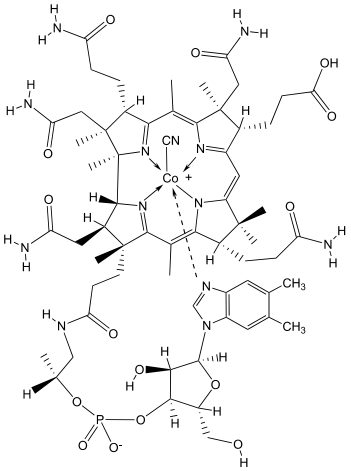
Fig. 3 LC/ESI–MS/MS chromatograms of corrinoid compounds purified from the head innards of *P. eous* Makarov, 1935. **a** TIC and mass chromatogram of the corrinoid compounds (*m/z* 678.29). **b**–**d** Mass spectra of the corrinoid compounds with retention times of 9.49 min (the magnified spectrum from *m/z* 678 to 680 is shown as an insert in **b**), 9.81 min (the magnified spectrum from *m/z* 678 to 681 is shown as an insert in **c**), and 10.04 min (the magnified spectrum from *m/z* 679 to 681 is shown as an insert in **d**), respectively. **e**–**g** MS/MS spectra of the peaks of the corrinoid compounds with retention times of 9.49 min (*m/z* 678.2894), 9.81 min (*m/z* 678.7849), and 10.04 min (*m/z* 679.2751), respectively. For abbreviations, see Fig. 1



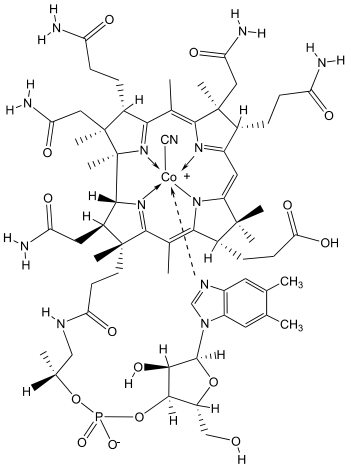
B_{12}



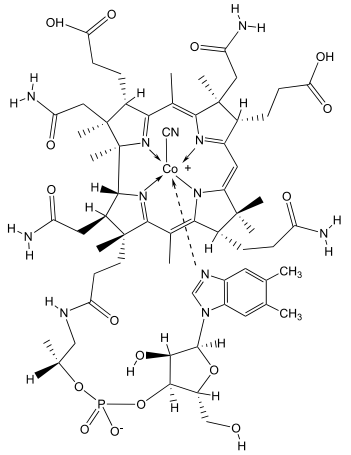
B_{12}-*b*-monocarboxylic acid



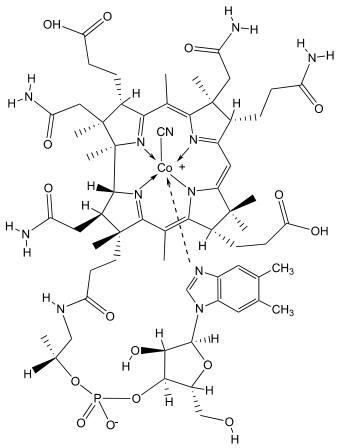
B_{12}-*d*-monocarboxylic acid



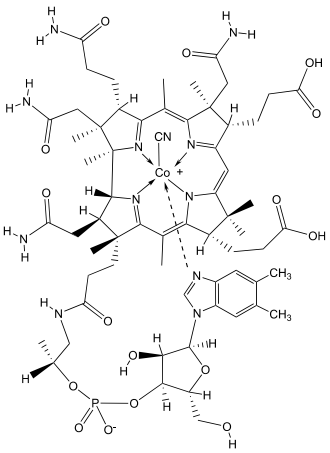
B_{12}-*e*-monocarboxylic acid



B_{12}-*bd*-dicarboxylic acid



B_{12}-*be*-dicarboxylic acid



B_{12}-*de*-dicarboxylic acid

Fig. 4 Chemical structures of unidentified B_{12} compounds A and B predicted using LC/ESI–MS/MS analysis. Compounds A and B were identified as B_{12}-monocarboxylic acid and a B_{12}-dicarboxylic acid, respectively. For abbreviations, see Fig. 1

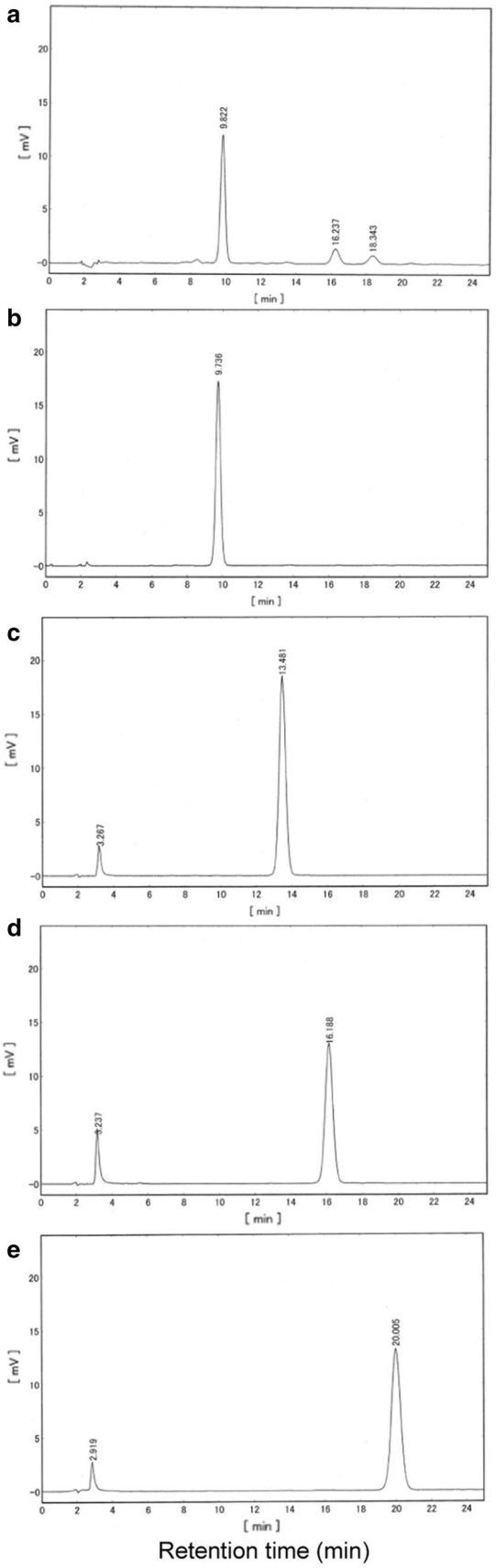
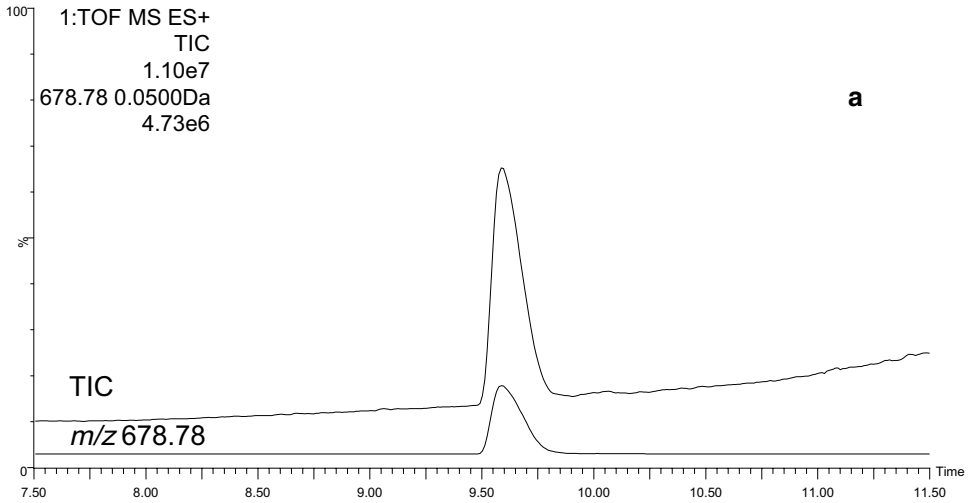


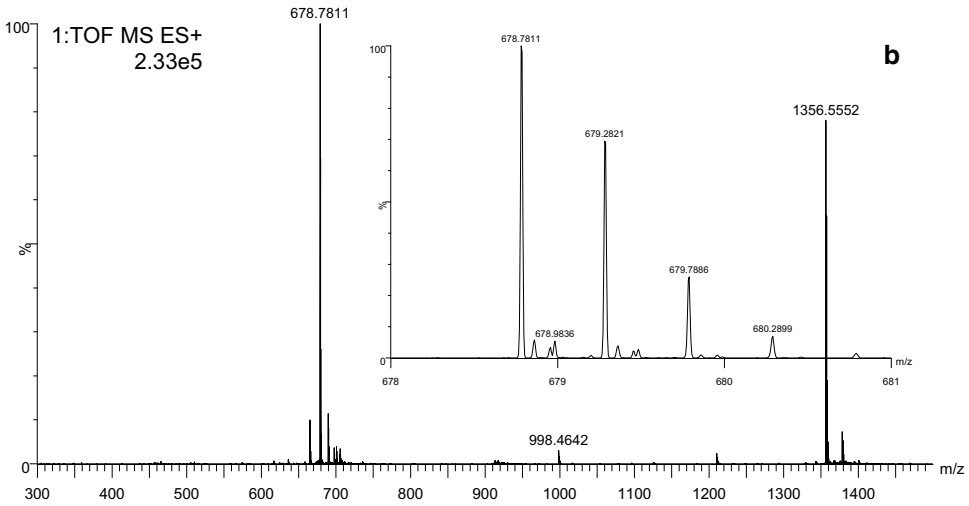
Fig. 5 High-performance liquid chromatography chromatograms of ▶ **a** B_{12} compounds present in head innard extract of *P. eous* Makarov, 1935, **b** B_{12}, **c** B_{12}-*b*-monocarboxylic acid, **d** B_{12}-*d*-monocarboxylic acid, and **e** B_{12}-*e*-monocarboxylic acid

We purified B_{12} compounds from the extracts of shrimp muscles and head innards using an immunoaffinity column, and the purified B_{12} compounds were identified using LC/ESI–MS/MS. The shrimp muscle extracts were characterized by the presence of only B_{12} while the extracts obtained from shrimp head innards contained three corrinoid compounds. These head innard extracts contained large amounts of B_{12} and smaller amounts of two unidentified B_{12} compounds denoted as compound A and compound B. The HPLC and LC/ESI–MS/MS analyses identified compound A as B_{12}-*d*-monocarboxylic acid (Figs. 5, 6). The identity of compound B could not be established completely and it was hypothetically identified as one of the B_{12}-dicarboxylic acids because of the presence of additional masses of two hydrogen atoms for B_{12} compounds having different corrin ring moieties. The content of the B_{12}-mono and -dicarboxylic acids varied from 17 to 65% of the total B_{12} content in shrimp head innards and their products (Table 2), which suggested that these B_{12} acid compound levels were dependent on the species sampled.

An intrinsic factor (IF) plays an important role in the gastrointestinal absorption of B_{12} in humans. This protein is known to have high specific binding for authentic B_{12}; however, it lacks any significant affinity towards B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids or B_{12}-*bde*-tricarboxylic acid (Kolhouse and Allen 1977). The low affinity of this IF protein for B_{12} mono- and tricarboxylic acids explains the poor absorption of these compounds following their oral administration in rabbits (Kolhouse and Allen 1977). Saido et al. (1993) reported that oral and intravenous administration of B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids did not show any improvement in the B_{12} levels of B_{12}-deficient rats. These studies strongly suggest that both B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxylic acids found in shrimp head innards might not be absorbed by the human intestine. However, shrimp head innards can be still considered a source of B_{12} for humans owing to their contents of B_{12}-mono and -dicarboxylic acids (17–65% of the total B_{12} content).

Anton et al. (1980) reported the formation of B_{12}-mono- and -dicarboxylic acids on mild acid hydrolysis of B_{12}. These B_{12} acids are derived from the propionamide side chains *b*, *d*, and *e*, which are more susceptible to hydrolysis than the acetamide side chains *a*, *c*, and *g* (Bernhauer et al. 1966) (Fig. 4). The presence of these B_{12} acid compounds in shrimp head innards is quite unusual. It is possible that trace elements are absorbed from sea water and accumulate in shrimp head innards (Kaymaci and Altun 2016), and might react with B_{12} to produce





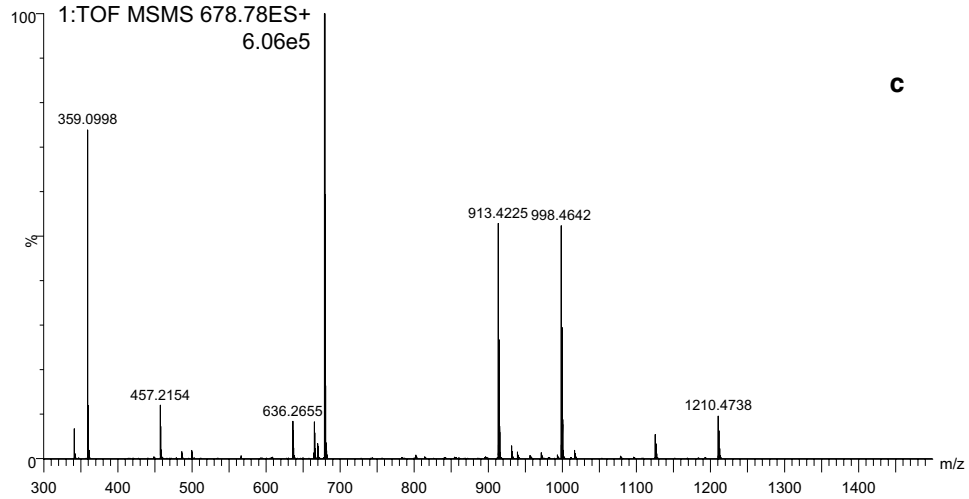


Fig. 6 LC/ESI–MS/MS chromatograms of B_{12}-*d*-monocarboxylic acid. **a** TIC and mass chromatogram of B_{12}-*d*-monocarboxylic acid (*m/z* 678.78), **b** mass spectrum of the corrinoid compounds with retention times of 9.6 min (insert magnified spectrum from *m/z* 678 to 681), **c** MS/MS spectrum showing the peak of the corrinoid compound with a retention time of 9.6 min (*m/z* 678.78). For abbreviations, see Fig. 1

Table 2 Relative contents (%) of B_{12} and compound A (B_{12}-*d*-monocarboxylic acid) and compound B (tentative B_{12}-dicarboxylic acids) in selected shrimp head innards and their products

	Relative content (%)		
	B_{12}	Compound A B_{12}-*d*-monocarboxylic acid	Compound B tentative B_{12}-dicarboxylic acids
Alaskan pink shrimp *P. eous*	34.6 ± 0.1	24.6 ± 0.2	40.8 ± 0.3
Kuro shrimp *A. lar*	73.9 ± 0.3	11.0 ± 0.2	15.0 ± 0.1
Shrimp head innards product A	82.3 ± 0.6	11.5 ± 0.2	6.1 ± 0.4
Shrimp head innards product B	78.2 ± 1.3	13.9 ± 0.2	7.9 ± 1.1

Data are presented as mean ± SEM of triplicates

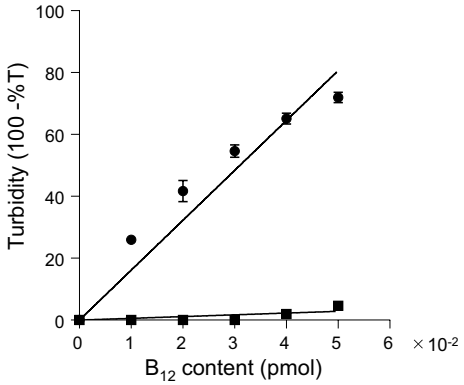


Fig. 7 Effect of authentic B_{12} (black circle) and B_{12}-*d*-monocarboxylic acid (black square) on growth of *L. delbrueckii* subsp. *lactis* ATCC 7830 expressed as turbidity as a function of dose. The experiment was performed in triplicate and results are given as mean ± SEM

these acid compounds. However, no detailed mechanism has been reported so far to explain this hypothesis.

Euglena gracilis Z, a B_{12}-dependent alga, has been widely utilized in microbiological assays for B_{12}. Supplementation of growth culture medium with B_{12}-*d*-monocarboxylic acid increased the growth of *Euglena* to similar levels to those observed in authentic B_{12}-supplemented culture medium (Watanabe et al. 1992). *L. delbrueckii* subsp. *lactis* ATCC 7830 is a bacterium commonly used to determine the B_{12} content of food. As shown in Fig. 7, B_{12}-*d*-monocarboxylic acid did not show any significant effect on the growth of this bacterium. There are no reports on the effect of B_{12}-dicarboxylic acids on the growth of *L. delbrueckii* subsp. *lactis* ATCC 7830. Since the structure of B_{12} changed more in the case of B_{12}-dicarboxylic acid compared to B_{12}-*d*-monocarboxylic acid, it is possible that B_{12}-dicarboxylic acids are inactive in this bacterium. The possible inactivity of B_{12}-*d*-monocarboxylic and B_{12}-dicarboxylic acids in *L. delbrueckii* subsp. *lactis* ATCC 7830 suggests that these compounds might not affect the B_{12} content of shrimp head innards.

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Author contributions N. O. and N. H. performed most of the experiments. Y. U. and S. T. analyzed the B_{12} compounds using LC/ESI–MS/MS and interpreted the results. N. O., T. B., and F. W. designed the experiments, interpreted the results, and wrote the manuscript. All authors reviewed and commented on the manuscript and approved the final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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