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Identification of vitamin B_{12} and pseudovitamin B_{12} from various edible shellfish using liquid chromatography–electrospray ionization/tandem mass spectrometry

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Abstract In this study, the vitamin B_{12} contents were analyzed in the edible portions of various shellfish (bivalves and snails). High vitamin B_{12} contents (30.5–53.3 μg/100 g wet weight) were detected in mussels, surf clams, bloody clams, and freshwater clams. However, scallops and abalone had extremely low vitamin B_{12} contents (0.1–1.1 μg/100 g wet weight) which was attributed to only the muscle portions being edible. These results suggest that high levels of vitamin B_{12} are accumulated in the viscera of shellfish. Vitamin B_{12} levels were also significantly higher in bivalves than in snails. The corrinoid compounds purified from all bivalves were identified as "true" vitamin B_{12} using liquid chromatography–electrospray ionization/tandem mass spectrometry. In edible snails, abalone, and pond snails, however, both vitamin B_{12} and pseudovitamin B_{12} (an inactive corrinoid) were observed to be the major and minor corrinoid compounds, respectively. Based on these results, we conclude that the whole bodies of these edible bivalves are excellent sources of vitamin B_{12} for humans.

Keywords Bivalve · Edible shellfish · Pseudovitamin B_{12} · Snails · Vitamin B_{12}

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Introduction

Vitamin B_{12} (B_{12}) compounds are synthesized only by certain bacteria which are primarily concentrated in the bodies of predatory animals relatively high in the natural food chain [1]. The usual dietary sources of B_{12} are animal food products (i.e., meat, milk, egg, fish, shellfish) [2]. Japanese people obtain most (approximately 84 %) of their daily B_{12} intake from fish and shellfish [3]. Shellfish that siphon large quantities of B_{12}-synthesizing bacteria from seawater and freshwater are excellent sources of B_{12} [4]. However, these B_{12}-synthesizing bacteria can also synthesize other corrinoids with a different base moiety in the lower ligand of the molecule [1, 5]. In our previous study [6], we extracted and purified corrinoid compounds from popular edible shellfish (clams, oysters, mussels) using silica gel thin-layer chromatography (TLC) and reversed-phase high performance liquid chromatography and identified the "true" B_{12} by NMR spectroscopy. However, our recent study using B_{12} antibody affinity chromatography and liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS) [7] indicates that the edible portion and viscera of abalone *Haliotis discus hannai* primarily contains pseudovitamin B_{12} (pseudo-B_{12}), which is inactive in humans. Moreover, the freshwater clam *Corbicula japonica* contains only a small amount of pseudo-B_{12} and unidentified corrinoid compounds [8]. Limited information is available on whether edible

shellfish (particularly their edible portions) contain B_{12} or other corrinoid compounds (such as pseudo-B_{12}) that are inactive in humans.

In this study, we identified B_{12} compounds from the edible portions of various shellfish (bloody clam, freshwater clam, mussel, scallop, surf clam, abalone, pond snail, and whelk) using LC/ESI–MS/MS. We found that all bivalves tested contained substantial amounts of true B_{12} but that inactive corrinoid compounds were also present in abalone and pond snail. These results indicate that the whole bodies of these bivalves are excellent sources of B_{12} for humans.

Materials and methods

Materials

B_{12} *cyanocobalamin* was obtained from Sigma (St. Louis, MO). Pseudovitamin B_{12} *7-adenyl cyanocobamide* was isolated from *Spirulina* sp. [5] and used in our study. Silica gel 60 TLC aluminum sheets were obtained from Merck (Darmstadt, Germany). All other reagents were of the highest commercially available purity. Raw shellfish (scallop *Mizuhopecten yessoensis*, mussel *Mytilus galloprovincialis*, surf clam *Pseudocardium sachalinense*, bloody clam *Anadara broughtonii*, freshwater clam *Corbicula japonica*, abalone *Haliotis diversicolor aquatilis*, whelk *Buccinum middendorffi*, and pond snail *Bellamya chinensis mallaeta*) were purchased from a local market in Tokyo, Japan ($n = 5$).

Extraction and assay of B_{12} compounds from shellfish

The edible portions (2 g) of the shellfish were suspended in 40 ml of distilled water and homogenized using a universal homogenizer (Polytron, Kinematica, Switzerland). The total corrinoid compounds were extracted from each sample by boiling at pH 4.5 and assayed using a microbiological method based on *Lactobacillus delbrueckii* ATCC7830, as previously described [5]. *L. delbrueckii* ATCC7830 can utilize deoxyribosides and deoxyribonucleotides (known to be an alkali-resistant factor) as well as B_{12}. The correct B_{12} values were therefore calculated by subtracting the values for the alkali-resistant factor from the values for the total B_{12} compounds.

TLC-bioautography assay using B_{12}-dependent *Escherichia coli* 215

A bioautography assay to detect corrinoid compounds was performed as previously described [9]. The B_{12} extracts [20-ml samples were prepared as described above and partially purified and concentrated using a Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, MA)] were washed 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 (VC960; TAITEC Corp., Saitama, Japan), and the residual fraction was dissolved in 1.0 ml of distilled water. Concentrated B_{12} extracts (1 µl) and B_{12} and pseudo-B_{12} (each 0.1 mg/l) were spotted onto the silica gel 60 TLC plates and developed in the dark using 2-propanol/NH_4OH (28 %)/water (7:1:2 v/v) at room temperature (25 °C). The TLC plate was dried and overlaid with agar-containing basal medium and precultured *E. coli* 215, followed by incubation at 30 °C for 20 h. The gel plate was subsequently sprayed with methanol solution containing 2,3,5-triphenyltetrazolium salt, and B_{12} compounds were visualized as red, which indicated *E. coli* growth.

Immunopurification of corrinoid compounds from edible shellfish

Each extract (40 ml) was partially purified and concentrated using a Sep-Pak® Plus C18 cartridge (Waters Corp) as described above. The eluate was evaporated in a centrifugal concentrator (VC960; TAITEC Corp), and the residual fraction was dissolved in 2.0 ml of distilled water. The purified extract was loaded onto an immunoaffinity column [EASI-EXTRACT® Vitamin B_{12} Immunoaffinity Column (P80); R-Biopharm AG, Darmstadt, Germany], and the corrinoids were purified following the manufacturer's protocol.

Identification of corrinoid compounds by LC/ESI–MS/MS

The B_{12} compounds were purified by passage through the immunoaffinity column, 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 any small particles. An aliquot (2 µl) of the filtrate was analyzed using a LC/MS IT-TOF (ion trap–time-of-flight) system coupled to an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Each purified corrinoid was injected into an Inert-Sustain column (3 µm, 2.0 × 100 mm; GL Science, Tokyo, Japan) and equilibrated with 100 % solvent A [0.1 % (v/v) acetic acid] and 0 % solvent B (100 % methanol) at 40 °C. Corrinoids 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). 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 B_{12} compounds. The ESI–MS system was operated in the positive ion mode,

and argon was used as the collision gas. Pseudo-B_{12} (*m/z* 672.7749) and B_{12} (*m/z* 678.2914) as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

Results

B_{12} contents of the edible portions of various shellfish

We analyzed the B_{12} contents of the edible portions of various shellfish that are commonly consumed in Japan using the *L. delbrueckii* ATCC 7830 microbiological assay method (Table 1). High B_{12} contents (30.5–53.3 μg/100 g wet weight) were detected in mussels, surf clams, bloody clams, and freshwater clams. Moderate B_{12} contents (10.5–21.4 μg/100 g wet weight) were observed in whelks and pond snails. However, scallops and abalone had extremely low B_{12} contents (0.1–1.1 μg/100 g wet weight). The B_{12} contents determined in our analyses are similar to those described in the Standard Tables of Food Composition in Japan [10].

Identification of corrinoid compounds from edible shellfish using the *E. coli* 215 bioautography assay

The corrinoids observed in all edible shellfish samples were analyzed using an *E. coli* 215 bioautogram after separation by silica gel 60 TLC. The corrinoids observed in all bivalve samples and whelks produced clear spots which had an R_f value identical to that of B_{12} (Fig. 1a), but not pseudo-B_{12}. The remaining snail samples yielded two spots, the R_f values of which were identical to those of pseudo-B_{12} and B_{12} (Fig. 1b).

Identification of corrinoid compounds purified from selected edible shellfish using LC/ESI–MS/MS

The bloody clam was selected from among the edible bivalves that contained high B_{12} levels for further analysis. A bloody clam extract was purified using a B_{12} immunoaffinity column and analyzed by LC/ESI–MS/MS. B_{12} and pseudo-B_{12} were eluted as peaks with retention times of 7.5 and 7.4 min, respectively (Fig. 2a-1, b-1). The mass spectrum of B_{12} indicated that a doubly charged ion with an *m/z* of 678.2889 $[M + 2H]^{2+}$ was prominent (Fig. 2a-2). The exact mass calculated from its formula ($C_{63}H_{88}CoN_{14}O_{14}P$) was 1354.5674, and the isotope distribution data showed that B_{12} was the major divalent ion under our LC/ESI–MS conditions. For pseudo-B_{12} with an exact mass of 1343.5375 ($C_{59}H_{83}CoN_{17}O_{14}P$), a doubly charged ion with an *m/z* of 672.7749 $[M + 2H]^{2+}$ was prominent (Fig. 2b-2). The MS/MS spectra of B_{12} and pseudo-B_{12} indicated that the dominant ions at *m/z* 359.0983 (Fig. 2a-3) and *m/z* 348.0674 (Fig. 2b-3), respectively, were

Table 1 Vitamin B_{12} contents of the edible portions of various edible shellfish[a]

Shellfish (portion analyzed)	Vitamin B_{12} content (μg/100 g wet weight)		Type of corrinoid compounds (relative percentage against total corrinoid peak areas)[c]
	Present study	Reference[b]	
Bivalves			
Scallop *Mizuhopecten yessoensis* (adductor muscle)	1.1 ± 0.2	2.0	Vitamin B_{12} (100 %)
Mussel *Mytilus galloprovincialis* (whole body)	30.5 ± 13.7	10.3	Vitamin B_{12} (100 %)
Surf clam *Pseudocardium sachalinense* (whole body)	41.7 ± 5.8	47.5	Vitamin B_{12} (100 %)
Bloody clam *Anadara broughtonii* (whole body)	42.9 ± 9.6	59.2[d]	Vitamin B_{12} (100 %)
Freshwater clam *Corbicula japonica* (whole body)	53.3 ± 15.8	62.4	Vitamin B_{12} (100 %)
Snails			
Abalone *Haliotis diversicolor aquatilis* (without viscera)	0.3 ± 0.1	3.2	Vitamin B_{12} (89 %), pseudovitamin B_{12} (11 %)
Whelk *Buccinum middendorffi* (whole body)	10.5 ± 3.6	6.5[d]	Vitamin B_{12} (100 %)
Pond snail *Bellamya (Cipangopaludina) chinensis mallaeta* (whole body)	21.4 ± 3.1	17.8	Vitamin B_{12} (83 %), pseudovitamin B_{12} (10 %), unidentified corrinoids (7 %)

Vitamin B_{12} was assayed in triplicate for each sample ($n = 5$), and the data are expressed as the mean ± standard deviation (SD)

[a] Vitamin B_{12} contents were determined using the *Lactobacillus delbrueckii* ATCC 7830 microbiological assay method, as described in the text

[b] Reference values were obtained from the Standard tables of food composition in Japan—2010 published by the Japan Council for Science and Technology, Ministry of Education, Culture, Sports, Science and Technology [10]

[c] As determined by liquid chromatography/mass spectrometry (LC/MS)

[d] Without viscera

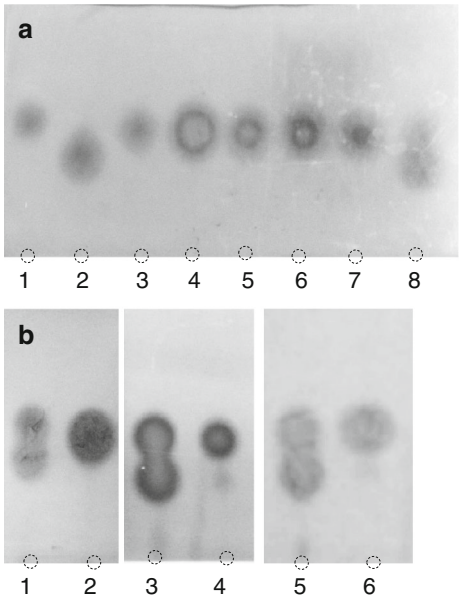

Fig. 1 Vitamin B_{12} (B_{12})-dependent *Escherichia coli* 215 bioautogram analysis of corrinoid compounds from the edible portions of various shellfish. **a** Bivalves. *Lanes*: *1* B_{12} *2* pseudo-B_{12} *3* fresh water clam (whole body), *4* bloody clam (whole body), *5* scallop (adductor muscle), *6* surf clam (whole body), *7* mussel (whole body), *8* mixture of B_{12} and pseudo-B_{12}. **b** Snails. *Lanes*: *1*, *3*, *5* Mixture of B_{12} and pseudo-B_{12}, *2* whelk (whole body), *4* abalone (without viscera), *6* pond snail. Each sample was spotted on the *circle* marked in the thin-layer chromatography (TLC) plate and developed. The data represent typical bioautograms from three independent experiments

attributable to the nucleotide moiety of each corrinoid compound. The corrinoids purified from the bloody clam sample were eluted to yield a single total ion peak with a retention time of 7.5 min, where the mass spectrum showed that the doubly charged B_{12} ion was formed at *m/z* 678.2916 (Fig. 3a-2). The MS/MS spectrum of the compound was identical to that of B_{12} (Fig. 3a-3). These results indicate that B_{12} is the predominant corrinoid compound in the bloody clam. As shown in Table 1, identical results were obtained with other edible bivalve samples.

In the compounds purified from abalone, ion peaks were detected at *m/z* 672.7749 and *m/z* 678.2914 for pseudo-B_{12} and B_{12}, respectively, and their retention times were identical to those of pseudo-B_{12} and B_{12} (Fig. 3b-1). The mass spectra at the retention times of 7.4 and 7.5 min showed that doubly charged ions were formed at *m/z* 672.7789 (Fig. 3b-2) and *m/z* 678.2920 (Fig. 3b-4) in both pseudo-B_{12} and B_{12}, respectively. The respective MS/MS spectrum of each compound was identical to that of pseudo-B_{12} (Fig. 3b-3) and B_{12} (Fig. 3b-5). Similar results were obtained with a pond snail sample (data not shown). Abalone and pond snails contained approximately 89 and 83 % of B_{12}, respectively, based on the respective ion peak areas. The results of our preliminary experiments also suggested that traces of other inactive corrinoid compounds (benzimidazolyl cyanocobamide, 5-hydroxybenzimidazolyl cyanocobamide, etc.) were present in the pond snail extract, but they could not be identified completely. These results indicate that B_{12} and pseudo-B_{12} were the major and minor corrinoid compounds, respectively, in abalone and pond snails.

Discussion

Some of the bivalves assayed in our study whose muscle and viscera were both edible contained considerably B_{12} content (30.5–53.3 μg/100 g wet weight). However, the B_{12} contents (0.1–1.1 μg/100 g wet weight) of the muscle portions of the scallops and abalone were considerably lower. Regardless of the shellfish species, the B_{12} content was higher in the viscera than in the muscle. Although bivalves siphon large quantities of B_{12}-synthesizing bacteria from seawater and freshwater, the majority of edible snails are herbivorous, and plants and seaweeds (except purple and green lavers) contain only trace amounts of B_{12} [2]. Thus, the vitamin B_{12} contents of shellfish may be attributable to their respective diet, because the production of B_{12} by intestinal bacteria is very low in shellfish [11]. In general, the bodies of edible bivalves contain much higher B_{12} levels than those of edible snails.

In our previous study, we showed that a considerable proportion (approximately 65 %) of the corrinoid compounds comprised pseudo-B_{12} in both the edible portion and viscera of abalone *Haliotis discus hannai*. In particular, the viscera contained substantial amounts of pseudo-B_{12} [7]. The muscle portion of abalone *Haliotis diversicolor aquatilis* contained most of the B_{12}, but the small amount of pseudo-B_{12} observed in abalone may have been derived from its viscera. These observations suggest that the viscera of certain edible snails (abalone and pond snails) contain substantial amounts of pseudo-B_{12} and, therefore, that they may not be suitable as B_{12} sources. However, pseudo-B_{12} was not detected in another snail, the whelk, in our study. Abalone and pond snails are herbivorous but whelk is carnivorous. The pseudo-B_{12} found in abalone and pond snails may be derived from their diets because various blue-green algae contain substantial amounts of pseudo-B_{12} [2].

Corrinoid compounds have been purified from most popular edible shellfish, including oyster, short-necked clams, and mussels (each whole body), which have been identified as B_{12} [6, 11, 12]. These observations and the results obtained in the present study indicate that edible bivalves generally contain B_{12}, but not pseudo-B_{12}. However, freshwater clams *Corbicula japonica* caught in Lake Togo (Tottori prefecture, Japan) were found to contain a small amount (10.4 %) of inactive corrinoid compounds

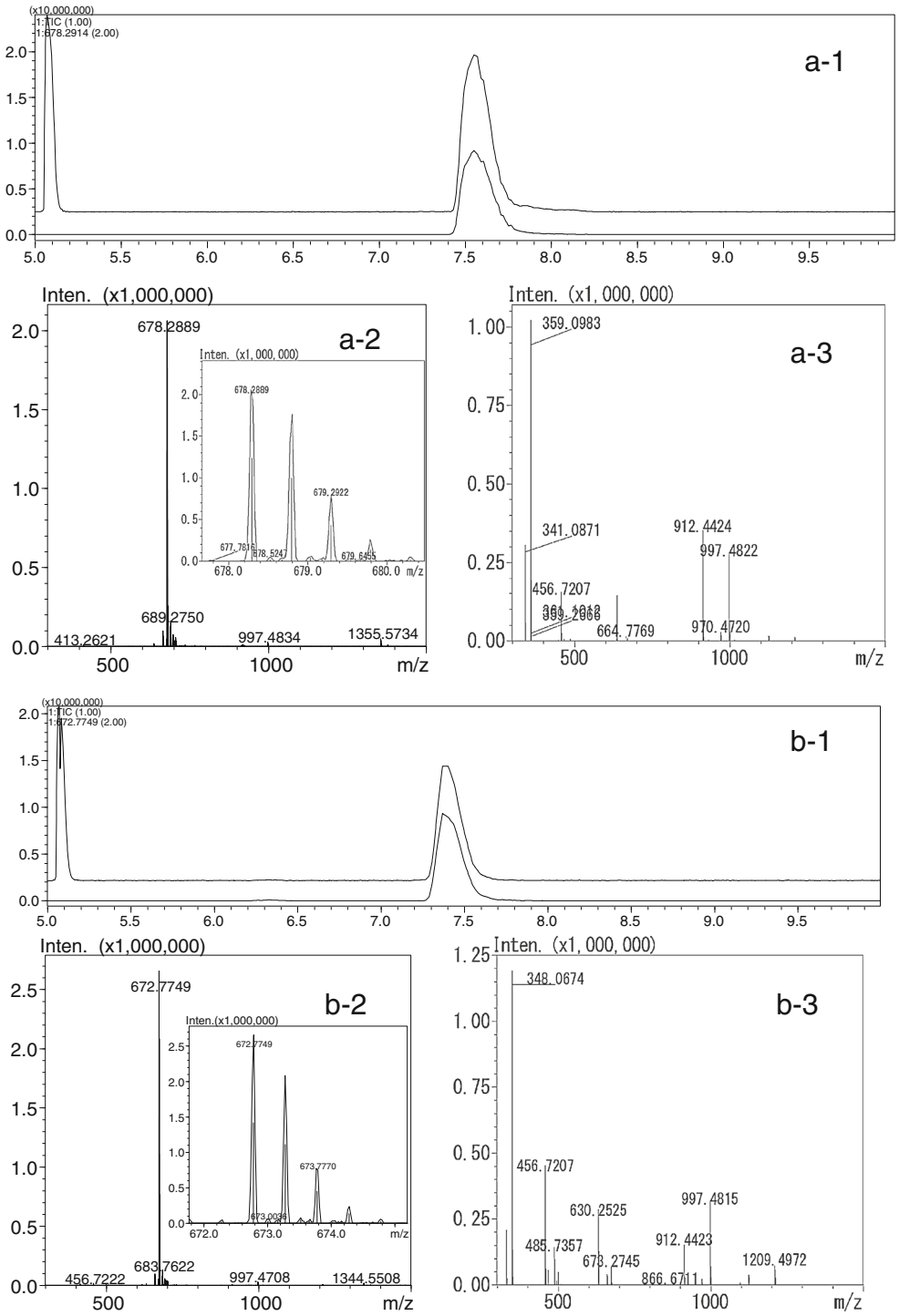

Fig. 2 Liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS) chromatograms of B_{12} and pseudo-B_{12}. B_{12} was analyzed by an LC/MS ion trap–time-of-flight (IT-TOF) (Shimadzu), as described in the text. **a-1** Total ion chromatogram (TIC; ×1) and reconstructed chromatogram of *m/z* 678.2914 (×2) for B_{12}. **a-2** Mass spectrum of the ion peak of B_{12}, where the magnified mass spectrum from *m/z* 678.0 to 680.0 is shown in the *insert*. **a-3** MS/MS spectrum of the B_{12} peak. **b-1** TIC (×1) and the reconstructed chromatogram at *m/z* 672.7749 (×2) for pseudo-B_{12}. **b-2** The mass spectrum of the ion peak of pseudo-B_{12}, where the magnified mass spectrum from *m/z* 678.0 to 680.0 is shown in the *insert*. **b-3** MS/MS spectrum of the pseudo-B_{12} peak

(pseudo-B_{12} and unidentified compounds) [8]. It remains unclear why these inactive corrinoid compounds were present in shellfish, but they may be attributable to the presence of bacteria that synthesize pseudo-B_{12} (and other inactive corrinoids) inside and/or outside their bodies. Thus, further detailed biochemical studies are required to

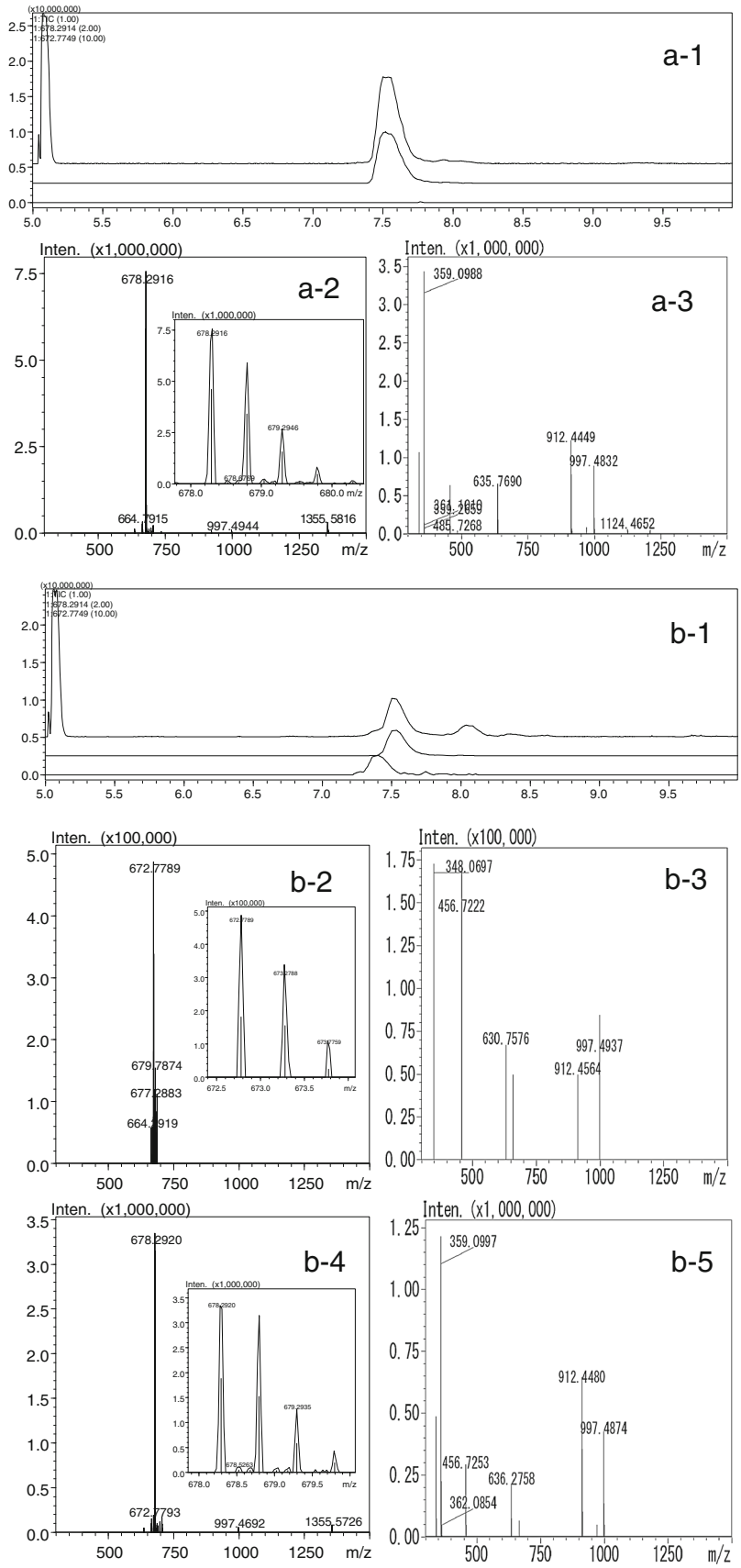

Fig. 3 LC/ESI–MS/MS chromatograms of the corrinoid compounds purified from the edible portions of bloody clams and abalone. **a-1** TIC (×1) and reconstructed chromatograms of the purified corrinoid compounds [*m/z* 678.2914 (×2)] from a bloody clam sample. **a-2** Mass spectrum of the corrinoid compounds purified at 7.5 min (*insert* magnified spectrum from *m/z* 678 to 680). **a-3** MS/MS spectrum of the *m/z* 678.2916 peak of the corrinoid compounds. **b-1** TIC (×1) and reconstructed chromatograms of the corrinoid compounds [*m/z* 678.2914 (×2) and *m/z* 672.7749 (×10)] purified from an abalone sample. **b-2**, **b-4** Mass spectra of the corrinoid compounds purified at 7.4 and 7.5 min, respectively (*inserts* magnified spectra, respectively, from *m/z* 678 to 680). **b-3**, **b-5** MS/MS spectra for the *m/z* 672.7789 and *m/z* 678.2920 peaks of the corrinoid compounds, respectively

elucidate the origins of these inactive corrinoid compounds. However, these minor and/or trace inactive corrinoid compounds may not be absorbed; therefore, they may not be available to humans because of their poor binding and uptake through the intrinsic factor (the highly specific B_{12}-binding protein) involved in the gastrointestinal absorption of B_{12} [13]. The consumption of approximately 5–8 g of edible bivalves, which contain high B_{12} levels (mean value 30–50 μg/100 g wet weight), could supply the recommended dietary allowance for adults (2.4 μg/day) [14]. Thus, the results obtained in our study indicate that even if certain edible bivalves contain small or trace amounts of various inactive corrinoid compounds, the whole bodies of bivalves, which we found to contain higher amounts of B_{12} than snails, would still be excellent sources of B_{12}.

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