Vitamin B_{12} (B_{12}) is synthesized only in certain bacteria and then concentrated mainly in the bodies of higher predatory organisms in the natural food chain system (1). Animal foods (i.e., meat, milk, egg, fish, and shellfish), but not plant foods, are considered to be the major dietary sources of B_{12} (2). Japanese people obtain most (~84%) of daily B_{12} intake from both fish and shellfish (3). Fish is also a good source of minerals, vitamins, proteins (with high biological values), and unsaturated lipids (containing eicosapentaenoic acid and docosahexaenoic acid) (4). Many studies (5–7) have suggested that regular fish intake prevents atherosclerosis, thrombosis, and cardiac diseases. Therefore, the trend of fish intake is spreading throughout the world.

Bennink and Ono (8) have reported appreciable loss (~33%) of B_{12} during cooking of raw beef. Our previous studies have demonstrated that loss of B_{12} significantly occurs in beef, pork, and milk during microwave heating (9). Although the effects of various cooking methods on the proximate composition, mineral, and vitamin (A, E, B_{1}, naiacin, and B_{6}) contents of certain fish fillets have been investigated (10), it is still unclear how much B_{12} is lost in raw fish meats during cooking; the lack of this information may be due to the difficulty of B_{12} assay in foods. If cooking processes lead to significant loss of B_{12} in the fish meats, it would be an important problem for assessment of the daily intake of B_{12} to prevent B_{12} deficiency. Here we describe the loss of B_{12} in a round herring’s (one of the most popular fishes) meat during various cooking treatments.

Materials and Methods

Materials. B_{12} was purchased from Sigma (St. Louis, Missouri, USA). A B_{12} assay medium for Lactobacillus delbrueckii subspecies lactis (formerly L. leichmannii) ATCC7830 was obtained from Nissui (Tokyo, Japan). A Shimadzu (Kyoto, Japan) ultraviolet/visible spectrophotometer (UVmini-1240) was used for measuring the turbidity of L. delbrueckii test cultures in the microbiological B_{12} assay. All other reagents used were of the highest purity commercially available.

Preparation for fish meats. Raw big-eye round herrings (Etrumeus teres) (48–75 g body weight) were purchased from local markets in Kochi City, Japan (Fig. 1). The head, viscera, and bones of the round herring were removed to prepare the edible portions (meats), which were used for the experiments.

Extraction and assay of vitamin B_{12}. B_{12} was extracted and assayed by the microbiological method with L. delbrueckii ATCC 7830 as described in the Standard Tables of Food Composition in Japan (11). Raw meats and viscera of the round herring were homogenized with a food processor (MILLSER-II IFM-200D, Iwatani Co., Tokyo, Japan). Two grams of each homogenate were used for B_{12} assay. Total B_{12} was extracted by boiling with 0.005% (w/v) KCN at pH 4.5 to convert various B_{12} compounds with different α-ligands (e.g., coenzyme forms of B_{12}) to cyanocobalamin (CN-B_{12}). A portion of the above total B_{12} extract was adjusted pH to 11.0 and then treated with an autoclave (MC-23, ALP Co., Ltd., Tokyo, Japan) at 121°C for 30 min in order to decompose B_{12} in the extract. The treated extract contains certain compounds (including deoxyribosides and deoxyribo-nucleotides) which are known as an alkali-resistant
factor. Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (alkali-resistant factor) as well as B<sub>12</sub>, the amount of true B<sub>12</sub> was calculated by subtracting the values of the alkali-resistant factor from the values of total B<sub>12</sub>.

In the case of cooking experiments, B<sub>12</sub> was extracted and assayed from the cooked round herring meats under the same conditions as described above.

The amounts of the alkali-resistant factor in the raw (or cooked) fish meats and viscera were determined to be <0.07 and <6.59 μg/100 g fresh weight, respectively.

**Cooking conditions.** The whole meats (30–50 g of fresh weight) of the round herring were treated with various cooking processes. The raw fish meats were cooked in a Hitachi (Tokyo, Japan) microwave oven MRH-530 (500 W) for 1.0 min, grilled in a conventional gas oven (RCK-10 M(a), Rinnai Co., Aichi, Japan) at 180˚C for 7.5 min, or steamed in a steamer for 4.5 and 9.0 min using a conventional gas range (RT-500GF-L, Rinnai Co.). In the boiling, the samples were heated in 200 mL of boiling water for 5.0 min or heated into 250 mL of water and boiled for 6.0 min (total treated times, 8.0 min). In the frying, the samples were treated in 800 mL of cooking oil at 180˚C for 2.0 and 4.0 min. In the case of vacuum-packed pouch cooking, the samples were packed in the pouches (HN-105, Asahi Kasei Pax Co., Tokyo, Japan) for vacuum-packed pouch cooking and then sealed using a vacuum sealing machine (at 99% vacuum) (V-380G, Tosei Electric Co., Shizuoka, Japan). The packed samples were cooked in an IH cooking heater (IC-D1, Sanyo, Tokyo, Japan) at 70˚C for 30 min; internal temperature during heating was at 75–76˚C. After heating, the samples were immediately chilled until reaching an internal temperature of 3˚C in 90 min.

The samples were weighted before and after this cooking, and then homogenized with the food processor. After B<sub>12</sub> was assayed as described above, total B<sub>12</sub> content found in total fish meats cooked or uncooked was calculated.

**Stability of B<sub>12</sub> in solution during various heating conditions.** Since B<sub>12</sub> (cyanocobalamin) was a heat-stable unnatural B<sub>12</sub> compound, hydroxocobalamin (OH-B<sub>12</sub>) was used as a naturally occurring cobalamin in the following experiments. Twelve micrograms of OH-B<sub>12</sub> per 100 g (or mL) solution (the same B<sub>12</sub> concentration as that of the raw round herring meats shown in Table 1) containing 10 mM potassium phosphate buffer, pH 7.0, was packed into a polyethylene bag (180×200×0.07 mm) and then heated by boiling and steaming for the indicated time courses under conditions similar to those described above. In the microwave heating, the OH-B<sub>12</sub> solution was poured into a 300 mL-glass flask, the top of the flask covered with a wrapping film, and the solution treated for the indicated time courses under the same conditions. In the case of vacuum-packed pouch cooking, the OH-B<sub>12</sub> solution was packed in pouches for vacuum-packed pouch cooking, treated with or without the vacuum sealing, and heated for the indicated time and temperature under the same conditions. The OH-B<sub>12</sub> solution (100 g) was directly treated for 7.5 min in a frying pan which was maintained at 180˚C (as grilled samples).

The treated OH-B<sub>12</sub> solution was concentrated with a Sep-pak Plus C<sub>18</sub> cartridge (Waters Corp., Milford, USA) which had been washed with 2 mL of 75% (v/v) ethanol and then equilibrated with 3 mL of distilled water. The C<sub>18</sub> cartridge was eluted with 1.5 mL of 75% (v/v) ethanol. The eluate was evaporated at low temperature with a centrifugal concentrator (Integrated Speed Vac<sup>®</sup> System ISS110, Savant Instruments Inc., NY, USA). The residual fraction was dissolved with 0.5 mL of distilled water. The concentrated OH-B<sub>12</sub> solution was analyzed by HPLC using a JASCO HPLC apparatus (PU-2080 Plus Pump, UV-2070 Plus Spectrophotometer, DG-2080-53 Degasser, CO-2065 column oven) and CDS ver. 5 chromatography processing system (LASoft, Ltd., Chiba, Japan). The sample (50 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 40˚C. The flow rate was 1.0 mL/min. The treated OH-B<sub>12</sub> solution and authentic OH-B<sub>12</sub> were isocratically eluted with the same solution and monitored by measuring absorbance at 351 nm. Retention time of authentic OH-B<sub>12</sub> was 2.7 min.

**Escherichia coli** 215 bioautogram and reversed-phase HPLC analysis. After a cooked round herring extract was concentrated and partially purified with a Sep-pack Plus C<sub>18</sub> cartridge (Waters Corp.), 2 μL of the purified B<sub>12</sub> extract and authentic B<sub>12</sub> (cyanocobalamin, 10 μg/L) were spotted on a silica gel 60 TLC sheet and developed with 2-propanol/NH<sub>4</sub>OH (28%/water (7 : 1 : 2 v/v) in the dark at 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, B<sub>12</sub> compounds were visualized as red in color indicating *E. coli* growth.

An aliquot (10 mL) of each extract was loaded onto an immunoaffinity column (EASI-EXTRACT<sup>®</sup> Vitamin B<sub>12</sub> Immunoaffinity Column (P80), R-Biopharm AG, Darmstadt, Germany) and then B<sub>12</sub> was purified according to the manufacturer’s recommended protocol. The purified B<sub>12</sub> solution was analyzed by HPLC using a
JASCO HPLC apparatus (PU-2080 Plus Pump, UV-2070 Plus Spectrophotometer, DG-2080-53 Degasser, CO-2065 column oven) and CDS ver. 5 chromatography processing system (LaSoft, Ltd.). 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 40°C. The flow rate was 1.0 mL/min. The B₁₂ compound and authentic B₁₂ were isocratically eluted with the same solution and monitored by measuring absorbance at 361 nm. Retention time of authentic B₁₂ was 10.4 min.

**Results and Discussion**

**B₁₂ contents of the raw round herring meats**

B₁₂ contents were determined in the meats and viscera of the round herrings using the microbiological B₁₂ assay method (Table 1). Amounts of B₁₂ (per 100 g of fresh meat weight) were three-times greater in the viscera (37.5±10.6 µg) than in the meats (12.2±2.1 µg), which value was similar to the raw fish meat B₁₂ content (14.2 µg) described in Standard Tables of Food Composition in Japan. 5th revised and enlarged edition (12). About 73% of total B₁₂ found in the whole fish

| Weight (g) | Vitamin B₁₂ contents (µg/100 g fresh weight) | Distribution (%) |
|-----------|---------------------------------------------|-----------------|
| Meats     | 41.4±1.4                                    |                  |
| Viscera   | 5.0±0.4                                     |                  |

The values represent mean±SE (n=10). **Distribution represents ratio of B₁₂ contents* found in the meat or viscera of each fish to the sum of them.

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| Meats     | 41.4±1.4                                    |                  |
| Viscera   | 5.0±0.4                                     |                  |

The values represent mean±SE (n=10). **Distribution represents ratio of B₁₂ contents* found in the meat or viscera of each fish to the sum of them.

Table 2. Effect of various cooking methods on vitamin B₁₂ contents of the meats of raw round herring.

| Changes in weight (g) | Vitamin B₁₂ contents (µg/meats) (% against meats without cooking)* | Cooking conditions |
|-----------------------|---------------------------------------------------------------|-------------------|
| Before                | After                                                         |                   |
| Raw (without cooking, n=10) | 41.4±1.4                                                   | 5.1±0.3           |
| Grilling (n=5)        | 46.8±1.7                                                      | 3.4±0.2           |
| Boiling-1 (n=3)       | 33.0±0.9                                                      | 1.9±0.3           |
| Meats                 | 40.1±1.2                                                      | 3.4±0.3           |
| Boiling-2 (n=5)       | 37.1±1.1                                                      | 4.2±0.1           |
| Steaming-1 (n=5)      | 44.8±1.3                                                      | 2.3±0.1           |
| Frying-1 (n=4)        | 42.8±2.4                                                      | 3.3±0.4           |
| Frying-2 (n=5)        | 48.3±2.0                                                      | 2.5±0.2           |
| Microwave (n=5)       | 38.3±0.8                                                      | 2.7±0.1           |
| Vacuum-packed pouch cooking (n=5) | 29.4±2.8                                                   | 3.6±0.5           |

The values represent mean±SE.

* These values were adjusted by using the ratio of mean weights of raw fish meats (control meats/meats used for cooking).
body (except for head and bones) was recovered in the meats. The raw meat of half a fish (about 21 g) can supply the recommended dietary allowance (2.4 μg of B12 per day) for adults (13).

Effects of various cooking methods on B12 contents of the raw round herring meats

To clarify how much B12 was lost in the raw fish meats during various cooking methods, B12 was extracted and analyzed from the cooked fish meats (Table 2). When the fish meats were treated under the appropriate cooking conditions, the B12 contents of the fish meats were significantly decreased up to about 59, 47, 41, 43, and 59% during the cooking by grilling (for 7.5 min), boiling (for 5.0 min), steaming (for 9.0 min), frying (for 4.0 min), and microwaving (1.0 min), respectively, but not at all during vacuum-packed pouch cooking. The stability of B12 was dependent on the treatment temperatures and times judging from the data of the steam and fry cooking. B12 content of broth in boiling with boiling water was about half of that in boiling with water; decrease in the leakage of B12 from the treated meats may be due to the meat surface proteins being denatured quickly by the boiling water.

Table 3 shows the loss of a naturally occurring B12 compound, OH-B12, solution (at the same B12 concentration as the raw round herring meats shown in Table 1) during various heat treatments as a model system. OH-B12 levels were the similar to those of the fish meat B12 for grilling, boiling, steaming (4.5 min), and vacuum-packed pouch cooking (70°C for 30 min), and greater for steaming (9.0 min) and microwaving the larger loss of B12 in the fish meats may be due to the B12 destruction stimulated by the interaction with the ingredients of the meats. As shown in Tables 2 and 3, the loss of B12 appears to be dependent on the temperature and time used in the conventional cooking.

As there is a lack of detailed data on nutritional aspects of vacuum-packed pouch cooking, it is difficult to evaluate whether the little (or no) loss of vitamins during vacuum-packed pouch cooking is only due to the lower temperature used or due to vacuum packaging (14). Petersen (15) has described the effects of the vacuum-packed pouch cooking, steaming, and boiling of broccoli florets on stability of vitamin C, vitamin B6,
and folate. The stability of all vitamins was lowest for boiling, while the stability was greatest for vacuum-packed pouch cooking and a little lower for steaming. Although stability of vitamin C is highly dependent on the degree of vacuum in the package, that of vitamin B₉ is independent on the degree of vacuum. Vitamins sensitive to oxidation have better stability in vacuum-packed pouch cooking than in conventional or traditional cooking. In the case of B₁₂, retention of B₁₂ in meat and fish dishes by vacuum-packed pouch cooking has been reported to be 87% (beef), 100% (veal), 100% (lamb), 100% (pork), 92% (salmon), and 72% (cod) (16). The results in Table 3 indicate that the loss of B₁₂ is not dependent on the vacuum or temperature (or both) used in vacuum-packed pouch cooking.

These results indicate that the usual cooking methods for fish meats (by grilling, frying, steaming, and microwaving) results in significant B₁₂ decrease (~50%), while vacuum-packed pouch cooking is an excellent method to prevent loss of B₁₂ during the cooking of fish meats.

Our previous studies have demonstrated that appreciable loss (~40%) of B₁₂ occurs in certain foods during microwave heating (9). Prolonged heat-treatments (boiling for 30 min or microwaving for 6 min) of authentic OH-B₁₂ solution have demonstrated the accelerated formation of B₁₂ degradation products, some of which are identified as the compounds with various changes to lower-ligand moiety (cobalt-coordinated nucleotide) (9, 17).

Since some of these B₁₂ degradation products are detectable by a B₁₂-dependent E. coli 2155 bioautogram and a reversed-phased HPLC (18), B₁₂ extracts of the fish meats treated by frying and microwaving were qualitatively analyzed (Fig. 2). The B₁₂ compound found in each extract was given as a single spot and peak, whose respective Rt values and retention times were identical to that of authentic B₁₂ according to the bioautogram and HPLC. These results suggest that no unidentified B₁₂ compounds were formed in the fish meats under these cooking conditions. The decreased B₁₂ contents of the various cooked fish meats appear to be due to the accelerated formation of B₁₂ by heating.

The results presented here indicate that the B₁₂ content of fish meats is decreased ~50% during normal cooking (grilling, boiling, frying, steaming, and microwaving); conveniently, 50% loss of B₁₂ should be estimated in assessment of the daily intake of B₁₂. We also demonstrate for the first time that vacuum-packed pouch cooking is an excellent method to prevent loss of B₁₂ during the cooking of round herring.

REFERENCES

1) Schneider Z, Stroinski A. 1987. Biosynthesis of vitamin B₁₂. In: Comprehensive B₁₂ (Scheider Z, Stroinski A, eds), p 93–110. Walter de Gruyter, Berlin.
2) Watanabe F. 2007. Vitamin B₁₂ sources and bioavailability. Exp Biol Med 232: 1266–1274.
3) Yoshino K, Inagawa M, Oshima M, Yokota K, Umesawa M, Endo M, Yamagishi K, Tanigawa T, Sato S, Shimamoto T, Iso H. 2005. Trends in dietary intake of folate, vitamin B₉, and vitamin B₁₂ among Japanese adults in two rural communities from 1971 through 2001. J Epidemiol 15: 29–37.
4) He M, Ke CH, Wang WX. 2010. Effects of cooking and subcellular distribution on the bioaccessibility of trace elements in two marine fish species. J Agric Food Chem 58: 3517–3523.
5) Hosomi R, Fukunaga K, Arai H, Nishiyama T, Yoshida M. 2009. Effects of dietary fish protein on serum and liver lipid concentrations in rats and the expression of hepatic genes involved in lipid metabolism. J Agric Food Chem 57: 9256–9262.
6) Iso H, Kobayashi M, Ishihara J, Sasaki S, Okada K, Kita Y, Kokubo Y, Tsugane S. 2006. Intake of fish and n3 fatty acids and risk of coronary heart disease among Japanese: the Japan Public Health Center-Based (JPHC) Study Cohort I. Circulation 113: 195–202.
7) Simopoulos AP. 2002. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 21: 495–505.
8) Bennink MR, Ono K. 1982. Vitamin B₁₂, E and D content of raw and cooked beef. J Food Sci 47: 1786–1792.
9) Watanabe F, Abe K, Fujita T, Goto M, Hiemori M, Nakano Y. 1998. Effects of microwave heating on the loss of vitamin B₁₂ in foods. J Agric Food Chem 46: 206–210.
10) Ersoy B, Özeren A. 2009. The effect of cooking methods on mineral and vitamin contents of African catfish. Food Chem 115: 419–422.
11) Resources Council, Science and Technology Agency. 1995. Vitamin B₁₂. In: Standard Tables of Food Composition in Japan-Vitamin K, B₉, and B₁₂, p 6–56. Resources Council, Science and Technology Agency, Japan, Tokyo.
12) The Council for Science and Technology. 2005. Report of the subdivision on Resources. In: Standard Tables of Food Composition in Japan, 5th revised and enlarged ed. Ministry of Education, Culture, Sports, Science and Technology, Japan, Tokyo.
13) Ministry of Health, Labour, and Welfare. Japan. 2009. Vitamin B₁₂. In: Dietary Reference Intakes for Japanese. 2010, p 159–161. Daiichi Shuppan Publishing Co., Ltd., Tokyo.
14) Schellekens M. 1996. New research issues in sous-vide cooking. Trend Food Sci Technol 7: 256–262.
15) Petersen MA. 1993. Influence of sous vide processing, steaming and boiling on vitamin retention and sensory quality in broccolini florets. Z Lebensm Unters Forsch 197: 375–380.
16) Creed PG. 1995. The sensory and nutritional quality of ‘sous vide’ foods. Food Control 6: 45–52.
17) Watanabe F, Abe K, Katsura H, Takenaka S, Nakano Y, Nakano M. 1998. Biological activity of hydroxo-vitamin B₁₂ degradation product formed during microwave heating. J Agric Food Chem 46: 5177–5180.
18) Watanabe F, Yabuta Y. 2011. Microbiological detection of vitamin B₁₂ and other vitamins. In: Fortified Foods with Vitamins—Analytical Concepts to Assure Better and Safer Products (Rychlik M, ed), p 165–171. Wiley-VCH Verlag & Co., Weisheim.