J Am Oil Chem Soc (2010) 87:963–972
DOI 10.1007/s11746-010-1578-x

ORIGINAL PAPER

An Assay Method for Determining the Total Lipid Content of Fish Meat Using a 2-Thiobarbituric Acid Reaction

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Received: 11 July 2009 / Revised: 9 January 2010 / Accepted: 26 February 2010 / Published online: 8 April 2010

Abstract A method for the determination of the total lipid content in fish meat was established using a 2-thiobarbituric acid (TBA) reaction, which had previously been used for the determination of lipid peroxides in animal tissues. In this method, an unspecific peroxidation of fish oils was created by omitting the addition of antioxidant to the reaction mixture during the TBA reaction, because fish meat is more sensitive to the TBA reaction due to its higher concentration of polyunsaturated fatty acids, especially docosahexaenoic acid and eicosapentaenoic acid, which can easily react artificially with TBA in the absence of an antioxidant in the assay system. As for a practical application of this method, we tried to optimize the assay procedures in the sampling, reaction, and detection steps of this method, and finally proposed a new standard procedure recommended for determining the total lipid content of fish using a TBA reaction. In order to confirm the accuracy of the new procedure, comparative evaluations for the lipid contents of commercially available fish, i.e., chub mackerel (*Scomber japonicus*) and saury (*Cololabis saira*) were made between the conventional procedure and the recommended TBA method. The lipid contents obtained by the two methods coincided well with high correlation. This method is relevant for total lipid content analysis of fish meat under restricted laboratory conditions.

Keywords Lipid content · Fish · Assay method · 2-Thiobarbituric acid (TBA) · Docosahexaenoic acid (DHA) · Eicosapentaenoic acid (EPA) · Lipid oxidation

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Introduction

Foods. including animal tissues, that contain polyunsaturated fatty acids are highly susceptible to lipid oxidation [1]. Lipid oxidation leads to the formation of hydroperoxides, which are very unstable and decompose to form secondary reaction products that adversely affect the flavor of food. The 2-thiobarbituric acid (TBA) assay is based on the reaction of TBA with bifunctional aldehydes, primarily malondialdehyde, derived from broken-down products from many oxidized molecules [2]. The resulting chromogen has the characteristic absorbance spectrum with a distinct peak at 532 nm in an acidic pH. The simplicity of this assay and its apparent reliability have led to it being widely accepted as an index of lipid oxidation.

Soon after the introduction of the TBA test, oxidation by the air of the lipids during the TBA reaction was found to produce misleading results [3]. The causes of inconsistent results when the TBA assay was applied to oils, especially fish oils which contain a lot of polyunsaturated fatty acids, were oxidation of the lipid samples by the air during the reaction [4, 5]. However, oxidation in air can be suppressed by the addition of antioxidants to the reaction system, and the use of an antioxidant such as butylated hydroxytoluene (BHT) is simple and a practical choice for routine TBA assays [4–6].

Liu et al. (1997) [7] examined the sensitivity of the TBA assay to detect TBA reacting substances generated from peroxidation of various unsaturated fatty acids, and suggested that (1) TBA reacting substance production is closely, but not strictly, related to the number of double bonds in unsaturated fatty acids; and (2) the lipid carbon chain length in unsaturated fatty acids is also relevant to the production of TBA reacting substances. For example, docosahexaenoic acid (DHA) produced the highest amount of TBA reacting substances, while oleic acid produced the lowest amount of TBA reacting substances. These findings are generally in agreement with that in fish samples, a higher lipid content produces higher TBA values, i.e., TBA reacting substances, because fish oils generally contain a lot of DHA [5].

Our idea of a positive utilization of unspecific peroxidation of fish oils by omitting the addition of BHT to the reaction mixture during the TBA reaction encouraged us to develop a new method for determining the total lipid content of fish samples. Until now, Folch's or Soxhlet extraction [8, 9] and precise weighing (gravimetry) has been the method used for the determination of the total lipid content of fish samples [10]. This conventional method is time consuming and requires hazardous organic solvents such as diethyl ether or chloroform and some special apparatuses such as Soxhlet extraction apparatus and evaporators. Since a good correlation of the values between the conventional method and the present method was observed in the present study, we propose this new method for the determination of the total lipid content of fish samples using a TBA reaction, and this method is relevant for total lipid content analysis of fish under restricted laboratory conditions.

Materials and Methods

Materials and Reagents

Fish, pork and beef were obtained from supermarkets in Shimonoseki, Yamaguchi Prefecture, Japan and the experiments were carried out while they were fresh. Tridocosahexaenoin, trilinolenin, triolein, tristearin and methyl docosahexaenoate were purchased from Funakoshi Co., Ltd (Tokyo, Japan). Methyl alpha-linolenate, methyl arachidonate and methyl eicosapentaenoate (all-*cis*-5, 8, 11, 14, 17-) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cotton seed oil, olive oil and other chemical reagents used in the experiments were purchased from Wako Pure Chemical Co. (Osaka, Japan), with the exception of those otherwise described.

Conventional Method for Total Lipid Content of Fish Meat

The conventional method for the determination of the total lipid content of fish meat samples was performed in the present study by chloroform–methanol (2:1) extraction according to Folch et al. [8] and precise weighing (gravimetry) of the extracted lipids as follows.

Fish meat was chopped in a food processor and 5.00 g of it was weighed and added to a 50-ml test tube. Thirty milliliters of chloroform–methanol (2:1) was added to the test tube and homogenized using a tissue homogenizer (Polytron, Kinematica; Central Scientific Commerce inc., Tokyo, Japan). Fragments of fish meat remaining on the blade were rinsed in the test tube with 4 ml of distilled water, and the mixture in the test tube was then centrifuged for 10 min at 3,000 rpm. The upper layer was removed by an aspirator and the remaining lower layer was filtered with a cotton plug. The volume of the solution obtained was adjusted to 50.0 ml by the addition of chloroform–methanol (2:1). This extracted lipid solution was sealed under a dry N_2 stream and stored at −50 °C until it was used. One thousand microliters of the extracted lipid solution was placed into the pre-weighed glass tube and the organic solvent was removed completely using a centrifugal concentrator (VC-96N) (TAITEC Co. Ltd., Koshigaya, Saitama Prefecture, Japan). The weight of the glass tube containing the lipids was measured accurately. The total lipid content (%) of the fish meat was calculated from the weight of the extracted lipids.

The New Assay Method for determining the Total Lipid Content of Fish Meat by TBA Reaction

Preparation of Standard Lipids from Fish Meat

The lipid preparation for each fish meat was conducted using the Folch's extraction method [8] described above. The extracted lipid fraction from each fish meat in chloroform–methanol (2:1) was sealed under a dry N_2 stream and stored at −50 °C. These lipid fractions remained stable for at least 6 months and were used as standard lipids for the TBA reaction.

TBA Reaction with Extracted or Standard Lipids Without the Addition of an Antioxidant

The chloroform–methanol (2:1) solution containing lipids (0.02–0.50 mg) was added into the glass test tube and the solvents were removed using a centrifugal concentrator at 40 °C. Lipids were dissolved by the addition of ethanol (0.20 ml) or 2-propanol (0.20 ml) into the tube and the

volume was adjusted to 1.00 ml by the addition of distilled water (0.80 ml). Furthermore, an acetate buffer solution, pH 3.5 (1.50 ml), and a 0.8% TBA solution in water (2.50 ml) were added to the tube and the mixture stirred vigorously. The mixture in the test tube was sealed with a screw cap and heated in a boiling water bath for 15 min. After cooling, the absorbance was measured at 532 nm using an ultraviolet–visible spectrophotometer (Hitachi UVmini-1240) (Hitachi Ltd., Tokyo, Japan). In some experiments, after cooling, the reaction product was analyzed by a high-performance liquid chromatography (HPLC) system equipped with a pump (Hitachi L-7100), detector (Hitachi L-4250 UV–VIS Detector), column (Intersil ODS-3, 4.6 × 250 mm) (GL Sciences Inc. Tokyo, Japan) and integrator (IATROCORDER TC-21) (Mitsubishi Chemical Co. Ltd., Tokyo, Japan). Analytical conditions of the HPLC were as follows: mobile phase; methanol–acetate buffer pH 5.6 (45:55), flow rate; 1.00 ml/min, wave length; 532 nm. The peak area of the product displaying absorption at 532 nm was measured. TBA reactions with standard oil samples, i.e., various triglycerides and fatty acid methyl esters, extracted lipids from pork or beef, olive oil and cotton seed oil were performed in accordance with the cases of extracted lipids of fish meats as mentioned above.

Preliminary Assay Procedure for Total Lipid Content of Fish Meat Using a TBA Reaction

In order to develop a simple assay procedure for detecting the total lipid content of fish meat using a TBA reaction, sampling and reaction were performed in the same test tube to reduce the number of operating steps in the assay procedure. Here, the chopped fish or animal meats (5.0–50 mg) were weighed and put in the glass test tube. Distilled water (1.00 ml) and an acetate buffer solution, pH 3.5 (1.50 ml), were added to the tube and stirred vigorously using a pencil type mixer (As One Co., Osaka, Japan). Next, a 0.8% TBA solution in water (2.50 ml) was added, and the TBA reaction was started by heating in a boiling water bath. Detection of the products was performed as described above.

Standard Procedure Finally Recommended for Total Lipid Content of Fish Meat by TBA Reaction

The assay for determining the total lipid content of fish meat by TBA reaction was finally set up as follows. A small mass of fish meat was quickly crushed in a small mortar and the cap of a micro tube (sampling tube 1.5 ml) (ASSIST Co. Ltd., Tokyo, Japan) was filled with the paste-like fish meat. The cap filled with fish meat was taken from the tube body and put into a 50-ml plastic tube containing 30 ml of 2-propanol. The mixture was stirred vigorously and centrifuged at 3000 rpm for 10 min. The aliquot (0.10–0.50 ml) of the supernatant was placed in a glass test tube and the volume was adjusted to 1.00 ml by the addition of distilled water. Furthermore, an acetate buffer solution, pH 3.5 (1.50 ml), and a 0.8% TBA solution in water (2.50 ml) were added into the tube and stirred vigorously. The mixture in the test tube was sealed with a screw cap and heated in a boiling water bath for 15 min. After cooling, absorbance was measured at 532 nm using the ultraviolet–visible spectrophotometer (Hitachi UVmini-1240). The absorbance of the blank reaction mixture was subtracted. The total lipid content of the fish was calculated from the calibration curves prepared by parallel TBA reactions of standard lipids extracted from the same fish species.

Determination of Fatty Acid Composition

Determination of fatty acid composition was performed according to Tanaka et al. [11]. The extracted lipid solutions of fish meat were transmethylated by sodium methoxide. The lipid solution (10.0 mg/tube) in the test tube was mixed with 0.50 ml of a 2.8% sodium methoxide solution and 0.10 ml of benzene, and heated using a heating block (MG-2200) (EYELA Inc., Tokyo, Japan) at 50 °C for 10 min. To the reaction mixture was then added 0.050 ml of acetic acid, 2.0 ml of distilled water and 4.0 ml of hexane, and the mixture was centrifuged at 2,500 rpm for 10 min. The upper layer was transferred into another test tube, the solvents were removed using a centrifugal concentrator, and the residue was redissolved in 0.10 ml acetone. The methylated fatty acids were analyzed by gas liquid chromatography (GLC) using a gas chromatograph system (shimadzu GC-14A) (Shimazu Co., Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (HRSS-10, 50 m × 0.25 mm i.d.) (Shinwa Chemical Industries, Kyoto, Japan). The column temperature was programmed for a linear increase of 3 °C/min from 150 to 220 °C, and was then maintained for 10 min. The injection and detector port temperatures were 250 and 300 °C, respectively. The methyl esters on the chromatogram were identified by conventional methods using the retention time of standard samples.

Determination of the Tocopherols Content

The tocopherols content was determined according to Ward et al. [12]. The residue of the extracted lipid solution of fish meat (10.0 mg lipid/tube) after the evaporation using a centrifugal concentrator was reconstituted with 0.050 ml of 1.0% sodium chloride in water and 1.0 ml of 3.0% pyrogallol in ethanol. This solution was saponified by the heating at 70 °C for 30 min after the addition of 60%

potassium hydroxide in water. After heating, 2.50 ml of 1.0% sodium chloride in water and 1.5 ml of ethyl acetate–*n*-hexane (1:9) were added to the reaction mixture and centrifuged at 2,500 rpm for 10 min. The upper layer was transferred into another tube and the lower layer was mixed again with 1.5 ml of ethyl acetate–*n*-hexane (1:9) and centrifuged at 2,500 rpm for 10 min. Both upper layers were combined, and the solvents were removed using a centrifugal concentrator. The residue was redissolved in 0.50 ml acetone and used for determination of tocopherols content using an HPLC system equipped with a pump (PU-980) (JASCO Co., Tokyo, Japan), a fluorescence detector (281-FP) (JASCO Co.), an integrator (D-2500) (Hitachi Ltd.), an injector (Rheodyne 7125) (MS Instruments, Inc., Osaka, Japan), a column oven (860-CO) (JASCO Co.) and a column (CAPCELL PAK C-18; 4.6 × 150 mm) (Shiseido Co., Tokyo, Japan). Analytical conditions of the HPLC were as follows: mobile phase; acetonitrile–methanol–water (97:1.5:1.5) with a flow rate of 1.00 ml/min, excitation wave length of 298 nm and a fluorescence wave length of 325 nm. The peak area of the product displaying fluorescence at 325 nm was measured.

Statistical Analysis

Statistical analysis of the correlation was performed using the software "Excel Statistics" (Esumi Co. Ltd., Tokyo, Japan).

Results and Discussion

TBA assays have long been used for the determination of lipid peroxides in animal tissues. Since lipids in samples are often oxidized and react with TBA in the heating step of the TBA assay, the addition of BHT, an antioxidant, into the assay system is required to prevent this spontaneous and undesirable oxidation effectively, as pointed out as being necessary by many reports [4–6, 13–17]. It is known that fish meat is more sensitive to the TBA reaction than animal meat [4, 5] because of its higher concentration of polyunsaturated fatty acids, especially DHA, which can easily react unspecifically with TBA in the absence of an antioxidant in the assay system [7]. Such information encouraged us to investigate the utilization of a TBA reaction for a total lipid content assay method in fish meat.

At first, we checked the reactivity of various types of oils with TBA. Figure 1**a** shows the results from 4 types of standard triglycerides. A very small amount of TBA products were produced in triolein and tristearin. On the other hand, in the case of trilinolenin and tridocosahexaenoin, the TBA product increases were dependent upon the concentration, and the increase in the case of tridocosahexanoin was about 18 times more than in the case of trilinolenin. The order of reactivity with TBA was as follows: tridocosahexanoin ≫ trilinolenin > triolein ∼ tristearin. Figure 1b shows the results from four types of standard unsaturated fatty acid methyl esters. The order of reactivity with TBA was as follows: methyl docosahexaenoate – methyl eicosapentaenoate ≫ methyl arachidonate > methyl *alpha*-linolenate. Figure 2a, b shows the results from six types of food oils, i.e., sardine (*Sandina pilchardus*) oil, yellowtail (*Seriola quinqueradiata*) oil, olive oil, cotton seed oil, pork oil (rib, fat) and beef oil (fillet, red). The order of reactivity with TBA was as follows: sardine oil – yellowtail oil ≫ cotton seed oil – pork oil (rib, fat) > olive oil – beef oil (fillet, red). These results suggest that the reactivity of various oils to TBA is related to the number of double bonds in the chemical structures of fatty acids contained in the oils, and that fish oils were much more sensitive to TBA reaction than vegetable and livestock oils. In addition, as shown in Table 1, the fatty acid composition of 16 types of fish meat, including yellowtail "buri" (*Seriola quinqueradiata*), yellowtail "hamachi" (*Seriola quinqueradiata*), yellowtail "yazu" (*Seriola quinqueradiata*), horse mackerel (*Trachurus japonicus*), flounder (*Paralichthys olivaceus*), salmon (*Oncorhynchus keta*), chub mackerel (*S. japonicus*), sea bass (*Lateolabrax japonicus*), silver-stripe round herring (*Spratelloides gracilis*), sea bream (*Pagrus major*), filefish (*Stephanolepis cirrhifer*), albacore (*Thunnus alalunga*), pollack (*Gadus macrocephalus*), cutlass fish (*Trichiurus japonicus*), saury (*C. saira*) and sardine (*Sardina pilchardus*), displayed high composition rates of n-3 poly-unsaturated fatty acids, i.e., DHA and EPA, which have six and five double bonds in their chemical structure as compared with that of livestock meats which does not include DHA or EPA. These results showed the idea that the assay method used to assess total lipid content of fish using a TBA reaction is available in fish meat but not in animal (livestock) meat. However, the correlation between TBA reaction values and total lipid contents should be strictly available in the same type of fish meats using the calibration curve made by the same fish meat oil, because the contents of DHA and EPA in various types of fish meats were strictly different from each other, as shown in Table 1. Moreover, Table 2 shows the concentrations of alpha-, beta-, gamma-, and delta-tocopherols in 14 types of fish meat as well as livestock meats, which are typical oil-soluble antioxidants. Alpha-tocopherol was only detected in fish meats and the *alpha*-tocopherol in yellowtail (hamachi) meat showed the highest content to be about 2.1 mg/100 g. In this case, the concentration of alpha-tocopherol in the TBA reaction mixture was calculated to be almost 17 nM. Astaxanthin is another oil-soluble antioxidant and its content in salmon meat, being highest

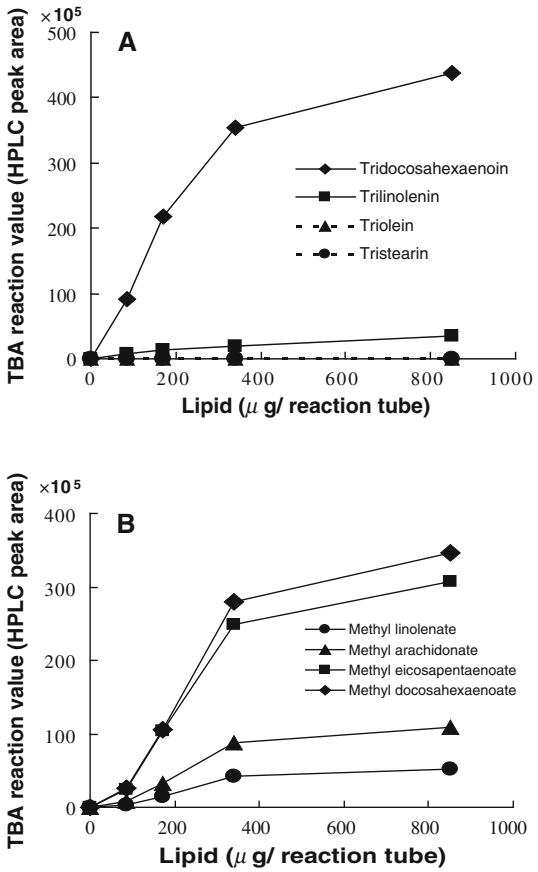

Fig. 1 The reactivity of various triglycerides (**a**) and fatty acid methyl esters (**b**) with TBA in the absence of antioxidants in the reaction mixture. Standard triglycerides and fatty acid methyl esters (0–0.850 mg/tube) were incubated with TBA in a boiling water bath without the addition of antioxidants as described in "Materials and Methods". The resulting reaction product was measured by HPLC. The abscissa and ordinate show the amounts of lipid and TBA reaction products, respectively. Each point shows the duplication mean

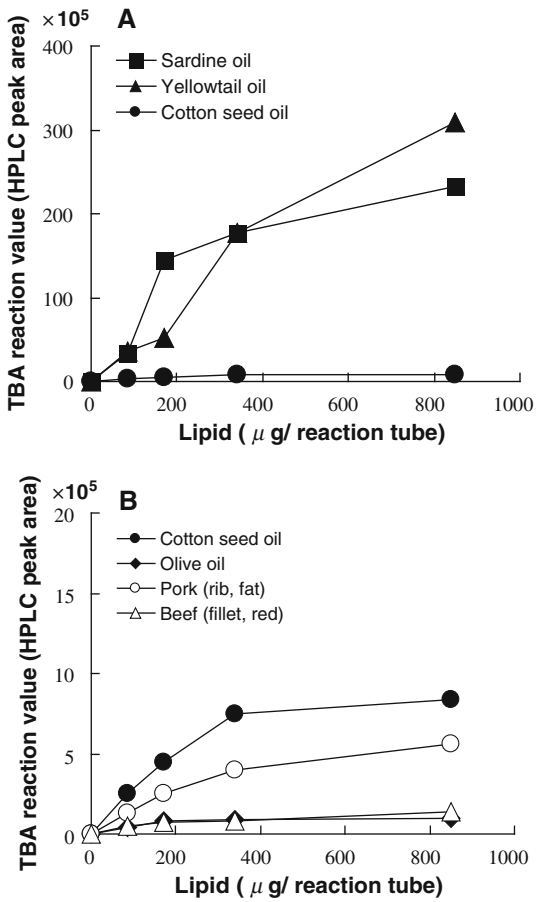

Fig. 2 The reactivity of various types of food oils, i.e., sardine oil (**a**), yellowtail oil (**a**), olive oil (**b**), cotton seed oil (**a**, **b**), pork oil (**b**) and beef oil (**b**), with TBA in the absence of antioxidants in the reaction mixture. Food oils (0–0.850 mg/tube) were incubated with TBA in a boiling water bath without the addition of antioxidants as described in "Materials and Methods". The resulting reaction product was measured by HPLC. The abscissa and ordinate show the amounts of lipid and TBA reaction products, respectively. Each point shows the duplication mean

among various fish meats, has been reported to be about 1.4 mg/100 g by Yamazaki et al. [18]. In this case, the concentration of astaxanthin in the TBA reaction mixture was calculated to be almost 8.3 nM. In fact, the TBA reaction of tridocosahexaenoin failed to be inhibited by these concentrations of alpha-tocopherol and astaxanthin (data not shown). Therefore, the effects of oil-soluble antioxidants, i.e., alpha-tocopherol and astaxanthin on the present assay method of fish total lipid content using a TBA reaction could be negligible practically.

As for practical application, we tried to optimize the assay procedure of this method as follows. For the measurement of the final TBA reaction product, the use of simpler ways like colorimetric measurement instead of chromatography, such as by HPLC, would be more suitable in workshops and laboratories having insufficient instrumentation. In addition, we originally tried to use homogenated samples of fish meat and omit the use of hazardous organic solvents or change hazardous organic solvents in the assay procedure to a type of organic solvent that is easy to handle. However, in the case of omitting the use of organic solvents, the final solution of the TBA reaction displaying a red color (532 nm) failed to be completely clear, despite efforts to solve the tissue residue by the addition of sodium chloride and several surfactants. As shown in Fig. 3a, the relationship between the amount of extracted fish (saury, *C. saira*) oil and the absorbance at 532 nm showed an extremely strong correlation ($R^2 = 0.9954$, $p < 0.01$). In contrast to the results from extracted oil, the reactivity of fish (saury, *C. saira*) meat homogenate with TBA showed a weaker correlation with the mass of the tissue ($R^2 = 0.5108$, not statistically significant) as shown in Fig. 3b. Therefore, in the early stages

Table 1 Fatty acid composition of fish meats, livestock meats and vegetable oils

Fats and oils	Fatty acids (%)				
	Saturated	Mono-unsaturated	Poly-unsaturated		
			n-3 + n-6	n-3	n-6
Fish meat oil					
Yellowtail (buri)	35.5	34.2	30.3	27.4	2.9
Horse mackerel	33.4	30.9	35.7	31.9	3.8
Flounder	28.5	31.6	39.9	34.6	5.3
Salmon	22.6	48.7	28.7	25.6	3.1
Chub mackerel	37.3	41.0	21.7	18.2	3.5
Sea bass	32.3	36.1	31.6	27.7	3.9
Silver-stripe round herring	44.0	24.0	32.0	28.0	4.0
Sea bream	33.3	35.8	30.9	27.1	3.8
Yellowtail (hamachi)	29.3	37.8	32.9	27.6	5.3
Filefish	33.3	16.7	50.0	33.3	16.7
Albacore	31.0	22.0	47.0	43.0	4.0
Pollack	21.4	21.4	57.2	50.1	7.1
Cutlass fish	35.1	42.8	22.1	19.6	2.5
Yellowtail (yazu)	31.9	33.6	34.5	30.4	4.1
Saury	22.0	54.2	23.8	21.0	2.8
Sardine	36.7	26.8	36.5	31.5	5.0
Livestock meat fat					
Pork (rib, fat)	40.7	46.7	12.6	0.5	12.1
Beef (fillet, red)	46.1	48.3	5.6	0.3	5.3
Vegetable oil					
Olive oil	14.1	78.2	7.7	0.7	7.0
Cotton seed oil	22.8	18.8	58.3	0.4	57.9

Each result is the mean of two measurements

of the assay, we introduced a step for extraction with an alcohol such as ethanol or 2-propanol, which are less harmful than the chloroform or diethyl ether and have been used frequently in food processing.

Figure 4a shows the effect of the sampling mass of fish (saury, *C. saira*) meat before the TBA reaction on the efficiency of lipid extraction with ethanol or 2-propanol. The results indicated that the sample size of the fish (saury, *C. saira*) meat should be acceptable between 0.15–0.50 g, since the efficiency of lipid extraction was not decreased throughout this range. In addition, we devised a simple and reproducible way to prepare the specific amount of fish meat required without utilizing a precise weighing machine. It was referred to as the "cap packing" method, and performed as follows: a mass of fish meat was quickly crushed by a small mortar and the cap of a plastic micro tube was filled with the paste-like fish meat. As shown in Fig. 4b, 20 samples for chub mackerel (*S. japonicus*) and 16 saury (*C. saira*) samples were weighed individually. The average weight of samples and standard deviations were 0.270 ± 0.009 and 0.273 ± 0.004 mg for chub mackerel and saury, respectively (Fig. 4). The coefficient

Table 2 Contents of alpha-, beta-, gamma- and delta-tocopherols in fish and livestock meats

Fish or livestock meats	Tocopherol (mg/100 g meat)			
	Alpha-	Beta-	Gamma-	Delta-
Yellowtail (buri)	2.0	0	0	0
Horse mackerel	0.4	0	0	0
Flounder	0.6	0	0	0
Salmon	1.3	0	0	0
Chub mackerel	0.9	0	0	0
Sea bass	1.2	0	0	0
Silver-stripe round herring	0.3	0	0	0
Sea bream	1.0	0	0	0
Yellowtail (hamachi)	2.1	0	0	0
Filefish	0.6	0	0	0
Albacore	0.7	0	0	0
Pollack	0.8	0	0	0
Cutlass fish	1.2	0	0	0
Yellowtail (yazu)	1.4	0	0	0
Pork (rib, fat)	0.6	0	0.1	0
Beef (fillet, red)	0.4	0	0	0

Each result is the mean of two measurements

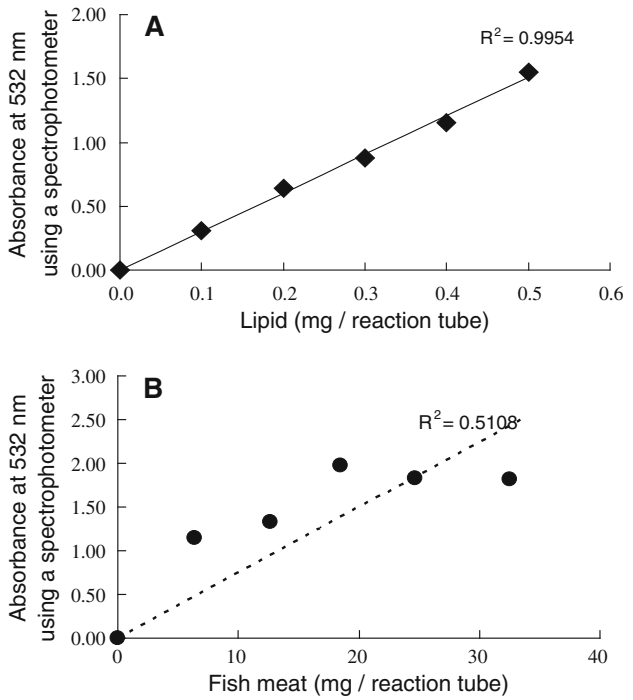

Fig. 3 The reactivity of extracted oil (**a**) and tissue of meat (**b**) with TBA in the absence of antioxidants in the reaction mixture. The extracted oil (0–0.50 mg/tube) and dispersed tissues (0–35 mg/tube) of the same fish (saury) were incubated with TBA in a boiling water bath without the addition of antioxidants as described in "Materials and Methods". The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. The value of R^2 shows the correlation coefficient. $R^2 = 0.9954$ ($p < 0.01$) for extracted oil and $R^2 = 0.5108$ (not statistically significant) for fish meat homogenate. Each point shows the duplication mean

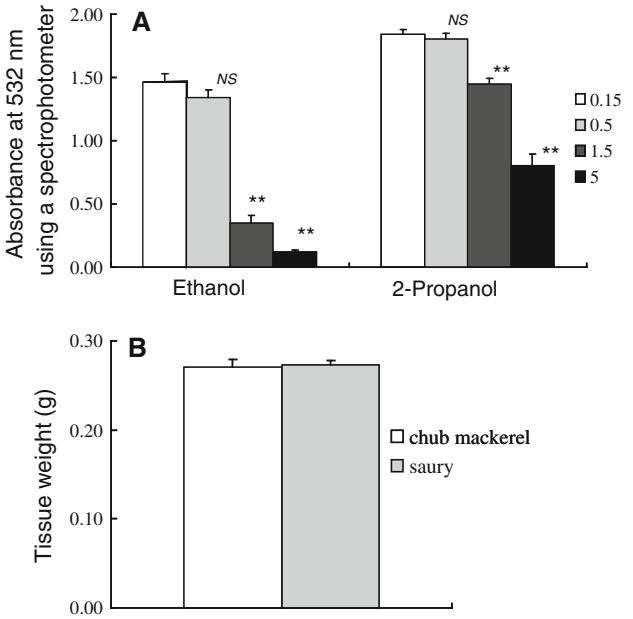

Fig. 4 The effect of the sampling amount of fish meat on the efficiency of lipid extraction with ethanol or 2-propanol before the TBA reaction (**a**), and the simple and accurate method sampling a certain amount of fish meat by "cap packing" without precise weight measurement machines (**b**). **a** The different amount of fish (saury) meat (□ 0.15, ◻ 0.50, ■ 1.50 and ■ 5.00 g) were put into the 50 ml plastic tube containing 30 ml of ethanol or 2-propanol and lipids were extracted by immersion for 10 min. The aliquot (□; 0.500, ◻; 0.150, ■; 0.050 and ■; 0.015 ml) of the extracted lipid solution was placed in a glass test tube and the volume was adjusted to be 0.500 ml by addition of ethanol or 2-propanol. Furthermore, 0.500 ml distilled water and 1.500 ml acetate buffer solution pH 3.5 were added into the tube and the mixture was incubated with TBA in a boiling water bath without the addition of antioxidants as described in "Materials and Methods". The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Data are expressed as mean ± S.D. ($n = 4$). $^{**}p < 0.01$ versus 0.15 g (fish meat) group. *NS* means not significant versus 0.15 g (fish meat) group. **b** The mass of fish (chub mackerel and saury) meat was quickly destroyed by a small mortar, and the cap part of a micro tube (Sampling tube 1.5 ml, ASSIST Co. Ltd., Tokyo, Japan) was filled with paste-like destroyed fish meat. The weight of the cap filled with fish meat was determined. Data are expressed as means ± S.D. ($n = 20$, chub mackerel; $n = 16$, saury). The coefficient of variation (CV) values of 20 runs for chub mackerel and 16 runs for saury were 3.3 and 1.5%, respectively

of variation (CV) values between 20 runs for chub mackerel and 16 runs for saury (i.e., inter-run CV values) were 3.3 and 1.5%, respectively (Fig. 4). The CV values within a run (i.e., intra-run CV values) were 0.4–5.6% for chub mackerel and 0.3–5.9% for saury, respectively. These results indicated that the weight of the samples without actually conducting any weighing procedure was approximately 0.270 g throughout all samples.

In addition, the extraction step using alcohol was optimized further as follows. Figure 5 shows the effect of the immersion time of fish (saury, *C. saira*) meat in ethanol or 2-propanol based on the efficiency of the lipid extraction (a), and the effect of centrifugation and filtration steps inserted after the extraction step on the following TBA reaction (b). The lipid extraction rates of samples at 15, 30 and 60 min of immersion time were not significantly different from that at 5 min of immersion time. The lipid extraction rate of samples with the centrifugation step was significantly larger than that with the filtration step or without the centrifugation and filtration steps ($p < 0.01$). These results showed that an immersion time of 5–60 min did not affect the efficiency of the lipid extraction, and that a centrifugation (3000 rpm, 10 min) step should be included.

Furthermore, the conditions for the TBA reaction such as the duration of heating and the retention time between the TBA addition and the start of heating were examined. Figure 6 shows that the retention time of 0–60 min did not affect the results of the TBA reaction (a), and that 15 min of heating should be enough to complete the TBA reaction (b). Throughout the examination of new procedures, 2-propanol seemed to be better than ethanol as an extraction solvent. To summarize what is mentioned above, the

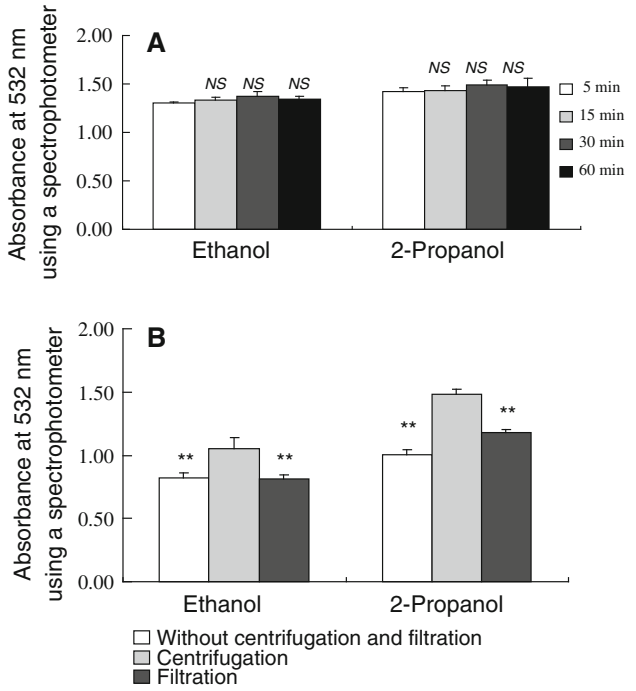

Fig. 5 The effect of the immersion time of the fish meat in ethanol or 2-propanol on the efficiency of lipid extraction before the TBA reaction (**a**), and the effect of the centrifugation and filtration step inserted after the extraction step on the following TBA reaction (**b**). **a** The fish (saury) meat (0.150 g) was put into a plastic tube containing 30 ml of ethanol or 2-propanol and lipids were extracted by immersion for 5, 10, 30 and 60 min. The extracted lipids (~0.20 mg/tube) were incubated with TBA as described in "Materials and Methods". The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Data are expressed as means ± S.D. ($n = 4$). *NS* means not significant versus 5 min (immersion time) group. **b** The fish (saury) meat (0.15 g) was put into a plastic tube containing 30 ml of ethanol or 2-propanol and lipids were extracted by immersion for 30 min. Before sampling of extracted lipids, centrifugation (3,000 rpm, 10 min) or filtration (pore size 0.2 micro meter; Whatman) of the extracted lipids solution were performed in the experiments. The extracted lipids (~0.20 mg/tube) were incubated with TBA as described in "Materials and Methods". The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Data are expressed as means ± S.D. ($n = 4$). $^{**}p < 0.01$ versus centrifugation group

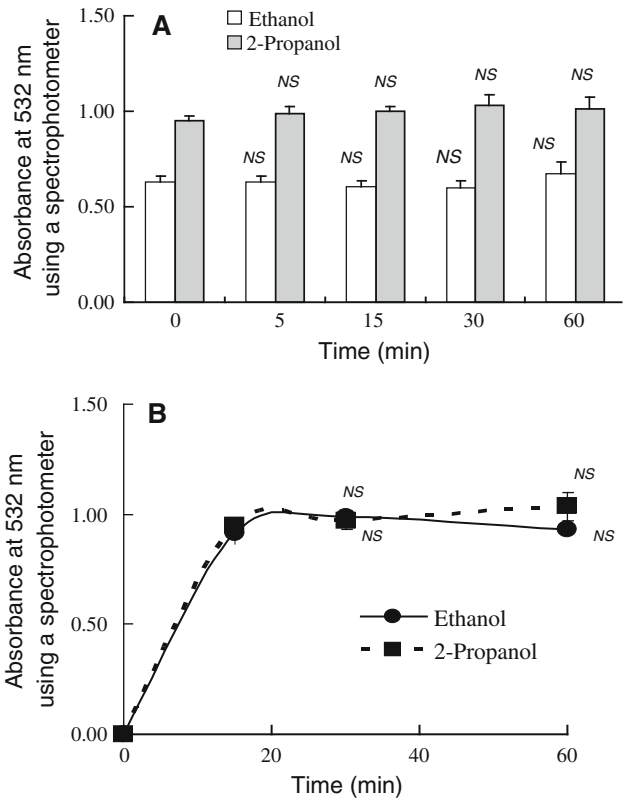

Fig. 6 The effects of pausing between the addition of TBA and heating (**a**), and the heating time (**b**) on the reactivity of the TBA reaction. **a** The extracted lipids (~0.150 mg/tube) of fish (saury) meat in ethanol or 2-propanol were incubated with TBA as described in "Materials and Methods". In the present experiment, the time interval between the addition of 0.8% TBA solution into the reaction mixture and the start of heating in a boiling water bath were set to be 0, 5, 15, 30 and 60 min. The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Data are expressed as means ± S.D. ($n = 4$). *NS* means not significant versus 0 min (time interval) group. **b** The extracted lipids (~0.150 mg/tube) of fish (saury) meat in ethanol or 2-propanol were incubated with TBA as described in "Materials and Methods". In the present experiment, the heating time in a boiling water bath were set to be 0, 15, 30 and 60 min. The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Data are expressed as means ± S.D. ($n = 4$). *NS* means not significant versus 15 min (heating time) group

assay procedure for the lipid content of fish meat using a TBA reaction was finally set. Figure 7 shows the flow chart of the recommended standard procedure for determining the total lipid content of fish by TBA reaction. The preparation of a calibration curve for each assay might be recommended.

In the next step, in order to confirm the accuracy of the procedure, a comparative evaluation for the determination of fish lipid content was made between the conventional procedure and the recommended TBA procedures. The total lipid contents of commercially available fish meat, i.e., chub mackerel (*S. japonicus*) [$n = 21$, body length (mean ± SD) 29.76 ± 1.95 cm, body weight (mean ± SD) 497.7 ± 32.5 g, 28 October 2008–21 January 2009] and saury (*C. saira*) [$n = 14$, body length (mean ± SD) 29.43 ± 1.28 cm, body weight (mean ± SD) 110.1 ± 8.7 g, 11 November 2008–26 December 2008] were evaluated using the new method. Figure 8 shows typical standard calibration graphs prepared by the plotting absorbance measurements at 532 nm for various concentrations of standard fish meat oils (chub mackerel and saury). The calibration curves were linear throughout the concentration ranges as shown in Fig. 8 ($R^2 = 0.9885$ and 0.9994, $p < 0.01$ and 0.01). Using the standard procedure of the TBA method mentioned above, lipid contents in meats of

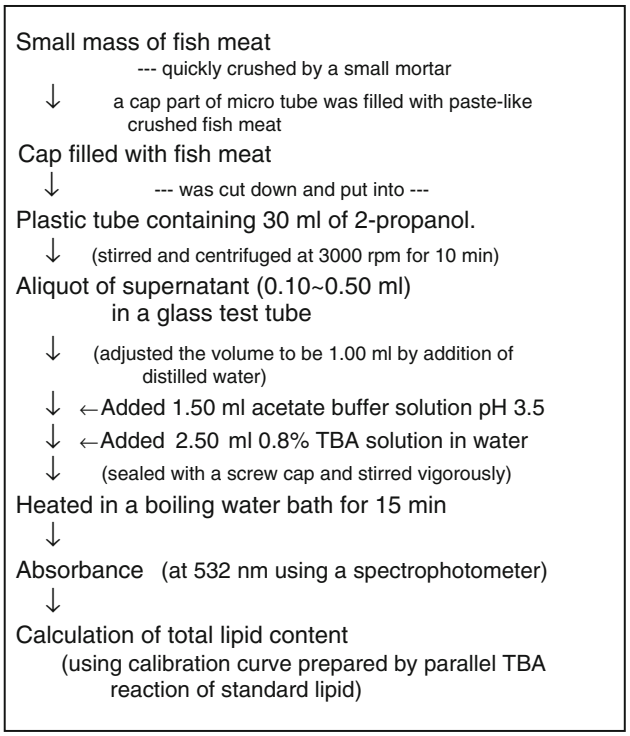

Fig. 7 Flow chart of assay procedure for lipid content of fish meat using the TBA reaction

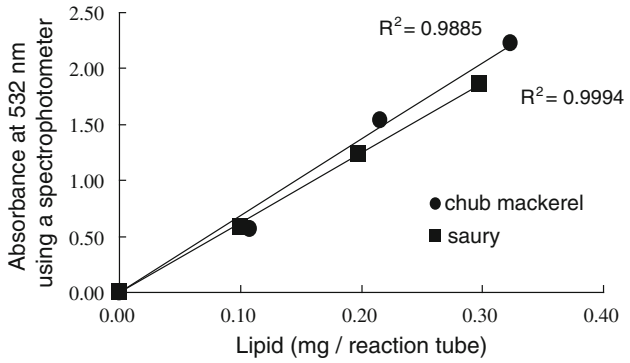

Fig. 8 Calibration curves for the determination of the total lipid content of fish prepared by TBA reactions of their standard lipids. The known amounts of standard lipids (0–0.30 mg/tube) of fish (chub mackerel and saury) were incubated with TBA as described in "Materials and Methods". The resulting reaction product was measured by absorbance at 532 nm using a spectrophotometer. Each result shows the duplication mean. The value of R^2 value shows the correlation coefficient. $R^2 = 0.9885$ ($p < 0.01$) for chub mackerel, $R^2 = 0.9994$ ($p < 0.01$) for saury

chub mackerel and saury were measured. As shown in Fig. 9, the levels of lipid content (%) in chub mackerel and saury meats were widely distributed across a broad range. The lipid content of the fish meats were found to be 0.5–20.3% for chub mackerel and 1.2–17.9% for saury. Furthermore, the results of the lipid content in chub mackerel and saury meats obtained by the conventional

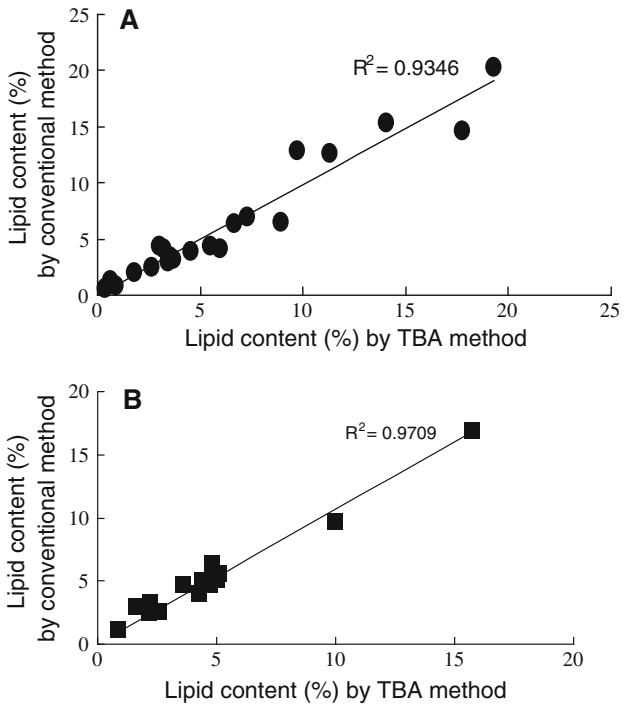

Fig. 9 Correlation between the conventional method and present TBA method for the determination of the lipid content of fish meat. The abscissa shows the lipid content of fish (**a**; chub mackerel, **b**; saury) measured by the conventional method described in "Materials and Methods". The ordinate shows the lipid content data of the same fish meat determined by the present TBA method. Each result shows the lipid content of individual fish which were purchased on separate days. The numbers for the determinate were 21 and 14 for chub mackerel and saury, respectively. The value of R^2 shows the correlation coefficient. $R^2 = 0.9346$ ($p < 0.01$) for chub mackerel, $R^2 = 0.9709$ ($p < 0.01$) for saury

method are also given in Fig. 9. The lipid content obtained by the two methods had almost the same values and showed very good correlations of $R^2 = 0.9346$ ($p < 0.01$) and 0.9709 ($p < 0.01$) for chub mackerel and saury, respectively. These results show the accuracy of the procedure and the possible future implementation of the TBA method instead of the conventional method.

In conclusion, a simple method using a TBA reaction for the determination of the total lipid content in fish meat has been designed. This method does not need precise weighing instrumentation or an apparatus for extraction and evaporation with hazardous organic solvents such as diethyl ether or chloroform. Since the present TBA method is simpler than the conventional method, it might be suitable for routine analysis under non-laboratory conditions such as in processing and market places as well as in laboratories of course.

Acknowledgments We would like to thank Ms. Makoto Ueno, Ms. Aya Akatsuka, Mr. Tomohide Matsuda and Mr. Kazuyuki Ishikawa for their technical assistance. This study was in part

supported by the Aid for the Young Scientist's Research (2004–2006) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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