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Extraction of Phospholipid-Containing Feed Oil by Propylalcohol

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The traditional propylalcohol extraction procedure was re-examined from the view point of marine phospholipid recovery.

Thirty sardines were wholly minced and subjected to the 1-propanol extraction, 2-propanol extraction, and chloroform/methanol extraction as a control. A portion of minced material was freeze dried and it was also subjected to the 1-propanol extraction.

Lipids extracted by propylalcohols contained some amounts of non-lipid contaminants in the lipid extract, though they were easily removed by centrifuge (1500×g) from the extract.

1-Propanol extraction from the wet material was considered to be the most promising way to extract phospholipid among the commonly used nontoxic solvents, and it amounted to 70% of the chloroform/methanol extraction. Phosphatidylcholine was the most prominent component among the phospholipid classes in all the extracts.

Comparing the quality of the extracts, just a slight difference was observed in the molecular species composition of the main lipid classes, *i.e.* the phosphatidylcholine and the triglyceride. Among the molecular species of phosphatidylcholine of the extracts, (16: 0, 22: 6) was the most prominent combination, followed by (16: 0, 20: 5).

The molecular species of triglyceride in the extracts were so complicated that it was hard to point out the most prominent combination.

The great thriving power of phosphatidylcholine (PC) that possesses the $\omega 3$ fatty acids in β position in its molecule had been born out by the larval fish and larval crustacea feeding tests.[1,2] In the field of medical science, cell differentiation inducting activity of this type of PC has come to notice,*2 and it is expected to be utilized as a medication for cancer (e.g. leucosis).[3]

Brockerhoff *et al.*,[4] Menzel and Olcott[5] originally observed that PC from cod *Gadus callarias*, scallop *Platopectin magellariccus*, lobster *Homarus americanus*, salmon *Oncorhynchus tshawytscha*, menhaden *Brevoortia tyrannus* and tuna *Thunnus alalunga* muscles are usually rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at the β position in its molecule. Takahashi *et al.*[6] also speculated about the binding position of highly unsaturated fatty acid in PC obtained from chum salmon *Oncorhynchus keta*, sardine *Sardinops melanosticta*, mackerel *Scomber japonicus*, big-eyed tuna *Parathunnus obeus*, brown sole *Limanda herzensteini*, sand flounder *Limanda punctatissima*, rock fish *Sebastes schlegeli*, Alaska pollack *Theraga chalcogramma*, blue shark *Prionace grauca*, mackerel shark *Lamna cornubica*, carp *Cyprinus carpio* and rainbow trout *Salmo gairdnerii irideus* muscles, and considered that those are mostly rich in β position in the PC molecule. From these observations, PC from marine sources might be considered as a valuable dietary source that could be supplied constantly throughout the year as for the feed oil of larval fish and larval crustacea. And may be utilized for the medication for cancer.

The barriers in obtaining phospholipid from marine sources are considered to be as follows; 1. Cooking/press process: Deterioration occurs and very poor in phospholipid recovery; 2. *n*-Hexane extraction procedure: Poor in phospholipid recovery; 3. Ethanol or methanol extraction procedure: Considerable amount of water soluble impurities appear, and those are hard to remove from the lipid extract; 4. *n*-Hexane/methanol extraction procedure: Undesirable phase separation occurs and makes hard to recover the clean lipids; 5. Chloroform/methanol extraction procedure: Though this is the most effective way of recovering phospholipids, even a trace amount of remaining chloroform changes into toxic compound. Or chloroform itself is carcinogenic.

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Until the early 1970s', 2-propanol was one of the most popular solvent in producing fish protein concentrate (FPC), although the problem in using this solvent was to remove the residual 2-propanol at the level of less than 250 ppm (FDA standard) without using steam.[7] Because of the high energy consumption and high cost of vacuum drying facilities, and also with the decrease of the demand of FPC, this 2-propanol extraction procedure went out of use in the 1980s'. Just recently, vacuum drying process has become one of the most expected drying process due to the advances in energy saving technology.

We have re-examined the traditional propylalcohol extraction procedure from the view point of marine phospholipid recovery, since propylalcohol is considered to be more hydrophobic than ethanol, and therefore, it is expected to recover more lipids and less water soluble contaminants. Expense of vacuum drying and solvent recovering processes are no longer a restrictive factor in obtaining valuable marine PC.

Materials and Methods

Extraction of Lipids

Thirty sardines *Sardinops melanosticta*, averaging 48.7 g, were captured using set net that has a mesh size of 20 mm, at the coast of Kamiiso-cho, Hokkaido in November, 1987. The whole sardine bodies were minced, and subjected to the following extraction procedures:

Procedure 1. Chloroform/methanol extraction (Control): The method of Bligh and Dyer[8] was modified as follows: To 100 g of minced whole body, 100 m*l* of chloroform and 200 m*l* of methanol were added and homogenized for two minutes at 5000 to 6000 rpm. Then, 100 m*l* of chloroform was added and homogenized for another thirty seconds. Finally, 100 m*l* of water was added and blended for further thirty seconds. The homogenate was filtered through a Toyo No. 2 filter paper (Toyo Roshi Kaisya, Ltd., Tokyo, Japan) using a Büchner funnel, and the residue was re-extracted with 100 m*l* of chloroform using a homogenizer for thirty seconds. All the glass wares, homogenizer cups and blades were rinsed with 30 m*l* of chloroform and was poured into the same (Toyo No. 2 filter paper) Büchner funnel. The combined filtrates were transferred into a separatory funnel and the filtrate container glass wares were also rinsed with 20 m*l* of chloroform. This solution was combined with the solution in the separatory funnel as well. The separatory funnel was kept at 5°C for over night. And then the lower layer appeared in this separatory funnel was filtered through a Toyo No. 3 filter paper that retained sodium sulfate anhydrous inside it. This filtrate was evaporated to give the constant weight, and the actual total lipid content of the sardine whole body was obtained.

Procedure 2. 1-Propanol extraction: To 100 g of minced whole body, 200 m*l* of 1-propanol was added and homogenized for one minute at 5000 to 6000 rpm. After suction filtration, the residue was re-extracted with 100 m*l* of 1-propanol. This re-extraction procedure was repeated for six times. All of the filtrates were combined, and evaporated to dryness so as to obtain the yield of the 1-propanol extraction.

Procedure 3. 2-Propanol extraction: All of the procedures were same with procedure 1 except that 2-propanol was used instead of 1-propanol.

Procedure 4. 1-Propanol extraction from dried material: 190 g of minced whole body was freeze dried by a freeze dryer (Model FD-5, Tokyo Rikakikai Co., Ltd.; 170×10^{-3} torr, never exceeding 20°C) up to the drying limit (it was 106 g). And to this dried material, 200 m*l* of 1-propanol was added. Subsequent treatments were exactly the same with above.

Lipid Composition Analysis

Lipid composition was determined by the TLC-Densitometric method (Kieselgel 60 for TLC, E. Merck, Darmstadt F. R. Germany; Cosmo Electro-Chrom Model F-808, Tokyo, Japan). The developing solvents used for TLC were *n*-hexane/diethyl ether/acetic acid (75: 25: 1, v/v) for the simple lipid composition analysis, and acetone/diethyl ether (4: 1, v/v) for over night, followed by chloroform/methanol/acetic acid/water (25: 15: 4: 2, v/v)[9] for the complexed lipid composition analysis. Phospholipid content was determined by multiplying 25 to the phosphorus content of the lipid which had been determined by the modified Fiske-Sabbarow method.[10]

Isolation of Triglyceride

Triglyceride (TG) was isolated by a preparative TLC (Kieselgel 60 for preparative TLC, 0.5 mm thickness, E. Merck, Darmstadt, F. R. Germany). The developing solvent used for this purpose was *n*-hexane/diethyl ether (7: 3, v/v). Bands on the TLC plate were visualized by 0.05% rhodamine 6G ethanol solution. And the TG band was scraped off, then extracted with diethyl ether, filtered

through a 0.45 μ type FP-45 fluoropore filter (Sumitomo Electric Ind., Ltd., Osaka, Japan) and subjected to HPLC for the molecular species analysis.

Molecular Species Analysis of Triglyceride

Molecular species of TG was analyzed by the following condition: Hitachi liquid chromatograph 638-50 (Hitachi Co., Ltd., Tokyo, Japan) was equipped with a Supersphere RP-18 reverse phase column packing cartridge that was inserted into LiChro CART® 250×4 mm column holder (E. Merck, Darmstadt, F. R. Germany). The sample loop was shortened to approximately 6–7 μl. The pipes that connect the injector, the column and the RI detector (Shodex SE-11, Showa Denko K. K., Tokyo, Japan) were shortened as possible to avoid solute dispersion. The mobile phase used was acetone/acetonitrile (1: 1 v/v) at a flow rate of 0.8 ml/min. Column temperature was ambient, though the whole chromatographic system was equipped at a room that has less fluctuation in temperature. Sharp peaks appeared on the HPLC chromatograms were collected at the top of the peaks manually from the out let of the RI detector. This was repeated for 4–5 times. And each collected fraction (nitrogen gas dried) was methyl esterified as follows by the method of Christopher & Glass as described by Prevot & Mordret[11] for the fatty acid composition analysis. Collected fractions were dissolved in 1 m*l* *n*-hexane, and 0.2 m*l* of methanolic 2N-NaOH solution was added. After shaking this mixture, it was stood for 20 seconds under 50°C and then 0.2 m*l* of methanolic 2N-HCl solution was added. The *n*-hexane layer was collected, then concentrated and subjected to GLC analysis. The analytical condition of GLC was as follows: instrument, Hitachi 063 gas chromatograph (Hitachi Co., Ltd., Tokyo, Japan); column, Unisole 3000 (Gasukuro Kogyo, Tokyo, Japan) 3 m×3 mm glass column; column temp., 220°C; injection temp., 270°C; detector, FID; carrier gas, N_2 40 ml/min.

By infering from the result of the fatty acid composition analysis of each peak, and by applying the RPI (relative retention potential index) theory presented in the previous paper,[12] the acyl combination (*i.e.* the molecular species) of each peak was determined.

Isolation of Phosphatidylcholine

Extracted lipids by propylalcohols were centrifuged (1500×g). And the transparent portion was subjected to silicic acid column chromatography which has been successfully done by Lands and Hart,[13] *i.e.* an aliquot amount of total lipid was dissolved into double volume of diethyl ether/ethanol (9: 1, v/v) and then applied to a silicic acid column (Mallinckrodt 100 mesh, Mallinckrodt Chemical Works, St. Louis, U.S.A.). The elutions were done through diethyl ether/ethanol (9: 1, v/v), diethyl ether/ethanol (1: 1, v/v), ethanol/methanol (9: 1, v/v) and finally by 100% methanol. The 100% methanol eluates were collected with a fraction collector, monitored by TLC (developing solvent: acetone/diethyl ether (4: 1, v/v) for over night followed by chloroform/methanol/acetic acid/water (25: 15: 4: 2, v/v)[9]), and PC fractions of more than 95% purity were collected.

Molecular Species Analysis of Phosphatidylcholine

Each isolated PC (purity>95%) was hydrolyzed with phospholipase C (*Clostridium perfringence*, P-L Biochemical Inc., Milwaukii, U.S.A.), according to the method of Renkonen.[14] It was done in the following manner. First, 50 to 100 mg of PC was dissolved into 10 to 15 m*l* of diethyl ether. Then, 10 to 15 m*l* of one molar Tris buffer (pH 7.3) including calcium chloride (0.25 mM) with 5 mg of phospholipase C was added to the diethyl ether solution. The head space gas of the container was filled with nitrogen gas and hydrolysis of PC was continued for four hours under room temperature (20–22°C). The hydrolyzate was washed several times with water and diglyceride was purified by a preparative TLC. The developing solvent used was *n*-hexane/diethyl ether (2: 3, v/v). Bands on the TLC plate were visualized by 0.05% rhodamine 6G ethanol solution. And the diglyceride band was scraped off, then extracted with diethyl ether. Acetylation of diglyceride was performed by adding an appropriate amount of acetic anhydride to the solution of diglyceride in pyridine, and by standing it for over night at room temperature.[15] Resulting acetyldiglyceride was purified by the method of preparative TLC by using the solvent system *n*-hexane/diethyl ether (7: 3, v/v). Bands on the plate were visualized by 0.05% rhodamine 6G ethanol solution. And the acetyldiglyceride band was scraped off, then extracted with diethyl ether. Finally, it was filtered through a 0.45 μ type FP-45 Fluoropore filter (Sumitomo Electric Industry, Ltd., Osaka, Japan) and subjected to HPLC. The acetyldiglycerides prepared as above were fractionated into major molecular species on the same HPLC system that were employed for the TG molecular species

analysis, except that the eluting solvent and the flow rate were 2-propanol/acetone/methanol/acetonitrile (10: 3: 30: 40, v/v) and 0.5 ml/min, respectively. Sharp peaks appeared on the HPLC chromatogram were collected and identified in the same manner as described in the above TG analysis description. The RPI calculation for the confirmation of individual molecular species was done according to the method of Takahashi and and Hirano.[16]

Peroxide Value and Acid Value Measurement

Peroxide value (POV) and acid value (AV) were determined according to the AOAC official method of analysis (fourteenth edition, method Nos. 28.025, 28.026 for the former and method Nos. 16.237, 16.238, 16.239 for the latter), though in case of POV, it was modified as follows: lipid amount, 30–100 mg; container, short neck flask (100 ml); chloroform/acetic acid volume, 5 ml; water volume, 15 ml; sodium thiosulfate concentration, 0.01 N; shaking during titration by nitrogen gas bubbling.

Proximate Composition Analysis

Moisture content was determined by heating the sample at 105°C for over night. Five per cent trichloroacetic acid (TCA) insoluble protein amount was determined by subtracting the 5% TCA soluble nitrogen amount from the total nitrogen content, and by using a conversion factor of 6.25. Total lipid content was determined by procedure 1 as described above.

Results and Discussion

The minced whole body of sardine examined in this study consisted of 63.2% of moisture, 13.4% of 5% TCA insoluble protein, and 17.9% of total lipid. Extracting lipids by propylalcohols resulted in some amount of non-lipid contaminants as shown in Fig. 1. 2-Propanol extract contained the largest amount of non-lipid compounds followed by 1-propanol extract and 1-propanol extract from the dried material. Though the amount of non-lipid material was relatively small in the extract of 1-propanol from dried material, AV was the highest among the four types of extracts (Table 1). In addition, orange coloration was observed, indicating a deterioration to some extent during the drying process. POV and AV of other extracts were also not so low even though the extracted lipids did not exhibit coloration. This was considered as an initiation stage of lipid oxidation owing to the relatively long period of solvent evaporation that was inevitable for obtaining the reliable lipid yield. Additionally, on the account of the adulteration of the internal organs that

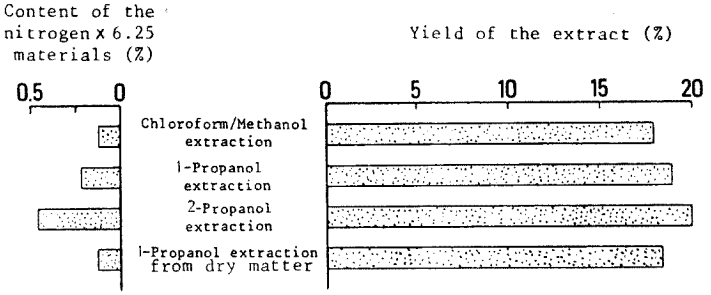

Fig. 1. Yields and contents of contaminants of chloroform/methanol extract, 1-propanol extract and 2-propanol extract of sardine whole body.
Contents of the impurities in the extracts are expressed as nitrogen amount in the extracts ×6.25.

Table 1. Peroxide value, acid value, and color of the chloroform/methanol extract, 1-propanol extract and 2-propanol extract of sardine whole body

Characteristics	Chloroform/ Methanol extract	1-Propanol extract	2-Propanol extract	Freeze-dried 1-Propanol extract
POV	2.2	5.0	6.1	5.2
AV	2.1 (1.6)	6.5 (3.5)	4.8 (2.9)	7.8 (5.8)
Color	Light yellow	Light yellow	Light yellow	Light orange

* Parentheses show the solvent acidity subtracted AV values.

are rich in hydrolytic enzymes, hydrolysis of lipids were unavoidable to some extent, resulting in a relatively high AV level. Adulteration of blood and fish skin that are rich in hem compound and lipoxygenase, respectively, might also facilitate lipid deterioration.[17] Acidity of the solvents used for the extraction of lipids was considered as one of the cause of high AV level. The parentheses in Table 1 show the solvent acidity subtracted AV values. These values are still over the desirable restrictive AV level ($AV<1$).[18] So treatment using NaOH or Na_2CO_3 might be an inevitable process. Though it is very hard to avoid lipid hydrolysis in the minced whole body of sardine, it might be feasible to prevent lipid oxidation to some extent by filling up the processing system with nitrogen gas.

It has been considered that phospholipid is an undesirable compound, since it hampers the subsequent refining process when the fish oil is obtained by the conventional way (e.g. cooking/press process). So it has been removed at the deguming process with other impurities.[19] As aforementioned, PC from marine sources has become notice as a valuable source as an effective feed oil and as a medication for cancer. Therefore, it is a sheer waste to discard PC in the fish oil as a "gum". PC does not hamper the refining process when the fish oil is extracted by propylalcohol (see Fig. 2).

Power[20] investigated the 2-propanol treatment process for producing high quality FPC. In his recommended process, hot 2-propanol (around 70–80°C) was used. This heat treatment process saves the consumption of the extractant

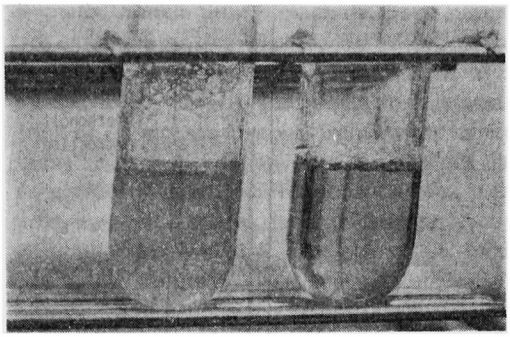

Fig. 2. Removal of impurities in the 1-propanol extract of sardine whole body by centrifuge ($1500\times g$).
Left: before centrifuge; Right: after centrifuge.

in a great deal. But it has to be pointed out that heat will damage the $\omega 3$ fatty acid containing lipids. From the view point of recovering undamaged PC, the extractant should always be kept cold (ambient), though in this case, as shown in this study, at least two to three times of extractant, compared with the traditional procedure, is required for the good recovery of PC. In the days of Powers',[20] high consumption of extractant was a decisive demerit. But today, owing to the advances in solvent recovering technology, and because of the revaluation of the $\omega 3$ fatty acid containing PC, large volume of extractant is no more a serious demerit.

The crucial points of the propylalcohol extraction were observed as follows: 1. Non-lipid materials that appeared as a turbid impurities in the solvent free lipid fraction was easily removed

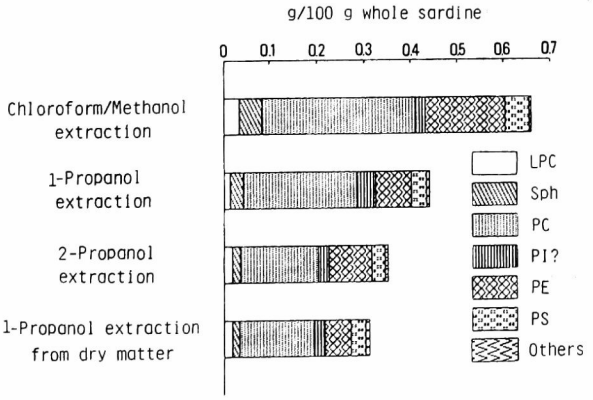

Fig. 3. Recovery of phospholipid classes in chloroform/methanol extract, 1-propanol extract and 2-propanol extract of sardine whole body.
PC: Phosphatidylcholin; PE: Phosphatidylethanolamine; PS: Phosphatidylserine; PI: Phosphatidylinositol; Sph: Sphingolipid; LPC: Lysophosphatidylcholine.

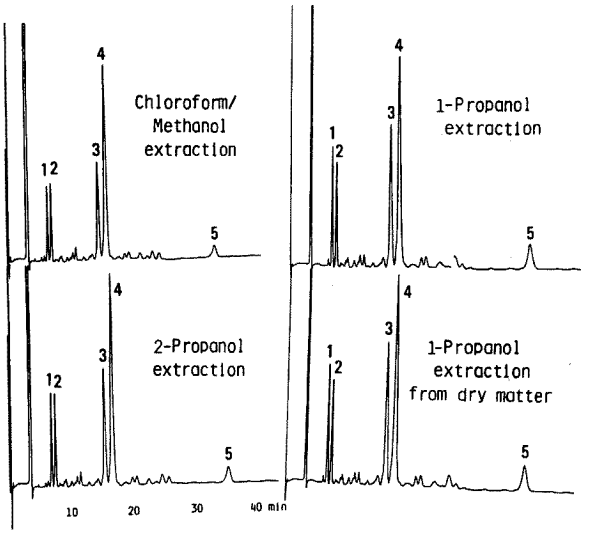

Fig. 4. Reverse phase high performance liquid chromatography of acetyldiglycerides derived from phosphatidylcholines which were obtained from chloroform/methanol extract, 1-propanol extract and 2-propanol extract of sardine whole body.
Chromatographic condition; column, Supersphere RP-18 3 μm, 250 × 4 mm; solvent, 2-propanol/acetone/methanol/acetonitrile (10: 3: 30: 40, v/v); flow, 0.5 ml/min; column temp., ambient; Detector, Shodex SE-11 RI monitor.
Numbered peaks are: 1. (20: 5, 22: 6), 2. (22: 6, 22: 6), 3. (16: 0, 20: 5), 4. (16: 0, 22: 6), 5. (16: 0, 18: 1).

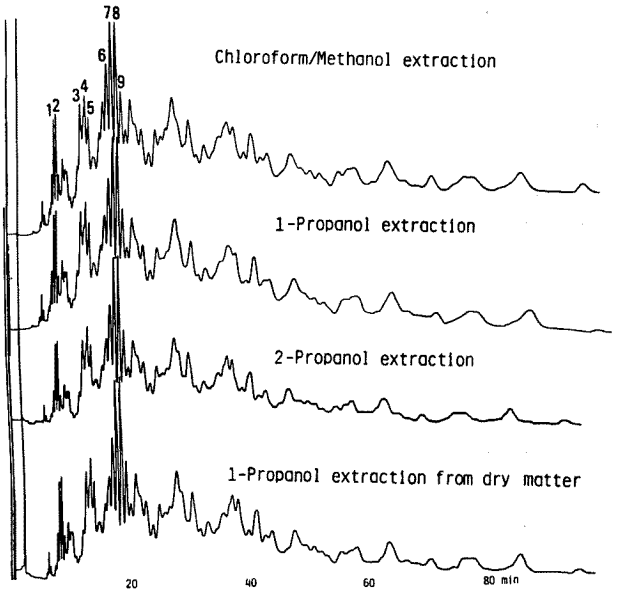

Fig. 5. Reverse phase high performance liquid chromatography of triglycerides obtained from chloroform/methanol extract, 1-propanol extract and 2-propanol extract of sardine whole body. Chromatographic conditions are the same with the legend of Fig. 4 except that the solvent system was acetone/acetonitrile (1: 1, v/v), and the flow rate was 0.8 ml/min.
Each numbered peak represents: 1. (20: 5, 20: 5, 20: 5), 2. (20: 5, 20: 5, 22: 6), 3. (19: 2, 20: 5, 20: 5), 4. (16: 1, 20: 5, 22: 6), 5. (14: 0, 20: 5, 22: 6) and (16: 1, 20: 5, 22: 6), 6. (18: 1, 20: 5, 22: 6), 7. (16: 0, 20: 5, 20: 5), 8. (16: 0, 20: 5, 22: 6), 9. (16: 1, 16: 1, 22: 6).

by centrifuge (1500×g) as shown in Fig. 2. 2. Phospholipid was recovered effectively. Specifically, 1-propanol extraction from the wet material was the most promising way in extracting phospholipid among the three propylalcohol extraction procedures as illustrated in Fig. 3. From the results shown in Fig. 3, we can considered that from one kg of whole sardine body (when total lipid content is around 18%), it is possible to obtain four to five grams of phospholipid by the 1-propanol extraction procedure from the wet material that accounts for 70% of the phospholipid amount that is actually contained in the whole sardine body. Among the phospholipid classes, PC was the most dominantly found lipid class. And no outstanding difference in phospholipid composition among the four types of extractions was observed (Fig. 3). Fig. 4 shows the elution pattern of PC molecular species by reverse phase HPLC. Though it is well expected, even at the PC molecular species level, there is no principal difference in quality among the four different ways of extractions. The dominantly found molecular species were (16: 0, 22: 6), (16: 0, 20: 5), (20: 5, 22: 6), (22: 6, 22: 6) and (16: 0, 18: 1). Compared with the chloroform/methanol extraction, extractions by propylalcohols are, if anything, relatively low in the recovery of (22: 6, 22: 6) species, while (16: 0, 18: 1) species exhibited a slightly better recovery. Fig. 5 shows the HPLC elution pattern of TG molecular species. The chromatographic patterns were very similar to each other all the more as well expected. There is not even a noticeable difference even at the TG molecular species level among the four different types of extraction procedures. Unfortunately, the resolution of HPLC was still not good enough to point out the most prominent TG molecular species contained. And it was hard to determine the accurate composition of them. The numbered sharp peaks were presumed as it is shown in the figure legend, though each peak contained 20–40% of other acyl combinations. Recently, Shukla *et al.** made the 2 μm particle size column packing feasible for HPLC. This type of HPLC column packing is expected to be utilized to particularly good effect for the separation of TG molecular species from marine sources in the near future. At any rate, it is obvious that no significant qualitative changes occur even at the lipid molecular species level during the propylalcohol extraction procedure. And we might conclude that though treatment of foods using propylalcohol is not permitted in Japan, it can be expected to be used as an effective extractant for obtaining the ω3 fatty acid rich phospholipid as a dietary source that could be supplied constantly throughout the year for larval fish and larval crustacea.

Appendix

Table 2 shows an estimate for phospholipid-containing feed oil production. Scaling up by two and a half times of the processing scale from 20 t/day to 50 t/day saves 31 to 32% of the expences.

Suppose that the plant will run for 10 years, the production cost will be 223 yen/g for the 20 t/day processing scale, and 136 yen/g for the 50 t/day processing scale. And by disregarding the expences for the facillity, the production cost will be 187 yen/g for the 20 t/day processing scale, and 115 yen/g for the 50 t/day processing scale.

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Table 2. An estimate for phospholipid-containing feed oil production

Processing scale of sardine	20 t/day	50 t/day
Operating days	264 days/year	
Whole amount of sardine per year	5,280 t	13,200 t
Facility	300,000,000 yen	510,000,000 yen
Energy and propylalcohol consumption	157,876,000 yen	272,748,000 yen
Yield of the feed oil	845 t	2,376 t

* Under publication in a chromatographic journal.

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