A Comparison of the Effects of Various Purification Treatments on the Oxidative Stability of Squid Visceral Oil

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ABSTRACT: Factors influencing the oxidative stability of refined squid visceral oil after various purification treatments were evaluated. Squid visceral oil was treated by refining, steam deodorizing, molecular distillation, Sep-Pak cartridge extraction, alumina adsorption, EDTA solution washing, and column chromatography, and the treated oils were compared for oxidative stability. Sep-Pak cartridge extraction and alumina adsorption were designed to eliminate phenolic compounds, α-tocopherol, phospholipids, and metal ions from the oil. The oxidative stability was measured using PV and increase in weight. The refined oil and the deodorized oil were the most stable, and the highly purified oil was the least stable. However, alumina-adsorbed oil that had been washed with EDTA was more resistant to oxidation than highly purified oil. When refined oil was passed through an activated carbon–Celite chromatography column, it could be separated into hexane, ether, and ethanol fractions. The ethanol eluate contained more α-tocopherol and phospholipids than the ether eluate. The addition of the ethanol eluate extracted from squid visceral oil to the highly purified oil resulted in excellent stability.

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KEY WORDS: Molecular distillation, oxidative stability, refining, squid visceral oil, steam deodorizing.

Squid visceral oil contains considerable amounts of PUFA such as EPA and DHA, and it could be utilized as a food supplement (1). However, it is also highly susceptible to oxidation because of the polyunsaturated nature of the FA. Autoxidation is one of the most important reactions that can occur in fish oil. Oxidation is related to toxicity and is an important factor with respect to human consumption of this type of product (2). Oxidative stability is one of the most critical aspects for the long-term storage quality of oil. Fish oil is made predominantly of TG; however, other minor components also present in fish oil are of great importance when considering oxidative stability (2).

Each purification process or treatment has a specific function and removes certain components, which can act as prooxidants or antioxidants (3–6). A deodorization or distillation processing step removes smaller and odorous organic compounds (6–8). High-vacuum distillation for deodorization reduces the PV of the fish oil (7). Ferrari *et al*. (8) showed that the level of total tocopherols decreased after deodorization. Boki *et al*. (9) described how hydroperoxides, aldehydes, and ketones were adsorbed on bleaching media, such as aluminum oxide, activated clay, activated carbons, and other commercial adsorbents. Investigation of the oxidative stability of soybean oil at the various stages of refining has shown that crude oil is the most stable and that highly purified soybean TG is the least stable (5,10).

Squid visceral oil is used as an animal feed ingredient in Taiwan. It contains many unsaponifiable compounds (1); therefore, the oil needs further refining to be suitable for use as a healthful food. Refined squid visceral oils are reportedly more resistant to oxidation than other fish oils because of their unsaponifiable component (1). However, the oxidative stability of squid visceral oils may also be influenced by minor constituents that are naturally present or through contamination during the refining processing. This study investigated the effects of different purification treatments, such as deodorization, molecular distillation, adsorption, solid phase extraction, and filtration, on the oxidative stability of squid visceral oil. Residual phenolics, α-tocopherol, phospholipids, and iron and copper ions were determined. This research also sought to clarify the relationship between the amount of oxidation-related components present and the oxidative stability of squid visceral oil by monitoring the PV and weight increase.

EXPERIMENTAL PROCEDURES

Samples. Crude squid visceral oils were obtained from the heat-rendering processing lines at the Ho-I factory (Kaoshiang, Taiwan). Samples were taken before and after each treatment and stored under nitrogen in a freezer until used. There were eight oils from different treatments of the crude oil: refined oil (**R** oil), deodorized oil (**D** oil), molecular-distilled oil (**MD** oil), alumina-adsorbed oil (**A** oil), Sep-Pak–extracted oil (**S** oil), alumina-adsorbed and Sep-Pak–extracted oil (**AS** oil), alumina-adsorbed and EDTA-washed oil (**AE** oil), and chromatographically purified oil (**CP** oil).

R oil is the refined and bleached oil described previously (1). **R** oil was purified further still to give **D** oil, **MD** oil, **A** oil, **S** oil, and **CP** oil. **A** oil was further purified to give **AS** and **AE** oil. Deodorization at 3–5.25 mbar was performed over various temperatures, and the deodorization intervals ranged from 0.5 (at 210°C) to 2 h (at 100°C). A modified laboratory-scale batch-type steam deodorizer similar to that described by Zehnder (11) and Heide-Jensen (12) was used. Molecular distillation provides effective short-path distilla-

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tion to remove volatile compounds. The short-path distillation system was produced by UIC Inc. (Type KDL-4; Alzenau, Germany). Operation conditions were: feed rate 10 mL/min, pressure 1–3 × 10^{-3} mbar, temperature 185°C, rolling rate 900/min. **A** oil was prepared by dry elution using column chromatography on alumina (neutral aluminum oxide; Merck, Darmstadt, Germany) as described by Yoshida *et al*. (13). To produce **AS** oil, a 10-g portion of the **A** oil was diluted with 50 mL hexane, then purified by passage through two continuous Florisil-type Sep-Pak cartridges (Waters Associates, Milford, MA). **AE** oil was prepared from **A** oil by washing six times with a 0.5 M aqueous solution of EDTA.

Oil fractionation. Refined squid visceral oil was fractionated by liquid chromatography over a column (60 × 4 cm) packed with 100 g of a 1:1 mixture of activated carbon (Sigma Chemical Co., St. Louis, MO) and Celite (Fluka Chemical Corp., Madrid, Spain). Thirty grams of **R** oil diluted in *n*-hexane was applied to the column, and elution was carried out using 1 L of *n*-hexane, then 500 mL of ether, and finally 400 mL of ethanol. The *n*-hexane eluate was designated **CP** oil. The total recovery of all three eluates was about 55.16%. The purity of the different fractions was evaluated by TLC-FID (Iatroscan TH-10; Iatron Co., Tokyo, Japan). The samples were then developed with hexane/ether/formic acid (85:15:0.1, by vol) on the rod (Chromarod S-II, Iatron Co.) in a closed TLC chamber. The chromatographed components were then quantitated using an FID.

Determination of total phenolics. A dilution of 1 mL Folin–Ciocalteu phenol reagent (Merck) made with 2 mL distilled water was mixed with the sample. After 30 min, the absorbance was read at 765 nm, using gallic acid as a standard for the calibration curve (14).

Determination of tocopherol. The free form of tocopherol was determined by analyzing the oil directly. Total tocopherol was determined by analyzing the oil after saponification. Then 100 mg of accurately weighed oil was placed in a 20 mL vial. To this was added 10 mL of 3% pyrogallol ethanolic solution and 1 mL 60% (wt/vol) sodium hydroxide solution. The contents were heated to 70°C for 30 min. The mixture was cooled to ~50°C, extracted using hexane, and the extract was evaporated to dryness under a stream of nitrogen. Tocopherols were analyzed by normal-phase HPLC and fluorimetric detection as follows: An HPLC pump (type 400; Applied Biosystems Inc., San Jose, CA), a 20-µL sample loop injector (type 7125; Rheodyne, Berkeley, CA), and a fluorescence detector (type 470; Waters) were used. The HPLC column was a Supelcosil LC-Si, 5 cm length, 4.6 mm i.d., particle size 5 µm (Supelco Inc., Bellefonte, PA). Elution was carried out using an *n*-hexane/isopropanol mixture (99.5:0.5, vol/vol). The detector wavelengths were 298 nm for excitation and 325 nm for emission. The internal standard was 2,2,5,7,8-pentamethyl-6-hydroxychroman.

Determination of phospholipids. Phospholipids were analyzed using TLC-FID as described in the *Oil fractionation* section above. Cholesterol acetate was used as an internal standard.

Determination of iron and copper. Each 1 g sample was pre-reacted with 1.5 mL of 18 M H_2SO_4 for 15 min, followed by two 2-mL additions of 16 M HNO_3 at 10 min intervals. The samples were then directly analyzed by atomic adsorption spectrometry (Shimadzu-6501; Shimadzu, Kyoto, Japan) as described by Martin-Polvillo *et al*. (15).

Oxidation experiment. One-milliliter portions were placed in 20 mL uncapped sample vials (i.d. 17.5 × 54 mm) and oxidized in the dark in a 37°C oven. Ether and ethanol eluate were added to the chemically pure oil at the original concentration, and the solvents were removed using nitrogen. Samples were weighed, then subsamples were withdrawn for duplicate PV determination. The ferric thiocyanate method (16) was used to determine the PV.

Statistical analyses. Results were reported as an average of two independent measurements. Each treatment was analyzed by one-way ANOVA. Duncan's multiple range test was applied to determine significance of differences between means.

RESULTS AND DISCUSSION

It has been reported that the type and amount of minor components in soybean oils vary with the operational conditions of the treatment (4). The quantities of total phenolic compounds, α-tocopherol, phospholipids, iron, and copper present in squid visceral oil after different treatments are shown in Table 1. Analyses of the seven types of squid visceral oils in this study showed that the total phenolic compounds ranged from 0 to 1.23 meq gallic acid/g. Molecular distillation removed almost 28% of phenolics from refined oil ($P < 0.05$). Refined squid visceral oil contained about 255 µg/g of α-tocopherol naturally, but contained virtually no β-, γ-, or δ-tocopherol (1). Most of the α-tocopherol was in the free form (81.5%) in squid visceral oil. The phospholipid concentration in refined squid visceral oil was 1.54 mg/g, and iron and copper contents were 38.03 and 8.67 µg/g, respectively.

Ferrari *et al*. (8) reported that the tocopherol content was markedly reduced during the deodorization process because

TABLE 1
Concentration of Total Phenolics, α-Tocopherol, Phospholipids, Iron, and Copper in Oils Treated in Various Ways[a]

Visceral oil treatment[b]	Total phenolics (meq/g)	α-Tocopherol (µg/g)	Phospholipids (mg/g)	Iron (µg/g)	Copper (µg/g)
R[c]	1.23[a]	255.3[a]	1.54[a]	38.03[a]	8.67[a]
D	1.22[a]	183.5[b]	1.13[b]	30.53[b]	9.11[a]
MD	0.88[b]	135.7[c]	1.09[b]	33.86[b]	7.98[b]
S	0.98[b]	241.2[a]	1.08[b]	29.53[b]	8.26[b]
A	0.58[c]	20.9[d]	0.77[c]	22.75[c]	8.04[b]
AS	ND[d]	ND	0.39[d]	10.26[d]	6.35[c]
AE	0.41[d]	13.3[d]	0.74[c]	ND	0.11[d]

[a]Values within each column bearing different superscript roman letters differ significantly at $P < 0.05$.
[b]**R**, refined oil; **D**, deodorized oil; **MD**, molecular-distilled oil; **S**, Sep-Pak–extracted oil; **A**, alumina-adsorbed oil; **AS**, alumina–adsorbed and Sep-Pak–extracted oil; **AE**, alumina-adsorbed and EDTA-washed oil.
[c]**R** oil contained 208 µg/g free α-tocopherol.
[d]ND, not detected.

tocopherols are volatile and heat labile. This was confirmed by the loss of α-tocopherol in **D** and **MD** oils as well as phospholipids; however, the process reduced iron and copper contaminations only slightly. The level of α-tocopherol in **D** oil decreased by 28%. The reduction was about 47% in **MD** oil after molecular distillation at 185°C. The residual α-tocopherol might be in the form of a low volatile nonfree form of α-tocopherol. There was a significant reduction in the phospholipids level (around 29%) in **MD** oil, and apparently the phospholipids were stripped into the condensate bottle during the high-vacuum operation. This phenomenon also might be due to adherence of phospholipids on the glass distillation chamber or tubing during molecular distillation.

Alumina, used for chromatography, and Sep-Pak cartridges reportedly adsorb polar compounds (9). It should be noted that refined oil after alumina adsorption (**A** oil) showed a significant decrease in the concentration of total phenolics concentration as shown in Table 1 ($P < 0.05$). The free form of α-tocopherol was almost completely removed by dry elution alumina chromatography (data not shown). Purification by alumina chromatography was more effective than that achieved using the Florisil-type Sep-Pak cartridge. A similar phenomenon was observed for phospholipids. Boki *et al*. (9) reported that such a decrease is due to the strong adsorption of polar compounds to the alumina phase. The results suggested that polar compound adsorption on alumina was irreversible but that this interaction was lower in the Sep-Pak cartridge, as indicated by the partial release of polar compounds from Florisil-type Sep-Pak cartridge when eluted with hexane. Of all the types of oils, the maximal reduction in α-tocopherol and phospholipids was observed in **AS** oil. Although a subsequent alumina and Sep-Pak treatment removed phenolics, α-tocopherol, and phospholipids completely, the iron and copper in squid visceral oil responded differently. The presence of metals in oil is undesirable because the metals can increase oxidation and decrease shelf life. Table 1 clearly indicates that refining, deodorization, alumina adsorption, and Sep-Pak solid extraction do not remove iron and copper efficiently. Alumina adsorption and EDTA solution washing (**AE** oil), however, could cause a decrease in the ferrous and cupric ions effectively. This effective removal of metal in **AE** oil can be explained by the chelating ability of EDTA.

Oxidative stability is greatly influenced by the presence of phenolic compounds, tocopherols, phospholipids, and trace metal in oils (4). Oil oxidation was monitored by two methods. PV was used to estimate hydroperoxides (Fig. 1), and weight gained was used to measure oxidation (Fig. 2). On the basis of PV (Fig. 1), the oxidative stabilities of the various purified oils varied considerably. They could be divided into two groups. The first group included **R**, **D**, and **S** oils that had higher PV in the initial period (0–8 d) and gave a plateau after 10 d. The second group comprised **MD**, **A**, **AE**, and **AS** oils, which had lower initial PV values that then increased significantly. Hydroperoxides were the primary products that were further reacted with other oxidized compounds or decomposed in the chain reaction of oxidation. The results with **R**, **D**, and **S** oils suggested that the formation and disappearance of hydroperoxides might reach equilibrium after 10 d. The higher PV in the initial period of **R**, **D**, and **S** oils can be explained by the prooxidant effect of α-tocopherol. α-Tocopherol contributes more to biologically active vitamin E potency than other tocopherol isomers (17); however, it is unstable both during processing and in storage (8). It also has the lowest antioxidant activity among the isomers (17).

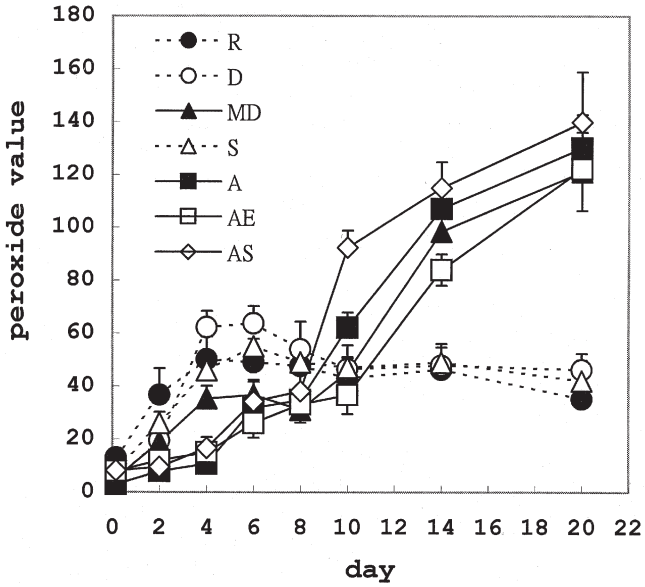

FIG. 1. Effect of various treatments on the PV of squid visceral oil after 37°C storage. **R** oil, refined oil; **D** oil, deodorized oil; **MD** oil, molecular-distilled oil; **A** oil, alumina-adsorbed oil; **S** oil, Sep-Pak–extracted oil; **AS** oil, alumina-adsorbed and Sep-Pak–extracted oil; **AE** oil, alumina-adsorbed and EDTA-washed oil. Data are expressed as mean ± SD ($n = 5$).

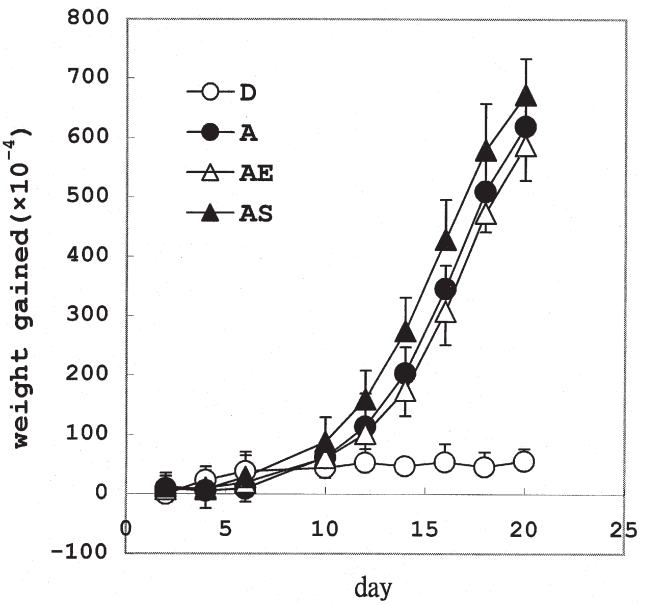

FIG. 2. Effect of various treatments on the weight gain of squid visceral oil after 37°C storage. See Figure 1 for abbreviations. Data are expressed as mean ± SD ($n = 5$).

α-Tocopherol was unable to inhibit oxidation (18) and behaved as a prooxidant at high concentrations based on the PV in squid visceral oil (1) and olive oil (2). When **R** oil, **D** oil, and **S** oil were compared for oxidative stability after 10 d, these oils showed outstanding stability in bulk storage. The stability was determined in a stress test at high surface area and at 37°C. Because of the prooxidative effect of α-tocopherol on the squid oil in the initial period, we suggest that antioxidant should be added back to purified oil when the oils are developed to produce an edible product for human consumption. The correlation statistics of the chemical components and the oxidative stabilities of the seven different squid visceral oils from various treatments are shown in Table 2. From the RSQ (square of correlation coefficient) values for PV, it is clear that a good correlation existed for α-tocopherol, less for total phenolics and phospholipids, and poor correlation for iron and copper. Thus, the oxidative stabilities of squid visceral oils were attributed mainly to the concentration of α-tocopherol, phenolics, and phospholipids. The greater oxidative stability of the **R**, **D,** and **S** oils can be explained by their higher content of natural antioxidants (α-tocopherol and phospholipids). Although the **MD**, **A**, **AE**, and **AS** oils had lower PV, these values increased significantly after an induction period of 10 d. Moreover, the deodorization and distillation processes were necessary for the removal of hydroperoxides, harmful components, and undesirable odorous volatiles from the oil to produce acceptable food grade oils with good stability (6,7).

Even though **A**, **AS,** and **AE** oil initially had a significantly lower PV than **R** or **D** oil, the hydroperoxide of high-purity oil increased quickly over 10 d of oxidation (Fig. 1). The oxidation results observed by PV showed that Sep-Pak cartridge extraction and alumina adsorption oil (**AS**) eliminated hydroperoxides initially. However, later the high-purity oil showed rapid oxidation. Our results for squid visceral oil are consistent with results for soybean oil (10) and vegetable oil (19).

The α-tocopherol content of oil has a significant impact on its oxidative stability. However, because of the hydroperoxide-induced effect of α-tocopherol in the initial period, we tried to use another method to investigate further the oxidative stabilities of oils that had lower contents of α-tocopherol. Oxygen was introduced into the oils during the oxidation study, and then the weight of the oxidized oils was measured. Weight gain determinations are different from PV, and the values of weight gained will increase continuously during the oxidation period. Figure 2 shows that **D** oil was more oxidatively stable than purified oils, and this can be attributed to the larger amount of tocopherol in this oil compared with **A**, **AS**, and **AE** oils. The results of weight gain (Fig. 2) agreed with the results from PV (Fig. 1). Figure 2 shows that oxidation of **AS** oil was the highest of all the oils tested throughout the process. **A** oil contained 0.58 meq/g phenolic compounds and 20.9 μg/g α-tocopherol and was found to be more stable than **AS** oil. Phospholipids are synergistic with tocopherol and other primary antioxidants (3). **AS** oil contained phospholipids at a concentration of 0.39 mg/g, but phospholipids cannot protect oil in the absence of α-tocopherol. **A** oil and **AS** oil were less stable than **AE** oil, which contained only traces of these natural antioxidants. The differences in oxidative stability between **A** oil and **AE** oil reflect the presence of metal ion catalysts, which were much lower in **A** and **AS** oils and lowest in **AE** oil. Thus, the greater oxidative stability of **AE** oil compared with **A** oil can be explained by its lower metal content. Evidently, the presence of metal ions reduces the antioxidant activity of the phenolic compounds and tocopherol. It also limits the synergistic effect of phospholipids naturally present in these oils.

R oil was fractionated into three parts—hexane, ether, and ethanol eluates—by using activated carbon–Celite column chromatography. The recoveries were 41.6, 13.3, and 0.29% for the hexane, ether, and ethanol eluates, respectively. The low recovery might be due to the adsorption of oil constituents in the column. The purified hexane eluate was designated **CP** oil and was monitored by TLC-FID (chromatogram not shown). Most of the impurities were removed, and TG was the main component in the **CP** oil. The total phenolics, α-tocopherol, and phospholipid contents of the three eluates were measured and are given in Table 3. Although there were traces of phenolic compounds in the **CP** oil, no significant antioxidant activity could be detected by TLC as described by Fujimoto *et al*. (20). Among the samples analyzed, the ethanol eluate had the highest level of α-tocopherol and phospholipids. Iron and copper were not detected in any of the three eluates. Phenolic compounds are of fundamental importance to the shelf life of oil (5). Phenolic compounds extracted from virgin olive oils increased the oxidative stability of refined, bleached, and deodorized olive oil (2). The ethanol

TABLE 2
Correlation Statistics of Total Phenolics, α-Tocopherol, Phospholipids, Iron, and Copper Relative to the PV Value at 20 d[a]

	Total phenolics	α-Tocopherol	Phospholipids	Iron	Copper
Intercept	147.01	139.30	188.66	143.51	141.35
Slope	–78.37	–0.40	–101.85	–2.23	–7.26
RSQ	0.70	0.86	0.65	0.42	0.23

[a]Calibration statistics data were from the **R** oil, **D** oil, **MD** oil, **S** oil, **A** oil, **AS** oil, and **AE** oil. Number of samples, $n = 7$. RSQ, square of correlation coefficient; see Table 1 for other abbreviations.

TABLE 3
Concentration of Total Phenolics, α-Tocopherol and Phospholipids in the Eluates from the Activated Carbon–Celite Column

	Recovery[a] (%)	Total phenolics (meq/g)	α-Tocopherol (μg/g)	Phospholipids (mg/g)
CP oil[b]	41.6	0.49	ND[c]	0.03
Ether eluate[d]	13.3	1.58	ND	3.81
Ethanol eluate	0.29	8.89	1054.3	63.29

[a]Recovery was calculated as dry weight of eluate/oil weight.
[b]CP oil was the hexane fraction of refined oil (R) from column chromatography.
[c]ND, not detected.
[d]Iron and copper were not detected in either eluate.

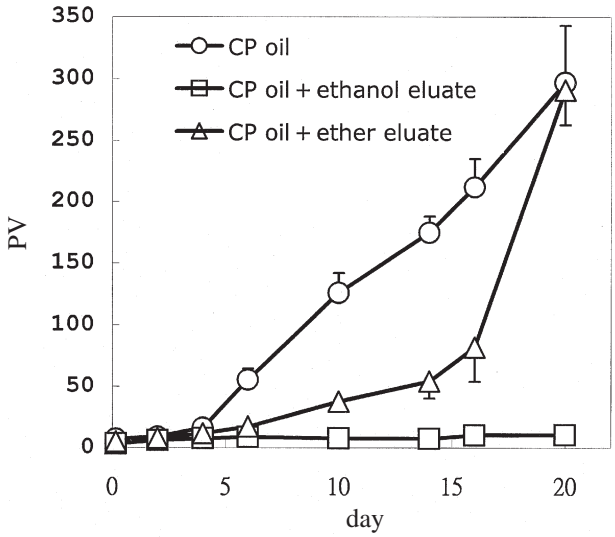

FIG. 3. Effect of ether and ethanol eluates on the PV of oil after 37°C storage. **CP** oil, hexane fraction of refined oil (**R**) from activated carbon–Celite chromatographic column. Data are expressed as mean ± SD (n = 5).

and ether eluates from refined squid visceral oil were added back to the **CP** oil separately at their original concentrations in **R** oil (0.5 and 24%, respectively). The hydroperoxide formation results showed that there was much greater antioxidant activity from the ethanol eluate than from the ether eluate (Fig. 3). Similar results were obtained when weight gain was measured (Fig. 4). Addition of the 0.5% ethanol eluate to **CP** oil resulted in no weight gain (i.e., no oxidation) over 20 d at 37°C. These results show that the antioxidants in the ethanol eluate are very important for the oxidative stability of squid visceral oil. This ethanol fraction is complex, and it is not yet clear which components contribute to the residual antioxidant activity besides the α-tocopherol and phospholipids. However, it is clear that because the **CP** oil is free of catalytic metal ions and other prooxidants in the ether fraction or adsorbed to the column, the introduction of antioxidants such as α-tocopherol and phospholipids found in the ethanol eluate resulted in increased oxidative stability.

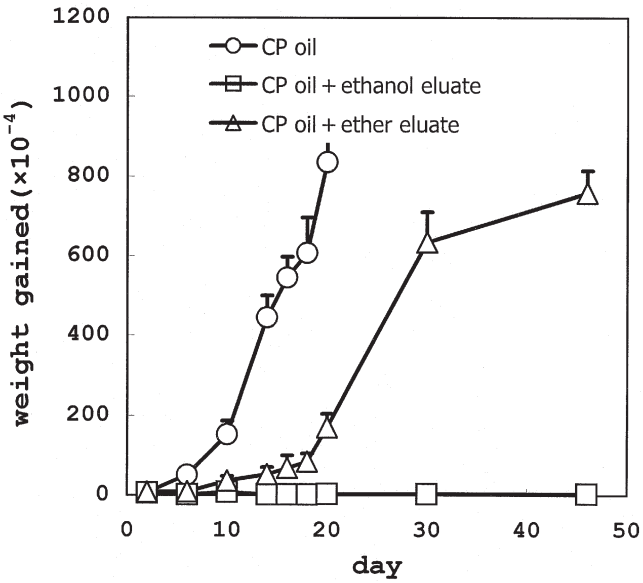

FIG. 4. Effect of ether and ethanol eluates on weight gain of oil after 37°C storage. See Figure 3 for abbreviation. Data are expressed as mean ± SD (n = 5).

It can be concluded that, over the oil purification process, there was a gradual decrease at each stage in the minor components of the oil. There was also a relationship between the oxidative stability of squid visceral oil and the presence of certain minor components, which acted as antioxidants (phenolic compounds, tocopherols, and phospholipids) or prooxidants (metal and hydroperoxide). To obtain the most oxidatively stable squid visceral oil, it is necessary to use an optimal combination of antioxidants while making sure the oil is free of prooxidants. We would therefore suggest that natural antioxidants be added back to highly purified squid visceral oil to develop a stable product.

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