Oxidative Deterioration of Flesh Lipids of Pacific Cod *(Gadus macrocephalus)*

W. T. ROUBAL, Food Science Pioneer Research Laboratory,
Bureau of Commercial Fisheries, Seattle, Washington

Abstract

Light flesh lipids of Pacific cod, composed chiefly of phosphatidyl ethanolamine and phosphatidyl choline, contain fatty acids rich in unsaturation. In model systems free of pro-oxidant the ethanolamine derivative exhibited a high rate of oxidation. Phosphatidyl choline, on the other hand, required an added pro-oxidant.

Cod porphyrins exhibited pro-oxidant effects not unlike those observed in other biological systems involving catalyzed lipid oxidation.

Neutral lipids exhibited very low rates of oxidation.

Introduction

Lipids of lean fish exist to a considerable extent as phospholipids (PL) intimately associated with muscle tissue. These lipids are generally less variable in composition than are the neutral lipids (NL) usually associated with fat depots and nonstructural functions.

Numerous studies have characterized the structural and metabolic roles of phospholipids (1–3). Other research indicates that they also play a role in the oxidative deterioration which arises in the processing and storage of food products (4,5). The close relation between these lipids and the cell provides reaction pathways whereby lipid peroxy free-radicals can directly attack remaining cellular constituents.

The object of this work was a) to provide information on the major lipids of the light flesh of Pacific true cod (*Gadus macrocephalus*), b) to measure their rates of oxidation in model systems, and c) to determine if cod porphyrins exhibit pro-oxidant properties.

Experimental

Pacific true cod caught in the Puget Sound area were chilled in ice and processed as soon as landed. A composite sample was prepared from several fish, utilizing light flesh only; no attempt was made to determine sex.

Lipid Extraction

Total lipid was extracted from 700 g of flesh by the procedure of Silk and DeKoning (6), but the acetone phase was retained. Water-soluble nonlipid impurities were removed by the diffusion technique of Folch et al. (7). Lipid contents were determined by evaporating aliquots in tared flasks under a stream of nitrogen.

Lipid-Class Separation

The lipid extract was evaporated under vacuum at 30–50C and dissolved in 50 ml of hexane. Then 450 milliliters of acetone were added, and the mixture was stored at −15C overnight in order to precipitate PL; the supernatant was decanted and saved. Phospholipid was dissolved in 10–15 ml of hexane and applied to a column packed with 60 g of Baker AR activated silica gel (Fig. 1). Hexane, pumped at the rate of 1 ml/min with a Milton Roy Mini pump, effected a rapid separation of PL from a small contamination of NL. Contaminants were then eluted with diethyl ether and retained for addition to the NL fraction. The acetone supernatant containing the bulk of the neutral lipids was evaporated under vacuum, applied to a fresh silica-gel column, and recovered in diethyl ether by eluting first with hexane.

Fractionation of Lipid Classes

The scheme of Hanahan et al., employing stepwise increases of chloroform in methanol, was used for PL fractionation (8). The column contained 60 g of activated silicic acid (Malinkrodt AR 100 mesh powder) slurried in $CHCl_3$:MeOH 7:1 v/v. Flow rate was maintained at 1 ml/min, collecting 8-ml fractions. Neutral lipids were fractionated on 60 g of Calbiochem Bio-Sil HA by employing a 2.5-cm I.D. conventional column. Flow rate was maintained at 1 ml/min with stepwise increases of diethyl ether in hexane according to Hirsch and Ahrens (9).

Thin-Layer Chromatography

Phospholipids were chromatographed on plates of Silica-Gel G according to the method of Skidmore and Entenman, using their solvent system No. 1 (10). A nonspecific sulfuric acid-dichromate spray was used for survey of spots; specific identifications were

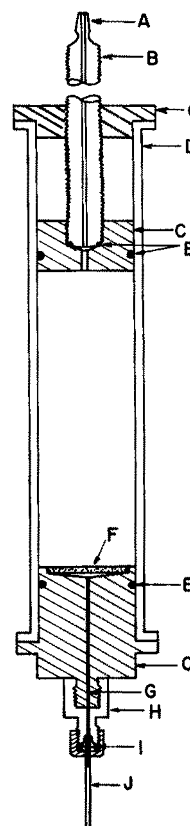

Fig. 1. Minimum dead-volume chromatographic column. *A*, machined luer connector; *B*, Teflon extension tube; *C*, Teflon; *D*, borosilicate glass column with tooled ends; *E*, fluorosilicic "O" rings; *F*, porous Teflon; *G*, No. 22 stainless steel hypodermic tubing; *H*, Crawford (Swagelok) connector No. 100-7-1, *I*, 1/16-in. nylon ferrules; and *J*, AWG No. 22 capillary Teflon tubing.

TABLE I

Neutral Lipids of Cod Flesh

Fraction	Identity	Percentage of neutral lipids
A	Paraffins and other hydrocarbons	24
B	Cholesterol esters	11
C	Unknown	~ 1
D	Triglycerides	28
E	Unknown	~ 1
F	Vitamin A esters plus some overlap fractions E and G	23
G	Pigments and other polar materials	12

made with selective spray reagents (10). Neutral lipids were chromatographed along with authentic materials on silica-gel G plates, using petroleum ether-diethyl ether-acetic acid 90:10:1 v/v/v. Hydroperoxides were detected by means of the starch iodide spray of Oette (11).

Chemical and Physical Studies

Phospholipids were assayed according to the procedure described by Hawk et al. (12). Uptake of oxygen by lipid samples was measured with a Warburg apparatus (Gilson Medical Electronics Model RWB-3). Phospholipids, based on 0.5 mg of PL phosphorus, or 10–15 mg of NL freed of solvent under a stream of nitrogen, were emulsified with 0.5 ml of pH 7, 0.05 M phosphate buffer, and 4 small glass beads in 20-ml Warburg flasks. In duplicate experiments stock buffer was made up 10^{-5} M in hemoglobin (Hb). All oxidations were conducted under oxygen at 37C.

Neutral lipids and PL were esterified according to the procedure of Gauglitz and Lehman (13), using 2–3 ml of sodium methoxide solution in test tubes. GLC analyses were conducted at 168C with a RESCO series 600 apparatus. Column packing consisted of 4% DEGS on Anachrom ABS 80–90 mesh; an argon detector was employed, and the carrier gas was maintained at 50 ml/min.

Results and Discussion

On a wet basis, extractable oils represented 1% of the samples used. Phospholipids represented 65% of the total lipid; remaining lipids belonged to the neutral lipid class. TLC of PL concentrates consistently gave five spots. Major phospholipids were represented by phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). Lesser phosphatides were represented by phosphatidic acid, phosphatidyl serine (PS), and sphingomyelin. A faintly positive Shiff test (PS, PC, and PE spots) indicated the presence of phosphatidal analogs. Phosphatidyl inositol was not detected. Cod PL was eluted from silicic acid in the same sequence as those from rat and beef liver as described by Hanahan et al. (8). In these initial studies the low content of PS, sphingomyelin, and phosphatidic acid prevented further characterization of these substances. Densitometric measurement of charred spots showed that PC, PE, and PS made up 60, 35, and ~4% of the total PL respectively.

The composition of neutral lipids is given in Table I. Bligh et al. have observed a similar lipid distribution in Atlantic cod (*Gadus morhua*). PC and PE also constitute the bulk of lipids present in this fish (14). The fatty acid composition of neutral, PC, and PE fractions from Pacific cod is presented in Table II. Analysis of the small amount of cholesterol esters was not attempted in the present study because of

TABLE II

Fatty Acid Composition of Cod Flesh Lipids

Fatty Acid Carbon No.: Double bonds	Phosphatidyl ethanolamine	Phosphatidyl choline	Triglycerides	Vitamin A esters
14:0	0.1	0.6	1.5	1.0
15:0	tr.[a]	0.2	0.2	0.2
16:0	6.9	18.4	15.0	18.5
16:1	0.7	12.0	5.0	1.0
17:0	1.1	0.6	4.7	1.0
17:1 + 16:2	0.4	0.3	0.2	0.5
18:0	3.8	0.7	3.3	10.0
18:1	11.3	9.7	19.5	1.0
18:2	0.8	0.7	3.5	3.6
18:3	0.5	0.5	0.4	tr.
18:4 – 20:1	2.6	0.7	8.8	tr.
Unknown	....	....	1.2	....
Unknown	....	....	2.1	....
Unknown	....	....	0.7	....
20:4ω6	1.5	1.6	7.2	tr.
20:4ω3	0.8	0.4	5.7	....
Unknown	. .	. . .	....	56.0[b]
20:5	20.6	21.5	6.8	1.0
Unknown	.	...	2.0	....
22:3	0.8	0.4	0.3	....
22:4	0.5	0.4	0.9	....
22:5	1.6	0.9	0.3	....
22:6	46.6	30.0	9.8	....

[a] tr., trace amount.
[b] Peak plus shoulder. Relative retention time of major peak does not correspond with those of 20:4ω3, 22:1, or 20:5.

the difficulty in obtaining workable samples. The data show that the fatty acid content is limited chiefly to phospholipids with lesser amounts in the triglyceride, vitamin A ester, and cholesterol ester fractions. The neutral lipid fraction was characterized by a moderately high content of palmitate and a moderate degree of unsaturation. Vitamin A esters consisted of the usual fatty acids through the C_{18} series; with one notable exception there was a complete lack of C_{20} and C_{22} fatty acids. By contrast, highly unsaturated fatty acids of the C_{20-22} series were particularly abundant in the phosphatide fractions.

In general, palmitate-to-stearate ratios appeared to be low for PE and high for PC. Shuster et al. have reported values of 0.3 and 5 for albacore tuna and 0.1 and 21 for yellowfin tuna PE and PC respectively (15). Similarly palmitate-to-stearate ratios for true cod PE and PC were 1.7 and 26.3 respectively. In another study, values for rock cod PE and PC were 1 and 11 respectively (16).

Data in Figure 2 indicate the rates of oxygen consumption for the lipids of cod flesh. The ready oxidizability of rechromatographed PE is at once apparent; this lipid oxidized rapidly both in the presence and the absence of hemoglobin. On the other hand, purified PC, free of hydroperoxides as shown by TLC, exhibited a long induction period. Only after a long time did uncatalyzed PC begin to show reactivity. The marked difference between PE and PC regarding pro-oxidant requirements is not understood; hemoglobin was added in order to simulate pro-oxidant conditions characteristic of many biological systems.

Mattsson and Swartling (17), utilizing lipids of milk, have attributed rapid oxidations in PL to fractions containing PE and PS. Of the individual PL they found PS to be most readily oxidized. Acosta et al. working with chicken likewise observed that it was PC which oxidized most rapidly during early stages but that this anomaly was caused by PS contamination (4). In the present work little PS eluted with PE; it appears that cod PE exhibits properties similar to the PS derived from other sources.

The ease with which PE undergoes oxidation suggests that this class of lipids is primarily the cause of the initial changes leading to rancidity in lean cod flesh. Lea observed that the PE of egg, spread

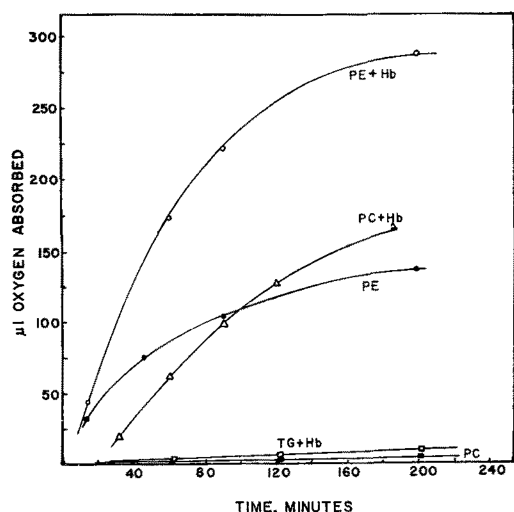

FIG. 2. The relation between lipid type and and ease of oxidation. Phosphatidyl ethanolamine + Hb-buffer (○); phosphatidyl ethanolamine + buffer only (●); phosphatidyl choline + Hb-buffer (△); phosphatidyl choline + buffer only (■); triglyceride + Hb-buffer (□).

in thin films at 37C, oxidized at a phenomenal rate; on the other hand, PC, which constitutes the bulk of egg phosphatide, oxidized at a much lower rate (18).

The curves shown in Figure 3 illustrate the particular lability of the 20:5, 22:4, and 22:6 fatty acids of cod PE. In these experiments hemoglobin was used at a concentration of 5 mg/flask. Data for 22:5 closely parallelled those for 22:4. That 20:5, 22:3, 22:4, and 22:5 underwent complete destruction whereas 22:6 approached a constant minimum value merits discussion. The lower initial content of all but 22:6 would, in the absence of other factors, explain their loss under actively oxidizing conditions. Similarly it might be expected that, although 22:6 might not undergo complete destruction within the 23-hr reaction period, it would not appear likely that a minimum plateau would be attained and maintained. This anomaly is explained as a consequence of the presence of protein within the reaction mixture. Studies have shown that actively oxidizing lipid-protein mixtures give rise to soluble as well as insoluble material, which comprises polymerized protein together with bound as well as trapped lipid (19,20). In the present investigation jellylike flakes of suspended material were observed early in the oxidation. Gelatinous materials continued to accumulate until the fifth hour, at which time the reaction mixture gelled to a stiff gummy mass. Had the insolubilization occurred in such a manner that the product remained suspended in buffer, the 22:6 would probably have been reduced to a low level through reaction with diffusable oxygen. It is evident that, although insolubilization was taking place, all but 22:6 were completely oxidized. The 22:6, initially present at a high level, was reduced to a low level, at which time it was occluded and removed from reaction.

During the 23-hr reaction period 18:1, 18:2, and saturated fatty acids remained fairly constant. Concurrently with the destruction of other fatty acids the content of 18:0 steadily increased; the content of 16:0 varied in an erratic fashion. Both 20:4ω6 and 20:4ω3 showed a gradual decrease with time. A small portion of the observed losses is attributed to complex formation between lipid and protein. Bound lipid is not easily recovered by conventional solvent extraction and escapes recovery.

Neutral lipids of cod were almost without reactivity

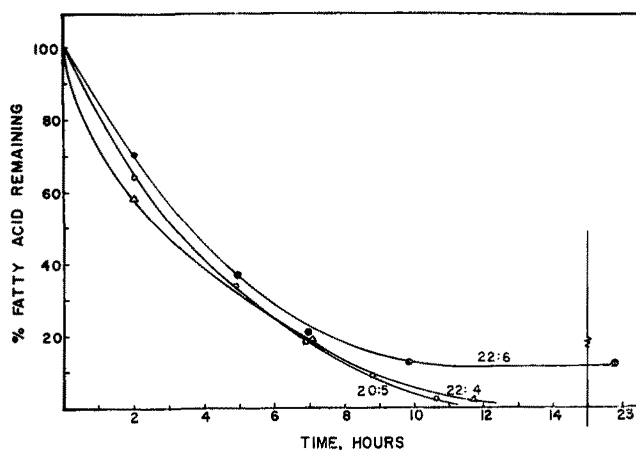

FIG. 3. Catalyzed oxidative destruction of the higher chain-length unsaturated fatty acids of cod phosphatidyl ethanolamine. 5 mg Hb/flask (0.5 ml buffer). 22:6 (●); 20:5 (○); 22:4 (△).

under the conditions employed. The triglyceride fraction with 10% 22:6 oxidized only slowly; vitamin A esters, with moderate unsaturation in the fatty acid portion, remained unreactive.

To determine to what extent endogenous porphyrin compounds of cod might enhance oxidation of PE, aqueous extracts of freeze-dried cod flesh were incorporated into the reaction media. The control consisted of PE in untreated buffer. Test mixtures consisted of an equal amount of PE in a 10% buffer extract at pH 8 with and without the addition of 10^{-4} M NaCN.

At the end of one hour the control had absorbed 310 μ $1O_2$; the test mixture less CN^- absorbed 524 μ $1O_2$. In contrast, the test mixture + CN^- absorbed 340 μ $1O_2$, a value approaching that of control. The marked increase in oxidation of PE + buffer extract less CN^- is attributed to extractable porphyrin compounds. Porphyrin compounds catalyze the cleavage of hydroperoxides and involve free-radical chain reactions; CN^- inhibits this process. The low content of cod porphyrins could very well enhance oxidative deterioration in stored cod, especially in processed foodstuffs that have undergone cellular disorganization and have been exposed to air.

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