LIPOXYGENASE ACTIVITY IN MENHADEN (BREVOORTIA TYRANNUS) AND ITS CONTRIBUTION TO OXIDATION OF OMEGA-3 POLYUNSATURATED FATTY ACIDS IN MENHADEN OIL

by

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(Abstract)

Menhaden is the major source of fish oil in the United States. Due to a high amount of polyunsaturated fatty acids which are highly susceptible to autoxidation, menhaden oil deteriorates rapidly, leading to objectionable off-odors and off-flavors. The purpose of this study was to investigate if the enzyme lipoxygenase is present in menhaden gill tissue and if it is a contributing factor in menhaden oil oxidation.

Peroxide, TBA and anisidine values of undeodorized and deodorized menhaden oils exhibited two maxima during 20 weeks of storage at 30°C. Peroxide values of the undeodorized oil peaked at week 1 with 6.71 meq/kg and at week 12 with 21.50 meq/kg, while in the deodorized oil it peaked at week 8 (9.28 meq/kg) and week 20 (18.71 meq/kg). TBA maxima were observed at week 2 (1416 μMol/kg) and week 12 (4951 μMol/kg) and at week 8 (1397 μMol/kg) and week 20 (4284 μMo/kg) for undeodorized and deodorized menhaden oil respectively. Anisidine values showed maxima at the same weeks. These results indicate that lipid peroxidation of the deodorized oil lagged a few weeks behind the undeodorized oil. In this study, the conjugated diene and fluorescence analyses were found to be poor indicators for monitoring lipid oxidation in menhaden oil.

Enzyme assays indicated that lipoxygenase activity is present in menhaden gill tissue with maximum activity at pH 9-10, resembling that of soybean lipoxygenase-1. A sensory panel judged omega-3 fatty acid ester concentrates treated with the enzyme extract as having a significantly ($p < 0.03$) stronger smell than the control ester for the first four weeks of an eight week study. However, no significant difference was found between the TBA values of the esters.

Of the 60 volatile compounds identified by GC-MS in the undeodorized menhaden

oil, 19 were aldehydes, 9 were alcohols and 8 were ketones. Volatiles that are potentially lipoxygenase derived, namely 2-octenal, 1-octen-3-ol, 2-nonenal, 2,6-nonadienal (E,Z), and 2,5-octadien-1-ol were among those identified in the undeodorized menhaden oil. The deodorized oil contained fewer total volatiles, and fewer aldehydes (6), ketones (1) and alcohols (8), but more long chain aliphatic compounds such as hydrocarbons, many of which were not possible to positively identify. No lipoxygenase derived volatiles were identified in the deodorized oil. Most of the volatiles in the omega-3 fatty acid ester concentrates were identified as esterified short chain fatty acids. No difference in the amount of total volatiles was found between four esters that were treated with and without the enzyme extract, a boiled enzyme extract and an enzyme extract that was inocculated with esculetin. However, in a repetition of just the control and the enzyme treated ester, a significantly ($p < 0.02$) higher amount of total volatiles was found in the enzyme treated ester, supporting the results of the sensory analysis. It was not possible to identify specific volatiles in the enzyme treated ester that were present in larger concentrations than in the other ester treatments. Volatiles identified in EPA and DHA ethyl esters were similar to those volatiles found in the undeodorized and deodorized menhaden oil as well as the omega-3 fatty acid ester concentrates, but no lipoxygenase derived volatiles were found.

While lipoxygenase activity was found in the gill extract of menhaden, and sensory analysis was able to distinguish between a control and an enzyme incubated oil, the enzymatic activity was low (apparent K_m = 16.7 mMol) and volatile analysis of various oils did not support the hypothesis that lipoxygenase is a major contributor to menhaden oil oxidation. Future research should include isolation and purification of menhaden gill lipoxygenase and the study of model systems to develop a better understanding of the contribution of lipoxygenase activity to oxidation of menhaden oil.

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1. Introduction

The world's harvest of fish, crustaceans and mollusks is approaching 100 million metric tons per year, of which approximately 30% is processed into fish meal and oil. About 1.44 million metric tons of fish oil were produced worldwide in 1990 (Bimbo and Crowther, 1992). In 1987, the U.S. produced 135.4 thousand metric tons of marine oil, of which almost 99% (133.8 thousand metric tons) was extracted from menhaden (FAO, 1989).

Fish and fish oil is of major interest to the health and research community because of mounting evidence for the health benefits of omega-3* fatty acids (Nettleton, 1990). More than 1500 scientific articles were published on omega-3 fatty acids between 1984 and 1989 (Nettleton, 1990). While nutritionists recommend an increase in or a partial substitution of meat dishes by fish, the high cost of fresh fish and the problem with spoilage during long transportation and storage as well as personal preferences, limit this option for many people (Barlow and Young, 1988; Rice, 1992).

With the approval of hydrogenated and partially hydrogenated menhaden oil as GRAS (U.S. Government, 1989) and the expectation that unhydrogenated menhaden oil will receive GRAS status in the near future (Bimbo and Crowther, 1992), sections of the fisheries industry are interested in developing menhaden oil into a major food ingredient in the U.S. A new market for menhaden products would greatly benefit the declining menhaden harvest and the fisheries industry in general. However, hydrogenated and partially hydrogenated menhaden oil have not yet found a market in the U.S. (Bimbo and Crowther, 1992), even though they are less expensive than many other commonly used oils (Bimbo, 1987). It is likely that food manufacturers would be interested in using fish oils in their products, if they could claim health benefits from the consumption of the oil. However, since the omega-3 fatty acids are lost upon hydrogenation of the oil this claim cannot be made, and since fish oil has the reputation of being a strong smelling oil, food manufacturers are probably not willing to experiment with this new food ingredient. The incorporation of refined unhydrogenated menhaden oil into other food products on the other hand still poses major problems with flavor stability due to the rapid oxidation of the

* omega-3 can be used interchangeably with n-3, both denote the first double bond being at the third carbon atom from the methyl end of the fatty acid;

omega-3 fatty acids (Schnepf et al., 1991; Park et al., 1989).

In order to be able to incorporate menhaden oil into foods, it is necessary to find ways to slow down the oxidative deterioration of the oil. The common approach of minimizing lipid oxidation in foods is finding an effective antioxidant system. This approach, however, appears to be limited for preserving omega-3 fatty acids in foods since no adequate antioxidant system has been found yet (Rice, 1992). Micro encapsulation is a method that has been used successfully for incorporating fish oil into foods without experiencing oxidative changes, but is limited due to high costs that are not expected to decrease in the near future (Rice, 1991). A third approach is to identify the initiators of oxidation and to develop means of controlling them.

Lipoxygenases, which are well known to cause oxidative deterioration of lipids in plant foods, were long thought to be of little importance in animal foods (Gardner, 1980). Instead heme-iron was considered the main initiator of autoxidation in animal foods (Tappel, 1962). Recent research, however, identified lipoxygenase to be a source of lipid oxidation and volatile development in trout (Hsieh, 1988), and has been suggested to be present in many other fish species (Josephson et al., 1984a). While most studies suggest that the volatiles derived from lipoxygenase activity in fish are not contributing to the fishy off-flavor (Karahadian and Lindsay, 1989b), lipoxygenase activity appears to increase early hydroperoxide formation (Josephson et al., 1987). Thus, lipoxygenase might play an active role in initiating lipid oxidation in menhaden by forming hydroperoxides which then trigger the chain reaction of autoxidation.

2. Review of Literature

2.1. Menhaden (Brevoortia)

Menhaden belongs to the herring family (Clupeidae) and is classified as a fatty fish, characterized by the high fat content of the flesh. There are at least four species belonging to the genus Brevoortia. B. tyrannus is caught on the east coast of the United States, while in the Gulf of Mexico the species B. patronus is harvested. On the coast of Paraguy, the main menhaden species is B. pectinata, and the species B. aureus is found along the coast line of Brazil, but has also been sighted off the coast of North Carolina (Goode, 1884; Harrison ,1931). Recently, two more species have been identified in the Gulf of Mexico, namely B. gunteri and B. smithi (Anonymous, 1988).

While menhaden is the popular name now commonly used, menhaden has had many other popular names, most of which were used within narrow geographical boundaries, and some of which are still in use today. The word menhaden is derived from a Narragonsett Indian word and means "that which enriches the earth". Other popular names were "Pogy", "Hard-Head", "Bay Fish" (Northern New England), "Mossbunker", "Bunker", "Marshbanker" (New York, New Jersey), "Alewife", "Pilcher", "Green-Tail" (Chesapeake), "Fatback" (Carolinas to Florida), "Yellow-Tail", and "Shiner" (Florida) (Goode, 1884).

The harvest of Brevoortia tyrannus on the east coast is limited to between May and October in order to prevent over-fishing and because menhaden is a migrating fish. The winter quarters of Brevoortia tyrannus are off the southeast coast of the U.S. in the Atlantic Ocean at a latitude of about 25°. In spring, the menhaden migrates to the coast and north, reaching a latitude of approximately 45° in the summer. There is evidence that menhaden migration depends on water temperature, since menhaden have been sighted only when waters reach a minimum temperature of 10°C, their preferred temperature, however, is around 15°C. The fish becomes very lean in the winter months, but during the migration north, the fish feeds constantly and gains weight mostly in form of oil. Menhaden is one of the most abundant fish species on the eastern coast of the United States (Goode, 1884; Harrison, 1931).

Menhaden is not used at present as a food fish mainly because of a bone structure that does not allow for easy deboning (Zapata Haynie Corporation, 1985). Nevertheless,

menhaden fishing has been in practice since it started in the early 19th century off the coast of Rhode Island. Today, the U.S. menhaden fishery, the main product of which is fishmeal, is located off the eastern and southeastern shore (Doody, 1981). In 1988, the United States produced 224.7 Million pounds of fish oil, and exported 149.3 Million pounds valued at 21.9 Million US$ (U.S. Department of Commerce, 1989). In foods menhaden oil has been almost exclusively used in hydrogenated forms as an ingredient in margarine in Europe (Young, 1986b; Doody, 1981). The use of fish oil in unhydrogenated form was never considered due to the rapid oxidation and off-flavor development of the oil (Young, 1985), until recently when omega-3 fatty acids were found to have potential therapeutic benefits in a number of chronic diseases (Kinsella, 1986, Pigott and Tucker, 1987; Simopoulos, 1986).

2.1.1 Historical Perspective

The value of menhaden as a fertilizer material was known to the Indians before the first settlers came to America. However, the colonists did not use menhaden as a fertilizer until the beginning of the 19th century. The manufacture of menhaden oil also commenced early in the 19th century. The oil was separated from the fish by putrefaction of the crushed fish yielding a dark brown foul smelling oil. Later, the use of open cooking kettles and shortly thereafter large cooking tanks was introduced resulting in an oil with considerably better quality (Harrison ,1931).

The commercial importance of menhaden was not realized until the end of the 19th century. In the middle of that century, only a few million fish were caught and mainly used for bait, fertilizer or were salted and exported to the West Indies. By the end of the century, however, about nine hundred million fish weighing approximately three hundred thousand tons were harvested and used in large quantities as fertilizer and as the major bait fish. At the end of the 19th century, menhaden was also used as a food fish, either in cured or canned form (Harrison, 1931; Goode ,1884). The production of menhaden oil during the last century is summarized in Table 1.

2.1.2. Catching, Handling and Processing

Menhaden schools are easily detectable since they feed close to the water surface. Spotter planes are used to locate the schools and to direct the fishing vessels to the fishing

Table 1. U.S. Menhaden oil production from 1875 to 1988

Year	Menhaden Oil (1,000 pounds)
1875	20,111
1880	15,262
1885	17,597
1890	22,044
1895	13,258
1901	28,593
1905	22,530
1910	28,340
1915	20,458
1921	46,954
1925	45,173
1930	23,934
1935	30,496
1940	43,310
1945	62,513
1950	76,575
1955	159,241
1960	183,403
1965	175,204
1970	177,470
1975	213,211
1980	291,434
1985	278,358
1986	332,017
1987	294,964
1988	217,493

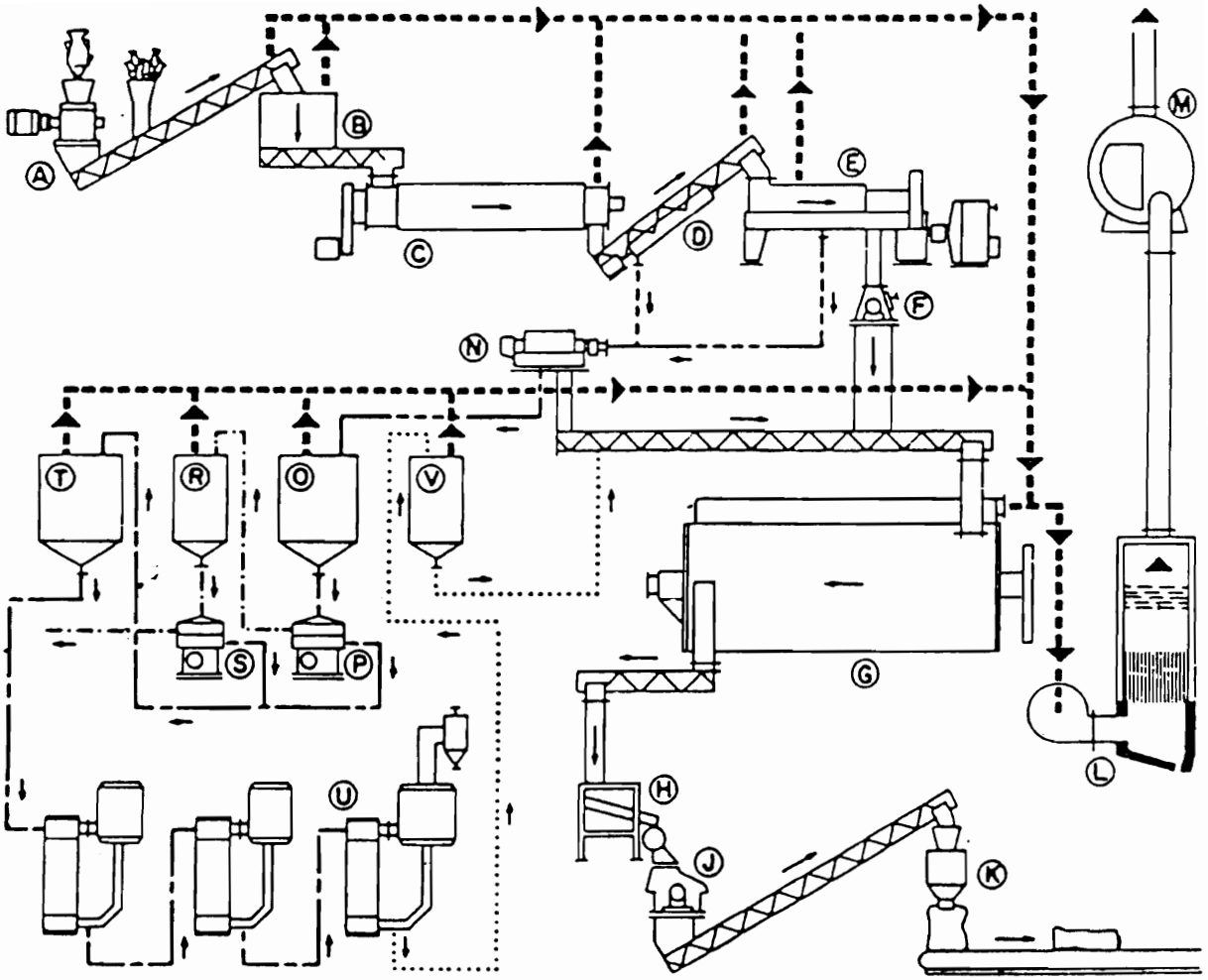
Source: Bimbo, 1989b

grounds (Doody, 1981). Menhaden is caught using two purse boats that encircle the school with a net. The net is closed, pursed and slowly retrieved so that the fish are caught in a very small area. The fish are then pumped into the stowage tanks of the vessel, while the sea water is separated from the fish and discharged (Bimbo, 1986; Zapata Haynie Corporation, 1985). A detailed description of the equipment can be found in Bulletin No. 18 published by the Gulf States Marine Fisheries Commission (Anonymous, 1988). The type of stowage on ship is typically bulk stowage in chilled water where menhaden is chilled less than food fishes (Doody, 1981). One catch is usually in the order of 10-60 tons. The vessels can hold 400-600 tons, thus can make several catches, before they return after 2-3 days to the processing plant (Bimbo, 1986). The layout of a common fish meal/oil plant is shown in Figure 1, and a detailed description can be found in the FAO Fisheries Technical Paper No. 142 (FAO Fishery Industries Division, 1986).

At the processing plant, the load of the vessel is pumped through a de-watering screen into large rotating drums and from there into storage. Menhaden oil is extracted by a wet reduction method. After storage, the fish is steam cooked, then pressed and dried to make fish meal. The liquid fraction which contains the oil, water and fish solids is decanted to separate most of the solids from the liquid and fed into a centrifugal separator that removes the oil from the water (Doody, 1981). After separation, the oil is winterized, degummed, neutralized, bleached and deodorized (Bimbo, 1986; Doody, 1981). A flow diagram of menhaden processing is shown in Figure 2.

2.1.3. Menhaden Products

As pointed out earlier, the main product of the menhaden fishery is fish meal used as an animal feed, mostly for poultry and pigs (78% of total fish meal consumption) (Bimbo and Crowther, 1992). The fish oil which must be considered a fish meal by-product, still finds its largest use in hydrogenated form as an ingredient in margarine in the European baking industry (Bimbo and Crowther, 1992), or is used for industrial lubricants, paints and varnishes in the US (Doody, 1981). However, research is currently being conducted to determine the applicability of the unhydrogenated oil in foods. On June 13, 1986, the National Fish Meal and Oil Association filed a petition with the U.S. Food and Drug Administration to consider unhydrogenated and hydrogenated fish oil as GRAS (Generally Recognized As Safe) substances (Barlow and Young, 1988; Bimbo, 1987). Hydrogenated



Vapour
Press liquor
Fish oil
Stickwater
Stickwater concentrate

A. Hasher	B. Feeder	C. Steam Cooker	D. Strainer Conveyor
E. Twin Screw Press	F. Wet Mill	G. Dryer	H. Vibrating Screen
J. Hammer Mill	K. Scales	L. Scrubber	M. Steam Boiler
N. Decanter	O. Buffer Tank	P. Centrifuge	R. Buffer Tank
S. Separator	T. Buffer Tank	U. Evaporator	V. Buffer Tank

Figure 1. Layout of a fish meal/oil plant; (FAO, Fishery Industries Division, 1986)

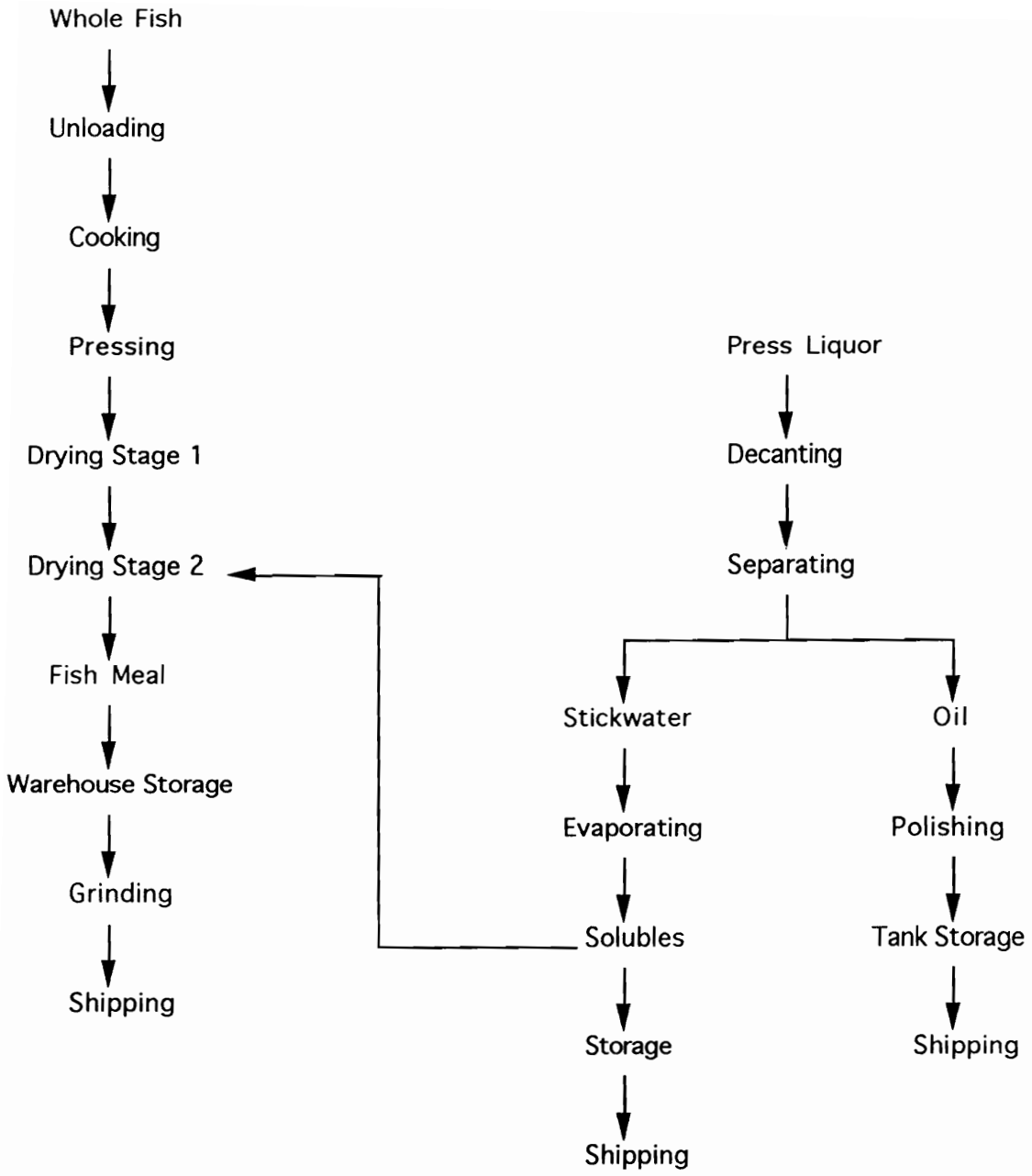


Figure 2. Schematic diagram of menhaden processing; (Bimbo and Crowther, 1992)

and partially hydrogenated menhaden oil were approved by the U.S. Food and Drug Administration as GRAS list substances in 1989 (U.S. Government, 1989), while the approval of unhydrogenated menhaden oil is not expected until late 1992 (Bimbo and Crowther, 1992). The GRAS affirmation of hydrogenated and partially hydrogenated menhaden oil has not as yet led to a major U.S. market (Bimbo and Crowther, 1992).

The water separated from the oil contains another by-product of the fish meal production. The evaporation of the bulk of this water leaves a residue consisting mainly of water and protein that is being used as pig feed (Doody ,1981). The use of menhaden in the form of fish protein concentrate has been investigated in the last few years. Fish protein concentrate for human consumption is similar to fish meal with some differences in the protein and oil content (Doody, 1981). However, large scale production of fish protein concentrate is currently not feasible, due to the necessity for higher sanitary standards than those currently employed for fish meal production (Bimbo, 1989a).

2.2. Fish Oil

In recent years, fish oil has received considerable attention because of its high content of omega-3 fatty acids which have been associated with lowering risk factors for cardiovascular diseases (Kinsella, 1987; Pigott and Tucker, 1987). Fish oil consists of over 90% triacylglycerides and is partially solid at 20°C (Young, 1986a). The omega-3 fatty acids found in fish oil are mainly eicosapentaenoic (C20:5) (EPA) and docosahexaenoic (C22:6) (DHA) acid (Kinsella, 1986). However, fish oil is a very complex oil in which more than 45 different fatty acids have been identified (Young, 1986a). The principal fatty acids of various marine oils are listed in Table 2, while other fatty acids that can be found in fish oils are listed in Table 3. Table 3, however, is not an exhaustive list.

Menhaden is the largest source of fish oil in the United States. Menhaden oil is an inexpensive oil that can compete economically with oils such as soybean oil or palm oil. In 1986, the price per metric ton of fish oil was $275 compared to $417 for soybean oil and $318 for palm oil (Bimbo, 1987).

2.2.1. Nutritional Implications

Fish oil became of interest to researchers in the 1970's, when studies showed lower

Table 2. Principal fatty acids of various marine oils (in % of total fatty acids)

Fatty acid	Herring	Norw.Pout	Mackerel	Sandeel	Menhaden	Sardine	Anchovy
C 14:0	7	6	8	7	9	8	9
C 16:0	16	13	14	15	20	18	19
C 16:1	6	5	7	8	12	10	9
C 18:1	13	14	13	9	11	13	13
C 20:1	13	11	12	15	1	4	5
C 22:1	20	12	15	16	0.2	3	2
C 20:5	5	8	7	9	14	18	17
C 22:6	6	13	8	9	8	9	9

Source: Barlow and Young, 1989

Table 3. Fatty acids found in fish oil

C 12:0	C 17:branched	C 19:branched	C 21:0
C 14:0	C 17:0	C 19:0	C 21:5 n*-2
C 14:1	C 17:1	C 19:1	C 22:0
C 15:branched	C 18:branched	C 20:0	C 22:1 n-11
C 15:0	C 18:0	C 20:1	C 22:2
C 16:0	C 18:1	C 20:2 n-9	C 22:3 n-3
C 16:1	C 18:2 n-9	C 20:2 n-6	C 22:4 n-3
C 16:2 n-7	C 18:2 n-6	C 20:3 n-6	C 22:5 n-3
C 16:2 n-4	C 18:2 n-4	C 20:3 n-3	C 22:6 n-3
C 16:3 n-4	C 18:3 n-6	C 20:4 n-6	C 23:0
C 16:3 n-3	C 18:3 n-3	C 20:4 n-3	C 24:0
C 16:4 n-4	C 18:4 n-3	C 20: n-5	C 24:1
C 16:4 n-1			

* **n** indicates the position of the first double bond from the methyl end of the fatty acid

Source: Barlow and Young, 1989

incidence of cardiovascular diseases in the Greenland Eskimo population as compared to the Danish Eskimos who differed mainly in their food behavior (Bang et al., 1976; Munro, 1983). The diet of the Greenland Eskimos is very high in fish oil which led researchers to question if there is any association between lowered risk of cardiovascular diseases and consumption of fish and omega-3 fatty acids (Shukla et al., 1980). A large amount of research has been conducted subsequently, and mechanisms have been proposed for the potential involvement of omega-3 fatty acids in reducing the risk of cardiovascular diseases (Higgs et al., 1986). Claims have even been made that omega-3 fatty acids are essential for the human body (Neuringer and Connor, 1986), and while evidence for this claim is mounting (von Schacky, 1987) it has not yet been proven (Kinsella, 1987). Evidence has also been found for additional benefits from increasing omega-3 fatty acid intake such as improvements in hypertension (Knapp, 1990), rheumatoid arthritis (Kremer et al. 1987), diabetes (Haines et al., 1986), migraine (McCarren et al., 1985), psoriasis (Bittner et al., 1988), malaria (Anonymous, 1989), certain cancers (Raloff, 1989), dermatitis (Bjørneboe et al. 1987), various intestinal diseases, nephritis, and various forms of trauma, such as burns (Harris, 1992).

A short review of the pathogenesis of atherosclerosis is necessary for understanding how omega-3 fatty acids can possibly reduce the risk of atherosclerosis. There are three current theories for the etiology of atherosclerosis. In the most accepted theory, atherosclerosis starts with an injury to the endothelium of an arterial vessel which can be due to a variety of causes such as infections, toxins or trauma. At the injury site, platelets are activated by the exposed muscle cells of the lesion, leading to the generation of thromboxane which causes platelet aggregation and the formation of thrombi at the vessel wall. The platelets also release platelet-derived growth factor which promotes growth of the smooth muscle and endothelial cells. The formation of leukotrienes by the platelets causes monocytes and smooth muscle cells of the intima to be attracted to the injury site, causing an inflammatory response of the tissue. Low density lipoprotein and other lipids are incorporated into these cells which become foam cells that form the bulk of the developing occlusion. These cells are sometimes referred to as the "fatty streak" due to their lipid incorporation and whitish appearance. The foam cells with the adhering thrombi and the proliferating connective tissue start depositing calcium and grow over time narrowing the vessel. While the lesion often heals by being covered with endothelial cells,

the outcropping of the vessel wall makes the site prone to future injuries, repeating the cycle and gradually building up an occlusive plaque. A floating thrombus usually occludes the vessel causing ischemia and myocardial infarction which results in arrhythmia and possibly fatal heart failure (Leaf and Weber, 1988; Keith, 1990).

The main physiological effects of omega-3 fatty acids in fish oil can be summarized as follows:

1. Omega-3 fatty acids are readily incorporated into phospholipids in membranes. Like arachidonic acid, EPA is released from platelet membranes by phospholipase A_2 upon membrane stimulation, and functions as a competitive inhibitor of cyclooxygenase. Cyclooxygenase is responsible for the formation of eicosanoids, such as thromboxane A_2 from arachidonic acid, or thromboxane A_3 from EPA. Thromboxane A_2 is a proaggregatory prostaglandin responsible for thrombi formation, whereas thromboxane A_3 has a much weaker proaggregatory effect. Therefore, the competitive inhibition of cyclooxygenase by EPA reduces the formation of thromboxane A_2 from arachidonic acid, and increases the formation of thromboxane A_3, decreasing the rate of thrombi formation
2. Prostacyclin (PGI_2) is an antiaggregatory and smooth muscle cell relaxant formed by lipoxygenase from arachidonic acid in the endothelial cells. The formation of PGI_2 from arachidonic acid is not markedly reduced by EPA. In addition, lipoxygenase forms a PGI_3 from EPA, which has the same effects as PGI_2. Therefore the formation of antiaggregatory prostacyclins is relatively enhanced compared to proaggregatory thromboxane formation.
3. The incorporation of omega-3 fatty acids into membrane phospholipids will increase flexibility of platelet and red blood cell membranes. An increase in membrane flexibility will decrease blood viscosity. Decreased blood viscosity reduces the aggravation of injured arterial walls, decreasing intimal hyperplasia. The higher flexibility of the platelet membranes also decreases their tendency to aggregate.

Omega-3 fatty acids also cause other physiological changes that may contribute to decreasing the risk of atherosclerosis:

a) Inhibition of delta-6-desaturase by EPA, thereby reducing the conversion of linoleic acid into arachidonic acid;

b) A decreased sensitivity to epinephrine-induced contraction of blood vessels;

c) An increased effect of endothelium-derived relaxing factor, augmenting the vessel relaxing effect of prostacyclin;

d) The prostaglandin D_3 formation from EPA increases c-AMP in platelets, which inhibits platelet aggregation;

e) The formation of leukotriene B_5 from EPA by lipoxygenase, which is less chemoattractive than the leukotriene B_4 that is formed from arachidonic acid, thereby decreasing the vascular response to ischemia;

f) And an increase in endogenous fibrinolytic activity by increasing the levels of tissue plasminogen activator, which causes disintegration of thrombi;

(Higgs et al., 1986; Leaf and Weber, 1988; vonSchacky, 1987; Weaver and Holub, 1988).

Based on these physiological effects, it is possible to consider the following effect of omega-3 fatty acids on the etiology of atherosclerosis: Decreased blood viscosity and blood pressure decrease the incidence of vessel wall injury (Leaf and Weber, 1988). Due to the increased formation of prostacyclins by the endothelial cells and the decreased production of thromboxane by the platelets, fewer and smaller thrombi will form at the vessel wall (Knapp et al., 1986). The reduced release of platelet-derived growth factor from the platelets will diminish intimal hyperplasia and the production of foam cells. A reduced formation of leukotriene will reduce the attraction of monocytes and leukocytes, decreasing the inflammatory response. The increased fibrinolytic activity will also prevent the formation of an occlusive plaque (Leaf and Weber, 1988).

2.2.2. Fish Oil in Foods

Fish oil has been used in foods to a large extent in Europe. It is mainly used in hydrogenated form in margarine by the baking industry (Bimbo and Crowther, 1992). Fish oils contain many different fatty acids and consequently a wide variety of triacylglycerides. Upon hydrogenation this variety even increases due to isomerization. Thus, hydrogenated fish oils are very heterogeneous and form a stable beta-prime crystal structure which is the optimal crystalline form for food fats (Barlow and Young ,1989).

The feasibility of incorporating deodorized, unhydrogenated fish oil into various foods was explored at the Leatherhead Foods Lab R.A. (Leatherhead, U.K.) (Barlow and

Young, 1988, 1989). While the results of this study were generally promising, the investigators concluded that the flavor stability of the products must be improved and that more research is needed to elucidate the mechanisms of off-flavor development in the oils and the food products (Barlow and Young 1988, 1989). These results were in stark contrast to findings of more recent studies. The Leatherhead Foods Lab R.A. found that 16.5% fish oil was an acceptable amount in frankfurters when they were analyzed by a sensory panel 21 days after processing (Barlow and Young, 1988, 1989). Park et al. (1989) on the other hand found frankfurters with 5% deodorized menhaden oil unacceptable after preparation, and did not even include them in a storage study. Schnepf et al. (1991) likewise found that including 10% of deodorized menhaden oil in a French salad dressing resulted in a significantly stronger off-flavor after 8 weeks when compared to salad dressing made from soybean oil. From research at the Leatherhead Foods Lab R.A. however, it was concluded that 10% fish oil is a feasible and acceptable amount in salad dressing (Barlow and Young, 1988, 1989). An important difference between these two studies was that in the research at the Leatherhead Foods Lab R.A. the dressings were analyzed by a sensory panel the day after preparation and were never stored for longer periods of time. Fish oil mayonnaise, the food item that caused the biggest problem for the Leatherhead Foods Lab R.A. (Barlow and Young, 1988, 1989) was not found to be different in sensory characteristics from a mayonnaise prepared with soybean oil after 14 weeks of storage at 2°C under nitrogen and with TBHQ (0.02%) (Hsieh and Regenstein, 1991). Another report cites that a commercial fat spread made of fish oil has been marketed with reasonable success in Denmark within the last year (Rice, 1991). Although there is evidence that fish oil can be incorporated into foods, it must be concluded that the results are quite controversial and that currently the incorporation of deodorized unhydrogenated menhaden oil into foods is difficult at best.

While presently the supply of high quality fish oil is limited (Rice, 1991), better large scale deodorization processes (Lin et al., 1990) or the refinement of fish oil using resins (Fernandez, 1986) might be beneficial for the oxidative stability of menhaden oil and might produce an acceptable food ingredient. One possibility for incorporating fish oils into foods is micro encapsulation. In this method, the oil is incorporated into the porous matrix of micro capsules, which are put into foods. The resulting product is a free flowing powder. Recent experiments have successfully used this method in a range of foods

(Vaelds-Frederiksen, 1991). A bread enriched with fish oil by this method has been marketed with good success since 1990 in Denmark (Nielsen, 1992). However, the major drawback to this method is its the very high cost that is not expected to decrease in the near future (Rice, 1991).

A different approach to incorporating fish oil or more precisely omega-3 fatty acids into foods is taken by the poultry industry. Feeding layer hens 1.5-3% refined menhaden oil in their diets increased omega-3 fatty acid content of the egg yolk without causing off-flavors (Huang et al., 1990; Van-Elswyk et al., 1992). The researchers attributed these findings to the stabilization of the omega-3 fatty acids by natural antioxidants in the eggs (Huang et al., 1990). However, while taste panelists were not able to taste a difference in hard boiled eggs, when scrambled, the eggs enriched with omega-3 fatty acids had a stronger flavor (Van-Elswyk et al., 1992). The incorporation of omega-3 fatty acids into the diets of poultry also increased the concentration of these fatty acids in the thigh muscle of the birds. However, off-flavor formation remained a problem in these experiments (Chanmugam et al., 1992). Similar approaches are being taken by the beef and pork industry with varying success (Bimbo and Crowther, 1992). Since farm raised fish does not contain omega-3 fatty acids, aquaculturists have also considered incorporating fish oil into the diets of their fish as well (International Association of Fish Meal Manufacturers, 1990).

The main problem for incorporating fish oil into foods remains the high susceptibility of the omega-3 fatty acids to peroxidation. Therefore, the oil has to be handled carefully during all stages of processing in order to minimize lipid peroxidation. In view of the rapid spoilage of fish oil, Singh and Chandra (1988) concluded that "the most important factor in the production of a high quality crude fish oil is the condition of the raw material at the time of processing". However, current harvesting and processing practices make menhaden oil susceptible to rapid oxidation (Hsieh T.C.-Y. et al., 1989). Even after deodorization, menhaden oil is much more prone to off-flavor development than other edible oils (Fujimoto, 1989). Research is needed to describe the mechanisms of fish oil oxidation, in order to develop or modify existing processing methods that will improve oxidative stability of the oil.

2.3. Lipid Oxidation

While lipid oxidation has only recently been associated with pathophysiological conditions (Maycock et al., 1989; Crooke and Wong, 1991), the quality deterioration of foods due to fat rancidity is well established (Nawar, 1985). Polyunsaturated fatty acids are particularly susceptible to free radical reactions due to the relative ease of abstraction of a hydrogen from the alpha carbon adjacent to a double bond forming an allyl radical. The abstracted hydrogen is commonly an allelic hydrogen that is one carbon removed from a double bond and has a lower bond energy than a methylene hydrogen due to the resonance stabilization by the double bond (Kanner et al., 1987; Hsieh and Kinsella, 1989b). The most accepted mechanism for lipid oxidation consists of three stages, initiation, propagation, and termination (Khayat and Schwall, 1983):

Initiation:	$RH + O_2$	——>	$R\cdot + H\cdot$
	ROOH	——>	$ROO\cdot\ H\cdot$
Propagation:	$R\cdot + O_2$	—fast—>	$ROO\cdot$
	$ROO\cdot + RH$	—slow—>	$ROOH + R\cdot$
Termination:	$R\cdot + R\cdot$	——>	RR
	$ROO\cdot + ROO\cdot$	——>	stable products
	$R\cdot + ROO\cdot$	——>	stable products

The chemical reactions involved in these stages are extremely complex and the products formed are very diverse. The initiation stage, which can occur as a result of the removal of the hydrogen by lipoxygenase or its abstraction by a peroxyl radical (propagation step 2) is considered to be the rate limiting step in lipid oxidation (Whitaker, 1991; Hsieh and Kinsella, 1989b). Several theories for the initiation of lipid oxidation exist, and it is now clear that the initiation of lipid oxidation is a multifaceted reaction (Nawar, 1985).

Singlet oxygen is considered to be of major importance in the initiation of the autoxidation of fatty acids. In the ground state, oxygen is in a triplet state with two unpaired electrons having the same directional spin. Singlet oxygen exists in two forms, either with two unpaired electrons having the opposite spin direction or with paired electrons also with opposite spin directions. Only singlet oxygen can react with fatty acids

due to the spin repulsion between singlet fatty acids and triplet oxygen. Singlet oxygen can be formed by enzymes, such as lipoxygenase or by photosentisization in the presence of natural pigments such as hematoporphyrin. Other initiators of autoxidation are superoxide anion radical, perhydroxyl radical, hydrogen peroxide and hydroxyl radical that are being formed by the reduction of oxygen to water. These compounds are formed enzymatically or non-enzymatically under biological conditions by sub cellular organelles. Transition metals (Fe, Cu) have also been found to play a role in the initiation step due to their ability to donate electrons to or to abstract hydrogens from polyunsaturated fatty acids (PUFA), thus creating a PUFA radical. They may also contribute indirectly by generating various oxygen species that will attack PUFAs. Other biological compounds, such as cytochrome P450, activated heme proteins and activated ADP-Fe^{3+} are sources of lipid oxidation. Enzymes, such as lipoxygenase, cyclooxygenase and peroxidases have also been identified as major contributors to the initiation of lipid oxidation (Kanner et al., 1987; Hsieh and Kinsella, 1989b).

The rate of oxidation is dependent on the predominant fatty acids in an oil. EPA and DHA oxidize much more rapidly without a distinct induction period than linoleic or linolenic acid under light irradiation. This difference in oxidation rate appears to be limited to the initiation stage, however, since no difference was found in oxidation rate of these four fatty acids during the propagation stage (Cho et al., 1987a). In the dark, a 3-4 day induction period was observed for EPA and DHA oxidation (Cho et al., 1987b). The hydroperoxides of EPA and DHA were found to be highly unstable, and polymerization of the secondary oxidation products occurred very early. Therefore, the peroxide value was not considered a good measure of oxidative deterioration of oils containing EPA and DHA, and the researchers suggested the determination of secondary oxidation products instead (Cho et al., 1987a, 1987b).

2.3.1. Role of Enzymes in Lipid Oxidation

Microsomal enzymes, peroxidases, cyclooxygenases and lipoxygenases are enzymes that have been associated with the initiation of lipid oxidation. Most microsomal enzymes, such as cytochrome P-450 have a broad substrate specificity and involve a prosthetic group such as NAD(P) which initiates the oxidation. Peroxidases interact with H_2O_2 which activates the ferric-heme enzyme to a higher redox state, which can then attack lipids

(Kanner et al., 1987).

These enzymes are generally activated in stress situations such as tissue disruption or injury (Gardner, 1980). In addition, cyclooxygenases and lipoxygenases carry out controlled lipid oxidation in prostaglandin and leukotriene synthesis. These enzymes are responsible for the introduction of oxygen into fatty acids such as arachidonic acid, thus creating active lipid hydroperoxides. If disturbed, these activated complexes could cause uncontrolled lipid peroxidation (Kanner et al., 1987; Hsieh and Kinsella, 1989b).

2.3.2. Prevention of Lipid Oxidation

The methods for controlling lipid oxidation can be divided into two categories. One category includes methods to control levels of oxygen and oxygen species, while the other category includes agents that control the level of free radicals and activated catalysts (Kanner et al., 1987).

The level of oxygen can be controlled by specific processing and packaging methods, such as vacuum packaging. In living biological systems proliferation of oxygen species is commonly controlled by the enzyme systems superoxide dismutase, catalase, and glutathione peroxidase. The use of enzyme systems, such as the glucose-oxidase/catalase system has been adopted for food preservation and is another way of controlling oxygen levels (Kanner et al., 1987). Similarly, one group of antioxidants are reducing agents, that control oxygen levels by being oxidized instead of the lipids. Ascorbyl palmitate, sulfides, ascorbic acid and erythorbic acid belong to this category of oxygen scavengers (Dziezak, 1986).

The second category consists of agents, such as certain antioxidants that reduce free radicals (Kanner et al., 1987). These antioxidants interrupt the free radical chain of oxidation by contributing hydrogen from a phenolic hydroxyl group and becoming a free radical themselves. These antioxidant-free radicals are stabilized by the conjugated double bonds of the phenol ring and do not initiate further oxidation of lipids. Several well known antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate (PG) and the homologues of tocopherol function in this manner (Dziezak, 1986). Chelating agents also belong to this second category and act by binding activated catalysts (Kanner et al., 1987). The complexing action is promoted by an unshared pair of electrons in their molecular structure (Dziezak,

1986). Chelating agents such as EDTA interfere with the initiation of lipid oxidation by tightly binding pro-oxidative transition metals such as iron and copper that can induce peroxidation (Dziezak, 1986; Hsieh and Kinsella, 1989b). Other chelating agents used in foods are citric acid and polyphosphates (Dziezak, 1986).

Pro-oxidative enzymes, such as cyclooxygenase can be inhibited either by specific inhibitors such as indomethacin or other enzyme systems such as catalase. Enzyme denaturation is another means of inhibiting cyclooxygenase or lipoxygenase activity. The most commonly used method to inhibit enzyme activity in foods systems is blanching (Williams et al., 1986). A recent study showed that a combination of preservation methods, including heat treatment at 80°C, effectively reduced spoilage in minced pelagic fish (Aguilera et al., 1992).

While tocopherols are natural antioxidant found in fish oils, recent research indicates that ascorbic acid and tocopherol in an oil/lecithin/water emulsion can delay the induction of fish oil oxidation 22fold as compared to an untreated control. Since the induction period with both antioxidants present was always longer than the sum of the induction periods of the individual antioxidants, the effect is considered a synergistic one. The researchers suggested a free radical interaction between the antioxidants as the reason for this synergistic effect. Even after 36 days, the treated fish oil emulsion did not exhibit an objectionable fish odor (Han et al., 1990, 1991; Yi et al., 1991). However, currently available antioxidant systems do not reduce fish oil peroxidation effectively enough to produce a shelf stable product (Rice, 1992)

2.3.3. Lipid Oxidation Analyses

Since lipid peroxidation is a chain reaction in which the products are constantly changing, any analysis will reflect the extent of lipid oxidation only at that specific point in time. In addition, no analysis can capture the true current condition of the lipid but reflects only a small portion of the compounds involved in and created by lipid peroxidation (Kim and LaBella, 1987). The change in fatty acid composition is an indirect measure of lipid oxidation. However, these changes are sometimes very small, and the method is not well suited for many foods (Melton, 1983). More commonly used measures are the peroxide value, the TBA value, fluorescent and conjugated diene measurements, the anisidine value, the Totox value, GC analysis, and non-quantitative measures such as the Kreis test

(Melton, 1983). While most of these chemical methods have been statistically compared to sensory evaluation results (Robards et al., 1988), hardly any information exists on the comparison of the chemical methods with each other (MacDonald et al., 1982). In one paper, peroxide values, TBA values and total carbonyl compounds were all found to be significantly correlated (Mac Donald et al., 1982).

Organoleptic Evaluation

Sensory evaluation is probably the most sensitive method to determine lipid oxidation, since oxidized lipid food system are easily recognizable by their off-flavors and -odors (Gray, 1978), and no combination of physical and chemical tests can equal the sensory assessment of the composite sensory attributes of foods (Robards et al. 1988). Considering that the consumer is also the ultimate judge of what is acceptable and what is unacceptable in regard to flavors and odors, it is also the ultimate assessment method (Robards et al., 1988). Unfortunately, sensory evaluation is costly and time intensive (Robards et al., 1988) and lacks high reproducibility when compared to chemical methods (Gray, 1978).

Peroxide Value

Hydroperoxides are the primary products of lipid oxidation. Most of these peroxides are labile and quickly break down to secondary products such as aldehydes. Therefore, the peroxide value increases in the beginning of lipid oxidation, reaches a maximum and then decreases again. Thus, the peroxide value is a measure of early lipid peroxidation, and its usefulness in prolonged storage studies is limited (Gray, 1978; Melton, 1983). The iodometric method published by the AOAC is the most commonly used method for peroxide measurement (Melton, 1983). The peroxide value, expressed as milliequivalents of peroxides per kilogram of sample is <1 for fresh oils >2.5 for excessive peroxidation and >7.5 when aldehydes and off-flavor development are present (Robards et al., 1988). This method is highly empirical, however, and any change in conditions may lead to variation in the results (Gray, 1978; Robards et al., 1988). The main sources of error in these methods are the absorption of iodine to double bonds and the liberation of iodine by oxygen present in the titrated solution t(Gray, 1978; Robards et al., 1988). Some studies report a good correlation of the peroxide value with flavor rancidity (Jeremiah, 1980; Fioriti, et al. 1974).

TBA Analysis

The TBA value has been extensively used in the food industry to determine peroxidation levels (Melton, 1983). However, it is also the most criticized test (Gray, 1978). There are many different methods that measure TBA value. Since the different methods will give different TBA values for the same oil, a direct comparison of TBA values is only possible when the same method is used (Melton, 1983). This is the reason why Kim and LaBella (1987) do not consider the TBA assay to be truly quantitative but to serve only as a relative index of lipid peroxidation. The TBA method detects malonaldehyde as well as those peroxides and secondary oxidation products that will release malonaldehyde during analysis (acid-heating) conditions (Kim and LaBella, 1987). The formation and liberation of malonaldehyde was shown to occur only in lipids with fatty acids containing more than three double bonds (Gray, 1978). However, TBA also reacts with other lipid oxidation products, such as the alka-2,4-dienals and other alkenals, forming a complex that absorbs at the same wavelength as the TBA-malonaldehyde complex (Melton, 1983; Robards et al. 1988). Thus, most recent literature use the terminology thiobarbituric acid reactive substances (TBARS) rather than simply TBA value for this methodology (Lubis and Buckle, 1990). However, only a small portion of the lipid loss in lipid peroxidation can be accounted for by the TBA value (Kim and Labella, 1987). While the TBA value measures secondary products of lipid oxidation, it does not necessarily continue to increase over the entire course of lipid oxidation (Melton, 1983). The TBA value parallels that of the hydroperoxides, both of which can reach a peak then decrease to non-oxidized levels (Kim and LaBella, 1987). TBA values have been found to correlate well with flavor scores in some studies, whereas in others they did not (Gray, 1978; Melton, 1983). While the TBA value is the most often used measure for lipid oxidation, it is not truly a quantitative measure and therefore not ideal for assessing lipid oxidation.

Carbonyl Compounds

The colorimetric detection of carbonyl compounds is another method to follow lipid oxidation. In this method carbonyls are converted into their dinitrophenylhyrazones. This method actually has to be divided into three methods, namely total carbonyls, total monocarbonyls and classes of monocarbonyls. It appears total carbonyl measurement is

the best method of the three (Melton, 1983). Many researchers found that total monocarbonyls increase with lipid oxidation, others detected decreases upon storage, and some found erratic changes in monocarbonyls, so that the method was found completely unsuitable to assess lipid oxidation (Melton, 1983; Mai and Kinsella, 1979). Carbonyl compounds also react with anisidine (p-methoxyaniline) (Gray, 1978). The anisidine value does not have a unit, but is defined as 100 times the absorbance of a solution resulting from the reaction of 1 g of oil in 100 mL of a mixture of solvent and p-anisidine, measured at 350 nm in a 1 cm cell (Robards et al. 1988). Values below 10 represent acceptable oils (Robards et al., 1988). According to List et al. (1974) and Warner et al. (1988) the anisidine value correlates well with flavor scores, while Robards et al. (1988) reports little correlation between carbonyl compounds and sensory scores.

Totox Value

The totox number is defined as the anisidine value plus twice the peroxide value (Robards et al. 1988). It has been widely used and is supposedly a good measure of early and late peroxidation, since the peroxide value assesses early stages and the anisidine value late stages of peroxidation (Rossell, 1989). Robards et al. (1988) considers the totox number a poor index however, since it is the sum of two measurements with incompatible units, and claims that the separate values provide more information .

Oxygen Absorption

Oxygen absorption has also been used frequently to follow lipid oxidation. The major drawback of this method is that other reactions, such as protein oxidation, also use up oxygen in food sytems. However, it is a suitable method in pure oil systems (Melton, 1983).

GC Analysis of Aldehydes

Originally hexanal formation in the headspace of oxidizing lipid foods had been used to follow lipid oxidation by GC (Melton, 1983), but since the techniques for GC headspace analysis are improving rapidly, new methods are constantly developed. A second GC method that is widely used and has been highly correlated with sensory scores is a direct GC method, that was developed by Dupuy and co-workers (Dupuy et al., 1971, 1976,

1977; Rayner et al., 1978; Legendre et al., 1979). For a more detailed discussion of the different GC methods, the reader is referred to section 2.3.4. (GC Analyses in Lipid Oxidation).

Fluorescence

The fluorescence of oxidizing lipids is based on the reaction of secondary carbonyl compounds with amino groups to form Schiff base complexes (Kim and LaBella, 1987). However, even in the absence of amino groups, auto fluorescence occurs during autoxidation of PUFAs (Gutteridge et al., 1978). The method is very sensitive and it has been shown that the results closely parallel oxygen absorption (Gray, 1978). However, fluorescence has mostly been used in biological tissue, such as cell membranes, but very little in food systems until recently. Thus, little information is available on fluorescence in food systems is (Melton, 1983). Also the excitation and emission maxima differ for various condensation products and thus do not allow for quantitative assessment of lipid oxidation (Gray, 1978).

Diene Conjugation

The method is based on the increased UV absorption of oxidizing polyunsaturated fatty acids, due to the change of methylene interrupted double bonds to conjugated double bonds. The absorption is strongest in the region between 230 and 275 nm, where dienes absorb strongest at 234 nm and trienes at 268 nm. The absorption changes are not correlated to the degree of oxidation since the effect on absorption changes differs for various fatty acids (Gray, 1978). The method, however, correlates well with peroxide values (St. Angelo et al., 1975).

Summary

Diene conjugation and TBA values are mostly derived from hydroperoxides, whereas fluorescence is derived from secondary carbonyl compounds. Thus, these measures are inversely related; with an increase of fluorescence one can observe a decrease in TBA and conjugated diene value (Kim and LaBella, 1987). Since no method can truly capture the oxidative state of a biological system, parallel and sequential determinations by a wide variety of methods are necessary to evaluate primary and secondary products in lipid

peroxidation (Kim and LaBella, 1987).

2.3.4. GC Analyses in Lipid Oxidation

There are numerous GC methods used for assessing lipid oxidation. The methods have to be distinguished between direct injection, static and dynamic headspace analysis. An overview of examples of the food application of these methods was compiled by Robards et al. (1988). The main criticism for all GC methods is the possibility of production of secondary oxidation products in the heated injector port instead of during storage (Robards et al., 1988).

Scholz and Ptak (1966) were the first to use direct injection for the analysis of volatiles. In 1971, Dupuy et al. published a simplified direct injection method for analyzing volatiles in oils. The method is based on the absorption of a small sample (2 μL) onto glass wool, which is positioned in the glass liner of the GC injector (Warner and Frankel, 1985) or in an external closed inlet device attachment (Carlat, 1990). The sample in the glass wool is then heated to 180°C, eluting the volatiles onto the column where they are cryogenically focused (Snyder et al., 1988). The main problem with direct injection is the possibility of column contamination since the sample is placed before the column, and traces can possibly leak through the glass wool onto the column (Robards et al., 1988).

Static headspace sampling in its simplest form probably comes the closest to real-life conditions, since a certain volume of headspace gas is injected into the GC. However, the low concentration of volatiles in the headspace of many samples and the possibility of absorption or condensation of volatiles in the syringe prevents this method from being applicable for many systems. Therefore, in static headspace systems the samples are first pre-heated to high temperatures in the sample vials. The liquid sample phase and the gas phase are brought into an equilibrium. The sample is then drawn under pressure and injected. Raising the temperature will enhance the sensitivity of the method, but introduces the previously mentioned problem of sample decomposition. In addition, the large injection volume (several mL) makes this method unsuitable for capillary column chromatography due to overloading and band broadening. A cryogenic trap at the beginning of the capillary column may focus the solutes sufficiently, or a gas stream splitter can be used to decrease the sample amount entering the column. This is of course only possible when sensitivity is not important (Hinshaw, 1990). Static headspace analysis has

been found to concentrate the low boiling volatile compounds (Snyder et al., 1988).

Dynamic headspace sampling, also known as purge and trap analysis, is based on the purging (stripping) of the sample with an inert gas, and then trapping the volatile compounds on a porous material, thus concentrating the volatiles. The volatiles are then eluted and injected into the column (Snyder et al., 1988). There are several modifications of the dynamic headspace method. In one modification, the samples are heated during purging and concentrated in a trap at room temperature, which is connected to the GC. The volatiles are then eluted by heating the trap. The volatiles can also be cryogenically focused during elution in the beginning of the column (Snyder et al., 1988). In another modification, which was used in this study, the volatiles are both purged and trapped at room temperature. They are then eluted with a solvent, such as hexane or ethyl ether, and the eluate is then concentrated by evaporating the solvent (Olafsdottir et al., 1985). While the first modification can potentially cause artificial volatile production or decomposition during purging, the second modification has the least problems with compound production or decomposition. However, low boiling substances are often masked in the chromatogram by the solvent peak (Hinshaw, 1990).

2.4. Lipoxygenases

Lipoxygenase (linoleate: oxygen oxidoreductase, E.C. 1.13.11.12) is known to be widely distributed in plants (Pinsky et al., 1971; Axelrod, 1974), where it is responsible for the development of off-flavors in various unblanched frozen vegetables (Whitaker, 1991). Different lipoxygenase isozymes have been identified in various plant tissues, e.g. just recently three new lipoxygenases were identified in soybeans (Kato et al., 1992) and most of them have a rather broad substrate specificity (Rokach, 1989; Crooke and Wong, 1991; Whitaker, 1991; Eriksson and Svenson, 1970; Anstis and Friend ,1974; Yoon and Klein, 1979; Ben-Aziz et al., 1970; Sanders et al., 1975; Nicolas et al., 1982; Wallace and Wheeler, 1979; Kermasha et al., 1991). Until recently, the importance of lipoxygenases in animal tissue was doubted, since lipid oxidation was considered to be due to various heme compounds (Gardner, 1980). However, the physiological importance of leukotrienes in the immune system of animals initiated investigations of lipoxygenase in animal tissue.

Lipoxygenase catalyzes the oxygenation of methylene-interrupted cis, cis 1,4-pentadiene systems producing a conjugated cis, trans 2,4-pentadienyl hydroperoxide.

Lipoxygenases abstract a methylene hydrogen from unsaturated fatty acids to produce an allyl free radical which is readily quenched by molecular oxygen forming hydroperoxides (Galliard and Chan, 1980; Maycock et al., 1989; Whitaker, 1991). While autoxidatively derived products are always racemic mixtures, lipoxygenase derived products show stereospecificity (Winkler et al., 1992), with the positional and stereoisomers varying widely between different isozymes and tissues (Kühn et al., 1987; Maycock et al., 1989). The position of the hydroperoxide group is used to classify lipoxygenases (Whitaker, 1991). Lipoxygenases that insert oxygen at the 5, 8, 9, 11, 12, and 15 position of various polyunsaturated fatty acids have been identified (Kanner et al., 1987).

While the stereo chemistry of lipoxygenases is well described, the reaction mechanism has not been established in detail yet (Maycock et al., 1989; Whitaker, 1991). It has been shown that the abstraction of a hydrogen precedes the insertion of oxygen. The hydrogen is always abstracted from the methylene group between two double bonds, and the oxygen is inserted at the first carbon atom of the double bond closest to the methyl end of the fatty acid (Whitaker, 1991). Since most lipoxygenases have non-heme iron as a prosthetic group (Vliegenthart and Veldink, 1980; Maycock et al., 1989; Whitaker, 1991), it is likely that the mechanism is based on a redox system where Fe^{3+} abstracts a hydrogen atom from the substrate forming an enzyme-substrate complex with an allylic radical and Fe^{2+}. Oxygen then oxygenates the enzyme bound substrate radical followed by an electron transfer that forms the hydroperoxide and regenerates the enzyme to its Fe^{3+} state (Maycock et al., 1989; Whitaker, 1991). Lipoxygenases might also be instrumental in degrading hydroperoxides to secondary products, where the ferrous form (Fe^{2+}) of lipoxygenase causes homolytic cleavage of the hydroperoxide (Galliard and Chan, 1980; Gardner, 1980). However, this theory is still debated (Gardner, 1980).

Most lipoxygenases have a lag period before they reach maximum velocity (Maycock et al., 1989; Whitaker, 1991) and require the presence of hydroperoxy fatty acids for activation (Hsieh and Kinsella, 1989b). Lipoxygenases from different sources and isozymes of the same species are reported to have their highest activity at neutral (Whitaker, 1991; Kermasha et al., 1991) or alkaline pH (Whitaker, 1991; Satoh et al., 1976). Soybean lipoxygenase-1 for instance has its highest activity at pH 9, while soybean lipoxygenase-2 peaks at pH 6.5 (Christopher et al., 1970). While lipoxygenases have a broad substrate specificity, most lipoxygenases with alkaline pH optima prefer free acids

and lipoxygenases with neutral pH optima prefer neutral compounds such as triacylglycerides (Gardner, 1980).

2.4.1. Lipoxygenase in Fish Lipid Oxidation

As early as the mid 30s, Lea (1937) and Banks (1937) provided evidence for an enzyme system that was involved in pork and fish oil oxidation. Banks (1937) observed that herring muscle catalyzed the oxidation of herring oil and that this effect was destroyed by heat. A few years later, Tarr (1947) observed that cooked salmon gave lower peroxide values than raw salmon. The first report of a lipoxidase (early nomenclature for lipoxygenase) present in the flesh of herring was published by Khan (1952).

In spite of the traditional opinion that enzymes do not play a major role in lipid oxidation in meats (Tappel, 1962), Fisher and Deng (1977) indicated that the proximity of non-heme iron containing enzymes to polyunsaturated lipids might be of importance in lipid oxidation in biological systems. Many research reports supported this theory. Bosund and Ganrot (1970) found that precooking decreased lipid oxidation (measured by TBA) in frozen fish. Similarly, Sen and Bhandary (1978) found that cooking decreased TBA values in refrigerated sardine, presumably due to the formation of water soluble antioxidative substances. The TBA value tended to decrease with an increase in processing temperatures (Sen and Bhandary, 1978), which is the opposite of Wang et al.'s (1991) findings who reported increasing TBA values with increasing temperatures. This agrees with early observations by Zipser and Watts (1961) that cooking rapidly increases TBA values in muscle tissue of mullet.

Some early studies indicated the possibility that a non-heme iron containing enzyme such as lipoxygenase is involved in the lipid oxidation in seafood. Liu (1970a,b) found that in shrimp non-heme iron is associated with the oxidation whereas in beef it is heme-iron. Also in 1970, Tsukuda reported the partial purification of a lipoxygenase-like enzyme with the ability to discolor carotenoids in fish skin (Tsukuda, 1970). However, other enzyme systems have been identified in fish that might also play a role in lipid oxidation. In 1983, Kanner and Kinsella identified myeloperoxidase isolated from fish leukocytes as a potential initiator of lipid peroxidation in biological tissues (Kanner and Kinsella, 1983). Other investigators reported enzymatic lipid oxidation that originate in microsomal fractions from lean and fatty fish tissues (McDonald et al., 1979; Slabyj and Hultin, 1982, 1984).

However, most studies implicate lipoxygenase as the important enzyme involved in lipid peroxidation in fish.

Several studies investigated lipid peroxidation in fish skin. Toyomizu et al. (1980) observed preferential lipid oxidation in skin tissue of various fatty fish species. This finding was attributed to the greater instability of skin lipids. The same results were found by Yamaguchi et al. (1984a,b) in other fatty and lean fish species. However, after mincing, lipid oxidation was highest in dark muscle tissue which led the authors to believe that the preferential lipid oxidation in skin was due to the aerobic conditions of skin tissue as compared to the anaerobic conditions in muscle tissue (Yamaguchi et al., 1984a,b). The occurrence of prooxidants in fish skin was hypothesized by Ke and co-workers (Ke and Ackman, 1976; Ke et al., 1977), since the fish skin lipids were more susceptible to oxidation than the fish meat tissue. After investigating prooxidants in fish skin, Yamaguchi and Toyomizu (1984) found a dioxygenase that accelerated linoleate oxidation. The enzyme had an optimum pH of 13 and contained iron in the molecule (Yamaguchi and Toyomizu, 1984).

In 1983, Josephson et al. stated that the similarity between the identified flavors in fresh white fish and those identified in various plant sources "strongly suggest a lipoxygenase-like enzyme system" in fish (Josephson et al., 1983). Another group of researchers (Cho et al., 1989a) found that oxygen absorbance and peroxide value were comparable for cooked and BHA-treated salted dried sardine for the first four weeks of storage at 5°C, while those of untreated sardine were significantly higher, and they concluded that the results suggested the importance of enzymatic oxidation in fish. These results agreed with the findings of a similar experiment using a model fish system (Cho et al., 1989b), in which raw fish skin and dark muscle was incubated with EPA. Fish skin had higher heat labile prooxidant activity than dark muscle with its abundance of heme, indicating enzymatic activity in fish skin (Cho et al., 1989b). Later, the same group of researchers (Mohri et al., 1990, 1992) was able to show the occurrence of an unusual heme-containing lipoxygenase in sardine skin which was fairly heat stable. This lipoxygenase showed higher reaction velocity with linoleic acid than with arachidonic acid with an optimal pH at 7 (Mohri et al., 1992). Since damage to fish tissue starts in the gill and skin tissue, Mohri et al. (1992) suggested that the existence of lipoxygenase in these tissues supports the theory that lipoxygenase is an important factor in fish deterioration.

Kinsella and co-workers also suggested that lipoxygenase extracted from skin as well as gill tissue of rainbow trout is a significant initiator of free radical formation and lipid peroxidation (German and Kinsella, 1985; German et al., 1986, Hsieh and Kinsella, 1986). They found evidence that the initiation of lipid peroxidation was due to postmortem lipoxygenase activity and not some non-enzymatic initiator (Hsieh and Kinsella, 1986) or some nonspecific enzymatic initiator such as cytochrome P450 (German and Kinsella, 1985) or cyclooxygenase (German et al., 1986). The trout gill lipoxygenase was heat labile and showed its highest activity at neutral pH (German and Kinsella, 1985; Hsieh and Kinsella, 1989b).

A recent study investigated the effect of heat treatments on lipoxygenase activity in lake herring skin (Wang et al., 1991). The researchers found that the enzyme was fully inactivated after 5 minutes of heating in a water bath at 80°C and inactivated by 50% after 1.5 minutes of heating. Higher TBA values for heated (80°C) and stored samples, however, indicated that any decrease in oxidative status due to enzyme inactivation was overcome by increased nonenzymatic oxidation due to heat. Heating at 60°C for 10 minutes, on the other hand, resulted in lower TBA values than those in the unheated control, presumably due to a decrease in enzymatic oxidation (Wang et al., 1991). Unfortunately, no data were presented on the effect of 60°C heating on lipoxygenase activity. While a new lipoxygenase was found just recently (German and Creveling, 1990), it appears that not every marine species contains lipoxygenase. For instance, no lipoxygenase activity was found in Eastern or Korean oysters in a study that determined various enzyme activities in marine foods (Burnette et al., 1979).

2.4.2. Volatiles in Fish Lipid Oxidation

The hydroperoxides formed in the initiation and propagation of lipid oxidation decompose into volatile aldehydes, ketones, alcohols, hydrocarbons, alkenes, alkenals, and alkenols by homolytic cleavage or enzymatic action such as by hydroperoxide lyase. For each hydroperoxide there are two positions for homolytic cleavage, one to each side of the alkoxy group. Considering the different fatty acids that fish oils are composed of and the large number of different hydroperoxides possible from these fatty acids and the possibility of rearrangement of double bonds, hydroxyl groups etc, a vast number of volatile compounds can be found in oxidizing fish oils (Hsieh and Kinsella, 1989b). Flavor

compounds in fish can be derived from many different pathways. Since the resulting flavor compounds from the different pathways are often identical, it is extremely difficult to distinguish between the source of the flavor compounds (Karahadian and Lindsay, 1989b).

In early studies, certain lipoxygenase derived volatiles were always noted irrespective of the source of the enzyme. These volatiles were hexanal, 2,4-decadienal, 2-heptenal, 2-octenal, pentanal, pentylfuran and 2,4-nonadienal. However, the distribution of the various volatiles and the occurrence of stereoisomers differed for the lipoxygenases (Gardner, 1980). In 1980, St. Angelo et al. incubated peanut lipoxygenase with linoleate or linoleic acid and analyzed the volatiles formed by direct GC-MS. In a closed (no oxygen access) system, the main volatiles found were pentane, hexanal, 2-pentylfuran and two isomers of 2,4 decadienal while in an open system three more compounds, namely pentanal, pentanol, and 2-heptanone were observed. After additional analyses, hexanal was the only product positively identified as a directly enzyme-derived decomposition product, while the other compounds were most likely due to thermal decomposition of the hydroperoxides formed (St. Angelo et al., 1980).

Hsieh and Kinsella (1989a) also determined major volatile oxidation products formed by trout lipoxygenase activity on EPA and arachidonic acid. They used a purge-and-trap system similar to the one used in this study, and employed enzyme inhibitors for a positive identification of lipoxygenase-derived volatiles. They found that 2-octenal, 1-octen-3-ol and 2-nonenal appear to be the three major volatiles derived from arachidonic acid, while 2,6-nonadienal, 1,5-octadien-3-ol and 2,5-octadien-1-ol were derived from EPA. These six compounds were not found when a lipoxygenase inhibitor such as esculetin was added to the model system (Hsieh and Kinsella, 1989a).

Some or all of these six volatile compounds have been identified in a variety of seafood species (Josephson et al., 1983, 1984a, 1985; Cha et al., 1992; Hsieh et al., 1989; St. Angelo et al., 1987; McGill et al., 1977). In 1989, Karahadian and Lindsay analyzed the volatiles of several refined and deodorized fish oils, including menhaden and were able to identify the above mentioned compounds in the oils (Karahadian and Lindsay, 1989a). Hsieh et al. (1989) also identified nonenal and 2,6-nonadienal in the volatile profile of menhaden oil, suggesting that lipoxygenase plays an active role in menhaden oil oxidation.

While evaluating the volatile compounds of several freshwater and three saltwater species, Josephson et al. (1984a) observed that 2-octenal, 2-nonenal and 2,6-nonadienal

were absent from the volatile spectrum of the saltwater fish species while 1-octen-3-ol and 1,5-octadien-3-ol were present. The researchers also did not find any evidence of 2,5-octadien-1-ol in Ocean perch, whereas it was present in cod and sole (Josephson et al., 1984a).

Josephson et al. (1983, 1984c) associated a cucumber-like odor with 2-nonenal and 2,6-nonadienal. The compounds 1-octen-3-ol, 1,5-octadien-3-ol and 2,5-octadien-1-ol were associated with a heavy, planty odor (Josephson et al., 1983, 1984c; Karahadian and Lindsay, 1989b). The compound 2,6-nonadienal is considered to be the main contributor to the green, melony flavor found in fish oils (Karahadian and Lindsay, 1989b).

One volatile that was previously associated with the objectionable fishy flavors was identified as 2,4,7-t,c,c decatrienal (Meijboom and Stroink 1972; Badings, 1973). Badings (1973) emphasized that the fishy off flavors result from the presence of many different compounds some of which do not impart a fishy flavor by themselves but rather act synergistically with others to give the typical painty, fishy off odor that is so objectionable in fish. These results were confirmed in later studies and other compounds such as 2,4,7-t,t,c decatrienal, 2,4,-t,t and 2,4-t,c decadienal as well as 2,4-t,c and 2,4-t,t heptadienals and hexanal were associated with the fishy off odors (Karahadian and Lindsay, 1989b).

Hardy et al. (1979) reported c-4 heptenal in cold stored cod as the primary source of off-flavors. When present at high levels, c-4 heptenal has been found to complement the fishy flavor of the decatrienals (Karahadian and Lindsay, 1989b). Earlier, Josephson and Lindsay (1987) demonstrated that hydration of lipoxygenase-derived 2,6-nonadienal to 3-hydroxy-6-nonenal and subsequent retro-aldol condensation (a reversion of the condensation of two aldehydes) will yield c-4 heptenal and ethanal in various seafood species.

In their attempt to elucidate the mechanism of the formation of the six compounds that Hsieh and Kinsella (1989a) later identified as derivatives of lipoxygenase activity, Josephson et al. (1984b) were able to inhibit the formation of these compounds in Emerald shiner after treating the minced fish with acetyl salicylic acid and stannous [tin(II)] chloride, cyclooxygenase and lipoxygenase inhibitors respectively. They also postulated pathways for the formation of 1,5-octadien-3-ol, 2,5-octadien-1-ol and 2,6-nonadienal from EPA *via* lipoxygenase activity (see Figure 3) (Josephson and Lindsay, 1986). They concluded that the enzyme-mediated formation of volatiles leads mainly to characteristic fresh fish aromas.

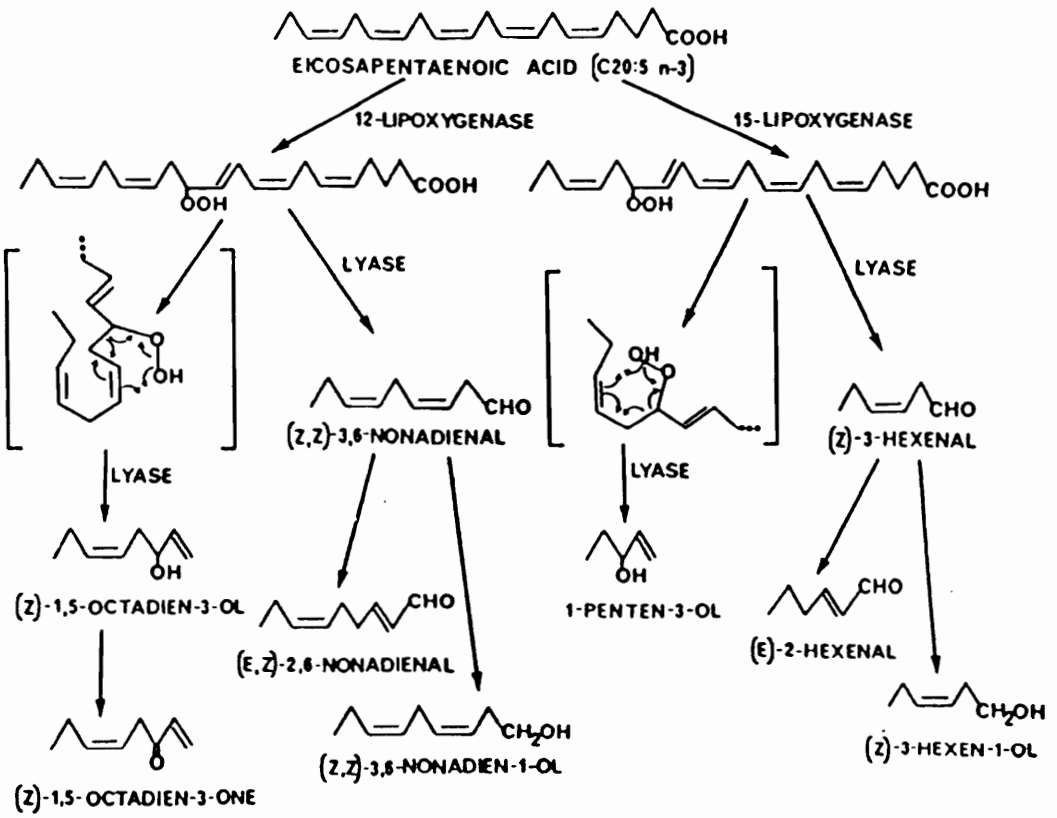


Figure 3. Postulated reaction mechanism for the formation of characterizing volatile aroma compounds in fresh fish from eicosapentaenoic acid (C20:5) via lipoxygenase and lyase action. (Josephson and Lindsay, 1986)

However, they also hypothesized that enzymatic formation of hydroperoxides can accelerate the formation of compounds contributing to the oxidized flavors in fish, through a "hydroperoxide-seeding" action. In this theory, lipid peroxidation is initiated by enzymatic activity. The enzymatically derived hydroperoxides are then responsible for the formation of other hydroperoxides, as shown by the previously described propagation stage, which then break-down to volatiles that contribute to the oxidized odor of fish (Josephson et al., 1984b; Josephson et al., 1985).

While studying volatiles in fresh and oxidized whitefish, Josephson et al. (1984c) found that the concentration of 1,5 octadien-3-ol, 2-nonenal, and 2,6-nonadienal is substantially decreased in oxidized whitefish, as compared to fresh whitefish, and concluded that further breakdown of these compounds occurs during oxidation. In a later study, Josephson et al. (1987) demonstrated that the formation not only of fresh-fish volatiles but also of classical autoxidation products is decreased when oxidases are inhibited. The researchers showed that microwaving of trout and emerald shiner resulted in substantially lower concentrations of hydroperoxides after 24 hours when compared to non-microwaved controls. They attributed these findings to the inactivation of oxidases, such as lipoxygenase. The accumulation of hydroperoxides was observed in later stages of oxidation and was attributed to the progression of classic autoxidative lipid oxidation. They concluded that early control of oxidative enzyme systems could decrease the development of autoxidatively-derived volatiles during processing and storage (Josephson et al., 1987). Thus, lipoxygenase-derived hydroperoxides can potentially catalyze the initiation of free radical autoxidation (Josephson and Lindsay, 1986).

On the other hand, the addition of lipoxygenase to shrimp has been considered in order to improve flavor. Kuo and Sun Pan (1991) were able to show that the addition of soybean lipoxygenase to shrimp increased the development of 5,8,11-tetradecatriene-2-one, a desirable flavor compound in shrimp and suggested that lipoxygenase might possibly improve the flavor of processed shrimp products.

In summary, while the volatiles that are directly derived from lipoxygenase activity cannot be associated with fishy and painty off-odors, but rather with fresh-fish, green grassy odors, the early formation of hydroperoxides by lipoxygenase may contribute to the accelerated formation of classic autoxidatively-derived volatiles that are responsible for the objectionable fishy, painty odors.

3. Statement of Problem and Justification of Study

The dietary intake of omega-3 fatty acids has been linked to the reduction of risk factors for various chronic diseases, such as coronary heart disease and hypertension (Kinsella, 1986). Fish oil is a good source of omega-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Kinsella, 1986). Most food fish species, such as cod or flounder, belong to the white fish category which have most of their fat stored in the liver and only insignificant levels in the muscle tissue, whereas in high-fat fishes such as tuna, salmon or menhaden, the muscle tissue contains most of the fat (Love, 1982). Since it is difficult to achieve the dietary intake of levels of omega-3 fatty acids believed to be beneficial solely by increasing fish consumption, the suggestion has been made for the incorporation of fish oil in other foods (Barlow and Young, 1988).

Menhaden is the largest source of fish oil worldwide (Doody, 1981). Current harvesting and processing practices and the high concentrations of polyunsaturated fatty acids (PUFAs) make menhaden oil susceptible to rapid oxidation which is the major reason why it has never been used as a food ingredient in an unhydrogenated form (Hsieh T.C.Y. et al., 1989). Recent investigations have shown that menhaden oil is difficult to include in foods such as salad dressings (Schnepf et al., 1991), or frankfurters (Park et al., 1989), due to a quickly developing objectionable fish flavor. A delay in the onset of or a decrease in lipid oxidation would help make menhaden oil a suitable food ingredient.

The direct reaction of oxygen with unsaturated fatty acids is thermodynamically favored but requires energy to be activated. This activation energy is reduced in an enzymatic reaction, and the initiation of the self-proliferating chain-reaction of free radicals that will result in the oxidative spoilage of oils is facilitated (German and Kinsella, 1985). Recently, lipoxygenase activity has been identified to be of possible major importance in the initiation of lipid oxidation in trout (German, 1984; Hsieh, 1988). Experiments have provided evidence that lipoxygenase derived volatiles can participate further in peroxidation promoting reactions *in vivo* (Kanner et al., 1987). While aroma volatiles directly derived from lipoxygenase activity do not contribute to the objectionable fishy odor of aged fish, the early formation of hydroperoxides by lipoxygenase might possibly be the major initiator of classic lipid autoxidation, leading to

the formation of these objectionable odors (Josephson and Lindsay, 1986). The lipoxygenase hydroperoxides might be responsible for the abstraction of a hydrogen from unoxidized fatty acids at various locations within the molecule, forming classic autoxidatively derived hydroperoxides which then degrade to off-odor causing aldehydes such as decatrienals.

Lipoxygenase might play a role in the initiation of lipid peroxidation in menhaden oil as early as the time of on-board storage of the fish in chilled sea water, since lipoxygenase has been found to retain high activity at near freezing temperatures (Hsieh and Kinsella, 1989b). If lipoxygenase can be shown to be a major contributor to initiation of lipid oxidation in menhaden, the destruction of the enzyme might be an appropriate way to delay the onset of lipid oxidation (Hsieh et al., 1988) and increase the oxidative stability of menhaden oil.

4. Objectives

The objectives of this study were to:

1) determine if lipoxygenase activity can be detected in menhaden gill tissue;

2) determine if lipoxygenase activity is a contributor to initiation of lipid oxidation in menhaden;

3) determine if volatile degradation products, derived from lipoxygenase activity, can be identified in undeodorized and deodorized menhaden oil;

4) determine if inactivation of menhaden lipoxygenase will decrease formation of classic lipid autoxidation volatiles;

5. Materials and Methods

This study was carried out in three stages. A diagram of the study design is shown in Figure 4. In stage one, the oxidative status of undeodorized and deodorized menhaden oils which had been processed in a commercial menhaden plant (Zapata Haynie, Reedville, VA) were monitored over a twenty week period. Both oils were processed identically at the processing plant. After being extracted from menhaden, the oils were winterized, degummed, neutralized and bleached. The only difference between the oils was that after bleaching, one of them was steam-deodorized in a laboratory of the processing plant by a method adopted from Bailey and Feuge (1943), removing low and high boiling volatiles. In stage two, the activity of gill lipoxygenase of menhaden on omega-3 fatty acid ester concentrates was determined. A comparison of the data from stage one and stage two was made in stage three in order to detect volatiles in the undeodorized and deodorized menhaden oil that are derived from lipoxygenase activity.

Stage 1

Upon arrival, the deodorized and undeodorized menhaden oils were stored under nitrogen at -70°C. In stage 1, the oils were stored in 18 (9 for each oil) 50 mL crimp-top vials (Supelco, Bellefonte, PA) under air in the dark at 30°C. The headspace in the vials was 10 mL or 20% of the amount of oil (the vials are defined as 50 mL vials, but hold a total volume of 60 mL). The oils were sampled at weeks 0, 1, 2, 3, 4, 8, 12, 16, and 20. The oxidative status of the oils was monitored by fluorescence (5.5.), diene conjugation (5.4.), thiobarbituric acid (TBA) (5.1.), peroxide (5.2.), and anisidine (5.3.) values. Volatile compounds in the oils were separated by gas chromatography and identified by gas chromatography-mass spectrometry (GC-MS), using dynamic headspace analysis (5.6.).

Stage 2

Five live menhaden were obtained from Lewis and Son Fisheries, Burgess, VA. Lipoxygenase was extracted from the gill tissue of menhaden as described by Hsieh and Kinsella (1986)(5.7.). Omega-3 fatty acid ester concentrates (Southeast Fisheries Science Center, Charleston, SC) (Appendix I) were incubated with the enzyme extract. Four

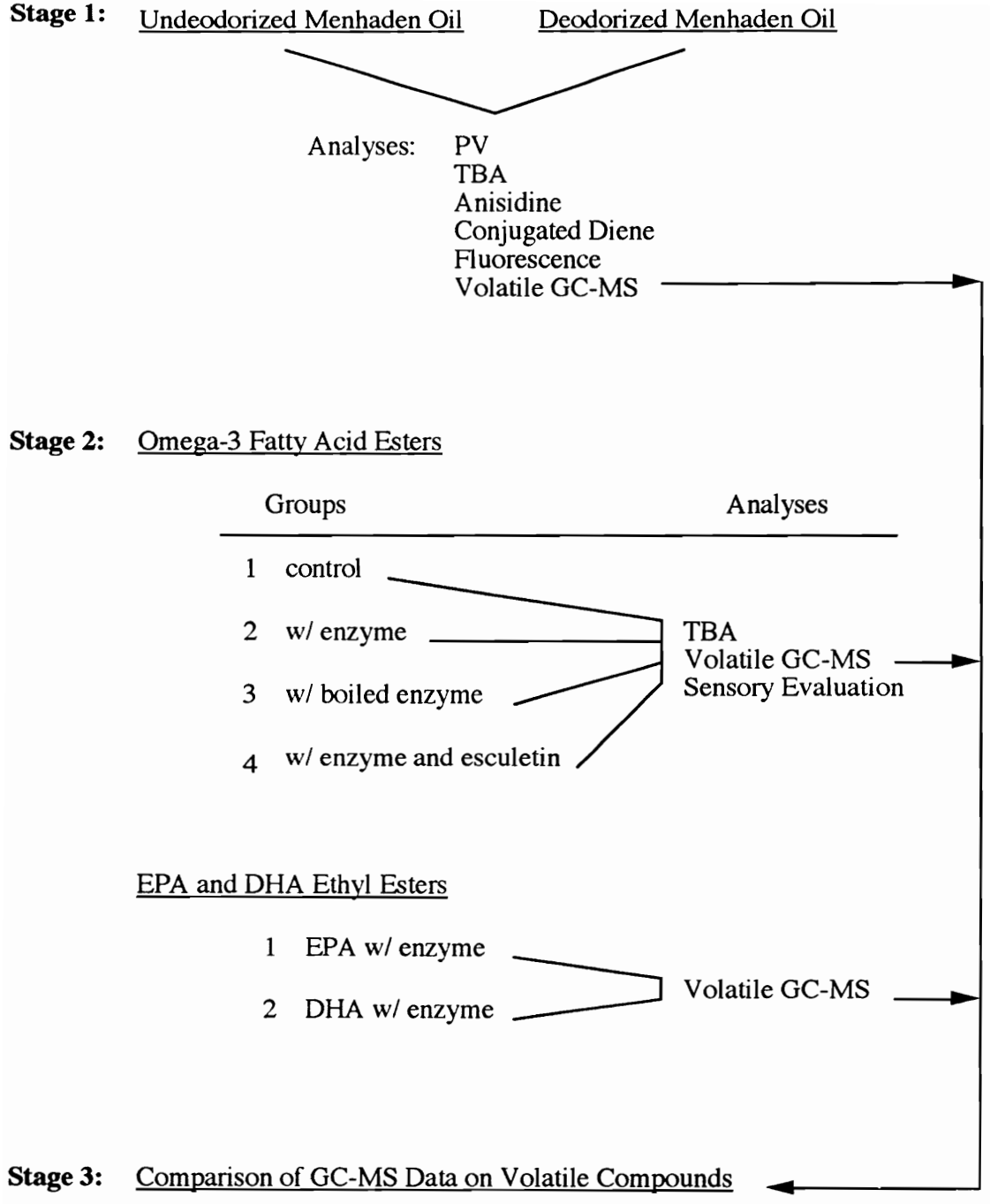


Figure 4. Diagram of the study design

treatment groups were set up, i.e. a control without the enzyme extract, a treatment group with the enzyme extract, a treatment group with the enzyme extract boiled for 10 minutes, and a treatment group with the enzyme extract and the lipoxygenase inhibitor esculetin (6,7-dihydroxy-2-hydro-1-benzopyran-2-one ≡ 6,7 dihydroxycoumarin) (Figure 5) (Aldrich, Milwaukee, WI).

The control group in stage 2 had just the buffer added so that in the case of separation of the oil and water phase the treatment group was not recognizable by sight by the sensory panelists. The enzyme extracts were added at a one percent concentration (v/v) to the ester concentrates. In order for the enzyme extracts to be miscible with the ester concentrates, 1 mL Tween 20 (polyoxyethylene-sorbitan monolaureate) (Sigma, St. Louis, MO) was added to 10 mL of the enzyme extract and the extract was added dropwise while stirring to the esters. For this study, 18.5 mL of the esters were stored at 30°C in 24 (6 for each treatment group) 20 mL crimp top vials where the headspace was adjusted to 20% of the amount of ester concentrates by using glass beads (the vials are defined as 20 mL vials, but hold a total volume of 26 mL). The concentrates were sampled at weeks 0, 1, 2, 3, 4, and 8 for volatile analysis by GC and GC-MS (5.6.) and determination of TBA value (5.1.). A 10 mL aliquot from the control group and the treatment group with the enzyme extract were simultaneously stored in glass petri dishes for sensory evaluation (5.10.).

As an addendum to the sensory part of the study, analysis of the control group and the treatment group with the enzyme extract was repeated. This part was necessary for two reasons: 1. the results of the sensory analysis earlier suggested considerable changes of the samples within the first week; 2. for the sensory part of the study, the oils were stored in petri dishes, while for the TBA and GC-MS analysis the oils were stored in closed vials, thus a comparison of the sensory data with the chemical data was not possible for that first part of the sensory analysis. For this second part, the control group with buffer and the treatment group with enzyme extract were prepared as described above. Eight 15 mL samples (four of each of the two groups) were stored in petridishes for sensory analysis (5.10.).

A third part of stage 2 was the incubation of EPA and DHA ethyl esters (Southwest Fisheries Science Center, Charleston, SC) with the enzyme extract. Volatile

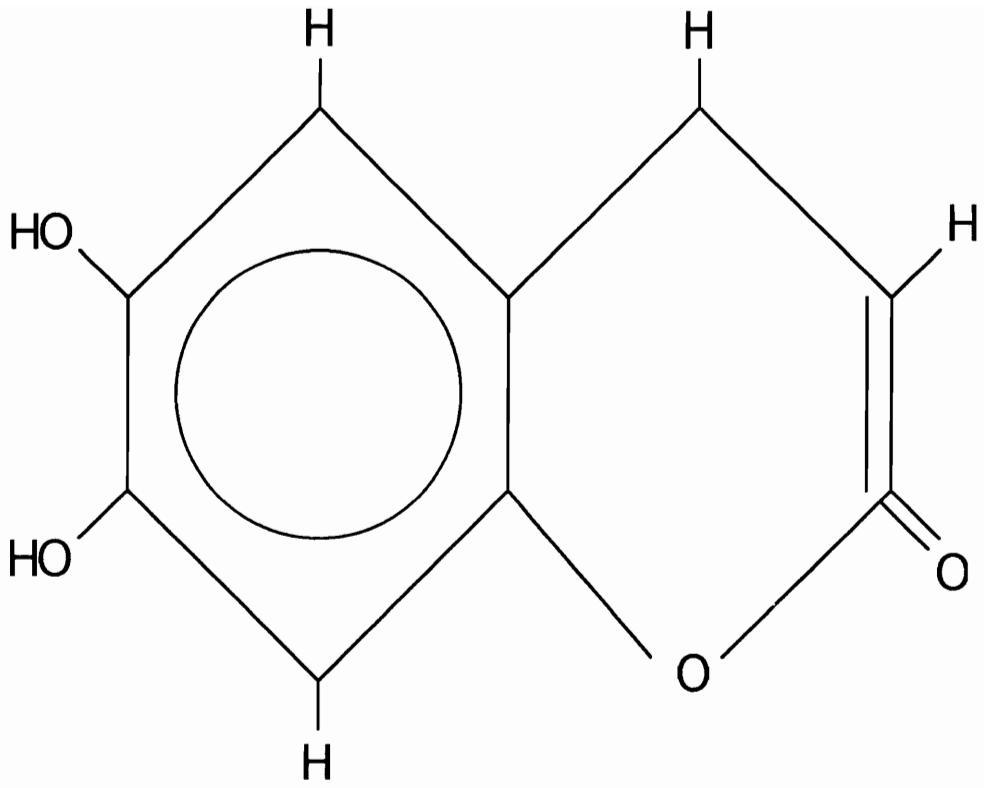


Figure 5. Esculetin (6,7 Dihydroxycoumarin) (Lidl, 1991-92)

compounds derived from the incubation of EPA and DHA ethyl esters with gill enzyme extracts were identified by GC-MS analysis (5.8.).

Stage 3

The contribution of lipoxygenase to the oxidative spoilage of menhaden oil was determined in stage 3. The results of the GC/MS analysis from stages 1 and 2 were compared in order to identify volatile compounds in the undeodorized and deodorized menhaden oil that may be derived from lipoxygenase activity. Additional information on lipoxygenase derived volatiles could be derived from the incubation of the n-3 fatty acid esters with and without lipoxygenase. If certain volatiles could be identified in the lipoxygenase incubated esters and were found absent in the boiled lipoxygenase extract and esculetin treated esters, it would be possible to conclude that these volatiles were most likely derived from lipoxygenase activity. By identifying these volatiles in the deodorized or undeodorized menhaden oil and by evaluating the overall volatile profile and identifying the off-flavor contributing volatiles in the esters and the oils, it was possible to draw conclusions as to the importance of lipoxygenase in menhaden oil peroxidation.

All reagents used in the analyses were reagent-grade unless otherwise stated. Water always refers to deionized-distilled water. A list of suppliers of reagents can be found in Appendix I.

5.1. Thiobarbituric Acid Value

Thiobarbituric acid (TBA) values were determined on the oil according to the procedure of Ke and Woyewoda (1979). In this procedure, malonaldehyde, a product of oxidation of fatty acids with two or more double bonds, reacts with TBA (Mallinckrodt, Paris, KY) to form a red-colored compound whose absorbance is measured at 538 nm. Methods for the preparation of the reagents and a standard curve can be found in Appendix II. The following procedure was used for determining the TBA value: One drop (15-20 mg) of sample was placed into a screw cap test tube and 10 mL TBA working solution was added. 0.01, 0.05, 0.1, 0.2, and 0.4 mL tetraethoxy-propane (TEP) (Aldrich, Milwaukee, WI) standard working solution was pipetted into screw cap test tubes and 10 mL TBA working solution. was added to the tubes. These standards

corresponded to 1, 5, 10, 20 and 40 nMol malonaldehyde. The tubes were vortexed for 10 to 15 seconds, and then heated for 45 minutes in a briskly boiling water bath. The tubes were then cooled under tap water, and 5 mL TCA (Sigma, St. Louis, MO) solution was added to the tubes, which were mixed by inversion. The tubes were vortexed for 6 minutes in a clinical centrifuge. The absorbance of the pink aqueous phase (top layer) was photometrically (Milton Roy, Spectronic 601) measured at 538 nm. The TBA value was calculated as TBA reactive substances (TBARS) using a standard curve and the following equation:

$$\text{TBARS} = \frac{\text{Curve Reading [nm]}}{\text{Sample Weight [g]}}$$

5.2. Peroxide Value

Peroxide values were determined using AOAC method 28.026 (AOAC, 1984). In this procedure, an acetic acid/chloroform solution (Fisher, Fair Lawn, NJ/Mallinckrodt, Paris, KY) and saturated potassium iodide (Mallinckrodt, Paris, KY) solution is added to oil samples. Excess iodine is titrated with 0.01 N sodium thiosulfate (Fisher, Fair Lawn, NJ). The peroxide value is expressed as milliequivalent of peroxide per kilogram of oil. Methods for the preparation of the reagents can be found in Appendix III. The following procedure was used for determining peroxide values: Five g of sample were weighed into a 250 mL Erlenmeyer flask. Thirty mL of acetic acid-chloroform solution was added and the flask was shaken until the oil was dissolved. Half a mL saturated potassium iodide was added and the flask was rotated rapidly for 15-20 seconds. The samples were allowed to stand in the dark for exactly one minute, then 30 mL of distilled water was added. The liberated iodine was titrated with standardized 0.01 N sodium thiosulfate while the flask was vigorously shaken. Approximately 1 mL of a 1% starch solution (Fisher, Fair Lawn, NJ) was added and the sample was titrated until the blue color disappeared. The peroxide value was calculated according to the following equation:

$$\text{P.V.} = \frac{(\text{S-B}) * \text{N} * 1000}{\text{Sample Weight [g]}}$$

where: S = mL sodium thiosulfate required for titrating the sample
B = mL sodium thiosulfate required for titrating the blank
N = Normality of sodium thiosulfate solution

5.3. Anisidine Value

Anisidine values were determined by method (#2.504) recommended by the International Union of Pure and Applied Chemistry (1987). In this method conjugated dienals react with para-anisidine (Sigma, St. Louis, MO) to form a yellow pigment which can be measured at 350 nm. The method for preparing the anisidine reagent can be found in Appendix IV. The following procedure was used for determining anisidine values: One g of oil was accurately weighed into a 25 mL volumetric flask, and dissolved in and made up to volume with iso-octane (spectroquality) (Mallinckroft, Paris, KY). The absorbance of the fat solution was measured against a blank of iso-octane at 350 nm (Milton Roy, Spectronic 601) in a quartz cuvette. Five mL of the fat solution was pipetted into one 10 mL screw cap test tube and 5 mL of plain iso-octane into another test tube (blank). One mL anisidine reagent was added to both test tubes. The tubes were vortexed and kept in the dark for exactly 10 minutes. The absorbance of the fat/anisidine solution was measured against the absorbance of the iso-octane/anisidine solution at 350 nm in a quartz cuvette. The anisidine values were calculated according to the following equation:

$$A.V. = \frac{25*[1.2*Ea)-Eb]}{\text{Sample Weight [g]}}$$

where: Ea = absorbance of fat solution in iso-octane without anisidine reagent
Eb = absorbance of fat solution in iso-octane with anisidine reagent

5.4. Conjugated Diene Determination

Oxidative deterioration of oils leads to conjugated diene formation which can be monitored by the UV absorbance changes between 220 and 320 nm (International Union of Pure and Applied Chemistry 1987, Method #2.505). In this method, 0.05 mL of the isooctane/oil solution for the anisidine analysis were further diluted with 5 mL iso-octane (trimethylpentane) (Mallinckrodt, Paris, KY). The absorption curve for wavelengths

between 225 and 245 and between 260 and 284 nm was plotted against pure iso-octane. A Perkin Elmer Lambda 3 UV/Visible spectrophotometer was used to monitor conjugated diene development. The absorbance readings were divided by the sample weight and averaged for the three samples. The method is not a quantitative method since different fatty acids have various effects on the changes in absorption, therefore no statistical analysis was performed on the conjugated diene value. The figures were compared qualitatively however, since the method can be used as a relative measure of oxidation (Gray, 1978).

5.5. Determination of Fluorescent Products Formation

Fluorescent peroxidation products in the oil were determined by a method adapted from Fletcher et al. (1973). In this method one drop (0.012 g) of the oil was dissolved in 25 mL chloroform (Mallinckrodt, Paris, KY) and the fluorescence (excitation at 330 nm, emission at 445 nm, excitation and emission slits 10 nm, sensitivity *0.1) of the solution was determined using a Perkin Elmer 650-10S Fluorescence Spectrophotometer. The excitation and emission wavelengths were chosen based on a preliminary study that determined the excitation and emission maxima of menhaden oil. Fifty ng/mL quinine sulfate dihydrate (Mallinckrodt, Paris, KY) in 0.1 N sulfuric acid (Fisher, Fair Lawn, NJ) was used as a standard. While the method is not considered a quantitative method the sample readings were adjusted according to the following equation:

$$\text{Fluorescent Products} = \frac{\text{Sample Reading}}{\text{Sample Weight [g]} * \text{Std. Reading}}$$

5.6. GC and GC-MS Analysis of Menhaden Oil Volatiles

The volatile compounds in menhaden oil were collected by a purge-and-trap technique using Tenax GR powder (2,6 diphenyl-p-phenylene-oxide polymer)(Alltech, Deerfield, IL). This dynamic head space method for the sampling of the menhaden oil volatiles was adapted from Olafsdottir et al. (1985). Fifteen mL of the oil and 10 μL of t-2 decenal (Bedoukian, Danbury, CT)(1% t-2 decenal in ethyl ether solution) as the internal standard (6.67 ppm) was placed in a 50 mL round-bottom flask. Nitrogen at a flow rate of 100 mL per minute was bubbled through the oil for 16 hours in order to release the volatiles. Volatiles were trapped in a collection tube filled with 100 mg of

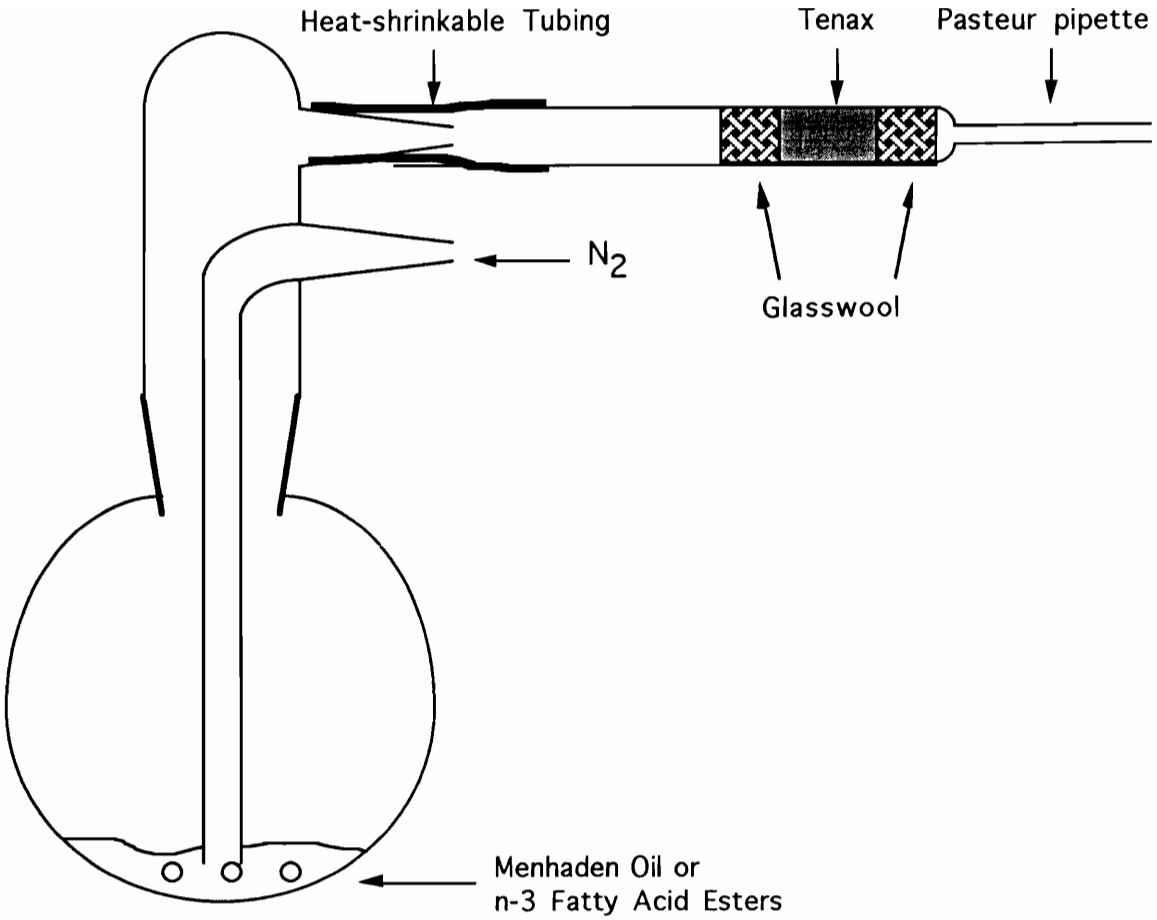


Figure 6. Extraction of Volatiles

Tenax GR. This purge-and-trap device is depicted in Figure 6. The collection tubes were fabricated using 6 inch pasteur pipettes (Fisher Scientific, Fair Lawn, NJ). The Tenax GR was held in place with silane treated glass wool (Alltech, Deerfield, IL). The volatiles were recovered from the Tenax GR by extracting them twice with 1 mL of ethyl ether (Mallinckrodt, Paris, KY). The solution was concentrated to 0.1 mL under a slow stream of nitrogen. 0.8 μL of the ethyl ether solution was then injected into the GC (Shimadzu GC 9A). A Supelcowax 10 (Supelco, Bellefonte, PA) capillary column (60 m, 0.25 mm ID, 0.25 μm film thickness) was used for the separation of the volatiles. The following temperature programming conditions were used: 50°C initial temperature held for 1 minute, 2°C per minute up to 150°C and then 4°C per minute to 220°C. Injector and flame ionization detector were both held at 240°C. The carrier gas was helium with a head pressure of 1.8 kg/cm^2 and a linear flow rate of 20 cm/sec.

For identifying volatiles, the following samples were subjected to GC-MS analysis: deodorized menhaden oil at week 0, 3 and 4, undeodorized menhaden oil at week 0 and 3, control n-3 fatty acid esters at week 0, enzyme treated fatty acid esters at week 1, boiled enzyme treated n-3 fatty acid esters at week 2, enzyme and esculetin treated n-3 fatty acid esters at week 3, enzyme treated EPA and enzyme treated DHA. The results of the GC-MS analysis were used to identify the volatiles in the other samples by carefully matching their retention times with those of the samples subjected to GC-MS. A GC/MS (HP 5790/VG Analytical 7070E) system was run under the same conditions used for the GC analysis. The ionization energy of the mass spectrometer (MS) was set at 70 eV and the ion acceleration voltage at 2000 V. The ion source temperature was 240°C, the transfer line temperature 280°C and the analyzer temperature 270°C.

Volatile compounds in the menhaden oil were identified by matching the mass spectra obtained with library mass spectra and by matching retention times with published references. The National Bureau of Standards mass spectra library with 42,000 entries was used for identifying volatiles. The library parameters are displayed in Appendix V. The library search was conducted in the "mixed search" mode since matching the library entries with a subspectrum of the unknown has been shown to be advantageous when the unknown spectrum is possibly that of a mixture (Heller et al., 1980). A computer program (Appendix VI) was used to perform a background

subtraction on the mass spectra obtained and to submit the resulting mass spectra to the library search. A background subtraction was necessary, since stray ions and the carrier gas would make a proper comparison of the spectra with those of the library impossible. It is difficult to find published figures about the percent agreement between the unknown and the library match required for the match to be considered correct. For one computer matching method, 60% agreement of the peaks between the unknown and the library spectrum was required (Ridley, 1972), and in the industry between 50% and 60% agreement is commonly used (Crvich, 1993). In this study, since a mixed search was performed, the library reference spectra had to match the unknown mass spectra by at least 75% in order to be considered as a correct match. Matches below 75% were called "unknown" compounds unless retention times/indices supported the match. A printed copy of the Wiley/NBS library of mass spectra (McLafferty and Stauffer, 1989) which includes a much larger number of mass spectra was perused in an attempt to identify these "unknown" compounds.

A true quantification of volatiles was not possible in this study, since the detector response rate of the various volatiles relative to that of the internal standard was not known. However, the concentration of volatiles could be estimated using the internal standard, by the following equation:

$$\text{Volatile Concentration} = \frac{\text{Area of Volatile} * 6.67 \text{ ppm}}{\text{Area of Internal Standard}}$$

5.7. Enzyme Extraction

Gill tissue (entire gills without cartilage) was removed from freshly killed menhaden. The tissue was homogenized in 20 mL of 0.1 M phosphate buffer (pH 7.4). The homogenate was poured into 5 mL cryogenic vials and immediately frozen in liquid nitrogen and stored at -70°C. After thawing, the homogenate was diluted 1:1 with 0.1 M phosphate buffer (pH 7.4) and then centrifuged (Sorvall RC-2) at 15,000 g at 4°C for 15 minutes to remove particulate matter and cellular organelles. The supernatant was used as the crude enzyme source for the incubation of the oils and the enzyme assays without further purification. The protein content of the dilute extract was determined by using a Sigma Diagnostic Protein Assay Kit (Procedure #P5656) (Sigma, St. Louis, MO) which

is based on Lowry's method with bovine serum albumin as standard (Lowry et al., 1951)(Appendix VII).

5.8. Volatile Lipoxygenase Degradation Products Analysis

One mL of EPA and DHA ethyl esters each (Southeast Fisheries Science Center, Charleston, SC) were incubated with the enzyme extract (50 μL = 0.84%) for 48 hours at room temperature. The volatile compounds derived from the hydroperoxides by ß-scission and homolytic cleavage were purged with nitrogen and trapped on Tenax GR as described in 5.6. with the only difference being that the purge time was increased to 36 hours since only one mL of the esters was available. The volatiles were eluted thrice with 1 mL ethyl ether, concentrated and injected into the GC (Shimadzu GC 9A) under the operating conditions previously described in 5.6. except that the analysis time was increased to 2 hours to ensure complete elution of high boiling volatiles. For identifying the volatiles the GC-MS system (HP 5790/VG Analytical 7070E) was operated under conditions identical to those described above (see section 5.6.).

5.9. Lipoxygenase Activity Assay

The method for determining the lipoxygenase activity in the gill and skin extracts was adapted from Ben Aziz et al. (1970) and Grossman and Zakut (1979). The method is based on the development of conjugated double bonds when linoleic acid (Sigma, St. Louis, MO) is incubated with lipoxygenase. The development of conjugated double bonds was monitored over time at 234 nm using a double beam Perkin Elmer Lambda 3 UV/Visible spectrophotometer. The methods for the preparation of the reagents are described in Appendix VIII. The following procedure was used for determining lipoxygenase activity: 2.49 mL of the substrate solution was pipetted into a quartz cuvette, and 0.01 mL of the enzyme extract was added. The solution was stirred and the changes in absorbance at 234 nm over time were recorded. This procedure was repeated for various substrate concentrations. For inhibitor studies, the esculetin (Aldrich, Milwaukee, WI) solution was added to the enzyme extract at the desired concentrations before adding the enzyme extract to the substrate solution. Enzyme activity was calculated as the change in absorbance at 234 nm per minute per mg protein using the following equation:

$$\text{Enzyme Activity} = \frac{\text{Change in Absorbance / sec} * 60\ [\text{sec}]}{\text{Protein Concentration [mg/mL]} * \text{Amount of Enzyme Extract [mL]}}$$

The concentration of linoleic acid ranged from 2.5 to 20 mMol. The experiment was also repeated with lower substrate concentrations ranging from 0.5 to 2.5 mMol using 0.1 mL enzyme extract. Lineweaver-Burk plots were used to demonstrate the effect of substrate concentration on the initial velocity of the lipoxygenase activity (Lineweaver and Burk, 1934). Esculetin (dihydroxycoumarin) was used for kinetic inhibition studies, since esculetin has been shown to be a lipoxygenase specific inhibitor that does not effect cyclooxygenase or other enzymes that are potentially present in the extract (Sekiya et al., 1982; Neichi et al., 1983; Serhan and Sheppard, 1990). Concentrations of 2.5 mMol to 20 mMol of substrate at pH 9 were used for inhibitor assays. The effect of esculetin at various concentrations ranging from 0.1 to 1.0 mMol on the lipoxygenase activity was determined by mixing the enzyme solution and esculetin solutions prior to the analysis. The pH dependency of the enzyme was analyzed by adjusting the pH of the substrate solution to the desired pHs of 4, 5, 6, 7, and 8 with citrate buffers and the pHs of 9, 10, and 11 with 0.1 N NaOH.

5.10. Sensory Analysis

Eighteen panelists were recruited from the staff and graduate students of the Department of Human Nutrition and Foods. All panelists were given an explanation of the study. In a training session each panelist evaluated three pairs of oils with different degrees of oxidation, in order to assess if they were able to distinguish between oxidized oils. In addition, this training session helped them to become familiar with study procedures. For the study, the samples (petridishes) were randomly coded with different three digit numbers for each panelist (drawn from a Random Number Table in Bhattacharya and Johnson, 1977). The analyses were conducted in a sensory room with individual booths in the Department of Human Nutrition and Foods. No special consideration was given to lighting since there was no difference in color between the esters. The evaluation sheet for the sensory study can be found in Appendix IX.

The omega-3 fatty acid ester concentrates were used for sensory analysis. A 10 mL aliquot from the control (without enzyme) and the treatment groups (with enzyme)

were placed into glass petridishes for this analysis. The panelists were asked to smell the two oils and judge which one of the oils had a stronger smell. This directed paired comparison test was performed in duplicate at 0, 1, 2, 3, 4, and 8 weeks of storage. This part of the study paralleled the GC and TBA analysis of the ester concentrates as described earlier in Stage 2. The TBA analysis was chosen for parallel analysis, since the TBA value correlated highly with sensory evaluation scores of various oils, when a threshold analysis, as was done in this study, was performed (Gray, 1978)

Eleven to 14 of the original 18 panelists were chosen based on availability to repeat the evaluation of four more sample pairs on days 0,1,2 and 7 for reasons given earlier (see paragraph 3 on page 40). The panelists analyzed the samples within the first hour after they were prepared (day 0) and again 6 hours later (day 1). The oils were always analyzed by all panelists within one hour. After a sample pair was analyzed by the panelists, the volatiles were extracted for GC-MS analysis (5.6.).

5.11. Statistical Analysis

The coefficient of variation was calculated for each method, with the exception of the conjugated diene and fluorescence methods. For the GC methods, the coefficient of variation was only calculated for the peak area.

An analysis of variance (ANOVA) in general linear models (GLM) with a Tukey's studentized range test using the Statistical Analysis Systems (SAS) (SAS Institute Inc., 1989) package for the IBM 3900 (CMS/VM) was used to determine significant differences in TBA, anisidine and peroxide values of the deodorized and undeodorized menhaden oil. Tukey's studentized range test is a very conservative test for pairwise comparison and was chosen because it controls the type 1 experimentwise error rate. Although multiple comparisons were made, with this method the overall (experimentwise) alpha remains at the chosen 0.05 level, whereas in other methods, such as Duncan's multiple range test, Fisher's protected LSD or repeated t-tests which control the comparisonwise error rate, the true experimental error can be a multitude of the chosen alpha level (SAS Institute, Inc., 1989).

Differences in TBA values and total volatiles of the four differently treated n-3 fatty acid esters were determined by using ANOVA in GLM with the Tukey's studentized range test for the separation of means. The results of the sensory evaluation were first

analyzed using ANOVA in GLM with week and panelist as the main effects to determine if panelists have an effect on the results. Then a Mantel-Haenszel Chi-Square was used to test the linear relationship between week and proportion of panelists choosing the oil with the enzyme extract. A correlation coefficient was determined to describe this relationship (SAS Institute, Inc., 1989). A one-tailed probability levels table was used to determine if panelists were able to find a significant difference between the oils for each week (Stone and Sidel, 1985). Sensory analysis was performed only on two esters (control and enzyme-treated), while the TBA value and the total volatiles were determined on all four esters, including the boiled and esculetin treated esters. Therefore, in order to compare the sensory results with the chemical analyses, a paired t-test was used to compare the total volatiles of the control and enzyme treated esters.

The second part of the sensory analysis was analyzed identically to the first part of the sensory analysis. A correlation coefficient for describing the relationship between day and proportion of panelists choosing the oil with the enzyme extract was determined and a one-tailed probability test was used to determine if panelists were able to distinguish between the oils for each day. A paired t-test was used to determine differences in total volatiles between the control and the enzyme treated ester used in the second part of the sensory analysis.

6. Results and Discussion

6.1. Peroxide Analysis

The peroxide values of the individual samples are given in Appendix X. The coefficient of variation for the peroxide value analysis was determined on four identical menhaden oil samples and was 6.1%. A large variance for the peroxide analysis has been observed by other researchers as well (Tsai et al., 1978). The results of the peroxide analysis are presented in Table 4 and graphically depicted in Figure 7. The results of the Tukey's studentized range test are also given in Table 4. The results of the ANOVA indicated that oil type, week and the interaction of week with oil type were significantly different at $p < 0.0001$. The significant interaction of the oil type with week can be seen in Figure 7 and is due to the different changes in the peroxide values for the two oils over time; i.e. the peroxide value of deodorized menhaden oil is higher at week 8 but lower at week 12 than that of undeodorized menhaden oil.

The peroxide value of the deodorized oil increased rapidly during the first three weeks, while that of the undeodorized oil rose sharply the first week and did not significantly change again until week 12. The peroxide value of the deodorized oil peaked at week 8 and 20, while the values for undeodorized oil peaked at week 1 and week 12. Since the deodorization process removes volatiles and peroxides from the oil, it effectively reduces lipid peroxidation (Lin et al., 1990). Therefore, it can be safely assumed that the undeodorized oil was more oxidized at the beginning of the study than the deodorized oil. This may be why the peroxide value of the undeodorized oil peaked earlier than that of the deodorized oil. This theory is supported by the fact that the undeodorized oil had higher peroxide values throughout most of the study.

The peroxide value is known to increase in the beginning of lipid peroxidation and to decrease during extended lipid peroxidation due to breakdown of peroxides to carbonyls (Gray, 1978), although continuous increases in peroxide value over extended time periods have been observed as well (Srikar et al., 1989). The decline of the peroxide value almost to the values observed at the beginning of a study has been well documented in the literature (Gray, 1978; Melton, 1983), and was observed in this study for the undeodorized oil at week 20.

Table 4. Mean Peroxide Values of the Deodorized and Undeodorized Menhaden Oil over 20 Weeks of Storage at 30°C

Week	Peroxide Value ± SD [meq/kg]	
	Deodorized MO*	Undeodorized MO
0	$1.58^{a} \pm 0.264$	$4.65^{a} \pm 0.225$
1	$1.81^{a} \pm 0.060$	$6.71^{a,b} \pm 0.100$
2	$3.31^{b} \pm 0.026$	$5.75^{b,c,d} \pm 0.006$
3	$5.63^{c} \pm 0.092$	$6.00^{b,c} \pm 0.072$
4	$4.66^{d} \pm 0.199$	$6.21^{b} \pm 0.072$
8	$9.28^{e} \pm 0.148$	$4.73^{a,d} \pm 0.006$
12	$3.67^{b} \pm 0.153$	$21.50^{e} \pm 0.173$
16	$3.50^{b} \pm 0.120$	$5.10^{a,c,d} \pm 0.597$
20	$18.71^{f} \pm 0.629$	$5.13^{a,c,d} \pm 0.852$

* deodorized and undeodorized menhaden oil were significantly different for all weeks

a,b,c,d,e,f different letters within the same column indicate significant differences between weeks respectively for deodorized and undeodorized menhaden oil

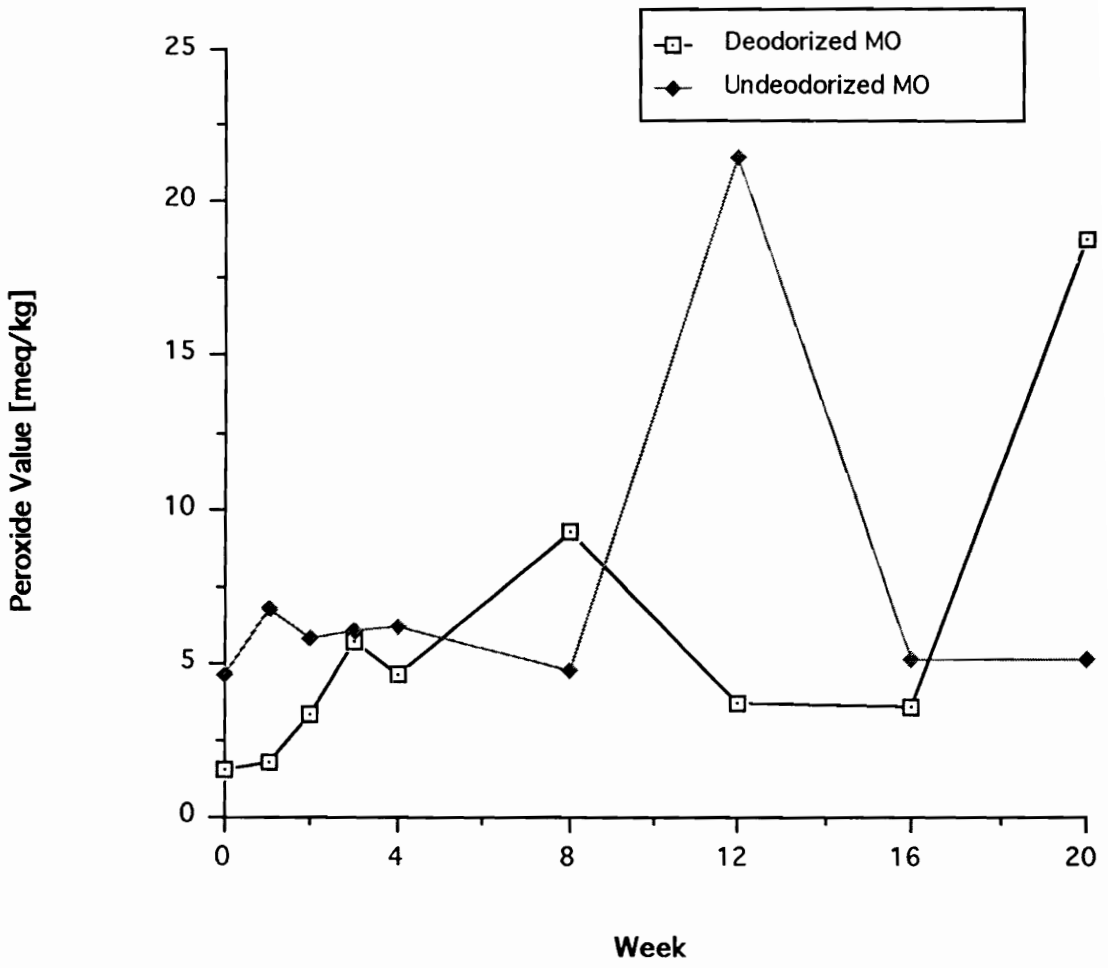


Figure 7. Mean peroxide values of deodorized and undeodorized menhaden oil over 20 weeks of storage at 30°C

Some researchers (Bosund and Ganrot, 1970; Sen and Bhandary, 1978; Cho et al., 1989a; Spencer, 1989; Pérez-Villarreal and Howgate, 1991) observed several peaks and erratic changes in the peroxide value over time. Cho et al. (1989a) found that the peroxide value increased in dried sardine fillets from day 0 to day 8, decreased slightly and then increased again at day 12, which they attributed to the early peroxidation of phospholipids. They based their conclusion on the findings of Igene et al. (1981), who observed that triglycerides were oxidized after initial oxidation of phospholipids. Neither deodorized nor undeodorized menhaden oil should contain phospholipids, since phospholipids are removed during the oil degumming. However, both oils contain significant amounts of n-3 fatty acids, which are more susceptible to oxidation than linolenic or linoleic acid (Cho et al., 1987a,b). Cho et al. (1987b) reported that, based on oxygen uptake, EPA and DHA are approximately five and eight times more susceptible to oxidation in the dark respectively than linolenic acid which is twice as susceptible as linoleic acid. In addition, the hydroperoxides formed from EPA and DHA were found to be very unstable (Fujimoto, 1989). Thus, the peroxides derived from EPA and DHA are degraded before the more stable fatty acids begin forming peroxides, which would explain the drop of the peroxide value between peaks. These results suggest that the two peroxide value peaks for both oils may be due to the difference in oxidative susceptibility of the various fatty acids found in menhaden oils.

It can be concluded that the undeodorized oil oxidized earlier during storage than the deodorized oil, and that both oils showed two peaks for the peroxide value, possibly due to a difference in oxidation susceptibility of the highly unsaturated n-3 fatty acids and other fatty acids. It was also observed that the two oils showed somewhat similar patterns of lipid peroxidation which however took place at different times, as assessed by peroxide value.

6.2. TBA Analysis

The TBA values of the individual samples are given in Appendix XI. The coefficient of variation for the TBA analysis was determined on nine identical menhaden oil samples and was 3.9%. The results of the TBA analysis are presented in Table 5 and graphically depicted in Figure 8. The results of the Tukey's standardized student range test are also given in Table 5. The ANOVA indicated that week and the interaction of

Table 5. Mean TBA Values of the Deodorized and Undeodorized Menhaden Oil over 20 Weeks of Storage at 30°C

Week	TBA Value ± SD [μMol/kg]	
	Deodorized MO	Undeodorized MO
0*	$282.9^{a} \pm 12.68$	$954.9^{a} \pm 71.55$
1*	$563.0^{a,b} \pm 73.40$	$1406.6^{a} \pm 69.89$
2*	$672.8^{a,b} \pm 26.06$	$1415.5^{a} \pm 54.30$
3	$953.8^{a,b} \pm 105.76$	$1154.7^{a} \pm 124.05$
4*	$1023.5^{a,b} \pm 68.87$	$1463.9^{a} \pm 111.75$
8*	$1396.7^{b} \pm 93.17$	$922.0^{a} \pm 112.34$
12	$3533.3^{c} \pm 251.94$	$4950.7^{b} \pm 1520.35$
16	$1401.1^{b} \pm 442.44$	$1649.4^{a} \pm 31.82$
20*	$4284.2^{c} \pm 784.24$	$1693.8^{a} \pm 82.87$

* deodorized and undeodorized menhaden oil were significantly different for these weeks

a,b,c different letters in the same column indicate significant differences between weeks respectively for deodorized and undeodorized menhaden oil

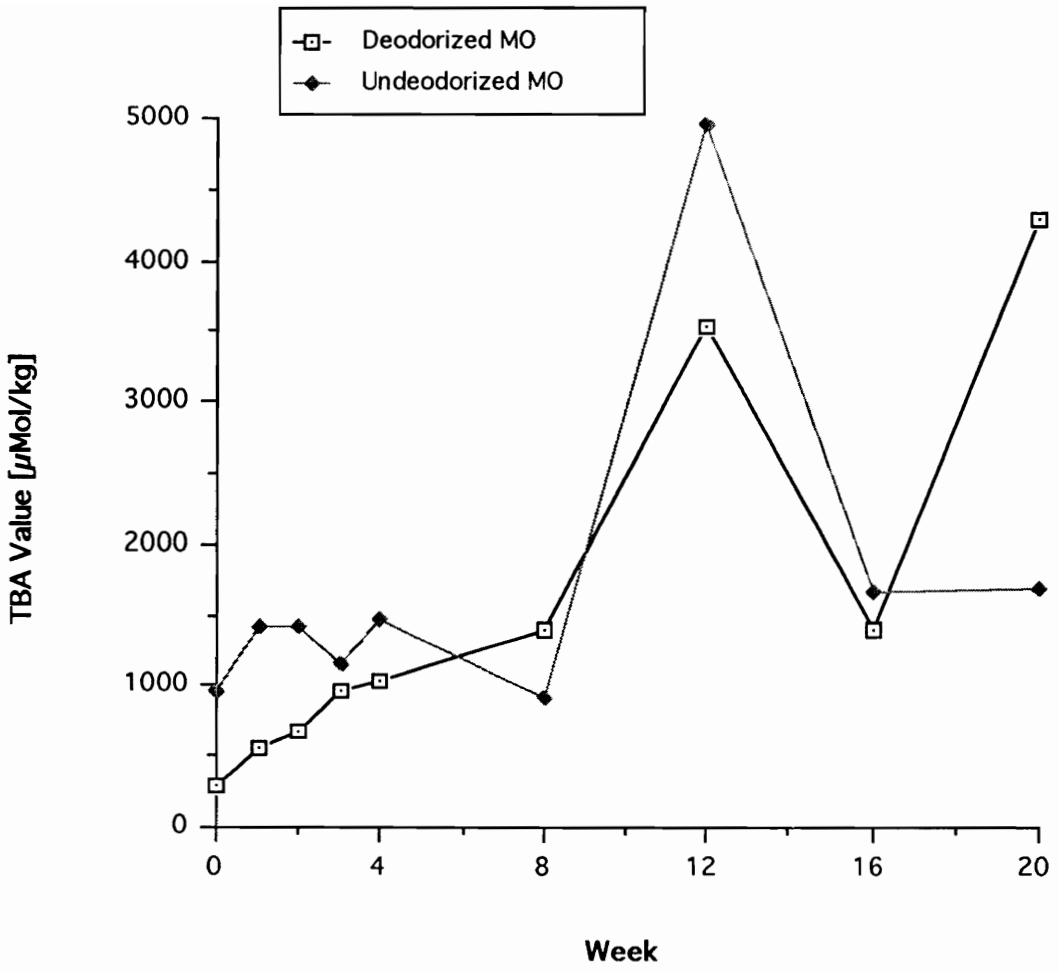


Figure 8. Mean TBA values of deodorized and undeodorized menhaden oil over 20 weeks of storage at 30°C

week with oil type were significantly different at $p < 0.0001$, whereas oil type was not significantly different. The reason for the significant interaction can be seen in Figure 8. It is caused by the TBA value of the deodorized oil being lower than that of the undeodorized oil throughout the study, except for weeks 8 and 20.

The TBA values showed a pattern similar to the peroxide values, with two maxima for each oil. Several researchers have observed a decrease in TBA values after reaching a peak during prolonged storage studies (Melton, 1983). The deodorized menhaden oil exhibited a gradual increase in TBA value for the first few weeks and then increased rapidly reaching a maximum at week 12. The TBA value then declined in week 16 and increased again at week 20. The TBA value for the undeodorized oil peaked early at week 4, then decreased and peaked again at week 12, after which it declined nearly to levels observed at the beginning of the study. The TBA value of the undeodorized menhaden oil was significantly greater than that of the deodorized menhaden oil at the beginning of the study (Week 0 - 2), which indicates that the undeodorized oil was more highly oxidized than the deodorized menhaden oil. This explains why the TBA value peaked earlier in the undeodorized oil than in the deodorized oil. Several researchers have also observed similar fluctuations in TBA values during storage (Sen and Bhandary, 1978; Kim and LaBella, 1987; Lubis and Buckle, 1990; Smith et al., 1990; Wang et al., 1991; Kelleher et al., 1992). Smith et al. (1990) found that the TBA value dropped to zero in stored salted-dried catfish. Lubis and Buckle (1990) observed a drop in the TBA value of salted sardines after an initial maximum at the beginning of a storage study. The TBA value then increased again and reached a second maximum at week 12 when the sardines were stored at 5 or at 20°C and at week 6 when stored at 30°C (Lubis and Buckle, 1990). Maruf et al. (1990) observed similar results in dried-salted mackerel. TBA values were highest at the beginning of the study, then decreased, reaching a minimum at week 10. TBA values then increased again, reaching a second maximum at week 15 and then decreased towards the end of the study reaching a second minimum at week 20. Since the TBA value measures malonaldehyde as well as those peroxides and secondary oxidation products that will release malonaldehyde during analysis (acid-heating) conditions (Kim and LaBella, 1987), TBA values may fluctuate for the same reasons as the peroxide values.

The similarity between the results of the peroxide and the TBA analysis has been reported in the literature as well (Kim and LaBella, 1987; Hasegawa et al., 1992). Kim and LaBella (1987) found a very close relationship between the TBA value and conjugated diene formation. The TBA value and the peroxide value have been shown to peak at the same time and to be closely correlated (Gray, 1978; Tsai et al., 1978). MacDonald et al. (1982) found a highly significant correlation ($r = 0.91$) between peroxide value and TBA values. This indicates that methods that assess early autoxidation, such as conjugated dienes, peroxide value and TBA value yield similar results. Other researchers, however, have not found a significant correlation between peroxide and TBA value. (Bosund and Ganrot, 1970)

It can be concluded from the measurement of TBA values that both oils showed a similar pattern of lipid oxidation and that the deodorized oil is oxidized later during storage than the undeodorized oil

6.3. Anisidine Analysis

The anisidine values of the individual samples are given in Appendix XII. The coefficient of variation for the anisidine analysis was determined on six identical menhaden oil samples and was 1.8%. The results of the anisidine analysis are presented in Table 6 and graphically depicted in Figure 9. The results of the Tukey's studentized range test can also be seen in Table 6. The ANOVA indicated that oil type, week and the interaction of week with oil type were significantly different at $p < 0.0001$. The interaction between week and oil type can be seen in Figure 9. While the anisidine value of the undeodorized oil decreased at week 16 and 20, it increased in the deodorized oil. Thus, changes in the anisidine value were different for the two oils over time, which is the reason for the interaction.

The anisidine test was introduced in 1974 and is a modification of the benzidine test. It has been found to correlate well with flavor scores of soybean oil (List et al., 1974). The anisidine value of the undeodorized menhaden oil was significantly higher than that of the deodorized oil throughout the study. However, the anisidine value of the undeodorized menhaden oil started decreasing towards the end of the study, while the anisidine value of the deodorized oil began to rise. Both of these trends were significant.

Table 6. Mean Anisidine Values of the Deodorized and Undeodorized Menhaden Oil over 20 Weeks of Storage at 30°C

Week	Anisidine Value ± SD	
	Deodorized MO*	Undeodorized MO
0	$13.40^{a} \pm 0.359$	$46.36^{a} \pm 0.917$
1	$15.00^{b,c} \pm 0.218$	$50.24^{b,c} \pm 0.611$
2	$14.43^{b} \pm 0.146$	$48.65^{b} \pm 0.267$
3	$15.22^{c,d} \pm 0.144$	$49.21^{b} \pm 0.818$
4	$15.66^{c,d,e} \pm 0.072$	$51.40^{c} \pm 0.259$
8	$18.39^{f} \pm 0.270$	$51.14^{c} \pm 0.220$
12	$16.42^{e} \pm 0.083$	$57.56^{d} \pm 0.137$
16	$15.81^{d,e} \pm 0.045$	$45.56^{a} \pm 0.413$
20	$26.02^{g} \pm 0.599$	$42.64^{e} \pm 0.741$

* deodorized and undeodorized menhaden oil were significantly different for all weeks

a,b,c,d,e,f,g different letters in the same column indicate significant differences between weeks respectively for deodorized and undeodorized menhaden oil

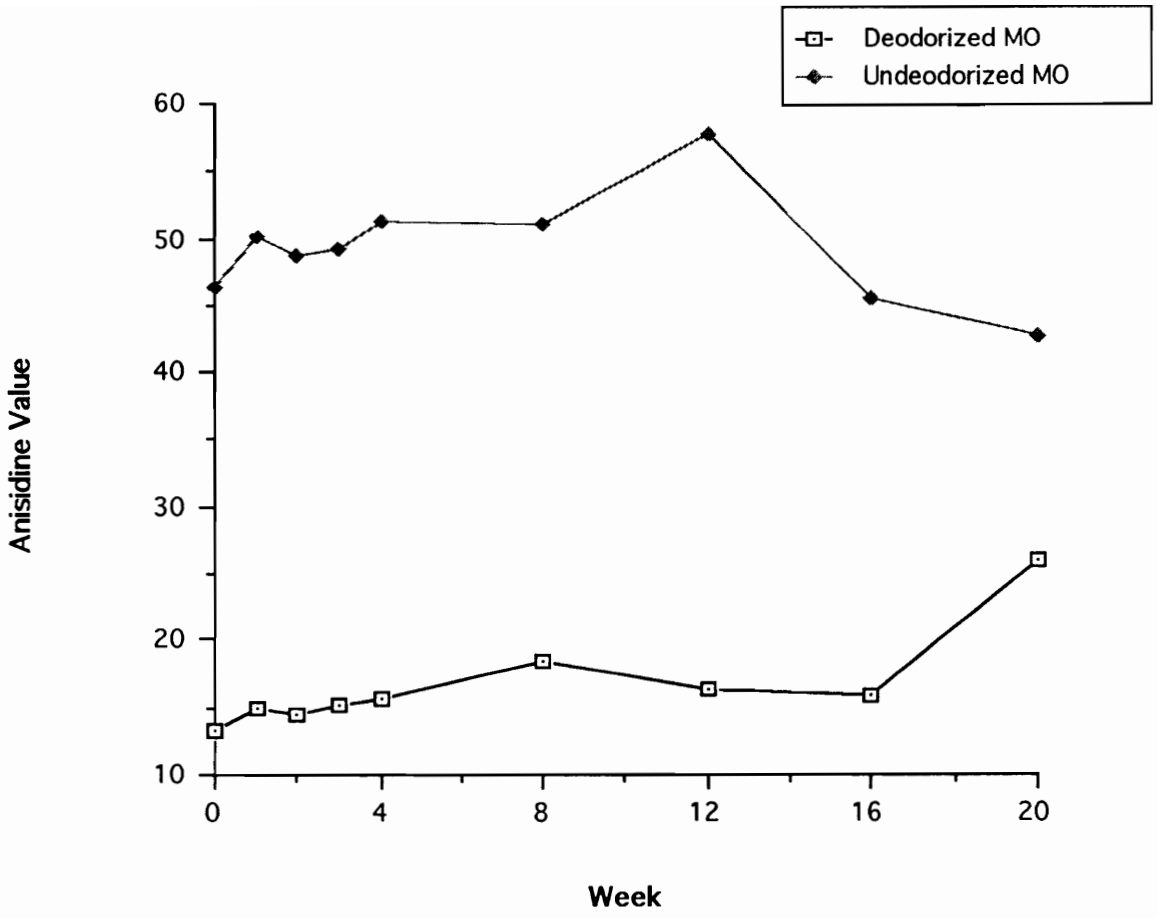


Figure 9. Mean anisidine values of deodorized and undeodorized menhaden oil over 20 weeks of storage at 30°C

Of the five lipid peroxidation methods used in this study, the anisidine value most clearly showed that the undeodorized oil was more highly oxidized than the deodorized oil. Smith et al., (1990) observed a decrease in anisidine value below values found in the fresh fish after storing salted-dried catfish for 10 weeks. The anisidine value is one of the methods for determining carbonyl compounds (Gray, 1978). Thus, it is a method that evaluates secondary lipid oxidation products in the oils. Carbonyl compounds decrease during the last stages of lipid oxidation due to formation of carbonyl polymers (Gutteridge et al., 1982). Since anisidine does not react with these polymers, anisidine values will decrease at this stage.

It can be concluded that the undeodorized oil was more oxidized than the deodorized oil throughout the study. It can also be concluded that at the end of the study, the undeodorized oil was starting to polymerize, which is one of the final stages of lipid peroxidation, while the deodorized oil entered a phase of increased carbonyl compound formation.

6.4. Conjugated Diene Analysis

The individual values for the conjugated diene analysis can be found in Appendix XIII. The results of this analysis are shown in Figures 10-13. Fatty acids with conjugated double bonds absorb light in the UV region from 230 - 375 nm. There are two absorbance maxima reported in the literature, the first one at 234 nm where conjugated dienes absorb most strongly and the second at 268 nm, where conjugated trienes absorb most strongly (Gray, 1978). While the deodorized menhaden oil exhibited highest absorbance at 242 nm (Appendix XIII) and not at 234 nm, the undeodorized menhaden oil did not exhibit any absorbance peak in the region between 225 and 247 nm (Appendix XIII). Privett and Blank (1962) also reported that they did not observe any absorption maxima in the ultraviolet spectrum of various fatty acid esters in the beginning of their study. However, eventually an absorption maximum developed at 233 nm (Privett and Blank, 1962). A triene conjugation peak was observed for both oils. The deodorized oil showed its highest absorbance at 274 nm (Appendix XIII) and the undeodorized oil showed a maximum absorbance at 270 nm (Appendix XIII). Very little change in absorbance was observed at any wavelength over the 20 week period for either of the oils (Figures 10-13, Appendix XIII).

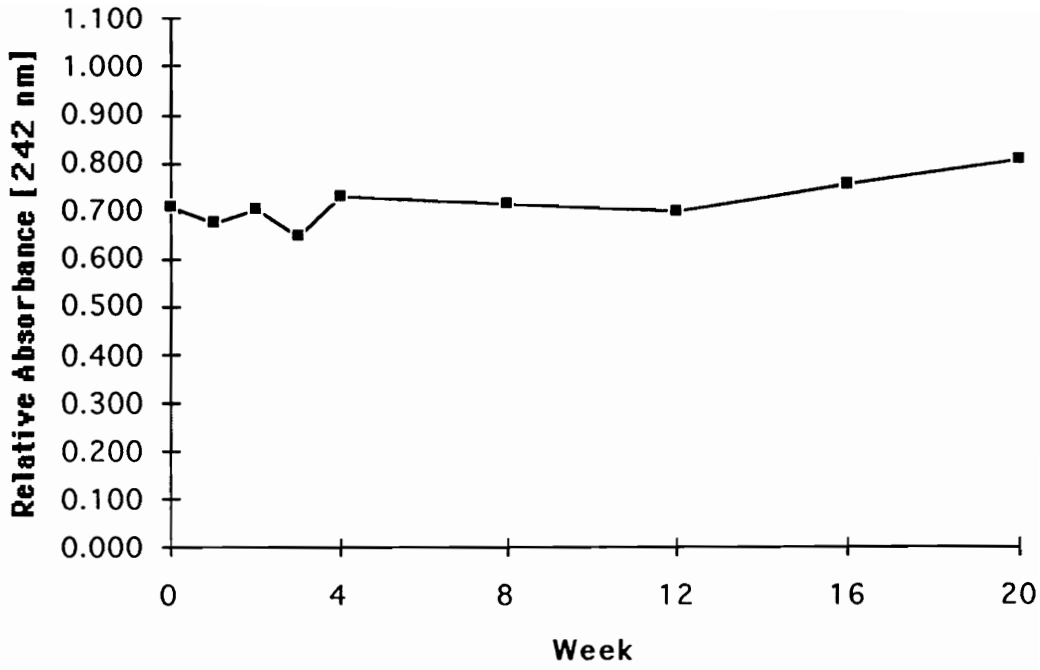


Figure 10. Relative absorbance of deodorized menhaden oil at 242 nm over 20 weeks of storage at 30°C

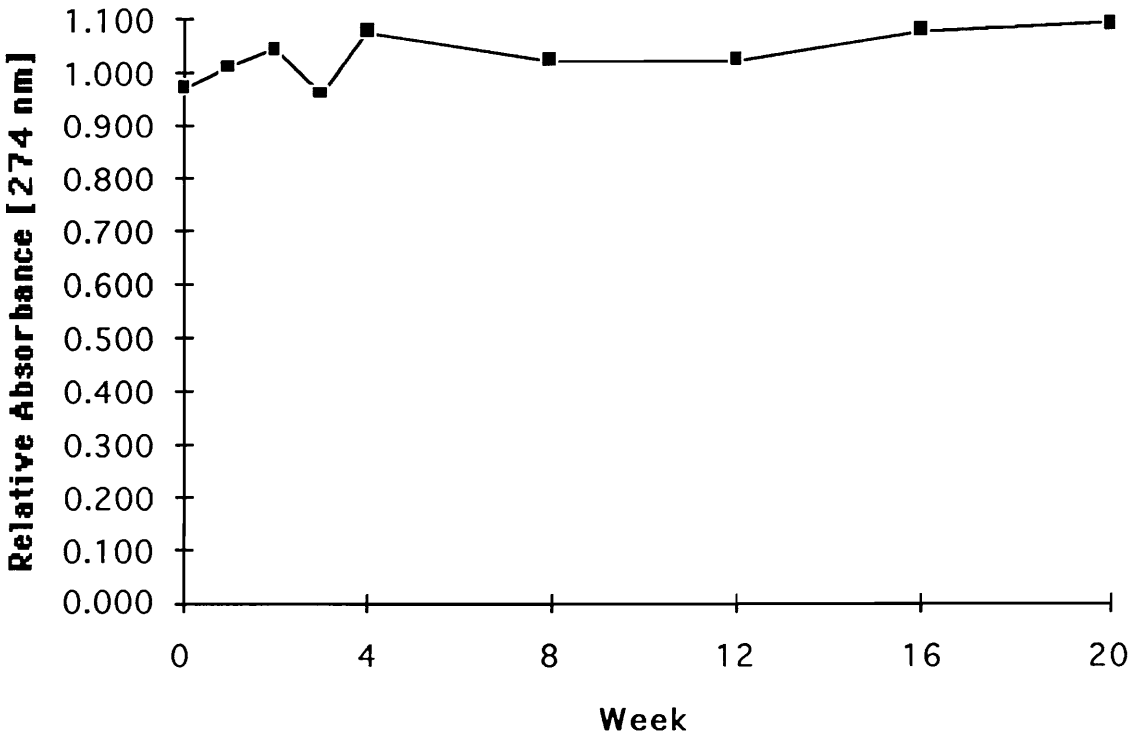


Figure 11 Relative absorbance of deodorized menhaden oil at 274 nm over 20 weeks of storage at 30°C

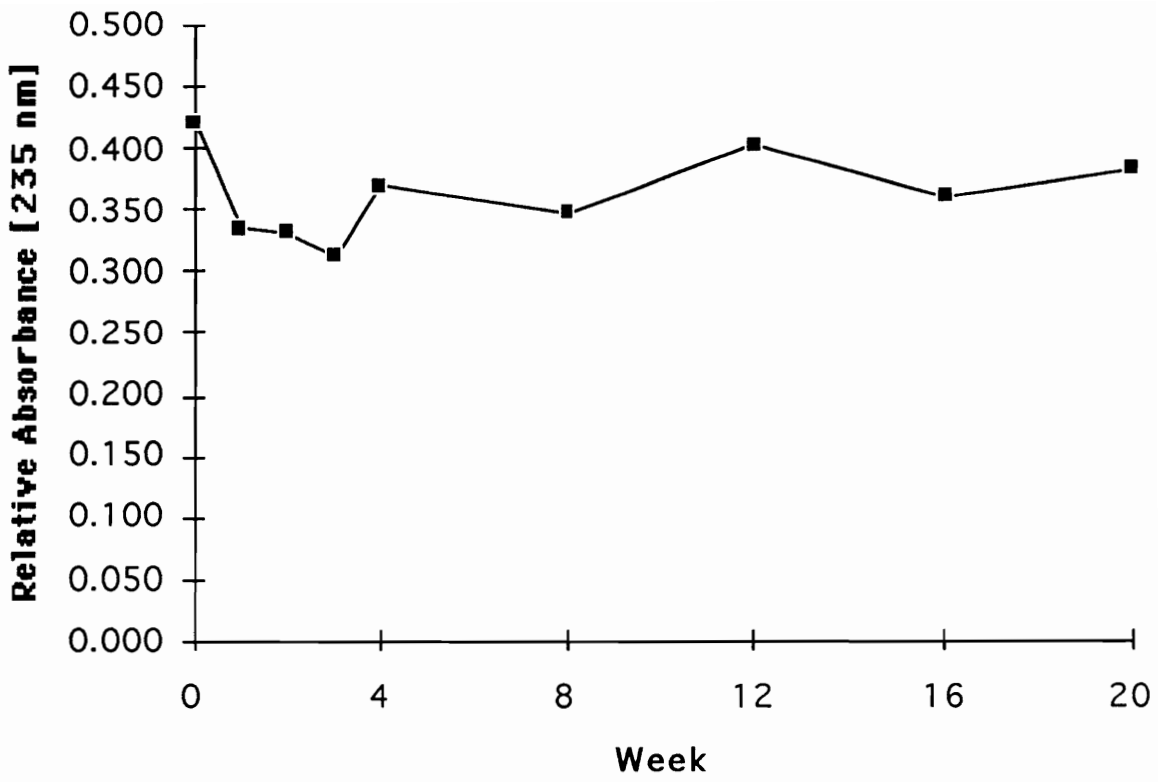


Figure 12. Relative absorbance of undeodorized menhaden oil at 235 nm over 20 weeks of storage at 30°C

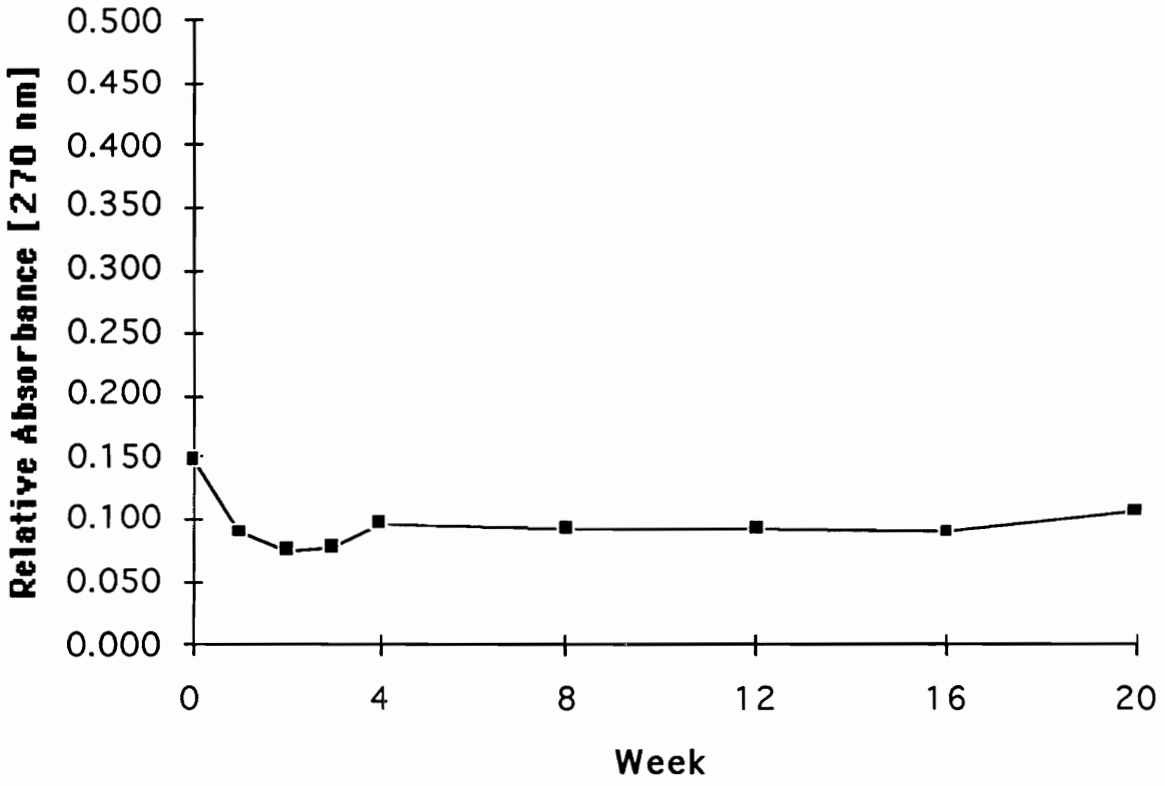


Figure 13. Relative absorbance of undeodorized menhaden oil at 270 nm over 20 weeks of storage at 30°C

Since little information about the performance of the conjugated diene method in foods is available in the literature, it is difficult to make comparisons (Gray, 1978). The application of this method for following autoxidation of linoleic and linolenic acid derivatives indicated that the method correlated well with TBA values (Kim and LaBella, 1987). The method has been successfully used to measure autoxidation in peanut butter (St. Angelo et al., 1975), and to compare the effect of various treatments on the autoxidation of EPA and DHA (Cho et al., 1987b; Cho et al., 1989b). The conjugated diene value closely followed oxygen absorption during autoxidation of EPA and DHA in the dark (Cho et al. 1987b). Cho et al., (1989b) also found that the conjugated diene method gave the same results as the peroxide value in a dry fish model system, and St. Angelo et al. (1975) concluded that the conjugated diene method can be used instead of or in addition to the peroxide value to determine staling of peanut butter. Lubis and Buckle (1990) on the other hand did not find the conjugated diene method useful in studying lipid oxidation in salted sardines, because they did not observe any changes in absorbance for the first 4-6 weeks of the study. However, towards the end of their study the absorbance started decreasing without ever showing a peak. Privett and Blank (1962) also observed that the conjugated dienes quickly reach a plateau just prior to the end of the induction period.

In this study, the conjugated diene analysis was of little use, since only small changes were observed in absorbance over time, which might be due to the temporary nature of the conjugated dienes in the oils. It must be concluded that the method as used in this study is not applicable for assessing lipid oxidation in menhaden oil.

6.5. Fluorescence Analysis

The individual values of the fluorescence analysis can be found in Appendix XIV. The results of the analysis are displayed in Table 7 and depicted in Figure 14. The fluorescence of oils that do not contain any amino groups is considered to be due to the formation of carbonyl polymers (Gutteridge et al., 1982; Kim and LaBella, 1987), which occurs in the later stages of lipid peroxidation (Gray, 1978). However, this method, like the conjugated diene method has not been widely used for assessing lipid oxidation in foods (Melton, 1983), but has found application in more recent studies on salted-dried catfish (Smith et al., 1990), mackerel (Maruf et al., 1990) salted sardines (Lubis and

Table 7. Average relative fluorescence values of deodorized and undeodorized menhaden oil over 20 weeks of storage at 30°C

Week	Deodorized MO	Undeodorized MO
0	62.449	111.009
1	63.038	112.669
2	62.137	109.893
3	54.958	98.5289
4	53.735	93.6735
8	55.869	92.1496
12	55.918	80.1048
16	50.591	85.1326
20	64.002	92.6368

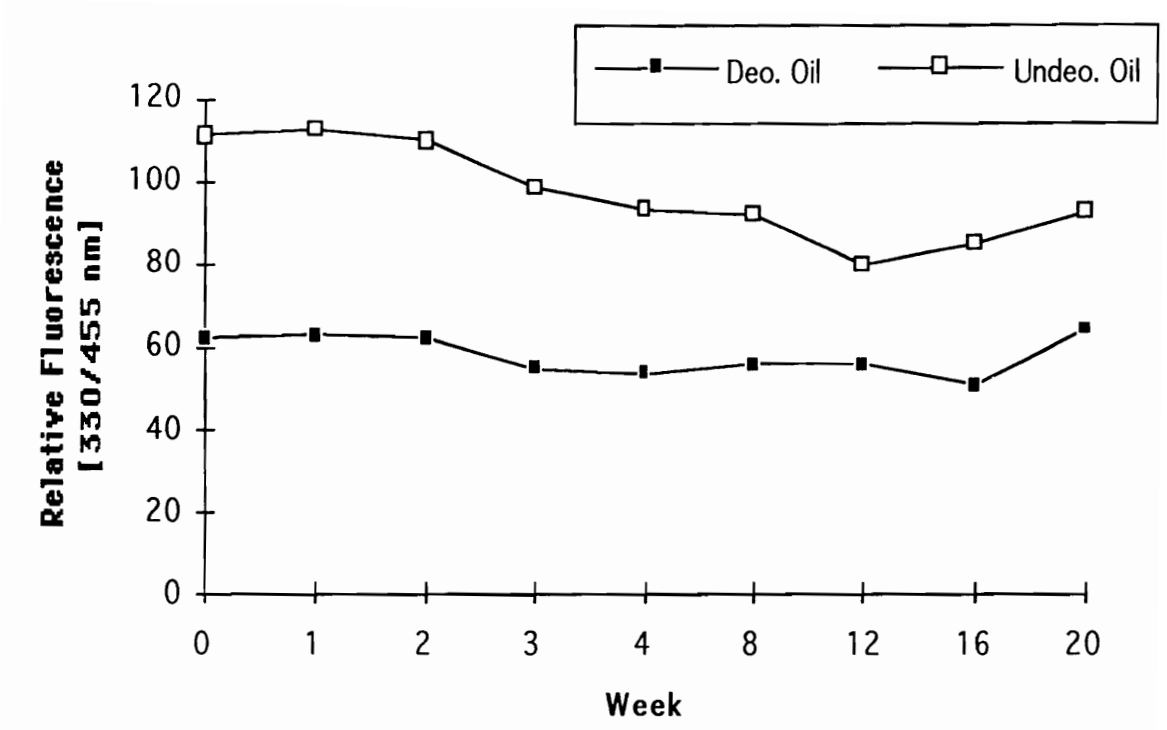


Figure 14. Relative fluorescence of deodorized and undeodorized menhaden oil over 20 weeks of storage at 30°C

Buckle, 1990), ripening avocados (Meir et al., 1991), and dried fish model systems (Hasegawa et al. 1992). It was very difficult to determine the fluorescence value in this study, due to a large fluctuation of the reading in the magnitude of ±5%. No explanation for this fluctuation can be given, since no peculiarities, such as air bubbles or particulates were noted.

The fluorescence of the undeodorized menhaden was considerably higher than that of the deodorized oil, which indicates that the undeodorized oil is more highly oxidized than the deodorized oil. For both oils, the fluorescence increased slightly in the beginning of the study, then showed a continuous slight decrease over several weeks to increase once again at the end of the study. Lubis and Buckle (1990) also found a relatively constant level of fluorescence in some salted sardine samples. Kim and LaBella (1987) observed an increase in fluorescence with increased fatty acid unsaturation. Thus, fluorescence values observed in these samples might be associated with their high degree of unsaturation. They also found an increase in fluorescence after hydroperoxides, as determined by conjugated diene absorption and TBA value, began to decrease (Kim and LaBella, 1987). Maruf et al. (1990) found a small but steady increase in fluorescence of dried-salted mackerel. The fluorescence observed by Davis (1982) and Davis and Reece (1982) in the flesh of various fish species was found to be associated with muscle proteins, and was not considered to be autofluorescence as described by Gutteridge et al. (1978). Smith et al. (1990) found fluorescence to be a good indicator of extended lipid peroxidation. In addition, they observed that more commonly used methods, such as peroxide, TBA or anisidine value, gave results erroneously indicating that after 10 weeks of storage at 30°C salted-dried catfish was less oxidized than fresh fish.

Fluorescence values started increasing at the end of the present study, when the TBA and peroxide values decreased to initial levels. In a very recent investigation with a dried fish model system, Hasegawa et al. (1992) observed two types of fluorescence, one at 360 (excitation) and 450 nm (emission) (type 1) and a second at 430 (excitation) and 500 nm (emission)(type 2). While the type 1 fluorescence decreased to very low levels within the first week, fluorescence type 2 increased at the end of the first week and then slowly decreased over the following week. Type 2 fluorescence correlated well with the results of the TBA analysis.

In general, the fluorescence analysis did not seem to be a good indicator of lipid oxidation in these menhaden oils, with the exception that the analysis confirmed that the undeodorized oil was more strongly oxidized than the deodorized oil. The determination of fluorescence type 2, as described by Hasegawa et al (1992), might have been useful in this study.

6.6. Sensory Analysis

Results from both parts of the sensory analysis can be found in Appendix XV, while a summary of the results is given in Table 8. In part 1, where 18 panelists evaluated the esters, the ANOVA in GLM indicated that the esters differed by week ($p = 0.0391$), but that differences in the responses of the individual panelists did not influence the results ($p = 0.4750$). The proportion of panelists choosing the oil with the enzyme is shown in Figure 15. The Mantel-Haenszel Chi-Square analysis verified that the relationship between week and the proportion of panelists was linear ($p = 0.002$), thus a correlation was the appropriate analysis. The correlation of the proportion of panelists choosing the oil with the enzyme extract with week was highly significant ($p = 0.0109$) with a correlation coefficient of -0.91344. The probability levels for accepting the hypothesis that the esters are different, when in fact they are not (alpha or type 1 error) are given in Table 8 for both sensory analyses. For the second part of the sensory analysis, the ANOVA in GLM indicated that the esters did not differ by day ($p = 0.1515$), and that the differences in the responses of individual panelists did not influence the results ($p = 0.3345$). The Mantel-Haenszel Chi Square confirmed the linearity ($p = 0.025$), but the correlation was not significant ($p = 0.2465$; $r = 0.75345$) (Figure 16).

For part one of the sensory analysis, the correlation of week with the proportion of panelists is negative because with increasing weeks the proportion of panelists that chose the ester with the enzyme extract decreased (Figure 15). Part two of the sensory analysis was basically a repetition of the first week of part one. However, more samples were analyzed during this first week in part two. An increase in the proportion of panelists that chose the ester with the enzyme extract over the seven days (Figure 16) was observed in part two, even though it was not significant. By combining the results of the two parts, it can be seen that the panelists were not able to distinguish between the esters for the first two days, but were able to do so after one week. Then for the next four weeks the

Table 8. Proportion of Panelists Choosing the Oil with the Enzyme Extract

Part 1			
Week	**n/N**	**Proportion**	**Probability**
0	32/36	0.8889	< 0.001
1	27/34	0.7941	< 0.001
2	25/36	0.6944	< 0.03
3	28/36	0.7778	< 0.001
4	26/36	0.7222	< 0.01
8	20/36	0.5556	> 0.05
Part 2			
Day	**n/N**	**Proportion**	**Probability**
0	4/11	0.3636	> 0.05
1	7/11	0.6364	> 0.05
2	8/11	0.7273	> 0.05
7	11/14	0.7857	< 0.05

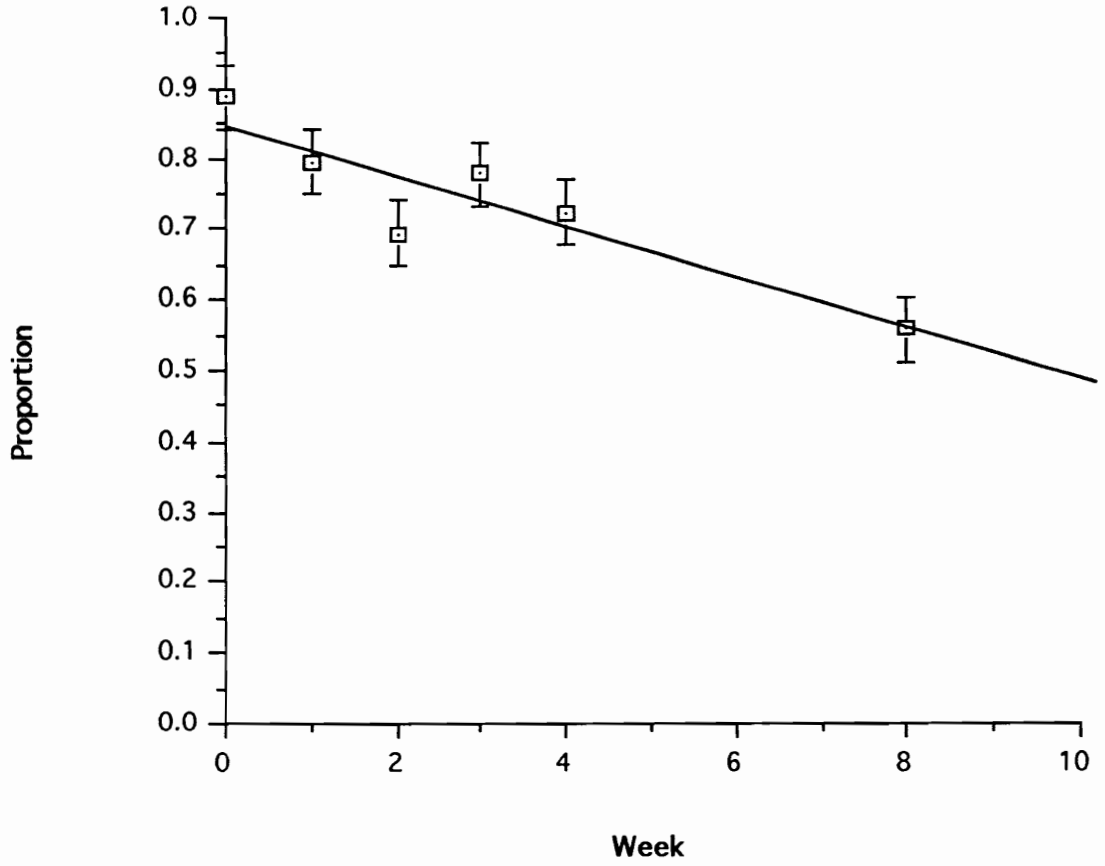


Figure 15. Correlation of Proportion (incl. Standard Error) of Panelists Choosing the Oil with the Enzyme Extract with Week (Part 1)

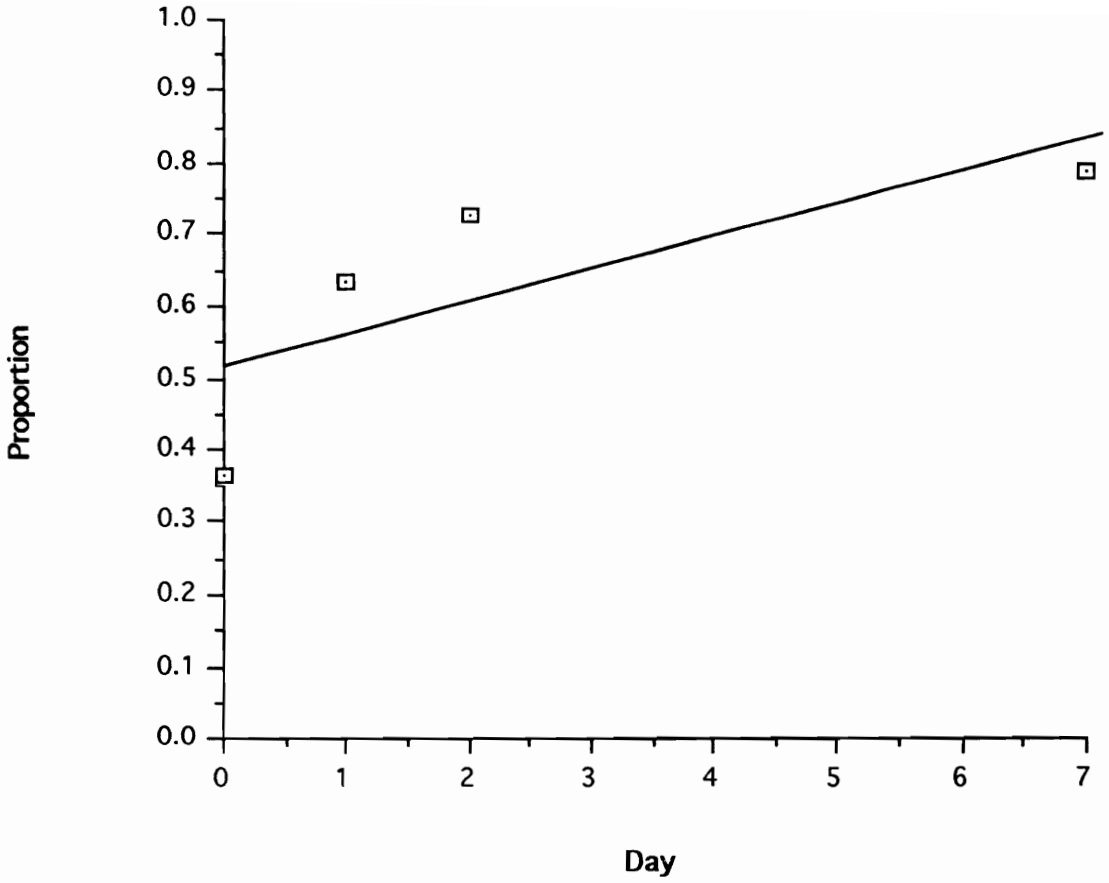


Figure 16. Correlation of Proportion of Panelists Choosing the Oil with the Enzyme Extract with Day (Part 2)

panelists continued to be able to detect a significant difference between the esters, while by week 8, this difference was not significant anymore.

Since the enzyme is extracted from menhaden, it was possible that the enzyme extract, even at a 1% level, contributes a fishy smell to the esters, that could have been picked up by the panelists. However, since the panelists were not able to distinguish between the esters at the very beginning of the study, it is likely that the 1% enzyme extract did not contribute a detectable smell of its own. However, within the first week the panelists were able to discriminate the ester with the enzyme extract as the stronger smelling ester, indicating a higher rate of lipid oxidation in this ester. Over time, this difference in the esters disappeared, and the panelists were not able to distinguish between the ester samples anymore. Since the only difference between the two samples was the addition of the lipoxygenase extract to one of them, it can be concluded that the extract changed the oxidation pattern of the esters, as assessed by sensory evaluation. Since after a short induction period, the panelists judged the ester with the extract as having a stronger odor than the ester without the extract, it is possible to conclude that the enzyme extract accelerated lipid peroxidation of the esters.

6.6.1. TBA Values of Esters Used for Sensory Evaluation

The TBA values of the individual ester samples are given in Appendix XVI. The results of the TBA analysis are presented in Table 9 and graphically depicted in Figure 17. The results of the ANOVA for the TBA values of the four treatments indicated that the treatments did not affect the TBA values. Tukey's studentized range test revealed a significant difference in TBA values of the esters only for week 2. The ester treated with the enzyme extract and esculetin had a significantly lower TBA value than the ester treated with the boiled or unheated lipoxygenase extract. However, the TBA value of the esters treated with the enzyme extract and esculetin was not lower than that of the control ester (Table 9). A paired t-test performed only on the control and the enzyme treated esters showed that there was no significant difference ($p = 0.5994$) in TBA value between the two esters used in the sensory analysis when all weeks were used in the analysis. Since the sensory panel found a difference between the esters from week 0 to week 4 but not for week 8, it was tried to delete week 8 from the analysis. This did not result in a

Table 9. TBA values of esters treated with and without enzyme extract, esculetin and boiled enzyme extract

Week	Control	Enzyme	Boiled Enzyme	Enz.+Esculetin
0	2629.4	2017.5	1828.8	1874.4
1	2257.2	2104.3	2044.2	2673.4
2	2084.9	2547.9	2631.3	1694.4*
3	756.4	913.9	970.9	804.1
4	1208.2	1253.5	1120.7	1101.3
8	3578.6	3271.8	3976.8	4092.3

* significantly ($p < 0.05$) lower than the TBA value of the "Enzyme" and "Boiled Enzyme" esters

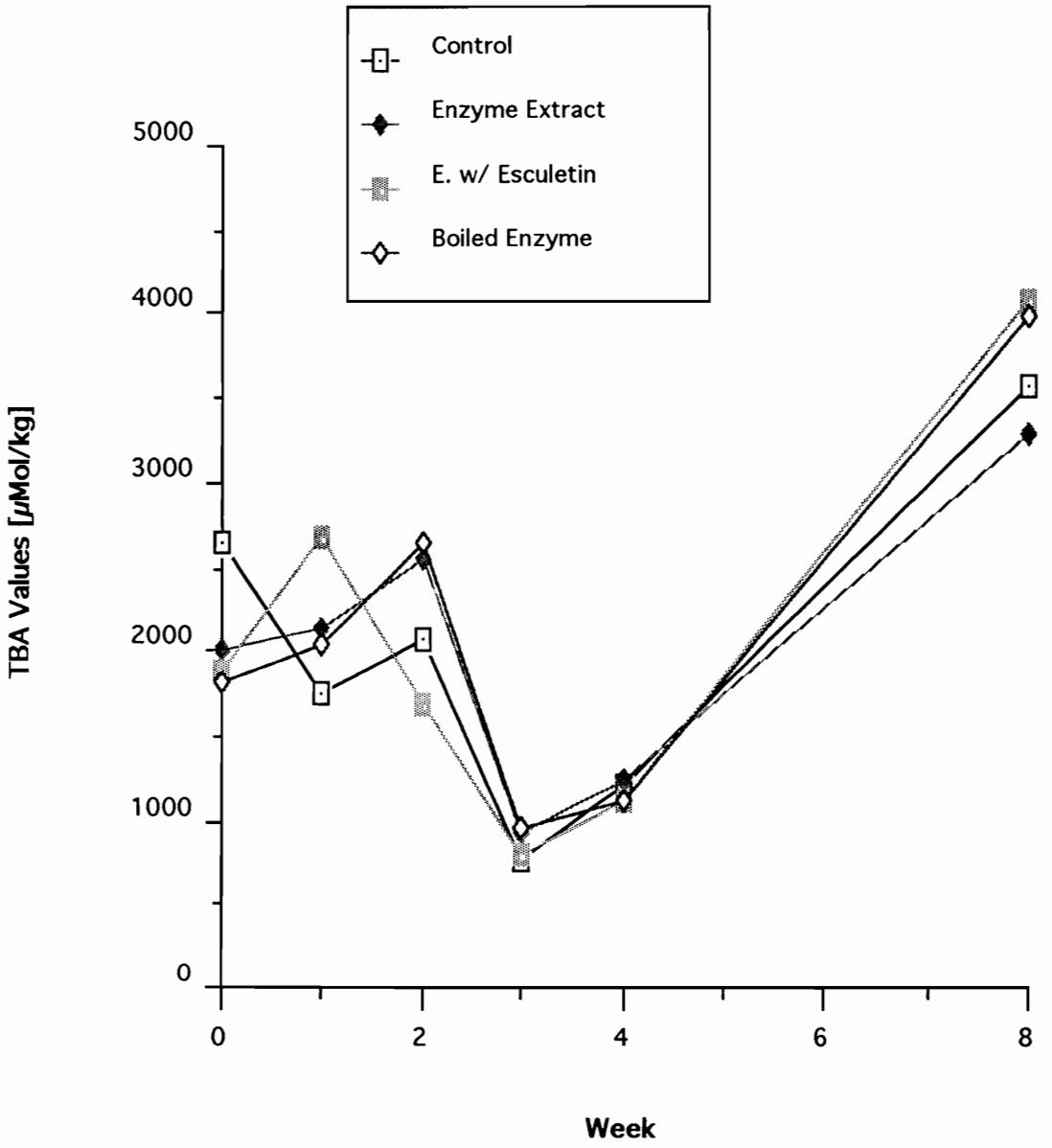


Figure 17. TBA values of n-3 fatty acid esters exposed to various treatments

significant difference ($p = 0.9261$) in TBA value between the esters either.

The TBA test did not reveal a difference in lipid peroxidation between the four esters. Thus, it must be concluded that the treatment of the esters with the unheated and boiled enzyme extract or with the esculetin inhibited enzyme extract did not affect their oxidative status. Since the TBA value correlated highly with sensory evaluation scores of various oils in a threshold analysis (Gray, 1978), the TBA analysis was the chemical analysis chosen to determine lipid peroxidation in the esters. The TBA analysis was performed only on the esters used in the first part of the sensory analysis, but not on the esters used in the second part due to an insufficient amount of esters. The paired t-test on the mean TBA values of the control and the enzyme treated esters revealed that the TBA value did not reflect the difference in the smell of the esters found by the sensory panelists. Sensory evaluation is considered the most sensitive of all lipid peroxidation assessment methods (Robards et al., 1988). It must be concluded that the differences in the esters were so small, that they were only distinguishable by sensory analysis but not by a chemical test such as TBA analysis. These findings imply that the chemical analyses commonly used to evaluate the lipid peroxidation status of oils might not be sensitive enough to capture small changes in oxidation that affect the sensory quality of the oils.

6.7. Lipoxygenase Activity

The menhaden gill lipoxygenase activity at various substrate concentrations is graphically depicted in Figure 18. As is typical for enzymatic reactions, the curve flattens at high substrate concentrations, approaching V_{max}. A double reciprocal plot (Lineweaver-Burk plot) (Figure 19) of the substrate concentration in Mol versus the reaction velocity in ΔAbsorbance $*$ min^{-1} $*$ mg protein^{-1}, reveals a linear relationship as is expected for an enzymatic reaction. The activity of menhaden gill lipoxygenase at various pHs is shown in Figure 20, and Figure 21 shows a Lineweaver-Burk plot of the enzyme activity at various esculetin concentrations. The data of the enzyme assays can be found in Appendix XVII.

From Figure 19, it is possible to calculate the K_m and V_{max} for the enzyme extract. While the y-intercept in a Lineweaver-Burk plot represents $1/V_{max}$, the x-intercept represents $-1/K_m$. Thus, the y-intercept value of 0.004 results in an apparent

V_{max} of 250 expressed as absorbance units per minute per mg protein, and the x-intercept at -60 results in an apparent K_m of 16.7 mMol. Ben-Aziz et al. (1970) observed that at higher substrate levels (>5 mMol) i.e. those used in this study, the rate of conjugated diene formation started declining within a minute, which could cause an overestimation of K_m. Therefore the experiment was repeated with lower substrate concentrations (0.5 -2.5 mMol) similar to those used in Ben-Aziz's study. The results are virtually identical to those found at higher substrate concentrations and are shown in Figure 19a. The x-intercept at -0.05 results in an apparent K_m of 20 mMol, and the y-intercept of 0.04 in an apparent V_{max} of 25 expressed as absorbance units per minute per mg protein (factor 10 is due to 0.1 mL of pipetted enzyme extract vs. 0.01 mL at higher substrate concentrations). Ben-Aziz's observation that the rate of conjugate diene formation over time declined much more rapidly at high substrate concentrations than at low substrate concentrations was also observed in this study (Figure 22), which can cause problems in accurately determining the initial rate of product formation when manual recording is used. However, since in this study the change in absorbance was recorded per second by a computer system, sufficient data points were available to accurately determine the initial rate of conjugated diene formation. It was also observed that at substrate concentrations below 0.5 mMol the conjugated diene formation was so slow that recording the change for quantification purposes was not possible.

Previous studies observed the K_m of different lipoxygenases to be between 20, 30, 40 μM and 2.8 mMol (Hsieh et al., 1988; German et al., 1986; Tappel et al., 1952; Al-Obaidy and Siddiqi, 1981). Ben-Aziz et al. (1970) calculated K_m values for soy bean lipoxygenase to be between 25 μMol and 0.61 mMol, based on the different Tween 20 concentrations used in the study, because Tween 20 was found to be a competitive inhibitor of lipoxygenase. The K_m of 0.61 mMol was found at the 0.25% level of Tween 20, which is the level that was used in this study. The K_m value found in this study was higher than those reported for other lipoxygenases, possibly because linoleic acid, which is found only in low concentrations in fish (Barlow and Young, 1989) is not a good substrate for menhaden lipoxygenase. Since the V_{max} is based on the protein concentration in the enzyme extract and there are many protein sources in the crude extract, a good comparison of the V_{max} value with those reported in the literature is not possible.

The crude gill lipoxygenase extract exhibited its highest activity at pH 9-10,

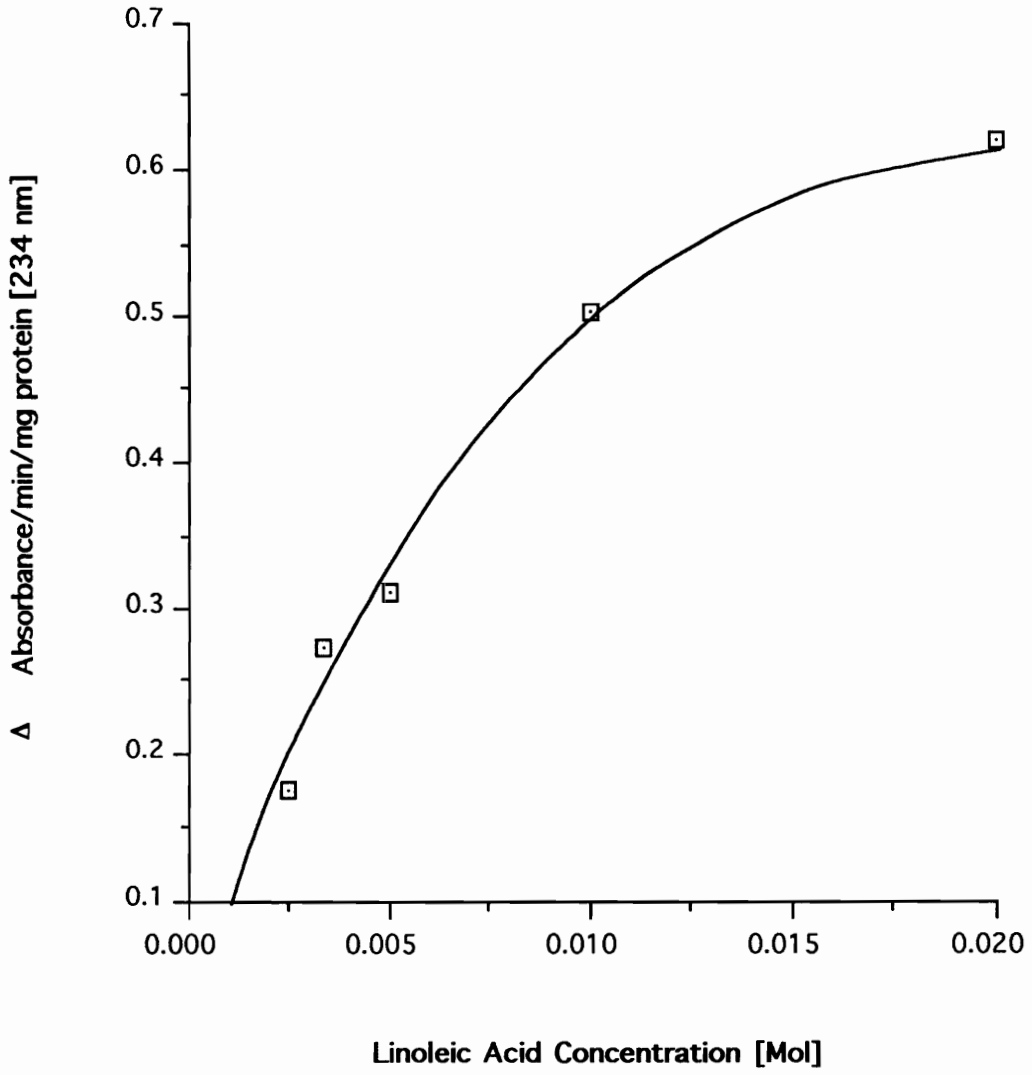


Figure 18. Lipoxygenase activity at various linoleic acid concentrations

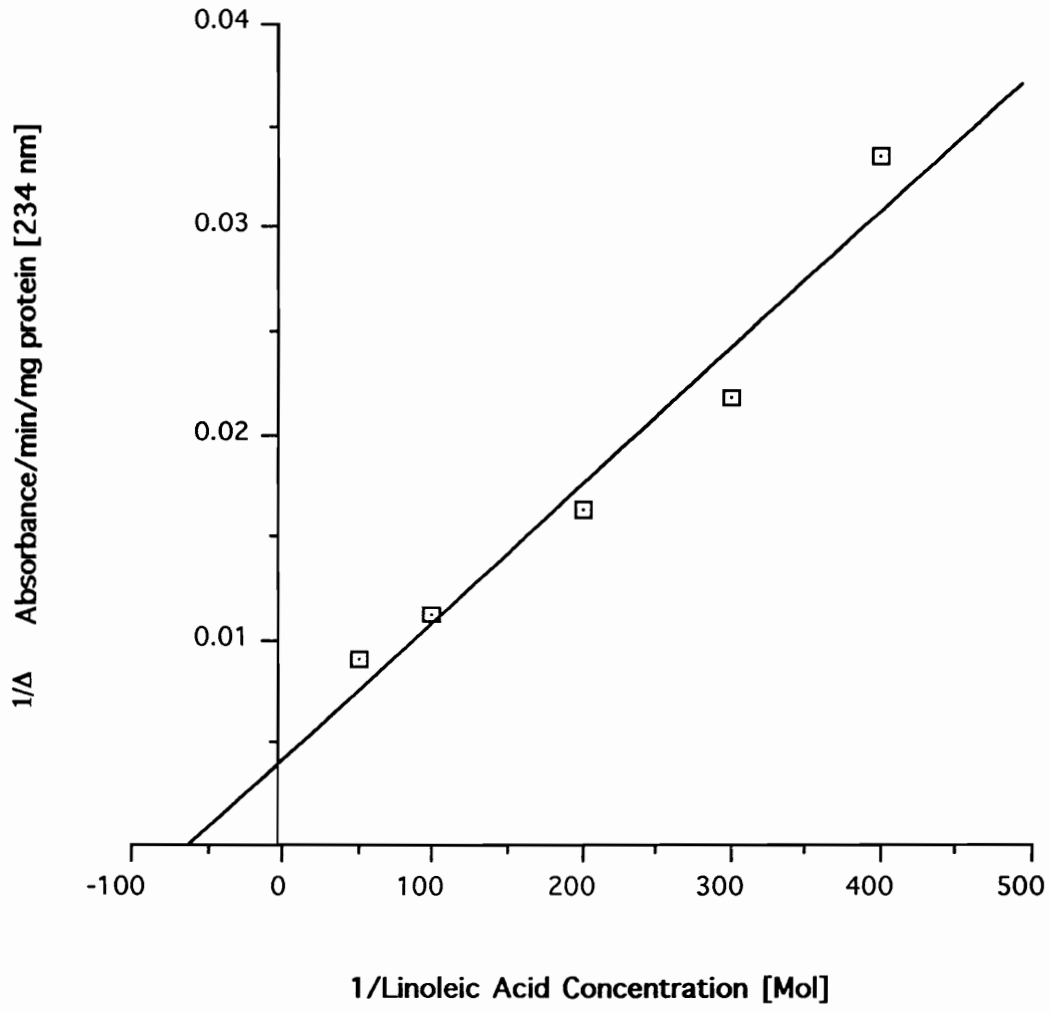


Figure 19. Lineweaver-Burk plot of lipoxygenase activity at high substrate concentrations

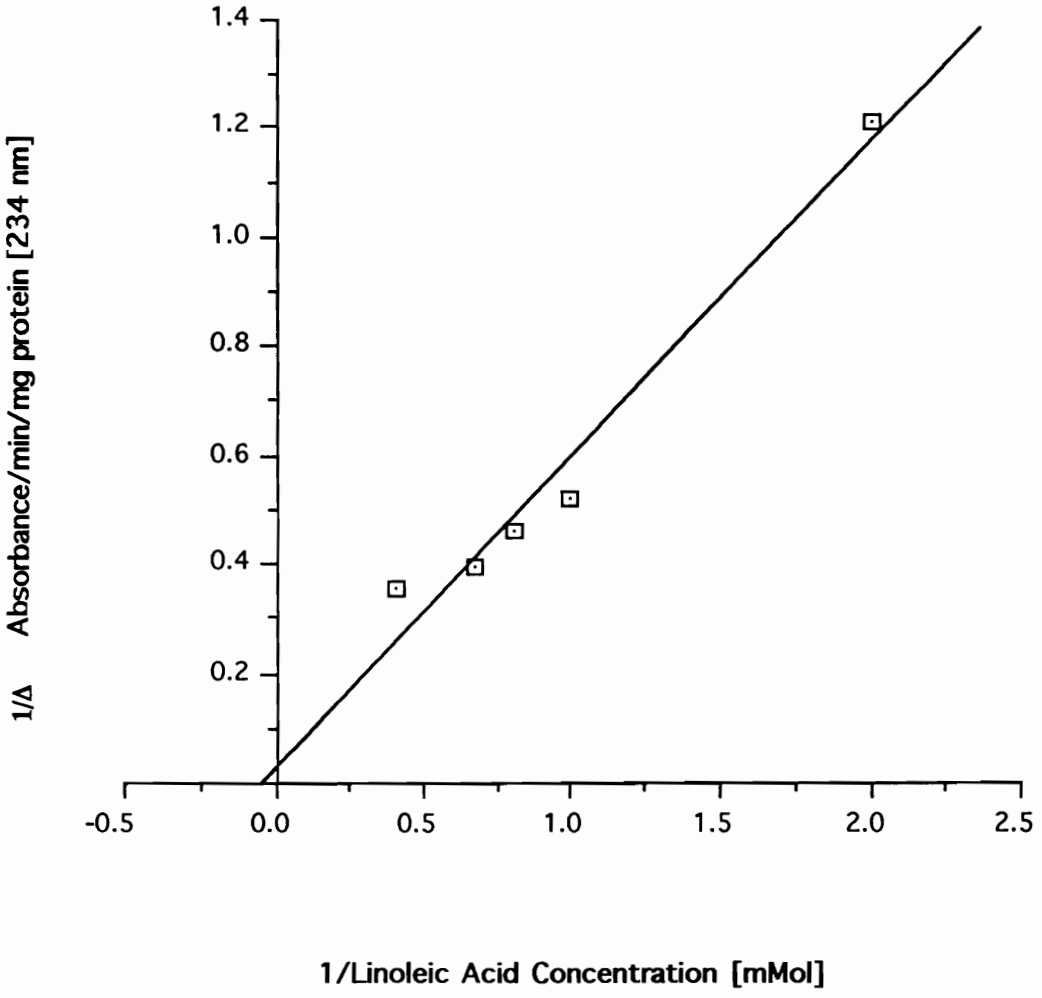


Figure 19a. Lineweaver-Burk plot of lipoxygenase activity at low substrate concentrations

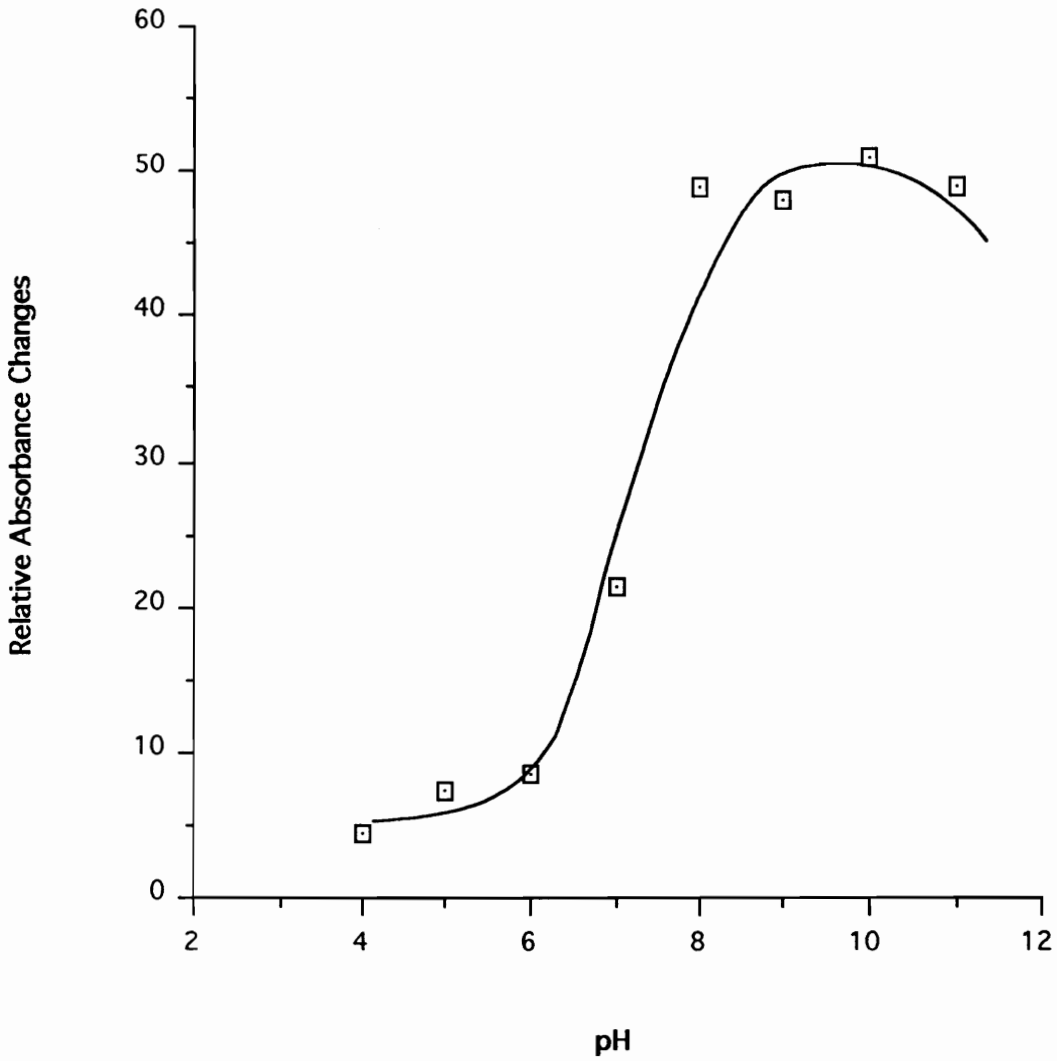


Figure 20. Lipoxygenase activity at various pHs

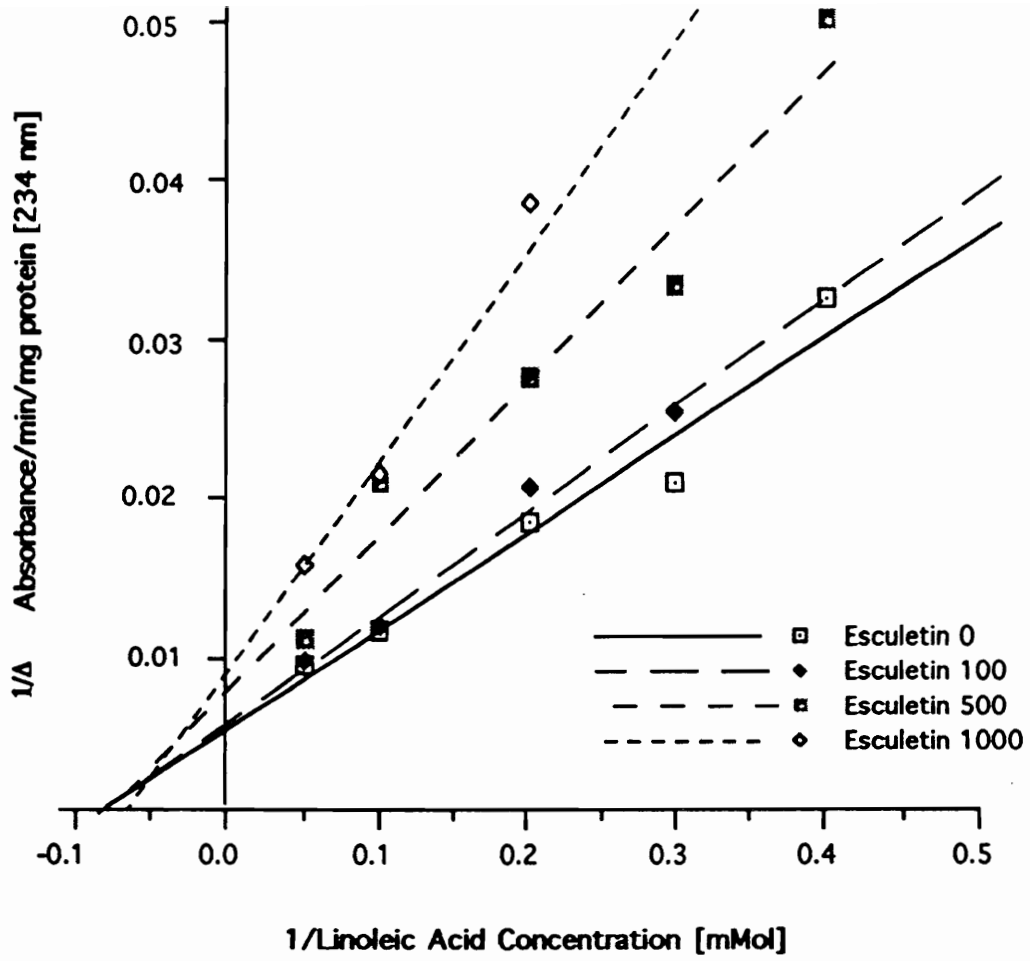


Figure 21. Lineweaver-Burk plot of lipoxygenase activity at various esculetin concentrations

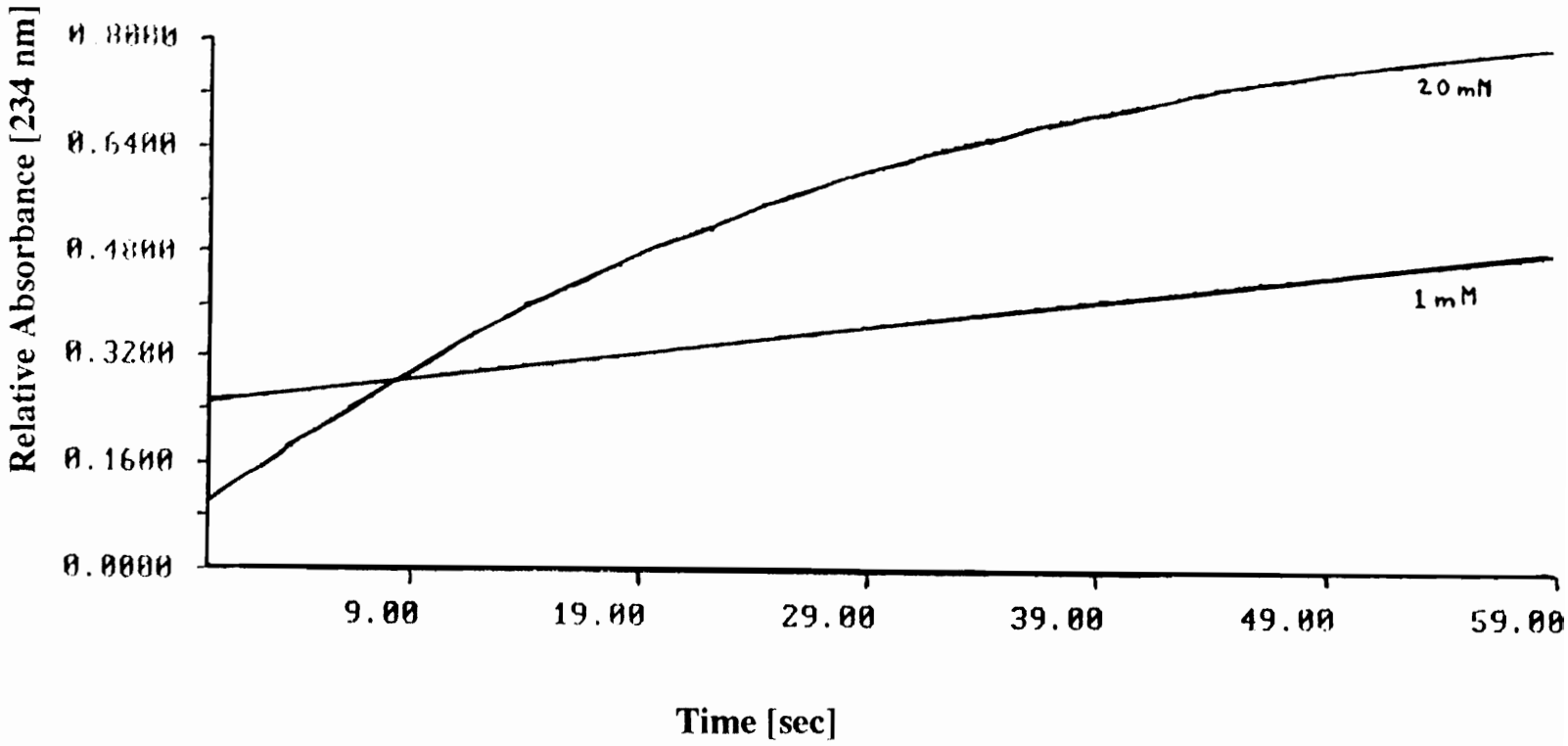


Figure 22. Time course of linoleate oxidation in the presence of the lipoxygenase extract at two different substrate concentrations

which is consistent with the literature reports that some lipoxygenases, such as the isozyme identified as lipoxygenase-1 in soybeans, show highest activity at alkaline pH (Christopher et al., 1970). However, it must be considered that the fish tissue under normal physiological conditions will not reach such alkaline levels. The 12-lipoxygenase characterized in trout gill had its highest activity at pH 7, but had a broad pH spectrum and was more sensitive to acidic pHs than to alkaline pHs (German et al., 1986; Hsieh et al., 1988). Non-heme iron as well as some heme-iron proteins have their highest catalytic effects at acidic pHs (Hsieh and Kinsella, 1989b; Ben-Aziz, et al. 1970), while this gill extract exhibited its highest activity at an alkaline pH. This is an indication that substrate oxidation was due to enzymatic activity in the extract and was not induced by non-heme or heme-iron which might also have been present in the unpurified extract.

According to the Lineweaver-Burk plot in Figure 21, esculetin is a non-competitive inhibitor of lipoxygenase activity, which is consistent with other reports in the literature (Sekiya et al., 1982). It has been shown that esculetin causes an instantaneous inhibition of lipoxygenase activity (Sekiya et al., 1982; Galpin et al., 1976) which indicates that esculetin does not function by chelating the iron atom of lipoxygenase (Sekiya et al., 1982). Inhibitors that function by chelating prooxidants, such as iron, show a lag time in their action (Dziezak, 1986). In a study that evaluated various catechols for their lipoxygenase inhibiting activities, Galpin et al. (1976) and did not find that the esculetin-like compound 7,8-dihydroxy-4-methylcoumarin inhibited soybean lipoxygenase by chelating the iron atom of the enzyme. Sekiya et al. (1982) found that the 6-carbon and 7-carbon hydroxyl groups of esculetin are important for lipoxygenase inhibition by coumarin derivatives. It has been hypothesized that esculetin function as a lipoxygenase inhibitor by scavenging free radicals (Sekiya et al., 1982; Neichi et al., 1983). This theory is supported by the fact that lipoxygenase requires hydroperoxides for its activation (Hsieh and Kinsella, 1989b).

6.8. Menhaden Oil Volatiles

A reproducibility analysis of the menhaden oil volatile extraction was done on four identical undeodorized menhaden oil samples. This analysis indicated that peak area was more reproducible than peak height based on the average standard deviation. The

standard deviation for peak area was dependent on the volatile compound and varied from 6.8 to 48.6 (coefficient of variation: 6.8% to 33.3%).

Representative GC chromatograms of an undeodorized and a deodorized menhaden oil sample are shown in Figure 23 and Figure 24 respectively. The volatiles identified in the undeodorized and deodorized menhaden oils including corresponding GC retention times and mass spectrometry scan numbers are given in Table 10 and Table 11 respectively. While in the GC analysis a large solvent peak prevented separation and therefore quantification of low boiling volatiles such as 2-methyl pentane or octane, the unique detection system of the mass spectrometer allowed the identification of the compounds. Peak areas of volatiles in undeodorized and deodorized menhaden oil can be found in Appendices XVIII and XIX respectively. Typical derivations and identifications of mass spectra of two volatiles (hexanal and benzaldehyde) are shown in Figure 25 and 26 respectively. The top box of the figures shows the mass spectrum of the unknown volatile. The second box represents the mass spectrum after subtracting the background and the third box shows the library match for the mass spectrum of the volatile. It can be seen that for a compound in high concentrations, such as hexanal, the background subtraction has little effect on the mass spectrum, whereas for a compound that is present in small concentrations the background subtraction was crucial for proper identification of the volatile.

A comparison of volatiles identified in this study with the findings in the literature is shown in Tables 12 and 13. A large number of volatiles that were previously identified in menhaden oil (Hsieh, T.C.-Y. et al., 1989; Karahadian and Lindsay, 1989a; Lin et al., 1990) were also found in this study (Table 12). Many additional volatiles, some of which have been found in the volatile spectrum of other seafood (Cha et al., 1992; St Angelo et al., 1987; Heil and Lindsay, 1988; Josephson et al., 1983, 1984b, 1984c, 1985, 1991) have also been identified in menhaden oil in this study (Table 13).

It was not possible to identify all compounds using the automated computer program linked to the mass spectrometer due to the following reasons:

1. the library match was less than 80% - the agreement between the unknown and the library match was below 80%, and while this match might be correct, without retention indices backing it up, the match was rejected;

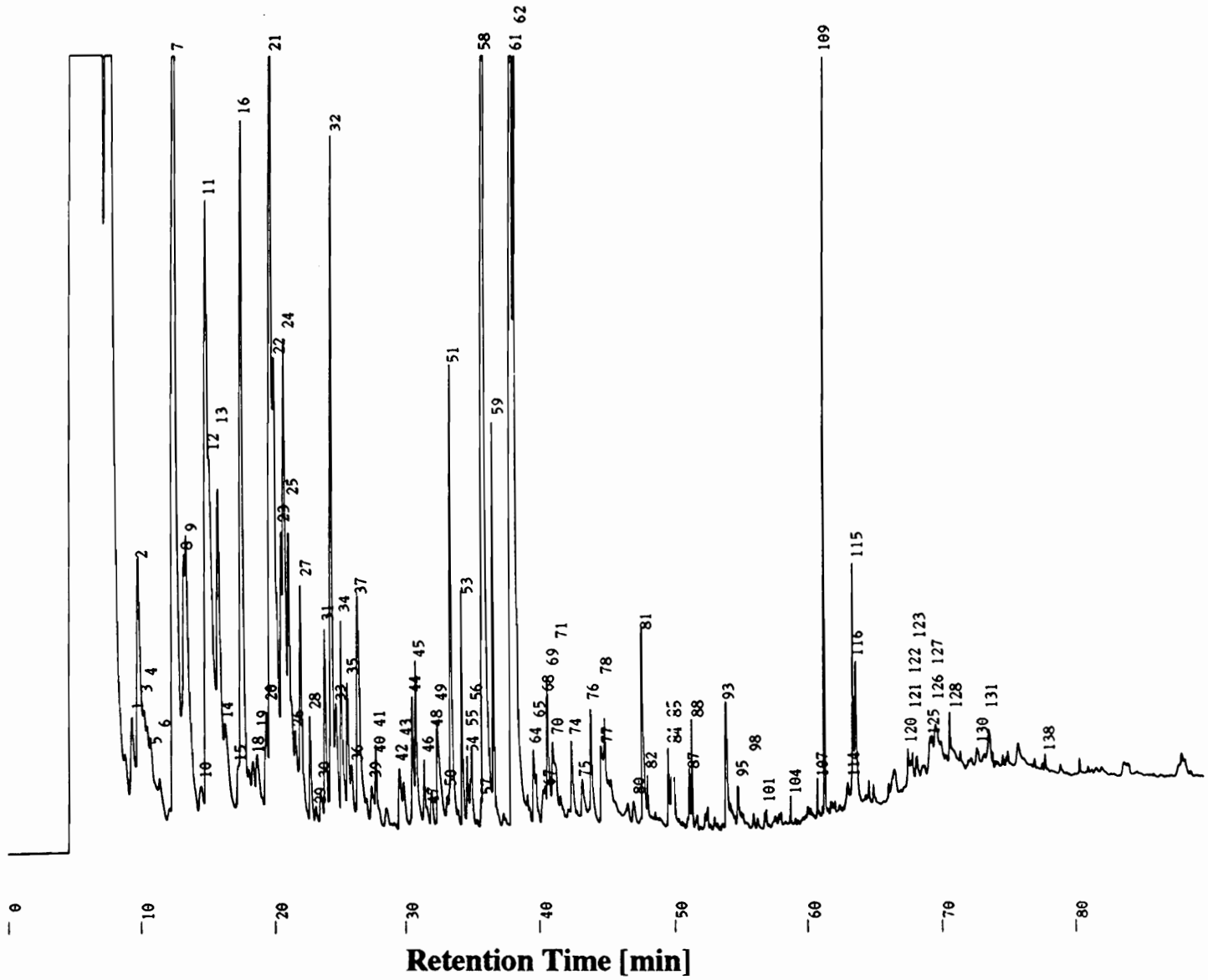


Figure 23. GC chromatogram of undeodorized menhaden oil volatiles at week 3 of storage

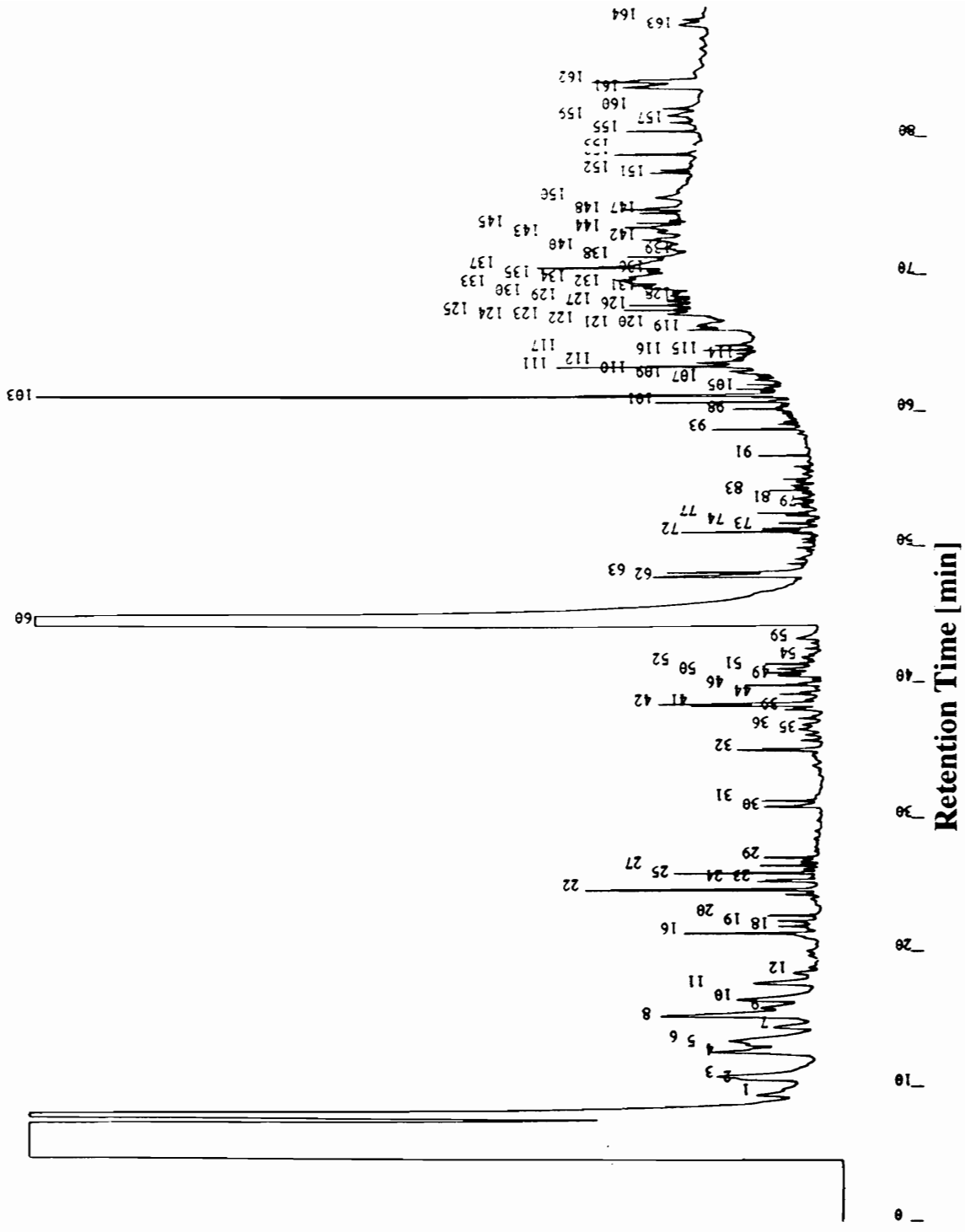


Figure 24. GC chromatogram of deodorized menhaden oil volatiles at week 3 of storage

Table 10. Volatiles identified in undeodorized menhaden oil

Compound Name	Peak No. Fig22	Week 0		Week 3	
		MS Scan	RT	MS Scan	RT
2-Methyl pentane	n.s.	189	n.s.	188	n.s.
Octane	n.s.	238	n.s.	237	n.s.
2-Octene	n.s.	266	n.s.	263	n.s.
Acetic Acid, Ethyl Ester	n.s.	283	n.s.	284	n.s.
3-Methyl 1,4-heptadiene	n.s.	301	n.s.	308	n.s.
3-Methyl 1,4-heptadiene	n.s.	311	n.s.	317	n.s.
Ethanol	n.s.	330	n.s.	n.p.	n.s.
Benzene	n.s.	n.p.	n.s.	331	n.s.
Pentanal	0	374	8.9	367	8.9
Decane	1	384	9.4	383	9.4
2,3-Dimethyl 1,4-hexadiene	2	409	9.9	407	9.9
1-Penten-3-one	3	424	10.3	420	10.3
3-Ethyl 1,4-hexadiene	4	432	10.6	429	10.5
Methyl benzene	5	450	10.8	446	10.8
Hexanal	7	510	12.5	507	12.5
1,3,6-Octatriene (E,E)	8	537	13.3	535	13.4
1,3,6-Octatriene (Z,E)	9	542	13.4	540	13.4
Ethyl benzene	10	582	14.5	583	14.5
2-Pentenal	11	593	15.0	589	14.8
1,4-Dimethyl benzene	12	612	15.2	610	15.2
1-Penten-3-ol	13	629	15.8	626	15.8
2-Methyl 2-pentenal	14	646	16.7	644	16.3
Heptanal	16	691	17.6	687	17.5
4,5-Dimethyl 1-hexene	18	718	18.5	716	18.4
4-Methyl 3-pentenal	19	726	18.8	724	18.7

(Table 10 cont'd. Volatiles identified in undeodorized menhaden oil)

Compound Name	Peak No. Fig22	Week 0		Week 3	
		MS Scan	RT	MS Scan	RT
2-Hexen-3-one	20	752	19.5	750	19.4
2-Hexenal	21	761	19.7	758	19.7
Unknown*§	22	779	20.0	778	20.0
Unknown§	23	799	20.6	798	20.6
4-Heptenal (Z)	24	805	20.8	803	20.8
Unknown*§	25	824	21.2	824	21.1
Unknown*§	27	839	22.1	838	22.0
Unknown*§	28	864	22.8	863	22.7
1,3,5-Trimethyl benzene	30	891	23.6	890	23.5
Octanal	31	901	23.9	899	23.9
Unknown*	32	925	24.4	924	24.4
2-Penten-1-ol (E)	34	937	25.2	936	25.1
2-Penten-1-ol (Z)	35	954	25.8	953	25.7
2-Heptenal (Z)	37	980	26.6	979	26.5
6-Octen-2-one	39	992	27.2	991	27.1
1-Hexanol	41	1020	27.9	1019	27.8
2-Methyl 2-cyclopenten-1-one	42	1081	29.7	1079	29.6
3-Hexen-1-ol (Z)	43	1091	30.0	1092	29.9
2-Nonanone	44	1111	30.6	1109	30.6
Nonanal	45	1122	30.9	1121	30.8
2,4-Hexadienal (E,E)	49	1151	32.5	1150	32.5
2-Octenal	51	1203	33.6	1202	33.5
1-Octen-3-ol	53	1225	34.4	1225	34.3
2,4-Heptadienal (E,Z)	58	1279	35.9	1277	35.9

(Table 10 cont'd. Volatiles identified in undeodorized menhaden oil)

Compound Name	Peak No. Fig22	Week 0		Week 3	
		MS Scan	RT	MS Scan	RT
Unknown§	59	1303	36.7	1302	36.7
Pentadecane	61	1327	38.1	1325	38.0
2,4-Heptadienal (E,E)	62	1341	38.4	1339	38.3
Benzaldehyde	65	1409	40.8	1409	40.8
2-Nonenal	68	1423	41.2	1422	41.2
Unknown	74	1448	41.6	1448	42.4
2,4-Octadienal (Z,Z)	75	1477	42.6	1476	42.6
3,5-Octadiene-2-one (E,E)	76	1496	43.4	1495	43.4
2,6-Nonadienal (E,Z)	77	1529	44.8	1528	44.7
2,4-Octadienal (E,E)	78	1537	45.1	1537	44.9
2-Decenal (Z) (Internal Std.)	81	1639	47.9	1638	47.8
Hexadecane	84	1687	49.8	1687	49.7
2,5 Octadien-1-ol	85	1696	50.1	1697	49.9
Heptadecane	88	1729	51.5	1715	51.3
1,4-Cyclohexadiene	93	1825	54.0	1826	53.9
5-Ethyl-2(5H)-furanone	95	1846	54.9	1847	54.8
2,4-Decadienal (E,E)	98	1909	57.0	1908	57.0
Antioxidant	109	2030	61.4	2031	61.3
1-Dodecanol	115	2081	63.5	2081	63.5

Italics: Lipoxygenase derived volatiles (Hsieh and Kinsella, 1989a)

Unknown*: Mass spectra of unknown shown in Figures 28 to 32

n.s.: these volatiles were not separated by GC but by GC-MS

£: coeluting compounds

§ coumpound also found in n-3 fatty acid ester concentrates

Table 11. Volatiles identified in deodorized menhaden oil

Compound Name	Peak No. Fig23	Week 0		Week 3		Week 4	
		MS Scan	RT	MS Scan	RT	MS Scan	RT
2-Methyl pentane	n.s.	188	n.s.	188	n.s.		
Octane[£]	n.s.	238	n.s.	238	n.s.		
Acetic Acid, Ethyl Ester	n.s.	286	n.s.	282	n.s.	372	n.s.
Ethanol	n.s.	310	n.s.	317	n.s.	412	n.s.
Benzene	n.s.	330	n.s.	331	n.s.	424	n.s.
Decane	1	378	9.2	379	9.3		
Methyl benzene	3	443	10.8	444	10.8		
5-Ethyl 2-methyl octane	n.s.	496	12.2	497	12.2		
Hexanal	4	506	12.6	506	12.6		
Undecane	5	517	13.1	517	13.1	624	n.i.
1,3,6-Octatriene[£]	6	530	13.5	531	13.5	666	13.5
Ethyl benzene	7	582	14.4	582	14.4		
1,4-Dimethyl benzene	8	606	15.3	608	15.3		
2-Methyl 2-pentenal	10	640	16.4	641	16.4		
1,3-Dimethyl benzene	11	695	17.5	696	17.5		
Unknown**	16	780	21.5	779	21.4		
Unknown**	18	791	22.0	790	21.9		
Unknown**	19	809	22.4	810	22.3		
Unknown**	20	824	22.8	823	22.8		
Ethenyl benzene	21	-	-	835	23.2		
Unknown**	22	892	24.8	891	24.7		
Unknown**	23 24	912	25.4 25.5	911	25.4 25.5		
Unknown**	25	932	26.0	931	26.0		
Unknown	26	940	26.2	939	26.2		

(Table 11 cont'd. Volatiles identified in deodorized menhaden oil)

Compound Name	Peak No. Fig23	Week 0		Week 3		Week 4	
		MS Scan	RT	MS Scan	RT	MS Scan	RT
Unknown	27	957	n.i.	958	26.6		
2-Heptenal£	29	976	27.1	975	27.1		
Octanoic Acid, Methyl Ester	30	-	n.i.	-	30.8	1292	30.8
2-Nonanone	31	1110	n.i.	1110	31.4		
Tetradecane	32	1197	35.2	1196	35.1		
Butanoic Acid	33	-	-	1216	35.8	1454	35.7
1-Octen-3-ol£	34	-	-	1229	36.2		
2,4-Heptadienal	36	-	-	1275	36.9		
Unknown**	42	1306	38.6	1304	38.5		
Pentadecane	44	1321	n.i.	1320	39.2		
2,4-Heptadienal (E,E)	45	-	n.i.	1336	39.4		
Unknown	46	1357	40.0	1356	39.9		
Dimethyl hexadecane	49	1377	n.i.	1376	40.5		
1,3-Butanediol	50	1396	40.8	1394	40.8	1656	40.8
Benzaldehyde	51	1402	n.i.	1405	41.0		
2-Nonenal£	52		41.5	1417	41.4		
2,3-Butanediol	59	-	-	-	-	1739	43.2
1,2-Propanediol	60	1546	44.3	1536	44.3	1784	44.4
2-Decenal (Z) (Internal Std.)	62	1637	47.9	1635	47.9		
Hexadecane	72	1685	51.3	1683	51.2		
Unknown*	77	1712	n.i.	1714	52.5		
Heptadecane	79	1744	53.2	1744	53.2		
Unknown*	83	1811	n.i.	1814	54.3		
Unknown*	91	1899	n.i.	1898	56.8		
Unknown* (Octadecane)	93	1963	58.9	1962	58.8		

(Table 11 cont'd. Volatiles identified in deodorized menhaden oil)

Compound Name	Peak No. Fig23	Week 0		Week 3		Week 4	
		MS Scan	RT	MS Scan	RT	MS Scan	RT
Unknown	98	1982	n.i.	1980	60.3		
2-Ethyl 1-Decanol	101	1998	60.8	1997	60.8		
Antioxidant	103	2029	61.3	2028	61.3		
1-Dodecanol	111	2077	63.5	2078	63.5		
1-Dodecene	112	2105	64.0	2104	63.8		
Tetradecanol	116	2112	n.i.	2113	64.6		
Unknown*	117	2118	65.0	2118	65.0		
Unknown* (Eicosane)	119	2168	n.i.	2168	66.1		
Ethoxy Benzoic Acid, Ethyl Ester	137	2316	70.8	2317	70.7		
Unknown*	142	2361	n.i.	2362	72.7		
Unknown*	144	2406	n.i.	2406	73.7		
Unknown*	145	2415	74.0	2416	73.9		
Unknown* (Docosane)	147	2433	74.7	2433	74.7		
Phenol Compound	148	-	75.0	2456	74.9		
Unknown	155	2570	80.6	2569	80.4		
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	160	-	-	2675	81.5		
Dodecanoic Acid	161	2717	n.i.	2723	83.6		

£ compounds not pure (i.e. separation not complete)

* Library matches for all these compounds were octadecane, eicosane, docosane, 2,6,10,14-Tetramethylhexadecane, 2,6,10,15-Tetramethylheptadecane, 2,6,10-Trimethyltetradecane, 10-methyleicosane, 2-methylhexadecane, 7,9-dimethyl hexadecane

** Library matches for all these compounds were 4,7-dimethylundecane, 2,6,11-trimethyldodecane, 3,7-dimethylundecane, 2,7,10-trimethyldodecane, 4,6-dimethyldodecane, 5-ethyl-2-methyl-octane, 3,8-dimethylundecane, 3,5-dimethyl undecane, 5,7-dimethylundecane, 2,7-dimethylundecane, 5-ethyl-5-methyl-decane, 3,6-dimethylundecane, 3,6-dimethyldecane

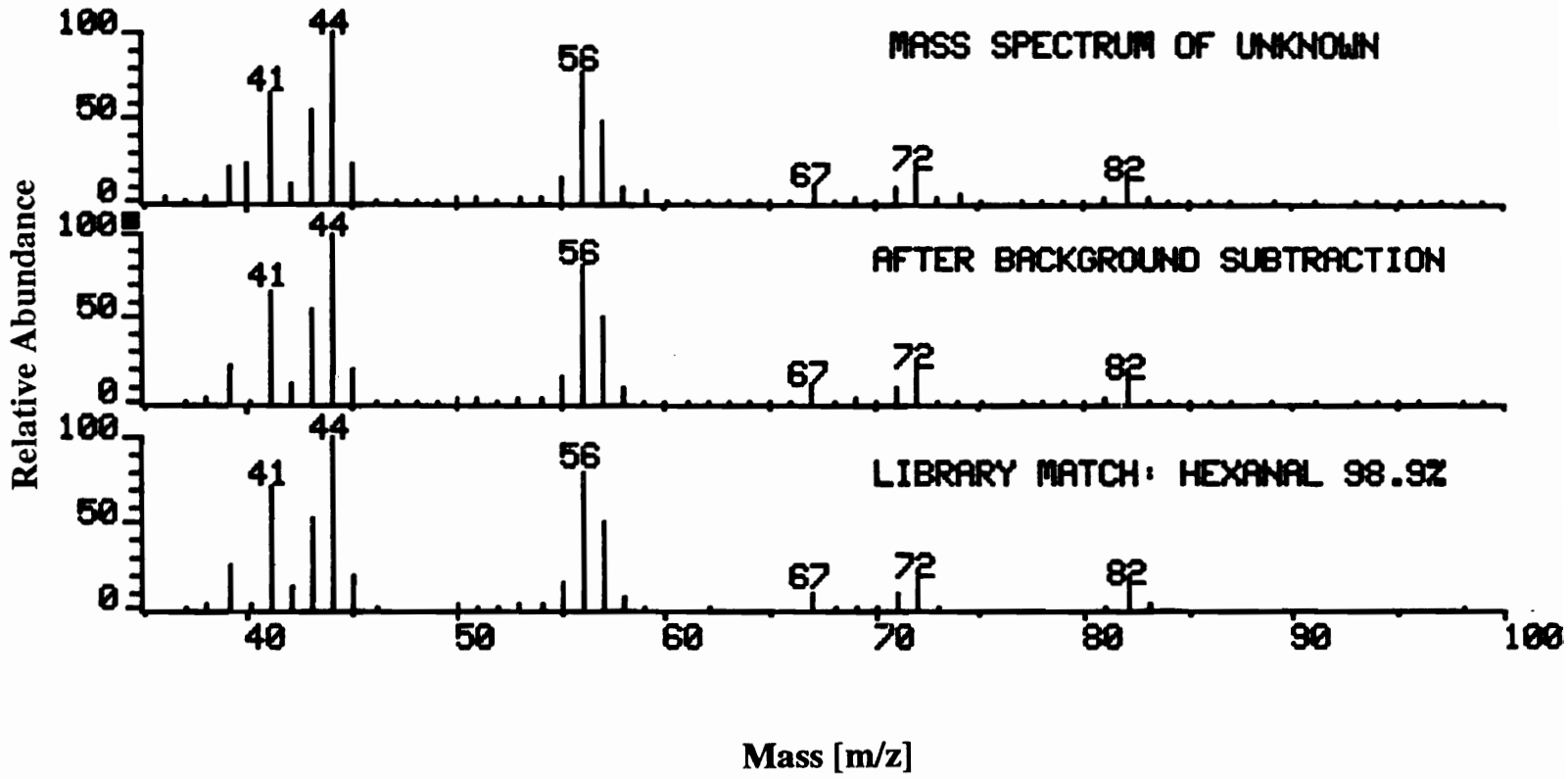


Figure 25. Mass spectrum of unknown volatile eluting at RT=12.5 minutes, before and after background subtraction with library match of hexanal

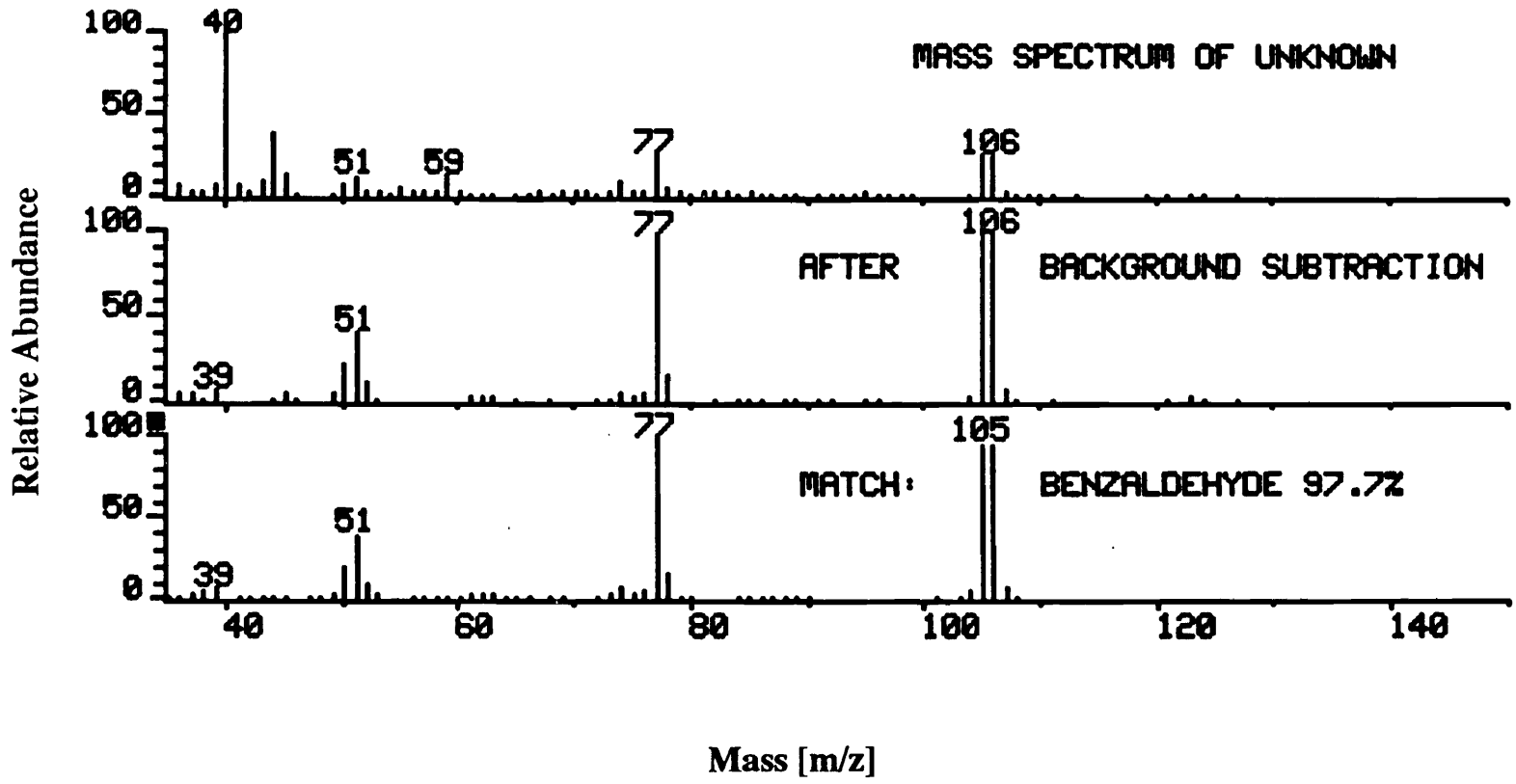


Figure 26. Mass spectrum of unknown volatile eluting at RT=40.4 minutes, before and after background subtraction with library match of benzaldehyde

Table 12. Volatiles identified in this study and reported in the literature for menhaden oil

Volatile Compound	MS Scan	Match [%]	1 [RI] *	2 [I_E] **	3 [RT] ***	4 [RT]
Octane	238	91.6	800		6.5	
Benzene	331	89.1			10.4	
Pentanal	374	55.7	979	3.31	12.3	15.13
Decane	384	83.6	1000	3.46	13.3	
2-Butanol	416	90.2			17.1	
1-Penten-3-one	424	80.1	1022	3.67	15.3	
Hexanal	510	95.8	1085	4.49	21.2	21.16
Undecane	521	76.6	1100		22.4	
1,3,5-Octatriene	542	83.1		4.69		
Ethyl benzene	582	92.4			25.8	
2-Pentenal (E)	593	94.3	1130	4.90		
1-Penten-3-ol	626	79.7		5.27		
Heptanal	691	91.2	1186	5.46	33.6	27.49
Dodecane	696	70.9	1200		35.2	
2-Hexenal	761	85.5	1218		37.9	
4-Heptenal (Z)	805	84.3		6.03		
1,3,5-Trimethyl benzene	890	74.6	1242		41.1	
Octanal	900	87.7	1290	6.52		
2-Penten-1-ol (E)	937	91.8		6.66	50.8	
2-Heptenal	980	89.1	1323	6.85	52.1	31.59
1-Hexanol	1020	94.6			56.3	
2-Nonanone	1111	88.4	1390	7.51		
Nonanal	1122	80.1	1395	7.55	61.7	41.48
2,4-Hexadienal (E,E)	1150	86.5	1402	7.60		
2-Octenal	1202	68.3	1429	7.91		38.49
1-Octen-3-ol	1225	85.4		8.10	69.4	
2,4-Heptadienal (E,Z)	1279	86.3	1467	8.24	71.2	34.35
Pentadecane	1325	87.2	1500		76.1	
2,4-Heptadienal (E,E)	1341	82.3	1493	8.52	74.8	35.00
3,5-Octadiene-2-one (E,Z)	1390	81.8		8.80		39.62
Benzaldehyde	1409	97.7	1521	8.89	78.5	
2-Nonenal	1423	77.9	1535	8.95	80.5	44.86
2,4-Octadienal (Z,Z)	1477	91.0	1563		83.5	
3,5-Octadiene-2-one (E,E)	1496	80.1	1570	9.30	84.5	
2,6 Nonadienal (E,Z)	1528	81.2	1587	9.44		
Propanediol	1530	95.1			86.2	
2,4-Octadienal (E,E)	1537	82.4	1590		87.1	

(Table 12. cont'd. Volatiles identified in this study and reported in the literature for menhaden oil)

Volatile Compound	MS Scan	Match [%]	1 [RI] *	2 [I_E] **	3 [RT] ***	4 [RT]
Hexadecane	1687	79.1	1600		88.3	
Heptadecane	1715	83.5	1700		101.1	
5-Ethyl-2(5H)-furanone	1846	87.5	1755			
2,4-Decadianal (E,E)	1908	77.3	1764	11.62		53.26
1-Dodecanol	2081	91.6			129.6	

* RI = Retention Index
** I_E = Retention Indices
*** RT = Retention Time

Italics: compounds identified as lipoxygenase derived by Hsieh and Kinsella, 1989a

1 Hsieh T.C.-Y. et al., 1989. (Undeodorized menhaden oil)
Supelcowax 10 (60m, 0.32mm i.d, 0.25μm film);
5 min hold at 40°C, 1°C/min to 175°C

2 Karahadian &Lindsay, 1989a. (Deodorized menhaden oil)
Carbowax 20M (60m, 0.25mm i.d.)
1 min hold at 50°C, 4°C/min to 220°C

3 Lin et al., 1990. (Crude menhaden oil)
Supelcowax 10 (60m, 0.25mm i.d, 0.25μm film);
5 min hold at 40°C, 1°C/min to 175°C, 5°C/min 195°C

4 St Angelo et al., 1987. (Crude menhaden oil)
SE 54 (no further information)
-30°C no hold, 10°C/min to 30°C, 2.5°C/min to 150°C, 5°C/min to 250°C

Table 13. Volatiles identified in this study and reported in the literature for various fish species other than menhaden

Volatile Compound	MS Scan	Match [%]	5 [I_E]*	6 [I_E]	7 [I_E]	8 [I_E]	9 [I_E]	10 [RI]**	11 [I_E]
Ethanol	317	98.9		2.66		2.66	3.00		
Pentanal	331	89.1		3.31	3.31				
Decane	374	55.7			3.46				
2,3-Dimethyl 1,4-hexadiene	409	88.0						1273	
2-Butanol	416	90.2					3.78		
1-Penten-3-one	424	80.1		3.67	3.67	3.67			
Hexanal	510	95.8	4.49	4.49	4.49		4.49	1080	4.49
Undecane	521	76.6		4.81	4.81			1098	
1,3,5-Octatriene	542	83.1		4.87		4.87	4.64		
2-Pentenal (E)	593	94.3		5.07		5.07	4.97	1127	
1,3-Dimethyl benzene	612	94.3		5.17	5.36				
1-Penten-3-ol	626	81.0	5.08	5.23	5.27	5.23	5.24	1156	5.03
2-Methyl 2-pentenal	646	85.0	5.24	5.30	5.21				5.37
Heptanal	691	91.2	5.51	5.50	5.46		5.46	1185	5.63
Dodecane	696	70.9	5.50		5.64				5.76
2-Hexenal	761	85.5	5.83	5.81			5.81	1217	
4-Heptenal (Z)	805	84.3					6.08	1242	
1,3,5 Trimethyl benzene	890	74.6		6.12	6.37				6.10
Ethenyl benzene	835	81.7	6.26						
Octanal	900	87.7	6.57		6.52		6.52	1290	6.52
2-Penten-1-ol (E)	937	91.8						1310	
2-Heptenal	980	89.1			6.85				6.84
1-Hexanol	1020	94.6	7.14	7.14	7.18		7.12	1351	7.18
2-Nonanone	1111	88.4			7.51		7.64	1391	
Nonanal	1122	80.1	7.62	7.62		7.62	7.68	1396	7.61
2,4 Hexadienal (E,E)	1150	86.5			7.60			1404	7.69
2-Octenal	1202	68.3			7.91				7.91
1-Octen-3-ol	1225	85.4	8.12	8.15	8.15	8.12	8.07	1450	8.15
2,4-Heptadienal (E,Z)	1279	86.3		8.24	8.24	8.24			
Pentadecane	1325	87.2		8.75	8.75				8.75
2,4-Heptadienal (E,E)	1341	82.3		8.52	8.52	8.52		1432	
3,5-Octadiene-2-one (E,Z)	1390	81.8		8.80	8.80				
Benzaldehyde	1409	97.7	8.69	8.91	8.73	8.69		1530	8.85
2-Nonenal	1423	77.9	9.00	9.00	8.95	9.00	9.00	1540	8.95
2,3-Butanediol	1495	91.7	9.15						
3,5-Octadiene-2-one (E,E)	1496	80.1		9.30	9.30	9.30		1575	9.21
2,6 Nonadienal (E,Z)	1528	81.2	9.43	9.43	9.44	9.43	9.46	1592	
Hexadecane	1687	79.1	9.74		9.70				9.72
2,5-Octadien-1-ol	1696	n.a.	10.34	10.34	10.35	10.34	10.36		10.40
Heptadecane	1715	83.5	10.67	10.67	10.65			1702	10.75

(Table 13 cont'd. Volatiles identified in this study and reported in the literature for various fish species other than menhaden)

Volatile Compound	MS Scan	Match [%]	5 [I_E]*	6 [I_E]	7 [I_E]	8 [I_E]	9 [I_E]	10 [RI]**	11 [I_E]
2,4-Decadianal (E,E)	1908	77.3	11.62	11.62			11.62	1815	
Octadecane	1941								12.36
Eicosane	2175								13.73
Tridecanol	2189	81.6							13.81
Docosane	2463								13.73
Dodecanoic acid	2723	97.2							18.1

* I_E = Retention Indices

** RI = Retention Index

Italics: compounds identified as lipoxygenase-derived by Hsieh and Kinsella, 1989a

5 Josephson et al., 1983. (Fresh Whitefish)
Carbowax 20M (60m, 0.31mm i.d.)
0.5 min hold 50°C, 5°C/min to 100°C, 10°C/min to 220°C

6 Josephson et al., 1984b. (Emerald Shiner)
Carbowax 20M (60m, 0.31mm i.d.)
5 min hold at 50°C, 5°C/min to 140°C, 10°C/min to 220°C

7 Josephson et al., 1984c. (Oxidized frozen Whitefish)
Carbowax 20M (60m, 0.25mm i.d.)
5 min hold at 50°C, 5°C/min to 140°C, 10°C/min to 220°C

8 Josephson et al., 1985. (Oysters)
Carbowax 20M (60m, 0.25mm i.d.)
5 min hold at 50°C, 5°C/min to 140°C, 10°C/min to 220°C

9 Josephson et al. 1991. (Crayfish waste)
Supelcowax 10 (60m; 0.25mm i.d.)
5 min hold at 50°C, 6°C/min to 140°C; 10°C/min to 220°C

10 Cha et al., 1992.
Supelcowax 10 (60m, 0.25mm i.d, 0.25μm film);
5 min hold at 40°C, 2°C/min to 175°C, 30 min hold, 5°C/min 195°C

11 Heil and Lindsay, 1988 (Walleye and Northern Pike)
Carbowax 20M (60m, 0.32mm i.d.)
1 min hold at 50°C, 5°C/min to 140°C, 10°C/min to 220°C)

2. the background subtraction was incorrect - this happened mainly due to difficulties of the computer system to identify the proper baseline for a particular peak and caused background ions not belonging to the compound of interest to be part of the mass spectrum, which made the mass spectrum match impossible;
3. possible coelution of two or more compounds occurred - the mass spectrum of the unknown peak contained ions from two volatiles, which rendered the mass spectrum match impossible;
4. the parent ion was missing or ignored by the program due to its small size - the parent ion is often important in proper identification, however the required electric ionization mode was not conducive for detecting the parent ion, especially in cases where the compound is fairly labile;

However, it was possible to overcome the problem described in point two for many cases by a more intensive interactive search where the background was subtracted after studying the total ion GC peak shape, and thus, to identify many of these volatiles.

A comparison of unknown mass spectra with mass spectra in a printed copy of the latest Wiley/NBS Library of Mass Spectra (McLafferty and Stauffer, 1989) did not give any additional information as to the nature of the unknowns, except for one compound, 2,5-octadien-1-ol, eluting after 50.0 minutes. This compound was not in the NBS library used by the computer system, but a comparison of the mass spectrum with that of other octadienol isomers in this library suggested that it also was an octadienol isomer. A comparison of the mass spectrum with the printed library revealed that the spectrum most closely matched that of 2,5-octadien-1-ol. The peak eluting at RT 36.7 also showed the major ions of an octadienol isomer. The retention time indicates that this compound could be 1,5-octadien-3-ol, but the presence of some other ions that can not be generated from 1,5-octadien-3-ol suggested that there is another compound coeluting at the same time. The elution peak for 2,4-decadienal, a volatile associated with the fishy flavor (Swoboda and Peers, 1977) was found after scanning the region of possible elution for the specific ions generated from 2,4-decadienal. The volatile 2,4-decadienal is mainly derived from the 9-, and 13- monohydroxyperoxides of linoleic acid (Chan et al., 1976), an acid of relative low abundance in menhaden oil (Bimbo, 1987). An ion-specific search was also conducted for decatrienal isomers, because they have been reported to be mainly responsible for the fishy odor (Meijboom and Stroink, 1973; Ke et al., 1975; Karahadian

and Lindsay, 1989a). While a scan for the specific ions of 2,4,7-decatrienal (Figure 27) showed that most of them are contained in a very small peak eluting at scan 2057 (RT = 62.5; Peak No. 114), it was not possible to confirm this peak as a decatrienal isomer due to the same problem observed for 1,5-octadien-3-ol, the large number of decatrienal unrelated ions. Crawford et al. (1976) also were not able to identify decatrienal in tuna oil and explained these findings by the labile nature of higher unsaturated aldehydes, resulting in a possible break down of the volatiles during oil extraction

The mass spectra of several unidentified volatiles that were present in relatively high concentrations in undeodorized menhaden oil are shown in Figures 28 to 32. Even though inferences can be made about the nature of these compounds from the ion patterns of the mass spectrum, the information is not sufficient to identify these compounds. The structural information that can be derived from low mass ions is relatively ambiguous, so that only general structure features can be recognized (McLafferty, 1973). In addition, since the mass analysis was not done in the high resolution mode, a mass fragment number can be ±1 of the displayed value, adding additional problems in identifying unknown compounds.

The ion fragments of the volatile in Figure 28 (RT=20.0) do not resemble any series of common fragment ions as described by McLafferty (1973). However, based on the fragment losses some deductions can be made. The loss of a CH_3-fragment (m/z =15) can be deduced from the occurrence of m/z =96 and m/z =81 ions, CH_2-fragments (m/z =14) can be deduced from the m/z =81 and m/z =67 ions, as well as the m/z =107 and 121 ions, indicating an aliphatic chain. The loss of at least two different CH-fragments (m/z =13) can be deduced from the m/z =67, 54, and 41 ions, indicating the presence of double bonds. The relative abundance of the ions as well as the number of ions separated by m/z =14 can give one experienced with mass spectroscopy indications as to the length and positioning of the alkyl-moieties of the compound. The compound also loses a mass fragment of m/z =17 (m/z 121 and 138), which corresponds to a hydroxyl group, indicating an alcohol. The small peak at 207 could be the parent ion, but is most probably just background noise. More likely, the parent ion is m/z =150. Based on the retention indices reported in the literature, the compound ought to be 2-pentylfuran (Karahadian and Lindsay 1989a; Hsieh, T.C.-Y., et al., 1989; Cha et al., 1992). A comparison of the mass spectrum of the volatile in Figure 28 with that of 2-pentylfuran

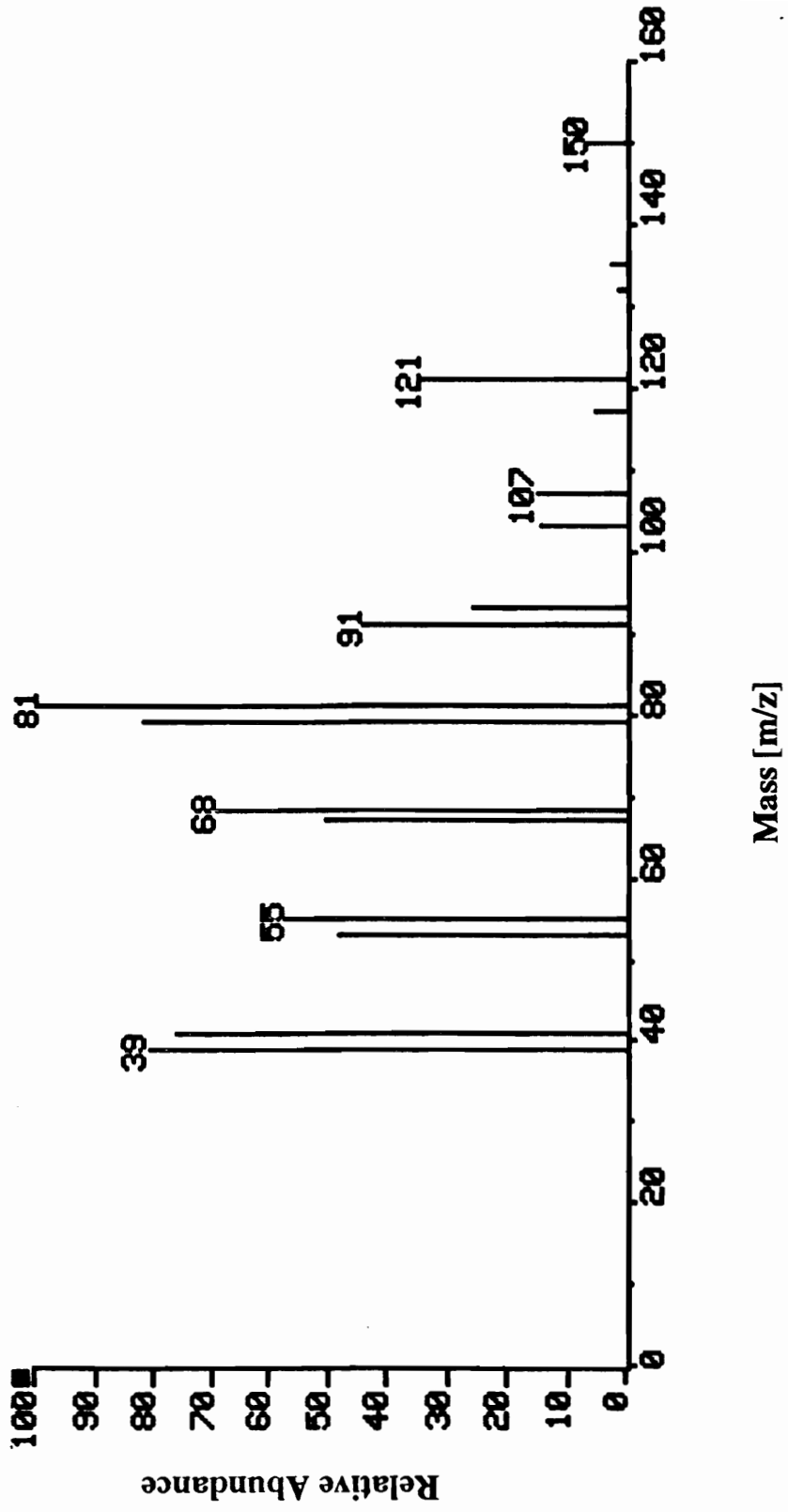


Figure 27. Mass spectrum of (E,E,Z) 2,4,7-Decatrienal (adapted from Seifert and Buttery, 1980)

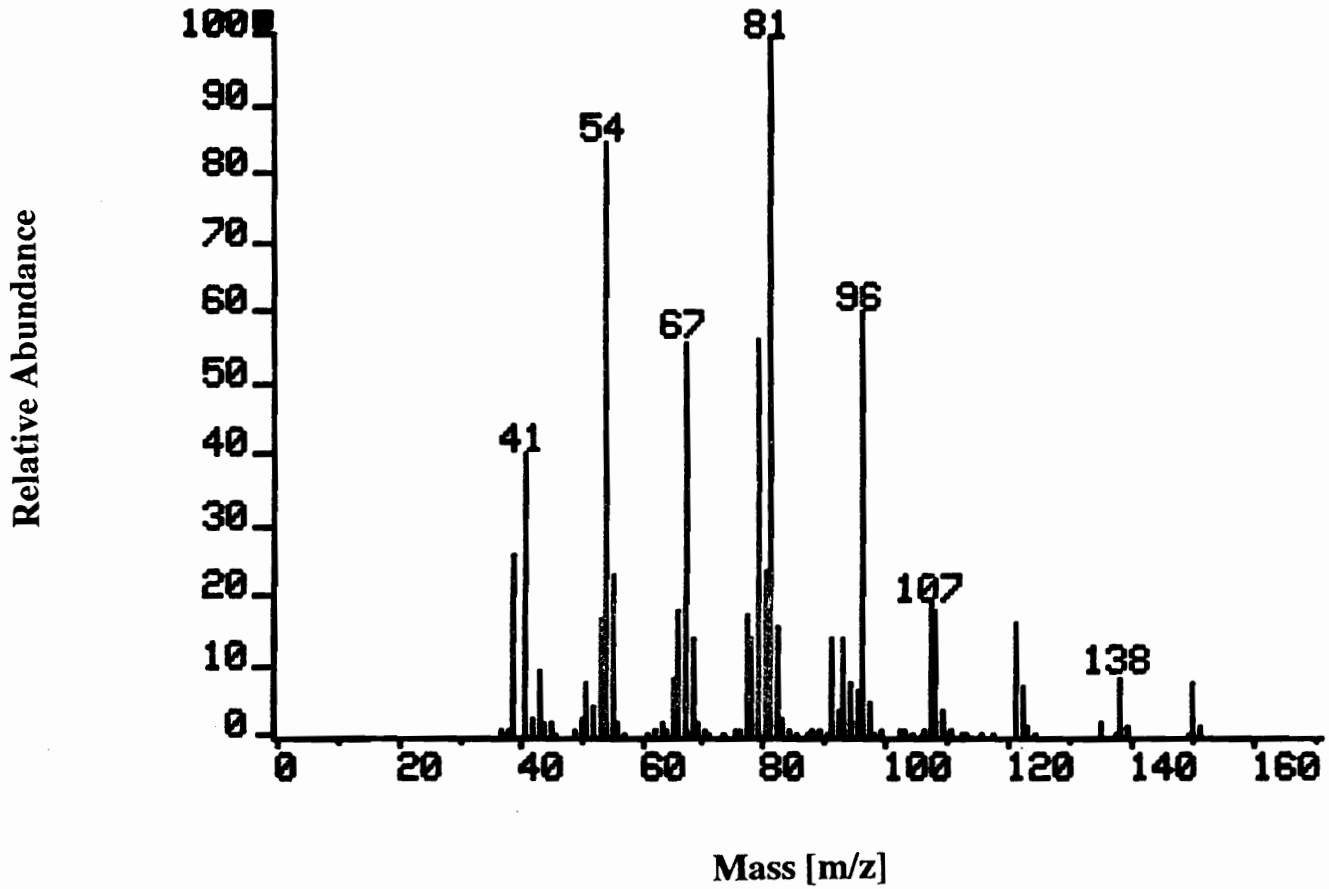


Figure 28. Mass spectrum of compound eluting at RT=20.0 minutes (Peak No. 22) in undeodorized menhaden oil

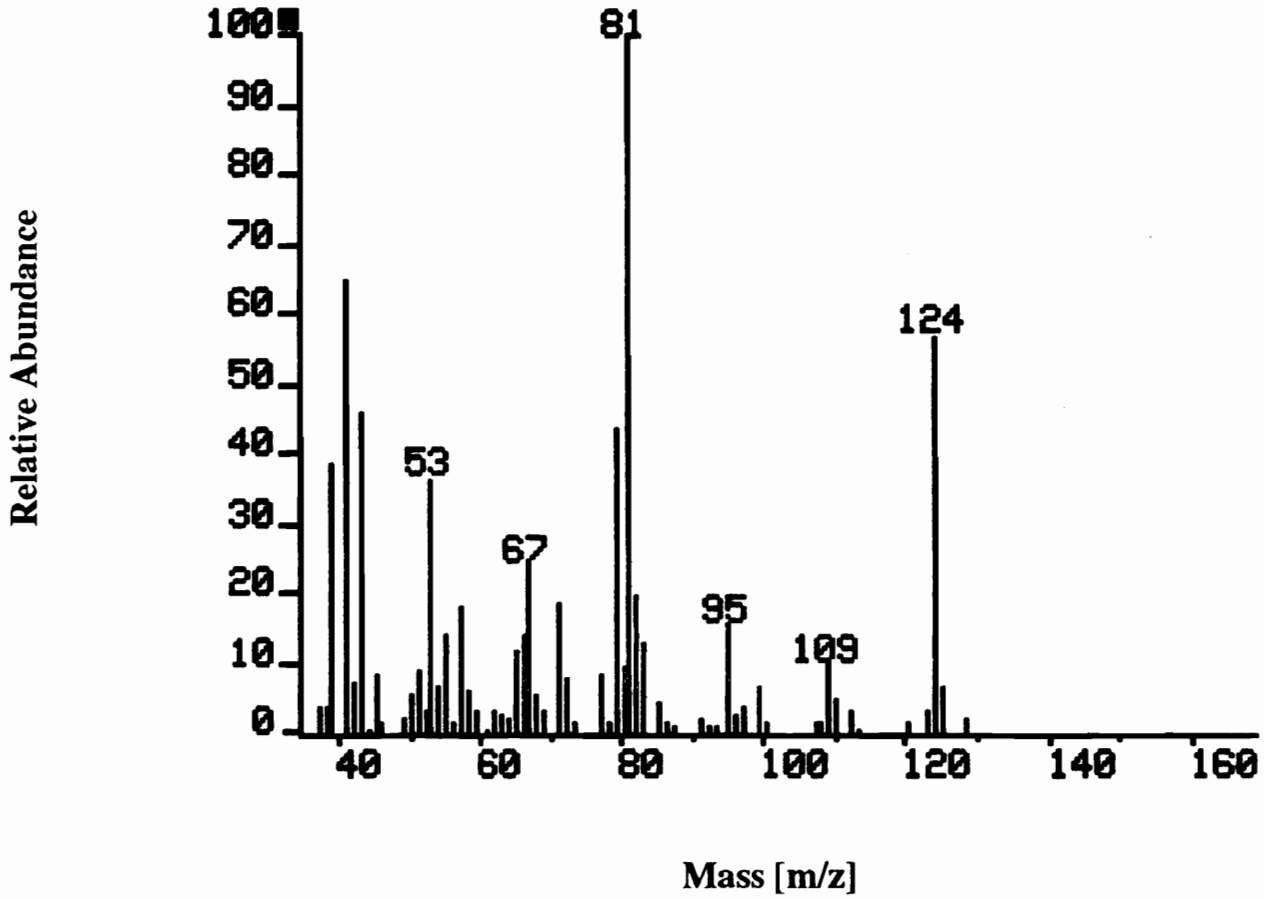


Figure 29. Mass spectrum of compound eluting at RT=21.1 minutes (Peak No. 25) in undeodorized menhaden oil

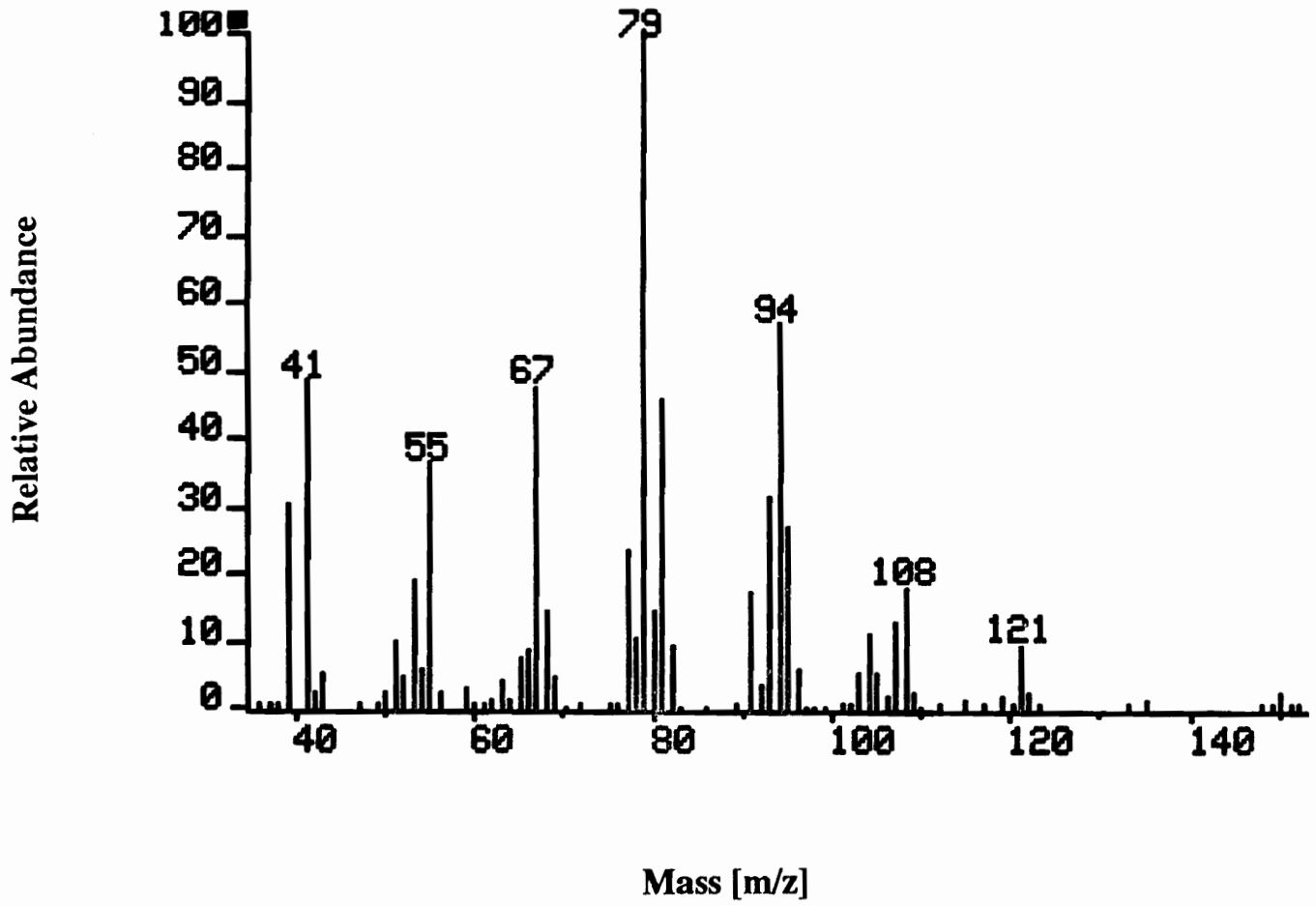


Figure 30. Mass spectrum of compound eluting at RT=22.0 minutes (Peak No. 27) in undeodorized menhaden oil

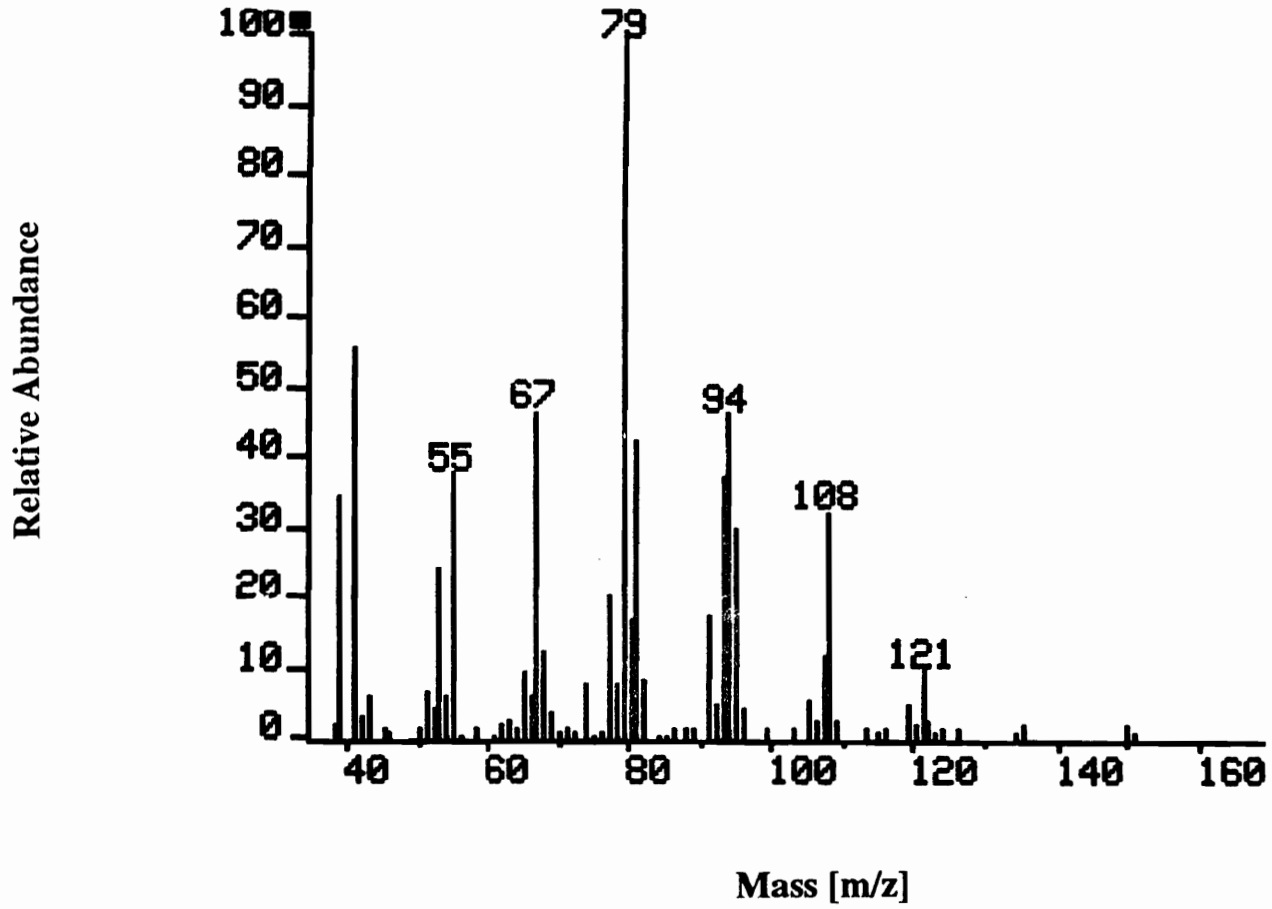


Figure 31. Mass spectrum of compound eluting at RT=22.8 minutes (Peak No. 28) in undeodorized menhaden oil

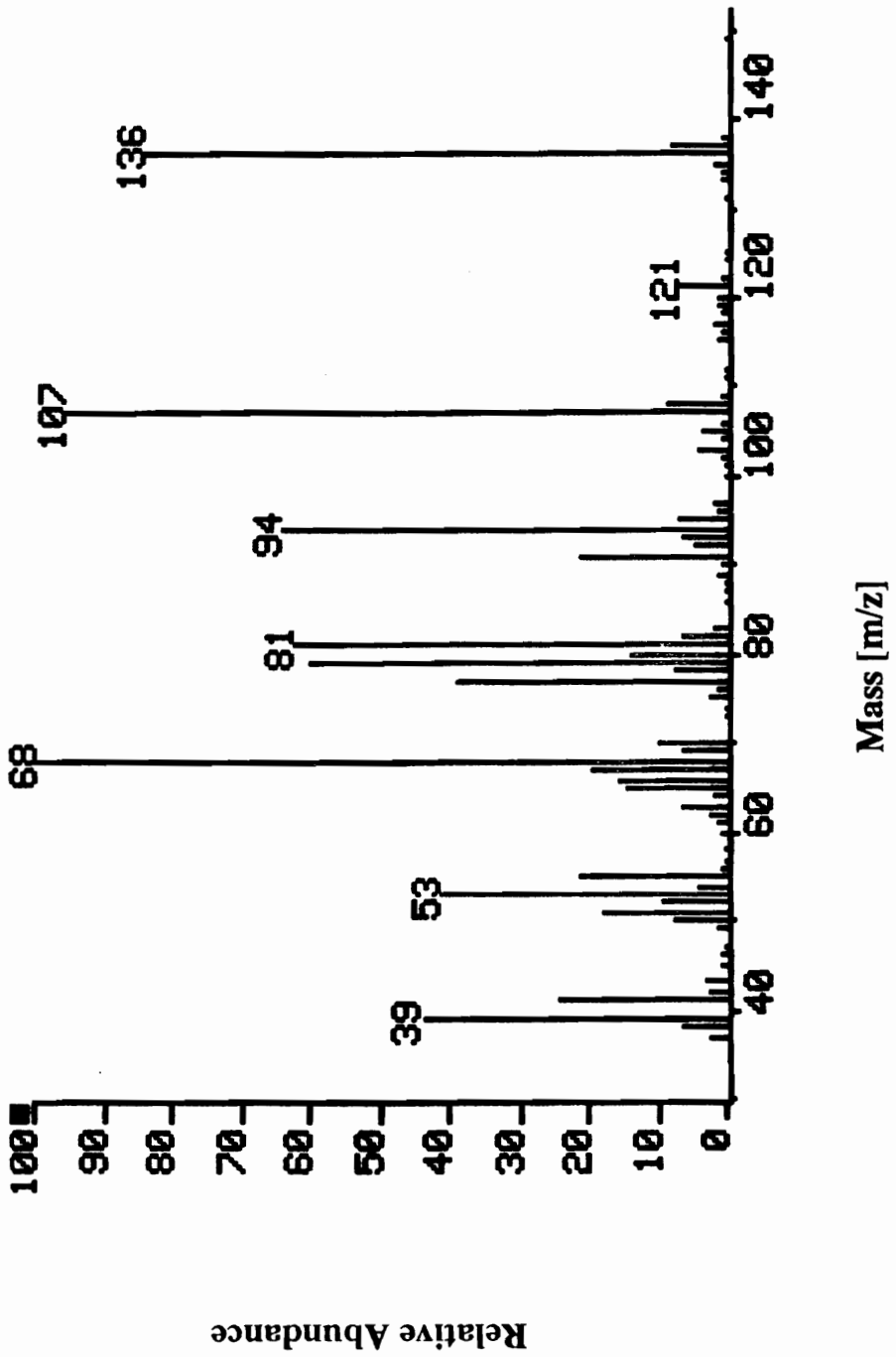


Figure 32. Mass spectrum of compound eluting at RT=24.4 minutes (Peak No. 32) in undeodorized menhaden oil

revealed that all the ions (*m/z* 138 (parent ion), 81, 67, 54, 41, and 39) formed by 2-pentylfuran are part of the unknown mass spectrum, but that other ions not formed from 2-pentylfuran are present as well, and that the relative abundance of the ions is different from that of 2-pentylfuran, indicating another coeluting compound.

The low mass end (*m/z* 39, 53, 67, 81, 95, and 109) of the volatile in Figure 29 (RT=21.2 minutes) is that of a typical ion fragmentation pattern of a diene, alkyne, or cycloalkene (McLafferty, 1973). However, none of the volatiles reported in the literature eluting at that retention time and not already identified in this study as well, belongs to any of these three groups or has a mass spectrum similarly to that in Figure 29.

The two compounds eluting at RT=22.0 and 22.8 minutes (Figures 30 and 31 respectively) have such a similar mass spectrum that they must be geometric isomers or enantiomers, since they differ only slightly in the relative abundance of the fragment ions. Unfortunately, these mass spectra also do not resemble any series of common fragment ions, and the only interpretation possible from the mass spectra is that they are hydrocarbons, since they lose various CH fragments.

The peak eluting at RT=24.4 minutes (Figure 32) could possibly include two compounds since the mass spectrum has fragment ions common to terpenes (fragments *m/z* 79, 107 and 121) as well as dienes, alkynes and cycloalkenes (fragments *m/z* 39, 53, and 81) (McLafferty, 1973). The fragments *m/z* 68, 81, 94, and 107 indicate that this volatile might contain three double bonds. Considering this information and the retention time, the unknown mass spectrum was specifically compared with that of 1,2,4-trimethyl benzene, but there was no resemblance in the fragmentation pattern.

Since a large number of studies have found hexanal to be the principal volatile formed in oxidizing lipids (Forss et al., 1960; Yu et al., 1961; Badings, 1970; Nobel and Nawar, 1971; Chan et al., 1976; Warner et al., 1978; Selke et al., 1980; Frankel et al., 1982; Josephson et al., 1984c; Snyder et al. 1991; Elizalde et al., 1991), it is often used as the volatile for monitoring lipid oxidation (Snyder et al. 1991; Elizalde et al., 1991). Hexanal is derived mainly from autoxidation, but can be formed through enzymatic activities as well and is also a secondary oxidation product, i.e. derived from 2,4-decadienal oxidation (Josephson et al, 1984c; Josephson and Lindsay 1986). In this study, hexanal was also found to be one of the major volatiles in undeodorized menhaden oil. Hexanal concentration was highest in week 1, decreased sharply in week 3, rose

again and decreased towards the end of the study (Figure 33). Hexanal can be formed autoxidatively or enzymatically, and its formation in freshly harvested fish appeared to be due to enzymatic activity while after storage it is most likely due to autoxidation. Therefore, these change in concentration could potentially be explained by enzymatic hexanal formation from week 0 to week 2 and after week 2 autoxidation was mainly responsible for hexanal formation.

Total volatiles in undeodorized menhaden oil doubled from week 0 to week 16 and then declined sharply. This result is in agreement with the findings for the anisidine value in undeodorized menhaden oil. The formation of carbonyl polymers would not only lower the anisidine value but also the amount of total volatiles. Quantities of individual volatiles varied considerably over the 20 weeks. The changes in concentration for selected volatiles in undeodorized menhaden oil and for total volatiles over 20 weeks are shown in Table 14.

While there is evidence that volatiles, such as hexanal, 2,4-heptadienals, and 2,4-decadienals are potentially lipoxygenase derived (Gardner, 1980), Josephson et al. (1984c) considered them to be classical autoxidation derived volatiles. Their relative increase and the disappearance of 6-nonen-1-ol and 3,6-nonadien-1-ol, as well as the decrease in (E)-2-nonenal and (E,Z)-2,6-nonadienal were considered the cause of the absence of fresh fish flavors from oxidized whitefish in favor of oxidized fishy flavors (Josephson et al., 1984c). Josephson et al., (1987) suggested that 2-nonenal and 2,6-nonadienal can be either enzymatically or nonenzymatically derived. However, they pointed out that in the case of enzymatic derivation of the two C_9 aldehydes, the enzyme-derived volatiles 3,6-nonadien-1-ol and 6-nonen-1-ol should be present as well. On the other hand, Josephson and Lindsay (1986) also stated that the C_9 volatiles in saltwater species occur in greater abundance in the aldehyde form than in the alcohol from. In this study, 6-nonen-1-ol and 3,6-nonadien-1-ol were not observed in the menhaden oil, but t-2-nonenal exhibited a remarkable change in concentration. It was very low in the beginning then increased sharply from week 3 to week 4 and decreased gradually from week 8 to week 20 (Figure 34). Since 6-nonen-1-ol and 3,6-nonadien-1-ol were not found in this study, it gives a slight indication that enzymatic derivation was not the prevalent mode of t-2-nonenal and t,c-2,6-nonadienal formation.

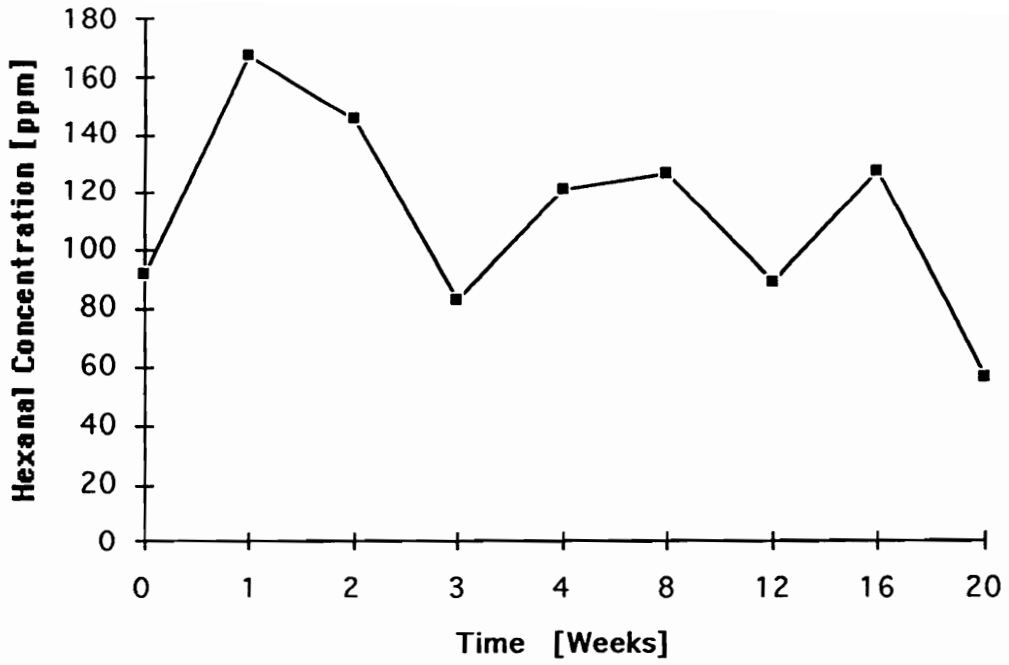


Figure 33. Change in hexanal concentration in the undeodorized menhaden oil over 20 weeks of storage at 30°C

Table 14. Changes in concentration of volatiles identified in undeodorized menhaden oil over 20 weeks of storage

Compound Name	Concentration [ppm] and Week of Storage				
	0	1	2	3	4
Decane	4.98	9.13	9.46	4.20	8.35
2,3-Dimethyl 1,4-hexadiene	19.18	39.83	37.05	18.69	42.79
1-Penten-3-one	2.66	7.73	6.40	3.21	0.00
3-Ethyl 1,4-hexadiene	0.98	5.03	4.22	2.38	0.00
5-Ethyl 2-methyl octane	0.00	0.34	0.24	0.00	2.00
Hexanal	91.92	167.27	145.75	82.63	121.14
1,3,6-Octatrienes (E,E and Z,E)	31.65	60.75	59.95	30.37	66.50
2-Pentenal	27.39	44.62	34.26	33.55	44.35
1,4-Dimethyl benzene	22.41	43.73	38.99	23.38	n.s.
1-Penten-3-ol	21.39	44.23	35.83	27.33	n.s.
2-Methyl 2-pentenal	6.07	12.95	11.96	8.25	0.00
Heptanal	50.47	86.51	85.22	29.24	72.64
4,5-Dimethyl 1-hexene	1.51	1.39	1.49	1.14	1.37
4-Methyl 3-pentenal	3.12	6.65	5.39	2.44	85.02
2-Hexen-3-one	6.11	11.38	10.72	5.39	10.89
2-Hexenal (E)	61.53	122.73	113.50	50.44	80.17
4-Heptenal (Z)	29.67	47.95	46.73	21.35	35.42
1,3,5-Trimethyl benzene	1.54	1.98	2.46	1.36	2.75
Octanal	9.60	14.03	14.62	8.17	42.67
2-Penten-1-ol (E)	8.52	13.88	13.02	11.37	12.37
2-Penten-1-ol (Z)	5.11	8.53	7.71	7.08	10.93
2-Heptenal (Z)	16.11	26.77	29.54	16.07	20.65
6-Octen-2-one	0.00	0.00	0.24	0.78	3.16
1-Hexanol	3.02	7.33	7.08	3.74	7.24
2-Methyl-2-cyclopenten-1-one	3.85	6.91	6.44	3.09	4.67
3-Hexen-1-ol (Z)	2.43	3.97	3.73	2.11	3.08
2-Nonanone	4.66	7.19	7.44	4.93	6.67
Nonanal	7.23	9.87	10.08	6.78	8.94
2,4-Hexadienal (E,E)	8.03	14.31	14.33	7.27	11.68
2-Octenal (E)	17.17	27.47	28.73	17.67	24.58
1-Octen-3-ol	5.45	8.80	9.80	6.26	9.36
2,4-Heptadienal (E,Z)	93.69	141.90	146.33	82.24	122.90
Pentadecane	72.45	111.08	83.87	60.11	73.74
2,4 Heptadienal (E,E)	39.12	60.16	28.92	39.98	72.81
3,5-Octadiene-2-one (E,Z)	3.23	4.85	5.44	2.78	3.68

(Table 14 cont'd. Changes in concentration of volatiles identified in undeodorized menhaden oil over 20 weeks of storage)

Compound Name	Concentration [ppm] and Week of Storage				
	0	1	2	3	4
Benzaldehyde	7.06	9.22	8.89	5.79	91.69
2-Nonenal	4.15	7.76	9.85	3.03	22.48
2,4-Octadienal (Z,Z)	3.64	5.38	4.94	3.41	5.71
3,5-Octadien-2-one	2.31	3.46	3.14	2.50	3.81
2,6-Nonadienal (E,Z)	6.38	6.38	7.22	4.14	4.50
2,4-Octadienal (E,E)	3.85	7.43	7.19	4.26	6.19
2-Decenal (Z) (Internal Standard)	6.67	6.67	6.67	6.67	6.67
Hexadecane	2.14	3.43	3.35	2.10	2.95
2,5-Octadien-1-ol	1.71	2.75	2.50	1.66	1.73
Heptadecane	2.21	3.71	2.77	2.58	3.10
5-Ethyl-2(5H)-furanone	2.14	2.97	2.64	1.65	4.44
2,4-Decadienal	0.54	1.44	0.90	0.34	1.08
1-Dodecanol	4.14	5.80	8.66	6.08	8.32
Total Volatile Area	4326903	5055734	5407352	4803161	5898202

(Table 14 cont'd. Changes in concentration of volatiles identified in undeodorized menhaden oil over 20 weeks of storage)

Compound Name	Concentration [ppm] and Week of Storage			
	8	12	16	20
Decane	6.19	4.57	7.31	3.96
2,3-Dimethyl 1,4-hexadiene	43.55	11.87	16.21	21.45
1-Penten-3-one	0.00	0.00	0.00	0.00
3-Ethyl 1,4-hexadiene	0.00	0.00	0.00	0.00
5-Ethyl 2-methyl octane	1.94	1.73	2.44	0.00
Hexanal	126.26	88.98	127.45	55.84
1,3,6-Octatrienes (E,E and Z,E)	63.41	81.97	69.04	30.78
2-Pentenal	22.71	42.22	22.95	6.93
1,4-Dimethyl benzene	16.56	41.73	72.65	10.04
1-Penten-3-ol	87.59	81.54	141.51	11.44
2-Methyl 2-pentenal	0.00	0.00	0.00	3.61
Heptanal	65.07	42.95	54.65	39.08
4,5-Dimethyl 1-hexene	1.08	2.30	0.00	0.60
4-Methyl 3-pentenal	95.40	99.01	116.21	5.97
2-Hexen-3-one	10.09	8.27	8.22	5.11
2-Hexenal (E)	77.51	70.17	62.03	48.22
4-Heptenal (Z)	31.98	19.42	34.67	22.38
1,3,5Trimethyl benzene	0.00	0.00	0.00	1.52
Octanal	48.56	51.18	65.02	7.95
2-Penten-1-ol (E)	13.35	9.97	13.54	5.29
2-Penten-1-ol (Z)	12.22	8.63	10.99	2.89
2-Heptenal (Z)	18.78	17.54	23.04	15.58
6-Octen-2-one	2.61	2.19	1.06	0.10
1-Hexanol	7.19	7.28	8.83	3.55
2-Methyl-2-cyclopenten-1-one	4.42	3.97	5.45	3.01
3-Hexen-1-ol (Z)	2.78	2.23	2.85	1.96
2-Nonanone	6.90	5.67	1.82	4.00
Nonanal	8.23	5.55	4.22	5.09
2,4-Hexadienal (E,E)	11.26	9.96	9.71	7.20
2-Octenal (E)	23.38	20.70	22.43	16.04
1-Octen-3-ol	9.17	9.88	9.26	5.97
2,4-Heptadienal (E,Z)	115.39	109.15	105.87	85.21
Pentadecane	77.95	73.30	68.20	61.67
2,4-Heptadienal (E,E)	53.84	42.21	36.80	32.95
3,5-Octadiene-2-one (E,Z)	11.31	9.49	6.70	4.24

(Table 14 cont'd. Changes in concentration of volatiles identified in undeodorized menhaden oil over 20 weeks of storage)

Compound Name	Concentration [ppm] and Week of Storage			
	8	12	16	20
Benzaldehyde	111.91	141.69	peaks not	5.57
2-Nonenal	22.94	17.23	separated	2.48
2,4-Octadienal (Z,Z)	9.35	7.17	6.36	3.39
3,5-Octadien-2-one	3.87	3.64	3.43	3.81
2,6-Nonadienal (E,Z)	7.67	9.23	12.81	3.37
2,4-Octadienal (E,E)	7.28	5.70	4.81	3.08
2-Decenal (Z) (Internal Standard)	6.67	6.67	6.67	6.67
Hexadecane	2.86	2.85	3.21	2.13
2,5-Octadien-1-ol	1.76	1.74	1.79	1.06
Heptadecane	2.70	2.12	2.45	1.89
5-Ethyl-2(5H)-furanone	2.65	3.14	2.59	1.14
2,4-Decadienal	1.62	2.61	2.77	0.35
1-Dodecanol	5.72	4.61	2.22	0.62
Total Volatile Area	7494378	9288356	9680695	5075052

n.s. compounds not separated

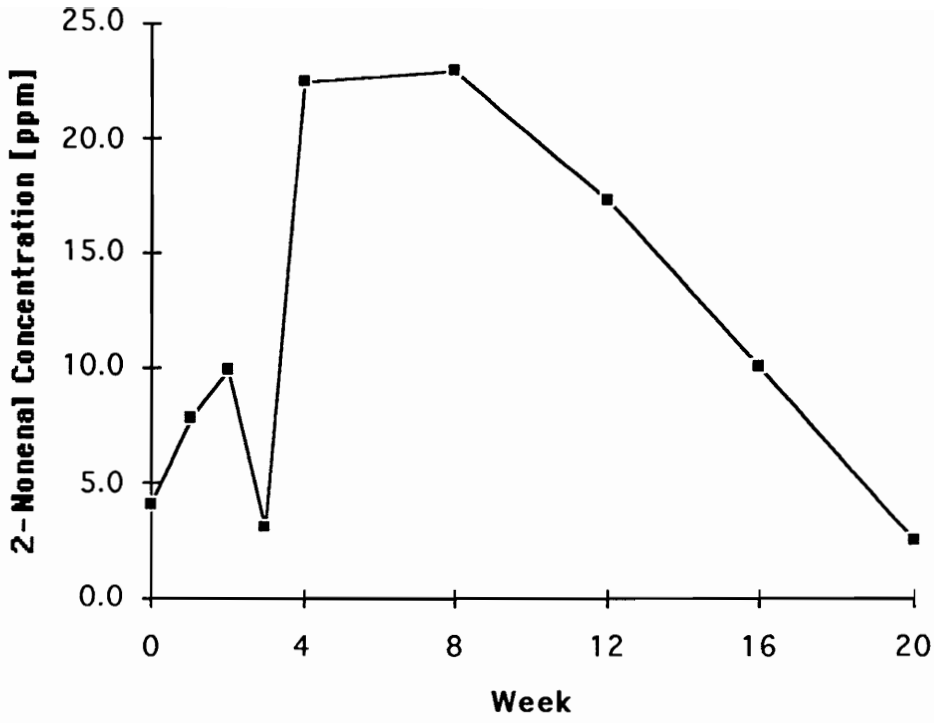


Figure 34. Changes in 2-nonenal concentration in undeodorized menhaden oil over 20 weeks of storage at 30°C

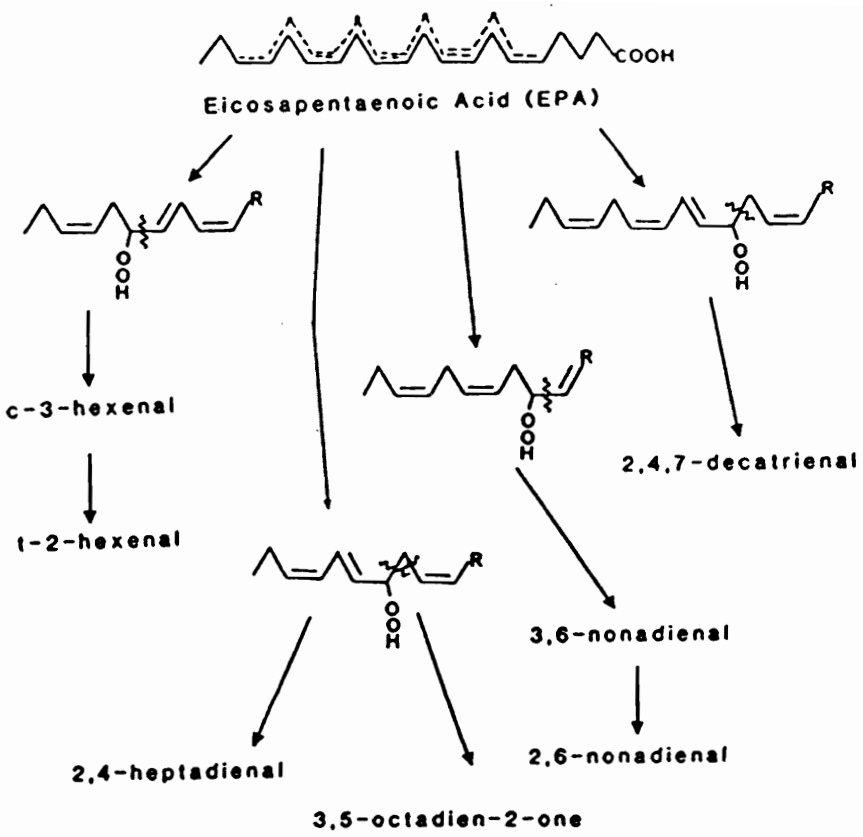


Figure 35. Postulated pathway for the formation of green grassy volatiles by autoxidation of EPA.
(Karahadian and Lindsay, 1989b)

Stansby and Jellinek (1965) described the odor of oxidizing menhaden oil to change from a green cucumber-like smell to a fishy, rancid smell. The green flavors are considered to be due to the volatiles c-3-hexen-1-ol, t-2-hexenal, and t,c-2,6-nonadienal. These volatiles can either be derived from lipoxygenase activity or autoxidation. Josephson and Lindsay (1986) postulated pathways for the lipoxygenase derived formation of these volatiles (see Figure 3), while a possible autoxidation derived pathway (Figure 35) was later postulated by Karahadian and Lindsay, (1989b). Hsieh and Kinsella (1989a) confirmed that one of these volatiles, t,c-2,6-nonadienal is derived from lipoxygenase activity. While Lin et al. (1990) was able to show the presence of c-3-hexen-1-ol in undeodorized menhaden oil, other studies were not able to detect it in menhaden oil (Karahadian and Lindsay, 1989a; Hsieh T.C.-Y. et al, 1989). In this study, all three compounds that contribute to the green flavor were identified in the undeodorized oil. T-2-hexenal exhibited its highest concentration at week 1, then decreased sharply at week 3, increased again at week 4 and slowly decreased towards the end of the study (Figure 36). C-3-hexen-1-ol showed a very similar pattern (Figure 37). The compound t,c-2,6-nonadienal on the other hand showed a steady increase until week 16 with a small dip between week 3 and 4 and then decreased sharply at week 20 (Figure 38). When derived from autoxidation, these volatiles are accompanied by elevated concentrations of compounds that contribute oxidized flavors, such as 2,4,7-decatrienals or c-4-heptenal (Karahadian and Lindsay, 1989b). However, c-4-heptenal can also be formed from t,c-2,6-nonadienal, an autoxidation or lipoxygenase-derived volatile (Josephson and Lindsay, 1987). C-4-heptenal is the volatile primarily responsible for the off-flavor in cold-stored cod (McGill et al., 1977) and was previously identified as the volatile compound contributing fishy odors to soybean and linseed oils (Seals and Hammond, 1970). In this study considerable amounts of c-4-heptenal but no decatrienals were found in the undeodorized menhaden oil, which is an indication that lipoxygenase activity is at least partly responsible for the formation of c-3-hexen-1-ol, t-2-hexenal, and t,c-2,6-nonadienal. The presence of the volatiles 2-octenal, 1-penten-3-ol, 1-octen-3-ol and 2,5-octadien-1-ol is also an indication of enzymatic activity, since they are enzymatically derived volatiles (Josephson et al., 1984a, 1986, 1987; Hsieh and Kinsella, 1989a).

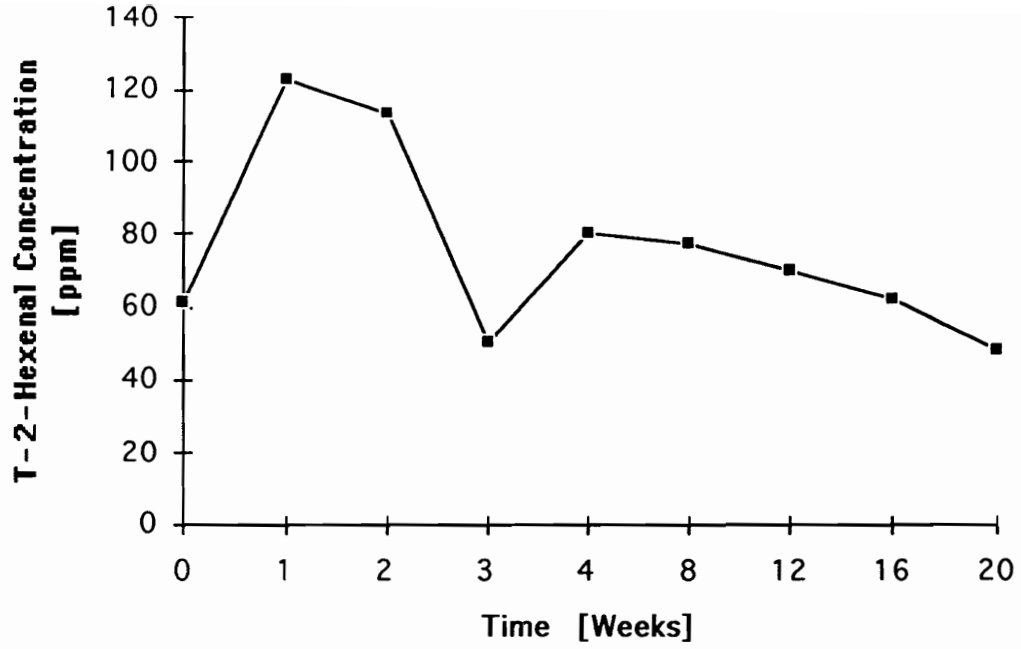


Figure 36. Changes in t-2-hexenal concentration in undeodorized menhaden oil over 20 weeks of storage at 30°C

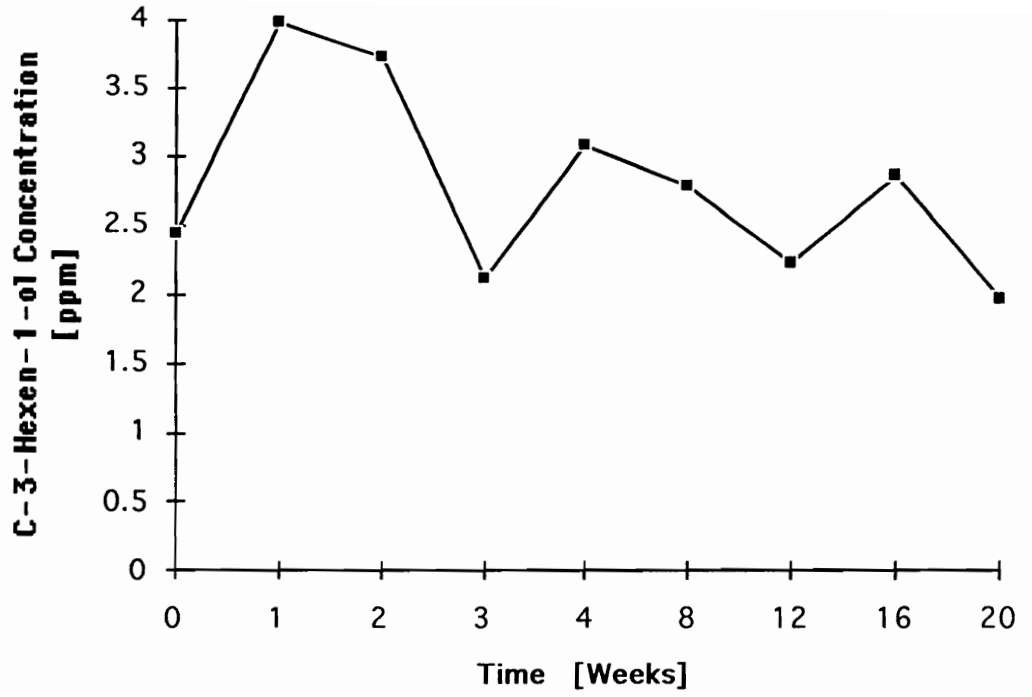


Figure 37. Changes in c-3-hexen-1-ol concentration in undeodorized menhaden oil over 20 weeks of storage at 30°C

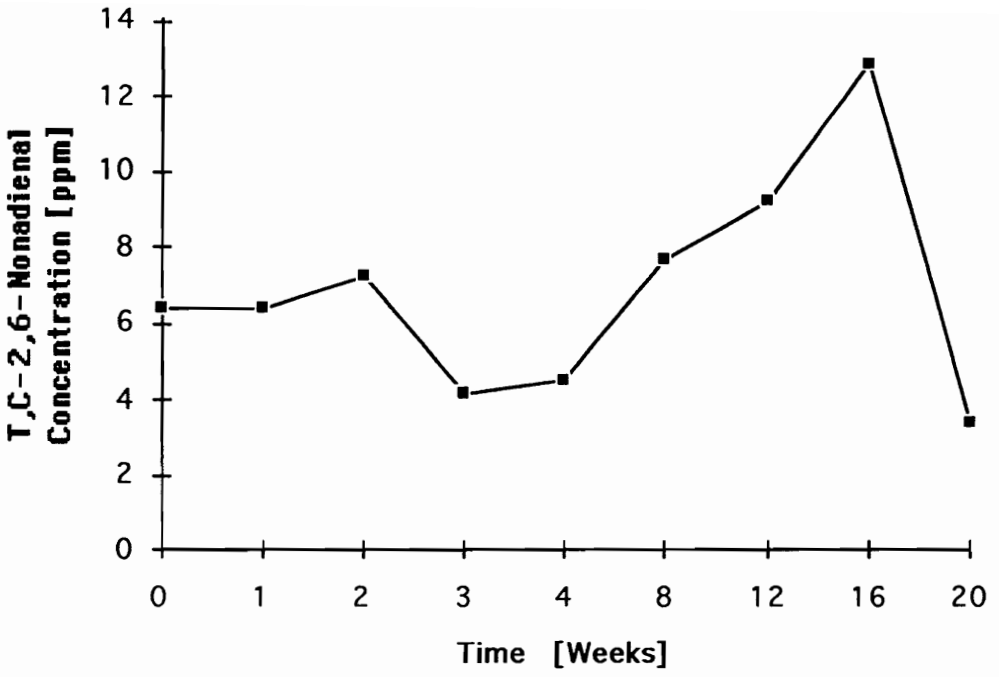


Figure 38. Changes in t,c-2,6-nonadienal concentration in undeodorized menhaden oil over 20 weeks of storage at 30°C

On the other hand, the role of autoxidation in the degradation of the undeodorized menhaden oil in this study is indicated by the presence of what are considered classical autoxidation volatiles, such as the isomers of 2,4-heptadienal and 3,5-octadiene-2-one as well as 2,4-decadienal (Josephson et al., 1984c, 1987). However, these autoxidation derived volatiles have also been shown to be produced by various plant lipoxygenases (Gardner, 1980). Heptadienals, decadienals and decatrienals are the compounds mainly associated with the rancid fishy and painty off-odors (Meijboom and Stroink, 1973; Ke et al., 1975; Swoboda and Peers, 1977; Josephson et al., 1984c; Karahadian and Lindsay, 1989a). Other investigators also found very high concentrations of heptadienal isomers in oxidizing fish oils (Karahadian and Lindsay, 1989a)

In general, volatiles derived from autoxidative processes are in abundance in the undeodorized menhaden oil, but a number of volatiles likely derived from enzymatic activity were identified as well. Many volatiles were found to be in higher amounts in this study than has been reported elsewhere. There are several possible reasons for this including: 1. the extraction time was considerably longer in this study than other studies, resulting in higher volatile extraction, 2. a larger amount of oil was used in this study than in most other studies, resulting in larger total amount of extracted volatiles, 3. the internal standard t-2-decenal might be less responsive to the FID detector than internal standards used in other studies leading to an overestimation of volatile concentrations, 4. the internal standard t-2-decenal might not have been completely extracted from the oil due to various reasons, such as its solubility in the solvent, resulting in an overestimation of volatile concentrations. In general, it must be considered that the use of an internal standard does not allow quantification since relative detector response rates vary considerably for different compounds, thus allowing only relative comparisons between volatile concentrations over time.

Deodorization caused considerable changes in the volatile spectrum of the menhaden oil. Relatively few of the volatiles present in the undeodorized oil were identified in the deodorized oil. Secondary volatiles especially, such as alcohols, aldehydes and ketones were absent. Most volatiles were also present in much lower concentration in the deodorized oil than in the undeodorized particularly in the beginning of the study. Therefore, a GC-MS analysis was repeated on a sample from week 4 of storage. However, since several months had passed since the extraction of the volatiles

before the GC-MS analysis on the week 4 sample was attempted, many volatiles had degraded and the additional information gained from this sample in regard to volatile identification was limited (see Table 11). These findings are in agreement with those of Lin et al. (1990), who showed that almost all volatiles found in the unprocessed menhaden oil were removed by deodorization. However, menhaden oil is highly susceptible to autoxidation, even after deodorization (Fujimoto, 1989), otherwise TBA, peroxide and anisidine values would not have increased during storage to the extent that they did in this study. In addition, the total volatiles in the deodorized oil increased considerably over a short time period (Table 15).

Except for relatively small amounts of 2-nonenal which can also be autoxidatively derived, none of the lipoxygenase derived volatiles were present in the deodorized oil. Since it is not possible to have lipoxygenase activity in the deodorized oil, these results were expected. It was impossible to identify many of the volatiles in the deodorized oil since the volatiles eluting between 20 and 30 minutes and the volatiles eluting between 45 and 70 minutes respectively had almost identical mass spectra. The library matches for all the unknown compounds marked with one asterisk (Table 11) were always octadecane, eicosane, docosane, 2,6,10,14-tetramethyl hexadecane, 2,6,10,15-tetramethyl heptadecane, 2,6,10-trimethyl tetradecane, 10-methyl eicosane, 2-methyl hexadecane, and 7,9-dimethyl hexadecane, while for the unknowns marked with two asterisks the library matches were always 4,7-dimethyl undecane, 2,6,11-trimethyl dodecane, 3,7-dimethyl undecane, 2,7,10-trimethyl dodecane, 4,6-dimethyl dodecane, 5-ethyl-2-methyl octane, 3,8-dimethyl undecane, 3,5-dimethyl undecane, 5,7-dimethyl undecane, 2,7-dimethyl undecane, 5-ethyl-5-methyl-decane, 3,6-dimethyl undecane, and 3,6-dimethyl decane. Since for all the compounds marked with one or two asterisks several matches were found to be >80%, it was not possible to make a positive identification of any of these compounds. It is difficult to make a proper identification of these compounds, since they are basically all long chain hydrocarbons, which have very similar daughter ions. For proper identification of these compounds, the parent ion and thus the molecular weight information is crucial. However, the particular mode of ionization used in this study, electrical ionization, makes it difficult to ascertain the parent ion. Using the retention indices of Heil and Lindsay (1988) and comparing their results with the results of this study, tentative identification could be made for octadecane, eicosane, and docosane.

Table 15. Changes in concentration of volatiles identified in deodorized menhaden oil over 20 weeks of storage

Compound Name	Concentration [ppm] and Week of Storage				
	0	1	2	3	4
Decane	1.13	2.74	2.09	1.81	7.02
Methyl benzene	2.53	6.54	6.01	6.29	8.58
Hexanal	0.64	2.47	2.50	2.14	11.50
Ethyl benzene	1.59	3.86	3.10	2.62	1.73
1,4-Dimethyl benzene	5.54	15.07	11.85	12.79	7.37
Dodecane	1.70	4.99	3.63	3.90	2.21
Ethenyl benzene	0.00	0.00	0.00	0.00	2.41
Octanal	0.00	0.00	0.00	0.00	55.86
Octanoic Acid, Methyl Ester	0.00	1.70	1.00	1.44	2.67
Tetradecane	1.03	2.54	2.37	2.74	2.78
Butanoic Acid	0.09	0.54	0.50	0.64	11.48
2,4-Heptadienal (E,Z)	0.00	0.54	0.38	0.66	4.23
Unknown	1.27	2.05	2.70	3.30	4.68
Pentadecane	0.24	0.67	0.61	0.92	1.47
2,4-Heptadienal (E,E)	0.14	0.32	0.30	0.39	0.70
Dimethyl hexadecane	0.77	2.37	1.56	1.64	1.99
1,3-Butanediol	0.42	1.54	1.28	1.43	210.79
Benzaldehyde	0.00	1.31	1.42	1.92	0.22
2-Nonenal	0.34	1.12	1.11	1.48	1.27
2,3-Butanediol	0.00	0.00	0.00	0.00	296.79
1,2-Propanediol	554.86	1217.73	1080.81	1001.02	898.68
Hexadecane	0.93	2.11	1.94	2.57	2.10
2-Ethyl 1-decanol	0.55	1.31	1.12	2.03	1.70
1-Dodecanol	1.02	4.80	4.45	6.42	5.35
1-Dodecene	1.21	6.06	2.70	2.66	6.58
Tetradecanol	0.81	0.61	0.72	0.88	3.45
Total Volatile Area	2126064	2647043	2399113	3173219	5153345

(Table 15 cont'd. Changes in concentration of volatiles identified in deodorized menhaden oil over 20 weeks of storage)

Compound Name	Concentration [ppm] and Week of Storage			
	8	12	16	20
Decane	8.52	2.23	13.47	0.25
Methyl benzene	0.86	0.26	0.00	1.65
Hexanal	12.85	6.20	22.47	n.s.
Ethyl benzene	1.07	1.27	2.19	0.10
1,4-Dimethyl benzene	7.11	2.93	118.02	4.61
Dodecane	2.28	1.77	5.18	2.73
Ethenyl benzene	2.26	1.07	4.14	0.00
Octanal	46.98	29.66	102.09	0.76
Octanoic Acid, Methyl Ester	3.47	9.12	19.60	0.54
Tetradecane	2.07	0.98	3.27	1.59
Butanoic Acid	11.14	4.60	15.34	0.34
2,4-Heptadienal (E,Z)	9.87	5.93	9.72	31.49
Unknown	3.45	2.36	8.13	5.09
Pentadecane	1.05	0.92	8.02	1.17
2,4-Heptadienal (E,E)	2.11	0.14	4.81	0.25
Dimethyl hexadecane	4.94	2.45	13.55	1.81
1,3-Butanediol	186.08	100.46	381.36	1.47
Benzaldehyde	n.i.	n.i.	n.i.	1.62
2-Nonenal	1.13	0.08	2.74	0.65
2,3-Butanediol	266.50	142.19	538.40	2.22
1,2-Propanediol	717.68	210.63	961.26	322.49
Hexadecane	0.41	0.70	1.85	1.81
2-Ethyl 1-decanol	0.71	0.71	1.37	1.05
1-Dodecanol	3.36	1.91	1.80	2.94
1-Dodecene	3.46	2.27	1.69	9.45
Tetradecanol	1.37	1.07	2.58	1.72
Total Volatile Area	5810132	5843255	6932020	3969449

n.i. peak not integrated
n.s. peak not separated

Sample mass spectrum of these unknowns (Figure 39 one asterisk; Figure 40 two asterisks) reveal that they all have long aliphatic chains which corresponds with the library matches. This can be seen by the continuous loss of a mass of 14 which is that of methylene (CH_2) (Figures 39 and 40). Long aliphatic compounds have high boiling points and thus are those least likely to be removed by steam deodorization.

The absence of these long aliphatic chains in the undeodorized oil is an indication that the undeodorized oil was more oxidized than the deodorized oil. These compounds were for the most part already oxidized in the undeodorized oil. Thus, if at all, they were present only in small concentrations causing them to be overlooked in the undeodorized oil as insignificant due to small peak size. In general total volatiles were significantly ($p = 0.0001$) lower in the deodorized oil than in the undeodorized throughout the study.

The compounds butanediol and popanediol were found in deodorized but not undeodorized menhaden oil. Lin et al. (1990) also observed 1,2 propanediol in deodorized but not undeodorized menhaden oil. They attributed it to the addition of Tenox 20A to the deodorized oil, since they found 1,2 propanediol in the headspace of Tenox 20A. In this study *tert.*-butylhydroquinone (TBHQ) and not Tenox 20A was used to stabilize the oils and n-3 fatty acid ester concentrates. The compound eluting at 61.3 minutes from the oils was matched with 2,6 Bis(1,1-Dimethyl ethyl)-4-methyl phenol, the only compound in the library, whose structure resembled that of TBHQ, and was assumed to be TBHQ, since the mass spectrum of TBHQ was not part of the mass spectra library. Lin et al. (1990) also showed that steam deodorization does not completely remove benzene-containing compounds, which is in agreement with the results of this study. Since Josephson et al. (1983) found trimethylbenzene and benzaldehyde in fresh fish and these compounds have been found in other foods as well, they suggested that the benzene-containing compounds have a common biochemical origin. However, Lin et al. (1990) suggested that the plastic containers the oils were stored in were the origin of the benzene-containing compounds. Since classical micro biologically derived volatiles such as butanedione, acetoin, acetic acid or butanediol (Josephson et al., 1983) were absent from the menhaden oils, microbial involvement in the production of volatiles in these oils is unlikely.

Changes in the concentration of volatiles identified in deodorized menhaden oil are shown in Table 15. Hexanal (Figure 41), the volatile often used for assessing oil

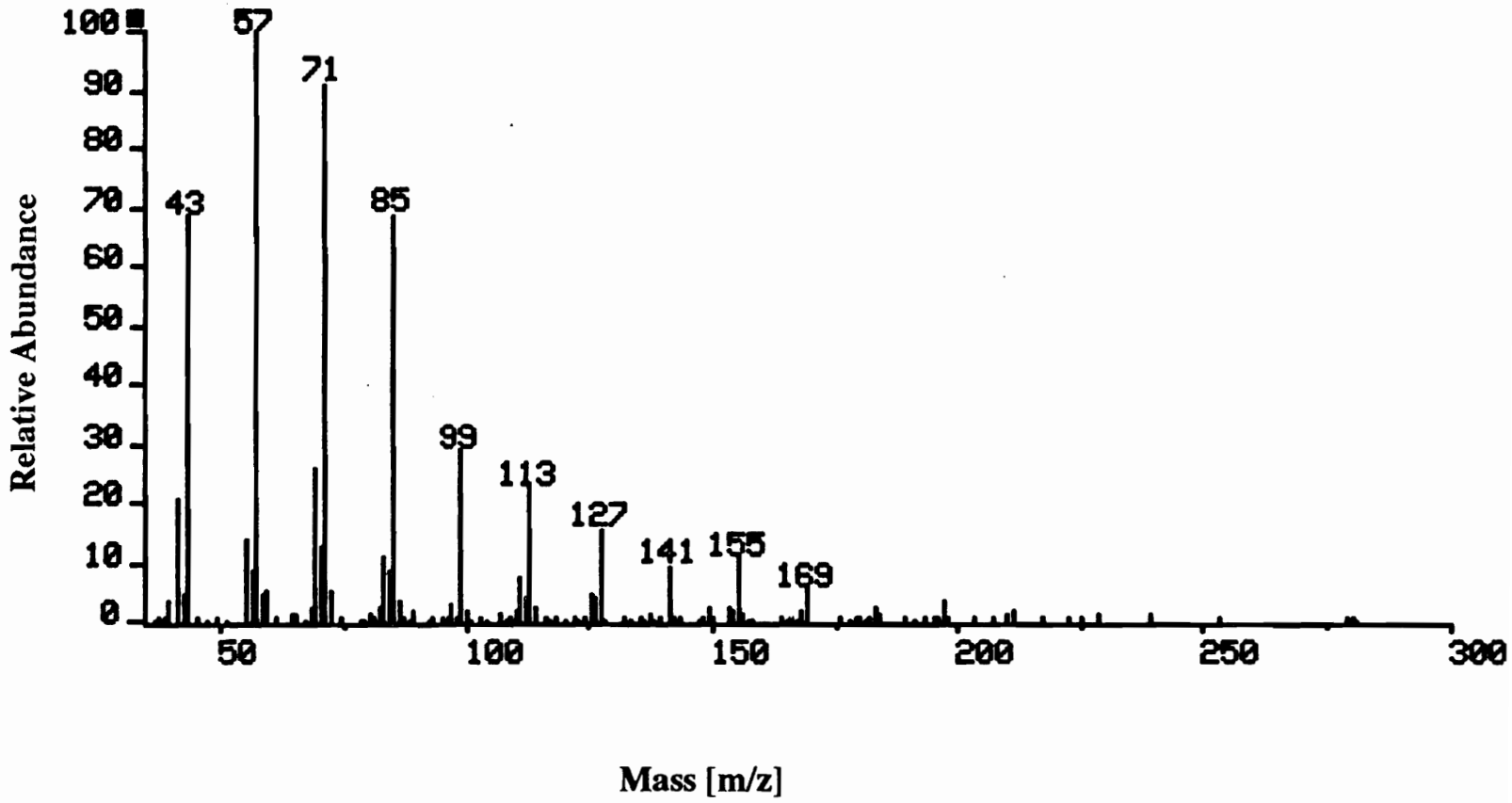


Figure 39. Sample mass spectrum of some of the unknowns marked with one asterisk (RT=65.0 minutes) in deodorized menhaden oil

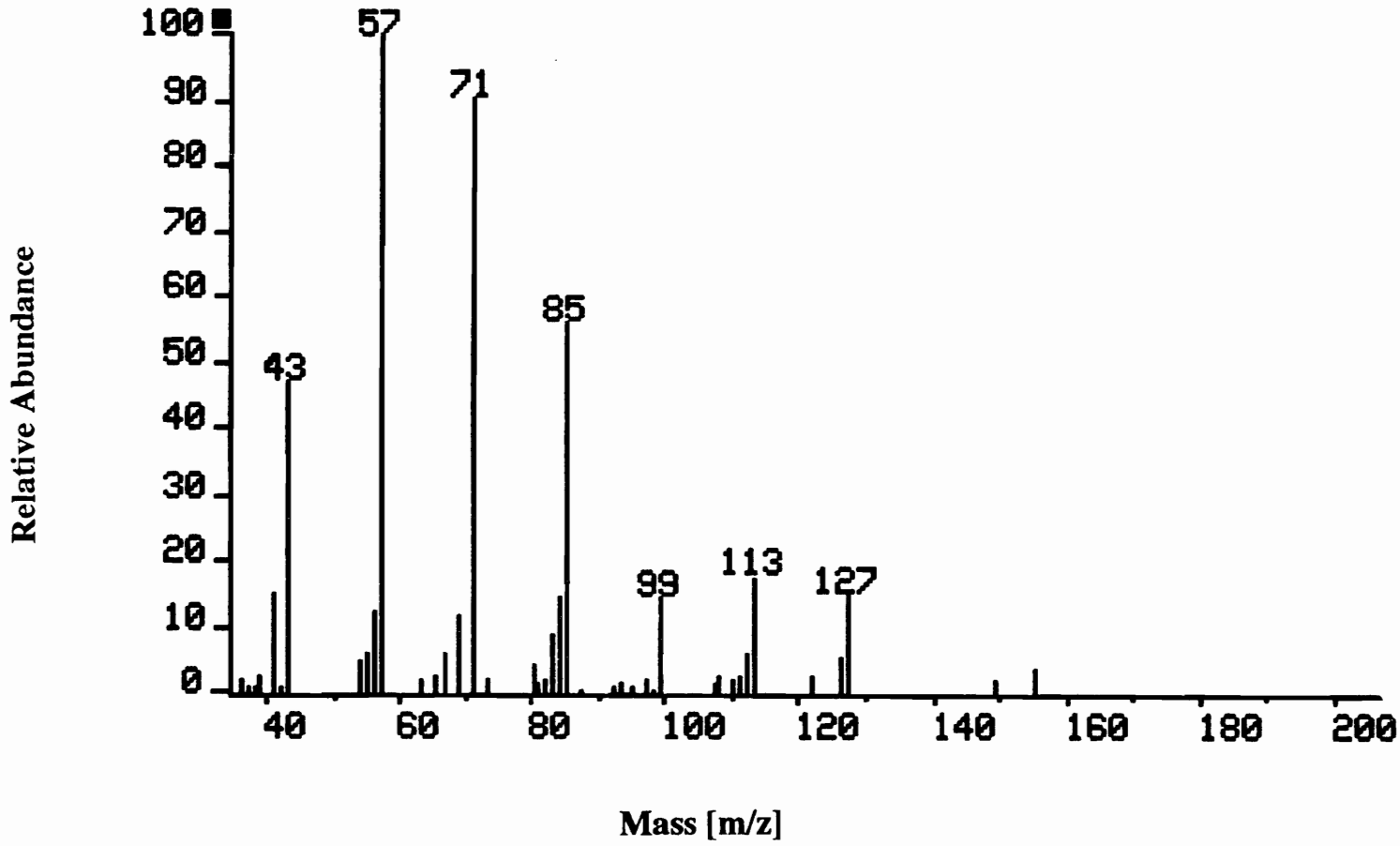


Figure 40. Sample mass spectrum of some of the unknowns marked with two asterisks (RT=21.9 minutes) in deodorized menhaden oil

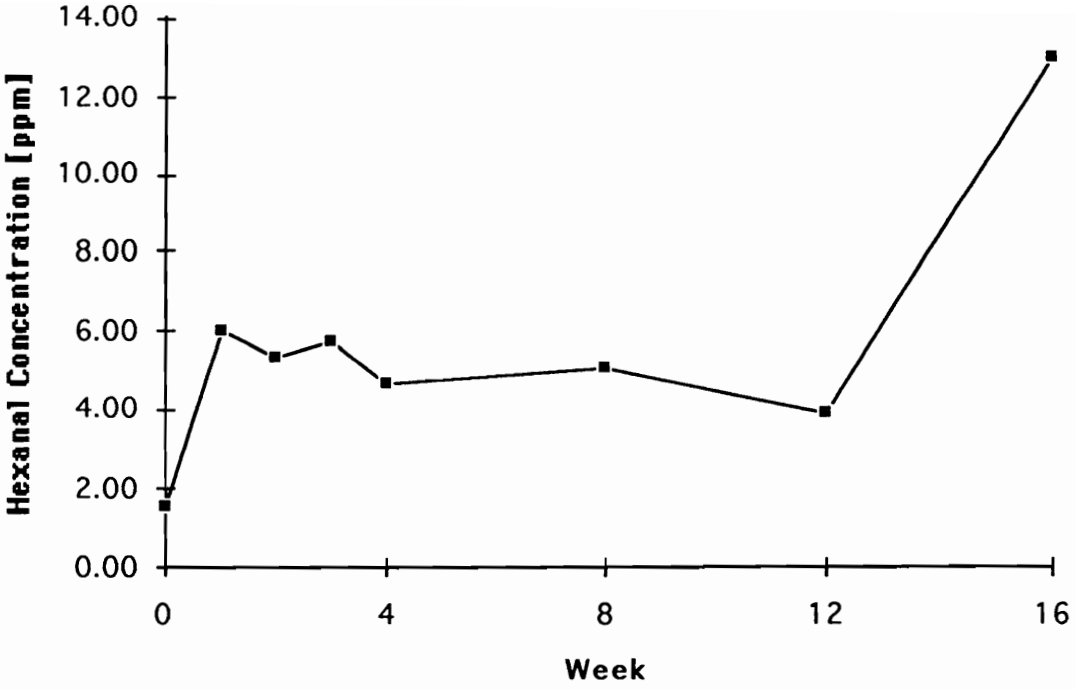


Figure 41. Hexanal concentration in deodorized menhaden oil over 16 weeks of storage

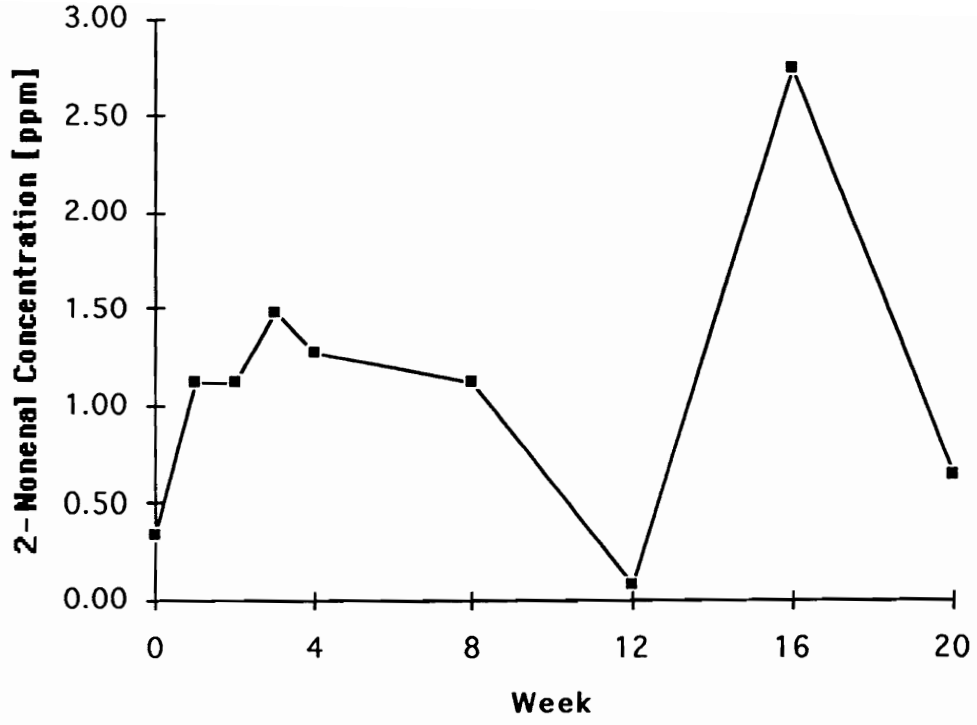


Figure 42. 2-Nonenal concentration in deodrized menhaden oil over 20 weeks of storage

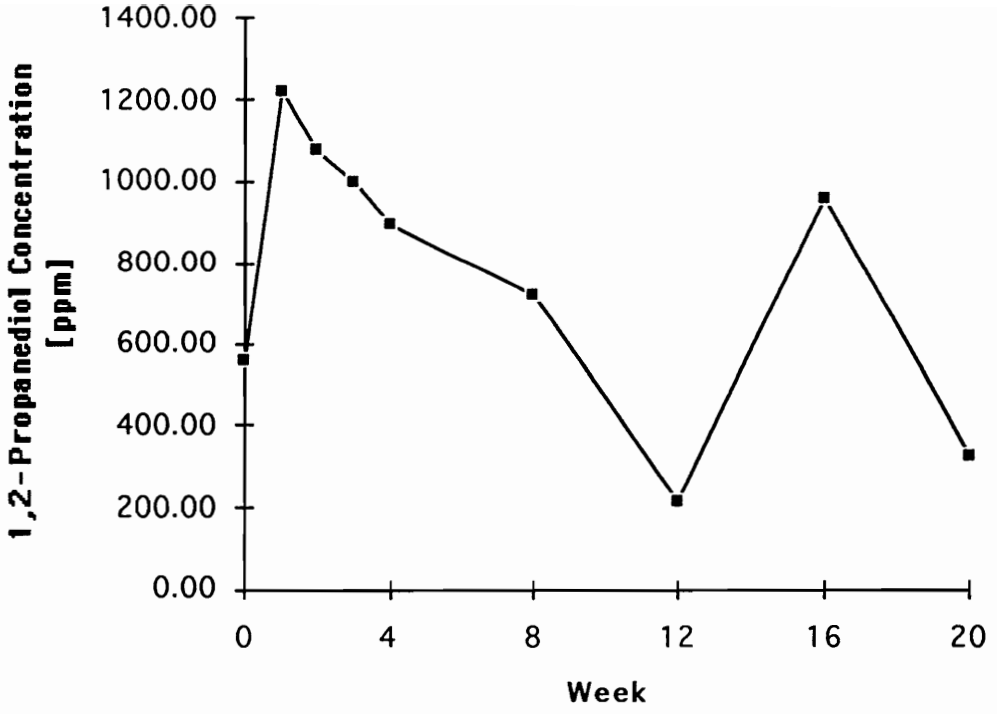


Figure 43. 1,2-Propanediol concentration in deodorized menhaden oil over 20 weeks of storage

deterioration increased considerably during the first week of storage, then remained stable until week 12, when it started rising rapidly. 2-Nonenal also increased during the first week, remained stable until week 8 and showed large fluctuations thereafter (Figure 42). The volatile in the highest concentration in the deodorized oil was 1,2-propanediol, which exhibited a peak at week 1, after which it steadily decreased with some fluctuations at the end of the study (Figure 43).

6.9. Volatiles of n-3 Fatty Acid Esters Concentrates

Representative chromatograms of the esters with the four different treatments are shown in Figure 44 to Figure 47 respectively. The n-3 fatty acid esters volatiles identified by GC-MS are displayed in Table 16, and changes in the concentration of selected volatiles are displayed in Table 17. Peak areas of volatiles in n-3 fatty acid ester concentrates can found in Appendix XX.

Considerable differences were observed between the volatiles found in the menhaden oils and the volatiles derived from the n-3 fatty acid esters. This can be attributed to the ethyl moiety esterified to the carboxyl group of the n-3 fatty acids. Many volatile compounds were ethyl esters of saturated and unsaturated short chain fatty acids. Autoxidation of ethyl linoleate was found to yield a large number of these short chain fatty acid esters (Henderson et al. 1980). However, some of the volatiles were identical to those observed in undeodorized and deodorized menhaden oil. Since the esters consisted of approximately 70% n-3 fatty acid esters, it is possible to assume that the volatiles found in the oils and the esters are derived from the degradation of the n-3 fatty acids. The n-3 fatty acid esters also gave rise to a large number of dioxo- and -oxy compounds, which were not identified by GC-MS, since there were no library entries for these compounds. Henderson et al. (1980) also identified a large number of volatile compounds from the autoxidation of linoleate whose origin and derivation could not be easily explained. They proposed reactions other than the ß-scission of the peroxides such as C-O bond cleavage, leading to isomerization, epoxidation, dihydroperoxide formation, cyclization and dimerization. The mass spectrum of one of the unknowns (RT = 22.1) was identified as 3-ethenyl-cyclooctene, 1,4-cyclooctadiene or as an -yne (having a triple bond) compound (marked with *) and is shown in Figure 48. Several of the unknown compounds in the esters are identical to unknown compounds found in the undeodorized

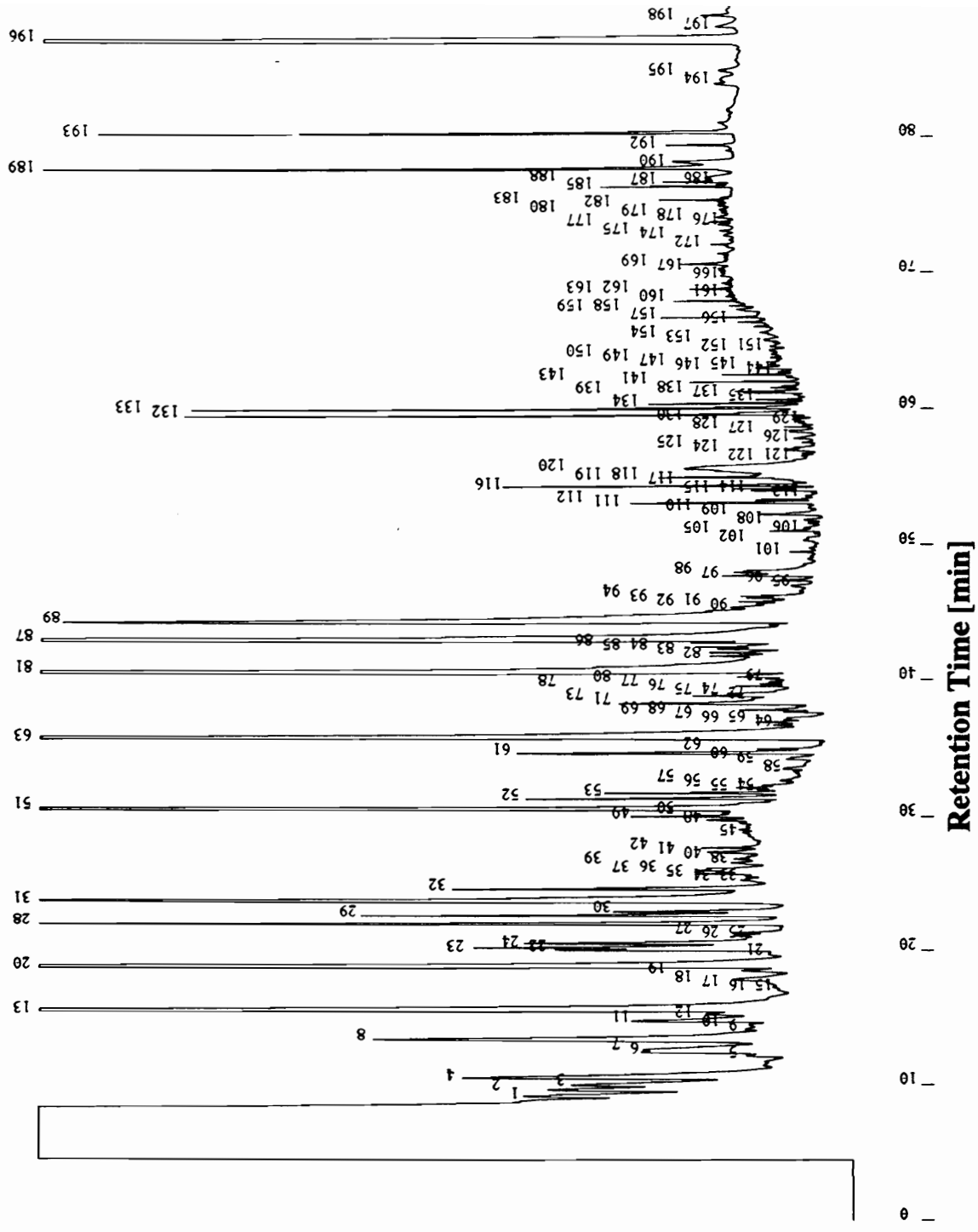


Figure 44. GC chromatogram of n-3 fatty acid esters without treatment (Control) at week 1 of storage

Figure 45. GC chromatogram of n-3 fatty acid esters treated with menhaden gill lipoxygenase extract at week 2 of storage

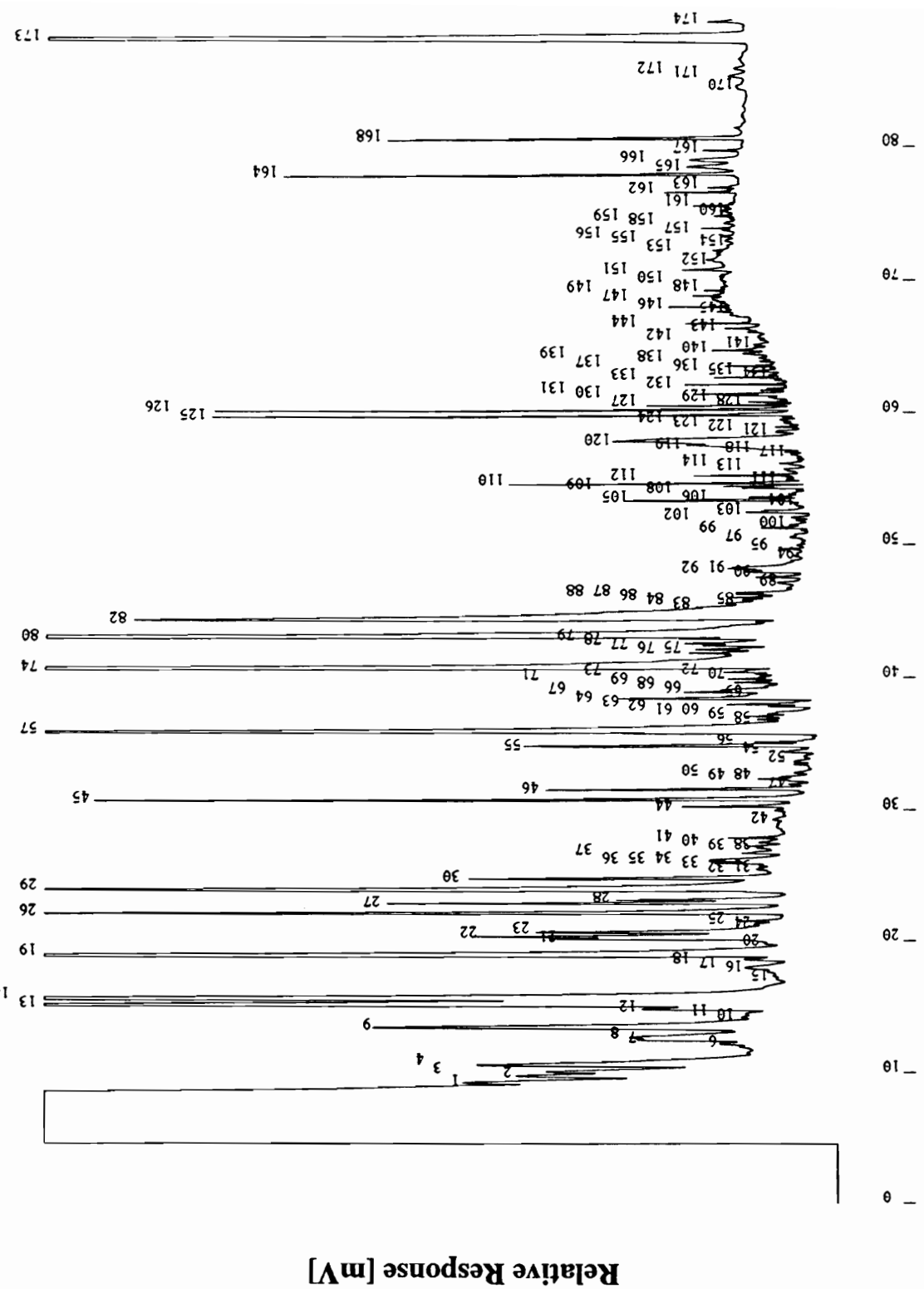


Figure 46. GC chromatogram of n-3 fatty acid esters treated with boiled menhaden gill lipoxygenase extract at week 3 of storage

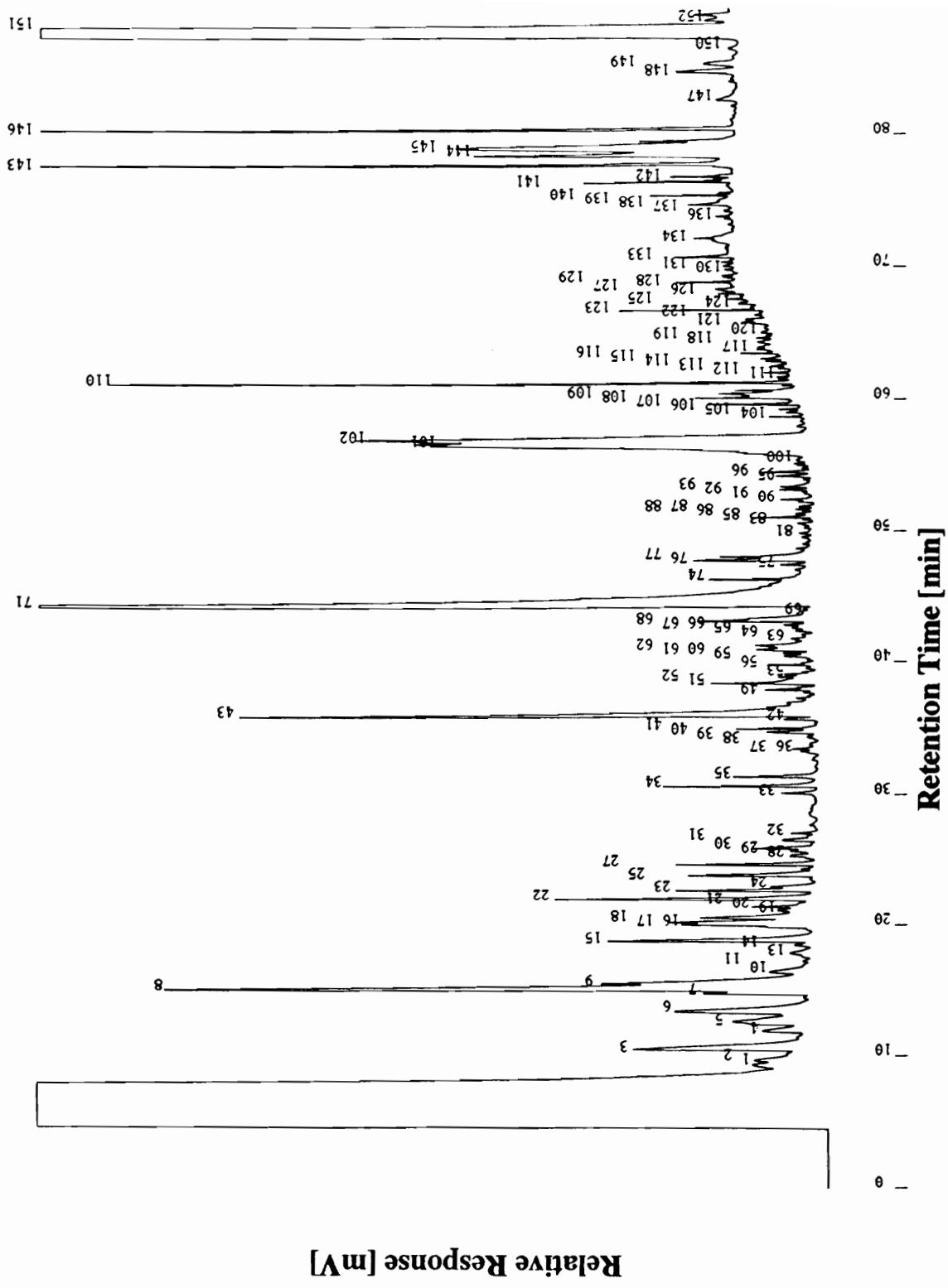


Figure 47. GC chromatogram of n-3 fatty acid esters treated with menhaden gill lipoxygenase extract and esculetin at week 4 of storage

Table 16. Volatiles identified in n-3 fatty acid ester concentrates

Compound Name/Sample[¥]	Peak No.	C	RT	Peak No.	E	RT	Peak No.	B	RT	Peak No.	I	RT
Formic Acid, Ethyl Ester (+)		**259**	n.s.		**257**	n.s.		**253**	n.s.		-	n.s.
2-Octene		**273**	n.s.		**272**	n.s.		**267**	n.s.		**272**	n.s.
Acetic Acid, Ethyl Ester (+)		**294**	n.s.		**292**	n.s.		288*	n.s.		293*	n.s.
3-Methyl 1,4-heptadiene		**315**	n.s.		**314**	n.s.		**309**	n.s.		**314**	n.s.
3-Methyl 1,4-heptadiene[£]		**325**	n.s.		-	n.s.		**319**	n.s.		**324**	n.s.
Ethanol		-	-		**324**	n.s.		-	-		-	-
Unknown**	1	**382**	9.2	1	**381**	9.2	1	**377**	9.3	1	**382**	9.3
Unknown**	2	**400**	9.7	2	**400**	9.7	2	**395**	9.8	2	**400**	9.8
2-Butanol	3	**417**	10.1	3	**416**	10.1	3	**412**	10.1	-	-	-
Butanoic Acid, Ethyl Ester (+)	4	**441**	10.6	4	**440**	10.6	4	**435**	10.7	3	**441**	10.6
2-Methyl-3-hexanone	-	-	-	-	-	-	5	487	12.0	4	**490**	11.9
Butanal	5	**502**	12.2	5	**502**	12.2	6	**497**	12.3	n.s.	502*	n.s.
Hexanal (+)	6	512	12.5	6	511	12.5	7	506	12.6	5	**512**	12.6
2-Pentanol	7	**521**	12.7	7	**521**	12.7	8	**516**	12.8	n.s.	523	n.s.
1,3,6-Octatriene (E,E)	8	**540**	13.4	8	**539**	13.4	9	**534**	13.5	6	**540**	13.4
Ethoxy ethene	9	**547**	14.1	9	**546**	14.1	10	**542**	14.3	n.s.	**548**	n.s.
Pentanoic Acid, Ethyl Ester (+)	11	**594**	14.8	11	**594**	14.8	11	**589**	14.6	7	**595**	14.8
1-Butanol	-	-	-	-	-	-	12	**596**	14.9	8	**602**	15.1
1,4-Dimethyl benzene	12	**615**	15.3	12	**614**	15.3	13	**610**	15.3	9	**615**	15.5
Unknown**	13	**620**	15.6	13	**620**	15.6	14	**616**	15.7	n.s.	**621**	n.s.
2-Methyl-2-pentenal	-	-	-	-	-	-	-	-	-	10	**648**	16.4
Unknown**	20	**730**	18.9	19	**730**	18.9	19	**727**	19.0	15	**731**	18.9
2-Hexenal	21	**763**	-	-	-	-	20	**759**	-	-	-	-
Unknown[§]	22	**781**	20.1	20	**781**	20.1	21	**776**	20.2	16	**781**	20.1
Unknown*[§]	23	**800**	20.3	21	**799**	20.3	22	**795**	20.4	17	**800**	20.3
Unknown[§]	24	**826**	20.6	22	**826**	20.6	23	**822**	20.8	18	**826**	20.6

(Table 16 cont'd. Volatiles identified in n-3 fatty acid ester concentrates)

Compound Name/Sample[¥]	Peak No.	C	RT	Peak No.	E	RT	Peak No.	B	RT	Peak No.	I	RT
Unknown*§	28	**839**	22.1	27	**839**	22.1	26	**835**	22.2	22	**840**	22.1
Unknown*§	29	**864**	22.8	28	**864**	22.8	27	**860**	22.9	23	**865**	22.8
Hexenoic- or Heptenoic Acid Ethyl Ester Isomer	30	**876**	23.1	29	**874**	23.0	28	**871**	23.2	24	**876**	23.1
3-Hexenoic acid,Ethyl Ester (E?)	31	**903**	23.9	30	**903**	23.8	29	**902**	24.1	25	**903**	24.0
3-Hexenoic acid,Ethyl Ester (Z?)	32	**932**	24.8	31	-	24.7	30	**928**	24.9	27	**932**	24.8
Heptenoic acid, Ethyl Ester (+)	51	**1101**	30.8	47	-	30.7	45	-	30.8	34	**1100**	30.7
5-Heptenoicacid, Ethyl Ester (E)	52	**1119**	31.5	49	-	31.4	46	**1114**	31.6	35	**1118**	31.5
Heptenoic acid, Ethyl Ester	53	**1144**	31.9	50	-	31.8	47	**1140**	31.9	-	-	-
Unknown**	61	**1228**	34.8	57	**1228**	34.8	55	**1225**	35.0	40	**1229**	34.9
2,4-Heptadienal (E,Z)	63	**1282**	35.9	60	**1281**	36.0	57	**1277**	36.1	43	**1281**	36.1
2,4-Heptadienal (E,E)	71	**1344**	38.4	65	1344	38.4	65	1338	38.6	51	**1343**	38.5
Benzaldehyde (+)		-		-	-	-	72	**1406**	40.5	n.i.	**1412**	n.i.
1,3-Butanediol	81	**1422**	40.7	68	**1422**	40.7	74	**1419**	40.8	61	1424	40.9
2,3-Butanediol	87	**1495**	43.1	84	**1495**	43.1	80	**1491**	43.3	68	**1495**	43.2
1,2-Propanediol	89	**1530**	44.4	86	**1529**	44.3	82	**1532**	44.6	71	**1530**	44.4
2-Decenal (Z) (Internal Std.)	97	1640	47.8	96	1639	47.8	91	1635	48.0	76	**1640**	47.8
Unknown**	111	**1775**	53.2	111	**1774**	53.2	105	**1771**	53.4	n.i.	**1775**	n.i.
Unknown**	116	**1811**	54.3	116	**1810**	54.5	110	**1806**	54.7	n.i.	**1812**	54.3
Unknown**	132	**1965**	59.7	134	**1963**	59.7	125	**1959**	59.9	107	**1964**	59.8
Unknown**	133	**1980**	60.2	135	**1978**	60.2	126	**1975**	60.4	108	**1979**	60.2
Antioxidant	141	**2033**	62.0	144	**2033**	62.1	131	**2033**	61.4	110	**2033**	61.4
Undecanoic Acid, Ethyl Ester(+)	157	-	66.8	162	-	66.8	144	-	66.9	123	**2175**	66.8
Ethoxy Benzoic Acid, Ethyl Ester	169	**2327**	70.8	176	**2325**	70.8	151	2320	71.0	133	**2325**	70.8

(Table 16 cont'd. Volatiles identified in n-3 fatty acid ester concentrates)

¥:	C = Control
	E = Ester treated with enzyme
	B = Ester treated with boiled enzyme
	I = Ester treated with enzyme and esculetin
RT	GC Retention Time
n.s.	not separated
n.i.	not integrated
-	compound not present

Bolded: identified by Scan Number and GC-MS

Unbolded: identified by Scan Number only

Unknown*	Matches for all compounds was 3-ethenyl-cyclooctene, 1,4-cyclooctadiene or -yne(triple bond) compounds below 80%
Unknown**	All peaks had two major ions: 45 and 73, which are common to all dioxolanes, methoxy- and ethoxy- propane, -butane and -pentane
Scan No.*	coeluting peaks
£	Ethanol might be coeluting in this peak, but is so insignificant that the match for 3-methyl 1,4-heptadiene is above 80%
§	Identical to compound found in undeodorized menhaden oil
+	Compounds identified after autoxidation of ethyl linoleate (Henderson et al. 1980)

Table 17. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage

Compound Name	Concentration [ppm] and Day			
	C* 0	E* 0	B* 0	I* 0
2-Butanol	21.01	27.45	31.48	59.94
Butanoic acid, Ethyl Ester	14.08	13.66	15.21	17.69
1,3,6-Octatriene (E,E)	77.47	95.77	87.64	143.28
Ethoxy ethene	4.48	7.49	1.95	5.19
Pentanoic acid, Ethyl Ester	9.48	12.35	5.44	0.00
1-Butanol	0.00	0.00	0.00	0.00
1,4-Dimethyl benzene	9.53	13.66	10.61	17.20
2-Methyl-2-pentenal	0.00	0.00	0.00	0.00
Hexenoic- or Heptenoic acid Ester	5.79	6.87	7.57	12.48
3-Hydroxy butanone	132.34	163.39	179.57	311.47
3-Hexenoic acid, Ethyl Ester (Z)	15.20	17.94	18.45	34.41
Heptenoic acid, Ethyl Ester	41.47	3.86	6.36	3.45
5-Heptenoic acid, Ethyl Ester (E)	2.78	3.53	3.75	5.99
4-or 6-Heptenoic acid, Ethyl Ester	2.36	0.65	0.78	1.33
2,4-Heptadienal (E,Z)	19.24	24.39	25.80	49.41
2,4-Heptadienal (E,E)	7.18	11.72	16.43	22.78
Benzaldehyde	8.90	13.55	14.27	25.15
1,3-Butanediol	436.67	545.83	607.61	1102.9
2,3-Butanediol	601.96	764.33	848.67	1553.0
1,2-Propanediol	307.20	478.24	608.82	1211.5
Undecanoic acid, Ethyl Ester	4.65	5.28	7.14	13.26
Ethoxy Benzoic acid, Ethyl Ester	5.25	7.80	8.43	22.62
Total Volatile Area	5173397	6239998	6867966	6091160

(Table 17 cont'd. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage)

Compound Name	Concentration [ppm] and Day			
	C 1	E 1	B 1	I 1
2-Butanol	16.68	16.55	23.66	9.61
Butanoic acid, Ethyl Ester	40.94	17.78	50.06	8.16
1,3,6-Octatriene (E,E)	80.41	66.49	108.44	36.63
Ethoxy ethene	1.08	5.11	1.54	2.83
Pentanoic acid, Ethyl Ester	22.26	9.48	30.37	5.83
1-Butanol	0.00	4.15	0.00	0.00
1,4-Dimethyl benzene	9.52	10.98	13.92	5.35
2-Methyl-2-pentenal	0.55	0.41	0.54	1.35
Hexenoic- or Heptenoic acid Ester	13.62	6.22	18.14	3.23
3-Hydroxy butanone	127.69	107.97	180.30	62.67
3-Hexenoic acid, Ethyl Ester (Z)	27.06	14.55	36.10	7.34
Heptenoic acid, Ethyl Ester	156.86	145.31	197.72	79.17
5-Heptenoic acid, Ethyl Ester (E)	14.26	3.93	18.36	1.92
4-or 6-Heptenoic acid, Ethyl Ester	8.81	7.41	14.06	5.77
2,4-Heptadienal (E,Z)	190.96	15.79	240.41	10.01
2,4-Heptadienal (E,E)	21.31	9.57	27.88	7.39
Benzaldehyde	9.07	5.89	11.49	6.07
1,3-Butanediol	322.42	350.30	440.42	208.42
2,3-Butanediol	450.70	488.36	611.77	288.92
1,2-Propanediol	161.35	299.96	238.38	123.71
Undecanoic acid, Ethyl Ester	3.51	3.48	5.68	3.16
Ethoxy Benzoic acid, Ethyl Ester	2.97	3.09	4.23	1.89
Total Volatile Area	7635817	5971189	6892015	6469148

(Table 17 cont'd. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage)

Compound Name	Concentration [ppm] and Day			
	C 2	E 2	B 2	I 2
2-Butanol	5.11	7.34	13.46	14.79
Butanoic acid, Ethyl Ester	11.58	10.01	34.06	16.91
1,3,6-Octatriene (E,E)	29.77	41.07	70.45	71.25
Ethoxy ethene	0.18	0.68	0.80	1.42
Pentanoic acid, Ethyl Ester	9.84	7.92	23.92	11.25
1-Butanol	0.00	0.00	0.00	0.00
1,4-Dimethyl benzene	2.75	5.09	7.10	9.18
2-Methyl-2-pentenal	0.13	0.16	0.34	0.24
Hexenoic- or Heptenoic acid Ester	6.32	3.56	15.85	5.31
3-Hydroxy butanone	47.48	60.41	121.46	101.02
3-Hexenoic acid, Ethyl Ester (Z)	11.85	8.46	29.78	14.19
Heptenoic acid, Ethyl Ester	50.45	36.74	62.79	49.20
5-Heptenoic acid, Ethyl Ester (E)	7.16	3.03	17.57	3.53
4-or 6-Heptenoic acid, Ethyl Ester	1.73	1.06	0.00	1.42
2,4-Heptadienal (E,Z)	106.98	8.84	263.03	16.92
2,4-Heptadienal (E,E)	4.66	8.24	21.82	8.72
Benzaldehyde	1.68	5.95	3.27	3.88
1,3-Butanediol	105.86	206.21	289.83	373.77
2,3-Butanediol	152.17	285.10	418.92	521.83
1,2-Propanediol	37.53	105.05	113.38	199.66
Undecanoic acid, Ethyl Ester	10.07	7.88	5.87	5.48
Ethoxy Benzoic acid, Ethyl Ester	0.87	3.92	3.23	4.18
Total Volatile Area	8027720	7219629	7762701	6359773

(Table 17 cont'd. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage)

Compound Name	Concentration [ppm] and Day			
	C 3	E 3	B 3	I 3
2-Butanol	19.53	18.11	25.50	19.42
Butanoic acid, Ethyl Ester	44.01	15.97	65.17	23.99
1,3,6-Octatriene (E,E)	89.49	64.87	110.14	75.74
Ethoxy ethene	1.10	1.32	1.38	2.30
Pentanoic acid, Ethyl Ester	20.08	7.95	21.58	6.46
1-Butanol	0.00	0.00	0.00	0.00
1,4-Dimethyl benzene	323.31	213.08	229.80	245.24
2-Methyl-2-pentenal	0.00	0.00	0.00	0.31
Hexenoic- or Heptenoic acid Ester	22.60	8.16	24.23	9.89
3-Hydroxy butanone	185.95	120.97	213.54	146.52
3-Hexenoic acid, Ethyl Ester (Z)	43.25	17.04	46.73	20.41
Heptenoic acid, Ethyl Ester	94.43	31.81	68.81	99.99
5-Heptenoic acid, Ethyl Ester (E)	23.96	7.13	26.60	7.43
4-or 6-Heptenoic acid, Ethyl Ester	3.09	1.62	1.98	9.41
2,4-Heptadienal (E,Z)	330.98	16.61	309.21	20.19
2,4-Heptadienal (E,E)	37.46	11.14	33.42	20.46
Benzaldehyde	7.67	11.60	15.95	13.89
1,3-Butanediol	440.01	413.75	580.06	508.16
2,3-Butanediol	491.05	576.39	816.97	714.94
1,2-Propanediol	143.88	186.12	236.61	299.72
Undecanoic acid, Ethyl Ester	9.10	4.86	7.11	4.72
Ethoxy Benzoic acid, Ethyl Ester	13.01	3.76	13.01	3.98
Total Volatile Area	10864620	7656782	7981307	10812275

(Table 17 cont'd. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage)

Compound Name	Concentration [ppm] and Day			
	C 4	E 4	B 4	I 4
2-Butanol	0.00	0.00	0.00	0.00
Butanoic acid, Ethyl Ester	32.75	19.12	32.00	19.69
1,3,6-Octatriene (E,E)	31.29	20.68	25.17	18.86
Ethoxy ethene	0.00	0.00	0.00	0.00
Pentanoic acid, Ethyl Ester	15.62	5.99	15.70	6.26
1-Butanol	0.00	0.00	0.00	0.00
1,4-Dimethyl benzene	45.13	77.46	39.08	63.89
2-Methyl-2-pentenal	3.33	3.95	3.28	4.60
Hexenoic- or Heptenoic acid Ester	11.75	2.95	12.07	2.66
3-Hydroxy butanone	40.87	8.01	41.42	7.84
3-Hexenoic acid, Ethyl Ester (Z)	21.27	7.29	21.28	6.79
Heptenoic acid, Ethyl Ester	32.01	7.35	32.69	6.95
5-Heptenoic acid, Ethyl Ester (E)	16.26	5.76	17.32	4.48
4-or 6-Heptenoic acid, Ethyl Ester	0.00	0.00	0.00	0.00
2,4-Heptadienal (E,Z)	217.26	56.36	208.51	53.46
2,4-Heptadienal (E,E)	18.13	7.58	16.79	6.95
Benzaldehyde	2.17	2.04	2.00	1.61
1,3-Butanediol	0.85	1.20	0.88	1.08
2,3-Butanediol	7.44	9.46	4.65	9.86
1,2-Propanediol	88.92	191.59	99.17	162.47
Undecanoic acid, Ethyl Ester	3.33	4.52	5.97	3.04
Ethoxy Benzoic acid, Ethyl Ester	2.73	2.54	1.83	2.30
Total Volatile Area	4478977	3761600	5889094	5120210

(Table 17 cont'd. Changes in concentration of volatiles identified in n-3 fatty acid ester concentrates with various treatments over 8 weeks of storage)

Compound Name	Concentration [ppm] and Day			
	C 8	E 8	B 8	I 8
2-Butanol	0.00	0.00	0.00	0.00
Butanoic acid, Ethyl Ester	11.74	21.22	30.64	23.22
1,3,6-Octatriene (E,E)	12.01	14.33	19.60	17.88
Ethoxy ethene	0.00	0.00	0.00	0.00
Pentanoic acid, Ethyl Ester	11.81	5.74	25.10	8.87
1-Butanol	0.00	0.00	0.00	9.84
1,4-Dimethyl benzene	0.00	14.27	0.00	1.57
2-Methyl-2-pentenal	1.17	4.66	3.53	3.39
Hexenoic- or Heptenoic acid Ester	1.05	2.52	10.93	3.67
3-Hydroxy butanone	21.26	7.96	38.19	11.78
3-Hexenoic acid, Ethyl Ester (Z)	10.77	7.21	19.93	8.90
Heptenoic acid, Ethyl Ester	17.07	7.43	29.75	10.84
5-Heptenoic acid, Ethyl Ester (E)	8.53	3.92	14.84	6.84
4-or 6-Heptenoic acid, Ethyl Ester	0.00	0.00	0.00	0.00
2,4-Heptadienal (E,Z)	101.38	49.71	178.33	71.62
2,4-Heptadienal (E,E)	11.57	7.68	19.05	9.43
Benzaldehyde	1.61	1.32	1.07	1.55
1,3-Butanediol	0.83	1.21	1.24	1.24
2,3-Butanediol	1.60	2.93	2.86	2.60
1,2-Propanediol	43.23	172.81	90.88	150.56
Undecanoic acid, Ethyl Ester	2.09	7.93	3.21	3.03
Ethoxy Benzoic acid, Ethyl Ester	4.05	6.68	3.52	2.12
Total Volatile Area	6234184	4897261	4989852	3744710

* C = Control
E = Enzyme treated
B = Boiled enzyme treated
I = Enzyme and esculetin treated

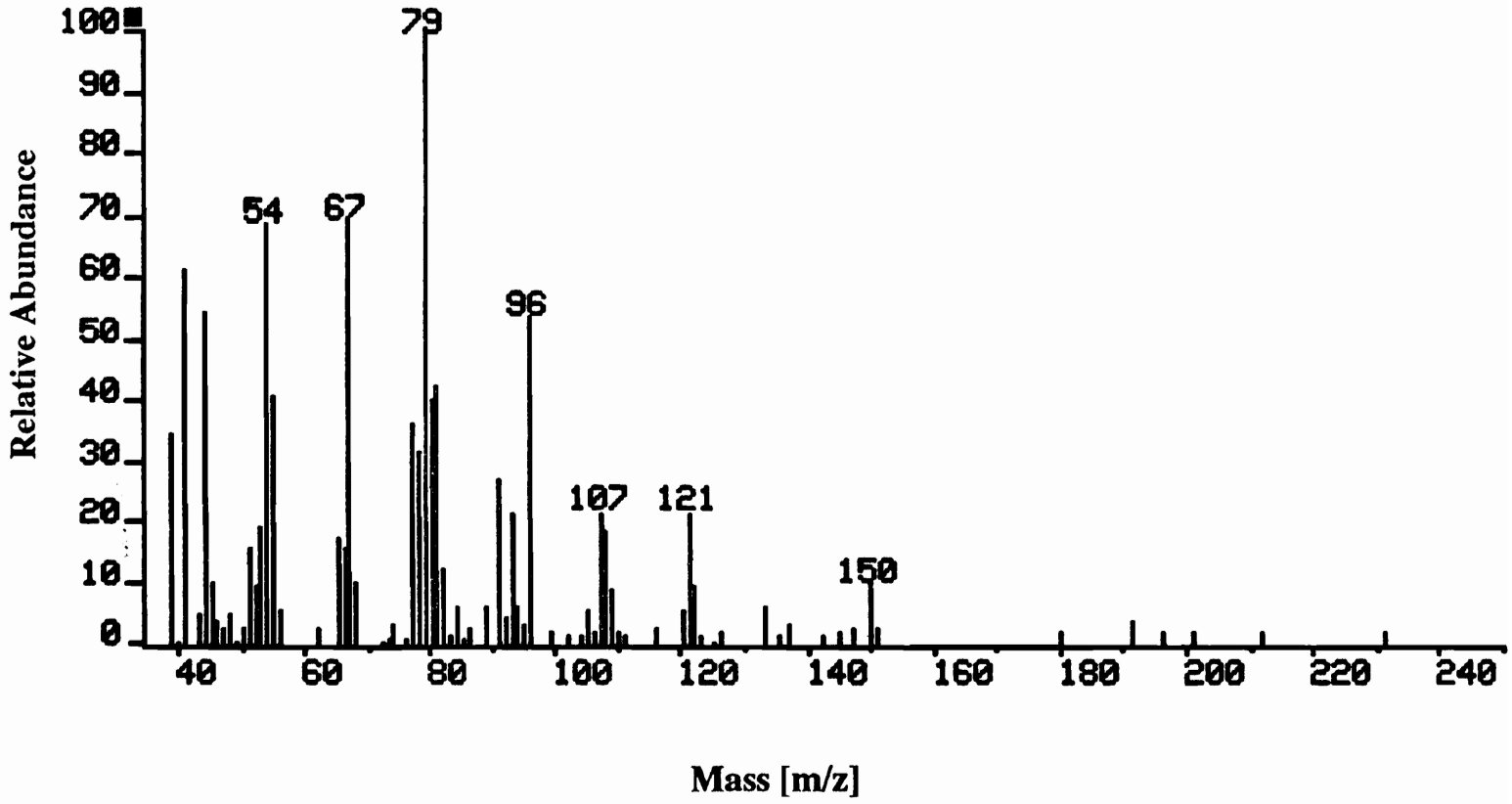


Figure 48. Mass spectrum of volatile eluting after 22.1 minutes in the n-3 fatty acid ester concentrates

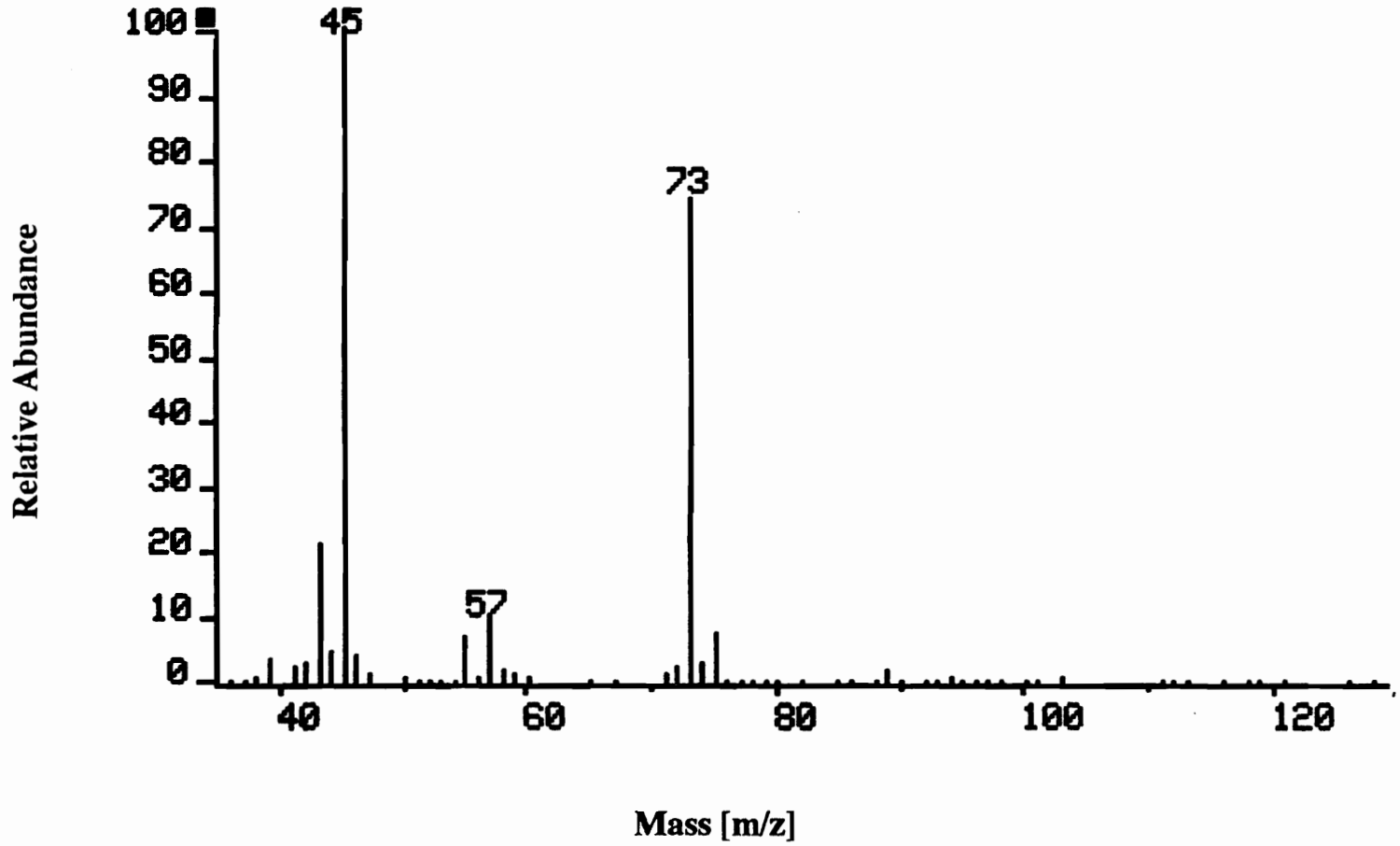


Figure 49. Mass spectrum of volatile eluting after 15.7 minutes in the n-3 fatty acid ester concentrates

and deodorized menhaden oils with the same retention time. These compounds are marked with § or §§ respectively in Table 16. A sample mass spectrum (RT = 15.7) of the unknowns repeatedly identified as dioxolanes or methoxy- or ethoxy- short chain aliphats (marked with **) is shown in Figure 49. The simplicity of the mass spectra (Figure 49) makes proper identification of these compounds virtually impossible, since a large number of various dioxolanes match these mass spectra.

Based on the ANOVA, there was no significant differences in the amount of total volatiles between the different treatments. However, the statistical analysis has limited validity since only one sample per week for each treatment was taken, thus a week effect could not be considered. The paired t-test on just the control and enzyme treated ester, which considers week effects due to the pairing of the samples by week, also did not show any significant differences ($p = 0.1086$) in total volatiles. Only two compounds appear to occur in higher concentrations in the enzyme treated ester than in the control. Ethoxy ethene seems to be slightly higher in the beginning of the study in the enzyme treated ester, while towards the end of the study propanediol begins to increase in the enzyme treated ester relative to the control. However, since these changes can also be seen in the ester with the esculetin inhibited enzyme extract and there is no likely pathway for the lipoxygenase derived formation of ethoxy ethene or propanediol, it is unlikely that lipoxygenase is responsible for these differences.

Changes in volatile concentrations observed in the esters used in the second part of the sensory analysis are shown in Table 18. Since these samples are those that were evaluated by the sensory panel, a comparison of the volatile analysis with the sensory results can be made. The sensory panel judged the samples to be significantly different after one week of incubation. These results are also reflected by the volatile analysis. As can be seen in Table 18, the total volatiles are higher in the enzyme-treated ester than in the control ester for all days except Day 0, which is the measurement taken after pipeting the enzyme into the oil without incubation time. When the paired t-test was used on all samples (Day 0 - Day 8), there was no significant difference ($p = 0.0659$) between the esters. However, when Day 0 was dropped from the analysis, which is appropriate since there cannot be any enzyme effect before incubation, the difference was statistically significant ($p = 0.0186$).

Table 18. Changes in volatiles identified in n-3 fatty acid ester concentrates used in Stage 2 of the sensory analysis

Compound Name	Concentration [ppm] and Day				
	C* 0	E* 0	C 1	E 1	C 2
Butanoic acid, Ethyl Ester	7.25	7.86	7.89	8.40	14.71
1,3,6-Octatriene (E,E)	5.35	5.74	6.12	8.44	9.39
Pentanoic acid, Ethyl Ester	1.79	0.00	2.11	2.07	4.15
1-Butanol	0.00	0.00	0.00	0.00	0.00
2-Methyl-2-pentenal	2.59	0.00	2.83	3.28	0.00
3-Hydroxy butanone	0.72	0.00	0.87	1.06	0.00
3-Hexenoic acid, Ethyl Ester (Z)	2.36	2.41	2.43	2.80	4.28
5-Heptenoic acid, Ethyl Ester (E)	0.47	0.44	0.46	0.47	0.96
2,4-Heptadienal (E,Z)	6.72	7.05	7.22	7.55	7.27
2,4-Heptadienal (E,E)	0.00	0.00	0.00	0.00	3.33
Benzaldehyde	0.77	0.44	0.89	0.49	0.61
1,3-Butanediol	0.16	0.00	0.15	0.17	0.73
2,3-Butanediol	0.00	0.00	1.74	4.26	4.88
1,2-Propanediol	275.17	251.15	288.82	312.68	577.66
Undecanoic acid, Ethyl Ester	2.92	2.58	2.60	2.06	3.33
Ethoxy Benzoic acid, Ethyl Ester	1.53	1.38	1.66	2.47	2.20
Total Volatile Area	2765631	2647737	3140477	3528692	1805206

Compound Name	Concentration [ppm] and Day				
	E 2	C 3	E 3	C 8	E 8
Butanoic acid, Ethyl Ester	16.49	6.59	5.72	8.13	10.06
1,3,6-Octatriene (E,E)	0.00	7.00	6.20	5.99	7.40
Pentanoic acid, Ethyl Ester	0.00	1.98	1.75	1.81	2.18
1-Butanol	0.00	0.00	0.00	491.65	640.44
2-Methyl-2-pentenal	0.00	2.63	2.30	0.00	2.69
3-Hydroxy butanone	0.00	0.76	0.65	0.00	0.00
3-Hexenoic acid, Ethyl Ester (Z)	5.53	2.41	2.07	2.83	3.71
5-Heptenoic acid, Ethyl Ester (E)	0.00	0.69	0.69	0.70	0.78
2,4-Heptadienal (E,Z)	6.22	6.45	6.25	6.24	6.60
2,4-Heptadienal (E,E)	4.82	1.78	1.77	2.23	3.21
Benzaldehyde	0.75	0.46	0.50	0.47	0.58
1,3-Butanediol	0.84	0.58	0.54	0.47	0.61
2,3-Butanediol	0.00	0.00	0.00	8.48	11.33
1,2-Propanediol	739.54	311.80	238.37	214.40	311.51
Undecanoic acid, Ethyl Ester	4.40	2.64	2.25	3.04	2.56
Ethoxy Benzoic acid, Ethyl Ester	4.50	2.73	2.95	3.58	6.93
Total Volatile Area	2465929	3108152	3755255	4839470	5075548

* C = Control E = Enzyme treated

While in the beginning most compounds had similar concentrations in the enzyme treated ester and the control ester, after 8 days virtually all compounds were higher in the enzyme treated ester. Since the increase in volatiles was a general increase rather than an increase of specific volatiles, these results are in support of the theory that lipoxygenase accelerates oxidation of the oil by promoting autoxidation through what has been called a hydroperoxide seeding action, but does not contribute a large amount of specific lipoxygenase derived volatiles.

Since the volatiles have different smells and different thresholds for sensory perception, the reason why the sensory panel was able to differentiate between the two esters after one week is not necessarily due to a general increase in volatiles but could be caused by the increase of only a few very specific volatiles that have low thresholds or a strong smell. However, in order to evaluate which volatiles are the major contributors, a sniff port on the GC would have been needed.

Since the esters that were analyzed by the sensory panel and those analyzed by GC in the first part of the sensory analysis were not truly identical due to the different storage conditions (see paragraph 3 on page 40), these results were found only in the second part of the sensory analysis.

6.10. Eicosapentanoic and Docosahexaenoic Acid Ethyl Ester Volatiles

The GC chromatograms of EPA- and DHA-ethyl ester incubated with menhaden gill lipoxygenase extract are shown in Figures 50/50a and 51/51a, respectively. The volatiles identified in the two samples are displayed in Table 19.

Autoxidation of methyl eicosapentaenoate has been shown to yield eight monohydroperoxide isomers, that is 5-, 8-, 9-, 11-, 12-, 14-, 15-, and 18- hydroperoxy eicosapentaenoate (Yamauchi et al., 1983), while docosahexaenoic acid yields 10 isomers, namely 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20- hydroperoxy docosahexaneoate isomers (Noble and Nawar, 1971; Van Rollins and Murphy, 1984). The aliphatic aldehydes identified by Noble and Nawar (1971) that were derived from the hydroperoxy docosahexaenoate isomers were propanal, hexanal, 3-hexenal, 2,4-heptadienal, 3,6-nonadienal, 2,4-decadienal, decatrienal, 3,6,9-dodecatrienal, tridecatetraenal, and 3,6,9,12-pentadecatetraenal. In a recent study, Boyd et al. (1992) identified propenal, propanal and acetaldehyde as the main volatiles in the head space of

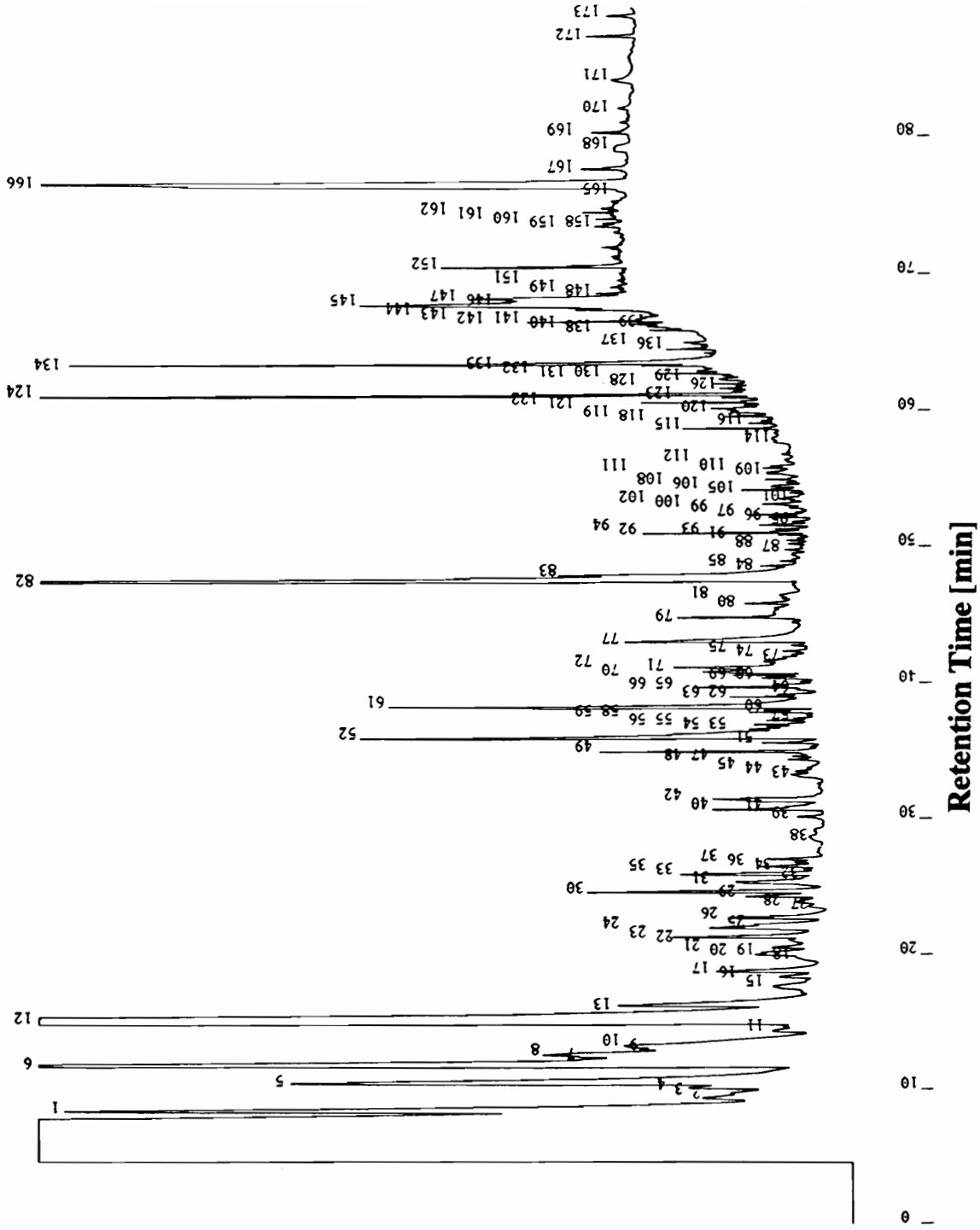


Figure 50. GC chromatogram of EPA ethyl ester incubated with menhaden gill lipoxygenase extract

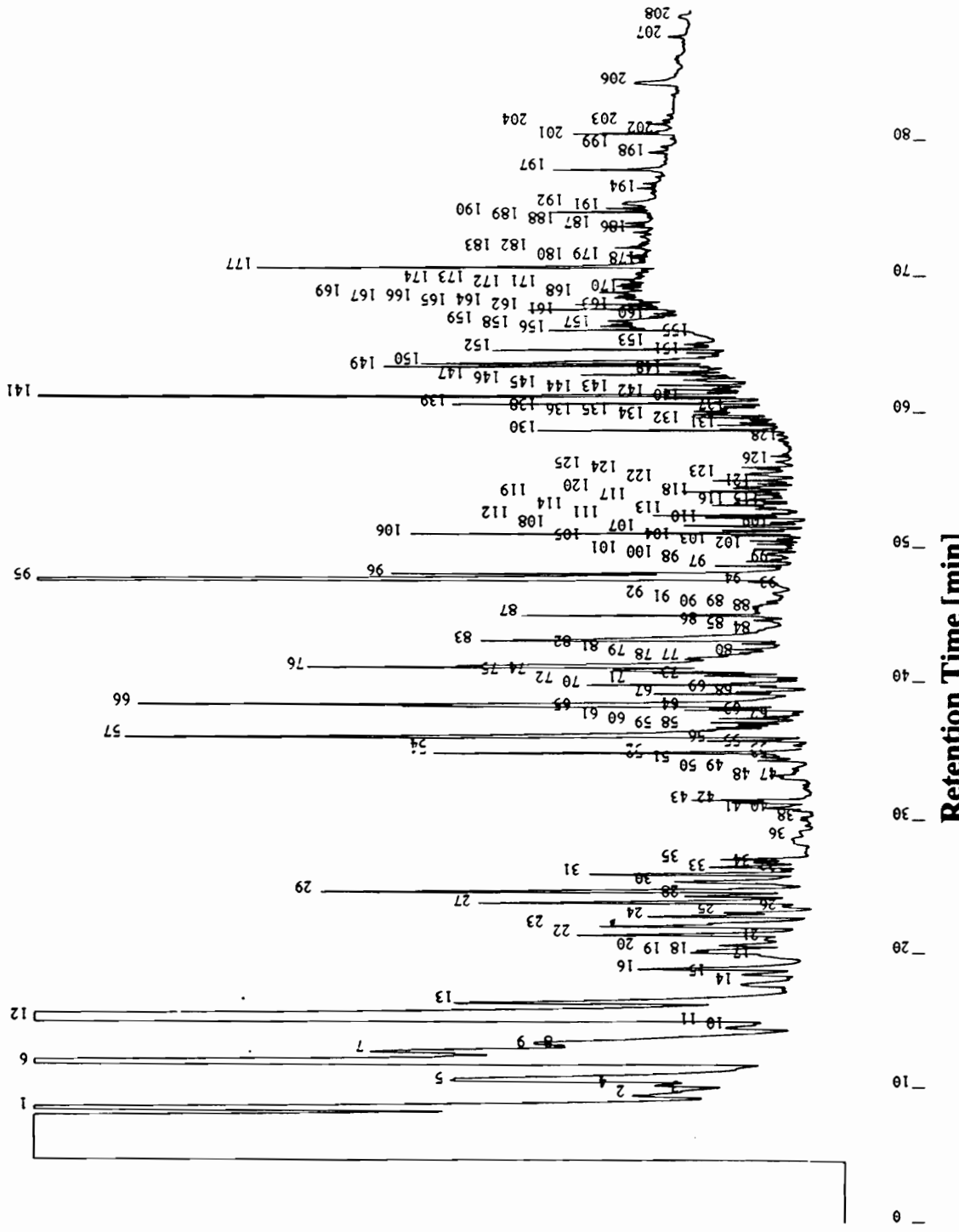


Figure 51. GC chromatogram of DHA ethyl ester incubated with menhaden gill lipoxygenase extract

Table 19. Volatiles identified in EPA- and DHA-ethyl ester incubated with menhaden gill lipoxygenase extract

Compound	EPA			DHA		
	Peak No.	RT	MS Scan	Peak No.	RT	MS Scan
Trimethyl hexane	n.i.	n.s.	n.p.	n.i.	n.s.	325
4-Methyl octane	n.i.	n.s.	n.p.	n.i.	n.s.	353
Acetic Acid, Ethyl Ester	n.i.	n.s.	377	n.i.	n.s.	381
3-Methyl 1,4-heptadiene	n.i.	n.s.	n.p.	n.i.	n.s.	404
2-Propanol	n.i.	n.s.	417	n.i.	n.s.	422
Benzene	1	8.5	432	1	8.5	437
2,4,6-Trimethyl decane	-	-	n.p.	2	9.5	535
2,6,8-Trimethyl decane	2	9.5	538	2	9.5	542
2,2,9 Trimethyl decane	-	-	n.p.	2	9.5	551
Methyl benzene	5	10.6	560	5	10.6	568
2,6,6-Trimethyl dodecane	-	-	n.p.	6	11.9	580
2,6,11-Trimethyl dodecane	6	11.9	619	6	11.9	623
Trimethyl dodecane Isomer	6	11.9	628	6	11.9	633
Hexanal	8	12.7	-	7	12.8	639
Dimethyl 1-octanol	9	13.1	-	8	13.1	662
2-Ethyl 4-pentenal	10	13.4	-	9	13.3	670
2-Butanone	10	13.4	-	9	13.3	679
Ethyl benzene	-	n.i.	-	10	14.5	742
1,4-Dimethyl benzene	12	15.2	750	12	15.2	755
3-Methyl 2-butanol	12	15.2	764	12	15.2	770
2-Methyl 2-pentenal	13	16.3	794	13	16.4	799
2-Buten-1-ol	15	17.7	846	-	-	n.p.
Unknown	-	-	n.p.	14	17.7	849
D-Limonene	16	n.i.	861	15	18.5	868

(Table 19 cont'd. Volatiles identified in EPA- and DHA ethyl ester incubated with menhaden gill lipoxygenase extract)

Compound	EPA			DHA		
	Peak No.	RT	MS Scan	Peak No.	RT	MS Scan
2-Pentanol	17	18.8	888	16	18.9	895
1,5 Cyclododecadiene	-	-	n.p.	17	19.9	940
3-Hepten-2-ol (Z)	19	20.1	937			
Unknown (ali)	-	-	n.p.	18	20.1	945
Unknown (ali)	21	20.6	955	20	20.6	963
Unknown (ali)	-	-	n.p.	22	21.5	994
4-Ethenyl cyclooctene	24	22.0	999	23	22.1	1005
3-Ethenyl cyclooctene	26	22.7	1028	24	22.8	1034
Unknown (ali)	27	n.i.	1064	26	23.6	1070
3-Hexenoic Acid, Ethyl Ester	-	-	n.p.	27	23.9	1084
Unknown (ali)	-	-	n.p.	28	24.4	1093
3-Hexenoic Acid, Ethyl Ester	-	-	n.p.	29	24.8	1117
4,6,8-Trimethyl-1-nonene	-	-	n.p.	30	25.5	1145
6-Heptenoic Acid, Ethyl Ester	39	n.i.	1300	-	-	n.p.
5-Heptenoic Acid, Ethyl Ester (E)	40	30.7	1319	-	-	n.p.
Nonanal	-	-	n.p.	41	30.9	1336
5-Heptenoic Acid, Ethyl Ester (Z)	42	31.4	1348	-	-	n.p.
Acetic Acid	49	35.0	1479	54	35.1	1485
2,4-Heptadienal (E,Z)	52	36.1	1508	57	36.1	1514
2-Ethyl-1-Hexanol	55	37.0	1530	60	37.1	1536
2,4-Heptadienal (E,E)	61	38.4	1578	66	38.5	1584
3,5 Octadiene-2-one (E,E)	65	39.8	1634	70	39.9	1639
Benzaldehyde	-	-	n.p.	75	40.9	1659
1,3-Butanediol	71	41.3	1671	76	41.2	1679

(Table 19 cont'd. Volatiles identified in EPA and DHA-ethyl ester incubated with menhaden gill lipoxygenase)

Compound	EPA			DHA		
	Peak No.	RT	MS Scan	Peak No.	RT	MS Scan
2-Nonanol	72	41.6	1687	-	-	n.p.
2-Methyl Butanoic Acid, Ethyl Ester	-	-	n.p.	80	42.5	1712
2,3-Butanediol	77	43.2	1748	83	43.2	1754
2-Decenal (Z) (Int. Std.)	82	47.7	1900	95	47.7	1903
Hexanoic Acid, Ethyl Ester	83	48.1	1923	96	48.2	1929
Antioxidant	124	61.3	2306	141	61.4	2316
2,4,6-Trimethyl Nonanoic Acid, Methyl Ester	152	70.8	2600	-	-	n.p.
2-Ethyl Undecanoic Acid, Methyl Ester	166	76.9	2772	-	-	n.p.

n.i. peak not integrated

n.s. peak not separated

n.p. peak not present

(ali) long aliphatic chain (positive identification not possible)

thermally oxidized EPA and DHA, of which propenal was the most abundant volatile. Other volatiles identified in that study were 2-buten-1-ol, butanal, 1-penten-3-ol, 2-pentenal, hexanal, and octanal. While hexanal was the major volatile in the headspace of thermally oxidized linoleic acid, it was only a minor volatile in the EPA and DHA samples (Boyd et al., 1992). Based on the monohydroperoxy isomers derived from EPA and DHA, it is possible to predict the aldehydes that should be found in autoxidized EPA and DHA. The aldehydes derived from 14-hydroperoxy EPA and 17-hydroperoxy DHA are shown as examples in Figures 52 and 53 respectively. The following aldehydes should be derived as the primary aldehydes from the eight monohydroperoxides of EPA: acetaldehyde, propanal, 2-pentenal, 3-hexenal, 2,4-heptadienal, 2,5-octadienal, 3,6-nonadienal, 2,4,7-decatrienal, 2,5,8-undecatrienal, 3,6,9-dodecatrienal, 2,4,7,10-tridecatetraenal, 3,6,9,12-pentadecatetraenal, and 2,4,7,10,13-hexadecapentaenal. The primary aldehydes derived from the monohydroperoxides of DHA are virtually the same since the locations of the double bonds from the methyl end of the acid are the same, with the exception, that DHA will have 3 more aldehydes, namely: 2,5,8,11-tetradecatetraenal, 3,6,9,12,15-octadecapentaenal, and 2,4,7,10,13,16-nonadecahexaenal. However, the longer chain aldehydes will most certainly not be detectable, since not only will monohydroperoxides occur, but multiple hydroperoxides, which will break down into smaller aldehydes. In addition, since ß-scission can also lead to hydroxyl groups, and isomerization, given the location of adjacent double bonds in EPA and DHA a multitude of additional aldehydes, ketones and alcohols are to be expected.

Several volatiles identified in the EPA and DHA ethyl esters were previously identified in the undeodorized and deodorized menhaden oil, as well as in the n-3 fatty acid ester concentrates. As was found for the n-3 fatty acid ester concentrates, a considerable number of shorter chain fatty acid ethyl esters were identified. It appears that hexenoic acid isomers are derived from EPA, while heptenoic acid isomers are derived from DHA. Classical volatiles such as 2,4-heptadienal and 3,5-octadiene-2-one which had been identified in the undeodorized and deodorized menhaden oils were present as well. Since 1,3 and 2,3-butanediol were found in the deodorized oil, the ester concentrates and the EPA and DHA ethyl esters, but not in the undeodorized oil, it is likely that they are either derived from a preservative incorporated in these oils, or that stripping the oils of volatiles favors the formation of these diols. No lipoxygenase

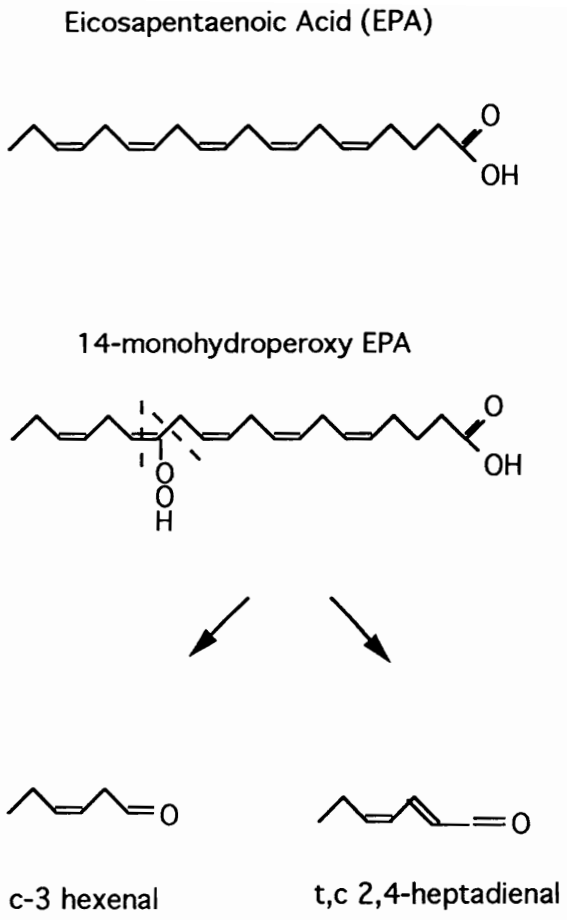


Figure 52. Formation of 14-monohydroperoxy-EPA and the resulting aldehydes

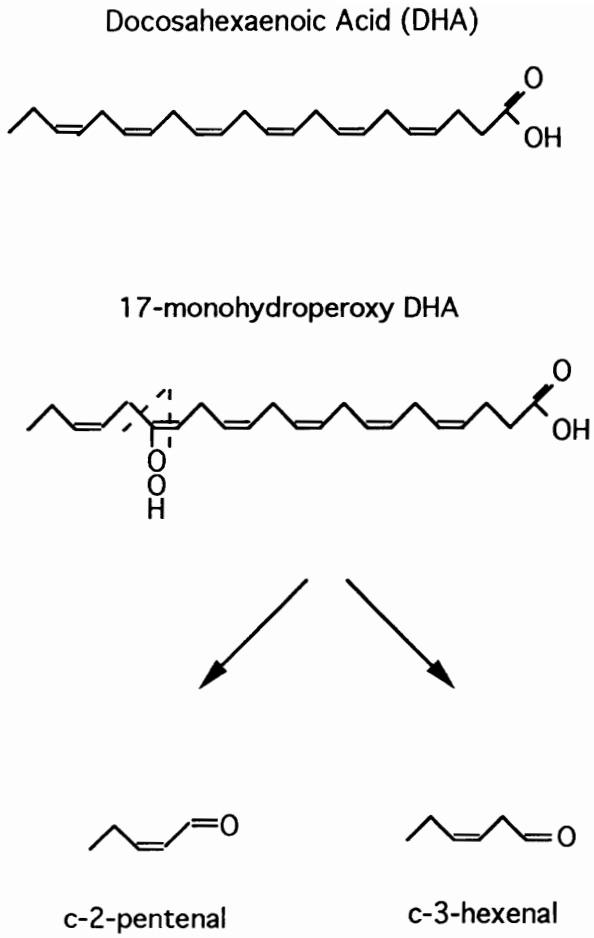


Figure 53. Formation of 17-monohydroperoxy-DHA and the resulting aldehydes

derived volatile was identified in the EPA and DHA esters. This could be due to the fairly low activity of the enzyme indicated by the high K_M. In addition, while the lipoxygenases with a pH maximum at pH 7 have been found to prefer esters as a substrate, lipoxygenases with pH maxima in the alkaline region show a higher affinity for free fatty acids (Gardner, 1980). Therefore, in order to better study volatiles derived from EPA and DHA, the use of free EPA and DHA and the isolation and purification of the enzyme will be necessary. The occurrence of D-limonene in the EPA and DHA ethyl ester could be of potential interest to the cancer research community. D-limonene has been found to be a strong anticarcinogen and current research focuses on the activity of D-limonene in decreasing the proliferation of mammary cancer (Crowell et al., 1991). A large number of studies has shown that menhaden oil and specifically n-3 fatty acids as well decrease mammary tumors in rats and mice (Gabor and Abraham, 1986; Carroll and Braden, 1986; Jurkowski and Cave, 1985; Karmali, et al., 1984; Kort et al., 1987). Recently, D-limonene has also been found to be effective against cancer of the gastrointestinal tract (Wattenberger and Coccia, 1991), a site where fish oil has been found to be effective as well (Nelson et al., 1988; Minoura et al., 1988). While the mechanism of the n-3 fatty acids in combating tumors is probably mainly through the formation of specific prostaglandin, the occurrence of a strong natural anticarcinogen in the volatile extract of these fatty acids suggests its potential involvement in their anticarcinogenic properties.

7. Conclusion

It can be concluded from the results of the various lipid peroxidation methods on the undeodorized and deodorized menhaden oil, that the undeodorized oil was received in a later stage of lipid oxidation than the deodorized oil. However, both oils showed virtually the same patterns of lipid oxidation over time. The results of the peroxide, TBA and anisidine analyses for the two oils are almost identical except that the lipid peroxidation of the deodorized oil lagged a few weeks behind the undeodorized oil. Thus, it must be concluded that deodorization of the menhaden oil delays the progress of lipid peroxidation, but does not change the overall pattern of lipid peroxidation.

Based on the enzyme activity studies, it can be concluded furthermore that lipoxygenase activity is present in menhaden gill tissue. The results of both sensory parts of the study indicate that menhaden gill lipoxygenase activity was responsible in causing a significantly stronger smell in the n-3 fatty acid ester concentrate treated with the enzyme extract. However, these results are not supported by the results of the volatile analysis of the various esters used in the first part of the sensory analysis (weeks 0 - 8). The total volatiles in the enzyme treated ester were neither different from those in the control, nor was it possible to identify particular volatiles that were present in higher concentration in the enzyme treated ester. However, in the second part of the sensory analysis (days 0-7), the corresponding volatile analysis indicated higher amounts of total volatiles in the enzyme treated ester compared to the control.

The first part of the sensory analysis with the corresponding volatiles analysis was supposed to identify specific lipoxygenase derived volatiles by inhibiting menhaden lipoxygenase activity by boiling and esculetin. This part of the study failed to corroborate the results of the sensory panel and the enzyme assays. It was not possible to identify specific volatiles in the enzyme treated esters that were present in higher concentrations than in the control ester in either of the two analysis parts. However, since in the second part of the sensory analysis virtually all volatiles were found to be in higher concentrations in the enzyme treated ester than in the control ester, it indicates that lipoxygenase activity might increase general autoxidation. It must be considered that the model system with the n-3 fatty acid ester concentrates resulted in considerable differences in the volatiles than those derived from free fatty acids in the undeodorized

and deodorized menhaden oil, which leads to the conclusion that it was not a good model system.

Since volatiles that were shown to be derived from lipoxygenase activity were present in the undeodorized menhaden oil, but not the deodorized menhaden oil, it is likely that lipoxygenase contributes to the oxidation of menhaden oil. However, just based on these volatiles, it is not possible to draw any conclusion about the relative contribution of lipoxygenase activity to menhaden oil oxidation, because it is difficult to specify the origin of these volatiles. A careful consideration of all the literature on lipoxygenase derived and autoxidatively derived volatiles indicates that it is not possible to assign any given volatile to a specific origin. Thus, a basic volatile profile does not give enough indication as to the true origin of the volatiles, unless very specific information is known about the various ratios of monohydroperoxides and resulting volatiles for the oil. In order to be able to evaluate the origin of specific volatiles, it is necessary to identify the stereospecificity of the enzyme.

While volatiles were identified in the EPA and DHA ethyl esters, which were previously identified in the undeodorized and deodorized menhaden oil as well as the n-3 fatty acid ester concentrates, no lipoxygenase derived volatiles were found. Thus, the results do not support the theory that lipoxygenase activity is a major contributor to menhaden oil oxidation. However, various other reasons, such as lowered enzyme activity after freezing and thawing a few times, or insufficient incubation time, could explain why these volatiles were not identified in the EPA and DHA esters.

7.1. Suggestions for Future Research

Improvements in the on-board storage of menhaden and in the menhaden oil extraction will most likely result in a higher quality oil. The fish should be stored at colder temperatures and the currently used wet rendering method could be replaced by a cold pressing method. While lipoxygenase appears to play a minor role in menhaden oil oxidation under current processing methods, a change in the extraction method might increase the importance of lipoxygenase. However, proteins, including lipoxygenase could then be removed by ultrafiltration.

The isolation and purification of menhaden lipoxygenase is an important step in the determination of the potential importance of lipoxygenase in menhaden oil oxidation.

A purified lipoxygenase will facilitate the proper identification of the stereospecificity of menhaden gill lipoxygenase by analyzing the monohydroperoxides and their relative amounts formed by menhaden gill lipoxygenase. Once it is known which monohydroperoxides are formed from lipoxygenase activity, it is possible to describe the likely volatiles that will result from these hydroperoxides. In order to be able to use this information to speculate on the contribution of the lipoxygenase derived volatiles to the total amount of volatiles found in menhaden oil, it is necessary to determine the ratio of monohydroperoxides in autoxidized EPA and DHA. Currently it is only known which monohydroperoxides are formed by autoxidation of EPA and DHA. It is then conceivable to generate a mathematical model that can project the volatiles and their relative amounts that are to be expected in an autoxidized oil. By knowing which stereospecific volatiles are likely to be contributed by lipoxygenase activity, the changing relative amounts of the volatiles can give indications as to the importance of lipoxygenase activity in the oxidation of the oil. In general, in order to get a better understanding of the complex question of fish oil oxidation, it is necessary to study isolated model systems rather than complex matrices such as oils, and to then piece together the information to develop an overall picture of fish oil oxidation.

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9. Appendices

Appendix I

Suppliers

Aldrich, Milwaukee, W.I.

- Esculetin
- Malonaldehyde (bis) diethylacetal
- 1,1,3,3 Tetraethoxypropane

Alltech Associates, Deerfield, IL

- Glass wool, silane treated
- Tenax GR

Bedoukian, Danbury, C.T.

- t-2 Decenal

Fisher, Fair Lawn, N.J.

- Acetic acid, glacial
- Sodium hydrogenphosphate (mono- and dibasic)
- Pasteur Pipettes, 6 inches
- Sulfuric acid

Mallinckrodt, Paris, K.Y.

- Iso-octane
- Quinine sulfate
- Chloroform
- Ethyl ether
- Thiobarbituric acid
- Potassium iodide
- Sodium thiosulfite, 0.01 N
- Starch solution, 1%

Nalgene, Rochester, N.Y.

5 mL cryovials

Sigma, St. Louis, M.O.

Tween 20

Diagnostic Protein Assay Kit

Linoleic Acid

Sodium sulfite

Trichloro acetic acid

p-Anisidine

Sodium hydroxide

Supelco, Bellefonte, P.A.

Crimp top vials (50 mL, 20 mL)

Supelcowax 10 (Length: 60 m, ID: 0.25 mm, Film: 0.25 μm)

US Department of Commerce, National Oceanic and Athmospheric Administration, National Marine Fisheries Service, Southeast Fisheries Science Center, Charleston, S.C.

N-3 Ethyl Ester Concentrates (Lot# L91150BF)

Eicosapentaenoic Acid Ethyl Ester (Lot# L91126BI.4)

Docosahexaenoic Acid Ethyl Ester (Lot# L91283BI.5)

Zapata Haynie Corp., Reedville, V.A.

SPMO™ Brand Menhaden Oil - Undeodorized (Lot# 6474 fresh)

SPMO™ Brand Menhaden Oil - Deodorized (Lot# 110790)

Appendix II

Reagents for Thiobarbituric Acid Value (Ke and Woyewoda, 1979)

Thiobarbituric acid (TBA) (4,6-Dihydroxy-2-mercapto pyrimidine) stock solution: Dissolve 2.88 g of TBA (Mallinckrodt, Paris, KY) in 50 mL water and make volume to 500 mL with glacial acetic acid (Fisher, Fair Lawn, NJ).

TBA working solution (fresh daily): Mix 90 mL TBA stock solution with 60 mL chloroform and 7.5 mL sodium sulfite solution.

Sodium sulfite solution (0.3 M): Dissolve 9.45 g of sodium sulfite (Sigma, St. Louis, MO) in 200 mL water and make volume to 250 mL with water.

Trichloroacetic acid solution(TCA)(0.28 M): Dissolve 22.876 g of TCA (Sigma, St. Louis, MO) in 450 mL water and make volume to 500 mL with water.

1,1,3,3 Tetraethoxypropane (TEP)(also: Malonaldehyde bis (diethylacetal)) standard stock and working solution (0.01 M): Dissolve 0.22 g of TEP (Aldrich, Milwaukee, WI) in 90 mL water and make volume to 100 mL with water (stock solution). Pipette 1 mL of stock solution into 100 mL volumetric flask and make to volume with water (working solution).

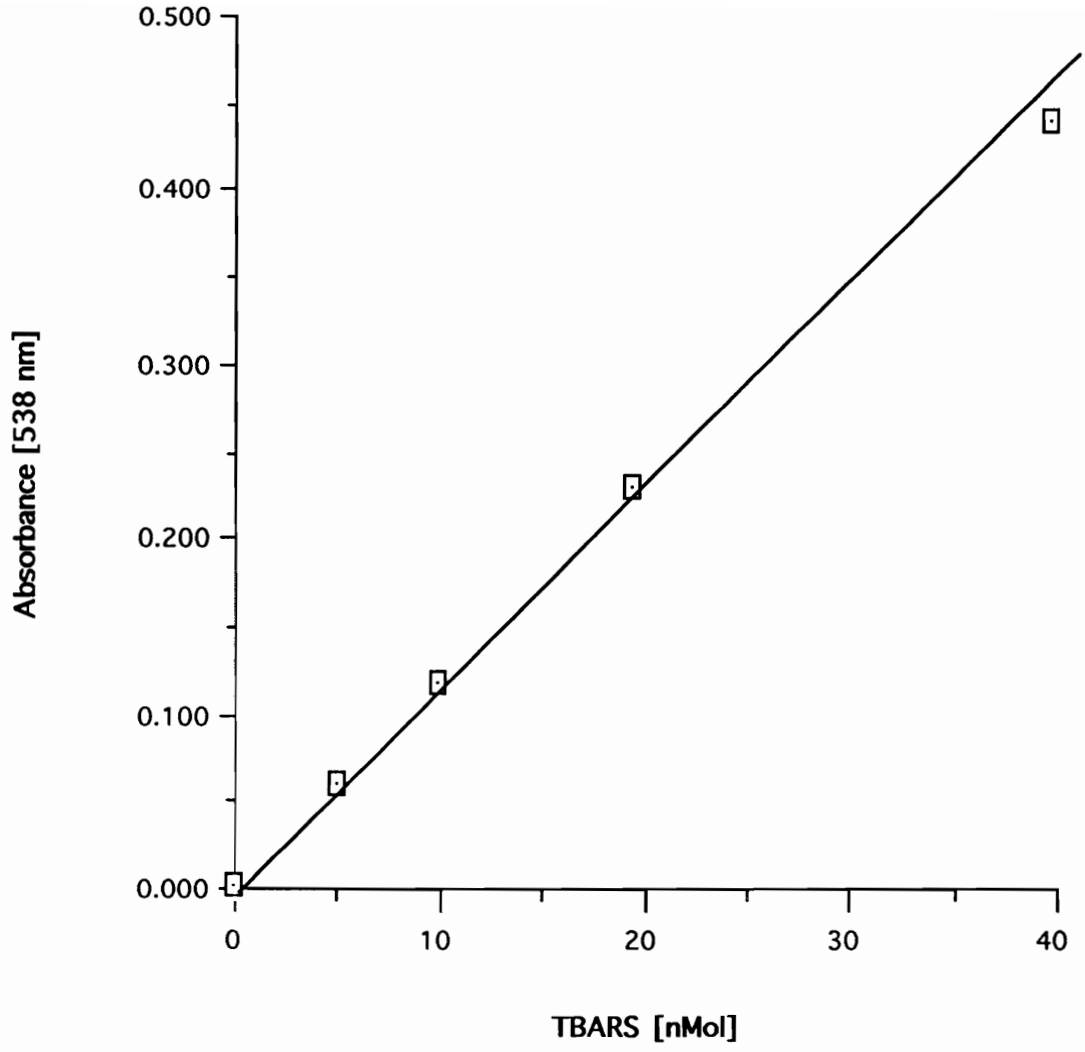

Figure 54. Standard curve for TBA-reactive substances

Appendix III

Reagents for Peroxide Value (AOAC Method 28.026)

Acetic acid-chloroform solution (fresh daily): Mix 18 mL of glacial acetic acid (Fisher, Fair Lawn, N.J.) with 12 mL of chloroform (Mallinckrodt, Paris, KY)

Potassium iodide solution, saturated (fresh daily): Dissolve 6 g of potassium iodide (Mallinckrodt, Paris, KY) in 5 mL distilled, freshly boiled water. Excess solid must remain.

Sodium thiosulfate solution: Purchase standardized 0.01 N solution (Mallinckrodt, Paris, KY).

Starch indicator solution (1%): Purchase standardized 1% solution (Mallinckrodt, Paris, KY).

Appendix IV

Reagents for Anisidine Value (International Union of Pure and Applied Chemistry 1987)

Anisidine (para-methoxy aniline) Reagent (fresh daily): In a 10 mL volumetric flask, dissolve 0.25 g para-anisidine (Sigma, St. Louis, MO; spectroquality) in 9 mL glacial acetic acid (Fisher, Fair Lawn, NJ), and fill to volume with glacial acetic acid.

Appendix V

Library Search Parameters

FL	Full Library	No
LL	Library Limits	0 - 24,000
MRG	Mass Range	35 - 250
NPM	Peaks to be Matched	3
FIT	Search Type	Mix
THR	Fit Threshold	0
MM	Min. in Main Search	20
EXM	Excludud Masses	None
RED	Reduce Data	Yes
NRP	Peaks per 100 amu	16
W1	Pass 1 Window	250
W2	Pass 2 Window	100
MR	Manual Reporting	Yes
RR	Rename Report	No
MOD	Background Edit Mode	L
LIW	List Weighted Intensity	NO
DF	Disable Filters	No
MWT	Molecular Weight	0 -250
E1	(C,H,N,O,S,CL) Range	C:0-30, H:0-50, N:0, O:0-5, S:0, CL:0
E2	(F,P,B,SI,BR) Range	F:0. P:0, B:0, SI:0, BR:0

Appendix VI

Computer Program for Library Search

```
.REPEAT FILE=FLIST
INT "FILE",SLIST
.WAIT INT
.REPEAT SCAN=SLIST
.%40="SCAN"
SUB "FILE"#%40,"FILE"S
.WAIT SUB
LIB "FILE"S#%40,LIBFIT/PSRD
.WAIT LIB
PLO SPE "FILE"S#%40,LIBFIT#1,LIBFIT#2
.WAIT PLO
.NEXT SCAN
```

Appendix VII

Protein Content (based on Lowry et al, 1951)

Sigma Diagnostic Protein Assay Kit (Sigma, St. Louis, MO) (Procedure #P5656)

Standards: 0.125, 0.25, 0.5, 0.75 and 1 mL of the protein standard solution were pipetted into test tubes. The standards were diluted to 1 mL with water. The standards correspond to 50, 100, 200, 300, and 400 mM protein.

Procedure: 0.1 mL sample was pipetted into a test tube. The volume was made up to 1 mL with water. One mL of Lowry reagent was added to all samples and standards. The solutions were vortexed and incubated at room temperature. After 20 minutes, 0.5 mL of Folin&Ciocalteu's phenol reagent was added under vortexing. The solutions were incubated for another 30 minutes. The absorbance of the samples and standards was measured at 500 nm.

Calculation: The protein concentration of the samples was determined using a standard curve (see next page) and by multiplying the curve reading with the dilution factor 10.

Protein Concentration = Curve Reading * Dilution Factor

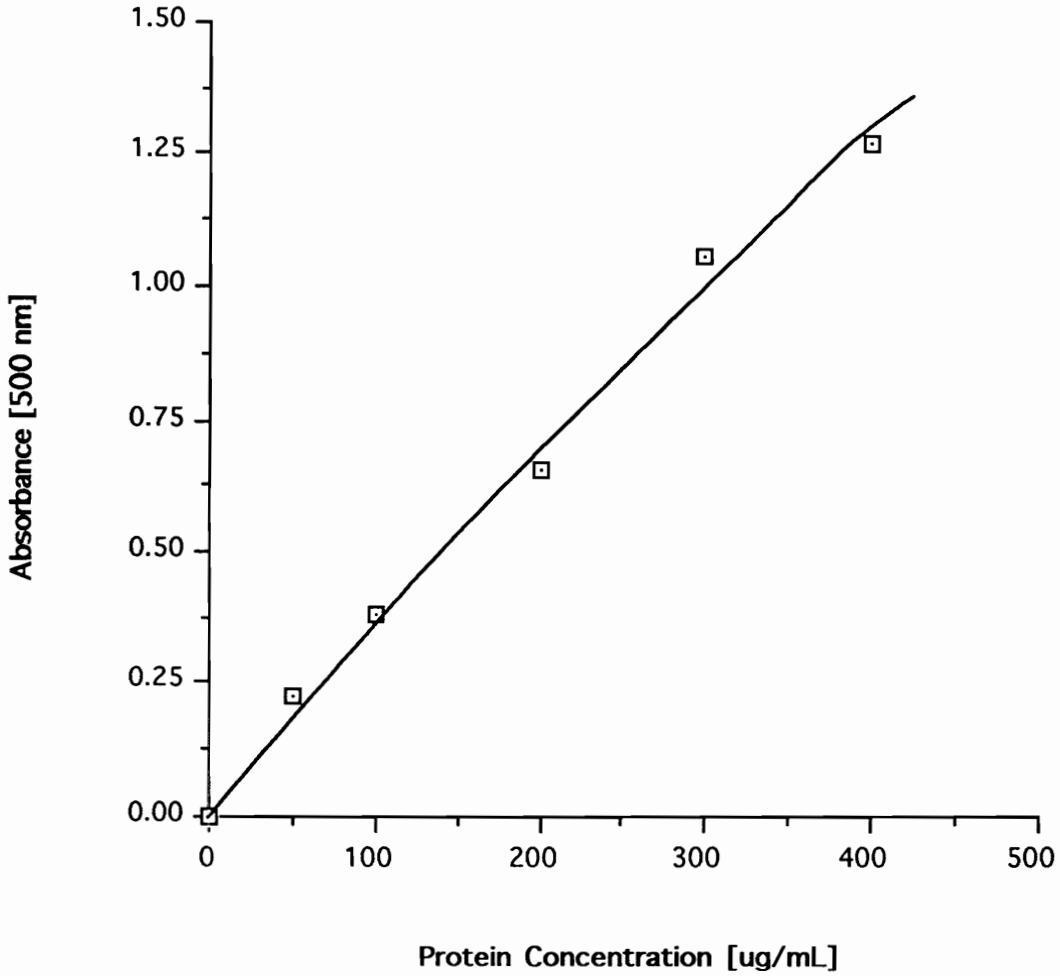

Figure 55. Standard curve for protein determination in the enzyme extract

Appendix VIII

Reagents for Lipoxygenase Activity (adapted from Ben-Aziz et al., 1970 and Grossman and Zakut, 1979)

Substrate Solutions: Prepare a 2.5 mM stock solution of linoleic acid by mixing 0.07 mL linoleic acid (Sigma, St. Louis, MO) with 0.25 mL Tween 20 (Sigma, St. Louis, MO) and slowly dripping the mixture into 100 mL 0.05 M Na_2HPO_4 (Fisher, Fair Lawn, NJ) under stirring. Adjust the pH to 9.0 with 0.1 N NaOH (Sigma, St. Louis, MO). Make a serial dilution with 0.05 M Na_2HPO_4 to get 1.5, 1.25, 1.0, 0.5, and 0.25 mM substrate solutions. Diluting the stock solution with 0.2 M citrate buffer solutions of desired pHs allows to determine enzyme activity at different pHs. Tween 20 facilitates the distribution of the fatty substrate and prevents the formation of an emulsion at low pH values. For pH ranges below 6 it might be required to use higher amounts of Tween 20.

Inhibitor Solutions: Dissolve 178.15 mg of esculetin (Aldrich, Milwaukee, WI) in a 200 mL volumetric flask with sodium phosphate buffer (pH 7.4) and make up to volume with buffer (5 mM). Make a serial dilution to get the desired inhibitor concentrations.

Appendix IX

Fatty Acid Ester Sensory Analysis

Date:_____ Panelist:_____

Please compare the two oil samples for their smell. Circle the number of the oil that has a stronger odor.

Method:

* Use the sample on the **left** first
* Lift the petridish to your nose
  (as close as possible without dipping your nose in)
* Uncover the dish
* Lightly swirl the dish under your nose
* Inhale
* Cover the dish and set it down
* Repeat with the second sample
* You can compare the oils as often as you want to
* Circle the number of the oil that is most odorous

If anything might have impaired your ability to analyze the oils properly (e.g. a common cold) please indicate the reason with a short note here:

Appendix X

Peroxide Values (meq/kg) of Deodorized and Undeodorized Menhaden Oil

	Deodorized Oil			Undeodorized Oil		
Week	1	2	3	1	2	3
0	1.71	1.28	1.76	4.90	4.46	4.60
1	1.80	1.75	1.87	6.71	6.61	6.81
2	3.28	3.33	3.32	5.75	5.76	5.75
3	5.55	5.61	5.73	6.05	5.92	6.04
4	4.44	4.70	4.83	6.23	6.13	6.27
8	n.a.	9.17	9.38	4.73	4.73	4.72
12	3.50	3.80	3.70	21.3	21.60	21.60
16	3.62	3.50	3.38	5.62	5.45	5.24
20	19.30	18.79	18.05	6.11	4.62	4.65

n.a. data point not available due to sample loss

Appendix XI

TBA Values (μMol/kg) of Deodorized and Undeodorized Menhaden Oil

	Deodorized Oil			Undeodorized Oil		
Week	1	2	3	1	2	3
0	268.9	294.2	285.1	951.7	885.0	1028.0
1	n.a.	614.9	511.1	1372.8	1360.1	1487.0
2	666.6	650.4	701.4	1358.2	1466.2	1422.0
3	1022.3	1007.1	832.0	1011.6	1220.1	1232.3
4	n.a.	1072.2	974.8	1563.8	1343.2	1484.6
8	1291.0	1432.0	1467.0	1043.0	821.0	902.0
12	n.a.	3711.4	3355.1	3580.6	4695.1	6586.3
16	1890.4	1283.7	1029.2	1612.8	1670.5	1664.9
20	3413.8	4503.0	4935.9	1470.6	1150.4	2460.3

n.a. data point not available due to sample loss

Appendix XII

Anisidine Values of Deodorized and Undeodorized Menhaden Oil

	Deodorized Oil			Undeodorized Oil		
Week	1	2	3	1	2	3
0	13.00	13.52	13.69	45.35	47.14	46.59
1	14.85	14.90	15.25	49.54	50.51	50.67
2	14.57	14.45	14.28	48.86	48.74	48.35
3	15.26	15.34	15.06	49.29	48.36	49.99
4	15.72	15.68	15.58	51.19	51.69	51.32
8	18.68	18.33	18.15	51.15	50.92	51.36
12	16.45	16.49	16.33	57.72	57.47	57.50
16	15.77	15.86	15.82	45.08	45.80	45.79
20	25.49	26.67	25.90	41.80	43.20	42.92

Appendix XIII

Conjugated Diene values* of deodorized and undeodorized menhaden oil over 20 Weeks

Undeodorized Menhaden Oil

λ [nm]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 8	Week 12	Week 16	Week 20
225	0.505	0.377	0.382	0.353	0.417	0.393	0.438	0.399	0.431
227	0.487	0.370	0.373	0.346	0.409	0.386	0.435	0.391	0.422
229	0.475	0.366	0.368	0.343	0.405	0.382	0.434	0.388	0.418
231	0.463	0.362	0.362	0.339	0.400	0.377	0.431	0.385	0.413
233	0.449	0.353	0.351	0.329	0.390	0.366	0.422	0.377	0.401
235	0.423	0.336	0.332	0.312	0.371	0.347	0.403	0.360	0.382
237	0.394	0.312	0.306	0.289	0.345	0.322	0.376	0.335	0.356
239	0.362	0.284	0.276	0.262	0.306	0.293	n.a.	0.306	0.325
241	0.329	0.255	0.247	0.235	0.281	0.263	0.310	0.275	0.291
242	0.313	0.240	0.230	0.220	0.265	0.247	0.292	0.259	0.275
243	0.296	0.224	0.215	0.205	0.248	0.231	0.274	0.241	0.257
244	0.278	0.208	0.198	0.190	0.230	0.214	0.254	0.222	0.238
245	0.256	0.192	0.181	0.173	0.211	0.197	0.234	0.202	0.219
247	0.219	0.158	0.145	0.140	0.173	0.161	0.192	0.163	0.179
260	0.138	0.081	0.067	0.070	0.088	0.084	0.086	0.080	0.095
262	0.139	0.083	0.068	0.071	0.089	0.085	0.086	0.081	0.096
263	0.140	0.084	0.068	0.072	0.090	0.086	0.086	0.081	0.097
264	0.141	0.084	0.069	0.072	0.090	0.087	0.087	0.082	0.098
266	0.144	0.086	0.071	0.075	0.093	0.089	0.089	0.085	0.101
268	0.147	0.089	0.074	0.077	0.096	0.092	0.092	0.088	0.105
270	0.149	0.091	0.075	0.079	0.097	0.094	0.093	0.089	0.108
272	0.149	0.091	0.076	0.079	0.097	0.094	0.093	0.088	0.108
273	0.148	0.091	0.075	0.079	0.096	0.093	0.092	0.087	0.108
274	0.147	0.090	0.074	0.078	0.094	0.092	0.090	0.086	0.107
275	0.146	0.088	0.073	0.076	0.093	0.090	0.089	0.085	0.105
276	0.145	0.087	0.071	0.075	0.091	0.089	0.087	0.083	0.103
278	0.143	0.084	0.068	0.072	0.088	0.086	0.084	0.081	0.100
280	0.141	0.081	0.064	0.069	0.085	0.082	0.080	0.079	0.097
282	0.138	0.078	0.062	0.066	0.082	0.079	0.076	0.076	0.094
284	0.134	0.075	0.058	0.063	0.078	0.075	0.072	0.073	0.092

* Average of three readings

n.a. data point not available

Deodorized Menhaden Oil

λ [nm]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 8	Week 12	Week 16	Week 20
225	0.626	0.578	0.605	0.551	0.624	0.611	0.602	0.639	0.688
227	0.632	0.592	0.617	0.578	0.636	0.626	0.615	0.654	0.706
229	0.649	0.611	0.638	0.586	0.662	0.649	0.637	0.678	0.733
231	0.670	0.633	0.662	0.608	0.686	0.673	0.659	0.705	0.761
233	0.687	0.651	0.681	0.625	0.652	0.692	0.676	0.726	0.783
235	0.697	0.661	0.693	0.636	0.718	0.703	0.686	0.739	0.796
237	0.698	0.664	0.697	0.640	0.722	0.706	n.a.	0.742	0.799
239	0.700	0.667	0.699	0.642	0.724	0.708	0.689	0.745	0.800
241	0.706	0.673	0.704	0.647	0.730	0.712	0.697	0.750	0.801
242	0.708	0.676	0.706	0.649	0.732	0.713	0.698	0.752	0.801
243	0.708	0.676	0.707	0.649	0.731	0.712	0.697	0.751	0.799
244	0.704	0.674	0.704	0.646	0.728	0.708	0.694	0.747	0.793
245	0.698	0.679	0.697	0.640	0.721	0.700	0.687	0.739	0.784
247	0.684	0.655	0.682	0.626	0.706	0.684	0.672	0.723	0.762
260	0.802	0.763	0.784	0.728	0.822	0.783	0.789	0.833	0.839
262	0.872	0.828	0.853	0.789	0.888	0.845	0.849	0.897	0.903
263	0.611	0.845	0.873	0.804	0.903	0.859	0.861	0.910	0.919
264	0.902	0.847	0.876	0.804	0.903	0.858	0.858	0.911	0.918
266	0.859	0.817	0.846	0.775	0.871	0.827	0.826	0.880	0.888
268	0.842	0.799	0.825	0.759	0.859	0.813	0.816	0.868	0.874
270	0.896	0.852	0.875	0.812	0.916	0.870	0.881	0.926	0.928
272	0.940	0.956	0.983	0.910	1.025	0.973	0.984	1.028	1.030
273	0.966	0.996	1.027	0.948	1.065	1.012	1.018	1.067	1.071
274	0.976	1.013	1.047	0.964	1.080	1.026	1.027	1.082	1.088
275	0.672	1.002	1.036	0.951	1.065	1.011	1.006	1.068	1.078
276	0.941	0.962	0.998	0.913	1.022	0.970	0.960	1.028	1.040
278	0.850	0.842	0.873	0.795	0.894	0.847	0.837	0.905	0.918
280	0.783	0.745	0.769	0.704	0.794	0.752	0.748	0.803	0.812
282	0.763	0.724	0.745	0.688	0.777	0.737	0.739	0.786	0.791
284	0.807	0.765	0.786	0.729	0.822	0.780	0.782	0.814	0.813

* Average of three readings

n.a. data point not available

Appendix XIV

Fluorescence Values of Deodorized and Undeodorized Menhaden Oil over 20 Weeks

Raw Fluroescence Readings

Wk	DS1*	Wt.**	DS2	Wt.	DS3	Wt.	US1	Wt.	US2	Wt.	US3	Wt.
0	108.7	.0267	46.8	.0115	49.3	.0117	84.8	.0117	77.8	.0106	85.8	.0117
1	100.9	.0255	53.3	.0131	47.4	.0119	91.5	.0131	85.1	.0119	82.8	.0113
2	49.5	.0121	51.3	.0123	50.3	.0124	95.3	.0129	93.4	.0129	95.5	.0133
3	55.5	.0133	42.2	.0100	52.5	.0130	68.6	.0091	91.5	.0124	87.7	.0119
4	49.4	.0120	49.0	.0116	50.9	.0125	86.4	.0119	86.3	.0119	90.5	.0127
8	67.2	.0146	63.6	.0138	63.7	.0139	93.0	.0123	100.9	.0136	98.7	.0127
12	39.1	.0110	44.7	.0120	44.5	.0130	65.0	.0130	57.8	.0120	71.7	.0130
16	48.6	.0143	42.9	.0126	47.2	.0139	73.4	.0129	85.3	.0153	74.9	.0127
20	50.8	.0134	55.7	.0147	52.1	.0139	69.3	.0125	69.7	.0129	70.3	.0129

* DS1 to US3 = Deodorized Oil Sample No.1 to Undeodorized Oil Sample No.3

** Wt. = Weight of the Preceding Sample in gram

Fluorescence Readings Adjusted for Sample Weight and Standard Reading

Week	Standard Reading	DS 1*	DS 2	DS 3	US 1	US 2	US 3
0	66.0	61.7	61.9	63.7	110.2	111.6	111.2
1	63.5	62.3	64.1	62.7	110.0	112.6	115.4
2	66.1	62.1	63.0	61.3	111.7	109.5	108.5
3	75.4	55.3	56.0	53.6	100.0	97.9	97.7
4	77.0	53.5	54.9	52.9	94.3	94.2	92.5
8	82.3	55.9	56.0	55.7	91.9	90.1	94.4
12	63.8	55.7	58.4	53.7	78.4	75.5	86.4
16	67.2	50.6	50.7	50.5	84.7	83.0	87.8
20	59.0	64.3	64.2	63.5	94.0	91.6	92.4

* DS1 to US3 = Deodorized Oil Sample No.1 to Undeodorized Oil Sample No.3

Appendix XV

Individual Responses of the Panelists (Part 1)

Panelist

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
0	2	2	2	2	1	2	1	2	2	1	2	2	1	2	2	2	2	2
1	2	1	2	2	1	2	1	1	-	2	2	1	2	2	1	2	2	1
2	2	2	1	1	2	0	1	1	1	1	2	1	2	1	1	2	2	2
3	2	2	1	2	2	2	2	1	2	2	1	2	1	2	0	2	1	1
4	2	1	2	2	2	0	2	0	1	1	2	2	2	2	2	2	1	1
8	1	2	1	1	2	0	1	1	1	1	1	0	1	2	1	1	1	2

0= panelist did not chose enzyme treated ester as the stronger smelling one

1= for one repetition panelist chose the enzyme treated ester as the stronger smelling one

2= for two repetitions panelist chose the enzyme treated ester as the stronger smelling one

-- = panelist was not available

Individual Responses of the Panelists (Part 2)

Panelist

Day	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	18
0	0	0	0	0	0	–	1	–	0	1	–	1	1	–	–	0
1	0	0	1	0	0	–	1	–	1	1	–	0	0	–	–	0
2	1	1	0	1	0	–	1	–	1	1	–	0	1	–	–	1
7	0	1	1	–	1	1	1	1	1	1	1	0	–	1	1	0

0 = panelist did not chose enzyme treated ester as the stronger smelling one

1 = panelist chose the enzyme treated ester as the stronger smelling one

-- = panelist was not available

Appendix XVI

TBA Values (μMol/kg) of n-3 Fatty Acid Esters

Week	Sample	Control	w/ Enzyme	w/ boiled Extract	w/ Esculetin
0	1	2401.2	2310.7	1802.6	1660.2
0	2	2857.6	1724.3	1854.9	2088.5
1	1	2090.9	2356.3	1841.0	2713.8
1	2	2423.5	1924.3	2247.3	2632.9
2	1	1903.0	2659.4	2722.4	1740.2
2	2	2266.8	2436.3	2540.2	1648.6
3	1	656.1	1013.9	970.9	589.0
3	2	856.6	813.9	n.a.	1019.1
4	1	1250.7	1268.0	1140.1	1042.1
4	2	1165.7	1239.0	1101.3	1160.4

n.a. data point not available due to sample loss

Appendix XVII

Enzyme Assay Data

Assay	Protein Content of Extract [μg/mL]	Linoleic Acid Concentration [mMol]	Average Absorbance Change per Minute
Lipoxygenase	1238.2	2.50	0.358
	1238.2	1.50	0.322
	1238.2	1.25	0.285
	1238.2	1.00	0.243
	1238.2	0.50	0.102
	1238.2	0.25	0.031
Esculetin 0	563.74	20	0.619
	563.74	10	0.502
	563.74	5	0.312
	563.74	3.3	0.274
	563.74	2.5	0.175
Esculetin 100	563.74	20	0.598
	563.74	10	0.485
	563.74	5	0.277
	563.74	3.3	0.226
	563.74	2.5	(0.124)
Esculetin 500	563.74	20	0.529
	563.74	10	0.272
	563.74	5	0.208
	563.74	3.3	0.172
	563.74	2.5	0.113
Esculetin 1000	563.74	20	0.365
	563.74	10	0.267
	563.74	5	0.148
	563.74	3.3	(0.085)
	563.74	2.5	(0.050)

Appendix XVIII

Peak Areas of Volatiles in Undeodorized Oil

Compound Name / Week	RT	Wk0-A1	Wk0-A2	Wk1-A1	Wk1-A2
Decane	9.4	24329	21149	33095	24554
2,3 Dimethyl 1,4 Hexadiene	9.9	90139	84781	138596	112234
1-Penten-3-one	10.3	11247	12818	22829	25401
3-Ethyl 1,4 Hexadiene	10.6	4255	4674	16173	15358
Methyl Benzene	10.8	0	0	8480	6596
5-Ethyl 2-Methyl Octane	12.1	n.i.	n.i.	2255	n.i.
Hexanal	12.5	399914	434236	521987	525036
1,3,6 Octatriene (E,E)	13.3	43588	44322	56681	59659
1,3,6 Octatriene(Z,E)	13.4	102293	98052	142996	122023
2-Pentenal	15	90288	154737	123307	154317
1,4-Dimethyl Benzene	15.2	100552	103228	136783	137000
1-Penten-3-ol	15.8	82495	110310	132264	143998
2-Methyl 2-Pentenal	16.4	25023	29871	41129	39982
Heptanal	17.6	245938	215340	307722	237822
4,5 Dimethyl 1-Hexene	18.4	4291	9146	5210	3557
4-Methyl 3-Pentenal	18.7	13235	15041	20712	20901
2-Hexen-3-one	19.4	28377	27250	39709	31959
2-Hexenal	19.6	282878	277362	416220	355520
Unknown	20	139700	151290	181229	143527
Unknown	20.5	51930	59266	65319	48773
4-Heptenal (Z)	20.7	138666	131741	165734	136117
Unknown	21.1	74354	84689	88159	86280
Unknown	22	47888	54666	58709	50891
Unknown	22.7	17748	18208	21624	16904
1,3,5 Trimethyl benzene	23.5	7205	6818	5822	6530
Octanal	23.8	43622	43709	52258	36447
Unknown	24.3	130571	130442	165222	117415
2-Penten-1-ol (E)	25.1	33337	43553	45519	41588
2-Penten-1-ol (Z)	25.7	19155	26796	28026	25546
2-Heptenal (Z)	26.5	70597	75693	105847	64088
6-Octen-2-one	27	0	0	0	0
1-Hexanol	27.8	15403	12322	27029	19290
2-Methyl 2-cyclopenten-1-one	29.6	18373	16796	23855	19636
3-Hexen-1-ol (Z)	29.9	11138	10998	14464	10620
2-Nonanone	30.5	21183	21206	25881	19491
Nonanal	30.8	31660	33953	34793	27440
2,4 Hexadienal (E,E)	32.4	38470	34797	50253	39926
2-Octenal (E)	33.5	76146	79841	93132	79582
1-Octen-3-ol	34.3	24975	24628	31326	24191
2,4 Heptadienal (E,Z)	35.8	426041	426406	522159	374525

Unknown	36.6	49835	52249	61100	47753
Pentadecane	38	329375	329788	423888	279630
2,4 Heptadienal (E,E)	38.3	164895	189458	179598	196110
3,5 Octadiene-2-one (E,Z)	39.6	14434	14900	17756	12910
Benzaldehyde	40.7	29140	34750	30816	27112
2-Nonenal	41.2	18332	19352	33614	15983
Unknown	41.6	2201	4507	4775	4193
2,4 Octadienal (Z,Z)	42.5	14095	18681	17704	16051
3,5 Octadiene-2-one (E,E)	43.9	8148	12556	10137	11433
2,6 Nonadienal	44.7	42773	16958	27445	13263
2,4-Octadienal (E,E)	45	15387	19348	25821	20977
2-Decenal (Z) (Internal Std.)	47.8	32486	28470	22118	19772
Unknown	48.2	2616	3352	2421	3475
Hexadecane	49.7	10163	9327	11796	9790
2,5 Octadien-1-ol	50	8147	7490	9362	7918
Heptadecane	51.4	10717	9465	9831	13210
Unknown	53.9	26382	28110	31346	25173
5-Ethyl-2(5H)-furanone	54.8	8986	10412	10022	8620
2,4 Decadienal	57	2556	2390	4512	4494
TBHQ	61.3	46710	60074	52117	66597
1-Dodecanol	63.4	18353	19239	16244	19889

Peak Areas of Volatiles in Undeodorized Oil (cont'd)

Compound Name / Week	RT	Wk2-A1	Wk2-A2	Wk3-A1	Wk3-A2
Decane	9.4	30734	36224	26466	18190
2,3 Dimethyl 1,4 Hexadiene	9.9	122125	139302	90450	101953
1-Penten-3-one	10.3	20628	24782	19223	peaks
3-Ethyl 1,4 Hexadiene	10.6	14163	15530	14232	not
Methyl Benzene	10.8	8970	10715	10763	separated
5-Ethyl 2-Methyl Octane	12.1	n.i.	2057	n.i.	n.i.
Hexanal	12.5	484400	542054	399436	451196
1,3,6 Octatriene (E,E)	13.3	62612	84211	67286	n.s.
1,3,6 Octatriene(Z,E)	13.4	128603	150895	114367	170025
2-Pentenal	15	115133	125511	200702	peaks not
1,4-Dimethyl Benzene	15.2	129414	145263	139861	separated
1-Penten-3-ol	15.8	117714	135351	124714	154911
2-Methyl 2-Pentenal	16.4	40977	42660	39562	45280
Heptanal	17.6	272617	332950	24713	248950
4,5 Dimethyl 1-Hexene	18.4	4313	6496	6430	5482
4-Methyl 3-Pentenal	18.7	16722	21834	11535	13535
2-Hexen-3-one	19.4	34021	42308	25783	29682
2-Hexenal	19.6	374565	426158	259092	263742
Unknown	20	144448	193733	156354	156356
Unknown	20.5	55310	69151	57631	peaks not
4-Heptenal (Z)	20.7	150471	181059	127739	separated
Unknown	21.1	87434	101952	90215	99348
Unknown	22	60412	75796	63487	57105
Unknown	22.7	19595	25100	21503	19037
1,3,5 Trimethyl benzene	23.5	7751	9818	7686	6608
Octanal	23.8	45706	58678	44866	40506
Unknown	24.3	143556	184650	141406	129137
2-Penten-1-ol (E)	25.1	42327	49860	55763	61485
2-Penten-1-ol (Z)	25.7	25062	29509	34329	38610
2-Heptenal (Z)	26.5	94550	115302	89470	78762
6-Octen-2-one	27	924	725	1494	6005
1-Hexanol	27.8	22167	28407	19675	19235
2-Methyl 2-cyclopenten-1-one	29.6	21034	24544	15868	16133
3-Hexen-1-ol (Z)	29.9	11985	14456	9986	11718
2-Nonanone	30.5	23240	29896	23572	27148
Nonanal	30.8	31705	40203	33988	36080
2,4 Hexadienal (E,E)	32.4	45366	56684	37844	37663
2-Octenal (E)	33.5	90326	114617	93704	90155
1-Octen-3-ol	34.3	30423	39697	33559	31696
2,4 Heptadienal (E,Z)	35.8	456990	588458	447976	410487
Unknown	36.6	57011	72118	55080	56412

Pentadecane	38	177750	464020	337590	292235
2,4 Heptadienal (E,E)	38.3	34137	200917	184446	225066
3,5 Octadiene-2-one (E,Z)	39.6	14791	25152	15907	13272
Benzaldehyde	40.7	27923	35556	28636	31141
2-Nonenal	41.2	31420	38658	15441	15986
Unknown	41.6	4435	6290	1194	8829
2,4 Octadienal (Z,Z)	42.5	15034	20489	16141	18873
3,5 Octadiene-2-one (E,E)	43.9	9484	13125	9932	15308
2,6 Nonadienal	44.7	26158	23583	16880	25049
2,4-Octadienal (E,E)	45	26716	22458	15167	27405
2-Decenal (Z) (Internal Std.)	47.8	19321	29095	39901	30557
Unknown	48.2	2683	2755	4629	6645
Hexadecane	49.7	10365	13596	10837	10901
2,5 Octadien-1-ol	50	7876	9928	7684	9337
Heptadecane	51.4	9095	10465	12380	14145
Unknown	53.9	27971	36760	27580	29426
5-Ethyl-2(5H)-furanone	54.8	8161	10731	7705	9186
2,4 Decadienal	57	2859	3560	2023	n.i.
TBHQ	61.3	58133	57273	83528	103762
1-Dodecanol	63.4	33379	25278	30291	32488

Peak Areas of Volatiles in Undeodorized Oil (cont'd)

Compound Name / Week	RT	Wk4-A1	Wk4-A2	Wk8-A1	Wk8-A2
Decane	9.4	23640	29001	31829	19142
2,3 Dimethyl 1,4 Hexadiene	9.9	113125	161040	185864	163124
1-Penten-3-one	10.3	peaks	peaks	peaks	peaks
3-Ethyl 1,4 Hexadiene	10.6	not	not	not	not
Methyl Benzene	10.8	separated	separated	separated	separated
5-Ethyl 2-Methyl Octane	12.1	5908	6578	6885	8299
Hexanal	12.5	361010	391973	486531	512169
1,3,6 Octatriene (E,E)	13.3	n.s.	n.s.	n.s.	n.s.
1,3,6 Octatriene(Z,E)	13.4	196443	217893	258689	246487
2-Pentenal	15	95745	200658	105316	peaks
1,4-Dimethyl Benzene	15.2	62652	peaks not	76778	not
1-Penten-3-ol	15.8	314820	separated	406215	separated
2-Methyl 2-Pentenal	16.4	0	0	0	0
Heptanal	17.6	214359	238362	282212	240432
4,5 Dimethyl 1-Hexene	18.4	3400	5517	4808	3870
4-Methyl 3-Pentenal	18.7	250941	278931	352672	398138
2-Hexen-3-one	19.4	32335	35447	42419	38253
2-Hexenal	19.6	241607	255200	321142	297579
Unknown	20	113142	138085	166028	142715
Unknown	20.5	41561	51076	62589	51353
4-Heptenal (Z)	20.7	103815	117343	134070	121590
Unknown	21.1	66755	76688	86064	89622
Unknown	22	45617	59310	69378	56387
Unknown	22.7	17204	22870	26502	21844
1,3,5 Trimethyl benzene	23.5	7603	9818	n.i.	n.i.
Octanal	23.8	124615	142048	180536	201880
Unknown	24.3	116715	136482	155133	139878
2-Penten-1-ol (E)	25.1	35068	42808	41578	61521
2-Penten-1-ol (Z)	25.7	30787	38154	39259	55446
2-Heptenal (Z)	26.5	60875	67896	79713	70653
6-Octen-2-one	27	9719	9757	10051	10602
1-Hexanol	27.8	20830	24577	31355	26403
2-Methyl 2-cyclopenten-1-one	29.6	14045	14913	18813	16628
3-Hexen-1-ol (Z)	29.9	9098	10063	12067	10278
2-Nonanone	30.5	18805	23272	26471	28045
Nonanal	30.8	24652	32028	35076	30843
2,4 Hexadienal (E,E)	32.4	34172	38799	47730	42417
2-Octenal (E)	33.5	68755	86598	95887	90479
1-Octen-3-ol	34.3	25398	34205	38920	34502
2,4 Heptadienal (E,Z)	35.8	350713	422090	489208	434719
Unknown	36.6	40314	52372	59417	52597

Pentadecane	38	218735	240240	333092	291695
2,4 Heptadienal (E,E)	38.3	185178	285525	246130	189434
3,5 Octadiene-2-one (E,Z)	39.6	14937	14547	25620	39229
Benzaldehyde	40.7	peaks not	362787	415579	465671
2-Nonenal	41.2	separated	88937	92327	90126
Unknown	41.6	1517	15014	13258	13405
2,4 Octadienal (Z,Z)	42.5	9622	30105	46894	29789
3,5 Octadiene-2-one (E,E)	43.9	9616	14580	17102	14074
2,6 Nonadienal	44.7	13452	14500	37315	25282
2,4-Octadienal (E,E)	45	17688	21193	31972	26608
2-Decenal (Z) (Internal Std.)	47.8	16815	26390	30932	23143
Unknown	48.2	6367	14391	13829	12824
Hexadecane	49.7	7336	11858	12756	10319
2,5 Octadien-1-ol	50	4130	7234	7732	6444
Heptadecane	51.4	7960	12053	13514	8644
Unknown	53.9	24540	25438	39893	25759
5-Ethyl-2(5H)-furanone	54.8	16087	9848	10916	10216
2,4 Decadienal	57	4101	2151	4729	7677
TBHQ	61.3	10259	15396	13753	15225
1-Dodecanol	63.4	23317	29214	24067	21720

Peak Areas of Volatiles in Undeodorized Oil (cont'd)

Compound Name / Week	RT	Wk12-A1	Wk12-A2	Wk16-A1	Wk16-A2
Decane	9.4	16606	31436	32982	34371
2,3 Dimethyl 1,4 Hexadiene	9.9	51780	71670	70786	79764
1-Penten-3-one	10.3	peaks	peaks	peaks	peaks
3-Ethyl 1,4 Hexadiene	10.6	not	not	not	not
Methyl Benzene	10.8	separated	separated	separated	separated
5-Ethyl 2-Methyl Octane	12.1	9546	8074	10528	12183
Hexanal	12.5	504637	401575	560194	621905
1,3,6 Octatriene (E,E)	13.3	n.s.	n.s.	n.s.	n.s.
1,3,6 Octatriene(Z,E)	13.4	403654	441025	303644	336600
2-Pentenal	15	322918	93523	peaks not	132331
1,4-Dimethyl Benzene	15.2	339168	69171	separated	418862
1-Penten-3-ol	15.8	351733	496670	612204	705321
2-Methyl 2-Pentenal	16.4	n.i.	n.i.	n.i.	n.i.
Heptanal	17.6	188888	257389	243546	261676
4,5 Dimethyl 1-Hexene	18.4	15275	7838	n.i.	n.i.
4-Methyl 3-Pentenal	18.7	548992	461394	peaks	670050
2-Hexen-3-one	19.4	40637	44599	not	47373
2-Hexenal	19.6	342089	381582	separated	357629
Unknown	20	157269	182921	peaks not	173225
Unknown	20.5	58898	66118	separated	59709
4-Heptenal (Z)	20.7	86370	115232	178044	130400
Unknown	21.1	96104	76626	88689	104680
Unknown	22	67536	83772	38661	77414
Unknown	22.7	27254	31092	27874	31160
1,3,5 Trimethyl benzene	23.5	n.i.	n.i	n.i.	n.i
Octanal	23.8	276865	246560	284083	319814
Unknown	24.3	142439	185354	159743	173923
2-Penten-1-ol (E)	25.1	58822	42378	55335	72394
2-Penten-1-ol (Z)	25.7	50092	37572	45433	57924
2-Heptenal (Z)	26.5	79307	102546	108478	101484
6-Octen-2-one	27	8351	14661	n.i.	12229
1-Hexanol	27.8	35251	39878	37832	44584
2-Methyl 2-cyclopenten-1-one	29.6	18156	22972	21975	29594
3-Hexen-1-ol (Z)	29.9	10583	12416	12093	14583
2-Nonanone	30.5	27251	31268	peaks not	10498
Nonanal	30.8	26968	30305	separated	24343
2,4 Hexadienal (E,E)	32.4	45185	58107	45202	43618
2-Octenal (E)	33.5	97656	116399	102958	102887
1-Octen-3-ol	34.3	46316	55920	40463	45501
2,4 Heptadienal (E,Z)	35.8	501316	629301	464367	518055
Unknown	36.6	62669	78305	60861	67252

Pentadecane	38	322490	439126	peaks not	393219
2,4 Heptadienal (E,E)	38.3	206168	229045	separated	212187
3,5 Octadiene-2-one (E,Z)	39.6	45354	peaks not	peaks not	38636
Benzaldehyde	40.7	870995	561098	peaks not	peaks not
2-Nonenal	41.2	n.i.	95672	separated	separated
Unknown	41.6	2259	16176	6198	2087
2,4 Octadienal (Z,Z)	42.5	20872	55322	26541	33119
3,5 Octadiene-2-one (E,E)	43.9	16521	21230	14215	17991
2,6 Nonadienal	44.7	55313	38176	78023	29659
2,4-Octadienal (E,E)	45	32011	26053	18769	27104
2-Decenal (Z) (Internal Std.)	47.8	31866	37028	25412	38458
Unknown	48.2	18451	12406	21797	15880
Hexadecane	49.7	13410	16058	15433	13634
2,5 Octadien-1-ol	50	8119	9902	8278	8111
Heptadecane	51.4	11041	10741	10917	11681
Unknown	53.9	37792	60266	58719	51187
5-Ethyl-2(5H)-furanone	54.8	15888	16376	10729	13651
2,4 Decadienal	57	14646	11946	14008	10782
TBHQ	61.3	14871	2278	13475	13127
1-Dodecanol	63.4	22018	n.i.	10770	9299

Peak Areas of Volatiles in Undeodorized Oil (cont'd)

Compound Name / Week	RT	Wk20-A1	Wk20-A2
Decane	9.4	25104	28802
2,3 Dimethyl 1,4 Hexadiene	9.9	146515	143265
1-Penten-3-one	10.3	peaks	peaks
3-Ethyl 1,4 Hexadiene	10.6	not	not
Methyl Benzene	10.8	separated	separated
5-Ethyl 2-Methyl Octane	12.1	0	0
Hexanal	12.5	435201	309667
1,3,6 Octatriene (E,E)	13.3	68285	70279
1,3,6 Octatriene(Z,E)	13.4	132815	146036
2-Pentenal	15	peaks not	50976
1,4-Dimethyl Benzene	15.2	separated	73927
1-Penten-3-ol	15.8	88453	64245
2-Methyl 2-Pentenal	16.4	25957	22531
Heptanal	17.6	252326	278237
4,5 Dimethyl 1-Hexene	18.4	3904	4253
4-Methyl 3-Pentenal	18.7	40811	39805
2-Hexen-3-one	19.4	33738	35563
2-Hexenal	19.6	314730	339252
Unknown	20	184005	199889
Unknown	20.5	58110	61799
4-Heptenal (Z)	20.7	144266	159573
Unknown	21.1	81936	54933
Unknown	22	84485	88566
Unknown	22.7	28342	28665
1,3,5 Trimethyl benzene	23.5	9758	10862
Octanal	23.8	52076	55678
Unknown	24.3	166071	174799
2-Penten-1-ol (E)	25.1	39334	31520
2-Penten-1-ol (Z)	25.7	22350	16269
2-Heptenal (Z)	26.5	101474	109835
6-Octen-2-one	27	623	803
1-Hexanol	27.8	22766	25410
2-Methyl 2-cyclopenten-1-one	29.6	19385	21428
3-Hexen-1-ol (Z)	29.9	12822	13745
2-Nonanone	30.5	26628	27606
Nonanal	30.8	33812	35135
2,4 Hexadienal (E,E)	32.4	45735	52122
2-Octenal (E)	33.5	107034	110158
1-Octen-3-ol	34.3	38912	42006
2,4 Heptadienal (E,Z)	35.8	561173	593696
Unknown	36.6	68327	71412

Pentadecane	38	403266	433045
2,4 Heptadienal (E,E)	38.3	217580	228873
3,5 Octadiene-2-one (E,Z)	39.6	28226	29145
Benzaldehyde	40.7	36908	38541
2-Nonenal	41.2	16005	17737
Unknown	41.6	5195	4396
2,4 Octadienal (Z,Z)	42.5	22146	23894
3,5 Octadiene-2-one (E,E)	43.9	25001	26642
2,6 Nonadienal	44.7	21789	24023
2,4-Octadienal (E,E)	45	22314	19118
2-Decenal (Z) (Internal Std.)	47.8	41703	49093
Unknown	48.2	3945	4815
Hexadecane	49.7	13914	15014
2,5 Octadien-1-ol	50	7106	7265
Heptadecane	51.4	11638	14141
Unknown	53.9	49279	54942
5-Ethyl-2(5H)-furanone	54.8	7440	8021
2,4 Decadienal	57	2376	2374
Antioxidant	61.3	38779	43107
1-Dodecanol	63.4	6829	1046

n.i. peak not integrated

n.s. peak not separated from adjacent peak

Appendix XIX

Peak Areas of Volatiles in Deodorized Oil

Compound Name	RT	Wk0-A1	Wk0-A2	Wk1-A1	Wk1-A2
Acetic Acid, Ethyl Ester	n.s.				
Ethanol	n.s.				
Benzene	n.s.				
Decane	9.4	3549	3828	5343	4472
Methyl Benzene	10.8	8352	8272	12669	10758
Hexanal	12.6	5387	4852	12454	9158
Undecane	13.1	5029	3070	6327	5069
Unknown	13.5	9526	6688	20228	17504
Ethyl Benzene	14.4	5098	5306	7448	6366
1,4-Dimethyl Benzene	15.3	18069	18261	31643	22830
2-Methyl 2-Pentenal	16.4	9055	9431	15277	12667
Dimethyl Benzene	17.6	n.i.	5048	10147	7814
Unknown**	21.4	3518	3364	5911	5412
Unknown**	21.9	907	888	1406	1250
Unknown**	22.3	1223	1107	2300	2088
Unknown**	22.8	1292	1252	1804	1680
Ethenyl Benzene	23.1	0	0	0	0
Octanal	23.9	0	0	0	0
Unknown**	24.7	7097	6427	10358	9133
Unknown**	26	4209	3936	5718	5462
Unknown	26.6	1657	1566	2287	2244
Unknown	26.8	668	612	1039	946
Unknown**	27.1	1276	1237	2041	1846
Octanoic Acid, Methyl Ester	30.8	0	0	3261	2803
Unknown**	31.4	785	681	1874	1747
Tetradecane	35.1	3621	3194	4416	4576
Butanoic Acid	35.8		558	1002	907
2,4 Heptadienal (E,Z)	36.2	0	0	1000	921
Unknown	38.5	4478	3936	3437	3790
Pentadecane	39.2	908	662	1159	1196
2,4 Heptadienal (E,E)	39.4	510	n.i.	623	524
Unknown	39.9	1712	1418	2297	2619
Dimethyl Hexadecane	40.5	2639	2422	4720	3800
1,3 Butanediol	40.8	1491	1260	2623	2810
Benzaldehyde	41.1	0	0	2415	2253
2-Nonenal	41.4	1182	1046	1991	1973
2,3 Butanediol	43.1	0	0	0	0
1,2 Propanediol	44.3	1943934	1721477	2244795	2090158
2-Decenal (Z) (Internal Std.)	47.9	24394	19859	13430	10557
Hexadecane	51.2	3651	2586	3147	4212

Unknown*	52.3	771	549	723	950
Unknown*	54.3	1004	736	1427	1556
Unknown*	56.8	1301	500	1531	2086
Unknown* (Octadecane)	58.8	1980	1000	1283	1609
Unknown	60.3	0	0	0	0
2-Ethyl 1-Decanol	60.8	2461	1281	1924	2623
Antioxidant	61.3	57317	40593	55299	64229
1-Dodecanol	63.5	2427	4123	7056	9645
1-Dodecene	63.8		7203	10116	11224
Tetradecanol	64.6	4686	1001	1059	1113
Unknown*	65	2121	582	1302	1282
Unknown* (Eicosane)	66.1	987	n.i.	1144	872
Ethoxy Benzoic Acid, Ethyl Ester	70.7	4170	10838	16377	6803
Unknown*	72.7	569	n.i.	6953	n.i.
Unknown*	73.7	733	n.i.	23337	1732
Unknown*	73.9	3811	1353	2237	1272
Unknown* (Docosane)	74.7	2287	1270	2334	957
Phenol Compound	74.9	2273	1741	3012	1767
Unknown?	80.3	7711	3020	2227	2729
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	81.4	562	n.i.	0	0
Dodecanoic Acid	83.6	7763	5644	6012	11097

Peak Areas of Volatiles in Deodorized Oil (cont'd)

Compound Name	RT	Wk2-A1	Wk2-A2	Wk3-A1	Wk3-A2
Acetic Acid, Ethyl Ester	n.s.				
Ethanol	n.s.				
Benzene	n.s.				
Decane	9.4	3683	3951	4574	4718
Methyl Benzene	10.8	10493	11427	15224	16971
Hexanal	12.6	10760	9538	n.s.	14436
Undecane	13.1	5351	5174	7996	6834
Unknown	13.5	18674	10873	24519	24124
Ethyl Benzene	14.4	6100	5567	6736	6715
1,4-Dimethyl Benzene	15.3	26462	19772	33948	31632
2-Methyl 2-Pentenal	16.4	12135	10822	16793	14854
Dimethyl Benzene	17.6	7656	6287	12203	7879
Unknown**	21.4	4835	4750	10648	8595
Unknown**	21.9	1309	1213	2824	2418
Unknown**	22.3	2112	1444	4052	3575
Unknown**	22.8	1745	1754	3155	2720
Ethenyl Benzene	23.1	0	0	0	0
Octanal	23.9	0	0	0	0
Unknown**	24.7	9779	9450	14880	12208
Unknown**	26	5417	5648	8252	6925
Unknown	26.6	2185	2315	3385	2853
Unknown	26.8	1072	972	1696	1493
Unknown**	27.1	1941	1895	3027	2625
Octanoic Acid, Methyl Ester	30.8	1923	1806	5655	1840
Unknown**	31.4	903	1066	2977	2016
Tetradecane	35.1	4526	4309	7713	6361
Butanoic Acid	35.8	1088	844	1833	1469
2,4 Heptadienal (E,Z)	36.2	1002	573	1879	1492
Unknown	38.5	5411	4796	9941	7049
Pentadecane	39.2	1192	1104	2822	1933
2,4 Heptadienal (E,E)	39.4	553	556	1145	854
Unknown	39.9	2179	2400	4201	3139
Dimethyl Hexadecane	40.5	3492	2612	4446	3992
1,3 Butanediol	40.8	2478	2323	3996	3326
Benzaldehyde	41.1	2665	2625	5391	4447
2-Nonenal	41.4	2078	2056	4277	3305
2,3 Butanediol	43.1	0	0	0	0
1,2 Propanediol	44.3	1833330	2080968	2723235	2413486
2-Decenal (Z) (Internal Std.)	47.9	19126	9118	17511	16686
Hexadecane	51.2	3480	3638	8146	5110
Unknown*	52.3	801	695	1817	1208

Unknown*	54.3	1476	1133	3116	2175
Unknown*	56.8	1121	791	2317	1610
Unknown* (Octadecane)	58.8	1565	1343	4931	2526
Unknown	60.3	0	0	4187	3435
2-Ethyl 1-Decanol	60.8	2399	1932	6716	3744
Antioxidant	61.3	52756	55315	97727	73739
1-Dodecanol	63.5	11953	6481	16997	15904
1-Dodecene	63.8	7748	n.i.	7849	5835
Tetradecanol	64.6	2585	738	3052	1517
Unknown*	65	4646	559	2160	2337
Unknown* (Eicosane)	66.1	1339	651	5039	3434
Ethoxy Benzoic Acid, Ethyl Ester	70.7	7546	4888	31969	22724
Unknown*	72.7	2496	37083	11580	10454
Unknown*	73.7	10164	6227	8468	10648
Unknown*	73.9	1734	1427	2031	2461
Unknown* (Docosane)	74.7	1545	1426	2438	1904
Phenol Compound	74.9	2808	2686	2960	735
Unknown?	80.3	3230	3149	4471	4534
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	81.4	0	0	1436	6214
Dodecanoic Acid	83.6	7273	8104	13342	8587

Peak Areas of Volatiles in Deodorized Oil (cont'd)

Compound Name	RT	Wk4-A1	Wk4-A2	Wk8-A1	Wk8-A2
Acetic Acid, Ethyl Ester	n.s.				
Ethanol	n.s.				
Benzene	n.s.				
Decane	9.4	10605	25737	25994	27708
Methyl Benzene	10.8	n.i.	29260	2483	0
Hexanal	12.6	n.s.	15963	14656	n.s.
Undecane	13.1	5065	7667	5532	peaks not
Unknown	13.5	66165	89538	107697	separated
Ethyl Benzene	14.4	3270	4958	3298	3432
1,4-Dimethyl Benzene	15.3	15737	17411	21617	23179
2-Methyl 2-Pentenal	16.4	n.i.	n.i.	n.i.	n.i.
Dimethyl Benzene	17.6	5963	2643	7070	7269
Unknown**	21.4	4715	6566	6722	7173
Unknown**	21.9	3855	2456	3024	3171
Unknown**	22.3	5095	6021	7794	8213
Unknown**	22.8	13862	7972	9500	8688
Ethenyl Benzene	23.1	4666	6687	6695	7551
Octanal	23.9	112864	145434	137072	160098
Unknown**	24.7	13915	19030	22587	22744
Unknown**	26	5114	7319	7570	6859
Unknown	26.6	1754	2207	2829	2310
Unknown	26.8	906	1018	6204	6703
Unknown**	27.1	4855	3289	2566	3264
Octanoic Acid, Methyl Ester	30.8	8205	1104	19058	1223
Unknown**	31.4	4036	3756	3844	3751
Tetradecane	35.1	5618	7219	6213	6824
Butanoic Acid	35.8	22493	31326	33841	36398
2,4 Heptadienal (E,Z)	36.2	11805	4201	30152	32009
Unknown	38.5	9635	11821	9958	11918
Pentadecane	39.2	4218	1212	1144	5838
2,4 Heptadienal (E,E)	39.4	851	2991	3725	10082
Unknown	39.9	3226	1710	2575	6770
Dimethyl Hexadecane	40.5	5435	2246	12776	18751
1,3 Butanediol	40.8	443499	512115	536962	641201
Benzaldehyde	41.1	n.i.	745	n.i.	n.i.
2-Nonenal	41.4	2348	3760	3103	4052
2,3 Butanediol	43.1	574160	825956	787537	896285
1,2 Propanediol	44.3	2043929	1863742	2118198	2416879
2-Decenal (Z) (Internal Std.)	47.9	10900	22742	19282	22943
Hexadecane	51.2	4466	4997	1328	1253
Unknown*	52.3	8248	10640	9918	12626

Unknown*	54.3	7598	6709	5738	6956
Unknown*	56.8	4761	4064	1811	1641
Unknown* (Octadecane)	58.8	2190	2738	648	2350
Unknown	60.3	10021	11986	7061	12173
2-Ethyl 1-Decanol	60.8	3933	3385	1171	3498
Antioxidant	61.3	17659	19392	9462	15027
1-Dodecanol	63.5	16437	2210	10106	11116
1-Dodecene	63.8	19730	3737	10951	10745
Tetradecanol	64.6	10236	2152	2984	5853
Unknown*	65	30881	1852	4979	3296
Unknown* (Eicosane)	66.1	4778	989	705	3069
Ethoxy Benzoic Acid, Ethyl Ester	70.7	30631	15062	4366	19132
Unknown*	72.7	36017	2009	932	8510
Unknown*	73.7	42646	2329	859	10194
Unknown*	73.9	7593	2460	1430	3667
Unknown* (Docosane)	74.7	6926	2118	n.i.	3280
Phenol Compound	74.9	21412	3119	1914	2030
Unknown?	80.3	3429	3125	4075	2514
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	81.4	7980	2034	n.i.	n.i.
Dodecanoic Acid	83.6	4739	16630	n.i.	4112

Peak Areas of Volatiles in Deodorized Oil (cont'd)

Compound Name	RT	Wk12-A1	Wk12-A2	Wk16-A1	Wk16-A2
Acetic Acid, Ethyl Ester	n.s.				
Ethanol	n.s.				
Benzene	n.s.				
Decane	9.4	16439	this	33561	25484
Methyl Benzene	10.8	1929	sample	0	0
Hexanal	12.6	28914	was	29699	27253
Undecane	13.1	7224	spilled	peaks not	peaks not
Unknown	13.5	122208	no	separated	separated
Ethyl Benzene	14.4	9411	peak	5072	4514
1,4-Dimethyl Benzene	15.3	21647	areas	283257	233414
2-Methyl 2-Pentenal	16.4	n.i.	are	n.i.	n.i.
Dimethyl Benzene	17.6	13107	available	10911	11664
Unknown**	21.4	6293		6328	5816
Unknown**	21.9	8339		2709	3996
Unknown**	22.3	15954		9173	8808
Unknown**	22.8	14531		9837	9122
Ethenyl Benzene	23.1	7872		9046	9034
Octanal	23.9	219073		236563	209795
Unknown**	24.7	26441		31869	23443
Unknown**	26	16571		9215	9991
Unknown	26.6	3653		8783	5122
Unknown	26.8	13561		7899	7793
Unknown**	27.1	5006		15803	5724
Octanoic Acid, Methyl Ester	30.8	723325		58463	28126
Unknown**	31.4	10451		4358	3483
Tetradecane	35.1	7209		7007	7237
Butanoic Acid	35.8	34010		34317	32680
2,4 Heptadienal (E,Z)	36.2	43773		20526	21847
Unknown	38.5	17450		16838	18589
Pentadecane	39.2	6767		18197	16859
2,4 Heptadienal (E,E)	39.4	1000		10693	10308
Unknown	39.9	7548		10400	11445
Dimethyl Hexadecane	40.5	18116		26156	32726
1,3 Butanediol	40.8	742015		853281	812080
Benzaldehyde	41.1	n.i.		n.i.	n.i.
2-Nonenal	41.4	626		6186	n.i.
2,3 Butanediol	43.1	1050279		1204357	1146773
1,2 Propanediol	44.3	1555816		2185266	2014841
2-Decenal (Z) (Internal Std.)	47.9	59595		15079	14059
Hexadecane	51.2	5175		3838	4239
Unknown*	52.3	12637		11845	11036

Unknown*	54.3	8965		7230	5949
Unknown*	56.8	5807		3000	1026
Unknown* (Octadecane)	58.8	2386		1972	2342
Unknown	60.3	11712		10763	11534
2-Ethyl 1-Decanol	60.8	5225		2527	3423
Antioxidant	61.3	8594		9848	12545
1-Dodecanol	63.5	14119		2635	5123
1-Dodecene	63.8	16786		3886	3485
Tetradecanol	64.6	7917		3085	7997
Unknown*	65	7622		742	n.i.
Unknown* (Eicosane)	66.1	2449		1083	963
Ethoxy Benzoic Acid, Ethyl Ester	70.7	4077		5105	7553
Unknown*	72.7	3681		n.i.	n.i.
Unknown*	73.7	2610		3228	1404
Unknown*	73.9	1970		3523	1861
Unknown* (Docosane)	74.7	1479		882	n.i
Phenol Compound	74.9	n.i.		1965	1917
Unknown?	80.3	3207		4183	2753
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	81.4	2830		529	599
Dodecanoic Acid	83.6	n.i.		n.i.	n.i.

Peak Areas of Volatiles in Deodorized Oil (cont'd)

Compound Name	RT	Wk20-A1	Wk20-A2
Acetic Acid, Ethyl Ester	n.s.		
Ethanol	n.s.		
Benzene	n.s.		
Decane	9.4	n.i.	1682
Methyl Benzene	10.8	11869	11264
Hexanal	12.6	n.s.	n.s.
Undecane	13.1	peaks not	peaks not
Unknown	13.5	separated	separated
Ethyl Benzene	14.4	971	n.i.
1,4-Dimethyl Benzene	15.3	26400	26210
2-Methyl 2-Pentenal	16.4	40099	31790
Dimethyl Benzene	17.6	14233	17489
Unknown**	21.4	11195	10128
Unknown**	21.9	91148	117380
Unknown**	22.3	32926	41840
Unknown**	22.8	12635	16087
Ethenyl Benzene	23.1	n.i.	n.i.
Octanal	23.9	5048	3404
Unknown**	24.7	10824	10880
Unknown**	26	12052	11822
Unknown	26.6	2674	2816
Unknown	26.8	16594	23194
Unknown**	27.1	8547	9286
Octanoic Acid, Methyl Ester	30.8	2658	3730
Unknown**	31.4	3881	4842
Tetradecane	35.1	9067	9031
Butanoic Acid	35.8	1847	2006
2,4 Heptadienal (E,Z)	36.2	155421	213157
Unknown	38.5	25936	33331
Pentadecane	39.2	6043	7555
2,4 Heptadienal (E,E)	39.4	1418	1483
Unknown	39.9	13147	17971
Dimethyl Hexadecane	40.5	8962	12166
1,3 Butanediol	40.8	8744	7899
Benzaldehyde	41.1	8710	9963
2-Nonenal	41.4	3744	3616
2,3 Butanediol	43.1	12318	13183
1,2 Propanediol	44.3	2011083	1601453
2-Decenal (Z) (Internal Std.)	47.9	43604	54589
Hexadecane	51.2	10351	10349
Unknown*	52.3	4079	3305

Unknown*	54.3	6284	1865
Unknown*	56.8	590	n.i.
Unknown* (Octadecane)	58.8	3429	2729
Unknown	60.3	12295	10255
2-Ethyl 1-Decanol	60.8	7228	4273
Antioxidant	61.3	67502	59275
1-Dodecanol	63.5	20412	11708
1-Dodecene	63.8	52505	55892
Tetradecanol	64.6	14672	3100
Unknown*	65	13946	2176
Unknown* (Eicosane)	66.1	26722	2509
Ethoxy Benzoic Acid, Ethyl Ester	70.7	34709	19165
Unknown*	72.7	n.i.	n.i.
Unknown*	73.7	peaks not	15865
Unknown*	73.9	separated	13608
Unknown* (Docosane)	74.7	peaks not	6095
Phenol Compound	74.9	separated	684
Unknown?	80.3	42275	n.i.
1,2-Benzenedicarboxylic Acid, Diisooctyl ester	81.4	45844	n.i.
Dodecanoic Acid	83.6	n.i.	n.i.

n.i. peak not integrated

Appendix XX

Peak Areas of Volatiles in N-3 Fatty Acid Esters

Compound Name	RT	C0	E0	B0	I0
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene	8.9				
Ethanol	8.9				
Unknown**	9.2	27147	28619	36636	30944
Unknown**	9.7	27089	28849	37403	31278
2-Butanol (R)	10.1	35878	41098	47570	43700
Butanoic Acid, Ethyl Ester	10.6	24047	20457	22980	12900
2-Methyl-3-Hexanone	11.9	0	0	0	0
Butanal	12.2	8101	8434	9170	7886
Hexanal	12.5	28496	30943	28449	25075
2-Pentanol	12.7	46565	51615	55485	44435
1,3,6 Octatriene (E,E)	13.4	132303	143398	132445	104461
Ethoxy-Ethene	14.1	7647	11221	2953	3781
Pentanoic Acid, Ethyl Ester	14.8	16192	18487	8223	n.i.
1-Butanol	15.0	0	0	0	0
1,4-Dimethyl Benzene	15.3	16272	20449	16036	12543
Unknown**	15.7	364349	420828	446552	389225
2-Methyl-2-Pentenal	16.6	0	0	0	0
Unknown**	18.9	487216	534523	607108	522627
Unknown*	20.7	5245	3999	3826	n.i.
Unknown*	21.4	6006	6647	5485	5935
Unknown*	22.1	14219	12124	8317	n.i.
Unknown*	22.8	11472	10748	12084	8652
Hexenoic- or Heptenoic Acid Ester	23.1	9890	10284	11436	9097
3-Hydroxy-Butanone	23.9	226016	244644	271376	227090
3-Hexenoic acid, Ethyl ester (Z)	24.8	25956	26864	27883	25090
Some Heptenoic acid, Ethyl Ester	30.7	70822	5776	9615	2512
5-Heptenoic acid, Ethyl Ester (E)	31.5	4740	5290	5669	4368
4-or 6-Heptenoicacid, Ethyl Ester	31.8	4028	973	1179	972
Unknown**	34.8	51972	57464	61195	56446
2,4-Heptadienal (E,Z)	35.9	32850	36512	38995	36021
2,4-Heptadienal (E,E)	38.4	12261	17541	24834	16612
Benzaldehyde	40.4	15201	20288	21566	18336
1,3 Butanediol	40.7	745746	817277	918243	804129
2,3 Butanediol	43.1	1028021	1144425	1282547	1132284
1,2 Propanediol	44.4	524633	716064	920070	883323
2-Decenal (Z) (Internal Std.)	47.8	11391	9987	10080	4863

Unknown**	53.2	24601	32952	30332	31123
Unknown**	54.3	6157	7518	7605	7166
Unknown**	59.7	50534	68297	60865	67228
Unknown**	60.2	46869	67359	54777	59397
Undecanoic Acid, Ethyl Ester	66.8	7945	7905	10796	9671
Ethoxy Benzoic Acid, Ethyl Ester	70.8	8973	11678	12733	16492

Peak Areas of Volatiles in N-3 Fatty Acid Esters (cont'd)

Compound Name	RT	C1	E1	B1	I1
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene£	8.9				
Ethanol	8.9				
Unknown**	9.2	32282	27810	30996	29216
Unknown**	9.7	40014	30909	35687	31050
2-Butanol (R)	10.1	41431	33573	41699	35737
Butanoic Acid, Ethyl Ester	10.6	101686	36059	88240	30344
2-Methyl-3-Hexanone	11.9	0	0	0	0
Butanal	12.2	8847	7857	8873	8136
Hexanal	12.5	33268	29673	peaks not	34950
2-Pentanol	12.7	55542	46165	separated	44760
1,3,6 Octatriene (E,E)	13.4	199712	134852	191132	136181
Ethoxy-Ethene	14.1	2678	10356	2722	10510
Pentanoic Acid, Ethyl Ester	14.8	55284	19228	53533	21675
1-Butanol	15.0	n.i.	8411	n.i.	n.i.
1,4-Dimethyl Benzene	15.3	23635	22275	24535	19882
Unknown**	15.7	417911	372054	423210	389659
2-Methyl-2-Pentenal	16.6	1357	836	959	5002
Unknown**	18.9	521572	468678	518542	502549
Unknown*	20.7	66556	10907	66165	6410
Unknown*	21.4	8693	6962	7734	8365
Unknown*	22.1	158771	29276	146146	19152
Unknown*	22.8	75101	16632	69081	12810
Hexenoic- or Heptenoic Acid Ester	23.1	33834	12616	31979	12010
3-Hydroxy-Butanone	23.9	317169	218987	317785	232988
3-Hexenoic acid, Ethyl ester (Z)	24.8	67202	29515	63628	27277
Some Heptenoicacid, Ethyl Ester	30.7	389611	294717	348485	294355
5-Heptenoicacid, Ethyl Ester (E)	31.5	35428	7967	32366	7149
4-or 6-Heptenoicacid, Ethyl Ester	31.8	21874	15023	24785	21452
Unknown**	34.8	56681	49708	55693	54577
2,4-Heptadienal (E,Z)	35.9	474304	32031	423727	37211
2,4-Heptadienal (E,E)	38.4	52940	19406	49146	27494
Benzaldehyde	40.4	22524	11939	20260	22567
1,3 Butanediol	40.7	800824	710464	776244	774899
2,3 Butanediol	43.1	1119452	990492	1078262	1074212
1,2 Propanediol	44.4	400771	608365	420145	459964
2-Decenal (Z) (Internal Std.)	47.8	16567	13528	11756	24799
Unknown**	53.2	32583	26865	29477	30285

Unknown**	54.3	10164	7287	8998	8884
Unknown**	59.7	67725	55281	58374	64925
Unknown**	60.2	60290	50932	53999	57411
Undecanoic Acid, Ethyl Ester	66.8	8724	7050	10011	11757
Ethoxy Benzoic Acid, Ethyl Ester	70.8	7365	6271	7455	7021

Peak Areas of Volatiles in N-3 Fatty Acid Esters (cont'd)

Compound Name	RT	C2	E2	B2	I2
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene£	8.9				
Ethanol	8.9				
Unknown**	9.2	31756	29870	28163	30012
Unknown**	9.7	38079	34272	36035	33242
2-Butanol (R)	10.1	39538	35721	34718	33377
Butanoic Acid, Ethyl Ester	10.6	89657	48728	87867	38174
2-Methyl-3-Hexanone	11.9	0	0	0	0
Butanal	12.2	9541	10186	8632	8774
Hexanal	12.5	34360	41309	29074	31115
2-Pentanol	12.7	51450	62785	47075	530028
1,3,6 Octatriene (E,E)	13.4	230446	199996	181722	160806
Ethoxy-Ethene	14.1	1421	3325	2073	3195
Pentanoic Acid, Ethyl Ester	14.8	76138	38576	61703	25384
1-Butanol	15.0	n.i.	n.i.	n.i.	n.i.
1,4-Dimethyl Benzene	15.3	21260	24803	18319	20708
Unknown**	15.7	391006	473703	348786	397917
2-Methyl-2-Pentenal	16.6	990	770	877	547
Unknown**	18.9	506957	603647	449451	510605
Unknown*	20.7	99414	22085	90399	10837
Unknown*	21.4	7186	7625	6464	7390
Unknown*	22.1	255039	53985	214274	27260
Unknown*	22.8	118500	29212	97831	16072
Hexenoic- or Heptenoic Acid Ester	23.1	48914	17311	40880	11982
3-Hydroxy-Butanone	23.9	367483	294173	313308	227996
3-Hexenoic acid, Ethyl ester (Z)	24.8	91691	41184	76826	32024
Some Heptenoicacid, Ethyl Ester	30.7	390483	178913	161974	111047
5-Heptenoicacid, Ethyl Ester (E)	31.5	55394	14773	45322	7973
4-or 6-Heptenoicacid, Ethyl Ester	31.8	13417	5180	n.i.	3204
Unknown**	34.8	55461	67041	48685	54786
2,4-Heptadienal (E,Z)	35.9	828060	43054	678463	38188
2,4-Heptadienal (E,E)	38.4	36047	40125	56272	19683
Benzaldehyde	40.4	12967	28980	8442	8766
1,3 Butanediol	40.7	819382	1004103	747612	843586
2,3 Butanediol	43.1	1177825	1388256	1080579	1177762
1,2 Propanediol	44.4	290469	511512	292469	450628
2-Decenal (Z) (Internal Std.)	47.8	51627	32479	17205	15054
Unknown**	53.2	29383	39804	24633	30367

Unknown**	54.3	10921	23741	17259	13719
Unknown**	59.7	57985	83696	56018	63177
Unknown**	60.2	53914	79767	44981	57449
Undecanoic Acid, Ethyl Ester	66.8	77931	38391	15148	12376
Ethoxy Benzoic Acid, Ethyl Ester	70.8	6770	19077	8321	9438

Peak Areas of Volatiles in N-3 Fatty Acid Esters (cont'd)

Compound Name	RT	C3	E3	B3	I3
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene£	8.9				
Ethanol	8.9				
Unknown**	9.2	36409	34528	28104	48107
Unknown**	9.7	50082	37440	37206	52361
2-Butanol (R)	10.1	51687	40791	38592	53823
Butanoic Acid, Ethyl Ester	10.6	116489	35971	98623	66471
2-Methyl-3-Hexanone	11.9	7346	8656	1933	4275
Butanal	12.2	15981	14690	7753	14170
Hexanal	12.5	41695	30697	26675	41923
2-Pentanol	12.7	59478	50236	49873	70636
1,3,6 Octatriene (E,E)	13.4	236852	146143	166684	209889
Ethoxy-Ethene	14.1	2912	2977	2094	6382
Pentanoic Acid, Ethyl Ester	14.8	53140	17914	32660	17897
1-Butanol	15.0	n.i.	n.i.	n.i.	n.i.
1,4-Dimethyl Benzene	15.3	855739	480061	347768	679565
Unknown**	15.7	594892	486518	440845	720729
2-Methyl-2-Pentenal	16.6	0	0	n.i.	850
Unknown**	18.9	717487	585792	548261	847342
Unknown*	20.7	110870	18332	74909	25622
Unknown*	21.4	10746	5651	8616	12938
Unknown*	22.1	288234	57419	171738	63946
Unknown*	22.8	136150	29679	79668	36680
Hexenoic- or Heptenoic Acid Ester	23.1	59829	18390	36665	27394
3-Hydroxy-Butanone	23.9	492155	272540	323155	406022
3-Hexenoic acid, Ethyl ester (Z)	24.8	114481	38385	70726	56551
Some Heptenoicacid, Ethyl Ester	30.7	249940	71672	104138	277070
5-Heptenoicacid, Ethyl Ester (E)	31.5	63425	16058	40256	20591
4-or 6-Heptenoicacid, Ethyl Ester	31.8	8167	3658	3003	26062
Unknown**	34.8	83313	59077	60041	90389
2,4-Heptadienal (E,Z)	35.9	876026	37416	467944	55955
2,4-Heptadienal (E,E)	38.4	99147	25106	50574	56692
Benzaldehyde	40.4	20292	26125	24138	38494
1,3 Butanediol	40.7	1164614	932143	877830	1408153
2,3 Butanediol	43.1	1299690	1298570	1236357	1981136
1,2 Propanediol	44.4	380828	419317	358074	830543
2-Decenal (Z) (Internal Std.)	47.8	17654	15027	10094	18483
Unknown**	53.2	41507	29813	28346	47715

Unknown**	54.3	25706	8779	10105	15191
Unknown**	59.7	83563	56388	61888	88459
Unknown**	60.2	76440	52817	56155	82669
Undecanoic Acid, Ethyl Ester	66.8	24082	10949	10757	13072
Ethoxy Benzoic Acid, Ethyl Ester	70.8	34447	8466	19693	11018

Peak Areas of Volatiles in N-3 Fatty Acid Esters (cont'd)

Compound Name	RT	C4	E4	B4	I4
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene	8.9				
Ethanol	8.9				
Unknown**	9.2	n.i.	9458	3844	9179
Unknown**	9.7	9347	9000	10045	9867
2-Butanol (R)	10.1	n.i.	n.i.	n.i.	n.i.
Butanoic Acid, Ethyl Ester	10.6	123025	68343	138421	76382
2-Methyl-3-Hexanone	11.9	13820	16049	13483	16790
Butanal	12.2	peaks	peaks	peaks	peaks
Hexanal	12.5	not	not	not	not
2-Pentanol	12.7	separated	separated	separated	separated
1,3,6 Octatriene (E,E)	13.4	117526	73918	108903	73155
Ethoxy-Ethene	14.1	0	0	0	0
Pentanoic Acid, Ethyl Ester	14.8	58658	21400	67925	24297
1-Butanol	15.0	n.i.	n.i.	n.i.	n.i.
1,4-Dimethyl Benzene	15.3	169494	276933	169039	247806
Unknown**	15.7	65917	85421	66993	87651
2-Methyl-2-Pentenal	16.6	12514	14113	14201	17842
Unknown**	18.9	56897	59140	57119	62357
Unknown*	20.7	108263	37127	140439	36198
Unknown*	21.4	11000	12880	13004	11310
Unknown*	22.1	267808	66898	321397	65665
Unknown*	22.8	117551	28819	137927	28987
Hexenoic- or Heptenoic Acid Ester	23.1	44142	10557	52195	10298
3-Hydroxy-Butanone	23.9	153498	28630	179192	30418
3-Hexenoic acid, Ethyl ester (Z)	24.8	79881	26069	92077	26325
Some Heptenoicacid, Ethyl Ester	30.7	120230	26272	141422	26953
5-Heptenoicacid, Ethyl Ester (E)	31.5	61067	20585	74919	17369
4-or 6-Heptenoicacid, Ethyl Ester	31.8	0	0	0	0
Unknown**	34.8	12397	10378	13148	11154
2,4-Heptadienal (E,Z)	35.9	816007	201498	901982	207355
2,4-Heptadienal (E,E)	38.4	68113	27112	72625	26975
Benzaldehyde	40.4	8164	7308	8666	6229
1,3 Butanediol	40.7	3202	4295	3809	4191
2,3 Butanediol	43.1	27936	33833	20125	38224
1,2 Propanediol	44.4	333983	684939	429008	630164
2-Decenal (Z) (Internal Std.)	47.8	25052	23845	28854	25870
Unknown**	53.2	3251	5059	4281	5266

Unknown**	54.3	6277	6688	8293	5911
Unknown**	59.7	9692	12643	13042	12821
Unknown**	60.2	12749	16130	15029	14212
Undecanoic Acid, Ethyl Ester	66.8	12519	16171	25820	11789
Ethoxy Benzoic Acid, Ethyl Ester	70.8	10260	9073	7904	8918

Peak Areas of Volatiles in N-3 Fatty Acid Esters (cont'd)

Compound Name	RT	C8	E8	B8	I8
Formic Acid, Ethyl Ester	n.s.				
Octene	n.s.				
Acetic Acid, Ethyl Ester	n.s.				
3-Methyl 1,4-Heptadiene	n.s.				
3-Methyl 1,4-Heptadiene£	8.9				
Ethanol	8.9				
Unknown**	9.2				
Unknown**	9.7	4007		4208	
2-Butanol (R)	10.1	n.i.	n.i.	n.i.	n.i
Butanoic Acid, Ethyl Ester	10.6	116612	87382	140868	79465
2-Methyl-3-Hexanone	11.9	0	0	0	0
Butanal	12.2	peaks	peaks	peaks	peaks
Hexanal	12.5	not	not	not	not
2-Pentanol	12.7	separated	separated	separated	separated
1,3,6 Octatriene (E,E)	13.4	119281	59002	90116	61216
Ethoxy-Ethene	14.1	0	0	0	0
Pentanoic Acid, Ethyl Ester	14.8	117257	23632	115419	30353
1-Butanol	15.0	n.i.	n.i.	n.i.	33682
1,4-Dimethyl Benzene	15.3	n.i.	58762	n.i.	5383
Unknown**	15.7	2050	14171	1660	6562
2-Methyl-2-Pentenal	16.6	11663	19188	16239	11610
Unknown**	18.9	5392	2686	5492	2728
Unknown*	20.7	155526	43634	139829	43865
Unknown*	21.4	14081	14377	15202	11462
Unknown*	22.1	355910	75995	304259	86395
Unknown*	22.8	214066	31969	129133	35996
Hexenoic- or Heptenoic Acid Ester	23.1	10388	10388	50243	12555
3-Hydroxy-Butanone	23.9	211104	32803	175596	40316
3-Hexenoic acid, Ethyl ester (Z)	24.8	106955	29689	91610	30463
Some Heptenoicacid, Ethyl Ester	30.7	169499	30587	136797	37112
5-Heptenoicacid, Ethyl Ester (E)	31.5	84731	16165	68214	23423
4-or 6-Heptenoicacid, Ethyl Ester	31.8	0	0	0	0
Unknown**	34.8	10575	13115	11474	7771
2,4-Heptadienal (E,Z)	35.9	1006711	204745	819877	245152
2,4-Heptadienal (E,E)	38.4	114935	31618	87571	32282
Benzaldehyde	40.4	15944	5451	4907	5289
1,3 Butanediol	40.7	8256	5000	5695	4228
2,3 Butanediol	43.1	15878	12063	13166	8885
1,2 Propanediol	44.4	429232	711723	417829	515331
2-Decenal (Z) (Internal Std.)	47.8	66233	27471	30666	22830
Unknown**	53.2	2753	n.i.	12169	6915

Unknown**	54.3	2353	4027	2112	1564
Unknown**	59.7	3078	3004	2233	2339
Unknown**	60.2	8962	5291	5096	4468
Undecanoic Acid, Ethyl Ester	66.8	20737	32667	14772	10373
Ethoxy Benzoic Acid, Ethyl Ester	70.8	40239	27530	16193	7273

Vita

Ingolf Grün was born July 22, 1962 in Holzminden, Germany, where he grew up and attended High School. In Spring of 1983, Ingolf began his studies of Home Economics and Nutrition at Justus-Liebig University, Giessen, Germany. After studying 7 semesters and finishing a thesis under Prof. Ibrahim Elmadfa as part of the diploma requirements in 1986, he participated in the International Student Exchange Program, and came to Virginia Polytechnic Institute and State University. In 1987, Ingolf became a regular Master's student in the Department of Human Nutrition and Foods with an emphasis in foods. He successfully defended his Master's thesis with the title "Determination of vitamin B-6, available lysine, and pyridoxyllysine in a new instant baby food product" under the supervision of Dr. William E. Barbeau in June 1989. In Fall of 1989, he enrolled in the Ph.D. program in the same department. For the academic year 1991-92, Ingolf received a Cunningham Fellowship from the university to work on his research involving lipoxygenase activity in menhaden. For many years, Ingolf was also working for the International Food and Nutrition Program with Dr. Marilyn Prehm as his supervisor. As an extracurricular activity, Ingolf served in the student governance as the treasurer of the Graduate Student Assembly from 1990 until 1992 besides other involvements. After finishing his Ph.D., Ingolf starts working as a postdoctoral research associate for Dr. Kent Stewart in the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute and State University.