AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented October 8, 1965

Title TOTAL FATTY ACID COMPOSITION OF AUTOXIDIZED ANCHOVY MEAL AND STUDIES OF RELATED MODEL SYSTEMS

Abstract approved
(Major professor)

The variation in fatty acid composition between the "bound" and "free" lipid in stored anchovy meal was determined using gas-liquid chromatographic analysis. A model system of fresh anchovy oil on powdered lactalbumin was also studied to determine the changes in extractability and fatty acid composition which occur during oxidation.

All of the lipid-protein systems examined were first extracted with hexane. The extracted protein was then extracted with choroform:: methanol (2:1 v/v), and finally digested in HCl and the hydrolysate extracted with diethyl ether. The chloroform : methanol extract and HCl digestion represented oil which was bound to the protein.

Each lipid extract was interesterified with methanol to yield the corresponding methyl esters, and these esters were then

analyzed by gas-liquid chromatography. Both quantitative and qualitative analysis was carried out using a diethylene glycol succinate packed column in a gas chromatograph equipped with a hydrogen flame detector.

The results of these investigations showed that the oil from anchovy meal differed markedly in fatty acid composition from the corresponding fresh oil. Although the types of fatty acids found were identical to those in the fresh oil, there was considerable quantitative variation. These differences were characterized by smaller amounts of polyunsaturated fatty acids in the meal, and correspondingly larger amounts of saturated and monoenoic fatty acids. The greatest change occurred in the acid, 20:5.

Anchovy meal contained a "bound"lipid fraction which varied in quantitative fatty acid composition with the readily extractable lipid. The fractions associated with the protein had higher proportions of 22:4, 22:5, and 22:6, with the acid digest fraction considerably lower in the monoenoic fatty acids.

The oxidation of anchovy oil and anchovy oil methyl esters on lactalbumin produced a reduction in hexane extractable lipid with a corresponding increase in lipid which was soluble only in chloroform : methanol or after HCl hydrolysis. These changes were more drastic when untreated anchovy oil was used. The oxidation of anchovy oil methyl esters on lactalbumin produced a reduction in

the amounts of polyunsaturated fatty acids present in the hexane extract. The other solvent extractions, however, yielded only higher molecular weight compounds which could not be analyzed by GLC. These observations led to the conclusion that the fatty acids formed compounds which interacted before being bound to the protein.

TOTAL FATTY ACID COMPOSITION OF AUTOXIDIZED ANCHOVY MEAL AND STUDIES OF RELATED MODEL SYSTEMS

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1966

APPROVED:

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Date thesis is presented October 8, 1965

Typed by Luanne Bayless

ACKNOWLEDGMENT

The author wishes to express his appreciation to Professor Russel O. Sinnhuber for his able guidance during the course of this investigation and the preparation of the thesis. The author also wishes to acknowledge T. C. Yu for his assistance in the operation of the gas chromatograph and his helpful suggestions concerning experimental procedures.

Funds for support of this research were made available through Grant 32241, United States Department of Interior, Bureau of Commercial Fisheries. The author would like to express his gratitude to this agency for making this study possible.

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TOTAL FATTY ACID COMPOSITION OF AUTOXIDIZED ANCHOVY MEAL AND STUDIES OF RELATED MODEL SYSTEMS

INTRODUCTION

Fish meal, made by the reduction of whole fish and fish scrap, is by far the largest and most important source of animal feed protein. The essential amino acids which make up its protein, along with vitamins and minerals, make fish meal a necessary component of poultry rations. During the manufacturing process and particularly during storage, the fat in fish meal, which is composed of highly unsaturated fatty acids, undergoes considerable autoxidation. The autoxidized lipid is believed to be largely unavailable as a food component. This results in a loss of energy value. In addition, the autoxidized fat may itself interact or produce substances which will react with amino acids to reduce the nutritive value of the protein. The difficulty of removal of this altered or "bound" lipid by the usual fat extraction procedure has been recognized for some time. It is only very recently, with the scientific advances in the feed industry and the possibility that a fish protein concentrate or fish flour might be used for human purposes, that attention has been focused on the lipid and particularly the "bound" lipid in fish meal. Its role in reducing protein availability and imparting fishy or rancid flåvors is now being carefully looked at by investigators all over the world.

Research on the formation, character, and removal of the "bound" lipid in fish meal has been concerned primarily with the

changes in extractability, protein digestibility, iodine number and peroxide value. Few studies have been made on the changes in fatty acid composition which occur in the lipid during storage.

The purpose of this research was to determine the differences in composition between the "bound" and "free" lipid present in a typical fish meal which had been oxidized during production and storage. The oil from this meal was compared with the fresh oil which was removed during preparation of the meal. A model system of anchovy oil on lactalbumin was also used to study the changes which occur in these lipid fractions during autoxidation. Gas chromatography was employed to carry out the quantitative and qualitative fatty acid analyses. Numerous extraction procedures were examined and procedures were developed for extraction and isolation of the "bound" lipid.

REVIEW OF LITERATURE

The Nature of Marine Oils

The chemical nature of fish oils is not unlike that of other naturally occurring fats and oils. That is, they are composed primarily of triglycerides with lesser amounts of compounds such as pigments, free fatty acids, hydrocarbons, waxes, sterols, phosphatides, fat soluble vitamins and glyceryl ethers (12, p. 46). The component fatty acids of fish oils are of the usual type with the straight-chained, even-numbered acids predominating. The unsaturated acids are also normal in that they are mainly cis, methylene interrupted polyenes (5).

Fish oils differ from other natural lipids mainly because of their fatty acid composition. Fish oils contain unusually large amounts of unsaturated fatty acids, with minor amounts of the so called "essential" fatty acids; linoleic, linolenic, and arachidonic (32, p. 10). While most other lipids are composed primarily of fatty acids having 14-20 carbons, many marine oils have as much as half of their component fatty acids with 20 and 22 carbons.

The unusual nature and complexity of these fish oils makes them an interesting challenge to the analytical chemist. This challenge, incidentally, has just begun to be resolved.

Autoxidation of Lipids

Farmer et al (25), in the original works concerning the oxidation of polyisoprenes, proposed that compounds most labile to oxidation were those possessing a methylene interrupted, non-conjugated system of unsaturation. The olefinic compounds studied were classified into four general groups with group I the most labile to oxidation and the other groups in order of decreasing lability.

I. $-CH=CH-CH_2-CH=CH-$

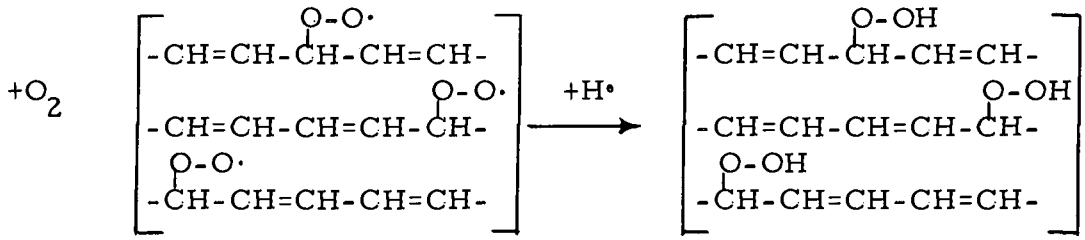
II. $-CH=CH-CH_2-CH_2-CH=CH-$

III. $-CH_2CH=CH-CH_2-$

IV. $-CH=CH-CH=CH-$

The reason for the lability of group I is the tendency of the hydrogens on the methylene group to be removed to form resonance stabilized free radicals. These free radicals then react with oxygen to form peroxy free radicals, which in turn add a hydrogen atom to form hydroperoxides. The general pattern of this type of oxidation was summarized by Bollard and Koch (16) and is shown below.

$$-CH=CH-CH_2-CH=CH- \xrightarrow{-H\cdot} \begin{bmatrix} -CH=CH-\dot{C}H-CH=CH- \\ -CH=CH-CH=CH-\dot{C}H- \\ -\dot{C}H-CH=CH-CH=CH- \end{bmatrix}$$



These hydroperoxides decompose to form a wide variety of secondary oxidation products (40). These include aldehydes, alcohols, ketones, epoxides, and oxygen linked copolymers.

When one considers that the normal polyunsaturated fatty acids in natural oils are included in Farmer's first grouping, it is not surprising to find that these acids comprise the major source of oxidation in lipids (70, p. 14). It also follows that marine oils, being highly unsaturated, should be extremely susceptible to oxidation.

Formation of Lipid-Protein Complexes Upon Autoxidation of Lipids

Studies Using Model Systems

During the oxidation of lipids, a variety of oxygen containing compounds are formed which may react with one another, with unchanged lipid, or with non-lipid substances. Of these non-lipid substances, proteins are perhaps most important because they have many reactive groups and are present in many foods containing lipids.

Oxidized lipids may react with proteins to form fairly stable

compounds. Examples of this would be the reaction between carbonyl groups and free amine groups (71), and the possible reaction between the peroxy free radicals of oxidized lipids and proteins. The latter reaction was proposed by Venolia and Tappel (72) and Desai and Tappel (21). Both reactions are characterized by the formation of brown colors (71, 72), insolubility in aqueous solutions, and difficulties in HCl hydrolysis (21).

Other stable lipid-protein complexes were observed by Fisher and Gurin (27) in plasma lipoprotein. The results of these studies showed that the fatty acids were bound to the protein far more firmly than would be expected with hydrophobic or electrostatic interactions. In all probability a covalent bond was involved.

Oxidizing lipids may also react with proteins to form complexes in which the mechanism for binding is largely physical bonds. Narayan and Kummerow (57, 58) and Narayan et al (59) studied the nature of these bonds in aqueous systems and concluded that the keto and epoxy groups were the most active. The hydroxy and hydroperoxy groups formed during oxidation were thought to be much less reactive in the formation of this type of complex. It was found that egg albumin and lactalbumin complexed most readily while no complex formed with casein (57). Acylation of the protein had very little effect on complexing ability. These authors suggested that the complexes may be formed through hydrogen bonding between the carbonyl

oxygen in the lipid and the hydrogen of the amide groups of the proteins, and also between the carbonyl oxygen in the protein and the hydrogen of the hydroxyl or hydroperoxide groups in the oxidized lipid. Tappel (71) also suggested that physical bonding may result when oxidized lipids reacted with themselves to form compounds which were then absorbed on the protein.

Pokorney (63) studied the formation of these complexes in a dry system of casein and the fatty acid ethyl esters of sunflower oil. He found that lipid-protein complexes were formed most readily if the lipid was allowed to oxidize in contact with the protein. Oxidized esters added to the protein were much less reactive. Since the iodine value of this bound lipid did not change appreciably during the course of oxidation, it was thought to arise through a specific reaction.

Pokorney used solubility changes to follow the course of the reaction. The unchanged esters were extracted using hexane, while the weakly bound complexes were dissolved using chloroform and methanol. The strongly bound complexes were dissolved by hydrolysis in HCl followed by extraction with carbon disulfide.

Studies Using Fish Meal

The oxidation of the lipid present in fish meal has been the subject of some interest. It is known that a certain fraction of the

lipid in aged fish meal is "bound" and cannot be extracted by ordinary lipid solvents. Stansby (66) suggested that an HCl digestion followed by extraction would remove this bound lipid. Almquist (11) studied this phenomenon further and noted that during normal storage of fish meal the amount of ether extractable lipid decreased markedly. The pepsin digestibility of the protein was also decreased. He concluded that this change in the lipid and protein was due to oxidation and complexing during storage.

More recently, March et al (50,51) reported a similar reduction in extractability along with an extreme drop in iodine value. An interesting aspect of these studies is that a much greater change in extractability and iodine value was observed in samples kept at very low temperatures (-20°C). A proposed explanation was that oxidation inhibitors may actually be formed or accumulated at higher storage temperatures. The meals stored at lower temperatures also yielded a high percentage of compounds that could not be split by HCl to yield an extractable product. This would suggest that this change at low temperatures occurs through a somewhat different mechanism than that observed at higher temperatures.

Other studies concerning the chemical and nutritive changes which occur in stored herring meal were carried out by Carpenter et al (17,18) and Lea et al (42,43). The latter group stated that the "bound" lipid recovered after acid hydrolysis was correlated to the

small amounts of lysine which became unavailable on storage of the meal in air. This drop in available lysine has been studied by many others (50, 51, 17, 18) and shown to have little effect on the nutritive quality and stability of the protein.

Gas-Liquid Chromatography of Fatty Acids

With the possible exception of the petroleum industry, no industry or technology has been effected by gas chromatography as much as that concerned with fats and oils. Through the proper use of gas chromatography, analyses which were formerly lengthy, inaccurate, and even impossible can be done routinely.

Gas chromatography was first described by Martin and Synge (54) in 1941, in their Nobel Prize winning paper on liquid-liquid partition chromatography. In this paper, the authors mentioned that the mobile phase could just as well be a vapor. More than ten years later, James and Martin (36) developed this idea and used it to separate volatile fatty acids from formic through dodecanoic, using titration as means of detection. In 1953, Cropper and Heywood (19) extended this technique by separating the even-numbered fatty acids from C_{12} to C_{22}. James and Martin soon noted that the methyl esters of fatty acids were more readily chromatographed because of their higher volatility, and their decreased tendency to dimerize and adsorb irreversibly on the column.

Another major contribution to the field of gas chromatography was the invention in 1956 of the gas density balance detector by Martin and James (53). The subsequent rapid expansion and improvement of this technique (37, 38, 14) was due primarily to the high sensitivity of this detection system which allowed the use of very small samples. The use of smaller samples greatly increased column efficiency by reducing band spreading due to column overload, and consequently making separations more distinct.

Another significant contribution was the development of polar stationary liquids by Lipsky and Landowne (44, 45) and Orr and Callen (60). These new liquids were superior to the non-polar silicone greases formerly used in that they would separate the methyl esters through C_{18} in less than 30 minutes. Furthermore, they could separate the unsaturated esters from the saturated esters with astounding ease. The best phases found were diethylene glycol succinate polyester, diethylene glycol adipate polyester, and many similar polymers. With the advent of these polyester liquid phases, routine analysis of fatty acids became possible.

There are several excellent reviews (61, 29, 26) which outline the present method as it has developed. Perhaps the most complete and useful of these is by Farquhar et al (26). This review, although almost six years old, outlines basic techniques and procedures which are still applicable.

Theory and Technique

A complete review of the factors influencing separation in gas-liquid chromatography, including the underlying theory and technique, would be much too lengthy to cover adequately here. There are, however, several reviews available which treat this subject in detail.

A comprehensive review of the theoretical aspects of gas-liquid chromatography was reported in two publications by Dal Nogare and Juvet (20) and Pecsok (62). A more practical review, concerning the effects of various operating conditions was published by Howard (34). A similar but more extensive review by Hardy and Pollard (31) outlined in some detail the principles of separation as well as the apparatus and technique used in gas-liquid chromatography.

Qualitative Aspects

Under conditions of constant temperature, flow rate, and column characteristics, the time necessary to elute a specific component is constant. For this reason, the qualitative analysis of a mixture of compounds can be based primarily on retention times. This is particularly true in the case of fatty acid methyl esters in that all components have identical functional groups. Because of this homogeneity, differences in chromatographic mobility are

dependent only on differences in the carbon chain.

Because of the slight variability in column performance, even under carefully controlled conditions, relative retention times are used instead of simple retention times. This parameter is constant over a much wider range of column conditions and is, therefore, more characteristic of each compound. The relative retention time is simply the retention time of the compound divided by the retention time of a known standard. In the case of fatty acids this standard is usually methyl stearate.

Several investigators have tried, with some success, to improve the precision of retention data by running chromatographs at two sets of conditions. The use of two chemically distinct stationary liquids for this purpose has been reported (35,46) as well as the use of two different temperatures (2). It has also been suggested that the use of fresh and aged columns of identical packing may be helpful (49).

Several other methods of expressing elution data have been proposed as an improvement over relative retention time. "Carbon number" proposed by Woodford and Van Gent (73), and "equivalent chain length" suggested by Miwa et al (56) are two closely related parameters which have been used for this purpose. Relative retention time, however, is still the most widely used system.

Ideally, identification of compounds in gas chromatography

should be effected by recovering each compound and identifying it by chemical means. Due to the complexity of lipid systems, and to the small samples necessary, this method is impractical in most cases. Identification by comparison of retention data with that of known standards is therefore the most widely used method.

In a complex lipid system, there exist many fatty acids for which known standards are not readily available. Fortunately, the structural homogeneity of most mixtures of fatty acids (5) makes possible some graphical methods of establishing a tentative identity. These fatty acid mixtures are homogeneous in that almost all have cis, methylene interrupted double bonds with either three, six, or nine carbons from the last double bond to the end of the carbon chain. This last group of carbon atoms is referred to as the "end carbon chain".

A linear relationship exists between the log retention times and the number of carbon atoms in homologous fatty acid methyl esters having identical numbers of double bonds and identical end carbon chains. This relationship provides a graphical aid, the use of which is discussed recently by Ackman and Burgher (6), Ackman (1,3,4) and Evans et al (24). A similar linear relationship has been suggested by Ackman (3,4) to exist among fatty acid methyl esters having identical numbers of carbon atoms, and identical end carbon chains, but differing in amount of saturation. In this case, the log

retention time is plotted against the number of double bonds. These plots, when used in conjunction with known standards can be a valuable aid in the establishment of tentative identities.

Ackman and Burgher (8) have also proposed that naturally occurring fatty acid mixtures containing compounds of well established identity may be used as a secondary reference standard for comparison of retention times. In their work, cod liver oil was chosen because it contained a wide variety of typical fatty acids, and was free of interfering contaminants.

Quantitative Aspects

The usual method of quantitation of fatty acid mixtures is measurement of the area under each component part and then dividing by the total area to obtain the percent composition. Methods used in estimating areas include triangulation, planimetry, or merely cutting out the peaks and weighing the paper (33).

A serious problem often arises from the fact that the detectors do not always give a linear response to concentration. In this case correlation factors must be calculated for each component and concentration (22, 39, 10). This is particularly true in cases where a broad range of fatty acids is to be analyzed.

In the case of the hydrogen flame detector, Ettre and Kabot (23) state that area response is very close to weight percent

composition, and that differences between saturated and unsaturated fatty acids in this respect is also very small. Due to the inherent variability of biological lipid systems, and the systematic error involved in calculating area, the non-linearity problem is usually of small significance. This is especially true when the very long or short chained fatty acids are minor components. In most cases it is sufficient to make multiple runs and report average compositions along with standard deviations. A detailed outline of these quantitative procedures has been made available by Horning et al (33).

Application to Marine Lipids

As mentioned earlier, the complexity of the lipids isolated from marine sources presents a challenging analytical problem. This problem is one to which gas-liquid chromatography is particularly adapted. The separations made possible by this analytical tool have resulted in at least provisional identification of the component fatty acids of many of these lipids. The progress in this field may be illustrated by observing that in 1955 Lovern (47, p. 51) reported in a review article that the number of fatty acids present in a fat such as cod liver oil was 19. In 1963, Ackman (7) using gas-liquid chromatography reported the presence of 46 fatty acids in cod liver oil. He also reported their percent composition as well as positional structure.

Probably the most extensively studied marine oil in recent years has been menhaden body oil. This oil was used by Stoffel et al (67, 68, 69) of the Rockefeller Institute because of its complexity. Their primary interest was in the improvement of separation techniques for the study of higher unsaturated fatty acids. In addition to this group, several other workers (13, 26) have studied menhaden oil, and its component fatty acids have been well characterized.

Some other marine oils which have been thoroughly analyzed by gas-liquid chromatography include cod liver oil (7, 8, 9), tuna (64, 65), sardine (41), and many more. A fairly extensive review has recently been published by Ackman (5) which cites 48 publications concerning the fatty acid composition of twelve species of fish. Perhaps the most complete reference to the composition of fish oils has recently been published by Gruger et al (30). In this paper the fatty acid composition of the oil from 21 species of marine fish, fresh water fish, and shellfish is reported.

Unfortunately, qualitative and quantitative analysis of marine oils makes use of many empirical methods, and offers in many cases only provisional identification of minor components. One cannot, however, expect a single analytical technique to give a complete picture. It is sufficient to say that gas-liquid chromatography, although not an exact tool, has catalyzed a rapid growth in lipid analysis by effecting rapid and complete separations which were formerly impossible.

EXPERIMENTAL

Raw Materials

Anchovy Oil

The anchovy oil (Engraulis rigens) used in this study was obtained from Star-kist Foods Inc., Terminal Island, California. This oil was of good quality with a light brown color and a peroxide value of 5.2.

Menhaden Oil

The menhaden oil (Brevoortia tyrannus) used in this study was obtained from the U.S. Department of Interior, Bureau of Commercial Fisheries, Technological Laboratory, Seattle,Washington. This oil had been decolorized during processing, and was pale yellow in color. The oil had a peroxide value of 15.7.

Fish Meal

The meal used in this research was also obtained from Star-kist Foods Inc., and had been prepared commercially from Peruvian anchovies (Engraulis rigens). This meal is used as a diet component for animal and poultry feeds, and had been stored one year at -18°C prior to analysis. The lipid portion of this meal was highly

oxidized, and varied in appearance, depending on the degree of extraction, from a red-brown, viscous oil to a dark brown, waxy solid.

Purified Protein

The protein used in this investigation was powdered lactalbumin obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Oxidation of Anchovy Oil On Lactalbumin

Anchovy Oil was mixed with powdered lactalbumin (0.75 gm oil per 10 gm protein) in a mortar and pestle until a smooth consistency was attained. This mixture was placed in petri dishes at a thickness of 5 - 8 mm, and oxidized at 50°C in the dark. Ten gram samples were taken periodically and analyzed.

This procedure was repeated using a methyl ester mixture instead of the untreated oil. These methyl esters were prepared from anchovy oil using the methanolysis procedure which is described in the appropriate section.

Extraction of Lipids

Preliminary Investigations

An extreme variability in the completeness of extraction was

observed when the fish meal was extracted with various organic solvents. For this reason a series of extractions were carried out using several different solvent systems and extraction conditions. From this investigation an appropriate solvent extraction system was chosen.

Extraction Procedure

Both the fish meal and lactalbumin samples were carried through identical extraction schemes to keep them as experimentally comparable as possible. These extractions were similar to those employed by Pokorney (63) which were designed to separate lipids roughly on the basis of their affinity for protein.

Ten grams of the lipid-protein mixture were initially extracted with 100 ml of hexane (redistilled, U.S.P. grade), and the protein removed by centrifugation for ten minutes at 3,000 rpm. The solvent was decanted off, and the protein extracted two more times with 50 ml of hexane. The combined solvent extract was then filtered through a short column (2.5 cm o.d.) containing 10 gm of celite 545[1] to remove suspended protein fines. The resulting clear solution was evaporated to dryness using a rotary vacuum evaporator, and the residue weighed. The lipid residue was then used

[1] Johns-Manville, celite 545, unsized. Treated with 1 N NaOH to decrease adsorptivity, and with 1 N HCl to remove traces of inorganic salts and metals.

directly for methanolysis.

The protein residue from the first extraction was extracted again using the solvent system described by Folch et al (28). This extraction utilized a chloroform : methanol (2:1, v/v) solvent, followed by the addition of 0.2 volumes water. The top phase of the resulting two-phase system contained non-lipid materials and was discarded. The lower phase was then carried through filtration followed by evaporation as in the hexane extraction.

Acid Digestion of Protein

The solvent extracted protein residues were digested in aqueous HCl to free any lipid which may have been "bound" to the protein. These digestions were carried out using gently refluxing 4 N HCl (5 ml per gm protein). Following digestion, the protein solutions were diluted with three volumes of water and extracted with three 50 ml portions of diethyl ether (U.S.P., distilled). The lipid extracted in this manner was reported as "bound" lipid.

The optimum length of digestion was determined by digesting extracted meal for various lengths of time and determining the amount of lipid which was recovered in each case. The effect of the length of digestion on fatty acid composition of the lipid was also determined. This was accomplished by digesting whole meal for various time periods, and determining the fatty acid composition of

the recovered lipid. These lipid compositions were compared to that obtained from an exhaustive soxhlet extraction of whole meal using azeotropic chloroform : methanol (87.3:12.7). This extraction was not complete, but the amount of lipid left on the meal was small and therefore did not contribute significantly to the overall composition of the total lipid. This extraction was therefore regarded as the total lipid in the meal for purposes of comparison.

Preparation of Methyl Ester Derivatives of Fatty Acids

The procedure used for the preparation of methyl esters was a base-catalyzed methanolysis using 0.1 N sodium methoxide (15, 48). In this procedure, one gram or less of lipid was dissolved in 20 ml of anhydrous ether and an equal volume of 0.1 N sodium methoxide in methanol added. The solution was then allowed to stand overnight at room temperature under nitrogen. The methyl esters were recovered by diluting the solution with five volumes of water, acidifying with dilute HCl to a bromcresol purple end point, and extracting with diethyl ether. The ether extract was then dried over sodium sulfate, the solvent removed partially under vacuum, and the last traces removed under a stream of nitrogen.

When methyl esters were to be prepared from highly oxidized samples, it was necessary to use dry benzene as the solvent in the

methanolysis reaction. This modification was necessary because of the insolubility of these samples in diethyl ether. It should also be noted that this reaction is an interesterification and is useful only with samples composed of triglycerides, phospholipids, and cholesterol esters. Hydrolyzed samples cannot be esterified in this manner.

To esterify the HCl digested samples, BF_3-methanol[2] was used (55). In this procedure, 200 mg or less of fatty acids was dissolved in 3 ml of the BF_3-methanol reagent. The solution was then heated over a steam bath for two minutes in a sealed container under nitrogen. The esters were recovered in the usual manner by diluting with water and extracting with diethyl ether.

Purification of Fatty Acid Methyl Esters

The oxidized oils used in this study yielded esters which were highly colored, and contained considerable amounts of polymeric and steroid impurities. To remove these impurities, the procedure of Luddy et al (48) was used. This method employed a column containing 10 gm of silicic acid : celite (8:2) which had been conditioned overnight at 110°C, and equilibrated with 4% water. A sample of 100-200 mg was placed on the column in hexane and eluted with 300

[2] BF_3-methanol reagent obtained from Applied Science Laboratories Inc. Contains 13% BF_3 in anhydrous methanol.

ml of 1% diethyl ether in hexane. The monomeric methyl esters were completely eluted and were a very pale yellow color. These esters were weighed and stored in vials at -18°C under nitrogen.

Hydrogenation of Fatty Acid Methyl Esters

The hydrogenation of various samples of methyl esters was carried out using the apparatus and procedure outlined by Farquhar et al (26). The hydrogenations were performed as an aid to qualitative analysis in the conformation of saturated and unsaturated components.

In this procedure, 50-100 mg of esters were dissolved in 10 ml of methanol and hydrogenated at low pressure using a catalyst of PtO_2. The saturated esters were recovered by filtering the solution, and evaporating the solvent under nitrogen. The esters were then used directly in gas chromatographic analysis.

Gas Chromatographic Analysis of Methyl Esters

Chromatographic Instrument

The gas chromatograph used in this research was an Aerograph, Model 600-B "Hi-Fi", equipped with a hydrogen flame detector. The recorder used was a Barber Coleman "Wheelco", Model 8000-2700-J1, with one millivolt full scale sensitivity and one

second full scale balancing speed.

Operating Parameters

The chromatographic instrument was set at an input impedance of 10^7 ohms, and an output sensitivity of 1-X. The flow rate of the nitrogen carrier gas was 20 ml/min. at 12 psi inlet pressure, and the hydrogen flow rate to the detector was also 20 ml/min. These flow rates were measured by a soap bubble flow meter. The air flow to the detector was approximately 300 ml/min. measured by water displacement. The column temperature was maintained in all cases at 185°C, with an injector temperature of 245°C. The recorder was operated at a chart speed of 30 in./hr.

Column Preparation

The solid support used in the chromatographic columns was celite 545 (Johns Manville) which had been size graded. The 120-140 mesh celite was selected and treated by the procedure described previously. The treated celite was again sized, and the 120-140 mesh used in the column packing.

The stationary liquid used in this investigation was diethylene glycol succinate polyester (DEGS)[3]. To coat the inert support, the

[3] C_6 Diethylene Glycol Succinate, Stabilized polyester. Obtained from Analabs, Inc., Hamden, Conn.

polyester was dissolved in a small volume of acetone, and the proper amount of the treated celite added. The mixture was stirred gently to avoid breaking the celite, and the bulk of the acetone removed by evaporation under a stream of nitrogen. The last traces of acetone were removed by heating the packing material in an oven at 100°C for 30 minutes.

The columns were packed by plugging one end of a section of 1/8 in. O.D. stainless steel tubing with glass wool, and attaching that end to an aspirator with rubber tubing. The packing was then added through the opposite end using a small funnel with the aid of a vibrating tool.

All analyses in this study were carried out using a seven foot column, containing 2.6 gm of 16.6% DEGS on 120-140 mesh treated celite 545. All columns were conditioned 48 hours at 200°C prior to use.

Qualitative Analysis

Identification of the fatty acid components was carried out primarily by comparison with known standards. For this purpose, pure esters of 14:0, 16:0, 16:1, 18:0, 18:1, and 20:4[4] were obtained

[4] Shorthand notation. The first number refers to the chain length of the fatty acid, and the second number refers to the number of double bonds.

from Applied Science Laboratory, State College, Pa. In addition, Purified esters of 8:0, 10:0, 12:0, 18:2, 18:3, 20:1, 20:5, 22:1, and 22:6 were obtained from the National Institutes of Health, Metabolism Study Section. The retention times of other saturated esters were established through hydrogenation. These standards were chromatographed at various intervals throughout the conduct of this investigation due to slight variations in retention times as the columns aged.

Other components were identified using linear plots of log retention time against carbon number. A secondary reference standard of menhaden body oil methyl esters was also used to establish probable identity of many minor components.

The retention times were expressed as relative retention times relative to methyl stearate. The measurement of retention time was accomplished by measuring the distance on the recording chart from a small ether peak to the apex of the component peak in question.

Quantitative Analysis

Quantitation of the methyl ester mixtures was carried out using the triangulation technique. Lines were drawn along both sides of each component peak intersecting the baseline and joining together at the top. The area of the resulting triangle was calculated, and

the percent composition calculated by dividing each individual area into the total area of all the peaks.

The linear response of the recorder was checked for the quantitative method used through the analysis of standard mixtures of known composition. The standards used were mixtures A, B, C, and D, supplied by the National Institutes of Health, Metabolism Study Section.

RESULTS AND DISCUSSION

Preliminary Investigations

Solvent Extractions

Of the various solvent systems and extraction conditions tested, a soxhlet extraction using azeotropic chloroform : methanol was found to be most efficient. However, an extraction using chloroform : methanol (2:1) at room temperature was found to be of comparable efficiency, and because of the milder conditions was selected for use in this research. A room temperature extraction using hexane was chosen to remove readily extractable lipid.

The extreme variability in extraction efficiency which was observed (Table 1) is characteristic of aged fish meal. This variability seems to be due to polar interactions between the lipid and protein resulting from oxidation of the lipid. Another possible contributing factor is the formation of oxidative and thermal polymers of altered solubility characteristics. This would account for the relative ineffectiveness of the non-polar solvents such as hexane.

HCl Digestion

A small portion of the oil in the meal was bound to the protein by a stronger interaction than that which was broken by polar

Table 1. Extraction of Whole Fish Meal With Various Lipid Solvents

Solvent	Extraction Conditions	Percent Lipid Recovered	Percent of Total Lipid
Hexane	Room temp.[1]	4.12	32.1
Ethyl ether	Room temp.	5.48	42.7
Ethanol	Room temp.	6.45	50.3
Ethanol:ether (1:1)	Room temp.	6.68	52.1
Ethanol:ether (3:1)	Room temp.	7.48	58.3
$CHCl_3$:MeOH (2:1)	Room temp.	11.61	90.6
Ethyl ether	10 hr soxhlet[2]	6.45	50.3
Acetone	10 hr soxhlet	8.88	69.3
Ethanol	10 hr soxhlet	10.90	85.0
$CHCl_3$:MeOH (87.3:12.7)	10 hr soxhlet	12.01	93.7
HCl digest[3]	As described in appropriate section	12.82	100.0

[1] All room temperature extractions used 10 ml/gm meal followed by two solvent washes of one half this volume.

[2] All soxhlet extractions used 10 gm samples of meal and approximately 40 solvent exchanges.

[3] This digestion was carried out on the residual protein from the $CHCl_3$:MeOH (87.3:12.7) soxhlet extraction, and the total of both lipid fractions reported. This represents the total lipid in the meal.

solvents. To extract this lipid it was first necessary to carry out an acid digestion of the meal. During the course of the digestion there was a gradual degradation of the lipid components of the meal and a reduction in the amount of lipid recovered. A digestion time of two hours with 4N HCl was found to be most desirable; yielding a maximum recovery of bound lipid (Figure 1) with no detectable change in fatty acid composition (Table 2).

As the digestion time was increased, a marked decrease in the fatty acids 16:1, 18:1, 18:2, 18:4, 20:5, and 22:6 was observed. These changes, however, did not take place until after at least eight hours of digestion. Another change observed was the appearance of two saturated compounds of unknown structure with relative retention times of 2.47 and 4.27. The formation of these compounds during digestion corresponds very closely with the disappearance of 16:1 and 18:1. The possibility exists, therefore, that these compounds are the products of a unique degradation of 16:1 and 18:1. Markley (52) describes the addition of HCl to monoenoic fatty acids by either an ionic or peroxide initiated free radical mechanism. Although the conditions for this type of reaction were not optimum in this case, the possibility of hydrohalogenation definitely exists. The further study of this reaction, using purified methyl oleate or palmitoleate, is indicated.

Table 2. Fatty Acid Composition of Total Fish Meal Lipid After Various Lengths of Digestion in 4N HCl.

Peak No.	Compound	Tr/Tr	Percent Composition 0 hr[1]	1 hr	2 hr	4 hr	8 hr	12 hr	18 hr
1	8:0	0.06	T	T	T	T	T	0.18	T
2	10:0	0.09	T	T	T	T	T	T	T
3	11:0	0.12	T	T	T	T	T	T	T
4	12:0	0.16	0.22	0.23	0.24	0.21	0.22	0.37	0.11
5	sat.	0.19	T	T	T	T	T	0.28	T
6	13:0	0.22	T	T	T	T	T	T	T
7	sat.	0.25	T	T	T	T	T	T	T
8	14:0	0.30	10.48	10.76	10.50	10.93	10.45	14.03	13.18
9	14:0br	0.35	0.28	0.27	0.24	0.20	0.30	0.20	0.21
10	15:0	0.40	0.98	1.07	1.01	1.10	1.07	1 39	0.88
11	sat.	0.47	T	T	T	T	T	T	0.18
12	16:0	0.56	29.88	29.51	30.03	29.63	31.05	29.71	40.61
13	16:1	0.66	11.11	10.94	10.61	11.02	10.38	7.69	7.29
14	17:0	0.73	1.59	1.54	1.61	1.72	1.28	2.01	1.16
15	17:0br	0.76	T	T	T	T	T	T	T
16	16:2	0.82	T	T	T	T	T	T	T
17	16:2	0.87	1.79	1.86	1.84	1.77	1.69	1.59	0.67
18	18:0	1.00	7.38	7.60	7.43	7.09	8.00	7.82	10.13
19	16:3	1.07	0.88	0.81	0.91	0.84	0.76	T	T
20	18:1	1.18	14.46	14.20	14.42	14.67	14.81	13.15	10.25
21	19:0	1.33	T	T	T	T	T	T	0.33
22	18:2	1.44	1.93	1.86	2.21	1.98	1.77	1.37	0.76
23	18:2	1.54	0.72	0.77	0.82	0.74	0.83	0.91	0.28
24	20:0	1.77	0.44	0.34	0.45	0.40	0.59	0.67	0.39
25	20:1	2.04	1.75	1.71	1.84	1.80	1.66	2.20	1.34
26	18:4	2.38	1.15	1.03	1.51	1.22	1.60	T	T
27	sat.	2.47	----	----	----	----	----	4.20	4.33
28	20:2	2.54	T	T	T	T	T	----	----
29	unsat.	3.03	T	----	T	T	T	T	T
30	unsat.	3.27	T	T	T	T	T	T	T
31	20:4	3.54	0.85	0.90	0.68	0.75	0.89	T	T
32	unsat.	3.75	T	T	T	T	T	----	----
33	unsat.	4.12	T	T	T	T	T	----	----
34	sat.	4.27	----	----	----	----	----	1 83	1.75
35	20:5	4.80	6.09	6.17	6.15	6.02	5.94	4.63	2.06
36	unsat.	5.30	----	----	T	T	----	----	T
37	unsat.	6.16	0.40	0.53	0.40	0.58	0.35	T	T
38	22:4	6.84	1.21	1.32	0.96	1.20	0.74	1.49	1.16
39	22:5	8.47	0.50	0.55	0.45	0.50	T	T	0.65
40	22:6	10.00	5.84	6.12	6.05	5.63	5.61	4.48	2.55

[1] Composition of $CHCl_3$:MeOH azeotrope extraction by soxhlet.

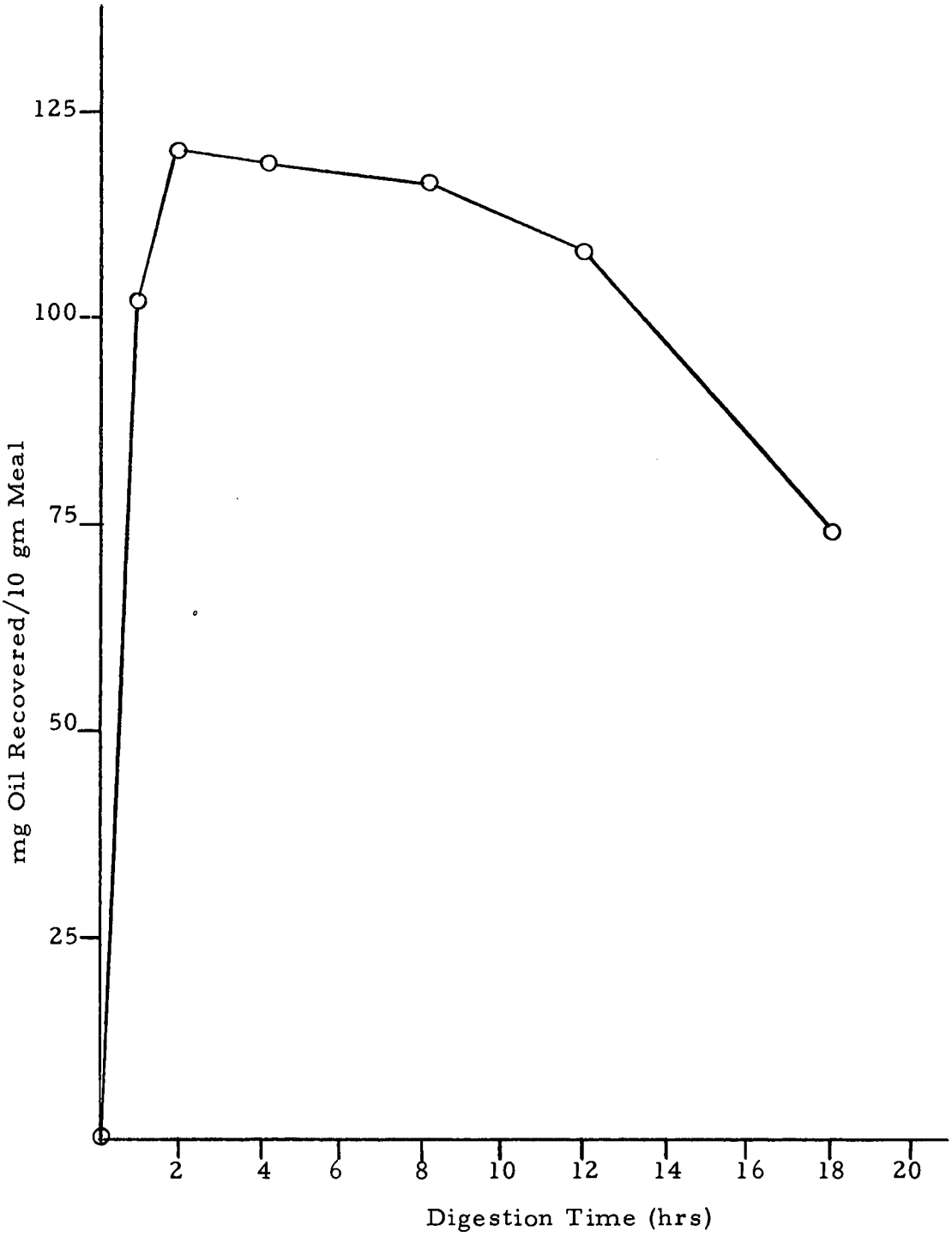


Figure 1. Effect of Acid Hydrolysis on the Recovery of "Bound" Lipid

Determination of Quantitative Precision and Accuracy

Quantitative fatty acid standards A, B, C, and D provided by the National Institutes of Health, Metabolism Study Section were used to determine the accuracy of the detection system. Quantitative results agreed with the stated composition data with a relative error of less than 3.1% for the major components (greater than 10% of the mixture), and less than 4.8% for the minor components (less than 10% of the mixture).

The precision of the quantitative method used was determined by chromatographing each standard in triplicate and calculating the standard deviation for each component peak. Using this procedure it was found that a standard deviation of less than 0.98 was obtained for the major components, and less than 0.54 for the minor components.

Establishment of Secondary Reference Standard

As an aid to qualitative analysis, a secondary reference standard of menhaden body oil was used. Through the use of primary standards, linear log plots, and the data published by Farquhar et al (26), structures were assigned to 27 compounds (Table 3). Other minor components were present in the mixture of esters but could not be identified and therefore were not reported.

Table 3. Summary of Compounds Identified in Menhaden Body Oil as a Secondary Reference Standard.

Peak No.	Compound	Tr/Tr	Standard Tr/Tr	% Comp.	Data of Farquhar et al (26) Structure[1]	% Comp.
4	12:0	0.16	0.16	0.15		0.10
7	14:0	0.30	0.30	9.43		7.20
8	14:0 br	0.35		0.05		0.10
9	15:0	0.41	0.40	0.85		0.50
11	16:0	0.56	0.57	19.09		17.00
12	16:1	0.68	0.66	11.12	9	9.80
13	17:0	0.74	0.73	0.96		0.40
14	17:0 br	0.78		T		T
15	16:2	0.83		T	6,9	T
16	16:2	0.89		2.23	9,12	2.00
17	18:0	1.00	1.00	2.98		3.10
18	16:3	1.10		1.61	6,9,12	1.30
19	18:1	1.19	1.17	14.71	9	14.50
20	19:0	1.35	1.33	T		T
21	18:2	1.46	1.47	2.48	9,12	2.00
22	18:2	1.58		0.81	6,9	0.70
23	20:0	1.78	1 78	0.44		0.10
24	18:3	1.92	1.95	0.83	6,9,12	1.30
25	20:1	2.03	2.09	2.04	11	2.10
26	18:4	2.42		3.15	6,9,12,15	3 20
27	20:2	2.52		0.40	11.14	1.20
28	20:4	3.56	3.56	0.80	5,8,11,14	0.60
29	20: un	4.17		0.85		1.20
30	20:5	4.88	4 87	13.69	5,8,11,14,17	12.50
31	22:4	6.55		0.23	7,10,13,16	T
32	22:5	8.48		1.36	7,10,13,16,19	2.00
33	22:6	9.95	10.00	8.23	4,7,10,13,16,19	8.90

[1] Number indicates position of double bond in the molecule

br = branched

un = unsaturated

The Fatty Acid Composition of Anchovy Oil

The separations achieved in the chromatographic analysis of anchovy oil are shown on Table 4 and Figure 2. Thirty-two components were clearly separated into individual peaks, and one more was identified on the basis of a dissymmetric peak (peaks No. 25, 26). Sixteen of these peaks were identified by comparison with known standards. The identities of these compounds were further substantiated by the use of the secondary reference standard. In addition, the identities of ten more compounds were provisionally established in this manner. Finally, through the use of linear log retention plots, the probable identity of 20 compounds was established. Hydrogenation was also used to confirm the identity of all saturated compounds, and to establish the presence of unsaturation in other compounds. Through the use of two or more of these four empirical methods, the identity of all but four of the component peaks was established. These unknown compounds, with the exception of peak No. 29, were all present in trace amounts.

The unknown compounds are classified on Table 4 as either saturated (sat.) or unsaturated (unsat.). Of these compounds, peak No. 29 merits some discussion. This peak was reported by Farquhar et al (26) in menhaden oil as 20:un, meaning that it had 20 carbons and was unsaturated. Due to its position in the chromatogram, this

Table 4. Retention Data and Percent Composition of Anchovy Oil Methyl Esters.

Peak #	Compound	Tr/Tr	Standard Tr/Tr	Probable Structure	% Composition
1	8:0	0.04	0.04		T
2	10:0	0.09	0.09		T
3	11:0**	0.13			T
4	12:0	0.17	0.16		0.13
5	13:0**	0.23			T
6	sat.	0.26			T
7	14:0	0.30	0.30		8.00
8	14:0br*	0.35			0.20
9	15:0	0.41	0.40		0.61
10	sat.	0.47			0.15
11	16:0	0.57	0.55		16.76
12	16:1	0.68	0.66	9	10.42
13	17:0	0.73	0.73		0.40
14	17:0br*	0.77			T
15	16:2	0.83		6,9	0.30
16	16:2	0.89		9,12	1.97
17	18:0	1.00	1.00		3.05
18	16:3	1.09		6,9,12	2.48
19	18:1	1.18	1.17	9	12.08
20	19:0	1.32	1.32		T
21	18:2	1.46	1.47	9,12	4.81
22	18:2*	1.56		6,9	0.70
23	20:0	1.78	1.78		0.45
24	20:1	2.02	2.09	11	1.66
25	18:4*	2.41		6,9,12,15	3.00
26	20:2*	2.55		11,14	T
27	unsat.	2.82			T
28	20:4	3.53	3.52		1.37
29	unsat.	4.15			0.72
30	20:5	4.88	4.87	5,8,11,14,17	20.94
31	22:4*	6.51		7,10,13,16	1.04
32	22:5*	8.40		7,10,13,16,19	2.40
33	22:6	9.89	10.00	4,7,10,13,16,19	6.37

[1] Number indicates position of double bond

* Identified through use of secondary reference standard

** Identified through use of log Tr/Tr plots

sat. = saturated unknown

unsat. = unsaturated unknown

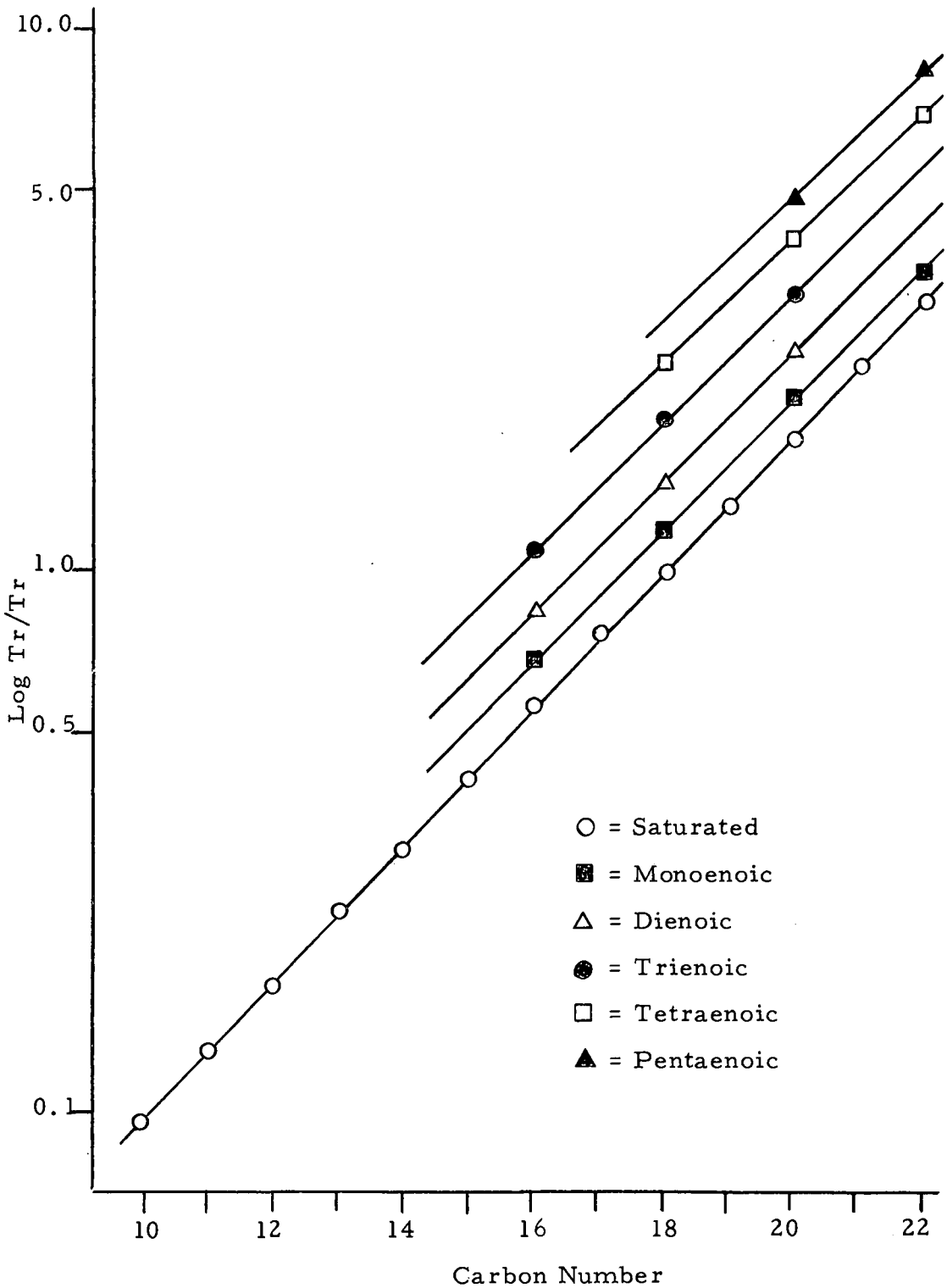


Figure 2. Semilog Plot of Tr/Tr vs. Chain Length of the Acid

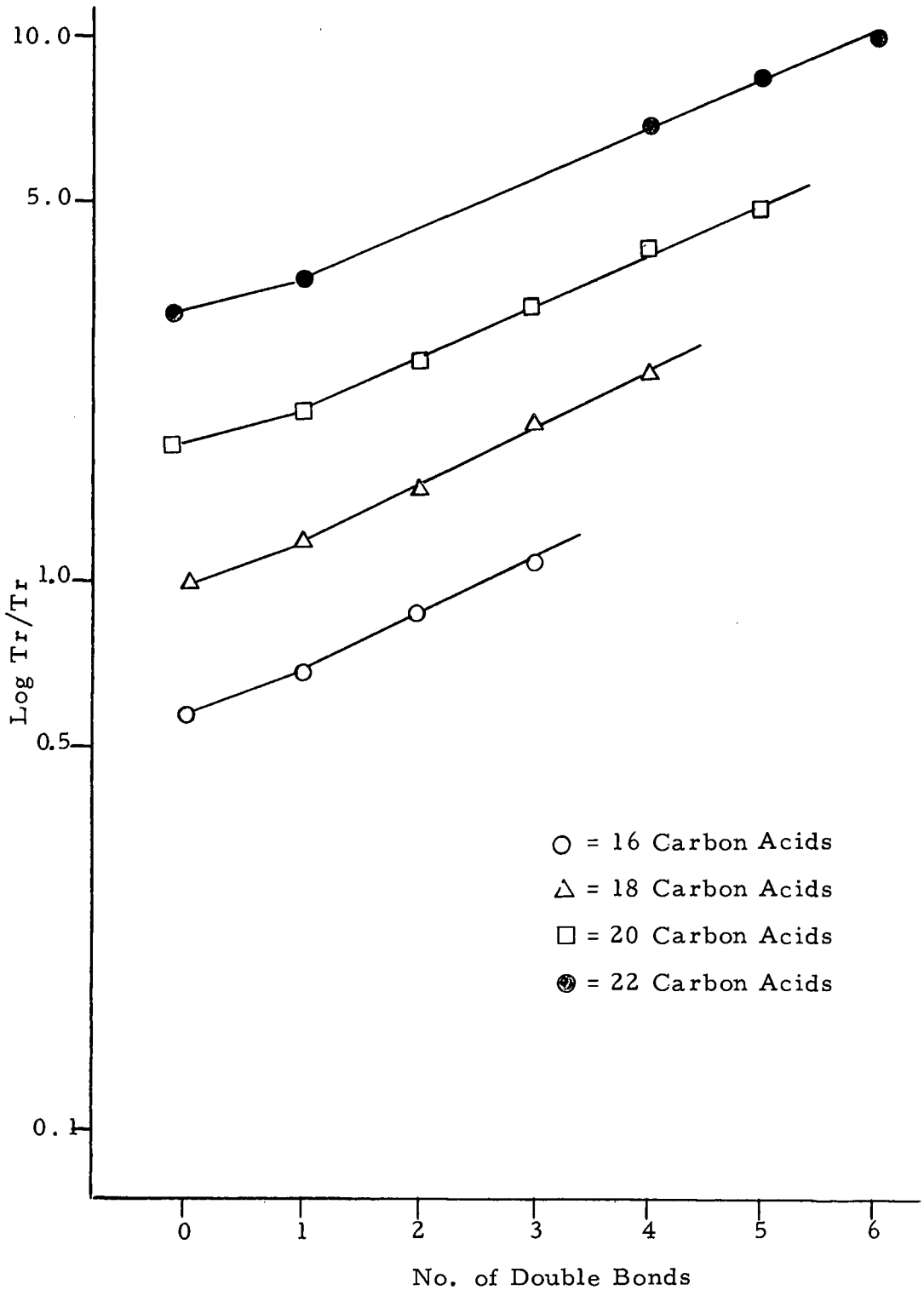


Figure 3. Semilog plot of Tr/Tr vs. Degree of Unsaturation of the Acid

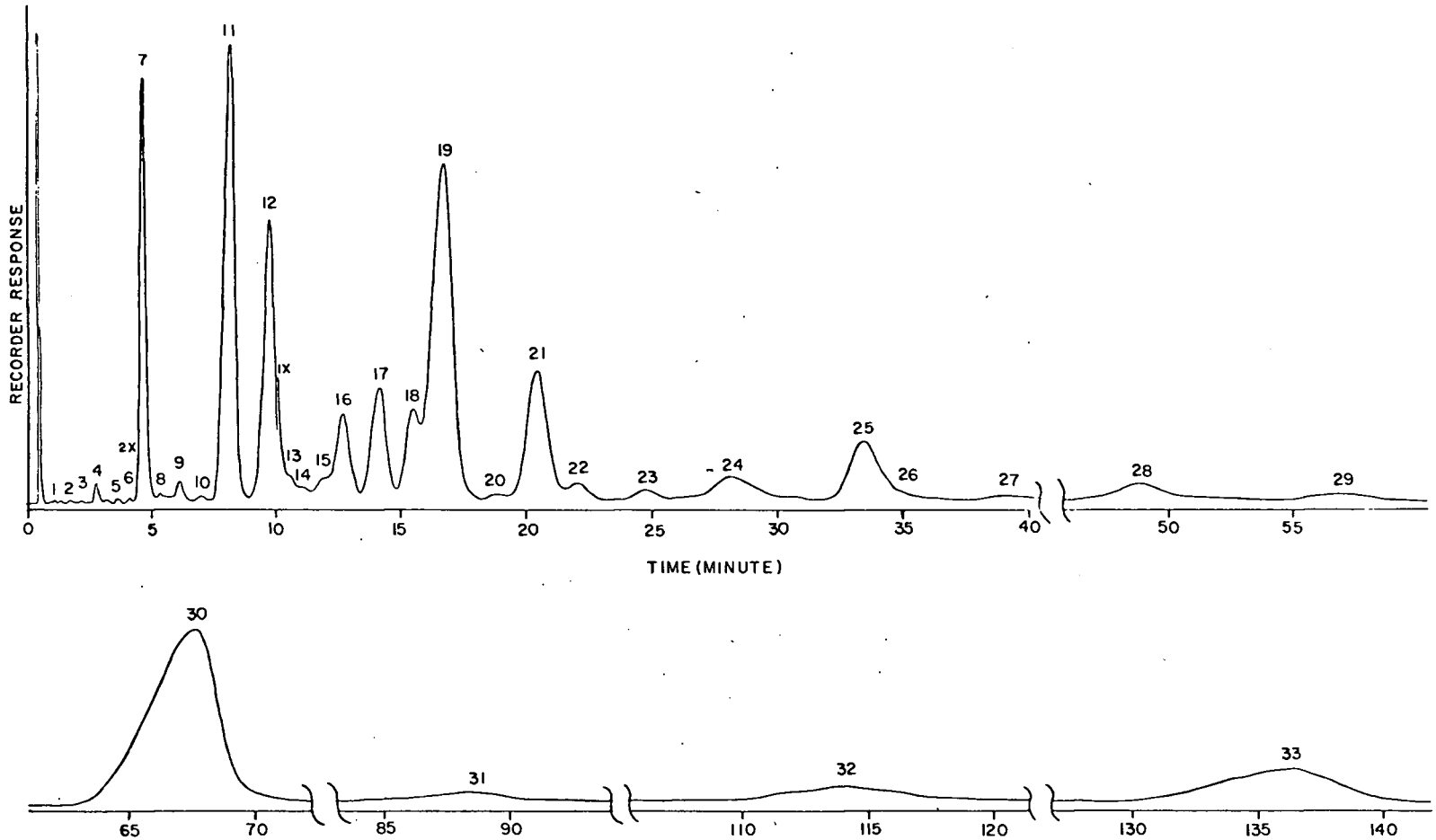


Figure 4. Gas Chromatogram of Fatty Acid Methyl Esters From Fresh Anchovy Oil

would mean either four or five double bonds. Stansby et al (66) reported this compound as 22:1. In this research (see Table 7) this compound was shown to disappear during atmospheric oxidation. This would indicate the presence of at least one 1,4-butadiene system; thereby ruling out 22:1. In the linear log retention plots, this compound fits the 20:4 position. A positional isomer of 20:4 may therefore be indicated.

Quantitative data indicates that this oil is highly unsaturated with unusually large quantities of 20:5 and 22:6. The saturated acids comprise only 29.75% of the total acids, with the monoenoic acids contributing an additional 24.16%. It is of interest to note that 35.77% of the component acids contain four or more double bonds. This explains the extreme susceptability of this oil to oxidation.

Fatty Acid Composition of Anchovy Meal Lipid

GLC analysis of the three solvent fractions from the fish meal revealed several differences in composition (Table 6). Compounds whose percent compositions varied notably were 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 22:4, 22:5, and 22:6. The most notable changes perhaps occurred in the 22-carbon, polyunsaturated fatty acids. As the extractions became more efficient, as with the Folch extraction and acid digestion, higher quantities of 22:4, 22:5, and 22:6 were found to be present in the triglycerides. This variation is significant

in that an incompletely extracted meal would contain oil which was higher in these compounds and would be quite easily oxidized. This could constitute a difficult problem in the production of an edible "fish flour" for human consumption.

Other changes can be generalized as a higher concentration of saturated acids with more complete extraction, and a corresponding decrease in the monoenoic acids. These changes do not significantly effect the properties of the oil with respect to oxidation, because both saturated and monoenoic acids are unreactive.

In general, oil extracted from the fish meal differed in fatty acid composition quite markedly from that of the fresh oil. The differences, for the most part, were typical of changes due to oxidation which occurs during preparation and storage of the meal. There was a drastic reduction in the amount of 20:5 in all the solvent extracts, as well as a reduction of 22:6 in the hexane extract. Percent compositions of 16:2, 16:3, 18:2, 18:4, 20:4, 22:4, and 22:5 were also reduced in the meal. Correspondingly, the percent composition of the saturated and monoenoic acids was higher in the meal.

Table 5. Quantitation of the Oil Extracted From Anchovy Meal and Recovered After Esterification and Column Purification

Solvent	% Oil Extracted	% Recovery After Esterification	% Recovery After Column Purification
Hexane	3.87	85.34	83.21
$CHCl_3$:MeOH (2 1)	7.80	65.08	60.22
HCl digest	1.21	77.61	67.75
Fresh Oil	100.00	98.80	97.96

Table 6. Retention Data and Percent Composition of the Fatty Acid Methyl Esters of Various Extractions of Fish Meal.

Peak No.	Compound	Hexane Extract		$CHCl_3$:MeOH Extract		HCl Digestion	
		Tr/Tr	% Comp.	Tr/Tr	% Comp.	Tr/Tr	% Comp.
1	8:0	0 04	T	0. 05	T	0. 04	T
2	sat.	0. 06	T	0. 06	T	0. 06	0. 15
3	10:0	0. 08	T	0. 09	T	0. 08	T
4	11:0	0. 12	T	0. 12	T	0. 11	T
5	12:0	0. 16	0. 24	0. 16	0. 15	0. 16	0. 31
6	sat.	0. 19	T	0. 19	T	0. 19	0. 44
7	13:0	0. 22	T	0. 22	T	0. 21	0. 15
8	sat.	0. 25	T	0. 25	T	0. 25	0. 15
9	14:0	0. 30	12. 20	0. 30	10. 16	0. 29	10. 61
10	14:0br	0. 34	T	0. 35	0. 45	0. 35	T
11	15:0	0. 40	0. 91	0 40	1. 00	0. 40	0. 96
12	sat.	0. 47	T	0. 47	T	0. 46	T
13	16:0	0. 57	25. 70	0. 56	28. 04	0. 56	32. 30
14	16:1	0. 67	14. 40	0. 67	10. 71	0. 67	8. 75
15	17:0	0. 74	0. 96	0. 73	1. 23	0. 73	1. 63
16	17:0br	0. 78	0. 15	0. 77	0. 23	0. 77	T
17	16:2	0. 83	0. 61	0. 82	0. 47	0. 82	T
18	16:2	0. 90	2. 11	0. 89	1. 73	0. 89	1. 71
19	18:0	1. 00	5. 86	1. 00	6. 89	1. 00	10. 11
20	16:3	1. 09	0. 67	1. 09	0. 80	1. 08	0. 54
21	18:1	1. 18	17. 01	1. 19	14. 83	1. 19	12. 14
22	19:0	----	----	1. 32	T	1. 33	T
23	18:2	1. 49	3. 19	1. 49	2. 20	1. 48	1. 75
24	18:2	1. 59	0. 69	1. 58	0. 64	1. 56	0. 88
25	20:0	1. 79	0. 46	1. 78	0. 55	1. 77	0. 55
26	20:1	2. 06	2. 17	2. 04	1. 70	2. 00	1. 88
27	18:4	2. 47	1. 33	2. 45	1. 02	2. 37	1. 32
28	20:2	2. 55	T	2. 57	T	----	----
29	20:3	3. 26	0. 90	3. 19	T	----	----
30	20:4	3. 67	0. 82	3. 60	0. 86	3. 50	0. 61
31	unsat.	4. 29	T	4. 25	T	4. 09	T
32	20:5	5. 00	7. 49	4. 94	7. 30	4. 76	5. 80
33	unsat.	----	----	5. 92	T	5. 41	T
34	22:4	6. 80	T	7. 17	1. 42	6. 70	T
35	22:5	8. 79	T	8. 64	0. 71	8. 31	1. 24
36	22:6	10. 38	2. 13	9. 52	6. 93	9. 75	6. 02

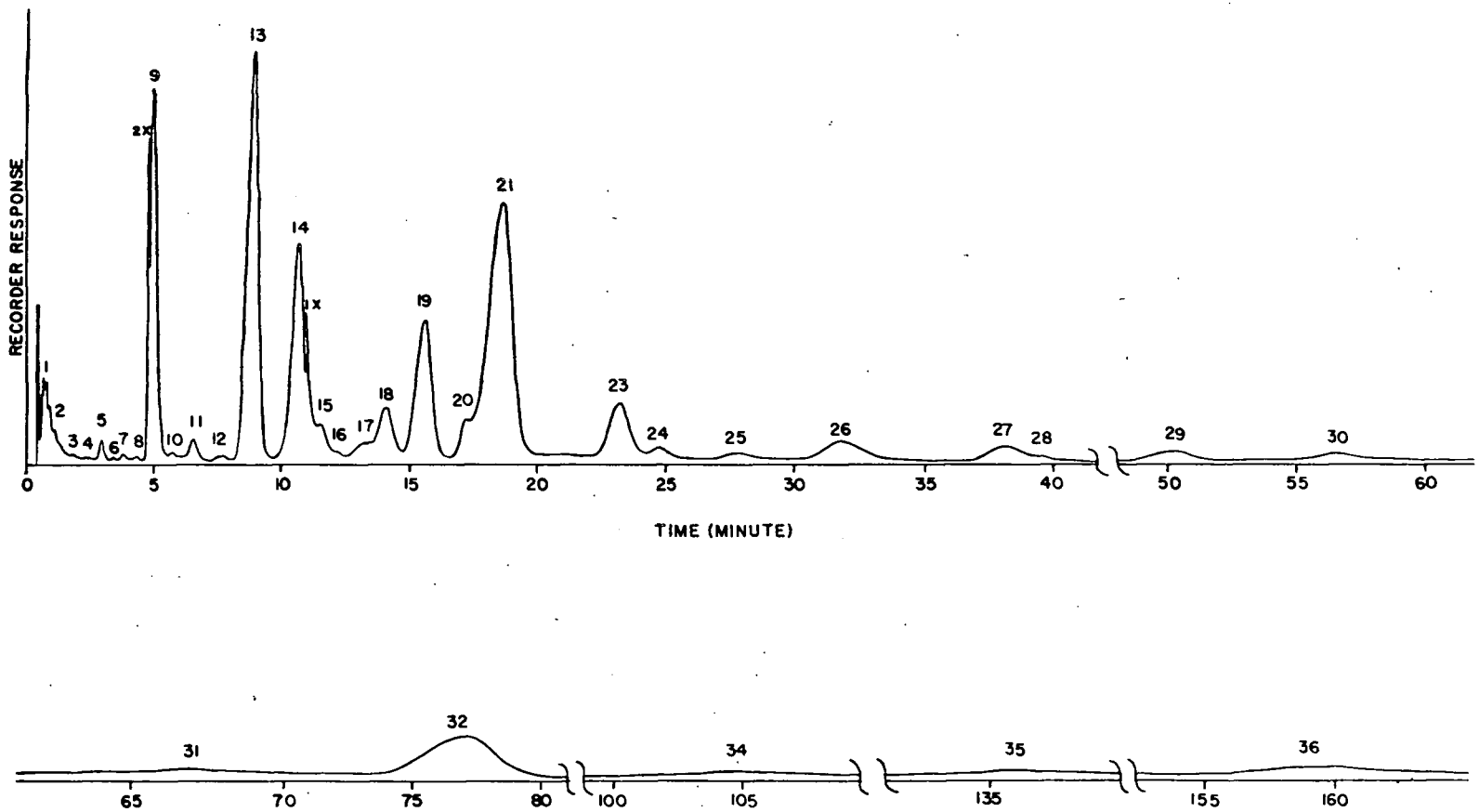


Figure 5. Gas Chromatogram of Fatty Acid Methyl Esters From Anchovy Meal (Hexane Extract)

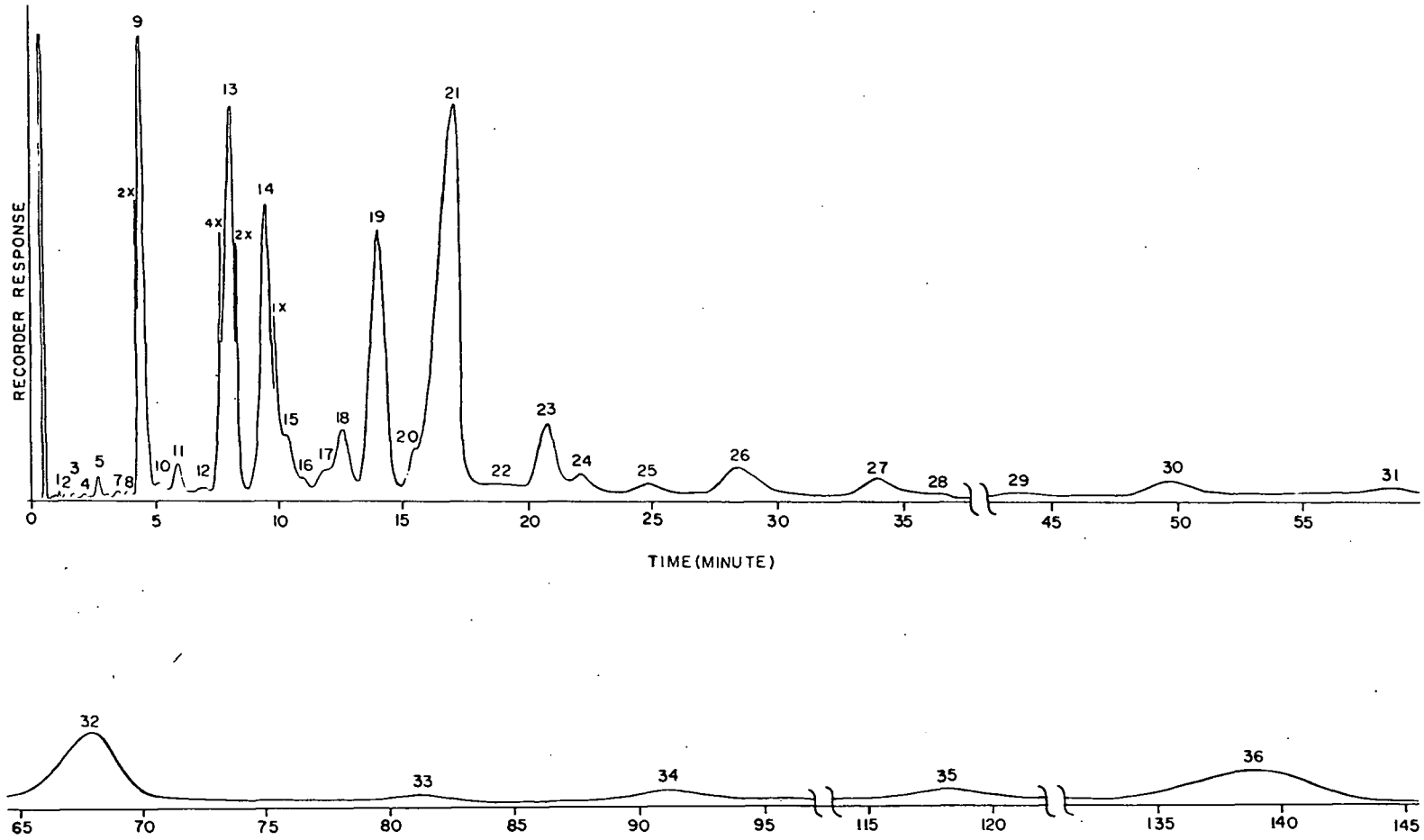


Figure 6. Gas Chromatogram of Fatty Acid Methyl Esters From Anchovy Meal ($CHCl_3$:MeOH Extract)

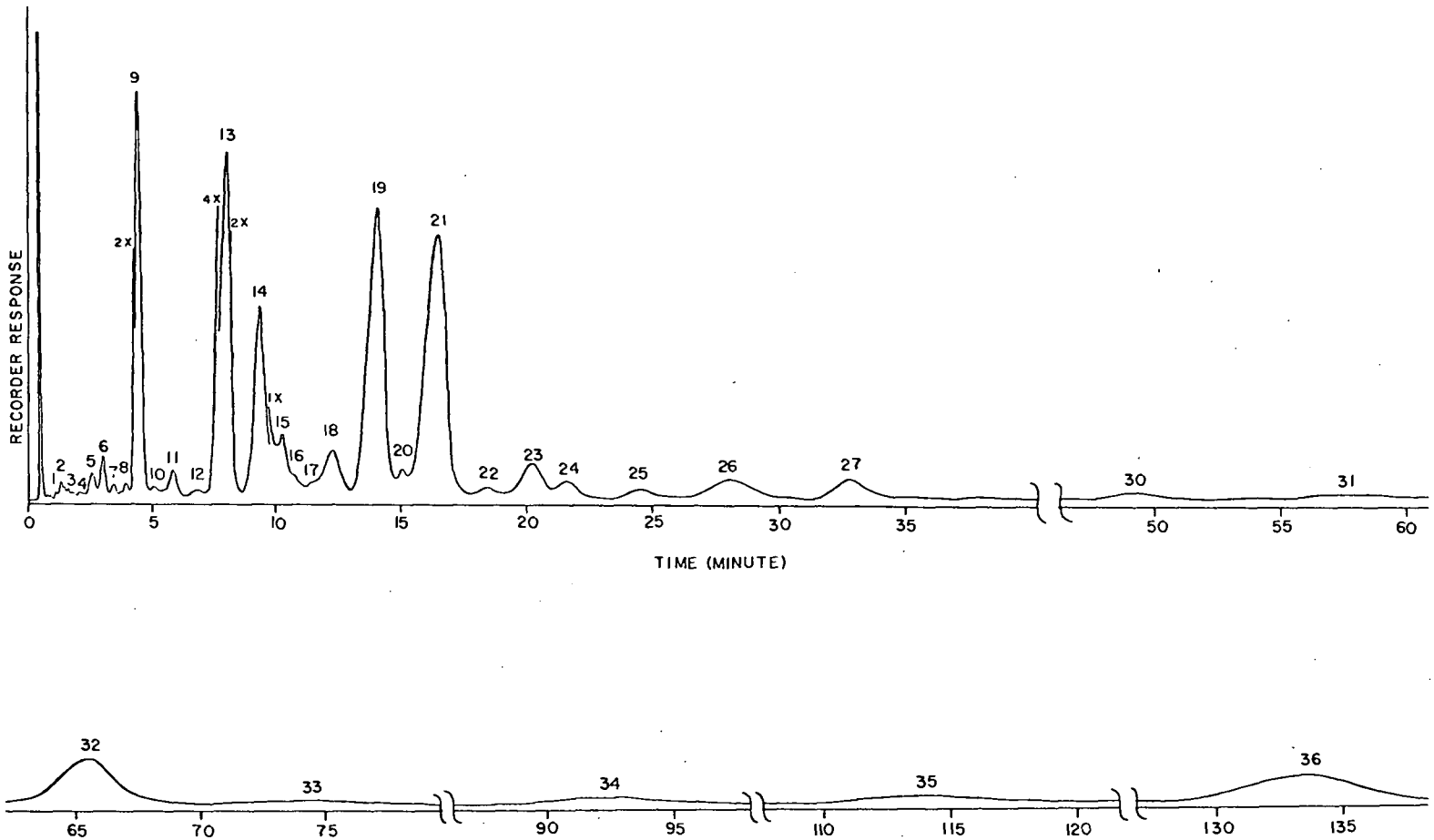


Figure 7. Gas Chromatogram of Fatty Acid Methyl Esters From Anchovy Meal (HCl Digest)

Oxidation of Anchovy Oil Methyl Esters on Lactalbumin

The oxidation of a mixture of anchovy oil methyl esters on dry lactalbumin, at a slightly elevated temperature, altered the extractability and fatty acid composition of the oil. The change in extractability was characterized by a fairly rapid reduction in hexane extractable lipid, and a corresponding increase in lipid which was soluble only in chloroform : methanol or soluble only after acid digestion. In all cases the oil extracted by hexane was of normal viscosity and nearly colorless, while all other extractions were red-brown in color and quite viscous.

As the oxidation proceeded, a gradual reduction in polyunsaturated fatty acids was observed in the hexane extract. The rate of reduction seemed to be directly related to the number of 1,4-butadiene systems present in each molecule. Under the conditions of this experiment, the changes in composition were completed after about 96 hours of oxidation. Although 22:6 was completely destroyed it is interesting to note that substantial amounts of 16:2, 18:2, 18:4, and 20:5 still remained.

The chloroform : methanol extract was found to contain primarily higher molecular weight compounds which were removed by column purification and did not show peaks in GLC analysis. The small amounts of monomeric methyl esters were nearly identical

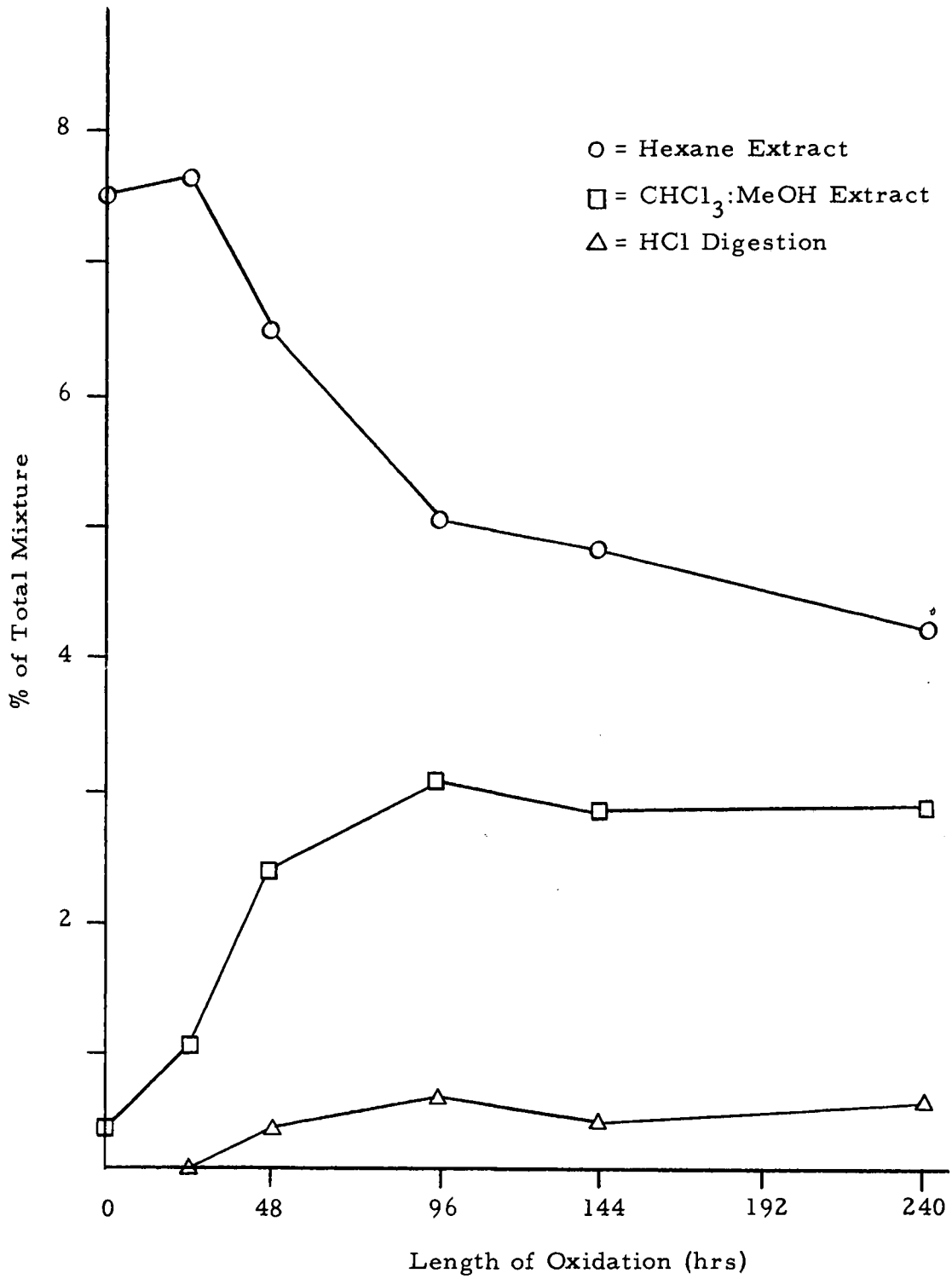


Figure 8. Change in Extractability of Anchovy Oil Methyl Esters During Oxidation on Lactalbumin

Table 7. Retention Data and Percent Composition of Anchovy Oil Methyl Esters After Various Lengths of Oxidation on Lactalbumin and Extraction With Hexane.

Peak No.	Compound	Tr/Tr	% Composition 0 hr	24 hr	48 hr	96 hr	144 hr	240 hr
1	8:0	0.06	T	T	T	T	----	----
2	10:0	0.09	T	T	T	T	T	----
3	11:0	0.12	T	T	T	T	T	----
4	12:0	0.17	0.14	0.13	0.15	0.13	0.14	T
5	sat.	0.20	T	T	0.11	T	T	----
6	13:0	0.23	T	T	T	T	T	----
7	sat.	0.27	T	T	T	T	T	----
8	14:0	0.31	8.02	8.05	10.98	11.83	10.19	4.80
9	14:0br	0.36	0.48	0.47	0.64	0.70	0.50	T
10	15:0	0.41	0.61	0.66	0.85	1.02	0.85	0.68
11	sat.	0.48	T	T	T	T	T	T
12	16:0	0.56	16.68	16.72	22.83	26.85	27.18	29.45
13	16:1	0.67	10.41	10.87	14.64	16.53	17.02	15.82
14	17:0	0.74	0.60	0.64	-.92	1.02	1.11	1.19
15	17:0br	0.77	T	T	T	T	T	T
16	16:2	0.83	0.41	0.51	0.59	T	T	T
17	16:2	0.89	2.07	2.17	2.27	2.41	2.38	2.33
18	18:0	1.00	3.05	3.25	3.79	5.24	5.43	6.35
19	16:3	1.09	2.48	2.45	1.94	1.77	1.65	1.12
20	18:1	1.19	12.13	12.88	16.50	18.72	20.12	23.84
21	19:0	1.32	T	T	T	T	T	T
22	18:2	1.46	4.80	4.84	3.70	2.89	2.83	2.80
23	18:2	1.57	0.75	0.90	0.55	0.79	0.84	0.91
24	20:0	1.78	0.41	0.43	0.50	0.41	0.46	0.43
25	20:1	2.04	1.67	1.69	1.82	2.26	2.41	2.67
26	18:4	2.42	2.91	2.83	1.97	1.37	1.31	1.53
27	20:2	2.48	T	T	T	T	T	T
28	unsat.	2.82	T	T	T	T	----	----
29	20:4	3.56	1.27	1.21	0.64	T	T	T
30	unsat.	4.17	0.62	0.49	T	T	T	T
31	20:5	4.86	20.84	19.12	11.82	6.20	6.03	6.09
32	22:4	6.50	1.00	0.80	T	T	----	----
33	22:5	8.40	2.45	2.68	0.68	T	----	----
34	22:6	9.88	6.22	6.20	2.12	T	----	----

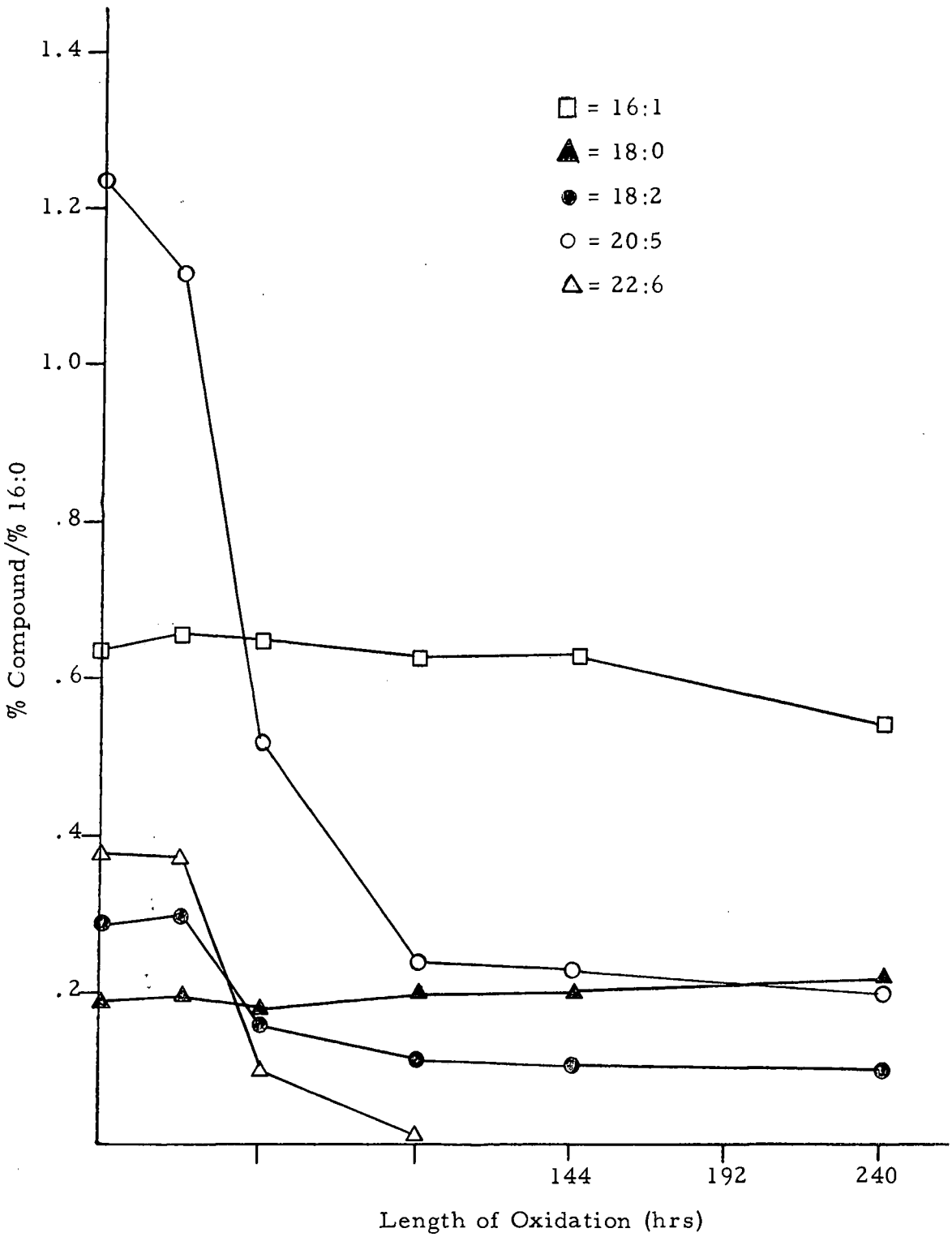


Figure 9. Changes in Composition, Relative to Palmitic Acid (16:0), of Anchovy Oil Methyl Esters Oxidized on Lactalbumin and Extracted With Hexane

in composition to the hexane extract and were therefore not reported. The HCl digested fraction consisted entirely of higher molecular weight compounds which did not show peaks in GLC analysis.

These observations strongly suggest that association of the lipid with the protein takes place only after the fatty acids have interacted to form oxygen linked dimers and polymers. This theory has been proposed by Tappel (71), and later supported by Lea et al (42) using fish meal.

Oxidation of Anchovy Oil on Lactalbumin

The oxidation of a mixture of anchovy oil and dry lactalbumin produced a change in extractability which was more immediate and severe than that observed with the methyl esters. The fatty acid composition also changed very rapidly. The observation that the reduction of fat extractability is more severe can be explained by noting that any fatty acid which becomes associated with the protein may carry two additional fatty acids linked through a triglyceride.

The fatty acid composition of the extracted oil after 24 hours of oxidation differed markedly from the composition of the fresh oil, the oxidized methyl esters, and the fish meal oil. The composition of the oil could be described as the result of extreme oxidation. The polyunsaturated fatty acids (three or more double bonds) were almost completely absent in all the solvent fractions, and

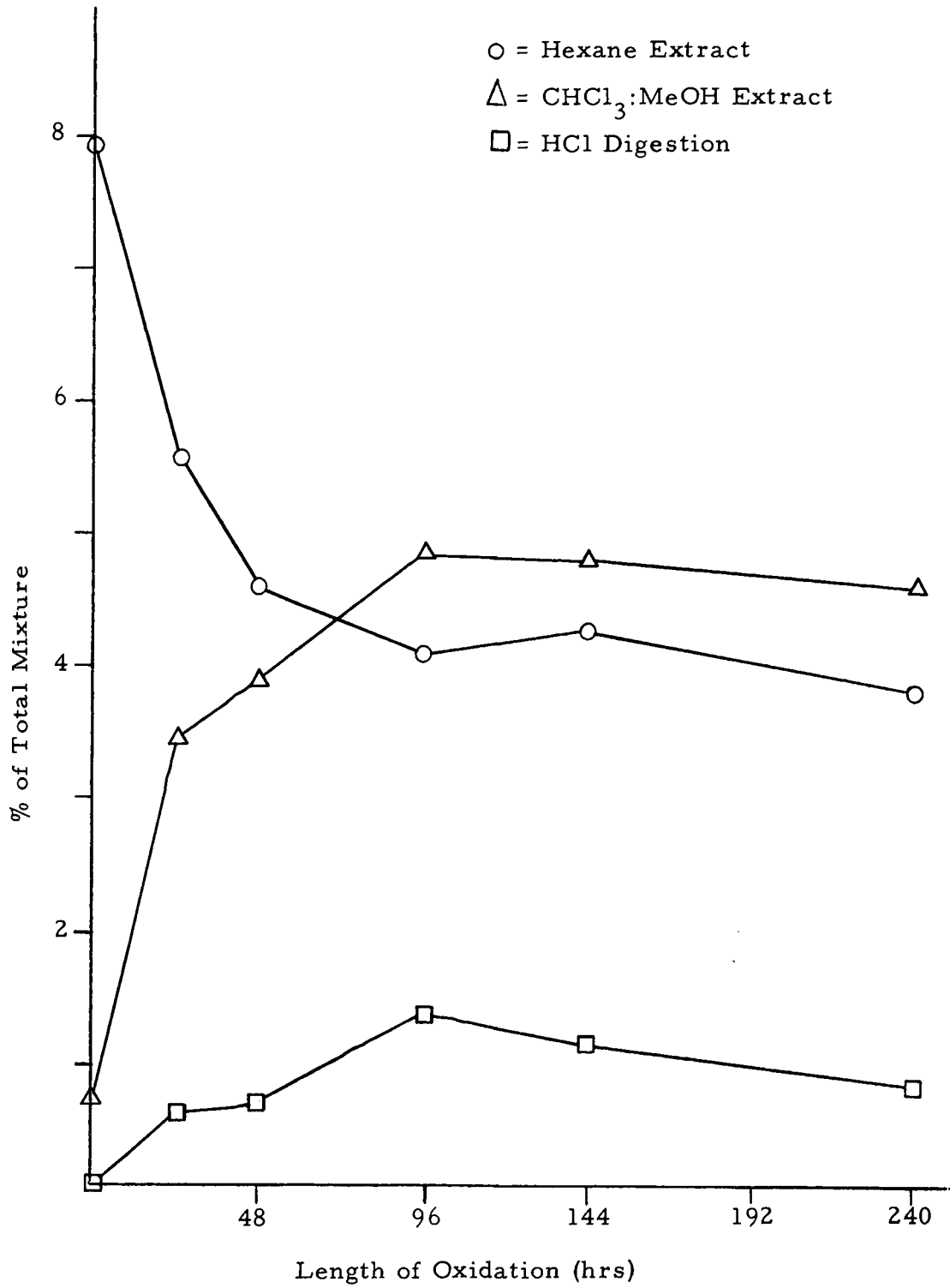


Figure 10. Change in Extractability of Anchovy Oil Oxidized On Dry Lactalbumin

large quantities of short chained acids were observed, which are not normally present in marine oils. These short chained acids could have been formed either through the direct oxidative cleavage of unsaturated fatty acids, or through the oxidation of aldehydes formed through oxidative cleavage. It must be recognized that accurate quantitative estimation of these more volatile acids was not possible due to the evaporation technique used in working up the methyl esters.

The fatty acid composition of the oxidized oil varied somewhat with completeness of extraction. The chloroform : methanol extract and HCl digest both contained considerably higher amounts of 18:2 and 18:3, but were somewhat lower in 16:1. The oxidation had proceeded too far for a good comparison to be made with the fish meal oil. There was, however, some consistancy in the observation that the more unsaturated acids were most concentrated in the least readily extractable portion of the oil.

An interesting aspect of the experiments using the model system was the extreme difference in oxidation rates observed between the oil and the methyl esters. This difference is possibly due to the removal of pro-oxidants present in the oil through the base treatment used in methanolysis.

Table 8. Fatty Acid Composition of Various Solvent Extractions of Anchovy Oil After 24 Hours of Oxidation On Dried Lactalbumin.

Peak No.	Compound	Tr/Tr	Percent Composition Hexane	$CHCl_3$:MeOH	HCl Digest
1	sat.	0.01	----	0.62	0.63
2	6:0*	0.02	0.51	0.58	0.57
3	8:0	0.04	0.38	0.61	0.60
4	9:0*	0.06	----	2.61	2.47
5	10:0	0.08	0.69	1.02	1.14
6	11:0*	0.11	T	0.21	0.19
7	12:0	0.16	0.98	1.43	1.51
8	13:0	0.21	T	T	T
9	sat.	0.25	T	T	T
10	14:0	0.30	11.73	8.06	8.03
11	14:0br	0.35	0.92	1.12	1.08
12	15:0	0.40	1.43	1.41	1.35
13	sat.	0.46	T	T	T
14	16:0	0.55	28.15	22.56	22.55
15	16:1	0.65	12.61	5.21	5.20
16	17:0	0.73	1.68	1.41	1.27
17	16:2	0.86	1.35	0.96	1.00
18	18:0	1.00	8.79	13.08	12.61
19	18:1	1.19	22.99	26.51	26.56
20	19:0	1.35	1.25	1.05	1.01
21	18:2	1.48	1.40	3.73	3.94
22	18:2	1.55	0.51	0.56	0.63
23	20:0	1.79	0.25	0.25	0.32
24	18:3	1.95	T	2.42	2.57
25	20:1	2.03	1.72	0.89	0.87
26	18:4	2.35	0.83	2.01	2.11
27	unsat.	2.80	T	T	T
28	20:4	3.45	T	T	T
29	20:5	4.80	1.78	1.69	1.80

* Identified through use of log Tr/Tr plots

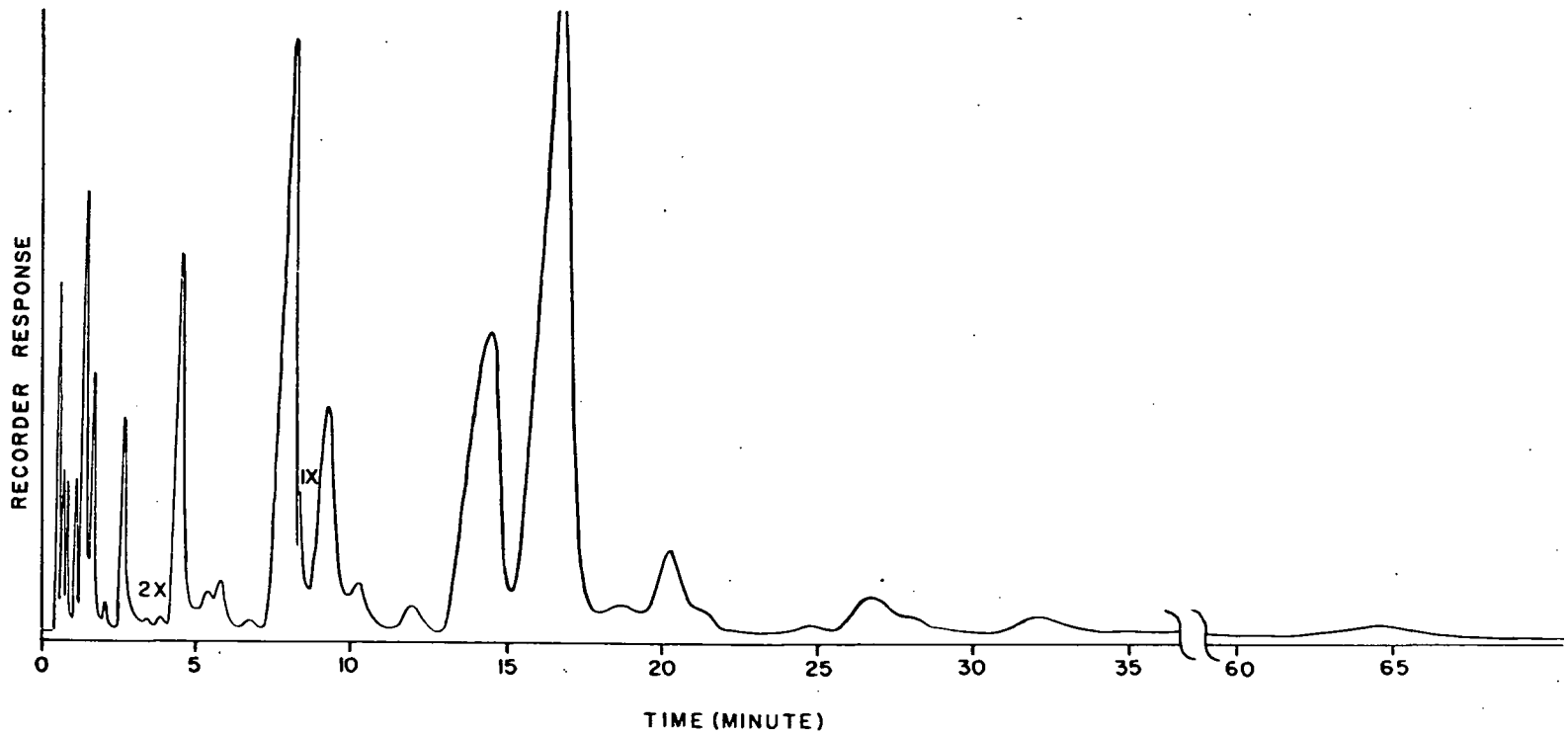


Figure 11. Typical Gas Chromatogram of Anchovy Oil Oxidized on Powdered Lactalbumin (24 Hours Oxidation; $CHCl_3$:MeOH Extract)

SUMMARY AND CONCLUSIONS

The component fatty acids of anchovy oil, and various solvent fractions of anchovy meal were examined by gas-chromatography. Model systems of anchovy oil and anchovy oil methyl esters mixed with powdered lactalbumin were oxidized and their oils also analyzed as a comparison with the fish meal.

An acid digestion procedure for the recovery of bound lipid was examined. The use of gently refluxing 4 N HCl for two hours was found to produce a maximum recovery of oil in a minimum time period with no change in fatty acid composition.

The use of a short silicic acid : celite column (8:2), equilibrated with 4% water was found to be satisfactory for removing contaminating materials such as pigments, sterols, and polymers from the methyl esters prepared in this research.

The gas-chromatographic analyses of the various oils studied revealed that:

1. HCl digestion for prolonged periods under reflux produces changes in fatty acid composition. These changes are characterized by a general destruction of polyunsaturated acids and the monoenoic acids, 16:1 and 18:1. Two saturated compounds of unknown structures are also formed. These compounds may result from the hydrochlorination of 16:1 and 18:1 either by an ionic or free radical

mechanism.

2. Stored anchovy meal contains an oil which differs markedly from its corresponding fresh fish oil. A drastic decrease in 20:5 is observed, as well as somewhat smaller reductions in 16:2, 16:3, 18:2, 18:4, 20:4, 22:4, 22:5, and 22:6. The highly unsaturated acids, 22:4, 22:5, and 22:6, are decreased mainly in the hexane extract of the meal.

3. Fish meal oil varies in composition with the completeness of extraction, with the higher unsaturated acids, 22:4, 22:5, and 22:6, being most concentrated in the least readily extractable fractions of the oil.

4. The oxidation of fatty acid methyl esters on powdered lactalbumin produces a reduction in extractability, and a typical destruction of polyunsaturated fatty acids in the hexane extractable lipid. The remaining lipid consists primarily of higher molecular weight compounds which are not detected by GLC.

5. The oxidation of untreated fish oil on powdered lactalbumin produces an extreme reduction in extractability, and rapid destruction of polyunsaturated fatty acids. The hexane extract of the oxidized oil was significantly lower in 18:2 and 18:3 and higher in 16:1.

The experiment using anchovy oil methyl esters on powdered lactalbumin leads to the conclusion that the oxidized oil becomes

associated with the protein only after reacting with itself to form oxygen linked dimers and polymers. Because of the large reduction in the amounts of 20:5 and 22:6 which occurs during oxidation, it may be assumed that these two fatty acids are largely responsible for the formation of the lipid-protein complexes.

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