COMMERCIAL FISHERIES REVIEW

November 1958 -Supplement Washington 25, D.C. Vol. 20, No. 11a

THE NONSAPONIFIABLE FRACTION OF MENHADEN OIL

William A. Mosher, Wiley H. Daniels,
Jack R. Celeste, and William H. Kelley*

ABSTRACT

Methods of separating the nonsaponifiable portion from menhaden oil were evaluated, and procedures for fractionating this portion then were investigated. In this latter work, three principal fractions were obtained: (1) squalene, (2) cholesterol and other steroids, and (3) color bodies.

INTRODUCTION

A fundamental study of the chemical identity of the components of fish-body oils was one of the primary objects of the contractual research program started by the U. S. Bureau of Commercial Fisheries. One phase of this study was to determine the nature of the components in the nonsaponifiable fraction of menhaden oil. Chemical studies on samples of the body oil of menhaden were conducted at the Chemistry Department of the University of Delaware for a period of two years.

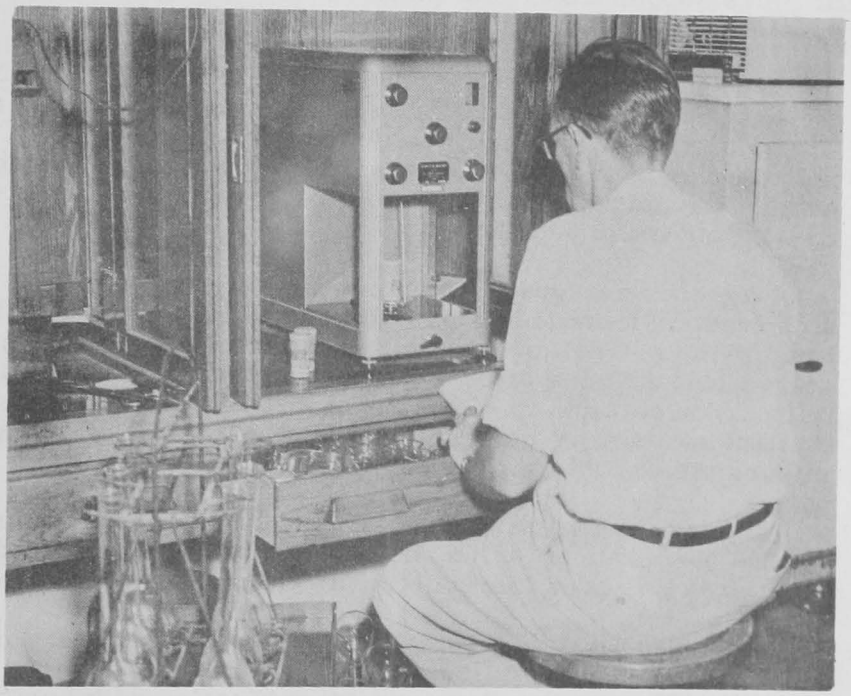

Fig. 1 - Various components of the nonsaponifiable portion of menhaden oil are quantitatively determined after selective fractionation.

EXPERIMENTAL AND RESULTS

SEPARATION OF NONSAPONIFIABLE PORTION FROM OIL: Initially, several methods of separation of the nonsaponifiable portion of the fish oils were studied. These were (1) saponification with barium hydroxide, aqueous potassium hydroxide, and methanolic and ethanolic potassium hydroxide; (2) acid hydrolysis by means of the "Twitchell" reagent, benzenestearosulfonic acid, and emulsifier that permits the acid hydrolyzing agent to contact the oil intimately; (3) enzymatic hydrolysis by the use of a lipase present in Ricinus castor bean. The same chemical products were obtained by the three methods used to separate the nonsaponifiable portion. This

*Researchers. The research reported in this paper was conducted at the University of Delaware, Department of Chemistry, under contract with the U. S. Bureau of Commercial Fisheries. It was financed by funds made available under provisions of Public Law 466, 83rd Congress, approved July 1, 1954, generally termed the Saltonstall-Kennedy Act. This article was prepared by Dr. Donald G. Snyder, Biochemist, Fishery Technological Laboratory, U. S. Bureau of Commercial Fisheries, College Park, Md., from progress reports submitted by the contractor to the Bureau.

identity of products indicates that the nonsaponifiable portion is relatively stable to acid, neutral, and strongly basic solutions.

EXPERIMENTAL AND RESULTS

SEPARATION OF NONSAPONIFIABLE PORTION FROM OIL: Initially, several methods of separation of the nonsaponifiable portion of the fish oils were studied. These were (1) saponification with barium hydroxide, aqueous potassium hydroxide, and methanolic and ethanolic potassium hydroxide; (2) acid hydrolysis by means of the "Twitchell" reagent, benzenestearosulfonic acid, and emulsifier that permits the acid hydrolyzing agent to contact the oil intimately; (3) enzymatic hydrolysis by the use of a lipase present in Ricinus castor bean. The same chemical products were obtained by the three methods used to separate the nonsaponifiable portion. This identity of products indicates that the nonsaponifiable portion is relatively stable to acid, neutral, and strongly basic solutions.

Of the methods studied, only the base-catalyzed hydrolysis of the fatty-acid esters present in menhaden oil was technically promising. The recovered oils from several hydrolyses were fractionally distilled and crystallized, but good isolation of compounds could not be attained. Separation by chromatography on alumina apparently was a more satisfactory and precise method.

The saponification of the oil for one hour with alcoholic potassium hydroxide gave the best results and the cleanest handling material. Several large batches of oil were saponified by this method. An average of 1.68 percent total materials unsaponified by potassium hydroxide was obtained.

FRACTIONATION OF NONSAPONIFIABLE PORTION: Solvent extractions of the unsaponifiable portion indicated that cholesterol and other sterols were present. The total steroid fraction was determined to be about 15 percent of the unsaponifiable portion of the oil, and the squalene fraction, to be about 23 percent. The balance of the nonsaponifiable portion was determined by chromatographic methods to consist of "color bodies."

The chromatographic method consisted of freeing a solution of the unsaponifiable fraction from solids by chilling and then concentrating the fraction by evaporating off the solvent on a steam bath. The ethereal extracts were dried and redissolved, and suitable aliquots were made for adsorption analyses on columns of alumina. The solution then was developed with petroleum ether. By this method, three distinct zones were noted: (1) bright yellow, (2) pink, and (3) faint yellow. Other eluates yielded varying bands of colors.

During the course of this study, it was noted that the nonsaponifiable fraction of the oil had a rancid, acrid odor entirely different from that of the whole oil. It was found that this odor increased with age owing to autoxidation.

As a result of further studies, a very satisfactory method was perfected for isolating the nonsaponifiable fraction of menhaden oil in large-scale laboratory apparatus. The process involved saponification with potassium hydroxide in water-ethanol for less than an hour. A continuous liquid-liquid extractor permitted convenient extraction of the nonsaponifiable fraction from the soap solution with ethyl ether. These large-scale saponifications yielded about 1 percent unsaponifiable material.

The steroid fraction was found to be about 90 percent cholesterol when determined by fractional crystallization from acetone and ethanol (2:3 v/v) and chromatographic adsorption on an alumina column. About 10 percent of the nonsaponifiable fraction was determined to be a concentrated fraction of color bodies. This

fraction was separated by adsorption analysis on an alumina column into three distinct oily fractions, which varied from golden yellow to red-brown.

In an effort to determine the best chromatographic method of separating the unsaponifiable fraction of menhaden oil preparatory to identifying the various fractions, numerous trials were conducted with relatively small columns, using various adsorbents and solvents. The adsorbents used were alumina, barium carbonate, silicic acid, and magnesium trisilicate. Solvents investigated with these adsorbents included petroleum ether, hexane, benzene, dichloromethane, ether, ethanol, ethyl acetate, and acetone. In many of the trials, the results obtained from chromatographic analysis with the various adsorbents and solvents could not be duplicated exactly; and in some trials, the results appeared contradictory. This lack of precision indicated that the original materials were changing in composition before or during the chromatographic procedures. Also, it was observed that fractionation of the nonsaponifiable components from menhaden oil was complicated by autoxidative changes during saponification and crystallization. These changes occurred even when special precautions were taken. The characteristic absorption spectra of several fractions were completely destroyed, for example, when the process was exposed to air. Thus, an investigation was attempted to separate chromatographically the entire nonsaponifiable fraction without the preliminary fractionation. A complete retention of spectral characteristics was obtained only when chromatographic experimental procedures were conducted under nitrogen.

Results from the initial study indicated no sharp separation of the steroid materials from the color body or carotene fraction, but the odor was reduced markedly. Squalene, however, was readily separated by this procedure. During the course of this work, it was found that a hydrocarbon and oxyginated fraction were obtained by partitioning the carotenoids between petroleum ether and methanol (90 percent).

SQUALENE: A satisfactory chromatographic technique was selected for separating the squalene from the whole nonsaponifiable fraction. The squalene was fractioned from petroleum ether on a column of alumina. The crude material was separated by the formation of hydrochlorides into three isomers that were found to be identical with squalene. The squalene was determined quantitatively in the eluates by iodometric titration procedures after chromatography on alumina. The percentage of squalene in the unsaponifiable fraction was determined to be about 5 percent.

STEROL: Attempts to isolate an impurity obtained previously in the sterol fractions were rather unsuccessful. Crude sterol fractions obtained from two recrystallizations were chromatographed on a column of alumina, and arbitrary eluant fractions were collected from benzene and methanol. Small amounts of impurity were obtained by this procedure. It was possible, however, that at least a part of the material considered as an impurity may have been squalene that was occluded in the crystalline mass of the crystallizing cholesterol. This possibility was likely, since both squalene and the impurity were eluted very early from the columns of alumina while one of the later arbitrary eluant fractions contained cholesterol of very high purity. The fraction that contained the very pure cholesterol was eluted with methanol and did not represent a 100-percent recovery of total cholesterol.

An attempt was made to determine total cholesterol in the unsaponifiable matter by precipitating gravimetrically the cholesterol with digitonin. Digitonin forms a molecular complex with cholesterol in the molecular proportion of 1 to 1. In this procedure, 100 grams of menhaden oil was saponified, and the unsaponified material was collected after drying under a hot stream of carbon dioxide. This material was dissolved in ether, and a small aliquot of the ether solution was dissolved further in ethanol. The digitonin was added next, and the resultant precipitant was dried to constant weight. By this method, the content of cholesterol in the unsaponifiable matter was determined to be somewhat more than 25 percent.

As was mentioned before, the cholesterol fraction could not be separated entirely from the color bodies or pigment fraction. At this time, other procedures were attempted to afford this separation. Columns of aluminum silicate were used with hexane, benzene, and methanol as developers. As it was determined, however, that all of the eluant fractions contained some cholesterol, the aluminum silicate was considered to be nonselective. No further work was attempted on the complete separation of the sterol and pigment fractions by chromatographic techniques.

COLOR BODIES: All further work consisted of trying to identify the pigment fractions of the unsaponifiable material of the oil. A sample of the menhaden oil was saponified, and the unsaponified matter was partitioned between petroleum ether and methanol. The petroleum ether layer was dried over anhydrous sodium sulfate, and the solvent was by a Renco solvent evaporator. The residue then was dissolved in petroleum ether and chromatographed in a column containing a 1 to 1 mixture of magnesium oxide and celite. By this method, no colored zones or bands were detected visually, but two bands were detected under ultraviolet light. These bands were eluted from the column with petroleum ether. The solvent then was removed, and the solid material was dissolved in petroleum ether as before. The spectra of these fractions indicated that more than one substance was present in each fraction.

In an attempt to isolate the individual substances in each fraction, the first fraction from above was rechromatographed on a column of alumina and developed with petroleum ether followed by a 50-percent mixture of petroleum ether and benzene. No visible results were observed when either of these solvents was used. Methanol next was used as a developer, and two bands were developed. One of these bands remained adsorbed on the column, and one eluted. The results from an analysis of the spectra of each band indicated that the materials still were not pure.

The band eluted from the column therefore was chromatographed on another column composed, in this case, of calcium hydroxide and was developed with petroleum ether. A narrow band was noted that separated from the bulk of the material and that was eluted from the column. Continued development with petroleum ether effected elution of the other band. A spectral analysis of the two bands indicated that the materials still were not pure. The band that initially was eluted from the calcium hydroxide column therefore was chromatographed in a column of 50-percent celite and magnesium and was developed with petroleum ether. One band was eluted, the solvent was removed, the residue was dissolved in n-hexane, and the visible spectrum was determined. The adsorption peaks obtained corresponded to the absorption peaks reported for alpha-carotene.

Testing the material dissolved in anhydrous chloroform with antimony trichloride resulted in the development of a blue color as is reported for alpha-carotene. The band that was eluted later from the calcium hydroxide column was treated in the same manner as was the first band that was eluted. Examination of the spectra of this material indicated absorption peaks that corresponded to peaks reported for gamma-carotene. The antimony trichloride test with this material also was positive. Thus, the presence of alpha and gamma carotene was indicated in the unsaponifiable material of menhaden oil. The second fraction that was chromatographed on the magnesium oxide-celite column and detected by ultraviolet light was not studied.

The material obtained in the methanol or hypophase during partition of the unsaponifiable matter between methanol and petroleum ether was investigated next. The solvent was removed and the residue was dissolved in petroleum ether. The solution then was chromatographed in an alumina column, with the use of petroleum ether as a developer. This step resulted in the development of six zones. The first four zones were close together and were removed physically from the column. These zones were eluted from the column with methanol, the solvent was evaporated, and the residue was dissolved in benzene. The residue dissolved in benzene was developed chromatographically on alumina. Five zones were found of varied colors.

Further development with benezene resulted in the elution of the two lower zones. The zones that remained were developed with a 20-percent acetone-benzene solution. The top zone, a yellow one, eluted but lost color on standing for several days. The second zone above split into a faintly yellow zone that remained and a yellow zone that moved rapidly with the developer. The rapidly moving zone was isolated. The spectrum obtained compared fairly well with the spectrum of xanthophyll in petrol and in hexane.

Continued development with 20-percent acetone-benzene resulted in the elution of a heavy yellow zone. No separation was obtained when this zone was rechromatographed in an alumina column with the use of benzene as a developer. When 5-percent acetone-benzene was used, however, five zones were developed. The fifth zone was eluted by further development with 5-percent acetone-benzene. The solvent was removed and replaced with n-hexane. The spectrum obtained compared fairly well with violaxanthin in petrol. The fourth zone also was eluted fairly easily with the same developer. This zone was not studied.

Further development for 2 days with the same solvent system resulted in the union of zones 3 and 2. When this united zone finally eluted, the fraction was arbitrarily split into two. Neither of these fractions was identified.

After this long period of development, the remaining zone on the column was split into three zones. The column then was extruded physically, and the three zones were separated and eluted separately with methanol. The spectra of the two lower zones corresponded very closely to that of zeaxanthin. The top zone and the various other zones obtained during the complete chromatographic procedure were not identified.

During the course of this phase of the study, it was suspected that the introduction of bulky groups into the carotenoid molecules would facilitate crystallization of the carotenoid molecules and thus afford a purer sample for chromatography. For this reason, the carotenoid molecules were acetylated. These acetylated carotenoids then were developed on alumina with various solvents, but none of the fractions obtained was crystalline in nature. Violaxanthin and xanthophyll again were indicated in certain fractions, but the absorption maximums apparently were not affected by acetylation.

At this time an attempt was made to remove the cholesterol by a direct-adsorption process utilizing "Florisil," 1/ a magnesium silicate adsorbent. This attempt was not successful.

SUMMARY

Chemical studies were conducted with samples of menhaden oil to determine the nature of the components in the nonsaponifiable fraction of the oil. Initially, three separate methods of hydrolysis were investigated to determine the best method of obtaining the nonsaponifiable fraction of the oil. Of these, alkaline hydrolysis of the oil with alcoholic potassium hydroxide was assessed as giving the best results and the cleanest handling material. By this method, nearly 2 percent of unsaponifiable material was obtained.

The unsaponified material was fractionated into three groups: (1) squalene, (2) cholesterol and other steroids, and (3) pigments. These three fractions comprise nearly 75 percent of the total nonsaponifiable fraction of the oil. The balance of the unsaponifiable material was in the form of oily substances that were not characterized.

The squalene amounted to about 10 percent of the nonsaponifiable fraction of the oil. Squalene may readily be separated from the fraction by chromatography

1/ Floridin Co., Tallahassee, Fla.

from petroleum ether on columns of alumina. The crude material was separated by the formation of hydrochlorides into three isomers that proved to be identical with ordinary squalene. The squalene was determined quantitatively by an iodometric titration procedure.

The steroid fraction amounted to about 30 percent of the nonsaponifiable matter. Results obtained by gravimetrically precipitating the cholesterol with digitonin indicate that the cholesterol comprises about 90 percent of the steroid fraction. A second sterol apparently is present as a minor constituent, but it could not be identified. Crystallization of the cholesterol from methanol resulted in an impure fraction, whereas chromatographic separation on alumina with methanol resulted in a very pure fraction. Several attempts were made to separate by chromatography the entire nonsaponifiable fraction directly without preliminary fractionation, but the cholesterol fraction could not be entirely separated from the color body or pigment fraction. Odor was reduced markedly, however, and color and the stability of color were improved by adsorptive fractionation.

The remaining nonsaponifiable portion of the oil was the color-body or pigment fraction. This fraction consists of a very complex mixture of substances of fairly high molecular weight. Most of these are the carotenes. The following were tentatively identified: alpha and gamma-carotene, zeaxanthin, violaxanthin, xanthophyll, and two oxygenated carotenes of undetermined structure. These materials can be separated by adsorptive fractionation procedures. The carotene pigments probably contribute to much of the color in menhaden oil and to the poor stability of the color.

During the course of this study, it was noted that the nonsaponifiable fraction of the oil had a rancid, acrid odor that increased with age, owing to autoxidation.

RED SNAPPER

The red snapper (Lutjanus aya) is a deep-water fish (found at depths of 20-60 fathoms) and concentrated in relatively confined areas. Snappers usually school a few feet off the sea bottom, but have been observed to surface. It is believed that they remain in one locality for considerable lengths of time. The sea floor over which the fish are found commonly consists of patches of hard limestone covered with live corals and grass. The number of such habitats is relatively small. Irregular bottom formations, such as depressions or elevations and folds, appear to be preferred habitats for red snappers. The better known red snapper fishing spots in the northern Gulf of Mexico are off Ft. Walton, Carrabelle, and Pensacola in Florida, and off the Texas coast.

--The Marine Laboratory,
University of Miami,
Coral Gables, Fla.