The free fatty acid content was the determining variable in this series. The aged samples had generally less lipoxidase than the fresh samples. The two aged flours showed the highest tocopherol content, but the aged semolina and flour contained severalfold more free fatty acids and correspondingly lower pigment stability.

The results of the experiments reported here indicate the importance of the amount of free fatty acids (chiefly linoleic) of wheat and its milled products upon the oxidative stability of the carotenoid pigments. This is not surprising, since ordinary oxidative rancidity of many fat-containing substances is accelerated by accompanying hydrolytic rancidity. Free fatty acids are more rapidly autoxidized than the esterified form of triglycerides. It would appear that free fatty acids constitute a prime substrate for wheat lipoxidase action.

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FISH OIL ODORS

Volatile Acids from Menhaden Oil

J. R. CHIPAULT and EVELYN McMEANS

The Hormel Institute, University of Minnesota, Austin, Minn.

The acidic constituents of a highly volatile fraction collected during molecular distillation of menhaden oil have been examined by paper chromatography. Tentatively identified were formic (or acetic), acrylic, propionic, crotonic, butyric, and valeric acids, and an unknown compound with a polarity greater than formic acid but less than pyruvic or lactic acids.

Oils carefully rendered from fresh fish tissues are relatively bland or possess, at most, a mild, not unpleasant odor. The strong, undesirable odors usually associated with marine oils are caused mainly by the use of tissues in various stages of decomposition and by deterioration of the oil after it has been prepared. The first factor gives dark oils containing relatively large quantities of nitrogen and with odors of a putrid character. The second effect is associated with autoxidation of the highly unsaturated fatty acids of the oil.

The great complexity of the carbonyl compounds that can be isolated from fish oils by various techniques has been reported (*14, 19, 20*). Furthermore, fishy odors and flavors have been detected in fats of nonmarine origin, such as soybean oil, beef tallow, butter, and other dairy products. Under certain conditions, fishy flavors can be reproducibly developed in butter (*13*). According to Forss *et al.* and Stark and Forss, the fishy flavor of butterfat results from a combination of compounds responsible for an oily flavor (*n*-hexanal, *n*-heptanal, and hex-2-enal) (*7*) and a metallic flavor (oct-1-en-3-one) (*16*). However, other workers (*4, 6, 10*) have isolated carbonyl compounds identical or very closely related to those of Forss *et al.* from butterfat which was oxidized but not considered fishy.

On the other hand, Davies and Gill (*3*) concluded that fishiness developed when highly unsaturated fatty acids and tertiary nitrogen compounds were present together under autoxidizing conditions. The concept that tertiary nitrogen is necessary for fishiness to occur is supported also by Stansby (*15*) and by observations that trimethylamine, triethylamine, and even ammonia, at low concentrations, have odors akin to those encountered in products with a mild degree of fishiness. Chang *et al.* (*2*) also consider that the fishy odor of hydrogenated soybean oil is due to noncarbonyl compounds.

These observations made it desirable to examine the volatile material obtainable from fish oils for compounds other than the carbonyls which are usually associated with the odors of oxidized fats. This report describes the acidic volatile compounds isolated from menhaden oil.

Experimental

Starting Materials. The highly volatile fraction used in this study was accumulated in a cold trap maintained at dry ice–acetone temperature during fractional molecular distillation of 5 gallons of cold-pressed menhaden oil. After a degassing period at 500 microns and 60° C., six distillate fractions were collected at a pressure of 17 to 18 microns and temperatures between 190° and 212° C.

A total of 25 ml. of volatile compounds was collected during the distillation of 5 gallons of menhaden oil. When the fraction was melted, it separated into a nearly colorless lower aqueous layer with a volume of approximately 8 ml. and 17 ml. of a dark upper organic phase.

Examination of Total Volatiles. Both aqueous and organic phases had similar sharp unpleasant odors which were described by four observers as a combination of putrid, slightly fishy, and very strongly rancid. Qualitative tests indicated that both layers contained acids and carbonyls, but a Kjeldahl determination showed that the lower aqueous layer contained more than 15 times the concentration of nitrogen found in the upper layer. Spot tests for amines were positive for the lower phase, but negative for the upper organic layer. Tests for sulfhydryl groups (*5*) were negative for both layers. As a result of these observations, it was decided to examine the lower aqueous layer more thoroughly.

Separation of Acids from Aqueous Phase. A 1-ml. portion of the aqueous layer was neutralized to a pH of 10 (pH paper) with 0.1*N* NaOH (6.9 ml.).

Table I. R_f Values of Volatile Acids of Menhaden Oil

Unknown Volatile Acids	No. of Chromatograms	R_f Value	Std. Dev.	Known Standard	No. of Chromatograms	R_f Value	Std. Dev.
1	6	0.13	0.010	Formic	3	0.21	0.010
2	6	0.20	0.013	Acetic	5	0.22	0.015
3	4	0.31	0.005	Acrylic	3	0.32	0.007
4	6	0.36	0.009	Propionic	5	0.35	0.010
5	6	0.45	0.016	Crotonic	3	0.44	0.010
6	6	0.51	0.016	Butyric	5	0.49	0.010
7	3	0.61	0.007	Valeric	5	0.64	0.018

The neutralized solution was transferred to flask *A* (Figure 1), which was then placed in a dry ice–acetone bath at −78° C. while the system was evacuated to a pressure of 50 microns. Stopcock *C* was closed and the dry ice bath was removed from flask *A* and placed around condensing tube *B*. Distillation was allowed to proceed overnight at room temperature. The next morning flask *A* was immersed in a bath at 50° C. for 1 hour to ensure complete removal of the neutral and basic volatile components which were condensed in tube *B* and studied separately.

Examination of Volatile Acids. The sodium salts of the volatile fatty acids remaining in flask *A* were dissolved in a few microliters of water and chromatographed on strips of Whatman No. 1 filter paper, using an ascending technique at room temperature.

The solvent used was the upper layer resulting when a mixture of 30 parts of propanol and 30 parts of butanol was thoroughly mixed and equilibrated with 20 parts of the ammonia buffer solution used by Kalbe (*8*) for separating dicarboxylic acids on paper. After 20 hours in a chromatographic jar, the papers were dried thoroughly in air at room temperature and dipped in a ninhydrin indicator solution (200 mg. of ninhydrin and 50 mg. of ascorbic acid dissolved in 100 ml. of absolute ethanol), as suggested by Burness and King (*1*). The dipped papers were allowed to dry at room temperature and the acids appeared within 1 hour as purple spots on a white background. However, the chromatograms were not stable and the background gradually darkened, so the acid spots became invisible after about 3 additional hours. Heating of the chromatograms in an oven at 100° C. hastened appearance of the acid spots but resulted, also, in a more rapid darkening of the background.

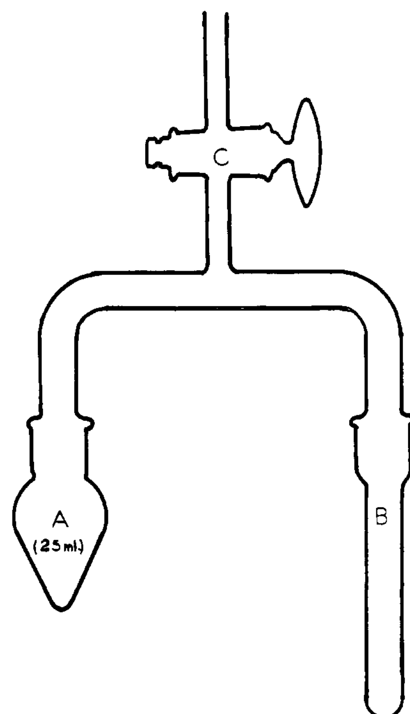

Figure 1. Apparatus for isolation of volatile acids from menhaden oil

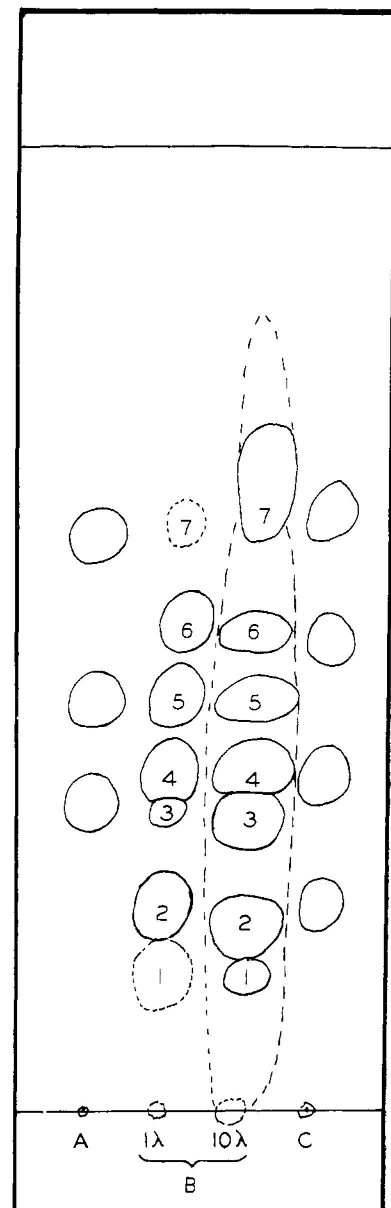

Figure 2. Paper chromatography of volatile short-chain acids

A. Acrylic, crotonic, and isovaleric acids
B. Volatile acids from menhaden oil
C. Acetic, propionic, butyric, and valeric acids

Results and Discussion

Several difficulties have been reported during paper chromatography of volatile fatty acids of low molecular weight as their nonvolatile alkali salts. The appearance of ghost spots due to impurities normally present in filter paper was eliminated by Kennedy and Barker (*9*) by thoroughly prewashing the paper. They treated the solution of the sodium salts of the fatty acids with ammonium sulfate to minimize interference, by sodium cations, with detection of the fatty acids of lower molecular weight. With the solvent system and indicator solution used here, these procedures became unnecessary, since no ghost spot was detected at any time and the sodium ions appeared as a compact yellow spot which remained very close to the base line and did not interfere with any of the fatty acids present.

Figure 2 is a tracing of a typical chromatogram of the volatile acids of menhaden oil compared with known standards. Seven spots were obtained when 1 to 10 μl. of solution were used. Larger amounts showed no additional spots but resulted in nearly complete merging of spots 1 with 2 and 3 with 4, and in considerable streaking of spot 7. With amounts less than 1 μl., spots 1 and 3 became somewhat better defined (although weaker), but spot 7 disappeared completely.

Table I shows average R_f values and standard deviations calculated from three to six chromatograms. The values for formic and acetic acids are almost identical and, when present together, these two compounds are not separated from each other. This is in agreement with the findings of many other workers, who failed to separate them with a number of solvent systems. Mukerjee (*12*), however, reported the separation of formate from acetate with methanol or a mixture of methanol and acetone containing 1 part per hundred of 30% ammonium hydroxide. For the present authors, however, these solvents failed to separate these two acids. Burness and King (*1*) pointed out that, with the exception of ammonium formate, the ammonium salts of the other fatty acids of low molecular weight require the presence of a reducing agent to react with ninhydrin, and ascorbic

acid has been added to the ninhydrin indicator solution for this purpose. Formate, however, acts as its own reducing agent and can be detected with a ninhydrin solution free from added reducing compounds, thus providing a means of differentiating it from acetic acid.

This property of formic acid was confirmed with known mixtures containing either formate or acetate. However, when a chromatogram of the unknown volatile acids from menhaden oil was dipped in the ninhydrin solution free from ascorbic acid, all seven spots appeared, indicating that some reducing impurities were carried along all through the chromatogram.

Spot 1, with an R_f value of 0.13, is unknown. It is somewhat more polar than formic or acetic acid, but less so than keto (pyruvic) or hydroxy (lactic) acids which do not migrate from their point of application with the solvents used here. The other spots correspond, respectively, to formic (or acetic), acrylic, propionic, crotonic, butyric, and valeric acids.

The presence of these acids in menhaden oil is easily explained through autoxidation of aldehydes which, in turn, result from degradation of hydroperoxides. Loury (*11*) has suggested that aldehydes with six to nine carbon atoms readily undergo a stepwise degradation through autoxidation to the corresponding moloxide, followed by scission to yield formic acid and the next lower aldehyde. The ease with which this scission occurs decreases with decreasing chain length and is almost nil with acetaldehyde and propionaldehyde. Based on these findings, therefore, spot 2 is more likely to be formic than acetic acid. Toyama and Matsumoto (*17*) and Toyama *et al.* (*18*) isolated saturated acids from formic to caproic from oxidized methyl esters of highly unsaturated sardine oil fatty acids. The conditions of oxidation and of peroxide decomposition were considerably more severe than those used to collect the volatiles studied here, perhaps explaining why no unsaturated acids were found by these workers.

Branched-chain acids such as isobutyric and isovaleric have the same R_f values as their straight-chain isomers. Therefore, spots 6 and 7 could represent branched isomers also, but this possibility appears rather remote. Presumably, the precursors for these compounds would have to be the branched-chain fatty acids. Although these compounds are known to occur in marine lipids, they comprise only about 1.5% of the total fatty acids of menhaden oil. Furthermore, they are not highly unsaturated and they would not be expected to undergo rapid oxidative degradation. Thus, the volatile acidic compounds expected to result from autoxidation of highly unsaturated fatty acids of fish oils are the straight-chain saturated and unsaturated acids of low molecular weight, tentatively identified in this study. These acids have strong, sharp, unpleasant odors, and their presence should contribute significantly to the over-all odor of menhaden oil, although none has a characteristic fishy odor.

Undoubtedly, acids of longer chain length occur also in oxidized menhaden oil but because of their low volatility and solubility in water they are not found in the fraction studied here. Valeric acid was detected only when large amounts of the acidic material were chromatographed. The higher homologs are either completely absent or present in quantities too small to be detected.

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MILK STALING

Phospholipids of Fresh Milk and of Sterile Whole Milk Concentrate

H. W. SPRECHER, F. M. STRONG, and A. M. SWANSON

Departments of Biochemistry and Dairy and Food Industries, College of Agriculture, Madison, Wis.

SEVERAL investigators have suggested that phospholipids may be involved in detrimental reactions in dairy products (*7, 16, 29, 30*). The susceptibility of these components to oxidative attack in model systems has been established for some time (*13, 28*). Lack of a better understanding of their behavior in various dairy products can be attributed to the difficulty encountered in separating them from the large amount of neutral lipids.

Investigators to date have taken advantage of the fact that upon separating milk, about 60% of the phospholipids go into the cream. Upon churning, these can readily be recovered from either the buttermilk (*23, 25*) or butter serum (*2, 3, 14, 19*) and it is these milk phospholipids which have been mainly studied to date. However, approximately 40% of the total milk phospholipids are not recovered by this procedure. While Rhodes and Lea (*23*) have presented evidence that no selective partitioning occurs when the cream is separated, it has never been established whether there might be selectivity in regard to the fatty acid composition of the phospholipids which do or do not go