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**Factors affecting peroxide types
in oxidizing fatty acid mixtures**

by

Donald Craig Johnson

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

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INTRODUCTION

The peroxide value test (43) has been used for many years to determine the stability and suitability of fats and oils. The test shows the total amount of peroxide present which oxidizes potassium iodide. But, in a fat or oil in which several unsaturated fatty acids are capable of forming peroxides, the peroxide test is incapable of showing the amount of each individual peroxide.

People frequently assume that fatty acids in mixtures will oxidize according to the relative rates of the pure individual fatty acid. But earlier work in this laboratory on natural oils (50) showed that individual peroxides present were not in the ratio those which would be expected from fatty acids being oxidized separately.

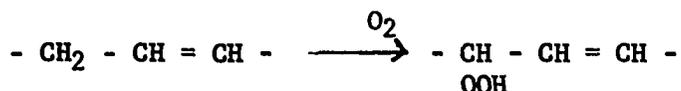
This work was undertaken to clarify the interaction among fatty acids, the effect of triglyceride structure, and/or other factors which influence the formation of individual peroxides in fatty acid mixtures.

REVIEW OF LITERATURE

Research has been carried out on the autoxidation of lipids for over one hundred years. Until 1940, the research was of limited value because it was mainly carried out on natural fatty materials, which are very complex. Therefore, it was impossible to understand the mechanisms of the numerous reactions occurring simultaneously (64).

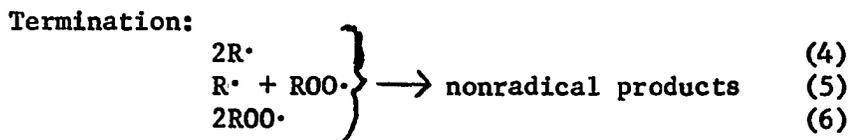
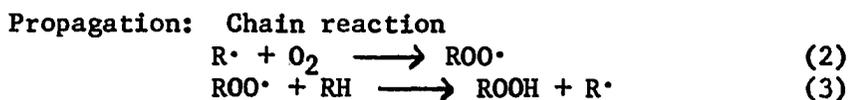
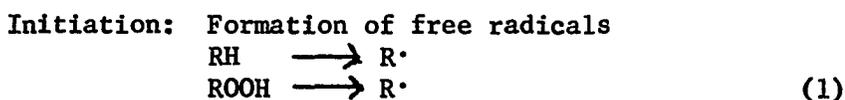
The early researchers believed the autoxidation of unsaturated fatty materials proceeded by direct addition of oxygen to the double bond, thus forming a cyclic peroxide. By studying the oxidation of simple, monounsaturated, nonfatty compounds, such as cyclohexene, information pertaining to the oxidation of monounsaturated lipid compounds was obtained. In 1928, Stephens (60) reported the isolation of a peroxide of cyclohexene. On the basis of the then accepted theories, he assumed that the product was saturated. Farmer and Sundralingam (16) established that Stephens' product was a hydroperoxide and that a double bond was present. They also found similar results with other compounds. The isolation of unsaturated hydroperoxides from oxidized olefins cast considerable doubt on the earlier concepts of autoxidation.

An extension of this work, mainly by Farmer's group (12, 13, 14, 15, 16, 17, 18, 19, 20) at the British Rubber Producers' Research Association proved that autoxidation usually proceeded through an attack at the methylene group adjacent to the double bond leading to the formation of allylic hydroperoxides:



According to Farmer's theory of autoxidation of all nonconjugated olefinic compounds, the addition of oxygen is by a free radical chain reaction on the carbon adjacent to the double bond.

There are three elementary reactions involved in the autoxidation of unsaturated fatty acids. These are initiation, propagation, and termination:



where RH represents an olefin with a α methylene hydrogen, $\text{R}\cdot$ is an alkyl free radical, and $\text{RO}_2\cdot$ is a peroxy free radical. The radical $\text{R}\cdot$ is the result of abstraction of a hydrogen from the fatty acid. The abstraction of hydrogen in reaction (3) occurs more readily if the radical ($\text{R}\cdot$) is stabilized by resonance. Due to the activation of the α methylene hydrogen by a double bond, unsaturated fatty acids oxidize to a greater extent than saturated fatty acids. In fatty acids where the allylic radical is formed, the hydroperoxide produced may differ in structure from its parent hydrocarbon.

The kinetics of olefin oxidation have been reviewed by Bateman (2),

Bolland (7), and more recently by Betts (5).

In typical fatty acid oxidation, the propagation sequence may occur a hundred or more times for every act of initiation or termination. The rate of oxygen consumption, therefore, equals the rate of propagation. If the rate constants for reactions (2) and (3) are k_o and k_p , respectively;

$$-\frac{d(O_2)}{dt} = \frac{d(ROOH)}{dt} = k_o (R\cdot) (O_2) = k_p (ROO\cdot) (RH)$$

The concentration of the alkylperoxyl radicals is minute, and its rate of change is near zero. If oxygen is not limiting, reaction (6) is the only important termination reaction. Applying stationary state principles; if the rate of initiation is R_i and the rate constant of reaction (6) is $2 k_t$;

$$\frac{d(ROO\cdot)}{dt} = 0 = R_i - 2k_t(ROO\cdot)^2$$

hence eliminating $(ROO\cdot)$ and giving a general rate equation:

$$-\frac{d(O_2)}{dt} = \frac{d(ROOH)}{dt} = k_p(RH) (R_i/2k_t)^{\frac{1}{2}}$$

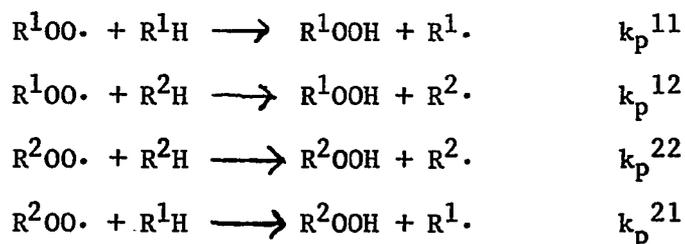
Under normal conditions, oxygen consumption and hydroperoxide yield agree closely (28, 39).

Oxygen consumption by fatty acids has been measured by many researchers (24, 31, 46, 61). Myers et al. (46) and Holman and Otto (31) found the acids to oxidize faster than the methyl esters. The energy required for the rupture of a carbon-hydrogen bond on the methylene group α to a double bond is believed to be considerably lower in a 1,4

diene system than in a monoene because the resonance energy of the pentadienyl radical is much higher, so the rate of autoxidation of linoleic is considerably faster than that of oleic acid. The rate of linolenic autoxidation is again faster than that of linoleic acid. Gunstone and Hilditch (24) and Holman and Otto (31) found rates of oxygen uptake of oleate: linoleate: linolenate of 1:10-12:25. The reliability of experiments on the rate of autoxidation of unsaturated fatty acids and esters is strongly dependent on the purity of the substrates used. Gunstone and Hilditch (23) have shown that the autoxidation of methyl oleate is strongly accelerated and the induction period much reduced in the presence of 1% methyl linoleate at 20C or 0.2% methyl linoleate at 50C.

Howard and Ingold (32), using a rotating sector apparatus, found the ratio of absolute rate constants (k_p) for oleate: linoleate: linolenate of 89:2100:3900. These authors did note that during oxidation the rates of oxygen uptake for linoleate and especially linolenate decreased with extent of oxidation even though R_i was constant. This may cause some error in k_p .

If two hydrocarbons, R^1H and R^2H are co-oxidized, four propagations must be considered (55). These are:



As derived by Russell (55), the relative rate equation is:

$$\frac{d(R^1H)}{d(R^2H)} = \frac{d(R^1OOH)}{d(R^2OOH)} = \frac{r_1(R^1H/R^2H) + 1}{r_2(R^2H/R^1H) + 1}$$

where

$$r_1 = k_p^{11}/k_p^{22}$$

$$r_2 = k_p^{22}/k_p^{21}$$

There have been no co-oxidation studies of fatty acids.

Although kinetic studies have been helpful, much of the present understanding of autoxidation mechanisms has come from a knowledge of the structures of the hydroperoxides formed during autoxidation. The primary objective in the characterization of fatty ester hydroperoxides is to establish the position of the hydroperoxide group and the unsaturation within the fatty acid chain. Because of the interference from and the instability of the hydroperoxide group, the reduction of the hydroperoxide group to the corresponding secondary alcohol is a fundamental step in the characterization of the fat hydroperoxide.

The reduction of peroxides has been investigated by many workers, (36, 50, 53). Potassium iodide, stannous chloride, sodium bisulfite and sodium borohydride have been most commonly used for this purpose.

Many forms of the iodometric reduction used by early workers were only qualitative, but some attempted to determine the iodine liberated. Lea (37) developed a method which involved heating the fat or oil with glacial acetic acid and chloroform in the presence of potassium iodide solution and titrating the liberated iodine with thiosulfate. Wheeler's

(67) procedure, which is used by the American Oil Chemists' Society, uses a saturated solution of potassium iodide and is performed in the presence of air at room temperature.

Stannous chloride was used to determine peroxides quantitatively (53). Hargrave and Morris (28) found yields ranging from 42-100% with stannous chloride. With sodium bisulfite, Knight and Swern (36) reported that some carbonyl groups were formed in the reduction of peroxides. Sodium borohydride was used to reduce linoleate hydroperoxides, yielding alcohols free of carbonyls (58). Lea (38) compared the iodometric, ferric thiocyanate (30), and 2,6-dichlorophenolindophenol (29) methods. He found yields of 71-91% for the iodometric method, but the yields in the other two methods were abnormally high in air, and obviously too low in its absence. Raghuveer (50) found only 60-80% of the theoretical amount of reduced hydroperoxide using stannous chloride and sodium borohydride, while he found essentially complete reduction with potassium iodide.

In the monounsaturated fatty acids, there are usually two α methylene groups which are the points of attack in the free radical chain reaction. Due to the stabilization by resonance, there is the possibility of the formation of four isomeric hydroperoxides.

In an extension of their earlier work on low molecular weight olefins, Farmer and Sutton (17) isolated nearly pure methyl oleate hydroperoxides by molecular distillation and chromatography. The isolated hydroperoxides contained the theoretical amount of peroxide oxygen. Oxidized methyl elaidate also yielded a hydroperoxide (62).

The first structural characterization of oleate hydroperoxides was carried out by Ross et al. (54). They found the isomers in decreasing amounts of methyl 9-hydroperoxido-10-octadecenoate, methyl-10-hydroperoxido-8-octadecenoate, methyl-8-hydroperoxido-9-octadecenoate, and methyl-9-hydroperoxido-10-octadecenoate. Knight et al. (35) and Swern et al. (63) found that autoxidation of methyl oleate induces a cis-trans isomerization of the double bond yielding the more stable trans hydroperoxides. In 1959, Privett and Nickell (48) showed that all four hydroperoxide isomers predicted by theory were formed in equal amounts.

Farmer and Sutton (17) showed that methyl oleate on autoxidation yielded a mixture of mono- and dihydroperoxides, the monohydroperoxide predominating. Swern et al. (63) examined several peroxide concentrates of methyl oleate; by using polarographic methods, they found the monohydroperoxides predominate, but as much as 28% of the peroxides were nonhydroperoxides. By following absorption of oxygen quantitatively and analyzing the peroxides formed, Saunders et al. (56) found only 90-95% of the peroxides could be accounted for as hydroperoxides.

In linoleic acid, there are three α methylene groups. The C_{11} methylene group is the preferential point of attack, supposedly because the intermediate formation of a C_{11} radical requires less energy than either the C_8 or C_{14} methylene group. The formation of three isomeric hydroperoxides would therefore be expected.

Farmer and Sutton (18) observed that conjugation of double bonds occurred in autoxidized fish oil. Bolland and Koch (8) estimated that 70% of the monhydroxide formed during autoxidation of ethyl linoleate

was conjugated diene isomers. Questions, as to whether three isomers (2 conjugated, 1 nonconjugated) or two conjugated isomers were formed, were answered by Bergström["] (4). He isolated the hydrogenated products from autoxidized linoleate and showed they contained 9- and 13-hydroxystearates. Khan et al. (33) autoxidized methyl linoleate under various conditions and found the hydroperoxides were composed of conjugated dienes.

The preponderance of conjugated hydroperoxides in autoxidized linoleate was well established by the studies of Cannon et al. (10) and Privett et al. (47), who isolated the hydroperoxides in high purity by countercurrent distribution. Infrared spectra showed that the hydroperoxides assumed a cis-trans and trans-trans configuration, the more stable trans-trans isomer being more prevalent at higher temperatures and levels of autoxidation. Both groups estimated that more than 90% of the isomers were conjugated. Sephton and Sutton (58) confirmed these results. Upon hydrogenation of the hydroxylinoleate isomers, they found equal amounts of the 9- and 13-hydroxystearates. They also reported the possible presence of cis-cis isomers. Banks et al. (1) using permanganate oxidation, agreed with the findings of Sephton and Sutton (58).

Khan et al. (34) reported a nonconjugated hydroperoxide when linoleate was autoxidized in the presence of chlorophyll. Cobern et al. (11) and Hall and Roberts (25) examined oxidized linoleate by mass spectroscopic and nuclear magnetic resonance techniques, respectively. Both groups found nonconjugated dienes present when the linoleate was oxidized in the presence of chlorophyll. Both groups also concluded that

autoxidation is invariably accompanied by double bond rearrangement and that for air oxidation (not catalyzed by chlorophyll) the rearrangement is always in the direction of conjugation.

The autoxidation of linolenic acid proceeds in a similar manner to that of linoleic acid. There are two methylene groups between double bonds, which are the point of attack in the free radical chain reaction. The formation of at least six isomeric monohydroperoxides can therefore be expected.

Fugger et al. (21) were unable to isolate monohydroperoxides from autoxidized methyl linolenate fractionated with a small countercurrent distribution apparatus. They concluded extensive polymerization occurred even under mild conditions of autoxidation. Privett et al. (49), also using countercurrent extraction, showed that methyl linolenate yielded a hydroperoxide containing 60% monomeric cis-trans conjugated diene monohydroperoxide. Privett et al. (49) and Frankel et al. (20) showed the hydroperoxides are conjugated dienes consisting primarily of cis-trans with some trans-trans configuration. Begemann et al. (3) isolated methyl linolenate monohydroperoxide and more polar peroxides. They identified four isomeric polar compounds containing two peroxide groups; a hydroperoxide group and a six membered ring peroxide. They proposed a mechanism for the formation of these compounds.

Raghuveer (50) developed a quantitative method to isolate and analyze peroxides from mixtures of fatty acids or natural oils. In soybean and olive oil, he found that the fatty acids oxidized at different rates than the data of pure methyl esters predicted.

Most of the previous work on fatty acid autoxidation has dealt with pure fatty acids. This work was undertaken to investigate the influence of fatty acids on each other during the formation of hydroperoxides. Also, the effect of glycerides structure and the presence of nonsaponifiables on the peroxides formed was checked.

EXPERIMENTAL INVESTIGATION

Materials and Methods

Soybean oil: Soybean oil was obtained from Anderson, Clayton and Company.

Safflower oil: Safflower oil was obtained from Nutritional Biochemicals Corporation.

Corn oil: Corn oil was obtained locally.

Castor oil: Castor oil was obtained from Fischer Scientific Company.

Linseed oil: Raw linseed oil was obtained from Archer Daniels Midland Company and refined with dilute alkali and activated carbon to remove impurities before use.

All glassware was soaked in 10% ammonium hydroxide.

Gas chromatography: Methyl esters were analyzed on a Beckman GC-5 chromatograph equipped with flame ionization detector. A six foot stainless steel column with 15% EGSS-X on Chrom Sorb P (Applied Science, Inc.) at 180C was used.

Methyl esters were prepared in methanol using 2% sulfuric acid as a catalyst, except where noted.

Methyl Oleate: Methyl oleate was isolated from olive oil methyl esters by the urea fractionation procedure of Raghuvver (50).

Methyl Linoleate: Methyl linoleate was prepared from safflower oil by urea fractionation. First 600 g of safflower oil fatty acids were dissolved in 2460 ml of methanol containing 953 g of urea and heated to

dissolve the urea. The solution was allowed to cool to room temperature and the urea adducts were removed by filtration. The filtrate was evaporated under reduced pressure until it solidified and this residue was dissolved in 2 N hydrochloric acid. The recovered fatty acids were esterified with methanol using 2% sulfuric acid as a catalyst. The product was washed free of acid with 5% sodium carbonate and finally with distilled water. The methyl linoleate was analyzed by gas chromatography and was found to be greater than 95% pure. Another urea fractionation was performed on these methyl esters with a ratio of urea: methanol: methyl esters of 1: 5: 1 respectively. The methyl esters recovered from the filtrate were found to be pure by gas chromatography.

Methyl linolenate: Methyl linolenate was prepared from linseed oil by urea fractionation (50). This yielded a product containing 75.7% linolenic, 20.9% linoleic and 3.4% oleic acid by gas chromatography. Methyl linolenate of high purity was prepared by the column chromatographic procedure of Hammond and Lundberg (27) except that a column 5.5 cm in diameter and 90 cm in length, filled to a height of 82 cm with a mixture of 80% silica gel 100 mesh (Mallinckrodt Chemical Company) and 20% Celite 545 (Fisher Scientific Company), was used.

Butylated methyl ricinoleate: Methyl ricinoleate was prepared as follows: Mixed methyl esters were obtained from castor oil by refluxing castor oil with methanol in the presence of sodium methoxide as a catalyst. The mixed methyl esters were butylated following the acetylation procedure of the American Oil Chemists' Society (42). Eighty seven milliliters of butyric anhydride (Eastman Kodak) were used. The esters were

thoroughly washed with warm water and dried over anhydrous sodium sulfate. The butylated methyl ricinoleate was purified by a urea fractionation scheme as follows: 10 g portions of methyl esters were dissolved in 170 ml of methanol containing 26 g urea. The mixture was cooled to room temperature with intermittent stirring during the first hour and kept at this temperature for 3 hr to let the urea complex crystallize. The urea complex was filtered and washed twice with 10 ml portions of methanol saturated with urea. Fraction 2 was obtained by adding 8 g of urea to the filtrate from fraction 1. The volume was maintained at 170 ml. The mixture was heated, stirred, cooled and filtered as before. Fractions 3, 4, and 5 were obtained similarly. The butylated methyl ricinoleate was recovered from the final filtrate by acidifying with 1 N hydrochloric acid and extracting the solution with 100 ml hexane. The solution was extracted a second time with 100 ml of diethyl ether and finally with a 100 ml of hexane. The combined extracts were then washed free of acid with distilled water and dried with sodium sulfate. The solvents were removed under reduced pressure in a rotary evaporator. The methyl esters were spotted on Silica Gel G thin-layer plates and developed in hexane and diethyl ether (80:20 ^v/_v). The plates were sprayed with 50% sulfuric acid saturated with potassium dichromate and then charred at 120C for 1 hr. The butylated methyl ricinoleate gave only one spot.

The methyl ester mixtures were prepared using pure methyl esters and methyl esters of natural oils. The ratios were checked by gas chromatography and adjusted as necessary. A short Widmer column

(12 cm) was used to distill the methyl ester mixtures at pressures of less than 0.1 mm immediately before oxidation.

Natural oils were deodorized in an all-glass apparatus (57) before they were oxidized.

Soybean oil and corn oil were randomized using 0.5% sodium methoxide. The reaction mixture was stirred with a magnetic stirrer; the pressure was kept below 1 mm and the temperature was maintained at 60C. After randomization, the mixture was washed with 5% acetic acid (to destroy catalyst), a 5% sodium carbonate solution, and finally with distilled water. After drying over sodium sulfate, the mixtures were deodorized (57).

All methyl ester mixtures and oils were autoxidized in 50 g lots in 300 ml Erlenmeyer flasks without stirring in a 28C incubator.

Samples were withdrawn periodically to determine the peroxide value (PV) by the method of Hamm et al. (26). When samples reached peroxide values of approximately 5, 10, 20, and 40, samples were withdrawn and the peroxides were reduced to alcohols by the iodometric method recommended in the Official Methods of the American Oil Chemists' Society (43), at which time the peroxide values were confirmed. The methyl esters were extracted 3 times with 100 ml portions of chloroform, washed with distilled water and twice with 5% sodium bicarbonate. After drying over sodium sulfate, the solvent was removed under reduced pressure. The hydroxymethyl esters formed by the reduction from hydroperoxides were then butylated with butyric anhydride following the acetylation method of the American Oil Chemists' Society (44). After 1 hr,

the reaction mixture was washed with warm water, cooled, and extracted 3 times with 100 ml portions of hexane. The methyl esters were washed with distilled water and dried over sodium sulfate. The solvent was evaporated under reduced pressure using a rotary evaporator.

Urea fractionation procedure: 10 g of butyric anhydride-treated methyl esters were heated in 170 ml methanol containing 42 g of urea. The solution was cooled to room temperature during the first 2 hr with occasional stirring. The solution was then placed in a 21C incubator for 3 hr, after which the solution was placed in a 7C incubator for 3 hr. This was done to allow complete crystallization at constant temperatures.

The solution was filtered through a cold Buchner funnel, and the crystals were washed twice with 10 ml portions of cold methanol saturated with urea. The filtrate was transferred to a separatory funnel and 100 ml of 1 N hydrochloric acid was added. One hundred milliliters of hexane were used to extract the solution. A second extraction was made with 100 ml of diethyl ether and finally a second 100 ml portion of hexane. The extracts were combined and washed free of acid with distilled water and dried over sodium sulfate. The solvents were removed under reduced pressure, and the methyl esters were transferred to a 10 ml volumetric flask and diluted to 10 ml with chloroform.

In the case of the natural oils, after reduction by iodide, methyl esters were prepared using sodium methoxide as a catalyst rather than sulfuric acid as done by Raghuveer (50). This was done to prevent dehydration of the hydroxy fatty acids (59, 66). The methyl esters were

butylated and urea fractionated as previously described.

Densitometer: A Model 525 Photovolt Densitometer and Model 43 Photovolt Varicord Recorder (Photovolt Corp., New York, N.Y.) were used for densitometry. The log scale of the recorder was used. A specially designed stage (6) for 20 x 20 cm chromoplates was used.

Thin-layer chromatography: Precoated Adsorbosil-5-plates (Applied Science Laboratories, Inc.) were used for all densitometer measurements. They were activated for $\frac{1}{2}$ hr at 100C before use. Locally prepared plates of Silica Gel G and Silica Gel G impregnated with 25% silver nitrate were used for preparative separations. These were air dried, then activated at 100C for 1 hr, and the silver nitrate plates were stored in a dark drying chamber.

Samples were applied as dilute solutions in chloroform along with the standards with a Hamilton microsyringe or, for preparative separations, with a streak applicator (Applied Science Laboratories, Inc.). The development was done in closed tanks lined with solvent soaked filter paper. After development, the spots or bands were located by spraying with 2', 7' dichlorofluorescein or by charring with 50% sulfuric acid saturated with potassium dichromate. In case of densitometry analyses, plates were charred for 1 hr at 120C. A linear relationship was obtained between peak area and sample size when there was less than 30.0×10^{-3} mg of material. The samples were applied to fall below this value. The standard was applied in 4 different concentrations, along with the samples to be analyzed. Both butylated methyl ricinoleate and methyl ricinoleate were used as standards.

For the butylated methyl esters both the Adsorbosil-5 and Silica Gel G plates were developed in hexane: diethyl ether (85:15 V/v).

As the silver nitrate plates could not separate the butoxy methyl esters of oleate and linoleate, the esters were debutylated in approximately 1 ml of methanol using several drops of 1 M sodium methoxide as a catalyst. The mixture was heated on a steam bath for 5 min, after which several drops of acetic acid and 1 ml of water were added. The esters were extracted with 2 ml of hexane, then with 2 ml of diethyl ether and again with 2 ml of hexane. The extracts were combined and washed with water. They were dried over sodium sulfate and the solvents were partially evaporated under reduced pressure. The hydroxy methyl esters were then spotted on Silica Gel G impregnated with 25% silver nitrate and developed in hexane: diethyl ether (40:60 V/v).

The hydroxy methyl esters of each degree of unsaturation were quantatized as above, but the solvent system was hexane: diethyl ether (60:40 V/v), and methyl ricinoleate was used as the standard.

RESULTS AND DISCUSSION

Modification of the procedure: The key step, in the analysis of hydroperoxide types in fats at low levels of oxidation, is their concentration from the common long-chain fatty acids from whence they are produced. In an earlier study in this laboratory, Raghuveer (50) accomplished this by reducing the hydroperoxides to alcohols and concentrating them by urea fractionation. Raghuveer found he had to acetylate the methyl esters of hydroxy fatty acids (such as methyl ricinoleate) to achieve satisfactory separation from the common long-chain fatty acid methyl esters. The procedure he devised involved several crystallizations and filtrations. I wanted a method with less handling of the sample to minimize losses and to reduce analysis time.

Because an acetyl side chain had helped separate hydroxy esters from common fatty esters, the use of longer side chains was explored. Methyl ricinoleate (castor oil) was used as a model compound and it was acylated with acetic, propionic, and butyric anhydride. The urea fractionation procedure described in the METHODS section for the isolation of butylated methyl ricinoleate was used. This involved five urea crystallizations. In the urea fractionation of the castor oil, it was found that the acetylated methyl ricinoleate was complexed in fraction 2, where propylated methyl ricinoleate did not complex until fraction 4 and the butylated methyl ricinoleate until fraction 5. This indicated that more rigorous conditions of urea fractionation could be performed on the butylated methyl ricinoleate, before it would form an

adduct. A longer side chain could modify the methyl ricinoleate enough to form an adduct with urea (65,68).

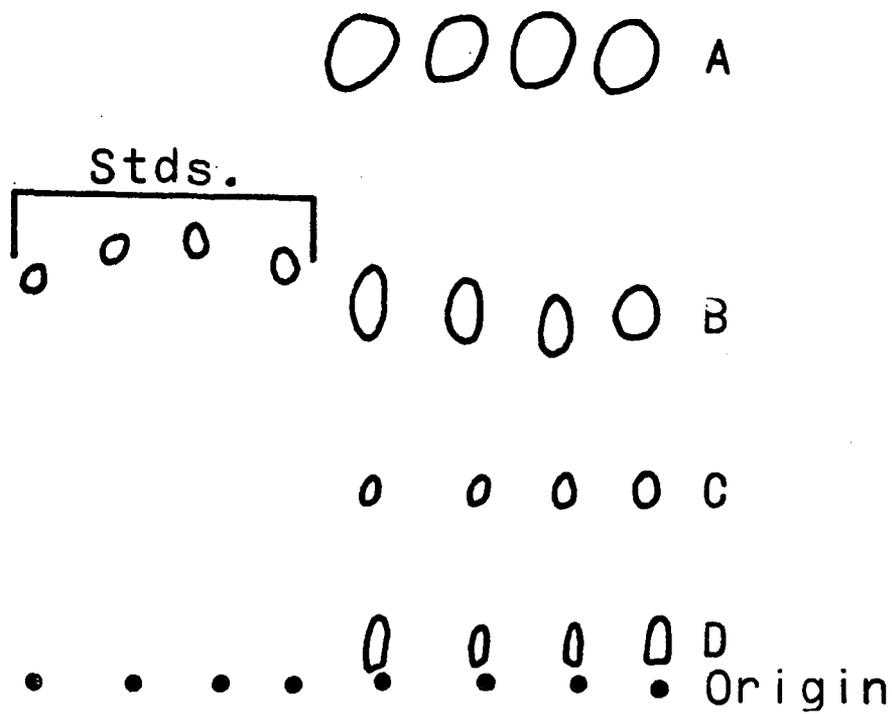
Instead of the time consuming repeated crystallizations at one temperature with increasing urea concentration as practiced by Raghuvver (50), the procedure of crystallization at decreasing temperatures described in the METHODS section was developed. The best results were obtained by cooling the sample at room temperature for 2 hr, followed by 3 hr in a 21C incubator and 3 hr in a 7C incubator. For example, when 60 mg of butylated methyl ricinoleate was added to corn oil methyl esters and the urea fractionation was performed in this way, quantitative recovery of the butylated methyl ricinoleate was attained. Only about 100-150 mg of other materials were present as shown by thin-layer chromatography. Cooling to lower temperatures did not remove any more of the unoxidized esters, and with the addition of more urea, small amounts of butylated methyl ricinoleate were included in the adducts.

When the reduction (43) and concentration procedures were tried on oxidized methyl esters, another spot was found near the butylated methyl esters on the thin-layer chromatogram. Infrared spectroscopy and mass spectrometry, showed the second spot to be acetoxy methyl esters. During the peroxide reduction, the fat was dissolved in acetic acid/chloroform (43). Evidently, residual acetic acid was being carried through the procedure and was competing with the butyric anhydride in the butylation reaction. The chloroform extracts were washed with 5% sodium bicarbonate to remove the acetic acid. After this, the procedure gave only one spot corresponding to butoxy methyl esters (Spot B, Figure 1)

Figure 1. Thin-layer chromatogram used for quantitation of butoxy methyl esters.

- A. Residual unoxidized methyl esters.
- B. Butoxy methyl esters.
- C. Mixed butyric-fatty acid anhydride.
- D. Unknown.

————— Solvent Front



by thin-layer chromatography.

Figure 1 shows a typical chromatogram used for quantitation of the butoxy methyl esters. Spot A is the residual unoxidized methyl esters not removed by the urea procedure. Spot C is probably a mixed anhydride of butyric and a fatty acid. When this spot is removed from the plate and transesterified with methanol and sodium methoxide, the product travels with the normal methyl esters (Spot A) on rechromatography. It normally is present in about 2% of the amount of the butoxy methyl esters, but its relative amount is increased by using a larger excess of butyric anhydride. It corresponds to the "other oxidized material" reported by Raghuvver. Spot D was equal to about 5% of the butoxy ester. It might be polymers or oxidation products other than hydroperoxides.

Raghuvver (50) was able to separate acetoxy methyl esters by degree of unsaturation by using silver ion thin-layer chromatography. This technique would not separate the butoxy methyl esters produced from oleate and linoleate. The only way to separate these compounds was to remove the butyl group and to separate the hydroxy-monoene and hydroxy-diene by silver ion thin-layer chromatography. Figure 2 shows a silver ion thin-layer chromatogram. The separation of hydroxy-monoene and hydroxy-diene was adequate by this procedure.

Saturated fatty acids: The first experiment was designed to see if saturated methyl esters influenced the amounts of monoene and diene peroxides formed when corn oil methyl esters were oxidized. The results of this experiment are given in Table 1.

The hydroxy esters traveled with either the hydroxy-monoene

Figure 2. Thin-layer chromatograph of hydroxy methyl esters on silver nitrate-Silica Gel G.

- A. 1. trans hydroxy-monoene.
2. cis hydroxy-monoene.
- B. hydroxy-diene.
- C. hydroxy-triene.
- D. methyl ricinoleate.

————— Solvent Front

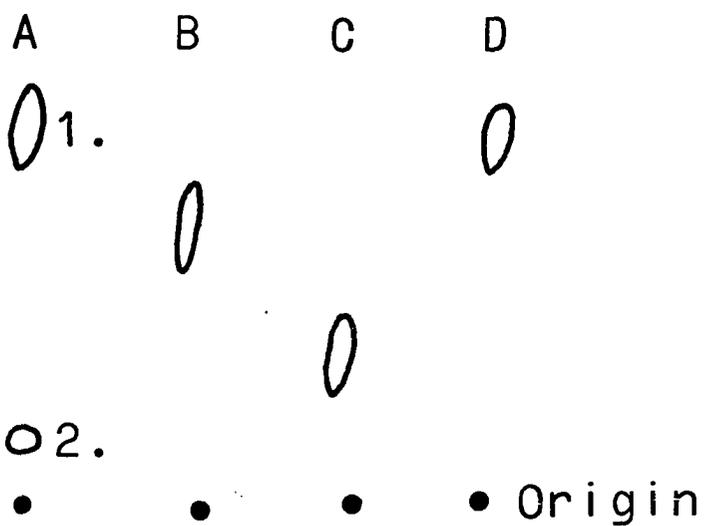


Table 1. TLC analysis of the effect of saturated methyl esters on the formation of peroxide types

| | | Sample L | | Composition | | Sample H | | | | | | | |
|--------|-----------------------------------|--|--------------------|--|-------|-----------------------|--------|-----------------------------------|--|-------|--|-------|-----------------------|
| | | 6.90% | | Palmitate | | 22.35% | | | | | | | |
| | | 0.00% | | Stearate | | 6.30% | | | | | | | |
| | | 46.55% | | Oleate | | 35.35% | | | | | | | |
| | | 46.55% | | Linoleate | | 36.00% | | | | | | | |
| P.V.-L | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | | Diene ² Peroxide (mg) | | Mono- ene Diene | P.V.-H | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | | Diene ² Peroxide (mg) | | Mono- ene Diene |
| | | | | 1.60 ⁵ | | | | | | | | | |
| 5.01 | 98.2 | 3.65 | 1.40 | 3.00 ⁶ | 1.22 | | 5.61 | 97.9 | 3.88 | 1.62 | 3.24 | 1.20 | |
| | | | 3.25 | | | | | | c 1.84 | 3.95 | | | |
| 10.43 | 104.0 | 8.90 | 3.25 | 6.60 | 1.34 | | 12.00 | 109.0 | t 8.20 | 10.14 | 4.00 | 7.95 | 1.28 |
| | | c 6.20 ³ | 8.20 | | | | | | c 6.21 | | 6.15 | | |
| 26.19 | 107.0 | t18.90 | 25.10 ⁴ | 7.80 | 16.00 | 1.57 | 21.65 | 95.4 | t12.25 | 18.46 | 6.21 | 12.36 | 1.49 |
| | | c10.65 | 17.05 | | | | | | c10.85 | | 11.68 | | |
| 44.09 | 97.3 | t26.40 | 37.05 | 18.40 | 34.45 | 1.08 | 37.03 | 99.9 | t23.00 | 33.85 | 12.22 | 23.90 | 1.42 |
| | | | | Ave. | | 1.30 | | | | | Ave. | | 1.35 |

¹Based on recovery of butylated methyl esters and the observed peroxide value using butylated methyl ricinoleate as a densitometric standard.

²Hydroxy methyl esters recovered using methyl ricinoleate as a densitometric standard.

³Cis monoene and trans monoene.

⁴Total monoene.

⁵Top figure is 13-hydroxy-diene, while lower figure is 9-hydroxy-diene.

⁶Total diene.

obtained from oxidized methyl oleate or the hydroxy-diene obtained from oxidized methyl linoleate on silver ion thin-layer plates. No evidence was found for saturated peroxides. Brodnitz et al. (9) had suggested that in the presence of polyunsaturated fatty acids, saturated fatty acids might autoxidize and form peroxides. My results confirmed the findings of Michalski and Hammond (45), who did not find saturated fatty acid oxidation products when C^{14} labelled stearic acid was oxidized with soybean oil.

Two spots were found when the hydroxy-diene esters were rechromatographed on Silica gel plates for quantitation (Figure 3). These are believed to correspond to the two isomeric hydroperoxides formed from linoleate (Figure 3). Graveland (22), using the same thin-layer system, found similar results. The R_f 's I observed agreed with his. Graveland, using infrared and mass spectroscopy, identified the compounds. The higher spot (R_f 0.43) was 13-hydroxy-9 cis, 11 trans-octadecadienoate, and the lower spot (R_f 0.35) was 9-hydroxy-10 trans, 12 cis-octadecadienoate. The two isomers were found in approximately equal amounts confirming the findings of Bergström (4) and Khan et al. (33). The molar absorbance of the butoxy-diene was 26×10^3 at 233 nm, which was in close agreement with the findings of Cannon et al. (10) and Privett et al. (47). Raghuveer (50) did not observe these two isomers using a similar thin-layer procedure, because resolution of the two isomers is not achieved using the acetoxy esters as he did.

At higher peroxide values, a slow moving spot was found on silver ion thin-layer chromatography. Raghuveer (50) sometimes found this

Figure 3. Thin-layer chromatograph used for the quantitation of the hydroxy methyl esters.

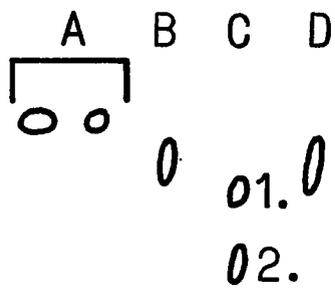
A. methyl ricinoleate standards.

B. hydroxy-monoene.

**C. 1. 13-hydroxy-diene
2. 9-hydroxy-diene.**

D. hydroxy-triene.

————— Solvent Front



product when methyl oleate was oxidized. He attributed the spot to cis-monoene-hydroperoxide. Others (35,36) have reported only the presence of trans double bonds in the hydroperoxides from methyl oleate and most of the oxidation product from methyl oleate is trans as shown by infrared spectroscopy. Because it is always produced in small amounts, there has never been enough of the slow moving product for spectroscopy.

The amount of saturated fatty acid methyl esters had no observable effect on the formation of hydroxy-monoene and hydroxy-diene methyl esters.

Nonsaponifiabiles: The effect of the nonsaponifiabiles on the formation of peroxide types was also examined. The nonsaponifiabiles were extracted from saponified corn oil and the fatty acids were converted to methyl esters which were then distilled before oxidation as described in the METHODS section. Methyl esters were also prepared from corn oil by transesterfication with a sodium methoxide catalyst. The esters were deodorized in all glass apparatus (57). The latter procedure does not remove or destroy the nonsaponifiabiles, but any hydroperoxides present are destroyed in the deodorization. Table 2 gives the quantitative analyses of peroxides formed.

The nonsaponifiabiles possibly had a small effect on the ratio of monoene/diene product, but if so, it is obscured by the experimental error. The only obvious difference was in the length of time it took the methyl esters to reach corresponding peroxide values. The methyl esters with the nonsaponifiabiles took over three times as long. This is because the nonsaponifiabiles include the tocopherol present in the corn oil.

Table 2. TLC analyses of the effect of nonsaponifiabiles on peroxide type formation

| Nonsaponifiabiles Removed | | | | Composition | | Nonsaponifiabiles Present | | | |
|---------------------------|-----------------------------------|--|---|------------------------------|--------|-----------------------------------|--|--|------------------------------|
| 13.84% | | | | Palmitate | | 11.55% | | | |
| 1.47% | | | | Stearate | | 1.35% | | | |
| 24.44% | | | | Oleate | | 25.25% | | | |
| 60.23% | | | | Linoleate | | 61.93% | | | |
| P.V.-R | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Mono- <u>ene</u> Diene | P.V.-P | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Mono- <u>ene</u> Diene |
| 6.45 | 99.1 | 2.06 | 3.40 ³ 3.89 7.29 ⁴ 7.41 | 0.28 | 5.01 | 100.0 | 1.80 | 2.75 2.88 5.63 8.18 | 0.32 |
| 10.53 | 100.0 | 2.48 | 7.68 15.09 16.95 | 0.17 | 13.03 | 98.6 | 3.75 | 8.03 16.21 9.95 | 0.23 |
| 25.05 | 101.0 | 6.05 | 15.60 32.55 | 0.19 | 16.37 | 98.8 | 8.15 | 9.63 19.58 22.00 | 0.41 |
| | | | Ave. | 0.21 | 36.65 | 96.9 | 11.98 | 23.08 45.08 Ave. | 0.26 0.30 |

¹Based on recovery of butylated methyl esters and the observed peroxide value using butylated methyl ricinoleate as a densitometric standard.

²Hydroxy methyl esters recovered using methyl ricinoleate as a densitometric standard.

³Top figure is 13-hydroxy-diene and lower figure is 9-hydroxy-diene.

⁴Total diene.

Oxidation of oleate-linoleate mixtures: With the findings that saturated fatty esters and nonsaponifiables had little or no effect on peroxide formation, a series of oleate-linoleate mixtures were oxidized and the peroxides formed were analyzed. The results are given in Table 3.

The table shows that the ratio of oleate-linoleate has no effect on the formation of the two hydroxy-diene isomers. They are essentially found in equal amounts at all concentrations.

At oleate concentrations of 46% and higher, and peroxide values greater than 20, the product believed to indicate the cis monoene hydroperoxide was found. It formed approximately 30% of the total hydroxy-monoene present in three of the mixtures, but in the mixture with the highest amounts of oleate, it accounted for only 12% of the hydroxy-monoene.

The results show that methyl oleate and methyl linoleate interact strongly when oxidized together. Oxygen uptake experiments on pure esters (24, 31) would predict a 1/10 ratio of hydroxy-monoene/hydro-diene, and Howard and Ingold's (32) results would predict a ratio of 1/23.

The results have been plotted in Figure 4. The graph shows that the formation of the hydroxy-diene is approximately directly proportional to linoleate concentration in the original methyl ester mixture at concentrations above 50% linoleate, but at lower concentrations, there is a significant deviation, giving higher proportions of oxidized linoleate.

In a binary mixture, there are four propagation reactions:

Table 3. TLC analysis of the formation of peroxide types in autoxidizing mixtures of oleate-linoleate

| Composition Original Methyl Ester Mixture | P.V. | Days to reach P.V. | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Diene in Oxidized Product % |
|---|-------|--------------------------|-----------------------------------|--|--|-----------------------------------|
| 18:1 = 91.23% | 7.18 | 14 | 95.0 | 6.75 | .95 ⁵ 1.50 2.45 ⁶ | 26.6 |
| 18:2 = 8.77% | 12.31 | 21 | 100.0 | 13.97 | 2.50 2.42 4.92 | 26.0 |
| | 24.45 | 25 | 98.7 | c 3.92 ³ t25.00 28.92 ⁴ | 5.05 4.83 9.88 | 25.4 |
| | 46.80 | 30 | 98.2 | c 7.05 t53.10 60.15 | 7.85 8.15 16.00 | 21.0 |
| 16:0 = 2.32% | 5.03 | 9 | 100.0 | 4.80 | 0.94 0.80 1.74 | 26.6 |
| 18:1 = 84.46% | 10.50 | 14 | 87.7 | 11.65 | 1.76 1.81 3.57 | 23.5 |
| 18:2 = 17.20% | 20.04 | 18 | 82.4 | c 4.00 t13.62 17.62 | 3.52 3.82 7.34 | 29.4 |
| | 42.96 | 23 | 101.0 | c10.25 t37.10 47.35 | 9.90 9.75 19.65 | 29.8 |

| | | | | | | | | | |
|---------------|-------|----|-------|--------|-------|-------|-------|-------|------|
| 16:0 = 1.95% | 5.02 | 13 | 98.1 | | 5.10 | 1.30 | 1.42 | 2.72 | 34.8 |
| 18:1 = 65.66% | 10.40 | 18 | 98.4 | | 10.55 | 2.80 | 2.75 | 5.55 | 34.5 |
| 18:2 = 32.48% | 21.51 | 24 | 99.0 | c 5.90 | | 5.50 | | | |
| | | | | t16.10 | 22.00 | 5.50 | 11.00 | | 33.0 |
| | 39.05 | 30 | 99.2 | c10.00 | | 10.60 | | | |
| | | | | t30.90 | 40.90 | 10.50 | 21.10 | | 35.4 |
| 16:0 = 6.90% | 5.01 | 7 | 98.2 | | 3.65 | 1.60 | 1.40 | 3.00 | 45.5 |
| 18:1 = 46.55% | 10.43 | 12 | 103.9 | | 8.90 | 3.25 | 3.35 | 6.60 | 42.6 |
| 18:2 = 46.55% | 26.19 | 16 | 107.3 | c 6.20 | | 8.20 | | | |
| | | | | t18.90 | 25.10 | 7.80 | 16.00 | | 39.0 |
| | 44.09 | 21 | 97.3 | c 9.65 | | 18.05 | | | |
| | | | | t25.40 | 35.05 | 18.40 | 36.45 | | 51.0 |
| 16:0 = 13.84% | 6.45 | 6 | 99.1 | | 2.06 | 3.40 | 3.89 | 7.29 | 78.0 |
| 18:0 = 1.47% | 10.53 | 11 | 100.0 | | 2.48 | 7.41 | 7.68 | 15.09 | 82.8 |
| 18:1 = 24.44% | 25.05 | 15 | 101.0 | | 6.05 | 16.95 | 15.60 | 32.55 | 82.4 |
| 18:2 = 60.23% | | | | | | | | | |

¹Based on recovery of butylated methyl esters and the observed peroxide value using butylated methyl ricinoleate as a densitometric standard.

²Hydroxy methyl esters recovered using methyl ricinoleate as a densitometric standard.

³Cis monoene and trans monoene.

⁴Total monoene.

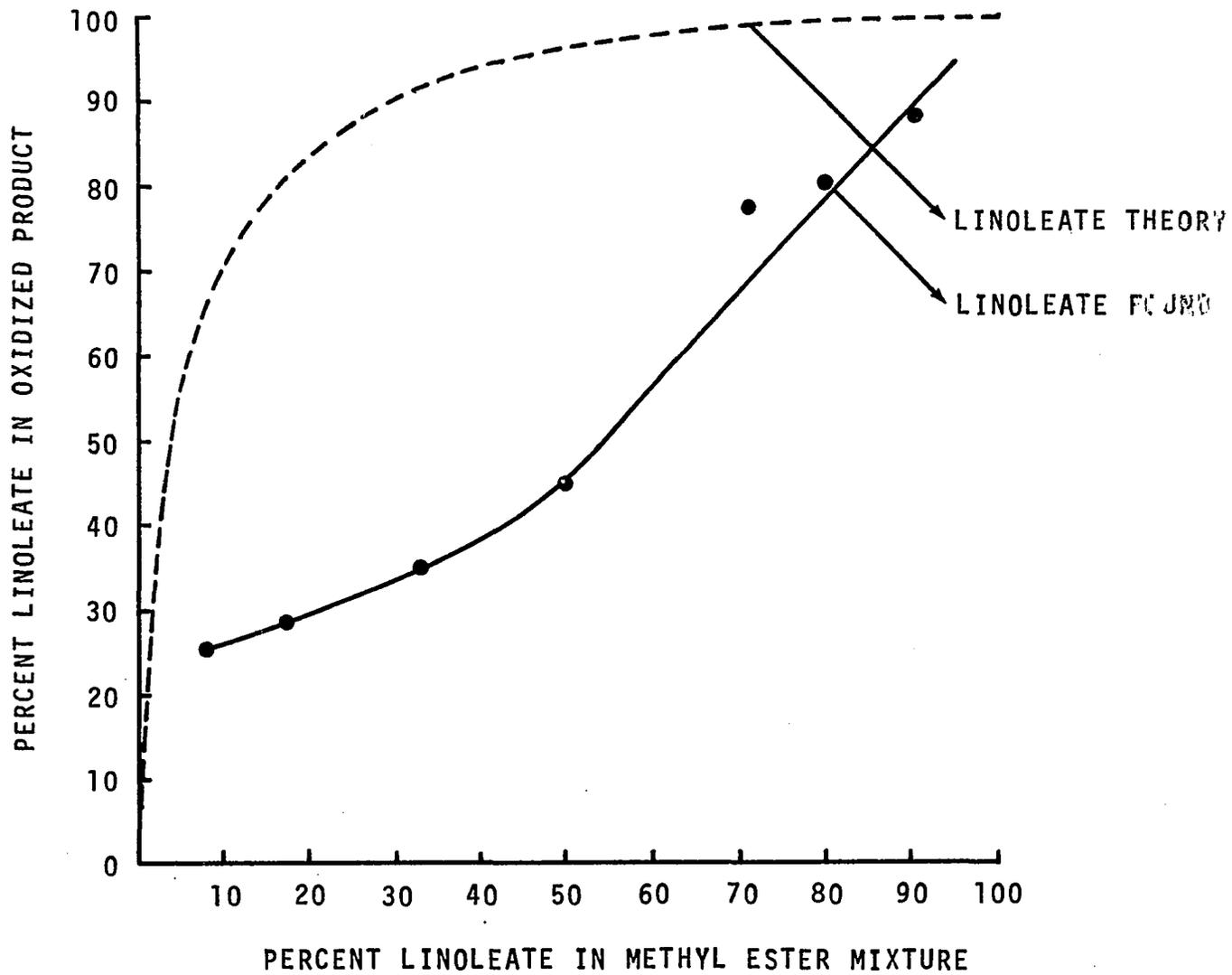
⁵Top figure is 13-hydroxy-diene, and lower figure is 9-hydroxy-diene.

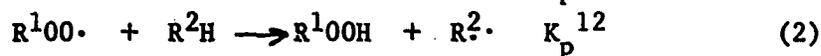
⁶Total diene.

Table 3 (Continued)

| Composition Original Methyl Ester Mixture | P.V. | Days to reach P.V. | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Diene in Oxidized Product % |
|---|-------|--------------------------|-----------------------------------|--|--|-----------------------------------|
| 16:0 = 1.74% | 4.27 | 3 | 97.1 | | 2.55 2.50 5.05 | 100.0 |
| 18:1 = 19.95% | 9.21 | 5 | 96.1 | 3.01 | 5.16 4.89 10.05 | 77.0 |
| 18:2 = 78.30% | 24.18 | 7 | 91.2 | 7.58 | 13.42 13.80 27.22 | 78.2 |
| | 45.11 | 10 | 99.4 | 10.22 | 28.30 27.10 55.40 | 84.4 |
| 16:0 = 3.01% | 4.65 | 2½ | 102.0 | 0.81 | 2.95 3.24 6.19 | 88.4 |
| 18:1 = 9.91% | 10.75 | 4 | 96.9 | 1.95 | 6.55 7.10 13.65 | 87.5 |
| 18:2 = 88.08% | | | | | | |
| | 38.33 | 7 | 96.4 | 5.81 | 24.05 24.05 48.10 | 89.2 |

Figure 4. Oxidation of linoleate at different ratios of linoleate/oleate concentrations. The dotted line indicates linoleate oxidation expected if no interactions occurred based on K_p 's from Howard and Ingold (32).





where R^1 represents oleate, and R^2 represents linoleate, and the K_p 's are rate constants. Assuming the termination reactions are all approximately equal, the ratio of olefin disappearance and hydroperoxide production is given by (5):

$$\frac{d(R^1H)}{d(R^2H)} = \frac{d(R^{100H})}{d(R^{200H})} = \frac{(R^{100\cdot}) K_p^{11}(R^1H) + K_p^{12}(R^2H)}{(R^{200\cdot}) K_p^{22}(R^2H) + K_p^{21}(R^1H)}$$

If the interconversion of peroxy radicals (Reaction 2 and 3) proceeds much more quickly than chain initiation or termination, the radical concentrations ($R^{100\cdot}$) and ($R^{200\cdot}$) may be eliminated by noting that $K_p^{12}(R^{100\cdot})(R^2H) = K_p^{21}(R^{200\cdot})(R^1H)$, hence:

$$\frac{d(R^1H)}{d(R^2H)} = \frac{d(R^{100H})}{d(R^{200H})} = \frac{r_1(R^1H/R^2H) + 1}{r_2(R^2H/R^1H) + 1}$$

where $r_1 = K_p^{11}/K_p^{12}$ and $r_2 = K_p^{22}/K_p^{21}$. This equation indicates that the ratio of oleate oxidized to linoleate oxidized should be a function of the oleate to linoleate ratio, but not a function of the extent of oxidation. Table 3 shows this was true, in general, the most obvious exception being the mixture containing 78.3% linoleate at peroxide value 1.74.

If $\frac{d(R^1H)}{d(R^2H)}$ is measured at several values of $(R^1H)/(R^2H)$, the reactivity ratios r_1 and r_2 can be found graphically (41). By this

method, r_1 was 1.3 and r_2 was 0.84 using the mixtures with 50% or more linoleate. The mixtures with high oleate percentages give values $r_1 = 0.17$ and $r_2 < 1$ (actually negative which is impossible). The reactivity coefficients show that the oleate peroxy radical ($R^1OO\cdot$) attacks oleate slightly better than linoleate, and that the linoleate peroxy radical ($R^2OO\cdot$) attacks oleate slightly better than linoleate. This is surprising, because work on hydrocarbons has indicated that the rates of the propagation reaction are usually dominated by the ease of abstraction of hydrogen from RH rather than the structure of the attacking peroxy free radical. But, if this were true, $r_1 = 0.045$ and $r_2 = 22$ from the data of Howard and Ingold (32), and this obviously is not so.

It would be interesting to carry out similar experiments with dilute solutions of fatty acids in solvents to see if the restraint of the aligned hydrocarbon chains in the liquid methyl esters somehow accounts for these surprising interaction effects. Howard and Ingold (32) apparently oxidized linoleate and linolenate in dilute solutions of chlorobenzene, while oleate was not. This might explain the large differences in K_p .

Oxidation of mixtures of oleate, linoleate and linolenate: Approximately equal amounts of oleate and linoleate methyl esters were added to methyl linolenate to give varying concentrations of linolenate. These were distilled and oxidized as before (METHODS section). The results of the hydroperoxide analysis are given in Table 4. Figure 5 shows the percentage of each individual ester in the total peroxides and the theoretical percentages assuming no interaction versus the

Table 4. Autoxidation products of mixtures of oleate, linoleate and linolenate methyl esters

| Composition Original Methyl Ester Mixture | P.V. | Days to reach P.V. | Re- ¹ cov- ery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Triene ² Peroxide (mg) |
|---|-------|--------------------------|-----------------------------------|--|---|---|
| 18:1 = 17.33% | 5.11 | 2 | 99.5 | 0.41 | 0.43 ³ 0.41 0.84 ⁴ | 6.60 |
| 18:2 = 15.93% | 14.97 | 3 | 99.5 | 1.30 | 1.20 1.15 2.35 | 19.31 |
| 18:3 = 66.73% | 24.04 | 4 | 97.4 | 2.00 | 1.85 1.99 3.84 | 30.78 |
| | 84.20 | 6 | 98.0 | 7.25 | 7.50 7.30 14.80 | 105.18 |
| 18:1 = 20.73% | 6.61 | 2 | 97.4 | 0.58 | 0.60 0.60 1.20 | 8.50 |
| 18:2 = 20.58% | 11.21 | 3½ | 99.5 | 1.03 | 1.00 0.99 1.99 | 14.20 |
| 18:3 = 58.65% | 29.05 | 5 | 99.2 | 2.06 | 2.80 2.50 5.30 | 37.31 |
| | 64.01 | 6 | 99.2 | 5.85 | 5.85 5.80 11.65 | 81.63 |
| 18:1 = 31.28% | 5.01 | 3 | 98.7 | 0.54 | 0.35 0.43 0.78 | 6.41 |
| 18:2 = 29.23% | 10.78 | 6 | 100.0 | 1.10 | 0.95 0.90 1.85 | 14.45 |
| 18:3 = 39.45% | 19.04 | 10 | 100.0 | 2.13 | 1.55 1.68 3.23 | 24.10 |

| | | | | | | | |
|---------------|-------|----|-------|-------|-------|-------|-------|
| 16:0 = 8.05% | 4.61 | 8 | 97.8 | 1.91 | 1.00 | | |
| | | | | | 1.05 | 2.05 | 3.00 |
| | | | | | 2.52 | | |
| 18:1 = 34.49% | 9.68 | 12 | 99.1 | 4.09 | 2.45 | 4.97 | 6.01 |
| | | | | | 5.40 | | |
| 18:2 = 35.63% | 21.75 | 17 | 97.5 | 9.90 | 5.30 | 10.70 | 13.05 |
| | | | | | 9.18 | | |
| 18:3 = 21.92% | 36.75 | 21 | 98.1 | 16.80 | 9.20 | 18.38 | 22.19 |
| | | | | | 1.20 | | |
| 18:1 = 43.78% | 4.96 | 6 | 98.6 | 2.09 | 1.10 | 2.30 | 2.45 |
| | | | | | 2.00 | | |
| 18:2 = 46.44% | 9.03 | 9 | 100.0 | 5.52 | 2.10 | 4.10 | 4.23 |
| | | | | | 4.51 | | |
| 18:3 = 9.76% | 19.42 | 15 | 88.8 | 9.50 | 4.82 | 9.33 | 10.00 |
| | | | | | 10.01 | | |
| | 38.09 | 20 | 98.9 | 18.25 | 9.90 | 19.91 | 18.88 |

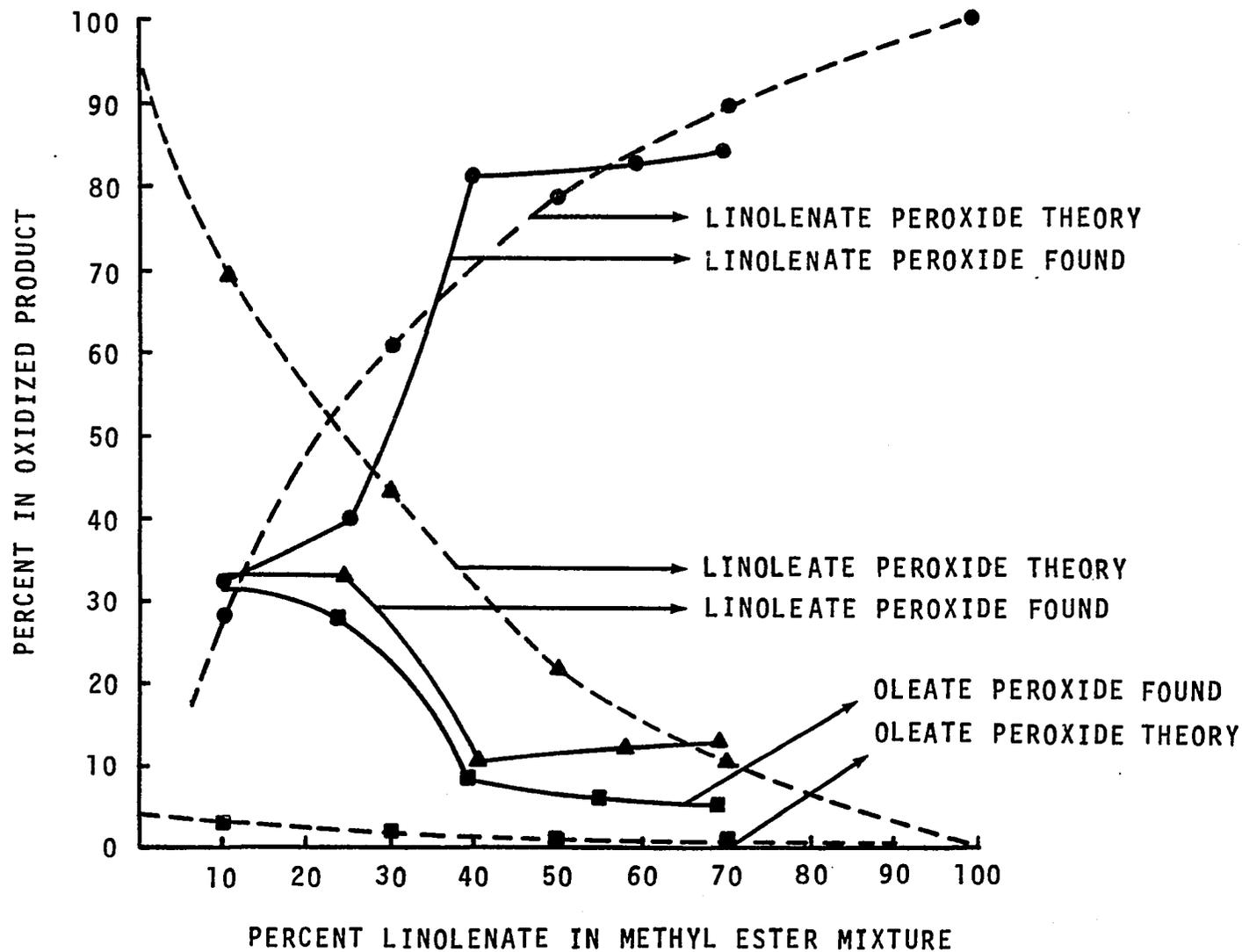
¹Based on recovery of butylated methyl esters and the observed peroxide value using butylated methyl ricinoleate as a densitometric standard.

²Hydroxy methyl esters recovered using methyl ricinoleate as a densitometric standard.

³Top figure is 13-hydroxy-diene and lower figure is 9-hydroxy-diene.

⁴Total diene.

Figure 5. Oxidation of different concentrations of linolenate in approximately equal amounts of oleate and linoleate. The dotted lines indicate the theoretical amounts of individual ester if no interaction occurred based on K_p 's from Howard and Ingold (32).



linolenate concentration.

Again, approximately equal amounts of the 9 and 13 hydroxy-diene were found under all conditions. No evidence of cis monoene hydroperoxide was found but the oleate percentage never exceeded 44%.

In the oxidation of a two compound mixture, there are four propagation steps; in a mixture with three compounds, there are nine propagation steps. A rate equation was derived:

Let x, y, z = oleate, linoleate and linolenate, respectively
 x', y', z' = oleate, linoleate and linolenate peroxy free radicals, respectively
 $a, b, c, d, e,$
 f, g, h, i = rate constants

The propagation steps are given by:

axx', dxy', gxz'
 byx', eyy', hyz'
 czx', fzy', izz'

The disappearance of each olefin is given by:

$$\begin{aligned} \mathcal{J}x &= axx' + dxy' + gxz' & (1a) \\ \mathcal{J}y &= byx' + eyy' + hyz' & (1b) \\ \mathcal{J}z &= czx' + fzy' + izz' & (1c) \end{aligned}$$

and since to have a steady state:

$$\begin{aligned} byx' + czx' &= dxy' + gxz' \\ dxy' + fzy' &= hyz' + byx' \\ gxz' + hyz' &= czx' + fzy' \end{aligned}$$

then:

$$\begin{aligned} \mathcal{J}x &= axx' + dxy' + gxz' = (ax + by + cz)x' & (2a) \\ \mathcal{J}y &= byx' + eyy' + hyz' = (dx + fz + ey)y' & (2b) \\ \mathcal{J}z &= czx' + fzy' + izz' = (gx + hy + iz)z' & (2c) \end{aligned}$$

and for x' :

$$x' = \frac{dxy' + gxz'}{by + cz} \quad (3)$$

From equations in group 2:

$$x' = \frac{dx}{ax + by + cz} \quad (4a)$$

$$y' = \frac{dy}{dx + fz + ey} \quad (4b)$$

$$z' = \frac{dz}{gx + hy + iz} \quad (4c)$$

and by substitution in equation 3 values of equations in group 4:

$$\begin{aligned} \frac{(by + cz) dx}{ax + by + cz} &= \frac{dx dy}{dx + fz + ey} + \frac{gx dz}{gx + hy + iz} \\ \frac{dx}{dy} \frac{by + cz}{ax + by + cz} &= \frac{dx}{dx + fz + ey} + \frac{gx \frac{dz}{dy}}{gx + hy + iz} \\ \frac{dx}{dy} \left(\frac{1}{\frac{ax}{by + cz} + 1} \right) &= \frac{1}{1 + \frac{fz + ey}{dx}} + \frac{dz}{dy} \frac{1}{1 + \frac{hy + iz}{gx}} \\ \frac{dx}{dy} &= \frac{\frac{ax}{by + cz} + 1}{\frac{fz + ey}{dx} + 1} + \frac{dz}{dy} \frac{\frac{ax}{by + cz} + 1}{\frac{hy + iz}{gx} + 1} \\ \frac{dx}{dy} &= \frac{\frac{1}{\frac{by}{ax} + \frac{cz}{ax}} + 1}{\frac{fz}{dx} + \frac{ey}{dx} + 1} + \frac{dz}{dy} \frac{\frac{1}{\frac{by}{ax} + \frac{cz}{ax}} + 1}{\frac{hy}{gx} + \frac{iz}{gx} + 1} \quad (5) \end{aligned}$$

The equation is solved in the same way to get:

$$\frac{dy}{dz} = \frac{\frac{1}{\frac{dx}{ey} + \frac{fz}{ey}} + 1}{\frac{gx}{hy} + \frac{iz}{hy} + 1} + \frac{dx}{dz} \frac{\frac{1}{\frac{dx}{ey} + \frac{fz}{ey}} + 1}{\frac{ax}{by} + \frac{cz}{by} + 1} \quad (6)$$

$$\frac{dz}{dx} = \frac{\frac{1}{\frac{ax}{cz} + \frac{by}{cz} + 1}}{\frac{gx}{iz} + \frac{hy}{iz} + 1} + \frac{dy}{dx} \frac{\frac{1}{\frac{ax}{cz} + \frac{by}{cz} + 1}}{\frac{dx}{fz} + \frac{ey}{fz} + 1} \quad (7)$$

By using the experimental data and calculating the ratios of the constants that best fit equation 5, the following values were found $c/a = 3$, $f/d = 1$, $h/g = 4$ and $i/g = 10$, assuming $a/b = 1.3$ and $e/d = 0.85$ as in the previous experiment. The values of the four ratios determined on the ternary mixture are subject to considerable error. It is also possible the values of the ratios are not the same for all ester ratios as was found with the binary mixtures. But taken at face value, they indicate that the oleate-peroxy radical attacked linolenate three times faster than oleate (c/a); the linoleate-peroxy radical attacked linolenate and oleate equally well (f/d); the linolenate-peroxy radical attacked linoleate four times faster than oleate (h/g); and the peroxy-linolenate radical attacked linolenate ten times faster than oleate (i/g).

That h/g and i/g must be larger than the other ratios is obvious from Figure 5, for the percentage of linolenate oxidized rises sharply with linolenate concentration. The percentage of linoleate oxidized shows a modest increase compared with oleate as the proportion of linolenate in the ester mixture increases.

The results indicate that there is something about the linolenic molecule that makes it more easily oxidized than oleate and linoleate. This may simply be the fact that it has two positions that may be attacked (C_{11} and C_{14}) that should have reactivities similar to the

single position (C₁₁) in linoleate. If the interaction rate equation constants took into account the concentrations in terms of active centers, the rate for linolenate might fall more into line with the others. It is also known that pure linolenate oxidizes about twice as fast as linoleate (24, 31).

It is surprising from a theoretical standpoint that linoleate is more like oleate in its behaviour than linolenate, and this implies that ease of abstraction of hydrogen from the α -positions of the various fatty acids does not dominate the rate of oxidation. But these results make considerable sense from a practical standpoint. It has long been known that a low percentage of linolenic acid was quite detrimental to the flavor of an oil. The relatively rapid rate of its oxidation could help it dominate the flavor of oils containing it. Also 2-pentylfuran, an oxidation product from linoleate, is believed to dominate the flavor of soybean oil, although the flavor instability of the oil is attributed to linolenic acid. The ability of linolenic acid to enhance the oxidation of linoleic may account for this paradox.

Oxygen uptake compared with peroxide values: The conclusions we have drawn depend on the assumption that most of the oxygen taken up by the fat is in the form of hydroperoxides and that the high concentrations of oleate peroxide found are not caused by its relative stability to destruction rather than its rate of production. Several researchers (28, 39) have found most of the oxygen taken up by fats is in the form of peroxides in the temperature range used.

To test this, measurements were conducted at 28C in a Warburg

respirometer using 5 and 10 g samples in large flasks (170 ml) with shaking. The samples were equilibrated for a $\frac{1}{2}$ hr before the manometers were closed. When the samples had attained previously selected levels of oxygen absorption (equal to peroxide values of 5, 10 and 20), they were immediately removed and the peroxide test (43) was performed. Table 5 contains the results of the experiment.

Table 5. Oxygen uptake of a fatty acid methyl ester mixture

| Composition of fatty acid methyl ester mixture | PV ¹ | PV ² | Time to reach PV | Recovery ³ % | Oleate ⁴ (mg) | Linoleate ⁴ (mg) | Oxidized Linoleate in oxidized product % |
|--|--------------------|-----------------|------------------|-------------------------|--------------------------|-----------------------------|--|
| 16:0 = 3.01% | 6.23 | 7.12 | 12 | 97.4 | 0.95 | 4.85 4.80 | 91.0 |
| 18:1 = 9.91% | 9.70 | 11.73 | 18 | 100.0 | 1.64 | 8.25 8.58 | 90.9 |
| 18:2 = 88.08% | 19.34 ⁵ | 19.47 | -- | ----- | ----- | ----- | ----- |
| | 21.17 ⁵ | 24.80 | 24 | 99.0 | 3.11 | 15.50 15.58 | 90.9 |

¹Peroxide value based on calculations of oxygen uptake.

²Peroxide value by iodide reduction (43).

³Based on recovery of butylated methyl esters using butylated methyl ricinoleate as a standard, and using the iodide reduction peroxide value as a basis for calculation.

⁴Based on hydroxy methyl esters using methyl ricinoleate as a standard.

⁵Due to limitation of oxygen in the flask two 5 g samples were used.

When five gram samples were used so that sufficient oxygen was present to give the desired peroxide value, two of them were combined and an average value taken for calculations to give enough product for the thin-layer analysis. The difference in peroxide values between oxygen uptake and iodide reduction is probably due to formation of

peroxides during the period of equilibrium. The samples oxidized faster than the corresponding samples in Table 3. The reason for the faster rate might be due to the difference in lighting between the dark incubator and the laboratory lights over the Warburg apparatus. The oxygen taken up by the fat is essentially converted to hydroperoxides. There are no obvious differences in the types of peroxides formed in the Warburg apparatus compared with the sample of similar composition run in the incubator. There is no evidence of extensive peroxide destruction under the conditions used.

Triglyceride structure and oxidation: The final experiment was to see if triglyceride structure had an effect on peroxide formation. To do this several vegetable oils were deodorized (57) and soybean and corn oil were also randomized. In randomization the fatty acids are detached from their original position on glycerol, then reformed in a random manner. This alters the concentration at the 1, 2 and 3 positions of glycerol. The fatty acid composition of the oils is given in Table 6.

Table 6. Fatty acid composition of methyl esters of oils by gas-liquid chromatography by percent

| Fatty acids | Safflower oil | Linseed oil | Soybean oil | Randomized | Corn oil | Randomized |
|-------------|---------------|-------------|-------------|-------------|----------|------------|
| | | | | Soybean oil | | Corn oil |
| 16:0 | 9.12 | 6.75 | 10.64 | 10.79 | 11.68 | 11.28 |
| 18:0 | 3.19 | 1.86 | 4.36 | 4.28 | 1.69 | 1.90 |
| 18:1 | 12.52 | 18.37 | 24.29 | 24.28 | 24.53 | 24.88 |
| 18:2 | 75.17 | 16.29 | 54.24 | 54.42 | 60.99 | 60.72 |
| 18:3 | | 56.73 | 6.82 | 6.21 | 1.35 | 1.19 |

Peroxide analysis of the oils before oxidation showed all to contain negligible amounts of hydroxy acids.

The results of autoxidation of the natural oils are given in Table 7. As can be seen by comparing Tables 3 and 7, the recoveries as butylated methyl esters are approximately 10% lower with triglycerides than with the pre-formed methyl esters. The methylation with sodium methoxide as a catalyst seems to interfere with or attack the hydroxy methyl esters. A sample of the safflower oil saponified and then methylated with 2% sulfuric acid as a catalyst, gave even lower recoveries (last row Table 7). Raghuvver (50) also found lower recoveries from soybean oil but not olive oil using 0.4% sulfuric acid as a catalyst compared with recoveries from methyl esters. Several groups (59, 66) have found that acid catalysts dehydrate hydroxy groups α to a double bond, but methylation with sodium methoxide is not supposed to dehydrate the double bond. I found dehydration when pure hydroxy-diene was refluxed with 2% methanolic hydrochloric acid. But with the safflower oil sample where small amounts (50-60 mg) of the hydroxy-acids were present in 10 g of fat, the dehydration was much less severe. Some preferential dehydration of the conjugated diene took place during the acid catalyzed formation of methyl esters from safflower oil. Eighty percent hydroxy-diene was recovered by this method while in the sodium methoxide catalyzed transesterification, 87% hydroxy-diene was recovered.

Raghuvver (50) used an acid catalyst because he saponified triglycerides to remove nonsaponifiables. In my work, the nonsaponifiables were not removed as they did not interfere with the thin-layer analysis.

Table 7. TLC analysis of the effect of triglyceride structure on the formation of peroxide types

| Oil | P.V. | Days to reach P.V. | Re- ¹ covery % | Monoene ² Peroxide (mg) | Diene ² Peroxide (mg) | Triene ² Peroxide (mg) |
|------------------------|--------------------|--------------------|---------------------------|------------------------------------|----------------------------------|-----------------------------------|
| Corn Oil | 4.05 | 15 | 89.7 | 2.25 | 1.70 1.60 3.30 6.35 | |
| | 9.12 | 26 | 87.2 | 9.21 | 6.50 12.85 | |
| Randomized Corn Oil | 6.75 | 3 | 83.5 | 2.99 | 2.81 2.85 5.66 6.92 | |
| | 16.10 | 5 | 88.8 | 7.48 | 6.99 13.99 | 0.53 |
| Soybean Oil | 3.98 | 13 | 90.0 | 1.02 | 0.85 0.85 1.70 1.40 | 2.68 |
| | 7.05 | 25 | 86.5 | 1.91 | 1.29 2.69 | 4.75 |
| Randomized Soybean Oil | 7.89 | 2 | 84.7 | 1.78 | 2.50 2.60 5.10 6.07 | 2.95 |
| | 18.15 | 4 | 88.3 | 4.93 | 6.00 12.07 | 7.23 |
| Linseed Oil | 5.91 | 1 | 89.7 | 0.85 | 2.40 2.12 4.62 5.40 | 2.83 |
| | 15.01 | 3 | 86.2 | 2.10 | 5.00 10.40 | 6.75 |
| Safflower Oil | 6.81 | 2 | 88.1 | 1.24 | 4.07 3.90 7.97 7.60 | |
| | 13.10 | 4 | 84.2 | 2.10 | 7.35 14.95 | |
| | 13.10 ³ | 4 | 72.2 | 2.80 | 5.90 5.31 11.21 | |

¹Based on recovery of butylated methyl esters and the observed peroxide value using butylated methyl ricinoleate as a densitometric standard.

²Hydroxy methyl esters recovered using methyl ricinoleate as a densitometric standard.

³Methyl esters prepared by using 2% sulfuric acid instead of sodium methoxide as a catalyst.

They do interfere with the analysis of Spot C, Figure 1, but since this material is now known to be an anhydride, its analysis is not important. It is difficult to remove nonsaponifiables and avoid the use of acid catalysts to form methyl esters.

With corn oil the oxidized product contained 59.5% hydroxy-diene and when compared with the graph (Figure 4) for oleate-linoleate mixtures at 73% (the percentage of linoleate in the oleate-linoleate fraction of corn oil), the point falls well below the curve. No hydroxy-triene was found. In the randomized corn oil, there was 65.5% hydroxy-diene in the oxidized product. Within experimental error, this value falls on the curve (Figure 4) for mixtures of oleate-linoleate. Randomized oils should behave more like methyl ester mixtures than the nonrandomized oils. At a peroxide value of 16.10, hydroxy-triene was found in small amounts. The randomized corn oil oxidized much faster than corn oil. The faster rate of oxidation of randomized corn oil agreed with the findings of Raghuvver and Hammond (51).

The thin-layer analysis of soybean oil and randomized soybean oil showed some surprising results. In the oxidized products of soybean oil, there was 18.9% hydroxy-monoene, 31.5% hydroxy-diene and 49.6% hydroxy-triene, while in randomized soybean oil there was 18.1% hydroxy-monoene, 51.9% hydroxy-diene and 30.0% hydroxy-triene. The randomized soybean oil oxidized much faster than the soybean oil (51).

Linseed oil had 10.4% hydroxy-monoene, 55.1% hydroxy-diene and 35.5% hydroxy-triene in the oxidized product. The hydroxy-diene is present in much larger amounts than expected from the methyl ester

mixtures, while the hydroxy-triene is much lower than expected.

Safflower oil with 13.5% hydroxy-monoene and 86.5% hydroxy-diene in the oxidized product would fall on the curve for methyl ester mixtures (Figure 4).

These results show that fatty acids present in triglycerides often oxidize in different proportions than would be predicted from oxidation of the corresponding methyl ester mixtures. This result seems to be more related to the distribution of the acyl groups on the glycerol than to the restraint of the glycerol itself, because in both the instances of soybean and corn oil, randomization caused a shift in oxidation pattern to one closer to that predicted from methyl esters. This supports the earlier findings of Raghuvver and Hammond (51) that glyceride distribution can make the local effective concentrations of an acyl group different from that calculated from its concentration in the whole oil.

SUMMARY

Mixtures of oleate and linoleate; oleate, linoleate and linolenate, and several vegetable oils were autoxidized in 50 g lots at 28C. Samples were withdrawn periodically to determine the peroxide value by the method of Hamm et al. (26). When the test showed peroxide values of approximately 5, 10, 20 and 40, samples were withdrawn from the methyl ester mixtures. The peroxides were reduced to alcohols by the iodometric method recommended in the official method of the American Oil Chemists' Society (43). The hydroxy acids were then butylated by butyric anhydride. After concentration by urea fractionation, the butylated reduced hydroperoxides were quantitatively analyzed by thin-layer chromatography. The recoveries of the butylated methyl esters varied from 93-102% based on butylated methyl ricinoleate as a standard.

Saturated fatty esters and nonsaponifiables had little or no effect on the peroxide types formed. In a series of mixtures of oleate and linoleate, it was found that the oleate and linoleate did not oxidize in the ratios predicted by research (24, 31) on the pure individual methyl esters. This indicates extensive interaction between the oxidizing species. At linoleate concentrations greater than 50%, the linoleate oxidized in direct proportion to its concentration, at lower linoleate concentrations a slightly greater proportion of the linoleate oxidized.

Mixtures of approximately equal amounts of oleate and linoleate with varying concentrations of linolenate were prepared. Again the methyl esters did not oxidize in the ratio predicted from pure methyl

esters (24, 31). An equation was derived to predict the interactions during oxidation and its constants were evaluated with the data. The linolenate was much more readily oxidized than linoleate and oleate, and it induced the linoleate to oxidize to a greater extent than found in the oleate and linoleate mixture.

Approximately equal amounts of 9- and 13-hydroxy-dienoate were found under all conditions, indicating that the 9- and 13-hydroperoxide of linoleate were formed in equal amounts. Cis-hydroxy-monoene was found when mixtures containing high concentrations of oleate were oxidized to peroxide values greater than 20.

The autoxidation of natural oils showed that glyceride structure also had an effect on peroxide types. Randomized soybean and corn oil when autoxidized formed peroxide types in approximately the proportions predicted by the mixtures of methyl esters, but quite different proportions were found among some of the natural fats and oils.

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