A Chromatographic Study of Autoxidized Methyl Linoleate*

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IT has long been known that the autoxidation of fats is accompanied by the formation of peroxidic compounds which more or less chart the extent of the spoilage of fats by oxidative processes. These peroxidic compounds resulting from fat autoxidation are therefore of great interest, but they have proved to be difficult to separate and characterize.

In a study of the autoxidation of methyl linoleate attempts were made to separate the oxidation products by means of chromatographic adsorption methods. The results of such attempts could be evaluated by a study of the ultraviolet absorption spectra and by determination of the peroxide content.

Bergström (1) and Bolland and Koch (2) have used alumina to adsorb the products resulting from the autoxidation of methyl linoleate at 37°C., but in neither case was the relationship between the peroxide value (PV) of the separated products of oxidation and their spectral properties clearly brought out.

In this study a specially prepared sodium aluminum silicate was used for the adsorbent, as described by Kraybill *et al.* (3). The columns were sectionalized and studied in light of the concepts pointed out by Strain (4) that the adsorbability of organic compounds is influenced primarily by the nature and the number of the polar groups in their molecules.

In the preliminary development of a chromatographic technique for this study the methyl esters of corn oil were used in order to conserve pure methyl linoleate. Because corn oil contains from 50-60% linoleic acid (5, 6), it follows that the peroxide values determined and the spectral characteristics observed would be due in a large measure to the oxidized linoleic acid in the corn oil.

Experimental

The sodium aluminum silicate used was found to be superior to silica gel for this study. The column (inside diameter one inch) was loosely packed with adsorbent and thoroughly wetted with isooctane. The esters were placed on the column as soon as the wetting solvent had entered the column. Development was accomplished by means of the same solvent without the aid of reduced pressure. In most cases 200 ml. of isooctane was sufficient to develop a column containing 24 g. of adsorbent and 5 g. of esters. As soon as the last of the 200 ml. of solvent had passed through the column, the contents were pushed out and divided into sections.

The division into sections was usually from the top of the column in the following sequence: (a) 0-1 inch (b) 1-3 inches, (c) 3-6 inches, and (d) 6-9 inches with the length of the last section varying with the total length of the column of adsorbent.

The esters were eluted from each section using 100 ml. of ethanol which had been purified for optical use. These eluates were then made up to definite volumes and aliquots used for spectrophotometric studies and determinations of the peroxide content. The concentrations of substances in the ethanol were determined by evaporation of aliquots under reduced pressure in a tared flask.

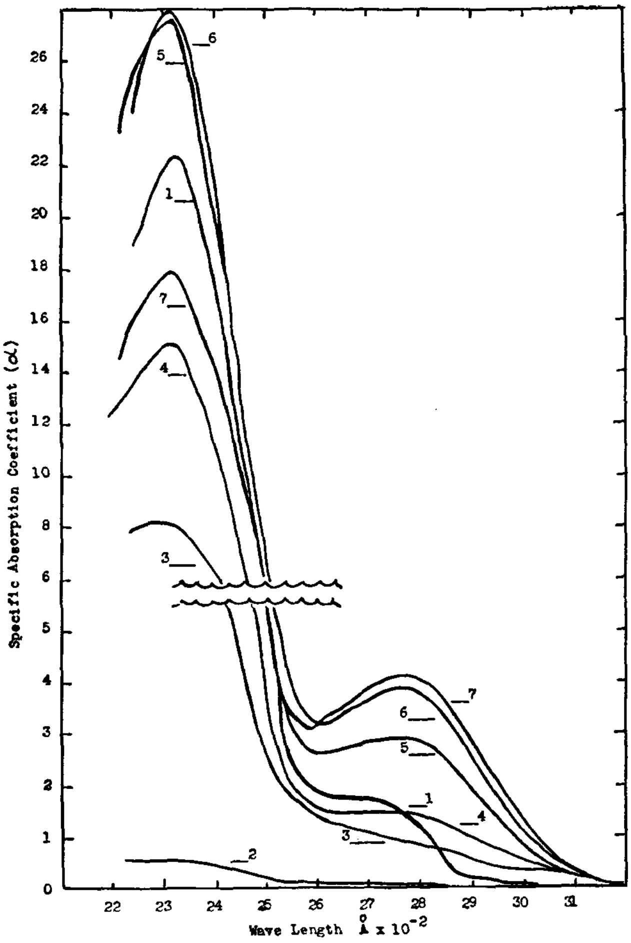

FIG. 1. *1.* Oxidized Esters, PV 1003; *2.* Ester in Percolate, PV 28; *3.* Ester on 0-1 in. section, PV 2125; *4.* Ester on 1-3 in. section, PV 2790; *5.* Ester on 3-5 in. section, PV 2470; *6.* Ester on 5-7 in. section, PV 2460; *7.* Ester on 7-8 in. section, PV 1660.

Discussion

Table I illustrates the fractionation obtained in a typical experiment. It shows that a sample of corn oil methyl esters oxidized to a PV of 1003 m.e./kg. was chromatographically separated into fractions with PV's ranging from 28 to 2790 m.e./kg. The PV of the esters was reduced from 1003 to 28 by being passed through the column and the materials retained on the column possessed much higher PV's than that of the original oxidized esters. (In another experiment, not described here, a fraction with a PV of 3800 was obtained from esters which had been oxidized to PV 263.)

Fig. 1 shows the ultraviolet absorption spectra of the fractions described in Table I. As evidenced by both the PV and the absorption spectra, the column very effectively adsorbed the major portion of the oxygenated substances. A comparison of the curve for the oxidized ester (curve 1) with that for

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TABLE I

Chromatographic Adsorption of Oxidized Methyl Esters of Corn Oil

Fraction	Wt. of Ester in Fraction (grams)	Peroxide Value m.e./kg.
Oxidized esters	5.35	1003
Ester in percolate	4.06	28
Ester on 0-1 inch section	0.22	2125
Ester on 1-3 inch section	0.29	2790
Ester on 3-5 inch section	0.25	2470
Ester on 5-7 inch section	0.27	2458
Ester on 7-8 inch section	0.18	1660
	5.29	

the ester in the percolate (curve 2) shows a relatively complete removal of ultraviolet-absorbing substances on passage through the column. The various adsorbed fractions exhibit maxima in both the 2750 and 2315 Å regions. The absorption at 2750 increased progressively from the top of the column to the bottom.

Studies on Oxidized Methyl Linoleate

The procedures described above were then applied to studies of oxidized methyl linoleate. Typical experimental observations were as follows: 13.30 g. of oxidized esters with a peroxide content of 5.10 m.e. corresponding to a PV of 377 m.e./kg. were put onto a column wet with isooctane. The 250 ml. of percolate contained 12.501 g. of ester with 0.084 m.e. peroxide corresponding to a PV of 7 m.e./kg. The contents were removed from the column and divided into sections from the top: (a) 0-1 inch, (b) 1-3 inches, (c) 3-6 inches, and (d) 6-10 inches each of which was eluted in a beaker with 100 ml. ethanol. Elution of the sections yielded:

Section (a) 0.1395 g. with a PV of 3500 m.e./kg.
Section (b) 0.2415 g. with a PV of 3280 m.e./kg.
Section (c) 0.2220 g. with a PV of 4000 m.e./kg.
Section (d) 0.3910 g. with a PV of 1000 m.e./kg.

Recovery was 13.495 for 13.30 g. put onto the column and 3.329 m.e. peroxide for 5.10 m.e. put on.

The effectiveness of the sodium aluminum silicate as an adsorbent for the oxygenated compounds resulting from the autoxidation of methyl linoleate is clearly shown. In all cases, regardless of the PV of the ester prior to passage onto the column, a concentration of oxygenated substances is demonstrated both by the very marked reduction in PV of the ester in the percolate and the high PV of the substances concentrated in the various sections of the adsorption column. For example, consider the experiment mentioned above. The oxidized ester went onto the column with a PV of 377 m.e./kg. and the non-adsorbed ester in the percolate had a PV of 7. Examination of the adsorbent then showed that the substances on the top 1-inch section of the column had a PV of 3500 while those on the next 2-inch section down the column had a PV of 3280, the next 3-inch section had a PV of 4000 and the lowest section had a PV of 1000. This indicates that the peroxidic substances are not only held on the column but are held in sections or bands. Assuming that the peroxidic compounds held on the upper sections of the column are the more highly oxygenated substances containing the secondary oxidation products which may be di-peroxides, hydroxy compounds, oxido compounds, keto compounds, and scission products, it would seem likely that the high peroxide content of the lower sections would be due mainly to the primary oxidation products, namely, the isomeric methyl hydroperoxido linoleates. Associated with the primary oxidation products would be some secondary products having only single oxygen-containing polar groups such as a doubly unsaturated ketone which may or may not be conjugated.

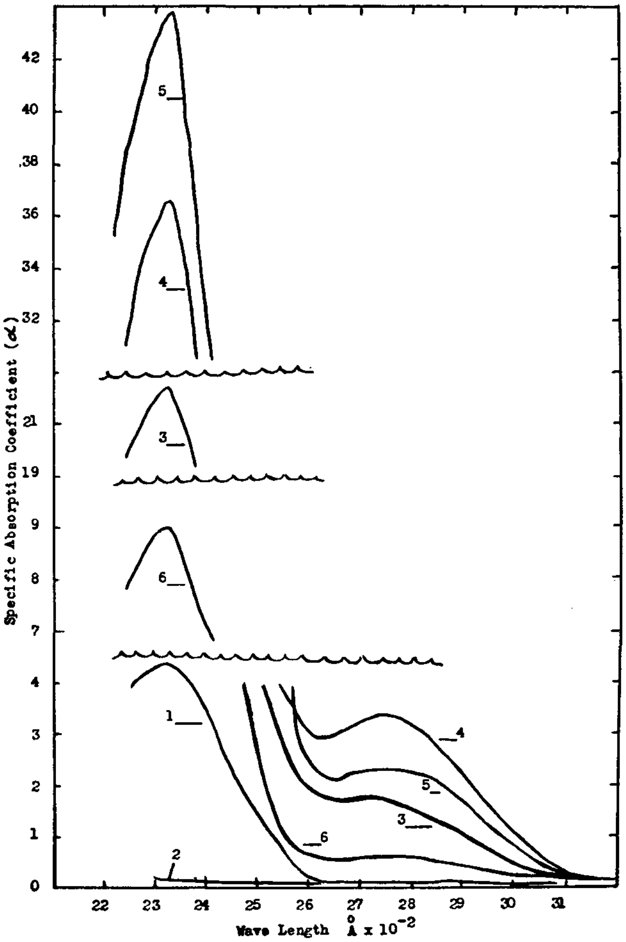


FIG. 2. *1.* Oxidized Ester, PV 377; *2.* Ester in Percolate, PV 7; *3.* Ester on 0-1 in. section, PV 3500; *4.* Ester on 1-3 in. section, PV 3280; *5.* Ester on 3-6 in. section, PV 4000; *6.* Ester on 6-10 in. section, PV 1000.

On the basis of peroxide value alone the substances eluted from section (c) in the experiment above with a PV of 4000 could be interpreted as being two-thirds oxidized, or rather that approximately two-thirds of the molecules are oxidized to a monohydroperoxide based on a theoretical PV of about 6125, thus indicating that a marked step toward separation and isolation of the primary oxidation products of methyl linoleate has been made.

Ultraviolet Absorption Studies of Oxidized Methyl Linoleate Adsorbed on Sodium Aluminum Silicate

Absorption spectra were determined on the oxidized esters both prior to and after passage through the columns and on the eluates from the various sections. The eluates were made up to volume and aliquots were taken for the dilutions required.

In Fig. 2 are shown the absorption curves for methyl linoleate oxidized to a PV of 377 as described above. The spectrum of the oxidized ester exhibits a maximum at 2315 Å (Curve 1). The holdup of oxygenated substances was very good as indicated by the low PV and also by the absorption curve for the ester in the percolate (Curve 2). Substances with extremely high PV's were adsorbed on the upper sections of the column. All of the fractions show well defined maxima at 2315 Å, indicating a large amount of diene conjugation. The most strongly adsorbed substances, i.e., those adsorbed at the top of the column, have a much lower absorption maximum than do those from other sections having a comparable PV. In the 2750 Å region, however, maximum absorption occurred with the substances on the 1-3 inch section of the column.

In Fig. 3 are shown the absorption spectra for an experiment in which the ester was oxidized to a PV of 990 m.e./kg. Again a strong maximum occurs at 2315 Å and strong absorption at 2760 Å. The ester in the percolate was not stripped as clean in this experiment as is shown by the maxima occurring at 2680 and 2315 Å. In this experiment maximum absorption and position on the adsorption column are in inverse order both in the 2315 and the 2760 Å regions. Note that the substances eluted from the bottom section have a high absorption coefficient.

In Fig. 4 are shown the absorption spectra for the ester sample oxidized to a PV of only 47 m.e./kg. prior to passage onto the column. A typical triene structure at 2680 Å is evident in the absorption curve for both the oxidized ester and the ester in the percolate with both showing an inflection at 2315 Å. This ester with the low PV was adsorbed on the column in such a manner that the maximum absorption both at 2760 and 2315 Å was greatest for the substances adsorbed on the top section and consecutively less for those adsorbed on the lower sections.

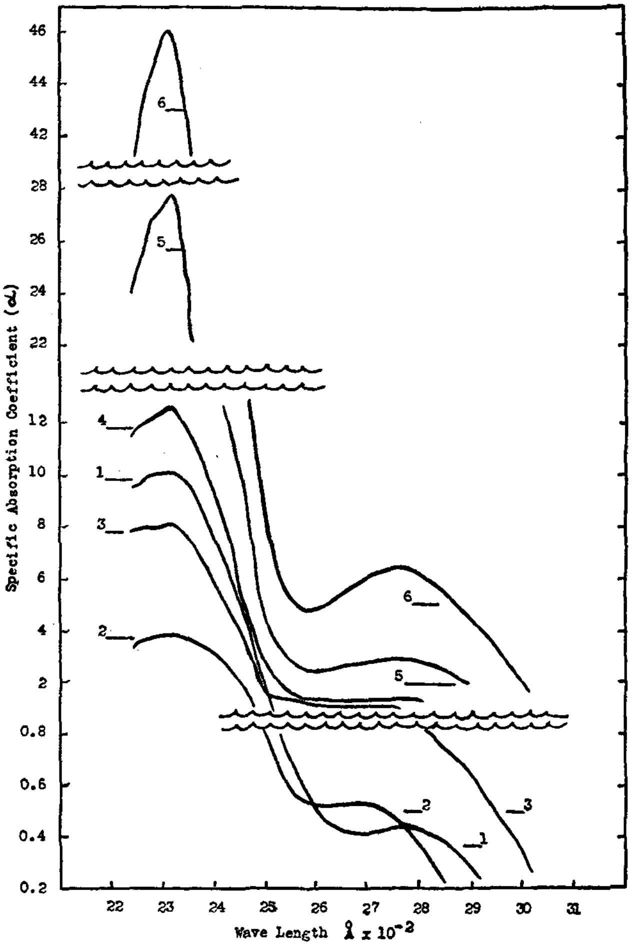


FIG. 3. *1.* Oxidized Ester, PV 990; *2.* Ester in Percolate, PV 20; *3.* Ester on 0-1 in. section, PV 1680; *4.* Ester on 1-3 in. section, PV 1860; *5.* Ester on 3-6 in. section, PV 1460; *6.* Ester on 6-9 in. section, PV 2170.

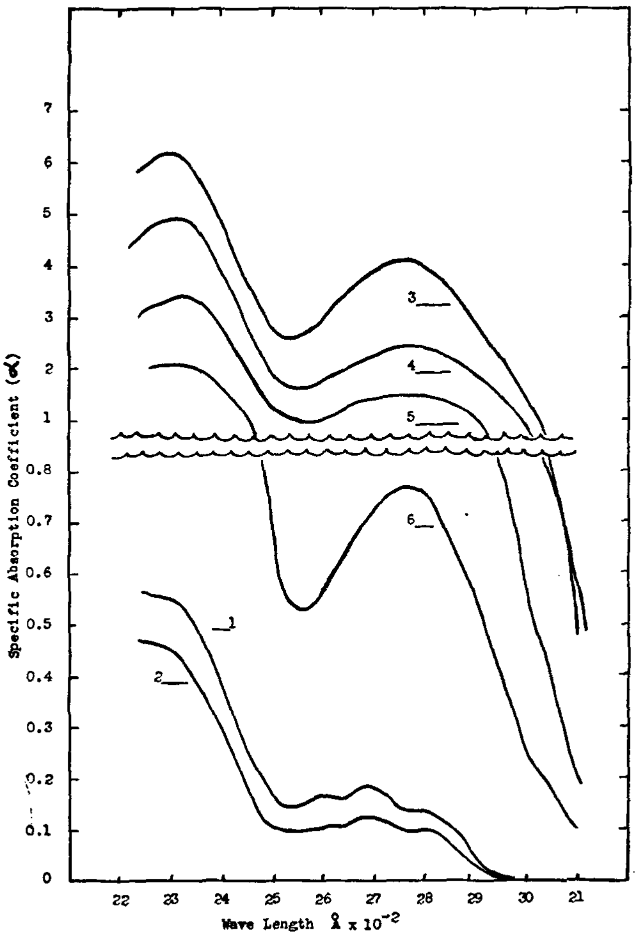
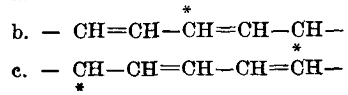


FIG. 4. *1.* Oxidized Ester, PV 47; *2.* Ester in Percolate, PV 15; *3.* Ester on 0-1 in. section, PV 409; *4.* Ester on 1-3 in, section, PV 427; *5.* Ester on 3-6 in. section, PV 236; *6.* Ester on 6-9 in. section, PV 194.

Discussion

As evidenced by both the PV determinations and the absorption curves for the example cited from the corn oil investigation, the most highly oxygenated substances (not necessarily those with the highest PV) seem to be concentrated on the upper portion of the column as would be expected from the concept that molecules with greatest polarity will be adsorbed more strongly and therefore should appear in the upper portion of the column. The less oxygenated and therefore the less polar substances should be found lower on the column. This same relationship has been found to hold with the adsorbed fractions from the oxidized methyl linoleate.

Reaction mechanisms proposed by Bolland and Koch (2) or by Farmer (7) may be used in an explanation of the formation of the primary oxidation products. Bolland and Koch postulated the attack of the methylene group between double bonds to form a free radical which is a resonance hybrid of three equivalent canonical structures:



9 10 11 12 13

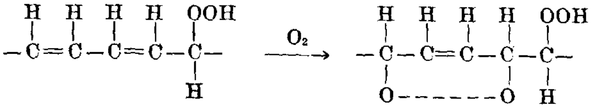
a. — $\mathrm{CH{=}CH{-}\underset{*}{CH}{-}CH{=}CH{-}}$

b. — $\mathrm{CH{=}CH{-}CH{=}CH{-}\underset{*}{CH}{-}}$

c. — $\mathrm{\underset{*}{CH}{-}CH{=}CH{-}CH{=}CH{-}}$

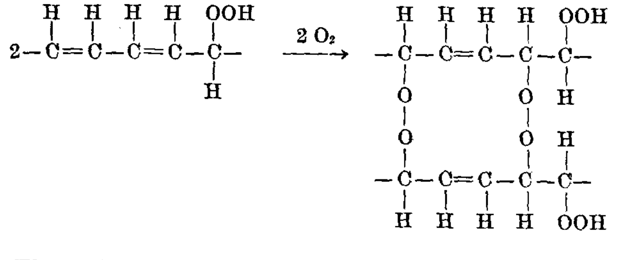
Hydroperoxides formed from the free radical would be expected to contain three isomeric forms, two-thirds of which would be conjugated assuming a random formation; but it seems likely that the conjugated forms would predominate because of their resonance energy. Farmer proposed a mechanism involving universal initiation of autoxidative attack in all the various kinds of olefinic systems by addition occurring at double bond centers, and formulation of the alpha-methylenic reaction characteristic of unconjugated olefins by an inter-molecular scheme. According to Farmer's scheme, the extent of actual addition to the double bonds of alpha-methylene systems would be relatively insignificant but would be sufficient to start the necessary reaction chains. Either of the reaction mechanisms proposed would then result in at least two-thirds of the molecules attacked by a single molecule of oxygen becoming diene-conjugated with the formation of a monohydroperoxide.

The monohydroperoxides formed by the schemes proposed above should absorb intensely in the conjugated-diene region and a number of the curves (not all shown here) for substances on the lower portions of their respective adsorption columns show a somewhat comparable relationship between absorption maxima at 2310 Å and PV. Those absorption curves for the substances found on the upper portions of the columns, and for which the PV's were high, were found to absorb much less intensely and in general to have a broadened absorption band instead of a well defined peak at 2310-20 Å. If a certain number of the conjugated monohydroperoxides should add oxygen by 1-4 addition as has been postulated for other conjugated systems (8), a high peroxide value, increased adsorbability, and decreased absorption in the diene region would be the result as we have observed. The postulated structure might then be:



$$-\overset{H}{C}=\overset{H}{C}-\overset{H}{C}=\overset{H}{C}-\underset{H}{\overset{OOH}{C}}- \xrightarrow{O_2} -\underset{O}{\overset{H}{C}}-\overset{H}{C}=\overset{H}{C}-\underset{O}{\overset{H}{C}}-\underset{H}{\overset{OOH}{C}}-$$

or



$$2-\overset{H}{C}=\overset{H}{C}-\overset{H}{C}=\overset{H}{C}-\underset{H}{\overset{OOH}{C}}- \xrightarrow{2\,O_2} \begin{array}{c} -\overset{H}{C}-\overset{H}{C}=\overset{H}{C}-\overset{H}{C}-\overset{OOH}{C}- \\ O \qquad\qquad O \quad H \\ O \qquad\qquad O \quad H \\ -\underset{H}{C}-\underset{H}{C}=\underset{H}{C}-\underset{H}{C}-\underset{OOH}{C}- \end{array}$$

The substances adsorbed on lower sections of the column show increasing absorption in the 2750 Å region. Since this absorption is in the region where conjugated dienones absorb (9, 10) and since the substances that give rise to this absorption come off the column with the monohydroperoxides which have one oxygenated group associated with a conjugated diene system, it appears probable that this absorption may be due to a conjugated dienone structure in the oxidized fatty acid molecule. Although isolation of a 2,4-dinitrophenylhydrazone of carbonyl compounds was not accomplished, reaction with this reagent produced an appreciable darkening and formation of a brownish-red color in solution of fractions considered to have appreciable quantities of the dienone substances. The same reagent in the presence of non-oxidized methyl linoleate produced only a pale yellow color in the solution. This structure may be formed by secondary decomposition of a hydroperoxide, by a dehydration reaction either prior to or after passage onto the column. It has also been suggested by Lundberg and Chipault (11) that the absorption in this region is due to a conjugated ketone formed from a common precursor of it and the hydroperoxide.

The absorption curves that we have obtained then can be used to explain the presence of at least four substances in the oxidized mixture: the two isomeric monohydroperoxides absorbing at 2315 Å, the high peroxide containing substances from the top of the column which absorb much less intensely and over a broader range in the diene region than the monohydroperoxides, and the conjugated dienones absorbing at 2750 Å. In this light it should be noted that Bergström (1) isolated the products of methyl linoleate (oxidized in the presence of inorganic salts as catalysts) on an alumina column and by means of hydrogenation identified two hydroxystearic acids corresponding to two monohydroperoxides, and a dihydroxystearic acid resulting from the further oxidation of the conjugated diene hydroperoxides. At the same time he reported the presence of a ketonic substance which he postulated was formed on the column.

Our observations on the autoxidized fat agree well with those of Bergström although it seems unlikely that all of the ketonic substances are formed on the column since there is strong absorption in the 2750 Å region by the oxidized esters before passage into the column. It may be that the absorption is due to triene conjugated substances as well as the ketonic substances since a triene structure has been observed in some fractions. This could account for the lack of a well-defined or real maximum in most of the oxidized samples.

Iodine numbers were determined on the substances eluted from sections of some of the columns. This procedure was used in an attempt to determine the relative unsaturation on the various sections. Based on the concept of the diene conjugated monohydroperoxides being adsorbed on the lower sections and the further oxygenated substances, presumably with lesser unsaturation, being adsorbed on the upper sections, the iodine values should be progressively greater for substances adsorbed on consecutive sections down the column. In all cases in which iodine numbers were determined the substances eluted from the top sections of the columns gave the lowest iodine number while those from the lower sections gave higher values and in general the values were progressively greater toward the bottom of the column. It is recognized that the absolute values for the iodine numbers may have little or no significance in this study, but the order of magnitude and the sequence seem to support the concepts outlined above.

An interesting observation on absorption maxima at 2315 Å can be made by comparing the greatest we have observed (Curve 6, Fig. 3) and the molecular extinction coefficient calculated from it with those listed by Holman (12). In the reference cited a table lists the E determined by plotting specific extinction coefficients *vs.* mols O_2 absorbed/mol ester. The greatest E for methyl linoleate is 14,000 representing

54.6% conjugation based on a molar extinction coefficient of 25,640 for 10,12-linoleic acid in ethanol. Some higher values are listed for ethyl linoleate based on samples with lesser oxygen content and therefore with less possibility for secondary oxidation products. The maximum absorption that we noted was for curve 6 in Fig. 3, which had a specific absorption coefficient of 46.2. This is equivalent to a molecular extinction coefficient of 15,061, representing 58.7% conjugation of the dienes. This represents isolation on the adsorbent of a more nearly pure monohydroperoxide than could be calculated from the extrapolated data used in the references on methyl linoleate.

Table II shows the relative spectral densities of the adsorbed fractions arranged in order of their positions on the adsorption columns. The spectral densities concerned are those at the 2750 and the 2315 Å wavelengths. The various experiments are arranged in the order of increasing peroxide values for the ester used prior to passage onto the adsorption column. The portion marked "1" possessed the greatest spectral density of that series and the portion marked "4" possessed the least density.

Note that, in the adsorption of the materials with relatively low peroxide values, the most strongly adsorbed substances (top section) possessed the greatest optical density while the least strongly adsorbed materials (bottom of column) possessed the least density.

Note also that as the peroxide value of the esters increased to the highest used, there occurred a most interesting "apparent" reversal of adsorption versus spectral density and that the most strongly adsorbed fraction finally is that one of smallest density, or which exhibits the lowest absorption maximum.

The table clearly shows the effect of formation and adsorption of secondary oxidation products on the absorption at the two analytical wavelengths. For the sample with low initial peroxide value in which the primary oxidation products would be expected to predominate, the absorption maxima are greatest for those substances adsorbed on the top section of the column.

As the initial peroxide value is increased, the number and kind of secondary oxidation products would be expected to increase, and these groups, being generally more polar, would be adsorbed at the top of the column thus crowding the less polar primary oxidation products down the column. Since the primary oxidation products absorb more strongly at 2315 Å than do the secondary products, the spectral density of substances adsorbed on the upper sections would decrease with addition of esters having a higher initial peroxide value. A complete reversal of density versus adsorption is noted as the initial peroxide value goes from 47 m.e./kg., the lowest PV used, to 990 m.e./kg., the highest PV used.

TABLE II

Relative Optical Densities of Adsorbed Fractions Arranged in Order of Their Relative Positions on the Columns in Various Stages of Oxidation

(Figures 1 to 4 indicate decreasing density)

Initial PV	Position on Adsorption Column							
	Rel. Density at 2315 Å				Rel. Density at 2750 Å			
	Top	Upper Mid.	Lower Mid.	Bottom	Top	Upper Mid.	Lower Mid.	Bottom
47	1	2	3	4	1	2	3	4
128	1	2	3	4	1	2	3	4
221	2	1	3	4	1	2	3	4
377	3	2	1	4	3	1	2	4
415	3	2	1	4	3	1	2	4
456	2	1	3	4	2	1	3	4
654	3	2	1	4	3	2	1	4
830	4	2	1	3	4	3	1	2
830	4	3	2	1	3	4	2	1
990	4	3	2	1	4	3	2	1

We believe that this table indicates clearly that we have demonstrated a method whereby it should be possible to separate primary oxidation products from secondary products, and to concentrate them as an aid to further study of the reactions occurring during fat oxidation.

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