TABLE IV
Thermal Polymerization of Fatty Acids and Their Methyl Esters

Heating time at 290°C.	Monomer	Polymer	Polar material
hr.	%	%	%
Methyl linoleate			
3	87.8	10.0	0.6
6	82.6	15.9	0.6
12	61.8	30.7	4.0
24	35.9	54.9	6.8
Alkali-conjugated methyl linoleate [a]			
2	67.9	22.2	4.4
4.5	47.4	42.7	6.9
6	41.8	49.9	8.2
8	34.7	54.4	8.2
Linoleic acid			
4	64.8	29.3	4.2
8	42.6	47.9	6.3
12	36.2	54.3	8.3
Alkali-conjugated linoleic acid [a]			
0	97.2	3.2	.....
1	58.1	34.1	5.3
2	40.1	45.2	9.4
4	31.3	53.6	9.4
6	27.4	53.0	15.4

[a] k 232 mμ = 77.0 (on methyl ester basis).

The method may be used to study basic fat processing and to follow compositional changes that occur in oils subjected to heat and deep fat-frying conditions. Other fields of applying the technique lie in studies on films and film-forming properties of oils produced by autoxidation and polymerization of heated or blown oils.

Acknowledgment

C.R. Scholfield supplied the alkali-conjugated linoleic acids.

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[Received July 27, 1960]

Analyses of Lipids and Oxidation Products by Partition Chromatography. Fatty Acid Hydroperoxides

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A liquid partition chromatographic method was developed to isolate and determine hydroperoxides in autoxidized fatty acids or their methyl esters. By the use of benzene containing 2 to 4% methanol as the mobile solvent, the hydroperoxides were separated from unoxidized fatty acids or methyl esters and from secondary and polymeric decomposition products. In the analyses of oxidized fatty acids, diethyl ether was necessary to elute the secondary decomposition products.

Saponification of autoxidized fatty esters destroyed the peroxides as determined iodometrically, but the resulting acids contained a fraction which was eluted in the same position as hydroperoxide acids. Evidence showed that this fraction is a monomeric hydroxy fatty acid containing conjugated *cis-trans* and *trans-trans* unsaturation.

Fatty ester hydroperoxides were isolated chromatographically in yields and purity comparable to those reported in the literature by countercurrent distribution. The concentrations of methyl linoleate hydroperoxide determined chromatographically were smaller than indicated by the peroxide value and diene conjugation of the autoxidized methyl linoleate.

ISOLATION of pure fat hydroperoxides is one of the most difficult steps in the elucidation of the mechanism of fat autoxidation. Early workers in this field obtained hydroperoxide concentrates by various methods, including molecular distillation (9) low-temperature crystallization (20), and adsorption chromatography (2,3,8,9). Those procedures generally give low yields of hydroperoxides because of varying degrees of decomposition. More recently, purer hydroperoxide concentrates were obtained in higher yields by countercurrent solvent distribution (5,13,14,17,22), urea fractionation (7), and reverse-phase partition chromatography (4,18).

This second paper in the series presents a liquid partition chromatographic method for the isolation and determination of pure fatty esters or fatty acid hydroperoxides. This method has proved useful for routine analyses and for isolations in fat autoxidation studies. The method was also applied to the determination of dimeric and polymeric products in oils (12), hydroxy fatty acids and esters, and partial glycerides (10).

Experimental

The fatty acids and their methyl esters used in this study were obtained from the Hormel Institute. Saponification of oxidized methyl esters was carried out according to the A.O.C.S. Method Ca 6b-53, and the acids were obtained by ether extraction of the acidified soaps.

The procedures for chromatography and titration of acids were the same as described for the determination of dimeric and polymeric acids (12). In highly oxidized fatty acid samples, ethyl ether was added to the column at the end of the run in order to elute the polar fraction of secondary oxidation products. The chromatographic fractionation of methyl esters was followed by collecting fractions in tared 10-ml. beakers, evaporating the solvent on a steam plate, and drying their contents to constant weight in a desiccator.

Autoxidations were carried out at 37°C. on 1- to

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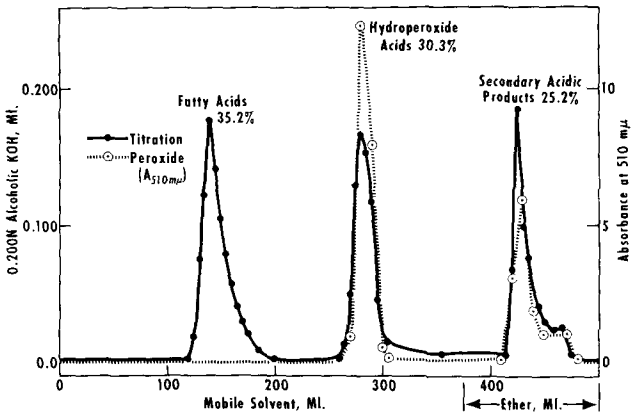

FIG. 1. Chromatographic fractionation of autoxidized linoleic acid (peroxide value 1134).

2-g. samples of esters either in a Warburg apparatus with shaking or in a manometric system, using a 50-ml. burette with magnetic stirring. To follow the chromatographic separation of hydroperoxides from unoxidized fatty acids and esters, peroxides were determined colorimetrically by the ferric thiocyanate method (15). The isolated hydroperoxide fractions were analyzed for peroxides by the iodometric method (13).

Ultraviolet absorption spectra were determined in absolute methanol with a Beckman DU spectrophotometer and a Cary recording spectrophotometer. Infrared spectra were determined with a Baird Atomic spectrophotometer, using sodium chloride cells with carbon disulfide as the solvent.

Results

In the initial chromatographic separations equilibrated mixtures of benzene, methanol, and water were studied, using the lower phase as immobile solvent and the upper phase as mobile solvent. In the analyses of oxidized fatty acids partial separations of hydroperoxides were obtained with benzene-methanol-water mixtures containing the minimum concentration of water (7%) required for a two-phase system. However reversal of phases occurred because the mixtures of benzene-methanol-water had similar densities at the concentrations used (1). Better and more reproducible separations were obtained by eliminating water from the mixture and by drying silicic acid in a 120°C. oven. Figures 1 and 2 show the fractionation of autoxidized linoleic acid and methyl esters of safflower oil, respectively, using 20% methanol in benzene as the immobile solvent and 2% methanol in benzene as the mobile solvent. Composition of the effluent was determined in control columns, without sample, by measuring the refractive indices of the eluted fractions. The composition of the effluent solvent remained constant, as shown by the dotted lines in Figure 3, for the first 275 ml., after which the concentration of methanol increased sharply. The hydroperoxide acids were eluted at this point, and the concentration of methanol (1.30%) remained constant up to 550 ml. of eluate. The retention volume of hydroperoxide acids corresponds to that of dimeric acids from thermally-decomposed hydroperoxides (12). The methyl linoleate and hydroperoxide esters were eluted sooner than the corresponding free acid and hydroperoxide acids and produced sharper and more symmetrically-distributed peaks. In the autoxidized esters a polar fraction probably containing secondary and polymeric oxidation products was eluted

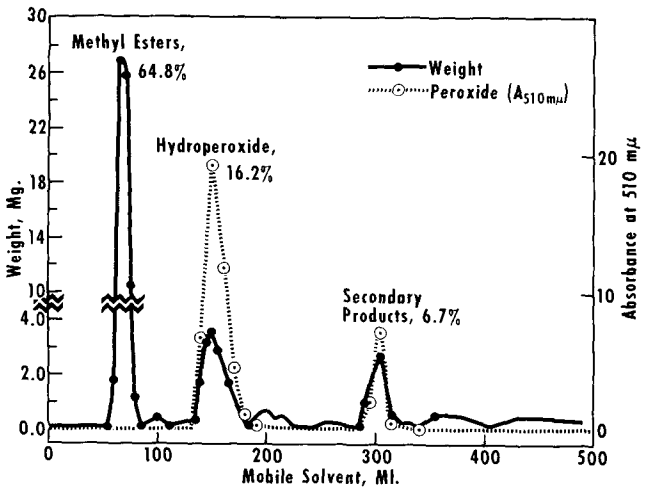

FIG. 2. Chromatographic fractionation of autoxidized methyl esters of safflower fatty acids (peroxide value 1770).

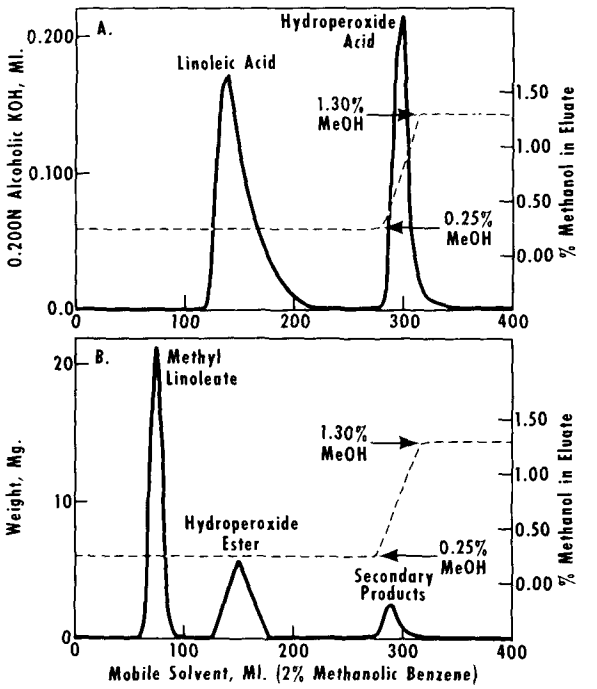

FIG. 3. Relationship between the change in composition of eluting solvent and the chromatographic separation of hydroperoxide acids and esters from (A) autoxidized linoleic acid and (B) autoxidized methyl linoleate.

TABLE I

Chromatographic Fractionation of Oxidized Linoleic Acid (Peroxide Value 1134)

Methanol in Benzene		Retention Volumes[a]		Methanol in Effluent[b]	
Mobile	Immobile	Fatty acids	Hydroperoxides	Before hydroperoxides	After hydroperoxides
%	%	ml.	ml.	%	
1	20	155–320	410–485	0.10	0.45
2	20	120–200	260–305	0.25	1.30
3	20	95–160	175–210	0.35	2.25
4	20	85–130	135–165	0.55	3.30
4	12	140—no separation		.....	.....
4	30	60—no separation		.....	.....

[a] Volumes given for the beginning and end of each concentration peak.
[b] Composition of effluents was determined in control columns without sample and compared with corresponding columns with samples.

in a position corresponding to the sharp increase in methanol concentration in the eluate.

Table I shows the fractionation of oxidized linoleic acid with varying concentrations of methanol in benzene in the mobile and immobile solvents. Linoleic acid was barely separated from its hydroperoxide by 20% methanol in benzene as the immobile solvent and 4% methanol in benzene as the mobile phase. Although the resolution of the hydroperoxide acids was improved by decreasing the methanol concentration in the mobile solvent from 4 to 2%, this change also broadened the fatty acid peak. Decreasing the methanol concentration in the immobile solvent from 20 to 12% or increasing it to 30% gave a poorer resolution of the hydroperoxide acids. There is an optimum concentration of methanol incorporated in the silicic acid which is necessary for the best resolution of hydroperoxide acid from unoxidized fatty acids.

Chromatographic analyses of linoleic acid autoxidized to different levels are given in Table II. The results show good replication of hydroperoxide values

TABLE II

Chromatographic Analyses of Autoxidized Linoleic Acid [a]

Peroxide value	Unoxidized acid	Hydroperoxide acid	Ether eluate	Total recovery
me./kg.	%	%	%	%
Sample A				
228	a) 94.8	4.2	.....	99.0
	b) 95.6	3.9	.....	99.5
1134	a) 35.2	30.3	25.2	90.7
	b) 33.5	29.6	28.5	91.6
	c) 39.0	30.9	27.1	97.0
	d)	31.7	.....	.....
Sample B [b]				
253	94.6	2.7	.....	97.3
115	94.3	3.9	.....	98.2
140	95.1	4.2	.....	99.3
243	94.9	4.7	.....	99.6
340	93.5	5.7	.....	99.2
450	93.1	6.2	0.7	100.0
528	91.4	7.1	1.2	99.7
750	89.5	9.4	1.6	100.5
1000	88.0	10.1	2.2	100.3

[a] 60°C., Warburg shaker.
[b] Peroxide values determined by ferric thiocyanate method (15).

and essentially quantitative recoveries of the acids. The amount of the polar fraction, eluted with diethyl ether, increased with the amount of autoxidation and represents probably secondary and polymeric reaction products. Elution of this polar fraction was necessary in the analyses of highly autoxidized acids to obtain quantitative recovery of the total sample added to the column.

Upon saponification, autoxidized methyl esters of fatty acids lost their peroxides as determined iodometrically with potassium iodide or colorimetrically with ferric thiocyanate. However the acids obtained by ether extraction of the acidified soaps gave a fraction which was eluted in the same position as the

TABLE III

Effect of Saponification on Chromatographic Analyses of Autoxidized Fatty Esters

Samples	Peroxide value	Unoxidized fraction	Hydroperoxide or hydroxy acid	Polar secondary products	Total recovery
	me./kg.	%	%	%	%
Safflower methyl esters	1770	64.8	16.2	6.7	87.7
After saponification	0	66.0	21.4	9.4	97.8
Methyl linoleate	1282	51.4	29.4	12.7	93.5
After saponification	0	51.4	31.7	13.7	96.8
Methyl linolenate	1544	83.4	7.9	4.1	95.4
After saponification	0	81.8	9.3	6.1	97.2

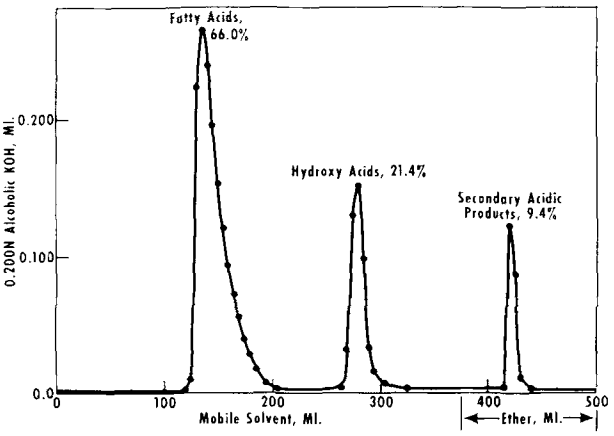

FIG. 4. Chromatographic fractionation of saponified-autoxidized safflower esters (peroxide value 1770 before saponification).

hydroperoxide acids from autoxidized linoleic acid (Figure 4). Table III shows that the yields of this fraction from the saponified esters were a little higher than the yields of hydroperoxides from the corresponding autoxidized methyl esters. The retention volume of this second fraction in saponified-autoxidized methyl esters was the same as that of ricinoleic acid and of monohydroxy stearic acid (10). The molecular weight of the second fraction from saponified-autoxidized methyl linoleate (P.V. 1282) after methylation with diazomethane was 360. This fraction had a high diene conjugation (k_{231} mμ = 74.9); its infrared spectrum showed the sharp band at 2.8 μ for hydroxyl ($k_{2.84} = 0.040$ compared to 0.054 l.g.$^{-1}$ cm.$^{-1}$ for methyl ricinoleate) and the double peaks at 10.17 and 10.56 for *trans-trans* and *cis-trans* conjugation. These results indicate that saponification of fatty ester hydroperoxides converts them to the corre-

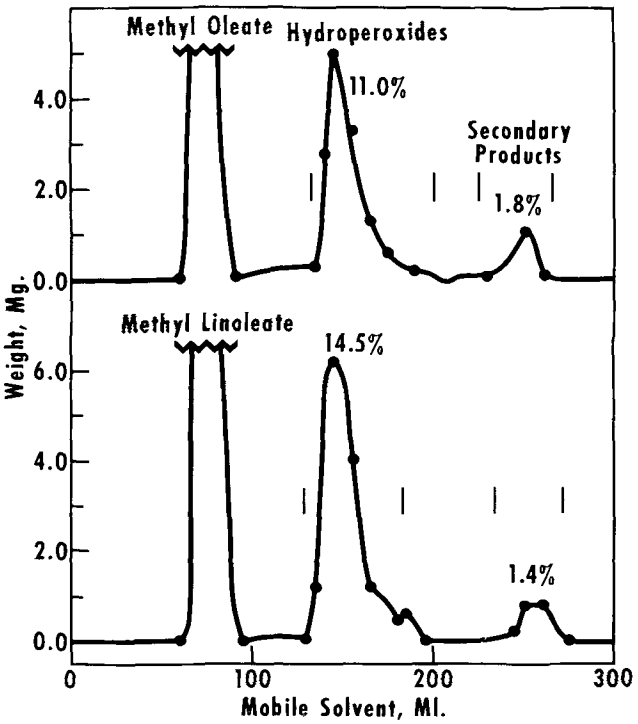

FIG. 5. Chromatographic fractionation of autoxidized methyl oleate (peroxide value 760) and linoleate (peroxide value 974).

sponding monomeric hydroxy acids without apparent change in their conjugated diene structure.

The chromatographic fractionation of autoxidized methyl oleate and linoleate is represented in Figure 5. The esters all showed the presence of a hydroperoxide fraction and a secondary polar fraction which increased at higher levels of autoxidation. Elution of the polar fraction with diethyl ether was not necessary for quantitative recoveries of methyl esters as it was with the fatty acids. Analytical data on these autoxidized fatty esters appear in Table IV. Yields

TABLE IV

Chromatographic Analyses of Autoxidized[a] Methyl Esters of Fatty Acids

Percentage of Hydroperoxide Calculated from			Chromatographic Fractions, %			Yield of hydroperoxide,[d] %
Oxygen absorbed	Peroxide value[b]	Diene[c]	Unoxidized ester	Hydroperoxide	Secondary products	
Methyl oleate						
15.8	12.4	(10.8)[e]	83.5	11.0	1.8	69.6
Methyl linoleate						
10.5	12.8	11.9	86.0	10.0	2.2	95.2
16.2	15.9	15.3	85.0	14.4	1.4	88.9
36.5	31.0	25.6	66.7	24.4	7.3	66.8

[a] Autoxidation at 37°C. under oxygen in a manometric system. The oxidation of methyl oleate was catalyzed by ultraviolet light.
[b] Theoretical peroxide value of fatty ester hydroperoxide taken as 6125 me./kg.
[c] Used k for linoleate hydroperoxide of 81.3 (5).
[d] Yield calculated from total oxygen absorbed and expressed as percentage of oxygen in fatty ester hydroperoxides.
[e] Percentage of isolated *trans*, using $k_{10.3\,\mu} = 0.471$ for methyl elaidate.

of hydroperoxides at equivalent levels of oxygen absorption were higher from methyl linoleate than from methyl oleate but were of the same order of magnitude as those obtained by Cannon *et al.* (5) by countercurrent solvent distribution. The percentage of methyl oleate hydroperoxide determined chromatographically agreed well with that based on spectral measurement of isolated *trans* in the autoxidized methyl oleate. The percentages of chromatographically-determined linoleate hydroperoxide were smaller than those based on the conjugated diene and peroxide values in the autoxidized ester because the secondary polar products contribute to the diene conjugation and peroxide values.

To determine their purity, hydroperoxide fractions were isolated chromatographically from various autoxidized fatty esters. The methyl oleate hydroperoxide fraction had a peroxide value of 5380 me./kg. and the strong band at 10.3 μ (k: 0.308) for isolated *trans*. The methyl linoleate hydroperoxide fractions had peroxide values in the range of 5100–5700 me./kg., and a high diene conjugation (k_{232} mμ = 73.2–77.9) which was shown by infrared to have the *cis-trans* and *trans-trans* configuration (peaks at 10.1 and 10.5 μ). These results agree well with those reported in the literature (5,16,18,19). The high purity and the good yields of linoleate hydroperoxides are taken as evidence that no decomposition results from the chromatographic treatment. A relatively pure hydroperoxide fraction was also isolated chromatographically from autoxidized methyl linolenate, and its characterization has been presented (11).

Discussion

As pointed out by Cassidy (6), it is sometimes difficult to make a clear distinction between partition and adsorption chromatography. Since about 90% of the methanol in the developer remains on the silicic acid column (Table I), it is evident that absorbed methanol is the actual stationary phase. If the methanol acts as a selective solvent for the components of oxidized fatty acids and esters, then the conditions for partition chromatography appear to exist in the chromatographic method reported in this paper. However variations in the proportions of methanol incorporated on the columns had distinct effects on the elution behavior of oxidized fatty acids. Smaller concentrations of methanol in the developer resulted in broader concentration peaks and increased tailing, which indicated a greater participation of adsorption than partition.

Both adsorption and partition effects undoubtedly occur. An optimum amount of methanol is apparently necessary to balance these effects and obtain desirable resolution of components of oxidized fatty acids. The simultaneous operation of partition and adsorption in chromatographic columns has been used by van Duin (21) to obtain certain separations not possible by either phenomenon alone. Early attempts to isolate fat hydroperoxides by adsorption chromatography (2) showed relatively low yields of hydroperoxides because decomposition resulted. The pure linoleate hydroperoxide fractions obtained in our work were comparable in yield to those from countercurrent solvent distribution (5). This similarity is a strong indication that the partition phenomenon is an important factor in the chromatographic separation. Partition chromatography affords additional selectivity and permits the separation of methyl linolenate hydroperoxide (11).

Acknowledgment

The authors thank E. Selke for determinations of ultraviolet and infrared spectra.

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[Received July 27, 1960]