Systematic Isolation and Identification of Minor Components in Heated and Unheated Fat[1]

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ABSTRACT

Although animal studies have shown that the chemical reactions occurring in fats during frying do not make them unsuited for food use, questions continue to arise about the products of these reactions and their significance. The purpose of this work was to identify as many as possible of the monomeric products that form when fats are heated in the air. Cottonseed oil was kept at 182 C for six 8 hr days; then it was converted to methyl esters. The distillable, non-urea-adductable fraction (DNUA) of the esters was separated into 96 fractions by gradient elution chromatography on silica gel. Each fraction was analyzed by gas liquid chromatography (GLC). One hundred thirty-six components were observed, corresponding to 0.42% of the fat. Many of the components were isolated by preparative GLC. Fifty-one were partially or completely characterized. The most abundant components of the DNUA were octadecenoate and octadecadienoate which had not been removed completely by urea adduction. Esters of alicyclic fatty acids made up 34% of the characterized material. These cyclic materials are probably responsible for the toxic effects that have been seen when DNUA was isolated and fed to rats at high dietary levels. Many of the other components appear to be of little biological interest. No components present in substantial amounts remain uncharacterized.

There is convincing evidence that used frying fats are not harmful to experimental animals, even when fed at high levels for long times (1). However such fats do contain trace amounts of substances which, when isolated and fed at high levels, have proved harmful to rats (2). We sought in this work to identify some of the myriad of substances that are present in heated fats, in the expectation that such knowledge would indicate which of the components deserve further attention.

The products formed by heating or oxidizing fats, or both, may be roughly classified as volatiles, monomers and polymers. The volatiles distill or steam distill out of the fat at atmospheric pressure during heating or frying. The nature of the volatile products has been reported in admirable detail (3-6). Although the dimers and polymers are much less amenable to investigation, they too have recently received much attention (7-14). Our efforts have been directed toward the monomeric products (15), since they appear to be the materials of greatest biological significance (16).

Numerous substances have been reported as monomeric, nonvolatile components of heated or oxidized fats (15-21), but it has never been clear whether the materials reported were the major components or were merely the ones most amenable to isolation; neither has any information been available concerning the total number of components or their levels. For this work we set out to investigate the components of a heated fat in a systematic way that would answer these concerns.

EXPERIMENTAL PROCEDURES

Preparation of Heated Fat

Commercially refined and bleached cottonseed (30% oleic, 45% linoleic) oil was chosen as the fat for this study. Seven liters of the oil in a 12 liter stainless steel kettle was kept at 182 C for six 8 hr days, with the fat being allowed to cool to room temperature overnight and during a weekend. In order to assess the extent of heat damage to the oil, 300 g of fresh potatoes were fried in it at the beginning of the heating schedule and again at the end. At

[1]Presented at the ISF-AOCS World Congress, Chicago, September 1970.

TABLE I

Analytical Values for the Heated and Unheated Cottonseed Oils

Analysis	Heated		Unheated
	Batch No. 1	Batch No. 2	
Acid value	0.2	0.3	< 0.1
Peroxide value	7.7	5.8	10.5
Conjugated diene, %	2.4	2.5	0.2
Conjugated triene, %	0.09	0.07	0.05
Iodine value	101	102	112
Linoleic by UV, %	46.1	47.0	53.3
Linolenic by UV, %	0.03	0.08	1.0
Refractive index	1.4710	1.4715	1.4685
Polar triglyceride	20.1	19.0	1.7
Lovibond color	70:10	70:15	16:1.6
Hydroxyl value	9.2	10.3	2.0
Fatty acid composition by gas liquid chromatography, %			
14:0	0.9	1.1	0.7
16:0	27.5	26.4	23.5
16:1	0.8	0.7	0.7
18:0	2.2	3.6	2.2
18:1	18.4	18.6	16.9
18:2	50.0	49.4	56.0
18:3	0.06	---	0.06

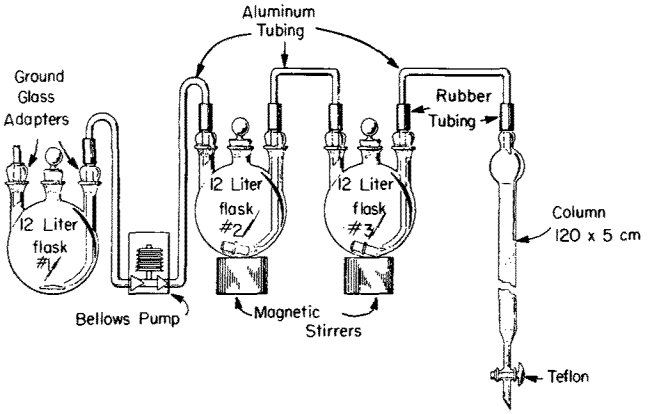

FIG. 1. Apparatus for gradient elution column chromatography.

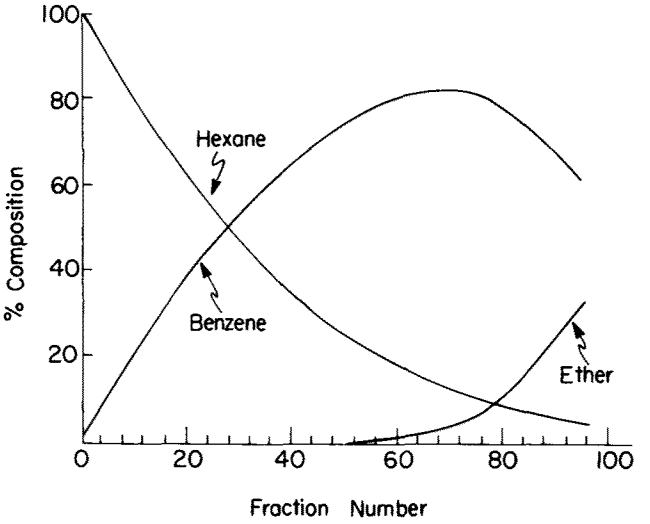

FIG. 2. Solvent composition for gradient elution chromatography.

the end of the heating schedule, the time required to brown the potatoes was approximately twice as great as it had been in the fresh oil, and there was severe foaming during the frying. These frying tests established that the fat was at or beyond the point at which it was no longer suitable for use in frying. Two such batches of heated oil were prepared (but no potatoes were fried in the second batch). After evaluation by the usual analytical methods (Table I), they were worked up separately as described below.

Preparation of Distillable Non-Urea-Adductable Fraction (DNUA)

The oil was first converted to methyl esters. Each batch, weighing ca. 6 kg, was heated at 80 C under 1 mm pressure for 2 hr to remove water and other volatiles. The oil was cooled to 60 C, and to it was added a solution made by dissolving 23 g sodium in 1400 ml methanol. The mixture was stirred 2 hr at 60 C, then allowed to settle. The lower layer was drawn off, and the upper layer was washed with 2% acetic acid solution until the washings remained below pH 6, then four times with water and once with saturated NaCl solution. Without transfer from the original reaction flask, the methyl esters were distilled. A forerun was collected over a boiling range of 77-165 C at 1 mm pressure (pot temperatures up to 193 C). The main distillate fraction was collected at 160-205 C at 1 mm (pot temperatures up to 250 C). From the two batches of oil, the forerun amounted to 1.1 and 1.2%, the distillate to 85.3 and 86.9%, and the residue to 12.9 and 12.0%, respectively.

Each batch of distillate was mixed with a warm (55 C) slurry of four times its weight of urea in four times its volume of alcohol (denatured ethanol formula 2B). After 30 min stirring the mixture was let cool slowly to room temperature and was filtered by suction. The filter cake was washed with urea-saturated alcohol until the washings were colorless. The filtrate was evaporated to small volume; then water and ether were added to facilitate the removal of urea from the fatty material. Evaporation of the ether solutions afforded DNUA in quantities corresponding to 2.8% and 2.5% of total methyl esters. DNUAs from the two batches were combined and adducted twice more, leaving 128 g of material (1.1% of the total esters). This residue was distilled at 1 mm pressure and pot temperatures of 165-310 C; it distilled at 75-217 C. The distillate amounted to 103 g or 0.86% of the total methyl esters.

For purposes of comparison, DNUA was prepared by the same procedure from unheated cottonseed oil. The unheated oil gave no low-boiling fraction. Residue from the first distillation amounted to 1.8%. The yield of DNUA was 6%, based on total methyl esters.

Gradient Elution Column Chromatography of the DNUAs

Gradient elution column chromatography was carried out on the DNUAs from both the heated and the unheated cottonseed oils. The apparatus used (see Fig. 1) was designed to avoid contamination of the solvents with materials leached from flexible tubing. All connections were made with aluminum tubing, which was fitted to the flasks through ground glass sleeve adapters. Seals were made by slipping heavy black rubber tubing over both the sleeves and the aluminum tubing; the solvents passed through the aluminum tubing without contacting the rubber. No stopcock grease was used. The pump was a Microflex Bellows pump, adjusted to deliver 30 ml/min. The column was packed with 1 kg of 100-200 mesh silica gel (Davison) previously deactivated by the addition of 5% water. The silica gel was slurried with hexane for column packing. All solvents had recently been distilled without contacting grease, rubber or plastic.

Fifty grams of DNUA from the heated oil (or 22 g from the unheated oil) was put on the column, and 4 liters of hexane was passed through. This removed all hexane-elutable material (0.55 g). For the beginning of the gradient, flask 3 was filled with hexane, flask 2 with hexane-benzene 1:1 v/v, and flask 1 with benzene. The benzene was pumped from flask 1 into flask 2, thereby displacing solvents from flasks 2 and 3 through the column, and gradually increasing the concentration of benzene in the eluting solvent. The eluate was collected from the column in 500 ml fractions. After flask 1 was emptied, it was refilled with benzene. When it was emptied the second time, a solution of benzene-ethyl ether 80:20 v/v was introduced. Finally it was refilled with ethyl ether, and when this solvent had been pumped into flask 2, the gradient was considered to be complete, and the column was stripped with 1.5 liters of ethyl ether. Figure 2 shows the calculated solvent composition for each fraction.

Each 500 ml portion of eluate was evaporated separately. Weights of the residues are plotted as chromatograms in Figure 3.

Gas Liquid Chromatography (GLC) of Fractions

A qualitative GLC analysis was performed on fractions 10-96 obtained from the gradient elution column chromatography of the DNUA from the heated cottonseed oil. This was done for the purpose of determining the appropriate fatty ester markers to be used for quantitative GLC analysis of the fractions, and to observe at what point the fractions no longer demonstrated adequate GLC response necessary for quantitation of components. The qualitative GLC analyses were performed on an Aerograph Hy Fi model 600B using a 10 ft x 1/8 in. 10% DEGS on 60/80 mesh Gas Chrom Q column at 190 C with 20 psig N_2

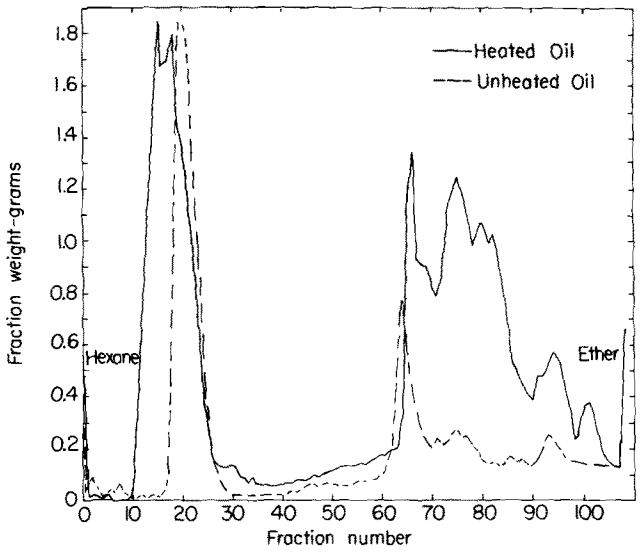

FIG. 3. Gradient elution chromatogram of distillable non-urea-adductable fraction from cottonseed oils.

pressure, or on a 5 ft x 1/8 in. 3% HI-EFF-8BP on 60/80 mesh Gas Chrom Q column at 180 C with 14 psig N_2 pressure. The column used for each analysis is shown in Table II. The fractions were also analyzed on an Aerograph A-90 using a 10 ft x 1/4 in. 5% SE30 on 60/80 mesh Chromosorb W column at 200 C with a helium flow rate of 75 ml/min and thermal conductivity detection.

For quantitative analysis a 20 mg sample was taken from each fraction, and to it was added 5 mg of a marker, chosen so its presence would not obscure any components of the sample. The following markers were used (column packing, fraction numbers, marker): polyester, 10-20 and 54-84, amyl laurate; 21-41 and 85-87, octyl palmitate; 42-53, hexyl palmitate; 88-96, heptyl oleate; silicone, 10-20 and 54-84, amyl laurate; 21-53, hexyl palmitate; 85-96, hexyl laurate. The samples containing the markers were then analyzed on the silicone column and on one of the polyester columns mentioned above. Retention times, peak heights, and widths at half height were measured for each peak, including the marker. These values were used to calculate the retention ratio of each peak, the per cent of the peak in the fraction (assuming level of substance is proportional to area of peak), and its per cent in the total DNUA. When markers other than amyl laurate were used, retention times relative to amyl laurate were calculated by multiplying the component-marker retention ratio by the previously established marker-amyl laurate retention ratio.

The retention ratio of each peak on polyester was plotted against the fraction number in which the peak occurred. Adjacent points were connected to produce the two dimensional chromatogram shown as Figure 4. Each spot on this diagram represents a single substance or a group of inseparable substances. The size of a spot indicates the extent to which the substance was spread out over various fractions of the column chromatogram; the size of the spot has no quantitative implications. Some judgment had to be exercised in deciding whether neighboring points represented the same substance or different substances. Most such uncertainties were resolved by additional information that became available as the work progressed.

Each fraction of the DNUA from the unheated oil was analyzed qualitatively by GLC. The results showed that certain components were present in both the heated and the unheated oils; these components are noted in Table II.

Isolation of Components

Representative fractions from the gradient elution chromatography of DNUA from heated fat were chosen to give adequate sampling of the different spots. The fractions were subjected to preparative GLC on the Aerograph A-90 instrument. For most fractions the initial separation was made by injecting 35-100 μliters of material onto a 17 ft x 1/4 in. column packed with 25% HI-EFF-8BP on 60/80 mesh Gas Chrom Q, operated at 210-230 C with 200 ml/min He flow. Components were condensed from the effluent gas stream in simple glass capillaries which were sealed until the contents were to be handled again. Many components were purified further by GLC on either a 20 ft x 1/4 in. column containing 25% AAEG on 60/80 mesh acid-washed Chromosorb W or on a 10 ft x 1/4 in. column packed with 10% HI-EFF-8BP on 60/80 mesh Gas Chrom Q. The former column was operated at 175-210 C, the latter at 190-230 C. The choice of which, if either, of these columns to use was based on small scale experimentation. Each component was finally purified by injecting 2-5 μliters of it onto a 10 ft x 1/4 in. column packed with 5% or 10% SE-30 on Gas Chrom Q.

No particular difficulty was encountered in isolating the components from fractions 12-50 except poor recovery of materials. Some of the compounds present in fractions 54-64 apparently decomposed partly during preparative gas chromatography. This was not unexpected, since these fractions had given poor material balances during quantitative analysis. Fortunately they contained only minor amounts of the DNUA.

Fractions 70-96 all contained hydroxyl compounds, as shown by their IR spectra, and it was necessary to use a silanized GLC support for their collection. Fractions 73, 78, 82, 85 and 86 were silylated prior to preparative GLC in order to make silyl ether derivatives of any hydroxyl compounds in the fraction. The silylation was performed by diluting the fraction with 50 times its volume of tetrahydrofuran, then adding 30 times its volume of hexamethyldisilizane and 1.5 times its volume of chlorotrimethylsilane. This mixture was allowed to stand at room temperature overnight; complete conversion of hydroxyl compounds to their silyl ether derivatives was achieved. Many of the compounds that had slightly different retention ratios relative to amyl laurate on GLC before silylation merged into a single peak as silyl ether derivatives. These materials were collected as one fraction from the silylated mixture.

Fractions 87-96 were not silylated even though their IR spectra showed absorption for hydroxyl function. Silylation of a 10 μliter sample from each of these fractions was performed and no change could be noted in the samples by GLC analysis.

Examination of Components

For those components where a large enough sample could be isolated, the material was examined by gas chromatography and by various spectroscopic techniques. GLC (on the analytical columns described above) showed that many of the isolated materials were apparently single substances; others clearly were not, but could not be further separated on a preparative scale. On a Perkin-Elmer 421 instrument, IR spectra were made of the isolates as liquid films between NaCl flats. NMR spectra were made on a Varian HR-100 instrument, using computer averaging of the signal where necessary. Mass spectra were recorded on an Atlas SM-1 double focusing instrument, and were converted to element maps by computer processing of the data.

Descriptions of Components

Table II shows for each individually discernible component its number (corresponding to the numbers in Fig. 4), the gradient elution chromatography fractions in which it appeared, its retention ratio on a polyester GLC column, and the level at which it occurred in the DNUA. Peaks that appeared in both the heated and unheated fat are marked in

TABLE II

Description of Components

Component number	Gradient elution fractions	Gas liquid chromatography retention ratio[a]		Parts per 10,000 of distillable non-urea-adductable fraction	Description
		On polyester[b]	On silicone[c]		
1	10-14	2.4-2.5[b]	2.1	23	Octadecenoate
2	10	9.1	---	0.08	---
3	10-17	10.0-10.6[b]	7.5	89	C_{23} esters
4[d]	11-57	2.8-3.2	2.13	1010	Octadecadienoate
5	11	9.75[b]	---	0.06	---
6	12-20	2.7[b]	2.3	210	Unsaturated cyclic ester
7	12	8.3[b]	---	1.4	---
8	13-17	2.1-2.2[b]	---	14	---
9[d]	13-20	2.2-2.3[b]	1.7	92	Unsaturated cyclic ester
10	13-20	3.9-4.1[b]	2.7	46	Unsaturated cyclic ester
11	13	4.7[b]	---	1.1	---
12	14-17	11.2-11.8[b]	7.0-7.2	40	---
13	20	3.4[b]	---	2.3	---
14[d]	21-33	0.75-0.83	0.58	24	Methyl tetradecadienoate
15[d]	21-30	1.8-1.9	1.17	10	Methyl hexadecadienoate
16[d]	21-29	2.4-2.6	1.55	56	Methyl heptadecadienoate
17	21-33	4.3-4.7	2.79	151	Unsaturated cyclic ester
18	22-28	5.6-6.0	3.0-3.5	7.5	Mixture of unsaturated esters
19[d]	24-36	0.46-0.56	---	1.3	---
20[d]	25-33	1.1-1.2	0.94	4.3	Trimethyl-2-pentadecanone
21	26-27	6.8-6.9	---	2.2	---
22	26-28	10.9-11.7	3.5-3.9	1.3	---
23	27-41	6.0-7.2	2.4	46	Methyl 4-(2-octylphenyl)butyrate
24	28-36	8.2-8.5	2.9	16	Mixture of 23 and 36
25	30-32	0.25-0.29	---	21	---
26	30-39	2.2-2.7	0.80	40	Methyl 8-phenyloctanoate
27	30-41	14.6-16.3	5.9	14	---
28	30-38	21.2-23.5	8.2	4.5	---
29[d]	31-38	1.5-1.8	0.57	6	Methyl 7-phenylheptanoate
30	32	9.5	---	0.08	---
31	32	10.1	---	0.08	---
32	34-37	0.60-0.69	---	0.4	---
33	34-44	0.95-1.05	0.42	3.1	Methyl 8-(2-furyl)octanoate
34	34	3.0	---	0.2	---
35	35-38	4.4-4.6	---	0.08	---
36	37-49	7.8-8.1	3.0	14	Cyclic keto-ester
37	38-42	0.25-0.28	---	0.3	---
38	38-42	0.44-0.54	---	0.16	---
39	38-42	0.72-0.79	---	0.18	---
40	38-40	7.0-7.3	---	0.27	---
41	38-43	28-30	8.2	3.0	Di-2-ethylhexyl phthalate
42[d]	39-51	1.6-1.8	---	2.1	---
43[d]	40-51	1.9-2.1	---	0.7	---
44	41-42	38-41	---	0.28	---
45	42-50	13.7-14.3	8.2	6.4	---
46	43	0.30	---	0.03	---
47	44-64	4.4-4.8	2.3	92.9	2-Alkyl-5-carbomethoxy-alkenyl-tetrahydrofuran
48	45-51	0.33-0.38	---	0.57	---
49	45-53	1.0-1.1	---	0.83	---
50	46-47	0.46-0.50	---	0.12	---
51	47-49	0.69-0.78	---	0.14	---
52	53-63	0.54-0.57	0.26	4.9	Mixture
53	54-62	15.0-16.4	---	4.0	---
54	55-59	29.4-30.8	5.0	3.3	Keto-ester
55	56-67	8.5-9.4	---	17.3	---
56	56	14.7	---	0.10	---
57	58-62	33.0-34.5	---	2.8	Keto-ester
58	58-64	10.0-10.6	---	10.6	---
59	59	7.0	---	0.1	---
60	60-64	8.3-8.7	---	2.6	---
61	61-64	7.2-7.7	---	1.0	---
62	63	9.6	---	0.82	---
63[d]	64-67	0.62-0.69	---	7.0	---
64	64-68	6.0-6.5	2.8	87.4	1,5-Epoxy ester
65	65	0.48	---	0.44	---
66	65-66	3.5-3.6	0.68	2.9	---
67	65	6.9	---	1.7	---
68	65-67	9.6-9.9	3.2	82.6	Cyclic keto-ester
69	66-68	12.4-13.2	3.7	34.8	Cyclic keto-ester
70	67-70	0.33-0.37	---	2.2	---
71[d]	67-69	0.45-0.50	---	3.1	---
72	67	15.1	---	3.5	---
73	67	16.7	---	0.95	---
74	68-71	4.5-4.7	1.2	6.7	---
75	68-71	7.7-8.0	3.2	16.3	Carbethoxypentylbenzoic acid
76	68-71	9.9-10.0	3.9	50.5	Cyclic unsaturated hydroxy ester
77	68-69	11.0-11.2	---	15.9	---

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Component number	Gradient elution fractions	Gas liquid chromatography retention ratio[a]		Parts per 10,000 of distillable non-urea-adductable fraction	Description
		On polyester[b]	On silicone[c]		
78	68-69	16.0-16.2	4.9	12.4	Keto-ester
79[d]	70-74	0.62-0.69	0.22	8.6	---
80[d]	71-78	0.45-0.50	0.22	10.1	---
81[d]	71-76	2.0-2.2	0.51	17.5	Hydroxy-keto-ester
82	71	5.60	---	0.40	---
83	71-73	15.6-17.3	4.3	62.7	Hydroxy-esters
84	72-75	10.8-11.1	3.4	72.4	Hydroxy-esters
85	72-74	12.9-13.6	3.4	72.9	Hydroxy-esters
86	72-73	17.5-18.0	4.8	16.2	Hydroxy-esters
87	72-74	23.9-25.3	5.9	50.7	Cyclic unsaturated hydroxy-esters
88	73-86	17.0-19.3	4.8	376	Hydroxy-esters
89	74-75	9.8-10.2	---	3.9	---
90	74-77	17.0-17.7	---	181	---
91	75-78	8.5-9.0	3.1	5.7	Hydroxy-ester
92	75-82	13.4-15.7	3.8	196	Cyclic unsaturated hydroxy-ester
93	75-77	19.3-20.3	4.3	5.5	Cyclic unsaturated hydroxy-ester
94	75-78	25.8-27.2	5.0	31.6	Hydroxy-ester
95	76-77	10.4-10.7	---	11.1	---
96	76-82	22.2-23.8	---	192	---
97	77-78	9.9-10.2	---	8.7	---
98	77	28.5	---	2.0	---
99	79-80	24.2-24.3	---	8.5	---
100	80-89	24.3-26.8	5.1	74.8	Cyclic unsaturated keto-ester
101	80	26.5	---	1.2	---
102	83-84	0.50	---	1.5	---
103	83-84	6.6-6.7	1.3	3.7	Methyl 8-(5-0xo-1-cyclopentenyl)-octanoate
104	83-84	20.0-20.4	---	28.4	Cyclic unsaturated hydroxy-ester
105	84-85	1.9-2.1	0.44	3.2	(not identified)
106	85-86	1.3	0.26	1.2	(not identified)
107	85-89	4.5-4.7	0.97	20.2	Methyl 7-(5-oxo-1-cyclopentenyl)-heptanoate
108	85-92	6.9-7.6	2.56	23.9	Mixture
109	85-89	21.6-23.7	3.91	28.7	Hydroxy-ester
110	85-86	34.2-34.9	4.76	4.4	Cyclic unsaturated keto-ester
111	86	0.73	---	0.3	---
112	86-88	0.8-0.9	---	1.1	---
113	86-91	1.4-1.7	0.42	49.9	Methyl 9-hydroxy-10-undecenoate
114	87-88	0.45-0.48	---	0.6	---
115	87-88	1.4-1.5	---	0.9	---
116	88-91	3.1-3.3	0.91	2.9	Methyl 6-(5-oxo-1-cyclopentenyl)-hexanoate
117	88-89	9.5-10.0	---	1.3	---
118	88-91	11.2-11.9	1.84	5.1	Methyl 8-(4-methoxy-5-oxo-1-cyclopentenyl)octanoate
119	89-95	0.6-0.7	0.34	2.3	---
120	89-96	1.1-1.2	0.34	6.0	---
121	89-96	1.8-2.1	---	4.12	Cyclic hydroxy-ester
122	89	6.4	---	0.8	---
123	89-90	32.8-33.4	5.24	3.6	---
124	90	1.0	---	0.05	---
125	90	2.8	---	0.2	---
126	90	50	---	0.8	---
127	91-92	0.48-0.50	---	0.2	---
128	91	0.87	---	0.2	---
129	91-96	2.4	---	59.8	Cyclic hydroxy-ester
130	91-93	40.0-40.6	---	16.0	---
131	93-95	62.7-63.6	---	21.1	Diketo-ester
132	95-96	0.49-0.54	---	1.2	---
133	95-96	0.83-0.84	---	21.0	Mixture
134	95-96	2.9-3.0	---	3.2	---
135	95-96	3.5-3.7	---	1.0	---
136	95	11.9	---	0.4	---

[a]Retention time of component/retention time of amyl laurate.

[b]HI-EFF-8BP except values marked (b) on DEGS.

[c]SE-30.

[d]A component having the same chromatographic and gas chromatographic behavior was seen in the unheated fat.

the table by a superscript letter (d) following the component number. Retention ratios on silicone are shown where they could be established unambiguously. A brief description is given for components that were isolated and examined further.

The brief descriptions of many components are supplemented by the discussion below, where information is presented in the following sequence for the various components: (a) The number corresponding to the component numbers in Table II; (b) Important IR peaks in cm^{-1}; (c) Mass spectra (MS) parent peaks, shown as empirical formulas. (The masses calculated for the empirical formulas differ from the masses observed on the photoplates by no more than 3 millimass units.); (d) In some cases the empirical formulas of important MS fragment ions; (e) NMR peaks expressed as τ values. Integral areas and multiplicity of the NMR peaks were appropriate for the structures deduced, and are not listed except where they are particularly significant; (f) For some components, additional information concerning the spectra of derivatives

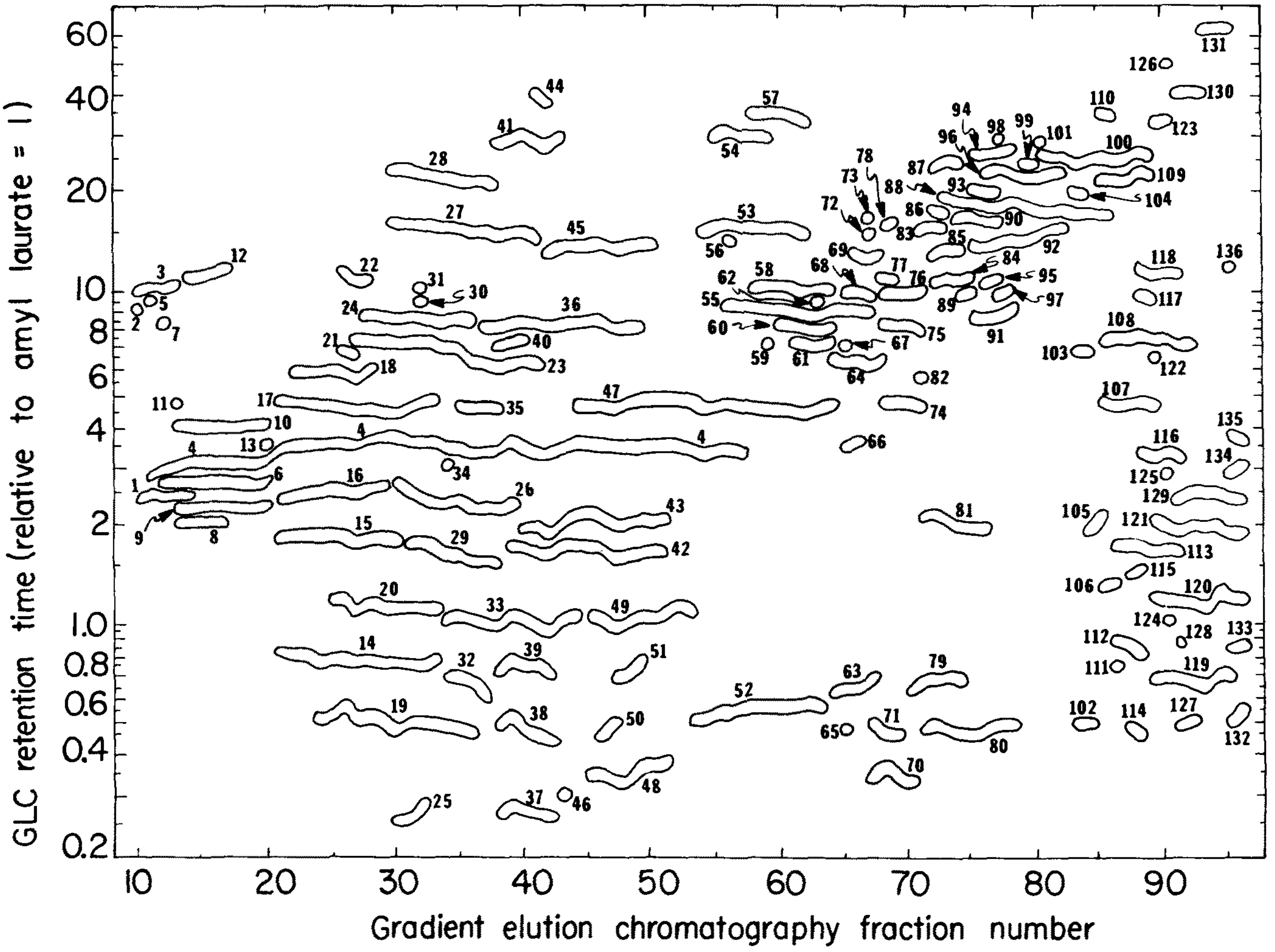


FIG. 4. Composite chromatogram of distillable non-urea-adductable fraction from heated oil.

made from the components. For the components that were isolated as trimethylsilyl ethers, all spectral information given is that of the derivatives.

1) *Methyl octadecenoate:* spectra closely resemble the spectra of methyl oleate containing minor amounts of methyl linoleate.

3) *Methyl tricosanoate and tricosenoate:* IR: 1740, 1165 (ester), 960 (*trans* unsaturation). MS: $C_{24}H_{48}O_2$, $C_{24}H_{46}O_2$ (after hydrogenation $C_{24}H_{48}O_2$), no evidence of branching. NMR 4.69 (-CH=CH-), 6.39 ($-COOCH_3$), 7.72 ($-CH_2COO-$), 8.05 ($-CH_2$-C=C-), 8.38 ($-CH_2$-C-COO- and $-CH_2$-C-C=C-), 8.75 ($-CH_2-$), 9.12 (CH_3-).

4) *Methyl octadecadienoate:* spectra resemble those of methyl linoleate, except that material isolated from the higher numbered fractions shows evidence of *trans* unsaturation (IR: 962).

6) *Methyl ester of cyclic unsaturated acid:* IR: 3040 (unsaturation), 1740, 1165 (ester). MS: $C_{19}H_{34}O_2$; after hydrogenation $C_{19}H_{36}O_2$. NMR: 4.40-4.57 (-CH=CH-), 6.30 ($-COOCH_3$), 7.68 ($-CH_2COO-$), 8.00 ($-CH_2$-C=C-), 8.2-8.4 ($-CH_2$-C-C=C- and $-CH_2$-C-COO-), 9.16 (CH_3-).

9) *Methyl ester of cyclic unsaturated acid:* IR: 1740, 1165 (ester). MS: $C_{19}H_{34}O_2$, $C_{18}H_{31}O$. NMR: 6.36 ($-COOCH_3$), 7.70 ($-CH_2$-COO-), 8.05 ($-CH_2$-C=C-), 8.35 ($-CH_2$-C-COO-), 8.70 ($-CH_2$-), 9.12 (CH_3). After hydrogenation, MS: $C_{19}H_{36}O_2$ with fragmentation pattern suggesting branching at C_8-C_{11} position. NMR: 6.35, 7.70, 8.4, 8.76, 9.12. Data suggest the structure $H(CH_2)_n$-C═══C-$(CH_2)_m$-$COOCH_3$, with m = 7-10 (ring closed by $(CH_2)_4$ between the two C atoms) and $n + m = 11$.

10) *Methyl ester of cyclic unsaturated acid:* IR: 3020 (unsaturation), 1740, 1165 (ester), 962 (*trans* double bond). MS: $C_{19}H_{34}O_2$; series $C_nH_{2n-1}O_2$ is present with n = 3-7; series C_nH_{2n+1} is absent above n = 5. NMR: Same as No. 6. Ozonolysis gave a compound having NMR: 6.40 ($-COOCH_3$, six protons), 7.73 ($-CH_2$-COO-, four protons), 8.4 ($-CH_2$-C-COO-, four protons), 8.70 ($-CH_2$-, eight protons). These data suggest the structure: C_5H_{11}-CH=CH-$(CH_2)_2$-CH————CH-$(CH_2)_2$-$COOCH_3$ (ring closed by $(CH_2)_3$ between the two CH atoms).

14) *Methyl tetradecadienoate:* IR: 3010 (unsaturation) 1740, 1150 (ester). MS: $C_{15}H_{26}O_2$ (after hydrogenation, $C_{15}H_{30}O_2$). NMR: 4.60 (-CH=CH-, four protons), 6.32 ($-COOCH_3$), 7.21 (-C=C-CH_2-C=C-), 7.66 ($-CH_2COO-$), 7.90 ($-CH_2$-C=C-), 8.28 ($-CH_2$-C-COO-), 8.68 ($-CH_2$-), 9.08 (CH_3-).

15) *Methyl hexadecadienoate:* IR and NMR similar to No. 14. MS: $C_{17}H_{30}O_2$.

16) *Methyl heptadecadienoate:* IR and NMR similar to No. 14. MS: $C_{18}H_{32}O_2$.

17) *Methyl ester of cyclic unsaturated acid:* spectra similar to No. 10.

20) *Trimethyl-2-pentadecanone:* IR: 1710 (ketone), 1375, 1365 (*gem*-dimethyl), 1162 (isopropyl). MS: $C_{18}H_{36}O$. NMR: 7.6 ($-CH_2$-CO-, two protons), 7.88 (-CO-CH_3), 8.5-8.75 (methylenes and methines), 9.12 ($-CH_3$, 12 protons). The MS fragmentation pattern suggests that the methyl groups may be in the 6, 10 and 14 positions, corresponding to the ketone's having originated as a degradation product of tocopherol.

23) *Methyl 4-(2'-octylphenyl)butyrate:* IR: 3060, 3020, 1660, 1645, 1490, 750, 665 (aromatic), 1740, 1170 (ester). MS: $C_{19}H_{30}O_2$, $C_{16}H_{23}O_2$, $C_{15}H_{21}O_2$, $C_{14}H_{19}O_2$,

$C_4H_7O_2$, $C_3H_6O_2$, $C_{18}H_{26}O$, $C_{14}H_{17}O$, $C_{13}H_{15}O$, $C_{11}H_{15}$, C_8H_9, C_7H_7 (presence of homologous fragments suggests mixture of isomers). NMR: 2.92 (aromatic), 6.38 ($-COOCH_3$), 7.44 (Ph-CH_2-), 7.72 ($-CH_2$-COO-), 8.38-8.67 (Ph-C-CH_2-, $-CH_2$-C-COO-, $-CH_2$-), 9.10 (CH_3-C-).

26) *Methyl 8-phenyloctanoate:* IR: 3090, 3070, 3030, 1600, 1495, 745, 700 (aromatic), 1740, 1170 (ester). MS: $C_{15}H_{22}O_2$, $C_3H_6O_2$, $C_8H_{15}O_2$, $C_{14}H_{18}O$, $C_{10}H_{13}$, C_8H_9, C_7H_7. NMR: 2.83 (aromatic, five protons), 6.38 ($-COOCH_3$), 7.43 (Ph-CH_2-), 7.73 ($-CH_2$-COO-), 8.25-8.40 (Ph-C-CH_2-, $-CH_2$-C-$COOCH_3$), 8.69 ($-CH_2$-).

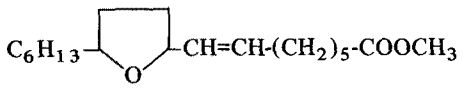
29) *Methyl 7-phenylheptanoate:* IR: 3090, 3065, 3030, 1500, 1495, 745, 695 (aromatic), 1738, 1170 (ester). MS: $C_{14}H_{20}O_2$, $C_7H_{13}O_2$, $C_3H_6O_2$, $C_{13}H_{17}O$, $C_{13}H_{15}$, $C_{11}H_{14}$, C_8H_9, C_7H_7, C_6H_5. NMR: 2.83 (aromatic, five protons), 6.39 ($-COOCH_3$), 7.43 (Ph-CH_2-), 7.72 ($-CH_2$-COO-), 8.40 (Ph-C-CH_2- and $-CH_2$-C-COO-), 8.75 ($-CH_2$-).

33) *Methyl 8-(2'-furyl)octanoate:* IR: 3025 (unsaturation), 1738 (ester), 1595, 1505 (aromatic), 1170 (ester), 882 (furan), 7.25 (tetramethylene). MS: $C_{13}H_{20}O_3$, $C_{13}H_{18}O_2$, $C_{12}H_{17}O_2$, $C_3H_6O_2$, $C_{12}H_{14}O$, $C_{10}H_{15}O$, $C_9H_{13}O$, $C_8H_{11}O$, $C_{10}H_{10}$, C_8H_9, C_6H_9. NMR: 2.83 (furan, 5-H), 3.78 (furan, 4-H), 4.07 (furan, 3-H), 6.36 ($-COOCH_3$), 7.42 ($-CH_2$- adjacent to ring), 7.72 ($-CH_2$-COO-), 8.36-8.51 ($-CH_2$-, β to ring, β to ester), 8.67 ($-CH_2$-).

36) *Cyclic keto-ester:* IR: 3140, 740 (aromatic contaminant), 1738, 1170 (ester), 1640 (ketone). MS: $C_{19}H_{34}O_3$, $C_{14}H_{23}O_3$, $C_{18}H_{31}O_2$, $C_{14}H_{21}O_2$, $C_{13}H_{19}O_2$, $C_{12}H_{19}O_2$, $C_{10}H_{17}O$, $C_{10}H_{15}$. NMR: 6.37 ($-COOCH_3$), 7.71 ($-CH_2COO$-), 8.00 ($-CH_2$-CO-C-, two protons), 8.40 ($-CH_2$-C-COO-), 8.70 ($-CH_2$-), 9.11 ($-CH_3$). After hydrogenation, IR: 3450 (OH), (1640 absent).

41) *2-Ethylhexyl phthalate:* spectra identical with those of authentic sample. This is presumed to be a contaminant.

47) *2-Alkyl-5-carbomethoxyalkenyl-tetrahydrofuran:* IR: 3030 (unsaturation), 1740, 1170 (ester), 1180 (ether), 725 (tetramethylene). MS: $C_{19}H_{34}O_3$, $C_{14}H_{23}O_3$, $C_{10}H_{17}O_3$, $C_{18}H_{31}O_2$, $C_8H_{15}O_2$, $C_4H_7O_2$, $C_3H_6O_2$, $C_{10}H_{17}O$, $C_6H_{11}O$, C_6H_{13}, C_8H_{13}, $C_{10}H_{15}$, $C_{11}H_{17}$. NMR: 4.40 (-CH=CH-), 6.0 (-C=C-CH-O, one proton), 6.38 ($-COOCH_3$), 6.1-6.6 (>CH-O-, one proton), 7.72 ($-CH_2$-COO-), 8.08 ($-CH_2$-C=C-), 8.70 ($-CH_2$-), 9.12 (CH_3-). After hydrogenation, MS: $C_{19}H_{36}O_3$; NMR: 6.40, 6.85 (>CH-O, two protons), 7.75, 8.4, 8.74, 9.14. These data suggest that the structure shown below may be a component of this material, along with isomers of it.



54) *Keto-ester:* IR: 1740, 1170 (ester), 1710 (ketone). MS: No parent peak. NMR: 6.38 ($-COOCH_3$, three protons), 7.74 ($-CO-CH_2$-, six protons), 8.2-8.4 ($-CH_2$-C-CO-, six protons), 8.75 ($-CH_2$-), 9.18 ($-CH_3$).

57) *Keto-ester:* spectra resemble those of No. 54.

64) *Methyl ester of 1, 5-epoxy acid:* IR: 3015 (unsaturation), 1740, 1170 (ester), 1190 (ether). NMR: 4.30 (vinyl), 5.90 (>CH-O-), 6.37 ($-COOCH_3$), 7.71 ($-CH_2$-CO-), 8.05 ($-CH_2$-C=C-), 8.40 ($-CH_2$-C-CO-), 8.68 ($-CH_2$-), 9.10 ($-CH_3$). MS: $C_{19}H_{34}O_3$, $C_{14}H_{23}O_3$, $C_{13}H_{19}O_2$, $C_{14}H_{21}O_2$, $C_{13}H_{17}O$, $C_{10}H_{17}O$, $C_{10}H_{15}$. After hydrogenation, MS: $C_{19}H_{36}O_3$, $C_{14}H_{25}O_3$, $C_{13}H_{21}O_2$, $C_{14}H_{23}O_2$, $C_{13}H_{19}O$, $C_{10}H_{19}O$, $C_{10}H_{17}$. The fragmentation pattern of the hydrogenated compound fits the structure C_5H_{11}-CH-$(CH_2)_3$-CH-$(CH_2)_7$-$COOCH_3$ (with O bridging the two CH groups) according to the information given by Abbot et al. (22). The structure of the isolated material is evidently the same, but with one double bond per molecule in undetermined position(s).

66) *Phthalate:* IR spectrum closely resembles spectra of phthalate esters. A presumed contaminant.

68) *Cyclic keto-ester:* IR: 1738, 1165 (ester), 1705 (ketone). MS: $C_{19}H_{34}O_3$. NMR: 6.37 ($-COOCH_3$), 7.66 and 7.72 (CH_2-CO-, six protons), 8.38 ($-CH_2$-C-CO-), 8.72 ($-CH_2$-), 9.11 (CH_3-).

69) *Cyclic keto-ester:* Spectra resemble those of No. 68.

75) *2-(5-Carboethoxypentyl) benzoic acid:* IR: 3100-3500 (OH), 1730 (ester carbonyl), 1695 (acid carbonyl), 1585, 1360 (carboxylate ion), 1600, 700-800, 690 (aromatic). UV in hexane: 223 (strong), 270 (weak). MS: No parent peak, $C_{14}H_{16}O_3$ (P-H_2O), $C_{13}H_{15}O_3$ (P-CH_3-O), $C_{11}H_{10}O_2$, $C_{11}H_{10}O$, $C_8H_7O_2$, $C_7H_{13}O_2$ (P-HOOC-C_6H_4), C_8H_8O, C_7H_5O, $C_4H_7O_2$, $C_3H_6O_2$. NMR: 0.28 (-COOH), 2.08, 2.43, 2.60, 3.09 (aromatic protons), 6.37 ($-COOCH_3$), 7.34 (Ph-CH_2-), 7.70 ($-CH_2$-COO-), 8.33 ($-CH_2$-C-COO- and $-CH_2$-C-Ph), 8.75 ($-CH_2$-). Data are consistent with the structure HOOC-C_6H_4-$(CH_2)_5$-$COOCH_3$. Positions of substitution on the ring were not ascertained.

76) *Methyl ester of cyclic unsaturated hydroxy-acid:* IR: 3100-3600 (OH), 1738, 1160 (ester), 965 (*trans* unsaturation). MS: $C_{19}H_{34}O_3$, $C_{19}H_{32}O_2$, $C_{18}H_{31}O_2$. NMR: 4.5-4.8 (-CH=CH-), 6.37 ($-COOCH_3$), 6.70 (-O-CH-), 7.72 ($-CH_2$-COO-), 8.00 ($-CH_2$-C=C-), 8.40 ($-CH_2$-C-COO- and $-CH_2$-C-C=C-), 8.70 ($-CH_2$-), 9.00 (-OH), 9.12 ($-CH_3$). The data indicate this is a methyl ester of a C_{18} acid containing one ring, one double bond, one hydroxyl group.

78) *Keto-ester:* IR: 1735, 1160 (ester), 1695-1720 (ketone). MS: $C_{19}H_{32}O_3$. NMR: 6.38 ($-COOCH_3$), 7.72-7.88 ($-CH_2$-CO-), 8.40 ($-CH_2$-C-CO-), 8.73 ($-CH_2$-), 9.12 ($-CH_3$). After hydrogenation: MS: $C_{19}H_{34}O_3$, $C_{19}H_{32}O_2$, $C_nH_{2n-1}O_2$ with n = 6-9.

81) *Hydroxy-keto-ester:* IR: 1735, 1160 (ester), 3100-3600 (-OH), 1675 (ketone). MS: No parent peak. NMR: insufficient sample.

83,86,88) *Hydroxy-esters collected as a mixture after silylation:* IR: 1735, 1160 (ester), 970 (*trans* unsaturation), 745, 840, 1245 (siloxane). MS: $C_{22}H_{42}O_3Si$ and $C_{22}H_{44}O_3Si$. NMR absorptions at 4.62 (vinyl) and 8.02 (allyl) were weak, suggesting that the isolate was a mixture of saturated and unsaturated substances.

84,85) *Hydroxy-esters collected as a mixture after silylation:* similar to No. 83, 86, 88.

87) *Cyclic unsaturated hydroxy-ester:* IR: 1738, 1165 (ester), 970 (*trans* unsaturation), 745, 835, 1245 (siloxane). MS: $C_{22}H_{42}SiO_3$ $C_{21}H_{39}SiO_3$, $C_{19}H_{32}O_2$, $C_9H_{21}OSi$. NMR: 4.60 (vinyl), 6.37 ($-COOCH_3$), 7.72 ($-CH_2$-COO-), 8.00 ($-CH_2C$=C-), 8.38 ($-CH_2$-C-COO- and $-CH_2$-C-C=C-), 8.72 ($-CH_2$-), 9.12 ($-CH_3$).

91) *Hydroxy-ester:* IR and NMR similar to No. 87. MS unsatisfactory.

92) *Cyclic unsaturated hydroxy-ester:* spectra similar to No. 87, except NMR spectrum also shows weak multiplet at 5.95 [-CH-(OSi)-].

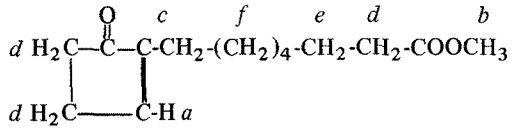
93) *Cyclic unsaturated hydroxy-ester:* resembles No. 92.

94) *Hydroxy-ester:* resembles No. 92, but impure.

100) *Cyclic unsaturated keto-ester:* IR: 1738, 1160 (ester), 1695, 1635 (carbonyl, disappeared on hydrogenation). MS: $C_{19}H_{32}O_3$, $C_{18}H_{28}O_2$, $C_{15}H_{21}O_2$, $C_{10}H_{17}O_3$, $C_{12}H_{19}O$, $C_{10}H_{15}O$, $C_8H_{11}O$, $C_7H_{10}O$. NMR: 6.37 ($-COOCH_3$), 7.6-7.9 ($-CH_2$-C=C- and $-CH_2$-C=O-; 10 protons), 8.4 ($-CH_2$-C-COO-), 8.70 ($-CH_2$-), 9.10 ($-CH_3$). These data fit the structure CH_3-$(CH_2)_2$-C≡≡≡C-C(O)-$(CH_2)_7$-$COOCH_3$ (with $(CH_2)_4$ bridging the triple bond) however the behavior of the material on chromatography suggests a much more polar structure. The IR spectrum shows some absorption at 3200-3600 (-OH), and the MS spectrum shows a weak peak at m/e 326 ($C_{19}H_{34}O_4$). It seems likely that the original component was an hydroxy-keto-ester which failed to silylate and which was dehy-

drated during isolation.

103) *Methyl 8-(5'-oxo-1'-cyclopentenyl)octanoate:* IR: 1735, 1165 (ester), 1700 (ketone), 1630 (unsaturation). MS: $C_{14}H_{22}O_3$, $C_{14}H_{20}O_2$, $C_{13}H_{19}O_2$, $C_{13}H_{18}O_2$, $C_{12}H_{18}O$, $C_{11}H_{17}O$, $C_8H_{15}O_2$, $C_9H_{13}O$, $C_8H_{11}O$, C_7H_9O, C_6H_8O, C_5H_6O. NMR:



a, 2.75; *b*, 6.39; *c*, 7.48; *d*, 7.61-7.95; *e*, 8.40; *f* 8.71

104) *Cyclic unsaturated hydroxy-ester:* similar to No. 92.

107) *Methyl 7-(5'-oxo-1'-cyclopentenyl)heptanoate:* similar to No. 103, but containing one less methylene group.

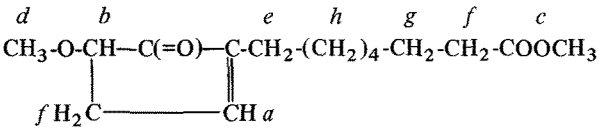
109) *Hydroxy ester:* IR: 3100-3600 (OH), 1735, 1165 (ester). MS: $C_{19}H_{32}O_2$, $C_nH_{2n-1}O_2$ where n = 3-8, C_nH_{2n+1} where n = 3-7. The presumed parent peak, $C_{19}H_{34}O_3$, was not seen; compounds containing secondary -OH groups dehydrate in the mass spectrometer. NMR: 6.38 (CH_3OOC-), 6.8 (-OH, moves to 7.0 at 50 C), 7.71 (-CH_2-COO-), 8.02 multiplet, two protons (?), 8.45 (-CH_2-C-COO-), 8.83 (-CH_2-), 9.12 (CH_3-).

110) *Cyclic unsaturated keto-ester:* resembles No. 100.

113) *Methyl 9-hydroxy-10-undecenoate:* IR: 1745, 1165 (ester), 3100-3600 (OH), 915, 985, 3090 (vinyl). MS of trimethylsilyl ether: $C_{15}H_{30}O_3Si$, $C_{14}H_{27}O_3Si$, $C_{14}H_{27}O_2Si$, $C_{13}H_{23}O_2Si$, $C_{10}H_{21}OSi$, $C_7H_{16}OSi$. $C_6H_{13}OSi$. NMR: 4.18 (-C=CH-), 4.92 (CH_2=C-), 5.96 (-C=C-CH(-O-)-C-), 6.38 (-$COOCH_3$), 7.47 (-OH, moves to 7.72 at 50 C), 7.73 (-CH_2-COO-), 8.48 (-CH_2-C-COO-), 8.70 (-CH_2-).

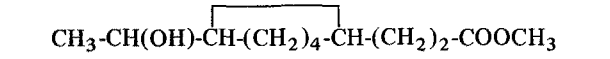
116) *Methyl 6-(5'-oxo-1'-cyclopentenyl)hexanoate:* similar to No. 107, but with one less methylene group.

118) *Methyl 8-(4'-methoxy-5'-oxo-1'-cyclopentenyl)octanoate:* IR: 1733, 1170 (ester), 1710 (ketone), 1635 (unsaturation), 1090 (ether). MS: $C_{15}H_{24}O_4$, $C_{14}H_{20}O_3$, $C_{13}H_{20}O_2$, $C_{13}H_{16}O_2$, $C_{12}H_{16}O$, $C_8H_{15}O_2$, $C_7H_9O_2$. NMR: Data fit the following structure:



a, 2.79; *b*, 5.50; *c*, 6.37; *d*, 6.61; *e*, 7.40; *f*, 7.6-7.9; *g*, 8.44; *h*, 8.67.

121) *Cyclic hydroxy-ester:* IR: 3200-3600 (-OH), 1735, 1165 (ester). MS: $C_{12}H_{20}O_2$ (after silylation, $C_{15}H_{30}O_3Si$), $C_{11}H_{17}O_2$, $C_{11}H_{16}O$, series $C_nH_{2n-1}O_2$, where n = 3-6. NMR: 5.95(>CH-O-), 6.38 (-$COOCH_3$), 7.65 (-OH, moves to 7.86 at 50 C), 7.75 (-CH_2-COO-), 8.3-8.9 (-CH_2-), 9.00 (CH_3-C-O-). These data are appropriate for a mixture, one component of which has the structure



129) *Cyclic hydroxy-ester:* resembles No. 121, but NMR shows terminal CH_3 at the usual 9.12 τ. Hence the structure is isomeric with the one shown for No. 121.

131) *Diketo-ester:* IR: 1730, 1135 (ester), 1640, 1795 (carbonyl). MS: No element map; parent peak at m/e 340 corresponds to $C_{20}H_{36}O_4$. NMR: 6.39 (-$COOCH_3$), 6.76 (singlet, one proton, impurity?), 7.49-7.80 (multiplet, 10 protons, -CH_2-C(O)-), 8.40 (-CH_2-C-CO-), 8.76 (-CH_2-), 9.14 (CH_3-).

DISCUSSION

In the DNUA from the heated fat 136 components were distinguished by GLC. The sum of their estimated levels amounted to 0.42% of the fat or 49% of the DNUA. Failure to account for a larger fraction of the DNUA probably arises from our use of semiquantitative procedures, from our inability to calibrate the GLC detectors for the unknown components, and from incomplete elution of the heavier and more polar components. For only a few of the gradient elution fractions did the sum of the gas chromatographic peaks account for all the material injected. Since poor recoveries were obtained for most of the gradient elution fractions, it is not likely that any large class of substances was overlooked; rather there appear to have been small losses of many components.

Fifty-one of the components were partially or completely characterized. Although these are less than half of the components observed, they make up ca. 83% of the quantity of the GLC eluates. Twenty-five per cent of the recognizable material consisted of octadecenoate and octadecadienoate that had not been removed by repeated urea adduction. An additional 24% of the material appeared to be open-chained oxidized substances, largely hydroxyl-containing and keto-containing fatty acid molecules, some of which had undergone chain cleavage. These are normal and familiar components of oxidized fats. And 34% of the recognizable components were identified as cyclic substances, mostly alicyclic, with smaller amounts of aromatic and heterocyclic materials. It seems likely that these cyclic materials are responsible for the oral toxicity of heated fat DNUA fractions as Crampton et al. (23) and Matsuo (24) postulated for heat-treated linolenate-containing oils.

The biological properties of heated fat components are naturally of paramount interest, but relatively little is known about them. Reports have recently been published on the metabolism of *o*-alkylbenzenealkanoic acids (25) and hydroxyalkanoic acids (26,27). The heterocyclic compounds described above are tetrahydropyrans and tetrahydrofurans, and probably do not justify further attention in view of the low levels at which they appear in the heated fat. The alicyclic compounds are like the ones reported in the past by several workers.

Since the pioneering work of Crampton and Matsuo, there has been recurring suspicion that the DNUA fraction of heated fat harbored some mysterious and potentially very harmful substance. Our work makes it clear that the greatest part of the DNUA is made up of the oxidation and cyclization products which have been known for some time, and that any other kinds of materials must be present at very low levels. We suspect that the already familiar cyclized fatty acids are the substances present in DNUA that are responsible for the toxic effects seen when DNUA has been isolated and fed at high levels to experimental animals.

From previously reported animal feeding studies we have concluded that fats that have been used for frying are perfectly wholesome and safe for ingestion as part of the normal human diet. None of the results found in the present study lead us to alter that conclusion.

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