Occurrence of Fatty Acid Methyl Esters in Walnut Kernel and Other Oils

Louis B. Rockland and Charles De Benedict

Cold-pressed oil extracted from English (Persian) walnuts, *Juglans regia*, was stripped of volatile constituents by molecular distillation at 150° C. The molecular distillate contained free fatty acids, β-sitosterol, other unidentified apparent steroids and a small amount of C_{16} and C_{18} fatty acid methyl esters (FAME). The latter were isolated by micropreparative gas chromatography and identified using infrared, NMR, and mass spectrometry. Special care was taken to preclude formation of FAME during sample preparation, isolation, and characterization. A sample of rancid kernels contained 30 times as much FAME as fresh kernels. FAME may be formed by deteriorative reactions within the kernel during storage.

Numerous organic esters have been identified in essential oils and volatile plant products. However, the presence of methyl and ethyl esters of short chain aliphatic acids in lipids has been reported only recently. Methyl butyrate and hexanoate have been reported in orange volatiles (Schultz *et al.*, 1967) and in irradiated and oxidized butterfat (Forss *et al.*, 1966; Merritt *et al.*, 1967). Evidence has also been presented for the occurrence of methyl decanoate and several ethyl esters among the volatile constituents in hops (Buttery *et al.*, 1967). Dhopeshwarkar and Mead (1962) reported methyl esters of higher fatty acids in normal guinea pig tissue lipids; Leikola *et al.*, (1965) found fatty acid methyl esters (FAME) in pancreas lipids; and Fischer *et al.* (1966) reported both natural and artificial methyl esters in liver lipid extracts. Sloan *et al.* (1968) reported that FAME represented 1 to 2% of total lipid in grasshopper eggs and suggested that they may have biological significance. Artifact methyl esters have also been reported by Lough *et al.* (1962) in extracts of storage tissue lipids. The presence of higher fatty acid methyl esters in plant lipids has not been reported previously. Since methyl esters may be produced during isolation and analysis of the ester fraction and previous characterizations of naturally occurring C_{18} methyl esters were indirect, it appeared important to preclude artifact formation and to demonstrate unequivocally the presence of FAME in walnut kernel oil. Evidence has also been obtained of the presence of FAME in peanut, filbert, almond, and other natural oils.

MATERIALS AND METHODS

Shelled, light-colored, budded Placentia, English walnut (*Juglans regia*) kernels from Northern California were employed. Fresh kernels were adjusted to their optimum moisture content of 3.8% (Rockland *et al.*, 1961), placed in sealed glass jars, and held at 5° C. until used. The rancid kernels had been stored in sealed glass jars for several years under ambient conditions. Peanut, almond, soybean, and other oils were obtained from arbitrary commercial sources and held at 5° C. in amber bottles.

Preparation of Oil Samples. Walnut kernels were chopped in a Henry nut slicer modified with all stainless steel fittings. Weighed, 50-gram portions of chopped kernels were pressed and extracted in a stainless steel filter-press assembly operated continuously at 1000 p.s.i. on a 2-inch plunger by means of an automatic, pneumatically driven hydraulic press (Rockland, 1969). The sparkling, clear oil was expressed into a light-shielded borosilicate bottle during a 2-hour period, flushed with nitrogen, and used within a few hours or stored in the dark at 5° C. for not more than 24 hours.

Molecular Distillation. Walnut and other oils were fractionated in a Kontes Model K-285600, falling film molecular still operated at 150° C. (boiling hexanol) and 10^{-2} torr. Distillation rate was adjusted to 10 ml. per hour for 50-ml. samples. Total contact time of the oil on the heated portion of the still was estimated to be 5 minutes or less.

Thin-Layer Chromatography. Brinkmann Silica gel F-254 precoated plates were employed. They were irrigated in blotter-lined, solvent saturated jars at 20° C. with hexane:methanol (98:2 v./v. at 20° C.). Generally four developments were used to improve separation and resolution. Plates were examined under visible and ultraviolet light both before and after spraying with concentrated sulfuric acid, after heat treatment at 120° C. for several minutes, and after charring at 250° C. Permanent records were obtained using a reflective type photocopy system.

Column Chromatography. Acid-washed, Merck Reagent Grade aluminum oxide (Al_2O_3) was employed for fractionation of molecular distillates. Two grams of alumina were dry packed in a 9.5-mm.-diameter glass chromatographic column and prewashed with 10 ml. of hexane:methanol (99.25:0.75 v./v. at 20° C.). The column was loaded with 100 to 150 mg. of distillate and developed with the same solvent.

Gas Chromatography. A modified Micro Tek Model 2500 R gas chromatograph was employed with the following operating parameters:

Column. 20-foot × 0.125-inch (0.101-inch I.D.) Weldrawn Type 304 Stainless steel.

Packing. 5% stabilized DEGS on 80/100-mesh Chromosorb G, acid washed, DMCS.

Detectors. Thermal conductivity and hydrogen flame ionization at 230° C.

Column temperature. 190° C.

Inlet and outlet block temperature. 295° C.

Carrier gas. Helium at 45 ml. per minute.

Hydrogen flow. 78 ml. per minute.

Flame combustion gases. Nitrogen at 170 ml. per minute and oxygen at 27 ml. per minute.

The effluent stream was split at the outlet block. The split ratio was adjusted by a capillary restrictor placed at the thermal conductivity (TC) cell outlet which caused 10% of the effluent stream to be diverted to the flame ionization detector. The main stream was routed through the TC cell, and the

Fruit and Vegetable Chemistry Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, 263 South Chester Avenue, Pasadena, Calif. 91106

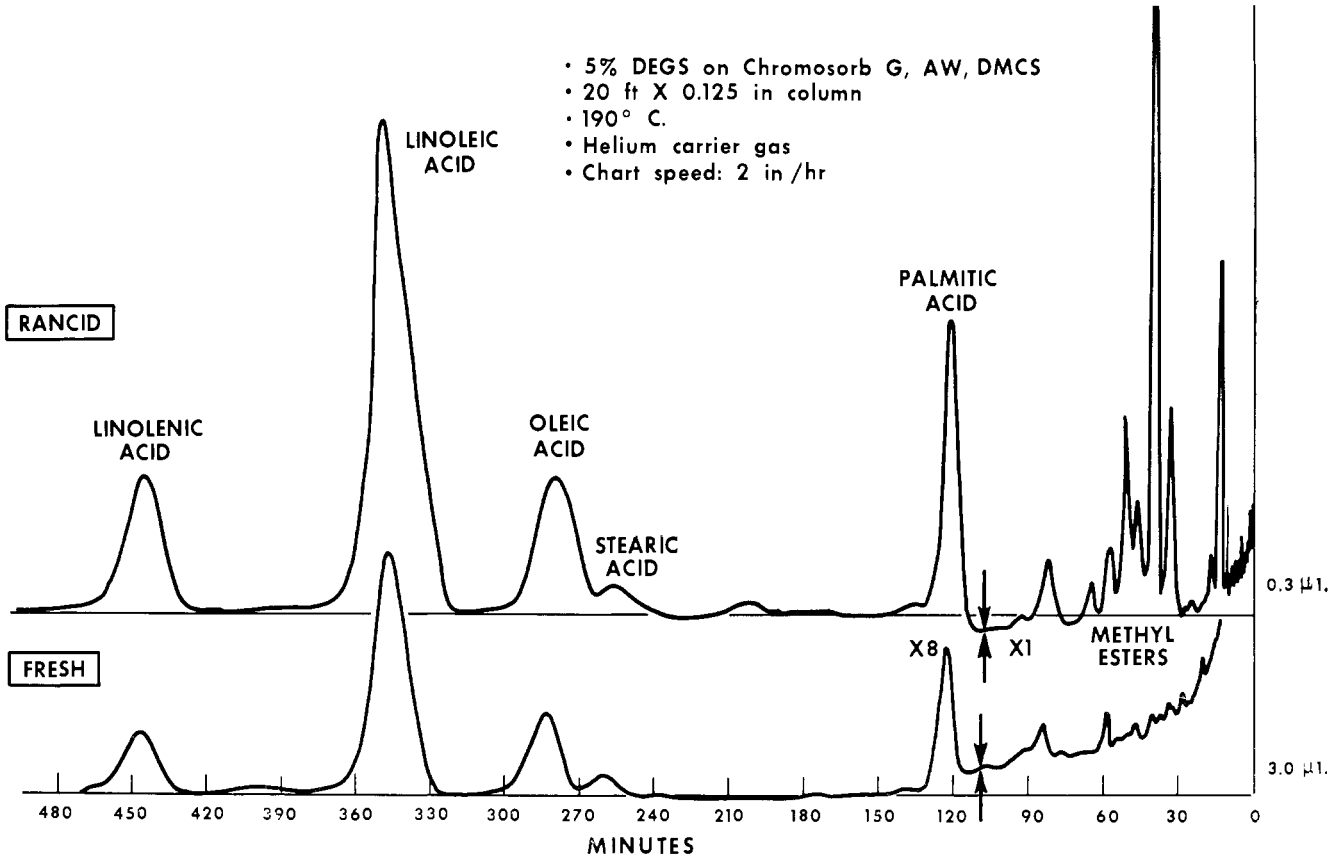

Figure 1. Gas chromatograms of molecular distillates of cold-pressed oils from fresh and rancid walnut kernels

eluate was trapped in a 1.5 × 100 mm. silicone-coated capillary tube which extended about 25 mm. into the TC outlet. The high sensitivity ionization detector permitted monitoring of the effluent stream during micropreparative isolations. For analytical purposes and maximum sensitivity, the TC outlet was capped and the entire effluent stream diverted to the flame detector.

Infrared Spectroscopy. A Wilks Model 12 double beam multiple ATR (attenuated total reflectance) unit was installed in a Perkin-Elmer Model 521 infrared spectrophotometer. Approximately 0.5 mg. or less of test material was dissolved in about 0.25 ml. of diethyl ether and spotted randomly in 1- to 2-µl. aliquots on a 2-mm. thick KRS-5, 45° crystal. After evaporation of the solvent, the crystal face was swabbed lightly with clean cotton to distribute the sample more uniformly over the surface and thereby intensify absorption bands. High resolution spectra were obtained with as little as 25 µg. of sample without scale expansion.

Nuclear Magnetic Resonance Spectroscopy. A Varian A-60 NMR spectrometer was employed. One- to two-milligram samples were dissolved in $CHCl_3$ containing a trace of trimethylsilane, filtered through a 0.45-micron filter and placed in standard 5-mm. NMR tubes filled to a height of 17 mm. or in a microtube containing 40 µl. of a 2 to 5% solution of sample.

Mass Spectrometry. The central two thirds of GLC peaks were trapped in capillary tubes and examined without further purification. Generally 100 to 500 µg. of sample were collected. Authentic samples, purified in the same manner by gas chromatography, and products isolated from walnut oil were analyzed in a Hitachi RMU 6 mass spectrometer by the West Coast Technical Service, San Gabriel, Calif.

RESULTS

Initially, molecular distillation was employed in an attempt to use the mildest conditions for the concentration of the highly volatile products of oxidative rancidity contained in cold-pressed oil from rancid walnut kernels. At 150° C., three fractions were obtained: a small amount of highly volatile material trapped between the high vacuum pump and the primary condenser of the molecular still; nondistillable residue of stripped oil which constituted 90% or more of the original oil; and distillate which condensed on the outer, air-cooled wall of the apparatus. The cold-trap material had a very offensive odor, dissipated rapidly at ambient temperature, and was difficult to handle and analyze in a reproducible manner. The nondistillable fraction had a clear yellow color and no significant odor.

Identification of Free Fatty Acids. Gas chromatography of the molecular distillate on a 5% DEGS column at 190° C. indicated two distinct groups of compounds: a series of small peaks eluted in 5 to 50 minutes; and a series of stronger peaks having a similar profile which required 2 to 8 hours to elute from the gas column (Figure 1). The chromatographic profiles of both groups were remarkably similar to those obtained from transesterified whole walnut oil. The slow group of compounds were identified readily as free fatty acids (FFA). Unequivocal identification of the FFA was obtained as follows: absolute and relative GLC retention time of each acid was compared to an authentic sample; esterification of molecular distillate with BF_3-propanol produced products which corresponded to authentic standard propyl esters and simultaneously eliminated the FFA peaks observed in the original gas chromatograms; thin-layer chromatograms showed a strong spot which corresponded to

Table I. Procedures Employed for Characterization of Fatty Acid Methyl Esters in Cold Pressed Walnut Oil

Peak No.	Compound	Relative Retention Time in Gas Chromatograph: Synthetic standard	Relative Retention Time in Gas Chromatograph: Walnut oil	Spectroscopy: ATR infrared spectra	Spectroscopy: Nuclear magnetic resonance spectra	Spectroscopy: Mass spectra	GLC Peaks Observed in Other Oils[a]
3	Methyl laurate	0.11	0.11	...	...	...	g
8	Methyl myristate	0.20	0.20	...	...	...	d, e, g, i, j
12	C_{15} methyl ester	0.32	0.32	...	...	...	a, f
13	Methyl palmitate	0.38	0.39	X	X	X	a to e, g to j
14	Methyl palmitoleate	0.42	0.42	...	...	...	a, d, e, f, i, j
21	Methyl stearate	0.75	0.75	X	...	X	a to f, j
22	Methyl oleate	0.82	0.82	X	X	X	a to g, i, j
24	Methyl linoleate	1.00	1.00	X	X	X	a to g, i
25	C_{19} methyl ester	1.16	1.16	...	...	...	a to g, j
26	Methyl linolenate	1.29	1.29	X	X	X	a to d, f, g, h

[a] a, Pecan; b, Almond; c, Filbert; d, Corn; f, Soybean; g, Safflower; h, Sunflower; i, Butter; j, Cod liver.

the locus of FFA (this spot was eliminated after esterification with *n*-propanol and replaced by a spot corresponding to fatty acid propyl esters); infrared spectra of the crude molecular distillate contained a strong absorption band for an acid carbonyl. It shifted to a lower wave number after exposure of the ATR coated plate to ammonia fumes which caused ionization of the fatty acid carboxyl groups.

Isolation and Identification of Methyl Esters. Thin-layer chromatography on Silica Gel G of the molecular distillate (Figure 2) obtained from cold-pressed oil of rancid walnuts yielded four major spots after four developments with hexane:methanol (98:2). Free fatty acids remained close to the origin. Traces of apparent glycerides were present at about R_f 0.8. A large spot and several minor spots observed between the glyceride and FFA areas were identified subsequently as β-sitosterol and other apparent steroids. An elliptical area just above the glycerides represented the mixture of FAME which was characterized as described below.

A portion of the molecular distillate was esterified with BF_3-propanol and the esterified products were chromatographed on TLC plates. Glycerides and FFA were absent and esters were concentrated in a large spot corresponding to the above-glyceride spot observed on TLC plates of untreated distillate. Gas chromatography of the esterified material showed a series of peaks which eluted from the column within 50 minutes. Steroids were absorbed on the gas chromatographic column and were not eluted from the column under the conditions employed. The profiles of the derived propyl esters and the unknown group of compounds in the original distillate were similar, except that the former were present in larger amounts. NMR spectra of crude molecular distillates of walnut oil contained strong peaks corresponding to methyl and ethylene protons and other groups characteristic to FFA, glycerides, and unsaturated compounds, as well as a small distinctive singlet corresponding to the methoxy (CH_3O) protons of methyl esters at 3.62 δ. The singlet was observed in spectra of various crude walnut oil distillates at 3.61 to 3.65 δ.

Addition of authentic C_{18} methyl esters enhanced the singlet peak at the same locus. The peak was also reinforced in concentrates of the unknown fraction prepared by removing FFA, glycerides, and other components. An alumina column was employed for enrichment of the methyl ester fraction. The less polar group was eluted just behind the solvent front with 0.75% methanol in hexane, separating it quantitatively

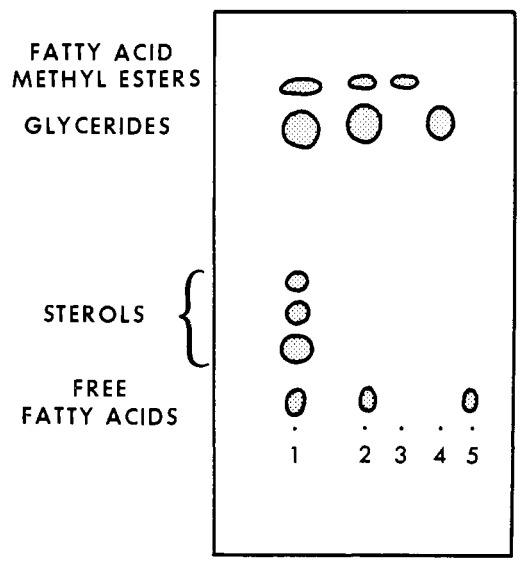

Figure 2. Thin-layer chromatogram of a molecular distillate of cold-pressed walnut oil. Four developments with hexane-methanol (98:2) on Brinkmann Silica Gel G plate and charred at 250° C with 50% sulfuric acid

1. Molecular distillate of cold-pressed oil
2. *Ad hoc* mixture of C_{16} and C_{18} free fatty acids (10%), walnut oil glycerides (80%), and synthetic C_{16} and C_{18} fatty acid methyl esters (10%)
3. *Ad hoc* mixture of synthetic C_{16} and C_{18} fatty acid methyl esters
4. Walnut oil glycerides
5. *Ad hoc* mixture of C_{16} and C_{18} fatty acids

from FFA, glycerides, and steroids. Gas chromatography of the crude distillate as well as the enriched fraction demonstrated that the major unknown peaks corresponded with authentic methyl esters (Table I). Individual methyl esters were isolated by micropreparative gas chromatography using 0.5- to 1.0-μl. aliquots of the enriched methyl ester fraction. Center portions containing no more than 50% of each GLC peak were collected on silicone-coated glass capillary tubes (2- × 100-mm. melting point tubes). Up to 15 separate collections were required to obtain sufficient amounts of the minor components including methyl palmitate and methyl stearate. A portion of each composite collection was rechromatographed in the same manner to determine its purity. It was estimated that each of the

Table II. NMR Spectroscopy of Synthetic and Naturally Occurring Fatty Acid Methyl Esters Isolated from Cold-Pressed Walnut Oil

Compound	Preparation	Principal Band Assignments (δ, cps) CH_3	CH_2	Alpha to C=C	Alpha to COOH	=C—C—C= H	CH_3O	H H C=C
Methyl palmitate	Synthetic	0.89	1.28	...	2.25	...	3.61	...
	Isolate	0.89	1.28	...	2.22	...	3.62	...
Methyl stearate	Synthetic	0.90	1.27	...	2.23	...	3.62	...
Methyl oleate	Synthetic	0.90	1.30	2.03	2.22	...	3.62	5.30
	Isolate	0.90	1.30	2.04	2.22	...	3.61	5.30
Methyl linoleate	Synthetic	0.90	1.33	2.04	2.22	2.74	3.62	5.30
	Isolate	0.91	1.31	2.02	2.23	2.70	3.63	5.31
Methyl linolenate	Synthetic	0.95	1.31	2.09	2.23	2.76	3.62	5.32
	Isolate	0.96	1.32	2.10	2.24	2.77	3.63	5.34

Table III. Infrared Spectroscopy of Synthetic and Naturally Occurring Fatty Acid Methyl Esters Isolated from Cold-Pressed Walnut Oil

Compound	Preparation	Principal Absorption Bands (frequency, cm.$^{-1}$) H H C=C stretch	CH_2 stretch	Ester carbonyl	CH_2 scissors	CH_3 ester deform.	Unassigned			$(CH_2)_4$ rocking
Methyl palmitate	Synthetic	...	2923s	1739s	1463m	1437m	...	...	885m	721m
	Isolate	...	2922s	1740s	1461m	1434m	...	...	880w	719w
Methyl stearate	Synthetic	...	2919s	1738s	1461m	1433m	1232m	1212m	882m	720m
	Isolate	...	2918s	1739s	1460m	1435m	1230m	1212m	885m	720m
Methyl oleate	Synthetic	3000m	2920s	1740s	1460m	1435m	...	...	...	720m
	Isolate	3005m	2923s	1741s	1462m	1435m	...	...	...	723m
Methyl linoleate	Synthetic	3002m	2922s	1738s	1460m	1432m	...	...	...	722m
	Isolate	3005m	2924s	1740s	1460m	1435m	...	...	...	723m
Methyl linolenate	Synthetic	3005m	2922s	1739s	1460m	1432m	...	...	...	722m
	Isolate	3007m	2928s	1740s	1461m	1435m	...	...	...	720m

Table IV. Distinguishing Mass Spectral Peaks Characterizing Fatty Methyl Esters Isolated from Walnut Oil[a]

Fragment	Principal Fragments (m/e) Observed for Methyl palmitate	Methyl stearate	Methyl oleate	Methyl linoleate	Methyl linolenate
C_3	43	43	41	...	...
C_4	55	55	55	55	55
C_5	...	...	...	67	67
$CH_3OCOHCH_2$	74, 75	74	74, 75	...	...
C_6	...	...	...	81, 82	79, 80, 81
$CH_3OCOH(CH_2)_2$	87	87	...	...	...
Tropolium	...	...	91	91	91
C_7	...	...	...	95, 96	93, 94, 95
C_8	...	...	...	...	108
$CH_3OCOH(CH_2)_5$	...	129	...	...	...
$CH_3OCOH(CH_2)_6$	143	143	...	...	...
M-$CH_3OCOH(CH_2)_4$	...	...	180	...	...
M-$CH_3OCOH(CH_2)$	...	...	220	...	...
M-69	...	...	...	...	223
M-56	...	...	...	...	236
M-43	227	255	...	...	...
M-CH_3O	239	267	264	263	261
M-C_2	...	269	...	...	...
M (Parent)	270	298	296	294	292

[a] The first two most intense peaks are indicated by double and single underlines, respectively.

isolated products was not less than 95% pure. Both infrared and NMR data corroborated this estimate of purity. Each of the five principal esters, palmitate, stearate, oleate, linoleate, and linolenate was obtained in sufficient amounts to obtain infrared, NMR, and mass spectra. These spectra were compared with those obtained under the same conditions for authentic synthetic products. Although the chemical and physical properties of this homologous group of compounds are very similar, individual differences in one or more characteristics permitted unequivocal identification of each of the five FAME. For example, the NMR spectra of methyl oleate (Table II) does not contain a signal at 2.74 δ corresponding to the methylene between two vinyl groups found in linoleate and linolenate, but contains vinyl protons at 5.30 δ which are not found in spectra of methyl esters of stearic and palmitic acids. Similarly, ATR infrared spectra aided in distinguishing palmitate and stearate from each other and from the unsaturated esters (Table III).

Although most of the absorption bands of the unsaturated esters are identical, absorption intensities in the 3000-cm.$^{-1}$ region assigned to vinyl stretching modes aided in distinguishing the three compounds from the saturated esters. Mass spectra of each of the five compounds are also sufficiently unique to distinguish each of the esters (Table IV). The

Table V. Molecular Distillate Yields from Various Samples of Cold-Pressed Walnut Oil

Sample	Yield, %
Fresh kernels	0.25
Commercial oil	0.33
Rancid kernels (2.5% moisture)	0.62
Rancid kernels (3.4% moisture)	0.36
Rancid kernels (4.8% moisture)	1.50
Commercial oil (hot-pressed)	2.10

Table VI. Composition of Molecular Distillates Obtained from Cold-Pressed Oils of Fresh and Rancid Walnut Kernels

Fraction	Fresh kernels	Rancid kernels
Glyceride (distillate residue)	99.8%	98.3%
Distillate	0.2%	1.7%
Steroids and unknowns	189 mg. %	756 mg. %
Free fatty acids	20 mg. %	879 mg. %
Methyl esters	0.7 mg. %	24 mg. %

Table VII. Composition of Methyl Ester Fraction of Walnut Oil Obtained from Moisture-Adjusted Kernels after Storage and Rancidity Development[a]

Material	Fresh Kernels %	Rancid Kernels after Storage at 2.5% H_2O %	3.4% H_2O %	4.8% H_2O %
Methyl esters in molecular distillate[b]	..	4	7	19
Peak 9 (methyl myristate)	2	Trace	Trace	Trace
Methyl palmitate	7	5	7	8
Methyl stearate	2	2	2	2
Methyl oleate	15	8	12	14
Methyl linoleate	54	37	52	62
Methyl linolenate	18	10	15	13
Peaks 29, 30, 31	0.5	10	3	0
Peak 33	0.3	20	5	0
Peak 34 and 35	1.0	8	4	0

[a] Calculated by integration of GLC peaks. [b] Proportion of FAME and FFA peaks observed on gas chromatogram of molecular distillate.

fragmentation patterns for palmitate and stearate were quite similar. However, the parent peaks, the M-43 and M-31 fragments, distinguished the two saturated esters. In general, the mass spectra of the FAME corresponded reasonably well with those reported by Christie and Holman (1966) and Ryhage and Stenhagen (1960).

Analysis of Fresh and Rancid Walnut Oils. Yields of molecular distillate obtained from random samples of walnut kernels varied widely. It was of interest to compare distillate yields and proportions of FFA, FAME, and other components from cold-pressed oils obtained from fresh and rancid kernels. Hot-pressed commercial oil and oils obtained from rancid kernels containing high moisture levels yielded more distillate (Table V). The distillates obtained from fresh kernels tended to be semisolid, while those obtained from rancid kernels were liquid. Higher sensitivity was also required for the detection of both FFA and FAME during GLC analysis of the oil from fresh kernels. It was suspected that large amounts of material in the fresh kernel distillates were not eluted from the GLC column. The addition of a measured amount of propyl palmitate, as an internal standard, to the distillate of both fresh and rancid kernel oil confirmed the presence of a major proportion of material which was not detected by gas chromatography. FFA and FAME constituted only about 10% of the fresh oil distillate and about 45% of the rancid oil distillate. Concurrent increases of FFA and steroids with rancidity development suggest that deteriorative processes may involve hydrolysis of steroid esters of FA. Possibly the free sterol may have been formed from β-sitosterol-D-glucoside which has been reported in walnuts by Jurd (1956). On TLC plates, material which did not elute from the GLC column was located in an area between the FFA and glycerides (Figure 2).

Chromatography of fresh walnut oil distillate on alumina with hexane:methanol (98:2 v./v.) permitted the separation of several grams of a white crystalline solid which precipitated spontaneously as it eluted from the column. The white solid was recrystallized twice from absolute methanol. On TLC plates it gave a single spot in the area between FFA and glycerides and a positive Lieberman-Burchard reaction for sterols. Elemental analyses, mass and infrared spectra of this compound were identical to those of an authentic sample of β-sitosterol. The melting point of a mixture of the unknown compound and β-sitosterol showed no depression. This compound constituted the major part of the distillate fraction which was not eluted from the gas chromatography column. Several apparently related compounds were isolated in microgram quantities using a combination of absorption and thin-layer chromatography procedures. Their mass spectra indicated molecular weights in the range of β-sitosterol.

The relative proportions of FFA and FAME, exclusive of steroids and other compounds which were not eluted from gas columns, were estimated by totalizing the corrected peak areas obtained from the individual FFA and FAME. Each peak area was converted to a weight percentage using conversion factors obtained from gas chromatograms of *ad hoc* mixtures. The proportions of FFA and FAME in the oil prepared from fresh and rancid kernels may be estimated from the data shown in Table VI. The FFA:FAME ratio varied only from about 30:1 for fresh kernels to 40:1 for rancid kernels, although the latter contained about 35 times as much total FAME and 45 times as much FFA. In a group of oil samples obtained from rancid kernels which had been stored at different moisture levels, the FAME:FFA proportions varied directly with moisture content (Table VII). No further work was conducted to characterize peaks 29, 30, 31, 33, 34, and 35. It is also of interest that the proportions of FFA in the fresh oil sample (not reported) paralleled the FFA proportions in the rancid sample stored at near optimum moisture (Rockland *et al.*, 1961).

Apparent Fatty Acid Methyl Esters in Other Oils. Molecular distillation of 50-ml. portions of a variety of natural oils yielded distillates ranging from traces up to 3%, and averaging about 2% of the whole oil, with the exception of sperm oil from which a 13% yield was obtained. Each distillate was chromatographed on alumina to concentrate the apparent FAME as described previously for walnut oil. GLC indicated the presence of apparent FAME in various products (Table I) although their proportions varied widely from those of walnut oil and from each other. TLC studies of the concentrates supported the GLC data for peanut, almond, filbert, soybean, and safflower oils. NMR spectra of peanut oil distillates contained a shift at $\delta = 3.62$, indicating the presence of methyl esters. Similar shifts were not observed in NMR spectra of crude distillates obtained from safflower, sunflower, or soybean oils.

DISCUSSION

The occurrence of 0.4 to 1.5 mg. % of FAME in walnut oil may be compared with the 4 to 5 mg. % FAME found in liver by Fischer *et al.* (1966). Because of the relatively small amount of FAME observed in walnuts, and the danger of artifact formation during sample preparation, extreme efforts were made to obtain unequivocal evidence for the presence of these compounds in cold-pressed walnut oil. The following studies were conducted in order to preclude the possibility that FAME in walnut oil may be artifacts.

Oil was extracted at ambient temperature in a clean, dry, stainless steel filter press, protected from light, held under nitrogen, and utilized within 24 hours; direct injection of relatively massive amounts of freshly prepared whole, cold-pressed walnut oil into the gas chromatograph produced peaks corresponding to authentic FAME. The injection of large amounts of whole oil into the gas chromatograph irreversibly changed the characteristics of the column and destroyed its further usefulness. Molecular distillation and liquid chromatography were utilized to remove products which interfered with gas chromatography.

Crude molecular distillates of cold-pressed walnut oil showed a small but distinct singlet in NMR spectra which corresponded with the CH_3O peak for methyl esters at 3.62 δ. Addition of an authentic C_{18} methyl ester to the crude distillate enhanced the singlet peak proportionately.

Injection of stripped glyceride residue into the gas chromatograph produced no peaks, indicating that the FAME could not have been formed by pyrolysis or other reactions within the gas chromatograph.

Repeated stripping of the nondistillable glyceride residue yielded no significant amount of distillate, precluding the possibility that the esters might have been formed during distillation.

FAME fractions were rechromatographed as many as three times on alumina columns without change in the proportions of individual esters or significant loss of material.

The presence of only nominal amounts of FAME in oil obtained from fresh kernels in comparison with thirty or more times as much in rancid samples may be considered further evidence opposed to the possibility that the FAME were artifacts produced during sample preparation. The present evidence suggests that only small amounts of FAME occur in fresh kernels and that deterioration of kernels results in the formation of both FFA and FAME. The proportions of the individual FAME are strikingly similar to the proportions of the fatty acids in both the FFA and glyceride fractions of walnut oil. Since the formation of FAME roughly parallels FFA formation, it is possible that FFA may not be formed exclusively through enzymatic or simple hydrolytic cleavage of glycerides. Further systematic studies of changes in FAME during deterioration and development of rancidity in walnut kernels are in progress.

ACKNOWLEDGMENT

The authors are indebted to Raymond Johnson for making available walnut kernels of known history, to Robert Horowitz and Robert Lundin for assistance in interpreting NMR data, and to Henry Yokoyama for an authentic sample of β-sitosterol.

LITERATURE CITED

Buttery, R. G., Black, D. R., Lewis, M. J., Ling, L., *J. Food Sci.* **32,** 414 (1967).
Christie, W. W., Holman, R. T., *Lipids* **1,** 176 (1966).
Dhopeshwarkar, G. A., Mead, J. F., *Proc. Soc. Exptl. Biol. Med.* **109,** 425 (1962).
Fischer, G. A., Sauk, J. J., Kabara, J. J., *Microchem. J.* **11,** 461 (1966).
Forss, D. A., Angelini, P., Bazinet, M. L., Merritt, C., Jr., *J. Amer. Oil Chem. Soc.* **44,** 141 (1966).
Jurd, L., *J. Org. Chem.* **21,** 759 (1956).
Leikola, E., Nieminen, E., Salomaa, E., *J. Lipid Res.* **6,** 490 (1965).
Lough, A. K., Felinski, L., Garton, G. A., *J. Lipid Res.* **3,** 478 (1962).
Merritt, C., Jr., Forss, D. A., Angelini, P., Bazinet, M. L., *J. Amer. Oil Chem. Soc.* **44,** 144 (1967).
Rockland, L. B., Swarthout, D. M., Johnson, R. A., *Food Technol.* **15,** 112 (1961).
Rockland, L. B., unpublished data, 1969.
Ryhage, R., Stenhagen, E., *J. Lipid Res.* **1,** 361 (1960).
Schultz, T. H., Black, D. R., Bomben, J. L., Mon, T. R., Teranishi, R., *J. Food Sci.* **32,** 698 (1967).
Sloan, C. L., Jackson, L. J., Baker, G. L., Henry, J. E., *Lipids* **3,** 455 (1968).

Received for review June 16, 1969. Accepted November 17, 1969. Presented at the Division of Agricultural and Food Chemistry, 155th Meeting, ACS, San Francisco, Calif., April 1968. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable. Supported in part by the Diamond Walnut Growers, Inc., Stockton, Calif., and the Walnut Control Board, San Mateo, Calif.