Determination and Occurrence of Oxofatty Acids in Fats and Oils

Daniel P. Schwartz* and **Aly H. Rady**[1]
Eastern Regional Research Center, ARS/USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118

A relatively simple method is detailed for the routine isolation and estimation of oxofatty acids (OFA) in lipids. The lipid in cyclohexane is transmethylated in a two-phase, 3.5 min procedure, and the carbonyls in the methyl ester fraction are derivatized with 2,4-dinitrophenylhydrazine (DNPH) in the presence of monochloroacetic acid (MCA). The derivatives are fractionated on alumina, and the OFA fraction is obtained and evaluated spectrophotometrically. A large variety of animal, plant, and marine lipids contained OFA ranging from <1 to > 50 μmoles/g. Data also show that (a) OFA are formed in naturally oxidizing fats and oils, and (b) strongly acidic conditions can cause elaboration of OFA in hydroperoxidized fats and oils.

KEY WORDS: Alumina fractionation, 2,4-dinitrophenylhydrazones, fat oxidation, monochloroacetic acid.

In conjunction with a project dealing with the effect of gamma irradiation of food on lipid composition, our laboratory undertook the development of methods for the examination of some minor classes that might be present in lipids. It was anticipated that if changes induced by irradiation were to occur, they would be more readily seen in a minor class as opposed to appearing in the usual fatty acids composing the bulk of the glycerides. To this end we have developed a relatively simple micro procedure for the isolation and estimation of the oxofatty acids (OFA) in glycerides. Observations were made in the 1950's (1,2) that some fats and oils contain relatively high concentrations of nonvolatile (compared to volatile) carbonyl compounds. Subsequently, the major nonvolatile carbonyl class in one fat (milkfat) was identified as an OFA (3), and identification of the OFA was eventually accomplished (4). Other than in milkfat, relatively few OFA have been reported to occur naturally in lipids (5–7). Ostensibly, this is due to a lack of suitable, routine methodology for their detection and isolation. Application of the method to be described to a fairly large number of lipid-containing materials has indicated that OFA are widespread.

MATERIALS AND METHODS

Cyclohexane (Burdick and Jackson, Muskegon, MI) was rendered carbonyl-free by scrubbing it over a 10 g bed of analytical grade Celite (Fisher Sci. Co., Malvern, PA), containing 0.5 g of 2,4-dinitrophenylhydrazine (DNPH) in 10 mL of 60% H_3PO_4 (8) overlaid with 20 g of Celite 545 containing 10 mL of conc. H_2SO_4 ground onto it (9). A chromatography tube (2 cm × 30 cm) with an attached reservoir was used. The flow was ~3L/24 hr. The scrubbed solvent was distilled. Benzene was purified by the method of Henick *et al.* (2) as modified by Craske and Edwards (10). Acidic alumina (Alfa Products, Danvers, MA) was partially deactivated by the addition of 8% water, shaking until all lumps were dispersed and equilibrating overnight. DNPH was recrystallized from n-butanol (1:35) and the crystals washed with purified cyclohexane. A benzene solution of DNPH was made by dissolving (with heat) 1 mg/mL and storing in the dark when not in use. A 16% solution of monochloroacetic acid (MCA) (Aldrich Chem. Co., Milwaukee, WI, highest purity) was prepared in benzene using low heat to effect solution. Both DNPH and MCA solutions are just saturated. Some crystals may appear with time depending on the temperature of the room. When this happened, the MCA solution was warmed to 40°C until dissolution of the crystals, and the solution taken prior to recrystallization. Ecosorb GL-119, a sulfonic acid resin on charged fibers, was obtained from Graver Chem. Co., Union, NJ, and used as received. Methanolic KOH (2N) was prepared using a freshly opened bottle of methanol (Burdick and Jackson). Aliquots of this solution were taken with a syringe through a septum. American Society for Testing and Materials (ASTM) sand was used as received. Large volume Pasteur pipettes (Fisher) were used as columns for the Ecosorb 119.

EXPERIMENTAL PROCEDURES

Isolation of lipids. Lipids were isolated as soon as their source arrived at the laboratory. When this was not feasible, the source was stored at −18°C. All isolated lipids were analyzed within one hour following extraction. Seeds and nuts (1–10 g) were ground in a coffee mill for 0.5–1.0 min. If the shell or hull was difficult to remove, the whole thing was ground; otherwise, they were first shelled. The ground material was transferred to a 200 mL centrifuge bottle or a 45 mL vial and covered with 5 parts of cyclohexane, making sure that all of the powder was wetted. After 20–30 min, the container was shaken and centrifuged at 3000 rpm for 5 min. The supernatant, if clear, was decanted through a 6.5 cm funnel containing a 6 mm glass bead in the stem that was covered with a little sand. If the supernatant was turbid, it was passed over a 0.2 g bed of Celite 545 contained in a large volume Pasteur pipette plugged with a 4 mm glass bead and containing sand to fill the tapered portion. Animal tissue and animal products (10–20 g) were homogenized for 0.5–1.0 min in a 200 mL centrifuge bottle with 2.5 parts of cyclohexane using a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was then treated as described above.

Transesterification. The procedure of Christopherson and Glass (11) was used with minor modification. This step was carried out rapidly to minimize saponification. The cyclohexane extract (~4 mL) containing 10–

[1]Present address: Radiobiology Department, Nuclear Research Center, Atomic Energy Authority, Inshas, Egypt.
*To whom correspondence should be addressed.

200 mg/mL of accurately weighed lipid was vortexed for 3.5 min with 0.5 mL of 2N methanolic KOH in a 9 mL vial with Teflon-lined screw cap. Distilled water (0.5 mL) was added, the vial inverted several times, and then centrifuged for 2 min at 4000 rpm. The upper phase was removed for analysis.

Derivatization of carbonyls. Three aliquots (usually 0.5, 0.75 and 1.0 mL) of the methyl ester solution in 5 mL screw cap vials were placed in a stream of nitrogen at room temperature until most of the cyclohexane was evaporated. DNPH solution (2 mL) and 0.5 mL of MCA solution were added and the vial kept in the dark for 1 hr.

Removal of excess DNPH and fractionation of derivatives. Alumina (5 g) was added in portions with shaking to ~6 mL of n-hexane contained in a glass column (32 cm to taper × 1.1 cm i.d.) plugged with glass wool. After settling, any alumina on the sides was washed down and ~0.5 cm of sand was added. A bed of Ecosorb 119 (200–250 mg) was prepared in a large volume Pasteur pipette that was plugged with a 4 mm glass bead and contained sand to fill the tapered portion. The resin was tamped lightly to just give a compact bed and the pipette was set atop the alumina tube. The reaction mixture was transferred to the top tube and let drain. The vial was rinsed with two 1 mL portions of cyclohexane and the rinsings transferred to the top column. The Pasteur pipette was removed and the alumina column was drained. The carbonyls containing no ester function were eluted with 35 mL of n-hexane:benzene (1.5:1) and discarded. The OFA band (~ halfway down the bed) was eluted with 35 mL of benzene:hexane (1.5:1). The solvent was evaporated with heat under a stream of nitrogen. The residue was scanned in both $CHCl_3$ and in cyclohexane from 420–320 nm at 600 nm/sec in a Beckman DU-70 spectrophotometer. A blank was run each time a new reagent solution was prepared.

RESULTS AND DISCUSSION

Stability of hydroperoxides. To minimize the possibility of artifact formation by reaction conditions, the quantitative aspects of the reaction of DNPH with OFA in the presence of organic acids of different strengths were studied. The acids were trichloroacetic (TCA), dichloroacetic (DCA), and monochloroacetic (MCA). When conditions were established for the quantitative reaction of methyl 12-oxostearate with DNPH in the presence of the acid catalyst, the effect of these conditions on the decomposition of four hydroperoxide-containing lipids, e.g., olive oil (PV = 900), soybean oil (PV = 138), safflower oil (PV = 80), and methyl linoleate (PV = 1100), was determined. TCA catalyzed the derivatization completely in 10 min but caused some decomposition of all hydroperoxidized lipids. DCA catalyzed the reaction to completion in 15 min but caused some decomposition of the hydroperoxides of safflower and soybean oils but not those of olive oil and methyl linoleate. MCA needed 1 hr to catalyze the reaction to completion but had no or only negligible effect on hydroperoxide decomposition of any of the lipids. All reactions were conducted at room temperature.

Model compound experiments. To check the quantitative aspects of the procedure (Table 1), methyl esters of oxostearates were put through the entire procedure, initially in the presence of a pure synthetic glyceride, 1-palmitoyl-2,3-distearoyl glycerol. When it was determined that the glyceride did not affect recovery, subsequent studies were done in the absence of the glyceride. A molar absorptivity of 22,500 was used to convert the spectrophotometer reading to concentration of OFA. This coefficient is an average determined from literature values calculated for a number of saturated and unsaturated non-conjugated aliphatic ketones and short-chain, unesterified oxo acids (12). When recoveries were less than quantitative (e.g., 3-oxo,17-oxo), the original OFA was subjected to thin-layer chromatography on silica gel G to estimate purity and the recoveries were adjusted accordingly. Dichloromethane was used as mobile phase and spots were revealed by charring. The plate was scanned at 440 nm using a Camag II thin-layer chromatography (TLC) scanning densitometer.

The range of positional isomers covered in Table 1 suggests that all other positions between the extremes should be recovered to a similar extent. The methyl 2-oxostearate was not recovered at all. The loss was

TABLE 1

Recovery of Methyl Oxostearates Subjected to Entire Procedure

Compound	Estimated purity[a] (%)	Amount assayed (μmoles)	Recovery[b] (%)	Absorption maximum of 2,4-dinitrophenylhydrazone in cyclohexane (nm)	Absorption maximum of 2,4-dinitrophenylhydrazone in $CHCl_3$ (nm)
Methyl 2-oxostearate	100	1.1	0	339	352
Methyl 3-oxostearate	82	0.8	97[c]	344	361
Methyl 5-oxostearate	100	1.2	100	348	365
Methyl 12-oxostearate	100	1.9	98	349	366
Methyl 17-oxostearate	90	1.0	96[c]	346	364

[a]Determined by densitometry of silica gel G plate after development with CH_2Cl_2 and charring.
[b]Average of >3 determinations using $\varepsilon = 22{,}500$.
[c]Corrected for non-oxofatty acid impurities.

traced to saponification of the ester bond during transmethylation with some simultaneous decarboxylation. Although of only academic interest, it should be mentioned that the 2-oxostearate could be quantitatively derivatized and isolated if the transmethylation step was omitted. Therefore, it is possible that the 2-oxofatty acids in glycerides could be determined if an acid-catalyzed transmethylation procedure is used, although this might be accompanied by artifact formation. Reaction of DNPH with the oxostearates listed in Table 1 was linear. Regression coefficients of 0.99 were obtained. Derivatization and isolation of the OFA fraction from a natural product (milkfat) also gave a linear response when concentration of derivative was plotted against the weight of milkfat analyzed (regression coefficient = 1.00). Under the conditions of derivatization described, linearity was only obtained up to a maximum of close to 2.0 μmoles of OFA, and it is assumed that this figure also holds true for the total carbonyls present in the sample to be derivatized. As a consequence, it is necessary, when examining a lipid quantitatively for OFA for the first time, that linearity be established. If linearity is not obtained, either the amount of lipid analyzed should be reduced, the volume of reagents increased, or both.

The absorption maxima of the oxostearates, excluding the 2-position, fell between 344–349 nm in cyclohexane and 361–366 nm in $CHCl_3$. The only absorption maxima of OFA DNPs in the literature are for short-chain unesterified oxo acids, e.g., 4-oxopentanoic (levulinic) and 3-oxobutyric acids. These had maxima of 365 nm and 360 nm in $CHCl_3$, respectively (12).

Analysis of fats and oils. The lipids and their sources analyzed are listed alphabetically in Table 2. Information on the source of the product is given in parentheses following the name. When oil follows the name, it indicates that it was a commercial oil either purchased in the market (mkt) or supplied by a processor (P). The absorption maximum found for the OFA DNPs in $CHCl_3$ and in cyclohexane is given. The absorption maxima of model saturated OFA were presented in Table 1. Unsaturated OFA in which the double bond(s) is or are not conjugated with the oxo group would be expected to have similar maxima. This is exemplified by the absorption maximum found for the OFA derivatives isolated from oiticica oil. The major OFA in this oil is 4-oxo-9,11,13-octadecatrienoic (licanic) acid. OFA with one double bond conjugated with the oxo group, and OFA with 2 or 3 double bonds conjugated with each other and also with the oxo group would be expected to have absorption maxima in $CHCl_3$ of 373–385, 388–407, and 400–415 nm, respectively, the exact maximum in any given class being dependent on the type and number of alkyl groups present at or near the double bonds (12). The absorption maximum obtained for any entry in Table 2 may also be the result of the ratio of saturated (or unsaturated, non-conjugated -enones or -dienones) to conjugated -enones and -dienones. The effect of admixtures of a saturated OFA DNP (methyl 5-oxostearate) with the OFA DNPs isolated from squash seed oil on the resultant absorption maximum was studied. These data are in Table 3 and indicate that as much as 20% contamination of the saturated OFA (λ $CHCl_3$ = 364.5) with squash seed oil methyl esters (λ $CHCl_3$ = 390.5 nm) only raised the absorption maximum by 2 nm. When the situation is reversed, however, a more significant shift is observed. Accordingly, it is not feasible to draw any conclusions as to the classification or purity of the OFA isolated from any entry in Table 2 based solely on the absorption maximum, although this characteristic, in some instances, might suggest a preponderance of one class over the others. For these reasons, also, an accurate quantitative figure for concentration of OFA in any of the entries in Table 2 cannot be given, except by chance. However, in view of the fact that the methodology employed is quantitative or nearly so, it was felt that despite the lack of more definitive analytical information, an approximate concentration of OFA in the lipids would be appropriate to report. This is included in Table 2, and the calculation is based on a molar absorptivity of 22,500, the coefficient used for saturated OFA or for OFA containing no double bonds conjugated with the oxo function. There are no molar absorptivities in the literature for OFA with one or more double bonds conjugated with the oxo group. However, there are molar absorptivities determined for monoenoic, dienoic, and trienoic conjugated ketones (25,000–35,000; 30,000–40,000; and 40,000–50,000 nm, respectively) (12). Assuming that these values would also be applicable to OFA, the figures in Table 2 besides being approximate would also be maximal. However, if all of the OFA in a given entry were saturated or contained double bonds not conjugated with the oxo group, the value in Table 2 would be correct.

The information in Table 2 indicates that OFA are widespread in nature, albeit in relatively low concentrations in many of the lipids examined. Only one entry (*Lesquerella fendleri*) was devoid of OFA. *Lesquerella fendleri* contains high concentrations of hydroxy fatty acids (13). Of the > 100 entries in Table 2, 17% had less than 1 μmole/g of extracted lipid. The possibility that *in vitro* lipid oxidation gave rise to part or all of the OFA isolated from some of the entries in Table 2 cannot be ruled out, especially in view of some of the low concentrations found in some of the lipids. It has been demonstrated that α,β-unsaturated-enones can be produced when oleic and elaidic acids are exposed to oxygen and CO^{++} (14,15).

On the other hand, it is known that OFA are produced in mammalian cells during the biosynthesis of some prostaglandins and also by the action of lipoxygenase and other enzymes in some plant tissue (16). Lipoxygenase-initiated oxidation of linoleic acid has also been shown to give rise to isomeric C_{18} conjugated dienones (17). Whether some of these are present in the fractions isolated in this study is under investigation.

OFA in oxidized lipids—MCA vs TCA as catalyst. It was pointed out earlier that exposure of highly peroxidized lipids to TCA leads to extensive destruction of hydroperoxides as measured by the PV. MCA, on the other hand, caused no or only negligible decomposition under the same conditions and its use was accordingly adopted. It was of academic interest to us to compare the values obtained using TCA and MCA as catalysts in the DNPH derivatization of OFA as this would give some idea of the magnitude of OFA generation from peroxidized lipids caused by exposure

TABLE 2

Oxofatty Acid Content of Fats and Oils Determined as 2,4-Dinitrophenylhydrazones

Source of fat or oil	Absorption maximum of 2,4-dinitrophenyl-hydrazones in cyclohexane (nm)	$CHCl_3$ (nm)	Approximate concentration (μmoles/g lipid)
Acorn (single green nut)	349	366	1.3 ± 0.0
Alfalfa seed	368	384	2.0 ± 0.0
Almond (closed shell)	350	367	0.4 ± 0.0
Almond (open shell)	350	367	0.6 ± 0.0
Amaranthus cruentus seed	371	384	2.2 ± 0.1
Apple seed (mixed varieties)	348	365	0.2 ± 0.0
Avocado (flesh)	350	366	0.1 ± 0.0
Beef heart (freshly killed steer)	349	366	3.2 ± 0.0
Beef kidney (freshly killed steer)	349	366	3.6 ± 0.0
Beef rump (freshly killed steer)	349	366	3.2 ± 0.1
Beef, ground (mkt)	349	366	2.3 ± 0.1
Beef, ground (same as above; pan-fried medium-well)	349	366	2.5 ± 0.1
Black currant seed (*Ribes nigrum*)	346	364	2.0 ± 0.1
Blue fish (fresh-caught)	348	366	0.3 ± 0.0
Brazil nut (mkt, unshelled)	349	367	0.6 ± 0.0
Brewer's yeast (mkt)	345	371	5.0 ± 0.0
Canola seed (>2 yrs old)	354	370	2.5 ± 0.1
Caper-spurge seed (*Euphorbia lathyrus*)	351	368	0.8 ± 0.0
Cashew nut (mkt, raw, purchase shelled)	358	372	1.5 ± 0.0
Cheese fat (Roquefort, mold-ripened; sheep's milk)	349	366	5.9 ± 0.1
Cheese fat (L'explorateur, mold-ripened, cow's milk)	349	366	6.3 ± 0.1
Cheese fat (Cheddar, >3 yrs old)	349	366	4.5 ± 0.0
Cheese, fat (Gouda, >1 yr old, goat's milk)	349	366	2.9 ± 0.0
Chia seed (mkt)	353	370	4.2 ± 0.1
Chicken (skin + subcutaneous fat, fresh-killed, feathers removed cold)	349	367	1.4 ± 0.1
Chicken (skin + subcutaneous fat, fresh-killed, feathers removed with heat)	349	367	1.1 + 0.0
Chicken (mkt, skin + subcutaneous fat)	349	367	3.5 ± 0.1
Chicken fat (mkt, jar)	353	367	4.8 ± 0.2
Coconut oil (crude) P	348	365	1.1 ± 0.0
Coconut oil (same as above, refined) P	348	365	1.2 ± 0.0
Cod-liver oil (mkt, analyzed when opened)	351	368	1.1 ± 0.0
Cod-liver oil (same as above, used, analyzed on expiration date)	369	386	3.1 ± 0.1
Coffee bean (green, Columbian, type A)	350	367	0.9 ± 0.0
Coffee bean (same as above, roasted)	357	374	1.7 ± 0.0
Corn oil (mkt)	360	375	3.8 ± 0.1
Corn oil (crude) P	368	382	3.0 ± 0.0
Corn oil (as above, refined) P	358	372	2.5 ± 0.0
Cottonseed oil (crude) P	367	378	1.3 ± 0.0
Cottonseed oil (refined) P	351	367	3.4 ± 0.0
Flax seed (mkt)	363	378	1.8 ± 0.0
French fried potates (fast food)	350	367	6.0 ± 0.0
Gooseberry seed (*Ribes grass-ularia*)	349	366	2.5 ± 0.1
Grape seed (red chancellor)	350	367	1.0 ± 0.0
Grape seed oil P	367	377	6.5 ± 0.2
Hazel nut (large Filbert)	348	364	0.4 ± 0.0
Human fat (from lower abdominal wall, live female)	350	366	0.8 ± 0.0
Jimsonweed seed (*Datura stramonium*)	349	367	8.2 ± 0.4
Lard (mkt)	349	366	4.7 ± 0.0
Lamb (rib chop, mkt)	348	366	4.3 ± 0.1
Lamb (same as above, broiled to medium-well)	348	366	3.3 ± 0.0

(Continued)

TABLE 2 Continued

Source of fat or oil	Absorption maximum of 2,4-dinitrophenylhydrazones in cyclohexane (nm)	$CHCl_3$ (nm)	Approximate concentration (μmoles/g lipid)
Lamb (same as above, drippings)	348	366	3.3 ± 0.0
Lesquerella fendleri seed	—	—	0.0 ±
Macadamia nut	348	366	1.1 ± 0.0
Maxepa oil (mkt, capsules)	351	369	1.9 ± 0.1
Milkfat (cow's, from ultra-pasteurized heavy cream)	349	366	4.0 ± 0.1
Milkfat (cow's from mkt butter)	349	366	6.1 ± 0.0
Milkfat (same as above, browned 1-2 min)	349	366	6.6 ± 0.1
Milkfat (pig)	353	367	2.3 ± 0.1
Milkfat (sheep)	349	365	2.8 ± 0.1
Milkfat (human)	352	366	0.8 ± 0.0
Morning Glory seed (*Ipomoea purpurea*, mkt)	349	366	55.5 ± 2.6
Mustard seed	352	365	1.3 ± 0.0
Oat seed	351	365	1.3 ± 0.0
Oiticica oil P	347	363	869.0 ± 17.2
Oiticica oil P	347	363	808.4 ± 22.0
Oiticica oil P	347	363	818.4 ± 31.3
Okra seeds (Clemson spineless)	379	391	6.9 ± 0.3
Olive oil (Extra Virgin, mkt)	366	379	2.1 ± 0.1
Olive oil (mkt)	365	377	3.6 ± 0.0
Onion seed	377	392	1.7 ± 0.0
Orchard grass seed	347	365	4.0 ± 0.3
Pea (dried green, mkt)	359	373	0.8 ± 0.0
Peanut (mkt, roasted in shell)	366	381	1.7 ± 0.0
Peanut oil (mkt)	366	381	3.0 ± 0.1
Peanut oil (crude) P	353	368	1.2 ± 0.0
Peanut oil (same as above, refined) P	352	368	1.5 ± 0.0
Pecan (unshelled, mkt)	348	365	0.9 ± 0.0
Pepper seed (Calif. Bell)	356	369	0.8 ± 0.0
Pignolia (shelled, mkt)	379	392	3.3 ± 0.2
Pistachio nut (unshelled)	355	368	1.0 ± 0.0
Purslane seed	367	385	1.1 ± 0.0
Quinoa seed (mkt)	370	384	2.8 ± 0.1
Rapeseed oil (low erucic acid)	362	372	3.8 ± 0.1
Red Clover seed	358	370	14.5 ± 0.4
Rice Bran (stabilized, 1-2 yrs old)	365	380	2.5 ± 0.2
Safflower oil (mkt)	365	378	1.3 ± 0.0
Sesame seed	349	366	0.3 ± 0.0
Soybean (Golden Harvest, seed >2 yrs old)	352	366	1.3 ± 0.1
Soybean oil (crude) P	353	366	1.5 ± 0.0
Soybean oil (same as above, refined) P	351	365	2.5 ± 0.0
Squash seed (winter, Table Queen Ebony)	379	390	30.9 ± 0.2
Sunflower oil (mkt)	366	382	2.0 ± 0.0
Tall fescue grass seed	362	379	4.7 ± 0.3
Timothy grass seed	349	363	3.3 ± 0.0
Tomato seed (var. Rutgers)	364	380	1.7 ± 0.0
Tung oil P	379	393	2.3 ± 0.0
Turkey skin (non-frozen bird, mkt)	349	363	3.2 ± 0.1
Velvet leaf seed (*Abutilon theophrasti*)	370	388	5.0 ± 0.1
Vernonia galamensis seed (flaked)	366	382	3.7 ± 0.1
Walnut, (unshelled, mkt)	351	368	0.5 ± 0.0
Wheat germ	353	368	0.4 ± 0.0
Wheat germ oil	365	381	1.4 ± 0.0

TABLE 3

Effect of Admixtures of the 2,4-Dinitrophenylhydrazones of Methyl 5-Oxostearate and the Oxofatty Acid Methyl Esters of Squash Seed Oil on Absorption Maxima

Methyl 5-oxostearate		Squash seed oil methyl esters	
% in mixture	λ in $CHCl_3$ (nm)	% in mixture	λ in $CHCl_3$ (nm)
100.0	364.5	100.0	390.5
85.7	366.0	85.7	385.5
80.0	366.5	80.0	383.0
75.0	368.0	75.0	382.0
66.6	368.5	66.5	379.5
50.0	373.5	50.0	373.5

to strongly acidic conditions (TCA is ~100 times stronger than MCA). The analytical procedures were identical except that 0.75 mL of a 17.2% benzene solution of TCA and a 10 min reaction time at room temperature were used compared to the standard procedure. Results were as follows (lipid, PV, MCA and TCA values in μmoles/g): milkfat, 217, 14.5, 15.7; olive oil, 246, 23.8, 28.9; safflower oil, 297, 32.7, 48.6. These results suggest that decomposition of hydroperoxidized lipids by strong acid leads to the formation of some OFA. However, the argument could be made that the higher values obtained with TCA may merely be due to increased reaction of some OFA with DNPH. Even though both MCA and TCA under their respective reaction conditions give quantitative or near quantitative yields of DNPs with the model compounds tested, the possibility exists that there are some unsaturated, branched, or hindered OFA that react more quantitatively with DNPH under TCA catalysis than under MCA catalysis. To determine whether this was the case, the following experiment was conducted. The safflower oil methyl esters in 5 mL of cyclohexane were passed over a 0.5 g bed of Celite 545 charged with N/1 H_2SO_4 (2.5 parts Celite 545: 1 part N/1 H_2SO_4) contained in a column 0.8 cm × 11 cm. Flow rate was ~1 mL/5 min. The effluent was analyzed by the standard (MCA) procedure. The OFA value rose from 32.7 to 47.1 μmoles/g, a figure near the TCA value of 48.6 μmoles/g and adding support that strong acid conditions may cause some elaboration of OFA from hydroperoxidized lipids.

The original milkfat, olive oil and safflower oil used in the above study had PVs of less than 1.0 and contained 3.2, 3.6 and 1.3 μmoles/g of OFA, respectively, determined by the MCA-catalyzed procedure. These lipids were permitted to oxidize at room temperature in their original containers under ordinary laboratory light for 2–3 years and then yielded the figures cited above. Thus, the OFA content increased 4.5, 6.6, and 25 times, respectively. The absorption maximum of the OFA isolated from the three oils increased from 15–18 nm.

Miscellaneous observations. A short-chain saturated aldehyde (formaldehyde), a ketone (acetone) and a dienal (2,4-pentadienal) DNPs were all removed with their homologues in the non-ester-containing carbonyl fraction during fractionation on the alumina bed. Thus, all members of these classes would also be in this fraction and will not interfere in the estimation of OFA.

The semialdehyde DNP, methyl azelaaldehydate, separated cleanly from model OFA DNPs on the alumina bed. Clean separation was also observed when the carbonyls in badly oxidized oils were derivatized and chromatographed. The semialdehyde fraction moves slower than the OFA methyl ester derivatives and can be obtained, if desired, by elution of the bed with 20 mL of benzene:hexane (3:1) following collection of the OFA zone. No semialdehyde zone was noted during fractionation of the derivatives from any of the entries in Table 2. In this regard, removal of unreacted DNPH with a cation exchange resin prior to fractionation of the DNPs facilitated visual examination of the alumina for zones moving slower than the OFA band. In addition, removal of excess reagent in this manner permitted the use of crude hexane and benzene for elution of the zones. It has been reported that derivatization can occur without acid catalysis on the surface of alumina when ketones come in contact with adsorbed DNPH (18). Use of Ecosorb 119 rather than a conventional cation exchange resin (19) for removal of DNPH is considered an improvement in that the beds are easier, faster and less expensive to prepare.

Each lipid was analyzed by TLC following the transmethylation step to ascertain whether this procedure was quantitative. In all but a few instances, no glycerides were detected. In those positive, only traces were seen even though exaggeratedly large amounts of the methyl ester solution were spotted.

The OFA fraction is obtained virtually lipid-free from the alumina column and can be subjected to TLC on silica gel G plates or on reverse phase plates. Presumably, it could also be subjected to analogous high performance liquid chromatography (HPLC) conditions with ultraviolet (UV) detection.

ACKNOWLEDGMENTS

We thank the late A.P. Tulloch and the Prairie Regional Laboratory, Saskatchewan, Canada, for the OFA, and we thank Lou-Ana Foods, Opelousas, LA; Polyesther Corp., Southampton, NY; California Almond Growers Assn., Sacramento, CA; General Foods Corp., Tarreytown, NY; The Viobin Corp., Monticello, IL; and Welch, Holme and Clark Co., Inc., Harrison, NJ, for samples of oils and their sources.

REFERENCES

1. Berry, N.W., and A.A. McKerrigan, *J. Sci. Food Agric. 9*:693 (1958).
2. Henick, A.S., M.F. Benca and J.H. Mitchell, *J. Am. Oil Chem. Soc. 31*:88 (1954).
3. Keeney, M., I. Katz and D.P. Schwartz, *Biochem. Biophys. Acta 62*:615 (1962).
4. Weihrauch, J.L., C.R. Brewington and D.P. Schwartz, *Lipids 9*:883 (1974).
5. Pohl, P., and H. Wagner, *Fette Seifen Anstrich. 74*:541 (1972).
6. Badami, R.C., and K.B. Patil, *Prog. Lipid Res. 19*:119 (1981).
7. Schulz, W., W. Francke and M. Boppre, *Biol. Chem. Hoppe-Seyler 369*:633 (1988).
8. Schwartz, D.P., and O.W. Parks, *Anal. Chem. 33*:1396 (1961).
9. Hornstein, I., and P.F. Crowe, *Ibid. 34*:1037 (1962).
10. Craske, J.D., and R.A. Edwards, *J. Chromatogr. 57*:265 (1971).
11. Christopherson, S.W., and R.L. Glass, *J. Dairy Sci. 52*:1280 (1970).

12. Braude, E.A., and E.R.H. Jones, *J. Chem. Soc.*, 498 (1945).
13. Mikolajczak, K.L., F.R. Earle and I.A. Wolff, *J. Am. Oil Chem. Soc. 39*:78 (1962).
14. King, G., *J. Chem. Soc.*, 2114 (1954).
15. King, G., *Ibid.*, 587 (1956).
16. Vick, B.A., and D.C. Zimmerman, *Plant Physiol. 75*:458 (1984).
17. Vioque, E., and R.T. Holman, *Arch. Biochem. and Biophys. 99*:522 (1962).
18. Pool, M.F., and A.A. Klose, *J. Am. Oil Chem. Soc. 28*:215 (1951).
19. Schwartz, D.P., A.R. Johnson and O.W. Parks, *Microchem. J. 6*:37 (1962).

[Received February 9, 1990; accepted May 12, 1990]