Quantitation and Occurrence of Hydroxy Fatty Acids in Fats and Oils

Daniel P. Schwartz* and **Aly H. Rady**[1]
USDA, Eastern Regional Research Center, ARS, Philadelphia, Pennsylvania 19118

A relatively simple method is detailed for the routine quantitation and isolation of hydroxy fatty acids (HFA) in the glycerides of lipids. The lipid in cyclohexane is transmethylated in a two-phase, 3.5 min procedure, and the hydroxyl compounds in the methyl ester fraction are derivatized with pyruvic acid chloride 2,6-dinitrophenylhydrazone in the presence of triethylenediamine. The derivatives are fractionated on alumina, and the HFA fraction is evaluated spectrophotometrically. A large variety of animal, plant and marine lipids contain HFA in concentrations ranging from <10 to >1000 μmoles/g lipid. The derivatives lend themselves admirably to purification techniques. A procedure for regenerating the parent HFA from the derivative is described.

KEY WORDS: Alumina fractionation, pyruvic acid chloride 2,6-dinitrophenylhydrazone, triethylenediamine.

In connection with a project dealing with the detection of meat subjected to low-dose gamma irradiation, this laboratory undertook the development of methods for examining several minor classes of fatty acids that may be present and/or generated in lipids. It was reasoned that if changes induced by gamma irradiation were to occur, they would more easily be detected in a minor as opposed to a major class. In this regard, a previous report from this laboratory (1) described the isolation and estimation of oxo fatty acids (OFA) in lipids. The present work details a method for the isolation and quantitation of the hydroxy fatty acids (HFA) in the glycerides of fats and oils. Although many more HFA than OFA have been isolated from natural products (2,3), no routine method has been described for both the isolation and quantitation of relatively low as well as high concentrations of HFA in glycerides. Application of the method outlined below to a relatively large number of fats, oils and lipid-bearing materials has revealed that HFA, like OFA, are widespread.

MATERIALS AND METHODS

All solvents were from Burdick and Jackson, Muskegon, MI, and were used as received. Pyruvic acid chloride 2,6-dinitrophenylhydrazone (PAC) was synthesized (4) and the crystals were stored at −18°C over anhydrous $CaCl_2$. It was later ascertained that the crystals could also be stored without drying agent at room temperature in opaque plastic or brown-glass containers for at least 1.5 years without loss of reactivity. A solution of PAC in dried [calcium hydride (CaH_2)] toluene was prepared (with heat) to contain 6.7 mg (23.5 μmoles) per mL and stored at 4–5°C when not in use. Triethylenediamine (1,4-diazabicyclo- [2.2.2] octane) (Aldrich Chemical Co., Milwaukee, WI) was used as received. A solution containing 84 mg (70 μmoles) per mL of dried toluene was stored over CaH_2. Acidic alumina (Alfa Products, Danvers, MA) was partially deactivated with 8% water, shaken until all lumps were dispersed and equilibrated 16 hr before use. Sodium methoxide (2N in methanol) was prepared from metallic sodium and MeOH from a freshly opened bottle. Aliquots of this solution were taken by syringe through a septum. Polyethylene glycol 400 (PEG) (J.T. Baker, Phillipsburg, NJ) on Celite 545 (Fisher Science Co., Malvern, PA) was prepared by grinding 2 mL of PEG on 9 g of Celite 545 in a mortar. Powdered CaH_2 was from Fluka AG (Buchs, Switzerland). Other materials were: ignited fine sand (Baker); 4 mm glass beads and disposable super Pasteur pipettes (Fisher).

EXPERIMENTAL PROCEDURES

Distilled water was used; screw caps were Teflon-lined; evaporations were done under nitrogen. Glassware for the reactants and reaction was dried at 130°C for 1 hr.

Isolation of lipids. Neutral lipids were isolated from their source as described previously (1). With some seeds, when only small amounts were available, the hulls were removed manually, and 1 part of the seed was ground in a mortar with 0.5 part of Celite 545 and 0.5 part of sand until homogeneous. The powder was transferred to a glass column and tamped semi-tightly, and the lipids were extracted by percolating cyclohexane (25 mL/≤ 3 g shelled seeds) over the bed.

Transmethylation. The cyclohexane solution (2–6 mL), containing up to 100 mg of accurately weighed lipid/mL, and 0.2 mL of sodium methoxide solution in a 9-mL vial was vortexed for 3.5 min at high speed. Citric acid solution (0.3 mL, 2 M) was immediately added. The vial was shaken vigorously for ~15 sec and then centrifuged at 3500 rpm for 3 min.

Derivatization. Three aliquots (usually 0.5, 0.75 and 1.0 mL) of the methyl ester solution in 5-mL screw cap vials were evaporated at room temperature until most or all of the cyclohexane was gone. PAC solution (0.5 mL) and 0.5 mL of dry toluene and 1–2 mg of CaH_2 were added. The vials were capped and stored in the dark until no hydrogen bubbles were visible. This normally took from 5–30 min, depending upon the amount of methyl ester in the aliquot. Triethylenediamine solution (0.2 mL) was added and the vial was let stand uncapped until the blood-red color faded to orange or yellow (~5–10 min).

Fractionation of derivatives. Alumina (5 g) was added to ~6 mL of n-hexane contained in a glass column (32 cm to taper × 1.1 cm i.d.). Alumina adhering to the sides was washed down with n-hexane, and ~0.5 cm of sand was added. The reaction mixture was transferred to the column, and the vial was rinsed with two 1-mL aliquots of n-hexane. After draining, the sides of the column were washed with 2 mL of n-hexane. The non-HFA derivatives (sterols, fatty alcohols) were eluted with 50–60 mL of

*To whom correspondence should be addressed at USDA, Eastern Regional Research Center, ARS, 600 E. Mermaid Lane, Philadelphia, PA 19118.

[1]Present address: Radiobiology Department, Nuclear Research Center, Atomic Energy Authority, Inshas, Egypt.

hexane:toluene (1.5:1). The HFA derivatives were then eluted with 40 mL of hexane:toluene (1:3). The solvent was evaporated completely with heat, and the residue was dissolved in 2 mL of hexane.

Removal of methanol derivative. The HFA fraction may contain small amounts of the derivative of methanol. Some methanol partitions into the cyclohexane layer during the transmethylation step and may not be completely removed during evaporation of the aliquots, and will be derivatized. The derivative chromatographs with the HFA derivatives. It was removed as follows. Approximately 0.5 g of the Celite-PEG powder was transferred to a super Pasteur pipette plugged with a 4-mm glass bead, and the tapered portion filled with sand. The packing was tamped moderately to give a compact bed. The solution of the residue was transferred to the bed, and the beaker was rinsed with 1-mL aliquots of hexane (usually 2) to transfer all color. The bed was washed with hexane (~3 mL) until all color below the methanol derivative band (near top of bed) was removed. The solvent was evaporated and the absorbance of residue was read in toluene at 404 nm. The concentration of HFA in the lipid was calculated from the following equation (Equation 1):

$$C = (A_s - A_b)/0.595 \times W \quad [1]$$

where C = μmoles HFA/g extracted lipid; A_s and A_b = absorption of sample and blank, respectively, in 10 mL; 0.595 = molar absorptivity in 10 mL, and W = weight of lipid analyzed in grams.

RESULTS AND DISCUSSION

Model compound analysis. To check the quantitative aspects of the procedure, methyl esters of HFA were dissolved in cyclohexane containing 20 mg/mL of pure, synthetic 1-stearoyl-2,3-dipalmitoyl glycerol and then subjected to the entire procedure, including the transmethylation step. Pertinent data are in Table 1, which shows that the 6-, 12-, and 17-hydroxy-stearates were recovered nearly quantitatively. The 3-hydroxy stearate and the 16-hydroxy palmitate were recovered less quantitatively. However, both compounds contained an impurity that was detected on thin-layer chromatograms. The impurity was fast-moving on silica gel G and diffuse in all solvent systems tried. Attempts to accurately quantitate the chromatograms by means of densitometry were unsuccessful due to diffusion of the impurity zone coupled with the disproportionality of the HFA and impurity zones when the plate was charred. The recovery figures for the 3-hydroxy stearate and 16-hydroxy palmitate accordingly are minimal. All hydroxyl positions between 6- and 17- in the stearates would be expected to give yields similar to those obtained with the 6-, 12- and 17-positions. The 2-hydroxy eicosanoate gave poor and variable recoveries. Variations in the derivatization procedure (*e.g.*, narrowing or broadening the ratio of PAC to triethylenediamine and heating the solution prior to or following addition of the base) did not significantly increase yields. Similar recoveries were obtained when the transmethylation step was omitted, thereby ruling out saponification as a major cause of the low and variable recoveries of the 2-hydroxy eicosanoate. A shorter-chain compound (2-hydroxy laurate) behaved similarly.

TABLE 1

Recovery of Methyl Esters of Hydroxy Fatty Acids Subjected to Entire Procedure

HFA	Amount analyzed (μmoles)	Recovery (% ± SD (n))
2-Hydroxyeicosanoate	0.8	30–50 (4)
3-Hydroxystearate	1.0	89.0 ± 1.1 (3)
6-Hydroxystearate	1.8	98.2 ± 0.5 (3)
12-Hydroxystearate	2.0	99.0 ± 0.4 (2)
17-Hydroxystearate	1.9	98.4 ± 0.8 (3)
16-Hydroxypalmitate	1.9	95.5 ± 0.9 (4)

Analysis of lipids. Sources of the lipids analyzed are listed alphabetically in Table 2. Information on the source of the product follows the name. If the word "seed," "nut," or "pit" is used it means that the seed, nut or pit was extracted in this laboratory to obtain the lipid. The letter (M) indicates that the product was purchased in a market. No letter means that the product was obtained privately. Table 2 reveals that all lipids contained HFA in a broad concentration range. Of the 77 products listed, 57 (74%) had <10 μmoles/g lipid. Castor oil contained the highest concentration, 2,863 μmoles/g oil. Assuming that all of the HFA in castor oil is ricinoleic acid (MW methyl ester = 312), the ricinoleic acid content calculates to 89.3%, a figure close to literature values obtained by other techniques (5). The second highest concentration of HFA was found in the seed oil of *Lesquerella fendleri* (variety unknown to us), 1,747 μmoles/g lipid. The major HFA identified in the seed oil of *L. fendleri* is lesquerolic acid (MW methyl ester = 340) and our figure calculates to 59.4%, a value which is close to the literature value (6).

HFA have been identified as one of the secondary oxidation products in both enzyme-catalyzed oxidation and nonenzymatically oxidized model systems (7–11) and also in some oxidized natural products (7,10). In Table 2, only a small number of entries were relatively fresh, *e.g.*, acorn, avocado, ground beef, black currant seed, blue fish, chicken, coconut flesh, flounder, gooseberry and pepper seeds. The ages of the remaining entries were either unknown to us, or known to be relatively old. This fact alone, however, does not necessarily mean that some or all of the HFA have arisen *via* oxidation after the formation of the product. Nevertheless, this possibility should be recognized, especially in view of the relatively low concentrations found in many of the entries.

Besides castor oil and *L. fendleri*, only one other entry in Table 2 has been analyzed for HFA. Using PAC and direct analysis of the glycerides of butteroil, Timmen *et al.* (12) found 8.1 μmoles/g, a figure slightly higher than the 7.4 μmoles/g reported in Table 2.

Transmethylation. The transmethylation procedure is a slight modification of that described by Christopherson and Glass (13) for the analysis of milkfat fatty acids. The procedure was effective, giving complete transmethylation of tristearin and even tribehenin when the latter was dissolved in toluene-cyclohexane. No partial glycerides could be detected by thin-layer chromatography (TLC) on silica gel G, (CH_2Cl_2 developer), even when exaggeratedly large volumes of the methyl ester solution was spotted.

Addition of citric acid solution at the end of the transmethylation reaction neutralizes the alkali, dropping the

TABLE 2

Concentration of Hydroxy Fatty Acids (HFA) in Fats and Oils

Source of lipid	HFA (μmoles/g)	Source of lipid	HFA (μmoles/g)
Acorn (one green nut)	5.1 ± 0.6	Lettuce (seed) (Great Lakes) (M)	76.4 ± 0.3
Alfalfa (seed) (M)	32.1 ± 0.3	Lily (seed) (Mardi Gras × Gold Eagle)	6.2 ± 0.4
Almond (nut) (M)	11.4 ± 0.1	Macadamia (nut) (M)	5.6 ± 0.2
Apple seed (mixed varieties)	3.6 ± 0.2	Maxepa oil (capsules) (M)	6.0 ± 0.2
Amaranthus cruentus (seed) (M)	6.6 ± 0.2	Meadowfoam oil (super refined)	0.3 ± 0.0
Avocado (flesh) (M)	4.4 ± 0.0	Mustard (seed) (M)	8.2 ± 0.2
Beef (ground, raw) (M)	6.0 ± 0.2	Okra (seed) (Clemson spineless) (M)	112.0 ± 1.1
Black currant (seed) *(Ribes nigrum)*	10.6 ± 1.5	Olive oil (extra virgin) (M)	2.7 ± 0.1
Bluefish (fresh-caught)	3.5 ± 0.0	Olive oil (virgin) (M)	2.4 ± 0.1
Brazil nut (M)	7.5 ± 0.2	Onion (seed) (early yellow Globe) (M)	50.1 ± 4.9
Butter oil (M)	7.4 ± 0.2	Peanut (Virginia)	7.1 ± 0.1
Candlenut	4.3 ± 0.1	Peanut (Spanish)	9.1 ± 0.1
Canola (seed)	9.6 ± 0.1	Peanut (Florunner)	9.4 ± 0.2
Cashew (nut) (M)	2.5 ± 0.1	Pecan (nut) (M)	3.7 ± 0.1
Castor oil (M)	2863.0 ± 28.5	Pepper (seed) (California Bell)	11.1 ± 1.3
Cheddar cheese (aged 1 year) (M)	8.2 ± 0.1	Pignolia (pine nut) (M)	7.7 ± 0.2
Chenopodium quinoa (seed) (low saponin)	7.4 ± 0.3	Pistachio (nut) (M)	9.2 ± 0.3
Chenopodium quinoa (seed) (high saponin)	7.4 ± 0.3	Poppy (seed) (M)	6.5 ± 0.2
Chia (seed) (M)	9.7 ± 0.1	Purslane (seed) (M)	1.8 ± 0.0
Chicken (skin + subcutaneous fat) (M)	2.7 ± 0.0	Red clover (seed) (M)	111.1 ± 0.2
Chocolate, unroasted (natural nibs)	1.7 ± 0.0	Rice bran (stabilized)	20.8 ± 0.3
Chocolate, above roasted (Dutch nibs)	1.9 ± 0.0	Safflower oil (M)	7.5 ± 0.0
Cod-liver oil (freshly opened bottle) (M)	1.6 ± 0.1	Sardine (from water-packed Norwegian) (M)	1.7 ± 0.0
Coconut (flesh) (M)	1.2 ± 0.0	Sesame oil (Japanese) (M)	3.3 ± 0.1
Corn oil (M)	3.0 ± 0.1	Soybean (Century)	6.6 ± 0.1
Cottonseed	33.0 ± 0.4	Soybean (Golden Harvest)	7.1 ± 0.1
Date (pit) (M)	4.6 ± 0.1	Sunflower oil (M)	3.5 ± 0.0
Dill (seed) (M)	8.5 ± 0.4	Sunflower (seed) (M)	2.7 ± 0.0
Evening primrose oil (capsules, age unknown) (M)	20.0 ± 0.3	Tall fescue (seed) (M)	19.8 ± 1.2
Evening primrose oil (capsules, freshly opened bottle) (M)	7.5 ± 0.1	Timothy grass (seed) (M)	19.2 ± 0.8
Fast food fat (mainly tallow, unused)	6.0 ± 0.1	Tomato (seed) (Rutgers) (M)	4.1 ± 0.1
Flaxseed (brown) (M)	28.6 ± 0.0	Trisun (seed)	8.7 ± 0.2
Flounder (fresh-caught)	3.2 ± 0.0	Trisun oil (crude)	3.6 ± 0.2
Gooseberry (seed) *(Ribes grass-ularia)*	11.7 ± 0.1	Trisun oil (cooking grade)	2.9 ± 0.0
Hazel nut (Filbert) (M)	3.5 ± 0.1	Trisun oil (salad grade)	2.1 ± 0.0
Ilomba (seed) *(Pycanthus angelous)*	30.5 ± 0.1	*Vernonia galamensis* oil (degummed, charcoal-treated)	24.5 ± 0.8
Lard (M)	5.1 ± 0.0	Walnut (M)	1.3 ± 0.1
Lesquerella fendleri (seed)	1747.0 ± 25.0	Wheat germ (raw powder) (M)	9.0 ± 0.2

pH to ~4.2. This value is sufficiently above the acidity that could cause decomposition of hydroperoxides with the accompanying possibility of artifact formation (1).

There is a small (~0.5%) loss of HFA during transmethylation, presumably *via* saponification as determined on the model compounds in Table 1. This loss appears to be unavoidable. Shortening the transmethylation reaction time to 3 min gives incomplete transesterification of tristearin. Incomplete transesterification is unacceptable because cyclohexane-soluble partial glycerides will react with PAC.

Derivatization. Sufficient reagent must be present in the reaction mixture to react with all hydroxyl groups, including HFA, sterols, fatty alcohols and polyhydroxy fatty acids, if present. Also, unesterified fatty acids complex PAC, reducing its effective concentration (14). PAC, like most acid chlorides, reacts with water in the presence of the catalyst. It is essential that linearity of the derivatization reaction of PAC with HFA be established with each lipid sample when it is analyzed for the first time. If linearity is not obtained with the 3 aliquots taken, the original methyl ester solution should be diluted until linearity is obtained.

Fractionation. Clean separation of sterol and fatty alcohol derivatives (not separable from each other) from the HFA derivatives was readily accomplished for each lipid analyzed. The sterol + fatty alcohol fraction was quantitated in most instances but the results will not be reported here. The HFA fraction was virtually free of colorless lipid but was usually contaminated with traces of the derivative of methanol. The latter was removed inexpensively on the PEG-Celite partition bed. All model HFA (Table 1) and also methyl ricinoleate and methyl ricinelaidate derivatives were eluted with the recommended (40 mL) of hexane:toluene (1:3). Essentially all of the HFA isolated from the entries in Table 2 were also eluted from the alumina bed within this volume. Occasionally, depending on HFA concentration, 5–10 mL more was required. Slower-moving zones were sometimes seen on the alumina bed above the HFA zone but were not eluted for analysis. These more polar derivatives may be di- and polyhydroxy-, epoxyhydroxy-, and oxohydroxy fatty acid esters.

Pyruvic acid chloride 2,6-dinitrophenylhydrazone and derivatives. PAC is a highly reactive acid chloride when its reaction is catalyzed by triethylenediamine (14). Primary, secondary and tertiary alcohols, primary and secondary amines and thiols all react essentially instantaneously (15). All primary and secondary alcohol derivatives have molar absorptivities close to each other, averaging 5,950 (4). The derivatives of alcohols are bright yellow with absorption maxima in benzene or toluene at 403–405 nm. Chromatographic fractionation of derivatives is readily followed visually. All derivatives contain the acidic hydrogen atom in the hydrazone linkage, which enables them to be strongly adsorbed by alkaline adsorbents (*e.g.*, MgO, $ZnCO_3$) and to exchange on a basic anion exchange resin (15) while undergoing a spectral shift from yellow to violet. These characteristics are highly advantageous when isolating and purifying HFA away from colorless lipids. Fractionation of derivatives of HFA model compounds (methyl 12-hydroxy stearate, methyl ricinoleate and methyl ricinelaidate and also purified HFA derivatives isolated from milkfat) by Ag^+ TLC on silica gel G has been carried out in this laboratory. Classes obtained in this way can be subjected to thin-layer and/or column partition chromatography with bonded phases to separate the classes into chainlengths.

Regeneration of derivatives to parent HFA. To identify a fractionated HFA derivative, it is helpful to obtain the parent HFA for mass spectral and other analytical techniques. Regeneration is readily accomplished as follows. Up to 1 μmole of derivative is dissolved in 6 mL of cyclohexane in a 9-mL vial. This solution is transmethylated by using the conditions specified earlier, except that citric acid solution is not added after vortexing. Following centrifugation, the upper layer (colorless) is transferred to a 9-mL vial, and 0.3 mL of 2N HCl is added. The vial is shaken vigorously, then centrifuged 2 min at 3,500 rpm. The upper layer contains the methyl ester of the parent HFA. Recoveries are ~70%.

Miscellaneous. The use of chloroform:methanol (2:1) to extract the lipids from their source was tried on several of the entries in Table 2. The HFA values obtained were not significantly higher than those obtained with the recommended cyclohexane extraction. This is probably because the aliquots analyzed for HFA were relatively small, normally 25–100 mg. It may also be because the cyclohexane-methanolic sodium methoxide system used for transmethylation may exclude polar lipids such as phospholipids.

Addition of CaH_2 to dry the reaction mixture should not significantly exceed the recommended 1–2 mg. If too much CaH_2 is added, flow through the alumina column may slow down due to the presence of H_2 bubbles generated when the CaH_2 is transferred with the reaction mixture and reacts with water in the alumina and eluting solvents. We conveniently dispensed the recommended amount of CaH_2 from a 15-mL polyethylene dropping bottle by squeezing it once.

The blank does not change for at least two months when the reagents are stored under the recommended conditions. The blank value is low, corresponding to ~0.02 μmole of HFA.

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