

The Formation of Oxo- and Hydroxy-Fatty Acids in Heated Fats and Oils¹

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A fast-food fat (mostly tallow), olive oil and safflower oil were heated in air for 4 d and periodically analyzed for oxo-fatty acids (OFA), monohydroxy-fatty acids (HFA) and polyhydroxy-fatty acids (PHFA). After transmethylation, the OFA were estimated as 2,4-dinitrophenylhydrazones, and the HFA and PHFA were quantitated as pyruvic acid 2,6-dinitrophenylhydrazone esters. At least half of the maximum concentration attained for OFA, HFA and PHFA was generated between 16–24 h of heating of each oil. Safflower oil contained greater concentrations of HFA and PHFA than either olive oil or the fast-food fat. The fast-food fat sample contained a greater concentration of OFA than did the other oils. The sum of the concentrations of OFA, HFA and PHFA at the time of maximum formation in the oils was approximately 260 $\mu\text{moles/g}$ at 48–72 h for safflower, 200 $\mu\text{moles/g}$ at 48–72 h for olive and 170 $\mu\text{moles/g}$ at 72 h for the fast-food fat.

KEY WORDS: Alumina fractionation, derivatization, 2,4-dinitrophenylhydrazones, pyruvic acid 2,6-dinitrophenylhydrazone esters.

Consequences of the prolonged heating of fats and oils have been reviewed recently. These include the safety and health aspects of ingesting such fats and oils (1), quality control (2), regulatory control (3), and the chemistry and physics of deep fat-frying operations (4). Also, the analytical methods for detecting and quantitating some of the known classes of compounds generated in the heating of fats and oils have been surveyed (5).

Recent publications from this laboratory (6,7) described methods for estimating oxo-fatty acids (OFA) as 2,4-dinitrophenylhydrazones (DNPs) and for quantitating hydroxy-fatty acids (HFA) as pyruvic acid 2,6-DNP esters. Both OFA and HFA occur in oxidized methyl oleate (8,9), but they have not been estimated or quantitated specifically in glycerides subjected to prolonged heating.

The methods for estimating the concentration of OFA and HFA were applied to two common oils and to a fast-food fat that were heated for four days. The investigation was undertaken to determine the amount of OFA and HFA generated during heating.

EXPERIMENTAL PROCEDURES

Safflower and olive (extra virgin) oils were obtained in a market in the Philadelphia, Pennsylvania, area. The fast-food fat (solid at room temperature) was supplied by a private, anonymous source and was purported to be mainly tallow containing a small amount of a vegetable oil. One 25-g sample of each lipid in uncovered 50-mL beakers was subjected to $180 \pm 2^\circ\text{C}$ in a circulating-air oven (Model

#OV-510A-2; Blue M, Inc., Blue Island, IL). Aliquots (≈ 1 g) were removed at periodic intervals and stored capped at -18°C until analyzed. Duplicate, or, if necessary, triplicate samples of accurately weighed stored aliquots were analyzed for OFA and HFA.

HFA. HFA were derivatized, isolated and quantitated as previously described (7). The method for HFA was modified to include the isolation and quantitation of the more polar HFA, polyhydroxy-fatty acids (PHFA). The fraction containing PHFA was eluted from the alumina with methylene chloride/toluene, 3:1 (50 mL) after the complete elution of the HFA fraction.

The assumption was made that the PHFA was mainly dihydroxy-fatty acids and that the *bis* derivative was formed exclusively. A molar absorptivity of 11,850 (10) was used in the calculation. The assumption that the PHFA fraction is mainly dihydroxy-fatty acids was based on the formation and chromatography of the *bis* derivative of pure methyl-*threo*-9,10-dihydroxystearate. The *bis* derivative was obtained exclusively and quantitatively under the conditions described for reaction of HFA with pyruvic acid chloride 2,6-DNP. The *bis* derivatives of both vicinal and nonvicinal alkanediols are also formed exclusively and quantitatively under the same conditions (10). The *bis* derivative of methyl-*threo*-9,10-dihydroxy stearate moved about half-way down the alumina bed after complete elution of the mono HFA with hexane/toluene, 1:3 (7). The *bis* derivative was then eluted completely with 50 mL of CH_2Cl_2 /toluene, 3:1. Epoxyhydroxy- and oxohydroxy-fatty acids are other classes of HFA that might be formed in thermally oxidized fats and oils. The derivative of epoxy ricinoleate (methyl-12-hydroxy 9,10-epoxystearate) was quantitatively obtained under the specified reaction conditions. It moved approximately halfway down the alumina bed following complete elution of the *bis* derivative of methyl-*threo*-9,10-dihydroxystearate. No sufficiently pure oxohydroxy fatty acid was available for study.

OFA. The method for OFA (6) was modified as follows. Transmethylation of the fat or oil was done with sodium methoxide as the catalyst, as described for the HFA (7), instead of 2N KOH. Thus, methyl esters for both the HFA and OFA analyses were obtained from one sample. In the chromatographic separation of the derivatized OFA fraction, two OFA bands were usually seen in the heated oil samples. Whenever possible, these bands were collected separately for spectrophotometric evaluation. In the modified procedure, the alumina column was eluted with 30 mL of hexane/toluene, 1.5:1, to remove the nonester-containing carbonyl derivatives, which were discarded. The column was next eluted with 20 to 25 mL of hexane/toluene, 1:1.5, to remove the first OFA fraction (band 1). The second OFA fraction (band 2) was then eluted with an additional 40 to 50 mL of hexane/toluene, 1:1.5.

RESULTS AND DISCUSSION

The fatty acid composition of the lipid samples is given in Table 1. The analysis indicates that the fast-food fat was probably an animal fat that contained a small

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TABLE 1

Major Fatty Acid Composition of Fat and Oils Used in Study

Fatty acid	Retention time ^a	(area %)		
		Fast-food	Olive oil	Safflower oil
14:0	10.2	3.3	0.0	0.1
16:0	15.3	26.0	12.6	7.5
18:0	22.9	21.0	2.7	2.4
18:1	25.6	35.9	74.1	6.5
18:2	29.5	1.6	6.4	76.7
18:3	33.7	0.3	0.4	0.3

^aBy gas-liquid chromatography of zero-time methyl esters on a 60 M × 0.25 mm fused-silica capillary with 0.20-mm film of SP 2340 (Supelco, Bellefonte, PA). Programmed in three stages: (i) 150–160°C at 0.5°C/min, (ii) 160–170°C at 1°C/min, (iii) 170–190°C at 1.5°C/min. Injector temperature = 198°C; detector temperature = 250°C; carrier gas = He.

percentage of a vegetable oil. Thus, a so-called "saturated fat" (fast-food), a predominantly monounsaturated oil (olive) and a polyunsaturated oil (safflower) were examined for thermally generated OFA, HFA and PHFA.

Results of the analyses are summarized in Tables 2, 3 and 4 for the fast-food fat, olive oil and safflower oil, respectively.

HFA formation. HFA formed rapidly during heating. More than 50% of the maximum concentration generated during heating was generated within the first 16 h. With the fast-food fat, the concentration of HFA peaked at ≈43 μmoles/g between 16–24 h, then remained essentially constant. With olive oil, maximum HFA formation occurred between 24–48 h and then leveled off. Maximum HFA

with safflower oil took place between 48–72 h. Further heating then resulted in a loss of HFA. By the end of the heating period, the concentration of HFA in the safflower oil had fallen to a concentration near that found at 16 h.

PHFA formation. As with the HFA, more than half of the maximum PHFA was produced within 16 h when the safflower oil was heated. With fast-food fat and olive oil, however, this occurred within 24 h. Maximum formation of PHFA occurred at 72, 88, and 72 h for the fast-food fat, olive oil and safflower oil, respectively. With continued heating, there was a decrease in the concentration of PHFA in the samples.

OFA. As with HFA and PHFA, OFA formed rapidly. With the fast-food fat sample, approximately the same

TABLE 2

Concentration of Oxo- (OFA) and Hydroxy-Fatty Acids (HFA) in a Heated Fast-Food Sample^a

Heating time (h)	HFA (μmoles/g oil)	PHFA ^a (μmoles/g oil)	OFA band 1 (μmoles/g oil)	Absorption maximum		OFA band 2 (μmoles/g oil)	Absorption maximum		Total OFA (μmoles/g oil)	Absorption maximum	
				C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)
				0	6.0 ± 0.4		<0.1	—		—	—
16	32.8 ± 0.4	10.7 ± 0.5	—	—	—	—	—	—	31.9 ± 0.5	352.0	369.5
24	43.3 ± 1.8	20.4 ± 0.2	—	—	—	—	—	—	50.6 ± 4.9	351.0	368.5
48	48.4 ± 0.7	28.0 ± 1.6	—	—	—	—	—	—	86.9 ± 1.7	351.0	368.5
72	44.6 ± 0.7	35.3 ± 0.2	—	—	—	—	—	—	93.6 ± 0.4	351.0	368.0
88	40.0 ± 0.9	22.8 ± 0.4	90.7 ± 1.0	349.5	367.0	13.9 ± 1.3	345.0	364.0	104.6	—	—
96	43.2 ± 1.8	22.9 ± 0.2	98.3 ± 0.7	347.5	364.0	2.5 ± 0.2	345.0	362.0	100.8	—	—

^aPolyhydroxy-fatty acids (along with HFA and OFA), average of closely agreeing duplicates and standard deviation from mean.

TABLE 3

Concentration of Oxo- (OFA) and Hydroxy-Fatty Acids (HFA) in a Heated Olive Oil Sample^a

Heating time (h)	HFA (μmoles/g oil)	PHFA ^a (μmoles/g oil)	OFA band 1 (μmoles/g oil)	Absorption maximum		OFA band 2 (μmoles/g oil)	Absorption maximum		Total OFA (μmoles/g oil)	Absorption maximum	
				C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)
				0	7.0 ± 0.2		3.0 ± 0.4	—		—	—
16	44.8 ± 0.5	20.3 ± 0.5	13.0 ± 0.5	351.5	369.5	10.8 ± 0.2	349.0	366.0	23.8	—	—
24	60.9 ± 0.9	28.4 ± 1.4	17.8 ± 0.5	351.0	370.0	16.6 ± 1.0	347.5	366.0	34.4	—	—
48	84.3 ± 0.3	47.0 ± 2.0	25.5 ± 0.8	351.0	368.5	24.7 ± 1.0	348.5	365.0	50.2	—	—
72	87.7 ± 1.5	48.6 ± 4.6	36.0 ± 0.4	350.5	363.5	26.8 ± 0.2	347.0	366.0	62.8	—	—
88	86.7 ± 0.7	54.2 ± 2.5	43.3 ± 0.7	350.0	366.5	23.2 ± 0.7	345.5	367.5	65.5	—	—
96	85.1 ± 4.0	34.9 ± 0.9	40.2 ± 1.1	350.0	368.0	27.0 ± 0.8	346.0	364.0	67.2	—	—

^aSee footnote to Table 2.

FORMATION OF OXO-/HYDROXY-FATTY ACIDS

TABLE 4

Concentration of Oxo- (OFA) and Hydroxy-Fatty Acids (HFA) in a Heated Safflower Oil

Heating time (h)	HFA ($\mu\text{moles/g}$ oil)	PHFA ^a ($\mu\text{moles/g}$ oil)	OFA band 1 ($\mu\text{moles/g}$ oil)	Absorption maximum		OFA band 2 ($\mu\text{moles/g}$ oil)	Absorption maximum		Total OFA ($\mu\text{moles/g}$ oil)	Absorption maximum	
				C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)		C ₆ H ₁₂ (nm)	CHCl ₃ (nm)
0	13.9 \pm 0.0	4.4 \pm 0.3	—	—	—	—	—	—	2.1 \pm 0.0	363.5	381.5
16	64.0 \pm 2.0	55.5 \pm 0.3	16.0 \pm 0.4	354.0	373.5	27.5 \pm 3.5	354.0	370.0	43.5	—	—
24	73.0 \pm 0.3	64.5 \pm 1.1	20.2 \pm 1.9	352.0	373.0	30.5 \pm 1.2	349.5	365.5	50.7	—	—
48	79.2 \pm 0.3	102.2 \pm 3.1	34.1 \pm 0.3	353.0	370.5	39.8 \pm 1.4	348.0	365.5	73.9	—	—
72	80.5 \pm 3.3	110.2 \pm 4.6	32.0 \pm 2.0	351.5	370.5	39.4 \pm 0.3	345.0	364.0	71.4	—	—
88	68.9 \pm 4.9	96.1 \pm 5.9	41.2 \pm 2.8	352.0	371.0	29.2 \pm 2.0	344.5	362.5	70.4	—	—
96	59.1 \pm 5.5	74.9 \pm 0.9	35.6 \pm 0.5	351.0	370.0	28.1 \pm 1.0	344.5	362.5	63.7	—	—

^aSee footnote to Table 2.

concentration of OFA as HFA was generated at 16 h of heating. With further heating, the concentration of OFA exceeded the concentration of HFA or PHFA. Furthermore, at 48 h and beyond, more OFA was present in the fast-food fat than HFA and PHFA combined. With the fast-food fat, OFA formation did not reach a maximum until 88 h. At this time, the OFA concentration in the fast-food fat exceeded the maximum concentration generated in either safflower or olive oils.

For olive and safflower oils, in which two OFA bands were always noted and collected, band 1, with a few exceptions, had a higher absorption maximum than did band 2. It is possible that the DNPs in band 2 are geometric isomers of some of the DNPs in band 1. Many DNPs form isomers that can be separated, in some instances, by both adsorption and/or partition chromatography. Van Duin (11) noted and discussed isomerism in short-chain saturated oxo acids. DNP isomerism was also observed in the OFA isolated from butter oil (12). That study showed that the chromatographic separation of the isomers of the saturated oxostearates depended on the proximity of the oxo group to the ester linkage. Oxo groups in the 2 to 8 positions were separable isomers; the other positions were not. The closer the oxo group was to the ester linkage, the more marked was the separation of the isomers.

The absorption wavelength maxima recorded for band 2 for all lipids suggest that the OFA are predominantly saturated or contain double bonds not conjugated with the carbonyl group (6). The absorption wavelength maxima also indicated that the oxo group is a ketone. Aldehyde esters of intermediate chainlength have been identified in oxidized model systems (13). Pure methyl-9-oxononanoate was examined in this study. Its DNP had an absorption maximum in cyclohexane and in CHCl₃ at 335 and 352 nm, respectively. It co-eluted with band 2 on the alumina bed. The fact that the significantly higher absorption maxima of the DNPs in band 2 is indicative of a ketone does not rule out the possible presence of aldehyde methyl esters in that zone. As much as 20% of one carbonyl class in another may not necessarily change the absorption maximum of the predominant class (6).

The absorption maxima for band 1 are generally higher than those for band 2 and slightly higher than the maximum recorded for the oxostearates (6). This indicates that

some of the OFA in this zone contain one or more double bonds conjugated with the carbonyl, but the bulk of the OFA are saturated or, more likely, contain double bonds not conjugated with the carbonyl.

When the concentrations of OFA, HFA and PHFA were added together for each heating time, a total of approximately 200 $\mu\text{moles/g}$ was the maximum found for olive oil (48–72 h), approximately 260 $\mu\text{moles/g}$ for safflower oil (48–72 h), and approximately 170 $\mu\text{moles/g}$ for the fast-food fat (72 h). Assuming an average molecular weight of 310 daltons, approximately 50–80 mg of the three classes was formed per g of oil in approximately 3 d.

Perkins and Van Akkeren (14) showed that intermittent (as opposed to continuous) heating of cottonseed oil accelerated the deterioration of the oil. The same was also apparent when water in the form of steam was introduced into the oil during heating when compared to the same oil heated without introduction of water. Assuming that these observations would also be true for the fats and oils used in the present study, the conditions (continuous heating in oils rendered essentially anhydrous during heating) would result in both a slower rate of formation and a lower concentration of OFA, HFA and PHFA than would be expected in an operation with intermittent heating and introduction of water *via* foods.

No attempt was made to identify any specific component of the OFA, HFA and PHFA fractions, although methods for doing this have been developed in this laboratory (12,15). It already has been shown that four α,β *cis* unsaturated and four α,β *trans* unsaturated HFA (and the same for OFA), generated in thermally oxidized methyl oleate, are those predicted from the decomposition of the hydroperoxides known to be formed in methyl oleate (9). In the fat and oils studied here, a complex array of members of HFA, OFA and PHFA is to be anticipated and their approximate compositional mix should be predictable.

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