

Formation of Oxo- and Hydroxyfatty Acids in Irradiated Chickens

ABSTRACT

Finely ground chicken skin and subcutaneous fat exposed to gamma radiation from ^{137}Cs at 0–2°C for up to 10 kGy generated oxofatty acids (OFA) and hydroxyfatty acids (HFA) in the glycerides. Both classes were determined as colored derivatives; OFA as 2,4-dinitrophenylhydrazones, and HFA as esters of pyruvic acid 2,6-dinitrophenylhydrazone. The concentration of OFA increased with increasing irradiation dose but not always linearly. Variations in the concentration of both classes were noted and some chicken lipids failed to form both classes. In the samples where OFA were generated in significant quantities, the absorption maximum of the derivatives shifted toward a higher wavelength with increasing dose due ostensibly to the formation of double bond(s) in conjugation with the oxo group. This shift in absorption maximum was initially considered to be a means for detecting irradiation as well as indicating the dose received with fair accuracy. However, in several instances irradiation of a chicken sample did not result in the formation of significant increases in OFA and therefore this method cannot be used as a definitive test.

INTRODUCTION

Gamma irradiation of natural foodstuffs produces a variety of changes in the major classes, e.g. proteins, carbohydrates and fats. In the latter, both oxidation and hydrolysis, scission and rearrangement of glycerides occurs producing new classes and/or augmenting existing ones. Nawar (1986a,b) compiled tables of volatile compounds and classes of compounds that have been identified as resulting from irradiation of both model systems and natural fats and oils. Although the volatile compounds produced by irradiation of lipids have been studied extensively, investigation of the non-volatile classes and compounds

generated by irradiation of food lipids has received only limited attention. Recent development in this laboratory of methods for the estimation of oxofatty acids (OFA) and for quantitation of hydroxyfatty acids (HFA) in the glycerides fats and oils, prompted us to examine the effect of relatively low doses of gamma irradiation of chicken lipids on the formation of these two classes.

MATERIALS AND METHODS

Reagents

2,4-Dinitrophenylhydrazine (DNPH) was obtained from Aldrich Chem. Co. (Milwaukee, WI)* and recrystallized from *n*-butanol (1:35). The crystals were washed with purified cyclohexane and stored at -18°C . Cyclohexane (Burdick & Jackson, Muskegon, MI) was purified as described by Schwartz & Rady (1990). Toluene was purified (Henick *et al.* 1954) as modified by Craske & Edwards (1971). A toluene solution of DNPH was made by dissolving 1 mg/ml with heating. The solution was stored in the dark when not in use. A 16% solution of monochloroacetic acid (MCA) (Aldrich) was prepared in toluene using low heat to effect solution. Both DNPH and MCA solutions were saturated. Some crystals may appear with time depending on the temperature of the room. When this occurred the MCA solution was warmed to 40°C until dissolution of the crystals, and the aliquot 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 taking precautions against loss of moisture. Methanolic KOH (2N) was prepared using a freshly opened bottle of methanol (Burdick & Jackson). Aliquots of this solution were taken with a syringe through a septum. Washed sand was purchased from J.T. Baker Co. (Phillipsburg, NJ). Large volume Pasteur pipettes (Fisher Scientific, King of Prussia, PA) were used as columns for Ecosorb GL-119. Pyruvic acid chloride 2,6-dinitrophenylhydrazone (PAC) was synthesized (Schwartz & Brewington, 1966) and a solution of it prepared (with heat) to contain 6.7 mg (23.5 μmoles) per ml of toluene. The solution was stored at 4°C when not in use. Triethylenediamine (1,4-diazabicyclo-[2,2,2] octane)(Aldrich) was used as received. A solution containing 84 mg (70 μmoles) per ml of dried (powdered calcium hydride (CaH_2 , Fluka, Buchs, Switzerland) toluene was stored over CaH_2 . Acidic alumina (Alfa Products, Danvers, MA) was partially deactivated with 8% H_2O , shaken until all lumps were dispersed and equilibrated overnight. Polyethylene glycol 400 (PEG) on Celite 545 (Fisher) was prepared by grinding 2 ml of PEG onto 9 g of Celite 545 in a mortar.

Materials and irradiation

Whole chickens (fryers) were purchased from local supermarkets. In some instances chickens were obtained from a butcher. These were presumably fresher.

*Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

In either case the skin and subcutaneous fat were carefully removed upon returning to the laboratory and frozen in crushed dry ice. Just prior to irradiation the frozen tissue was ground to a 'powder' in a bowl cutter (Pettinatti *et al.*, 1983) or in a Waring blender both in the presence of dry ice. The 'powder'-dry ice mixture was transferred to a crystallizing dish and the dry ice permitted to sublimate completely at 4°C. The lipid 'powder' was heat-sealed in low air permeable Kenfield pouches (IKD All-Vak #13)(Rosemont, IL) in the presence of air. Samples were irradiated with gamma rays from a ¹³⁷cesium source at a dose rate of 0.114 kGy/min. Irradiation was conducted between 0–2°C, and samples received up to 10 kGy. The pouches were stored at –18°C until analyzed. Analyses were always completed within 1 week.

Extraction of neutral lipids

Irradiated or control 'powder' (1 part) in a 200 ml screwcap centrifuge bottle was dispersed in 5 parts of cyclohexane using a Polytron (Kinematica, Lucerne, Switzerland) for ~30 s. at ambient temperature and then centrifuged for 5 min at 3000 rpm. The supernatant was decanted over a small bed of sand supported by a 6 mm glass bead lodged at the junction of the stem and cone of a 3" glass funnel. If turbidity was present in the supernatant, it was decanted over a small layer of Celite 545 (Fisher) instead of sand. Aliquots of the clear filtrate were taken to constant weight, at 40°C under a stream of N₂, to establish lipid concentration.

Transesterification

Approximately 5 ml of filtrate containing up to 100 mg of accurately weighed lipid per ml and 0.5 ml of 2N methanolic KOH was vortexed for 3.5 min in a 9 ml vial. The vial was immediately centrifuged (clinical, 1 min) and the upper phase promptly removed.

Estimation of OFA

The procedure was essentially that of Schwartz & Rady (1990). Three aliquots of the methyl ester solution were pipetted into 5 ml screw cap vials and the cyclohexane evaporated under a N₂ stream at room temperature. The aliquots were normally multiples of the lowest volume taken (e.g. 0.25, 0.50, 0.75 ml). DNPH solution (2 ml) and 0.5 ml of MCA solution were added and the vials capped (Teflon-lined) and stored 1 hr in the dark. Alumina (5 g) was added 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 adhering to the sides were washed down and ~0.5 cm of sand was added. A bed of Ecosorb GL-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 and the pipette was set atop the alumina tube. The Ecosorb GL-119 removed excess DNPH. The reaction mixture was transferred to the top tube and permitted to drain. The vial was rinsed with two 1 ml portions of cyclohexane. The Pasteur pipette was removed and the carbonyls containing no ester function were eluted from the alumina with 35 ml of

n-hexane:toluene (1.5:1) and discarded. The OFA band was then eluted with 35 ml of toluene:hexane (1.5:1). After removal of solvent, the residue was scanned in cyclohexane from 320–420 nm at 600 nm/min in a Beckman DU-70 spectrophotometer. A blank was run each time a new reagent solution was prepared. Estimation of concentration was done using the following equation.

$$C = (A_s - A_b)/2.25 \times W \quad (1)$$

where C = μ moles OFA/g extracted lipid, A_s and A_b = absorption of sample and blank, respectively, in 10 ml. 2.25 = Molar absorptivity in 10 ml, and W = weight of lipid analyzed in grams.

Quantitation of HFA

Three aliquots of the methyl ester phase were taken as above for OFA analyses. After evaporation of solvent, PAC solution (0.5 ml), 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 or only traces of hydrogen bubbles were visible. Triethylenediamine solution (0.2 ml) was added and the vial was left standing uncapped until the blood-red color faded to orange or yellow (~5–10 min). The reaction mixture was transferred to an alumina column prepared as above but with no Ecosorb GL-119 column in tandem. Two 1 ml aliquots of cyclohexane were used to wash the vial free of color. The non-HFA derivatives (e.g. sterols, fatty alcohols) were eluted with 40–50 ml of hexane:toluene (1.5:1) and discarded. The HFA derivatives were then eluted with 40 ml of hexane:toluene (1:3). After evaporation of the solvent with heat under N_2 , the residue was dissolved in 2 ml of hexane. The HFA fraction contains small amounts of the derivative of methanol which was removed as follows: Celite-PEG powder (~0.5 g) was transferred to a large volume Pasteur pipette plugged with a 4 mm glass bead and the tapered portion filled with sand. The powder was tamped moderately to give a compact bed. The hexane solution of the HFA residue was transferred to the bed, and the beaker was rinsed with 1 ml aliquots of *n*-hexane (usually 2 ml) to transfer all color. The bed was washed with hexane (~3 ml) until all of the color below the methanol derivative band (near top of bed) was removed. The solvent was evaporated and the absorbance of the residue was read in toluene at 404 nm. The concentration of HFA was calculated using equation 1, substituting 0.595 for molar absorptivity in 10 ml.

RESULTS AND DISCUSSION

The data in Table 1 show that OFA may be formed when ground chicken lipids are irradiated, although not always in a linear relationship with dose. The concentration given in Table 1 should be considered only as an estimate, as it is not possible without additional analyses to determine the ratio of (a) saturated (and unsaturated, non-conjugated with the oxo group) to (b) α , β -enones and to (c) α , β , γ , δ -dienones (Schwartz & Rady, 1990). The latter two classes have higher molar absorptivities than that used in the calculations in equation 1. Thus the figures in Table 1 are correct only if all of the OFA are in category (a) and would be lower if (b) and/or (c) are also present.

TABLE 1
Formation of Oxofatty Acids in Irradiated Chicken Lipids

Chicken		0 kGy	1 kGy	2 kGy	3 kGy	4 kGy	5 kGy	10 kGy
#1'	$\mu\text{m/g}$	3.5	4.3		6.4			13.7
	abs. nm*	349.6		N.I.	362.0	N.I.	N.I.	366.6
#2	$\mu\text{m/g}$	3.8						9.3
	abs. nm	350.0	N.I.	N.I.	N.I.	N.I.	N.I.	364.4
#3	$\mu\text{m/g}$	3.9						8.5
	abs. nm	349.6	N.I.	N.I.	N.I.	N.I.	N.I.	363.5
#4	$\mu\text{m/g}$	3.1	3.8	4.2	5.1	5.4	6.7	9.6
	abs. nm	349.6	351.7	352.2	355.3	360.0	362.4	364.7
#5	$\mu\text{m/g}$	2.6	3.0	3.7	3.8	6.0	6.5	6.7
	abs. nm	349.6	350.0	352.0	352.0	361.3	363.1	—
#6	$\mu\text{m/g}$	3.3	3.5	4.2	4.9	6.6		9.3
	abs. nm	349.6	350.2	352.0	353.1	361.9	N.I.	364.3

*Represents absorbance (nm) maximum in cyclohexane.
N.I. = not irradiated.

Along with the increase of OFA with dose, there is also an increase in the absorption maximum of the derivatives. This increase indicates that the ratio of OFA in categories (b) and/or (c) to (a) is becoming larger. In the unirradiated control the absorption maximum never exceeded 350 nm. The DNPs, for example, of methyl 12-oxostearate, methyl 5-oxostearate and methyl 4-oxo-9,11,13 octadecatrienoate have absorption maxima at 349 nm, 348 nm and 347 nm, respectively (Schwartz & Rady, 1990). It was initially thought that the absorption maximum of the OFA DNPs might be used as a means of detecting the irradiation of chicken. Indeed, in some blind trials, it was possible not only to select the irradiated samples, but it was also possible to determine the irradiation dose the sample had received with fair accuracy. However, this possibility was short-lived when several samples failed to generate OFA upon irradiation even at 10 kGy doses. The reason for this was never established despite a number of experiments designed to eliminate variables such as placement of, and number of samples in the irradiator, volume of air in the pouches, etc. It is possible that some chicken lipids contain sufficient antioxidants, either native or introduced through feed (Lin *et al.*, 1989) to account for the aberrant behavior, but this was conjecture and was not fully explored.

Table 2 gives the results obtained on two chickens in which OFA were generated in both instances and HFA in only chicken #7. Other samples of chicken also showed formation of both classes while some samples showed formation of neither class. In no instance were HFA generated and OFA were not.

Chicken skin and subcutaneous fat when not ground but irradiated up to 10 kGy as described for 'powdered' chicken lipids, never generated OFA and HFA to the same extent. The explanation for this is probably due to the decrease in total surface area exposed to air in the unground lipids.

'Powdered' chicken lipids that were incapable of forming OFA and HFA upon irradiation readily formed these classes when thermally oxidized. For example, when 5 g of chicken fat (obtained by rendering the 'powder' was spread

TABLE 2
Formation of Oxofatty Acids and Hydroxyfatty Acids in Irradiated Chicken Lipids

Chicken		0 kGy	3 kGy	5 kGy	6 kGy	10 kGy
#7'	OFA					
	$\mu\text{m/g}$	3.6	4.3		5.9	9.3
	abs. nm*	350.0	355.0	N.I.	361.0	365.0
#7	HFA					
	$\mu\text{m/g}$	1.9	2.8	N.I.	3.3	5.2
#8	OFA					
	$\mu\text{m/g}$	1.9	2.8	3.7		5.1
	abs. nm	350.0	351.5	356.0	N.I.	362.0
#8	HFA					
	$\mu\text{m/g}$	2.2	2.8	2.9	N.I.	2.6

*Represents wavelength (nm) at absorbance maximum in cyclohexane.
N.I. = not irradiated.

over the bottom of a 400 ml beaker and heated uncovered for 2 hr at 180–182°C in a circulating air oven, 34 μmoles OFA/g of oil (λ_{max} cyclohexane = 352.0) and 71 μmoles HFA/g of oil were generated. It should be noted that despite the formation of a much higher concentration of OFA in the heated chicken fat relative to that produced in irradiated chicken 'powder' (Table 1), the absorption maximum in the latter was considerably higher (at 4–10 kGy) than that determined in the thermally oxidized chicken fat. Heated safflower, fast food (mainly tallow) and olive oils gave an OFA DNP absorption maximum near that of the above chicken oil even over a prolonged (96 hr) heating period (Schwartz *et al.*, in press). This suggests that either lipid oxidation by irradiation takes a course different than thermal oxidation or else the virtual, if not complete absence of water in fat or oil heated in an open vessel at high temperature has a strong influence on the products of oxidation. The presence of water introduced as steam into heated cottonseed oil accelerated deterioration of the oil (Perkins & Van Akkern, 1965) but no mechanism was given.

CONCLUSION

Gamma irradiation of 'powdered' chicken lipids may produce both OFA and HFA in the glycerides. In some trials OFA increased with increasing irradiation dose, but not necessarily proportionally. When this occurred there was also a shift in the absorption maximum of the OFA DNPs toward a higher wavelength indicating the formation of increasing concentration of unsaturated OFA with one or more double bonds in conjugation with the oxo group. In these samples the absorption maximum could be used as an irradiation marker and correlated with dose received with fair accuracy. However, in view of the fact that not all 'powdered' chicken lipid generated OFA in significant amounts upon irradiation, the possibility of using the absorption maximum of the OFA DNP's for detection was negated.

REFERENCES

- Craske, J. D. & Edwards, R. A. (1971). *J. Chromatogr.*, **57**, 265.
- Henick, A. S., Benca, M. F. & Mitchell, J. H. (1954). *J. Am. Oil Chem. Soc.*, **31**, 88.
- Lin, C. F., Gray, J. I., Asghar, A., Buckley, D. J., Booren, A. M. & Flegal, C. J. (1989). *J. Food Sci.*, **54**, 1457.
- Nawar, W. W. (1986a). In *Preservation of Food by Ionizing Radiation*, Vol. II, ed. E. S. Josephson & M. S. Peterson. CRC Press, Boca Raton, Florida, p. 75.
- Nawar, W. W. (1986b). *Food Rev. Intl.*, **2**, 45.
- Perkins, E. G. & Van Akkeren, L. A. (1965). *J. Am. Oil Chem. Soc.*, **43**, 782.
- Pettinatti, J. D., Ackerman, S. A., Jenkins, R. K., Happich, M. L. & Phillips, J. G. (1983). *J. Assoc. Off. Anal. Chem.*, **66**, 759.
- Schwartz, D. P. & Brewington, C. R. (1966). *Microchem. J.*, **11**, 430.
- Schwartz, D. P. & Rady, A. (1990). *J. Am. Oil Chem. Soc.*, **67**, 635.
- Schwartz, D. P. & Rady, A. (1992). *J. Am. Oil Chem. Soc.*, **69**, 170.
- Schwartz, D. P., Rady, A. & Casteneda, S. *J. Am. Oil Chem. Soc.*, In press.