

Quantification of Three Cholesterol Oxidation Products in Raw Meat and Chicken

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ABSTRACT

Raw veal, beef, pork, and chicken muscle tissues were extracted by a modified dry column procedure in which silicic acid was incorporated in the trap of the column. This method separated cholesterol derivatives from the bulk of neutral lipids, phospholipids and cholesterol. Isolation of sterols by preparative thin layer chromatography followed by quantification by direct on-column capillary gas chromatography permitted measurement of 7-ketocholesterol, cholesterol 5 α , 6 α -epoxide, and cholesterol 5 β ,6 β -epoxide at concentrations less than 1 ppm. All muscle tissues contained the three cholesterol products in measurable quantities. 7-Ketocholesterol constituted more than 50% of the oxidation products in all samples.

INTRODUCTION

CHOLESTEROL, an essential component of animal tissue, is well known to be susceptible to oxidation (Smith, 1981, 1987; Maerker, 1987), and cholesterol oxidation products have been detected repeatedly in animal-derived food products (Missler et al., 1985; Nourooz-Zadeh and Appelqvist, 1989; Park and Addis, 1985, 1987). Recent studies in our laboratories (Lakritz and Maerker, 1989) revealed that exposure of cholesterol, in an aqueous environment, to gamma radiation led to generation of certain cholesterol oxidation products in ratios different than those produced by autoxidation. The specific cholesterol derivatives involved were 7-ketocholesterol (3 β -hydroxycholesterol-5-en-7-one), the α -epoxide (cholesterol 5 α ,6 α -epoxide) and the β -epoxide (cholesterol 5 β ,6 β -epoxide). Our earlier work suggested that the unusual relative amounts of these products may be characteristic of the effects of gamma radiation. The hypothesis required testing in actual animal tissue. However, analytical methods available to accomplish the test were tedious, and/or not sensitive enough.

Before such a test could be conducted, we needed to determine the amounts of the three compounds, 7-ketocholesterol, cholesterol α -epoxide and cholesterol β -epoxide, that would be normally found in meat and poultry. Furthermore, because the amounts of cholesterol oxidation products generated by low dose gamma radiation were expected to be extremely small, it was important to develop methodology capable of measuring endogenous cholesterol oxidation products consistently and of measuring increases due to gamma radiation.

Park and Addis (1985) attempted but could not detect 7-ketocholesterol in raw beef and beef products, but found the compound in 3-year old freeze-dried pork and broiled beef steak (Park and Addis, 1987). Sander et al. (1989) reported ppm quantities of 7-ketocholesterol and the two epoxides in dehydrated chicken, beef and turkey, but the method, which involved Folch extraction (Folch et al., 1957) and overnight cold saponification was somewhat cumbersome. The methods proposed by other investigators (Csallany et al., 1989; Nourooz-Zadeh and Appelqvist, 1989; De Vore, 1988; and Higley et al., 1986) were also not suitable for our purposes for lack of specificity, sensitivity or other reasons.

Our objectives was to develop methodology that permitted determination of specific cholesterol oxidation products in meat and poultry below 1 ppm. Application of the method to various muscle tissues would provide information on amounts of such compounds normally present in muscle foods to serve as a basis of comparison for later studies (determination in irradiated foods).

MATERIALS AND METHODS

Materials and reagents

Cholesterol (primary standard grade) was purchased from Eastman Kodak Co. (Rochester, NY); 7-ketocholesterol (3 β -hydroxycholesterol-5-en-7-one), α -epoxide (cholesterol 5 α ,6 α -epoxide) and 6-ketocholestanol (5 α -cholestan-3 β -ol-6-one) from Sigma Chemical Co. (St. Louis, MO); and β -epoxide (cholesterol 5 β ,6 β -epoxide) from Research Plus, Inc. (Bayonne, NJ). Celite 545 was purchased from Fisher Scientific (Malvern, PA) and silicic acid (100 mesh) from Aldrich Chemical Co. (Milwaukee, WI). Triolein, practical grade, was purchased from Sigma Chemical Co. (St. Louis, MO) and purified as reported previously (Maerker and Unruh, 1986). Thin layer chromatography (TLC) plates, silica gel G (500 μ m), prescored 20 \times 20 cm were from Analtech, Inc. (Newark, DE). All solvents used were "distilled in glass grade", and chemicals were of reagent grade quality. Beef (tenderloin), chicken (breast), pork (center cut chops) and veal (various cuts) were purchased from retail stores, and skin and visible fat were trimmed.

Extraction procedure

A modification of the "dry column procedure" (Maxwell et al., 1980; Zubillaga and Maerker, 1984) was used. A glass column was packed with a ground, homogenous mixture containing 10.0 g silicic acid, 9.0g Celite 545 and 1.0g CaHPO₄·2H₂O and compacted by tapping it on a rubber base. To 10.0 g comminuted meat trimmed of visible fat was added 40.0g granular, anhydrous sodium sulfate and 15.0g Celite 545. The three components were ground thoroughly in a mortar, and the resulting dry powder was transferred to the same glass column quantitatively with tapping on a rubber base after addition. A steel rod with flattened circular end was used to press the column packing in place. The column was wetted with 75 mL hexane; ethyl acetate (9:1,v/v) and eluted with 300 mL of the same solvent at about 2 mL/min with positive nitrogen pressure. The eluate (Fraction 1) containing the bulk of the neutral lipids and cholesterol was discarded. Ethyl acetate (150 mL) was used to elute and collect cholesterol oxidation products at 1 mL/min (Fraction 2), while the bulk of the phospholipids remained on the column. Fraction 2 was dried on the rotary evaporator and reconstituted in 250 μ l ethyl acetate.

Thin layer chromatography (TLC)

Plates were washed by development with chloroform: methanol (2:1,v/v) and activated overnight at 115°C in an air oven. The ethyl acetate concentrate of Fraction 2 was applied as a band to the middle 15 cm section of the plate. Cholesterol and cholesterol oxide bands were located by spotting 10 μ L of an ethyl acetate solution containing 6.0 μ g cholesterol and 3.0 μ g α -epoxide per microliter on the end 2.5 cm sections of the plate.

The plate was developed with benzene: ethyl acetate (60:40,v/v), and the end sections were snapped off, sprayed with 50% H₂SO₄ and charred on a hot plate. The cholesterol oxide band from the middle section of the plate was scraped into a test tube and extracted 3 \times with 5, 5, and 2 mL ethyl acetate, vortexing and centrifuging each time. The combined were filtered through a 0.2 μ m Nylon 66 filter, evap-

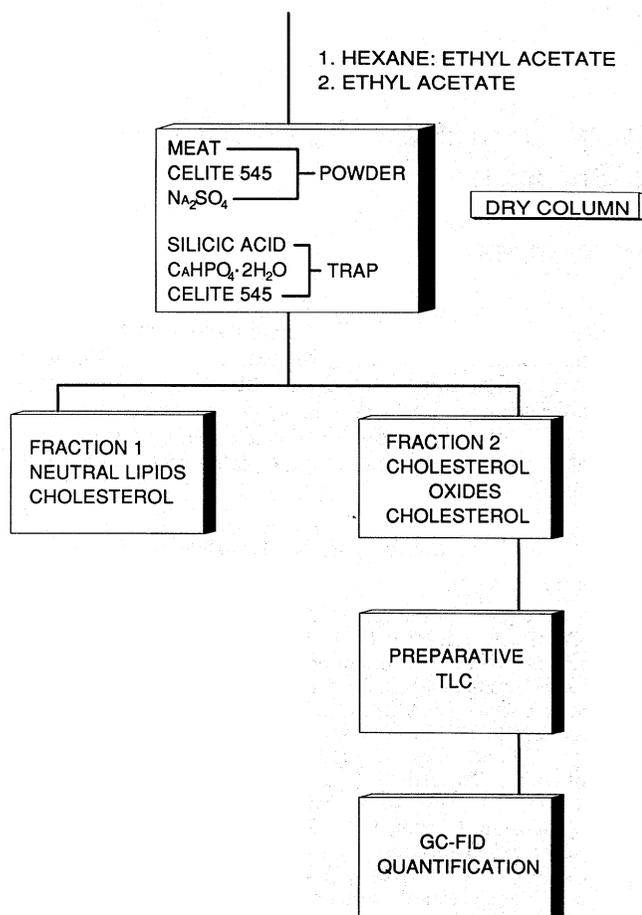


Fig. 1—Isolation and measurement of cholesterol oxidation products in meat and chicken.

orated to dryness, and reconstituted in 250 μ L ethyl acetate for GC analysis.

Gas chromatography (GC)

Analysis of cholesterol oxides was on a Varian 3600 gas chromatograph with on-column capillary injection and FID detection. A 0.20 mm i.d. \times 25 m bonded phase 5% phenylsilicone column with 0.33 μ m film thickness (Hewlett-Packard Ultra #2) was used. Helium was carrier gas at a head pressure of 20 psi. Initial injection temperature 70°C was held for 0.5 min and then increased to 275°C at 50°/min and held at 275°C for 10 min. The initial oven temperature of 100°C was held for 3 min, increased at 30°/min to 260°C and at 0.6°/min to 280°C. The temperature was then increased to 300°C at 30°/min and held at 300°C for 10 min. The detector temperature was 325°C.

An aliquot (100 μ L) of concentrated (250 μ L) TLC extract was evaporated to dryness, internal standard 6-ketocholestanol (4 μ L, 1 μ g/ μ L in ethyl acetate) was added, the solvent was removed under nitrogen, the sample was reconstituted in 100 μ L ethyl acetate, and 1 μ L was injected.

RESULTS & DISCUSSION

THE METHOD used to isolate and measure cholesterol oxidation products is outlined in Fig. 1. The purpose of the procedure was to quantify three compounds, 7-ketocholesterol, the α -epoxide and the β -epoxide, each at a level of 3 μ g or less per 10g meat. Before quantification, the cholesterol oxidation products had to be freed from the usual amounts of lipids present in 10g meat: 0.5–1.0g neutral lipids, about 80 mg polar lipids and about 7 mg cholesterol (Marmer and Maxwell, 1981).

Previous work in our laboratory (Maxwell et al., 1980; Zubillaga and Maerker, 1984) had demonstrated the effectiveness

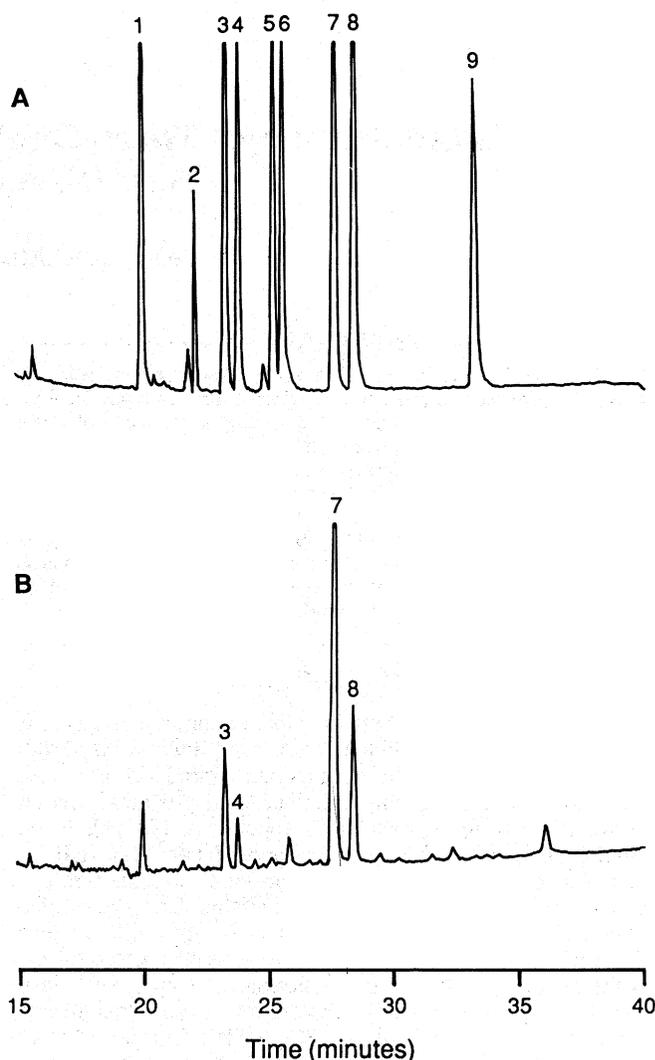


Fig. 2—Gas chromatograms of cholesterol oxidation products. (A) Nine component test mixture. (B) Cholesterol oxides in pork chops. 1-Cholest-5-en-3 β -ol (cholesterol); 2-Cholesta-3,5-dien-7-one; 3-Cholesterol 5 β , 6 β -epoxide; 4-Cholesterol 5 α ,6 α -epoxide; 5-Cholest-5-ene-3 β ,7 α -diol (7 α -hydroxycholesterol); 6-Cholest-5-ene-3 β ,7 β -diol (7 β -hydroxycholesterol); 7-5 α -Cholestan-6-one (6-ketocholestanol, internal standard); 8-3 β -Hydroxy-5-en-7-one (7-ketocholesterol); 9-5 α -Cholestane-3 β ,5,6 β -triol.

and convenience of the dry column extraction procedure in separation of neutral and polar lipids from meat tissue. Other research (Maerker and Jones, 1991) had also established that cholesterol and its oxidation products were eluted with neutral lipids, rather than polar lipids, in the dry column extraction procedure. Other researchers, who had chosen alternative lipid isolation procedures such as the Folch method (Folch et al., 1957), had used silicic acid columns to separate cholesterol oxidation products from bulk lipids (Park and Addis, 1985; Missler et al., 1985; Lakritz and Maerker, 1989).

In our current work, we planned to combine the silicic acid column with the dry column to achieve maximum separation of cholesterol oxidation products from the meat tissue and the bulk lipids in a single step. Initially, separation and recovery of lipids from a silicic acid column was studied. The model lipid mixture consisted of triolein (40 mg), cholesterol (2 mg) and 7-ketocholesterol (1 mg), and the silicic acid column (6 g) was prepared according to Lakritz and Maerker (1989). Elution with hexane: ethyl acetate (50 ml, 9:1, v/v) gave 96% recovery of triolein and cholesterol, while elution with ethyl acetate (50 mL) gave complete recovery of 7-ketocholesterol.

Table 1—Content of three cholesterol oxidation products in muscle tissue

Sample		Cholesterol oxide ($\mu\text{g}/10\text{ g}$)			
Type	Number ^a	7-Ketone ^b	β -Epoxide ^c	α -Epoxide ^d	Total
Veal	3	2.2 \pm 0.3	0.87 \pm 0.2	0.33 \pm 0.2	3.4 \pm 0.3
Pork	4	0.63 \pm 0.08	0.41 \pm 0.1	0.23 \pm 0.04	1.26 \pm 0.2
Beef	3	8.29 \pm 0.4	3.67 \pm 0.7	1.34 \pm 0.3	13.3 \pm 1.1
Chicken	7	1.29 \pm 0.3	0.58 \pm 0.3	0.66 \pm 0.3	2.53 \pm 0.6

^a All samples analyzed in duplicate.

^b 3 β -Hydroxycholest-5-en-7-one.

^c Cholesterol 5 β ,6 β -epoxide.

^d Cholesterol 5 α ,6 α -epoxide.

Table 2—Storage effects on content of three cholesterol oxidation products in beef

Storage (days) ^a	Cholesterol oxide ($\mu\text{g}/10\text{ g}$)			
	7-Ketone ^b	β -Epoxide ^c	α -Epoxide ^d	Total
7	10.2 \pm 1.6	4.4 \pm 0.55	1.3 \pm 0.20	15.8 \pm 2.3
14	13.7 \pm 1.2	5.4 \pm 0.20	3.7 \pm 1.4	22.8 \pm 2.5
21	25.5 \pm 3.5	7.6 \pm 1.1	3.6 \pm 0.10	36.7 \pm 2.6

^a Temp = 0–4°C.

^b 3 β -Hydroxycholest-5-en-7-one.

^c Cholesterol 5 β ,6 β -epoxide.

^d Cholesterol 5 α ,6 α -epoxide.

n=2

In subsequent experiments the silicic acid was combined with sodium sulfate and Celite 545 in the body of the dry column, and various combinations of hexane and ethyl acetate were tested in elution of mixtures of triolein, cholesterol and 7-ketocholesterol. Presence of silicic acid in the body of the column, however, considerably slowed the elution rate. Placement of the silicic acid into the CaHPO₄/Celite trap resolved the flow-rate problem and led to effective retention of cholesterol oxidation products prior to elution with ethyl acetate.

In operation of the dry column procedure on meat samples most of the neutral lipids and the bulk of the cholesterol were eluted with hexane: ethyl acetate (9:1, v/v) into Fraction 1. This fraction contained no cholesterol oxidation products as judged by TLC. Fraction 2, obtained by elution with ethyl acetate, contained the cholesterol oxidation products, along with minor amounts of neutral lipids, cholesterol and polar lipids. The solid residue of Fraction 2 usually weighed 10 mg or less. The modified dry column procedure removed all water, as do other lipid extraction procedures, but also increased the concentration of the cholesterol oxidation products in lipids by a factor of 100 or greater.

In TLC 7-ketocholesterol and the two isomeric cholesterol epoxides were well resolved by benzene: ethyl acetate (6:4, v/v) from other lipids (Maerker and Bunick, 1986; Zulack and Maerker, 1989) but developed as a single spot or band. In our current work, multiple extraction of the scraped TLC band with ethyl acetate gave quantitative recovery of the cholesterol derivatives. Filtration of the extracts was necessary to avoid deposition of silica fines on the GC column. The cholesterol oxidation products were injected into the GC without prior derivatization as reported earlier (Maerker and Unruh, 1986). As illustrated in Fig. 2, the internal standard, 6-ketocholestanol, was well resolved from 7-ketocholesterol and from the two 5,6-epoxides. The latter two also showed good base-line separation.

Table 1 shows the results of analysis of veal, pork, beef, and chicken (without skin) meat purchased at local retail outlets. The method indicated all samples contained the three cholesterol oxidation products of interest, a reflection of the greater sensitivity of the method. Pork contained the lowest amounts of cholesterol derivatives, and α -epoxide levels as low as 200 ng/10g (20 ppb) were measured frequently. Beef contained much higher levels of oxidation products, especially 7-ketone and β -epoxide, than the others for reasons that are not clear.

This may have been related to the higher myoglobin content of beef.

Possibly, the cholesterol oxidation products measured in this study may not have been present in the original tissue but could have been formed during preparation of samples. Such artifact formation, if prominent, would be most easily detectable in pork muscle, which is inherently low in these compounds (Table 1). To test the possibility of artifact formation, we added 16 mg of cholesterol (twice the amount normally present) to 10 g of the pork muscle tissue. The sample was prepared by the normal procedure along with another sample without added cholesterol. Examination of cholesterol oxide content of the two samples showed no significant differences in amounts of any of the three oxidation products. Artifact formation during work-up was therefore not likely an important factor in measurement of the cholesterol derivatives. On storage in the refrigerator (0–4°C) amounts of cholesterol oxides increased with time, as shown in Table 2. All three cholesterol oxidation products increased over the three week period. 7-Ketocholesterol was the principal product and constituted well over 50% of the mixture, so that the ratio 7-ketone/total epoxides remained greater than unity.

To test the procedure, known amounts of cholesterol oxidation products (purchased standards) were applied as ethyl acetate solutions to the meat before grinding in amounts equal to about ten times those present in the meats. The content of the solution was measured directly by GC, and the meat without added cholesterol oxidation products was also analyzed. Recoveries of 7-ketocholesterol and of the α -epoxide were 96.7 \pm 3.5% and 96.8 \pm 3.0%, respectively. Recovery of β -epoxide, however, was 62.0 \pm 13%. Hydration of both epoxides has been reported to result in formation of the triol (5 α -cholestane-3- β ,5,6 β -triol). In aqueous acidic media the β -epoxide is converted to the triol much more readily than the α -epoxide (Maerker and Bunick, 1986). Examination of the Fraction 2 eluate from meat to which the β -epoxide had been added indeed showed the presence of a substantial amount of triol, which, however, was not quantitated. Triol was apparently absent in eluates from meat to which 5,6-epoxides had not been added. This appeared to indicate that cholesterol epoxides normally found in meat were protected from contact with the acid aqueous medium by the hydrophobic property of the cell membrane.

These results showed that cholesterol oxidation products in meat and chicken could be measured at levels well below 1 ppm and that 7-ketocholesterol and the isomeric 5,6-epoxides occur in such tissues with the amounts of the ketone exceeding those of the epoxides. Sander et al. (1989) reported the health effects of cholesterol oxidation products in foods. Whether the very low levels of cholesterol oxidation products reported here have any biological significance is not known.

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