Evaluation of Oxidative Degradation of Cholesterol in Food and Food Ingredients: Identification and Quantification of Cholesterol Oxides

Jae Eun Pie, Khira Spahis, and Christine Seillan*

Laboratoire des Sciences de la Consommation, INRA-CRJ, 78350 Jouy en Josas, France

Cholesterol oxidation derivatives display a wide range of undesirable biological properties, and their presence in foodstuffs has raised much concern and attention. We have developed a method allowing quick, simple, and reliable quantification of cholesterol oxidative degradation in food. After lipid extraction and mild saponification, the unsaponifiable fractions from food samples are deposited on thin-layer chromatography plates and developed in hexane–ether (70:30); cholesterol oxides, migrating in a single band, are resolved from cholesterol, and both areas are scraped and analyzed as trimethylsilyl derivatives by capillary gas chromatography. 19-Hydroxycholesterol and cholestanol are used as internal standards for the quantification of cholesterol oxides and of cholesterol, respectively. We have shown that moderate heating of butter leads to the formation of cholesterol oxides, in amounts increasing with temperature, length of heating, and storage times. Cholesterol oxides are also present in commercial egg powder, egg mixes, and butter cookies or cakes. This method is a useful tool in assessing the quantitative importance of cholesterol oxides in the human diet and their formation during food processing or storing.

Cholesterol can easily undergo oxidation in air, and the many substances derived from this oxidation, the so-called cholesterol oxides or oxysterols, have recently received much attention in view of their biological activities (Smith, 1981). Some cholesterol oxides display angiotoxic properties both in vivo and in vitro (Imai et al., 1976; Jacobson et al., 1985; Matthias et al., 1987) or act as mutagenic or carcinogenic compounds (Sevanian and Peterson, 1986; Raaphorst et al., 1987). Their presence in processed food has attracted much concern since cholesterol oxides have been detected in several food ingredients such as egg powder, milk powder, or heated tallow (Nourooz-Zadeh and Appelqvist, 1988; Park and Addis, 1986), and they are readily absorbed and incorporated into circulating lipoproteins (Peng et al., 1987). Thus, it is important to assess the daily ingestion of oxysterols from the diet. However, progress in this field has been hampered by difficulties associated with the isolation and analysis of these compounds. Gas–liquid chromatography (GLC) has been used in several studies (Park and Addis, 1985; Nourooz-Zadeh and Appelqvist, 1987; van de Bovenkamp et al., 1988), but complete resolution of major cholesterol oxides is not always achieved; high-performance liquid chromatography methods have been developed (Csiky, 1982; Kou and Holmes, 1985; Sugino et al., 1986), but oxysterols are poorly detected by UV spectrometry. Moreover, because of their low concentrations in lipid extracts from food samples rich in cholesterol, it is necessary to separate the oxysterols from cholesterol to facilitate proper quantification: the isolation and concentration steps are long and tedious procedures that generally include several steps of liquid chromatography (Csiky, 1982; Higley et al., 1986; van de Bovenkamp et al., 1988).

We have developed a method that provides quick and simple isolation and quantification of the major cholesterol oxides. Data obtained from several food ingredients are reported.

MATERIALS AND METHODS

Reagents. Cholesterol, cholestanol, cholestanetriol, cholesterol α-epoxide, 20-hydroxycholesterol, 7-ketocholesterol, and 19-hydroxycholesterol were purchased from the Sigma Chemical Co. (St. Louis, MO). Cholesterol β-epoxide and 26(R)- and 26(S)-hydroxycholesterol came from Research Plus (Bayonne, NJ), and 25-hydroxycholesterol and 7α- and 7β-hydroxycholesterol were obtained from Steraloids Inc. (Wilton, NH). All standards were found to be pure upon purchase, as determined by means of GLC analysis.

Lipid Extraction. For all food samples except butter, which was saponified without extraction, the extraction procedure was as follows: 0.3 g of egg powder was diluted with 10 mL of ethanol containing 0.002% butylated hydroxytoluene (Sigma). The mixture was vigorously stirred, and 10 mL of dichloromethane and internal standards (20 μg of 19-hydroxycholesterol and 1 mg of cholestanol) were added. After 1 h stirring, the mixture was filtered in a funnel over cotton and rinsed with 10 mL of ethanol–dichloromethane (1:1). The organic extract was evaporated under vacuum, resuspended in 15 mL of diethyl ether, and filtered again. For the egg powder or egg mixes, the last filtration step was necessary to remove any contaminating traces of powder. This step could be omitted for the cookies or cakes.

Egg mixes (6–12 g) and samples of cookies (2 g) were prepared for extraction, and the volumes of solvents were adjusted accordingly. The amounts of internal standards were the same as mentioned for egg powder, except that, for cookies or cakes, 10 μg of 19-hydroxycholesterol was added.

Isolation of Cholesterol and Cholesterol Oxides. Lipid extracts from egg powder (0.12–0.15 g of lipids), egg mixes (0.12–0.17 g), or cakes (0.2 g) or 0.5 g of gently melted butter with the internal standards was saponified with 20 mL of 1 N KOH in methanol; the mixture was stirred for 10 min at 40 °C and left overnight at room temperature. The unsaponifiable fraction was extracted twice with 25 mL of diethyl ether after addition of 20 mL of doubly distilled water. The pooled organic fractions were rinsed at least three times with doubly distilled water until the pH of the aqueous phase was neutral.

The unsaponifiable fraction was evaporated under vacuum to dryness, resuspended into 0.5 mL of chloroform, and deposited on a silica thin-layer chromatography (TLC) plate (Merck;

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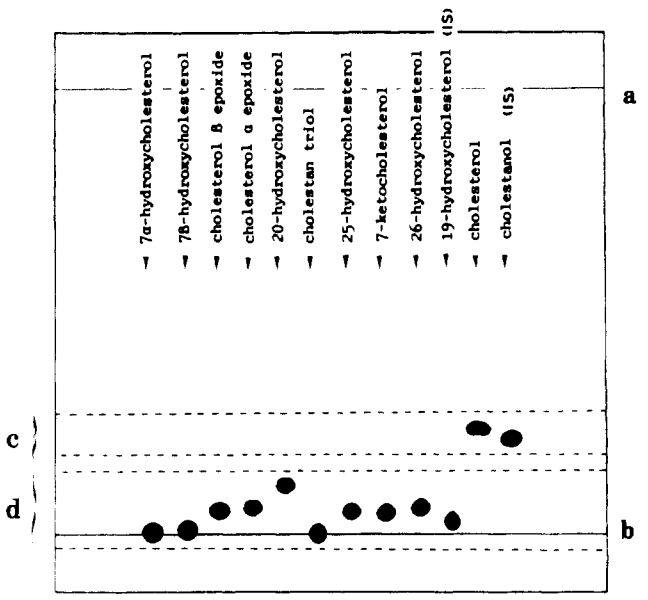

Figure 1. Thin-layer chromatography of standards. Standards were deposited and developed in hexane–ether (70:30). For the samples, two bands of silica were scraped and extracted separately. IS = internal standard. Key: a, solvent front; b, deposit area; c, cholesterol band; d, oxysterol band.

20 × 20 cm, silica gel 60 without fluorescence indicator, gel thickness 0.25 mm). The plate was developed with hexane–ether (70:30) until the solvent front moved to 2 cm from the top of the plate. The plate was dried and sprayed with Rhodamin (Sigma; 1 mg/mL in ethanol). Oxysterols and cholesterol areas were scraped and recovered separately, and silica was extracted twice with 10 mL of diethyl ether. Combined extracts were dried under nitrogen. A 0.3-mL portion of pyridine–hexamethyldisilazane–trichloromethylsilane (1:1:1) (Merck) was added, and derivatization was achieved within 2–3 h at room temperature. Remaining derivatization reagents were evaporated under nitrogen, and 0.2 mL of hexane was added.

Gas Chromatography. Quantitative analysis of cholesterol and cholesterol oxides was performed on a Girdel 30 gas chromatograph (Suresnes, France) with a flame ionization detector, a Shimadzu C-R5A integrator, and a 30-m fused silica capillary DB 5 column (J&W Scientific, Folsom, CA) with a film thickness of 1.0 μm. The oven temperature was 280 °C for the analysis of cholesterol oxides and 270 °C for the analysis of the cholesterol. Other conditions: carrier gas, helium; flow rate, 1 mL/min; pressure, 12 psi; injector temperature, 300 °C; detector temperature, 310 °C.

Mass Spectrometry. Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed to confirm the identity of peaks obtained with GLC analysis. The analysis was carried out on a Dani 3800HR-VG 70E-PDP8A digital mass spectrometer with a CPsil 5CB column (Chrompack, France); (25 m × 0.22 mm (i.d.), film thickness 0.12 μm). Flow rate of helium was 1 mL/min. Samples were injected when the oven temperature was 30 °C. After 1 min the temperature was raised to 140 °C and increased to 280 °C (10 °C/min). The injector and interface temperatures were 280 °C. Mass spectra were obtained by electron impact ionization within a mass range of m/z 20–700. Scan speed was 0.5 s/decade. The accelerating voltage was 6 kV. Ionization energy was 70 eV.

RESULTS AND DISCUSSION

Isolation and Analysis of Oxysterols and Cholesterol. The major cholesterol oxides were separated from cholesterol by TLC. Figure 1 shows a typical migration of standards on the TLC plate. Most of the cholesterol oxides studied remained close to the deposit area, including 19-hydroxycholesterol, an internal standard chosen to quantify oxysterols. 20-Hydroxycholesterol migrated with the highest R_f. Cholesterol and cholestanol, which

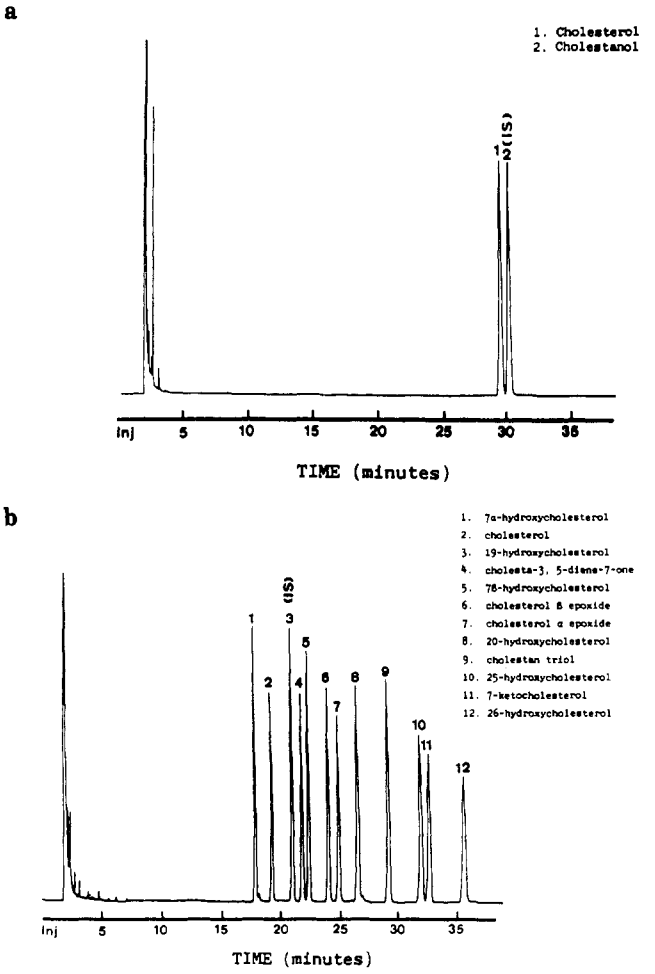

Figure 2. Gas–liquid chromatography profiles of standards: a, cholesterol; b, cholesterol oxides. The GLC conditions were as described under Materials and Methods. IS = internal standard.

was the internal standard for the quantification of cholesterol, were fully resolved from the oxysterols. When samples were analyzed, two bands of silica were scraped and extracted after migration as indicated in the figure.

Figure 2 represents the GLC profile of standards. Under the conditions employed, a distinct separation of cholesterol from cholestanol allowed us to properly quantify cholesterol in the samples. All the major cholesterol oxides were fully resolved from each other (Figure 2b): we obtained good resolution of cholestanetriol and 7-ketocholesterol and the cholesterol α- and β-epoxides, which were previously found difficult to resolve (Park and Addis, 1985; Bascoul et al., 1986). Cholesterol did not interfere with any of the oxides, but it had to be separated from the oxysterols because of the high concentration in the samples. These chromatographic conditions did not provide resolution between the R and S epimers of 26-hydroxycholesterol.

Figure 3 shows the response linearity obtained for 7-ketocholesterol, 7α-hydroxycholesterol, and cholesterol. Excellent linear response was recorded for each cholesterol oxide, the weight ratio varying from 0 to 2. Table I summarizes the relative response factors for each cholesterol oxide.

Recoveries. To assess the recoveries of the different oxides, the following experiment was performed: 20-μg portions of the different oxysterols were added in 0.5 g of fresh butter (containing negligible amounts of cholesterol oxides; see below). The sample was processed through the entire procedure and finally analyzed by GLC. An aliquot of the mixture of standards was derivatized and

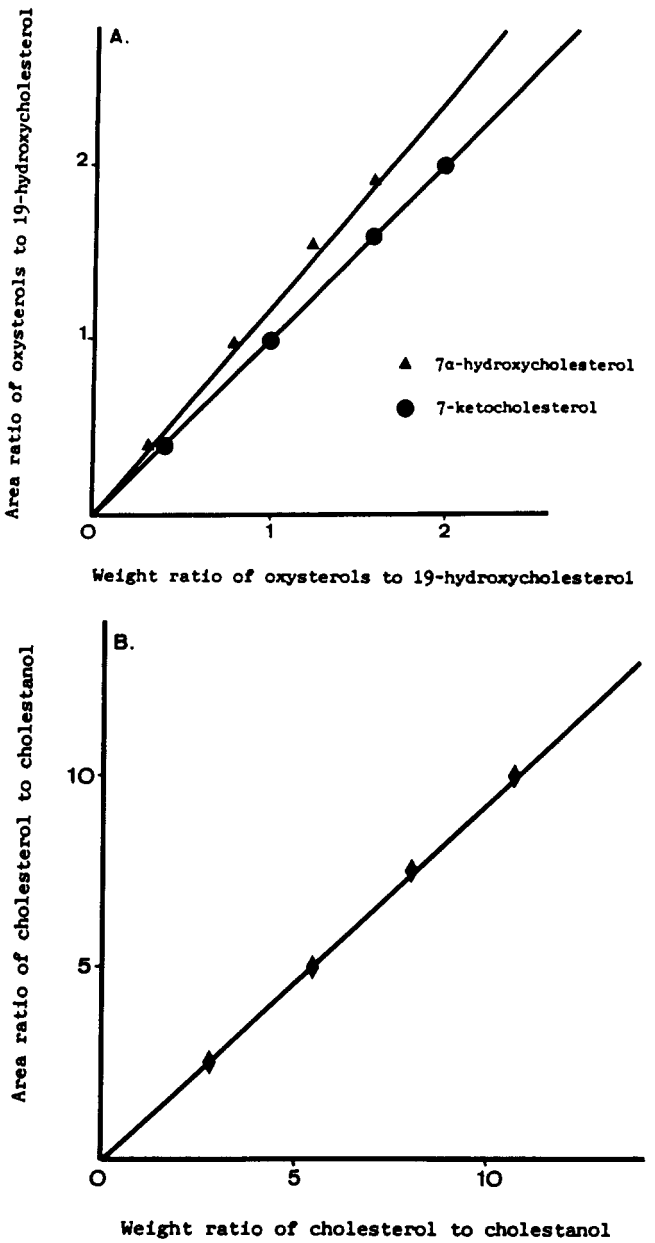

Figure 3. Response linearity of 7α-hydroxycholesterol and 7-ketocholesterol (panel A) and of cholesterol (panel B). Various amounts of sterols were added to fixed amounts of cholestanol (200 μg) and 19-hydroxycholesterol (20 μg).

Table I. Relative Response Factors for Trimethylsilyl Derivatives of Cholesterol Oxides and Cholesterol (Mean ± SD, n = 5) and Recoveries of Cholesterol and Oxysterols (Mean ± SD, n = 4)

	response factor	recovery
19-hydroxycholesterol	1.00	100.0
7α-hydroxycholesterol	1.05 ± 0.03	100.5 ± 9.9
7β-hydroxycholesterol	0.99 ± 0.01	107.7 ± 4.8
cholesterol α-epoxide	1.10 ± 0.03	118.5 ± 2.4
cholesterol β-epoxide	1.18 ± 0.04	118.2 ± 19.5
7-ketocholesterol	1.20 ± 0.02	103.5 ± 6.0
20-hydroxycholesterol	1.06 ± 0.04	117.7 ± 4.1
25-hydroxycholesterol	1.14 ± 0.05	104.0 ± 1.4
cholestanetriol	1.13 ± 0.04	64.9 ± 11.4
cholestanol	1.00	100.0
cholesterol	0.92 ± 0.01	102.4 ± 3.7

injected. The areas of the peaks in the butter sample were compared with the peaks obtained with the mixture of pure compounds, and recoveries were calculated, assuming that the recovery of 19-hydroxycholesterol was 100%. Table I reports the recoveries obtained. Cholestanetriol had the lowest recovery: experiments performed with pure compound showed that the substance was not degraded but poorly extracted from the silica after the TLC separation. 20-Hydroxycholesterol and cholesterol epoxides were slightly overestimated in the analysis, probably because they were extracted more effectively from silica than 19-hydroxycholesterol. All other oxysterols were recorded satisfactorily, which demonstrates that they were not degraded or lost during previous steps. There has been some controversy about the possible damages to oxysterols due to saponification (Park and Addis, 1986; van de Bovenkamp et al., 1988), particularly to 7-ketocholesterol. Our data show that none of the oxides studied were damaged during sample processing.

The detection limit in these conditions was 0.1 μg/g of sample. The recovery of cholesterol was assessed by adding 400 μg of cholesterol in 0.5 g of butter and calculating the amount recovered after deletion of the cholesterol contained in the sample. Results are shown in Table I.

Application to Food and Food Ingredients. As has already been shown in several studies, heated animal fats contain measurable amounts of cholesterol oxides (Park and Addis, 1986; Bascoul et al., 1986; Jacobson, 1987). We applied our method to heated butter, a daily staple in the European diet. Butter was heated during different periods of time at different temperatures, relevant to the conditions of daily cooking. As represented in Table II, if cholesterol oxides were barely detectable in fresh butter, indicating that no artefactual oxidation had occurred during sample processing, the amounts of all the oxysterols present were the result of increasing the temperature and length of heating time.

Parts a and b of Figures 4 show typical chromatographic profiles of fresh butter and butter heated at 180 °C for 10 min, respectively.

We studied the effect of storage on cholesterol degradation. Results reported in Table II indicate that the longer the storage at −20 °C, the more intense the degradation. The amount of each cholesterol oxide gradually increased with length of storage: after 6 months, the percentage of polar sterols over cholesterol was twice that of heated fresh butter. It seems that stored butter is more susceptible to oxidation than fresh fat.

In all cases, the most abundant oxides were, in decreasing order, 7-ketocholesterol, the cholesterol epoxides, and 7α- and 7β-hydroxycholesterol. 25-Hydroxycholesterol was found in small amounts in our study, much smaller than reported in a previous work (Csiky, 1982). The degradation of cholesterol is lower than the degradation observed in heated tallows (Park and Addis, 1986; Bascoul et al., 1986), but in these studies the fat was heated for very long periods of time (56–400 h).

We then measured the amount of oxides in commercial cookies or cakes, selected for the high percentage of butter announced by the manufacturer (13–30%). Results are expressed in Table III. Measurable amounts of oxides were found in all the products tested. Butter cake 4, in spite of a low percentage of butter, had the highest quantity of cholesterol oxides: whole milk powder was one of the ingredients in this product, and since the presence of oxysterols in this ingredient has been demonstrated (Nourooz-Zadeh and Appelqvist, 1988), this could explain the high amount of oxides observed. Therefore, cooking conditions of cakes or cookies lead to significant degradation of cholesterol.

Another food ingredient in which the presence of oxy-

Table II. Quantification of Cholesterol Oxides and Cholesterol in Butter[a]

sample	treatment	cholesterol and its oxides: cholesterol	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol	cholesterol α-epoxide	cholesterol β-epoxide	7-keto-cholesterol	20-hydroxy-cholesterol	25-hydroxy-cholesterol	cholestane-triol	total amt oxides	total amt oxides/cholesterol, %
fresh butter	unheated	2.80 ± 0.01	ND	ND	ND	ND	TR	ND	ND	ND		
	heated for 10 min at 170 °C	2.79 ± 0.05	1.17 ± 0.02	1.70 ± 0.05	0.95 ± 0.14	4.75 ± 0.15	5.16 ± 0.14	TR	TR	TR	13.73	0.49
	heated for 10 min at 180 °C	2.72 ± 0.04	1.61 ± 0.01	2.32 ± 0.04	1.30 ± 0.07	4.28 ± 0.18	5.12 ± 0.25	TR	TR	TR	14.63	0.54
	heated for 20 min at 180 °C	2.64 ± 0.05	3.93 ± 0.04	4.58 ± 0.32	2.90 ± 0.31	7.26 ± 0.25	8.63 ± 0.01	TR	TR	TR	27.30	1.03
butter stored at −20 °C for 3 months	unheated	2.60 ± 0.01	ND	ND	ND	ND	0.97 ± 0.01	ND	ND	ND	0.97	0.04
	heated for 10 min at 170 °C	2.58 ± 0.01	1.95 ± 0.04	2.49 ± 0.02	1.65 ± 0.12	4.52 ± 0.02	8.48 ± 0.04	TR	TR	0.20 ± 0.01	19.29	0.75
	heated for 10 min at 180 °C	2.56 ± 0.01	2.88 ± 0.07	3.84 ± 0.11	2.13 ± 0.02	6.55 ± 0.02	9.50 ± 0.07	0.57 ± 0.01	0.49 ± 0.01	0.33 ± 0.01	26.29	1.03
	heated for 20 min at 180 °C	2.56 ± 0.01	4.13 ± 0.07	5.73 ± 0.15	2.83 ± 0.04	9.21 ± 0.03	10.78 ± 0.23	0.62 ± 0.01	0.61 ± 0.01	0.44 ± 0.02	34.55	1.35
butter stored at −20 °C for 6 months	unheated	2.67 ± 0.04	0.22 ± 0.02	ND	ND	1.52 ± 0.01	0.42 ± 0.18	ND	ND	ND	2.16	0.08
	heated for 10 min at 170 °C	2.63 ± 0.02	1.64 ± 0.10	1.93 ± 0.04	1.00 ± 0.10	4.38 ± 0.55	5.01 ± 0.16	TR	TR	TR	13.96	0.53
	heated for 10 min at 180 °C	2.57 ± 0.06	4.49 ± 0.11	5.60 ± 0.26	2.94 ± 0.18	8.18 ± 0.35	9.70 ± 0.14	TR	TR	TR	30.91	1.20
	heated for 20 min at 180 °C	2.53 ± 0.06	8.88 ± 0.34	14.88 ± 0.40	7.40 ± 0.65	18.45 ± 2.07	14.35 ± 0.42	TR	TR	TR	63.96	2.53

[a] Results are expressed in milligrams per gram of sample for the cholesterol and in micrograms per gram of sample for the oxides. Means ± SD (n = 3). ND = not detectable. TR = trace.

Table III. Quantification of Cholesterol Oxides and Cholesterol in Butter Cakes or Cookies[a]

sample	butter, %	cholesterol and its oxides: cholesterol	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol	cholesterol α-epoxide	cholesterol β-epoxide	7-keto-cholesterol	20-hydroxy-cholesterol	25-hydroxy-cholesterol	cholestane-triol	total amt oxides	total amt oxides/cholesterol, %
butter cake 1	25	14.6 ± 0.01	2.89 ± 0.21	0.77 ± 0.06	2.17 ± 0.11	9.02 ± 0.65	15.5 ± 0.32	TR	TR	TR	30.85	0.21
butter cake 2	25	16.06 ± 0.05	2.96 ± 0.12	3.30 ± 0.17	3.37 ± 0.16	11.03 ± 0.56	16.86 ± 0.63	1.18 ± 0.22	1.18 ± 0.33	0.75 ± 0.01	40.63	0.25
butter cake 3	22	16.10 ± 0.07	3.65 ± 0.27	4.35 ± 0.28	3.64 ± 0.77	12.49 ± 0.74	16.81 ± 0.67	0.97 ± 0.01	1.09 ± 0.01	0.37 ± 0.01	43.37	0.27
butter cake 4	13	15.21 ± 0.24	3.17 ± 0.27	3.26 ± 0.32	4.48 ± 0.41	14.51 ± 0.04	15.61 ± 0.18	1.69 ± 0.08	0.97 ± 0.01	TR	43.69	0.29
croissant	unknown	6.01 ± 0.26	0.70 ± 0.04	0.65 ± 0.06	1.42 ± 0.21	2.54 ± 0.43	4.55 ± 0.5	TR	0.51 ± 0.05	0.63 ± 0.01	11.00	0.18
butter cookie 1	28	10.45 ± 0.26	1.81 ± 0.03	1.17 ± 0.17	0.95 ± 0.09	5.38 ± 0.08	7.47 ± 0.24	TR	TR	TR	16.78	0.16
butter cookie 2	30	7.98 ± 0.08	1.56 ± 0.03	1.37 ± 0.08	1.92 ± 0.14	7.34 ± 0.02	6.76 ± 0.19	TR	TR	TR	18.95	0.24

[a] Results are expressed in milligrams/10 g of sample for cholesterol and micrograms/10 g of sample for the oxides. Means ± SD (n = 3). TR = trace.

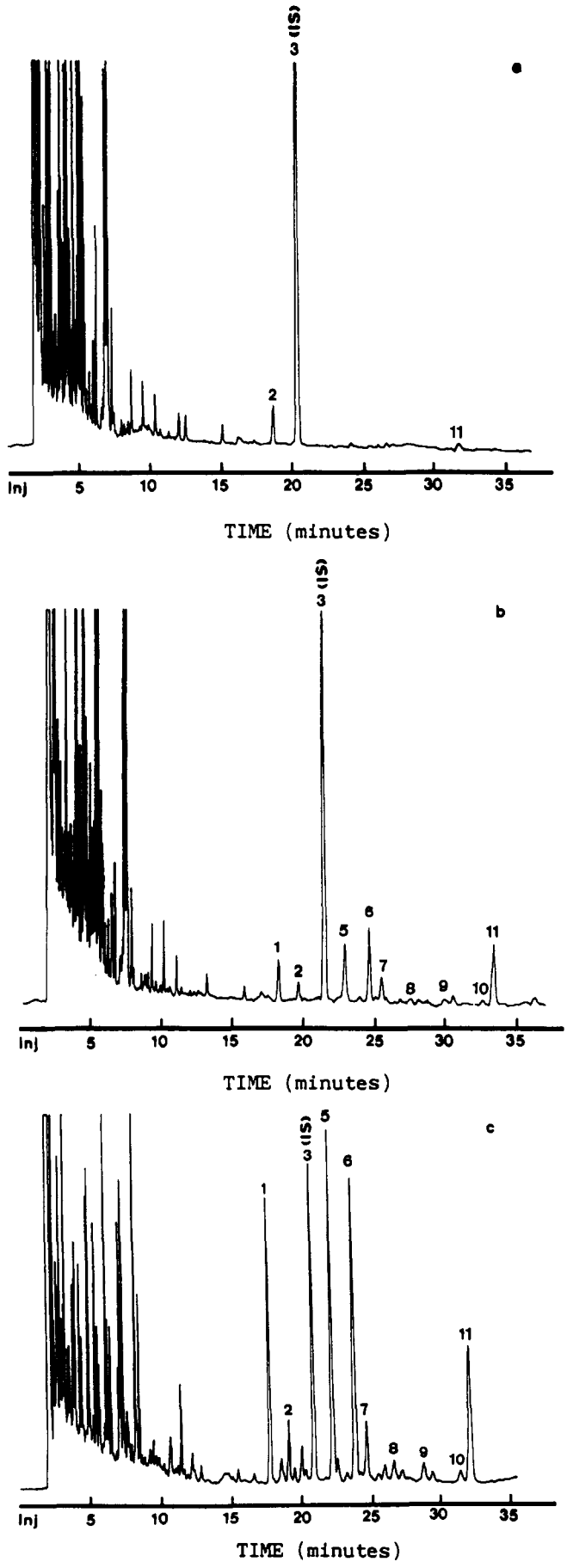

Figure 4. Gas–liquid chromatography profiles of food samples: a, unheated fresh butter; b, butter heated at 180 °C for 10 min; c, whole egg powder. The numbers used to identify the oxysterols are the same as in Figure 2. IS = internal standard.

sterols has been reported for some time is egg powder. Oxysterols have been detected in egg powder either stored (Sugino et al., 1986) or exposed to light (Herian and Lee, 1985) for long periods of time. We have measured oxysterols in several samples of powders to be used by the food industry. Table IV summarizes the data obtained. Cholesterol oxides were present in high quantity, representing 1.24–1.47% of cholesterol. The most abundant compounds were 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, and the cholesterol epoxides. In a previous study, the epoxide epimers were found at levels of 0.1–0.2% of cholesterol in egg powder (Sugino et al., 1986), and our results are consistent with these observations. A substance with a retention time similar to that of 20-hydroxycholesterol was noted in measurable amounts in all the samples, and cholestanetriol and 25-hydroxycholesterol were present in trace amounts. Figure 4c shows a typical GLC profile obtained with a sample of egg powder.

We analyzed the cholesterol oxide content of several commercial egg mixes purchased in local department stores. As shown in Table IV, cholesterol oxides were indeed present in the mixes, in quantities increasing with the percentage of egg powder announced by the manufacturer. Egg mix 4 was cooked in a standard kitchen oven, according to the instructions on the package. The amount of oxysterols was drastically increased after cooking. Consequently, the final quantity of oxysterols per serving was 360 μg.

Identification of Substances by GLC–MS. Several food samples such as heated butter, egg powder, and cooked egg mix 4 were analyzed by mass spectrometry to confirm the presence of cholesterol oxides.

Mass spectra of the peaks observed in the samples were compared to mass spectra of pure standards. The following substances could be identified in all the samples analyzed: 7α- and 7β-hydroxycholesterol (m/z 351, 366, 456, 546), 7-ketocholesterol (m/z 367, 382, 457, 472), and cholesterol α- and β-epoxide (m/z 366, 384, 456, 474). Other oxysterols were present in amounts too low to be detected and identified by this method.

Our observations show that cholesterol oxides are present in common food ingredients and that their formation is the consequence of not only prolonged periods of heating or storing but also daily cooking. Data obtained from samples demonstrate that the amount of cholesterol oxides can frequently reach 1% of total cholesterol, and occasionally up to 2%.

The level of cholesterol oxide formation is related to not only temperature and length of heating time as already reported (Park and Addis, 1986; Bascoul et al., 1986) but also to storage conditions, as demonstrated by our results obtained with butter stored at −20 °C. The use of frozen foods has increased in industrialized countries, and in view of our data, it would be of interest to quantify the level of cholesterol oxides in a foodstuff that has been heated, then frozen for storage, and heated again for terminal consumption.

Newly developed foodstuffs are composed of ingredients that have been processed, dried, or stored before use, and one may wonder whether this trend may not lead to an increase of the proportions of cholesterol oxides in the diet. Oxysterols display angiotoxicity at very low doses: Jacobson et al. (1985) have demonstrated that pigeons fed a diet containing 0.5% cholesterol and cholestanetriol at 0.3% of cholesterol by weight had more coronary stenosis and calcium accumulation in the aortic tissues than pigeons fed a diet containing 0.5% choles-

Table IV. Quantification of Cholesterol and Cholesterol Oxides in Egg Powder and Egg Mixes[a]

sample	whole egg powder in egg mixes, %	cholesterol and its oxides								total amt oxides	total amt oxides/cholesterol, %
		cholesterol	7α-hydroxycholesterol	7β-hydroxycholesterol	cholesterol α-epoxide	cholesterol β-epoxide	7-ketocholesterol	20-hydroxycholesterol	25-hydroxycholesterol	cholestanetriol	
egg yolk powder		18.81 ± 0.06	43.15 ± 2.26	47.35 ± 0.62	32.34 ± 3.96	68.50 ± 0.68	46.20 ± 0.01	8.02 ± 0.14	2.49 ± 0.15	TR	248.05	1.32
whole egg powder 1		13.30 ± 0.06	34.38 ± 0.36	43.27 ± 1.63	16.58 ± 0.46	62.67 ± 0.68	29.57 ± 0.69	4.78 ± 0.01	3.04 ± 0.04	TR	294.29	1.46
whole egg powder 2		13.30 ± 0.54	21.56 ± 0.53	23.05 ± 0.28	47.27 ± 0.10	40.27 ± 0.35	22.43 ± 1.00	9.07 ± 0.62	TR	TR	163.65	1.24
whole egg powder 3		14.01 ± 0.29	33.11 ± 0.46	38.51 ± 0.32	42.52 ± 2.28	53.23 ± 0.64	32.73 ± 1.05	8.84 ± 1.13	TR	TR	205.94	1.47
egg mix 1	2.0	39.18 ± 0.22	31.74 ± 0.26	34.63 ± 1.01	11.29 ± 1.14	67.73 ± 0.16	50.68 ± 0.11	6.64 ± 0.55	TR	2.88 ± 0.32	205.55	0.52
egg mix 2	2.5	49.21 ± 0.05	37.13 ± 3.63	35.51 ± 4.99	62.98 ± 1.36	66.57 ± 2.99	50.44 ± 1.51	13.62 ± 1.75	9.8 ± 0.01	TR	276.1	0.56
egg mix 3	3.0	58.39 ± 0.30	48.16 ± 1.00	44.07 ± 1.27	78.13 ± 4.61	90.79 ± 6.16	73.34 ± 3.83	12.02 ± 0.29	TR	TR	346.51	0.59
egg mix 4	5.7	83.21 ± 0.29	101.05 ± 0.11	116.17 ± 4.80	43.33 ± 5.80	206.51 ± 0.64	156.81 ± 4.13	27.27 ± 1.21	TR	9.67 ± 0.67	660.81	0.79
egg mix 4, cooked		73.34 ± 1.89	130.38 ± 4.63	171.72 ± 6.53	94.27 ± 3.35	217.25 ± 7.20	245.61 ± 6.00	93.75 ± 1.41	16.86 ± 2.20	16.24 ± 2.10	906.08	1.25

[a] Results are expressed in milligrams per gram of egg powder and milligrams/100 g of egg mixes for the cholesterol and in micrograms per gram of egg powder and micrograms/100 g of egg mixes for the cholesterol oxides. Means ±SD ($n = 3$). TR = trace.

terol alone. This study suggests that it is important that cholesterol oxides, obligatory substances in processed food, do not reach a certain level in the diet: it is thus important to understand better the consequences of food processing on cholesterol degradation and to assess the presence of cholesterol oxides in the human diet. Further studies are undertaken to answer this question.

ABBREVIATIONS USED

Cholest-5-en-3β-ol, cholesterol; 5α-cholestan-3β-ol, cholestanol; cholest-5-ene-3β,7α-diol, 7α-hydroxycholesterol; cholest-5-ene-3β,7β-diol, 7β-hydroxycholesterol; cholest-5-ene-3β,19-diol, 19-hydroxycholesterol; cholest-5-ene-3β,20α-diol, 20-hydroxycholesterol; cholest-5-ene-3β,25-diol, 25-hydroxycholesterol; (25*R*)-cholest-5-ene-3β,26-diol, (26*R*)-hydroxycholesterol; (25*S*)-cholest-5-ene-3β,26-diol, (26*S*)-hydroxycholesterol; cholestane-3β,5α,6β-triol, cholestanetriol; 5,6α-epoxy-5α-cholestan-3β-ol, cholesterol α-epoxide; 5,6β-epoxy-5α-cholestan-3β-ol, cholesterol β-epoxide; cholest-5-en-3β-ol-7-one, 7-ketocholesterol.

ACKNOWLEDGMENT

We are grateful to Drs. Gaudin and Virelizier (Centre d'Études Nucléaires, Saclay, France) for performing the GLC–MS analysis.

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Received for review July 5, 1989. Accepted November 21, 1989.

Determination and Qualitative Confirmation of Melengestrol Acetate Residues in Beef Fat by Electron Capture Gas Chromatography and Gas Chromatographic/Chemical Ionization Mass Spectrometry

Eli E. Neidert,*,† Ronald G. Gedir,‡ Leo J. Milward,§ Craig D. Salisbury,‡ Narine P. Gurprasad,⊥ and Peter W. Saschenbrecker†

Health of Animals Laboratory, Agriculture Canada, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 2R3, Agri-Food Safety Division, Agriculture Canada, 2255 Carling Avenue, Ottawa, Ontario, Canada K1A 0Y9, Laboratory Services Division, Agriculture Canada, 102 11th Avenue S. E., Calgary, Alberta, Canada T2G 0X5, and Environment Canada, Room 210, 4999 98th Avenue, Edmonton, Alberta, Canada T6B 2X3

A method for the detection of melengestrol acetate in beef fat is presented. Crude extracts are purified by preparation thin-layer chromatographic techniques. Residues of melengestrol acetate in the range 1-100 ppm are quantitated. Mass spectral confirmation is possible without additional cleanup of the sample extract. Test results obtained on Canadian beef heifers for 1982-1984 are presented.

Current Canadian regulations (Agriculture Canada (No. 46), 1984) allow the use of melengestrol acetate, MGA (17-hydroxy-6-methyl-16-methylenepregna-4,6-diene- 3,20-dione acetate), at a rate of 0.40 mg/animal per day. It is administered as a feed additive and restricted to intact heifers 181 kg and over. A withdrawal period of 48 h prior to slaughter is required.

At present a zero tolerance exists for MGA residues in foods (Campbell, 1978). With current sensitive analytical techniques, detectable residues may occur in spite of proper withdrawal (Ryan and Dupont, 1975).

Various residue detection techniqueshave been developed with spectrophotometric (Krzeminski et al., 1968), gas chromatographic with flame ionization (Duggan, 1968, 1969), and gas chromatographic with electron capture (Krzeminski and Cox, 1973; Krzeminski et al., 1976; Ryan and Dupont, 1975) detection principles. Only the method of Ryan and Dupont has the required sensitivity to detect MGA residues at the low levels that might occur in animal tissues (Krzeminski et al., 1981). The methods employing electron capture detection suffer from quantitation problems due to the severe effect of coextractants on the gas chromatographic base line.

In this paper, a relatively simple cleanup technique is described, which allows quantitation of MGA residues in the range 1-100 μg/kg in animal fat. Direct injection into a gas chromatograph/mass spectrometer without further cleanup was possible, thus allowing unambiguous identification of the residue.

This method was used in a 2-year survey of MGA residue levels in the fat tissue of Canadian slaughter heifers. During 1982-1984 671 samples were collected from Canadian packing plants for analysis. In 1982-1983, MGA levels ranged from less than 2 to 20.4 μg/kg with a mean of 3.4 μg/kg. In 1983-1984, MGA levels ranged from less than 2 to 28.7 μg/kg, with a mean of 2.8 μg/kg.

EXPERIMENTAL SECTION

Materials. Melengestrol acetate (minimum 99% purity) was provided by Upjohn Co., Kalamazoo, MI. Precoated silica gel thin-layer chromatography plates with preabsorbant spotting area (Whatman LK5F) were purchased from Terochem Laboratories Ltd., Edmonton, Alberta, Canada T6E 3A4. All solvents used were glass-distilled residue grade.

Apparatus. The gas chromatograph was a Hewlett-Packard 5880 equipped with a ^{63}Ni high-temperature electron capture detector. The mass spectrometer was a Finnigan 4021 capable of positive/negative multiple ion monitoring and operating in both electron impact and chemical ionization modes.

Standard Solutions. Stock solutions A and B were prepared as previously described (Krzeminski et al., 1976) except that solution B used acetone instead of methanol. A 5-mL portion of solution B was diluted to 200.0 mL with acetone (solution C, 2.5 μg/mL) and prepared daily as needed. Portions of 1, 2, 3, and 4 mL each of solution C were diluted to 10.0 mL

† Health of Animals Laboratory, Agriculture Canada.
‡ Agri-Food Safety Division, Agriculture Canada.
§ Laboratory Services Division, Agriculture Canada.
⊥ Environment Canada.

0021-8561/90/1438-0979$02.50/0 Published 1990 by the American Chemical Society