METHODS

# Quantification of Oxysterols in Dutch Foods: Egg Products and Mixed Diets

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**A sensitive and specific method is described for quantifying various cholesterol oxidation products in foodstuffs, including 7β-hydroxycholesterol, cholesterol-α-epoxide, cholestane-triol, 7-ketocholesterol and 25-hydroxycholesterol. A chloroform-methanol extract of the food was fractionated over two successive silica columns. Two fractions containing different classes of oxysterols were then analyzed as trimethylsilyl derivatives by capillary gas liquid chromatography, using on-column injection and a temperature gradient from 70 to 200°C. The detection limit was about 0.5 μg/g dry weight for egg yolk powder. Fresh egg yolk contained only 1.2 μg/g of total oxides per g dry weight, showing that artifactual oxidation during the procedure was minimal. Recovery of 5 pure oxysterols added to egg yolk at levels of 6.5 and 10 μg/g was between 93 and 102%. In commercial egg yolk and whole egg powder stored for one year, total amounts of oxysterols ranging from 21 to 137 μg/g dry weight were found. In duplicates of mixed Dutch diets, total amounts ranged from 3.6 to 6.2 μg/g dry weight. Duplicates containing mostly fried and baked foods did not have higher levels than duplicates in which foods had been prepared by boiling or left raw. We conclude that a normal mixed diet provides only minor amounts of cholesterol oxidation products.**
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Cholesterol oxidizes spontaneously in air, yielding a variety of oxidation products. At least 30 oxidation products of cholesterol have been reported (1). Many of these have potent biological effects (2). Some are cytotoxic and angiotoxic and may play a role in atherogenesis (3). Certain oxysterols may also be carcinogenic or mutagenic (4–6).

Foods containing cholesterol, particularly those that have been exposed to heat and air during processing or have been stored at ambient temperature, might contain autoxidation products of cholesterol. However, little information is available on the actual presence of cholesterol oxidation products in foods. In egg products, cholestane-triol (7), 7-hydroxycholesterol isomers (8,9) and cholesterol-α-epoxide (10) have been found, but often only after irradiation with UV light. A recent paper (11) reported that no oxysterols could be detected in fresh egg yolk. Spray-dried egg yolk powder contained traces of oxysterols when fresh or stored for 2 months at 4°C, but prolonged storage gave lipids extracts that contained variable levels (0–12 ppm) of various oxidation products. Variable amounts of cholesterol oxidation products have also been found in lard (12), in anhydrous milk fat and nonfat dry milk stored for 2 years at ambient temperature (13) and in heated beef tallow (14,15).

This paper describes a method for the quantitative determination of some cholesterol oxidation products in foods. It employs an isolation and prefractionation step using silica column chromatography. Oxysterols from food products containing high amounts of lipids and a relatively low cholesterol level can thus be purified and enriched in an adequate way before capillary gas liquid chromatography. The levels of some cholesterol oxidation products in fresh egg yolk, dehydrated egg and milk products stored for various periods of time, and in some mixed Dutch diets were estimated with this method.

## METHODS AND MATERIALS

*Overview.* The food is extracted with chloroform-methanol, and lipids other than sterols are removed by column chromatography on silica column I. The sterol fraction is then applied to silica column II, which serves to separate cholesterol (usually present to excess) from oxysterols. The latter are collected in two fractions of different polarity, each of which is then analyzed by capillary gas chromatography. Analysis of two samples in duplicate, plus appropriate standards and controls, takes one technician about two days.

*Food samples.* Whole egg powder, egg yolk powder, desugared whole egg powder and desugared egg yolk powder were obtained from four different companies in the Netherlands. Because not all companies could provide all four products, the total number of samples analyzed was only 11. Samples were stored in polyethylene bags in a refrigerator at 4°C for approximately one year. In addition to these products, an egg yolk powder stored for four years at ambient temperature was also available. A subsample of this egg yolk powder was irradiated with UV light (254 nm) with a Camag universal UV-lamp (type TL-900) at a distance of 10 cm for three weeks. During this period the powder was mixed with a spatula once a day.

Two samples of dry full-fat milk stored for two and seven years, and three mixed human diets prepared for a study on the carcinogenicity of different diets in rats were also analyzed for cholesterol oxidation products. This latter study aimed at determining the effects on tumor incidence of baking, frying and grilling, and of adding extra fruits and vegetables to the diet. The diets were duplicates of the average diet consumed in The Netherlands. They were kitchen-prepared in batches of several hundred kg and then freeze-dried and pelleted. For the first diet, all foods were left raw; for the second diet,

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Abbreviations: Cholestane-triol, cholestan-3,5,6-triol (3β, 5α, 6β)-; cholestanol, cholestan-3-ol (5α, 3β)-; cholesterol-α-epoxide, cholestan-3-ol,5,6-epoxy- (3β,5α, 6α)-; cholesterol-β-epoxide, cholestan-3-ol,5,6-epoxy- (3β,5β,6β)-; 7α-hydroxycholesterol, cholest-5-en-3,7-diol (3β,7α)-; 7β-hydroxycholesterol, cholest-5-en-3,7-diol (3β,7β)-; 20-hydroxycholesterol, cholest-5-en-3,20-diol (3β)-; 25-hydroxycholesterol, cholest-5-en-3,25-diol (3β)-; 7-ketocholesterol, cholest-5-en-3-ol-7-one (3β)-.

products were fried, baked or grilled when appropriate. The third diet was equal in composition to the second one, but contained added fruits and vegetables of supposedly anticarcinogenic action. A commercial pelleted rat chow was also analyzed.

*Solvents, reagents and column materials.* Chloroform (99.5%, v/v) from Janssen Chimica (Brussels, Belgium) (No. 15.821.10) with 0.75% (v/v) ethanol was used for chromatography on column I (Fig. 1). For the fractionation on column II, HPLC grade chloroform stabilized with 2-methyl-2-butene was used from Merck (Darmstad, West Germany) (no. 2444), as were methanol (No. 6009), and acetone (No. 14). Hexane (No. RH 1002) was obtained from Rathburn (Walkerburn, Scotland). Silylation reagents, hexamethyldisilazane (No. 84770) and trimethylchlorosilane (No. 88530) were from Pierce Eurochemie (Oud-Beijerland, The Netherlands) and dried pyridine (No. 7463) was from Merck. The purity of all solvents was checked by chromatography and found to be satisfactory.

Silica gel 60 (Merck) (No. 7734) of particle size 63–200 μm was used for column I. It was purified and activated by suspending 500 g in 1 liter 3 M HCl for 60 min and then washed with deionized water until neutral, dried overnight at 110–120°C and stored in a polyethylene bottle.

For column II, silica gel for low-pressure column chromatography from Baker Chemical B.V. (Deventer, The Netherlands) (No. 7024-1) was used as purchased. The average particle size was 40 μm (30–60 μm) and the average pore diameter 6 nm. Cholestane-triol (No. 4700), 7β-hydroxycholesterol (No. 6430), 7α-hydroxycholesterol (No. 6420), cholesterol-α-epoxide (No. 4130), 7-ketocholesterol (No. 6970) and 25-hydroxycholesterol (No. 6510) were from Steraloids Inc., Wilton, NH; 5α-cholestane (No. 17060) was from Pfaltz and Bauer, Inc., Stanford, CT; and betulin (No. B-9757) was from Sigma Chemical Co., St. Louis, MO.

*Saponification.* Saponification followed by extraction of the unsaponifiable fraction has often been used in the determination of sterols in foods (16–18). It has been reported (10), however, that up to 75% of the cholesterol-α-epoxide was lost during saponification. Therefore we subjected a cholesterol sample dating back to 1963, a UV-irradiated egg yolk powder and a mixture of pure oxysterols to two degrees of saponification. The oxysterol mixture contained 7-ketocholesterol, 25-hydroxycholesterol and cholestane-triol. Mild saponification with 100 ml 2 M ethanolic NaOH per l to 2 g of each sample was carried out at ambient temperature for one night, and normal saponification was done under reflux in a boiling water bath for 30 min under conditions, as have been reported elsewhere (19).

*Extraction of lipids.* An aliquot of dry sample containing 1–1.5 g lipids was transferred to a 150 ml wide-mouth conical flask and 100 ml Folch reagent (chloroform/methanol, 2:1, v/v) was added. The mixture was then homogenized with a Polytron Model PT 10/35 homogenizer (Kinematica, GmbH, Lucerne, Switzerland) at speed 5 for about 15 sec. After 10 min of equilibration, the extract was filtered through a defatted folded filter paper (MN 615¼, Macherey-Nagel, Düren, Germany). The residue was washed three times with Folch reagent and the combined filtrate was dried under vacuum with a rotary film evaporator. The vacuum of the evaporator was released with nitrogen gas. The lipids were redissolved in 5 ml of chloroform.

*Silica gel column chromatography.* For the isolation of cholesterol and oxysterols, a glass column (length 150 mm, o.d. 40 mm) equipped with a teflon stopcock and connected by a glass joint to a glass solvent reservoir was used (column I). The column was packed using a slurry of 50 g activated and purified Silica Gel 60 in chloroform. After settling of the adsorbent, the bed was vibrated with a hand vibrator to remove remaining air bubbles. Then the silica gel was washed with 75 ml chloroform, at a flow rate of 2–3 ml/min. The lipid extracted was transferred to the column, and two portions of 5 ml of chloroform were used for rinsing the flask and then added to the column.

The neutral lipids were eluted with 175 ml of chloroform and the sterols and their oxidation products were then eluted with 150 ml of acetone. The phospholipids remained on the column. The acetone eluent was collected into a flat-bottom extraction flask and evaporated to dryness under vacuum with a rotary evaporator. The vacuum of the evaporator was released with nitrogen gas and the residue was redissolved immediately in a 3-ml chloroform-acetone mixture (98:2, v/v).

For the prefractionation of oxysterols, a 10 g silica gel column (column II) was prepared as follows. In a separation funnel, 10 g of silica for flash chromatography was mixed with chloroform acetone (98:2, v/v) to form a slurry and was left at room temperature for 15 min. Then it was shaken again and the slurry poured into a glass column of length 250 mm and o.d. 13 mm, which was connected by a ball joint to a solvent reservoir. The column contained a small plug of glass wool covered by a thin layer of sea sand in chloroform-acetone mixture (98:2, v/v) on top of the teflon stopcock at the bottom of the tube. After settling of the adsorbent, the column was vibrated with a hand vibrator and rinsed with 50 ml chloroform-acetone solvent mixture (98:2, v/v). A flow rate of 2–3 ml/min was maintained by a nitrogen pressure of about 50 kPa on the solvent reservoir. The solvent level was allowed to descend to the top of the bed, and the sterols were layered on top of the adsorbent, using 3 ml rinsing aliquots of the chloroform-acetone (98:2, v/v) mixture. The column was then successively eluted with 100 ml of three different chloroform-acetone mixtures: A (98:2, v/v), B (80:20, v/v) and C (50:50, v/v).

Cholesterol eluted in fraction A. So did cholestanol and, therefore, quantification of this compound was not possible. Cholesterol-α-epoxide, 7-ketocholesterol, 25-hydroxycholesterol and the major part of the 7β-hydroxycholesterol eluted in fraction B. The cholestane-triol and a minor part of 7β-hydroxycholesterol eluted in fraction C. Common plant sterols eluted in the cholesterol fraction (fraction A) and, thus, did not interfere with the later gas-chromatographic separation of oxysterols (data not shown).

Fraction A was discarded, and fractions B and C were collected separately in extraction flasks. Then 1 ml of an internal standard solution containing 20 μg of each standard component (5α-cholestane and betulin) was added to both fractions. The solvents were evaporated under vacuum with a rotary thin-film evaporator. The vacuum was released with nitrogen gas, and the residues were redissolved in chloroform and transferred to a 1 ml conical vial for derivatization.

*Derivatization.* The residue was dried under nitrogen. Then 0.5 ml of pyridine hexamethyldisilazane trichloromethylsilane (10:2:1, v/v) was added. The vial was closed with a screw top sealed with teflon. It was left to stand for 30 min at ambient temperature. After removal of the excess of pyridine under a stream of nitrogen, 400 μl of hexane was added. The vial was shaken and centrifuged, and 0.5 μl of the hexane solution was injected into the gas chromatograph.

*Gas liquid chromatography.* A Packard Instruments (Delft, The Netherlands) gas liquid chromatograph Model 433 with a flame ionization detector, a digital integrator and a capillary on-column injector from Chrompack (Middelburg, The Netherlands) was used. It was equipped with a 25 m × 0.22 mm fused silica capillary WCOT column CP Sil5CB (Chrompack), with a film thickness of 0.12 μm and a coating efficiency of about 90%. The oven temperature program was: initial temperature 70°C for 2 min followed by a rise to 200°C at a rate of 15°C/min and, then, by another rise at a rate of 10°C/min to an upper temperature of 295°C. The oven was held at this upper temperature for various lengths of time until all components were eluted. Other conditions were: carrier gas, hydrogen; pressure, 150 kPa; make-up gas, nitrogen with flow rate 15/min; and detector temperature, 325°C.

Prior to the analysis of samples, the system was calibrated with a mixture of pure compounds, and response factors relative to the average response of betulin and 5α-cholestane were calculated. If the betulin or 5α-cholestane peaks could not be identified due to interference from other unknown compounds, then response factors were calculated relative to only one of these compounds.

*Mass spectrometry.* To confirm the tentative gas chromatographic identification, some samples were also analyzed on a Finnigan-MAT 8200 mass spectrometer (Finnigan Corp., Cincinnati, OH) equipped with a Finnigan-MAT ss200 data system.

A Varian 3700 gas chromatograph (Varian Associates Inc., Sunnyvale, CA) fitted with a 25 m × 0.5 mm i.d. CP-Sil5 glass capillary column (Chrompack, 4330 EW Middelburg, The Netherlands) was attached to the mass spectrometer through an open split interface. Helium was used as carrier gas at a flow rate of 6 ml/min. The initial oven temperature was 200°C and was increased to 320°C at a rate of 40°C/min. The injector and interface temperatures were 250°C.

Spectra were obtained by electron impact ionization within a mass range of 20–700 m/e. The scan speed was 1 sec/decade. Background subtraction and renormalization to the most intense peak were performed. Peaks were identified by comparing their mass spectra with those of the pure compounds.

## RESULTS AND DISCUSSION

*Effect of saponification.* The results of normal and mild saponification of the old cholesterol sample, the UV-irradiated egg yolk powder and the mixture of pure oxysterols showed that 7-ketocholesterol is very sensitive to alkaline hydrolysis. Only 11–40% of the original amount was recovered under mild saponification conditions, but saponification at 100°C caused a total disappearance of 7-ketocholesterol. 25-Hydroxycholesterol was not very sensitive to alkaline hydrolysis (recovery range, 89–121%), while cholestane-triol was somewhat sensitive to both mild and normal saponification (recovery range, 78–90%). Tsai et al. (17) reported that up to 75% of the cholesterol-α-epoxide was lost during saponification. Therefore, no saponification was used. The error introduced by not saponifying is probably small, as only some 10% of the cholesterol in foods is esterified (20).

*Silica chromatography.* Silica gel column I served to remove the nonsterol lipids. From an egg yolk powder containing 62.5% of weight as lipid, 79.7% of these lipids were recovered in the chloroform fraction, and the acetone fraction contained 3.7% of the lipids and 80–90% of the total amount of cholesterol.

Column II was used to remove the bulk of cholesterol and to prefractionate the oxidation products. For samples with a highly complex composition such as egg yolk such prefractionation proved necessary, as certain oxysterols could not be separated by only gas chromatography.

*Gas liquid chromatography.* In Figures 1 and 2 a gas chromatogram of an egg yolk sample is shown. In between the two internal standard peaks, 5α-cholestane and betulin, a large number of peaks was present. Various constituents were first identified tentatively by their retention time relative to 5α-cholestane and betulin. The retention times and response factors are given in Table 1. The mean standard deviation of 0.009 for response factors relative to betulin is smaller than the standard deviation of 0.027 found for response factors relative to 5α-cholestane, because for some samples, the 5α-cholestane was not separated well from other unknown components. The separation of 7-ketocholesterol and 25-hydroxycholesterol was also critical. Due to changes of the capillary column with time, these two components sometimes could no longer be separated after the column had been in use for a prolonged period. The problem could be solved by inserting a 5-min halt at 270°C into the final part of the temperature program.

As for oxysterols not listed in Table 1, 20-hydroxycholesterol was never detected in foods and was therefore not studied extensively. We were unable to quantitate 7α-hydroxycholesterol, because this peak partially coincided with that of cholesterol. In addition, the number of oxysterols that we could quantitate was limited to those for which pure standards were available. Therefore, we could identify the 5,6α-, but not the 5,6β-isomer of cholesterol

TABLE 1

**Retention Times of Various Cholesterol Oxidation Products Upon GLC and Response Factors[a]**

| Cholesterol oxidation product | Retention time | Relative response factor[a] | |
|---|---|---|---|
| | | 5α-cholestane | Betulin |
| 5α-Cholestane | 19.80 | 1.000 | — |
| 7β-Hydroxycholesterol | 23.33 | 0.926 ± 0.022 | 0.906 ± 0.005 |
| Cholesterol-α-epoxide | 23.48 | 1.234 ± 0.037 | 1.207 ± 0.015 |
| Cholestane-triol | 26.90 | 1.074 ± 0.026 | 1.038 ± 0.013 |
| 7-Ketocholesterol | 25.15 | 1.100 ± 0.025 | 1.077 ± 0.005 |
| 25-Hydroxycholesterol | 25.50 | 0.931 ± 0.025 | 0.911 ± 0.008 |
| Betulin | 27.50 | — | 1.000 |

[a]The relative response factor equals (concentration of component/peak area of component)/(concentration of internal standard/peak area of standard); mean ± SD of 3 measurements is given. Factors are relative to the two internal standards, 5α-cholestane and betulin.

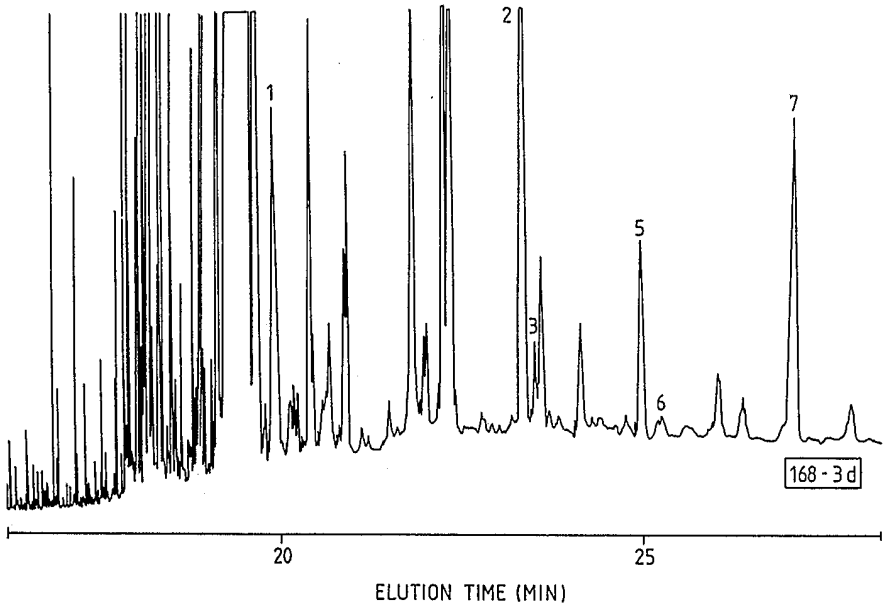


**FIG. 1. Gas chromatogram of cholesterol oxidation products in egg yolk powder: fraction B, containing the less polar oxysterols. 1, 5α-cholestane (internal standard); 2, 7β-hydroxycholesterol; 3, cholesterol-α-epoxide; 5, 7-ketocholesterol; 6, 25-hydroxycholesterol; 7, betulin (internal standard).**

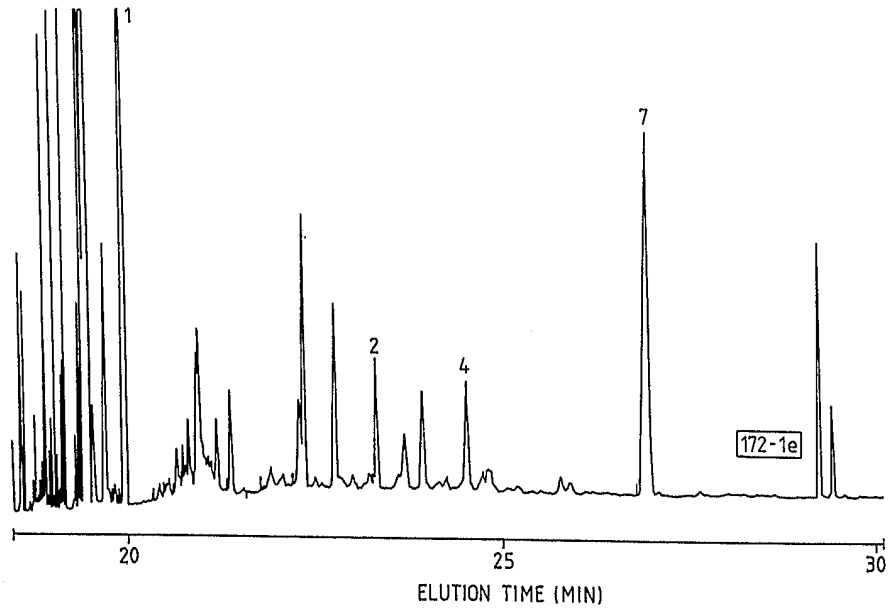


**FIG. 2. Gas chromatogram of cholesterol oxidation products in egg yolk powder: fraction C, containing the more polar oxysterols. 1, 5α-cholestane (internal standard); 2, 7β-hydroxycholesterol; 4, cholestane-triol; 7, betulin (internal standard).**

epoxide. As the gas chromatography column separated mainly by boiling point, there is a possibility that the α and the β isomer of cholesterol epoxide eluted as one peak. Alternatively, the unidentified peaks in the oxysterol region of the chromatogram (Figs. 1 and 2) could contain this or other unidentified oxysterols.

The stability of the various oxidation products on the column was very good, as indicated by the low standard deviations of the relative response factors. Low standard deviations were obtained only if the sample was injected directly on the column at a temperature of 70°C. If we used a more conventional splitless flash heater at 280–300°C for sample, injection we found large standard deviations, possibly caused by variable decomposition of oxysterols in the flash heater (data not shown).

*Recovery, precision and detection limit.* A standard mixture containing 20 μg each of cholesterol-α-epoxide, cholestane-triol, 25-hydroxycholesterol and 200 μg of cholesterol

was taken through the full procedure. Recoveries were between 86 and 98% (Table 2). The same oxysterols were added in known amounts (6.5 and 10 μg/g) to egg yolk powder. Recoveries (Table 2) varied between 93 and 98% at the 6.5 μg/g level and between 94 and 102% at the 10 μg/g level.

The reproducibility for independently worked-up samples is given in Table 3. The coefficient of variation tended to be lower, the higher the level of the analyte. For 25-hydroxycholesterol, a concentration of 0.3 μg/g with a coefficient of variation of 72% was found. Thus, this is about the lowest level of oxysterol that can be detected with our method in samples with highly complex matrices such as egg yolk.

*Identification.* Both the gas chromatographic separation and the detector response were poorer for the GC–MS analyses than for the routine gas chromatographic analyses. The use of splitless on-column injection in the latter was probably a major factor in providing a high yield and good separation.

The m/e values of typical fragments in the mass spectra of pure compounds and of oxysterol fractions from egg yolk powder were compared with published data (1). This provided identification of the trimethylsilylether of cholesterol-α-epoxide (m/e 474, 456 and 384), of cholestane-triol (m/e 546, 456, 403, 367 and 321) and of a 7-hydroxycholesterol (m/e 546, 456, 233 and 208); the geometry of the latter (7α or 7β) could not be determined. The quality of the GC–MS analyses proved insufficient to confirm or reject the identifications of the other oxysterols in egg yolk powder.

*Levels of oxysterols in foods.* Table 4 gives the levels of oxidation products in egg yolk and whole egg powder, and in dry milk. The very low content of oxysterols in fresh egg yolk indicates that artifactual oxidation of cholesterol during sample work up was minimal; the oxysterol concentration of 1 μg/g should be contrasted with the total cholesterol content of about 12,000 μg present in 1 g of egg yolk. A similar result was found by Nourooz-Zadeh and Appelqvist (11). The levels of 7β-hydroxycholesterol (12.8 to 78.0 μg/g) and cholesterol-α-epoxide (4.2 to 46.0 μg/g) found by us in nonirradiated samples of egg yolk and whole egg powder stored one to four years prior to analysis were higher than those found by Nourooz-Zadeh and Appelqvist (11) who found concentrations between 0 and 9.8 μg/g (7β-hydroxycholesterol) and between 0 and 2.5 μg/g (cholesterol-α-epoxide) in samples stored up to one and a half years, and concentrations of 46.8 μg/g (7β-hydroxycholesterol) and of 9.4 μg/g (cholesterol-α-epoxide) in a sample stored for eight years. The levels of cholestane-triol, 7-ketocholesterol and 25-hydroxycholesterol found by us in egg yolk and whole egg powder were generally low (below 15 μg/g), although these too were higher than those of Nourooz-Zadeh and Appelqvist (11). The effect of storage on the concentrations

**TABLE 2**

**Recovery of Cholesterol Oxidation Products From a Mixture of Pure Compounds and From Egg Yolk Powder[a]**

| Cholesterol oxidation product | Mean percentage recovery | | |
|---|---|---|---|
| | Standard compounds | Added to egg yolk powder | |
| | 20 μg each (n = 5)[b] | 6.5 μg/g (n = 2)[c] | 10 μ/g (n = 4)[b] |
| 7β-Hydroxycholesterol | 86 ± 12 | 95 (85–105) | 102 ± 7 |
| Cholesterol-α-epoxide | 92 ± 6 | 98 (92–103) | 99 ± 6 |
| Cholestane-triol | 90 ± 5 | 95 (91–99) | 94 ± 3 |
| 7-Ketocholesterol | 98 ± 9 | 98 (95–101) | 96 ± 4 |
| 25-Hydroxycholesterol | 94 ± 7 | 93 (92–94) | 97 ± 1 |

[a]To which pure compounds had been added at two different levels. The samples underwent the entire extraction and column chromatography procedure prior to gas chromatography. The standard mixture also contained 200 μg cholesterol.

[b]Mean ± SD.

[c]Mean (range).

**TABLE 3**

**Reproducibility of the Determination of Cholesterol Oxidation Products in Commercial Whole Egg and Egg Yolk Powder**

| Cholesterol oxidation product | n | Mean (range) (μg/g) | SD (μg/g) | Coefficient of variation (%) |
|---|---|---|---|---|
| 7β-Hydroxycholesterol | 12 | 36.8 (11.9–78.0) | 2.18 | 5.9 |
| Cholesterol-α-epoxide | 11 | 4.4 (1.8–11.2) | 0.75 | 17.0[a] |
| Cholestane-triol | 12 | 2.8 (0.3–13.9) | 0.48 | 17.2 |
| 7-Ketocholesterol | 12 | 16.9 (4.2–46.0) | 1.31 | 7.7 |
| 25-Hydroxycholesterol | 12 | 0.3 (0.0–0.5) | 0.21 | 71.8 |

[a]If one outlier was excluded, the coefficient of variation was 8.6%.

TABLE 4

**Concentration of Cholesterol Oxidation Products in Foods and in Duplicate Diets[a]**

| Commodity | 7-Hydroxycholesterol | Cholesterol-α-epoxide | Cholestane-triol | 7-Ketocholesterol | 25-Hydroxycholesterol |
|---|---|---|---|---|---|
| | | | (μg/g as is) | | |
| Fresh egg yolk | 0.3 | 0.7 | 0.0 | 0.2 | 0.0 |
| Egg yolk powder | | | | | |
| Manufacturer 1 | 12.8 | 5.8 | 0.3 | 1.8 | 0.3 |
| Manufacturer 2 | 45.1 | 26.3 | 0.4 | 6.5 | 2.6 |
| Manufacturer 2[b] | 74.1 | 46.0 | 0.5 | 9.4 | 7.3 |
| Manufacturer 3 | 16.9 | 8.1 | 0.0 | 2.8 | 1.6 |
| Manufacturer 3[b] | 16.8 | 6.7 | 0.1 | 1.8 | 1.0 |
| Manufacturer 4 | 22.3 | 9.7 | 0.5 | 3.4 | 0.5 |
| Manufacturer 4[c] | 78.0 | 25.2 | 0.5 | 4.2 | 13.9 |
| Manufacturer 4[d] | 507 | 2522 | 62 | 200 | 860 |
| Whole egg powder | | | | | |
| Manufacturer 1 | 21.2 | 6.4 | 0.0 | 3.2 | 0.4 |
| Manufacturer 2 | 64.9 | 35.8 | 0.5 | 11.2 | 2.3 |
| Manufacturer 2[b] | 36.8 | 19.4 | 0.2 | 8.1 | 1.4 |
| Manufacturer 4[b] | 40.8 | 9.6 | 0.4 | 3.6 | 1.9 |
| Manufacturer 4[c] | 11.9 | 4.2 | 0.0 | 1.9 | 0.3 |
| Dry full-fat milk | | | | | |
| Manufacturer 1[e] | 2.9 | 1.2 | 0.3 | 0.5 | 0.8 |
| Manufacturer 2[f] | 3.9 | 4.1 | <0.1 | 1.5 | 0.1 |
| Commercial rat feed | 0.3 | 0.04 | 0.0 | 0.3 | 0.0 |
| Commercial rat feed with extra fruit and vegetables | 0.1 | 0.02 | 0.0 | 0.2 | 0.0 |
| Duplicate Dutch diet, raw | 2.3 | 0.7 | 0.1 | 1.1 | 0.0 |
| Duplicate Dutch diet, baked/fried/grilled | 1.7 | 0.8 | 0.1 | 1.0 | 0.0 |
| Duplicate Dutch diet, baked/fried/grilled, plus extra fruit and vegetables | 2.2 | 1.7 | <0.1 | 2.0 | 0.2 |

[a]Duplicate diets had been freeze-dried and pelleted and stored for one year at 4°C, unless indicated otherwise.
[b]Desugared.
[c]After storage at ambient temperature for 4 years.
[d]After irradiation of sample c with UV light for three weeks.
[e]After 7 years of storage.
[f]After 2 years of storage.

of oxysterols was unclear. In egg yolk powder the concentrations increased upon long-term storage, but in whole egg powder they decreased. It might be that differences in storage temperature at the manufacturers are responsible for this (11). UV-irradiation considerably increased the concentrations of all oxysterols studied.

The total concentration in dry full-fat milk stored between two and seven years did not exceed 10 μg/g. Levels of cholesterol oxidation products in commercial rat chow were also very low (Table 4).

In duplicates of the average diet eaten in the Netherlands, oxysterol levels ranged from 0.0–0.2 μg/g for 25-hydroxycholesterol to about 2 μg/g for 7β-hydroxycholesterol. The levels were not higher in duplicate diets made up of fried, baked and grilled products than in duplicate diets made up of the equivalent raw foods. Thus, the amount of cholesterol oxidized during the frying, baking or grilling of foods appears to be very small. As the daily food intake for an average person is about 500 g, the average Dutchman will ingest about 1 mg of 7β-hydroxycholesterol and 0.5 mg of cholesterol-α-epoxide per day. The diets to which fruits and vegetables of supposedly anticarcinogenic activity had been added, contained somewhat higher levels of 7-ketocholesterol (2.0 μg/g compared with 1.1 and 1.0 μg/g for the other two mixed diets without the extra fruits and vegetables added). In view of the freeze drying and pelleting that our samples had undergone these figures may be considered upper limits. On the other hand, the chromatograms of these duplicate diets did contain quite a few peaks that escaped identification, and one could theorize that normal foods might contain appreciable amounts of oxysterols other than those identified. However, the oxysterols studied by us are those naturally formed from cholesterol under a range of conditions, and it seems unlikely that the reaction of oxygen with cholesterol in foods would produce an entirely different range of products. Therefore we suggest that the most plausible load of oxysterols in the average Dutch diet is at most a few mg per day. Whether such amounts contribute to the development of chronic diseases is at present not known.

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