Identification and Quantification of Cholesterol Oxidation Products in Canned Tuna

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ABSTRACT: Cholesterol oxidation in tuna canned in brine was studied. Gas chromatography–mass spectrometry was used for the detection of the seven major cholesterol oxidation products originating from both direct and indirect oxidation. The total amount of cholesterol oxidation products in the analyzed samples varied considerably, ranging between 40 and 350 μg/g lipids, with the exception of an anomalous sample, that reached a 1600 μg/g level. The lipid content ranged between 0.5 and 1 g/100 g wet product. As most samples did not exceed 100 μg/g lipids, it is possible to assume that the total content of cholesterol oxidation products can be kept below this value if good manufacturing conditions are used, together with a careful choice of the best tuna cuts. The application of principal component analysis to the detected variables confirmed that 7-ketocholesterol is a useful index of the whole oxidation process.

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KEY WORDS: Canned tuna, cholesterol oxidation, GC–MS, 7-ketocholesterol.

Cholesterol is present in many foodstuffs of animal origin and may undergo oxidation during industrial processes that require high temperatures. More than 70 cholesterol oxidation products (COP) have been identified so far, and some of them have a toxic activity (1–6).

Cholesterol oxidation may develop both directly, if oxygen is present, and indirectly, if other oxidizers are present in food. The direct oxidation pathway leads mainly to 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol, together with other minor COP. Indirect oxidation, whose major products are 5α- and 5β-epoxycholesterols and cholestanetriol, is favored by the presence of unsaturated fatty acids in the lipid fraction, such as in fish, whose oil is particularly rich in polyunsaturated fatty acids (PUFA).

Previous studies (7) reported the formation of COP in some processed marine foods, but they also emphasized the need for further studies. The industrial production of canned fish generally involves two high-temperature steps that unavoidably lead to lipid oxidation: precooking of fish and its sterilization in cans. The former step may be carried out by pressure or steam cooking or by baking in a slow to moderate oven; it improves the appearance, taste, flavor, and texture of the final product. The latter step is generally carried out in a pressure canner and aims mainly at the complete destruction of *Clostridium botulinum*. Since canned tuna is consumed nearly all over the world, a study of cholesterol oxidation in this kind of product is certainly useful.

In this study, several COP were detected in samples of tuna canned in brine. This product is obtained by adding only water, salt, and sometimes spices to fish. Thus, it contains only lipids derived from fish, which may be oxidized and contribute to indirect cholesterol oxidation. Tuna canned in vegetable oils will be the subject of another paper. To evaluate the development of the two different oxidation pathways, gas chromatography–mass spectrometry (GC–MS) was used to detect the major COP resulting both from direct oxidation (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol) and from indirect oxidation (5α-epoxycholesterol, 5β-epoxycholesterol, and cholestanetriol). Particular attention was paid to the 7-ketocholesterol content, because it might be considered a useful marker (8–11) of the total oxidative process. On the other hand, summing the amounts of the seven detected COP, which is close to the total amount of COP, allows a more careful evaluation of the effects of preserving technologies on cholesterol oxidation.

MATERIALS AND METHODS

Chemicals. Analytical-grade solvents were supplied by Merck (Darmstadt, Germany), absolute pyridine (over molecular sieves) by Fluka (Buchs, Switzerland), and Sylon BFT® [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane] by Supelco Inc. (Bellefonte, PA). Diethyl ether (Merck) was deperoxidized before analysis by molecular sieves (Deperox supplied by Fluka) (12).

The 5,6α-epoxy-cholestan-3β-ol (5α-epoxycholesterol), 3β-hydroxycholest-5-ene-7-one (7-ketocholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol), and cholest-5-ene-3β,19-diol (19-hydroxycholesterol) were supplied by Sigma Chemicals Co (St. Louis, MO); cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) and 5,6β-epoxy-cholestan-3β-ol (5β-epoxycholesterol), by Steraloids Inc. (Wilton, NH).

Samples. Eighteen samples of tuna canned in brine were purchased from local shops. Cans had two different sizes, and their content of drained product ranged between 56 and 150 g.

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Lipid extraction. The entire content of each can was drained and crushed into small pieces in a mortar. Lipids were then extracted from an accurately weighed (± 0.0001 g) 10-g sample by chloroform/methanol 2:1 (vol/vol) (13). The homogenate was filtered through paper. After washing the crude extract with 0.05 N NaCl, the mixture was allowed to separate overnight into two clear phases in a separatory funnel. The lower layer was collected in a 50-mL round-bottomed flask, and the solvent evaporated under vacuum at room temperature.

Cold saponification and extraction of nonsaponifiable products. The compound 19-hydroxycholesterol (1.8 μg) was added to 60–80 mg of the extracted lipids in a 50-mL round-bottomed flask, and cold saponification was carried out in the dark by 10 mL 1 N KOH in methanol (14). Any warming of the alkaline methanolic solution was carefully avoided (15). Unsaponifiables were then extracted (14) and evaporated to dryness in a Rotavapor at 20°C.

Enrichment of COP from total unsaponifiables by solid phase extraction (SPE). The extracted unsaponifiables were dissolved in 1 mL *n*-heptane/2-propanol (98:2, vol/vol); 0.5 mL of this solution was applied to a 500-mg SPE Florisil cartridge (International Sorbent Technology, Hengoed, United Kingdom), prewashed with 3 mL of *n*-heptane. The cartridge was washed with 4 mL *n*-heptane/2-propanol (98:2, vol/vol); the retained sterol oxides were then eluted by 3 mL acetone.

Derivatization. The oxysterol-containing acetone fraction was dried under a stream of nitrogen. Then 20 μL of dry pyridine and 80 μL of Sylon BFT were added; the mixture was allowed to stand overnight.

GC–MS. GC–MS was used for verification of peak assignments [total ion current (TIC) mode] and for quantification [single ion monitoring (SIM) mode] of COP as trimethylsilyl (TMS) ethers. Pulsed splitless injection (injection pulsed pressure 21 psi, injection pulsed time 1 min, splitless time 1 min) was performed at 290°C in an HP 6890 GC System coupled with an HP 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA). Sample (1 μL) was injected onto a 30 m × 0.25 mm i.d. × 0.25 μm film thickness HP5MS fused-silica capillary column (Agilent Technologies) at an oven temperature of 90°C and a helium flow rate of 0.8 mL/min. The oven temperature was kept at 90°C for 1 min; then it was programmed at 20°C/min to 280°C and at 4°C/min to 300°C (kept for 16 min) under flow-controlled conditions (constant flow 0.8 mL/min). A 5-min post-run at 310°C was then performed. The mass spectrometer interface temperature was set to 310°C. The temperature of the ion source was 230°C, electron energy 70 eV, and quadrupole temperature 150°C.

Method viability test. Recoveries were checked by running a solution of COP and 19-hydroxycholesterol standards through all experimental procedures. Recoveries were greater than 90% for all the detected COP.

Statistical analysis. A pattern recognition analysis of the data set was performed using the principal component analysis (PCA), as implemented in Q-PARVUS 3.0 (16).

RESULTS AND DISCUSSION

Direct exposure to light was carefully avoided during sample preparation and sample cleanup procedures. Overnight derivatization at room temperature was used to obtain quantitative silylation. Pulsed splitless injection was chosen because it allows a rapid sample introduction into the column, thus limiting COP degradation at high temperature during splitless injection. The TMS derivatives of COP were well resolved using the described chromatographic conditions. Mass spectrometric detection of the compounds was achieved after electron impact ionization. TIC acquisition and verification of the spectral match against authentic standards allowed identification of each compound. The derivative of cholestanetriol was identified as its bis-TMS ether (17). The major ions obtained for each compound are summarized in Table 1. Each of these ions has been described previously (18). COP quantification was achieved by SIM acquisition of the target ions reported in Table 1, using 19-hydroxycholesterol as internal standard. At least two qualifier ions were used for each compound in order to verify their percentage ratio to target ion (Table 1). A multilevel calibration was performed for each detected COP in the range 1–5 and 4–30 pmol; R^2 values are reported in Table 1. As the 5,6β-epoxycholestan-3β-ol standard was highly impure, for this compound the calibration of its α isomer was used. The limits of detection (19) of COP ranged between 0.5 and 4 pmol (0.1–0.8 μg/g lipid).

TABLE 1
Characteristic Ions in the Electron Impact Mass Spectra of the Trimethylsilyl Derivative of COP and Values of R^2 Obtained by Target Ion Calibration[a]

Cholesterol oxide	Major ions	Target ion	Qualifier ions	R^2
7α-Hydroxycholesterol	546, 456, 129	456 $[M - 90]^+$	546, 531, 457	1.000
19-Hydroxycholesterol	531, 456, 366, 353, 145	353	457, 456, 366	I.S.
7β-Hydroxycholesterol	546, 456, 129	456 $[M - 90]^+$	546, 531, 457	0.998
5β-Epoxycholesterol	474, 459, 445, 384, 366	474 $[M]^+$	475, 366	N.D.
5β-Epoxycholesterol	474, 459, 445, 384, 366	474 $[M]^+$	475, 366	1.000
Cholestanetriol	546, 456, 403, 321, 129	403	547, 546, 456	0.988
25-Hydroxycholesterol	456, 327, 271, 131, 129	131	457, 456, 271	0.999
7-Ketocholesterol	472, 457, 382, 367, 129	472 $[M - 90]^+$	473, 367	0.999

[a]I.S., internal standard; N.D., not detected; COP, cholesterol oxidation products.

TABLE 2
Minimum and Maximum, Mean, and Relative Standard Deviation Values of the Detected COP

Cholesterol oxide	Minimum–maximum (μg/g in lipids)	Mean (μg/g in lipids)	RSD[a] (%)
1 7α-Hydroxycholesterol	5.4–37.3	17.3	48.4
2 7β-Hydroxycholesterol	7.0–50.1	21.5	53.7
3 5β-Epoxycholesterol	3.1–52.8	18.5	86.7
4 5α-Epoxycholesterol	2.6–25.8	9.4	71.4
5 Cholestanetriol	2.6–41.5	14.3	74.4
6 25-Hydroxycholesterol	0.7–2.7	1.5	34.6
7 7-Ketocholesterol	10.1–139.2	37.4	86.7
8 TOTAL COPS	37.7–328.9	119.9	65.5
9 Total COP from direct oxidation	23.8–229.4	50.5	65.0
10 Total COP from indirect oxidation	13.9–108.8	30.9	73.1

[a]RSD, relative standard deviation. See Table 1 for other abbreviation.

Three individual measurements per sample were performed; the relative standard deviation (RSD) of each COP was generally lower than 7%. The 7-ketocholesterol and cholesterol epoxides often showed the highest RSD values.

Table 2 reports the minimum and maximum detected amounts of each COP, together with their mean and RSD, in 17 samples of tuna canned in brine. Samples were produced by 12 different manufacturers that make up more than 90% of the Italian market. Moreover, Table 2 reports the range of the sums of COP derived from the two different oxidation pathways (direct and indirect) and the range of the total COP amount. In this table, as in Figure 1, the 18th sample was not considered, because it contained more than 1600 μg/g (in lipids) of total COP and it would have strongly affected the mean and RSD values (Table 1) and the Y-scale (Fig. 1). This sample already appeared finely minced, a condition that extends the contact with oxygen and the development of the oxidation process. Several brown areas were also evident which indicates the use of inferior-quality fish parts. For these reasons, this sample may be considered anomalous. This hypothesis was confirmed by the analysis of other samples of the same brand, which showed total and partial COP amounts of the same magnitude as the other analyzed samples and had a clear color.

In accordance with data previously reported (20,21) for other foodstuffs, the amounts of 7-ketocholesterol, 7β-hydroxycholesterol, and 5β-epoxycholesterol were often higher with respect to the other detected COP. Nevertheless, considerable amounts of cholestanetriol, which is considered the most toxic among COP (1), were detected. On the other hand, low amounts of 25-hydroxycholesterol, which also shows a marked cytotoxic activity, were detected.

Particular attention must be paid to the detected values of 7-ketocholesterol, since this compound appears to be an excellent marker for the entire oxidative process. The histograms in Figure 1 show that 7-ketocholesterol is an excellent index of both direct and total cholesterol oxidation. The correlation coefficient between 7-ketocholesterol and the COP sum is equal to 0.978.

Figure 1 also shows that in most cases direct prevailed over indirect oxidation; nevertheless, the high PUFA content also caused extensive indirect oxidation.

To confirm the key role of 7-ketocholesterol as an oxidation marker, a multivariate analysis of the data set was carried out by building a data matrix composed of 17 rows (the 17 tuna samples) and 8 columns (the 7 detected COP and their sum). Then, the autoscaled data were analyzed using PCA in order to extract, visualize, and rationalize as much of the useful information as possible. Figure 2 represents the biplot (projection of the object scores and of the variable loadings) on the first two principal components, which explain about the 91.3% of the total information.

The plot analysis emphasizes a separation between the objects whose COP amounts exceed 200 mg/g lipids and the other objects along the first component, which is mainly built by 7-ketocholesterol (variable 7) and the COP sum (variable 8). These two variables provide almost exactly the same information, again confirming our assumption that 7-ketocholesterol can monitor the entire oxidation process.

The second principal component seems to be related to the oxidation pathways (direct and indirect oxidation).

In summary, the total amount of COP varies considerably, and two groups can be singled out in the analyzed samples. In the first group, total COP were less than 120 μg/g lipids, whereas the three samples having a COP content exceeding 200 μg/g lipids, together with the anomalous one, form the

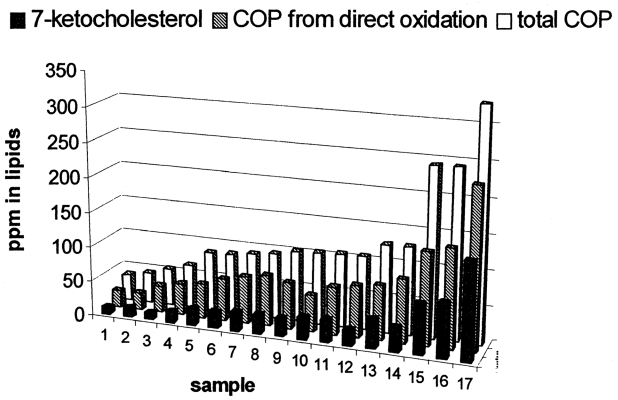

FIG. 1. Contents of 7-ketocholesterol, cholesterol oxidation products (COP) from direct oxidation, and total COP of the analyzed samples.

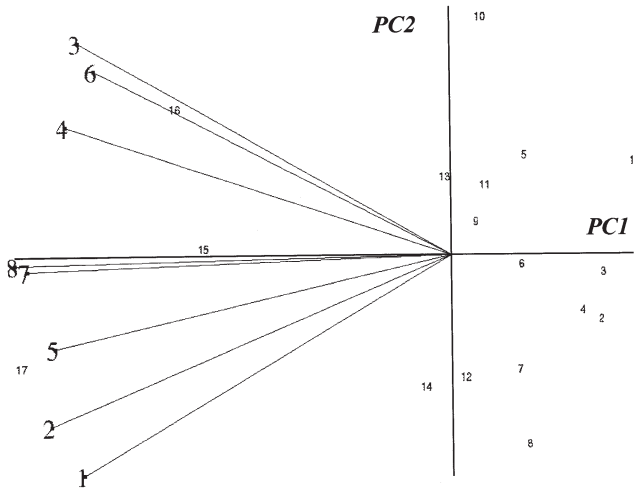

FIG. 2. Biplot on the first two principal components (PC) PC1 vs. PC2. The 17 objects are identified by a small numerical index, the eight variables (the first eight variables listed in Table 2) by a large numerical index.

second group. The variability of COP content was not observed among different-sized products; thus, it may be ascribed to the different technologies employed for canned tuna manufacturing. Nevertheless, the different quality of fish cuts might also play an important role in the development of cholesterol oxidation. The manufacturing of crumbled and fat-rich meat, for example, with its high content of PUFA and cholesterol, might favor the development of cholesterol oxidation. However, the total COP amounts detected in this study were lower than those previously reported for canned fish (7); but, in the latter study, quantification of COP was achieved by GC–MS analysis of the total unsaponifiable fraction. Thus, it could have been affected by the presence of cholesterol and other major unsaponifiable compounds.

The content of cholesterol oxidation products in the tuna samples appeared quite high if compared to the lipid content of the sample. Fortunately, present manufacturing technologies often involve a large reduction in the fish lipid content before canning. The lipid fraction of the canned tuna samples was ≅0.5–1%; thus, the recorded values of COP amounted to approximately 0.3–3 μg/g of canned tuna. In the anomalous sample, the total content of COP was about 16 μg/g. Such high quantities could be of possible concern. However, as the total COP content of most samples did not exceed 100 μg/g lipids, it is possible to conclude that COP amount could be kept below this value if good manufacturing conditions are used together with a careful choice of the best tuna cuts.

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