Effect of Synthetic Antioxidants on Cholesterol Stability During the Thermal-Induced Oxidation of a Polyunsaturated Vegetable Oil

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ABSTRACT: As a molecule with an unsaturated bond, cholesterol is prone to oxidation. Cholesterol oxidation products (COP) are found in many common foods and have been shown to be atherogenic, cytotoxic, mutagenic, and possibly carcinogenic. Efforts to reduce the formation of oxidation products are considered important during the manufacture and processing of foods. The effect of synthetic antioxidants on cholesterol oxidation has not been extensively studied. We assayed the effect of five commonly used antioxidants—BHT, BHA, the *n*-propyl ester of 3,4,5-trihydroxy benzoic acid (PG), TBHQ, and 6-ethoxy-1,2-dihydro-2,4-trimethylquinoline (EQ)—on cholesterol stability when oxidation is induced in a Rancimat 679 instrument by bubbling air through the sample at 150°C. The sample consisted of 200 mg cholesterol dispersed in 100 g of a polyunsaturated vegetable oil (soybean oil). Formation of six COP was measured at the induction period, and at the 50 and 100 μS conductivity values. Under the experimental conditions, BHT and TBHQ were the most effective inhibitors of cholesterol oxidation. BHA and EQ were less effective, and PG was unable to prevent cholesterol oxidation. Synthetic antioxidants were more effective in preventing COP formation at the nucleus of the cholesterol structure than at the lateral chain.

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KEY WORDS: Cholesterol, cholesterol oxidation products, synthetic antioxidants, vegetable oil oxidation.

The lability of cholesterol (CHO) has been amply documented, and well over 60 products resulting from autooxidation, photooxidation, and enzymatic action have been described (1,2). CHO, either as a solid, in suspension, or in solution, when exposed to air at room temperature is slowly oxidized (3). CHO oxidation products (COP) are found in many common foods and have been shown to be atherogenic, cytotoxic, mutagenic, and possibly carcinogenic (2,4). CHO autooxidation is more rapid at elevated temperatures and in the presence of phospholipids and/or TG (5). As an unsaturated lipid, CHO is autooxidized in the presence of molecular oxygen and light by a free radical process that forms chemically labile hydroperoxides. These hydroperoxides are decomposed to form secondary oxidation products. When food containing CHO is exposed to heat and air during processing and/or storage, there is a significant increase in the level of COP. When heated alone, CHO behaves as a very stable molecule (6). However, when it is heated with polyunsaturated TG, such as some vegetable or marine oils, the sterol decomposes rapidly to form diverse COP (6).

Antioxidants are organic lipid- or water-soluble substances of either synthetic or natural origin that can prevent or delay the development or the progress of PUFA oxidation (7). Antioxidants can scavenge the active forms of oxygen involved in the initiation step of the oxidation, or break the oxidative chain reaction by reacting with the FA peroxy radicals to form stable antioxidant radicals that are either too unreactive for further reactions or else form nonradical products (8). The protective effect of antioxidants on TG oxidation in a wide variety of fats and oils is amply documented (9). Since CHO oxidation proceeds *via* a free radical mechanism similar to PUFA oxidation, antioxidants used to inhibit general fat and oil oxidation may also be able to prevent or retard CHO oxidation. However, the effect of synthetic antioxidants on CHO oxidation has not been extensively studied, and those results have been diverse and sometimes contradictory. Morgan and Armstrong (10) demonstrated that BHT, BHA, and the *n*-propyl ester of 3,4,5-trihydroxybenzoic acid (propyl gallate, PG) are somewhat effective in slowing hydrogen peroxide-induced CHO oxidation of spray-dried egg yolk at room temperature for several months. Huber *et al.* (11) showed a significant inhibitory effect of BHA on COP formation when spray-dried egg yolk oxidation was catalyzed by Cu^{2+} during accelerated storage at 60°C. Maerker and Unruh (12) observed that BHT is unable to inhibit CHO oxidation during hot saponification of TG. The same antioxidant cannot inhibit formation of COP from irradiated egg yolk powder (13). The effect of two other important antioxidants—TBHQ and 6-ethoxy-1,2-dihydro-2,4-trimethylquinoline (ethoxyquin, EQ)—on CHO oxidation has not yet been evaluated.

The present investigation was undertaken to evaluate the effectiveness of BHT, BHA, PG, TBHQ, and EQ to inhibit CHO oxidation during the accelerated thermal-induced oxidation of a polyunsaturated vegetable oil.

MATERIAL AND METHODS

Materials. CHO (cholest-5-en-3β-ol) of the highest purity available, and the following GC standards: 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol) (7α-OH); 7β-hydroxycholesterol (cholest-5-en-3β,7β-diol) (7β-OH); 7-ketocholesterol (3β-hy-

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droxycholest-5-en-7-one) (7-keto); cholestanetriol (cholestane-3β,5α,6β-triol) (5α-3OH); 20-hydroxycholesterol (5-cholestene-3β,20-diol) (20-OH); 25-hydroxycholesterol (5-cholestene-3β,25-diol) (25-OH); and 5α-cholestane (as internal standard), were purchased from Sigma Chemical Co. (St. Louis, MO). Sylon BFT (99% bis-trimethylsilyltrifluoroacetamide + 1% trimethylchlorosilane) was purchased from Supelco Inc. (Bellefonte, PA). All other chemicals were purchased from Merck Quimica Chilena (Santiago, Chile). Highly refined soybean oil was a gift of Watt's Alimentos SA (Santiago, Chile). Commercial BHT (99.5%), BHA (98%), PG (99%), and TBHQ (99.5%) were obtained at the local market (Santiago, Chile) from different suppliers: BHT, BHA, and PG (Eastman Chemical Co.) were purchased from Molypak SA (Santiago, Chile); TBHQ (Eastman Chemical Co.) was purchased from Quimica Alfa; and EQ (98%) (Monsanto Co., Santiago, Chile) adsorbed in vermiculite was purchased from Novus (Santiago, Chile).

Methods. Commercial CHO was subjected to silicic acid column chromatography (silica gel 60, 70–230 mesh, 18 × 200 mm; Merck) to separate possible COP derived from extraction and/or from further processing and handling of CHO according to the procedure of Kim and Nawar (5) and Osada *et al.* (6). Purity of COP-free CHO preparation was assessed by TLC (Kieselgel 60, 0.25 mm thickness; Merck), evidenced by a single spot revealed by spraying 3% copper acetate/8% phosphoric acid and when compared to the CHO standard (14). COP-free CHO fractions were maintained in sealed ampoules in the dark until use.

Recently refined soybean oil, free of antioxidant and maintained under N_2 atmosphere, was mixed with COP-free CHO (200 mg/100 g oil). The sterol was dissolved by gentle rotary agitation at 40°C. Once CHO was dissolved, the oil was assayed for oxidation in the presence of either BHT, BHA, PG, TBHQ, or EQ (200 mg/kg oil). Oil oxidation was induced by a Rancimat 679 instrument (Metrohm, Herisau, Switzerland). Oil samples (6 mL) were placed in each of the six Rancimat reaction vessels (three vessels for the control and three for the assay), and oxidation was induced at 150°C with an air flow of 20 L/h and in the induction period (IP) operation mode (15). Once the IP was established, oil samples were maintained in the same experimental conditions until the oxidation reached a conductivity value of either 50 or 100 μS. Time to reach these conductivity values varied from 3 to 22 h, depending on the antioxidant assayed. Controls were carried out under the same conditions with antioxidant-free oil.

COP formation after each oxidation assay was assessed by capillary GC according to the procedure of Addis *et al.* (16), with some minor modifications. At the end of each assay, a 15-mL sample of oxidized oil was collected by mixing the contents of three Rancimat reaction vessels (volume: 5 mL each) for the extraction of CHO and COP by a cold saponification procedure. One hundred fifty microliters of 5% wt/vol solution of 5α-cholestane in chloroform was added to the oxidized oil as internal standard. Saponification was performed by adding 40 mL of 1 N KOH in methanol, and the solution was maintained for 20 h at 20°C under a N_2 stream. Nonsaponifiables were then extracted with 30 mL of anhydrous ethyl ether and dried over Na_2SO_4. The dried extracts were filtered with Whatman no. 1 filter paper, which was re-extracted with another 30 mL of ethyl ether and filtered again. The combined filtrates were evaporated to 0.5 mL in a rotary vacuum evaporator (Büchi Rotavapor, Flawil, Switzerland). Trimethylsilyl ester derivatives were formed by the addition of 300 μL pyridine and 150 μL Sylon BTZ (13). GC analyses of COP, as trimethylsilyl ester derivatives, were made in a Hewlett-Packard 5890 Series II GC, equipped with an FID. The GC conditions were: Ultra 1 Hewlett-Packard (Avondale, PA) capillary column (50 m × 0.2 mm i.d., 0.33 μm film thickness); temperature programming from 180 to 285°C; and detector at 300°C.

Statistical analysis. Data were analyzed statistically by Student's *t*-test. Mean values were obtained by averaging six independent measurements and expressed ±SD. Differences between different average measurements were considered significant at $P < 0.05$. Those values that are significantly different from the respective control are indicated by (s).

RESULTS AND DISCUSSION

Soybean oil oxidation induced by the Rancimat instrument under our experimental conditions had a very short induction period (IP = 2.5 ± 0.12 h), and the addition of 200 mg/dL of cholesterol slightly shortened the IP (2.35 ± 0.15 h). Addition of antioxidants in some cases substantially modified the IP for oxidation, the time varying according to the type of antioxidant added to the oil. Table 1 shows the IP obtained for the different antioxidants and the times (h) where the 50 and 100 μS conductivity values were reached. BHT and TBHQ were highly effective as antioxidants (IP: 12.17 and 13.14 h, respectively), PG showed the lowest antioxidant activity (IP: 2.85 h), and BHA and EQ exhibited an intermediate antioxidant behavior (IP: 9.12 and 8.31, respectively). Times to reach the 50 and 100 μS values were different for each antioxidant, and a correlation between the conductivity values and the IP value was observed.

Table 2 shows the remaining CHO concentration and the COP formed at the IP, and at the 50 and 100 μS conductivity values for the oil in the presence of each antioxidant assayed. In the control and in each of the antioxidant-containing oil

TABLE 1
Time Course (h) Necessary to Reach the IP and the 50 and 100 μS Conductivity Values for Soybean Oil (oil) + Cholesterol (CHO) in the Presence of Synthetic Antoxidants[a]

	IP (h)	50 μS	100 μS
Control (oil + CHO)	2.35 ± 0.15	3.18 ± 0.18	4.02 ± 0.20
+ BHT	12.17 ± 0.24	18.21 ± 0.72	21.34 ± 0.54
+ BHA	9.12 ± 0.12	11.03 ± 0.55	13.93 ± 0.32
+ PG	2.85 ± 0.17	4.87 ± 0.23	6.92 ± 0.27
+ TBHQ	13.14 ± 0.32	19.19 ± 0.47	21.90 ± 0.56
+ EQ	8.31 ± 0.19	11.91 ± 0.22	14.75 ± 0.37

[a]Values represent the mean of six assays ± SD. IP, induction period; PG, propyl gallate; EQ, ethoxyquin.

TABLE 2
COP Formation from the Oxidation of Soybean Oil + CHO at the IP and the 50 and 100 µS Conductivity Values in the Presence of BHT, BHA, PG, TBHQ, and EQ[a]

	Control		BHT		BHA		PG		TBHQ		EQ	
	IP	50 µS	100 µS	IP	50 µS	100 µS	IP	50 µS	100 µS	IP	50 µS	100 µS	IP	50 µS	100 µS
CHO	1890 ± 85	1272 ± 78	778 ± 92	1890 ± 88	1780 ± 49 (s)	1510 ± 68 (s)	1808 ± 110	1470 ± 58 (s)	1147 ± 86 (s)	1830 ± 109	1195 ± 49	710 ± 29	1895 ± 59	1872 ± 78 (s)	1792 ± 92 (s)	1920 ± 145	1792 ± 47 (s)	1090 ± 72 (s)
7α-OH	ND	58 ± 17	126 ± 42	ND	ND (s)	ND (s)	ND	36 ± 13	116 ± 27	ND	62 ± 21	138 ± 29	ND	ND (s)	ND (s)	ND	52 ± 24	114 ± 37
7β-OH	ND	ND	59 ± 12	ND	ND	32 ± 17	ND	ND	36 ± 12	ND	ND	57 ± 12	ND	ND	ND (s)	ND	ND	62 ± 21
7-Keto	ND	93 ± 24	246 ± 38	ND	54 ± 12 (s)	72 ± 22 (s)	ND	67 ± 18	151 ± 27 (s)	ND	86 ± 17	179 ± 21	ND	ND (s)	ND (s)	ND	79 ± 12	168 ± 15 (s)
5α-3OH	ND	85 ± 28	152 ± 37	ND	ND (s)	ND (s)	ND	69 ± 24	79 ± 17 (s)	ND	82 ± 24	149 ± 17	ND	ND (s)	52 ± 14 (s)	ND	85 ± 18	108 ± 11 (s)
20-OH	ND	210 ± 68	306 ± 98	ND	82 ± 22 (s)	163 ± 48 (s)	ND	174 ± 58	217 ± 41	ND	202 ± 59	298 ± 61	ND	91 ± 32 (s)	108 ± 41 (s)	ND	225 ± 72	214 ± 38
25-OH	ND	274 ± 62	346 ± 58	ND	57 ± 12 (s)	124 ± 51 (s)	ND	193 ± 61	242 ± 37	ND	248 ± 15	334 ± 28	ND	22 ± 13 (s)	72 ± 29 (s)	ND	172 ± 59	234 ± 37 (s)

[a]Values are presented, in µg/mL, as the mean ± SD (n = 6). ND, not detected; for other abbreviations see Table 1.
[b](s), significantly different from the respective control.

assays, there is no CHO oxidation, and therefore no COP are formed before the IP is reached. However, after this time, at the 50 and 100 µS conductivity values, different amounts of COP form in the control. Therefore, under our experimental conditions, CHO oxidation parallels oil oxidation in the absence of any added antioxidant. BHT produces an important inhibition of COP formation. Low amounts of the oxidation products form after the addition of the antioxidant; 7α-OH and 5α-3OH are still undetectable at the 100 µS conductivity value. BHA can inhibit CHO oxidation, but with much less efficiency than BHT. In this case, at the 100 µS conductivity value, only the 7-keto and 5α-3OH amounts are significantly different from the control. PG was unable to prevent CHO oxidation, thus allowing the formation of all COP assayed, both at the lower and at the higher conductivity value, and with no difference in the amount formed from the control. TBHQ was the best of all the antioxidants assayed at inhibiting CHO oxidation. This antioxidant can prevent the formation of 7α-OH, 7β-OH, 7-keto, and 5α-3OH at the 50 µS conductivity value, allowing the formation of only a small amount of 20-OH and 25-OH. At the 100 µS value, a small amount of 5α-3OH formed, and higher amounts of 20-OH and 25-OH formed that were significantly different from the control amounts. EQ exhibited a very similar behavior to BHA, showing significant differences only for 7-keto and 5α-3OH formation at 100 µS. Possible inclusion of phytosterol oxidation products, derived from soybean oil oxidation, in the monitoring of COP was minimized because the amount of phytosterols that remained after oil processing was very low, and also because GC retention times for these oxidation products are different from those of the COP assayed.

The synthetic antioxidants assayed can prevent CHO oxidation, but to a different degree. In our experimental conditions the ranking for effectiveness is TBHQ > BHT > BHA ≈ EQ > PG. There is also a good correlation between the capacity of each antioxidant to delay the induction of oil oxidation (IP, Table 1) and the efficiency to inhibit COP formation. That is, the higher the IP observed for each individual antioxidant is, the lower the level of CHO oxidation and the amount and variety of COP formed after the different level of oil oxidation (50 and 100 µS values). However, although CHO oxidation is parallel to the oil oxidation, the kinetics of this oxidation may not be the same for the TG and for the sterol. At the IP, i.e., when oil oxidation is initiated, CHO is not oxidized; when the 100 µS value is reached, evidencing a high level of oil oxidation, 40% of the CHO, on average, remains unoxidized (Table 2, control). It has been proposed that the mechanism for sterol oxidation must be similar to the mechanism for TG oxidation (5). TG appear to oxidize first, thus providing free radicals and peroxides. These may trigger a faster but delayed oxidation of CHO (11). However, it is difficult to use our model to support these proposals because the great difference in TG concentration compared to CHO concentration may determine different oxidation kinetics for each molecule. These results may in turn obscure the start of sterol oxidation. It is interesting that in the control assays and in the antioxidant assays, 20-OH and 25-OH are always the most prevalent

oxidation products. We recently demonstrated that α- and γ-tocopherols can almost selectively inhibit the formation of 20-OH and 25-OH COP by unilamelar phospholipid/cholesterol-containing liposomes when oxidation is induced by Fe^{2+} (17). This result is in contradiction to those of the present report. However, the procedures applied for TG and phospholipid oxidation and the antioxidants assayed in both experimental models are not the same. One can speculate why some synthetic antioxidants can preferentially inhibit the oxidation of the sterol at the nuclear structure of CHO rather than at the lateral chain. 7α-OH, 7β-OH, 7-keto, and 5α-3OH are oxidation products formed adjacent to the 5,6 double bond of the sterol nuclear structure. These oxidation products are formed through intermediary structures such as 7-hydroperoxide cholesterol (which results in the formation of the 7α-OH, 7β-OH, and 7-keto products), and 5,6-epoxycholesterol (which forms 5α-3OH). The mechanism for the formation of these COP requires the previous formation of free radicals either at the 5- or the 7-carbon position of the sterol structure. Formation of these free radicals may be enhanced by the 5,6 double bond, and antioxidants can prevent, although with different efficiency, the formation of the free-radical intermediary products. Formation of the 20-OH and 25-OH products may occur through a direct free-radical reaction, without intermediary product formation, which allows the fast incorporation of molecular oxygen into the 20- or 25-OH carbon of the lateral chain. This reaction can be strongly affected by temperature, with free radical scavenger molecules, such as the synthetic antioxidants assayed, being less efficient to prevent this oxidation. Therefore, temperature-induced oxidation of CHO may favor the formation of oxidation products at the lateral chain. The different efficiency of the antioxidants assayed to prevent the formation of the 7α-OH, 7β-OH, 7-keto, and 5α-3OH products depends on their individual free radical scavenger capabilities and also on their thermal stability. We have recently demonstrated that there is a good correlation between the thermal stability of synthetic antioxidants and their effectiveness to prevent thermal-induced oxidation of oils (18). Finally, it must be emphasized that the high temperature applied to accelerate the course of oil oxidation and COP formation, most closely resemble those of deep-fat frying operations, but with longer periods of heating (minutes for frying vs. hours for our model). This may mean that the formation of toxic products such as 20-OH or 25-OH produced in our model would probably not be seen in typical food processing applications.

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