Research Article

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Antioxidant addition improves cholesterol and astaxanthin stability in dry salted shrimp

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Abstract

BACKGROUND: Traditional production of dry salted shrimp enhances cholesterol oxidation and astaxanthin degradation in the product. The aim of this study was to evaluate the effect of addition of the antioxidants butylhydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) to cooked shrimp on the formation of cholesterol oxidation products (COPs) and astaxanthin degradation during solar drying of shrimp.

RESULTS: The added antioxidants significantly inhibited COPs formation after the product was boiled in brine. Smaller amounts of COPs were formed in antioxidant-treated shrimps (∼−23%) as compared to untreated samples. The antioxidants continued to significantly inhibit COPs formation (∼−39%) during sun drying. Similarly, TBHQ and BHT reduced by 51.3% and 37.2%, respectively, the degradation rate of astaxanthin, favoring a higher retention of this carotenoid in the final product.

CONCLUSION: The use of the antioxidants BHT and TBHQ in the preparation of dry salted shrimp significantly inhibited the formation of COPs after cooking raw shrimp and during direct solar drying. They also protected astaxanthin contained in the cooked shrimp from photodegradation. These results are technologically relevant because it is possible to prepare a product with a higher content of astaxanthin and lower the presence of hazardous COPs.

Supporting information may be found in the online version of this article.

Keywords: dry salted shrimp; cholesterol oxidation; astaxanthin degradation; antioxidants; butylhydroxytoluene; *tert*-butylhydroquinone

INTRODUCTION

Cholesterol is a steroid of great importance for the development of animal organisms since it is a fundamental constituent of cell membranes. This sterol is susceptible to oxidation, producing compounds that have proven to be harmful for human health, and have been denominated cholesterol oxidation products (COPs), or oxysterols. In recent decades, COPs have attracted attention because of their adverse effects on wellbeing, including carcinogenic, mutagenic and cytotoxic activities.[1] In addition, it has been reported that they are involved in onset and progression of several chronic degenerative conditions, such as atherosclerosis, neurodegenerative disorders, diabetes, renal failure and inflammatory bowel diseases.[1] An important part of the COPs present in human plasma originates from exogenous sources such as foods.[2-4] COPs can be absorbed into the intestinal tract by a mechanism similar to that of cholesterol. These COPs can therefore enter the bloodstream along with cholesterol from the diet and bile, and thus form part of the chylomicrons structure.[3] The main sources of COPs are foods rich in cholesterol that are subjected to different treatments that favor their oxidation. It is therefore very important to prevent their formation during food processing and storage. Although it is known that heat, light, radiation, oxygen, moisture and pro-oxidant agents are factors that favor the production of COPs in food, the typical processing conditions make it very difficult to prevent their occurrence.[2,4,5]

The presence of native antioxidant compounds (such as tocopherols) or added ones (such as butylhydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)) have shown significant inhibitory activity both in model systems as well in some foods.[6,7] To further contrast cholesterol oxidation during food storage, suitable packaging material (that transmits light with wavelengths between 490 and 589 nm, and/or with aluminum foil), packaging conditions (protective atmosphere or vacuum) and low-

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ultraviolet (low-UV) lighting conditions (emission > 390 nm) have also proved helpful.[8,9]

Oxidation of cholesterol in foods can be initiated at high temperatures, by direct exposure to UV light or through a type I photo-oxidation reaction, giving rise to hydroperoxides (primary oxidation products).[10] Singlet oxygen ($^1O_2$) formed from triplet oxygen via a type II photo-oxidation reaction can also generate hydroperoxides. These primary oxidation products can follow either a monomolecular oxidation pathway that produces 7-hydroxy derivatives (7α-OH and 7β-OH) and 7-ketocholesterol (7-KC), or a biomolecular oxidation pathway that generates 5,6-epoxy derivatives (α-CE and β-CE) and cholestanetriol (CT).[11,12] On the other hand, tertiary hydrocarbons (positions 20 and 25) present in the side chain are also prone to oxidation, yielding hydroperoxides and subsequently the corresponding hydroxy derivatives.[13]

In particular, shrimp is a product from fisheries and aquaculture with a high commercial value worldwide and is characterized by a high cholesterol content that varies from 930 to 1540 mg kg^{-1}.[14] The worldwide trade of this crustacean is mainly done either fresh or frozen. Nevertheless, some products such as dry salted shrimp are manufactured in different countries (such as Mexico and Brazil) for use in the preparation of traditional dishes. The preparation of dry salted shrimp involves the following steps: (i) boiling in brine; (ii) drying by direct exposure to the sun for 3–5 days; and (iii) packaging in plastic bags.[15] It has been shown that during this process large amounts of COPs are formed mainly during solar drying and storage. In this sense, the content of COPs can reach concentrations of up to 372 ± 16.3 mg kg^{-1} lipids after 32 h of solar drying and 886 ± 97.9 mg kg^{-1} lipids after 90 days of storage.[15] In addition, a significant decrease (75%) in astaxanthin content has been reported in the dry salted shrimp after a 32 h solar drying period.[16] The above is important if it is taken into account that astaxanthin is a carotenoid with high antioxidant activity (both *in vitro* and *in vivo*) related to several beneficial effects in human health.[17] Thus, its degradation during solar drying not only represents the elimination of a barrier that restricts lipid oxidation in salted dried shrimp, but also limits the health benefits that this functional compound would provide to the consumer. Carotenoids degrade through free-radical-mediated autoxidation reactions, so their chemical characteristics and the surrounding environmental conditions will promote or inhibit their degradation.[18]

Given that cholesterol oxidation also proceeds via a free radical mechanism, antioxidants that are frequently employed to inhibit this type of oxidation could also prevent or delay astaxanthin degradation.[7,18] In this sense, it has been reported that the use of antioxidants such as TBHQ inhibit the photodegradation of β-carotene in diluted emulsions exposed to light.[18] Hence the use of antioxidants could effectively limit astaxanthin degradation as well and reduce the formation of COPs in shrimps during cooking and solar drying. In addition, to the best of our knowledge, there are no scientific reports that deal with strategies to limit COPs formation during processing of this food product; moreover, as mentioned, the use of such antioxidants may also help in preserving astaxanthin, which is of utmost importance because of its effects on consumer health and on product color, one of the main quality parameters of dry salted shrimp. Therefore, the aim of the present study was to evaluate the effect of the addition of BHT and TBHQ to cooked shrimp on the formation of COPs and astaxanthin degradation during solar drying.

MATERIALS AND METHODS

Materials

Fresh white shrimps (*Litopenaeus vannamei*) were obtained from a local seafood market in Veracruz (Mexico). The average weight of individual shrimp was 25.9 ± 2.5 g (35–43 shrimps kg^{-1}).

Shrimp processing and sampling

The experimental design is summarized in Fig. 1.

Boiling in brine

Five kilograms of raw shrimps were used for the present study. Raw shrimps were graded, washed with tap water and divided into three groups prior to cooking (in triplicate). Before preparation, shrimps were kept at 2 °C in crushed ice. Raw whole shrimps were boiled for 15 min in a brine that contained 125 g kg^{-1} salt. The shrimp-to-brine ratio was 1:4 on weight basis. After boiling, shrimp immersed in the brine were cooled to 70 °C and the corresponding antioxidant solution (50 mg mL^{-1} BHT or 12.5 mg mL^{-1} TBHQ in ethanol) was added; the antioxidant/fresh product ratio considered was 4 g kg^{-1} for BHT and 1 g kg^{-1} for TBHQ. These antioxidant/product ratios were established considering a preliminary study, where the differences between BHT and TBHQ in relation to the degree of solubility in water, boiling temperature and susceptibility to degradation during heating were considered. In this way, it was found that the BHT/product ratio of 4 g kg^{-1} and TBHQ/product ratio of 1 g kg^{-1} resulted in an antioxidant concentration in the product within the limit suggested by the Codex Alimentarius for products similar to dried salted shrimp.[19]

Finally, the mixture of shrimps, brine and antioxidants were manually stirred for 10 min and then the shrimps were removed from the brine. Subsequently, the cooked shrimps were allowed to reach room temperature. A control treatment was included in the experimental design in which the shrimps were boiled in brine under the same conditions and, after being cooked, were added with a similar volume of ethanol without antioxidants.

Sun drying

After being placed over a plastic mesh, cooked shrimps were sun-dried for 8 h d^{-1}, for a total of 4 days. To guarantee an even sun drying on both sides of the shrimps, they were flipped every 2 h. Solar drying of shrimps was carried out from 9:00 to 17:00 h (UTC − 5) during the month of May. After every 8 h of sun exposure, the shrimps were stored under dark conditions at 21 °C in plastic containers with tight-fitting lids until the next day, to start a new cycle of 8 h of direct solar drying.

Sampling

Samples were taken during processing as follows: (i) before boiling; (ii) after boiling in brine (boiled shrimp); and (iii) every 8 h during sun drying. Each sample consisted of four randomly selected shrimps taken from each of the stages described above. Each sample was minced with a blade-type blender and maintained at −20 °C until analysis.

Reagents and chemicals

Solvents of analytical grade were supplied by Teqsiquim (Mexico City, Mexico). Astaxanthin standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Most COP standards were supplied by Sigma-Aldrich (Mexico City, Mexico), except for 7α-hydroxycholesterol (7α-HC) and 19-hydroxycholesterol (internal standard for COP quantification), which were purchased from Steraloids (Newport, CT, USA).

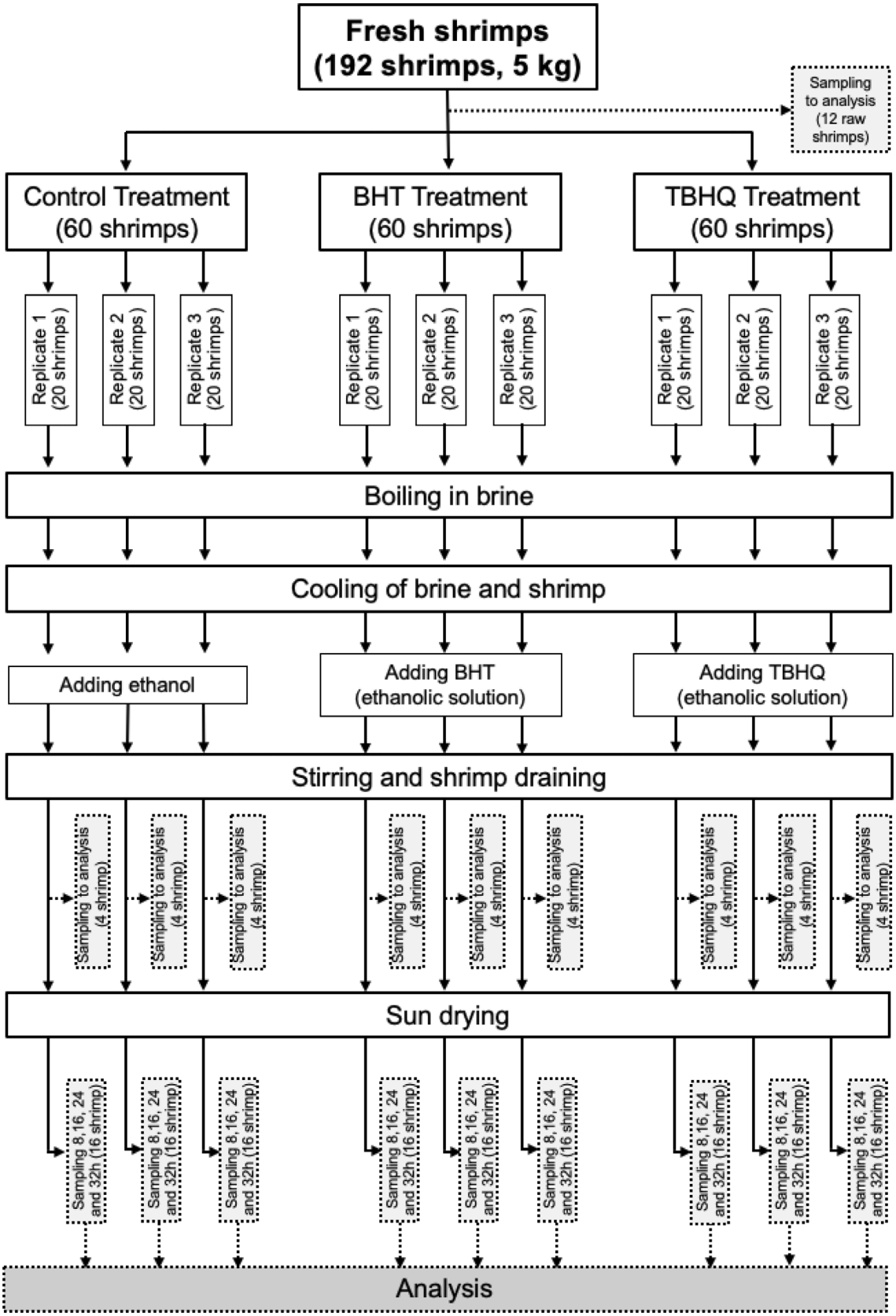

Figure 1. Sampling design of the study.

NH_2 solid-phase extraction (SPE) cartridges (500 mg stationary phase/3 mL) were supplied by Alltech (Mountain View, CA, USA). The silylation mixture (dried pyridine–hexamethyldisilazane–trimethylchlorosilane, 5:2:1 by volume) was prepared with Sigma-Aldrich reagents. BHT and TBHQ were also purchased from Sigma-Aldrich.

Methods

Moisture analysis

Moisture content was determined using AOAC method 950.46.[20]

Lipid extraction

Lipid extraction was carried out according to Boselli *et al.*,[21] using 16 g (dry basis, DB) of the ground shrimp mix. The fat content was gravimetrically determined. The lipid extract was stored at −20 °C in *n*-hexane–isopropanol (3:2, v/v) until analysis.

Analysis of cholesterol and its oxidation products

Cholesterol and its oxidation products were extracted and purified according to Soto-Rodríguez *et al.*[22] About 250 mg of the lipid extract was mixed with 200 μL of a solution of 5α-cholestane (5.0 mg mL^{-1} in *n*-hexane) and 25 μL of a solution of 19-hydroxycholesterol (0.5 mg mL^{-1} in *n*-hexane–isopropanol, 3:2, v/v), which were used as internal standards for the quantification of cholesterol and COPs, respectively. After drying under nitrogen flow, the sample was then subjected to cold saponification for 18 h under shaking in the dark.[22] The unsaponifiable matter was extracted, taken to dryness, and dissolved in 1 mL *n*-hexane–isopropanol (3:2, v/v). One-tenth of the unsaponifiable fraction was used for cholesterol quantification, while the residual (9/10) was subjected to NH_2 SPE for COPs purification.[23] Before analysis, both cholesterol and COPs fractions were silylated at 40 °C for 15 min, dried under nitrogen flow, and dissolved in 100 μL and 20 μL *n*-hexane, respectively.[24] One microliter of the silylated samples was injected into a gas chromatograph coupled to a flame ionization detector and analyzed under the same chromatographic conditions reported by Hernández-Becerra *et al.*[16] Peak identification of cholesterol and COPs was performed by comparing the peak retention times with those of the corresponding standards and by spiking the samples with a small amount of cholesterol and a COPs standard mixture, respectively. Quantification of cholesterol and COPs from gas chromatographic data was carried out with the internal standard method, using the corresponding internal standards (5α-cholestane and 19-hydroxycholesterol) and calculating the relative response factors.

Astaxanthin analysis

Astaxanthin content was determined as reported by Tolasa *et al.*[25] About 10 g of sample was extracted three times with 40 mL BHT cool solution (0.5 g L^{-1}) in acetone for 2 min and centrifuged (4000 × *g* at 4 °C for 5 min). The collected extracts were mixed with 40 mL *n*-hexane and 100 mL aqueous NaCl solution (5.0 g L^{-1}), shaken manually, and left standing until phase separation. The upper layer was collected and made up to 50 mL with *n*-hexane. The absorption spectrum of the *n*-hexane-soluble compounds was recorded at 472 nm using a photodiode array spectrophotometer (model 8453, Agilent Technologies Inc., Waldbronn, Germany). A standard curve of astaxanthin was prepared using solutions with a concentration range from 1.0 to 6.0 μg mL^{-1} of astaxanthin. The content of astaxanthin was calculated from the regression equation of the standard curve ($y = 0.2066x + 0.0271$; $r^2 = 0.9995$).

Analysis of BHT and TBHQ

BHT and TBHQ analysis was carried out according to Saad *et al.*[26] About 10 g of sample was homogenized with acetonitrile–methanol (1:1, v/v) using an Ultra-Turrax IKA T25 digital homogenizer (Ika-Werke, Staufen, Germany). The homogenate was placed in an ultrasonicator bath for 15 min, vortexed, and centrifuged at 1200 × *g* for 10 min at 4 °C. The upper layer was kept at −20 °C for 2 h, and 10 μL of the clear portion was injected into a Waters high-performance liquid chromatograph (600 solvent delivery system, 717plus autosampler, and a 2487 UV–visible detector; Waters, Milford, MA, USA), which was coupled to a C18 column (250 mm × 4.6 mm, 5 μm particle size; Alltech, Columbia, MD, USA). Acetonitrile (solvent A) and acid–water (1:100, v/v) (solvent B) were used as mobile phase. The elution program started with a flow of 10% A and 90% B (1.0 mL min^{-1}, 10 min); then it passed to gradient mode from 10% to 100% of A in 5 min (1.5 mL min^{-1}) and kept at 100% of A for 25 min. The UV detection of BHT and TBHQ was performed at 280 nm. Identification of the peaks was made by comparing the peak retention times with those of commercial standards. A standard curve (1–300 mg L^{-1}) was prepared for each antioxidant ($y = 3328.5x$; $r^2 = 0.9974$ for BHT and $y = 6199.1x$; $r^2 = 0.9980$ for TBHQ).

Kinetic analysis of astaxanthin degradation

It has been reported that the astaxanthin contained in shrimp is photodegraded during solar drying. The degradation of this carotenoid follows first-order reaction kinetics,[15] represented as

$$\ln(C/C_0) = -kt$$

where C_0 is the initial concentration of astaxathin (mg kg^{-1} shrimp DB); C is the concentration of astaxanthin (mg kg^{-1} shrimp DB) at any time t; k is the rate constant (h^{-1} or d^{-1}); and t is time (hours or days). A plot of $\ln(C/C_0)$ *versus* t was constructed to estimate the value of k by linear regression.

Statistical analysis

Data in Tables 1 and 2, as well as in Figs 2 and 3, are reported as mean values of three replicates and their corresponding standard deviations. One-way analysis of variance and Tukey's honest significant multiple comparison test were used to determine statistical differences between samples ($P < 0.05$). Statistical analysis of the data was performed using the software STATISTICA v. 6.0 (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Main physicochemical changes and astaxanthin content in shrimp after boiling in brine

Shrimp is a very perishable product, so cooking in brine is necessary to reduce the microbial load and thus lower the risk of deterioration caused by the microorganisms through sun drying. During cooking in brine, the product undergoes significant physicochemical modifications. One such change is the so-called cooking loss, which consists of product shrinkage with consequent dripping and modification of the moisture, lipid and protein content. In the present study, the observed cooking loss was 29.2%, which is similar to data reported in our previous study.[16] Table 1 shows a significant decrease in moisture content in the shrimp after cooking, where no significant differences were found between shrimp treated with antioxidants (BHT or TBHQ) and the control. As with cooking loss, the moisture content of cooked shrimp was similar to that reported in our previous studies.[16,27]

An important bioactive compound present in this type of crustacean is astaxanthin, which can be found free, esterified or complexed with proteins.[28] During cooking, part of the astaxanthin located in the shell of this crustacean can be expelled to the cooking medium along with proteins and then form polymeric

Table 1. Effect of antioxidants BHT and TBHQ added to shrimp on the content of moisture (g kg^{-1} WB), astaxanthin (mg kg^{-1} DB), lipids (g kg^{-1} DB), cholesterol (g kg^{-1} lipids), oxidized cholesterol (%), single and total COPs (mg kg^{-1} lipids) after boiling

	Raw	Control	BHT	TBHQ
Moisture	750.4 ± 0.7a	612.8 ± 8.4b	626.4 ± 7.0b	634.3 ± 0.1b
Astaxanthin content	144.4 ± 0.4a	132.1 ± 3.6b	126.2 ± 0.9b	126.7 ± 3.8b
Lipids content	99.9 ± 1.5a	98.4 ± 9.6a	93.8 ± 3.0a	85.7 ± 11a
Cholesterol	73.3 ± 2.7a	67.2 ± 5.3a	71.1 ± 3.8a	70.0 ± 3.2a
Oxidized cholesterol	0.044 ± 0.002c	0.111 ± 0.003a	0.082 ± 0.006b	0.081 ± 0.007b
COPs				
7α-HC	4.9 ± 1.7a	10.1 ± 0.3a	11.5 ± 2.2a	6.7 ± 4.6a
7β-HC	10.2 ± 1.5a	14.7 ± 6.0a	17.2 ± 0.1a	18.4 ± 7.3a
β-CE	7.3 ± 1.1c	31.2 ± 1.8a	13.3 ± 2.5b	10.5 ± 0.9bc
α-CE	1.9 ± 0.3a	2.5 ± 2.5a	3.8 ± 0.8a	4.3 ± 1.6a
CT	ND	4.3 ± 0.4a	2.7 ± 2.7a	6.9 ± 1.2a
25 HC	2.4 ± 0.3a	1.7 ± 0.4a	1.9 ± 0.1a	1.9 ± 0.5a
7-KC	5.3 ± 1.9b	10.2 ± 0.5a	7.5 ± 0.7ab	8.1 ± 1.5ab
Total COPs	32.2 ± 0.8a	74.7 ± 6.1c	57.9 ± 1.9b	56.9 ± 2.6b

Each entry represents the mean ± standard deviation of three replicates. Means in the same row followed by different lower-case letters are significantly different ($P < 0.05$).

Table 2. Effect of antioxidants BHT and TBHQ added to shrimp on the content of moisture (g kg^{-1} WB), astaxanthin (mg kg^{-1} DB) lipids (g kg^{-1} DB), cholesterol (g kg^{-1} lipids), oxidized cholesterol (%), single and COPs (mg kg^{-1} lipids) after 32 h of sun drying

	Control	BHT	TBHQ
Moisture	160.4 ± 0.9a	167.1 ± 9.3a	168.1 ± 0.4a
Astaxanthin content	42.6 ± 1.6b	63.4 ± 7.4a	71.3 ± 1.7a
Lipids content	100.2 ± 13.4a	86.1 ± 1.6a	88.0 ± 0.4a
Cholesterol	65.5 ± 3.6b	72.5 ± 10.9b	72.4 ± 5.4b
Oxidized cholesterol	0.64 ± 0.09a	0.34 ± 0.02b	0.37 ± 0.06b
COPs			
7α-HC	95.6 ± 12.2a	49.1 ± 12.1b	60.7 ± 13.4b
7β-HC	98.0 ± 7.8a	60.9 ± 11.7b	64.3 ± 2.4b
β-CE	99.8 ± 7.6a	68.2 ± 9.5b	66.1 ± 1.4b
α-CE	29.7 ± 1.4a	16.6 ± 1.6b	16.6 ± 1.2b
CT	8.5 ± 1.1a	4.4 ± 0.4b	6.6 ± 1.3ab
25 HC	9.3 ± 2.0a	4.8 ± 0.0b	6.2 ± 1.8ab
7-KC	79.2 ± 8.7a	40.3 ± 7.9b	47.4 ± 8.8b
Total COPs	420.1 ± 40.7b	244.4 ± 43.2a	268.1 ± 30.3a

Each entry represents the mean ± standard deviation of three replicates. Means in the same row followed by different lower-case letters are significantly different ($P < 0.05$).

aggregates or bind to other macromolecules.[29] It has been reported that astaxanthin expelled into the brine during shrimp cooking is influenced by the brine concentration and the cooking time used. This could be related to the denaturation and partial solubilization of the carotenoprotein complex within the brine.[27] In this sense, Niamnuy *et al.*[30] found a significant decrease in the content of myofibrillar, sarcoplasmic and stromal proteins in shrimp boiled in saline, indicating that this type of protein could be dissolved in the saline solution. This explains the significant decrease in astaxanthin content observed between raw and cooked shrimp (Table 1). However, no significant differences were observed in the content of this carotenoid between cooked shrimp treated with the antioxidants and the control. The above indicates that the loss of astaxanthin in the raw product mainly occurred during cooking, prior to the incorporation of the antioxidants. Therefore, treatments that prevent astaxanthin being expelled into the cooking medium should be investigated in future research. The incorporation of antioxidants into the product before cooking should also be tested to increase astaxanthin retention and reduce its degradation during cooking.

Cholesterol oxidation in shrimp after boiling in brine

On the other hand, there were no significant changes in total lipid and cholesterol content attributed to shrimp cooking or the use of antioxidants in the product (Table 1). However, there was a significant increase in the total content of COPs in the boiled shrimp caused by the cooking of these crustaceans (Table 1). It was observed that the total content of COPs increased from 32.2

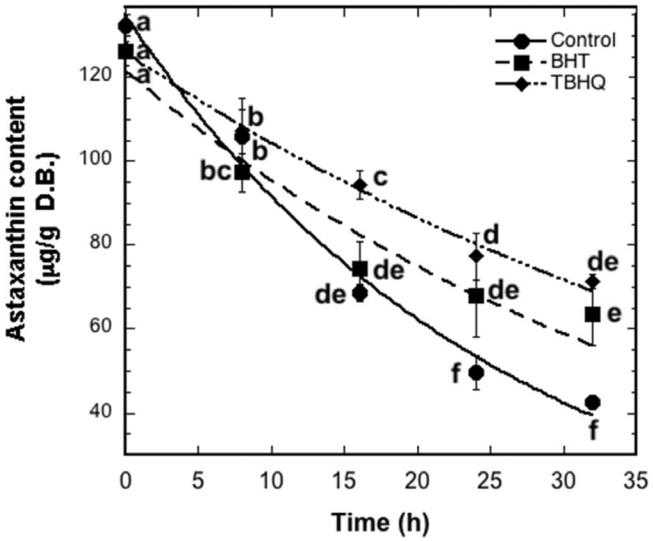

Figure 2. Kinetics of astaxanthin degradation in boiled shrimp during sun drying. Key: control, BHT, TBHQ, best fit. Entries represent the mean values ± standard deviation of three replicates. Means with different lower-case letters (a–f) are significantly different ($P < 0.05$).

± 0.8 mg kg^{-1} lipids in raw shrimp to 74.7 ± 6.1, 57.9 ± 1.9 and 56.9 ± 2.6 mg kg^{-1} lipids in the control, BHT and TBHQ treatments, respectively (Table 1). Similarly, a higher proportion of oxidized cholesterol (as percentage of total cholesterol) was observed in cooked shrimp compared to the raw product (Table 1). It is known that the thermal treatments carried out on cholesterol-rich products, as well as other related factors, favor the oxidation of this sterol, causing the appearance of different COPs. Souza *et al.*[31] reported that shrimp salting carried out by cooking into brine significantly increased the total content of COPs in this type of food product. In this regard, it is important to highlight the pro-oxidant effect of NaCl on lipid fractions in marine products. The above is proposed by Osinchak *et al.*,[32] who identified the chloride ion as the active component

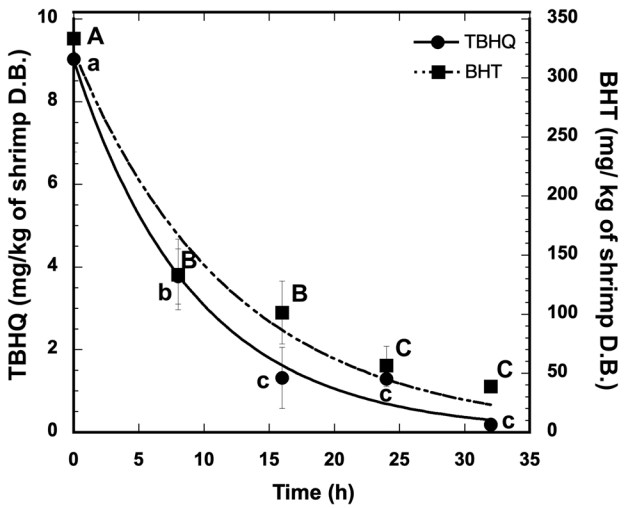

Figure 3. Kinetics of BHT and TBHQ degradation in boiled shrimp during sun drying. Abbreviations: TBHQ, BHT, best fit. Entries represent the mean values ± standard deviation of three replicates. Means with different lower-case letters (a–c) or different upper-case letters (A–C) are significantly different ($P < 0.05$).

responsible for the promotion of lipid oxidation in model systems and marine products.

The total amount of COPs present in shrimps prior to their direct solar drying derives from cholesterol oxidation in two previous stages. The first stage is when raw shrimps are immersed in the boiling brine and kept there for the defined cooking time. The second occurs when the product is removed from the brine, drained and kept on a table for the time needed to reach room temperature. This is evident when the addition of antioxidants (TBHQ and BHT) to the product, after being cooked in brine, inhibited the formation of COPs in this second stage, reaching a total content of COPs that was significantly lower than the control shrimp (Table 1). Based on the total content of COPs formed in shrimp not treated with antioxidants after their cooking, it was observed that the use of BHT and TBHQ inhibited the formation of COPs in the product by 22.4% and 23.8%, respectively. The cholesterol oxidation ratio (%) was also significantly lower in the samples treated with TBHQ and BHT compared to the control samples (Table 1). The values of oxidized cholesterol (as %) found in the present study were lower than those reported by Souza *et al.*[31] However, in a different study conducted by Sampaio *et al.*,[33] it was reported that the percentage of oxidized cholesterol in dried salted shrimp samples processed in different periods of the year was variable (1.31×10^{-8} to 3.35%). The researchers argued that the variability observed was also attributed to the different manufacture variables (brine concentration, cooking time and drying conditions). The above can also explain the difference in the percentage of oxidized cholesterol observed in this study compared to that found in previous reports.

Antioxidants can scavenge active forms of oxygen involved in the initiation step of lipid oxidation or break the oxidative chain of reactions.[7] Synthetic antioxidants such as BHT and TBHQ have the ability to donate a hydrogen atom to free radicals, forming stable low-energy radicals and thereby inhibiting the propagation of the oxidation reactions.[34] In this respect, the efficiency of this type of antioxidant to inhibit COPs formation in foods has been evaluated. Shozen *et al.*[35] found that BHA was very efficient in reducing the formation of COPs in anchovies during brine cooking and subsequent drying. Valenzuela *et al.*[36] reported that TBHQ and BHT were also highly effective in inhibiting the thermo-oxidation of cholesterol dispersed in soybean oil, which had been heated at 150 °C for 22 min.

Table 1 shows that raw shrimp contained small amounts of the following COPs: 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5β,6β-epoxycholesterol (β-CE), 5α,6α-epoxycholesterol (α-CE), 25-hydroxycholesterol (25-HC) and 7-KC. The presence of COPs in raw shrimp may be caused by autoxidation, photo-oxidation and/or enzymatic oxidation prior to cooking. A similar finding was described by our research group in previous studies performed on salted dry shrimp.[16,27] Similarly, the presence of different COPs in other raw marine products has been reported.[35] Brine cooking of shrimp caused a significant increase in the concentrations of β-CE, CT and 7-KC (Table 1). In fact, a fourfold increase in β-CE content was observed after shrimp cooking. However, the use of TBHQ or BHT maintained the concentration of this COP at similar levels to those observed in the raw product (Table 1). It has been established that both β-CE and α-CE are produced by epoxidation of cholesterol, which occurs by a bimolecular reaction between hydroperoxyl radicals and cholesterol.[11] Considering the above, BHT and TBHQ, capable of scavenging free radicals, very likely reduced the presence of hydroperoxyl radicals in the environment, resulting in inhibition of the formation of this type of COP.

In the case of 7-KC, it was found that it increased from 5.3 ± 1.9 to 10.2 ± 0.5 mg kg^{-1} of lipids for the control treatment, observing only a slight but not significant inhibitory effect of the added antioxidants on the presence of this COP (Table 1).

Astaxanthin changes during sun drying

During direct solar drying of cooked shrimps, the latter underwent a series of relevant physicochemical changes. One of these changes was the decrease in their moisture content derived from water evaporation. After 32 h of direct exposure to the sun, the product reached a moisture content of 160.4 g kg^{-1} (wet basis, WB), and there were no significant differences between the shrimps treated with the antioxidants and the control (Table 2).

Regarding the lipid and cholesterol content of the samples, these components did not change after direct sun exposure (Tables 1 and 2). Similarly, there were no significant differences between the control and the antioxidant-treated products after 32 h of solar drying (Table 2).

One of the most notorious changes detected in cooked shrimp during sun drying was the decrease in their astaxanthin content. In this study, the astaxanthin content in control shrimps decreased from 132.1 ± 3.6 to 42.6 ± 1.6 mg kg^{-1} of product (DB) after 32 h of direct solar drying. This decrease corresponds to a 68% loss, which is close to the 75% reported in our previous study where cooked shrimps were exposed to solar radiation for 40 h.[16] This significant reduction in astaxanthin content is explained by its susceptibility to photobleaching, which has already been reported.[37] However, incorporation of BHT and TBHQ into cooked shrimps inhibited the photodegradation of this carotenoid after 32 h of solar drying. This is evident from the significantly higher concentration of astaxanthin in shrimp treated with antioxidants compared to the control (Table 2).

The protective effect of BHT and TBHQ on astaxanthin photodegradation was observed after 16 h of direct exposure to the sun (Fig. 2). It has been established that degradation of astaxanthin in salted dried shrimp follows first-order kinetics.[16,30] The protective effect of antioxidants on astaxanthin photodegradation was also reflected in the rate constant (k) of this reaction. Table 3 depicts the main kinetic parameters estimated for astaxanthin photodegradation reaction in the different treatments. It must be noted that the k-values for the shrimps treated with BHT and TBHQ were smaller than for the control samples. The use of TBHQ in shrimp halved the degradation constant of astaxanthin during solar drying compared with the control shrimps.

Astaxanthin is the main carotenoid present in shrimp and responsible for their red-orange tonality after cooking. Considering that color is one of the main attributes in the quality assessment of dry-salted shrimp, retaining such color shade is important. The protective effect of antioxidants on astaxanthin in shrimp was also reflected by a greater intensity of red color in the treated samples compared to the control shrimps (Supporting Information, Fig. S1). The above aspect and results are of great importance to manufacturers of dry shrimp.

It has been established that carotenoids such as astaxanthin play a protective role in cells against the harmful effects of light, singlet oxygen ($^1O_2$) and photosensitizing pigments (Sens). Thus, these carotenoids generally act as antioxidants in processes of photo-oxidation of proteins, lipids and vitamins.[17] $^1O_2$ plays a major role in photo-oxidation processes and is generally formed by the reaction between a sensitizing compound in triplet state (^{3}Sens*) and the oxygen in the ground state (O_2). Carotenoids can accept the energy from $^1O_2$ and thus reduce the oxygen back to its ground state. Instead, the carotenoid with the received energy from $^1O_2$ reaches a triplet state (^{3}Car*); the latter is capable of releasing the acquired energy to the environment and so return to its original state.[38] This form can also undergo autoxidation, forming its corresponding epoxide and decreasing its concentration in the medium.[39] Carotenoids derived from their photodegradation may also produce other compounds, such as apocarotenoids.[38]

Sensitizing compounds (Sens) are key players in $^1O_2$ formation, which can absorb UV–visible light and transform them into their excited singlet state (^{1}Sens*). Subsequently, by an intersystem crossing mechanism,^{1}Sens* changes to its excited triplet state (^{3}Sens*). Sensitizing compounds can be found endogenously in different biological systems; examples of these are porphyrins, chlorophyll, bilirubin and riboflavin.[38] The presence of riboflavin in shrimp has been reported to vary from 0.32 to 0.36 mg kg^{-1} of crude product and from 0.18 to 0.26 mg kg^{-1} of cooked product.[40] Considering the above, the presence of this sensitizer in shrimp could favor the formation of $^1O_2$ during solar drying and thus promote photo-oxidation of different compounds, such as astaxanthin. Polyphenolic synthetic antioxidants, such as BHT, have been reported to be efficient $^1O_2$ scavengers, because they are an easy target for this type of reactive oxygen species.[41] This would explain the inhibitory effect of BHT and TBHQ on the degradation of astaxanthin in cooked shrimp during solar drying (Table 2 and Fig. 2).

COPs formation during sun drying

Cholesterol is also susceptible to oxidation during direct solar drying of cooked shrimp. In the present study, the content of total COPs found in cooked control shrimp changed from 74.7 ± 6.1 to 420.1 ± 40.7 mg kg^{-1} lipids after 32 h of solar drying (Tables 1 and 2). Similarly, the percentage of oxidized cholesterol increased from 0.1% to 0.6% in the control sample; such values are close to those reported by Souza *et al.*[31] for two batches of dried salted shrimp (0.882% and 0.833%). This small difference could be attributed to the different cooking conditions used in both studies; in fact, Souza *et al.*[31] boiled the shrimps for 10 min in 300 g kg^{-1} brine, being thus cooked in 30% less time but with a more concentrated brine (× 2.4) than that in the present work. COPs with the highest increase observed were 7α-HC, 7β-HC and β-CE, followed by 7-KC and α-CE. On the other hand, 25-HC and CT were present at the lowest concentrations (Table 2). This is consistent with our previous study on salted dry shrimp.[16] However, after 32 h of direct solar drying, a significantly lower COPs content was measured in shrimp treated with BHT and TBHQ, compared to the control (Table 2). Furthermore, no significant differences were observed between products treated with the antioxidants. The estimated reduction in the COPs content caused by antioxidant incorporation was 39%. Similarly, Shozen *et al.*[35] evaluated the effect of BHA addition (66 g kg^{-1} of product) on the formation of COPs in cooked anchovies subjected to drying and found a significant reduction of 37.9% in the content of the main COPs. Despite the different experimental conditions employed, Valenzuela *et al.*[36] found that both BHT and TBHQ were able to inhibit COPs formation in cholesterol that had been dispersed in soybean oil and subjected to thermally induced oxidation (150 °C for 22 min). Table 2 shows the significant inhibition caused by BHT and TBHQ on the formation of most COPs (7α-HC, 7β-HC, β-CE, 7-KC and α-CE). However, no significant differences were found in the single COPs concentrations in samples treated with BHT compared to those treated with TBHQ.

Table 3. First-order kinetics of astaxanthin degradation in boiled shrimp during sun drying

	C_0 (mg kg^{-1} DB)	k (h^{-1})	R^2
Control	134.5	0.0384	0.99
BHT	121.6	0.0241	0.95
TBHQ	125.9	0.0187	0.99

Different COPs formed during food processing can be produced through several pathways. Some routes proceed via free radical mechanisms, where factors such as elevated temperatures, UV light, the presence of pro-oxidant agents and triplet oxygen can trigger the auto-oxidation of cholesterol.[11] Another pathway involves photosensitized oxidation of cholesterol, which requires $^1O_2$ as promoting factor; the latter is produced when sensitizing compounds are exposed to light and interact with triplet oxygen.[11] During sun drying of cooked shrimp, the prevailing conditions favor the oxidation of cholesterol by free radical mechanisms or through the action of $^1O_2$. In both cases, antioxidants can remove free radicals, as well as scavenge $^1O_2$, thus inhibiting cholesterol oxidation. An important aspect to consider during the use of antioxidants as food additives is their legal regulation and recommended limits of use. The Food and Drug Administration (FDA) allows the use of both BHT and TBHQ as additives for direct addition to food. In the case of emulsion stabilizers for shortenings, the content of BHT must not exceed 200 mg kg^{-1}.[42] For TBHQ, the content of this antioxidant should not exceed 0.2 g kg^{-1} of the fat or oil content of the food.[43] The overall standard for food additives (CODEX STAN 192-1995) of the Codex Alimentarius suggests that the maximum level allowed for BHT in dried and salted crustaceans is 200 mg kg^{-1}.[19] In the case of TBHQ, the Codex does not indicate the maximum level allowed for this type of product. However, it is specified that for processed meat the maximum allowed level is 100 mg kg^{-1}.[19] In this study, the concentration of BHT and TBHQ reached after application to cooked shrimp, was 333.3 ± 3.7 and 9.03 ± 2.2 mg kg^{-1} DB, respectively. As can be noted, the concentration of TBHQ in shrimp is lower than the maximum recommended level, but this is not the case for BHT; the higher accumulation of the latter could be attributed to its higher steric hindrance and thus its lower antioxidant activity as compared to TBHQ.[44] However, it can be observed in Fig. 3 that the presence of both antioxidants in the product decreased significantly during direct solar drying. In fact, after 8 h of solar drying, BHT concentration in the product reached ~40 mg kg^{-1}, a value below the maximum recommended level. BHT and TBHQ decrease in the product during solar drying is consistent with data reported by Ohshima *et al.*,[45] who analyzed the antioxidant potential of BHA, BHT, TBHQ and tocopherols in horse mackerel (*Trachurus japonicus*) during its fermentation, drying and storage. Like astaxanthin, degradation of BHT and TBHQ in shrimp during solar drying followed first-order kinetics. In the case of BHT, the estimated degradation constant (k) was 8.23 × 10^{-2} h^{-1} (R^2 = 0.98), whereas for the TBHQ k was 10.74 × 10^{-2} h^{-1} (R^2 = 0.97). The different degradation rates of BHT and TBHQ during the sun drying of shrimp could be caused by a greater participation of TBHQ in the processes of: (i) quenching free radicals generated during the initiation stage of oxidation in the unsaturated lipids and cholesterol; and (ii) quenching $^1O_2$ formed during photooxidation promoted by photosensitizers such as riboflavin. Polyphenolic antioxidants are known to react with peroxyl radicals, forming stable resonant radicals and different adducts.[40] On the other hand, it has been reported that TBHQ, BHA and BHT are capable of quenching (at different rates) $^1O_2$ formed through photosensitizers present in the environment.[46,47] All of the above could explain the decrease in the concentration of the antioxidants in the surrounding environment at different rates.

Degradation of the antioxidants evaluated in this study acquires toxicological relevance in the case of BHT. This is because different reports relate this antioxidant and its metabolites with different toxicological effects observed in animals.[48] In recent decades, many toxicological studies have been conducted to determine the safety of this compound. In this regard, the reported data are controversial, indicating either beneficial effects, harmful, or no effects.[46] TBHQ has also been studied toxicologically to establish its safe use in food. The results in this case indicate that the use of this antioxidant, at the recommended levels, is safe.[49,50]

Although the scientific evidence collected in recent years in relation to the use of synthetic antioxidants is still controversial, there is a current tendency to decrease their use in foods. However, the use of TBHQ during food processing, such as salted dried shrimp, could represent an alternative to reduce the formation of COPs, whose hazardous effects on human health have already been widely proved.

CONCLUSIONS

The use of the antioxidants BHT and TBHQ in the preparation of dry salted shrimp significantly inhibited the formation of COPs (~−23%) after brine-cooking of raw shrimp and during direct solar drying (~−39%). They also protected astaxanthin contained in the cooked shrimp from photodegradation during sun drying, thus reducing the rate of loss of this carotenoid by 51.3% and 37.2% using TBHQ and BHT, respectively. All the obtained data suggest a beneficial effect of the tested antioxidants, especially TBHQ, on the main oxidative processes undergone by cholesterol and astaxanthin. These results are technologically relevant, because the addition of these antioxidants led to the preparation of salted, sun-dried shrimps with a final higher content of astaxanthin and a lower amount of the hazardous COPs as compared to untreated samples.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ABBREVIATIONS

7α-HC	7α-hydroxycholesterol
7β-HC	7β-hydroxycholesterol
α-CE	5α, 6α-epoxycholesterol
β-CE	5β, 6β-epoxycholesterol
7-KC	7-ketocholesterol
20-HC	20-hydroxycholesterol

25-HC	25-hydroxycholesterol
BHT	butylhydroxytoluene
COPs	cholesterol oxidation products
CT	cholestanetriol
TBHQ	tert-butylhydroquinone

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