The Effects of Bleached and Unbleached Rosemary Oleoresins on Light-Sensitized Oxidation of Soybean Oil[1]

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Bleached and unbleached forms of a rosemary oleoresin (RO) in stripped and nonstripped soybean oil behave both as antioxidant and prooxidant in a light-induced oxidative system. At 0.02 and 0.05% levels, RO had the greatest antioxidant activity, while at 0.01 and 0.5% levels it had the highest prooxidant activity in both stripped and nonstripped soybean oil. Treatment of both soybean oil systems with tertiary butylhydroquinone controlled light-induced oxidation of the oil better than did the oleoresin treatments. The prooxidant activity of the 0.5% RO level was probably due to an excess of prooxidant components being carried into the oil at that level, whereas the reduced antioxidant activity at 0.01% was probably due to the low initial level of active antioxidant components being added to the oil.

KEY WORDS: Bleached rosemary oleoresin, light-induced oxidation, nonstripped soybean oil, stripped soybean oil, tertiary butylhydroquinone.

A crude rosemary (*Rosmarinus officinalis* L.) oleoresin (RO) is currently being sold as a flavoring with the added feature that it exhibits antioxidant activity. Gray *et al.* (1) reported that RO reduces warmed-over flavors and reduces lipid oxidation of restructured beef steaks. Bracco *et al.* (2) found that rosemary extract is effective in stabilizing potato flakes against oxidation. Barbut *et al.* (3) demonstrated the antioxidative activity of RO in turkey breakfast sausages. Although RO has proven to be an effective antioxidant in autoxidized systems, no data have been reported on its effects on light-induced oxidation (photooxidation).

Light-induced oxidation of fats has been recognized since the turn of the century, but the mechanism by which this oxidation occurs was not determined until the late 1960s. The mechanism by which photooxidation most often occurs in fats and oils involves the absorption of light energy by a sensitizer (*e.g.*, chlorophyll and pheophytin) causing the formation of an excited sensitizer in the singlet state ($^1S^*$). This sensitizer can either drop back to the ground state or, more importantly, can undergo intersystem crossing to the higher, less stable, vibrational levels of the triplet state sensitizer ($^3S^*$) (4,5). $^3S^*$ can then undergo an intersystem crossing and transfer its energy to the lowest vibrational energy state of oxygen (most stable, triplet—$^3O_2$). This transfer of energy causes the $^3O_2$ to go to a higher vibrational energy state defined as singlet oxygen ($^1O_2$) (4–8). Being more electrophilic than $^3O_2$, $^1O_2$ will attack moieties of high electron density (*e.g.*, C=C), resulting in peroxy radicals and ultimately hydroperoxides. The entire process by which light stimulates the production of hydroperoxides and their decomposition into free radicals and to the initiation of autocatalytic oxidation can be defined as light-induced oxidation (9).

During the expression of oil from soybeans, residual pigments and extraneous matter become part of the oil. Usuki *et al.* (10) found that chlorophyll and pheophytin levels in refined, bleached and deodorized soybean oil (SBO) ranged from 0.3 to 15.3 ppm and from 56.7 to 100.8 ppm, respectively. Both chlorophyll and pheophytin have been found to be sensitizers in SBO, thus acting as prooxidants in photooxidation systems (6,11–13). Prooxidants are compounds that cause an acceleration of oxidation of a substrate, either in autocatalytic or photooxidative systems; therefore, the presence of chlorophyll and pheophytin in an oil can have detrimental effects, especially if the oil is exposed to light (14). On the other hand, antioxidants are compounds that inhibit autocatalytic oxidation by donaton of hydrogen to free radicals (15). Tertiary butylhydroquinone (TBHQ), a phenolic antioxidant, was developed for use in highly polyunsaturated systems where other synthetic antioxidants fail (15). Tocopherols can also act as antioxidants in autocatalytic conditions but, unlike TBHQ, can also act as $^1O_2$ quenchers (15,16). A $^1O_2$ quencher is a compound that reduces the $^1O_2$ into the more stable $^3O_2$ (16). β-Carotene is an active $^1O_2$ quencher in SBO (17).

This study was performed to determine whether a commercial RO and a bleached RO (not commercially available) would inhibit light-induced oxidation in stripped and nonstripped SBO. Since SBO naturally contains both photosensitizers and antioxidants, including $^1O_2$ quenchers, the chlorophyll, pheophytin, β-carotene and α-tocopherol were removed to eliminate their effects on the light-induced oxidation of the oil. Nonstripped SBO was used to determine possible synergism of β-carotene and α-tocopherol in the SBO with the RO.

MATERIALS AND METHODS

Analysis of SBO components. α-Tocopherol, β-carotene and chlorophyll standards were obtained from Sigma Chemical Co. (St. Louis, MO). Tocopherol determination was performed with the high-performance liquid chromatography (HPLC) method of Carpenter (18). The oil samples were prepared by diluting 5 g oil with 1.5% isopropyl alcohol in hexane to a final volume of 100 mL. Tocopherol standards were made up in 1.5% isopropyl alcohol in hexane (1000, 200, 100, 20, 10, 1 and 0.1 ppm) and monitored at 280 nm to establish a standard curve. Tocopherol separation was performed on a 3.9 mm i.d. × 300 mm μPorasil™ column with 10 μm particle diameter (Waters Associates, Millford, MA) by using a mobile phase of 1.5% isopropyl alcohol in HPLC-grade hexane (filtered through 0.45 μm) at a flow rate of 1.0 mL/min. A Waters model 6000A solvent delivery system was used, and sample injections (20 μL) were made with a model 7125 Rheodyne (Cotati, CA) sample injection valve. A Waters model 440 detector, set at 280 nm, and a Spectra Physics 4270A (San Jose, CA) integrator were used for quantitation.

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Chlorophyll and carotene were determined by using modifications of the Association of Official Analytical Chemists' spectrophotometric methods (19,20). The oil samples were prepared by dissolving 5.0 g SBO in 10 mL hexane and then filtering through a column (10 mm i.d. × 300 mm) packed with a mixture of florisil and magnesia (1:1, w/w) with a gradient elution of hexane (100%); hexane/acetone (90:10); acetone (100%) and methanol (100%). The receiving flask was changed prior to elution with hexane/acetone to eliminate excessive oil in the final sample. The receiving flask was changed a second time prior to the elution with acetone (100%) and methanol (100%). The acetone and methanol fractions were combined and then evaporated in nitrogen and reconstituted in 10 mL ethyl ether prior to chlorophyll absorbance measurements. Chlorophyll standards (100, 10, 5, 2, 1, 0.5, 0.2 and 0.01 ppm) were dissolved in ethyl ether, and absorbencies of the samples and standards were measured at 660 and 642.5 nm. The chlorophyll content was determined by using the following equation:

$$\text{total chlorophyll} = 7.12\ A_{660} + 16.8\ A_{642.5} \quad [1]$$

The hexane/acetone (90:10) was evaporated by using nitrogen, and the dried sample was reconstituted in 10 mL hexane/acetone (90:10) prior to carotene absorbance measurements. Carotene standards (100, 10, 5, 2, 1, 0.5, 0.2 and 0.01 ppm) were made up in hexane/acetone (90:10, vol/vol), and the absorbencies of the samples and standards were measured at 436 nm.

Antioxidants. TBHQ was obtained from Eastman Chemical Products Inc. (Kingsport, TN). An RO (Herbolox® Seasoning, type 0) was obtained from Kalsec® Inc. (Kalamazoo, MI). Product literature indicated that the RO contained a mixture of vegetable oil and mono- and diglycerides with chlorophyll substantially removed. The tocopherol, chlorophyll and carotene contents for the RO were determined by the same methods as used in the SBO analysis.

Stripping of SBO. Commercial SBO was purchased from a local supermarket in 64-oz containers. The oil was stored in the dark at −18°C until needed. The oil was stripped by following the method of Kiritsakis and Dugan (21). The oil was filtered, under vacuum, through a mixture of Tonsil Optimum Extra® (L.A. Solomon Co., Port Washington, NY); activated charcoal, Norit SG® (EM Industries, Inc., Cherry Hill, NJ); Hyflo Super Cel® (Fisher Scientific Co., Fairlawn, NJ); and 60–100 mesh Florisil® (Fisher Scientific Co.) blended in a ratio of 1:0.70:0.50:0.35 (w/w), respectively. The stripping (bleaching) material was packed into a 300 mm × 25 mm i.d. glass column plugged with cotton and capped with a 1-cm layer of Infusorial Earth® (Fisher Scientific Co.). The column was conditioned by percolating 150 mL hexane through the packing material. A 150-mL oil sample (1:1.5, vol/vol, oil to hexane) was then added, and the column was washed with 150 mL hexane after the oil sample eluted from the column. The conditioning, eluting and washing of the column were done under nitrogen. The oil was stripped three times, resulting in a visually clear oil. Both the stripped SBO in hexane and nonstripped SBO were stored in a freezer (−18°C) until needed.

Bleaching of RO. The commercially available RO is pigmented and could contain residual chlorophyll that might change the rate of oxidation. A study was conducted with the nonbleached RO in both stripped and nonstripped SBOs. From this, a second study was conducted with a bleached RO to determine the effects of this pigmentation/chlorophyll on the light-induced oxidation of both the stripped and nonstripped SBOs. RO was bleached by the procedure described for the stripping of SBO.

Oxidation of SBOs. Prior to the beginning of each study, the hexane was evaporated under vacuum at 35°C from the stripped SBO. Samples (100 g) of stripped or nonstripped SBO were weighed into 110-mL glass jars, then appropriate amounts of either bleached or unbleached RO (0.01, 0.02, 0.05, 0.1 or 0.5%) or TBHQ (0.02%) were added. Each sample was thoroughly mixed to assure complete dispersion of the antioxidants. Hexane was mixed with nonstripped oil and evaporated to act as a control to determine the effect of hexane on the oxidation of the oil. A total of fourteen treatments, seven for the stripped and seven for the nonstripped oil, not including a hexane treatment, were prepared. The study was replicated three times. The jars were covered with clear plastic wrap and placed randomly under two 15-W cool fluorescent lamps at an illuminance level of 4200 lux at 25 ± 1°C. To create uniform lighting, aluminum foil was placed in the open areas between the sides of the jars and the bottom of the lamps. Peroxide values (PVs) were determined every 12 h during a 60-h light exposure by using the American Oil Chemists' Society Method Cd-8-53 (22).

During sampling, the oil samples were removed sequentially and were returned immediately to the same position under the radiation source after sampling. The entire sampling and peroxide determination was completed in less than 1.5 h. The oil samples were then subjected to an additional 12 h of illumination before PVs were taken again.

Statistical analysis. The data were statistically analyzed by analysis of variance in which the least significant means (23) were used to determine the significance ($P < 0.05$) between the mean values of the treatments in both the stripped and nonstripped SBO test systems.

RESULTS AND DISCUSSION

Two studies were conducted to determine the effects of bleached and unbleached rosemary oleoresin (BRO and URO, respectively) on the light-sensitized oxidation in both stripped and nonstripped SBO. For ease of discussion, however, the results will be presented based upon type of oil.

Stripped SBO contained less than 0.014 ppm chlorophyll, less than 0.03 ppm β-carotene and less than 20 ppm tocopherols (Table 1). The nonstripped SBO had a chlorophyll content of 0.095 ppm, which is comparable to the chlorophyll content in commercially refined SBO reported by Usuki *et al.* (10). Nonstripped SBO had a carotene content of 0.12 ppm, which was much lower than the carotene content of crude, unrefined oil at 28–30 ppm, in which 3% (0.9 ppm) was carotene, as reported by Snyder and Kwon (24). The total tocopherol content of 129 ppm in the nonstripped SBO was lower than the range of 580–1530 ppm reported for refined SBO (24,25).

When stripped SBO was treated with the URO, all treatments had antioxidant activity when compared to

TABLE 1

Composition of Stripped and Nonstripped Soybean Oils (SBOs) and Bleached and Nonbleached Rosemary Oleoresin (RO)

Sample	Carotene (ppm)	Chlorophyll (ppm)	Tocopherol (ppm)
Nonstripped SBO	0.120	0.095	129
Stripped SBO	0.030	0.014	<20
Nonbleached RO	104.000	4.210	390
Bleached RO	0.204	0.670	79

the control (Fig. 1). The TBHQ treatment had PVs that were significantly ($P < 0.05$) lower than those of the URO treatments and the control after 12 h and remained significantly ($P < 0.05$) lower throughout the duration of the study. Within the URO treatments, the 0.02 and 0.05% URO were not significantly ($P > 0.05$) different from each other, and the 0.1 and 0.5% URO treatments were not significantly ($P > 0.05$) different from each other. However, the treatments containing 0.02 and 0.05% URO had PVs that were significantly ($P < 0.05$) lower than the PV of the 0.1 and 0.5% URO treatments. The 0.01% URO treatment was not significantly ($P > 0.05$) different from the 0.02 and 0.05% URO treatments at 36 h. However, after 36 h there as a significant ($P < 0.05$) increase in the PV of the 0.01% URO treatment at 48 and 60 h; this treatment was not significantly ($P > 0.05$) different from the treatments containing 0.1 and 0.5% URO.

There are several possible reasons for the diverse actions of RO in light-induced oxidation of stripped SBO. First, although the URO was, according to product literature, "substantially free" of chlorophyll, our analysis found a chlorophyll content of 4.21 ppm (Table 1). This low level may have been sufficient to cause a slight increase in the rate of oxidation. Although the chlorophyll content of the oleoresin was determined, a lot-to-lot variation was not established, and it may be that the results in this study could vary dependent on the composition of the RO used as treatments. Secondly, because of their emulsifying properties, the presence of mono- and diglycerides in the URO could reduce the surface tension between the lipid and oxygen, allowing for oxidation to occur more readily (D.B. Min, personal communication). Although not quantitated in this study, it is possible that the mono- and diglycerides contained in the RO (product literature) may contribute to the greater oxidation, especially at higher levels of RO. In the case of the 0.01% URO treatment, there may have been a limited supply of active antioxidant compounds; by 36 h, they could have been depleted, and the prooxidants in the oleoresin were then able to function more readily.

When stripped SBO was treated with the bleached BRO and exposed to fluorescent light, the 0.01% BRO treatment had prooxidant activity after 36 h (Fig. 2). The stripped SBO control in this study developed a lower PV over time than did the stripped control in the previous study. The reasons for this difference may be because the stripped SBOs were from different sources and separate

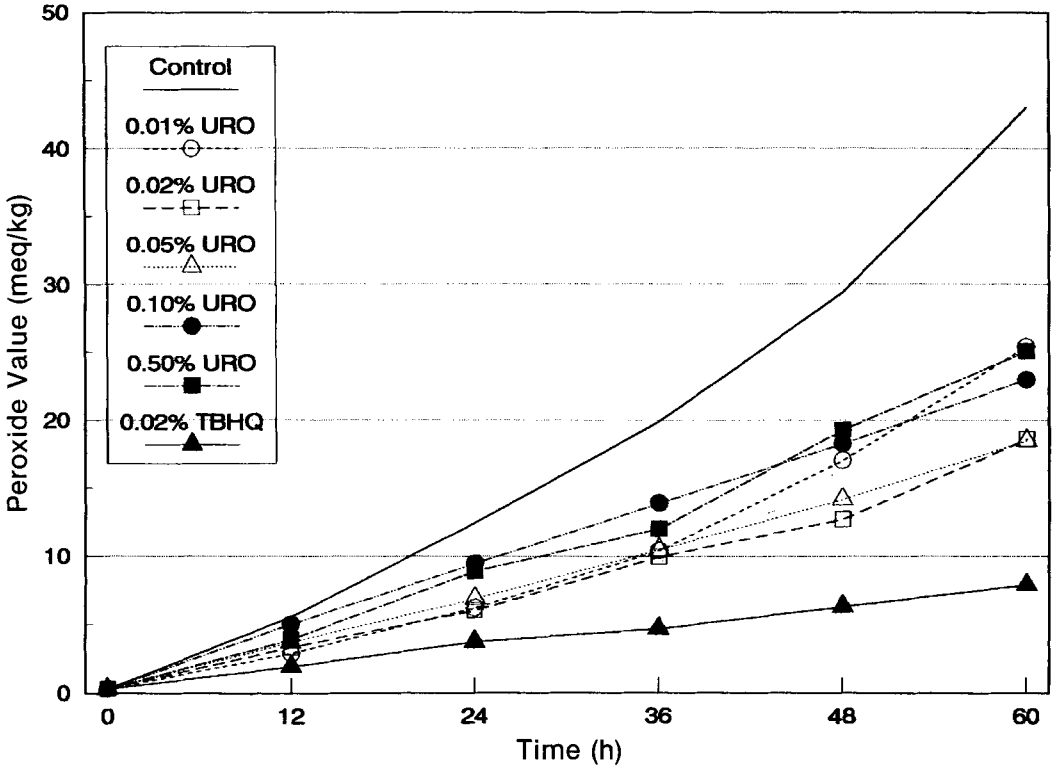

FIG. 1. The effect of an unbleached rosemary oleoresin (URO) and tertiary butylhydroquinone (TBHQ) on light-induced oxidation, as measured by peroxide values, of stripped soybean oil over time (60 h).

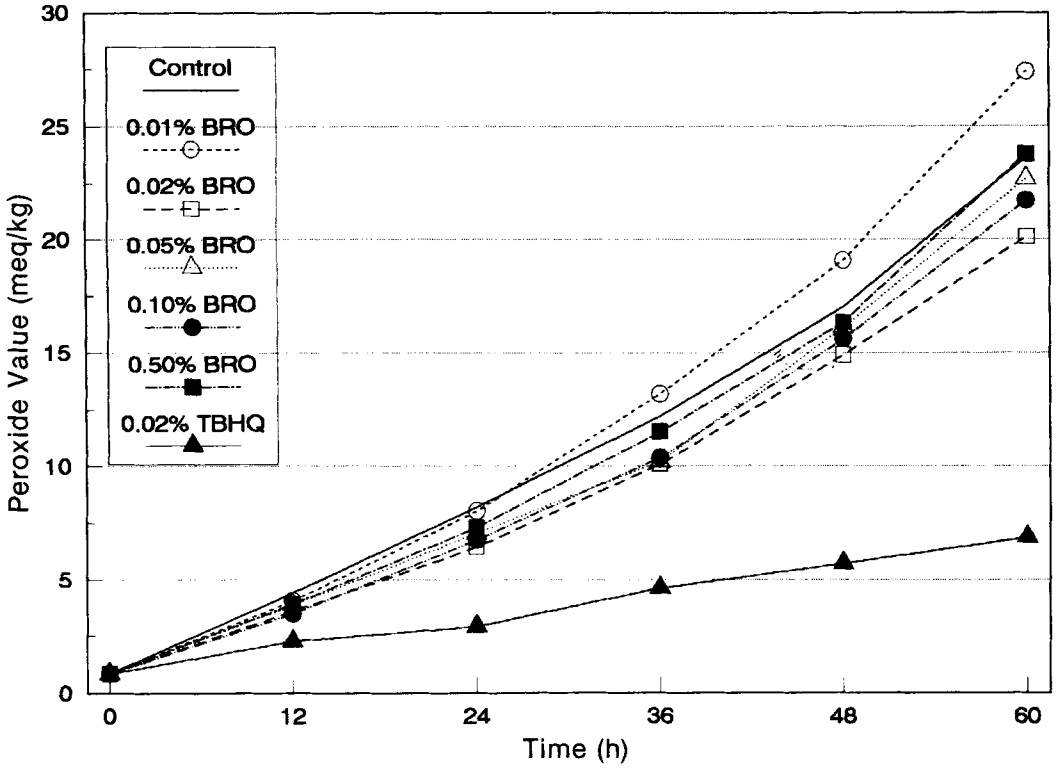

FIG. 2. The effect of a bleached rosemary oleoresin (BRO) and tertiary butylhydroquinone (TBHQ) on light-induced oxidation, as a measure of peroxide value of stripped soybean oil over time (60 h).

strippings. The PVs of the 0.01% BRO treatment were significantly ($P < 0.05$) higher than all other treatments after 36 h throughout the duration of the study. The 0.02, 0.05 and 0.1% BRO treatments had slight to marginal antioxidant activity when compared to the control (Fig. 2). The treatment containing 0.02% BRO had PVs that were significantly ($P < 0.05$) lower than the control, and the 0.5% BRO treatment at 60 h but was not significantly ($P > 0.05$) different from the 0.1% BRO treatment throughout the study. The 0.1 and 0.05% BRO treatments had PV that were not significantly ($P > 0.05$) different from the control and from the 0.5% BRO treatment throughout the study (Fig. 2). The data indicate that until 48 h, all levels of BRO, except 0.01%, had slight to marginal antioxidant activity. At 60 h, the 0.5% BRO treatment had no antioxidant activity whereas the other three treatments maintained antioxidant activity, with 0.02% BRO having the greatest antioxidant activity. The TBHQ treatment had significantly ($P < 0.05$) lower PV than all other treatments after 24 h, and this level increased only slightly throughout the duration of the study (Fig. 2). The TBHQ had the highest antioxidant activity of the treatments tested. An explanation for the prooxidant activity of the BRO in the stripped SBO could be that the stripping of the oil reduced the level of natural antioxidants (*i.e.*, tocopherols, carotenoids) in the SBO, and this, in combination with the reduced levels of active antioxidant components in the BRO (*i.e.*, rosmanol, carnosol, rosmariquinone, *etc.*), may have acted to reduce the BRO effectiveness.

In the nonstripped SBO system treated with the URO, the URO treatments had primarily prooxidant activity that appeared to be concentration-dependent (Fig. 3). The lower levels of URO addition (0.01 and 0.02%) were not significantly ($P > 0.05$) different from each other or the control throughout the duration of the study. The 0.1 and 0.05% BRO treatments were not significantly ($P > 0.05$) different from each other throughout the duration of the study; however, they had significantly ($P < 0.05$) higher PV than did the control and the lower levels of URO. The 0.5% URO had significantly ($P < 0.05$) higher PV than all other treatments throughout the duration of the study. TBHQ had PVs that were significantly ($P < 0.05$) lower than all other tratments after 24 h and for the rest of the study time (Fig. 3). Perhaps, prooxidant constituents (*i.e.*, mono- and diglycerides, and chlorophyll) in the URO were able to function, thus causing an increased oxidation in some of the URO treatments.

When the nonstripped SBO was treated with the BRO and exposed to fluorescent light (Fig. 4), throughout the 60 h of study, the 0.01 and 0.5% BRO treatments and the control were not significantly ($P > 0.05$) different. In contrast, the 0.02, 0.05 and 0.1% BRO treatments were not significantly ($P > 0.05$) different from each other throughout the study but were significantly ($P < 0.05$) lower than the 0.1 and 0.5% BRO and the control after 48 h (Fig. 4). The TBHQ treatment was not significantly ($P > 0.05$) different from any of the other treatments at 24 h. At 36 h, TBHQ had significantly ($P < 0.05$) lower PV than the 0.01 and 0.5% BRO treatments and the

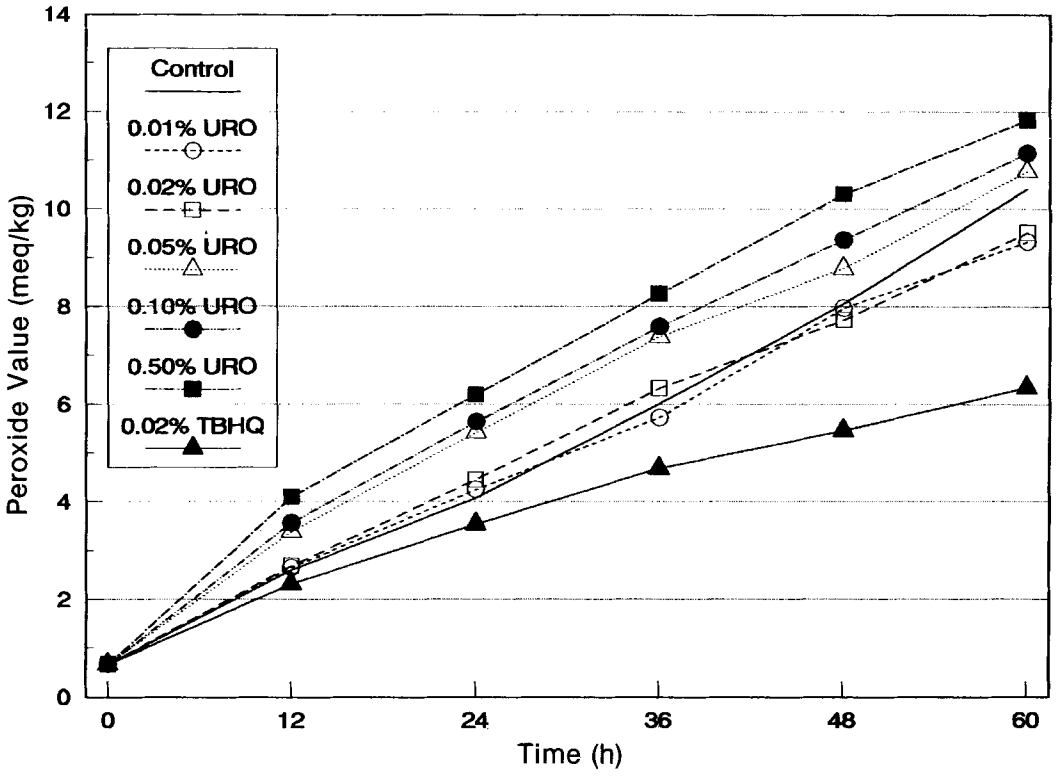

FIG. 3. The effect of an unbleached rosemary oleoresin (URO) and tertiary butylhydroquinone (TBHQ) on light-induced oxidation, as measured by peroxide values, of nonstripped soybean oil over time (60 h).

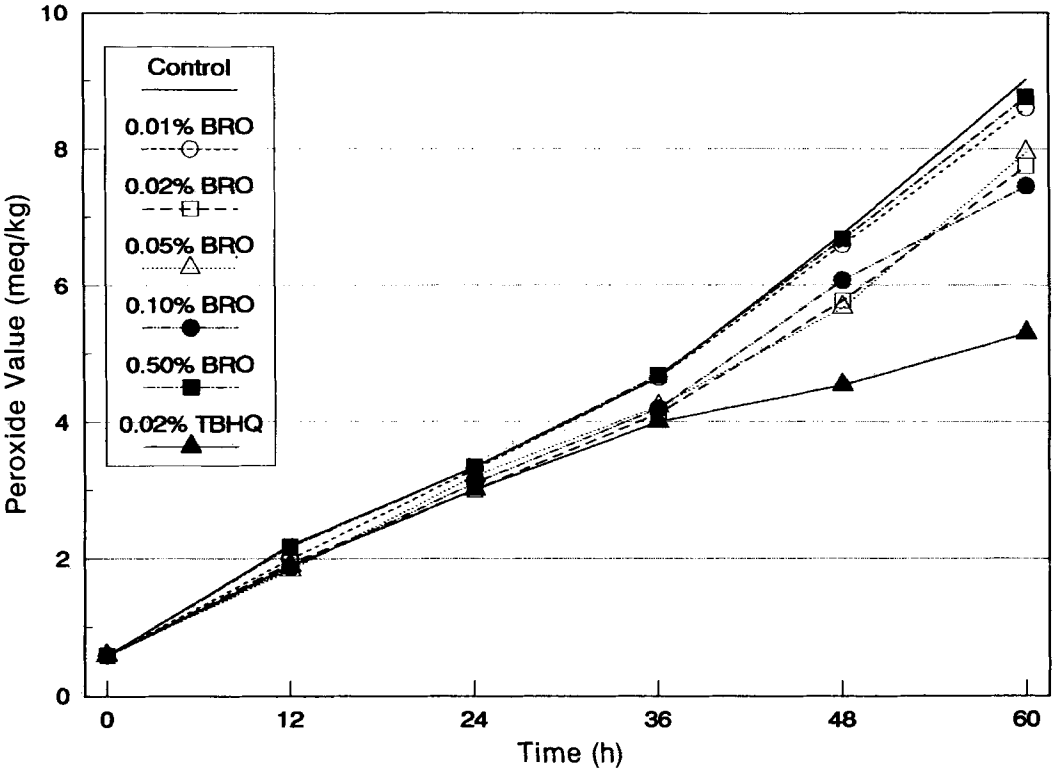

FIG. 4. The effect of a bleached rosemary oleoresin (BRO) and tertiary butylhydroquinone (TBHQ) on light-induced oxidation, as measured by peroxide values, of nonstripped soybean oil over time (60 h).

control but was not significantly ($P > 0.05$) different from the other treatments (Fig. 4). The treatment containing TBHQ had significantly ($P < 0.05$) lower PV than all other treatments after 48 h. Bleaching of the oleoresin may have reduced mono- and diglyceride levels associated with the BRO, which could account for the reduced oxidation of the nonstripped SBO. As before, the lower concentration level of antioxidant (0.01% BRO) may have been the reason for the poor antioxidant activity.

The addition to and the removal of hexane in nonstripped SBO was done to determine whether the presence of residual hexane in the stripped oil had an effect on the oxidation. No effect was found, indicating that residual hexane was not a factor contributing to the oxidation rates.

In both stripped and nonstripped SBO, the TBHQ treatment had significantly ($P < 0.05$) greater antioxidant activity than did BRO and URO. Because TBHQ is an antioxidant that functions by donating hydrogen to the free radical, thus stabilizing the radical species (15), the data indicate that free-radical oxidation occurred during the 60-h test period. Although a light-induced system (photooxidation) was being studied, after initiation and the formation of hydroperoxides, autoxidation can begin to contribute to the rate of oxidation; therefore, differentiation from photooxidation is difficult unless product formation is monitored. Antioxidant activity of RO at the 0.02 and 0.05% levels indicated that the RO may act as a free radical acceptor but not to the same degree as the TBHQ. The $^1O_2$ quenching activity may have occurred in the initial stages of the study, but this is only speculation because no study was completed to determine the exact mechanism.

In general, the nonstripped SBO had lower PV than did the stripped SBO, likely because the stripping of the SBO reduced the tocopherol and β-carotene concentration, both of which are antioxidants. Nonstripped SBO treated with BRO had the least oxidation (lowest PV), when compared to nonstripped SBO treated with URO, throughout the 60-h study. This same pattern was observed in the stripped SBO systems where URO treatment produced, in general, PVs that were slightly greater than those of stripped SBO treated with BRO. This increased oxidation could be due to the higher monoglyceride level that is being carried into the SBO system from the URO.

To determine the true effectiveness of rosemary antioxidants, pure systems must be established and utilized. One such method is to synthesize pure antioxidant components of rosemary and compare these to the oleoresin.

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