Evaluation of Antioxidants for Cod Liver Oil by Chemiluminescence and the Rancimat Method

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ABSTRACT: Commercial blends of natural antioxidants, *viz.*, tocopherol concentrates, rosemary extracts, sage extracts, and lecithins, were tested for their ability to stabilize cod liver oil. The antioxidants were tested by using the Rancimat apparatus at 80°C and by a method based on hypochlorite-activated chemiluminescence analysis of samples stored at 35°C for 24 h in light. In addition, a stability study at 5°C in the dark for 8 wk, under conditions realistic for normal consumption of cod liver oil was carried out. A low correlation ($r = 0.339$) was found between Rancimat induction times and chemiluminescence data for the sixteen antioxidant systems tested, probably due to temperature differences, and different ways of detecting oxidation products. Based on Rancimat induction times, δ-tocopherol-rich antioxidants and lecithin had the best stabilizing effect. However, based on the chemiluminescence method, the tocopherols acted as prooxidants, while tocopherols with lecithin increased the stability. Both Rancimat and chemiluminescence data showed stabilizing effects with rosemary and sage extracts, but no synergistic effect between the herbal extracts and lecithin or tocopherol was observed. Analyses of oil aged at 5°C for 8 wk showed the highest stability for cod liver oil containing rosemary extracts. The tocopherol mixtures showed only a minor effect on the stability. Ranking of antioxidants varied considerably depending on the method used, and increasing the temperature seemed to decrease the usefulness of the method. Antioxidant evaluation has to be done by using as many evaluation methods as possible under conditions relevant for normal storage and use.

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KEY WORDS: Antioxidant evaluation, autoxidation, chemiluminescence, cod liver oil, lecithin, natural antioxidants, Rancimat, rosemary, sage, sensory, tocopherol.

Unsaturated fatty acids are easily autoxidized, and oxidative rancidity is the most critical factor affecting shelf life of marine lipids. During autoxidation, a number of compounds may be produced from the initially formed hydroperoxides (1). Many of these are low-molecular weight compounds with strong odor and taste, which cause the characteristic sensory attributes of rancid lipids (2). Substantial amounts of high-molecular weight compounds may be formed in marine lipids, with polymeric triacylglycerols as the major end products (3).

Autoxidation can be inhibited or retarded by adding antioxidants, which may act as initiation inhibitors or chain-breaking substances (4). Several accelerated methods for testing resistance to oxidation and prediction of shelf life of lipids have been developed at elevated temperatures (40–150°C) and oxygen supply. The Sylvester Test (5), the Oxygen Bomb Method (6), the Induction Time Graph (7), and the Oxidograph (8) determine the induction time (IT) by measuring the oxygen uptake or pressure drop caused by oxidation. In the Schaal Oven Test (9) and the Active Oxygen Method (AOM or Swift Test) (10,11), IT of the process is determined by measuring oxidation products formed. In the automated version of AOM (12–14), now known as the Rancimat method, IT is measured by the conductivity increase from the formation of volatile acids (15). Data from the Rancimat apparatus correlated well with the manual AOM test (16), but in general it gave longer IT than the Sylvester test (17), probably due to measurement of only secondary products by the Rancimat method (17). Reproducibility of the Rancimat apparatus was satisfactory (18). Although volatile components as well as some antioxidants may be swept away from the system by the air stream, the method is used extensively.

Several researchers have questioned if the data from tests carried out at highly elevated temperatures are relevant for prediction of shelf life under normal storage conditions (19–21). Recently, we have shown that hypochlorite-activated chemiluminescence (CL) could be a useful method for antioxidant evaluation (22). Due to the high sensitivity and the ability to detect small changes in degree of oxidation, the antioxidants can be evaluated at 35°C within 24 h by this method. In the present study, the Rancimat method and storage at 35°C in the light with hypochlorite-activated CL were used to evaluate cod liver oil (CLO) that contained different natural antioxidant systems. The results were compared with analyses of CLO stored at 5°C for 8 wk.

EXPERIMENTAL PROCEDURES

Chemicals. CLO without antioxidants was supplied by Peter Möller avd. av ORKLA A.S. (Oslo, Norway). Covi-ox T70

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[tocopherol concentrate (70%) with typical distribution 12% *d*-α-tocopherol, 2% *d*-β-tocopherol, 56% *d*-γ-tocopherol, and 30% *d*-δ-tocopherol] was obtained from Henkel (Düsseldorf, Germany); Grindox 1020 [tocopherol mixture (7%), ascorbyl palmitate (20%), lecithin (33%), emulsifier (33%), and vegetable oil (7%)] from Grindsted (Brabrand, Denmark); Herbalox O (rosemary extract in vegetable oil), Herbalox W (rosemary extract, lecithin, diacylglycerols, and monoacylglycerols in vegetable oil), Herbalox DS (sage extract in vegetable oil), and Herbalox WDS (sage extract, lecithin, diacylglycerols, and monoacylglycerols in vegetable oil) from Kalsec (Kalamazoo, MI); lecithin IV-S, lecithin XV-E, and *dl*-α-tocopherol from Sigma (St. Louis, MO); Pristene 180 [tocopherol concentrate (70%) with more than 80% γ- and δ-tocopherol in vegetable oil], Pristene RO (rosemary extract, diacylglycerols, and monoacylglycerols in vegetable oil) and Pristene 180/RO (a 1:1 mixture of Pristene 180 and Pristene RO) from UOP (Des Plaines, IL); Ronoxan A [*dl*-α-tocopherol (5%), ascorbyl palmitate (25%), and lecithin (70%)] and Ronoxan FS 20 [*dl*-α-tocopherol (1%), ascorbyl palmitate (5%), and lecithin (32%) in peanut oil (62%)] from F. Hoffmann-La Roche (Basle, Switzerland); Tenox GT-2 [tocopherol concentrate (70%) with typical distribution 12% *d*-α-tocopherol, 65% *d*-γ-tocopherol, and 23% *d*-δ-tocopherol in vegetable oil] from Eastman Chemical Company (Kingsport, TN); and *d*-δ-tocopherol from Eisai (Tokyo, Japan). All other chemicals used were of *pro analysi* quality delivered by Sigma, Aldrich (Steinheim, Germany), or Merck (Darmstadt, Germany), except the solvents that were of high-performance liquid chromatography grade and obtained from Rathburn Chemicals (Walkerburn, Scotland).

Methods. CL was determined on a Bio-Orbit 1251 Luminometer (Turku, Finland) equipped with dispenser SVD and dispenser controller DC. The light emission was recorded for 60 s at 30°C, after injection of 200 μL hypochlorite solution (1.0 M in 0.1 M NaOH) to samples containing 100 μL oil dissolved in 500 μL *tert*-butanol. The results are presented either as counts corrected for the change in CL after antioxidant addition (22), or relative CL-change (Eq. 1).

$$(CL_{1(sample)} - CL_{0(sample)})/CL_{1(reference)} - CL_{0(reference)}) \times 100 \quad [1]$$

Values below 100 indicate a stabilization effect, while values above 100 show increased oxidation relative to pure CLO. Peroxide values (PV) were measured by iodometric titration (23), and anisidine values (AV) were measured according to IUPAC 2.504 (24). Sensory evaluations were performed by two persons trained at Peter Möller avd. av ORKLA A.S. The oils were presented to the panel directly from the bottles stored at 5°C.

The samples were prepared by addition of antioxidant systems to CLO under stirring. Some mixtures had to be dissolved at 40°C. Antioxidant evaluation at 80°C was conducted in a Metrohm Rancimat 679 (Metrohm Instruments, Herisau, Switzerland), with sample size of 2.5 g and air flow rate set to 20 L/h. Increased ITs compared to pure CLO indicate a stabilization effect. The oxidation experiments at 35°C were performed in open beakers (4.2 cm i.d.) with stirring for 24 h. Sample size was 40 g, and the beakers were exposed to an artificial daylight fluorescent tube (420–750 nm, 1200 lux). The tests at 5°C were carried out in close bottles with air in the headspace in the dark for 8 wk. Sample size was 250 mL from the start, and every working day, 5-mL samples of oil (recommended daily dosage of CLO due to the content of vitamins A and D) were withdrawn for analysis. The results are averages of two analyses.

RESULTS AND DISCUSSION

In this study, the ability of different antioxidant systems to stabilize CLO was evaluated. The antioxidant systems used were commercial blends from natural sources, *viz.* tocopherol concentrates, rosemary extracts, sage extracts, and lecithins, with only one production lot of each (Table 1). To test the reproducibility of the Rancimat method, we measured ITs of two samples of CLO with and two samples of CLO without Covi-ox T70 ten times. The IT of pure CLO was 2.9 ± 0.1 h, and of CLO with Covi-ox T70 6.7 ± 0.2 h. The difference was highly significant ($P < 0.0001$).

Measured by the Rancimat apparatus at 80°C, all antioxidant systems (1.5 mg/g oil) increased IT compare to pure CLO (Table 1). δ-Tocopherol gave the longest IT (11.1 h), while α-tocopherol gave IT = 4.7 h and pure CLO IT = 2.9 h. The difference between the tocopherols agreed with previous findings from experiments conducted at slightly elevated temperatures (25). The effect may be explained by stability differences between the tocopherols toward oxidation, which makes the more reactive α-tocopherol available as an antioxidant for a shorter period of time (26). Tenox GT-2 was the most effective tocopherol mixture with IT = 10.3 h. Surprisingly, this mixture is reported to have the same total composition as Covi-ox T70 and Pristene 180 (Table 1), which gave IT of 6.7 and 8.5 h, respectively. The observed differences may be due to minor variations in the composition. Tocopherol-based antioxidants with ascorbyl palmitate and lecithin [Ronoxan A (IT = 8.1 h), Ronoxan FS 20 (IT = 7.4 h), and Grindox 1020 (IT = 6.7 h)] gave IT values between Covi-ox T70 and Tenox GT-2. These results may be due to different contents of lecithin in these blends because both soybean and egg lecithin increased the IT (9.1–11.0 h). Synergistic effects between tocopherols and lecithin are often reported for accelerated tests (25,27), but no conclusion regarding such effects could be drawn from the Rancimat data.

Both the rosemary extracts (Herbalox O and Pristene RO) and the sage extract (Herbalox DS) (Table 1) gave IT (2.9–3.7 h) close to the value for pure CLO. The corresponding blends with lecithin [Herbalox W (IT = 7.6 h) and Herbalox WDS (IT = 6.9)], however, gave IT comparable to Ronoxan A. Again, the enhanced IT may be due to the lecithin content and not the herbal extract. Although synergistic effects between α-tocopherol and rosemary extract have been reported (28), mixing Pristene RO with the toco-

TABLE 1
Effect of Natural Antioxidants (1.5 mg/g oil) on the Stability of Cod Liver Oil Tested by the Rancimat Method and by Relative Change in Chemiluminescence (CL) in Stored Oil (for 24 h)

Sample[a]	Rancimat induction time (h)[b] (80°C, dark)	CL (relative change)[c] (35°C, light)
Pure cod liver oil	2.9	100
Tocopherols		
Covi-ox T70[d]	6.7	99
Pristene 180[e]	8.5	146
Tenox GT-2[f]	10.3	151
dl-α-Tocopherol (99%)	4.7	130
d-δ–Tocopherol (90%)	11.1	127
Tocopherols with lecithin		
Ronoxan A[g]	8.1	35
Ronoxan FS 20[h]	7.4	55
Grindox 1020[i]	6.7	56
Herbal extracts		
Herbalox O[j]	3.7	75
Pristene RO[k]	2.9	74
Pristene 180/RO[l]	7.6	107
Herbalox DS[m]	3.5	65
Herbal extracts with lecithin		
Herbalox W[n]	7.6	75
Herbalox WDS[o]	6.9	74
Lecithin		
Soybean lecithin (40%)	11.0	90
Egg lecithin (60%)	9.1	108

[a]Composition given by the manufacturer.
[b]Standard deviation <2%.
[c]Standard deviation <5%.
[d]Tocopherol concentrate (70%) with typical distribution 12% *d*-α-tocopherol, 2% *d*-β-tocopherol, 56% *d*-γ-tocopherol, and 30% *d*-δ-tocopherol (Henkel, Düsseldorf, Germany).
[e]Tocopherol concentrate (70%) with more than 80% γ- and δ-tocopherol in vegetable oil (UOP, Des Plaines, IL).
[f]Tocopherol concentrate (70%) with typical distribution 12% *d*-α-tocopherol, 65% *d*-γ-tocopherol, and 23% *d*-δ-tocopherol in vegetable oil (Eastman Chemical Company, Kingsport, TN).
[g]*dl*-α-Tocopherol (5%), ascorbyl palmitate (25%), and lecithin (70%) (F. Hoffman-La Roche, Basle, Switzerland).
[h]*dl*-α-Tocopherol (1%), ascorbyl palmitate (5%), and lecithin (32%) in peanut oil (62%) (F. Hoffman-La Roche).
[i]Tocopherol mixture (7%), ascorbyl palmitate (20%), lecithin (33%), emulsifier (33%), and vegetable oil (7%) (Grinsted, Brabrand, Denmark).
[j]Rosemary extract in vegetable oil (Kalsec, Kalamazoo, MI).
[k]Rosemary extract, diacylglycerols, and monoacylglycerols in vegetable oil (UOP).
[l]A 1:1 mixture of Pristene 180 [tocopherol concentrate (70%) with more than 80% γ- and δ-tocopherol in vegetable oil] and Pristene RO (rosemary extract, diacylglycerols, and monoacylglycerols in vegetable oil).
[m]Sage extract in vegetable oil (Kalsec).
[n]Rosemary extract, lecithin, diacylglycerols, and monoacylglycerols in vegetable oil (Kalsec).
[o]Sage extract, lecithin, diacylglycerols, and monoacylglycerols in vegetable oil (Kalsec).

pherol extract Pristene 180 reduced the IT compared to Pristene 180 alone. This effect is probably caused by a dilution of the active compounds in Pristene 180.

Stabilization effects, based on the experiments conducted at 35°C with CL measurements, showed unaltered or decreased stability of tocopherol-based antioxidants without lecithin compared to pure CLO (Table 1). Both α- and δ-tocopherol were found to act as prooxidants, with almost identical CL-values (relative CL change of 130 and 127, respectively), in spite of different Rancimat ITs (4.7 h and 11.1 h, respectively). Tenox GT-2 (relative CL change 151), which gave one of the longest IT in the Rancimat experiments, showed the highest prooxidative effect. Best stabilization was found for tocopherol mixtures with lecithin and ascorbyl palmitate, with Ronoxan A as the most effective mixture (relative CL change 35). Lecithins alone had minor effects on stability (relative CL change 90 and 108). This is in contrast to the results obtained by the Rancimat method.

Rosemary (Herbalox O and Pristene RO) and sage (Herbalox DS) extracts showed a stabilizing effect with relative CL change in the range of 65 to 75. No synergistic effect between the herbal extracts and lecithin was observed. Neither was any synergistic effect between rosemary extracts and tocopherols found, as the mixture Pristene RO/180 gave decreased stability compared to the rosemary extract Pristene RO alone (relative CL change 107 vs. 74).

Based on Rancimat measurements, increased antioxidant concentration gave increased IT for all the antioxidant systems tested (Fig. 1A), but the relative effect of the antioxidants was maintained. However, based on the CL method, the picture is more complex, depending on the antioxidant system used (Fig. 1B). A low correlation (correlation coefficient $r = 0.339$) was found between Rancimat ITs and relative CL change for the sixteen antioxidant mixtures tested (Fig. 2). The temperature difference between these two methods is 45°C, and this influences the mechanism and degradation rate of the polyunsaturated fatty acids (19,29,30). Assessment of the oxidation products formed is also different. IT determination by Rancimat is based on detection of volatile acids (15), while in the CL procedure, the autoxidation products detected are mainly hydroperoxides (31,32) and, to a lesser extent, secondary products (22). The Rancimat method was performed in the dark, while the CL method was performed in the light. Light is known to influence initiation and propagation of the oxidation process (33). The wavelength of the light used in this experiment ($\lambda > 420$ nm) and the absence of photosensitizers in the purified CLO make formation of singlet oxygen or photolysis of already formed hydroperoxides unlikely (34).

The present study showed differences between results from accelerated shelf life testing at 80 and 35°C. Therefore, we decided to carry out a stability study at 5°C for 8 wk in the dark under conditions that are typical for normal consumption of CLO. A number of different antioxidant systems in the range 0.188–1.500 mg/g oil were tested, and the results from CLO containing tocopherol concentrates with and without lecithin (Covi-ox T70 and Ronoxan A) and rosemary extracts with and without lecithin (Herbalox O and Herbalox W) are presented below. With all antioxidant systems used, the highest concentration gave the best stabilizing effect. The oxidation level was assessed by PV, AV, CL, and sensory evaluation.

PV measurements (Fig. 3A) showed an excellent stabilizing effect of the rosemary extracts Herbalox W and O, with PV of 3 and 4 meq/kg after 8 wk, compared to PV of 32

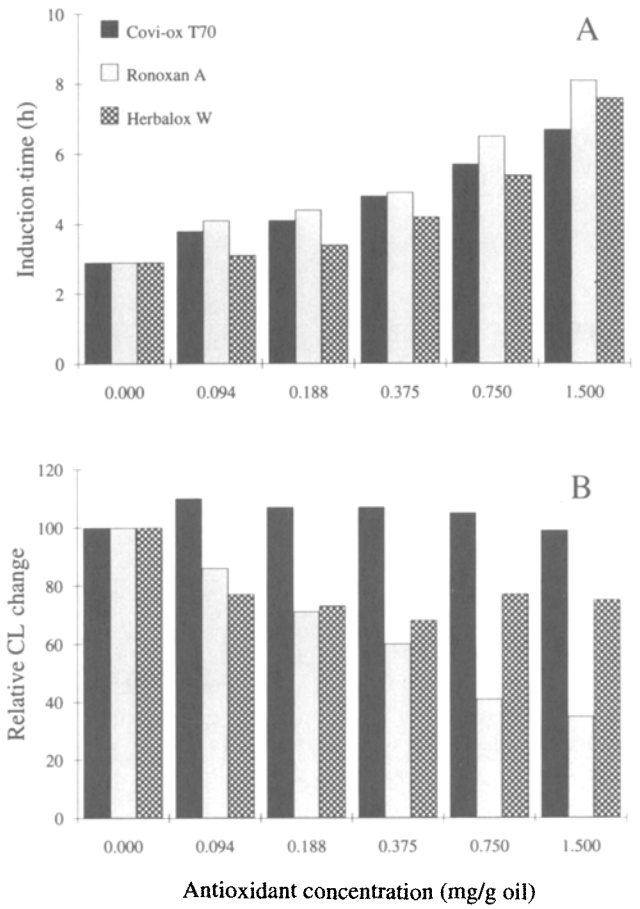

FIG. 1. Antioxidant activity of different concentrations of Covi-ox T70 (Henkel, Düsseldorf, Germany), Ronoxan A (F. Hoffman-La Roche, Basle, Switzerland) and Herbalox W (Kalsec, Kalamazoo, MI) on cod liver oil; A, measured as induction time on Metrohm Rancimat at 80°C in dark; B, measured as relative chemiluminescence (CL) change in oils stored at 35°C for 24 h in light.

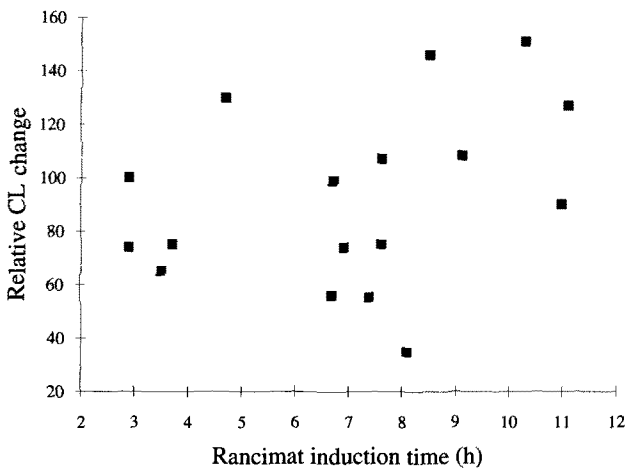

FIG. 2. Correlation (r = 0.339) between Rancimat induction time at 80°C and relative chemiluminescence (CL) change in oils stored at 35°C for 24 h of the antioxidant systems tested (1.5 mg/g cod liver oil).

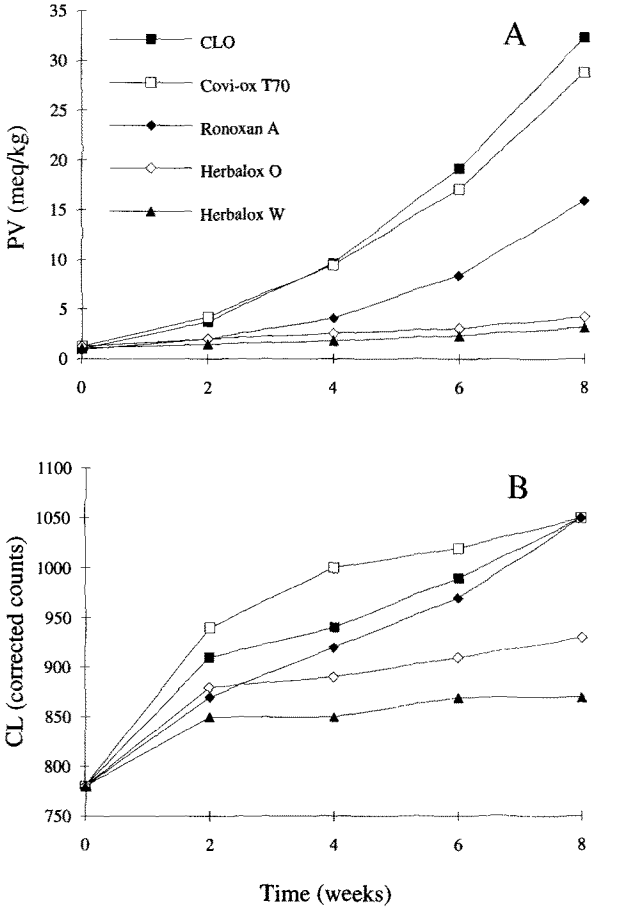

FIG. 3. Tests of cod liver oil (CLO) with Covi-ox T70, Ronoxan A, Herbalox O (Kalsec, Kalamazoo, MI) or Herbalox W (1.5 mg/g oil) conducted at 5°C for 8 wk in dark; A, peroxide value (PV); B, chemiluminescence (CL) change. See Figure 1 for other company sources.

meq/kg for the pure CLO. The tocopherol mixture Covi-ox T70 showed only a minor stabilizing effect, while the tocopherol-based antioxidant with lecithin (Ronoxan A) gave a stabilizing effect intermediate to the tocopherol concentrate and the rosemary extracts.

Ranking of the antioxidants based on the CL data was dependent on time (Fig. 3B), giving profiles of the graphs different from the ones based on peroxide measurements. Based on CL measurements, in the beginning of the test Ronoxan A gave a relative increase in stability compared to the pure oil, but this effect disappeared after two weeks of storage. Covi-ox T70 showed a prooxidative effect when measured by this method. This observation is most probably due to the different oxidation products detected by the two methods. AV increased slightly during the storage period, with only minor differences between the antioxidants tested (not shown).

The sensory evaluations were not in accordance with the data from the chemical tests. Herbalox O and Ronoxan A were evaluated as best, while Herbalox W was graded lower than the pure CLO (not shown). This latter observation was ascribed to the flavor of rosemary in the oil.

TABLE 2
Ranking of Some Natural Antioxidants[a] (1.5 mg/g oil) for Cod Liver Oil

Antioxidant	Test method		
	Rancimat[b] (80°C)	CL[c] (35°C, 24 h)	PV[d] (5°C, 8 wk)
Covi-ox T70	3	4	4
Ronoxan A	1	**1**	**3**
Herbalox O	4	2	2
Herbalox W	2	2	**1**

[a]For composition and company sources, see Table 1, highest stability is **1**.
[b]From Rancimat IT at 80°C in dark (Table 1).
[c]Based on chemiluminescence (CL) data from stability experiments conducted at 35°C in light for 24 h (see Table 1).
[d]Based on peroxide value (PV) of cod liver oil stored at 5°C in dark for 8 wk (Fig. 3).

Shelf-life determination of CLO containing different antioxidant mixtures is highly dependent on the method used (Table 2). Increasing the temperature of the test system from the normal storage temperature of CLO (5°C) seemed to decrease the usefulness of the method. This was especially marked for weak antioxidants (e.g., tocopherols), where the reaction mechanisms are temperature-sensitive (21). Assessment of oxidation should therefore be done by as many evaluation methods possible, preferably under conditions relevant for normal storage and use. Further investigations on the influence of antioxidants on the formation of different autoxidation products are currently under way.

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