Antioxidant Activity and Stability of 6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid

W.M. CORT, Product Development Department[1], and **J.W. SCOTT**, Chemical Research Department, and **M. ARAUJO, W.J. MERGENS, M.A. CANNALONGA, M. OSADCA,** and **H. HARLEY**, Product Development Department, and **D.R. PARRISH**, Chemical Research Department, and **W.R. POOL**, Experimental Pathology and Toxicology Department, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110

ABSTRACT

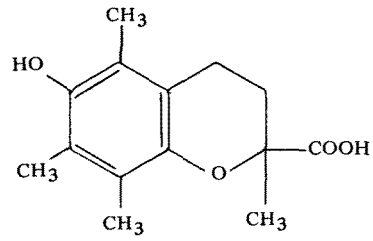
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid in thin layer tests in vegetable oils and animal fats has two-four times the antioxidant activity of butylated hydroxytoluene and butylated hydroxyanisole and is more active than propyl gallate, nordihydroquaiaretic acid, ascorbyl palmitate, and α- and γ-tocopherols. It is also more active than tertiary butylhydroquinone in chicken and pork fats and corn, peanut, sunflower, and safflower oils in thin layer tests. Tertiary butylhydroquinone is slightly more active in corn, soybean, cottonseed, and peanut oils in the active oxygen method, probably due to decomposition of the chroman at 98 C. This decomposition is inhibited by ascorbic acid. The chroman is synergized by ascorbyl palmitate-thiodipropionate and ascorbic acids. Active oxygen method values of 190 hr are obtained in combination with the latter. A spectrofluorometric assay for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid has been developed. The compound is 95-100% stable for 2 months at room temperature and 45 C, and a low level of toxicity is found. The copper and iron breakdown products of the chroman have been determined. A possible relationship between these products, protection of the chroman by ascorbic acid and ethylenediaminetetraacetic acid, and a mechanism of action is presented.

INTRODUCTION

6-Hydroxychromans, in particular the tocopherols, have been studied as antioxidants for many years. Recently, we have shown (1) that the natural (RRR) and all rac α-tocopherols have equivalent activity and that neither has increased activity above 20 mg percent. Compared to food-grade antioxidants, tocopherols have poor activity in vegetable oils but are better in animal fats. This selective activity may be due to a relatively high efficiency in the protection of oleic acid as compared to linoleic acid. It has been shown (2) that γ-tocopherol is a better antioxidant than the α-homologue.

Skinner, et al., (3) tested a number of tocopherol analogues with β-carotene as substrate. In this test, tocopherol was 1.5 times as active as the analogues, but, since this test was conducted on a molar basis, 1.7 times more tocopherol actually was used. On a wt basis, then, their activities are similar. In addition, we have shown (1) that the ratio of activity of antioxidant varies with the substrate. Antioxidant activity on carotenoids and on vitamin A does not presage comparable activity in oils or fats.

In a previous publication (4), we have described the synthesis and antioxidant evaluation of a number of 6-hydroxychromans. It was our unexpected finding that a carboxylic acid group at C-2 of the chroman gave compounds that were markedly superior to any other 6-hydroxychromans. Structure-activity studies indicated that 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [1], recently assigned the trivial name of Trolox C, was the most effective compound. It is shown below:



[1] Trolox C

It is the purpose of this paper to compare the activity of this compound to currently used food-grade antioxidants in

[1]Present address: Quality Control Department.

TABLE I

Comparative Antioxidant Activity[a]

Antioxidants	Oxygen analyzer RT % NDGA	Thin layer days[b], 45 C		AOM hr, 98 C soybean oil
		Soybean oil	Chicken fat	
Trolox [1]	100	17-36	35+	27
BHT	85	9	15	6.5
BHA	140	8	20	5
PG	85	15	NR	22
AP	58	11	10	11.5
TBHQ	85	20-37	34	32
NDGA	100	17	25	22
α-Tocopherol	7	6	13	5
γ-Tocopherol	9	7	29	5
None	0	5	5	5

[a]RT = room temperature, NDGA = nordihydroquaiaretic acid, BHT = butylated hydroxytoluene, BHA = butylated hydroxyanisole, PG = propyl gallate, AP = ascorbyl palmitate, and TBHQ = tertiary butylhydroquinone.

[b]To reach peroxide values of 70 meq/kg in vegetable oils and 20 meq/kg in animal fats. Thin layer and active oxygen method (AOM) antioxidants at .02%, oxygen analyzer 100 mcg/test.

TABLE II

45 C Antioxidant Efficiency—Thin Layer Days to Reach 70 Peroxide Value meq/kg

Antioxidant[a]	Substrate oil			
	Corn	Peanut	Sunflower	Safflower
Trolox C	25	34	27	39
TBHQ	24	30	27	33
PG	21	26	19	16
BHT	13	15	9	10
BHA	15	15	8	8
None	12	15	6	6

[a]All at .02% (legal limit). Soybean oil, see Table I. TBHQ = tertiary butylhydroquinone, PG = propyl gallate, BHT = butylated hydroxytoluene, and BHA = butylated hydroxyanisole.

several test systems; namely thin layer tests in vegetable oils and animal fats, active oxygen method (AOM), and a hemoglobin-catalyzed emulsion peroxidation. Solubility, assay procedure, stability, synergistic reactions, and a possible mode of action will be presented.

EXPERIMENTAL PROCEDURES

Compounds

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (I, Trolox C) was prepared as previously described (4). 2-Hydroxy-2-methyl-4-(3,5,6-trimethylbenzoquinone-2-yl) butanoic acid [2] was obtained by the oxidation of [1] with ferric chloride in a two phase mixture of ether and aqueous methanol (5). Oxidation of an ethereal suspension of [1] with potassium ferricyanide in aqueous sodium hydroxide solution (6) or with bromine and potassium bromide in aqueous sodium bicarbonate solution (7) gave a mixture of the quinone [2] and 6,8aH-dihydro-2,5,7,8-tetramethyl-6-oxo-chroman-2-carboxylic acid lactone [3]. Washing an ethereal solution of the products with dilute sodium bicarbonate solution gave the pure lactone [3]. Compounds [2] (mp 117-119 C from ether/30-60 C petroleum ether) and [3] (mp 59.5-62 C from the same solvent pair) were characterized fully by UV, IR, NMR, and mass spectrometry, and by carbon and hydrogen analyses.

Stripped safflower oil was prepared on a rotary molecular still at 270 C. It contained less than 0.5 mg% tocopherols. All the oils were tested as received to conform to specifications. These include free fatty acid value below 0.2, saponification value of 190, correct iodine value, specific gravity, color and rancidity measurements, and analysis for the absence of butylated hydroxytoluene (BHT). All the fats and oils were stored under nitrogen at 5 C and retested for peroxides at the onset of each experiment, and only those below 4 meq/kg were used. Lard was prepared by rendering pork fat as previously described (1).

Antioxidant Testing

In the thin layer test, a layer of 0.20 ml fat or oil in a 50 ml beaker (37 mm diameter) at 45 C was titrated daily to determine the peroxide values. The AOM test was performed at 98 C in the standard manner (8) in a specially constructed oil bath (Sargent). The hemoglobin-catalyzed peroxidation of a 10% safflower oil emulsion (oxygen analyzer test [9]) was measured with a Beckmann 777 oxygen analyzer. The ability of test compounds to prevent the removal of oxygen from solution (peroxidation) is expressed as percent of a standard, in this case, nordihydroquaiaretic acid (NDGA). All experiments reported herein have been performed in duplicate on at least two different days.

TABLE III

Synergism of Trolox C[a]

Additive[b]	Hr to reach 70 PV activity alone	Activity in combination with Trolox C at .02%
None	5	27
.02% AP	11	35[c]
.02% AP + .02% TDPA	30	69
.2% AP	27	47[c]
.2% AP + .02% TDPA	67	98
.1% Ascorbic acid	150	190
.05% Trolox C	38	---
.1% Trolox C	53	---

[a]Active oxygen method—soybean oil at 98 C. PV = peroxide value. Meq/kg.

[b]AP = ascorbyl palmitate and TDPA = ascorbyl palmitate plus palmitate plus thiodipropionate.

[c]Total less than sum of both compounds.

TABLE IV

Active Oxygen Method Hr to Reach 70 Peroxide Value at 98 C

Antioxidants[a]	Substrate oils			
	Corn	Cottonseed	Peanut	Lard
None	13	11	12	3
BHT	14	15	22	30
BHA	13	15	13	45
PG	26	21	36	70
TBHQ	34	38	52	42
Trolox C	28	36	36	73
Trolox C + .05% AA	90	45	164	150
Trolox C + .1% AA	96	66	170	200

[a]All antioxidants at .02% (except ascorbic acid [AA]) 1 = .05% AA and 2 = .1% AA. BHT = butylated hydroxytoluene, BHA = butylated hydroxyanisole, PG = propyl gallate, and TBHQ = tertiary butyhydroquinone.

Assay Methods

The antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [1] was measured by fluorescence with a Farrand spectrofluorometer by excitation at 290 nm and reading the peak emission at 330 nm. Alcoholic solutions were used directly without preextraction. Oils were dissolved in hexane and extracted with a mixture of alcohol-water (1:1 v/v) which removed the tocopherols in the hexane phase; the alcohol-water phases were assayed. Samples were subdiluted in both 0.1% ammonium hydroxide in methanol-water and in 0.1 N HCl in methanol-water and read within 5 min. The latter reading was subtracted from the ammonium hydroxide reading. Recovery of the chroman from soybean oil and coconut oil was 93-99%, whereas recovery from alcoholic solutions was 98-100%.

The quinone [2] had no fluorescence. At 270 nm, the $E^{1\%}_{1cm}$ of this compound was 715, whereas the chroman [1] had very little absorption at this wavelength. Thus, the quinone [2] was determined by UV assay after subtracting absorption due to chroman as previously determined by fluorescence. TLC assays also were used to check for qualitative identification of breakdown products.

Tocopherol was measured by the Emmerie Engel (EE) reaction and by the gas liquid chromatographic (GLC) method of Rudy, et al. (10). The C-32 hydrocarbon was used as an internal standard for the GLC test. A 5 mcg sample was injected into an Aerograph 1700 flame ionization detector equipped with 4 ft stainless steel x 1/8 in. inside diameter column containing 10% SE 30 on 80-100 aeropak and a Hewlett Packard electronic integrator. The α-tocopherol peak appeared in 800 sec, γ-tocopherol in 670 sec, p-tocopheroquinone in 820 sec, and the internal standard in 1100 sec.

TABLE V

Physico-Chemical Data 6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid

Solubility at 25 C	
Solvents	Percent
Water	.053
Ethanol USP	16.0
Propylene glycol	1.6
Medium chain triglyceride	0.7
Vegetable oils	.17
Diethyl ether	2.56
Petroleum ether	.04
Chloroform	1.08
Methanol, 2-propanol Acetone, benzyl alcohol	Greater 10.0

Formula wt 250.32
mp 189-195 C
UV peak 291 $E^{1\%}_{1cm}$ = 120
Appearance: white to light yellow power
Odor:none
Taste:bland

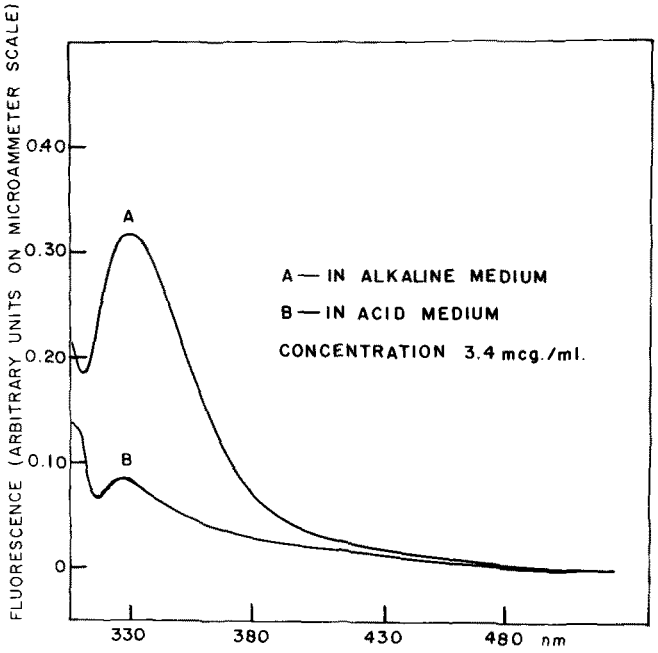


FIG. 1. Emission spectra of Trolox C.

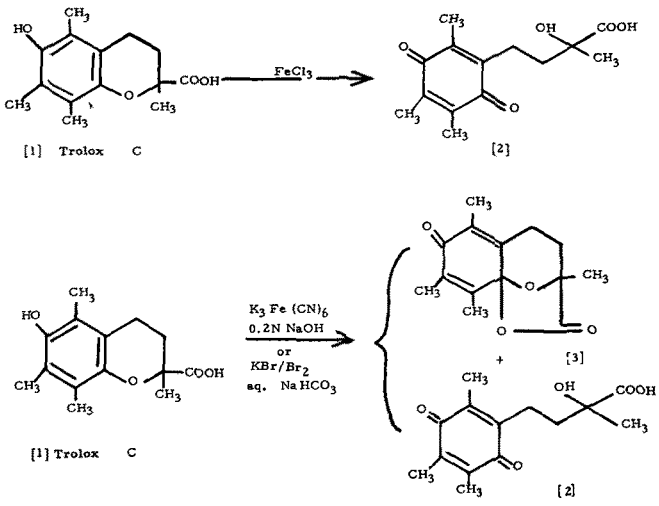


FIG. 2. Oxidation products formed from Trolox C.

Solubility was measured by mixing overnight and subsequent Swinny filtration (0.22 μ pore size) and measuring the concentration of the compound in solution by the spectrofluorometric assay.

The LD_{50} values were obtained using 10 animals/dose level, and the animals were observed for 5 days. The compound was ground in a mortar and suspended in 5% gum acacia solution for administration. The LD_{50} values were calculated by the method of Miller and Tainter (12).

RESULTS AND DISCUSSION

Antioxidant Activity

Table I exhibits comparative antioxidant activity of Trolox C to other antioxidants that have been used in foods in the U.S. The chroman is two-four times more active than BHT and butylated hydroxyanisole (BHA) in the thin layer test and AOM and is more active than propyl gallate (PG), ascorbyl palmitate (AP), and NDGA (no longer legal in the U.S.) in all test systems. It is a great deal more active than α- or γ-tocopherol. It is equal to, or better than, tertiary butylhydroquinone (TBHQ), the newest antioxidant, in the thin layer test but is slightly less active in the AOM. It is better than TBHQ in the emulsion test. In chicken and pork fat (not shown) tested in thin layers, the chroman was more active than TBHQ and the tocopherols.

Further comparisons in an assortment of vegetable oils—corn, peanut, sunflower, safflower, and soybean—are presented in Table II. In all cases, the chroman is better than BHA, BHT, and PG, and is competitive with TBHQ. In corn and safflower oils, the chroman is better; in sunflower oil, they are equivalent; and in soybean oil, TBHQ is slightly better.

To increase activity on soybean oil in the AOM, the chroman was studied in combinations. In the first column of Table III, the activity is given for the additive alone and in the second column in combinations with Trolox C. AP increased the AOM values but to a value less than the sum of the individual compounds. AP plus thiodipropionate (TDPA) does give a true synergism, and AOM hr as high as 98 were obtained. The most interesting combination was 0.1% ascorbic acid (AA) with .02% chroman, which gave an AOM value of 190.

Most of our results have been obtained at .02%, which is the legal limit for most antioxidants. However, increasing the levels of the chroman to 0.05% (Table III) results in 35 AOM and to 0.1% in 53 AOM hr.

Table IV presents results from the AOM using corn, cottonseed, and peanut oils and lard as substrates. After 25 hr, the oils were assayed for the chroman by a spectrofluorometric procedure (vide infra) and showed a 25% loss of the compound, whereas the vegetable oils containing added AA and the lard showed no loss. The destruction of the chroman in these vegetable oils explains why it is less active than TBHQ. However, the addition of AA stabilized the chroman, and the combination with AA is very active.

Physical Properties, Stability

The solubility of the chroman, as well as its physicochemical characteristics, appears in Table V. The maximum absorption at 291 nm and an EE reaction were predictable from tocopherol data. The EE reaction, as expected, was equal to tocopherol on a molar basis (1.7 times more active

TABLE VI

6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid Copper and Iron Decomposition

		mcg/ml in 50% Ethanol, 50% H_2O		
		8 Days room temperature		15 Days room temperature
Additives chelate	Metal	Chroman	Quinone [2]	Chroman
0	0	1030	0	940
0	50 ppm Cu^{++}	600	430	210
Na_2EDTA[2]		1080	0	1080
Citric acid		790	250	580
EDTA acid		1080	0	1060
Ascorbic acid		1080	0	1080
0	50 ppm Fe^{+++}	900	150	790
Na_2EDTA		1050	0	980
Citric acid		960	90	850
EDTA acid		945	100	890
Ascorbic acid		1080	0	1080

[a]EDTA = ethylenediaminetetraacetic acid.

on a wt basis).

A spectrophotometric method (see "Experimental Procedures" for details) was developed which allowed accurate determination of solubilities. Fluorescent spectra are given in Figure 1, and a typical linear response is given from 1-6 mcg. The solubility in alcohol at 16% is useful in preparation of bulk solutions. The solubility in vegetable oils is not as high as one might like, but the compound is soluble at 0.17% which is well above the legal limit for most antioxidants in the U.S. The compound is an off-white, odorless, tasteless solid. This is in contrast to the viscous liquid tocopherols and the odiferous phenolic characteristics of BHT and BHA.

The stability in oils was studied using the spectrofluorometric procedure. In soybean oil at a concentration of 0.099 and 0.02%, retention for 2 months at room temperature was 97-100%. Retention at 45 C was 95-100%. As expected, retention at 70 C was less (80-89%). In coconut oil, retention for 2 months at 45 C was 96% and at 70 C was 92%. Solutions in USP alcohol for 6 months at 16 and 5.0% have been completely stable in dark bottles.

As part of our study of the stability of Trolox C, we looked at potential oxidation products of this compound (Fig. 2). With $FeCl_3$, we obtained the expected quinone [2]. With $K_3Fe(CN)_6$ (or KBr/Br_2 [7]), a mixture of [2] and the novel lactone [3] was obtained. Under similar conditions, the tocopherols and their analogues give mixtures of dimers and trimers (6, 12-14). No such compounds arising from Trolox C could be found by thin layer chromatography (TLC). Neither oxidation product had antioxidant properties. The hydroquinone from reduction of [2] was only very weakly active (AOM value [soybean oil] of 6).

We previously have found (15) that tocopherol is unstable in the presence of Cu^{++} or Fe^{+++}. As a result, experiments involving interactions of these metals with Trolox C were performed (Table VI). In 15 days, a 1080 mcg/ml solution in 50% ethanol containing 50 ppm Cu^{++} or 50 ppm Fe^{+++} suffered an 80 and 20% loss, respectively, of the chroman, as measured spectrofluorometrically. Both of these catalyzed oxidations were stopped completely by AA. From data on tocopherols, one would expect that Fe^{+++} would promote oxidation to the quinone [2], and Cu^{++} also produced the quinone [2]. Ethylenediaminetetraacetic acid (EDTA) stopped the Cu^{++} oxidation but only partially inhibited the Fe^{+++} oxidation. TLC mixtures indicated only the chroman [1] and the quinone [2] and no other breakdown products.

TABLE VII

Acute Toxicity 6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid[a]

Species	Route	LD_{50} ± standard error mg/kg
Mice	PO	1630 ± 154
	IP	1700 ± 117
	SC	1930 ± 167
Adult rats	PO	4300 ± 318
	IP	1800 ± 170
Neonatal rats	PO	1120 ± 117
Rabbits	PO	>2000

[a]Animals (10) each dose level; administered suspended in 5% acacia solution all routes. Carrier is nontoxic. PO = oral, IP = interperitoneal, and SC = subcutaneous. CF-IS mice, wt 17-25 g; Charles River CD rats, wt 130-175 g; albino rabbits, wt 2.3-2.7 kg; and Charles River CD rats, less than 24 hr old, wt 6-9 g.

Toxicity

The LD_{50} toxicity is given in Table VII and is well above 1500 mg/kg. Trolox C also has been fed to 3 dogs in doses pyramiding daily up to 320 mg/kg over 14 days. The dogs appeared normal and the white and red cell counts, hematocrit, hemoglobin, and differential leucocyte counts, as well as blood chemistry, including blood urea nitrogen, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase, and plasma glucose, all were within normal limits. However, at this time, the compound *has not been released for human consumption.*

TABLE VIII

Residual Antioxidant after Thin Layer Oxidation in Soybean Oil, 45 C, 2 Months

Antioxidant[a]	Peroxides formed	Residual antioxidant (%)	Percent loss antioxidant
.05% Trolox C	120	.042	20
.02% Trolox C + .2% AA	20	.018	10
1.0% All rac-α-tocopherol	650	0.7	30
1.0% BHT	36	0.8	20

[a]AA = ascorbic acid and BHT = butylated hydroxytoluene.

Mechanism of Action

The mechanism of action of autoxidation of fats and oils has been classified as a free radical chain reaction for many years. Phenolic antioxidants have been said to act as electron or hydrogen donors which quench electron mobility. It has been stated (16) that ultimately the antioxidants are swamped, and, thus, oxidation proceeds. However, as is shown in Table VIII, this swamping occurs while much of the antioxidant remains. This apparent anomaly may be explained by the theory of Olcott and Lin (17) that the "true" antioxidants are free radical one-electron oxidation products of the antioxidant. These free radicals usually are hindered and, thus, do not initiate radical processes; rather, they combine with the oil-free radicals to quench such processes. The preparation of stable one-electron oxidation products of an α-tocopherol model compound recently has been demonstrated (18).

The "free radicals as antioxidant" theory also yields an explanation for the synergism seen in mixtures of Trolox C and AA. Fe^{+++} and Cu^{++} are known (19) to lead to the oxidation and destruction of organic free radicals. AA would keep these metals in lower oxidation states (20), thus protecting the antioxidant. EDTA also would protect the antioxidant by removing the metal ions by chelation.

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