Antioxidant for Fats and Oils from Canary Seed: Sterol and Triterpene Alcohol Esters of Caffeic Acid

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ABSTRACT

Several kinds of seeds used as bird feed were extracted successively with hexane, ether and methanol. In the antioxidant test with extracts, the ether extract from canary seeds showed the highest activity. The antioxidant fraction separated from it by thin layer chromatography showed excellent activity for lard and sardine oil. The effective components were identified by gas chromatography and gas chromatography-mass spectrometry of the hydrolyzed products as the esters of caffeic acid with cycloartenol, gramisterol, sitosterol and campesterol with the minor amounts of 24-methylenecycloartanol, obtusifoliol, brassicasterol and Δ7-stigmastenol.

INTRODUCTION

Caffeic acid and its esters have been found in a wide variety of plant materials and their excellent antioxidant activity is well known (1). However, most of them are water-soluble substances which have low solubility in fats and oils and have a bitter taste. These properties have inhibited their use as antioxidants by direct addition to edible oils.

In this study we found a lipid-soluble antioxidant which consists of sterol and triterpene alcohol esters of caffeic acid in canary seeds (*Phalaris canariensis, Gramineae*). The seeds were products of Australia and possibly contain small amounts of *P. tuberosa* and *P. arundinaccea*.

Lipid-soluble caffeic acid esters have been found in oats (2-5) and in *Sophora subprostrata* Chun and T. Chen roots (6), Leguminosae (Chinese drug: San Dou Gen). In oats, 24 phenolic antioxidant components have been detected by thin layer chromatography (TLC). Eight of them were identified as the mixed esters of caffeic and ferulic acid with fatty alcohols, α, ω-fatty diols and ω-hydroxy fatty acids with 26 and 28 carbons. From the *S. subprostrata*, C_{20}-C_{26} (mainly C_{22}) fatty alcohol esters of caffeic acid have been isolated. The antioxidant from oats is presumed to have lower activity, since it contains ferulic acid esters which are less effective than caffeic acid esters (4). San Dou Gen is a special material for medical use and contains the caffeic acid esters at a lower level. Canary seeds are available in large quantity, since they are a popular bird feed. Another advantage of the antioxidant from canary seeds over the aliphatic esters of caffeic acid is higher melting point, which facilitates solvent removal at higher temperatures and refining by recrystallization. Based on subjective testing the antioxidant does not have appreciable bitterness. Consequently, the canary seed extract has potential as an antioxidant for edible oils.

Sterol and triterpene alcohol esters of caffeic acid were found in the natural products for the first time in this study, although the occurrence of the corresponding esters of ferulic acid (methyl ether of caffeic acid at 3-OH) in vegetable oils and related materials has been reported in many papers (7).

MATERIALS AND METHODS

Antioxidant Test

The antioxidant test was performed by the oven test using lard and sardine oil. A glass tube (50 ml, 3 cm id) with a flat bottom containing 3 ± 0.01 g oil and 3 or 1.5 ± 0.01 mg extract was placed into an incubator kept at 60 ± 1 C. The lard, which included no antioxidant, was obtained from a lard refining plant. The sardine oil, obtained from an industrial plant, was used after refining by column chromatography with silicic acid as packing material and *n*-hexane as eluent. The periodical change of the peroxide value (POV) was measured using colorimetric iodine method (8).

Effects of antioxidants were indicated by the protection factor (PF_n) described elsewhere (9) and shown by the following equation: $PF_n = T_n/T_n^\circ$. In this equation, T_n is the time for POV of fats to attain n and T_n° is the T_n in the control test. Values of PF_n are obtained from the curve of the periodical change of POV. At the ends of the induction periods, POV values were measured at intervals of ca. 10-40 hr.

Thin Layer Chromatography

In all TLC, Silica Gel G plates (20 x 20 cm) were used, and the thicknesses of the layers for analytical and preparative TLC were 0.25 and 0.5 mm, respectively. In the analytical TLC, hexane/ether/acetic acid (80:20:1, v/v/v) was used in development. All spots were visualized after spraying with 50% sulfuric acid with subsequent charring at 100 C. In preparative TLC, development was carried out using the solvents shown in Figure 1 and the bands were detected under ultraviolet (UV) light after spraying with Rhodamine 6G (0.1% ethanol solution). Each band was scraped and extracted with ether.

Spectroscopy

UV spectra were obtained with a Hitachi 124 spectrophotometer (Hitachi Seisakusho Co. Ltd., Tokyo), infrared (IR) spectra were run in KBr pellets on a Nippon Bunkō Kōgyo Co. Ltd., Tokyo). The 100-MHz proton magnetic resonance (PMR) spectra were recorded with Nippon Denshi FX-100 (Nippon Denshi Co. Ltd., Tokyo) in $CDCl_3$ with $Si(CH_3)_4$ as a reference marker.

Gas Liquid Chromatography (GLC) and Gas Chromatography-Mass Spectrometry (GC-MS)

GLC was done with a Shimadzu GC 6AMPF with a flame ionization detector and an integrator Shimadzu E1A (Shimadzu Seisakusho Co. Ltd., Kyoto). GLC of sterols and triterpene alcohols were run with a 1.5 m x 3 mm glass column packed with 2% OV-17 on 80/100 mesh Chromosorb W at column temperature 270 C. GLC of the trimethylsilylated (TMS) product from the phenolic acid, the fraction C in Figure 1, was done under the same conditions except the column temperature (190 C) after conversion to TMS derivatives with the reagent, dry pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1, v/v/v) (10). GLC of methyl esters of the fatty acid from the hexane extract of canary seeds and sardine oil were run with a 2 m x 3 mm glass column packed with 3% SILAR 10 C on 100/120 mesh Gas Chrom Q at a column temperature of 190 C, with 10% diethyleneglycol succinate polyester on 80/100

mesh Chromosorb W at a column temperature of 170 C, respectively. In every run, the carrier gas was nitrogen.

GC-MS was done with a Hitachi M60 instrument coupled to a computer Hitachi 002B. The GLC in GC-MS of sterols and triterpene alcohols and the TMS product of C were performed on a 2 m x 3 mm glass column packed with 2% OV-17 on 80/100 mesh Gas Chrom Q. The column temperature was 250 C for the sterols and triterpene alcohols and 200 C for the TMS product of C. The spectra were taken at an ionizing voltage of 20eV; carrier gas was helium.

RESULTS AND DISCUSSION

Antioxidant Activity of the Extracts

Table I shows the antioxidant activities of the extracts from the common species of the bird feed. The seeds were obtained at a grain store in Hakodate and were ground by an electric mill. A 100-g sample of the powder was extracted successively with 250 ml hexane, ether and methanol, respectively, in a Soxhlet extractor for 5 hr. The solvents were evaporated from the extract solution in a rotatory evaporator below 40 C. All operations were run in the dark to protect the extract from light-induced isomerization and all solvents were prepared by distillation of reagent-grade products.

Since the ether extract from canary seeds showed the highest activity, separation and characterization of the effective components from it were investigated.

Separation of the Antioxidant from Canary Seeds

Ground canary seeds (3.9 kg) was extracted at room temperature successively with 3 7.5 ℓ portions of hexane and ether. A yellowish oil (27.9 g) was obtained as the ether extract. Separation of the antioxidant was carried out by the procedures shown in Figure 1. The ether and hexane extracts were analyzed by TLC using hexane/ether/acetic acid (80:20:1, v/v/v) for developing. The hexane extract consisted of triacylglycerols as shown by TLC. The identification was made by agreement of the location of the spot and complete overlapping with a triacyglycerol specimen in the mixed development. In the analysis of the ether extract, 7 spots were detected by spraying of H_2SO_4 and heating on the TLC plate. The second spot from the origin line indicated green fluorescence by UV radiation and a green color

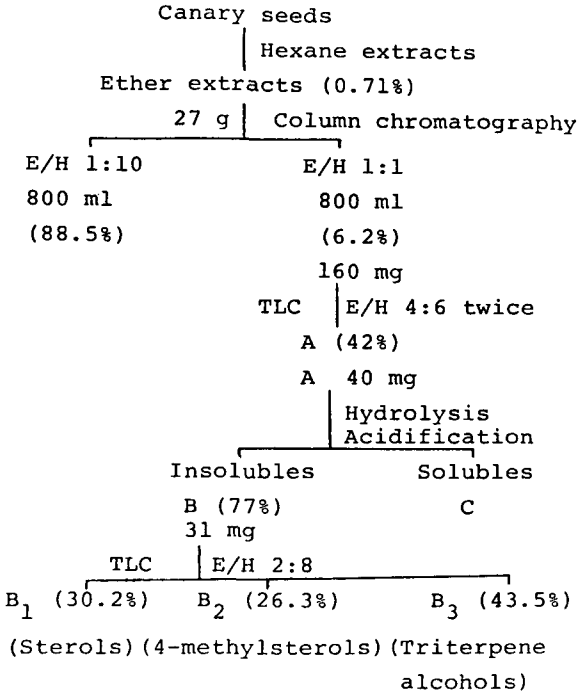

FIG. 1. Separation of the antioxidant and its hydrolyzed products. An abbreviation E/H 4:6 means ether/hexane 4:6, v/v solvent for development in chromatography.

by spraying with $FeCl_3$ reagent for the detection of phenols. The spot was made visible as a light brown spot by spraying with titanium chromogenic reagent (20% $TiCl_4$ in conc. HCl), which can specifically identify the phenolic compounds possessing *ortho*-dihydroxy groups (11). The spot also showed a pale color on a red background after spraying with DPPH reagent (0.001% solution of 1,1-diphenyl-2-picrylhydrazine, Nakarai Kagaku-Yakuhin Co. Ltd., Kyoto) for the detection of the antioxidants with radical scavenging ability (12,13).

The ether extract (300 mg) was separated by TLC using 5 plates of 0.5-mm thickness and the same solvent for development, and 7 bands were detected similarly. The

TABLE I

Antioxidant Activity of Seed Extracts[a]

Common name (Japanese)	Genus and species	Solvent	Yields (%)[b]	PF_{20}[c]	PF_{50}	PF_{100}
Canary seed	*Phalaris canariensis*	Hexane	5.10	1.08	1.07	1.05
		Ether	1.10	1.65	1.59	1.62
		MeOH	2.48	1.35	1.21	1.22
Hemp seed	*Cannabis sativa*	Ether	2.83	0.88	0.97	0.98
		MeOH	2.82	1.36	1.28	1.28
Italian millet (AWA)	*Setaria italica*	Ether	0.44	1.02	1.00	1.02
		MeOH	1.70	1.14	1.14	1.15
Common millet (Kibi)	*Panicum miliaceum*	Ether	0.30	1.00	1.00	1.01
		MeOH	1.47	1.18	1.13	1.12
Banyard millet (Hie)	*Panicum Crus-galli*	Ether	0.71	1.12	1.20	1.12
		MeOH	1.50	1.17	1.12	1.13
Niger Seed	*Guizotia abyssinica*	Ether	3.47	1.08	1.05	1.06
		MeOH	3.66	1.23	1.22	1.25
γ-Tocopherol		———	———	0.88	1.59	1.85
BHA		———	———	1.59	1.92	2.81
BHT		———	———	10.36	10.28	9.40

[a]The raw extracts were examined by the oven test using lard. The extract (0.1%) was added.

[b]The yield (%) from the seeds.

[c]Protection factor, $PF_n = T_n/T_n^\circ$. T_n and T_n° are the times for POV of lard to attain n. T_{20}° = 212, T_{50}° = 263 and T_{100}° = 281 (hr).

extracts from each band with ether were subjected to the antioxidant test with BHT, BHA and γ-tocopherol and the results are shown in Table II. Only the extract from the second band past the origin line showed significantly high antioxidant activity as expected from the color reaction of the qualitative TLC. Table III shows the antioxidant activity of the extract from the second band for sardine oil, compared with those of BHT, BHA and γ-tocopherol. The data also show that the extract has a qualitatively excellent activity.

The procedures for separation of the antioxidant on a large scale are shown in Figure 1. The ether extract (27 g) was fractionated first by chromatography on a silicic acid column (2.8 x 31 cm, Wakogel C-200, Wako Junyaku-Kōgyō Co. Ltd., Osaka) and the concentrate was then refined twice by preparative TLC using ether/ hexane, 4:6 for development. The refined product (A) was a white solid with a melting point of 170 C. It showed only one spot in the same location with that of the extract from the second band in TLC on a same plate. The addition of 3 drops each of 0.001 M $SrCl_2$ solution and NH_3 saturated methanol to 1 ml of methanol solution of the fraction A (ca. 1 mg) produced a brown precipitate. This showed the presence of an *ortho*-dihydroxy group on an aromatic ring in the component A (14).

Spectra of the Antioxidant

The UV spectrum of A was analogous to that of caffeic acid esters reported elsewhere (3). It showed maxima at 217, 236, 243, 300 and 328 nm with intensities 278.5, 178.5, 182.8, 240.2 and 305.5 in $E^{1cm}_{1\%}$, respectively. The location of the maxima was in fair agreement with reported data; dodecyl caffeate 246, 300 and 332 nm (6), and ethyl caffeate 218, 246 and 331 nm (4). The bathochromic shift of the peaks peculiar to *ortho*-dihydroxy phenolic compounds was observed by the addition of H_3BO_3 and sodium acetate to the sample (15). The solution, made up to 10 ml with 2 ml of H_3BO_3 saturated solution, 2 ml of the sample solution and the necessary amount of ethanol, and saturated with a little excess of sodium acetate, showed the maxima at 254, 260, 306 and 352.5 nm with intensities 196.6, 193.2, 178.6 and 384.6 in $E^{1cm}_{1\%}$, respectively.

The IR spectrum of A showed 3490 and 3300 cm^{-1} OH stretching bands. The 3300 cm^{-1} band supported the presence of the intramolecular hydrogen bond which is normal in *ortho*-dihydroxy phenolic compounds. It also showed the strong 2960 and 2800 cm^{-1} CH stretching bands for methyl and methylene groups, the weak 3040 cm^{-1} CH stretching band of an aromatic ring, 1683 cm^{-1} C=O stretching band and 979 cm^{-1} CH deformation of CH=CH band in *trans*-CH=CHCOOR (6), and 863 cm^{-1} 1H and 815 cm^{-1} vicinal 2H out-of-plane deformation of the substituted benzene ring.

The PMR spectrum of A indicated signals at δ 6.70-7.40 (3H one sharp singlet and 3 broad peaks) assigned to protons on the benzene ring, and δ 6.28 and δ 7.56 (each 1H doublet J=16 Hz) assigned to H_a and H_b in *trans* $Ar\text{-}CH_a{=}CH_bCOOR$ (16). The signals in the region of δ 7-10 showed the usual patterns of steryl esters.

Since the spectral data showed the presence of an ester group in the antioxidant component, it was hydrolyzed and the components of acidic and neutral products were subsequently investigated.

Compositions of Hydrolyzed Products

Fraction A (40 mg) was heated with 2 ml of 20% KOH aqueous solution in a sealed tube under a nitrogen atmosphere at 100 C for 1 hr. After cooling, the solution was acidified with diluted HCl and the insoluble material (B, 30 mg) was collected by filtration. It was applied along a line on a 0.5-mm Silica Gel G plate and developed with hexane/ ether, 8:2. Three bands, B_1-B_3, were detected under UV

TABLE II

Antioxidant Activity of the Fractions from Ether Extract of Canary Seeds[a]

Fraction	Yield (%)[b]	PF_{20}	PF_{50}	PF_{100}
1[c]	5.8	1.46	1.32	1.26
2	4.9	8.89	8.86	8.18
3	1.2	1.38	1.36	1.29
4	2.2	1.63	1.54	1.44
5	1.7	1.00	0.98	0.98
6	7.9	1.08	1.16	1.05
7	76.2	1.02	1.11	1.00

[a]The experimental conditions are identical to those for Table I. Each fraction (0.05%) was added.

[b]Percentage in the ether extract.

[c]Separated by TLC. The number for the fraction is same as that of the spots in TLC.

TABLE III

Antioxidant Activity in Sardine Oil[a]

Antioxidant	Added (%)	PF_{20}	PF_{50}	PF_{100}
Canary seeds	0.05	2.93	3.00	3.47
γ-Tocopherol	0.05	0.93	1.26	1.82
BHA	0.02	1.80	1.74	2.31
BHT	0.02	2.00	1.88	2.37

[a]The oven test at 60 C using 3 g sardine oil having the following composition; 14:0—7.5%; 14:1—0.1%; 15.0—0.4%; 16:0—18.4%; 16:1—9.8%; 17:0+16:2—0.4%; 17:1—0.6%; 18:0—3.5%; 18:1—16.9%; 18:2ω6—0.3%; 18:3ω3+20:0—0.8%; 20:1—2.7%; 18:4ω3—2.4%; 22:0—1.6%; 22:1—3.2%; 20:5ω3—15.7%; 24:1—0.7%; 22:5ω6—0.9%; 22:5ω3—1.8%; 22:6ω3—11.1%. T°_{20}=7.5; T°_{50}=17.0; T°_{100}=24.5 (hr).

light after spraying with Rhodamine 6G solution, and were then extracted with ether. The extracts from each were identified as sterol (B_1), 4-methylsterol (B_2) and triterpene alcohol (B_3) fractions, respectively, by comparing their location on a TLC plate with those of reference specimens obtained from the unsaponifiables of wheat germ oil.

The filtrate obtained from the hydrolyzed products of A after removing of B was extracted with ethyl acetate 3 times. The extract (C) was recovered by distillation of the solvent after dehydration with anhydrous Na_2SO_4. The fractions, B, B_1-B_3 and C were subjected to subsequent GLC and GC-MS analyses.

Table IV shows the components identified in B by GLC. Most of the identifications were made by agreement with the reference specimens of their relative retention times and the complete overlapping in the mixed injection of B_1-B_3. Some of the peaks overlapped on the gas chromatogram of B, but they appeared separately in those of B_1-B_3. Sterol, 4-methylsterol and triterpene alcohol fractions from the unsaponifiables of wheat germ oil and the mixture of cycloartenol and 24-methylenecycloartanol (donated by A. Kato, National Chemical Laboratory for Industry, Tsukuba, Japan) were used as the reference specimens. The former showed the peaks for campesterol, sitosterol, obtusifoliol, gramisterol (24-methylenelophenol), cycloartenol and 24-methylenecycloartanol as described elsewhere (17,18). All were identified in B. Brassicasterol and Δ7-stigmastenol were identified by agreement of their relative retention times with those reported in the literature (17). The rapid development of color in the B_2 Liebermann-Burchard reaction, typical for Δ7-sterols (19), supported the presence of gramisterol (4-α-methyl-24-methylene-Δ7-cholestene-3β-ol). The components found in B are normally found in the unsaponifiables of several vegetable oils (17,18).

In GC-MS, the components of peaks 3,6,7 and 9 of Table IV showed their molecular ion peaks (M^+) at m/e 400, 414, 412 and 426, respectively, with their fragment ion peaks of M^+-15 (CH_3), M^+-18 (H_2O) and M^+-33 (CH_3 + H_2O). The components of both 3 and 6 showed (M^+-side chain) ion peaks at m/e 273 with (M^+-side chain-H_2O) ion peaks at m/e 255. Each also showed the peaks characteristic for Δ5-sterol (M^+-H_2O-67 [C_5H_7] and M^+-H_2O-93 [C_7H_9]) at 315 and 289 for peak 3 and 329 and 303 for peak 6. These data support identification of components 3 and 6 as respectively, campesterol and sitosterol. Peak 7 showed M^+-side chain at 287 with M^+-side chain-H_2O at 269. The mass numbers of the molecular ion and the fragment ion are evidence that the component of peak 7 chiefly consists of gramisterol. The component of peak 9 showed the fragment ion peaks for sterol having a 9,19-cyclopropane ring and a 4,4-dimethyl group; m/e 286 (M^+-$C_9H_{16}O$ [A ring + 19 CH_2]) and 339 (M^+-$C_5H_{11}O$ [by scission between 1-C and 2-C, and between 4-C and 5-C]). It also showed m/e 297 peak (M^+-side chain-H_2O) and m/e 295 peak (M^+-side chain-H_2O-2H). The results support identification of the component in peak 9 as cycloartenol (20-22).

The TMS product of C showed only one GLC peak, which was identified as the TMS derivative of caffeic acid by comparing the relative retention time and complete overlapping in the mixed injection of it with the reference specimen. Further identification was made by comparing its fragmentation pattern with that of the reference specimen (caffeic acid, reagent grade, Tokyo Kasei Organic Chemicals Co. Ltd., Tokyo) by GC-MS. The mass spectra of the sample showed M^+ ion at m/e 396 (intensity, 89.0), M^+-CH_3 at m/e 381 (24.1), M^+-$(CH_3)_3SiO$ at m/e 307 (5.8) and a parent ion, M^+-2$(CH_3)_3SiO$, at m/e 218 (100). The results are in agreement with the data obtained by the reference specimen.

Conclusively, the antioxidant components in canary seeds were identified as the esters of caffeic acid with cycloartenol, gramisterol, sitosterol and campesterol as main components and 24-methylenecycloartanol, obtusifoliol, brassicasterol and Δ7-stigmasternol as minor components.

Characteristics of the Antioxidant

This study presented the antioxidant activity of the caffeic acid esters only for lard and sardine oil at 60 C by an oven test. However, the excellent antioxidant activities of caffeic acid and its esters have been reported under the various conditions in the previous papers. The extract from oat containing caffeic and ferulic acid esters, and synthesized dodecyl caffeate and caffeic acid were reported to be effective as antioxidants for oat oil at 100 C (4) and for corn oil at 90 C (23) by an oxygen absorption method, respectively. Recently, chlorogenic and caffeic acids were shown spectrophotometrically to be effective antioxidants in the coupled oxidation of β-carotene and linoleic acid in comparison with ferulic and *p*-coumaric acids and others in the oxidation at 37 C (24). Therefore, the antioxidant from canary seeds would be similarly effective for vegetable oils. The data in Tables I and II show that the content of the caffeate in the fatty oil of canary seeds is ca. 0.5%. The

TABLE IV

Compositions of Sterols and Triterpene Alcohols from the Caffeic Acid Esters in Canary Seeds

Peak no.	Fraction[a]	Component	Relative RT[b] Found	Relative RT[b] Reported[c]	Peak area (%)
1	B_1	Cholesterol	0.64	0.61	t
2	B_1	Brassicasterol	0.72	0.69	0.3
3	B_1	Campesterol	0.82	0.81	14.1
4	B_2	Unknown	0.86	0.88	0.6
5	B_2	Obtusifoliol	0.96	0.95	3.7
6	B_1	Sitosterol	1.00	1.00	15.7
7	B_2	Gramisterol	1.12	1.13	21.7
8	B_1	Δ7-Stigmastenol	1.18	1.18	0.1
9	B_3	Cycloartenol	1.24	1.23	38.1
10	B_3	24-Methylene-Cycloartenol	1.37	1.38	5.4
11	B_3	Unknown	1.49	———	0.3

[a]The symbols are the same with those for the spots in TLC of the hydrolyzed products of B: B_1—sterols, B_2—4-methylsterols and B_3—triterpene alcohols.

[b]Relative retention times obtained with OV-17.

[c]Data from references 17 and 18.

fatty acid compositions of the fatty oil (the hexane extract) obtained by GLC are 16:0—11.1%, 18:0—0.7%, 18:1—31.3%, 18:2—54.5% and 18:3—2.4%. The composition is typical in the oleic-linoleic oils such as sunflower oil (25). The high antioxidant content in the canary seeds would play an important role to protect the highly unsaturated oil in the seeds from oxidation. The curves of the periodical change of POV for the oven test of lard containing the caffeates A, BHA, and γ-tocopherol are shown in Figure 2. The curve for γ-tocopherol gradually shifted higher even in the low POV region at the initial stage of oxidation. The pattern of the curves for the caffeates resembled those for BHT: they are held at the low level of POV during the induction period and rise rapidly at the end of the period, as shown in Figure 2. The characteristics of the antioxidant are also demonstrated by the PF_n values in Tables I-III.

Experiments to determine the practical use of knowing the effect of synergists and the organoleptic taste test are in progress.

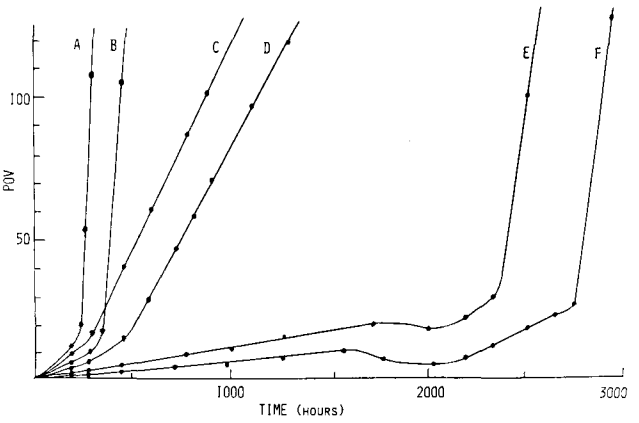

FIG. 2. Effect of the antioxidants on rate of autoxidation of lard in the oven test at 60 C. A: control; B: ether extract from canary seeds; C: γ-tocopherol; D: BHA; E: caffeate (fraction A); F: BHT. Each was added at 0.05% except for BHA and BHT (0.1%).

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