Antioxidant Effects of d-Tocopherols at Different Concentrations in Oils During Microwave Heating

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The effects of d-tocopherols at different concentrations (50 to 1000 ppm) on the oxidative stability of ethyl linoleate and tocopherol-stripped oils were investigated under microwave heating conditions. Purified substrate oils were prepared by aluminum oxide column chromatography. After the addition of tocopherols (α-, β-, γ- or δ-) to the oils, peroxide, carbonyl and *p*-anisidine values were measured in the samples after heating in a microwave oven. Further, the residual amount of tocopherol homologues in the oils after heating was determined by high-performance liquid chromatography for evaluation of their effects at different concentrations on oxidative deterioration. Microwave heating resulted in some acceleration in the oxidation of the purified substrate oils. Optimum concentrations of tocopherols required to increase oxidative stability were 100 ppm for α-, 150–200 ppm for β- or γ- and 500 ppm for δ-tocopherol, respectively. The antioxidant effect of tocopherols decreased in the order $\alpha > \beta \fallingdotseq \gamma > \delta$ at each level, in all substrates. Therefore, α-tocopherol was consumed first, followed by β- or γ-tocopherol, and δ-tocopherol was consumed more slowly. The tocopherols had no further significant antioxidant activity ($P > 0.05$) at concentrations higher than 500 ppm.

KEY WORDS: Anisidine value, antioxidant effects, carbonyl value, high-performance liquid chromatography, microwave heating, peroxide value, purified vegetable oils, tocopherol homologues.

Tocopherols, in addition to possessing vitamin E function (1), are the major natural antioxidants in foods and are important for the stability of vegetable oils. This antioxidant aspect of tocopherol chemistry has been studied extensively (2–8), especially with regard to the relative antioxidant activities of α-, γ- and δ-tocopherols, the forms commonly found in vegetable oils. *In vitro*, their antioxidant efficacy decreases in the order $\gamma > \delta \gg \alpha$, whereas α-tocopherol is most effective as vitamin E (9). At high concentration, α-tocopherol can cause a prooxidant effect *in vitro* and consequently increase the autoxidation rate of fatty acids (10,11). There is some difficulty, however, in adequately evaluating the antioxidant activity of tocopherols because this activity is frequently influenced by the types of oils and fats used as substrates, the concentration of tocopherols and the method of evaluation. Moreover, there appears to be widespread confusion concerning both the relative and the absolute antioxidant effectiveness *in vitro* of individual tocopherols that make up vitamin E. Thus, some workers (2,12) have reported that γ-tocopherol is more effective than α-tocopherol at preventing *in vitro* lipid oxidation. Others (13) have reported that relative effectiveness depends on the experimental conditions.

Tocopherols are not volatile, as are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and they don't cause off-flavor, as does tertiary butylhydroquinone (TBHQ), or discoloration, as does lecithin at higher temperatures (14); therefore, they can be used for stabilization of heated oils. Microwave ovens are considered among the most energy-efficient types of ovens, and foods exposed to microwaves are heated as the result of molecular excitation (15). The application of microwave processing in both home and institutional meal preparation has increased because of its speed and convenience as compared to conventional cooking methods (16,17). Relatively little information is available on the fate of tocopherol homologues themselves during fat oxidation, even though this information could be important in evaluating possible changes in vitamin E content during cooking or storage. Although tocopherols have received much attention as important antioxidants, not only in foods and vegetable oils (18,19), but also in biological systems (20,21), the detailed effects of various tocopherols at different concentrations on oxidative stability of purified vegetable oils have not been reported.

The present study was designed to evaluate the effects of α-, β-, γ- and δ-tocopherols at different concentrations on oxidative stability of purified substrate oils when heated in a microwave oven. Therefore, it was important to use tocopherol-stripped oil with impurities removed to investigate precisely the antioxidant effectiveness of individual tocopherols during microwave heating. In this paper, all substrate oils were purified by column chromatography and contained no tocopherols.

EXPERIMENTAL PROCEDURES

Ethyl ester. Commercially available ethyl linoleate without additives (Wako Pure Chemical Ind. Ltd., Osaka, Japan) was twice refined by silicic acid column chromatography immediately before use. Purity of the ethyl ester after chromatography was better than 99.5% in gas chromatographic analysis. Chemical indices after purification were not detectable for peroxide value, less than 0.2 for carbonyl value and 0.1 for anisidine value.

Vegetable oils. In Japan (22), rapeseed oil is the major edible oil, followed by soybean oil, and they are used in shortenings, margarines and cooking oils. Recently, the consumption of palm oil has been increasing rapidly. Therefore, we chose these three oils with different degrees of unsaturation [by iodine values (IV)]. Refined rapeseed (IV = 108.5), soybean (IV = 132.0) and palm (IV = 52.5) oils were purchased from Nacalai Tesque Inc. (Kyoto, Japan). In accordance with the manufacturer's statement, the oils were degummed, bleached, alkali-refined and deodorized, and were free of added antioxidants and preservations. Tocopherol-stripped oils were prepared from these oils by aluminum oxide column chromatography (23) immediately prior to use. The aluminum oxide was activated at 200°C for 10 h before use after washing fully with deionized water. For solid samples (palm oil) at

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ambient temperature (25°C), the column was heated with a ribbon heater (40°C), and the sample was simultaneously released with a flow of nitrogen gas. Free fatty acids, phospholipids and chlorophylls of sample oils before and after purification were determined by AOCS Methods Ca 5a-40, Ca 19-86 and Cc 13d-55, respectively (24). Tocopherols in commercial and tocopherol-stripped oils were determined by normal-phase high-performance liquid chromatography (HPLC) as described later. Fatty acid methyl esters were prepared from tocopherol-stripped oils as outlined earlier (25). The compositions were analyzed by a Shimadzu Model GC-14A (Shimadzu Instruments Inc., Kyoto, Japan) as described previously (26).

Tocopherols. The α-, β-, γ- and δ-tocopherols were purchased from Eisai Co. (Tokyo, Japan). In accordance with the manufacturer's statement, all the tocopherols were of the d-form (RRR-), and purity of each tocopherol was above 98.5% as determined by HPLC. Each tocopherol was added directly to the purified ethyl linoleate or tocopherol-stripped oils as an *n*-hexane solution (50–1000 ppm), which was stirred at 25°C for 20 min to ensure complete dissolution of the tocopherols in the oils. The hexane was removed by evaporation under a stream of nitrogen before microwave heating. A control sample with no added tocopherols was prepared under the same conditions as described above. 2,2,5,7,8-Pentamethyl-6-hydroxychroman (Eisai Co.) was used as internal standard for determination of tocopherol homologues.

Microwave heating treatment. Purified substrate (ethyl ester or tocopherol-stripped) oils containing 50, 100, 200, 500 or 1000 ppm α-, β-, γ- or δ-tocopherol were separately prepared. Each 5.0-g prepared sample was placed in a 25-mL brown bottle and sealed with polyethylene film. All oil samples were prepared in replicate and then simultaneously heated at a frequency of 2,450 MHz for each time period in a microwave oven, as reported previously (27). Treatment time varied from 4 to 20 min, at intervals of 4 min. The temperatures of the oils immediately after each treatment were determined with a chromel-alumel thermocouple as described earlier (28). A control sample was prepared for each exposure time, under the same conditions, without added tocopherols.

HPLC. A 0.5-g portion of each oil sample, before and after microwave heating, was placed in a 5-mL brown volumetric flask and diluted with the mobile phase for HPLC as described below. The tocopherol analyses were performed in a Shimadzu Model LC-6A HPLC (Shimadzu Instruments Inc.) connected to a Shimadzu Chromatopac C-R6B recording data processor. The chromatographic system consisted of a normal-bonded phase (5 μm) Shim-pack CLC-SIL (M) column (250 × 4.6 mm i.d.; Shimadzu) protected by a 1-cm amino guard column (Shim-pack G). The mobile phase was *n*-hexane/1,4-dioxane/ethanol (490:10:1, vol/vol/vol) at 2.0 mL/min. Samples (5 μL) were injected with a fully loaded 20-μL loop. The tocopherols were monitored with a fluorescence spectrophotometer set at excitation wavelength 296 nm and emission wavelength 320 nm and quantitated by comparison to the content before microwave heating. The other HPLC conditions were as described previously (27).

Evaluation of effects of tocopherols on oxidation of substrate oils. After fixed time intervals, the carbonyl values and *p*-anisidine values of the heated oils were determined by JOCS methods 2•4•22-73 and 2•4•26-81 (29). Peroxide values and IVs were determined by AOAC methods 28•022 and 28•020 (30).

Statistical analysis of experimental data. To illustrate the relative stability of tocopherols during microwave heating, the values before treatment were normalized to 100. Each reported value is the means of four measurements from two replicates. The data were subjected to one-way analysis of variance with a randomized complete block design to partition the effect of different parameters (31). Significant differences among treatment means were separated by using Duncan's multiple range test, at a level of $P < 0.05$ (32).

RESULTS AND DISCUSSION

Fatty acid composition of oils after purification. The commercially available vegetable oils passed through the alumina columns were termed purified vegetable oils. The purified oils contained no tocopherols, chlorophylls, free fatty acids or phospholipids (data not shown). These results agree with those of Yoshida *et al.* (27). Table 1 presents the profiles of the fatty acid compositions of purified oils before microwave heating. The fatty acid compositions of commercial and purified (tocopherol-stripped) oils were not significantly different ($P > 0.05$). The highest degree of unsaturation as defined in Table 1 (33) was shown by soybean oil (1.54), followed by rapeseed (1.39) and palm (0.55) oils.

Relative stability of tocopherols in a model system. In the first experiment, the relative stability of individual tocopherols during microwave heating was compared at different concentrations in ethyl linoleate as a model system. Figure 1 shows the tocopherol stability at different concentrations (50 to 500 ppm) in ethyl linoleate when heated in a microwave oven. A significant change ($P < 0.05$) in tocopherols was observed after heating, and the change depended on the differences in the types and amounts of tocopherols. The greater the tocopherol level,

TABLE 1

Fatty Acid Composition of Tocopherol-Stripped Vegetable Oils Before Microwave Heating[a]

	Oil		
Fatty acid	Rapeseed	Soybean	Palm
12:0	—[b]	—	1.6
14:0	0.1	0.1	4.8
16:0	4.4	11.2	44.6
16:1	0.3	0.1	0.3
18:0	1.7	3.7	3.7
18:1	58.7	23.6	36.0
18:2	22.9	53.7	8.5
18:3	11.2	7.5	0.5
20:0	0.2	0.1	—
22:0	0.2	—	—
22:1	0.3	—	—
Saturates	6.6	15.1	54.7
Unsaturates	93.4	84.9	45.3
Degree of unsaturation[c]	1.39	1.54	0.55

[a] Each value is an average of three determinations and expressed as wt% of total fatty acid methyl esters.

[b] Not detected.

[c] Degree of unsaturation is calculated as [%palmitoleic + %oleic + %erucic + %linoleic × 2 + % linolenic × 3] ÷ 100.

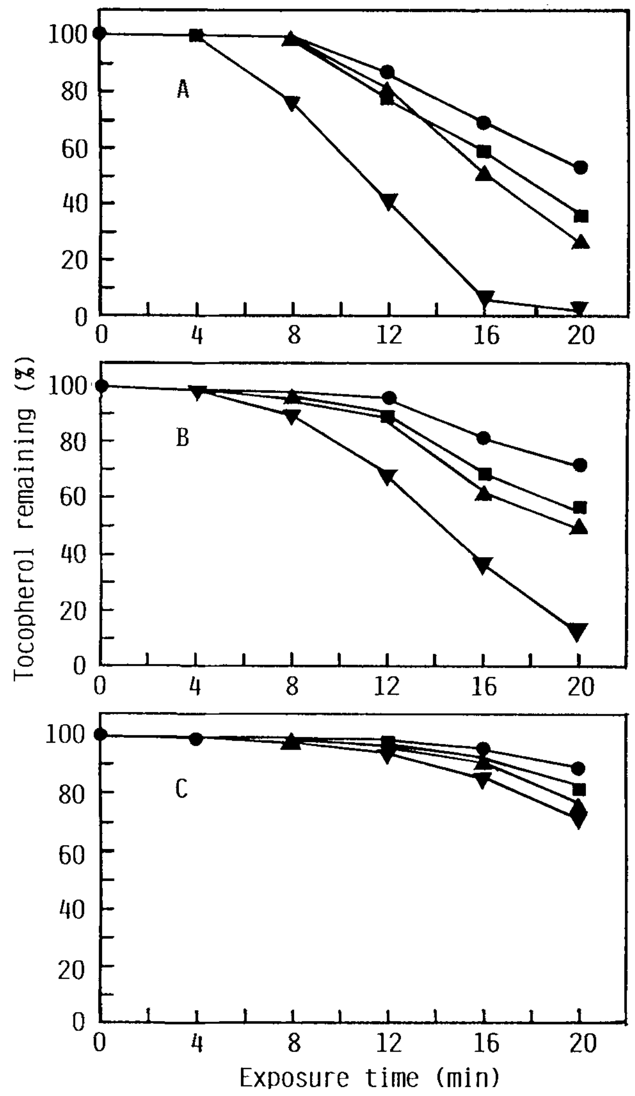

FIG. 1. Effects of microwave heating (frequency 2,450 MHz) on loss of d-tocopherols at different concentrations in ethyl linoleate: A, 50 ppm; B, 100 ppm; C, 500 ppm. ▼, α-Tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

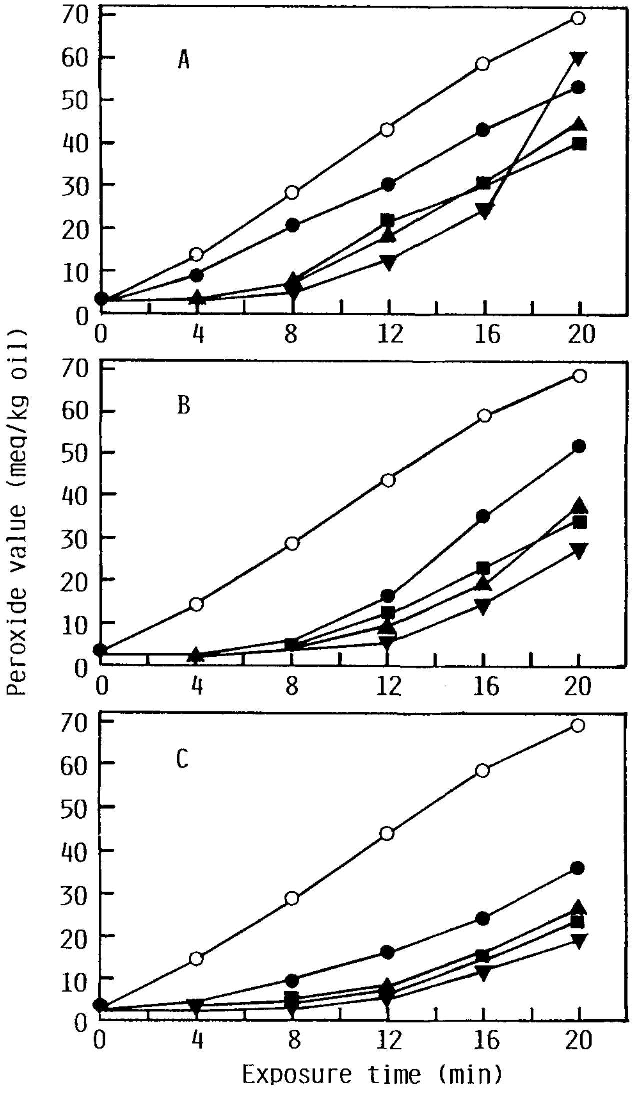

FIG. 2. Effects of different concentrations of d-tocoperhols on peroxide values of ethyl linoleate during microwave heating (frequency 2,450 MHz): A, 50 ppm; B, 100 ppm; C, 500 ppm. ○, Control; ▼, α-tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

the less was the loss of individual tocopherols. Also, with longer exposure to microwave energy, the loss became significantly smaller ($P < 0.05$) with increased levels of tocopherols: At 500 ppm, over 70% of their original levels were still retained after 20 min of heating. Data generated at concentrations from 500 to 1000 ppm demonstrated similar patterns to those obtained at 500 ppm (omitted from this paper). The highest oxidative rate was seen in α-, followed by β- or γ- and δ-tocopherols. This order did not depend on the amounts of the added tocopherols. However, the lower the level of tocopherol added to ethyl linoleate, the greater was the reduction in the amount of tocopherol. At 50 ppm, α-tocopherol was reduced to only 8% of the initial level after 16 min of heating and, thereafter, fast oxidation must have taken place because it was depleted at 20 min (Fig. 1).

Oxidative stability of ethyl linoleate after addition of tocopherols. The oxidation of ethyl linoleate during microwave heating after the addition of individual tocopherols was measured by peroxide, carbonyl and anisidine values (Figs. 2–4, respectively). All tocopherols had some effect in stabilizing ethyl linoleate, which increased with concentration in ethyl linoleate. The rate of peroxide formation decreased after the addition of tocopherols, and the decrease became greater as the concentration of the tocopherol in ethyl linoleate increased. The highest antioxidant activity was seen in α-, followed by β- or γ- and δ-tocopherols. These effects became more pronounced when the concentration was increased from 100 to 500 ppm. At 100 ppm, α-, β- or γ-tocopherol prolonged the induction period and protected ethyl linoleate appreciably, whereas δ-tocopherol seemed significantly ($P < 0.05$) less effective than the others.

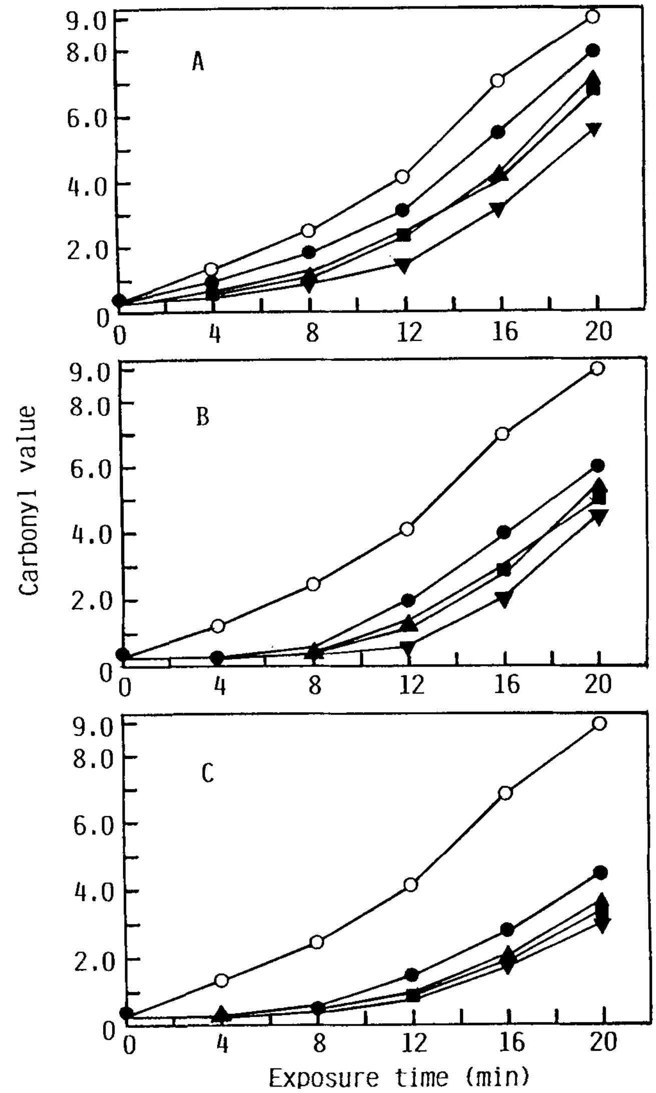

FIG. 3. Effects of different concentrations of d-tocopherols on carbonyl values of ethyl linoleate during microwave heating (frequency 2,450 MHz): A, 50 ppm; B, 100 ppm; C, 500 ppm. ○, Control; ▼, α-tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

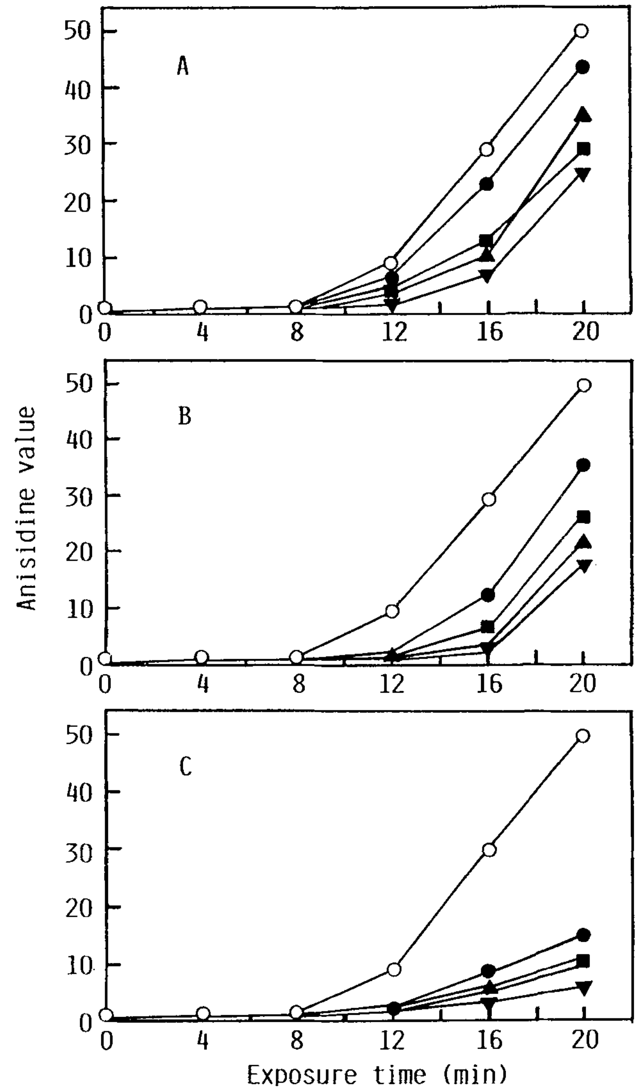

FIG. 4. Effects of different concentrations of d-tocopherols on anisidine values of ethyl linoleate during microwave heating (frequency 2,450 MHz): A, 50 ppm; B, 100 ppm; C, 500 ppm. ○, Control; ▼, α-tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

When purified ethyl linoleate was heated with microwave energy, the lower the amount of tocopherol in the oil, the greater became the carbonyl and anisidine values as a secondary indicator of oxidative deterioration (Figs. 3 and 4). The antioxidant activity of tocopherols was more significantly ($P < 0.05$) effective at concentrations above 100 ppm than at 50 ppm. Figures 3 and 4 show that α-tocopherol at all levels was significantly ($P < 0.05$) more effective than the others in stabilizing ethyl linoleate, followed by β- or γ- and δ-tocopherols in a decreasing order. Peroxide and carbonyl values were not statistically significant ($P > 0.05$) between β- and γ-tocopherols. No appreciable change in anisidine value was observed up to 8 min of heating, but this changed rapidly thereafter. The low anisidine value, however, refers only to minor progress in the secondary reactions of ethyl linoleate. All tocopherols suppressed the formation of anisidine-reactive compounds. The efficiency decreased in the order $\alpha > \beta \fallingdotseq \gamma > \delta$-tocopherols. In general, the peroxide values do not represent the absolute state of oxidation of an oil because of the transitory nature of the peroxides (34). But, from the results shown in Figures 2–4, peroxide value adequately represented the changes in oxidation of the oils with the tocopherol additives.

Comparison of oxidative stability of tocopherols in purified rapeseed oil. In the second experiment, to clarify the antioxidant effects of tocopherols in purified oils during microwave heating, each tocopherol was added to purified rapeseed oil at levels ranging from 50 to 1000 ppm. Figure 5 shows the α-, β- and δ-tocopherol stabilities at different concentrations in the oil when heated in a microwave oven. When added within the concentration range from 500 to 1000 ppm, 80% of their original levels still remained after 20 min of heating. However, there were

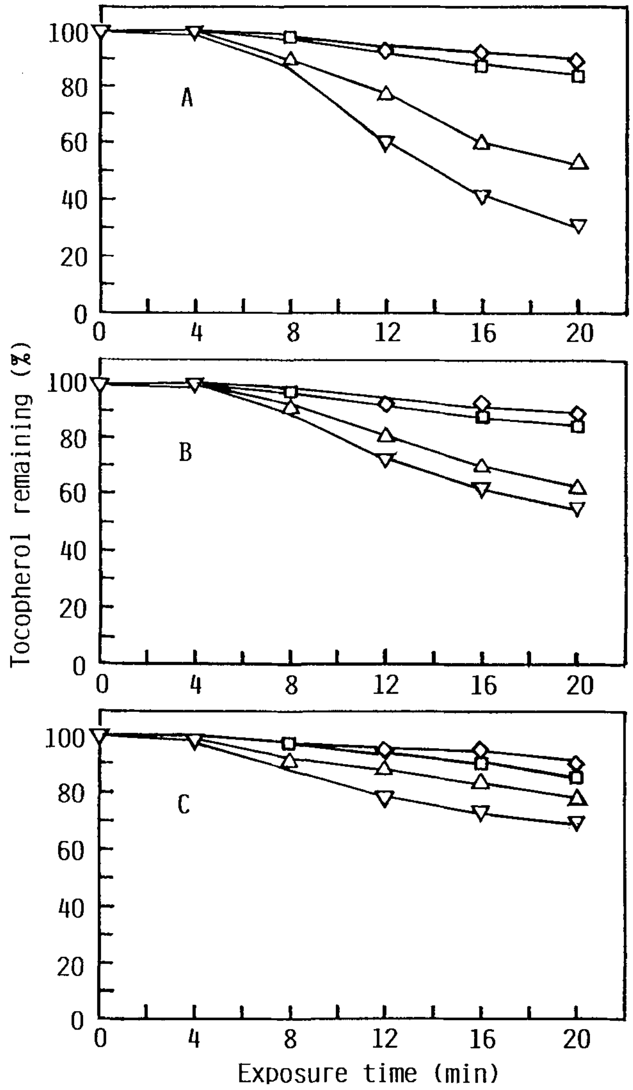

FIG. 5. Effects of microwave heating (frequency 2,450 MHz) on loss of d-tocopherols at different concentrations in tocopherol-stripped rapeseed oil: A, α-tocopherol; B, β-tocopherol; C, δ-tocopherol; ▽, 50 ppm; △, 100 ppm; □, 500 ppm; open diamond, 1000 ppm. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

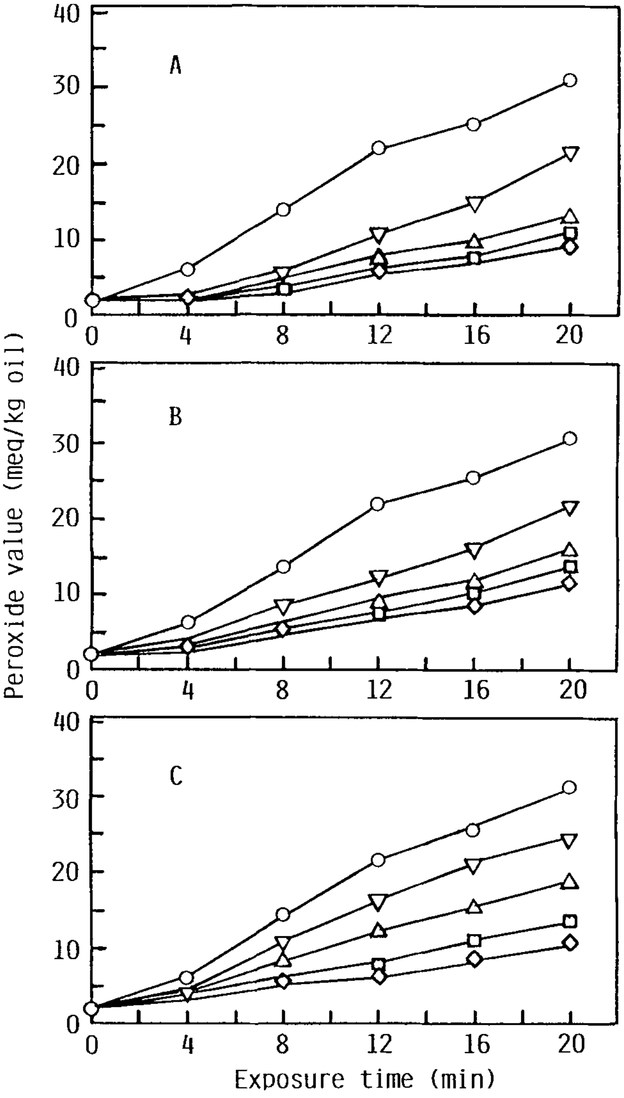

FIG. 6. Effects of different concentrations of d-tocopherols on peroxide values of tocopherol-stripped rapeseed oil during microwave heating (frequency 2,450 MHz): A, α-tocopherol; B, β-tocopherol; C, δ-tocopherol; ○, control; ▽, 50 ppm; △, 100 ppm; □, 500 ppm; open diamond, 1000 ppm. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

significant differences ($P < 0.05$) among homologues in the loss of tocopherol at concentrations of 50 and 100 ppm during microwave heating. The highest relative stability at both concentrations was seen in δ-, followed by β- and α-tocopherols in a decreasing order. After 20 min of heating, δ-, β- and α-tocopherols at 50 ppm were retained at 70, 60 and 30% of the original concentrations, respectively, and at 100 ppm were retained at 80, 65 and 55%, respectively, of the original concentrations. These trends are in agreement with those for purified ethyl linoleate as mentioned previously (Fig. 1). The data at the concentrations for γ-tocopherol were omitted from Figure 5 because they were not significantly different ($P > 0.05$) from the results of β-tocopherol.

Figure 6 shows the effects of different tocopherol concentrations on peroxide values of purified rapeseed oil when heated in a microwave oven. The tocopherols significantly ($P < 0.05$) inhibited the development of peroxides of the oil during microwave heating. At concentrations of greater than 100 ppm, the efficiency of α- or β-tocopherol became significantly ($P < 0.05$) more than that of the control or of δ-tocopherol. The efficiency of δ-tocopherol as an antioxidant was apparent at different concentrations (50–1000 ppm), and was not much greater ($P > 0.05$) than that of α- or β-tocopherol.

Relationship between oxidative stability and antioxidative activity of tocopherols in purified oils. Figure 7 shows the effects of microwave heating on oxidative stabilities of tocopherols at 200 ppm in three purified vegetable oils. The addition of tocopherols at a level of 200 ppm to these purified oils resulted in the retardation of oxidative deterioration when heated in a microwave oven. The extent of oxidative deterioration was suppressed by α-, followed by γ- or β- and δ-tocopherols. The antioxidative

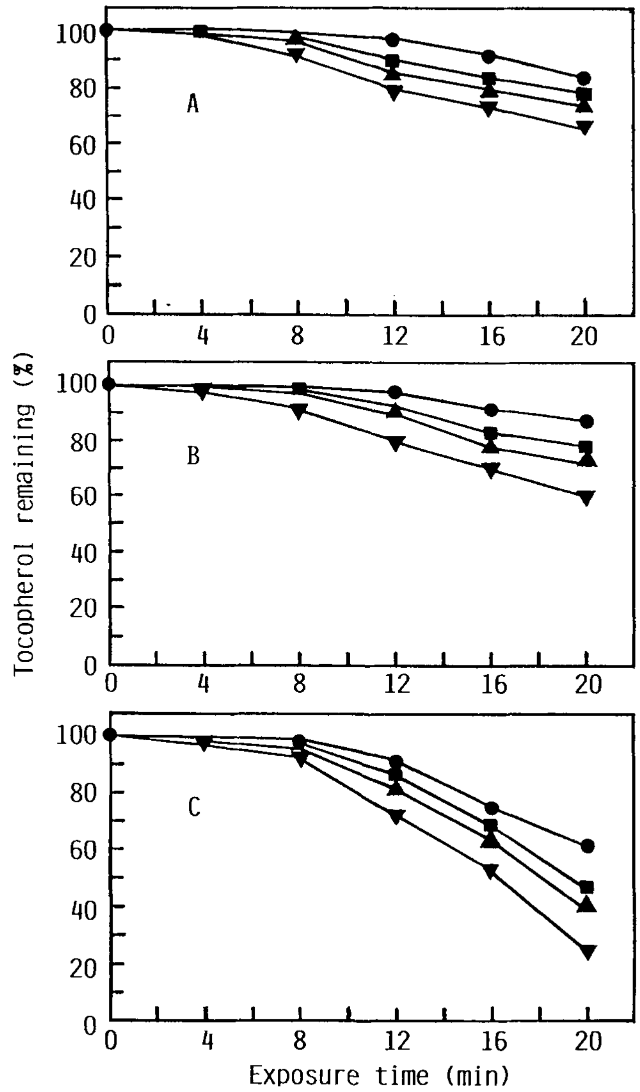

FIG. 7. Effects of microwave heating (frequency 2,450 MHz) on loss of d-tocopherols at 200 ppm levels in tocopherol-stripped oils: A, rapeseed; B, soybean; C, palm: ▼, α-tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

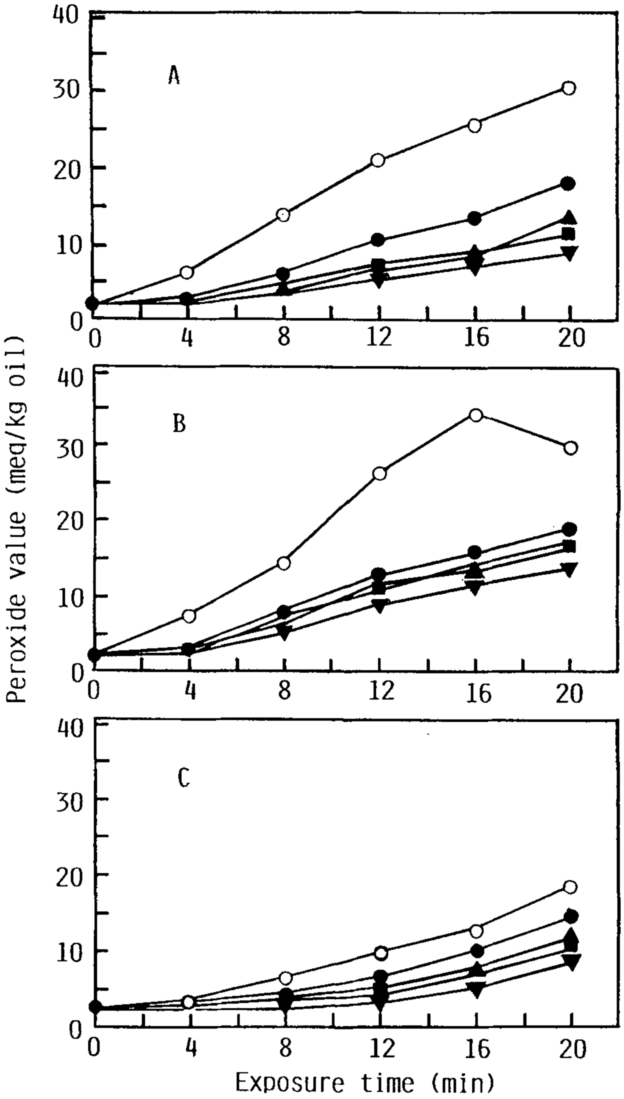

FIG. 8. Effects of d-tocopherols at 200 ppm levels on peroxide values of tocopherol-stripped oil during microwave heating (frequency 2,450 MHz): A, rapeseed; B, soybean; C, palm: ○, control; ▼, α-tocopherol; ▲, β-tocopherol; ■, γ-tocopherol; ●, δ-tocopherol. All data points represent the means of four measurements from two replicates, and the standard errors are within the size of the symbols.

effectiveness was significantly ($P < 0.05$) greater in the rapeseed and soybean oils than in the palm oil (Fig. 8). Judging from the remaining amounts of tocopherols (Fig. 7), the highest rate of loss was shown in palm oil, followed by soybean or rapeseed oil, and the oxidative stability decreased in the order $\delta > \gamma \fallingdotseq \beta > \alpha$-tocopherols. In a previous paper (35), we reported that the levels of fatty acids increased in purified vegetable oils when heated in a microwave oven, and that with increased free fatty acid levels, the reduction of tocopherols became greater in palm oil than in rapeseed or soybean oil.

Figure 8 shows the effects of tocopherols at 200 ppm levels on peroxide values of the three purified oils during heating. The tocopherols significantly ($P < 0.05$) inhibited the peroxide value levels. The addition of tocopherols to rapeseed or soybean oil was significantly ($P < 0.05$) more effective than was the addition to palm oil because of relatively low peroxide values that developed in the palm oil control. The effective order had the same trend as described above (Fig. 3).

The effectiveness of tocopherols as lipid antioxidants has been attributed mainly to their ability to break chain reactions by reacting with fatty acid peroxy radicals. α-Tocopherol is the most reactive chain-breaking, phenolic antioxidant known (36). Burton and Ingold (4) reported that the rate of scavengers for peroxy radicals by β- and γ-tocopherols was two-thirds, and δ-tocopherol one-fourth, that of α-tocopherol.

In conclusion, the results suggest that the desirable optimum concentrations of individual tocopherol homologues as antioxidants in purified oils are: 100 ppm for α-, 150–200 ppm for β- or γ- and 500 ppm for δ-tocopherol, respectively. Recently, the Codex Committee on Fats and Oils amended the food additive provisions of all Codex Standards for Fats and Oils and established the following maximum levels in mg/kg (ppm): BHT, 75; TBHQ, 120; BHA, 175 and tocopherols, 500 (37). Comparing these limits to tocopherol contents used in this study, we can see that optimum levels of all the tocopherols were within the limits.

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