Natural Antioxidants Produced in Oxidized Lipid/Amino Acid Browning Reactions

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ABSTRACT: 1-Substituted pyrroles (**1** and **2**) and 1-substituted 2-(1′-hydroxypropyl)pyrroles (**3-5**) were produced in reactions between a lipid peroxidation product, 4,5(*E*)-epoxy-2(*E*)-heptenal, and the amino acid lysine. The antioxidative activity of compounds **1–5** was studied. Oxidative stability was evaluated in refined soybean oil containing compounds **1–5**, butylated hydroxytoluene (BHT), *n*-propyl gallate or L-lysine, at concentrations of 50–200 ppm. Oils were either oxidized at 60°C and oxidation products determined by the thiobarbituric acid-reactive substances assay, or they were oxidized at 110°C by the Rancimat method. Although both methods gave similar results, greater differences were observed at 60°C than at 110°C. Addition of compounds **1–5**, L-lysine, BHT, and propyl gallate significantly ($P < 0.01$) protected the oil against oxidation. The effectiveness order found was: L-lysine << compounds **3–4** < compounds **1–2** < compound **5** ≈ BHT << propyl gallate. *JAOCS 72*, 1571–1575 (1995).

KEY WORDS: Antioxidative activity, BHT, nonenzymatic browning reactions, oxidative stress, oxidized lipid/amino acid reactions, propyl gallate, Rancimat, 1-substituted-pyrroles, TBARS.

Controlling oxidation in natural and processed foods is a difficult aspect of food preservation, even in low-fat foods (1–3). Lipid oxidation not only produces characteristic undesirable odors and flavors, but also decreases the nutritional quality and safety of foods by formation of secondary reaction products during cooking and processing (4–6). Protection of foods against lipid oxidation usually involves exclusion of oxygen by packing in vacuum or inert gases and/or the addition of antioxidants. Antioxidants frequently used include synthetic (mostly phenols) and natural compounds; however, none of them has been completely satisfactory. Butylated hydroxyanisole and butylated hydroxytoluene (BHT), although effective in foods, are suspected to be carcinogenic (7,8). In addition, tocopherols and ascorbic acid, which are now widely used as safe, natural antioxidants, have lower antioxidant activities than do synthetic antioxidants (9). Therefore, much research has been conducted to find safe antioxidants with high activity from natural sources (10,11). Moreover, many other studies have been dedicated to isolate and characterize components, normally present in foods, that possess antioxidative properties. For example, the antioxidative properties of Maillard reaction products have long been known (12), but, a much less developed area has been the study of antioxidative properties of oxidized lipid/protein reaction products.

It is well known that oils and fats are less susceptible to oxidation when mixed with whey powder, wheat flour, casein, or amino acids, and heated at temperatures ranging from 100 to 300°C (13–15). These findings suggest that antioxidant compounds are produced from these heated mixtures, most likely by interaction of the initial lipid oxidation products with reactive groups of amino acids. The compounds responsible for the observed reduction in oxidation have not been fully characterized. Macku and Shibamoto (15) collected the headspace volatiles produced in a mixture of corn oil and glycine, heated at 180°C, and found some heterocyclic compounds that showed antioxidative activity. However, no studies have been dedicated to the nonvolatile fraction of oxidized lipid/amino acid reaction mixtures.

Recent studies from this laboratory identified a compound, 4,5(*E*)-epoxy-2(*E*)-heptenal (EH), that was reactive with the amino groups of amines, amino acids, and proteins (16). EH was first isolated from a Cu/α-tocopherol-induced autoxidation of either butterfat or cod liver oil (17) and, later, as a thermal decomposition product of methyl linolenate hydroperoxides (18). The reaction of EH with L-lysine in a model system has been studied, and different 1-substituted 2-(1′-hydroxypropyl)pyrroles and 1-substituted pyrroles were identified (19). These 1-substituted 2-(1′-hydroxypropyl)pyrroles polymerized spontaneously in solution *via* a reaction that was characterized (20), and the polymers produced exhibited color and fluorescence analogous to the melanoidins isolated from sugar/amino acid reactions. This similarity in color and fluorescence, produced in both oxidized lipid/amino acid and sugar/amino acid reactions, also was observed when both systems were irradiated in a microwave oven (21). In a search for new natural antioxidants, the objective of the present study was to investigate the antioxidative activity of the compounds characterized in the EH/lysine system.

MATERIALS AND METHODS

Materials. Refined soybean oil was obtained from our pilot plant (Instituto de la Grasa, CSIC, Sevilla, Spain). EH was

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prepared from 2(*E*),4(*E*)-heptadienal as described by Swoboda and Peers (17). 2(*E*),4(*E*)-Heptadienal, L-lysine monohydrochloride, and 3-chloroperoxybenzoic acid were purchased from Aldrich Chemical (Milwaukee, WI). 2-Thiobarbituric acid monohydrate was purchased from Merck (Darmstadt, Germany). BHT and *n*-propyl gallate were purchased from Sigma Chemicals Co. (St. Louis, MO). Other analytical-grade reagents and solvents were purchased from commercial sources.

Chemical synthesis of EH/lysine reaction products. 1-(5′-Amino-1′-carboxypentyl)pyrrole (**1**), (Scheme 1), 1-(5′-amino-5′-carboxypentyl)pyrrole (**2**), 1-(5′-amino-1′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole (diasteromers **3** and **4**), and 1-(5′-amino-5′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole (**5**) were isolated by preparative high performance liquid chromatography from an EH/lysine reaction mixture incubated for 16 h at 25°C. Fractionation of the reaction mixture was carried out according to the previously described method (19), and purity and identity of the isolated fractions was studied by ^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry. Compounds **1–5** were chromatographically pure and were prepared just before carrying out antioxidative activity measurements.

Measurement of antioxidative activity. Oxidative stability of refined soybean oil without antioxidants was compared with refined oil samples containing a lyophilized EH/lysine reaction mixture, compounds **1–5**, BHT, *n*-propyl gallate, or L-lysine added at concentrations of 50–200 ppm. Antioxidative activity of the compounds was evaluated by two methods.

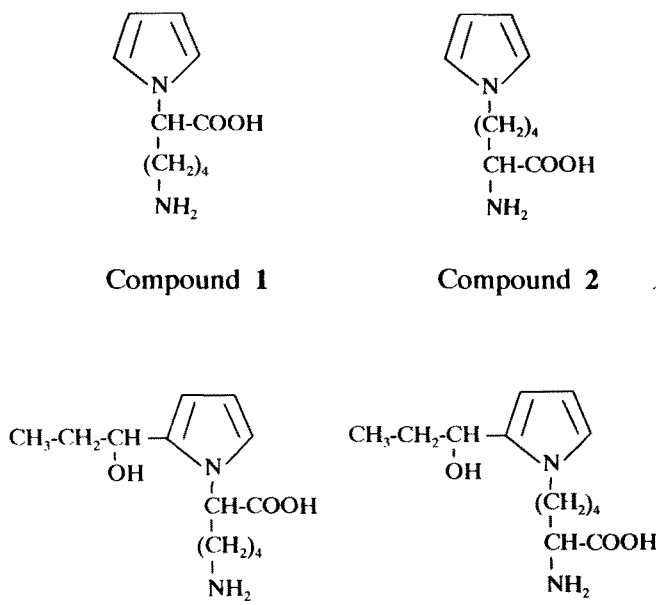
In the first method, oil samples (10 g) were weighed into 90 × 20-mm Petri dishes and oxidized for 72 h in air in the dark at 60°C. Peroxidation was evaluated periodically by the thiobarbituric acid-reactive substances (TBARS) assay as described by Kosugi *et al.* (22). For comparison purposes, a protection index (PI) was defined according to the following equation:

$$PI = 100 - [100 \times (\text{TBARS sample} - \text{TBARS gallate})/(\text{TBARS control oil} - \text{TBARS gallate})] \quad [1]$$

This equation, slightly modified, was used previously for the calculation of an inhibition percentage in a peroxidation process (23). If PI is equal to 100, the compound is as effective as gallate. A PI equal to zero indicates a compound with no protective effect. A PI < 0 indicates a prooxidant effect. This index cannot be applied at the initial time because TBARS of control oil should be higher than TBARS of gallate.

In the second method, oil samples (2 g) were heated at 110°C in a Metrohm Rancimat (Metrohm AG, Herisau, Switzerland). A continuous airstream (15 L/h) was passed through the heated sample, and the volatiles were absorbed in a conductivity cell. Conductivities were continuously monitored until a sudden rise signified the end of the induction period.

Statistical analysis. All results are means of three replicates unless otherwise indicated. Statistical comparisons between several groups were made by analysis of variance. When significant *F* values were obtained, group differences were evaluated by the Student-Newman-Keuls test (24). All statistical procedures were carried out by using *Primer of Biostatistics: The Program* (McGraw-Hill, Inc., New York). Significance level is $P < 0.05$ unless otherwise indicated.



SCHEME 1

RESULTS

An EH/lysine reaction mixture, incubated for 16 h, was lyophilized, added to the oil, and tested for antioxidative activity in a Rancimat apparatus. Table 1 shows the induction periods obtained for untreated oil and oil treated with 60 ppm of either the reaction mixture, BHT, or the individual compounds. The EH/lysine reaction mixture significantly ($P < 0.01$) protected the oil by increasing the induction period by 6.7% compared to the untreated control oil. This induction period was 2.5% longer than the induction period observed for oil treated with BHT.

The EH/lysine reaction mixture was then fractionated into 1-substituted pyrroles (**1** and **2**) and 1-substituted 2-(1′-hydroxypropyl)pyrroles (**3-5**), and the products tested for antioxidative activity. Table 1 shows the induction periods obtained for untreated oil and oil treated with 60 ppm of compounds **1–5**. Compounds **1–2** significantly ($P < 0.01$) increased the induction period compared to the control. The protection was similar for the two compounds and to the one exhibited by the whole reaction mixture. Compounds **3–5** also increased the Rancimat induction period as compared to the control. However, induction periods obtained for these com-

TABLE 1
Rancimat Induction Times of Control Soybean Oil and Soybean Oils Containing 4,5(*E*)-Epoxy-2(*E*)-Heptenal/Lysine Reaction Mixture, Butylated Hydroxytoluene (BHT), or Compounds 1–5 at a Level of 60 ppm[a]

Compound tested	Induction period[b] (h)	n[c]
None (control)	11.29 ± 0.26[d]	19
Reaction mixture	12.05 ± 0.27[e,f]	8
BHT	11.76 ± 0.29[f,g]	8
1	12.32 ± 0.62[e]	5
2	12.05 ± 0.40[e,f]	6
3	11.42 ± 0.25[d,g]	6
4	11.47 ± 0.27[d,g]	6
5	11.54 ± 0.70[d,f]	5

[a]Values are expressed as mean ± SD.
[b]Means in the same column with different superscript letters (d–g) are significantly different ($P < 0.05$).
[c]Number of samples.

pounds were lower than those for BHT, the reaction mixture, and compounds **1–2**.

A second procedure was used for testing the antioxidative activity of compounds **1–5**. Figure 1 shows TBARS production in soybean oil heated in an oven at 60°C for up to 72 h. The major protection observed, at the three levels tested, was by propyl gallate. Protection exhibited by compounds **1** and **2** at 50 (Fig. 1A), 100 (Fig. 1B), and 200 (Fig. 1C) ppm was similar, within the two compounds, as well as to the activity exhibited by BHT. L-Lysine also provided significant protection ($P < 0.01$), but this protection was significantly ($P < 0.01$) lower than activity of compounds **1–2**. The PI values obtained after incubating the oil 72 h at 60°C showed propyl gallate to have the major protection (PI = 100), and all other compounds showed lower activity (Table 2). Protection by compounds **1–2** was similar, although significantly ($P < 0.01$) lower than the observed values for BHT. PI was 25–26% at 50 ppm, 33–38% at 100 ppm, and 56–58% at 200 ppm for compounds **1–2**. PI for BHT were 31, 38, and 61%, respectively, at the three concentrations assayed. L-Lysine also significantly ($P < 0.01$) protected the oil at 50 ppm, although its PI was low (6%), but this protection increased at 100 ppm (16%) and 200 ppm (28%).

Figure 2 shows TBARS values obtained for the oil treated with compounds **3–5** at 50 (Fig. 2A), 100 (Fig. 2B), and 200 (Fig. 2C) ppm. Analogously to compounds **1–2**, the antioxidant activity exhibited by these compounds was similar to BHT and lower than propyl gallate. PI values obtained for samples incubated for 72 h at 60°C are presented in Table 2. Compounds **3–5** significantly ($P < 0.01$) protected soybean oil against oxidation, and this protection was slightly lower than that exhibited by compounds **1–2** at 50 ppm. However, at 100 and 200 ppm treatment levels, compound **5** was a better antioxidant ($P < 0.05$) than compounds **1** and **2**. The calculated PI for compounds **3–5** were 17–22% at 50 ppm, and these percentages increased to 28–43% at 100 ppm, and to 45–59% at 200 ppm. Diasteromers **3–4** always protected similarly at the three levels assayed, suggesting that isomerism

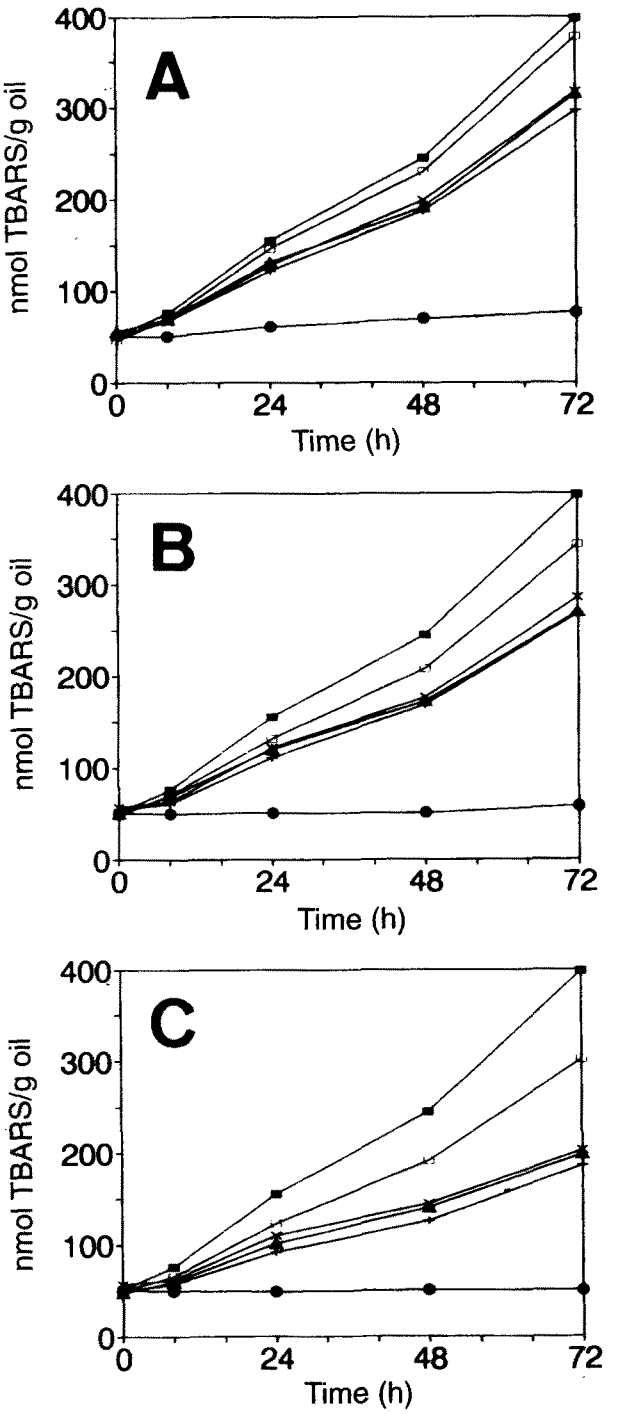


FIG. 1. Effects of compounds **1** (x) and **2** (▲), L-lysine (□), butylated hydroxytoluene (+), and propyl gallate (●), at 50 (A), 100 (B), and 200 (C) ppm, on soybean oil oxidation (■) measured as thiobarbituric acid-reactive substances (TBARS) formation. Results represent the mean of three assays.

was not an important factor in the antioxidative activity. In contrast to compounds **1–2**, the position of the carboxyl group seemed to play some role in these compounds, and protection of compound **5** was significantly ($P < 0.01$) higher than compounds **3–4** at 100 and 200 ppm.

TABLE 2
Protection Index Calculated for Soybean Oils Containing Compounds 1–5, Lysine, BHT, or Propyl Gallate After Incubating for 72 h at 60°C[a]

Compound tested	Compound level (ppm)		
	50	100	200
None	0	0	0
1	25	33	56
2	26	38	58
3	22	28	45
4	17	29	47
5	18	43	59
Lysine	6	16	28
BHT	31	38	61
Propyl gallate	100	100	100

[a]Abbreviation as in Table 1.

DISCUSSION

Results showed that, analogously to Maillard browning reaction products, some oxidized lipid/amino acid browning reaction products also played a role in the antioxidative activity of foods. When the EH/lysine reaction mixture was lyophilized and tested for antioxidative activity, it significantly ($P < 0.01$) increased the induction period of a soybean oil when added at a level of 60 ppm. The compounds produced in the EH/lysine reaction mixture were mainly 1-substituted pyrroles (**1–2**), as well as 1-substituted 2-(1′-hydroxypropyl)pyrroles (**3–5**). All of them exhibited a protective effect that was equivalent to BHT. When the protective effect was compared with the effect produced by propyl gallate, compounds **1–5** had PI values of about 17–25% at 50 ppm, 28–43% at 100 ppm, and of 45–59% at 200 ppm. Therefore, these compounds have an antioxidant effect. This antioxidative activity seemed to be mainly related to the heterocyclic structure (common for all of them), and only 10–15% differences in the antioxidative activity (as compared with propyl gallate) were observed with the introduction of different substituents at positions 1 or 2 of the pyrrole ring. Antioxidative activity of 1-methylpyrrole was previously observed by Macku and Shibamoto (15).

Although conclusions obtained with the two procedures used in this study were similar, the differences observed in TBARS values in the system oxidized at 60°C were much higher than those obtained with the Rancimat method at 110°C. These results might be related to the limitations of high-temperature stability tests previously described by Frankel (1). Pyrroles at high temperatures and in the presence of air are likely to be degraded. Therefore, the evaluation of antioxidative activity of these compounds was better when it was carried out under the conditions of 60°C than under Rancimat conditions at 110°C.

Results obtained in this study agreed with previous findings on antioxidative properties of amino acids (25–27). However, the antioxidative activity in this study was considerably increased when the amino acids reacted with lipid oxidation products to yield pyrroles. These heterocyclic compounds, which have been shown to be produced during *in vitro* oxidation of polyunsaturated fatty acids in the presence of amino compounds (Ref. 28; Zamora, R., and F.J. Hidalgo, unpublished results), might produce additional stability in foods during their processing or storage. There is an increased interest in the potential beneficial effects obtained from con-

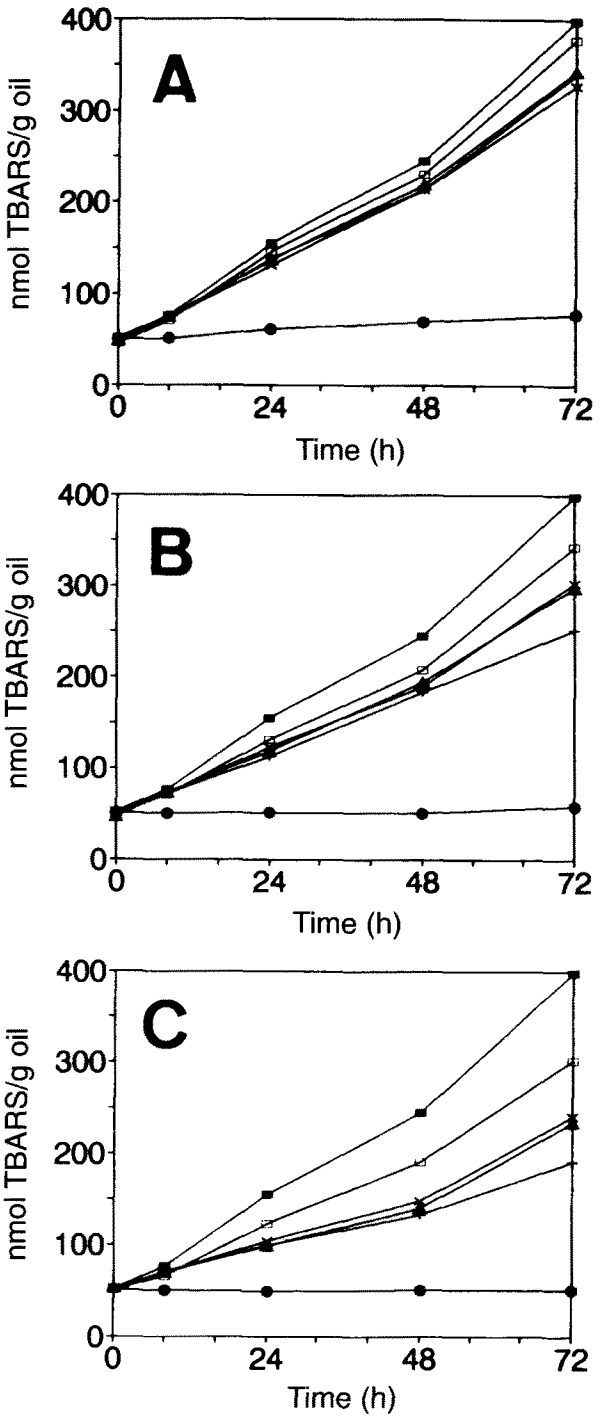


FIG. 2. Effects of compounds **3** (×), **4** (▲), and **5** (+), L-lysine (□), and propyl gallate (●), at 50 (A), 100 (B), and 200 (C) ppm, on soybean oil oxidation (■) measured as TBARS formation. Results represent the mean of three assays. See Figure 1 for abbreviation.

suming antioxidant-rich nutrients (29–31). The results obtained in this study suggest that, in addition to the well known antioxidants, other compounds also are able to act as antioxidants, and they might either be taken unsuspectedly in the diet or even produced during normal *in vivo* oxidative stress. Therefore, oxidized lipid–protein reactions, in addition to the well known negative quality and nutritional consequences in food (32), might also play a positive role by blocking toxic lipid peroxides both *in vitro* and *in vivo* and, at the same time, produce amino acid derivatives with increased antioxidative activity. These derivatives would decrease or prevent new lipid peroxidation. Additional studies are being carried out to determine the proportion in which pyrrole derivatives are produced in foods, and to study their antioxidative role in the presence of other antioxidants.

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