Antioxidative Activity of (*E*)-2-Octenal/Amino Acids Reaction Products

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The effects of several compounds, previously identified in nonenzymatic browning reactions between oxidized lipids and amino acids, on protecting bulk vegetable oils against oxidation were evaluated to investigate if these naturally formed products may contribute to the overall antioxidative activity of foods. Two amino acid residue analogs, *N*-(carbobenzyloxy)-L-histidine (*Z*-His) and N^2-(carbobenzyloxy)-L-lysine (*Z*-Lys), and their products of reaction with (*E*)-2-octenal, the Michael adduct **1** and two pyridinium salts, **2** and **3**, were added at three levels of concentration (50, 100, and 200 ppm) to soybean oil which was oxidized under air in the dark at 60 °C. Oil peroxidation was evaluated by using the thiobarbituric acid-reactive substances (TBARS) assay. Compounds **1**–**3** significantly decreased TBARS formation on soybean oil ($p < 0.05$) and showed diverse activity as compared with synthetic antioxidants. The order of effectiveness obtained was **1** ≪ **2** ≈ butylated hydroxytoluene ≈ **3** < propyl gallate. These results suggest that some reactions between oxidized lipids and amino acids may contribute to the antioxidant activity of foods in addition to the development of color and fluorescence.

Keywords: *Antioxidative activity; nonenzymatic browning; lipid peroxidation; lysine modification; histidine modification*

INTRODUCTION

The Maillard reaction between reducing sugars and amino acids involves condensation, dehydration, and polymerization (Hodge, 1953). As a result of this complex reaction, a variety of byproducts, intermediates, and brown pigments (melanoidins) are produced, which may contribute to the flavor, antioxidative activity, and color of food (Yamaguchi et al., 1981).

The antioxidative activity of Maillard reaction products was first observed by Franzke and Iwainsky (1954), who reported the effect of browning reaction products on the oxidative stability of margarine. Later, the formation of antioxidative Maillard reaction products from model systems was extensively studied (Park and Kim, 1983; Lingnert and Ericksson, 1980); more recently, volatile products of this reaction have also been shown to possess antioxidative activity (Elizalde et al., 1991; Eiserich and Shibamoto, 1994).

In addition to reducing sugars, other carbonyl compounds, and particularly lipid peroxidation products, are also able to react with amino groups, producing brown macromolecular pigments with properties similar to those of melanoidins (Eriksson, 1987; Karel, 1984). In this context, previous research from this laboratory has shown that volatile short-chain aldehydes having a 4,5-epoxy-1-oxo-2-pentene system, which are produced during lipid peroxidation, are very reactive with amines, amino acids, and proteins, producing brown color and fluorescence (Hidalgo and Zamora, 1993a). The development of color and fluorescence in this system is a complex polymerization reaction that involves in a first step the formation of 1-alkyl-2-(1′-hydroxyalkyl)pyrroles (Zamora and Hidalgo, 1994). The later polymerization of these compounds produces the melanoidin-like polymers which are responsible for the color and fluorescence observed (Hidalgo and Zamora, 1993b).

Analogously to this similarity found in color and fluorescence development on carbonyl–amine reactions derived from both sugars and lipid peroxidation products, the formation of products with antioxidative properties in the reactions between oxidized lipids and amino acids might be expected. Thus, it is well-known that oils and fats are less susceptible to oxidation when they are mixed with whey powder, wheat flour, casein, or amino acids and then heated at temperatures ranging from 100 to 300 °C (Lips, 1951; Janicek and Pokorny, 1961; Kawashima et al., 1977; Dworschák and Szabó, 1986). Using headspace extracts of heated vegetable oil/amino acid model systems, Macku and Shibamoto (1991a,b) identified several sulfur- and nitrogen-containing heterocycles that possessed antioxidative activity. However, the potential antioxidative activity of nonvolatile low molecular weight products of these reactions has been much less studied.

In the present study, several compounds previously identified in the reaction of the lipid peroxidation product (*E*)-2-octenal with lysine and histidine analogs (Alaiz and Girón, 1994; Alaiz and Barragán, 1994) were evaluated for antioxidative activity.

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's pilot plant (Instituto de la Grasa, CSIC, Sevilla, Spain). 2-Thiobarbituric acid monohydrate (TBA) was purchased from Merck (Darmstadt, Germany). (*E*)-2-Octenal and N^2-(carbobenzyloxy)-L-lysine (*Z*-Lys) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Butylated hydroxytoluene (BHT), *n*-propyl gallate, and *N*-(carbobenzyloxy)-L-histidine (*Z*-His) were purchased from Sigma Chemical Co. (St. Louis, MO). MN-Kiesegel 60 (0.063–0.2 mm particle size) for column chromatography and Alugram analytical plates (20 × 20 cm) with fluorescent indicator for TLC were obtained from Macherey Nagel (Düren, Germany). Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

Synthesis of *N*-(Carbobenzyloxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine Dihydrate (Isomeric Mixtures) (1). Compound **1**, as a mixture of two Michael adducts (structures for the compounds used in this study are given in

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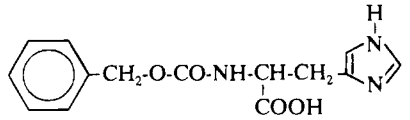
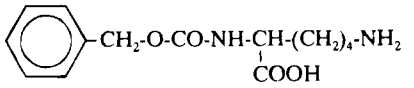


Z-His



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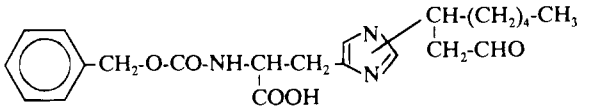
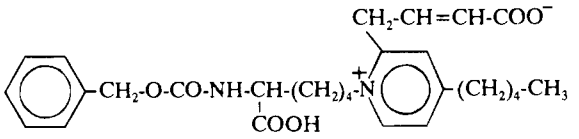
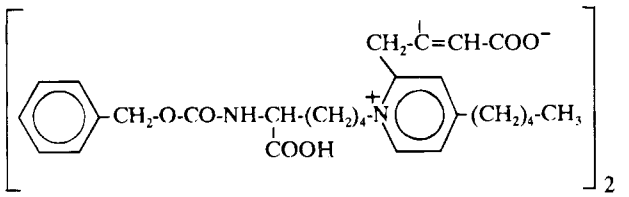


Compound 1



Compound 2



Compound 3

Figure 1. Chemical structures of compounds tested for antioxidative activity in this study.

Figure 1), was obtained by reaction of *Z*-His with (*E*)-2-octenal (Alaiz and Girón, 1994). A reaction mixture (100 mL) containing 25 mM *Z*-His, 25 mM (*E*)-2-octenal, and 100 mM sodium phosphate (pH 7.0) was incubated at 37 °C for 24 h. After that time, the mixture was freezed-dried and the obtained residue treated with 5 mL of methanol. The inorganic salts formed were filtered out, and the solution was concentrated using a stream of nitrogen. The residue was then fractionated by column chromatography using Kiesegel 60 (50 g) as adsorbent and chloroform–methanol (2:1) as eluent. The collected fractions were examined by TLC, using 1-propanol–water (16:1) as eluent and UV light (254 nm) or 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole spray reagent (Dickinson and Jacobsen, 1970) for detection. TLC pure crystalline compound **1** was obtained with a yield of 14.4%. It afforded a single spot of $R_f = 0.30$ in TLC.

Syntheses of 1-(N^2-(Carbobenzyloxy)-L-lysyl)-2-[3′-carboxy-2′-(*E*)-propen-1′-yl]-4-pentylpyridinium Betaine (2) and Bis[1-(N^2-carbobenzyloxy)-L-lysyl]-2-(3′-carboxy-2′-propene-1′-diyl)-4-pentylpyridinium Betaine (Isomeric Mixture) (3). These quaternary pyridinium salts (Figure 1) were obtained by reaction of *Z*-Lys with (*E*)-2-octenal (Alaiz and Barragán, 1994). A reaction mixture (400 mL) containing 25 mM *Z*-Lys, 25 mM (*E*)-2-octenal, and 100 mM sodium phosphate (pH 7.0) was incubated at 37 °C for 24 h. After that time, the reaction mixture was freezed-dried and the residue treated with 25 mL of methanol. The inorganic salts

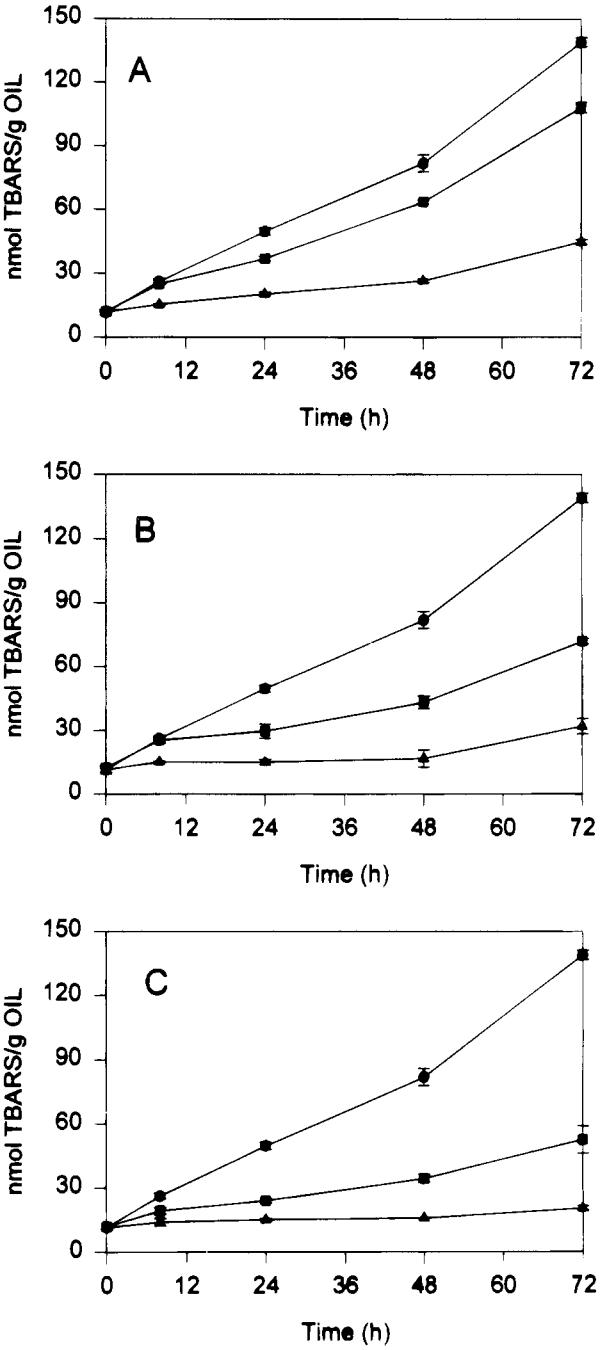


Figure 2. Effects of BHT (■) and propyl gallate (▲), at 50 (A), 100 (B), and 200 (C) ppm, on soybean oil oxidation (●) measured as thiobarbituric acid-reactive substances (TBARS) formation. Results represent the mean ± SD of three assays.

formed were filtered out, and the solution was concentrated using a stream of nitrogen. The obtained residue was extracted first with 25 mL of diethyl ether to remove the lipids and then with 25 mL of chloroform. The chloroformic extract was concentrated under nitrogen and then fractionated by column chromatography using Kiesegel 60 (50 g) as adsorbent and chloroform–methanol (2:3) as eluent. The collected fractions were examined by TLC, using 1-propanol–water (16:1) as eluent and UV light (254 nm) or molybdatophosphoric acid hydrate pulverization reagent for detection. TLC pure crystalline compounds **2** and **3** were obtained with yields of 3.1 and 6.1%, respectively. They afforded single spots of $R_f = 0.26$ and 0.08, respectively, in TLC.

Measurement of Antioxidative Activity of BHT, Propyl Gallate, Z-His, Z-Lys, and Compounds 1–3. Stripped soybean oil with no antioxidant was compared with samples containing BHT, propyl gallate, *Z*-His, *Z*-Lys, and compounds **1–3**, added at concentrations of 50, 100, and 200 ppm. Oil

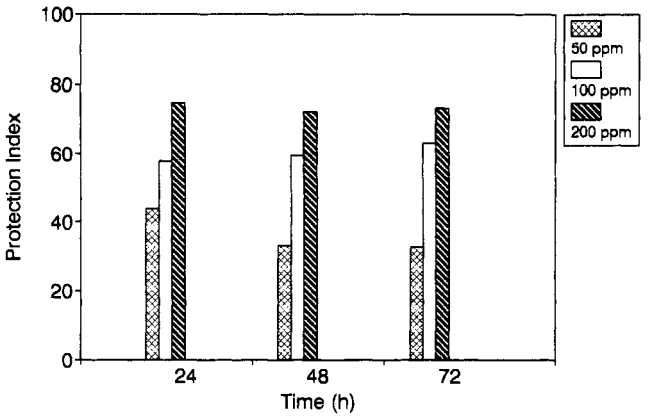


Figure 3. Effect of incubation time on the protection index (PI) of BHT. PI values were calculated from the TBARS values using the formula described under Experimental Procedures.

samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 72 h under air in the dark at 60 °C. Peroxidation was evaluated periodically by using the thiobarbituric acid-reactive substances (TBARS) assay as described by Kosugi et al. (1989). For comparison purposes, a protection index (PI) was defined according to the following equation:

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

This equation, slightly modified, was used previously for the calculation of an inhibition percentage in a peroxidation process (Hidalgo et al., 1990). PI equal to 100 meant that the compound tested was as effective as gallate. PI equal to zero meant that the compound tested had no protective effect. A PI < 0 meant that the compound tested had a prooxidant effect. This index can only be applied at $t > 0$, because TBARS of oil should be higher than TBARS of gallate.

Statistical Analysis. All results are expressed as mean values of three experiments. Statistical comparisons between two groups were made using Student's t test. With several groups, ANOVA was used. When significant F values were obtained, group differences were evaluated by the Student–Newman–Keuls test (Snedecor and Cochran, 1980). All statistical procedures were carried out using *Primer of Biostatistics: The Program* (McGraw-Hill: New York). Significance level is $p < 0.05$ unless otherwise indicated.

RESULTS AND DISCUSSION

Antioxidative Activity of BHT and Propyl Gallate. BHT and propyl gallate were first used to test the selected procedure for antioxidant activity measurement and for reference in the comparison with the data obtained for the different compounds tested. BHT and propyl gallate are representative of nonpolar lipophilic antioxidants and polar antioxidants with high hydrophilic–lipophilic balance, respectively (Frankel et al., 1994; Porter, 1993). Figure 2 shows the TBARS produced in the oil samples treated with 50 (A), 100 (B), and 200 (C) ppm of BHT and propyl gallate. Propyl gallate always slowed the development of TBARS much more effectively than BHT, and, therefore, it should be considered more effective. These results are in agreement with the general rule postulated by Porter (Porter, 1980, 1993; Porter et al., 1989) that in foods of low surface-to-volume ratio (e.g., the soybean oil used) polar antioxidants, such as propyl gallate, are more effective than nonpolar antioxidants. The decrease in TBARS production of propyl gallate was significant after only 8 h at a concentration of 50 ppm. The significant

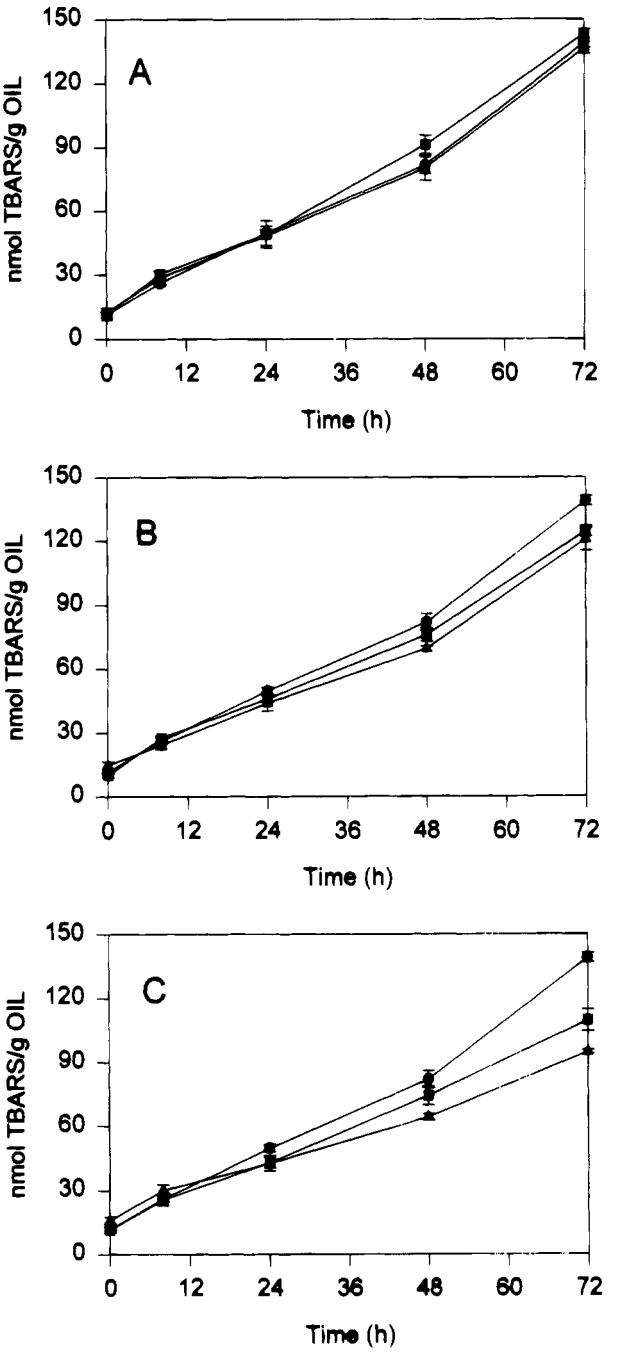


Figure 4. Effects of Z-His (■) and compound **1** (▲), at 50 (A), 100 (B), and 200 (C) ppm, on soybean oil oxidation (●) measured as thiobarbituric acid-reactive substances (TBARS) formation. Results represent the mean ± SD of three assays.

decrease in TBARS for samples treated with BHT was observed after 24 h of incubation at levels of 50 and 100 ppm and after 8 h when 200 ppm was used. The PI for BHT was calculated at the different times and concentrations used. At $t \geq 24$ h, the PI obtained for BHT, and also for many of the compounds studied, did not suffer big changes as a function of time. Figure 3 shows the values of PI obtained for BHT at the three concentrations studied at 24, 48, and 72 h. These values of PI at the different concentrations and times gave a good idea of the effectiveness of the antioxidant at that concentration and time and were used in the comparisons among the different compounds. Thus, BHT was about 60–70% less effective than propyl gallate at 50 ppm at the three times, 40% at 100 ppm, and only 25–30% at 200 ppm.

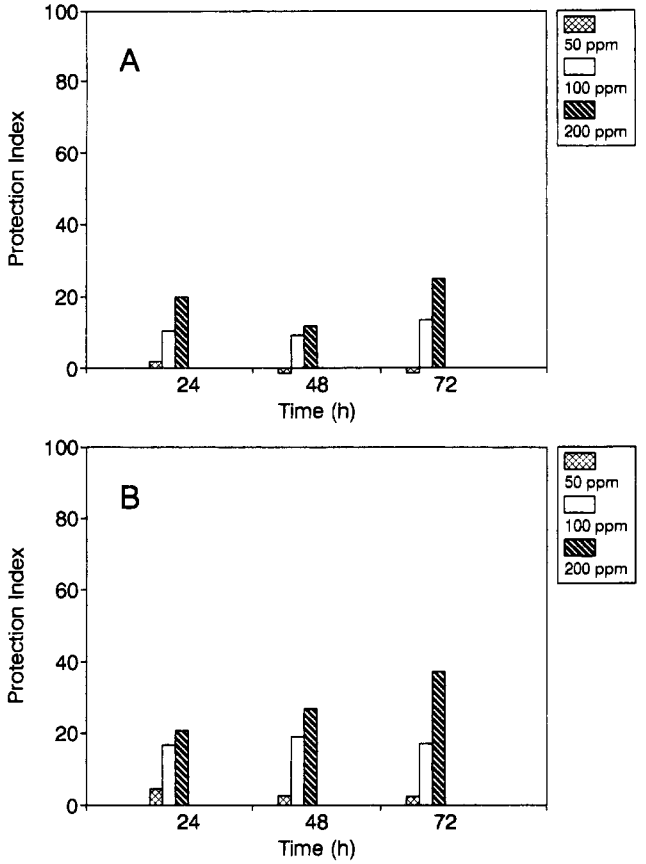


Figure 5. Effect of incubation time on the protection index (PI) of *Z*-His (A) and compound **1** (B). PI values were calculated from the TBARS values using the formula described under Experimental Procedures.

Antioxidative Activity of *Z*-His and Compound 1. Figure 4 shows the development of TBARS as a function of time for soybean oil treated with *Z*-His and compound **1** at 50 (A), 100 (B), and 200 (C) ppm. Amino acids have been reported to act as antioxidants or prooxidants or to have no effect on the oxidation of lipids (Chen and Nawar, 1991). *Z*-His was assayed to test if the protecting group of the amino acid had some effect on the antioxidative activity and if the hypothetical antioxidative activity of compound **1** was due to the starting amino acid or to the (*E*)-2-octenal/amino acid reaction product itself. *Z*-His did not decrease the production of TBARS as a function of the time at 50 ppm. However, it exhibited a significant protection on TBARS production at 100 ppm after 72 h and at 200 ppm after 24 h. Its PI values at these two concentrations were 10 and 15–25, respectively (Figure 5A). The Michael adduct **1** produced in the reaction of *Z*-His with (*E*)-2-octenal was slightly better as antioxidant. It also did not decrease TBARS production at 50 ppm and showed significant differences at 100 ppm after 48 h and at 200 ppm after 24 h. However, TBARS production of oil treated with 200 ppm of compound **1** was significantly lower than TBARS production of oil treated with the same concentration of *Z*-His after 48 h of oxidation. Its PI values at these last two concentrations were 20 and 20–40, respectively (Figure 5B). Therefore, the reaction of *Z*-His with (*E*)-2-octenal produced an adduct that effectively protected soybean oil against TBARS production. However, this protection was small at the concentrations used in this study. At 200 ppm, it had only 20–40% of the effectiveness of gallate when BHT had 70–75% effectiveness.

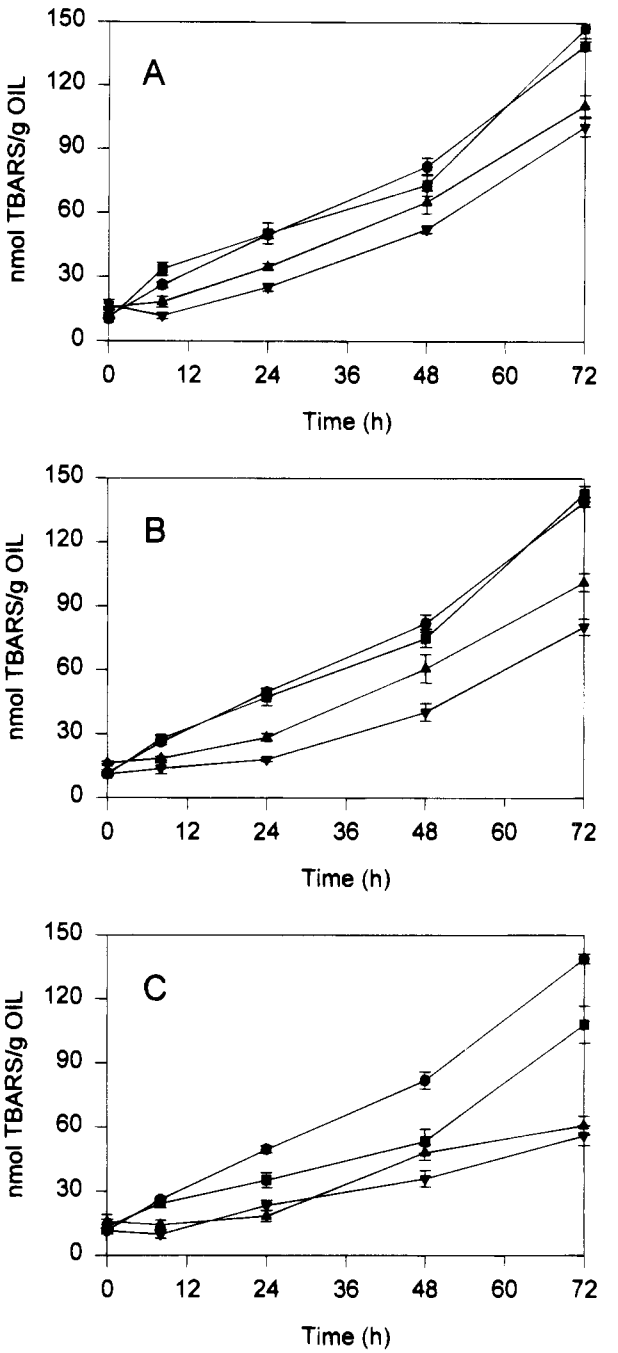


Figure 6. Effects of *Z*-Lys (■), compound **2** (▲), and compound **3** (▼), at 50 (A), 100 (B), and 200 (C) ppm, on soybean oil oxidation (●) measured as thiobarbituric acid-reactive substances (TBARS) formation. Results represent the mean ± SD of three assays.

Antioxidative Activity of *Z*-Lys and Compounds 2 and 3. Analogously to *Z*-His, *Z*-Lys was also tested for antioxidative activity (Figure 6). *Z*-Lys only decreased significantly TBARS production at 200 ppm after 24 h. Its PI was 30–40 at this concentration (Figure 7A). Its reaction with (*E*)-2-octenal produced two pyridinium salts, compounds **2** and **3**, that protected much more efficiently than the *Z*-Lys itself. These reaction products decreased significantly TBARS production at 50 ppm after only 8 h. Their PI values at the three concentrations studied were 30–50, 35–60, and 50–90, respectively, for compound **2**, and 40–80, 60–90, and 70–80, respectively, for compound **3**. Both compounds **2** and **3** protected more efficiently than BHT at short oxidation times (between 8 and 48 h) and were

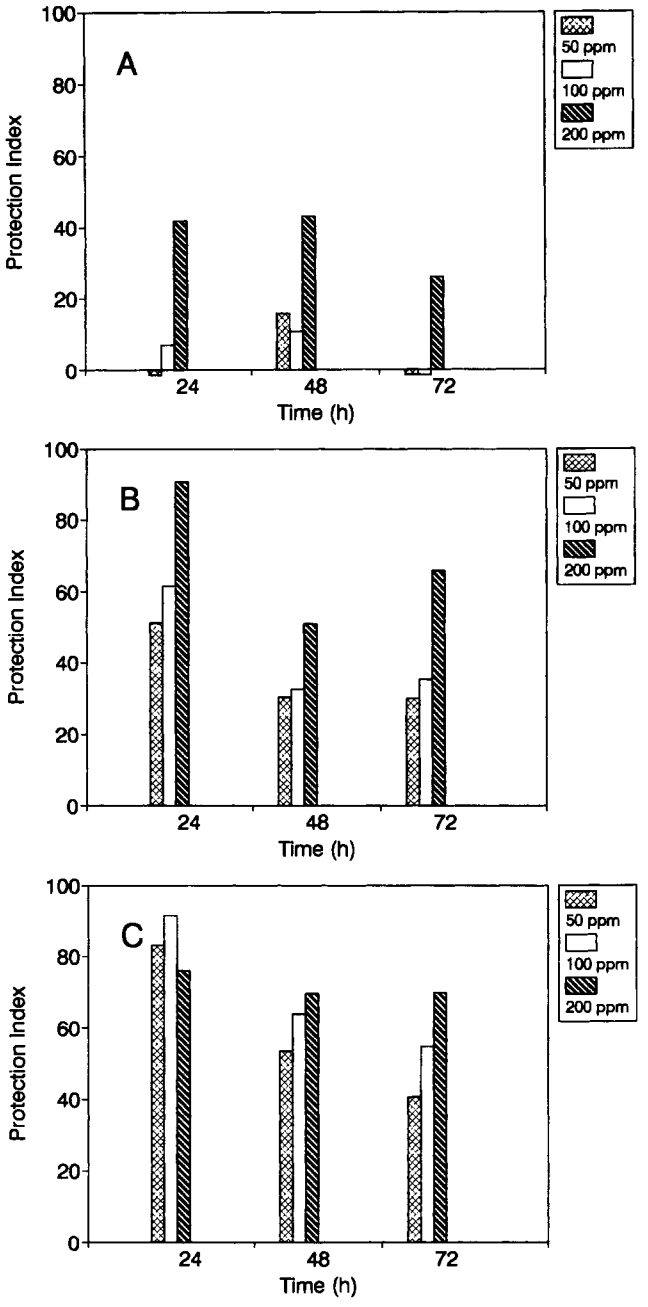


Figure 7. Effect of incubation time on the protection index (PI) of Z-Lys (A), compound **2** (B), and compound **3** (C). PI values were calculated from the TBARS values using the formula described under Experimental Procedures.

comparable with it at higher times. Compound **3** protected more effectively than compound **2** at 50 and 100 ppm for oxidation times higher than 24 h.

The above results suggest that, in addition to contributing to the color and fluorescence development in foods, the reactions between oxidized lipids and amino acids may also contribute to the antioxidative activity of foods. Because these oxidized lipid/amino acid reaction products are produced during the peroxidation process, it is unclear if this antioxidative activity has a role in slowing the peroxidative process or in preventing further oxidations. Additional studies are being carried out to define the antioxidative role of these compounds in the overall products formed during nonenzymatic browning.

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