Contribution of the Formation of Oxidized Lipid/Amino Acid Reaction Products to the Protective Role of Amino Acids in Oils and Fats

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The antioxidative activity of *n*-octylamine, *N*-methylheptylamine, and *N*,*N*-dimethylhexylamine in a soybean oil was evaluated in order to investigate if this antioxidative activity might be related to their different abilities to produce oxidized lipid/amine reaction products (OLARP). The three amines were added at five levels of concentration 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. When added at 100 or 200 ppm, *n*-octylamine and *N*-methylheptylamine, but not *N*,*N*-dimethylhexylamine, decreased TBARS production in the soybean oil. The three amines, when added at higher concentrations, exhibited a high antioxidative activity, and no big differences among them were observed. Because only primary and secondary amines are able to produce antioxidative OLARP, the differences observed on antioxidative activity at 100 and 200 ppm are in agreement with a contribution of the protective effect of OLARP formation. This hypothesis was confirmed by detection of some OLARP in a soybean oil incubated in the presence of 200 ppm of *n*-octylamine for 240 h. In addition to OLARP formation, other processes should also be implicated so that the antioxidative activities observed for the three amines at higher concentrations could be explained.

Keywords: *Antioxidative activity; antioxidative mechanism; nonenzymatic browning; lipid peroxidation; aliphatic amines*

INTRODUCTION

Amino acids have been reported to be either antioxidants or prooxidants or to have no effect on the oxidation of lipids, which is explained, in part, by the diversity of oils and systems used for their evaluation as antioxidants (Chen and Nawar, 1991; Gopala-Krishna and Prabhakar, 1994; Taylor and Richardson, 1980). The primary amino group of amino acids seems to play a major role in their antioxidative properties, and protonation of these primary amino groups has been reported to accelerate lipid oxidation (Farag et al., 1978; Riisom et al., 1980). The mechanisms involved on the antioxidative activity of amino acids, however, are not well-understood.

Previous research from this laboratory has shown that addition of oxidized lipids/amino acids reaction products (OLAARP) efficiently reduced peroxidation in a soybean oil (Alaiz et al., 1995a,b). Because hydroperoxide formation and decomposition occur simultaneously in lipids (Gardner, 1987) and, also, OLAARP formation takes place at the same time, unoxidized lipids, lipid hydroperoxides, secondary products, and OLAARP exist simultaneously in foods. Therefore, OLAARP may act as antioxidants at the same time that they are being produced (Alaiz et al., 1995c).

In the present study, the contribution of OLAARP formation to the protective role of amino acids was evaluated by analyzing the antioxidative activity of *n*-octylamine, *N*-methylheptylamine, and *N*,*N*-dimethylhexylamine in a soybean oil. These three amines are lipid soluble; they have similar boiling points, the same molecular weight, and only one nitrogen atom and might be representative of the different ways in which amino groups may be constituents of amino acids and proteins. *n*-Octylamine has a primary amino group and may represent amino acid and protein free amino groups. *N*-Methylheptylamine has a secondary amino group and may represent protein amino acid residues like proline or, with significant differences, histidine, among others. Finally, *N*,*N*-dimethylhexylamine has a tertiary amino group, and it has no common equivalent in amino acid residues. In addition, only primary and secondary amino groups are able to react with oxidized lipids producing covalent compounds (see, for example: Alaiz and Barragán, 1995; Alaiz and Girón, 1994; Hidalgo and Zamora, 1993; Zamora and Hidalgo, 1994), which exhibited antioxidative properties (Alaiz et al., 1996). Therefore, if formation of OLAARP contributes to the protective role of amino groups, primary, secondary, and tertiary amino groups should exhibit significant differences in their antioxidative activities.

EXPERIMENTAL PROCEDURES

Materials. Soybean oil was obtained from our Institute's Pilot Plant (Instituto de la Grasa, CSIC, Sevilla, Spain). 2-Thiobarbituric acid monohydrate was purchased from Merck (Darmstadt, Germany). *n*-Octylamine, *N*-methylheptylamine, *N*,*N*-dimethylhexylamine, (*E*)-2-octenal, and (*E*)-2-(*E*)-4-heptadienal were obtained from Aldrich Chemical Co. (Milwaukee, WI). Butylate hydroxytoluene (BHT) and *n*-propyl gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents and solvents used were analytical grade and were purchased from reliable commercial sources. (*E*)-4,5-Epoxy-(*E*)-2-heptenal was prepared as described previously (Zamora and Hidalgo, 1992).

Measurement of Antioxidative Activity. Stripped soybean oil with no antioxidant was compared with samples containing *n*-octylamine, *N*-methylheptylamine, *N*,*N*-dimethylhexylamine, BHT, and propyl gallate, added at concentrations of 100, 200, 500, 1000, and 2000 ppm. Oil samples (10 g) were weighted into 90 × 20 mm Petri dishes and oxidized for 240 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:

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

PI = 100 meant that the compound tested was as effective as gallate. PI = 0 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 the TBARS of oil should be higher than the TBARS of gallate.

Reaction of (*E*)-4,5-Epoxy-(*E*)-2-heptenal and (*E*)-2-Octenal with *n*-Octylamine. Two standard mixtures were prepared between one oxidized lipid [(*E*)-4,5-epoxy-(*E*)-2-heptenal or (*E*)-2-octenal] (0.107 mmol) and *n*-octylamine (0.214 mmol) in chloroform (1 mL). These mixtures were incubated overnight at 37 °C and then studied by gas chromatography coupled with mass spectrometry (GC-MS). GC-MS analyses were conducted with a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced, *via* an open coupling system, to an AEI-MS/70VG mass spectrometer (VG Analytical, Manchester, U.K.). A DB-5 fused-silica capillary column (J&W Scientific, Folsom, CA), 30 m × 0.25 mm i.d. was used in all of the experiments. The column temperature was programmed from 50 (2 min) to 250 °C at 4 °C/min. The MS conditions were described previously (Hidalgo and Zamora, 1995).

Oxidized Lipid/Amine Reaction Products (OLARP) Formation in Soybean Oil Treated with *n*-Octylamine. To confirm that OLARP formation contributes to the protective role of amino groups, some OLARP were detected in the soybean oil incubated samples. The above oil samples, which were incubated for 240 h in the presence and in the absence of 200 ppm of *n*-octylamine, were fractionated by solid-phase extraction (SPE) chromatography using 6 mL octadecyl columns (J. T. Baker Inc., Phillipsburg, NJ). Samples (1 g) were dissolved in 4 mL of hexane and passed through the column. The column was washed with hexane (10 mL), and then polar compounds, including OLARP, were extracted with methanol (3 mL). This sequence was used repeatedly until the whole sample (9 g) was fractionated. Purified fractions were studied directly by GC-MS.

Statistical Analysis. All antioxidative activity measurements are expressed as mean values of three experiments. 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 (Snedecor and Cochran, 1980). All statistical procedures were carried out using the Primer of Biostatistics: The Program (McGraw-Hill, Inc., New York). The significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Antioxidative Activity of Primary, Secondary, and Tertiary Amines. Figure 1 shows the effect of *n*-octylamine, *N*-methylheptylamine, and *N*,*N*-dimethylhexylamine, added at a concentration of 200 ppm, on TBARS production in a soybean oil heated at 60 °C. The figure also includes, for comparison purposes, untreated oil and oil treated with 200 ppm of either BHT or propyl gallate. The three amines significantly protected the oil for the first 96 h. However, after that time, protection of the tertiary amine was progressively lower, and it did not show significant protection after 192 h (PI = 4). Although contribution of other effects should not be excluded, these results may be interpreted on the basis of OLARP formation. For the first 4 days, hydroperoxide formation and decomposition were being produced in the oil, which exhibited a TBARS increase during this period. At the same time, OLARP production should also be taking place by reaction of the secondary products of lipid oxidation with the amino groups of primary and secondary amines. After 4 days of oxidation, the OLARP concentration should be sufficient to significantly protect the oil against oxidation. Thus, both primary and secondary amines efficiently protected the oil for the whole heating period.

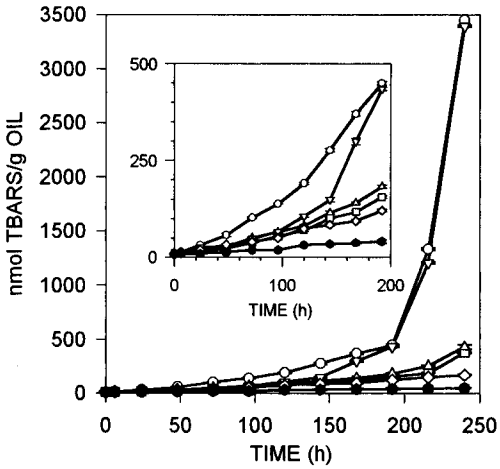

Figure 1. Effects of *n*-octylamine (□), *N*-methylheptylamine (△), *N*,*N*-dimethylhexylamine (▽), BHT (◊), and propyl gallate (●), added at 200 ppm, on soybean oil oxidation (○) measured as TBARS formation. Results represent the mean ± the standard deviation of three assays. (Inset) Expanded scale.

Although both primary and secondary amines exhibited antioxidative activity, there were small but significant differences among them. Thus, protection exhibited by *n*-octylamine was significantly higher than that exhibited by *N*-methylheptylamine after only 24 h, and this difference was maintained for the whole heating period.

Figure 1 also shows the effect of common antioxidants BHT and propyl gallate in slowing TBARS production. BHT protected analogously to *n*-octylamine for the first 120 h, and this protection was significantly higher at longer incubation times. At the end of the heating period the PI for BHT and *n*-octylamine were 96 and 90, respectively. As expected from previous studies (Alaiz et al., 1995a), propyl gallate always exhibited the highest protection in accordance 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.

To confirm that OLARP production was taking place during sample incubation, final oil samples were analyzed for OLARP production. Previously, model systems involving some compounds produced during oil oxidation were studied. Because mechanisms for OLARP formation are better known in reactions involving primary amino groups, only model reactions with *n*-octylamine were studied.

Reaction of (*E*)-4,5-Epoxy-(*E*)-2-heptenal with *n*-Octylamine. The reaction between epoxyalkenals and primary amino groups has been studied in this laboratory, and the reaction mechanism is well-characterized (Hidalgo and Zamora, 1993; Zamora and Hidalgo, 1995). This reaction always produces two types of pyrrole derivatives: 1-substituted pyrroles and 1-substituted 2-(1′-hydroxyalkyl)pyrroles, which are the main products of this reaction. In addition, polimerization of 1-substituted 2-(1′-hydroxyalkyl)pyrroles and formation of other products, which corresponded to decomposition products of the aldehyde, could also be detected. Figure 2 shows the total ion chromatogram of GC-MS analysis for the reaction mixture of (*E*)-4,5-epoxy-(*E*)-2-heptenal with *n*-octylamine in chloroform after stir-

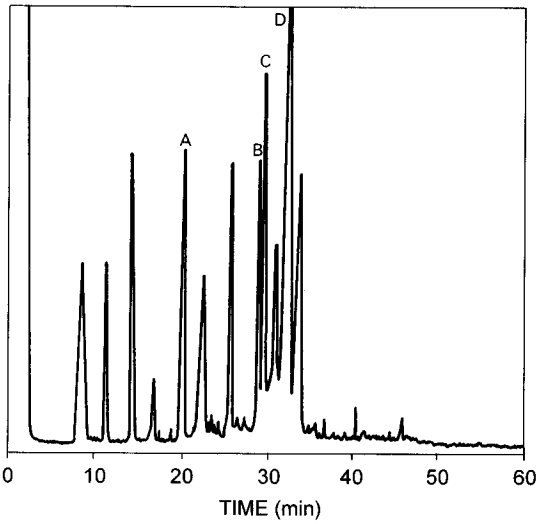

Figure 2. Total ion chromatogram of GC-MS analysis for the reaction mixture of (*E*)-4,5-epoxy-(*E*)-2-heptenal and *n*-octylamine in chloroform after overnight incubation at 37 °C.

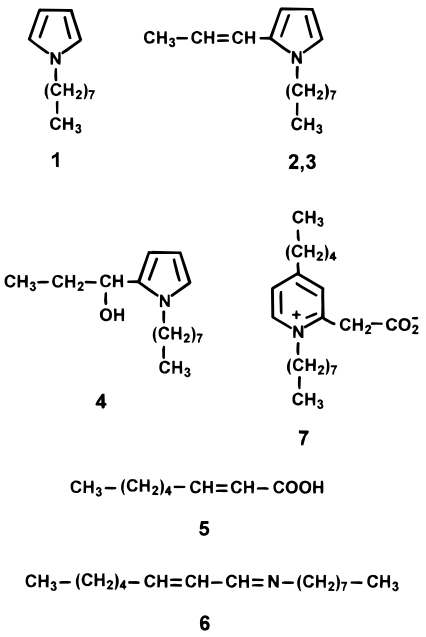

Figure 3. Chemical structures of compounds identified in this study.

ring overnight at 37 °C. The pyrrole derivatives could be easily identified by analogy of their MS with the mass spectra of previously characterized similar compounds. Thus, peaks A−D corresponded to 1-*n*-octylpyrrole (**1**), 1-*n*-octyl-2-[(*E* or *Z*)-1-propenyl]pyrrole (**2**), 1-*n*-octyl-2-[(*Z* or *E*)-1-propenyl]pyrrole (**3**), and 2-(1-hydroxypropyl)-1-*n*-octylpyrrole (**4**), respectively. Figure 3 shows the structures of the different compounds identified in this study.

Peak A was identified as 1-*n*-octylpyrrole (**1**). GC, R_t 20.5 min. GC-MS *m*/*z* (relative intensity, ion structure): 179 (65, M^+), 164 (5, M^+ − methyl), 150 (19, M^+ − ethyl), 136 (27, M^+ − propyl), 122 (41, M^+ − butyl), 108 (31, M^+ − pentyl), 94 (73, M^+ − hexyl), 81 (100, 1-methylpyrrole), 80 (100, M^+ − heptyl), and 67 (55, pyrrole − 1).

Peaks B and C were identified as 1-*n*-octyl-2-(1-propenyl)pyrrole (diastereomers **2** and **3**). The dehydration reaction of compound **4** produced diasteromers **2** and **3**, which were fractionated by GC. This dehydration was also observed previously in an analogous compound: 1-butyl-2-(1-hydroxyhexyl)pyrrole (Zamora and Hidalgo, 1995). Compounds **2** and **3** exhibited similar MS. GC, R_t 29.2 and 29.8 min for compounds **2** and **3**, respectively. GC-MS *m*/*z* (relative intensity, ion structure): 219 (54, M^+), 204 (18, M^+ − methyl), 190 (23, M^+ − ethyl), 176 (17, M^+ − propyl), 162 (17, M^+ − butyl), 148 (23, M^+ − pentyl), 134 (42, M^+ − hexyl), 121 (89, 1-methyl-2-(1-propenyl)pyrrole), 120 (82, M^+ − heptyl), and 106 (100, 2-(1-propenyl)pyrrole − 1).

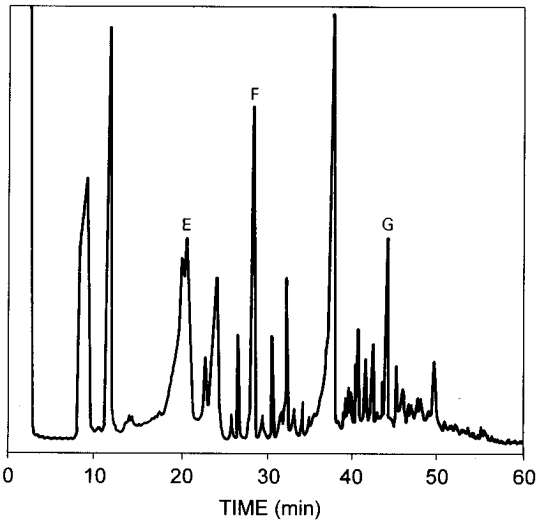

Figure 4. Total ion chromatogram of GC-MS analysis for the reaction mixture of (*E*)-2-octenal and *n*-octylamine in chloroform after overnight incubation at 37 °C.

Peak D was identified as 2-(1-hydroxypropyl)-1-*n*-octylpyrrole (**4**). GC, R_t 32.6 min. GC-MS *m*/*z* (relative intensity, ion structure): 237 (3, M^+), 219 (88, M^+ − H_2O), 208 (19, M^+ − ethyl), 204 (30, M^+ − H_2O − methyl), 190 (35, M^+ − H_2O − ethyl), 176 (32, M^+ − H_2O − propyl), 162 (29, M^+ − H_2O − butyl), 148 (38, M^+ − H_2O − pentyl), 134 (68, M^+ − H_2O − hexyl), 121 (100, 1-methyl-2-(1-propenyl)pyrrole), 120 (100, M^+ − H_2O − heptyl), and 106 (100, 2-(1-propenyl)pyrrole − 1).

Reaction of (*E*)-2-Octenal with *n*-Octylamine. Reactions involving (*E*)-2-octenal are more complex than those involving epoxyalkenals because alkenals produce more slowly stable compounds with amines. In addition, these aldehydes are decomposed in solution, and alkenals and decomposition products react among them producing new compounds, including lipidic polymers. Therefore, the reaction between (*E*)-2-octenal and *n*-octylamine produced many compounds, and only some of them could be identified by the similarity of their MS with those previously characterized in analogous reactions (Alaiz and Barragán, 1995). Figure 4 shows the total ion chromatogram of GC-MS analysis for the reaction mixture of (*E*)-2-octenal and *n*-octylamine in chloroform after stirring overnight at 37 °C. The structures of identified compounds are given in Figure 3.

Peak E was identified as (*E*)-2-octenoic acid (**5**). GC, R_t 20.8 min. Identification of this compound was carried out by computer-matching with the reference mass spectra of the NBS Data Base installed on the VG Station used for MS acquisition and processing.

Peak F was tentatively identified as 1-(octylimino)-2-octene (**6**). GC, R_t 28.3 min. GC-MS *m*/*z* (relative intensity, ion structure): 237 (12, M^+), 222 (26, M^+ − methyl), 208 (34, M^+ − ethyl), 194 (45, M^+ − propyl),

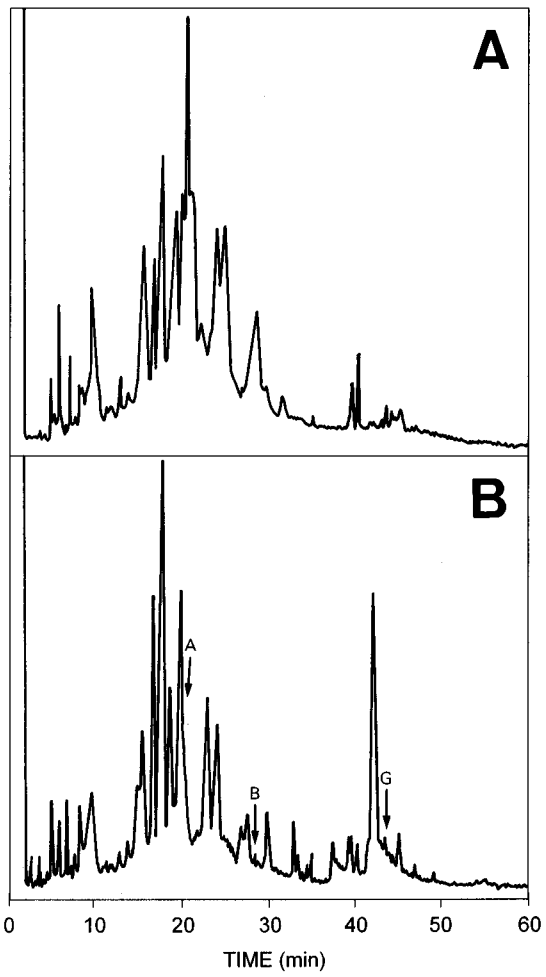

Figure 5. Total ion chromatograms of GC-MS analyses for (A) soybean oil and (B) soybean oil treated with 200 ppm of *n*-octylamine. Oils were heated at 60 °C for 240 h and then fractionated chromatographically to eliminate unreacted triglycerides previously injected in the chromatograph.

180 (11, $M^+ -$ butyl), 166 (6, $M^+ -$ pentyl), 152 (7, $M^+ -$ hexyl), 138 (23, $M^+ -$ heptyl), and 124 (100, $M^+ -$ octyl).

Peak G was tentatively identified as 2-(1-carboxymethyl)-1-octyl-4-pentylpyridinium betaine (**7**). GC, R_t 44.0 min. GC-MS *m*/*z* (relative intensity, ion structure): 319 (11, M^+), 290 (10, $M^+ -$ ethyl), 276 (15, $M^+ -$ propyl), 262 (23, $M^+ -$ butyl), 249 (21, (1-octylpyridinium-2-acetate), 248 (100, $M^+ -$ pentyl), 206 (29, $M^+ -$ octyl), and 150 (17, 249 − heptyl).

Detection of OLARP in the Soybean Oil Incubated Samples. Samples incubated for 240 h were fractionated chromatographically to eliminate unreacted triglycerides and then studied by GC-MS. Figure 5 shows the total ion chromatograms of GC-MS analyses for a control oil and an oil incubated in the presence of 200 ppm *n*-octylamine. Both chromatograms were similar, and main peaks corresponded to oxidized fatty acid derivatives. However, the presence of several OLARP could be detected. Thus, peaks A, B, and G, corresponded to 1-*n*-octylpyrrole (**1**), 1-*n*-octyl-2-(1-propenyl)pyrrole (**2**), and 2-(1-carboxymethyl)-1-octyl-4-pentylpyridinium betaine (**7**), respectively, according to their R_t and MS. In addition, the presence of other unidentified OLARPs could also be detected.

Effect of Amine Concentration on Antioxidative Activity. The effect of amine concentration on TBARS production in a soybean oil heated at 60 °C is shown in

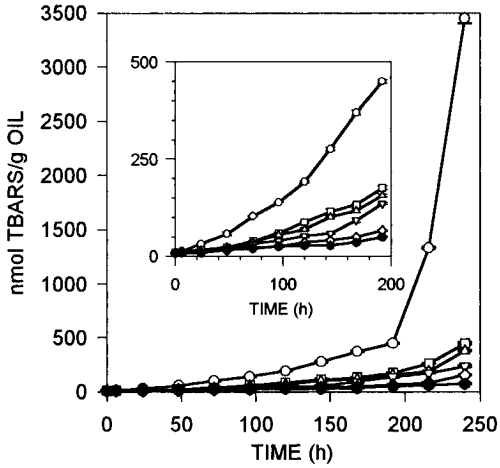

Figure 6. Effect of *n*-octylamine added at 100 (□), 200 (△), 500 (▽), 1000 (◊), and 2000 (●) ppm, on soybean oil oxidation (○) measured as TBARS formation. Results represent the mean ± the standard deviation of three assays. (Inset) Expanded scale.

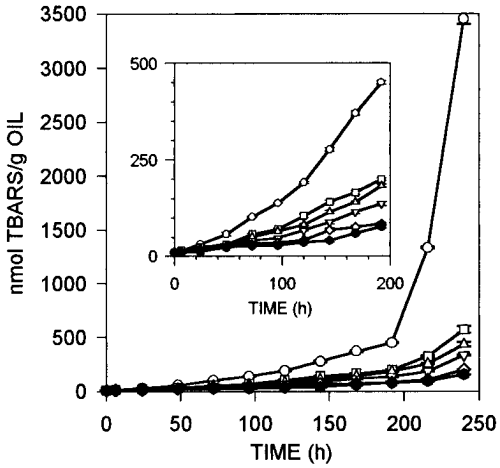

Figure 7. Effect of *N*-methylheptylamine added at 100 (□), 200 (△), 500 (▽), 1000 (◊), and 2000 (●) ppm, on soybean oil oxidation (○) measured as TBARS formation. Results represent the mean ± the standard deviation of three assays. (Inset) Expanded scale.

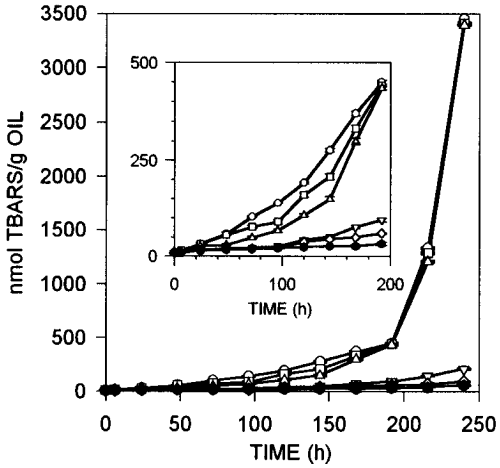

Figure 8. Effect of *N*,*N*-dimethylhexylamine added at 100 (□), 200 (△), 500 (▽), 1000 (◊), and 2000 (●) ppm, on soybean oil oxidation (○) measured as TBARS formation. Results represent the mean ± the standard deviation of three assays. (Inset) Expanded scale.

Figures 6−8. An increase in the concentration of the amine always produced a diminution on TBARS production, but this diminution depended on the amine

type. Thus, Figure 6 shows the effect of *n*-octylamine on TBARS production. This amine exhibited a significant protection at the five assayed concentrations after only 6 h. At higher incubation times, significant differences were observed among the five concentrations assayed. Thus, after 24 h, the amine added at 500, 1000, and 2000 ppm exhibited a higher protection than the amine added at 100 and 200 ppm, and, after 96 h, significant differences on TBARS production were observed among all of the concentrations assayed.

The effect of *N*-methylheptylamine on TBARS production is shown in Figure 7. *N*-Methylheptylamine did not show any protection for the first 24 h, but differences among concentrations increased at higher times. Analogously to primary amines, after 96 h significant differences on TBARS production were observed among all of the concentrations assayed. Secondary amines always exhibited a lower protection than primary amines at the five concentrations assayed.

The effect of *N*,*N*-dimethylhexylamine on TBARS production is shown in Figure 8. Differently from the other two amines, this tertiary amine exhibited two different behaviors depending on the concentration assayed. At 100 and 200 ppm, *N*,*N*-dimethylhexylamine showed a small protection for the first 168 h, and no protection at higher incubation times. On the contrary, at 500, 1000, and 2000 ppm, the protection exhibited by *N*,*N*-dimethylhexylamine was very high and was similar to that exhibited by *n*-octylamine. At these concentrations, the assayed primary and tertiary amines exhibited the same antioxidative activity, and this activity was significantly higher than that exhibited by the secondary amine.

DISCUSSION

The results obtained in this study show that, at 100 and 200 ppm, *n*-octylamine, *N*-methylheptylamine, and *N*,*N*-dimethylhexylamine exhibited significant differences in their antioxidative activities that may be related to their different abilities to produce antioxidative OLARP. This hypothesis has been confirmed by detecting the formation of some OLARP derived from two lipid peroxidation products, (*E*)-4,5-epoxy-(*E*)-2-heptenal and (*E*)-2-octenal, in oil incubated in the presence of 200 ppm of *n*-octylamine. In addition, previous studies have shown that these OLARP exhibited antioxidative activity in soybean oil (Alaiz et al., 1995a,b), and therefore, they should be contributing to the higher stability of the oil treated with *n*-octylamine. Although no studies have been carried out with secondary amines, because the reaction mechanisms are less understood, these reactions should also produce antioxidative OLARP (Alaiz et al., 1996). On the contrary, no reaction with the tertiary amine is expected, and therefore, no protective effect of OLARP should be awaited.

Although OLARP formation during oil oxidation in the presence of amino compounds has been shown in this study, this should not be the unique mechanism for the antioxidative activity exhibited by compounds having amino groups. When the study was carried out with a concentration of amine higher than 200 ppm, there was not a clear relation between the amine type and the antioxidative activity observed. Therefore, additional studies are needed to understand the antioxidative activity exhibited by these compounds at high concentration.

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