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Effect of the Pyrrole Polymerization Mechanism on the Antioxidative Activity of Nonenzymatic Browning Reactions

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The present investigation was undertaken to study how the antioxidative activity (AA) of nonenzymatic browning reactions is changing at the same time that the browning (by the pyrrole polymerization mechanism) is being produced. The antioxidative activities of eight model pyrroles (pyrrole, 1-methylpyrrole, 2,5-dimethylpyrrole, 1,2,5-trimethylpyrrole, 2-acetylpyrrole, 2-acetyl-1-methylpyrrole, pyrrole-2-carboxaldehyde, and 1-methyl-2-pyrrolecarboxaldehyde) as well as the browning reaction of 2-(1-hydroxyethyl)-1-methylpyrrole (HMP) and the dimer (DIM) produced during HMP browning were determined. The results obtained suggest that the AAs observed in nonenzymatic browning reactions are the result of the AAs of the different oxidized lipid/amino acid reaction products formed. Thus, the different pyrrole derivatives produced in these reactions had different AAs, and the highest AAs were observed for alkyl-substituted pyrroles without free α-positions. Because some of these pyrrole derivatives are implicated in nonenzymatic browning production and this browning production implies the loss of hydroxyl groups and the transformation of some pyrroles with one type of substitution into others, changes in AA during browning production were observed, and the resulting DIM derivative was more antioxidant than HMP or higher polymers. These results explain the AA observed in fatty acid/protein mixtures after slight oxidation and suggest that, when the pyrrole polymerization mechanism is predominant, slightly browned samples may be more antioxidant than samples in which nonenzymatic browning has been highly developed.

KEYWORDS: Carbonyl−amine reactions; lipid oxidation; Maillard reaction; natural antioxidants; nonenzymatic browning; pyrrole derivatives; pyrrole polymerization

INTRODUCTION

Lipids are important components that contribute very significantly to the nutritional and sensory value of almost all kinds of foods. This contribution is predominantly related to the contents, distribution in the food matrix, chemical composition, and reactivity of the lipids, as well as to their changes due to processing, and the interactions with other food components (*1*). When lipids are oxidized, sensory quality and nutritive value of food products are deteriorated. For this reason, lipid oxidation is a great economic concern to the food industry, and, therefore, numerous studies have been carried out to control the implicated processes (*2−5*).

Nowadays, antioxidants are commonly used as food additives to extend the shelf life of oils and fatty foods during storage and processing. The antioxidants to be used are determined by various factors including legislation, effectiveness, and cost. In addition, consumer preference for natural additives has encouraged the development of natural antioxidants. Thus, much research has been conducted to find safe antioxidants with high activity from natural resources (*6−10*). Furthermore, other studies have been dedicated to isolate and characterize components, normally present in foods, that possess antioxidative properties. In this context, the antioxidative properties of Maillard reaction products have long been known (*11−13*) and, more recently, also the antioxidative activity of the nonenzymatic browned products formed in oxidized lipid/protein reactions (*14−16*). Nevertheless, neither the relative antioxidative activities of many of the different oxidized lipid/amino acids reaction products (OLAARPs) nor how these activities are changing in parallel to the oxidative process are known.

Different studies have shown that when the lipid oxidation process takes place in the presence of proteins, OLAARPs are always produced (*17*). In fact, OLAARPs play a significant role in the nonenzymatic browning process, and model studies carried out in this laboratory have pointed to a pyrrole formation and polymerization mechanism (**Figure 1**) as being responsible, at least partially, for the nonenzymatic browning produced as a consequence of oxidized lipid/amino acid reactions (*18*). The key intermediates in this mechanism are *N*-substituted 2-(1-hydroxyalkyl)pyrroles (**I**). These compounds have been shown to be produced in the reaction of 4,5-epoxy-2-alkenals with the amino groups of amino acids and proteins (*19*, *20*), and their formation is always accompanied by the production of *N*-substituted pyrroles (**II**). These last compounds are relatively stable and have been found in more than 20 fresh food products,

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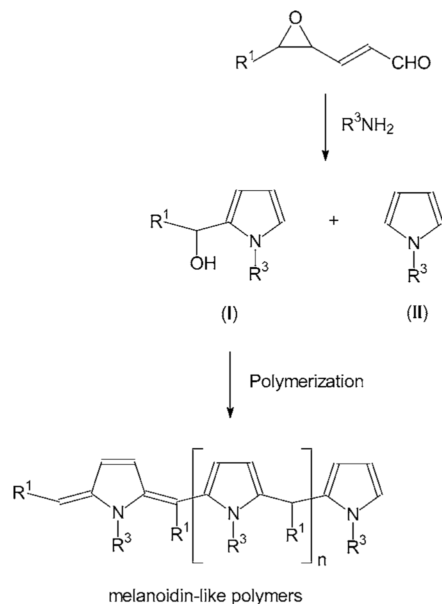


Figure 1. Mechanism for nonenzymatic browning produced as a consequence of 2-(1-hydroxyalkyl)pyrrole polymerization.

including meats, fishes, vegetables, and nuts (*21*). However, the *N*-substituted 2-(1-hydroxyalkyl)pyrroles are unstable and polymerize rapidly and spontaneously to produce brown macromolecules with fluorescent melanoidin-like characteristics (*22*).

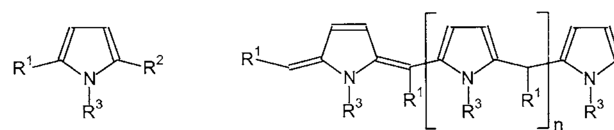
According to this mechanism, hydroxylalkylpyrroles with at least one unsubstituted α-position are produced in a first step, and these monomers evolve alkyl-substituted pyrroles with no free α-positions. The present study was undertaken to analyze comparatively the antioxidative activities of different pyrrole derivatives, including those formed in the first steps of pyrrole polymerization, in an attempt to clarify how the antioxidative activity of nonenzymatic browning reactions is changing at the same time that the browning (by the pyrrole polymerization mechanism) is being produced.

EXPERIMENTAL PROCEDURES

Materials. Pyrrole, 1-methylpyrrole, 2,5-dimethylpyrrole, 1,2,5-trimethylpyrrole, 2-acetylpyrrole, 2-acetyl-1-methylpyrrole, pyrrole-2-carboxaldehyde, and 1-methyl-2-pyrrolecarboxaldehyde were purchased from Aldrich (Milwaukee, WI). Structures for the different pyrroles employed in this study are collected in **Figure 2**. Other reagents and solvents were of analytical grade and were purchased from reliable commercial sources.

2-(1-Hydroxyethyl)-1-methylpyrrole (HMP) and its dimer produced during nonenzymatic browning, 1,1′-dimethyl-5-ethylidene-2,2′-dipyrrylmethylmethene (DIM), were prepared from 2-acetyl-1-methylpyrrole according to a previously described procedure (*22*), which was modified. Briefly, 2-acetyl-1-methylpyrrole (4 mmol) was reduced with sodium borohydride (4 mmol) in methanol (5 mL) for 1.5 h at room temperature. The reaction produced quantitatively HMP, but this compound partially polymerized during column chromatography fractionation on silica gel, and DIM was also isolated. Column chromatography was carried out using diethyl ether/hexane (1:1) as eluent and was followed by thin-layer chromatography with diethyl ether/hexane (7:3). Identities of HMP (R_f 0.46) and DIM (R_f 0.38) were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and/or gas chromatography−mass spectrometry (GC-MS).

Measurement of Antioxidative Activity in Pyrroles. Antioxidative activity was determined following a described procedure (*23*, *24*), which was modified. Briefly, a linolenic acid substrate solution was prepared by adding linolenic acid (0.125 mL) to a mixture of oxygen-free borate buffer (2.5 mL of 50 mM, pH 9.0) and Tween 20 (0.125 mL). Then,



Compound	n	R^1	R^2	R^3
Pyrrole	-	H	H	H
1-Methylpyrrole	-	H	H	CH_3
2,5-Dimethylpyrrole	-	CH_3	CH_3	H
1,3,5-Trimethylpyrrole	-	CH_3	CH_3	CH_3
2-Acetylpyrrole	-	CH_3CO	H	H
2-Acetyl-1-methylpyrrole	-	CH_3CO	H	CH_3
Pyrrole-2-carboxaldehyde	-	CHO	H	H
1-Methyl-2-pyrrolecarboxaldehyde	-	CHO	H	CH_3
HMP	-	$CH_3CH(OH)$	H	CH_3
DIM	0	CH_3	-	CH_3
TRI	1	CH_3	-	CH_3
TET	2	CH_3	-	CH_3

Figure 2. Structures of the different pyrrole derivatives employed in this study. Abbreviations: DIM, dimer produced in the polymerization reaction of HMP; HMP, 2-(1-hydroxyethyl)-1-methylpyrrole; TRI, trimer produced in the polymerization reaction of HMP; TET, tetramer produced in the polymerization reaction of HMP.

aqueous sodium hydroxide solution was added until a clear solution was obtained, and the mixture was diluted with oxygen-free borate buffer (50 mM, pH 9.0) to 25 mL and made up to 50 mL with oxygen-free, distilled water. To determine the antioxidative activity, the sample (60 μL of a solution containing 0.5−10 mg/mL in 60% ethanol) was added to a solution of oxygen-saturated phosphate buffer (3 mL of 0.2 M, pH 6.75), hydrogen peroxide (100 μL of 16 mM), ferrous sulfate (100 μL of 16 mM containing 15 mM EDTA), and linolenic acid substrate (1.0 mL) and incubated for 10 min at room temperature. After that time, 1 mL of the obtained solution was pipetted into disposable cuvettes containing a solution (2.0 mL) of the color reagent consisting of dimethylformamide (8%), Triton X-100 (1.4%), hemoglobin (56 mg/L), and benzoyl leucomethylene blue (130 μM) in phosphate buffer (0.2 M, pH 5.0). After an incubation time of 30 min, the absorbance was measured at 666 nm against the buffer/color reagent blank. The results were related to the absorption of an aqueous standard solution of Trolox (1 mM) and were expressed as Trolox equivalent (TE) values.

Development of Nonenzymatic Browning by Pyrrole Polymerization. Methanolic solutions of HMP and DIM (10 mg) were pipetted into test tubes and taken to dryness to form a thin film. These tubes were incubated under an inert atmosphere at 37 °C in the dark for different periods of time. At the end of the incubation period, the sample was dissolved in methanol (1 mL) and the color determined. Aliquots of these solutions were also employed for GC-MS analyses and antioxidative activity determinations.

Color Determination. The colors of the solutions were determined spectrophotometrically using a Shimadzu UV-2401 PC UV−vis spectrophotometer. Color differences (ΔE) at the different periods of time were calculated from the determined CIELAB L^* a^* b^* values according to Hunter (*25*)

$$\Delta E = [(L^* - 100)^2 + (a^*)^2 + (b^*)^2]^{1/2}$$

by referring the determined values to an ideal colorless solution of $L^* = 100$ and $a^* = b^* = 0$.

GC-MS. Incubated samples (100 μL), containing *cis*-3-nonen-1-ol (25 μL of a solution of 1.35 mg/mL in methanol) as internal standard, were studied by GC-MS. GC-MS analyses were conducted with a Hewlett-Packard 6890 GC Plus coupled with an Agilent 5973 MSD (mass selective detector, quadrupole type). A fused-silica HP5-MS capillary column (30 × 0.25 mm i.d.; coating thickness = 0.25 μm)

Table 1. Antioxidative Activity of Pyrroles[a]

compound	concentration		
	0.5 mg/mL (30 μg)	2 mg/mL (120 μg)	10 mg/mL (600 μg)
pyrrole	0.10 ± 0.02b	0.31 ± 0.04b	0.72 ± 0.02b
1-methylpyrrole	0.09 ± 0.02b	0.28 ± 0.04bc	0.64 ± 0.07b
2,5-dimethylpyrrole[b]	0.90 ± 0.13c	0.87 ± 0.09d	1.03 ± 0.11c
1,2,5-trimethylpyrrole[c]	1.01 ± 0.02c	0.91 ± 0.08d	1.12 ± 0.06c
2-acetylpyrrole	0.02 ± 0.05b	0.18 ± 0.03bc	0.28 ± 0.05d
2-acetyl-1-methylpyrrole	0.01 ± 0.06b	0.13 ± 0.01c	0.29 ± 0.03d
pyrrole-2-carboxaldehyde	0.04 ± 0.11b	0.20 ± 0.03bc	0.37 ± 0.05d
1-methyl-2-pyrrolecarboxaldehyde	0.05 ± 0.03b	0.24 ± 0.11bc	0.30 ± 0.09d

[a] Values are mean ± SE for three experiments and are given in Trolox equivalents (TE). The amount of compound introduced in the assay is given in parentheses. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Its antioxidative activities were 0.87 and 0.20 TE at 0.1 and 0.001 mg/mL, respectively. [c] Its antioxidative activities were 0.93 and 0.31 TE at 0.1 and 0.001 mg/mL, respectively.

was used. Working conditions were as follows: carrier gas, helium (1 mL/min at constant flow); injector, 250 °C; oven temperature, from 70 (1 min) to 240 ° C at 5 °C/min and then to 325 °C at 10 °C/min; transfer line to MSD, 280 °C; ionization EI, 70 eV.

Measurement of Antioxidative Activity during Nonenzymatic Browning. Antioxidative activity was determined as described previously by using the incubated samples (100 μL), which were diluted with 150 μL of methanol/water (3:2). Thirty microliter aliquots of the diluted samples, which contained 120 μg of the tested compounds, were employed for antioxidative activity determinations.

Statistical Analysis. All results are expressed as mean values ± standard error (SE) of three independent experiments for model pyrroles and, at least, six independent experiments for pyrrole polymerization. Statistical comparisons among different groups were made using ANOVA. When significant F values were obtained, group differences were evaluated by the Student−Newman−Keuls test (*26*). All statistical procedures were carried out using Primer of Biostatistics: The Program (McGraw-Hill, Inc., New York). Significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Measurement of Antioxidative Activity in Pyrroles. Eight different model pyrroles were assayed for antioxidative activity. They included both unsubstituted and *N*-substituted pyrroles as well as pyrroles with alkyl, aldehyde, and ketone groups at position 2 or 5 of the pyrrole ring. Their antioxidative activities in the assayed system are shown in **Table 1**. There was a very different behavior between pyrroles with no free α-position and those in which at least one of the α-positions was unsubstituted. The highest antioxidative activity was exhibited by 2,5-dimethylpyrrole and 1,2,5-trimethylpyrrole, both having no free α-positions. Both compounds had similar antioxidative activities among them, which were independent of concentration in the range of 0.5−10 mg/mL, and all obtained values were similar to that of 1 mM trolox (TE = 1). Their antioxidant activities decreased at lower concentrations and were 0.87 and 0.20 TE for 2,5-dimethylpyrrole at 0.1 and 0.001 mg/mL, respectively, and 0.93 and 0.31 TE for 1,2,5-trimethylpyrrole at 0.1 and 0.001 mg/mL, respectively. The next stronger antioxidants were pyrrole and 1-methylpyrrole, both unsubstituted at the α-position. These compounds exhibited an almost negligible antioxidative activity when assayed at 0.5 mg/mL, but their antioxidative activities increased with concentration, and ∼0.7 TE values were obtained when assayed at 10 mg/mL. Finally, the existence of an oxygenated group decreased the antioxidative activity of pyrrole derivatives, and the lowest antioxidative values were observed for 2-acetylpyrrole, 2-acetyl-1-methylpyrrole, pyrrole-2-carboxaldehyde, and 1-methyl-2-pyrrolecarboxaldehyde. These last compounds exhibited similar antioxidative activities among them, and they were also similar to pyrrole and 1-methylpyrrole at 0.5 mg/mL. However, although this antioxidative activity increased with concentration, this increase was lower than that exhibited by pyrrole and 1-methylpyrrole, and the antioxidative activity exhibited by these oxygenated compounds at 10 mg/mL was ∼0.3 TE. All of these results suggested that the antioxidative activity of a nonenzymatic browning reaction might be changing at the same time that the different pyrroles are either being produced or disappearing to evolve higher polymers.

Change in the Antioxidative Activity during the Polymerization of HMP. HMP is an ideal model to study nonenzymatic browning because the mechanism for browning production has been well characterized (*22*) and it takes place at a relatively slow rate. **Figure 3A** shows the GC chromatogram of pure HMP. Although this compound was obtained with a high purity, as indicated by both ^{1}H and ^{13}C NMR spectroscopy, its GC-MS spectra presented several peaks. Thus, in addition to the two peaks corresponding to HMP (one corresponding to the compound dehydrated in the injector port of the chromatograph and the other corresponding to HMP itself), some small peaks corresponding to dimers (DIM), trimers (TRI), and tetramers (TET) could also be observed. They were likely produced in the injector port of the chromatograph. Mass spectra of HMP as well as DIM, TRI, and TET are collected in **Figure 4**.

When HMP was incubated under an inert atmosphere in the dark at 37 °C, the chromatograms changed slightly and the decrease of the monomers was accompanied by the formation of polymers (**Figure 3B**). **Figure 5** shows the evolution of HMP, DIM, TRI, and TET as a function of incubation time. HMP decreased almost linearly for the first 5 h and then more slowly. After 24 h, the concentration of HMP was ∼50% of the initial and it did not seem to experience any changes for the next 6 h. This decrease in HMP was a consequence of its polymerization and, therefore, polymer formation was observed. Thus, increases for TRI and TET concentrations were observed during the whole incubation period. However, DIM concentration increased significantly for the first 2−3 h and then decreased to a value that was slightly lower than the initial one. These results suggested that HMP produced DIM in a first step and that both HMP and DIM continued polymerizing to produce TRI, TET, and higher polymers. In addition, the polymerization seemed to be produced mainly on the DIM rather than in higher polymers.

These results were confirmed when the changes suffered by DIM upon incubation were studied. Thus, **Figure 3C** shows the GC-MS chromatogram corresponding to untreated DIM. The chromatogram showed the absence of monomers but the

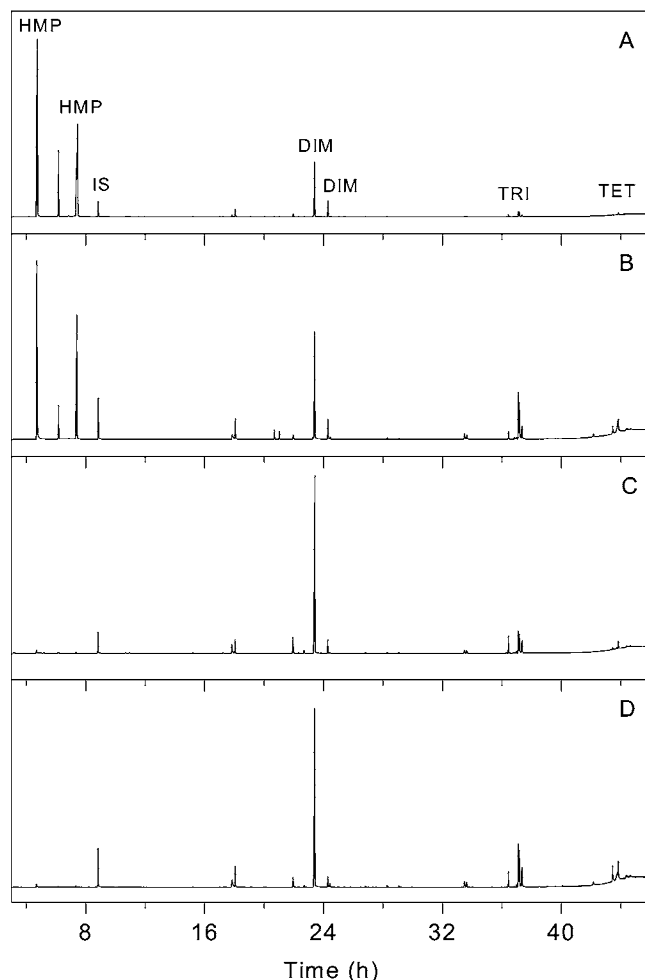


Figure 3. Total ion GC-MS chromatogram obtained for (A) pure 2-(1-hydroxyethyl)-1-methylpyrrole (HMP), (B) HMP incubated for 29 h, (C) unincubated dimer produced in the polymerization reaction of HMP (DIM), and (D) DIM incubated for 29 h. Abbreviations: DIM, dimer produced in the polymerization reaction of HMP; HMP, 2-(1-hydroxyethyl)-1-methylpyrrole; IS, internal standard (*cis*-3-nonen-1-ol); TRI, trimer produced in the polymerization reaction of HMP; TET, tetramer produced in the polymerization reaction of HMP. Structures for the different compounds are given in **Figure 2**.

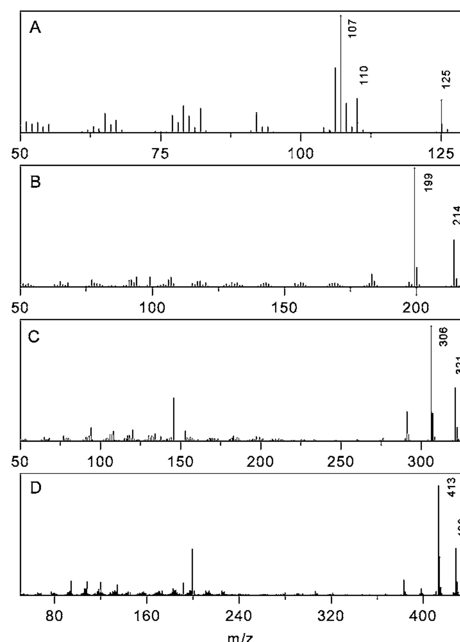


Figure 4. Mass spectra of (A) 2-(1-hydroxyethyl)-1-methylpyrrole (HMP), (B) dimer produced in the polymerization reaction of HMP (DIM), (C) trimer produced in the polymerization reaction of HMP (TRI), and (D) tetramer produced in the polymerization reaction of HMP (TET). Structures for the different compounds are given in **Figure 2**.

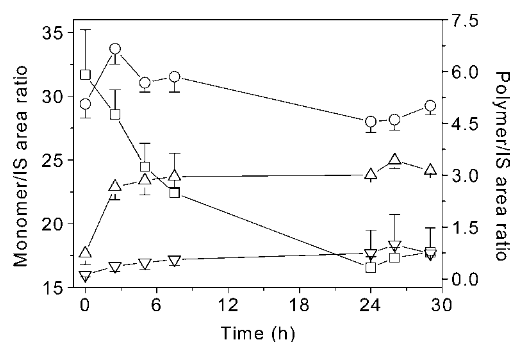


Figure 5. Time course of monomer disappearance and polymer formation during 2-(1-hydroxyethyl)-1-methylpyrrole (HMP) incubation under an inert atmosphere in the dark at 37 °C. Compounds determined were (□) 2-(1-hydroxyethyl)-1-methylpyrrole (HMP), (○) dimer produced in the polymerization reaction of HMP, (△) trimer produced in the polymerization reaction of HMP, and (▽) tetramer produced in the polymerization reaction of HMP. Values are the mean ± SE for six experiments. Structures for the different compounds are given in **Figure 2**.

presence of a certain quantity of TRI. These last polymers might be present in the initial DIM because these compounds and higher polymers are not easily fractionated by absorption chromatography. In addition, it is unclear that DIM may be converted into TRI (see below). When DIM was incubated under an inert atmosphere in the dark at 37 °C, it rapidly polymerized, analogously to HMP (**Figure 3D**). **Figure 6** shows the evolution of HMP, DIM, TRI, and TET as a function of incubation time. Analogously to HMP, DIM also decreased linearly for the first 5 h and, then, much more slowly. This decrease was not accompanied by an increase in TRI, which also decreased with time. On the other hand, an increase in TET was observed. TET increased almost linearly for the first 5 h; its concentration then stabilized for a long time period and, finally, decreased. These results suggested a transformation of DIM into TET and, then, into higher polymers.

The polymerizations produced in all of these reactions, confirmed by GC-MS for small polymers and suggested for bigger ones, were also in agreement with color formation in these reactions, which has been shown to be a consequence of the formation of long-chain polymers (*22*). **Figure 7** shows the color development in both HMP and DIM incubations. Color difference increased linearly ($r = 0.986$, $p < 0.0001$) in HMP incubated under an inert atmosphere in the dark at 37 °C. This increase was not so clearly observed in DIM, although it increased in a way parallel to DIM disappearance (**Figure 6**). In fact, DIM decrease and color formation in DIM incubation were correlated ($r = -0.95$, $p < 0.001$).

These conversions of some pyrroles into others were very related to the changes produced in the antioxidative activities exhibited by HMP and DIM when incubated under an inert atmosphere in the dark at 37 °C (**Figure 8**). Thus, HMP and DIM exhibited antioxidative activities of 0.30 and 0.69 TE, respectively, when employing 120 μg of sample. The antioxidative activity of HMP increased almost linearly for the first 5 h (TE values were doubled after that time) and, then, decreased to arrive at 1.5 times the initial TE value. This behavior seemed

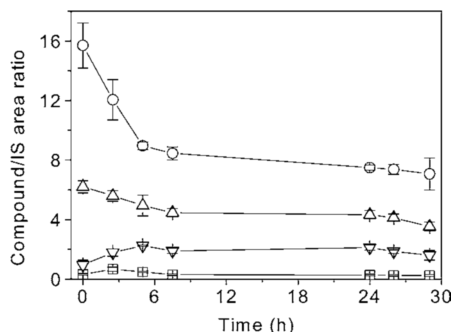


Figure 6. Time course of reactions produced during incubation under an inert atmosphere in the dark at 37 °C of the dimer produced in the polymerization reaction of 2-(1-hydroxyethyl)-1-methylpyrrole. Compounds determined were (□) 2-(1-hydroxyethyl)-1-methylpyrrole (HMP), (○) dimer produced in the polymerization reaction of HMP, (△) trimer produced in the polymerization reaction of HMP, and (▽) tetramer produced in the polymerization reaction of HMP. Values are the mean ± SE for six experiments. Structures for the different compounds are given in **Figure 2**.

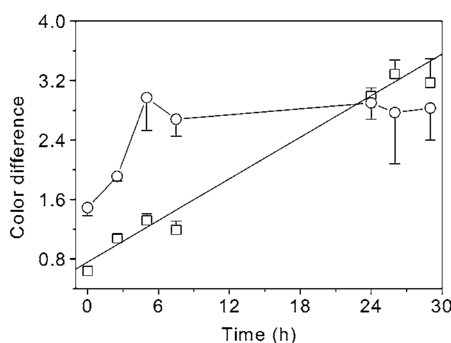


Figure 7. Color development in the incubation under an inert atmosphere in the dark at 37 °C of (□) 2-(1-hydroxyethyl)-1-methylpyrrole (HMP) and (○) the dimer produced in the polymerization reaction of HMP. Values are the mean ± SE for six experiments.

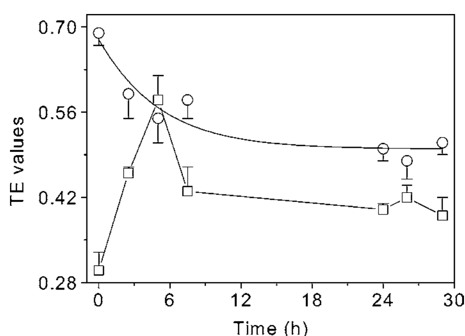


Figure 8. Antioxidative activity of the incubations under an inert atmosphere in the dark at 37 °C of (□) 2-(1-hydroxyethyl)-1-methylpyrrole (HMP) and (○) the dimer produced in the polymerization reaction of HMP. Values are the mean ± SE for six experiments.

to be a consequence of the different compounds that were being produced in the reaction. Thus, during the first few hours of incubation, the formation of short polymers was favored (**Figure 5**). Because these compounds have no hydroxyl groups and fewer free α-positions than monomers, they should be more antioxidative. As the incubation progressed, these small polymers were transformed into higher polymers. This increase in polymer chain length or further reactions that may take place in parallel to pyrrole polymerization should decrease the antioxidative activity. These results are also in agreement with those obtained during DIM incubation. As expected for a dimer with two pyrrole rings without oxygenated functions and one of them with no free α-position, DIM was ~2.5 times more antioxidative than HMP. The incubation of DIM under an inert atmosphere in the dark at 37 °C produced a decrease in the antioxidative activity, which was parallel to DIM disappearance observed by GC-MS (**Figure 6**). In fact, both decreases were correlated ($r = 0.937$, $p = 0.0018$). These results confirmed that DIM was a better antioxidant than higher polymers.

DISCUSSION

The results obtained in this study suggest that the mechanisms implicated in nonenzymatic browning are closely related to the antioxidative activity exhibited by OLAARPs. Among the different products formed in the reactions between oxidized lipids and amino acids and proteins, different pyrrole derivatives have been shown to be produced in the reaction of amino groups with 4,5-epoxy-2-alkenals (*27*), 4-hydroxy-2-alkenals (*28*), 4-oxo-2-alkenals (*29*), unsaturated epoxyoxo fatty acids (*30*), and lipid hydroperoxides (*31*), among others. All of these types of pyrrole derivatives with different substituents in the pyrrole ring also play a significant role in the antioxidative activity of foods. However, the different pyrrole derivatives have different antioxidative activities, and the highest antioxidative activities have been observed for nonoxygenated pyrrole derivatives with no free α-positions.

Because some of these pyrrole derivatives are implicated in nonenzymatic browning production and this browning production implies the loss of hydroxyl groups and the transformation of some pyrroles with one type of substitution into others, changes in antioxidative activity during browning production should be expected. The results obtained in this study agree with this hypothesis. In addition, the above results suggest that not all of the different polymers produced have similar antioxidative activities, although all of them are believed to be similarly substituted at the α-positions. In fact, the dimer was found to be the most antioxidative derivative. The reason for the observed differences in the antioxidative activities among the different polymeric pyrroles is unclear at present.

The above results explain the antioxidative activity observed in polyunsaturated fatty acid/protein mixtures after slight oxidation (*32*). They also suggest that slightly browned samples may be more antioxidative than samples in which nonenzymatic browning has been highly developed, an effect that has been observed, for example, in coffee roasting (*33*) or black tea beverage storage (*34*). In addition, all of these results point out that the antioxidative activity observed in a nonenzymatic browning reaction is the sum of the antioxidative activities of the different compounds present in the sample.

ABBREVIATIONS USED

DIM, dimer produced in the polymerization reaction of HMP; EDTA, ethylenediaminetetraacetic acid; GC-MS, gas chromatography−mass spectrometry; HMP, 2-(1-hydroxyethyl)-1-methylpyrrole; NMR, nuclear magnetic resonance; OLAARPs, oxidized lipid/amino acid reaction products; TE, Trolox equivalents; TET, tetramer produced in the polymerization reaction of HMP; TRI, trimer produced in the polymerization reaction of HMP.

ACKNOWLEDGMENT

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