Contribution of Pyrrole Formation and Polymerization to the Nonenzymatic Browning Produced by Amino−Carbonyl Reactions

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Recent studies have hypothesized that pyrrole formation and polymerization may be contribute to the nonenzymatic browning produced in both oxidized lipid/protein reactions and the Maillard reaction. To develop a methodology that would allow investigation of the contribution of this browning mechanism, the kinetics of formation of color, fluorescence, and pyrrolization in 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine and linolenic acid/lysine model systems were studied. In both cases similar kinetics for the three measurements were observed at the two temperatures assayed (37 and 60 °C), and there was a high correlation among color, fluorescence, and pyrrolization measurements obtained as a function of incubation time. Because the color and fluorescence production in the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine system is a consequence of pyrrole formation and polymerization, the high correlations observed with the unsaturated fatty acid also suggest a contribution of the pyrrole formation and polymerization to the development of color and fluorescence observed in the fatty acid/lysine system. Although the contribution of other mechanisms cannot be discarded, all of these results suggest that when the pyrrole formation and polymerization mechanism contributes to the nonenzymatic browning of foods, a high correlation among color, fluorescence, and pyrrolization measurements should be expected.

Keywords: *Nonenzymatic browning; Maillard reaction; oxidized lipid/protein reactions; amino−carbonyl reactions; pyrrole polymerization; color; fluorescence; oxidative stress*

INTRODUCTION

Nonenzymatic browning reactions of amino acids and proteins with carbohydrates and oxidized lipids cause modification of food during storage and processing together with simultaneous formation of both deleterious and beneficial compounds (Friedman, 1996; Hutchings, 1994; Narayan, 1998; Sapers, 1993). They embrace a whole network of different reactions that are only partially understood due to the high reactivities of the reactants and products, the intertwining reaction routes, and the diversity of products (Ames et al., 1999; Ikan, 1996; Namiki, 1988; O'Brien et al., 1998). The products that have been characterized are preponderantly those that are stable and do not undergo further change to any great extent either in the reaction mixture or during isolation or purification (Ledl and Schleicher, 1990). Such compounds can serve as indicator substances for certain reaction pathways in the nonenzymatic browning. However, difficulties are encountered in the isolation of reactive intermediates, because they are present only in very low concentrations in the reaction mixture and they usually react further during isolation. Nevertheless, these compounds are precisely those of the greatest significance for these reactions because they play an important role in the formation of browning products, aroma compounds, and high molecular weight substances.

Model studies carried out in this laboratory have identified two N-substituted 2-(1-hydroxyalkyl)pyrroles (compound **I** with $R^2 = H$ in Figure 1) as potential key intermediates for the nonenzymatic browning produced between oxidized lipids and amino acids and proteins (Hidalgo and Zamora, 1993). 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, and they are always accompanied by the formation of N-substituted pyrroles (**II**) (Zamora and Hidalgo, 1994, 1995). These last compounds are much more stable and have been found in >20 fresh food products, including meats, fishes, vegetables, and nuts (Zamora et al., 1999). However, the determination of N-substituted 2-(1-hydroxyalkyl)pyrroles is much more complex because they polymerize rapidly and spontaneously to produce brown macromolecules with fluorescent melanoidin-like characteristics (Hidalgo and Zamora, 1993). This polymerization reaction, which is shown in Figure 1, was characterized for 2-(1-hydroxyethyl)-1-methylpyrrole and produces sequentially dimers, trimers, tetramers, and, lately, melanoidin-like polymers (Hidalgo and Zamora, 1993).

One possibility for studying the formation of N-substituted 2-(1-hydroxyalkyl)pyrroles, and its later polymerization, in oxidized lipid/amino acid mixtures is to follow the pyrrole formation and polymerization in these mixtures. Pyrrole rings can be easily determined by using the Ehrlich reagent (Hidalgo et al., 1998). However, this procedure has been neither used nor demonstrated to be useful for the study of pyrrole polymerization. The present investigation was undertaken to apply the Ehrlich reaction to the study of pyrrole formation and polymerization in oxidized lipid/amino acid mixtures to evaluate the contribution of pyrrole polymerization to the nonenzymatic browning produced in these mixtures.

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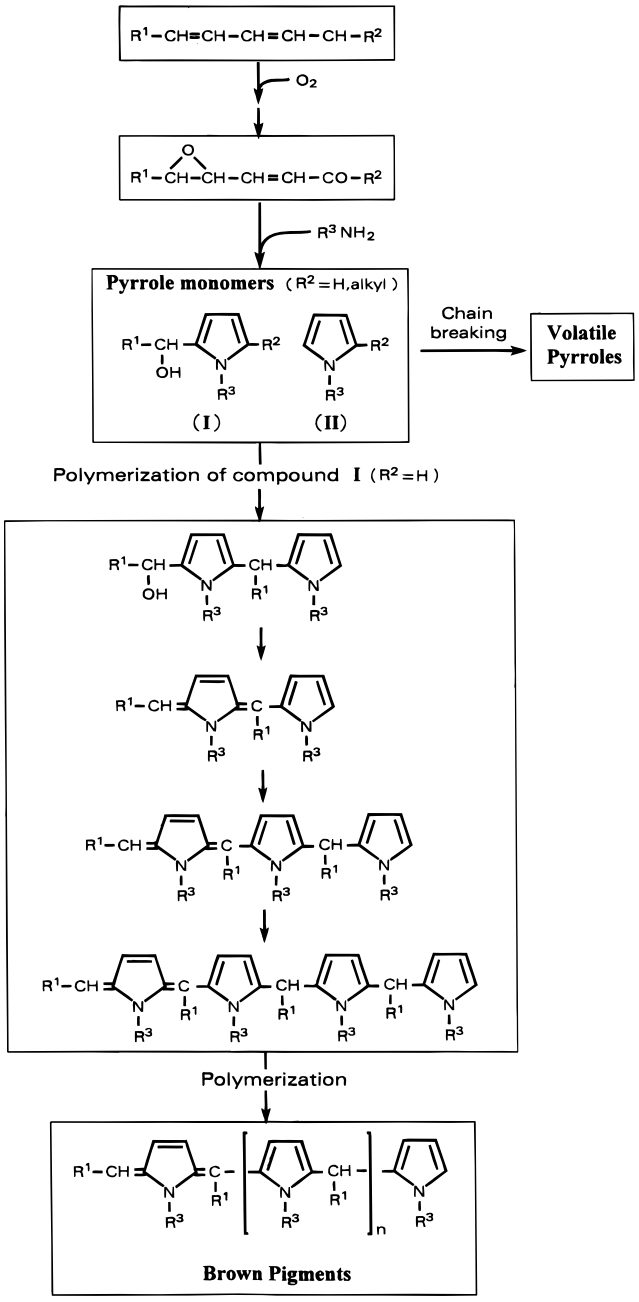
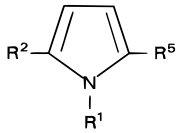


Figure 1. Pyrrole formation and polymerization mechanism. Pyrroles are produced in the reaction between oxidized fatty acids having the 4,5-epoxy-1-oxo-2-pentene group (including 4,5-epoxy-2-alkenals) and primary amino groups of amines, amino acids, and proteins. Nonenzymatic browning is a consequence of the polymerization of N-substituted 2-(1-hydroxyalkyl)pyrroles (**I**). N-Substituted pyrroles (**II**) have been detected in fresh food products (Zamora et al., 1999) as well as volatile pyrroles (Zamora et al., 1994).

EXPERIMENTAL PROCEDURES

Materials. 4,5(*E*)-Epoxy-2(*E*)-heptenal was prepared in a manner analogous to that of 4,5(*E*)-epoxy-2(*E*)-decenal (Zamora and Hidalgo, 1995). Pyrrole (**III**), 1-methylpyrrole (**IV**), 2,5-dimethylpyrrole (**V**), 1,2,5-trimethylpyrrole (**VI**), 2-acetylpyrrole (**VII**), 2-acetyl-1-methylpyrrole (**VIII**), pyrrole-2-carboxaldehyde (**XI**), 1-methyl-2-pyrrolecarboxaldehyde (**XII**), and 1,5-dimethyl-2-pyrrolecarbonitrile (**XIII**) were purchased from Aldrich (Milwaukee, WI). Structures for the model 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.



Compound	R^1	R^2	R^5
III	H	H	H
IV	Me	H	H
V	H	Me	Me
VI	Me	Me	Me
VII	H	$COCH_3$	H
VIII	Me	$COCH_3$	H
IX	H	$CH(OH)CH_3$	H
X	Me	$CH(OH)CH_3$	H
XI	H	CHO	H
XII	Me	CHO	H
XIII	Me	CN	Me
XIV	Me	CHO	Me

Figure 2. Chemical structures of model pyrroles used in this study.

2-(1-Hydroxyethyl)pyrrole (**IX**) and 2-(1-hydroxyethyl)-1-methylpyrrole (**X**) were prepared from compounds **VII** and **VIII**, respectively, according to the procedure described previously for the synthesis of compound **X** (Hidalgo and Zamora, 1993). Briefly, compounds **VII** and **VIII** (4 mmol) were reduced with sodium borohydride (4 mmol) in methanol (5 mL) for 1.5 h at room temperature. The reactions were fractionated by column chromatography with ether/hexane (1:1) as eluent, and pure compounds **IX** and **X** were obtained (60 and 98%, respectively); their identities were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Spectroscopic characterization of compound **X** was described previously (Hidalgo and Zamora, 1993). Spectral data obtained for compound **IX**: ^{1}H NMR [$(CD_3)_2CO$] δ 1.49 d (3H, J = 6.5 Hz, H2′), 4.86 q (1H, J = 6.5 Hz, H1′), 5.98 m (1H, H3), 6.01 q (1H, J = 2.8 Hz, H4), 6.67 dt (1H, $J_{H3,H5}$ = 1.7 Hz, $J_{H5,NH}$ = 2.6 Hz, $J_{H4,H5}$ = 2.6 Hz, H5), and 9.13 s, br (1H, NH); ^{13}C NMR [$(CD_3)_2CO$] δ 23.61 q (C2′), 63.93 d (C1′), 104.33 d (C3), 107.80 d (C4), 117.56 d (C5), and 136.78 s (C2); MS of the trimethylsilyl derivative (70 eV), *m*/*z* (%, ion structure) 183 (7, M^+), 168 (38, $M^+ - CH_3$), 93 [76, $M^+ - (CH_3)_3SiOH$], and 75 (100).

1,5-Dimethyl-2-pyrrolecarboxaldehyde (**XIV**) was prepared by reduction of compound **XIII** with lithium aluminum hydride. A solution of 240 mg (2 mmol) of compound **XIII** in diethyl ether (1.2 mL) was treated with 50 mg of $AlLiH_4$, which was added in small amounts. The reaction mixture was allowed to react for 15 min at room temperature, then diluted with 4 mL of ether, and, finally, treated slowly with methanol to eliminate the excess of hydride. The solids were removed, and the resulting solution was evaporated and fractionated by column chromatography on silica gel 60 using hexane/acetone (4:1) as eluent. Pure compound **XIV** was obtained (51.2 mg, 21%): TLC, R_f 0.39 (hexane/acetone, 4:1); ^{1}H NMR [$(CD_3)_2$-CO] δ 2.25 s (3H, CH_3), 3.83 s (3H, NCH_3), 6.01 dd (1H, $J_{H4,CHO}$ = 0.6 Hz, $J_{H3,H4}$ = 3.9 Hz, H4), 6.84 d (1H, $J_{H3,H4}$ = 3.9 Hz, H3), and 9.40 d (1H, $J_{H4,CHO}$ = 0.6 Hz, CHO); ^{13}C NMR [$(CD_3)_2$-CO] δ 11.87 q (CH_3), 32.27 q (NCH_3), 110.02 d (C4), 124.68 d (C3), 132.76 s (C2), 141.26 s (C5), and 178.78 d (CHO); MS (70 eV), *m*/*z* (%, ion structure) 123 (100, M^+), 122 (100, $M^+ - 1$), 108 (43, $M^+ - CH_3$), 94 (89, $M^+ - CHO$), 67 (58), and 53 (84).

Determination of Absorption Maxima and Extinction Coefficients of Ehrlich Adducts of Model Pyrroles. Model pyrroles were dissolved in water and derivatized with *p*-(dimethylamino)benzaldehyde (Mattocks, 1968; Liddell et al., 1993) using the conditions described previously (Hidalgo et

al., 1998). Briefly, the solution of pyrrole in water (800 μL) was introduced into a 1.5 mL microtube and treated with 128 μL of 2% *p*-(dimethylamino)benzaldehyde in 3.5 M HCl/ethanol (4:1). The tube was closed and heated at 45 °C for 30 min, and, finally, the maximum at 450−600 nm was determined spectrophotometrically.

Reaction of 4,5(*E*)-Epoxy-2(*E*)-heptenal with Lysine. Lysine (41 mg, 0.28 mmol) was dissolved in 7 mL of 0.3 M sodium phospate buffer, pH 7.4, and treated with 18 mg (0.14 mmol) of 4,5(*E*)-epoxy-2(*E*)-heptenal. The reaction was maintained at 37 or 60 °C, and, at different intervals of time, samples (500 μL) were withdrawn for analytical determinations. Samples were extracted with 500 μL of $CHCl_3$/MeOH (2:1) and centrifuged at 2250*g* for 5 min, and the aqueous phase was employed for the determination of color, fluorescence, and pyrrole content.

The color of the solutions was determined by using the weighted-ordinate method (Hunter, 1973). Tristimulus values (*X*, *Y*, *Z*) were calculated from the transmittances (*T*) obtained in a Beckman spectrometer. Transmittances were recorded at constant intervals (10 nm) from 400 to 700 nm using 1 cm glass cells. These readings were then converted by means of a computer program into the corresponding tristimulus and CIELAB *L* a* b** color values (CIE, 1978). The difference of color (Δ*E*) between CIELAB *L* a* b** determined at the initial time and that determined at each time was calculated, according to Hunter (1973), using the following equation:

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

Fluorescence spectra were recorded on a Perkin-Elmer LS-5 fluorescence spectrometer of 25−50 μL of aqueous phase diluted to 2.5 mL with 50 mM sodium phosphate buffer, pH 7.4. A slit width of 5 nm was used, and the instrument was standardized with quinine sulfate (0.1 μM in 0.1 N H_2SO_4) to give fluorescence intensity of 100 at 450 nm, when excitation was at 350 nm. Results are given for 25 μL of sample.

Determination of pyrrole amino acids was carried out as described previously. The pyrrole content was determined spectrophotometrically at the maximum at ∼564 nm by using an extinction coefficient of 37000.

Reaction of Linolenic Acid with Lysine. Lysine (41 mg, 0.28 mmol) was dissolved in 7 mL of 0.3 M sodium phospate buffer, pH 7.4, and treated with 39 mg (0.14 mmol) of linolenic acid. The reaction was maintained at 37 or 60 °C, and, at different intervals of time, samples (500 μL) were withdrawn for determination of color, fluorescence, and pyrrole content. Samples were extracted with 500 μL of $CHCl_3$/MeOH (2:1) and centrifuged at 2250*g* for 5 min. The aqueous phase was employed for the determinations, which were carried out as described above. The only difference was the measurement of pyrrole amino acids, which were determined spectrophotometrically at the maximum at ∼525 nm by using an extinction coefficient of 60000.

RESULTS

Determination of Absorption Maxima and Extinction Coefficients of Ehrlich Adducts of Model Pyrroles. As a first step in the determination of pyrrole formation and polymerization using the Ehrlich reagent, different model pyrroles were derivatized with *p*-(dimethylamino)benzaldehyde and studied spectrophotometrically. The reaction of a pyrrole ring with the Ehrlich reagent always produced at least one main maximum of absorbance at 500−600 nm. Both the position and the intensity of this maximum depended on the number and the type of the substituents in the pyrrole ring. Table 1 collects the absorption maxima and extinction coefficients of model pyrroles **III—XIV**. Most of the pyrroles that had at least one proton in the α-position exhibited the main maximum at 553−565 nm. However, for compound **IX**, this maximum appeared at 541 nm. In addition, compounds **VII** and **VIII** also exhibited a similar maximum at 511−517 nm, but the two maxima had very small extinction coefficients. On the contrary, if there was no proton at the α-position, the main maximum of the Ehrlich adduct appeared at 521−528 nm. Additionally, some derivatives exhibited other maxima at wavelengths slightly below 500 nm. It occurred with the pyrrole and the derivatives containing ketone and nitrile groups. Thus, the pyrrole exhibited a second maximum at 496 nm, which had an extinction coefficient lower than the main maximum. This maximum was also present in compounds **VII**, **VIII**, and **XIII** but with much smaller extinction coefficients.

Table 1. Absorbance Maxima and Extinction Coefficients of Ehrlich Adducts Obtained from Model Pyrroles

	absorbance maximum (extinction coefficient)[a]		
compound	at 450−500 nm	at ∼520 nm	at ∼560 nm
III	496 (18000)		557 (37000)
IV			565 (38000)
V		521 (65000)	
VI		523 (56000)	
VII	481 (4)	511 (5)	557 (3)
VIII	485 (20)	517 (24)	563 (15)
IX			541 (2500)
X			557 (6800)
XI			553 (100)
XII			563 (800)
XIII	490 (230)	521 (240)	
XIV		528 (5900)	

[a] Wavelength in nm; extinction coefficient in M^{-1}.

The highest extinction coefficients were obtained when only hydrogen or alkyl groups were present as substituents. These were compounds **III—VI**, and the extinction coefficients ranged from 37000 for compounds **III** and **IV** to 60000 for compounds **V** and **VI**. The introduction of a hydroxyalkyl group decreased the extinction coefficient, and it was smaller if the substituent was an aldehyde. Nevertheless, the smallest extinction coefficients were observed when the substituent was a ketone.

These results suggested that in pyrrole mixtures, for which the maximum observed is the sum of the maxima of the different compounds, this maximum would mainly be the sum of the unsubstituted pyrroles and the pyrroles substituted only with alkyl groups because these are the pyrroles with higher extinction coefficients. By using an extinction coefficient of 37000 (for the maximum at ∼560 nm) or 60000 (for the maximum at ∼520 nm) in pyrrole mixtures, it is possible to determine a minimum concentration of pyrroles in the mixture, which will mainly correspond to the sum of the unsubstituted and alkyl-substituted pyrroles present. In the case of pyrrole polymerization, 2-(1-hydroxyalkyl)pyrroles are converted to alkyl-substituted polypyrroles (Hidalgo and Zamora, 1993), and these last compounds should contribute significantly to the maxima of the Ehrlich adducts.

Reaction of 4,5(*E*)-Epoxy-2(*E*)-heptenal with Lysine. To study how the nonenzymatic browning produced as a consequence of pyrrole formation and polymerization may be followed by using the Ehrlich reagent, the reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal with lysine was selected because it has been well characterized and it is known that the color and fluorescence produced are a consequence of the formation of N-substituted 2-(1-hydroxyalkyl)pyrroles and

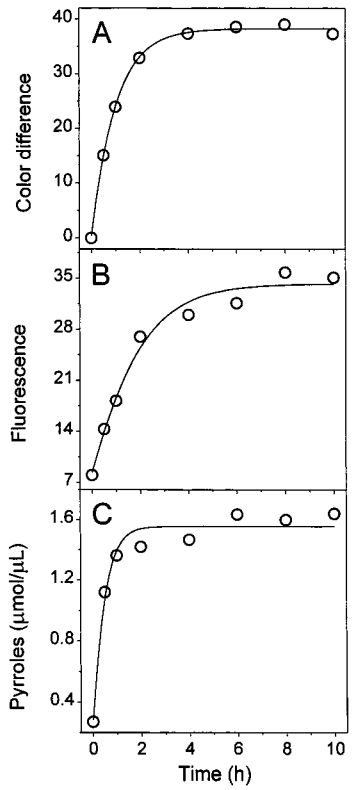


Figure 3. Time course of (A) color, (B) fluorescence, and (C) pyrrolization in a reaction of 4,5(*E*)-epoxy-2(*E*)-heptenal and lysine at 37 °C.

their subsequent polymerization (Hidalgo and Zamora, 1995a; Zamora and Hidalgo, 1994).

When a 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction was heated at 37 °C, the production of color, fluorescence, and pyrrole was observed. Figure 3 shows the time course of these three measurements. Analogously to color and fluorescence, Ehrlich adducts increased with incubation time and always exhibited analogous absorbance spectra with the main maximum at ~564 nm (data not shown). The measurements of color, fluorescence, and pyrrolization followed very similar kinetics, which could be adjusted by using the Boltzmann equation (Microcal Origin, v. 4.10, Microcal Software, Northampton, MA)

$$y = [(A_1 - A_2)/(1 + e^{(x-x_0)/dx}] + A_2 \qquad (2)$$

where A_1 is the initial Y value, A_2 is the final Y value, x_0 is the x value at Y_{50}, and dx is the width. The dx values obtained for color, fluorescence, and pyrrolization curves were 0.98, 1.49, and 0.43 h, respectively, suggesting that although the three measurements changed parallelly, the pyrrolization was produced slightly faster than the formation of color and both of them were a bit faster than fluorescence. These results are in agreement with the mechanism of color and fluorescence production suggested for this reaction: the reaction of an epoxyalkenal with lysine produces in a first step both N-substituted 2-(1-hydroxyalkyl)pyrroles and N-substituted pyrroles, and the rapid polymerization of the hydroxyalkylpyrroles is the origin of the color and fluorescence produced (Hidalgo and Zamora, 1993; Zamora and Hidalgo, 1994). Nevertheless, because both pyrrole formation and polymerization is almost simultaneous and pyrrole polymerization is likely to increase Ehrlich adducts, very good correlations were observed among the three measurements (Table 2).

Analogous results were obtained at 60 °C (Figure 4). This temperature increased the reaction rates when compared with the results obtained at 37 °C, but analogous Ehrlich adducts were produced. In addition, similar kinetics for the formation of color, fluorescence, and pyrroles were also observed. Analogously to the results obtained at 37 °C, color, fluorescence, and pyrrolization measurements could also be adjusted by using the Boltzmann equation (eq 2). The widths of the curves were 0.25, 0.64, and 0.17 h for color, fluorescence, and pyrrolization, respectively. As expected because the reaction occurred more rapidly, these values were smaller than those obtained at 37 °C but they increased following a similar order. In addition, color, fluorescence, and pyrrolization measurements were highly correlated analogously to those observed at 37 °C (Table 2).

All of these results suggest that, when nonenzymatic browning is a consequence of pyrrole production and polymerization, as occurs in the epoxyalkenal/lysine system, analogous kinetics for these three measurements should be expected. This can be analyzed by determining the correlations among these three measurements, which should be very high. In addition, and by using the Boltzmann equation, some small differences in the width of the adjusted curves should also be expected. In this case, the smallest dx values should correspond to the curve of pyrrolization, because pyrrole formation occurs prior to the development of color and fluorescence. In addition, formation of color seems also to be slightly faster than fluorescence production.

Reaction of Linolenic Acid with Lysine. Analogously to the reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal and lysine, the reaction between linolenic acid and lysine also produced color, fluorescence, and pyrrolization. However, the Ehrlich adducts produced in this reaction exhibited the main absorbance maximum at ~526 nm. This maximum suggested that trisubstituted pyrroles were produced preferentially from long-chain fatty acids. Figure 5 collects the results obtained for a linolenic acid/lysine reaction mixture heated at 37 °C. Similar to that observed in the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction mixture, analogous kinetics for color, fluorescence, and pyrrole production were also observed in this fatty acid/lysine system. In this case, the reaction took place more slowly because the oxidation of the fatty acid had to be produced prior to its reaction with the amino acid. When the curves were adjusted by using the Boltzmann equation (eq 2), the curve widths obtained were 99.73, 199.22, and 80.53 h for color, fluorescence, and pyrrolization, respectively. As expected, these values were much higher than the values obtained for the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction mixture. However, they followed an order similar to that obtained for the epoxyalkenal/lysine system, and the correlations among color, fluorescence, and pyrrolization measurements were also very high (Table 2).

When the linolenic acid/lysine reaction mixture was studied at 60 °C (Figure 6), the production of color, fluorescence, and pyrroles was faster than at 37 °C, but analogous kinetics for the three measurements were also observed. In addition, the three curves could be adjusted by using the Boltzmann equation (eq 2), and the dx values obtained were 108.00, 156.04, and 68.45 h, for color, fluorescence, and pyrrolization, respectively.

Table 2. Correlations among Color, Fluorescence, and Pyrrolization

		correlation coefficient (significance)		
reaction	temp, °C	color/fluorescence	color/pyrrolization	fluorescence/pyrrolization
epoxyalkenal/lysine	37	0.964 (0.000115)	0.883 (0.0037)	0.955 (0.000223)
	60	0.977 (<0.0001)	0.956 (<0.0001)	0.986 (<0.0001)
linolenic acid/lysine	37	0.983 (<0.0001)	0.976 (<0.0001)	0.989 (<0.0001)
	60	0.984 (<0.0001)	0.945 (<0.0001)	0.975 (<0.0001)

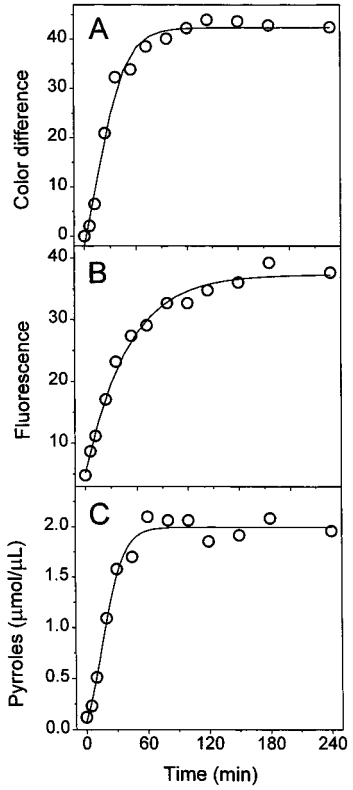


Figure 4. Time course of (A) color, (B) fluorescence, and (C) pyrrolization in a reaction of 4,5(*E*)-epoxy-2(*E*)-heptenal and lysine at 60 °C.

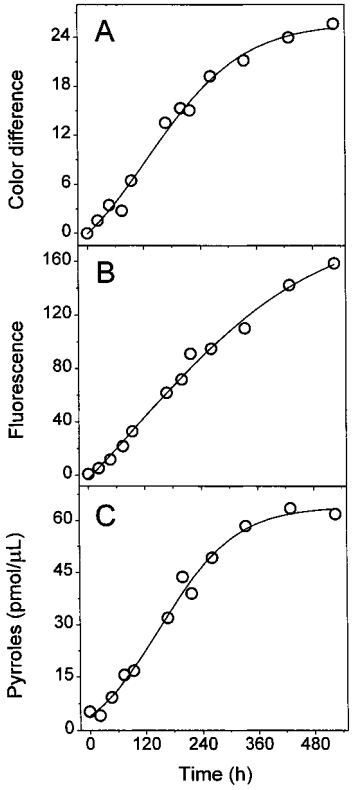


Figure 5. Time course of (A) color, (B) fluorescence, and (C) pyrrolization in a reaction of linolenic acid and lysine at 37 °C.

Therefore, and analogous to the results obtained at 37 °C, the smallest d*x* values corresponded to pyrrolization, followed by color and fluorescence. In addition, there were very good correlations among color, fluorescence, and pyrrolization measurements (Table 2).

DISCUSSION

The first mechanism proposed for the nonenzymatic browning produced by oxidized lipid/protein reactions was a repeated aldol condensation of the Schiff bases produced between the carbonyl compounds derived from unsaturated lipids and the protein-free amino groups (Belitz and Grosch, 1999; Frankel, 1998; Gardner, 1979; Montgomery and Day, 1965). It has not been until much more recently that an additional mechanism has also been proposed, suggesting that a pyrrole formation and polymerization was taking place and contributed to the production of both color and fluorescence in these reactions (Hidalgo and Zamora, 1993, 1995a; Zamora and Hidalgo, 1994). The main problem for testing if either of these mechanisms was occurring in foods was the lack of methods that allowed one to follow these polymerization reactions, which might also be influenced by the polymerization described for oxidized lipids to produce brown oxypolymers (Buttkus, 1975; Khayat and Schwall, 1983; Venolia and Tappel, 1958) and by the formation of colored low molecular weight compounds (Chio and Tappel, 1969; Kikugawa and Ido, 1984; Nakamura et al., 1998).

The results obtained in the present study propose a methodology, which may be applied to food systems, to investigate the contribution of pyrrole formation and polymerization mechanism to the nonenzymatic browning produced by oxidized lipid/protein reactions. By studying the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine model reaction, in which color and fluorescence have been shown to be a consequence of pyrrole formation and polymerization (Hidalgo and Zamora, 1993, 1995a; Zamora and Hidalgo, 1994), it has been found that the formation of color, fluorescence, and pyrrolization followed parallel kinetics. In addition, the correlation among these three measurements was always very high independent of the temperature of the reaction, suggesting that if pyrrole polymerization is the main mechanism responsible for the formation of color and fluorescence in these reactions, a very high correlation among color, fluorescence, and pyrrolization should be expected.

Very high correlations were also observed in the reaction between linolenic acid and lysine, suggesting that pyrrole polymerization was also contributing to the nonenzymatic browning produced in this system. Al-

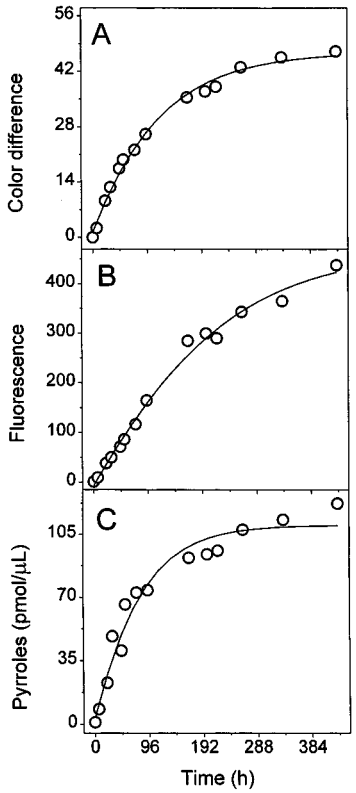


Figure 6. Time course of (A) color, (B) fluorescence, and (C) pyrrolization in a reaction of linolenic acid and lysine at 60 °C.

though oxidized fatty acids were previously found to react with the ϵ-amino group of lysine residues to produce both pyrrolized fatty acids and short-chain pyrroles (Hidalgo et al., 1995b,c; Zamora and Hidalgo, 1995), this is the first time that the formation of pyrroles is determined when an unoxidized fatty acid is incubated in the presence of an amino acid.

Additional confirmation that the pyrrole formation and polymerization were taking place was obtained from the curves adjusted by using the Boltzmann equation (eq 2). The width of the curves obtained for the pyrrolization of the amino acid was always the smallest of the three, suggesting that the formation of pyrroles is step immediately prior to the formation of color and fluorescence, which is in accordance with the mechanism proposed. In fact, the maximum value of pyrroles was attained prior to the maximum value of color or fluorescence, suggesting that pyrrole formation, and perhaps some polymerization, finished before the maximum color or fluorescence was reached. This maximum color and fluorescence might be related to an increase in the conjugation, and this might not influence significantly the formation of Ehrlich adducts.

With the data obtained in this study it is not possible to conclude if pyrrole formation and polymerization are the only, or even the main, mechanisms involved in the development of color and fluorescence in the linolenic acid/lysine system. Considering that the two studied reactions at the two assayed temperatures are comparable, the reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal and lysine produced ~15 times more pyrroles than the linolenic acid/lysine reaction, but the color difference obtained by the epoxyalkenal was only double than attained with the fatty acid. This might be a consequence of either additional mechanisms contributing to the formation of color in the linolenic acid/lysine system or other pyrroles different from those produced in the alkenal/lysine system and with a much higher browning capacity are being produced in the fatty acid/lysine reaction. Both mechanisms described by these hypotheses are likely to contribute to the nonenzymatic browning of this system. In fact, most of pyrroles obtained from linolenic acid were different from the pyrroles obtained from the epoxyalkenal because their Ehrlich adducts exhibited different maxima. On the other hand, the linolenic acid/lysine system produced >5 times the fluorescence produced by the epoxyalkenal. Additional studies are needed to isolate the pyrroles produced in the linolenic acid/lysine systems and to investigate how these pyrroles contribute to the formation of color and fluorescence in these reactions.

All of these results may also be useful for studying the color and fluorescence produced in the Maillard reaction between carbohydrates and proteins. In these latter reactions, a pyrrole polymerization mechanism, similar to that proposed for the oxidized lipid/protein reactions, has also been proposed (Tressl et al., 1998a,b), which might be competing with the polymerization of Amadori compounds also suggested (Kato and Tsuchida, 1981; Olsson et al., 1981). If pyrrole polymerization is contributing to the development of color and fluorescence in the Maillard reaction, good correlations among color, fluorescence, and pyrrolization should also be found in sugar/protein systems. These studies are being developed at present in this laboratory.

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LITERATURE CITED

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