Influence of Irradiation Time, pH, and Lipid/Amino Acid Ratio on Pyrrole Production during Microwave Heating of a Lysine/(*E*)-4,5-Epoxy-(*E*)-2-heptenal Model System

Rosario Zamora* and Francisco J. Hidalgo

Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Several lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal reaction mixtures, at various pHs and lipid/amino acid ratios, were irradiated for 15, 50, and 100 s in a microwave oven and then fractionated by high-performance liquid chromatography to better understand the nonenzymatic browning in foods by lipid peroxidation products during microwave heating. Peak areas of the characterized fractions were measured using chromatograms obtained at 250 nm. The irradiation time significantly decreased the peak area of the starting epoxyenealdehyde **1** and increased the peak areas of the formed 1-alkylpyrroles (**3**, **4**), 1-alkyl-2-(1′-hydroxypropyl)pyrroles (**5**, **6**), and 1-alkyl-2-(1′-propenyl)-pyrrole (**7**). Compounds **3**, **4**, and **7** showed a certain dependence on irradiation time, pH, and lipid/amino acid ratio. On the contrary, the formation of compounds **5** and **6** did not show a clear dependence on such variables, suggesting that these compounds were intermediates and were involved in further reactions. These results, and previous studies on nonirradiated samples, suggest a role of 1-alkyl-2-(1′-hydroxypropyl)pyrroles in the development of color and fluorescence in this system, when subject to microwave irradiation.

Keywords: *Microwave irradiation; pyrrole products; carbonyl–amine reactions; nonenzymatic browning; fluorescence development*

INTRODUCTION

Previous studies have shown that the model system formed by (*E*)-4,5-epoxy-(*E*)-2-heptenal and lysine may be useful to understand the nonenzymatic browning of foods via lipids (Hidalgo and Zamora, 1993a). This model system consists of a lipid peroxidation product, derived from an $n-3$ pentaenoic fatty acid (Swoboda and Peers, 1976), and lysine, an amino acid that is usually lost during deterioration of foods by peroxidizing lipids (Karel, 1984; Hurrel and Finot, 1985). It may be representative of systems rich in polyunsaturated fatty acids in which short-chain secondary oxidation products are easily produced and are able to react with amino groups of amines, amino acids, and proteins. (*E*)-4,5-Epoxy-(*E*)-2-heptenal, and other epoxyenealdehydes with this 4,5-epoxy-1-oxo-2-pentene system, particularly (*E*)-4,5-epoxy-(*E*)-2-decenal, seems to be easily produced during oxidative deterioration of lipids. They have been detected, among others, in many systems including oxidized butterfat (Swoboda and Peers, 1978), bread (Schieberle and Grosch, 1994), boiled trout (Milo and Grosch, 1993), and edible oils (Guth and Grosch, 1993).

(*E*)-4,5-Epoxy-(*E*)-2-heptenal is able to react with amino groups producing different 1-alkyl-2-(1′-hydroxypropyl)pyrroles and 1-alkylpyrroles which have been isolated and characterized in the reaction of the epoxyenealdehyde with butylamine (Hidalgo and Zamora, 1993b) and lysine (Zamora and Hidalgo, 1994). The produced 1-alkyl-2-(1′-hydroxypropyl)pyrroles were very unstable and spontaneously undergo polymerization reactions, which were responsible for the development of color and fluorescence (Hidalgo and Zamora, 1993b).

When the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine model system was irradiated in a microwave oven, the production of color and fluorescence was observed, and the browning rate was higher in this system than in an analogous glucose/lysine system (Zamora and Hidalgo, 1992). These results suggested that certain lipids might play an important role in the browning produced in microwaves at low or moderate irradiation times and also that these reactions might be useful as browning inducers in prepared foods, heated by microwaves. The characterization of the products formed in the model system upon microwave irradiation (Hidalgo and Zamora, 1995) revealed that both 1-alkyl-2-(1′-hydroxypropyl)pyrroles and 1-alkylpyrroles previously identified in incubated reactions at room temperature were also produced during microwave heating. In addition, 1-alkyl-2-(1′-hydroxypropyl)pyrroles, responsible for color and fluorescence formation in these reactions, undergo dehydration to produce 1-alkyl-2-(1′-propenyl)pyrroles, which were found for the first time. The objective of this paper was to study the influence of irradiation time, pH, and lipid/amino acid ratio on the formation of the nonenzymatic browning products and to analyze how the production of this type of compound might be related to the development of color and fluorescence in this model system.

EXPERIMENTAL PROCEDURES

Materials. (*E*)-4,5-Epoxy-(*E*)-2-heptenal was prepared from (*E*)-2-(*E*)-4-heptadienal as described by Swoboda and Peers (1978). (*E*)-2-(*E*)-4-Heptadienal and L-lysine were purchased from Aldrich Chemical Co. (Milwaukee, WI), and 3-chloroperoxybenzoic acid was from Fluka Chemie AG (Buchs, Switzerland). All other chemicals used were of analytical grade and were purchased from reliable commercial sources.

Sample Preparation. Sample preparation for high-performance liquid chromatography (HPLC) was analogous to the sample preparation for color and fluorescence measurements described by Zamora and Hidalgo (1992), but the irradiated samples (100 μL) were diluted with 400 μL of 0.1 M potassium phosphate, adjusted to pH 6.0, and then injected in the liquid chromatograph. The microwave oven and irradiation conditions used were the same as those in a previous study (Zamora and Hidalgo, 1992).

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of a 126 programmable delivery

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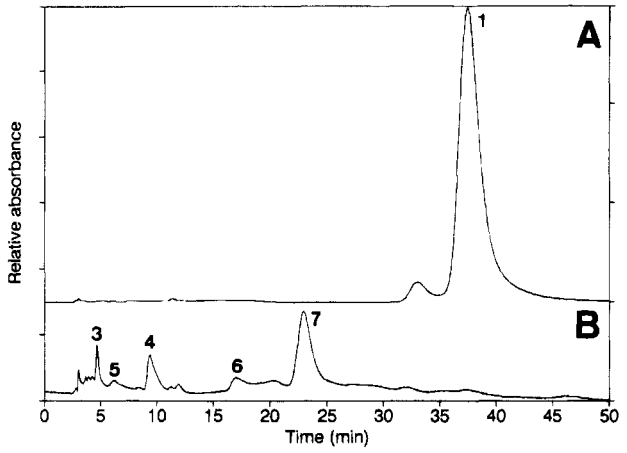


Figure 1. High-performance liquid chromatographic elution pattern of an (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine, pH 7, reaction mixture irradiated at the high setting of a microwave oven for 15 (A) and 100 s (B). The UV detection was carried out at 250 nm.

module and a 168 diode array detector module (Beckman, Fullerton, CA). Data acquisition and processing were effected with the software system Gold 7.1 version (Beckman). Comparison of UV spectra of the different peaks was carried out by using the software Scan Graphics (Beckman). Separations were realized by a Spherisorb ODS2 3-μm 25 × 0.46-cm column, using a loop of 20 μL and a flow rate of 0.8 mL/min. A temperature of 40 °C and UV wavelengths of 220 and 250 nm were selected in all experiments. Chromatograms at other wavelengths were obtained using the software ArrayView 1.0 version (Beckman).

Statistical Analysis. 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 the *Primer of Biostatistics: The Program* (McGraw-Hill: New York).

RESULTS

When a mixture of (*E*)-4,5-epoxy-(*E*)-2-heptenal and lysine was irradiated in a microwave oven, the development of color and fluorescence was observed (Zamora and Hidalgo, 1992). This was parallel to the formation and disappearance of several peaks in the high-performance liquid chromatogram. Figure 1, parts A and B, shows the high-performance liquid chromatograms obtained for mixtures at pH 7 irradiated for 15 and 100 s, respectively. Several pyrrole derivatives (compounds **3**–**7**), which were isolated and characterized, were formed during irradiation. This formation

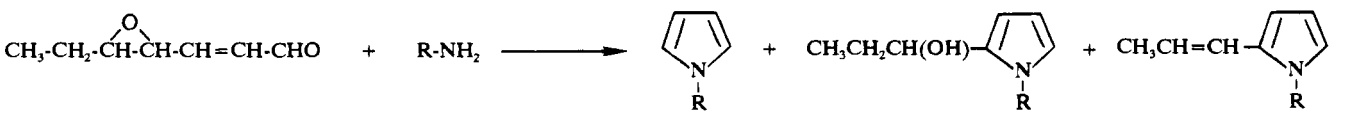
Table 1. Influence of Exposure Time on (*E*)-4,5-Epoxy-(*E*)-2-heptenal/Lysine Reaction Products at pH 7[a]

	exposure time		
compd	15 s	50 s	100 s
1	89480 ± 7400[b]	8823 ± 2427[c]	0 ± 1[c]
3	18 ± 7[b]	216 ± 6[c]	276 ± 11[d]
4	9 ± 4[b]	283 ± 28[c]	488 ± 33[d]
5	69 ± 4	80 ± 7	120 ± 36
6	82 ± 34	163 ± 70	273 ± 51
7	0 ± 1[b]	112 ± 25[b]	1761 ± 170[c]

[a] Irradiated samples were fractionated on a Spherisorb ODS2 column using the conditions described under Experimental Procedures. Peak areas are given for the chromatogram at 250 nm and are expressed in 100 μV s^{-1}. Values are mean ± SEM for three determinations. Means in the same row with different superscripts are significantly different ($p < 0.05$) as assessed by the Student–Newman–Keuls test.

was parallel to the disappearance of the starting epoxyenealdehyde **1**. The numbers of peaks in the high-performance liquid chromatogram are in accordance with the compounds identified previously, and their structures are shown in Figure 2.

The areas of the different peaks separated by high-performance liquid chromatography on an ODS2 column, using a reaction mixture at pH 7 and several irradiation times, are presented in Table 1. These areas were calculated for the chromatogram obtained at 250 nm. This chromatogram was better for peak area determination than the one obtained at 220 nm because it allowed an easier quantification of compounds **5**–**7**. These last compounds showed a small absorbance at 220 nm. Because different compounds with different chromophores were implicated, peak areas were only comparable for the same compound at different irradiation times or for different compounds with the same chromophoric group (i.e., compounds **3** and **4** and **5** and **6**). The peak area of starting epoxyenealdehyde **1** diminished significantly for the first 50 s, and this peak disappeared in latter stages. The formation of 1-alkylpyrroles **3** and **4** was gradual, and the peak area in the high-performance liquid chromatogram increased significantly from 15 to 50 s and also from 50 to 100 s. After 100 s of irradiation, the formation of compound **4** was significantly higher ($p = 0.004$) than formation of compound **3** (about 77%). The formation of 1-alkyl-2-(1′-hydroxypropyl)pyrroles **5** and **6** increased also as a function of irradiation time, but the differences were not significant at $p < 0.05$. Analogously to 1-alkylpyrroles, after 100 s of irradiation, the corresponding derivative of the ϵ-amino group of lysine (compound **6**) was higher than compound **5** by about 130% ($p = 0.07$). Finally,



1 2 3,4 5,6 7

Compound	R	Compound	R
2	$(CH_2)_4CH(NH_2)COOH$	**5**	$CH(COOH)(CH_2)_4NH_2$
3	$CH(COOH)(CH_2)_4NH_2$	**6**	$(CH_2)_4CH(NH_2)COOH$
4	$(CH_2)_4CH(NH_2)COOH$	**7**	$CH(COOH)(CH_2)_4NH_2$

Figure 2. Structures of the compounds identified from the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture irradiated at the high setting of a microwave oven. Compounds were isolated by semipreparative HPLC and identified by ^{1}H and ^{13}C NMR and MS.

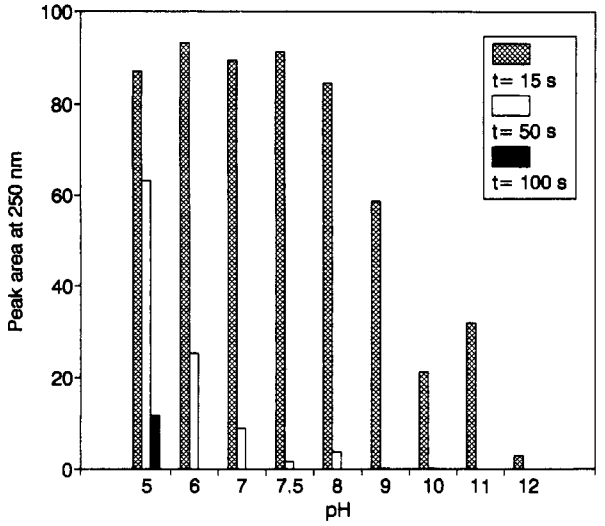


Figure 3. Effect of pH on peak area ($\times 10^3$) obtained for the epoxyenealdehyde **1** in the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture after microwave irradiation for 15, 50, and 100 s.

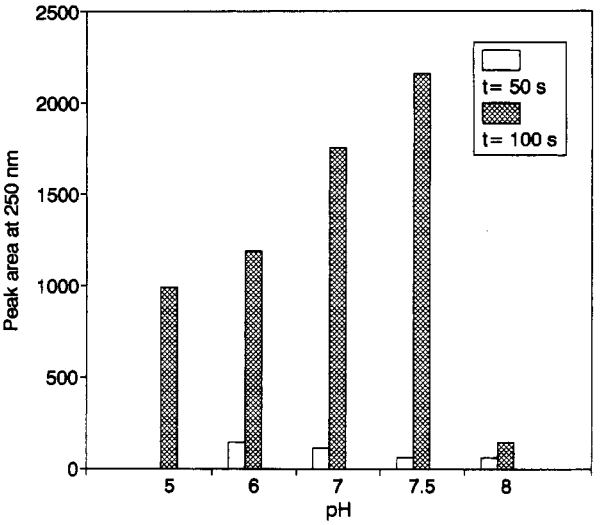


Figure 5. Effect of pH on peak area obtained for the 1-alkyl-2-(1′-propenyl)pyrrole **7** produced in the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture after microwave irradiation for 50 and 100 s.

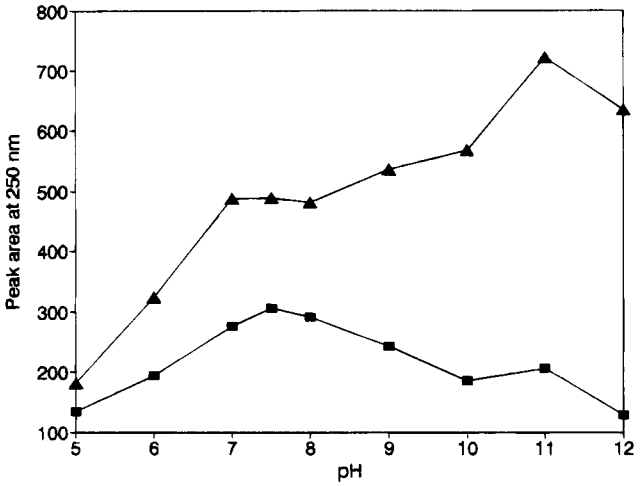


Figure 4. Effect of pH on peak area obtained for the 1-alkylpyrroles **3** (■) and **4** (▲) produced in the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture after microwave irradiation for 100 s.

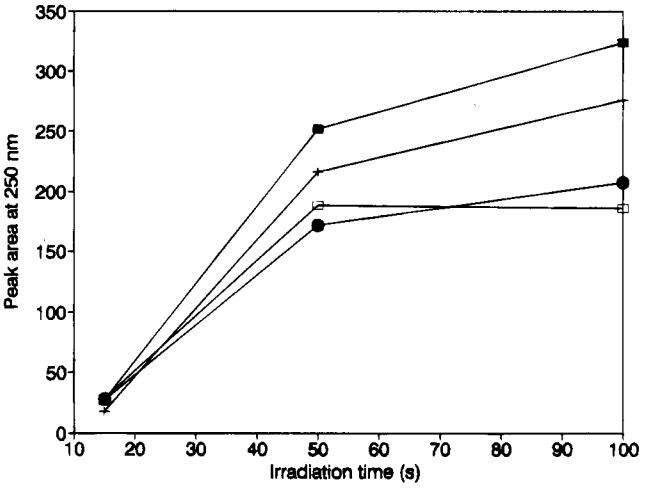


Figure 6. Effect of epoxyenealdehyde/lysine ratio [1:4 (■), 1:2 (+), 1:1 (●), and 2:1 (□)] on formation of 1-alkylpyrrole **3** as a function of irradiation time.

the formation of 1-alkyl-2-(1′-propenyl)pyrrole **7** was only significant in the last stages of the irradiation (from 50 to 100 s). This increase in peak areas of reaction products as a function of irradiation time was observed at all pHs and lipid/amino acid ratios assayed.

The pH played an important role in the disappearance of the starting epoxyenealdehyde and in the formation of some pyrrole derivatives. Figure 3 shows the peak areas obtained for compound **1** from pH 5 to 12 at the three irradiation times studied. In samples irradiated for 15 s, no significant changes in the concentration of the epoxyenealdehyde were observed at pH ≤7.5. However, higher pHs decreased the epoxyenealdehyde present after 15 s of irradiation, suggesting that at these pHs the amino groups of lysine reacted spontaneously with the epoxyenealdehyde and no irradiation was needed.

The reaction of the epoxyenealdehyde with lysine mainly produced the 1-alkylpyrroles **3** and **4**. Figure 4 shows the formation of compounds **3** and **4** after 100 s of irradiation. Compound **3** was mainly produced at pH 7−8, and the formation of compound **4** was higher at high pHs. Compound **4** was produced in a higher proportion than compound **3**, and this proportion increased with pH. Thus, compound **4** was 35% higher than compound **3** at pH 5, and this increased to 400% at pH 12.

Peak areas found for 1-alkyl-2-(1′-hydroxypropyl)-pyrroles **5** and **6** did not show a clear tendency as a function of pH, and both compounds were formed similarly at the different pHs. Compound **6** was produced in a higher proportion than compound **5**, and this ratio was approximately constant at the different pHs.

Figure 5 shows the production of 1-alkyl-2-(1′-propenyl)pyrrole **7** from pH 5 to 8. This compound increased from pH 5 to 7.5 and was not produced in a first instance at higher pHs. At pH >8, another compound appeared at the same position in the high-performance liquid chromatogram, but it failed to give the characteristic maximum at 270 nm of compound **7**. This compound, which presented a maximum at 230 nm, could not be characterized.

Analogously to pH, the epoxyenealdehyde/lysine ratio also influenced the production of several reaction products. Figure 6 shows that an increase in the proportion of lysine increased the production of compound **3**. The same effect was observed for compound **4**. However, an

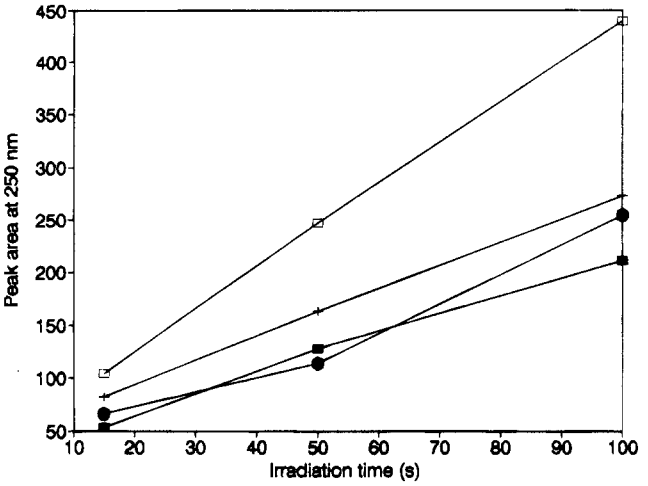


Figure 7. Effect of epoxyenealdehyde/lysine ratio [1:4 (■), 1:2 (+), 1:1 (●), and 2:1 (□)] on formation of 1-alkyl-2-(1′-hydroxypropyl)pyrrole **6** as a function of irradiation time.

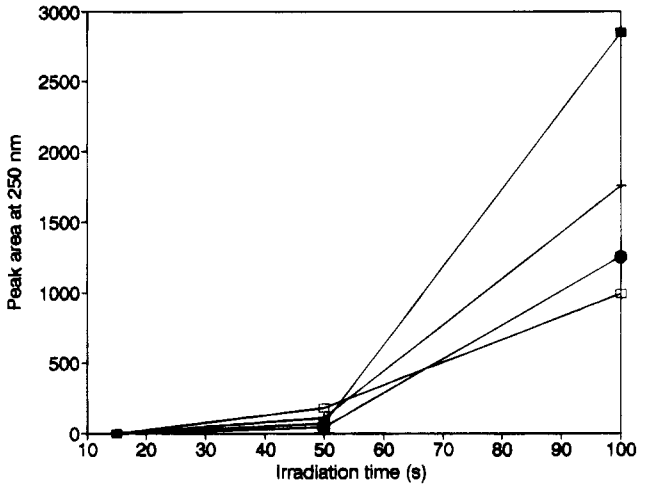


Figure 8. Effect of epoxyenealdehyde/lysine ratio [1:4 (■), 1:2 (+), 1:1 (●), and 2:1 (□)] on formation of 1-alkyl-2-(1′-propenyl)-pyrrole **7** as a function of irradiation time.

increase in the proportion of epoxyenealdehyde did not produce any effect in the production of compound **3** or **4**.

The influence of epoxyenealdehyde/lysine ratio on the production of compound **6** was the opposite of that for the production of 1-alkylpyrroles **3** and **4**. Figure 7 shows that an increase in the proportion of the epoxyenealdehyde increased the formation of compound **6**, but an increase in the proportion of lysine did not have a clear effect on the production of this compound. The results obtained for the production of compound **5** were not so clear, most probably because this compound was involved in the production of compound **7**, in addition to the production of color and fluorescence.

The epoxyenealdehyde/lysine ratio also influenced the production of compound **7**, and it had a behavior analogous to that of compounds **3** and **4** (Figure 8). An increase in the proportion of lysine increased the production of this compound, and the increase in the proportion of epoxyenealdehyde had not a clear effect.

DISCUSSION

When an (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture was irradiated in a microwave oven for different periods of time, the development of color and fluorescence was observed (Zamora and Hidalgo, 1992), and this was parallel to the formation of several pyrrole monomers and the disappearance of the starting epoxyenealdehyde. The major compounds of the reaction were 1-alkylpyrroles **3** and **4**. These compounds, which were difficult to detect at low irradiation times, increased significantly with irradiation time. The rate of formation was higher for compound **3** at pH 7–8 and for compound **4** at pH 11. This is supposed to be a consequence of the pK_a of lysine, because the amino group involved in the reaction needs to be in the basic form to react with the carbonyl group. At pH 7–8, an important part of the α-amino group of lysine is in the basic form, and therefore it is preferred for the reaction. At higher pHs, both α- and ϵ-amino groups are mainly in the basic form. At these pHs, the ϵ-amino group is preferred. This might be related to either the steric effect of the carboxyl group or the inductive effect of this group, which is electron-withdrawing and, therefore, attracts the electrons of the nitrogen and diminishes its reactivity. Both compounds **3** and **4** were produced in a higher extent when a higher proportion of lysine was used. This suggests a certain role of the lysine excess in liberating the supposedly formed propanal, most probably by reacting with the aldehyde and shifting the equilibrium.

The influence of irradiation time, pH, and aldehyde/lysine ratio on the formation of 1-alkyl-2-(1′-hydroxypropyl)pyrroles **5** and **6** was not clear. The irradiation time increased the formation of both compounds by 100–200%. However, this increase was much lower than the one observed for compounds **3** and **4** (1500–5000%). The pH did not produce a clear effect on the formation either compound, and only an increase in the proportion of the epoxyenealdehyde increased the formation of compound **6**. These effects are probably a consequence of the processes in which these compounds are involved. Previous studies have shown that 1-alkyl-2-(1′-hydroxypropyl)pyrroles are responsible for color and fluorescence formation in the reaction of (*E*)-4,5-epoxy-(*E*)-2-heptenal with amines and amino acids at low or moderate temperatures, via a polymerization reaction (Hidalgo and Zamora, 1993b; Zamora and Hidalgo, 1994). The results found in this study suggest that these compounds are also responsible for color and fluorescence formation when these reactions occur during microwave irradiation. The much lower increase observed during the formation of compounds **5** and **6** than for compounds **3** and **4**, which are supposed to be final compounds in these reactions, suggests that compounds **5** and **6** might be involved in further reactions such as polymerization reactions that develop the color and fluorescence observed. The important dependence of epoxyenealdehyde concentration on production of compound **6**, and also on color and fluorescence development (Zamora and Hidalgo, 1992), is additional evidence for the contribution of 1-alkyl-2-(1′-hydroxypropyl)-pyrroles to the overall browning and fluorescence produced in this model system.

The study of influence of irradiation time, pH, and aldehyde/lysine ratio on the formation of 1-alkyl-2-(1′-propenyl)pyrrole **7** showed that this compound was produced mainly in the last stages of the irradiation period and suggested that long exposure to microwave irradiation or very high temperatures were needed for the dehydration reaction, which is supposed to produce this compound. This might be the explanation for its absence in nonheated systems (Zamora and Hidalgo, 1994). Therefore, this compound might be characteristic

of microwave irradiation, and its presence can be related to severely irradiated samples. However, several facts related with the formation of this compound remain to be clarified. The 1-alkyl-2-(1′-propenyl)pyrrole derived from the ϵ-amino group of lysine has not been found (it should have the same UV spectrum as that of compound **7**, a spectrum that is very characteristic and easy to detect). In addition, compound **7** does not seem to be produced, in a first instance, at pH >8, suggesting that the basic catalysis is not involved in this dehydration. Finally, an increase in the proportion of lysine increases the formation of compound **7**, and an increase in the proportion of the epoxyenealdehyde does not seem to have a clear effect on the final concentration of compound **7**. This might be a consequence of the overall reactions that produce color and fluorescence in this model system.

Analogously to nonirradiated systems, the reaction of (*E*)-4,5-epoxy-(*E*)-2-heptenal with lysine during microwave irradiation produced mainly 1-alkylpyrroles and 1-alkyl-2-(1′-hydroxypropyl)pyrroles. The pH greatly influenced the amino group of lysine susceptible for reaction. However, color and fluorescence were much more influenced by lipid/amino acid ratio. At higher concentrations of epoxyenealdehyde, 1-alkyl-2-(1′-hydroxypropyl)pyrroles were produced to a higher extent. Therefore, it should be easy for these molecules to react among them, producing polymers, color, and fluorescence. A higher color and fluorescence were observed at higher concentrations of epoxyenealdehyde (Zamora and Hidalgo, 1992). On the contrary, at higher concentrations of lysine, a lower proportion of 1-alkyl-2-(1′-hydroxypropyl)pyrroles was produced because the excess of lysine reacted with the produced propanal and the formation of 1-alkylpyrroles **3** and **4** was favored. This should decrease the polymerization rate of 1-alkyl-2-(1′-hydroxypropyl)pyrroles (present at low concentrations), favoring the dehydration reaction.

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