Nonenymatic Browning, Fluorescence Development, and Formation of Pyrrole Derivatives in Phosphatidylethanolamine/ Ribose/Lysine Model Systems

FRANCISCO J. HIDALGO, FATIMA NOGALES, AND ROSARIO ZAMORA

ABSTRACT: Browning, fluorescence, and pyrrole formation were determined in 4 mixtures of phosphatidylethanolamine (PE), ribose (RI), and/or lysine (lys) incubated at 60 °C to study the contribution of lipid oxidation to food browning when lipid oxidation products are formed in the presence of other food components. The 4 assayed systems (PE/lys, PE/RI, RI/lys, and PE/RI/lys) were also extracted with chloroform-methanol (2:1) to distinguish between the nonenzymatic browning produced with the amino group of PE and that produced with the amino groups of lys. Under the assay conditions, both the RI and the oxidized products produced in the fatty acid chains of PE contributed to the development of browning in the assayed systems. However, RI and oxidation products of PE exhibited different reactivities for the different amino groups involved in these reactions. Thus, the oxidized lipids reacted with the amino group of PE with preference for the amino group of lys, and this preference was reversed for RI. These results suggest that the contribution of both carbohydrates and lipid oxidation products to food browning may be distinguished. In addition, determination of oxidized lipid/ethanolamine derivatives in phospholipids may be an alternative procedure to evaluate oxidative damage in foods.

Keywords: lipid oxidation, amino phospholipids, amino-carbonyl reactions, pyrroles, food browning

Introduction

Lipid oxidation is a major cause of food spoilage and is undesirable from a sensory acceptability and economic point of view (Nawar 1996; Akoh and Min 2002; Gordon 2004). Lipid oxidation produces different lipid oxidation products that are able to modify different food components. Among them, the chemical reactions of oxidized lipids with amines, amino acids, and proteins have received considerable attention because they constitute important deteriorative mechanisms that also produce changes in the functional properties, nutritive value, flavor, and color of foods (Gardner 1979; Ericksson 1987; Friedman 1996).

Oxidized lipid/amino group reactions in foods are only partially understood at present because of the high reactivities of reactants and products, the intertwining reaction routes, and the diversity of products (Hidalgo and Zamora 2000a). Nowadays, the best understood reactions are likely the chemical modifications of amines, amino acids, and proteins produced by model oxidized lipids, namely malondialdehyde (Chio and Tappel 1969; Kikugawa and others 1984), 4,5-epoxy-2-alkenals (Hidalgo and Zamora 1993a, 2000b; Zamora and Hidalgo 2005a), 4-hydroxy-2-nonenal (Salomon and others 2000), 4-oxo-2-nonenal (Zhang and others 2003), unsaturated epoxyoxo fatty acids (Hidalgo and Zamora 1995), and levuglandin E_2 (Salomon and others 1997). On the other hand, the reactions produced by incubating unmodified lipids in the presence of other food components are only partially understood because different reactions are competing and the effects observed are consequences of all these reactions.

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In an attempt to study food browning produced by the reaction of lipid oxidation products with amino acids in the presence of other food components, such as carbohydrates, this study describes the development of browning and fluorescence in systems containing phospholipids, amino acids, and carbohydrates. This study also determined formation of pyrrole derivatives because pyrroles are common products in the reaction of different lipid oxidation products with the primary amino groups of amines, amino acids, and proteins (Figure 1). In addition, the pyrrole amino acid ε-*N*-pyrrolylnorleucine has been found in more that 20 fresh food products (Zamora and others 1999).

Materials and Methods

Materials

Model systems analyzed included different mixtures of phosphatidylethanolamine (PE), ribose (RI), and lysine (lys). As a model phospholipid, soybean PE was selected because it is highly unsaturated, has a free amino group that can compete with the amino acid for the lipid oxidation (or sugar degradation) products produced, and can form relatively stable vesicles that may facilitate its reaction with water-soluble components. In addition, reactions may be easily stopped by phospholipid extraction with a polar solvent. RI was selected as a model carbohydrate because pentoses have a high reactivity for the Maillard reaction (Namiki and Hayashi 1983), and lys is an amino acid usually lost in both carbohydrate/protein and oxidized lipid/protein reactions.

PE was isolated from soybean lecithin by column chromatography on silicic acid/celite (2:1) using chloroform/methanol (3:1) as eluent, as described previously (Zamora and Hidalgo 2003). Other reagents and solvents were of analytical grade and were purchased from reliable commercial sources.

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PE/RI/lys model systems

Four model systems were studied: PE/lys, as a model for oxidized lipid/amino acid reactions with 2 different amino groups; PE/RI, as a model for a Maillard reaction between a carbohydrate and the amino group of a phospholipid in competition with the reaction between the phospholipid amino group and the lipid oxidation products produced; RI/lys, as a model for a classical Maillard reaction; and PE/RI/lys, as a model where the different reactions may take place simultaneously. The phospholipid (100 mg, 135 μmol) was suspended in 10 mL of 50 m*M* sodium phosphate buffer (pH 7.4), sonicated at a low power until a homogeneous emulsion was obtained, treated with the other components of the model system (675 μmol of each component), and finally, incubated at 60 °C for 4 d. For the RI/lys system, the phospholipid was not added but the procedure was identical. At different incubation times, samples (1 mL) were extracted twice with 2 mL of chloroform-methanol (2:1), and the resulting organic and aqueous extracts analyzed for browning, fluorescence, and pyrrole formation. PE was quantitatively recovered with the described procedure (Zamora and others 2005), and organic extracts were composed by PE and its derivatives (oxidized phospholipids and reaction products with the phospholipid amino group).

Analytical measurements

Organic extracts were taken to dryness, weighed, and dissolved in chloroform-methanol (2:1) to obtain 0.5% (w/v) solutions. Aqueous extracts (1.8 mL) were analyzed without any further treatment.

Color changes were determined spectrophotometrically using a Shimadzu UV-2401 PC UV-vis spectrophotometer Shimadzu Scientific Instruments, Columbia Md., U.S.A.). The yellowness index (YI) at the different periods of time was calculated from the determined CIELAB $L^*a^*b^*$ values according to Francis and Clydesdale (1975) as follows:

$$YI = 142.86 \times (b^*/L^*)$$

Fluorescence spectra were recorded with a Perkin-Elmer LS-5 fluorescence spectrophotometer (PerkinElmer, Boston, Mass., U.S.A.) using 10-μL samples diluted to 3 mL with methanol. A slit width of 10 nm was used, and the instrument was standardized with quinine sulfate (0.1 μ*M* in 0.1 *N* H_2SO_4) to give a fluorescence intensity of 100 at 450 nm, when excitation was done at 350 nm.

Pyrroles were determined with *p*-(dimethylamino)benzaldehyde following previously described procedures in both aqueous (Hidalgo and others 1998) and organic (Hidalgo and others 2004) extracts. Values are given in nmol of pyrrole per mL of reaction mixture in both aqueous and organic extracts.

Statistical analyses

All results are expressed as mean values ± SD of 3 experiments. Data were analyzed using Curve Fitting Functions of Microcal Origin® 7.0 (Microcal Software Inc., Northampton, Mass., U.S.A.).

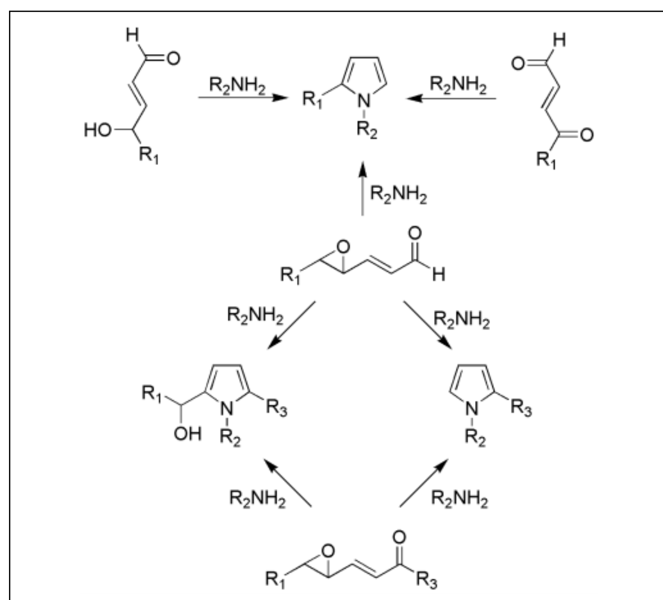

Figure 1—Formation of pyrrole derivatives in the reaction of lipid oxidation products with amino compounds.

Results and Discussion

Browning, fluorescence, and pyrrole development in a PE/lys model system

When an emulsion of PE and lys in sodium phosphate buffer was incubated for 4 d at 60 °C, the development of browning and fluorescence, and the conversion of some amino groups into pyrrole groups in both PE and lys was observed. Figure 2 shows the time-courses of YI, fluorescence, and pyrrolization produced in this system. This figure also includes the values observed in both organic and aqueous extracts. Browning, fluorescence, and pyrrolization produced in the organic and aqueous extracts of this model corresponded to the changes suffered by the amino group of the phospholipid and the amino groups of the amino acid, respectively (Zamora and others 2005).

Browning development in the non-extracted sample followed zero-order reaction kinetics ($r = 0.998$; $P = 0.00011$) and was described by Eq. 1:

$$YI = YI_0 + kt \quad (1)$$

where YI_0 represents the intercept, k is the rate constant, and t is the time. The values for YI_0 and k were 3.16 and 7.03/d^{-1}, respectively. Analogous zero-order kinetics were observed in both organic ($r = 0.999$; $P < 0.0001$) and aqueous extracts ($r = 0.96$; $P = 0.01$). YI_0 and k were 1.54 and 6.18/d^{-1}, and 1.62 and 0.85/d^{-1} for organic and aqueous extracts, respectively.

Fluorescence development was very similar to YI development and also exhibited zero-order reaction kinetics in the non-extracted sample ($r = 0.998$; $P < 0.0001$), and the organic ($r = 0.998$; $P < 0.0001$) and the aqueous ($r = 0.93$; $P = 0.02$) extracts. It was described by Eq. 2:

$$F = F_0 + kt \quad (2)$$

F_0 and k values for the non-extracted sample and for the organic and aqueous extracts were 9.97 and 18.89/d^{-1}, 5.62 and 17.35/d^{-1}, and 4.35 and 1.54/d^{-1}, respectively.

Most of fluorescence was linked to the phospholipid, analogously to browning. In fact, browning and fluorescence developments were correlated in the non-extracted sample ($r = 0.999$; $P < 0.001$), and the organic ($r = 0.998$; $P = 0.00011$), and aqueous extracts ($r = 0.94$; $P = 0.02$).

Analogous zero-order reaction kinetics were also observed when formation of pyrrole rings was determined in the non-extracted sample ($r = 0.999$; $P < 0.0001$), and the organic ($r = 0.992$; $P = 0.00089$) and aqueous ($r = 0.992$; $P = 0.00083$) extracts, and all of them were described by Eq. 3:

$$P = P_0 + kt \quad (3)$$

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P_0 and k values for the non-extracted sample, and the organic and aqueous extracts were 7.71 and 33.90 nmol mL^{-1} d^{-1}, 10.02 and 22.14 nmol mL^{-1} d^{-1}, and 0.31 and 11.76 nmol mL^{-1} d^{-1}, respectively.

Pyrrolization of PE was also higher to pyrrolization of lys, analogously to browning and fluorescence development. In fact, pyrrolization was correlated with browning development in the non-extracted sample ($r = 0.996$; $P = 0.00033$), and the organic ($r = 0.987$; $P = 0.0015$) and aqueous extracts ($r = 0.965$; $P = 0.008$), and with fluorescence development in the non-extracted sample ($r = 0.997$; $P = 0.00025$), and the organic ($r = 0.989$; $P = 0.0014$) and aqueous extracts ($r = 0.953$; $P = 0.012$).

The zero-order reaction kinetics observed for browning, fluorescence, and pyrrolization in this system were analogous to those observed in a 4,5-epoxy-2-heptenal/lysine model system (Hidalgo and Zamora 1993b) or in more complex systems involving fatty acids and proteins (Hidalgo and Zamora 2002). In addition, a high correlation among browning, fluorescence, and pyrrolization has always been observed when browning is a consequence of the pyrrole polymerization mechanism both in model systems (Zamora and others 2000) and foods (Hidalgo and others 2001). This mechanism is believed to be a major mechanism for browning and fluorescence development in oxidized lipids/amino groups reactions (Hidalgo and Zamora 1993a).

Browning, fluorescence, and pyrrolization were mainly produced in the organic extract, therefore indicating that the phospholipid contributes more than the amino acid to the observed changes. Because browning, fluorescence, and pyrrolization development in this system is a consequence of the reaction between the lipid oxidation products produced in the phospholipid fatty acid chains and the amino groups of both PE and lys (Zamora and others 2005), these results suggest that the reaction takes place in the amino group of the phospholipid with preference to the amino groups of the amino acid, in spite of the much higher concentration of amino groups of lysine (there were about 10 amino groups of lysine per amino group of PE in the reaction). This may be a consequence of the proximity of the place of generation of the lipid oxidation products and the amino group of PE. It also suggests that phospholipids should be analyzed with preference to amino acids and proteins when determining the development of these reactions in foods. Therefore, the determination of damaged phospholipids might be used to evaluate oxidative damage in foods as an alternative to other methodologies (Lynch and others 2001; Wold and others 2002).

Browning, fluorescence, and pyrrole development in a PE/RI model system

When lys was replaced by RI, the system also developed browning and fluorescence, and the conversion of the amino group of PE into a pyrrole group was also observed. Figure 3 shows the time-courses of YI, fluorescence, and pyrrolization produced in this system. This figure also includes the values observed in both organic and aqueous extracts.

Different from the PE/lys system described previously, browning, fluorescence, and pyrrolization in the PE/RI system followed neither zero-order nor 1st-order reaction kinetics. However, analogously to other model systems that also developed browning, fluorescence, and pyrrolization (Zamora and others 2000), the obtained data could be fitted ($r^2 > 0.9992$) by using the Boltzmann Eq. 4:

$$y = [(A_1 - \ _2)/(1 + e^{(x - x_0)/dx}] + \ _2 \qquad (4)$$

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 of the curve.

The absence of amino groups in the water-soluble fraction caused that most of the browning, fluorescence, and pyrrolization was linked to the phospholipid. However, small increases in browning and fluorescence development and in pyrrolization were ob-

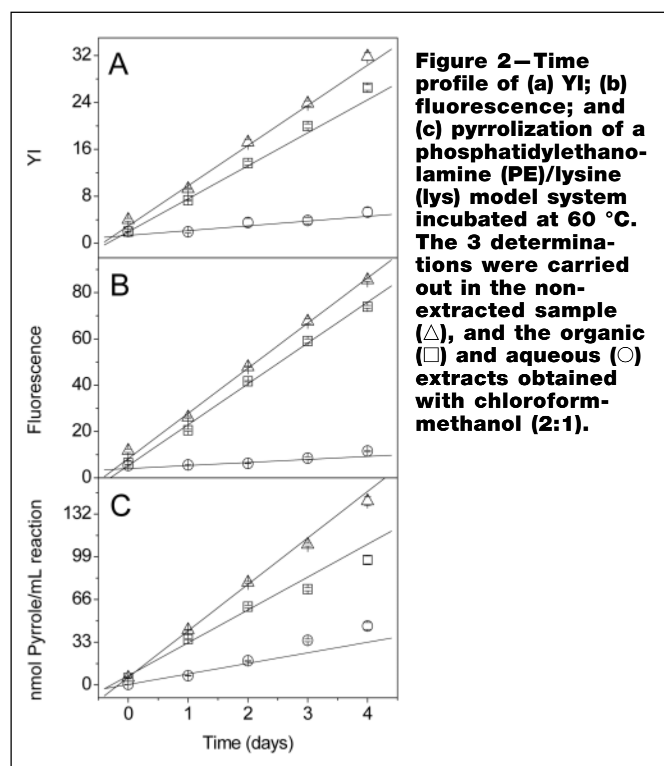

Figure 2—Time profile of (a) YI; (b) fluorescence; and (c) pyrrolization of a phosphatidylethanolamine (PE)/lysine (lys) model system incubated at 60 °C. The 3 determinations were carried out in the non-extracted sample (△), and the organic (□) and aqueous (○) extracts obtained with chloroform-methanol (2:1).

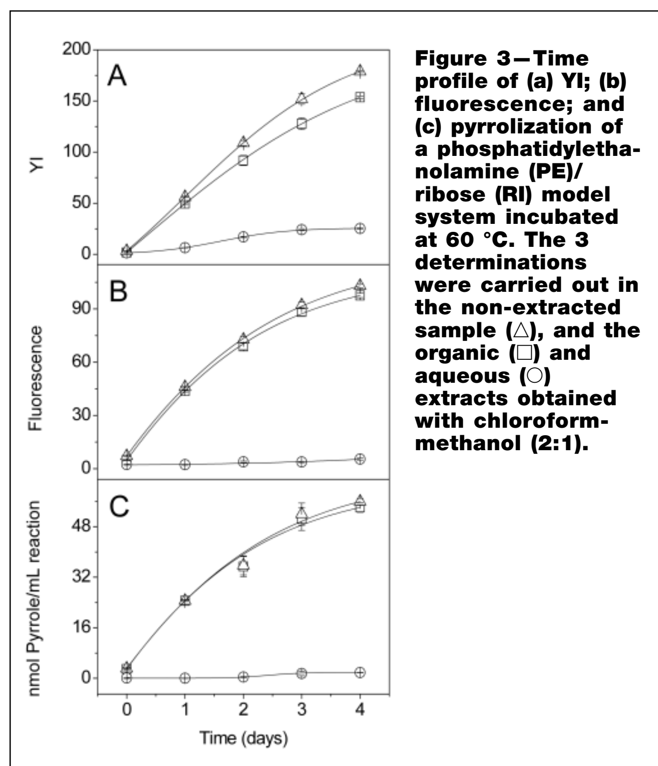

Figure 3—Time profile of (a) YI; (b) fluorescence; and (c) pyrrolization of a phosphatidylethanolamine (PE)/ribose (RI) model system incubated at 60 °C. The 3 determinations were carried out in the non-extracted sample (△), and the organic (□) and aqueous (○) extracts obtained with chloroform-methanol (2:1).

served in the aqueous fraction. These last increases might be related to the breakage of the phospholipid that should produce small amounts of water soluble amino derivatives. In addition, these 3 measurements were highly correlated. Thus, browning development was correlated with fluorescence in the non-extracted sample ($r = 0.993$; $P = 0.00069$), and the organic ($r = 0.992$; $P = 0.00089$) and aqueous extracts ($r = 0.993$; $P = 0.00069$), and with pyrrolization in the non-extracted sample ($r = 0.993$; $P = 0.00074$), and the organic ($r = 0.992$; $P = 0.00089$) and aqueous extracts ($r = 0.993$; $P = 0.00074$); and fluorescence development was correlated with pyrrolization in the non-extracted sample ($r = 0.996$; $P = 0.00034$), and the organic ($r = 0.997$; $P = 0.00019$) and aqueous extracts ($r = 0.871$; $P = 0.05$).

Two reactions are contributing to the nonenzymatic browning produced in the PE/RI system: the Maillard reaction between the sugar and the amino group of PE, and the reaction of fatty acid chain oxidation products with the amino group of PE. Because browned products produced by both reactions are presumably linked to the phospholipid, it is not possible to distinguish between them with extraction. However, by comparison of the results obtained for PE/RI and PE/lys systems, the contribution of the Maillard reaction between the sugar and the amino group of PE to the development of browning, fluorescence, and pyrrolization may be hypothesized. The final values, calculated according to Eq. 4, for the reaction of fatty acid chain oxidation products with the amino group of PE in the PE/lys system (the organic extract of the PE/lys model system) were 48.5 for YI, 84.8 for fluorescence, and 124 nmol of pyrrole per mL of reaction. In addition, the final values for the PE/RI system, calculated according to Eq. 4, were 203 for YI, 111.8 for fluorescence, and 61.5 nmol of pyrrole per mL of reaction. Therefore, the Maillard reaction between the sugar and the amino group of PE should be responsible for most of the browning produced. On the other hand, the reaction of fatty acid chain oxidation products with the amino group of PE seems to be responsible for a significant portion of the pyrrolization observed.

Browning, fluorescence, and pyrrole development in a RI/lys model system

The RI/lys system also developed browning, fluorescence, and pyrrolization when incubated for 4 d at 60 °C (Figure 4). However, in contrast to the previously described model systems, browning and fluorescence developments and amino acid pyrrolization were produced only in the aqueous extracts. In addition, these reactions were produced much more rapidly than those in which the oxidation of the fatty acid chains of PE was needed (PE/lys and PE/RI model systems).

Analogously to the previously described systems, the browning, fluorescence, and pyrrolization were highly correlated. Thus, browning development was correlated with fluorescence in the non-extracted sample ($r = 0.991$; $P = 0.001$), and the aqueous extract ($r = 0.991$; $P = 0.001$), and with pyrrolization in the non-extracted sample ($r = 0.995$; $P = 0.00041$), and the aqueous extract ($r = 0.995$; $P = 0.00041$); and fluorescence development was correlated with pyrrolization in the non-extracted sample ($r = 0.999$; $P < 0.0001$), and the aqueous extract ($r = 0.999$; $P < 0.0001$). As expected, browning, fluorescence, and pyrrolization of organic extracts did not exhibit any significant increase during incubation.

The RI/lys system very rapidly developed higher browning, fluorescence, and pyrrolization than the previously described PE/RI system. Although there were about 10 amino groups of lys per amino group of PE, the different kinetics observed for the PE/RI and RI/lys systems suggest a higher preference of RI for the amino groups of lys than for the amino group of PE.

Browning, fluorescence, and pyrrole development in a PE/RI/lys model system

When an emulsion of PE, RI, and lys in sodium phosphate buffer was incubated for 4 d at 60 °C, the development of browning and fluorescence, and the conversion of some amino groups into pyrrole groups in both PE and lys was observed. Figure 5 shows the time-courses of YI, fluorescence, and pyrrolization produced in this system. This figure also includes the values observed in both organic and aqueous extracts, which corresponded to the changes suffered by the phospholipid and the amino acid, respectively.

In this system, all previously described reactions (PE/lys, PE/RI, and RI/lys) took place. However, browning, fluorescence, and pyrrolization were considerably more important in the aqueous extracts than in the organic layers, more likely as a consequence of both the higher molar content of RI and lys than PE, and the temperature used. In fact, the time-courses obtained for the total browning, fluorescence, and pyrrolization of non-extracted samples in this system were similar to those obtained for the RI/lys model system. Although oxidized lipid/amino acid reactions have been described to affect the Maillard reaction course (Zamora and Hidalgo 2005b), this was not observed under the conditions described, most likely as a consequence of the different reaction rates: the RI/lys Maillard reaction was finished before production of a significant amount of either oxidized lipids/PE or RI/PE reactions.

The contribution of both the Maillard reaction between the sugar and the amino group of PE and the reaction of fatty acid chain oxidation products with the amino group of PE could be observed in the organic extracts. In these extracts browning, fluorescence, and pyrrolization could be fitted by using Eq. 4 ($r^2 > 0.995$). The final values for the organic extracts of the PE/RI/lys system, calculated according to Eq. 4, were 82.1 for YI, 47.9 for fluorescence, and 42.7 nmol of pyrrole per mL of reaction. These values were mostly lower than those obtained in both PE/lys and PE/RI, which may be related to the well-known antioxidative activity of Maillard reac-

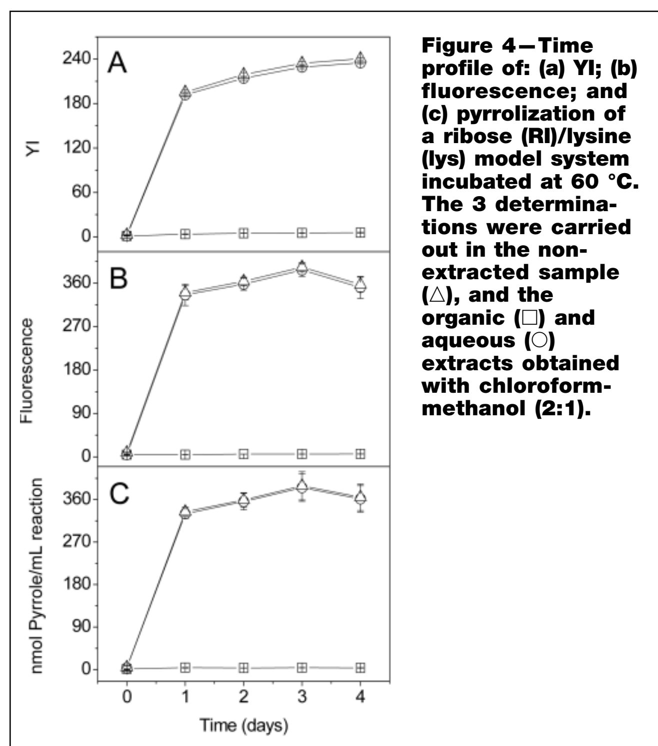

Figure 4—Time profile of: (a) YI; (b) fluorescence; and (c) pyrrolization of a ribose (RI)/lysine (lys) model system incubated at 60 °C. The 3 determinations were carried out in the non-extracted sample (△), and the organic (□) and aqueous (○) extracts obtained with chloroform-methanol (2:1).

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tions products (Manzocco and others 2001; Lee and Shibamoto 2002).

When the correlations among browning, fluorescence, and pyrrolization were studied, the results obtained indicated that correlations were better for the organic extracts than for the total and the aqueous extracts. Thus, browning development was correlated with fluorescence in the non-extracted sample ($r = 0.915$; $P = 0.029$), and the organic ($r = 0.993$; $P = 0.00069$) and aqueous extracts ($r = 0.914$; $P = 0.030$), and with pyrrolization in the non-extracted sample ($r = 0.986$; $P = 0.002$), and the organic ($r = 0.992$; $P = 0.00079$) and aqueous extracts ($r = 0.990$; $P = 0.001$); and fluorescence development was correlated with pyrrolization in the non-extracted sample ($r = 0.963$; $P = 0.009$), and the organic ($r = 0.992$; $P = 0.00083$) and aqueous extracts ($r = 0.955$; $P = 0.011$).

Conclusions

The previous results suggest that both the oxidized products produced in the fatty acid chains of amino phospholipids and the carbohydrates are contributing to the development of browning in systems composed of phospholipids, carbohydrates, and amino acids. However, carbohydrates and oxidation products of amino phospholipids exhibited different reactivities for the different amino groups involved in these reactions. Thus, the oxidized lipids reacted with the amino group of amino phospholipids with preference to the amino group of the amino acid and this preference was reversed for the carbohydrates. These results suggest a strategy for distinguishing the contribution of different food components to food browning. In addition, the analysis of the phospholipid fraction should allow detecting the production of carbonyl-amine reaction products in higher amounts than those produced with amino acids and proteins.

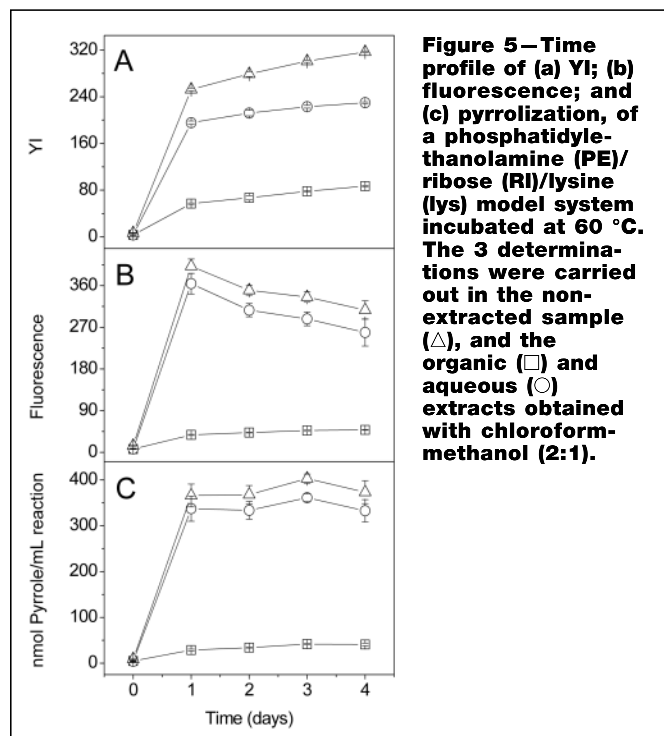

Figure 5—Time profile of (a) YI; (b) fluorescence; and (c) pyrrolization, of a phosphatidylethanolamine (PE)/ribose (RI)/lysine (lys) model system incubated at 60 °C. The 3 determinations were carried out in the non-extracted sample (△), and the organic (□) and aqueous (○) extracts obtained with chloroform-methanol (2:1).

Acknowledgments

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