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Photosensitizer Activity of Model Melanoidins

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This study investigates the potential of melanoidins, the brown pigments formed during Maillard reaction in thermally processed foods, to act as photosensitizers. Seven model melanoidins obtained from different amino and carbonyl compounds were irradiated in a photoreactor or exposed to sunlight. Changes in the ultraviolet−visible spectra and photobleaching were registered in all studied melanoidin systems, and reactive oxygen species were quantified. The data suggest a UV-A-dependent production of singlet oxygen via type II photoreaction and low levels of superoxide radical via type I reaction. The significance of these melanoidin-bound photosensitizers for food stability and quality is discussed.

KEYWORDS: Melanoidins; Maillard reaction; photosensitizers; singlet oxygen; superoxide radical; hydroperoxides; photobleaching

INTRODUCTION

Melanoidins are the brown pigments formed during the reaction of reducing sugars with amino groups, derived from amino acids, amines, and proteins. Their chemical structure is largely unknown, but their functional properties have been a matter of intensive study because of their high abundance in heat-treated foods. Melanoidin formation contributes to the aroma and color development of processed foods, as well as the food texture and beverage viscosity.

The melanoidins are considered as final products of a process known as nonenzymatic browning or Maillard reaction. A similar reaction between reducing sugars and biological molecules takes place under physiological conditions, a process usually called glycation. Glycation has a significant impact on the aging of living organisms and the pathology of some diseases. Experimental data obtained argue that specific chromophores in glycated proteins can function as photosensitizers when they absorb UV-A radiation. They use the absorbed energy to enable chemical reactions to take place. Photoactivation of a sensitizer by UV-A results in the formation of its triplet state. In type I photodynamic reaction (chemical sensitization) the activated photosensitizer directly reacts with a substrate molecule via electron or hydrogen atom transfer and gives rise to free radical formation. Type I reaction may involve the formation of superoxide anion radical by electron transfer from the excited photosensitizer to oxygen. In type II photosensitized oxidation (physical sensitization) the excited sensitizer reacts with oxygen to form singlet oxygen that then reacts with substrates to form oxidized products.

Production of reactive oxygen species during UV-A irradiation of glycated proteins has been documented by several research groups (*1*−*4*). The photosensitizer properties of melanoidins, however, have not been studied yet. Because melanoidin-containing foods are very often exposed to sunlight in an oxygen atmosphere and because the consequences of potential photosensitizer activity of melanoidins could be of importance for food stability and food safety, investigations on the interaction between melanoidins and solar radiation energy are in order.

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MATERIALS AND METHODS

The following reagents were delivered from Sigma (St. Louis, MO): glycine (Gly), arginine (Arg), glutamic acid (Glu), phenylalanine (Phe), glucose (Glc), fructose (Fru), ascorbic acid (AsA), xylose (Xyl), carnosine, diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD), catalase, NaN_3, and Chelex resin. *N*,*N*-Dimethylnitrosoaniline and deuterium oxide were obtained from Aldrich (Milwaukee, WI). Double-distilled water was used for preparation of all solutions. The phosphate buffers were treated with Chelex chelating resin to remove the contaminations of transition metal ions. UV−vis spectra were scanned using a Cary 1 spectrophotometer (Varian). The instrument software was used to obtain the action spectra (difference between the spectra before and after irradiation).

Melanoidin Preparation. Seven model melanoidins, Glc-Gly, Glc-Glu, Glc-Arg, Glc-Phe, Gly-Fru, Gly-AsA, and Gly-Xyl, were obtained according to the COST 919 action protocol (*5*). Briefly, equal amounts (0.05 mol) of amino acid and carbohydrate components were dissolved in 20−100 mL of water and freeze-dried. The solid residue was heated in an oven at 125 °C for 2 h. The water-soluble fraction of Maillard reaction products obtained was dialyzed through 12-kDa dialysis tubing, and the retained melanoidins were used in all further studies. Coffee brew was obtained according to the COST 919 standard procedure. Briefly, 100 g of ground middle-roasted coffee beans was stirred with 300 mL of hot water (75 °C) for 20 min, filtered through Whatman no. 4 paper filter, defatted by extraction with dichloromethane, and dialyzed as described for the model melanoidins.

Irradiation Conditions. Samples of melanoidins prepared in either distilled water, deuterium oxide, or 10 mM NaN_3 were exposed to sunlight at noon (12:30−3:00 p.m.) in glass cuvettes sealed with Teflon caps.

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Irradiation was carried out using a Heraeus laboratory photoreactor equipped with a 150 W medium-pressure mercury lamp TQ 150. It emits light having a spectrum containing the UV-A and visible portion of sunlight. The local intensity maximum of the emitter in the UV-A region is at $\lambda_{max} = 366$ nm. A water-jacketed Pyrex filter was used to cool the emitter and cut the lamp emission below 300 nm. The amount of melanoidins was normalized by their absorbance at 366 nm—the wavelength of UV-A lamp maximal emission. Samples for irradiation typically contained a melanoidin a having final absorbance at 366 nm equal to 0.5 AU. The solutions (total volume = 10 mL) were continuously stirred during the 30-min irradiation.

Quantification of Reactive Oxygen Species. Singlet oxygen was quantified by the decrease in absorbance of *N*,*N*-dimethyl-4-nitroso-aniline at 440 nm as described by Kraljic and El Mohsni (*6*) both in the absence and in the presence of 10 mM NaN_3, a singlet oxygen quencher. The amount of produced singlet oxygen was calculated by considering $\Delta A_{440} = 0.087$ as equivalent to 1.0 μmol/mL of singlet oxygen generated (*7*).

A PeroXOquant Quantitative Peroxide Assay Kit (Pierce Biotechnology, Rockford, IL) was used to quantify the hydroperoxides formed after irradiation according to the manufacturer's recommendations (1:10 sample/working reagent ratio). The assay is based on the oxidation of ferrous to ferric ion by hydroperoxides. In an acidic solution Fe^{3+} complexes with the Xylenol Orange dye to yield a purple product with a maximum absorbance at 560 nm. The concentration of peroxides in each sample was calculated by reference to a standard curve obtained from hydrogen peroxide solutions in the concentration range of 10−50 μM. The readings for dark controls, albeit negligible, were subtracted from those of irradiated samples.

Superoxide radical production was calculated by the increase in absorbance of 120 μM cytochrome *c* at 550 nm in the presence or absence of 300 units of SOD (*8*). Aliquots of catalase solution were added in 5-min intervals to destroy the hydrogen peroxide formed. The reducing power of model melanoidins was measured by the rate of ferric ion reduction and chelation of the reduced ferrous ion in a colored complex with a specific chromogen—Ferene S. Melanoidin solution in acetate buffer of pH 4.5 (0.8 mL) was mixed with 0.1 mL of 1 mM Ferene S in the same buffer and 0.1 mL of 1 mM $FeCl_3$ in 0.01 M HCl. The changes in the absorbance at 595 nm were followed over 10 min, and the rate of ferric ion reduction was calculated using the molar absorptivity of ferrous complex equal to 33 850 M^{-1} cm^{-1} (*9*).

All data were obtained from triple experiments and given as means ± standard deviation.

RESULTS

Definition of the Standard Unit of Melanoidin (SUM). The melanoidins are polydisperse materials with poorly characterized structures. This feature, as well as problems with melanoidin solubility after lyophilization, hinders the quantification of melanoidin solutions by means of commonly used types of concentration. Therefore, it is difficult to compare meaningfully those properties of melanoidins that depend on their concentration. Color intensity measured by the absorbance at specific wavelengths, for example, 345 and 420 nm, has been used in other comparative studies (*10*, *11*). In this study the absorbance at the wavelength of maximal lamp emission, 366 nm, was set as referent. Each milliliter of melanoidin solution, which had absorption at 366 nm equal to 0.5 absorption unit (AU), was referred to as a standard unit of melanoidin (SUM). For comparison the brown index at 420 nm in the studied melanoidin solutions was equal to 0.269 ± 0.026 AU.

Action Spectra. Irradiation of the Glc-Arg melanoidin solution in a photoreactor led to a linear loss of absorbance at 366 nm within the first 30 min of exposure and almost complete bleaching after 6 h of irradiation. Similarly, the irradiation of other model melanoidins caused an extensive bleaching of yellow chromophores. The action spectra (the difference between the spectra of melanoidin before and after irradiation)

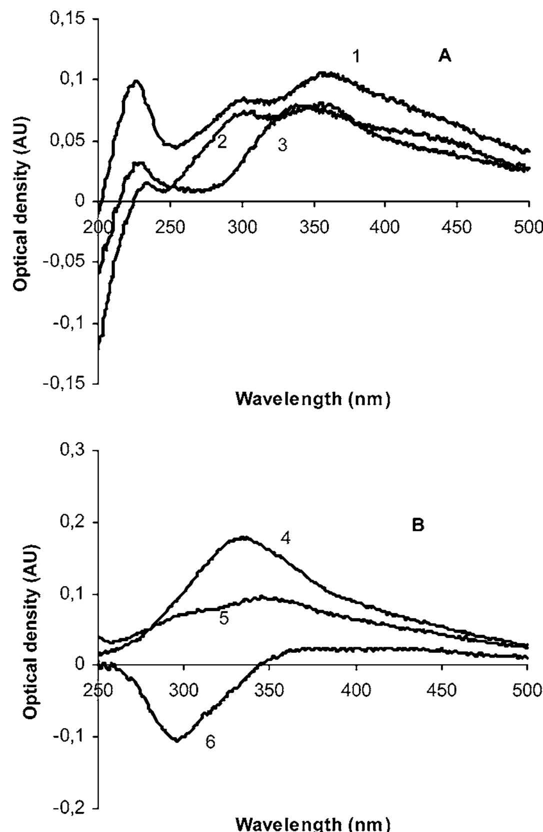

Figure 1. Action spectra of model melanoidins obtained as difference between the initial spectrum of each melanoidin and that after 30 min of irradiation: 1, Gly-Glc; 2, Gly-Fru; 3, Gly-Xyl; 4, Glc-Arg; 5, Glc-Glu; 6, Glc-Phe.

show that the most significant changes in the absorption of melanoidins Gly-Glc, Gly-Fru, and Gly-Xyl occur in the region of 300−500 nm with a maximum at ∼350 nm (**Figure 1A**). The maximal loss of absorption for Glc-Arg and Glc-Glu was shifted to shorter wavelengths, whereas Glc-Phe increased the absorbance at 300 nm as a result of irradiation (**Figure 1B**). The action spectrum of Gly-AsA represents a single sharp peak with a maximum at 273 nm (spectrum not shown).

Bleaching of Melanoidins upon Sunlight Exposure. Singlet oxygen ($^1O_2$) has a longer lifetime in D_2O than in H_2O, and this observation has served as a probing for the involvement of $^1O_2$ in photooxidation reactions. Sunlight exposure of both Glc-Arg and coffee brew in D_2O increased the extent of bleaching compared to the same samples prepared in H_2O (**Figure 2**).

However, the exposure of the solutions above prepared in 10 mM NaN_3, a well-known quencher of singlet oxygen, blocked the photobleaching. These observations suggest that type II photochemistry, involving the interaction of sensitizing structures in melanoidins with O_2 generating $^1O_2$, is occurring. The action spectrum of Glc-Arg with sunlight bleaching showed that, similarly to UV-A lamp irradiation, the most intense loss of absorbance was at 335 nm.

Singlet Oxygen Production and Formation of Hydroperoxides on Melanoidins. The strongest generator of singlet oxygen was Glc-Arg, producing 2.00 μmol/mL over 30 min of irradiation (**Figure 3**). The lowest concentration of $^1O_2$ was generated by Gly-AsA, 0.21 μmol/mL. Melanoidins obtained from Gly and different carbohydrates (Glc, Fru, and Xyl) slightly varied in their ability to produce singlet oxygen.

Hydroperoxides are the common products of the reaction between singlet oxygen and compounds bearing double bonds.

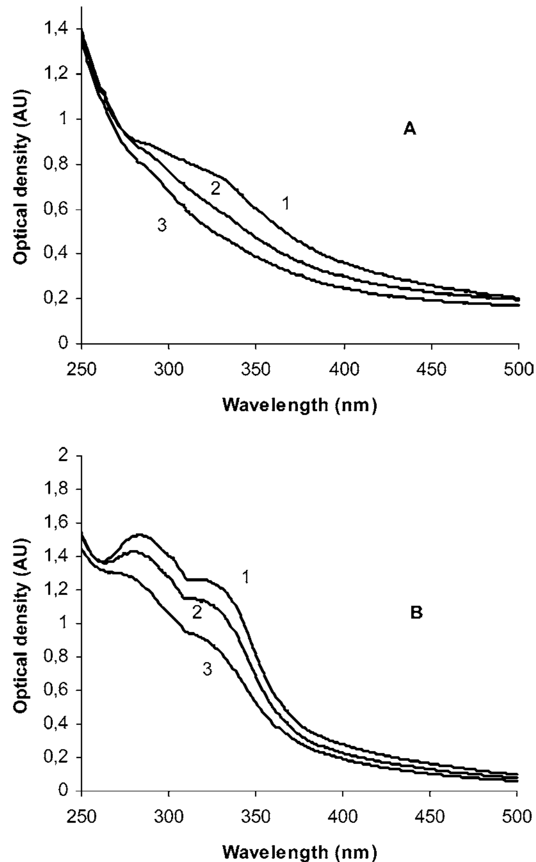

Figure 2. UV–vis spectra of melanoidins after 2.5 h of sunlight exposure: (**A**) Glc-Arg; (**B**) coffee brew; 1, dark control; 2, melanoidin in H_2O; 3, melanoidin in D_2O.

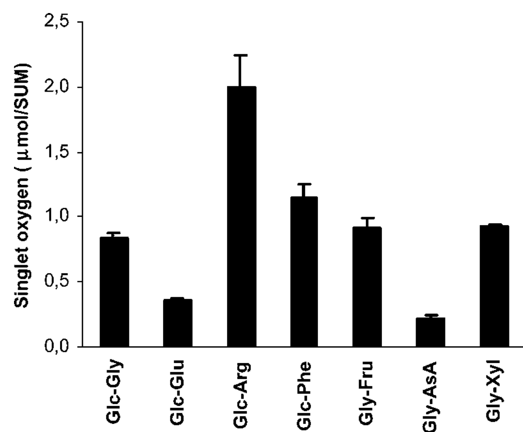

Figure 3. Quantities of singlet oxygen produced by different melanoidin solutions during 30 min of irradiation in a photoreactor.

Dark controls did not show any reactivity toward the reagents used in the assay, whereas micromolar concentrations of hydroperoxides were attained in all irradiated melanoidin solutions. In some cases the production of $^1O_2$ correlated well with the level of hydroperoxides (Glc-Glu, Glc-Gly, Gly-Fru, and Gly-Xyl), but in other cases there was no direct correlation (**Figure 4**).

Superoxide Anion Radical Production. The results in **Figure 5** show that the amount of superoxide radical produced by melanoidin-bound photosensitizers during the course of illumination was 2 orders of magnitude lower than the amount of singlet oxygen detected. Steady initial readings of the absorbance at 550 nm were achieved in all model systems but

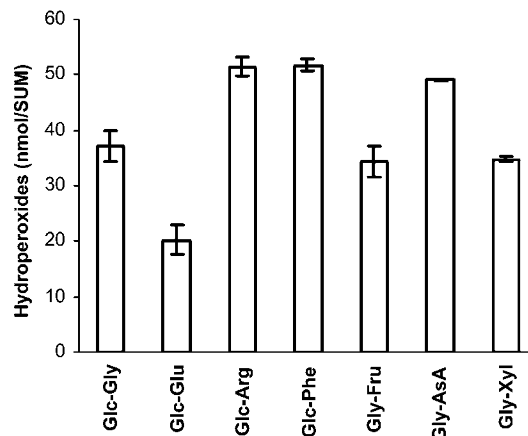

Figure 4. Amount of hydroperoxides detected in solutions of model melanoidins after 30 min of irradiation in a photoreactor.

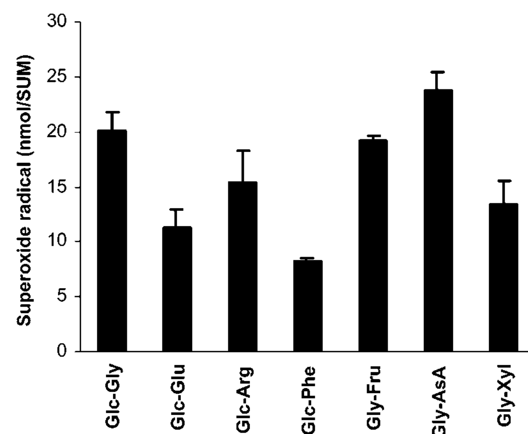

Figure 5. Quantities of superoxide anion radical produced during 30 min of irradiation of model melanoidins in a photoreactor.

one—Gly-AsA. Immediately after mixing of this melanoidin with the reagents, the absorbance at 550 nm started increasing gradually and reached a constant value 15−20 min later. This reading was taken as the initial one before irradiation.

The good reducing power of the Gly-AsA model system was confirmed by the rate of ferric ion reduction. The change of absorbance at 595 nm for this melanoidin was linear over the first 10 min and then logarithmically retarded. Thus, the rate of ferric ion reduction (nanomoles of ferric ions reduced per minute) followed within the first 10 min of the assay was considered as a measure for the reducing power of all studied melanoidins. The results are shown in **Figure 6**.

DISCUSSION

Herein reported experiments univocally demonstrated that the melanoidins are molecules that have the special property of not only absorbing light energy but using this energy to carry out chemical reactions; that is, they act as photosensitizers. It should be pointed out that the photosensitizer is not destroyed during type II reaction but returns to its ground state without chemical alteration and is able to repeat the process of energy transfer to oxygen many times. If type I photoreaction takes place, the photosensitizer may be oxidized. Because the production of singlet oxygen (type II reaction) predominated over the direct reduction pathway (type I reaction) in all studied model melanoidins, the action spectra (**Figure 1**) show the spectra of the structures most sensitive to oxygen in the melanoidin molecules rather than the spectrum of a photosensitizer.

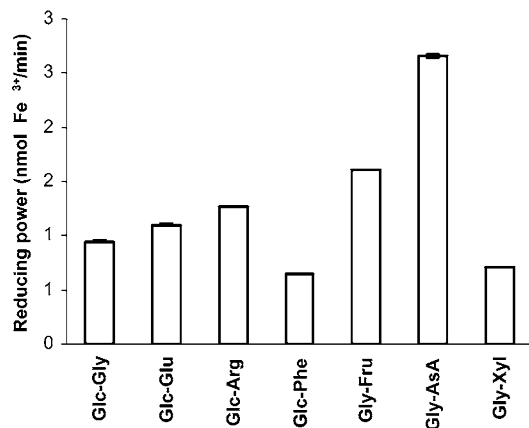

Figure 6. Reducing power of model melanoidins measured by the rate of ferric ion reduction.

The melanoidins are brown substances that absorb radiation energy of the UV region and to a lesser extent the visible light (>380 nm). The most intense emission of the lamp used lies in the UV-A (320−380 nm) region, and the radiation below 300 nm was cut off. For probability reasons the structures most likely to be responsible for melanoidin photosensitizer activities absorb in the UV-A region. The melanoidin photosensitizers produced millimolar concentrations of singlet oxygen in all studied model systems as assayed by *N*,*N*′-dimethylnitrosoaniline bleaching. On the other hand, the melanoidins possess active oxygen scavenging properties (*12*, *13*). Thus, the concentration of hydroperoxides could be more informative about the real consequences of singlet oxygen production since they are a result of overlapping effects of both, the photosensitizer activity and oxygen scavenging ability of the particular melanoidin.

Melanoidins possess reducing ability that is thought to correlate with their antioxidant properties (*14*). The simultaneous presence of reductant and photosensitizer in a reaction mixture subjected to irradiation could give rise to products of type I photodynamic reaction. Generally <1% of photosensitizer triplet reactions with molecular oxygen produce superoxide anion radical by an electron-transfer reaction (*15*). In the melanoidin solutions tested the quantities of superoxide radical varied between 0.7 and 11% of that of singlet oxygen. Like other polyfunctional macromolecules, different structural moieties of melanoidins may be involved in either the color formation, photosensitization, reactive oxygen scavenging, or reducing power. Therefore, any direct correlation between two or more of these properties could be rather accidental than applicable. Nevertheless, the superoxide radical production correlated well with the reducing capacity of melanoidins ($r = 0.783$).

The set of model melanoidins synthesized allowed the influence of both the carbonyl and amino components in the photosensitizer properties of melanoidins to be investigated. Data presented in **Figure 3A** show that the amino acid plays a determinant role in the photosensitizer activity of the model melanoidins. The photosensitizer properties of melanoidins could also shed some light on their structure. Among the studied model melanoidins, this obtained from AsA and Gly has more special characteristics compared to these produced in the reaction of carbohydrates and amino acids. Gly-AsA has a well-defined maximum at 273 nm in its UV−vis spectrum, which has been reported by other groups as well (*16*). After irradiation, this maximum was extensively diminished, whereas the absorbance at higher wavelengths was not significantly affected. The production of singlet oxygen in the presence of Gly-AsA was very low. There are two explanations of this finding: either this melanoidin does not possess photosensitizer structures, which is unlikely provided the good photosensitizer properties of AsA-glycated proteins (*3*), or some structural elements in the Gly-AsA melanoidin compete with carnosine in scavenging singlet oxygen and form stable peroxides in the assay used to quantify this reactive oxygen. The latter assumption is supported by the level of formed hydroperoxides that are comparable to those detected in other model systems. Ascorbic acid is a good quencher of singlet oxygen, and its photooxygenation gives rise to hydroperoxide intermediates with further decomposition to oxalate esters (*17*). The very high reducing power of Gly-AsA demonstrated by the reduction of ferric ions and the suitable redox potential for direct cytochrome *c* reduction argue that Gly-AsA contains a reductone-like structure. The same structural element, the enediol functional group, is responsible for the antioxidant and radical scavenging properties of ascorbic acid. Some authors have suggested that part of AsA incorporates into melanoidins through its C1 atom (*18*), whereas others have proposed a quick oxidation of AsA to dehydroascorbic acid, which in the presence of amino acids undergoes Strecker degradation to 2-amino-2-deoxy-L-ascorbic acid (scorbamic acid), which similarly to AsA is a reductone. Scorbamic acid produces yellow and red pigment before being incorporated into brown polymers (*19*). Several other intermediates of the Maillard reaction with AsA participation have been identified (*20*, *21*).

Irradiation of model melanoidins with a mercury lamp having maximal emission in the UV-A region caused an extensive photobleaching of all investigated model systems. Similar bleaching with a similar action spectrum was observed after exposure of melanoidin solutions to sunlight. The experiments carried out in D_2O confirmed the involvement of singlet oxygen in the decolorization of melanoidins. The browning of thermally processed foods is highly desirable and is intimately associated in the consumer's mind with a delicious, high-grade product, for example, baked bread, roasted coffee, and fried meat. In some cases, however, the melanoidin color is a serious drawback. The references related to decolorization are focused on microbial-assisted removal of melanoidin-like materials from industrial wastes following, for example, sugar refining (*22*), as well as decolorization of teeth (*23*). The photobleaching of melanoidins seems to be an unexplored field for a possible solution in cases when the intense melanoidin color is undesirable.

Generation of active oxygen species from melanoidins during UV-A irradiation could be a possible mechanism for food damage exposed to direct sunlight. The deleterious effect of produced reactive oxygen can be diminished by both antioxidants naturally presenting in the foods, for example, carotenoids, vitamin C, and phenolic compounds, and the melanoidins that are known to act as antioxidative substances in common foods. The antioxidative properties of Maillard reaction products were first observed in the early 1950s (*24*). It is supposed that the mechanism of melanoidin antioxidant action is either direct oxygen scavenging (*13*), trapping of electrophilic reactive species, radical scavenging (*25*), reducing properties of some melanoidin structures (*26*), metal chelation (*27*), or synergies.

Singlet oxygen reacts with various types of double bonds and initiates oxidation of unsaturated fatty acids (*28*). Both types of photodynamic reactions are coupled with Fenton-type chemistry (generation of hydroxyl radical in the presence of reduced transition metal ions). The hydroxyl radical is highly reactive and able to abstract a hydrogen atom from a wide range of organic compounds, generating a carbon-centered free radical. These reactions are of particular importance for the stability of

foods. Oxidation reactions in foods are not limited to the lipid components; reactions involving proteins, carbohydrates, and amino acids, as well as depletion in the levels of natural antioxidant molecules, are also extremely important for the quality, safety, and nutrition value of the foods.

Finally, these findings about the photosensitizer activity of melanoidins could urge a re-estimation of the harmlessness of sunless tanning products. They utilize dihydroxyacetone as a carbonyl component that quickly forms melanoidins on the skin surface (*29*). However, in the absence of additional sun protectors or antioxidants, the photosensitizing structures in these melanoidins could be harmful to the skin.

ABBREVIATIONS USED

Arg, arginine; AsA, ascorbic acid; DTPA, diethylenetriaminepentaacetic acid; Fru, fructose; Glc, glucose; Glu, glutamic acid; Gly, glycine; Phe, phenylalanine; SOD, superoxide dismutase; SUM, standard unit of melanoidin.

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