Volatile Antioxidants Formed from an L-Cysteine/D-Glucose Maillard Model System

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The microwave-induced volatile compounds formed from an L-cysteine/D-glucose Maillard model system at different pHs were evaluated for antioxidative activity using a newly developed method. The dichloromethane extracts of pH 9 and 5 showed the strongest antioxidative effects. Fractionation of the pH 9 extract by column chromatography revealed three fractions possessing antioxidative activity. 2,4,5-Trimethyloxazole, 2,4,5-trimethylthiazole, 4,5-dimethylthiazole, oxazole, thiazole, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) were identified in these fractions as volatile antioxidants. The strongest antioxidative activity was displayed by DMHF, followed by the oxazoles, whereas the thiazoles showed only slight activity. Trimethylpyrazine, another major volatile compound produced in the Maillard reaction, did not show any antioxidative activity. The antioxidative activities of these heterocyclic volatiles were directly dependent upon their concentrations.

INTRODUCTION

The antioxidative activity of the Maillard reaction products (MRPs) was first observed by Franzke and Iwainsky (1954), when they reported on the oxidative stability of margarine following the addition of products from the reaction of glycine and glucose. It has further been shown that antioxidative materials are formed as the result of heating foods. Griffith and Johnson (1957) showed that using glucose instead of sucrose in cookie dough enhances browning and antioxidative stability. Furthermore, Yamaguchi et al. (1964) found that the addition of glucose and amino acids to cookie dough prior to baking significantly increases stability. Dworschák and Szabó (1986) showed that antioxidative materials were formed during the frying of wheat flour and potatoes in meal preparation.

The formation of antioxidative MRPs from model systems has been extensively studied (Kirigaya et al., 1968; Park and Kim, 1983; Lingnert and Ericksson, 1980). Yamaguchi and Fujimaki (1974) observed that the antioxidative effect of MRPs from the reaction of glycine and xylose was comparable to the effect of butylated hydroxyanisole (BHA) but lower than that of butylated hydroxytoluene (BHT). Rhee and Kim (1975) observed antioxidative activity from the products of a glucose caramelization-type browning reaction, suggesting that amino groups are not always necessary for the formation of antioxidative materials.

Due to the complexity of the mechanisms involved in the Maillard reaction and the uncertainty of melanoidin formation, the exact structures of those compounds responsible for the antioxidative effect have not yet been fully determined. The early formation of a reductone-like compound by decomposition of Amadori products has been described by Hodge and Rist (1953) and was thought to contribute significantly to the antioxidative activity of the MRPs. Evans et al. (1958) reported on the antioxidative effect of several aminohexose reductones on vegetable oil. When fractionating MRPs by gel filtration, Yamaguchi et al. (1981) found strong antioxidative effect in fractions containing melanoidins with molecular weights of about 4500.

Identification of Maillard reaction antioxidants has focused primarily on the higher molecular weight melanoidins. The Maillard reaction, however, also produces hundreds of volatile compounds that are responsible for the aroma of cooked food. The objective of this study, then, was to identify volatile compounds from a Maillard model system that possessed antioxidative properties.

EXPERIMENTAL PROCEDURES

Materials. L-Cysteine, D-glucose, heptanal, nonanal, 2,4,5-trimethyloxazole, 2,4,5-trimethylthiazole, 4,5-dimethylthiazole, trimethylpyrazine, oxazole, thiazole, nonadecane, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) were purchased from Aldrich Chemical Co. (Milwaukee, WI). BHA and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO), and dichloromethane was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). All authentic chemicals were purchased from reliable commercial sources. Kieselgel 60 (silica gel) was purchased from EM Science (Darmstadt, Germany).

Sample Preparation. The method of sample preparation was adapted from Yeo and Shibamoto (1991a). L-Cysteine (0.05 mol) and D-glucose (0.05 mol) were dissolved in 30 mL of deionized water, and the pH of each solution was adjusted to 2, 5, 7, and 9 with either 6 N HCl or 6 N NaOH. The solutions were then brought to a final volume of 50 mL with deionized water and covered with Saran brand plastic wrap. The four solutions were irradiated at the high setting of a 700-W microwave oven for 15 min. At 4-min intervals, the irradiation was interrupted and the samples were rotated 90° to ensure uniform irradiation. The irradiation time coincided with the onset of browning, whereas further irradiation led to charring and sample combustion.

After microwave irradiation, each brown mass was dissolved in approximately 100 mL of deionized water and allowed to cool to room temperature. The resulting solutions were adjusted to pH 8 with 6 N NaOH to enhance the extraction efficiency of nitrogen-containing heterocyclic compounds. The aqueous solution was extracted with 50 mL of dichloromethane using a liquid–liquid continuous extractor for 6 h and then dried over anhydrous sodium sulfate for 12 h. After removal of sodium sulfate, the dichloromethane extract was concentrated to 1 mL by fractional distillation.

Fractionation of MRPs by Column Chromatography. The dichloromethane extracts were fractionated by a 30 cm × 2.5 cm (i.d.) glass column packed with silica gel. Each fraction was eluted with 90 mL of solvent, beginning with 100% pentane and increasing the eluant polarity for each successive elution up to 100% dichloromethane. The column was further eluted with 100% methanol. Seven fractions were collected, and their final

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0021-8561/92/1440-1982$03.00/0

volume was adjusted to 100 mL with the appropriate solvent mixture. A 50-mL aliquot of each fraction was concentrated to 1 mL by fractional distillation and stored at −5 °C for subsequent experiments.

Identification of Volatile MRPs. The volatile reaction products produced from the L-cysteine/D-glucose model system upon microwave irradiation were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Identification of the volatile compounds was performed by comparing the mass spectra and GC retention indices to those of authentic compounds.

Instruments. A Quasar Model MQ 7796 AW, 700-W Easy-Matic Cooking microwave oven was used for sample irradiation.

A Hewlett-Packard (HP) Model 5890 gas chromatograph equipped with a flame ionization detector (FID) and a 60 m × 0.25 mm i.d. DB-Wax bonded-phase fused-silica capillary column (J&W Scientific, Folsom, CA) was used for sample analysis. Peak areas were integrated using a Spectra Physics integrator (SP 4290). The GC was run with an injector temperature of 220 °C and a detector temperature of 250 °C. The oven temperature was held at 60 °C for 4 min and then programmed to 180 °C at 4 °C/min, which was further held for 30 min. The linear velocity of the helium carrier gas flow was 26.5 cm/s, with a split ratio of 1:40.

A HP Model 5890 GC interfaced to a VG Trio II mass spectrometer was used for MS identification of the GC components using the same column and oven conditions stated above. Mass spectra were obtained by electron impact ionization at 70 eV and a source temperature of 150 °C. The spectral data were recorded on a VG 11-250 computer data system.

Measurement of Antioxidative Activity. The antioxidative activity of the Maillard reaction products was evaluated according to a method similar to that developed by Macku and Shibamoto (1991). The antioxidative activities of the following extracts and compounds were measured: BHA and α-tocopherol (5, 10, and 50 ng/μL); dichloromethane extracts from each pH (5 μL); 100 μL of each column chromatographic fraction of the pH 9 extract; standards of 2,4,5-trimethyloxazole, 2,4,5-trimethylthiazole, 4,5-dimethylthiazole, DMHF, and trimethylpyrazine (10, 50, and 100 ng/μL); and oxazole and thiazole (5, 10, and 50 ng/μL). The above extracts and standards were added to solutions of heptanal and nonanal (5000 ng/μL). Nonadecane (80 ng/μL) was added as a gas chromatographic internal standard, and the resulting solutions were brought to a 5-mL final volume with dichloromethane. The solutions were transferred to small vials and stored at room temperature. The headspace of each vial was purged with air every 2 days. Controls containing only the aldehydes, the internal standard, and dichloromethane were prepared for each experiment. The experimental vials and controls were periodically analyzed by GC.

Carboxylic acids formed from aldehyde oxidation were measured by the same GC and a column as previously described, except that the column length was changed to 30 m. The initial oven temperature was programmed from 110 to 180 °C at a rate of 6 °C/min. The GC peak areas of heptanoic acid and nonanoic acid obtained at various time intervals were divided by the GC peak area of the internal standard (nonadecane, 80 ng/μL) to calculate a peak area ratio. The peak area ratio was then plotted against the corresponding time interval to produce graphical representations of carboxylic acid formation.

RESULTS AND DISCUSSION

Heptanal was readily oxidized to heptanoic acid in the dichloromethane solutions. However, the presence of α-tocopherol (Figure 1) and BHA (Figure 2) inhibited this transformation. Increasing amounts of each antioxidant inhibited the heptanal/heptanoic acid conversion for increasing periods of time. Results obtained using nonanal were consistent with those found using heptanal in all experiments. BHA exhibited stronger antioxidative activity than α-tocopherol, which is also consistent with other studies.

Figure 3 shows the antioxidative activity of 5-μL aliquots of each pH extract. The order of antioxidative effect of

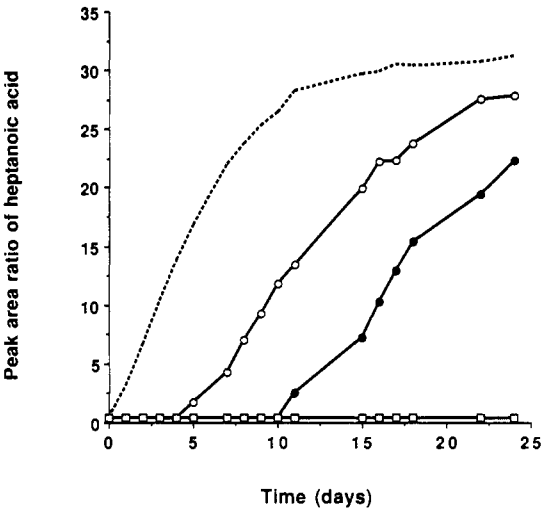

Figure 1. Antioxidative activity of various concentrations (nanograms per microliter) of α-tocopherol: control (- - -); 5 (○); 10 (●); 50 (□). Peak area ratio of heptanoic acid is equal to the GC peak area of heptanoic acid divided by the GC peak area of nonadecane.

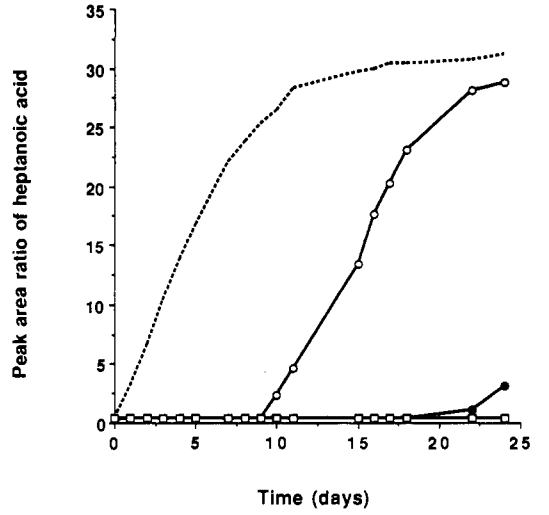

Figure 2. Antioxidative activity of various concentrations (nanograms per microliter) of BHA: control (- - -); 5 (○); 10 (●); 50 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

the extracts from the samples was as follows: pH 9 > pH 5 > pH 2 > pH 7. The Maillard reaction is catalyzed under both slightly acidic and basic conditions and may account for the stronger activity displayed by samples with pH 9 and 5. Glycosylamine formation and subsequent Amadori rearrangement early in the Maillard reaction are catalyzed by weakly acidic conditions (Namiki, 1988). Basic conditions (pH 9) have also been shown to stimulate browning and volatile production in a Maillard model system (Yeo and Shibamoto, 1991a). Yeo and Shibamoto (1991b) reported that electrolyte concentrations of 0.1 M generated maximum quantities of major volatile compounds following microwave irradiation of a Maillard model system. Adjusting the pH of the Maillard samples in the present investigation to 5 and 9 with HCl and NaOH, respectively, produced electrolyte concentrations of approximately 0.1 M. The combination of the above factors may explain the higher antioxidative effects of pH 9 and 5.

The sample obtained at pH 9 was further investigated because it showed strong antioxidative activity. To isolate the antioxidant(s) formed from the L-cysteine/D-glucose

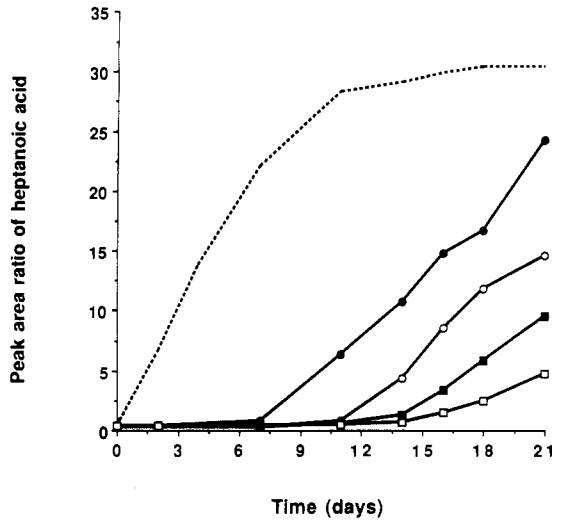

Figure 3. Antioxidative activity of L-cysteine/D-glucose extracts (5 μL) at different pHs: control (- - -); pH 2 (○); pH 5 (■); pH 7 (●); pH 9 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

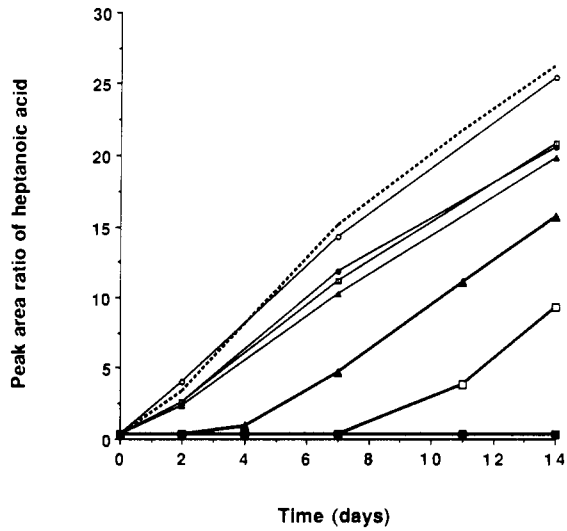

Figure 4. Antioxidative activity of column chromatographic fractions (100 μL) of the pH 9 extract: control (- - -); fraction 1 (○); fraction 2 (●); fraction 3 (▲); fraction 4 (⊡); fraction 5 (△); fraction 6 (□); fraction 7 (■). Peak area ratio of heptanoic acid is as defined in Figure 1.

reaction, each column chromatographic fraction of pH 9 was evaluated for antioxidative activity. Figure 4 shows the antioxidative effects of each fraction. Fraction 7 showed the strongest antioxidative effect among the seven fractions tested. Subsequent GC and GC/MS analyses of this fraction led to the positive identification of oxazoles, thiazoles, furanones, and pyrazines, findings that were consistent with those of other studies (Zhang and Ho, 1991; Yeo and Shibamoto, 1991a). Table I lists the volatile compounds identified in fraction 7 of the pH 9 extract. 2,4,5-Trimethyloxazole, 2,4,5-trimethylthiazole, 4,5-dimethylthiazole, trimethylpyrazine, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone were chosen from this fraction as potential antioxidants and were tested for antioxidative activity.

The results of the antioxidation tests for 2,4,5-trimethylthiazole and 4,5-dimethylthiazole are shown in Figures 5 and 6, respectively. The data from these two experiments suggest that 4,5-dimethylthiazole and 2,4,5-trimethylthiazole possess comparable antioxidative activities. This fact may suggest that the methyl substituent

Table I. Volatiles Identified in Column Chromatographic Fraction 7 (pH 9) of the L-Cysteine/D-Glucose Maillard Model System

compound	Kovats index (DB-Wax)[a]	peak area,[b] %	identification RT[c]	identification MS[d]
4,5-dimethyloxazole	1156	3.1		+
2,4,5-trimethyloxazole	1211	0.3	+	+
thiazole	1247	0.3	+	+
2,4-dimethylthiazole	1268	1.0		+
2,5-dimethylthiazole	1347	1.2	+	+
4,5-dimethylthiazole	1359	0.8	+	+
2,4,5-trimethylthiazole	1398	0.7	+	+
2-methylpyrazine	1268	0.9	+	+
2,5-dimethylpyrazine	1318	1.2	+	+
2,6-dimethylpyrazine	1322	0.9	+	+
2-ethylpyrazine	1325	0.8	+	+
2,3-dimethylpyrazine	1338	1.1	+	+
2-ethyl-6-methylpyrazine	1367	0.7	+	+
trimethylpyrazine	1396	2.3	+	+
2-acetylpyrrole	1957	1.0		+
2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	2047	20.1	+	+
2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	2278	29.0		+

[a] Kovats index values calculated on a DB-Wax capillary column. [b] Calculated from GC peak area after solvent peak area is subtracted from total area. [c] Identification performed by comparing retention times with those of authentic compounds. [d] Identification performed by comparing the mass spectra with those of authentic compounds.

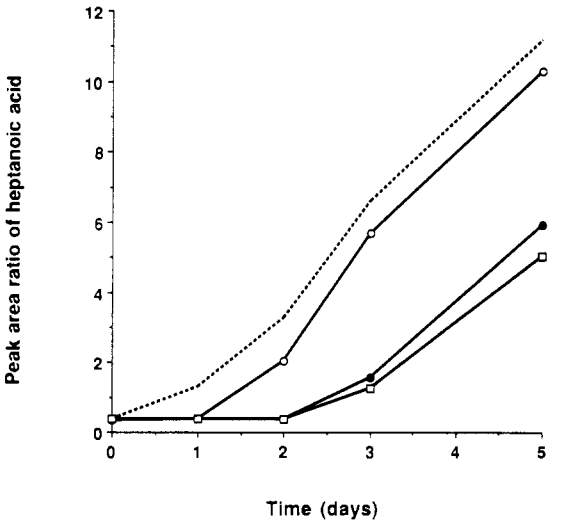

Figure 5. Antioxidative activity of various concentrations (nanograms per microliter) of 2,4,5-trimethylthiazole: control (- - -); 10 (○); 50 (●); 100 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

at the 2-position on the heterocyclic ring does not play a significant role in the antioxidative mechanism of the thiazole compounds. In both cases, concentrations of 50 and 100 ng/μL exhibited similar effects, suggesting a threshold concentration for maximum antioxidative potential in this testing system.

Figure 7 illustrates the results of the antioxidative assay for 2,4,5-trimethyloxazole. Increasing the concentration of 2,4,5-trimethyloxazole in the heptanal solution resulted in a subsequent increase in the amount of time oxidation was inhibited. These results indicate that 2,4,5-trimethyloxazole is a stronger antioxidant than 2,4,5-trimethylthiazole in this antioxidation test. 4,5-Dimethyloxazole was found in greater abundance than 2,4,5-trimethyloxazole in the same fraction but was unavailable from commercial sources for use in testing. The results from the comparison of the di- and trimethylthiazoles may

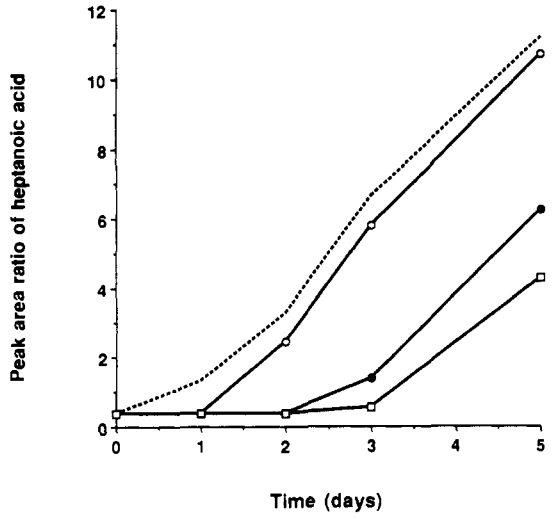

Figure 6. Antioxidative activity of various concentrations (nanograms per microliter) of 4,5-dimethylthiazole: control (- - -); 10 (○); 50 (●); 100 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

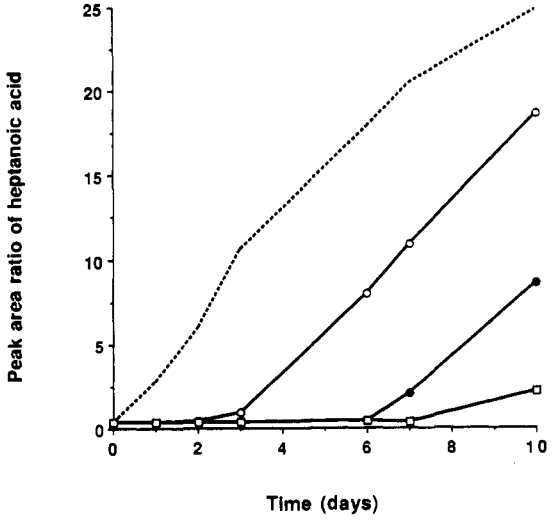

Figure 7. Antioxidative activity of various concentrations (nanograms per microliter) of 2,4,5-trimethyloxazole: control (- - -); 10 (○); 50 (●); 100 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

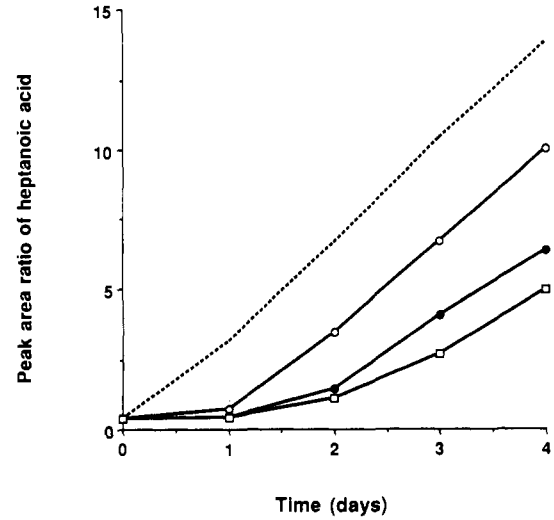

Figure 8. Antioxidative activity of various concentrations (nanograms per microliter) of thiazole: control (- - -); 5 (○); 10 (●); 50 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

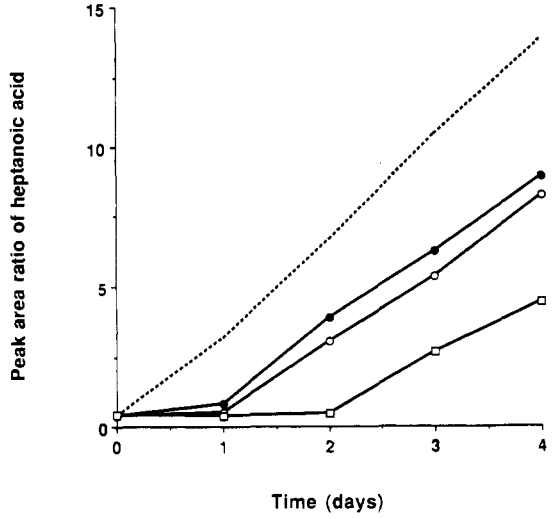

Figure 9. Antioxidative activity of various concentrations (nanograms per microliter) of oxazole: control (- - -); 5 (●); 10 (○); 50 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

suggest that 2,4,5-trimethyloxazole and 4,5-dimethyloxazole have similar antioxidative strengths.

To further understand the antioxidative properties of the thiazole and oxazole compounds, unsubstituted analogs of the above compounds were tested for antioxidative effects. The results of this experiment are shown in Figures 8 and 9 for thiazole and oxazole, respectively. Both of the unsubstituted heterocyclic compounds showed antioxidative activity. In the case of oxazole, the substituted compound (2,4,5-trimethyloxazole) showed considerably higher activity than the unsubstituted compound. The unsubstituted thiazole compound, on the other hand, showed antioxidative activity comparable to that of its substituted analogs.

Figure 10 shows the results of antioxidative testing on 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF). Increased concentrations of DMHF inhibited the oxidation for increasing periods of time, which is consistent with the antioxidants discussed thus far. Figure 11 illustrates the concentration relationship between DMHF (50 ng/μL) and heptanoic acid in the dichloromethane solution. The complete disappearance of DMHF after 15 days coincided with the formation of heptanoic acid, which clearly indicates that oxidation of heptanal was inhibited in the presence of DMHF. A comparison of the results of DMHF (Figure 10) and 2,4,5-trimethyloxazole (Figure 7) at 50 ng/μL shows that the former inhibited the oxidation of heptanal for more than twice as long as the latter, indicating the antioxidative superiority of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone over 2,4,5-trimethyloxazole in this assay.

Many alkylpyrazines were identified in the fraction of pH 9 (Table I). Formation of these volatile compounds under basic conditions has been observed in several studies (Leahy and Reineccius, 1989; Shibamoto and Bernhard, 1977). Trimethylpyrazine, a model alkylpyrazine, did not exhibit antioxidative activity, as shown in Figure 12. Increasing the concentration of trimethylpyrazine did not change its activity.

Many of the heterocyclic compounds identified in the present investigation possess aromatic characteristics. In both five- and six-membered heterocyclic compounds, six π-electrons are delocalized over the ring through resonance. Figure 13 shows the π-electron densities of furan, oxazole, thiazole, and pyrazine (Mahanti, 1977). In the case of

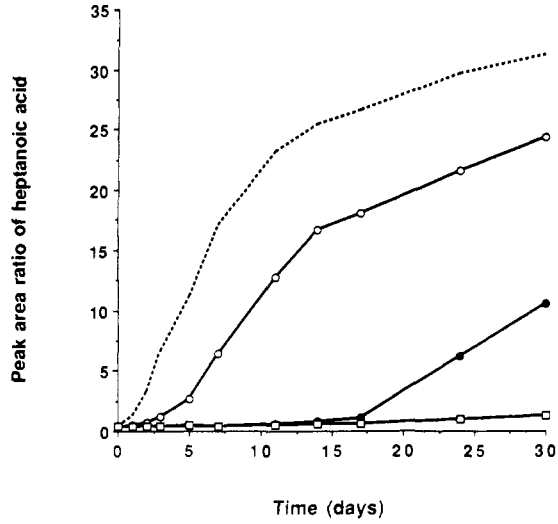

Figure 10. Antioxidative activity of various concentrations (nanograms per microliter) of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone: control (- - -); 10 (○); 50 (●); 100 (□). Peak area ratio of heptanoic acid is as defined in Figure 1.

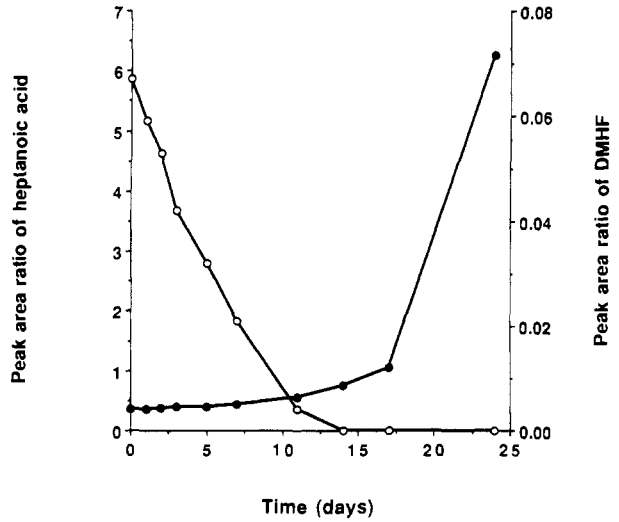

Figure 11. Relative concentrations of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) (○) and heptanoic acid (●) over time in a heptanal solution containing DMHF at 50 ng/μL. Peak area ratio of heptanoic acid is as defined in Figure 1. Peak area ratio of DMHF is equal to the GC peak area of DMHF divided by the GC peak area of nonadecane.

pyrazine, the two nitrogen atoms withdraw π-electrons from the remaining four carbon atoms, rendering them π-electron deficient. However, in the case of furan, oxazole, and thiazole, the electrons are distributed among only five atoms, which subsequently results in carbon atoms that are π-electron excessive. The higher π-electron densities of the five-membered heterocyclic compounds render them open to electrophilic addition by radical species. By the same token, the π-electron-deficient pyrazine does not readily undergo electrophilic addition, which explains its inability to exhibit antioxidative activity.

Dogan et al. (1990) found that hydroxyl radicals (OH•) react with oxazoles by exclusive addition to the carbon at the 5-position of the ring, producing an adduct containing an allylic radical at the 4-position. This can be explained by the higher π-electron density at the 5-position of the oxazole ring. This type of addition leaves the unsubstituted oxazole with a secondary allylic radical, whereas OH• addition to an oxazole substituted at the 4-position (2,4,5-trimethyloxazole and 4,5-dimethyloxazole) would result in a more stable tertiary allylic radical (Figure 14).

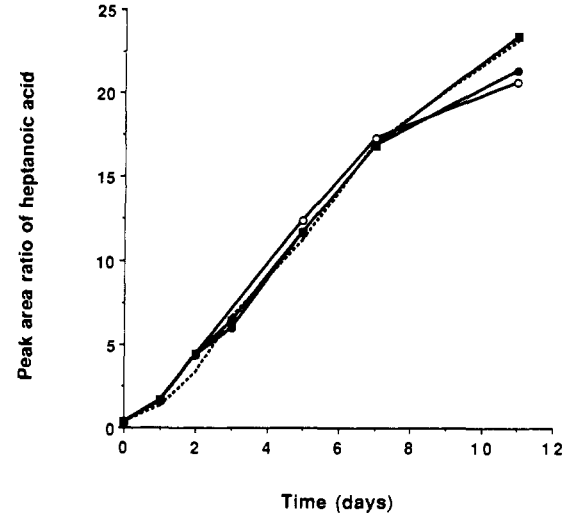

Figure 12. Antioxidative activity of various concentrations (nanograms per microliter) of trimethylpyrazine: control (- - -); 10 (○); 50 (■); 100 (●). Peak area ratio of heptanoic acid is as defined in Figure 1.

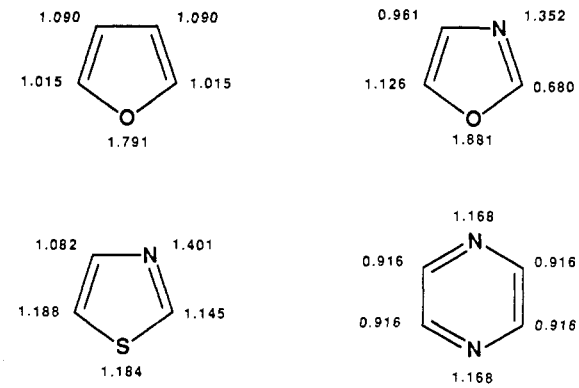

Figure 13. π-Electron densities of some five- and six-membered heterocyclic compounds identified in the Maillard model system. Values were calculated by Mahanti (1977) using the Hückel molecular orbital (HMO) method.

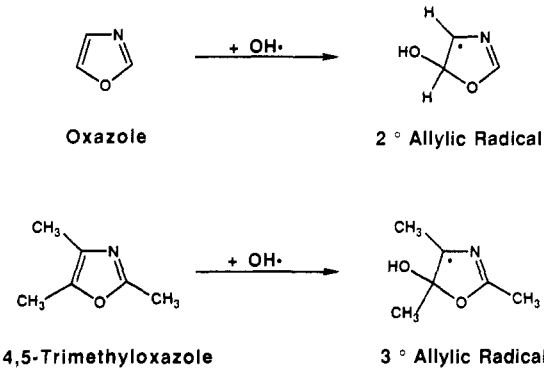

Figure 14. Comparison of OH• addition to oxazole and to 2,4,5-trimethyloxazole resulting in formation of secondary and tertiary allylic radicals, respectively. The mechanism of oxazole adduction by OH• is as proposed by Dogan et al. (1990).

This explains the stronger antioxidative activity of 2,4,5-trimethyloxazole (Figure 7) as compared to that of its unsubstituted analog (Figure 9).

Giese (1983) has shown that radical addition to alkenes is greatly accelerated when electron-withdrawing substituents are attached to the alkene. Furthermore, electron-withdrawing groups in the trans configuration render the alkenes even more susceptible to radical addition. Analogously, these trends observed for alkenes can be applied

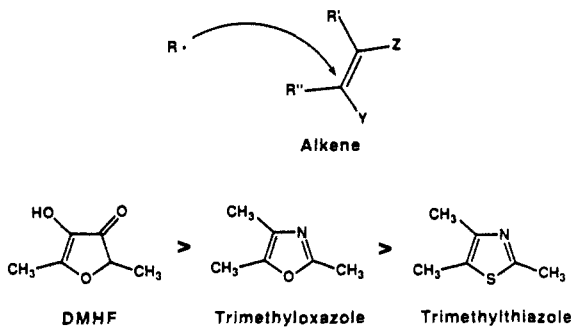

Figure 15. Effects of electron-withdrawing substituents (Y and Z) on alkene radical addition and its application to heterocyclic volatile compounds. The rate of expected radical addition parallels the antioxidative strength of the heterocyclic compounds.

to the heterocyclic antioxidants identified in this study, as shown in Figure 15. In the case of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, the electron-withdrawing effects of the oxygen heteroatom and the hydroxyl substituent oriented in the trans configuration allow for rapid radical addition. The oxygen heteroatom in oxazole is more electronegative than the sulfur in thiazole, which suggests that radical addition to the former will proceed at a faster rate. The derived relative rates at which heterocyclic compounds undergo radical addition are consistent with the strength of antioxidative activity. Consumption of reactive radical species through addition to furanones, oxazoles, and thiazoles, then, seems to be a plausible antioxidative mechanism of these five-membered heterocyclic compounds.

The volatiles found to possess antioxidative properties in the present investigation are major compounds produced from the Maillard reaction and are found in a variety of foods. Thiazole and oxazole compounds have been found in roasted foods and beverages such as coffee, cocoa, and peanuts and in several meat products (Maga, 1978; Lee et al., 1981). Alkyloxazoles have also been identified in the volatiles collected from french-fried potatoes (Carlin et al., 1986). Moreover, the oxazoles and thiazoles were shown to be formed from a Maillard model system in significantly higher amounts in microwaved samples as compared to samples that were thermally heated (Yeo and Shibamoto, 1991c). In addition to its widespread occurrence in cooked foods, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone has been identified in fruits such as pineapples, strawberries, guava, and mango (Wu et al., 1991; Pickenhagen et al., 1981; Idstein and Schreier, 1985).

Identification of volatile antioxidants (oxazoles, thiazoles, and furanones) produced from a Maillard model system is a novel finding. The antioxidative strengths of the individual volatiles were weaker than those of either α-tocopherol or BHA. However, their combined effects with each other and with other volatiles in the Maillard reaction mixture may be comparable to that of known antioxidants. This study suggests that foods and beverages containing the identified volatile antioxidants may be resistant to oxidative degradation. The application of these findings is limited, though, and further studies must be conducted on real food systems to fully evaluate the antioxidative potential of these heterocyclic compounds.

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Received for review March 23, 1992. Accepted July 27, 1992.

Registry No. 4,5-Dimethyloxazole, 20662-83-3; 2,4,5-trimethyloxazole, 20662-84-4; thiazole, 288-47-1; 2,4-dimethylthiazole, 541-58-2; 2,5-dimethylthiazole, 4175-66-0; 4,5-dimethylthiazole, 3581-91-7; 2,4,5-trimethylthiazole, 13623-11-5; 2-methylpyrazine, 109-08-0; 2,5-dimethylpyrazine, 123-32-0; 2,6-dimethylpyrazine, 123-32-0; 2-ethylpyrazine, 13925-00-3; 2,3-dimethylpyrazine, 5910-89-4; 2-ethyl-6-methylpyrazine, 13925-03-6; trimethylpyrazine, 14667-55-1; 2-acetylpyrrole, 1072-83-9; 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, 3658-77-3; 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, 28564-83-2; oxazole, 288-42-6.