Inhibition of Malonaldehyde and Acetaldehyde Formation from Blood Plasma Oxidation by Naturally Occurring Antioxidants

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The inhibitory effect of 2″-*O*-glycosylisovitexin (2″-O-GIV), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), L-ascorbic acid (vitamin C), and 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol) (probucol) on malonaldehyde (MA) and acetaldehyde formation from horse blood plasma oxidized with Fenton's reagent was determined by gas chromatography. 2″-O-GIV, DMHF, and L-ascorbic acid inhibited MA formation at a level of 100 nmol by 60, 22, and 22%, respectively; probucol did not show any inhibitory activity on MA formation. Improved inhibitory activity toward MA formation was observed when 2″-O-GIV or DMHF was mixed with L-ascorbic acid in equal amounts. Approximately 80−90% of acetaldehyde formation was inhibited by 300 nmol of 2″-O-GIV and probucol, whereas DMHF required 1000 nmol to exhibit the same level of inhibition.

Keywords: *Malonaldehyde; acetaldehyde; blood plasma; natural antioxidants*

Food components that possess such biological characteristics as anticarcinogenicity, antimutagenicity, antioxidative activity, and antiaging activity have recently received much attention as a third functional component of foods, after nutrients and flavor compounds. Among these functions, antioxidants found in natural foodstuffs have been investigated most intensively as constituents preventing diseases associated with oxidative damage.

Active oxygen species such as hydroxy radicals may lead to many biological complications, including carcinogenesis, mutagenesis, aging, and atherosclerosis (Halliwell and Gutteridge, 1989). For example, the accumulation of cholesterol esters is reportedly caused by the oxidation of blood plasma lipids (Retsky et al., 1993). Therefore, the oxidation of blood plasma lipids is strongly associated with atherosclerosis and endothelial dysfunction (Schmidt et al., 1994; Tesfamariam, 1994).

The mechanisms causing these diseases by lipid peroxidation are not yet well understood. One hypothesis is that reactive carbonyl compounds, such as malonaldehyde (MA) and acetaldehyde, formed from lipid peroxidation produce abnormal adducts with biological substances, including DNA and RNA (Feinman, 1988; Esterbauer et al., 1991). In fact, a strong relationship between atherosclerosis and MA and acetaldehydes formed from lipid peroxidation has been reported (Glavind et al., 1952).

Humans are constantly exposed to reactive oxygen species produced either by natural phenomena such as ultraviolet light or by anthropogenic activities (for example, automobile exhaust). Therefore, supplementing antioxidants to scavenge undesirable reactive oxygen species is very important to prevent in vivo oxidative damage. Natural plant foodstuffs are one of the most important suppliers of antioxidants (Namiki, 1990).

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In the present study, the inhibitory effect of natural plant antioxidants 2″-*O*-glycosylisovitexin (2″-O-GIV), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), and L-ascorbic acid (vitamin C) on acetaldehyde and MA formation from horse blood plasma lipids oxidized with Fenton's reagent was determined by gas chromatography (GC). A commercial antiatherosclerosis drug, probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was also tested according to the same method.

EXPERIMENTAL PROCEDURES

Materials. Butylated hydroxytoluene (BHT), sodium dodecyl sulfate (SDS), L-ascorbic acid (vitamin C), probucol, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Benzalkonium chloride (BC) was bought from ICN Biomedicals, Inc. (Aurora, OH). DMHF, 2-methylpyrazine, malonaldehyde bis(diethyl acetal), *N*-methylhydrazine, and ferrous chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Protein dye (Coomassie Blue) was purchased from Bio-Rad Laboratories (Richmond, CA). 2″-O-GIV was isolated from young green barley leaves (*Hordium vulgare* L. var. nudum Hook) harvested 2 weeks after germination according to a method previously reported (Osawa et al., 1992). The structures of 2″-O-GIV, DMHF, vitamin C, and probucol are shown in Figure 1.

Standard stock solutions (1 mL each) for GC analysis were prepared by dissolving 2-methylpyrazine and 2,4,5-trimethylthiazole in dichloromethane (10 mg/mL) and stored at 5 °C until used. Authentic *N*-methylpyrazole (NMP) was synthesized according to the method reported by Umano et al. (1988), and authentic 2-methylthiazolidine was synthesized according to the method reported by Yasuhara and Shibamoto (1991). Sodium malonaldehyde was synthesized according to a method previously described by Lacombe et al. (1990).

Blood plasma was prepared from horse blood (20-year-old male quarter horse) by centrifugation at 5000 rpm for 30 min at 4 °C. The blood plasma was frozen on dry ice immediately after preparation and stored at −80 °C until use.

Protein Analysis in Blood Plasma. The Coomassie Blue dye-binding assay (Bio-Rad Laboratories) was used to determine plasma protein concentrations (Bradford, 1976). A 10 μL aliquot of appropriately diluted plasma protein was added to the dye reagent, and then absorbance at 594 nm was

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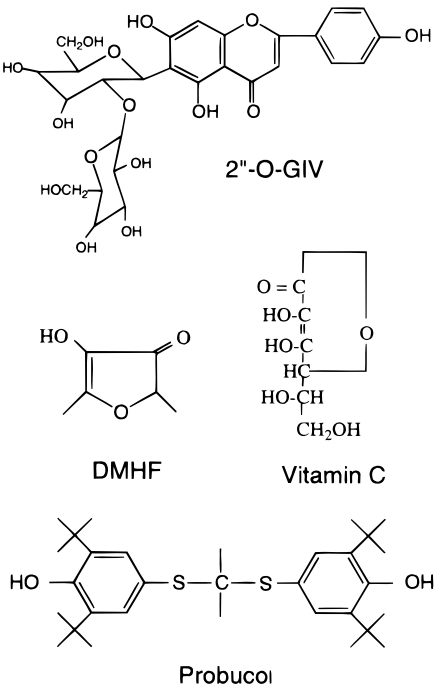

Figure 1. Structures of chemicals used in the present study.

measured versus a reagent blank, using an HP 8452A diode array UV spectophotometer. A standard curve using bovine serum albumin was used to calculate plasma protein concentration.

Oxidation of Blood Plasma with Fenton's Reagent in the Presence or Absence of Testing Chemicals. Blood plasma was oxidized according to the method reported previously (Ichinose et al., 1989). An aqueous solution (5 mL) containing 50 μL of blood plasma (860 μg of protein), 0.25 mmol phosphate buffer (pH 7.4), 3 μmol of ferrous chloride, 0.5 mmol of hydrogen peroxide, 0.75 mmol of potassium chloride, and 0.2% of surfactant BC or SDS was incubated with various amounts of 2″-O-GIV, DMHF, vitamin C, probucol, 2″-O-GIV + vitamin C, and DMHF + vitamin C for 16 h at 37 °C in a 20-mL test tube. The oxidation of samples was stopped by adding 50 μL of 4% BHT ethanol solution. The sample tubes were covered with aluminum foil during incubation to avoid any influence of light.

Analysis of MA as 1-Methylpyrazole. MA formed in the samples was analyzed according to a GC method reported previously (Ichinose et al., 1989; Wong et al., 1994). The MA was reacted with *N*-methylhydrazine (NMH), and the resulting derivative, 1-methylpyrazole, was analyzed with 2-methylpyrazine as an internal standard by a GC equipped with a fused silica capillary column and a nitrogen−phosphorus detector (NPD). The experiment was replicated three times.

Analysis of Acetaldehyde as 2-Methylthiazolidine. Acetaldehyde formed in the samples was analyzed according to a GC method reported previously (Miyake and Shibamoto, 1993, 1995) with slight modification. Acetaldehyde was reacted with cysteamine at room temperature for 24 h. The reaction mixture was extracted with 10 mL of dichloromethane using a liquid−liquid continuous extractor. After the extract was dried over anhydrous sodium sulfate, the resulting derivative, 2-methylthiazolidine, was analyzed with 2,3,5-trimethylthiazole as an internal standard by a GC equipped with a fused silica capillary column and an NPD. The experiment was replicated three times.

Recovery Test on MA and Acetaldehyde from Blood Plasma. MA (200 nmol as sodium salt) was spiked into 50 μL of horse blood plasma, and acetaldehyde (300 nmol) was spiked into 30 μL of horse blood plasma. Samples were prepared and analyzed for MA and acetaldehyde by GC as described above. The experiment was replicated three times.

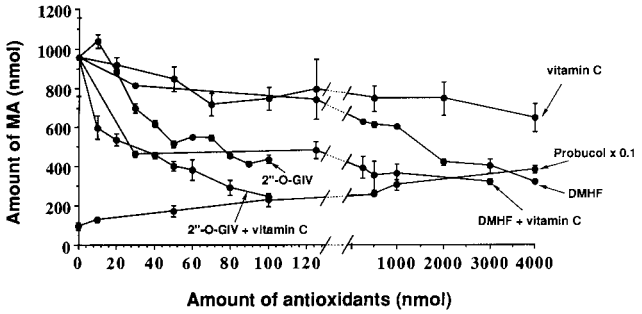

Figure 2. Inhibitory activity of 2″-O-GIV, DMHF, probucol, and L-ascorbic acid toward MA formation from blood plasma upon oxidation.

Instrumental Analysis. A Hewlett-Packard (HP) model 5890A gas chromatograph equipped with a 30 m × 0.25 mm i.d. (d_f = 1 μm) DB-1 bonded-phased fused silica capillary column (J&W Scientific, Folsom, CA) was used for quantitative analysis of 2-methylthiazolidine, and an HP model 5890A gas chromatograph equipped with a 30 m × 0.25 mm i.d. (d_f = 1 μm) DB-Wax column was used for quantitative analysis of NMP. The NPD and injector temperatures were 250 °C. The linear velocity of the helium carrier gas was 30 cm/s with a split ratio of 20:1. The oven temperature was programmed from 60 to 180 °C at 4 °C/min with a final holding time of 10 min. Peak areas were integrated with a Spectra Physics SP 4290 integrator.

An HP model 5890 series II GC interfaced to an HP model 5971 mass spectrometer was used to confirm the MA derivative, NMP, and acetaldehyde derivative, 2-methylthiazolidine, in the sample. The GC conditions were the same as for the GC described above. The mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 250 °C.

RESULTS AND DISCUSSION

In the present study, recovery efficiencies of MA and acetaldehyde from horse blood plasma were 82.1 ± 1.3 and 87.5 ± 1.2%, respectively. The values are mean ± standard deviation (n = 3). Protein concentration in the blood plasma was 17.2 μg/μL. When 50 μL of blood plasma (860 μg of protein) was oxidized, 957 ± 199 nmol of MA was formed. The value is mean ± standard deviation (n = 3). The control sample (860 μg of blood plasma) contained 60−80 nmol of MA, indicating that a certain amount of MA was present in the blood plasma prior to oxidation. When 30 μL of blood plasma (516 μg of protein) was oxidized, 135.0 ± 7.21 nmol of acetaldehyde was formed. The value is mean ± standard deviation (n = 3).

Figure 2 shows the inhibitory activity of 2″-O-GIV, DMHF, probucol, and L-ascorbic acid toward MA formation in horse blood plasma upon oxidation. Results of the mixtures of 2″-O-GIV or DMHF and L-ascorbic acid in equal amounts are also shown in Figure 2. The addition of 100 nmol of 2″-O-GIV inhibited MA formation by 60%. On the other hand, DMHF and vitamin C inhibited MA formation by only 20% at the same level (100 nmol). When doses were increased, the activity of DMHF steadily increased, but vitamin C activity did not change significantly. DMHF at 4000 nmol exhibited activity comparable to that of 100 nmol of 2″-O-GIV. Activity of 2″-O-GIV in doses higher than 100 nmol was not examined because of its solubility in the testing system.

When 2″-O-GIV and DMHF were mixed with vitamin C, an increase in their antioxidative activities was observed. A mixture of 2″-O-GIV (50 nmol) and vitamin

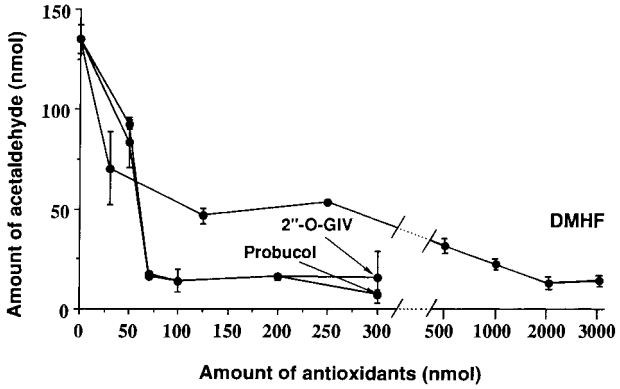

Figure 3. Inhibitory activity of 2″-O-GIV, probucol, and DMHF toward acetaldehyde formation from blood plasma upon oxidation.

C (50 nmol) inhibited MA formation by 75%. The activity was ~5% higher than that of 2″-O-GIV alone. A mixture of DMHF (25 nmol) and vitamin C (25 nmol) inhibited MA formation by 63%; however, the mixture did not increase activity significantly when the total dose was increased up to 4000 nmol. These results indicate that the inhibitory activities of 2″-O-GIV and DMHF are enhanced by the addition of vitamin C. Detailed mechanisms of these phenomena are not yet well understood. It has been known that L-ascorbic acid and α-tocopherol act synergistically to inhibit lipid peroxidation (Sharma and Buettner, 1993). It has been proposed that the mechanism by which this occurs is that L-ascorbic acid reacts with an α-tocopherol radical to regenerate α-tocopherol, and then the resulting L-ascorbic acid radical is reduced back to L-ascorbic acid by NADH (Packer et al., 1979). This set of reactions may explain the results observed in the present study.

The results obtained from probucol were unexpected. Addition of probucol increased MA formation. The values shown in Figure 2 are $^1/_{10}$ of actual values. When 860 μg of blood plasma was oxidized in the presence of 100 and 4000 nmol of probucol, 2296.8 ± 326 and 3875.5 ± 194 nmol of MA were formed, respectively, suggesting that probucol may have pro-oxidative activity. However, this hypothesis can be ruled out because probucol significantly inhibited acetaldehyde formation as reported in the following paragraph. Therefore, MA may be produced from probucol during the incubation of blood plasma. In fact, an aqueous solution of probucol (3 μmol) produced 2.6 μmol of MA upon oxidation with Fenton's reagent (Miyake and Shibamoto, 1998).

Figure 3 shows the respective inhibitory activity of 2″-O-GIV, probucol, and DMHF toward acetaldehyde formation from blood plasma upon oxidation. Because the structure of probucol is similar to that of BHT (Figure 1), it would be expected that probucol would have antioxidative activity and consequently inhibit the formation of lipid peroxidation products, including acetaldehyde and MA. As shown in Figure 3, 2″-O-GIV and probucol exhibited quite similar activities. The formation of acetaldehyde was inhibited nearly 90% in the presence of 300 nmol of either 2″-O-GIV or probucol. The inhibitory activity of DMHF toward acetaldehyde formation was much less than that of either 2″-O-GIV or probucol, at lower levels. However, when the level increased up to 2000 nmol, DMHF inhibited acetaldehyde formation by 88%, which is comparable to the activity obtained by either 2″-O-GIV or probucol at the 300 nmol level.

The highly effective antioxidative activity of 2″-O-GIV has been shown in various lipid peroxidation systems: ethyl linoleate or squalene/Fenton's reagent (Osawa et al., 1992; Kitta et al., 1992); ethyl arachidonate/Fenton's reagent or UV light (Nishiyama et al., 1993); phospholipids or fish liver oil/Fenton's reagent (Nishiyama et al., 1994); and ω-3 fatty acids/Fenton's reagent (Ogata et al., 1996). In the present study, 2″-O-GIV was also effective toward the inhibition of blood plasma oxidation. 2″-O-GIV has not been reported in foods. It is present in a commercial health food product prepared from young green barley leaves. Therefore, it is possible to expect in vivo concentrations obtained through food intake, but discussion of a commercial product is not scientifically relevant.

DMHF was first found in nonenzymatic browning reaction products in the early 1960s (Hodge et al., 1963). Later it was reported as a characteristic flavor chemical formed in carbohydrate caramelization and dehydration reactions (Hodge, 1967). DMHF has been found as a flavor component in various fruits, such as pineapples (Rodin et al., 1965), strawberries (Ohloff, 1969), and grapes (Rapp et al., 1980). More recently, it has been isolated from Swiss cheese (Preininger and Grosch, 1994); cultures of bacteria, *Lactobacillus helveticus* (Preininger and Grosch, 1995); and Maillard reaction products (Blank and Fay, 1996). Therefore, it is presumed that DMHF is ingested through the foods described above. The antioxidative activity of DMHF has never been reported prior to this study. Discovery of antioxidative activity in a flavor chemical, DMHF, is interesting because recently the antioxidative activity of flavor chemicals such as coffee flavor components has begun to receive much attention as a source of biologically active substances (Singhara et al., 1998).

L-Ascorbic acid (vitamin C) is a well-known naturally occurring antioxidant, and its numerous chemical and biological activities have been reported elsewhere. For example, it reportedly protected against free radical damage and cured scurvy (Krinsky, 1979). This compound was used to compare its activity with that of other tested compounds in the present study.

Probucol was also tested in the blood plasma system because it is a commercial drug that is used widely for clinical treatment of hypercholesterolemia; it is an effective antioxidant transported in lipoproteins (U.S. Department of Health and Human Services, 1988), including LDL, and blocks the oxidative modification of LDL in vitro (Parthasarathy et al., 1986). Even though amounts of MA formed in samples with probucol are considerably different from those formed in samples with 2″-O-GIV, the results obtained from the acetaldehyde analysis (Figure 3) are almost identical. Therefore, it is hypothesized that the naturally occurring antioxidant, 2″-O-GIV, may inhibit diseases such as atherosclerosis associated with oxidative damage as effectively as probucol. Moreover, this antioxidant might be more effective if it is used with vitamin C.

LITERATURE CITED

Blank, I.; Fay, L. B. Formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone through Maillard reaction based on pentose sugars. *J. Agric. Food Chem.* **1996**, *44*, 531−536.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Esterbauer, G. H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **1991**, *11*, 81−128.

Feinman, S. E. Structure-activity relationships of formaldehyde. In *Formaldehyde Sensitivity and Toxicity*; Feinman, S. E., Ed.; CRC Press: Boca Raton, FL, 1988; pp 197−204.

Glavind, J.; Hartmann, S.; Clemmesen, J.; Jessen, K. E.; Dam, H. Studies on the role of lipoperoxides in human pathology. II. The presence of peroxidized lipids in the atherosclerotic aorta. *Acta Pathol. Microbiol. Scand.* **1952**, *30*, 1−6.

Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Clarendon Press: Oxford, U.K., 1989; pp 1, 51−57.

Hodge, J. Origin of flavor in foods nonenzymatic browning reactions. In *Chemistry and Physiology of Flavors*; Schultz, H. W., Day, E. A., Libbey, L. M., Eds.; AVI Publishing: Westport, CT, 1967; pp 465−491.

Hodge, J. E.; Fisher, B. E.; Nelson, E. C. Dicarbonyls, reductones, and heterocyclics produced by the reactions of reducing sugars with secondary amine salts. *Am. Soc. Brew. Chem. Proc.* **1963**, *1963*, 84−92.

Ichinose, T.; Miller, M. G.; Shibamoto, T. Gas chromatographic analysis of free and bound malonaldehyde in rat liver homogenates. *Lipids* **1989**, *24*, 895−898.

Kitta, K.; Hagiwara, Y.; Shibamoto, T. Antioxidative activity of an isoflavonoid, 2″-O-glycosylisovitexin isolated from green barley leaves. *J. Agric. Food Chem.* **1992**, *40*, 1843−1845.

Krinsky, N. I. Carotenoid protection against oxidation. *Pure Appl. Chem.* **1979**, *51*, 649−660.

Lacombe, A.; Kermasha, S.; van de Voort, R. F.; Mills, L. B. Preparation and purification of malonaldehyde sodium salt. *J. Agric. Food Chem.* **1990**, *38*, 418−423.

Miyake, T.; Shibamoto, T. Quantitative analysis of acetaldehyde in foods and beverages. *J. Agric. Food Chem.* **1993**, *41*, 1968−1970.

Miyake, T.; Shibamoto, T. Formation of acetaldehyde from L-ascorbic acid and related compounds in various oxidation systems. *J. Agric. Food Chem.* **1995**, *43*, 1669−1672.

Miyake, T.; Shibamoto, T. Formation of malonaldehyde in the presence of probucol, an anti-atherosclerosis drug. *Food Chem. Toxicol.* **1998**, in press.

Namiki, M. Antioxidants/antimutagens in food. *Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 273−300.

Nishiyama, T.; Hagiwara, Y.; Hagiwara, H.; Shibamoto, T. Inhibition of malonaldehyde formation from lipids by an isoflavonoid isolated from young green barley leaves. *J. Am. Oil Chem. Soc.* **1993**, *70*, 811−813.

Nishiyama, T.; Hagiwara, Y.; Hagiwara, H.; Shibamoto, T. Formation and inhibition of genotoxic glyoxal and malonaldehyde from phospholipids and fish liver oil upon lipid peroxidation. *J. Agric. Food Chem.* **1994**, *42*, 1728−1731.

Ogata, J.; Hagiwara, Y.; Hagiwara, H.; Shibamoto, T. Inhibition of malonaldehyde formation by antioxidants from ω-3 polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.* **1996**, *73*, 653−656.

Ohloff, G. Chemistry of odoriferous and flavoring substances. *Fortschr. Chem. Forsch.* **1969**, *12*, 185−251.

Osawa, T.; Katsuzaki, H.; Hagiwara, Y.; Hagiwara, H.; Shibamoto, T. A novel antioxidant isolated from young green barley leaves. *J. Agric. Food Chem.* **1992**, *40*, 1135−1138.

Packer, J. E.; Slater, T. F.; Wilson, R. L. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* **1979**, *278*, 737−738.

Parthasarathy, S.; Young, S. G.; Wiztum, J. L.; Pittman, R. C.; Steinberg, D. Probucol inhibits oxidative modification of low density lipoprotein. *J. Clin. Invest.* **1986**, *77*, 641−644.

Preininger, M.; Grosch, W. Evaluation of key odorants of the neutral volatiles of emmentaler cheese by the calculation of odour activity values. *Food Sci. Technol.-Lebens.-Wissen. Technol.* **1994**, *27*, 237−244.

Preininger, M.; Grosch, W. Determination of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) in cultures of bacteria. *Z. Lebensm.-Unters. Forsch.* **1995**, *201*, 97−98.

Rapp, A.; Knipser, W.; Engel, L.; Ullemeyer, H.; Heimann, W. Off-flavor compounds in the berry and wine aroma. *Vitis* **1980**, *19*, 13−23.

Retsky, K. L.; Freeman, M. W.; Frei, B. Ascorbic acid oxidation products protect human low density lipoprotein against atherogenic modification—Antioxidant rather than prooxidant activity of vitamin C in the presence of transition metal ions. *J. Biol. Chem.* **1993**, *268*, 1304−1309.

Rodin, J. O.; Himel, R. M.; Silverstein, R. M.; Leeper, R. W.; Gortner, W. A. Volatile flavor and aroma components of pineapple (I) isolation and tentative identification of 2,5-dimethyl-4-hydroxy-3(2H)-furanone. *J. Food Sci.* **1965**, *30*, 280−285.

Schmidt, A. M.; Hori, O.; Brett, J.; Yan, D. D.; Wauitier, J. L.; Stern, D. Cellular receptors for advanced glycation end products—implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler. Thrombosis* **1994**, *14*, 1521−1528.

Sharma, M. K.; Buettner, G. R. Interaction of vitamin-C and vitamin-E during free radical stress in plasma—an ESR Study. *Free Radical Biol. Med.* **1993**, *14*, 649−653.

Singhara, A.; Macku, C.; Shibamoto, T. Antioxidative activity of brewed coffee extracts. In *Functional Foods: Disease Prevention*; Proceedings of the 213th National Meeting of the American Chemical Society, San Francisco, CA, 1998; in press.

Tesfamariam, B. Free radicals in diabetic endothelial cell dysfunction. *Free Radical Biol. Med.* **1994**, *16*, 383−391.

Umano, K.; Dennis, K. J.; Shibamoto, T. Analysis of free malonaldehyde in photoirradiated corn oil and beef fat via a pyrazole derivative. *Lipids* **1988**, *23*, 811−814.

U.S. Department of Health and Human Services. *Report 331*; National Toxicology Program: Research Triangle Park, NC, 1988.

Wong, J. W.; Ebeler, S. E.; Rivkah-Isseroff, R.; Shibamoto, T. Analysis of malondialdehyde in biological samples by capillary gas chromatography. *Anal. Biochem.* **1994**, *220*, 73−81.

Yasuhara, A.; Shibamoto, T. Determination of volatile aliphatic aldehydes in the headspace of heated food oils by derivatization with 2-aminoethanethiol. *J. Chromatogr.* **1991**, *547*, 291−298.

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