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Improved Malonaldehyde Assay Using Headspace Solid-Phase Microextraction and Its Application to the Measurement of the Antioxidant Activity of Phytochemicals

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A modified malonaldehyde (MA) assay for antioxidant activity, which involves derivatization and headspace solid-phase microextraction (HS-SPME) was developed and validated. The recovery of MA as 1-methylpyrazole (product of MA and *N*-methylhydrazine) from a headspace of an aqueous solution containing MA, buffer, surfactant, and cod liver oil using HS-SPME with a PDMS/DVB fiber was 91.3 ± 3.38%. MA was analyzed by a gas chromatograph with a nitrogen−phosphorus detector, and its detection limit was 0.0103 nmol/mL. The antioxidant activities of natural compounds were determined as the percentage inhibition of MA formed from cod liver oil oxidized by Fenton's reagents in the above aqueous solution. Sesamol inhibited MA formation most (86.1%), followed by eugenol (84.4%), capsaicin (80.7%), ethylvanillin (45.3%), and vanillin (31.6%) at a level of 50 μg/mL. This method did not require any organic solvents and is a simple, fast, and a highly sensitive method for MA determination.

KEYWORDS: Natural antioxidants; cod liver oil; headspace solid-phase microextraction (HS-SPME); lipid peroxidation; malonaldehyde (MA) assay

INTRODUCTION

Solid-phase microextraction (SPME) is a newly developed device for the extraction of volatile and semivolatile compounds from liquid phases or the headspace of liquid or solid phases (*1*). SPME has been widely used for the analysis of drugs, pesticides, aroma compounds, and volatile aldehydes in environmental samples, food, and biological matrices (*2*, *3*). Headspace SPME (HS-SPME) is a clean technique compared to immersion SPME and is suitable for biological matrices and food samples (*4*).

It has been common practice to analyze lipid peroxidation products to investigate the role of oxidants and antioxidants in diseases because oxidation caused by free radicals, reactive oxygen species, and reactive nitrogen species leads to lipid peroxidation, membrane damage, and degeneration of proteins and DNA in organic systems (*5*).

Among many oxidative degradation products of lipids, malonaldehyde (MA) is the most widely used as a biomaker of lipid peroxidation associated with various diseases including cancer, atherosclerosis, and aging (*6*). However, the sample preparation process for MA analysis is extremely difficult because MA is highly reactive and water soluble. Preparation of stable derivatives to solve these problems has been developed. One of the most commonly and widely used MA derivatives is thiobarbituric acid adduct. The resulting adduct is determined by a colorimeter (*7*, *8*). Because this method is not specific for MA, the results obtained by this method occasionally overestimate the presence of MA, and there exists an inconsistency between the studies using different model systems (*9*). Recently, a more specific and highly sensitive method for MA analysis has been developed (*10*−*13*).

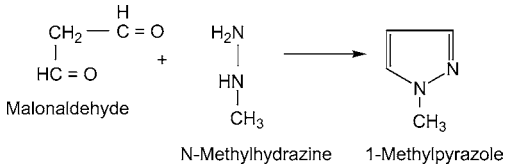

Figure 1. 1-Methylpyrazole formation from malonaldehyde and *N*-methylhydrazine.

This method involves the derivatization of MA with *N*-methylhydrazine into a stable 1-methylpyrazole (1-MP) under mild conditions (**Figure 1**); subsequently, 1-MP is determined by a gas chromatograph (GC) equipped with a nitrogen−phosphorus detector (NPD). One drawback of this method is the use of a liquid−liquid continuous extraction, which provides satisfactory recovery efficiency, but the process is considerably tedious. Moreover, because a halogenated solvent—such as dichloromethane—damages the NPD, the solvent must be changed to a nonhalogenated one, such as ethyl acetate, before it is injected into the GC for quantitative analysis.

In the present study, HS-SPME was applied to prepare samples for MA analysis formed in lipids to improve the sample preparation steps in lipid peroxidation studies. The improved

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method was validated by monitoring the formation and inhibition of MA formation in a cod liver oil/Fenton's reagent system.

MATERIALS AND METHODS

Materials. Cod liver oil fatty acid methyl esters, H_2O_2, $FeCl_2(4H_2O)$, *N*-methylhydrazine, malonaldehyde tetrabutylammonium salt, 1-MP, 2-methylpyrazine, butylated hydroxytoluene (BHT), α-tocopherol, sesamol [1,3-benzodioxol-5-ol (9CI)], eugenol [phenol, 2-methoxy-4-(2-propenyl)- (9CI)], capsaicin [6-nonenamide, *N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-, (6*E*)- (9CI)], vanillin [benzaldehyde, 4-hydroxy-3-methoxy- (6CI,8CI,9CI)], ethylvanillin [benzaldehyde, 3-ethoxy-4-hydroxy- (6CI,8CI,9CI)], catechin [2H-1-benzopyran-3,5,7-triol, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-, (2*R*,3*S*)- (9CI)], quercetin [4*H*-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-, dihydrate (9CI)], pyrrole [1*H*-pyrrole (9CI)], 2-acetylpyrrole [ethanone, 1-(1*H*-pyrrol-2-yl)- (9CI)], tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane hydrochloride, KCl, sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Buffer solutions (pH 4.00, 7.00, and 10.00), dimethyl sulfoxide, and 10% trichloroacetic acid solution (TCA) were purchased from Fisher Co. (Pittsburgh, PA). The Chemical Abstracts Service Index names for the phytochemicals tested in this study are provided in parentheses. Procedures for careful handling of *N*-methylhydrazine must be followed because of its toxicity. It must be handled in the form of a dilution throughout the experiments.

SPME Assemblies. Polydimethylsiloxane fiber (PDMS, 100 μm thickness), polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB, 65 μm thickness), carboxen/polydimethylsiloxane fiber (CAR/PDMS, 65 μm thickness), and a fiber assembly holder were purchased from Supelco, Inc. (Bellefonte, PA).

Time Course Profiles of SPME Fibers. Stock solutions of 1-MP (2 mM) and a GC internal standard 2-methylpyrazine (42 mM) were prepared in deionized water. An aqueous solution (5 mL) containing 500 μL of 0.05 M Tris buffer (pH 7.4), 100 μL of 1-MP stock solution, and 50 μL of 2-methylpyrazine stock solution were placed in a 20 mL glass tube, and the tube was capped with a rubber stopper. Each of three SPME fibers was inserted into the headspace above the solution and exposed for certain periods at room temperature. Extraction times were 1, 5, 10, 20, and 30 min. The fiber was retrieved and injected into the GC immediately for quantification. Desorption time was set at 5 min. Peak areas of 1-MP and 2-methylpyrazine were quantified using GC-NPD in triplicates.

pH Dependence of 1-MP on HS-SPME Extraction. To 5 mL of buffer solutions (pH 4, 7, 10) were added 50 μL of 1-MP stock solution and 100 μL of 2-methylpyrazine stock solution in a 20 mL glass tube. The tube was capped with a stopper. A PDMS/DVB fiber was inserted into the headspace above the solution and exposed for 10 min at room temperature. The fiber was retrieved and injected into the GC. Peak areas of 1-MP and 2- methylpyrazine in the buffer solutions were determined using GC-NPD in triplicates.

Limit of Quantitation, Recovery Efficiency, and Matrix Effect of SDS and CFAME. Standard 1-MP solutions (0, 10, 20, 40, 80, and 200 μM) were prepared in 0.05 M Tris buffer (pH 7.4). Each 5 mL of standard 1-MP solution containing 200 μg of 2-methylpyrazine, as an internal standard, was placed in a 20 mL glass tube with a stopper. A PDMS/DVB fiber was inserted into the headspace above the solution and exposed for 10 min at room temperature. The fiber was retrieved and injected into the GC. Peak areas of 1-MP and 2-methylpyrazine were determined using GC-NPD in triplicates. A standard curve for 1-MP was constructed with concentrations of 1-MP and peak area ratios of 1-MP/2-methylpyrazine and used for quantification of 1-MP. The quantification limit was measured using a 10 μM standard TMP solution seven times and was calculated using the following equation:

$$\text{quantification limit} = (\text{standard deviation/slope of standard curve}) \times 10$$

The recovery efficiency and matrix effect were determined with aqueous solutions (5 mL) containing 40 μM malonaldehyde tetrabutylammonium salt, 0.05 mM Tris buffer (pH, 7.4), with and without SDS (0.2%), and cod liver oil (10 μL). To the solutions were added 10 μL of *N*-methylhydrazine and 50 μL of 2-methylpyrazine stock solution, and then they were stirred magnetically for 1 h at room temperature. A blank solution without MA was prepared simultaneously. The derivative, 1-MP, was extracted by HS-SPME with a PDMS/DVB fiber as previously described. MA was quantified as 1-MP using GC-NPD in triplicates.

Thiobarbituric Acid (TBA) Assay. A TBA assay was performed to compare with the newly developed HS-SPME assay according to the procedure described previously with minor modifications (*14*). A TCA−TBA−HCl reagent was prepared by mixing 20 mL of 10% TCA solution, 20 mL of 0.67% TBA solution, 800 μL of 5 N HCl, and 2000 μL of 1% SDS. A 500 μM TMP stock solution was prepared by dissolving 20.5 mg of TMP with deionized water in a 250 mL volumetric flask. One hundred microliters of samples or standard TMP solutions (0, 10, 25, 50, and 100 μM) and 2.0 mL of the TCA−TBA−HCl reagent were placed into a 15 mL disposable centrifuge tube. After the centrifuge tube was vortex-mixed for 2 s, the mixture was heated for 1 h at 80 °C in a water bath. The mixture was cooled in an ice bath for 10 min, and the absorbance at 532 nm (A_{532}) was measured using a Hewlett-Packard 8452A diode array spectrophotometer in triplicates. The recovery efficiency and matrix effect were determined with aqueous solutions (0.1 mL) containing 40 μM malonaldehyde tetrabutylammonium salt and 0.05 mM Tris buffer (pH, 7.4), with and without SDS (0.2%), and cod liver oil (0.2 μL). The quantification limit was measured using a 10 μM standard TMP solution seven times and was calculated as the equation shown above.

Antioxidant Activity of Natural Compounds. The antioxidant activities of natural compounds were determined by analyzing the percentage inhibition of MA formation from cod liver oil upon oxidation induced with Fenton's reagent using a method previously reported with some modification (*15*). An aqueous solution (5 mL) containing 10 μL of cod liver oil, 0.05 M Tris buffer (pH 7.4), 0.5 μmol of H_2O_2, 1.0 μmol of $FeCl_2$, 0.75 mmol of KCl, 0.2% of SDS, and 50 μL of solutions of natural antioxidant in a 20 mL test tube with a phenolic cap was incubated for 16 h at 37 °C in a water bath equipped with a shaker (Precision Scientific Inc., Chicago IL). The aqueous solution was vortex-mixed for 10 s before incubation. The concentrations of the natural compounds were set at 2, 0.5, and 0.1%, which provided final concentrations of 200, 50, and 10 μg/mL. Ethanol was used as the solvent, except dimethyl sulfoxide was used for catechin and quercetin. Instead of antioxidant solutions ethanol or dimethyl sulfoxide (50 μL) was added for a negative control. Known antioxidants, α-tocopherol and BHT, were used to compare antioxidant activity to that of the natural compounds tested. The sample tubes were covered with aluminum foil during incubation to avoid photo-oxidation induced by light. After incubation, 50 μL of 4% BHT solution in ethanol was added to stop oxidation (*16*); it was confirmed by sampling controls at 2, 4, and 6 h after derivatization, and the addition of BHT efficiently stopped the lipid peroxidation for 6 h. Samples were further derivatized to 1-MP by adding 10 μL of *N*-methylhydrazine, and 50 μL of 2-methylpyrazine stock solution (4 mg/mL) were added as a GC internal standard. The tube was capped with a rubber stopper, and stirred magnetically for 1 h at room temperature. The derivative, 1-MP, was extracted by HS-SPME with a PDMS/DVB fiber as previously described. The mixture was stirred gently during extraction to maintain homogeneity of the solution; however, agitation was not necessary for HS-SPME extraction. MA was quantified as 1-MP using GC-NPD in triplicates. The percentage inhibition was calculated using the following equation:

$$\text{inhibition (\%)} = \{(1 - \text{MA formed in sample/MA formed in control})\} \times 100\ (\%)$$

The mass spectral data of 1-MP are as follows: *m*/*z* (relative intensity, %) 82 (M^+, 100), 81 (43), 54 (28), 53 (12), and 42 (19).

Instrumentation. An Agilent Technologies model 6890N GC equipped with a ZB-WAX fused-silica capillary column (30 m × 0.25 mm × 25 um) (Phenomenex, Torrance, CA) and a nitrogen−phosphorus detector was used for quantification of 1-MP. The GC oven temperature was held at 60 °C for 1 min and then programmed to

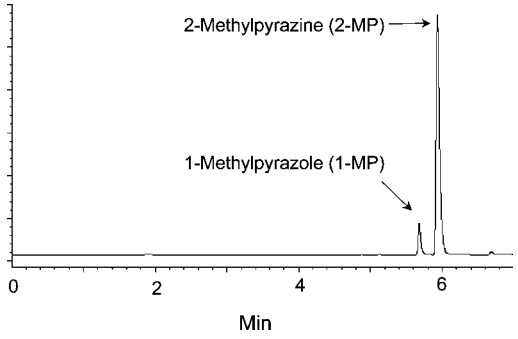

Figure 2. Representative gas chromatogram of 1-MP and 2-methylpyrazine (internal standard) obtained by HS-SPME with a PDMS/DVB fiber.

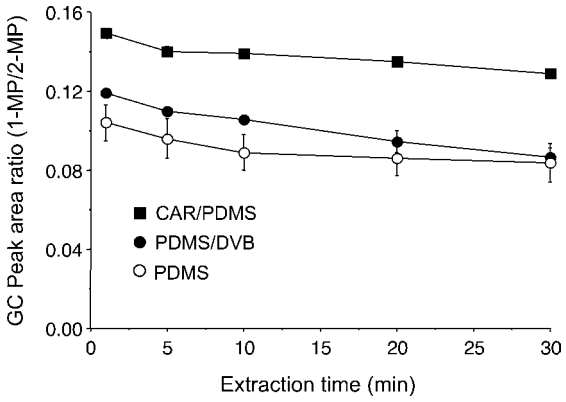

Figure 3. Time course of peak area ratio of 1-MP/2-methylpyrazine by HS-SPME using a PDMS/DVB fiber and a CAR/PDMS fiber. Values are mean ± standard deviation ($n = 3$).

180 °C at 12 °C/min and held for 1 min. The injector temperature was 200 °C. The detector temperature was 300 °C. The helium carrier gas flow rate was 1 mL/min.

A Hewlett-Packard model 5890 series II GC interfaced to a HP 5971 mass spectrometer was used to confirm 1-MP formed from cod liver oil. The GC conditions were the same as for the GC described above. The mass spectra were obtained by electron impact ionization at 70 eV at an ion source temperature of 250 °C.

RESULTS AND DISCUSSION

There has been no report on the determination of MA using either immersion SPME or HS-SPME techniques. Because MA is polar and reactive at the high temperatures used for a GC injector, the HS-SPME method cannot be applied directly. Derivatization and SPME methods have been commonly used for polar and highly reactive substances (*17*). We have recently quantified MA in oxidized oils using solid-phase extraction and GC-NPD after derivatization to 1-MP (*18*). Because 1-MP has a relatively high volatility at room temperature, the use of HS-SPME for 1-MP analysis is promising for the quantification of MA. The objectives of this study were to develop a fast and specific assay for MA based on derivatization and HS-SPME and to apply the assay for investigation of antioxidant activities of natural compounds upon lipid peroxidation.

A representative gas chromatogram of a 1-MP standard solution by HS-SPME with a PDMS/DVB fiber is shown in **Figure 2**. **Figure 3** shows results of the experiments on time courses of extraction profiles with three SPME fibers. The peak area ratio between 1-MP and 2-methylpyrazine was plotted against extraction time. 2-Methylpyrazine was chosen as an internal standard because of the high sensitivity with a NPD and the comparable retention time with 1-MP. The peak area ratio of 1-MP/2-methylpyrazine did not change during 30 min of exposure. The result suggests that the use of the internal standard, 2- methylpyrazine, was preferential for quantification of 1-MP. Judged by coefficient variants ($n = 3$) for 10 min, PDMS/DVB fiber (1.65%) and CAR/PDMS fiber (1.22%) showed good precision. The resolution for a CAR/PDMS fiber (4.10) was smaller than that for a PDMS/DVB fiber (5.25), and thus a PDMS/DVB fiber was chosen for further investigation. The extracted amount of analyte on the SPME fiber is a function of the extraction time and proportional to the initial concentration of the analyte in liquid phase (*19*). Therefore, quantification of the analyte could be accomplished under nonequilibrium conditions at a fixed extraction time. An extraction time of 10 min was chosen to provide a short analysis cycle (15 min/sample) for screening of the antioxidant activity of natural products.

The peak area ratio of 1-MP/2-methylpyrazine was 1.16 ± 0.003 at pH 4, 1.13 ± 0.004 at pH 7, and 1.12 ± 0.018 at pH 10. The values are mean ± standard deviation ($n = 3$). The samples were prepared using HS-SPME with a PDMS/DVB fiber. Peak area ratio was relatively independent with the pH between 4 and 10. The pH independence of HS-SPME for 1-MP is consistent with the pK_a of the corresponding conjugated acids, which is 2.19 for 1-MP (*20*) and 0.37 for 2-methylpyrazine (*21*). The results suggest that the HS-SPME method is applicable to the quantification of 1-MP at pH between 4 and 10 and that the micromolar level of MA can be precisely quantified using 2-methylpyrazine as an internal standard, at the physiological pH of 7.4.

The standard curve for 1-MP was prepared with the 1-MP standard solutions containing 2-methylpyrazine, and the regression equation of $y = 0.0027x - 0.0006$ (where y is peak area ratio and x is 1-MP concentration) was obtained ($R^2 = 0.9999$). The quantification limit was calculated using the 10 μM 1-MP solution and found to be 0.0103 nmol/mL. The recovery efficiency of MA (200 nmol) was determined as 86.7 ± 2.6%. Accuracy and precision were sufficient for the quantification of MA in aqueous samples. Because lipids such as linoleic acid, fish oils, and low-density lipoproteins are essentially contained in the MA assay for lipid peroxidation, the matrix effect of cod liver oil and SDS on recovery efficiency was examined. When 0.2% SDS was added, the recovery efficiency of MA was 92.1 ± 2.7%. When 0.2% SDS and 10 μL of cod liver oil were added, the recovery efficiency of MA was 91.3 ± 3.4%. The addition of SDS and cod liver oil did not exhibit a matrix effect on the 1-MP quantification.

The newly developed MA assay was compared with the TBA assay for validation. The standard curve for the TBA−MA adduct was constructed with the TMP standard solutions, and the regression equation of $y = 0.0082x - 0.0379$ (where y is A_{532} and x is TMP concentration) was obtained ($R^2 = 0.9989$). The quantification limit was calculated using the 10 μM TMP solution and found to be 0.310 nmol/mL. The recovery efficiency of MA (200 nmol) was determined to be 88.3 ± 4.8%. Accuracy and precision were satisfactory for the quantification of MA in aqueous samples; however, the quantification limit obtained by TBA assay was 30-fold higher than that of the HS-SPME assay. The matrix effect of cod liver oil and SDS on recovery efficiency was also examined. When 0.2% SDS was added, the recovery efficiency of MA was 89.1 ± 3.3%. When 0.2% SDS and 10 μL of cod liver oil were added, the recovery efficiency of MA was determined as 100.2 ± 3.6%. Compared to the new assay, the addition of cod liver oil resulted in an ∼10% increase of the 1-MP quantification limit by the TBA assay. The increase can be presumably attributed to TBA

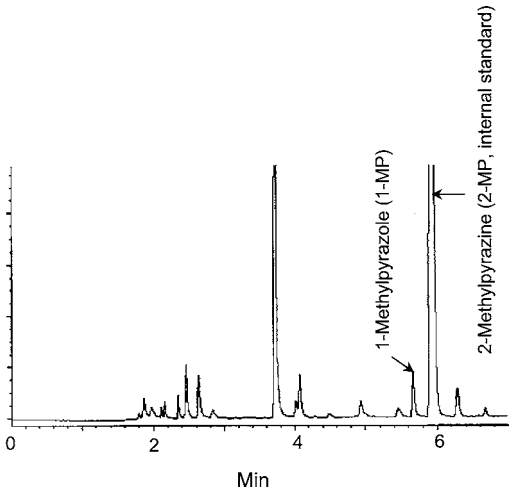

Figure 4. Representative gas chromatogram of oxidized cod liver oil sample obtained by HS-SPME with a PDMS/DVB fiber.

reactive substances other than MA that are formed from cod liver oil during derivatization under high temperature and acidic conditions (*22*). Because the derivatizing condition for the MA assay is mild (under neutral pH for 1 h at room temperature), the newly developed MA assay has the advantage of the formation of fewer artifacts over the TBA assay for studying lipid peroxidation.

Antioxidant activities of natural compounds were examined by measuring the inhibition of MA formation from cod liver oil oxidized with Fenton's reagent. Cod liver oil fatty acid methyl esters are a mixture of poly-unsaturated fatty acids, mono-unsaturated fatty acids, and saturated fatty acids. Cod liver oil contains ~30% of long-chain poly-unsaturated fatty acids, such as docosahexaenoic acid [$CH_3CH_2(CH{=}CHCH_2)_6CH_2$-$COOH$] and eicosapentaenoic acid [$CH_3CH_2(CH{=}CHCH_2)_5$-CH_2CH_2COOH], which are considerably susceptible to autoxidation. Fenton's reagent has been widely used as an oxidant to induce lipid peroxidation for model systems. A representative gas chromatogram of oxidized cod liver oil sample after derivatization and HS-SPME with PDMS/DVB is shown in **Figure 4**.

When a typical natural antioxidant, α-tocopherol, was examined for its antioxidative activity by this method, the inhibitory effect toward MA formation was 69.3 ± 3.3% at the level of 10 μg/mL, 85.9 ± 1.8% at the level of 50 μg/mL, and 90.1 ± 1.7% at the level of 200 μg/mL. The blank and control samples contained 5.91 ± 1.43 and 311 ± 74 nmol of MA, respectively. The antioxidant activity of α-tocopherol was in agreement with the values previously reported by Lee and Shibamoto (*23*). The results suggest that the oxidation condition and HS-SPME condition were appropriate for the investigation of antioxidant activities of natural compounds.

The modified MA assay in the present study was applied to examine the antioxidant activity of natural compounds (capsaicin, catechin, eugenol, ethylvanillin, quercetin, sesamol, vanillin, 2-acetylpyrrole, and pyrrole) with various concentrations (10, 50, and 200 μg/mL). Capsaicin is a natural ingredient in hot chili pepper (*24*). Catechin and quercetin are flavonoids found in green tea, vegetables, and fruits such as onions and apples (*25*−*27*). Sesamol is a lignan found in sesame seeds and oil (*28*). Eugenol is an aroma component of clove and eucalyptus (*29*). Vanillin is a major component of vanilla essential oil (*30*); however, ethylvanillin is not found in natural vanilla essence but is found in cork (*31*). Two pyrrole compounds are found in the volatile constituents of coffee aroma (*32*). In addition to

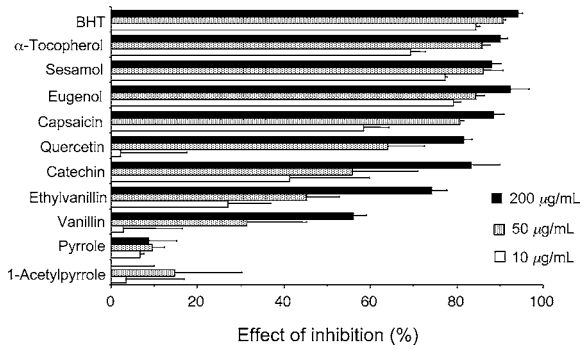

Figure 5. Inhibition effects of natural compounds and known antioxidants on MA formation from oxidized cod liver oil. Error bars indicate standard deviations.

the natural compounds, α-tocopherol and BHT were examined as standard antioxidant compounds.

The inhibitory effects of natural compounds and BHT on MA formation from cod liver oil determined by using the modified MA assay are shown in **Figure 5**. A known synthetic antioxidant, BHT, inhibited MA formation by 90.7% at a level of 50 μg/mL. Sesamol inhibited MA formation most (86.1%), followed by eugenol (84.5%) and capsaicin (80.7%) at a level of 50 μg/mL. Sesamol and eugenol showed nearly the same antioxidant activities as α-tocopherol (85.9%) at the same concentration in the present test system. Sesamol and eugenol showed inhibitory effects on lipid peroxidation induced by Fe^{2+} in the previous paper (*33*). Eugenol has also been reported to have an antioxidant activity on lipid peroxidation of rat liver mitochondria (*34*) and cod liver oil (*23*). Capsaicin was reported to show an antioxidant property on lipid hydroperoxide formation of linoleic acid (*35*).

Quercetin (64.0%), catechin (55.9%), ethylvanillin (45.3%), and vanillin (31.6%) showed moderate inhibitory effects at a level of 50 μg/mL, whereas quercetin and vanillin exhibited low antioxidant activities at a concentration of 10 μg/mL. It was reported that quercetin and catechin moderately inhibited MA formation from ethyl arachidonate oxidized with Fenton's reagent (*36*). Vanillin inhibited lipid peroxidation of the rat brain homogenate (*37*). The results in this study confirmed that quercetin, catechin, and vanillin have moderate antioxidant effect on MA formation. The antioxidant activity of ethylvanillin has not been reported, and this study is the first report on the antioxidant activity of ethylvanillin.

Two heterocyclic compounds, pyrrole and 2-acetylpyrrole, showed no antioxidant activities even at the highest concentration, 200 μg/mL. The standard deviations for 2-acetylpyrrole appeared to be high; however, the antioxidant activities at the three concentrations were not significant. Pyrrole and 2-acetylpyrrole have been reported to have antioxidant activity at a concentration of 50 μg/mL using a hexanal/hexanoic acid assay (*38*, *39*). The inconsistency is presumed to be due to the difference of oxidants and environments between the MA assay used in this study and the hexanal/hexanoic acid assay. The former MA assay utilized hydroxyl radicals as oxidants and was performed in a hydrophilic emulsion system; however, the latter used molecular oxygen as oxidant and was performed in a homogeneous and less polar medium, dichloromethane. The results suggest that pyrroles do not work as antioxidants under severe oxidative conditions such as Fenton's reaction. The partition effect of entities strongly affected the antioxidant activities in the previous study (*40*). Hydrophilic substrates such as Trolox showed good antioxidant activities under a hydrophobic environment, bulk oil systems; however, they did not

exhibit good activity under emulsion systems. The logarithms of the partition coefficient (log *P*) of the tested compounds are BHT (4.17) > α-tocopherol (3.36) > capsaicin (3.31) > eugenol (2.59) > quercetin (2.26) > ethylvanillin (1.61) > sesamol (1.52) > vanillin (1.17) > pyrrole (0.75) > catechin (0.38) > 2-acetylpyrrole (− 0.17), in decreasing order. The small log *P* of pyrroles appears to explain the low antioxidant activities measured by the MA assays because of the partition effect. Interestingly, catechin has a small log *P* value but showed a moderate activity on the inhibition of MA.

Because both catechin and quercetin contain an *o*-dihydroxyphenyl group in the B-ring, their antioxidant properties have been ascribed to donating hydrogen and chelating metal ions. In this study they exhibited similar antioxidant activities at concentrations of 50 and 200 μg/mL. Catechin exhibited higher antioxidant activities than quercetin at a concentration of 10 μg/mL. This may be because catechin possesses a higher chelating ability than quercetin and has a stronger cytoprotective effect on the iron-loaded hepatocyte system (*41*). The results in the present study suggest that the antioxidant effect of catechin is attributed in part to chelating iron ions, which prevent the decomposition of lipid peroxides and reduce the Fenton's reaction (*40*).

HS-SPME was successively applied to prepare samples for MA analysis in a complex lipid matrix for the first time. The assay did not require any solvent for extraction, which is required by most other chromatographic methods. The assay also did not require vigorous conditions such as low pH and high temperature for derivatization, which is essential for the TBA assay—thus, it has the advantage of less formation of artifacts. The HS-SPME method is simple and applicable for automation, which would provide a fast and specific assay for MA. In addition, it may be suitable for assessing the antioxidant activity of complex samples, such as blood and urine as well as food.

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