JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Novel Low-Density Lipoprotein (LDL) Oxidation Model: Antioxidant Capacity for the Inhibition of LDL Oxidation

YI-FANG CHU† AND RUI HAI LIU*,†,§

Department of Food Science and Institute of Comparative and Environmental Toxicology, Cornell University, Ithaca, New York 14853

A novel model of peroxyl radical initiated low-density lipoprotein (LDL) oxidation (LDL oxidation model for antioxidant capacity, or LOMAC) was developed to assess the free radical scavenging capacity of antioxidants and the extracts of natural products. A water-soluble free radical initiator, 2,2′-azobis-(amidinopropane) dihydrochloride, was used at physiological temperature (37 °C) to generate peroxyl radicals to catalyze lipid oxidation of LDL isolated from human plasma samples. Headspace hexanal, a major decomposition product of LDL oxidation, was measured by a headspace gas chromatograph as an indicator of antioxidant capacity of different concentrations of pure antioxidants (vitamins C and E) and the extracts of natural products (fresh apple phytochemical extracts). All vitamin C and E and apple extract concentrations tested resulted in increasing partial suppression and delay of LDL oxidation. On the basis of the median effective dose (EC_{50}) calculated for each compound or extract tested, the LOMAC value of 100 g of apple against LDL oxidation was equivalent to 1470 mg of vitamin E or to 402 mg of vitamin C. This study shows that the LOMAC assay can be routinely used to analyze or screen antioxidants or phytochemical extracts against LDL oxidation to prevent cardiovascular disease. The food-specific LOMAC values will be very useful as a new alternative biomarker for future epidemiological studies of cardiovascular disease.

KEYWORDS: Low-density lipoprotein; LDL oxidation model; antioxidant; phytochemicals

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States and most industrialized countries and is rapidly becoming a primary cause of mortality worldwide. In the United States, the annual CVD death rate exceeds 1 million, with an annual economic cost estimated at over $350 billion, the largest single national health cost compared to all others (*1*). Epidemiologic studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with a reduced risk of chronic diseases including CVD (*2*−*5*). Bioactive non-nutrient plant compounds in fruits, vegetables, and other plant foods, known as phytochemicals, are thought to be responsible for the reduced risk (*6*).

Elevated blood low-density lipoprotein (LDL) levels accelerate the formation of atherosclerotic plaque, leading to heart attacks and strokes, and have been a major subject of research for decades. An increase in plasma LDL levels leads to an increase in the adhesion of circulating monocytes onto arterial endothelial cells and to an increased rate of entry of LDL into artery walls, giving rise to a higher steady concentration of LDL in the intimal region. In the intima, LDL, without protection from sufficient antioxidants, can be oxidized by free radicals to become modified LDL. Oxidized LDLs are more atherogenic than native LDL, serve as chemotactic factors in the recruitment of circulating monocytes (precursors of macrophages), and are rapidly engulfed by macrophages and smooth muscle cells (SMCs) to generate foam cells. The uptake of oxidized LDL by macrophages and SMCs is not through the classical restrictive LDL receptor pathway, but instead by a distinct route through scavenger receptors where excessive levels can be attained due to the receptors' feed-forward control mechanism (*7*). Macrophages and SMCs proliferate in the subendothelial region, rapidly accumulate lipids and cholesterol from highly oxidized LDL, and develop into foam cells. Gruel-like, lipid-laden foam cell accumulation in the blood vessel, forming fatty streaks, would cause further endothelial injury and lead to advanced lesion, or thrombus. Rupture of thrombus would lead to myocardial infarction or stroke (*8*). Because oxidized LDLs play a significant role in the initiation and acceleration of the atherosclerotic process, a therapeutic approach aiming at reducing the oxidization of LDL is a strategy to prevent CVD. Dietary antioxidant phytochemicals from fruits and vegetables are capable of elevating plasma antioxidant levels (*9*) and thus can potentially lower the risk of CVD by reducing the degree and amount of oxidative modification of LDL.

Freshly prepared LDL has been widely used to test and screen the prooxidation/antioxidation behavior of pure compounds or

* Address correspondence to this author at the Department of Food Science, Stocking Hall, Cornell University, Ithaca, NY 14853-7201 [telephone (607) 255-6235; fax (607) 254-4868; e-mail RL23@cornell.edu].
† Department of Food Science.
§ Institute of Comparative and Environmental Toxicology.

10.1021/jf040099j CCC: $27.50
Published on Web 10/07/2004

of complex mixtures (*10*, *11*). Frankel et al. (*12*) found that phenolic substances in red wine strongly inhibited LDL oxidation in vitro. They applied a previously developed and refined model (*11*, *13*, *14*) using copper to catalyze LDL oxidation. Hexanal, an indicative lipid decomposition end-product, was quantitated to show the degree of oxidation or inhibition of oxidation. Hexanal is volatile and can be accurately and rapidly quantitated by headspace gas chromatography (GC). More frequently used spectrophotometric methods for lipid oxidation typically analyze multiple intermediates or end-products, which are difficult to analyze due to lack of specificity and interference between compounds. Therefore, measurement of a single indicator compound, such as hexanal, can avoid these issues. Use of copper as a catalyzing agent in the LDL oxidation model does not directly parallel a biological system as circulating copper in human blood is scant and mostly embedded in a copper-containing protein called ceruloplasmin, which is low in pro-oxidant activities (*15*). Besides copper, iron has been commonly used as a catalyst to initiate in vitro peroxidation. In contrast to copper, iron, in the form of hemoglobin, is abundant in the blood and can be released under stress conditions through trivial hemolysis. However, iron is less frequently applied in in vitro LDL oxidation studies due to poor solubility of iron in phosphate-containing buffers (*16*). Therefore, it is necessary to develop an assay to study the antioxidant activities of natural or synthetic compounds on LDL oxidation similar to that found under physiological conditions.

The objective of this study is to develop a novel peroxyl radical initiated LDL oxidation model for assessing the antioxidant activity of natural or synthetic antioxidants and the extracts of natural products. This assay is based on measuring hexanal, one of the major end-products of lipid oxidation, produced from reaction between LDL and peroxyl radicals. Peroxyl radicals will be generated by thermal homolysis of 2,2′-azobis(amidinopropane) dihydrochloride (ABAP) rather than by transition metals. ABAP decomposes slowly at 37 °C, pH 7.4, and releases a steady flux of water-soluble peroxyl radicals at physiological conditions similar to that in the human body (*17*). Peroxyl radicals attack unsaturated fatty acids in LDL to initiate lipid oxidation and to form hydroperoxide. Hexanal is generated from the decomposition of hydroperoxide and is quantitated by headspace GC analysis. Hexanal production correlates well with the oxidation of polyunsaturated fatty acid (PUFA) in LDL and reflects the degree of LDL oxidation in vitro (*11*−*13*). Peroxyl radical initiated LDL oxidation is more similar to the LDL oxidation at physiological condition in humans when compared to the Cu^{2+}-induced LDL oxidation model. The partial competitive inhibition of hexanal production (the potency of the analyte to scavenge free radicals) is the basis of this assay. We believe the LDL oxidation model for antioxidant capacity (LOMAC) proposed here will serve as a novel model for routine assessment of antioxidants preventing LDL oxidation and may provide decisive information for dietary modifications for the prevention of human CVD.

MATERIALS AND METHODS

Chemicals. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), potassium bromide (KBr), sodium chloride (NaCl), Dulbecco's modified phosphate-buffered saline (PBS), and disodium ethylenediaminetetraacetate (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Acetone and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). Spectra/Por 2 dialysis membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA). ABAP was obtained from Wako Chemicals (Richmond, VA). All reagents used were of analytical grade.

Apple Extraction. Fresh apples (Red Delicious) purchased from Cornell Orchard (Cornell University, Ithaca, NY) were cleaned and dried before extraction using the method reported previously from our laboratory (*18*, *19*). Briefly, 100 g of the edible part of fresh apples was weighed and homogenized with chilled 80% acetone (1:2 w/v) for 5 min using a chilled Waring blender. The sample was then further homogenized for an additional 3 min using a Polytron homogenizer. The homogenates were filtered through no.2 Whatman filter paper under a vacuum and then were evaporated at 45 °C until ∼90% of the filtrate had been evaporated. The filtrate was then recovered with water to a final volume of 50 mL and stored at −40 °C until use.

LDL Isolation. LDL ($1.063 > d > 1.019$ g/mL, 15 °C) was isolated by sequential ultracentrifugation (*20*). Whole blood (100 mL) from individual normolipidemic healthy donors was collected after overnight fasting into vacutainer tubes containing EDTA. Blood was centrifuged at 1600*g* for 20 min at 15 °C using a Beckman GS-6R centrifuge. Plasma was collected and then stored at −80 °C until use. Such storage does not affect the lag time or rate of LDL oxidation (*21*). Before analysis, plasma containing EDTA (density ≈ 1.011 g/mL) was adjusted to $d = 1.019$ g/mL using a $d = 1.346$ g/mL KBr/NaCl solution and saline. Ultracentrifugation was then carried out for 3 h at 15 °C with a Beckman L5-50 ultracentrifuge and an SW 41 Ti rotor at 39000 rpm (187728*g*). Chylomicron, very low-density lipoprotein (VLDL), and intermediate-density lipoprotein (IDL) forming a distinct layer on the top were removed by aspiration. The infranate was transferred to a new tube and adjusted to $d = 1.063$ g/mL. LDL was then separated by ultracentrifugation using the same conditions (41 Ti, 187728*g*) at 15 °C for 12 h. The LDL fraction was collected from the top by aspiration and then dialyzed at 4 °C in the dark using Spectra/Por 2 dialysis membranes against four exchanges of deaerated PBS (pH 7.4) containing 0.1% EDTA. The dialyzed solution was then filter-sterilized (0.45 μm) and stored as a stock solution at 4 °C under nitrogen in the dark until use. The protein concentration of freshly prepared, dialyzed LDL was determined according to the Lowry method (*22*) using bovine serum albumin as standards.

LDL Oxidation Model for Antioxidant Capacity (LOMAC). All reactions were carried out in a final volume of 1 mL of solution in 4 mL Teflon/silicon septum-sealed vials. LDL solution, different concentrations of antioxidant vitamin C and vitamin E or apple extracts, and PBS solution at pH 7.4 were added into individual vials with a combined volume of 900 μL. For each reaction, 50 μg of LDL was used. A commensurate volume of LDL stock solution to add into the vial was calculated on the basis of the protein content measured after LDL isolation. Reactions were initiated by injecting 100 μL of ABAP, followed by immediately placing vials into an orbital shaker/incubator (Forma Scientific, Marietta, OH) at 37 °C for the thermal homolysis of ABAP to generate peroxyl radicals. The inhibition degree of hexanal formation from LDL oxidation in the presence of antioxidant competitors was determined by headspace GC (Hewlett-Packard, Avondale, PA, model 5890 equipped with a flame ionization detector) with an HP3396 integrator. Hexanal was separated by a Supelcowax-10 fused silica capillary column (60 m × 0.53 mm i.d., 0.5 μm film thickness; Supelco, Inc., Bellefonte, PA) with a 2.4 mL/min helium flow. GC conditions were as follows: injector temperature, 180 °C; detector temperature, 200 °C; oven program, held at 40 °C for 2 min, increased at 20 °C/min to 140 °C, and then held for 1 min. Antioxidant activity was measured at 2, 4, 6, and 8 h for vitamin E or at 3, 6, and 9 h for vitamin C and apple extracts with four different concentrations. The LOMAC value for the inhibition of LDL oxidation was calculated by integrating the area under the kinetics curve for each concentration and then quantified by the following equation, where $\int$SA and $\int$CA are the integrated areas from the sample and control reactions, respectively:

$$\text{LOMAC} = 100 - (\int \text{SA}/\int \text{CA}) \times 100$$

If a sample concentration possesses a higher free radical scavenging capacity, the area under the curve ($\int$SA) would be smaller, and by this calculation the LOMAC value would be higher. Because values obtained from tested samples are relative to that of the control from the same experiment run, variations caused by LDL susceptibility difference, instrument sensitivity, or reagents can be minimized. The

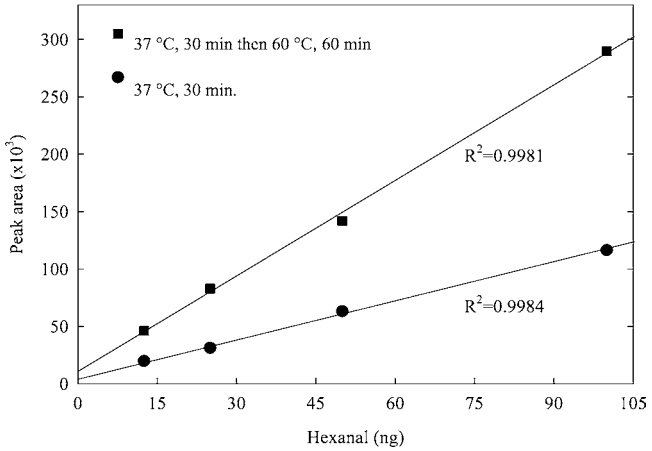

Figure 1. Effect of incubation conditions on headspace GC hexanal analysis. Hexanal (12.5, 25, 50, or 100 ng) was used as standard. Two incubation conditions were applied: (1) headspace equilibrium condition (37 °C for 30 min, followed by 60 °C for 60 min); (2) physiologically relevant condition (37 °C for 30 min).

dose required to cause a 50% inhibition of LDL oxidation (EC_{50}) was calculated for vitamins C and E and apple extracts. On the basis of the EC_{50}, the LOMAC value of apple (or any potential analyte) can be expressed as vitamin C or vitamin E equivalents.

RESULTS

Hexanal Analysis by Headspace GC. Headspace hexanal was compared at two conditions: (1) incubated at 37 °C for 30 min and then at 60 °C for 60 min and (2) incubated at 37 °C for 30 min (**Figure 1**). For standards, 12.5, 25, 50, or 100 ng of hexanal was first dissolved in 50 μL of DMSO and then brought to 1 mL with PBS in individual testing vials. For condition 1, hexanal was incubated at 37 °C for 30 min and then at 60 °C for 60 min to reach equilibrium in the vial headspace; this protocol was based on previous studies (*13*) that showed, when thermostated at 60 °C, the amount of hexanal leveled off after 55 min and with no observed decomposition of hexanal standard. As shown as the upper line in **Figure 1**, headspace hexanal determined after equilibrium that was reached under these conditions showed a positive linear correlation with hexanal standards in the solution with $R^2 = 0.9981$, verifying saturated headspace hexanal is able to indicate the amount in the solution. However, the 60 °C incubation will interfere with the interpretation of LDL oxidation at the physiological temperature of 37 °C. Therefore, we quantified headspace hexanal at the physiological temperature of 37 °C without equilibrium at 60 °C for 60 min. The results showed that hexanal can be detected at 37 °C in a linear dose-dependent manner with a strong positive relationship ($R^2 = 0.9984$), indicating the hexanal analyzed at 37 °C can as well accurately reflect the hexanal in the reaction solution, although the slope is lower than that with equilibrium at 60 °C for 60 min.

Antioxidant Capacity of Vitamin C for Inhibition of LDL Oxidation. Figure 2 shows the kinetics of hexanal formation with different concentrations of vitamin C. No vitamin C was added to the control, but three concentrations of vitamin C (56.8, 283.9, and 567.8 μM) were used for the antioxidation analyses. The area under the curve for each concentration represents the quantity of hexanal produced and hence indicates the degree of LDL lipid oxidation. As shown in **Figure 2**, the smallest area under the curve was observed for the highest vitamin C concentration (567.8 μM) calculated as a 95.7% inhibition of LDL oxidation as compared to the control. Partial inhibitions

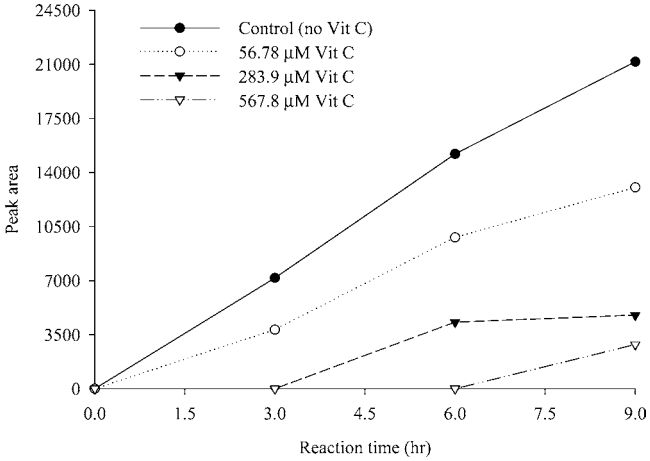

Figure 2. Kinetics of hexanal formation from LDL oxidation in the presence of different concentrations of vitamin C. The area under the curve for each concentration represents the quantity of hexanal produced and hence indicates the degree of LDL lipid oxidation when compared to the control. Larger area represents higher degree of LDL oxidation.

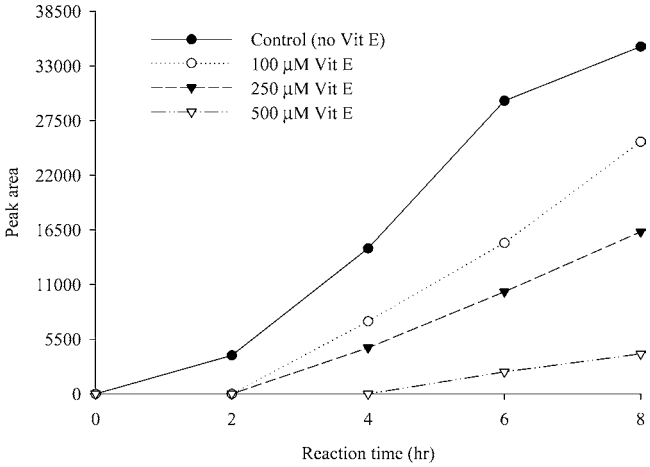

Figure 3. Kinetics of hexanal formation from LDL oxidation in the presence of different concentrations of vitamin E.

of LDL oxidation at 79.7 and 38.9% were observed for 283.9 and 56.8 μM vitamin C, respectively (**Figure 5**). In addition to the observed suppression or retardation of LDL oxidation for all vitamin C concentrations tested, it was also evident that vitamin C resulted in delays of oxidation (prolonged lag time) at doses of 283.9 and 567.8 μM for 3 and 6 h, respectively, indicating vitamin C is a potent antioxidant to prevent peroxyl radical induced LDL oxidation. The EC_{50} of vitamin C against LDL oxidation was calculated as 84.1 μM.

Anitoxidant Capacity of Vitamin E for Inhibition of LDL Oxidation. Figure 3 shows the kinetics of hexanal formation with three different concentrations of vitamin E (100, 250, and 500 μM) plus control with the absence of vitamin E. The area under the curve for each concentration indicates the quantity of hexanal produced from peroxyl radical induced LDL lipid oxidation. As shown in **Figure 3**, the smallest area under the curve was for the highest vitamin E concentration (500 μM) calculated as a 93.5% inhibition of LDL oxidation as compared to the control. Partial inhibitions of LDL oxidation at 64.8 and 46.3% were observed for 250 and 100 μM vitamin E, respectively (**Figure 5**). Similarly to vitamin C, both retardation and delay of LDL oxidation were observed with vitamin E. Retardation of LDL oxidation was observed for vitamin E at all concentrations tested, and delays of oxidation were observed

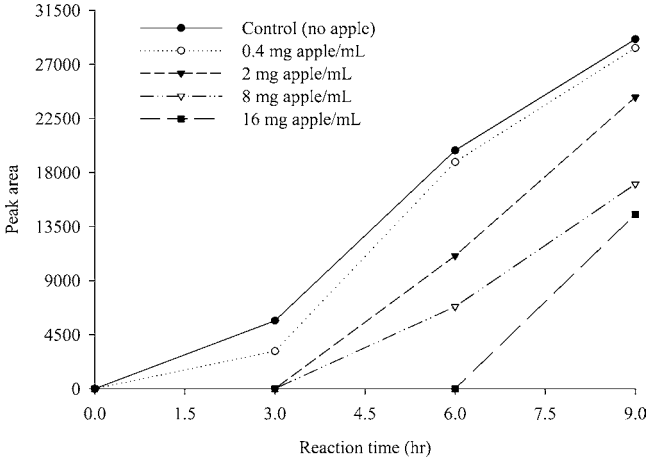

Figure 4. Kinetics of hexanal formation from LDL oxidation in the presence of different concentrations of apple extracts.

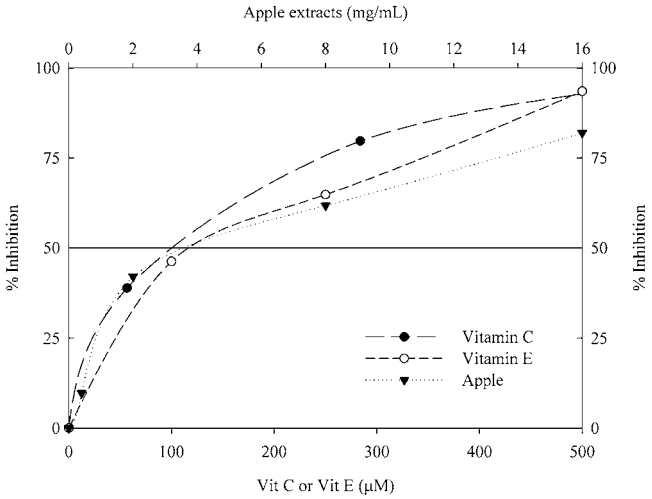

Figure 5. Inhibition percentage of different concentrations of vitamin C, vitamin E, and apple extracts.

for all vitamin E doses at 100, 250, and 500 μM for 2, 2, and 4 h, respectively. The EC_{50} for vitamin E against LDL oxidation was 125.7 μM.

Anitoxidant Capacity of Apple Extracts for Inhibition of LDL Oxidation. Apples of the Red Delicious variety previously reported to exhibit potent antioxidant activity (*19*) were selected, and the effect of apple phytochemical extracts on LDL oxidation was studied. **Figure 4** shows the kinetics of hexanal formation with different concentrations of apple extracts. Four concentrations of apple (0.4, 2, 8, and 16 mg of apple/mL) were used for analysis when compared to the control without apple extract. As shown in **Figure 4**, the smallest area under the curve was exhibited by the highest concentration of apple (16 mg of apple/mL) calculated as a 81.9% inhibition of LDL oxidation as compared to the control. Partial inhibitions of LDL oxidation at 61.7, 42.1, and 9.7% were observed for 8, 2, and 0.4 mg of apple/mL, respectively (**Figure 5**). Similar to the pure antioxidants (vitamin C and E), suppression of LDL oxidation was observed at all apple extract concentrations tested; delays of oxidation (prolonged lag time) were also observed at doses of 2, 8, and 16 mg of apple/mL for 3, 3, and 6 h, respectively. The EC_{50} for apples against LDL oxidation was 3.7 mg/mL. On the basis of the EC_{50} values of vitamin E and apple extracts, the vitamin E equivalent for apple extracts was calculated. The LOMAC value of 1 g of apples is equivalent to 34.1 μM vitamin E. Therefore, the LOMAC value of 100 g of apple against LDL oxidation is equivalent to 1470 mg of vitamin E. Similarly, the antioxidant activity of 100 g of apple preventing LDL oxidation is equivalent to 402 mg of vitamin C. Given that the average vitamin E and C contents are 0.2 and 5.7 mg/100 g of apples, respectively (*23*), almost all of the antioxidant activity in apples must be due to phytochemicals.

DISCUSSION

Since 1919, CVD has been the leading cause of death in the United States and claims more lives each year than the next five leading causes of death combined (*1*). Oxidization of LDL in humans has been implicated to stimulate key events in atherosclerosis (*24*). Cells in humans are constantly exposed to a variety of oxidizing agents, some of which are even necessary for life. The key factor is to maintain a balance between oxidants and antioxidants in order to sustain optimal physiological conditions in the body (*6*). Overproduction of oxidants can cause imbalance, leading to oxidative modification of LDL and consequent increased risk of CVD. The modification of LDL particles is the result of oxidation of PUFA. Formation of fatty acid hydroperoxides from PUFA is followed by fragmentation of fatty acids to short-chain aldehydes such as malondialdehyde (*25*) that chemically and structurally modify apoprotein B and alter the binding specificity of LDL molecules (*26*). The susceptibility of LDL to oxidative stress has been characterized by measuring thiobarbituric acid-reacting substance (TBARS) (*27*), conjugated dienes (*28*), fluorescent intensity (*29*), and peroxide value (*30*), yet these methods monitor mixtures of reaction end-products and hence lack specificity. More specific assessment of oxidative modification of LDL has been reported by determining individual decomposition compounds such as hexanal (*14*). The significance of hexanal as a marker for lipid oxidation (or antioxidant efficiency studies) is that the formation of hexanal is usually an order of magnitude higher than that of most other secondary oxidation products (*31*). Here, we report a simple, reliable GC assay to assess the LDL protecting (free radical scavenging) capacity of pure compounds or mixtures of antioxidants. Freshly prepared human LDL serves as a target for oxidation in this system, and hexanal is quantitated to provide information on the degree of oxidation (or inhibition of oxidation). Hexanal is reactive and might react with other compounds in the system and compromise its yield. However, alterations in yield due to this cross-reactivity would be corrected by control reactions providing background calibration. Some general problems can also be avoided, such as conditions of LDL isolation and storage, LDL composition difference, and metal ion contamination. In addition, in the presence of antioxidant, the formation of hexanal is reduced in two major ways, suppression (retardation) of reaction or delay of oxidation (prolonged lag time). Both inhibition patterns can be observed in this study (**Figures 2−4**), showing that both synthetic and natural, water-soluble and lipid-soluble antioxidants can be determined and characterized by this assay. Although the antioxidant mechanisms rendered by each form of antioxidants are different, the calculation of antioxidant values would not be affected by such variations because of the objectivity of area-under-curve calculation.

This assay is effective in assessing both water- and lipid-soluble antioxidants as the correlations between antioxidant concentrations and inhibition rate are >95% for vitamins C and E and for apple extracts. ABAP is generally applied in aqueous systems (*32*) and is routinely used in our laboratory to evaluate water-soluble natural antioxidants. In this study, LDL oxidation was inhibited in a dose-dependent manner after exposure to

vitamin E, suggesting that the free radical scavenging capacity of lipid-soluble antioxidants can be accurately measured. Barclay et al. (*33*) showed that, in the micellar phase of sodium dodecyl sulfate, ABAP partitions 91% into the micelles, a distribution very close to the 95% partition observed for linoleic acid. Therefore, it is suggested (*34*) that free radicals from ABAP are generated within or at the surface of the membrane micellar system. Lipoproteins are water-soluble macromolecules functioning as micelles to transport lipid-soluble components in the bloodstream. The experimental results confirmed that lipid-soluble antioxidants behaved normally in this assay, largely due to the versatility of ABAP, and this method is capable of measuring both hydrophilic and lipophilic antioxidants in preventing LDL oxidation.

For large-scale screening, this method can be modified to test one time point at 8 h using different concentrations of antioxidant. A drawback to this modification would be the loss of understanding about the antioxidant pattern or kinetics of inhibition, but this adjustment would allow large-scale, preliminary screening of potential antioxidants.

The LOMAC assay is proposed here to assess the antioxidant capacity of natural or synthetic compounds based on their potency to inhibit peroxyl radical initiated LDL oxidation. Compared to copper-initiated oxidation systems, peroxyl radical induced LDL oxidation is more relevant to physiological conditions. Hexanal, a marker of lipid oxidation and decomposition, is quantitated at 37 °C using headspace GC to determine the degree of LDL lipid oxidation. Determination of hexanal at 37 °C is more relevant to the physiological temperatures when compared to previous analysis requiring equilibrium at 60 °C for 60 min. In addition, both hydrophilic and lipophilic antioxidants can be evaluated using this assay. We believe the LOMAC assay proposed here will serve as a novel model for routine LDL oxidation prevention or suppression assessment of antioxidants and other natural compounds and furnish crucial information on dietary modifications for the prevention of human CVD.

ACKNOWLEDGMENT

We thank Dr. Robert S. Parker for help with blood collection and Dr. Bud C. Tennant and Dr. James R. Jacob for their technical assistance on LDL isolation.

LITERATURE CITED

(1) American Heart Association. *Heart Disease and Stroke Statistics—2003 Update*; American Heart Association: Dallax, TX, 2002.

(2) Knekt, P.; Jarvinen, R.; Reunanen, A.; Maatela, J. Flavonoid intake and coronary mortality in Finland: a cohort study. *Br. Med. J.* **1996**, *312*, 478−481.

(3) Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease—the Zutphen elderly study. *Lancet* **1993**, *342*, 1007−1011.

(4) Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *134*, 1106−1114.

(5) Willet, W. C. Diet and health: what should we eat? *Science* **1994**, *254*, 532−537.

(6) Liu, R. H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **2003**, *78*, 517S−520S.

(7) Suzuki, H.; Kurihara, Y.; Takeya, M.; Kamada, N.; Kataoka, M.; Jishage, K.; Ueda, O.; Sakaguchi, H.; Higashi, T.; Suzuki, T.; Takashima, Y.; Kawabe, Y.; Cynshi, O.; Wada, Y.; Honda, M.; Kurihara, H.; Aburatani, H.; Doi, T.; Matsumoto, A.; Azuma, S.; Noda, T.; Toyoda, Y.; Itakura, H.; Yazaki, Y.; Horiuchi, S.; Takahashi, K.; Kruijt, J. K.; vanBerkel, T. J. C.; Steinbrecher, U. P.; Ishibashi, S.; Maeda, N.; Gordon, S.; Kodama, T. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* **1997**, *386*, 292−296.

(8) Lusis, A. J. Atherosclerosis. *Nature* **2000**, *407*, 233−241.

(9) Miller, E. R.; Appel, L. J.; Risby, T. H. Effect of dietary patterns on measures of lipid peroxidation—results from a randomized clinical trial. *Circulation* **1998**, *98*, 2390−2395.

(10) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. The role of lipid-peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **1992**, *13*, 341−390.

(11) Frankel, E. N.; German, J. B.; Davis, P. A. Headspace gas chromatography to determine human low-density lipoprotein oxidation. *Lipids* **1992**, *27*, 1047−1051.

(12) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density-lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(13) Frankel, E. N.; Hu, M. L.; Tappel, A. L. Rapid headspace gas-chromatography of hexanal as a measure of lipid peroxidation in biological samples. *Lipids* **1989**, *24*, 976−981.

(14) Frankel, E. N.; Tappel, A. L. Headspace gas chromatography of volatile lipid peroxidation products from human red blood cell membranes. *Lipids* **1991**, *26*, 479−484.

(15) Burkitt, M. J. A critical overview of the chemistry of copper-dependent low-density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. *Arch. Biochem. Biophys.* **2001**, *394*, 117−135.

(16) Kuzuya, M.; Yamada, K.; Hayashi, T.; Funaki, C.; Naito, M.; Asai, K.; Kuzuya, F. Oxidation of low-density lipoprotein by copper and iron in phosphate buffer. *Biochim. Biophys. Acta* **1991**, *1084*, 198−201.

(17) Barclay, L. R. C.; Locke, S. J.; Macneil, J. M.; Vankessel, J.; Burton, G. W.; Ingold, K. U. Autoxidation of micelles and model membranes—quantitative kinetic measurements can be made by using either water-soluble or lipid-soluble initiators with water-soluble or lipid-soluble chain-breaking antioxidants. *J. Am. Chem. Soc.* **1984**, *106*, 2479−2481.

(18) Sun, J.; Chu, Y. F.; Wu, X. Z.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **2002**, *50*, 7449−7454.

(19) Eberhardt, M. V.; Lee, C. Y.; Liu, R. H. Antioxidant activity of fresh apples. *Nature* **2000**, *405*, 903−904.

(20) Havel, R. J.; Eder, H. A.; Bragdon, J. H. Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **1955**, *34*, 1345−1353.

(21) Kerry, N.; Abbey, M. The isoflavone genistein inhibits copper and peroxyl radical mediated low-density lipoprotein oxidation in vitro. *Atherosclerosis* **1998**, *140*, 341−347.

(22) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(23) USDA Nutrient Database for Standard Reference, 2003.

(24) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol—modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−924.

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

(26) Steinbrecher, U. P. Oxidation of human low-density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **1987**, *262*, 3603−3608.

(27) Steinbrecher, U. P.; Parthasarathy, S.; Leake, D. S.; Witztum, J. L.; Steinberg, D. Modification of low-density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low-density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3883−3887.

(28) Parthasarathy, S.; Khoo, J. C.; Miller, E.; Barnett, J.; Witztum, J. L.; Steinberg, D. Low-density lipoprotein rich in oleic-acid Is protected against oxidative modification—Implications for dietary prevention of atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3894−3898.

(29) Koller, E.; Quehenberger, O.; Jurgens, G.; Wolfbeis, O. S.; Esterbauer, H. Investigation of human plasma low-density lipoprotein by 3-dimensional fluorescence spectroscopy. *FEBS Lett.* **1986**, *198*, 229−234.

(30) Jessup, W.; Rankin, S. M.; Dewhalley, C. V.; Hoult, J. R. S.; Scott, J.; Leake, D. S. α-Tocopherol consumption during low-density-lipoprotein oxidation. *Biochem. J.* **1990**, *265*, 399−405.

(31) Snyder, J. M.; Frankel, E. N.; Selke, E. Capillary gas-chromatographic analyses of headspace volatiles from vegetable oils. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1675−1679.

(32) Paul, T.; Young, M. J.; Hill, I. E.; Ingold, K. U. Strand cleavage of supercoiled DNA by water-soluble peroxyl radicals. The overlooked importance of peroxyl radical charge. *Biochemistry* **2000**, *39*, 4129−4135.

(33) Barclay, L. R. C.; Baskin, K. A.; Locke, S. J.; Schaefer, T. D. Benzophenone-photosensitized autoxidation of linoleate in solution and sodium dodecyl-sulfate micelles. *Can. J. Chem.−Rev. Can. Chim.* **1987**, *65*, 2529−2540.

(34) Pryor, W. A.; Cornicelli, J. A.; Devall, L. J.; Tait, B.; Trivedi, B. K.; Witiak, D. T.; Wu, M. D. A rapid screening-test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.* **1993**, *58*, 3521−3532.

Received for review March 1, 2004. Accepted August 6, 2004.

JF040099J