Natural Antioxidants in Grapes and Wines

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The concentrations of phenolics of three grape varieties and two red wines were determined. The red grape variety and the red wines contain phenolics at concentrations of 920 mg/kg and 1800 and 3200 mg/L, respectively. The antioxidative effects of wine phenolics on the catalysis of lipid peroxidation by biological catalysts such as myoglobin, cytochrome *c*, iron ascorbate, and copper ions were estimated. Lipid peroxidation catalyzed by myoglobin, cytochrome *c*, and iron ascorbate was inhibited (I_{50}) by wine phenolics at concentrations of 0.2, 0.35, and 0.9 μg of phenolics/mL. The antioxidative effects of wine phenolics were determined also in a system containing low-density lipoproteins (LDL) oxidized *ex vivo* by Cu^{2+} ions. The inhibition of LDL oxidation by wine phenolics was compared with that by α-tocopherol. The results show I_{50} inhibitions of less than 1 μM for wine phenolics and 2 μM for α-tocopherol, respectively. The nutritional implications of natural antioxidants at high concentration in grapes, wines, and byproducts, and their utilization in foods, are discussed.

INTRODUCTION

Recently, there is a considerable interest in the food industry and in preventive medicine in the development of "natural antioxidants" from plant material (Loliger, 1991). Earlier, Chipault *et al.* (1956) and others (Chang *et al.*, 1977; Dugan, 1980; Loliger, 1991) evaluated the antioxidant properties of many plants and found that rosemary and sage provided the most effective extracts. A natural food antioxidant extracted from rosemary is now on the market. Natural antioxidants are also found in various vegetables (Pratt, 1965), soybean (Pratt and Birac, 1979), citrus peel (Harel and Kanner, 1984), sesame seed (Budowski, 1964), olives (Sheabar and Neeman, 1988), carob pod (Farr *et al.*, 1988), and green tea leaves (Zhao *et al.*, 1989). This area was recently reviewed by Namiki (1990), Pratt and Hudson (1990), Schuler (1990), and Loliger (1991).

Plant tissues are the main biological systems that synthesize α-tocopherol, ascorbic acid, and carotenoids, but in addition they are also rich in a wide variety of phenolic compounds (Dugan, 1980; Horesteen, 1983; Namiki, 1990; Loliger, 1991). Common foods of plant origin contain a variety of hydroxylated flavonoids and other phenolics in amounts ranging from traces to several grams per kilogram of fresh weight (Dugan, 1980; Pratt and Hudson, 1990; Macheix *et al.*, 1990). Flavonoids and other plant phenolics have been reported to have multiple biological effects such as antioxidant activity (Chipault *et al.*, 1956; Chang *et al.*, 1977; Hudson and Lewis, 1983; Bors and Saran, 1987), anti-inflammatory action (Lands and Hanel, 1982; Landolfi *et al.*, 1984; Moroney *et al.*, 1988), inhibition of platelet aggregation (Van-Wauwe and Gossenc, 1983), and antimicrobial activities (Dugan, 1980; Pratt and Hudson, 1990).

Grapes, wines, and grape byproducts contain large amounts of phenolic compounds, mostly flavonoids, at high concentrations of 1000–1800 mg/mL (Singleton, 1982; Brasseur *et al.*, 1986; Macheix *et al.*, 1990).

A large part of the phenolics in grapes, wines, and byproducts may act as antioxidants. Some of these compounds may act selectively at very low concentrations to inhibit *ex vivo* LDL oxidation *in vitro* (Frankel *et al.*, 1993).

The objective of this study was to quantify antioxidants and their effects on the inhibition of lipid peroxidation by several catalysts, such as myoglobin, cytochrome *c*, iron ascorbate, and copper ions.

MATERIALS AND METHODS

Myoglobin (type 1) from equine skeletal muscle, cytochrome *c* (horse heart), ascorbic acid, linoleic acid, β-carotene, α-tocopherol, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO); ferric chloride was from Mallinckrodt Chemical Works (St. Louis, MO), and hydrogen peroxide (30%) and trichloroacetic acid were from Merck (Darmstadt, Germany).

Grapes (var. Thomson seedless, Flame seedless, and Black seedless; see Table 1) were purchased from an outdoor market. Wines (Cabernet Sauvignon and Petite Sirah without added sulfite) were distilled to remove alcohol by vacuum at 40 °C and diluted to the original concentration by distilled water. The grape berries (20 g) were homogenized in the presence of ethanol (80 mL) for 1 min at room temperature and centrifuged at 4 °C for 15 min. The ethanolic extract which contained the grape phenolics was diluted by distilled water before being tested in a model system. Each control sample contained the same concentration of ethanol and not higher than 5%.

The concentration of wine phenolics was estimated by analyzing for total phenol by the Folin–Ciocalteu procedure of Singleton and Rossi (1965) and expressing results in micrograms per milliliter or molar equivalents (μM*) of quercetin (MW 338), a naturally occurring polyphenol.

Four milliliters of wine (Cabernet Sauvignon) was freeze-dried and solubilized in 1 mL of methanol. This extract (25 μL) was subjected to thin-layer chromatography on 20/20 cm glass plates, coated with 0.5 mm silica of gel 60 F254, and developed with chloroform–methanol (9:1 v/v). The separated compounds were located and identified by visualizing plates with Folin–Ciocalteu reagent to detect phenol groups and with a solution of linoleate–β-carotene to detect antioxidant active compounds. Antioxidants protecting against the bleaching of β-carotene gave orange spots (Harel and Kanner, 1984).

β-Carotene–linoleate oxidizing activity was assayed colorimetrically (Kanner, 1979). The technique consists of following the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contained 1.5 mL of buffered carotene–linoleate mixture at pH 7.0, 0.1–0.4-mL active fractions, and distilled water in a mixture as follows: β-carotene, 14 μM; linoleate, 2 mM; linoleic acid

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Table 1. Phenolic Compound Concentrations in Several Grape Varieties and Wines[a]

grape/wine	phenolics, mg/L[b]
Thomson seedless	260
Flame seedless	850
Black seedless	920
Cabernet Sauvignon	1800
Petite Sirah	3200

[a] Phenolic compounds from grapes were extracted with 80% ethanol (without distillation of the ethanol) and diluted by distilled water before being tested in model systems. [b] Wine, liter; grapes, kilogram.

hydropeoxides (2 μM); Tween 20, 0.05%, phosphate buffer, pH 7.0, 0.1 M; DETA (diethylenetriaminepentaacetic acid), 0.5 mM (except the model catalyzed by Fe–ascorbic acid). The blank sample contained all of the reagents except β-carotene.

The assay of diene conjugation was carried out according to the method described by Ben-Aziz *et al.* (1970). The reaction mixture contained linoleic acid (0.05%) and DETA (0.5 mM) in 0.1 M phosphate buffer at pH 7.0. Both assay methods employed a double-beam recording spectrophotometer.

Low-density lipoproteins were prepared from blood collected in EDTA by venipuncture (1.5 mg/mL) from two normolipidemic nonsmoking male volunteers (aged 41 and 64) and centrifuged at 150 g at 4 °C to prepare plasma. Plasma LDL was prepared as described previously (Frankel et al., 1993). Prior to the oxidation experiments LDL were exhaustively dialyzed with deoxygenated phosphate-buffered (10 mM, pH 7.4) saline (100 mM) for 24 h. The final concentration of each sample was diluted to the same protein content (0.25 mg/mL LDL) with phosphate-buffered saline (10 mM). The oxidative susceptibility of LDL was evaluated by the accumulation of conjugated dienes at 234 nm (Esterbauer et al., 1989). LDL was oxidized with 8 μM $CuSO_4$ at room temperature.

Spectral changes showing the stoichiometric reduction of ferryl myoglobin radical with phenolic compounds extracted from wine were determined spectrophotometrically at 545 and 575 nm, after interaction of equimolar concentrations of metmyoglobin with H_2O_2 (50 μM).

RESULTS

Grapes and wines contain large amounts of phenolic compounds. The concentration of phenolics, estimated by analyzing total phenols in three grape varieties and wines, is presented in Table 1. The results show that the red grape variety and red wines (Cabarnet Sauvignon and Petite Sirah) contain high concentrations of phenolics: 920 mg/kg and 1800 and 3200 mg/L, respectively. In contrast, the green Thomson grapes contain only 260 mg/kg phenolics.

Wine phenolics were separated by TLC and identified by visualizing plates with Folin–Ciocalteu reagent and with a solution of linoleate-β-carotene. All of the phenolic compounds, identified by the Folin–Ciocalteu reagent, also showed an antioxidative effect by protecting β-carotene from oxidation. The most active compounds were separated and identified at R_f 0.01, 0.25, 0.5, and 0.74 (spots 1, 3, 6, 8) (Figure 1).

The co-oxidation of β-carotene catalyzed by myoglobin was inhibited significantly by diluted grape extracts and wine (Figure 2). The I_{50} inhibition of β-carotene oxidation was approximately 0.2 μg of phenolics/mL for all grape varieties tested in this study. The I_{50} inhibition was higher in grape extracts than in red wine (I_{50} = 0.35 μg of phenolics/mL).

Lipid peroxidation of linoleic acid was also estimated by accumulation of conjugated diene hydroperoxides (Figure 3). The peroxidation was catalyzed by myoglobin (2.5 μM) and cytochrome *c* (2.5 and 5.0 μM). Wine phenolics inhibited cytochrome *c* lipid peroxidation at an

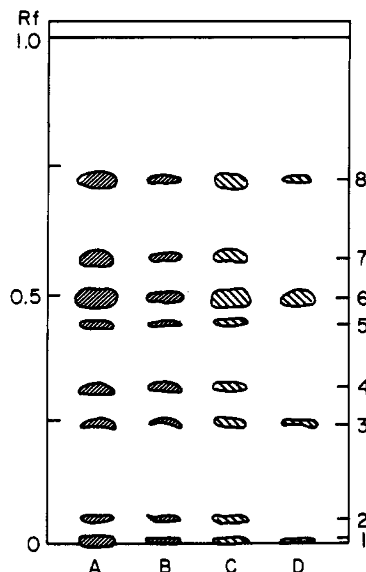


Figure 1. Thin-layer chromatography of wine (Cabernet Sauvignon) phenolics separated on a glass plate coated with 0.5 mm of silica gel 60. (A, C) 25 μL of wine phenolics; (B, D) 10 μL of wine phenolics; (▨) identified by Folin–Ciocalteu reagent; (▧) identified by linoleic acid–β-carotene (1 mM/50 μM in chloroform).

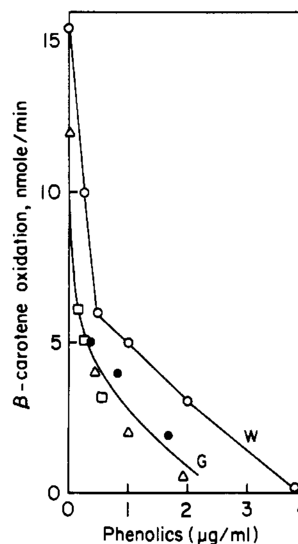


Figure 2. Inhibition by grapes (G) and wine (W) phenolics of linoleate–β-carotene oxidation catalyzed by myoglobin (2.5 μM) at pH 7.0, 23 °C. Results were calculated for the first minute of reaction time. (○) Wine (Cabernet Sauvignon); (□) grapes, Thomson seedless; (●) Flame seedless; (△) Black seedless.

I_{50} of 0.9 μg of phenolics/mL and myoglobin-dependent lipid peroxidation at an I_{50} of 0.35 μg of phenolics/mL. Similar results were obtained when wine phenolics inhibited co-oxidation of β-carotene–linoleic acid by the same catalysts (Figure 4).

The inhibition of wine phenolics of β-carotene–linoleic acid oxidation, at pH 4.5, in the presence of an iron "redox cycle" catalyst [Fe (50 μM)–ascorbic acid (200 μM)] and myoglobin (2.5 μM) is shown in Figure 5. The inhibitino by wine phenolics (I_{50}) of β-carotene oxidation by myoglobin is 0.35 μg of phenolics/mL, similar to that of pH 7.0; however, the I_{50} for the Fe–AA system was 3.75 μg/mL, almost 10-fold higher.

The antioxidative effects of wine phenolics were also studied in a system containing human LDL which oxidized *ex vivo* by Cu^{2+} ions. In this system LDL peroxidation by Cu^{2+} ions was estimated by the accumulation of conjugated

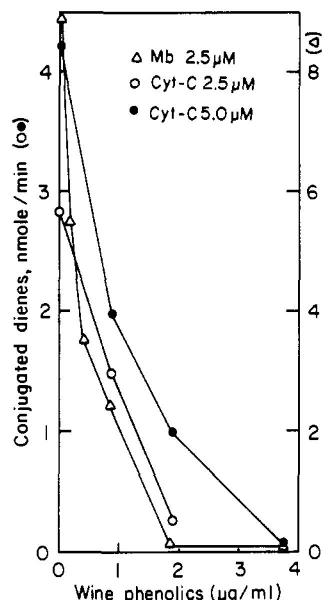


Figure 3. Inhibition of lipid peroxidation (conjugated dienes) by wine phenolics (Cabernet Sauvignon) induced with myoglobin and cytochrome *c* at pH 7.0, 23 °C. Results were calculated for the first minute of reaction time.

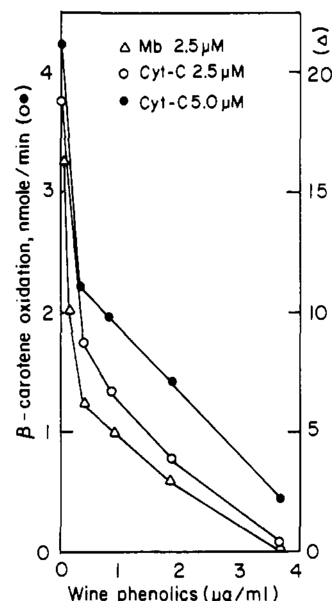


Figure 4. Inhibition of lipid peroxidation (β-carotene oxidation) by wine phenolics (Cabernet Sauvignon) induced with myoglobin and cytochrome *c* at pH 7.0, 23 °C. Results were calculated for the first minute of reaction time.

dienes. The inhibition of LDL oxidation by wine phenolics was compared with that of α-tocopherol (Figure 6). The results show an I_{50} inhibition of less than 1 μM* for wine phenolics and 2 μM for α-tocopherol. Increasing the concentration of Cu^{2+} ions in the same system did not decrease I_{50} inhibition by wine phenolics (results not shown).

To better understand the mechanism by which wine phenolics inhibit lipid peroxidation catalyzed by myoglobin and cytochrome *c*, the changes in the spectra between 210 and 550 nm were assessed (Figures 7 and 8). Each line denotes the spectra at intervals of 30 s. Myoglobin increased linoleic acid peroxidation; the rate of hydroperoxide accumulation at 234 nm increased during the first few minutes and decreased after 3 min of incubation. The heme group of myoglobin absorbs at 410 nm, but during catalysis it breaks down and disappears completely

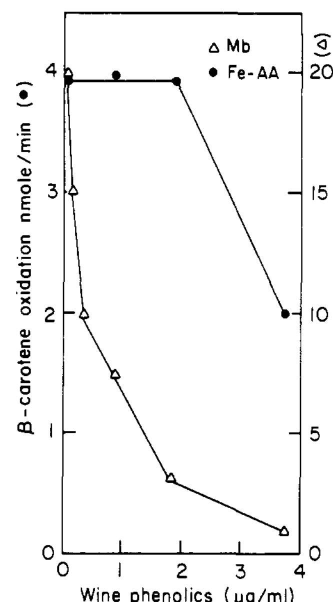


Figure 5. Inhibition by wine phenolics (Cabernet Sauvignon) of lipid peroxidation (β-carotene oxidation) induced with myoglobin and iron–ascorbic acid at pH 4.5, 23 °C. Results were calculated for the first minute of reaction time. (Δ) Myoglobin (2.5 μM); (●) iron (50 μM) + ascorbic acid (200 μM).

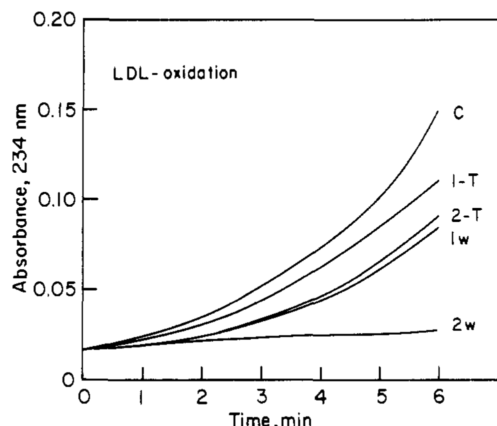


Figure 6. Inhibition by wine phenolics (Petite Sirah) and α-tocopherol of low-density lipoprotein peroxidation by copper ions at pH 7.4, 23 °C. C, control; 1-T, 1 μM α-tocopherol, 2-T, 2 μM α-tocopherol; 1w, 1 μM wine phenolics; 2w, 2 μM wine phenolics.

after 5 min. The breakdown products which absorb at 280 nm accumulate with time. Wine phenolics reduce the breakdown of the heme–myoglobin (at 410 nm) and not only eliminate conjugated diene accumulation (at 234 nm) but also decrease the amount of the hydroperoxides showing a "pseudo" peroxidase activity (Figure 7b).

Cytochrome *c* catalyzes linoleic acid oxidation (Figure 8a), its catalysis increasing with time (234 nm), and the heme absorption is very stable (420 nm). The addition of wine phenolics to this system (Figure 8b) prevented accumulations of conjugated dienes, without showing a specific pseudo peroxidase activity.

The stoichiometric reduction of ferryl myoglobin by wine phenolics is shown in Figure 9. Ferryl myoglobin was generated by the interaction of equimolar amounts of metmyoglobin and hydrogen peroxide (Kanner and Harel, 1985; Harel *et al.*, 1988). Interaction of equimolar concentrations of myoglobin and hydrogen peroxide (50 μM) to produce oxoferryl radical is shown by an increase in maxima at 547 and 575 nm. Metmyoglobin depletion is shown by a decrease in maxima at 500 and 630 nm,

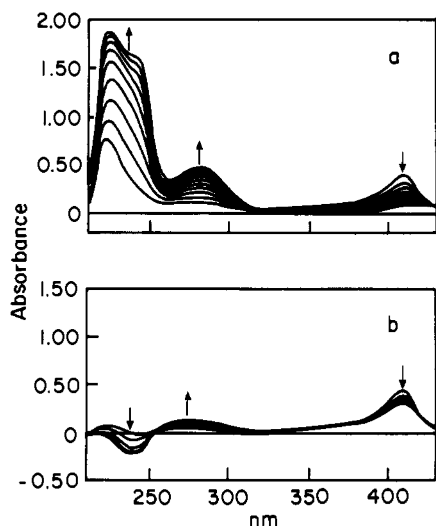


Figure 7. Spectral changes during linoleic acid (2 mM) lipid peroxidation induced by myoglobin (2.5 μM) (a) without or (b) with 3 μg/mL wine phenolics at pH 7.0, 23 °C.

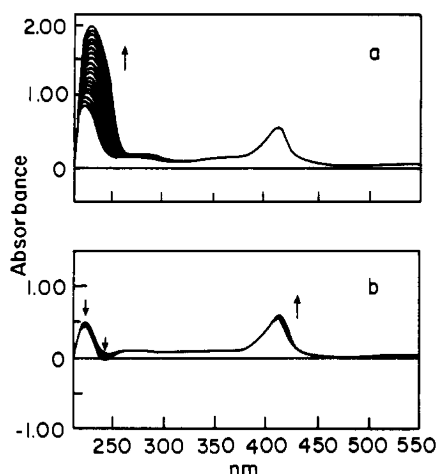


Figure 8. Spectral changes during linoleic acid (2 mM) lipid peroxidation induced by cytochrome *c* (2.5 μM) (a) without or (b) with 3 μg/mL wine phenolics at pH 7.0, 23 °C.

(Figure 9a). Reduction of ferryl myoglobin radical in the presence of wine phenolics is shown in Figure 9b by the decrease in maxima at 545 and 575 nm and increase in maxima at 500 and 630 nm. The product of reduction of ferryl to metmyoglobin by 50 μM wine phenolics is shown in Figure 9c. The inset shows the linear relationship between molar concentration of ferryl radical and wine phenolics.

Wine phenolics at an amount of 17 μg/mL completely reduced 50 μM ferryl myoglobin. This finding supports our approximation of phenolic concentrations in wine on the basis of an average molecular weight of approximately 330. By this calculation almost 50 μM wine phenolics reduce 50 μM ferryl myoglobin.

DISCUSSION

Recently much attention has focused on the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanisms of their action. Phenolic compounds, which occur widely in plants, were considered for a long period of time to be antioxidants (Chipault *et al.*, 1956; Pratt and Hudson, 1990). Grapes are rich in phenolic compounds; most of them are present in the peel (Singleton, 1982). Our results confirm a variation in phenolic content among three varieties, from 260 to 930 mg/kg of fresh weight. In red wines this concentration ranges from 1800 (Carbernet Sauvignon) to 3200 mg/L (Petite Sirah) phenolics. These results are in agreement with those published by others (Singleton, 1982; Macheix *et al.*, 1990).

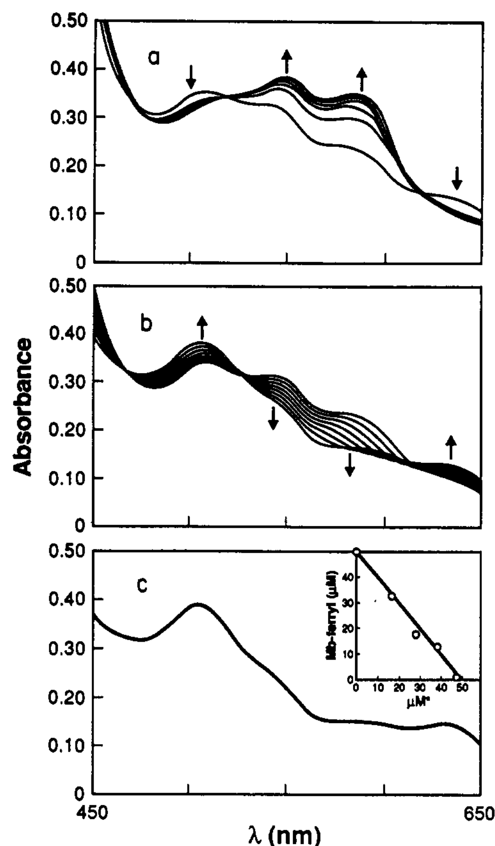
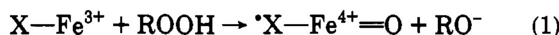


Figure 9. Reduction of ferryl myoglobin by wine phenolics at pH 7.0: spectral changes showing the stoichiometric reduction of oxoferryl radical of myoglobin with wine phenolic compounds. (a) Interaction of equimolar concentrations of myoglobin and hydrogen peroxide (50 μM) to produce oxoferryl radical as shown by increase in maxima at 545 and 575 nm. Metmyoglobin depletion is shown by the decrease in maxima at 500 and 630 nm. (b) Reduction of oxoferryl radical in the presence of wine phenolics (17 μg/mL) as shown by the decrease in maxima at 545 and 575 nm and increase in maxima at 500 and 630 nm. (c) Product of reduction of oxoferryl to metmyoglobin by 50 μM* of wine phenolics (17 μg/mL); (inset) linear relationship between molar concentration of oxoferryl myoglobin and wine phenolics. μM*, wine phenolics calculated on the basis of quercetin molecular weight (336).

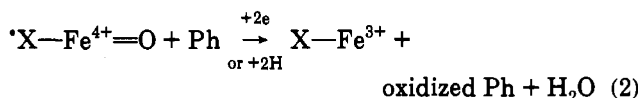
The antioxidative effects of grapes and wine phenolics were determined in our study by several systems, containing different catalysts, to increase our understanding of their activities (Halliwell, 1990). These catalysts include iron pentacoordinated (myoglobin), a form which would be activated to ferryl species (Kanner and Harel, 1985), hexacoordinated iron cytochrome *c* sites (Harel *et al.*, 1988), "free" iron, and "free" Cu ions. Very effective antioxidants were found in all grape varieties and wines tested in our study, and they corresponded to the concentration of phenolics in the system. All of the spots detected as antioxidants, after TLC separation followed by the linoleate–β-carotene spray method, had been previously detected as phenolic compounds by the Folin reagent.

Wine phenolics show higher antioxidative effects in the model systems catalyzed by myoglobin than those catalyzed with cytochrome *c*. We believe that this effect has developed because myoglobin, in the presence of wine phenolics, works partly like a peroxidase which breaks

down hydroperoxides to nonradical species by the following reactions:



$$X{-}Fe^{3+} + ROOH \rightarrow {}^{\bullet}X{-}Fe^{4+}{=}O + RO^{-} \quad (1)$$



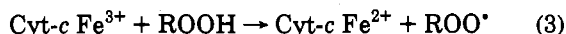
$${}^{\bullet}X{-}Fe^{4+}{=}O + Ph \underset{\text{or } +2H}{\overset{+2e}{\rightarrow}} X{-}Fe^{3+} + \text{oxidized Ph} + H_2O \quad (2)$$

where Ph represents phenolic compounds ${}^{\bullet}X{-}Fe^{4+}{=}O$ represents ferryl species, and X represents heme protein.

There is much evidence to support our suggestion that (a) myoglobin during interaction with hydroperoxides produces ferryl species (Walker *et al.*, 1983), (b) ferryl myoglobin is reduced by wine phenolics (Figure 8), and (c) hydroperoxides, determined by the conjugated diene products absorbing in the range 230–240 nm, decrease catalytically during incubation of linoleic acid with myoglobin and wine phenolics (Figure 7b).

In the model system, catalyzed by myoglobin, wine phenolics not only prevent lipid peroxidation, as evidenced by decreasing accumulation of conjugated dienes and β-carotene oxidations, but also stabilize the heme from breakdown by free radicals (Figure 7), and myoglobin may work as a pseudo peroxidase for a long period of time. Therefore, these results are very similar to those from our previous studies, showing that myoglobin and ascorbic acid worked as an antioxidant system preventing generation of hydroxyl radicals from H_2O_2 during the Fenton reaction (Harel and Kanner, 1989). In contrast, myoglobin without reducing compounds act as a catalyst and its prooxidative effect decreases with time, probably because of the breakdown of the heme (Harel *et al.*, 1988).

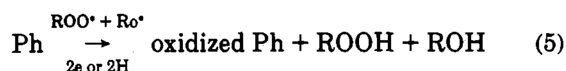
The iron in cytochrome *c* is bound to the polypeptide chain at all six coordinations sites. Iron Cyt-*c* interacts with lipid peroxides without producing an intermediate compound (Harel *et al.*, 1988). We assume that ferric Cyt-*c* catalyzes lipid peroxidation by reactions 3 and 4 (Kanner and Harel, 1985):



$$\text{Cyt-}c\ Fe^{3+} + ROOH \rightarrow \text{Cyt-}c\ Fe^{2+} + ROO^{\bullet} \quad (3)$$

$$\text{Cyt-}c\ Fe^{2+} + ROOH \rightarrow \text{Cyt-}c\ Fe^{3+} + RO^{\bullet} + HO^{-} \quad (4)$$

In this system wine phenolics may prevent lipid peroxidation by interacting with peroxyl and alkoxy radicals as described in



$$Ph \underset{2e \text{ or } 2H}{\overset{ROO^{\bullet} + Ro^{\bullet}}{\rightarrow}} \text{oxidized Ph} + ROOH + ROH \quad (5)$$

The higher antioxidative reactivity of wine phenolics in the myoglobin system seems to derive from the greater efficiency in working with ferryl species than with peroxyl or alkoxyl radicals, formed in the system catalyzed by Cyt-*c*.

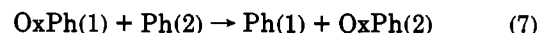
Lipid peroxidation by iron–ascorbic acid is inhibited by wine phenolics at concentrations 10- and 5-fold higher than that catalyzed by myoglobin or Cyt-*c*, respectively. Ferrous ion in this system reduces hydroperoxides to alkoxy radicals, which seem to be the main species responsible for peroxidation and β-carotene bleaching. The iron redox cycle generates by itself several oxygen species such as perhydroxyl radical (HO_2) and hydroxyl radical (${}^{\bullet}HO$) (Scarpa *et al.*, 1983; Kanner *et al.*, 1986), both of which could oxidize phenolic antioxidants and seem to reduce the efficiency of wine phenolics as antioxidants toward lipid peroxidation.

Low-density lipoprotein oxidation catalyzed by copper ions, which stimulates the same primary reactions involved in the initiation of atherosclerosis (Jacobson *et al.*, 1985; Peng *et al.*, 1985; Steinberg *et al.*, 1989; Addis and Warner, 1991), was inhibited by relatively low concentrations of wine phenolics (less than 1 μM) more effectively than by α-tocopherol.

The inhibition of LDL oxidation by several flavonoids, catalyzed by copper ions, has been demonstrated by others (DeWhalley *et al.*, 1990; Mangiopane *et al.*, 1992; Negre-Salvayre and Salvayre, 1992). Mangiopane *et al.* (1992) showed that catechin, a naturally occurring flavonoid, inhibits LDL oxidation only in a range around 100 μM. However, other flavonoids, such as hypolactin and gossypetin, were found to inhibit LDL oxidation at I_{50} of approximately 10 μM.

In most of our model systems, wine phenolics acted to inhibit lipid peroxidation with an I_{50} of less than 1 μM* (calculated from the stoichiometric reduction of ferryl myoglobin). This high antioxidant activity could be attributed to synergistic effects in a mixture of natural phenolic compounds. Several very active antioxidants may preferentially interact with ferryl or free radicals. The resulting oxidized phenolics (OxPh) are then reduced and possibly regenerated by less active phenolics by

$$Ph(1) + ROO^{\bullet} \rightarrow OxPh(1) + ROOH \quad (6)$$



$$OxPh(1) + Ph(2) \rightarrow Ph(1) + OxPh(2) \quad (7)$$

Circulating LDL are one of the fundamental targets of deleterious oxidation *in vivo*, resulting in the accumulation of atherogenic lipoproteins *in vivo*. The so-called French paradox indicates that the inhabitants of some regions of France have a lower rate of coronary heart disease (CHD) than that found in the United States despite consumption of fats at comparable levels, which are correlated to increased risk of heart attacks. This paradox has been attributed, in part, to the routine consumption of wine, and many researchers have attempted to correlate the low incidence of CHD with alcohol consumption (Colditz *et al.*, 1985; Rimm *et al.*, 1991; Renaud and De Lorgeril, 1992). We believe that the nonalcoholic compounds, the phenolic antioxidants, which are very abundant in red wine, may protect the French from CHD via prevention of LDL oxidation and inhibition of platelet aggregation (Frankel *et al.*, 1993).

As is known, polyphenols (e.g., catechin and quercetin) are absorbed by the gut into the bloodstream (Das, 1971; Hollman *et al.*, 1993). At the present time, grapes and nonfermented grape products are not considered particularly nutritious, since they do not contain large amounts of specific vitamins. However, their content of natural antioxidants represents a major beneficial property which may improve their market value in the future.

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